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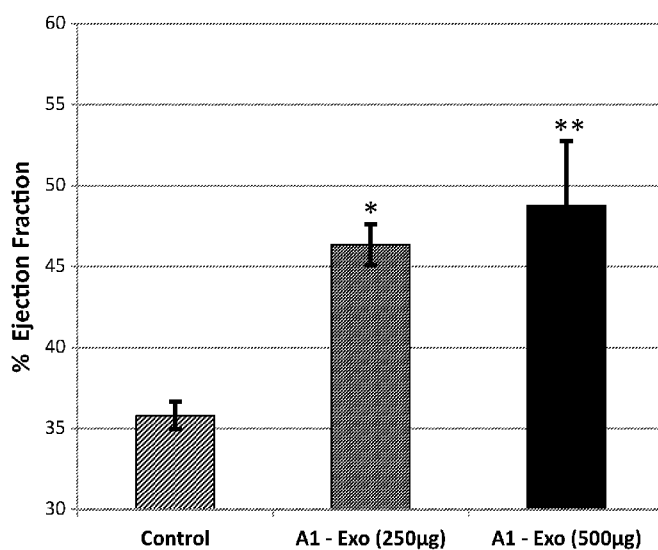
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(54) Title: URODELE EXOSOMES AS THERAPEUTIC AGENTS

Fig. 14

(57) Abstract: Described herein are composi-
 tions and techniques related to generation and
 therapeutic application of urodele cell derived
 exosomes. Urodeles such as newts and salaman-
 ders, are highly regenerative animals. There is in-
 creasing evidence that exosomes are vital actors
 potentiating repair and regenerative activity.
 Here, it is described that urodele derived cellular
 exosomes are bioactive in mammals, and promote
 rat cardiomyocyte proliferation, increase SDF-1
 secretion by human dermal fibroblasts, and im-
 prove functional recovery after myocardial infarct
 in rats. Described herein are compositions and
 techniques allowing for protection against and/or
 reversal of disease pathology in heart and vascu-
 lar disease.



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URODELE EXOSOMES AS THERAPEUTIC AGENTS**GOVERNMENT SUPPORT**

This invention was made with government support under NIH R01 HL124074 awarded by The National Institutes of Health. The government has certain rights in the invention.

FIELD OF THE INVENTION

Described herein are compositions and techniques allowing for protection against and/or reversal of disease pathology in heart and vascular disease.

15

BACKGROUND

The death of cardiac myocytes is a major cause of myocardial infarct and heart failure. The potential of cardiac regeneration in adult mammals has been suggested by the emerging possibility of endogenous regeneration persisting into later development, contrary to existing views that such mechanisms do not persist after birth. Additionally, it is now established that what had been considered terminally differentiated cells actually possess significant plasticity, as demonstrated by studies reporting dedifferentiation of somatic cells into pluripotent stem cells, or transdifferentiation into cardiac myocytes. Other types of stem cells, such as cardiosphere-derived cells (CDCs) have shown a proven therapeutic benefit by possibly tapping into the aforementioned repair and regeneration mechanisms. Current understanding of their salutary benefit indicates that indirect mechanisms are responsible. Rather than simply replacing or supplanting damaged cells, cellular exosomes (the lipid bilayer nanovesicles secreted by cells when multivesicular endosomes fuse with the plasma membrane) are now understood as central actors in the maintenance, repair and regeneration processes. It is therefore suggested that endogenous cardiac regeneration mechanisms may similarly be governed, and could be exploited by, therapeutic approaches involving exosomes.

Understanding the contours of such mechanisms in mammals may benefit significantly from the remarkable cardiac regenerative processes of urodeles such as newts and salamanders, organisms that demonstrate a remarkable survival capacity even after removal of up to 20% of the heart by transection of the ventricular apex. Notably, in these organisms, pre-existing cardiac myocytes adjacent to the site appear to undergo a process of dedifferentiation, characterized by dissolution of sarcomeric structures, increasing amounts of deoxyribonucleic acid (DNA) synthesis consistent with proliferation, and appearance of

5 newly generated cardiac myocytes capable of functional integration. Remarkably, this replacement of cardiac tissue has been reported as appearing without permanent scarring. By recapitulating developmental cardiogenesis, it appears such organisms are capable of complete morphological and functional regeneration. By deciphering the molecular pathways underlying these mechanisms, new therapeutic avenues for application in mammals
10 may become feasible.

Described herein are compositions and methods involving exosomes derived from urodeles, which are demonstrated as capable of improving heart function in mammals. Secreted exosomes from a newt cell line are bioactive in mammals, as shown to promote rat cardiomyocyte proliferation, increase SDF-1 secretion by human dermal fibroblasts, and
15 improve functional recovery after myocardial infarct.

SUMMARY OF THE INVENTION

Described herein is a composition, including a plurality of exosomes isolated from urodele cells. In other embodiments, the plurality of exosomes comprise one or more
20 exosomes with a diameter of about 30 nm to 300 nm and are about 2-5 kDa. In other embodiments, the plurality of exosomes comprise one or more exosomes with a diameter of about 40 nm to 100 nm and are about 3 kDa. In other embodiments, the plurality of exosomes comprise one or more exosomes including one or more microRNAs selected from the group consisting of: miR-1469, miR-762, miR-574-3p, miR-574-5p, miR-3197, miR-4281, miR-
25 1976, miR-1307, miR-1224-3p, miR-187, miR-3141, miR-1268, miR-155, miR-122, miR-638, miR-3196, miR-223, miR-4267, miR-1281, miR-885-5p, miR-663, miR-let-7b, miR-29d, miR-144, miR-let-7e 143, miR-lrt-7g, miR-17a, miR-96, miR-125a-5p, miR-128, miR-720, miR-21, miR-9, miR-26b, miR-29b, miR-30c, miR-30b, miR-191, and miR-1b. In other embodiments, the plurality of exosomes comprise one or more exosomes including miR-96,
30 miR-29b, and miR-191. In other embodiments, the plurality of exosomes comprise one or more exosomes including one or more messenger RNAs selected from the group consisting of: PGM5-AS1, NKD2, SLC43A1, PHF20L1, DLNZ, AMH, ARHGEF15, SCARF1, CPSF2, CEP170B, C16orf11/PRR35, CPSF6, RNF39, C22orf26/PRR34, TMEM64, PRDM16, CHST7, ADCYAP1, VSIG10L, MUC22, SRMS, SAP30L, GAS6-AS1, ZFAND5, GAGE13,
35 LCN12, NR5A1, HLA6, LY6E, and SPIN4. In other embodiments, the urodele cells comprise *Notophthalmus viridescens* cells. In other embodiments, the *Notophthalmus viridescens* cells comprise A1 cell line. In other embodiments, the urodele cells are dedifferentiating, transdifferentiating and/or proliferating when isolating the plurality of

5 exosomes. In other embodiments, the composition includes a pharmaceutically acceptable carrier.

Further described herein is a method of treating a heart related disease, including administering a composition including a plurality of exosomes isolated from urodele cells to
10 a subject, thereby treating the subject. In other embodiments, the plurality of exosomes comprise one or more exosomes with a diameter of about 30 nm to 300 nm and are about 2-5 kDa. In other embodiments, the plurality of exosomes comprise one or more exosomes with a diameter of about 40 nm to 100 nm, and are about 3 kDa. In other embodiments, administering a composition includes 1×10^8 or more exosomes in a single dose. In other
15 embodiments, the single dose is administered multiple times to the subject. In other embodiments, the subject has a chronic disease. In other embodiments, administering a composition includes one or more of intra-arterial infusion, intravenous infusion, and injection. In other embodiments, the subject is diagnosed as afflicted with a heart related disease prior to administering the composition. In other embodiments, the urodele cells
20 comprise *Notophthalmus viridescens* cells. In other embodiments, the *Notophthalmus viridescens* cells comprise A1

BRIEF DESCRIPTION OF FIGURES

Figure 1. Differential Expression of microRNAs in Cardiosphere-Derived Cell (CDC) Exosomes. (Fig. 1A) MicroRNA analysis of CDC-derived exosomes demonstrate
25 the differential cargo contents of exosomes based on parental cellular origin. Fold changes of microRNA abundance in CDC exosomes compared to normal human dermal fibroblasts (NHDF) exosomes (n = 4 independent experiments). Total RNA (including microRNAs) was isolated from CDC exosomes and NHDF exosomes. qRT-PCR was performed on an
30 microRNA array. **(Fig. 1B)** Venn diagram showing the variable microRNA profile between CDC and NHDF exosomes. Font size reflects the magnitude of differential expression of each microRNA.

Figure 2. Isolation of Exosomes from CDCs. (Fig. 2A) Graphical representation of
exosome isolation and purification for exosomes. **(Fig. 2B)** Cell viability (calcein) and cell
35 death (Ethidium homodimer-1) assay performed on CDCs over the 15 day serum-free conditioning period. **(Fig. 2C)** Representative images of CDCs before and after serum-free conditioning.

Figure 3. Heat Map or microRNA PCR Array Identifies Mir-146a as a Highly Differentially Expressed microRNA. Heat map showing fold regulation differential abundance data for transcripts between CDC exosomes and NHDF exosomes overlaid onto the PCR Array plate layout.

Figure 4. MicroRNAs reported to be upregulated following cardiac injury. Heat map showing urodele microRNAs conserved with human, frog, pipid frog, zebrafish microRNAs that are enriched 7 and 21 days post-injury (dpi). From Witman *et al.*, “miR-128 regulates non-myocyte hyperplasia, deposition of extracellular matrix and Islet1 expression during newt cardiac regeneration.” *Dev Biol.* 2013 Nov 15;383(2):253-63.

Figure 5. Culturing of A1 cell line. (Fig. 5A) Representative light (top panels) and confocal microscopy images (bottom panels) demonstrating A1 cells in growth media (left panels) and under serum-free conditions (right panels). Immunofluorescent images of blastema marker 12/101 under the normal and serum-starved conditions. Scale bar = 50µm. **(Fig. 5B)** Nanosight histogram of A1 exosome diameter and number **(Fig. 5C)** Graphical representation of exosome size and concentration per milliliter **(Fig. 5D)** Amount of isolated total RNA per microvesicle as determined by Nanodrop (RNA Concentration) and Nanosight (particle number) for A1, hCDC (OD35220) and Normal Human Dermal Fibroblasts (NHDF) exosomes following three days of serum-starvation of cells in culture. Values given in picograms per microliter.

Figure 6. Newt exosome characterization. (Fig. 6A) Profile of RNA types identified by MAVERIX Biomix of all RNA isolated from A1 exosomes and mapped to the Human genome. **(Fig. 6B)** Proteomic profile of total protein of A1 exosomes; analyzed by FUNRICH Functional Enrichment Analysis Tool software displaying relative protein abundances by cellular function.

Figure 7. qPCR, protein profiles A1 cargo contents. Comparison of **(Fig. 7A)** RNA and **(Fig. 7B)** Protein profiles from A1, hCDC, and NHDF exosomes following 3 days of serum-starvation of cell culture.

Figure 8. qPCR validation of A1 exosome-unique messenger RNAs identified by RNA sequencing. A1 exosome RNA was polyadenylated and converted to cDNA using a universal 3' adapter. The genes were detected using a forward primer specific for the gene and a universal 3' reverse primer for the end of the transcript. The forward primer sequences used in the qPCR validation experiments are shown in Table 1. Total RNA isolation from the A1 exosome was performed using the miRNeasy kit (Qiagen) as per manufacturer's instructions. A total amount of 100ng RNA was converted to cDNA using the Quantimix kit

5 (SBI) and PCR was performed using SYBR Green on ABI 7900HT detection system. The data were analyzed using the $\Delta\Delta C_t$ method.

Figure 9. Newt exosome surface protein characterization. Percentage of exosomes positive for protein surface markers as determine by MACSQuant Flow Cytometer (Miltenyi). **(Fig. 9A)** Secondary antibody-Alexa 488nm-treated of exosome (background control). **(Fig. 9B)** Positive Thrombospondin antibody-treated exosome counts (~79% of total exosome counts) **(Fig. 9C)** Postive Periostin antibody-treated exosome counts (~60% of total exosome counts) **(Fig. 9D)** Positive Fibronectin antibody-treated exosome counts (~52% of total exosome counts) **(Fig. 9E)** Positive CD81 antibody-treated exosome counts (~22% of total exosome counts) **(Fig. 9F)** Graphical representation of MACSQuant Flow Cytometry data.

Figure 10. Protein confirmation. Validation of proteomic analysis: Periostin identification by Western Blot. **(Fig. 10A)** PonceauS staining of PVDF membrane containing transferred total protein (40 μ g/lane) of A1, CDC and NHDF cells and exosomes. **(Fig. 10B)** PVDF membrane treated with rabbit polyclonal anti-periostin antibody (1:1000) overnight followed by goat anti-rabbit secondary antibody conjugated to horseradish peroxidase (1:5000). Blot was developed using enhanced chemiluminescence substrate (ECS) and bioluminescence captured using a Biorad Gel Dock Imager.

Figure 11. *In vitro* studies: cardiomyocyte proliferation. **(Fig. 11A)** Representative confocal images of EdU-positive Neonatal rat ventricular cardiomyocytes (NRVMs) following A1 exosome treatment. The green channel is EdU (5-ethynyl-2'-deoxyuridine; 10 μ M), a thymidine analog incorporated into DNA during DNA synthesis. The red channel is actin staining and the blue channel is DAPI nuclear stain. **(Fig. 11B)** Quantification of EdU-positive NRVMs after exposure to NHDF exosomes or A1 exosomes. Experiments were performed in triplicate. **(Fig. 11C)** Fold changes in expression of genes related to cell proliferation in NRVMs following treatment of A1 exosomes versus NHDF exosomes. Experiments were performed in triplicate.

Figure 12. *In vitro* studies: cardiomyocyte apoptosis. Flow Cytometry data of Annexin V Apoptotic marker. **(Fig. 12A)** Flow cytometry plots of Annexin V-stained NRVMs following 24hr treatment with A1 exosomes or NHDF exosomes and then 24hrs of ischemic injury (100 μ M H₂O₂). **(Fig. 12B)** Graphical representation of Annexin V- positive NRVMs treated with NHDF exosomes or A1 exosomes

Figure 13: *In vitro* studies: secreted factors. Expressed or Secreted Factors following Exosome exposure **(Fig. 13A)** ELISA performed from the supernatant collected

5 from NHDFs in culture exposed to NHDF exosomes or A1 exosomes for the cell proliferation-associated marker, SDF-1. Experiments were performed in triplicate. **(Fig. 13B)** ELISA performed from the supernatant collected from NHDFs in culture exposed to NHDF exosomes or A1 exosomes for the angiogenesis marker, VEGF. Experiments were performed in triplicate. **(Fig. 13C)** Western blot of collagen expression in A1 exosome-treated NHDFs versus NHDF exosome-treated cells (left panel) and pooled data of triplicate experiments (right panel).

Figure 14. *In vivo* studies: heart function ejection fraction as measured by echocardiography. Functional analysis of ejection fraction in female Wistar-Kyoto rat hearts three weeks following MI and 250µg or 500µg A1 exosome or 250µg NHDF exosome treatments. For ejection fraction evaluation 2D parasternal views were used to measure end-systolic and end-diastolic volumes. All images were analyzed on an offline computer with the appropriate VEVO 7 VisualSonics Software installed.

Figure 15. *In vivo* studies: heart morphology. Heart morphology of female Wistar-Kyoto rats subjected to permanent proximal left anterior descending coronary artery ligation and immediately injection of 250µg or 500µg A1 exosome or 250µg NHDF exosome treatments into the area at risk. **(Fig. 15A)** Representative brightfield images of stained rat heart sections (Masson's trichrome) three weeks after MI and NHDF or A1 exosome treatments **(Fig. 15B)** Analysis of the percent viable area three weeks following MI and exosome treatment **(Fig. 15C)** Analysis of the percent scar area three weeks following MI and exosome treatment **(Fig. 15D)** Analysis of the infarct wall thickness three weeks following MI and exosome treatment

Figure 16. *In vivo* studies: cardiomyocyte morphology. Cardiomyocyte diameter following MI and PBS or A1 exosome treatment **(Fig. 16A)** Representative confocal images of serial sections taken from paraffin-fixed left ventricle of hearts treated with 250µg or 500µg A1 exosome or 250µg NHDF exosome treatments. The heart tissue is stained with wheat germ agglutinin. **(Fig. 16B)** Graphical representation of cardiomyocyte size in microns; four animals per group.

Figure 17. *In vivo* studies: cardiomyocyte proliferation. Representative confocal images and graphical representation of cell proliferation six days following MI and NHDF (250µg) or A1 (250µg or 500µg) exosome treatments. Animals were injected within the intraperitoneal cavity with BrdU (5-bromo-2'-deoxyuridine) at days 0, 2, and 4 days post MI and the hearts were collected on day 6. Anti-sarcomeric actin was used as a secondary marker and DAPI marked the nucleus. The graph represents the number of BrdU-positive

5 nuclei within cardiomyocytes from each treatment group; four animals per group, scale bars = 100µm.

Figure 18. *In vivo*: microvasculature quantitation. Representative confocal images of heart tissue from the three treatment groups (NHDF (250µg) or A1 (250µg or 500µg)) three weeks after MI and treatment. Tissue was stained with smooth muscle actin to
10 measure microvessels and Von Willebrand Factor to measure capillaries. Scale bar = 100µm. Graphical data displays pooled measurements of microvessel and capillary density; n = 4 animals per treatment group.

Figure 19. Immunology: monocyte infiltration. Representative brightfield images of heart tissue stained with Hematoxylin and eosin from each of the three treatment groups,
15 three weeks after MI and treatment. Arrows indicate infiltrating monocyte clusters within the tissue. Graphical data shows the average number of monocytes counted per image field; 5 slides per heart, 4 animals per treatment group.

