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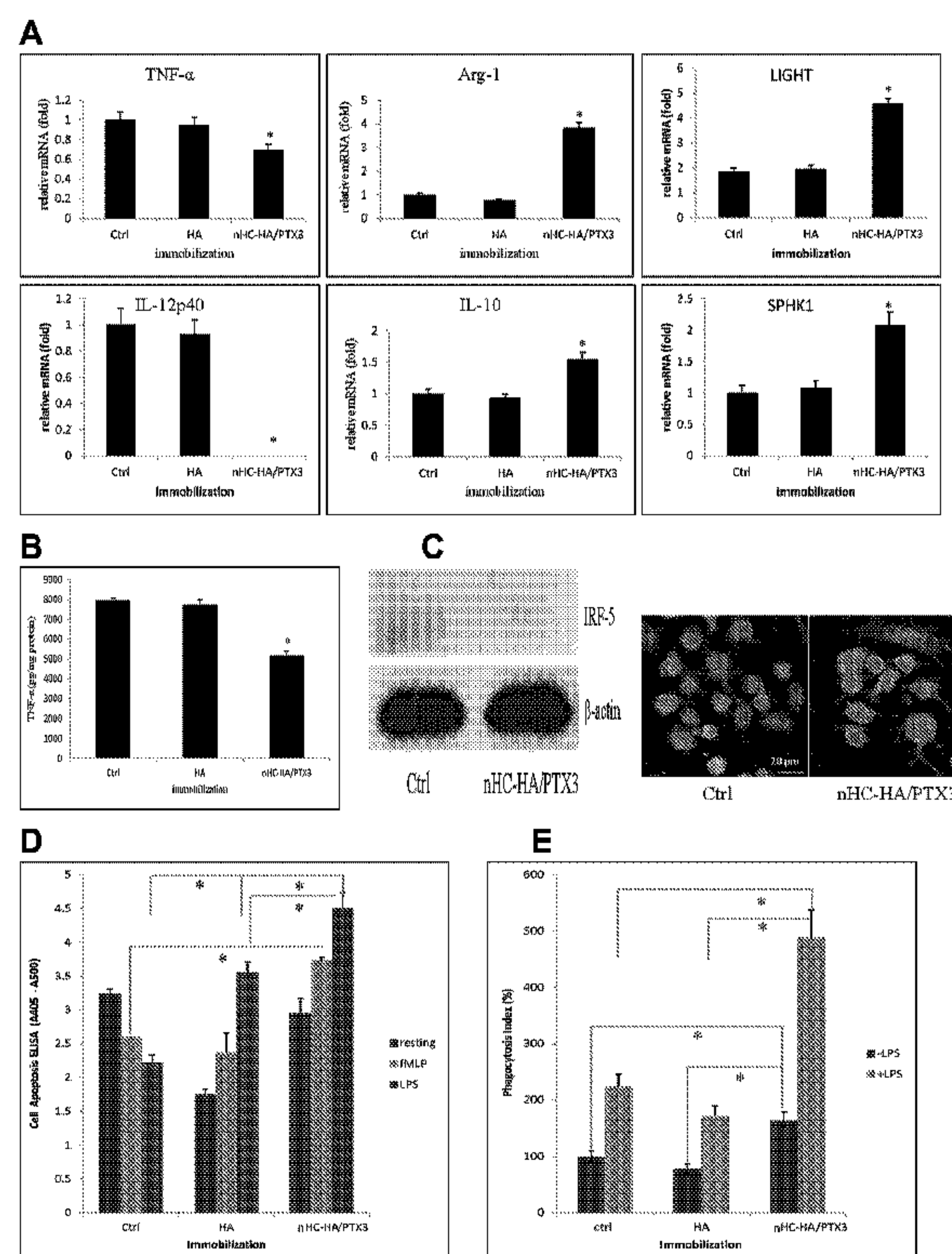
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(54) Titre : COMPOSITIONS CONTENANT DES COMPLEXES HC-HA/PTX3 ET PROCEDES D'UTILISATION ASSOCIES

(54) Title: COMPOSITIONS CONTAINING HC-HA/PTX3 COMPLEXES AND METHODS OF USE THEREOF

FIG. 3



(57) **Abrégé/Abstract:**

Provided herein are methods for the production of native and reconstituted hyaluronan (HA) complexes containing pentraxin-3 (PTX3) and heavy chain 1 (HC1) of inter alpha inhibitor (IaI). Compositions containing the complexes and therapeutic methods using the complexes are provided. Combinations and kits for use in practicing the methods also are provided.

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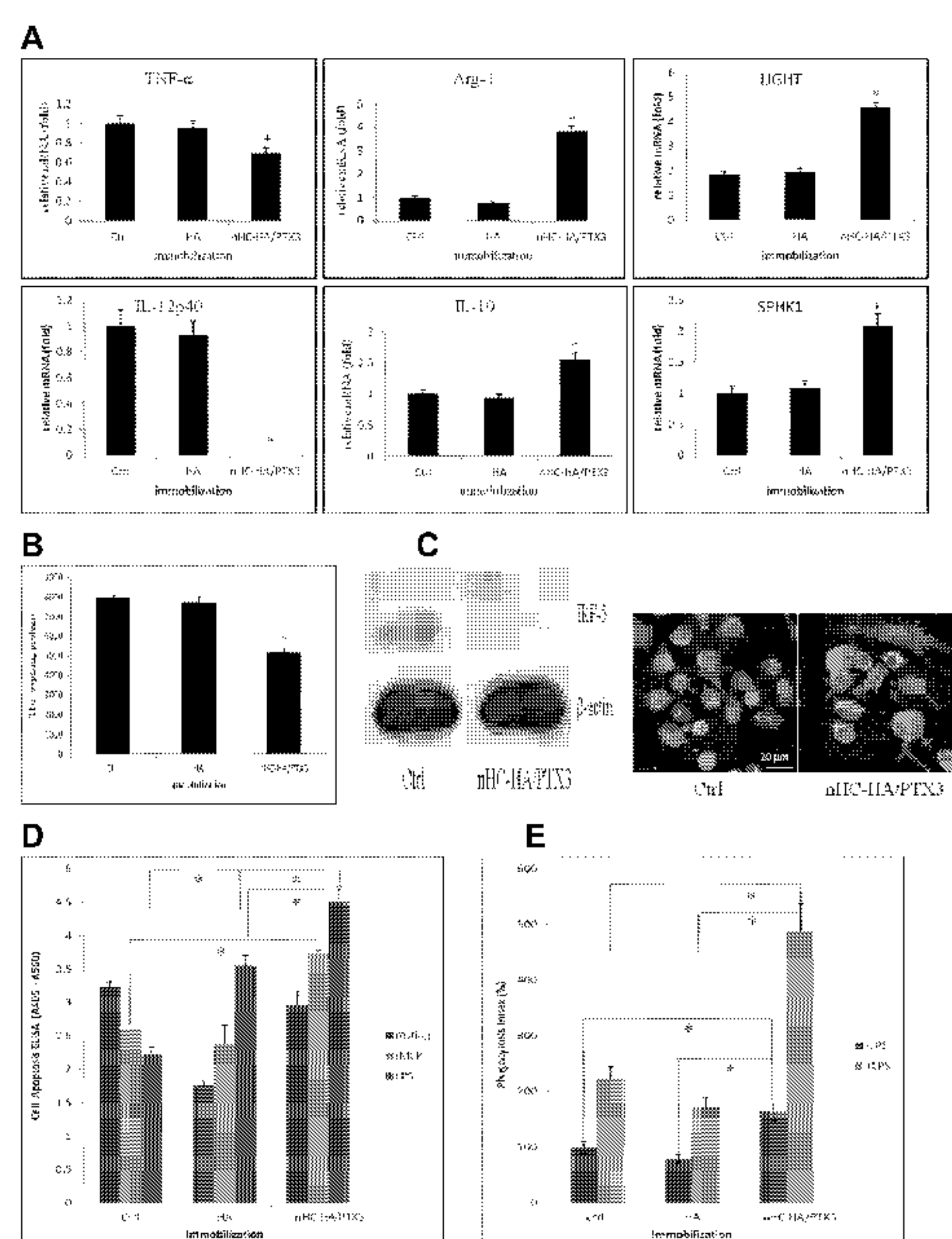
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(54) Title: COMPOSITIONS CONTAINING HC-HA/PTX3 COMPLEXES AND METHODS OF USE THEREOF

(57) Abstract: Provided herein are methods for the production of native and reconstituted hyaluronan (HA) complexes containing pentraxin-3 (PTX3) and heavy chain 1 (HC1) of inter alpha inhibitor (I α I). Compositions containing the complexes and therapeutic methods using the complexes are provided. Combinations and kits for use in practicing the methods also are provided.

FIG. 3



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COMPOSITIONS CONTAINING HC-HA/PTX3 COMPLEXES AND METHODS OF USE THEREOF

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Application Serial No. 61/670,571, filed on July 11, 2012, which is hereby incorporated by reference in its entirety.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

[0002] Funding for the work described herein was provided in part by federal research grants RO1 EY06819, R44 EY017497, and R43 EY021045 from the National Eye Institute, the National Institutes of Health, Bethesda, MD, USA.

INCORPORATION BY REFERENCE OF SEQUENCE LISTING SUBMITTED AS A TEXT FILE VIA EFS-WEB

[0003] The instant application contains a Sequence Listing, which has been submitted as a computer readable text file in ASCII format via EFS-Web and is hereby incorporated in its entirety by reference herein. The text file, created date of July 8, 2013, is named 34157-732-601SEQ.txt and is 462 KB in size.

BACKGROUND OF THE INVENTION

[0004] The amniotic membrane (AM) is an avascular membranous sac that is filled with amniotic fluid surrounding the fetus. The AM, like the placenta, is derived from the epiblast formed during development of the fertilized egg. The AM forms the innermost membrane surrounding the fetus in the amniotic cavity. In placental mammals, the umbilical cord (i.e., the *funiculus umbilicalis*) connects the developing fetus to the placenta. The umbilical cord is made up of amniotic membrane (UCAM) and Wharton's Jelly. The amniotic membrane forms the outer layer of the umbilical cord. The UCAM functions to regulate the fluid pressure within the UC. Wharton's Jelly is a gelatinous substance within the umbilical cord, largely made up of mucopolysaccharides (hyaluronic acid and chondroitin sulfate). It also contains some fibroblasts and macrophages. The umbilical cord further comprises two arteries (the umbilical arteries) and one vein (the umbilical vein), buried within the Wharton's Jelly.

SUMMARY OF THE INVENTION

[0005] Described herein are methods for the identification of HC-HA/PTX3 complexes in fetal tissues, such as amniotic membrane and umbilical cord. Also, described herein are methods for the isolation of native HC-HA/PTX3 complexes from fetal tissues, such as amniotic membrane and umbilical cord. Also described herein are methods for the production of reconstituted HC-HA/PTX3 complexes. Also described herein are methods for the use of native and reconstituted HC-HA/PTX3 complexes provided herein.

[0006] Described herein, in certain embodiments, are methods of producing HC-HA/PTX3 complexes. In some embodiments, the methods comprise isolating a native HC-HA/PTX3 (nHC-HA/PTX3) complex from an extract prepared from a tissue or cell. In some embodiments, the methods comprise generating a reconstituted HC-HA/PTX3 (rcHC-HA/PTX3) complex.

[0007] Described herein, in certain embodiments, are methods of isolating native HC-HA/PTX3 (nHC-HA/PTX3) complexes from amniotic tissues, such as umbilical cord or amniotic membrane. In some embodiments, the nHC-HA/PTX3 complexes are isolated from an isolated cell. In some embodiments, the nHC-HA/PTX3 complexes are isolated from a cultured cell. In some embodiments, the nHC-HA/PTX3 complexes are isolated from a stem cell. In some embodiments, the nHC-HA/PTX3 complexes are isolated from a water soluble fraction of an extract prepared from a tissue, such as umbilical cord or amniotic membrane. In some embodiments, the water soluble fraction is extracted with an isotonic salt solution. In some embodiments, the nHC-HA/PTX3 complexes are isolated from a water insoluble fraction of an extract prepared from a tissue, such as umbilical cord or amniotic membrane. In some embodiments, the insoluble fraction is extracted with GnHCl.

[0008] Described herein, in certain embodiments, are methods of producing a reconstituted HC-HA/PTX3 (rcHC-HA/PTX3) complex *in vitro*, (a) contacting (i) high molecular weight hyaluronan (HMW HA) immobilized to a solid support, and (ii) pentraxin 3 (PTX3) protein, to form an immobilized complex of PTX3 and HMW HA (immobilized PTX3/HA); and (b) contacting the immobilized PTX3/HA with an inter- α -inhibitor (I α I) protein comprising heavy chain 1 (HC1) and Tumor necrosis factor α -stimulated gene 6 (TSG-6) to form an immobilized rcHC-HA/PTX3 complex. In some embodiments, steps (a) and (b) of the method are performed sequentially in order. In some embodiments of the method, the method comprises contacting high molecular weight hyaluronan (HMW HA) with a pentraxin 3 (PTX3) protein, inter- α -inhibitor (I α I) protein comprising heavy chain 1 (HC1) and Tumor necrosis factor α -stimulated gene 6 (TSG-6) simultaneously. In some embodiments of the method, TSG-6 catalyzes the covalent linkage of I α I HC1 to HA. In some embodiments, the method further comprises removing unbound PTX3 protein

following step (a) and prior to performing step (b). In some embodiments, the method further comprises removing unbound TSG-6 following step (b). In some embodiments, the PTX3 protein used in the methods is a native PTX3 protein isolated from a cell. In some embodiments, the cell is a mammalian cell. In some embodiments, the cell is a human cell. In some embodiments, the cell is an amniotic membrane cell. In some embodiments, the cell is an umbilical cord cell. In some embodiments, the cell is an amniotic membrane cell from an umbilical cord. In some embodiments, the amniotic membrane cell is an amniotic epithelial cell. In some embodiments, the amniotic membrane cell is an umbilical cord epithelial cell. In some embodiments, the amniotic membrane cell is an amniotic stromal cell. In some embodiments, the amniotic membrane cell is an umbilical cord stromal cell. In some embodiments, the PTX3 protein is a recombinant protein. In some embodiments, the PTX3 protein used in the methods comprises a polypeptide having the sequence set forth in SEQ ID NO: 33 or a polypeptide having at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% sequence amino acid identity to the polypeptide having the sequence set forth in SEQ ID NO: 33. In some embodiments, the PTX3 protein used in the methods comprises a polypeptide having the sequence set forth in any of SEQ ID NOS: 32-45 or a species variant or allelic variant thereof. In some embodiments, the PTX3 protein used in the methods is a multimeric protein. In some embodiments, the PTX3 protein used in the methods is a homomultimer (i.e. a multimeric protein consisting of two or more identical components). In some embodiments, the PTX3 homomultimer is a dimer, trimer, tetramer, pentamer, hexamer, or octamer. In some embodiments, the PTX3 homomultimer is an octamer. In some embodiments, the PTX3 protein comprises a modified multimerization domain or a heterogeneous multimerization domain. In some embodiments, the TSG-6 protein used in the methods is a native TSG-6 protein isolated from a cell. In some embodiments, the cell is a mammalian cell. In some embodiments, the cell is a human cell. In some embodiments, the cell is an amniotic membrane cell. In some embodiments, the cell is an umbilical cord cell. In some embodiments, the cell is an amniotic membrane cell from an umbilical cord. In some embodiments, the amniotic membrane cell is an amniotic epithelial cell. In some embodiments, the amniotic membrane cell is an umbilical cord epithelial cell. In some embodiments, the amniotic membrane cell is an amniotic stromal cell. In some embodiments, the amniotic membrane cell is an umbilical cord stromal cell. In some embodiments, the TSG-6 protein is a recombinant protein. In some embodiments, the TSG-6 protein comprises a polypeptide having the sequence set forth in SEQ ID NO: 2 or a polypeptide having at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% sequence amino acid identity to the polypeptide having the sequence set forth in SEQ ID NO: 2. In some embodiments, the TSG-6 protein used in the methods comprises a polypeptide having the sequence set forth in any of SEQ ID NOS: 1-31 or a species

variant or allelic variant thereof. In some embodiments, the HC1 used in the methods comprises a polypeptide having the sequence set forth in SEQ ID NO: 47 or a polypeptide having at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% sequence amino acid identity to the polypeptide having the sequence set forth in SEQ ID NO: 47. In some embodiments, inter- α -inhibitor (I α I) protein used in the methods as a source of HC1 also comprises HC2 and bikunin linked by a chondroitin sulfate chain. In some embodiments, the HC1 comprises a polypeptide having the sequence set forth in any of SEQ ID NOS: 46-47 or a species variant or allelic variant thereof. In some embodiments, HC2 comprises a polypeptide having the sequence set forth in SEQ ID NO: 49 or a polypeptide having at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% sequence amino acid identity to the polypeptide having the sequence set forth in SEQ ID NO: 49. In some embodiments, the HC2 used in the methods comprises a polypeptide having the sequence set forth in any of SEQ ID NOS: 48-49 or a species variant or allelic variant thereof. In some embodiments, bikunin comprises a polypeptide having the sequence set forth in SEQ ID NO: 53 or a polypeptide having at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% sequence amino acid identity to the polypeptide having the sequence set forth in SEQ ID NO: 53. In some embodiments, the bikunin used in the methods comprises a polypeptide having the sequence set forth in any of SEQ ID NOS: 52-53 or a species variant or allelic variant thereof. In some embodiments, the I α I protein used in the methods is isolated from blood, serum, plasma, amniotic membrane, chorionic membrane, amniotic fluid, or a combination thereof. In some embodiments, the I α I protein used in the methods is isolated from serum. In some embodiments, the I α I protein used in the methods is isolated from human serum. In some embodiments, the I α I protein used in the methods is produced by a mammalian cell. In some embodiments, the cell is a human cell. In some embodiments, the cell is an amniotic membrane cell. In some embodiments, the cell is an umbilical cord cell. In some embodiments, the cell is an amniotic membrane cell from an umbilical cord. In some embodiments, the amniotic membrane cell is an amniotic epithelial cell. In some embodiments, the amniotic membrane cell is an umbilical cord epithelial cell. In some embodiments, the amniotic membrane cell is an amniotic stromal cell. In some embodiments, the amniotic membrane cell is an umbilical cord stromal cell. In some embodiments, the I α I and TSG-6 protein are contacted at a molar ratio of 1:1, 2:1, 3:1, 4:1, 5:1, 6:1, 7:1, 8:1, 9:1, 10:1, 15:1, or 20:1. In some embodiments, the I α I and TSG-6 protein are contacted at a molar ratio of 3:1. In some embodiments of the method, the weight average molecular weight of the HMW HA is between about 500 kDa and about 10,000 kDa, between about 800 kDa and about 8,500 kDa, between about 1100 kDa and about 5,000 kDa, or between about 1400 kDa and about 3,500 kDa. In some embodiments of the method, the weight average molecular weight of the HMW HA is 3,000 kDa. In some embodiments, HMW HA is

immobilized by direct linkage to a solid support. In some embodiments of the method, HMW HA is immobilized by indirect linkage to a solid support. In some embodiments of the method, HMW HA is immobilized by covalent attachment to the solid support. In some embodiments of the method, HMW HA is immobilized by non-covalent attachment to the solid support. In some embodiments of the method, HMW HA is immobilized by linkage to a solid support via a cleavable linker. In some embodiments, the linker is a chemically or enzymatically cleavable linker. In some embodiments, the method further comprises dissociation of the rcHC-HA/PTX3 complex from the solid support following step (b). In some embodiments, dissociation comprises cleavage of a cleavable linker. In some embodiments, the method further comprises purification of the dissociated rcHC-HA/PTX3 complex. In some embodiments, purification comprises affinity purification, centrifugation, filtration, chromatography or a combination thereof. In some embodiments of the method, PTX3, IaI HC1 or TSG-6 polypeptides comprise an affinity tag. In some embodiments, the affinity tag is selected from among a hemagglutinin tag, a poly-histidine tag, a myc tag, a FLAG tag, a glutathione-S-transferase (GST) tag. In some embodiments, HMW HA is immobilized by binding HMW HA to an intermediary polypeptide. In some embodiments, the intermediary polypeptide is covalently attached to the solid support. In some embodiments, binding HMW HA to the intermediary polypeptide is non-covalent. In some embodiments, the intermediary polypeptide is an HA binding protein (HABP). In some embodiments, the intermediary polypeptide is an HABP selected from among HAPLN1, HAPLN2, HAPLN3, HAPLN4, aggrecan, versican, neurocan, brevican, phosphacan, TSG-6, CD44, stabilin-1, stabilin-2, or a portion thereof sufficient to bind HA. In some embodiments, the intermediary polypeptide is versican. In some embodiments, the intermediary polypeptide comprises a link module. In some embodiments, the intermediary polypeptide comprises a link module of HAPLN1, HAPLN2, HAPLN3, HAPLN4, aggrecan, versican, neurocan, brevican, phosphacan, TSG-6, CD44, stabilin-1, or stabilin-2. In some embodiments, the intermediary polypeptide comprises a link module of versican. In some embodiments, the intermediary polypeptide comprises a polypeptide set forth in any of SEQ ID NOS: 54-99. In some embodiments, the intermediary polypeptide comprises a polypeptide linker. In some embodiments, the linker is attached to the solid support. In some embodiments, the method further comprises dissociation of the rcHC-HA/PTX3 complex from the intermediary polypeptide following step (b). In some embodiments, dissociation of the rcHC-HA/PTX3 complex from the intermediary polypeptide comprises contacting the complex with a dissociation agent. In some embodiments, the dissociation agent is guanidine hydrochloride or urea. In some embodiments, the dissociation agent is about 4 M to about 8M guanidine hydrochloride. In some embodiments, the intermediary polypeptide or linker comprises a proteolytic cleavage

sequence. In some embodiments, dissociation of the rcHC-HA/PTX3 complex comprises cleaving the intermediary polypeptide or linker at proteolytic cleavage sequence. In some embodiments, cleaving comprises contacting the proteolytic cleavage sequence with a protease. In some embodiments, the protease is selected from among furin, 3C protease, caspase, matrix metalloproteinase and TEV protease.

[0009] Described herein, in certain embodiments, are methods of producing a reconstituted HC-HA/PTX3 (rcHC-HA/PTX3) complex *in vitro*, comprising contacting a PTX3/H A complex immobilized to a solid support with inter- α -inhibitor (I α I) protein comprising heavy chain 1 (HC1) and TSG-6. In some embodiments, the PTX3/H A complex is produced by contacting high molecular weight hyaluronan (HMW HA) with a pentraxin 3 (PTX3) protein under conditions effective to form a complex of PTX3 and HMW HA (PTX3/H A), wherein the HMW HA is immobilized to a solid support. In some embodiments, the method further comprises removing unbound PTX3 protein prior to contacting the PTX3/H A complex with I α I and TSG-6. In some embodiments, the method further comprises removing unbound TSG-6. In some embodiments, the PTX3 protein is a recombinant protein. In some embodiments, the PTX3 protein comprises a polypeptide having the sequence set forth in SEQ ID NO: 33 or a polypeptide having at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% sequence amino acid identity to the polypeptide having the sequence set forth in SEQ ID NO: 33. In some embodiments, the PTX3 protein used in the methods is a multimeric protein. In some embodiments, the PTX3 protein used in the methods is a homomultimer. In some embodiments, PTX3 homomultimer is a dimer, trimer, tetramer, pentamer, hexamer, octamer. In some embodiments, the PTX3 homomultimer is an octamer. In some embodiments, PTX3 comprises a modified multimerization domain or a heterogeneous multimerization domain. In some embodiments, TSG-6 is a recombinant protein. In some embodiments, TSG-6 comprises a polypeptide having the sequence set forth in SEQ ID NO: 2 or a polypeptide having at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% sequence amino acid identity to the polypeptide having the sequence set forth in SEQ ID NO: 2. In some embodiments, HC1 comprises a polypeptide having the sequence set forth in SEQ ID NO: 47 or a polypeptide having at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% sequence amino acid identity to the polypeptide having the sequence set forth in SEQ ID NO: 47. In some embodiments, I α I protein also comprises HC2. In some embodiments, HC2 comprises a polypeptide having the sequence set forth in SEQ ID NO: 49 or a polypeptide having at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% sequence amino acid identity to the polypeptide having the sequence set forth in SEQ ID NO: 49. In some embodiments, the I α I protein also comprises bikunin. In some embodiments, bikunin comprises a polypeptide having the sequence set

forth in SEQ ID NO: 53 or a polypeptide having at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% sequence amino acid identity to the polypeptide having the sequence set forth in SEQ ID NO: 53. In some embodiments, IaI also comprises a chondroitin sulfate chain. In some embodiments, the IaI protein is a recombinant protein. In some embodiments, the IaI protein is isolated from blood, plasma, serum, amniotic membrane, chorionic membrane, amniotic fluid, or a combination thereof. In some embodiments, the IaI protein is isolated from serum. In some embodiments, the IaI protein is isolated from human serum. In some embodiments, the IaI and TSG-6 protein are contacted at a molar ratio of 1:1, 2:1, 3:1, 4:1, 5:1, 6:1, 7:1, 8:1, 9:1, 10:1, 15:1, or 20:1. In some embodiments, the IaI and TSG-6 protein are contacted at a molar ratio of 3:1. In some embodiments, the weight average molecular weight of the HMW HA is between about 500 kDa and about 10,000 kDa, between about 800 kDa and about 8,500 kDa, between about 1100 kDa and about 5,000 kDa, or between about 1400 kDa and about 3,500 kDa. In some embodiments, the weight average molecular weight of the HMW HA is 3,000 kDa. In some embodiments, HMW HA is immobilized by direct linkage to a solid support. In some embodiments, HMW HA is immobilized by indirect linkage to a solid support. In some embodiments, HMW HA is immobilized by covalent attachment to the solid support. In some embodiments, HMW HA is immobilized by non-covalent attachment to the solid support. In some embodiments, the method further comprises dissociation of the rcHC-HA/PTX3 complex from the solid support. In some embodiments, the method further comprises purification of the dissociated rcHC-HA/PTX3 complex. In some embodiments, purification comprises affinity purification, centrifugation, filtration, chromatography or a combination thereof. In some embodiments, the PTX3, IaI HC1 or TSG-6 polypeptides comprise an affinity tag. In some embodiments, the affinity tag is selected from among a hemagglutinin tag, a poly-histidine tag, a myc tag, a FLAG tag, a glutathione-S-transferase (GST) tag. In some embodiments, HMW HA is immobilized by binding HMW HA to an intermediary polypeptide. In some embodiments, the intermediary polypeptide is covalently attached to the solid support. In some embodiments, the binding HMW HA to the intermediary polypeptide is non-covalent. In some embodiments, the intermediary polypeptide is an HA binding protein (HABP). In some embodiments, the intermediary polypeptide is an HABP selected from among HAPLN1, HAPLN2, HAPLN3, HAPLN4, aggrecan, versican, neurocan, brevican, phosphacan, TSG-6, CD44, stabilin-1, stabilin-2, or a portion thereof sufficient to bind HA. In some embodiments, the intermediary polypeptide is versican. In some embodiments, the intermediary polypeptide comprises a link module. In some embodiments, the intermediary polypeptide comprises a link module of HAPLN1, HAPLN2, HAPLN3, HAPLN4, aggrecan, versican, neurocan, brevican, phosphacan, TSG-6, CD44, stabilin-1, or stabilin-2. In some

embodiments, the intermediary polypeptide comprises a link module of versican. In some embodiments, the intermediary polypeptide comprises a polypeptide set forth in any of SEQ ID NOS: 54-99. In some embodiments, the intermediary polypeptide comprises a polypeptide linker. In some embodiments, the linker is attached to the solid support. In some embodiments, the method further comprises dissociation of the rcHC-HA/PTX3 complex from the intermediary polypeptide. In some embodiments, dissociation of the rcHC-HA/PTX3 complex from the intermediary polypeptide comprises contacting the complex with a dissociation agent. In some embodiments, the dissociation agent is guanidine hydrochloride or urea. In some embodiments, the dissociation agent is about 4 M to about 8M guanidine hydrochloride. In some embodiments, the intermediary polypeptide or linker comprises a proteolytic cleavage sequence. In some embodiments, dissociation comprises cleaving the intermediary polypeptide or linker at proteolytic cleavage sequence. In some embodiments, cleaving comprises contacting the proteolytic cleavage sequence with a protease. In some embodiments, the protease is selected from among furin, 3C protease, caspase, matrix metalloproteinase and TEV protease.

[0010] Described herein, in certain embodiments, are reconstituted HC-HA (rcHC-HA/PTX3) complexes produced by any of the methods provided herein for generating rcHC-HA/PTX3 complexes.

[0011] Described herein, in certain embodiments, are reconstituted HC-HA/PTX3(rcHC-HA/PTX3) complexes comprising high molecular weight hyaluronan (HMW HA), PTX3, and IαI HC1, wherein the rcHC-HA/PTX3 complexes promote the M2 polarization of a macrophage. Described herein, in certain embodiments, are native HC-HA/PTX3(nHC-HA/PTX3) complexes comprising high molecular weight hyaluronan (HMW HA), PTX3, and IαI HC1, wherein the nHC-HA/PTX3 complexes promote the M2 polarization of a macrophage. Described herein, in certain embodiments, are methods for inducing the M2 polarization of macrophages comprising contacting a macrophage with an rcHC-HA/PTX3 or isolated nHC-HA/PTX3 complex.

[0012] Described herein, in certain embodiments, are reconstituted HC-HA/PTX3(rcHC-HA/PTX3) complexes comprising high molecular weight hyaluronan (HMW HA), PTX3, and IαI HC1, wherein the rcHC-HA/PTX3 complexes reduce the expression of IL-12p40 in LPS-stimulated macrophages, wherein the level of IL-12p40 expressed by LPS-stimulated macrophages is lower when the LPS-stimulated macrophages are contacted the rcHC-HA/PTX3 complexes compared to the level of IL-12p40 expressed by LPS-stimulated macrophages in the absence of the rcHC-HA/PTX3 complexes. In some embodiments, the level of IL-12p40 mRNA is reduced. In some embodiments, the level of IL-12p40 protein is reduced.

[0013] Described herein, in certain embodiments, are native HC-HA/PTX3(nHC-HA/PTX3) complexes comprising high molecular weight hyaluronan (HMW HA), PTX3, and IαI HC1, wherein the nHC-HA/PTX3 complexes reduce the expression of IL-12p40 in LPS-stimulated macrophages, wherein the level of IL-12p40 expressed by LPS-stimulated macrophages is lower when the LPS-stimulated macrophages are contacted the nHC-HA/PTX3 complexes compared to the level of IL-12p40 expressed by LPS-stimulated macrophages in the absence of the nHC-HA/PTX3 complexes. In some embodiments, the level of IL-12p40 mRNA is reduced. In some embodiments, the level of IL-12p40 protein is reduced.

[0014] Described herein, in certain embodiments, are methods for reducing the level of IL-12p40 expressed by LPS-stimulated macrophages comprising, contacting an LPS-stimulated macrophages with an rcHC-HA/PTX3 or isolated nHC-HA/PTX3 complex. In some embodiments, the level of IL-12p40 mRNA is reduced. In some embodiments, the level of IL-12p40 protein is reduced.

[0015] Described herein, in certain embodiments, are reconstituted HC-HA/PTX3 (rcHC-HA/PTX3) complexes comprising high molecular weight hyaluronan (HMW HA), PTX3, and IαI HC1, wherein the rcHC-HA/PTX3 complexes reduce the expression of IL-12p70 protein in LPS-stimulated macrophages, wherein the amount of IL-12p70 protein expressed by LPS-stimulated macrophages is lower when the LPS-stimulated macrophages are contacted the rcHC-HA/PTX3 complexes compared to the amount of IL-12p70 protein expressed by LPS-stimulated macrophages in the absence of the rcHC-HA/PTX3 complexes.

[0016] Described herein, in certain embodiments, are native HC-HA/PTX3 (nHC-HA/PTX3) complexes comprising high molecular weight hyaluronan (HMW HA), PTX3, and IαI HC1, wherein the nHC-HA/PTX3 complexes reduces the expression of IL-12p70 protein in LPS-stimulated macrophages, wherein the amount of IL-12p70 protein expressed by LPS-stimulated macrophages is lower when the LPS-stimulated macrophages are contacted the nHC-HA/PTX3 complexes compared to the amount of IL-12p70 protein expressed by LPS-stimulated macrophages in the absence of the nHC-HA/PTX3 complexes.

[0017] Described herein, in certain embodiments, are methods for reducing the level of IL-12p70 protein expressed by LPS-stimulated macrophages comprising, contacting an LPS-stimulated macrophages with an rcHC-HA/PTX3 or isolated nHC-HA/PTX3 complex.

[0018] Described herein, in certain embodiments, are reconstituted HC-HA/PTX3 (rcHC-HA/PTX3) complexes comprising high molecular weight hyaluronan (HMW HA), PTX3, and IαI HC1, wherein the rcHC-HA/PTX3 complexes reduce the expression of IL-23 in LPS-stimulated macrophages, wherein the level of IL-23 expressed by LPS-stimulated macrophages is lower when the LPS-stimulated macrophages are contacted the rcHC-HA/PTX3 complexes compared to the

level of IL-23 expressed by LPS-stimulated macrophages in the absence of the rcHC-HA/PTX3 complexes. In some embodiments, the level of IL-23 mRNA is reduced. In some embodiments, the level of IL-23 protein is reduced.

[0019] Described herein, in certain embodiments, are native HC-HA/PTX3 (rcHC-HA/PTX3) complexes comprising high molecular weight hyaluronan (HMW HA), PTX3, and IαI HC1, wherein the complexes reduces the expression of IL-23 in LPS-stimulated macrophages, wherein the level of IL-23 expressed by LPS-stimulated macrophages is lower when the LPS-stimulated macrophages are contacted the nHC-HA/PTX3 complexes compared to the level of IL-23 expressed by LPS-stimulated macrophages in the absence of the nHC-HA/PTX3 complex. In some embodiments, the level of IL-23 mRNA is reduced. In some embodiments, the level of IL-23 protein is reduced.

[0020] Described herein, in certain embodiments, are methods for reducing the level of IL-23 expressed by LPS-stimulated macrophages comprising, contacting an LPS-stimulated macrophages with an rcHC-HA/PTX3 or isolated nHC-HA/PTX3 complex. In some embodiments, the level of IL-23 mRNA is reduced. In some embodiments, the level of IL-23 protein is reduced.

[0021] Described herein, in certain embodiments, are reconstituted HC-HA/PTX3 (rcHC-HA/PTX3) complexes comprising high molecular weight hyaluronan (HMW HA), PTX3, and IαI HC1, wherein the rcHC-HA/PTX3 complexes increase the expression of IL-10 in LPS-stimulated macrophages, wherein the level of IL-10 expressed by LPS-stimulated macrophages is higher when the LPS-stimulated macrophages are contacted the rcHC-HA/PTX3 complexes compared to the level of IL-10 expressed by LPS-stimulated macrophages in the absence of the rcHC-HA/PTX3 complexes. In some embodiments, the level of IL-10 mRNA is increased. In some embodiments, the level of IL-10 protein is increased.

[0022] Described herein, in certain embodiments, are native HC-HA/PTX3 (nHC-HA/PTX3) complexes comprising high molecular weight hyaluronan (HMW HA), PTX3, and IαI HC1, wherein the complexes increase the expression of IL-10 in LPS/IFN γ -stimulated macrophages, wherein the amount of IL-10 expressed by LPS/IFN γ -stimulated macrophages is higher when the LPS-stimulated macrophages are contacted the nHC-HA/PTX3 complexes compared to the amount of IL-10 expressed by LPS/IFN γ -stimulated macrophages in the absence of the nHC-HA/PTX3 complexes. In some embodiments, the level of IL-10 mRNA is increased. In some embodiments, the level of IL-10 protein is increased.

[0023] Described herein, in certain embodiments, are methods for increasing the level of IL-10 expressed by LPS-stimulated macrophages comprising, contacting an LPS-stimulated macrophages

with an rcHC-HA/PTX3 or isolated nHC-HA/PTX3 complex. In some embodiments, the level of IL-10 mRNA is increased. In some embodiments, the level of IL-10 protein is increased.

[0024] Described herein, in certain embodiments, are reconstituted HC-HA/PTX3 (rcHC-HA/PTX3) complexes comprising high molecular weight hyaluronan (HMW HA), PTX3, and IαI HC1, wherein the rcHC-HA/PTX3 complexes promote apoptosis of LPS-stimulated neutrophils but do not promote apoptosis in resting neutrophils. In some embodiments, a reconstituted HC-HA/PTX3 (rcHC-HA/PTX3) complex, comprising high molecular weight hyaluronan (HMW HA), PTX3, and IαI HC1, promotes apoptosis of LPS-stimulated neutrophils wherein the number of LPS-stimulated neutrophils that are apoptotic in a sample of LPS-stimulated neutrophils is higher when the sample is contacted the rcHC-HA/PTX3 complex compared to the number of LPS-stimulated neutrophils that are apoptotic in the sample in the absence of the rcHC-HA/PTX3 complex.

[0025] Described herein, in certain embodiments, are native HC-HA/PTX3 (nHC-HA/PTX3) complex comprising high molecular weight hyaluronan (HMW HA), PTX3, and IαI HC1, wherein the nHC-HA/PTX3 complexes promote apoptosis of LPS-stimulated neutrophils but do not promote apoptosis in resting neutrophils. In some embodiments, a nHC-HA/PTX3 complex, comprising high molecular weight hyaluronan (HMW HA), PTX3, and IαI HC1, promotes apoptosis of LPS-stimulated neutrophils wherein the number of LPS-stimulated neutrophils that are apoptotic in a sample of LPS-stimulated neutrophils is higher when the sample is contacted the nHC-HA/PTX3 complex compared to the number of LPS-stimulated neutrophils that are apoptotic in the sample in the absence of the nHC-HA/PTX3 complex.

[0026] Described herein, in certain embodiments, are methods for inducing apoptosis of LPS-stimulated neutrophils comprising, contacting an LPS-stimulated neutrophil with an rcHC-HA/PTX3 or isolated nHC-HA/PTX3 complex.

[0027] Described herein, in certain embodiments, are reconstituted HC-HA/PTX3 (rcHC-HA/PTX3) complexes comprising high molecular weight hyaluronan (HMW HA), PTX3, and IαI HC1, wherein the rcHC-HA/PTX3 complexes promote phagocytosis of apoptotic neutrophils, wherein the number of apoptotic neutrophils that are phagocytosed by LPS-stimulated macrophages in a sample of apoptotic neutrophils and LPS-stimulated macrophages is higher when the sample is contacted with the rcHC-HA/PTX3 complexes compared to the number of neutrophils that are phagocytosed by LPS-stimulated macrophages in the sample in the absence of the rcHC-HA/PTX3 complexes. Described herein, in certain embodiments, are reconstituted HC-HA/PTX3 (rcHC-HA/PTX3) complexes comprising high molecular weight hyaluronan (HMW HA), PTX3, and IαI HC1, wherein the complexes promote phagocytosis of apoptotic neutrophils,

wherein the number of apoptotic neutrophils that are phagocytosed by resting macrophages in a sample of apoptotic neutrophils and resting macrophages is higher when the sample is contacted with the rcHC-HA/PTX3 complexes compared to the number of neutrophils that are phagocytosed by resting macrophages in the sample in the absence of the rcHC-HA/PTX3 complexes.

[0028] Described herein, in certain embodiments, are native HC-HA/PTX3 (nHC-HA/PTX3) complexes comprising high molecular weight hyaluronan (HMW HA), PTX3, and IαI HC1, wherein the nHC-HA/PTX3 complexes promotes phagocytosis of apoptotic neutrophils, wherein the number of apoptotic neutrophils that are phagocytosed by LPS-stimulated macrophages in a sample of apoptotic neutrophils and LPS-stimulated macrophages is higher when the sample is contacted with the nHC-HA/PTX3 complexes compared to the number of neutrophils that are phagocytosed by LPS-stimulated macrophages in the sample in the absence of the nHC-HA/PTX3 complexes. Described herein, in certain embodiments, are native HC-HA/PTX3 (nHC-HA/PTX3) complexes comprising high molecular weight hyaluronan (HMW HA), PTX3, and IαI HC1, wherein the nHC-HA/PTX3 complexes promotes phagocytosis of apoptotic neutrophils, wherein the number of apoptotic neutrophils that are phagocytosed by resting macrophages in a sample of apoptotic neutrophils and LPS-stimulated macrophages is higher when the sample is contacted with the nHC-HA/PTX3 complexes compared to the number of neutrophils that are phagocytosed by resting macrophages in the sample in the absence of the nHC-HA/PTX3 complexes.

[0029] Described herein, in certain embodiments, are methods inducing phagocytosis of apoptotic neutrophils comprising, contacting a sample containing apoptotic neutrophils and LPS-stimulated or resting macrophages with an rcHC-HA/PTX3 or isolated nHC-HA/PTX3 complex.

[0030] Described herein, in certain embodiments, are reconstituted HC-HA/PTX3 (rcHC-HA/PTX3) complexes comprising high molecular weight hyaluronan (HMW HA), PTX3, and IαI HC1, wherein the rcHC-HA/PTX3 complexes promote attachment of LPS-stimulated macrophages to at least the same level as a native HC-HA/PTX3 (nHC-HA/PTX3) complex isolated from human umbilical cord, human amniotic membrane, or a combination of nHC-HA/PTX3 complexes from both human umbilical cord and human amniotic membrane, wherein attachment comprises the contacting LPS-stimulated macrophages to the rcHC-HA/PTX3 or nHC-HA/PTX3 complexes immobilized to a solid support. In some embodiments, the nHC-HA/PTX3 is isolated from human umbilical cord. In some embodiments, the nHC-HA/PTX3 is isolated from human amniotic membrane. In some embodiments, the nHC-HA/PTX3 is isolated from a combination of nHC-HA/PTX3 complexes from both human umbilical cord and human amniotic membrane.

[0031] In some embodiments, the weight average molecular weight of the HMW HA of the nHC-HA/PTX3 or rcHC-HA/PTX3 complex is between about 500 kDa and about 10,000 kDa, between

about 800 kDa and about 8,500 kDa, between about 1100 kDa and about 5,000 kDa, or between about 1400 kDa and about 3,500 kDa. In some embodiments, contains, the weight average molecular weight of the HMW HA of the nHC-HA/PTX3 or rcHC-HA/PTX3 complex is about 3,000 kDa.

[0032] In some embodiments, HC1 of the nHC-HA/PTX3 or rcHC-HA/PTX3 complex is covalently linked to HA.

[0033] In some embodiments, the PTX3 protein of the rcHC-HA/PTX3 complex is a recombinant protein. In some embodiments, PTX3 of the rcHC-HA/PTX3 complex comprises a polypeptide having the sequence set forth in SEQ ID NO: 33 or a polypeptide having at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% sequence amino acid identity to the polypeptide having the sequence set forth in SEQ ID NO: 33. In some embodiments, the PTX3 protein used in the methods is a multimeric protein. In some embodiments, the PTX3 protein used in the methods is a homomultimer. In some embodiments, the PTX3 homomultimer is a dimer, trimer, tetramer, pentamer, hexamer, or octamer. In some embodiments, the PTX3 homomultimer is a trimer, tetramer, or octamer. In some embodiments, the PTX3 homomultimer is an octamer.

[0034] In some embodiments, the IaI HC1 of the rcHC-HA/PTX3 complex comprises a polypeptide having the sequence set forth in SEQ ID NO: 47 or a polypeptide having at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% sequence amino acid identity to the polypeptide having the sequence set forth in SEQ ID NO: 47. In some embodiments, the IaI HC1 of the rcHC-HA/PTX3 complex is a recombinant protein.

[0035] In some embodiments, the rcHC-HA/PTX3 complex comprises TSG-6. In some embodiments, the TSG-6 protein is a recombinant protein. In some embodiments, the TSG-6 protein comprises a polypeptide having the sequence set forth in SEQ ID NO: 2 or a polypeptide having at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% sequence amino acid identity to the polypeptide having the sequence set forth in SEQ ID NO: 2.

[0036] In some embodiments, the PTX3, IaI HC1 or TSG-6 polypeptides of the rcHC-HA/PTX3 complex comprise an affinity tag. In some embodiments, the affinity tag is selected from among is selected from among a hemagglutinin tag, a poly-histidine tag, a myc tag, a FLAG tag, a glutathione-S-transferase (GST) tag.

[0037] Described herein, in certain embodiments, is a pharmaceutical composition, comprising an nHC-HA/PTX3 or rcHC-HA/PTX3 complex described herein or produced by the methods provided herein. In some embodiments, pharmaceutical composition further comprises a pharmaceutically acceptable carrier or excipient. In some embodiments, pharmaceutical composition is in the form of a solution, suspension, powder, ointment, tablet, capsule, or an

aerosol. In some embodiments, pharmaceutical composition is in the form of a solid, a cross-linked gel, or a liposome. In some embodiments, pharmaceutical composition is in the form of a cross-linked hyaluronan hydrogel. In some embodiments, pharmaceutical composition comprises a natural polymer. In some embodiments, natural polymer comprises fibronectin, collagen, laminin, keratin, fibrin, fibrinogen, hyaluronic acid, heparan sulfate, chondroitin sulfate, or combinations thereof. In some embodiments, pharmaceutical composition further comprises an anti-inflammatory agent, an anti-scarring agent, an anti-neoplastic agent, a chemotherapeutic agent, an immunosuppressive agent, a cytotoxic agent, an antimicrobial agent, or a combination thereof.

[0038] Described herein, in certain embodiments, is a use of an nHC-HA/PTX3 or rcHC-HA/PTX3 complex described herein or produced by the methods provided herein for the production of a medicament.

[0039] Described herein, in certain embodiments, is a combination comprising: (a) an nHC-HA/PTX3 or rcHC-HA/PTX3 complex described herein or produced by the methods provided herein; and (b) an anti-inflammatory agent, an anti-scarring agent, an anti-neoplastic agent, a chemotherapeutic agent, an immunosuppressive agent, a cytotoxic agent, an antimicrobial agent or a combination thereof.

[0040] Described herein, in certain embodiments, are methods of treatment, comprising administering a pharmaceutical composition comprising an nHC-HA/PTX3 or rcHC-HA/PTX3 complex described herein or produced by the methods provided herein.

[0041] Described herein, in certain embodiments, are methods of preventing or reversing scar formation or fibrosis in a tissue, comprising administering to the subject in need thereof an effective amount of an nHC-HA/PTX3 or rcHC-HA/PTX3 complex described herein or produced by the methods provided herein. In some embodiments, the method comprises contacting the tissue with an effective amount of the nHC-HA/PTX3 or rcHC-HA/PTX3 complex. In some embodiments, the scar is a dermatitis scar, a keloid scar, contracture scar, a hypertrophic scar, or a scar resulting from acne. Described herein, in certain embodiments, is a use of an nHC-HA/PTX3 or rcHC-HA/PTX3 complex described herein or produced by the methods provided herein to reduce or prevent scarring. In some embodiments, administration of an nHC-HA/PTX3 or rcHC-HA/PTX3 complex described herein or produced by the methods provided herein reduces or prevents scarring or fibrosis by decreasing or inhibiting TGF- β signaling in the tissue.

[0042] Described herein, in certain embodiments, are methods of preventing or reducing inflammation in a subject in need thereof, comprising administering to the subject an effective amount of an nHC-HA/PTX3 or rcHC-HA/PTX3 complex described herein or produced by the methods provided herein. In some embodiments, the method comprises contacting inflamed tissues

with the nHC-HA/PTX3 or rcHC-HA/PTX3 complex. In some embodiments, the inflammation is acute inflammation or chronic inflammation. In some embodiments, the subject has an inflammatory disorder. In some embodiments, the inflammatory disorder is a macrophage-mediated inflammatory disorder, a Th-17-mediated immune disorder or a T-cell mediated inflammatory disorder. In some embodiments, the subject has an autoimmune disorder, an allergy, a leukocyte defect, an infection, graft versus host disease, tissue transplant rejection, or combinations thereof. In some embodiments, the inflammatory disorder is rheumatoid arthritis. In some embodiments, the inflammatory disorder is an inflammatory disorder of the eye. In some embodiments, the inflammatory disorder is conjunctivitis, keratitis, blepharitis, blepharoconjunctivitis, scleritis, episcleritis, uveitis, retinitis, or choroiditis. In some embodiments, the acute inflammation is caused by myocardial infarction, stroke, endotoxin shock or sepsis. In some embodiments, the subject has atherosclerosis. In some embodiments, the subject has cancer. In some embodiments, the subject has inflammation of a solid tumor. In some embodiments, the subject is administered the nHC-HA/PTX3 or rcHC-HA/PTX3 complex in combination with an additional anti-inflammatory agent. In some embodiments, the additional anti-inflammatory agent is selected from among an anti-TGF- β antibody, an anti-TGF- β receptor blocking antibody, an anti-TNF antibody, an anti-TNF receptor blocking antibody, an anti-IL1 β antibody, an anti-IL1 β receptor blocking antibody, an anti-IL-2 antibody, an anti-IL-2 receptor blocking antibody, an anti-IL-6 antibody, an anti-IL-6 receptor blocking antibody, an anti IL-12 antibody, an anti IL-12 receptor blocking antibody, an anti-IL-17 antibody, anti-IL-17 receptor blocking antibody, an anti-IL-23 antibody, or an anti-IL-23 receptor blocking antibody. In some embodiments, the Type 1 interferon is IFN- α or IFN- β . Described herein, in certain embodiments, is a use of an nHC-HA/PTX3 or rcHC-HA/PTX3 complex described herein or produced by the methods provided herein to reduce or prevent inflammation.

[0043] Described herein, in certain embodiments, are methods of treating a skin wound or ulcer in a subject in need thereof, comprising administering to the subject an effective amount of an nHC-HA/PTX3 or rcHC-HA/PTX3 complex described herein or produced by the methods provided herein. In some embodiments, the methods comprise contacting the skin wound or ulcer with an effective amount of the nHC-HA/PTX3 or rcHC-HA/PTX3 complex. Described herein, in certain embodiments, is a use of an nHC-HA/PTX3 or rcHC-HA/PTX3 complex described herein or produced by the methods provided herein to treat a skin wound or ulcer. In some embodiments, the skin wound or ulcer is a non-healing ulcer.

[0044] Described herein, in certain embodiments, are methods of promoting or inducing bone formation in a subject in need thereof, comprising administering to the administering to the subject an effective amount of the rcHC-HA/PTX3 complex or nHC-HA/PTX3 complex described herein

or produced by the methods provided herein. In some embodiments, the subject has arthritis, osteoporosis, alveolar bone degradation, Paget's disease, or a bone tumor. Described herein, in certain embodiments, is a use of an nHC-HA/PTX3 or rcHC-HA/PTX3 complex described herein or produced by the methods provided herein to promote or induce bone formation in a subject.

[0045] Described herein, in certain embodiments, are methods of preventing or reducing abnormal angiogenesis in a subject in need thereof, comprising administering to the subject an effective amount of an nHC-HA/PTX3 or rcHC-HA/PTX3 complex described herein or produced by the methods provided herein. In some embodiments, the subject has wet age-related macular degeneration (wARMD) or diabetic proliferative retinopathy. In some embodiments, the subject has cancer. In some embodiments, the subject has a solid tumor. In some embodiments, the subject is administered the nHC-HA/PTX3 or rcHC-HA/PTX3 complex in combination with an anti-cancer therapy. In some embodiments, the anticancer therapy comprises administration of an anti-neoplastic agent, a cytotoxic agent, an anti-angiogenic agent, a chemotherapeutic agent, or radiation therapy. In some embodiments, the anticancer therapy is administered sequentially, concurrently or intermittently with the nHC-HA/PTX3 or rcHC-HA/PTX3 complex. Described herein, in certain embodiments, is a use of an nHC-HA/PTX3 or rcHC-HA/PTX3 complex described herein or produced by the methods provided herein to reduce or prevent angiogenesis.

[0046] Described herein, in certain embodiments, are methods of preventing transplant rejection in a transplant recipient, comprising administering to the transplant recipient an effective amount of an nHC-HA/PTX3 or rcHC-HA/PTX3 complex described herein or produced by the methods provided herein. In some embodiments, the method comprise contacting the transplant with the nHC-HA/PTX3 or rcHC-HA/PTX3 complex. In some embodiments, the nHC-HA/PTX3 or rcHC-HA/PTX3 complex is administered before a transplantation procedure, after a transplantation procedure, or during a transplantation procedure. In some embodiments, the nHC-HA/PTX3 or rcHC-HA/PTX3 complex is administered in combination with an immunosuppressive agent. Described herein, in certain embodiments, is a use of an nHC-HA/PTX3 or rcHC-HA/PTX3 complex described herein or produced by the methods provided herein to prevent transplant rejection in a transplant recipient. In some embodiments, the transplant is a corneal transplant.

[0047] Described herein, in certain embodiments, are methods of inducing stem cell expansion in a subject in need thereof, comprising administering to the subject an effective amount of an nHC-HA/PTX3 or rcHC-HA/PTX3 complex described herein or produced by the methods provided herein. In some embodiments, the method comprise contacting the stem cell with the nHC-HA/PTX3 or rcHC-HA/PTX3 complex. Described herein, in certain embodiments, is a use of an nHC-HA/PTX3 or rcHC-HA/PTX3 complex described herein or produced by the methods

provided herein to induce stem cell expansion. In some embodiments, administration of an nHC-HA/PTX3 or rcHC-HA/PTX3 complex described herein or produced by the methods provided herein induces stem cell expansion by suppression of TGF- β signaling and/or upregulation of BMP signaling pathways. In some embodiments, administration of an nHC-HA/PTX3 or rcHC-HA/PTX3 complex described herein or produced by the methods provided herein induces stem cell expansion by reprogramming differentiated cells into stem cells (or induced progenitor cells, iPSCs).

[0048] Described herein, in certain embodiments, is a use of an nHC-HA/PTX3 or rcHC-HA/PTX3 complex described herein or produced by the methods provided herein for cell therapy.

[0049] Described herein, in certain embodiments, are methods of cell therapy in a subject in need thereof, comprising administering to the subject a composition comprising an effective amount of an nHC-HA/PTX3 or rcHC-HA/PTX3 complex described herein or produced by the methods provided herein in combination with a therapeutic cell. In some embodiments, the therapeutic cell and the nHC-HA/PTX3 or rcHC-HA/PTX3 complex are administered locally to a damaged tissue. In some embodiments, the therapeutic cell and the nHC-HA/PTX3 or rcHC-HA/PTX3 complex are administered systemically. In some embodiments, the therapeutic cell is a stem cell. In some embodiments, the therapeutic is a stem cell. In some embodiments, the cell therapy comprises administration of a stem cell. In some embodiments, the stem cell is a mesenchymal stem cell. In some embodiments, the stem cells are induced progenitor stem cells. In some embodiments, the cell therapy comprises administration of differentiated cells. In some embodiments, therapeutic cell is an insulin producing cell. In some embodiments, the insulin producing cell is an islet cell. In some embodiments, the subject has diabetes mellitus type 1.

[0050] Described herein, in certain embodiments, are methods of cell therapy in a subject in need thereof, comprising administering to the administering to the subject an effective amount of an nHC-HA/PTX3 or rcHC-HA/PTX3 complex described herein or produced by the methods provided herein in combination with a cell contained in a cell delivery device. In some embodiments, the cell is contained in a microcapsule. In some embodiments, the nHC-HA/PTX3 or rcHC-HA/PTX3 complex is attached to the microcapsule. In some embodiments, the microcapsule is administered locally to a damaged tissue. In some embodiments, the therapeutic cell and the nHC-HA/PTX3 or rcHC-HA/PTX3 complex are administered systemically. In some embodiments, the therapeutic cell is a stem cell. In some embodiments, the therapeutic is a stem cell. In some embodiments, the cell therapy comprises administration of a stem cell. In some embodiments, the stem cell is a mesenchymal stem cell. In some embodiments, the stem cells are induced progenitor stem cells. In some embodiments, the cell therapy comprises administration of differentiated cells.

In some embodiments, therapeutic cell is an insulin producing cell. In some embodiments, the insulin producing cell is an islet cell. In some embodiments, the subject has diabetes mellitus type 1.

[0051] Described herein, in certain embodiments, are methods of preventing or reducing pain in a subject in need thereof, comprising administering to the subject an effective amount of an nHC-HA/PTX3 or rcHC-HA/PTX3 complex described herein or produced by the methods provided herein, wherein the pain is caused by chemical burn, severe bacterial keratitis, Stevens-Johnson syndrome, toxic epidermal necrolysis, irradiation of ocular tumors. Described herein, in certain embodiments, is a use of an nHC-HA/PTX3 or rcHC-HA/PTX3 complex described herein or produced by the methods provided herein to reduce pain in a subject, wherein the pain is caused by chemical burn, severe bacterial keratitis, Stevens-Johnson syndrome, toxic epidermal necrolysis, irradiation of ocular tumors.

[0052] Described herein, in certain embodiments, are methods of inducing or promoting tissue regeneration in a subject in need thereof, comprising administering to the subject an effective amount of an nHC-HA/PTX3 or rcHC-HA/PTX3 complex described herein or produced by the methods provided herein. In some embodiments, the methods comprise contacting damaged tissue of the subject with an effective amount of the nHC-HA/PTX3 or rcHC-HA/PTX3 complex. In some embodiments, the tissue is bone or gum, corneal tissue, or conjunctival tissue. In some embodiments, the nHC-HA/PTX3 or rcHC-HA/PTX3 complex is administered in combination with a therapeutic cell, a plurality of therapeutic cells or a tissue transplant. In some embodiments, the tissue transplant is an allograft or an autograft. In some embodiments, the nHC-HA/PTX3 or rcHC-HA/PTX3 complex is administered in combination with a tissue based therapy. Described herein, in certain embodiments, is a use of an nHC-HA/PTX3 or rcHC-HA/PTX3 complex described herein or produced by the methods provided herein to induce or promote tissue regeneration in a subject.

[0053] Described herein, in certain embodiments, are methods of treating fibrosis in a subject in need thereof, comprising administering to the subject an effective amount of an nHC-HA/PTX3 or rcHC-HA/PTX3 complex described herein or produced by the methods provided herein. In some embodiments, the treatment inhibits or prevents scarring. Described herein, in certain embodiments, is a use of an nHC-HA/PTX3 or rcHC-HA/PTX3 complex described herein or produced by the methods provided herein to treat fibrosis in a subject.

[0054] Described herein, in certain embodiments, are methods of treating obesity or insulin resistance in a subject in need thereof, comprising administering to the subject an effective amount of an nHC-HA/PTX3 or rcHC-HA/PTX3 complex described herein or produced by the methods

provided herein. In some embodiments, the treatment inhibits or decreases the amount of M1 adipose tissue macrophages in the subject. Described herein, in certain embodiments, are methods of inhibiting or decreasing the amount of M1 adipose tissue macrophages in a subject in need thereof, administering to the subject an effective amount of an nHC-HA/PTX3 or rcHC-HA/PTX3 complex described herein or produced by the methods provided herein. In some embodiments, the subject has been diagnosed with obesity or insulin-resistance. Described herein, in certain embodiments, is a use of an nHC-HA/PTX3 or rcHC-HA/PTX3 complex described herein or produced by the methods provided herein to treat obesity or insulin resistance in a subject.

[0055] Described herein, in certain embodiments, are methods of treating conjunctivochalasis in a subject in need thereof, comprising administering to the subject an effective amount of an nHC-HA/PTX3 or rcHC-HA/PTX3 complex described herein or produced by the methods provided herein. In some embodiments, the methods comprise contacting the conjunctiva of the subject with an effective amount of the nHC-HA/PTX3 or rcHC-HA/PTX3 complex. Described herein, in certain embodiments, is a use of an nHC-HA/PTX3 or rcHC-HA/PTX3 complex described herein or produced by the methods provided herein to treat conjunctivochalasis in a subject.

[0056] Described herein, in certain embodiments, are cell cultures comprising a substrate suitable for culturing a cell and a rcHA/PTX3 complex or the nHC-HA/PTX3 complex described herein or produced by the methods provided herein. In some embodiments, the rcHA/PTX3 complex or the nHC-HA/PTX3 complex immobilized to the substrate.

[0057] Described herein, in certain embodiments, are methods of treatment, wherein the subject is administered an effective amount of an nHC-HA/PTX3 or rcHC-HA/PTX3 complex described herein or produced by the methods provided herein in combination with an additional therapeutic agent. In some embodiments, the additional therapeutic agent is selected from an interferon, an anti-tumor necrosis agent, an interleukin-1 (IL-1) receptor antagonist, an interleukin-2 (IL-2) receptor antagonist, an interleukin-6 (IL-6) receptor antagonist, an interleukin-12 (IL-12) receptor antagonist, an interleukin-17 (IL-17) receptor antagonist, an interleukin-23 (IL-23) receptor antagonist, a cytotoxic agent, an antimicrobial agent, an interleukin, an immunomodulatory agent, an antibiotic, a T-cell co-stimulatory blocker, a disorder-modifying anti-rheumatic agent, an immunosuppressive agent, an anti-lymphocyte antibody, an anti-angiogenesis agent, a chemotherapeutic agent, an anti-neoplastic agent, an anti-metabolite, an Akt inhibitor, an IGF-1 inhibitor, an angiotensin II antagonist, a cyclooxygenase inhibitor, an heparanase inhibitor, a lymphokine inhibitor, a cytokine inhibitor, an IKK inhibitor, a P38MAPK inhibitor, an anti-apoptotic pathway inhibitor, an apoptotic pathway agonist, a PPAR agonist, an inhibitors of Ras, a telomerase inhibitor, a protease inhibitor, a metalloproteinase inhibitor, an aminopeptidase

inhibitor, a SHIP activator and combinations thereof. In some embodiments, the antimicrobial agent is an antiviral, antibacterial or antifungal agent.

[0058] Described herein, in certain embodiments, are methods of treatment, wherein the subject is administered an effective amount of an nHC-HA/PTX3 or rcHC-HA/PTX3 complex described herein or produced by the methods provided herein and the subject is a mammal. In some embodiments, the mammal is a human.

[0059] Described herein, in certain embodiments, are methods of treatment, wherein the nHC-HA/PTX3 or rcHC-HA/PTX3 complex is attached to a solid surface. In some embodiments, the solid surface is a surface or a portion thereof of a nanoparticle, a bead, a microcapsule or implantable medical device.

[0060] Described herein, in certain embodiments, is are medical devices, comprising a substrate coated with an nHC-HA/PTX3 or rcHC-HA/PTX3 complex described herein or produced by the methods provided herein. In some embodiments, the substrate comprises at least one of a stent, a joint, a screw, a rod, a pin, a plate, a staple, a shunt, a clamp, a clip, a suture, a suture anchor, an electrode, a catheter, a lead, a graft, a dressing, a pacemaker, a pacemaker housing, a cardioverter, a cardioverter housing, a defibrillator, a defibrillator housing, a prostheses, an ear drainage tube, an ophthalmic implant, an orthopedic device, a vertebral disk, a bone substitute, an anastomotic device, a perivascular wrap, a colostomy bag attachment device, a hemostatic barrier, a vascular implant, a vascular support, a tissue adhesive, a tissue sealant, a tissue scaffold, and an intraluminal device.

[0061] Described herein, in certain embodiments, is a device comprising an nHC-HA/PTX3 or rcHC-HA/PTX3 complex described herein or produced by the methods provided herein immobilized on a surface. In some embodiments, the surface is a polystyrene, polyethylene, silica, metallic or polymeric surface. In some embodiments, the nHC-HA/PTX3 or rcHC-HA/PTX3 complex is attached to a microcapsule. In some embodiments, the nHC-HA/PTX3 or rcHC-HA/PTX3 complex is attached to a nanoparticle. In some embodiments, the nHC-HA/PTX3 or rcHC-HA/PTX3 complex is attached to a bead, a chip, a glass slide, or a filter. In some embodiments, the nHC-HA/PTX3 or rcHC-HA/PTX3 complex is attached to a contact lens. In some embodiments, the nHC-HA/PTX3 or rcHC-HA/PTX3 complex is attached to a surgical implant or prosthesis. In some embodiments, the implant or prosthesis is an artificial joint, a bone implant, a suture, or a stent. In some embodiments, the artificial joint is an artificial hip joint, an artificial knee, an artificial glenohumeral joint, or an artificial knee. In some embodiments, the stent is a coronary stent, a ureteral stent, a urethral stent, a prostatic stent, esophageal stent, or a bone stent.

[0062] Described herein, in certain embodiments, is are medical devices comprising: a structure adapted for implantation into a patient, wherein a surface or a portion of a surface of the structure is attached to comprising an nHC-HA/PTX3 or rcHC-HA/PTX3 complex described herein or produced by the methods provided herein. In some embodiments, attachment comprises covalent or non-covalent attachment of the nHC-HA/PTX3 or rcHC-HA/PTX3 complex to the surface or portion of a surface of the structure. In some embodiments, attachment comprises coating the surface or a portion of a surface of the structure with a composition containing the nHC-HA/PTX3 or rcHC-HA/PTX3 complex. In some embodiments, the structure is a vascular stent, an artificial joint, a suture, or a microcapsule. In some embodiments, the nHC-HA/PTX3 or rcHC-HA/PTX3 complex inhibits the formation of a bacterial biofilm. In some embodiments, the microcapsule contains a therapeutic cell. In some embodiments, the therapeutic cell is a stem cell.

[0063] Described herein, in certain embodiments, are methods for modulating macrophage activity comprising contacting a macrophage with an nHC-HA/PTX3 or rcHC-HA/PTX3 complex described herein or produced by the methods provided herein in an amount sufficient to reduce or inhibit the expression of IL-12 or IL-23 but also promote the expression of IL-10 in polarizing macrophages from M1 to M2 phenotype. In some embodiments, the macrophage has been stimulated with a pro-inflammatory mediator. In some embodiments, the pro-inflammatory mediator is lipopolysaccharide (LPS), tumor necrosis factor α (TNF- α), interferon-gamma (IFN γ) or a combination thereof. In some embodiments, the nHC-HA/PTX3 or rcHC-HA/PTX3 complex contacts the macrophage in vivo in a subject. In some embodiments, the subject is a mammal. In some embodiments, the mammal is a human. In some embodiments, the method is performed in vitro. Described herein, in certain embodiments, are methods of treatment comprising administration of macrophages that have been modulated by the method for modulating macrophage activity provided herein.

[0064] Described herein, in certain embodiments, is a kit, comprising an nHC-HA/PTX3 or rcHC-HA/PTX3 complex described herein or produced by the methods provided herein, a device for administration of the composition and, optionally, instructions for administration.

[0065] Described herein, in certain embodiments, is a combination or mixture comprising: (a) a complex of PTX3 pre-bound to HMW HA (PTX3/HA); (b) an inter- α -inhibitor (I α I) protein comprising heavy chain 1 (HC1); and (c) TSG-6.

[0066] Described herein, in certain embodiments, is a combination or a mixture comprising: (a) a complex of TSG-6 pre-bound to HC-HA; and (b) PTX3.

[0067] Described herein, in certain embodiments, are methods of producing a reconstituted HC-HA/PTX3 (rcHC-HA/PTX3) complex in vitro, comprising: (a) contacting (i) high molecular weight

hyaluronan (HMW HA) immobilized to a solid support, (ii) an inter- α -inhibitor (I α I) protein comprising heavy chain 1 (HC1) and (iii) TSG-6 to form an rcHC-HA complex pre-bound to TSG-6; and (b) contacting rcHC-HA complex pre-bound to TSG-6 with a pentraxin 3 (PTX3) protein to form an rcHC-HA/PTX3 complex.

[0068] Described herein, in certain embodiments, are methods of producing a reconstituted HC-HA/PTX3 (rcHC-HA/PTX3) complex *in vitro*, comprising contacting (i) high molecular weight hyaluronan (HMW HA) immobilized to a solid support, (ii) pentraxin 3 (PTX3) protein, (iii) inter- α -inhibitor (I α I) protein comprising heavy chain 1 (HC1) and (iv) Tumor necrosis factor α -stimulated gene 6 (TSG-6) to form an immobilized rcHC-HA/PTX3 complex.

[0069] Described herein, in certain embodiments, is a complex comprising immobilized HA bound to PTX3.

[0070] Described herein, in certain embodiments, are methods of producing a complex comprising immobilized HA bound to PTX3 *in vitro*, comprising contacting high molecular weight hyaluronan (HMW HA) with a PTX3 protein under conditions effective to form a complex of PTX3 and HMW HA (PTX3/HA), wherein the HMW HA is immobilized to a solid support. In some embodiments, the PTX3 protein is a native PTX3 protein isolated from a cell. In some embodiments, the cell is a mammalian cell. In some embodiments, the cell is a human cell. In some embodiments, the cell is an amniotic membrane cell. In some embodiments, the cell is an umbilical cord cell. In some embodiments, the cell is an amniotic membrane cell from an umbilical cord. In some embodiments, the amniotic membrane cell is an amniotic epithelial cell. In some embodiments, the amniotic membrane cell is an umbilical cord epithelial cell. In some embodiments, the amniotic membrane cell is an amniotic stromal cell. In some embodiments, the amniotic membrane cell is an umbilical cord stromal cell. In some embodiments, the PTX3 protein is a recombinant protein. In some embodiments, the PTX3 protein comprises a polypeptide having the sequence set forth in SEQ ID NO: 2 or a polypeptide having at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% sequence amino acid identity to the polypeptide having the sequence set forth in SEQ ID NO: 2. In some embodiments, the PTX3 protein used in the methods is a multimeric protein. In some embodiments, the PTX3 protein used in the methods is a homomultimer. In some embodiments, the PTX3 homomultimer is a dimer, trimer, tetramer, pentamer, hexamer, octamer. In some embodiments, the PTX3 homomultimer is a trimer, tetramer, or octamer. In some embodiments, the PTX3 homomultimer is an octamer. In some embodiments, the PTX3 comprises a modified multimerization domain or a heterogeneous multimerization domain. In some embodiments, the immobilizing HMW HA comprises non-covalent attachment to the solid support. In some embodiments, the immobilizing HMW HA comprises binding HMW HA

to an intermediary polypeptide. In some embodiments, the intermediary polypeptide is covalently attached to the solid support. In some embodiments, binding HMW HA to the intermediary polypeptide is non-covalent. In some embodiments, the intermediary polypeptide is an HA binding protein (HABP). In some embodiments, the intermediary polypeptide is an HABP selected from among HAPLN1, HAPLN2, HAPLN3, HAPLN4, aggrecan, versican, neurocan, brevican, phosphacan, TSG-6, CD44, stabilin-1, stabilin-2, or a portion thereof sufficient to bind HA. In some embodiments, the intermediary polypeptide is versican. In some embodiments, the intermediary polypeptide comprises a link module. In some embodiments, the intermediary polypeptide comprises a link module of HAPLN1, HAPLN2, HAPLN3, HAPLN4, aggrecan, versican, neurocan, brevican, phosphacan, TSG-6, CD44, stabilin-1, or stabilin-2. In some embodiments, the intermediary polypeptide comprises a link module of versican. In some embodiments, the intermediary polypeptide comprises a polypeptide set forth in any of SEQ ID NOS: 54-99. Described herein, in certain embodiments, is a PTX3/HA complex produced by the preceding method. Described herein, in certain embodiments, is a pharmaceutical composition, comprising the PTX3/HA complex produced by the preceding method. Described herein, in certain embodiments, is a use of the PTX3/HA complex for the production of a medicament. Described herein, in certain embodiments, are methods of treatment comprising administration of the PTX3/HA complex for the prevention or inhibition of scarring, inflammation, angiogenesis, cancer, diabetes, obesity, or fibrosis.

[0071] Described herein, in certain embodiments, are methods for inducing or maintaining pluripotency in a cell, comprising culturing the cell with an nHC-HA/PTX3 complex or rcHC-HA/PTX3 complex, thereby inducing or maintaining pluripotency in a cell. In some embodiments, the cell heterogeneously expresses a protein selected from among Sox2, myc, Oct4 and KLF4. In some embodiments, the cell heterogeneously expresses one, two, or three proteins selected from among Sox2, myc, Oct4 and KLF4. In some embodiments, the nHC-HA/PTX3 complex or rcHC-HA/PTX3 complex is immobilized. In some embodiments, the cell is an adult differentiated cell. In some embodiments, the cell is a fibroblast. In some embodiments, the cell is a human corneal fibroblast. In some embodiments, the cell is an embryonic stem cell, an adult stem cell, a fetal stem cell, or an induced pluripotent stem cell. In some embodiments, the cell is a limbal epithelial progenitor cell, a limbal stromal niche cell, an umbilical cord stem cell, an amniotic membrane stem cell or an adipose stem cell. In some embodiments, the nHC-HA/PTX3 complex is an amniotic membrane nHC-HA/PTX3 complex. In some embodiments, the nHC-HA/PTX3 is an umbilical cord nHC-HA/PTX3 complex. In some embodiments, the methods further comprise purifying the nHC-HA/PTX3 complex by performing ultracentrifugation on an amniotic membrane

extract. In some embodiments, the methods further comprise purifying the nHC-HA/PTX3 complex by performing ultracentrifugation on an amniotic membrane extract prepared in a PBS buffer to produce a nHC-HA/PTX3 extract (i.e. nHC-HA/PTX3 soluble). In some embodiments, the methods further comprise purifying the nHC-HA/PTX3 complex by performing ultracentrifugation on an amniotic membrane extract prepared in a GnHCl buffer to produce an nHC-HA/PTX3 extract (i.e. nHC-HA/PTX3 soluble). In some embodiments, the methods further comprise purifying the nHC-HA/PTX3 complex by performing ultracentrifugation on an umbilical cord extract. In some embodiments, the umbilical cord extract comprises umbilical cord amniotic membrane, umbilical cord stroma, Wharton's jelly, or any combination thereof. In some embodiments, the methods further comprise purifying the nHC-HA/PTX3 complex by performing ultracentrifugation on an umbilical cord extract prepared in a PBS buffer to produce a nHC-HA/PTX3 extract (i.e. nHC-HA/PTX3 soluble). In some embodiments, the methods further comprise purifying the nHC-HA/PTX3 complex by performing ultracentrifugation on an umbilical cord extract prepared in a GnHCl buffer to produce an nHC-HA/PTX3 extract (i.e. nHC-HA/PTX3 soluble). In some embodiments, the methods further comprise performing two, three or four rounds of ultracentrifugation. In some embodiments, the methods further comprise performing four rounds of ultracentrifugation. In some embodiments, the nHC-HA/PTX3 or rcHC-HA/PTX3 complex comprises PTX3. In some embodiments, the nHC-HA/PTX3 or rcHC-HA/PTX3 complex comprises a small leucine rich proteoglycan (SLRP). In some embodiments, the nHC-HA/PTX3 or rcHC-HA/PTX3 complex comprises PTX3 and a small leucine rich proteoglycan (SLRP). In some embodiments, the small leucine-rich proteoglycan is selected from among decorin, biglycan, fibromodulin, lumican, PRELP (proline arginine rich end leucine-rich protein), keratocan, osteoadherin, epipycan, and osteoglycin. In some embodiments, the small leucine-rich proteoglycan is covalently attached to a glycosaminoglycan. In some embodiments, the glycosaminoglycan is keratan sulfate.

[0072] It is to be understood that the foregoing general description and the following detailed description are exemplary and explanatory only and are not restrictive of any subject matter claimed.

BRIEF DESCRIPTION OF THE DRAWINGS

[0073] **FIG. 1** exemplifies purification of native HC-HA/PTX3 (nHC-HA/PTX3) complexes from amniotic membrane extract (AME) and analysis of protein composition and the size of HA. (A-B) Total protein and HA concentrations in fractions obtained by CsCl/4 M guanidine HCl ultracentrifugation. (C) HA stained by Stains-all dye in 0.5% agarose gel. (D-J) Analysis of proteins present in nHC-HA/PTX3 by Western blot using antibodies against HC1 (D and F), PTX3 (E and

G), HC2 (H), HC3 (I), bikunin (J), TSG-6 (K) or TSP-1 (L). NaOH or N = Treatment with 0.05 N NaOH at 25 °C for 1 h. HAase or H = Treatment with 20 units/ml HAase at 60 °C for 2 h (F-K). Bar graph of relative protein amount (M) determined by dot assays using antibodies against various antigens including IGFBP 1-3, PF4 or TIMP-1.

[0074] FIG. 2 exemplifies CD44 and TLR4 receptors mediate the attachment of LPS-Stimulated macrophages to immobilized nHC-HA/PTX3. (A) Cell attachment. RAW264.7 cells (100 μ l of 2.5×10^5 cells/ml) were seeded in immobilized HA (2 μ g/well) or nHC-HA/PTX3 (2 μ g/well) (n=3) and stimulated with LPS (1 μ g/ml). After incubation for 90 min, unattached cells were removed and attached cells were counted by the CyQuant assay. The scale bar represents 100 μ m. An asterisk (*) indicates p values < 0.05 (HA or nHC-HA/PTX3 vs. PBS control or nHC-HA/PTX3 vs. HA). (B) Cell viability. LPS-stimulated RAW264.7 cells were incubated on immobilized PBS control, HA, or nHC-HA/PTX3 for 24 h (n=3). The cell viability was measured by MTT assay. No significant differences (all p values > 0.05) in the cell viability among cells on these immobilized substrates were observed. (C) CD44 and TLR4 Receptors are responsible for attachment of LPS-stimulated macrophages to immobilized nHC-HA/PTX3. RAW264.7 cells (2.5×10^5 /ml) were pre-incubated with the blocking antibody against CD44, TLR2, TLR4, integrin α v, β 1, β 2, or β 3 or RGD peptides, along with the isotype control antibodies or a RGD control peptide, on ice for 30 min (n=3). After adding LPS (1 μ g/ml), cells were incubated for 90 min and the cell attachment assay was done the same as described in A. An asterisk (*) indicates p value < 0.05.

[0075] FIG. 3 exemplifies polarization of LPS-stimulated macrophages toward M2 phenotype by immobilized nHC-HA/PTX3. (A) Relative mRNA expression of M1 (TNF- α , IL-12p40) or M2 (IL-10, Arg-1, LIGHT, and SPHK1) markers in macrophages bound to PBS control or immobilized HA or nHC-HA/PTX3 as determined by quantitative PCR. (B) Relative TNF- α protein amounts as determined by ELISA. (C) Western blot (left) and cytolocalization (right) by immunofluorescence staining of IRF5, which is a M1 marker, in macrophages bound to PBS control or immobilized nHC-HA/PTX3. (D) Apoptosis of resting, fMLP- or LPS-stimulated neutrophils following incubation with immobilized nHC-HA/PTX3. An asterisk (*) indicates p < 0.05. (E) Phagocytosis of apoptotic neutrophils by resting or LPS-stimulated macrophages. An asterisk (*) indicates p < 0.05.

[0076] FIG. 4 exemplifies the role of CD44 in maintaining M2 macrophage polarization on immobilized nHC-HA/PTX3. (A) Relative mRNA expression of M1 (IL-12p40) and M2 (IL-10, LIGHT, and SPHK1) macrophage markers following binding of macrophages, pre-incubated with PBS or blocking antibodies to CD44, TLR4, or CD44/TLR4, to nHC-HA/PTX3 as determined by qPCR. An asterisk (*) indicates p < 0.05 compared to no antibody treatment (none) in the same

group. (B) IL-12 and IL-10 protein amounts as determined by ELISA. An asterisk (*) indicates $p < 0.05$ compared to no antibody treatment (none) in the same group.

[0077] FIG. 5 nHC-HA/PTX3(4th) promotes the cell aggregation, but both nHC-HA/PTX3(2nd) and nHC-HA/PTX3(4th) inhibit the production of IL-12p40 and IL-23 proteins in IFN- γ /LPS-stimulated macrophages RAW264.7 cells (2.5×10^5 /ml) were cultivated on immobilized substrates (PBS as the control) and stimulated with IFN- γ /LPS for 4 h (A) or 24 h (B and C). (A) Cell morphology at 4 h after seeding. Alternatively, cells are stimulated with LPS for 24 h and proteins of IL-10 and IL-12p70 in the cell culture supernatants were measured by respective ELISAs (B and C). p values are indicated in B and C.

[0078] FIG. 6 exemplifies dose-dependent and covalent coupling of HMW HA and nHC-HA/PTX3 to surfaces of 96 well CovaLink™ plates. (A) HA ELISA of bound HMW HA and nHC-HA/PTX3 after removal of unbound HMW HA and nHC-HA/PTX3. (B) HA ELISA of bound and unbound HA from HMW HA and nHC-HA/PTX3.

[0079] FIG. 7 exemplifies dose-dependent binding of TSG-6 to immobilized HA (iHA) and resistance to various dissociating and reducing agents. (A) TSG-6 bound to iHA as measured by TSG-6 ELISA. (B) TSG-6 bound to iHA as measured by TSG-6 ELISA following treatment with 6M Guanidine HCl, 8M Guanidine HCl, 2% SDS, 100 mM DTT, or 25 mM NaOH.

[0080] FIG. 8 exemplifies dose-dependent binding of PTX3 to immobilized HA (iHA) and resistance to various dissociating and reducing agents. (A) PTX3 bound to iHA as measured by PTX3 ELISA. (B) PTX3 bound to iHA as measured by PTX3 ELISA following treatment with 6M Guanidine HCl, 8M Guanidine HCl, 2% SDS, 100 mM DTT, or 25 mM NaOH.

[0081] FIG. 9 exemplifies the lack of competition or synergy between TSG-6 and PTX3 for binding iHA. The relative absorbance as measured by ELISA is shown for bound TSG-6 or PTX3 for incubation of each factor alone with iHA or combined incubation with iHA. No statistical significance is found between alone and combined for either TSG-6 or PTX3 binding to iHA ($p > 0.05$).

[0082] FIG. 10 exemplifies partial inhibition of PTX3 binding to iHA pre-bound with TSG-6 and lack of inhibition of TSG-6 binding to iHA pre-bound with PTX3. The figure shows TSG-6 and PTX3 ELISA results of subsequent binding of TSG-6 or PTX3 for pre-bound TSG-6/iHA (A) or pre-bound PTX3/iHA (B). P values are indicated in (A) and no statistical significance was found among groups in (B).

[0083] FIG. 11 exemplifies attachment of LPS-stimulated RAW264.7 macrophages to PBS (control), HA (iHA), nHC-HA/PTX3, TSG-6/iHA or PTX3/iHA. Cells were photographed 24 after incubation.

[0084] FIG. 12 exemplifies relative gene expression in RAW264.7 macrophages following incubation on PBS (control), HA (iHA), nHC-HA/PTX3, TSG-6/iHA or PTX3/iHA. Total RNAs were isolated and mRNA expression of IL-12p40 (A) and IL-10 (D) were measured by quantitative PCR. Alternatively, cells were stimulated with LPS (B and E) or IFN- γ /LPS (C) for 24 h, and protein expression of IL-12p70 (B), IL-23 (C), and IL-10 (E) in cell culture media were measured using respective ELISAs. An asterisk (*) indicates $p < 0.05$ compared to the control.

[0085] FIG. 13 exemplifies efficiency of TSG-6 free in solution versus bound TSG-6 for transferring HC1 and HC2 from I α I to iHA. (A, B) Relative bound HC1 (A) or I α I (B) following simultaneous or sequential addition of TSG-6 and I α I to iHA as determined by respective ELISA. An asterisk (*) indicates $p < 0.05$ in the same TSG-6 concentration when added simultaneously and sequentially. (C) Western blot of samples from A digested with hyaluronidase (HAase) and analyzed with anti-TSG-6 antibody. (D) Relative HC1 and PTX3 bound to iHA following simultaneous incubation of PTX3 and I α I with iHA as determined by ELISA.

[0086] FIG. 14 exemplifies complexes formed in solution following simultaneous incubation of I α I and TSG-6 with or without PTX3. (A-D) Western blot with antibodies against HC1 (A), HC2 (B), TSG-6 (C), or bikunin (D). HAase = treatment with hyaluronidase. (E) Illustration of TSG-6 interaction with I α I. (F) Illustration of the inhibition of HC2•TSG-6 formation by PTX3. (G) Western blot with antibody against I α I.

[0087] FIG. 15 exemplifies complexes formed on iHA following simultaneous incubation of I α I and TSG-6 with or without PTX3. After washes with 8 M GnHCl and PBS, bound HC1, TSG-6, and PTX3 were measured by respective ELISAs (A, D, F). An asterisk (*) indicates $p < 0.05$ compared to PTX3 at 1 μ g/ml. The complexes were washed again with 8 M GnHCl and PBS and bound components were digested with 1 unit/ml of hyaluronidase for 2 h. The digested samples were analyzed by Western blot with antibodies against HC1 (B), HC2 (C), TSG-6 (E), and PTX3 (G).

[0088] FIG. 16 exemplifies complexes formed on iHA following sequential addition of I α I with TSG-6 followed by PTX3. Bound HC1, TSG-6, and PTX3 were measured by respective ELISAs (A, D, F). The complexes were washed again with 8 M GnHCl and PBS and bound components were digested with 1 unit/ml of hyaluronidase for 2 h. The digested samples were analyzed by Western blot with antibodies against HC1 (B), HC2 (C), TSG-6 (E), PTX3 (G).

[0089] FIG. 17 exemplifies complexes formed on iHA following sequential addition of PTX3 followed by I α I with TSG-6. Bound HC1, TSG-6, and PTX3 were measured by respective ELISAs (A, C, E). The complexes were washed again with 8 M GnHCl and PBS and bound components

were digested with 1 unit/ml of hyaluronidase for 2 h. The digested samples were analyzed by Western blot with antibodies against PTX3 (B), TSG-6 (D), HC1 (F), and HC2 (G).

[0090] FIG. 18 exemplifies attachment of LPS-stimulated RAW264.7 macrophages to PBS (control), HA (iHA), nHC-HA/PTX3, (I α I/TSG-6/PTX3)/iHA (I α I, TSG-6 or PTX3 is simultaneously bound to iHA), (I α I/TSG-6)/PTX3/iHA (sequential addition of PTX3 to iHA pre-incubated with I α I and TSG-6), or (PTX3)/I α I/TSG-6/iHA (sequential addition of I α I and TSG-6 to iHA pre-incubated with PTX3). Cells were photographed 24 after incubation.

[0091] FIG. 19 exemplifies gene expression in RAW264.7 macrophages cultivated on immobilized substrates and stimulated with LPS. Total RNAs were isolated and expression of IL-10 and IL-12p40 mRNAs was measured by quantitative PCR (A and C). IL-10 and IL-12p70 proteins in the cell culture supernatants were measured by respective ELISAs (B and D). (E) IL-23 proteins in the cell culture supernatants of resting RAW264.7 cells (none) or with stimulation of IFN- γ (200 units/ml), LPS (1 μ g/ml), IFN- γ /LPS, LPS with immune complex (LPS/IC) or IL-4 (10 ng/ml) for 24 h as measured by IL-23 ELISA. (F) IL-23 in the cell culture supernatants of RAW264.7 cells cultivated on immobilized substrates and stimulated with IFN- γ /LPS for 24 h as measured by IL-23 ELISA. An asterisk (*) indicates $p < 0.05$.

[0092] FIG. 20 exemplifies immunostaining of HA, PTX3, TSG-6, HCs and bikunin in human umbilical cord (A) or amniotic membrane (B). Frozen sections of human umbilical cord were probed with biotinylated HABP with or without HAase digestion and with antibodies against PTX3 and TSG-6, and chain-specific antibodies against I α I and P α I components as indicated. Nuclei were counter-stained with Hoechst 33342 (blue). Epi, Epithelium. Bar represents 100 μ m.

[0093] FIG. 21 exemplifies a comparison of PTX3 levels in sequential PBS and GnHCl extract from AM, CH and UC. A, each lane contains 2 μ g HA in lanes 2 and 3 and 20 μ g total proteins in lanes 4-11. B, each lane contains 40 μ g total proteins in lanes 3-10.

[0094] FIG. 22 exemplifies a comparison of HC1, bikunin and I α I in sequential PBS and GnHCl extracts from AM, CH and UC. Each lane contains 20 μ g total protein in A and C and 40 μ g total protein in B and D-F except positive control.

[0095] FIG. 23 exemplifies a comparison of TSG-6 in AM and UC GnHCl extract. Each lane contains 40 μ g total proteins except positive TSG-6 control.

[0096] FIG. 24 exemplifies Western blot analysis of PTX3 (A), HC1 (B), HC2 (C), and TSG-6 (D) in 1-4th AM HC•HA complex. Each lane contains 4 μ g HA except positive control.

[0097] FIG. 25 exemplifies Western blot analysis of TSP-1 in AME, AM GnHCl and 1-4th HC-HA complex. Lanes 3, 4, 10 and 11, each lane contains 30 μ g total proteins. Lanes 5-8 and 12-15, each lane contains 4 μ g HA.

[0098] FIG. 26 exemplifies Western blot analysis of PTX3 (A), HC1 (B), HC2 (C), HC3 (D) and TSG-6 (E) in 4th UC HC-HA complex. Each lane contains 4 μ g HA except positive control.

[0099] FIG. 27 exemplifies a comparison of HC1 (A) and PTX3 (B) in 4th HC-HA complex from PBS and GnHCl extract. Each lane contains 4 μ g HA except positive control.

[00100] FIG. 28 exemplifies a comparison of 4th HC-HA complex from PBS and GnHCl in agarose gel. Each lane contains 15 μ g HA except positive HA control.

[00101] FIG. 29 exemplifies Coomassie blue staining for SDS-PAGE gel of GnHCl HC-HA and PBS HC-HA. A. AM PBS and GnHCl HC-HA. B. UC PBS and GnHCl HC-HA. Each lane contains 30 μ g HA.

[00102] FIG. 30 exemplifies that keratan sulfate and osteoadherin were present in AM GnHCl HC-HA but not in PBS HC-HA. A. Coomassie blue staining. Each lane contains 30 μ g HA. B and C. Western blot for keratan sulfate (B) and osteoadherin (C). Each lane contains 4 μ g HA. D, Immunostaining for keratan sulfate in AM.

[00103] FIG. 31 exemplifies deglycosylation and analysis of AM GnHCl HC-HA by SDS-PAGE with (Coomassie Blue) CB staining or Western blots. A. Coomassie blue staining. Each lane contains 30 μ g HA except lane 6 which contains 5 μ g HA. B-H. Western blots for Osteoadherin (B), Decorin (C, D), Biglycan (E, F), Keratan sulfate (G) and PTX3 (H). H: hyaluronidase; C: chondroitinase (Cabc); K: keratinase (keratan sulfate endo- β -galactosidase); T: TFMSA (trifluoromethanesulfonic acid). Each lane contains 4 μ g HA.

[00104] FIG. 32 exemplifies Decorin and biglycan were abundantly present in UC GnHCl HC-HA but not in PBS HC-HA. Keratan sulfate, osteoadherin and bikunin also were present in UC GnHCl HC-HA but not in PBS HC-HA except for keratan sulfate. A. Coomassie blue staining. Each lane contains 30 μ g HA except lane 6 which contains 5 μ g HA. B-H. Western blots for Decorin (B), Biglycan (C), Bikunin (D), PTX3 (E), Keratan sulfate (F) and Osteoadherin (G). H: hyaluronidase; C: chondroitinase (Cabc); K: keratinase (keratan sulfate endo- β -galactosidase). Each lane contains 4 μ g HA.

[00105] FIG. 33 exemplifies immunolocalization of PTX3 in human AM. Frozen sections of human fetal membrane were probed with anti-PTX3, biotinylated HABP with or without HAase digestion and with chain-specific antibodies against IgG components. Nuclei were counter-stained with Hoechst 33342 (blue). AM, amniotic membrane; Epi, Epithelium; CH, chorion. Bar, represents 100 μ m.

[00106] FIG. 34 exemplifies presence of PTX3 in AM soluble extract and purified HC-HA complex. Purified PTX3, AM extract (AME) and AM HC-HA complex were treated with or without 50 mM NaOH at 25 °C for 1 h or hyaluronidase (HAase) at 37 °C for 1 h before Western

blotting using anti-PTX3 (A) and anti-HC1 (B) antibodies and analysis on 0.5% agarose gel electrophoresis before staining with Stains-all dye (C). PTX3 species and its multimeric form were found in AM soluble extract and purified HC-HA complex. M, protein ladder markers.

[00107] FIG. 35 exemplifies expression of PTX3 mRNA and protein by AMECs and AMSCs. RNA and protein were extracted from human skin fibroblasts (Skin Fib.), and both AMECs and AMSCs. Expression of PTX3 mRNA (A) and protein in supernatants and cell lysates (B) was compared. PTX3 siRNA transfection was performed to verify the expression of PTX3 in AMECs and AMSCs (C).

[00108] FIG. 36 exemplifies morphological changes of human skin fibroblasts (HSF, A), AMSC (B) and AMEC (C) after agarose overlay. HSF, AMSC and AMEC were cultured in both serum-free and serum-containing conditions with or without a 3% agarose overlay for five days and the cell morphology were photographed. Scale bar, 50 μ m.

[00109] FIG. 37 exemplifies that agarose overlay decreased the release of HA into culture media by HSF, AMSC and AMEC cultures. The HA concentration were measured by ELISA assay in culture media from HSF, AMSC and AMEC with or without agarose overlay in both serum-free and serum-containing conditions.

[00110] FIG. 38 exemplifies immunolocalization of PTX3, HA and HC1 in cell cultures with an agarose overlay. HSF, AMSC and AMEC were cultured with an agarose overlay with or without TNF treatment and probed for hyaluronan (*red*), PTX3 (green, A-F; red, J-L) and HC1 (*green*) (nuclei are *blue*). Colocalization of HC1 with HA were found in all cultures, but colocalization of PTX3 with HA or HC1 were only found in AMSC and AMEC. Scale bar, 50 μ m.

[00111] FIG. 39 exemplifies HC-HA/PTX3 complex in AMSC but not HSF under agarose overlay. GnHCl extracts of cell layers from agarose overlayed HSF and AMSC cultures were subjected to Western blot for PTX3 with or with NaOH treatment. M, protein ladder markers.

[00112] FIG. 40 exemplifies reconstitution of HC-HA/PTX3 complex on immobilized HA *in vitro*. iHA (~ 14 μ g/ml), IaI (5 μ g/ml), and TSG-6 (12 μ g/ml) were incubated simultaneously without or with PTX3 (1, 5, or 20 μ g/ml) for 2 h at 37 °C. For sequentially, iHA (~ 14 μ g/ml), IaI (12 μ g/ml), and TSG-6 (12 μ g/ml) were incubated in the reaction buffer for 2 h at 37 °C, then PTX3 (1, 5, or 20 μ g/ml) were added and incubated for another 2 h at 37 °C. After washes with 8 M GnHCl and PBS, iHA with bound components were digested with 1 unit/ml of hyaluronidase for 2 h at 60 °C. The samples were analyzed by Western blot with antibodies against IaI (A), TSG-6 (B) and PTX3 (C).

[00113] FIG. 41 exemplifies cell morphology of human corneal fibroblasts up to D3 cultured on DMEM/10% FBS for 2 Days (A) or DMEM/10% FBS for 2 Days $\pm$ TGF- β 1 for 3 Days (B).

[00114] **FIG. 42** exemplifies soluble HC-HA (PBS) inhibits TGF β 1 but activates TGF β 3 signaling while insoluble HC-HA (GnHCl) activates both TGF β 1 and TGF β 3 signaling under serum-free conditions and is further enhanced with TGF β 1 stimulation.

[00115] **FIG. 43** exemplifies both soluble HC-HA (PBS) and insoluble HC-HA (GnHCl) inhibit TGF β R2 and TGF β R3 expression under challenge of TGF β 1. A, TGF β R mRNA expression. B, TGF β R protein expression.

[00116] **FIG. 44** exemplifies inhibition of nuclear translocation of pSMAD2/3 signaling by HC-HA inhibition of TGF β 1 signaling.

[00117] **FIG. 45** exemplifies HC-HA inhibition of alpha smooth muscle actin formation.

[00118] **FIG. 46** exemplifies BMP6 transcript was activated by HA and soluble/insoluble HC-HA. Addition of TGF β 1 activates transcript expression of BMP6 on plastic but dramatically activates mRNA expression of BMP4/6 in HCF on HA and both soluble and insoluble HC-HA.

[00119] **FIG. 47** exemplifies HC-HA but not HA activates transcript expression of BMPR1A in HCF challenged with TGF β 1, while additional TGF β 1 non-specifically activates mRNA expression of BMPR1B and BMPR2 in HCF.

[00120] **FIG. 48** exemplifies both soluble HC-HA (PBS) and insoluble HC-HA (GnHCl) activates BMP4/6 signaling via pSMAD1/5/8.

[00121] **FIG. 49** exemplifies activation of SMAD/1/5/8 resulted in upregulation of its downstream gene, inhibitor of DNA binding 1, 3 and 4 (ID1, ID3 and ID4), downstream targets of BMP signaling.

[00122] **FIG. 50** exemplifies HC-HA (PBS) and HC-HA (GnHCl) promote Keratocan mRNA expression by 14- and 16-fold respectively, which was significantly downregulated by additional TGF β 1.

[00123] **FIG. 51** exemplifies HC-HA (PBS) and HC-HA (GnHCl) promote keratocan protein expression.

[00124] **FIG. 52** exemplifies HCF express more ESC markers on 4X HC-HA (PBS and insoluble HC-HA (GnHCl) than on plastic and when the cells were challenged with addition of TGF β 1.

[00125] **FIG. 53** exemplifies cell viability of MC3T3-E1 cells as measured by MTT.

[00126] **FIG. 54** exemplifies mineralization of MC3T3-E1 cells as measured by Alizarin Red Staining.

[00127] **FIG. 55** exemplifies morphology of MC3T3-E1 cells treated with HC-HA (A) or AMP (B) on Day 13 of induction.

[00128] **FIG. 56** exemplifies Alizarin Red staining of MC3T3-E1 cells treated with HC-HA (A) or AMP (B) on day 13 of induction.

[00129] FIG. 57 exemplifies quantitative analysis of ARS staining of MC3T3-E1 cells treated with HC-HA (A) or AMP (B) on day 13 of induction.

[00130] FIG. 58 exemplifies ALP activity (IU/L) of HC-HA (A) and AMP (B) treated cells on day 13 of induction.

[00131] FIG. 59 exemplifies phase contrast microscopy of morphological changes in MC3T3-E1 cells following induction from Day 3. (A) Uninduced cells were cultured in α MEM w/ 10% FBS for 7 days. (B) MC3T3-E1 cells were cultured to confluence in flat bottom 96-well plates one day after seeding (Day 0). Cells were then induced with ascorbic acid and β -glycerophosphate.

[00132] FIG. 60 exemplifies phase contrast microscopy of morphological changes in induced MC3T3-E1 cells treated with HC-HA (A) or AMP (B) from Day 1 to Day 7.

[00133] FIG. 61 exemplifies phase contrast microscopy of spindle ring formation in induced MC3T3-E1 cells from Day 3 of induction.

[00134] FIG. 62 exemplifies phase contrast microscopy of spindle ring formation in induced MC3T3-E1 cells treated with HC-HA (A) and AMP (B) (Day 0 to Day 6).

[00135] FIG. 63 exemplifies ARS staining of induced MC3T3-E1 cells treated with HC-HA and AMP and Extraction with GnHCl.

[00136] FIG. 64 exemplifies ARS Extraction and Quantitation of MC3T3-E1 cells with Acetic Acid and 10% Ammonium Hydroxide. ARS extracts through acetic acid treatment were neutralized with 10% ammonium hydroxide then added to 96-well clear bottom assay plates (A) for reading on a spectrophotometer (B). * denotes statistical significance.

[00137] FIG. 65 exemplifies ARS Extraction and Quantitation of MC3T3-E1 cells with GnHCl. ARS extracts through GnHCl treatment were added to 96-well clear bottom assay plates (A) for reading on a spectrophotometer (B). * denotes statistical significance.

[00138] FIG. 66 exemplifies ARS staining (A) and quantitation (B) of MC3T3-E1 cells treated with HC-HA on D19 (D18 of Induction). ARS staining was conducted on D19 of culturing (D18 Induction).

[00139] FIG. 67 exemplifies ARS staining (A) and quantitation (B) of MC3T3-E1 cells treated with AMP. ARS staining was conducted on D19 of culturing (D18 Induction).

[00140] FIG. 68 exemplifies a spindle like ring observed in induced MC3T3-E1 cells on Day 14 of induction.

[00141] FIG. 69 exemplifies phase contrast microscopy of cell morphology of induced MC3T3-E1 cells treated with AMP after 14 days of induction cultured (A) without a transwell or (B) with a transwell.

[00142] FIG. 70 exemplifies ARS Staining of induced MC3T3-E1 cells at D14 of induction.

[00143] FIG. 71 exemplifies quantitation of ARS Staining of induced MC3T3-E1 cells at D14 of induction.

[00144] FIG. 72 exemplifies ARS staining and quantitation of MC3T3-E1 cells treated with AMP (A) Phase contrast picture and ARS staining picture MC3T3-E1 cells taken on Day 21 culturing (Day 20 of induction). (B) ARS staining was quantified on Day 21 of culture (Day 20 of induction). The * symbol denotes statistical significance of $p < 0.05$.

[00145] FIG. 73 illustrates a map of different progenitor differentiation to osteoblast, with common factors added to culture medium shown.

[00146] FIG. 74 exemplifies ARS staining and quantitation in MC3T3-E1 cells. (A) Phase contrast micrographs with or without ARS staining of HUVEC, hBMMSCs, and hAM stromal stem cells from Day 4 to Day 21. (B) Quantitation of ARS staining. The * symbol denotes statistical significance of $p < 0.05$ when compared to the negative control.

[00147] FIG. 75 illustrates a timeline of osteogenesis in MC3T3-E1 cells.

[00148] FIG. 76 exemplifies ARS staining and quantitation in MC3T3-E1 cells. (A) Cell morphology and ARS staining of MC3T3-E1 cells treated with AMP (Day 1, 2, 7, 10). (B) ARS Quantitation of MC3T3-E1 cells treated with AMP (Day 1, 2, 7, 10) The * symbol denotes statistical significance of $p < 0.05$.

[00149] FIG. 77 exemplifies a timeline of cell viability and proliferation through MTT assay. (A) MTT Assay of MC3T3-E1 cell viability and metabolic activity on Day 1, 2, and 4. The * symbol denotes statistical significance of $p < 0.05$ from Day 1. (B) BrdU Assay of MC3T3-E1 cell proliferation on Day 1, 2, and 16. The * symbol denotes statistical significance of $p < 0.05$ from Day 1.

[00150] FIG. 78 exemplifies mRNA expression by QPCR for various genes tested following differentiation induction with or without AMP treatment. (A) hMSC and (B) MC3T3-E1 cell expression.

[00151] FIG. 79 exemplifies phase microscopy and quantitation of ARS Assay. MC3T3-E1 cells deposited mineralization for the duration of the experiment, 8 days. ARS was then used for qualitative analysis. (A) CovaLink-NH 96-well plate in which the PBS, HA, and HC-HA were immobilized. (B) Conventional 96-well plate with the negative control and AGM (inductive agents) and AMP as positive controls. (C) Quantitation of ARS assay. The * symbol denotes statistical significance of $p < 0.05$ from Day 1.

[00152] FIG. 80 exemplifies mRNA expression by QPCR for various genes tested following differentiation induction with or without HC-HA/PTX3 treatment. (A) Master transcription factors Runx2 and Sox9 for osteogenesis and chondrogenesis, Day 1, 7, and 14. (B) Bone morphogenic

proteins (BMPs) expression on Day 14. (C) Chondrogenic marker Collagen 2 (COL2) and osteogenic marker alkaline phosphatase (ALPL), expressed on Days 7 and 14. (D) Hypertrophic markers Collagen 10 (COL10) and MMP13 expressed on Day 14. (E) Osteogenic markers Collagen 1 (COL1), Osterix (OSX) and Bone Sialoprotein (BSP) on Day 14.

[00153] FIG. 81 exemplifies phase microscopy (A) and quantitation (B) of ARS Assay following differentiation induction with or without HC-HA/PTX3 (soluble or insoluble) treatment on Day 14.

[00154] FIG. 82 exemplifies CD4⁺ T cell activation and differentiation. Under different stimuli, Naïve CD4⁺ T helper cell (Th) is differentiated into Th1, Th2, Th17, or Treg and secreted different cytokines. Th1-type cytokines (e.g., IFN- γ and IL-2) tend to produce the pro-inflammatory responses.

[00155] FIG. 83 exemplifies the procedure to measure cell proliferation and cytokine production. Splenocytes were isolated from OT-II mice that express a transgenic TCR specific for ovalbumin (OVA), and stimulated with OVA up to 4 days. Cell proliferation was measured by BrdU labeling and expression of cytokines (IFN- γ and IL-2) was measured by the respective ELISA.

[00156] FIG. 84 exemplifies nHC-HA/PTX3 inhibition of CD4⁺ T cell proliferation. Splenocytes isolated from Ova T cell receptor transgenic mice were stimulated with OVA (0 -10 μ M) for 4 days. AM extract (AME, 25 μ g/ml) and nHC-HA/PTX3 (25 μ g/ml) inhibited the clone growth of activated T cells induced by increasing OVA concentrations (top). Proliferation of splenocytes treated with HA, AME, or nHC-HA/PTX3 and labeled with CFSE for 4 days was measured by flow cytometry. Both AME and nHC-HA/PTX3 dose-dependently inhibited the cell proliferation (middle). 25 μ g/ml nHC-HA/PTX3 inhibited the proliferation of CD4⁺ T cell labeled with BrdU (bottom) (*, $p < 0.05$ compared to the control).

[00157] FIG. 85 exemplifies nHC-HA/PTX3 suppression of Th1-type cytokines IFN- γ and IL-2. Splenocytes treated with PBS, 25 μ g/ml HA, 25 μ g/ml AME, or 25 μ g/ml nHC-HA/PTX3 were stimulated with 10 μ M OVA for 4 days. IFN- γ and IL-2 in culture supernatants were measured by the respective ELISA. Both AME and nHC-HA/PTX3 suppressed the production of IFN- γ and IL-2 (* $p < 0.05$ compared to the control).

[00158] FIG. 86 exemplifies nHC-HA/PTX3 reduction of macrophage (labeled with enhanced green fluorescent protein, or EGFP) influx. LPS (2 μ l of 2 μ g/ml) was injected into the corneal stroma of Mafia mouse. Immediately, 5 μ l of PBS or nHC-HA/PTX3 (1 mg/ml) was injected into each quadrant of one cornea from the same mouse through the subconjunctival tissue. The influx of EGFP⁺ macrophages is monitored using in vivo intravital microscopy at day 1, 2, 3, and 6 after LPS treatment (top). Alternatively, mouse corneas were treated with PBS or nHC-HA/PTX3 simultaneously with LPS (pretreatment (-) or three days before LPS treatment (pretreatment (+))).

At day 4 after LPS treatment, cells in corneas were isolated by collagenase digestion and sorted into EGFP⁻ or EGFP⁺ (macrophages) by FACS (* and **, $p < 0.05$ and $p < 0.01$ compared to the control, respectively).

[00159] FIG. 87 exemplifies nHC-HA/PTX3 polarization of macrophages toward a M2 phenotype. mRNA expression of M2 markers (Arg-1 and IL-10) and M1 markers (IL-12p40 and IL-12p35) in macrophages (EGFP⁺) infiltrated to LPS-elicited murine corneas was quantitated by qPCR (* and **, $p < 0.05$ and $p < 0.01$ compared to the control, respectively).

[00160] FIG. 88 exemplifies nHC-HA/PTX3 improvement of corneal allograft survival. The survival of murine corneal allograft was significantly improved by injection of nHC-HA/PTX3 at one quadrant subconjunctival site (10 μ g /time, two times /a week, top left), but even dramatically better by injection of nHC-HA/PTX3 at four quadrant subconjunctival sites (20 μ g /time, two times / a week, top right). Bottom, photographs of PBS (post operative day 21, left) or nHC-HA/PTX3 treatment (post operative day 40, right) in corneal allografts.

[00161] FIG. 89 exemplifies macrophage classical M1 activation (e.g., induced by IFN- γ and/or TLR ligands such as LPS) to express high levels of proinflammatory cytokines (such as TNF- α , IL-12, and IL23), which activate Th1 and Th17 lymphocytes leading to many chronic inflammatory diseases.

[00162] FIG. 90 exemplifies LPS-elicited macrophage infiltration to murine corneas with treatment of PBS (control), HC-HA/PTX3, or AMP. Mafia mice (macrophages are EGFP⁺) with LPS intrastromal injection (5 μ g) for both eye. OS was treated with PBS, OD was treated with either HC-HA (2 injection sites) (A), HC-HA (4 injection sites) (B), AMP (2 injection sites) (C), AMP (4 injection sites) (D), each injection was 5 μ l. Treatment was one time right after LPS injection. Images were taken on day 1, day 2, day 3 and day 6. Cells were counted based on the intensity of green fluorescence.

[00163] FIG. 91 exemplifies LPS-elicited macrophage infiltration to murine corneas and their subtypes (M1 and M2) with treatment or pretreatment of PBS (control), nHC-HA/PTX3, or AMP. Pretreatment and treatment of Mafia mice are the same as described in FIG. 86. On Day 4, cornea buttons are cut and digested with collagenase at 37 °C for 2h. EGFP-positive/negative cells are sorted out by FACS. The ratio of EGFP-positive macrophages to EGFP-negative cells is calculated and used as an arbitrary unit to determine the extent of macrophage infiltration (A). Total RNA was extracted from sorted EGFP-positive macrophages and converted to cDNAs. The expression of Arg-1, IL-10, IL-12p40, and IL-12p35 is measured by quantitative PCR (B).

[00164] **FIG. 92** Diagram of injection site. The injection locations will be subconjunctiva close to fornix nHC-HA/PTX3 or AMP can reduce DS-induced ALKC in murine experimental dry eye model.

[00165] **FIG. 93** exemplifies HC-HA activation of IGF1-HIF1 α -VEGF signaling to promote angiogenesis. HC-HA induces 2- to 6-fold increase of IGF1 mRNA and 2-fold increase of VEGF mRNA when the cells are in rest condition. HC-HA induces 5- to 12-fold increase of IGF1 mRNA and 5- to 9-fold increase of VEGF mRNA when the cells are challenged by TGF β (10 ng/ml). n=4, *p<0.05, **p<0.01. IGF1, Insulin-like growth factor 1; HIF1 α , Hypoxia-inducible factor 1-alpha; VEGF, Vascular endothelial growth factor.

DETAILED DESCRIPTION OF THE INVENTION

Certain Terminology

[00166] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as is commonly understood by one of skill in the art to which the claimed subject matter belongs. All patents, patent applications, published applications and publications, GENBANK sequences, websites and other published materials referred to throughout the entire disclosure herein, unless noted otherwise, are incorporated by reference in their entirety. In the event that there is a plurality of definitions for terms herein, those in this section prevail. Where reference is made to a URL or other such identifier or address, it is understood that such identifiers can change and particular information on the internet can come and go, but equivalent information is known and can be readily accessed, such as by searching the internet and/or appropriate databases. Reference thereto evidences the availability and public dissemination of such information.

[00167] As used herein, ranges and amounts can be expressed as “about” a particular value or range. About also includes the exact amount. Hence “about 5 μ g” means “about 5 μ g” and also “5 μ g.” Generally, the term “about” includes an amount that would be expected to be within experimental error.

[00168] As used herein, a reconstituted HC-HA/PTX3 (rcHC-HA/PTX3) complex is an HC-HA/PTX3 complex that is formed by assembly of the component molecules of the complex *in vitro*. The process of assembling the rcHC-HA/PTX3 includes reconstitution with purified native proteins or molecules from biological source, recombinant proteins generated by recombinant methods, or synthesis of molecules by *in vitro* synthesis. In some instances, the purified native proteins used for assembly of the rcHC-HA/PTX3 are proteins in a complex with other proteins (i.e. a multimer, a multichain protein or other complex). In some instances, PTX3 is purified as a multimer (e.g. a homomultimer) from a cell and employed for assembly of the rcHC-HA/PTX3 complex.

[00169] As used herein, a purified native HC-HA/PTX3 (nHC-HA/PTX3) complex refers to an HC-HA/PTX3 complex that is purified from a biological source such as a cell, a tissue or a biological fluid. Such complexes are generally assembled *in vivo* in a subject or *ex vivo* in cells, tissues, or biological fluids from a subject, including a human or other animal.

[00170] As used herein, a PTX3/HA complex refers to an intermediate complex that is formed by contacting PTX3 with immobilized HA. In the methods provided herein, the PTX3/HA complex is the generated prior to the addition of HC1 to HA.

[00171] As used herein, "hyaluronan," "hyaluronic acid," or "hyaluronate" (HA) are used interchangeably to refer to a substantially non-sulfated linear glycosaminoglycan (GAG) with repeating disaccharide units of D-glucuronic acid and N-acetylglucosamine (D-glucuronosyl-N-acetylglucosamine).

[00172] As used herein, the term "high molecular weight" or "HMW," as in high molecular weight hyaluronan (HMW HA), is meant to refer to HA that has a weight average molecular weight that is greater than about 500 kilodaltons (kDa), such as, for example, between about 500 kDa and about 10,000 kDa, between about 800 kDa and about 8,500 kDa, between about 1100 kDa and about 5,000 kDa, or between about 1400 kDa and about 3,500 kDa. In some embodiments, the HMW HA has a weight average molecular weight of 3000 kDa or greater. In some embodiments, the HMW HA has a weight average molecular weight of 3000 kDa. In some embodiments, the HMW HA is Healon® with a weight average molecular weight of about 3000 kDa. In some embodiments, HMW HA has a molecular weight of between about 500 kDa and about 10,000 kDa. In some embodiments, HMW HA has a molecular weight of between about 800 kDa and about 8,500 kDa. In some embodiments, HMW HA has a molecular weight of about 3,000 kDa.

[00173] As used herein, the term "low molecular weight" or "LMW," as in low molecular weight hyaluronan (LMW HA), is meant to refer to HA that has a weight average molecular weight that is less than 500 kDa, such as for example, less than about 400 kDa, less than about 300 kDa, less than about 200 kDa, about 200-300 kDa, or about 1-300 kDa.

[00174] As used herein, pentraxin 3, or PTX3, protein or polypeptide refers to any PTX3 protein, including but not limited to, a recombinantly produced protein, a synthetically produced protein, a native PTX3 protein, and a PTX3 protein extracted from cells or tissues. PTX3 include multimeric forms (e.g. homomultimer) of PTX3, including, but not limited to, dimeric, trimeric, tetrameric, pentameric, hexameric, tetrameric, octameric, and other multimeric forms naturally or artificially produced.

[00175] As used herein, Tumor necrosis factor Stimulated Gene-6 (TSG-6) refers to any TSG-6 protein or polypeptide, including but not limited to, a recombinantly produced protein, a

synthetically produced protein, a native TSG-6 protein, and a TSG-6 protein extracted from cells or tissues.

[00176] As used herein, inter- α -inhibitor (I α I) refers to the I α I protein comprised of light chain (i.e., bikunin) and one or both heavy chains of type HC1 or HC2 covalently connected by a chondroitin sulfate chain. In some embodiments, the source of I α I is from serum or from cells producing I α I e.g., hepatic cells or amniotic epithelial or stromal cells or umbilical epithelial or stromal cells under a constitutive mode stimulation by proinflammatory cytokines such as IL-1 or TNF- α .

[00177] As used herein, a "hyaluronan binding protein", "HA binding protein", or "HABP" refers to any protein that specifically binds to HA.

[00178] As used herein, "link module" means a hyaluronan-binding domains.

[00179] As used herein, "biological activity" refers to the *in vivo* activities of an nHC-HA/PTX3 or rcHC-HA/PTX3 complex or physiological responses that result upon *in vivo* administration of an nHC-HA/PTX3 or rcHC-HA/PTX3 complex or a composition or mixture containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex. Biological activity, thus, encompasses therapeutic effects and pharmaceutical activity of nHC-HA/PTX3 or rcHC-HA/PTX3 complexes and compositions and mixtures thereof.

[00180] As used herein, the terms "subject", "individual" and "patient" are used interchangeably. None of the terms are to be interpreted as requiring the supervision of a medical professional (e.g., a doctor, nurse, physician's assistant, orderly, hospice worker). As used herein, the subject is any animal, including mammals (e.g., a human or non-human animal) and non-mammals. In one embodiment of the methods and compositions provided herein, the mammal is a human.

[00181] As used herein, the terms "treat," "treating" or "treatment," and other grammatical equivalents, include alleviating, abating or ameliorating one or more symptoms of a disease or condition, ameliorating, preventing or reducing the appearance, severity or frequency of one or more additional symptoms of a disease or condition, ameliorating or preventing the underlying metabolic causes of one or more symptoms of a disease or condition, inhibiting the disease or condition, such as, for example, arresting the development of the disease or condition, relieving the disease or condition, causing regression of the disease or condition, relieving a condition caused by the disease or condition, or inhibiting the symptoms of the disease or condition either prophylactically and/or therapeutically. In a non-limiting example, for prophylactic benefit, an rcHC-HA/PTX3 complex or composition disclosed herein is administered to an individual at risk of developing a particular disorder, predisposed to developing a particular disorder, or to an individual reporting one or more of the physiological symptoms of a disorder.

[00182] As used herein, “placenta” refers to the organ that connects a developing fetus to the maternal uterine wall to allow nutrient uptake, waste elimination, and gas exchange via the maternal blood supply. The placenta is composed of three layers. The innermost placental layer surrounding the fetus is called amnion. The allantois is the middle layer of the placenta (derived from the embryonic hindgut); blood vessels originating from the umbilicus traverse this membrane. The outermost layer of the placenta, the chorion, comes into contact with the endometrium. The chorion and allantois fuse to form the chorioallantoic membrane.

[00183] As used herein, “chorion” refers to the membrane formed by extraembryonic mesoderm and the two layers of trophoblast. The chorion consists of two layers: an outer formed by the trophoblast, and an inner formed by the somatic mesoderm; the amnion is in contact with the latter. The trophoblast is made up of an internal layer of cubical or prismatic cells, the cytotrophoblast or layer of Langhans, and an external layer of richly nucleated protoplasm devoid of cell boundaries, the syncytiotrophoblast. The avascular amnion is adherent to the inner layer of the chorion.

[00184] As used herein, “amnion-chorion” refers to a product comprising amnion and chorion. In some embodiments, the amnion and the chorion are not separated (i.e., the amnion is naturally adherent to the inner layer of the chorion). In some embodiments, the amnion is initially separated from the chorion and later combined with the chorion during processing.

[00185] As used herein, “umbilical cord” refers to the organ that connects a developing fetus to the placenta. The umbilical cord is composed of Wharton's jelly, a gelatinous substance made largely from mucopolysaccharides. It contains one vein, which carries oxygenated, nutrient-rich blood to the fetus, and two arteries that carry deoxygenated, nutrient-depleted blood away.

[00186] As used herein, “placental amniotic membrane” (PAM) refers to amniotic membrane derived from the placenta. In some embodiments, the PAM is substantially isolated.

[00187] As used herein, “umbilical cord amniotic membrane” (UCAM) means amniotic membrane derived from the umbilical cord. UCAM is a translucent membrane. The UCAM has multiple layers an epithelial layer, a basement membrane; a compact layer; a fibroblast layer; and a spongy layer. It lacks blood vessels or a direct blood supply. In some embodiments, the UCAM comprises Wharton's Jelly. In some embodiments, the UCAM comprises blood vessels and/or arteries. In some embodiments, the UCAM comprises Wharton's Jelly and blood vessels and/or arteries.

[00188] As used herein, the terms “purified”, “and “isolated” mean a material (e.g., nHC-HA/PTX3 complex) substantially or essentially free from components that normally accompany it in its native state. In some embodiments, “purified” or “isolated” mean a material (e.g., nHC-HA/PTX3 complex) is about 50% or more free from components that normally accompany it in its

native state, for example, about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% free from components that normally accompany it in its native state.

Overview: nHC-HA/PTX3 and rcHC-HA/PTX3 Complexes

[00189] Hyaluronan (HA) is a substantially non-sulfated linear glycosaminoglycan (GAG), composed of repeating disaccharide subunits units of D-glucuronic acid and N-acetyl-D-glucosamine via GlcUA- β 1,3-GlcNAc- β 1,4-linkages. HA is synthesized by HA synthases (e.g., HAS1, HAS2, and HAS3) and deposited into the extracellular matrix, where it contributes to the structural integrity of tissues and also regulates many cellular process via interaction with proteins, including cell surface receptors. The molecular weight of HA typically ranges in size from about 200 to about 10,000 kDa. Normal levels of HA are maintained in tissues through a balance of biosynthesis by HAS enzymes and catabolism by hyaluronidases, such as Hyal1.

[00190] High molecular weight HA (HMW HA), typically greater than 500 kDa, promotes cell quiescence and structural integrity of such tissues as the cartilage and the vitreous body (humor) in the eye, and is associated with scarless fetal wound healing. In certain instances, HMW HA inhibits the gene expression of pro-inflammatory mediators and angiogenesis.

[00191] In certain pathogenic conditions, HMW HA is degraded into smaller fragments and oligosaccharides (e.g., via hyaluronidase or free radical oxidation). LMW HA fragments stimulate vascular endothelial cell proliferation, migration, collagen synthesis, sprout formation, and angiogenesis in rat skin, myocardial infarction, and cryo-injured skin graft model by promoting the gene expression of pro-inflammatory and pro-angiogenic mediators.

[00192] The biological functions of HA are mediated though interaction of HA with HA-binding proteins (HABPs), also called hyaladherins. Such proteins include, but are not limited to, tumor necrosis factor- α -stimulated gene 6 (TSG-6), aggrecan, versican, neurocan, brevican, LYVE-1, CD44, and inter- α - inhibitor (I α I). In some instances, HABPs comprise a link module domain that binds to HA. TSG-6, aggrecan, versican, neurocan, brevican, LYVE-1 and CD44 are exemplary HABPs that contain a link module.

[00193] I α I comprises two heavy chains (HC1 and HC2), both of which are linked through ester bonds to a chondroitin sulfate chain that is attached to a light chain (i.e., Bikunin). In some instances, HA forms a covalent complex (hereinafter, "HC-HA") with one or both of the HCs of I α I by covalent linkage to the I α I heavy chains. In certain instances, the I α I is found in serum and/or obtained from cells producing I α I, e.g., hepatic cells or amniotic epithelial or stromal cells or

umbilical epithelial or stromal cells under a constitutive mode stimulation by proinflammatory cytokines, such as IL-1 or TNF- α

[00194] In certain instances, TSG-6 facilitates the transfer of, catalyzes the transfer of, and/or transfers the HC1 and HC2 of IaI to HA. TSG-6 forms stable complexes with immobilized HA (TSG-6•HA) resulting in the transfer HC1 and HC2 to HA to form an HC-HA complex and release of TSG-6 from the complex. The expression of TSG-6 is often induced by inflammatory mediators such as TNF- α and interleukin-1 and during inflammatory-like processes such as ovulation and cervical ripening.

[00195] Amniotic membrane (AM) modulates adult wound healing and facilitates tissue regeneration. In certain instances, AM promotes epithelialization while suppressing stromal inflammation, angiogenesis and scarring. AM has been used successfully as a surgical graft or temporary biological patch for the treatment of ophthalmic conditions which require corneal and conjunctival surface reconstruction, including, but not limited to, persistent epithelial defect, deep corneal ulcer, infectious keratitis, symptomatic bullous keratopathy, acute Stevens Johnson Syndrome/Toxic Epidermal Necrolysis (SJS/TEN), limbal stem cell deficiency, pterygium, pinguecula, conjunctivochalasis, symblepharon, formix reconstruction, and conjunctival tumors.

[00196] The avascular stromal matrix of AM contains high amounts of HA and constitutively expresses IaI (Zhang et al. (2012) *J. Biol. Chem.* 287(15):12433-44). HMW HA in AM forms nHC-HA complexes (He et al. (2009) *J. Biol. Chem.* 284(30):20136-20146). As shown herein in the Examples provided, this nHC-HA complex also contains pentraxin 3, PTX3 (FIG. 1), and hence it is referred to herein as “nHC-HA/PTX3 complex.” Native HC-HA/PTX3 complexes extracted from the AM exhibit suppression of TGF- β promoter activity, promotion of macrophage cell death, and suppression of blood vessel development. The nHC-HA/PTX3 complexes of the AM thus serve an active role in AM’s anti-inflammatory, antiscarring and antiangiogenic actions.

[00197] As described herein, nHC-HA/PTX3 complexes are also found in the umbilical cord (UC). The UC HC-HA/PTX3 complexes differ in their biochemical composition with respect to HA content and the presence and/or relative abundance of various components of the complex, including proteoglycans, such as small leucine rich proteoglycans (SLRPs). In some embodiments, the SLRP is decorin, biglycan and/or osteoadherin. As described herein, the complexes also differ in content with respect to the presence of particular sulfated glycosaminoglycans, such keratan sulfate. In addition, as described herein, complexes isolated from AM or UC using different extraction methods (e.g., PBS versus GnHCl extraction) resulted in complexes with different biochemical compositions and biological properties. In certain instances, it is found that complexes

isolated from an insoluble fraction by GnHCl extraction from umbilical cord tissue exhibit improved properties.

[00198] PTX3 is a multimeric protein that has been shown to interact directly with TSG-6 and IaI HCs. PTX3 is upregulated in response to inflammatory regulators and has been shown to play an important role in the organization of HA in the extracellular matrix of the cumulous oophorous during oocyte maturation. As demonstrated herein, PTX3 also is found within nHC-HA complexes (i.e. nHC-HA/PTX3) of the amniotic membrane and umbilical cord and plays a critical role in M2 macrophage polarization.

[00199] M1 macrophages, or classically activated proinflammatory macrophages are induced by interferon (IFN) alone or in combination with lipopolysaccharide (LPS) or tumor necrosis factor (TNF) α . M1 macrophages are typically characterized by high expression of interleukin-12 (IL-12) and IL-23 and low levels of IL-10. In contrast, M2 macrophages or “alternatively activated” macrophages display wound healing and tissue regenerative properties and are characterized by low IL-12/IL-23 and high IL-10 or about the same ratio of IL-12 to IL-10. In certain instances, M2 macrophages also have a high expression of TGF- β .

[00200] The examples provided herein demonstrate that PTX3 binds directly to immobilized HA as evidenced by resistance to dissociating agents. It is demonstrated herein that *in vitro* reconstituted complexes of HA bound to PTX3 exhibit different properties compared to *in vitro* reconstituted complexes of HA bound to TSG-6. For example, in some embodiments, a PTX3/HA complex promotes attachment of LPS-stimulated macrophages without aggregation and induces expression of IL-10 in an LPS-stimulated macrophage. In some embodiments, a PTX3/HA complex disclosed herein increases the expression of IL-10 by about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100% in an LPS-stimulated macrophage compared to IL-10 expression the absence of a PTX3/HA complex. In contrast, in some embodiments, the TSG-6/HA complex reduces cell attachment and promotes aggregation of LPS-stimulated macrophages and does not induce the expression of IL-10 in an LPS-stimulated macrophage. In addition, in some embodiments, TSG-6 pre-bound to HA inhibits subsequent binding of PTX3 to the complex. In some embodiments, both TSG-6/HA complex and PTX3/HA complex decreased expression of IL-12 in an LPS-stimulated macrophage. In some embodiments, a PTX3/HA complex or TSG-6/HA complex disclosed herein reduces or inhibits the expression of IL-12 by about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100% in an LPS-stimulated macrophage compared to IL-12 expression the absence of a PTX3/HA complex or TSG-6/HA complex. In some embodiments, both TSG-6/HA complex and PTX3/HA complex increased expression of IL-23 in

an LPS/IFN γ -stimulated macrophage. In some embodiments, a PTX3/HA complex or TSG-6/HA complex disclosed herein reduces or inhibits the expression of IL-23 by about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100% in an LPS/IFN γ -stimulated macrophage compared to IL-23 expression the absence of a PTX3/HA complex or TSG-6/HA complex.

[00201] In addition, it is demonstrated herein that *in vitro* reconstituted rcHC-HA/PTX3 complexes possess different biological activities dependent on whether the rcHC-HA/PTX3 complex is formed with HA pre-bound to TSG-6 in the presence of I α I followed by addition of PTX3 or HA pre-bound to PTX3 followed by addition of TSG-6 with I α I. Exemplary methods for reconstitution of rcHC-HA/PTX3 complexed formed with HA pre-bound to TSG-6 or HA pre-bound to PTX3 are provided herein. In some embodiments, rcHC-HA/PTX3 complexes formed with immobilized HA pre-bound to TSG-6 result in aggregation of LPS-stimulated macrophages. In some embodiments, rcHC-HA/PTX3 complexes formed with immobilized HA pre-bound to PTX3 promote attachment of LPS-stimulated macrophages without aggregation.

[00202] In some embodiments, rcHC-HA/PTX3 complexes formed with immobilized HA pre-bound to PTX3 decrease or inhibit expression of M1 macrophage markers such as IL-12 and IL-23. In some embodiments, rcHC-HA/PTX3 complexes formed with immobilized HA pre-bound to PTX3 decrease expression of IL-12 in an LPS-stimulated macrophage compared to IL-12 expression the absence of rcHC-HA/PTX3 complex. In some embodiments, an rcHC-HA/PTX3 complex disclosed herein reduces or inhibits the expression of IL-12 by about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100% in an LPS-stimulated macrophage compared to IL-12 expression the absence of rcHC-HA/PTX3 complex. In some embodiments, rcHC-HA/PTX3 complexes formed with immobilized HA pre-bound to PTX3 decrease or inhibit expression of IL-23 in an LPS/IFN γ -stimulated macrophage compared to IL-12 expression the absence of rcHC-HA/PTX3 complex. In some embodiments, rcHC-HA/PTX3 complexes formed with immobilized HA pre-bound to PTX3 decrease or inhibit expression of IL-23 by about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100% in an LPS/IFN γ -stimulated macrophage compared to IL-23 expression the absence of rcHC-HA/PTX3 complex. In some embodiments, rcHC-HA/PTX3 complexes formed with immobilized HA pre-bound to PTX3 replicate the activity of nHC-HA/PTX3 complexes isolated from amniotic membrane.

[00203] In some embodiments, rcHC-HA/PTX3 complexes formed with immobilized HA pre-bound to TSG-6 decrease or inhibit expression of M1 macrophage markers such as IL-12, but increase expression of IL-23. In some embodiments, rcHC-HA/PTX3 complexes formed with

immobilized HA pre-bound to TSG-6 decrease or inhibit expression of IL-12. In some embodiments, reHC-HA/PTX3 complexes formed with immobilized HA pre-bound to TSG-6 increase expression of IL-23.

[00204] Provided herein are methods of producing reconstituted HC-HA/PTX3 complexes using immobilized HA pre-bound to PTX3 and uses thereof. Also provided herein are complexes of immobilized HA pre-bound to PTX3 and uses thereof. Also provided herein are methods of producing reconstituted HC-HA/PTX3 complexes using immobilized HA pre-bound to TSG-6 and uses thereof. In some embodiments, the reconstituted HC-HA/PTX3 complexes provided herein are administered to treat a wide variety of diseases or conditions, including, but not limited to, the treatment, such as the inhibition, reduction, prevention or lowering the risk, of inflammation, immune reaction leading to autoimmune or immune rejection, adhesion, scarring, angiogenesis, conditions requiring cell or tissue regeneration, tissue reperfusion injury due to ischemia, including myocardial infarction and stroke, and the symptoms caused thereby. In some embodiments, the reconstituted HC-HA/PTX3 complexes provided herein are administered to treat inflammation. In some embodiments, the reconstituted HC-HA/PTX3 complexes provided herein are administered to treat scarring. In some embodiments, the reconstituted HC-HA/PTX3 complexes provided herein are administered to treat angiogenesis. In some embodiments, the reconstituted HC-HA/PTX3 complexes provided herein are administered to treat immune reaction leading to autoimmune or immune rejection. In some embodiments, the reconstituted HC-HA/PTX3 complexes provided herein are administered to treat conditions requiring inhibition of cell adhesion. In some embodiments, the reconstituted HC-HA/PTX3 complexes provided herein are administered to treat conditions requiring cell or tissue regeneration.

[00205] In addition, the examples provided herein demonstrate the ability of HC-HA/PTX3 complexes to maintain stem cells in an undifferentiated state as well as induce adult differentiated fibroblasts to younger progenitors in a human corneal fibroblasts model. Human corneal fibroblasts are differentiated from keratocytes and upon addition of exogenous TGF- β 1, they further differentiate into scar-forming myofibroblasts. The data provided herein demonstrate that culturing the cells in the presence of HC-HA prevented cells from differentiating into myofibroblasts under TGF- β 1 stimulation. In the absence of TGF- β 1, HC-HA/PTX3 complexes revert human corneal fibroblasts into keratocytes expressing keratocan and CD34. In the presence of TGF- β 1 human corneal fibroblasts are further reprogrammed into younger progenitors that lack of keratocan expression and but express a number of neural crest cell markers such as Osr2, FGF10, and Sox9 and embryonic stem cell markers, such as c-myc, KLF4, Nanog, nestin, Oct 4, Rex-1, Sox-2, and SSEA-4.

[00206] The transcription factors Sox2, Oct4, c-Myc, and KLF4 are known play an important role in the induction of progenitor stem cells (iPSCs) from adult differentiated cells. Accordingly, in some embodiments, HC-HA/PTX3 complexes provided herein are employed to reprogram adult differentiated cells into iPSCs. In some embodiments, induction of iPSCs using an HC-HA/PTX3 complex in combination with one or more of Sox2, Oct4, c-Myc, and KLF4 is performed with a much higher efficiency than the conventional methods that use these four transcription factors without HC-HA/PTX3. In some embodiments, addition of HC-HA/PTX3 complex facilitates stem cell induction by turning off TGF- β signaling to prevent differentiation and by turning on BMP signaling to facilitate reprogramming into young progenitor cells such as iPSCs. In some embodiments, addition of HC-HA/PTX3 complex facilitates stem cell induction by reprogramming cells into younger progenitors and induction of stem cell markers. In some embodiments, addition of HC-HA/PTX3 complex helps maintain stem cell characteristics during ex vivo expansion, thus eliminating the need of using feeder layers made of murine embryonic fibroblasts. Hence, in some embodiments, HC-HA/PTX3 complex is used as a carrier or scaffold to help deliver stem cells that have been ex vivo expanded into the human patients to promote the efficacy of said stem cell therapies.

Methods of Production of isolated nHC-HA/PTX3 Complexes

[00207] Disclosed herein are methods of generating isolated native HC-HA/PTX3 complexes (nHC-HA/PTX3).

[00208] In some embodiments, the isolated nHC-HA/PTX3 complex is isolated from an amniotic tissue. In some embodiments, the isolated nHC-HA/PTX3 complex is isolated from an amniotic membrane or an umbilical cord. In some embodiments, the isolated nHC-HA/PTX3 complex is isolated from fresh, frozen or previously frozen placental amniotic membrane (PAM), fresh, frozen or previously frozen umbilical cord amniotic membrane (UCAM), fresh, frozen or previously frozen placenta, fresh, frozen or previously frozen umbilical cord, fresh, frozen or previously frozen chorion, fresh, frozen or previously frozen amnion-chorion, or any combinations thereof. Such tissues can be obtained from any mammal, such as, for example, but not limited to a human, non-human primate, cow or pig.

[00209] In some embodiments, the nHC-HA/PTX3 is purified by any suitable method. In some embodiments, the nHC-HA/PTX3 complex is purified by centrifugation (e.g., ultracentrifugation, gradient centrifugation), chromatography (e.g., ion exchange, affinity, size exclusion, and hydroxyapatite chromatography), gel filtration, , or differential solubility, ethanol precipitation or by any other available technique for the purification of proteins (See, e.g., Scopes, Protein Purification Principles and Practice 2nd Edition, Springer-Verlag, New York, 1987; Higgins, S. J.

and Hames, B. D. (eds.), Protein Expression: A Practical Approach, Oxford Univ Press, 1999; and Deutscher, M. P., Simon, M. I., Abelson, J. N. (eds.), Guide to Protein Purification: Methods in Enzymology (Methods in Enzymology Series, Vol 182), Academic Press, 1997, all incorporated herein by reference).

[00210] In some embodiments, the nHC-HA/PTX3 is isolated from an extract. In some embodiments, the extract is prepared from an amniotic membrane extract. In some embodiments, the extract is prepared from an umbilical cord extract. In some embodiments, the umbilical cord extract comprises umbilical cord stroma and/or Wharton's jelly. In some embodiments, the nHC-HA/PTX3 complex is contained in an extract that is prepared by ultracentrifugation. In some embodiments, the nHC-HA/PTX3 complex is contained in an extract that is prepared by ultracentrifugation using a CsCl/4-6M guanidine HCl gradient. In some embodiments, the extract is prepared by at least 2 rounds of ultracentrifugation. In some embodiments, the extract is prepared by more than 2 rounds of ultracentrifugation (i.e. nHC-HA/PTX3 2nd). In some embodiments, the extract is prepared by at least 4 rounds of ultracentrifugation (i.e. nHC-HA/PTX3 4th). In some embodiments, the nHC-HA/PTX3 complex comprises a small leucine-rich proteoglycan. In some embodiments, the nHC-HA/PTX3 complex comprises HC1, HA, PTX3 and/or a small leucine-rich proteoglycan.

[00211] In some embodiments, ultracentrifugation is performed on an extract prepared by extraction in an isotonic solution. In some embodiments, the isotonic solution is PBS. For example, in some embodiments the tissue is homogenized in PBS to produce a homogenized sample. The homogenized sample is then separated into a soluble portion and insoluble portion by centrifugation. In some embodiments, ultracentrifugation is performed on the soluble portion of the PBS-extracted tissue. In such embodiments, the nHC-HA/PTX3 purified by ultracentrifugation of the PBS-extracted tissue called an nHC-HA/PTX3 soluble complex. In some embodiments, the nHC-HA soluble complex comprises a small leucine-rich proteoglycan. In some embodiments, the nHC-HA/PTX3 soluble complex comprises HC1, HA, PTX3 and/or a small leucine-rich proteoglycan.

[00212] In some embodiments, ultracentrifugation is performed on an extract prepared by direct guanidine HCl extraction (e.g. 4-6 M GnHCl) of the amniotic membrane and/or umbilical cord tissue. In some embodiments, the GnHCl extract tissues is then centrifuged to produce GnHCl soluble and GnHCl insoluble portions. In some embodiments, ultracentrifugation is performed on the GnHCl soluble portion. In such embodiments, the nHC-HA/PTX3 purified by ultracentrifugation of the guanidine HCl-extracted tissue is called an nHC-HA/PTX3 insoluble complex. In some embodiments, the nHC-HA insoluble complex comprises a small leucine-rich

proteoglycan. In some embodiments, the nHC-HA/PTX3 insoluble complex comprises HC1, HA, PTX3 and/or a small leucine-rich proteoglycan.

[00213] In some embodiments, ultracentrifugation is performed on an extract prepared by further guanidine HCl extraction of the insoluble portion of the PBS-extracted tissue. For example, in some embodiments the tissue is homogenized in PBS to produce a homogenized sample. The homogenized sample is then separated into a soluble portion and insoluble portion by centrifugation. The insoluble portion is then further extracted in guanidine HCl (e.g. 4-6 M GnHCl) and centrifuged to produce a guanidine HCl soluble and insoluble portions. In some embodiments, ultracentrifugation is performed on the guanidine HCl soluble portion. In such embodiments, the nHC-HA/PTX3 purified by ultracentrifugation of the guanidine HCl-extracted tissue is called an nHC-HA/PTX3 insoluble complex. In some embodiments, the nHC-HA insoluble complex comprises a small leucine-rich proteoglycan. In some embodiments, the nHC-HA/PTX3 insoluble complex comprises HC1, HA, PTX3 and/or a small leucine-rich proteoglycan.

[00214] In some embodiments, the method of purifying the isolated nHC-HA/PTX3 extract comprises: (a) dissolving the isolated extract (e.g. prepared by the soluble or insoluble method described herein) in CsCl/4-6M guanidine HCl at the initial density of 1.35 g/ml, to generate a CsCl mixture, (b) centrifuging the CsCl mixture at 125,000 x g for 48 h at 15 °C, to generate a first purified extract, (c) extracting the first purified extract and dialyzing it against distilled water to remove CsCl and guanidine HCl, to generate a dialysate. In some embodiments, the method of purifying the isolated extract further comprises (d) mixing the dialysate with 3 volumes of 95% (v/v) ethanol containing 1.3% (w/v) potassium acetate at 0 °C for 1 h, to generate a first dialysate/ethanol mixture, (e) centrifuging the first dialysate/ethanol mixture at 15,000 x g, to generate a second purified extract, and (f) extracting the second purified extract. In some embodiments, the method of purifying the isolated extract further comprises: (g) washing the second purified extract with ethanol (e.g., 70% ethanol), to generate a second purified extract/ethanol mixture; (h) centrifuging the second purified extract/ethanol mixture, to generate a third purified extract; and (i) extracting the third purified extract. In some embodiments, the method of purifying the isolated extract further comprises: (j) washing the third purified extract with ethanol (e.g., 70% ethanol), to generate a third purified extract/ethanol mixture; (k) centrifuging the third purified extract/ethanol mixture, to generate a forth purified extract; and (l) extracting the forth purified extract. In some embodiments, the purified extract comprises an nHC-HA/PTX3 complex.

[00215] In some embodiments, the nHC-HA/PTX3 complex is purified by immunoaffinity chromatography. In some embodiments, anti HC1 antibodies, anti-HC2 antibodies, or both are

generated and affixed to a stationary support. In some embodiments, the unpurified HC-HA complex (i.e., the mobile phase) is passed over the support. In certain instances, the HC-HA complex binds to the antibodies (e.g., via interaction of (a) an anti-HC1 antibody and HC1, (b) an anti-HC2 antibody and HC2, (c) an anti-PTX antibody and PTX3, (d) an anti-SLRP antibody and the SLRP, or (e) any combination thereof). In some embodiments the support is washed (e.g., with PBS) to remove any unbound or loosely bound molecules. In some embodiments, the support is then washed with a solution that enables elution of the nHC-HA/PTX3 complex from the support (e.g., 1% SDS, 6M guanidine-HCl, or 8M urea).

[00216] In some embodiments, the nHC-HA/PTX3 complex is purified by affinity chromatography. In some embodiments, HABP is generated and affixed to a stationary support. In some embodiments, the unpurified nHC-HA/PTX3 complex (i.e., the mobile phase) is passed over the support. In certain instances, the nHC-HA/PTX3 complex binds to the HABP. In some embodiments the support is washed (e.g., with PBS) to remove any unbound or loosely bound molecules. In some embodiments, the support is then washed with a solution that enables elution of the HC-HA complex from the support.

[00217] In some embodiments, the nHC-HA/PTX3 complex is purified by a combination of HABP affinity chromatography, and immunoaffinity chromatography using anti HC1 antibodies, anti-HC2 antibodies, anti-PTX3 antibodies, antibodies against a SLRP or a combination of SLRPs, or any combination of antibodies thereof.

[00218] In some embodiments, the nHC-HA/PTX3 complex is purified from the insoluble fraction as described herein using one or more antibodies. In some embodiments, the nHC-HA/PTX3 complex is purified from the insoluble fraction as described herein using anti-SLRP antibodies.

[00219] In some embodiments, the nHC-HA/PTX3 complex is purified from the soluble fraction as described herein. In some embodiments, the nHC-HA/PTX3 complex is purified from the soluble fraction as described herein using anti-PTX3 antibodies.

[00220] In some embodiments, the nHC-HA/PTX3 complex comprises a small leucine rich proteoglycan (SLRP). In some embodiments, the nHC-HA/PTX3 complex comprises a class I, class II or class II SLRP. In some embodiments, the small leucine-rich proteoglycan is selected from among class I SLRPs, such as decorin and biglycan. In some embodiments, the small leucine-rich proteoglycan is selected from among class II SLRPs, such as fibromodulin, lumican, PRELP (proline arginine rich end leucine-rich protein), keratocan, and osteoadherin. In some embodiments, the small leucine-rich proteoglycan is selected from among class III SLRPs, such as epipycan and osteoglycin. In some embodiments, the small leucine-rich proteoglycan is selected from among bikunin, decorin, biglycan, and osteoadherin. In some embodiments, the small leucine-rich protein

comprises a glycosaminoglycan. In some embodiments, the small leucine-rich proteoglycan comprises keratan sulfate.

Methods of Production of rcHC-HA/PTX3 Complexes

[00221] Disclosed herein are methods of generating reconstituted HC-HA/PTX3 complexes (rcHC-HA/PTX3) with or without SLRPs. Also disclosed herein are rcHC-HA/PTX3 complexes and intermediate combinations of components generated by such methods.

[00222] In some embodiments, a method for generating reconstituted HC-HA/PTX3 complexes comprises (a) contacting immobilized high molecular weight hyaluronan (HMW HA) with pentraxin 3 (PTX3) under suitable conditions to form a PTX3/HA complex, and (b) contacting the PTX3/HA complex with I α I and Tumor necrosis factor-Stimulated Gene-6 (TSG-6). Provided herein are rcHC-HA/PTX3 complexes produced by such method. In some embodiments, TSG-6 catalyzes the transfer of heavy chain 1 (HC1) of inter- α -inhibitor (I α I) to HA. In some embodiments, HC1 of I α I forms a covalent linkage with HA. In some embodiments, the steps (a) and (b) of the method are performed sequentially in order.

[00223] In some embodiments, a method for generating reconstituted HC-HA/PTX3 complexes comprises contacting a PTX3/HA complex with I α I and TSG-6. In some embodiments, TSG-6 catalyzes the transfer of heavy chain 1 (HC1) of inter- α -inhibitor (I α I) to HA. Provided herein are rcHC-HA/PTX3 complexes produced by such method. In some embodiments, HC1 of I α I forms a covalent linkage with HA.

[00224] In some embodiments, a method for generating a complex of HA bound to PTX3 comprises contacting immobilized high molecular weight hyaluronan (HMW HA) with pentraxin 3 (PTX3) under suitable conditions to form a PTX3/HA complex. Provided herein are PTX3/HA complexes produced by such method.

[00225] In some embodiments, a method for generating reconstituted HC-HA/PTX3 complexes comprises (a) contacting immobilized high molecular weight hyaluronan (HMW HA) with I α I and TSG-6 to HA to form an HC-HA complex pre-bound to TSG-6 and (b) contacting the HC-HA complex with pentraxin 3 (PTX3) under suitable conditions to form an rcHC-HA/PTX3 complex. Provided herein are rcHC-HA/PTX3 complexes produced by such method. In some embodiments, HC1 of I α I forms a covalent linkage with HA. In some embodiments, the steps (a) and (b) of the method are performed sequentially in order. In some embodiments, the method comprises contacting an HC-HA complex pre-bound to TSG-6 with PTX3.

[00226] In some embodiments, the method comprises first contacting high molecular weight hyaluronan (HMW HA) with pentraxin 3 (PTX3) under suitable conditions to form a PTX3/HA complex, then contacting the PTX3/HA complex with I α I and TSG-6.

[00227] In some embodiments, the IαI protein and TSG-6 protein are contacted to the complex at a molar ratio of about 1:1, 2:1, 3:1, 4:1, 5:1, 6:1, 7:1, 8:1, 9:1, 10:1, 15:1, or 20:1 (IαI:TSG-6). In some embodiments the ratio of IαI:TSG-6 ranges from about 1:1 to about 20:1, such as about 1:1 to about 10:1, such as about 1:1 to 5 about:1, such as about 1:1 to about 3:1. In some embodiments, the ratio of IαI:TSG-6 is 3:1 or higher. In some embodiments, the ratio of IαI:TSG-6 is 3:1.

[00228] In some embodiments, the steps (a) and (b) of the method are performed sequentially in order. In some embodiments, the method comprises contacting a PTX3/HA complex with IαI and TSG-6.

[00229] In certain instances, TSG-6 interacts with IαI and forms covalent complexes with HC1 and HC2 of IαI (i.e. HC1•TSG-6 and HC2•TSG-6). In certain instances, in the presence of HA, the HCs are transferred to HA to form rcHC-HA. In some embodiments, a TSG-6•HC1 complex is added to pre-bound PTX3/HA complex to catalyze the transfer of HC1 to HA. In some embodiments, the method comprises first contacting immobilized high molecular weight hyaluronan (HMW HA) with pentraxin 3 (PTX3) under suitable conditions to form a PTX3/HA complex, then contacting the PTX3/HA complex with a HC1•TSG-6 complex. In some embodiments, a combination of HC1•TSG-6 complex and HC2•TSG-6 complex is added to a PTX3/HA complex.

[00230] In some embodiments, the step of contacting PTX3 to immobilized HMW HA occurs for at least 10 minutes, at least 30 minutes, at least 1 hour, at least 2 hours, at least 3 hours, at least 4 hours, at least 5 hours, at least 6 hours, at least 12 hours, or at least 24 hours or longer. In some embodiments, the step of contacting PTX3 to immobilized HMW HA occurs for at least 2 hours or longer. In some embodiments, the step of contacting PTX3 to immobilized HMW HA occurs for at least 2 hours. In some embodiments, the step of contacting PTX3 to immobilized HMW HA occurs at 37 °C. In some embodiments, the step of contacting PTX3 to immobilized HMW HA occurs in 5 mM MgCl₂ in PBS.

[00231] In some embodiments, the step of contacting the PTX3/HA complex with IαI and TSG-6 to HA occurs for at least 10 minutes, at least 30 minutes, at least 1 hour, at least 2 hours, at least 3 hours, at least 4 hours, at least 5 hours, at least 6 hours, at least 12 hours, or at least 24 hours or longer. In some embodiments the step of contacting the PTX3/HA complex with a HC1•TSG-6 complex and/or a HC2•TSG-6 complex occurs for at least 10 minutes, at least 30 minutes, at least 1 hour, at least 2 hours, at least 3 hours, at least 4 hours, at least 5 hours, at least 6 hours, at least 12 hours, or at least 24 hours or longer. In some embodiments the step of contacting the PTX3/HA complex with a HC1•TSG-6 complex and/or a HC2•TSG-6 complex occurs for at least 2 hours or longer. In some embodiments the step of contacting the PTX3/HA complex with a HC1•TSG-6

complex and/or a HC2•TSG-6 complex occurs for at least 2 hours. In some embodiments the step of contacting the PTX3/HA complex with a HC1•TSG-6 complex and/or a HC1•TSG-6 complex occurs at 37 °C. In some embodiments the step of contacting the PTX3/HA complex with a HC1•TSG-6 complex and/or a HC1•TSG-6 complex occurs in 5 mM MgCl₂ in PBS.

[00232] In some embodiments, the method comprises contacting high molecular weight hyaluronan (HMW HA) with a pentraxin 3 (PTX3) protein, inter- α -inhibitor (I α I) protein comprising heavy chain 1 (HC1) and Tumor necrosis factor α -stimulated gene 6 (TSG-6) simultaneously under suitable conditions to form a HC-HA/PTX3 complex. In some embodiments, the contacting the HMW HA with PTX3, I α I and TSG-6 occurs for at least 10 minutes, at least 30 minutes, at least 1 hour, at least 2 hours, at least 3 hours, at least 4 hours, at least 5 hours, at least 6 hours, at least 12 hours, or at least 24 hours or longer. In some embodiments the step of contacting the HMW HA, PTX3, I α I, and TSG-6 occurs at 37 °C. In some embodiments the step of contacting the HMW HA, PTX3, I α I, and TSG-6 occurs in 5 mM MgCl₂ in PBS.

[00233] In some embodiments, the method comprises contacting high molecular weight hyaluronan (HMW HA) with a pentraxin 3 (PTX3) protein, inter- α -inhibitor (I α I) protein comprising heavy chain 1 (HC1) and Tumor necrosis factor α -stimulated gene 6 (TSG-6) sequentially, in any order, under suitable conditions to form a HC-HA/PTX3 complex. In some embodiments, the contacting the HMW HA with PTX3, I α I and TSG-6 occurs for at least 10 minutes, at least 30 minutes, at least 1 hour, at least 2 hours, at least 3 hours, at least 4 hours, at least 5 hours, at least 6 hours, at least 12 hours, or at least 24 hours or longer. In some embodiments the step of contacting the HMW HA, PTX3, I α I, and TSG-6 occurs at 37 °C. In some embodiments the step of contacting the HMW HA, PTX3, I α I, and TSG-6 occurs in 5 mM MgCl₂ in PBS.

[00234] In some embodiments, the methods for production of an rcHC-HA/PTX3 complex further comprises addition of one or more small leucine rich proteoglycans (SLRPs). In some embodiments, a method for generating reconstituted HC-HA/PTX3 complexes comprises (a) contacting immobilized high molecular weight hyaluronan (HMW HA) with pentraxin 3 (PTX3) under suitable conditions to form a PTX3/HA complex, (b) contacting the PTX3/HA complex with I α I and Tumor necrosis factor-Stimulated Gene-6 (TSG-6) and (c) contacting the PTX3/HA complex with one or more SLRPS. Provided herein are rcHC-HA/PTX3 complexes produced by such method. In some embodiments, TSG-6 catalyzes the transfer of heavy chain 1 (HC1) of inter- α -inhibitor (I α I) to HA. In some embodiments, HC1 of I α I forms a covalent linkage with HA. In some embodiments, the steps (a), (b), and (c) of the method are performed sequentially in order. In some embodiments, the steps (a), (b), and (c) of the method are performed simultaneously. In some embodiments, the step (a) of the method is performed and then steps (b) and (c) of the method are

performed sequentially in order. In some embodiments, the step (a) of the method is performed and then steps (b) and (c) of the method are performed simultaneously.

[00235] In some embodiments, a method for generating reconstituted HC-HA/PTX3 complexes comprises (a) contacting immobilized high molecular weight hyaluronan (HMW HA) with IαI and TSG-6 to HA to form an HC-HA complex pre-bound to TSG-6, (b) contacting the HC-HA complex with pentraxin 3 (PTX3) and (c) contacting the HC-HA complex with one or more SLRPS under suitable conditions to form an rcHC-HA/PTX3 complex. Provided herein are rcHC-HA/PTX3 complexes produced by such method. In some embodiments, HC1 of IαI forms a covalent linkage with HA. In some embodiments, the method comprises contacting an HC-HA complex pre-bound to TSG-6 with PTX3. In some embodiments, the steps (a), (b), and (c) of the method are performed sequentially in order. In some embodiments, the steps (a), (b), and (c) of the method are performed simultaneously. In some embodiments, the step (a) of the method is performed and then steps (b) and (c) of the method are performed sequentially in order. In some embodiments, the step (a) of the method is performed and then steps (b) and (c) of the method are performed simultaneously.

[00236] In some embodiments, the SLRP is selected from among a class I, class II or class II SLRP. In some embodiments, the SLRP is selected from among class I SLRPs, such as decorin and biglycan. In some embodiments, the small leucine-rich proteoglycan is selected from among class II SLRPs, such as fibromodulin, lumican, PRELP (proline arginine rich end leucine-rich protein), keratocan, and osteoadherin. In some embodiments, the small leucine-rich proteoglycan is selected from among class III SLRPs, such as epipycan and osteoglycin. In some embodiments, the small leucine-rich proteoglycan is selected from among bikunin, decorin, biglycan, and osteoadherin. In some embodiments, the small leucine-rich protein comprises a glycosaminoglycan. In some embodiments, the small leucine-rich proteoglycan comprises keratan sulfate.

[00237] PTX3

[00238] In some embodiments, PTX3 for use in the methods is isolated from a cell or a plurality of cells (e.g., a tissue extract). Exemplary cells suitable for the expression of PTX3 include, but are not limited to, animal cells including, but not limited to, mammalian cells, primate cells, human cells, rodent cells, insect cells, bacteria, and yeast, and plant cells, including, but not limited to, algae, angiosperms, gymnosperms, pteridophytes and bryophytes. In some embodiments, PTX3 for use in the methods is isolated from a human cell. In some embodiments, PTX3 for use in the methods is isolated from a cell that is stimulated with one or more proinflammatory cytokines to upregulate PTX3 expression. In some embodiments, the proinflammatory cytokine is IL-1 or TNF-α.

[00239] In some embodiments, PTX3 for use in the methods is isolated from an amniotic membrane cell. In some embodiments, PTX3 for use in the methods is isolated from an amniotic membrane cell from an umbilical cord. In some embodiments, the amniotic membrane cell is stimulated with or more proinflammatory cytokines to upregulate PTX3 expression. In some embodiments, the proinflammatory cytokine is IL-1 or TNF- α .

[00240] In some embodiments, PTX3 for use in the methods is isolated from an umbilical cord cell. In some embodiments, the umbilical cord cell is stimulated with or more proinflammatory cytokines to upregulate PTX3 expression. In some embodiments, the proinflammatory cytokine is IL-1 or TNF- α .

[00241] In some embodiments, PTX3 for use in the methods is isolated from an amniotic epithelial cell. In some embodiments, PTX3 for use in the methods is isolated from an umbilical cord epithelial cell. In some embodiments, the amniotic epithelial cell or umbilical cord epithelial cell is stimulated with or more proinflammatory cytokines to upregulate PTX3 expression. In some embodiments, the proinflammatory cytokine is IL-1 or TNF- α .

[00242] In some embodiments, PTX3 for use in the methods is isolated from an amniotic stromal cell. In some embodiments, PTX3 for use in the methods is isolated from an umbilical cord stromal cell. In some embodiments, the amniotic stromal cell or umbilical cord stromal cell is stimulated with or more proinflammatory cytokines to upregulate PTX3 expression. In some embodiments, the proinflammatory cytokine is IL-1 or TNF- α .

[00243] In some embodiments, PTX3 for use in the methods is a native PTX3 protein isolated from a cell. In some embodiments, the cell is stimulated with or more proinflammatory cytokines to upregulate PTX3 expression. In some embodiments, the proinflammatory cytokine is IL-1 or TNF- α .

[00244] In some embodiments, PTX3 is prepared by recombinant technology. In some embodiments, PTX3 is expressed from a recombinant expression vector. In some embodiments, nucleic acid encoding PTX3 is operably linked to a constitutive promoter. In some embodiments, nucleic acid encoding PTX3 is operably linked to an inducible promoter. In some embodiments, PTX3 is expressed in a transgenic animal. In some embodiments, PTX3 is a recombinant protein. In some embodiments, PTX3 is a recombinant protein isolated from a cell. In some embodiments, PTX3 is a recombinant protein produced in a cell-free extract.

[00245] In some embodiments, PTX3 is purified from amniotic membrane, umbilical cord, umbilical cord amniotic membrane, chorionic membrane, amniotic fluid, or a combination thereof. In some embodiments, PTX3 is purified from amniotic membrane cells. In some embodiments, the amniotic membrane cell is an amniotic epithelial cell. In some embodiments, the amniotic

membrane cell is an umbilical cord epithelial cell. In some embodiments, the amniotic membrane cell is an amniotic stromal cell. In some embodiments, the amniotic membrane cell is an umbilical cord stromal cell. In some embodiments, the amniotic membrane cell is stimulated with or more proinflammatory cytokines to upregulate PTX3 expression. In some embodiments, the proinflammatory cytokine is IL-1 or TNF- α .

[00246] In some embodiments, PTX3 is not isolated from a cell or a plurality of cells (e.g., a tissue extract).

[00247] In some embodiments, PTX3 comprises a polypeptide having the sequence set forth in SEQ ID NO: 33 or a variant thereof having at least 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% sequence amino acid identity to the polypeptide having the sequence set forth in SEQ ID NO: 33. Exemplary variants include, for example, species variants, allelic variants and variants that contain conservative and non-conservative amino acid mutations. In some embodiments, PTX3 comprises a fragment of PTX3 sufficient to bind to HA and facilitate the formation of rcHC-HA/PTX3 complex. In some embodiments, PTX3 comprises Glu18 to Ser277 of human PTX3. Variants of PTX3 for use in the provided methods include variants with an amino acid modification that is an amino acid replacement (substitution), deletion or insertion. In some embodiments, such modification improves one or more properties of the PTX3 polypeptides such as improving the one or more therapeutic properties of the rcHC-HA/PTX3 complex (e.g., anti-inflammatory, anti-immune, anti-angiogenic, anti-scarring, anti-adhesion, regeneration or other therapeutic activities as described herein).

[00248] In some embodiments PTX3 protein is obtained from a commercial source. An exemplary commercial source for PTX3 is, but is not limited to, PTX3 (Catalog No. 1826-TS; R&D Systems, Minneapolis, MN).

[00249] In some embodiments, the PTX3 protein used in the methods is a multimeric protein. In some embodiments, the PTX3 protein used in the methods is a homomultimer. In some embodiments, the homomultimer is a dimer, trimer, tetramer, hexamer, pentamer, or octamer. In some embodiments, the PTX3 homomultimer is a trimer, tetramer, or octamer. In particular embodiments, the PTX3 homomultimer is an octamer. In some embodiments, the multimerization domain is modified to improve multimerization of the PTX3 protein. In some embodiments, the multimerization domain is replaced with a heterogeneous multimerization domain (e.g., an Fc multimerization domain or leucine zipper) that when fused to PTX3 improves the multimerization of PTX3.

[00250] *TSG-6*

[00251] In some embodiments, TSG-6 for use in the methods is isolated from a cell or a plurality of cells (e.g., a tissue extract). Exemplary cells suitable for the expression of TSG-6 include, but are not limited to, animal cells including, but not limited to, mammalian cells, primate cells, human cells, rodent cells, insect cells, bacteria, and yeast, and plant cells, including, but not limited to, algae, angiosperms, gymnosperms, pteridophytes and bryophytes. In some embodiments, TSG-6 for use in the methods is isolated from a human cell. In some embodiments, TSG-6 for use in the methods is isolated from a cell that is stimulated with one or more proinflammatory cytokines to upregulate TSG-6 expression. In some embodiments, the proinflammatory cytokine is IL-1 or TNF- α .

[00252] In some embodiments, TSG-6 for use in the methods is isolated from an amniotic membrane cell. In some embodiments, TSG-6 for use in the methods is isolated from an amniotic membrane cell from an umbilical cord. In some embodiments, TSG-6 for use in the methods is isolated from an amniotic membrane cell that is stimulated with one or more proinflammatory cytokines to upregulate TSG-6 expression. In some embodiments, the proinflammatory cytokine is IL-1 or TNF- α .

[00253] In some embodiments, TSG-6 for use in the methods is isolated from an umbilical cord cell. In some embodiments, TSG-6 for use in the methods is isolated from an umbilical cord cell that is stimulated with one or more proinflammatory cytokines to upregulate TSG-6 expression. In some embodiments, the proinflammatory cytokine is IL-1 or TNF- α .

[00254] In some embodiments, TSG-6 for use in the methods is isolated from an amniotic epithelial cell. In some embodiments, TSG-6 for use in the methods is isolated from an umbilical cord epithelial cell. In some embodiments, TSG-6 for use in the methods is isolated from an amniotic epithelial cell or an umbilical cord epithelial cell that is stimulated with one or more proinflammatory cytokines to upregulate TSG-6 expression. In some embodiments, the proinflammatory cytokine is IL-1 or TNF- α .

[00255] In some embodiments, TSG-6 for use in the methods is isolated from an amniotic stromal cell. In some embodiments TSG-6 for use in the methods is isolated from an umbilical cord stromal cell. In some embodiments, TSG-6 for use in the methods is isolated from an amniotic stromal cell or an umbilical cord stromal cell that is stimulated with one or more proinflammatory cytokines to upregulate TSG-6 expression. In some embodiments, the proinflammatory cytokine is IL-1 or TNF- α .

[00256] In some embodiments, TSG-6 for use in the methods is a native TSG-6 protein isolated from a cell. In some embodiments, the cell is stimulated with or more proinflammatory cytokines to

upregulate TSG-6 expression. In some embodiments, the proinflammatory cytokine is IL-1 or TNF- α .

[00257] In some embodiments, TSG-6 is prepared by recombinant technology. In some embodiments, TSG-6 is expressed from a recombinant expression vector. In some embodiments, nucleic acid encoding TSG-6 is operably linked to a constitutive promoter. In some embodiments, nucleic acid encoding TSG-6 is operably linked to an inducible promoter. In some embodiments, TSG-6 is expressed in a transgenic animal. In some embodiments, TSG-6 is a recombinant protein. In some embodiments, TSG-6 is a recombinant protein isolated from a cell. In some embodiments, TSG-6 is a recombinant protein produced in a cell-free extract.

[00258] In some embodiments, TSG-6 is purified from amniotic membrane, amniotic membrane, chorionic membrane, amniotic fluid, or a combination thereof. In some embodiments, PTX3 is purified from amniotic membrane cells. In some embodiments, the amniotic membrane cell is an amniotic epithelial cell. In some embodiments, the amniotic epithelial cell is an umbilical cord epithelial cell. In some embodiments, the amniotic membrane cell is an amniotic stromal cell. In some embodiments, the amniotic membrane cell is an umbilical cord stromal cell. In some embodiments, the amniotic membrane cell is stimulated with or more proinflammatory cytokines to upregulate TSG-6 expression. In some embodiments, the proinflammatory cytokine is IL-1 or TNF- α .

[00259] In some embodiments, TSG-6 is not isolated from a cell or a plurality of cells (e.g., a tissue extract).

[00260] In some embodiments, TSG-6 comprises a fragment of TSG-6 that is sufficient to facilitate or catalyze the transfer HC1 of IaI to HA. In some embodiments, TSG-6 comprises the link module of TSG-6. In some embodiments, TSG-6 comprises amino acids Trp18 through Leu277 of TSG-6. In some embodiments, TSG-6 comprises a polypeptide having the sequence set forth in SEQ ID NO: 2 or a variant thereof having at least 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% sequence amino acid identity to the polypeptide having the sequence set forth in SEQ ID NO: 2. Exemplary variants include, for example, species variants, allelic variants and variants that contain conservative and non-conservative amino acid mutations. Natural allelic variants of human TSG-6 include, for example, TSG-6 containing the amino acid replacement Q144R. Variants of TSG-6 or HA binding fragments thereof for use in the provided methods include variants with an amino acid modification that is an amino acid replacement (substitution), deletion or insertion. In some embodiments, such modification improve one or more properties of the TSG-6 polypeptides such as improved transfer of HC1 of IaI to HA or improved release of the TSG-6 polypeptide from the rcHC-HA/PTX3 complex following transfer of HC1 of IaI to HA.

[00261] In some embodiments, TSG-6 comprises an affinity tag. Exemplary affinity tags include but are not limited to a hemagglutinin tag, a poly-histidine tag, a myc tag, a FLAG tag, a glutathione-S-transferase (GST) tag. Such affinity tags are well known in the art for use in purification. In some embodiments, such an affinity tag incorporated into the TSG-6 polypeptide as a fusion protein or via a chemical linker. In some embodiments, TSG-6 comprises an affinity tag and the unbound TSG-6 is removed from the rcHC-HA/PTX3 complex by affinity purification.

[00262] In some embodiments TSG-6 protein is obtained from a commercial source. An exemplary commercial source for TSG-6 is, but is not limited to, TSG-6 (Catalog No. 2104-TS R&D Systems, Minneapolis, MN).

[00263] *IαI*

[00264] In some embodiments, the IαI comprises an HC1 chain. In some embodiments, the IαI comprises an HC1 and an HC2 chain. In some embodiments, the IαI comprises an HC1 and bikunin. In some embodiments, the IαI comprises an HC1, and HC2 chain and bikunin. In some embodiments, the IαI comprises an HC1, and HC2 chain and bikunin linked by a chondroitin sulfate chain.

[00265] In some embodiments, IαI is isolated from a biological sample. In some embodiments the biological sample is a biological sample from a mammal. In some embodiments, the mammal is a human. In some embodiments, the biological sample is a blood, serum, plasma, liver, amniotic membrane, chorionic membrane or amniotic fluid sample. In some embodiments, the biological sample is a blood, serum, or plasma sample. In some embodiments, the biological sample is a blood sample. In some embodiments, the biological sample is a serum sample. In some embodiments, the biological sample is a plasma sample. In some embodiments, the IαI is purified from human blood, plasma or serum. In some embodiments, IαI is isolated from human serum. In some embodiments, IαI is not isolated from serum. In some embodiments, IαI for use in the methods is produced in an amniotic membrane cell. In some embodiments, IαI for use in the methods is produced in an umbilical cord cell. In some embodiments, IαI for use in the methods is produced in an amniotic membrane cell from an umbilical cord. In some embodiments, IαI for use in the methods is produced in an amniotic epithelial cell. In some embodiments, IαI for use in the methods is produced in an umbilical cord epithelial cell. In some embodiments, IαI for use in the methods is produced in an amniotic stromal cell. In some embodiments, IαI for use in the methods is produced in an umbilical cord stromal cell. In some embodiments, IαI for use in the methods is produced in a hepatic cell. In some embodiments, IαI is prepared by recombinant technology.