Figure 20. Immunology: macrophage infiltration. Representative confocal images of heart tissue stained with the macrophage marker CD68 from each of the three treatment
20 groups, three weeks after MI and treatment. Graphical data shows the average number of monocytes counted per image field; 5 slides per heart, 4 animals per treatment group. The difference in macrophage number between groups was not statistically significant.

Figure 21. Immunology: T-cell infiltration. Representative confocal images of heart tissue stained with antibodies against CD8+ (green) and CD4+ (red) T-cell markers,
25 from each of the three treatment groups, three weeks after MI and treatment. Graphical data shows the average number of CD8+ and CD4+ T-cells counted per image field; 5 slides per heart, 4 animals per treatment group. The difference in T-cell infiltration (CD8+ and CD4+) following 500µg of A1 exosome treatment from the NHDF exosome treatment was not statistically significant.

30 **Figure 22. miR-9-5-p induced cell proliferation.** Cardiomyocyte proliferation following treatment with miR-9-5p mimic or control microRNA (**Fig. 22A**) Representative confocal image of NRVMs in culture, treated with 25nM miR-9-5p microRNA mimic and EdU (10µM) for 24 hours. Cardiomyocytes were stained with sarcomeric actin and nuclei were stained with DAPI. Scale bar = 50µm. (**Fig. 22B**) Graphical representation of the
35 percentage of EdU-positive cardiomyocytes in culture, treated with the 25nM miR-9-5p mimic or 25nM control microRNA. A total of 10 images were acquired and analyzed in each of the groups. Experiments were repeated in triplicate. (**Fig. 22C**) Gene expression changes related to cell proliferation in NRVMs following treatment with miR-9-5p mimic or

control microRNA. Data shows fold change of gene expression in miR-9-5p-treated NRVMs relative to control microRNA-treated NRVMs.

Figure 23. miR-9-5p-induced secretion in NHDFs. (Fig. 23A) Graphical representation of SDF-1 secretion by NHDFs into the supernatant in picograms per milliliter. The graphs show secretion by NHDFs treated with 25nM and 50nM concentrations of the miR-9-5p mimic or control microRNA. The experiment was repeated in triplicate. **(Fig. 23B)** Graphical representation of VEGF secretion by NHDFs into the supernatant in picograms per milliliter. The graphs show secretion by NHDFs treated with 25nM and 50nM concentrations of the miR-9-5p mimic or control microRNA. The experiment was repeated in triplicate.

Figure 24. Quantification of EdU-positive NRVMs after exposure to NHDF exosomes or hCDC exosomes (OD35220). Experiments were performed in triplicate.

Figure 25. NHDF microRNAs expression post A1 exosome treatment. miRNA isolation from the A1 exosome-treated NHDFs was performed using the miRNeasy kit (Qiagen) as per manufacturer's instructions. A total amount of 100ng RNA was converted to cDNA using the miScript II RT Kit and PCR was performed using SYBR Green on ABI 7900HT detection system. The miScript RNA plates used were purchased from SA Biosciences. The data were analyzed using the $\Delta\Delta C_t$ method. Fold change of microRNA expression in NRVMs is shown relative to untreated NHDFs.

Figure 26. Tgf β /smad pathway gene expression following A1 or NHDF exosome treatment in NHDFs Fibrosis and the Tgf β /smad pathway. Western blot of A1 exosome-treated NHDFs compared to the NHDF exosome-treated NHDFs. A total amount of 10-20 μ g of protein was separated on 4-12% SDS-polyacrylamide gels and transferred onto nitrocellulose blotting membranes. Blocking of the membranes was performed using 5% non-fat milk in PBS containing 0.5% Tween 20 followed by incubation with TGFBR1 (1:500 Novus), TGFBR2 (1:500 Novus), phosphorylated smad4 (1:500 Sigma Aldrich), phosphorylated smad2/3 (1:500 Sigma Aldrich) and total smad2 (1:500 Abcam). Following washing with TBS-Tween buffer and incubation with appropriate HRP-labeled secondary antibodies protein detections was performed using ECL kit (Thermo Scientific). Image J was used for quantification of the bands. Fold protein changes was normalized to values of control cells. Pooled data showed no differences in p-smad2/3 and p-smad4 **(Fig. 26A)** and Tgf β -receptor 2 expression **(Fig. 26B)** between treatments. Data are mean $\pm$ SEM (the experiments were performed in triplicate).

5 **Figure 27. Echocardiography of infarcted rat hearts following A1 and NHDF
exosome treatments.** Additional echocardiographic parameters evaluated. For functional
analysis data transthoracic echocardiography was performed in all animals 3 weeks post MI
and treatment. All images were analyzed on an offline computer with the appropriate VEVO
7 VisualSonics Software installed. The groups analyzed were the NHDF exosome control, the
10 low dose newt-exosomes and the high-dose newt-exosomes. N=4 rats in each group. **(Fig.
27A)** Representative echocardiographic long axis images from each of the 3 groups
highlighting the infarct. **(Fig. 27B)** Graphical representation of left ventricle end-diastolic
volumes of infarcted rat hearts following NHDF exosome or A1 exosome treatments **(Fig.
27C)** Graphical representation of left ventricle end-systolic volumes of infarcted rat hearts
15 following NHDF exosome or A1 exosome treatments **(Fig. 27D)** Graphical representation of
fractional shortening of infarcted rat hearts following NHDF exosome or A1 exosome
treatments.

**Figure 28: Native miR-9-5p expression levels and downstream target gene
expression.** **(Fig. 28A)** Quantitative PCR of miR-9-5p from neonatal (P1) and 2 month-old
20 rat hearts. microRNA was extracted from neonatal Sprague Dawley rat hearts at P1 and at 2
months of age using miRNEasy minikit (Qiagen) according to manufacturer's instructions.
Data shows fold difference in miR-9-5p expression in adult hearts relative to neonatal rat
hearts. **(Fig. 28B)** PCR array of miR-9-5p target genes in NHDFs following A1 exosome-
treatment **(Fig. 28C)** PCR array of miR-9-5p target genes in NHDFs following miR-9-5p
25 treatment. All experiments described above were performed in triplicate.

DETAILED DESCRIPTION

All references cited herein are incorporated by reference in their entirety as though
fully set forth. Unless defined otherwise, technical and scientific terms used herein have the
30 same meaning as commonly understood by one of ordinary skill in the art to which this
invention belongs. Allen *et al.*, *Remington: The Science and Practice of Pharmacy* 22nd ed.,
Pharmaceutical Press (September 15, 2012); Hornyak *et al.*, *Introduction to Nanoscience and
Nanotechnology*, CRC Press (2008); Singleton and Sainsbury, *Dictionary of Microbiology
and Molecular Biology* 3rd ed., revised ed., J. Wiley & Sons (New York, NY 2006); Smith,
35 *March's Advanced Organic Chemistry Reactions, Mechanisms and Structure* 7th ed., J. Wiley
& Sons (New York, NY 2013); Singleton, *Dictionary of DNA and Genome Technology* 3rd
ed., Wiley-Blackwell (November 28, 2012); and Green and Sambrook, *Molecular Cloning: A
Laboratory Manual* 4th ed., Cold Spring Harbor Laboratory Press (Cold Spring Harbor, NY

2012), provide one skilled in the art with a general guide to many of the terms used in the present application. For references on how to prepare antibodies, see Greenfield, *Antibodies A Laboratory Manual 2nd ed.*, Cold Spring Harbor Press (Cold Spring Harbor NY, 2013); Köhler and Milstein, *Derivation of specific antibody-producing tissue culture and tumor lines by cell fusion*, Eur. J. Immunol. 1976 Jul, 6(7):511-9; Queen and Selick, *Humanized immunoglobulins*, U. S. Patent No. 5,585,089 (1996 Dec); and Riechmann *et al.*, *Reshaping human antibodies for therapy*, Nature 1988 Mar 24, 332(6162):323-7.

One skilled in the art will recognize many methods and materials similar or equivalent to those described herein, which could be used in the practice of the present invention. Indeed, the present invention is in no way limited to the methods and materials described.

For purposes of the present invention, the following terms are defined below.

As used in the description herein and throughout the claims that follow, the meaning of “a,” “an,” and “the” includes plural reference unless the context clearly dictates otherwise. Also, as used in the description herein, the meaning of “in” includes “in” and “on” unless the context clearly dictates otherwise.

Adult newts can regenerate amputated cardiac tissue (and whole limbs) without fibrosis, unlike adult mammals which lack such regenerative capacity. Based on the well-known regenerative capacity of urodeles for limb replacement, early studies sought to identify potentially similar mechanisms of organ replacement and/or repair. Adult newts possess the ability to regenerate a new lens following lentectomy. After removal of the lens, pigmented epithelial cells of the iris are able to transdifferentiate and subsequently change phenotype by differentiating into lens cells. Similarly, following complete transection spinal cord injury, the newt spine and tail regenerates and eventual restored use of hind limbs. Most recently, organisms such as the red-spotted newt (*Notophthalmus viridescens*) have been demonstrated as possessing cardiomyocytes capable of transdifferentiating into different cell types. Induced heart injury leads severe reduction of sarcomeric proteins in the myocardium. Such loss of the heavy myosin chain, various troponin proteins demonstrate partial de-differentiation of adult newt cardiomyocytes as an initial step during regeneration. Thereafter, appearance of markers such as Phospho-H3, indicative of G2 phase of cell cycle proliferation. Interestingly, the newt cardiomyocytes implanted into regenerating limbs lose their cardiac phenotype and acquire skeletal muscle or chondrocyte fate. Of great interest is whether these mechanisms occur by differentiated cells re-entering the cell cycle, recruitment of progenitor cells, or some combination of the two.

5 Notably, previous reports for urodele regeneration have highlighted an important role for microRNAs. Newt lens regeneration is specific to transdifferentiation of the epithelial cells of the dorsal iris, but not the ventral iris. This remarkable specificity appears to be due in-part to microRNA upregulation in one cell type compared to another, with a further interesting observation that hierarchal microRNA network regulation, not individual
10 microRNAs appear to be responsible for their regulatory function. Interestingly, certain reported microRNAs functional in newts, such as let-7 family, have also been documented in other reprogramming systems, such as in stem cells, suggesting common molecular signatures for reprogramming processes across organisms.

 What is largely unknown for urodele regeneration is the role of microRNAs in cardiac
15 regeneration and if microRNA activity is mediated by exosomes. It has been reported that during newt cardiac regeneration, microRNA miR-1 is down-regulated when hyperplasia is elevated and later returns to homeostatic levels when remodelling is occurring, suggesting a role in controlling cardiac cell fate. By contrast, microRNA miR-21 has been described as being upregulated in expression during the hyperplastic and remodelling phases of cardiac
20 regeneration, possibly indicating elevated microRNA miR-21 expression within cardiac fibroblasts as inducing production of an extracellular matrix scaffold, which is required for reconstitution of the myocardium. In the context of human heart disease, human cardiosphere-derived cells (CDCs) are known to improve myocardium and vasculature and increasing evidence establishes that stem cell-derived exosomes, including those produced by
25 CDCs, contain microRNAs that are important cellular actors in mediating these processes. Of great interest is understanding whether the microRNA activity of urodeles is similarly contained within exosomes, for which confirmation of such activity could offer benefits to human therapy.

 More specifically, exosomes, the secreted lipid vesicles containing a rich milieu of
30 biological factors, provide powerful paracrine signals by which stem cells effectuate their biological effects to neighboring cells, including diseased or injured cells. Through the encapsulation and transfer of protein, bio-active lipid and nucleic acid “cargo”, there is increasing recognition that these natural delivery devices are capable of inducing significant phenotypic and functional changes in recipient cells that lead to activation of regenerative
35 programs. The role of such indirect mechanisms to effectuate therapeutic benefits is suggested by evidence that after stem cell administration and clearance from delivery sites in tissue and organs, regeneration processes nevertheless persist and arise from endogenous tissues. The “paracrine hypothesis” of stem cell regenerative activity has created a paradigm

5 shift by which clinical applications based on exosomes secreted by the stem cells may prove superior, or provide distinct advantages, when compared to transplant and delivery of stem cells themselves. Stem cell-derived exosomes have been identified and isolated from supernatants of several cell types with demonstrated therapeutic potential, including mesenchymal stromal (MSC), (bone marrow stem cells) mononuclear (MNC), immune cells
10 (dendritic and CD34+) and human neural stem cells (hNSCs). As described, human cardiosphere-derived cells (CDCs) are known to improve myocardium and vasculature, for which exosomes and their microRNA cargo content appear to be important cellular actors.

Exosome-based, “cell-free” therapies, in contrast to cell therapy, provide distinct advantages in regenerative medicine. Generally, their production under defined conditions
15 allows for easier manufacture and scale-up opportunity. They further obviate safety issues as non-viable entities, with reduced or non-existent immunogenic or tumorigenic potential. For example, manufacture of exosomes is akin to conventional biopharmaceutical product manufacture, allowing for standardization in production and quality control for dosage and biological activity testing. The durability of exosomes in culture allows for the acquisition of
20 large quantities of exosomes through their collection from a culture medium in which the exosomes are secreted over periods of time. In addition, exosome encapsulation of bioactive components in lipid vesicles allows protection of contents from degradation *in vivo*, thereby potentially negating obstacles often associated with delivery of soluble molecules such as cytokines, growth factors, transcription factors and RNAs. Further, exosomes are likely to be
25 less immunogenic than parental cells, as a result of a lower content of membrane-bound proteins, including MHC complex molecules. Replacing the administration of live cells with their secreted exosomes, mitigates many of the safety concerns and limitations associated with the transplantation of viable replicating cells.

30 **General Features of Exosomes.** Secreted by a wide range of cell types, exosomes are lipid bilayer vesicles that are enriched in a variety of biological factors, including cytokines, growth factors, transcription factors, and coding and non-coding nucleic acids. Exosomes are found in blood, urine, amniotic fluid, interstitial and extracellular spaces. These exocytosed vesicles of endosomal origin can range in size between 30-300 nm, including sizes of 40-100
35 nm, and possess a cup-shaped morphology, as revealed by electron microscopy. Their initial formation begins with inward budding of the cell membrane to form endosomes, which is followed by invagination of the limiting membrane of late endosomes to form multivesicular bodies (MVB). Fusion of the MVB with the plasma membrane results in the release of the

5 internal vesicles to the extracellular space, through the formation of vesicles thereafter known as exosomes.

As described, the “cargo” contents of exosomes reflect their parental cellular origin, as containing distinct subsets of biological factors in connection with their parent cellular origin, including the cell regulatory state when formed. Exosomes contain a biological
10 milieu of different proteins, including cytokines and growth factors, coding and noncoding RNA molecules, all necessarily derived from their parental cells. In addition to containing a rich array of cytosolic derivatives, exosomes further express the extracellular domain of membrane-bound receptors at the surface of the membrane.

It is now well-established that exosomes are involved in intercellular communication
15 between different cell types, but much remains to be discovered in regard to the mechanisms of their production within parental cells of origin and effects on target recipient cells. Exosomes have been reported to be involved in numerous cellular, tissue and physiological processes, including immune modulating processes, angiogenesis, migration of endothelial cells in connection with tumor growth, or reducing damage in ischemia reperfusion injury.
20 Because exosomes contain cargo contents reflecting the parental cell type and its cellular regulatory state at time of production, the resulting composition of exosomes play a critical role in determining their function. Increasing evidence suggests that exosomes secreted by cells, such as cardiosphere-derived cells (CDCs), are capable of reproducing the therapeutic benefits of their parental cells, or possibly, are indispensable in effectuating such therapeutic
25 benefits. By extension, the remarkable regenerative potential of urodeles suggests the possibility of isolating and deploying their potent biological factors to spur cardiac regeneration for human therapies. As described some measure of urodele regeneration may occur through differentiated cells re-entering the cell cycle, recruitment of progenitor cells, or some combination of the two, and in this regard, isolating exosomes from urodele cells
30 undergoing dedifferentiation, transdifferentiation, and/or proliferation, may serve to enrich those particular factors (e.g., microRNAs) critical to regeneration and repair processes.

Importantly, the described encapsulation and formation processes necessarily create heterogeneity in exosome compositions based on parental cellular origin and regulatory state at time of formation. Nevertheless, generic budding formation and release mechanisms
35 establish a common set of features as a consequence of their origin, such as endosome-associated proteins (e.g., Rab GTPase, SNAREs, Annexins, and flotillin), proteins that are known to cluster into microdomains at the plasma membrane or at endosomes (four transmembrane domain tetraspanins, e.g., CD63, CD81, CD82, CD53, and CD37), lipid raft

5 associated proteins (e.g., glycosylphosphatidylinositol-anchored proteins and flotillin), cholesterol, sphingomyelin, and hexosylceramides, as examples.

In addition to these core components reflecting their vesicle origin, a critical property of exosomes is a demonstrated capability to contain both mRNA and microRNA associated with signaling processes, with both cargo mRNA being capable to translation in recipient
10 cells, or microRNA functionally degrading target mRNA in recipient cells. Other noncoding RNAs, capable for influencing gene expression, may also be present in exosomes. While the processes governing the selective incorporation of mRNA or microRNA populations into exosomes is not entirely understood, it is clearly that RNA molecules are selectively, not randomly incorporated into exosomes, as revealed by studies report enrichment of exosome
15 cargo RNAs when compared to the RNA profiles of the originating cells. Given the growing understanding of how such RNA molecules play a role in disease pathogenesis and regenerative processes, the presence of RNA molecules in exosomes and apparent potency in effecting target recipient cells suggests critical features that can be deployed in therapeutic approaches.