[00266] In some embodiments, HC1 of IαI is isolated from a biological sample. In some embodiments the biological sample is a biological sample from a mammal. In some embodiments,

the mammal is a human. In some embodiments, the biological sample is a blood, serum, plasma, liver, amniotic membrane, chorionic membrane or amniotic fluid sample. In some embodiments, the biological sample is a blood, serum, or plasma sample. In some embodiments, the biological sample is a blood sample. In some embodiments, the biological sample is a serum sample. In some embodiments, the biological sample is a plasma sample. In some embodiments, the HC1 of IaI is purified from human blood, plasma or serum. In some embodiments, IaI is isolated from human serum. In some embodiments, HC1 of IaI is not purified from serum. In some embodiments, HC1 of IaI is prepared by recombinant technology. In some embodiments, HC1 of IaI is purified from hepatic cells. In some embodiments, HC1 of IaI is purified from amniotic membrane cells. In some embodiments, HC1 of IaI is purified from amniotic epithelial cells or umbilical cord epithelial cells. In some embodiments, HC1 of IaI is purified from amniotic stromal cells or umbilical cord stromal cells.

[00267] In some embodiments, HC1 comprises a polypeptide having the sequence set forth in SEQ ID NO: 47 or a polypeptide having at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% sequence amino acid identity to the polypeptide having the sequence set forth in SEQ ID NO: 47.

[00268] In some embodiments, HC2 of IaI is isolated from a biological sample. In some embodiments the biological sample is a biological sample from a mammal. In some embodiments, the mammal is a human. In some embodiments, the biological sample is a blood, serum, plasma, liver, amniotic membrane, chorionic membrane or amniotic fluid sample. In some embodiments, the biological sample is a blood, serum, or plasma sample. In some embodiments, the biological sample is a blood sample. In some embodiments, the biological sample is a serum sample. In some embodiments, the biological sample is a plasma sample. In some embodiments, the HC2 of IaI is purified from human blood, plasma or serum. In some embodiments, HC2 of IaI is isolated from human serum. In some embodiments, HC2 of IaI is isolated from human serum. In some embodiments, HC2 of IaI is not isolated from blood serum. In some embodiments, HC2 of IaI is prepared by recombinant technology. In some embodiments, HC2 of IaI is purified from hepatic cells. In some embodiments, HC2 of IaI is purified from amniotic membrane cells. In some embodiments, HC2 of IaI is purified from amniotic epithelial cells or umbilical cord epithelial cells. In some embodiments, HC2 of IaI is purified from amniotic stromal cells or umbilical cord stromal cells.

[00269] In some embodiments, HC2 comprises a polypeptide having the sequence set forth in SEQ ID NO: 49 or a polypeptide having at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or

99% sequence amino acid identity to the polypeptide having the sequence set forth in SEQ ID NO: 49.

[00270] In some embodiments, IaI comprises bikunin. In some embodiments, bikunin comprises a polypeptide having the sequence set forth in SEQ ID NO: 53 or a polypeptide having at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% sequence amino acid identity to the polypeptide having the sequence set forth in SEQ ID NO: 53. In some embodiments, IaI comprises a chondroitin sulfate chain.

[00271] HA

[00272] In some embodiments, HA is purified from a cell, tissue or a fluid sample. In some embodiments, HA is obtained from a commercial supplier (e.g., Sigma Aldrich or Advanced Medical Optics, Irvine, CA (e.g., Healon)). In some embodiments, HA is obtained from a commercial supplier as a powder. In some embodiments, HA is expressed in a cell. Exemplary cells suitable for the expression of HA include, but are not limited to, animal cells including, but not limited to, mammalian cells, primate cells, human cells, rodent cells, insect cells, bacteria, and yeast, and plant cells, including, but not limited to, algae, angiosperms, gymnosperms, pteridophytes and bryophytes. In some embodiments, HA is expressed in a human cell. In some embodiments, HA is expressed in a transgenic animal. In some embodiments, HA is obtained from a cell that expresses a hyaluronan synthase (e.g., HAS1, HAS2, and HAS3). In some embodiments, the cell contains a recombinant expression vector that expresses an HA synthase. In certain instances, an HA synthase lengthens hyaluronan by repeatedly adding glucuronic acid and N-acetylglucosamine to the nascent polysaccharide as it is extruded through the cell membrane into the extracellular space.

[00273] HA for use in the methods is typically high molecular weight (HMW) HA. In some embodiments, the weight average molecular weight of HMW HA is greater than about 500 kilodaltons (kDa), such as, for example, between about 500 kDa and about 10,000 kDa, between about 800 kDa and about 8,500 kDa, between about 1100 kDa and about 5,000 kDa, or between about 1400 kDa and about 3,500 kDa. In some embodiments, the weight average molecular weight of HMW HA is about 3000 kDa.

[00274] Additional Components

[00275] In some embodiments, one or more additional components are added to generate an rcHC-HA/PTX3 complex. In some embodiments, a small leucine rich proteoglycan (SLRP) is added to generate an rcHC-HA/PTX3 complex. In some embodiments, the SLRP is a class I, class II or class II SLRP. In some embodiments, the SLRP is selected from among class I SLRPs, such as decorin and biglycan. In some embodiments, the SLRP is selected from among class II SLRPs, such as

fibromodulin, lumican, PRELP (proline arginine rich end leucine-rich protein), keratocan, and osteoadherin. In some embodiments, the SLRP is selected from among class III SLRPs, such as epipycan and osteoglycin. In some embodiments, the SLRP is selected from among bikunin, decorin, biglycan, and osteoadherin. In some embodiments, the SLRP comprises a glycosaminoglycan. In some embodiments, the SLRP comprises keratan sulfate.

[00276] *HA Immobilization*

[00277] In some embodiments, HMW HA is immobilized by any suitable method. In some embodiments, HMW HA is immobilized to a solid support, such as culture dish, bead, a column or other suitable surfaces, such as, for example, a surface of an implantable medical device or a portion thereof or on a surface that is subsequently connected to or combined with an implantable medical device as described herein. In some embodiments, HMW HA is immobilized directly to the solid support, such as by chemical linkage. In some embodiments, HMW HA is attached indirectly to the solid support via a linker or an intermediary protein. Numerous heterobifunctional cross-linking reagents that are used to form covalent bonds between amino groups and thiol groups and to introduce thiol groups into proteins, are known to those of skill in this art. In some embodiments, HMW HA is immobilized directly to the solid support via crosslinking to the solid support. In some embodiments, HMW HA is immobilized directly to the solid support without crosslinking to the solid support. In some embodiments, HMW HA is immobilized directly to the solid support as a coating. In some embodiments, HMW HA is immobilized to a Covalink™-NH surface. In some embodiments, HMW HA is immobilized directly to the solid support as a coating. In some embodiments, HMW HA is immobilized to a Covalink™-NH surface for about 16 h at 4 °C.

[00278] In some embodiments, the method comprises immobilizing HMW HA to a solid surface via direct linkage to a solid support (i.e. without an intermediary protein). In some embodiments, the solid support is washed to remove unbound HMW HA prior to contacting the immobilized HA with PTX3. In some embodiments, the solid support is washed with washes of 8M GnHCl and PBS to remove unbound HMW HA prior to contacting the immobilized HA with PTX3.

[00279] In some embodiments, the method comprises immobilizing HA to a solid surface via an intermediary protein or a linker. In some embodiments, the linker is a peptide linker. In some embodiments, the intermediary protein is an HA binding protein (HABP). In some embodiments, HABP is first attached to a solid support (e.g., by cross-linking, chemical linkage or via a chemical linker). In some embodiments, the solid support comprising HABP is then contacted with HA (e.g., HMW HA) to immobilize HA to the solid support via binding of the HABP to HA. In some embodiments, the solid support is washed to remove unbound HMW HA prior to contacting the

immobilized HMW HA with PTX3. In some embodiments, the solid support is washed with washes of 8M GnHCl and PBS to remove unbound HMW HA prior to contacting the immobilized HA with PTX3.

[00280] In some embodiments, the method comprises immobilizing HA to a solid surface via attachment of a peptide linker to the solid support and attachment HA to the peptide linker. In some embodiments, the peptide linker comprises a protease cleavage site.

[00281] In some embodiments, the method comprises immobilizing HA to a solid surface via attachment of a cleavable chemical linker, such as, but not limited to a disulfide chemical linker.

[00282] In some embodiments, the HABP selected for use in the methods is an HABP that is dissociated from HA following formation of the rcHC-HA/PTX3 complex. In some embodiments, the HABP non-covalently binds to HA. In some embodiments, the method further comprises dissociating the rcHC-HA/PTX3 complex from HABP using one or more dissociating agents. Dissociating agents for the disruption of non covalent interactions (e.g., guanidine hydrochloride, urea and various detergents, e.g., SDS) are known in the art. In some embodiments the dissociating agent is urea. In some embodiments the dissociating agent is guanidine hydrochloride. In some embodiments, the dissociation agent is about 4M to about 8M guanidine-HCl. In some embodiments, the dissociation agent is about 4M, about 5M, about 6M, about 7M, about 8M guanidine-HCl. In some embodiments, the dissociation agent is about 4M to about 8M guanidine-HCl in PBS at pH 7.5.

[00283] In some embodiments, such dissociating agents are employed to dissociate the rcHC-HA/PTX3 complex from an intermediary HABP. An HABP for use in the methods typically is selected such that the binding affinity for HA is strong enough to permit assembly of the rcHC-HA/PTX3 complex but is dissociated from the rcHC-HA/PTX3 complex with a suitable dissociation agent. In some embodiments the dissociating agent is guanidine hydrochloride.

[00284] Exemplary HABPs for use with the methods provided herein include, but are not limited to, HAPLN1, HAPLN2, HAPLN3, HAPLN4, aggrecan, versican, neurocan, brevican, phosphacan, TSG-6, CD44, stabilin-1, stabilin-2, or portions thereof (e.g., link modules thereof) sufficient to bind HA. In some embodiments, the HABP comprises a polypeptide having the sequence set forth in any of SEQ ID NOS: 54-99 or a polypeptide having at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% sequence amino acid identity to the polypeptide having the sequence set forth in any of SEQ ID NOS: 54-99. In some embodiments, the HABP is versican. In some embodiments, the HABP is a recombinant protein. In some embodiments, the HABP is a recombinant mammalian protein. In some embodiments, the HABP is a recombinant human protein. In some embodiments, the HABP is a recombinant versican protein or a portion thereof sufficient to bind to HA. In some

embodiments, the HABP is a recombinant aggrecan protein or a portion thereof sufficient to bind to HA. In some embodiments, the HABP is a native HABP or a portion thereof sufficient to bind to HA. In some embodiments, the native HABP is isolated from mammalian tissue or cells. In some embodiments, the HABP is isolated from bovine nasal cartilage (e.g. HABP from Seikagaku which contains the HA binding domains of aggrecan and link protein).

[00285] In some embodiments, the HABP comprises a link module of HAPLN1, HAPLN2, HAPLN3, HAPLN4, aggrecan, versican, neurocan, brevican, phosphacan, TSG-6, CD44, stabilin-1, or stabilin-2. In some embodiments, the HABP comprising a link module comprises a polypeptide having the sequence set forth in any of link domains of SEQ ID NOS: 54-99 or a polypeptide having at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% sequence amino acid identity to the polypeptide having the sequence set forth in any of link domains of SEQ ID NOS: 54-99. In some embodiments, the HABP comprises a link module of versican. In some embodiments, the HABP comprising a link module is a recombinant protein. In some embodiments, the HABP comprising a link module of versican is a recombinant protein.

[00286] In some embodiments, the or intermediary protein, such as an HABP, contains a proteolytic cleavage sequence that is recognized by and is hydrolyzed by a site specific protease, such as furin, 3C protease, caspase, matrix metalloproteinase or TEV protease. In such embodiments, assembled rcHC-HA/PTX3 complexes are released from the solid support by contacting the immobilized complexes with a protease that cleaves the specific cleavage sequence.

[00287] In some embodiments, the rcHC-HA/PTX3 complex is purified. In some embodiments, the rcHC-HA/PTX3 complex is purified by any suitable method or combination of methods. The embodiments described below are not intended to be exclusive, only exemplary.

[00288] In some embodiments, the rcHC-HA/PTX3 complex is purified by chromatography (e.g., ion exchange, affinity, size exclusion, and hydroxyapatite chromatography), gel filtration, centrifugation (e.g., gradient centrifugation), or differential solubility, ethanol precipitation or by any other available technique for the purification of proteins.

[00289] In some embodiments, the rcHC-HA/PTX3 complex is purified by immunoaffinity chromatography. In some embodiments antibodies are generated against a component of the rcHC-HA/PTX3 complex (e.g., anti-HC1, anti-PTX, an antibody against one or more SLRPs of the rcHC-HA/PTX3 complex, e.g., anti-bikunin, anti-decorin, anti-biglycan, or anti-osteoaderin) and affixed to a solid support. In some embodiments, the unpurified rcHC-HA/PTX3 complex (i.e., the mobile phase) is passed over the support. In certain instances, the rcHC-HA/PTX3 complex binds to the antibodies. In some embodiments, the support is washed (e.g., with PBS) to remove any unbound or loosely bound molecules. In some embodiments, the support is then washed with a solution that

enables elution of the rcHC-HA/PTX3 complex from the support (e.g., 1% SDS, 6M guanidine-HCl, or 8M urea). In some embodiments, the dissociating agent is removed from the dissociated rcHC-HA/PTX3 complex. In some embodiments, the dissociating agent is removed from the dissociated rcHC-HA/PTX3 complex by a method including, but not limited to, ion-exchange chromatography, dialysis, gel filtration chromatography, ultrafiltration, or diafiltration.

[00290] In some embodiments, the rcHC-HA/PTX3 complex is purified by affinity chromatography. In some embodiments, an HABP is employed to bind to the rcHC-HA/PTX3 complex for purification of the complex and affixed to a stationary support. In some embodiments, the unpurified rcHC-HA/PTX3 complex (i.e., the mobile phase) is passed over the support. In certain instances, the rcHC-HA/PTX3 complex binds to the HABP. In some embodiments the support is washed (e.g., with PBS) to remove any unbound or loosely bound molecules. In some embodiments, the support is then washed with a solution (e.g., a dissociating agent) that enables elution of the rcHC-HA/PTX3 complex from the support. In some embodiments, the dissociating agent is removed from the dissociated rcHC-HA/PTX3 complex by a method including, but not limited to, ion-exchange chromatography, dialysis, gel filtration chromatography, ultrafiltration, or diafiltration.

[00291] In some embodiments, the rcHC-HA/PTX3 complex is purified by a combination of HABP affinity chromatography, and immunoaffinity chromatography using antibodies against one or more components of the rcHC-HA/PTX3 complex.

[00292] In some embodiments, one or more components of the rcHC-HA/PTX3 complex disclosed herein comprise an affinity tag (e.g., a fusion protein of PTX3 or HC1 with an affinity tag). Exemplary affinity tags that are incorporated into one or more components of the rcHC-HA/PTX3 complex in some embodiments include, but are not limited to, a hemagglutinin tag, poly-histidine, a myc tag, a FLAG tag, or glutathione-S-transferase sequence. In some embodiments, the ligand for the affinity tag is affixed to the solid support. In some embodiments, the unpurified rcHC-HA/PTX3 complex is passed over the support. In certain instances, the rcHC-HA/PTX3 complex binds to the ligand. In some embodiments the support is washed (e.g., with PBS) to remove any unbound or loosely bound molecules. In some embodiments, the support is then washed with a solution that enables elution of an rcHC-HA/PTX3 complex disclosed herein from the support. In some embodiments, the elution agent is removed from the dissociated rcHC-HA/PTX3 complex by a method including, but not limited to, ion-exchange chromatography, dialysis, gel filtration chromatography, ultrafiltration, or diafiltration.

[00293] In some embodiments, the PTX3, TSG-6, and/or HC1 are conjugated to a label. A “label” refers to a detectable compound or composition which is conjugated directly or indirectly to a

polypeptide so as to generate a labeled polypeptide. In some embodiments, the label is detectable by itself (e.g., radioisotope labels or fluorescent labels) or, in the case of an enzymatic label, catalyzes chemical alteration of a substrate compound composition which is detectable. Non-limiting examples of labels include fluorogenic moieties, dyes, fluorescent tags, green fluorescent protein, or luciferase.

[00294] Methods of Assessing the Activity of nHC-HA/PTX3 and rcHC-HA/PTX3 complexes

[00295] The properties of nHC-HA/PTX3 and rcHC-HA/PTX3 complexes provided herein are assessed by any suitable method including, *in vitro* and *in vivo* methods. Exemplary *in vitro* methods are provided herein and include, but are not limited, to cell culture methods that assess the ability of nHC-HA/PTX3 or rcHC-HA/PTX3 complexes to promote attachment of macrophages to the immobilized nHC-HA/PTX3 or rcHC-HA/PTX3 complexes, to inhibit or reduce aggregation of macrophages, to promote apoptosis of neutrophils, macrophage phagocytosis of apoptotic neutrophils, and M2 polarization of stimulated macrophages. In some embodiments, the macrophages used in the assay are stimulated, such as by exposure to LPS or IFN- γ . In some embodiments, the gene or protein expression in stimulated macrophages is assessed following contact with nHC-HA/PTX3 or rcHC-HA/PTX3 complexes. In such methods of assessing activity of nHC-HA/PTX3 or rcHC-HA/PTX3 complex, a suitable control is employed for comparison. In some embodiments, the control is the absence of treatment with an nHC-HA/PTX3 or rcHC-HA/PTX3 complex (i.e. a negative control).

[00296] In some embodiments, the activity of an rcHC-HA/PTX3 complex is compared to the activity of a native HC-HA/PTX3 complex. In some embodiments the native HC-HA/PTX3 is isolated from amniotic membrane.

[00297] In some embodiments, gene expression in treated macrophages is assessed by PCR, RT-PCR, Northern blotting, western blotting, dot blotting, immunohistochemistry, chromatography or other suitable method of detecting proteins or nucleic acids. In some embodiments, the level of expression of IL-10, IL-12, IL23, LIGHT and SPHK1 is assessed.

[00298] Exemplary *in vitro* methods for assessing the activity of an nHC-HA/PTX3 or rcHC-HA/PTX3 complex provided herein include, but are not limited, to animal models of various disease and conditions. A variety of animal models are available and well-known in the art for diseases and conditions, including, but not limited to, animal models (e.g. rodent and primate models) for various inflammatory and autoimmune diseases and disorders including, but not limited to, ischemia reperfusion injury, type 1 and type 2 diabetes, inflammatory diseases, collagen induced arthritis, rheumatoid arthritis, antigen induced autoimmune disease such as collagen induced arthritis and myelin peptide-induced experimental allergic encephalomyelitis,

inflammatory bowel disease (IBD)/ulcerative colitis, multiple sclerosis, surgically induced osteoarthritis and nephritis, psoriasis, inflammatory skin diseases, LPS-induced endotoxic shock, LPS-induced lung injury, allergic rhinitis, liver injury, chronic stress, asthma, and xenograft and allograft models for various cancers.

[00299] In some embodiments, the animal model is a rodent model of inflammation such as chronic graft-versus-host disease (cGVHD), HSV1-induced necrotizing stromal keratitis, or high-risk corneal transplantation. In some embodiments, reduction of inflammation by nHC-HA/PTX3 or rcHC-HA/PTX3 treatment is assessed by the measuring the proliferation and activation of T cells and the production of immune cytokines such as IL-1 α , IL-2, IL-6, IFN- γ , and TNF- α . In some embodiments, the animal model is a rodent model of scarring such as excimer laser-assisted photorefractive keratectomy (PRK). Exemplary methods for the use of such animal models are provided in the Examples provided herein.

[00300] In some embodiments, the animal model is a genetic model of inflammatory and autoimmune diseases and disorder that contains one or more genetic modifications that cause the disease or disorder. In some embodiments, such models are obtained from a commercial source. In some embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex provided herein is administered to an animal model of a particular disease or condition and the ability of the rcHC-HA/PTX3 complex to inhibit or reduce one or more symptoms of the disease or condition is assessed.

Pharmaceutical Compositions

[00301] Disclosed herein, in certain embodiments, are pharmaceutical compositions comprising nHC-HA/PTX3 or rcHC-HA/PTX3 complexes described herein. Disclosed herein, in certain embodiments, are pharmaceutical compositions comprising nHC-HA/PTX3 or rcHC-HA/PTX3 complexes produced by the methods provided herein. In some embodiments, the pharmaceutical compositions are formulated in a conventional manner using one or more physiologically acceptable carriers including excipients and auxiliaries which facilitate processing of an nHC-HA/PTX3 or rcHC-HA/PTX3 complex into preparations which are suitable for pharmaceutical use. Proper formulation is dependent upon the route of administration selected. Any of the well-known techniques, carriers, and excipients can be used as suitable and as understood in the art.

[00302] Disclosed herein, in certain embodiments, is a pharmaceutical composition comprising an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein. In some embodiments, the pharmaceutical composition further comprises at least one pharmaceutically acceptable carrier. In some embodiments, the pharmaceutical composition further comprises an adjuvant, excipient, preservative, agent for delaying absorption, filler, binder, adsorbent, buffer, and/or solubilizing

agent. Exemplary pharmaceutical compositions that are formulated to contain an nHC-HA/PTX3 or rcHC-HA/PTX3 complex provided herein include, but are not limited to, a solution, suspension, emulsion, syrup, granule, powder, ointment, tablet, capsule, pill or an aerosol.

[00303] *Dosage Forms*

[00304] In some embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is administered as an aqueous suspension. In some embodiments, an aqueous suspension comprises water, Ringer's solution and/or isotonic sodium chloride solution. In some embodiments, an aqueous suspension comprises a sweetening or flavoring agent, coloring matters or dyes and, if desired, emulsifying agents or suspending agents, together with diluents water, ethanol, propylene glycol, glycerin, or combinations thereof. In some embodiments, an aqueous suspension comprises a suspending agent. In some embodiments, an aqueous suspension comprises sodium carboxymethylcellulose, methylcellulose, hydroxypropylmethyl-cellulose, sodium alginate, polyvinyl-pyrrolidone, gum tragacanth and/or gum acacia. In some embodiments, an aqueous suspension comprises a dispersing or wetting agent. In some embodiments, an aqueous suspension comprises a naturally-occurring phosphatide, for example lecithin, or condensation products of an alkylene oxide with fatty acids, for example polyoxyethylene stearate, or condensation products of ethylene oxide with long chain aliphatic alcohols, for example heptadecaethylene-oxycetanol, or condensation products of ethylene oxide with partial esters derived from fatty acids and a hexitol such as polyoxyethylene sorbitol monooleate, or condensation products of ethylene oxide with partial esters derived from fatty acids and hexitol anhydrides, for example polyethylene sorbitan monooleate. In some embodiments, an aqueous suspension comprises a preservative. In some embodiments, an aqueous suspension comprises ethyl, or n-propyl p-hydroxybenzoate. In some embodiments, an aqueous suspension comprises a sweetening agent. In some embodiments, an aqueous suspension comprises sucrose, saccharin or aspartame.

[00305] In some embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is administered as an oily suspension. In some embodiments, an oily suspension is formulated by suspending the active ingredient in a vegetable oil (e.g., arachis oil, olive oil, sesame oil or coconut oil), or in mineral oil (e.g., liquid paraffin). In some embodiments, an oily suspension comprises a thickening agent (e.g., beeswax, hard paraffin or cetyl alcohol). In some embodiments, an oily suspension comprises sweetening agents (e.g., those set forth above). In some embodiments, an oily suspension comprises an anti-oxidant (e.g., butylated hydroxyanisol or alpha-tocopherol).

[00306] In some embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is formulated for parenteral injection (e.g., via injection or infusion, including intraarterial, intracardiac, intradermal, intraduodenal, intramedullary, intramuscular, intraosseous,

intraperitoneal, intrathecal, intravascular, intravenous, intravitreal, epidural and/or subcutaneous). In some embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is administered as a sterile solution, suspension or emulsion.

[00307] In some embodiments, a formulation for parenteral administration includes aqueous and/or non-aqueous (oily) sterile injection solutions of an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein, which in some embodiments, contain antioxidants, buffers, bacteriostats and/or solutes which render the formulation isotonic with the blood of the intended recipient; and/or aqueous and/or non-aqueous sterile suspensions which in some embodiments, include a suspending agent and/or a thickening agent. In some embodiments, a formulation for parenteral administration includes suitable stabilizers or agents which increase the solubility of an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein to allow for the preparation of highly concentrated solutions.

[00308] In some embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is administered as an oil-in-water micro-emulsion where the active ingredient is dissolved in the oily phase. In some embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is dissolved in a fatty oil (e.g., sesame oil, or synthetic fatty acid esters, (e.g., ethyl oleate or triglycerides, or liposomes. In some embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is dissolved in a mixture of soybean oil and/or lecithin. In some embodiments, the oil solution is introduced into a water and glycerol mixture and processed to form a micro-emulsion.

[00309] In some embodiments, a composition formulated for parenteral administration is administered as a single bolus shot. In some embodiments, a composition formulated for parenteral administration is administered via a continuous intravenous delivery device (e.g., Deltec CADD-PLUS™ model 5400 intravenous pump).

[00310] In some embodiments, a formulation for injection is presented in unit dosage form, e.g., in ampoules or in multi-dose containers, with an added preservative. In some embodiments, a formulation for injection is stored in powder form or in a freeze-dried (lyophilized) condition requiring only the addition of the sterile liquid carrier, for example, saline or sterile pyrogen-free water, immediately prior to use.

[00311] In some embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is formulated for topical administration. Topical formulations include, but are not limited to, ointments, creams, lotions, solutions, pastes, gels, films, sticks, liposomes, nanoparticles. In some embodiments, a topical formulation is administered by use of a patch, bandage or wound dressing.

[00312] In some embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is formulated as composition is in the form of a solid, a cross-linked gel, or a liposome. In some embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is formulated as an insoluble cross-linked hydrogel.

[00313] In some embodiments, a topical formulation comprises a gelling (or thickening) agent. Suitable gelling agents include, but are not limited to, celluloses, cellulose derivatives, cellulose ethers (e.g., carboxymethylcellulose, ethylcellulose, hydroxyethylcellulose, hydroxymethylcellulose, hydroxypropylmethylcellulose, hydroxypropylcellulose, methylcellulose), guar gum, xanthan gum, locust bean gum, alginates (e.g., alginic acid), silicates, starch, tragacanth, carboxyvinyl polymers, carrageenan, paraffin, petrolatum, acacia (gum arabic), agar, aluminum magnesium silicate, sodium alginate, sodium stearate, bladderwrack, bentonite, carbomer, carrageenan, carbopol, xanthan, cellulose, microcrystalline cellulose (MCC), ceratonia, chondrus, dextrose, furcellaran, gelatin, ghatti gum, guar gum, hectorite, lactose, sucrose, maltodextrin, mannitol, sorbitol, honey, maize starch, wheat starch, rice starch, potato starch, gelatin, sterculia gum, polyethylene glycol (e.g. PEG 200-4500), gum tragacanth, ethyl cellulose, ethylhydroxyethyl cellulose, ethylmethyl cellulose, methyl cellulose, hydroxyethyl cellulose, hydroxyethylmethyl cellulose, hydroxypropyl cellulose, poly(hydroxyethyl methacrylate), oxypolygelatin, pectin, polygeline, povidone, propylene carbonate, methyl vinyl ether/maleic anhydride copolymer (PVM/MA), poly(methoxyethyl methacrylate), poly(methoxyethoxyethyl methacrylate), hydroxypropyl cellulose, hydroxypropylmethyl-cellulose (HPMC), sodium carboxymethyl-cellulose (CMC), silicon dioxide, polyvinylpyrrolidone (PVP: povidone), or combinations thereof.

[00314] In some embodiments, a topical formulation disclosed herein comprises an emollient. Emollients include, but are not limited to, castor oil esters, cocoa butter esters, safflower oil esters, cottonseed oil esters, corn oil esters, olive oil esters, cod liver oil esters, almond oil esters, avocado oil esters, palm oil esters, sesame oil esters, squalene esters, kikai oil esters, soybean oil esters, acetylated monoglycerides, ethoxylated glyceryl monostearate, hexyl laurate, isohexyl laurate, isohexyl palmitate, isopropyl palmitate, methyl palmitate, decyloleate, isodecyl oleate, hexadecyl stearate decyl stearate, isopropyl isostearate, methyl isostearate, diisopropyl adipate, diisohexyl adipate, dihexyldecyl adipate, diisopropyl sebacate, lauryl lactate, myristyl lactate, and cetyl lactate, oleyl myristate, oleyl stearate, and oleyl oleate, pelargonic acid, lauric acid, myristic acid, palmitic acid, stearic acid, isostearic acid, hydroxystearic acid, oleic acid, linoleic acid, ricinoleic acid, arachidic acid, behenic acid, erucic acid, lauryl alcohol, myristyl alcohol, cetyl alcohol, hexadecyl alcohol, stearyl alcohol, isostearyl alcohol, hydroxystearyl alcohol, oleyl alcohol, ricinoleyl alcohol, behenyl alcohol, erucyl alcohol, 2-octyl dodecanyl alcohol, lanolin and lanolin derivatives,

beeswax, spermaceti, myristyl myristate, stearyl stearate, carnauba wax, candelilla wax, lecithin, and cholesterol.

[00315] In some embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is formulated with one or more natural polymers. In some embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is formulated with a natural polymer that is fibronectin, collagen, laminin, keratin, fibrin, fibrinogen, hyaluronic acid, heparan sulfate, chondroitin sulfate. In some embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is formulated with a polymer gel formulated from a natural polymer. In some embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is formulated with a polymer gel formulated from a natural polymer, such as, but not limited to, fibronectin, collagen, laminin, keratin, fibrin, fibrinogen, hyaluronic acid, heparan sulfate, chondroitin sulfate, and combinations thereof. In some embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is formulated with a cross-linked polymer. In some embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is formulated with a non-cross-linked polymer. In some embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is formulated with a non-cross-linked polymer and a cross-linked polymer. In some embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is formulated with cross-linked hyaluronan gel. In some embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is formulated with an insoluble cross-linked HA hydrogel. In some embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is formulated with non-cross-linked hyaluronan gel. In some embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is formulated with a collagen matrix. In some embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is formulated with a fibrin matrix. In some embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is formulated with a fibrin/collagen matrix.

[00316] In some embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is formulated for administration to an eye or a tissue related thereto. Formulations suitable for administration to an eye include, but are not limited to, solutions, suspensions (e.g., an aqueous suspension), ointments, gels, creams, liposomes, niosomes, pharmacosomes, nanoparticles, or combinations thereof. In some embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein for topical administration to an eye is administered spraying, washing, or combinations thereof. In some embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is administered to an eye via an injectable depot preparation.

[00317] As used herein, a “depot preparation” is a controlled-release formulation that is implanted in an eye or a tissue related thereto (e.g., the sclera) (for example subcutaneously, intramuscularly,

intravitreally, or within the subconjunctiva). In some embodiments, a depot preparation is formulated by forming microencapsulated matrices (also known as microencapsulated matrices) of an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein in biodegradable polymers. In some embodiments, a depot preparation is formulated by entrapping an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein in liposomes or microemulsions.

[00318] A formulation for administration to an eye has an ophthalmically acceptable tonicity. In certain instances, lacrimal fluid has an isotonicity value equivalent to that of a 0.9% sodium chloride solution. In some embodiments, an isotonicity value from about 0.6% to about 1.8% sodium chloride equivalency is suitable for topical administration to an eye. In some embodiments, a formulation for administration to an eye disclosed herein has an osmolarity from about 200 to about 600 mOsm/L. In some embodiments, a formulation for administration to an eye disclosed herein is hypotonic and thus requires the addition of any suitable to attain the proper tonicity range. Ophthalmically acceptable substances that modulate tonicity include, but are not limited to, sodium chloride, potassium chloride, sodium thiosulfate, sodium bisulfite and ammonium sulfate.

[00319] A formulation for administration to an eye has an ophthalmically acceptable clarity. Examples of ophthalmically-acceptable clarifying agents include, but are not limited to, polysorbate 20, polysorbate 80, or combinations thereof.

[00320] In some embodiments, a formulation for administration to an eye comprises an ophthalmically acceptable viscosity enhancer. In some embodiments, a viscosity enhancer increases the time a formulation disclosed herein remains in an eye. In some embodiments, increasing the time a formulation disclosed herein remains in the eye allows for greater drug absorption and effect. Non-limiting examples of mucoadhesive polymers include carboxymethylcellulose, carbomer (acrylic acid polymer), poly(methylmethacrylate), polyacrylamide, polycarbophil, acrylic acid/butyl acrylate copolymer, sodium alginate and dextran.

[00321] In some embodiments, a formulation for administration to an eye is administered or delivered to the posterior segments of an eye (e.g., to the retina, choroid, vitreous and optic nerve). In some embodiments, a topical formulation for administration to an eye disclosed herein for delivery to the posterior of the eye comprises a solubilizing agent, for example, a glucan sulfate and/or a cyclodextrin. Glucan sulfates which are used in some embodiments include, but are not limited to, dextran sulfate, cyclodextrin sulfate and β -1,3-glucan sulfate, both natural and derivatives thereof, or any compound which temporarily binds to and be retained at tissues which contain fibroblast growth factor (FGF), which improves the stability and/or solubility of a drug, and/or which improves penetration and ophthalmic absorption of a topical formulation for administration to an eye disclosed herein. Cyclodextrin derivatives which are used in some

embodiments as a solubilizing agent include, but are not limited to, α -cyclodextrin, β -cyclodextrin, γ -cyclodextrin, hydroxyethyl β -cyclodextrin, hydroxypropyl γ -cyclodextrin, hydroxypropyl β -cyclodextrin, sulfated α -cyclodextrin, sulfated β -cyclodextrin, sulfobutyl ether β -cyclodextrin.

[00322] In some embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is formulated for rectal or vaginal administration. In some embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is administered as a suppository. In some embodiments, a composition suitable for rectal administration is prepared by mixing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein with a suitable non-irritating excipient which is solid at ordinary temperatures but liquid at the rectal temperature and will therefore melt in the rectum to release the drug. In some embodiments, a composition suitable for rectal administration is prepared by mixing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein with cocoa butter, glycerinated gelatin, hydrogenated vegetable oils, mixtures of polyethylene glycols of various molecular weights or fatty acid esters of polyethylene glycol.

[00323] In certain embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex described herein is optionally incorporated within controlled release particles, lipid complexes, liposomes, nanoparticles, microspheres, microparticles, nanocapsules or other agents which enhance or facilitate localized delivery to the skin. An example of a conventional microencapsulation process for pharmaceutical preparations is described in U.S. Pat. No. 3,737,337, incorporated herein by reference for such disclosure.

[00324] *Dosages*

[00325] The amount of pharmaceutical compositions administered is dependent in part on the individual being treated. In instances where pharmaceutical compositions are administered to a human subject, the daily dosage will normally be determined by the prescribing physician with the dosage generally varying according to the age, sex, diet, weight, general health and response of the individual, the severity of the individual's symptoms, the precise disease or condition being treated, the severity of the disease or condition being treated, time of administration, route of administration, the disposition of the composition, rate of excretion, drug combination, and the discretion of the prescribing physician.

[00326] In some embodiments, the dosage of an nHC-HA/PTX3 or rcHC-HA/PTX3 complex is between about 0.001 to about 1000 mg/kg body weight/day. In some embodiments, the amount of nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is in the range of about 0.5 to about 50 mg/kg/day. In some embodiments, the amount of nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is about 0.001 to about 7 g/day. In some embodiments, the amount of nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is about 0.01 to about 7 g/day. In some

embodiments, the amount of nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is about 0.02 to about 5 g/day. In some embodiments, the amount of nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is about 0.05 to about 2.5 g/day. In some embodiments, the amount of nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is about 0.1 to about 1 g/day.

[00327] In some embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is administered, before, during or after the occurrence of a disease or condition. In some embodiments, a combination therapy is administered before, during or after the occurrence of a disease or condition. In some embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is administered with a combination therapy before, during or after the occurrence of a disease or condition. In some embodiments, the timing of administering the composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 disclosed herein varies. Thus, in some examples, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is used as a prophylactic and is administered continuously to subjects with a propensity to develop conditions or diseases in order to prevent the occurrence of the disease or condition. In some embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is administered to a subject during or as soon as possible after the onset of the symptoms. In some embodiments, the administration of an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is initiated within the first 48 hours of the onset of the symptoms, preferably within the first 48 hours of the onset of the symptoms, more preferably within the first 6 hours of the onset of the symptoms, and most preferably within 3 hours of the onset of the symptoms. In some embodiments, the initial administration is via any route practical, such as, for example, an intravenous injection, a bolus injection, infusion over 5 minutes to about 5 hours, a pill, a capsule, transdermal patch, buccal delivery, or combination thereof. An nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is preferably administered as soon as is practicable after the onset of a disease or condition is detected or suspected, and for a length of time necessary for the treatment of the disease, such as, for example, from about 1 month to about 3 months. In some embodiments, the length of treatment varies for each subject, and the length is determined using the known criteria. In some embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein or a formulation containing a complex is administered for at least 2 weeks, preferably about 1 month to about 5 years, and more preferably from about 1 month to about 3 years.

[00328] In some embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is administered in a single dose, once daily. In some embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is administered in multiple doses, more than once per day. In some embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is

administered twice daily. In some embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is administered three times per day. In some embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex is administered four times per day. In some embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is administered more than four times per day.

[00329] In some embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is administered for prophylactic and/or therapeutic treatments. In therapeutic applications, in some embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is administered to an individual already suffering from a disease or condition, in an amount sufficient to cure or at least partially arrest the symptoms of the disease or condition. Amounts effective for this use will depend on the severity and course of the disease or condition, previous therapy, the individual's health status, weight, and response to the drugs, and the judgment of the treating physician.

[00330] In prophylactic applications, in some embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is administered to an individual that is at risk of a particular disorder. Such an amount is defined to be a "prophylactically effective amount or dose." In such use, the precise amounts also depend on the individual's state of health, weight, and other physical parameters of the individual.

[00331] In the case wherein the individual's condition does not improve, upon the doctor's discretion an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is administered chronically, that is, for an extended period of time, including throughout the duration of the individual's life in order to ameliorate or otherwise control or limit the symptoms of the individual's disease or condition.

[00332] In some embodiments, in cases where the individual's status does improve, upon the doctor's discretion, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is administered continuously or the dose of drug being administered is temporarily reduced or temporarily suspended for a certain length of time (i.e., a "drug holiday"). In some embodiments, the length of the drug holiday varies between 2 days and 1 year, including by way of example only, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, 10 days, 12 days, 15 days, 20 days, 28 days, 35 days, 50 days, 70 days, 100 days, 120 days, 150 days, 180 days, 200 days, 250 days, 280 days, 300 days, 320 days, 350 days, or 365 days. In some embodiments the dose reduction during a drug holiday is from 10%-100%, including, by way of example only, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100%.

[00333] Once improvement of the individual's conditions has occurred, a maintenance dose is administered if necessary. In some embodiments, subsequently, the dosage or the frequency of

administration, or both, is reduced, as a function of the symptoms, to a level at which the improved disease, disorder or condition is retained. In some embodiments, individuals require intermittent treatment on a long-term basis upon any recurrence of symptoms.

[00334] In some embodiments, the pharmaceutical composition described herein is in unit dosage forms suitable for single administration of precise dosages. In unit dosage form, the formulation is divided into unit doses containing appropriate quantities of an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein. In some embodiments, the unit dosage is in the form of a package containing discrete quantities of the formulation. Non-limiting examples are packaged tablets or capsules, and powders in vials or ampoules. In some embodiments, aqueous suspension compositions are packaged in single-dose non-reclosable containers. In some embodiments, multiple-dose reclosable containers are used, in which case it is typical to include a preservative in the composition. In some embodiments, formulations for parenteral injection are presented in unit dosage form, which include, but are not limited to ampoules, or in multi dose containers, with an added preservative.

[00335] The daily dosages appropriate for an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein are, for example, from about 0.01 to 2.5 mg/kg per body weight. An indicated daily dosage in the larger mammal, including, but not limited to, humans, is in the range from about 0.5 mg to about 100 mg, conveniently administered in divided doses, including, but not limited to, up to four times a day or in extended release form. Suitable unit dosage forms for oral administration include from about 1 to 50 mg active ingredient. The foregoing ranges are merely suggestive, as the number of variables in regard to an individual treatment regime is large, and considerable excursions from these recommended values are not uncommon. In some embodiments, the dosages are altered depending on a number of variables, not limited to the activity of an nHC-HA/PTX3 or rcHC-HA/PTX3 complex used, the disease or condition to be treated, the mode of administration, the requirements of the individual subject, the severity of the disease or condition being treated, and the judgment of the practitioner.

[00336] In some embodiments, the toxicity and therapeutic efficacy of such therapeutic regimens are determined by standard pharmaceutical procedures in cell cultures or experimental animals, including, but not limited to, the determination of the LD₅₀ (the dose lethal to 50% of the population) and the ED₅₀ (the dose therapeutically effective in 50% of the population). In some embodiments, the dose ratio between the toxic and therapeutic effects is the therapeutic index and it is expressed as the ratio between LD₅₀ and ED₅₀. nHC-HA/PTX3 or rcHC-HA/PTX3 complexes exhibiting high therapeutic indices are preferred. In some embodiments, the data obtained from cell culture assays and animal studies is used in formulating a range of dosage for use in human. The

dosage of an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein lies preferably within a range of circulating concentrations that include the ED₅₀ with minimal toxicity. In some embodiments, the dosage varies within this range depending upon the dosage form employed and the route of administration utilized.

[00337] In some embodiments, the pharmaceutical compositions of nHC-HA/PTX3 or rcHC-HA/PTX3 complexes are packaged as articles of manufacture containing packaging material, a pharmaceutical composition which is effective for prophylaxis and/or treating a disease or condition, and a label that indicates that the pharmaceutical composition is to be used for treating the disease or condition. In some embodiments, the pharmaceutical compositions are packaged in unit dosage forms contain an amount of the pharmaceutical composition for a single dose or multiple doses. In some embodiments, the packaged compositions contain a lyophilized powder of the pharmaceutical compositions, which is reconstituted (e.g., with water or saline) prior to administration.

[00338] *Medical Device and Biomaterials Compositions*

[00339] In some embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is assembled directly on a surface of or formulated as a coating for an implantable medical device. Methods for covalent attachment of hyaluronan to surfaces such as, but not limited to, metallic, polymeric, ceramic, silica and composite surfaces is well-known in the art and in some embodiments, is employed in conjunction with the methods provided herein for the assembly of nHC-HA/PTX3 or rcHC-HA/PTX3 complexes on such surfaces (see e.g., U.S. Pat. Nos. 5,356,433; 5,336,518, 4,613,665, 4,810,784, 5,037,677, 8,093,365). In some embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex is assembled directly on a surface of an implantable medical device or a portion thereof. In some embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex that has been generated according the methods provided herein is purified and then attached directly on a surface of an implantable medical device or a portion thereof. In some embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex that has been generated according the methods provided herein is purified and then formulated as a coating for attachment to the medical device or a portion thereof. In some embodiments, the coating is applied directly to the surfaces or is applied to a pretreated or coated surface where the pretreatment or coating is designed to aid adhesion of the coating to the substrate. In some embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex that has been generated according the methods provided herein is purified and then attached to a medical device or a portion thereof that has been coated with a substance that promotes the attachment of the nHC-HA/PTX3 or rcHC-HA/PTX3 complex. For example, in some embodiments, the medical device or a portion thereof is coated with an adhesive polymer that

provides functional groups on its surface for the covalent attachment of hyaluronan of the nHC-HA/PTX3 or rcHC-HA/PTX3 complex. In some embodiments, a coupling agent, such as, but not limited to carbodiimide is employed to attach the nHC-HA/PTX3 or rcHC-HA/PTX3 complex to the polymer coating. In some embodiments, photoimmobilization is employed to covalently attach an nHC-HA/PTX3 or rcHC-HA/PTX3 complex that has been generated according the methods provided herein to medical device or a portion thereof. In some embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex that has been generated according the methods provided herein is attached to a medical device or a portion thereof using a spacer molecule that comprises a photochemically or thermochemically reactive group.

[00340] In some embodiments, the coating formulations comprising an nHC-HA/PTX3 or rcHC-HA/PTX3 complex are applied to the substrate by for example dip-coating. Other methods of application include, but are not limited to, spray, wash, vapor deposition, brush, roller, curtain, spin coating and other methods known in the art.

[00341] Exemplary implantable medical devices include, but are not limited to an artificial joint, orthopedic device, bone implant, contact lenses, suture, surgical staple, surgical clip, catheter, angioplasty balloon, sensor, surgical instrument, electrode, needle, syringe, wound drain, shunt, urethral insert, metal or plastic implant, heart valve, artificial organ, lap band, annuloplasty ring, guide wire, K-wire or Denham pin, stent, stent graft, vascular graft, pacemaker, pellets, wafers, medical tubing, infusion sleeve, implantable defibrillator, neurostimulator, glucose sensor, cerebrospinal fluid shunt, implantable drug pump, spinal cage, artificial disc, ocular implant, cochlear implant, breast implant, replacement device for nucleus pulposus, ear tube, intraocular lens, drug delivery system, microparticle, nanoparticle, and microcapsule.

[00342] In particular embodiments, the implantable medical device is an implant or prosthesis comprising an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein. In particular embodiments, the prosthesis is an artificial joint. In some embodiments, the prosthesis is an artificial hip joint, artificial knee, an artificial glenohumeral joint, an artificial ankle.

[00343] In particular embodiments, the implant is a stent. In particular embodiments, the implant is a coronary stent, a ureteral stent, a urethral stent, a prostatic stent, a bone stent, or an esophageal stent. In particular embodiments, the implant is a bone implant, such as, but not limited to, an osseointegrated implant or a craniofacial prosthesis (e.g., an artificial ear, orbital prosthesis, nose prosthesis).

[00344] In some embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is assembled directly on a microparticle or a nanoparticle for delivery of the nHC-HA/PTX3 or rcHC-HA/PTX3 complex to a subject (see, e.g., WO 03/015755 and US2004/0241248).

[00345] In some embodiments, the nHC-HA/PTX3 or rcHC-HA/PTX3 complexes provided herein are attached to, assembled on, or provided as a coating on the surfaces of or portions thereof of any such implantable medical devices as described herein or known in the art. In some embodiments the nHC-HA/PTX3 or rcHC-HA/PTX3 complex elutes from the coating and into the surrounding tissue following implantation.

[00346] In some embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is assembled directly on a scaffold, a microparticle, a microcapsule or microcarrier employed for the delivery of a biomaterial, such as a stem cell or an insulin producing cell. In some embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is attached to the microcapsule or assembled directly on a microcapsule. In some embodiments, the nHC-HA/PTX3 or rcHC-HA/PTX3 complex is combined with a material used to form the microcapsule and a microcapsule is generated that contains the nHC-HA/PTX3 or rcHC-HA/PTX3 complex. In some embodiments, the nHC-HA/PTX3 or rcHC-HA/PTX3 complex is used to coat the inner surface of the microcapsule. In some embodiments, the nHC-HA/PTX3 or rcHC-HA/PTX3 complex is used to coat the outer surface of the microcapsule. In some embodiments, the nHC-HA/PTX3 or rcHC-HA/PTX3 complex is used to coat the inner and outer surface of the microcapsule.

[00347] Exemplary materials for encapsulating cells include, but are not limited to, thermosetting hydrogels, such as agarose, alginate, and artificial polymers such as poly(NiPAAm-co-AAC), poly(ethylene glycol) (PEG) and PEG derivatives such as PEG diacrylate and oligo(poly(ethylene glycol)) fumarate. Methods for the culturing and microencapsulation of stem cells are known in the art in some embodiments, are employed to generate microcapsules containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex provided herein.

[00348] In some embodiments the microcapsule contains a cell, a plurality of cells or other biological material. In some embodiments, the cell or cells are stem cells, such as, but not limited to, mesenchymal stem cells. In some embodiments, the cell or cells are differentiated cells, such as, but not limited to, insulin-producing cells. In some embodiments, the cell or cells are autologous cells (i.e. cells that are from or derived from the recipient of the cells). In some embodiments, the cell or cells are allogeneic cells (i.e. cells that are not from or derived from the recipient of the cells). In some embodiments, the microcapsule contains a cell, a plurality of cells or other biological material and the inner surfaces of the microcapsule are coated with nHC-HA/PTX3 or rcHC-HA/PTX3 complex provided herein. In some embodiments the microcapsule contains a cell, a plurality of cells or other biological material and the outer surfaces of the microcapsule are coated with nHC-HA/PTX3 or rcHC-HA/PTX3 complex provided herein. In some embodiments the microcapsule contains a cell, a plurality of cells or other biological material and the outer and inner

surfaces of the microcapsule are coated with nHC-HA/PTX3 or rcHC-HA/PTX3 complex provided herein. In some embodiments the microcapsule is administered to treat a disease or condition. Exemplary diseases and conditions and methods of treatment for which a microcapsule can be administered are described elsewhere herein and include but are not limited to inflammatory and immune related diseases.

Methods of Treatment

[00349] Disclosed herein, in certain embodiments, are methods of treating an individual in need thereof, comprising administering to the individual nHC-HA/PTX3 or rcHC-HA/PTX3 complexes described herein. Disclosed herein, in certain embodiments, are methods of treating an individual in need thereof, comprising administering to the individual nHC-HA/PTX3 or rcHC-HA/PTX3 complexes produced by the methods described herein. The following are non-limiting examples of methods of treatment comprising administration of an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein. In some embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is used to inhibit at least one of the following: scarring, inflammation, immune reaction leading to autoimmune or immune rejection, adhesion, angiogenesis and conditions requiring cell or tissue regeneration. In some embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is used to promote wound healing. In some embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is used to promote stem cell expansion. In some embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is used to promote tissue regeneration.

[00350] In some embodiments, the methods of treating an individual in need thereof, comprising administering to the individual nHC-HA/PTX3 or rcHC-HA/PTX3 complexes described herein by any suitable method. In some embodiments, the methods of treating an individual in need thereof, comprising administering to the individual nHC-HA/PTX3 or rcHC-HA/PTX3 complexes described herein by any suitable route of administration. Suitable methods for administration will depend on the disease or condition to be treated. In some embodiments, the nHC-HA/PTX3 or rcHC-HA/PTX3 complexes are administered locally to the site of treatment. In some embodiments, the nHC-HA/PTX3 or rcHC-HA/PTX3 complexes are administered systemically. Exemplary methods for administration of the nHC-HA/PTX3 or rcHC-HA/PTX3 complexes provided herein include but are not limited to parenteral, enteral, subcutaneous, percutaneous, transdermal, intradermal, intravenous, topical, inhalation, or implantation.

[00351] *Scarring*

[00352] Described herein, in certain embodiments, are methods of preventing, reducing, or reversing scarring in a subject in need thereof, comprising administering to the subject a composition comprising an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein.

[00353] As used herein, “scarring” refers to the formation of a scar. In one aspect, the scar is a hypertrophic scar, or keloid scar, or a scar resulting from acne. As used herein, a “scar” is an area of fibrous tissue that results from the overproduction of collagen. In certain instances, wound healing comprises the migration of fibroblasts to the site of injury. In certain instances, fibroblasts deposit collagen. In certain instances, fibroblasts deposit excess collagen at the wound site, resulting in a scar.

[00354] In certain instances, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein prevents or inhibits TGF- β signaling. In certain instances, TGF- β regulates the extracellular matrix by stimulating fibroplasia and collagen deposition and inhibiting extracellular matrix degradation (by up-regulating the synthesis of protease inhibitors). In certain instances, preventing or inhibiting the expression of TGF- β results in the prevention of or a reduction in intensity of a scar. In some embodiments, administering an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein prevents or reduces scarring.

[00355] In some embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein inhibits or prevents the ability of fibroblasts to differentiate into myofibroblasts. In some embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein reverts differentiated myofibroblasts to fibroblasts.

[00356] In some embodiments, a method disclosed herein is used to prevent, reduce or reverse the formation of a scar. In some embodiments, a method disclosed herein comprises administering an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein to an individual with a disorder that results in scarring (e.g., dermatitis scar, a keloid scar, contracture scar, a hypertrophic scar, or a scar resulting from acne). In some embodiments, a method disclosed herein comprises administering an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein to an individual in need thereof before or after trauma. In some embodiments, a method disclosed herein comprises administering an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein to an individual in need thereof before or after surgery.

[00357] In some embodiments, a method disclosed herein is used to prevent or reduce the formation of a scar on an eye or on the surrounding tissue. In some embodiments, a method disclosed herein comprises administering an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein to an individual with a disorder that results in scarring of the eye or surrounding tissue (e.g., retinopathy of prematurity). In some embodiments, a method disclosed herein comprises

administering an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein to an individual in need thereof before or after trauma to an eye or the surrounding tissue. In some embodiments, a method disclosed herein comprises administering an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein to an individual in need thereof before or after surgery to an eye or the surrounding tissue.

[00358] *Inflammation*

[00359] Described herein, in certain embodiments, are methods of preventing or reducing inflammation in a subject in need thereof, comprising administering to the subject a composition comprising an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein. As used herein, “inflammation” means physiological responses resulting from the migration of plasma and/or leukocytes (e.g., lymphocytes, macrophages, granulocytes, and neutrophils) to the site of an infection or trauma (e.g., blunt force trauma, penetrating trauma, or surgery).

[00360] In certain instances, leukocytes secrete cytokines following contact with an antigen. As used herein, “cytokines” are signaling proteins or glycoproteins. In certain instances, a cytokine binds to a cell-surface receptor. In certain instances, cytokines induces the chemotaxis of leukocytes to the site of an infection. In certain instances, cell surface receptors on a leukocyte detect chemical gradients of a cytokine. In certain instances, a leukocyte follows the gradient to the site of infection. In certain instances, the binding of a cytokine to a cell-surface receptor results in the upregulation or downregulation of certain genes and their transcription factors. In certain instances, changes in gene expression results in the production of cytokines, an increase in the production of cytokines, or an increase in the presentation of cell surface receptors.

[00361] By way of non-limiting example, cytokines include interleukins IL-1, IL-6, IL-8, MCP-1 (also known as CCL2), and TNF- α . Interleukin 1 is present in the body in two isoforms: IL-1 α and IL-1 β . In certain instances, the presence of IL-1 increases the expression of adhesion factors on endothelial cells. This, in turn, enables the transmigration of leukocytes to the site of infection. In certain instances, IL-8 induces the chemotaxis of leukocytes. In certain instances, TNF- α induces the chemotaxis of leukocytes. In certain instances, MCP-1 recruits leukocytes to sites of tissue injury and infection.

[00362] In some embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein suppresses the production of and/or activity of cytokines. In certain instances, a decrease in the concentration cytokines reduces or prevents inflammation by decreasing the number of leukocytes and/or the rate at which leukocytes migrate to the site of an injury.

[00363] In some embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein induces apoptosis of a leukocyte (e.g., a macrophage, neutrophil, or lymphocyte). In some

embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein decreases the number of activated leukocytes or the rate at which leukocytes are activated. In certain instances, a decrease in the concentration of leukocytes reduces or prevents inflammation by decreasing the number (e.g., facilitate death of such cells via apoptosis) of cells that migrate to the site of an injury.

[00364] In some embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein inhibits the polarization of a macrophage to an inflammatory phenotype (i.e. an M1 phenotype). In some embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein reduces or inhibits the expression of IL-12 or IL-23 in stimulated macrophages. In some embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein promotes the polarization of stimulated macrophages to a regulatory or wound healing M2 phenotype. In some embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein inhibits or reduces inflammation in a subject. In some embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein inhibits or reduces tissue damage caused by an inflammatory response in a subject. In some embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex inhibits or reduces tissue damage caused by a condition or disease that induces inflammation in a subject.

[00365] In some embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is administered to a subject having inflammation. In some embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is administered to a subject having acute inflammation. In some embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is administered to a subject having chronic inflammation. In some embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is administered to a subject having an inflammatory disorder. In some embodiments, the inflammatory disorder is a macrophage mediated inflammatory disorder. In some embodiments, the inflammatory disorder is a T-cell mediated inflammatory disorder. In some embodiments, the inflammatory disorder is a Th-17 mediated immune disorder.

[00366] In some embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is administered to a subject having an acute inflammatory response. In some embodiments, the acute inflammatory response is caused by, for example, an allergy, sepsis, endotoxic shock or ischemia, such as but not limited to, myocardial infarction and stroke. In some embodiments, the acute inflammatory response is the result of bacterial infection, a protozoal infection, a protozoal infection, a viral infection, a fungal infection, or combinations thereof. In some embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex inhibits or reduces acute inflammation. In some embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex inhibits or reduces tissue damage caused by acute inflammation. In some embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3

complex inhibits or reduces tissue reperfusion injury due to ischemia, including myocardial infarction and stroke,

[00367] In some embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is administered to a subject having chronic inflammation that is associated with the activation of lymphocytes via adaptive immunity. In some embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is administered to a subject having a Th1 response. In some embodiments the Th1 response leads to immune rejection of biological transplant. In some embodiments, the transplant is an allograft transplant. In some embodiments, the transplant is an autologous transplant. In some embodiments, the inflammatory disorder is graft versus host disease or tissue transplant rejection. In some embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex inhibits or reduces chronic inflammation in a subject.

[00368] In some embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is administered to a subject having chronic inflammation that is associated with a Th17 immune response associated with an inflammatory disorder. In some embodiments, the inflammatory disorder is an autoimmune disorder or a leukocyte defect.

[00369] In some embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is administered to a subject having an inflammatory disorder that is acute disseminated encephalomyelitis; Addison's disease; ankylosing spondylitis; antiphospholipid antibody syndrome; autoimmune hemolytic anemia; autoimmune hepatitis; autoimmune inner ear disease; bullous pemphigoid; Chagas disease; chronic obstructive pulmonary disease; coeliac disease; dermatomyositis; diabetes mellitus type 1; diabetes mellitus type 2; endometriosis; Goodpasture's syndrome; Graves' disease; Guillain-Barré syndrome; Hashimoto's disease; idiopathic thrombocytopenic purpura; interstitial cystitis; systemic lupus erythematosus (SLE); metabolic syndrome, multiple sclerosis; myasthenia gravis; myocarditis, narcolepsy; obesity; pemphigus vulgaris; pernicious anemia; polymyositis; primary biliary cirrhosis; rheumatoid arthritis; schizophrenia; scleroderma; Sjögren's syndrome; vasculitis; vitiligo; Wegener's granulomatosis; allergic rhinitis; prostate cancer; non-small cell lung carcinoma; ovarian cancer; breast cancer; melanoma; gastric cancer; colorectal cancer; brain cancer; metastatic bone disorder; pancreatic cancer; a lymphoma; nasal polyps; gastrointestinal cancer; ulcerative colitis; Crohn's disorder; collagenous colitis; lymphocytic colitis; ischaemic colitis; diversion colitis; Behçet's syndrome; infective colitis; indeterminate colitis; inflammatory liver disorder, ischemia, myocardial infarction, stroke, endotoxin shock, septic shock; rheumatoid spondylitis, ankylosing spondylitis, Gouty arthritis, polymyalgia rheumatica, Alzheimer's disorder, Parkinson's disorder, epilepsy, AIDS dementia, asthma, adult respiratory distress syndrome, bronchitis, cystic fibrosis, acute leukocyte-

mediated lung injury, distal proctitis, Wegener's granulomatosis, fibromyalgia, uveitis, conjunctivitis, psoriasis, eczema, dermatitis, smooth muscle proliferation disorders, meningitis, shingles, encephalitis, nephritis, tuberculosis, retinitis, atopic dermatitis, pancreatitis, periodontal gingivitis, coagulative Necrosis, liquefactive necrosis, fibrinoid necrosis, neointimal hyperplasia, or combinations thereof.

[00370] In some embodiments, the inflammatory disorder is an inflammatory disorder of an eye or the surrounding tissue. In some embodiments, the inflammatory disorder is conjunctivitis. In certain instances, conjunctivitis results from exposure to an allergen. In certain instances, conjunctivitis results from a bacterial infection. In some embodiments, the inflammatory disorder is keratitis. As used herein, “keratitis” is a disorder characterized by inflammation of the cornea. In some embodiments, the inflammatory disorder is keratoconjunctivitis (i.e., a combination of conjunctivitis and keratitis (i.e., corneal inflammation)). In some embodiments, the inflammatory disorder is blepharitis. As used herein, “blepharitis” is an ophthalmic disorder characterized by inflammation of the eyelid margins. In some embodiments, the inflammatory disorder is blepharoconjunctivitis (i.e., a combination of conjunctivitis and blepharitis (i.e., inflammation of an eyelid)). In some embodiments, the inflammatory disorder is scleritis. As used herein, “scleritis” is a disorder characterized by inflammation of the sclera. In some embodiments, the inflammatory disorder is episcleritis. As used herein, “episcleritis” is an inflammatory disorder of the episclera characterized by hyperaemia, and chemosis. In some embodiments, the inflammatory disorder is uveitis. As used herein, “uveitis” is an inflammatory disorder of the uvea. In some embodiments, the disorder is retinitis. As used herein, “retinitis” is an inflammatory disorder of a retina. In some embodiments, the disorder is choroiditis. As used herein, “choroiditis” is an inflammatory disorder of the uvea, ciliary body and the choroid.

[00371] *Abnormal Angiogenesis*

[00372] Disclosed herein, in certain embodiments, are methods of preventing or reducing angiogenesis in a subject in need thereof, comprising administering to the subject a composition comprising an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein. As used herein, “angiogenesis” means the formation of new blood vessels. In certain instances, angiogenesis facilitates the growth and metastasis of a tumor. Further, in certain instances, abnormal angiogenesis is the basis of wet age-related macular degeneration (wARMD) and diabetic proliferative retinopathy. In certain instances, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein prevents or reduces angiogenesis.

[00373] In certain instances, the binding of a ligand to the VEGF receptor-2 (VEGFR-2) starts a tyrosine kinase signaling cascade that stimulates the production of factors that variously stimulate

vessel permeability (eNOS, producing NO), proliferation/survival (bFGF), migration (ICAMs/VCAMs/MMPs) and finally differentiation into mature blood vessels. In certain instances, following binding of VEGFR-2 to its ligand, endothelial cells form tube structures resembling capillaries.

[00374] As used herein, “wet Age Related Macular Degeneration”, “wARMD”, or “wet ARMD” means a disorder of an eye characterized by the proliferation of blood vessels from the choroid. In certain instances, wet ARMD causes vision loss due blood and protein leakage below the macula. In certain instances, bleeding, leaking, and scarring from these blood vessels cause irreversible damage to the photoreceptors and rapid vision loss if left untreated.

[00375] As used herein, “diabetic proliferative retinopathy” means a disorder of an eye characterized by incompetence of the vascular walls. In certain instances, the lack of oxygen in the retina results in angiogenesis along the retina and in the vitreous humour. In certain instances, the new blood vessels bleed, cloud vision, and destroy the retina.

[00376] In certain instances, the proliferation of capillaries supplies a tumor with nutrients, allowing the tumor to expand. In certain instances, the proliferation of capillaries enables the rapid removal of cellular waste enabling tumor growth. In certain instances, angiogenesis facilitates metastasis. In certain instances, the proliferation of capillaries increases the chances that a cancerous cell will be able to enter a blood vessel and thus establish a new tumor at a new site.

[00377] Exemplary cancer types that are treated in some embodiments using an nHC-HA/PTX3 or rcHC-HA/PTX3 complex described herein include, but are not limited to, Acute Lymphoblastic Leukemia, Acute Myeloid Leukemia, Adrenocortical Carcinoma, AIDS-Related Cancers, AIDS-Related Lymphoma, Anal Cancer, Astrocytoma, Basal Cell Carcinoma, Bile Duct Cancer, Bladder Cancer, Bladder Cancer, Bone Cancer, Brain Stem Glioma, Brain Tumor, Breast Cancer, Bronchial Adenomas, Burkitt’s Lymphoma, Carcinoid Tumor, Carcinoma, Central Nervous System Lymphoma, Cerebellar Astrocytoma, Cervical Cancer, Chronic Lymphocytic Leukemia, Chronic Myelogenous Leukemia, Chronic Myeloproliferative Disorders, Colon Cancer, Colorectal Cancer, Cutaneous T-Cell Lymphoma, Endometrial Cancer, Ependymoma, Esophageal Cancer, Extragonadal Germ Cell Tumor, Eye Cancer, Intraocular Melanoma, Eye Cancer, Retinoblastoma, Gallbladder Cancer, Gastrointestinal Carcinoid Tumor, Gastrointestinal Stromal Tumor (GIST), Germ Cell Tumor (Extracranial), Germ Cell Tumor (Extragonadal), Germ Cell Tumor (Ovarian), Gestational Trophoblastic Tumor, Glioma, Hairy Cell Leukemia, Head and Neck Cancer, Hepatocellular (Liver) Cancer, Hodgkin’s Lymphoma, Hypopharyngeal Cancer, Hypothalamic and Visual Pathway Glioma, Intraocular Melanoma, Islet Cell Carcinoma (Endocrine Pancreas), Kaposi’s Sarcoma, Kidney (Renal Cell) Cancer, Laryngeal Cancer, Leukemia (Acute

Lymphoblastic), Leukemia (Acute Myeloid), Leukemia (Chronic Lymphocytic), Leukemia (Chronic Myelogenous), Lip and Oral Cavity Cancer, Liver Cancer, Lung Cancer (Non-Small Cell), Lung Cancer (Small Cell), Lymphoma, (Cutaneous T-Cell), Lymphoma (Non-Hodgkin's), Malignant Fibrous Histiocytoma of Bone/Osteosarcoma, Medulloblastoma, Melanoma, Merkel Cell Carcinoma, Mesothelioma, Metastatic Squamous Neck Cancer with Occult Primary, Multiple Endocrine Neoplasia Syndrome, Multiple Myeloma/Plasma Cell Neoplasm, Mycosis Fungoides, Myelodysplastic Syndromes, Myelodysplastic/Myeloproliferative Diseases, Myelogenous Leukemia, Myeloid Leukemia, Myeloproliferative Disorders, Nasal Cavity and Paranasal Sinus Cancer, Nasopharyngeal Cancer, Neuroblastoma, Oral Cancer, Oropharyngeal Cancer, Osteosarcoma/Malignant Fibrous Histiocytoma of Bone, Ovarian Cancer, Ovarian Epithelial Cancer, Ovarian Germ Cell Tumor, Ovarian Low Malignant Potential Tumor, Pancreatic Cancer, Parathyroid Cancer, Penile Cancer, Pheochromocytoma, Pineoblastoma and Supratentorial Primitive Neuroectodermal Tumors, Pituitary Tumor, Plasma Cell Neoplasm/Multiple Myeloma, Pleuropulmonary Blastoma, Prostate Cancer, Rectal Cancer, Retinoblastoma, Rhabdomyosarcoma, Salivary Gland Cancer, Sarcoma (Kaposi's), Sarcoma (uterine), Sezary Syndrome, Skin Cancer (non-Melanoma), Skin Cancer (Melanoma), Skin Carcinoma (Merkel Cell), Small Intestine Cancer, Soft Tissue Sarcoma, Squamous Cell Carcinoma, Stomach (Gastric) Cancer, T-Cell Lymphoma, Testicular Cancer, Thymoma, Thyroid Cancer, Trophoblastic Tumor, Gestational, Urethral Cancer, Uterine Cancer, Endometrial, Uterine Sarcoma, Vaginal Cancer, Visual Pathway and Hypothalamic Glioma, Vulvar Cancer, Waldenström's Macroglobulinemia, and Wilms' Tumor.

[00378] *Wound Repair and Tissue Regeneration*

[00379] In some embodiments, a pharmaceutical compositions containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is used as a wound covering or is used to facilitate wound repair. In some embodiments, the tissue was damaged, compromised, or lost due to an injury (e.g., a burn; a surgical incision; an area of necrosis resulting from an infection, trauma, or a toxin; a laceration). In some embodiments, the tissue was damaged, compromised, or lost due to a burn. In some embodiments, the tissue was damaged, compromised, or lost due to a wound (e.g., an incision, laceration, abrasion). In some embodiments, the tissue was damaged, compromised, or lost due to necrosis. In some embodiments, the tissue was damaged, compromised, or lost due to ulceration.

Burns

[00380] In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is applied to a burn. In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed

herein is applied to a first degree burn. In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is applied to a second degree burn. In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is applied to a third degree burn. In some embodiments, the pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex is applied to a substrate prior to be placed on the burn.

[00381] *Wounds*

[00382] In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is applied to a wound in the skin (e.g., an incision, laceration, abrasion, ulcer, puncture, penetration). In some embodiments, the wound is an ischemic wound. In some embodiments, the pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex is applied to a substrate prior to being placed on the wound. In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein treats the wound.

[00383] In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is applied to an incision in an organ (e.g., the skin, brain, stomach, kidneys, liver, intestines, lungs, bladder, trachea, esophagus, vagina, ureter, and blood vessel walls). In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is applied to a surgical incision. In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is applied to the site of a colon resection. In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is applied to the site of a gastrectomy. In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is applied to the site of a breast surgery (e.g., breast reduction surgery, breast augmentation surgery, and mastectomy). In some embodiments, the pharmaceutical composition containing nHC-HA/PTX3 or rcHC-HA/PTX3 complex is applied to a substrate prior to being placed on the wound.

[00384] In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is used as a covering over an incision in the skin (e.g., an incision to the epidermis, dermis, and/or hypodermis). In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is used to repair or supplement the skin following hemorrhoid surgery. In some embodiments, the pharmaceutical composition containing nHC-HA/PTX3 or rcHC-HA/PTX3 complex is applied to a

substrate prior to being placed on the wound. In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein treats the wound.

[00385] *Necrosis*

[00386] In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is used as a protective graft over an area of necrotic tissue (e.g., from an infection). In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is used as a protective graft over an area of necrotic skin. In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is placed on an area of necrotic tissue. In some embodiments, the pharmaceutical composition containing nHC-HA/PTX3 or rcHC-HA/PTX3 complex is applied to a substrate prior to being placed on the necrotic tissue. In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein treats the necrotic tissue.

[00387] *Ulcer*

[00388] In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is used as a protective covering over an ulcer. In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein treats the ulcer. In some embodiments, the ulcer is a diabetic ulcer, such as a diabetic foot or leg ulcer. In some embodiments, the ulcer is an ischemic wound. In some embodiments, the pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex is applied to a substrate prior to being placed on the ulcer. In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein treats the ulcer. In some embodiments the ulcer is a non-healing ulcer. For example, in some embodiments that non-healing ulcer is a wound or ulcer on the skin that has been present for about 3-4 weeks duration without healing.

[00389] In some embodiments, the ulcer is a foot ulcer (e.g., a diabetic foot ulcer or an arterial insufficiency ulcer). In some embodiments, treating a foot ulcer comprises (a) preparing the wound (e.g., debriding the wound); and (b) placing a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein on the wound. In some embodiments, treating a foot ulcer comprises (a) preparing the wound (e.g., debriding the wound); (b) placing a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein on the wound; and (c) covering the pharmaceutical composition with a protective barrier (e.g., a silvercell dressing, metipiel, gauze, or a bandage). In some embodiments, the pharmaceutical composition containing nHC-HA/PTX3 or rcHC-HA/PTX3 complex is applied to a substrate prior

to be placed on the ulcer. In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein treats the ulcer.

[00390] In some embodiments, the ulcer is a venous stasis (VS) ulcer. In some embodiments, treating a VS ulcer comprises (a) preparing the wound (e.g., debriding the wound); and (b) placing a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein on the wound. In some embodiments, treating a VS ulcer comprises (a) preparing the wound (e.g., debriding the wound); (b) placing a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein on the wound; and (c) covering the pharmaceutical composition with a protective barrier (e.g., a wound veil, antimicrobial dressing, gauze, or a bandage). In some embodiments, the pharmaceutical composition containing nHC-HA/PTX3 or rcHC-HA/PTX3 complex is applied to a substrate prior to being placed on the wound. In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein treats the ulcer.

[00391] In some embodiments, the ulcer is a corneal ulcer (i.e., ulcerative keratitis). In some embodiments, treating a corneal ulcer comprises (a) preparing the wound (e.g., debriding the wound); and (b) placing a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein on the wound. In some embodiments, treating a corneal ulcer comprises (a) preparing the wound (e.g., debriding the wound); (b) placing a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein on the wound; and (c) covering the pharmaceutical composition with a protective barrier (e.g., a contact lens or a bandage). In some embodiments, the pharmaceutical composition containing nHC-HA/PTX3 or rcHC-HA/PTX3 complex is applied to a substrate prior to being placed on the wound. In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein treats the ulcer.

[00392] *Therapeutic Cell Therapies*

[00393] In some embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is administered in combination with a cell therapy. In some embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is co-administered with a therapeutic cell. Therapeutic cells include any cell that exhibits a therapeutic property for treatment of a disease or disorder. In some embodiments, the therapeutic cell is a recombinant cell that heterologously expresses one or more therapeutic gene products. In some embodiments, the therapeutic cell is a transplanted cell. In some embodiments, the therapeutic cell is a stem cell. In some embodiments, the therapeutic cell is a cell that expresses one or more stem cell markers (e.g. Oct-3/4 (Pou5f1), Sox2, c-Myc, and Klf4).

[00394] In some embodiments, the cell therapy is a stem cell transplant. In some embodiments, the nHC-HA/PTX3 or rcHC-HA/PTX3 complex is administered to promote expansion of stem cells of the transplant and tissue regeneration. In some examples, the nHC-HA/PTX3 or rcHC-HA/PTX3 complex is employed to reduce or inhibit inflammation, scarring, and abnormal angiogenesis caused by a stem cell transplant. In some embodiments, nHC-HA/PTX3 or rcHC-HA/PTX3 complex is employed to maintain the stem cell characteristics during ex vivo expansion by substituting feeder layers. In some embodiments, nHC-HA/PTX3 or rcHC-HA/PTX3 complex is employed to reprogram a differentiated cell to a stem cell. In some embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is employed to expand and culture stem cells *in vitro* for subsequent transplant into a subject.

[00395] In some embodiments, the stem cell therapy is an embryonic stem cell therapy. In some embodiments, the stem cell therapy is an adult stem cell therapy. In some embodiments, the stem cell therapy is a mesenchymal stem cell therapy. In some embodiments, the stem cell therapy is administered for the treatment of a disease or disorder, such as, but not limited to, cardiovascular disease, cancer, diabetes, spinal cord injury, neurodegenerative disease, Alzheimer's disease, Parkinson's disease, multiple sclerosis, Amyotrophic lateral sclerosis, Duchenne Muscular Dystrophy, muscle damage or dystrophy, stroke, burns, lung disease, retinal disease, kidney disease, osteoarthritis, and rheumatoid arthritis.

[00396] In some embodiments, stem cells are used to treat diabetes mellitus. Type 1 diabetes results from autoimmune-mediated destruction of insulin-secreting β cells in the islets of Langerhans of the pancreas. Type 2 diabetes results from systemic insulin resistance and reduced insulin secretion by pancreatic β cells. Stem cells have been shown *in vitro* to differentiate into insulin-producing cells (see e.g., Schuldiner et al. (2000) *Proc. Natl. Acad. Sci. USA*. 97:11307–11312; Guo et al., (2009) *Endocr Rev* 30:214-227). Thus, in some embodiments, stem cells, including ESCs and ASCs, and their derivatives, such as partially differentiated stem cells, are used in stem cell therapy for regeneration of pancreatic β cells.

[00397] In some embodiments, stem cells or differentiated cells employed for therapy are encapsulated in a microcapsule device. In some embodiments, the microcapsule comprises an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein. In some embodiments the nHC-HA/PTX3 or rcHC-HA/PTX3 complex is covalently attached to the microcapsule. In some embodiments the nHC-HA/PTX3 or rcHC-HA/PTX3 complex is assembled on the surface of the microcapsule, such as the inner or out surface, or both. In some embodiments the nHC-HA/PTX3 or rcHC-HA/PTX3 is formulated to coat the microcapsule. In some embodiments, the microcapsule comprises pores to allow the passage of nutrients to cells into the microcapsule and/or allows secreted proteins and

molecules (e.g., insulin) by the encapsulated cells to flow out of the microcapsule. In some embodiments, the cells are first immobilized on a microcarrier, such as a bead coated with Matrigel® and then encapsulated within the microcapsule. Methods for the encapsulation of cells, such as stem cells are known in the art and are described, for example, in Serra et al. (2011) *PLoS ONE* 6(8):e23212. In some embodiments, any method for the encapsulation of cells is employed in conjunction with the methods provided herein.

[00398] In some embodiments, allogeneic therapeutic stem cells (e.g., insulin producing islet cells) are encapsulated in a microcapsule device for production of insulin. In some embodiments, the nHC-HA/PTX3 or rcHC-HA/PTX3 complex promotes the expansion of the stem cells. In some embodiments, the nHC-HA/PTX3 or rcHC-HA/PTX3 complex, inhibits or reduces an inflammatory response against the microcapsule containing the stem cells employed for therapy of diabetes mellitus. In some embodiments, the micro capsule comprises an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein. In some embodiments, the nHC-HA/PTX3 or rcHC-HA/PTX3 complex inhibits or reduces an inflammatory response against the microcapsule containing the stem cells.

[00399] Soft Tissue Uses

[00400] Disclosed herein, in certain embodiments, is the use of an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein for repairing, reconstructing, replacing, or supplementing a recipient's damaged, compromised, or missing soft tissue (e.g., tendons). In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is applied directly to the tissue. In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is administered in conjunction with cell or tissue based therapies. In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is mixed with a cell, a plurality of cells, or a tissue and is administered as part of a tissue based therapy. In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is used to coat a cell, a plurality of cells, or a tissue and is administered as part of a tissue based therapy.

[00401] In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is used as a covering over an incision in soft tissue (e.g., eyelids form the tissue plane between different layers of soft tissue). In some embodiments, the pharmaceutical composition containing nHC-HA/PTX3 or rcHC-HA/PTX3 complex is applied to a substrate and then used as a covering over an incision in soft tissue (e.g., eyelids form the tissue plane between different layers of soft tissue).

[00402] In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is used as structural (tectonic) support for soft tissue. In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein prevents adhesion in joint or tendon repairs.

[00403] In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is used in the repair a tendon or joint (such as rotator cuff repairs, hand tendon repairs). In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is used to reinforce a tendon or joint. In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is used to prevent adhesion of a healing tendon to surrounding tissue, tendons or joints. In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is used to prevent the formation of scar tissue on a tendon.

[00404] In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is applied to a substrate and the substrate/nHC-HA/PTX3 or substrate/rcHC-HA/PTX3 complex is used to augment smaller tendons and ligaments of the foot and ankle, including the posterior tibial tendon, the peroneal tendons, the flexor and extensor tendons, and the ligaments of the lateral ankle complex. In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is applied to a substrate and the substrate/nHC-HA/PTX3 or substrate/rcHC-HA/PTX3 complex is used to reinforce primary repair of the quadriceps and patellar tendons surrounding the knee. In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is applied to a substrate and the substrate/nHC-HA/PTX3 or substrate/rcHC-HA/PTX3 complex is used as a periosteal patch for bone graft in joint replacement. In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is applied to a substrate and the substrate/nHC-HA/PTX3 or substrate/rcHC-HA/PTX3 complex is used to augment deficient hip and knee capsular tissue following total joint revision surgery.

[00405] In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is applied to a substrate and the substrate/nHC-HA/PTX3 or substrate/rcHC-HA/PTX3 complex is used in the repair of a torn rotator cuff. In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is applied to a substrate and the substrate/nHC-HA/PTX3 or substrate/rcHC-HA/PTX3 complex is used as a patch over a rotator cuff muscle or tendon (e.g., the

supraspinatus tendon). In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is applied to a substrate and the substrate/nHC-HA/PTX3 or substrate/rcHC-HA/PTX3 complex is used to reconstruct a rotator cuff muscle or tendon (e.g., the supraspinatus tendon). In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is applied to a substrate and the substrate/nHC-HA/PTX3 or substrate/rcHC-HA/PTX3 complex is used to augment a rotator cuff muscle or tendon (e.g., the supraspinatus tendon). In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is applied to a substrate and the substrate/nHC-HA/PTX3 or substrate/rcHC-HA/PTX3 complex is used to reinforce a rotator cuff muscle or tendon (e.g., the supraspinatus tendon). In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is applied to a substrate and the substrate/nHC-HA/PTX3 or substrate/rcHC-HA/PTX3 complex is used to prevent adhesion of soft tissue to a rotator cuff muscle or tendon (e.g., the supraspinatus tendon).

[00406] In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is used in the repair gingiva. In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is used in the repair gingival recession. In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is applied to a substrate and used as a patch over gingiva. In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is applied to substrate and used as a patch over an exposed tooth root surface. In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is used to reconstruct gingiva. In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is used to augment gingiva. In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is used to reinforce gingiva. In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is used to prevent adhesion of soft tissue to gingiva.

[00407] In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex described herein is applied to a substrate and the substrate/nHC-HA/PTX3 or substrate/rcHC-HA/PTX3 complex is used as a protective graft over an incision or tear in the fascia. In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is applied to a substrate and the

substrate/nHC-HA/PTX3 or substrate/rcHC-HA/PTX3 complex is used as structural (tectonic) support the fascia. In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is applied to a substrate and the substrate/nHC-HA/PTX3 or substrate/rcHC-HA/PTX3 complex is used as a replacement or supplement for the fascia. In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is applied to a substrate and the substrate/nHC-HA/PTX3 or substrate/rcHC-HA/PTX3 complex is used to repair a hernia (e.g., to repair the fascia). In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is applied to a substrate and the substrate/nHC-HA/PTX3 or substrate/rcHC-HA/PTX3 complex is used to repair an inguinal hernia. In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is applied to a substrate and the substrate/nHC-HA/PTX3 or substrate/rcHC-HA/PTX3 complex is used to repair a femoral hernia. In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is applied to a substrate and the substrate/nHC-HA/PTX3 or substrate/rcHC-HA/PTX3 complex is used to repair an umbilical hernia. In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is applied to a substrate and the substrate/nHC-HA/PTX3 or substrate/rcHC-HA/PTX3 complex is used to repair an incisional hernia. In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is applied to a substrate and the substrate/nHC-HA/PTX3 or substrate/rcHC-HA/PTX3 complex is used to repair a diaphragmatic hernia. In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is applied to a substrate and the substrate/nHC-HA/PTX3 or substrate/rcHC-HA/PTX3 complex is used to repair a Cooper's hernia, an epigastric hernia, an hiatal hernia, a Littre's hernia, a lumbar hernia, a Maydl's hernia, an obturator hernia, a pantaloon hernia, a paraesophageal hernia, a paraumbilical hernia, a perineal hernia, a properitoneal hernia, a Richter's hernia, a sliding hernia, a sciatic hernia, a spigelian hernia, a sports hernia, a Velpeau hernia, or a Amyand's hernia.

[00408] In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is applied to a substrate and the substrate/nHC-HA/PTX3 or substrate/rcHC-HA/PTX3 complex is used to repair a spinal disc herniation. In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is applied to a substrate and the substrate/nHC-HA/PTX3 or substrate/rcHC-HA/PTX3 complex is used as a protective graft over an incision or tear in a spinal

disc. In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is applied to a substrate and the substrate/nHC-HA/PTX3 or substrate/rcHC-HA/PTX3 complex is used as a protective graft over an incision or tear in an annulus fibrosis. In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is applied to a substrate and the substrate/nHC-HA/PTX3 or substrate/rcHC-HA/PTX3 complex is used as structural (tectonic) support a spinal disc. In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is applied to a substrate and the substrate/nHC-HA/PTX3 or substrate/rcHC-HA/PTX3 complex is used as structural (tectonic) support an annulus fibrosis. In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is applied to a substrate and the substrate/nHC-HA/PTX3 or substrate/rcHC-HA/PTX3 complex is used as a replacement or supplement for a spinal disc. In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is applied to a substrate and the substrate/nHC-HA/PTX3 or substrate/rcHC-HA/PTX3 complex is used as structural (tectonic) support a spinal disc. In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is applied to a substrate and the substrate/nHC-HA/PTX3 or substrate/rcHC-HA/PTX3 complex is used as a replacement or supplement for an annulus fibrosis.

[00409] In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is applied to a substrate and the substrate/nHC-HA/PTX3 or substrate/rcHC-HA/PTX3 complex is used over an incision in the brain, or in one (or all) of the meninges (i.e., the dura mater, the pia mater, and/or the arachnoid mater). In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is applied to a substrate and the substrate/nHC-HA/PTX3 or substrate/rcHC-HA/PTX3 complex is used as structural (tectonic) support for one (or all) of the meninges (i.e., the dura mater, the pia mater, and/or the arachnoid mater). In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is applied to a substrate and the substrate/nHC-HA/PTX3 or substrate/rcHC-HA/PTX3 complex is used as a replacement for one (or all) of the meninges (i.e., the dura mater, the pia mater, and/or the arachnoid mater).

[00410] In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is applied to a substrate and the substrate/nHC-HA/PTX3 or substrate/rcHC-HA/PTX3 complex is used over an incision in a lung or in the pleura.

In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is applied to a substrate and the substrate/nHC-HA/PTX3 or substrate/rcHC-HA/PTX3 complex is used as structural (tectonic) support for the pleura. In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is applied to a substrate and the substrate/nHC-HA/PTX3 or substrate/rcHC-HA/PTX3 complex is used as a replacement for the pleura.

[00411] In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is applied to a substrate and the substrate/nHC-HA/PTX3 or substrate/rcHC-HA/PTX3 complex is used over an incision in a tympanic membrane. In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is applied to a substrate and the substrate/nHC-HA/PTX3 or substrate/rcHC-HA/PTX3 complex is used as structural (tectonic) support for a tympanic membrane. In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is applied to a substrate and the substrate/nHC-HA/PTX3 or substrate/rcHC-HA/PTX3 complex is used as a replacement for a tympanic membrane.

[00412] In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is applied to a substrate and the substrate/nHC-HA/PTX3 or substrate/rcHC-HA/PTX3 complex is used as a protective graft over an incision in the heart or the pericardium. In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is applied to a substrate and the substrate/nHC-HA/PTX3 or substrate/rcHC-HA/PTX3 complex is used as structural (tectonic) support for the pericardium. In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is applied to a substrate and the substrate/nHC-HA/PTX3 or substrate/rcHC-HA/PTX3 complex is used as a replacement for the pericardium.

[00413] In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is applied to a substrate and the substrate/nHC-HA/PTX3 or substrate/rcHC-HA/PTX3 complex is used as a protective graft over an incision in the peritoneum. In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is applied to a substrate and the substrate/nHC-HA/PTX3 or substrate/rcHC-HA/PTX3 complex is used as structural (tectonic) support for the peritoneum. In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or

rcHC-HA/PTX3 complex disclosed herein is applied to a substrate and the substrate/nHC-HA/PTX3 or substrate/rcHC-HA/PTX3 complex is used as a replacement for the peritoneum.

[00414] *Ophthalmic Uses*

[00415] Disclosed herein, in certain embodiments, is the use of a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein for repairing, reconstructing, replacing, or supplementing a recipient's damaged, compromised, or missing ocular tissue.

[00416] *Treatment of Glaucoma*

[00417] As used herein, "Glaucoma" means a disorder characterized by the loss of retinal ganglion cells in the optic nerve. In certain instances, glaucoma partially or fully results from an increase in intraocular pressure in the anterior chamber (AC). Intraocular pressure varies depending on the production of liquid aqueous humor by the ciliary processes of the eye and the drainage of the aqueous humor through the trabecular meshwork.

[00418] Glaucoma Drainage Devices (GDD) are medical devices that are implanted into an eye to relieve intraocular pressure by providing an alternative pathway for the aqueous humor to drain. If left uncovered, a GDD tube will erode and leave the eye susceptible to intraocular infection. Thus, the GDD tube needs to be covered. Currently, patches used to cover GDD tubes are made from pericardium, sclera and cornea. These patches are about 400-550 microns thick. The thinness of these patches results in their melting by 25% in 2 years potentially leaving the shunt tube exposed again.

[00419] In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is applied to a substrate and the substrate/nHC-HA/PTX3 or substrate/rcHC-HA/PTX3 complex is used to cover GDD tubes. In some embodiments, the substrate/nHC-HA/PTX3 or rcHC-HA/PTX3 complex is 300-600 microns thick. In some embodiments, the substrate/nHC-HA/PTX3 or substrate/rcHC-HA/PTX3 complex does not melt by 25% in 2 years.

[00420] *Treatment of Ocular Ulcers*

[00421] In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is applied to a substrate and the substrate/nHC-HA/PTX3 or substrate/rcHC-HA/PTX3 complex is used to cover persistent epithelial defects and/or ulcers in eyes.

[00422] In some embodiments, the base of the ulcer is debrided with surgical sponges and the poorly adherent epithelium adjacent to the edge of the ulcer is removed (e.g., to the section of the eye where the epithelium becomes quite adherent). In some embodiments, a pharmaceutical

composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is applied to a substrate and the substrate/nHC-HA/PTX3 or substrate/rcHC-HA/PTX3 complex is transferred to the recipient eye. In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is applied to a substrate and the substrate/nHC-HA/PTX3 or substrate/rcHC-HA/PTX3 complex is then secured to the eye by sutures (e.g., interrupted 10-0 nylon sutures or running 10-0 nylon sutures) with the suture knots being buried. In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is applied to a substrate and the substrate/nHC-HA/PTX3 or substrate/rcHC-HA/PTX3 complex is secured to the eye by use of fibrin glue. In some embodiments, a protective layer is applied over the substrate/nHC-HA/PTX3 or substrate/rcHC-HA/PTX3 complex or the entire eye (e.g., a contact lens). In some embodiments, the substrate/nHC-HA/PTX3 or substrate/rcHC-HA/PTX3 complex further comprises an antibiotic (e.g., neomycin, polymyxin b sulfate and dexamethasone).

[00423] *Conjunctival, scleral, lid, and orbital rim surface reconstruction*

[00424] In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is applied to a substrate and the substrate/nHC-HA/PTX3 or substrate/rcHC-HA/PTX3 complex is used in conjunctival, scleral, lid, and orbital rim surface reconstruction. In some embodiments, damage to the conjunctival surface results from symblepharon lysis; surgical removal of tumor, lesion, and/or scar tissue; excimer laser photorefractive keratectomy and therapeutic keratectomy; or combinations thereof.

[00425] *Coronary Uses*

[00426] Disclosed herein, in certain embodiments, is the use of a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein for repairing, reconstructing, replacing, or supplementing a recipient's damaged, compromised, or missing coronary tissue.

[00427] *Prevention of Ischemia Reperfusion Damage*

[00428] Disclosed herein, is the use of a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex described herein for the inhibition or reduction of tissue damage resulting from acute inflammation caused by ischemia, such as, for example myocardial infarction or stroke. In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is administered to a subject having an ischemic condition, such as, but not limited to myocardial infarction or stroke.

[00429] *Coronary Artery Bypass*

[00430] Disclosed herein, is the use of a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex described herein in coronary artery bypass surgery. In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is applied to a substrate and the substrate/nHC-HA/PTX3 or substrate/rcHC-HA/PTX3 complex is grafted onto a coronary artery to bypass a section of the artery that is characterized by atherosclerosis.

[00431] *Heart Valves*

[00432] In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is applied to a substrate and the substrate/nHC-HA/PTX3 or substrate/rcHC-HA/PTX3 complex is applied over a heart valve. In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is applied to a substrate and the substrate/nHC-HA/PTX3 or substrate/rcHC-HA/PTX3 complex is used as structural (tectonic) support for a heart valve. In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is applied to a substrate and the substrate/nHC-HA/PTX3 or substrate/rcHC-HA/PTX3 complex is used as a replacement for a heart valve.

[00433] *Veins and Arteries*

[00434] In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is applied to a substrate and the substrate/nHC-HA/PTX3 or substrate/rcHC-HA/PTX3 complex is applied to a vein or artery. In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is applied to a substrate and the substrate/nHC-HA/PTX3 or substrate/rcHC-HA/PTX3 complex is used as structural (tectonic) support for a vein or artery.

[00435] *Nerve Uses*

[00436] Disclosed herein, in certain embodiments, is the use of a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein for repairing, reconstructing, replacing, or supplementing a recipient's damaged, compromised, or missing nerve tissue.

[00437] In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is applied to a substrate and the substrate/nHC-HA/PTX3 or substrate/rcHC-HA/PTX3 complex is used as a covering over a nerve (e.g., a peripheral nerve). In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is applied to a substrate and the substrate/nHC-HA/PTX3 or substrate/rcHC-HA/PTX3 complex is used as a covering over a nerve

graft, nerve transfer, or a repaired nerve. In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is applied to a substrate and the substrate/nHC-HA/PTX3 or substrate/rcHC-HA/PTX3 complex is used as a covering over an incision in a nerve (e.g., a peripheral nerve). In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is applied to a substrate and the substrate/nHC-HA/PTX3 or substrate/rcHC-HA/PTX3 complex is used as structural (tectonic) support for a nerve (e.g., a peripheral nerve). In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein prevents adhesion in nerve repair.

[00438] In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is applied to a substrate and the substrate/nHC-HA/PTX3 or substrate/rcHC-HA/PTX3 complex is used as a non-constricting encasement for injured nerves. In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex described herein prevents or minimizes scar formation, encapsulation, chronic compression, tethering of a nerve, and nerve entrapment. In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex described herein prevents or minimizes neuroma formation. In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex described herein prevents or minimizes the migration of endogenous growth factors (i.e. Nerve Growth Factor) present during nerve repair.

[00439] *Spinal Uses*

[00440] Disclosed herein, in certain embodiments, is the use of a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex described herein during spinal surgery.

[00441] In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex described herein is used during a laminectomy. In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex described herein is used to reduce or prevent epidural fibrosis and/or scar adhesions following spinal surgery (e.g., laminectomy). In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex described herein is implanted between dura mater and overlying tissue following spinal surgery (e.g., laminectomy). In some embodiments, implanting a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex described herein between dura mater and overlying tissue following spinal surgery (e.g., laminectomy) reduces or prevents migration of fibroblasts to the dura mater and collagen deposition on the dura mater.

[00442] In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex described herein is used to reduce or prevent the development of proliferative scarring following spinal surgery (e.g., laminectomy). In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex described herein is used to reduce or prevent the development of a postoperative (e.g., postlaminectomy) epidural/peridural/perineural scar. In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex described herein is used to reduce or prevent the development of proliferative scarring following spinal surgery (e.g., laminectomy). In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is used to reduce or prevent the development of a postlaminectomy membrane.

[00443] In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex described herein is used to reduce or prevent the development of extradural compression or dural tethering following spinal surgery (e.g., laminectomy). In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex described herein is used to reduce or prevent the development of tethered nerve roots following spinal surgery (e.g., laminectomy). In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex described herein is used to reduce or prevent the development of arachnoiditis following spinal surgery (e.g., laminectomy).

[00444] In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein further comprises morselized bone tissue. In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein comprising morselized bone tissue is used during a spinal fusion procedure. In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein comprising morselized bone tissue is implanted between adjacent vertebrae. In some embodiments, implantation of a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein comprising morselized bone tissue between two adjacent vertebrae promotes fusion of the vertebrae.

[00445] In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is used as a protective graft over an incision in the dura mater. In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is applied to a substrate and the substrate/nHC-HA/PTX3 or substrate/rcHC-HA/PTX3 complex is used as structural (tectonic) support for the dura mater. In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or

rcHC-HA/PTX3 complex disclosed herein is applied to a substrate and the substrate/nHC-HA/PTX3 or substrate/rcHC-HA/PTX3 complex is used as a replacement for the dura mater.

[00446] *Miscellaneous Uses of an nHC-HA/PTX3 or rcHC-HA/PTX3 complex*

[00447] In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is applied to a patch or wound dressing.

[00448] In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is used as a dermal filler. In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is injected into subdermal facial tissues. In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is injected under wrinkles and aging lines of the face (e.g., nasolabial folds, melomental folds, "crow's feet" and forehead wrinkles). In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is used for lip augmentation. In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is injected into the lips.

[00449] In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is used to treat arthritis (e.g., osteoarthritis, rheumatoid arthritis, septic arthritis, ankylosing spondylitis, spondylosis). In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is injected into an arthritic joint (e.g., a knee).

[00450] In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is used to treat arthritis in the foot. In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is used to treat arthritis of the first metatarsophalangeal (MTP) joint (e.g., hallux rigidus). In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is administered to a MTP joint following dorsal cheilectomy. In some embodiments, administration of the pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein reduces one or more adverse symptoms associated with hallux rigidus or a dorsal cheilectomy procedure (e.g., scarring, joint stiffness, swelling, inflammation, and pain).

[00451] In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is used to treat one or more symptoms associated with a bone spur (e.g., scarring, joint stiffness, swelling, inflammation, and pain).

[00452] In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is used to inhibit bone resorption in an individual in need thereof. In some embodiments, the individual has arthritis, osteoporosis, alveolar bone degradation, Paget's disease, or a bone tumor. In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex is injected into a joint. In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex is contacted with a bone (e.g., by use of a wound dressing or bandage). In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex coats a bone stent, bone implant, or bone prosthesis (e.g., an osseointegrated implant). As used herein, an "osseointegrated implant" means a three dimensional implant containing pores into which osteoblasts and supporting connective tissue migrates. In some embodiments, the bone stents are inserted into the intramedullary canal of a bone. In some embodiments, the bone stent is placed in the sinus tarsi. In some embodiments, the bone stent is placed in a knee or joint. In some embodiments, the bone stent is placed in a bone fracture. In some embodiments, the bone stent is expandable or contractible.

[00453] In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is used to promote or induce bone formation in an individual in need thereof. In some embodiments, the individual has arthritis, osteoporosis, alveolar bone degradation, Paget's disease, or a bone tumor. In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex is injected into a joint. In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex is contacted with a bone (e.g., by use of a wound dressing or bandage). In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex coats a bone stent, bone implant, or bone prosthesis (e.g., an osseointegrated implant). As used herein, an "osseointegrated implant" means a three dimensional implant containing pores into which osteoblasts and supporting connective tissue migrates. In some embodiments, the bone stents are inserted into the intramedullary canal of a bone. In some embodiments, the bone stent is placed in the sinus tarsi. In some embodiments, the bone stent is placed in a knee or joint. In some embodiments, the bone stent is placed in a bone fracture. In some embodiments, the bone stent is expandable or contractible.

[00454] In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is used to inhibit osteoclast differentiation. In some embodiments, the individual has arthritis, osteoporosis, alveolar bone degradation, Paget's disease, or a bone tumor. In some embodiments, a pharmaceutical composition containing an nHC-

HA/PTX3 or rcHC-HA/PTX3 complex is injected into a joint. In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex is contacted with a bone (e.g., by use of a wound dressing or bandage). In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex coats a bone stent, bone implant, or bone prosthesis (e.g., an osseointegrated implant). As used herein, an “osseointegrated implant” means a three dimensional implant containing pores into which osteoblasts and supporting connective tissue migrates. In some embodiments, the bone stents are inserted into the intramedullary canal of a bone. In some embodiments, the bone stent is placed in the sinus tarsi. In some embodiments, the bone stent is placed in a knee or joint. In some embodiments, the bone stent is placed in a bone fracture. In some embodiments, the bone stent is expandable or contractible.

[00455] In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is used to promote mineralization by osteoblasts in an individual in need thereof. In some embodiments, the individual has arthritis, osteoporosis, alveolar bone degradation, Paget’s disease, or a bone tumor. In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex is injected into a joint. In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex is contacted with a bone (e.g., by use of a wound dressing or bandage). In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex coats a bone stent, bone implant, or bone prosthesis (e.g., an osseointegrated implant). As used herein, an “osseointegrated implant” means a three dimensional implant containing pores into which osteoblasts and supporting connective tissue migrates. In some embodiments, the bone stents are inserted into the intramedullary canal of a bone. In some embodiments, the bone stent is placed in the sinus tarsi. In some embodiments, the bone stent is placed in a knee or joint. In some embodiments, the bone stent is placed in a bone fracture. In some embodiments, the bone stent is expandable or contractible.

[00456] In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is used to balance bone resorption and bone formation in an individual in need thereof. In some embodiments, the individual has arthritis, osteoporosis, alveolar bone degradation, Paget’s disease, or a bone tumor. In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex is injected into a joint. In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex is contacted with a bone (e.g., by use of a wound dressing or bandage). In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-

HA/PTX3 complex coats a bone stent, bone implant, or bone prosthesis (e.g., an osseointegrated implant). As used herein, an “osseointegrated implant” means a three dimensional implant containing pores into which osteoblasts and supporting connective tissue migrates. In some embodiments, the bone stents are inserted into the intramedullary canal of a bone. In some embodiments, the bone stent is placed in the sinus tarsi. In some embodiments, the bone stent is placed in a knee or joint. In some embodiments, the bone stent is placed in a bone fracture. In some embodiments, the bone stent is expandable or contractible.

[00457] In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is used to treat an orthodontic or a periodontal condition. In some embodiments, the periodontal condition is selected from gingivitis, gingival recession or periodontitis. In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is used as an anti-inflammatory or used to promote osseointegration or healing. In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is used in combination with a dental implant to promote implant osseointegration, anti-inflammation, and healing.

[00458] In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein to treat hoarseness or voice disorders. In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is used for injection laryngoplasty to repair vocal cords.

[00459] In some embodiments, a pharmaceutical composition containing an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is coated onto a medical implant (e.g., a stent). In some embodiments, a medical implant/nHC-HA/PTX3 or implant/rcHC-HA/PTX3 complex disclosed herein is implanted into an individual in need thereof, wherein the nHC-HA/PTX3 or rcHC-HA/PTX3 complex is partially or fully released into the individual. In some embodiments, the medical implant is a stent (e.g., a bone stent or a coronary stent). In some embodiments, the medical implant is a bone stent. In some embodiments, the medical implant is a coronary stent.

Combinations

[00460] In some embodiments, the compositions and methods described herein are used in conjunction with a second therapeutic agent. In some embodiments, the compositions and methods described herein are used in conjunction with two or more therapeutic agents. In some embodiments, the compositions and methods described herein are used in conjunction with one or more therapeutic agents. In some embodiments, the compositions and methods described herein are used in conjunction with 2, 3, 4, 5, 6, 7, 8, 9, 10 or more therapeutic agents.

[00461] In some embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein and a second therapeutic agent are administered in the same dosage form. In some embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein and a second therapeutic agent are administered in separate dosage forms.

[00462] In some embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein and a second therapeutic agent are administered concurrently (e.g., simultaneously, essentially simultaneously or within the same treatment protocol).

[00463] In some embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein and a second therapeutic agent are administered sequentially. In some embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is administered before or after the second therapeutic agent. In some embodiments, the time period between administration of an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein and a second active agent ranges from a few minutes to several hours, depending upon the properties of each pharmaceutical agent, such as potency, solubility, bioavailability, plasma half-life and kinetic profile of the pharmaceutical agent. In some embodiments, circadian variation of the target molecule concentration determines the optimal dose interval. In some embodiments, the timing between the administration of an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein and a second active agent is about an hour, about 2 hours, about 3 hours, about 4 hours, about 5 hours, about 6 hours, about 7 hours, about 8 hours, about 9 hours, about 10 hours, about 11 hours, about a day, about 2 days, about 3 days, about 4 days, about 5 days, about 6 days, about a week, about 2 weeks, about 3 weeks, about a month, or longer.

[00464] In some embodiments, the co-administration of an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein results in a lower required dosage for the nHC-HA/PTX3 or rcHC-HA/PTX3 complex than the required dosage when administering an nHC-HA/PTX3 or rcHC-HA/PTX3 complex alone. In some embodiments, the co-administration of a second therapeutic agent results in a lower required dosage for the second agent than the required dosage when administering the second agent alone. Methods for experimentally determining therapeutically-effective dosages of drugs and other agents for use in combination treatment regimens are known and described in the art. For example, the use of metronomic dosing, i.e., providing more frequent, lower doses in order to minimize toxic side effects, has been described extensively in the art. Combination treatment further includes periodic treatments that start and stop at various times to assist with the clinical management of the individual.

[00465] In some embodiments, the combination treatment nHC-HA/PTX3 or rcHC-HA/PTX3 complex and one or more additional therapeutic agents is modified. In some embodiments, the

combination treatment is modified, whereby the amount of the nHC-HA/PTX3 or rcHC-HA/PTX3 complex is increased relative to the amount of a second therapeutic agent. In some embodiments, the combination treatment is modified, whereby the amount of the nHC-HA/PTX3 or rcHC-HA/PTX3 complex is decreased relative to the amount of a second therapeutic agent. In some embodiments, the combination treatment is modified, whereby the amount of is a second therapeutic agent increased relative to the amount of the nHC-HA/PTX3 or rcHC-HA/PTX3 complex. In some embodiments, the combination treatment is modified, whereby the amount of is a second therapeutic agent decreased relative to the amount of the nHC-HA/PTX3 or rcHC-HA/PTX3 complex.

[00466] In some embodiments, the second therapeutic agent is selected from cytotoxic agents, an antimicrobial agents, anti-angiogenesis agents, a chemotherapeutic agent, anti-neoplastic agents or radiation therapy. In some embodiments, the second therapeutic agent is selected from alkylating agents, anti-metabolites, epidophyllotoxins; antineoplastic enzymes, topoisomerase inhibitors, procarbazines, mitoxantrones, platinum coordination complexes, biological response modifiers and growth inhibitors, hormonal/anti-hormonal therapeutic agents, hematopoietic growth factors, aromatase inhibitors, anti-estrogens, anti-androgens, corticosteroids, gonadorelin agonists, microtubule active agents, nitrosoureas, lipid or protein kinase targeting agents, immunomodulatory drugs (IMiDs), protein or lipid phosphatase targeting agents, anti-angiogenic agents, Akt inhibitors, IGF-I inhibitors, FGF3 modulators, mTOR inhibitors, Smac mimetics, HDAC inhibitors, agents that induce cell differentiation, bradykinin 1 receptor antagonists, angiotensin II antagonists, cyclooxygenase inhibitors, heparanase inhibitors, lymphokine inhibitors, cytokine inhibitors, IKK inhibitors, P38MAPK inhibitors, HSP90 inhibitors, multikinase inhibitors, bisphosphonate, rapamycin derivatives, anti-apoptotic pathway inhibitors, apoptotic pathway agonists, PPAR agonists, RAR agonists, inhibitors of Ras isoforms, telomerase inhibitors, protease inhibitors, metalloproteinase inhibitors, aminopeptidase inhibitors, SHIP activators - AQX-MN100, Humax-CD20 (ofatumumab), CD20 antagonists, IL2-diphtheria toxin fusions, or combinations thereof. In some embodiments, the antimicrobial agent is an antiviral, antibacterial or antifungal agent. Non-limiting exemplary antibacterial agent(s) include those classified as aminoglycosides, beta lactams, quinolones or fluoroquinolones, macrolides, sulfonamides, sulfamethaxozoles, tetracyclines, streptogramins, oxazolidinones (such as linezolid), clindamycins, lincomycins, rifamycins, glycopeptides, polymyxins. lipo-peptide antibiotics, as well as pharmacologically acceptable sodium salts, pharmacologically acceptable calcium salts, pharmacologically acceptable potassium salts, lipid formulations, derivatives and/or analogs of the above. Non-limiting exemplary classes of antifungal agents include imidazoles or triazoles such as clotrimazole,

miconazole, ketoconazole, econazole, butoconazole, omoconazole, oxiconazole, terconazole, itraconazole, fluconazole, voriconazole (UK 109,496), posaconazole, ravuconazole or flutrimazole; the polyene antifungals such as amphotericin B, liposomal amphoterecin B, natamycin, nystatin and nystatin lipid formulations; the cell wall active cyclic lipopeptide antifungals, including the echinocandins such as caspofungin, micafungin, anidulfungin, cilofungin; LY121019; LY303366; the allylamine group of antifungals such as terbinafme. Yet other non-limiting examples of antifungal agents include naftifine, tolnaftate, mediocidin, candicidin, trichomycin, hamycin, aurefungin, ascosin, ayfatin, azacolutin, trichomycin, levorin, heptamycin, candimycin, griseofulvin, BF-796, MTCH 24, BTG-137586, pradimicins (MNS 18184), benanomycin; ambisome; nikkomycin Z; flucytosine, or perimycin. Non-limiting examples of antiviral agents include cidofovir, amantadine, rimantadine, acyclovir, gancyclovir, pencyclovir, famciclovir, foscarnet, ribavirin, or valcyclovir. In some embodiments, the antimicrobial agent is an innate immune peptide or proteins. Some exemplary classes of innate peptides or proteins are transferrins, lactoferrins, defensins, phospholipases, lysozyme, cathelicidins, serprocidins, bacteriocidal permeability increasing proteins, amphipathic alpha helical peptides, and other synthetic antimicrobial proteins. In some embodiments, the antimicrobial agent is an antiseptic agent.

[00467] In some embodiments, the second therapeutic agent is selected from ARRY-797, dacarbazine (DTIC), actinomycins C₂, C₃, D, and F₁, cyclophosphamide, melphalan, estramustine, maytansinol, rifamycin, streptovaricin, doxorubicin, daunorubicin, epirubicin, idarubicin, detorubicin, carminomycin, esorubicin, mitoxantrone, bleomycins A, A₂, and B, camptothecin, Irinotecan, Topotecan, 9-aminocamptothecin, 10,11 -methylenedioxycamptothecin, 9-nitrocamptothecin, bortezomib, temozolomide, TAS103, NPI0052, combretastatin, combretastatin A-2, combretastatin A-4, calicheamicins, neocarcinostatins, epothilones A B, C, and semi-synthetic variants, Herceptin, Rituxan, CD40 antibodies, asparaginase, interleukins, interferons, leuprolide, and pegaspargase, 5-fluorouracil, fluorodeoxyuridine, ptorafur, 5'-deoxyfluorouridine, UFT, MITC, S-1 capecitabine, diethylstilbestrol, tamoxifen, toremefine, tolmodex, thymitaq, flutamide, fluoxymesterone, bicalutamide, finasteride, estradiol, trioxifene, dexamethasone, leuproelin acetate, estramustine, droloxifene, medroxyprogesterone, megestrol acetate, aminoglutethimide, testolactone, testosterone, diethylstilbestrol, hydroxyprogesterone, mitomycins A, B and C, porfiromycin, cisplatin, carboplatin, oxaliplatin, tetraplatin, platinum-DACH, ormaplatin, thalidomide, lenalidomide, CI-973, telomestatin, CHIR258, Rad 001, SAHA, Tubacin, 17-AAG, sorafenib, JM-216, podophyllotoxin, epipodophyllotoxin, etoposide, teniposide, Tarceva, Iressa, Imatinib, Miltefosine, Perifosine, aminopterin, methotrexate, methopterin, dichloro-methotrexate, 6-mercaptopurine, thioguanine, azattuoprine, allopurinol, cladribine, fludarabine, pentostatin, 2-

chloroadenosine, deoxycytidine, cytosine arabinoside, cytarabine, azacitidine, 5-azacytosine, gencitabine, 5-azacytosine-arabinoside, vincristine, vinblastine, vinorelbine, leurosine, leurosidine and vindesine, paclitaxel, taxotere and/or docetaxel.

[00468] In some embodiments, the second active agent is niacin, a fibrate, a statin, a Apo-A1 mimetic polypeptide (e.g., DF-4, Novartis), an apoA-I transcriptional up-regulator, an ACAT inhibitor, a CETP modulator, Glycoprotein (GP) IIb/IIIa receptor antagonists, P2Y12 receptor antagonists, Lp-PLA2-inhibitors, an anti-tumor necrosis factor (TNF) agent, an interleukin-1 (IL-1) receptor antagonist, an interleukin-2 (IL-2) receptor antagonist, an interleukin-6 (IL-6) receptor antagonist, an interleukin-12 (IL-12) receptor antagonist, an interleukin-17 (IL-17) receptor antagonist, an interleukin-23 (IL-23) receptor antagonist, a cytotoxic agent, an antimicrobial agent, an immunomodulatory agent, an antibiotic, a T-cell co-stimulatory blocker, a disorder-modifying anti-rheumatic agent, a B cell depleting agent, an immunosuppressive agent, an anti-lymphocyte antibody, an alkylating agent, an anti-metabolite, a plant alkaloid, a terpenoids, a topoisomerase inhibitor, an anti-tumor antibiotic, a monoclonal antibody, a hormonal therapy (e.g., aromatase inhibitors), or combinations thereof.

[00469] In some embodiments, the second active agent is an anti-TGF- β antibody, an anti-TGF- β receptor blocking antibody, an anti-TNF antibody, an anti-TNF receptor blocking antibody, an anti-IL1 β antibody, an anti-IL1 β receptor blocking antibody, an anti-IL-2 antibody, an anti-IL-2 receptor blocking antibody, an anti-IL-6 antibody, an anti-IL-6 receptor blocking antibody, an anti IL-12 antibody, an anti IL-12 receptor blocking antibody, an anti-IL-17 antibody, anti-IL-17 receptor blocking antibody, an anti-IL-23 antibody, or an anti-IL-23 receptor blocking antibody.

[00470] In some embodiments, the second active agent is niacin, bezafibrate; ciprofibrate; clofibrate; gemfibrozil; fenofibrate; DF4 (Ac-D-W-F-K-A-F-Y-D-K-V-A-E-K-F-K-E-A-F-NH₂); DF5; RVX-208 (Resverlogix); avasimibe; pactimibe sulfate (CS-505); CI-1011 (2,6-diisopropylphenyl [(2, 4,6-triisopropylphenyl)acetyl]sulfamate); CI-976 (2,2-dimethyl-N-(2,4,6-trimethoxyphenyl)dodecanamide); VULM1457 (1-(2,6-diisopropyl-phenyl)-3-[4-(4'-nitrophenylthio)phenyl] urea); CI-976 (2,2-dimethyl-N-(2,4,6- trimethoxyphenyl)dodecanamide); E-5324 (n-butyl-N'-(2-(3-(5-ethyl-4-phenyl-1H-imidazol-1-yl)propoxy)-6-methylphenyl)urea); HL-004 (N-(2,6-diisopropylphenyl) tetradecylthioacetamide); KY-455 (N-(4,6- dimethyl-1-pentylindolin-7-yl)-2,2-dimethylpropanamide); FY-087 (N-[2-[N'-pentyl-(6,6-dimethyl-2,4-heptadiynyl)amino]ethyl]-(2-methyl-1-naphthyl-thio)acetamide); MCC-147 (Mitsubishi Pharma); F 12511 ((S)-2',3',5'-trimethyl-4'-hydroxy-alpha-dodecylthioacetanilide); SMP-500 (Sumitomo Pharmaceuticals); CL 277082 (2,4-difluoro-phenyl-N[[4-(2,2-dimethylpropyl)phenyl]methyl]-N-(heptyl)urea); F-1394 ((1s,2s)-2-[3-(2,2-dimethylpropyl)-3-nonylureido]aminocyclohexane-1-yl

3-[N-(2,2,5,5-tetramethyl-1,3-dioxane-4-carbonyl)amino]propionate); CP- 113818 (N-(2,4-bis(methylthio)-6-methylpyridin-3-yl)-2-(hexylthio)decanoic acid amide); YM-750; torcetrapib; anacetrapid; JTT-705 (Japan Tobacco/Roche); abciximab; eptifibatide; tirofiban; roxifiban; variabilin; XV 459 (N(3)-(2-(3-(4-formamidinophenyl)isoxazolin-5-yl)acetyl)-N(2)-(1-butyloxycarbonyl)-2,3-diaminopropionate); SR 121566A (3-[N-{4-[4-(aminoiminomethyl)phenyl]-1,3-thiazol-2-yl}-N-(1-carboxymethylpiperid-4-yl) aminol propionic acid, trihydrochloride); FK419 ((S)-2-acetylamino-3-[(R)-[1-[3-(piperidin-4-yl) propionyl] piperidin-3-ylcarbonyl] amino] propionic acid trihydrate); clopidogrel; prasugrel; cangrelor; AZD6140 (AstraZeneca); MRS 2395 (2,2-Dimethyl-propionic acid 3-(2-chloro-6-methylaminopurin-9-yl)- 2-(2,2-dimethyl-propionyloxymethyl)-propyl ester); BX 667 (Berlex Biosciences); BX 048 (Berlex Biosciences); darapladib (SB 480848); SB-435495 (GlaxoSmithKline); SB-222657 (GlaxoSmithKline); SB-253514 (GlaxoSmithKline); alefacept, efalizumab, methotrexate, acitretin, isotretinoin, hydroxyurea, mycophenolate mofetil, sulfasalazine, 6-Thioguanine, Dovonex, Taclonex, betamethasone, tazarotene, hydroxychloroquine, sulfasalazine, etanercept, adalimumab, infliximab, abatacept, rituximab, trastuzumab, anti-CD45 monoclonal antibody AHN-12 (NCI), Iodine-131 Anti-B1 Antibody (Corixa Corp.), anti-CD66 monoclonal antibody BW 250/183 (NCI, Southampton General Hospital), anti-CD45 monoclonal antibody (NCI, Baylor College of Medicine), antibody anti-anb3 integrin (NCI), BIW-8962 (BioWa Inc.), antibody BC8 (NCI), antibody muJ591 (NCI), indium In 111 monoclonal antibody MN-14 (NCI), yttrium Y 90 monoclonal antibody MN-14 (NCI), F105 Monoclonal Antibody (NIAID), Monoclonal Antibody RAV12 (Raven Biotechnologies), CAT-192 (Human Anti-TGF-Beta1 Monoclonal Antibody, Genzyme), antibody 3F8 (NCI), 177Lu-J591 (Weill Medical College of Cornell University), TB-403 (BioInvent International AB), anakinra, azathioprine, cyclophosphamide, cyclosporine A, leflunomide, d-penicillamine, amitriptyline, or nortriptyline, chlorambucil, nitrogen mustard, prasterone, LJP 394 (abetimus sodium), LJP 1082 (La Jolla Pharmaceutical), eculizumab, belimumab, rhuCD40L (NIAID), epratuzumab, sirolimus, tacrolimus, pimecrolimus, thalidomide, antithymocyte globulin-equine (Atgam, Pharmacia Upjohn), antithymocyte globulin-rabbit (Thymoglobulin, Genzyme), Muromonab-CD3 (FDA Office of Orphan Products Development), basiliximab, daclizumab, riluzole, cladribine, natalizumab, interferon beta-1b, interferon beta-1a, tizanidine, baclofen, mesalazine, asacol, pentasa, mesalamine, balsalazide, olsalazine, 6-mercaptopurine, AIN457 (Anti IL-17 Monoclonal Antibody, Novartis), theophylline, D2E7 (a human anti-TNF mAb from Knoll Pharmaceuticals), Mepolizumab (Anti-IL-5 antibody, SB 240563), Canakinumab (Anti-IL-1 Beta Antibody, NIAMS), Anti-IL-2 Receptor Antibody (Daclizumab, NHLBI), CNTO 328 (Anti IL-6 Monoclonal Antibody, Centocor), ACZ885 (fully

human anti-interleukin-1beta monoclonal antibody, Novartis), CNTO 1275 (Fully Human Anti-IL-12 Monoclonal Antibody, Centocor), (3S)-N-hydroxy-4-({4-[(4-hydroxy-2-butynyl)oxy]phenyl} sulfonyl)-2,2-dimethyl-3-thiomorpholine carboxamide (apratatstat), golimumab (CNTO 148), Onercept, BG9924 (Biogen Idec), Certolizumab Pegol (CDP870, UCB Pharma), AZD9056 (AstraZeneca), AZD5069 (AstraZeneca), AZD9668 (AstraZeneca), AZD7928 (AstraZeneca), AZD2914 (AstraZeneca), AZD6067 (AstraZeneca), AZD3342 (AstraZeneca), AZD8309 (AstraZeneca),), [(1R)-3-methyl-1-({(2S)-3-phenyl-2-[(pyrazin-2-ylcarbonyl)amino]propanoyl} amino)butyl]boronic acid (Bortezomib), AMG-714, (Anti-IL 15 Human Monoclonal Antibody, Amgen), ABT-874 (Anti IL-12 monoclonal antibody, Abbott Labs), MRA(Tocilizumab, an Anti IL-6 Receptor Monoclonal Antibody, Chugai Pharmaceutical), CAT-354 (a human anti-interleukin-13 monoclonal antibody, Cambridge Antibody Technology, MedImmune), aspirin, salicylic acid, gentisic acid, choline magnesium salicylate, choline salicylate, choline magnesium salicylate, choline salicylate, magnesium salicylate, sodium salicylate, diflunisal, carprofen, fenoprofen, fenoprofen calcium, flurobiprofen, ibuprofen, ketoprofen, nabutone, ketolorac, ketorolac tromethamine, naproxen, oxaprozin, diclofenac, etodolac, indomethacin, sulindac, tolmetin, meclofenamate, meclofenamate sodium, mefenamic acid, piroxicam, meloxicam, celecoxib, rofecoxib, valdecoxib, parecoxib, etoricoxib, lumiracoxib, CS-502 (Sankyo), JTE-522 (Japan Tobacco Inc.), L-745,337 (Almirall), NS398 (Sigma), betamethasone (Celestone), prednisone (Deltasone), alclometasone, aldosterone, amcinonide, beclometasone, betamethasone, budesonide, ciclesonide, clobetasol, clobetasone, clocortolone, cloprednol, cortisone, cortivazol, deflazacort, deoxycorticosterone, desonide, desoximetasone, desoxycortone, dexamethasone, diflorasone, diflucortolone, difluprednate, fluclorolone, fludrocortisone, fludroxycortide, flumetasone, flunisolide, fluocinolone acetonide, fluocinonide, fluocortin, fluocortolone, fluorometholone, fluperolone, fluprednidene, fluticasone, formocortol, formoterol, halcinonide, halometasone, hydrocortisone, hydrocortisone aceponate, hydrocortisone buteprate, hydrocortisone butyrate, loteprednol, medrysone, meprednisone, methylprednisolone, methylprednisolone aceponate, mometasone furoate, paramethasone, prednicarbate, prednisone, rimexolone, tixocortol, triamcinolone, ulobetasol; cisplatin; carboplatin; oxaliplatin; mechlorethamine; cyclophosphamide; chlorambucil; vincristine; vinblastine; vinorelbine; vindesine; azathioprine; mercaptopurine; fludarabine; pentostatin; cladribine; 5-fluorouracil (5FU); floxuridine (FUDR); cytosine arabinoside; methotrexate; trimethoprim; pyrimethamine; pemetrexed; paclitaxel; docetaxel; etoposide; teniposide; irinotecan; topotecan; amsacrine; etoposide; etoposide phosphate; teniposide; dactinomycin; doxorubicin; daunorubicin; valrubicine; idarubicine; epirubicin; bleomycin; plicamycin; mitomycin; trastuzumab; cetuximab; rituximab;

bevacizumab; finasteride; goserelin; aminoglutethimide; anastrozole; letrozole; vorozole; exemestane; 4-androstene-3,6,17-trione ("6-OXO"; 1,4,6-androstatrien-3,17-dione (ATD); formestane; testolactone; fadrozole; or combinations thereof.

[00471] In some embodiments, the second therapeutic agent is an antibiotic. In some embodiments, the second therapeutic agent is an anti-bacterial agent. In some embodiments, the second therapeutic agent is amikacin, gentamicin, kanamycin, neomycin, netilmicin, streptomycin, tobramycin, paromomycin, geldanamycin, herbimycin, loracarbef, ertapenem, doripenem, imipenem, cilastatin, meropenem, cefadroxil, cefazolin, cefalotin, cefalexin, cefaclor, cefamandole, cefoxitin, defprozil, cefuroxime, cefixime, cefdinir, cefditoren, cefoperazone, cefotaxime, cefpodoxime, ceftazidime, ceftibuten, ceftizoxime, ceftriaxone, cefepime, ceftobiprole, teicoplanin, vancomycin, azithromycin, clarithromycin, dirithromycin, erythromycin, roxithromycin, troleandomycin, telithromycin, spectinomycin, aztreonam, amoxicillin, ampicillin, azlocillin, carbenicillin, cloxacillin, dicloxacillin, flucloxacillin, mezlocillin, meticillin, nafcillin, oxacillin, penicillin, piperacillin, ticarcillin, bacitracin, colistin, polymyxin B, ciprofloxacin, enoxacin, gatifloxacin, levofloxacin, lomefloxacin, moxifloxacin, norfloxacin, ofloxacin, trovfloxacin, mafenide, prontosil, sulfacetamide, sulfamethizole, sulfanimilimide, sulfsalazine, sulfsioxazole, trimethoprim, demeclocycline, doxycycline, minocycline, oxtetracycline, tetracycline, arspenamine, chloramphenicol, clindamycin, lincomycin, ethambutol, fosfomycin, fusidic acid, furazolidone, isoniazid, linezolid, metronidazole, mupirocin, nitrofurantoin, platensimycin, pyrazinamide, quinupristin/dalfopristin, rifampin, tinidazole, and combinations thereof.

[00472] In some embodiments, the second therapeutic agent is an anti-viral agent. In some embodiments, the second therapeutic agent is acyclovir, famciclovir, valacyclovir, abacavir, aciclovir, adfovir, amantadine, amprenavir, arbidol., atazanavir, artipla, brivudine, cidofovir, combivir, edoxudine, efavirenz, emtricitabine, enfuvirtide, entecavir, fomvirsen, fosamprenavir, foscarnet, fosfonet, ganciclovir, gardasil, ibacitabine, imunovir, idoxuridine, imiquimod, indinavir, inosine, integrase inhibitors, interferons, including interferon type I (e.g. IFN α and IFN β), interferon type II, interferon type III, lamivudine, lopinavir, loviride, MK-0518, maraviroc, moroxydine, nelfinavir, nevirapine, nexavir, nucleoside analogues, oseltamivir, penciclovir, peramivir, pleconaril, podophyllotoxin, protease inhibitors, reverse transcriptase inhibitors, ribavirin, rimantadine, ritonavir, saquinavir, stavudine, tenofovir, tenofovir disoproxil, tipranavir, trifluridine, trizivir, tromantadine, truvada, valganciclovir, vicriviroc, vidarabine, viramidine, zalcitabine, zanamivir, zidovudine, and combinations thereof.

[00473] In some embodiments, the second therapeutic agent is an anti-fungal agent. In some embodiments, the second therapeutic agent is amroline, utenafine, naftifine, terbinafine,

flucytosine, fluconazole, itraconazole, ketoconazole, posaconazole, ravuconazole, voriconazole, clotrimazole, econazole, miconazole, oxiconazole, sulconazole, terconazole, tioconazole, nikkomycin Z, caspofungin, micafungin, anidulafungin, amphotericin B, liposomal nystatin, pimarinic, griseofulvin, ciclopirox olamine, haloprogin, tolnaftate, undecylenate, clioquinol, and combinations thereof.

[00474] In some embodiments, the second therapeutic agent is an anti-parasitic agent. In some embodiments, the second therapeutic agent is amitraz, amoscanate, avermectin, carbadox, diethylcarbamizine, dimetridazole, diminazene, ivermectin, macrofilaricide, malathion, mitaban, oxamniquine, permethrin, praziquantel, prantel pamoate, selamectin, sodium stibogluconate, thiabendazole, and combinations thereof.

[00475] *Combinations with cells and tissues*

[00476] In some embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is co-administered with a cell, a plurality of cells or a tissue.

[00477] In some embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is co-administered with a therapeutic cell. In some embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is co-administered with a tissue transplant. In some embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is co-administered with a stem cell transplant. In some embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is co-administered with an organ transplant.

[00478] In some embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is administered concurrently (e.g., simultaneously, essentially simultaneously or within the same treatment protocol) with a tissue transplant. In some embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is administered before or after a tissue transplant. In some embodiments, the time period between administration of an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein and the tissue transplant ranges from a few minutes to several hours, depending upon the properties of each pharmaceutical agent, such as potency, solubility, bioavailability, plasma half-life and kinetic profile of the pharmaceutical agent. In some embodiments, circadian variation of the target molecule concentration determines the optimal dose interval. In some embodiments, the timing between the administration of an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein and a second active agent is about less than an hour, less than a day, less than a week, or less than a month.

[00479] In some embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is co-administered with a tissue transplant and an immunosuppressive agent. In some embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is co-administered with a tissue

transplant and a calcineurin inhibitor (e.g., cyclosporin or tacrolimus); an mTOR inhibitor (sirolimus; everolimus); an anti-proliferative agent (azathioprine or mycophenolic acid); a corticosteroid (e.g., prednisolone or hydrocortisone); a monoclonal anti-IL-2R α receptor antibody (e.g., basiliximab or daclizumab); a polyclonal anti-T-cell antibodies (e.g., anti-thymocyte globulin (ATG) or anti-lymphocyte globulin (ALG)); or combinations thereof.

[00480] In some embodiments, a tissue is coated with an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein. In some embodiments, a plurality of stem cells are coated with an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein. In some embodiments, an organ is coated with an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein. In some embodiments, coating a tissue with an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein prevent a tissue from being acted upon by the host immune system.

[00481] In some embodiments, an organ, tissue, or plurality of stem cells is contacted with an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein. In some embodiments, an organ, tissue, or plurality of stem cells is contacted with a composition comprising an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein. In some embodiments, the composition has a pH of about 7.0 to about 7.5. In some embodiments, the composition has a pH of 7.4. In some embodiments, the composition further comprises potassium, magnesium, and raffinose. In some embodiments, the composition further comprises at least one of adenosine, glutathione, allopurinol, and hydroxyethyl starch. In some embodiments, the composition is UW solution supplemented with an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein.

[00482] In some embodiments, the organ, tissue, or plurality of stem cells are contacted with an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein for about 30 minutes, about 1 hour, about 2 hours, about 3 hours, about 4 hours, about 5 hours, about 6 hours, about 12 hours, about 24 hours, about 36 hours, or about 48 hours. In some embodiments, the contacting occurs at a temperature that protects tissues and vascular conditioning (e.g., less than ambient temperature). In some embodiments, the contacting occurs at 4°C.

[00483] *Medical device combinations*

[00484] In some embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is co-administered with a medical device. In some embodiments, medical device or a portion thereof is contacted with an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein. In some embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein is use to coat a medical device or a portion thereof as described elsewhere herein. In some embodiment, administration of an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein in combination with a medical device reduces, inhibits or prevents inflammatory reactions against the implanted

medical device. In some embodiment, administration of an nHC-HA/PTX3 or rcHC-HA/PTX3 complex disclosed herein in combination with a medical device reduces, inhibits or prevents the formation of infectious biofilms that are produced by microorganism growth on the implanted medical device (i.e. chronic biofilm infection). Exemplary of such biofilms are those produced by bacteria, such as but not limited to, *Staphylococcus aureus*.

Articles of Manufacture and Kits

[00485] The articles of manufacture provided herein contain packaging materials. Packaging materials for use in packaging pharmaceutical products are well known to those of skill in the art. Examples of pharmaceutical packaging materials include, but are not limited to, blister packs, bottles, tubes, inhalers, inhalers (e.g., pressurized metered dose inhalers (MDI), dry powder inhalers (DPI), nebulizers (e.g., jet or ultrasonic nebulizers) and other single breath liquid systems), pumps, bags, vials, containers, syringes, bottles, and any packaging material suitable for a selected formulation and intended mode of administration and treatment. In some embodiments, the pharmaceutical composition is incorporated in, applied to or coated on a medical device, such as implants, catheters, artificial joints, stents, valves, nanoparticles, or microcapsules.

[00486] In some embodiments, the pharmaceutical compositions or combinations provided herein are provided as kits. Kits optionally include one or more components such as instructions for use, devices and additional reagents (e.g., sterilized water or saline solutions for dilution of the compositions and/or reconstitution of lyophilized protein), and components, such as tubes, containers and syringes for practice of the methods. Exemplary kits include the pharmaceutical compositions or combinations provided herein, and optionally include instructions for use, a device for administering the pharmaceutical compositions or combinations to a subject, a device for detecting the nHC-HA/PTX3 or rcHC-HA/PTX3 complexes in a subject, a device for detecting the nHC-HA/PTX3 or rcHC-HA/PTX3 complexes in samples obtained from a subject, and a device for administering an additional therapeutic agent to a subject.

[00487] The kit can, optionally, include instructions. Instructions typically include a tangible expression describing the nHC-HA/PTX3 or rcHC-HA/PTX3 complexes and, optionally, other components included in the kit, and methods for administration, including methods for determining the proper state of the subject, the proper dosage amount, dosing regimens, and the proper administration method for administering the nHC-HA/PTX3 or rcHC-HA/PTX3 complexes. In some embodiments, instructions include guidance for monitoring the subject over the duration of the treatment time

[00488] In some embodiments, kits include a pharmaceutical composition described herein and an item for diagnosis. For example, such kits include an item for measuring the concentration, amount or activity of the selected nHC-HA/PTX3 or rcHC-HA/PTX3 complexes in a subject.

[00489] In some embodiments, kits provided herein include a device for administering the nHC-HA/PTX3 or rcHC-HA/PTX3 complexes to a subject. In some embodiments, any of a variety of devices known in the art for administering medications to a subject -is included in the kits provided herein. Exemplary devices include, but are not limited to, an inhaler (e.g., pressurized metered dose inhaler (MDI), dry powder inhaler (DPI), nebulizer (e.g., jet or ultrasonic nebulizers) and other single breath liquid system), a hypodermic needle, an intravenous needle, a catheter, and a liquid dispenser such as an eyedropper. Typically the device for administering the nHC-HA/PTX3 or rcHC-HA/PTX3 complexes of the kit will be compatible with the desired method of administration of the nHC-HA/PTX3 or rcHC-HA/PTX3 complexes.

Expansion of Stem Cell Cultures

[00490] Disclosed herein, in certain embodiments, are methods of expanding an isolated stem cell on a substrate that comprises an nHC-HA/PTX3 or rcHC-HA/PTX3 complex provided herein. As described herein, HC-HA/PTX3 complexes promote the aggregation of stem cells, prevent differentiation of the cells and preserves expression of stem cell markers.

[00491] In some embodiments, the expansion on an nHC-HA/PTX3 complex or rcHC-HA/PTX3 complex preserves expression of one or more of embryonic stem cell (ESC) markers (e.g. Oct4, Nanog, Sox2 (SRY (sex determining region Y)-box 2), Rex1 (Zfp42), SSEA4 (stage-specific embryonic antigen-4), MYC/c-Myc and *KLF4*, pericyte markers (e.g. NG2 (neuron-glia antigen 2/Chondroitin sulfate proteoglycan 4(CSPG4)), PDGFR- β (Platelet-derived growth factor receptor B), and α -SMA (α -smooth muscle actin)), and angiogenic markers (e.g. CD133/2, FLK-1 (VEGF-R2, Ly-73), vWF (von Willebrand factor), CD34, CD31 (PECAM-1) and CD146). In some embodiments, the expression of the stem cell marker is determined by conventional methods, such as for example, protein expression analysis (e.g. Western blotting, immunofluorescence, immunohistochemistry, fluorescence activated cell sorting) or mRNA analysis (e.g. polymerase chain reaction (PCR) or Northern).

[00492] In some embodiments, nHC-HA/PTX3 or rcHC-HA/PTX3 suppresses TGF- β signaling in a cultured cell. In some embodiments, nHC-HA/PTX3 or rcHC-HA/PTX3 suppresses TGF- β signaling in a cultured stem cell. In some embodiments, suppression of TGF- β signaling refers to a decrease in the activity or expression of one or more proteins or markers in the TGF- β cell signaling pathway such as pSMAD 2/3 signaling, α smooth muscle formation, in a cell in the

presence of the nHC-HA/PTX3 or rcHC-HA/PTX3 complex compared to the absence of the nHC-HA/PTX3 or rcHC-HA/PTX3 complex.

[00493] In some embodiments, nHC-HA/PTX3 or rcHC-HA/PTX3 induces BMP signaling in a cultured cell. In some embodiments, nHC-HA/PTX3 or rcHC-HA/PTX3 induces BMP signaling in a cultured stem cell. In some embodiments, suppression of TGF- β signaling refers to an increase in the activity or expression of one or more proteins in the BMP signaling pathway, such as BMP-2, BMP-4, BMP-6 and pSMAD1/5/8, in a cell in the presence of the nHC-HA/PTX3 or rcHC-HA/PTX3 complex compared to the absence of the nHC-HA/PTX3 or rcHC-HA/PTX3 complex.

[00494] In some embodiments, the isolated stem cell cultured on nHC-HA/PTX3 or rcHC-HA/PTX3 is an embryonic stem cell. In some embodiments, the isolated stem cell cultured on nHC-HA/PTX3 or rcHC-HA/PTX3 is an adult stem cell. In some embodiments, the isolated stem cell cultured on nHC-HA/PTX3 or rcHC-HA/PTX3 is a fetal stem cell. In some embodiments, the isolated stem cell cultured on nHC-HA/PTX3 or rcHC-HA/PTX3 is an induced pluripotent/progenitor stem cell (iPSC).

[00495] In some embodiments, the isolated stem cell cultured on nHC-HA/PTX3 or rcHC-HA/PTX3 is a mesenchymal stem cell. In some embodiments, the isolated stem cell cultured on nHC-HA/PTX3 or rcHC-HA/PTX3 is an adipose stem cell (ASC). In some embodiments, the isolated stem cell cultured on nHC-HA/PTX3 or rcHC-HA/PTX3 is an umbilical cord stem cell. In some embodiments, the isolated stem cell cultured on nHC-HA/PTX3 or rcHC-HA/PTX3 is an amniotic membrane stem cell. In some embodiments, the isolated stem cell cultured on nHC-HA/PTX3 or rcHC-HA/PTX3 is a limbal cell, such as a limbal niche cell or a limbal epithelial progenitor cell. In some embodiments, the isolated stem cell cultured on nHC-HA/PTX3 or rcHC-HA/PTX3 is an endothelial stem cell. In some embodiments, the isolated stem cell cultured on nHC-HA/PTX3 or rcHC-HA/PTX3 is a hematopoietic stem cell. In some embodiments, the isolated stem cell is a bone marrow stem cell. In some embodiments, the isolated stem cell cultured on nHC-HA/PTX3 or rcHC-HA/PTX3 is a neural stem cell. In some embodiments, the isolated stem cell cultured on nHC-HA/PTX3 or rcHC-HA/PTX3 is an endothelial progenitor cell. In some embodiments, the isolated stem cell cultured on nHC-HA/PTX3 or rcHC-HA/PTX3 is a skeletal muscle stem cell. In some embodiments, the isolated stem cell cultured on nHC-HA/PTX3 or rcHC-HA/PTX3 is a mammary stem cell. In some embodiments, the isolated stem cell cultured on nHC-HA/PTX3 or rcHC-HA/PTX3 is an intestinal stem cell.

[00496] In some embodiments, the isolated stem cell cultured on nHC-HA/PTX3 or rcHC-HA/PTX3 is an induced pluripotent/progenitor stem cell (iPSC). In some embodiments, the isolated stem cell cultured on nHC-HA/PTX3 or rcHC-HA/PTX3 is an induced pluripotent stem cell

derived from an adult differentiated or partially differentiated cell. In some embodiments, the isolated stem cell cultured on nHC-HA/PTX3 or rcHC-HA/PTX3 is an induced pluripotent stem cell derived from a fibroblast. In some embodiments, the isolated stem cell cultured on nHC-HA/PTX3 or rcHC-HA/PTX3 is an induced pluripotent stem cell derived from a human corneal fibroblast.

[00497] In some embodiments, the isolated stem cell cultured on nHC-HA/PTX3 or rcHC-HA/PTX3 is derived from a fetal tissue, such as placental tissue or an umbilical cord tissue. In some embodiments, the isolated stem cell cultured on nHC-HA/PTX3 or rcHC-HA/PTX3 is derived from amniotic membrane. In some embodiments, the isolated stem cell cultured on nHC-HA/PTX3 or rcHC-HA/PTX3 is derived from adipose tissue. In some embodiments, the isolated stem cell cultured on nHC-HA/PTX3 or rcHC-HA/PTX3 is derived from limbal tissue. In some embodiments, the isolated stem cell cultured on nHC-HA/PTX3 or rcHC-HA/PTX3 is derived from bone marrow. In some embodiments, the isolated stem cell cultured on nHC-HA/PTX3 or rcHC-HA/PTX3 is derived from endothelial tissue. In some embodiments, the isolated stem cell cultured on nHC-HA/PTX3 or rcHC-HA/PTX3 is derived from limbal tissue. In some embodiments, the isolated stem cell cultured on nHC-HA/PTX3 or rcHC-HA/PTX3 is derived from neural tissue. In some embodiments, the isolated stem cell cultured on nHC-HA/PTX3 or rcHC-HA/PTX3 is derived from limbal tissue. In some embodiments, the isolated stem cell cultured on nHC-HA/PTX3 or rcHC-HA/PTX3 is derived from skeletal muscle. In some embodiments, the isolated stem cell cultured on nHC-HA/PTX3 or rcHC-HA/PTX3 is derived from the skin. In some embodiments, the isolated stem cell cultured on nHC-HA/PTX3 or rcHC-HA/PTX3 is derived from the digestive system. In some embodiments, the isolated stem cell cultured on nHC-HA/PTX3 or rcHC-HA/PTX3 is derived from the pancreas. In some embodiments, the isolated stem cell cultured on nHC-HA/PTX3 or rcHC-HA/PTX3 is derived from the liver. In some embodiments, the isolated stem cell cultured on nHC-HA/PTX3 or rcHC-HA/PTX3 is derived from the olfactory mucosa. In some embodiments, the isolated stem cell cultured on nHC-HA/PTX3 or rcHC-HA/PTX3 is derived from a germ cell population. In some embodiments, the isolated stem cell cultured on nHC-HA/PTX3 or rcHC-HA/PTX3 is derived from blood. In some embodiments, the isolated stem cell cultured on nHC-HA/PTX3 or rcHC-HA/PTX3 is derived from umbilical cord blood.

[00498] In some embodiments, the HC-HA/PTX3 complex is an nHC-HA/PTX3 isolated from amniotic membrane or umbilical cord. In some embodiments, the HC-HA/PTX3 complex is a reconstituted HC-HA complex. In some embodiments, HA is covalently linked to HC. In some embodiments, the HC of IaI is heavy chain 1 (HC1). In some embodiments, the HC-HA complex comprises pentraxin 3 (PTX3).

[00499] In some embodiments, the nHC-HA/PTX3 or rcHC-HA/PTX3 complex comprises a small leucine rich proteoglycan (SLRP). In some embodiments, the nHC-HA/PTX3 or rcHC-HA/PTX3 complex comprises a class I, class II or class II SLRP. In some embodiments, the nHC-HA/PTX3 or rcHC-HA/PTX3 complex comprises PTX3 and a small leucine rich proteoglycan (SLRP). In some embodiments, the small leucine-rich proteoglycan is selected from among class I SLRPs, such as decorin and biglycan. In some embodiments, the small leucine-rich proteoglycan is selected from among class II SLRPs, such as fibromodulin, lumican, PRELP (proline arginine rich end leucine-rich protein), keratocan, and osteoadherin. In some embodiments, the small leucine-rich proteoglycan is selected from among class III SLRPs, such as epipycan and osteoglycin.

[00500] In some embodiments, the isolated stem cell is expanded on a substrate comprising immobilized nHC-HA/PTX3 or rcHC-HA/PTX3. In some embodiments, the immobilized nHC-HA/PTX3 or rcHC-HA/PTX3 comprises one or more small leucine rich proteoglycans (SLRPs). In some embodiments, the SLRP is selected from among bikunin, decorin, biglycan, and osteoadherin. In some embodiments, the small leucine-rich protein comprises a glycosaminoglycan. In some embodiments, the small leucine-rich proteoglycan comprises keratan sulfate.

[00501] In some embodiments, the isolated stem cell is expanded in a culture medium comprising nHC-HA/PTX3 or rcHC-HA/PTX3 complex. In some embodiments, the nHC-HA/PTX3 or rcHC-HA/PTX3 comprises one or more small leucine rich proteoglycans (SLRPs). In some embodiments, the SLRP is selected from among bikunin, decorin, biglycan, and osteoadherin. In some embodiments, the small leucine-rich protein comprises a glycosaminoglycan. In some embodiments, the small leucine-rich proteoglycan comprises keratan sulfate. In some embodiments, the medium is embryonic stem cell medium, modified embryonic stem cell medium, supplemented hormonal epithelial medium, and/or a combination thereof. In some embodiments, the medium is supplemented with one or more growth factors. In some embodiments, the medium is supplemented with EGF, bFGF and/or LIF. In some embodiments, the medium is supplemented with an inhibitor of Rho-associated kinase (ROCK inhibitor).

[00502] Inducing and Maintaining Pluripotency

[00503] Disclosed herein, in certain embodiments, are methods of inducing pluripotency in a cell or maintaining pluripotency of a stem cell on a substrate that comprises an nHC-HA/PTX3 or rcHC-HA/PTX3 complex. As described herein, HC-HA/PTX3 complexes assist in the maintenance of stem cell marker expression and prevent differentiation of the cells over successive passages of a stem cell population. In addition, as described herein, HC-HA/PTX3 complexes promote the induction of stem cell properties in a differentiated or partially differentiated population of cells.

[00504] In certain embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex promotes or induces pluripotency of a differentiated or partially differentiated cell. In certain embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex promotes or induces pluripotency of a differentiated or partially differentiated cell compared to a differentiated or partially differentiated cell cultured in the absence of an nHC-HA/PTX3 or rcHC-HA/PTX3 complex. In an exemplary method, a differentiated cell or partially differentiated cell is cultured on a substrate comprising nHC-HA/PTX3 or rcHC-HA/PTX3 complex, whereby pluripotency is induced in the cell.

[00505] In certain embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex further promotes or induces pluripotency of a stem cell. In certain embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex further promotes or induces pluripotency of a stem cell compared to a stem cell cultured in the absence of an nHC-HA/PTX3 or rcHC-HA/PTX3 complex. In an exemplary method, a stem cell is cultured on a substrate comprising nHC-HA/PTX3 or rcHC-HA/PTX3 complex, whereby pluripotency is maintained in the stem cell. In an exemplary method, a stem cell is cultured on a substrate comprising nHC-HA/PTX3 or rcHC-HA/PTX3 complex, whereby pluripotency is further induced in the stem cell.

[00506] Using genetic reprogramming with protein transcription factors, pluripotent stem cells equivalent to embryonic stem cells have been derived from human adult skin tissue. iPSC cells are typically derived by transfection of certain stem cell-associated genes into non-pluripotent cells, such as adult fibroblasts. Transfection is typically achieved through viral vectors, such as retroviruses, where the pluripotency gene is operably linked to a promoter for gene expression. Four key pluripotency genes essential for the production of pluripotent stem cells are Oct-3/4 (Pou5f1), Sox2, c-Myc, and Klf4. Other genes can enhance the efficiency of induction. In some studies, Oct4, Sox2, Nanog, and Lin28 have been employed to induce pluripotency. In certain instances, after 3–4 weeks, small numbers of transfected cells begin to become morphologically and biochemically similar to pluripotent stem cells, and are typically isolated through morphological selection, doubling time, or through a reporter gene and antibiotic selection.

[00507] In some embodiments, methods are provided for inducing pluripotency in a differentiated or partially differentiated cell using heterologous expression of fewer than four of the essential transcription factors Oct-3/4 (Pou5f1), Sox2, c-Myc, and Klf4. In some embodiments, a method for inducing pluripotency is provided where use of an nHC-HA/PTX3 or rcHC-HA/PTX3 complex enhances the induction of pluripotency of a differentiated or partially differentiated cell that expresses at least one of Oct-3/4 (Pou5f1), Sox2, c-Myc, and/or Klf4 by heterologous gene transfer. In some embodiments, a method for inducing pluripotency is provided where use of an nHC-HA/PTX3 or rcHC-HA/PTX3 complex enhances the induction of pluripotency of a differentiated

or partially differentiated cell that expresses one, two or three factors selected from among Oct-3/4 (Pou5f1), Sox2, c-Myc, and/or Klf4 by heterologous gene transfer. In some embodiments, a method for inducing pluripotency is provided where use of an nHC-HA/PTX3 or rcHC-HA/PTX3 complex enhances the induction of pluripotency of a differentiated or partially differentiated cell that expresses Oct-3/4 (Pou5f1) by heterologous gene transfer. In some embodiments, a method for inducing pluripotency is provided where use of an nHC-HA/PTX3 or rcHC-HA/PTX3 complex enhances the induction of pluripotency of a differentiated or partially differentiated cell that Sox2 by heterologous gene transfer. In some embodiments, a method for inducing pluripotency is provided where use of an nHC-HA/PTX3 or rcHC-HA/PTX3 complex enhances the induction of pluripotency of a differentiated or partially differentiated cell that expresses c-Myc by heterologous gene transfer. In some embodiments, a method for inducing pluripotency is provided where use of an nHC-HA/PTX3 or rcHC-HA/PTX3 complex enhances the induction of pluripotency of a differentiated or partially differentiated cell that expresses Klf4 by heterologous gene transfer.

[00508] In some embodiments, a differentiated or partially differentiated cell is transduced to express one or more of Oct-3/4 (Pou5f1), SOX2, c-Myc, and Klf4; and the transduced cell is cultured on a substrate comprising an nHC-HA/PTX3 or rcHC-HA/PTX3 complex. In some embodiments, a differentiated or partially differentiated cell is transduced to express at least one of Oct-3/4 (Pou5f1), SOX2, c-Myc, and Klf4; and the transduced cell is cultured on a substrate comprising an nHC-HA/PTX3 or rcHC-HA/PTX3 complex. In some embodiments, a differentiated or partially differentiated cell is transduced to express one, two or three of Oct-3/4 (Pou5f1), SOX2, c-Myc, and Klf4; and the transduced cell is cultured on a substrate comprising an nHC-HA/PTX3 or rcHC-HA/PTX3 complex. In some embodiments, a differentiated or partially differentiated cell is transduced to express Oct-3/4 (Pou5f1), SOX2, c-Myc, and Klf4; and the transduced cell is cultured on a substrate comprising an nHC-HA/PTX3 or rcHC-HA/PTX3 complex. In some embodiments, a differentiated or partially differentiated cell is transduced to express Oct-3/4 (Pou5f1); and the transduced cell is cultured on a substrate comprising an nHC-HA/PTX3 or rcHC-HA/PTX3 complex. In some embodiments, a differentiated or partially differentiated cell is transduced to express SOX2; and the transduced cell is cultured on a substrate comprising an nHC-HA/PTX3 or rcHC-HA/PTX3 complex. In some embodiments, a differentiated or partially differentiated cell is transduced to express c-Myc; and the transduced cell is cultured on a substrate comprising an nHC-HA/PTX3 or rcHC-HA/PTX3 complex. In some embodiments, a differentiated or partially differentiated cell is transduced to express Klf4; and the transduced cell is cultured on a substrate comprising an nHC-HA/PTX3 or rcHC-HA/PTX3 complex. In some embodiments, the

cell is transduced to express one or more additional genes, such as for example, Nanog, Fbx15, ERas, ECAT15-2, Tcl1, and β -catenin.

[00509] In some embodiments, a differentiated or partially differentiated cell is transduced with a viral vector containing one or more genes encoding one or more of Oct-3/4 (Pou5f1), SOX2, c-Myc, and Klf4. In some embodiments, a differentiated or partially differentiated cell is transduced with two or more viral vectors containing one or more genes encoding one or more of Oct-3/4 (Pou5f1), SOX2, c-Myc, and Klf4.

[00510] Various methods for the induction, culturing and maintenance of induced pluripotent stem cells and assessment of the pluripotency of induced stem cells, including assessment of stem cell markers and induction of different cell lineages, are well known in the art and include, for example, methods described in U.S. Pat. Nos. 7,682,828, 8,048,999, 8,211,697, 7,951,592, and US Pat. Pubs. 2009/0191159 and 2010/000375.

[00511] In some embodiments, the nHC-HA/PTX3 or rcHC-HA/PTX3 complex reduces to time of induction of pluripotency in the transduced cell compared to a transduced cell cultured in the absence of nHC-HA/PTX3 or rcHC-HA/PTX3 complex. In some embodiments, the nHC-HA/PTX3 or rcHC-HA/PTX3 complex increases the percentage of transduced cells that are induced to pluripotency in a population of transduced cells compared to transduced cells cultured in the absence of nHC-HA/PTX3 or rcHC-HA/PTX3 complex compared to a transduced cell cultured in the absence of nHC-HA/PTX3 or rcHC-HA/PTX3 complex. In some embodiments, the nHC-HA/PTX3 or rcHC-HA/PTX3 complex enhances the level of pluripotency in the transduced cell. In some embodiments, the nHC-HA/PTX3 or rcHC-HA/PTX3 complex decreases the number of heterologous transcription factors required for induction of pluripotency in the transduced cell.

[00512] In some embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex provided herein inhibits TGF β 1 signaling in a differentiated cell, a stem cell, or an iPSC. In some embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex provided herein inhibits nuclear translocation of SMAD2 or SMAD3 in a differentiated cell, a stem cell, or an iPSC. In some embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex provided herein inhibits alpha smooth muscle actin formation in a differentiated cell, a stem cell, or an iPSC. In some embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex provided herein activates BMP4 signaling in a differentiated cell, a stem cell, or an iPSC. In some embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex provided herein activates BMP6 signaling in a differentiated cell, a stem cell, or an iPSC. In some embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex provided herein induces expression of an embryonic cell marker in a differentiated cell, a stem cell, or an iPSC. In some embodiments, an nHC-HA/PTX3 or rcHC-HA/PTX3 complex provided herein induces expression of c-myc,

KLF-4, Nanog, nestin, Oct4, Rex-1, Sox-2, and SSEA-4 in a differentiated cell, a stem cell, or an iPSC.

EXAMPLES

[00513] The following examples are included for illustrative purposes only and are not intended to limit the scope of the claimed subject matter.

Example 1. Purification of Native HC-HA/PTX3 (nHC-HA/PTX3) Complexes from Human Amniotic Membrane Extracts (AME)

[00514] Preparation of Amniotic Membrane Extract (AME) and AM Powder (AMP)

[00515] Frozen human AM obtained from Bio-tissue (Miami, FL) was washed 2-3 times with PBS to remove the storage medium. To prepare AME, AM was transferred to a sterile 50 ml centrifuge tube and centrifuged at 4 °C for 5 min at $5000 \times g$ to remove the excess fluid. AM was weighed ($\sim 10 \text{ mg/cm}^2$), transferred to a 100 mm or 150 mm sterile Petri dish, and frozen in the air phase of a liquid nitrogen container for 20 min before being sliced into small pieces with a disposable scalpel and homogenized with Tissue-Tearor (Biospec Products, Inc., Bartlesville, OK) in PBS. The homogenate was mixed at 4 °C for 30 min and centrifuged at $48,000 \times g$ for 30 min. The supernatant was collected, designated as AME, and used for nHC-HA/PTX3 purification or stored at -80 °C.

[00516] To prepare lyophilized AM powder (AMP), AM frozen in a -80 °C freezer was transferred to and lyophilized in a bench top lyophilizer (Freezone 4.5, Labconco, Kansas City, MO) for 16 hours. The lyophilized AM was then micronized into its matrix form (AMP) by a Mixer Mill (Retsch, Newtown, PA). AMP was stored at below -20 °C for further analyses.

[00517] Purification of native HC-HA/PTX3 (nHC-HA/PTX3) Complex

[00518] AME was dissolved in CsCl/4M guanidine HCl mixture at an initial density of 1.35 g/ml, and centrifuged at $125,000 \times g$ for 48 h at 15 °C. A total of 15 fractions (0.8 ml/fraction) were collected from the top to the bottom of each tube. Total protein concentration for each fraction was determined by BCA Protein Assay Kit. Hyaluronan (HA) concentration for each fraction was determined by an ELISA-based HA Quantitative Test Kit from Corgenix (Westminster, CO) (FIG. 1A). Fractions #8-15, which contain HA but no detectable proteins, were pooled, and used for a second ultracentrifugation. A sample of the pooled fractions (designated AM 1st) was saved for analysis. The pooled fractions were adjusted with CsCl/4M guanidine HCl at an initial density of 1.40 g/ml, centrifuged, and fractionated in the same manner as described above (FIG. 1B).

Fractions #3-15, which contained HA but no detectable proteins, were pooled (designated AM 2nd)

and dialyzed against distilled water to remove CsCl and guanidine HCl. The dialysate was lyophilized the same manner as for AMP describe above. Alternatively, the dialysate was mixed with 3 volumes of 95% (v/v) ethanol containing 1.3% (w/v) potassium acetate at 0 °C for 1 h. After centrifugation at $15,000 \times g$, the pellet was washed with 70% (v/v) ethanol and centrifuged again. The pellet was briefly dried by air, stored at -80 °C. The powder and pellet were designated as nHC-HA/PTX3 complex.

[00519] In some instances, the pooled sample went through three or four times of ultracentrifugation. In these ultracentrifugations, only fractions #7-12 were pooled and the initial density of CsCl/4M guanidine HCl is at 1.42 g/ml. After the third or fourth ultracentrifugation, the pooled fractions #7-12 is designated nHC-HA/PTX3 (3rd) or nHC-HA/PTX3 (4th).

[00520] AME pooled fractions after 1st, 2nd, 3rd, or 4th ultracentrifugations were treated with or without 0.05 N NaOH at 25 °C for 1 h. Pooled fractions from 1st, 2nd, 3rd, or 4th ultracentrifugation also were digested with 20 units/ml Hyaluronidase (HAase) (Seikagaku Biobusiness Corporation, Tokyo, Japan) at 60 °C for 2 h.

[00521] Samples from the pooled fractions and the NaOH and HAase treated samples were then run on 0.5% agarose gels and analyzed by staining with Stains-all dye (FIG. 1C) or by Western blot using antibodies against IαI heavy chain 1 (HC1) (FIGS. 1D and 1F), pentraxin 3 (PTX3) (FIGS. 1E and 1G), IαI heavy chain 2 (HC2) (FIG. 1H), IαI heavy chain 3 (HC3) (FIG. 1I), bikunin (FIG. 1J), TNF-stimulated gene 6 (TSG-6) (FIG. 1K), thrombospondin-1 (TSP-1) (FIG. 1L) or IGFBP 1-3 and PF4 (FIG. 1M), of which the latter two were analyzed by protein dot assays using human angiogenesis arrays (each array contains 56 different angiogenic proteins, R&D Systems, Minneapolis, MN). Briefly, 1.5 ml of human AM extract (25 µg/ml HA) and purified nHC-HA/PTX3(2nd) (25 µg/ml HA) were incubated separately with the detection antibodies pre-coated on the membrane overnight at 4°C, followed by incubation with the secondary antibody. Signals were detected with chemiluminescent light exposed to x-ray film. The array data on developed x-ray film were quantitated by scanning the film on a transmission-mode scanner, and the array image file was analyzed by ImageJ1.46 software (National Institutes of Health, Bethesda, MD).

[00522] Biochemical characterization showed that nHC-HA/PTX3 is composed of high molecular weight HA (HMW HA) (FIG. 1C) covalently linked to heavy chain 1 (HC1) of IαI and PTX3. Both HC1 and PTX3 in nHC-HA/PTX3 are released only after treatment of hyaluronidase (HAase) or NaOH (FIGS. 1D-G), demonstrating that HC1 is linked to HA by ester bonds as reported.

[00523] In contrast, nHC-HA/PTX3 does not contain HC2 (FIG. 1H), HC3 (FIG. 1I, a band at ~12 kDa detected only after NaOH treatment is likely non-specific), bikunin (FIG. 1J), TSG-6 (FIG. 1K) and TSP-1 (FIG. 1L). Insulin-like growth factor binding protein-1-3 (IGFBP1-3) and platelet

factor 4 (PF4) are detected by protein dot assay (similar to ELISA) in nHC-HA/PTX3(2nd) (FIG. 1J); it remains unclear whether they are still in nHC-HA/PTX3(4th).

Example 2. Preparation of immobilized HA (iHA) by Covalent Linkage

[00524] A series of hyaluronan (HA) amounts (0, 0.25, 0.5, 1.0, 2.5, 5, 10, and 25 µg/well) from HMW HA (Healon, Advanced Medical Optics, Santa Ana, CA) or nHC-HA/PTX3(2nd) was added to the coupling solution containing Sulfo-NHS (0.184 mg/ml) and EDAC (0.123 mg/ml) (Both were from Thermo Fisher Scientific, Rockford, IL) and incubated in Covalink™-NH 96 well plates (Thermo Fisher Scientific Inc.), for 16 h at 4 °C. After three washes of 8 M Guanidine-HCl (GnHCl) followed by washes with PBS, the coupled HA from HMW HA or nHC-HA/PTX3 was measured quantitatively by HA ELISA from Corgenix (Westminster, CO) according to the manufacturer's protocol (FIG. 6A). HMW HA and nHC-HA/PTX3 purified from AM are dose-dependently and covalently coupled to surfaces of Covalink-NH 96 wells. The resultant iHA or immobilized nHC-HA/PTX3 is resistant to washes by 8 M Guanidine HCl. HA of HMW HA or nHC-HA/PTX3 was maximally coupled at 2 µg/well HA equivalent input (FIG. 6A).

[00525] To determine coupling efficiency, HA from HMW HA or nHC-HA/PTX3 was coupled to Covalink™-NH per well of the 96 well plates, and unbound and bound HA from HMW HA or nHC-HA/PTX3 were measured by HA ELISA (FIG. 6B). 2 µg of HA from HMW HA or nHC-HA/PTX3 was added to the coupling solution containing Sulfo-NHS (0.184 mg/ml) and EDAC (0.123 mg/ml) [1-ethyl-3-(3-dimethylaminopropyl) carbodiimide] in H₂O and incubated in Covalink™-NH 96 well plates (Thermo Fisher Scientific, Rockford, IL), for 16 h at 4 °C. Both HA coupled to wells or unbound in washed solution (pooled) were measured with HA ELISA from Corgenix (Westminster, CO) according to the manufacturer's protocol. The total amount of HA in each well in either coupled or unbound is divided by the input HA amount (2 µg/well) to calculate coupling efficiency or unbound percentage. The average coupling efficiency was determined to be 70.5 ± 13.4 % for HMW HA and 69.0 ± 5.7 % for nHC-HA/PTX3 (FIG. 6B). So, 2 µg/well input HA results in approximately 1.4 µg iHA.

Example 3. Activity of Purified Native HC-HA/PTX3(nHC-HA/PTX3) Complexes

[00526] Attachment of LPS-stimulated Macrophages to Immobilized nHC-HA/PTX3

[00527] RAW264.7 cells (100 µl of 2.5×10⁵ cells/ml) [American Type Culture Collection (ATCC), Manassas, VA] in DMEM/10% FBS (Life Technologies, Grand Island, NY) were seeded in 96-well plates containing immobilized HA (Advanced Medical Optics, Santa Ana, CA, 2 µg/well), nHC-HA/PTX3(2 µg/well) or PBS control and stimulated with Lipopolysaccharide (LPS) (1 µg/ml)

(n=3) [LPS-EB Ultrapure, InvivoGen, San Diego, CA]. Immobilization of HA and nHC-HA/PTX3 on the surface of Covalink-NH 96 well was performed similarly as described above. In brief, Covalink-NH 96-well plates were sterilized in 70% alcohol for 2 h, washed 3 times with distilled water, and added with 100 μ l of 0.184 mg/ml Sulfo-NHS (Thermo Fisher Scientific, Rockford, IL) and 0.123 mg/ml of EDAC (Thermo Fisher Scientific, Rockford, IL) in distilled water containing 20 μ g/ml HA or nHC-HA/PTX3 per 96-well plate (PBS control wells contain all reagents except for HA and nHC-HA/PTX3). The plate was incubated at 4 °C overnight or at 25 °C for 2 h before the coupling solution was removed, washed 3 times with PBS containing 2 M NaCl and 50 mM MgSO₄, and followed by 3 washes with PBS. After incubation for 90 min, unattached cells were removed and attached cells were photographed and counted by the CyQuant assay (FIG. 2A). A greater than 3-fold increase in the attachment of LPS-stimulated macrophages was observed for the wells containing immobilized nHC-HA/PTX3 compared to the control wells. Wells containing immobilized HA inhibited the attachment of LPS-stimulated macrophages.

[00528] The cell viability of attached LPS-stimulated macrophages was then examined. LPS-stimulated RAW264.7 cells (100 μ l of 2.5×10^5 cells/ml) were incubated in DMEM/10% FBS on immobilized PBS control, HA, or nHC-HA/PTX3 for 24 h as described above (n=3). Following incubation, the cell viability of the attached macrophages was measured by MTT assay. No significant differences (all p values > 0.05) in the cell viability among cells on these immobilized substrates were observed (FIG. 2B).

[00529] The ability of blocking antibodies and peptides to inhibit attachment of LPS-stimulated macrophages to immobilized nHC-HA/PTX3 was then examined. RAW264.7 cells (at concentration of 2.5×10^5 cells/ml) were pre-incubated in DMEM/10% FBS with the blocking antibodies against CD44 (10 μ g/ml), TLR2 (10 μ g/ml), TLR4 (10 μ g/ml), integrin α_v (20 μ g/ml), β_1 (20 μ g/ml), β_2 (20 μ g/ml), or β_3 (20 μ g/ml) or RGD peptides (SDGRG, RGDS, GRGDS, all at 1 mg/ml), along with the isotype control antibodies [rat IgG (10 μ g/ml), mouse IgG (10 μ g/ml), or Armenian hamster IgG (20 μ g/ml)] or a RGD control peptide (1 mg/ml), on ice for 30 min (n=3). (Antibodies to CD44 and rat IgG were from BD Pharmingen, San Diego, CA; antibodies to TLR2, TLR4, and mouse IgG were from InvivoGen, San Diego, CA; antibodies to integrin α_v , β_1 , β_2 , β_3 , and Armenian hamster IgG were from Biolegend, San Diego, CA; RGD peptides were from Sigma-Aldrich, St Louis, MO). After adding LPS (1 μ g/ml), cells were seeded into plates containing immobilized HA (2 μ g/well), nHC-HA/PTX3 (2 μ g/well) or PBS control and incubated for 90 min (n=3). After incubation, unattached cells were removed and attached cells were photographed and counted by the CyQuant assay (FIG. 2C). The results showed that antibodies to CD44 and TLR4 significantly inhibited attachment of LPS-stimulated macrophages demonstrating that these

receptors are involved in the attachment of LPS-stimulated macrophages to immobilized nHC-HA/PTX3.

[00530] Polarization of LPS-stimulated Macrophages

[00531] Polarization of LPS-stimulated macrophages toward the M1 or M2 phenotype by immobilized nHC-HA/PTX3 was examined by determining the expression of genes encoding M1 and M2 markers by RNA and protein analysis.