20 Importantly, the natural bilayer membrane encapsulation of exosomes provides a protected and controlled internal microenvironment that allows cargo contents to persist or migrate in the bloodstream within tissues without degradation. Their release into the extracellular environment, allows for interaction with recipient cells via adhesion to the cell surface mediated by lipid-ligand receptor interactions, internalization via endocytic uptake, or
25 by direct fusion of the vesicles and cell membrane. These processes lead to the release of exosome cargo content into the target cell. The net result of exosome-cell interactions is modulation of genetic pathways in the target recipient cell, as induced through any of several different mechanisms including antigen presentation, the transfer of transcription factors, cytokines, growth factors, nucleic acid such as mRNA and microRNAs. In the stem cell
30 context, embryonic stem cell (ESC)-derived exosomes have been demonstrated to shuttle/transfer mRNA and proteins to hematopoietic progenitors. Other studies have shown that adult stem cell-derived exosomes also shuttle selected patterns of mRNA, microRNA and pre-microRNA associated with several cellular functions involved in the control of transcription, proliferation and cell immune regulation.

35

Isolation and Preparation of Exosomes. Exosome isolation relies on exploiting their generic biochemical and biophysical features for separation and analysis. For example, differential ultracentrifugation has become a leading technique wherein secreted exosomes

5 are isolated from the supernatants of cultured cells. This approach allows for separation of
exosomes from nonmembranous particles, by exploiting their relatively low buoyant density.
Size exclusion allows for their separation from biochemically similar, but biophysically
different microvesicles, which possess larger diameters of up to 1,000 nm. Differences in
floatation velocity further allows for separation of differentially sized exosomes. In general,
10 exosome sizes will possess a diameter ranging from 30-300 nm, including sizes of 40-100
nm. Further purification may rely on specific properties of the particular exosomes of
interest. This includes, for example, use of immunoadsorption with a protein of interest to
select specific vesicles with exoplasmic or outward orientations.

Among current methods (differential centrifugation, discontinuous density gradients,
15 immunoaffinity, ultrafiltration and high performance liquid chromatography (HPLC),
differential ultracentrifugation is the most commonly used for exosome isolation. This
technique utilizes increasing centrifugal force from 2000xg to 10,000xg to separate the
medium- and larger-sized particles and cell debris from the exosome pellet at 100,000xg.
Centrifugation alone allows for significant separation/collection of exosomes from a
20 conditioned medium, although it is insufficient to remove various protein aggregates, genetic
materials, particulates from media and cell debris that are common contaminants. Enhanced
specificity of exosome purification may deploy sequential centrifugation in combination with
ultrafiltration, or equilibrium density gradient centrifugation in a sucrose density gradient, to
provide for the greater purity of the exosome preparation (floatation density 1.1-1.2g/ml) or
25 application of a discrete sugar cushion in preparation.

Importantly, ultrafiltration can be used to purify exosomes without compromising
their biological activity. Membranes with different pore sizes - such as 100 kDa molecular
weight cut-off (MWCO) and gel filtration to eliminate smaller particles - have been used to
avoid the use of a nonneutral pH or non-physiological salt concentration. Currently available
30 tangential flow filtration (TFF) systems are scalable (to >10,000L), allowing one to not only
purify, but concentrate the exosome fractions, and such approaches are less time consuming
than differential centrifugation. HPLC can also be used to purify exosomes to
homogeneously sized particles and preserve their biological activity as the preparation is
maintained at a physiological pH and salt concentration.

35 Other chemical methods have exploit differential solubility of exosomes for
precipitation techniques, addition to volume-excluding polymers (e.g., polyethylene glycols
(PEGs)), possibly combined additional rounds of centrifugation or filtration. For example, a
precipitation reagent, ExoQuick®, can be added to conditioned cell media to quickly and

5 rapidly precipitate a population of exosomes, although re-suspension of pellets prepared via this technique may be difficult. Flow field-flow fractionation (FIFFF) is an elution-based technique that is used to separate and characterize macromolecules (e.g., proteins) and nano- to micro-sized particles (e.g., organelles and cells) and which has been successfully applied to fractionate exosomes from culture media.

10 Beyond these techniques relying on general biochemical and biophysical features, focused techniques may be applied to isolated specific exosomes of interest. This includes relying on antibody immunoaffinity to recognizing certain exosome-associated antigens. Conjugation to magnetic beads, chromatography matrices, plates or microfluidic devices allows isolating of specific exosome populations of interest as may be related to their
15 production from a parent cell of interest or associated cellular regulatory state. Other affinity-capture methods use lectins which bind to specific saccharide residues on the exosome surface.

Exosome-Based Therapies. Important goals of developing exosome-based therapy
20 are creation of “cell-free” therapies, wherein the benefits of cell therapeutics can be provided with reduced risks or in scenarios in which cell therapy would be unavailable, or capturing the potential regenerative potential of urodeles for applications in mammals.

Without being bound by any particular theory, the Inventors believe that the therapeutic effects of stem cells or regenerative potential of urodeles can be reproduced by
25 exosomes. In fact, focused application of exosomes may actually provide superior results for the following reasons. Firstly, the retention of delivered stem cells has been shown to be short lived. Second, the quantity of local release of exosomes from a stem cell is limited and occurs only as long as the cell is retained. Thirdly, the quantity of exosomes delivered can be much higher (i.e., high dosing of its contents). Fourth, exosomes can be readily taken up by
30 the cells in the local tissue milieu. Fifth, issues of immunogenicity are avoided. Sixth, repeated doses of exosomes is feasible, while impractical/potentially dangerous for stem cells as they impact the microvasculature. Seventh, application of biological factors enriched in other species and vital to their regenerative potential, as extendible to mammalian species. The use of cell-based-exosome therapy has the potential to impact directly on the pathology
35 in heart disease and related conditions by reversing the course of the disease, as opposed to palliative or preventive measures. Such approaches focused on *bona fide* regenerative of diseased or dysfunctional tissue, representing a major therapeutic breakthrough in both direct repair of injured tissue and in generation of support vasculature that ultimate supports the

5 development and homeostasis of regenerated tissue. Such approaches are not addressed by the current pharmacologic tools currently employed.

While stem cell therapy for heart disease and related conditions has long been a promising concept for addressing such issues, they depend highly on successful delivery into the myocardial area of need. General principles from such techniques (e.g., concentration, 10 timing of delivery, and sustained bioavailability) are applicable to exosome-based therapy. However, a key benefit of exosome based therapy is that the central challenges limiting cellular transplants are largely obviated (e.g., cell engraftment of cells and prolonged survival of the transplanted cells). For example, a key limitation of cell delivery is providing a sufficient number of cells to maximize therapeutic effect, such cells being susceptible to 15 clearance and washout. Furthermore, the regenerative effects of delivered cells may further rely on migration and homing mechanisms to potentiate their stem cell activity at the site of injury. Physiological or biochemical barriers may effectively eliminate administered cells moving to sites of repair. Unlike cell therapy, the Inventors believe higher concentrations of biological agents to the local tissue milieu is possible via exosomes, and that repeated 20 administration of such exosomes may maximize tissue regeneration and repair in a manner that would be infeasible for cell therapy.

Generally, exosome based therapy can delivered via a number of routes: intravenous, intracoronary, and intramyocardial. Exosomes, also allow for new delivery routes that were previously infeasible for cell therapy, such as inhalation. Benefits and drawbacks of these 25 various approaches are described below.

Intravenous delivery technique can occur through a peripheral or central venous catheter. As the simplest delivery mode, this technique avoids the risk of an invasive procedure. However, intravenous may be regarded as a comparatively inefficient and less localized delivery method, as a high percentage of infused cell exosomes may become 30 sequestered in organs such as the lung, liver, or spleen. Such sequestration may results in few or no cellular exosomes reaching coronary circulation or have unintended systemic effects following their distribution. Exosomes reaching the site of injury may also face additional obstacles when migrating across or effectuating signaling across cells in the arterial or capillary wall. Importantly, this route is unlikely to exist as an option for patients 35 with occluded arteries, unless there are sufficient routes of collateral coronary artery circulation exist.

By contrast, an approach that may be preferential involves intracoronary cell infusion. As delivered through the central lumen of a balloon catheter positioned in the coronary

5 artery, exosomes can be administered with coronary flow. In some instances, balloon occlusion is used to introduce flow interruption as a means to minimize washout of the therapeutic. A key advantage of the intracoronary approach is selective, local delivery of cells to the myocardial area of interest, thereby limiting risks of systemic administration. Coronary delivery requires that the target myocardium be subtended by a patent coronary
10 artery or identifiable collateral vessel and therefore performed following percutaneous coronary intervention (PCI). In some therapeutic contexts, such as acute myocardial infarct, the relative ease of delivery following standard catheter intervention to re-establish coronary flow is a highly attractive opportunity for intracoronary delivery.

In another approach, direct intramyocardial delivery via injection into the
15 myocardium via a transepicardial or transendocardial entry. While this epicardial approach allows for direct visualization of the infarcted myocardium for accurate targeting of delivery, it requires open-heart surgery. Targeted injections can also be obtained by an endocardial approach, which obviates the need for surgery and has been applied as a stand-alone procedure, but the lack of direct visualization presents some difficulties. Further, existing
20 studies of direct injection into the myocardium may result in delivery only to relatively small myocardial areas, resulting in nonuniform distribution within the recipient heart. Intramyocardial injection of CSCs would be difficult to achieve clinically on a widespread basis, and a limitation of both epicardial and endocardial approaches is the risk of perforation. Nevertheless, such direct injection techniques can be used in instances wherein transvascular
25 delivery is not possible, such as patients with an ischemic cardiomyopathy and occluded coronary artery.

An alternative intravenous mode may be retrograde coronary sinus delivery. This approach relies on catheter placement into the coronary sinus, inflation of the balloon, and exosome administered by infusion at pressures higher than coronary sinus pressure (e.g., 20
30 mL), thereby allowing for retrograde perfusion of cells into the myocardium. Like intracoronary delivery, exosomes could be required to migrate across or effectuating their signaling across the arterial or capillary wall.

Realizing these benefits requires an improved understanding of whether exosomes
35 secreted by cells such as those derived from urodeles possess salutary benefits of cells such as CSCs, are alone capable of reproducing therapeutic benefits of their parental cells, or possibly indispensable in these processes. This can be investigated by isolating exosomes from a newt mesodermal cell line, and evaluated their bioactivity in rat models. Confirming

5 the role of exosomes in such processes will allow their application in new therapeutic approaches, including “cell-free” use in subjects for which cellular transplant or administration is unavailable (e.g., late stage heart disease). Pharmacological, device-based intervention or surgery may not provide significant options for such subjects. There is a great need in the art for identifying means by which to deliver the benefits of stem cell
10 regeneration, without resorting to mechanisms involving administration or transplant of the cell themselves.

Described herein is a composition including a plurality of exosomes. In certain embodiments, the plurality of exosomes is generated by a method including providing a
15 population of cells, and isolating a plurality of exosomes from the population of cells.

In various embodiments, the cells are from amphibians within the order Caudata (also described as urodeles). For example, this includes species within the family *Salamandridae* (also known as newts). Various non-limiting examples include *Notophthalmus viridescens* and *Ambystoma mexicanum*. In other embodiments, the cells are cultured as a cell line
20 capable of serial passaging. This includes, for example, the A1 cell line of *Notophthalmus viridescens*. In other embodiments, the cells are stem cells, progenitors, precursors, and/or mesenchyme.

In various embodiments, the plurality of exosomes is isolated from the supernatants of the population of cells. This includes, for example, exosomes secreted into media as
25 conditioned by a population of cells in culture, further including cell lines capable of serial passaging. In certain embodiments, the cells are cultured in a serum-free media. In certain embodiments, the cells in culture are grown to 10, 20, 30, 40, 50, 60, 70, 80, 90, or 90% or more confluency when exosomes are isolated. In certain embodiments, the population of cells has been genetically manipulated. This includes, for example, knockout (KO) or
30 transgenic (TG) cell lines, wherein an endogenous gene has been removed and/or an exogenous introduced in a stable, persistent manner. In certain embodiments, the cells are genetically modified to express endothelial nitric oxide synthase (eNOS), vascular endothelial growth factor (VEGF), SDF-1 (stromal derived factor), IGF-1 (insulin-like growth factor 1), HGF (hepatocyte growth factor). This further includes transient knockdown of one or more
35 genes and associated coding and non-coding transcripts within the population of cells, via any number of methods known in the art, such as introduction of dsRNA, siRNA, microRNA, etc. This further includes transient expression of one or more genes and associated coding and non-coding transcripts within the population of cells, via any number of methods known in

5 the art, such as introduction of a vector, plasmid, artificial plasmid, replicative and/or non-replicative virus, etc. In other embodiments, the population of cells has been altered by exposure to environmental conditions (e.g., hypoxia), small molecule addition, presence/absence of exogenous factors (e.g., growth factors, cytokines) at the time, or substantially contemporaneous with, isolating the plurality of exosomes in a manner altering
10 the regulatory state of the cell. For example, one may add a differentiation agent to a population of stem cells, progenitors and/or precursors in order to promote partial or full differentiation of the cell, and thereafter derive a plurality of exosomes. In various embodiments, altering the regulatory state of the cell changes composition of one or more exosomes in the plurality of exosomes. This includes, for example, isolating a plurality of
15 exosomes from cells, such as urodele cells, undergoing dedifferentiation, transdifferentiation, and/or proliferation.

In various embodiments, the plurality of exosomes includes one or more exosomes that are about 10 nm to about 250 nm in diameter, including those about 10 nm to about 15 nm, about 15 nm to about 20 nm, about 20 nm to about 25 nm, about 25 nm to about 30 nm,
20 about 30 nm to about 35 nm, about 35 nm to about 40 nm, about 40 nm to about 50 nm, about 50 nm to about 60 nm, about 60 nm to about 70 nm, about 70 nm to about 80 nm, about 80 nm to about 90 nm, about 90 nm to about 95 nm, about 95 nm to about 100 nm, about 100 nm to about 105 nm, about 105 nm to about 110 nm, about 110 nm to about 115 nm, about 115 nm to about 120 nm, about 120 nm to about 125 nm, about 125 nm to about 130 nm, about
25 130 nm to about 135 nm, about 135 nm to about 140 nm, about 140 nm to about 145 nm, about 145 nm to about 150 nm, about 150 to about 200 nm, about 200 nm to about 250 nm, about 250 nm or more.

In various embodiments, the plurality of exosomes includes one or more exosomes expressing a biomarker. In certain embodiments, the biomarkers are tetraspanins. In other
30 embodiments, the tetraspanins are one or more selected from the group including CD63, CD81, CD82, CD53, and CD37. In other embodiments, the exosomes express one or more lipid raft associated proteins (e.g., glycosylphosphatidylinositol-anchored proteins and flotillin), cholesterol, sphingomyelin, and/or hexosylceramides.

In several embodiments, the plurality of exosomes includes one or more exosomes
35 containing a biological protein. In various embodiments, the biological protein includes transcription factors, cytokines, growth factors, and similar proteins capable of modulating signaling pathways in a target cell. In various embodiments, the biological protein is capable of facilitating regeneration and/or improved function of a tissue. In other embodiments, the

5 biological protein is capable of modulating a pathway related to vasodilation, such as prostacyclin and nitric oxide, and/or vasoconstrictors such as thromboxane and endothelin-1 (ET-1). In various embodiments, the biological protein is capable of modulating pathways related to Irak1, Traf6, toll-like receptor (TLR) signaling pathway, NOX-4, SMAD-4, and/or TGF- β . In other embodiments, the biological protein is capable of mediating M1 and/or M2
10 immune responses in macrophages. In other embodiments, the biological protein related to exosome formation and packaging of cytosolic proteins such as Hsp70, Hsp90, 14-3-3 epsilon, PKM2, GW182 and AGO2. In certain embodiments, the exosomes express CD63, HSP70, CD105 or combinations thereof. In other embodiments, the exosomes do not express CD9 or CD81, or express neither. For example, plurality of exosomes can include one or
15 more exosomes that are CD63+, HSP+, CD105+, CD9-, and CD81-.

In other embodiments, the plurality of exosomes includes one or more exosomes containing a signaling lipid. This includes ceramide and derivatives. In other embodiments, the plurality of exosomes includes one or more exosomes containing a coding and/or non-coding nucleic acid.

20 In several embodiments, the plurality of exosomes includes one or more exosomes containing microRNAs. In various embodiments, these microRNAs can include miR-146a, miR22, miR-24, miR-210, miR-150, miR-140-3p, miR-19a, miR-27b, miR-19b, miR-27a, miR-376c, miR-128, miR-320a, miR-143, miR-21, miR-130a, miR-9, miR-185, and/or miR-23a. In several embodiments, the plurality of exosomes includes one or more exosomes
25 enriched in at least one of miR-146a, miR-22, miR-24. Enrichment can be measured by, for example, comparing the amount of one or more of the described microRNAs when derived from cells providing salutary benefit in a therapeutic setting (e.g., urodele-derived cells, cardiosphere-derived cells (CDCs) compared to cells that do not provide such a salutary benefit (e.g., fibroblasts). Enrichment may also be measured in absolute or relative
30 quantities, such as when compared to a standardized dilution series.

In other embodiments, the plurality of exosomes can include one or more exosomes containing microRNAs. This includes various microRNAs known in the art, such as miR-23a, miR-23b, miR-24, miR-26a, miR27-a, miR-30c, let-7e, mir-19b, miR-125b, mir-27b, let-7a, miR-19a, let-7c, miR-140-3p, miR-125a-5p, miR-132, miR-150, miR-155, mir-210,
35 let-7b, miR-24, miR-423-5p, miR-22, let-7f, and/or miR-146a, further including microRNAs evolutionarily conserved with the aforementioned microRNAs.

In other embodiments, the plurality of exosomes can include one or more exosomes containing microRNAs. This includes various microRNAs known in the art, such as miR-

5 1469, miR-762, miR-574-3p, miR-574-5p, miR-3197, miR-4281, miR-1976, miR-1307, miR-1224-3p, miR-187, miR-3141, miR-1268, miR-155, miR-122, miR-638, miR-3196, miR-223, miR-4267, miR-1281, miR-885-5p, miR-663, miR-let-7b, miR-29d, miR-144, miR-let-7e
10 143, miR-lrt-7g, miR-17a, miR-96, miR-125a-5p, miR-128, miR-720, miR-21, miR-9, miR-26b, miR-29b, miR-30c, miR-30b, miR-191, and miR-1b. In other embodiments, the one or more microRNAs comprise one or more exosomes including miR-96, miR-29b, and miR-191.