[00532] RAW264.7 cells (100 μ l of 2.5×10^5 cells/ml) in DMEM/10% FBS were seeded in 96-well plates containing immobilized HA (2 μ g/well), nHC-HA/PTX3 (2 μ g/well) or PBS control and stimulated with LPS (1 μ g/ml) for 4 h (n=3). Following incubation, unattached cells were removed and total RNAs were extracted from the attached cells. The mRNA expression of M1 markers (Tumor necrosis factor alpha (TNF- α) (Mm00443258_m1) and interleukin 12 subunit p40 (IL-12p40) (Mm00434165_m1)) and M2 markers (interleukin-10 (IL-10) (Mm00439614_m1), Arginase-1 (Arg-1) (Mm00475988_m1), LIGHT/TNSF14 (Mm00444567_m1, and Sphingosine kinase-1 (SPHK1) (Mm0044884_g1)) were measured by quantitative real-time PCR with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Mm99999915_g1) as the endogenous control. The real-time PCR was performed on 7300 Real-time PCR System (Applied Biosystems, Foster City, CA). The amplification program consisted of 10 min of initial activation at 95 °C followed by 40 cycles of 15 sec denaturation at 95 °C, and 1 min annealing and extension at 60 °C. The relative gene expression data was analyzed by the comparative CT method ($\Delta\Delta$ CT). All assays were performed in triplicate; the results were normalized by GAPDH as an internal control. All primers were from Applied Biosystems. Significant induction of expression of the M2 markers IL-10, Arg-1, LIGHT, and SPHK1 compared to the control was observed in cells attached to immobilized nHC-HA/PTX3, but not HA (FIG. 3A). In addition, expression of both M1 markers, TNF- α and IL-12p40, was reduced.

[00533] The amount of secreted TNF- α protein was measured in culture supernatants of cells treated with LPS (1 μ g/ml) stimulation for 4 h in DMEM/10% FBS on plates containing immobilized HA (2 μ g/well), nHC-HA/PTX3 (2 μ g/well) or PBS control as described above (n=3). The amount of TNF- α was measured by ELISA according to the manufacturer's protocol (R&D Systems, Minneapolis, MN).

[00534] A reduced amount of TNF- α was observed in the cell culture supernatants of cells incubated on plates containing immobilized nHC-HA/PTX3 compared to PBS control (FIG. 3B). No change in the amount of TNF- α was observed on the immobilized HA plate.

[00535] High expression of IRF-5 is characteristic of M1 macrophages. IRF-5 directly activates transcription of the genes encoding IL-12p40, IL-12p35 and IL-23p19 and represses the gene

encoding IL-10. Expression of IRF-5 protein and its cytolocalization on immobilized nHC-HA/PTX3 was examined. Cells seeded on the immobilized control or nHC-HA/PTX3 were stimulated with LPS (1 μ g/ml) for 4 or 24 h in DMEM/10% FBS. The expression of IRF-5 protein in cell lysates (LPS stimulation for 24 h) was detected by Western blot (FIG 3C, left) (primary antibody: abcam, Cambridge, MA; secondary antibody, DAKO, Carpinteria, CA). In a parallel experiment, cells (LPS stimulation for 4 h) were fixed and immunostained with anti-IRF-5 antibody. The cytolocalization of IRF-5 was examined by confocal immunofluorescence microscopy (LSM 700 confocal microscope, Zeiss, Oberkochen, Germany) (FIG 3C, right). Immobilized nHC-HA/PTX3 reduced expression and prevented nuclear localization of IRF-5. These results are consistent with the suppression of the M1 phenotype by immobilized nHC-HA/PTX3.

[00536] Apoptosis of Activated Neutrophils and Macrophage Phagocytosis of Apoptotic Neutrophils

[00537] Neutrophils were isolated from the normal human peripheral blood using the dextran density [Lymphocyte Poly(R), Cedarlane USA, Burlington, NC] centrifugation according to the manufacturer's instruction. Isolated neutrophils were seeded at 2×10^6 cells/ml in IMDM (Iscoe's Modified Dulbecco's Medium, Life Technologies, Grand Island, NY) on immobilized HA (2 μ g/well), nHC-HA/PTX3(2 μ g/well) or PBS control and treated with PBS (resting), N-formyl-methionyl-leucyl-phenylalanine (fMLP) (1 μ M) (Sigma- Aldrich, St Louis, MO) or LPS (1 μ g/ml) for 24 h (n=3). Apoptosis of neutrophils was determined by Cell Death Detection ELISA (Roche Applied Science, Indianapolis, IN) in cell lysates according to the manufacturer's protocol. Immobilized nHC-HA/PTX3, but not HA, promotes apoptosis of fMLP or LPS-activated neutrophils but not resting neutrophils (FIG. 3D).

[00538] Phagocytosis of apoptotic neutrophils by resting or LPS-stimulated macrophages was then examined. RAW264.7 cells (1×10^5 cells/ml) were cultivated in DMEM/10% FBS on the immobilized HA (2 μ g/well), nHC-HA/PTX3 (2 μ g/well) or PBS control without or with LPS (1 μ g/ml) stimulation for 6 days (n=3). The cell culture medium was then removed, and 100 μ l of 2×10^6 cells/ml of apoptotic neutrophils in IMDM (prepared by treatment of isolated resting human neutrophils with roscovitine (20 μ M) (Sigma- Aldrich, St Louis, MO) for 8 h) were added to each well containing resting or LPS-stimulated macrophages. After incubation for 30 min at 37 °C, each well was washed three times with the cold PBS, and cell lysates (including macrophages and phagocytosed neutrophils) were collected to determine human myeloperoxidase (MPO) activity by the ELISA assay to measure phagocytosed neutrophils by macrophages. Cell lysates were collected and subjected to human myeloperoxidase (MPO) ELISA assay (n=4) (R&D Systems, Minneapolis,

MN) according to the manufacturer's protocol. MPO was then normalized with total protein measured by BCA protein assay (Thermo Fisher Scientific, Rockford, IL) in respective cell lysate and expressed as phagocytosis index. The phagocytosis index of resting cells without LPS (-LPS) stimulation was defined as 100 % in this experiment. Immobilized nHC-HA/PTX3, but not HA, promoted phagocytosis of apoptotic neutrophils by either resting or LPS-treated macrophages (FIG. 3E).

[00539] These results demonstrate that immobilized nHC-HA/PTX3 (2nd) enhances the apoptosis of activated neutrophils and phagocytosis of apoptotic neutrophils by macrophages.

[00540] Analysis of Receptors Involved in Polarization of M2 Macrophages by Immobilized nHC-HA/PTX3

[00541] In order to determine the involvement of particular receptors in M2 macrophage polarization, quantitative mRNA expression of M1 and M2 macrophage markers in the presence or absence of receptor blocking antibodies was performed. RAW264.7 cells (2.5×10^5 cells/ml) in DMEM/10% FBS were pre-incubated with PBS (control) or blocking antibodies to CD44 (10 μ g/ml), TLR4 (10 μ g/ml), or CD44/TLR4 (each at 10 μ g/ml) for 30 min on ice (n=3). Cells were then stimulated with LPS (1 μ g/ml) and incubated at 37 °C for 4 h on immobilized HA (2 μ g/well), nHC-HA/PTX3 (2 μ g/well) or PBS control. Total RNAs were extracted from the total cells. The relative mRNA expression of M1 marker (IL-12p40) and M2 markers (IL-10, LIGHT, and SPHK1) were determined by quantitative PCR with GAPDH as the endogenous control as described above (FIG. 4A). Expression of IL-12p40 was abolished while that of IL-10, LIGHT and SPHK1 was promoted by immobilized nHC-HA/PTX3, but not HA. This expression pattern was inhibited more by the CD44 blocking antibody than the TLR4 blocking antibody. In contrast, expression of IL-12p40 and IL-10 transcript by immobilized HA was affected more by the blocking antibody against TLR4 than that against CD44.

[00542] Protein expression of IL-12 and IL-10 also was determined. Cell culture supernatants were collected from cells cultivated on immobilized HA (2 μ g/well), nHC-HA/PTX3 (2 μ g/well) or PBS control treated as described above except for 24 h (instead of 4h) (n=3). The amount of IL-12 or IL-10 protein in the cell culture supernatants was determined by ELISAs (Biolegend, San Diego, CA) according to the manufacturer's protocol (FIG. 4B). Expression of IL-12 protein is abolished while that of IL-10 protein is markedly promoted by immobilized nHC-HA/PTX3. This expression pattern is inhibited by the blocking antibody against CD44. In contrast, expression of IL-12 protein is promoted while that of IL-10 is suppressed by immobilized HA, and the expression pattern was affected more by the blocking antibody against TLR4.

[00543] Comparison of nHC-HA/PTX3(2nd) and nHC-HA/PTX3(4th) complexes

[00544] nHC-HA/PTX3 (2nd) and nHC-HA/PTX3 (4th) complexes were compared by examining the ability of each complex to induce cell aggregation of macrophages (indicative of poor cell attachment) and/or promote M2 macrophage polarization. RAW264.7 cells (2.5×10^5 cells/ml) were cultivated in DMEM/10 % FBS on immobilized HA (2 μ g/well), nHC-HA/PTX3 (2 μ g/well) or PBS control and stimulated with 200 units/ml IFN- γ /1 μ g/ml LPS (Both were from InvivoGen, San Diego, CA) for 4 h or 24 h (n=3). After 4 hours, cell aggregation was examined by light microscopy and photographed (FIG. 5A). Immobilized nHC-HA/PTX3 (4th), but not nHC-HA/PTX3 (2nd), promotes cell aggregation of macrophages, indicating that nHC-HA/PTX3 (4th) does not promote cell attachment to the plate while nHC-HA/PTX3(2nd) does.

[00545] After 24h, samples were obtained from cell culture supernatants and IL-12p40 protein and IL-23 protein concentration was measured by respective ELISAs (Biolegend, San Diego, CA) according to the manufacturer's protocol (FIG. 5B and 5C). Both nHC-HA/PTX3(2nd) and nHC-HA/PTX3(4th) inhibit the production of IL-12p40 and IL-23 proteins in IFN- γ /LPS -stimulated macrophages.

Example 4: *In vitro* binding of TSG-6 and PTX3 to Immobilized HA (iHA) in the absence of α I

[00546] Binding of TSG-6 to iHA

[00547] Immobilized HA (2 μ g/well input) was prepared as described above. A series of human TSG-6 (overexpression in mouse myeloma cell line NS0 with Trp18 to Leu277 of human TSG-6, with a C terminal 10 His tag, Accession # P98066; R&D Systems, Minneapolis, MN, Cat. No. 2104-TS) concentrations (0, 0.24, 1.2, 6, 12, and 24 μ g/ml, 100 μ l volume per well) were incubated with iHA for 2 h at 37 °C in the reaction buffer (5 mM MgCl₂ in PBS, pH 7.5). Unbound TSG-6 was removed by washes of 8 M Guanidine-HCl and PBS. The bound TSG-6 was measured by modified TSG-6 ELISA (R&D Systems, Minneapolis, MN). Because TSG-6 was already bound to iHA coupled in wells, the steps of incubating samples with pre-coated TSG-6 antibody were omitted. The subsequent steps were according to the manufacturer's protocol (FIG. 7A). TSG-6 dose-dependently bound iHA and reached its maximal binding capacity at about 6 μ g/ml (or 0.6 μ g in 0.1 ml of the reaction solution) when iHA was about 1.4 μ g (2 μ g HA per well based on the coupling efficiency of ~70%). The molar ratio of TSG-6 to HA was about 37:1 based on TSG-6 being 35 kDa and HA being ~3,000 kDa.

[00548] The ability of TSG-6/iHA complex to resist dissociation was then examined. iHA (2 μ g/well input) was prepared as described above. TSG-6 (6 μ g/ml in 100 μ l) was incubated with iHA for 2 h at 37 °C. Unbound TSG-6 was removed by washes with PBS (as a control) or with

different dissociating or reducing agents: 6M Guanidine HCl/PBS, 8M Guanidine HCl/PBS, 2% SDS/PBS, 100 mM DTT/PBS, and 25 mM NaOH/H₂O. The bound TSG-6 was measured by modified TSG-6 ELISA as described above (FIG. 7B). The formed TSG-6/iHA complex was stable and was resistant to the treatment with the various dissociating or/and reducing agents. No statistical significance was noted among all groups.

[00549] Binding of PTX3 to iHA

[00550] Immobilized HA (2 µg/well input) was prepared as described above. A series of PTX3 (overexpression in mouse myeloma cell line NS0 with Glu18 to Ser277 of human PTX3, with a C terminal 6 His tag, Accession # P26022; R&D Systems, Minneapolis, MN) concentrations (0, 0.04, 0.2, 1, 5, and 25 µg/ml, 100 µl volume per well) were incubated with iHA for 2 h at 37 °C in the reaction buffer (5 mM MgCl₂ in PBS, pH 7.5). Unbound PTX3 was removed by washes with 8M GnHCl and PBS. The bound PTX3 was measured by modified PTX3 ELISA (R&D Systems, Minneapolis, MN). Because PTX3 was already bound to iHA coupled in wells, the steps of incubating samples with pre-coated PTX3 antibody were omitted. The subsequent steps were according to the manufacturer's protocol (FIG. 8A). PTX3 dose-dependently bound iHA and reached the maximal binding capacity at about 5 µg/ml (or 0.5 µg in 0.1 ml of the reaction solution) when iHA was about 1.4 µg (2 µg HA per well based on the coupling efficiency of 70%) The molar ratio of PTX3 to HA was about 24:1 based on PTX3 being 45 kDa and HA being ~3,000 kDa.

[00551] The ability of PTX3/iHA complex to resist dissociation was then examined. iHA (2 µg/well input) was prepared as described above. PTX3 (5 µg/ml in 100 µl) was incubated with iHA for 2 h at 37 °C. Unbound PTX3 was removed by washes with PBS (as a control) or with different dissociating or reducing agents: 6M Guanidine HCl/PBS, 8M Guanidine HCl/PBS, 2% SDS/PBS, 100 mM DTT/PBS, and 25 mM NaOH/H₂O. The bound PTX3 was measured by modified PTX3 ELISA as described above (FIG. 8B). The formed PTX3/iHA complex was stable and was resistant to the treatment with the various dissociating or/and reducing agents. No statistical significance was noted among all groups.

[00552] Simultaneous Binding of TSG-6 and PTX3 to iHA

[00553] iHA (2 µg/well input) was prepared as described above. 6 µg/ml of TSG-6 and 5 µg/ml of PTX3 (concentrations for maximal binding as described above) were incubated with iHA either alone or combined in the reaction buffer (5 mM MgCl₂ in PBS, pH 7.5). The bound TSG-6 or PTX3 was measured by respective modified ELISA as described above. There was no competition or synergy for binding to iHA by TSG-6 or PTX3 when both proteins were incubated with iHA simultaneously as compared to that when TSG-6 or PTX3 was added alone ($p > 0.05$). These data

indicated that the binding sites on iHA for TSG-6 and PTX3 are different and might not overlap (FIG. 9).

[00554] Sequential Binding of TSG-6 and PTX3 to iHA

[00555] Sequential addition of TSG-6 and PTX3 to iHA was examined to determine whether pre-bound TSG-6 or PTX3 would inhibit binding of the other protein to iHA. 6 µg/ml of TSG-6 or 5 µg/ml of PTX3 were pre-bound to iHA, prepared as described above. After washes with 8M GnHCl and PBS, serial concentrations of PTX3 (0, 5, or 5 µg/ml) or TSG-6 (0, 1.2, or 6 µg/ml) were subsequently incubated with pre-bound TSG-6/iHA or pre-bound PTX3/iHA in the reaction buffer (5 mM MgCl₂ in PBS, pH 7.5)), respectively. The subsequently bound TSG-6 and PTX3 were measured by respective modified ELISAs.

[00556] Pre-bound TSG-6 (6 µg/ml) partially prevented subsequent PTX3 from binding to iHA (FIG. 10A) ($p = 0.05$ and 0.01 when subsequent PTX3 was added at 1 µg/ml and 5 µg/ml, respectively) (FIG. 5A). Pre-bound PTX3 (5 µg/ml) did not interfere with the subsequent TSG-6 binding to iHA ($p = 0.56$ and 0.74 when subsequent TSG-6 was added at 1.2 µg/ml and 6 µg/ml, respectively) (FIG. 10B). These data indicate that iHA is structurally changed after TSG-6 binding so that it interferes with subsequent PTX3 binding.

Example 5: Attachment of LPS-stimulated Macrophages to Immobilized TSG-6/iHA and PTX3/iHA complexes

[00557] Covalink-NH 96 wells were covalently coupled with PBS (control), HA (iHA), or native HC-HA/PTX3 (nHC-HA/PTX3) as described above. TSG-6 (6 µg/ml) or PTX3 (5 µg/ml) was then added and bound to iHA. RAW264.7 macrophages (100 µl of 1×10^5 cells/ml) in DMEM/10% FBS were seeded into each coupled well and treated with 1 µg/ml LPS. After incubation for 24 h, cell morphology was photographed.

[00558] Macrophages attached poorly (i.e., resulting in more aggregated cells) to iHA when compared to the plastic control. Such attachment was further hampered by TSG-6/iHA, resulting in high number of and larger aggregations of cells. In contrast, PTX3/iHA promoted cell attachment, resulting in a similar pattern to that shown on immobilized nHC-HA/PTX3 (FIG. 11).

Example 6: Regulation of M1 and M2 Markers by TSG-6/iHA and PTX3/iHA complexes

[00559] RAW264.7 cells were cultivated as in Example 5 and stimulated with 1 µg/ml LPS for 4 h on PBS (control), immobilized HA (iHA), TSG-6/iHA, PTX3/iHA, or nHC-HA/PTX3. Total RNAs were isolated from cells and mRNA expression of the M2 marker IL-10 and the M1 marker IL-12p40 were measured by quantitative PCR (FIGS. 12A and 12D) as described above.

Alternatively, the cells were stimulated with 1 $\mu\text{g/ml}$ LPS (FIGS. 12B and 12E) or IFN- γ /LPS (FIG. 12C) for 24 h, and protein expression of IL-10, IL-12p70, and IL-23 were measured in cell culture media using respective ELISAs.

[00560] The expression of IL-12p40 mRNA (one of two subunits of IL-12p70) was not significantly changed on iHA (none) compared to that of the control (Ctrl) ($p > 0.05$) (FIG. 12A). In contrast, IL-12p40 mRNA was significantly upregulated on TSG-6/iHA ($p < 0.01$), but significantly downregulated on PTX3/iHA and nHC-HA/PTX3 ($p < 0.05$) (FIG. 12A). The expression of IL-12p70 protein, however, was only detectable without any significant difference ($p > 0.05$) on the control or iHA alone, but is undetectable on TSG-6/iHA, PTX3/iHA, and nHC-HA/PTX3 (FIG. 12B). IL-12p40 also serves as a subunit of IL-23. It was observed that expression of IL-23 protein was significantly upregulated on TSG-6/iHA and PTX3/iHA ($p < 0.01$), but is undetectable on nHC-HA/PTX3 ($p < 0.05$) (FIG. 12C). These data indicate that both TSG-6/iHA and PTX3/iHA are effective in suppressing IL-12 but not IL-23.

[00561] The expression of IL-10 mRNA by RAW264.7 cells was not significantly changed on iHA alone compared to the control ($p > 0.05$), but was significantly upregulated on TSG-6/iHA, PTX3/iHA, and nHC-HA/PTX3 ($p < 0.05$) (FIG. 12D). The expression of IL-10 protein, however, is only significantly upregulated on PTX3/iHA similar to the positive control of immobilized nHC-HA/PTX3 ($p < 0.05$) (FIG. 12E). These data suggest that following different patterns of cell attachment (Example 5), resultant cells exhibit different functions, and that PTX3/iHA is more effective than TSG-6/iHA in upregulating IL-10

Example 7: *In vitro* transfer of HC1 and HC2 from I α I to Immobilized HA

[00562] Covalink-NH 96 wells were covalently coupled with PBS (control), HA (iHA), or native HC-HA/PTX3 (nHC-HA/PTX3) as described above. Serial TSG-6 concentrations (0, 0.24, 1.2, 6, 12 $\mu\text{g/ml}$ in 100 μl) were individually incubated with iHA in the reaction buffer (5 mM MgCl_2 in PBS, pH 7.5). Human I α I (5 $\mu\text{g/ml}$) (prepared from human plasma according to Blom et al. (1999) *J. Biol. Chem.* 274, 298–304) was added either simultaneously with TSG-6 or sequentially (2 h later). Bound HC1, HC2 (antibodies to HC1 and HC2 were from abcam, Cambridge, MA), or I α I (DAKO, Carpinteria, CA) was measured by respective modified ELISA similar to TSG-6 and PTX3 ELISAs described above.

[00563] The data show that the amount of HC1 (FIG. 13A) or I α I (FIG. 13B) bound to iHA is lower at higher TSG-6 concentrations (6 and 12 $\mu\text{g/ml}$) when TSG-6 was pre-bound to iHA with subsequent addition of I α I than when TSG-6 and I α I were added simultaneously. HC2 was not detected in the samples (data not shown). The wells were incubated with hyaluronidase to digest

the bound HA and to release bound proteins from HA and these samples were analyzed by Western blot with anti-TSG-6 antibody (R&D Systems, Minneapolis, MN); the amount of TSG-6 bound to iHA was lower when IαI was simultaneously added with TSG-6 than when IαI was subsequently added (i.e. after TSG-6 was bound to iHA, FIG. 13C). When 5 μg/ml of PTX3 and 5 μg/ml of IαI were incubated with iHA simultaneously, PTX3 but not IαI was bound to iHA (FIG. 13D).

[00564] These data indicate that TSG-6 free in solution is more efficient than TSG-6 bound to iHA in transferring HC1 from IαI onto iHA (FIGS. 13A and 13B). More TSG-6 is bound to iHA when TSG-6 is pre-bound to iHA alone than when TSG-6 and IαI are incubated with iHA simultaneously (FIG 13C), indicating that IαI prevents TSG-6 from binding to iHA if added simultaneously with TSG-6, and that TSG-6 might have a higher affinity in binding to IαI than to iHA. In addition, PTX3 free in solution or bound to iHA does not transfer HCs from IαI to iHA (FIG. 13D).

Example 8: Effect of PTX3 on the formation of HC1•TSG-6 and HC2•TSG-6 Complexes

[00565] IαI (40 μg/ml) and TSG-6 (6 μg/ml) were incubated in the reaction buffer (5 mM MgCl₂ in PBS, pH 7.5) for 2 h at 37 °C without or with PTX3 (20 μg/ml or 120 μg/ml). The reaction samples were analyzed by Western blot with antibodies against HC1 (FIG. 14A), HC2 (FIG. 14B), TSG-6 (FIG. 14C), bikunin (abcam, Cambridge, MA) (FIG. 14D), and PTX3 (data not shown). In solution without HMW HA, TSG-6 forms HC1•TSG-6 and HC2•TSG-6 complexes, and generates HMW IαI (FIGS. 14A and 14B). The formation of HMW IαI is illustrated in FIG. 14E. This data indicates that both HC1 and HC2 are transferred by TSG-6 in solution to HMW HA.

[00566] Simultaneous addition of PTX3 dose-dependently inhibits formation of HC2•TSG-6 but not HC1•TSG-6 (FIGS. 14A and 14B). In contrast, HMW IαI containing HC2 is increased while HMW IαI containing HC1 is decreased (FIGS. 14A and 14B). TSG-6 forms dimers when added in solution without HMW HA with or without PTX3 (FIG. 14C). These findings indicate that 1) both HC1 and HC2 in IαI form either a complex with TSG-6 or a HMW IαI via TSG-6's action; 2) PTX3 interferes with the above process differently regarding the transfer of HC1 and HC2 by TSG-6. There is no truncated form of HC1 or HC2. The inhibition of HC2•TSG-6 by PTX3 is illustrated in FIG. 14F.

[00567] A glycosylated form and a glycosylated and chondroitin sulfate-conjugated form of bikunin about 40 kDa and 45 kDa, respectively, were released by TSG-6 and PTX3, respectively (FIG. 14D). This data is consistent with the published data showing that both TSG-6 and PTX3 interact with IαI, and also suggests that TSG-6 and PTX3 interact differently with IαI resulting in different outcome of bikunin.

[00568] In a separate experiment, HMW HA (250 µg/ml), IαI (40 µg/ml), and TSG-6 (6 µg/ml) were incubated in solution for 24 h at 37 °C without or with PTX3 (1, 2.5, and 5 µg/ml) in the reaction buffer (5 mM MgCl₂ in PBS, pH 7.5). The reaction samples were analyzed by Western blot with antibodies against IαI (FIG. 14G). In solution with HMW HA but without PTX3, HCs from IαI were completely transferred to HMW HA by TSG-6. In the presence of PTX3, TSG-6-mediated HCs transfer was dose-dependently inhibited, resulting in the accumulation of LMW intermediates (~ 130 kDa and likely consisting of HC1-TSG-6) or unprocessed pre-IαI (~130 kDa, unprocessed IαI (220 kDa), and HMW IαI (retained in loading wells) (FIG. 14G). These data are consistent with the finding that PTX3 specifically prevents the formation of HC2-TSG-6, resulting in the inhibition of HC2 transfer and possible HC1 transfer.

Example 9: Formation of Reconstituted HC-HA/PTX3 (rcHC-HA/PTX3) Complexes *in vitro* from Immobilized HA with Simultaneous Addition of TSG-6, PTX3, and IαI

[00569] Immobilized HA (~ 14 µg/ml or 1.4 µg in 100 µl in each well) was prepared as described in Example 3. IαI (5 µg/ml) and TSG-6 (12 µg/ml) were incubated simultaneously on iHA with or without PTX3 (1, 5, or 20 µg/ml) for 2 h at 37 °C the reaction buffer (5 mM MgCl₂ in PBS, pH 7.5). After washes with 8 M GnHCl and PBS, bound HC1, TSG-6, and PTX3 were measured by respective modified ELISAs (FIGS. 15A, 15D, and 15F, respectively). The wells were washed again with 8 M GnHCl and PBS, and iHA with bound components were digested with 1 unit/ml of hyaluronidase for 2 h at 60 °C in 10 mM acetate buffer with 75 mM NaCl, pH 6.0. The samples were analyzed by Western blot with antibodies against HC1 (FIG. 15B), HC2 (FIG. 15C), TSG-6 (FIG. 15E), and PTX3 (FIG. 15G).

[00570] Simultaneous addition of TSG-6, PTX3 and IαI to iHA resulted in rcHC-HA/PTX3 complexes containing HMW HC1 but not HC2, and truncated HC1 and HC2 (FIGS. 15A-C). PTX3 dose-dependently reduced the amount of HMW HC1 in the complex. The data show that PTX3 dose-dependently interfered with the transfer of HC1 and HC2 to iHA by TSG-6 (FIGS. 15A-C), resulting less HC1/truncated HC1 and truncated HC2 (FIGS. 15B and 15C). TSG-6 monomer was decreased while HMW TSG-6 (either multimeric or complexed with PTX3 and/or HCs) did not change (FIGS. 15D and E).

[00571] The data also indicate that PTX3 does not interfere with TSG-6 bound to iHA in the presence or absence of IαI. The published data suggest that TSG-6 forms dimers with iHA when a smaller MW HA is tested (Baranova et al. (2011) *J Biol Chem.* 286(29):25675-86). The data present herein indicate that TSG-6 is complexed in HMW HC-HA/PTX3 complex in the presence of IαI. Because the free TSG-6 is decreased by PTX3 in a dose-dependent fashion, it further

indicates that PTX3 promotes the binding of TSG-6 into HC-HA/PTX3 complex in the presence of IaI. Under this situation, the majority of PTX3 exists as multimeric forms in HC-HA/PTX3 complex, similar to what has been observed in nHC-HA/PTX3, with a declining amount of monomer, dimers or trimers (FIG. 15F and G).

Example 10: Effect of Sequential Addition of PTX3 to Reconstituted HC-HA/PTX3 (rcHC-HA/PTX3) Complexes formed *in vitro* with TSG-6 and IaI on Immobilized HA

[00572] Immobilized HA (~ 14 µg/ml) was prepared as described in Example 3. IaI (5 µg/ml) and TSG-6 (12 µg/ml) were incubated on iHA in the reaction buffer (5 mM MgCl₂ in PBS, pH 7.5) for 2 h at 37 °C. After removing unbound IaI and TSG-6, reaction buffer with or without PTX3 (1, 5, or 20 µg/ml) was incubated with the pre-bound HCs and TSG-6 for 2 h at 37 °C. After washes with 8 M GnHCl and PBS, bound HC1, TSG-6, and PTX3 were measured by respectively ELISAs (FIGS. 16A, 16D, and 16F, respectively). The wells were then washed again with 8 M GnHCl. PBS control and iHA with bound components were then digested with 1 unit/ml of hyaluronidase for 2 h at 60 °C. The samples were analyzed by Western blot with antibodies against HC1 (FIG. 16B), HC2 (FIG. 16C), TSG-6 (FIG. 16E), PTX3 (FIG. 16G)

[00573] When PTX3 is added subsequently after TSG-6 and HCs have been pre-bound to iHA, PTX3 dose-dependently reduces HC1 transfer to the HMW complex (both intact HC1 and truncated HC1 are reduced) but increases the amount of truncated HC2 in the complex. Consistent with the data shown in Example 7, bound TSG-6 is less efficient than free TSG-6 in transferring HCs to iHA.

[00574] Similar to the data shown in Example 8, PTX3 also dose-dependently reduced the HMW TSG-6 and monomeric TSG-6 (FIG. 16D and 16E), indicating that subsequent addition of PTX3 continuously depletes pre-bound TSG-6. PTX3, however, is no longer able to be incorporated in TSG-6/HC-HA complex (FIG. 16F and 16G). Because pre-bound TSG-6 in iHA also partially prevents PTX3 from binding to iHA (see Example 4), this finding indicates that formation of a rcHC-HA/PTX3 complex by TSG-6 and IaI is structurally different from TSG-6/iHA in the extent that PTX3 binding to iHA is completely excluded.

Example 11: Formation of Reconstituted HC-HA/PTX3 (rcHC-HA/PTX3) Complexes *in vitro* with Pre-bound PTX3 on Immobilized HA and Sequential Addition of TSG-6 and IaI

[00575] Immobilized HA (~ 14 µg/ml) was prepared as described in Example 3. PTX3 (5 µg/ml) and iHA were incubated in the reaction buffer for 2 h at 37 °C in the reaction buffer (5 mM MgCl₂ in PBS, pH 7.5). After removing unbound PTX3, the reaction buffer containing TSG-6 (6 µg/ml)

and IαI (5, 25, and 125 μg/ml) were incubated for 2 h at 37 °C. After washes with 8 M GnHCl and PBS, bound HC1, TSG-6, and PTX3 were measured by respectively ELISAs (FIGS. 17A, 17C, and 17E, respectively). The wells were then washed again with 8 M GnHCl. PBS or iHA with bound components were digested with 1 unit/ml of hyaluronidase for 2 h at 60 °C in 10 mM acetate buffer with 75 mM NaCl, pH 6.0. The samples were analyzed by Western blot with antibodies against PTX3 (FIG. 17B), TSG-6 (FIG. 17D), HC1 (FIG. 17F), and HC2 (FIG. 17G).

[00576] In the presence of IαI and TSG-6, pre-bound PTX3 dose-dependently increased ELISA immunoreactivity for PTX3 and the amount of multimeric PTX3 but decreased that of monomeric PTX3 in HC-HA/PTX3 complex (FIGS. 17A and 17B). This data indicates that multimeric PTX3 promotes immunoreactivity by this antibody.

[00577] Pre-bound PTX3 dose-dependently excluded monomeric TSG-6 while decreasing TSG-6 in the rcHC-HA/PTX3 complex (FIGS. 17C and 17D). The significant reduction of bound TSG-6 (both monomer and HMW forms) is detected when a molar ratio of IαI to TSG-6 is 3:1, where bound multimeric PTX3 is maximized as well.

[00578] There was no significant change in bound HC1 based on HC1 ELISA data (FIG. 17E). The transfer of HC2 was dose-dependently increased by increasing IαI concentrations.

Example 12: Comparison of Macrophage Cell Attachment Activity Between Reconstituted HC-HA/PTX3 (rcHC-HA/PTX3) Complexes Formed *in vitro* with Pre-bound TSG-6 Versus Pre-bound PTX3 on Immobilized HA

[00579] Covalink-NH 96 wells were covalently coupled with PBS (control), HA (iHA), or nHC-HA/PTX3 as described in Example 3. IαI (5 μg/ml), TSG-6 (6 μg/ml) or PTX3 (5 μg/ml) were simultaneously or sequentially bound to iHA as follows: (1) (IαI/TSG-6/PTX3)/iHA: IαI, TSG-6, and PTX3 were simultaneously incubated with iHA for 2 h at 37 °C in the reaction buffer; (2) (IαI/TSG-6)/PTX3/iHA: IαI and TSG-6 were first incubated with iHA for 2 h at 37 °C in the reaction buffer. After removed the unbound IαI/TSG-6, washed with 8 M GnHCl and PBS, PTX3 was added and incubated for 2 h at 37 °C in the reaction buffer; (3) (PTX3)/IαI/TSG-6/iHA: PTX3 was first incubated with iHA for 2 h at 37 °C in the reaction buffer. After removed the unbound PTX3, washed with 8 M GnHCl and PBS, IαI/TSG-6 was added and incubated for 2 h at 37 °C in the reaction buffer. Following formation of the complexes, 100 μl of RAW264.7 cells (1×10^5 cells/ml) were seeded into each coupled well and treated with 1 μg/ml LPS. After incubation for 24 h, cell morphology was photographed.

[00580] Macrophages attached poorly to iHA as the control. In the presence of IαI, simultaneous or pre-bound TSG-6 to iHA ((IαI/TSG-6/PTX3)/iHA or (IαI/TSG-6)/PTX3/iHA) inhibits cell

attachment and promotes cell aggregation (FIG. 18) similar to the condition without IαI (see Example 5). In contrast, pre-bound PTX3 to iHA [(PTX3)/IαI/TSG-6/iHA] promotes cell attachment similar to pre-bound PTX3 without IαI as shown in Example 5. The latter resembles the positive control of nHC-HA/PTX3 (FIG. 18).

Example 13: Comparison of Regulation of M1 and M2 Marker Expression Between Reconstituted HC-HA/PTX3 (rcHC-HA/PTX3) Complexes Formed *in vitro* with Pre-bound TSG-6 Versus Pre-bound PTX3 on Immobilized HA

[00581] Expression of IL-10 and IL-12p40 in Macrophages Cultivated on rcHC-HA/PTX3 Complexes

[00582] RAW264.7 cells were cultivated in DMEM/10% FBS on immobilized substrates and stimulated with 1 µg/ml LPS for 4 h as described in Example 12. Total RNAs were isolated and expression of IL-10 and IL-12p40 mRNAs was measured by quantitative PCR as described above (FIGS. 19A and 19C). Alternatively, cells were stimulated with 1 µg/ml LPS for 24 h and IL-10 and IL-12p70 proteins in the cell culture supernatants were measured by respective ELISAs (FIGS. 19B and 19D).

[00583] Compared to the PBS control, expression of IL-10 mRNA was not significantly changed by iHA ($p = 0.56$), but was significantly upregulated on complexes formed by simultaneous addition of TSG-6, IαI, and PTX3 on iHA (IαI/TSG-6/PTX3(a) in FIG 19) ($p = 0.0008$). Similarly, expression of IL-10 mRNA was significantly upregulated on complexes formed by pre-bound TSG-6 to iHA with subsequent addition of IαI and PTX3 (IαI/TSG-6/PTX3(b) in FIG 19) ($p = 0.04$) and the positive control nHC-HA/PTX3 ($p = 0.008$). Expression of IL-10 mRNA was significantly higher on nHC-HA/PTX3 than on IαI/TSG-6/PTX3(a) ($p = 0.04$), but not significantly higher than on IαI/TSG-6/PTX3(b) ($p = 0.55$). In contrast, expression of IL-10 mRNA was not significantly upregulated by on complexes formed by pre-bound PTX3 to iHA (IαI/TSG-6/PTX3(c) in FIG. 19) ($p = 0.74$) (FIG. 19A). Expression of IL-10 protein, as measured by ELISA, was only significantly upregulated by nHC-HA/PTX3 ($p = 0.03$) (FIG. 19B).

[00584] Compared to the control, expression of IL-12p40 (IL-12p40 is one of two subunits of IL-12p70 and the other subunit is IL-12p35) mRNA was not significantly changed by iHA ($p = 0.1$). In contrast, expression of IL-12p40 mRNA was significantly upregulated on complexes formed by simultaneous addition of TSG-6, IαI, and PTX3 on iHA (IαI/TSG-6/PTX3(a) in FIG 19) ($p = 0.05$) and on complexes formed by pre-bound TSG-6 to iHA (IαI/TSG-6/PTX3(b) in FIG 19) ($p = 0.04$). In contrast, expression of IL-12p40 mRNA was completely abolished on complexes formed by pre-bound PTX3 (IαI/TSG-6/PTX3 (c) in FIG. 19) and significantly downregulated by nHC-HA/PTX3

($p = 0.01$). There was a statistical significance difference between the latter two conditions ($p = 0.04$) (FIG. 19C). Compared to the control, expression of IL-12p70 protein was not significantly changed by iHA ($p = 0.32$), but significantly downregulated on complexes formed by pre-bound PTX3 (I α I/TSG-6/PTX3 (c)) ($p = 0.03$) (FIG. 19D). In contrast, expression of IL-12p70 protein was abolished on complexes formed by simultaneous addition of TSG-6, I α I, and PTX3 on iHA (I α I/TSG-6/PTX3 (a)), on complexes formed by pre-bound PTX3 (I α I/TSG-6/PTX3 (c)), and nHC-HA/PTX3 ($p = 0.05$, 0.02 , and 0.01 , respectively).

[00585] Expression of IL-23 in Macrophages Cultivated in the Presence of Various Stimuli

[00586] In a separate experiment, IL-23 protein in the cell culture supernatants of resting RAW264.7 cells (none) or with stimulation of IFN- γ (200 units/ml), LPS (1 μ g/ml), IFN- γ /LPS, LPS (1 μ g/ml) with immune complex or IC (LPS/IC) [IC contained 150 μ g/ml IgG-opsonized OVA (IgG-OVA) and was made by mixing a tenfold molar excess of rabbit anti-OVA IgG (Cappel, Durham, NC) to OVA (Worthington Biochemical Corp., Lakewood, NJ) for 30 min at 25 °C], or IL-4 (10 ng/ml) (R&D Systems, Minneapolis, MN) in DMEM/10% FBS for 24 h was measured. IL-23 protein in the cell culture supernatants was measured by IL-23 ELISA (Biolegend, San Diego, CA) according to the manufacturer's protocol (FIG. 19E). IL-23 protein was undetectable in the cell culture supernatant of resting RAW264.7 cells and in those of cells under stimulation for 24 h by LPS (1 μ g/ml), LPS with immune complex (LPS/IC), or IL-4 (10 ng/ml), but became detectable under stimulation for 24 h by IFN- γ (200 units/ml) and IFN- γ /LPS (FIG. 19E).

[00587] Expression of IL-23 in Macrophages Cultivated on rcHC-HA/PTX3 Complexes

[00588] In a separate experiment, RAW264.7 cells were cultivated on immobilized substrates as described above and stimulated with IFN- γ /LPS for 24 h. IL-23 in the cell culture supernatants was measured by IL-23 ELISA as described above (FIG. 19F).

[00589] Compared to the control, IL-23 protein in the cell culture supernatant of RAW264.7 cells with stimulation of 200 units/ml IFN- γ / 1 μ g/ml LPS for 24 h was not significantly affected by iHA ($p = 0.02$), but was significantly upregulated on complexes formed by simultaneous addition of TSG-6, I α I, and PTX3 on iHA (I α I/TSG-6/PTX3 (a)) ($p = 0.002$) and on complexes formed by pre-bound TSG-6 to iHA (I α I/TSG-6/PTX3 (b)) ($p = 0.0005$). In contrast, IL-23 protein is completely abolished on complexes formed by pre-bound PTX3 (I α I/TSG-6/PTX3 (c)) ($p = 0.05$) similar to nHC-HA/PTX3 ($p = 0.05$) (FIG. 19F).

Example 14: Use of HC-HA/PTX3 for the Treatment of Chronic Graft versus Host Disease

[00590] Allogeneic hematopoietic stem cell transplantation (HSCT) is a potentially curative treatment for hematological malignancies. However, chronic graft-versus-host disease (cGVHD)

remains a major complication. GVHD causes several ocular manifestations in 45–60%, among which dry eye is the most frequent complication, occurring in nearly 50% of allogeneic HSCT recipients. In fact, dry eye is a distinctive sign and symptom for the diagnosis of cGVHD. Patients with cGVHD manifest either early-stage mild dry eye disease related to cGVHD or the so called ‘distinctive feature of cGVHD’ according to the NIH consensus conference classification. Two types of dry eye after HSCT have been noted; one had severe ocular surface and tear function damage with decreased reflex tearing that occurs soon after the onset of dry eye, whereas the other is mild with normal reflex tearing. Dry eye typically occurs 6 months after the transplantation and the severity has been reported to be correlated with the presence of cGVHD and meibomian gland disease. The onset of cGVHD-related severe dry eye is earlier than that of mild dry eye. For example, severe dry eye occurs 6.8 ± 2.5 months after HSCT, while mild dry eye occurs 13.2 ± 9.1 months after HSCT. A comparative study of 50 eyes of 25 post-HSCT patients and 28 eyes of 14 age-matched healthy controls showed that MG obstruction, decreased corneal sensitivity, enhanced tear evaporation rate, decreased conjunctival GCD, increased conjunctival squamous metaplasia and inflammatory cells were noted more in cGVHD-related dry eyes than the normal controls and post-HSCT without dry eye subjects. Furthermore, the conjunctival inflammatory cells were significantly higher in severe dry eyes compared with mild dry eyes ($P < 0.03$). Moreover, most severe dry eye patients had systemic cGVHD, whereas only a few patients in the mild dry group had systemic cGVHD. Those findings indicated the different pathologic processes in cGVHD-related severe and mild dry eye disease. Because comprehensive ocular surface alteration was noted in post-HSCT patients, regardless of whether they had cGVHD-related dry eye or not, their results suggest that the extent of inflammatory process seems to have a pivotal role in the outcome of the cGVHD-related dry eye. The conjunctival brush cytology specimens showed considerably increased inflammatory cell numbers in both cGVHD-related severe dry eye and mild dry eye patients compared with normal controls and post-HSCT without dry eye subjects. Moreover, the number of inflammatory cells in severe dry eye specimens was significantly higher than in mild dry eye specimens. Furthermore, many inflammatory markers expressed in biopsy samples of the conjunctiva and lacrimal gland from cGVHD-related dry eye patients, confirming that inflammation is involved in the pathogenesis of cGVHD-related dry eye.

[00591] One likely cause of generating a number of scarring complication in cGVHD is via EMT of conjunctival basal epithelia and lacrimal gland myoepithelia as a result of cytokines released by chronic inflammation because of infiltrating donor lymphocytes. Previously, it has been recognized that inflammation and excessive fibrosis are prominent histologic features of chronic graft-versus-host disease (cGVHD), but the mechanism underlying these changes remains unknown. cGVHD

manifests features resembling scleroderma, exhibiting prominent fibrosis in skin lesions, pulmonary fibrosis, and chronic immunodeficiency. Clinical features of ocular cGVHD include onset of dry, gritty, or painful eyes, cicatricial conjunctivitis including subconjunctival fibrovascular tissue formation, and scleral shortening, which is characteristic feature of conjunctival fibrosis. In addition to sclerotic features in skin lesions, mucosal atrophy in the mouth, strictures or stenosis in the upper to mid third of the esophagus, joint stiffness or contracture due to sclerosis, and bronchitis obliterans in lung together indicate the characteristic features of systemic GVHD-mediated fibrosis. The main histologic findings in the affected exocrine gland and mucosal membrane are marked fibrosis of the interstitium and a prominent increase in the number of fibroblasts, accompanied by mild lymphocytic infiltration. Clinically, the severity of the dry eye is correlated with the degree of fibrotic change, rather than with the amount of lymphocytic infiltration, indicating that excessive extracellular matrix accumulation primarily contributes to the exocrine dysfunction. The fibroblasts at the interstitium also play a role in inflammation, by attaching to lymphocytes and expressing human leukocyte antigen class II and costimulatory molecules. These findings together indicate that fibroblasts play an important role in the pathogenesis of cGVHD. Moreover, we have found that the accumulated fibroblasts in the lacrimal gland of cGVHD patients have a chimeric status. Thus, fibroblasts originating from circulating donor-derived precursors and recipient derived fibroblasts may participate in the excessive fibrosis in patients with cGVHD by interacting T cells. It remains unknown whether controlling inflammation by suppressing T cell infiltration will lead to less cicatricial complication in cGVHD.

[00592] Previously, donor-derived fibroblasts were detected by combining immunohistochemistry and Y-chromosome fluorescent in situ hybridization (FISH) methods in human cGVHD tissue samples. Using a murine model of cGVHD established by Zhang et al. ((2002) *J Immunol.* 168:3088-3098), the above finding can be reproduced. In this model, the tear volume begins to decrease at 3 weeks after transplantation. Early fibrosis around lacrimal gland ducts and progressive fibrosis are detected as early as 3 weeks after transplantation, and gradually progress for up to 8 weeks in a similar fashion to human samples. We have performed this experiments over 20 times to create both GVHD and control groups with success, resulting in overall reproducibility is 70-80% based on analysis of lacrimal gland tissue samples and tear volumes.

[00593] In a typical transplantation experiment, 7- to 8-wk-old male and female B10.D2 (H-2d) and BALB/c (H-2d, Sankyo Laboratory, Ltd) mice are used as donors and recipients, respectively, using added spleen cells as a source of mature T cells. Briefly, female recipient mice are lethally irradiated with 700 cGy from a Gammacel 137Cs source (J. L. Shepherd & Associates, San Fernando, CA). Approximately 6 h later they are injected by tail vein with male donor bone

marrow (1×10^6 /mouse) and spleen (2×10^6 /mouse) cells suspended in RPMI 1640 (BioWhittaker, Walkersville, MD). A control group (syngeneic BMT) consists of female BALB/c recipient mice that receive the same number of male BALB/c spleen and bone marrow cells. (Zhang et al. (2002) *J Immunol.* 168:3088-3098). For HC-HA/PTX3 treatment, HC-HA/PTX3 complexes are administered via subconjunctival injection at predetermined times following bone marrow transplantation, such as 7, 14, 21 and 28 days following bone marrow transplantation.

[00594] Effects of treatment are assessed using assays including, but not limited to measurement of lacrimal gland fibrosis using Mallory staining, determining the number of activated fibroblasts per field using HSP47, a collagen specific molecular chaperon, as a marker of activated fibroblasts, measure lacrimal tear production under pilocarpine stimulation using a cotton thread test, and determining the level of fibrogenic cytokines such as HSP47, IL-4, IL-6, and TGF-beta using RT-PCR.

[00595] It is expected that treatment with HC-HA/PTX3 complexes will result in the reduction of lacrimal gland fibrosis in the mouse model. HC-HA/PTX3 complexes are then administered in the clinical setting by means of subconjunctival injection to human subjects for the treatment of dry eye caused by cGVHD.

[00596] In some examples, the HC-HA/PTX3 complex used in the method of treatment described herein is an isolated native HC-HA/PTX3 complex (nHC-HA/PTX3). In some examples, the nHC-HA/PTX3 is isolated from umbilical cord tissue. In some examples, the nHC-HA/PTX3 is isolated from amniotic membrane. In some examples, the HC-HA/PTX3 complex used in the method of treatment described herein is a reconstituted HC-HA/PTX3 complex (rcHC-HA/PTX3).

Example 15: Use of HC-HA/PTX3 for the Treatment of Inflammation in a Mouse Model

[00597] In this example, anti-inflammatory efficacy is tested in a murine model of HSV-1 necrotizing corneal stromal keratitis. A total of 240 female BALB/c mice (6 - 8 week old) obtained from Charles River Wiga (Sulzfeld, Germany) are anesthetized by intraperitoneal injection of 2 mg ketamine HCl and 400 ng mepivacaine HCl. For each mouse, the central cornea of one eye is then be scratched in a crisscross pattern with 8 horizontal and 8 vertical scratches using a 27-gauge needle under a surgical microscope. Each injured cornea is applied with a 5 μ l suspension containing 1×10^5 plaque forming units of HSV-1 viruses (KOS strain), which are routinely propagated on Vero cells, stored at -80°C , and quantified by standard plaque assay. On Day 14 after HSV-1 inoculation, mouse corneas that have developed severe ulcerating stromal keratitis are included for the study (about 50% yield) and subdivided into three groups, each consisting of 40 corneas (n=6 for clinical examination, n=5 for histology, n=5 for immunostaining, n=6 for cytokine

ELISA, n=5 for TUNEL, and n=10 for flow cytometry, and n=3 for attrition/backup). The non-infected fellow eyes are used as the negative control group. The positive control group receive tarsorrhaphy using 2 10-0 nylon sutures to close eyelids. The experimental HC-HA/PTX3 group receive the same tarsorrhaphy as the positive control and topical application 4 times a day of a composition containing purified HC-HA/PTX3 complex. The experimental HA group receives the same tarsorrhaphy but topical application of composition of HA alone four times a day. After 2 days, tarsorrhaphy is removed in all three groups. Using an operation microscope (Zeiss, Germany), the severity of stromal inflammation of each cornea is evaluated by a score of 0 to 4+, with 1+ having less than 25%, 2+ less than 50%, 3+ less than 75%, and 4+ between 75 and 100% corneal opacity with corneal neovascularization, edema, and thinning. After euthanasia by CO₂ chamber followed by cervical dislocation, 5 corneas from each group are subjected to frozen sectioning immunostaining using primary antibodies to CD11b (neutrophils and macrophages), F4/80 (macrophages), Gr-1 (PMNs), and CD3 (T cells) (see Methods), and another 5 corneas from each group are submitted to hematoxylin-eosin staining and TUNEL staining. In addition, corneal homogenates prepared from 6 corneas from each group are subjected to ELISA measurement of IL-1 α , IL-2, IL-6, IFN- γ and TNF α levels. Cells released by collagenase from 10 corneas of each group are prepared for flow cytometry to quantitate viable cells by MTT assay and apoptotic cells by the Annexin V-PE Apoptosis Detection Kit (BD-Pharmingen, Heidelberg, Germany).

[00598] It is expected that 50% mouse HSV-1-infected corneas will develop severe corneal stromal keratitis (inflammation), edema, and ulceration in two weeks after inoculation to be included for the study. Two days later, the non-infected corneas will remain normal, while infected corneas in the control group will maintain similar severe inflammation when tarsorrhaphy is removed. Similar to the control group, corneas in the experimental HA group will exhibit similar severe inflammation. In contrast, corneas of the experimental HC-HA/PTX3 group will show reduction of inflammation, which will be correlated with and substantiated by significant reduction of inflammatory (PMN/macrophages) and immune (T-cells) infiltration based on histology and immunostaining to CD11b, F4/80, Gr-1, and CD3, by significant reduction of inflammatory and immune cytokines such as IL-1 α , IL-2, IL-6, IFN- γ , and TNF- α based on ELISA and by a significant increase of TUNEL-positive cells in corneal tissues and of dead (MTT) and apoptotic cells released by collagenase from the cornea (flow cytometry using Annexin-V/7-AAD) when compared to the positive control group and the experimental HA group. Collectively, these data support the notion that the HC-HA/PTX3 complex exerts a clinical anti-inflammatory efficacy in this murine HSV-1 model.

[00599] In some examples, the HC-HA/PTX3 complex used in the method of treatment described herein is an isolated native HC-HA/PTX3 complex (nHC-HA/PTX3). In some examples, the nHC-HA/PTX3 is isolated from umbilical cord tissue. In some examples, the nHC-HA/PTX3 is isolated from amniotic membrane. In some examples, the HC-HA/PTX3 complex used in the method of treatment described herein is a reconstituted HC-HA/PTX3 complex (rcHC-HA/PTX3).

Example 16: Use of HC-HA/PTX3 for the Inhibition of Scarring in a Rabbit Model

[00600] In this example, anti-scarring efficacy tested in a rabbit model of excimer laser-assisted photorefractive keratectomy (PRK). A total of 30 New Zealand white albino rabbits with body weight (BW) of 2.5-3.0 kg, either sex, are used and subdivided into three groups (n=10 each): the non-PRK control group, the PRK HA group, and the PRK HC-HA/PTX3 group. Before PRK and for all CMTF examinations, rabbits are anesthetized by intramuscular injection of 5 mg/kg BW xylazine and 30 mg/kg BW ketamine and topically by 0.5% tetracaine HCl ophthalmic solution (Ortopics Laboratories Corp., Fairton, NJ). For the two PRK groups, the corneal epithelium of one eye of each animal is manually be removed by gentle scraping with a blunt spatula in an area just larger than the ablation zone, the denuded stroma is irrigated with normal saline, and excess fluid is removed gently with a cellulose sponge. A standard 6 mm diameter, 9.0 D PRK myopic correction PRK is performed using a LaddarVision Excimer Laser (Alcon, Ft. Worth, TX) to achieve a predicted theoretical stromal ablation depth of 118 μm . Immediately following PRK and thereafter, the PRK HC-HA/PTX3 Group is applied with a composition containing the HC-HA/PTX3 complex while the PRK HA group is applied with composition containing HA alone, both four times a day thereafter for a total of 3 weeks. In addition, all PRK-treated eyes is instilled topical 0.1% sodium diclofenac (one drop immediately post-PRK) and 0.3% gentamicin sulfate (three times daily for 3 days).

[00601] *In vivo* CMTF is performed on all operated eyes (n=6 from each group) prior to PRK and at one, two, three, and 4 weeks, two months and 4 months post-PRK using a modified Tandem Scanning Confocal Microscope (Tandem Scanning Corporation, Reston, VA) with a 24X surface-contact objective. Following a standard confocal examination of corneal morphology, video camera setting (gain, kilovolts, and black level) are switched to manual and kept constant during the study to allow direct comparison of all scans. CMTF is performed as a continuous, z-axis scan through the entire cornea. Corneal, epithelial, and stromal thickness are mapped within the central 3 mm zone by performing 10 consecutive CMTF-scans in areas covering all regions. Only data obtained from the thinnest stromal region corresponding to the center of the photoablation profile are used for subsequent calculations. CMTF-profiles based on image intensity depth are generated from

CMTF videorecordings. Corneal light reflectivity is measured by CMTF-profiles and expressed in arbitrary units (U) defined as $\mu\text{m} \times \text{pixel intensity}$ as an estimate of corneal haze.

[00602] To identify and measure the presence of stromal fibrotic tissue, three PRK-treated animals from each group (control and treated) are vitally stained with 0.5% 5-(4,6-dichlorotriazinyl) aminofluorescein (DTAF) dissolved in 0.2 M sodium bicarbonate as previously reported. After 2 min staining, eyes are thoroughly rinsed to remove excess dye before administration of topical antibiotics. At 4 months post-PRK animals is euthanized by intravenous injection of sodium pentobarbital (120 mg/kg BW). Following euthanasia, all corneas are fixed in situ by anterior chamber perfusion of 2% paraformaldehyde in PBS, pH 7.2, for 3 min, excised, placed in fresh fixative, and stored at 4 °C. Tissue is then by embedded in OCT, snap frozen in liquid nitrogen and sectioned using a cryomicrotome. Tissue is serially stepped section to identify the central and deepest part of the photoablation, and subjected to immunostaining using antibodies to keratocan, CD3434, FITC-conjugated phalloidin, ED-A fibronectin, S-100A4 and α -smooth muscle actin (α -SMA) to correlate changes of corneal haze (by CMTF light reflectivity) with phenotypic changes from keratocytes to fibroblasts or myofibroblasts. Additionally, in those eyes that are stained with DTAF, the thickness of the fibrotic tissue that is deposited is measured by determining the distance between the basal epithelial cells and the DTAF stained corneal tissue which represents the original, undamaged corneal stroma.

[00603] It is expected that *in vivo* confocal microscopy will reveal characteristic epithelial, basal lamina, stromal and endothelial characteristics, which will correlate well with well-defined peaks that change in intensity and position over time when *in vivo* CMTF-profiles are analyzed for corneas of the non-PRK control as well as for those receiving PRK. According to the published data, the PRK-treated corneas will exhibit four peaks originating from the superficial epithelium, photoablated stromal surface, layers of spindle-shaped fibroblasts, and endothelium, while those of the non-PRK control will exhibit three peaks originating from superficial epithelium, basal lamina, and endothelium at one week post-PRK. It is expected that there will not be much difference between the two experimental groups at one week post-PRK. At 2 weeks post-PRK, the experimental PRK HA group will show an increasing intensity of the peak close to the photoablated stromal surface due to ongoing cell migration of spindle-shaped fibroblasts. However, it is expected that the intensity of the repopulating fibroblasts (by the height of the peak) will be much reduced in the experimental PRK HC-HA/PTX3 group. During the period from 3 weeks to four months post-PRK, the peak corresponding to the layer of spindle-shaped fibroblasts will merge with the peak originating from the photoablated stromal surface because of the completion of repopulation of the acellular anterior stroma in the experimental PRK HA group, and will result in

a dramatic increase of the reflectivity of the peak corresponding to the photoablated stromal surface. In contrast, there will not be such a dramatic increase of the reflectivity in the experimental PRK HC-HA/PTX3 group. Such a difference of light reflectivity can also be quantitated by calculating the area of the CMTF-peaks originating from specific intra-corneal structures. It is expected that the experimental PRK HA group will exhibit a substantial linear increase in reflectivity intensity within the first 2 to 3 weeks post-PRK and a slow linear decline in reflectivity thereafter. In contrast, it is expected that there will be a significant decrease of reflectivity in the experimental PRK HC-HA/PTX3 group during both periods. Collectively, these CMTF data support that HC-HA/PTX3 complex exerts an inhibitory effect of keratocytes activation, migration and cell recruitment during repopulation of the acellular anterior stroma, explaining why corneal light scattering (haze) is reduced similar to anti-TGF- β antibodies previously reported. As a result, there is less cellularity and reflectivity of activated, migrating, intra-stromal wound healing keratocytes, and less deposition of new stromal extracellular matrix and a faster establishment of a normal quiescent keratocytes population in the anterior stroma. This conclusion will be corroborated by a significant reduction of activated keratocytes (F-actin in keratocan expressing cells), fibroblasts (cytoplasmic staining of S-100A4, membrane expression of ED-A fibronectin), and myofibroblasts (nuclear expression of S100A4 and cytoplasmic expression of α -SMA) in the experimental PRK HC-HA/PTX3 group when compared to the experimental PRK HA group during the period of 2 to 3 weeks post-PRK. It also is expected that there will be a significant reduction of the distance between the basal epithelial cells and the DTAF stained corneal tissue in the PRK HC-HA/PTX3 group, indicative of significantly less fibrotic tissue, when compared to the PRK HA group.

[00604] In some examples, the HC-HA/PTX3 complex used in the method of treatment described herein is an isolated native HC-HA/PTX3 complex (nHC-HA/PTX3). In some examples, the nHC-HA/PTX3 is isolated from umbilical cord tissue. In some examples, the nHC-HA/PTX3 is isolated from amniotic membrane. In some examples, the HC-HA/PTX3 complex used in the method of treatment described herein is a reconstituted HC-HA/PTX3 complex (rcHC-HA/PTX3).

Example 17: Use of HC-HA/PTX3 for the Treatment of Atherosclerosis

[00605] In this example, an HC-HA/PTX3 complex generated by the methods described herein is administered for the treatment of atherosclerosis.

[00606] Atherosclerosis includes the involvement an inflammatory cell population, in particular macrophages. A macrophage phenotypic switch is observed during disease progression. In atherosclerosis, circulating monocytes are recruited to sites of fatty deposit accumulation within the

vascular intima and subintima via CCR2 and endothelial adhesion mediated mechanisms. Upon arrival these cells become activated and differentiate into macrophages. The fatty deposits then begin to mature into plaques with continued recruitment of inflammatory cells, smooth muscle cells, and the production of extracellular matrix. The initial infiltrating macrophage population in early atherosclerosis is heterogeneous, but possesses a predominantly M2-like phenotype. Concurrent with lesion progression and expansion, a switch to a predominantly M1 phenotype has been observed. This phenotypic switch may be due to the phagocytosis of excess oxidized low-density lipoproteins (LDL) within the plaque by macrophages and the production of IFN- γ by local Th1 cells, resulting in the development of foam cell macrophages. Foam cell macrophages exhibit a highly activated phenotype leading to production of pro-inflammatory mediators and MMPs that destabilize the plaques, potentially leading to thromboembolism. Therapies which prevent the M2 to M1 switch or deplete M1 macrophages selectively are of clinical utility for the stabilization of atherosclerotic plaques.

[00607] An HC-HA/PTX3 complex generated by the methods described herein is administered to a subject having atherosclerosis. The HC-HA/PTX3 complex employed, for example, to coat implantable medical devices, such as a stent, for implantation in at or near the site of inflammation. Treatment of atherosclerosis with an HC-HA/PTX3 complex is expected to decrease inflammation and prevent thromboembolism.

[00608] In some examples, the HC-HA/PTX3 complex used in the method of treatment described herein is an isolated native HC-HA/PTX3 complex (nHC-HA/PTX3). In some examples, the nHC-HA/PTX3 is isolated from umbilical cord tissue. In some examples, the nHC-HA/PTX3 is isolated from amniotic membrane. In some examples, the HC-HA/PTX3 complex used in the method of treatment described herein is a reconstituted HC-HA/PTX3 complex (rcHC-HA/PTX3).

Example 18: Use of HC-HA/PTX3 for the Treatment of Obesity and Insulin Resistance

[00609] In this example, an HC-HA/PTX3 complex generated by the methods described herein is administered for the treatment of obesity and insulin resistance.

[00610] Adipose tissue macrophages (ATM) comprise a significant proportion of the cellular component of adipose tissue in both lean and obese states. In normal humans, ATMs make up as much as ten percent of the cellular constituents of the tissue. In comparison, in obese subjects that number rises to as much as 40%. In normal, non-obese subjects, ATMs have a polarized M2 phenotype characterized by increased baseline STAT6 and PPAR- γ expression. These cells play an important and beneficial role in nutrient metabolism. The deficiency in PPAR- γ leads to impaired M2 macrophage function and susceptibility to diet-induced inflammation and insulin resistance. In

contrast, ATMs accumulate in the adipose tissue during obesity have a strongly polarized pro-inflammatory M1 phenotype. These cells produce high levels of TNF α , IL-6, and IL-1 β , all of which are also observed in increased levels of adipose tissue from insulin resistant individuals. High levels of pro-inflammatory mediators locally impair the function of resident insulin processing cells.

[00611] An HC-HA/PTX3 complex generated by the methods described herein is administered to a subject suffering from obesity or insulin resistance. An HC-HA/PTX3 complex is administered, for example, as a solution or gel for treatment. It is expected that treatment with an HC-HA/PTX3 complex will promote a phenotypic switch of adipose tissue macrophages (ATM) from a pro-inflammatory M1 phenotype to an M2 phenotype and restore normal insulin processing.

[00612] In some examples, the HC-HA/PTX3 complex used in the method of treatment described herein is an isolated native HC-HA/PTX3 complex (nHC-HA/PTX3). In some examples, the nHC-HA/PTX3 is isolated from umbilical cord tissue. In some examples, the nHC-HA/PTX3 is isolated from amniotic membrane. In some examples, the HC-HA/PTX3 complex used in the method of treatment described herein is a reconstituted HC-HA/PTX3 complex (rcHC-HA/PTX3).

Example 19: Use of HC-HA/PTX3 for the Treatment of Type 1 Diabetes

[00613] In this example, an HC-HA/PTX3 complex generated by the methods described herein is administered for the treatment of type 1 diabetes.

[00614] Diabetes mellitus type 1 (Type 1 diabetes, T1DM, IDDM, or, formerly, juvenile diabetes) is a form of diabetes mellitus that results from autoimmune destruction of insulin-producing beta cells of the pancreas. The subsequent lack of insulin leads to increased blood and urine glucose. The classical symptoms are polyuria (frequent urination), polydipsia (increased thirst), polyphagia (increased hunger), and weight loss.

[00615] An HC-HA/PTX3 complex generated by the methods described herein is administered to a subject suffering from Type 1 diabetes in the form of microcapsules containing autologous or allogeneic insulin-producing cells coated with HC-HA/PTX3 complex. The microcapsules are administered to a subject for example, by injection. It is expected that treatment with the HC-HA/PTX3 coated microcapsules will allow for the production of insulin that is released in the subject and prevent or reduce inflammatory responses against the cell therapy or microcapsule, thereby alleviating the Type 1 diabetes and the symptoms thereof.

[00616] In some examples, the HC-HA/PTX3 complex used in the method of treatment described herein is an isolated native HC-HA/PTX3 complex (nHC-HA/PTX3). In some examples, the nHC-HA/PTX3 is isolated from umbilical cord tissue. In some examples, the nHC-HA/PTX3 is isolated

from amniotic membrane. In some examples, the HC-HA/PTX3 complex used in the method of treatment described herein is a reconstituted HC-HA/PTX3 complex (rcHC-HA/PTX3).

Example 20: Use of HC-HA/PTX3 for the Treatment of Fibrosis

[00617] In this example, an HC-HA/PTX3 complex generated by the methods described herein is administered for the treatment of fibrosis or fibrotic disorder.

[00618] The progressive fibrotic diseases, such as idiopathic pulmonary fibrosis (IPF), hepatic fibrosis and systemic sclerosis, are tightly regulated by macrophages. 'Pro-fibrotic' macrophages exhibit M1 properties and produce various mediators, including TGF β 1, PDGF and insulin-like growth factor 1, that directly activate fibroblasts and myofibroblasts, which control ECM deposition. Pro-fibrotic macrophages also produce MMPs, TIMPs, and IL-1 β . IL-1 β stimulates TH17 cells to produce IL-17, an important inducer of bleomycin-induced pulmonary fibrosis, a fibrotic disorder with characteristics that are similar to those of IPF. The production of IL-10, RELM α and ARG1 by M2-like macrophages suppress fibrosis.

[00619] An HC-HA/PTX3 complex generated by the methods described herein is administered to a subject suffering from fibrosis or a fibrotic disorder. An HC-HA/PTX3 complex is administered, for example, as a solution, gel or as a coating on an implantable medical device. It is expected that treatment with an HC-HA/PTX3 complex will decrease the M1 macrophages and activation of fibroblasts and myofibroblasts and increase the amount M2 macrophages present at the affected site(s) in the subject thereby suppressing the fibrosis and symptoms thereof, such as scarring.

[00620] In some examples, the HC-HA/PTX3 complex used in the method of treatment described herein is an isolated native HC-HA/PTX3 complex (nHC-HA/PTX3). In some examples, the nHC-HA/PTX3 is isolated from umbilical cord tissue. In some examples, the nHC-HA/PTX3 is isolated from amniotic membrane. In some examples, the HC-HA/PTX3 complex used in the method of treatment described herein is a reconstituted HC-HA/PTX3 complex (rcHC-HA/PTX3).

Example 21: Use of HC-HA/PTX3 for the Treatment of Chronic Inflammation

[00621] In this example, an HC-HA/PTX3 complex generated by the methods described herein is administered for the treatment of a chronic inflammatory condition, such as rheumatoid arthritis.

[00622] Many autoimmune diseases, including rheumatoid arthritis, involve inflammatory responses to autoantibodies that activate Fc receptors to trigger mast-cell and macrophage activation, and neutrophil invasion. This leads to an intense local inflammatory response and, if not resolved, to tissue damage over time with cycles of repair and destruction. In rheumatoid arthritis, CSF1 is produced constitutively by synovial fibroblasts and recruits tissue-infiltrating monocytes

and macrophages. In addition, locally produced CSF1, together with RANK1, induces the differentiation of monocytes to osteoclasts, which trigger bone loss.

[00623] An HC-HA/PTX3 complex generated by the methods described herein is administered to a subject suffering from a chronic inflammatory condition, such as rheumatoid arthritis. An HC-HA/PTX3 complex is administered, for example, as a solution, gel or as a coating on an implantable medical device. It is expected that treatment with HC-HA/PTX3 will suppress M1 proinflammatory macrophages, induce neutrophil apoptosis, and inhibit osteoclast differentiation, thereby treating the inflammatory condition and the symptoms thereof.

[00624] In some examples, the HC-HA/PTX3 complex used in the method of treatment described herein is an isolated native HC-HA/PTX3 complex (nHC-HA/PTX3). In some examples, the nHC-HA/PTX3 is isolated from umbilical cord tissue. In some examples, the nHC-HA/PTX3 is isolated from amniotic membrane. In some examples, the HC-HA/PTX3 complex used in the method of treatment described herein is a reconstituted HC-HA/PTX3 complex (rcHC-HA/PTX3).

Example 22: Use of HC-HA/PTX3 for the Treatment of Acute Inflammatory Response

[00625] In this example, an HC-HA/PTX3 complex generated by the methods described herein is administered for the treatment of an acute inflammatory response caused by a condition such as myocardial infarction, stroke or sepsis. An HC-HA/PTX3 complex generated by the methods described herein is administered to a subject having an acute inflammatory response caused by a condition such as myocardial infarction, stroke or sepsis. An HC-HA/PTX3 complex is administered, for example, as a solution by intravenous infusion. It is expected that the HC-HA/PTX3 complex will decrease or prevent damage caused by acute inflammation by suppression of M1 inflammatory macrophages.

[00626] In some examples, the HC-HA/PTX3 complex used in the method of treatment described herein is an isolated native HC-HA/PTX3 complex (nHC-HA/PTX3). In some examples, the nHC-HA/PTX3 is isolated from umbilical cord tissue. In some examples, the nHC-HA/PTX3 is isolated from amniotic membrane. In some examples, the HC-HA/PTX3 complex used in the method of treatment described herein is a reconstituted HC-HA/PTX3 complex (rcHC-HA/PTX3).

Example 23: Use of HC-HA/PTX3 for the Treatment of Cancer

[00627] In this example, an HC-HA/PTX3 complex generated by the methods described herein is administered for the treatment of cancer.

[00628] The participation of large numbers of inflammatory cells in tumor development and progression has been observed and is commonly described as “smoldering inflammation”. These

observations have led to a well-established link between inflammatory cells, macrophages in particular, and cancer. It was initially thought that development of oncogenes resulted in the hallmark microenvironment of cancer, in which transformed cells secrete cytokines and chemokines that promote tissue development and prevent apoptosis as well as suppress cytotoxic immune responses (termed the “intrinsic pathway”). It is now recognized that another pathway leading to tumorigenesis exists. This “extrinsic pathway” is initially characterized by a chronic pro-inflammatory environment resulting from a persistent microbial infection, autoimmune disease, or other etiology of unknown origin. The chronic production of large quantities of inflammatory mediators in these cases can lead to tumor cell proliferation and survival or to the induction of genetic instabilities in normal cells, with resultant expression of oncogenes and production of immune suppressive cytokines. Thus, early tumor development is, in many instances, characterized by a polarized inflammatory, M1-like macrophage environment.

[00629] An HC-HA/PTX3 complex generated by the methods described herein is administered to a subject having a cancer, such as a solid tumor cancer. An HC-HA/PTX3 complex is administered, for example, as a solution, gel or as a coating on an implantable medical device for topical, injective, or implantive application. Because HC-HA/PTX3 can suppress M1 macrophage polarization, it is expected that treatment with HC-HA/PTX3 will inhibit or prevent cancers or their progress into late stage phenotypes.

[00630] In some examples, the HC-HA/PTX3 complex used in the method of treatment described herein is an isolated native HC-HA/PTX3 complex (nHC-HA/PTX3). In some examples, the nHC-HA/PTX3 is isolated from umbilical cord tissue. In some examples, the nHC-HA/PTX3 is isolated from amniotic membrane. In some examples, the HC-HA/PTX3 complex used in the method of treatment described herein is a reconstituted HC-HA/PTX3 complex (rcHC-HA/PTX3).

Example 24: Use of HC-HA/PTX3 for the Treatment of Non-healing Skin Wounds or Ulcers

[00631] In this example, an HC-HA/PTX3 complex generated by the methods described herein is administered for the treatment of a non-healing wound or ulcer on the skin.

[00632] A non-healing wound or ulcer on the skin that has been present for about 3-4 weeks duration, without healing is called non-healing ulcer. Diseases that commonly cause non-healing ulcers are vascular disease, diabetes, skin cancers and some infections.

[00633] An HC-HA/PTX3 complex generated by the methods described herein is administered to a subject having a non-healing wound or ulcer on skin. An HC-HA/PTX3 complex is administered, for example, as a solution, gel topically or subcutaneously for the treatment at the site of the wound

or ulcer. It is expected that treatment with HC-HA/PTX3 will promote the healing of the wound or ulcer by promoting the M2 phenotype of wound healing and tissue regenerative macrophages.

[00634] In some examples, the HC-HA/PTX3 complex used in the method of treatment described herein is an isolated native HC-HA/PTX3 complex (nHC-HA/PTX3). In some examples, the nHC-HA/PTX3 is isolated from umbilical cord tissue. In some examples, the nHC-HA/PTX3 is isolated from amniotic membrane. In some examples, the HC-HA/PTX3 complex used in the method of treatment described herein is a reconstituted HC-HA/PTX3 complex (rcHC-HA/PTX3).

Example 25: Use of HC-HA/PTX3 for the Treatment of High Risk Corneal Transplants

[00635] In this example, HC-HA/PTX3 is administered for the treatment of high risk corneal transplants. Mafia mice, which EGFP⁺ macrophages are intrastromally injected with LPS (5 µg per eye) for both eyes. In each eye, OS (oculus sinister; left eye) is treated with PBS (2 or 4 injection sites) while OD (oculus dexter, right eye) is treated one time with HC-HA/PTX3 (2 or 4 injection sites; 5 µl of 1 mg/ml HA composition containing HC-HA/PTX3 per injection site) immediately after LPS injection. Images of whole corneas are taken with *in vivo* intravital microscopy on day 1, day 2, day 3, day 4, day 5, day 6, and day 7. EGFP-positive cells are counted based on the intensity of green fluorescence to determine the level of EGFP infiltration. In a mouse model of corneal transplantation, injection of HC-HA/PTX3 into subconjunctival sites is expected to reduce inflammation (i.e. infiltration of macrophages) and improve the survival rate of transplanted corneas when compared to PBS vehicle control.

[00636] In some examples, the HC-HA/PTX3 complex used in the method of treatment described herein is an isolated native HC-HA/PTX3 complex (nHC-HA/PTX3). In some examples, the nHC-HA/PTX3 is isolated from umbilical cord tissue. In some examples, the nHC-HA/PTX3 is isolated from amniotic membrane. In some examples, the HC-HA/PTX3 complex used in the method of treatment described herein is a reconstituted HC-HA/PTX3 complex (rcHC-HA/PTX3).

Example 26: Distribution of HA, PTX3, TSG-6, HC1, HC2, HC3 and Bikunin in Umbilical Cord (UC)

[00637] In this example, the *in vivo* distribution of HA, PTX3, TSG-6, HC1, HC2, HC3 and bikunin was detected in umbilical cord (UC) by immunostaining. UC tissue frozen sections were subjected to immunostaining for HA, PTX3, TSG-6 and various components of IαI including HCs and bikunin. UC consisted of a layer of epithelium and a stroma composed of a sub-amnion layer and a Wharton's jelly that contained three vessels, i.e., one vein and two arteries (FIG. 20a, with one artery vessel, phase). Strong positive HA staining was observed in the UC epithelium, sub-

amnion layer and Wharton's jelly, and weak HA staining in the vessel wall (FIG. 20a, HA). With HAase digestion, the aforementioned HA staining disappeared (FIG. 20a, HA (+ HAase)), conforming the specific staining for HA.

[00638] Strong positive immunostaining of PTX3 was present in Wharton's jelly, and weak PTX3 staining in sub-amnion layer and epithelium (FIG. 20a, PTX3). HAase digestion did not enhance PTX3 staining in the sub-amnion layer and the epithelium (not shown), suggesting that the weak PTX3 staining was not due to the masking effect by HA. Positive PTX3 staining was also observed in the endothelium of vessels (not shown) but not in the vessel wall of arteries and vein.

[00639] Both TSG-6 and bikunin were present in the whole UC with TSG-6 mainly present in cells and around cells and more TSG-6 was in epithelium and subamnion compared with Wharton's jelly. HC1 had a similar localization as HA except that the epithelium and the vessel wall had faint HC1 staining. Weak to no HC2 and HC3 staining was present in the epithelium but not in the stroma of UC. These results showed that UC produced abundant HA, PTX3, TSG-6, HC1 and bikunin, and disproportionally less HC2 and HC3 when compared to AM. It was determined that UC constitutively expressed the above proteins, and HC-HA/PTX3 complex.

[00640] Furthermore, PTX3 was present in UC with a different distribution pattern from what has been reported in AM. More PTX3 was present in the UC Wharton's jelly and less in epithelium and sub-amnion. In contrast, more PTX3 was in the epithelium and the stromal compact layer in AM. UC had a similar pattern to AM in the following markers: more HA was present in the whole stroma of UC and little in epithelium of UC. This was similar in the distribution pattern of HA in AM. TSG-6 was mainly localized in epithelial and subamnion cells of UC, and bikunin was found in the whole UC.

Example 27: Comparison of the extracts of PBS and GnE sequentially obtained from AM and UC

[00641] This example determined whether the insoluble part after PBS extract from AM still contained any PTX3, TSG-6 and IαI as well as HC-HA/PTX3 complex. Proteins were extracted from the insoluble part of AM by 4 M GnHCl after PBS extraction to see whether there was PTX3, TSG-6 and IαI. In addition, UC with PBS was also sequentially extracted with 4 M GnHCl to detect PTX3, TSG-6, HCs and bikunin in these two different extractions.

[00642] According to the method described in He et al. (2009) *J. Biol. Chem.* 284:20136-20146), AM, CH and UC were homogenized with a blender in cold PBS at 1:1 (g/ml) for AM or 1:1.5 (g/ml) for UC, and mixed at 4 °C for 1 h. The mixture was centrifuged at 48,000 g at 4 °C for 30 min. The supernatants of PBS extract were designated as AME, CHE and UCE, respectively. In

addition, Wharton's jelly mixture from UC was also extracted by PBS and such extract was named UJE. The insoluble pellet of AM, CH, UC and UC jelly mixture after PBS extraction were further extracted by 4 M GnHCl buffer (100 mM sodium acetate, pH 5.8, 4M GnHCl, 10 mM EDTA, 1% Triton X-100) at 4 °C for 24 h. After centrifugation at 48,000 g, at 4 °C for 30 min, the supernatants were collected and named AMGnE, CHGnE, UCGnE and UJGnE, respectively. The HA and protein concentrations in each extraction were detected by HA ELISA and BCA assay, respectively.

[00643] GnHCl further extracted abundant HA and proteins from the insoluble pellet after PBS extraction

[00644] The HA and protein concentrations in sequential PBS and GnHCl extracts are summarized in Table 1 where the HA/protein ratio was also compared between the two extracts. In general, 4 M GnHCl further extracted abundant proteins and HA from the insoluble pellet of AM, CH, UC and UC jelly mix after PBS extraction. GnHCl buffer extracted more proteins but less HA from the insoluble pellet than PBS. However, UCGnE still contained similar amount of proteins and HA to AME and CHE. That is, UC contained more HA than AM and CH in both PBS and GnHCl extracts.

Table 1. Quantitation of proteins and HA in 4 M GnHCl extracts (GnE) from insoluble pellets of AM, CH, UC and UC jelly mix after PBS extract

	HA ($\mu\text{g/ml}$)	Protein ($\mu\text{g/ml}$)	HA/protein Ratio ($\mu\text{g}/\mu\text{g}$)
AME I103	75.7	1014.3	0.074633
AME J021	61.5	5353.0	0.011489
AMGnE I103+J021	47.7	6097.5	0.007823
CHE I103	78.9	6161.7	0.012805
CHGnE I103	24.9	7021.7	0.003546
UCE 1 I103	453.5	8523.8	0.053204
UCGnE 1 I103	88.2	1925.7	0.045802
UCE 2 5001	421.3	7471.3	0.056389
UCGnE 2 5001	79.6	2670.2	0.029811
UCJE	277.5	5135.1	0.05404
UCJGnE	57.2	4955.1	0.011544

[00645] Monomer, dimer, and HMW PTX3 was present at higher amounts in PBS extraction of UC cells compared to AM cells. But HMW PTX3 was present in higher amounts in GnHCl extraction in AM cells.

[00646] Analysis of the AME with anti-PTX3 antibody revealed a band of ~45 kDa corresponding to the size of the native PTX3 monomer, and a HMW band at the bottom of the loading well (FIG. 21A, lane 4). NaOH treatment did not affect the 45 kDa band, but completely eliminated the HMW band, resulting in a HMW smear of PTX3 (FIG. 21A, lane 5), which is a notable feature of PTX3 in HC-HA complex with NaOH treatment where no monomer PTX3 but a 90 kD dimer was detected with or without NaOH treatment (FIG. 21A, lane 2 and 3). The HMW smear of PTX3 represented the complexes formed between PTX3 and HC-HA. CHE had the same pattern of PTX3 band, but there was less of a smear of PTX3 after NaOH treatment, consistent with the immunostaining result. Placenta extract had the same result with CHE. Notably, PTX3 existed more in a dimer and HMW smear besides monomer in UCE₂ and their intensity further increased after NaOH treatment. Similar to AME, UCE also generated HMW smearing pattern, more so than AME. These results showed that UCE contain more PTX3 than AME, while CHE and placenta extracts contained little PTX3.

[00647] Compared with AME (FIG. 21A), AMGnE showed a strong HMW PTX3 smear, weak dimer and monomer levels of PTX3, and the intensity of HMW PTX3 smear was further increased after NaOH treatment (FIG. 21B, lane 3 and 4), which showed that AMGnE contained more HMW PTX3 than AME. More PTX3 was present in water-insoluble part of AM. CHGnE only had a HMW band in the loading well but no HMW PTX3 smear regardless of treatment with or without NaOH. UCGnE and UJGnE had the same pattern of PTX3 with or without NaOH treatment as AMGnE except the intensity of PTX3 smear was a little weaker than that in AMGnE (FIG. 21B, lane 3 and 4). The intensity of PTX3 smear in UCGnE was also lower than that in UCE, which showed that UCGnE contained less HMW PTX3 than UCE; that is more PTX3 was present in the water soluble part of UC.

[00648] The above results showed that both AM and UC contained HMW PTX3. In AM more HMW PTX3 was water-insoluble and could be extracted by GnHCl after PBS extraction, while more HMW PTX3 in UC was water soluble that could be mostly extracted by PBS.

[00649] Both IαI and HC1 mostly in AM PBS extract but in UC most IαI in PBS extract while HC1 in GnHCl extract, while more bikunin present in UCGnE than in UCE with no difference in others' two extracts

[00650] FIG. 22 shows that an 80 kDa HC1 band was present in all PBS extracts except UCE and in all GnE extracts except AMGnE. This band was increased by NaOH in all PBS extracts but not in GnE, showing that AM contained both free and bound water soluble HC1 (i.e. ester bound to HA in HC-HA and to bikunin in IαI) that was released by NaOH in agreement with Zhang et al. (2012) *J. Biol. Chem.* 287:12433-12444. UC contained water soluble bounded HC1 that was released by

NaOH, and also contained water insoluble free HC1 bound to water insoluble extracellular components that were dissociated by SDS and 2-ME but not affected by NaOH. This was consistent with strong positive HC1 staining in UC. The HMW HC1 band was present in all PBS and GnHCl extracts in loading wells and was decreased by NaOH, showing that it was the HC-HA complex. The HMW HC1 band was weaker in UCGnE than in UCJGnE, illustrating that Wharton's jelly contained more water insoluble HC-HA complex. Free I α I was found in all PBS extracts but not in all GnE extracts, suggesting that it was water soluble. However, free P α I was found in all PBS and GnHCl extracts, suggesting P α I had a different interaction with I α I. More bikunin was found in UCGnE than in UCE with no difference in other two extracts, highlighting that most bikunin was bound to other water insoluble molecules in UC and that this was indicative of a unique function.

[00651] TSG-6 was present in the HMW complex of AM but not UC GnHCl extract

[00652] FIG. 23 showed that the 35 kDa TSG-6 band, which has been reported in AME (Zhang et al. (2012) *J. Biol. Chem.* 287:12433-12444), was present in AMGnE but not all other extracts, showing that TSG-6 was absent in GnE of UC. This band was not affected by NaOH, confirming that TSG-6 was not bound to HMW species that can be cleaved by NaOH. However, the HMW TSG-6 band was found in AMGnE and CHGnE, but not in UCGnE and UJGnE. Furthermore, this band was not changed by NaOH, showing that TSG-6 was strongly bound to HMW species. TSG-6 was not detected in HC-HA purified by 4X ultracentrifugation from GnE, suggesting that although TSG-6 is still bound in the insoluble matrix, it is separable by GnHCl during ultracentrifugation.

[00653] In summary, GnHCl further extracted abundant HA and proteins from the insoluble part of AM and UC after PBS extract. UC contained more HA than AM and CH in both PBS and GnHCl extracts. HMW PTX3 were at a higher level in AM GnHCl extract and at a higher level in UC PBS extract. More HMW PTX3 was retained in the insoluble part after PBS extract. HC1 was mostly in AM PBS extract but not in UC GnHCl extract. HMW TSG-6 was in AMGnE but not in UCGnE and UJGnE, showing that TSG-6 was still bound in the insoluble matrix but separable by GnHCl during ultracentrifugation.

Example 28: Purification of the HC-HA complex by four successive ultracentrifugations from AM and UC PBS extract and detection of the presence of PTX3, HCs, Bikunin, and TSG-6 in the HC-HA complexes

[00654] In this example, HC-HA complex was purified by four successive ultracentrifugations from AME and UCE, and the presence of PTX3 as well as HCs, bikunin and TSG-6 were detected in the AM and UC HC-HA complex by Western blot.

[00655] The 4th AM HC-HA complex contained more HMW PTX3 and HC1, and was purer than the 2nd and 3rd HC-HA complex.

[00656] With the anti-PTX3 antibody, Western blot analysis of 1-4th AM HC-HA showed a 90 kDa band (dimer), compared to the monomer which was found in PBS extracts. This showed that the dimer status was resolved by further extraction in 4M GnHCl through ultracentrifugation, revealing and a HMW band in gel top in 1st, 2nd, 3rd and 4th HC-HA complex (FIG. 24a). Compared with purified PTX3 control, the 90 kDa band was PTX3 dimer, and the high molecular weight band was PTX3-containing HC-HA complex. After HAase treatment, the 90 kDa band did not change in all HC-HA complexes, but the HMW smear band was vaguely detected in 3rd and 4th fractions. That is, from 1st to 4th, the HMW band gradually disappeared and a smear gradually appeared which was more intensified in 4th HC-HA complex. There was no 45 kDa PTX3 monomer band in all HC-HA complexes. The results showed that HC-HA complex contained multimeric form of PTX3 that is able to bind to HC-HA, and with the number of times of ultracentrifugation increasing, the PTX3-containing HC-HA complex became more purified. The existence of 90 kDa PTX3 dimer in HC-HA complex with or without HAase showed that: 1) PTX3 dimer present in HC-HA was dissociated by SDS and 2-ME, and 2) the HMW PTX3 was resistant to SDS and 2-ME. The PTX3 dimer being a product due to 2ME is further confirmed below (FIG. 24a).

[00657] With anti-HC1 antibody, an 80 kDa HC1 band was detected only in earlier 1st and 2nd fractions from all the four HC-HA complexes (FIG.24b). After HAase treatment, the HC1 band intensified, and several smaller bands also appeared in 1st to 3rd HC-HA complexes. The results showed that purified HC-HA complex did not contain free HC, and HC-HA was made of HC1. In agreement with above PTX3 Western blot results, with increasing times of ultracentrifugation, the HC-HA complex became more purified. No HC2 (FIG.24c), HC3, and TSG-6 (FIG. 24d) were found in all HC-HA complexes.

[00658] TSP-1 was only present in AM GnHCl extract but not in PBS extract and 1-4th HC-HA complex

[00659] Thrombospondin-1 (TSP-1) was only detected as trimer in AM GnHCl extract but not in PBS extract and 1-4th HC-HA complex (FIG. 25). After HAase treatment it appeared as a smear, illustrating that TSP-1 was water insoluble and strongly bound to HC-HA. However, such a binding in the insoluble matrix to HC-HA can be dissociated by GnHCl and CsCl.

[00660] Like 4th AM HC-HA complex, 4th UC HC-HA complex contained PTX3 and HC1 but not HC2, HC3, bikunin and TSG-6 and the lack of 2ME did not generate PTX3 dimer but yielded HC1

[00661] With the anti-PTX3 antibody, Western blot analysis of 4th UC HC-HA with or without HAase treatment showed a similar PTX3 band pattern in 4th UC HC-HA complex (FIG. 26a, lanes 5 and 6) to that in 4th AM HC-HA complex (FIG. 26a, lanes 3 and 4). When the sample buffer did not contain 2-ME, the 90 kDa PTX3 dimer disappeared in UC HC-HA complex with or without HAase (FIG. 26a, lanes 7 and 8), showing that the appearance of 90 kDa PTX3 dimer in HC-HA complex was due to the reduction of PTX3 bound to HC-HA by 2-ME in the sample buffer. With anti-HC1 antibody, only a high molecular weight HC-HA band was detected in 4th UC HC-HA complex (FIG. 26b, lane 6) similar to that in 4th AM HC-HA complex (FIG. 26b, lane 4). After HAase the HC-HA band disappeared and the HC1 band increased (lane 7) like 4th AM HC-HA complex (lane 5), though its intensity was a little weaker than that in 4th AM HC-HA. This showed that HC1 formed a complex with S-S with PTX3. The stronger band with a MW slightly higher than generic HC1 appeared when the sample buffer did not contain 2-ME, showing that HC1 was linked to PTX3 via S-S. No HC2, HC3 (FIG. 26c), bikunin (FIG. 26d) and TSG-6 (FIG. 26e) was detected in 4th AM and UC HC-HA complex.

Example 29: Purification of HC-HA complex from total AM and UC GnHCl extract by four successive ultracentrifugations and comparison with PBS extractions

[00662] This example determined that more HC-HA complex from AM and UC could be obtained, the HC-HA complex had a more reasonable constitution of PTX3 and HC-HA, and that it had more effective therapeutic role in the clinic. AM and UC was extracted by 6M GnHCl buffer (200 mM Tris-HCl, pH 8.0, 6M GnHCl, 10 mM EDTA, 10mM aminocaproic acid, 10mM N-ethylmaleimide, 2 mM PMSF): GnHCl extraction from AM and UC was performed by adding 6M GnHCl buffer to AM and UC powder at 1:4 (g/ml). Samples were mixed overnight at 4 °C, and centrifuged at 48,000 g, at 4 °C for 30 min. The supernatants were GnHCl extracts. The 4th HC-HA complex was purified from AM and UC GnHCl extract using the same procedure as that for the 4th HC-HA purification from PBS extract. Characterization of HC-HA complex was performed by Western blotting to examine PTX3, HCs, bikunin, TSG-6 and likely other proteins. An agarose gel of HC-HA was run to see HA content and molecular weight.

[00663] GnHCl extracted more HC-HA complex from AM and UC than PBS.

[00664] The GnHCl extracts from AM and UC were named AMEG and UCEG, and their HA and protein contents were detected by BCA assay and HA ELISA, respectively. 4th HC-HA complex was purified from GnHCl extract and their HA and protein contents were similarly detected. Table 2 summarizes the contents of protein and HA in both PBS and GnHCl extracts and their 4th HC-HA complex. The results showed that AM and UC GnHCl extracts contained more HA and have a

higher HA/protein ratio compared to relative PBS extract. Furthermore, more HC-HA complex was purified from GnHCl extract.

Table 2. Quantitation of proteins and HA in extracts and 4th HC-HA from AM and UC.

Sample	Tissue weight/Extract buffer volume (g / ml)	HA (μg/ml)	Protein (μg/ml)	HA/protein Ratio (μg/μg)	Relative 4 th HC-HA	
					HA (μg/ml)	Protein (μg/ml)
AME-PBS J021	1:1	61.5	5353.0	0.011	4	undetectable
AMEG G021	1:4	431.4	3762.5	0.115	11	undetectable
UCE-PBS I103	1:1.5	453.5	8523.8	0.053	32	89.1
UCEG G021	1:4	442.0	4750.0	0.093	44	13
UCmixE-PBX	1:4	277.5	5135.1	0.054	20	undetectable
UCmixEG	1:6	441.6	4730.0	0.093	40	undetectable

[00665] AM 4th GnHCl HC-HA complex contained more HC1 and HMW PTX3 but contained less than the PBS HC-HA with or without HAase or NaOH treatment, and both PBS and GnHCl HC-HA did not contain TSP-1.

[00666] With the anti-HC1 antibody, GnHCl HC-HA showed a HMW band in the loading well as PBS HC-HA, but HAase digestion only released weaker HC1 (FIG. 27a, lanes 6 and 7). NaOH treatment also released a weaker HC1 band that had a little higher MW than that released by HAase (FIG. 27a, lane 8), which was not seen in PBS HC-HA after NaOH treatment. These results showed that GnHCl HC-HA contained HC1 but the amount was less than PBS HC-HA. Similarly, not like PBS HC-HA, with anti-PTX3 antibody, GnHCl HC-HA only showed dimer PTX3 but no notable HMW PTX3 smear with or without HAase digestion (FIG. 27b, lanes 6 and 7). NaOH also resulted in a HMW and dimer PTX3 appearance. These results showed that GnHCl HC-HA contained less HMW PTX3 than PBS HC-HA.

[00667] Similar to HC1 blot, a higher MW dimer of PTX3 occurred after NaOH than HAase in GnHCl HC-HA. These results collectively indicated that NaOH released an ester bond that links HC1 to HA and could be associated with PTX3. Because GnHCl HC-HA had more HA content than PBS HC-HA, the GnHCl HC-HA complex contained HA that was not bounded by PTX3 or HC1 resulting in the decrease of actual HC-HA/PTX3 complex content in the purified products and less HC1 and HMW PTX3 in it. TSP-1 was not detected in PBS HC-HA with anti-TSP-1. It should be noted that TSP-1 was also not detected in GnHCl HC-HA. Because GnHCl extract contains

TSP-1, these results showed that TSP-1 dissociated by ultracentrifugation, so it was not present in GnHCl HC-HA.

[00668] Agrose gel showed abundant HA in GnHCl HC-HA

[00669] PBS HC-HA showed a “continuous HA smear from the top loading well to the bottom of agarose gel, and HAase completely abolished the HA smear (FIG. 28, lanes 3 and 4). GnHCl HC-HA showed a band in the loading well and a HA smear, which started from the 4,570 kDa location to the bottom of the agarose gel (FIG. 28, lane 5). HA in GnHCl HC-HA had a break between the loading well to the beginning of the HA smear, although its intensity was stronger than that in PBS HC-HA. Furthermore, HAase did not completely abolish the HA smear and HMW HA band in GnHCl HC-HA (FIG. 28, lane 6). The top fractions (1-6 fractions) from GnHCl extract after the 4th ultracentrifugation also showed the same HA smear pattern as the “bottom fractions” of GnHCl HC-HA (FIG. 28, lanes 7 and 8). These results showed that GnHCl HC-HA contained more HMW HA (with a MW smaller than PBS HC-HA) but lacked a portion of HMW HA smear that corresponded to the lack of HMW PTX3 smear in the Western blot of GnHCl HC-HA. This indicated that the missing HMW HA smear, which was present in PBS HC-HA, was at least partly formed by crosslinking of PTX3 and HC-HA, and that the HMW HA in the loading well of GnHCl HC-HA was complexed with components other than PTX3

[00670] GnHCl HC-HA contained some proteins that are not found in PBS HC-HA by Coomassie blue staining

[00671] FIG. 29A shows that bands in the top loading well, a major 140 kDa and some minor 70 kDa, doublet 55 kDa and 20 kDa bands, were present in AM GnHCl HC-HA and in the top fractions of GnHCl HC-HA but not in all other PBS HC-HA. This showed that AM GnHCl HC-HA contained some proteins that were absent in PBS HC-HA. In addition, a 90 kDa and 25 kDa band were also visualized in the GnHCl HC-HA top fractions. Because Western blot detected HC1 in PBS HC-HA, the HC1 should also be present in PBS HC-HA by Coomassie blue staining. The reason why it was not present in PBS HC-HA was due to the fact that the loaded HC-HA did not enter the gel due to over-loading. The 140 kDa showed as a broad band suggesting it contained sugar moieties. In addition, HAase did not affect these bands, showing that these species were dissociated by SDS and 2-ME.

[00672] Compared to AM GnHCl HC-HA, UC GnHCl HC-HA showed bands in the top loading well, a 90 kDa, 70 kDa, doublet 55 kDa, 35 kDa and 20 kDa bands (FIG. 29B, lanes 4 and 5). A faint 140 kDa band was also present in it. These bands were not affected by HAase. In addition, the top fractions of GnHCl HC-HA also showed a smear from top well to the site of 200 kDa which decreased after HAase treatment. All the bands were absent in UC PBS HC-HA. These results

showed that UC GnHCl HC-HA also contained some proteins that were absent in PBS HC-HA, and UC GnHCl HC-HA was different from AM GnHCl HC-HA regarding the protein bands they contained.

[00673] The above results showed that GnHCl HC-HA was different from PBS HC-HA from both AM and UC in the following aspects: (1) GnHCl HC-HA contained less HC1 and HMW PTX3 than PBS HC-HA (from Western blot) while like PBS HC-HA, TSG-6, HC2 and HC3 were also not present in GnHCl HC-HA. (2) GnHCl HC-HA contained more HMW HA but lacked a piece of HMW HA that corresponded to the HMW PTX3 smear in Western blotting shown by PBS HC-HA (from agrose gel). (3) GnHCl HC-HA contained some proteins mainly with MW 140 kDa that were not found in PBS HC-HA (from Coomassie blue staining gel).

[00674] In summary, GnHCl extracted more HA and proteins from AM and UC tissue, resulting in a higher HA/protein ratio compared to PBS extract. More HC-HA complex (according to HA content) was purified from the GnHCl extract for both AM and UC. GnHCl HC-HA contained HC1 and HMW PTX3 but much less than in PBS HC-HA for both AM and UC. GnHCl HC-HA lacked a species of HMW HA smear in the agrose gel that corresponded to the HMW PTX3 smear in Western blotting shown by PBS HC-HA. GnHCl HC-HA contained some proteins that are not found in PBS HC-HA.

Example 30: Determination of the identity of unknown protein bands in GnHCl HC-HA complex purified from AM and UC

[00675] This example determined the identify of unknown bands in GnHCl HC-HA by running SDS-PAGE gels followed by either CB staining or Western blot analysis of GnHCl HC-HA from AM and UC with or without deglycosylation. The sample was lyophilized AM and UC 4x HC-HA (contained 30 μ g HA) from both PBS and GnHCl extracts. Lyophilized HC-HA were incubated with 50 μ l TFMS and 20 μ l anisole on ice for 3 h and neutralized with TFMS with 125 μ l N-ethylmorpholine. Samples were precipitated with 5-10 volumes of acetone overnight at -20 C or for 1 h at -80 C. Samples were centrifuged and the dried pellet was dissolved in SDS sample loading buffer for electrophoresis. Enzymatic deglycosylation with keratinase (Endo- β -galactosidase) was performed to remove keratan sulfate chain and N-linked oligosaccharides, or with Chondroitinase (Cabc) to remove chondroitin sulfate chain. HC-HA (contained 30 μ g HA) was incubated with 0.1 U/ml keratinase in 50 mM sodium acetate, pH 5.8, at 37 C for 2h, or incubated with 5 U/ml Cabc in PBS at 37 C for 2h. An SDS-PAGE gel was run to test for CB staining, followed by Western blot analysis.

[00676] Keratan sulfate and osteoadherin were present in AM GnHCl HC-HA but not in PBS HC-HA.

[00677] Western blot analysis was performed. The results are shown in FIG. 30.B and C. Western blot with anti-keratan sulfate antibody showed a broad 70 kDa (60-80 kDa) band in AM GnHCl HC-HA with or without HAase digestion (FIG. 30B, lanes 6-8), but not in PBS HC-HA, which showed that this ~70 kD keratin sulfate proteoglycan was responsible for the positive immunostaining shown in FIG. 30D. This 70 kD band corresponded to the same band noted in GnHCl HC-HA with or without HAase treatment shown in Coomassie Blue stained gel (FIG. 30A).

[00678] To further determine if this 70 kD keratin sulfate proteoglycan is a SLRPs, anti-lumican, anti-fibromodulin, and anti-osteoadherin antibodies were used in the Western blot. The anti-osteoadherin antibody recognized a 60 kD band, but not a 70 kD band in GnHCl HC-HA with or without HAase digestion (FIG. 30C, lanes 6-8), but not in PBS HC-HA. Osteoadherin with keratin sulfate chain has a molecular mass of ~80 kD, while its non-keratin sulfate protein is ~60 kD. A keratin sulfate band in the size of 60 kD was detected, but only in a broad size of 70 kD, which showed that the 60 kD band detected by anti-osteoadherin was non-keratan sulfate osteoadherin. AM GnHCl HC-HA contained non-keratan sulfate osteoadherin that tightly associated with HC-HA and withstood 4 times ultracentrifugations in the presence of 6M GnHCl and cesium chloride. But, it was released by SDS and 2-ME in the sample buffer. The results also showed that the 70 kD keratin sulfate proteoglycan was not osteoadherin. There was no lumican and fibromodulin detected in GnHCl HC-HA, which showed that the 80 kD proteoglycan is neither lumican nor fibromodulin.

[00679] Deglycosylation and analysis of AM GnHCl HC-HA

[00680] HC-HA was deglycosylated by TFMSA to remove all glycans using keratinase and chondroitinase to remove specific glycans to see whether there were any changes to the 140 kD and ~80 kD bands in AM GnHCl HC-HA, and further determine whether the ~80 kD keratin sulfate proteoglycan was Keratocan, PRELP or Osteoglycan. As a first step confirming the effect of the above deglycosylation, Coomassie Blue staining was performed.

[00681] In FIG. 31A, the Coomassie Blue (CB) stained gel, AM GnHCl HC-HA (FIG. 31A, lane 2) showed the same bands of dominant 140 kDa, 70 kDa, doublet 50 kDa, 20 kDa and a weak 35 kDa band as well as a HMW band in the top of gel. K/C/H did not greatly affect these bands except for generating a major 80 kDa, a weak 100 kDa, and 30 kDa bands appearance (FIG. 31A, lane 3). This pattern was similar to that derived from the top fraction of AM GnHCl HC-HA under C/K/H (FIG. 31A, lane 6). These results showed that the 140 kDa, 70 kDa and 55 kDa bands were not keratan sulfated and/or chondroitin sulfated, and that there were other keratan sulfated and/or

chondroitin sulfated proteins in GnHCl HC-HA that were released as a major 80 kDa species after C/K/H. TFMSA treatment led to disappearance of all of the above bands except the 20 kDa band, and generated a clear new 50 kDa band and a HMW smear (FIG. 31A, lane 4). TFMSA/H made the smear disappear resulting in a new 25 kDa band but did not change the 50 kDa band (FIG. 31A, lane 5). This result showed 140 kDa, 70 kDa, 55 kDa and 80 kDa band were the same species of 50 kDa that with different amounts of glycan chains.

[00682] To determine whether the 50 kDa band originated from 60 kDa osteoadherin, Western blot analysis was performed with an anti-osteoadherin antibody. The result showed a 60 kDa species in AM GnHCl HC-HA (FIG. 31B, lane 4), consistent with the finding shown in FIG. 31C. C/K/H did not change its molecular mass (FIG. 31B, lanes 5 and 6), but TFMSA with or without HAase (T/H) treatment completely changed it into a 55 kDa species with less intensity (FIG. 31, lanes 7 and 8). The top fraction of AM GnHCl HC-HA with T/H showed a stronger band but a smaller MW without the intensity change compared to without T/H (FIG. 31B, lanes 9 and 10). These results further confirmed that AM GnHCl HC-HA contained osteoadherin, which was free of keratan sulfate and chondroitin sulfate. The reason why the intensity of osteoadherin band decreased after TFMSA treatment was due to (1) the protein was degraded by TFMSA; (2) it was blocked by other large amount of proteins with the same MW that were also released after TFMSA treatment. Osteoadherin was not detectable in PBS HC-HA without any treatment, but a doublet bands of 60 kDa appeared after TFMSA/HAase treatment, showing that PBS HC-HA contained a minute amount of osteoadherin, which was tightly bound to HA.

[00683] Western blot with an anti-decorin antibody showed a very strong broad 140 kDa species (80-160 kDa) and a weak doublet 50 kDa species in AM GnHCl HC-HA (FIG. 31C, lane 4; 31D, lanes 4 and 5) but not in PBS HC-HA (FIG. 31C, lane 2; 31D, lanes 2 and 3). The broad 140 kDa species corresponded to decorin with one chondroitin sulfate or dermatan sulfate chain and different number of glycans while doublet 50 kDa species likely corresponded to the less glycosylated decorin. Because HAase did not affect decorin species, it showed they can be released by SDS and 2-ME. The above notion was confirmed by C/H, which increased a 70 kDa species (FIG. 31C, lane 5), and by C/H/K, which gave the same result (FIG. 31B, lane 6). Hence, the 70 kDa species was the chondroitin-free decorin. This 70 kDa species was a minor component because the major broad 140 kDa species was not greatly changed by either C/H or C/H/K. TFMSA treatment completely deleted the broad 140 kDa species meanwhile gave rise to a major 43 kDa species that corresponded to deglycosylated decorin core protein, and minor 95 kDa, 80 kDa and a weak 30 kDa species (FIG. 31C, lane 7). TFMSA/H treatment showed the same species pattern as TFMSA alone except that the intensity of all these species was enhanced, showing that decorin was

tightly bound to HA. TFMSA/H also resulted in a release of a faint 43 kDa species from PBS HC-HA, showing that AM PBS HC-HA also contained a minute amount of decorin that was tightly bound to HA. The top fraction of AM GnHCl HC-HA with or without TFMSA/H showed the same pattern as the bottom fraction with the intensity stronger than the latter (FIG. 31C, lanes 9 and 10), which showed that the top fraction also contained abundant decorin.. The above results showed that the major 140 kDa, 70 kDa and doublet 50 kDa species in CB staining gel are formed by decorin via CS and mostly non-CS and non-KS.

[00684] Unlike decorin, Western blot with an anti-biglycan antibody showed a HMW smear with one strong area at 400 kDa, and a weak 45 kDa species in AM GnHCl HC-HA with or without HAase (FIG. 31E lane 4; 2F lanes 4 and 5) but not in PBS HC-HA (FIG. 31D, lane 2; F, lanes 2 and 3). The 45 kDa species corresponded to a biglycan core protein, while the HMW smear was glycosylated biglycan with two chondroitin sulfate or dermatan sulfate chains. HAase intensified the 400 kDa area with less HMW smear above 400 kDa, which showed some biglycan were also bound to HC-HA. C/H or C/K/H did not greatly change the HMW smear and 45 kDa species, but increased a 70 kDa species (FIG. 31E, lanes 5 and 6) that was likely the chondroitin-free biglycan. Because the amount of 70 kDa species was very small and the major HMW smear was not greatly changed by the above treatments, most biglycans in AM GnHCl HC-HA were not associated with CS or KS. TFMSA treatment completely deleted the HMW smear and gave rise to a major 45 kDa species that corresponded to deglycosylated decorin core protein, and a weak 95 kDa, 80 kDa and 30 kDa species (FIG. 31E, lane 7), which suggested the existence of biglycan in AM GnHCl HC-HA. The 95kDa and 80 kDa species were partly deglycosylated biglycan, while the 30 kDa species was degraded biglycan. TFMSA together with HAase treatment showed the same species pattern as TFMSA alone except that the intensity of all these species enhanced, which suggested that biglycan was also tightly bound to HA. The top fraction of AM GnHCl HC-HA with or without TFMSA/HAase showed the same species pattern as the bottom fraction with the intensity stronger than the latter (FIG. 31E, lanes 9 and 10), which showed the top fraction also contained abundant biglycan. Western blot analysis with an anti-keratan sulfate antibody showed the presence of the 70 kDa keratan sulfated protein in AM GnHCl HC-HA with or without keratinase or chondroitinase treatment without shifting the molecular size (FIG. 31G), which suggested that keratinase did not completely eliminate the keratan sulfate or the amount of KS was minute in this species. Western blot with anti-PTX3 antibody showed an increased HMW PTX3 smear which was shown in AM GnHCl HC-HA with K and even more with K/H (FIG. 31H, lanes 6 and 8) compared to that with or without HAase alone digestion (FIG. 31G, lanes 4 and 5). Chondroitinase had no such effect, which indicated that some KS-containing species were bound to PTX3 in GnHCl HC-HA. The

same results were also obtained from Western blot analysis of UC GnHCl HC-HA with or without keratinase digestion (see below FIG. 32G). Western blots confirmed that there was no Fibromodulin, Lumican, Keratocan, PRELP, Osteoglycin, epiphygan, Periostin and TSG-6 as well as Bikunin in AM GnHCl HC-HA.

[00685] In summary, AM GnHCl HC-HA contained abundant decorin and biglycan that were bound to HC-HA, but PBS HC-HA contained only faint decorin and no biglycan. AM GnHCl HC-HA contained osteoadherin and keratan sulfate-containing species, while PBS HC-HA did not. A very small amount of decorin and biglycan in AM GnHCl HC-HA contained chondroitin sulfate chain.

[00686] Deglycosylation and analysis of UC GnHCl HC-HA showed abundant present of Decorin and biglycan in UC GnHCl HC-HA but not in PBS HC-HA. Keratan sulfate, osteoadherin and bikunin were also present in UC GnHCl HC-HA.

[00687] CB staining (FIG. 32A) showed the same bands of 160 kDa, 90 kDa, 70 kDa, doublet 50 kDa, 35 kDa and 20 kDa bands in the top loading well in UC GnHCl HC-HA (FIG. 32A, lane 4). C/H or C/H/K did not greatly affect these bands except resulting in the appearance of a major 80 kDa and a weak 30 kDa band (FIG. 32A, lanes 5 and 6). TFMSA treatment decreased all above bands except the 20 kDa band but increased a major 50 kDa band, a minor 80 kDa band and a HMW smear (FIG. 32A, lane 7). TFMSA/H made the smear and 80 kDa band disappear but resulted in a weak 25 kDa band that appeared and decreased the intensity of the newly formed 50 kDa band (FIG. 32A, lane 8). These results are similar to that obtained from AM GnHCl HC-HA (FIG. 31A), showing that UC GnHCl HC-HA had a similar constitution with AM GnHCl HC-HA. The top fraction of UC GnHCl HC-HA with or without TFMSA/H showed the same pattern as the bottom fraction (FIG. 32A, lanes 9 and 10), indicating they had the same components as the bottom fraction. UC PBS HC-HA without deglycosylation only showed a HMW band in the loading well and below it, as well as a 20 kDa band. TFMSA/H deleted the HMW band but mainly increased a 50 kDa band beside a weak 80 kDa and a 25 kDa band, suggesting that UC PBS HC-HA contained some glycosylated protein that only was released by complete deglycosylation and HA degradation.

[00688] Western blot analysis with an anti-decorin antibody showed a broad 160 kDa species in UC GnHCl HC-HA with or without HAase (FIG. 32B, lanes 4 and 5) but not in PBS HC-HA (FIG. 32B, lanes 2 and 3), indicating that UC GnHCl HC-HA, like AM GnHCl HC-HA, also contained decorin. Its molecular mass was different than that in AM GnHCl HC-HA due to a different level of glycosylation. HAase greatly decreased the 160 kDa species, showing that it was bound to HC-HA. Keratinase with or without HAase also decreased the intensity of 160 kDa species, indicating it also contained some KS. Notably, C with or without HAase digestion led to

the 160 kDa species and top well species disappearing, but gave rise to a strong 50 kDa and 90 kDa species as well as a HMW smear, showing the decorin in UC GnHCl HC-HA was mainly chondroitin sulfate in comparison to that in AM GnHCl HC-HA, where less of them contained chondroitin sulfate chain. These results further confirmed that UC GnHCl HC-HA contained decorin, and that decorin in UC differed from AM in (1) glycosylation, (2) type of glycosaminoglycan is attached, and (3) the overall amount.

[00689] Western blot with anti-biglycan antibody showed a strong HMW species in top well, a HMW smear at 400 kDa area and 140 kDa area, and a 45 kDa species in UC GnHCl HC-HA with or without HAase (FIG. 31C, lanes 4 and 5) but not in PBS HC-HA (FIG. 31C, lanes 2 and 3). HAase intensified the 400 kDa area without affecting the HMW species in the well, suggesting that some species was tightly bound to HA. Keratinase with or without HAase did not greatly change above species except decreasing the 45 kDa species (FIG. 32C, lanes 6 and 8), suggesting that the 45 kDa species contained KS but most others do not. Chondroitinase alone abolished the HMW species in top well and reduced the intensity of 400 kDa area, but increased a strong broad 50 kDa species, 100 kDa species and 28 kDa species with smear between them (FIG. 32C, lane 7). Chondroitinase plus HAase had the same results with the whole smear more intensified and the 28 kDa species disappeared (FIG. 32C, lane 9). These results suggested that similar to decorin, biglycan in UC GnHCl HC-HA mainly brings chondroitin sulfate chain. This finding is different from that in AM HC-HA where less was chondroitin sulfated. Also most biglycans form HMW complex in HC-HA with some bound to HC-HA.

[00690] Western blot with anti-bikunin antibody showed a broad 35 kDa band in UC GnHCl HC-HA with or without HAase digestion but not in PBS HC-HA (FIG. 32D, lanes 4 and 5). The 35 kDa band corresponds to the MW of native bikunin. Keratinase with or without HAase sharpened this 35 kDa band sharp but did not change its MW (FIG. 32D, lanes 6 and 8), while chondroitinase with or without HAase digestion changed the 35 kDa bikunin into a 25 kDa core bikunin (FIG. 32D, lanes 7 and 9), further confirming the existence of bikunin in UC GnHCl HC-HA, and contained CS as reported. Because a HMW smear also formed after chondroitinase treatment, it suggested that bikunin is tightly bound to HC-HA via CS. These results were different from AM GnHCl HC-HA that did not contain bikunin.

[00691] Western blot with anti-PTX3 antibody showed a increased HMW PTX3 smear in UC GnHCl HC-HA after H, K, C, and especially with K/H (FIG. 32E, lanes 6 and 8) compared to that only with or without HAase digestion (FIG. 32E, lanes 4 and 5). These results confirmed the existence of HMW PTX3 in UC GnHCl HC-HA, and its strong binding in GnHCl HC-HA. Furthermore, such a strong binding was further helped by the presence of KS-containing species, of

which the identity remains unclear. It also explained that our previous data (without enzymatic digestion) might have underestimated the amount of HMW PTX3 in UC GnHCl HC-HA.

[00692] Western blot with anti-keratan sulfate antibody showed a HMW species in the well and a faint 55 kD band in UC PBS HC-HA. HAase did not change this band but made a 60 kDa band more obvious (FIG. 32F, lanes 2 and 3). However, a 140 kD band was recognized by anti-keratan sulfate antibody in UC GnHCl HC-HA with or without HAase digestion (FIG. 32F, lanes 4 and 5). Keratinase with or without HAase treatment did not delete the 140 kDa band, but increased mainly a 35 kDa band and several other bands between them including a 60 kDa and a 55 kDa bands seen in PBS HC-HA (FIG. 32F, lanes 6 and 8).

[00693] Chondroitinase with or without HAase treatment also did not delete the 140 kDa band, but increased a 90 kDa band as well as a 60 kDa and a 55 kDa bands seen in PBS HC-HA (FIG. 32F, lanes 7 and 9). Chondroitinase treatment also resulted in a HMW smear appearance that decreased after HAase treatment, suggesting UC GnHCl HC-HA contained abundant chondroitin sulfated proteins beside keratan sulfated proteins. It was clear that the keratan sulfated protein in UC GnHCl HC-HA (140 kDa) had a different MW with that in AM GnHCl HC-HA (~80 kDa), and maybe it was due to the different amount of glycan in the chain.

[00694] With anti-osteoaderin antibody a major 60 kD band and a weak 80 kD band were detected in UC GnHCl HC-HA (FIG. 32G, lanes 4) but not in PBS HC-HA. These two bands were not obviously affected by Keratinase or chondroitinase or HAase. The 80 kD should be keratin sulfated osteoaderin, while ~60 kD should be non-keratin sulfated osteoaderin. The results suggested that UC GnHCl HC-HA contained both keratin sulfated and non-keratin sulfated osteoaderin.

[00695] In summary, no Fibromodulin, Lumican, Keratocan, PRELP, Osteoglycin, epiphygan, Periostin and TSG-6 were detected in UC GnHCl HC-HA. UC GnHCl HC-HA contained Decorin, biglycan, Osteoaderin, keratan sulfate and Bikunin. Biglycan and decorin were abundant in it, while PBS HC-HA did not contain an abundance of these species.

[00696] Summary

[00697] We purified HC-HA complex by 4x ultracentrifugation from both PBS and GnHCl extract of AM and UC. The HC-HA purified from GnE was quite different from the HC-HA purified from PBS extract in yield and chemical constitution (see Table 1). In quantity, HC-HA purified from GnE contained more HA than that purified from PBS. In chemical constitution: GnHCl HC-HA contained more HMW HA (with a MW slightly smaller than PBS HC-HA) but lacked a piece of HMW HA that corresponded to the HMW PTX3 smear in Western blotting shown by PBS HC-HA (from agarose gel). With or without HAase digestion GnHCl HC-HA contained less HCl and HMW

PTX3 than PBS HC-HA, but after keratinase plus HAase digestion, more PTX3 was detected (from Western blot), suggesting the HMW PTX3 was tightly bound to keratan sulfated proteins in GnHCl HC-HA. Neither PBS HC-HA nor GnHCl HC-HA contained TSG-6, HC2 and HC3. Bikunin was present in UC GnHCl HC-HA but not in UC PBS HC-HA and both AM PBS and GnHCl HC-HA. AM GnHCl HC-HA contained abundant Decorin, relatively more so than UC GnHCl HC-HA. Both AM and UC PBS HC-HA contained a faint amount of decorin. AM and UC GnHCl HC-HA contained abundant Biglycan, especially in UC GnHCl HC-HA. No biglycan was present in PBS HC-HA. AM and UC GnHCl HC-HA contained Osteoadherin. AM and UC PBS HC-HA did not contain Biglycan, keratan sulfate-containing species, Fibromodulin, Lumican, Keratocan, PRELP, Osteoglycin, epiphycan, Periostin, and Osteopondin, TSP-1. There was no Fibromodulin, Lumican, Keratocan, PRELP, Osteoglycin, epiphycan, Periostin, and Osteopondin, TSP-1, Asporin in AM and UC GnHCl HC-HA. GnHCl HC-HA contained visible protein bands mainly with MW 200 kDa, 80 kDa, and 60 kDa that were not found in PBS HC-HA (from Coomassie blue staining gel).

Table 3. Summary comparison of 4x HC-HA complex purified from PBS and GnHCl extract.

Components	4 th PBS HC-HA		4 th GnHCl HC-HA	
	AM	UC	AM	UC
Wet weight (g) from one placenta	23.8 ± 4.7	48.2 ± 17.2	24.6 ± 9.4	33.5
Total HA content (μg)	208 ± 100	2800 ± 1697	872 ± 378	6561
HC1	+++	++	+	+
PTX3	+++	++	++ by keratinase	++ by keratinase
TSG-6, HC2, HC3	-	-	-	-
Bikunin	-	-	-	+
Decorin	+/-	-	+++++	++
Biglycan	-	-	+++++	+++
Keratan sulfate	-	-/+	++	+
Osteoadherin	-	-	++	+
TSP-1*	-	-	-	-
Osteopondin	-	-	-	-
Asporin	-	-	-	-
Fibromodulin,	-	-	-	-
Lumican	-	-	-	-
Osteoglycan	-	-	-	-
Keratocan	-	-	-	-
Testican	-	-	-	-
Epiphycan	-	-	-	-
Periostin	-	-	-	-

*Note that this information is not the same as extract, suggesting that there is TSP-1 that is dissociable by ultracentrifugation.

Table 4. Summary comparison of the extract of PBS and GnE sequentially obtained from AM and UC.

	PBS extract		GnHCl extract	
	AM	UC	AM	UC
HA content ($\mu\text{g/ml}$)	<		<	
HCl	>		<	
PTX3	<		>	
TSG-6	+	unknown	+	-
TSP-1	-	-	+	+

Example 31: Constitutive Expression of PTX3 by Human Amniotic Membrane Stromal Cells Leads to HC-HA/PTX3 Complex Formation

[00698] In this example, PTX3 expression in HC-HA purified from AM and its effect on HC-HA/PTX3 complex formation in AM was examined.

[00699] Experimental Procedures

[00700] 1. Materials

[00701] Guanidine hydrochloride, cesium chloride, EDTA, anhydrous alcohol, potassium acetate, sodium acetate, sodium chloride, sodium hydroxide, Tris base, Triton X-100, 3-(N,N-Dimethyl palmityl ammonio) propanesulfonate (Zwittergent³⁻¹⁶), protease inhibitor mixture (including 4-(2-aminoethyl)- benzenesulfonyl fluoride hydrochloride, aprotinin, bestatin hydrochloride, E-64, leupeptin, and pepstatin A) and phenylmethanesulfonyl fluoride were obtained from Sigma-Aldrich (St. Louis, MO). *Streptomyces* hyaluronidase (HAase) and biotinylated HA-binding protein (HABP) were from Seikagaku Biobusiness Corporation (Tokyo, Japan). Dulbecco's modified Eagle's medium, Ham's F12 nutrient mixture, fetal bovine serum, Hank's balanced salt solution, gentamicin, amphotericin B and RIPA buffer were purchased from Invitrogen (Grand Island, NY). Slide-A-Lyzer Dialysis Cassettes (3.5K MWCO) was from Fisher Scientific (Pittsburgh, PA). BCA Protein Assay Kit was from Pierce (Rockford, IL). HA Quantitative Test Kit was from Corgenix (Westminster, CO). 4–15% gradient acrylamide ready gels and nitrocellulose membranes were from Bio-Rad (Hercules, CA). IgA was prepared in our laboratory from human plasma, according to the published method (1,38). PTX3 mAb (MNB4) and pAb were from Enzo Life Sciences, Inc. (Plymouth, PA). Recombinant human TNF, recombinant human Pentraxin 3/TSG-14 and human/mouse TSG-6 MAb (MAB2104) were from R&D Systems (Minneapolis, MN). Mouse anti-human ITIH1 polyclonal antibody against full length ITIH1 and rabbit anti-human ITIH2 polyclonal antibody against amino acids 124-321 were from Abcam Inc. (Cambridge, MA).

HiPerFect Transfection Reagent and RNeasy Mini RNA isolation Kit was from QIAGEN (Valencia, CA). Small interfering RNA (siRNA) oligonucleotides for targeting endogenous human PTX3 (ACACUUGAGACUAAUGAAAGAGAGA) and non-targeting siRNA control oligonucleotides (scrambled RNA) siRNA were from OriGene Technologies, Inc (Rockville, MD). Western LightingTM Chemiluminescence Reagent was from PerkinElmer, Inc. (Waltham, MA). The ultracentrifuge (LM8 model, SW41 rotor) was from Beckman Coulter, Inc. (Fullerton, CA).

[00702] 2. Cell Cultures and Agarose Overlay

[00703] Human tissue was handled according to the Declaration of Helsinki. The fresh human placenta was obtained from healthy mothers after elective cesarean deliveries in the Baptist Hospital (Miami, FL) via an approval (Protocol Number 03-028) by the Baptist Health South Florida Institutional Review Board. Primary human AM epithelial and stromal cells (designated as AMEC and AMSC, respectively) were isolated from fresh placenta as previously described (Chen et al. (2007) *Stem Cells*. 25: 1995-2005; Li et al. (2008) *J. Cell. Physiol.* 215:657-664) and cultured in supplemental hormonal epithelial medium (SHEM, which consisted of DMEM/F12 (1:1, v/v), 5% (v/v) FBS, 0.5% (v/v) dimethyl sulfoxide, 2 ng/ml EGF, 5 µg/ml insulin, 5 µg/ml transferrin, 5 ng/ml sodium selenite, 0.5 µg/ml hydrocortisone, 0.1 nM cholera toxin, 50 µg/ml gentamicin, 1.25 µg/ml amphotericin B) (Chen et al. (2007) *Stem Cells* 25:1995-2005; Chen et al. (2011) *Tissue Eng Part C Methods* 17:537-548) under a humidified atmosphere of 5% CO₂ at 37 °C. The culture medium was changed every 2 days. Cells at 80% confluence were switched to DMEM/F12 containing 0.5% FBS for 48h to let the cells become quiescent and then treated with 20 ng/ml TNF or 20 ng/ml IL-1β for 4h or 24 before subject to RT-PCR and Western blot analysis. For agarose overlay culture, AMEC, AMSC and HSF are seed in 12-well (1x10⁵ cells/well) and 6-well (2x10⁵ cells/well) plate at a density of 2x10⁴ /cm² in SHEM. The medium are changed at day 1 to serum-free SHEM containing 5% KnockOut serum replacement and 1mM 2-phospho-L-ascorbic acid and incubated for another 2 days. After removal of the medium, 3% agarose (low melting-type, Type VII, Sigma, A9045) in DMEM/F12 with 1mM 2-phospho-L-ascorbic acid was over-layered at 1 ml or 0.5 ml to achieve a 1mm thick gel layer at room temperature for 5-10 min before adding 3 ml or 1.5 ml of serum-free SHEM media with or without 5 ng/ml TNF per 6 or 12 well plate, respectively. Cells were harvested without intervening media changes on Days 5.

[00704] 3. siRNA Transfection

[00705] AMEC and AMSC were cultured in SHEM in six-well plates till 80% confluence. Cells were switched to DMEM/F12 with 0.5% FBS for 48h and were transfected with PepMuteTM siRNA Transfection Reagent with 100 nM of PTX3 siRNA or scrambled (sc) RNA. After 48 h, cells were harvested and subjected to RT-PCR and Western blot analysis.

[00706] 4. Purification of HC-HA Complex from AM and Serum-free Cultures by Ultracentrifugation

[00707] HC-HA complex was purified from AM and cell cultures as previously described (He et al. (2009) *J. Biol. Chem.* 284: 20136-20146; Yoneda et al. (1990) *J. Biol. Chem.* 265:5247-5257; He et al. (2008) *Invest. Ophthalmol. Vis. Sci.* 49:4468-447532). In brief, cryopreserved human AM, obtained from Bio-tissue, Inc. (Miami, FL), was sliced into small pieces, frozen in liquid nitrogen, and ground to fine powder by a BioPulverizer. The powder was mixed with cold phosphate-buffered saline (PBS) buffer at 1:1 (g/ml). The mixture was kept at 4 °C for 1 h with gentle stirring and then centrifuged at 48,000 g for 30 min at 4 °C. The supernatant (designated as AM extract) was then mixed with a 8 M guanidine HCl/PBS solution (at 1:1 ratio of v/v) containing 10 mM EDTA, 10 mM aminocaproic acid, 10 mM N-ethylmaleimide, and 2 mM PMSF and adjusted to a density of 1.35 g/ml (AM extract) or 1.40 g/ml (cell extract) with cesium chloride, respectively, and subjected to isopycnic centrifugation at 35,000 rpm, 15 °C, for 48 h. The resultant density gradients were fractioned into 12 tubes (1 ml/tube), in which the contents of HA and proteins were measured using HA Quantitative Test Kit and BCA Protein Assay Kit, respectively. Fractions from the first ultracentrifugation, which contained most HA were pooled, brought to a density of 1.40 g/ml by addition of CsCl, ultracentrifuged, and fractionated in the same manner as described above. Fractions from the second ultracentrifugation, which contained HA but no detectable proteins, were pooled and continue to the third and the forth ultracentrifugation in a density of 1.42 g/ml by addition of CsCl. Fractions from the second and the forth ultracentrifugation were dialyzed in distill water and then precipitated twice with 3 volumes of 95% (v/v) ethanol containing 1.3% (w/v) potassium acetate at 0 °C for 1 h. After centrifugation at 15,000 g, the pellet was briefly dried by air, stored at -80 °C and designated as AM 2nd HC-HA and 4th HC-HA, respectively.

[00708] 5. Immunostaining

[00709] Human fetal membrane containing AM and chorion section as well as cell cultures with or without an agarose overlay were fixed with 4% paraformaldehyde at room temperature for 15 min, permeabilized with 0.2% (v/v) Triton X-100 in PBS for 20 min. After blocking with 0.2% (w/v) bovine serum albumin in PBS for 1 h, sections were incubated with biotinylated HABP (for HA, 5 µg/ml), anti-PTX3, anti-HC1 or anti-HC2 antibodies (all diluted 1:200 in blocking solution) overnight in a humidity chamber at 4 °C. After washing with PBS, they were incubated with Alexa Fluor 488 Streptavidin (for HA, diluted 1:100), or respective secondary antibodies (i.e., Alexa Fluor 488 anti-mouse IgG, or Alexa Fluor 555-conjugated anti-rat IgG,) for 1 h at room temperature. Isotype-matched nonspecific IgG antibodies were used as a control. Alternatively, sections were treated with 50 U/mL *Streptomyces* HAase at 37 °C for 4 h before fixation. Nuclei

were stained by Hoechst 33342, and images were obtained using a Zeiss LSM700 confocal laser scanning microscope (Zeiss, Germany).

[00710] 6. Real-time PCR

[00711] Total RNA was extracted from cell cultures using RNeasy Mini RNA isolation Kit. The cDNA was reverse-transcribed from 1 µg of total RNA using a Cloned AMW First-Strand cDNA synthesis kit with oligo(dT) primer. First-strand cDNAs were amplified by qPCR using AmpliTaq Gold Fast PCR Master Mix and the specific PTX3 primers (46-48). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene expression was used to normalize the amounts of the amplified products.

[00712] 7. Western blot

[00713] Culture supernatants were collected, and cell lysates were obtained by washing cells six times with cold PBS followed by incubating in RIPA buffer at 4 °C for 1 h with gentle stirring and centrifugation at 14,000 g for 30 min at 4 °C. Protein concentrations in culture supernatants and cell lysates were quantified with a BCA Protein Assay Kit. Samples were incubated in 50 mM NaOH for 1 h at 25 °C or dissolved in 0.1 M sodium acetate buffer (pH 6.0) and incubated at 60 °C for 1 h with or without 20 units/ml of *Streptomyces* HAase. They then were resolved by SDS-PAGE on 4-15 % (w/v) gradient acrylamide ready gels under denaturing and reducing conditions and transferred to a nitrocellulose membrane. The membrane was then blocked with 5% (w/v) fat-free milk in 50 mM Tris-HCl, pH 7.5, buffer containing 150 mM NaCl and 0.05% (v/v) Tween-20 followed by sequential incubation with different primary antibodies followed by their respective HRP-conjugated secondary antibodies. Immunoreactive proteins were visualized by Western LightingTM Chemiluminescence Reagent.

[00714] Results

[00715] Positive PTX3 Staining in AM epithelium and the Compact Stroma

[00716] Immunofluorescence staining using anti-human PTX3 antibody was performed on cross-sections of fresh human fetal membrane, which consisted of a layer of epithelium and an avascular stroma, which can be further subdivided into a compact layer and a spongy layer, and the subjacent cell-rich chorion (FIG. 33, phase). Positive PTX3 staining was found in the apical surface of the epithelium and the compact stroma. In contrast, PTX3 staining was markedly attenuated in the spongy stroma and the chorion (FIG. 33, PTX3). HAase digestion did not enhance PTX3 staining in the latter, suggesting that the weak PTX3 staining in these two areas was not due to the masking effect. Strong positive HA immunostaining was found in AM stroma and relatively weak staining in AM epithelium using a biotinylated HABP (FIG. 33, HA), while a weak staining of HA was noted in the upper layer of the chorion subjacent to the AM stroma but strong staining in the lower

layer of the chorion. This staining disappeared when the tissue section was pre-digested by HAase (FIG. 33, HA (+HAase)) suggesting that HA staining was specific. Immunostaining of individual HC, also revealed a positive staining in AM epithelium, stromal cells and matrix, and chorion (FIG. 33, HC1 and HC2). These results suggested the presence of PTX3 in AM predominantly in the compact stroma and the epithelium.

[00717] Presence of PTX3 in AM Soluble Extract and Purified HC-HA Complex

[00718] To investigate further the presence of PTX3 in AM, we first performed Western blotting analyses of AM extract obtained by an isotonic salt buffer before and after 50mM NaOH treatment to cleave ester bonds. Recombinant PTX3 appeared as 45 kDa, 90 kDa, 180 kDa and HMW species (FIG. 34, lane 2). Soluble AM extract revealed 45 kDa and a HMW species at the bottom of the loading well (mostly not entering the gel) (FIG. 34, lane 3). NaOH treatment did not affect the 45 kDa species, but completely eliminated the HMW species, resulting in a HMW smear of PTX3 (FIG. 34, lane 4). These results suggested that PTX3 was present as a monomer and a HMW complex in the AME. Because the latter could be dissociated into a HMW smear by NaOH that can cleave off the ester covalent linkage between HC-HA, the HMW PTX3-containing species in the loading well might be associated with HC-HA.

[00719] To further confirm whether PTX3 was associated with AM HC-HA complex, we purified HC-HA complex by two and four successive ultracentrifugations from the AM soluble extract as previously reported (He et al. (2009) *J. Biol. Chem.* 284:20136-20146.; Zhang et al. (2012) *J. Biol. Chem.* 287:12433-12444) and performed Western blotting analyses with or without HAase digestion. Contrasted to the monomer found in soluble AME, a 90 kDa species corresponding to the size of the native PTX3 dimer was shown in AM 2nd and 4th HC-HA complex besides a HMW band at the bottom of the loading well (FIG. 34, lanes 5 and 7). In addition, a HMW smear was also seen weak in 2nd HC-HA and strong in 4th HC-HA complex. After HAase treatment, the 90 kDa dimer remained in both HC-HA complexes, but the HMW smear was intensified in 4th HC-HA and increased in 2nd HC-HA with the disappearance of HMW band in gel top (FIG. 34, lanes 6 and 8) similar to the results seen in AME. The existence of 90 kDa PTX3 dimer in HC-HA complex with or without HAase was found to be caused by dissociation from HC-HA caused by 2-ME as elimination of 2-ME resulted in the absence of this 90 kDa species (not shown). These results suggested that HC-HA complex contained PTX3 that was bind to HC-HA to form the HC-HA/PTX3 complex despite four times of ultracentrifugation.

[00720] Western blot analysis with anti-HC1 antibody showed the presence of HC-HA complex as a HMW species at the bottom of the loading well that disappeared upon HAase digestion (FIG. 34, lanes 10-13), and the presence of HC1 in the HC-HA complex that was released from HC-HA

complex after HAase digestion (FIG. 34, lanes 11 and 13). We did not detect HC2 nor TSG-6 (not shown). These results collectively confirmed that HC-HA purified from AM only contained HC1. We also noted a prime difference between 2nd and 4th HC-HA complexes, that is, a free 80 kDa HC1 band was detected only in the 2nd HC-HA complex (FIG. 34, lane 11), but not 4th HC-HA complex, suggesting that the latter did not contain free HC1. The presence of HA in 4th HC-HA were verified by agarose gel electrophoresis to display as a continuous HA smear from the top loading well to the bottom of the gel, and that such a smearing was resolved by HAase digestion.

[00721] Expression of PTX3 mRNA and Protein by AMEC and AMSC

[00722] We then determined that PTX3 was synthesized by AM epithelial and stromal cell cultures. We cultured these cells as reported (Chen et al. (2007) *Stem Cells* 25(8):1995-2005; Li et al. (2008) *J Cell Physiol.* 215(3):657-64; Zhang et al. (2012) *J. Biol. Chem.* 287:12433-12444) and extracted total RNA for RT-PCR and proteins for Western blot analysis, and compared them to human skin fibroblasts (HCF), which were reported to express PTX3 mRNA and protein only under the stimulation of pro-inflammatory cytokine such as TNF and IL-1. As expected, qRT-PCR results showed that expression of PTX3 transcript was low in resting HSF, but upregulated by TNF and IL-1 β (FIG. 35A). Although expression of PTX3 transcript in resting AMEC and AMSC was also low, it was dramatically elevated by TNF or IL-1 β (FIG. 35A). Western blotting analysis confirmed that PTX3 protein was low in lysates (45 kDa) and undetectable in media in resting HSF (FIG.35B, lane 2) but was detected in lysates but not media after addition of TNF or IL-1 β for 24 h. In contrast, PTX3 protein was detectable in resting AMEC and notably increased by TNF or IL-1 β in both lysates (45 kDa) and media (45 kDa and 90 kDa) (FIG.35B, lanes 5, 6 and 7), with TNF being more effective than IL-1 β . The same finding was noted in AMSC (FIG.34B, lanes 8, 9 and 10). To verify that the 75 kDa and 135 kDa bands in lysates and the 50 kDa band in media from all cells were nonspecific because these bands did not changes under TNF or IL-1 β , PTX3 siRNA transfection was performed and indeed downregulated the 45 kDa species in lysis and both 45 kDa and 90 kDa species but not these non-specific bands. (FIG. 35C). These results collectively suggested that PTX3 was indeed synthesized and secreted by resting AM cells and was further upregulated by proinflammatory stimuli.

[00723] Production of HC-HA/PTX3 Complex by AM Stromal Cells

[00724] Previous studies have shown that HC-HA (i.e. SHAP-HA) complex can be isolated from the cell layer of cultured mouse dermal fibroblasts in a medium supplemented with FBS, and that the isolated HC-HA contains both HC1 and HC2 of IaI derived from FBS (Yoneda et al. (1990) *J. Biol. Chem.* 265:5247-5257; Huang et al. (1993) *J. Biol. Chem.* 268:26725-26730). However, we reported that AM cells produce HC-HA by using their endogenously generated IaI (Zhang et al.

(2012) *J. Biol. Chem.* 287:12433–12444). Because AM cells synthesized PTX3 protein which was further increased by TNF and IL-1, we aimed to determine whether they produced HC-HA that also contained PTX3. We used HSF as a control, which expressed PTX3 only under pro-inflammatory stimuli, e.g. TNF and IL-1, in a serum-containing condition (Yoneda et al. (1990) *J. Biol. Chem.* 265:5247-5257; Huang et al. (1993) *J. Biol. Chem.* 268:26725-26730) (FIG. 35) and compared to AMECs and AMSCs cultured in both serum-free and serum-containing condition with or without TNF. We also overlaid 3% agarose over cell monolayers because this method has been found to entrap secreted procollagen at or near the cell surface, rather than into the media on keratocyte cultures (Hassell et al. (2008) *Experimental Eye Research.* 87:604-611; Etheredge et al. (2010) *Matrix Boil.* 29:519-524). After 5 days of overlay, HSF, AMSC and AMEC became more compact especially in the serum-containing condition (FIG. 36). The epithelial morphology became more distinct in AMEC.

[00725] We then determined whether 3% agarose overlay was also effective in entrapping secreted HA by measuring the HA concentration in culture media by HA ELISA assay. Without agarose overlay, the HA level was readily detectable in the serum-free medium of both AMSC and AMEC but not in that of HSF. TNF significantly increased the HA level of all three cell cultures (FIG. 37). The above pattern was further promoted in the serum-containing medium. Agarose overlay reduce the HA level more than 50% in both serum-free and serum-containing conditions among all three cultures. These results suggested that agarose overlay indeed reduced the HA level into culture medium.

[00726] To determine when secreted HA was indeed entrapped in the extracellular matrix after agarose overlay, we performed double immunostaining for PTX3/HA, HC1/HA, and HC1/PTX3 with biotin-labeled HABP, specific antibody to HC1 and HC2, and two different anti-PTX3 antibodies, i.e. MNB4 and biotin-labeled pAb, respectively. In the serum-free condition, positive HA staining was noted in the pericellular region in HSF, while PTX3 staining was negative (FIG. 38A). With TNF stimulation, positive PTX3 staining was observed in the cytoplasm (FIG. 38B), confirming inducible expression of PTX3 by TNF in HSF. TNF did not greatly increase the HA staining intensity but induced a cable-like structure (FIG. 38G) similar to what has been reported in cultured renal proximal tubular epithelial cells (Selbi et al. (2006) *Kidney International.* 70: 1287-1295). No colocalization of PTX3 with HA was observed, suggesting that PTX3 was not associate with HA in HSF after TNF stimulation. In contrast, positive HA staining was detected in resting AMSC as a fibrillar network on the cell surface and extracellular matrix as was colocalized with HA in the extracellular matrix (FIG. 38C and 6K). TNF further increased PTX3 staining intensity and the amount of HA fibrils (FIG. 38D and 38K).

[00727] In resting AMEC, positive HA staining was also found in extracellular spaces with some fibrillar appearance but only in sporadic areas where cells were not as compact (FIG. 38E and 38L). PTX3 and HA colocalization was also observed in AMEC. TNF treatment further increased PTX3 staining intensity and the amount of HA fibrils (FIG. 38F). These results further confirmed that PTX3 was constitutively expressed by AMSC and AMEC, and its expression can be further increased by TNF. Weak positive HC1 staining was observed only in some HSF with (FIG. 38G and 38J) or without (not shown) TNF treatment, and was colocalized with HA especially in HA cable-like structure (FIG. 38G). However, we did not note colocalization between HC1 and PTX3 (FIG. 38J). In contrast, strong positive HC1 staining was noted in AMSC and colocalized with HA on the cell membrane (FIG. 38H) and colocalized with PTX3 in the cytoplasm (FIG. 38K). AMEC also showed the strong positive HC1 staining and colocalization with HA in the cell membrane (FIG. 38I) and with PTX3 in the cytoplasm and the cell membrane (FIG. 38L). Collectively, these results suggested that HA-rich matrix was effectively trapped by agarose overlay in AMSC, AMEC and HSF, and contained both HC1 and PTX3 only in AMSC and AMEC in the serum-free condition, further confirming that such HC-HA/PTX3 complex was synthesized by endogenous I α I.

[00728] To further confirm the formation of HC-HA/PTX3 complex under agarose overlay, we extracted cell layer with 6M GnHCl and performed Western blot analysis in AMSC and HSF cultures under both serum-free and serum-containing condition with or without NaOH treatment. Serum-free HSF showed a 170 kDa and a 140 kDa species but not 45 kDa species corresponding to the control PTX3 with or without TNF stimulation and NaOH treatment, suggesting that these two bands were not specific (FIG. 38D, lane 2-5). Serum-containing HSF with TNF showed a faint 140 kDa species and some small MW species similar to those seen in serum-free HSF (FIG. 39, lanes 11 and 12). In contrast, serum-free AMSC showed a weak HMW PTX3 smear, a 90 kDa and a 45 kDa species (FIG. 39, lane 6), of which the later two corresponded to PTX3 dimer and monomer, respectively (FIG. 39, lane 2). These results were similar to those seen in AME (FIG. 34). The same non-specific 170 kDa species and some small molecular species as seen in serum-free HSF were also observed. NaOH treatment increased the HMW PTX3 smear but did not affect the PTX3 monomer and dimer (FIG. 39, lane 7) and other species. TNF increased the HMW PTX3 smear, PTX3 dimer and monomer as well as other species (FIG. 39, lane 8 and 9). Serum-containing AMSC with TNF showed strong HMW PTX3 smear, 90 kDa PTX3 dimer and 45 kDa PTX3 monomer (FIG. 39, lane 13). NaOH treatment greatly increased the HMW PTX3 smear and two species at 60 kDa and 50 kDa (FIG. 39, lane 14). Because NaOH breaks the ester bond between HC and HA leading to dissolution of HC-HA, these results suggested that HMW PTX3 was

released from HC-HA complex. Collectively, above results suggested that AMSC but not HSF produced HC-HA complex containing PTX3.

[00729] *In Vitro* Reconstitution of HC-HA/PTX3 Complex (rcHC-HA/PTX3)

[00730] To further confirm how HC-HA/PTX3 complex could be generated, we would like to reconstitute HC-HA/PTX3 complex *in vitro* with HA, TSG-6, I α I and PTX3. We first immobilized HA on plastic, and successfully added recombinant TSG-6, purified I α I or serum as a source of I α I. It has been reported that TSG-6 can form stable TSG-6/HA complex by binding to HA that is immobilized on a solid surface (Wisniewski et al. (2005) *J Biol Chem.* 280:14476-84), and that both free and HA-bound TSG-6 can transfer HCs from I α I to immobilized HA to form HC-HA (Colon (2009) *J Biol Chem.* 284:2320-31). Western blot using an anti-I α I antibody did not detect any species in control iHA alone as expected (FIG. 40A, lane 2). When I α I and TSG-6 were simultaneously added to iHA, a weak 220 kDa I α I, 85 kDa HC2, a strong ~80 kDa HC1 and a 50 kDa species were detected (FIG. 40A, lane 3), suggesting that both HC1 and HC2 were transfer to iHA in the presence of TSG-6 to form HC-HA. Comparison of the band intensity of HC1 to that of HC2 suggested that more HC1 were transferred to iHA than HC2 by TSG-6, resulting in a truncated 50 kDa species. When PTX3 were simultaneously with I α I and TSG-6 to iHA, the intensity of I α I species decreased but the HC1 intensity increased in a PTX3 dose-dependent manner (FIG. 40A, lanes 4-6), meanwhile HC2 was not detectable, suggesting that PTX3 preferentially promoted transfer of HC1 but not HC2 to immobilized HA catalyzed by TSG-6. When PTX3 were added after simultaneous addition of I α I and TSG-6, HC1 but not I α I or HC2 was detected, and the HC1 intensity was also increased in a PTX3 dose-dependent manner (FIG. 40A, lanes 7-9), confirming that PTX3 promoted transfer of HC1 but not HC2 to immobilized HA. These results suggested that PTX3 uniquely promoted transfer of predominately HC1 to immobilized HA to form HC1-HA complex regardless whether it was simultaneously or sequentially. Western blot using the anti-TSG-6 antibody showed that both 35 kDa TSG-6 monomer and 75 kDa dimer were detected in rcHC-HA formed by simultaneous or sequential addition of PTX3 in midst of I α I and TSG-6 on iHA. Because the intensity of the TSG-6 band decreased in a PTX3 dose-dependent, suggesting that TSG-6 bound to immobilized HA could be competed out by PTX3 (FIG. 40B). The Western blot using the anti-PTX3 antibody showed a prominent HMW PTX3 smear in the loading well with faint tetramer and dimer bands when PTX3 was added simultaneously with I α I and TSG-6, and that their intensity increased in a PTX3 dose-dependent manner (FIG. 40C, lanes 4-6). This finding suggested that PTX3 was preferentially and strongly bound to rcHC-HA complex in which the binding was resistant to 8 M GnHCl wash. In contrast, when PTX3 was added sequentially after addition of I α I and TSG-6, we did not detect

HMW PTX3 and tetramer and dimer species (FIG. 40C, lanes 7-9), suggesting that the binding between PTX3 and preformed rcHC-HA PTX3 was not strong to withstand 8 M GnHCl wash. Hence, these *in vitro* reconstitution experiments suggested that PTX3 must have been produced simultaneously with HA, IαI and TSG-6 *in vivo* to allow formation of HC-HA/PTX3. This interpretation was supported by immunocolocalization of HA, HC, and PTX3 in *in vivo* tissue sections as well as extracellular matrix formed by AMSCs.

Example 33: Effects of HC-HA complexes on TGFβ1 Signaling

[00731] Immobilized HC-HA inhibits TGFβ1 signaling by downregulating expression of TGF-β1 but upregulating TGF-β3 signaling. Such inhibition of TGFβ1 signaling can withstand the challenge by addition of serum or exogenous TGF-β1 because of further suppression of TGFβRII and TGFβRIII. Consequently, immobilized HC-HA prevents corneal fibroblasts from myofibroblast differentiation by inhibiting SMAD2/3 signaling and expression of alpha smooth muscle formation. This action is also potent enough to revert corneal fibroblasts to keratocytes. HC-HA(Insoluble (I)) differs from HC-HA(Soluble (S)) in consisting of additional small leucine rich proteins (SLRPs), and activates expression of TGFβ1 and BMP signaling by upregulating expression of BMPs and their receptors, thus activating pSMAD1/5/8, which together further promotes formation of aggregation. These actions can further de-differentiate corneal fibroblasts to neural crest progenitors.

[00732] In this example the effect of immobilized soluble and insoluble HC-HA on TGF-β signaling in human corneal fibroblasts with or without exogenous TGFβ1 challenge was examined. In addition the effect of HC-HA complexes on SMAD2/3 signaling and suppression of αSMA expression was tested.

[00733] Experimental and clinical studies support an anti-scarring therapeutic action by cryopreserved amniotic membrane (AM). Our studies demonstrated that heavy chain-hyaluronan complex (HC-HA) is uniquely produced by and can be purified from AM and suppresses the TGF-β1 promoter activity in human corneal fibroblasts. It is unclear whether HC-HA suppresses TGF-β1 mRNA and protein expression and promotes TGF-β3 mRNA and protein expression known to counteract TGF-β1 signaling, and if so, whether such inhibition of TGFβ signaling by HC-HA is translated in to suppression of nuclear translocation of pSMAD2/3.

[00734] Mouse keratocytes can maintain an undifferentiated state (expressing keratocan) in serum-free DMEM/ITS on plastic or in DMEM/10% FBS if cultured on AM. Treatment of keratocytes in serum-free DMEM/ITS with 10% serum or 10 ng/ml TGF-β1 induces Smad2/3 phosphorylation and nuclear localization (3 h) and α-SMA expression (5 days). The activation of Smad2/3 and α-SMA in keratocytes on AM with either serum or TGF-β1 treatment is suppressed (Kawakita et al. (2005))

J Biol Chem. 280(29):27085-92). Our studies demonstrated HC-HA suppresses the TGF- β 1 promoter activity in human corneal fibroblasts. It was expected that pSmad2/3 signaling and α -SMA formation will be suppressed by HC-HA.

[00735] Human corneal fibroblasts (or Limbal niche cells, p3) were seeded on plastic dishes with or without immobilized HA, soluble HC-HA (PBS) (2X or 4X) or insoluble HC-HA (GnHCl) (2X or 4X) for 48 h as described above. The cells were then treated with or without TGF β 1 for 24 h before being harvested for mRNA quantitation and determination of SMAD2/3 signaling. For determination of protein of TGF β receptors, the cells were treated with or without TGF- β 1 for 48 h before collection of protein samples to allow time for protein expression from the expressed mRNA. For TGF- β 1 ELISA, the cells were treated with or without TGF- β 1 for 24 h, and then cultured in the fresh medium for 24 (and 48) h. The supernatants were collected for TGF- β 1 ELISA. For TGF- β 2 and TGF- β 3 ELISA, the cells were treated with or without TGF- β 1 for 48 h. The supernatants were collected for TGF- β 2 and TGF- β 3 ELISA. Phase contrast images were taken up to 72 h for various cultures.

[00736] The following experiments were performed:

[00737] 1. mRNA semiquantitation for TGF- β 1, TGF- β 2, TGF- β 3 and their receptors by real-time PCR: used for estimation of mRNA transcript expression of TGF- β family and their receptors. Real-time RT-PCR profile consisted of 10 minutes of initial activation at 95°C, followed by 40 cycles of 15 seconds denaturation at 95°C, and 1 minute annealing and extension at 60°C.

[00738] 2. Determination of α -smooth muscle formation and SMAD2/3 signaling by immunostaining: performed to monitor α -smooth muscle formation and SMAD2/3 signaling using standard immunostaining procedure.

[00739] The experimental groups for experiments 1 and 2 were:

PBS
PBS+TGF- β 1
HA
HA+TGF- β 1
2X HC-HA PBS
2X HC-HA PBS+TGF- β 1
4X HC-HA PBS
4X HC-HA PBS+TGF- β 1
2X HC-HA Gn
2X HC-HA Gn+TGF- β 1
4X HC-HA Gn
4X HC-HA Gn+TGF- β 1

[00740] 3. Quantitation of TGF β R by Western blotting: used to quantitate protein concentration of TGF β RI, TGF β RII, and TGF β RIII using their corresponding antibodies (R&D Systems). The loading sequence was as follows:

MW marker	Plastic	HA	4X HC-HA PBS	4X HC-HA Gn	Plastic + TGF- β 1	HA + TGF- β 1	4X HC-HA PBS + TGF- β 1	4X HC-HA Gn + TGF- β 1
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[00741] 4. ELISA for Quantitation of TGF β s in the Medium: The Quantikine Human TGF- β 1 and TGF- β 2 ELISA Kits from R&D Systems and TGF- β 3 ELISA Kit from Norvus Biologicals are solid phase ELISAs designed to measure TGF- β 1, TGF- β 2 and TGF- β 3 in acid activated cell culture supernatants, serum, plasma, and urine. They contain recombinant human TGF- β 1, TGF- β 2 and TGF- β 3 and have been shown to quantitate the recombinant factors accurately. Results obtained using natural TGF- β 1, TGF- β 2 TGF- β 3 showed linear curves that were parallel to the standard curves obtained using the recombinant kit standards. These kits were used to determine TGF- β 1, TGF- β 2 and TGF- β 3 in the medium. The experimental groups for experiment 4 were:

PBS
PBS+TGF- β 1
HA
HA+TGF- β 1
4X HC-HA PBS
4X HC-HA PBS +TGF- β 1
4X HC-HA Gn
4X HC-HA Gn+TGF- β 1

[00742] Results

[00743] HCF seeded in DMEM/10%FBS formed aggregates by 72 h only on insoluble HC-HA (FIG. 41). Such aggregation persisted after 24 h serum starvation in DMEM/ITS (insulin/transferrin/selenium) medium. The cells were then cultivated into three different culture media: A - DMEM/ITS for 48 h; B - 24 h DMEM/ITS, 24 h DMEM/10% FBS; C- 24 DMEM/ITS, 24 h DMEM/ITS with 10 ng/ml TGF- β 1. The cells cultured in HC-HA form small aggregates on 4X HC-HA (Gn), but not in other culture conditions (FIG. 41). However, after seeding for 2 hours in DMEM/10% FBS on immobilized substrates, only HCF on the control attached well. Cells on HA, HC-HA [HC-HA (4X, PBS) and 4X, GnHCl] were all rounded, suggesting they were not well attached. After incubation for 72 hours, all cells were attached well, however, there were much less cells on immobilized HC-HAs. The number of cells on these substrates was greater on HC-HA (4X, PBS) than on HC-HA (4X, GnHCl). HCF started to form aggregates on HC-HA (Gn) after 24 h of culture and condensed to bigger aggregates after 72 h of culture. After stimulation of TGF β 1, we observed aggregation of HCF cultured on HC-HA (4X, PBS; 4X, Gn).

[00744] In summary, HC-HA (Gn) promotes aggregation of HCF with/without challenge of TGF β 1 while HC-HA (PBS) promotes aggregation of HCF under challenge of TGF β 1. The significance of aggregation is unknown.

[00745] In DMEM/ITS, expression of , TGF β 1 and TGF β 3 transcripts were elevated by HC-HA (Gn), but TGF β 3 transcript was elevated by HC-HA (PBS) (FIG. 42). As expected in auto-induction, TGF β 1 and TGF β 3 mRNA were increased by 4- and 2-fold, respectively, in HCF cultured on plastic by TGF β 1 challenge, with corresponding increase of TGF β 1 protein from 60 pg/ml to 105 pg/ml (TGF β 3 protein was not detected in the experiment due experimental error). Under serum-free conditions, soluble 4X HC-HA reduced TGF β 1 protein expression. Insoluble HC-HA also decreased secreted TGF β 1 despite its promotion of TGF β 1 mRNA expression although still higher than the control cultured in DMEM/ITS. In addition, notable suppression of TGF β RII and TGF β RIII by both soluble and insoluble HC-HA was observed. Consequently, autocrine TGF β signaling was suppressed in HCF cultured on either soluble or insoluble HC-HA but paracrine TGF β signaling is preserved in HCF cultured on insoluble HC-HA. Furthermore, both soluble and insoluble 4X HC-HA upregulated TGF β 3 mRNA expression by 3-fold under serum-free conditions without TGF β 1 stimulation, which is known to counteract TGF- β 1 signaling. Under stimulation by TGF β 1, TGF β 3 mRNA expression is increased by 5- and 8-fold when HCF were cultured on soluble and insoluble HC-HA respectively, indicating that soluble and insoluble HC-HA strongly promotes TGF β 3 transcript expression. These results also indicate that HC-HA purified from AM promotes AM's anti-scarring action by not only suppressing TGF β 1 signaling but also by marked upregulation of TGF β 3 expression. From our results, it appears that HC-HA (PBS and Gn) does not affect TGF β 2 expression at both mRNA and protein levels. In summary, HC-HA (PBS) inhibits TGF β 1, but activates TGF β 3 signaling in HCF challenged with TGF β 1 while HC-HA (Gn) activates both TGF β 1 and TGF β 3 signaling.

[00746] In the plastic control, TGF β RII mRNA was upregulated by 8-fold under TGF β challenge (FIG. 43). TGF β II and TGF β III receptor mRNA was upregulated by HC-HA (PBS and Gn) by 2- to 8-fold respectively in serum-free condition, but completely inhibited under TGF β 1 challenge. The same result is noted for HA as well. Corresponding protein expression of TGF β RII and TGF β RIII was downregulated by 3- and 3-, and 2- and 3-fold respectively when HCF cultured on HC-HA (PBS and Gn respectively) were challenged by TGF β . Under this situation, these protein expression was not changed by HA. Such downregulation may partially explain the mechanism of the anti-inflammatory and anti-scarring effect by AM. In summary, mRNA expression of TGF β R2 and TGF β R3 was increased when HCF were cultured on HA and HC-HA (PBS and Gn).

[00747] Immunostaining indicated that HC-HA (PBS and Gn) inhibited pSMAD2/3 nuclear translocation in both DMEM/ITS with and without TGF β 1 challenge (FIG. 44). Such an effect was more apparent with TGF β . This finding further confirmed that suppression of TGF β 1, TGF β RII and TGF β RIII was translated into suppression of SMAD-mediated signaling.

[00748] In addition, immunostaining results indicate that both soluble and insoluble HC-HAs inhibited α -SMA formation after challenge of TGF β 1, further supporting that such inhibition of TGF- β 1 signaling by soluble and insoluble HC-HA inhibits differentiation of HCF into myofibroblast with or without addition of TGF β 1 (FIG. 45).

[00749] In summary, soluble HC-HA downregulates TGF- β 1 but upregulates TGF- β 3 expression, while insoluble HC-HA upregulates both TGF- β 1 and TGF- β 3 expression in HCF under serum-free and TGF β 1 challenging conditions. Because both soluble and insoluble HC-HA downregulated expression of both TGF β RII and TGF β RIII, these changes resulted in inhibition of TGF β signaling as evidenced by the lack of nuclear translocation of pSMAD2/3 and suppression of alpha smooth muscle formation.

Example 34: Effects of HC-HA complexes on BMP Signaling

[00750] In this example, the effect of immobilized HC-HA with additional TGF- β 1 on BMP signaling was examined. The activation of BMP signaling via activation of pSMAD1/5/8 also was determined.

[00751] BMPs constitute a subgroup of TGF β superfamily including BMP1-3, BMP3b, BMP4-7, BMP8a, BMP8b, BMP9-15. BMP binds type II receptors (ALK2, ALK3, or ALK6), which activates type I receptor to phosphorylates Smad1, Smad5, and Smad8, resulting in nuclear translocation of pSmad1/5/8 (reviewed in Massague 2000; Herpin, 2007). It was not clear which specific BMPs and BMP receptors are present in HCF, and if so, whether BMP signaling can be activated by HC-HA (PBS and Gn) and additional TGF β 1 when the TGF β signaling is suppression, and if so, which forms of BMPs and BMP receptors play a major role in controlling BMP signaling and whether such an activation of BMP signaling is via pSMAD1/5/8.

[00752] Human corneal fibroblasts were seeded on plastic with or without immobilized HA or HC-HA PBS (4X) or HC-HA Gn (4X) for 48 h, and then treated with or without TGF β 1 for 24 h for mRNA quantitation and determination of pSMAD1/5/8 as described above. For determination of protein of BMP receptors, the cells were treated with or without TGF- β 1 for 48 h before collection of protein samples to allow for protein expression. For BMP ELISA, the cells were treated with or without TGF- β 1 for 48 h. The supernatants were collected for BMP ELISA. Phase contrast images were taken up to 72 h for various cultures.

[00753] The following experiments were performed on the cultures:

[00754] 1. mRNA semiquantitation for BMPs and their receptors by real-time PCR: used for estimation of mRNA transcript expression of BMP family and their receptors.

[00755] 2. Determination of α -smooth muscle formation and SMAD1/5/8 signaling by immunostaining: performed to monitor α -smooth muscle formation and SMAD2/3 signaling by immunostaining.

[00756] The experimental groups for experiment 1 and 2 are:

PBS
PBS+TGF- β 1
HA
HA+TGF- β 1
4X HC-HA PBS
4X HC-HA PBS +TGF- β 1
4X HC-HA Gn
4X HC-HA Gn+TGF- β 1

[00757] 3. Quantitation of BMPRs by Western blotting using BMPR1A, BMPR1B and BMPR2 antibodies: used to quantitate protein concentration of BMPR1A, BMPR1B and BMPR2, respectively. The loading sequence was as follows:

MW marker	Plastic	HA	4X HC-HA PBS	4X HC-HA Gn	Plastic+ TGF- β 1	HA+ TGF- β 1	4X HC-HA PBS + TGF- β 1	4X HC-HA Gn+ TGF- β 1
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[00758] 4. ELISA for Quantitation of BMPs in the Medium: We used BMP ELISA kits (R&D Systems) to determine BMPs in the medium. The experimental groups for experiment 4 are:

PBS
PBS+TGF- β 1
HA
HA+TGF- β 1
4X HC-HA PBS
4X HC-HA PBS +TGF- β 1
4X HC-HA Gn
4X HC-HAGn+TGF- β 1

[00759] Results

[00760] Under the resting state, HA and both HC-HA (PBS) and HC-HA (Gn) activate transcript expression of BMP6 by 7- and 4-fold, respectively (FIG. 46). In the presence of TGF β 1, HA and both HC-HA (PBS) and HC-HA (Gn) activate transcript expression of BMP4 by 6-, 11- and 6-fold,

and mRNA expression of BMP6 by 30-, 37- and 46-fold respectively in HCF, indicating that HA and both soluble and insoluble HC-HA can upregulate BMP4/6 expression, while additional TGF β 1 further dramatically upregulated BMP4 and BMP6 signaling. BMP7 and BMP9 were not detected.

[00761] While TGF β 1 itself did not activated transcript expression of BMPR1A, both HC-HA (PBS) and HC-HA (Gn), but not HA activate transcript expression of BMPR1A by 7- and 3-fold respectively in the presence of TGF β 1, indicating that BMPR1A may play a major role in HC-HA+TGF β 1 activated BMP signaling (FIG.47). In addition, TGF β 1 activates BMPR1B by 3-fold, and BMPR2 by 3-fold, but by 4-fold on plastic with or without HA and both HC-HA (PBS) and HC-HA (Gn), indicating that TGF β 1 itself non-specifically activates mRNA expression of BMPR1B and BMPR2. HC-HA (PBS) or HC-HA (Gn) enhances transcript expression of BMPR2 to 4-fold. BMP-BMPR1A is expected to activate SMAD1/5/8 signaling while BMP-BMPRII activates non-SMAD signaling.

[00762] Immunofluorescence results indicate that TGF β 1 itself moderately activates nuclear translocation of pSMAD1/5/8 in HCF despite the substrate used (FIG. 48). HC-HA (PBS and Gn) strongly facilitates activation of BMP4/6 signaling via nuclear translocation of pSMAD1/5/8, as evidenced by more nuclei having pSMAD1/5/8 and a much stronger nuclear staining of pSMAD1/5/8.

[00763] ID1 is a helix-loop-helix (HLH) protein that can form heterodimers with members of the basic HLH family of transcription factors, a known downstream gene regulated by SMAD1/5/8 signaling. Our results demonstrated that activation of SMAD1/5/8 resulted in 4- and 8-fold upregulation of ID1 mRNA when HCF were cultured on HC-HA (PBS) and HC-HA (Gn) respectively, indicating that SMAD1/5/8 signaling in HCF is indeed activated by HC-HA + TGF β (FIG. 49). Since ID1 has no DNA binding activity and therefore can inhibit the DNA binding and transcriptional activation ability of basic HLH proteins with which it interacts, we expect that ID1 plays an important role in cell growth, senescence, and differentiation.

Example 35: Effects of HC-HA complexes on myofibroblast differentiation and reversion of human corneal fibroblasts to keratocytes or younger progenitors

[00764] Keratocytes, a unique population of neural crest-derived cells embedded in the corneal stroma, express keratan sulfate-containing proteoglycans including cornea-specific keratocan. Keratocan (Kera) is a cornea-specific keratan sulfate proteoglycan (KSPG) in the adult vertebrate eye. It belongs to the small leucine-rich proteoglycan (SLRP) gene family and is one of the major components of extracellular KSPG in the vertebrate corneal stroma. Corneal KSPGs play a pivotal

role in matrix assembly, which is accountable for corneal transparency. Lumican constitutes about half of corneal KSPG. Most of the remaining corneal keratan sulfate modifies keratocan. In adult tissues, keratocan is limited to corneal stroma, and keratocan expression is considered a phenotypic marker for keratocytes (Liu et al.(2003) *J. Biol Chem.* 278(24):21672-7; Carlson et al. (2005) *J Biol Chem.* 280(27):25541-7). On plastic dishes, human, bovine and rabbit keratocytes lose their characteristic dendritic morphology and keratocan expression when cultured in serum-containing media (Espana et al. (2003) *Invest Ophthalmol Vis Sci.* 44 (12): 5136-41 ; Espana et al. (2004) *Invest Ophthalmol Vis Sci.*45(9):2985-91). These exposed cells downregulate the expression of keratan sulfate-containing proteoglycans, keratocan and CD34 ,and upregulate that of chondroitin-dermatan sulfate-containing proteoglycans and α -SMA, indicating that those cells become more differentiated.

[00765] Previous studies have shown that human (Espana et al. (2003) *Invest Ophthalmol Vis Sci.* 44 (12): 5136-41 ; Espana et al. (2004) *Invest Ophthalmol Vis Sci.*45(9):2985-91) and murine (Kawakita et al. (2005) *J Biol Chem.* 280(29):27085-92) keratocyte can maintain their phenotype without differentiation into α -SMA-expressing myofibroblasts when cultured on the AM stromal surface even when TGF- β is added in a serum-containing medium due to downregulation of the Smad signaling pathway. The amniotic membrane stroma can maintain keratocan expressing in cultures and prevent keratocytes from differentiating into myofibroblasts (Kawakita et al. (2005) *J Biol Chem.* 280(29):27085-92) . The keratocyte maintained a dendritic morphology, continued to express corneal stroma-specific keratocan for at least 5 passages (at 1:2 split), and did not express α -SMA under serum containing conditions or addition of TGF- β 1(Espana et al. (2004) *Invest Ophthalmol Vis Sci.*45(9):2985-91). Murine keratocytes can also be expanded on AM for at least 8 passages without losing their normal phenotype and that suppression of Smad-mediated TGF- β signaling pathway is pivotal in maintaining keratocan-expressing phenotype (Kawakita et al. (2005) *J Biol Chem.* 280(29):27085-92). In this example, it was examined whether immobilized HC-HA can do the same, and if so, whether additional TGF β 1 can affect their outcome.

[00766] Results

[00767] HA upregulated Keratocan mRNA expression by 4-fold (FIG. 50). Human corneal fibroblasts were seeded on plastics with or without immobilized HA for 48 h, starved without serum for 24 h, and then treated with or without TGF β 1 for 24 h before being harvested for mRNA quantitation and determination of SMAD2/3 signaling. For determination of protein of TGF β receptors, the cells were treated with or without TGF- β 1 for 48 h before collection of protein samples because the protein expression lags behind of mRNA expression. For TGF- β 1 ELISA, the cells were treated with or without TGF- β 1 for 24 h, and then cultured in the fresh medium for 24

(and 48) h. The supernatants were collected for TGF- β 1 ELISA. For TGF- β 2 and TGF- β 3 ELISA, the cells were treated with or without TGF- β 1 for 48 h. The supernatants were collected for TGF- β 2 and TGF- β 3 ELISA. As expected, immobilized HC-HA promoted mRNA expression of Keratocan by 14- and 16-fold, indicating those HCF are much younger when they were cultured on HC-HA without TGF β 1. After TGF β 1 challenge, the mRNA expression of Keratocan was downregulated dramatically on plastic and HA. However, keratocan expression was still maintained at 3-fold on HC-HA(Soluble, PBS). Expression of keratocan was absent on HC-HA(Insoluble, Gn). We expect that the resultant phenotype on HC-HA (I) should be even more younger than keratocytes.