In other embodiments, the plurality of exosomes can include one or more exosomes containing microRNAs. This includes various microRNAs known in the art, such as miR-17, miR-21, miR-92, miR92a, miR-29, miR-29a, miR-29b, miR-29c, miR-34, miR-34a, miR-150, miR-451, miR-145, miR-143, miR-144, miR-193a-3p, miR-133a, miR-155, miR-181a, miR-214, miR-199b, miR-199a, miR-210, miR-126, miR-378, miR-363 and miR-30b, and miR-499. Other microRNAs known in the art include miR-92, miR-17, miR-21, miR-92, miR92a, miR-29, miR-29a, miR-29b, miR-29c, miR-34, miR-34a, miR-150, miR-451, miR-145, miR-143, miR-144, miR-193a-3p, miR-133a, miR-155, miR-181a, miR-214, miR-199b, miR-199a, miR-126, miR-378, miR-363 and miR-30b, and/or miR-499, further including microRNAs evolutionarily conserved with the aforementioned microRNAs.

In several embodiments, isolating a plurality of exosomes from the population of cells includes centrifugation of the cells and/or media conditioned by the cells. In several embodiments, ultracentrifugation is used. In several embodiments, isolating a plurality of exosomes from the population of cells is via size-exclusion filtration. In other embodiments, isolating a plurality of exosomes from the population of cells includes use of discontinuous density gradients, immunoaffinity, ultrafiltration and/or high performance liquid chromatography (HPLC).

In certain embodiments, differential ultracentrifugation includes using centrifugal force from 1000-2000xg, 2000-3000xg, 3000-4000xg, 4000-5000xg, 5000-6000xg, 6000-7000xg, 7000-8000xg, 8000-9000xg, 9000-10,000xg, to 10,000xg or more to separate larger-sized particles from a plurality of exosomes derived from the cells.

In other embodiments, isolating a plurality of exosomes from the population of cells includes use of filtration or ultrafiltration. In certain embodiments, a size exclusion membrane with different pore sizes is used. For example, a size exclusion membrane can include use of a filter with a pore size of 0.1-0.5 μ M, 0.5-1.0 μ M, 1-2.5 μ M, 2.5-5 μ M, 5 or more μ M. In certain embodiments, the pore size is about 0.2 μ M. In certain embodiments, filtration or ultrafiltration includes size exclusion ranging from 100-500 daltons (Da), 500-1

5 kDa, 1-2 kDa, 2-5 kDa, 5-10 kDa, 10-25 kDa, 25-50 kDa, 50-100 kDa, 100-250 kDa, 250-500 kDa, 500 or more kDa. In certain embodiments, the size exclusion is for about 2-5 kDa. In certain embodiments, the size exclusion is for about 3 kDa. In other embodiments, filtration or ultrafiltration includes size exclusion includes use of hollow fiber membranes capable of isolating particles ranging from 100-500 daltons (Da), 500-1 kDa, 1-2 kDa, 2-5
10 kDa, 5-10 kDa, 10-25 kDa, 25-50 kDa, 50-100 kDa, 100-250 kDa, 250-500 kDa, 500 or more kDa. In certain embodiments, the size exclusion is for about 2-5 kDa. In certain embodiments, the size exclusion is for about 3 kDa. In other embodiments, a molecular weight cut-off (MWCO) gel filtration capable of isolating particles ranging from 100-500 daltons (Da), 500-1 kDa, 1-2 kDa, 2-5 kDa, 5-10 kDa, 10-25 kDa, 25-50 kDa, 50-100 kDa,
15 100-250 kDa, 250-500 kDa, 500 or more kDa. In certain embodiments, the size exclusion is for about 2-5 kDa. In certain embodiments, the size exclusion is for about 3 kDa. In various embodiments, such systems are used in combination with variable fluid flow systems.

In other embodiments, isolating a plurality of exosomes from the population of cells includes use of tangential flow filtration (TFF) systems are used purify and/or concentrate the
20 exosome fractions. In other embodiments, isolating a plurality of exosomes from the population of cells includes use of (HPLC) can also be used to purify exosomes to homogeneously sized particles. In various embodiments, density gradients as used, such as centrifugation in a sucrose density gradient or application of a discrete sugar cushion in preparation.

25 In other embodiments, isolating a plurality of exosomes from the population of cells includes use of a precipitation reagent. For example, a precipitation reagent, ExoQuick®, can be added to conditioned cell media to quickly and rapidly precipitate a population of exosomes. In other embodiments, isolating a plurality of exosomes from the population of cells includes use of volume-excluding polymers (e.g., polyethylene glycols (PEGs)) are
30 used. In another embodiment, isolating a plurality of exosomes from the population of cells includes use of flow field-flow fractionation (FIFFF), an elution-based technique.

In certain embodiments, isolating a plurality of exosomes from the population of cells includes use of one or more capture agents to isolate one or more exosomes possessing specific biomarkers or containing particular biological molecules. In one embodiment, one or
35 more capture agents include at least one antibody. For example, antibody immunoaffinity recognizing exosome-associated antigens is used to capture specific exosomes. In other embodiments, the at least one antibody are conjugated to a fixed surface, such as magnetic beads, chromatography matrices, plates or microfluidic devices, thereby allowing isolation of

5 the specific exosome populations of interest. In other embodiments, isolating a plurality of exosomes from the population of cells includes use of one or more capture agents that is not an antibody. This includes, for example, use of a “bait” molecule presenting an antigenic feature complementary to a corresponding molecule of interest on the exosome surface, such as a receptor or other coupling molecule. In one embodiment, the non-antibody capture agent
10 is a lectin capable of binding to polysaccharide residues on the exosome surface.

In various embodiments, the cells are from amphibians within the order Caudata (also described as urodeles). For example, this includes species within the family *Salamandridae* (also known as newts). Various non-limiting examples include *Notophthalmus viridescens* and *Ambystoma mexicanum*. In other embodiments, the cells are cultured as a cell line
15 capable of serial passaging. This includes, for example, the A1 cell line of *Notophthalmus viridescens*. In other embodiments, the cells are stem cells, progenitors, precursors, and/or mesenchyme. As disclosed above, in some embodiments, synthetic exosomes are generated, which can be isolated by similar mechanisms as those above. In various embodiments, the composition that is a plurality of exosomes is a pharmaceutical composition further including
20 a pharmaceutically acceptable carrier.

In various embodiments, the plurality of exosomes range in size from 30 to 300 nm. In various embodiments, the plurality of exosomes range in size from 40 to 100 nm. In certain embodiments, the plurality of exosomes cells are from amphibians within the order Caudata (also described as urodeles). In certain embodiments, the plurality of exosomes
25 includes one or more exosomes that are CD63+, CD105+, or both. In various embodiments, the exosomes include microRNAs miR-146a, miR22, miR-24, miR-210, miR-150, miR-140-3p, miR-19a, miR-27b, miR-19b, miR-27a, miR-376c, miR-128, miR-320a, miR-143, miR-21, miR-130a, miR-9, miR-185, and/or miR-23a. In other embodiments, the plurality of exosomes can include one or more exosomes containing microRNAs. This includes various
30 microRNAs known in the art, such miR-1469, miR-762, miR-574-3p, miR-574-5p, miR-3197, miR-4281, miR-1976, miR-1307, miR-1224-3p, miR-187, miR-3141, miR-1268, miR-155, miR-122, miR-638, miR-3196, miR-223, miR-4267, miR-1281, miR-885-5p, miR-663, miR-let-7b, miR-29d, miR-144, miR-let-7e 143, miR-lrt-7g, miR-17a, miR-96, miR-125a-5p, miR-128, miR-720, miR-21, miR-9, miR-26b, miR-29b, miR-30c, miR-30b, miR-191, and
35 miR-1b. In other embodiments, the one or more microRNAs comprise one or more exosomes including miR-96, miR-29b, and miR-191. In other embodiments, the exosomes are 2-5 kDa, such as 3 kDa. Other examples or embodiments relating to the composition and

5 techniques involving exosomes are presented, in PCT Pub. No. WO 2014/028,493, which is fully incorporated herein by reference.

Described herein is a method of treatment. In various embodiments, the method is a method of treating heart related disease, including administering a composition including a plurality of exosomes isolated from urodele cells to a subject, thereby treating the subject. In various embodiments, the method includes selecting a subject in need of treatment, administering a composition including a plurality of exosomes to the individual, wherein administration of the composition treat the subject. In certain embodiments, the subject is in need to treatment for a disease and/or condition involving tissue damage or dysfunction. In other embodiments, the disease and/or condition involving tissue damage or dysfunction is pulmonary disease. In other embodiments, the disease and/or condition involving tissue damage or dysfunction is heart disease. In other embodiments, the plurality of exosomes includes exosomes including one or more microRNAs. In other embodiments, the the subject is diagnosed as afflicted with a heart related disease prior to administering the composition.

20 In certain embodiments, the plurality of exosomes is generated by a method including providing a population of cells, and isolating a plurality of exosomes from the population of cells. In various embodiments, the cells are from amphibians within the order Caudata (also described as urodeles). For example, this includes species within the family *Salamandridae* (also known as newts). Various non-limiting examples include *Notophthalmus viridescens* and *Ambystoma mexicanum*. In other embodiments, the cells are cultured as a cell line capable of serial passaging. This includes, for example, the A1 cell line of *Notophthalmus viridescens*. In other embodiments, the cells are stem cells, progenitors, precursors, and/or mesenchyme. In certain embodiments, the exosomes are synthetic.

In various embodiments, the plurality of exosomes is derived from urodele cells. In other embodiments, the plurality of exosomes includes exosomes including one or more biological molecules. In other embodiments, the plurality of exosomes including exosomes enriched for one or more biological molecules when derived from urodele compared to exosome derived from non-urodele sources. In various embodiments, the one or more biological molecules are proteins, growth factors, cytokines, transcription factors and/or morphogenic factors. In other embodiments, the plurality of exosomes including exosomes enriched for one or more biological molecules includes microRNAs, further including microRNAs that are enriched when derived from urodele compared to exosome derived from non- urodele sources. In various embodiments, these microRNAs can include miR-146a,

5 miR22, miR-24, miR-210, miR-150, miR-140-3p, miR-19a, miR-27b, miR-19b, miR-27a, miR-376c, miR-128, miR-320a, miR-143, miR-21, miR-130a, miR-9, miR-185, and/or miR-23a. In several embodiments, the plurality of exosomes includes one or more exosomes enriched in at least one of miR-146a, miR-22, miR-24. In other embodiments, the plurality of exosomes can include one or more exosomes containing microRNAs. This includes
 10 various microRNAs known in the art, such as miR-1469, miR-762, miR-574-3p, miR-574-5p, miR-3197, miR-4281, miR-1976, miR-1307, miR-1224-3p, miR-187, miR-3141, miR-1268, miR-155, miR-122, miR-638, miR-3196, miR-223, miR-4267, miR-1281, miR-885-5p, miR-663, miR-let-7b, miR-29d, miR-144, miR-let-7e 143, miR-lrt-7g, miR-17a, miR-96, miR-125a-5p, miR-128, miR-720, miR-21, miR-9, miR-26b, miR-29b, miR-30c, miR-30b, miR-15
 15 191, and miR-1b. In other embodiments, the one or more microRNAs comprise one or more exosomes including miR-96, miR-29b, and miR-191.

In various embodiments, administration of the plurality of exosomes alters gene expression in the damaged or dysfunctional tissue, improves viability of the damaged tissue, and/or enhances regeneration or production of new tissue in the individual. In various
 20 embodiments, the quantities of exosomes that are administered to achieved these effects range from 1×10^6 to 1×10^7 , 1×10^7 to 1×10^8 , 1×10^8 to 1×10^9 , 1×10^9 to 1×10^{10} , 1×10^{10} to 1×10^{11} , 1×10^{11} to 1×10^{12} , 1×10^{12} or more. In other embodiments, the numbers of exosomes is relative to the number of cells used in a clinically relevant dose for a cell-therapy method. For example, it has been demonstrated that 3mL / 3×10^5 human cardiac-derived
 25 cells (CDCs), is capable of providing therapeutic benefit in intracoronary administration, and therefore, a plurality of exosomes as derived from that number of cells in a clinically relevant dose for a cell-therapy method. In various embodiments, administration can be in repeated doses. For example, defining an effective dose range, dosing regimen and route of administration, may be guided by studies using fluorescently labeled exosomes, and
 30 measuring target tissue retention, which can be >10X, >50X, or >100X background, as measured 5, 10, 15, 30, or 30 or more min as a screening criterion. In certain embodiments, >100X background measured at 30 mins is a baseline measurement for a low and high dose that is then assessed for safety and bioactivity (e.g., using MRI endpoints: scar size, global and regional function). In various embodiments, single doses are compared to two, three,
 35 four, four or more sequentially-applied doses. In various embodiments, the repeated or sequentially-applied doses are provided for treatment of an acute disease and/or condition. In various embodiments, the repeated or sequentially-applied doses are provided for treatment of a chronic disease and/or condition.

5 In various embodiments, administration of exosomes to the subject occurs through any of known techniques in the art. In some embodiments, this includes percutaneous delivery and/or injection into heart or skeletal muscle. In other embodiments, myocardial infusion is used, for example, the use of intracoronary catheters. In various embodiments, delivery can be intra-arterial or intravenous. Additional delivery sites include any one or
10 more compartments of the heart, such as myocardium, associated arterial, venous, and/or ventricular locations. In certain embodiments, administration can include delivery to a tissue or organ site that is the same as the site of diseased and/or dysfunctional tissue. In certain embodiments, administration can include delivery to a tissue or organ site that is different from the site of diseased and/or dysfunctional tissue. In certain embodiments, the delivery is
15 via inhalation or oral administration. In various embodiments, administration of exosomes can include combinations of multiple delivery techniques, such as intravenous, intracoronary, and intramyocardial delivery.

In various embodiments, administration of the plurality of exosomes alters gene expression in the damaged or dysfunctional tissue, improves viability of the damaged tissue,
20 and/or enhances regeneration or production of new tissue in the individual. In various embodiments, administration of the exosomes results in functional improvement in the tissue. In certain embodiments, the damaged tissue is pulmonary, arterial or capillary tissue. In several embodiments, the damaged or dysfunctional tissue includes cardiac tissue.

In various embodiments, the plurality of exosomes modulate smad pathway activity, including for example, smad4 and smad 2/3. In various embodiments, the plurality of
25 exosomes increase cardiomyocyte proliferation. In various embodiments, the plurality of exosomes are capable of modulating SDF-1, VEGF and/or collagen expression. In various embodiments, the plurality of exosomes is capable of enhancing infiltration of monocytes, macrophages, and T-cells.

30 For example, in certain embodiments in which pulmonary, arterial, capillary, or cardiac tissue is damaged or dysfunctional, functional improvement may comprise increased cardiac output, contractility, ventricular function and/or reduction in arrhythmia (among other functional improvements). For example, this may include a decrease in right ventricle systolic pressure. For other tissues, improved function may be realized as well, such as
35 enhanced cognition in response to treatment of neural damage, improved blood-oxygen transfer in response to treatment of lung damage, improved immune function in response to treatment of damaged immunological-related tissues. In other embodiments, the disease and/or condition involving tissue damage or dysfunction is pulmonary tissue, including

5 pulmonary, arterial or capillary tissue, such as the endothelial lining of distal pulmonary arteries. In other embodiments, the disease and/or condition involving tissue damage or dysfunction is heart disease.

In various embodiments, administration of the plurality of exosomes alters gene expression in the damaged or dysfunctional tissue, improves viability of the damaged tissue,
10 and/or enhances regeneration or production of new tissue in the individual. In various embodiments, administration of the exosomes results in functional improvement in the tissue. In several embodiments, the damaged or dysfunctional tissue includes skeletal muscle tissue.

For example, in certain embodiments in which skeletal muscle tissue is damaged or dysfunctional, functional improvement may include increased contractile strength, improved
15 ability to walk (for example, and increase in the six-minute walk test results), improved ability to stand from a seated position, improved ability to sit from a recumbent or supine position, or improved manual dexterity such as pointing and/or clicking a mouse.

In various embodiments, the damaged or dysfunctional tissue is in need of repair, regeneration, or improved function due to an acute event. Acute events include, but are not
20 limited to, trauma such as laceration, crush or impact injury, shock, loss of blood or oxygen flow, infection, chemical or heat exposure, poison or venom exposure, drug overuse or overexposure, and the like. In certain embodiments, the damaged tissue is pulmonary, arterial or capillary tissue, such as the endothelial lining of distal pulmonary arteries. In other embodiments, the damaged tissue is cardiac tissue and the acute event includes a myocardial
25 infarction. In some embodiments, administration of the exosomes results in an increase in cardiac wall thickness in the area subjected to the infarction.

In other embodiments, damaged or dysfunctional tissue is due to chronic disease, such as for example congestive heart failure, including as conditions secondary to diseases such as emphysema, ischemic heart disease, hypertension, valvular heart disease, connective tissue
30 diseases, HIV infection, liver disease, sickle cell disease, dilated cardiomyopathy, infection such as Schistosomiasis, diabetes, and the like. In various embodiments, the administration can be in repeated doses, such as two, three, four, four or more sequentially-applied doses. In various embodiments, the repeated or sequentially-applied doses are provided for treatment of an acute disease and/or condition. In various embodiments, the repeated or sequentially-
35 applied doses are provided for treatment of a chronic disease and/or condition.