[00768] Correspondingly, immobilized HC-HA(I/S) promoted protein levels of Keratocan by 8- and 10-fold, indicating those HCF are indeed reverted to keratocytes when they were cultured on HC-HA(S/I) (FIG. 51). We did not see any corresponding keratocan protein expression by other treatments tested, including HA (4-fold increase of keratocan mRNA) and 4X HC-HA (PBS) (3-fold increase of keratocan mRNA), indicating such a moderate increase of keratocan mRNA was not enough to promote corresponding protein expression of keratocan.

Example 36: Effects of HC-HA complexes on ESC marker expression in HCF

[00769] Example 35 showed a strong evidence that HCF was not only prevented from undergoing myofibroblast differentiation under the challenge by exogenous TGF- β 1 but also reverted back to keratocytes with expression of keratocan with or without exogenous TGF- β 1. We thus examined whether HCF could be further reprogrammed into younger progenitors, especially when seeded on immobilized HC-HA(insoluble, GnHCl) with exogenous TGF- β 1, which has been shown to suppress TGF- β signaling, promote BMP signaling, but turning off keratocan expression. We examine the expression of a number of markers found in ESC and endothelial progenitors and pericytes, which as we have recently reported to be found in angiogenesis progenitors. To further look into the potential reprogramming of HCF under these conditions modulated by HC-HA, we also examined expression of the four key transcription factors, i.e., Sox2, Oct4, c-Myc, and KLF4, which have been reported to play a key role of reprogramming adult differentiated fibroblasts into iPSCs.

[00770] Results

[00771] Examination of gene expression was performed on the HCF cultures described above in Example 35. The results indicated that HCF expressed more (2- to 6-fold) ESC markers such as cMYC, KLF-4, Nanog, nestin, Oct4, Rex-1, SOX-2 and SSEA-4 on 4X HC-HA, and 2- to 4-fold more ESC markers even when HCF were challenged by exogenous TGF- β 1 when compared to the

plastic control ($p < 0.05$, $n = 3$) (FIG. 52). These results suggest that HC-HA, especially HC-HA (insoluble), can reprogram HCF into younger progenitors.

Example 37: HC-HA In Solution Inhibits Osteogenesis by Affecting MC3T3-E1 Cell Viability and Differentiation

[00772] In this example, we assessed the effect of HC-HA (soluble fraction) and HA in solution on the viability and differentiation of undifferentiated MC3T3-E1 cells. MC3T3-E1 is an osteoblastic cell line established from C57BL/6 mouse. MC3T3-E1 cells have the capacity to differentiate into osteoblasts and osteocytes and have been demonstrated to form calcified bone tissue *in vitro*.

[00773] MC3T3-E1 cells were cultured in the complete medium (α -MEM, 10% FBS, 100 units/ml Penicillin, and 100 μ g/ml streptomycin) with various concentrations of HA (1, 5, 25, 100 μ g/ml) or HC-HA (1, 5, 25 μ g/ml), with PBS as a vehicle control, and seeded in plastic cell culture treated 96 wells at 1.6×10^4 cells/ml. Cell viability was measured by MTT assays. The result showed that absorbance at 550 nm increased from 24 to 48 h for all conditions except for 25 μ g/ml HC-HA, suggesting that cell proliferation proceeds normally in the control, HA from 1 to 100 μ g/ml, and HC-HA from 1 to 5 μ g/ml (FIG. 53).

[00774] Next, the effect of HC-HA or HA on MC3T3-E1 differentiation into osteoblasts was examined. MC3T3-E1 cells were re-suspended in non-induction medium (1.6×10^5 /ml) and seeded in 96 wells and incubated until confluence. The non-induction medium was removed and induction medium 1 (complete medium with 0.2 mM ascorbic acid 2-phosphate and 10 mM glycerol 2-phosphate, manufacturer's instruction for *in vitro* osteogenesis assay kit, cat# ECM810, Millipore) was added. After 12 days of differentiation, Alizarin Red staining was used to measure and quantify osteoblast mineralization. The result showed that AR was indeed promoted by the induction medium in the control. Consistent with a prior report (Kawano (2011) *Biochemical and Biophysical Research Communications*. 405: 575–580), 100 μ g/ml HA but not 25 μ g/ml HA further promoted AR staining when compared to the control. In contrast, AR staining was not reduced by 5 μ g/ml HC-HA ($p = 0.11$), but significantly reduced by 25 μ g/ml HC-HA ($p = 0.00002$) (FIG. 54). These results suggested that increasing HC-HA concentrations during induction also reduced bone formation.

Example 38: Dose dependent response for HC-HA and AMP on osteoblast differentiation using MC3T3-E1 model system.

[00775] Previous findings showed that HC-HA and AMP dose-dependently inhibit osteoclast differentiation from RAW264.7 cells induced by RANKL (*see* International PCT Publication No. WO 2012/149486). AMP (Amniotic Membrane Powder) is a lyophilized and then pulverized form of the amniotic membrane. In this example, IC₅₀ of HC-HA and AMP for osteogenesis was determined and compares to that for osteoclastogenesis.

[00776] Alkaline phosphatase (ALP) assay and Alizarin Red Staining (AR-S) are two assays used to measure differentiation of MC3T3-E1 cells. ALP is excreted by osteoblasts and has long been a widely recognized biochemical marker for osteoblast activity (Sabokbar (1994) *Bone Miner.* 27(1):57-67), and hence serves as an early marker for osteogenesis. Alizarin Red (AR) dye forms a chelate with calcium and hence AR-S is used to visualize mineralization. Because ARS dye can be easily extracted, it can also be converted into quantitation of mineralization (Gregory et al. (2005) *Analytical Biochemistry* 329: 77-84).

[00777] **Part A**

[00778] **Experimental Design:**

[00779] ***MC3T3-E1 Culture***

[00780] The model system of MC3T3-E1 cells from Millipore *In Vitro* Osteogenesis Kit which consists of the base medium of α -MEM (Invitrogen, Cat. # ICM810) containing 10% FBS, 100 units/ml Penicillin, and 100 μ g/ml streptomycin, was employed. Cells were seeded at 50,000 cells/cm² and cultured in 95% air and 5 % CO₂ at 37.0°C in a 100 mL cell culture dish and passaged before confluence. Once sufficient cell numbers were obtained then cells were seeded at 1.6×10^5 cells/ml in 96-well culture dish with 100 μ L volume of the base medium per well (52 wells). Each concentration was done in 4 wells with 2 wells for ALP assay and 2 wells for AR-S staining. Cells were cultured at 37°C in 5% CO₂ humidified air and the medium was changed every 48-72 hrs until confluence.

[00781] The dosing range to be investigated was derived from the preliminary data performed in osteoblast differentiation (see above) as well as the dose-response curves for HC-HA and AMP against osteoclast differentiation. Because HC-HA at 25 μ g/ml significantly inhibited MC3T3-E1 cell proliferation and differentiation into osteoblasts and completely inhibited osteoclast differentiation from RAW264.7 cells (*see* International PCT Publication No. WO 2012/149486), it was selected as the highest concentration. Because 5 μ g/ml HC-HA showed less than 50% inhibition, suggesting that the IC₅₀ of HC-HA for osteoblast differentiation might be higher than the IC₅₀ in P-214, HC-HA concentrations in the range of 0.1, 0.5, 1, 5, 10, and 25 μ g/ml were selected. Based on preliminary data on HC-HA, AMP at the following concentrations: 1, 5, 25, 125, 250 μ g/ml was selected. Because the ALP activity peaks at Day 12 of differentiation in

MC3T3-E1 cells (Maeda (2004) *Journal of Cellular Biochemistry* 92:458–471; Wang, (2008) *J Dent Res.* 87(7):650-654), we chose to study osteogenesis at Day 12 after induction.

[00782] Upon confluence, the medium from each well was replaced with 100 μ L of Osteogenesis Induction Medium #1. Osteogenesis Induction Medium contains 0.2 mM ascorbic acid 2-phosphate and 10 mM β -glycerolphosphate (*in vitro* osteogenesis assay kit, cat# ECM810, Millipore). 10 μ L of working solutions of AMP and HC-HA were added into the Induction Medium #1. (Stock solutions of AMP (AMP-4; Lot #CB102971, *see* International PCT Publication No. WO 2012/149486) (5 mg/ml) and HC-HA (He et al. (2009) *J. Biol. Chem.* 284(30): 20136–20146) (250 μ g/ml) in PBS were made and diluted accordingly with appropriate culture medium (Osteogenesis Induction Medium #1) for each experimental concentration (0.1, 0.5, 1, 5, 10, and 25 μ g/ml for HC-HA and 1, 5, 25, 125, 250 μ g/ml for AMP)). The medium was changed every 3 days.

[00783] On Differentiation Day 6, replace medium with 100 μ L fresh Osteogenesis Induction Medium #2 containing ascorbic acid, β -glycerolphosphate, and melatonin. 10 μ L of working solutions of AMP and HC-HA were added into the 100 μ L the Osteogenesis Induction Medium #2 (0.2 mM ascorbic acid 2-phosphate, 10 mM glycerol 2-phosphate and 50 nM Melatonin Solution, manufacturer's instruction for *in vitro* osteogenesis assay kit, cat# ECM810, Millipore) to make the final experimental concentrations in culture wells. The medium was changed every 2-3 days. Samples were then assayed with ALP assay (H-156) and ARS Staining Assay following the manufacturer's instructions (*In Vitro* Osteogenesis Assay Kit, (Catalog #ECM810)).

[00784] *Alizarin Red S Staining*

[00785] The culture medium from each well was aspirated without disturbing the cells. The cells were washed 1X with 200 μ L PBS. The cells were fixed by adding 100 μ L 70% ethanol and incubating at R.T. for 15 min. Fixative was then removed and the cells were rinsed 3X (5 min each) with an excess of distilled water without disturbing the cell monolayer. Water was removed and 100 μ L/well Alizarin Red Stain Solution was added. The wells were incubated at R.T. for 1 h. Excess dye was removed, and the cells were washed 4X with deionized water (gentle rocking for 5 min with each wash). 0.1 – 0.15 mL water was added to each well to prevent cells from drying. The stained cells were photographed under microscope.

[00786] Excess water was then removed from each well. 100 μ L 10% acetic acid was added to each well and incubated with shaking for 30 min. The loosely attached monolayer was carefully removed with cell scraper and the cells and acetic acid were transferred to a 1.5 mL microcentrifuge tube and vortexed vigorously for 30 min. The samples were heated to 85°C for 10 min, then transfer to ice for 5 min to cool the tubes. The slurry in the tubes was centrifuged at 20,000xg for 15 min. 400 μ L of supernatant was removed and transferred to a new 1.5 mL

microcentrifuge tube. The pH was neutralized with 150 μ L 10% ammonium hydroxide to within range of 4.1 – 4.5. 150 μ L of each sample was added to a transparent bottom 96-well plate and read at OD405. A plot of Alizarin Red concentration vs. OD405 was made.

[00787] *ALP Assay (BioAssay Systems: QuantiChrom ALP Assay Kit, Cat. #: DALP-250)*

[00788] Cells in each well (96-well plate) were washed with PBS and lysed in 100 μ L 0.2% Triton X-100 in distilled water by shaking for 20 min at R.T. 200 μ L distilled water and 200 μ L Calibrator solution (supplied by kit) were transferred into separate wells for controls. 50 μ L samples were transferred into separate wells. 150 μ L Working solution (200 μ L Assay Buffer, 5 μ L Mg Acetate (5 mM), 2 μ L pNPP liquid substrate (10 mM)) was added to the sample wells (final reaction volume was 200 μ L). The plate was tapped briefly to mix. OD₄₀₅ was read at 0 min and 4 min on plate reader.

[00789] Results

[00790] *Phase Contrast Microscopy*

[00791] The negative control maintained a hexagonal shape through 13 days of induction (FIG. 55). The monolayer was smoother than the positive control, suggesting that more cells or more pile up of cells occurred in the latter. Cells in the positive control became fusiform in shape after beginning of induction. With more time, a spindle-like ring developed along the edge (~ 2-3 mm away) of the plastic culture around the 4th day of induction.

[00792] From 0.1 μ g/ml to 10 μ g/ml of HC-HA, the cell monolayer did not differ from that of the positive control, suggesting that HC-HA at these concentrations did not negatively affect the induction (FIG. 55A). Like the positive control, cells also developed a fusiform shape and the monolayer developed a spindle-like ring. However, at 25 μ g/ml, a dramatic decrease in cell density and change in cell morphology was observed on D13 of induction.

[00793] At concentrations above 25 μ g/ml, AMP deposited particles that settled on the monolayer (FIG. 55B). Because AMP concentration was replenished after each medium change, AMP deposit on top the monolayer increased through induction period. Treatment with AMP below 125 μ g/ml did not affect cell morphology as cells also developed fusiform shapes with spindle rings, suggesting that AMP did not negatively influence induction. At concentrations above 125 μ g/ml, AMP particle density increased to the extent that obscured visual observation of spindle ring formation. However, cell density and morphology remained unchanged from that of the positive controls, also confirming that AMP did not negatively affect induction.

[00794] *Alizarin Red Staining*

[00795] The negative control yielded a blue-gray background color with parts of the monolayer showing a light pink. In contrast, the positive control yielded a rose pink background (FIG. 56).

[00796] 0.1 $\mu\text{g/ml}$ HC-HA yielded a rust red color with the visible spindle ring staining red-brown, which was dramatically different from that of the positive control (FIG. 56A). This trend continued from 0.5 $\mu\text{g/ml}$ to 1 $\mu\text{g/ml}$ with a slight lightening of color at 10 $\mu\text{g/ml}$, suggesting that the mineralization was maintained from 0.1 to 5 $\mu\text{g/ml}$ and that there might be a dose-dependent relationship between 0 and 0.1 $\mu\text{g/ml}$. At 25 $\mu\text{g/ml}$ HC-HA, the rust red background disappeared and returned to a light purple-pink with noticeable white gaps in between the cell junction.

[00797] AMP at 1 $\mu\text{g/ml}$ to 125 $\mu\text{g/ml}$, yielded a dose dependent change of color from a lighter rust brown (1 $\mu\text{g/ml}$), which appeared to be lighter than 0.1 $\mu\text{g/ml}$ HC-HA, suggesting that the dosing response was more gradual, to a rust red background (5 $\mu\text{g/ml}$ & 25 $\mu\text{g/ml}$), red-brown (125 $\mu\text{g/ml}$), and dramatically increased to dark red-brown at 250 $\mu\text{g/ml}$ (FIG. 56B). It is noted that AMP particles found on the top of cell monolayers treated with more than 5 $\mu\text{g/ml}$ of AMP, and the particle size was smaller than the cell itself, and appeared to match the color of the stained background.

[00798] By quantitative analysis of OD value, ARS staining with 0.1 $\mu\text{g/ml}$ HC-HA treatment increased 3x from positive control with statistical significance ($p < 0.05$) (FIG. 57A). Some variation in concentrations 0.1 $\mu\text{g/ml}$ to 10 $\mu\text{g/ml}$ was observed, which may be attributed to small sample size ($N=2$). A dramatic decrease OD value at 25 $\mu\text{g/ml}$ HC-HA treatment was observed. For AMP, treatment with 125 $\mu\text{g/ml}$ of AMP more than doubled the amount of mineralization from positive control and was statistically significant ($p < 0.05$) (FIG. 57B). A small decrease in OD value from the positive control was seen in 1 $\mu\text{g/ml}$ AMP, and from 1 $\mu\text{g/ml}$ to 25 $\mu\text{g/ml}$, there was a small dose dependent increase in OD values. OD₄₀₅ decreased in 250 $\mu\text{g/ml}$ AMP compared to 125 $\mu\text{g/ml}$. Some variation in may be attributed to small sample size ($N=2$).

[00799] *ALP Staining*

[00800] In both AM derivatives group, the negative control showed 5-fold more ALP activity than the positive control, which could have occurred from loss of sample in the negative control, which decreased the sample size (FIG. 58). The smaller sample size contributed to the standard deviation value being 8x higher for the negative control than the positive control, and this much larger variation could contribute to the increase.

[00801] Treatment of induced MC3T3-E1 with any amount of HC-HA decreased ALP activity compared to the positive and negative control (FIG. 58A). ALP activity varied between the different concentration groups until 25 $\mu\text{g/ml}$ where ALP activity significantly dropped. It is worthwhile to note that there was very little variation from the mean in each of the experimental groups and the positive control.

[00802] At 1 $\mu\text{g/ml}$, ALP activity was almost 4-fold higher than the positive control (FIG. 58B). This phenomenon was blocked at 5 $\mu\text{g/ml}$ and 25 $\mu\text{g/ml}$ with ALP activity decreased almost 3x from the positive control. Increasing the concentration to 125 $\mu\text{g/ml}$ to 250 $\mu\text{g/ml}$ restored ALP activity to levels close to 1 $\mu\text{g/ml}$. It thus appeared that ALP activity was not congruent with the amount of mineralization.

[00803] **Results Summary**

[00804] Compared to the negative control, the positive control exhibited more fusiform cells and formed “ring” around the edge of the plastic well (FIG. 55A), a change of color by alizarin staining (FIG. 56A), and a detectable but not significant change of OD₄₀₅ (FIG. 57A). Previously, MC3T3-E1 cells seeded at 5×10^4 cells/35 mm plastic dish also revealed formation of layered collagen fibrils after day 4, layered fibrils by day 18, and formation of nodular regions by day 21 of induction. (Sudo (1983) *J. Cell. Biol.* 96: 191-198). This prior study did not note the same ring formation as we observed. [Alternatively, they may interpret the ring as layered collagen fibril. If this were the case, the ring area should be prone for mineralization.]

[00805] Alizarin red staining has been described as a crimson red color in the literature to signify mineralization. Mineralization and osteoblastic nodules were described to be stained a deep red and the intensity of color increased with mineralization (Wang, (2006) *Biotechnol. Prog.* 22(6):1697-701; Zhao, 2007). ARS staining is read at 405 nm, which corresponds to a violet color in the visible spectrum. Unlike our results, color photographs of ARS staining of MC3T3-E1 mineralization from published data did not show a rust-red or red-brown color in the monolayer. The darker color in our results indicates more ARS staining and thus more osteogenesis compared with published results. The amount of OD change in ARS staining and quantitation varied depending on the culture conditions and cell type. Human mesenchymal stem cells (hMSCs) cultured in 6-well ($10 \text{ cm}^2/\text{well}$) for 30 days attained an increase from OD₄₀₅ of 0.5 to 4 (Gregory et al. (2005) *Analytical Biochemistry* 329: 77-84). MC3T3-E1 cells cultured for 28 days (α -MEM, ascorbic acid, β -glycerolphosphate) in 24-well plates attained an OD of 0.6. However, Day 16 and Day 26 OD were much lower than Day 28 at below 0.05 and 0.2, respectively (Burkhardt (2006) University of Basel, Master Thesis). The lack of dramatic color change in the positive control might be due to the time of D13, which was too short for ARS although it is optimal for ALP.

[00806] 0.1 $\mu\text{g/ml}$ HC-HA also induced “ring change” (FIG. 55A), a clear increase of color (FIG. 56A), and 3X higher OD value than the positive control ($p < 0.05$) (FIG. 57A), suggesting that HC-HA at the lower dose promotes mineralization and that a dose-response curve exists between 0 and 0.1 $\mu\text{g/ml}$.

[00807] HC-HA from 0.5 to 10 µg/ml also showed “rings” (FIG. 55A), maintained the same color as 0.1 µg/ml (FIG. 56A), and yielded OD₄₀₅ without a statistical significance. HC-HA at 25 µg/ml decreased cell density, changed the cell morphology, lost “ring” (FIG. 55A), did not yield any color change (resembling the negative control) (FIG. 56A), and generated negligible OD₄₀₅ (like the negative control).

[00808] AMP from 25 µg/ml left particles (FIG. 55B), from 1 µg/ml increased the color with a positive dose-responsive relationship (FIG. 56B), but OD₄₀₅ showed an increase that was not statistically significant until 125 µg/ml AMP ($p < 0.05$), and then declined at 250 µg/ml, which was not consistent with the color change. Unlike HC-HA, AMP at the higher dose did not cause any ill effects on cell morphology.

[00809] **Part B**

[00810] The Alizarin Red Staining method was then improved by increasing the sample size and incorporating the methodology with Gregory et al. ((2005) *Analytical Biochemistry* 329: 77-84) used for Alizarin staining of human MSC and other methods known in the art. A comparison of the method of Gregory et al, our previous method described above, and the new method outlined in this example is provided in the table below. Previous studies showed that MC3T3-E1 differentiation under induction can be subdivided into three stages, i.e., proliferation (day 1 to 9), ECM formation (day 9 to day 16), and mineralization (deposit minerals in formed ECM) (day 16+). (Quarles et al. (1992) *Bone Miner Res.* 7(6):683-92; Hong et al. (2010) *Exp Cell Res.* 316(14):2291-300). To compare studies from different groups, it was thus important to time the event starting from confluence as Day 0.

Table 5.

	Gregory et al. 2005	Previous Method	New Extraction Method
Cell Type	Human MSC	MC3T3-E1	MC3T3-E1
Culturing conditions	5000 cells per cm ² (6-well) αMEM, 20% FCS, streptomycin, penicillin, glutamine; induction: sodium glycerolphosphate, ascorbic acid, dexamethasone	3.1x10 ⁴ cells/cm ² (96- well) αMEM, 10% FBS, streptomycin, penicillin, glutamine; induction: β- glycerolphosphate, ascorbic acid, melatonin	3.1x10 ⁴ cells/cm ² (96- well) αMEM, 10% FBS, streptomycin, penicillin, glutamine; induction: β- glycerolphosphate, ascorbic acid, melatonin
ARS Time	Day 0 – Day 30	Day 18	Day 18
ARS Staining & Quant. Steps			
1.	Wash with PBS	← Same	← Same
2.	Fix in 10%	Fix in 4%	Fix 4%

	formaldehyde RT, 15 min.	Paraformaldehyde RT, 15 min.	Paraformaldehyde RT, 15 min
3.	Wash 2x with dH ₂ O	←	←
4.	1mL/well 40mM ARS RT, gentle shaking, 20 min.	100 µL/well 40mM ARS per well RT gentle shaking for 1 h.	←
5.	Wash 4X with dH ₂ O, shaking (5min). Remove excess water (tip plate)	←	←
6.	View by phase microscopy.	←	←
7. *	Add 800 µL/well 10% acetic acid RT, shaking (30 min)	Add 300 µL/well 10% acetic acid RT, shaking (30 min)	Incubate samples with 350µL/well 4M guanidine HCl @ 37°C O/N
8.	Scrape monolayers w/ cell scraper Transfer → 1.5-mL tube	←	Transfer 150 µL solution in duplicate to read
9.	Vortex for 30 s	←	
10.	Overlay the slurry with 500 µL mineral oil, heat to 85 °C for 10 min	Heat to 85°C for 10 min (with the cap on).	
11.	Transfer to ice → open when cooled.	←	
12.	Centrifuged at 20,000g for 15min	←	
13. *	Remove 500 µL of 800 µL supernatant to a new 1.5-mL tube.	Remove 255 µL of 300 uL supernatant to a new 1.5-mL tube.	
14.	Neutralize pH with 200 µL of 10% ammonium hydroxide (pH 4.1 - 4.5)	Neutralize pH with 95 µL 10% ammonium hydroxide (pH 4.1 – 4.5)	
15. *	Add 150 µL in triplicates in opaque-walled, transparent-bottomed 96-well plates. Read at 405 nm.	Add 150 µL of standard/sample in doubles to opaque-walled, transparent bottom 96-well plate. Read at 405 nm.	Read at 490 nm in opaque-walled, transparent bottom 96-well plate.

*Steps with differences between the 3 methods for ARS staining and quantitation.

[00811] Experimental Design:

[00812] Cells were cultured and stimulated in differentiation medium as described in Part A and Table 5. Differentiation medium was changed every 3 days for 18 days. 0.1 µg/ml HC-HA and 125 µg/ml AMP were employed for the assay. The ARS assay was performed as described in Part A with the changes as noted in Table 5.

[00813] Results**[00814] *Cell Morphology and Ring Formation***

[00815] Uninduced cells attained a flat cuboidal shape after seeding (FIG. 59A). Cell border became more defined on Day 4 with raised edges, and some cells developed fusiform shapes on Day 6. No spindle cells or multiple layers developed.

[00816] From Day 1 to Day 3, cells maintained a cuboid shape and monolayer remained flat (FIG. 59B). By day 3, cell borders became more distinct and cell edges became raised. In addition, small round cell-like structures were visible on the monolayer (indicated by black circle). Cell morphology changed by day 4 with the appearance of fusiform shaped cells and cells organized in multiple layers. The appearance of the small round cell-like structures continued to increase through Day 6 and Day 7.

[00817] Cell morphology change in induced cells treated with HC-HA mirrored the positive control's changes (FIG. 60A). HC-HA did not leave particles on the monolayer like AMP. Like the positive control, small round cell-like structures (shown in black circle) appeared on Day 3 and continued to increase to Day 7.

[00818] AMP particles (noted with black arrow) settled on top of the monolayer and obstructed observation of the monolayer underneath (FIG. 60B). Areas devoid of AMP particles on Day 0 and Day 1 showed round and cuboidal shapes. On Day 2, some fusiform shaped cells appeared on the monolayer. It was difficult to identify and distinguish the small round cell-like structures from smaller AMP particles and the development of these structures remains unknown. On Day 5, fusiform shapes elongated to form spindle-like cells. On Day 6, long spindle cells formed web-like interactions with AMP particles (shown in black circle).

[00819] On Day 3, fusiform shaped cells developed near the edge of the well (1 – 2 mm from the edge) (FIG. 61A). Spindle cells and ring formation did not develop from Day 4 onwards. Cells appeared to overlap each other at the edge and grow into fusiform shapes. From Day 0 to Day 2, there are no spindle-like cells along the well edge (FIG. 61B). On Day 3, similar fusiform cells piled up in a ring configuration were noted in the edge. From Day 5, these cells were concentrated as a prominent ring about 2 mm from the edge. On Day 6, monolayers show detachment from the plastic surface along certain areas near the edge (indicated by white →).

[00820] Cells remained smooth and cuboidal along the edge until Day 2 (FIG. 62A). Fusiform shaped cells developed near the edge on Day 2. Some small round cell-like structures were also visible near the edge at the time. Spindle-like cells developed on Day 3 and continued to thicken in a ring around the well edge to Day 5. Monolayer detachment from the plastic well was observed on Day 6 in areas near the edge (noted with white arrow). Fusiform shaped cells appeared near the well edge on Day 2 (FIG. 62B). Spindle-like cells developed from the edge on Day 3 (about 1 to 2 mm away) and a ring of spindle-like cells formed by Day 5. Monolayer detachment from the plastic well was observed on Day 5 in some areas near the edge. Detachment continued on Day 6, but the monolayers did not detach as much as the HC-HA treated cells and the positive control cells.

[00821] *ARS Staining and Quantitation*

[00822] The negative controls monolayer stained a light pink color in some areas (FIG. 63). The positive control stained a light pink in the center but showed a bright crimson red color in the spindle ring area, indicating MC3T3-E1 cells deposit mineralization heavily in ring rather than the rest of the monolayer. Both intensity and color of staining in the center monolayer and the spindle ring between 0.1 $\mu\text{g/ml}$ HC-HA and positive control was the same. AMP particles on top of the cells stained a red-brown color. Visual observation of cells underneath was obstructed by the stained particles, but openings showed lack of prominent cell monolayer with some sparse cells staining a light pink color similar to the negative control. Since AMP treated cells did not show a visible spindle ring, and the ARS did not stain a crimson red around the edge similar to the positive control.

[00823] GnHCl treatment solubilized the cell matrix and removed crimson red ARS dye while leaving the monolayer intact in both the positive control and HC-HA treated cells. GnHCl digested and denatured the cell protein, leaving the extracellular matrix behind. In the AMP experimental group, AMP particle density decreased but most particles still remained on the bottom of the well. With the long culture time, AMP particles may form tight interactions with the ECM matrix that was not dissolved by the GnHCl . The particles that once stained a bright red-brown now showed a light brown like the color AMP naturally exhibits. A distinct monolayer structure, however, was not observed; this supported the observation of a monolayer of cells in the gaps between the AMP. The cells may have migrated from the monolayer into the AMP particles and used it as a scaffold for differentiation and mineralization.

[00824] ARS standard showed a progression from crimson red to a cream pink color through serial dilution from 2 mM to 31.3 μM (FIG. 64A). There was a noticeable color change between HC-HA and AMP treated samples. HC-HA treated cell extracts showed a clear cream color while AMP treated cell extracts showed a light cream pink color. The positive control also showed the same

color and level of intensity of color as the HC-HA treated cells, while the negative control showed lighter color and resembled the blank (not shown). The OD₄₀₅ values stayed in the same range as the values from in the previous example (FIG. 64B). Compared to the negative control, the positive control showed a statically significant 2-fold decrease in OD. HC-HA-treated (0.1 µg/ml) cells showed slight increase in OD than the positive control, but the difference is not statistically significant. AMP (125 µg/ml) treatment slightly decreased OD values when compared to the positive control, but this decrease was not statistically significant.

[00825] ARS standard showed a progression from crimson red to a cream pink color through serial dilution from 2 mM to 31.3 µM (FIG. 65A). The negative control extract showed a light cream color slightly darker than the blank (not shown). The positive control showed a light golden color and the color was visibly darker than the negative control. HC-HA also showed a light golden color at the same intensity as the positive control. AMP treated extracts showed an orange-golden color that was darker than both the positive and HC-HA extract groups. OD₄₀₅ values stayed in the same range with the highest at around 0.25 (FIG. 65B). Negative controls showed a negligible OD value close to 0. The positive controls and HC-HA (0.1 µg/ml) treated extracts showed an average OD value close to 0.05. AMP-treated (125 µg/ml) extracts showed a statically significant 5-fold increase in OD ($P = 0.039$) from both the positive and HC-HA treated groups.

[00826] Summary

[00827] Cell Morphology

[00828] MC3T3-E1 cells cultured in α MEM w/ 10% FBS grew to confluence and developed cuboidal shape. Like findings in Aim #1 and #2, cells did not differentiate without the addition of ascorbic acid, β -glycerolphosphate, and melatonin. Without induction medium, spindle cells and spindle rings did not form (FIG. 59). MC3T3-E1 cells were induced into differentiation with ascorbic acid, β -glycerolphosphate and melatonin. After seeding, a smooth monolayer formed with cuboidal shaped cells. After 3 days of induction, cells attained a fusiform shape. By day 5, cells elongated and became spindle-like.

[00829] In this example, spindle rings developed on Day 3 of induction, with spindle like cells forming 1 to 2 mm from the well wall. On Day 6, detachment of the monolayer from the well edge and plastic bottom was observed (FIG. 60). Small round cell-like structures developed on the monolayer beginning on day 2 and increased in number to day 6 (FIG. 59). They did not float in the culture medium and were firmly attached in the monolayer, resting mostly in between the cell borders. These structures may represent matrix vesicles (MVs) are extracellular, membrane-invested particles located at sites of initial calcification in cartilage and bone. Matrix vesicle synthesis occurs through budding and pinching-off of vesicles from specific regions of the outer

plasma membranes of differentiating growth plate chondrocytes and osteoblasts (Anderson et al. (2003) *Curr Rheumatol Rep.* 5(3):222-6).

[00830] Treatment with HC-HA did not alter MC3T3-E1 cell morphological change through differentiation (FIG. 60). Formation of fusiform shaped cells, spindle-like cells and spindle ring (FIG. 61) followed the reported time course of the positive control. AMP particles settled on top of the cell monolayer similar to previous findings (FIG. 60). This impeded full observation of the cell monolayer and cell morphology change through induction. However, some observations were noted through openings where AMP particles did not settle. Unlike HC-HA, AMP treatment sped up cell morphological change and some fusiform cells were visible a day earlier on induction Day 2. AMP treated cells formed a spindle ring similar to HC-HA treated cells and the positive control (FIG. 62). However, the monolayer detached earlier (FIG. 62) than the other two experimental groups (on Day 5 instead of Day 6).

[00831] *ARS Staining and Quantitation*

[00832] ARS staining showed drastically different color than previous reported (FIG. 66). ARS stained a light pink instead of blue-grey color in the negative control cell monolayers. ARS staining also showed a bright crimson red concentrated in the spindle ring instead of a rust-brown color from previous results in the positive controls and HC-HA treated cells. AMP particles stained a red-brown instead of a dark brown, and the cells underneath was stained light pink instead of a rust-brown. Degradation of ARS Solution may have contributed to the color change.

[00833] After Acetic Acid extraction, the monolayer still appeared to have significant amount of stained color left. Acetic Acid is not effective in completely removing ARS stain from the monolayer. With Acetic Acid extraction, there was a statistically significant difference between the OD values of the positive and negative control (FIG. 68) that matched the visual observation of more ARS staining in the positive control (FIG. 66). However, acetic acid extraction was not effective in showing a statistically significant increase in AMP's OD compared to the positive and HC-HA groups (FIG. 66) despite visual observation of AMP groups having more color in the assay extracts than the other two groups (FIG. 67). AMP particle deposits may have made it more difficult to remove ARS from the AMP groups. Mineralized matrix and cells could also may have interactions with the AMP particles that hinder acetic acid extraction of ARS.

[00834] GnHCl removed ARS from cell monolayer more completely than Acetic Acid treatment (FIG. 69). Additionally, color was solubilized in GnHCl solution without the use of a cell scraper to remove the monolayer. Both methods seem to have left particles invisible to the naked eye and unaffected by centrifuge. For GnHCl , this could be calcium and dissolved matrix that forms fine

particles. This caused variations between the duplicates in each sample. Reading at 670 nm to remove the particles solved this problem for the GnHCl extraction method.

[00835] GnHCl extraction could not establish a quantifiable statistical significance between the negative and positive control (FIG. 71) to match the visual observation of the ARS staining. GnHCl extraction also showed that 0.1 µg/ml HC-HA did not promote more mineralization in differentiating MC3T3-E1 cells (FIG. 71), and this matched the results of the Acetic Acid extraction. From looking at the assay extract colors in 96-wells, the positive control and HC-HA wells showed no difference in color or intensity (FIG. 70).

[00836] GnHCl extraction showed that 125 µg/ml AMP promoted more mineralization in differentiating MC3T3-E1 cells than the positive control (FIG. 71). This matched both the ARS staining of the monolayer (FIG. 66) and the visual observation of the extracts in 96-well assay plates (FIG. 70), where AMP showed a much deeper golden-yellow color than the HC-HA or positive control.

[00837] From these results, GnHCl is the better extraction method because it removes ARS stain more completely from the monolayer and leaving it intact; there is less technical error because the monolayer does not need to be scraped from the well. The extract color can be quantified through spectrophotometer reading.

[00838] Part C

[00839] The preliminary study above did not exhibit statistically significant results on the dose-response curves for HC-HA and AMP on osteoblast differentiation due to the small sample size and incomplete development of the ARS assay. This suggested that 0.1 µg/ml HC-HA may enhance mineralization. Furthermore, HC-HA at 10 µg/ml to 25 µg/ml might affect cell viability and reduce mineralization. The dose curve for HC-HA should include a lower concentration below 0.1 µg/ml as well as concentrations above 10 µg/ml. Unlike HC-HA, AMP at 5 µg/ml to 125 µg/ml may promote mineralization. In this experiment, the dose response of HC-HA and AMP was retested using the method of Part B. In this experiment, ARS stain was used to assay at Day 15, and the revised protocol using 10% acetic acid to stain and quantitate was employed.

[00840] Experimental Design

[00841] The same model system as shown in Part B based on 3T3-E1 cells was used by seeding 3×10^4 cells/cm²/well in 96 well plates in αMEM medium w/ 10% FBS. Upon confluence, cells were induced to differentiation by adding ascorbic acid, β-glycerolphosphate, melatonin induction medium. For each condition, N=3 was tested. After confluence (Day 0 = seeding), HC-HA was added at 0.02 µg/ml, 0.1 µg/ml, 1 µg/ml, 5 µg/ml, and 25 µg/ml while AMP was added at 1 µg/ml, 5 µg/ml, 25 µg/ml, and 125 µg/ml. For ARS staining, the modified method of Part B was employed.

[00842] Results

[00843] Negative control MC3T3-E1 cells did not develop spindle-like shapes or spindle rings throughout culturing (FIG. 66A). The monolayer center and peripheral stained a beige color. The positive control cells developed fusiform and spindle-like cells, with the appearance of spindle rings around D5 of culturing (D4 of induction). ARS staining showed a light maroon color in the center and was mostly concentrated in the spindle rings with a dark crimson color. Increasing HC-HA concentration to 1.25 µg/ml had no effect on cell morphology or ARS staining intensity and pattern. At 2.5 µg/ml, MC3T3-E1 cells' spindle ring started to degrade and the ARS color changed from crimson to a red-brown color. By 20 µg/ml, cells lost their fusiform and spindle shapes; cell edges were also less defined and raised. Cells density decreased and the monolayer did not appear raised like before. GnHCl successfully extracted the ARS dye and the coefficient of variation in OD₄₅₀ values ranged from 5% to 19%. The positive control showed statistically significant increased OD values (FIG. 66B).

[00844] Negative control MC3T3-E1 cells did not develop spindle-like shapes or spindle rings throughout culturing (FIG. 67A). The monolayer center and peripheral stained a beige color. The positive control cells developed fusiform and spindle-like cells, with the appearance of spindle rings around D5 of culturing (D4 of induction). ARS staining showed a light maroon color in the center of the monolayer. AMP treatment left AMP particles that settled on top of the cell monolayer and obscured observation of the monolayer from concentration 62.5 µg/ml upwards. MC3T3-E1 cells treated with only AMP and no induction showed no spindle rings along the edge and ARS staining showed a dark crimson with a light pink background. From 7.8 µg/ml to 31.25 µg/ml, the AMP particles did not completely cover the monolayer and cells showed fusiform and spindle shapes. Along the edge, spindle rings could be seen. ARS staining showed the center monolayer staining a light maroon and the dye concentrated along the spindle ring to a dark crimson color like the positive control. GnHCl successfully extracted the ARS dye and the coefficient of variation in OD₄₅₀ values ranged from 3% to 10% (FIG. 67B).

[00845] Results Summary**[00846] Cell morphology/ARS staining**

[00847] Similar to previous results, MC3T3-E1 differentiation progresses from cells changing from cuboidal shape to fusiform and spindle shapes. With increasing induction time, spindle rings form along the well edge (~ 2mm away) and contracts the monolayer with time. Unlike the positive control, the negative control never developed spindle-like cells or spindle ring. While the negative control monolayer stained a uniform beige color, the positive control showed a light maroon/pink in the center and concentrated dark crimson in the spindle rings.

[00848] At 10 $\mu\text{g/ml}$ HC-HA, cell morphology changed from lower concentrations, with less cells showing the fusiform and spindle shapes. Cell density decreased and the monolayer looked less raised. At 20 $\mu\text{g/ml}$ HC-HA, cell density decreased dramatically and few cells were spindle shaped. The monolayer looked smooth like the negative control. For both concentrations, these changes were noticed starting on D5 of culturing and induction. In both concentrations, the spindle rings were either poorly formed or non-existent. Increasing HC-HA concentration caused the disintegration of the spindle ring at a concentration of 2.5 $\mu\text{g/ml}$ and upwards.

[00849] In all AMP treated cells, areas around where AMP particles settled stained dark crimson. Around the edge of the well, no monolayer could be seen through the openings of AMP particles. Uninduced MC3T3-E1 treated with 125 $\mu\text{g/ml}$ AMP showed staining similar to induced MC3T3-E1 treated with 125 $\mu\text{g/ml}$ AMP. The cells with settled AMP stained dark crimson in patches, with no observation of a stained monolayer underneath. From 7.8 $\mu\text{g/ml}$ to 15.6 $\mu\text{g/ml}$ AMP, cell morphology was visible. Cells showed fusiform and spindle shapes with spindle rings formed along the edge of the well. ARS staining resembled the positive control with light maroon color in the center and dark crimson in the spindle ring areas.

[00850] GnHCl treatment was successful in extracting ARS dye from the stained monolayer. The extraction showed a statistically significant ($p < 0.01$) 2-fold increase in OD_{450} from negative control to positive control. HC-HA treated cells showed a trend of decreasing mineralization with increasing HC-HA concentration. At 10 $\mu\text{g/ml}$ and 20 $\mu\text{g/ml}$ HC-HA, there was a statistically significant ($p < 0.05$) decrease in mineralization from the positive control. AMP dose-dependently increased mineralization of differentiating MC3T3-E1 cells.

[00851] Uninduced cells treated with AMP also showed mineralization, and it would appear AMP induced and promoted mineralization more than positive control ($p < 0.01$). Also, at 125 $\mu\text{g/ml}$ AMP, treatment to uninduced cells showed more mineralization than cells cultured in induction medium ($p < 0.05$).

Example 39. Effect of AMP on osteoblast differentiation

[00852] Although ARS staining showed a clear dose-dependent increase of staining by AMP, and exhibited a statistically significant increase in mineralization at 125 $\mu\text{g/ml}$, it was unclear whether such a change is caused by non-specific binding of ARS to AMP. Because AMP acted differently from HC-HA especially at the high dose, i.e., promoting mineralization but not inhibiting it, it was important to rule out whether AMP's action depends on cell direct contact with AMP. This question was addressed by the use of transwells with a 3 μm pore size, which is sufficient for HC-HA to pass through but small enough to preclude AMP particles. Because the available transwell

plate with this pore size fits in a 24-well plate, the culture conditions of the assay were altered accordingly. A concentration of 125 µg/ml AMP was employed for the assay.

[00853] Experimental Design:

[00854] MC3T3-E1 cells (Cells at P2; ATCC, catalog number: CRL-2593™) were seeded onto 12-well flat, transparent bottomed wells at a density of 1×10^5 cells/ml. The same AMP stock solution (AMP-4; Lot #CB102971) as used above was prepared as 5 mg/ml in PBS. For wells without Transwells, 17.5 µL of AMP stock was added in 0.7 ml of the culture medium (base or induction medium) to achieve 125 µg/ml AMP concentration in either α-MEM w/ 10% FBS (without induction) or induction medium #1 followed by #2 (with induction). For wells with Transwells, 17.5 µL of AMP stock was added to directly into center of Transwell membrane in the same manner as described above. Culture mediums (α-MEM with 10% FBS; Induction Medium #1 and #2 as described above) were changed every 3 days after D0 of induction. ARS staining and quantitation procedures were performed as described above except that cell monolayers were fixed with 4% paraformaldehyde instead of 70% ethanol, and stained for 2 h instead of 1 h.

[00855] Results

[00856] All induced cells developed ring formation on D4 of induction (FIG. 68). Rings were composed of layers of cells around the edge of the plastic wells, about 2-3 mm away. They grew in layers, curled up, and then the monolayer detached in many wells from the plastic. Without induction, cells maintained mostly hexagonal shapes with some fusiform shapes with increasing culture period, and the monolayer remained smooth compared to induced cells (FIG. 69). Induction rendered the cells with a fusiform shape and the monolayer became raised and borders between cells became more distinct. With induction, by day 4, a spindle-like ring was observed developing along the edge of the culture plate. Treatment with AMP did not affect cell viability; nor did it affect the ring formation, suggesting that AMP did not negatively affect induction. The addition of transwells did not affect cell growth or morphology.

[00857] Without transwells, AMP at 125 µg/ml left particles settled on the cell monolayer (FIG. 69A). Without induction, AMP, by itself, did not cause cells to develop spindle shape and did not generate ring, thus suggesting that AMP alone was not sufficient to cause induction, similar to the negative control. With induction, 125 µg/ml of AMP did cause the cell morphology change like the positive control, suggesting that AMP itself did not negatively affect induction. Through transwells, 125 µg/ml AMP left negligible particles on the cell monolayer (FIG. 69B). Without induction, AMP was not sufficient in inducing cells to differentiate and cells resembled the negative control. With induction and 125 µg/ml of AMP, cells developed fusiform and spindle-like shapes and by D14 resembled the positive control, again suggesting that AMP did not negatively affect induction.

[00858] The negative control yielded a taupe background color with patches of light pink in the monolayer, which differed from the blue-gray color of the negative control in the previous experiment (FIG. 70). Without induction but with transwell, monolayers stained a taupe background similar to the negative control even when treated with AMP, suggesting that AMP itself did not cause induction. Without transwell, compared to the negative control, there were zones exhibiting a notable decrease of background color, suggesting that AMP particles settled on monolayer might have blocked the color (marked by **), and AMP itself did not generate positive color suggesting mineralization.

[00859] The positive control yielded rose pink with the ring stained rust red, which was darker than rose-red color from the positive control in the previous experiment. The addition of a transwell did not affect color of the positive control, confirming that transwell itself did not affect induction. With AMP but without transwell, monolayers yielded a stronger rust red color than the positive control, with the ring stained darker, suggesting that AMP exerted additional positive induction. In contrast, with the transwell, the intensity of the background color decreased to the level of the positive control while the ring maintaining the same color, suggesting that transwell exerted a negative influence on AMP's effect on induction.

[00860] Quantitation results were not adequate in providing statistical significance, and did not match visual analysis of ARS staining (FIG. 71). However, the overall trend suggested that the positive control showed more mineralization than the negative control. Furthermore, there was a trend suggesting the less OD₄₀₅ when transwell was included in the presence of AMP and induction.

[00861] ***Results Summary***

[00862] The ring formation (FIG. 69A) was easy to observe presumably because the size of dish was bigger. However, probably due to the change of fixative, the background color for the negative control was different from the prior experiment. Furthermore, there was a more dramatic color change between negative and positive controls, especially in the ring area (FIG. 70). The introduction of a transwell in the positive control did not affect cell morphology (FIG. 69B) or ARS staining color (FIG. 70).

[00863] Without induction, AMP without transwell clearly blocked the positive ARS staining (FIG. 70), and AMP with transwell showed the same color as the negative control. AMP itself does not cause non-specific ARS and does not cause any induction. With induction, AMP without transwell caused more color than the positive control. In contrast, AMP with transwell seemed to yield the same color as the positive control.

[00864] Previous ARS quantitation results showed 125 µg/ml AMP promoting 3X more mineralization than positive control ($p < 0.05$) (FIG. 67), but not so in FIG. 71.

[00865] There was no difference in cell morphology between cells directly treated with AMP and cells treated with AMP through transwells (FIG. 69A, 69B). Both cell groups developed ring formation and visual observation showed no perceivable difference between the ring structures. ARS staining showed negligible difference in background color between the two experimental groups. However, cells directly treated with AMP showed a more diffuse ring formation, which could be a scattering effect from the AMP particles (FIG. 70).

[00866] AMP does not need direct contact with MC3T3-E1 cells to affect mineralization. While it was unclear from this experiment AMP promotes mineralization, AMP has been shown to not affect cell morphology or cell viability (FIG. 69).

[00867] **Part B**

[00868] Our results showed that 125 µg/mL AMP significantly increased mineralization of MC3T3-E1 cells (FIG. 65) when compared to the positive control on Day 15 of growth and differentiation. AMP was delivered directly in culture medium and AMP particles settled on top of the cell monolayer; therefore, AMP's effect in Aim 1B necessitated direct contact.

[00869] In Part A, we sought to investigate whether AMP's effect is through acting as a scaffold for the differentiating MC3T3-E1 cells or if factors are released from the particles to promote mineralization. However, the small sample size and technical errors in ARS stain extraction with 10% acetic acid affected the data and no statistical significance was found. The experiment was repeated using the improved technical methods of ARS staining and extraction developed in Example 38 with 4M guanidine HCl.

[00870] **Experimental Design:**

[00871] MC3T3-E1 cells were seeded at 3×10^4 cells/cm²/well in 24-well with α MEM medium plus 10% FBS as described above. Upon confluence, cells were induced to differentiation by adding ascorbic acid, β -glycerolphosphate, melatonin. For each condition, N=3 was tested. Day 0 counted as the day of cell seeding, and induction followed after cell confluence. Total induction time = 15 Days. There were two experimental groups: AMP directly added to induction medium and AMP delivered through transwell. AMP concentration was kept the same as before at 125 µg/ml. A negative control (without induction) was added with or without AMP but without insert. ARS staining and quantitation on Day 15 of induction were performed as described above.

[00872] **Results**

[00873] Without induction medium, negative control cells maintained hexagonal shapes with some fusiform shapes (FIG. 72A). Spindle-like shapes were not observed, and no spindle ring formed along the periphery. ARS stained the monolayer a light pink. With induction, positive control cells attained spindle like shapes. Cell borders were more prominent and raised; a spindle ring formed

along the periphery near the well edge. The monolayer center stained a maroon color, and the ARS stain concentrated in the spindle ring with an intense crimson red color. Treatment with AMP directly caused AMP particles to settle on the monolayer and obscured the morphology of the cells. However, near the culture well edge, gaps between the AMP particles showed lack of a prominent monolayer underneath in both groups. There was no difference in cell morphology between AMP treatment alone and AMP treatment with induction. The cells that were visible were spindle like in shape. No spindle ring like the positive control was observed. ARS staining showed a crimson red staining in the center with reddish-brown staining along the periphery. Staining color and patterns were indistinguishable between the induction and no-induction groups. Treatment with AMP through a transwell did not produce AMP particle settlement on the monolayer. Cells were elongated and spindle-like, with spindle ring formation along the well edge. Like the positive control, the monolayer center stained a maroon color, and ARS dye concentrated in the spindle ring with an intense crimson red color. GnHCl successfully extracted the ARS dye and the coefficient of variation in OD₄₅₀ values ranged from 2% to 15% (FIG. 72B).

[00874] *Results Summary*

[00875] *Morphology/ARS staining*

[00876] The negative control cultured in α -MEM w/ 10% FBS for 21 days did not develop spindle-like cells or a spindle ring along the edge. The positive control, after 20 days of induction with AA, β -glycerophosphate, and melatonin, MC3T3-E1 cells developed spindle-like cells and a spindle ring around the edge of the well (FIG. 72). ARS color and stain pattern of the cell monolayer were different between the negative and positive controls. The negative control monolayer failed to collect as much dye as the positive control and showed a uniform light pink color. The positive control monolayer center stained a maroon color and the ARS dye concentrated in the spindle ring into an intense crimson color (FIG. 72).

[00877] Treatment with AMP directly in culture medium obscured observation of cell monolayer in AMP and AMP with induction groups due to settlement of AMP particles. However, there was no observation of spindle rings along the well edge, and some cells along the edge showed spindle-like morphology. Openings through the AMP particles showed lack of monolayer underneath. ARS staining showed no difference in color or pattern between the AMP only group and the AMP with induction group.

[00878] Treatment with AMP through transwells did not produce AMP particles on top of the monolayer. Cell morphology was similar to the positive control, with spindle-like cells and spindle rings forming. The ARS staining color and pattern also resembled the positive control, with the

background monolayer staining maroon and the ARS stain concentrating in the spindle rings in a crimson red color.

[00879] *ARS Quantitation*

[00880] GnHCl was necessary and sufficient in extracting ARS staining from monolayer.

Comparing the controls, there was a statistically significant 2-fold increase in OD₄₅₀ from the negative control to the positive control ($p < 0.01$). Also, there was about a 6-fold increase in OD from the negative/positive control and AMP+ induction. There is a 6.5x increase in surface area from 96-well to 24-well, and this could account for the increase in OD. We also conducted ARS staining and quantitation on D20 of culturing, which increased the culturing period by 2 days and could have also increased mineralization.

[00881] AMP alone induced statistically significant 10-fold ($p < 0.01$) and 3-fold ($p < 0.01$) increase in OD₄₅₀ values from the negative and positive controls, respectively. Thus, AMP alone was sufficient in inducing and promoting differentiation. AMP with induction slightly decreased the OD₄₅₀ from AMP alone ($p < 0.05$), but showed a 3-fold increase in OD from the positive control ($p < 0.01$). The induction medium hindered differentiation and mineralization.

[00882] AMP delivered through transwells with induction showed an OD that was 3-times lower than AMP delivered directly with induction ($p < 0.01$), and it showed a slightly lower OD than the positive control ($p < 0.05$). AMP requires direct contact to promote differentiation. Without direct contact, AMP inhibits MC3T3-E1 mineralization.

Example 40: Effect of AMP on induction of osteogenesis in MSCs

[00883] Our results have shown that AMP promotes MC3T3-E1 differentiation when in contact with the pre-osteoblasts. However, it was unclear how AMP affects the growth and differentiation of less differentiated and less committed cell lines to the osteoblastic lineage, such as Mesenchymal stem cells (MSCs).

[00884] MC3T3-E1 cells are pre-osteoblasts, unipotent, and therefore require only supplements to push along their differentiation towards osteoblasts. Other progenitor cell lines such as embryonic stem cells (ESCs) or mesenchymal stem cells (MSCs) are less differentiated and are oligopotent and pluripotent, respectively. Thus, by studying the effect of AMP on MSCs derived from different areas of the human body, we can better understand the role of AMP in osteoblast differentiation programming and the factors involved. This investigation would allow us to narrow down which cell types AMP can affect and what effects it has on inducing osteogenesis in different progenitor cells.

[00885] **Experimental Design**

[00886] The following cell lines were used: MC3T3-E1 (ATCC, Manassas, VA), Mesenchymal stem cells derived from human bone marrow cells (Lonza, Walkersfield, MD), Limbal Niche cells (Tissue Tech, Miami, FL), human Amniotic Membrane (hAM) stromal cells (Tissue Tech, Miami, FL), and human umbilical cord vein endothelial cells (HUVEC) (ATCC, Manassas, VA).

[00887] Cells were seeded at 3×10^4 cells/cm²/well in α MEM medium plus 10% FBS in 96-well plastic culture plates. Upon confluence, cells were induced to osteoblast differentiation by adding ascorbic acid, β -glycerolphosphate, and melatonin (AGM). For each condition, N=5 was tested. Total induction time = 20 Days. Day 0 counted as the day of cell seeding, and induction followed after cell confluence. Each cell type had 3 experimental groups: negative control, positive control, and AMP treatment only. For AMP treatment, an AMP Concentration of 125 μ g/ml was used. The medium (100 μ L) was changed every 3 days for culture time of 20 days. A negative control with AMP (without induction or cells) was added. ARS staining and quantitation were performed as described above on D20. Extracts were then read at 450 nm.

[00888] Results

[00889] HUVEC cells formed a net-like pattern of cell growth by Day 4 (FIG. 74A). However, there was significant cell death with dead cells settled on top of the network of cells until Day 21. Most of the HUVEC cells could not be fixed with 10% Paraformaldehyde and the few cells stained with ARS showed a dark brown color. Although AMP settled on top of the HUVEC cells and covered the network of cells, the AMP particles detached from the plastic well with the cells upon ARS staining; the few remaining AMP particles also stained a dark brown.

[00890] hBM MSCs, without induction, maintained a long fibroblastic shape (FIG. 74A). With induction, MSCs became elongated with more raised cell edges by Day 4. By Day 10, induced MSCs developed spindle-like cells, and cells grew overlapping layers with each other on the monolayer. On Day 17, the overlapping spindle cells formed a dense ring about 5 mm from the center of well. ARS staining showed that the un-induced MSCs monolayer stained a cream color, and the spindle ring stained a red-orange color. AMP-treated MSCs contained AMP particles that covered the monolayer. With time, the monolayer retracted around concentrated areas of AMP particles. ARS staining showed a deep red-brown color.

[00891] hAM stromal stem cells, without induction, maintained a rectangular shape (FIG. 74A). By Day 4, with induction, cell morphology changed and cells elongated with some developing fusiform shapes. AMP particles settled and covered some of the monolayer by Day 4 in AMP-treated stroma cells. The cells not covered by AMP particles in Day 4 were rectangular in shape. By Day 17, cells not covered by AMP particles were elongated similar to induced cells in the positive control. By Day 21, AMP particles covered the well and cell morphology could not be

observed. ARS staining showed the un-induced cells staining a cream color, while the induced cells stained a light pink color. The AMP-treated cells stained a deep red-brown color similar to the AMP-treated hBM MSCs.

[00892] The extract with 4M GnHCl yielded the coefficient of variation in OD₄₅₀ values ranged from 2% to 15% (FIG. 74B).

[00893] Results Summary

[00894] The results indicated that no mineralization was noted in the negative control of HUVEC with either inductive agent or AMP. For both hBM MSC and hAM stromal stem cells, mineralization was promoted by the inductive agent, which was less than that promoted by AMP.

Example 41: Effect of AMP on mineralization and cell proliferation during MC3T3-E1 differentiation

[00895] MC3T3-E1 cells undergo three main stages before becoming a mature osteoblast: proliferation, matrix deposition/maturation, and mineralization (FIG. 75). Our results have shown that AMP promotes mineralization in MC3T3-E1 cells. In viewing the ARS staining, it shows that cells treated with AMP stained a darker and denser (FIG. 72) than the positive and negative controls. Additionally, there was a lack of monolayer underneath the AMP particles (FIG. 77) when the AMP particles covered the monolayer. One possibility was that AMP is acting as a scaffold for MC3T3-E1 cells and this interaction in the 3D matrix allowed cells to grow and mineralize. Thus, AMP may be promoting mineralization by increasing proliferation of MC3T3-E1 cells.

[00896] Experimental Design

[00897] MC3T3-E1 cells were seeded at 3×10^4 cells/cm²/well in 24-well with α MEM medium plus 10% FBS as described above. Upon confluence, cells were induced to differentiation by adding ascorbic acid, β -glycerolphosphate, melatonin. For each condition, N=3 was tested. Day 1 counted as the day of cell seeding, and induction followed after cell confluence. Total induction time = 20 Days. Four time-points were sampled: D1, D2, D7, D10, D13, D20. Each time-point had four groups: negative control, positive control, AMP treatment only, AMP + (w/ induction). The AMP concentration employed was 125 μ g/ml.

[00898] For the proliferation assay measured by MTT assay, the culture period was 9 days for proliferation. 4 timepoints were sampled: D1, D2, D4, and D9. Each time-point had 3 groups: cells only, AMP only, AMP + cells. The AMP concentration employed was 125 μ g/ml.

[00899] Results

[00900] Mineralization

[00901] On Day 1, cells were seeded for 24 hours and were not treated with induction medium or AMP (FIG. 76). Cells were round and appear more raised on the monolayer. ARS staining showed a light beige color with little to no mineralization. On Day 2, without induction, negative control cells became hexagonal. ARS stained the monolayer a light peach color. With induction, positive control cells looked identical to negative control with little mineralization and ARS staining a light peach color. Treatment with AMP caused MC3T3-E1 cell morphology to change, and fusiform shaped cells and spindle cells were observed. ARS staining showed increase in mineralization from Day 1 and the monolayer stained a light pink color. The area where AMP particles settled, however, stained a reddish-brown color. AMP treatment with induction also changed some cell morphology. Fusiform shaped cells were present and the monolayer also stained a light pink color with areas around AMP particles staining reddish-brown. On Day 7, negative control cells appeared more hexagonal and the cell boundaries are more defined. There is an increase in mineralization and the monolayer stained a light beige color. The positive control, with induction, developed spindle shaped cells and spindle rings. ARS also stained a deep pink color with more mineralization. Treatment with AMP and AMP with induction caused AMP particles to settle and cell morphology could not be viewed. A cell monolayer with individual cells could not be seen. ARS showed areas around AMP particles staining a red-brown color and the monolayer staining a light pink. GnHCl successfully extracted the ARS dye and the coefficient of variation in OD_{450} values ranged from 6% to 16%.

[00902] Results Summary

[00903] Negative control MC3T3-E1 cells show increase in ARS staining and therefore mineralization with increasing cell culturing after Day 2 (FIG. 76). Similar to previous results, positive control MC3T3-E1 cells cultured with induction medium underwent cell morphology change and developed fusiform shaped cells and spindle rings by Day 7 (FIG. 76). After 1 Day of AMP treatment (D2), with or without induction, a change in cell morphology could be seen. Cells become spindle and even fibroblast shaped (FIG. 76A). This change was not observed in the negative or positive control. Additionally, this change was observed in induced MC3T3-E1 cells after at least 4 days of culturing (FIG. 76A).

[00904] AMP treated and AMP with induction treated cells showed statistically significant increase in mineralization from the positive control by Day 2, as shown through ARS quantitation (FIG. 76B). The monolayer stained a light pink color, but small areas where AMP particles settled showed increased mineralization and stained a deep red-brown color (FIG. 76A). This increase in mineralization continued throughout the culturing period.

[00905] There was no increase in promoting mineralization with AMP only when compared to AMP with induction treatment.

[00906] Proliferation

[00907] To determine whether AMP promoted mineralization via promoting cell proliferation, MC3T3-E1 cells were seeded at 3×10^4 cells/cm²/well in 96-well with α MEM medium plus 10% FBS. Upon confluence, the AMP group was treated with fresh 125 μ g/ml AMP added every 3 days in the culture medium. The MTT assay was conducted on Day 1, 2 and 4, while the BrdU assay was conducted on Day 1, 2, and 16.

[00908] In untreated MC3T3-E1 cells, cell viability increases from Day 1 to Day 4 (FIG. 77A). In AMP-treated cells, cell viability decreased on Day 2 and then more than doubled on Day 4, following the trend of the cells only group. BrdU assay showed decrease in cell proliferation following Day 1 in both the cells only group and AMP-treated cell group (FIG. 77B). Cell proliferation in the cells only group decreased by more than half by Day 2 and continued to decrease on Day 16. In AMP-treated cells, cell proliferation showed a statistically significant decrease only by Day 16. These findings suggest that AMP did not promote proliferation during the culturing period of 16 days.

[00909] Results Summary

[00910] Cell viability increased in MC3T3-E1 cells from Day 1 until Day 4 as shown through MTT assay. AMP treated MC3T3-E1 cells showed a decrease in cell viability from Day 1 to Day 2 but followed an upwards trend like the untreated cells. However, cell viability on day 4 in the AMP-treated cells were around half of the untreated MC3T3-E1 cells. Cell proliferation, as measured through BrdU, decreased from Day 1 all the way to Day 16. Unlike the AMP-treated cells, cell proliferation decreased by more than half by Day 2 in the untreated MC3T3-E1 cells. By Day 16, AMP-treated and untreated MC3T3-E1 cells exhibited the same levels of cell proliferation.

Example 42: Identification of genes expressed during the early stages of Osteogenesis in AMP-treated MC3T3-E1 and hBM MSCs.

[00911] Human bone marrow mesenchymal stem cells (hBMMSCs) are multipotent and can differentiate into multiple tissue types such as osteoblasts, chondrocytes, and adipocytes (Born, 2012 *J Cell Biochem.* 113(1):313-21.). When transplanted in vivo, they are capable of forming new bone, and in vitro, hBM MSCs can be directed towards Osteogenesis by cultivating in β -glycerophosphate, ascorbic acid, vitamin D₃, and low doses of dexamethasone. Osteogenesis for hBMMSCs is regulated by the expression of osteoblast-associated genes, including specific transcription factors, adhesion molecules and proteins of the ECM (Born (2012) *J Cell*

Biochem. 113(1):313-21; Vater (2011) *Acta Biomater.* 7(2):463-77.). The progression to mature osteoblast mirrors that of MC3T3-E1 and occurs with the loss of cellular expansion capacity, the increase of osteogenic markers expression, and the mineralization of the ECM (Born (2012) *J Cell Biochem.* 113(1):313-21). First, cells initiate the synthesis of the ECM with expression of collagen I (Col I). Simultaneously, bone-specific alkaline phosphatase (bALP) expression increases and by Day 4, significant increase in ALP levels in the induced cells (6×10^4 cells/60mm culture dish) from the control could be observed (Born (2012) *J Cell Biochem.* 113(1):313-21; Jaiswal (1997) *J Cell Biochem.* 64:295–312). As differentiation continues, cells produce proteins such as bone sialo protein (BSP), Osteopontin, Osteonectin and osteocalcin. Finally, mineralization of the ECM, much like osteogenesis in MC3T3-E1 cells, indicates a mature osteoblast.

[00912] In this example, genes and transcription factors expressed in the early stages of Osteogenesis in three cell lines: MC3T3-E1 cells and hBM MSC cells were determined. Our results have shown that a statistically significant increase in mineralization could be seen in AMP-treated MC3T3-E1 cells compared to the positive control by Day 2 (Day 1 of treatment). Therefore, the experiment focused on the early stages of Osteogenesis to identify AMP's effect on specific genes after treatment.

[00913] Experimental Design

[00914] MC3T3-E1 or human Bone Marrow MSC cells were seeded at 3×10^4 cells/cm²/well in 24-well plates with α MEM medium plus 10% FBS as described above. Upon confluence, cells were induced to differentiation by adding ascorbic acid and β -glycerolphosphate. For each assay at each timepoint, N=2 was tested. There were 4 time-points sampled: D0 (after confluence but before induction/treatment), D1, D2, D4, and D6. Each time-point had three groups: negative control, positive control, AMP treatment only. AMP concentration used was 125 μ g/ml.

[00915] Results

[00916] hMSC expression

[00917] AMP induces robust endogenous expression of BMP2 and BMP6 transcripts within 24 hrs of culturing (60 and 5 folds, respectively) (FIG. 78A). BMP2 reached its peak at D1 (120 folds) and maintained a high level (between 60 to 10 folds) until D4 before showing a decline. In contrast, BMP6 peaked at 4 h and then showed a gradual decline from D1 on. As a comparison, AMP did not change expression level of BMP4, BMP7 and BMP9. Nonetheless, AG induced a mild upregulation of BMP2 and BMP6 (1-2 fold) only at D4 and notable upregulation of BMP4 (4-10 folds) after D1. As a comparison, AGM did not change expression of BMP7 and BMP9 either.

[00918] Runx2 peaked at D1 for AMP but D2 for AGM (FIG. 78A). Both groups then had a gradual decline followed by another peak at Day 6. Runx2 induces the differentiation of

multipotent mesenchymal cells into immature osteoblasts, directing the formation of immature bone. Furthermore, Runx2 triggers the expression of major bone matrix genes during the early stages of osteoblast differentiation, but Runx2 is not essential for the maintenance of these gene expressions in mature osteoblasts (Komori (2010) *Adv Exp Med Biol.* 658:43-9).

[00919] Both ALP and Sox9 peaked at D4, with the level upregulated by AMP being less than AGM (FIG. 78A). This trend is consistent with the view that ALP and Sox9 are downstream of Runx2. ALP is expressed in the early phase of osteogenesis and creates an alkaline environment which causes calcium to come out of solution and crystallize.

[00920] AMP upregulated VEGF which peaked at 4 h and D4 but AGM upregulated VEGF only at D4 (FIG. 78A). AMP upregulated CXCR4 while AGM upregulated SDF1 at D1, with a rapid decline for the former but a slower one for the latter. Kortesidis et al. ((2005) *Blood.* 105(10):3793-801) suggest that SDF-1 may act to localize primitive uncommitted BMSSC populations within their perivascular niche until required to proliferate and differentiate in response to environmental cues that may act to disrupt SDF-1/CXCR4 interactions. In 7 preconditioning experiments of MSCs, expression of CXCR4 is normally around 2 to 4 fold increase Cencioni et al. ((2012) *Cardiovasc Res.* 94(3):400-7) however our results with AMP showed a 60 fold increase after Day 1.

[00921] SDF-1 expression was very high in all groups of the MSCs, although it does not appear so in FIG. 78A. SDF-1 was seen around Cycle 20 whereas GAPDH was seen around Cycle 17. From the literature it is known stromal cell-derived factor-1 activates adhesion molecules on progenitor cells, and mAb against SDF-1 inhibits transendothelial migration of hematopoietic progenitor cells (Imai et al. (1999) *Blood* 93(1):149-56). SDF-1 activates CXCR4+/CD34+ cells and leads to their adhesion and transendothelial migration (Bhakta et al. (2006) *Cardiovasc Revasc Med.* 7:19-24.). GAPDH expression did increase in the AMP group from Day 2 to Day 6, therefore one would assume proliferation. This trend was not seen in the other samples. Furthermore, BMP9 was not detectable (after Cycle 40), CXCR4 was detected around Cycle 33, VEGF was detected around Cycle 19, and SOX-9 was detected around Cycle 27. BMP4 was detected around Cycle 28, BMP7 was not detected (after Cycle 40), BMP2 was detected around Cycle 20, Runx2 was around Cycle 25, BMP6 was detected around Cycle 27, and ALPL was detected around Cycle 24.

[00922] *MC3T3-E1 expression*

[00923] It is known that MSC are multipotent as where osteoprogenitors are already pushed farther down the lineage pathway and should only produce bone formation. Contrary from what was expected, BMP2 expression was barely expressed in Mouse cells especially compared to human MSC (FIG. 79). BMPs have been shown to be inefficient in promoting osteogenesis in

human, yet are more than capable in Mouse cells (Osyzka et al. (2004) *Cells Tissues Organs*. 176(1-3):109-19., Skarzynska et al. (2011) *Connect Tissue Res*. 52(5):408-14). Expression of BMP-2 is not prominent in MC3T3 cells as indicated by gene array (Beck et al. (2001) *Cell Growth & Differentiation* 12: 61-83). The differences seen between human and rodent cells may indicate that, unlike Smad1 in human cells, rodent Smad1 does not undergo ERK linker phosphorylation during osteogenesis. Alternatively, Smad1 activity in rodent cells may not be suppressed by ERK-mediated linker phosphorylation (Skarzynska et al. (2011) *Connect Tissue Res*. 52(5):408-14). BMP-induced osteogenesis in poorly responsive human MSC requires modulation (inhibiting) of ERK and phosphatidylinositol 3-kinase (PI3-K) pathways; inhibiting the insulin/IGF-I-activated PI3-K/AKT pathway decreases BMP-induced alkaline phosphatase and osteopontin expression in serum-free cultures of human MSC, but increases BMP activation of Smads (Osyzka et al. (2005) *Endocrinology*. 2005 (8):3428-37).

[00924] Runx2 also was shown to be different in the MC3T3 cells compared to hMSCs. There is clearly an incident on Day 2 that causes an upregulation of genes in MC3T3 cells. The AGM groups appeared to have opposite effects of the AMP group, which may be related to Pi3K, MAPK pathways. Ibsp, also known as Bone Sialprotein (BSP), is upregulated on Day 2 in AMP but not expressed other times. In the positive group, BSP is highly expressed after Day 2.

[00925] Bglap-rs1, also known as Osteocalcin (OCN), is also upregulated on Day 2 in AMP but not expressed elsewhere. Consistent with BSP, OC is highly expressed after Day 2 in positive group. Through analysis of these results one would think BSP and OCN need to be upregulated since they are known to be expressed by mature osteoblasts. Runx2 regulates the expression of Col1, BSP and OCN in MC3T3, which is consistent with our results. Runx2 increases on Day 2, as does BSP and OCN, and is not expressed elsewhere. In summary, AMP downregulates osteogenic gene expression but promotes mineralization.

Example 43: Effect of nHC-HA/PTX3 purified from AM on mineralization using MC3T3-E1 model system.

[00926] Previous experiments have clearly demonstrated AMP's unique properties to promote mineralization independent of inducing agents such as ascorbic acid, β -glycerol phosphate, and melatonin (AGM). In these experiments, AMP was added to the murine osteoblast progenitor cell line (MC3T3) in the presence or absence of AGM to determine the potency of the AMP. Alizarin Red Staining with the GnHCl extraction method was also used at multiple time points for further quantification. It was concluded AMP not only enhanced mineralization independent of AGM, but also enhanced mineralization at a faster rate. Further investigation of AMP showed that the

osteoinductive effect exhibited by AMP requires direct cell contact. That is, under direct contact with AMP, cells will have accelerated and enhanced mineralization without the introduction of AGM.

[00927] Previous studies showed that MC3T3-E1 differentiation under induction can be subdivided into three stages, i.e., proliferation (day 1 to 9), ECM formation (day 9 to day 16), and mineralization (deposit minerals in formed ECM) (day 16+) (Quarles et al. (1992) *J Bone Miner Res.* 7(6):683-92.; Hong et al. (2010) *Exp Cell Res.* 316(14):2291-300). However our previous Aim 4 has shown mineralization can be easily detected within 7 to 10 days using AMP. Therefore we will perform our ARS assay at day 8 in order to determine if nHC-HA/PTXS purified from AM is responsible for promoting mineralization. Day 8 was picked because results should be noticeable as seen in Aim 4 and the media will only have to be replaced on day 0, day 3 and day 6.

[00928] It is already known that nHC-HA/PTX3 is responsible for amniotic membrane's known anti-inflammatory, anti-angiogenic and anti-scarring therapeutic actions. It is our hypothesis that immobilized HC-HC/PTX3 is responsible for AMP's effect on promoting mineralization. Because it also contains HA, we will also compare nHC-HA/PTX3 to HA to see the putative effect is uniquely present in nHC-HA/PTX3 but not in HA. Hyaluronan (HA) is an unsulfated glycosaminoglycan consisting of a single repeating disaccharide unit. It is an important component in connective tissue promoting matrix assembly and tissue hydration. Luben et al speculated HA acts as a calcium binding agent to act as a barrier to the diffusion of enzymes away from the resorption site or to regulate the mobility of osteoclasts. Stern and Raisz stated "hyaluronic acid seems to be the most appropriate to study because it has been clearly linked to bone resorption. By the nature of its hygroscopic properties HA can occupy 10,000 times its own volume. Thus, HA allows proliferating cells to avoid inhibitory contacts. Hyaluronic acid synthesis precedes mitosis and dissociates the dividing cell from its substratum, permitting cell movement (Balazs (2001) *Am J Physiol Regulatory Integrative Comp Physiol* 280: R466-R472).

[00929] Experimental Design:

[00930] Murine MC3T3-E1 cells (C-136) were taken from liquid nitrogen freezer and grown on 100mm dish (five dishes) in α MEM medium (10ml per 100 mm dish) plus 10% FBS changed every 3 days till 80% confluence $\sim 1.5 \times 10^6$ cells [$4 \times (3.1 \times 10^4) \times 9 = 1,116,000$ cells]. Cells were then seeded at 3.1×10^4 cells/cm²/96 plastic well. Medium (100 μ l per 96 well) was replaced every 2-3 days, i.e., at Day 0(Wed), 2(Fri), and 5(Mon) and cultures will be terminated at Day 8. N=4 was tested per condition.

[00931] A summary of the Groups was as follows:

[00932] Control Groups:

[00933] Negative Control: Nothing on conventional 96 well plate

[00934] Positive Control 1: AGM on conventional 96 well plate

[00935] Positive Control 2: 125 µg/ml of AMP added every 3 days on conventional 96 well plate

[00936] Experimental Groups:

[00937] Negative Control: Covalink-NH 96 Well Plate

[00938] Experimental Group 1: AGM added every 3 days added to Covalink-NH 96 Well Plate

[00939] Experimental Group 2: 20 µg/ml of HA immobilized on Covalink-NH 96 Well Plate

[00940] Experimental Group 3: 20 µg/ml of HA immobilized on Covalink-NH 96 Well Plate with AGM added every 3 days (H-124)

[00941] Experimental Group 4: 20 µg/ml of nHC-HC/PTX3 immobilized on Covalink-NH 96 Well

[00942] Experimental Group 5: 20 µg/ml of nHC-HC/PTX3 immobilized on Covalink-NH 96 Well Plate with AGM added every 3 days

[00943] For the AGM groups: On Days 0 and 3, Osteogenesis induction media #1 (ascorbic acid, β-glycerolphosphate) was replaced. On Day 6, Osteogenesis induction media #2 (ascorbic acid, β-glycerolphosphate, melatonin) was replaced. On Day 0, only 0.2ml of 10X Induction media was made. On Days 3 and 6, 10ml of the Osteogenesis induction media was prepared fresh. Instructions for Induction Medias obtained from In Vitro Osteogenesis Assay Kit (Millipore).

[00944] Induction media #1: 9.88ml of αMEM medium plus 10%, 20µl Ascorbic Acid 2-Phosphate 500X (Millipore, Part. 2004011), 100µl Glycerol 2-Phosphate 100X (Millipore, Part. 2004011).

[00945] Induction media #2: 9.87ml of αMEM medium plus 10%, 20µl Ascorbic Acid 2-Phosphate 500X (Millipore, Part. 2004011), 100µl Glycerol 2-Phosphate 100 X (Millipore, Part. 2004011), 10µl Melatonin 50uM (Millipore, Part. 2004011). Add 500ul dH2O to 6 ug of melantonin supplied.

[00946] ARS Staining and Quantification was performed as described above. Pictures were taken at 10X using Nikon Eclipse CFI60.

[00947] Results

[00948] The MC3T3-E1 cells were cultured on the different well plates for 8 days. On Day 8, phase contrast pictures were taken of the wells and can be seen below (FIG. 79A, 79B). Negative control wells showed round cells and the ARS stained a very light pink. Wells with induction media showed much brighter red color and spindle cells were seen on the periphery of the wells. The ARS staining was seen more in abundance in the outer periphery ring. Treatment with AMP showed a crimson red after ARS treatment and the cells were rather hard to see because the AMP

settled on top of them. No aggregation was seen during the experiment (even though aggregation has been seen with other cell types on immobilized HC-HA.) Microscopy pictures were taken on Days 6, 7 and 8.

[00949] Guanidine hydrochloride extraction method was used on Day 8 and the well plates were incubated overnight. GnHCl was able to extract the ARS dye although it was hard to tell by the naked eye; all the wells seemed to have the same light pink/red color. The ARS extraction was quantified at 450nm because the plate reader did not have the capabilities of reading at 490nm or closer. Results can be seen in FIG. 79C. The * symbol denotes statistical significance of $p < 0.05$. (+ denotes with AGM, - denotes without AGM)

[00950] In agreement with previous results, AMP was able to successfully promote mineralization without the need of induction agents. This experiment only lasted 8 days so the results aren't as noticeable as in Aim 4 which lasted 20 days. All conditions that were treated with AGM showed an increase in mineralization from their negative control counterpart. Our results also show that immobilized nHC-HA/PTXS is not responsible for promoting mineralization in AMP. Therefore there must be another active component of AMP that is promoting mineralization.

Example 44: Effect of HC-HA/PTX3 (PBS) and HC-HA/PTX3 (Gn) on endochondral ossification

[00951] Master transcription factors for osteogenesis and chondrogenesis (Runx2 and Sox9, respectively) were expressed by cells in both HC-HA conditions through the 14 day culture period. HC-HA/PTX3, both soluble and insoluble, were able to promote the expression of BMP2 and to an extent BMP6 without osteoinductive agents AGM (i.e., ascorbic acid, glycerolphosphate, melatonin) commercially provided (see below).

[00952] Chondrogenic marker Collagen 2 was highly expressed by HC-HA/PTX3 (PBS) without the need for AGM. HC-HA/PTX3 (Gn) with the addition of AGM also was able to upregulate Collagen 2. Osteogenic markers (BSP, ALPL, Osx) were upregulated by the HC-HA conditions on Day 14 thus confirming a transition from a cartilage to bone genotype.

[00953] Experimental Design:

[00954] Culture conditions: Human bone marrow-derived mesenchymal stem cells purchased from Lonza (Basel, Switzerland) were taken from liquid nitrogen freezer and grown on 100mm dish in the same medium changed every 3 days till 80% confluence. Cell culture medium was α MEM containing 10% fetal bovine serum and antibiotics. Culture medium (10ml per 100 mm dish) was changed every 3 days, and cells subpassaged at 80% confluence until they reach the above desirable cell numbers. For the experiment the cells were seeded at 3.1×10^4 cells/96 plastic well on

immobilized HA, HC-HA (PBS) or HC-HA (Gn) with or without osteoinductive agents Ascorbic Acid 2- Phosphate, Glycerol 2-Phosphate and Melatonin (AGM). The final concentration of AGM added was 0.2mM, 10mM and 50nM, respectively. AGM was added simultaneously when cells were seeded (D0) and mRNA was extracted at Days 1, 7 and 14. In order to quantify gene expression, qPCR was performed. Culture medium (100 ul per 96 well) was replaced every 3 days.

[00955] A summary of the experimental Groups was as follows:

[00956] Negative Control: Covalink-NH 96 Well Plate

[00957] Experimental Group 1: AGM added every 3 days added to Covalink-NH 96 Well Plate

[00958] Experimental Group 2: 20 µg/ml of HA immobilized on Covalink-NH 96 Well Plate

[00959] Experimental Group 3: 20 µg/ml of HA immobilized on Covalink-NH 96 Well Plate with AGM added every 3 days

[00960] Experimental Group 4: 20 µg/ml of 4X nHC-HC/PTX3 immobilized on Covalink-NH 96 Well Plate

[00961] Experimental Group 5: 20 µg/ml of 4X nHC-HC/PTX3 immobilized on Covalink-NH 96 Well Plate with AGM added every 3 days

[00962] Experimental Group 6: 20 µg/ml of 4X nHC-HC/PTX3 (GuHCl extraction) immobilized on Covalink-NH 96 Well Plate

[00963] Experimental Group 7: 20 µg/ml of 4X nHC-HC/PTX3 (GuHCl extraction) immobilized on Covalink-NH 96 Well Plate with AGM added every 3 days

[00964] For the AGM induction groups: On Days 0 and 3, Osteogenesis induction media #1 (ascorbic acid, glycerolphosphate) replaced the media. On Day 6, Osteogenesis induction media #2 (ascorbic acid, glycerolphosphate, melatonin) will replace the media. On Day 0, 10X Induction media was made. On Days 3 and 6, 10ml of the Osteogenesis induction media was prepared fresh. Instructions for preparation of Induction Media obtained from In Vitro Osteogenesis Assay Kit (Millipore).

[00965] Induction media #1: 9.88ml of αMEM medium plus 10%, 20µl Ascorbic Acid 2-Phosphate 500X (Millipore, Part. 2004011), 100µl Glycerol 2-Phosphate 100X (Millipore, Part. 2004011)

[00966] Induction media #2: 9.87ml of αMEM medium plus 10%, 20µl Ascorbic Acid 2-Phosphate 500X (Millipore, Part. 2004011), 100µl Glycerol 2-Phosphate 100 X (Millipore, Part. 2004011), 10µl Melatonin 50uM (Millipore, Part. 2004011). Add 500ul dH2O to 6 ug of melantonin supplied.

[00967] mRNA was extracted from cells on Days 1, 7, and 14 and gene expression was determined by QPCR (FIG. 80A-E). The following genes were assayed: Osteogenesis markers

Runx2, alkaline phosphatase (ALPL), markers Collagen 1 (COL1), Osterix (OSX) and Bone Sialoprotein (BSP) and chondrogenesis markers Sox9 and Collagen 2 (COL2), hypertrophic markers Collagen 10 (COL10) and MMP13. ARS staining and quantification was performed on Day 14 cultures as described above (FIG. 81A, 81B).

[00968] AGM upregulated BMP4 on plastic. HA upregulated BMP4 (early) but downregulated (late) BMP6 and did not affect BMP2 (FIG. 80B). No literature data suggests HA itself upregulates BMP. However, addition of AGM upregulated BMP2 and BMP6.

[00969] 4X Soluble HC-HA initially upregulated BMP4 but downregulated BMP4 late (like HA) and markedly upregulated BMP2 (like AMP, but without transient BMP6) (FIG. 80B). In contrast, addition of AGM did not change the expression pattern of BMPs.

[00970] 4X Insoluble HC-HA initially upregulated BMP4 but downregulated BMP4 (late) (like HA) and markedly upregulation of BMP2 (like Soluble HCHA) (FIG. 80B). [Identical to Soluble] Similarly, addition of AGM did not change the expression pattern.

[00971] Our results show soluble HC-HA and insoluble HC-HA were able to form bone differentiation and mineralization through an endochondral mechanism. Expression of bone markers (Col1, Osx, ALP, and BSP) were apparent as were expression of chondrocyte markers (Col2) and hypertrophy markers (Col10, MMP13) (FIG. 80A-E). The difference between these HC-HA conditions is that insoluble HC-HA was able to promote greater amplitude of gene expressions and more noticeable bone nodules (even without AGM) while soluble HC-HA requires AGM (data not shown). Thus, HC-HA/PTX3, both soluble and insoluble, were able to promote the expression of BMP2 without osteoinductive agents AGM.

[00972] HA without AGM also showed chondrogenic markers (COL2) but also showed signs of bone formation with ARS defined mineralization and a slight increase in ALP and OC. However, HC-HA/PTX3 (PBS) had greater chondrogenic expression and higher expression of bone markers ALP, Osx and BSP than HA. Yet neither of these conditions expressed significant hypertrophic markers. HC-HA/PTX3 (Gn) expressed much greater expression of ALP, OSX and BSP than the two aforementioned conditions. Hypertrophic marker MMP13 was also expressed as was slight expression of chondrogenic marker COL2.

[00973] HA plus AGM promotes osteogenesis with increased BMP2, ALP, Osx, BSP and OC expression and exhibits hypertrophic markers COL10 and MMP13. However, HA produced less bone specific mRNA expression and bone nodule formation than HC-HA groups. Another key difference was HA downregulated SOX9 but increased BMP6 expression late.

[00974] All prior data indicates insoluble HCHA is the strongest inducer of bone and, more importantly, induces an endochondral mechanism.

Example 45. nHC-HA/PTX3 Suppresses Inflammatory and Immune Responses and Improves Murine Corneal Allograft Survival

[00975] Experimental and clinical studies have shown that amniotic membrane (AM), AM extract, and nHC-HA/PTX3 [a covalent complex formed by heavy chain (HC) of inter- α -trypsin inhibitor (I α I) and hyaluronan (HA)] suppress pro-inflammatory responses. This example demonstrates that nHC-HA/PTX3/PTX3 can regulate T cell responses and reduce murine corneal allograft rejection.

[00976] T cell activation may be assessed by proliferation and production of various cytokines (FIG. 82). In this instance, splenocytes were isolated from OT-II mice that express a transgenic TCR specific for ovalbumin (OVA), and stimulated with OVA up to 4 days (FIG. 83). Cell proliferation was measured by BrdU labeling and expression of cytokines (IFN- γ and IL-2) was measured by the respective ELISA. nHC-HA/PTX3 but not HA at 1 mg/ml significantly suppressed the proliferation (FIG. 84) and production of IFN- γ and IL-2 (FIG. 85) in splenocytes with OVA peptide at day 2 and day 4 (all $p < 0.05$). Furthermore, corneal T cells were activated in vivo by LPS injection.

[00977] Optimization of injection sites, volume, and frequency with nHC-HA/PTX3 before or during intracorneal injection of LPS was determined by influx of EGFP-positive macrophages into corneas of Mafia mice. The injection regimen was further optimized by giving 5 μ l at each injection between subconjunctiva and fornix to all four quadrants. At day 4 after nHC-HA/PTX3 treatment, corneas were digested with 820 units/ml of collagenase at 37 °C for 1 h. EGFP- and EGFP+ cells were isolated by FACS. Pretreatment of nHC-HA/PTX3 3 days prior to LPS injection significantly suppressed the influx of EGFP+ macrophages to LPS-insulted corneas (9.1 ± 0.3 vs. 12.3 ± 0.4 , nHC-HA/PTX3 vs PBS, $p = 0.02$) (FIG. 86). Importantly, even though EGFP+ macrophages did migrate into corneas, some of them were polarized into M2 phenotype as suggested by significant up-regulation of Arg-1 and IL-10 but down-regulation of IL-12 ($p < 0.05$) (FIG. 87). mRNA expression of Arg-1, IL-10, and IL-12 were measured by qPCR. Finally, allogeneic corneal transplantation was performed using wild-type BALB/c mice as recipients and C57BL/6 mice as donors, and its outcome scored by graft clarity measured twice a week using slit lamp biomicroscopy. Grafts that received two consecutive scores ≥ 3 without resolution were considered rejected. Compared to PBS control, allograft rejection was significantly suppressed by injection of 10 μ l nHC-HA/PTX3 at one quadrant twice a week ($p < 0.05$), and further reduced by injection with 5 μ l at 4 quadrants twice a week ($p < 0.002$) (FIG. 88).

[00978] These experiments demonstrate that nHC-HA/PTX3 significantly suppresses murine corneal allograft rejection. The mechanism of this action may be contributed by nHC-HA/PTX3's ability to down-regulate pro-inflammatory macrophages and to suppress T cell immune response.

Example 46: Treatment of Mouse Dry Eye Caused by Desiccating Stress by nHC-HA/PTX3 and AMP

[00979] Dry eye, also known as dysfunctional tear syndrome, is a common ocular surface disease with high prevalence and significant morbidity worldwide. It is an autoimmune-based inflammatory disease characterized by chronic auto-reactive T cell-mediated inflammation and dysfunction of the lacrimal function unit (LFU; cornea, conjunctiva, lacrimal glands, and meibomian glands). Sjögren's syndrome (SS) is a prevalent chronic autoimmune disorder characterized by infiltration of salivary and lacrimal glands by mononuclear cells, causing secondary destruction of the parenchymal tissue.

[00980] Keratoconjunctivitis sicca (KCS) in SS is a severe and potentially sight-threatening ocular surface epithelial disease characterized by infiltrating CD4⁺ T cells producing IL-17 and interferon (IFN)- γ . Compounds that inhibit T cell activation (e.g., cyclosporine A) attenuate dry eye disease in both animals and humans. Macrophages may undergo classical M1 activation (e.g., by IFN- γ and/or TLR ligands such as LPS) to express high levels of proinflammatory cytokines (such as TNF- α , IL-12, and IL23), which activate Th1 and Th17 lymphocytes (Fig 89) leading to many chronic inflammatory diseases. This example demonstrates that nHC-HA/PTX3 and AMP administration may be useful in the treatment of such conditions.

[00981] Infiltration of macrophages into corneas is inhibited with four injection sites for each eye

[00982] MAFIA mice permit *in vivo* tracking of macrophage influx as they are labeled with EGFP. These mice were used to determine if nHC-HA/PTX3 or AMP prevents LPS-induced macrophage influx to the cornea, a model for keratitis. LPS was injected between subconjunctiva and fornix at a suitable volume of 5 μ l or less at each injection site. Eyes of MAFIA mice (macrophages are EGFP⁺) were intrastromally injected with LPS (5 μ g per eye). In each eye, OS was treated with PBS (2 or 4 injection sites) while OD was treated one time with either nHC-HA/PTX3 (2 or 4 injection sites; 5 μ l of 1 mg/ml HA in nHC-HA/PTX3 per injection site) or AMP (2 or 4 injection sites; 5 μ l of 10 mg/ml protein in AMP per injection site). The treatment was immediately after LPS injection. Images of whole corneas were taken with *in vivo* intravital microscopy on day 1, day 2, day 3 and day 6. EGFP-positive cells were counted based on the intensity of green fluorescence.

[00983] At Day 1, EGFP-positive macrophages are detected in the most corneal peripheral area after LPS injection with PBS treatment. Treatment with either nHC-HA/PTX3 or AMP did not significantly increase or decrease macrophages in corneas.

[00984] At Day 2, the macrophages in corneas with PBS treatment increased significantly ($p < 0.05$) from Day 1, so did with treatment of nHC-HA/PTX3 (2 and 4 injection sites, $p < 0.05$) and of AMP (2 and 4 injection sites, $p < 0.05$). Specifically, more macrophages were infiltrated in corneas treated with 2 injections of nHC-HA/PTX3 than those with PBS treatment ($p > 0.05$), but less are in corneas with 4 injections of nHC-HA/PTX3 ($p > 0.05$). For AMP treatment, 2 injections had no significant effect but 4 injections slightly decreased the infiltration of macrophages, suggesting 4 injections of either nHC-HA/PTX3 or AMP for each eye is necessary to have an effect on reducing the infiltration of macrophages.

[00985] At Day 3, the infiltration of macrophages continued with treatment of PBS, nHC-HA/PTX3, and AMP.

[00986] There was no inhibition of the infiltration with treatment of either 2 injections, 4 injections of nHC-HA/PTX3, or 2 injections of AMP. The only treatment shows the inhibition is the 4 injection of AMP ($p < 0.05$).

[00987] At Day 6, the infiltration of macrophages decreased. However, no treatment of HC-HA/PTX3 or AMP had a significant inhibitory effect compared to control.

[00988] These data showed EGFP-positive macrophages continue to infiltrate into LPS-injected corneas from Day 1 to Day 3 and peaks at Day 4 or Day 5, then decline at Day 6. This is consistent with the previous reported data (FIG. 90). The infiltration of macrophages was slightly inhibited by treatment with 4 injections of nHC-HA/PTX3 per eye at Day 2 or with 4 injection of AMP at Day 2 and Day 3. This suggests AMP has a better potency than nHC-HA/PTX3 in blocking the influx of macrophages elicited by LPS.

[00989] Pretreatment with AMP significantly inhibits the macrophage infiltration incited by injuries due to additional injections if followed by subsequent injections of either nHC-HA/PTX3 or AMP

[00990] The left eye (OS) of each MAFIA mouse was pretreated with PBS (5 μ l) or AMP (5 μ l of 10 mg/ml protein) at 4 sites of subconjunctival/fornix as defined above. The right eye (OD) of each mouse was left untreated. Three days later, each eye was injected with LPS (5 μ g) to the cornea and immediately followed by treatment with PBS (5 μ l), HC-HA/PTX3 (5 μ l of 1 mg/ml HA), or AMP (5 μ l of 10 mg/ml protein) at 4 sites. The infiltration of EGFP⁺ macrophages was counted using in vivo intravital microscopy, which did not disclose any significance ($P > 0.05$) in reducing

macrophage influx to mouse corneas (data not shown). We then investigated the accuracy of this quantitative method by in vivo fluorescence microscopy.

[00991] To measure more accurately the infiltrated macrophages and examine the resultant macrophage phenotype (e.g., M1 vs. M2), we decided to quantitate EGFP-positive macrophages by subjecting the corneas removed at Day 4 to collagenase digestion and FACS. EGFP-positive macrophages were then normalized by EGFP-negative cells as a ratio to assess the extent of macrophage infiltration. In groups with no pretreatment, LPS injection caused macrophage infiltration in the PBS control group (FIG. 91, A, the blue bar). This infiltration was significantly inhibited by nHC-HA/PTX3 (9.1 ± 0.3 vs. 12.3 ± 0.4 , $p=0.02$) or AMP (2.1 ± 0.1 vs. 12.3 ± 0.4 , $p=0.02$). AMP treatment was better than nHC-HA/PTX3 treatment in inhibition of the macrophage infiltration ($p=0.02$).

[00992] In groups with pretreatment, LPS injection caused significant macrophage infiltration in PBS control group compared with no pretreatment (37.2 ± 1.3 vs. 12.3 ± 0.4 , $p=0.01$) (FIG. 91, A, the red bar). This difference was expected as 4 subconjunctival injections made during pretreatment caused injury that elicited inflammation, which augmented macrophage influx to the cornea which was later on treated by LPS. Nonetheless, this dramatic increased infiltration was completely inhibited by AMP pretreatment followed with either nHC-HA/PTX3 (8.2 ± 0.3 vs. 37.2 ± 1.3 , $p=0.02$) or AMP treatment (2.3 ± 0.1 vs. 37.2 ± 1.3 , $p=0.02$). Again, AMP pretreatment followed by AMP treatment was better than AMP pretreatment followed by nHC-HA/PTX3 treatment in inhibiting macrophage infiltration (2.3 ± 0.1 vs. 8.2 ± 0.3 , $p=0.02$). However, there was no significant difference in inhibition between the group with no pretreatment and the group with pretreatment of AMP for either nHC-HA/PTX3 or AMP ($p > 0.05$). qPCR data (FIG. 91, B) show pretreatment of nHC-HA/PTX3 decreases M1 markers if IL-12p40 and IL-12p35 while increases M2 markers of Arg-1. AMP treatment and pretreatment significantly decreases IL-12p40 and IL-12p35 but greatly increases Arg-1 and IL-10. In all, AMP pretreatment can completely eliminate the macrophage infiltration incited by additional injuries during the pretreatment. Such an effect is sustained by subsequent injection of either nHC-HA/PTX3 or AMP simultaneously with LPS injection. This benefit is noted 4 days later, and AMP is more potent than nHC-HA/PTX3.

[00993] nHC-HA/PTX3 or AMP can reduce DS-induced ALKC in a murine experimental dry eye model.

[00994] Design

[00995] Species: C57BL/6 mice

[00996] Endpoints: corneal epithelial barrier function (OGD staining)

[00997] Sample Size: 15 mice per group

[00998] Groups: 2 controls and 3 treatment groups (PBS, nHC-HA/PTX3, and AMP): 1) Non-dry eye, untreated control (UT) – kept in a separate vivarium room; 2) Experimental dry eye, untreated control (EDE); 3) PBS; 4) nHA-HC/PTX; 5)AMP.

[00999] Desiccating Stress (DS)

[001000] The desiccating stress model is created by pharmacological cholinergic blockade of tear secretion and exposure to an air draft and low humidity in an environmentally controlled room for 5 days (Monday-Friday). Mice are placed in specially designed perforated cages which consist of regular mouse cages that have their sides replaced by a wire mesh to allow air flow through the cage. Each cage is placed in front a constant air flow (electrical fan). Lacrimal gland secretion is inhibited by subcutaneous administration of scopolamine (0.5mg in 0.2mL, Sigma-Aldrich) 4 times per day for 5 days (8:30am, 11:30am, 1:30pm, 4:30pm). Humidity in the environmentally controlled room is maintained at ~25-30% relative humidity, which is achieved by 4 portable dehumidifiers and a dehumidifier unit in the ceiling.

[001001] Treatment Procedure (5-Day Protocol)

[001002] During each experiment, 3 controls are included:

[001003] Untreated control, consists of a group of mice that are kept in the vivarium, under relative humidity of 40~70%. These mice are never exposed to DS nor receive any topical treatment.

[001004] Dry eye control, which consists of a group of mice that are placed in the environmental dry eye chamber but receive no treatment.

[001005] Vehicle control, consists of a group of mice that are subjected to DS but receive PBS.

[001006] In addition, two experimental groups are included: nHA-HC/PTX3 and AMP

[001007] For injection, we used nHC-HA/PTX3 (containing 1 mg/ml HMW HA) and AMP (containing 10 mg/ml total protein) with PBS as the vehicle control. All solutions (PBS, nHC-HA/PTX3, and AMP) were drawn into a tuberculin syringe with 30 G. The injection locations are subconjunctiva close to fornix (Fig 92). Four (4) injections at 3, 6, 9, and 12 o'clock with 5 µl per injection sites were administrated. The diffused solution completely covered the whole peripheral of conjunctiva and caused a minimum conjunctival or globe congestion/swelling (If any, it should disappear in 15 min) which impeded eye closure and corneal surface breakdown or inflammation. The injection was administrated at Day 1 and Day 3 (for all reagents), making a total of 2 times. This injection protocol is summarized in Table 6.

Table 6. Experimental groups and required reagents for nHC-HA/PTX3 or AMP reduction of DS-induced ALKC in murine experimental dry eye model.

Group	Treatment	# Mice	Injection sites x volume x days x eyes x mice
1	UT	15	NA

2	EDE	15	NA
3	EDE + PBS	15	4 x 5 μ l x 5 x 2 x 15 = 3.0 ml
4	EDE + nHC-HA /PTX3 (1 mg/ml)	15	4 x 5 μ l x 5 x 2 x 15 = 3.0 ml
5	EDE + AMP (10 mg/ml)	15	4 x 5 μ l x 5 x 2 x 15 = 3.0 ml

[001008] Measurement of Corneal Staining

[001009] On the morning of 5th day, mice received one s.c. dose of scopolamine after measurement of tear volume. 2 hours after that scopolamine dose corneal staining was performed using Oregon Green Dextran (OGD-488), which is a conjugated fluorescent dye of a 70kDa molecular size (Molecular Probes). The procedure consisted of instillation of 0.5 μ l of OGD on the cornea using a glass capillary pipette, 1 minute before euthanasia. Mice were euthanized by inhalation of isoflurane anesthetic gas followed by cervical dislocation. Eyes were then rinsed with 2 ml of BSS. Excess liquid was carefully blotted from the ocular surface with filter papers without touching the cornea. Digital images of both eyes were captured under 470 nm excitation and 488 nm emission wave lengths using a Nikon SMZ-1500 stereo microscope with CoolSnap HQ₂ cooled CCD camera, with an exposure time of 1 second. Both eyes from each animal were evaluated. The fluorescence intensity in a fixed region of interest (a 1-mm diameter circle) in the central cornea was measured in 3 digital images using Nikon Elements software and data is stored in a database (Excel, Microsoft). Results were presented as mean $\pm$ standard deviation of gray levels. Results from 3 separate experiments were averaged for statistical comparisons of groups.

[001010] The effects of nHC-HA/PTX3 and lyophilized amniotic membrane powder (AMP) on levels of T helper cell pathway mediators were compared in an experimental dry eye (EDE) model created in C57BL/6 mice for 5 days. Expression of Th1 (IL-12, IFN- γ and T-Bet), Th-17 (IL-23, IL-17, ROR- γ t, IL-6, TGF- β 1, MMP-3 and MMP-9) and Th2 (IL-4, IL-13 and GATA3) related factors were measured in the corneal epithelium and conjunctiva in the following groups by real-time PCR.

[001011] Statistical analysis

[001012] Statistical analysis was performed using GraphPad Prism 5.0 software (GraphPad Inc). One-way analysis of variance (ANOVA) was used to determine overall differences among groups, followed by a post-hoc test (Tukey's post hoc). An unpaired t-test is used to evaluate statistical differences between 2 experimental groups.

Example 47: HC-HA activates IGF1-HIF1 α -VEGF signaling to promote angiogenesis, which is further promoted by addition of TGF β 1 in human corneal fibroblasts.

[001013] In this example, the effect of HC-HA complexes on the induction of angiogenic markers in human corneal fibroblasts was examined.

[001014] Human corneal fibroblasts (3000 cells/well in a 96-well plate) were seeded on plastic dishes with or without immobilized HA, soluble HC-HA (PBS) (4X) or insoluble HC-HA (GnHCl) (4X) for 48 h as described above. The cells were then treated with or without TGF β 1 for 24 h before being harvested for mRNA quantitation of IGF1, HIF1 α and VEGF. The experimental groups were:

PBS
PBS+TGF- β 1
HA
HA+TGF- β 1
4X HC-HA PBS
4X HC-HA PBS+TGF- β 1
4X HC-HA Gn
4X HC-HA Gn+TGF- β 1

[001015] Total RNAs were extracted using RNeasy Mini Kit (Qiagen) and were reverse transcribed using High Capacity Reverse Transcription Kit (Applied Biosystems). cDNA of each cell component was amplified by real-time RT-PCR using specific primer-probe mixtures and DNA polymerase in 7000 Real-time PCR System (Applied Biosystems). Real-time RT-PCR profile consisted of 10 minutes of initial activation at 95°C, followed by 40 cycles of 15 seconds denaturation at 95°C, and 1 minute annealing and extension at 60°C. The identity of each PCR product (IGF1, HIF1 α and VEGF) was confirmed size determination using 2% agarose gels followed by ethidium bromide staining together with PCR marker according to EC3 Imaging System (BioImaging System).

[001016] HC-HA induced a 2- to 6-fold increase of IGF1 mRNA and 2-fold increase of VEGF mRNA when the cells were in resting conditions (FIG. 92). In contrast, HC-HA induced 5- to 12-fold increase of IGF1 mRNA and 5- to 9-fold increase of VEGF mRNA when the cells were challenged by TGF β (10 ng/ml). VEGF has been demonstrated to be a major contributor to angiogenesis, increasing the number of capillaries in a given network. VEGF activation is controlled by upstream regulators such as IGF1 and HIF1 α . Our results demonstrate that HC-HA activates IGF1-HIF1 α -VEGF network to promote angiogenesis, which is further promoted by addition of TGF β 1 in human corneal fibroblasts.

[001017] While preferred embodiments have been shown and described herein, it will be obvious to those skilled in the art that such embodiments are provided by way of example only. Numerous variations, changes, and substitutions may now occur. It should be understood that various

alternatives to the embodiments described herein can be employed in practicing the described methods. It is intended that the following claims define the scope of the embodiments and that methods and structures within the scope of these claims and their equivalents be covered thereby.

CLAIMS

WHAT IS CLAIMED IS:

1. A method for producing a reconstituted HC-HA/PTX3 (rcHC-HA/PTX3) complex *in vitro*, comprising contacting (i) high molecular weight hyaluronan (HMW HA) immobilized to a solid support, (ii) pentraxin 3 (PTX3) protein, (iii) inter- α -inhibitor (I α I) protein comprising heavy chain 1 (HC1) and (iv) Tumor necrosis factor α -stimulated gene 6 (TSG-6) to form an immobilized rcHC-HA/PTX3 complex.
2. The method of claim 1, comprising:
 - (a) contacting (i) high molecular weight hyaluronan (HMW HA) immobilized to a solid support, and (ii) pentraxin 3 (PTX3) protein, to form an immobilized complex of PTX3 and HMW HA (immobilized PTX3/HA); and
 - (b) contacting the immobilized PTX3/HA with an inter- α -inhibitor (I α I) protein comprising heavy chain 1 (HC1) and Tumor necrosis factor α -stimulated gene 6 (TSG-6) to form an immobilized rcHC-HA/PTX3 complex.
3. The method of claim 1, comprising:
 - (a) contacting (i) high molecular weight hyaluronan (HMW HA) immobilized to a solid support, (ii) an inter- α -inhibitor (I α I) protein comprising heavy chain 1 (HC1) and (iii) TSG-6 to form an rcHC-HA complex pre-bound to TSG-6; and
 - (b) contacting rcHC-HA complex pre-bound to TSG-6 with a pentraxin 3 (PTX3) protein to form an rcHC-HA/PTX3 complex.
4. The method of any of claims 1-3, wherein TSG-6 catalyzes the covalent linkage of I α I HC1 to HA.
5. The method of claim 2, further comprising removing unbound PTX3 protein following step (a) and prior to performing step (b).
6. The method of any of claims 2-5, further comprising removing unbound TSG-6 following step (b).
7. The method of any of claims 1-6, wherein the PTX3 protein is native PTX3 protein or recombinant PTX3 protein, and the TSG-6 protein is native TSG-6 protein or recombinant TSG-6 protein.
8. The method of any of claims 1-7, wherein I α I protein further comprises HC2, bikunin, a chondroitin sulfate chain, or any combinations thereof.
9. The method of any of claims 1-8, wherein I α I protein is isolated from blood, serum, plasma, amniotic membrane, chorionic membrane, amniotic fluid, or a combination thereof.

10. The method of claim 7-9, wherein IαI protein, native TSG-6 protein, and/or native PTX3 protein is produced by an amniotic membrane cell.
11. The method of claim 10, wherein the amniotic membrane cell is an amniotic epithelial cell, amniotic stromal cell, umbilical cord epithelial cell, or umbilical cord stromal cell.
12. The method of any of claims 1-11, wherein PTX3 is a homomultimer.
13. The method of claim 12, wherein the PTX3 homomultimer is an octamer.
14. The method of any of claims 1-13, wherein PTX3 comprises a modified multimerization domain or a heterogeneous multimerization domain.
15. The method of any of claims 1-14, wherein the PTX3, IαI HC1 or TSG-6 polypeptides comprise an affinity tag.
16. The method of claim 15, wherein the affinity tag is selected from among a hemagglutinin tag, a poly-histidine tag, a myc tag, a FLAG tag, a glutathione-S-transferase (GST) tag.
17. The method of any of claims 1-16, wherein the IαI and TSG-6 protein are contacted at a molar ratio of 3:1.
18. The method of any of claims 1-17, wherein the weight average molecular weight of the HMW HA is 3,000 kDa.
19. The method of any of claims 1-18, wherein the HMW HA is immobilized by direct linkage to a solid support.
20. The method of any of claims 1-18, wherein the HMW HA is immobilized by indirect linkage to a solid support.
21. The method of any of claims 1-18, wherein the HMW HA is immobilized by linkage to a solid support via a cleavable linker.
22. The method of claim 21, wherein the cleavable linker is a chemically or enzymatically cleavable linker.
23. The method of any of claims 1-18, wherein the HMW HA is immobilized by covalent or non-covalent attachment to the solid support.
24. The method of any of claims 1-18, wherein the HMW HA is immobilized by binding HMW HA to an intermediary polypeptide.
25. The method of claim 24, wherein the intermediary polypeptide is covalently attached to the solid support.
26. The method of claim 24 or claim 25, wherein the HMW HA is bound to the intermediary polypeptide by a non-covalent bond.

27. The method of any of claims 24-26, wherein the intermediary polypeptide is an HA binding protein (HABP).

28. The method of claim 27, wherein the HABP is selected from among HAPLN1, HAPLN2, HAPLN3, HAPLN4, aggrecan, versican, neurocan, brevican, phosphacan, TSG-6, CD44, stabilin-1, stabilin-2, or a portion thereof sufficient to bind HA.

29. The method of claim 27, wherein the HABP is versican.

30. The method of any of claims 24-29, wherein the intermediary polypeptide comprises a link module.

31. The method of claim 30, wherein the link module is a link module of HAPLN1, HAPLN2, HAPLN3, HAPLN4, aggrecan, versican, neurocan, brevican, phosphacan, TSG-6, CD44, stabilin-1, or stabilin-2.

32. The method of any of claims 24-31, wherein the intermediary polypeptide comprises a polypeptide linker.

33. The method of claim 32, wherein the polypeptide linker is attached to the solid support.

34. The method of any of claims 24-33, wherein the intermediary polypeptide comprises a proteolytic cleavage sequence.

35. The method of any of claims 1-22, further comprising dissociation of the immobilized rcHC-HA/PTX3 complex to form an dissociated rcHC-HA/PTX3 complex.

36. The method of claim 35, wherein dissociation of the immobilized rcHC-HA/PTX3 complex comprises cleavage of a cleavable linker.

37. The method of claim 35, wherein dissociation of the immobilized rcHC-HA/PTX3 complex comprises contacting the immobilized rcHC-HA/PTX3 complex with a dissociation agent.

38. The method of claim 37, wherein the dissociation agent is guanidine hydrochloride or urea.

39. The method of any of claims 35-38, wherein the method further comprises purification of the dissociated rcHC-HA/PTX3 complex.

40. The method of claim 39, wherein purification comprises affinity purification, centrifugation, filtration, chromatography or a combination thereof.

41. The method of any of claims 1-40, further comprising contacting the immobilized PTX3/HA complex or rcHC-HA/PTX3 complex with a small leucine rich proteoglycan (SLRP).

42. The method of claim 41, wherein the SLRP is selected from among decorin, biglycan, fibromodulin, lumican, PRELP (proline arginine rich end leucine-rich protein), keratocan, osteoadherin, epipycan, and osteoglycin.

43. The method of claim 41, wherein the SLRP is selected from among decorin, biglycan, and osteoglycin.

44. The method of any of claims 41-43, wherein the SLRP is covalently attached to a glycosaminoglycan.

45. The method of claim 44, wherein the glycosaminoglycan is keratan sulfate.

46. A reconstituted HC-HA (rcHC-HA/PTX3) complex produced by the method of any of claims 1-45.

47. A reconstituted HC-HA (rcHC-HA/PTX3) complex comprising high molecular weight hyaluronan (HMW HA), (ii) pentraxin 3 (PTX3) protein, and (iii) inter- α -inhibitor (I α I) protein comprising heavy chain 1 (HC1).

48. The rcHC-HA/PTX3 of claim 47 immobilized to a solid support.

49. An isolated native HC-HA/PTX3 (nHC-HA/PTX3) complex purified from an amniotic membrane extract or an umbilical cord extract.

50. The isolated nHC-HA/PTX3 complex of claim 49, wherein the umbilical cord extract or amniotic membrane extract is prepared from amniotic membrane, umbilical cord amniotic membrane, umbilical cord stroma or any combinations thereof.

51. The isolated nHC-HA/PTX3 complex of claim 49 or claim 50, wherein the nHC-HA/PTX3 complex is purified by ultracentrifugation of the amniotic membrane extract or umbilical cord extract.

52. The isolated nHC-HA/PTX3 complex of claim 51, wherein the amniotic membrane extract or umbilical cord extract is a water insoluble extract.

53. The isolated nHC-HA/PTX3 complex of claim 52, wherein the water insoluble extract is extracted with guanidine hydrochloride.

54. The isolated nHC-HA/PTX3 complex of any of claims 51-53, further comprising a small leucine rich proteoglycan (SLRP).

55. The isolated nHC-HA/PTX3 complex of claim 54, wherein the SLRP is selected from among decorin, biglycan, fibromodulin, lumican, PRELP (proline arginine rich end leucine-rich protein), keratocan, osteoadherin, epipycan, and osteoglycin.

56. The isolated nHC-HA/PTX3 complex of claim 54, wherein the SLRP is selected from among decorin, biglycan, and osteoglycin.

57. The isolated nHC-HA/PTX3 complex of any of claims 54-56, wherein the SLRP is covalently attached to a glycosaminoglycan.

58. The isolated nHC-HA/PTX3 complex of claim 57, wherein the glycosaminoglycan is keratan sulfate.

59. The isolated nHC-HA/PTX3 complex of any of claims 49-51, wherein the amniotic membrane extract or umbilical cord extract is a water soluble extract.

60. The isolated nHC-HA/PTX3 complex of claim 59, wherein the water soluble extract is extracted with an isotonic salt solution.

61. The isolated nHC-HA/PTX3 complex of claim 60, wherein the isotonic salt solution is PBS.

62. The rcHC-HA/PTX3 complex of any of claims 46-48 or isolated nHC-HA/PTX3 complex of any of claims 49-61, wherein the complex promotes the M2 polarization of a macrophage.

63. The rcHC-HA/PTX3 complex of any of claims 46-48 or isolated nHC-HA/PTX3 complex of any of claims 49-61, wherein:

(i) the rcHC-HA/PTX3 or isolated nHC-HA/PTX3 complex reduces the expression of IL-12p40 in LPS-stimulated macrophages, wherein the level of IL-12p40 expressed by LPS-stimulated macrophages is lower when the LPS-stimulated macrophages are contacted with the rcHC-HA/PTX3 or isolated nHC-HA/PTX3 complex compared to the level of IL-12p40 expressed by LPS-stimulated macrophages in the absence of the rcHC-HA/PTX3 or isolated nHC-HA/PTX3 complex;

(ii) the rcHC-HA/PTX3 or isolated nHC-HA/PTX3 complex reduces the expression of IL-12p70 protein in LPS-stimulated macrophages, wherein the amount of IL-12p70 protein expressed by LPS-stimulated macrophages is lower when the LPS-stimulated macrophages are contacted the rcHC-HA/PTX3 or isolated nHC-HA/PTX3 complex compared to the amount of IL-12p70 protein expressed by LPS-stimulated macrophages in the absence of the rcHC-HA/PTX3 or isolated nHC-HA/PTX3 complex;

(iii) the rcHC-HA/PTX3 or isolated nHC-HA/PTX3 complex reduces the expression of IL-23 in LPS-stimulated macrophages, wherein the level of IL-23 expressed by LPS-stimulated macrophages is lower when the LPS-stimulated macrophages are contacted the rcHC-HA/PTX3 or isolated nHC-HA/PTX3 complex compared to the level of IL-23 expressed by LPS-stimulated macrophages in the absence of the rcHC-HA/PTX3 or isolated nHC-HA/PTX3 complex;

(iv) the rcHC-HA/PTX3 or isolated nHC-HA/PTX3 complex increases the expression of IL-10 in LPS-stimulated macrophages, wherein the level of IL-10 expressed by LPS-stimulated

macrophages is higher when the LPS-stimulated macrophages are contacted the rcHC-HA/PTX3 or isolated nHC-HA/PTX3 complex compared to the level of IL-10 expressed by LPS-stimulated macrophages in the absence of rcHC-HA/PTX3 or isolated nHC-HA/PTX3 complex;

(v) the rcHC-HA/PTX3 or isolated nHC-HA/PTX3 complex promotes apoptosis of LPS-stimulated neutrophils, wherein the number of LPS-stimulated neutrophils that are apoptotic in a sample of LPS-stimulated neutrophils is higher when the sample is contacted the rcHC-HA/PTX3 or isolated nHC-HA/PTX3 complex compared to the number of LPS-stimulated neutrophils that are apoptotic in the sample in the absence of the rcHC-HA/PTX3 or isolated nHC-HA/PTX3 complex;

(vi) the rcHC-HA/PTX3 or isolated nHC-HA/PTX3 complex promotes phagocytosis of apoptotic neutrophils, wherein the number of apoptotic neutrophils that are phagocytosed by LPS-stimulated or resting macrophages in a sample of apoptotic neutrophils and LPS-stimulated or resting macrophages is higher when the sample is contacted the rcHC-HA/PTX3 or isolated nHC-HA/PTX3 complex compared to the number of neutrophils that are phagocytosed by LPS-stimulated or resting macrophages in the sample in the absence of the rcHC-HA/PTX3 or isolated nHC-HA/PTX3 complex.

64. The rcHC-HA/PTX3 complex of any of claims 46-48 or isolated nHC-HA/PTX3 complex of any of claims 49-61, wherein:

(i) the rcHC-HA/PTX3 complex or isolated nHC-HA/PTX3 complex reduces the level of IL-12p40 mRNA or IL-12p40 protein;

(ii) the rcHC-HA/PTX3 complex or isolated nHC-HA/PTX3 complex reduces the level of IL-12p70 protein;

(iii) the rcHC-HA/PTX3 complex or isolated nHC-HA/PTX3 complex reduces the level of IL-23 mRNA or IL-23 protein;

(iv) the rcHC-HA/PTX3 complex or isolated nHC-HA/PTX3 complex increases the level of IL-10 mRNA or IL-10 protein; or

any combination of (i)-(iv) thereof.

65. The rcHC-HA/PTX3 complex of any of claims 46-48, wherein the complex promotes attachment of LPS-stimulated macrophages to at least the same level as a native HC-HA/PTX3 (nHC-HA/PTX3) complex isolated from human amniotic membrane, wherein attachment comprises the contacting LPS-stimulated macrophages to the rcHC-HA/PTX3 or isolated nHC-HA/PTX3 complexes immobilized to a solid support.

66. A method for inducing the M2 polarization of macrophages comprising contacting an LPS-stimulated macrophage with an rcHC-HA/PTX3 or isolated nHC-HA/PTX3 complex.

67. The method of claim 66, wherein the rcHC-HA/PTX3 or isolated nHC-HA/PTX3 complex is rcHC-HA/PTX3 complex of any of claims 46-48 or isolated nHC-HA/PTX3 complex of any of claims 49-61.

68. The method of claim 66 or claim 67, wherein contacting a macrophage with an rcHC-HA/PTX3 or isolated nHC-HA/PTX3 complex:

- (i) reduces the level of IL-12p40 expressed by the macrophage;
- (ii) reduces the level of IL-12p70 expressed by the macrophage;
- (iii) reduces the level of IL-23 expressed by the macrophage; or
- (iv) increases the level of IL-10 expressed by the macrophage.
- (v) induces apoptosis of LPS-stimulated neutrophils;
- (vi) promotes phagocytosis of apoptotic neutrophils; or
- any combination of (i)-(vi) thereof.

69. A pharmaceutical composition, comprising an rcHC-HA/PTX3 complex of any of claims 46-48 or isolated nHC-HA/PTX3 complex of any of claims 49-61 and a pharmaceutically acceptable excipient.

70. The pharmaceutical composition of claim 69, further comprising fibronectin, collagen, laminin, keratin, fibrin, fibrinogen, hyaluronic acid, heparan sulfate, chondroitin sulfate, or combinations thereof.

71. The pharmaceutical composition of claim 69 or claim 70, further comprising an anti-inflammatory agent, an anti-scarring agent, an anti-neoplastic agent, a chemotherapeutic agent, an immunosuppressive agent, a cytotoxic agent, an antimicrobial agent or a combination thereof.

72. The pharmaceutical composition of any of claims 69-71, wherein the composition is a solution, suspension, gel, powder, ointment, tablet, capsule, or an aerosol.

73. The pharmaceutical composition of claim 72, wherein the gel is a cross-linked gel, or a liposome.

74. The pharmaceutical composition of claim 72, wherein the gel is a cross-linked hyaluronan hydrogel.

75. Use of an effective amount of an rcHC-HA/PTX3 complex of any of claims 46-48 or isolated nHC-HA/PTX3 complex of any of claims 49-61 for preventing or reversing scar formation or fibrosis in a tissue, comprising contacting the tissue with an effective amount of an rcHC-HA/PTX3 complex of any of claims 46-48 or isolated nHC-HA/PTX3 complex of any of claims 49-61.

76. The use of claim 75, wherein the scar is a dermatitis scar, a keloid scar, contracture scar, a hypertrophic scar, or a scar resulting from acne.

77. Use of an effective amount of an rcHC-HA/PTX3 complex of any of claims 46-48 or isolated nHC-HA/PTX3 complex of any of claims 49-61 for preventing or reducing inflammation in a subject in need thereof.

78. The use of claim 77, wherein the inflammation is acute inflammation or chronic inflammation.

79. The use of claim 77 or 78, wherein the inflammation is a macrophage-mediated inflammation, Th-17-mediated inflammation or T-cell mediated inflammation.

80. The use of any of claims 77-79, wherein the inflammation is associated with an autoimmune disorder, an allergy, a leukocyte defect, an infection, graft versus host disease, tissue transplant rejection, or combinations thereof.

81. The use of any of claims 77-79, wherein the inflammation is associated with rheumatoid arthritis.

82. The use of any of claims 77-79, wherein the inflammation is associated with conjunctivitis, keratitis, blepharitis, blepharoconjunctivitis, scleritis, episcleritis, uveitis, retinitis, or choroiditis.

83. The use of any of claims 77-79, wherein the inflammation is associated with myocardial infarction, stroke, endotoxin shock or sepsis.

84. The use of any of claims 77-79, wherein the inflammation is associated with atherosclerosis.

85. The use of any of claims 77-79, wherein the subject has cancer.

86. The use of any of claims 77-85, for administration in combination with an additional anti-inflammatory agent to the subject.

87. The use of claim 86, wherein the additional anti-inflammatory agent is selected from among an anti-TGF- β antibody, an anti-TGF- β receptor blocking antibody, an anti-TNF antibody, an anti-TNF receptor blocking antibody, an anti-IL1 β antibody, an anti-IL1 β receptor blocking antibody, an anti-IL-2 antibody, an anti-IL-2 receptor blocking antibody, an anti-IL-6 antibody, an anti-IL-6 receptor blocking antibody, an anti IL-12 antibody, an anti IL-12 receptor blocking antibody, an anti-IL-17 antibody, anti-IL-17 receptor blocking antibody, an anti-IL-23 antibody, or an anti-IL-23 receptor blocking antibody.

88. The use of claim 87, wherein the Type 1 interferon is IFN- α or IFN- β .

89. Use of an effective amount of an rcHC-HA/PTX3 complex of any of claims 46-48 or isolated nHC-HA/PTX3 complex of any of claims 49-61 for treating a skin wound or ulcer in a subject in need thereof.

90. The use of claim 89, wherein the skin wound or ulcer is non-healing.

91. Use of an effective amount of an rcHC-HA/PTX3 complex of any of claims 46-48 or isolated nHC-HA/PTX3 complex of any of claims 49-61 for promoting or inducing bone formation in a subject in need thereof.

92. The use of claim 91, wherein the subject has arthritis, osteoporosis, alveolar bone degradation, Paget's disease, or a bone tumor.

93. Use of an effective amount of an rcHC-HA/PTX3 complex of any of claims 46-48 or isolated nHC-HA/PTX3 complex of any of claims 49-61 for preventing or reducing unwanted angiogenesis in a subject in need thereof.

94. The use of claim 93, wherein the unwanted angiogenesis is associated with wet age-related macular degeneration (wARMD) or diabetic proliferative retinopathy

95. The use of claim 93, wherein the unwanted angiogenesis is associated with cancer.

96. The use of claim 93, wherein the subject has a solid tumor.

97. The use of any of claims 95-96, for administration in combination with an anti-cancer therapy.

98. The use of claim 97, wherein the anti-cancer therapy comprises administration of an anti-neoplastic agent, a cytotoxic agent, an antimicrobial agent, an anti-angiogenic agent, a chemotherapeutic agent, or radiation therapy.

99. Use of an effective amount of an rcHC-HA/PTX3 complex of any of claims 46-48 or isolated nHC-HA/PTX3 complex of any of claims 49-61 for inhibiting or preventing transplant rejection in a transplant recipient.

100. The use of claim 99, for administration before a transplantation procedure, after a transplantation procedure, or during a transplantation procedure.

101. The use of claim 99 or claim 100, for administration in combination with an immunosuppressive agent.

102. A method of inducing stem cell expansion, comprising contacting a stem cell *in vitro* with an effective amount of an rcHC-HA/PTX3 complex of any of claims 46-48 or isolated nHC-HA/PTX3 complex of any of claims 49-61.

103. Use of an effective amount of an rcHC-HA/PTX3 complex of any of claims 46-48 or isolated nHC-HA/PTX3 complex of any of claims 49-61 in combination with a therapeutic cell for providing a cell therapy to a subject in need thereof.

104. The use of claim 103, further comprising contacting a damaged tissue with the rcHC-HA/PTX3 complex or isolated nHC-HA/PTX3 complex and the therapeutic cell.

105. The use of claim 103, wherein the rcHC-HA/PTX3 complex or isolated nHC-HA/PTX3 complex and therapeutic cell are formulated in a single composition or separate compositions.

106. The use of claim 103, wherein the therapeutic cell is contained in a microcapsule.

107. The use of claim 104, wherein the rcHC-HA/PTX3 complex or isolated nHC-HA/PTX3 complex is attached to the microcapsule.

108. The use of any of claims 103-107, wherein the therapeutic cell is a stem cell.

109. The use of claim 108, wherein the therapeutic cell is an adult stem cell or an induced pluripotent stem cell.

110. The use of claim 108, wherein the therapeutic cell is a mesenchymal stem cell, limbal epithelial progenitor cell, a limbal stromal niche cell, an umbilical cord stem cell, an amniotic membrane stem cell or an adipose stem cell

111. The use of any of claims 103-108, wherein therapeutic cell is a differentiated cell.

112. The use of any of claims 103-108, wherein therapeutic cell is an insulin producing cell.

113. The use of claim 112, wherein the insulin producing therapeutic cell is an islet cell.

114. The use of claim 112 or claim 113, wherein the subject has diabetes mellitus type 1.

115. Use of an effective amount of an rcHC-HA/PTX3 complex of any of claims 46-48 or isolated nHC-HA/PTX3 complex of any of claims 49-61 for inducing or promoting tissue regeneration in a subject in need thereof.

116. The use of claim 115, wherein the tissue is bone or gum, corneal tissue, or conjunctival tissue.

117. The use of claim 115 or 116, for administration in combination with a therapeutic cell or a tissue transplant.

118. The use of claim 117, wherein the tissue transplant is an allograft or an autograft.

119. Use of an effective amount of an rcHC-HA/PTX3 complex of any of claims 46-48 or isolated nHC-HA/PTX3 complex of any of claims 49-61 for inhibiting or decreasing the amount of M1 adipose tissue macrophages in a subject.

120. The use of claim 119, wherein the subject is diagnosed with obesity or insulin-resistance.

121. Use of an effective amount of an rcHC-HA/PTX3 complex of any of claims 46-48 or isolated nHC-HA/PTX3 complex of any of claims 49-61 for conjunctivochalasis in a subject in need thereof.

122. A cell culture, comprising a substrate suitable for culturing a cell and an rcHC-HA/PTX3 complex of any of claims 46-48 or isolated nHC-HA/PTX3 complex of any of claims 49-61.

123. The cell culture of claim 122, wherein the rcHA/PTX3 complex or the nHC-HA/PTX3 complex is immobilized to the substrate.

124. A medical device, comprising a substrate coated with an rcHC-HA/PTX3 complex of any of claims 46-48 or isolated nHC-HA/PTX3 complex of any of claims 49-61.

125. The medical device of claim 124, wherein the substrate comprises at least one of a stent, a joint, a screw, a rod, a pin, a plate, a staple, a shunt, a clamp, a clip, a suture, a suture anchor, an electrode, a catheter, a lead, a graft, a dressing, a pacemaker, a pacemaker housing, a cardioverter, a cardioverter housing, a defibrillator, a defibrillator housing, a prostheses, an ear drainage tube, an ophthalmic implant, an orthopedic device, a vertebral disk, a bone substitute, an anastomotic device, a perivascular wrap, a colostomy bag attachment device, a hemostatic barrier, a vascular implant, a vascular support, a tissue adhesive, a tissue sealant, a tissue scaffold, and an intraluminal device.

126. The medical device of claim 124, wherein the substrate is a microcapsule, nanoparticle, or a bead.

127. The medical device of claim 126, wherein microcapsule contains a stem cell.

128. The medical device of claim 124, wherein the substrate is a contact lens.

129. The medical device of claim 124, wherein the substrate is a surgical implant or prosthesis.

130. The medical device of claim 129, wherein the implant or prosthesis is an artificial joint, a bone implant, a suture, or a stent.

131. The medical device of claim 130, wherein the artificial joint is an artificial hip joint, an artificial knee, an artificial glenohumeral joint, or an artificial knee.

132. The medical device of claim 130, wherein the stent is a coronary stent, a ureteral stent, a urethral stent, a prostatic stent, esophageal stent, or a bone stent.

133. The medical device of any of claims 124-132, wherein the rcHC-HA/PTX3 complex or isolated nHC-HA/PTX3 complex inhibits the formation of a bacterial biofilm.

134. A method for modulating macrophage activity comprising contacting a macrophage with an rcHC-HA/PTX3 complex of any of claims 46-48 or isolated nHC-HA/PTX3 complex of any of claims 49-61 in an amount sufficient to (i) reduce or inhibit the expression of IL-12 or IL-23 or (ii) increase the expression of IL-10.

135. The method of claim 134, wherein the macrophage has been stimulated with a pro-inflammatory mediator.

136. The method of claim 135, wherein the pro-inflammatory mediator is lipopolysaccharide (LPS), tumor necrosis factor α (TNF- α), interferon-gamma (IFN γ) or a combination thereof.

137. The method of any of claims 134-136, wherein the rcHC-HA/PTX3 complex or isolated nHC-HA/PTX3 complex contacts the macrophage *in vitro*.

138. A kit, comprising a composition comprising an rcHC-HA/PTX3 complex of any of claims 46-48 or isolated nHC-HA/PTX3 complex of any of claims 49-61, a device for administration of the composition and, optionally, instructions for administration.

139. A method for inducing or maintaining pluripotency in a cell, comprising culturing the cell with an nHC-HA/PTX3 complex or an rcHC-HA/PTX3 complex, thereby inducing or maintaining pluripotency in the cell.

140. The method of claim 139, wherein the cell heterogeneously expresses a protein selected from among Sox2, myc, Oct4 and KLF4.

141. The method of claim 139, wherein the cell heterogeneously expresses one, two, or three proteins selected from among Sox2, myc, Oct4 and KLF4.

142. The method of claim 139, wherein the nHC-HA/PTX3 complex or rcHC-HA/PTX3 complex is immobilized onto a substrate.

143. The method of claim 139, wherein the cell is an adult differentiated cell.

144. The method of claim 139, wherein the cell is a fibroblast.

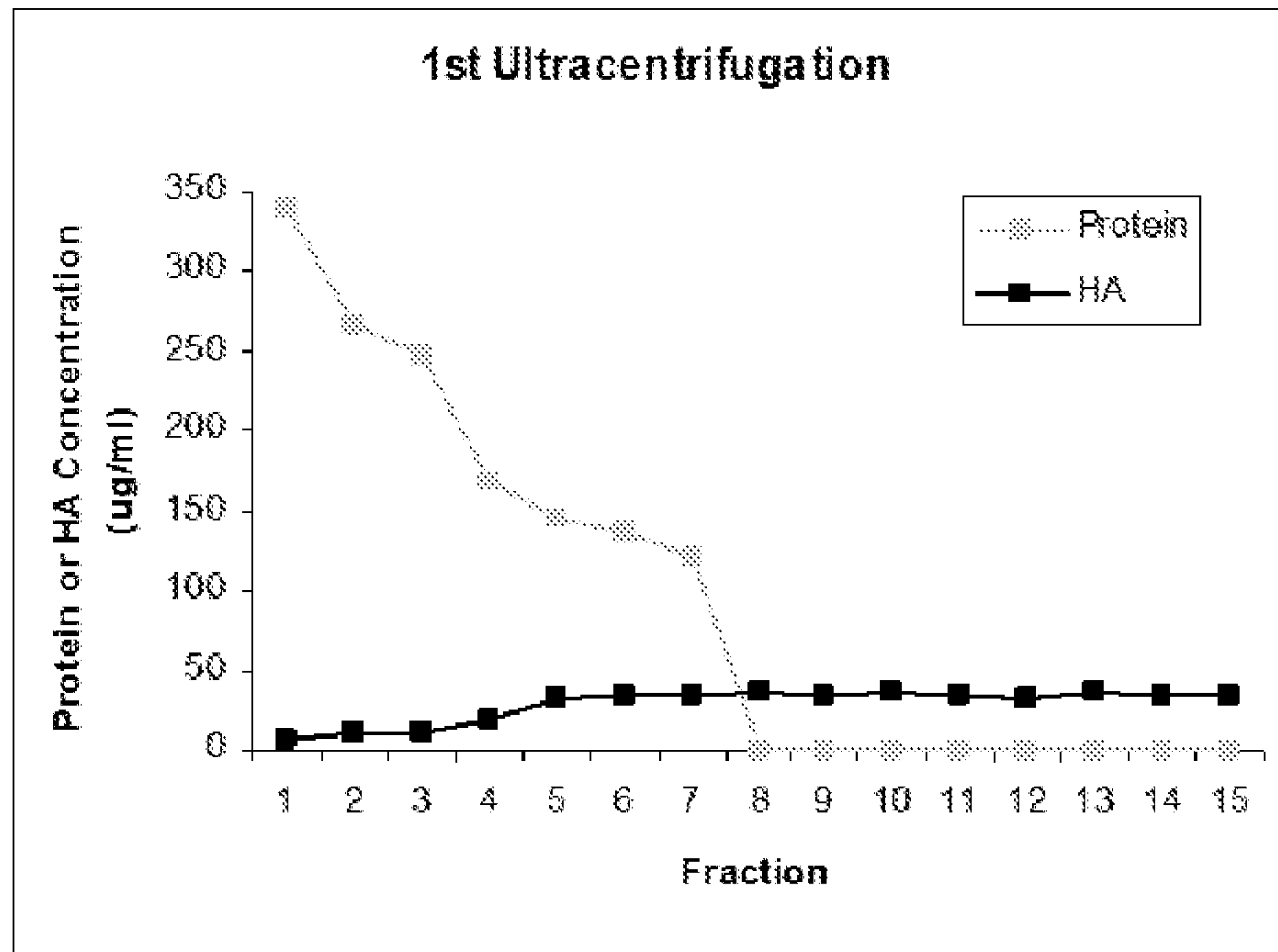
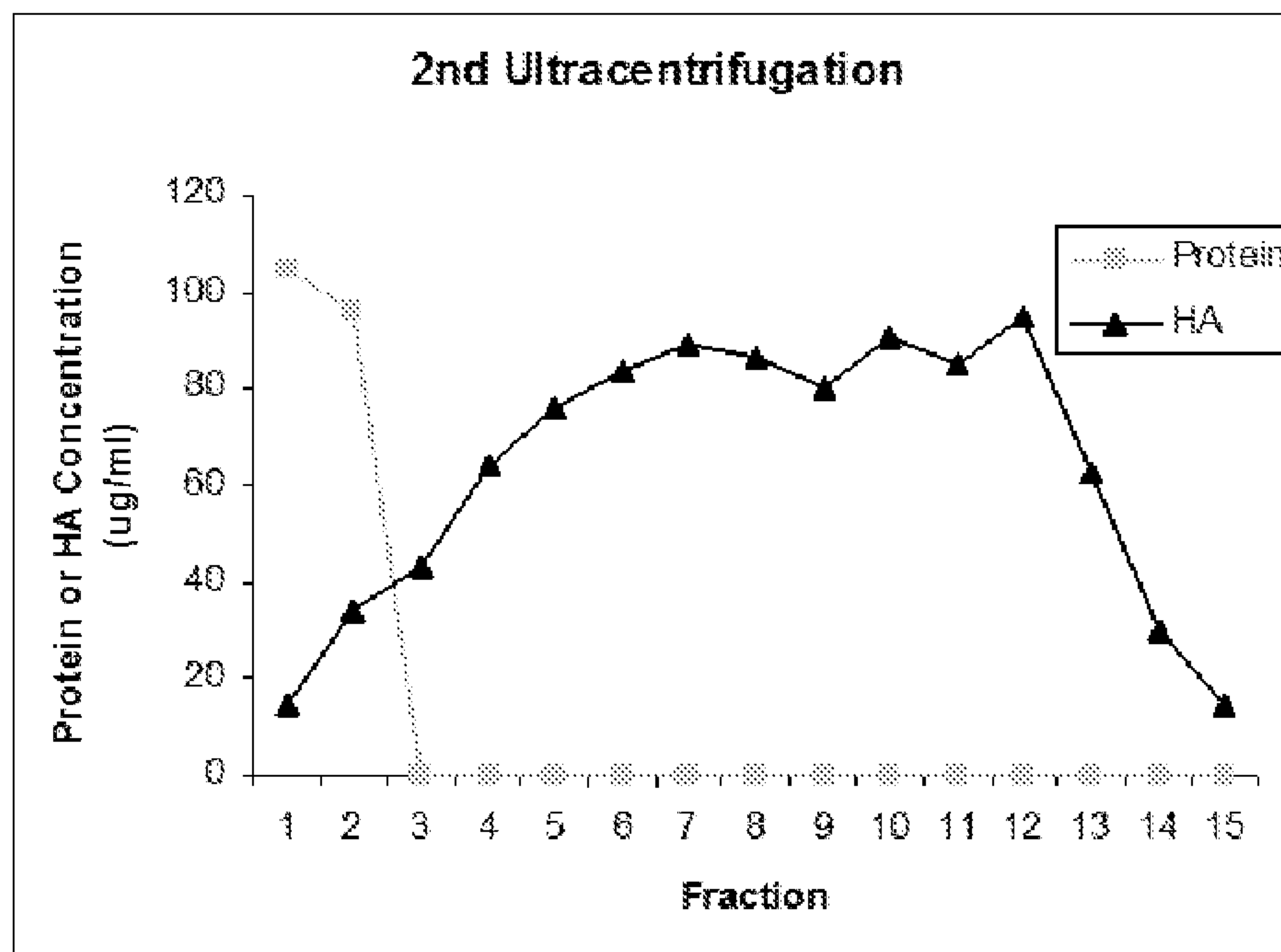
145. The method of claim 144, wherein the cell is a human corneal fibroblast.

146. The method of claim 139, wherein the cell is an adult stem cell, or an induced pluripotent stem cell.

147. The method of claim 139, wherein the cell is a limbal epithelial progenitor cell, a limbal stromal niche cell, an umbilical cord stem cell, an amniotic membrane stem cell or an adipose stem cell.

148. The method of any of claims 139-147, wherein the nHC-HA/PTX3 complex or rcHC-HA/PTX3 complex is an rcHC-HA/PTX3 complex of any of claims 46-48 or isolated nHC-HA/PTX3 complex of any of claims 49-61.

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FIG. 1**A****B**

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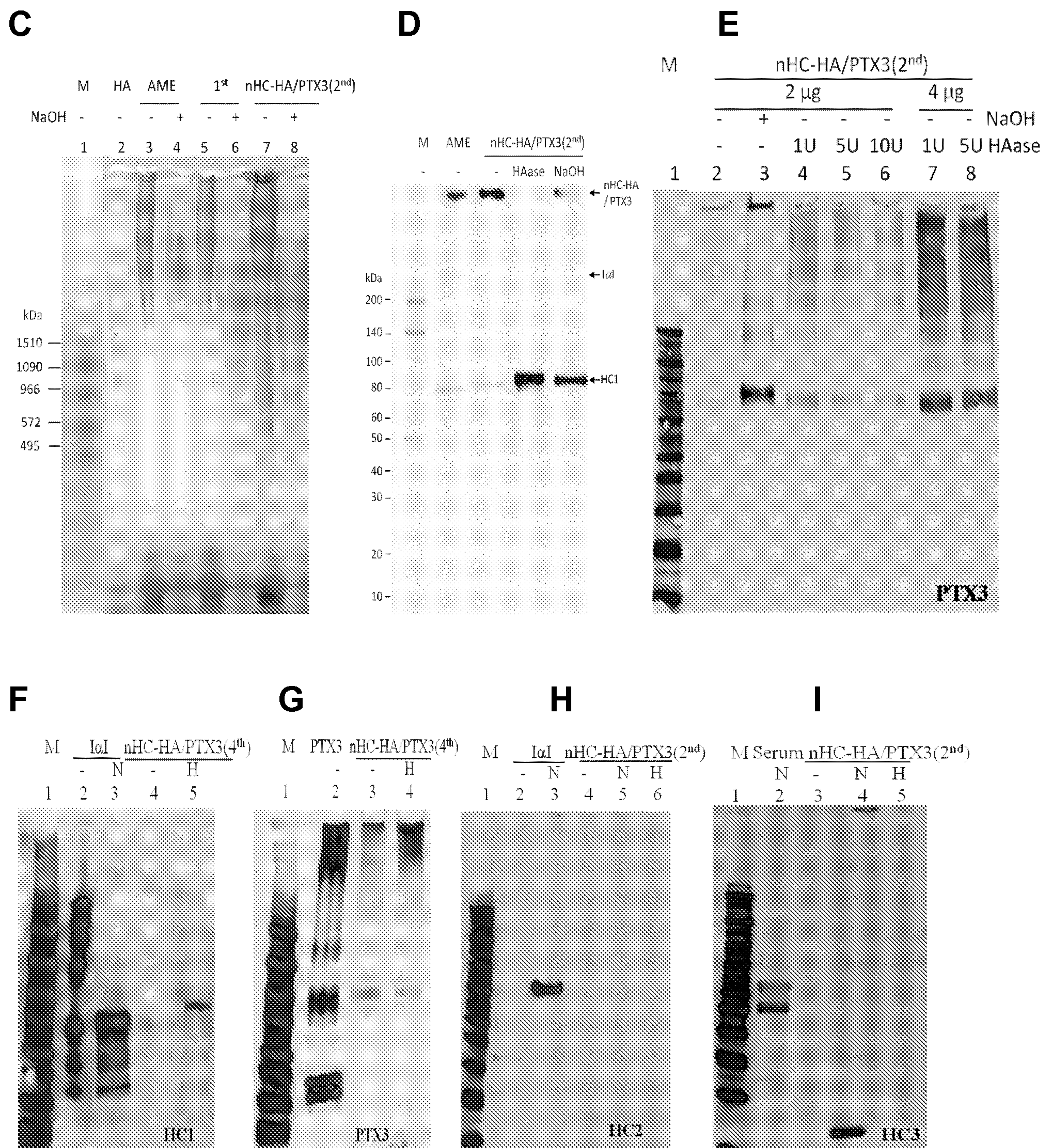
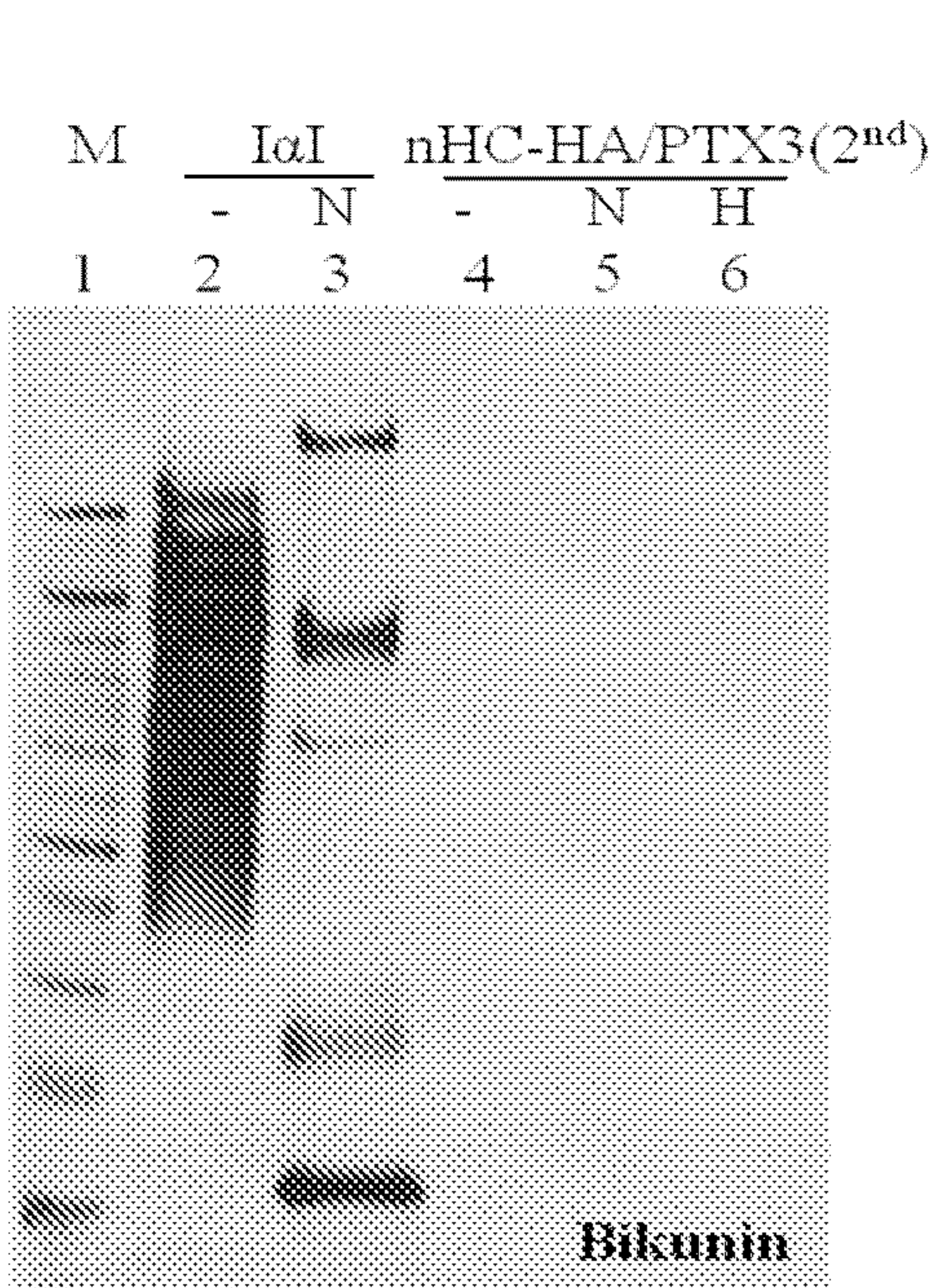
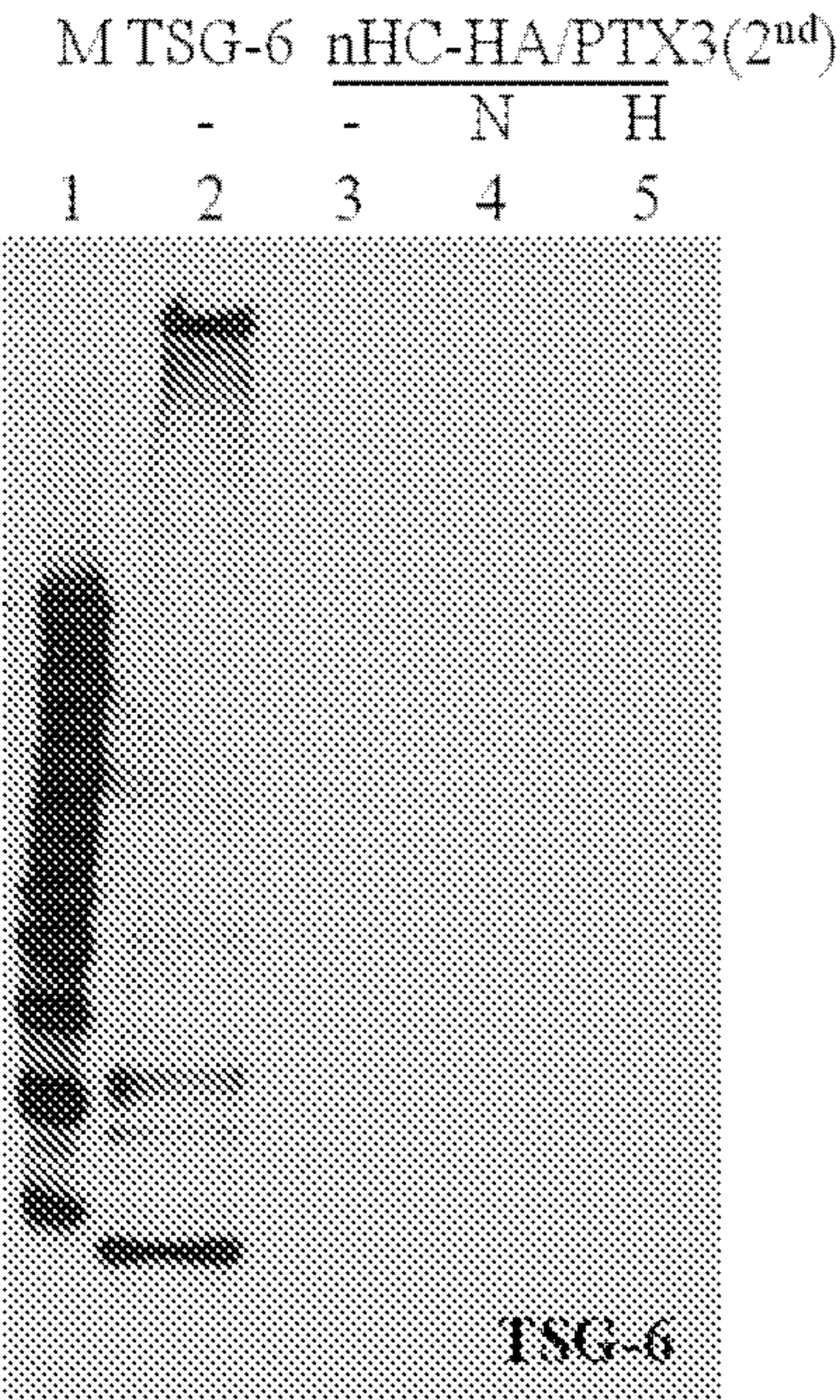
FIG. 1 (cont'd)

FIG. 1 (cont'd)