In other embodiments, damaged or dysfunctional tissue is in the brain and due to an acute event or chronic disease, such as traumatic head injury, stroke or neurodegenerative conditions such as Alzheimer's, Parkinson's, Huntington's, ALS or similar conditions,

5 wherein exosomes, including urodele-derived exosomes are capable of to delivering microRNAs and other exosome cargo by crossing the blood-brain barrier. This includes, for example, exosomes administered at a site that is not the site of damaged or dysfunctional tissue, such as delivery of exosome cargo contents to injured brain when administered via the intravenous or intra-arterial routes.

10 Other sources of damage also include, but are not limited to, injury, age-related degeneration, cancer, and infection. In several embodiments, the regenerative cells are from the same tissue type as is in need of repair or regeneration. In several other embodiments, the regenerative cells are from a tissue type other than the tissue in need of repair or regeneration.

In certain embodiments, the method of treating a heart related disease, includes
15 administering a composition including a plurality of exosomes isolated from urodele cells to a subject, thereby treating the subject. In certain embodiments, the method of treatment includes, selecting a subject in need of treatment for a pulmonary disease and/or condition, administering a composition including a plurality of exosomes to the individual, wherein administration of the composition treat the subject. In certain embodiments, the method of
20 treatment includes, selecting a subject in need of treatment for a heart related disease and/or condition, administering a composition including a plurality of exosomes to the individual, wherein administration of the composition treat the subject. In various embodiments, the heart related disease and/or condition includes heart failure. In various embodiments, the plurality of exosomes range in size from 30 to 300 nm. In various embodiments, the
25 plurality of exosomes range in size from 40 to 100 nm. In certain embodiments, the plurality of exosomes urodele-derived cell exosomes. In certain embodiments, the plurality of exosomes includes one or more exosomes that are CD63+, CD105+, or both. In various embodiments, the exosomes include microRNAs miR-146a, miR22, miR-24, miR-210, miR-150, miR-140-3p, miR-19a, miR-27b, miR-19b, miR-27a, miR-376c, miR-128, miR-320a,
30 miR-143, miR-21, miR-130a, miR-9, miR-185, and/or miR-23a. In other embodiments, the plurality of exosomes can include one or more exosomes containing microRNAs. This includes various microRNAs known in the art, such as miR-1469, miR-762, miR-574-3p, miR-574-5p, miR-3197, miR-4281, miR-1976, miR-1307, miR-1224-3p, miR-187, miR-3141, miR-1268, miR-155, miR-122, miR-638, miR-3196, miR-223, miR-4267, miR-1281,
35 miR-885-5p, miR-663, miR-let-7b, miR-29d, miR-144, miR-let-7e 143, miR-lrt-7g, miR-17a, miR-96, miR-125a-5p, miR-128, miR-720, miR-21, miR-9, miR-26b, miR-29b, miR-30c, miR-30b, miR-191, and miR-1b. In other embodiments, the one or more microRNAs comprise one or more exosomes including miR-96, miR-29b, and miR-191. In other

5 embodiments, the exosomes are 2-5 kDa, such as 3 kDa. In other embodiments, administering a composition includes a dosage of 1×10^8 , 1×10^8 to 1×10^9 , 1×10^9 to 1×10^{10} , 1×10^{10} to 1×10^{11} , 1×10^{11} to 1×10^{12} , 1×10^{12} or more exosomes. In other embodiments, the numbers of exosomes is relative to the number of cells used in a clinically relevant dose for a cell-therapy method. For example, it has been demonstrated that 3mL / 3×10^5 CDCs, is capable of providing therapeutic benefit in intracoronary administration, and therefore, a plurality of exosomes as derived from that number of cells in a clinically relevant dose for a cell-therapy method. In various embodiments, administering a composition includes multiple dosages of the exosomes. In various embodiments, the repeated or sequentially-applied doses are provided for treatment of an acute disease and/or condition. In various embodiments, the repeated or sequentially-applied doses are provided for treatment of a chronic disease and/or condition. In other embodiments, administering a composition includes myocardial infusion. In other embodiments, administering a composition includes use of an intracoronary catheter. In other embodiments, administration of a composition includes intra-arterial infusion. In other embodiments, administration of a composition includes intravenous infusion. In other embodiments, administering a composition includes percutaneous injection. In other embodiments, injection includes injection into heart or skeletal muscle. In other embodiments, administration of a composition includes inhalation. In other embodiments, exosome therapy is provided in combination with standard therapy for a disease and/or condition. This may include co-administration of the exosomes with a therapeutic agent.

Described herein is a method of administering a plurality of exosomes including selecting a subject and administering a composition including a plurality of exosomes to the subject, wherein administration consists of one or more of: intra-arterial infusion, intravenous infusion, and injection. In other embodiments, injection includes percutaneous injection. In other embodiments, injection includes injection into heart or skeletal muscle.

In certain embodiments, administering a composition includes 1×10^8 or more exosomes in a single dose. In other embodiments, administering a composition includes a dosage of 1×10^8 , 1×10^8 to 1×10^9 , 1×10^9 to 1×10^{10} , 1×10^{10} to 1×10^{11} , 1×10^{11} to 1×10^{12} , 1×10^{12} or more exosomes. In other embodiments, the numbers of exosomes is relative to the number of cells used in a clinically relevant dose for a cell-therapy method. For example, it has been demonstrated that 3mL / 3×10^5 CDCs, is capable of providing therapeutic benefit in intracoronary administration, and therefore, a plurality of exosomes as

5 derived from that number of cells in a clinically relevant dose for a cell-therapy method. For example, it has been demonstrated that 3mL / 3×10^5 CDCs, is capable of providing therapeutic benefit in intracoronary administration, and therefore, a plurality of exosomes as derived from that number of cells in a clinically relevant dose for a cell-therapy method. In various embodiments, administration can be in repeated doses. For example, defining an
 10 effective dose range, dosing regimen and route of administration, may be guided by studies using fluorescently labeled exosomes, and measuring target tissue retention, which can be >10X, >50X, or >100X background, as measured 5, 10, 15, 30, or 30 or more min as a screening criterion. In certain embodiments, >100X background measured at 30 mins is a baseline measurement for a low and high dose that is then assessed for safety and bioactivity
 15 (e.g., using MRI endpoints: scar size, global and regional function).

In certain embodiments, a single dose is administered multiple times to the subject. In certain embodiments, the multiple administrations to the subject includes of two or more of intra-arterial infusion, intravenous infusion, and injection. In other embodiments, injection includes percutaneous injection. In other embodiments, injection includes injection into heart
 20 or skeletal muscle.

In certain embodiments, the plurality of exosomes include one or more exosomes with a diameter of about 40 nm to 100 nm and at least about 3 kDa. In various embodiments, the cells are from amphibians within the order Caudata (also described as urodeles). For example, this includes species within the family *Salamandridae* (also known as newts).
 25 Various non-limiting examples include *Notophthalmus viridescens* and *Ambystoma mexicanum*. In other embodiments, the cells are cultured as a cell line capable of serial passaging. This includes, for example, the A1 cell line of *Notophthalmus viridescens*. In other embodiments, the cells are stem cells, progenitors, precursors, and/or mesenchyme. In certain embodiments, the plurality of exosomes includes one or more exosomes including
 30 one or more microRNAs selected from the group consisting of: miR-146a, miR22, miR-24, miR-210, miR-150, miR-140-3p, miR-19a, miR-27b, miR-19b, miR-27a, miR-376c, miR-128, miR-320a, miR-143, miR-21, miR-130a, miR-9, miR-185, and miR-23a. In certain embodiments, the one or more microRNAs include miR-146a, miR22, and miR-24. In other embodiments, the plurality of exosomes can include one or more exosomes containing
 35 microRNAs. This includes various microRNAs known in the art, such as miR-1469, miR-762, miR-574-3p, miR-574-5p, miR-3197, miR-4281, miR-1976, miR-1307, miR-1224-3p, miR-187, miR-3141, miR-1268, miR-155, miR-122, miR-638, miR-3196, miR-223, miR-4267, miR-1281, miR-885-5p, miR-663, miR-let-7b, miR-29d, miR-144, miR-let-7e 143,

5 miR-lrt-7g, miR-17a, miR-96, miR-125a-5p, miR-128, miR-720, miR-21, miR-9, miR-26b, miR-29b, miR-30c, miR-30b, miR-191, and miR-1b. In other embodiments, the one or more microRNAs comprise one or more exosomes including miR-96, miR-29b, and miR-191. In certain embodiments, the plurality of exosomes includes one or more exosomes that are CD63+, CD105+, or both. In certain embodiments, the subject has a heart related disease and/or condition. In certain embodiments, the heart related disease and/or condition includes myocardial infarct. In certain embodiments, the heart related disease and/or condition includes heart failure. In certain embodiments, the heart failure is associated with Duchenne muscular dystrophy.

15 In other embodiments, the subject has a brain related disease and/or condition, such as damaged or dysfunctional brain or other neural tissue. In some embodiments, the brain related disease and/or condition is due to an acute event such as traumatic head injury or stroke. In other embodiments, the brain related disease and/or condition is due to chronic disease, such as neurodegenerative diseases including such as Alzheimer's, Parkinson's, Huntington's, ALS or similar conditions. In various embodiments, the intra-arterial infusion, intravenous infusion, and/or injection of a plurality of exosomes, including urodele-derived exosomes, is capable of delivering microRNAs and other exosome cargo by crossing the blood-brain barrier. In certain embodiments, administration is at the site of diseased and/or dysfunctional tissue. In certain embodiments, administration is not at the site of diseased and/or dysfunctional tissue.

25

Further described herein is a method of improving cardiac performance in a subject including, selecting a subject, administering a composition including a plurality of exosomes to the individual, wherein administration of the composition improves cardiac performance in the subject. In some embodiments, this includes a decrease in right ventricle systolic pressure. In other embodiments, there is a reduction in arteriolar narrowing, or pulmonary vascular resistance. In other embodiments, improving cardiac performance can be demonstrated, by for example, improvements in baseline ejection volume. In other embodiments, improving cardiac performance relates to increases in viable tissue, reduction in scar mass, improvements in wall thickness, regenerative remodeling of injury sites, enhanced angiogenesis, improvements in cardiomyogenic effects, reduction in apoptosis, and/or decrease in levels of pro-inflammatory cytokines.

35 In certain embodiments, the method of improving cardiac performance includes, selecting a subject in need of treatment for a heart related disease and/or condition,

5 administering a composition including a plurality of exosomes to the individual, wherein administration of the composition treat the subject. In various embodiments, the heart related disease and/or condition includes heart failure. In various embodiments, the plurality of exosomes range in size from 30 to 300 nm. In various embodiments, the plurality of exosomes range in size from 40 to 100 nm. In certain embodiments, the plurality of

10 exosomes are urodele cell derived exosomes. In certain embodiments, the plurality of exosomes includes one or more exosomes that are CD63+, CD105+, or both. In various embodiments, the exosomes include microRNAs miR-146a, miR22, miR-24, miR-210, miR-150, miR-140-3p, miR-19a, miR-27b, miR-19b, miR-27a, miR-376c, miR-128, miR-320a, miR-143, miR-21, miR-130a, miR-9, miR-185, and/or miR-23a. In various embodiments,

15 the exosomes include microRNAs miR-1469, miR-762, miR-574-3p, miR-574-5p, miR-3197, miR-4281, miR-1976, miR-1307, miR-1224-3p, miR-187, miR-3141, miR-1268, miR-155, miR-122, miR-638, miR-3196, miR-223, miR-4267, miR-1281, miR-885-5p, miR-663, miR-let-7b, miR-29d, miR-144, miR-let-7e 143, miR-lrt-7g, miR-17a, miR-96, miR-125a-5p, miR-128, miR-720, miR-21, miR-9, miR-26b, miR-29b, miR-30c, miR-30b, miR-191, and

20 miR-1b. In other embodiments, the one or more microRNAs comprise one or more exosomes including miR-96, miR-29b, and miR-191. In other embodiments, the exosomes are 2-5 kDa, such as 3 kDa. In other embodiments, administering a composition includes a dosage of 1×10^8 , 1×10^8 to 1×10^9 , 1×10^9 to 1×10^{10} , 1×10^{10} to 1×10^{11} , 1×10^{11} to 1×10^{12} , 1×10^{12} or more exosomes. In other embodiments, the numbers of exosomes is relative

25 to the number of cells used in a clinically relevant dose for a cell-therapy method. For example, it has been demonstrated that 3mL / 3×10^5 CDCs, is capable of providing therapeutic benefit in intracoronary administration, and therefore, a plurality of exosomes as derived from that number of cells in a clinically relevant dose for a cell-therapy method. In various embodiments, administering a composition includes multiple dosages of the

30 exosomes. In various embodiments, the repeated or sequentially-applied doses are provided for treatment of an acute disease and/or condition. In various embodiments, the repeated or sequentially-applied doses are provided for treatment of a chronic disease and/or condition. In other embodiments, administering a composition includes percutaneous injection. In other embodiments, administering a composition includes injection into heart or skeletal muscle.

35 In other embodiments, administering a composition includes myocardial infusion. In other embodiments, administering a composition includes use of an intracoronary catheter. In other embodiments, administration a composition includes intra-arterial or intravenous delivery. Additional delivery sites include any one or more compartments of the heart, such

5 as myocardium, associated arterial, venous, and/or ventricular locations. In certain
embodiments, administration can include delivery to a tissue or organ site that is the same as
the site of diseased and/or dysfunctional tissue. In certain embodiments, administration can
include delivery to a tissue or organ site that is different from the site of diseased and/or
dysfunctional tissue. In certain embodiments, the delivery is via inhalation or oral
10 administration. In various embodiments, administration of exosomes can include
combinations of multiple delivery techniques, such as intravenous, intracoronary, and
intramyocardial delivery. In other embodiments, exosome therapy is provided in
combination with standard therapy for a disease and/or condition. This may include co-
administration of the exosomes with a therapeutic agent.

Example 1

Sources of Exosomes

As described, a critical scientific and medical question is understanding whether stem
cells might be helpful in not only preventing or ameliorating disease and/or conditions, but
20 actually capable of treating heart disease and related conditions via regeneration and repair of
damaged cells and promotion of vascular cell growth. It is suggested that therapeutic effects
of stem cells via regeneration can be significantly enhanced by directly delivering exosomes
produced by such stem cells as an alternative to delivering the cell themselves. There is
increasing evidence that, for example, cardiosphere derived cellular exosomes are indeed
25 capable of delivering therapeutic benefits of their parental cell type. This includes, for
example, intracoronary delivery in ischemia/reperfusion (IR) injury, percutaneous injection in
a myocardial infarct mode, intravenous infusion of CDC exosomes for pulmonary arterial
hypertension (PAH) is capable of noticeable benefits, as shown via echocardiography. There
is further evidence that key biological factors delivered by these exosomes include
30 microRNAs.

Based on the well-known regenerative capacity of urodeles for limb replacement, it is
of interest to understand whether their extraordinary capacity for replacement and/or repair
are mediated by similar processes involving exosomes and if such features can be applied in
human therapy. As described urodele regeneration have highlighted an important role for
35 microRNAs, but without an understanding if such microRNAs exhibit their effects by
processes involving cellular exosomes. Moreover, it is entirely unknown if isolation and
administration of such urodele exosome, and their biological factors such as microRNAs, can
find application in mammals such as humans.

5

*Example 2**Media Conditioning and Exosome Purification*

Urodele cell line, such as A1 cell line isolated from mesenchyme, is utilized as a cell line capable of serial passaging in culture. One can also isolate exosomes from normal human dermal fibroblasts (NHDF), cells that have been previously utilized as controls providing no salutary benefit, as a control, or cardiosphere derived cells (CDCs) as a comparison for efficacy of therapeutic benefit. Cells can be conditioned in serum-free media for 15 days at 100% confluence. Aspirated media is then centrifuged at 3,000xg for 15 min to remove cellular debris. Exosomes were then isolated using Exoquick Exosome Precipitation Solution (Figure 2).

Exosome pellets are resuspended in the appropriate media and used for assays. Expression of the conserved exosome marker CD63 can be verified using ELISA. RNA content of exosome pellets can also be quantified using a Nanodrop spectrophotometer. Exosomal RNA degradation is performed by suspending exosome pellets in 2 ml of PBS. To one sample, 100 μ l of Triton X-100 (Sigma Aldrich) is added to achieve 5% triton concentration. Exosomes are treated with 0.4 mg/ml RNase A treatment for 10 min at 37°C. Samples are further treated with 0.1 mg/ml Proteinase K for 20 min at 37°C. RNA is purified from samples using an microRNA isolation kit. RNA levels are measured using Nanodrop.

Example 3

25

Mass Spectrometry Analysis on Exosome Pellets

Proteins are prepared for digestion using the filter-assisted sample preparation (FASP) method. Concentrations were measured using a Qubit fluorometer (Invitrogen). Trypsin is added at a 1:40 enzyme-to-substrate ratio and the sample incubated overnight on a heat block at 37°C. The device is centrifuged and the filtrate collected. Digested peptides are desalted using C18 stop-and-go extraction (STAGE) tips. Peptides are fractionated by strong anion exchange STAGE tip chromatography. Peptides are eluted from the C18 STAGE tip and dried. Each fraction can be analyzed with liquid chromatography-tandem mass spectrometry. Samples are loaded to a 2 cm 3 100 μ m I.D. trap column. The analytical column was 13 cm 3 75 μ m I.D. fused silica with a pulled tip emitter. The mass spectrometer is programmed to acquire, by data-dependent acquisition, tandem mass spectra from the top 15 ions in the full scan from 400 to 1,400 m/z. Mass spectrometer RAW data files are converted to MGF format using msconvert. MGF files are searched using X!Hunter against the latest spectral library available on the GPM at the time. MGF files are also searched using

5 X!!Tandem using both the native and k-score scoring algorithms and by OMSSA. Proteins are required to have one or more unique peptides with peptide E-value scores of 0.01 or less from X!!Tandem, 0.01 or less from OMSSA, 0.001 or less and theta values of 0.5 or greater from X!Hunter searches, and protein E-value scores of 0.0001 or less from X!!Tandem and X!Hunter. Myocyte Isolation Neonatal rat cardiomyocytes (NRCMs) were isolated from 1- to
10 2-day-old Sprague Dawley rat pups and cultured in monolayers as described.

Example 4

Analysis of Exosome MicroRNAs Content

Towards investigating the basis of the therapeutic benefit of CDC exosomes, the
15 Inventors previously compared their microRNA repertoire to that of NHDF exosomes using a PCR microarray of the 88 best-defined microRNAs, and it was shown that the microRNA content of the two cell types differed dramatically. Forty-three microRNAs were differentially present in the two groups; among these, miR-146a was the most highly enriched in CDC exosomes (262-fold higher than in NHDF exosomes; Figures 1A, 1B, and 3).

20 A similar approach can be deployed to evaluate possible factors in urodele derived cellular exosomes. Information regarding microRNAs in the urodeles such as newts is quite limited, yet it has been reported that microRNA microarray screen with detection probes complementary to *Xenopus*, zebrafish and human miRNAs, such as μ Paraflo™, is capable of gauging differentially expressed microRNAs to identify those microRNAs involved in
25 cardiac regeneration. Witman *et al.*, “miR-128 regulates non-myocyte hyperplasia, deposition of extracellular matrix and Islet1 expression during newt cardiac regeneration.” *Dev Biol.* 2013 Nov 15;383(2):253-63, which is fully incorporated by reference herein.

Recently, the therapeutic effects of microRNAs such as miR-146a, as derived from CDCs, have been shown as mediate some of the therapeutic benefits of CDC exosomes. For
30 example, miR-146a leads to thicker infarct wall thickness and increased viable tissue in a mouse model of myocardial infarct. Ibrahim, *et al.*, “Exosomes as critical agents of cardiac regeneration triggered by cell therapy.” *Stem Cell Reports.* 2014 May 8;2(5):606-19, which is fully incorporated by reference herein. Separately it has been reported in that miR-128 regulates non-myocyte hyperplasia via Islet1 expression during newt cardiac regeneration.

35

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*Example 5**Intracoronary Infusion of Exosome Therapeutic*

An example of the described technique, exosomes are isolated from human CDCs as described using a technique such as ExoQuick® precipitation in order to generate a composition include a population of exosomes ranging in size from 30-100 nm that are enriched in biological agents capable of cardiac repair (e.g., proteins, surface antigens such as CD105, microRNAs such as miR-146a). A single dose, such as 3mL / 3×10^5 CDCs, can be delivered to a subject in need of treatment for a heart related diseases and/or conditions, which can include both acute and chronic diseases and/or conditions. Importantly, the above results indicate that exosomes provide both cardioprotective and regenerative effects, thereby providing multiple timepoints for administration ranging from immediately after an acute event (e.g., myocardial infarct) or at much later timepoints such as weeks and/or months during the progression of chronic disease (e.g., congestive heart disease).

Such administration may occur as a single dose or a series of repeated doses, and it is understood that dosages may be provided by variable routes of administration combined together. Administration may be via intracoronary infusion as delivered through the central lumen of a balloon catheter positioned in the coronary artery, such as via over-the-wire balloon catheter, with a subtended by a patent coronary artery. Subsequent repeat doses can also be via intracoronary infusion, but may rely on other methods of administration (e.g., intravenous infusion).

A variety of techniques may be relied upon to evaluate the therapeutic effects of exosome therapy. This includes echocardiographic assessment, wherein wall thickness, ejection volume or a variety of other parameters may indicate cardiac improvement. Other examples include hemodynamic measurement.

30

*Example 6**Urodele Derived Cellular Exosomes Are Bioactive in Mammals and Exhibit Cardioprotective Function*

The Inventors discovered that urodele derived cellular exosomes from the A1 cell line of *Notophthalmus viridescens* are bioactive in mammals. In particular, exosomes isolated via the above described methods are capable of promoting rat cardiomyocyte proliferation, increase SDF-1 secretion by human dermal fibroblasts, and improve functional recovery after myocardial infarct in rats.

Specifically, A1 cells, derived from the amputated limb buds of *Notophthalmus viridescense*, were expanded in culture. Further description can be found for example, in Ferreti and Brockes, "Culture of newt cells from different tissues and their expression of a regeneration-associated antigen" *J Exp Zool.* 1988 Jul;247(1):77-91. Culture of newt cells from different tissues and their expression of a regeneration-associated antigen, which is fully incorporated by reference herein.

Exosomes were isolated by polyethylene glycol precipitation of A1-conditioned serum-free media (or media conditioned by human dermal fibroblasts (DF) as a control) followed by centrifugation. Bioactivity was tested *in vitro* on neonatal rat ventricular myocytes (NRVM), and *in vivo* on acute myocardial infarction in Wistar-Kyoto rats (250µg or 500µg of A1-exosomes or vehicle (placebo) injected intramyocardially). Functional and histological analyses were performed 3 weeks after therapy.

A1-conditioned media yielded $\sim 2.8 \pm 1$ Billion particles/ml of 129 ± 1.1 nm diameter. *In vitro*, A1-exosomes increased the proliferative capacity of NRVM compared to DF-exosomes ($4.98 \pm 0.89\%$ vs $0.77 \pm 0.33\%$, $p=0.035$). Priming of DFs with A1-exosomes increased SDF-1 secretion compared to DF-exosomes (755 ± 117 pg/ml vs 368 ± 21 pg/ml, $p=0.03$). *In vivo*, both A1-exosome doses increased cardiac function compared to placebo (EF= $46 \pm 1\%$ in 250µg, $49 \pm 4\%$ in 500µg vs $36 \pm 1\%$ in placebo, $p=0.045$ by ANOVA). Scar size was markedly decreased ($11 \pm 1\%$ in 250µg, $9 \pm 2\%$ in 500µg vs $18 \pm 2\%$ in placebo, $p=0.006$ by ANOVA), and infarct wall thickness was increased after A1-exosome treatment (1.7 ± 0.11 mm in 250µg, 1.85 ± 0.16 mm in 500µg vs 1.17 ± 0.11 mm in Placebo, $p=0.01$ by ANOVA). Donor-specific antibodies were present at barely detectable levels in the serum of animals that had been injected with A1-exosomes.

Newt exosomes stimulate rat cardiomyocyte proliferation and improve functional and structural outcomes in rats with myocardial infarction. Characterization of the RNA and protein content of newt exosomes, provides clues regarding conserved (or newt-unique) molecular mediators of therapeutic benefit.

Example 7

Discussion

The Inventors' previous work has demonstrated that exosomes reproduce cardiosphere derived therapeutic regeneration, via paracrine mechanisms involving exosomes, for which their cargo content of microRNAs, have the ability to alter cell behavior. Here, the Inventors have extended these studies by demonstrated urodele derived

5 cellular exosomes are bioactive in mammals, and may contain microRNAs similarly capable of delivering a salutary benefit.

Of great interest will be deciphering whether urodele derived exosome bioactivity in mammals is due to a particular repertoire of microRNAs unique to their parental cell of origin. It has been shown that microRNAs, such as miR-146a appear to play an important
10 part in mediating the effects of CDC exosomes, but alone may not suffice to confer comprehensive therapeutic benefit. Other microRNAs in the repertoire may exert synonymous or perhaps synergistic effects with miR-146a. For instance, miR-22 (another microRNA highly enriched in CDC exosomes) has been shown to be critical for adaptive responses to cardiac stress. Likewise, miR-24 (also identified in CDC exosomes) modulates
15 cardiac fibrosis by targeting furin, a member of the profibrotic TGF- β signaling pathway; overexpression of miR-24 in a model of MI decreased myocardial scar formation.

Further, miR-128 in the newt *Notophthalmus viridescens* has been reported as elevated when cardiac hyperplasia is at its peak following injury, with a localised expression pattern for miR-128 in the cardiomyocytes and non-cardiomyocytes in close proximity to the
20 regeneration zone and a regulatory role for miR-128 in proliferating non-cardiomyocyte populations and extracellular matrix deposition, possibly via interaction with Islet1. Based on the reported results described herein, the bioactivity of urodeles in mammals provides compelling avenues for which the extraordinary regenerative potential of urodeles may find application in new therapeutic avenues for heart disease in mammals.

25 Whereas dissection of the active principles within CDC exosomes is worthwhile, deconstruction of the nanovesicles may be counterproductive from a therapeutic perspective. CDC exosomes are naturally cell permeant, and their lipid bilayer coat protects their payloads from degradation as particles shuttle from cell to cell, so that the intact particles themselves may be well suited for disease applications.

30 Based on what is known for CDC paracrine mechanisms involving exosomes, secretion of a medley of individual growth factors and cytokines that collectively produce diverse benefits may be a possible mechanism for their effects and those in urodele regeneration. Yet the involvement of master-regulator microRNAs within exosomes would help tie together the various effects without postulating complex mixtures of numerous
35 secreted protein factors. Such mechanisms are at least partially suggested by what is known regarding regulator microRNAs in newt lens regeneration. Moreover, microRNAs are known to confer long-lasting benefits and fundamental alterations of the injured microenvironment. As possibly unleashing rich signaling information conferred by a cell type that is the first

- 5 shown to be capable of producing regeneration in a setting of “permanent” injury, and confer the same extraordinary properties of urodele cellular regeneration in mammals.

Example 8

Newt exosomes are bioactive on mammalian heart, enhancing proliferation of rat cardiomyocytes and improving recovery after myocardial infarction

10

Adult newts can regenerate amputated cardiac tissue (and whole limbs) without fibrosis, unlike adult mammals which lack such regenerative capacity. Exosomes are nanoparticles which mediate intercellular communication and play a critical role in therapeutic regeneration.

15

A1 cells, derived from the amputated limb buds of *Notophthalmus viridescens*, were expanded in culture. Exosomes from these cells were assayed for bioactivity both in vitro on neonatal rat ventricular myocytes (NRVMs), and in vivo on acute myocardial infarction in Wistar-Kyoto rats. Functional and histological analyses were performed 3 weeks after therapy. The A1 exosomes were analyzed for both RNA using next-generation sequencing and protein cargo using mass spectrometry.

20

A1-conditioned media yielded exosomes that increased the proliferative capacity of NRVMs and significantly increased cardiac function and infarct wall thickness as compared to placebo controls. A1 exosome RNA included numerous miRNAs, lncRNAs and mRNAs that have homology to known mammalian exosome RNA cargo, as well as newt-specific sequences. Proteomic analysis revealed a plethora of contents, some of which are homologues of proteins known to be present in human exosomes.

25

Despite being separated by 300 million years of evolution, newt exosomes are bioactive on the mammalian heart. Newt exosomes stimulate rat cardiomyocyte proliferation and improve functional and structural outcomes in rats with myocardial infarction. Characterization of the RNA and protein content of newt exosomes is beginning to provide clues regarding conserved (or newt-unique) molecular mediators of therapeutic benefit.

30

Example 9

Culturing of A1 cell line, exosome characterization

35

The Inventors cultured A1 cell line, under normal under normal and serum-starved conditions and were able to utilize nanosight technology to visualize exosome diameter and number, as shown in Figure 5.

- 5 Initial observations were followed by additional exosome characterization, including RNA and proteomic profiling, along with functional classification as shown in Figure 6. RNA profiling was validated using qRT-PCR as shown in Figures 7 and 8. qRT-PCR primers used as shown in Table 1.

Table 1. Primer Sequences

Assay Name	Tail Assay Sequence
SAP30L	GGCTCTGTACTGTTGAAAGGCTCTC [SEQ ID NO: 1]
GAS6-AS1	GTGCGCTGCCGCTACGATGTTTG [SEQ ID NO: 2]
SCARF1	TCCAGGCCAGTTGGCCAG [SEQ ID NO: 3]
PGM5-AS1	GATCTACAGCACAGCATGGTGAC [SEQ ID NO: 4]
SLC43A1	CCTCTCTGGTGCTGCTGCTTG [SEQ ID NO: 5]
LY6E	GTTTCTGCCACTTCCGGGTCTAG [SEQ ID NO: 6]
CPSF2	TGCTTGGCACTTCTTCCTGCT [SEQ ID NO: 7]
PRDM16	GAAAGCCTAGGAGAAGCCGATTTG [SEQ ID NO: 8]
ARHGEF15	CAGGACTGACACTGTCACCCTG [SEQ ID NO: 9]
TOR3A	GTCAGTGTAGGCCAAGACTTATGGTC [SEQ ID NO: 10]
SRMS	GAGCGTACGGGTCCACGTG [SEQ ID NO: 11]
CEP170B	CGTTCTCAGGTGGTCTTGTGGC [SEQ ID NO: 12]
VSIG10L	GGCTTGCTGCTGCTGTCTTCAG [SEQ ID NO: 13]
MUC22	GAGTCACGACAACCACCCTCAG [SEQ ID NO: 14]
NKD2	ATGAGGCCCTGGAGTGCGC [SEQ ID NO: 15]
FAM101A	CAGAGATGCTGTCTGGTTAAACAAAGG [SEQ ID NO: 16]
CHST7	GATGCAGTTCAATTGCATGGTTTG [SEQ ID NO: 17]
AMH	CACGTGCCCAACATGGTGGC [SEQ ID NO: 18]
TMEM64	GAATTGCAAAGTGATTGCTGCAAAC [SEQ ID NO: 19]
ADCYAP1	GAAATGCCAAGAGCCTTAACTG [SEQ ID NO: 20]
RNF39	GGAATACTTCAGACATCTCCAACT [SEQ ID NO: 21]
DMRTA2	CCGCAGCGGAAAACGCTGC [SEQ ID NO: 22]
C22orf26/PRR34	CTGGAGTTCAGGAGGAACCAG [SEQ ID NO: 23]
CPSF6	GCCATTGTCGGCCTTAACTGC [SEQ ID NO: 24]
FAM3C	CCAGGGATTGTGAAATAAATGTTGCAG [SEQ ID NO: 25]
GAGE13	GAAGACACGTTGAAATGATGCAGGC [SEQ ID NO: 26]
NR5A1	CCACAGCCCTGAAACGAGAAG [SEQ ID NO: 27]
DNLZ	AGACGGAGCCGAGCTGACTC [SEQ ID NO: 28]
PHF20L1	CCTAACACTCATCTTATTTCCACTGTC [SEQ ID NO: 29]
ZFAND5	CCAGAGTATTTCTGTTACAGCCATAG [SEQ ID NO: 30]
LCN12	GCCAGCGTCTGTTGAAGGATG [SEQ ID NO: 31]
C16orf11/PRR35	CACACAGGGACACTGGAGGTC [SEQ ID NO: 32]
SPIN4	CTTGTTGACTGTACTATTCAGATGGC [SEQ ID NO: 33]
HLA-G	GCTGGGATGTCTCCGTCTCTG [SEQ ID NO: 34]

5 Protein profiling was validated using flow cytometry as shown in Figure 9, and western blot as shown in Figure 10.

Example 10

In vitro studies

10 Neonatal rat ventricular cardiomyocytes (NRVMs) were treated with A1-derived exosomes or control, with analysis of gene expression as shown in Figure 11. Cardiomyocyte apoptosis was measured using flow cytometry and Annexin V staining, 24 hours after ischemic injury, as shown in Figure 12. Further analysis via ELISA analysis of expressed or secreted after application of exosomes, including supernatant collected from NHDFs in
15 culture exposed to NHDF exosomes or A1 exosomes for the cell proliferation-associated marker, SDF-1, and western blot detection of collagen, as shown in Figure 13.

Example 11

In vivo studies

20 A variety of in vivo studies were performed including measurement of echocardiographic measurement of heart function ejection fraction in female Wistar-Kyoto rat hearts three weeks following myocardial infarct, with variable dosages of 250µg or 500µg A1 exosome or 250µg NHDF exosome treatments for control, as shown in Figure 14. Kyoto rats subjected to permanent proximal left anterior descending coronary artery ligation
25 and immediately injection of 250µg or 500µg A1 exosome or 250µg NHDF exosome treatments into the area at risk, as shown in Figure 15.

Further measurements were made of heart sections, percent viable area three weeks following MI and exosome treatment, percent scar area three weeks and infarct wall thickness three weeks following MI and exosome treatment, as shown in Figure 15.

30 Morphological analysis included cardiomyocyte diameter following MI and PBS or A1 exosome treatment as shown in Figure 16. Proliferation of cardiomyocyte proliferation was also measured using BrdU staining, anti-sarcomeric actin as a secondary marker and DAPI nucleus labeling as shown in Figure 17. In addition, microvasculature was quantified, including tissue staining of smooth muscle actin as shown in Figure 18.

5

*Example 12**Immunology studies*

Monocyte infiltration was measured including identification of infiltrating monocyte clusters as shown in Figure 19 with the numbers of monocytes increasing significantly with A1 exosome treatment and escalating with increased dosage. Macrophage infiltration was also measured, including heart tissue stained with the macrophage marker CD68, with graphical data shows the average number of monocytes counted per image field as shown in Figure 20. T-cell infiltration was also measured using heart tissue stained with antibodies against CD8+ (green) and CD4+ (red) T-cell markers, with graphical data shows the average number of CD8+ and CD4+ T-cells counted per image field as shown in Figure 21. Notably, an increase in CD4+ cells was observed with A1 exosome treatment.

*Example 13**microRNAs*

Of interest is understanding the microRNA populations that may be responsible for mediating the observed effects of exosome application. Following A1 exosome treatment, NHDFs demonstrated enrichment of miR-9, a microRNA regulating cardiac hypertrophy also found to be enriched in CDCs (Figure 1B). Specifically, NHDF microRNAs expression was measured post A1 exosome treatment. miRNA isolation from the A1 exosome-treated NHDFs was performed and measured using qRT-PCR as shown in Figure 25. Additionally, native miR-9-5p expression levels and downstream target gene expression was measured as shown in Figure 28. This included quantitative PCR of miR-9-5p from neonatal (P1) and 2 month-old rat hearts with microRNA extracted from neonatal Sprague Dawley rat hearts at P1 and at 2 months of age and PCR array of miR-9-5p target genes in NHDFs following A1 exosome-treatment PCR array of miR-9-5p target genes in NHDFs following miR-9-5p treatment as shown in Figure 28.