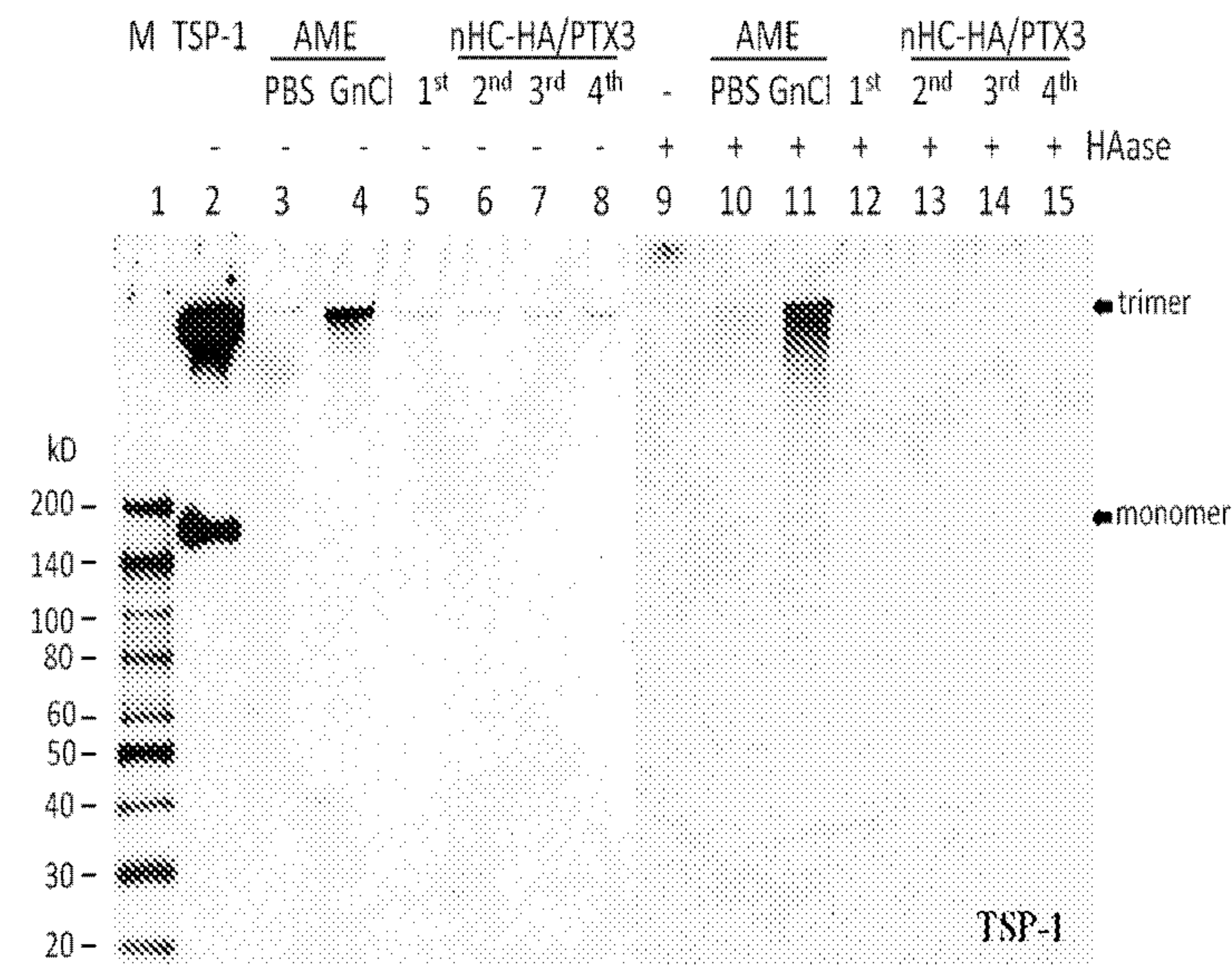
J



K



L



M

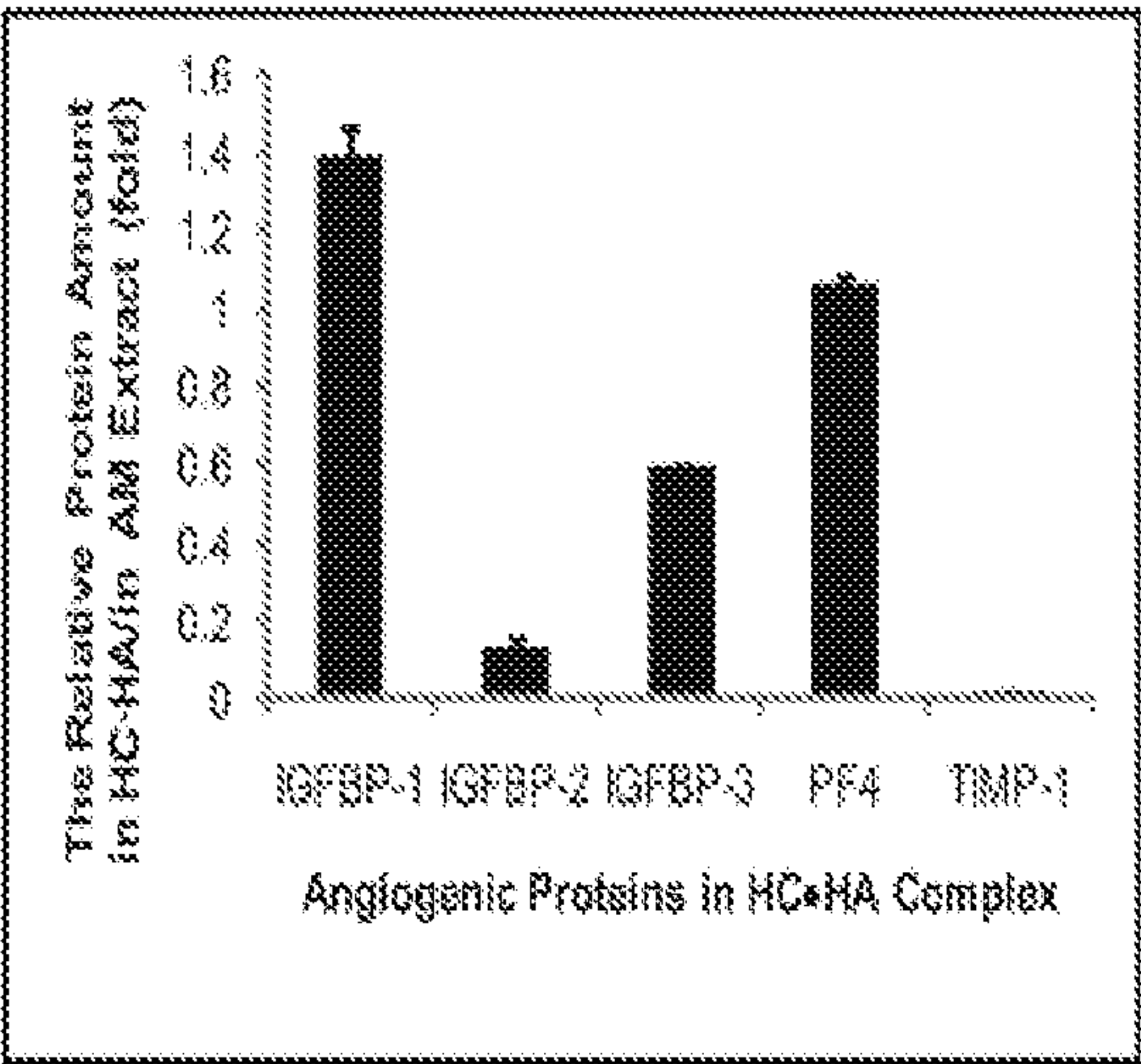
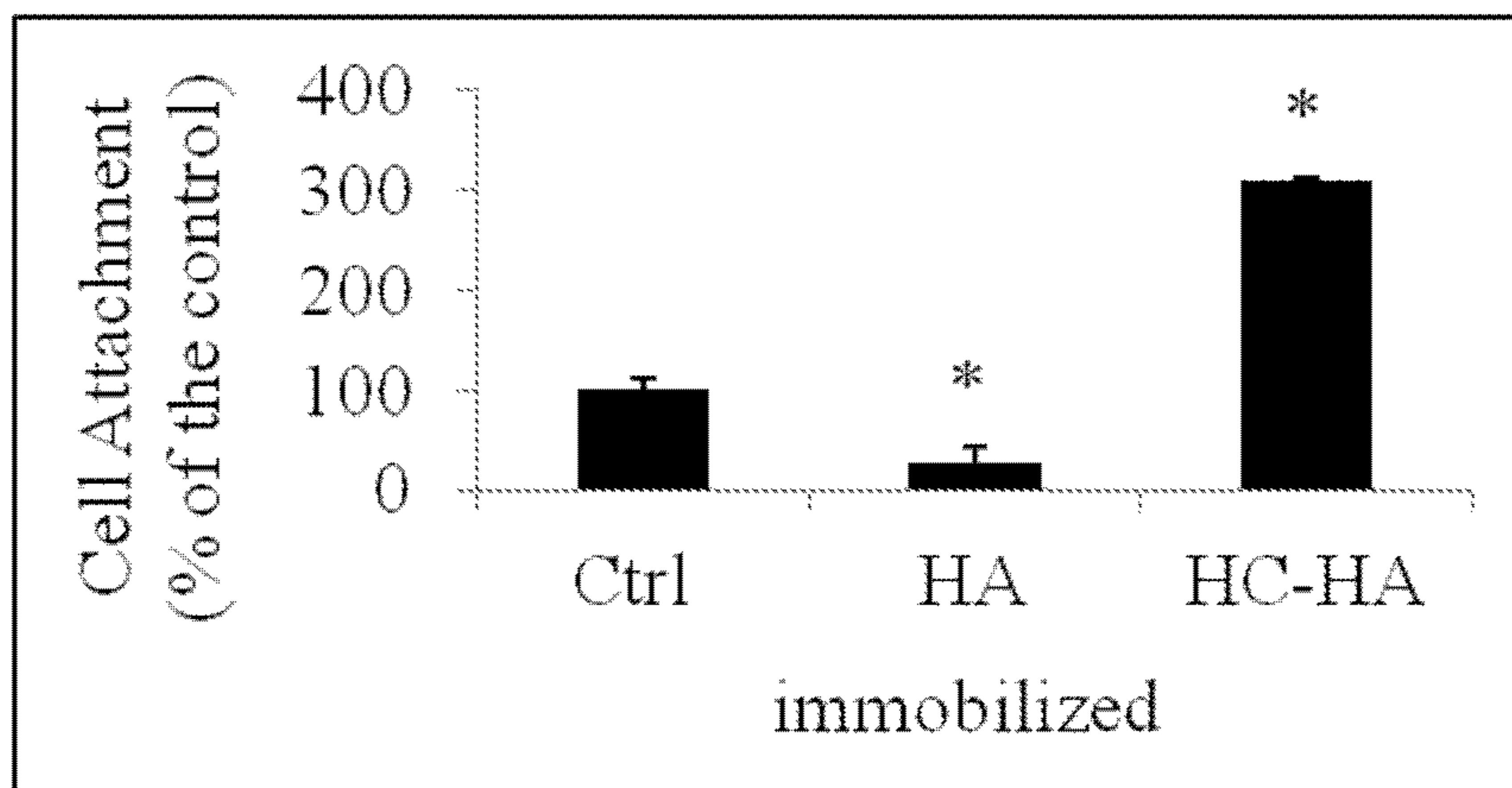
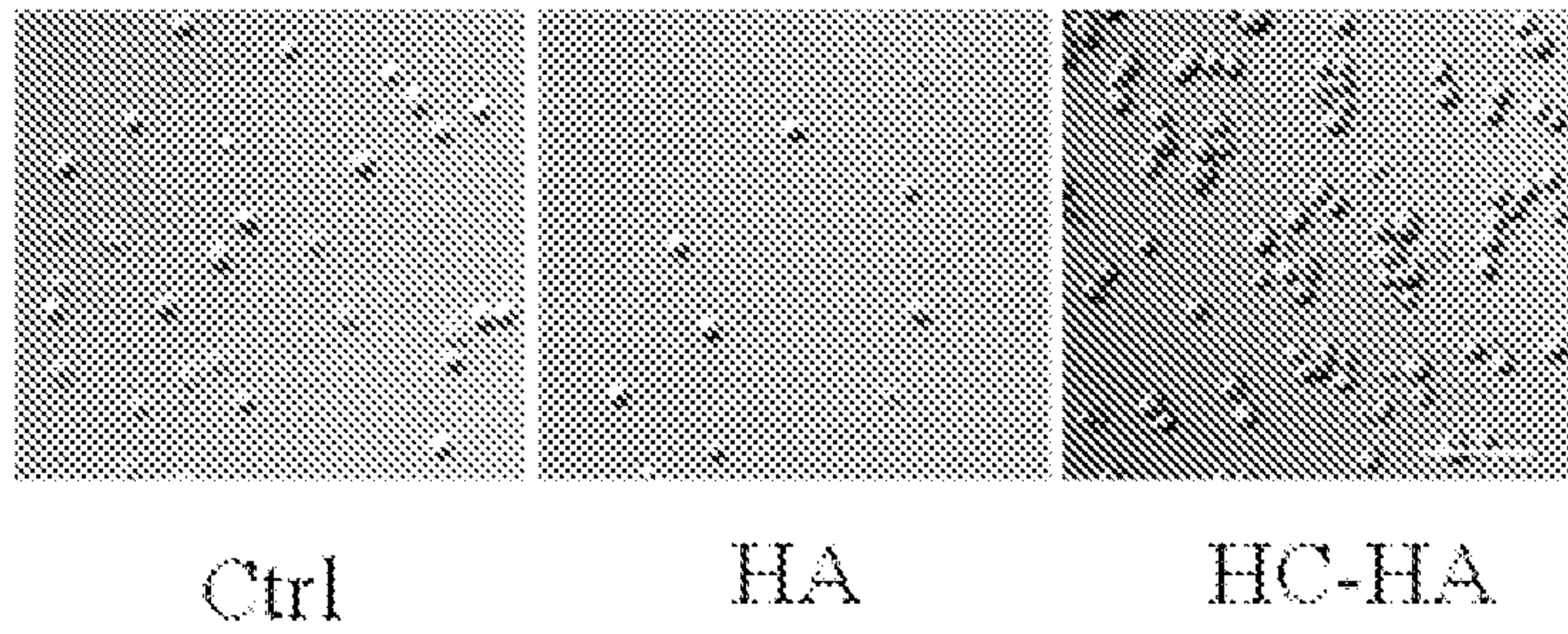
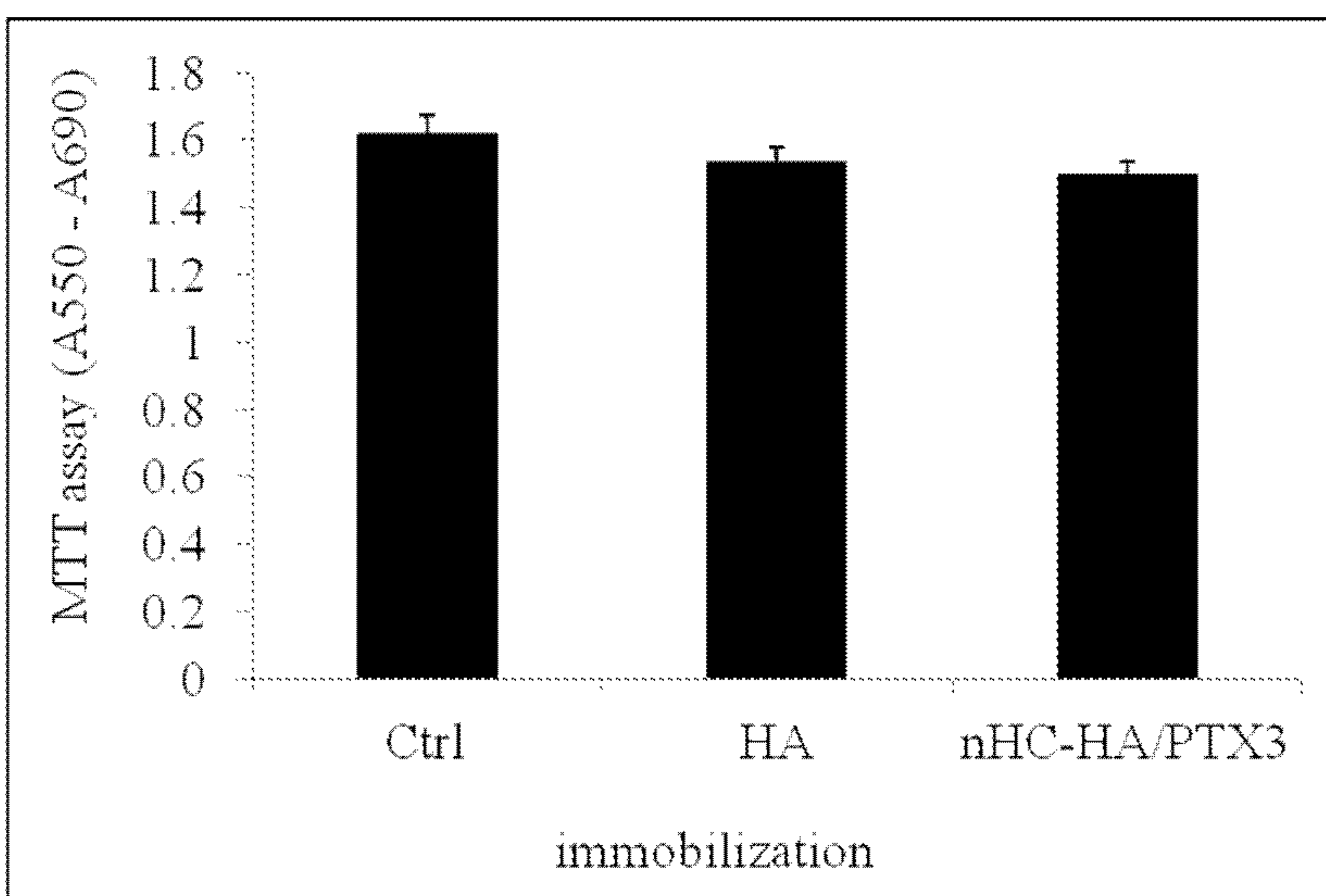
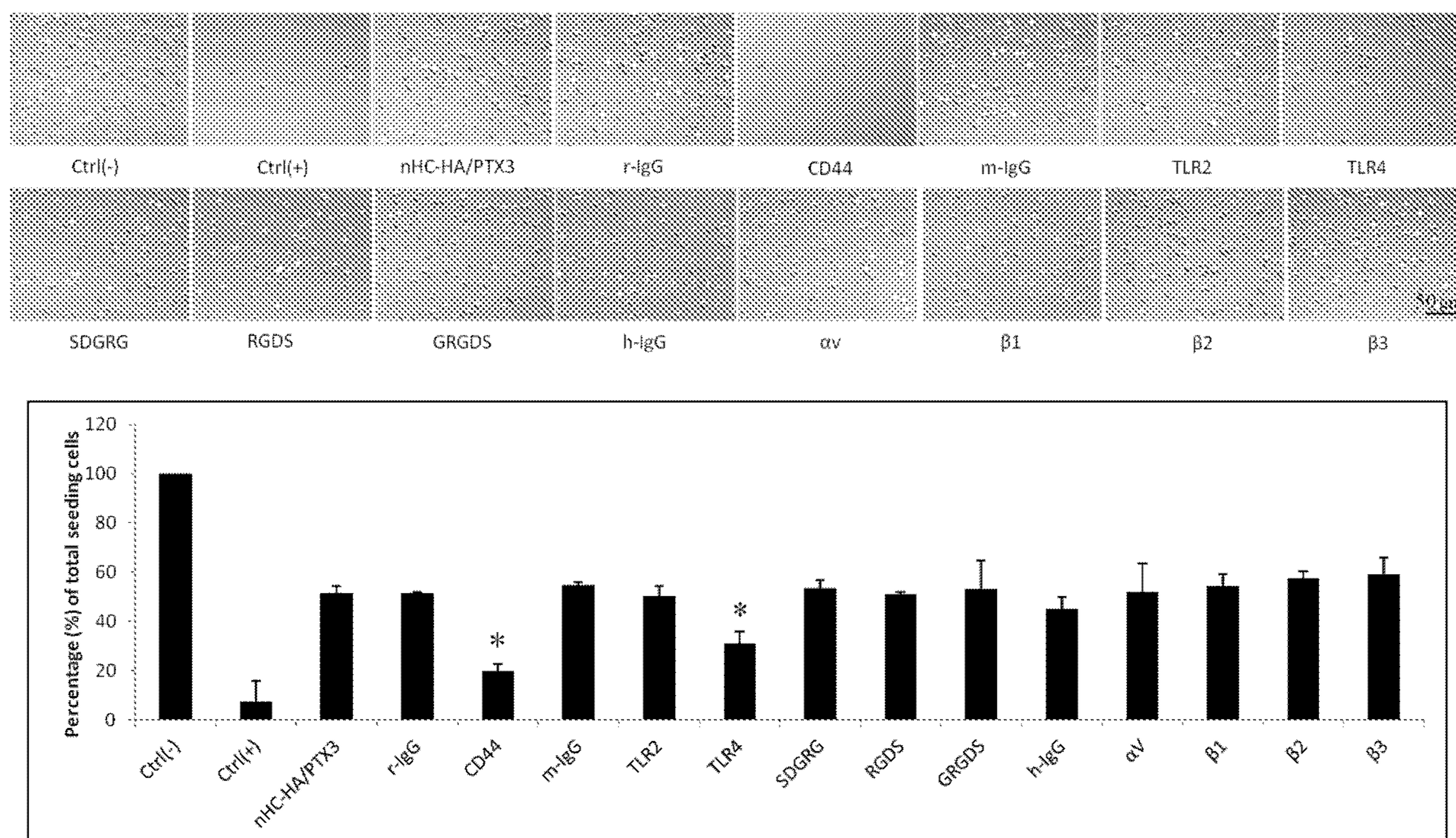
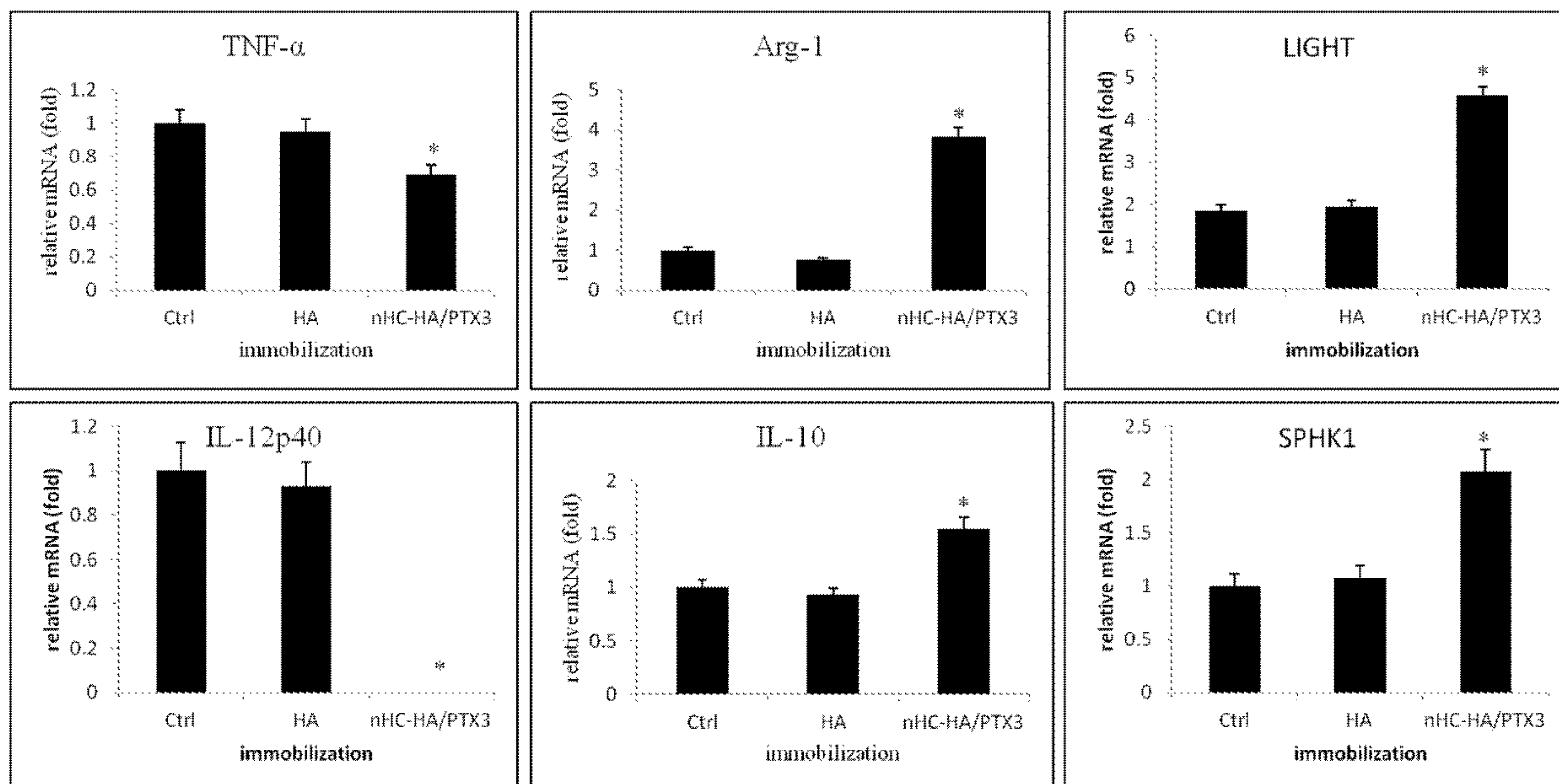
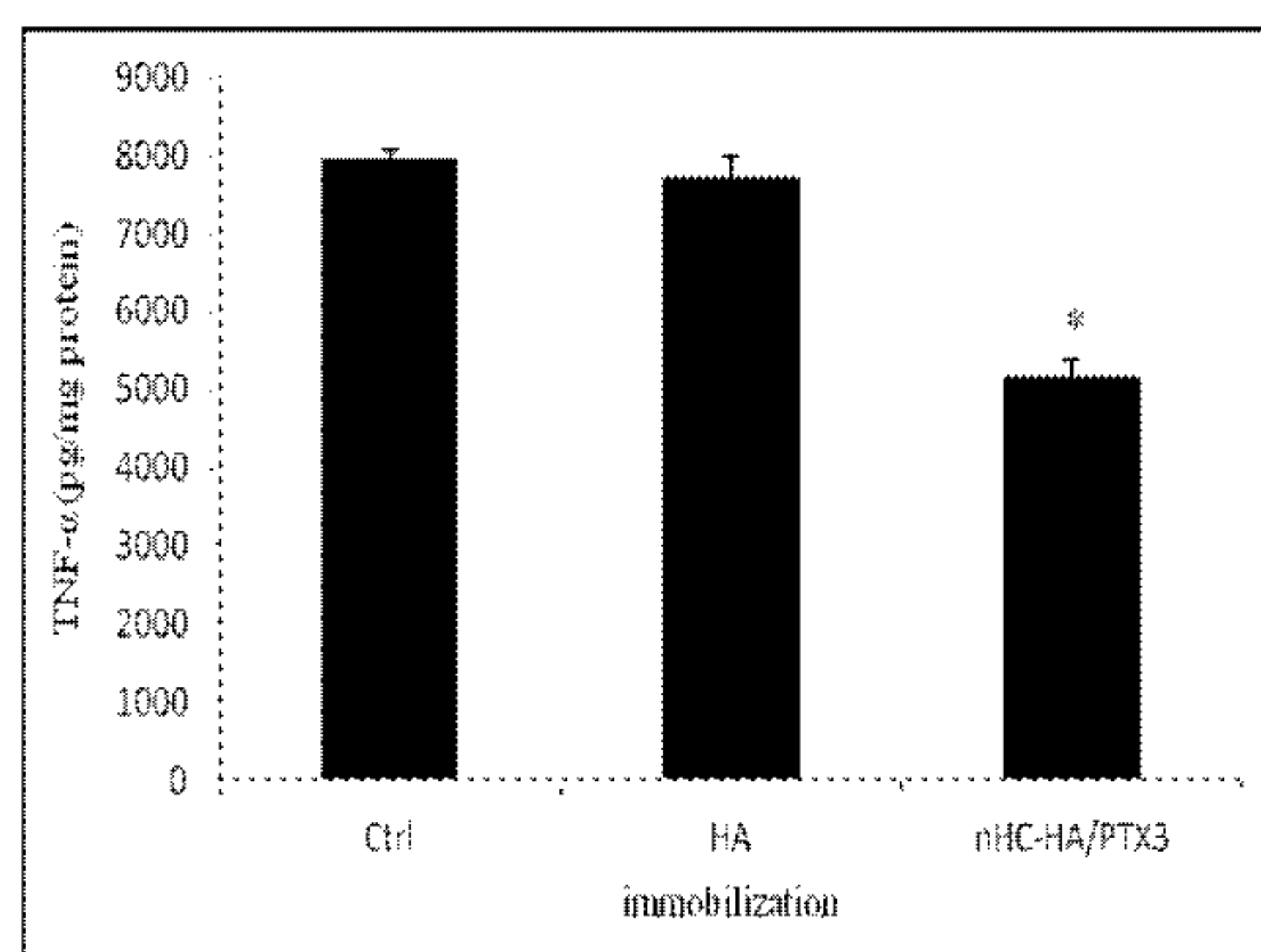
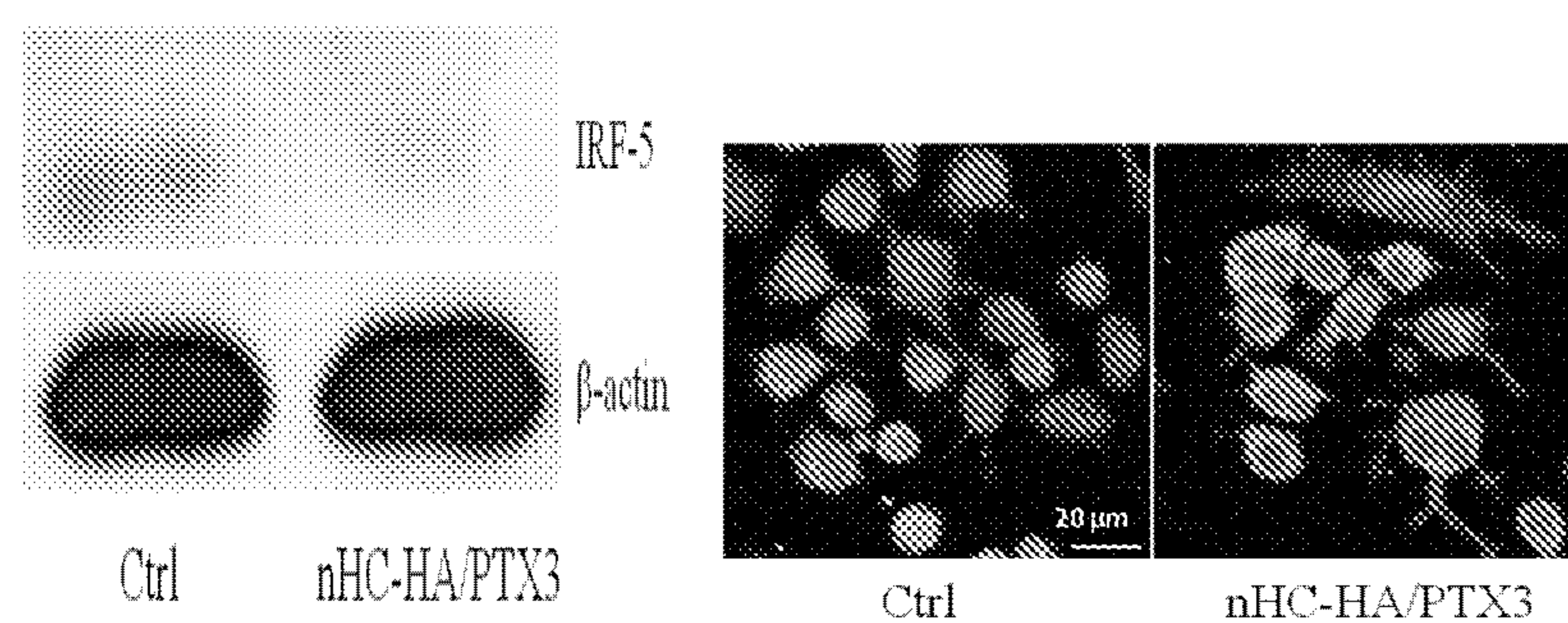
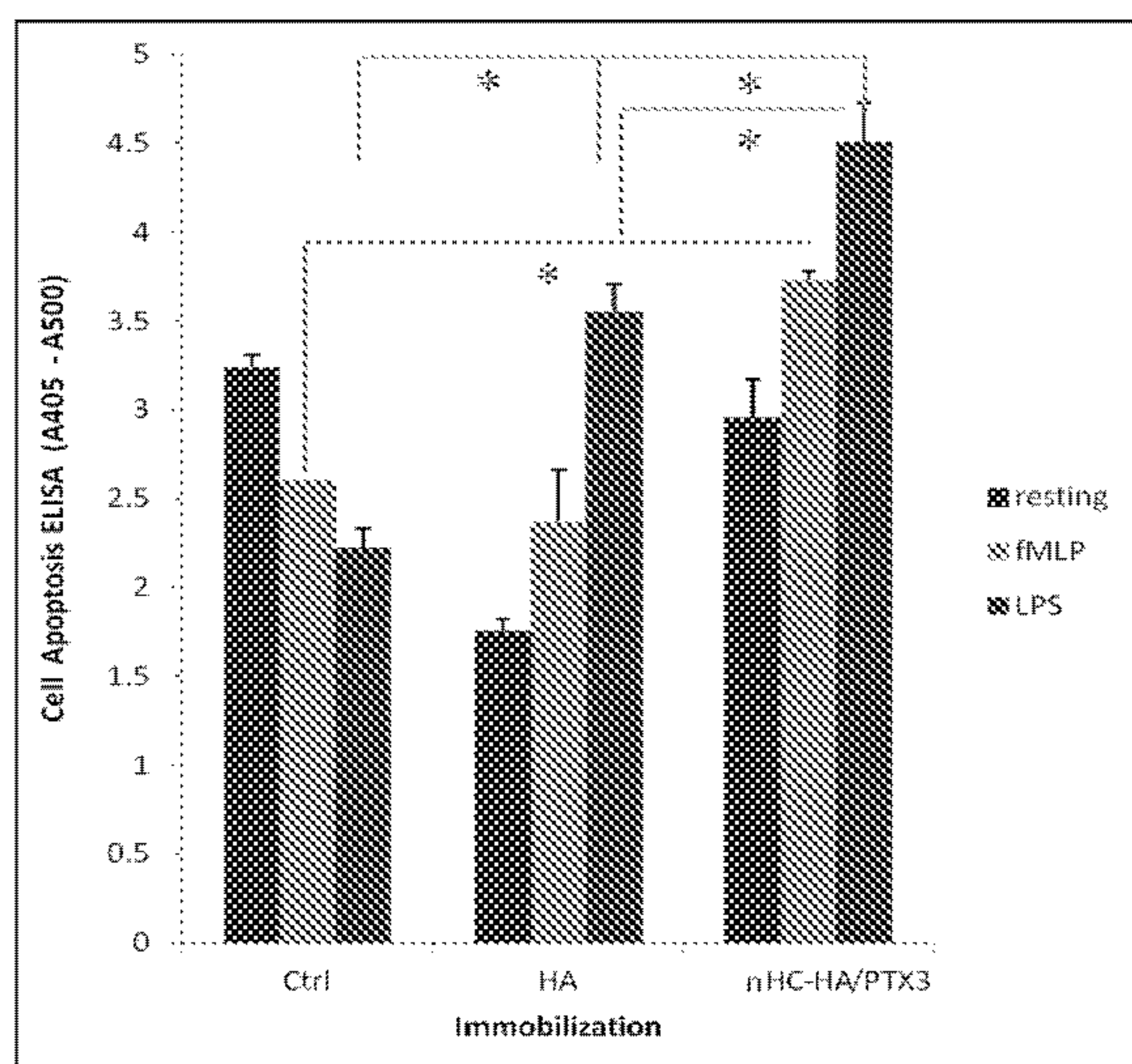
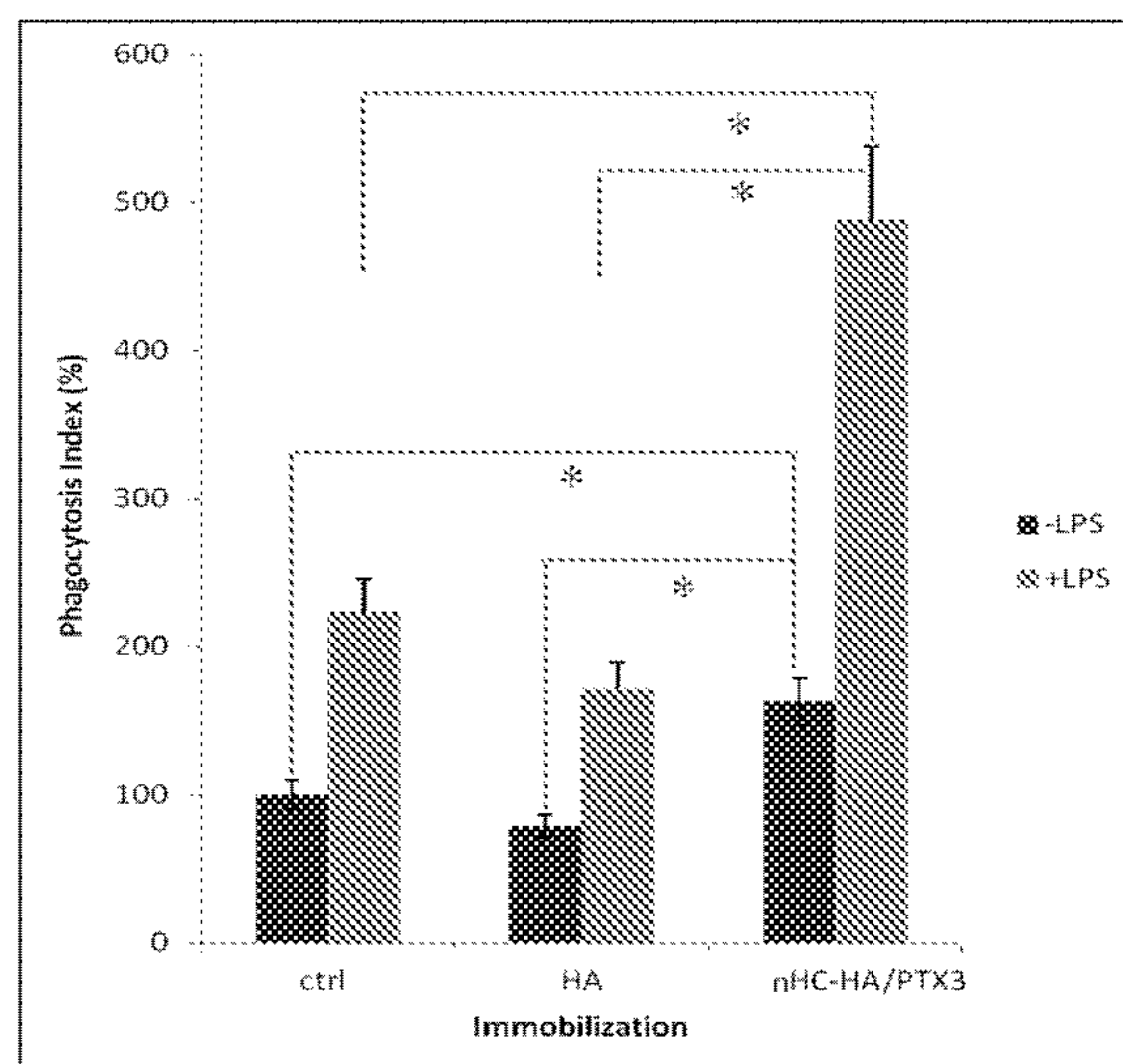


FIG. 2**A****B**

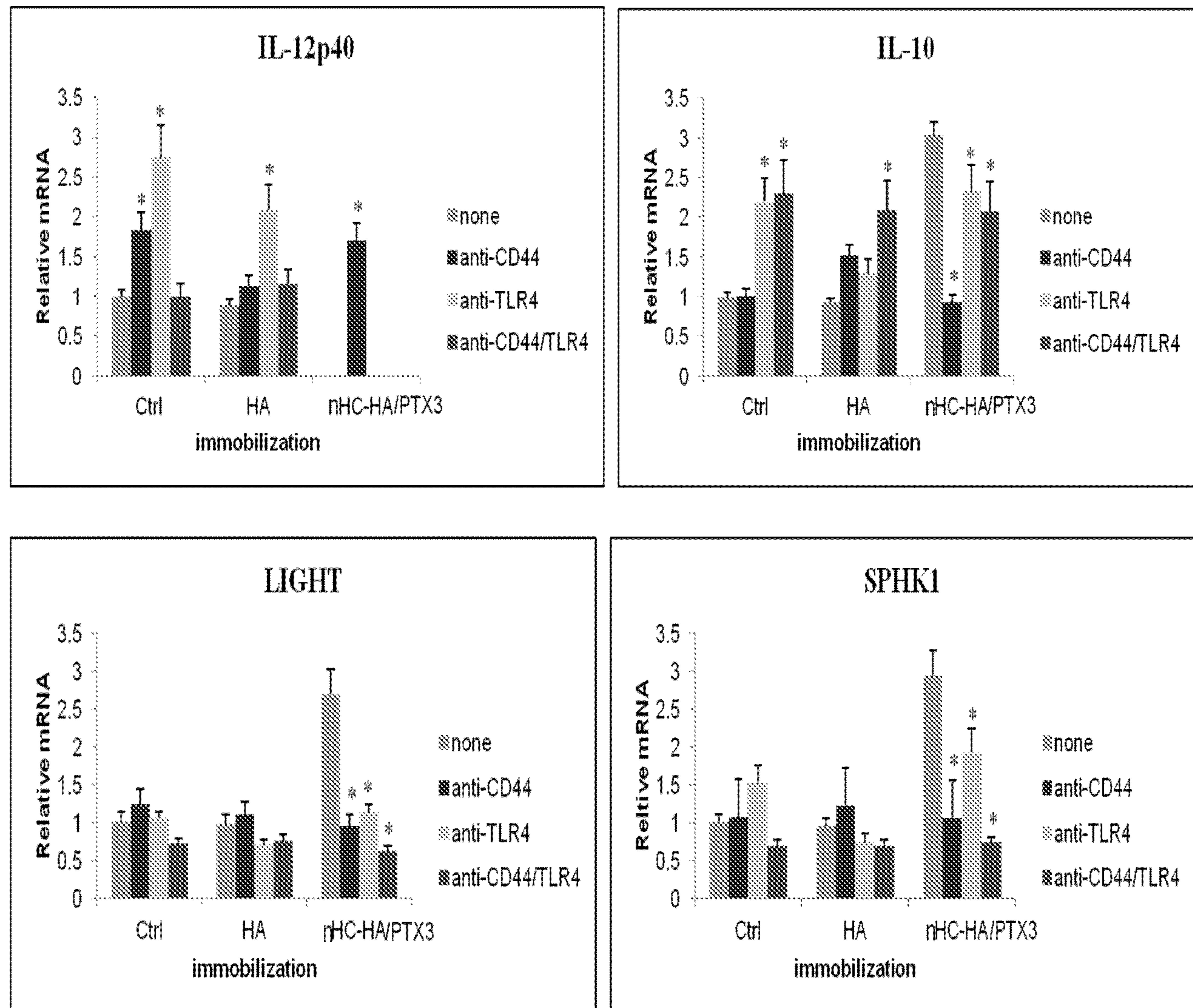
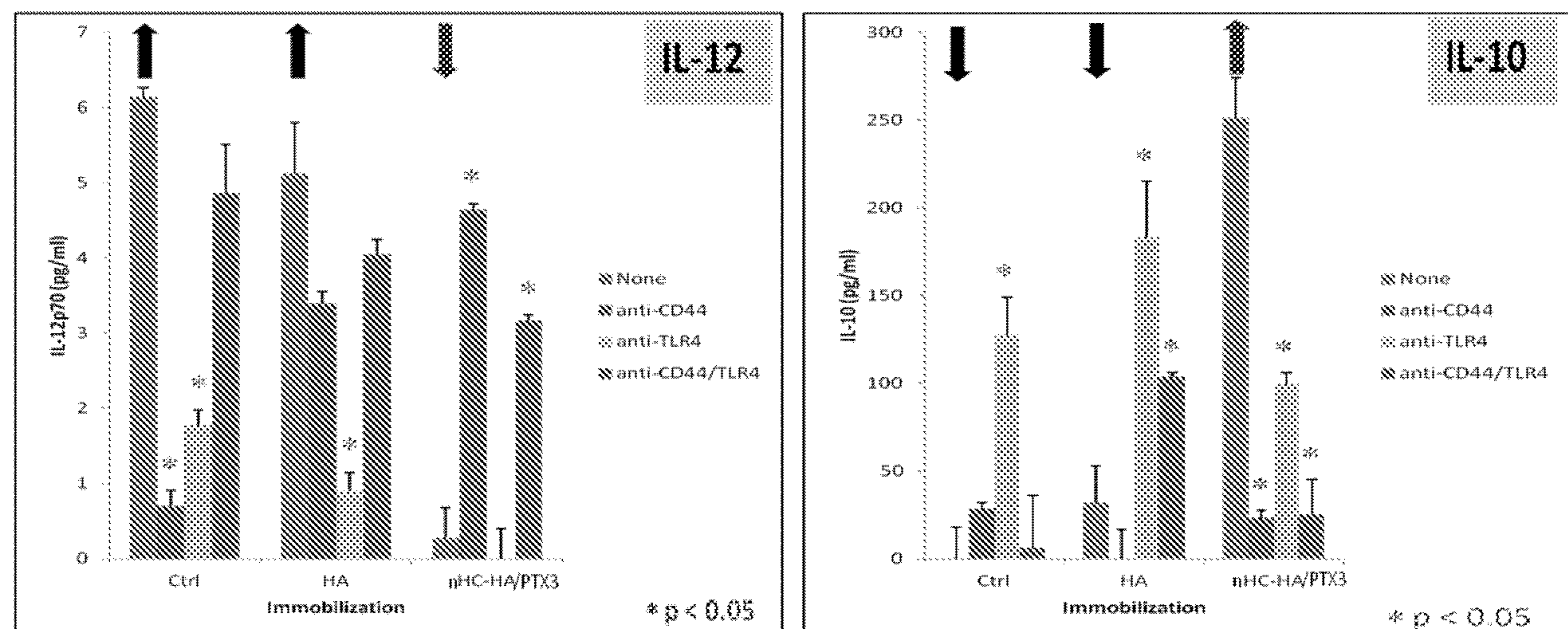
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FIG. 2 (cont'd)**C**

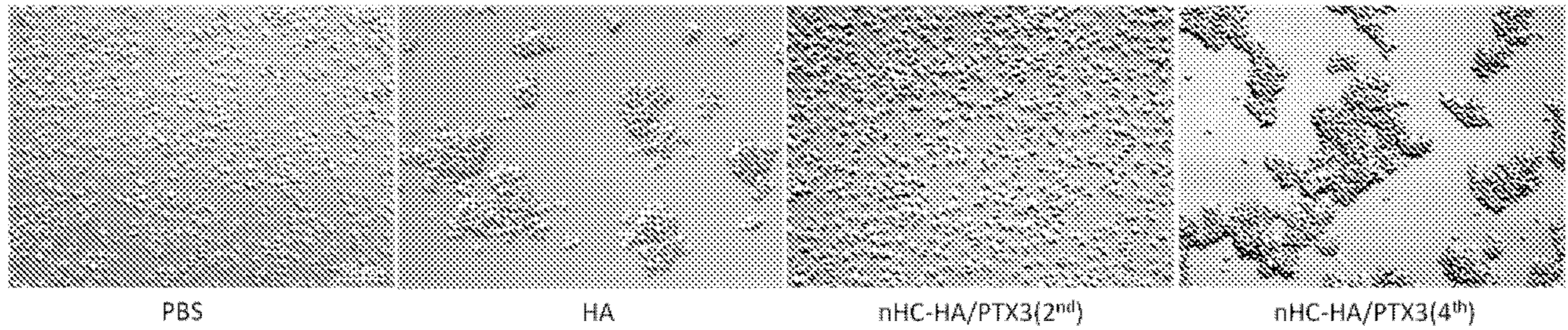
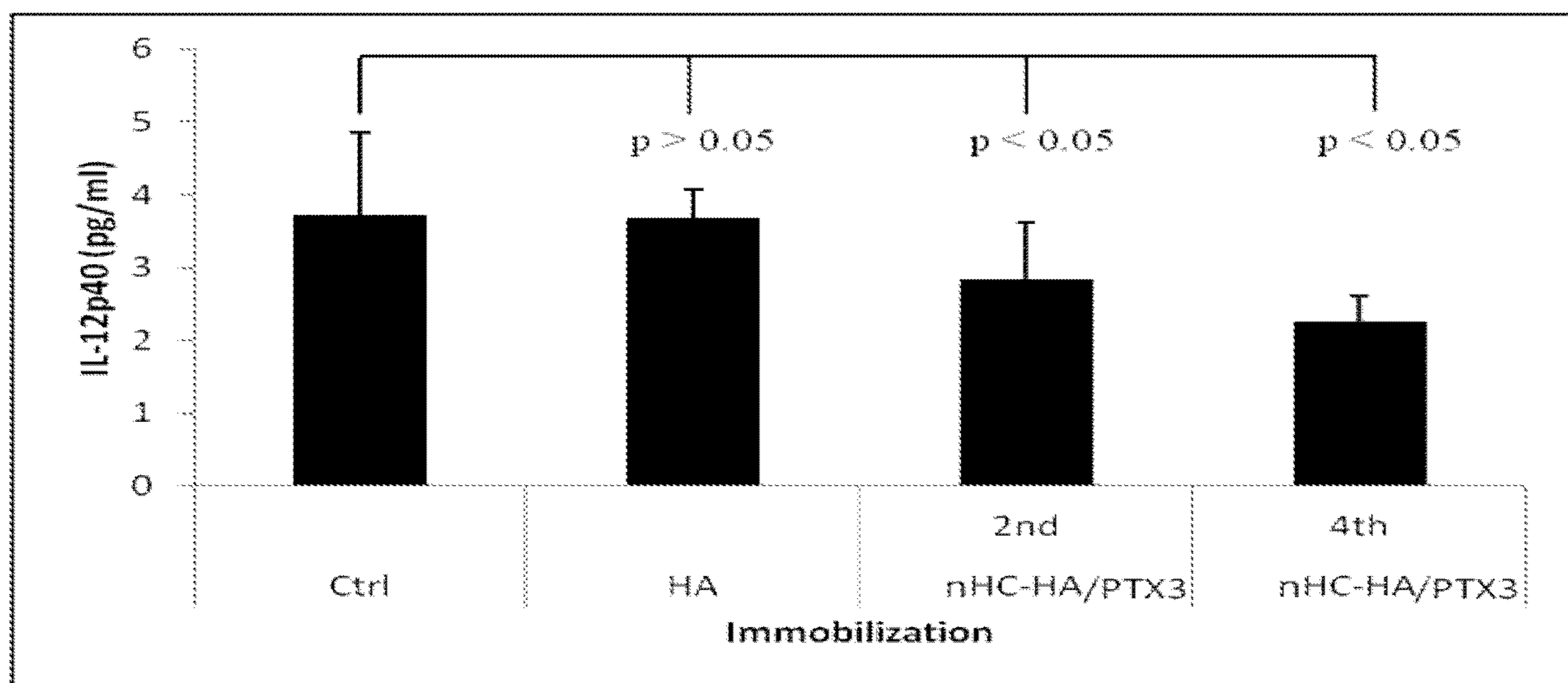
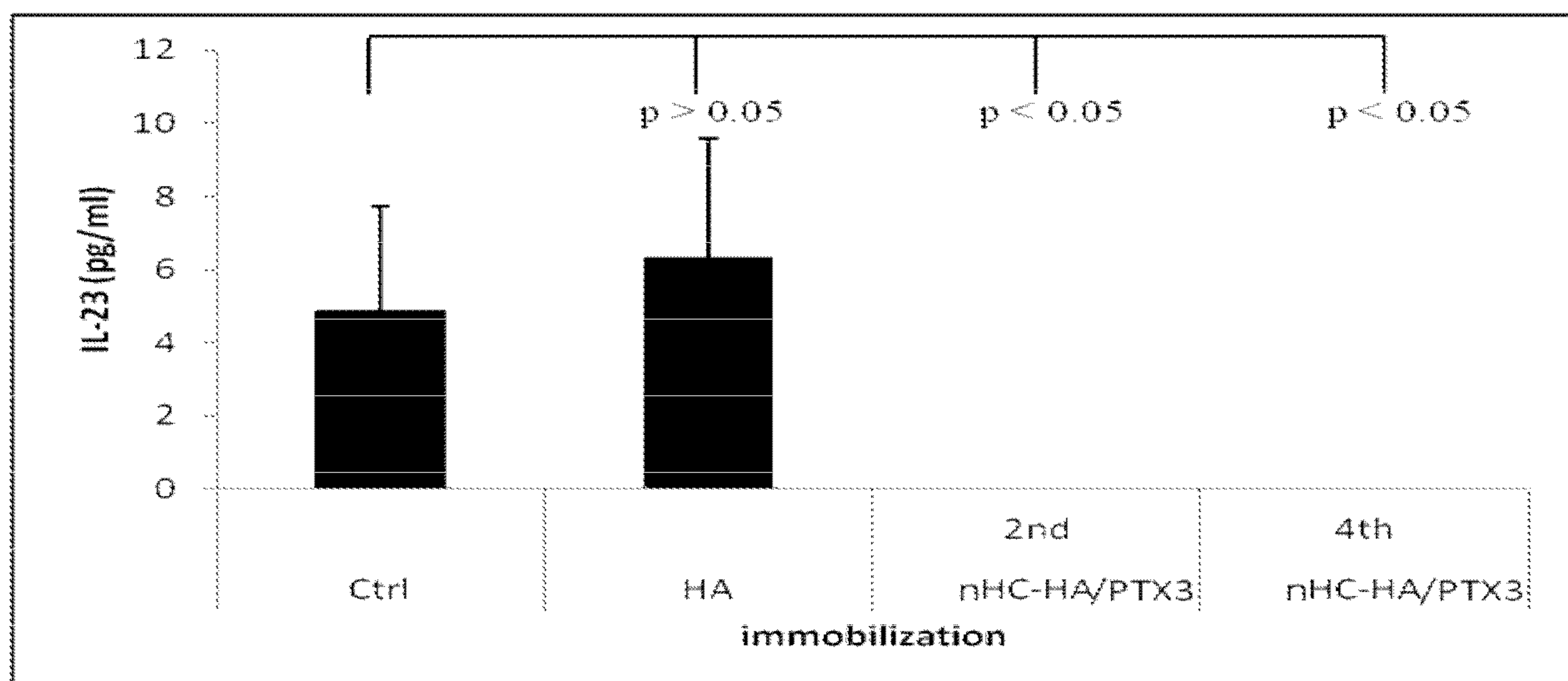
6/112

FIG. 3**A****B****C****D****E**

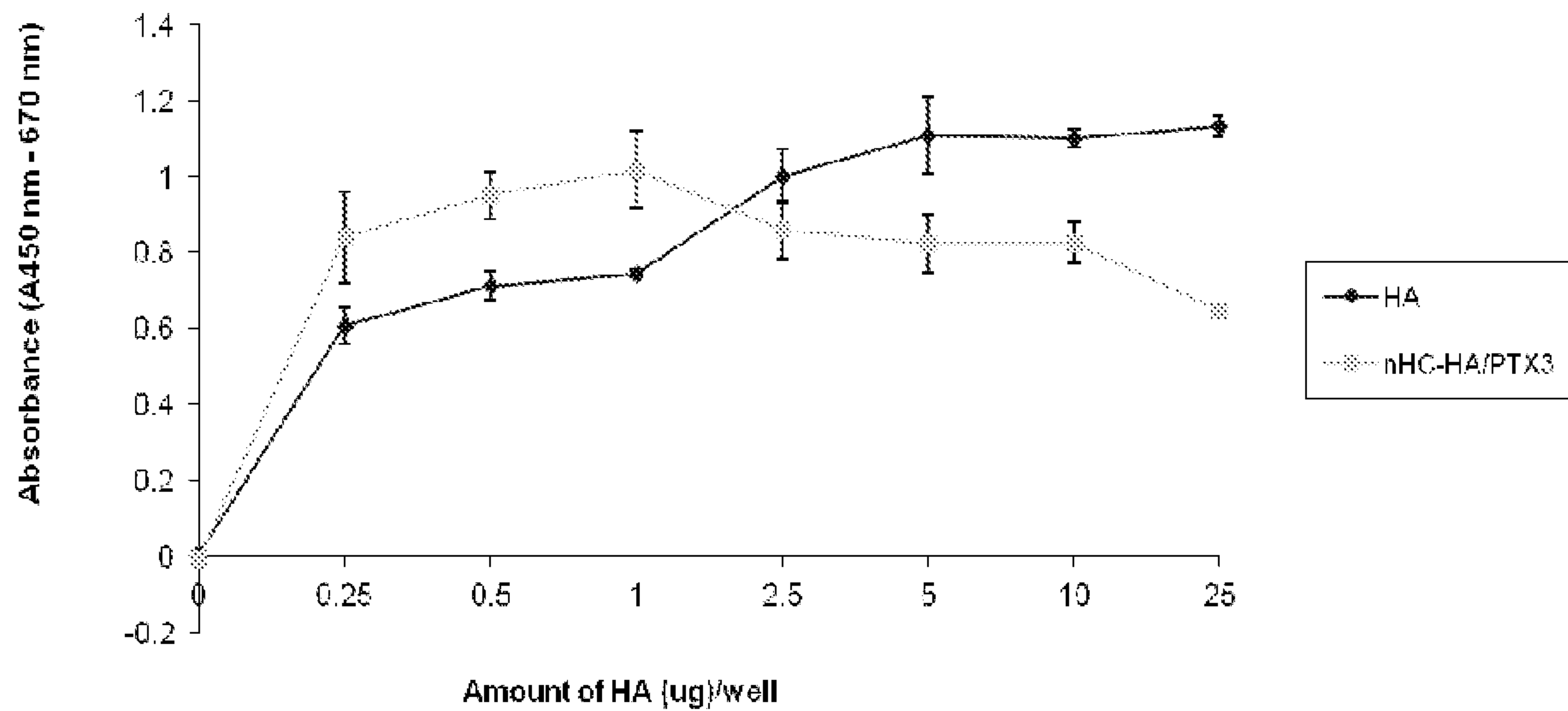
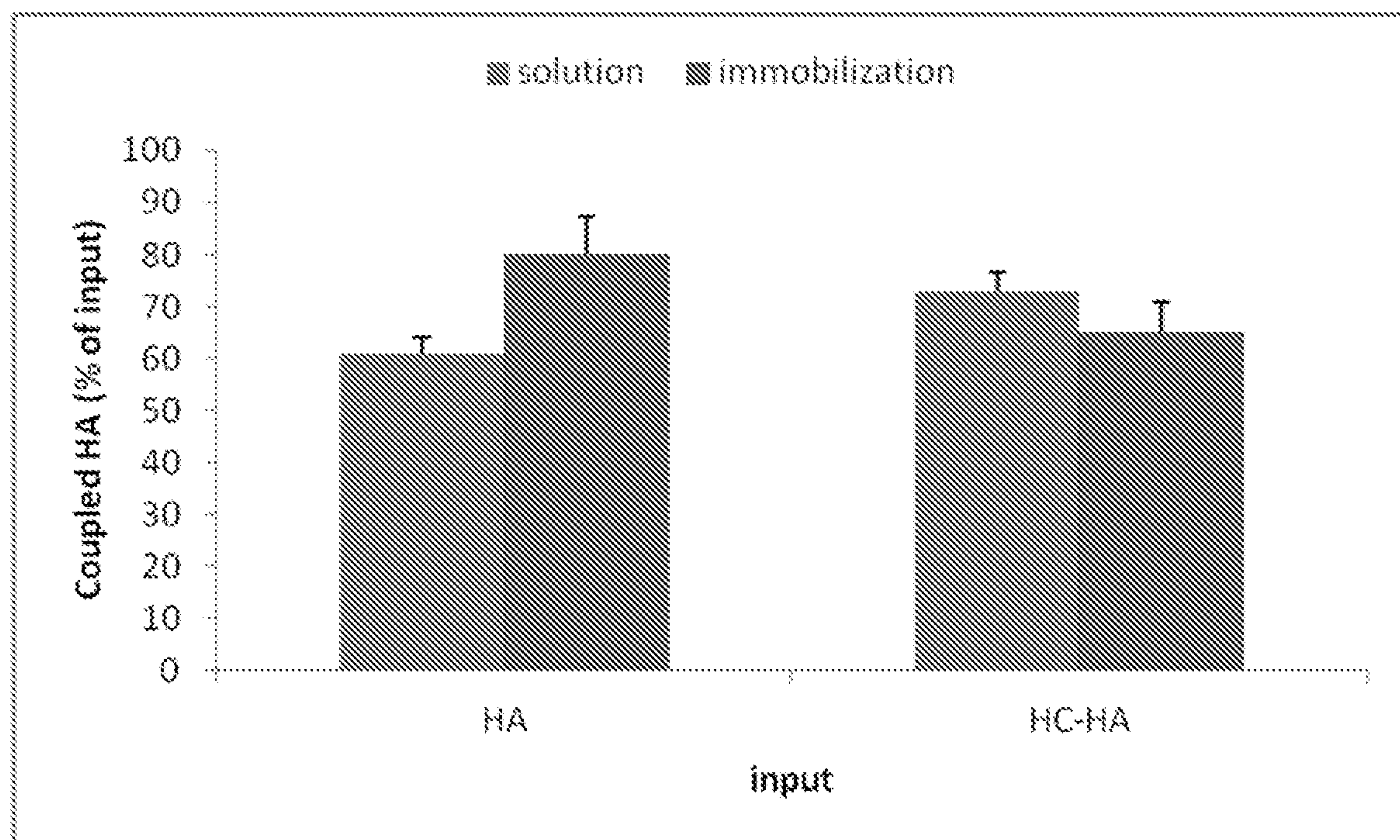
7/112

FIG. 4**A****B**

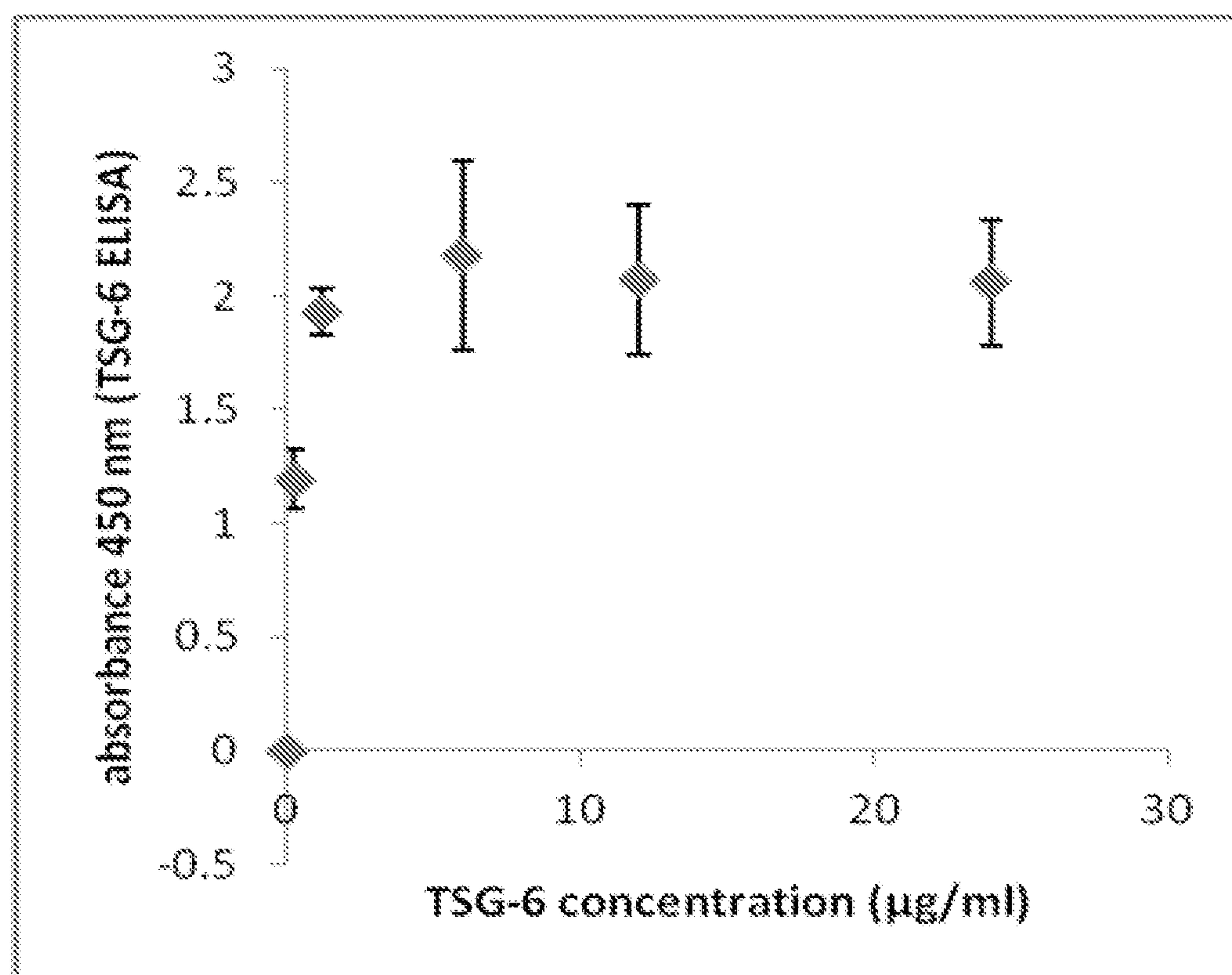
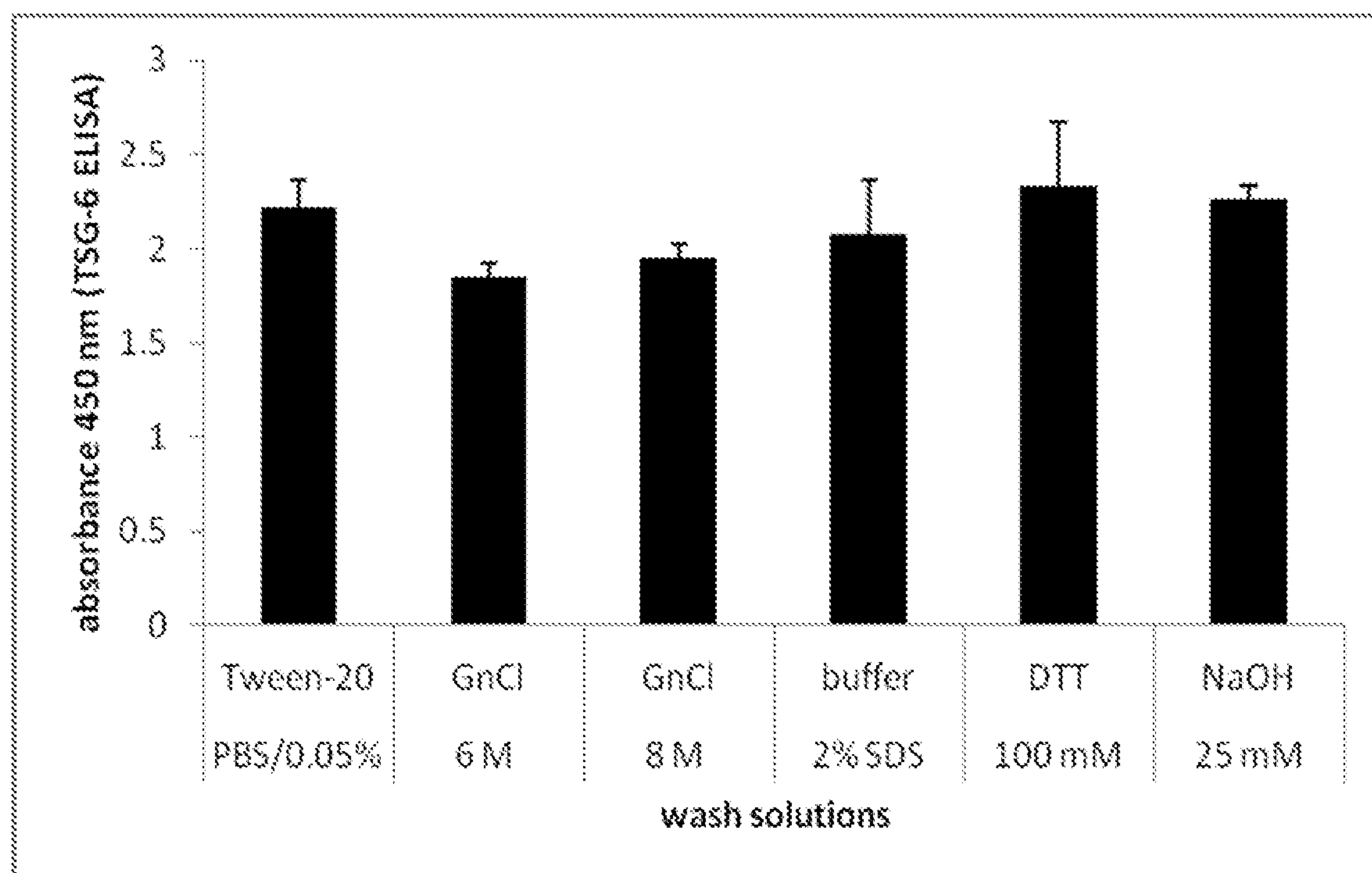
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FIG. 5**A****B****C**

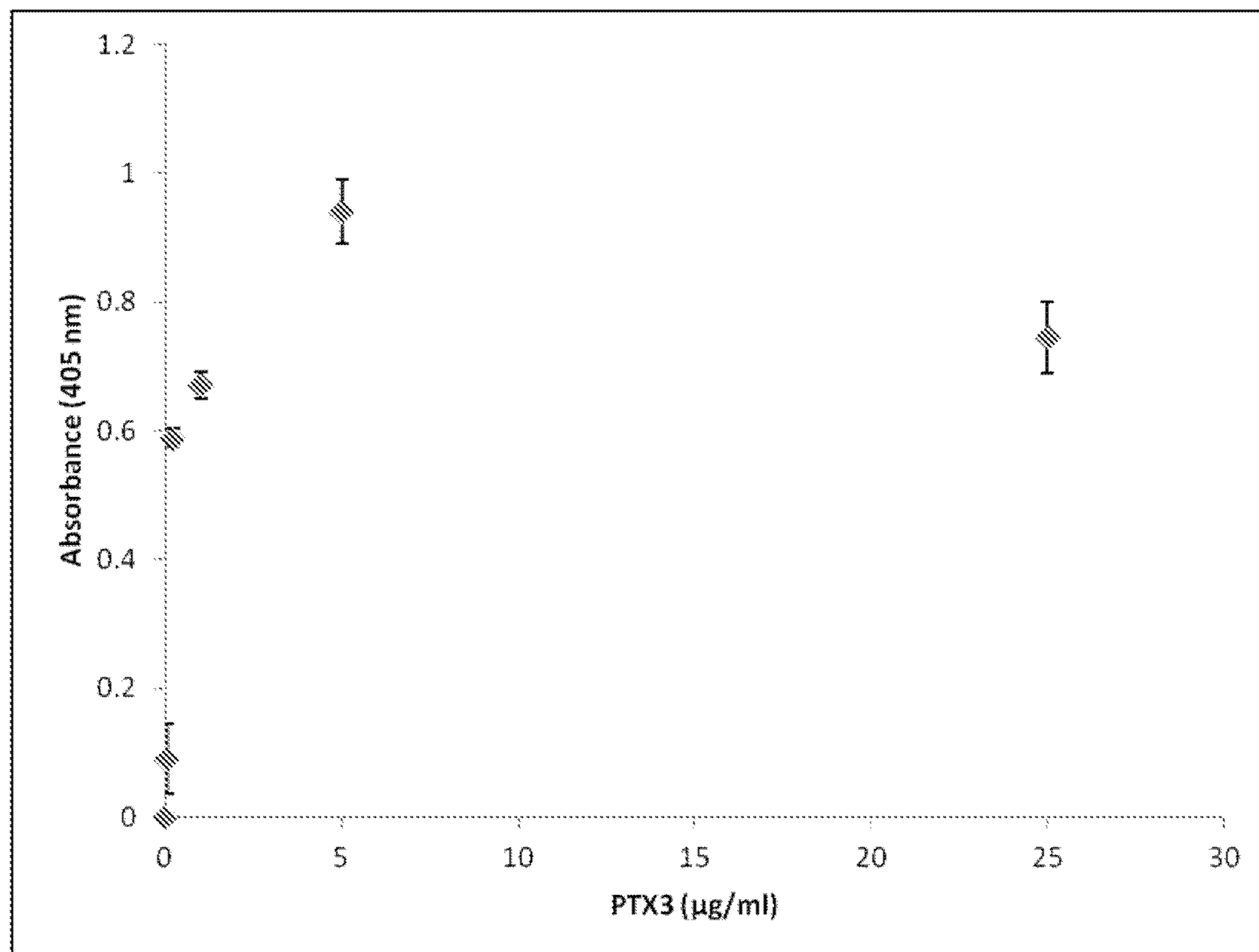
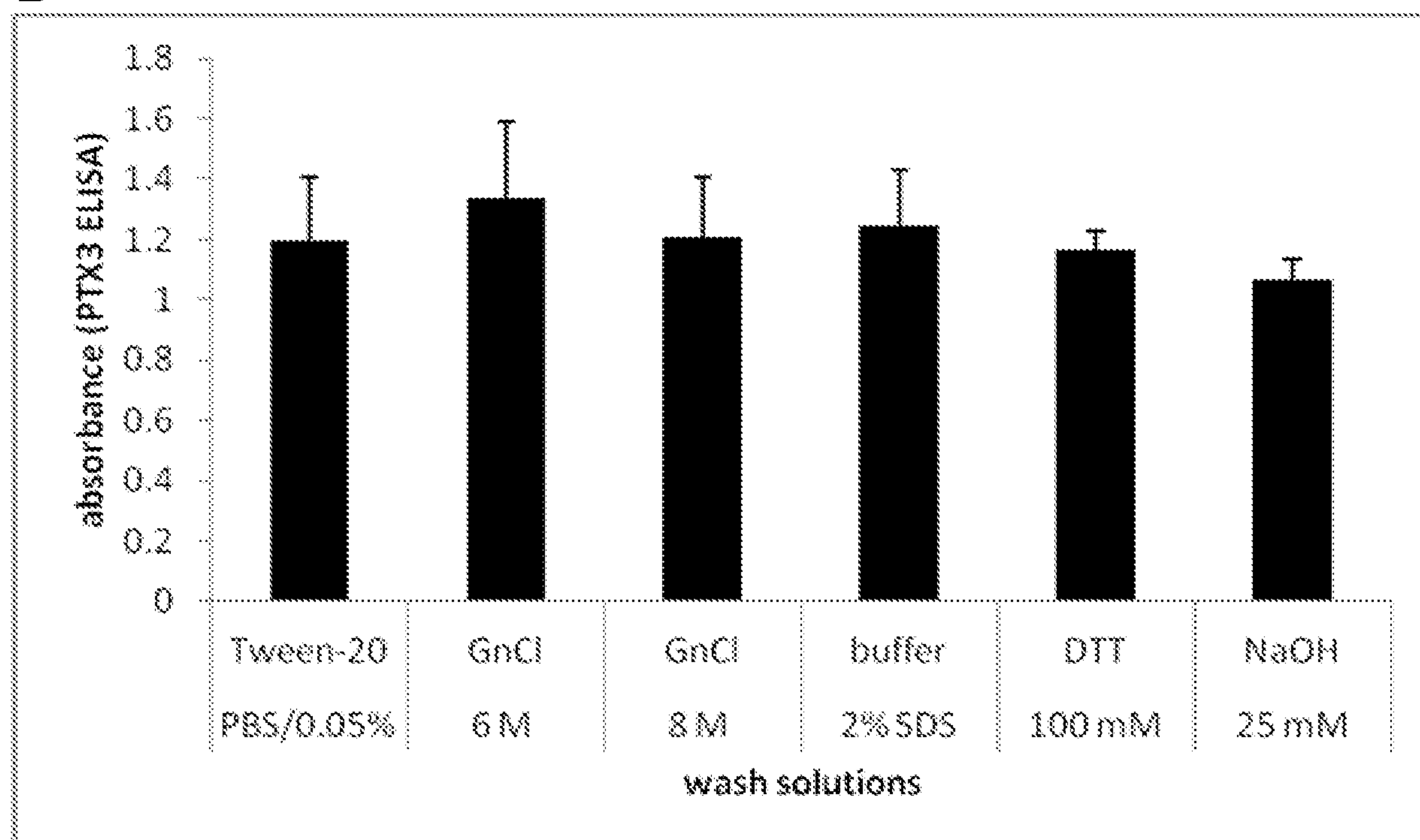
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FIG. 6**A****B**

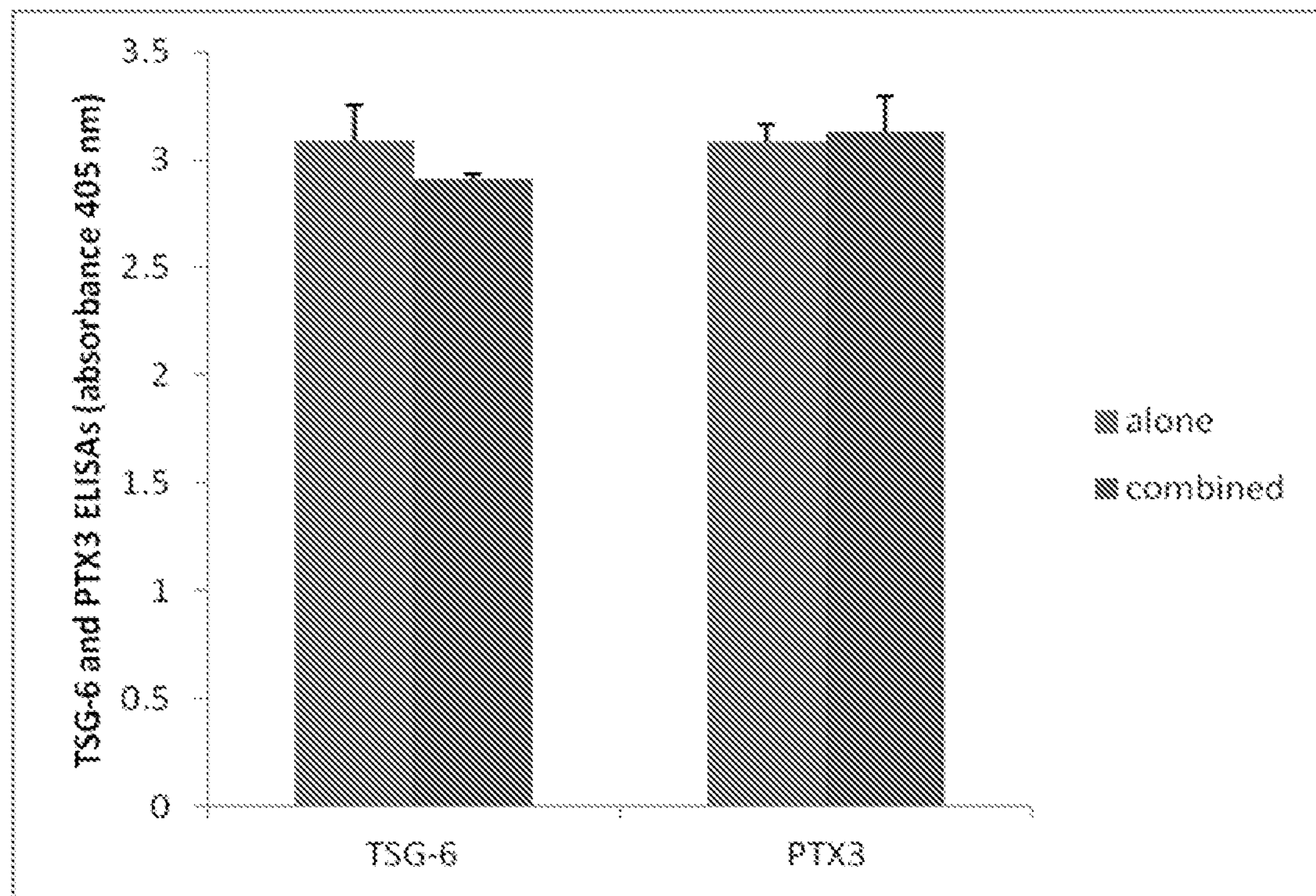
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FIG. 7**A****B**

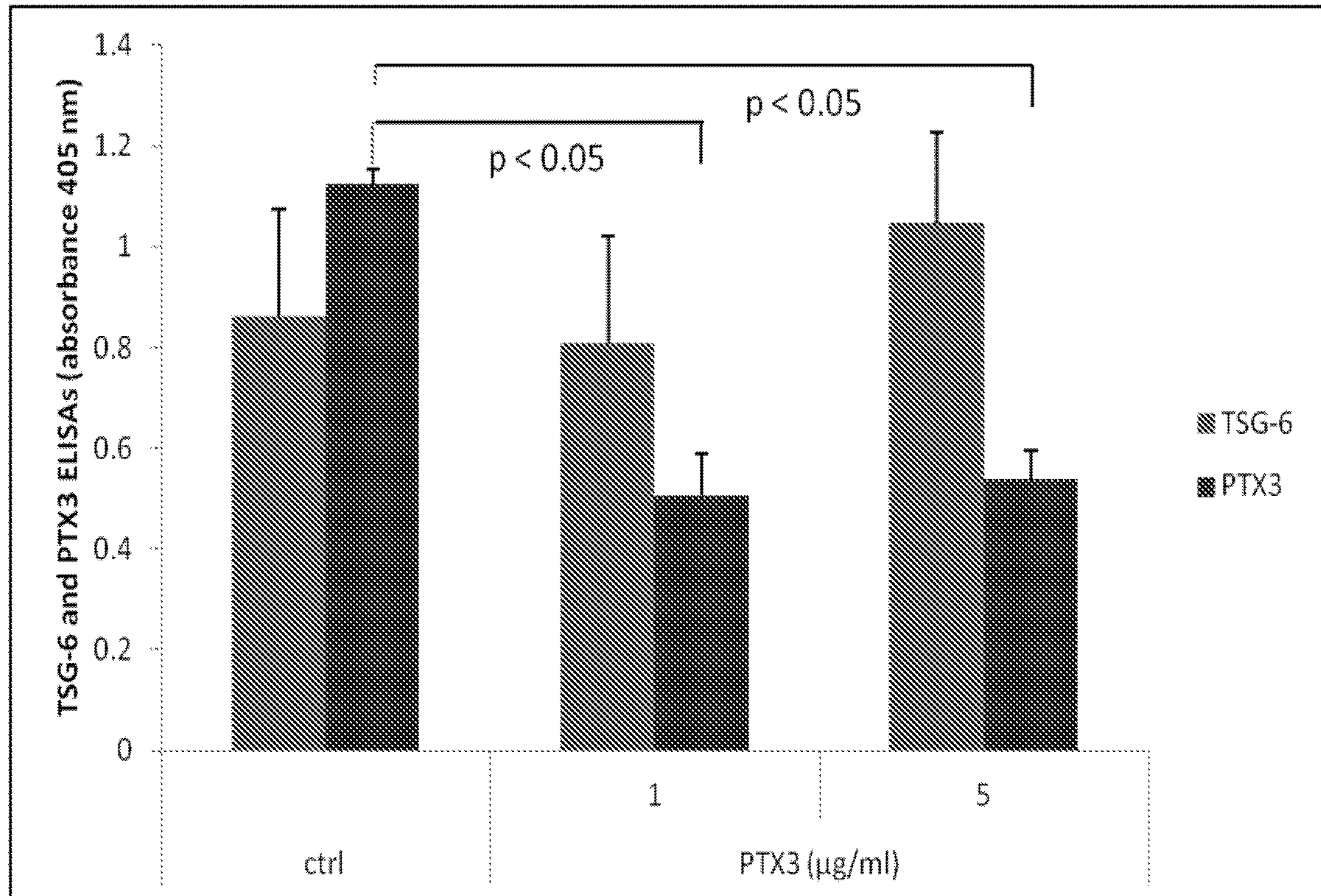
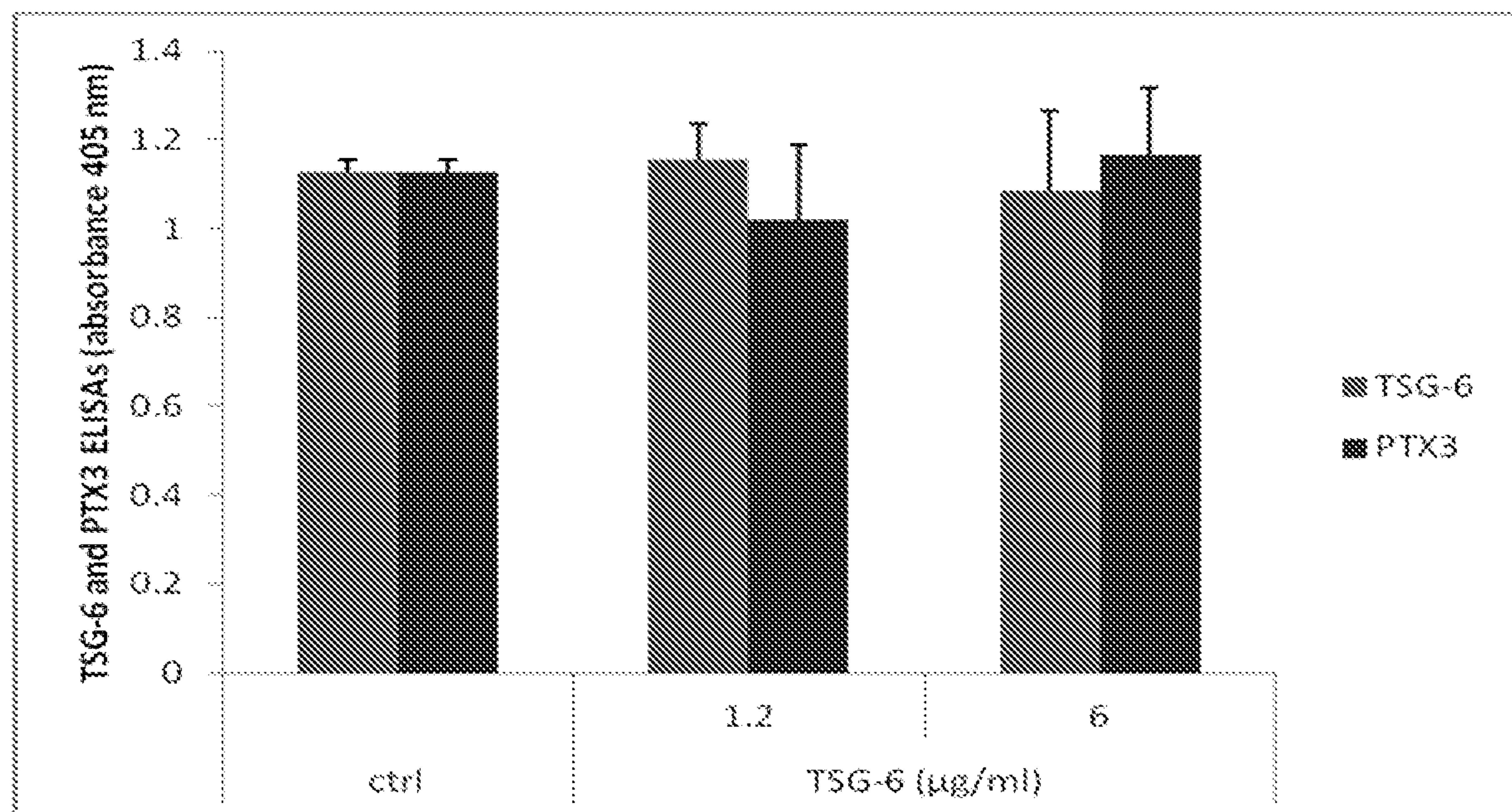
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FIG. 8**A****B**

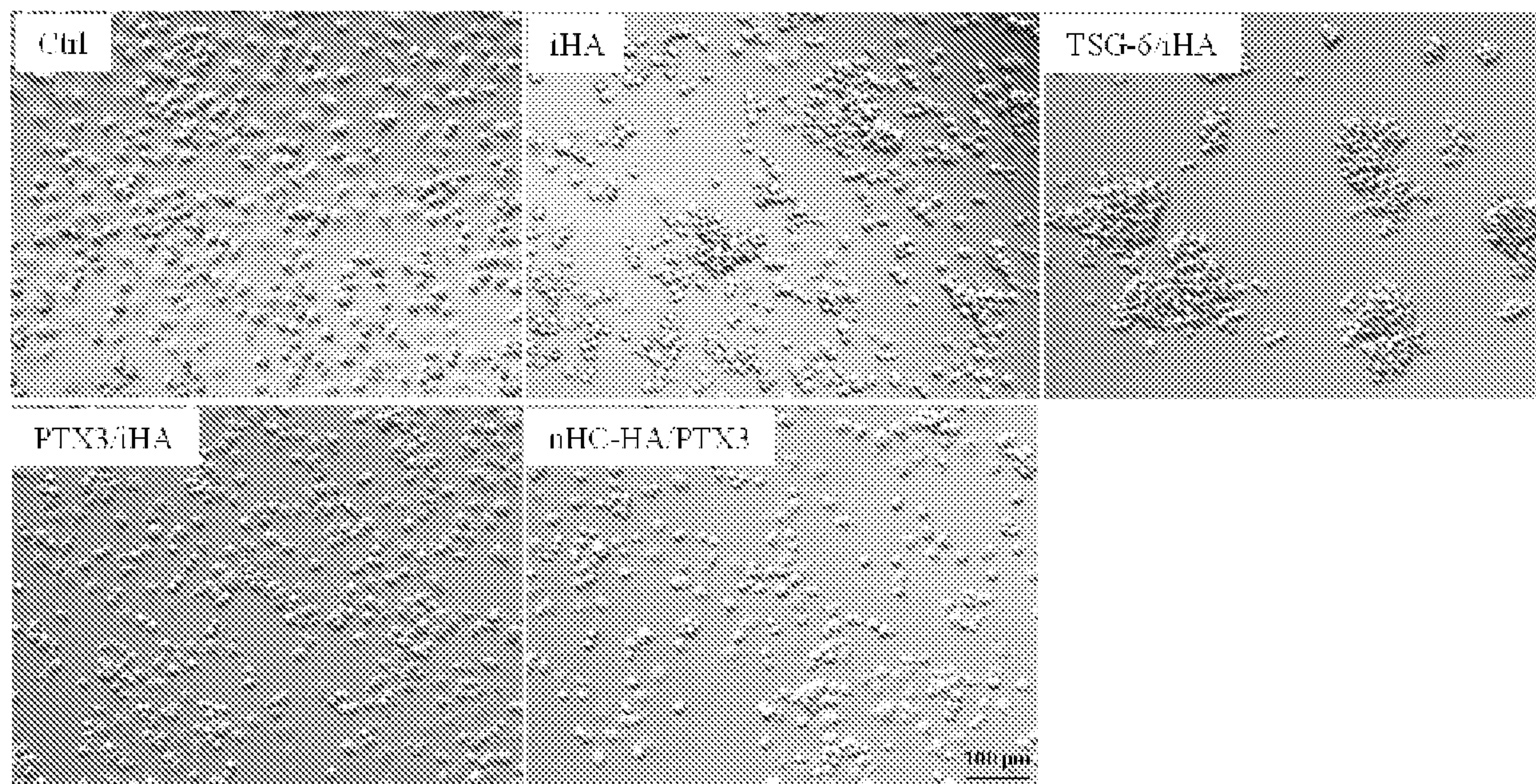
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FIG. 9

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FIG. 10**A****B**

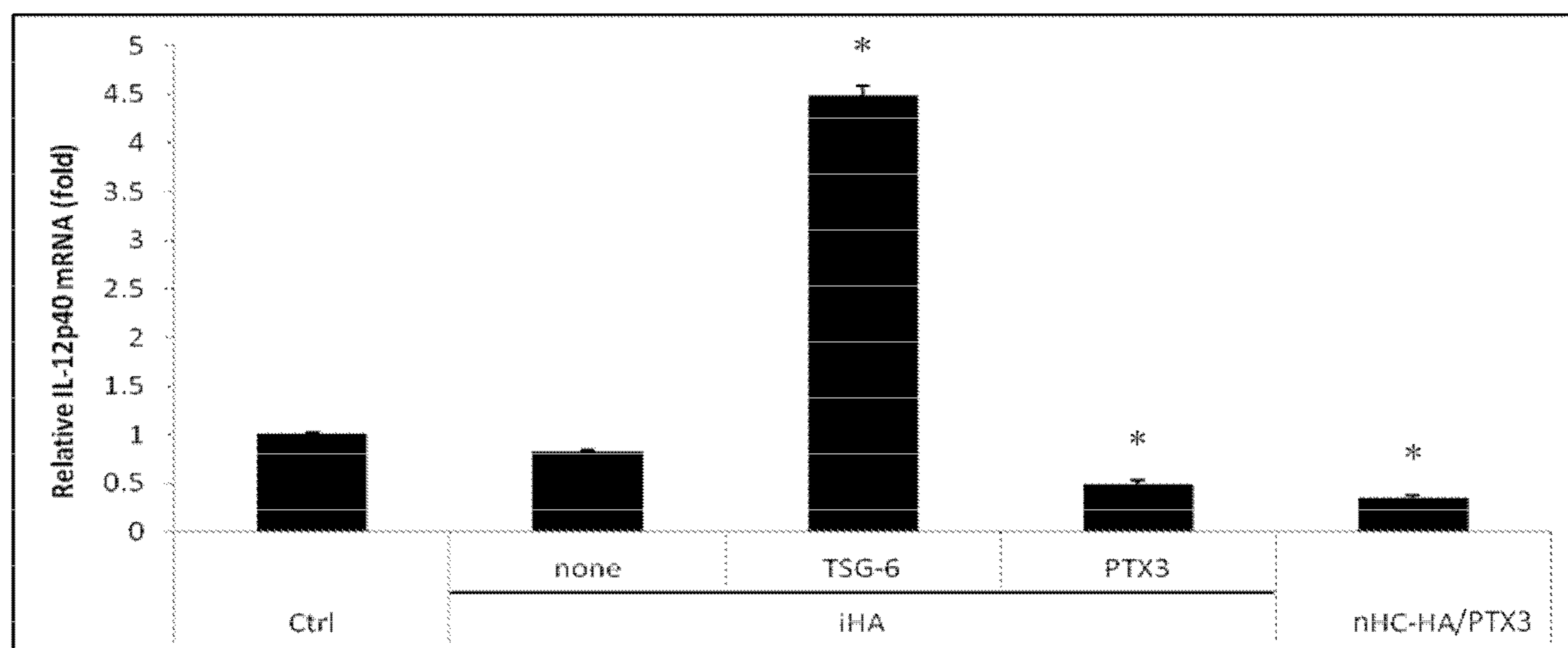
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FIG. 11

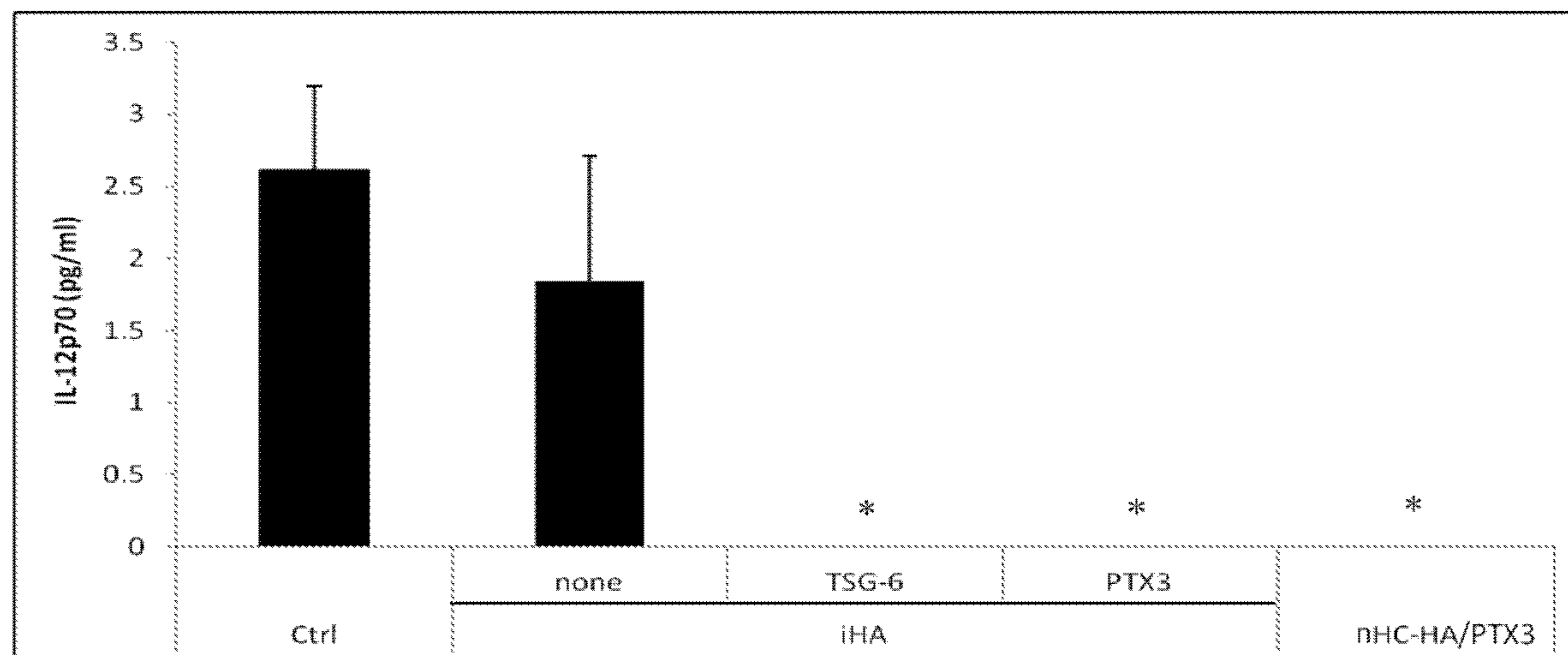
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FIG. 12

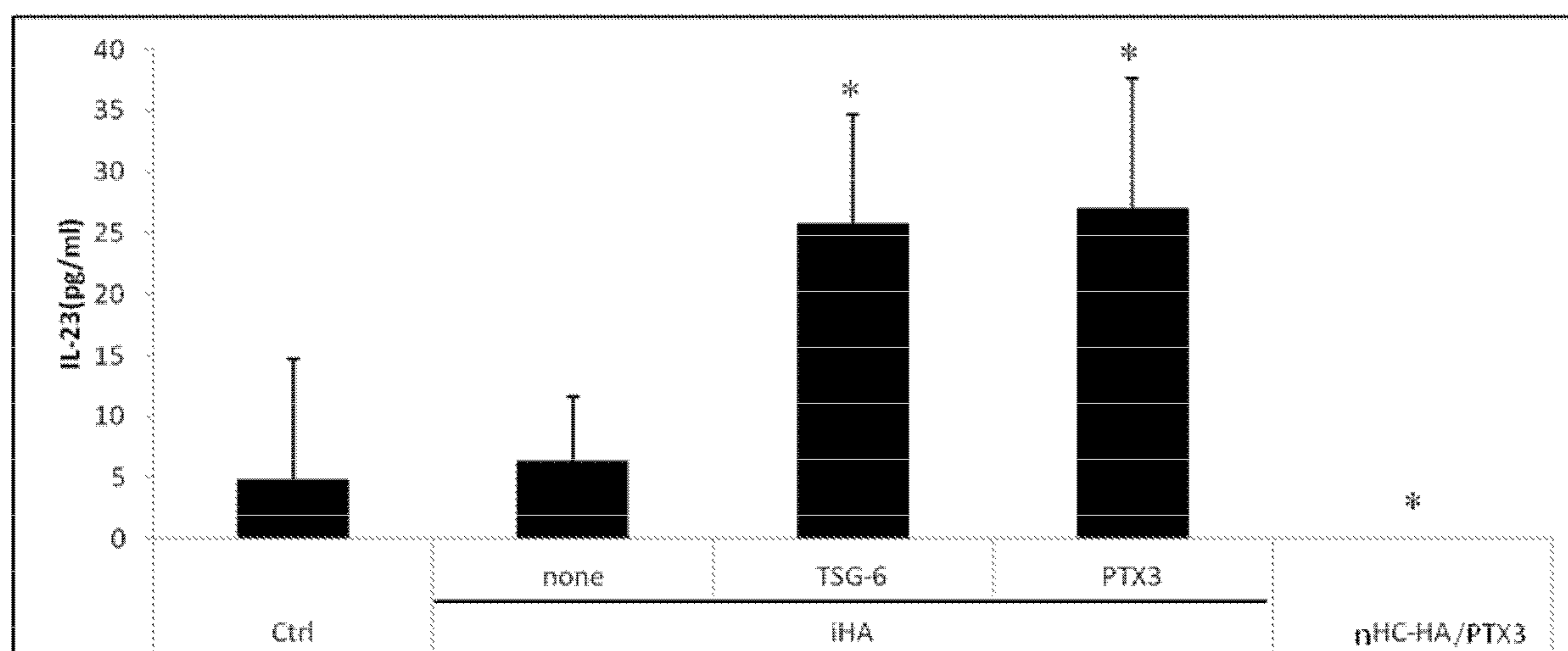
A



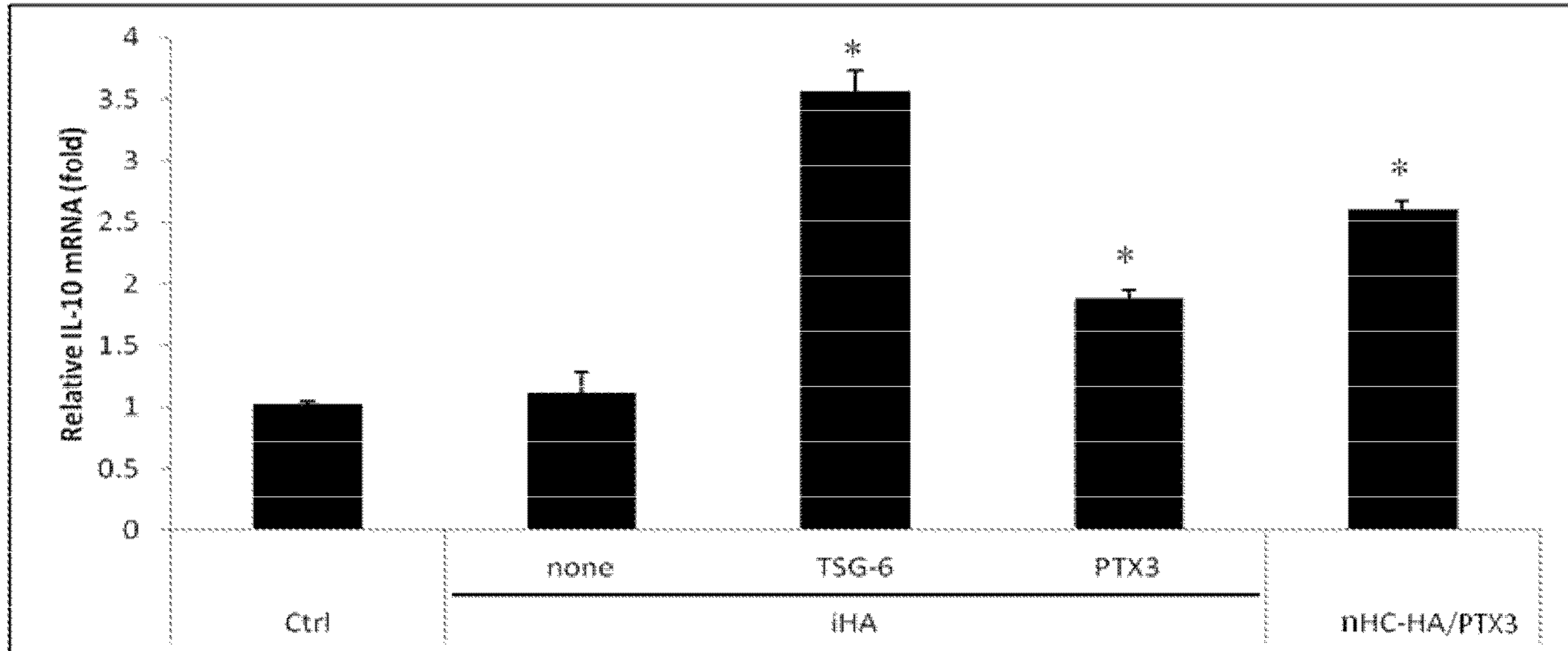
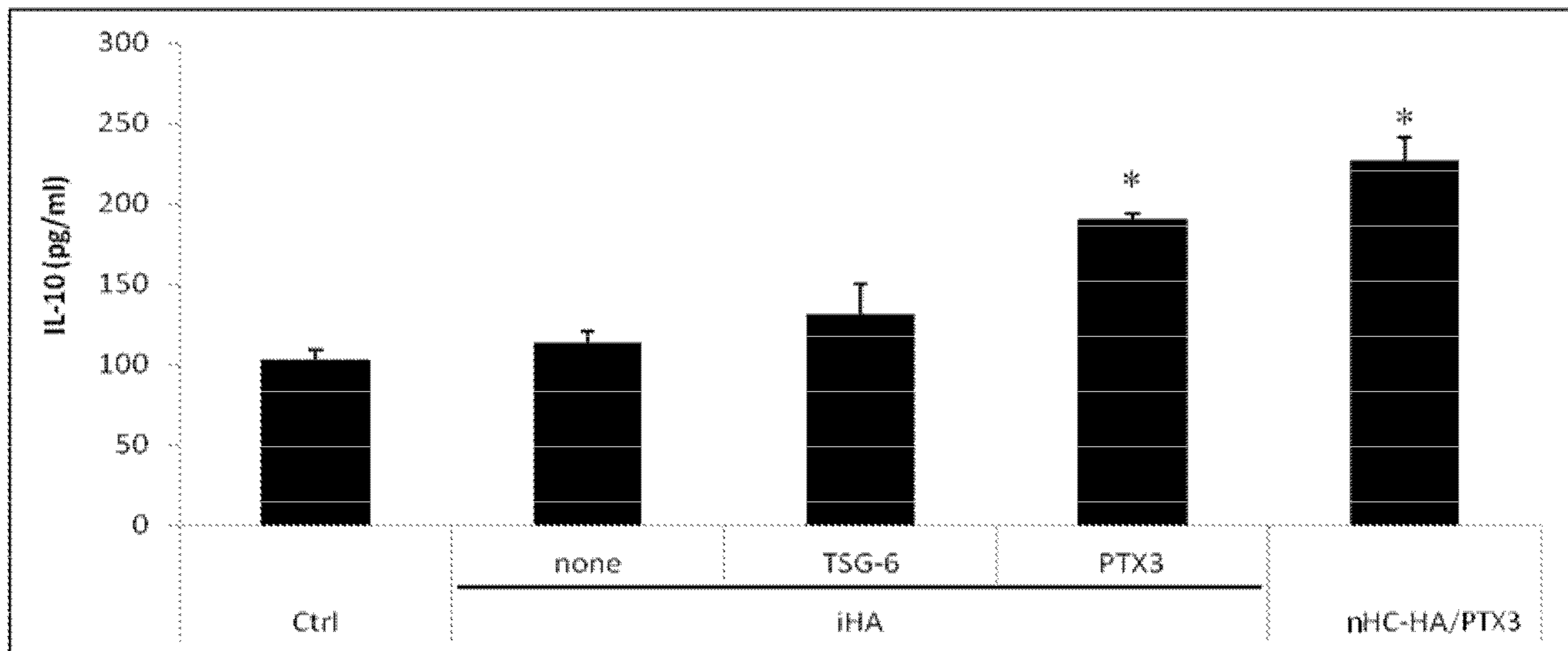
B



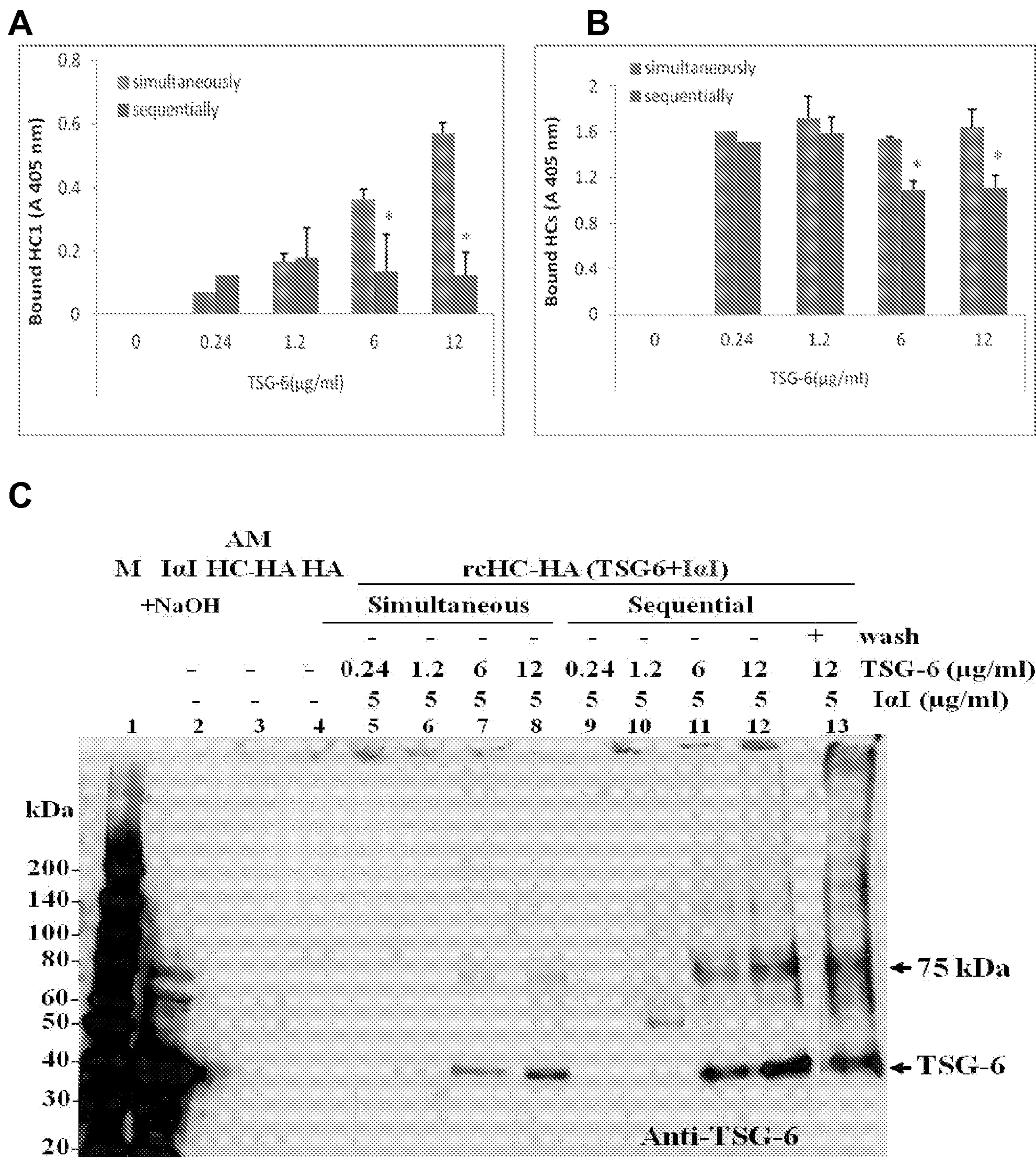
C



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FIG. 12 (cont'd)**D****E**

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FIG. 13

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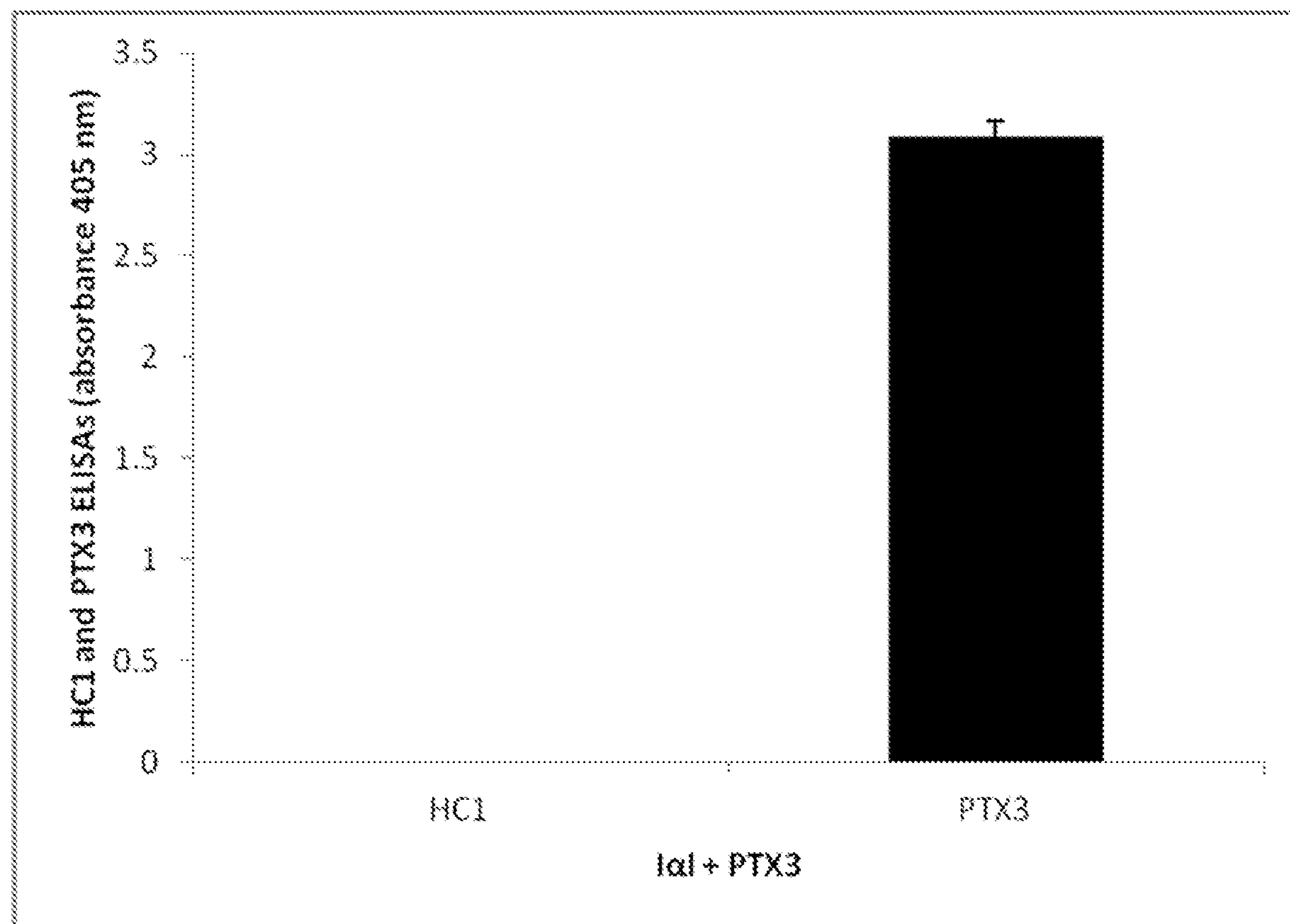
FIG. 13 (cont'd)**D**

FIG. 14

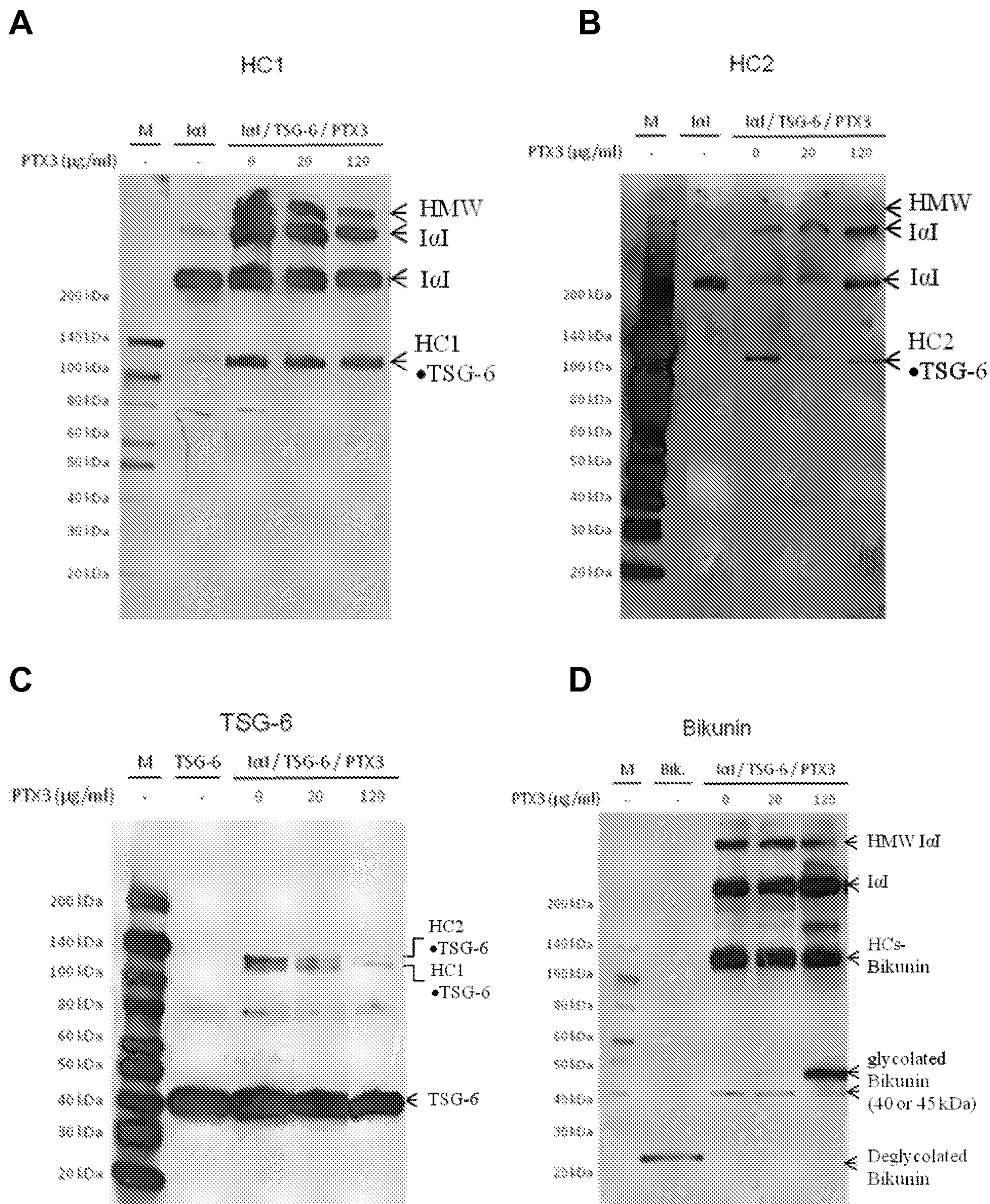
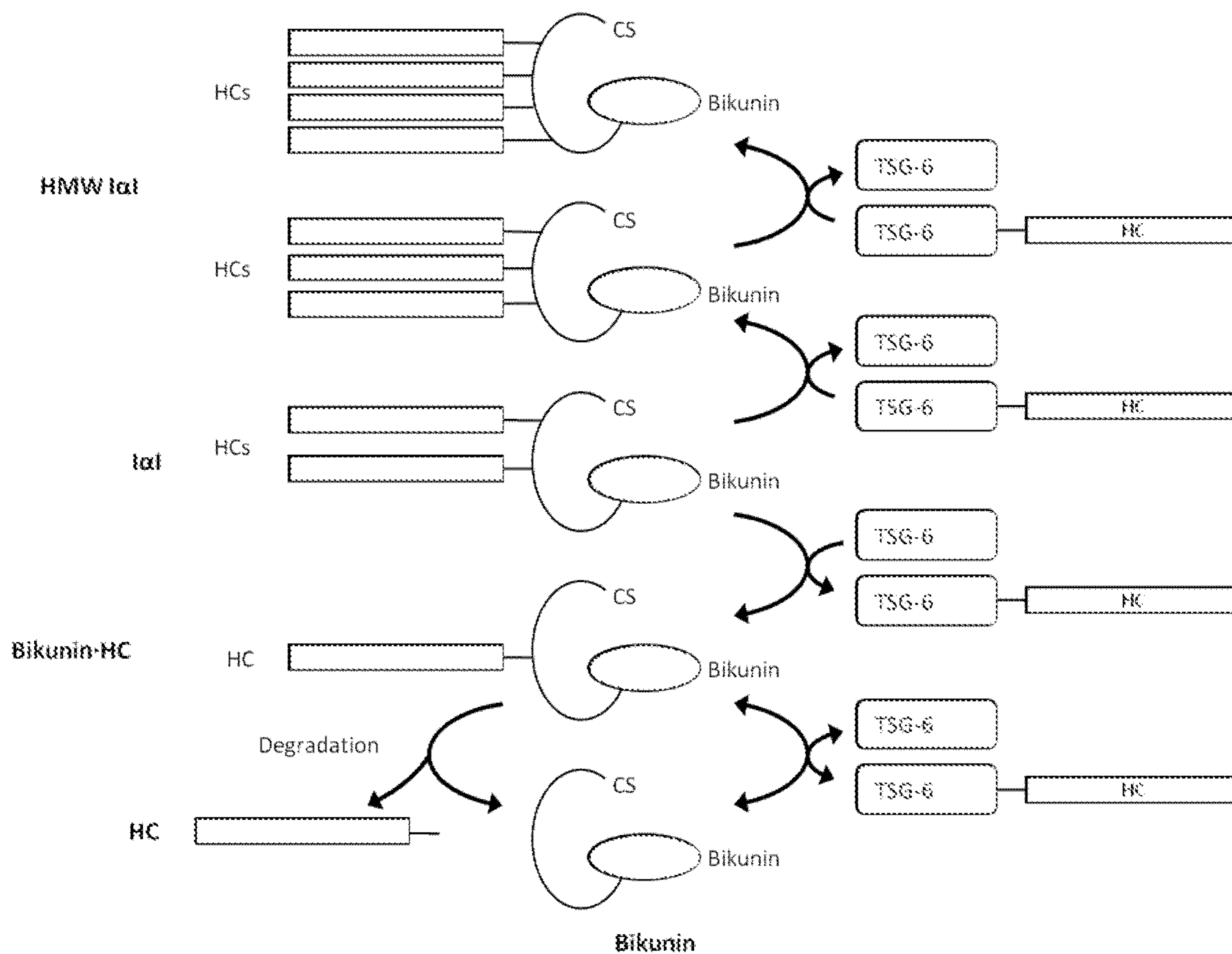
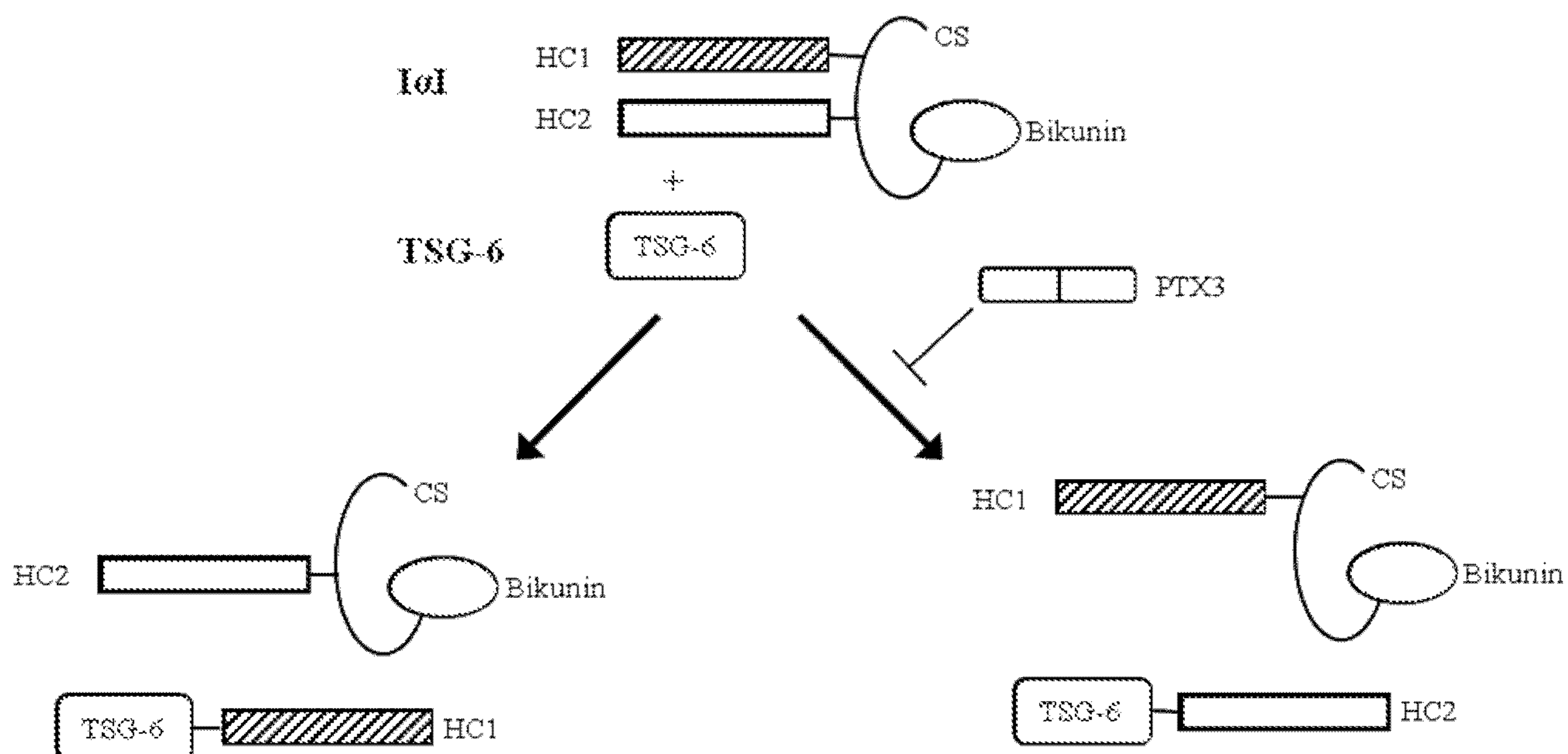


FIG. 14 (cont'd)**E****F**

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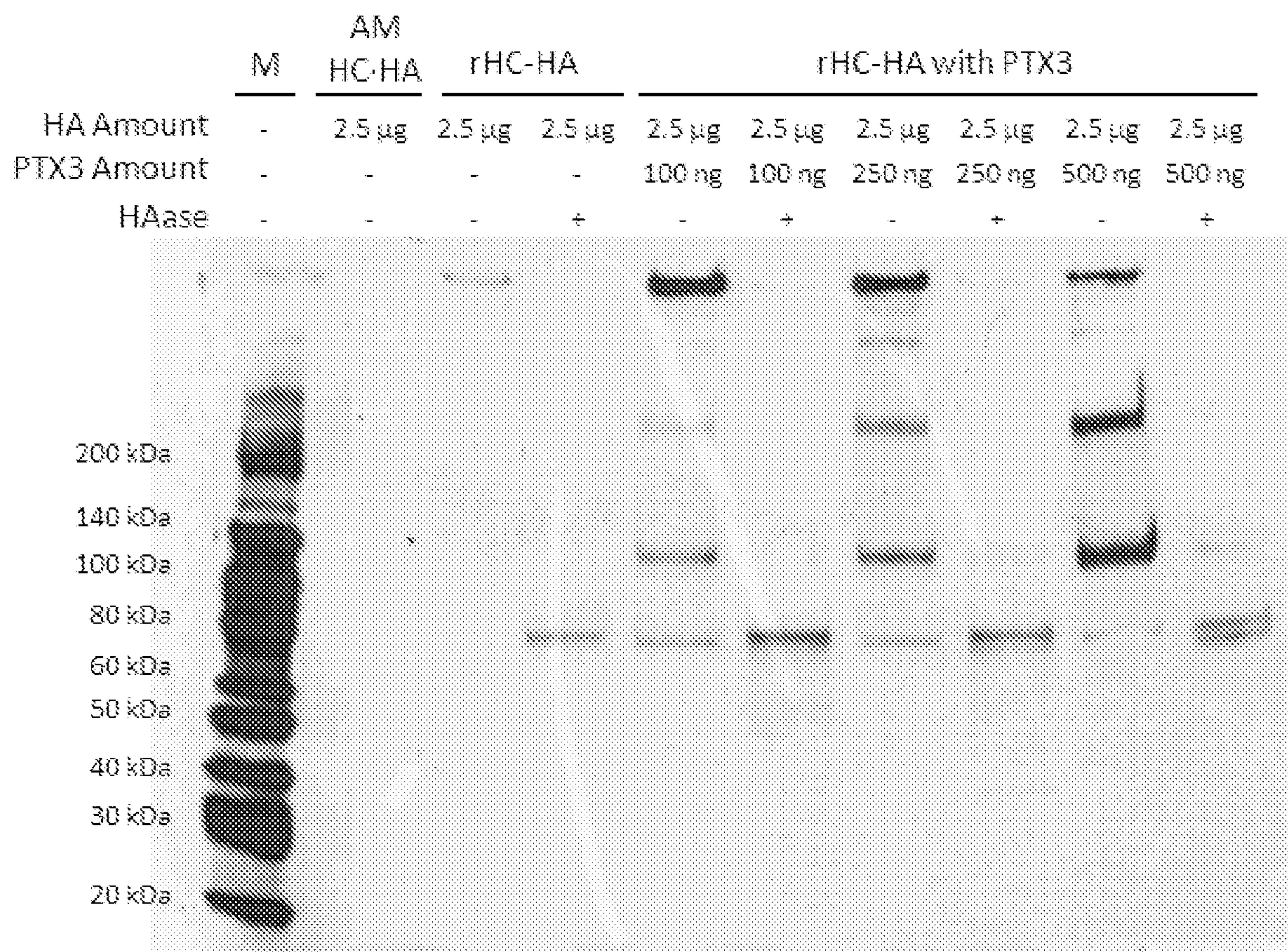
FIG. 14 (cont'd)**G**

FIG. 15

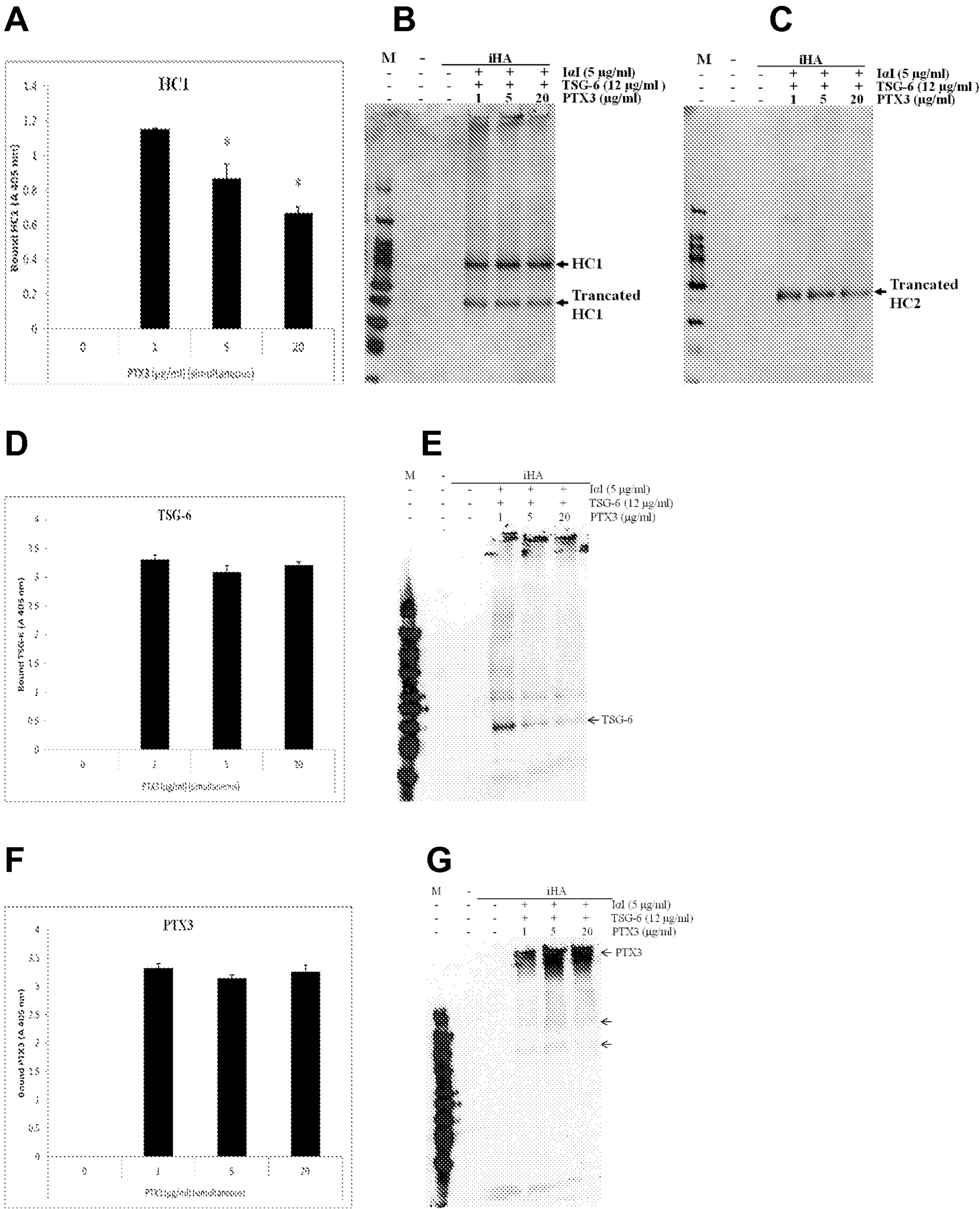


FIG. 16

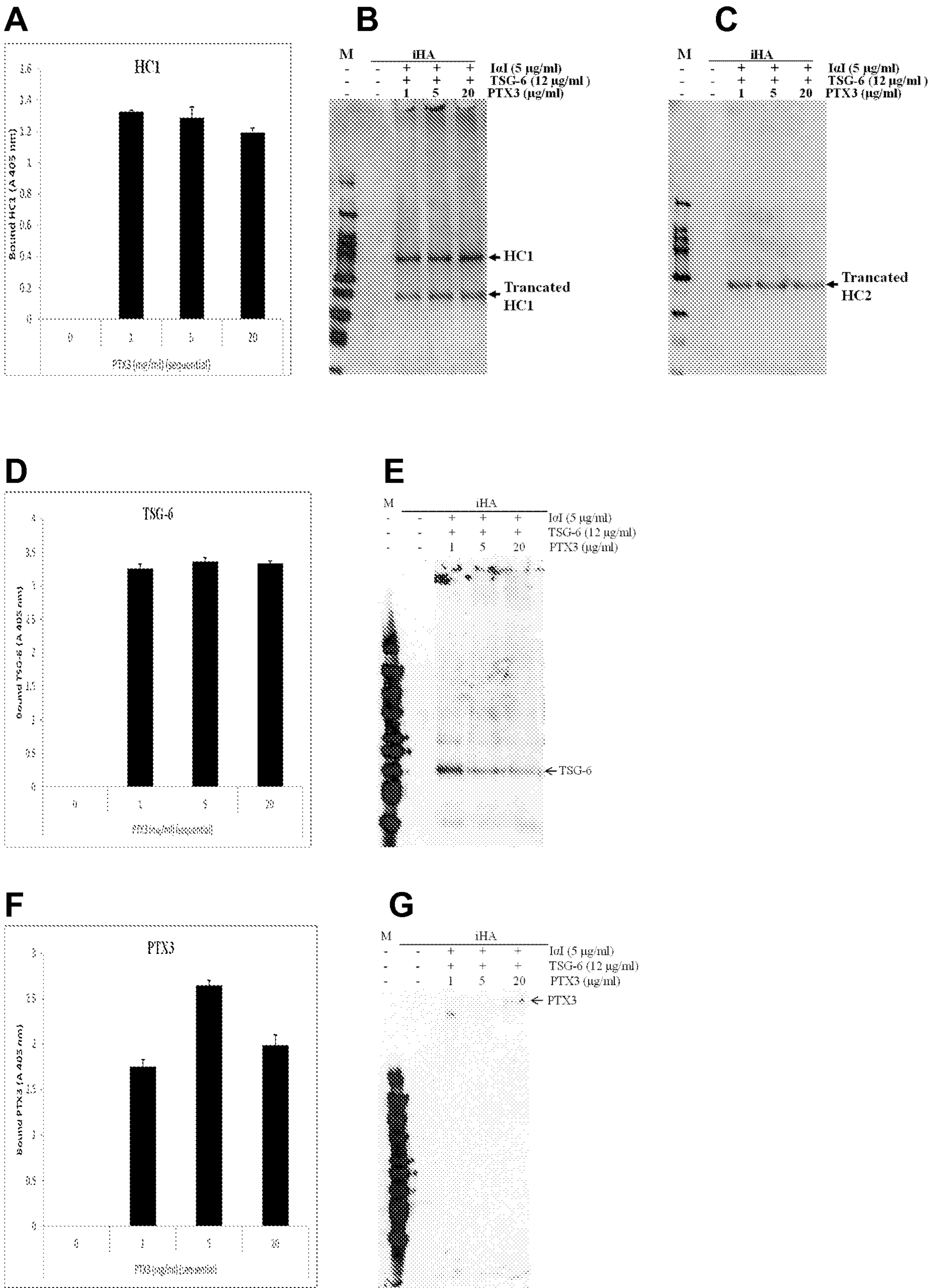
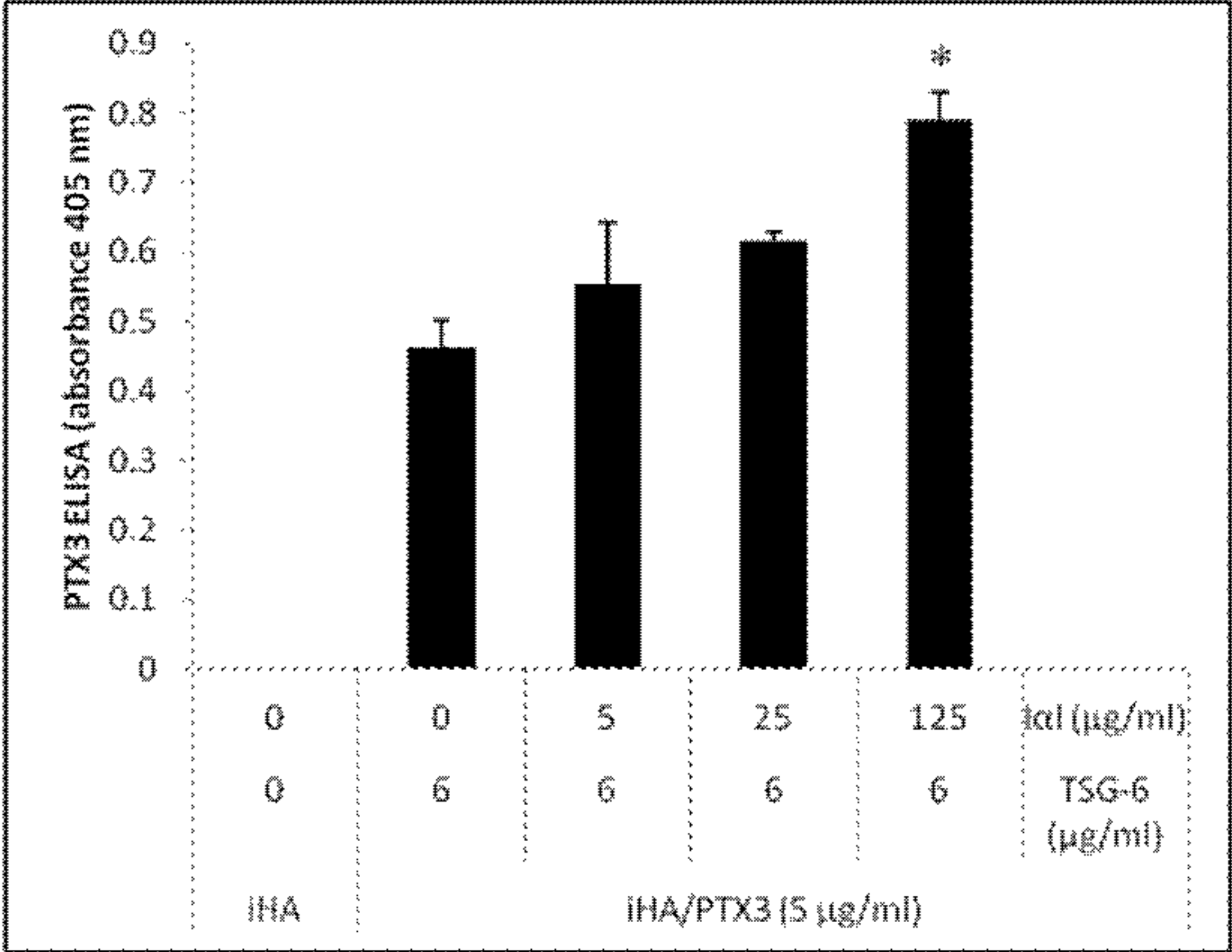
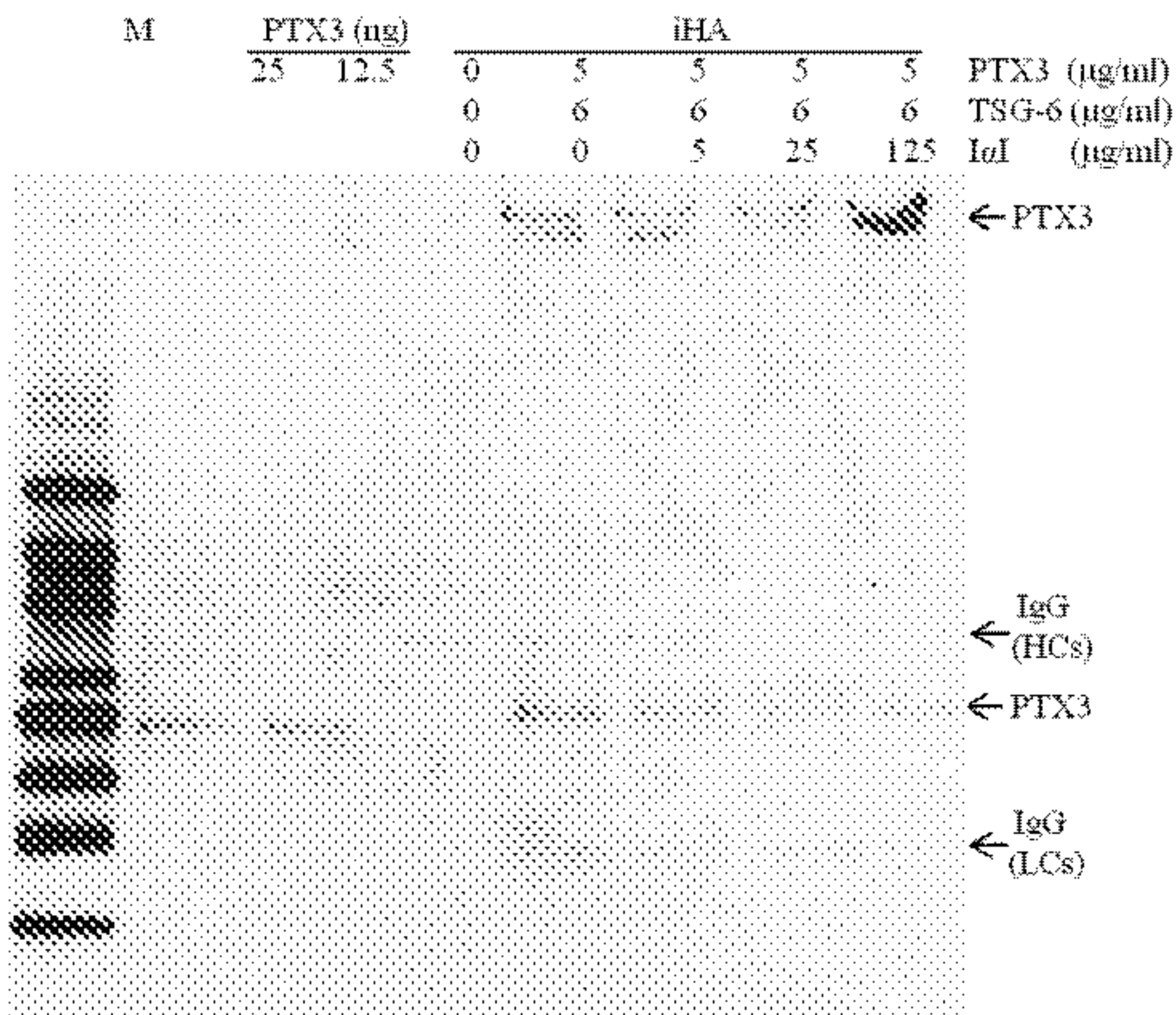


FIG. 17

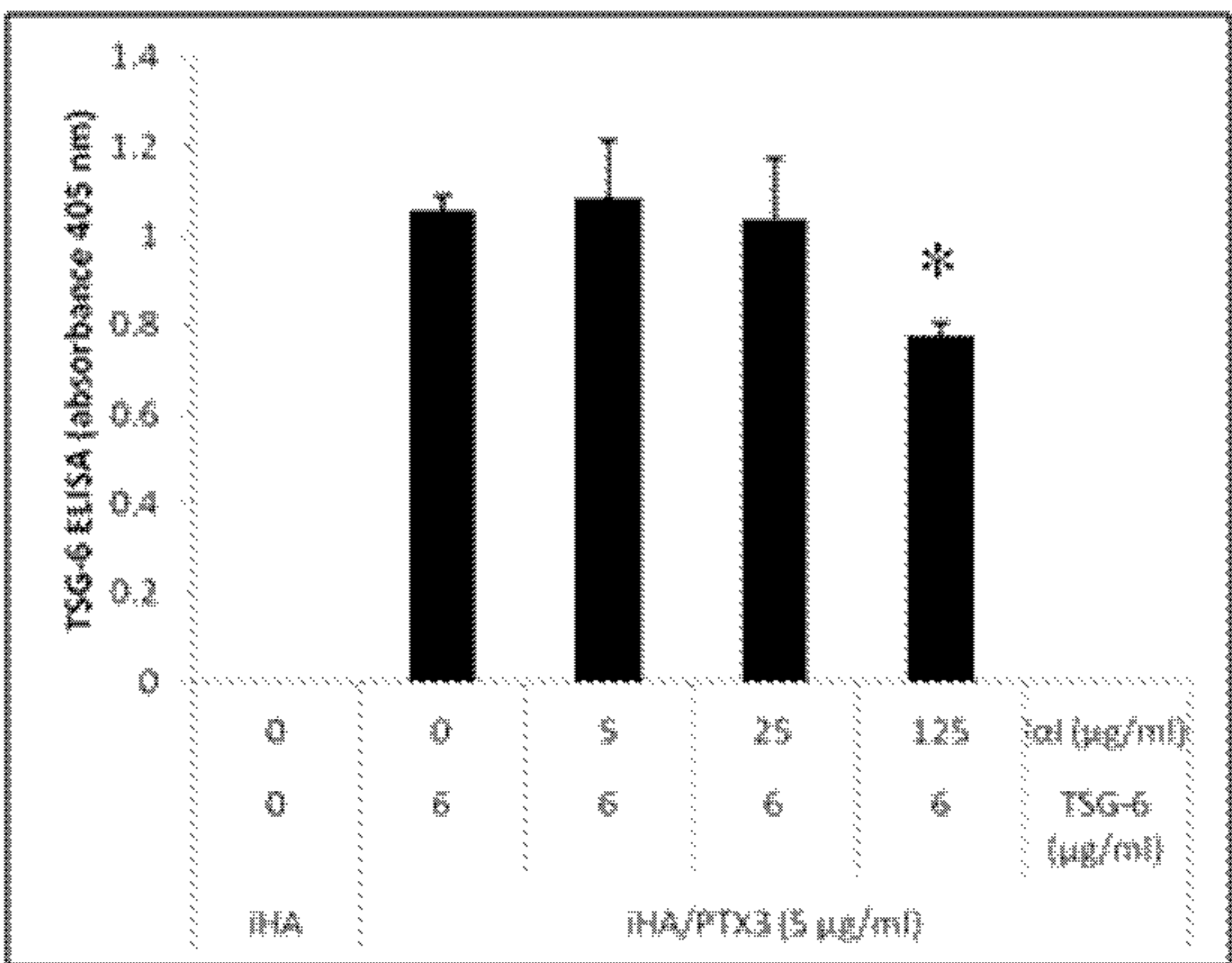
A



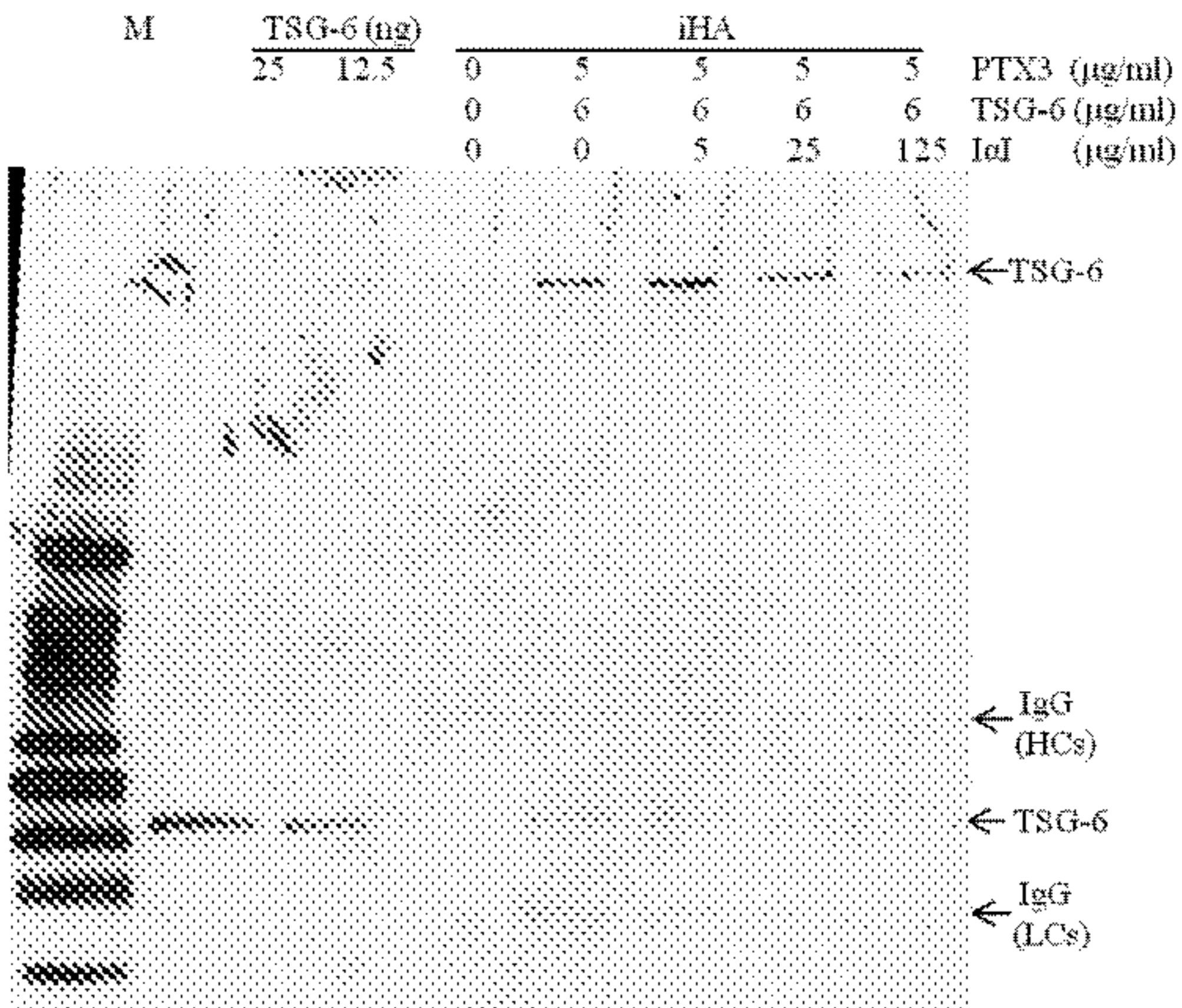
B



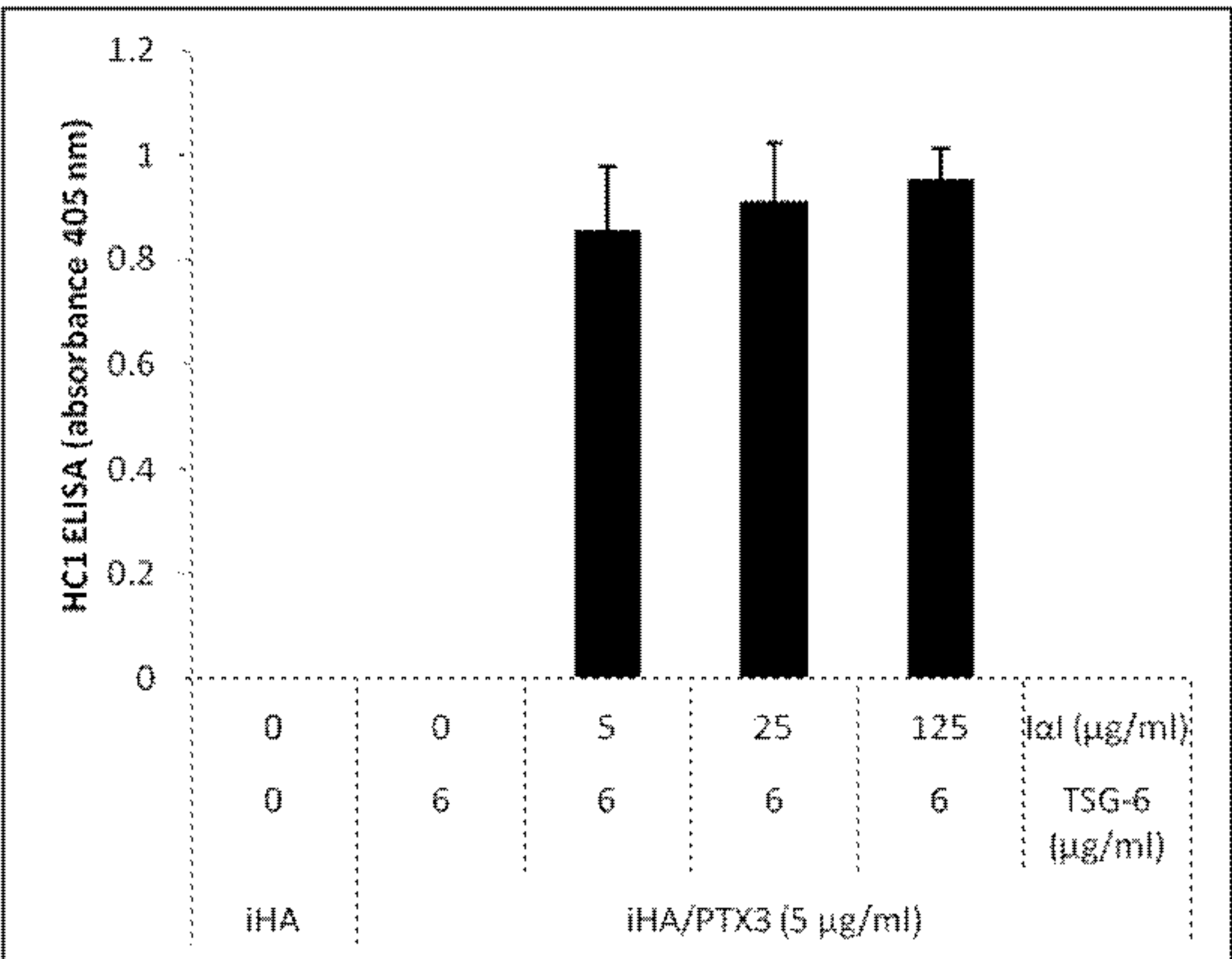
C



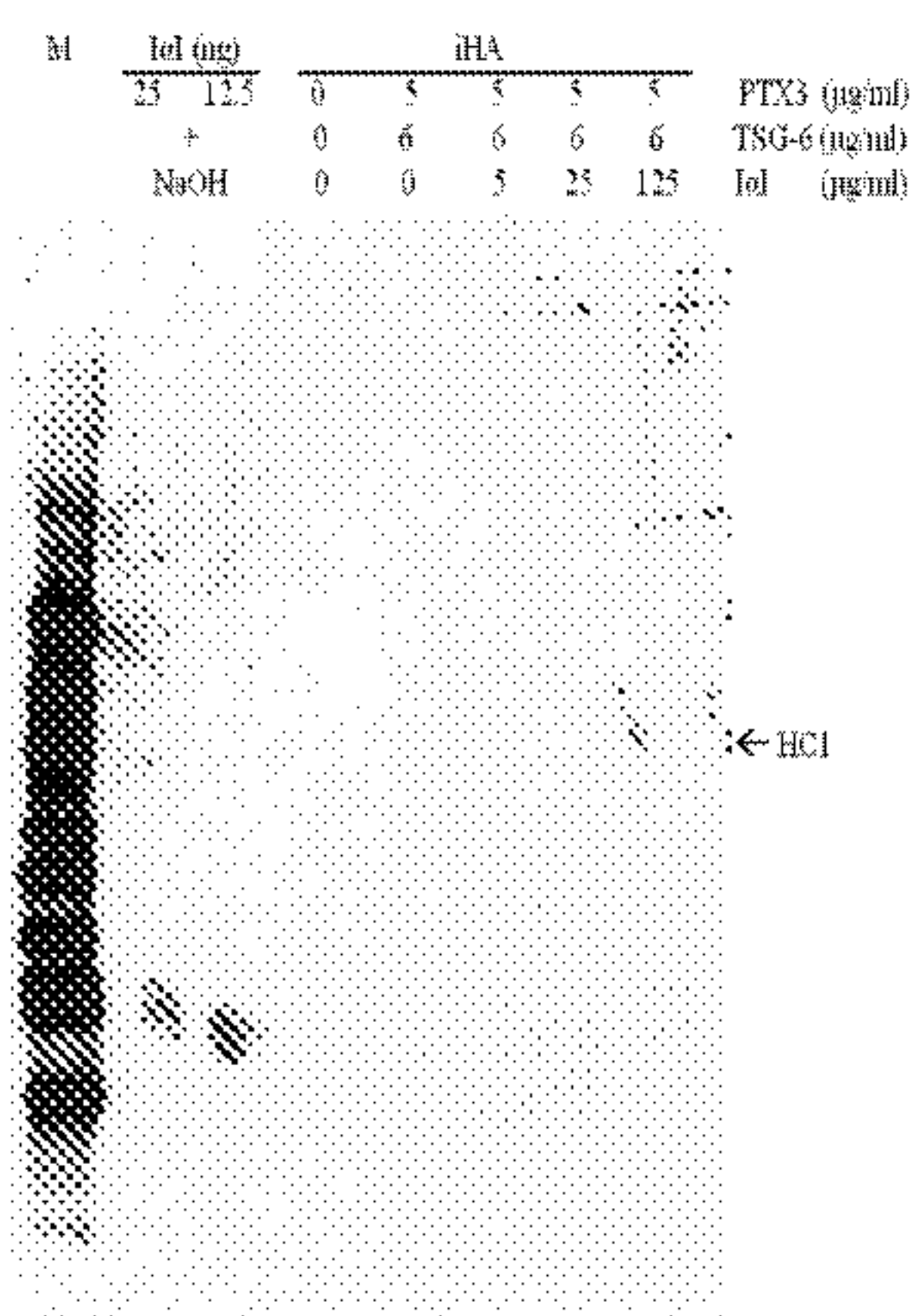
D



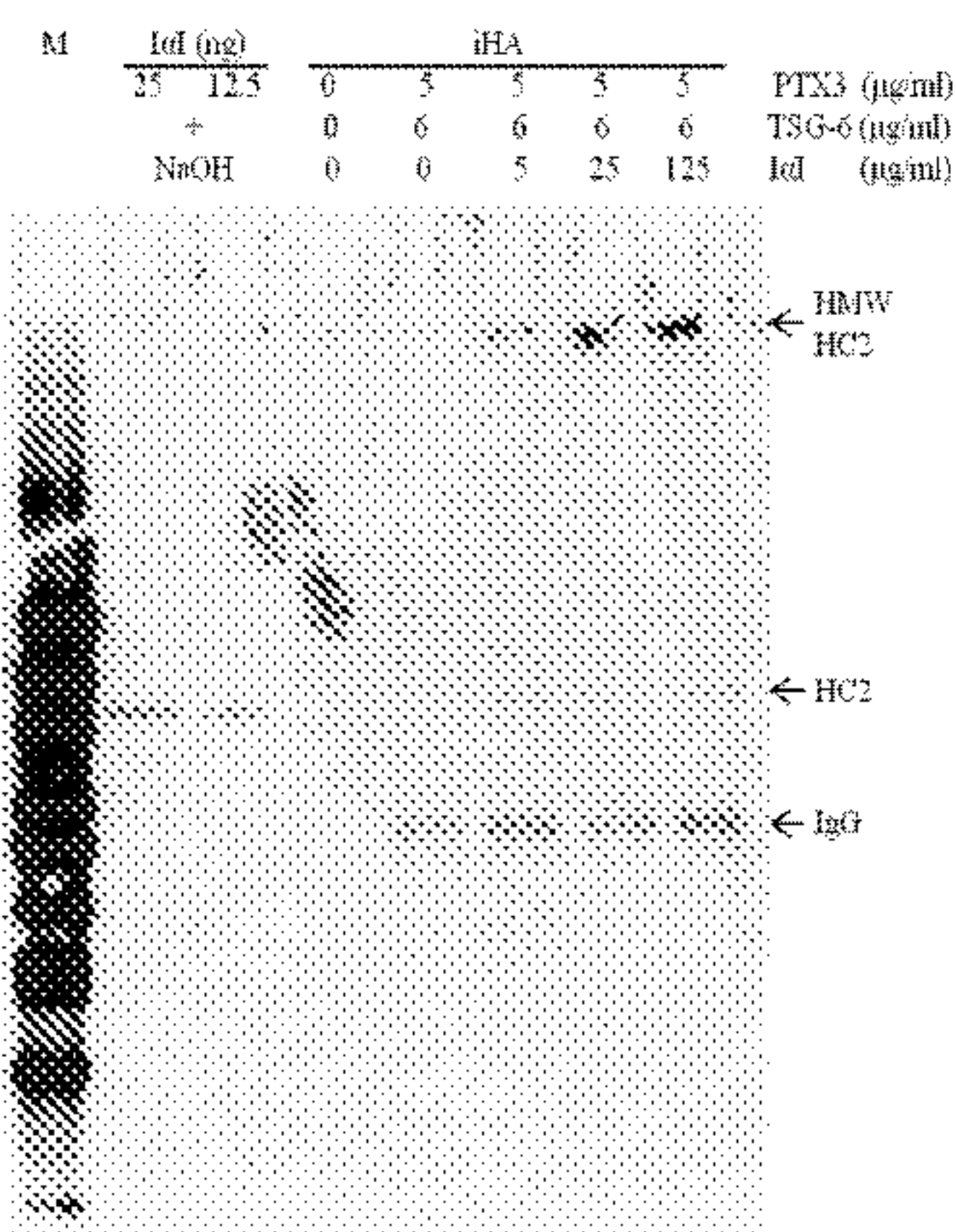
E



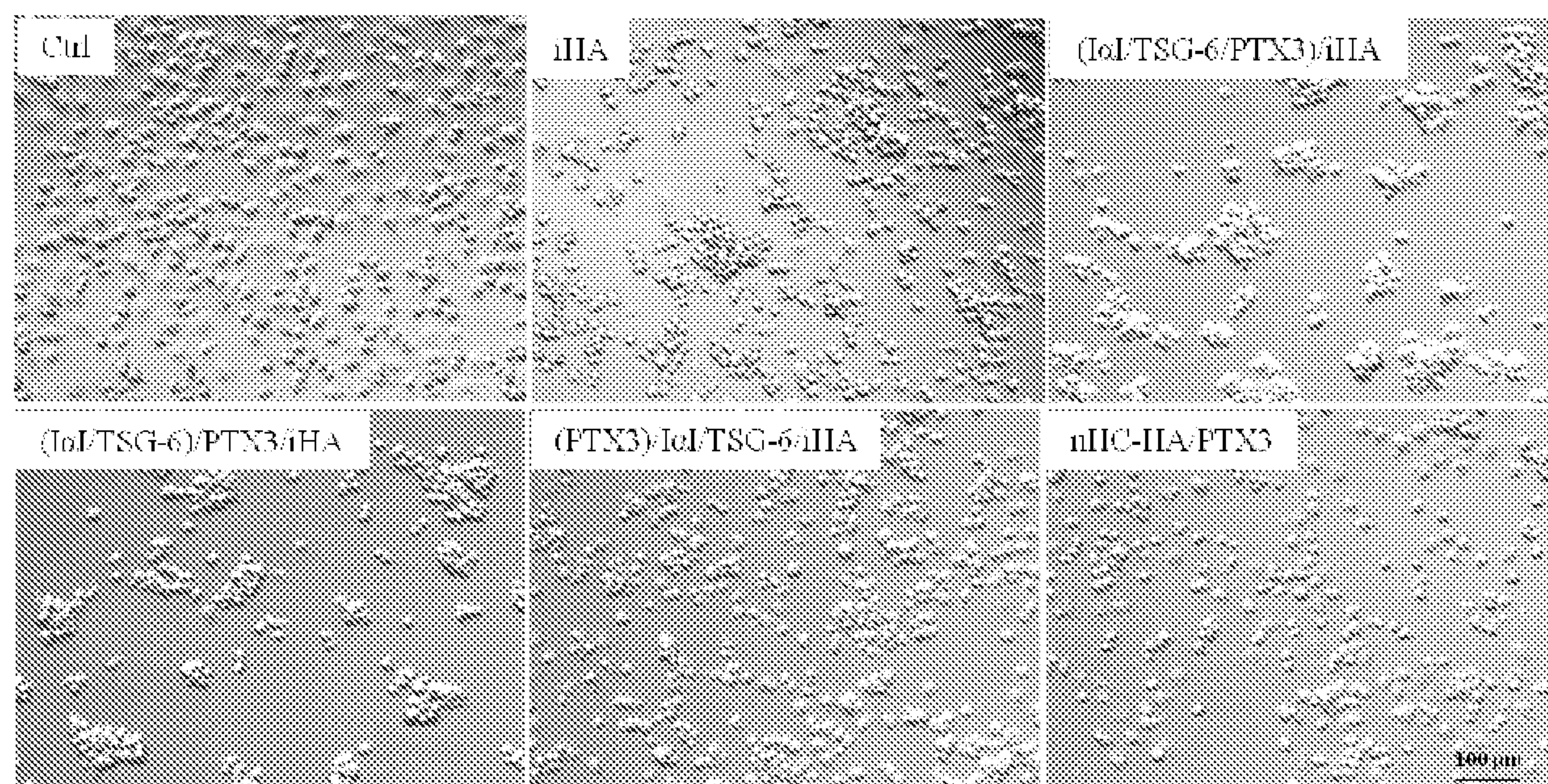
F



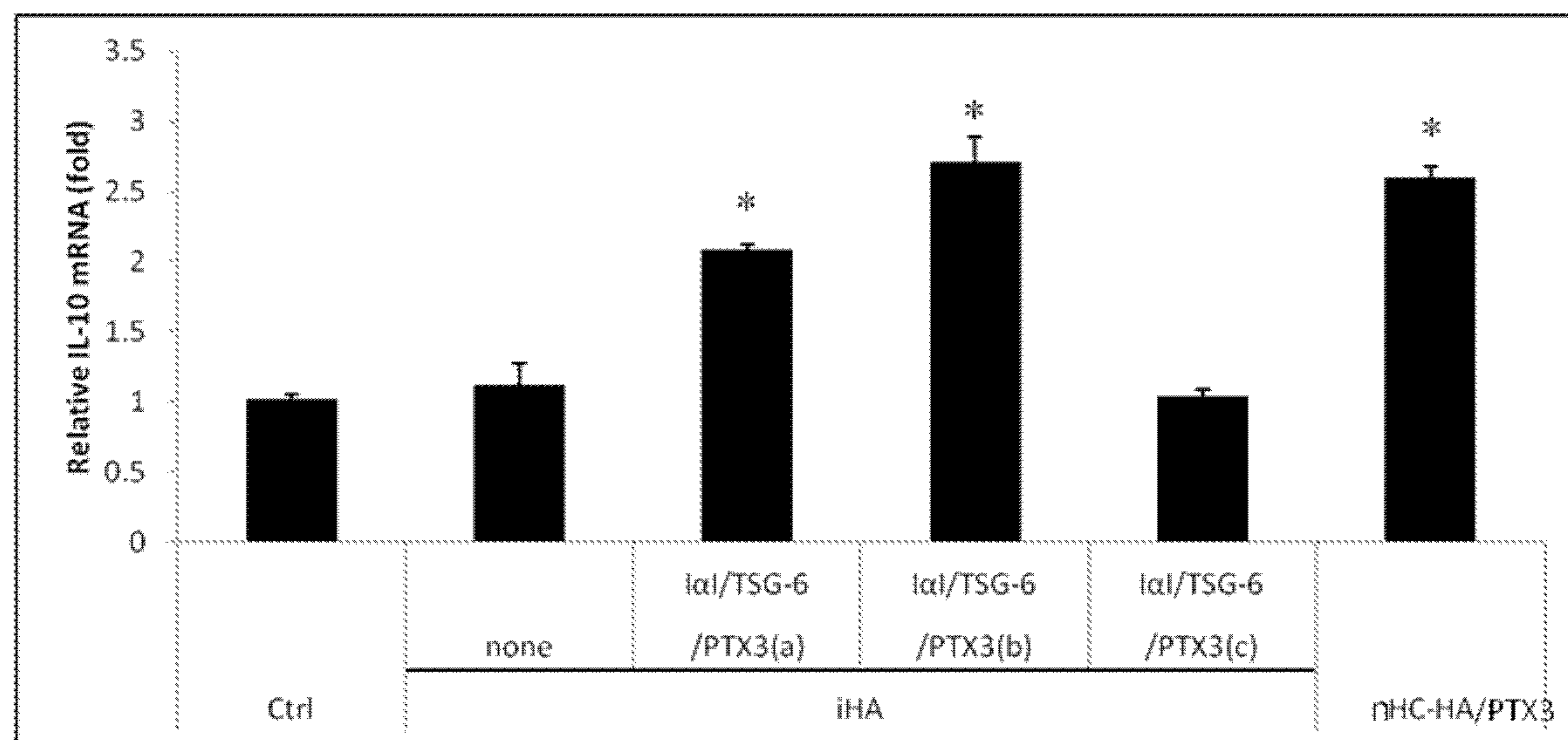
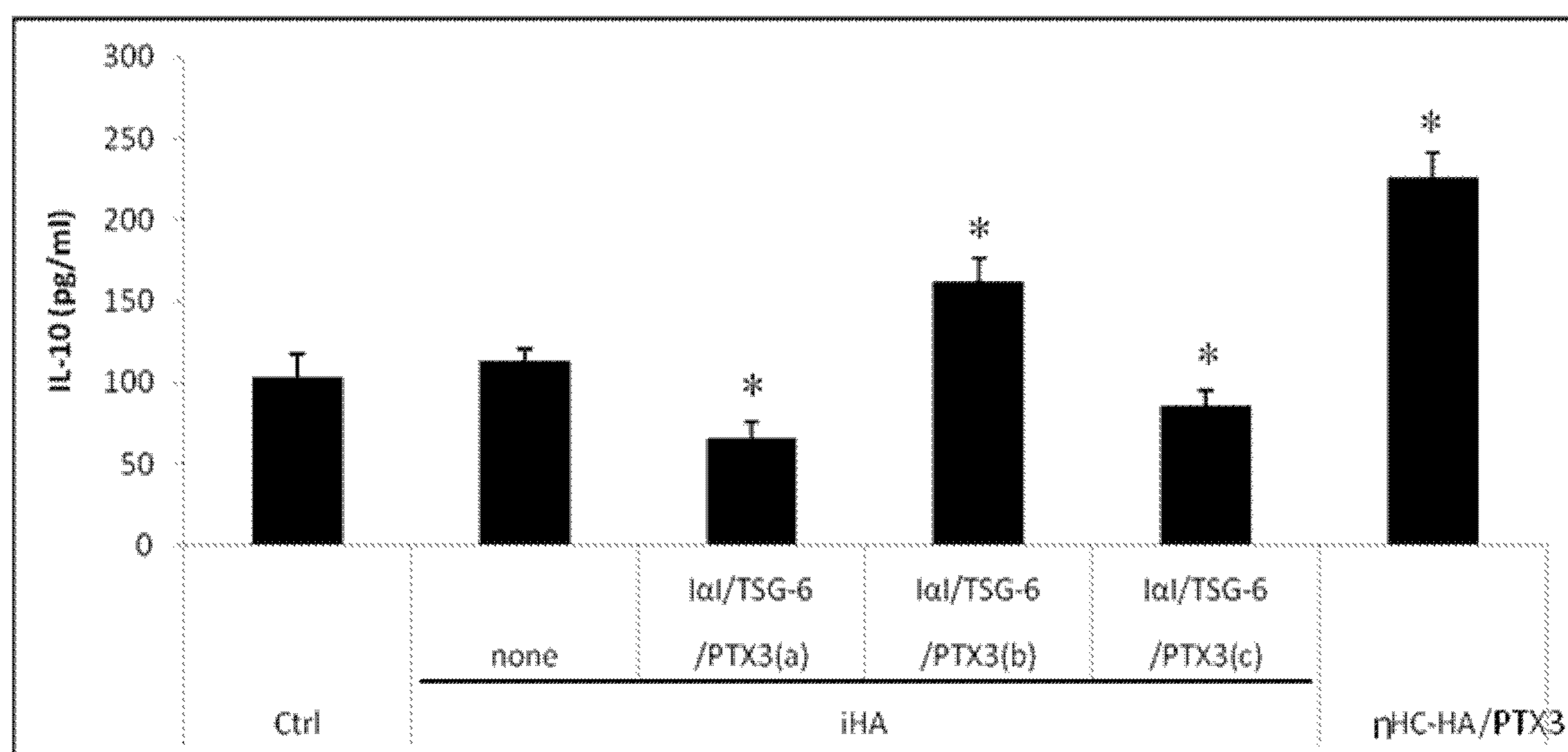
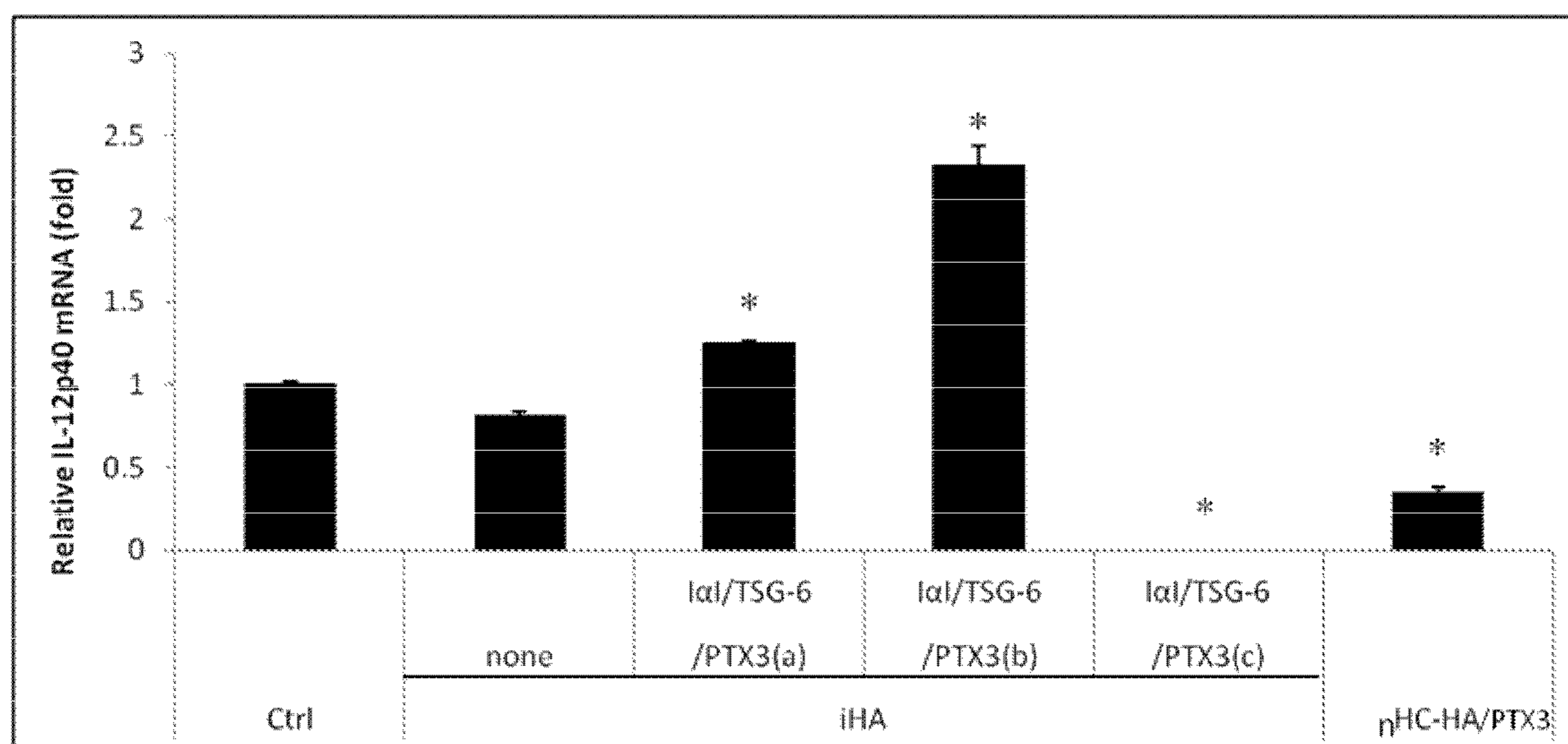
G



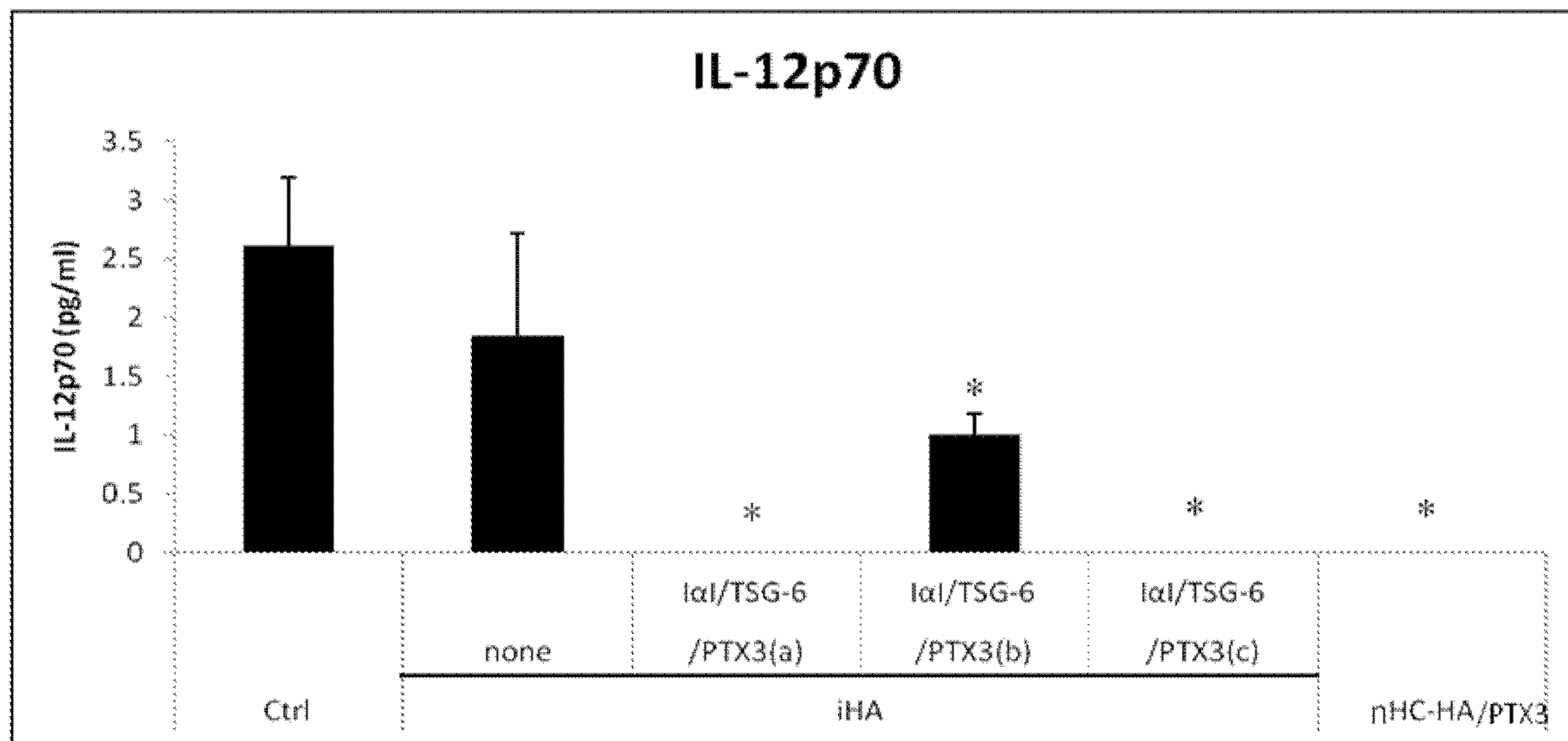
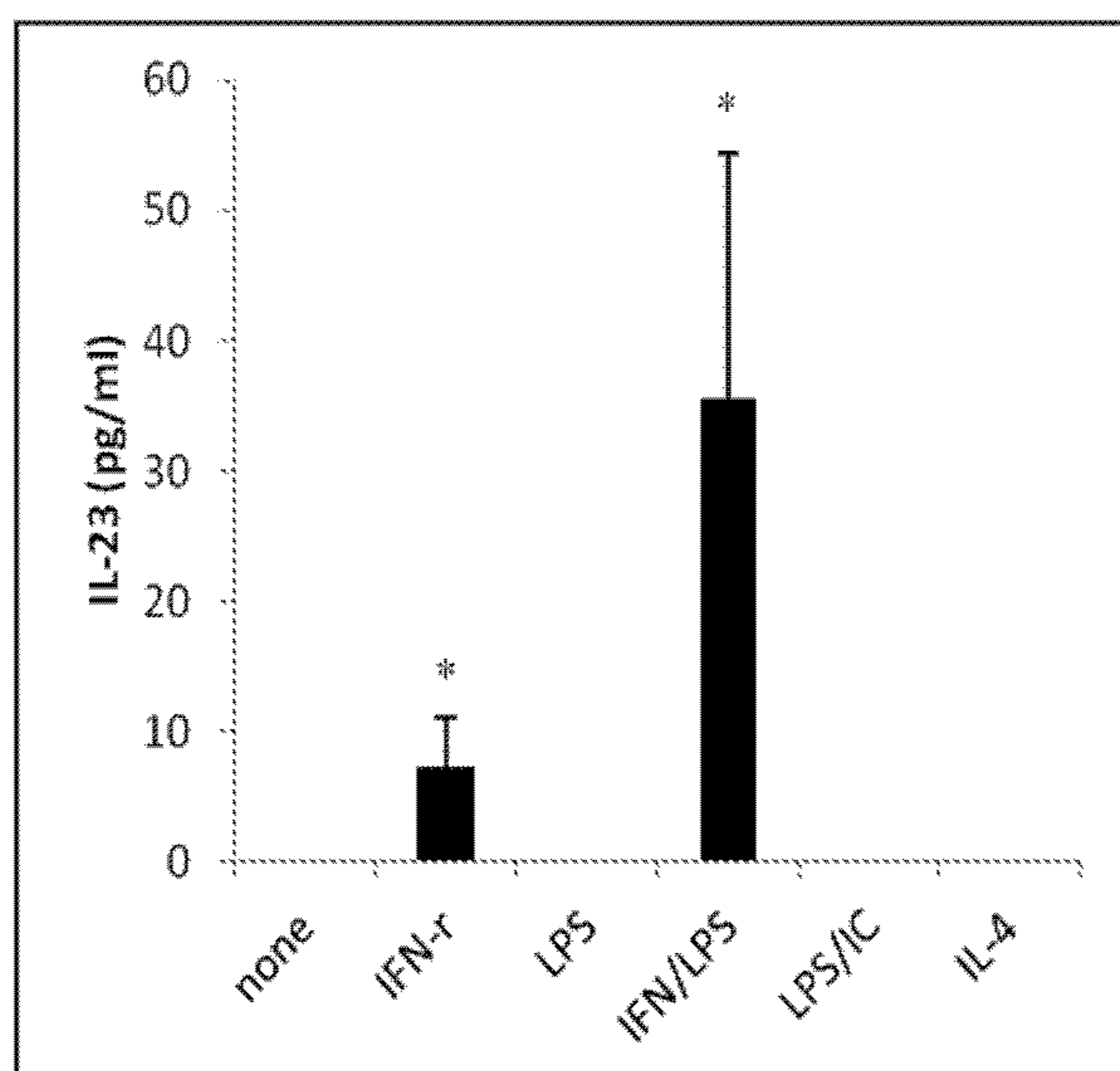
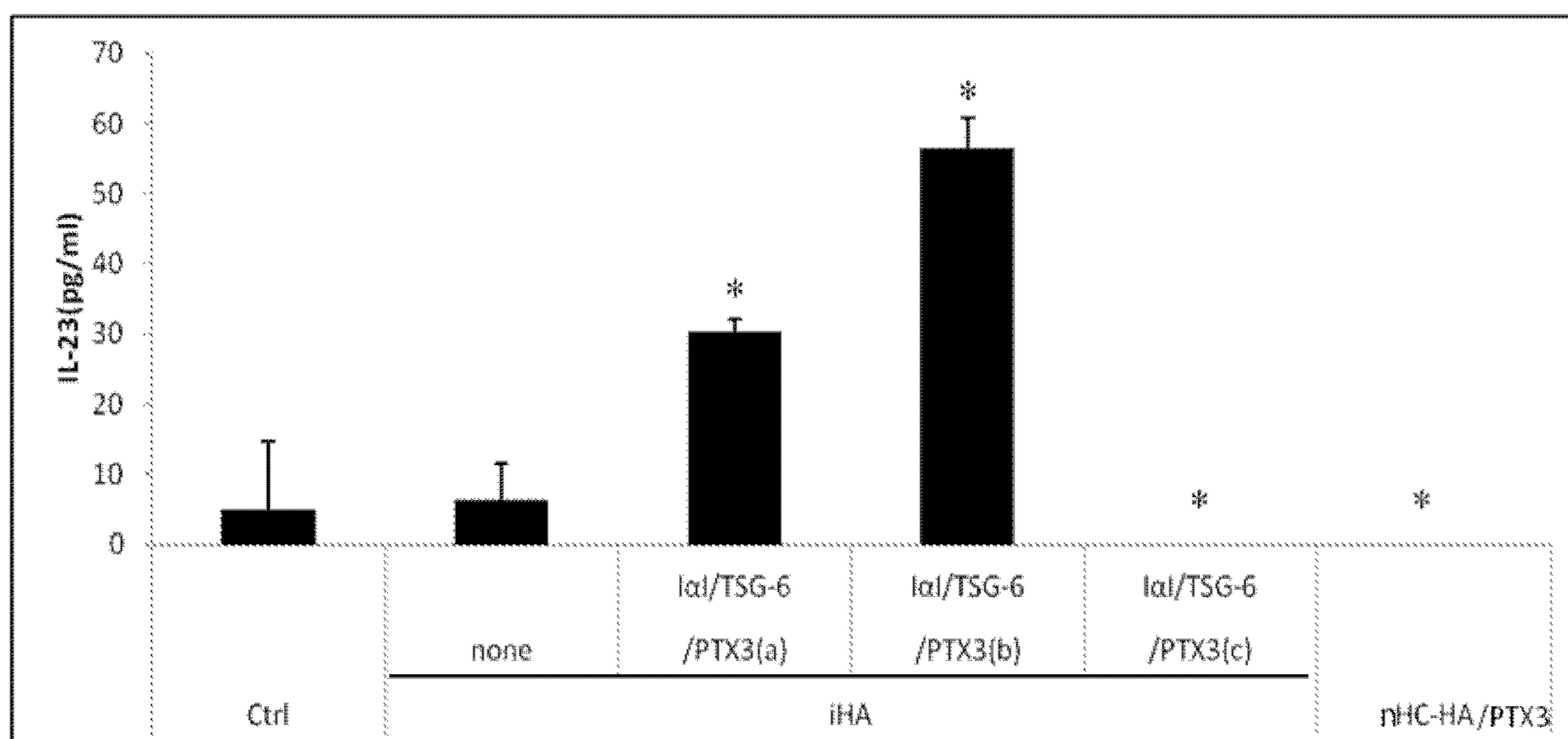
25/112