To investigate potential effects of miR-9, the Inventors added miR-9 (miR-9-5p specifically) to *in vitro* cultured cells. Specifically, cardiomyocyte proliferation was measured following treatment with miR-9-5p mimic or control microRNA. NRVMs in culture were treated with 25nM miR-9-5p microRNA mimic and EdU (10 μ M) for 24 hours, stained with sarcomeric actin and DAPI nuclei staining with cell proliferation increasing following miR-9-5p. Gene expression changes related to cell proliferation in NRVMs following treatment with miR-9-5p mimic or control microRNA as measured, results are

5 shown in Figure 22. Importantly, it was observed that miR-9-5p was capable of inducing SDF-1 secretion in NHDFs, as shown in Figure 23. 5-ethynyl-2'-deoxyuridine (EdU) can rapidly and sensitively label proliferating cardiac cells in developmental and pathological states. The Inventors measured EdU-positive NRVMs after exposure to NHDF exosomes or hCDC exosomes as shown in Figure 24.

Example 14

Tgfb β /smad pathway

10 Interestingly, A1 exosome treated NHDFs appear to at least partially mediate their effects via the Tgfb β /smad pathway. Gene expression following A1 or NHDF exosome treatment in NHDFs Fibrosis and the Tgfb β /smad pathway was measured as shown in Figure 15 26.

Example 15

Echocardiography of infarcted rat hearts following A1 and NHDF exosome treatments.

20 For functional analysis, of interest was echocardiography of infarcted rat hearts following A1 and NHDF exosome treatments. Additional echocardiographic parameters were evaluated and performed in all animals 3 weeks post MI and treatment and groups analyzed were the NHDF exosome control, the low dose newt-exosomes and the high-dose newt-exosomes. N=4 rats in each group as shown in Figure 27.

25 The various methods and techniques described above provide a number of ways to carry out the invention. Of course, it is to be understood that not necessarily all objectives or advantages described may be achieved in accordance with any particular embodiment described herein. Thus, for example, those skilled in the art will recognize that the methods 30 can be performed in a manner that achieves or optimizes one advantage or group of advantages as taught herein without necessarily achieving other objectives or advantages as may be taught or suggested herein. A variety of advantageous and disadvantageous alternatives are mentioned herein. It is to be understood that some preferred embodiments specifically include one, another, or several advantageous features, while others specifically 35 exclude one, another, or several disadvantageous features, while still others specifically mitigate a present disadvantageous feature by inclusion of one, another, or several advantageous features.

5 Furthermore, the skilled artisan will recognize the applicability of various features from different embodiments. Similarly, the various elements, features and steps discussed above, as well as other known equivalents for each such element, feature or step, can be mixed and matched by one of ordinary skill in this art to perform methods in accordance with principles described herein. Among the various elements, features, and steps some will be
10 specifically included and others specifically excluded in diverse embodiments.

Although the invention has been disclosed in the context of certain embodiments and examples, it will be understood by those skilled in the art that the embodiments of the invention extend beyond the specifically disclosed embodiments to other alternative embodiments and/or uses and modifications and equivalents thereof.

15 Many variations and alternative elements have been disclosed in embodiments of the present invention. Still further variations and alternate elements will be apparent to one of skill in the art. Among these variations, without limitation, are sources of urodele derived cells, the use of alternative sources such as cells derived directly from urodeles or urodele cell lines, or from urodele derived cells undergoing dedifferentiation, transdifferentiation, and/or
20 proliferation, exosomes produced by such cells, method of isolating, characterizing or altering exosomes produced by such cells, and the particular use of the products created through the teachings of the invention. Various embodiments of the invention can specifically include or exclude any of these variations or elements.

In some embodiments, the numbers expressing quantities of ingredients, properties
25 such as concentration, reaction conditions, and so forth, used to describe and claim certain embodiments of the invention are to be understood as being modified in some instances by the term “about.” Accordingly, in some embodiments, the numerical parameters set forth in the written description and attached claims are approximations that can vary depending upon the desired properties sought to be obtained by a particular embodiment. In some
30 embodiments, the numerical parameters should be construed in light of the number of reported significant digits and by applying ordinary rounding techniques. Notwithstanding that the numerical ranges and parameters setting forth the broad scope of some embodiments of the invention are approximations, the numerical values set forth in the specific examples are reported as precisely as practicable. The numerical values presented in some
35 embodiments of the invention may contain certain errors necessarily resulting from the standard deviation found in their respective testing measurements.

In some embodiments, the terms “a” and “an” and “the” and similar references used in the context of describing a particular embodiment of the invention (especially in the

5 context of certain of the following claims) can be construed to cover both the singular and the plural. The recitation of ranges of values herein is merely intended to serve as a shorthand method of referring individually to each separate value falling within the range. Unless otherwise indicated herein, each individual value is incorporated into the specification as if it were individually recited herein. All methods described herein can be performed in any
10 suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (e.g. "such as") provided with respect to certain embodiments herein is intended merely to better illuminate the invention and does not pose a limitation on the scope of the invention otherwise claimed. No language in the specification should be construed as indicating any non-claimed element essential to
15 the practice of the invention.

Groupings of alternative elements or embodiments of the invention disclosed herein are not to be construed as limitations. Each group member can be referred to and claimed individually or in any combination with other members of the group or other elements found herein. One or more members of a group can be included in, or deleted from, a group for
20 reasons of convenience and/or patentability. When any such inclusion or deletion occurs, the specification is herein deemed to contain the group as modified thus fulfilling the written description of all Markush groups used in the appended claims.

Preferred embodiments of this invention are described herein, including the best mode known to the inventors for carrying out the invention. Variations on those preferred
25 embodiments will become apparent to those of ordinary skill in the art upon reading the foregoing description. It is contemplated that skilled artisans can employ such variations as appropriate, and the invention can be practiced otherwise than specifically described herein. Accordingly, many embodiments of this invention include all modifications and equivalents of the subject matter recited in the claims appended hereto as permitted by applicable law.
30 Moreover, any combination of the above-described elements in all possible variations thereof is encompassed by the invention unless otherwise indicated herein or otherwise clearly contradicted by context.

Furthermore, numerous references have been made to patents and printed publications throughout this specification. Each of the above cited references and printed publications are
35 herein individually incorporated by reference in their entirety.

In closing, it is to be understood that the embodiments of the invention disclosed herein are illustrative of the principles of the present invention. Other modifications that can

- 5 be employed can be within the scope of the invention. Thus, by way of example, but not of limitation, alternative configurations of the present invention can be utilized in accordance with the teachings herein. Accordingly, embodiments of the present invention are not limited to that precisely as shown and described.

THE CLAIMS

1. A composition, comprising:
a plurality of exosomes isolated from urodele cells.
2. The composition of claim 1, wherein the plurality of exosomes comprise one or more exosomes with a diameter of about 30 nm to 300 nm and are about 2-5 kDa.
3. The composition of claim 2, wherein the plurality of exosomes comprise one or more exosomes with a diameter of about 40 nm to 100 nm and are about 3 kDa.
4. The composition of claim 1, wherein the plurality of exosomes comprise one or more exosomes comprising one or more microRNAs selected from the group consisting of: miR-1469, miR-762, miR-574-3p, miR-574-5p, miR-3197, miR-4281, miR-1976, miR-1307, miR-1224-3p, miR-187, miR-3141, miR-1268, miR-155, miR-122, miR-638, miR-3196, miR-223, miR-4267, miR-1281, miR-885-5p, miR-663, miR-let-7b, miR-29d, miR-144, miR-let-7e 143, miR-lrt-7g, miR-17a, miR-96, miR-125a-5p, miR-128, miR-720, miR-21, miR-9, miR-26b, miR-29b, miR-30c, miR-30b, miR-191, and miR-1b.
5. The composition of claim 4, wherein the plurality of exosomes comprise one or more exosomes comprising miR-96, miR-29b, and miR-191.
6. The composition of claim 1, wherein the plurality of exosomes comprise one or more exosomes comprising one or more messenger RNAs selected from the group consisting of: PGM5-AS1, NKD2, SLC43A1, PHF20L1, DLNZ, AMH, ARHGEF15, SCARF1, CPSF2, CEP170B, C16orf11/PRR35, CPSF6, RNF39, C22orf26/PRR34, TMEM64, PRDM16, CHST7, ADCYAP1, VSIG10L, MUC22, SRMS, SAP30L, GAS6-AS1, ZFAND5, GAGE13, LCN12, NR5A1, HLA6, LY6E, and SPIN4.
7. The composition of claim 1, wherein the urodele cells comprise *Notophthalmus viridescens* cells.
8. The composition of claim 7, wherein the *Notophthalmus viridescens* cells comprise A1 cell line.

9. The composition of claim 1, wherein the urodele cells are dedifferentiating, transdifferentiating and/or proliferating when isolating the plurality of exosomes.
10. The composition of claim 1, further comprising a pharmaceutically acceptable carrier.
11. A method of treating a heart related disease, comprising:
administering a composition comprising a plurality of exosomes isolated from urodele cells to a subject, thereby treating the subject.
12. The method of claim 11, wherein the plurality of exosomes comprise one or more exosomes with a diameter of about 30 nm to 300 nm and are about 2-5 kDa.
13. The method of claim 12, wherein the plurality of exosomes comprise one or more exosomes with a diameter of about 40 nm to 100 nm, and are about 3 kDa.
14. The method of claim 12, wherein administering a composition comprises 1×10^8 or more exosomes in a single dose.
15. The method of claim 14, wherein single dose is administered multiple times to the subject.
16. The method of claim 15, wherein the subject has a chronic disease.
17. The method of claim 11, wherein administering a composition comprises one or more of intra-arterial infusion, intravenous infusion, and injection.
18. The method of claim 11, wherein the subject is diagnosed as afflicted with a heart related disease prior to administering the composition.
19. The method of claim 11, wherein the urodele cells comprise *Notophthalmus viridescens* cells.
20. The composition of claim 19, wherein the *Notophthalmus viridescens* cells comprise A1 cell line.

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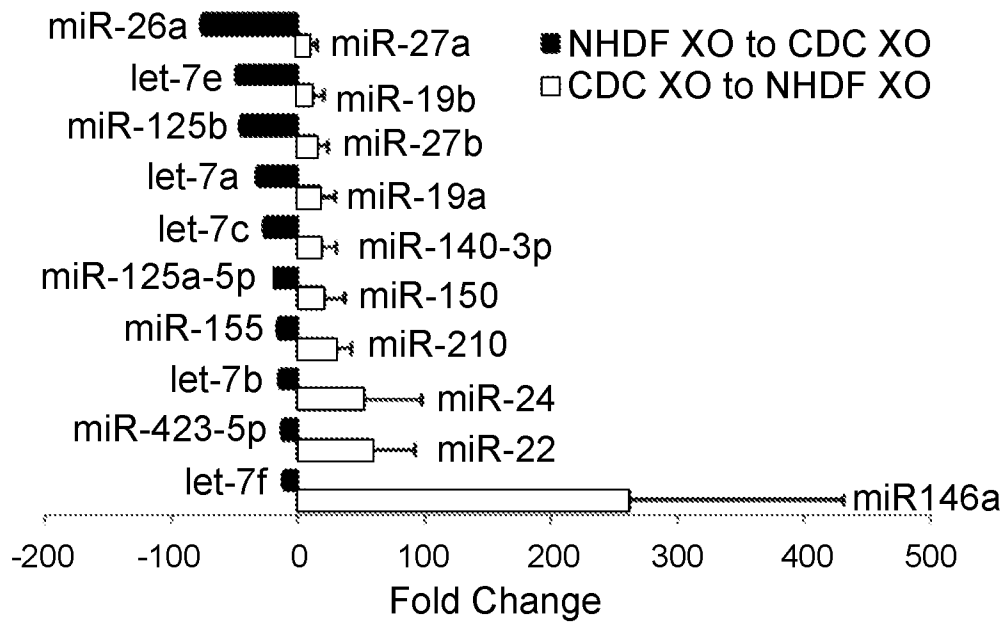
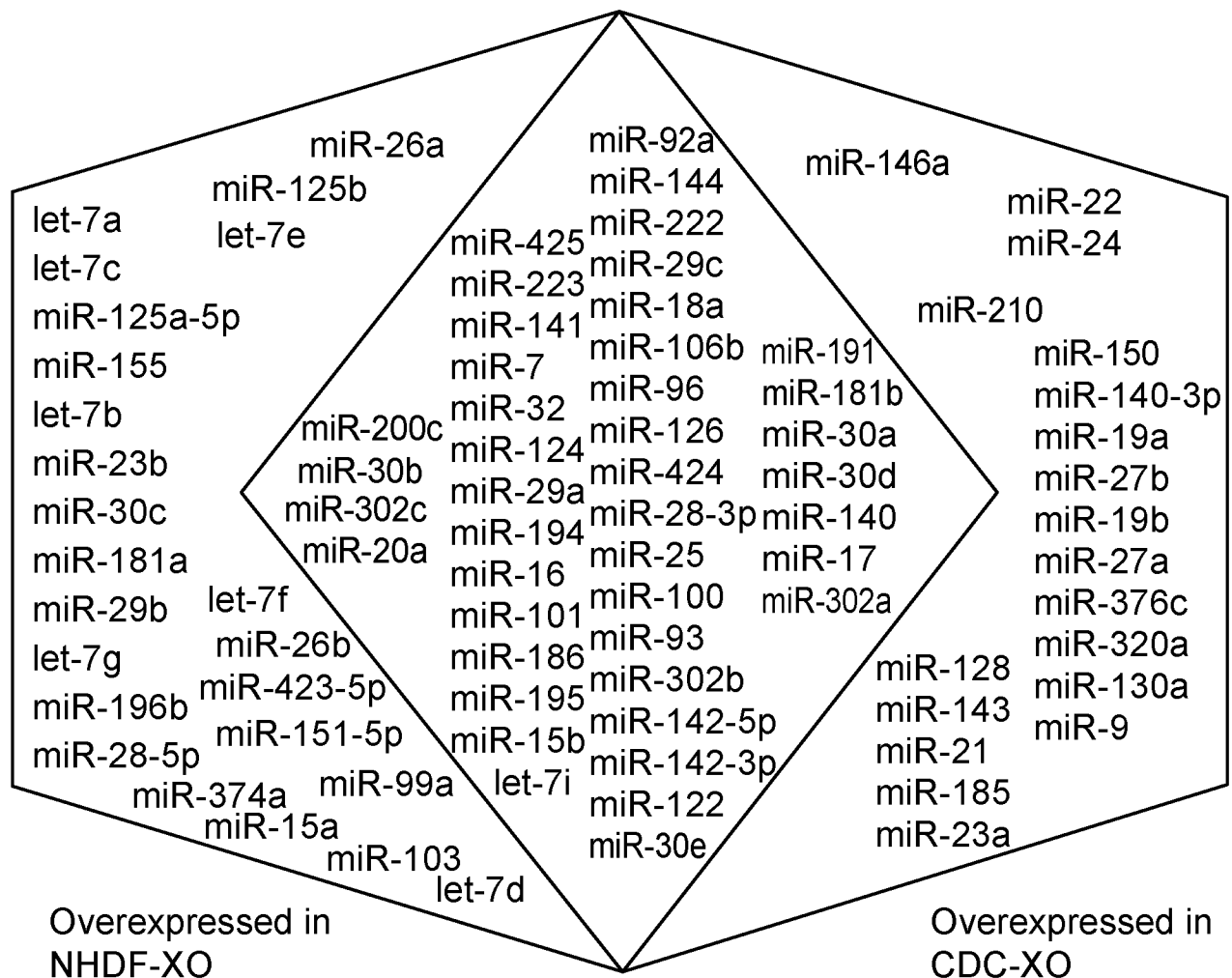
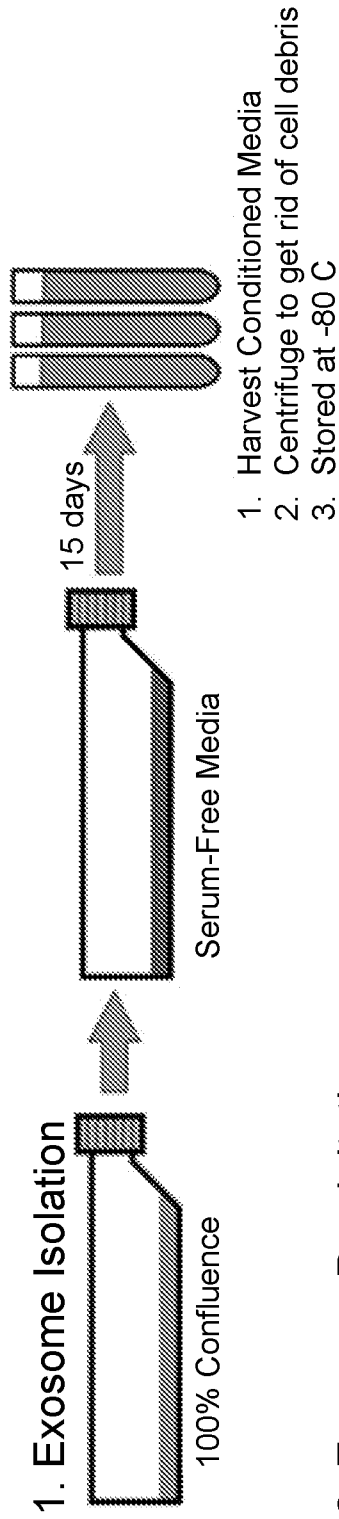
Fig. 1A**Fig. 1B**