FIG. 18

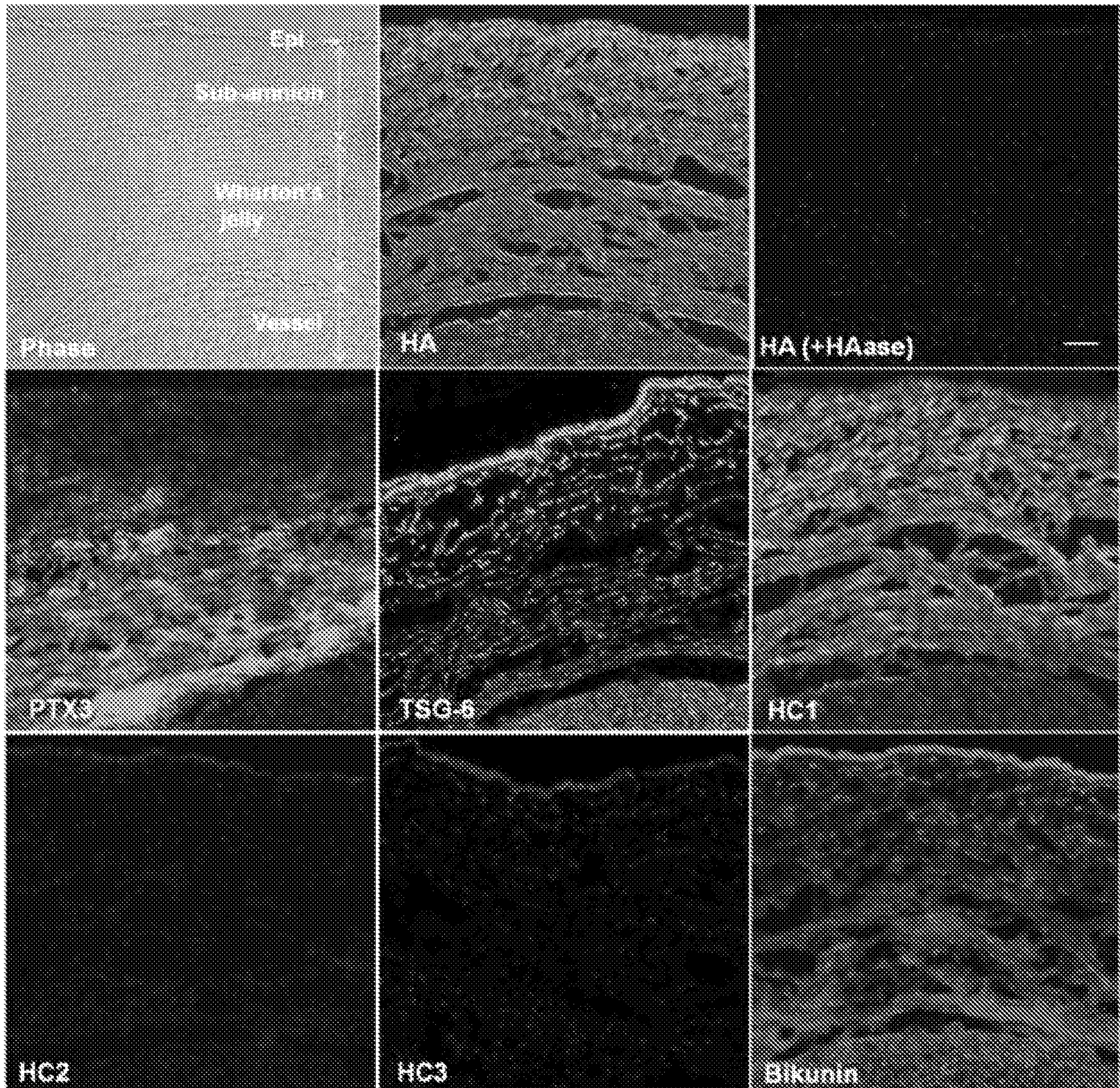
26/112

FIG. 19**A****B****C**

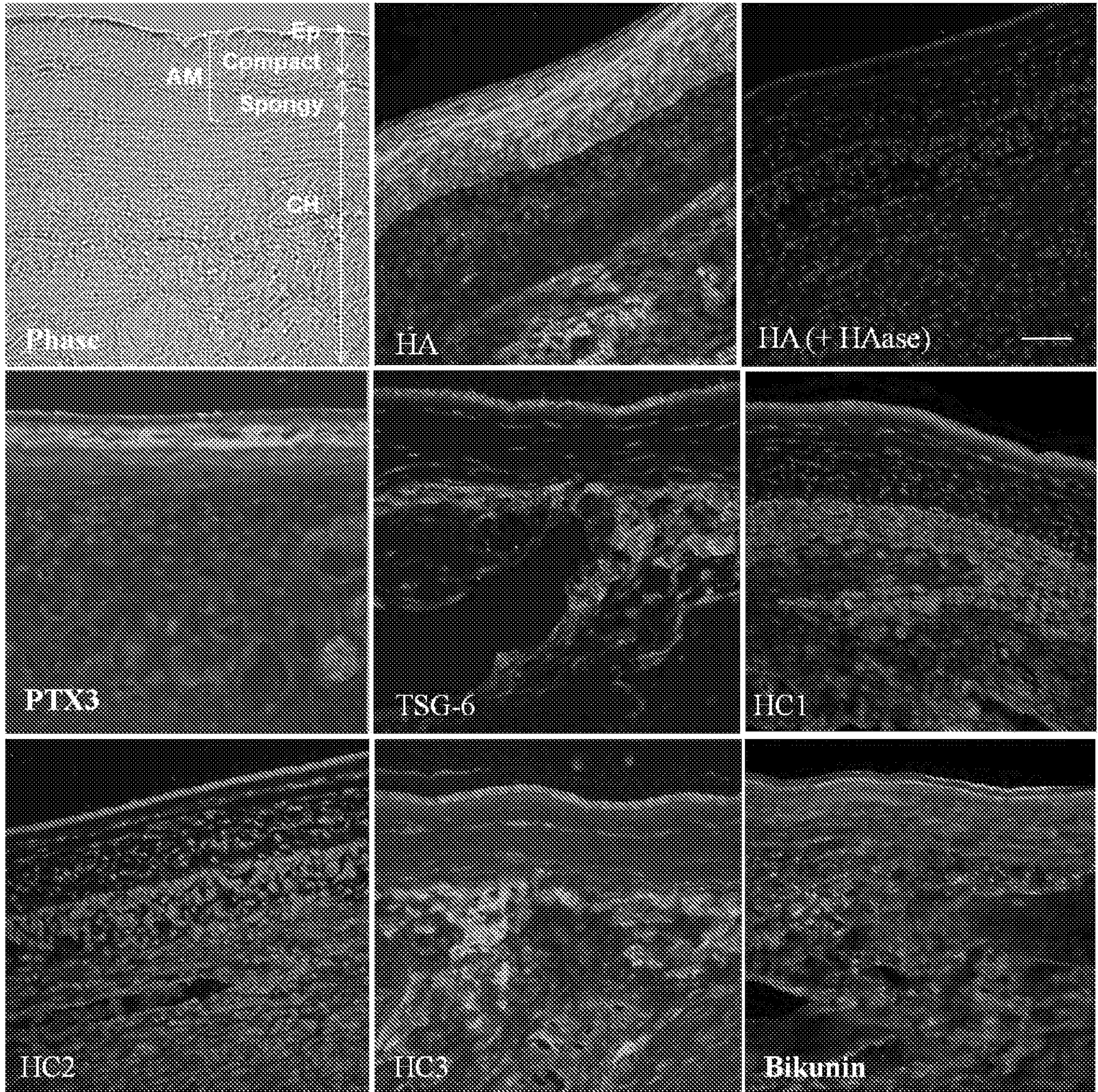
27/112

FIG. 19 (cont'd)**D****E****F**

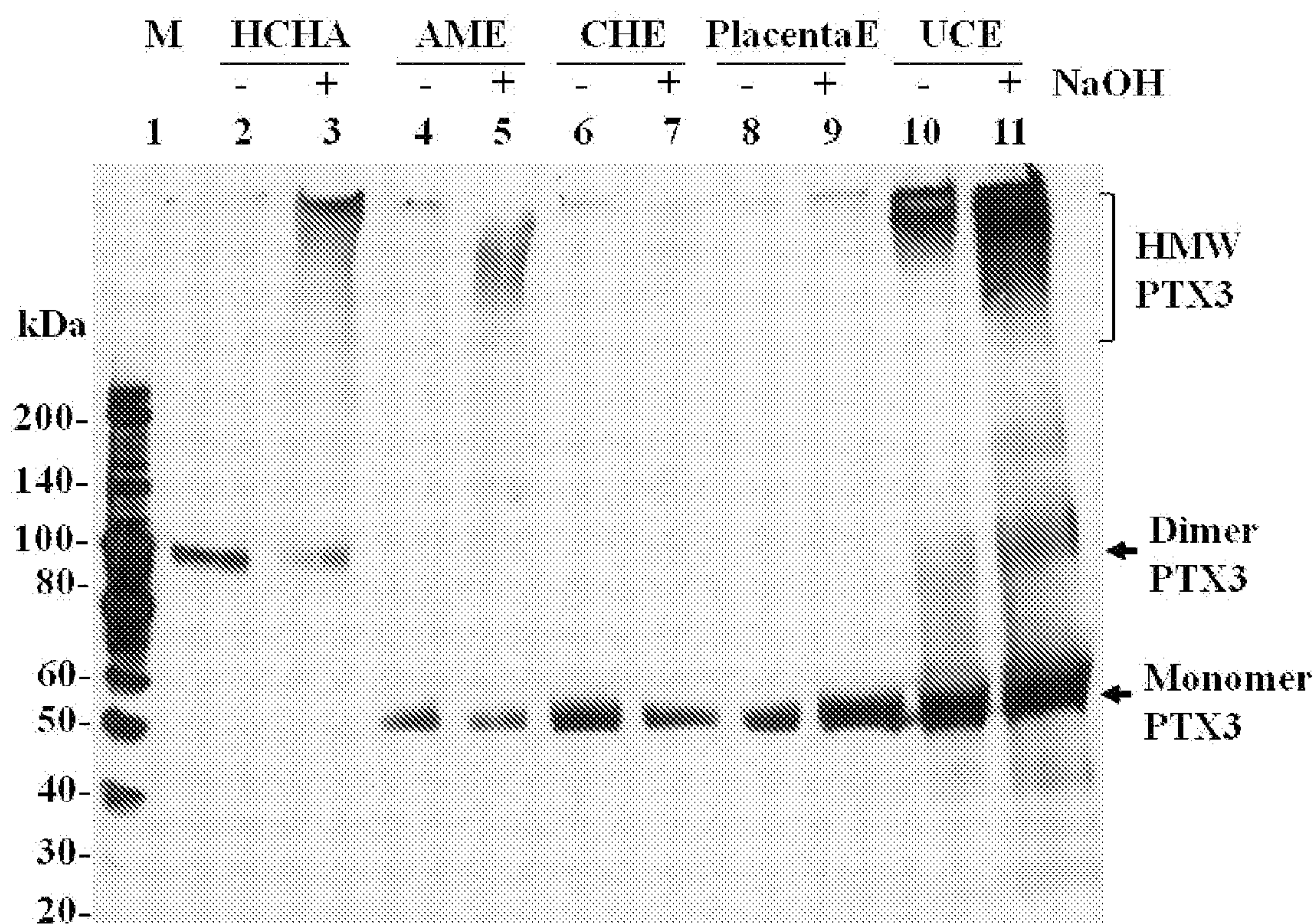
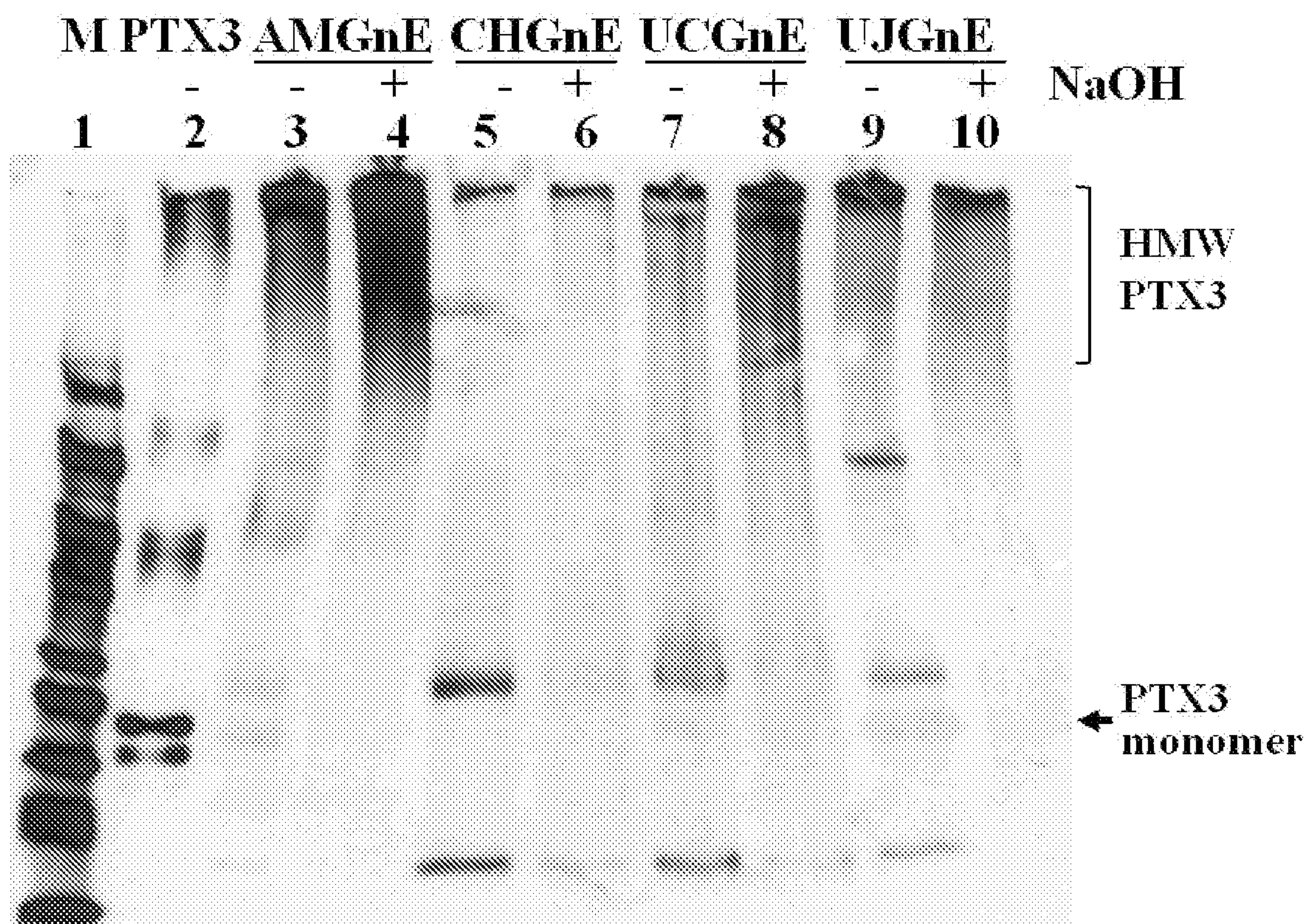
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FIG. 20A**Umbilical Cord**

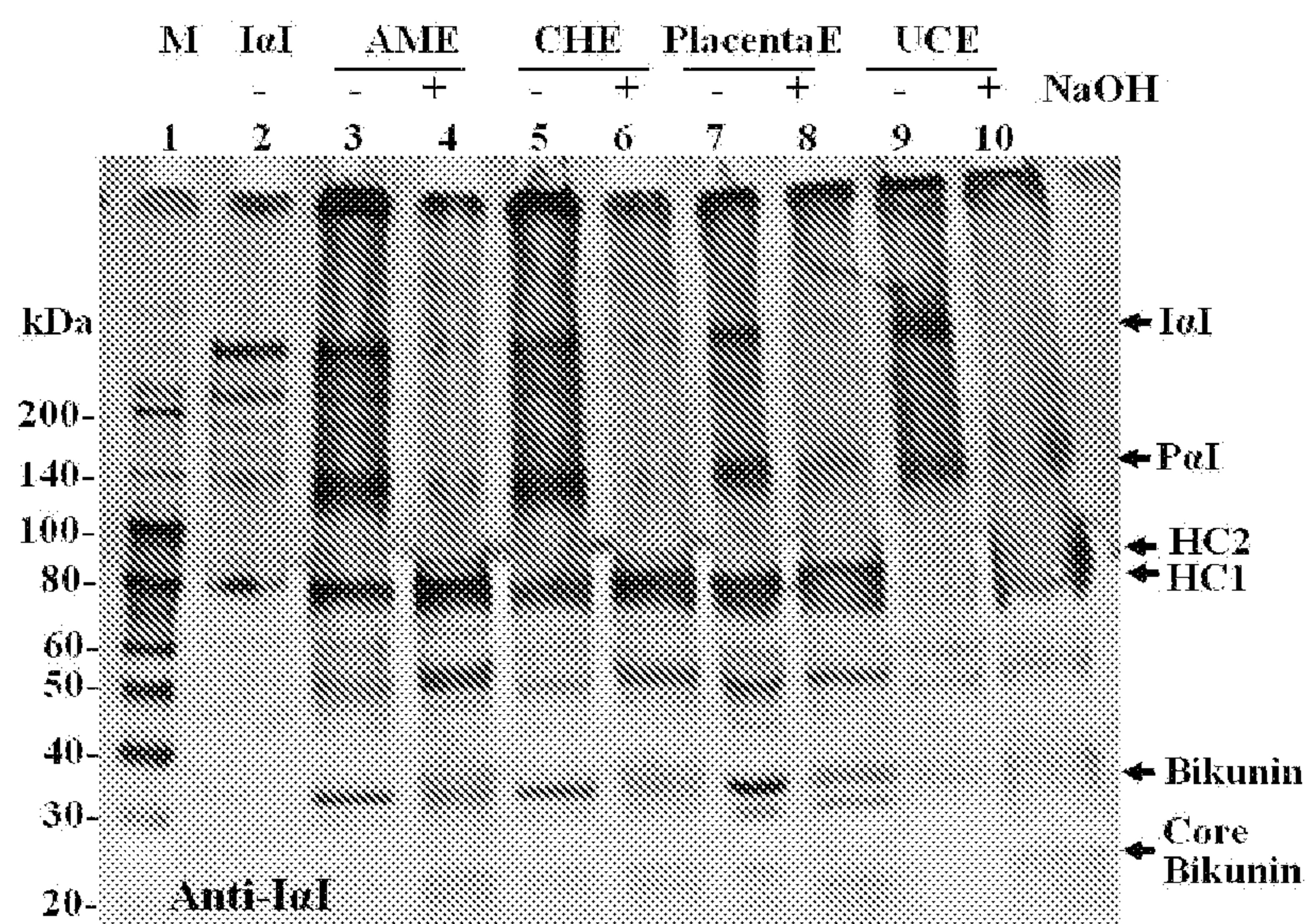
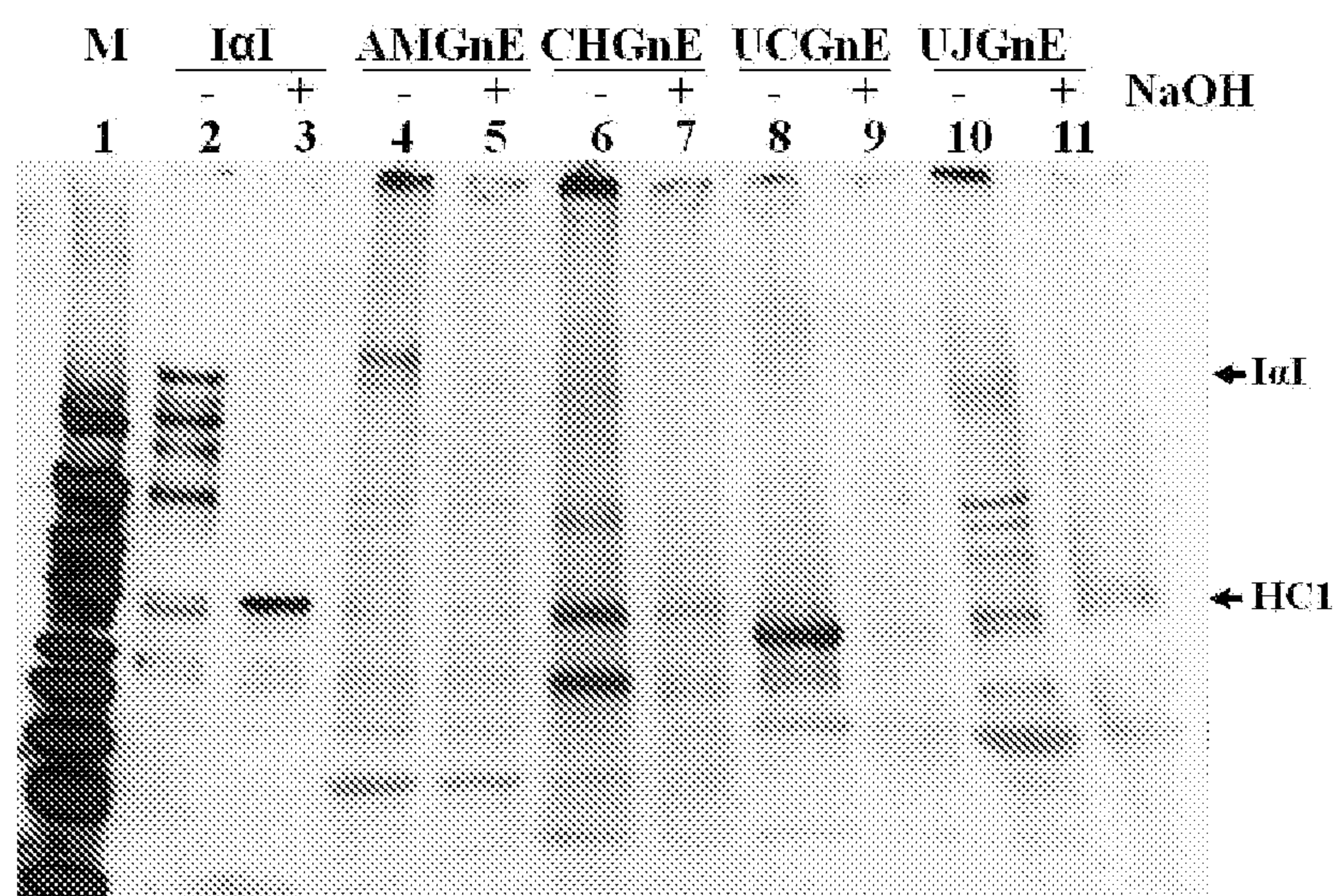
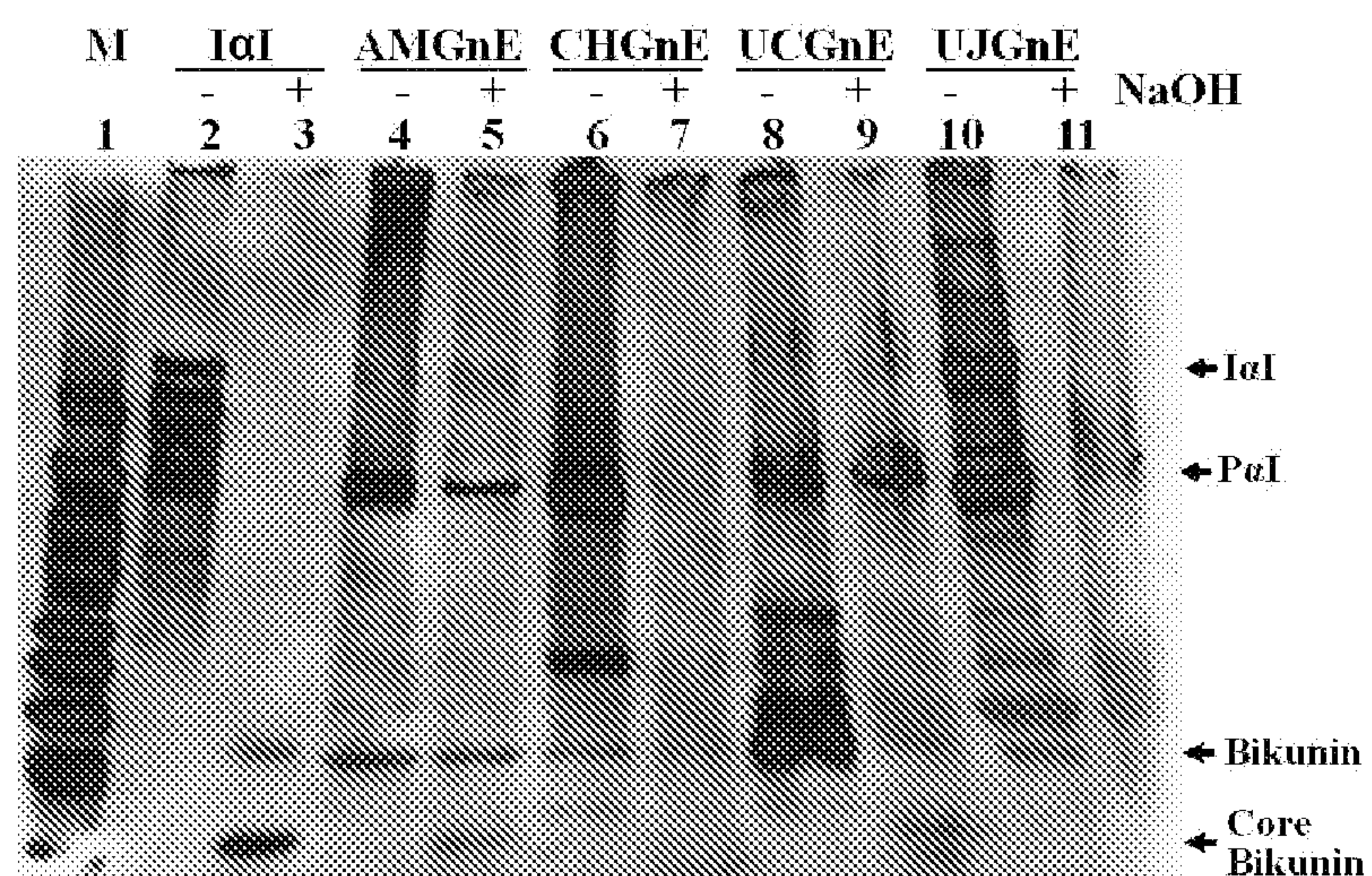
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FIG. 20B**Amniotic Membrane**

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FIG. 21**A****B**

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FIG. 22**A****B****C**

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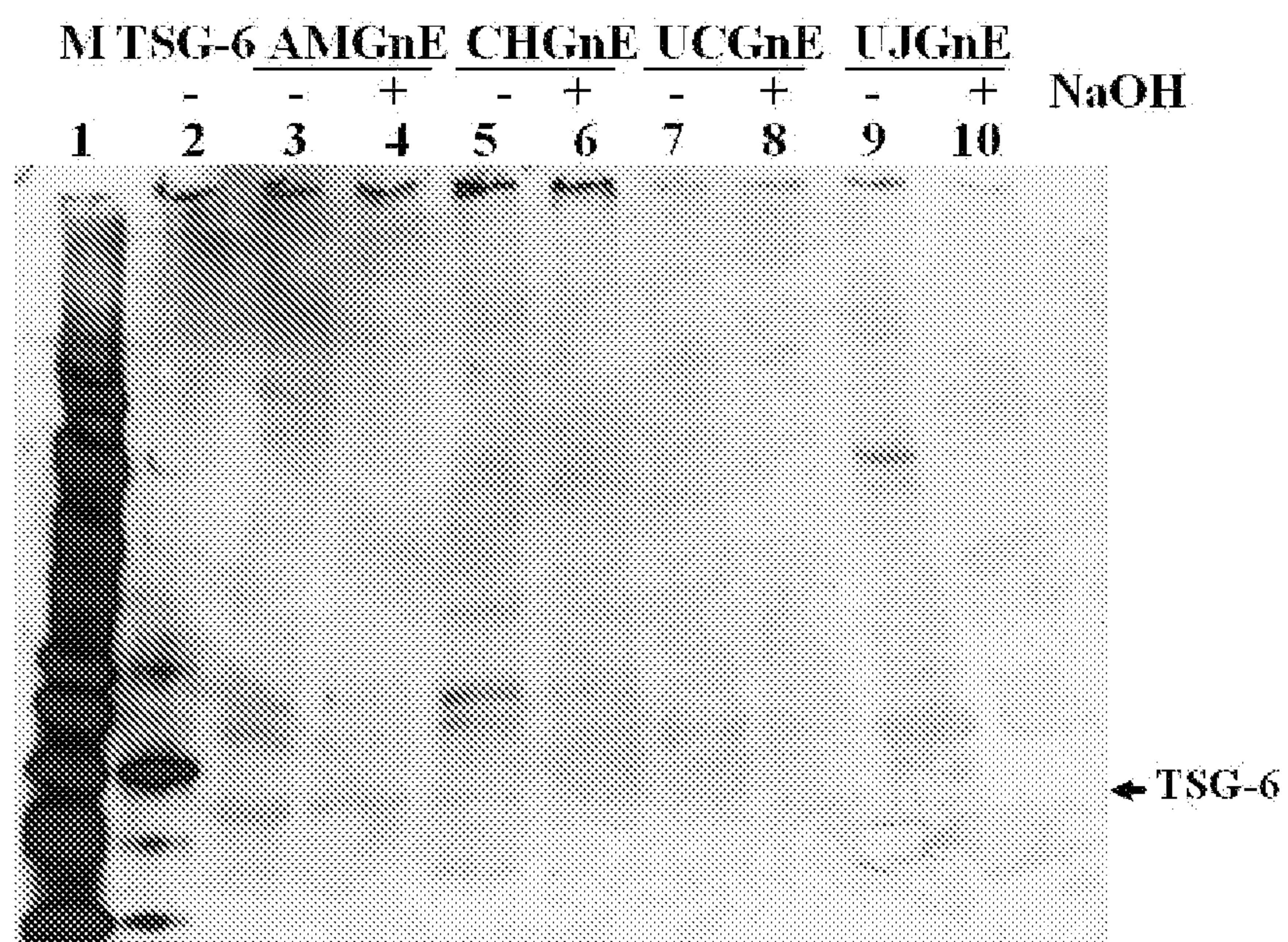
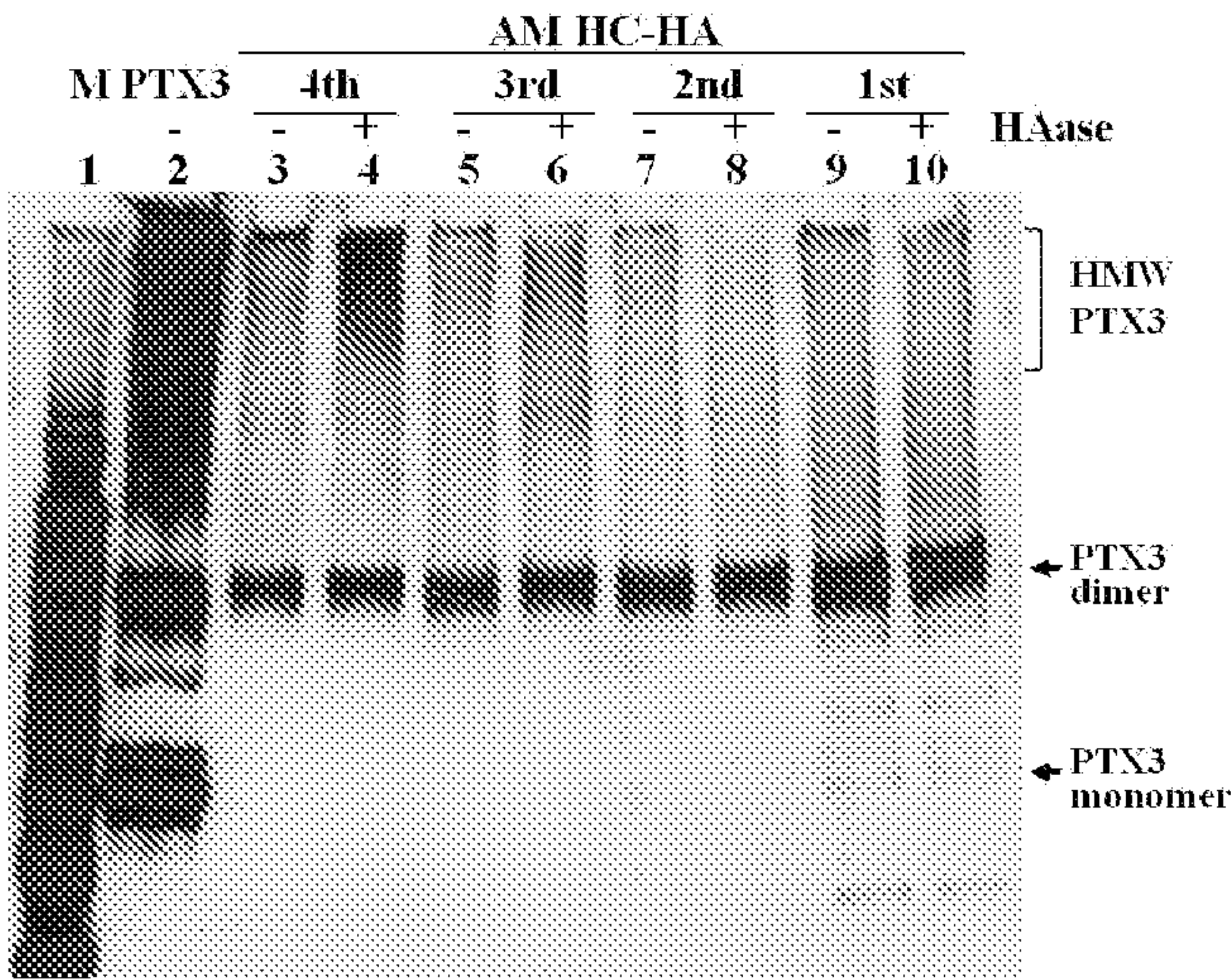
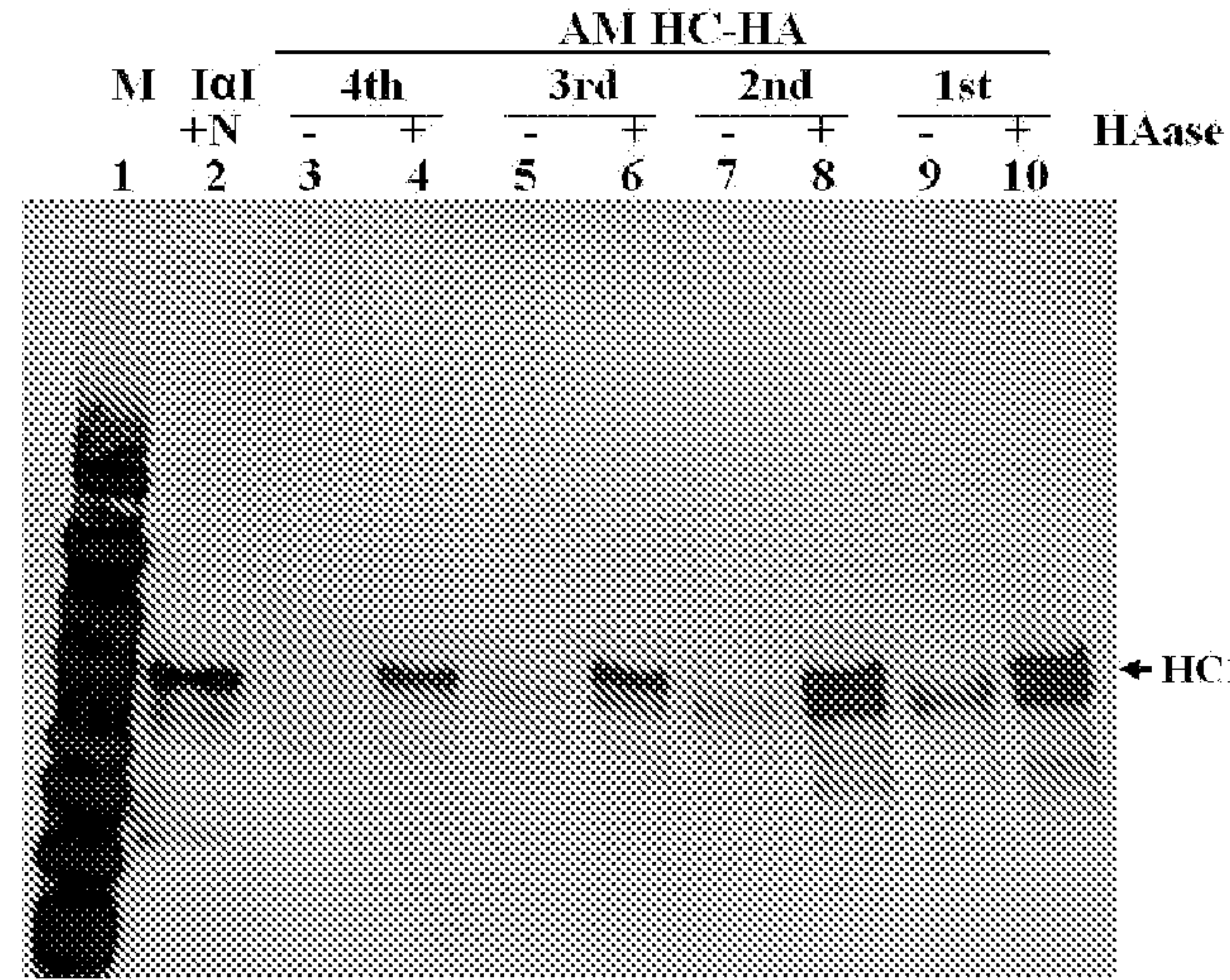
FIG. 23

FIG. 24

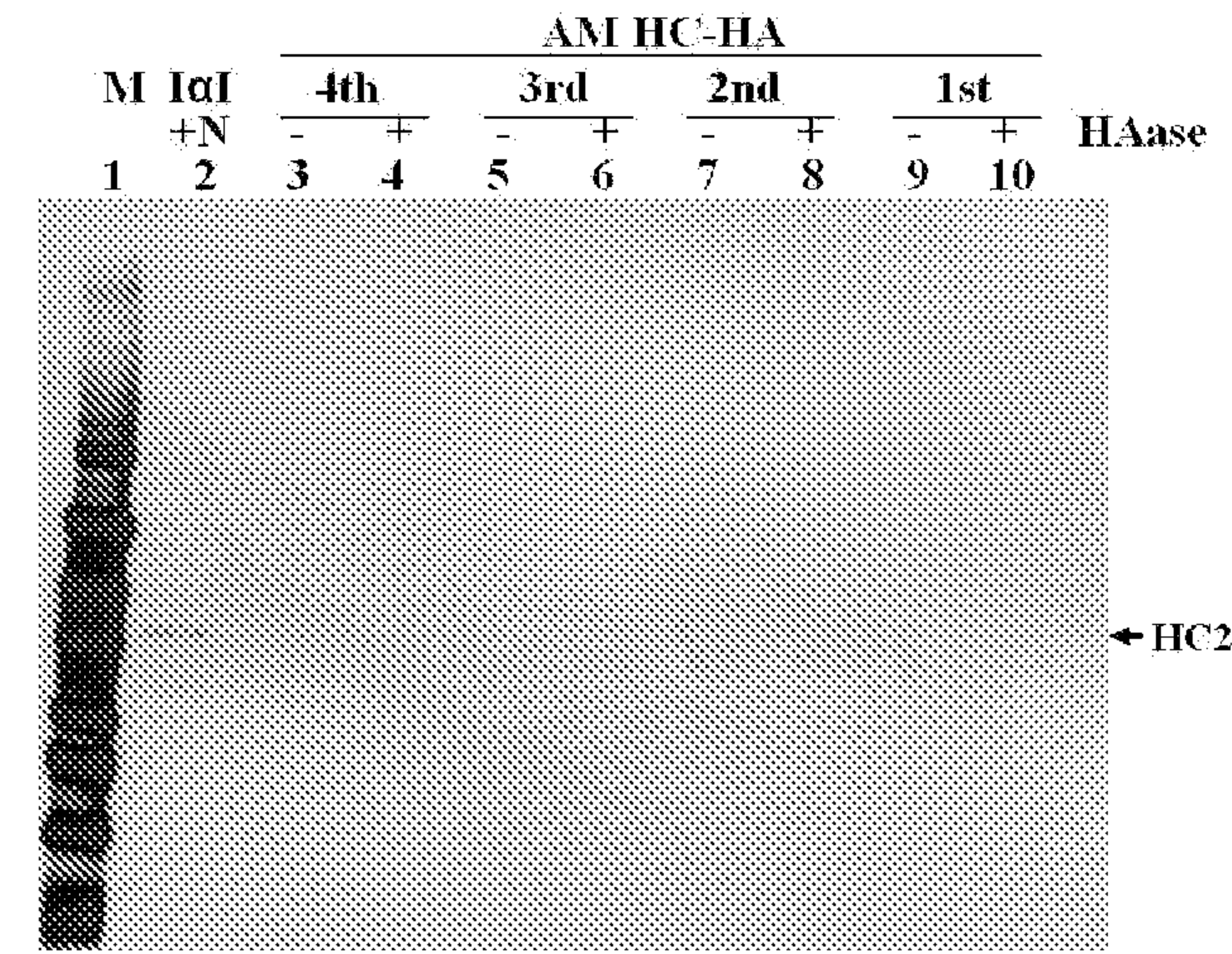
A



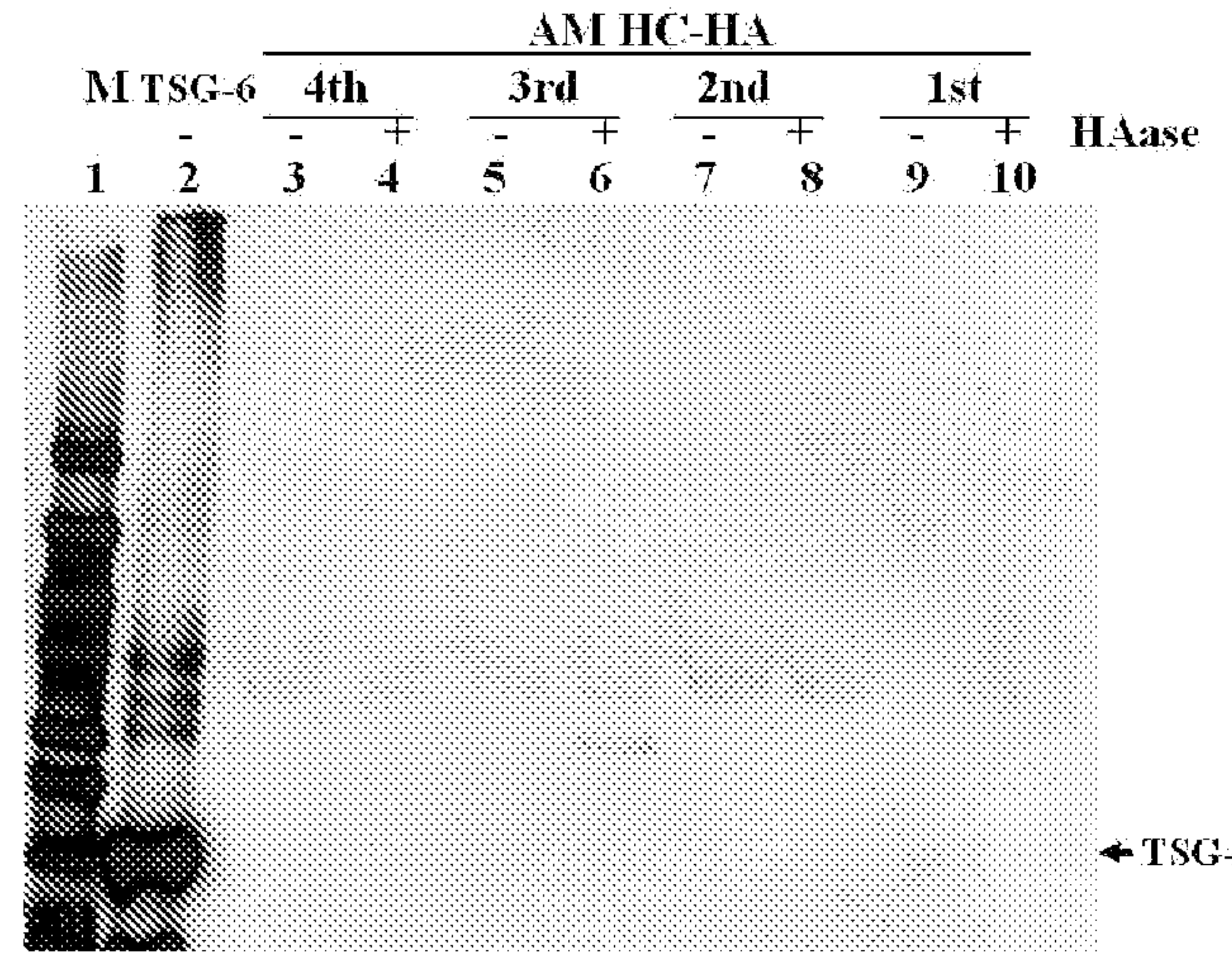
B



C



D



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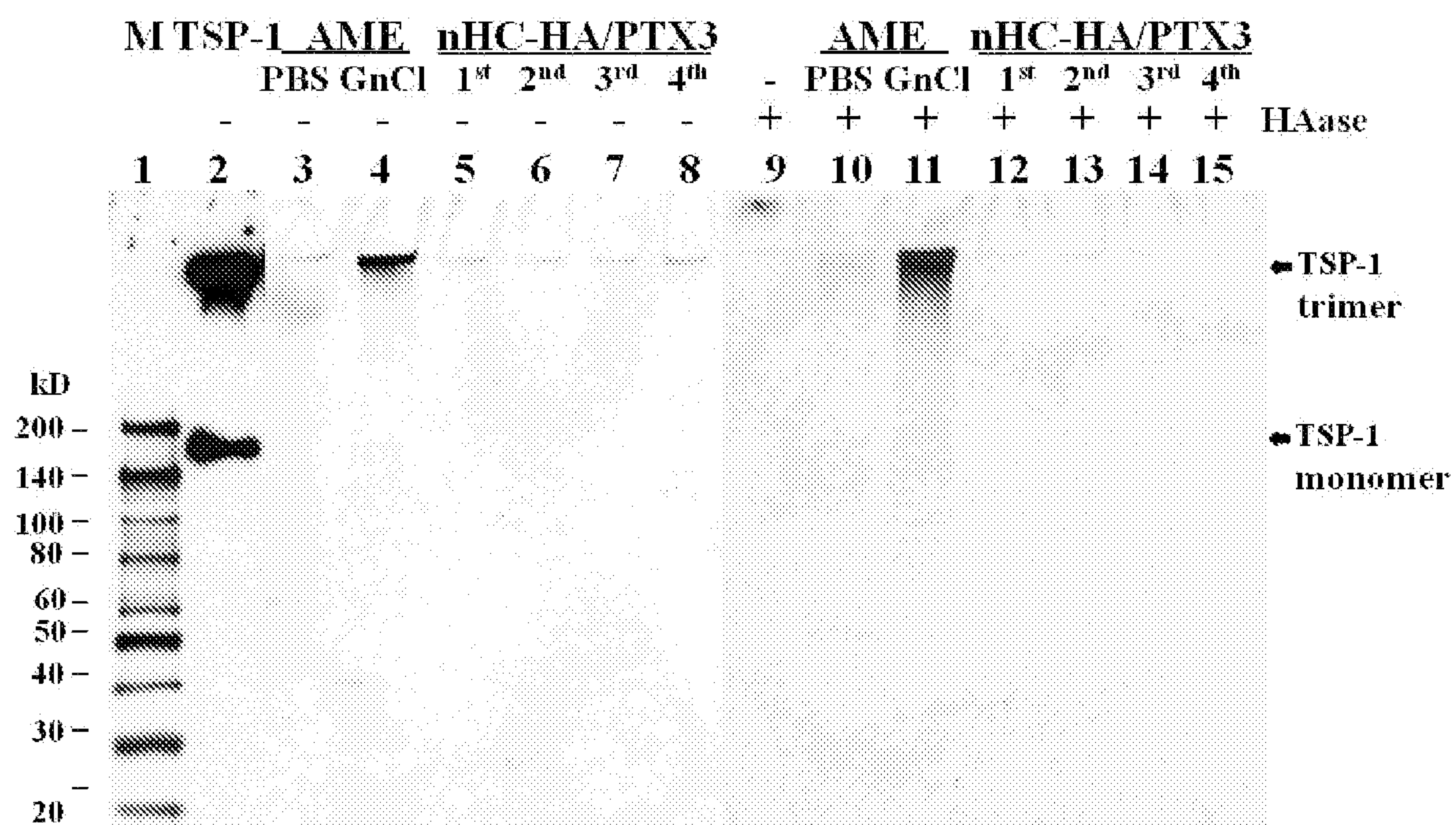
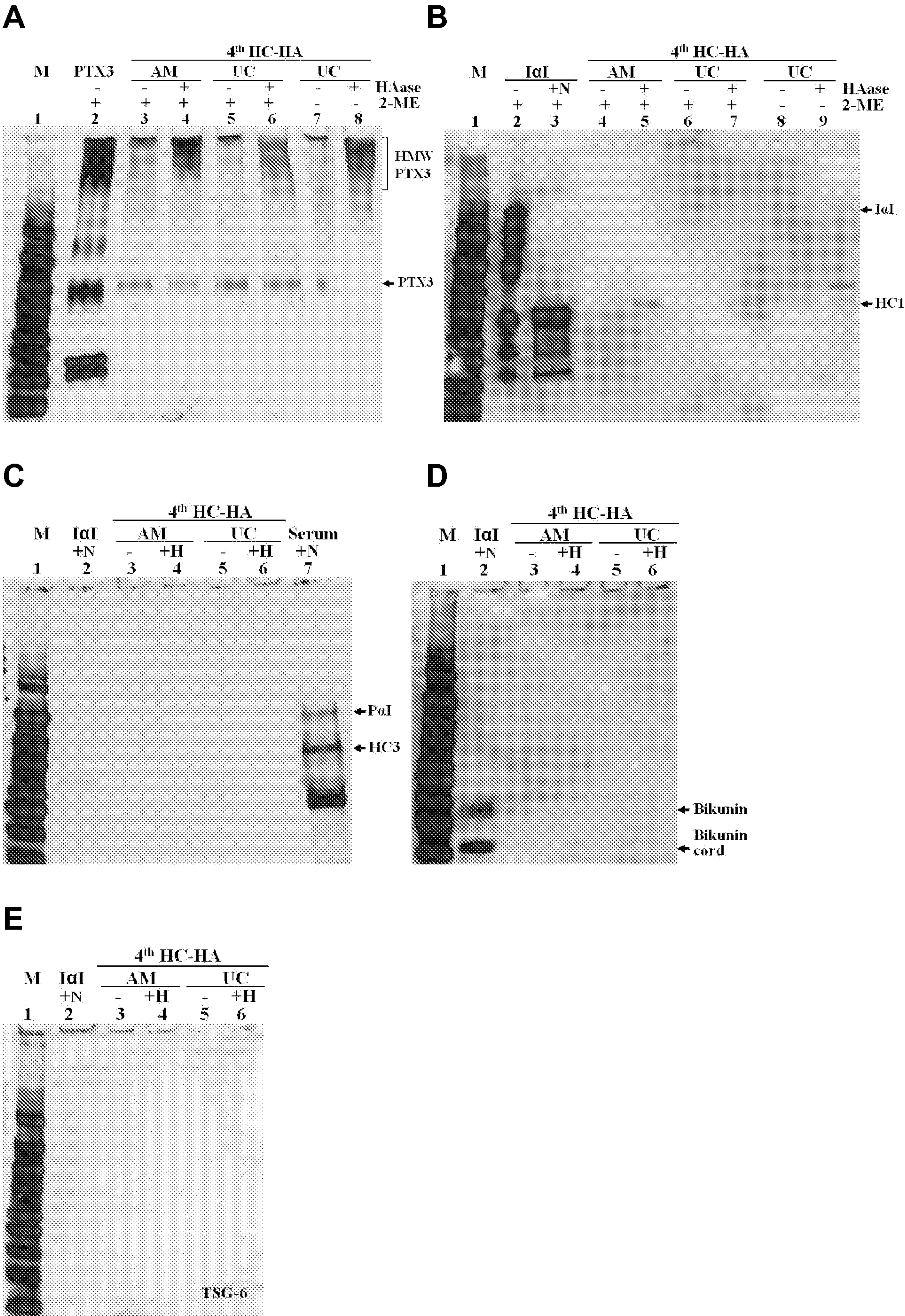
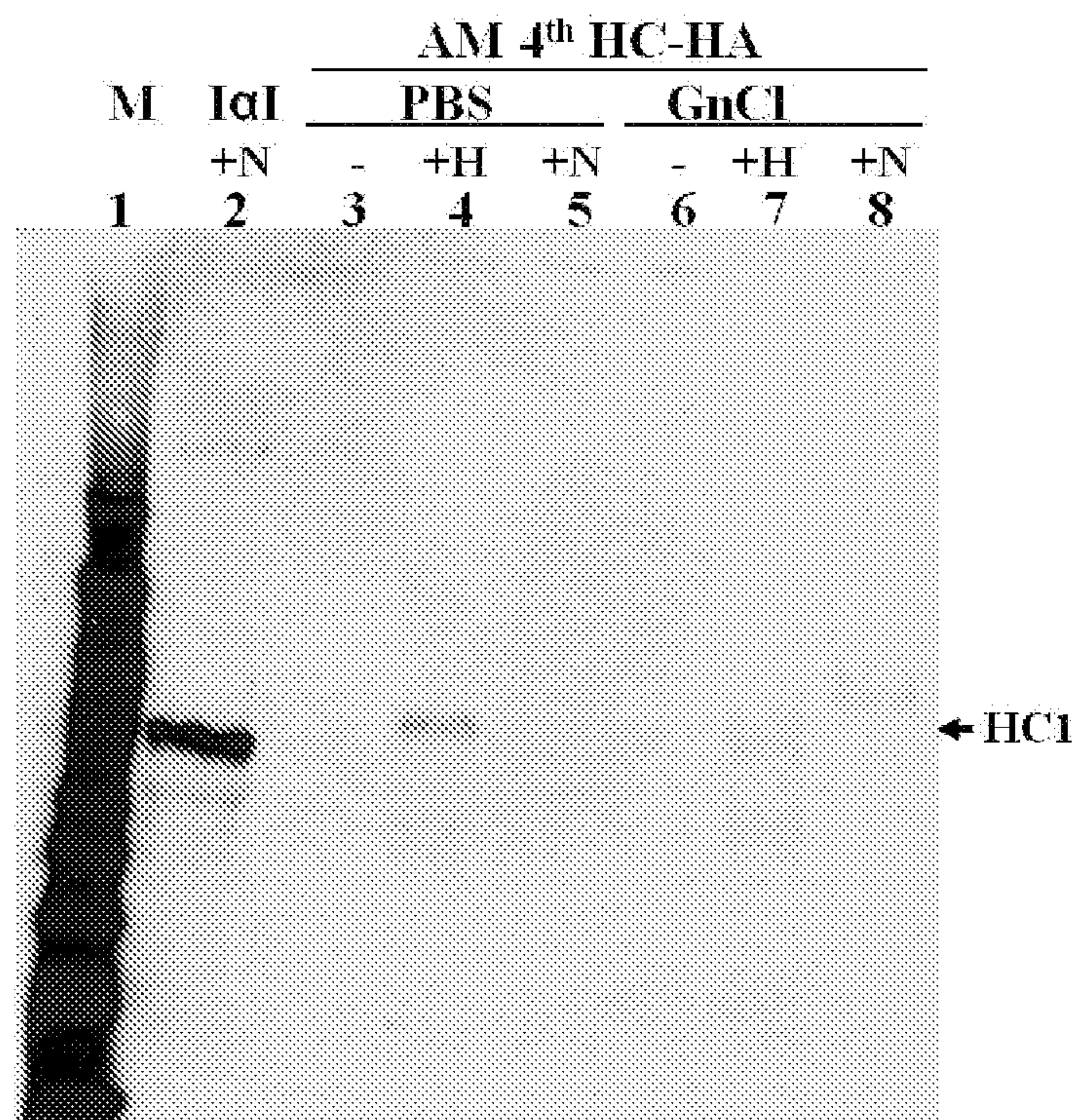
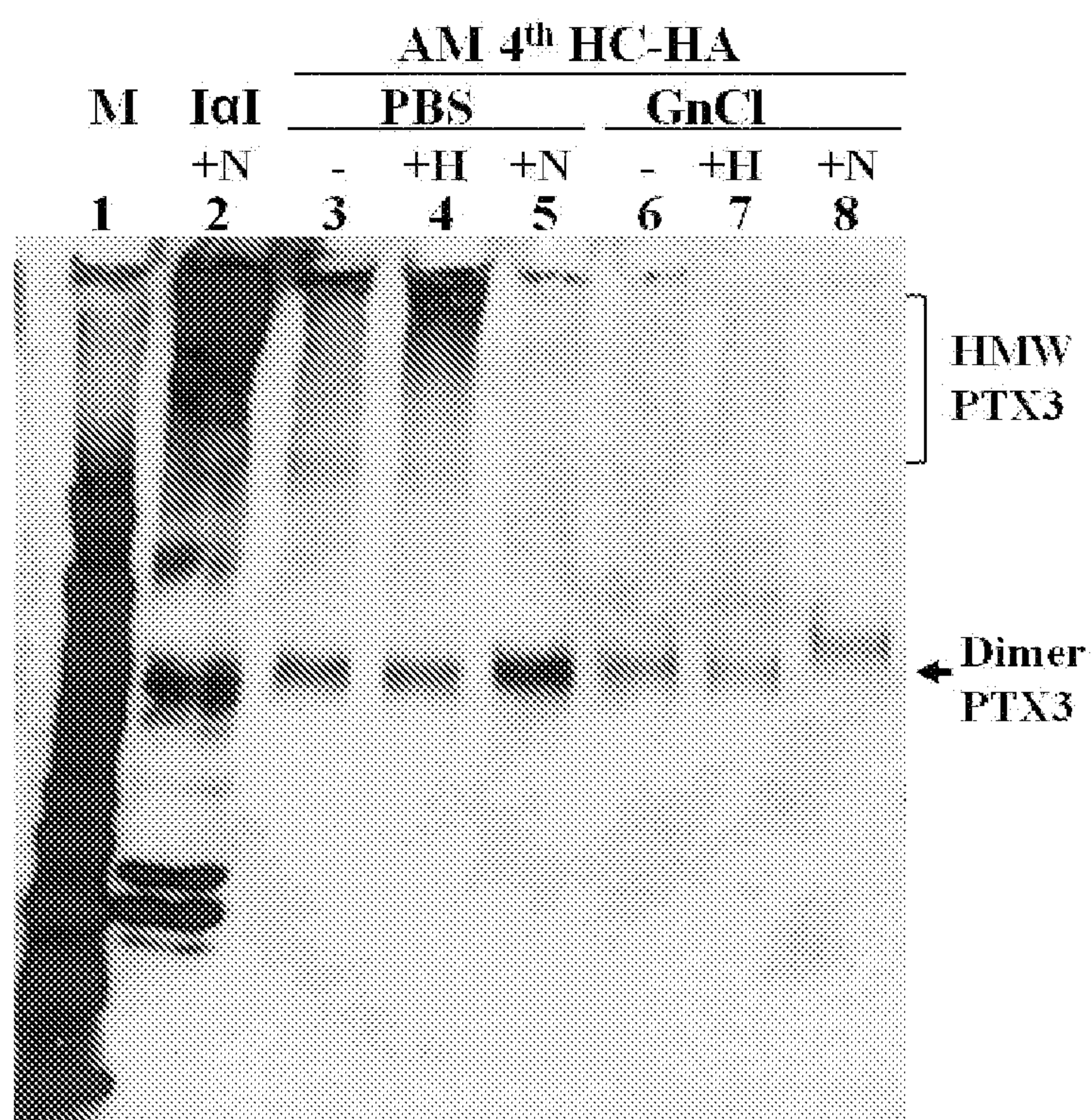
FIG. 25

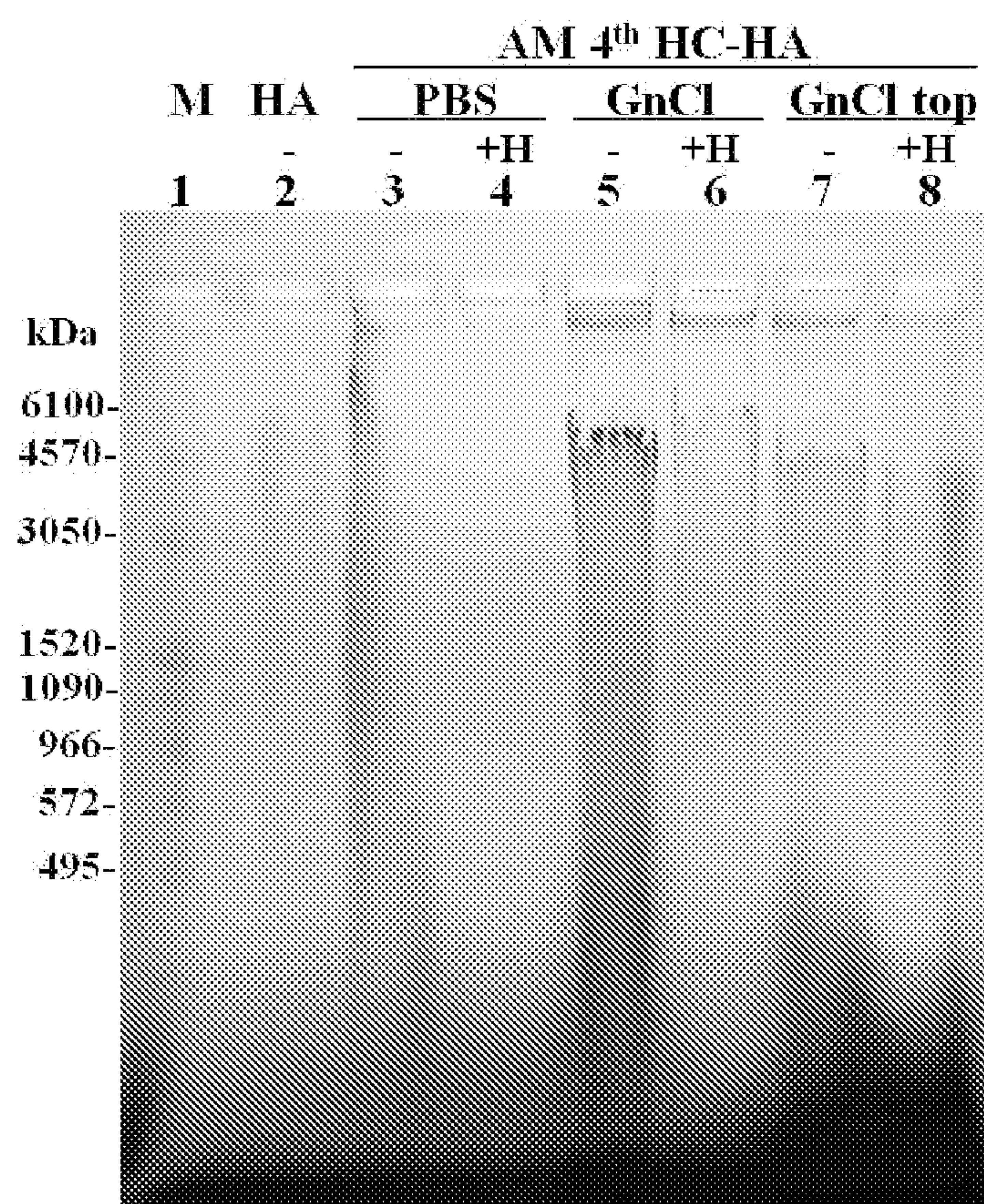
FIG. 26



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FIG. 27**A****B**

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FIG. 28

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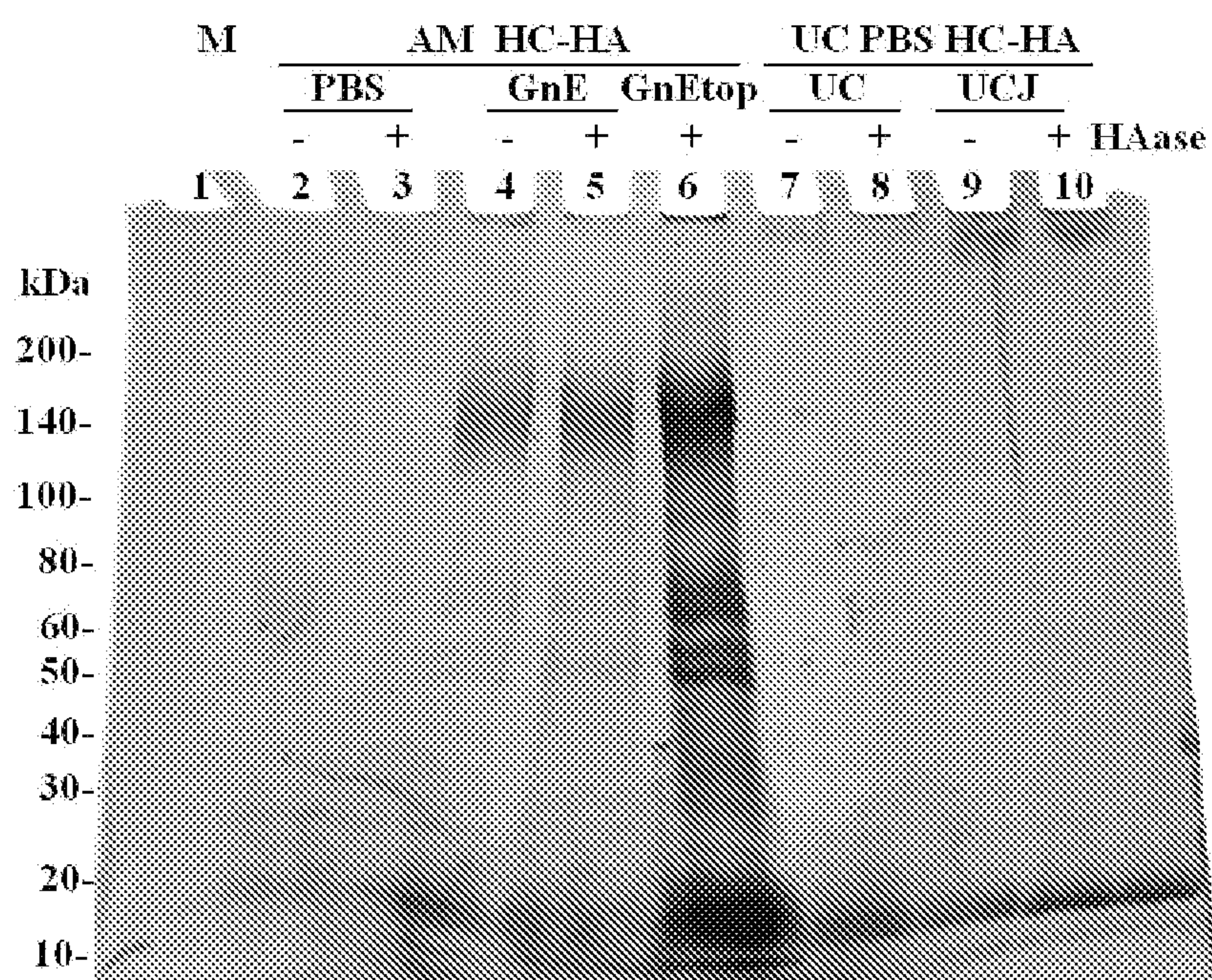
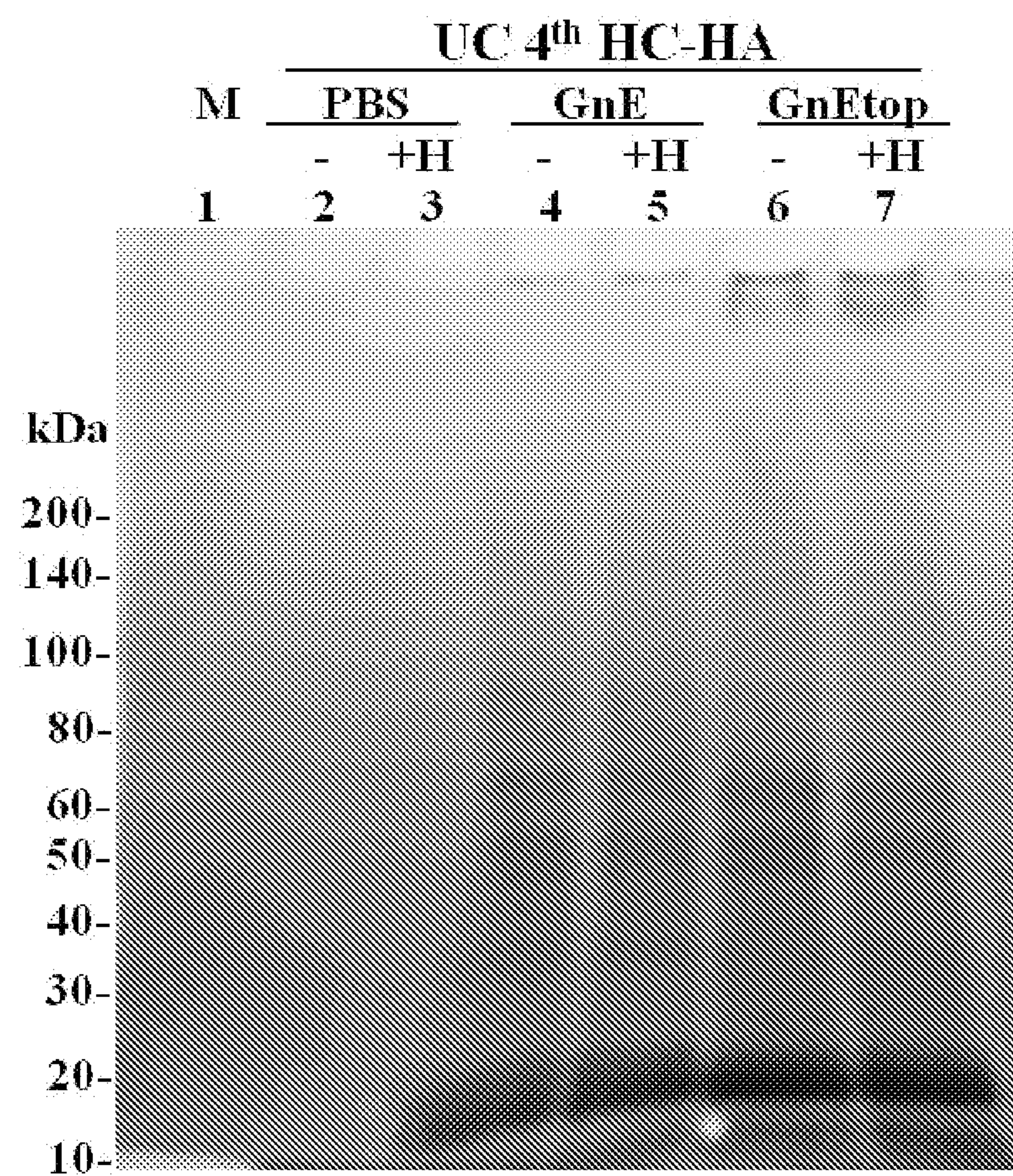
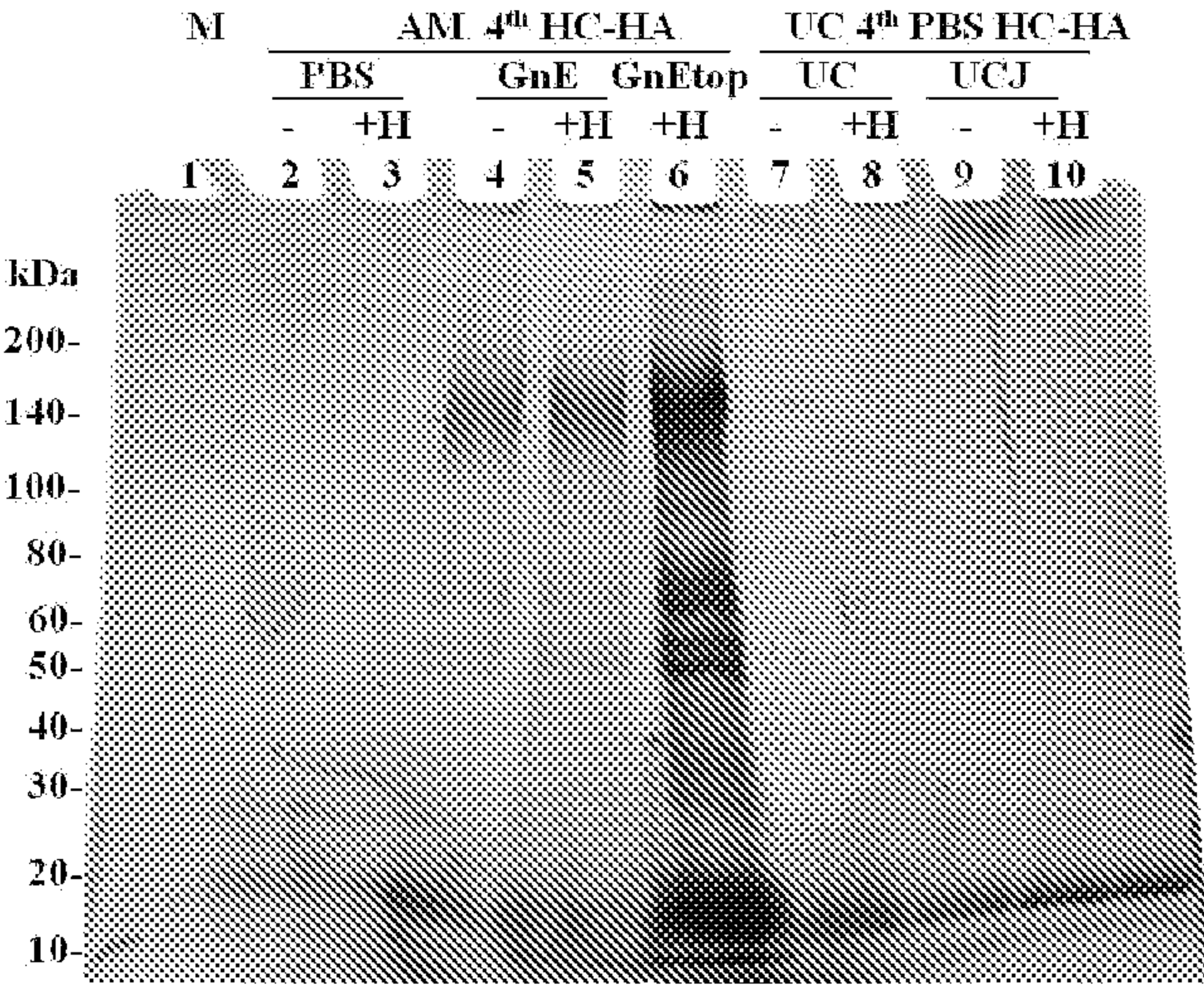
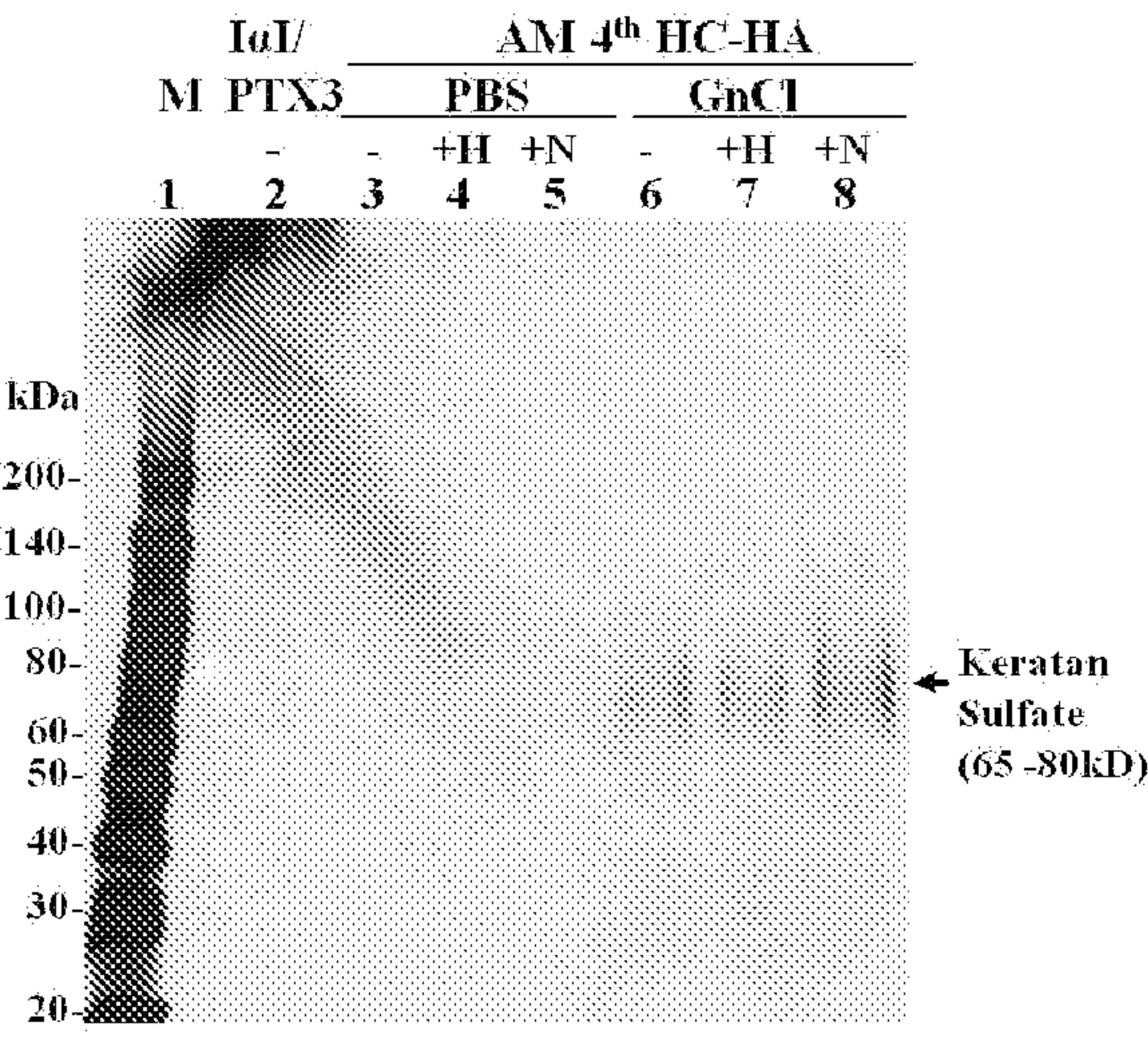
FIG. 29**A****B**

FIG. 30

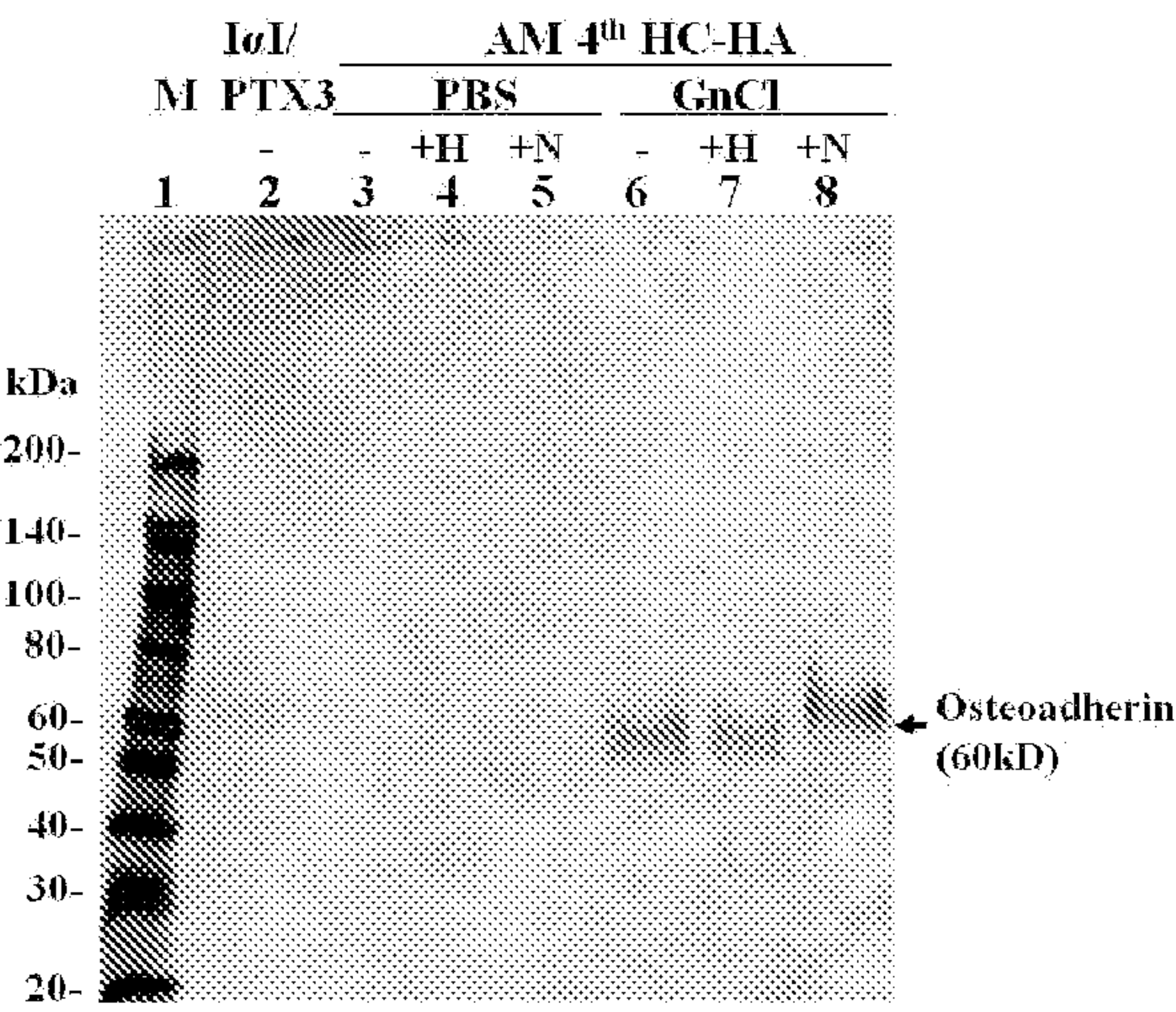
A



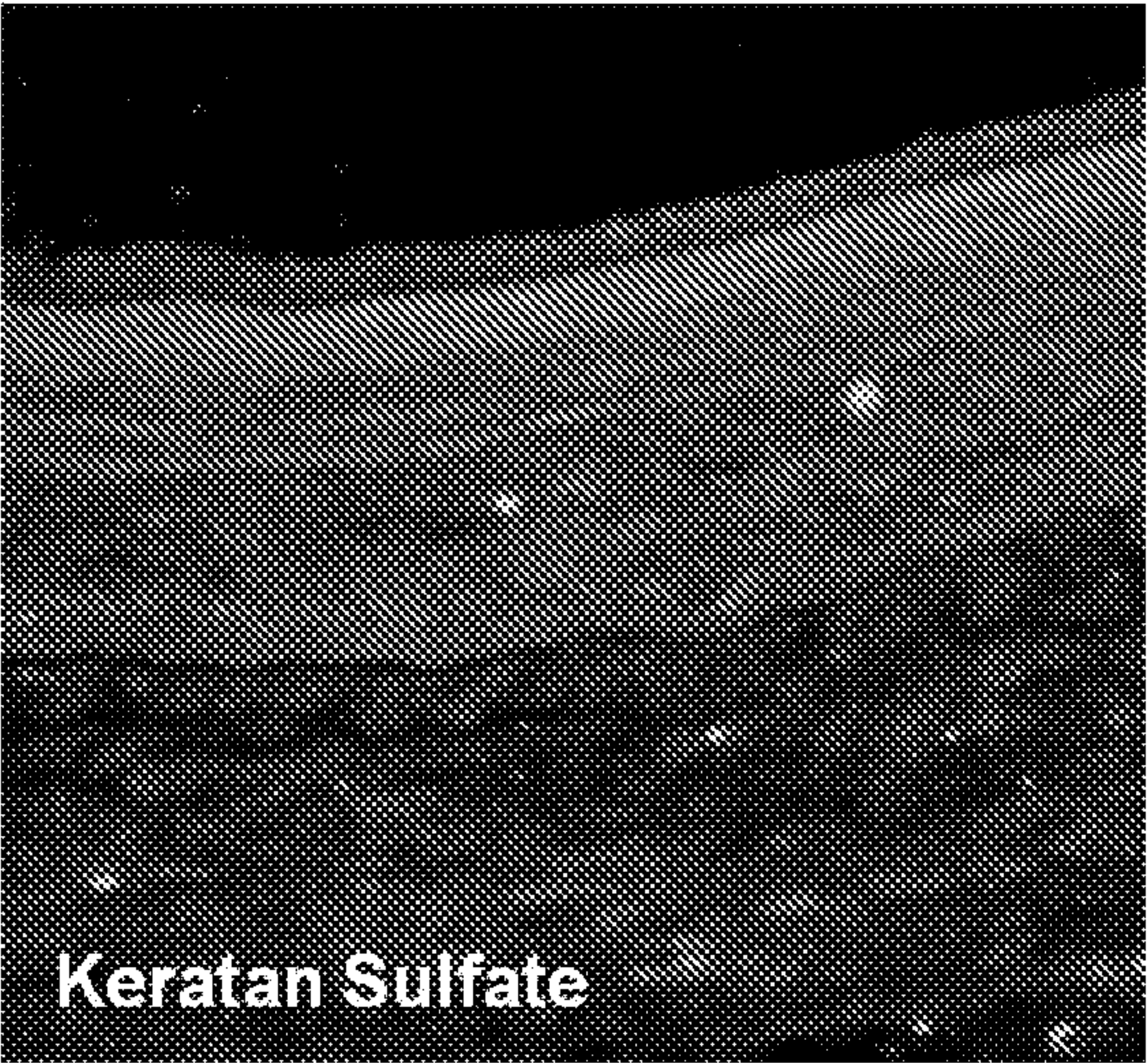
B



C

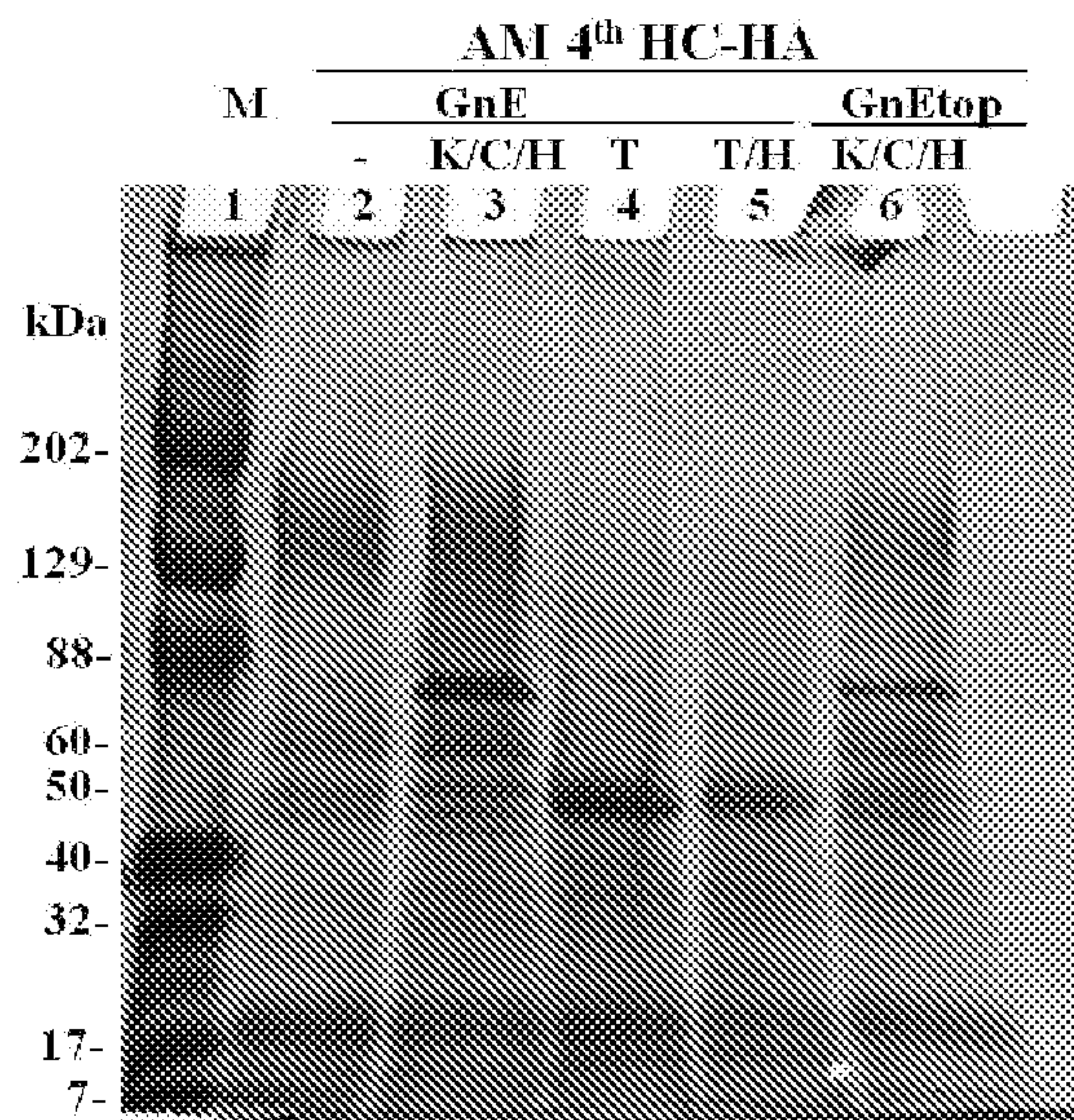
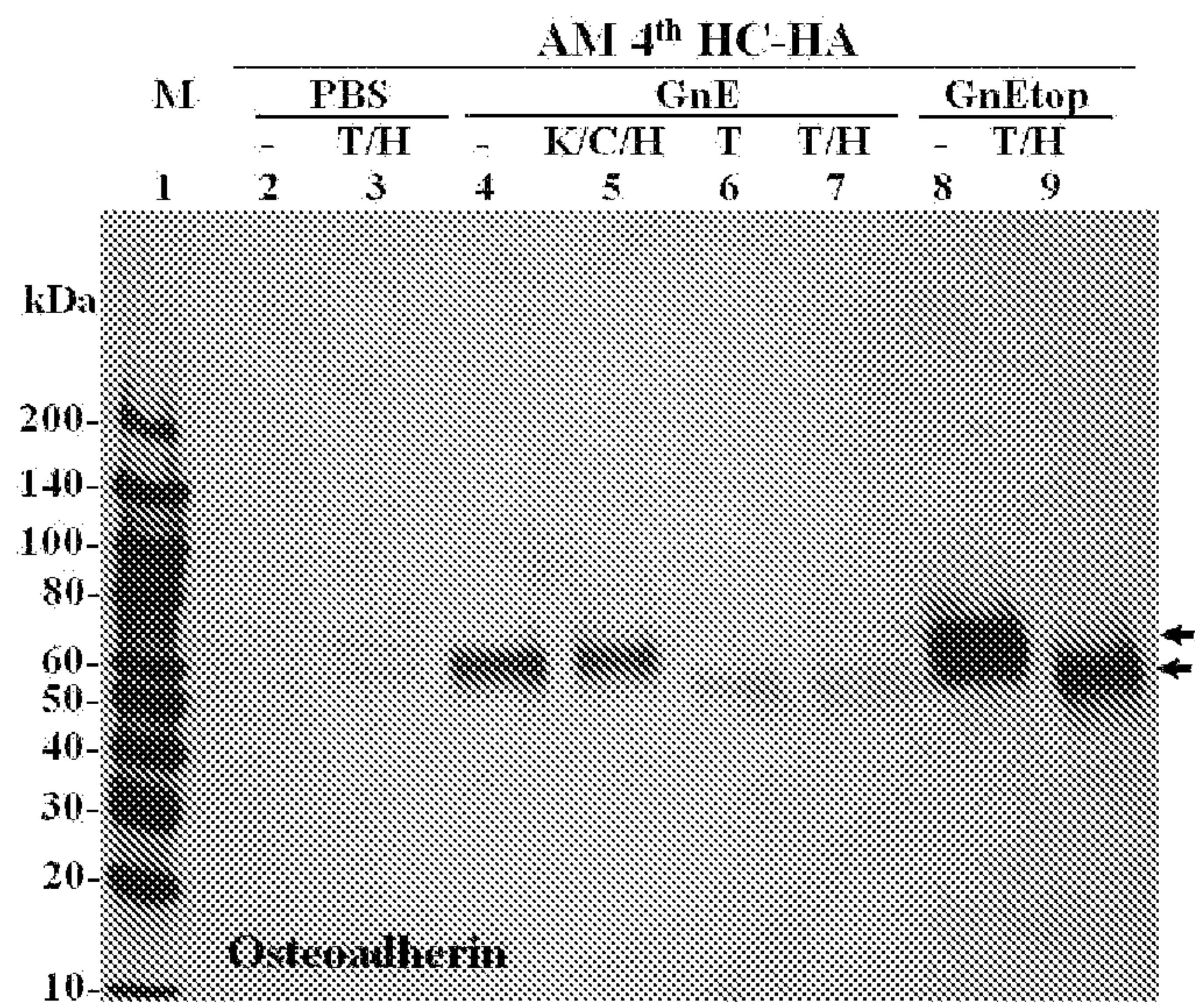
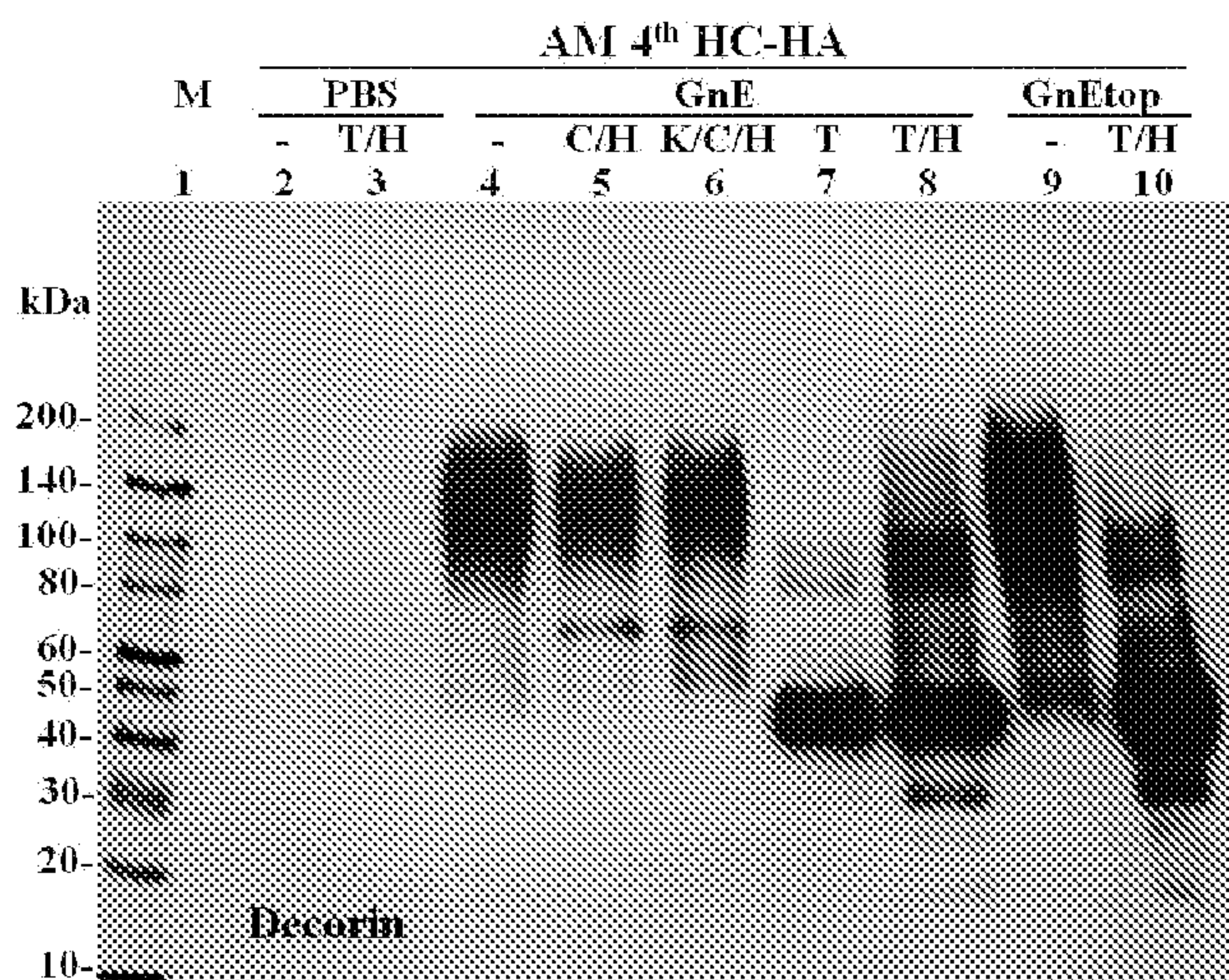
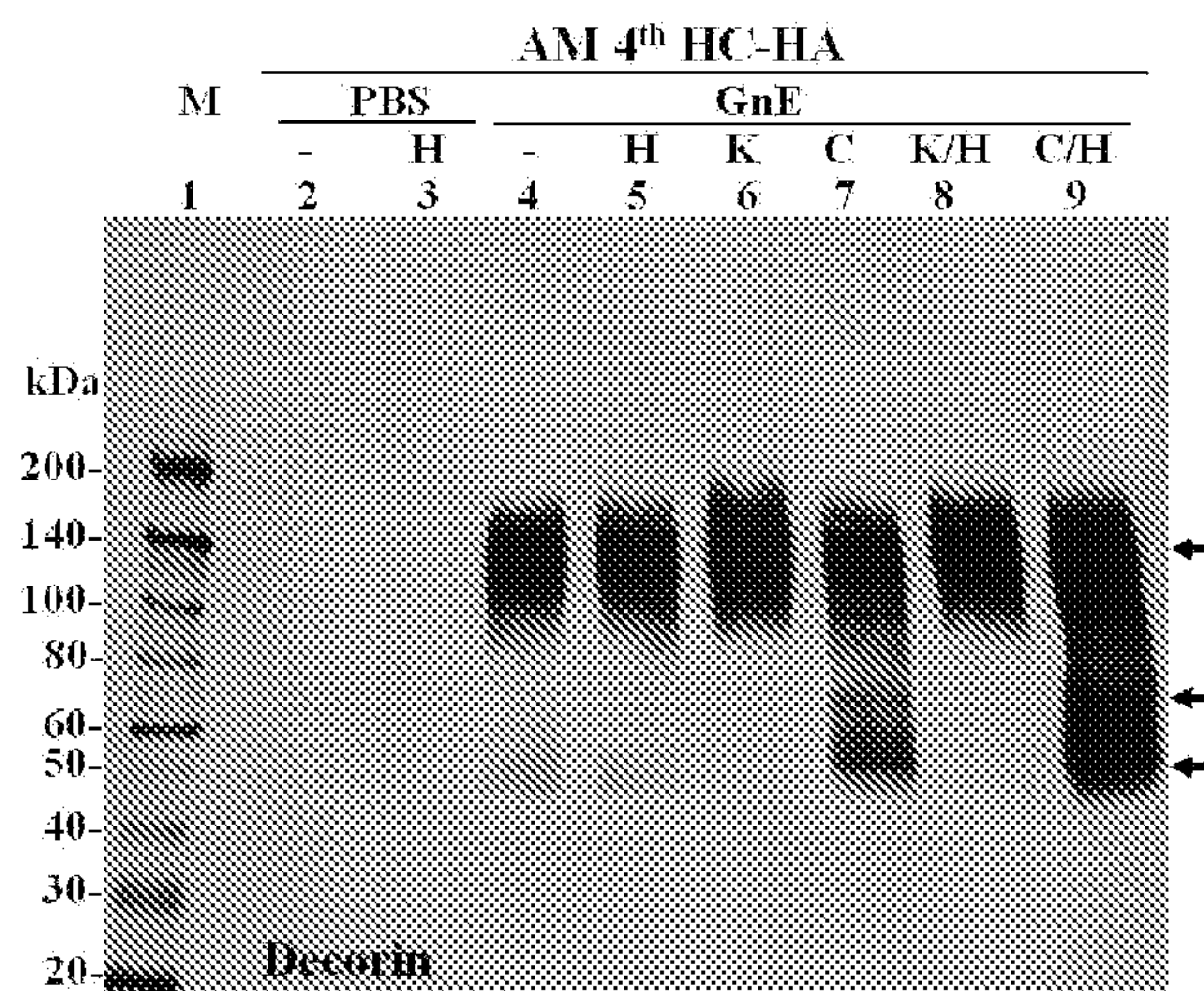


D

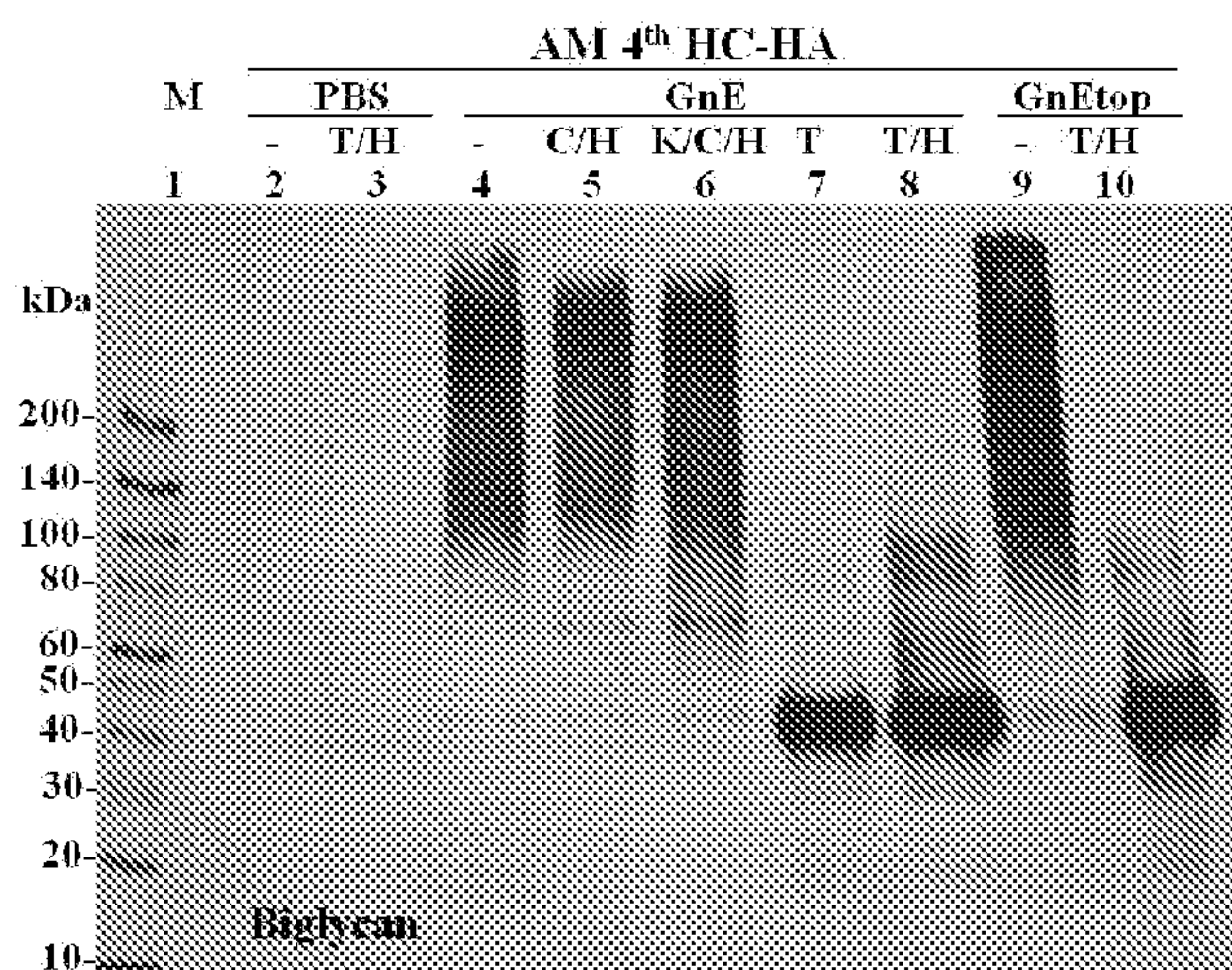
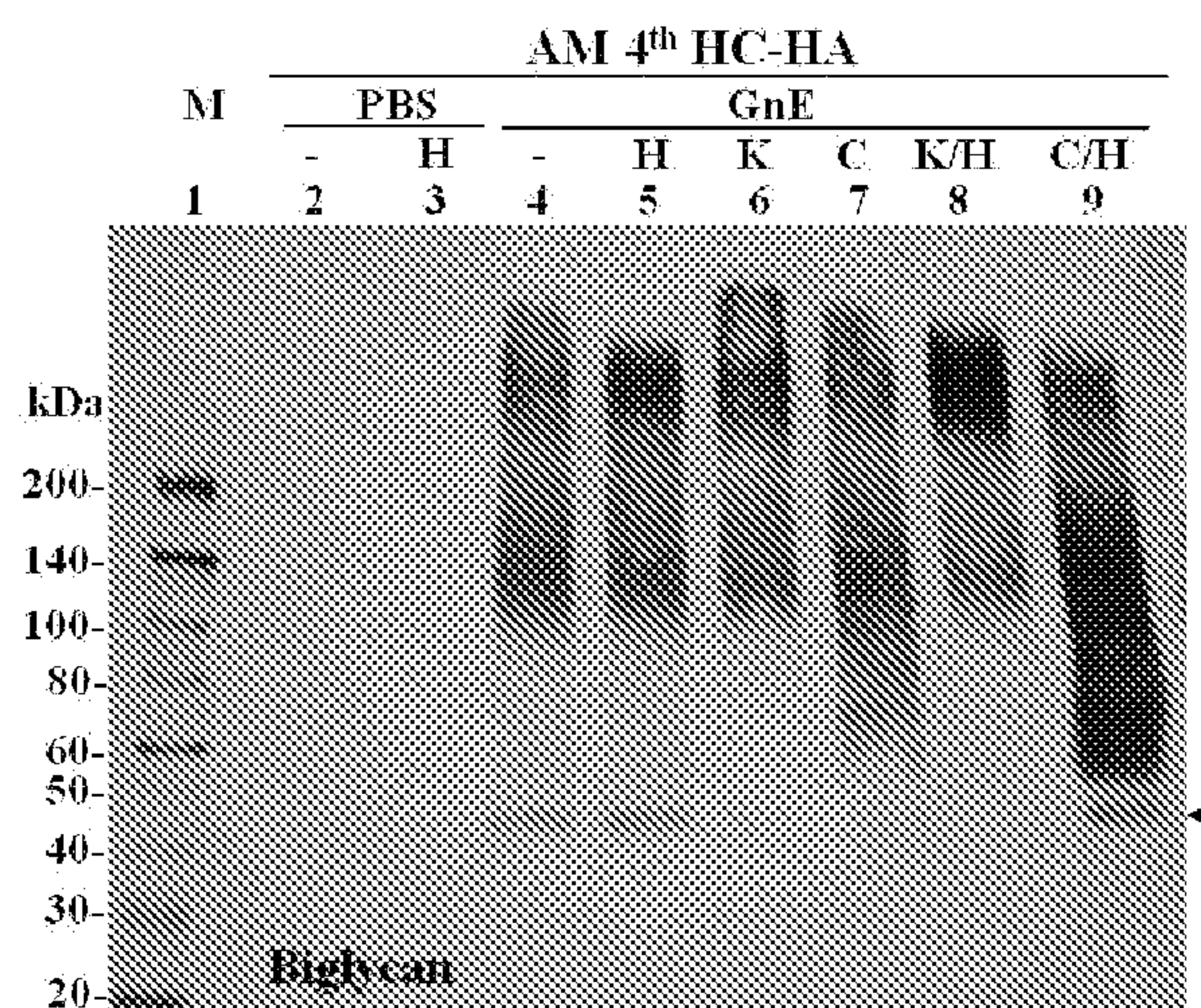
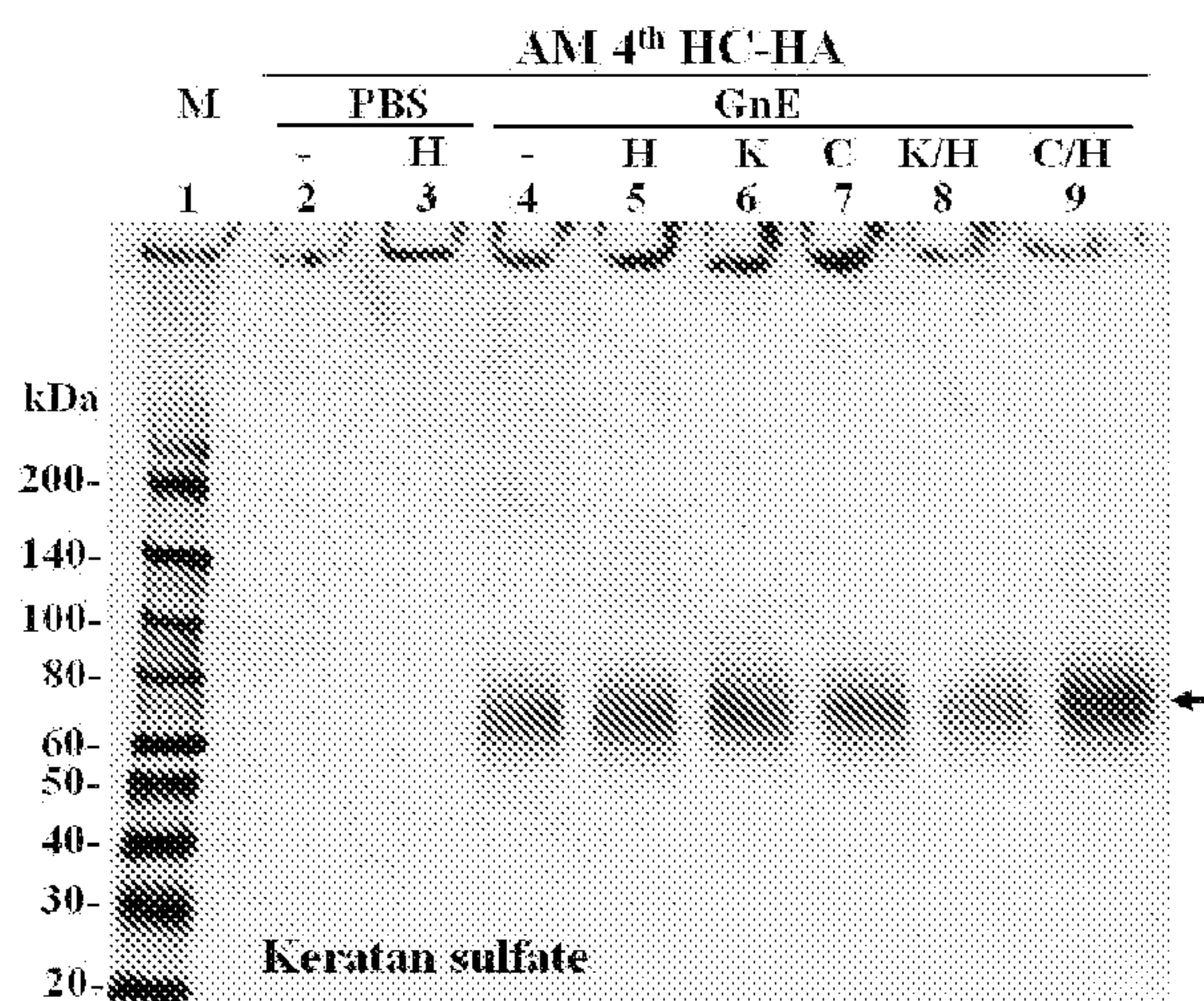
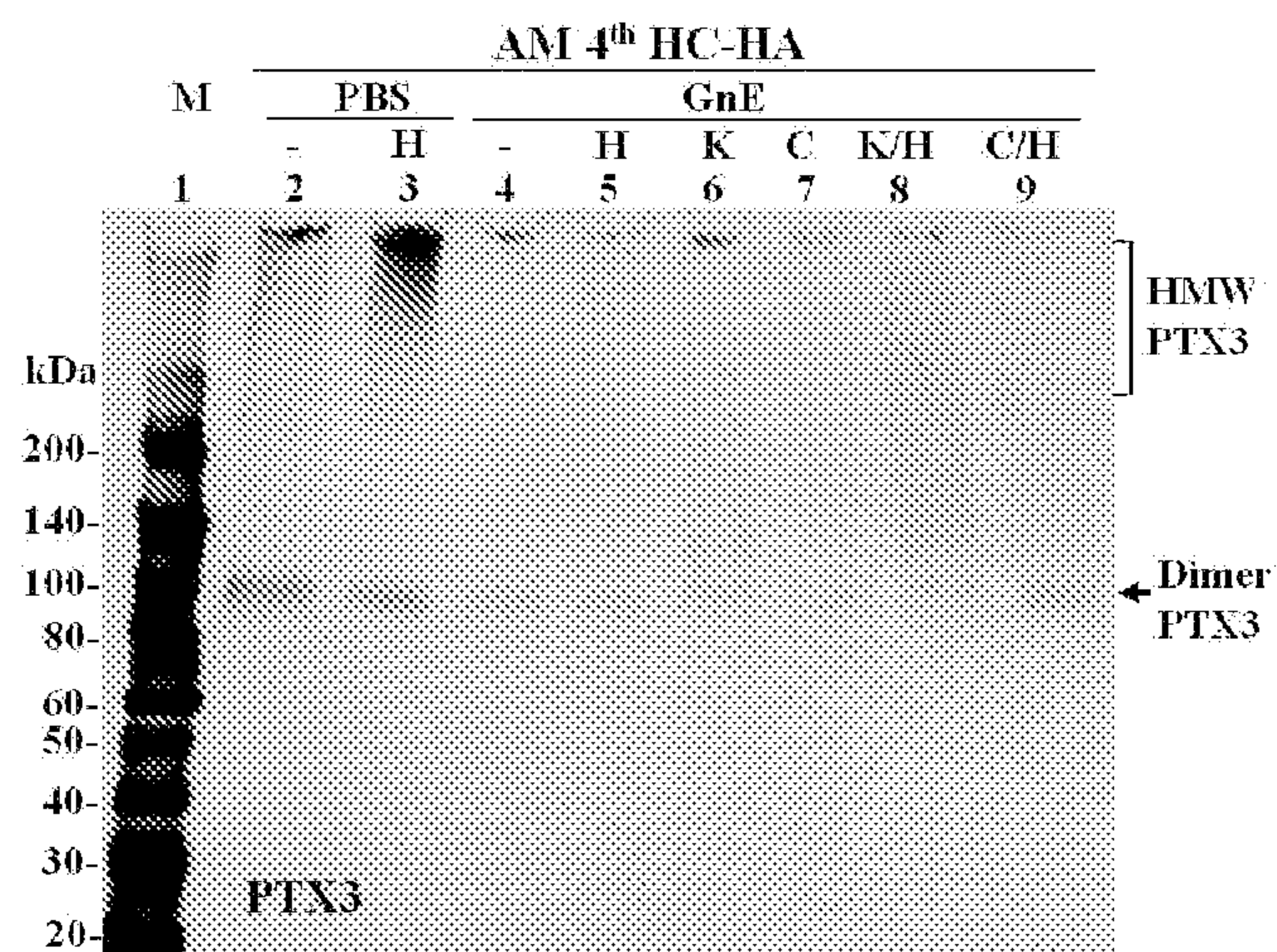


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FIG. 31

A**B****C****D**

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FIG. 31 (cont'd)**E****F****G****H**

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FIG. 32

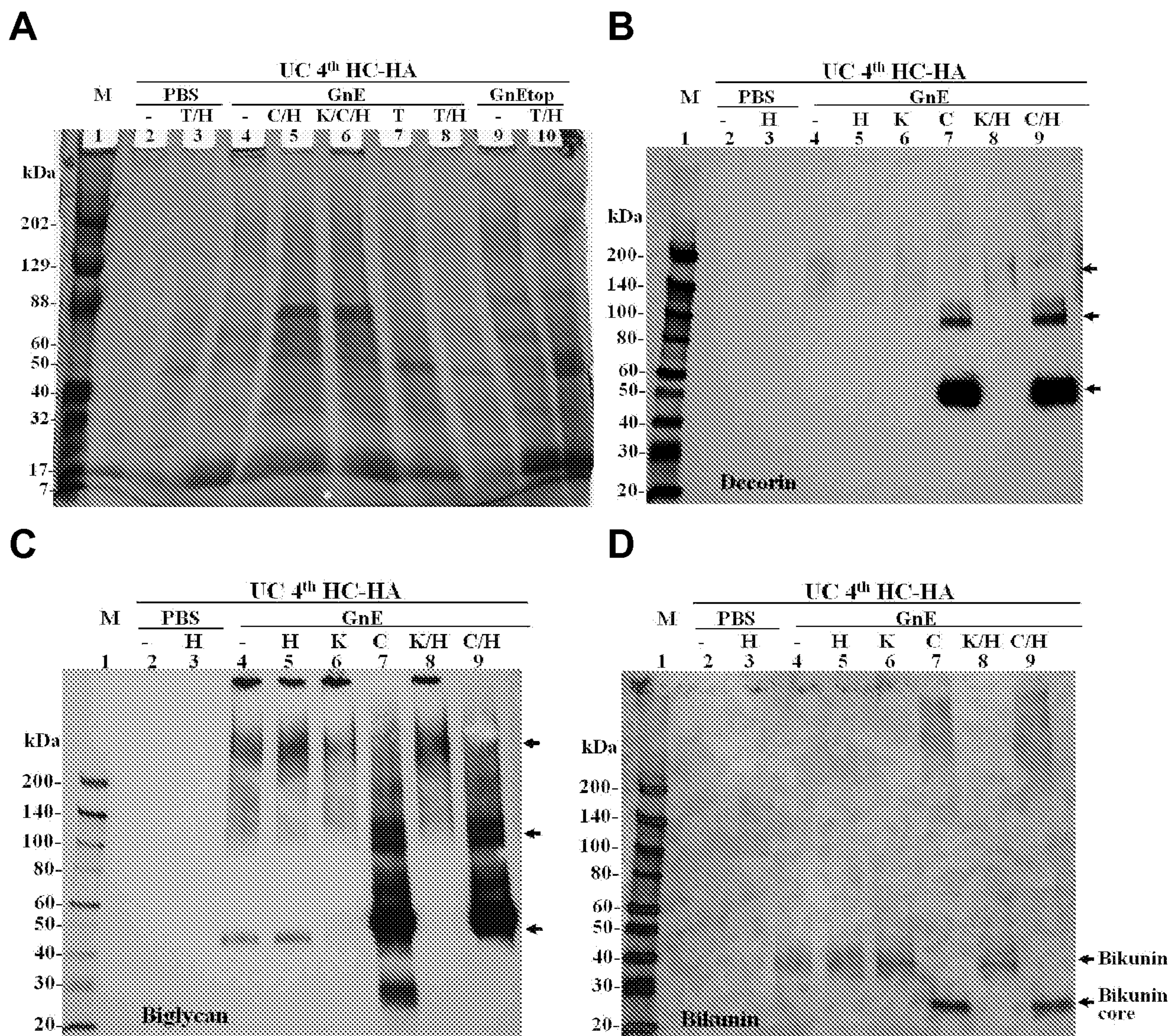
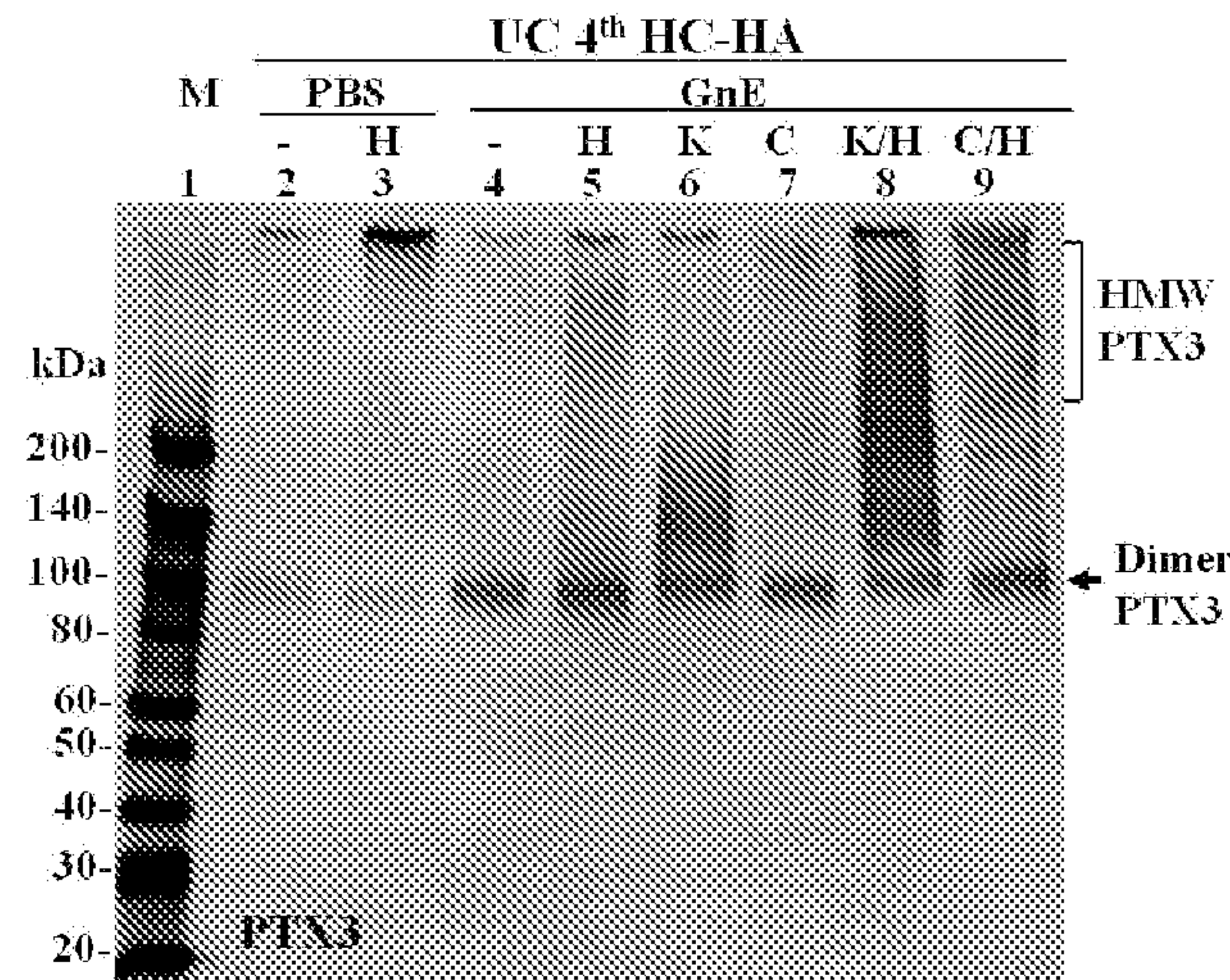
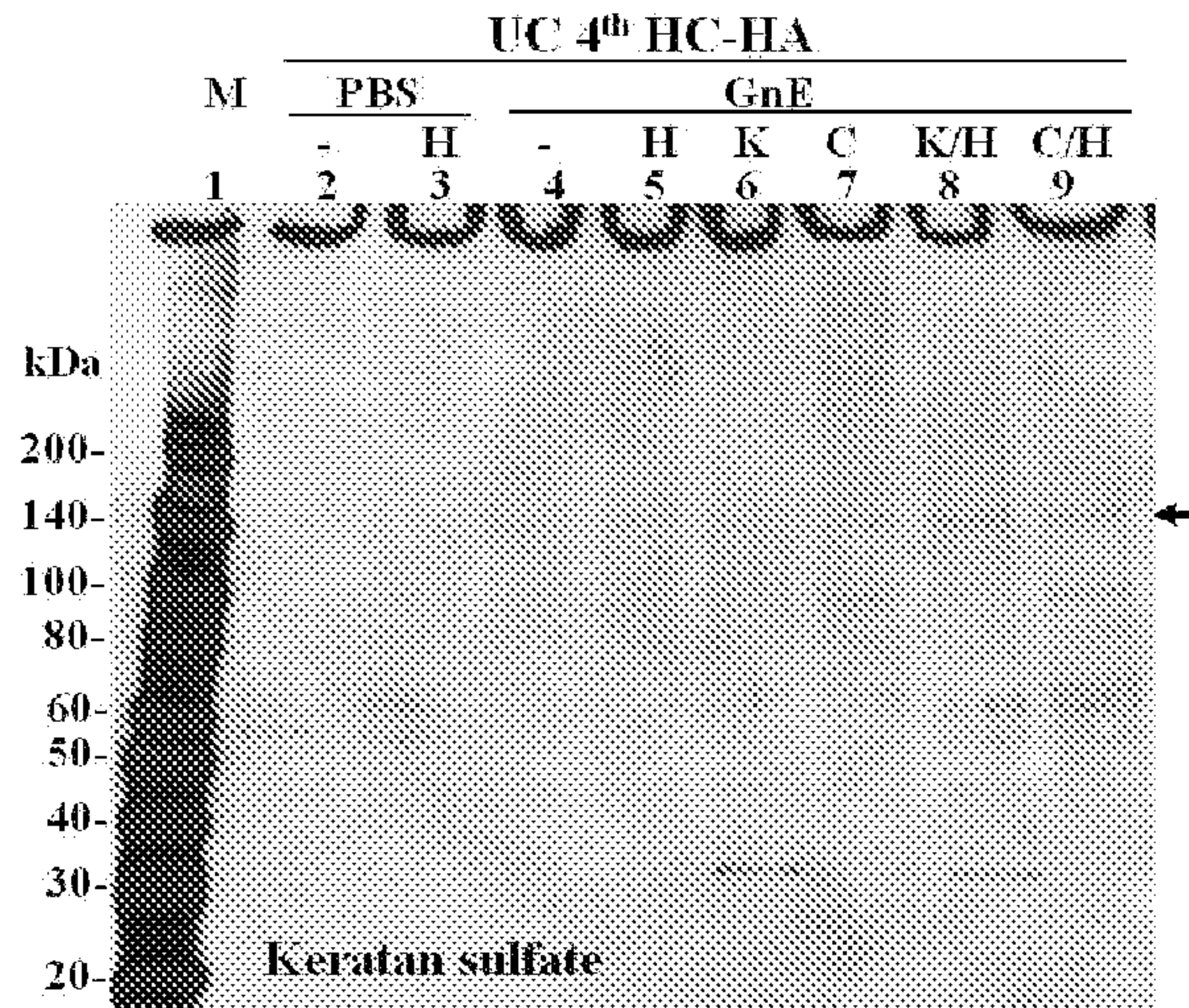


FIG. 32 (cont'd)

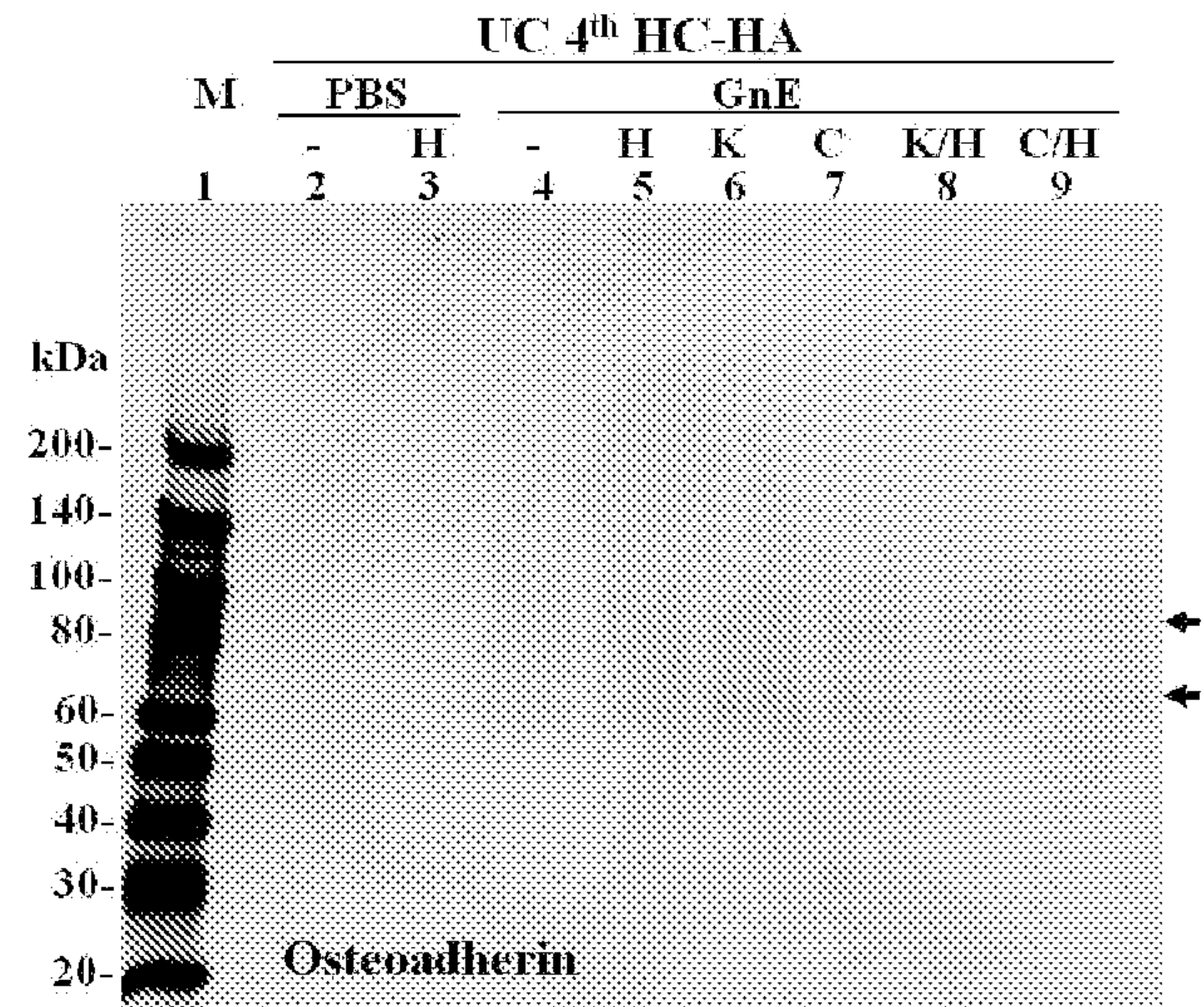
E



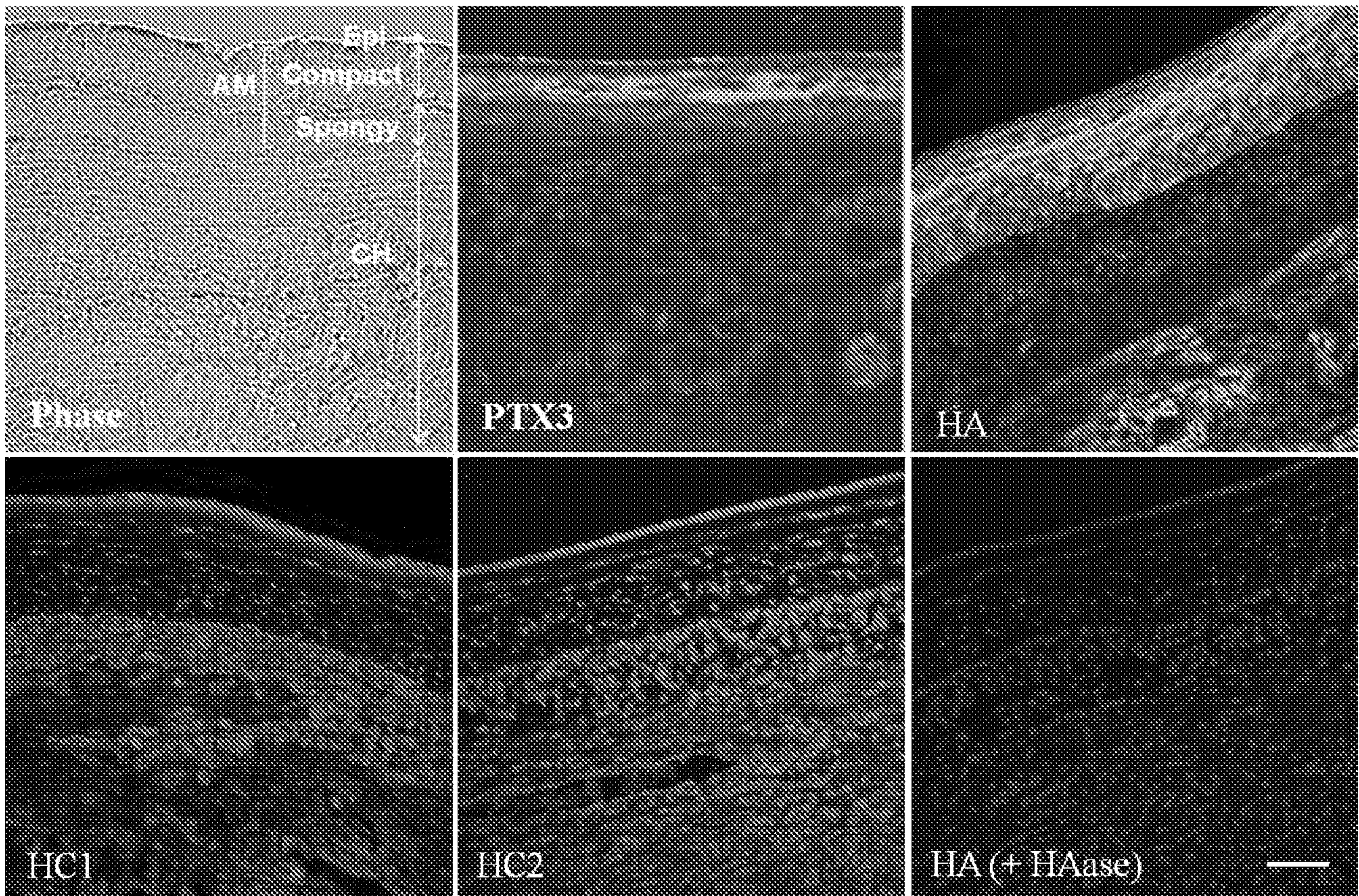
F



G

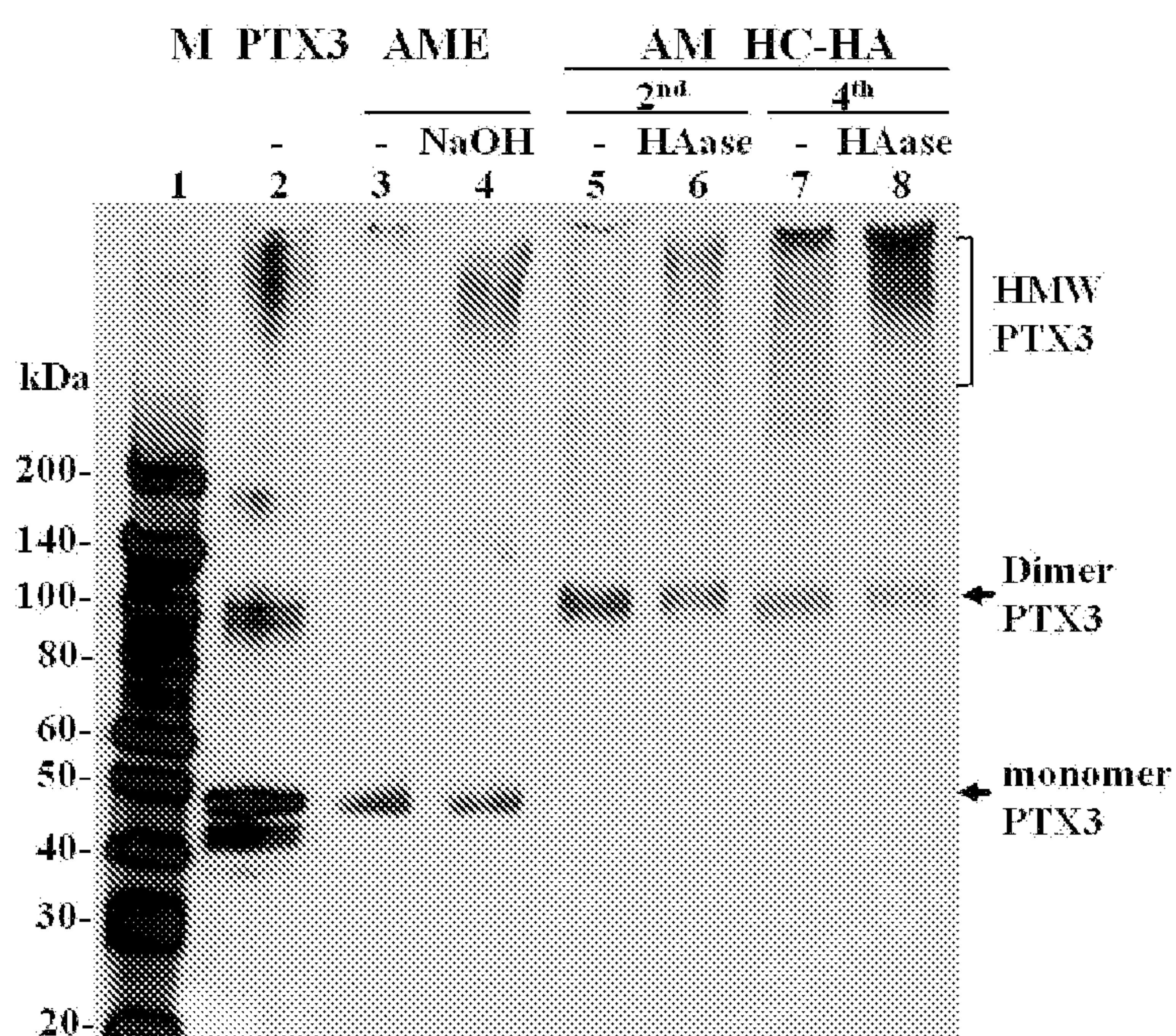
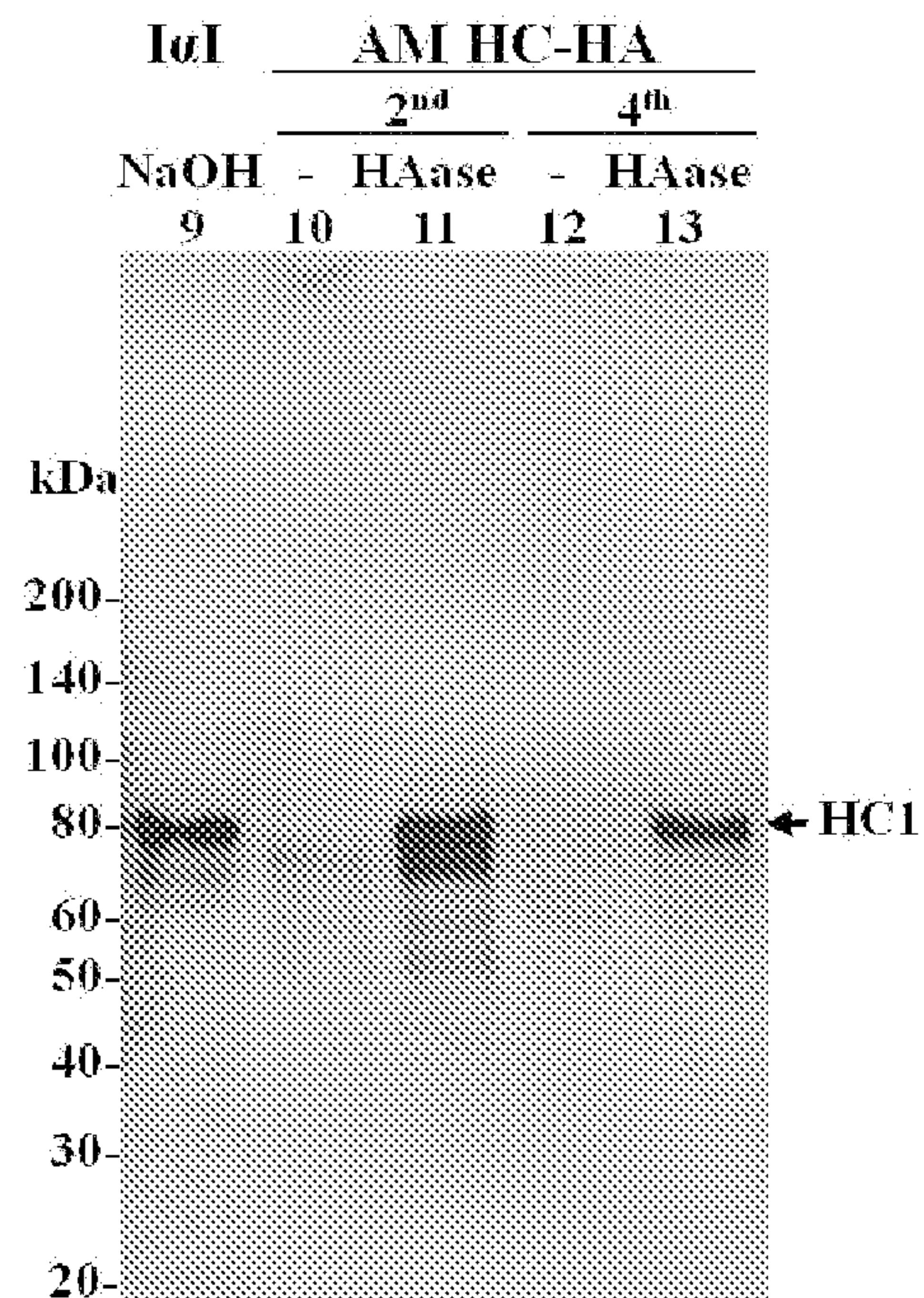
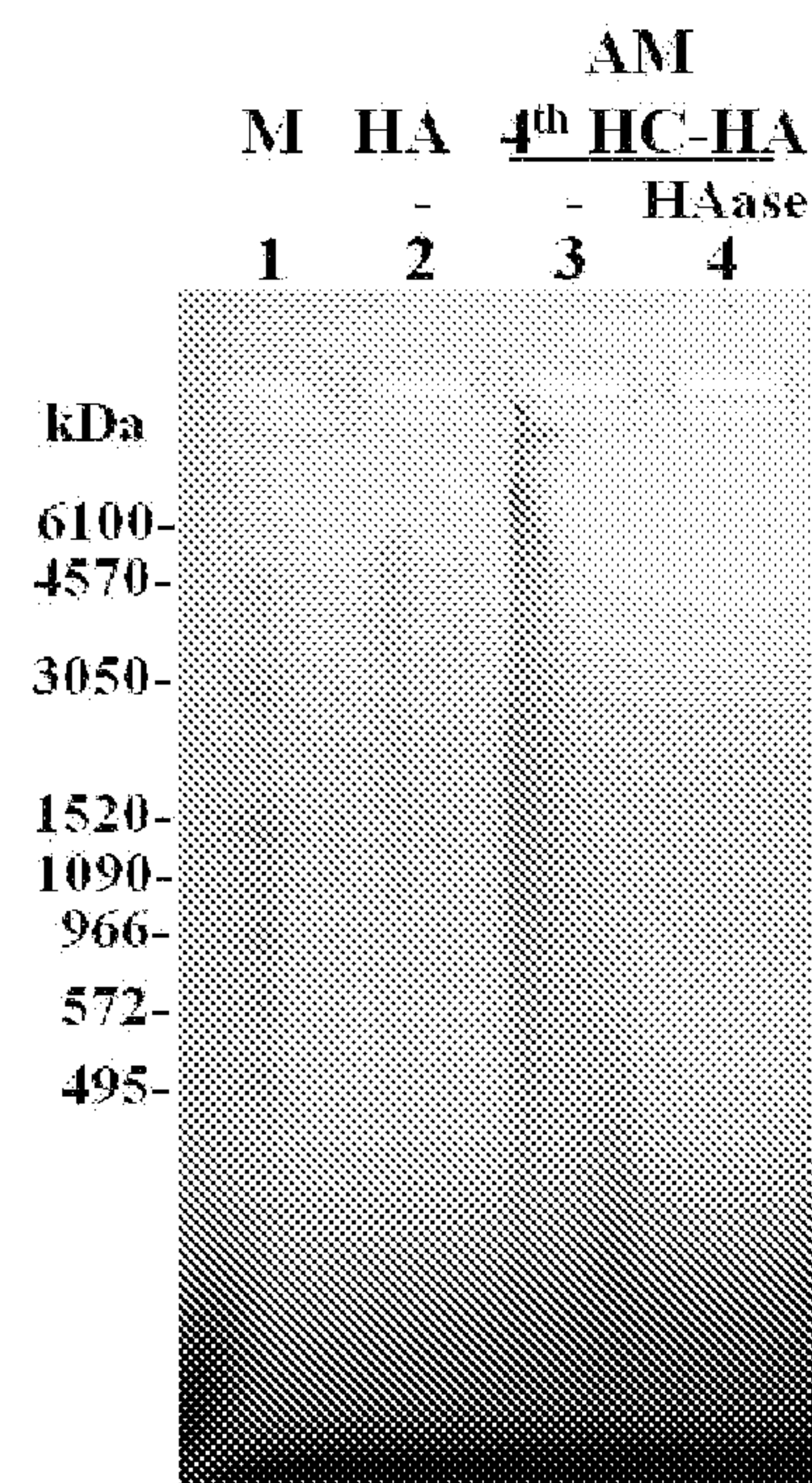


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FIG. 33

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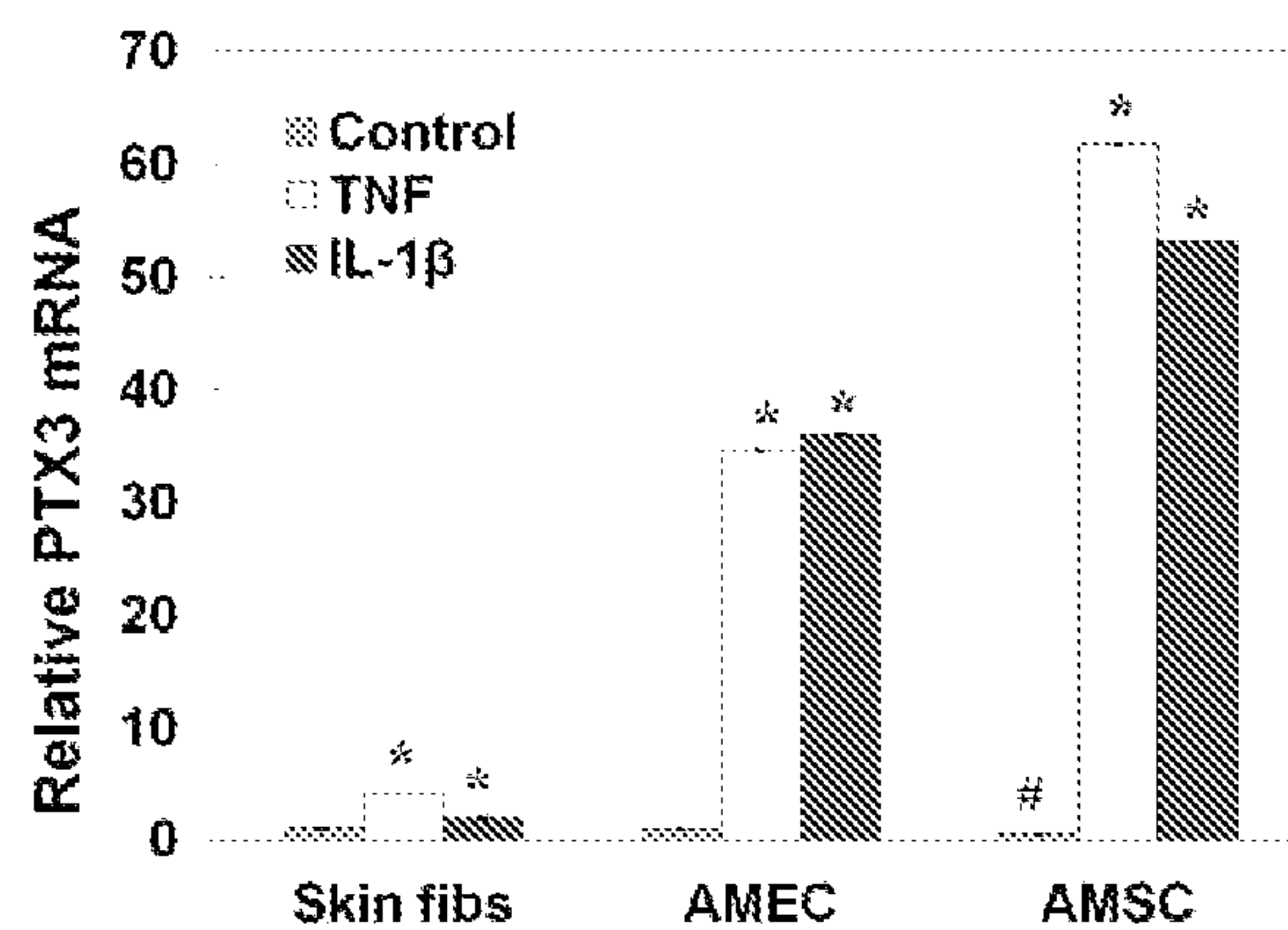
FIG. 34

A**B****C**

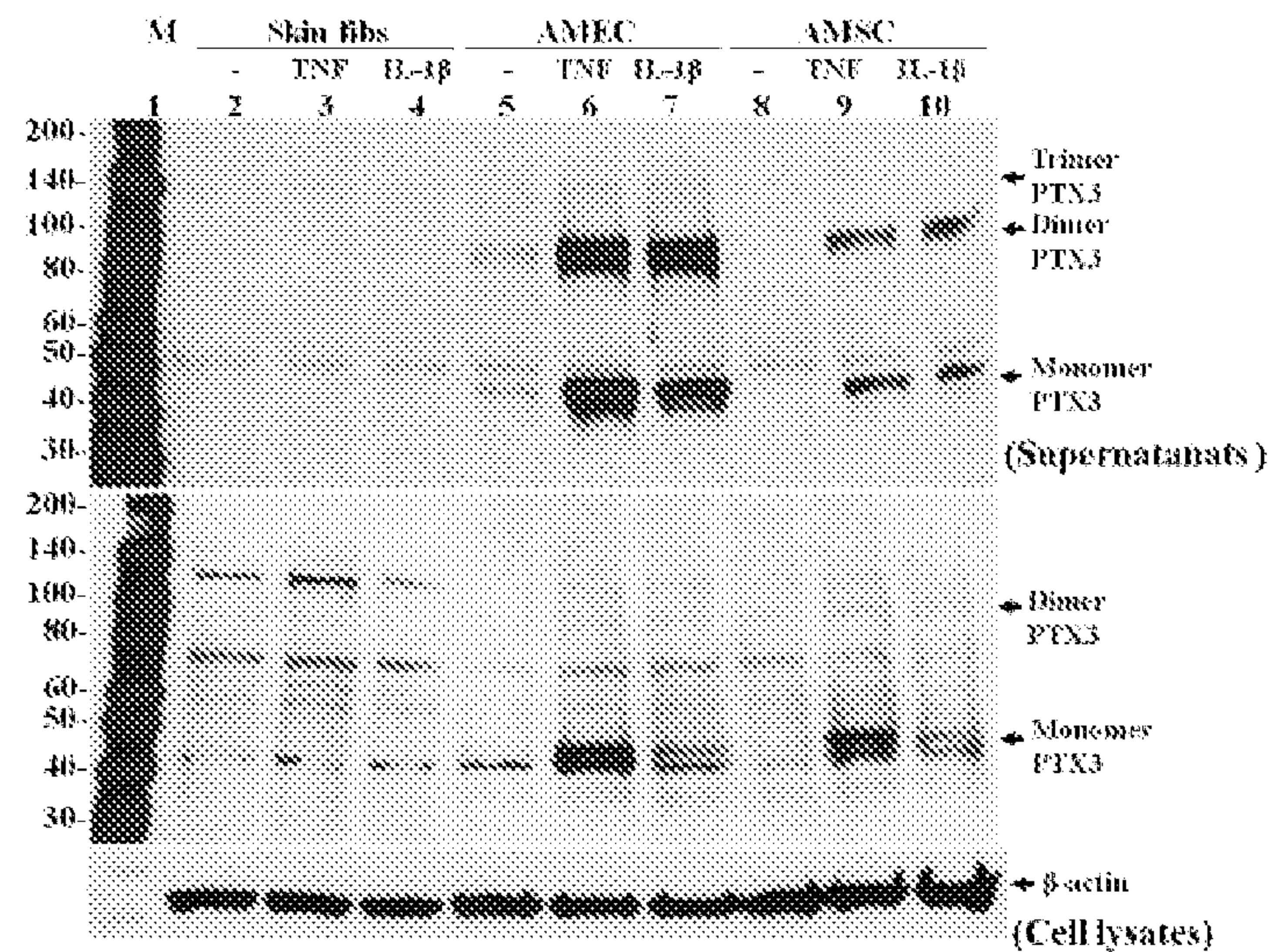
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FIG. 35

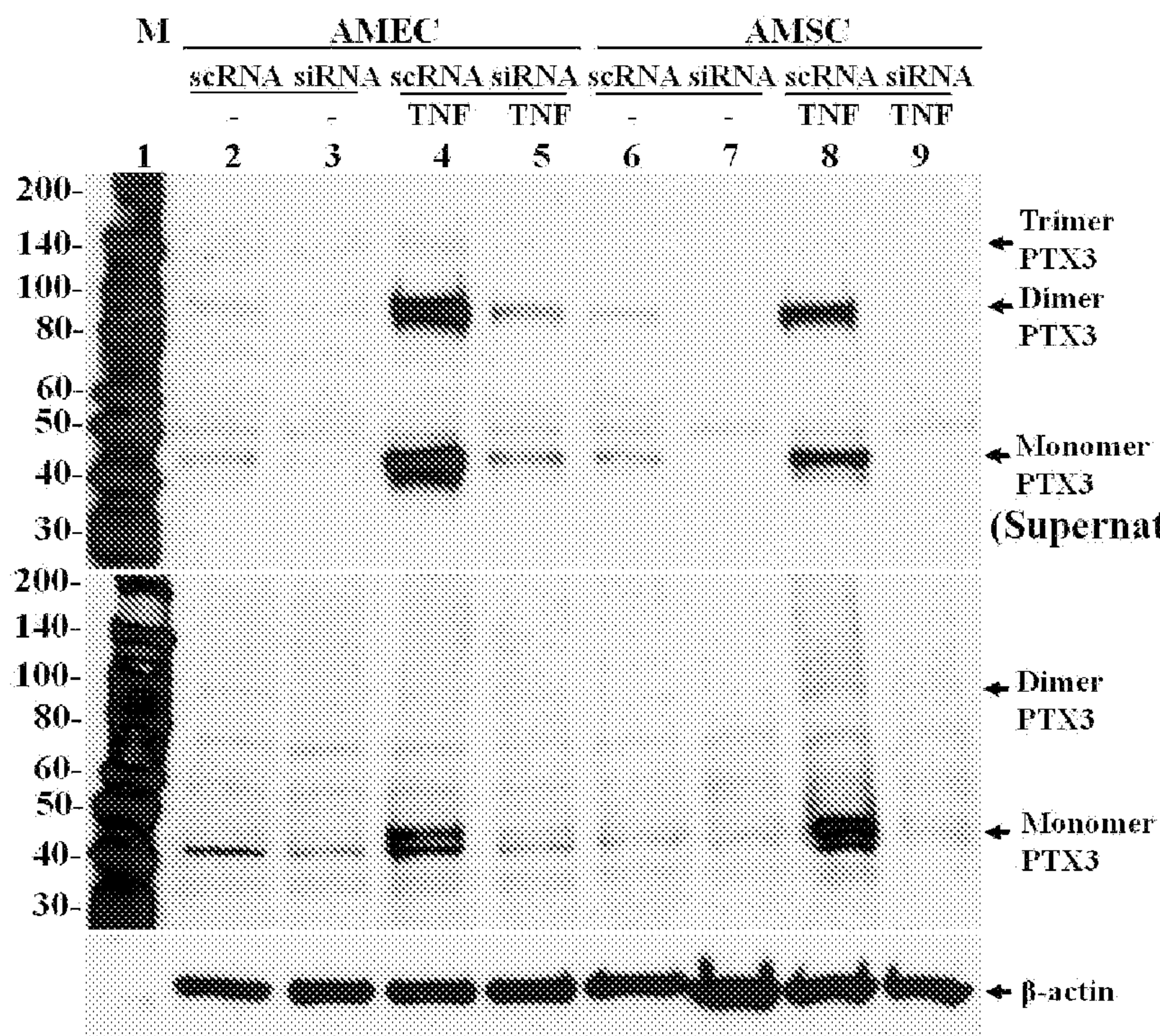
A



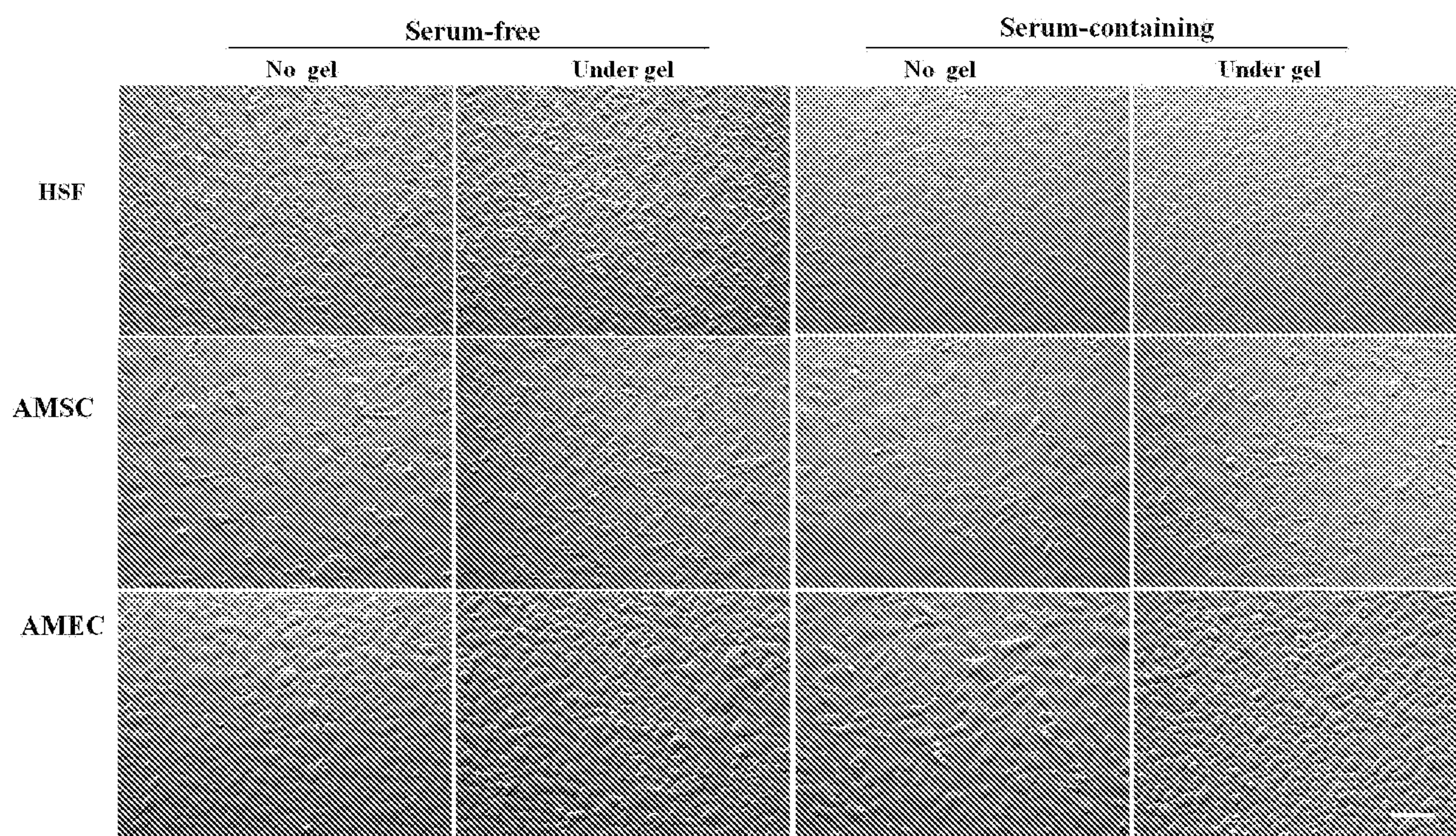
B



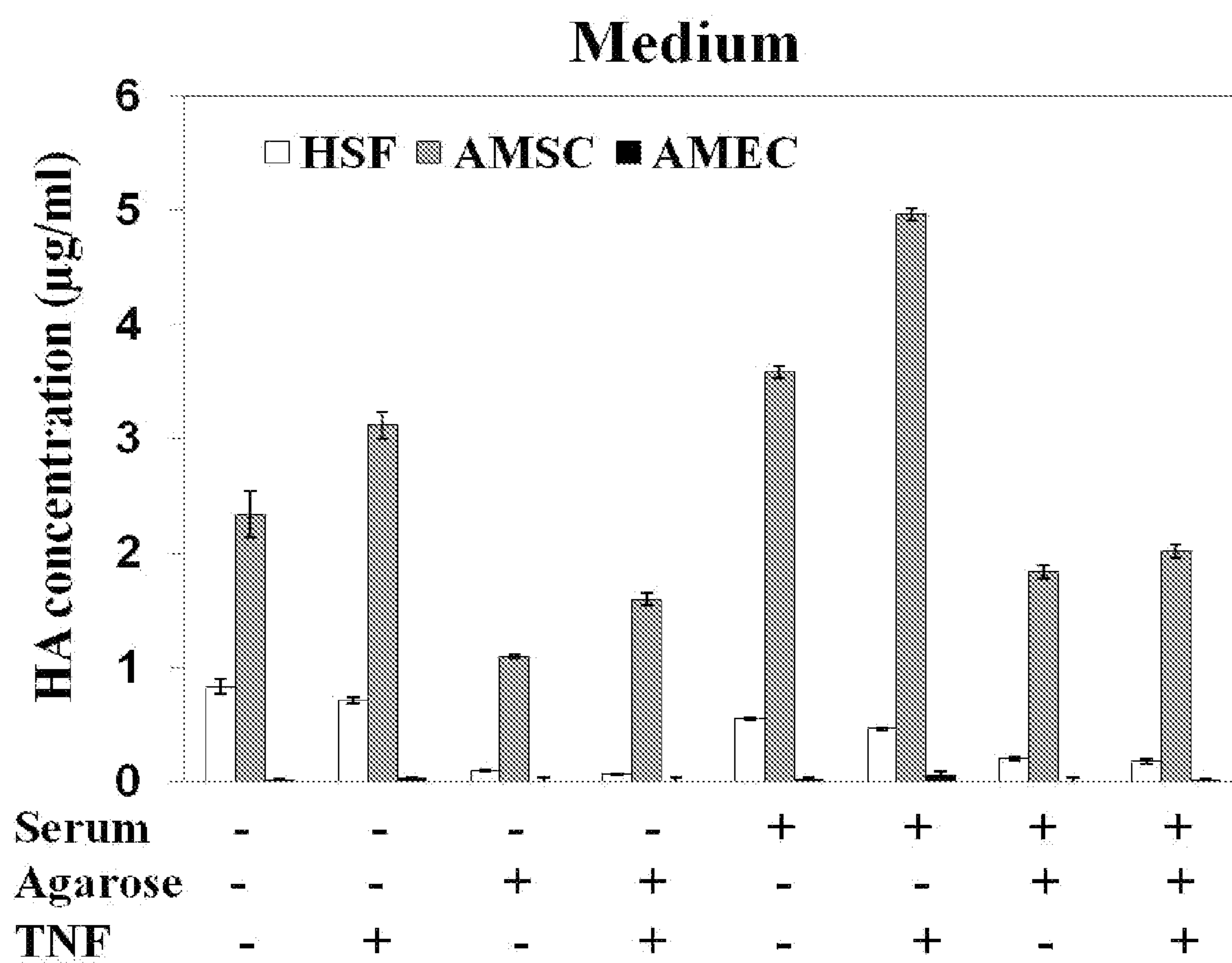
C



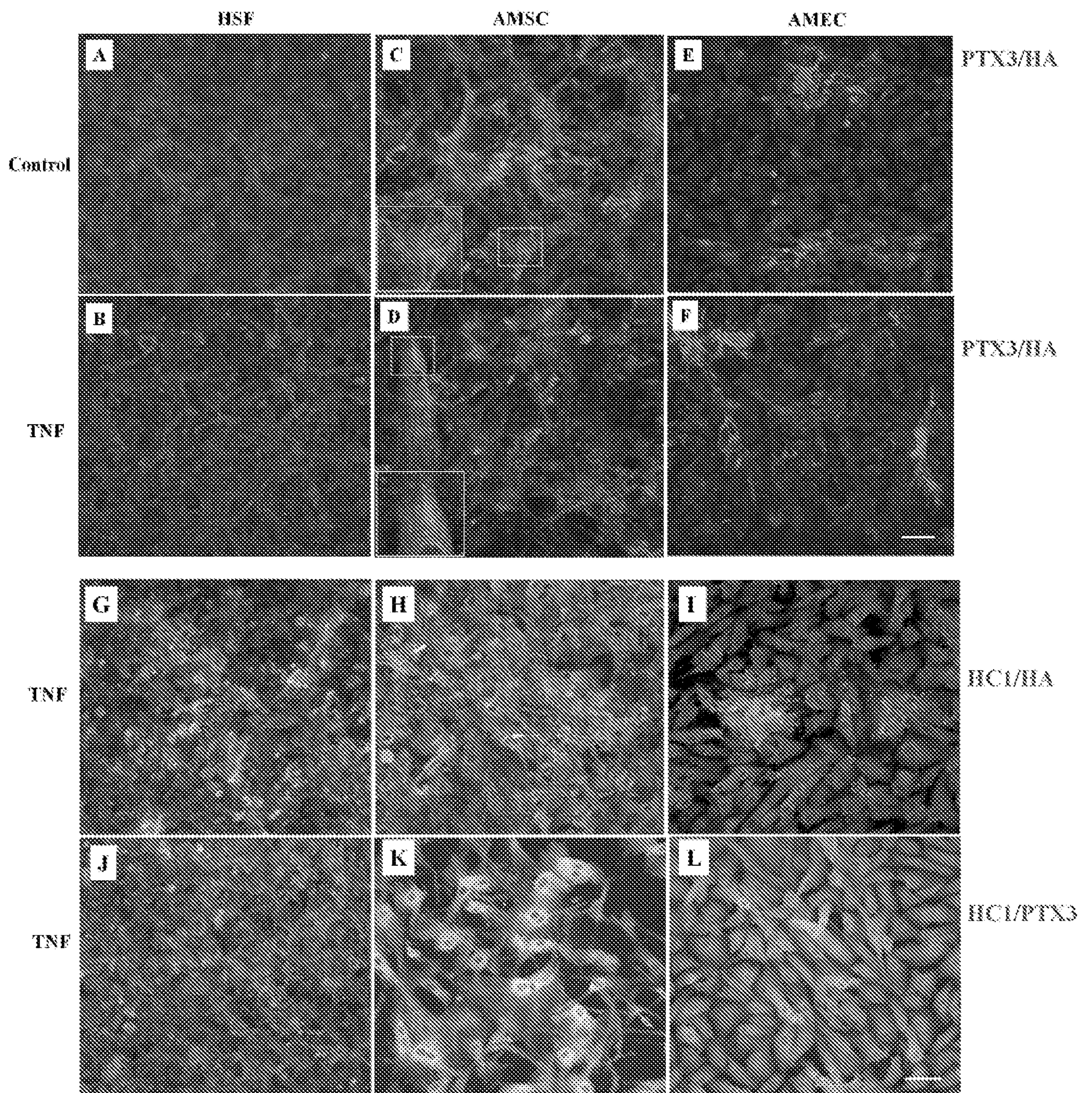
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FIG. 36

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FIG. 37

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FIG. 38

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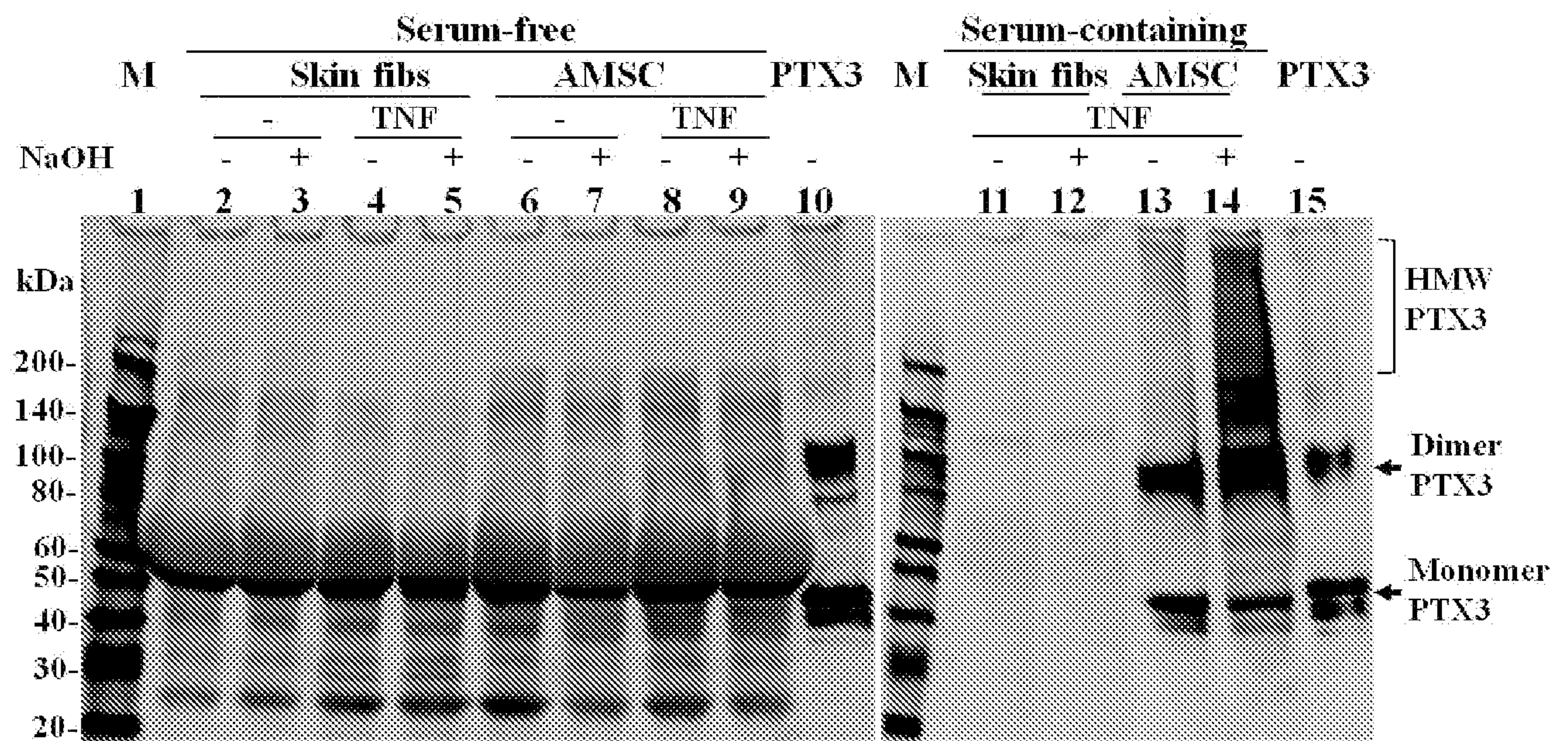
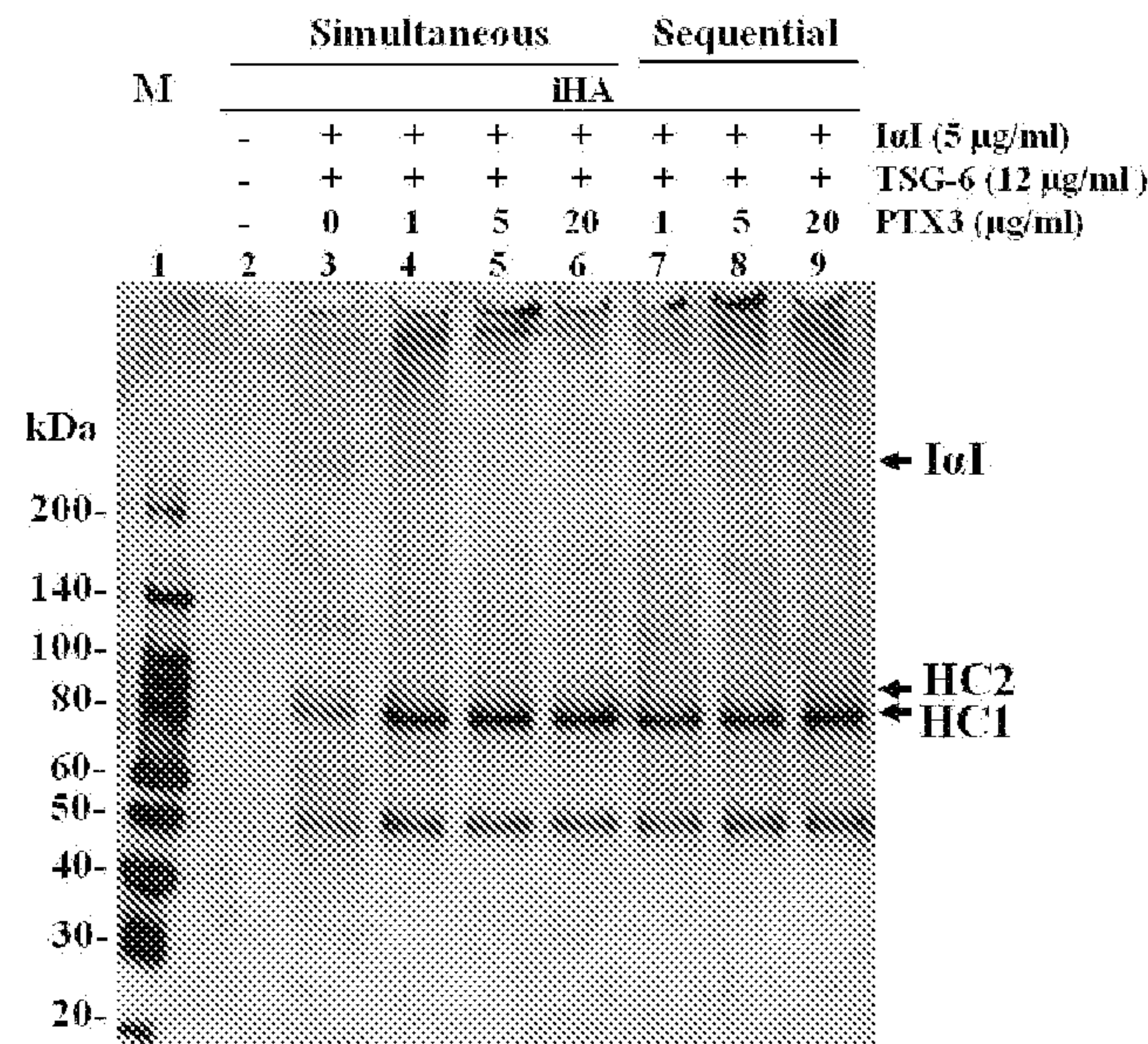
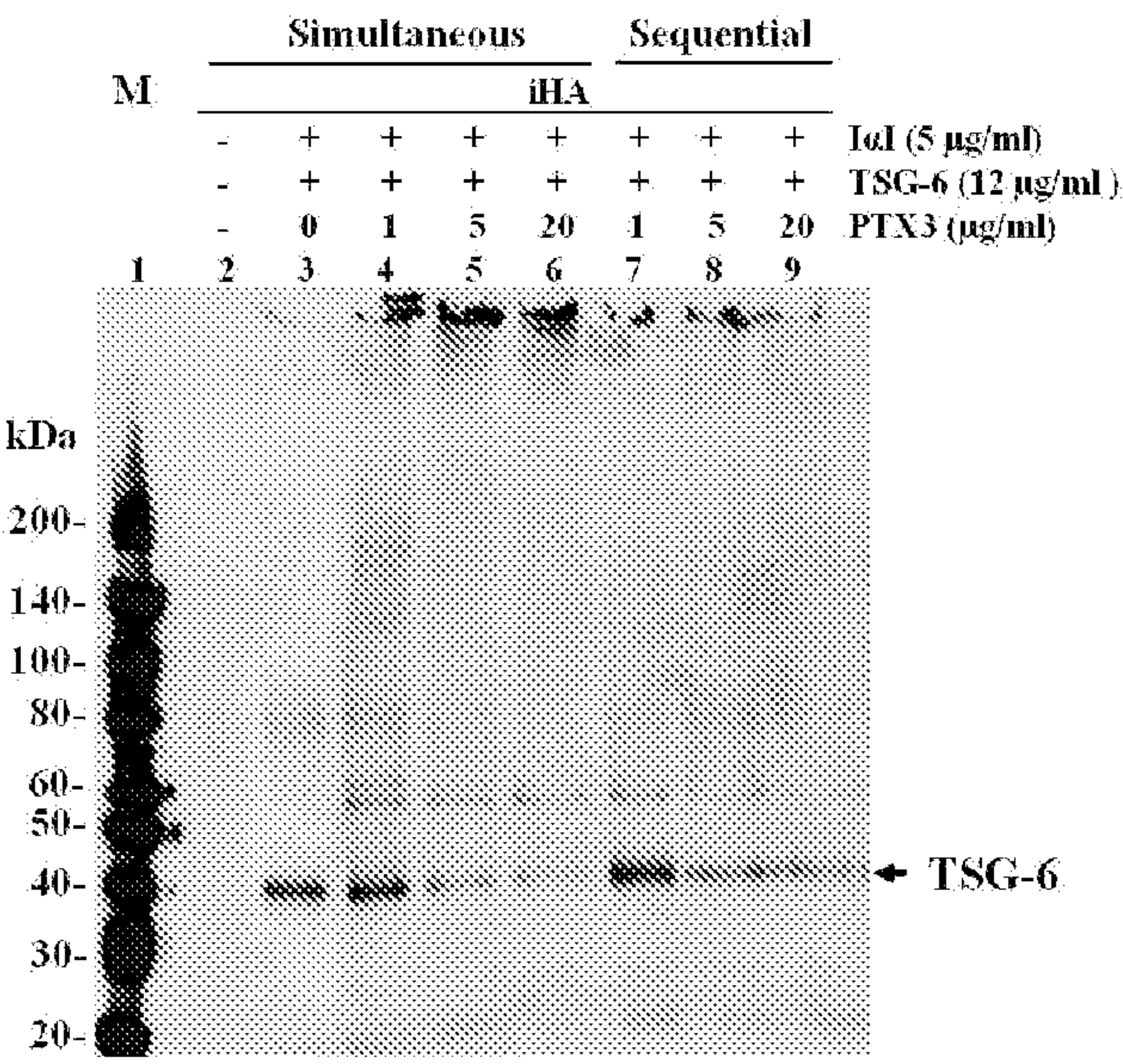
FIG. 39

FIG. 40

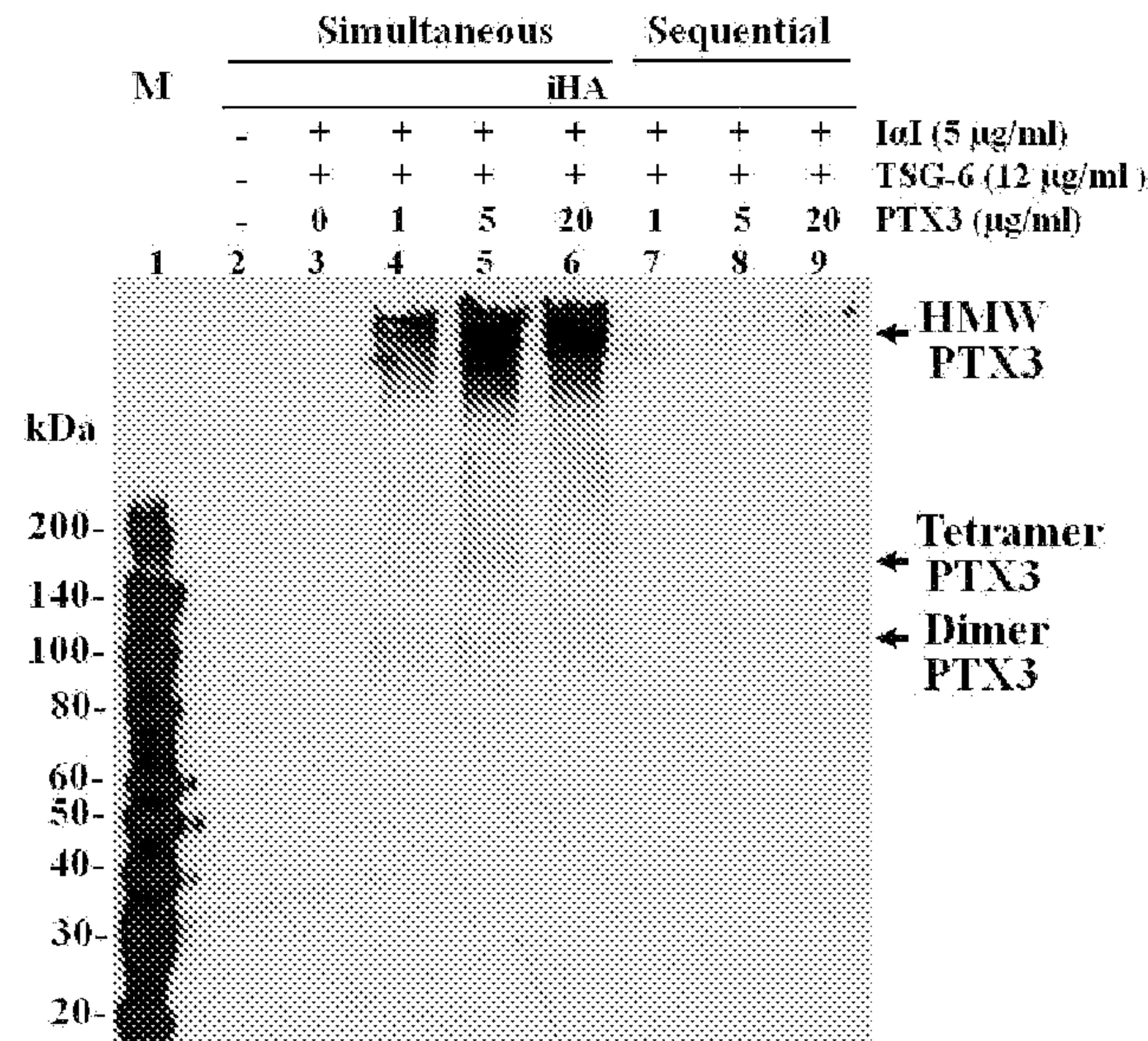
A



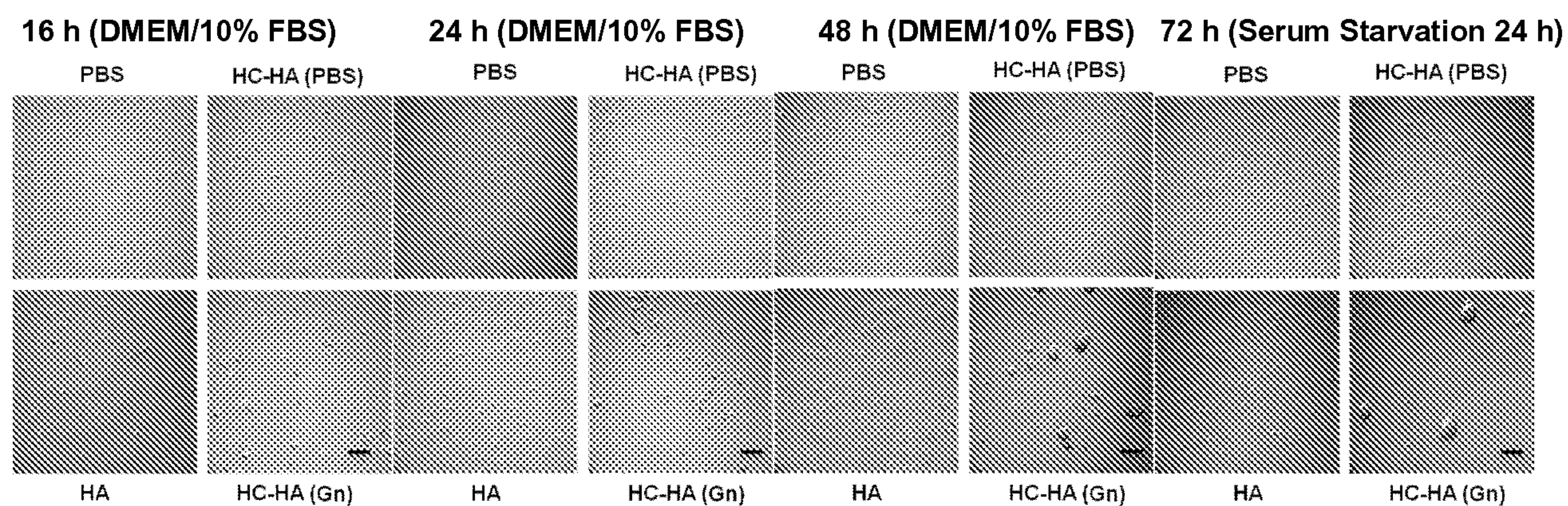
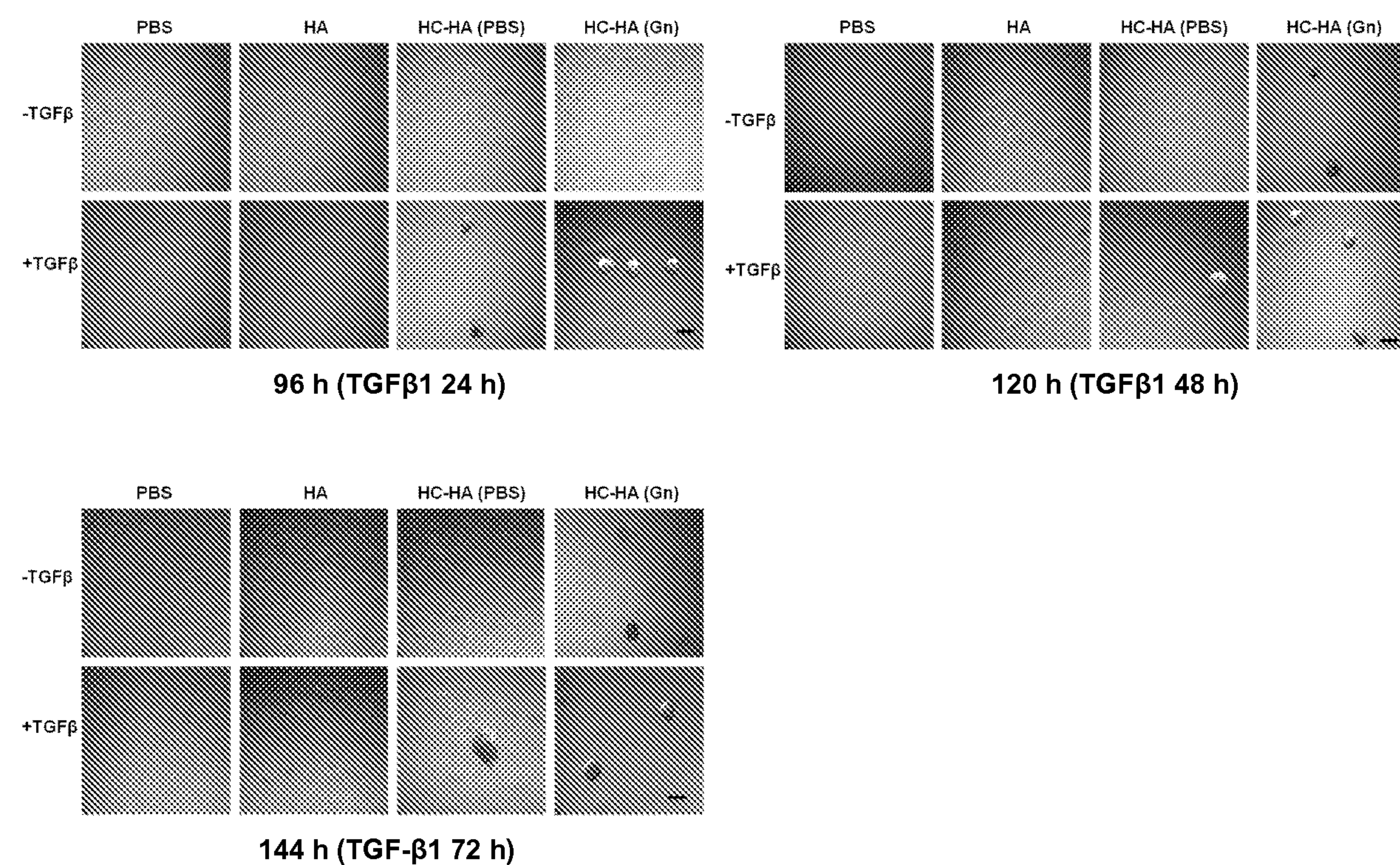
B



C

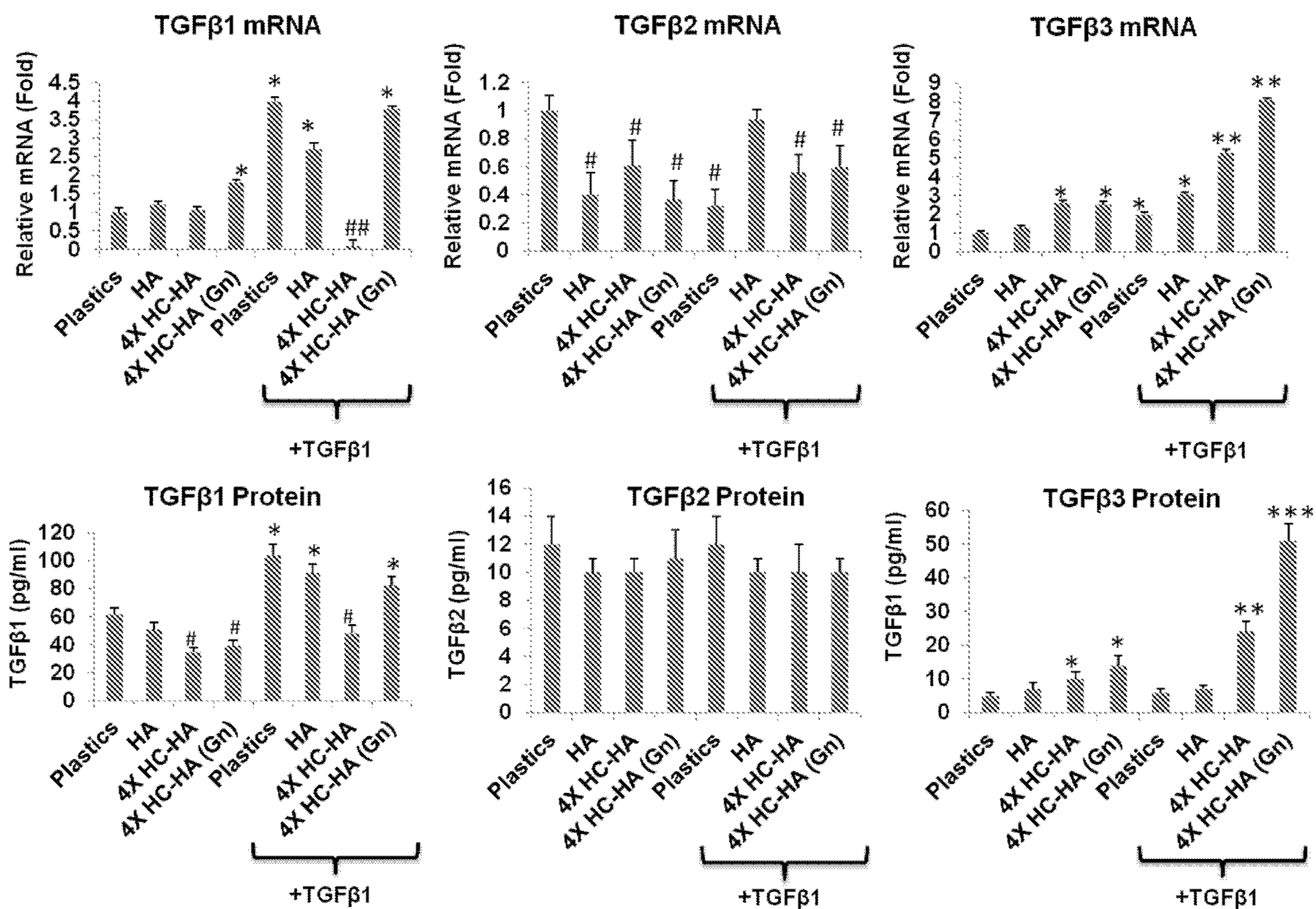


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FIG. 41**A****B**

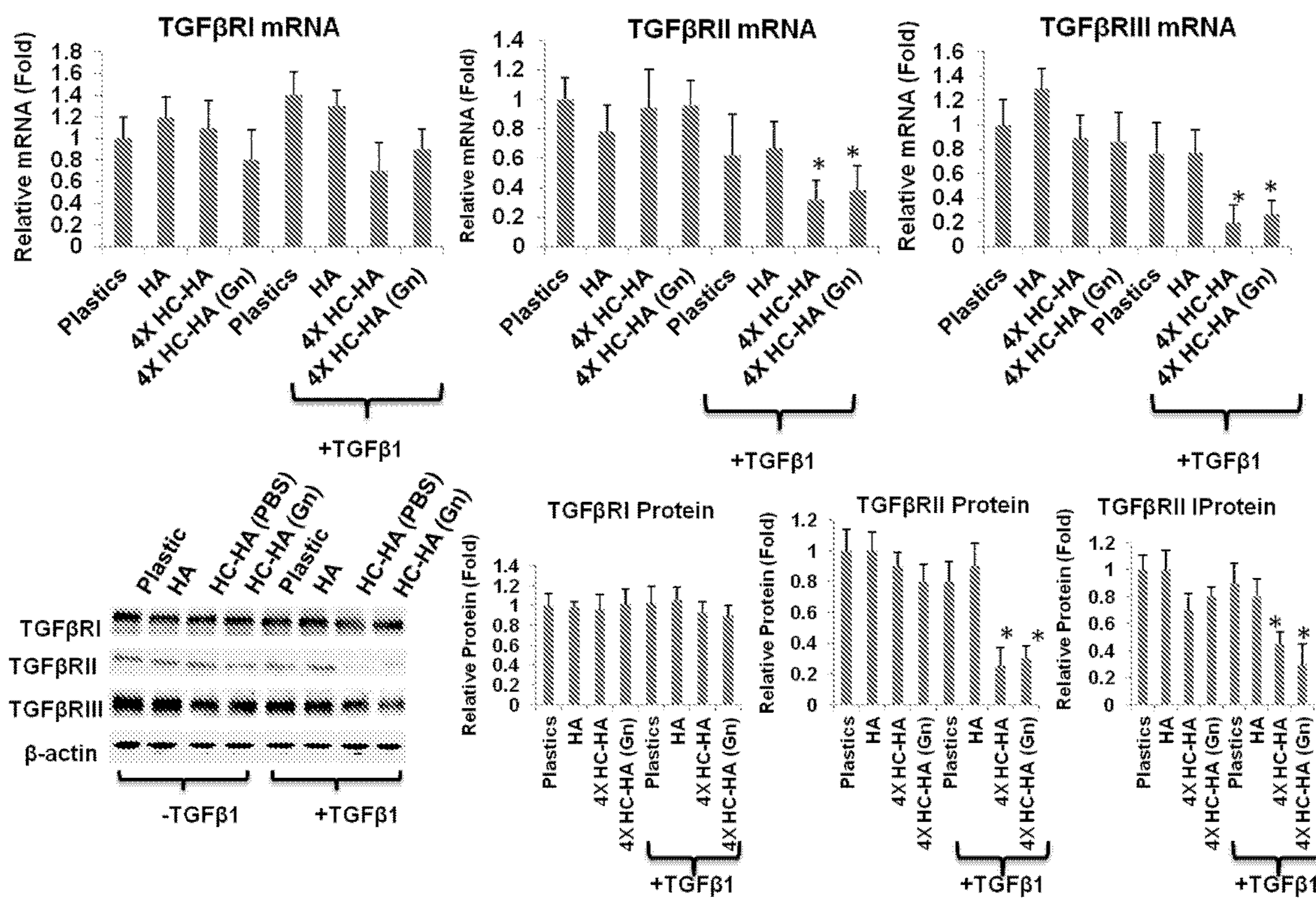
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FIG. 42

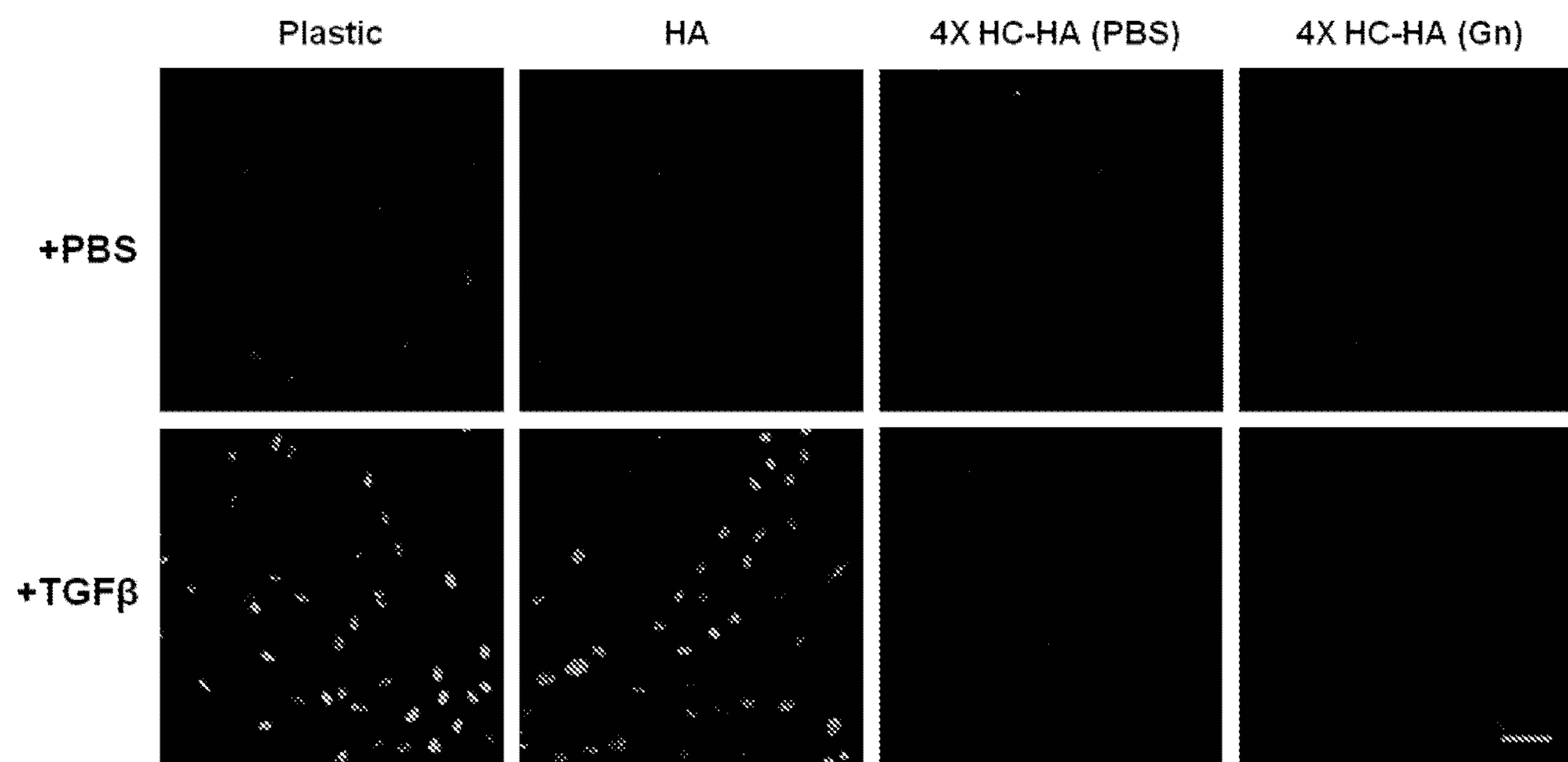


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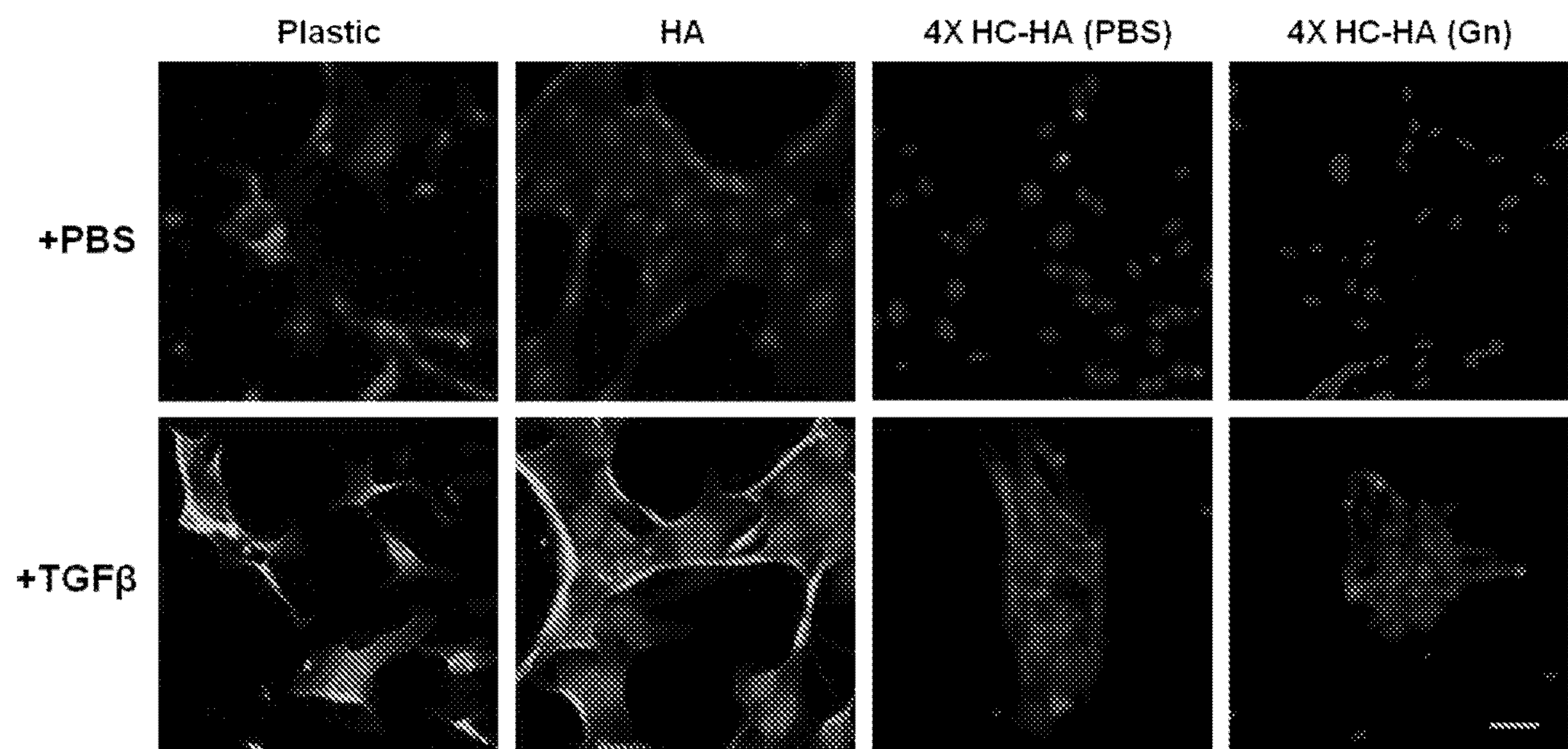
FIG. 43



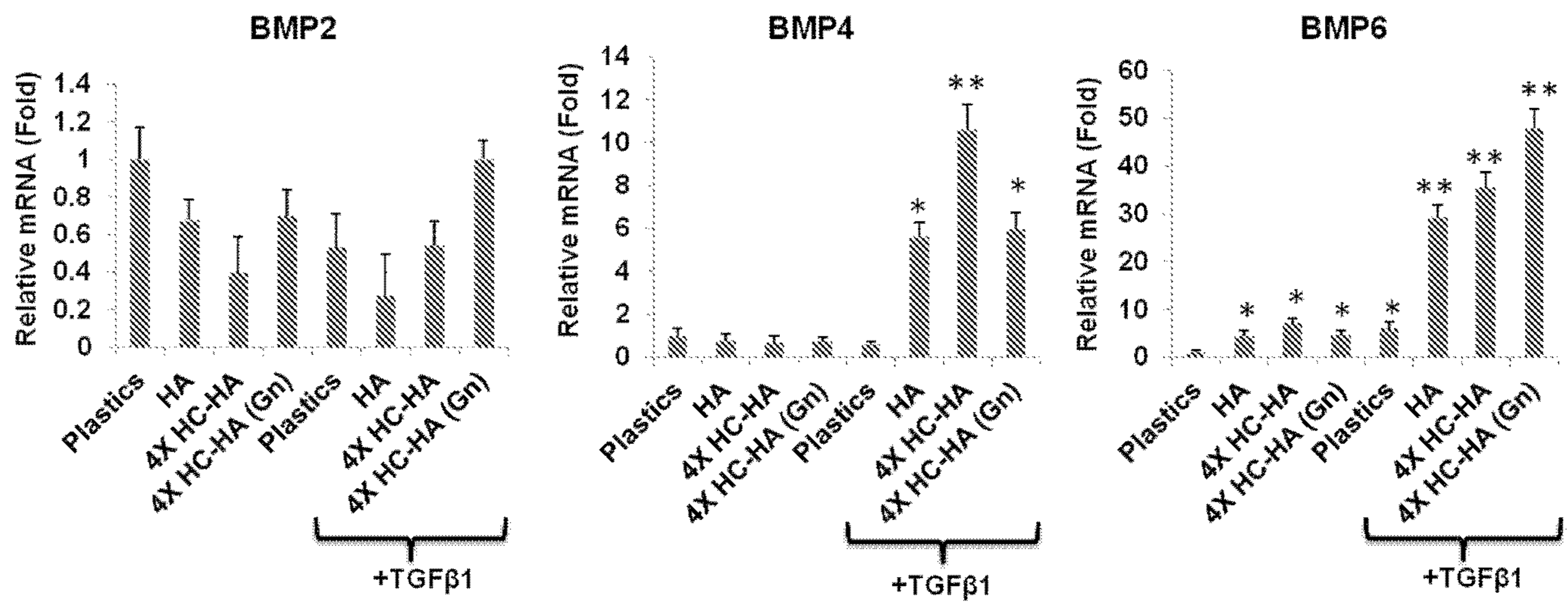
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FIG. 44

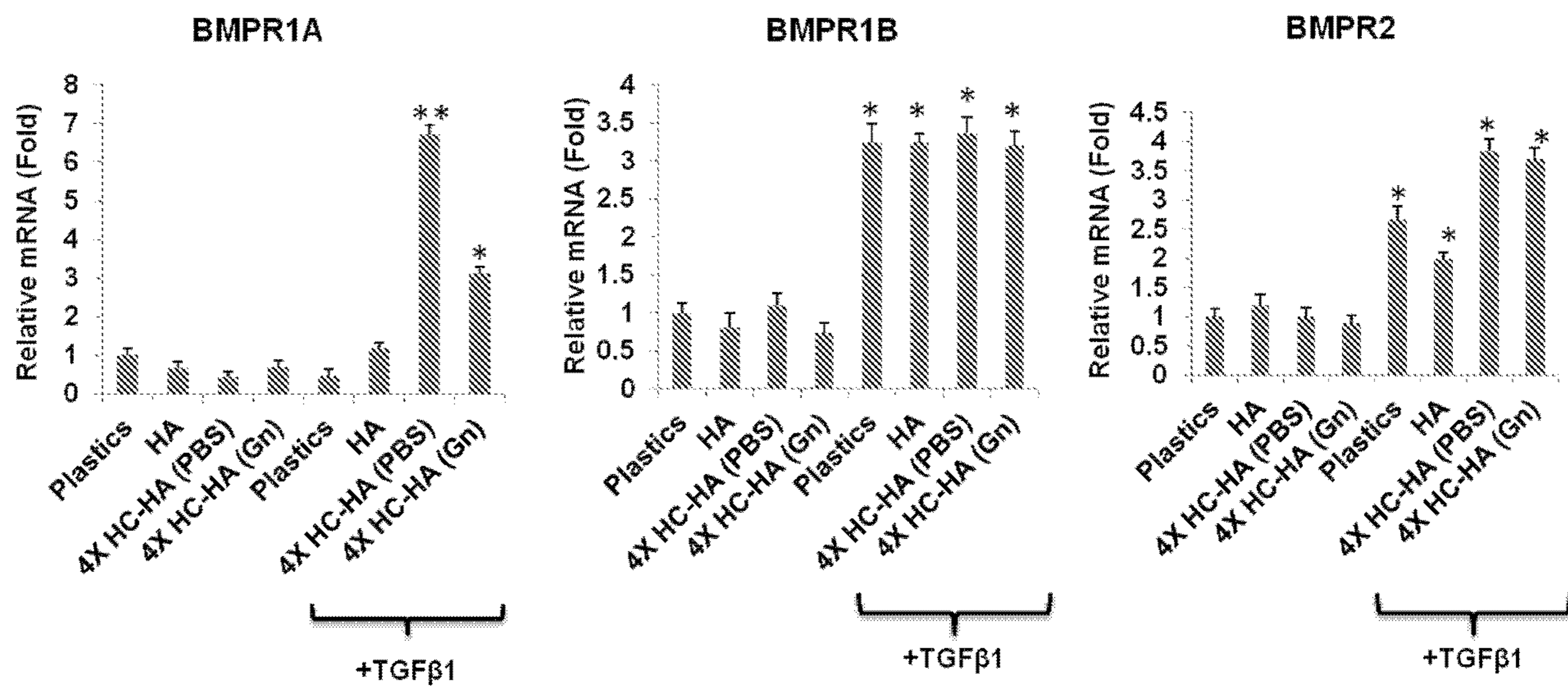
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FIG. 45

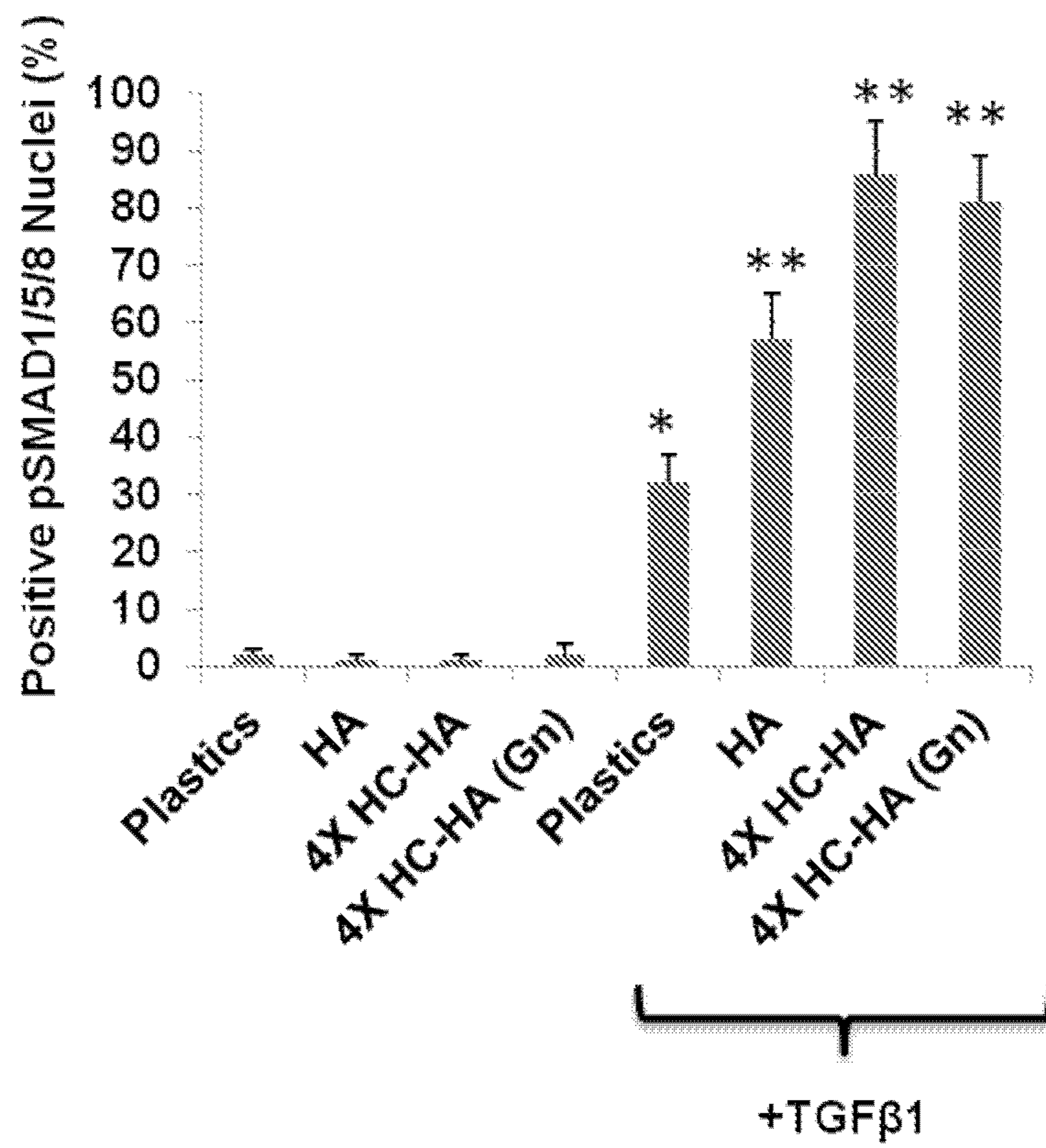
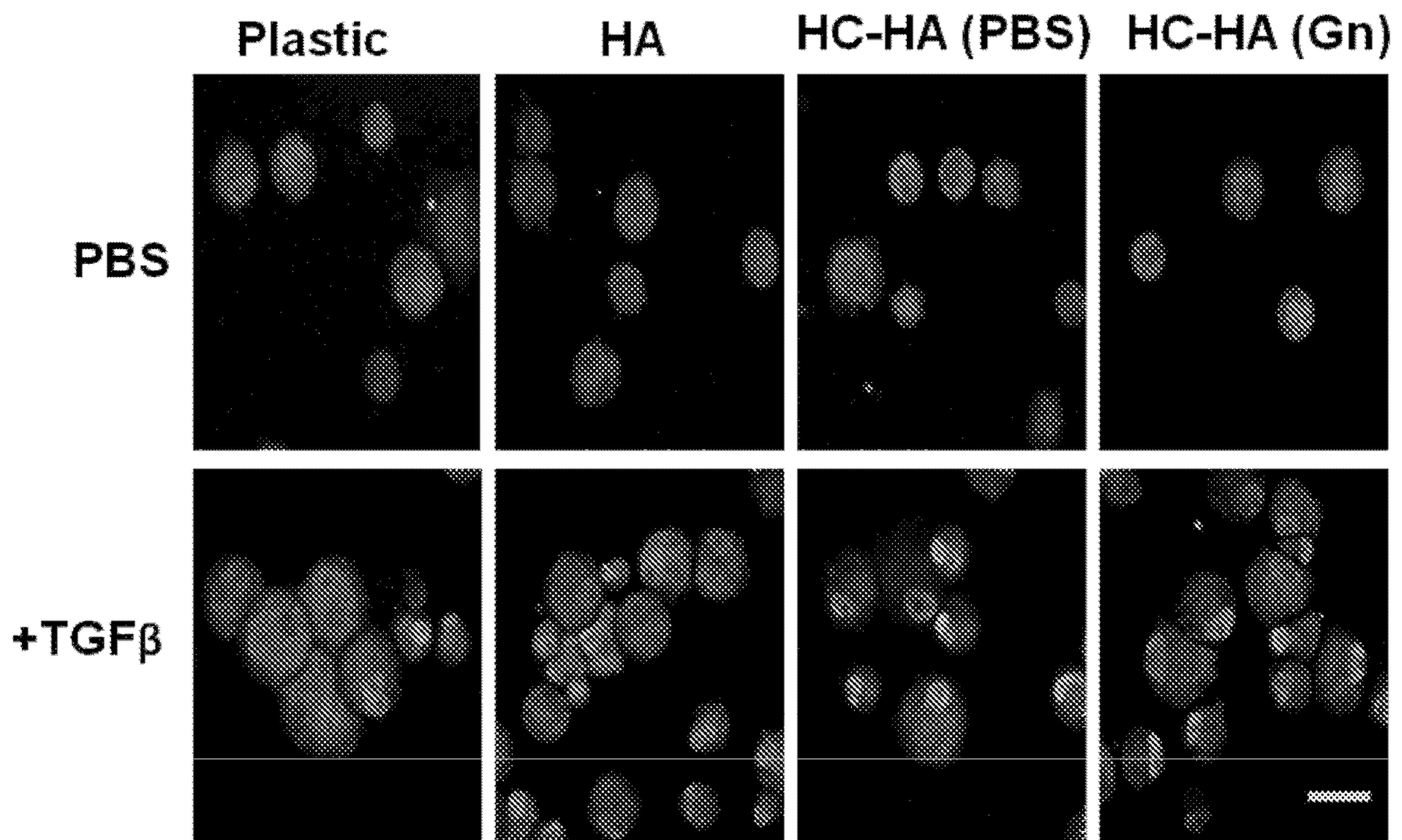
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FIG. 46

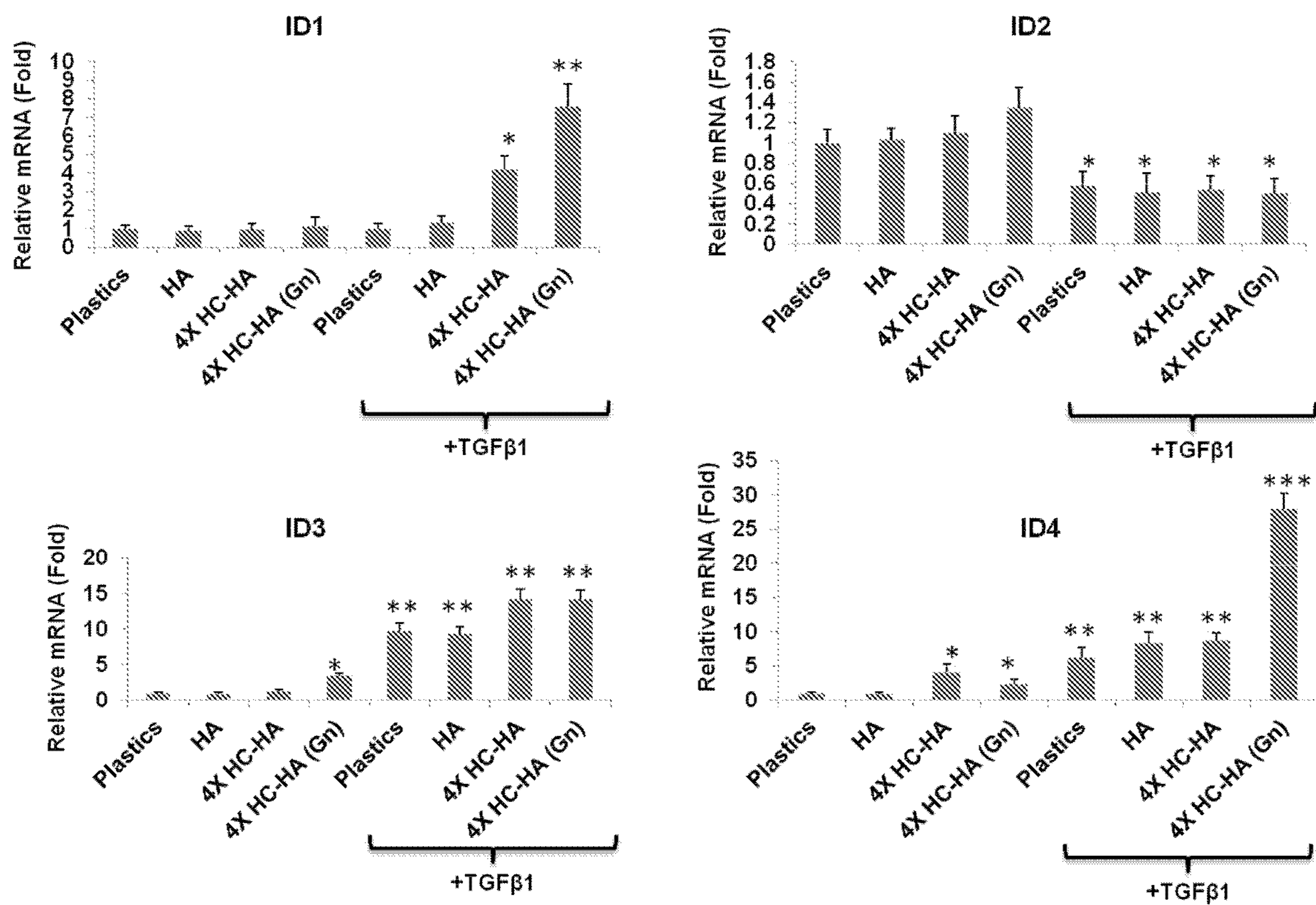
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FIG. 47

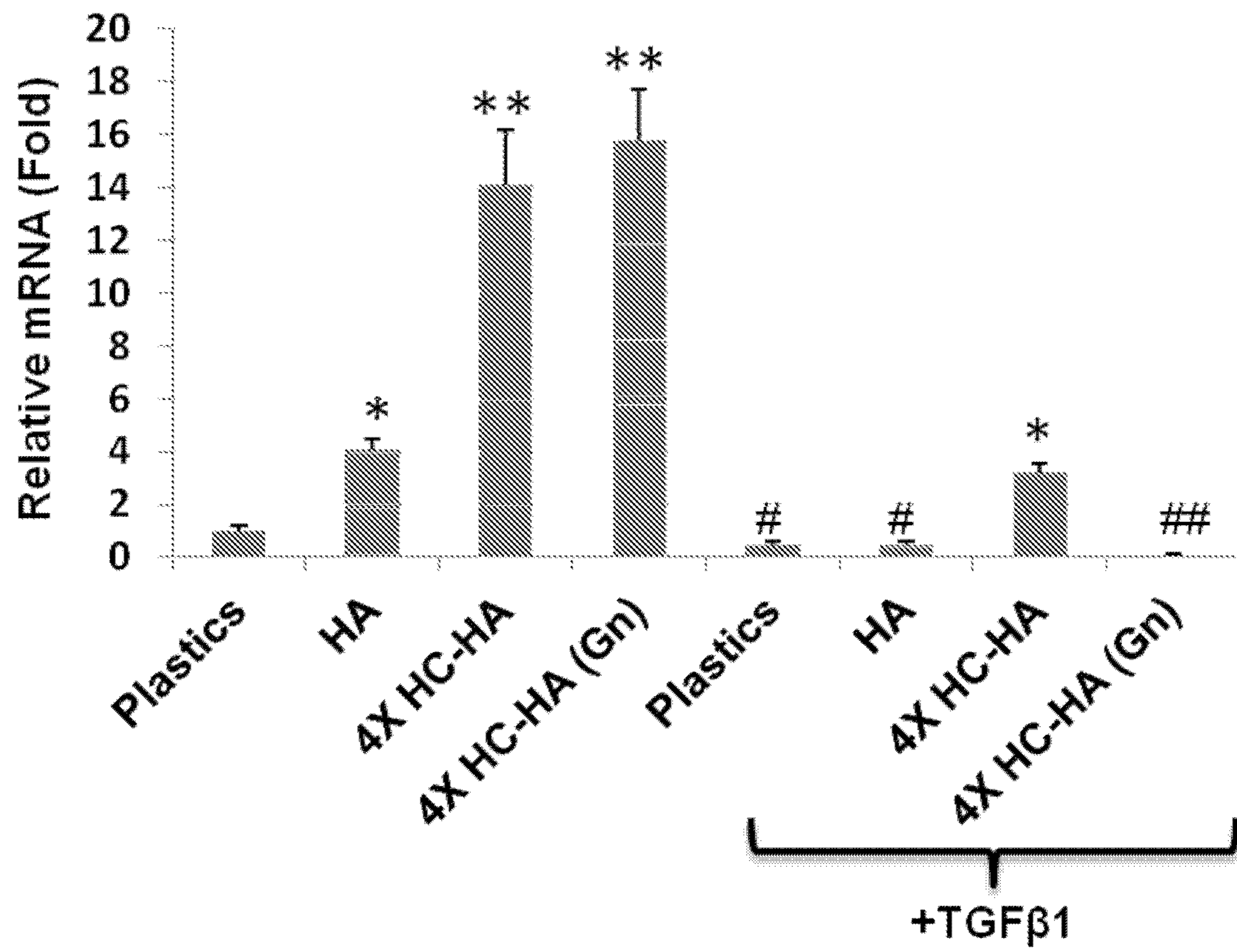
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FIG. 48

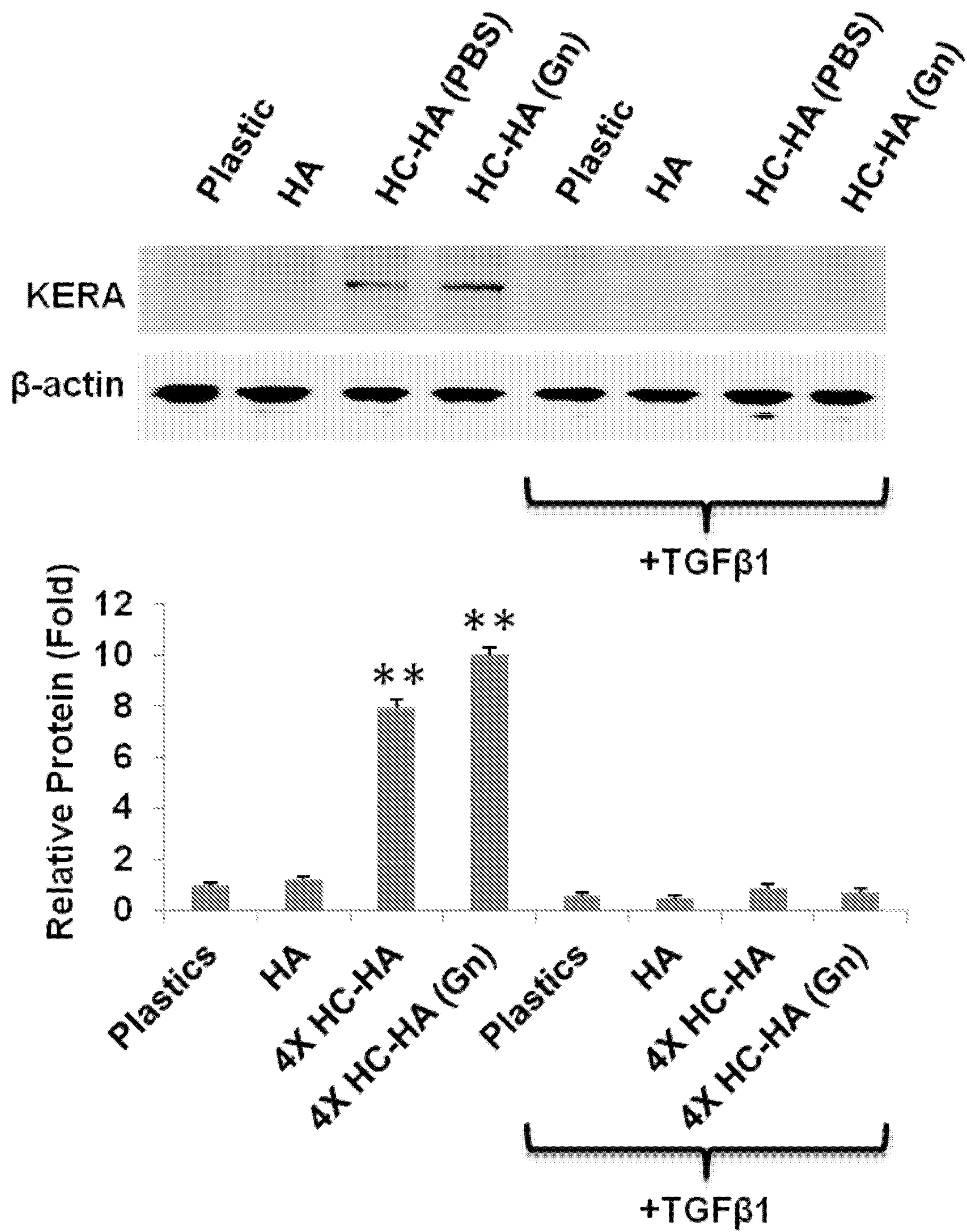
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FIG. 49

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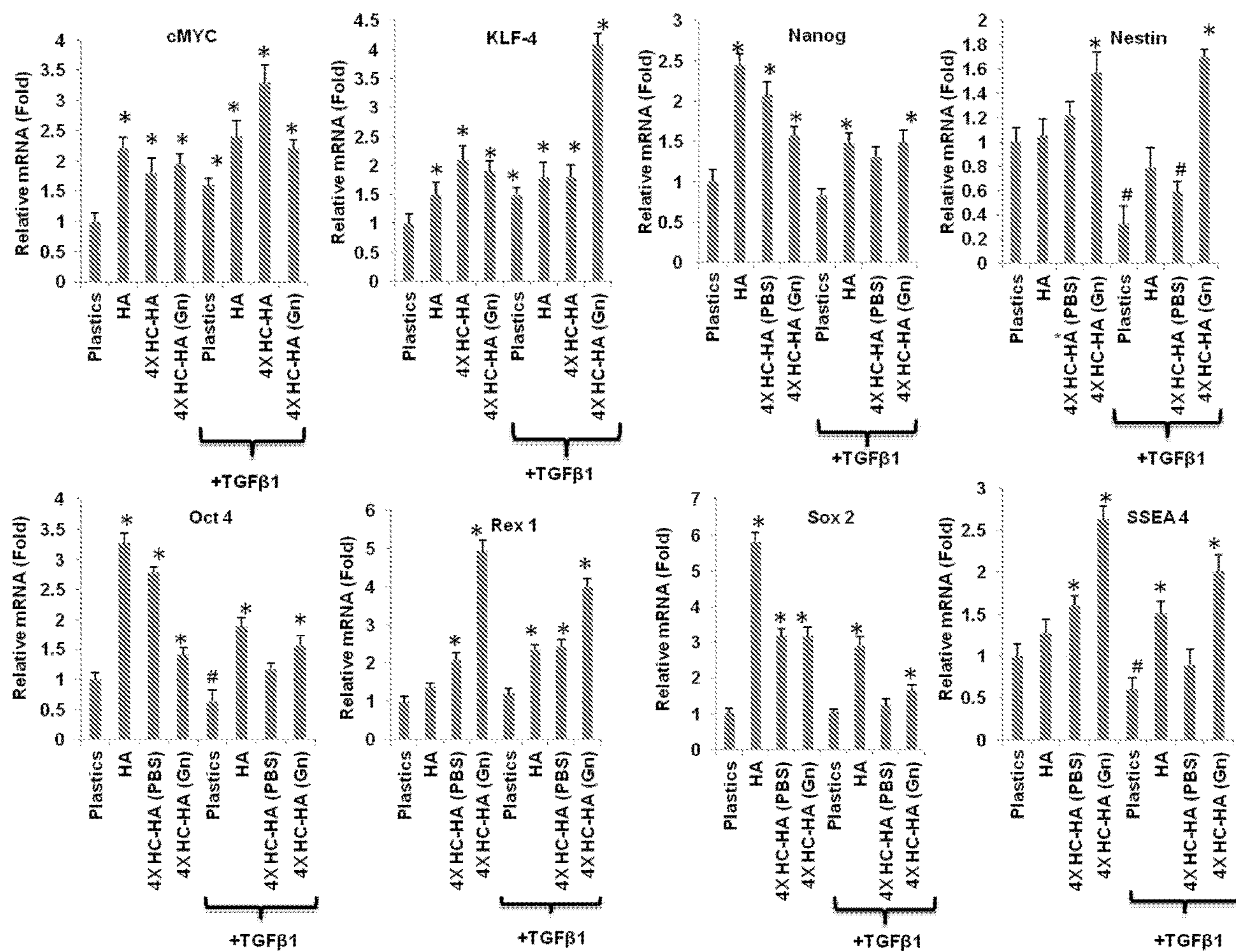
FIG. 50

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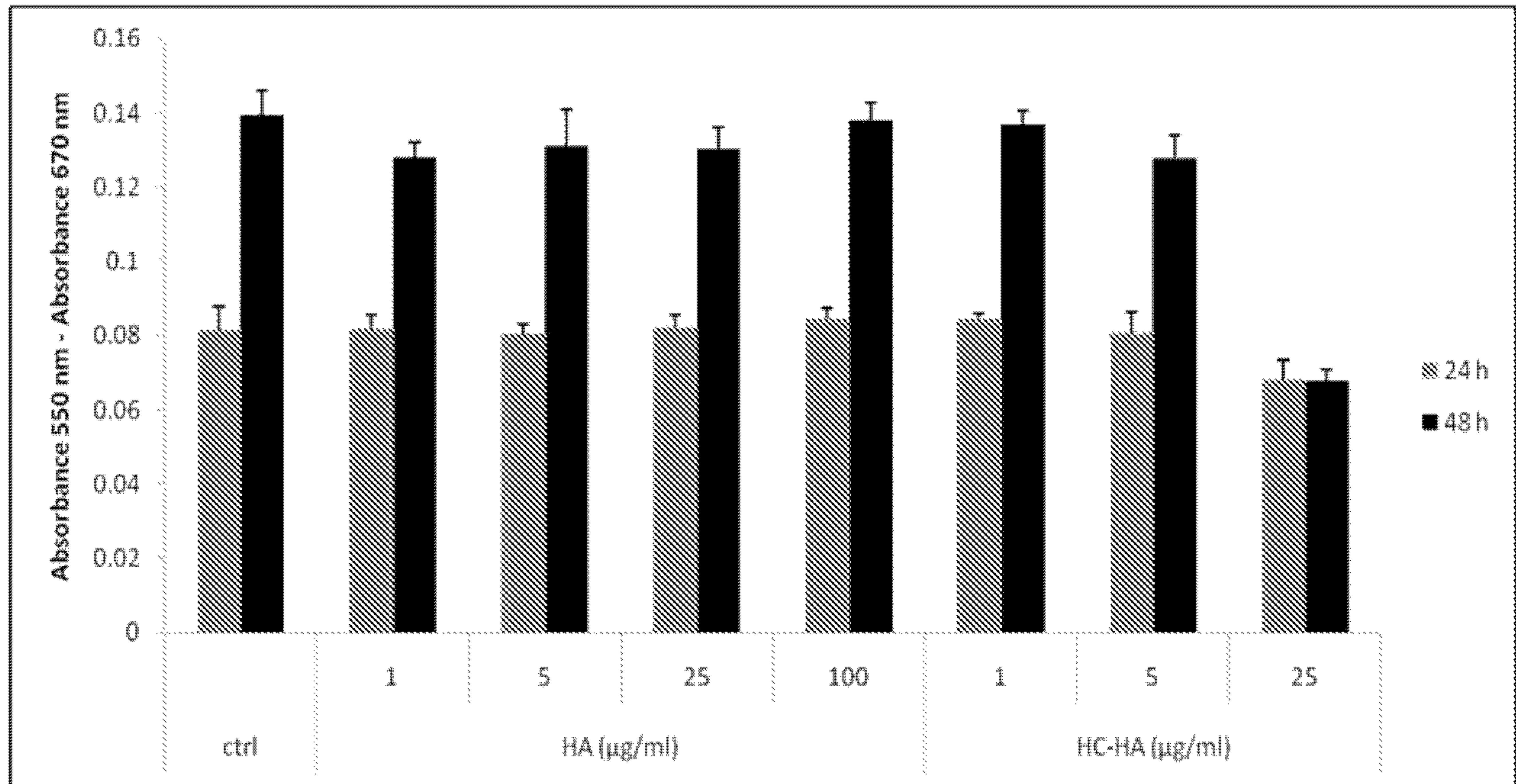
FIG. 51

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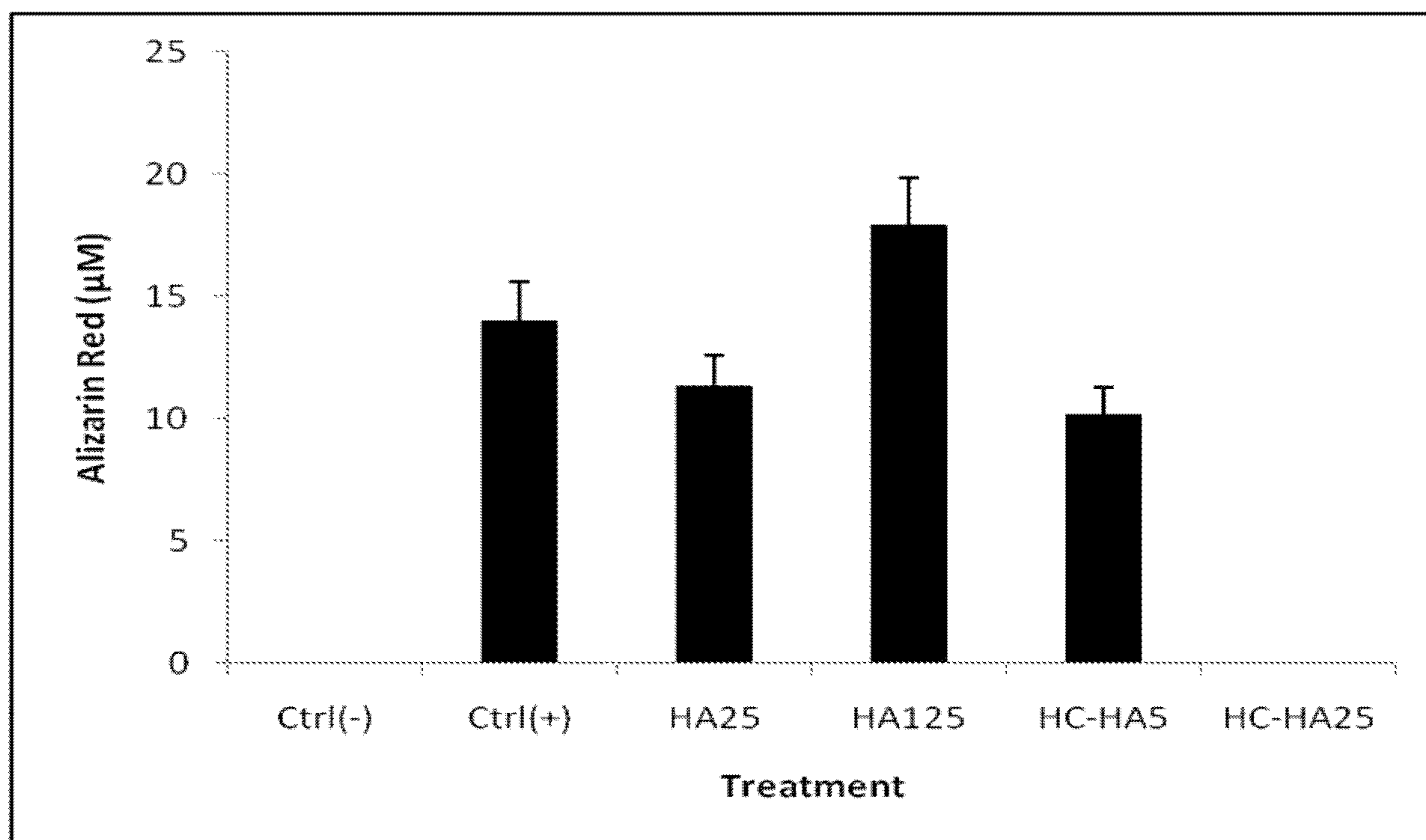
FIG. 52



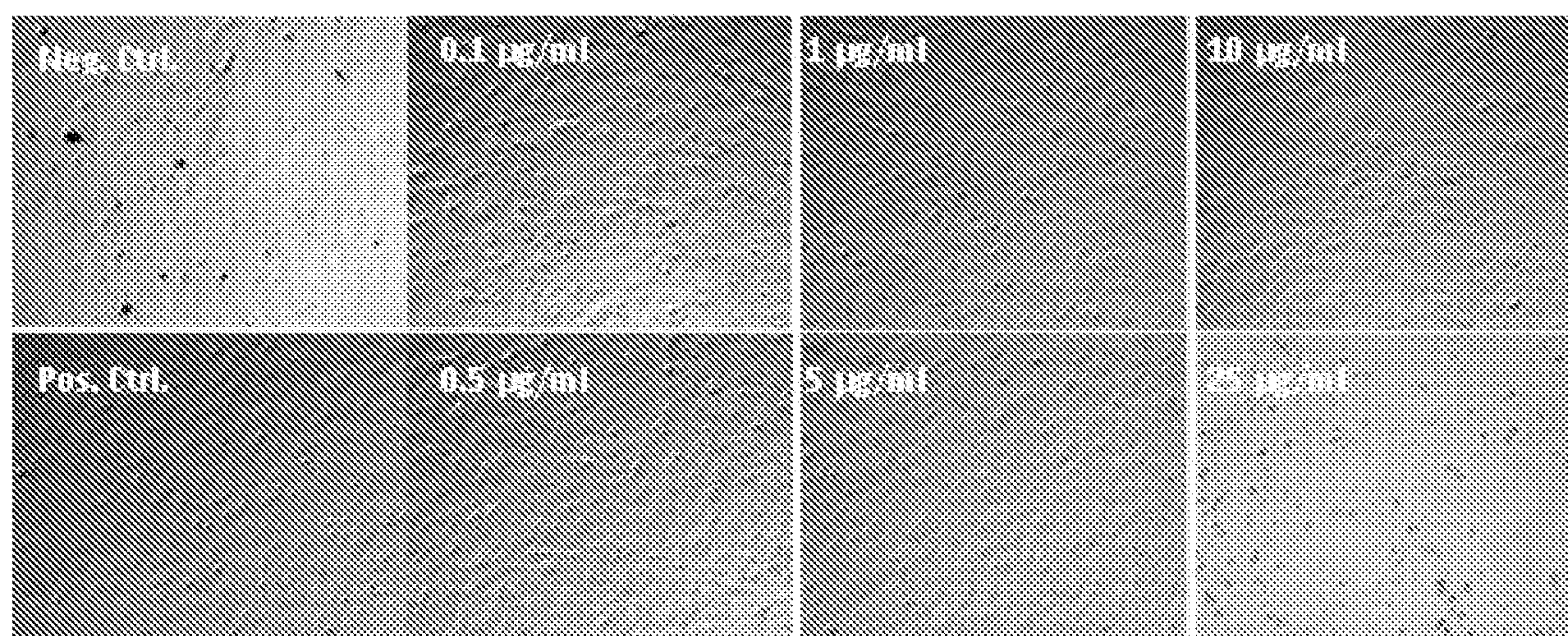
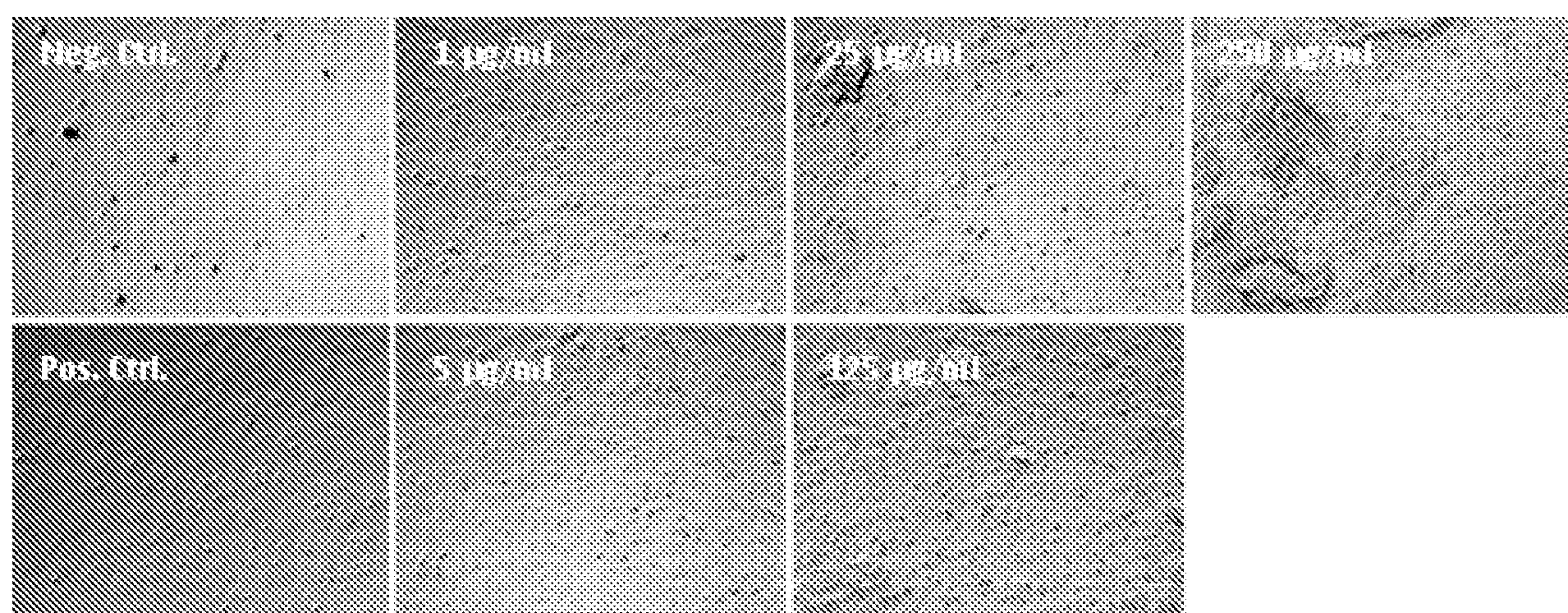
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FIG. 53

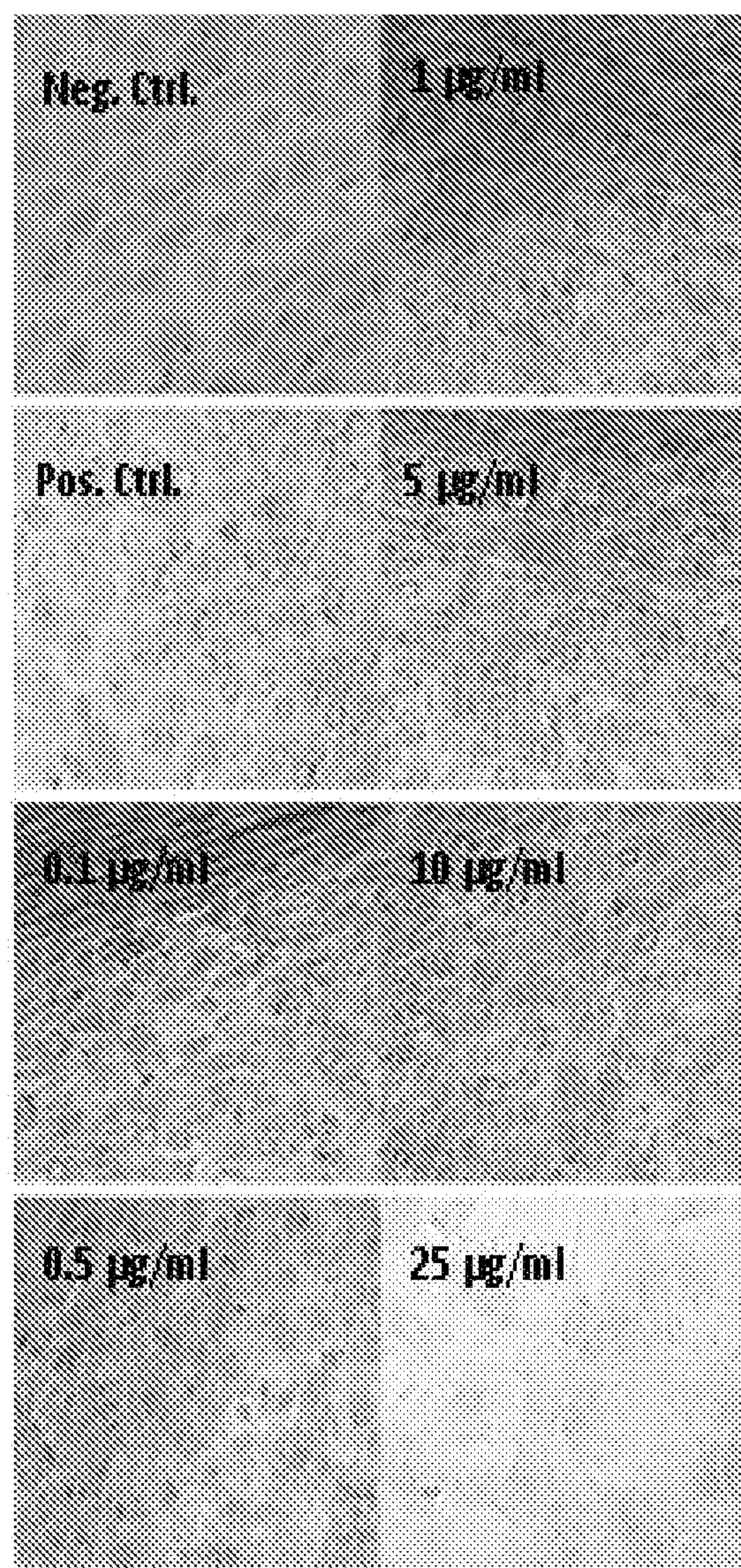
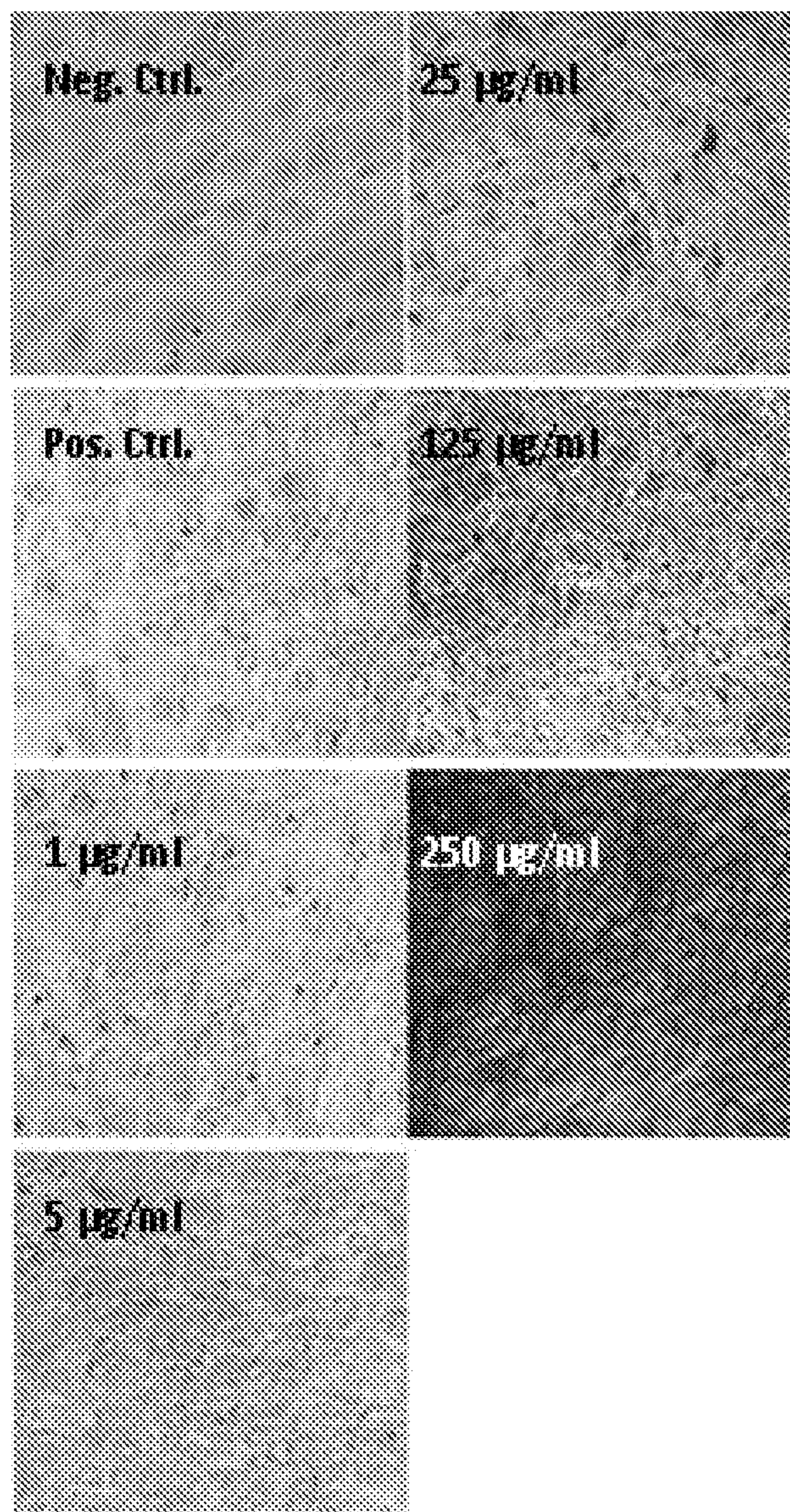
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FIG. 54

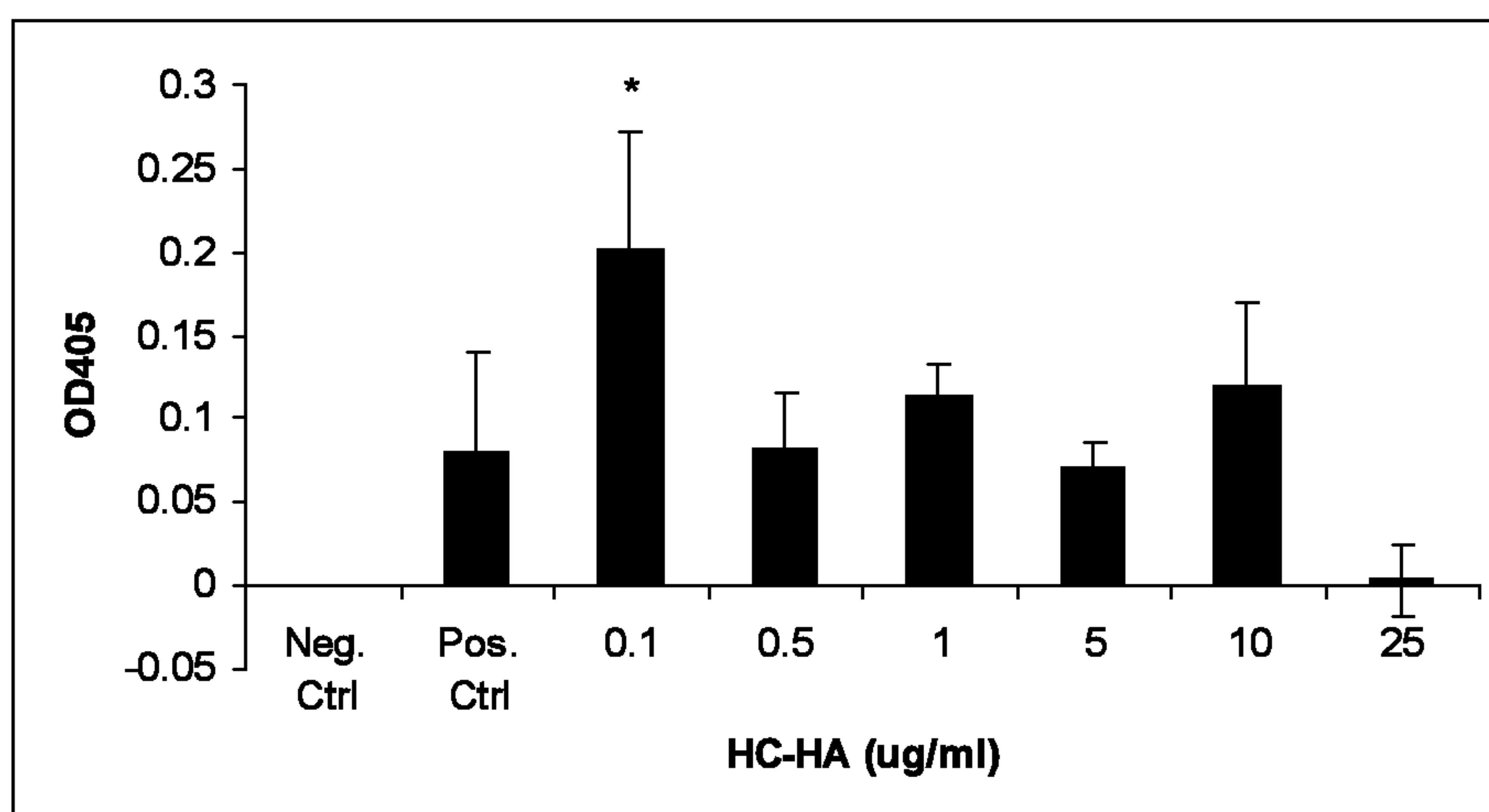
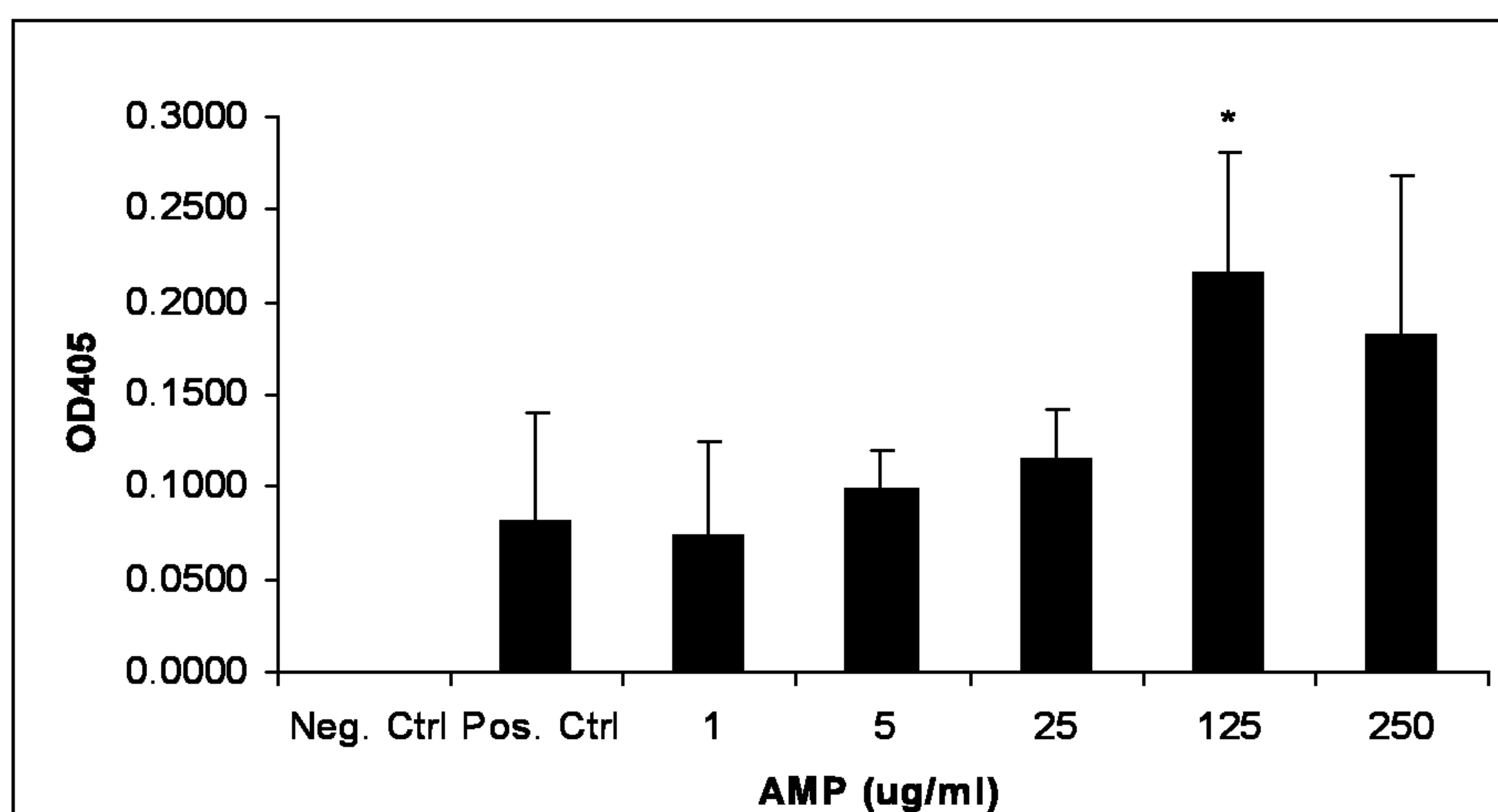
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FIG. 55**A****B**

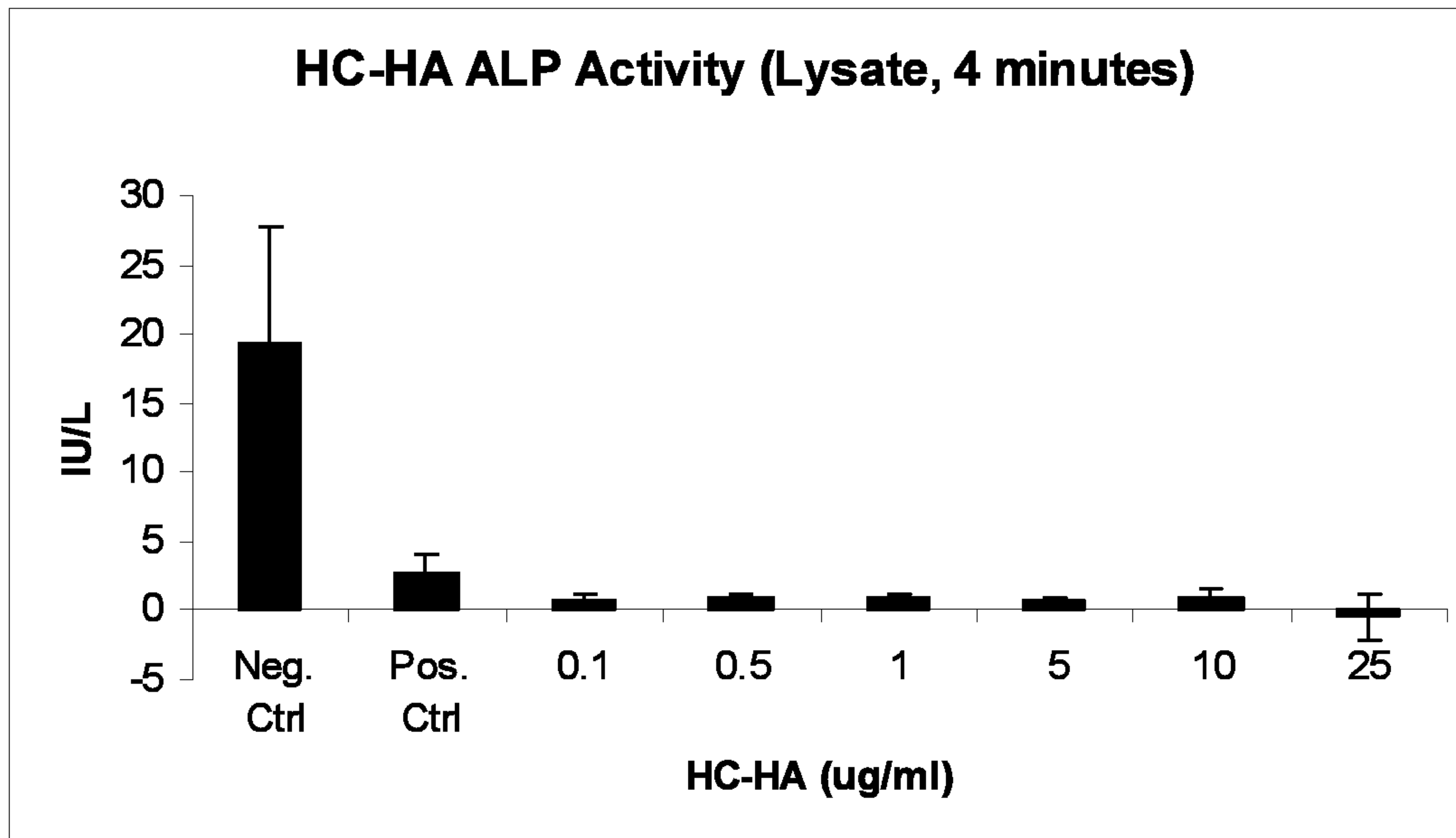
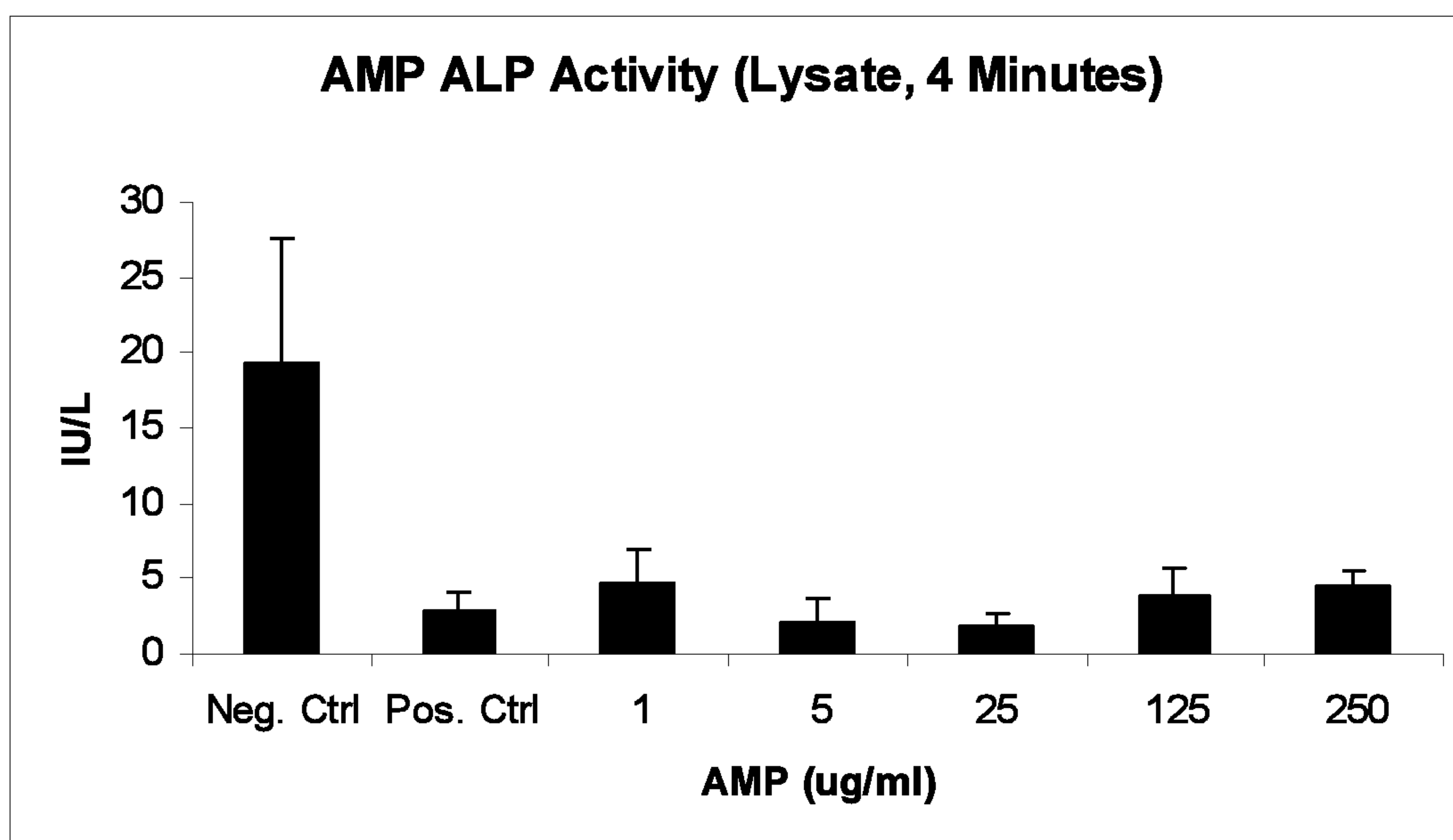
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FIG. 56**A. HC-HA****B. AMP**

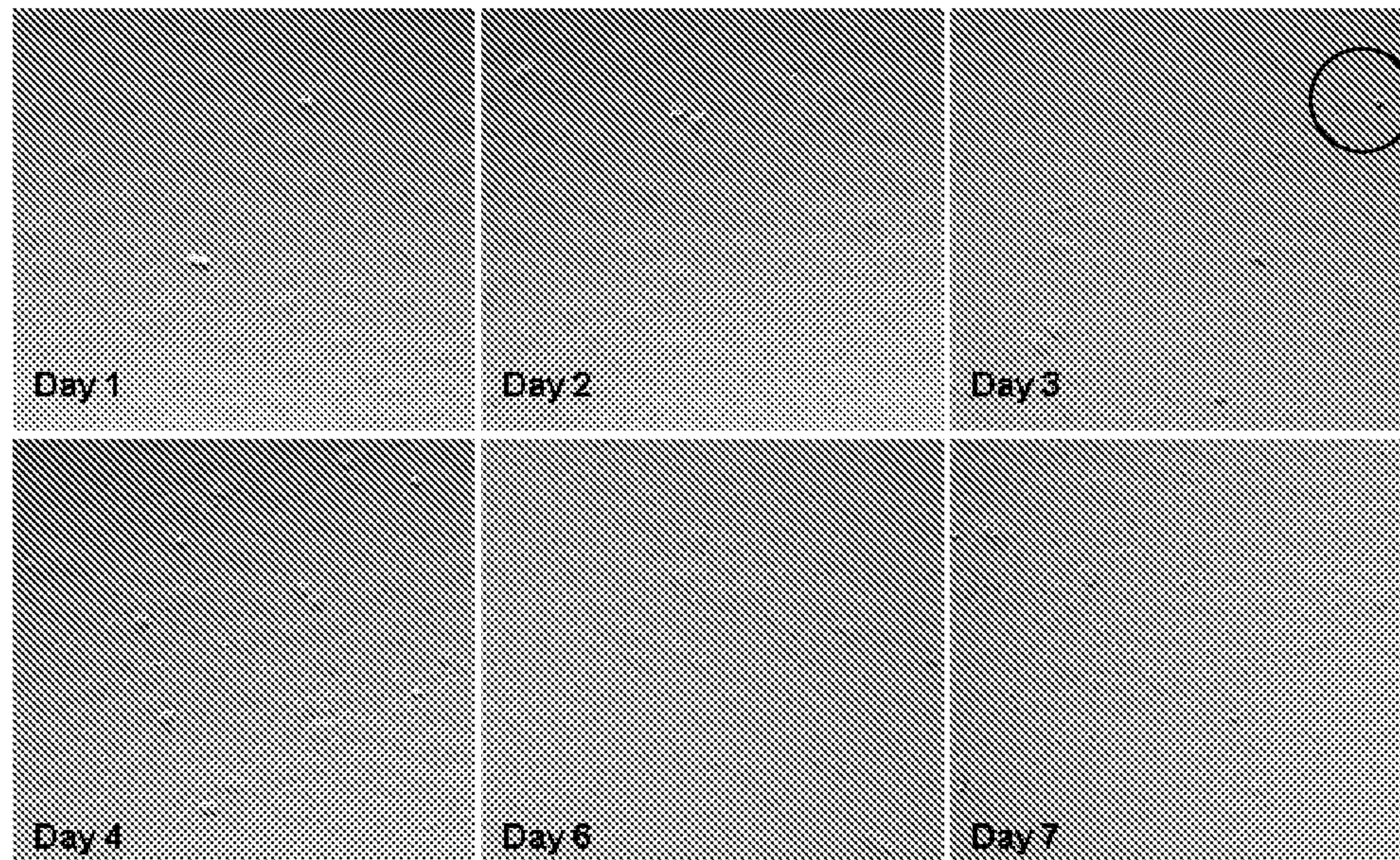
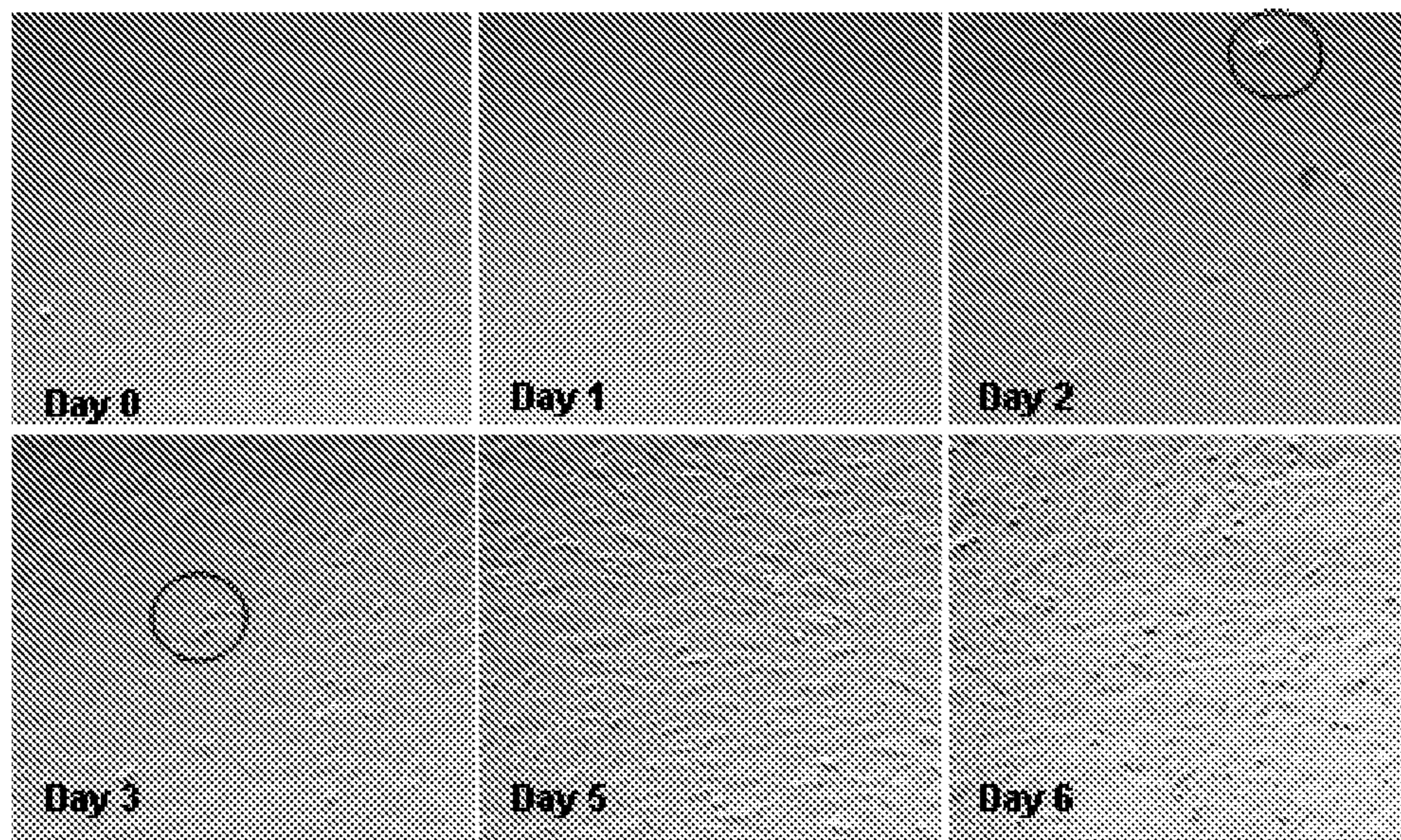
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FIG. 57**A. HC-HA****B. AMP**

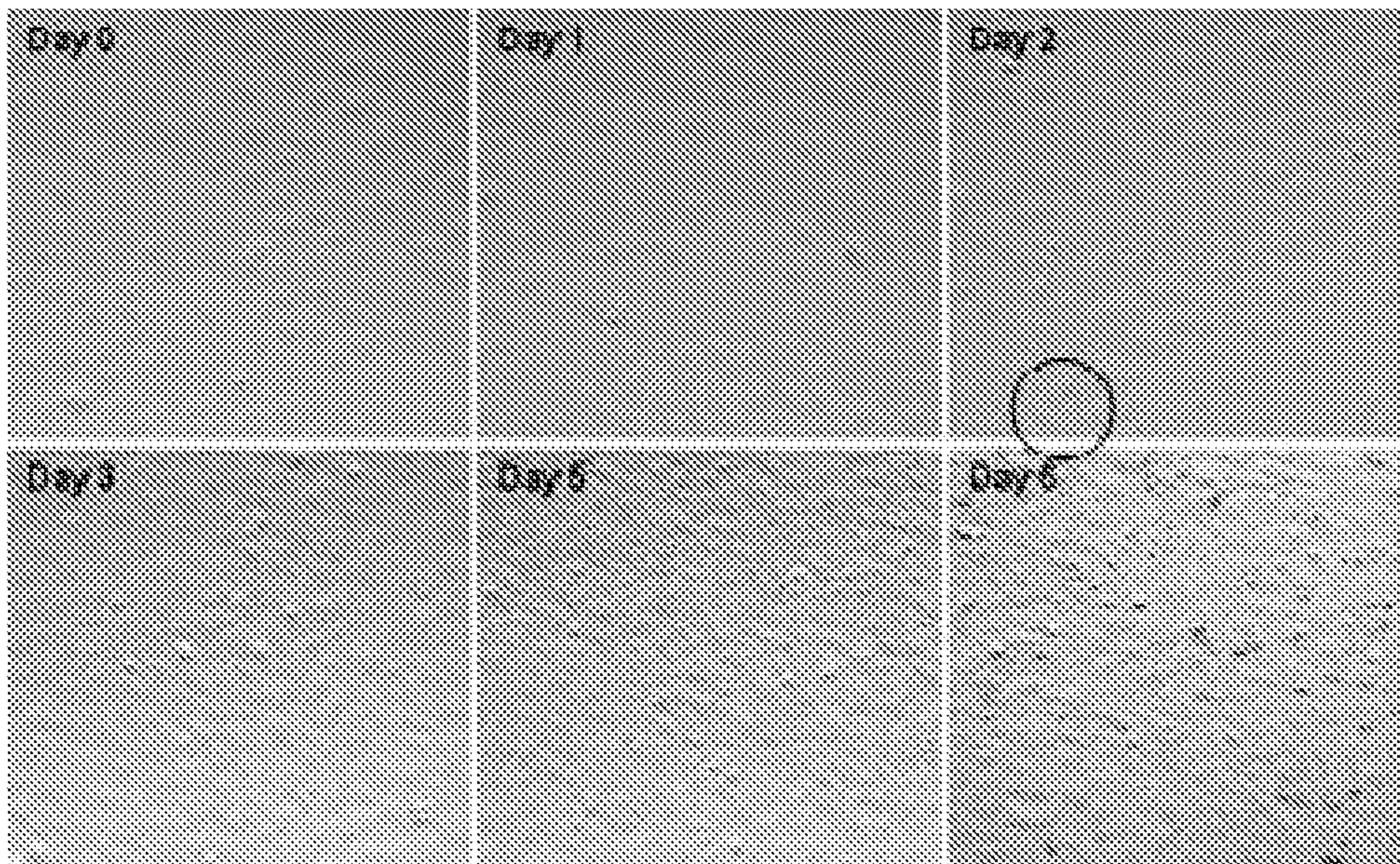
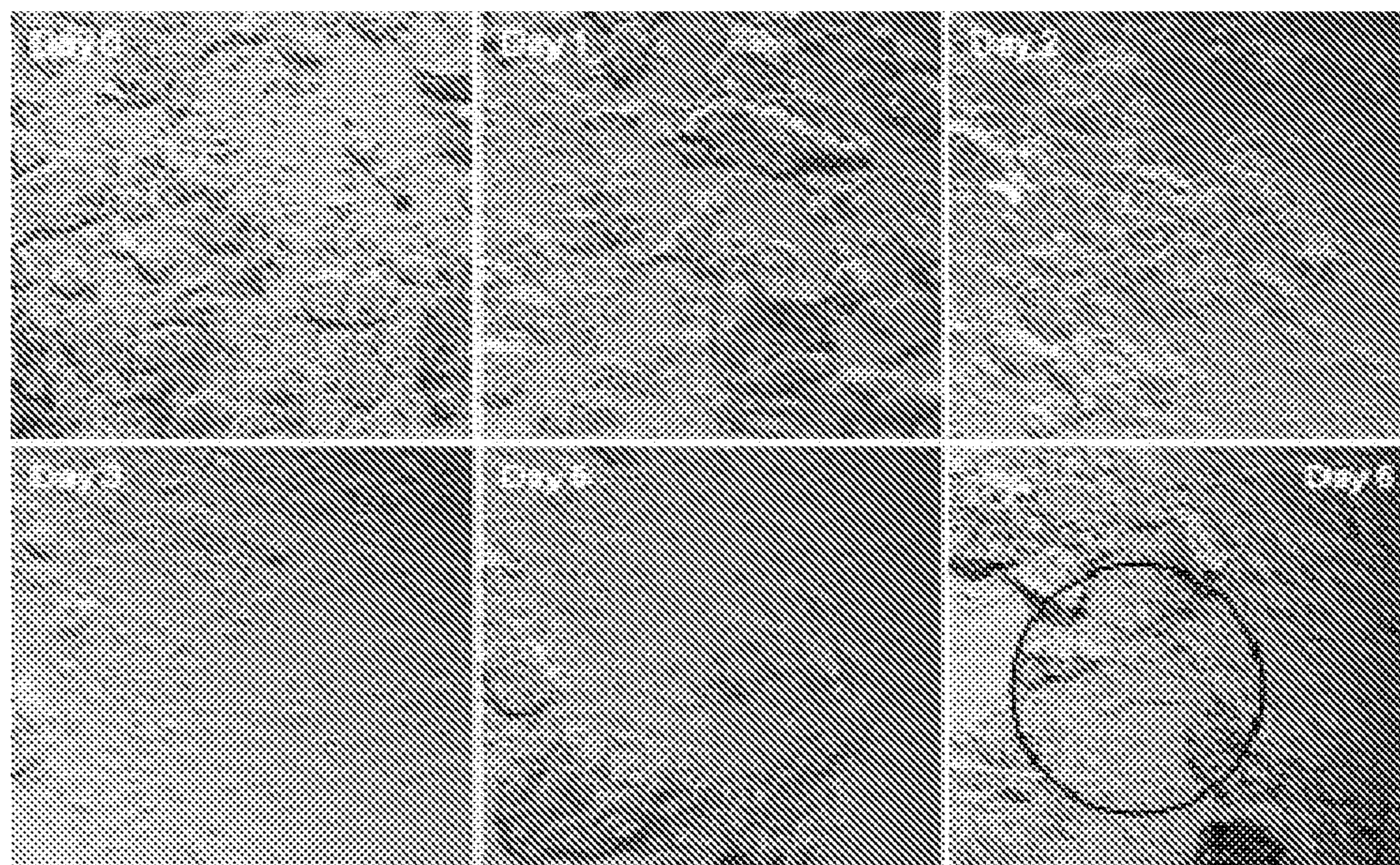
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FIG. 58**A. HC-HA****B. AMP**

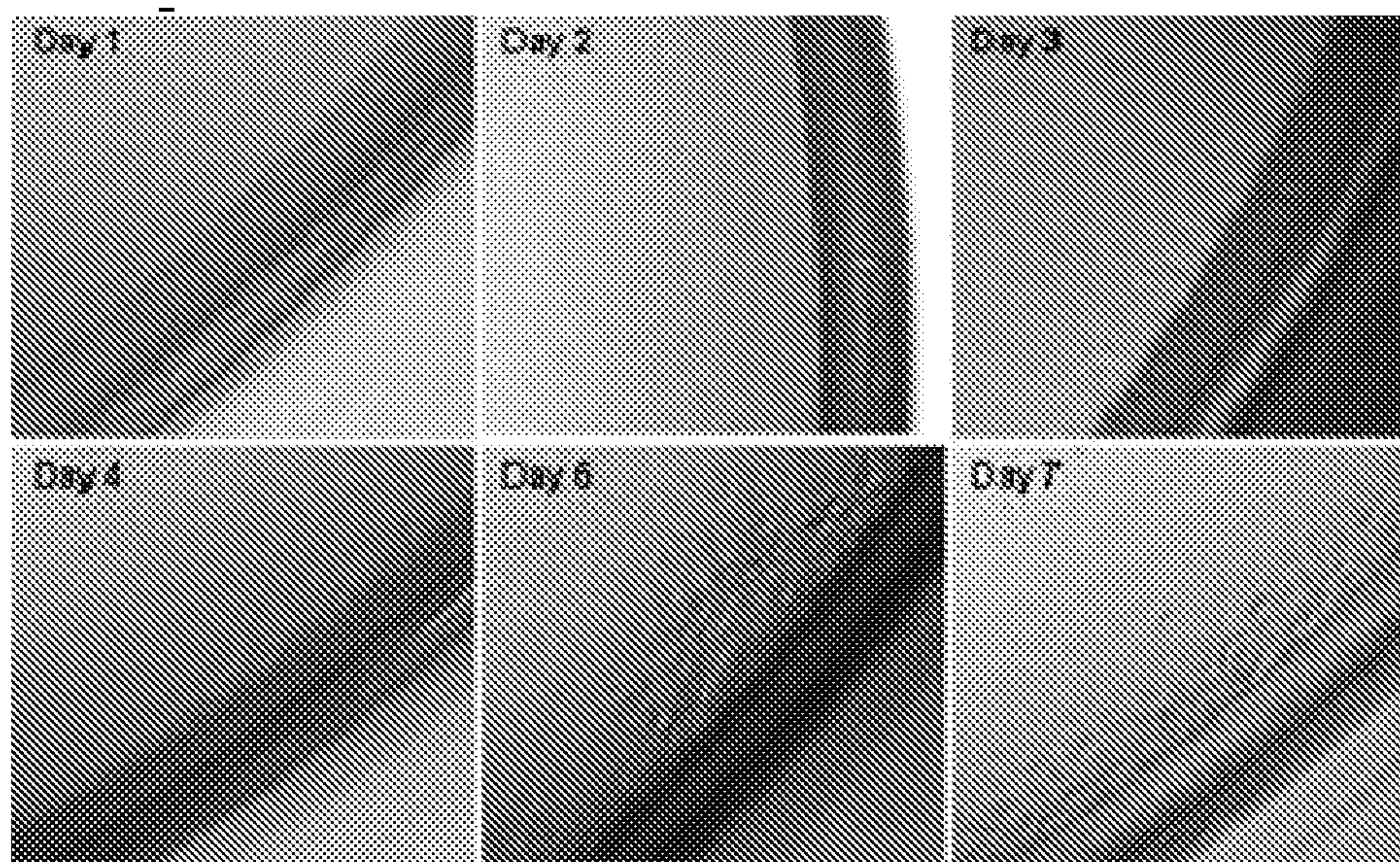
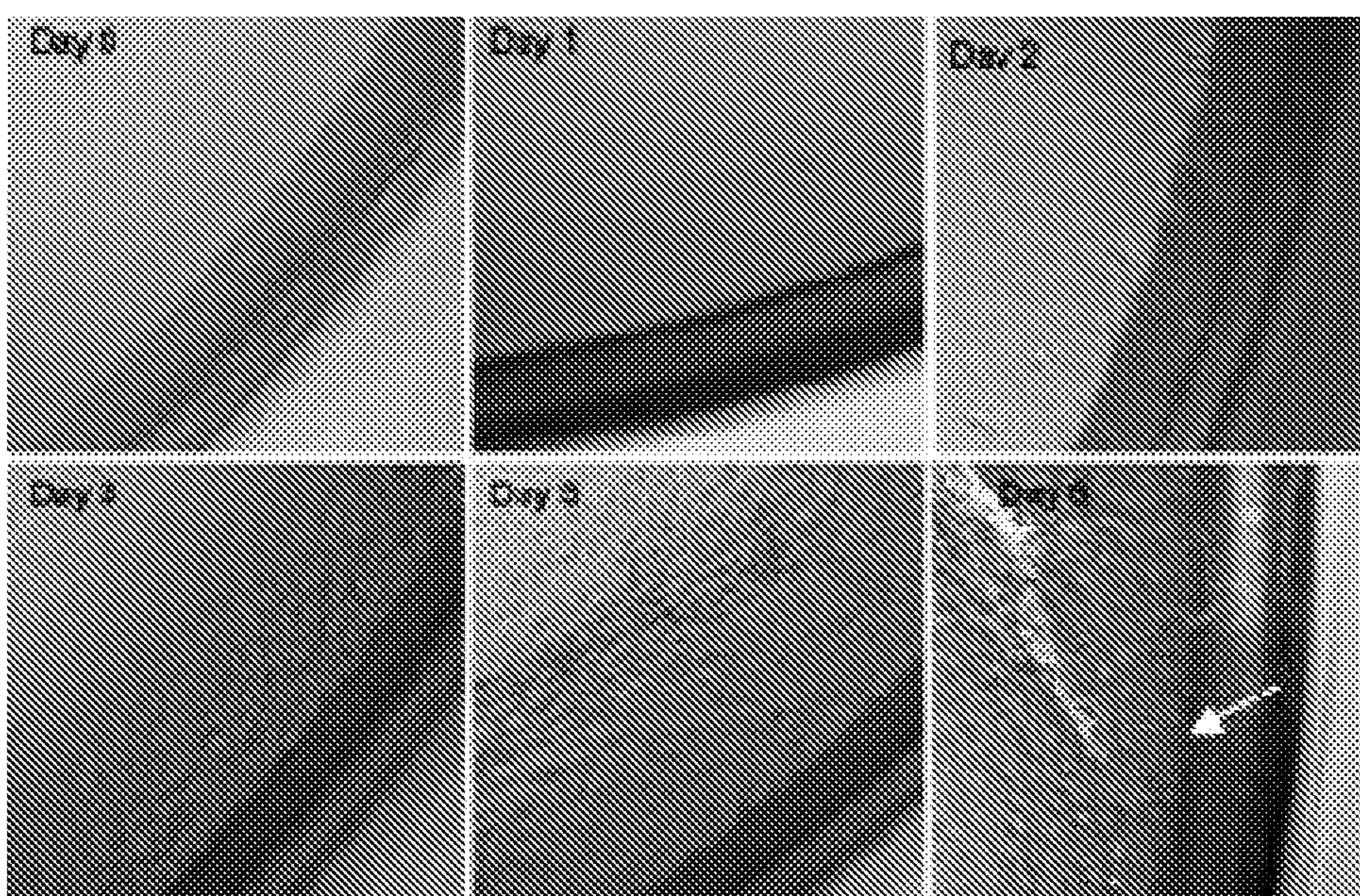
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FIG. 59**A. Negative Control****B. Positive Control**

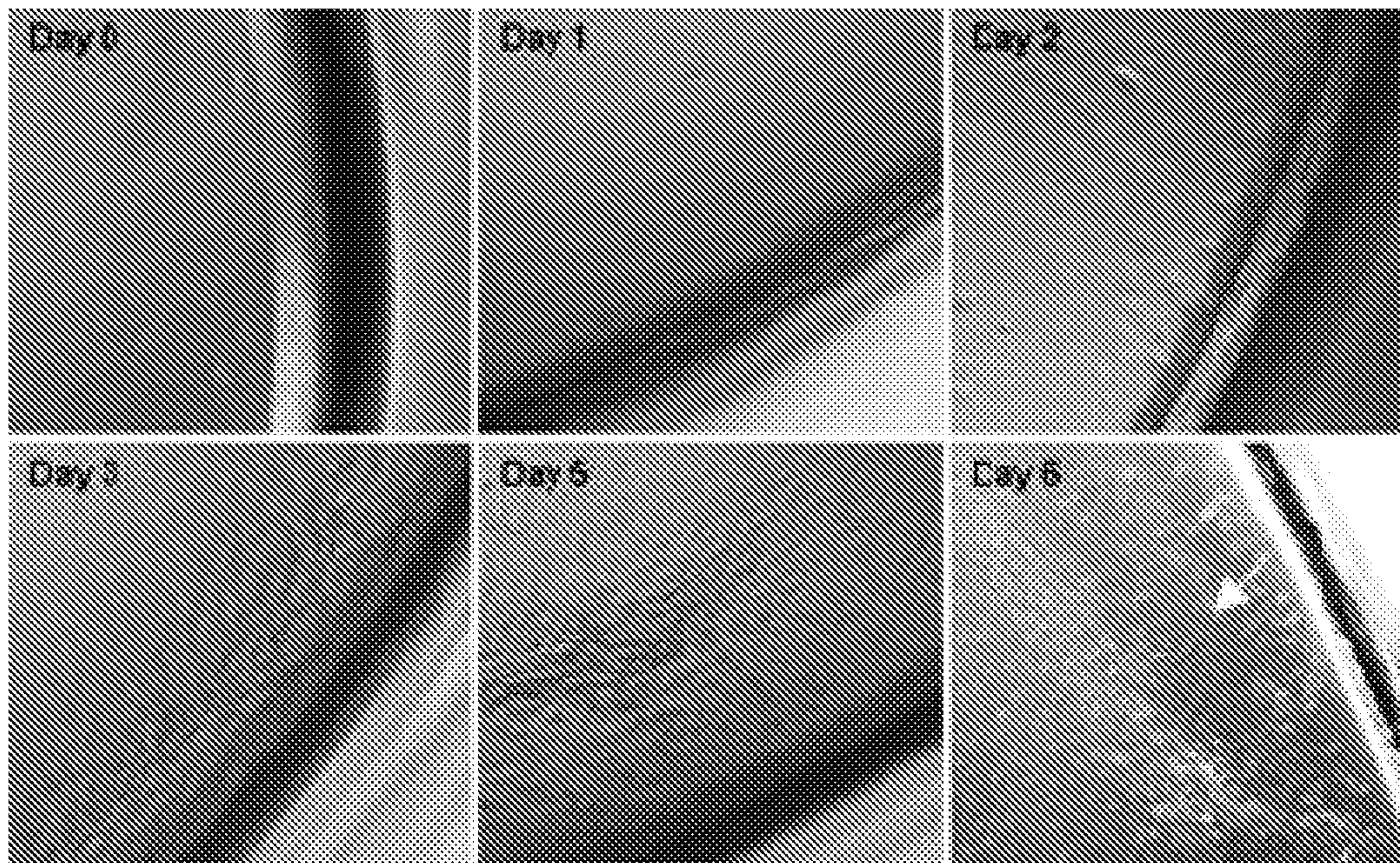
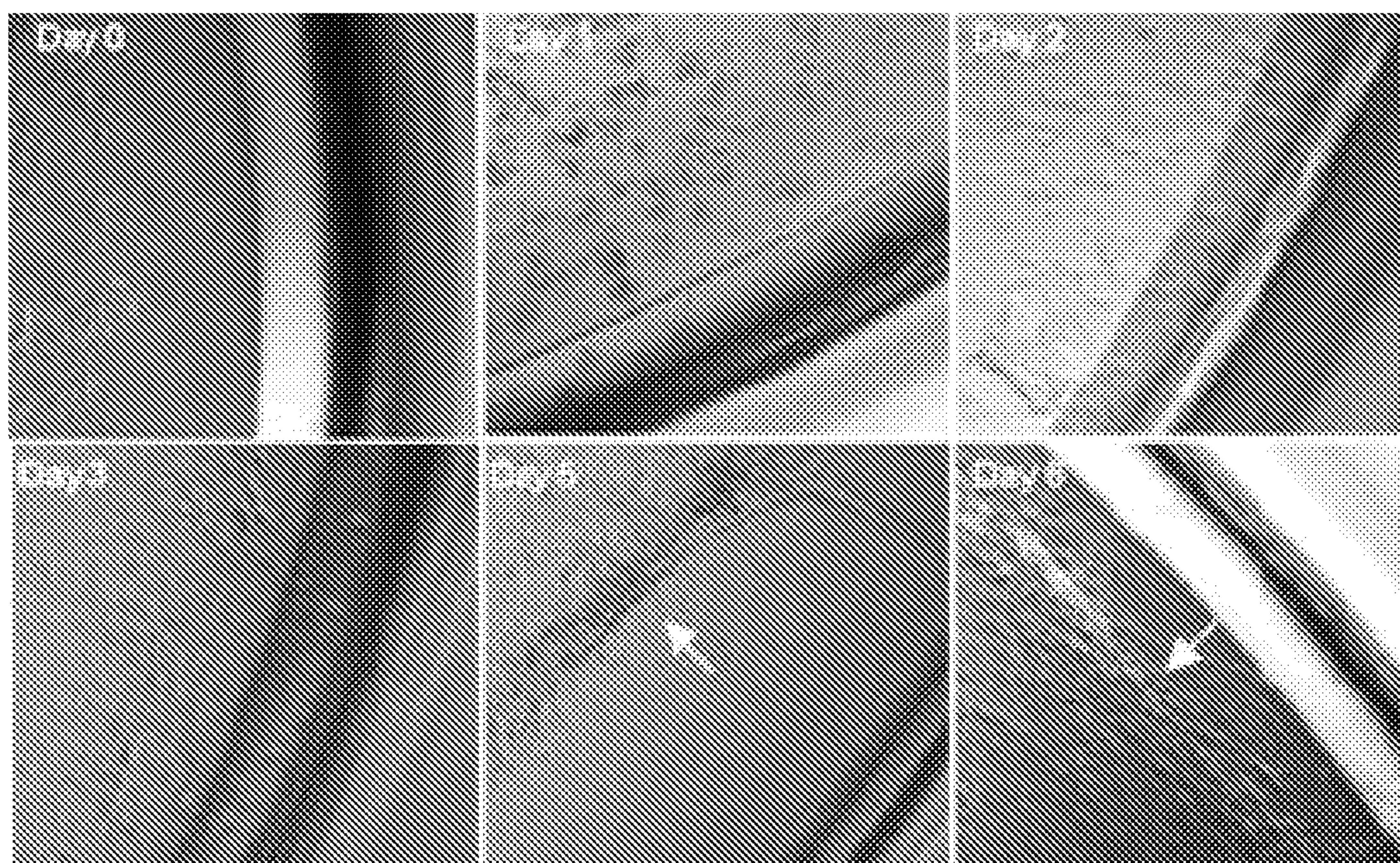
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FIG. 60**A. HC-HA****B. AMP**

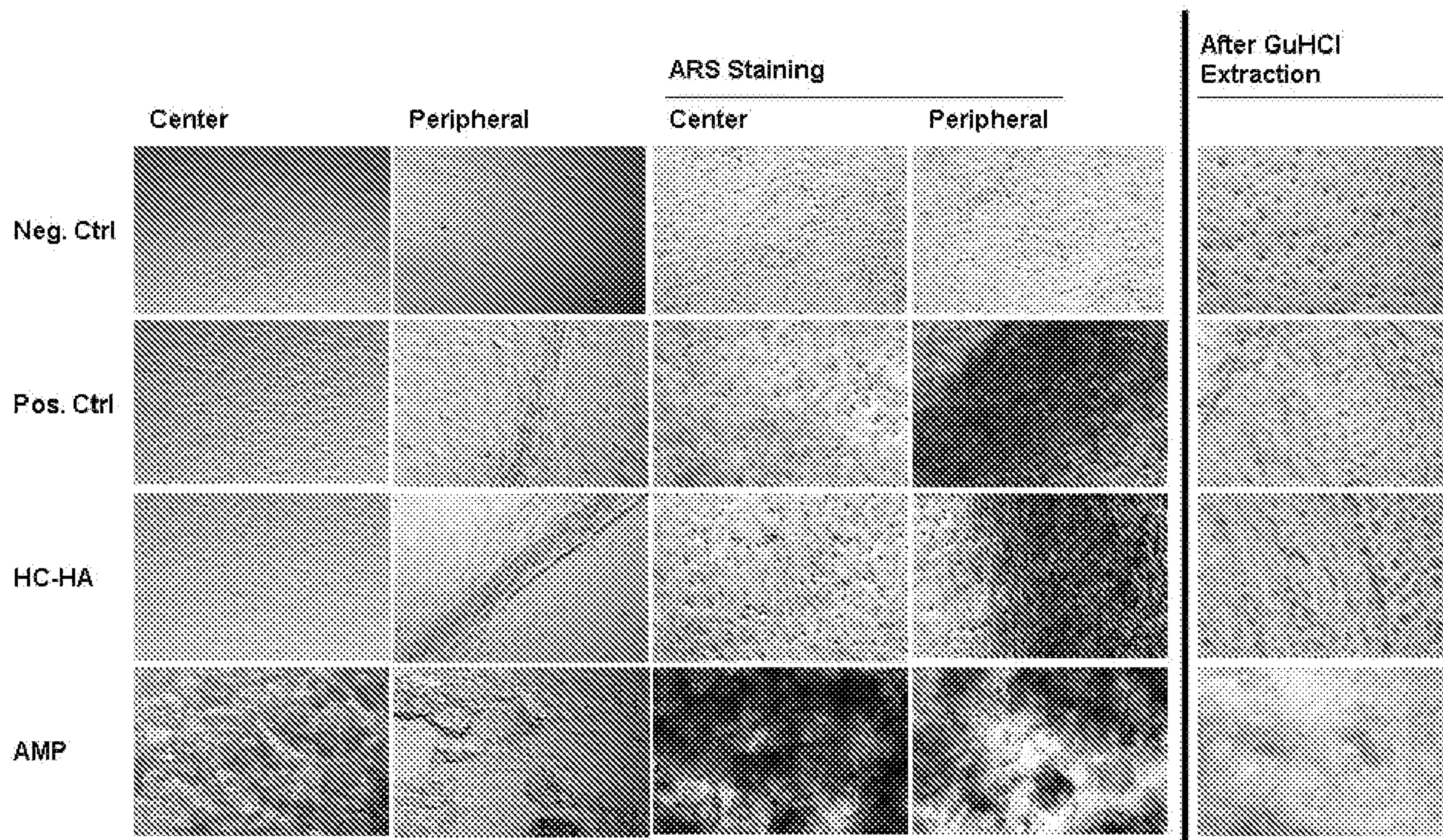
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FIG. 61**A. Negative Control****B. Positive Control**

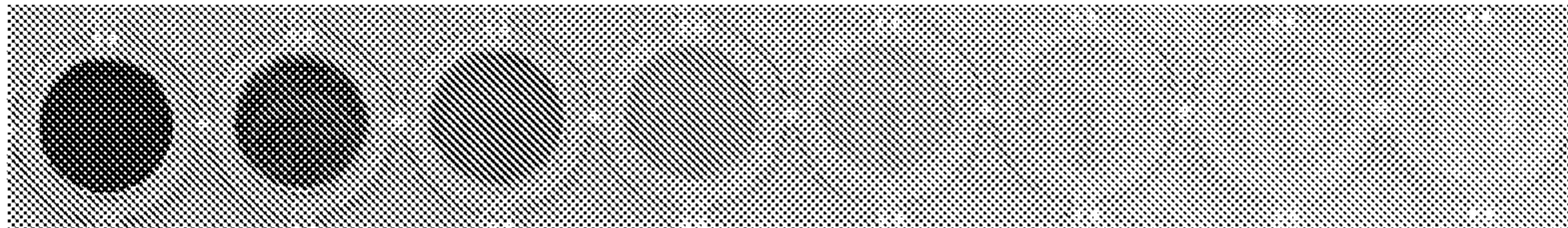
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FIG. 62**A. HC-HA****B. AMP**

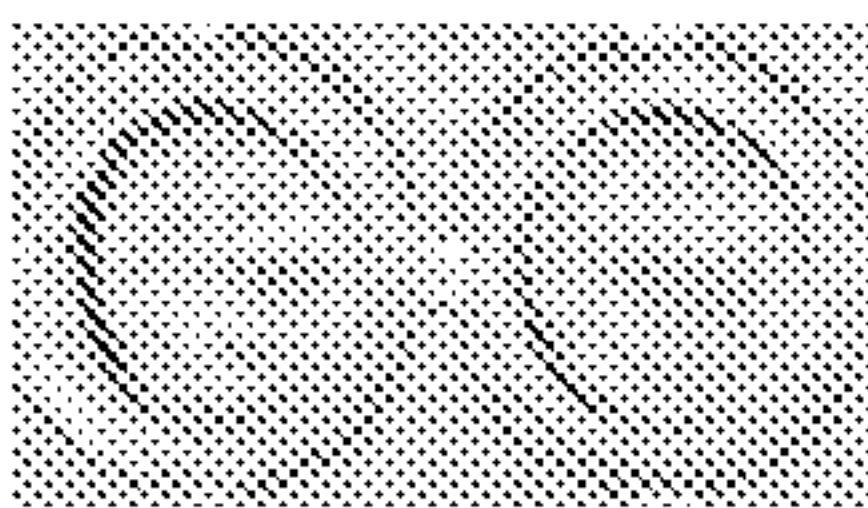
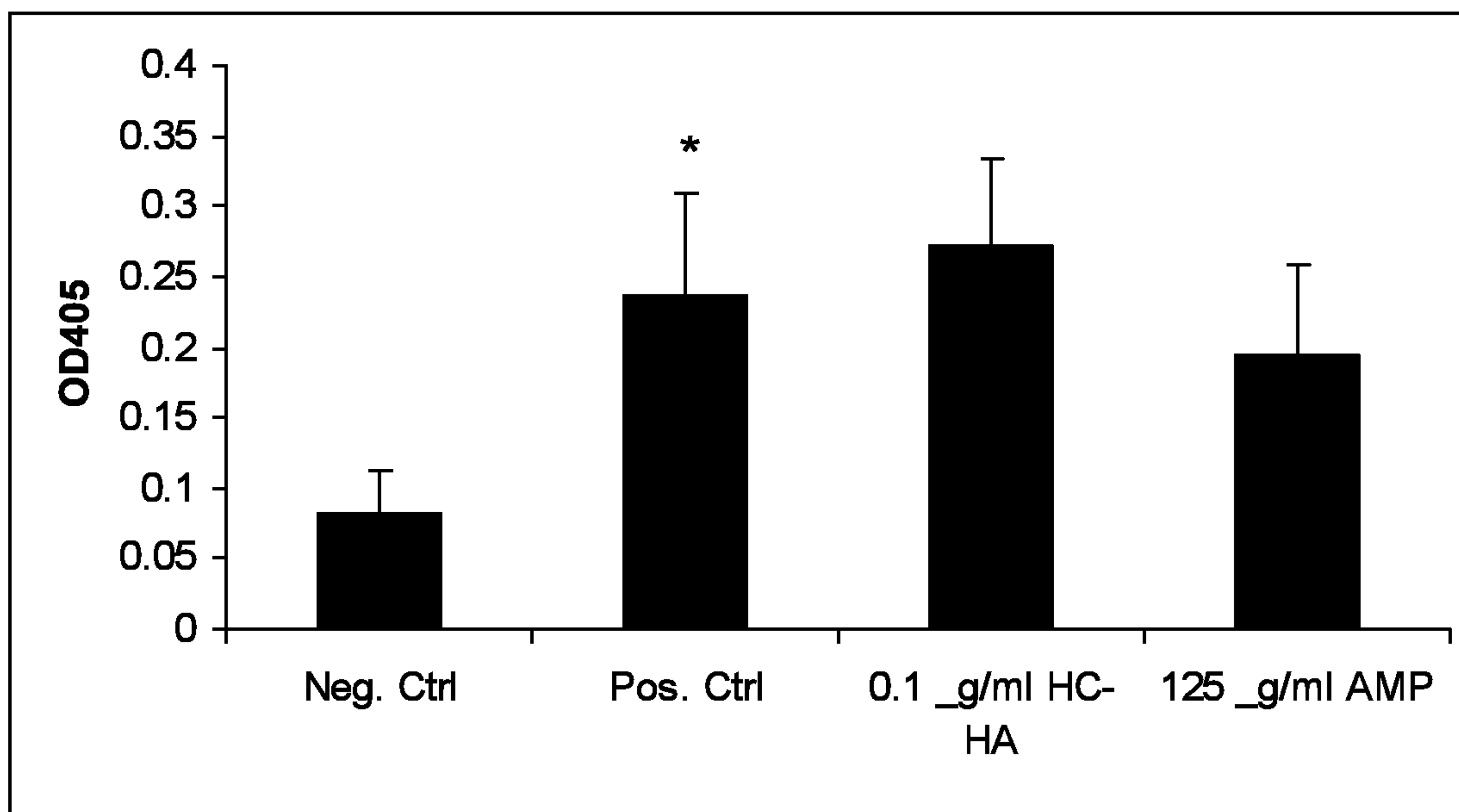
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FIG. 63

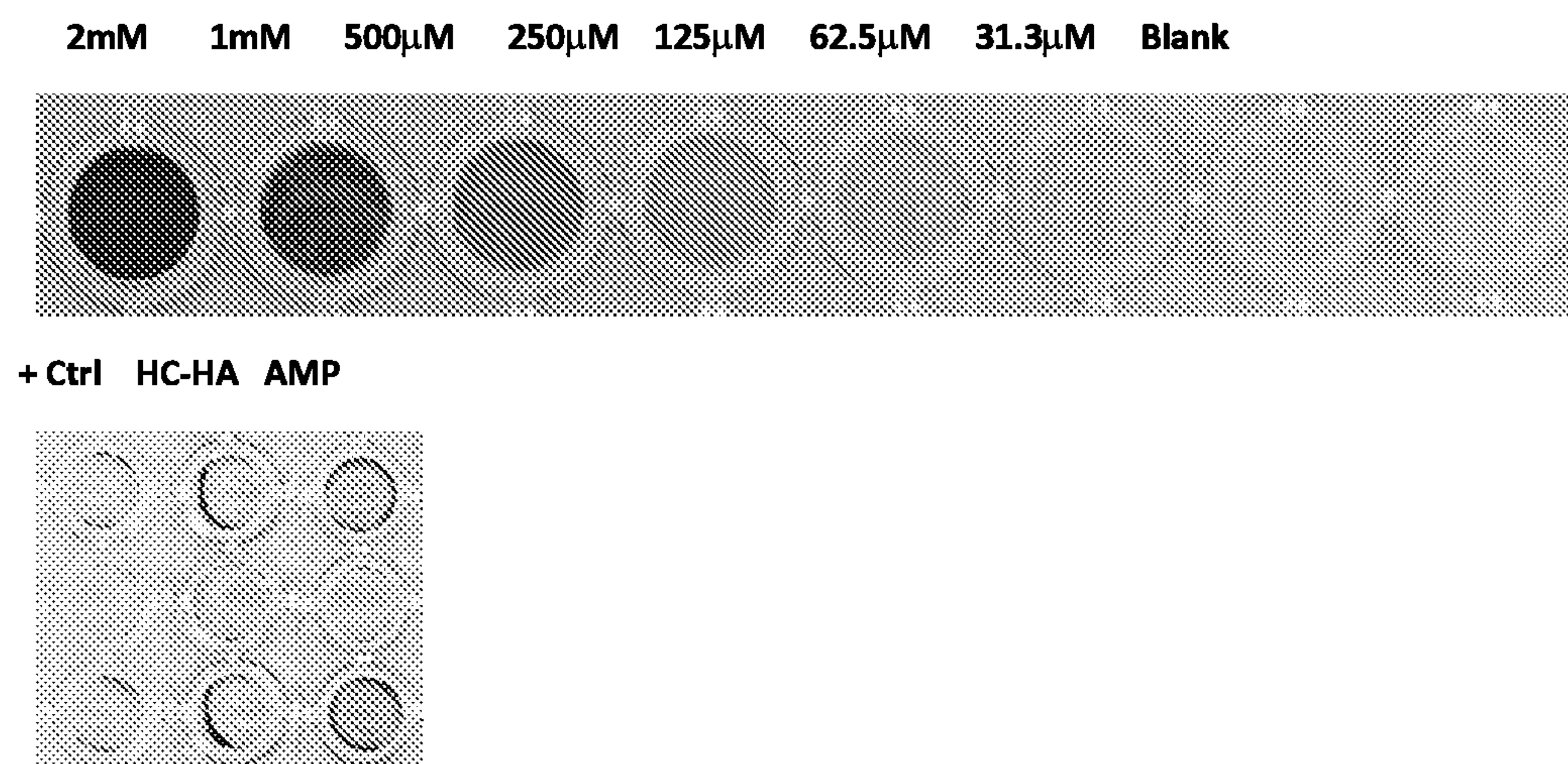
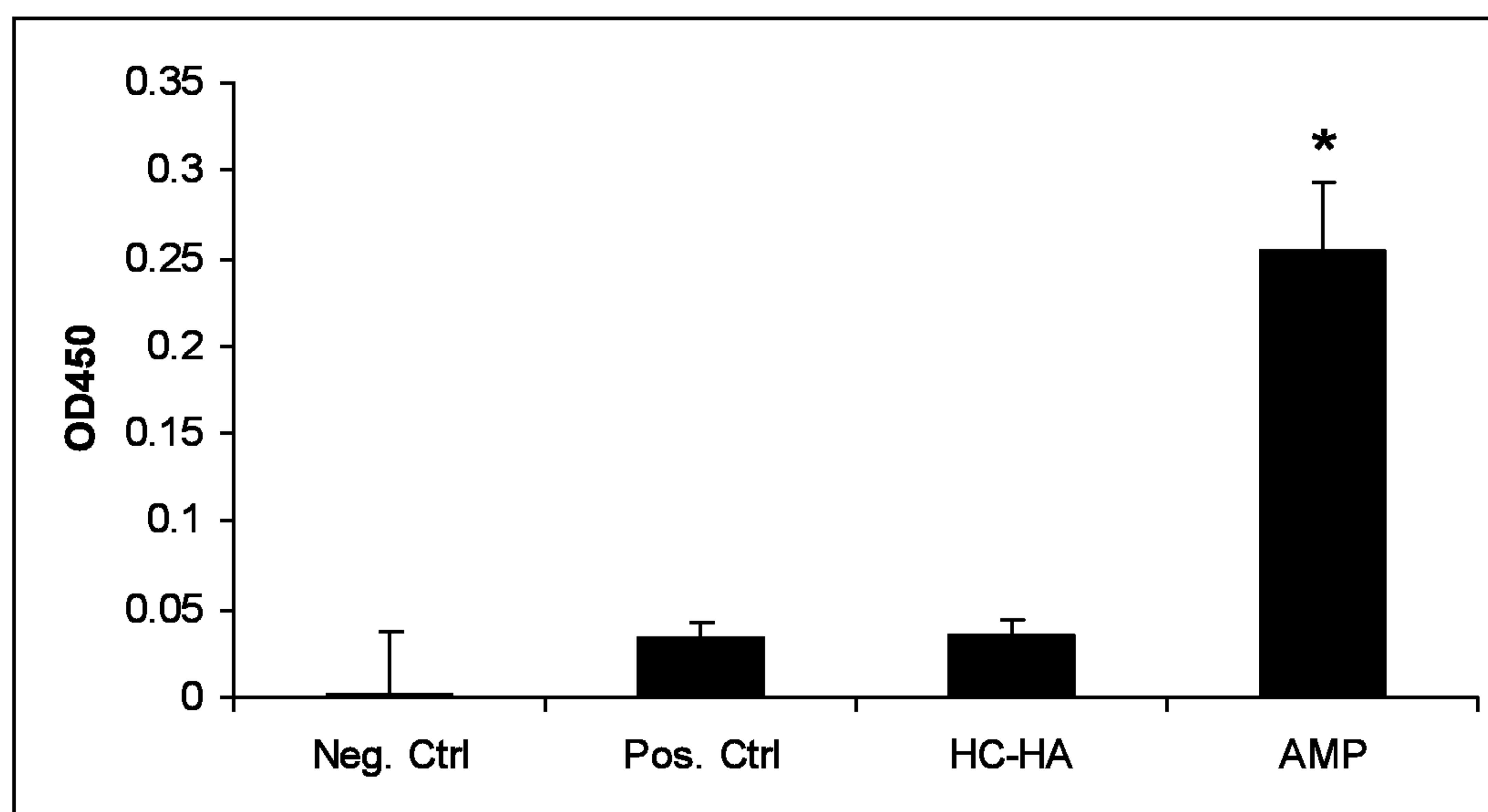
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FIG. 64**A**2mM 1mM 500 μ M 250 μ M 125 μ M 62.5 μ M 31.3 μ M Blank

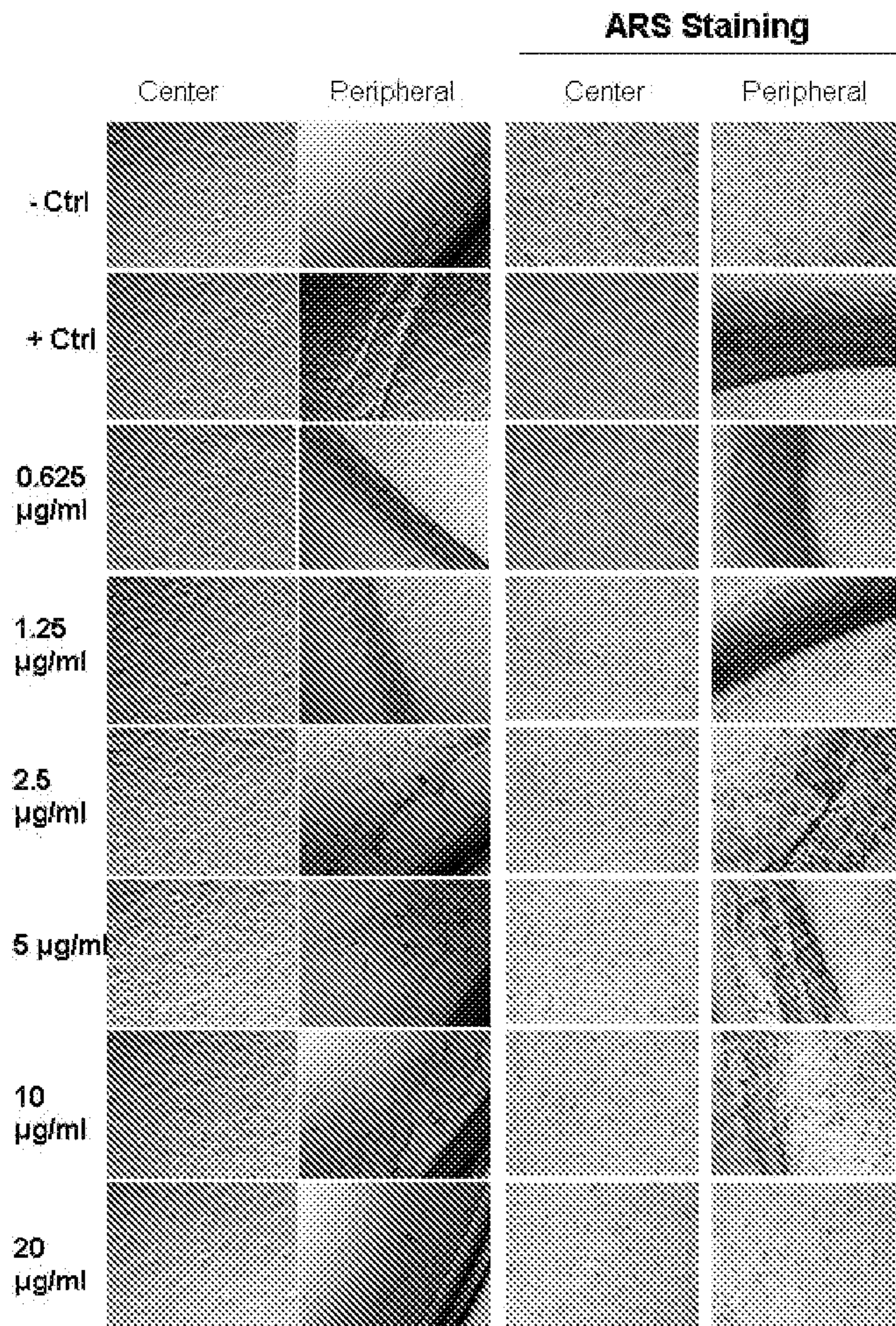
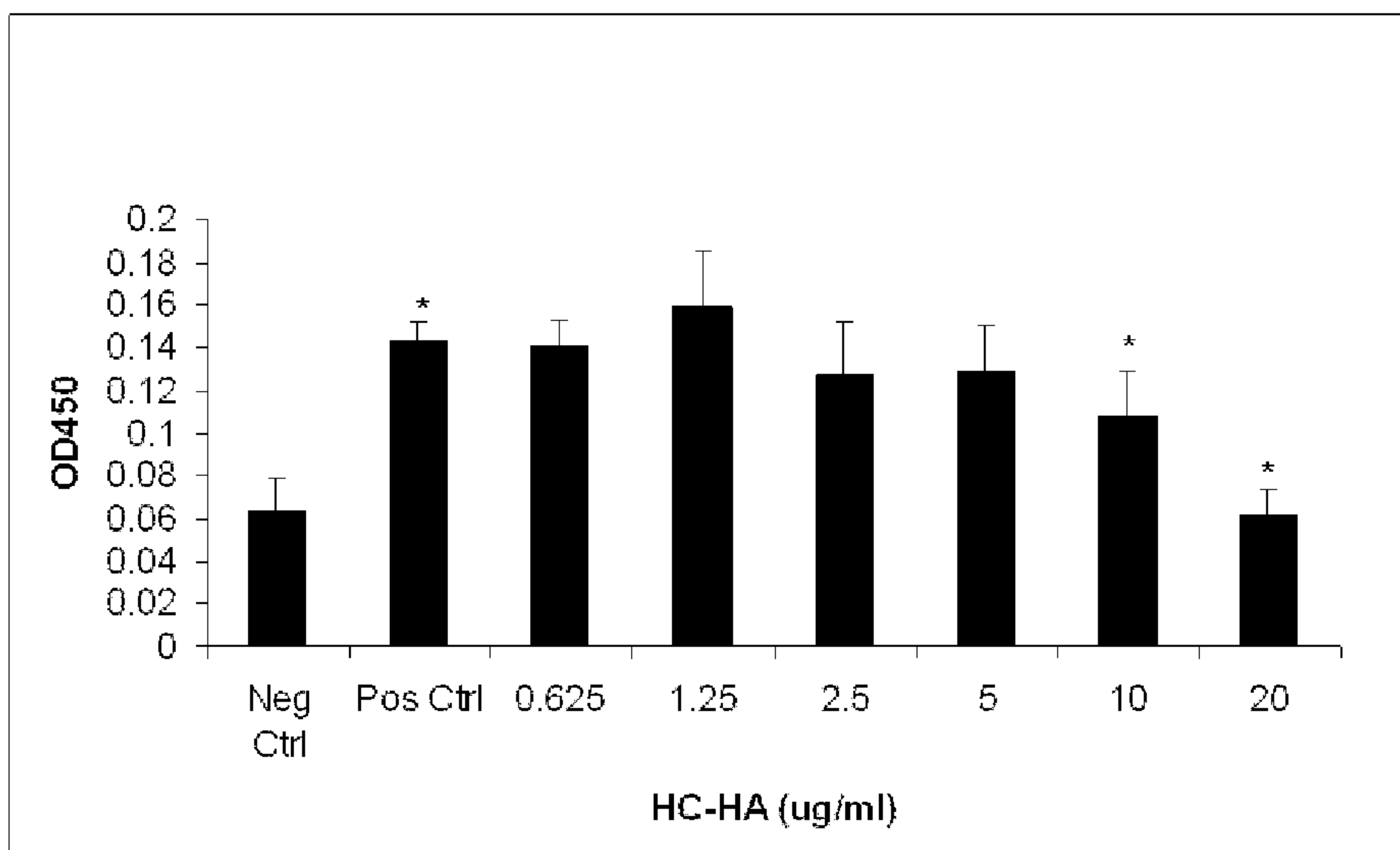
HC-HA AMP

**B**

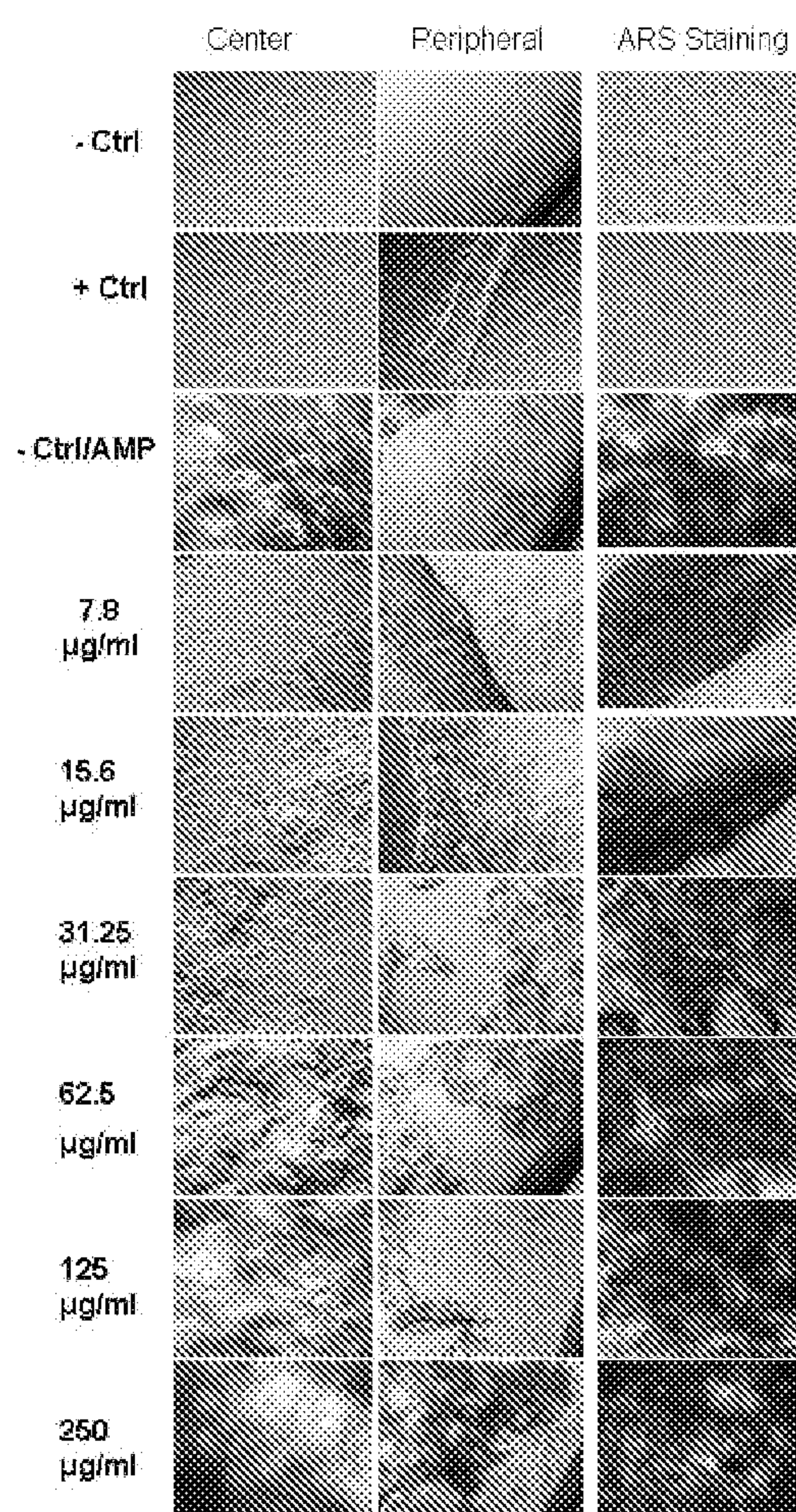
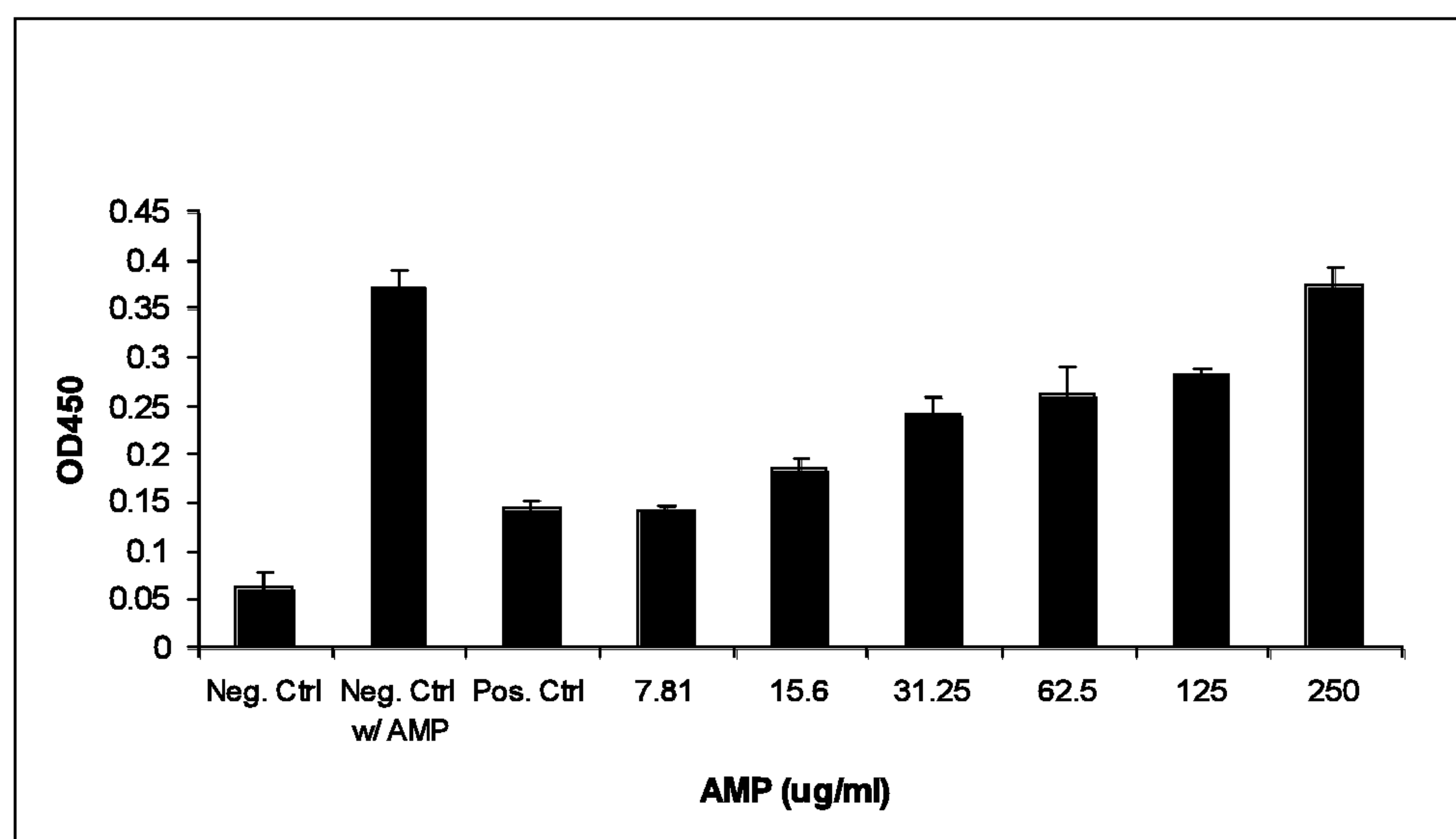
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FIG. 65**A****B**

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FIG. 66**A****B**

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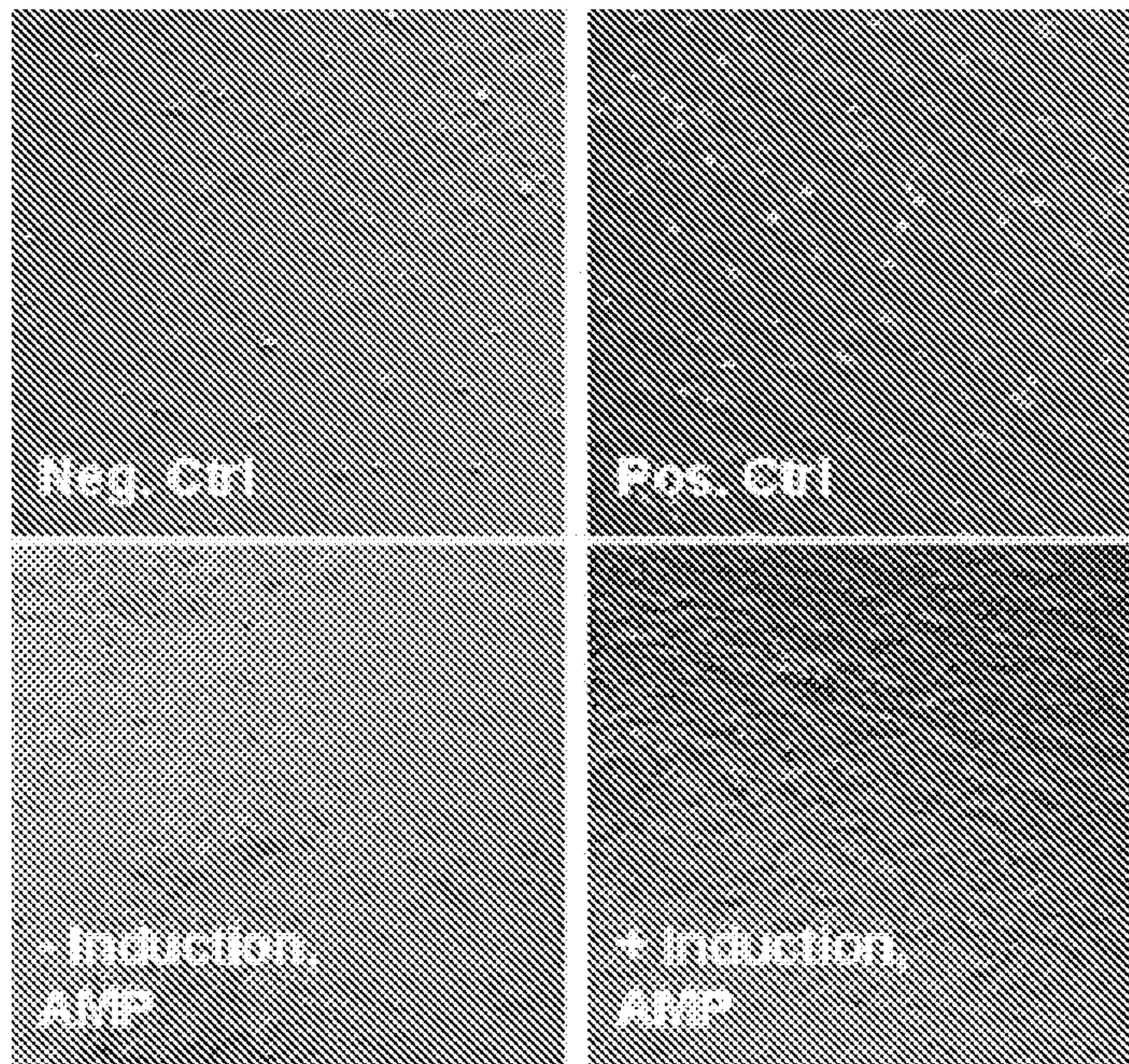
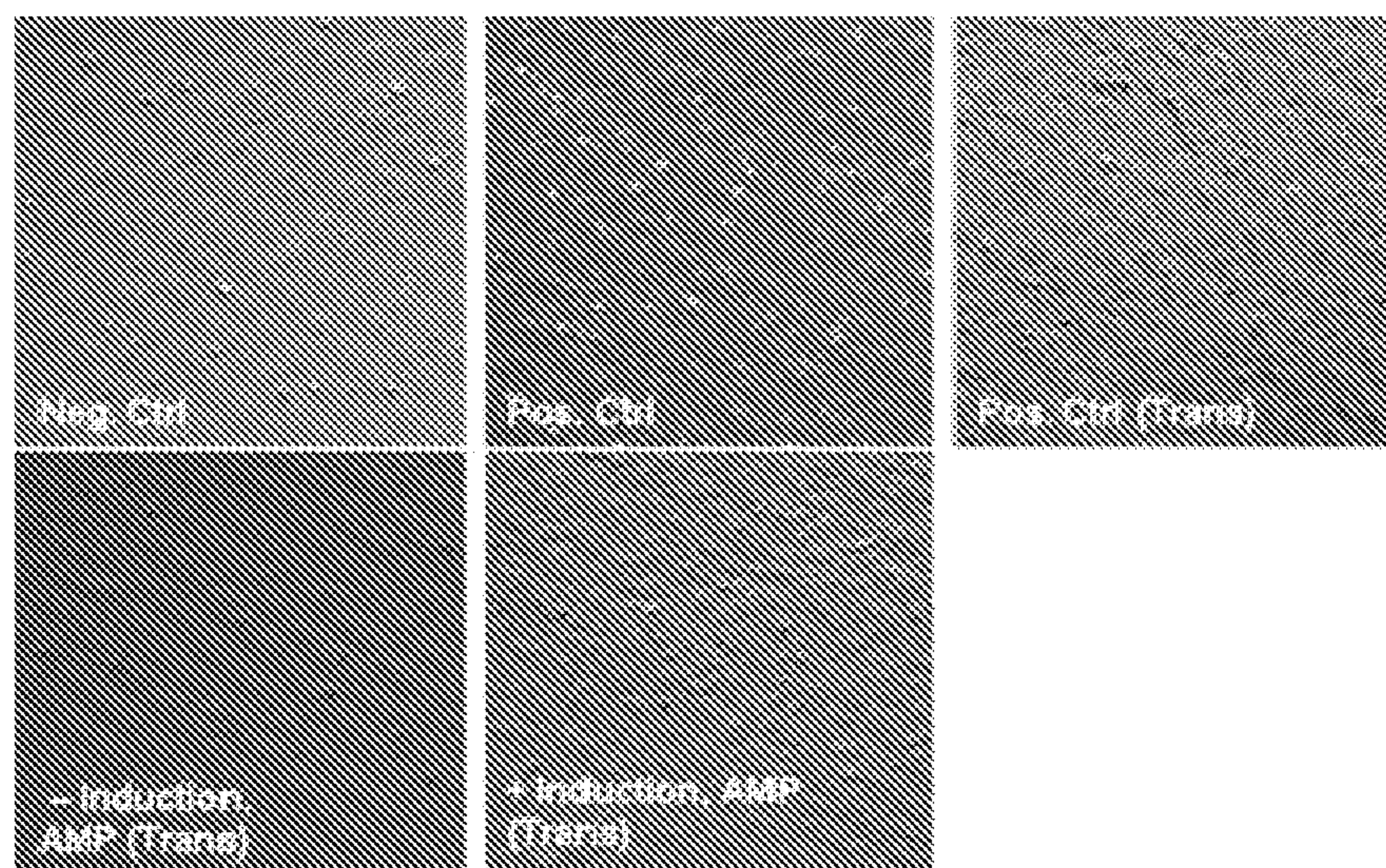
FIG. 67**A****B**

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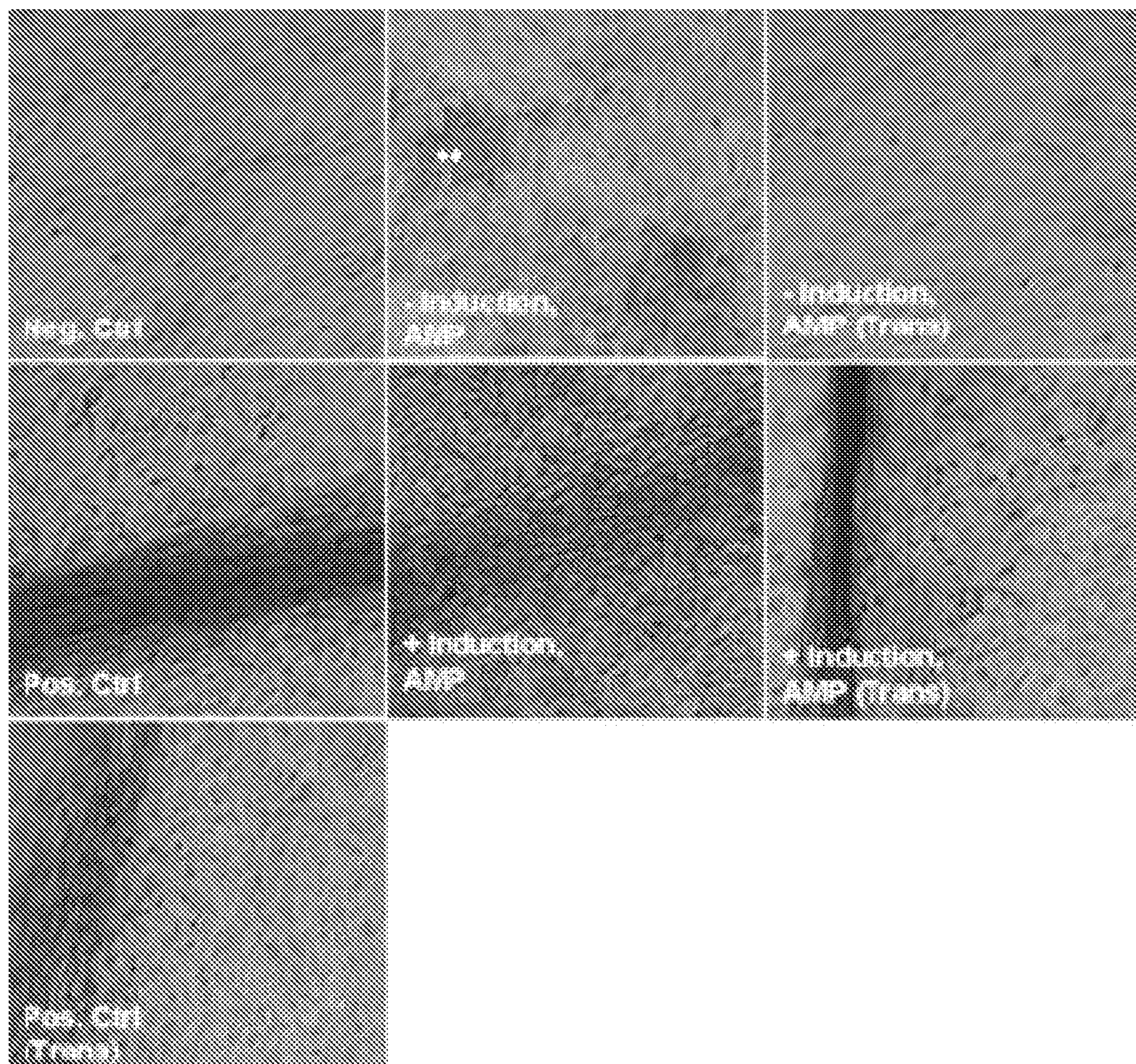
FIG. 68



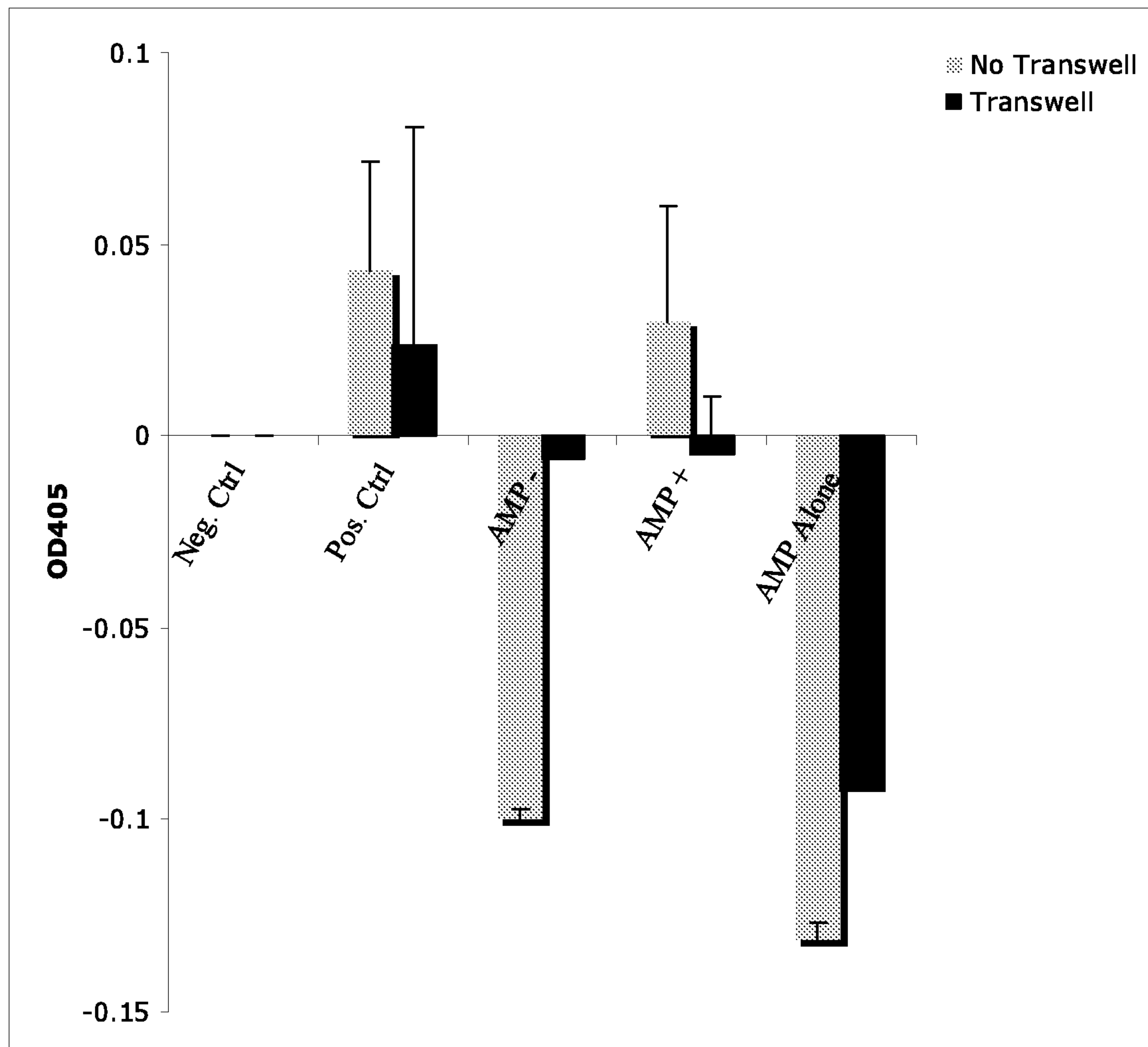
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FIG. 69**A. No Transwell****B. Transwell**

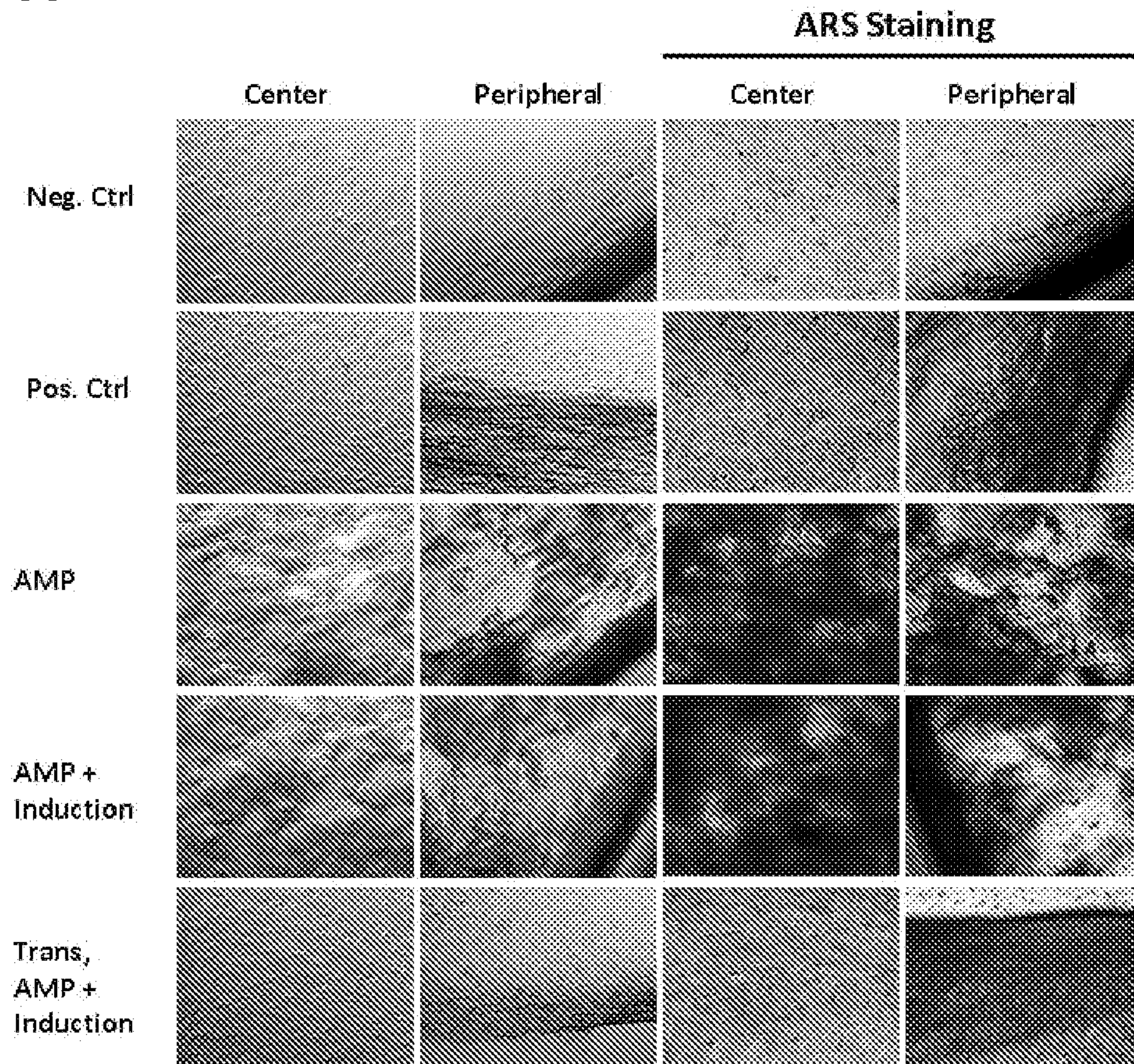
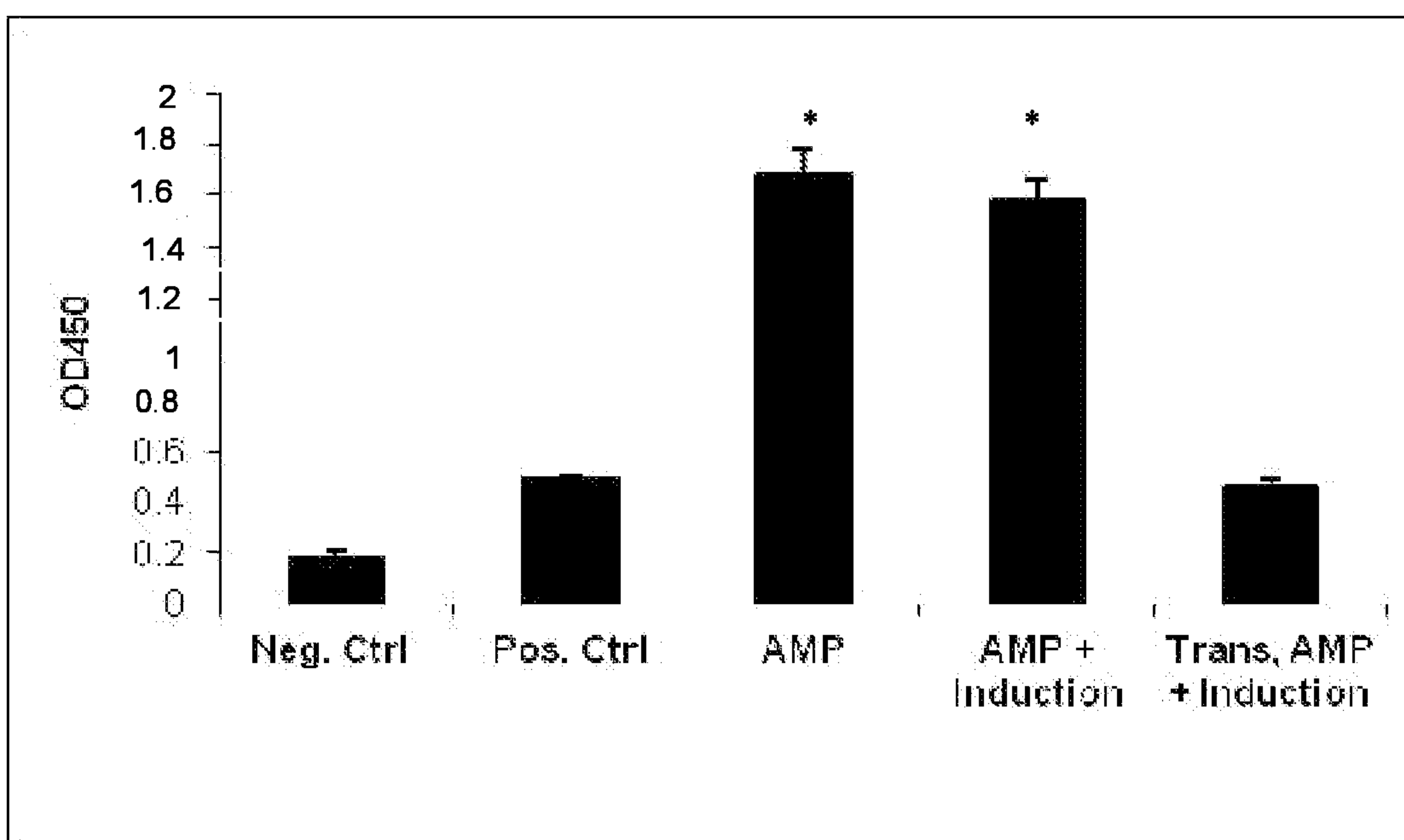
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FIG. 70

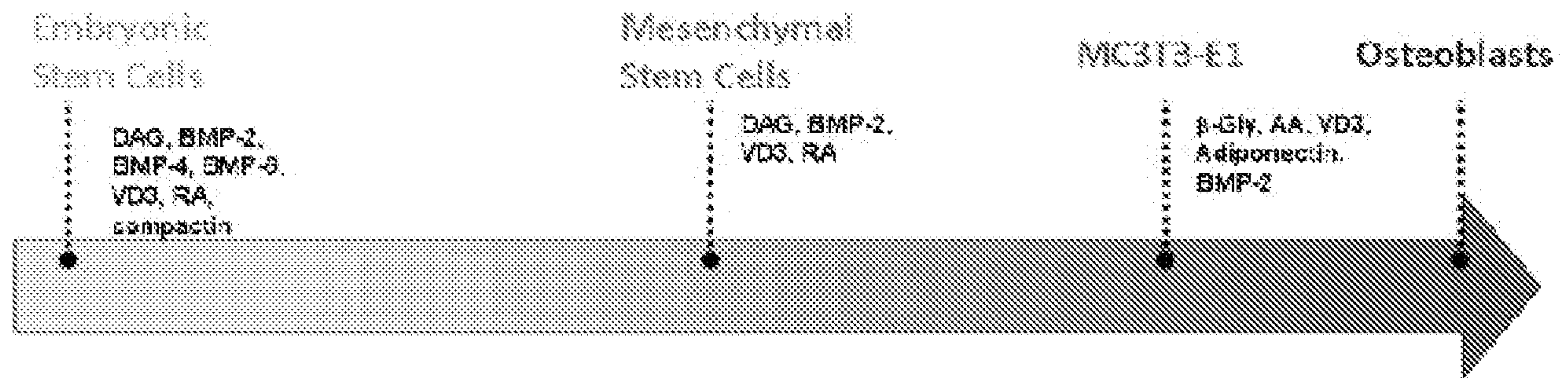
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FIG. 71

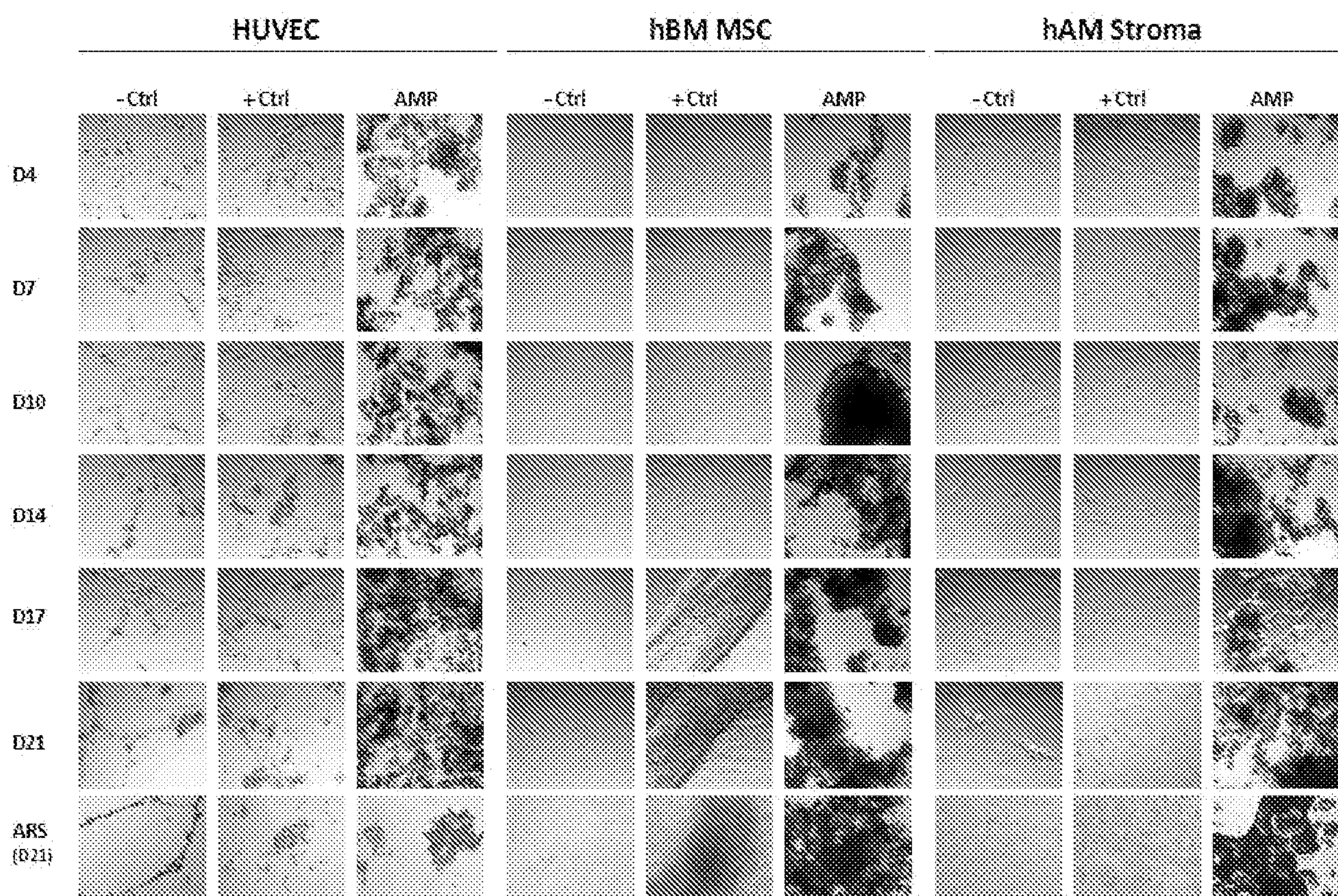
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FIG. 72**A****B**

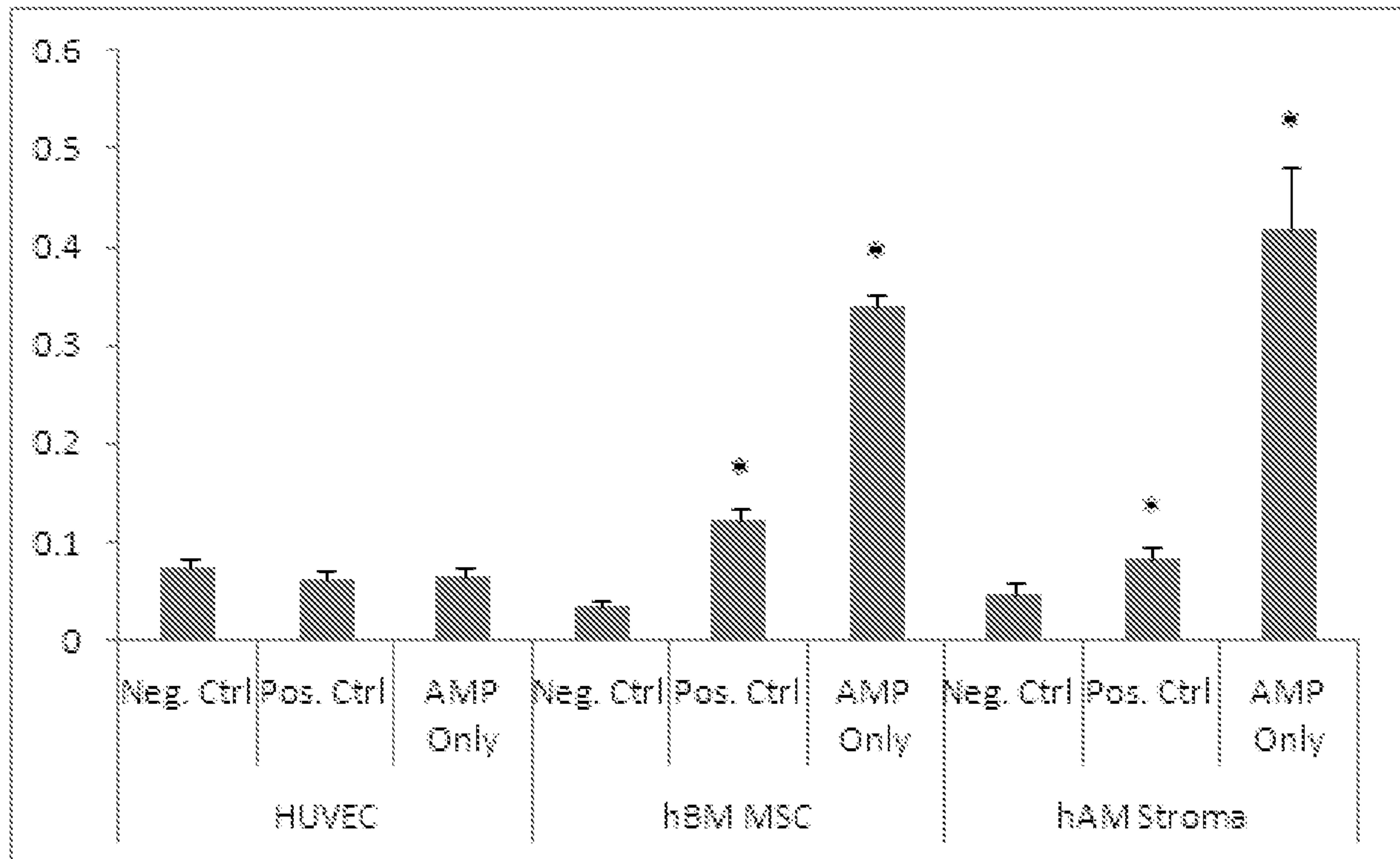
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FIG. 73

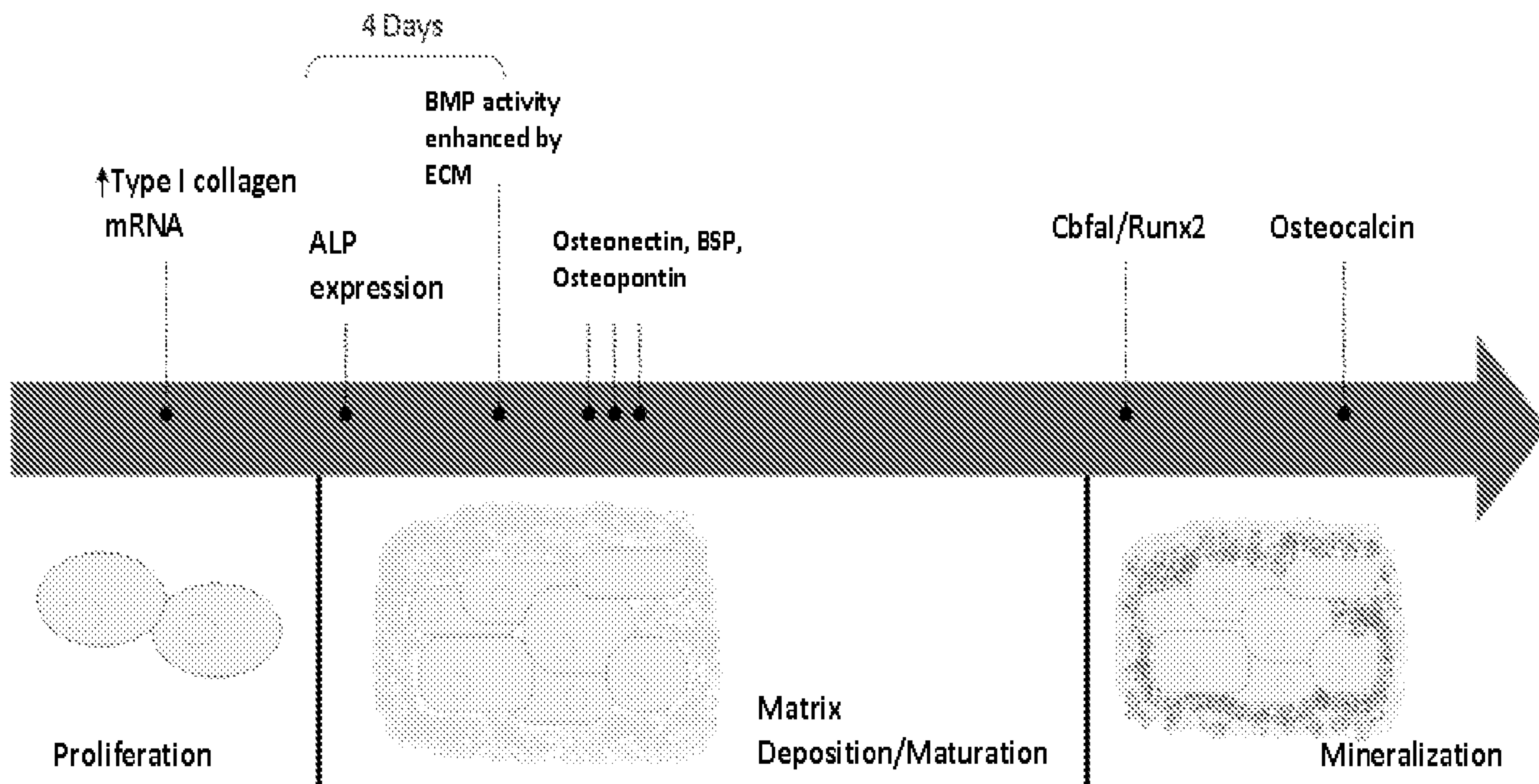
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FIG. 74**A**

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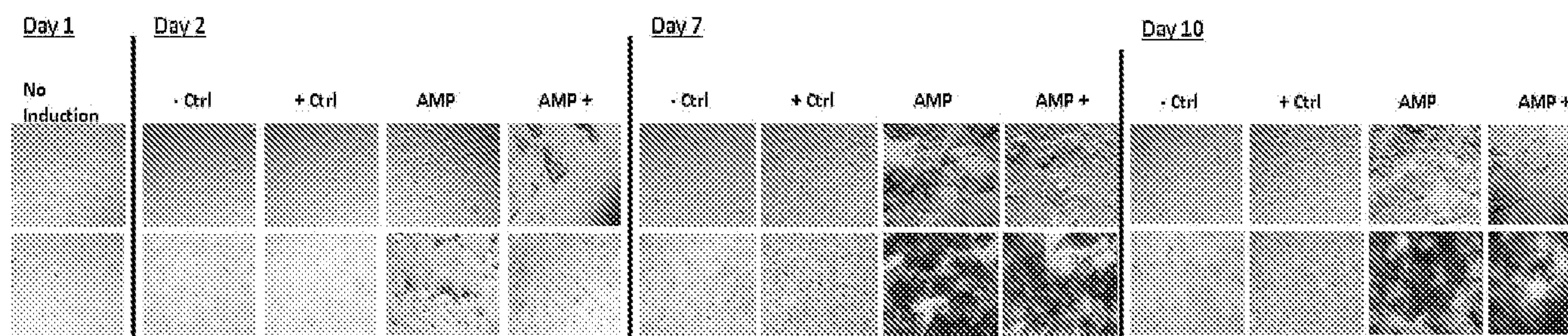
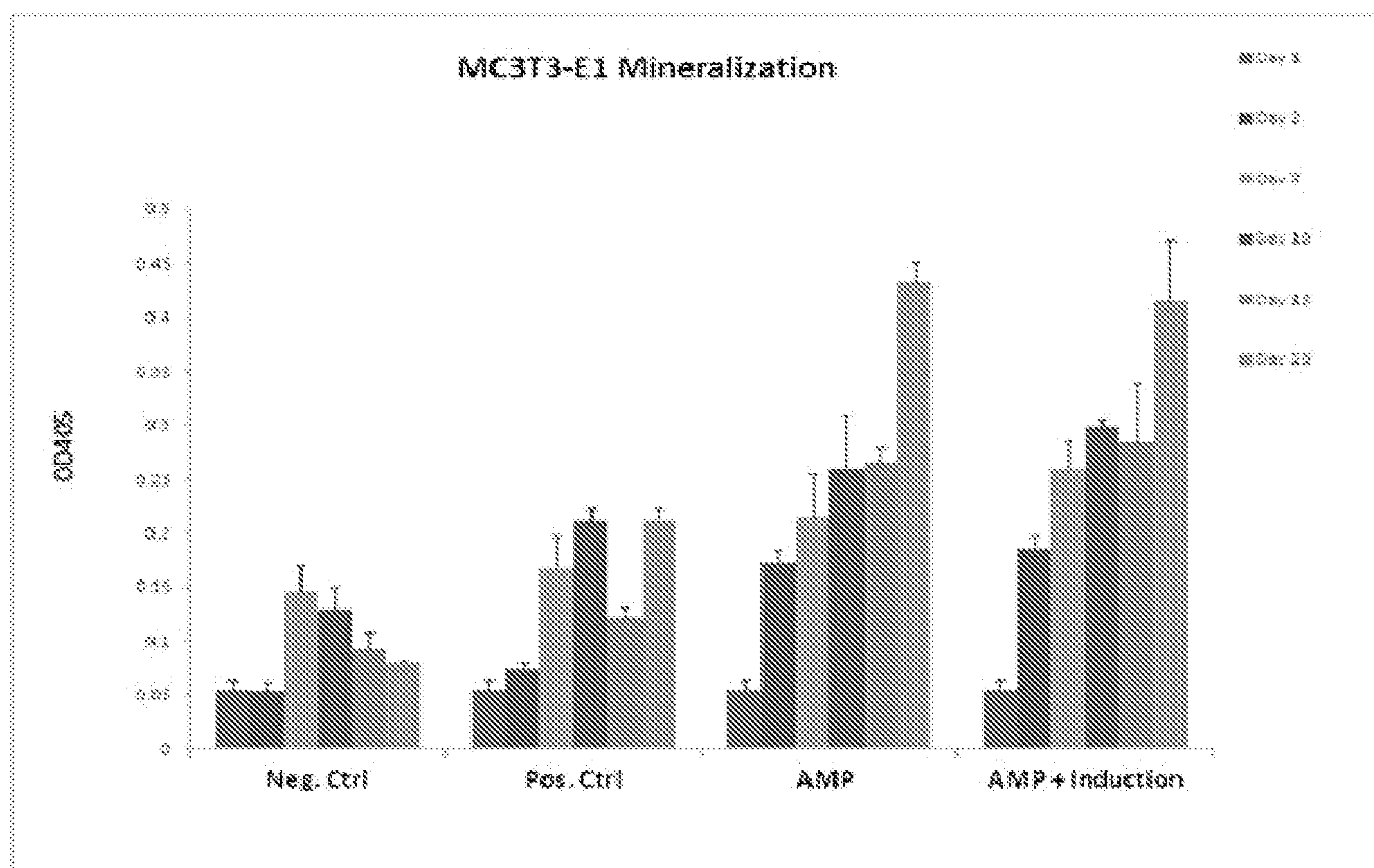
FIG. 74 (cont'd)**B**

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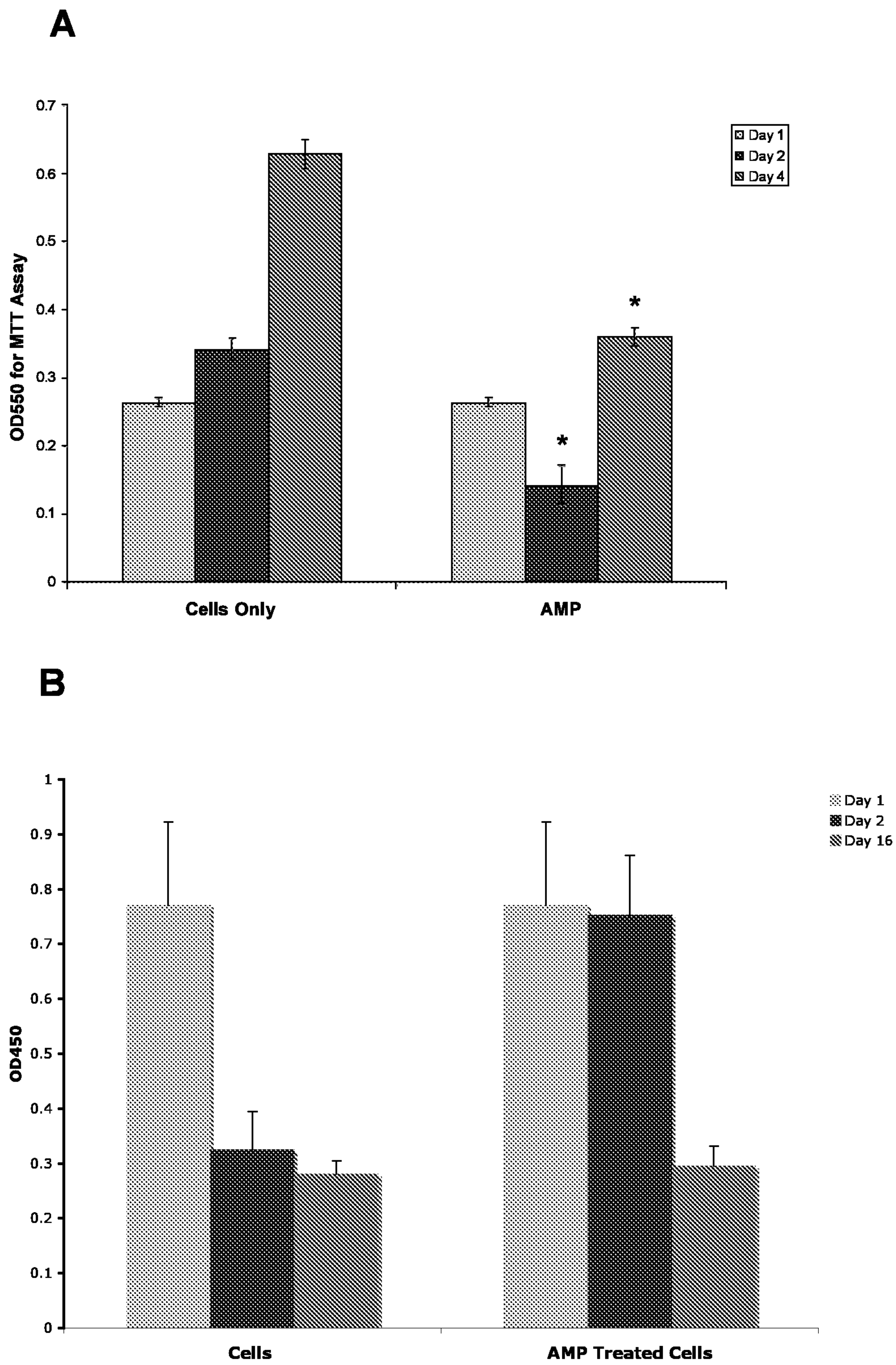
FIG. 75

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FIG. 76

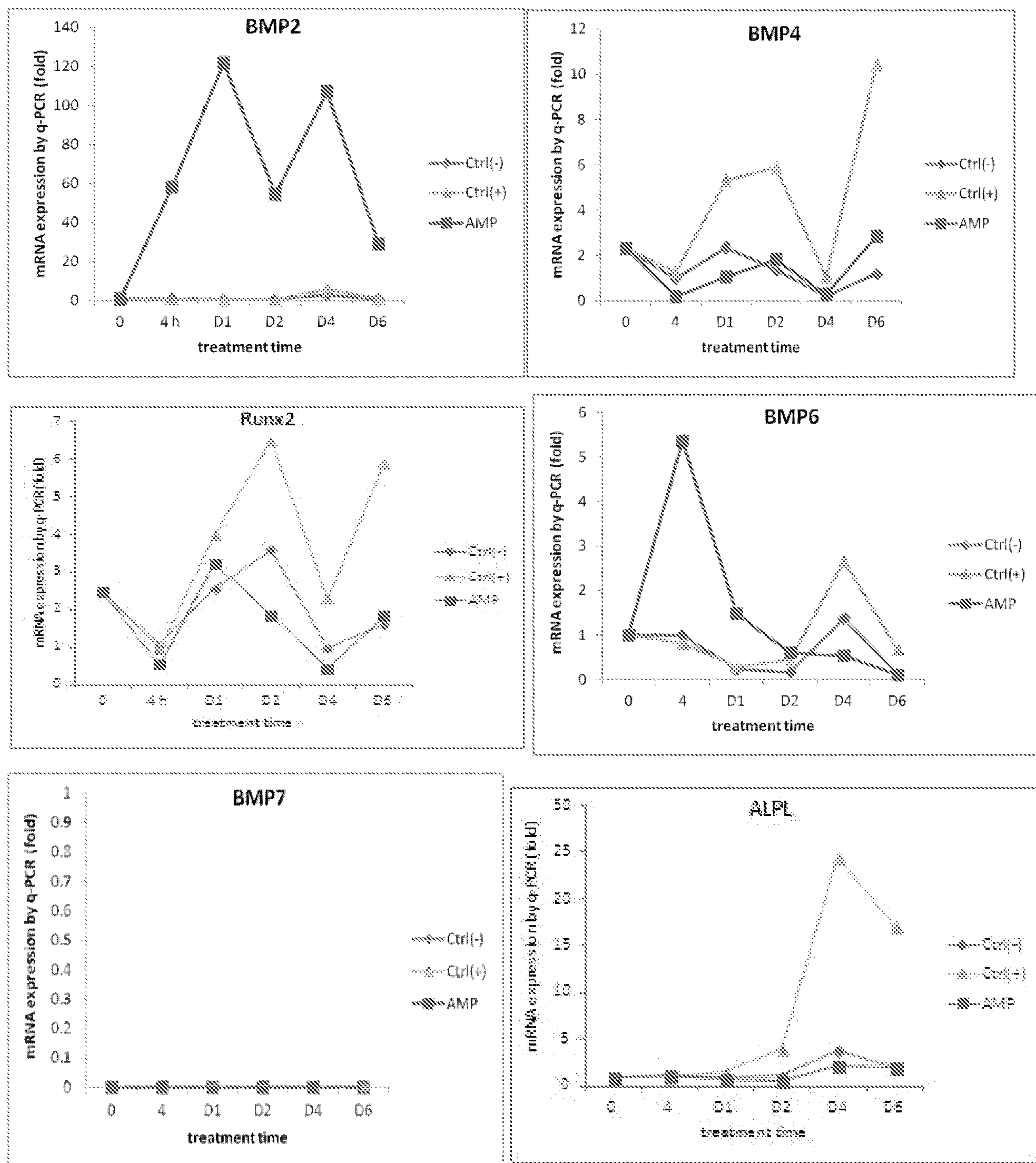
A**B**

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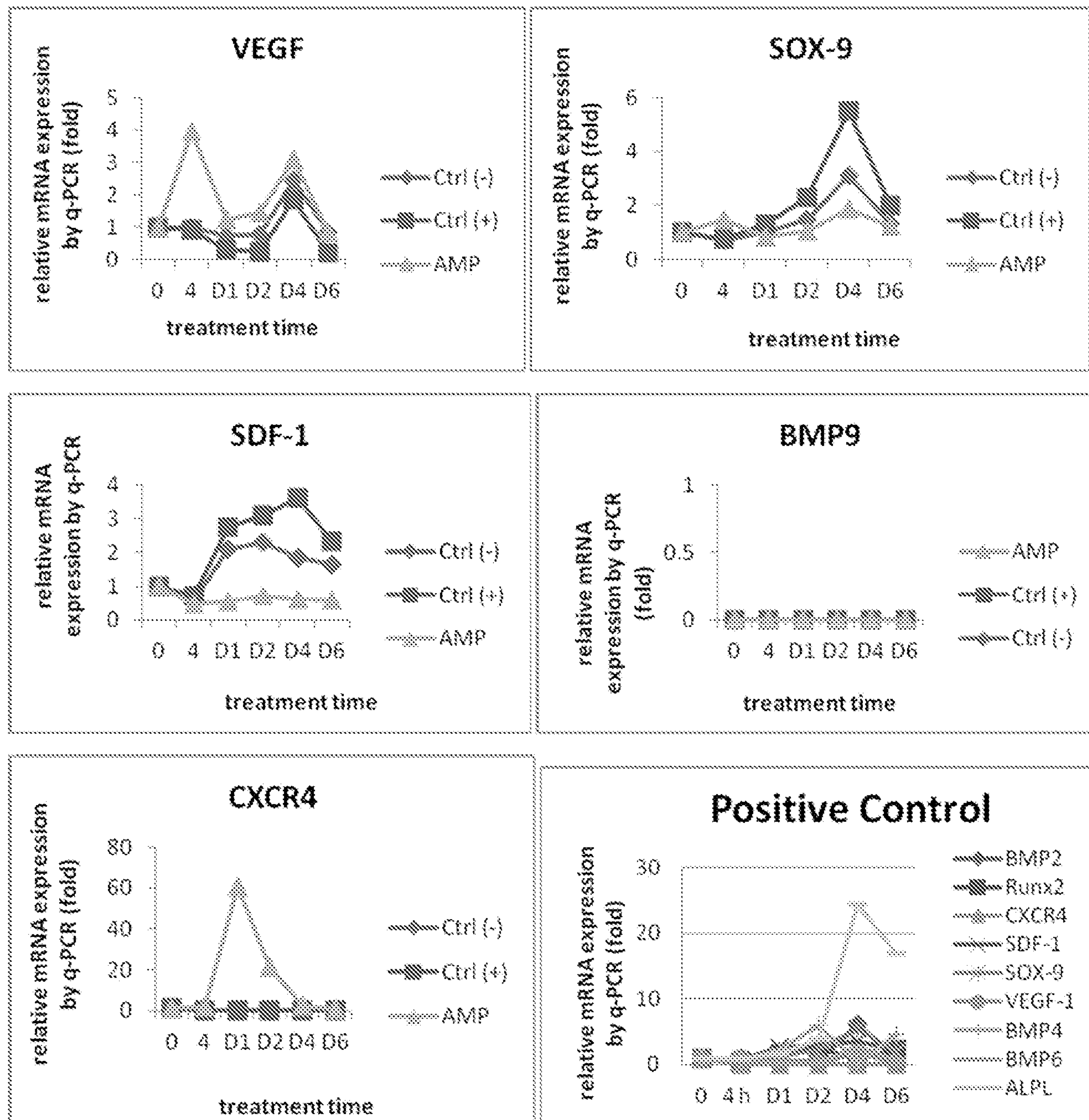
FIG. 77

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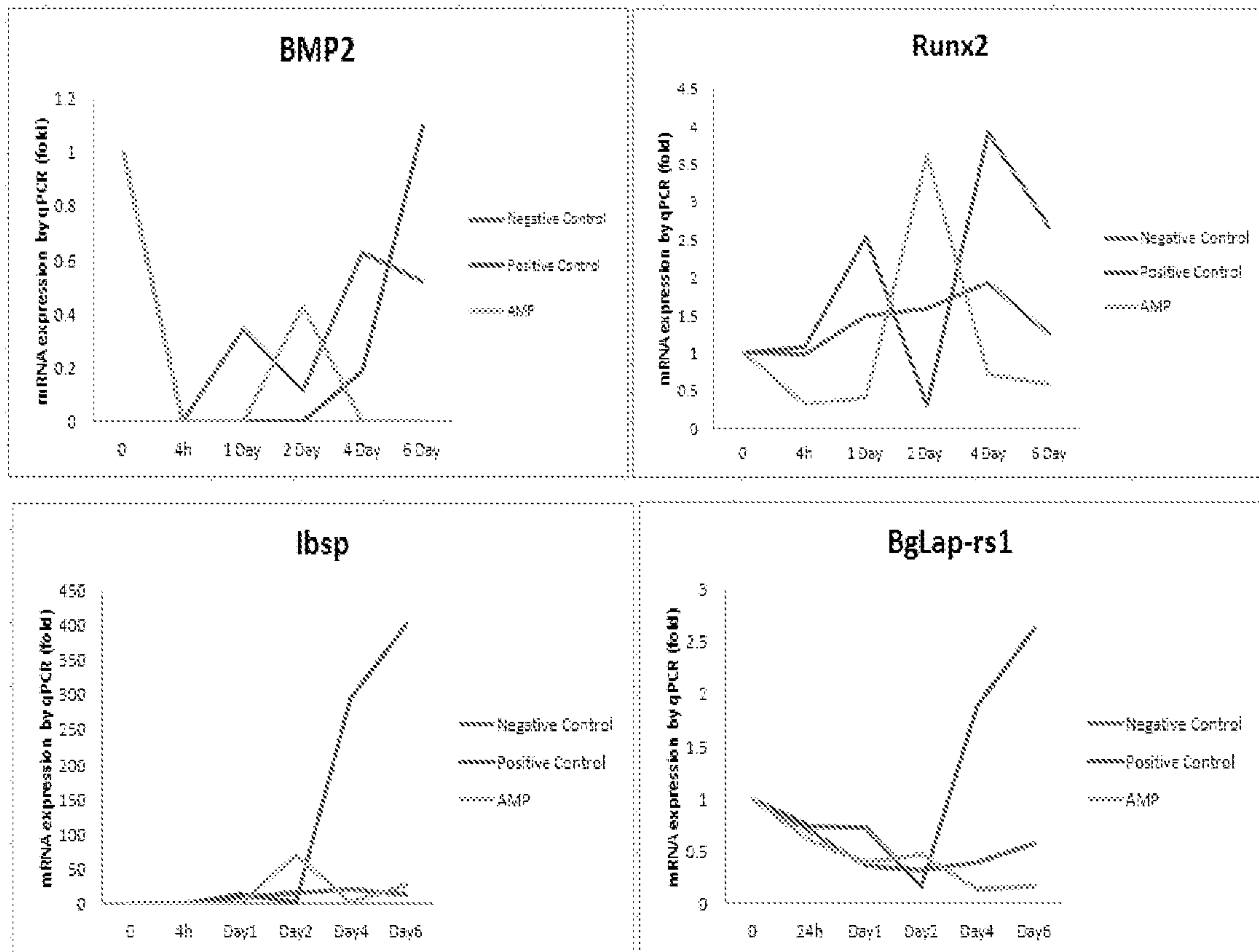
FIG. 78A



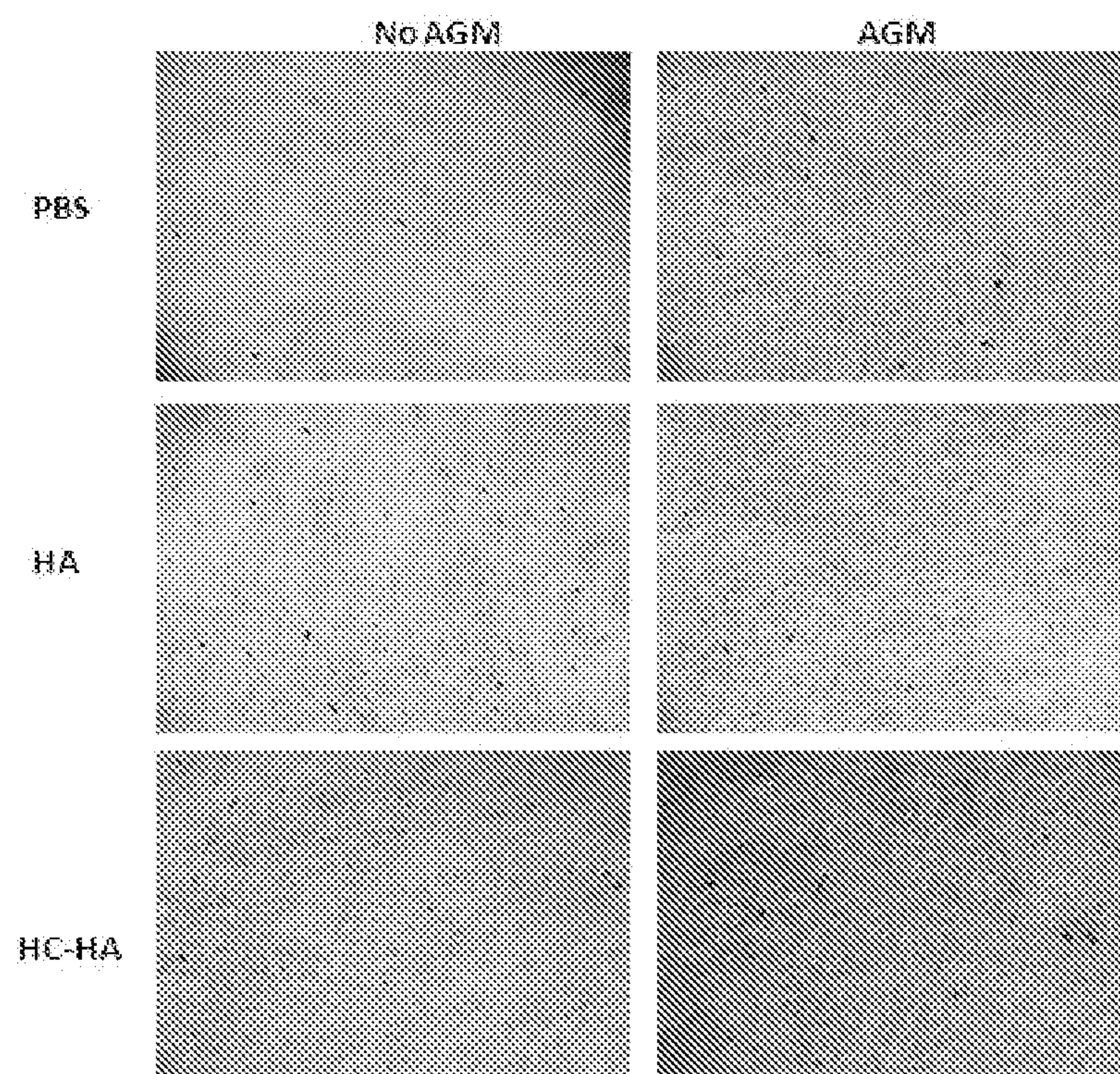
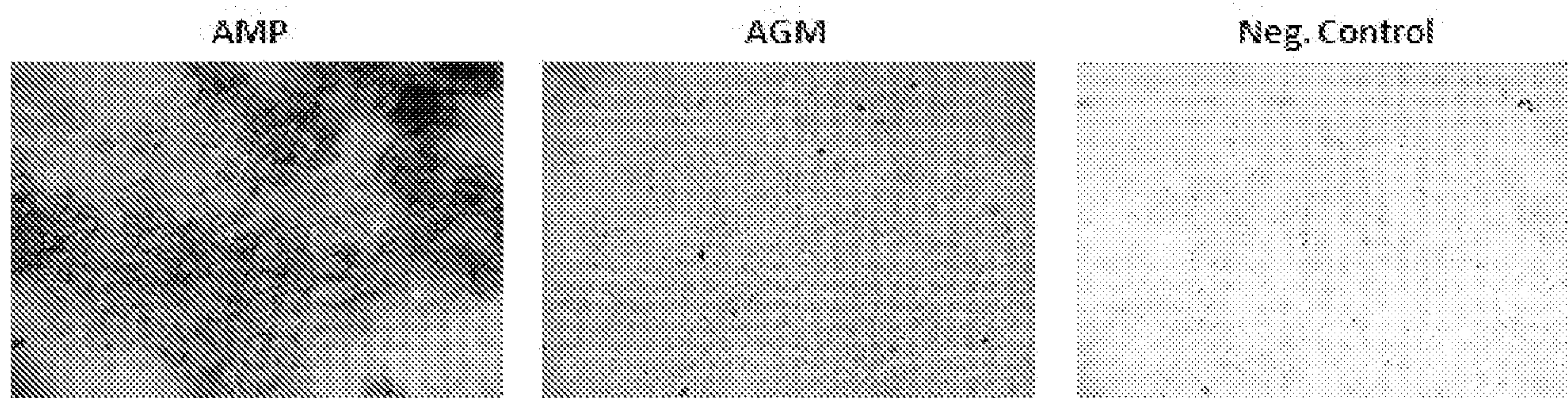
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FIG. 78A (cont'd)

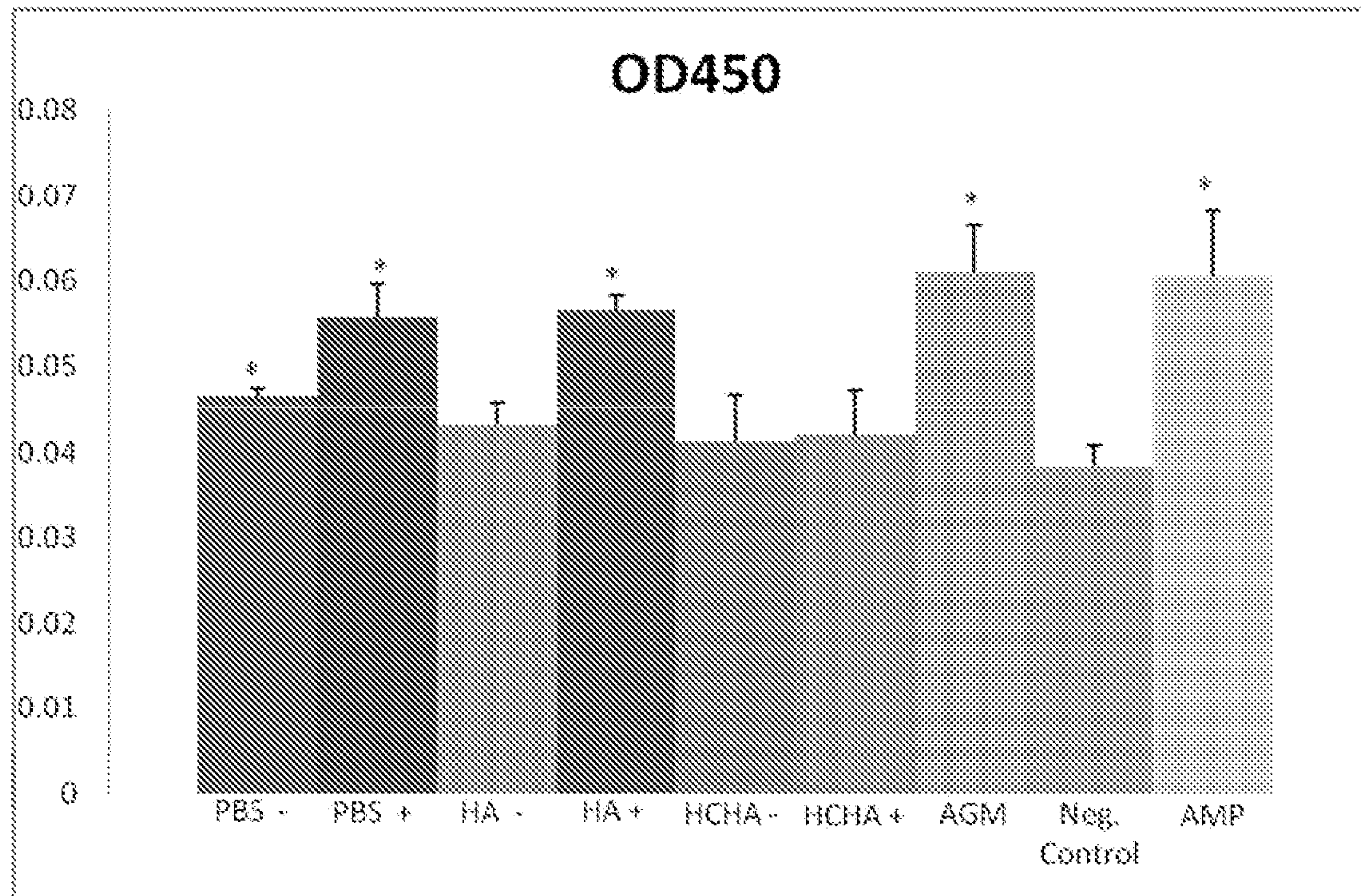
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FIG. 78B

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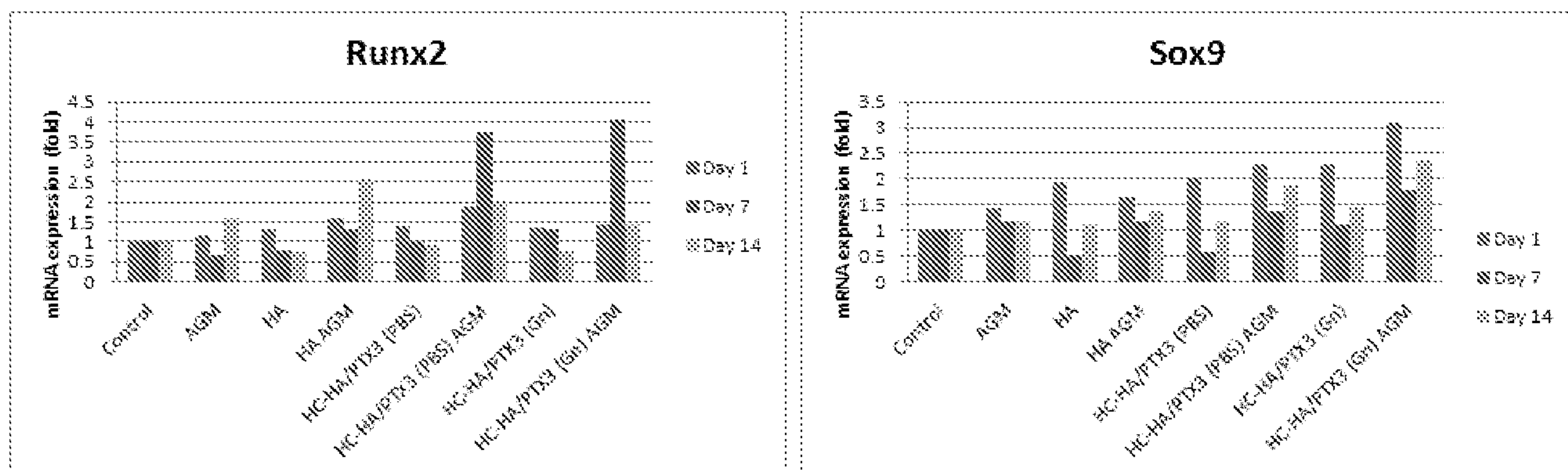
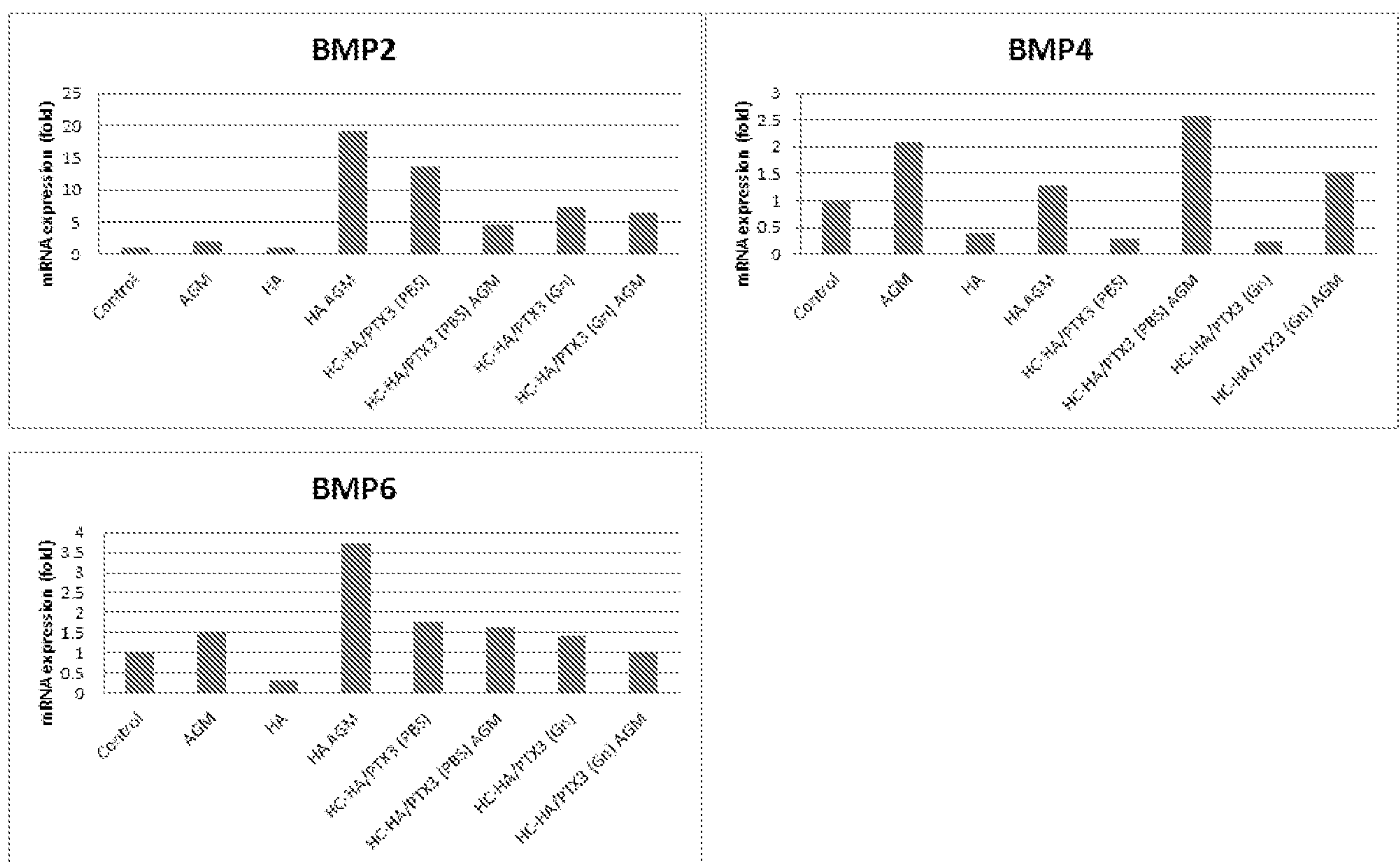
FIG. 79**A****B**

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FIG. 79 (cont'd)**C**

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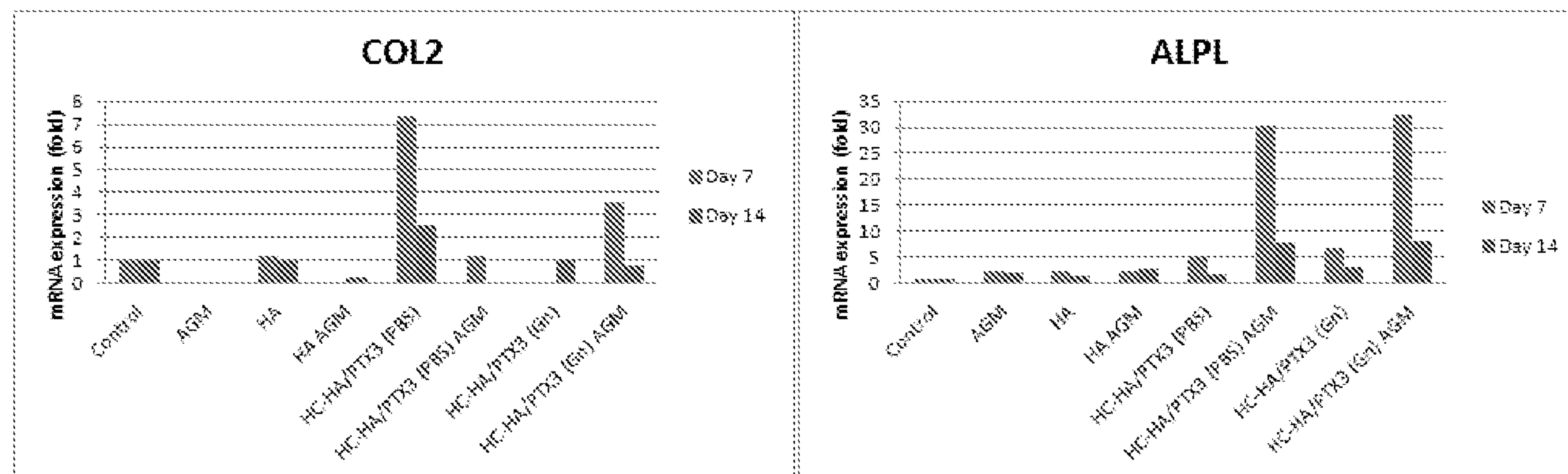
FIG. 80

A**B**

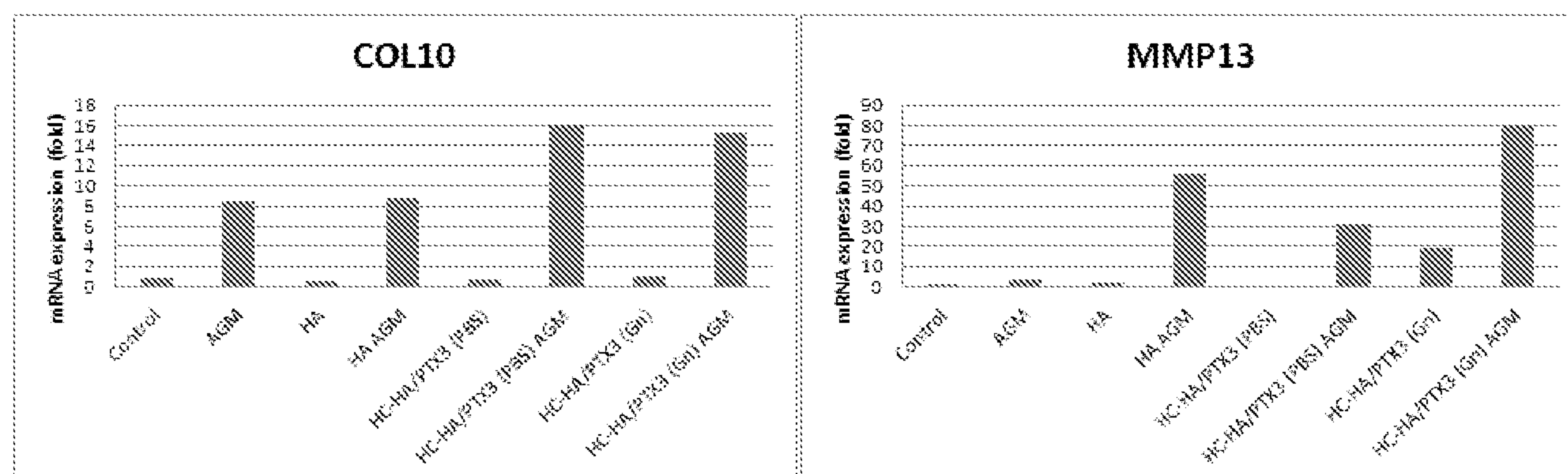
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C

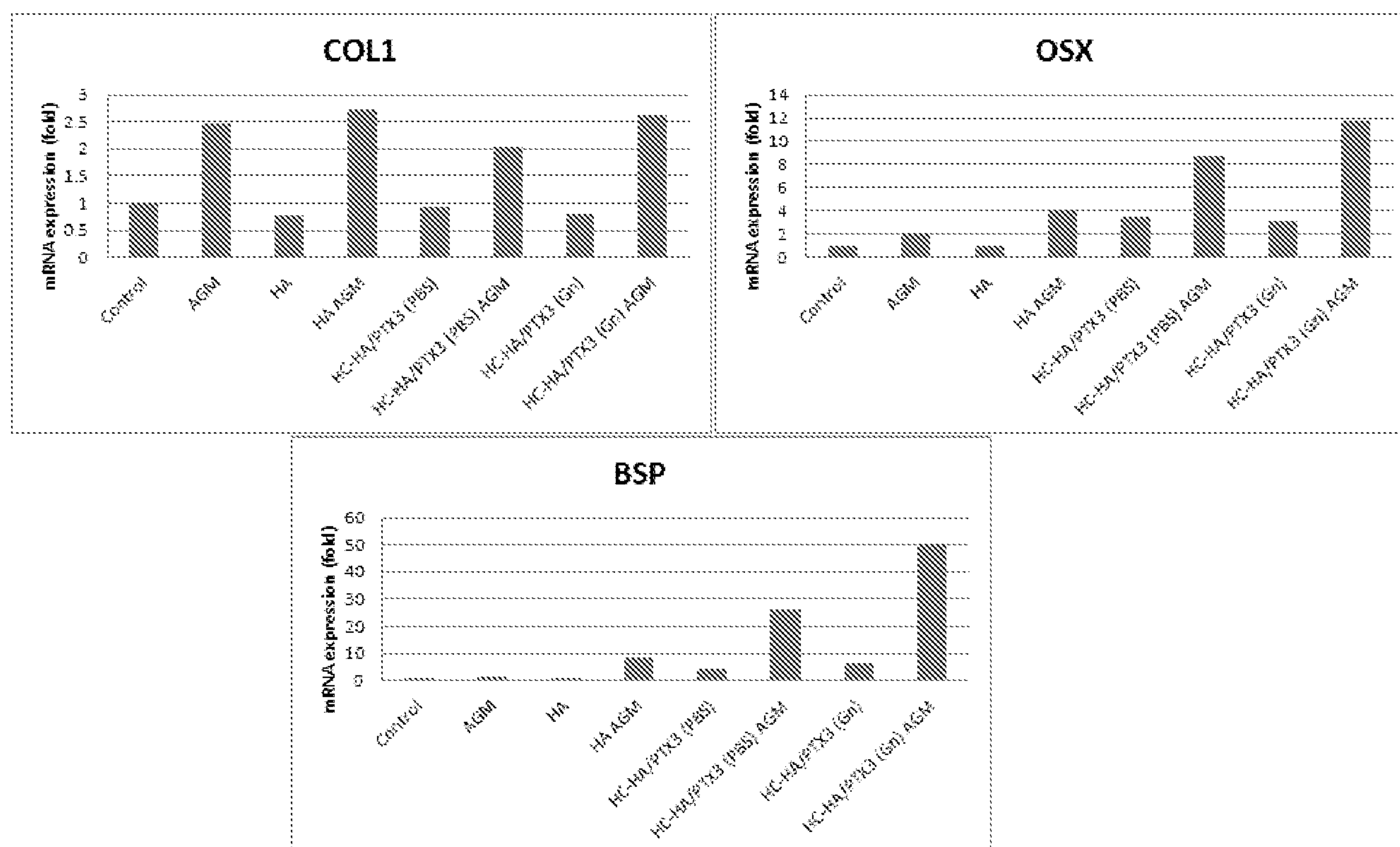
FIG. 80 (cont'd)



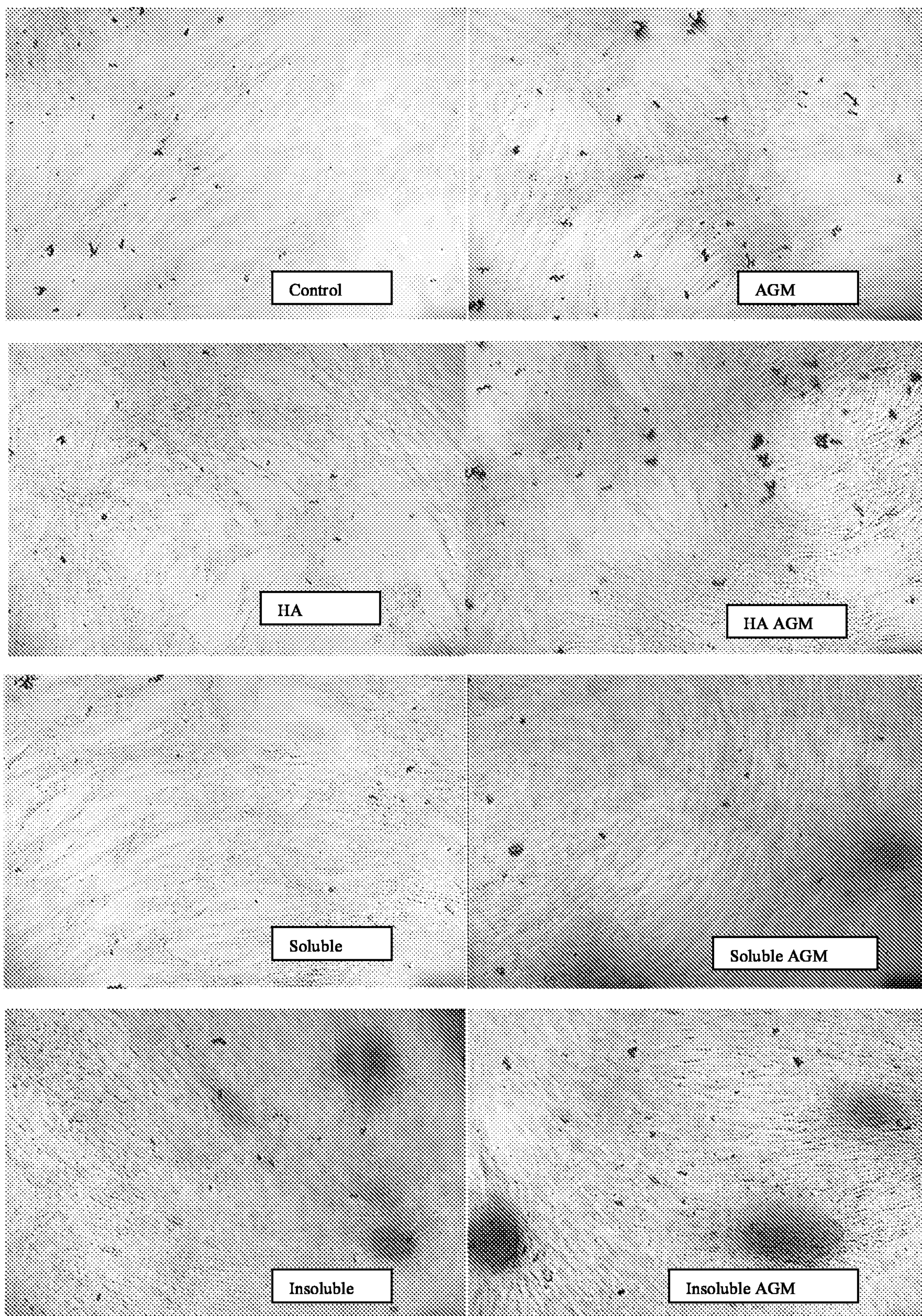
D



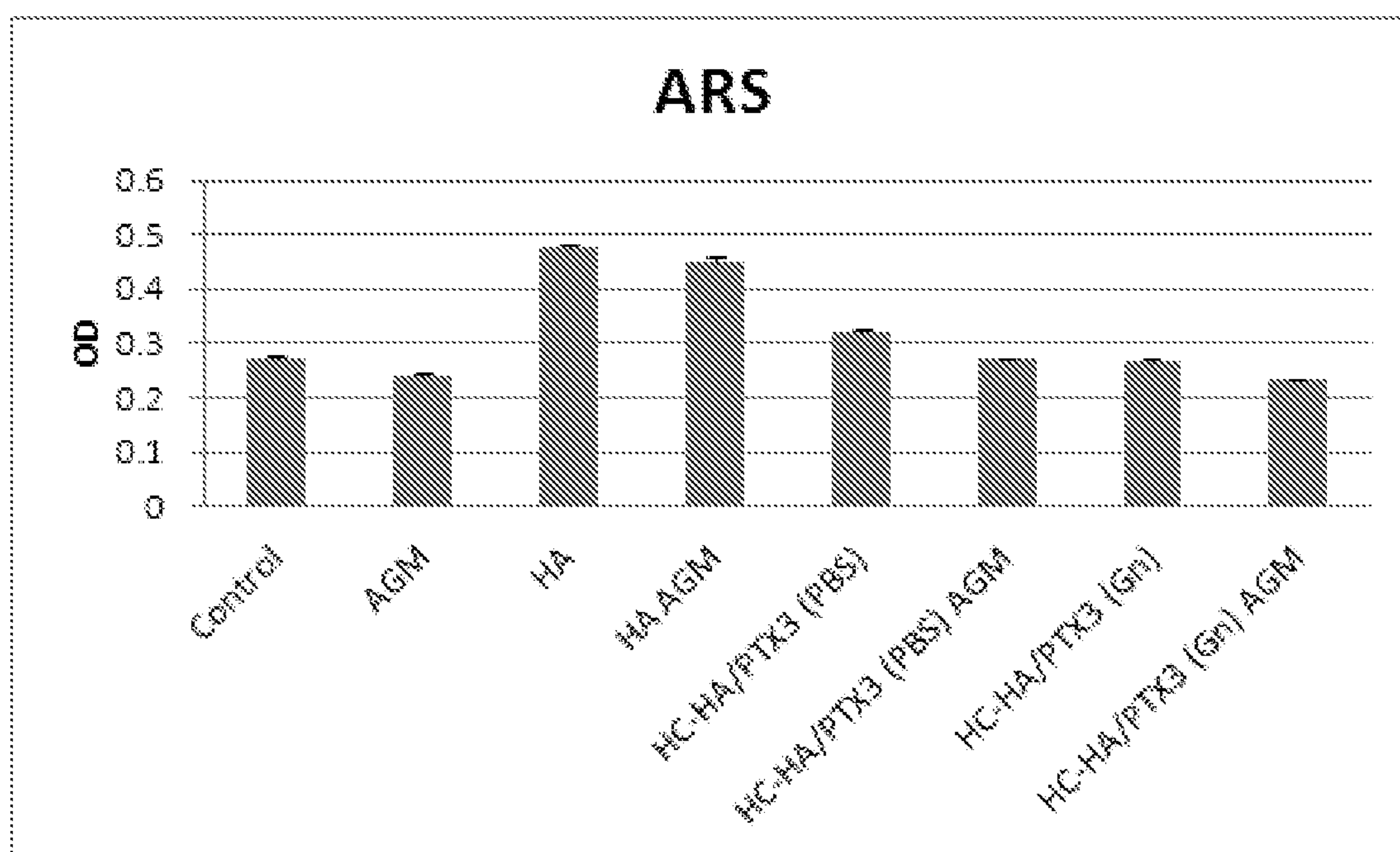
E



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FIG. 81A

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FIG. 81B

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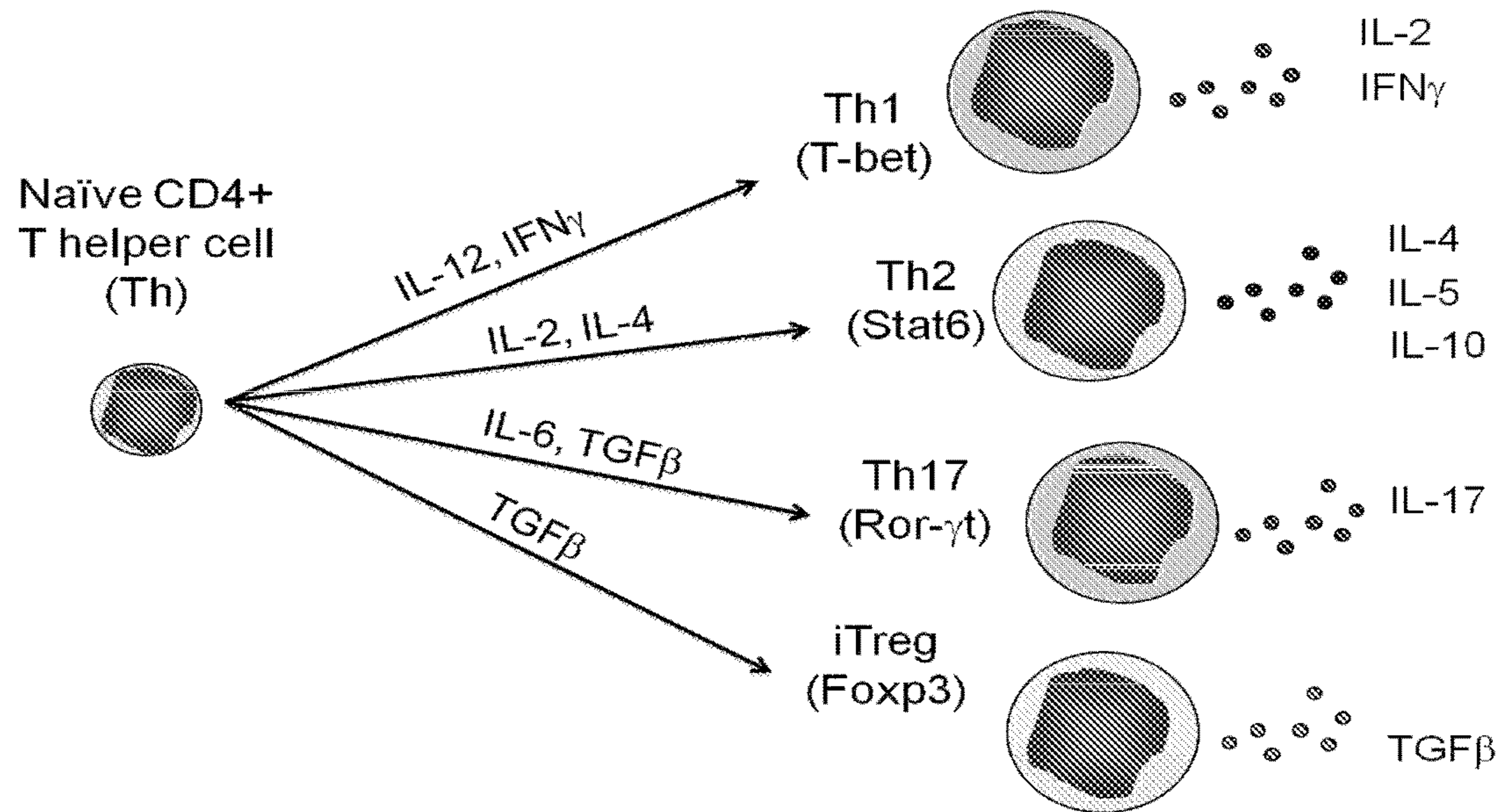
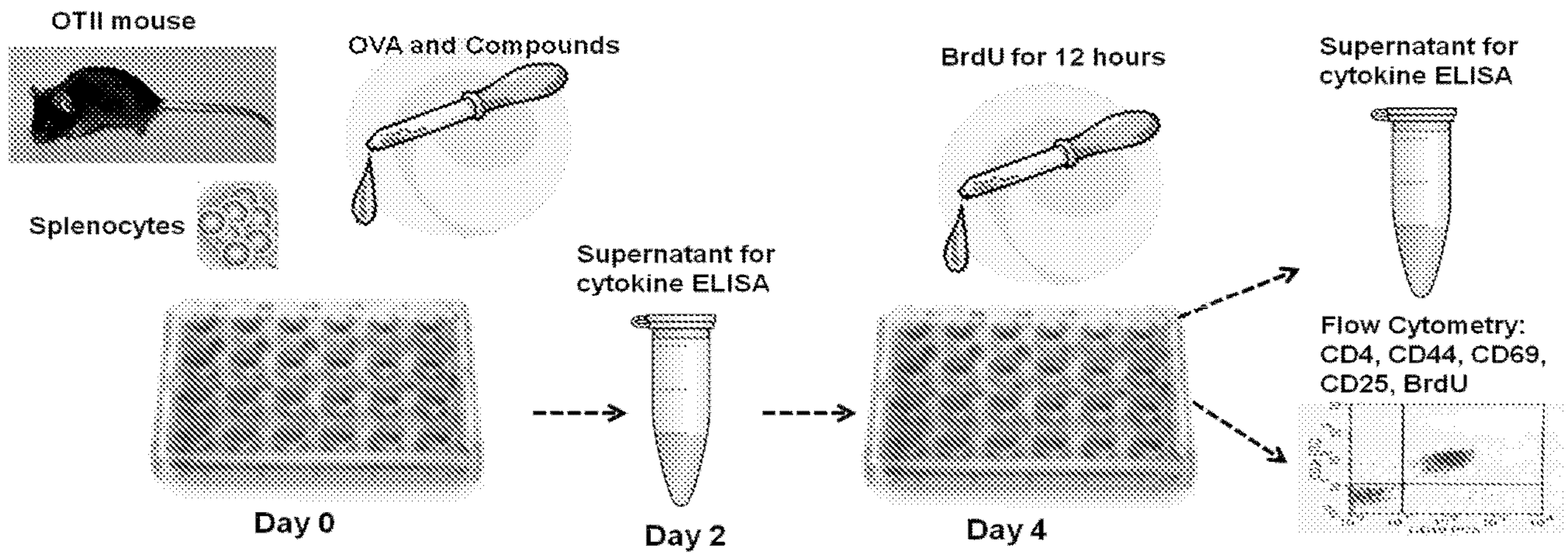
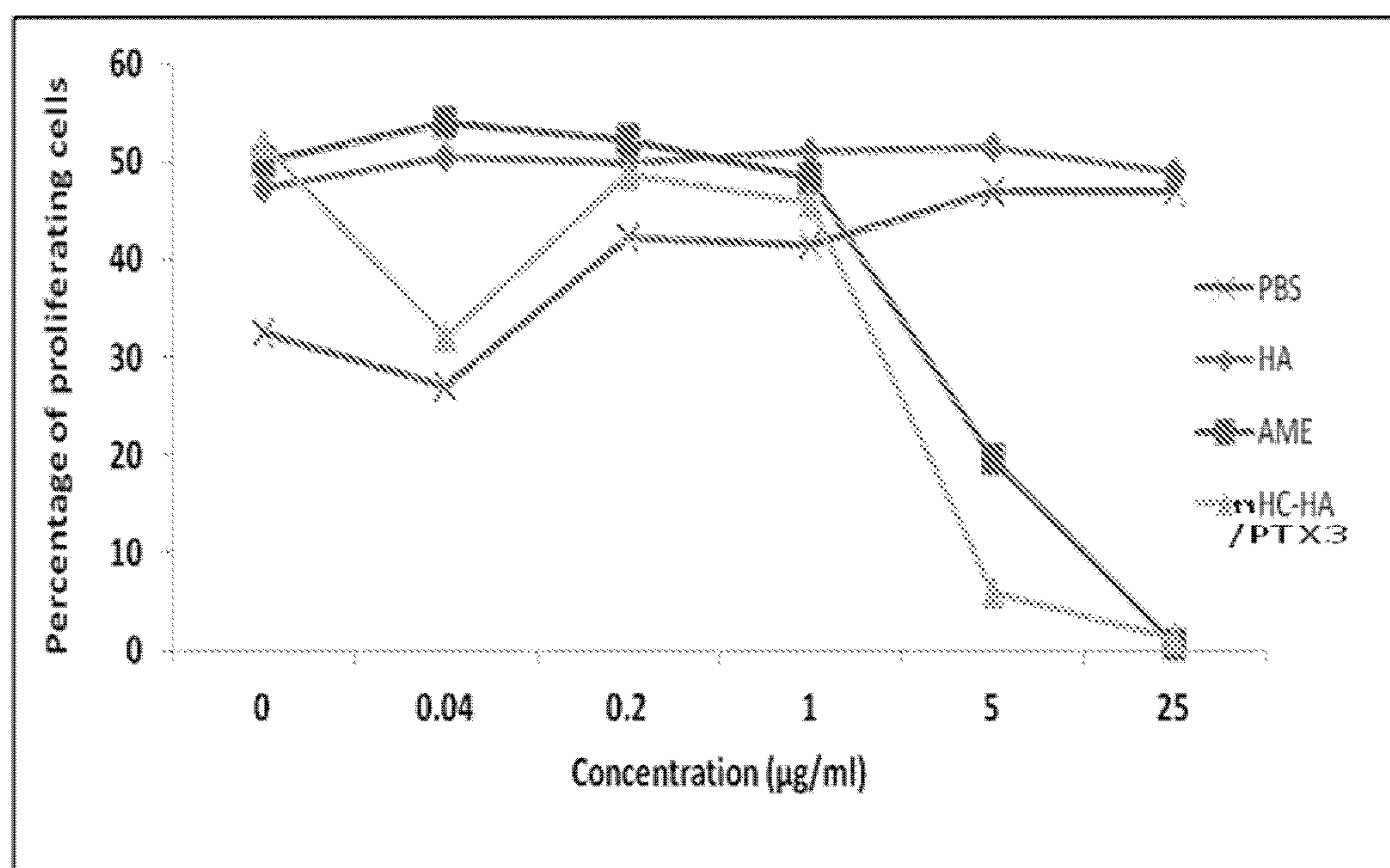
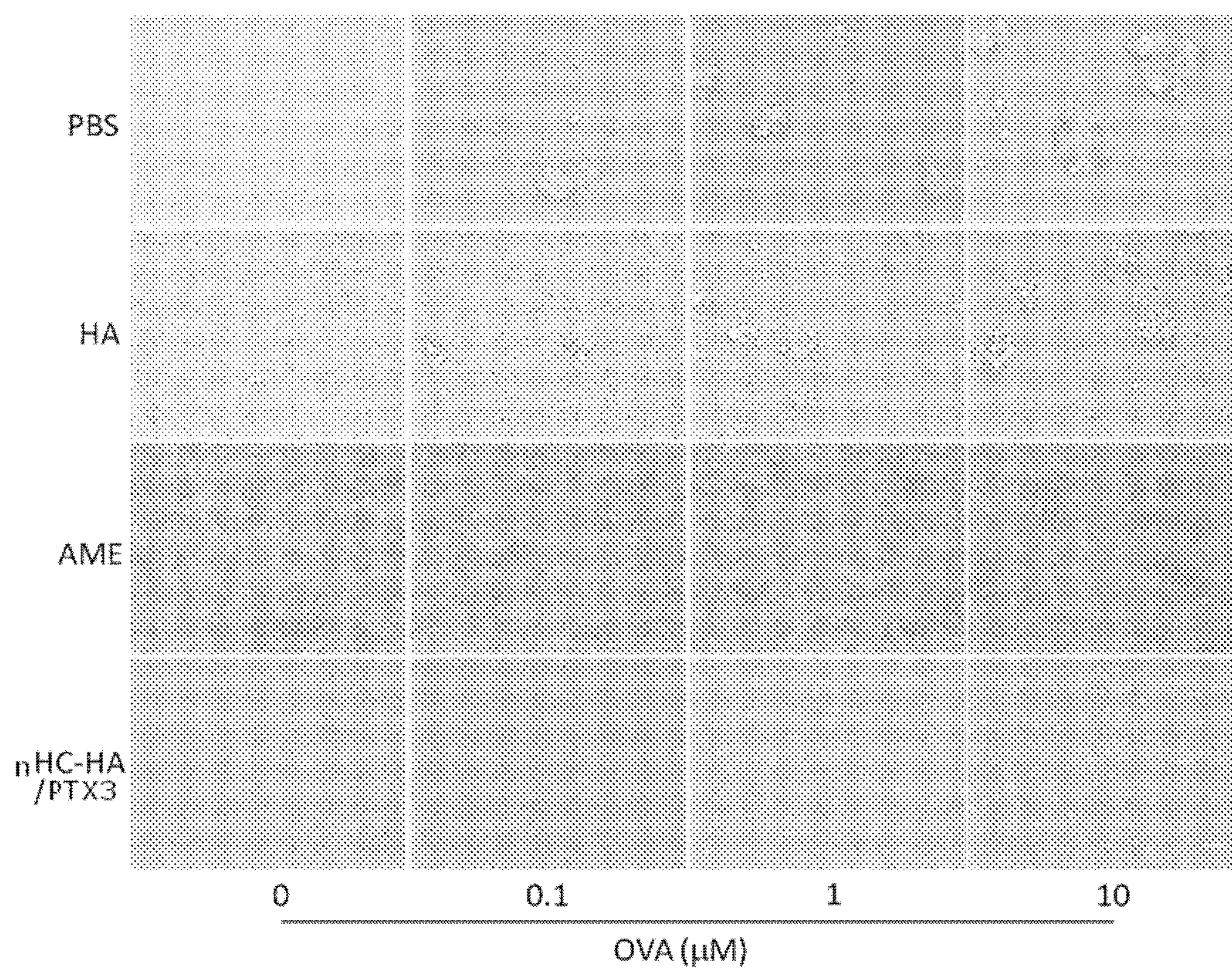
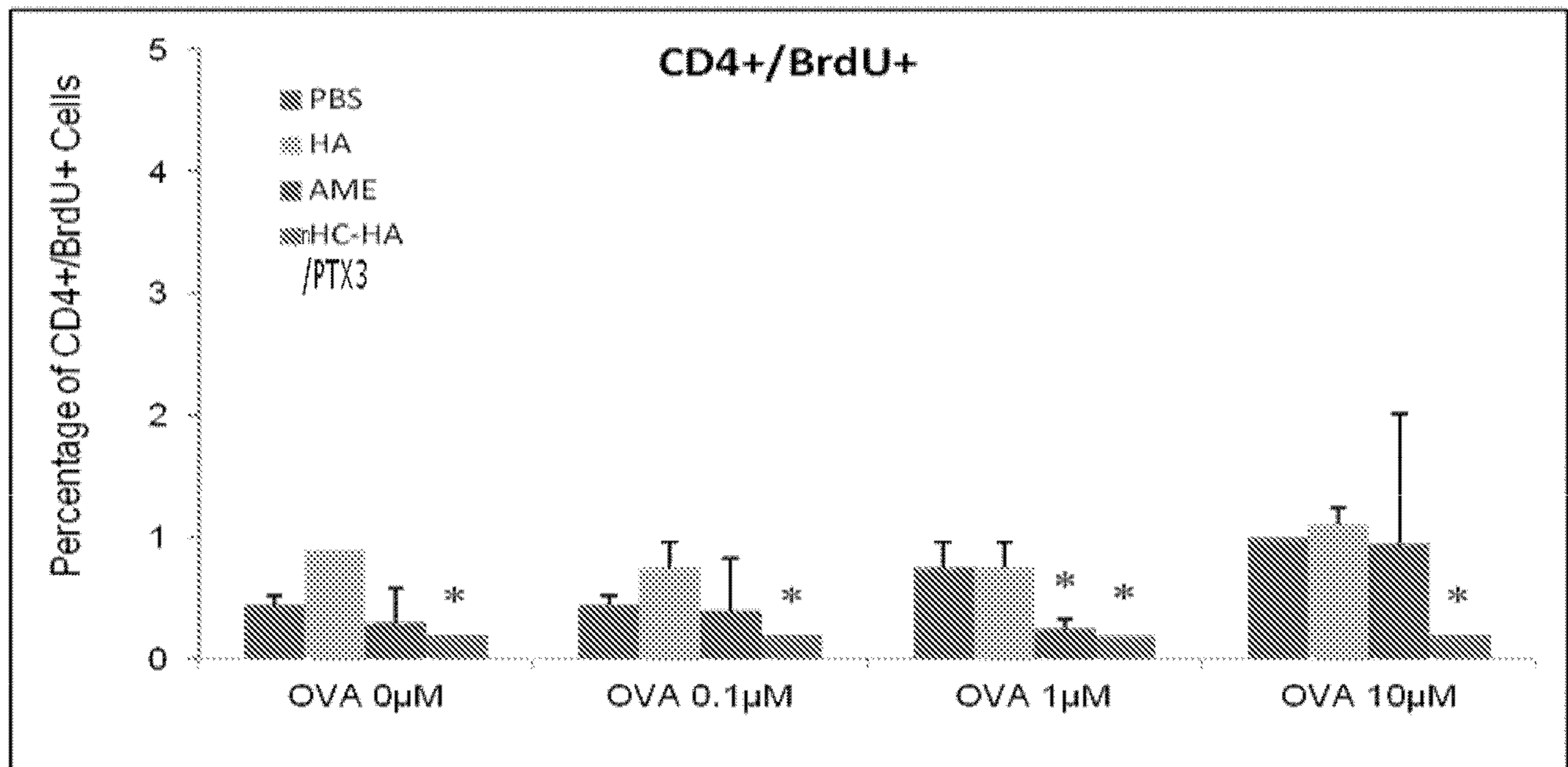
FIG. 82

FIG. 83

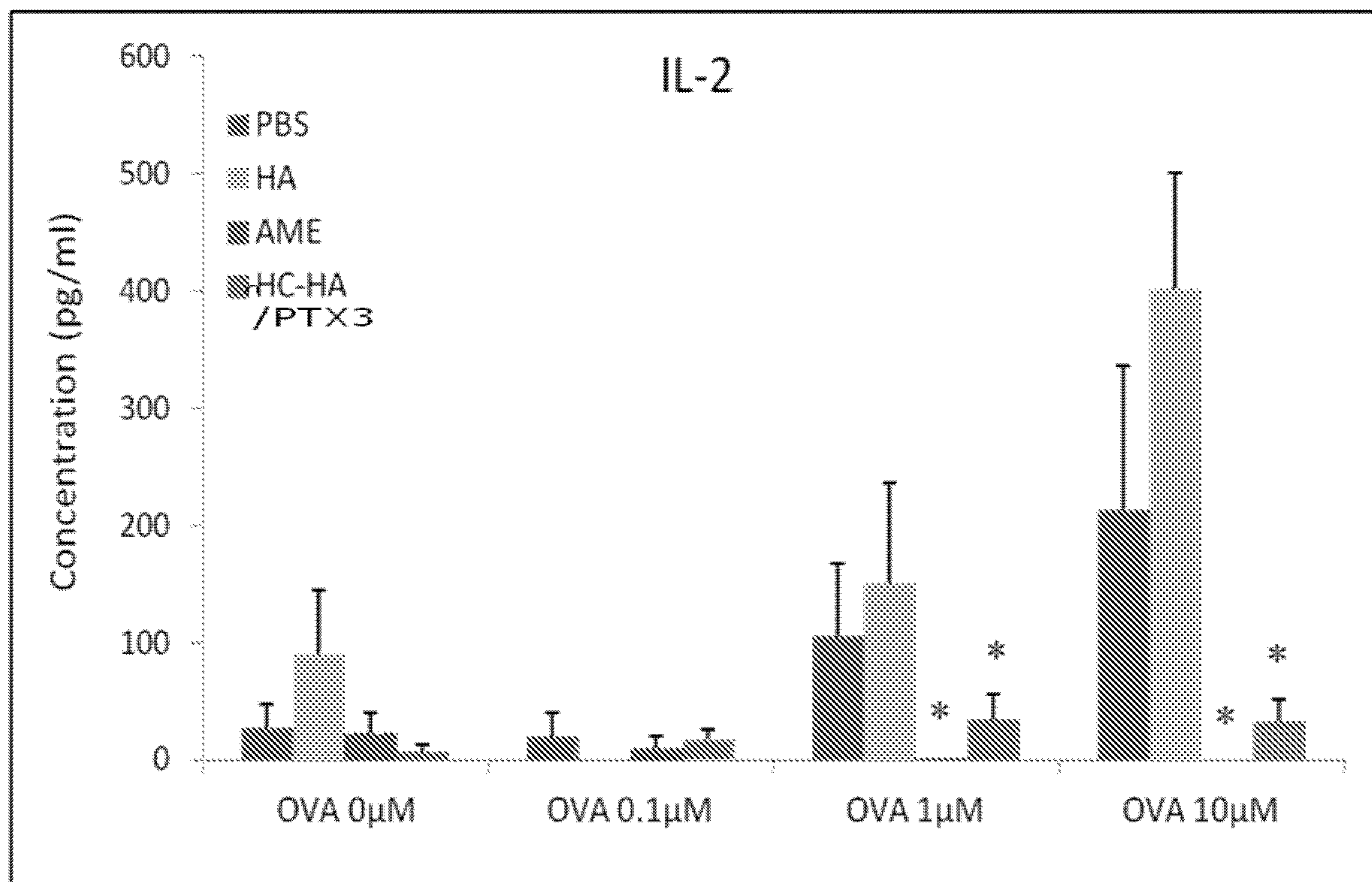
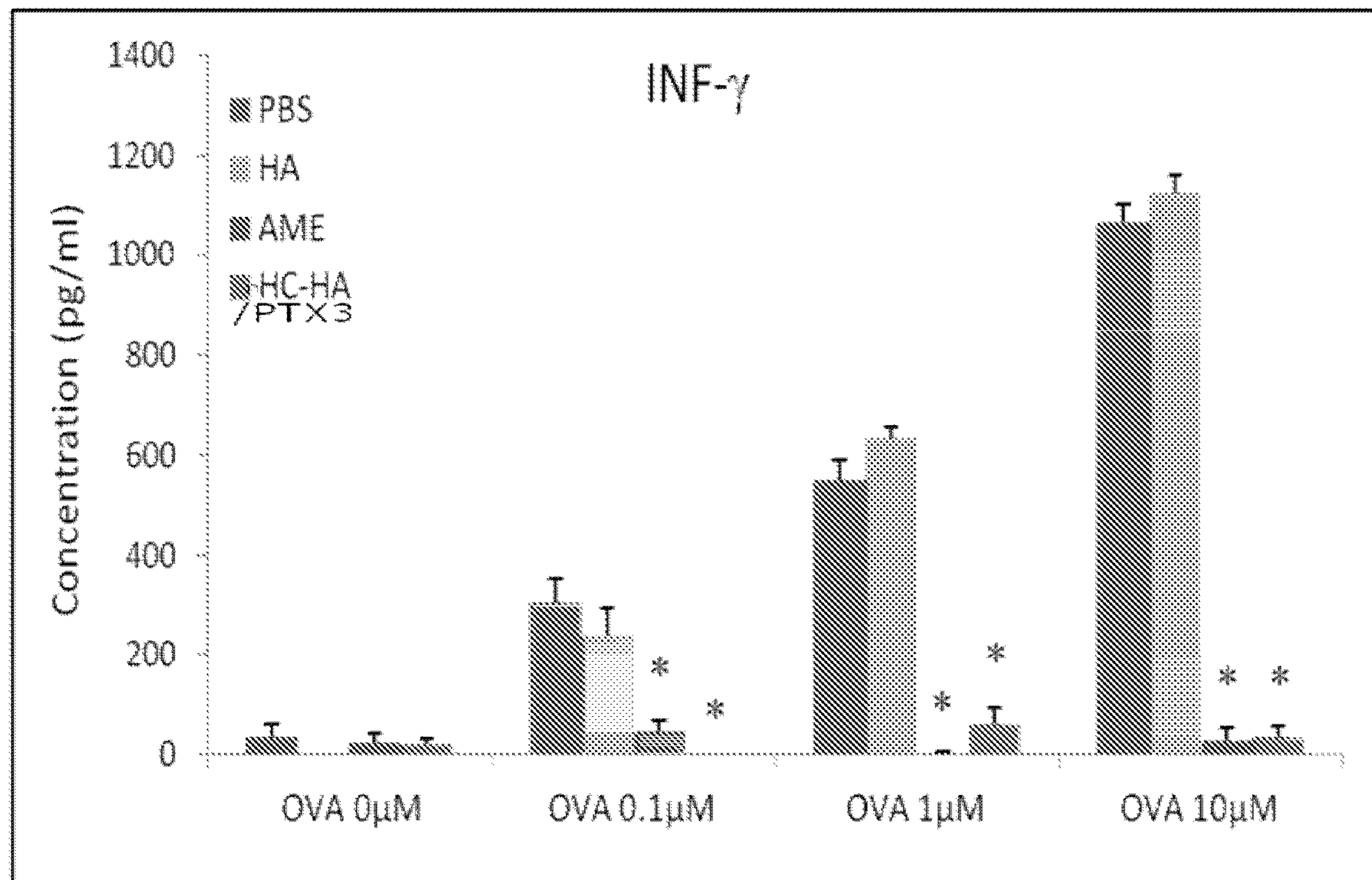
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FIG. 84

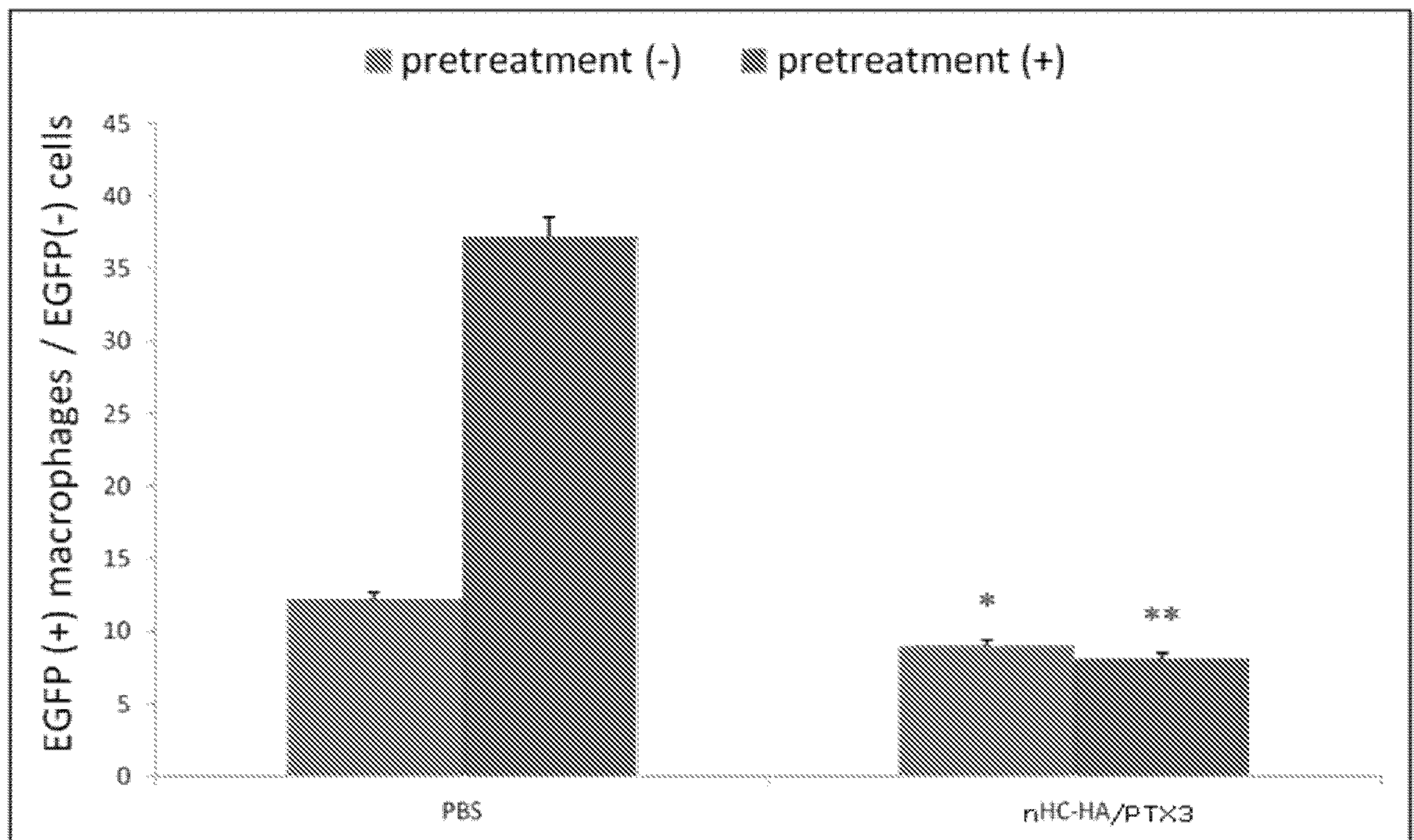
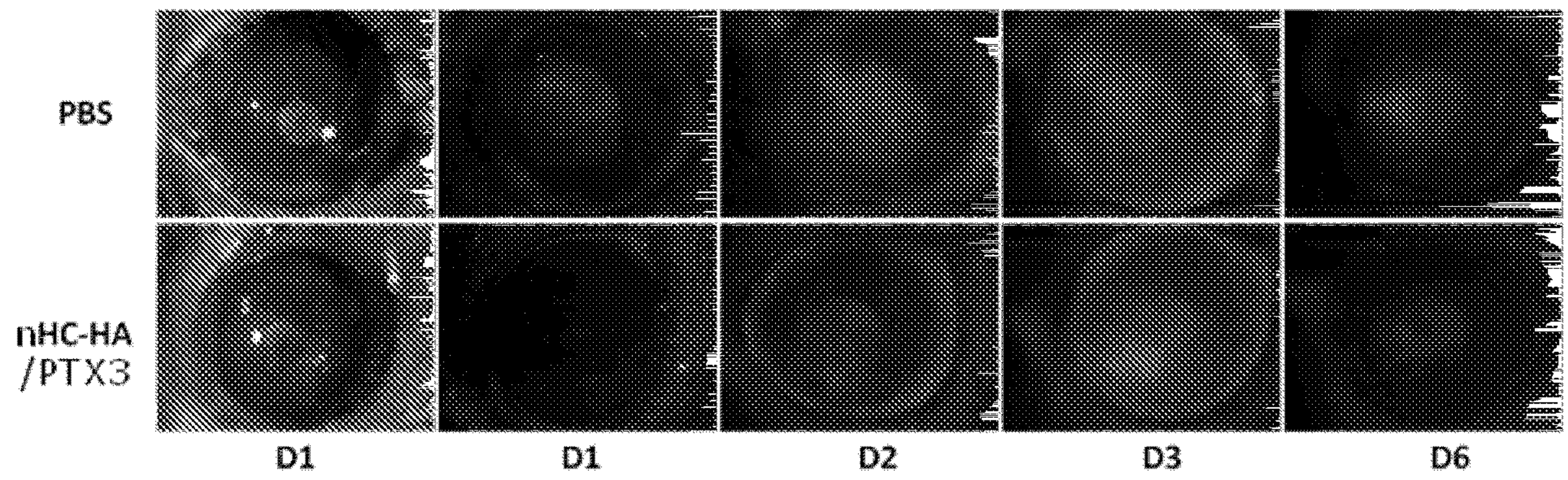
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FIG. 84 (cont'd)

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FIG. 85

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FIG. 86

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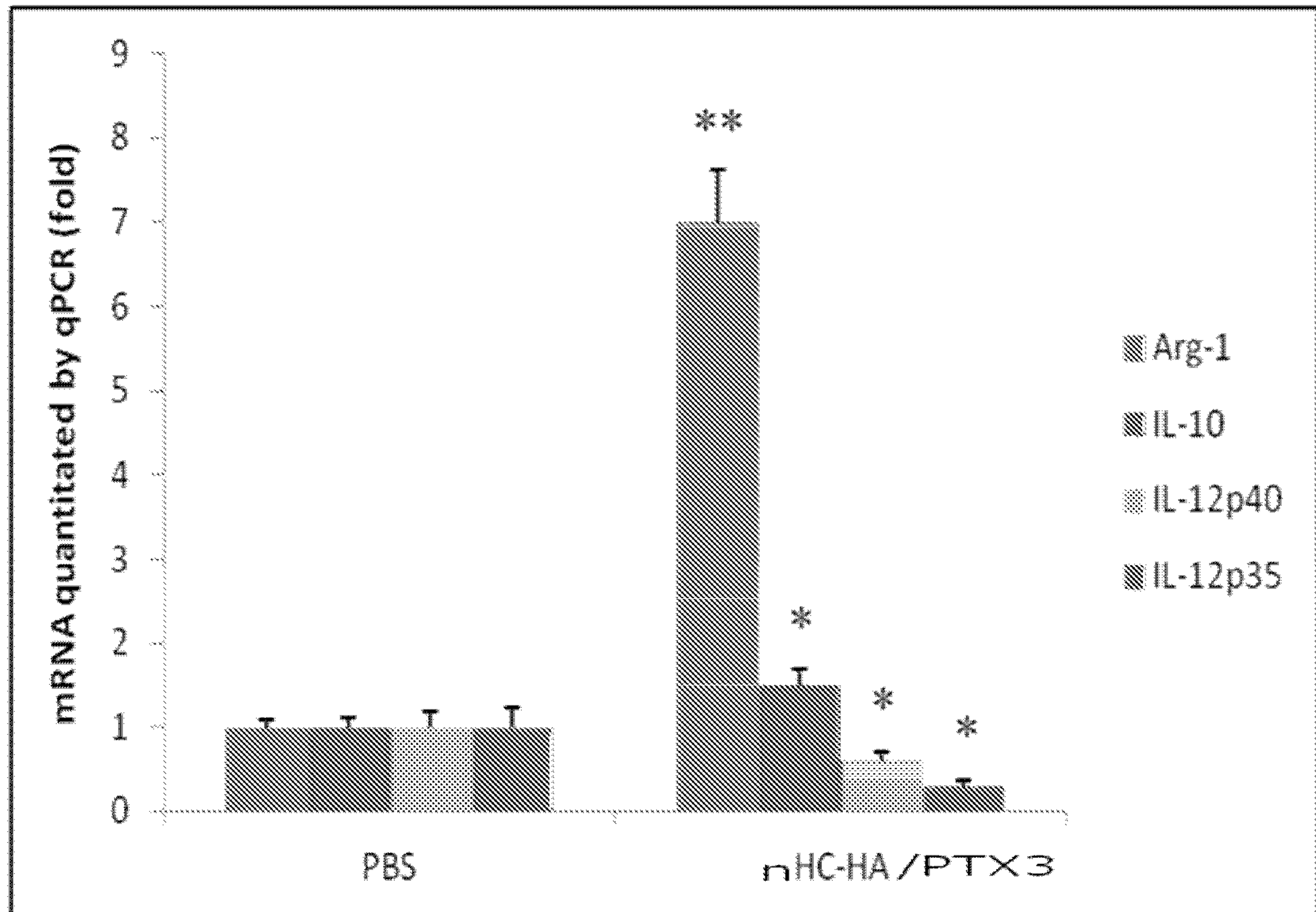
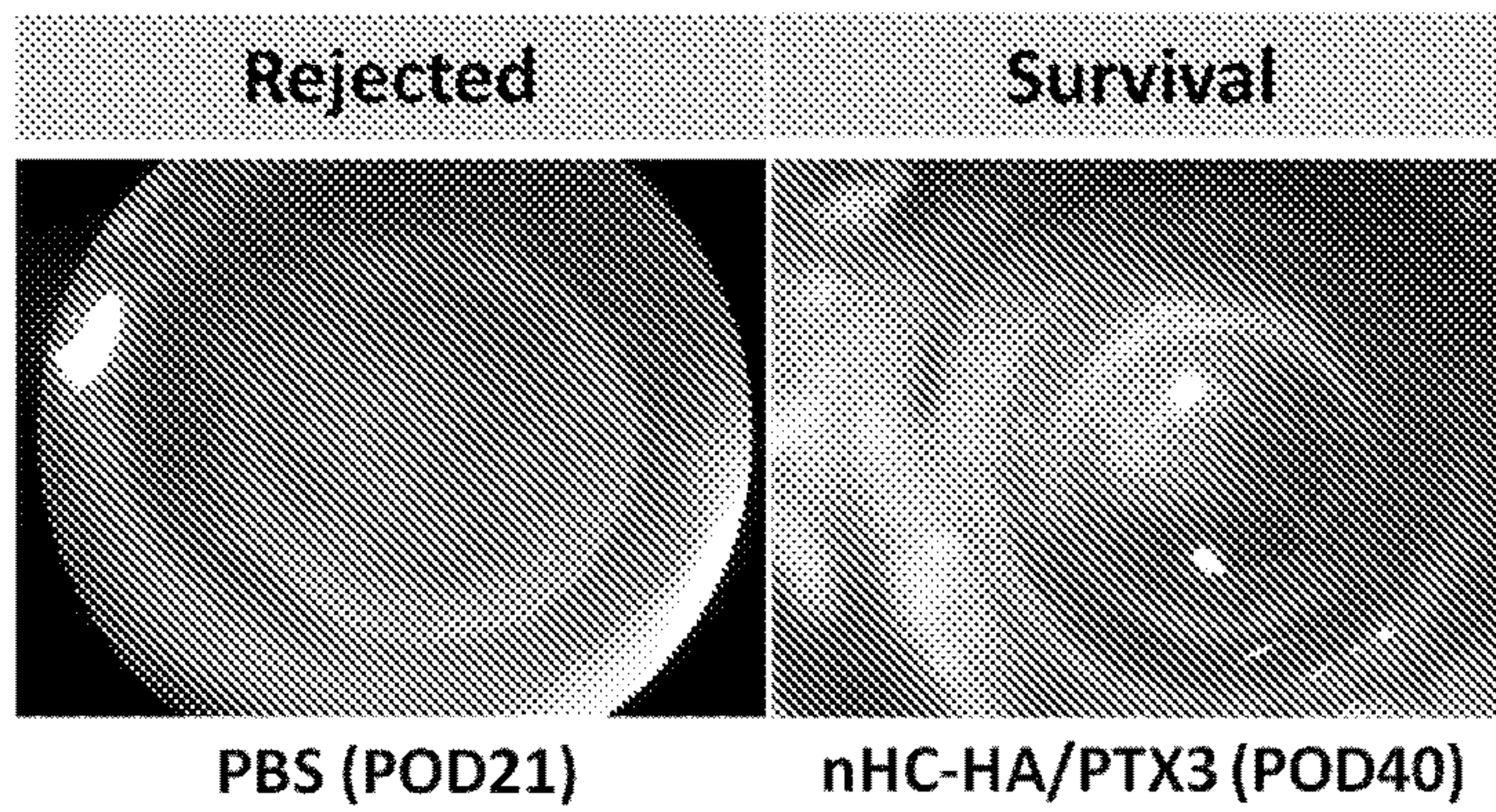
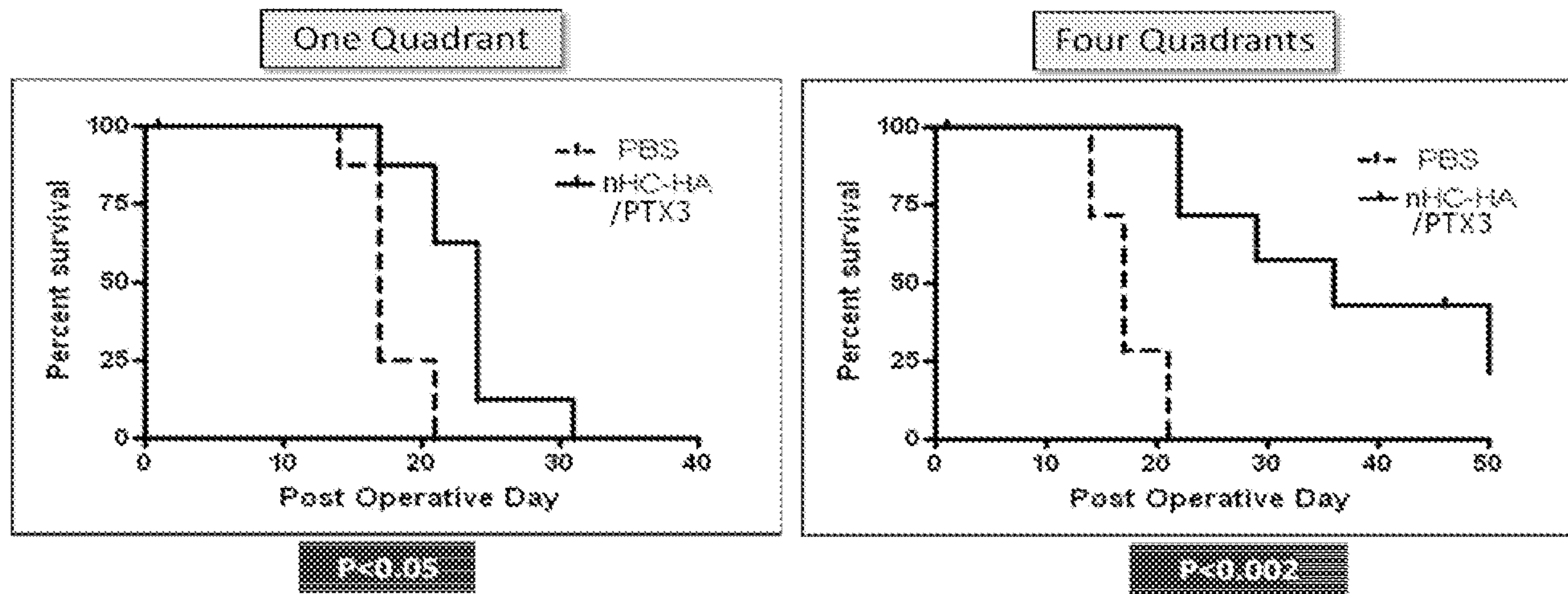
FIG. 87

FIG. 88

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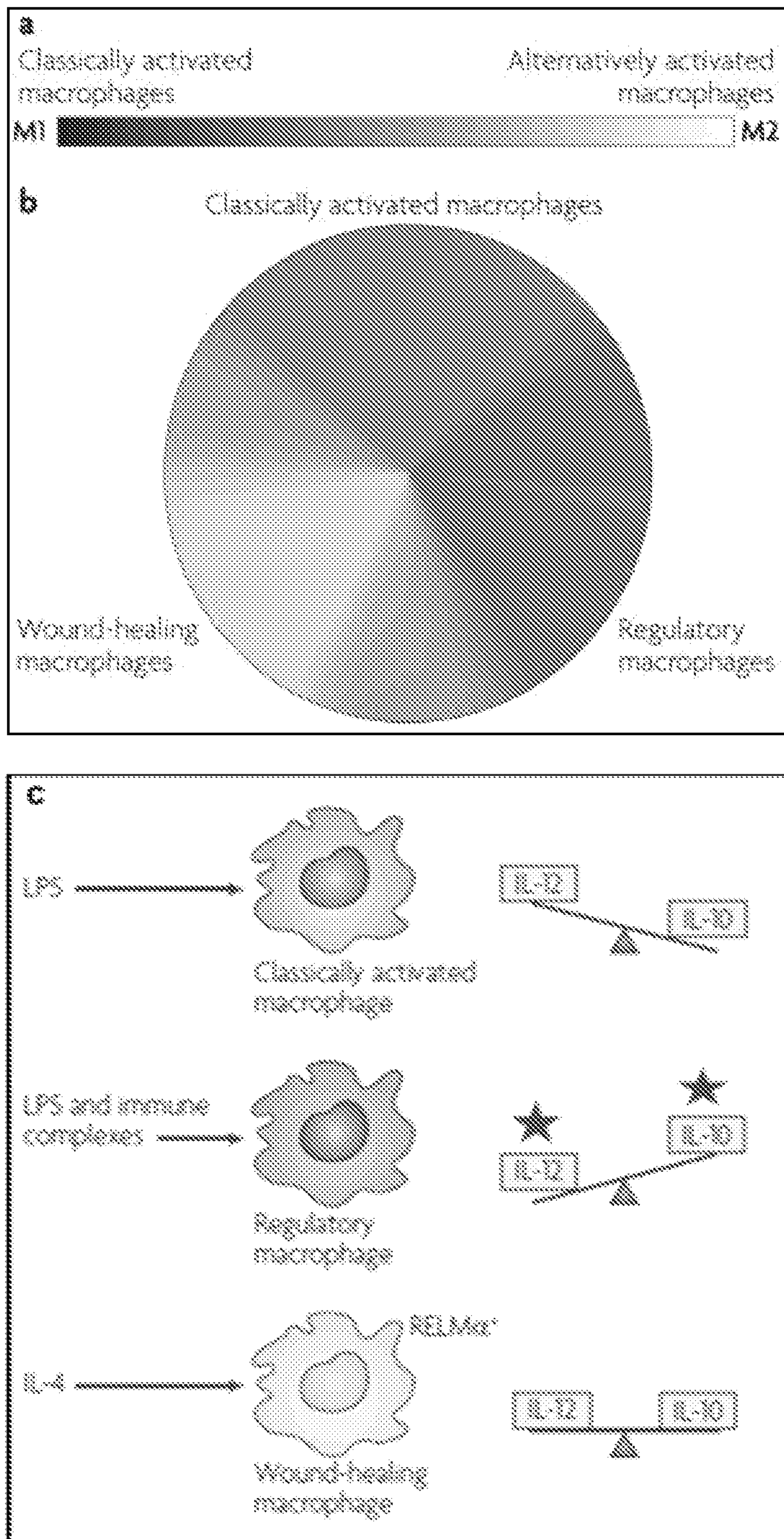
FIG. 89

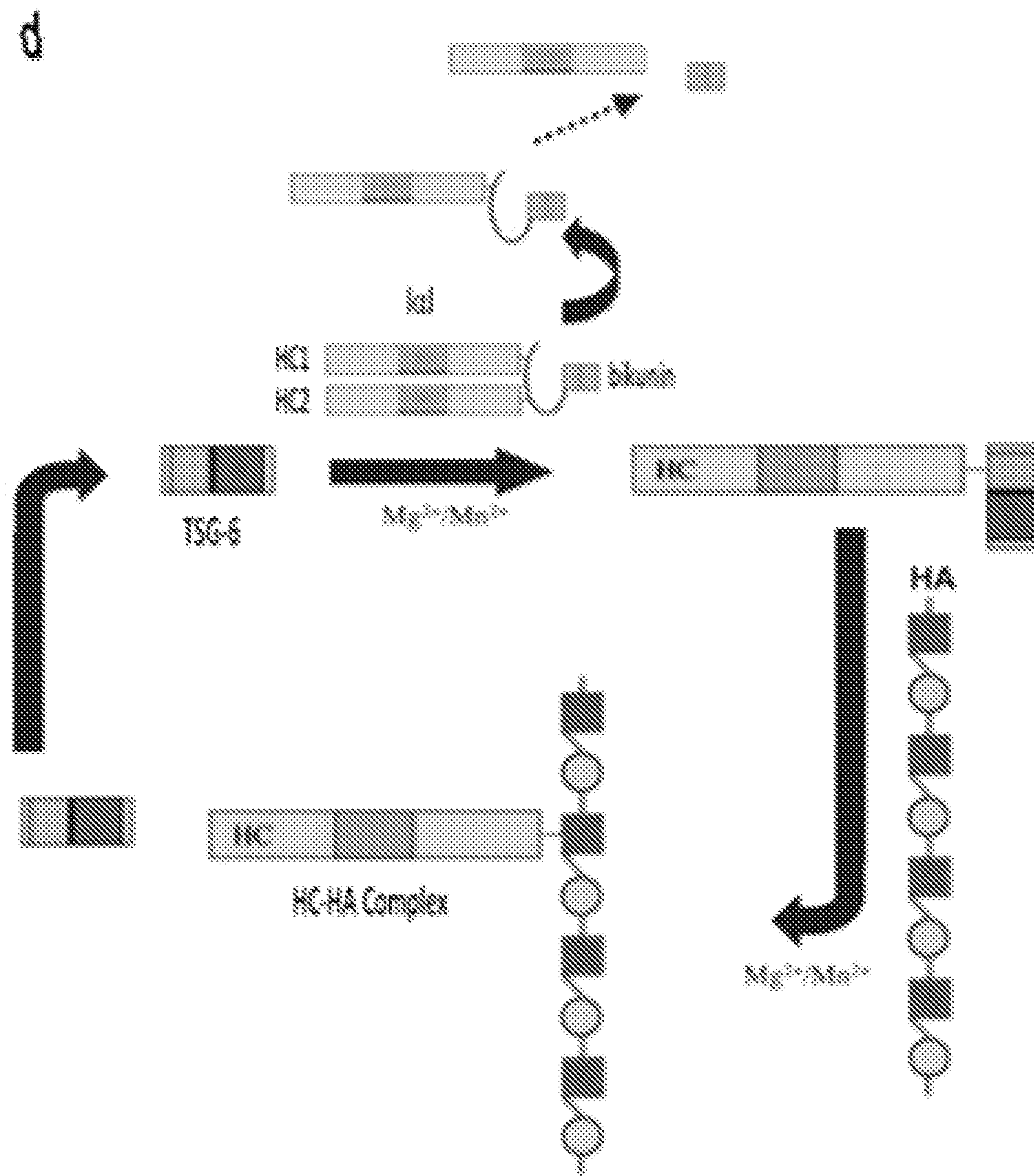
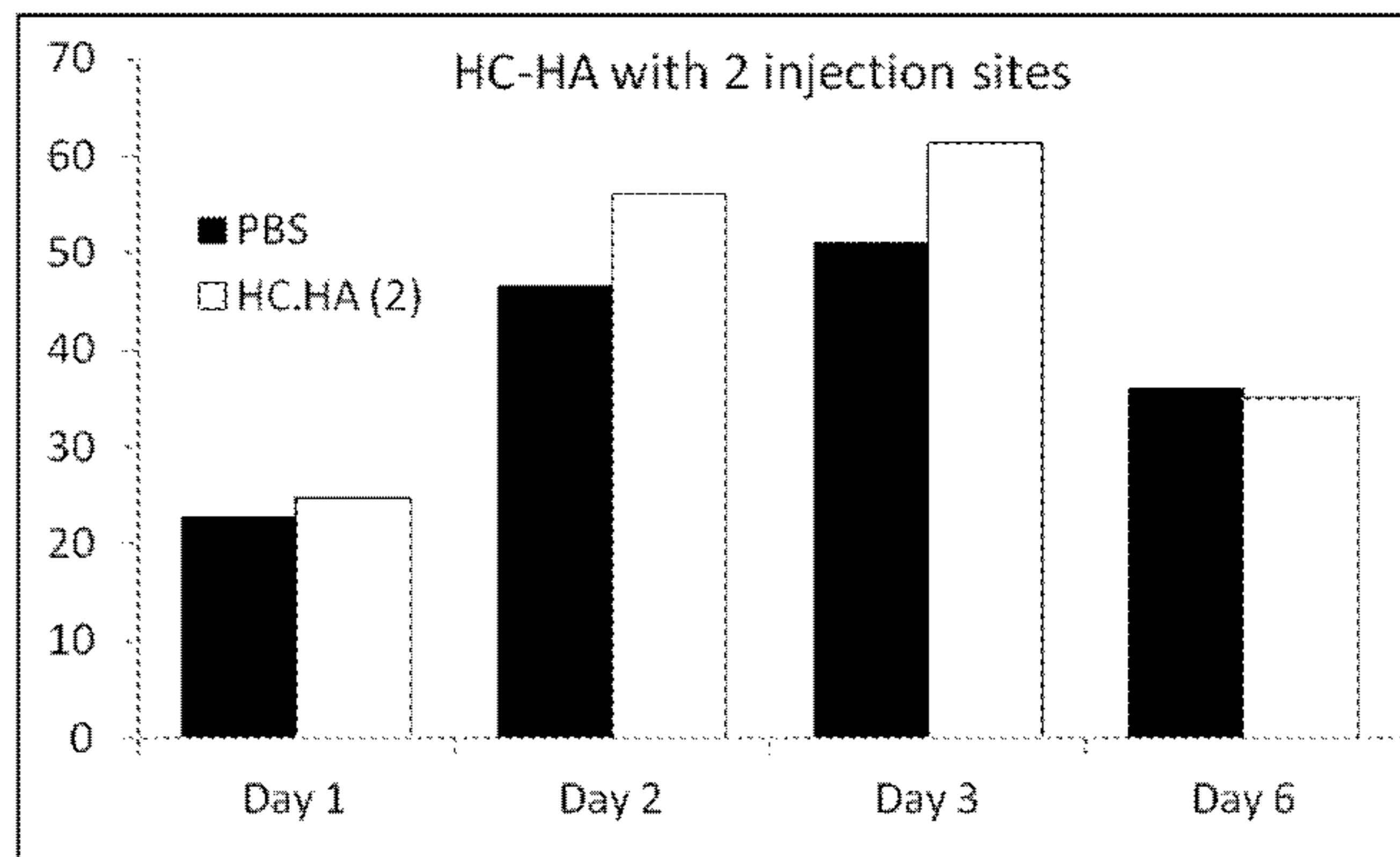
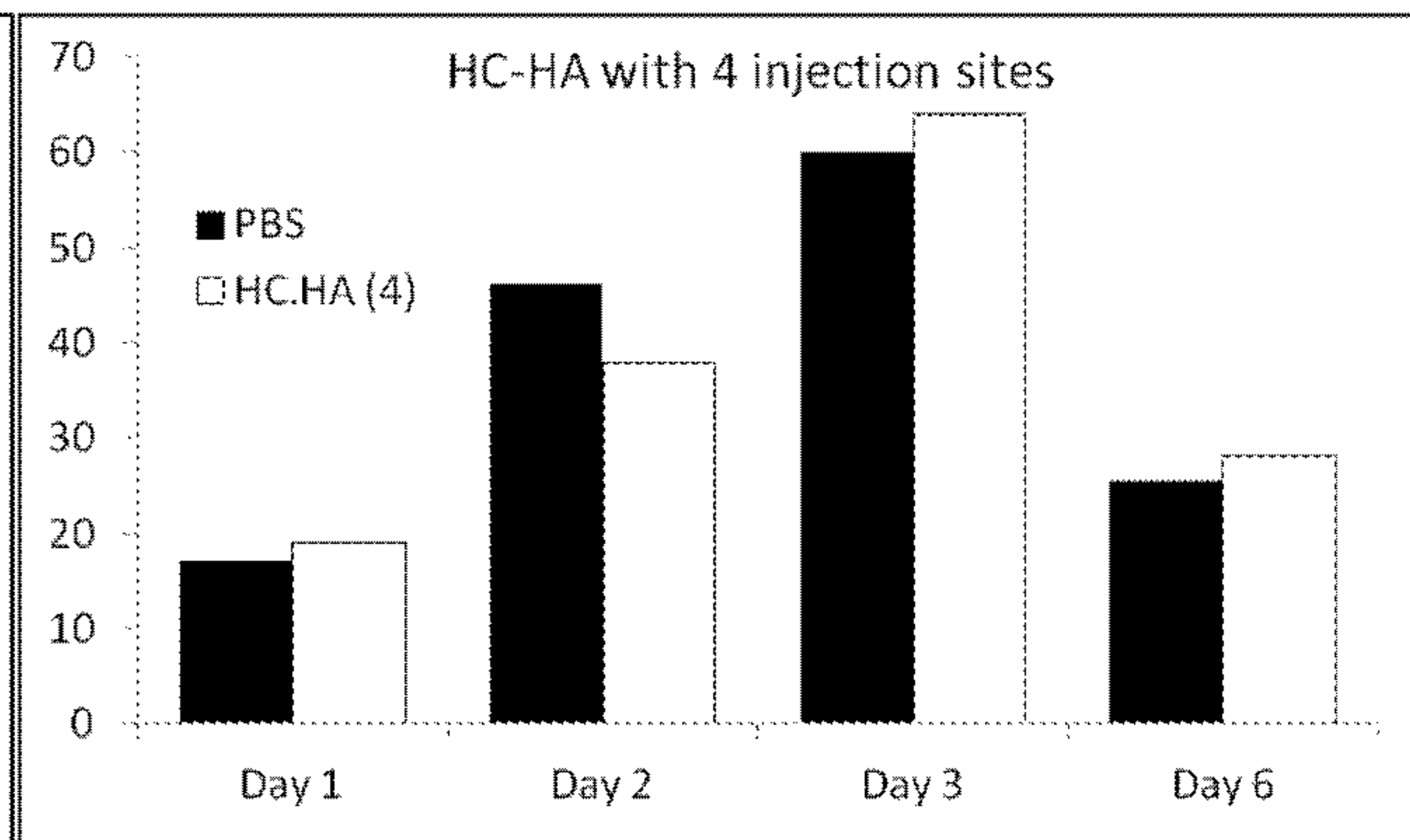
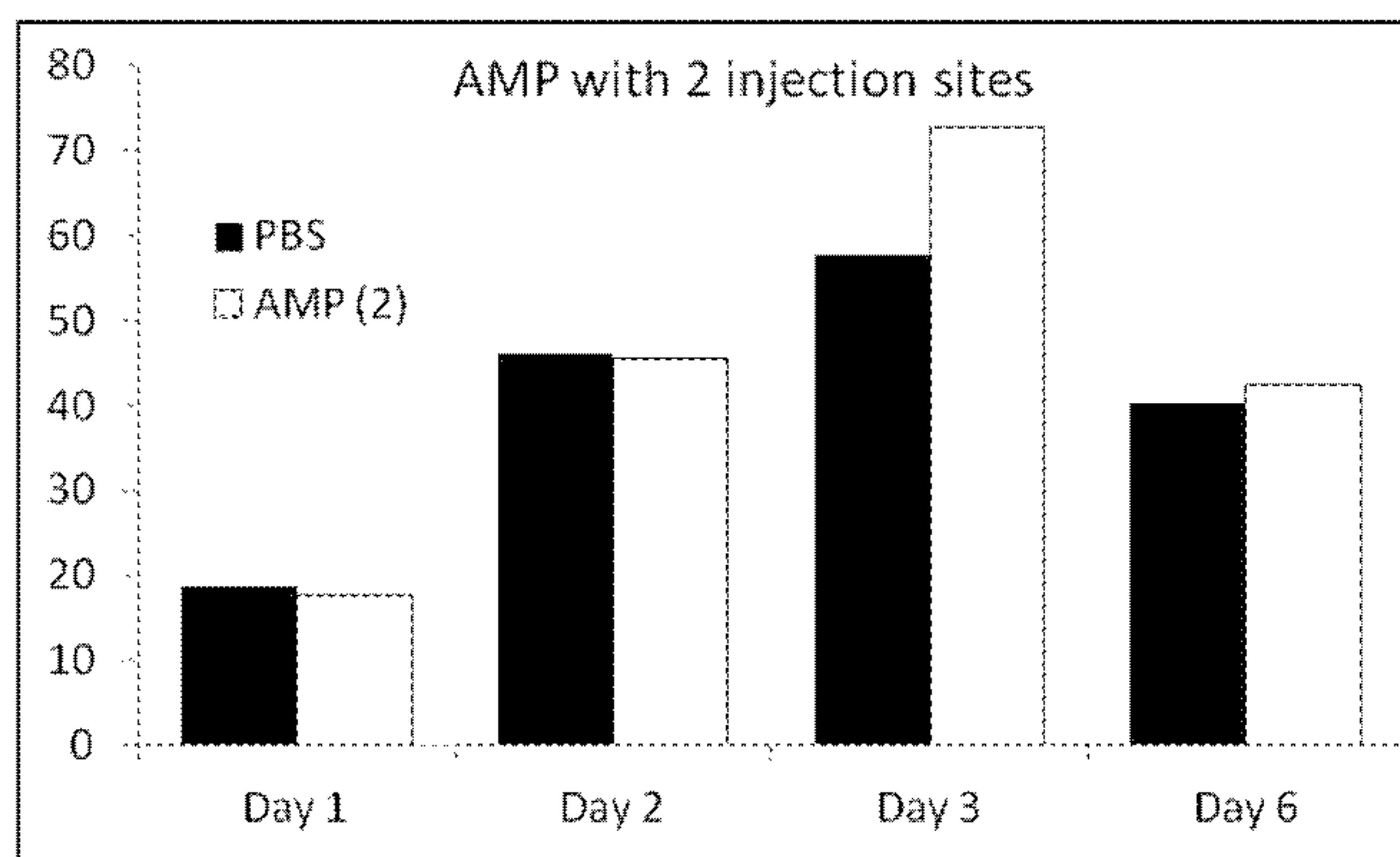
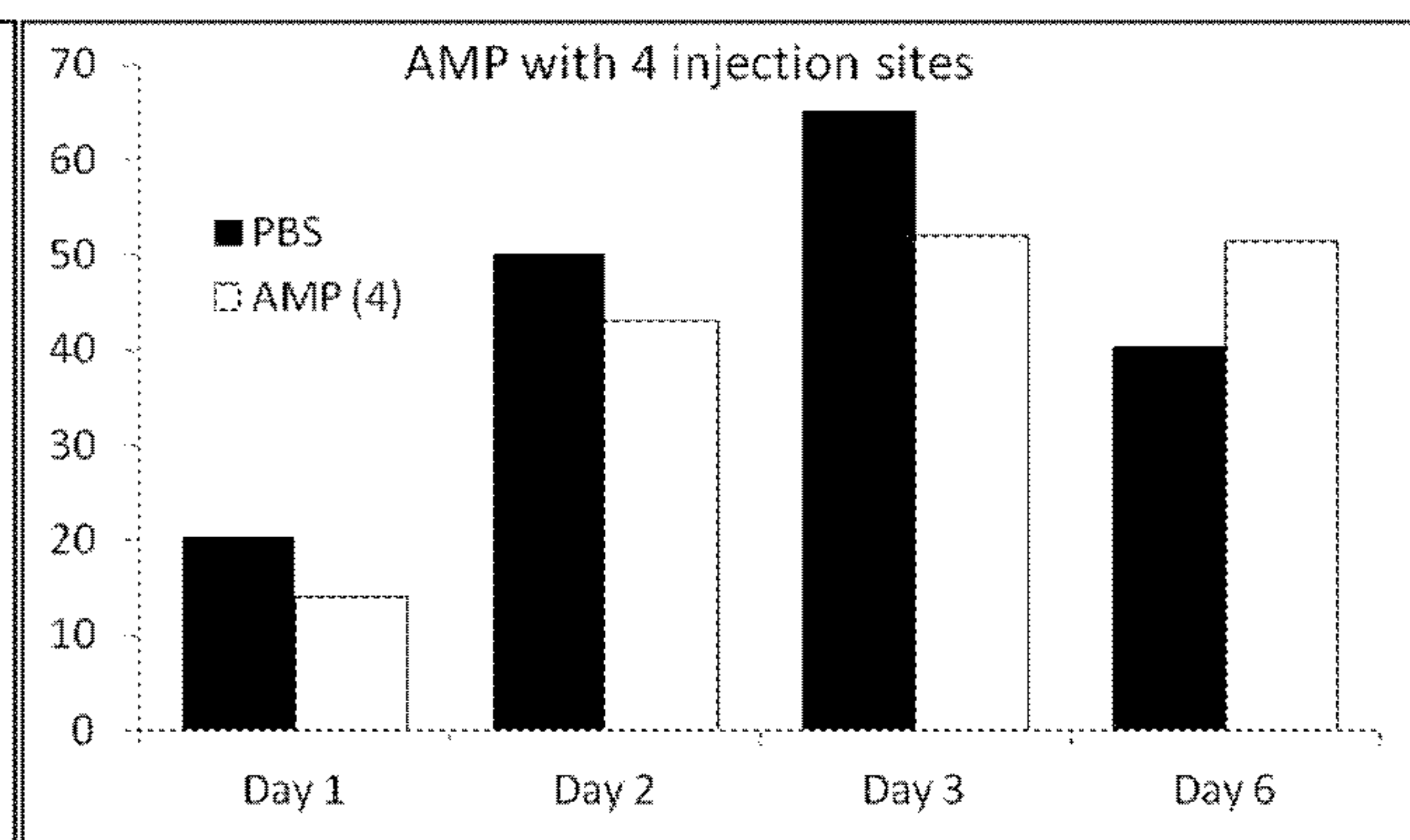
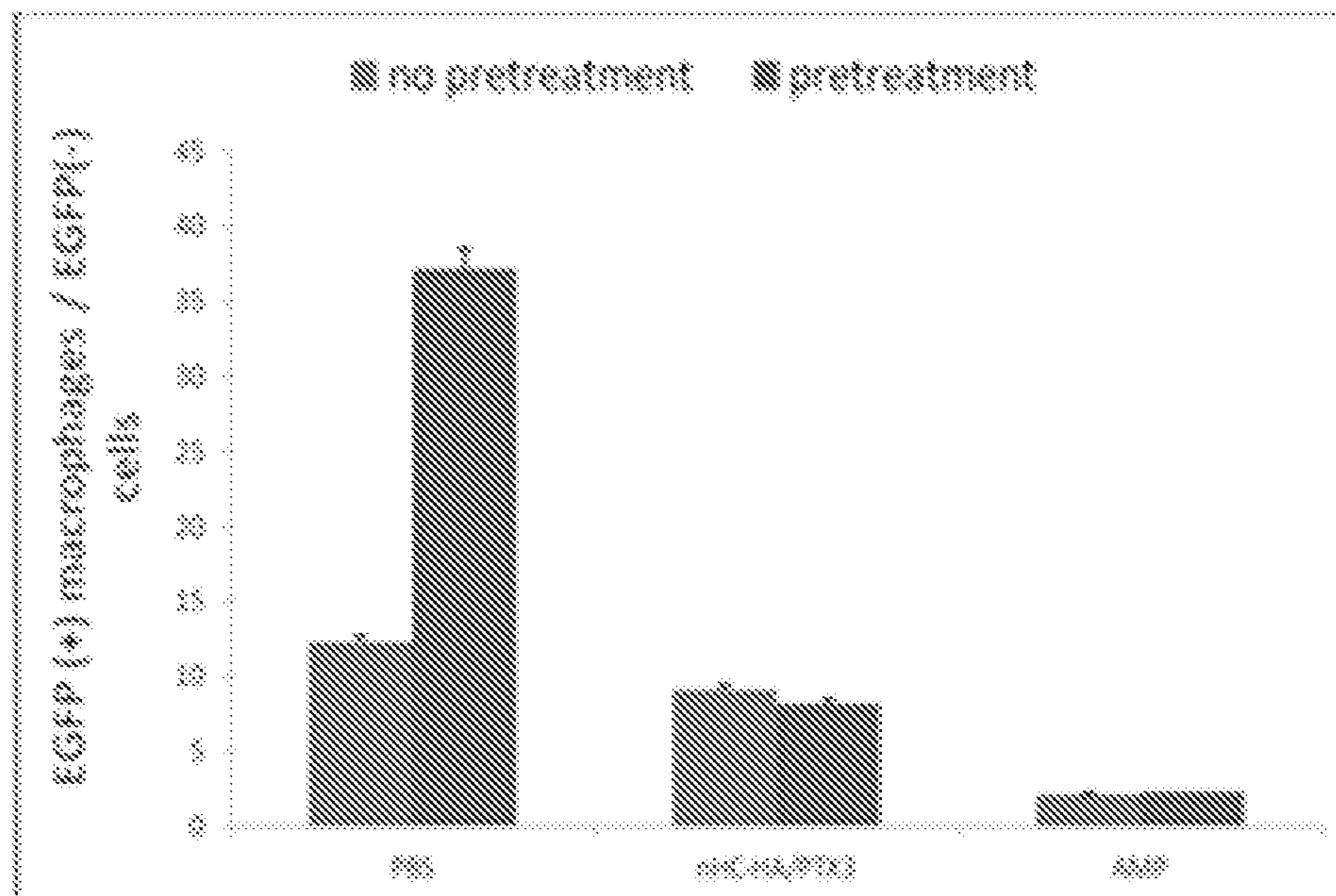
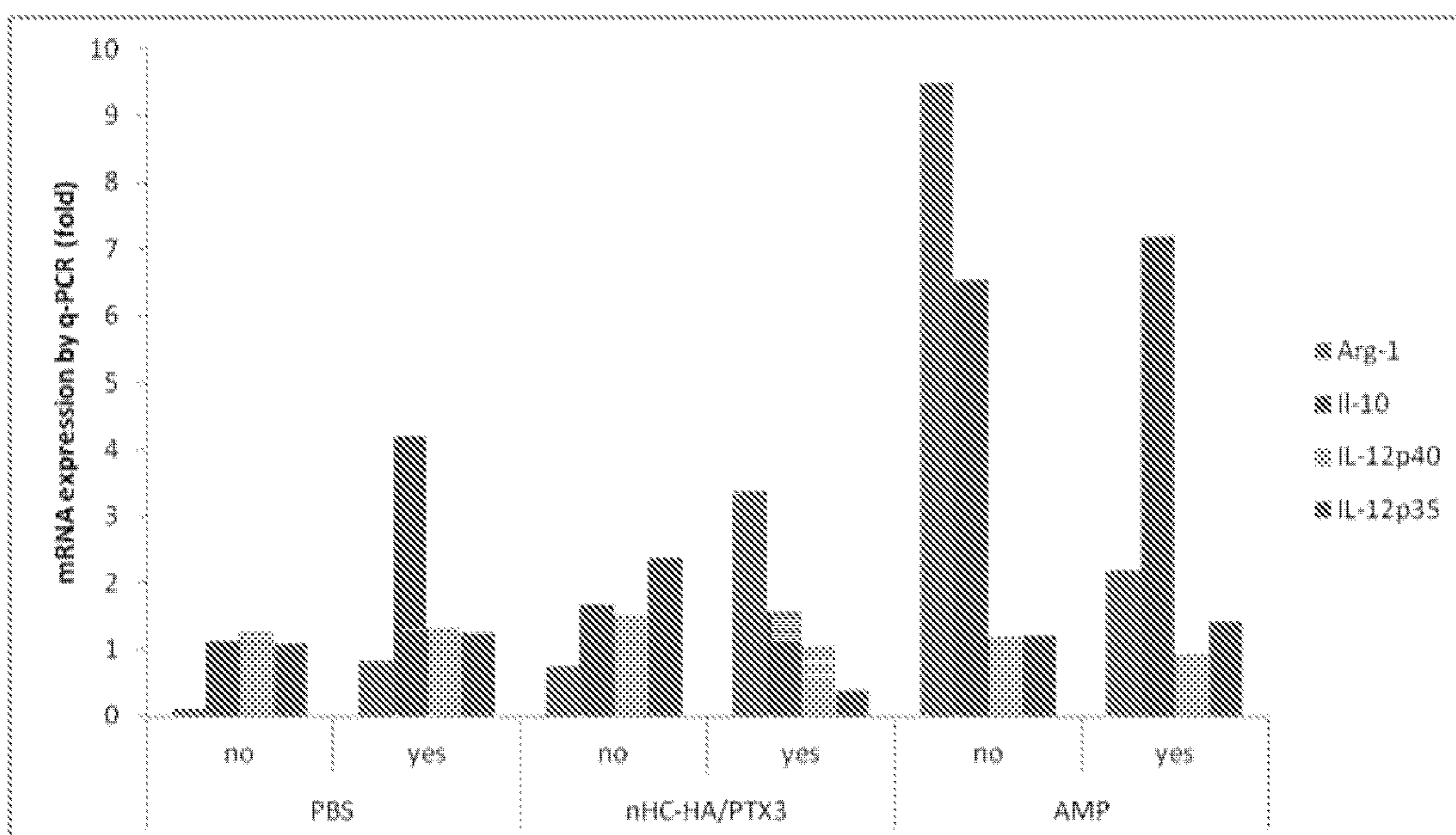
FIG. 89 (cont'd)

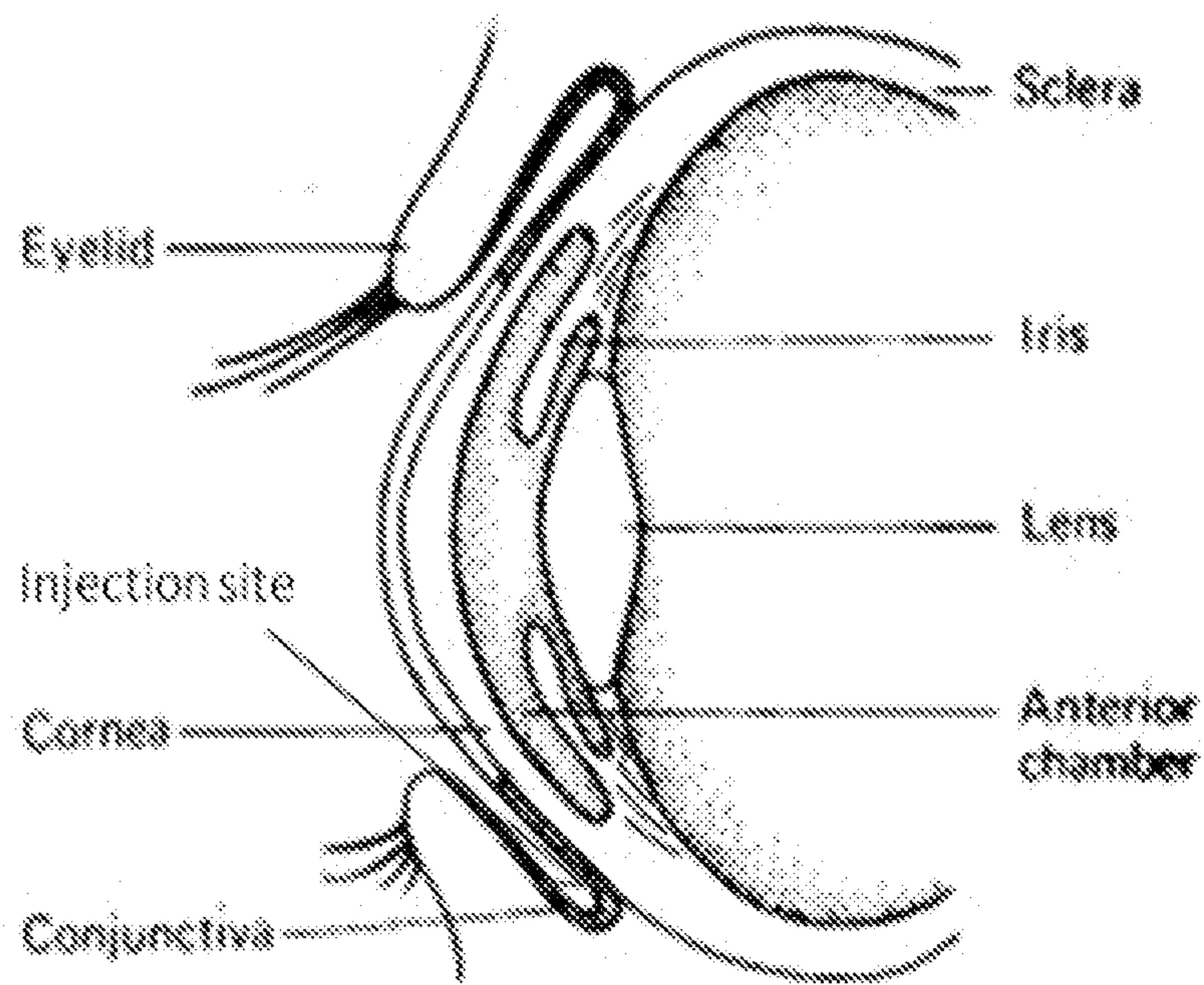
FIG. 90**A****B****C****D**

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FIG. 91

A**B**

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FIG. 92

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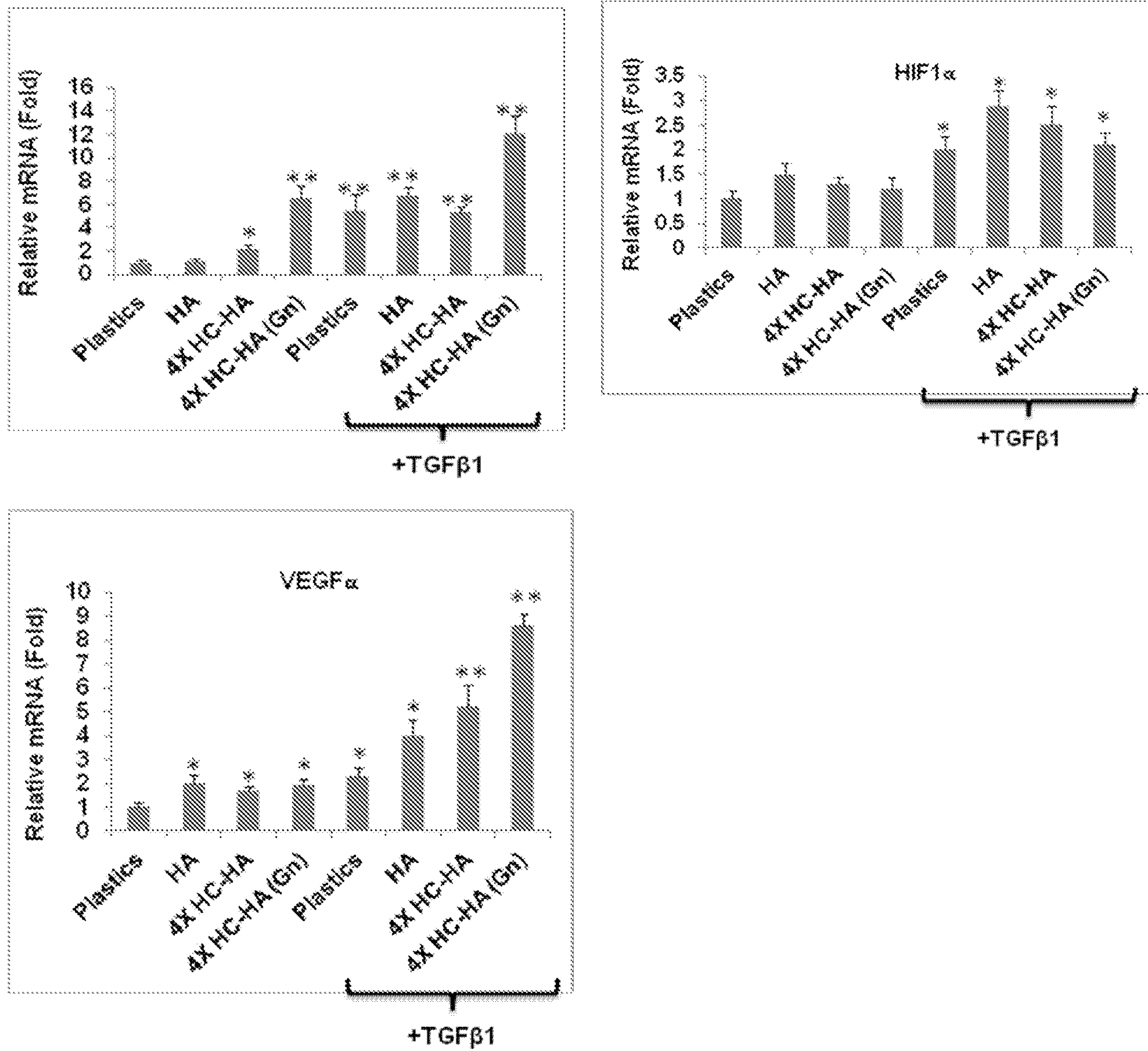
FIG. 93

FIG. 3