Fig. 2A



2. Exosome Precipitation

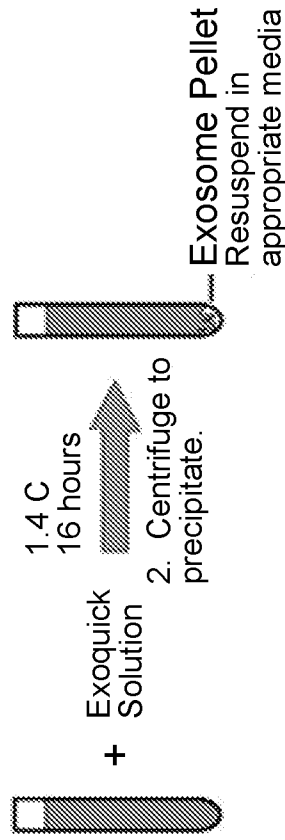


Fig. 2B

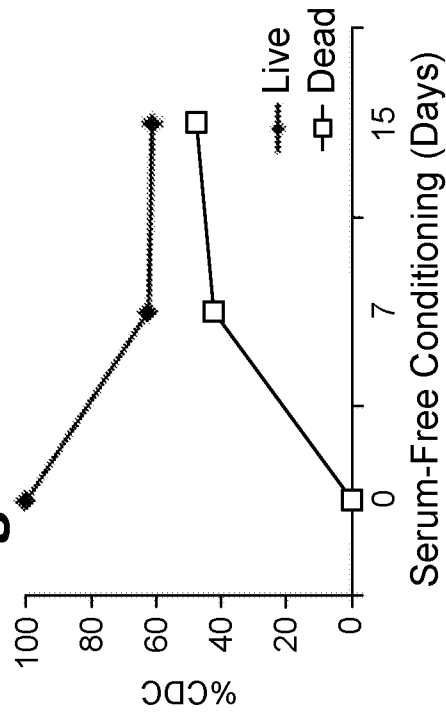
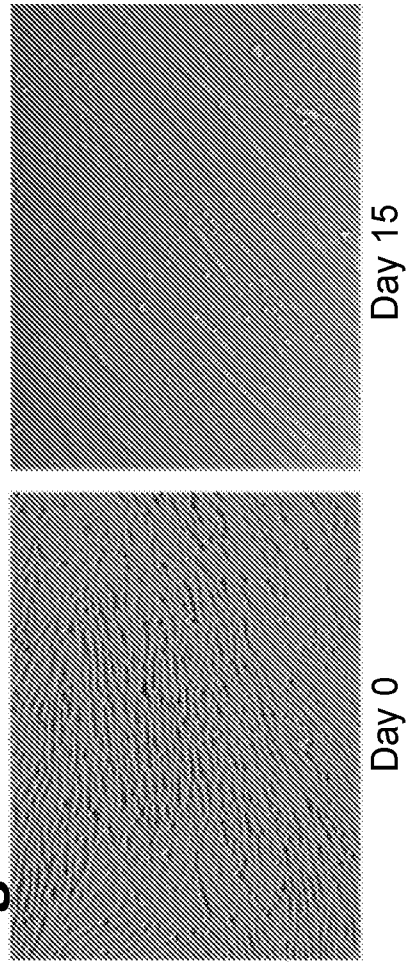


Fig. 2C



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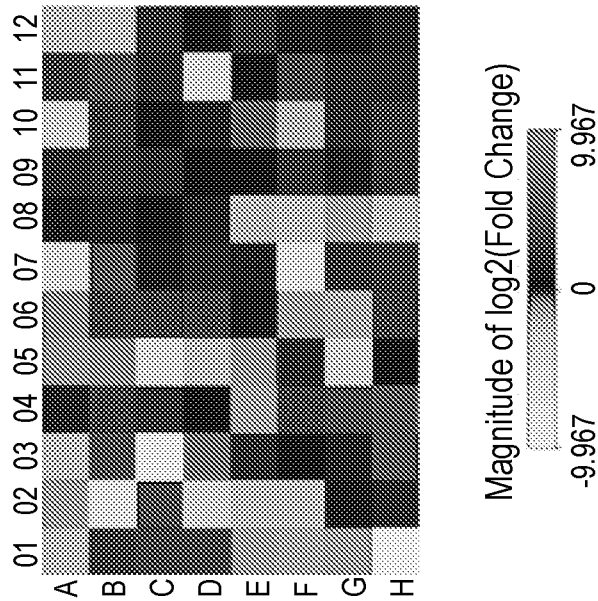


Fig. 3

A01	hsa-miR-142-5p	A02	hsa-miR-16	A03	hsa-miR-142-3p	A04	hsa-miR-21	A05	hsa-miR-15a	A06	hsa-miR-29b	A07	hsa-let-7a	A08	hsa-miR-126	A09	hsa-miR-143	A10	hsa-let-7b	A11	hsa-miR-27a	A12	hsa-let-7f
B01	hsa-miR-9	B02	hsa-miR-26a	B03	hsa-miR-24	B04	hsa-miR-30e	B05	hsa-miR-181a	B06	hsa-miR-29a	B07	hsa-miR-124	B08	hsa-miR-144	B09	hsa-miR-30d	B10	hsa-miR-19b	B11	hsa-miR-22	B12	hsa-miR-122
C01	hsa-miR-150	C02	hsa-miR-32	C03	hsa-miR-155	C04	hsa-miR-140-5p	C05	hsa-miR-125b	C06	hsa-miR-141	C07	hsa-miR-92a	C08	hsa-miR-424	C09	hsa-miR-191	C10	hsa-miR-17	C11	hsa-miR-130a	C12	hsa-miR-20a
D01	hsa-miR-27b	D02	hsa-miR-26b	D03	hsa-miR-146a	D04	hsa-miR-200c	D05	hsa-miR-99a	D06	hsa-miR-19a	D07	hsa-miR-23a	D08	hsa-miR-30a	D09	hsa-let-7i	D10	hsa-miR-93	D11	hsa-let-7c	D12	hsa-miR-106b
E01	hsa-miR-101	E02	hsa-let-7g	E03	hsa-miR-425	E04	hsa-miR-15b	E05	hsa-miR-28-5p	E06	hsa-miR18a	E07	hsa-miR-25	E08	hsa-miR-23b	E09	hsa-miR-302a	E10	hsa-miR-186	E11	hsa-miR-29c	E12	hsa-miR-7
F01	hsa-let-7d	F02	hsa-miR-30c	F03	hsa-miR-181b	F04	hsa-miR-223	F05	hsa-miR-320a	F06	hsa-miR-374a	F07	hsa-let-7e	F08	hsa-miR-151-5p	F09	hsa-miR-374b	F10	hsa-miR-196b	F11	hsa-miR-140-3p	F12	hsa-miR-100
G01	hsa-miR-103	G02	hsa-miR-96	G03	hsa-miR-307b	G04	hsa-miR-194	G05	hsa-miR-125a-5p	G06	hsa-miR-423-5p	G07	hsa-miR-376c	G08	hsa-miR-195	G09	hsa-miR-222	G10	hsa-miR-28-3p	G11	hsa-miR-128	G12	hsa-miR-302c
H01	hsa-miR-423-3p	H02	hsa-miR-185	H03	hsa-miR-30b	H04	hsa-miR-210	H05	SNORD48														

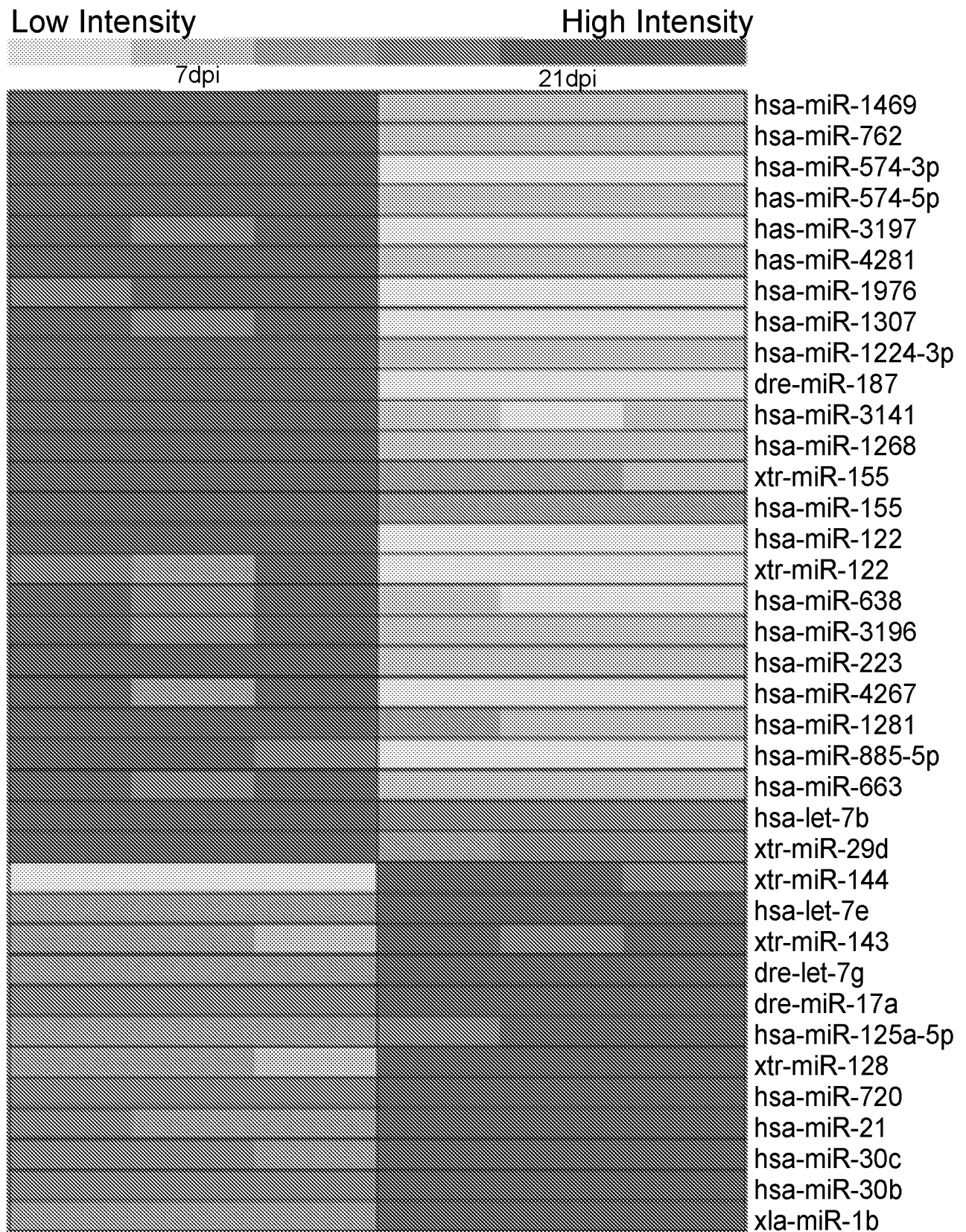
Fig. 4

Fig. 5A

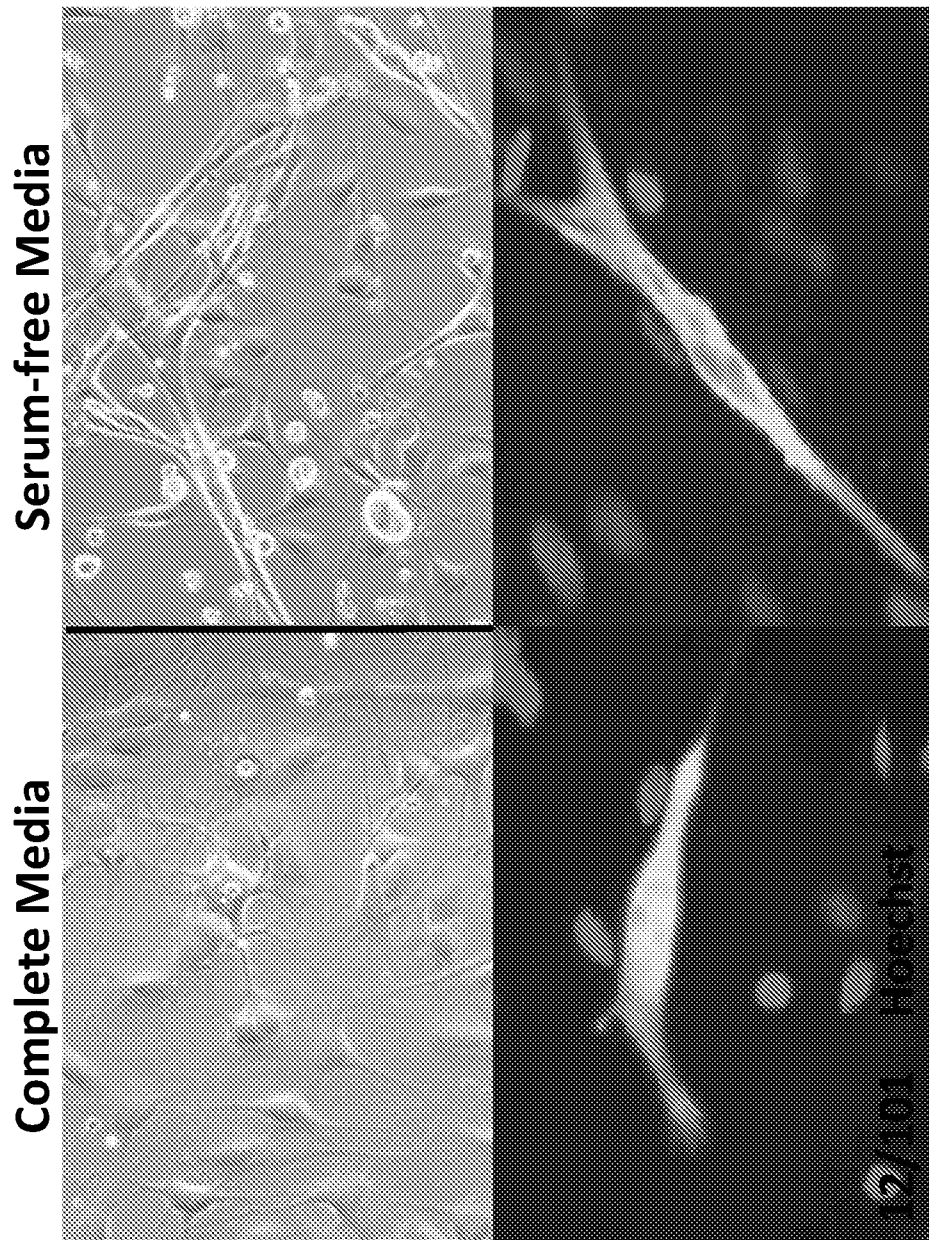


Fig. 5C

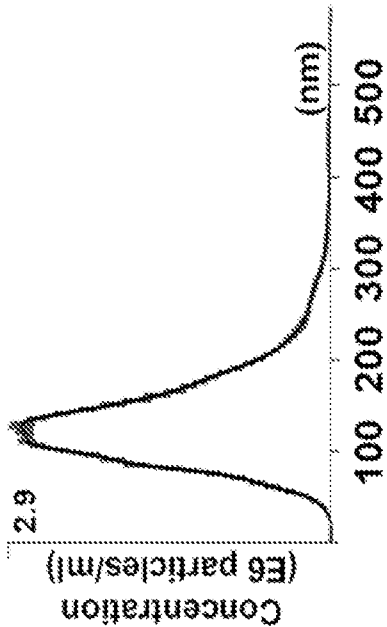


Fig. 5B

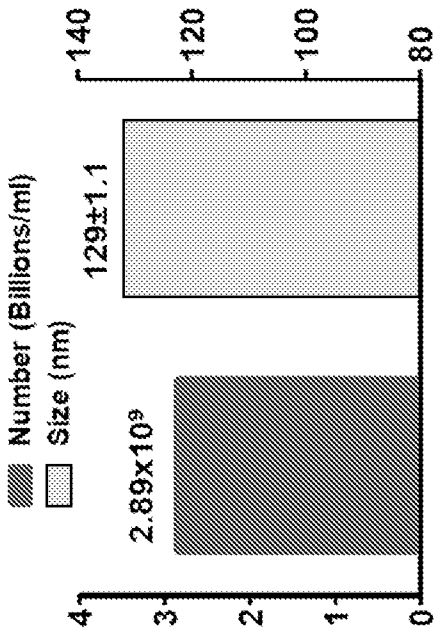
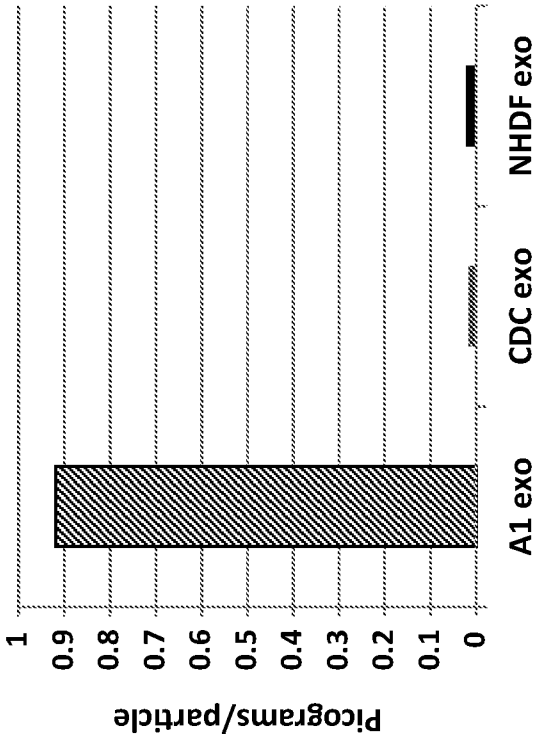


Fig. 5D



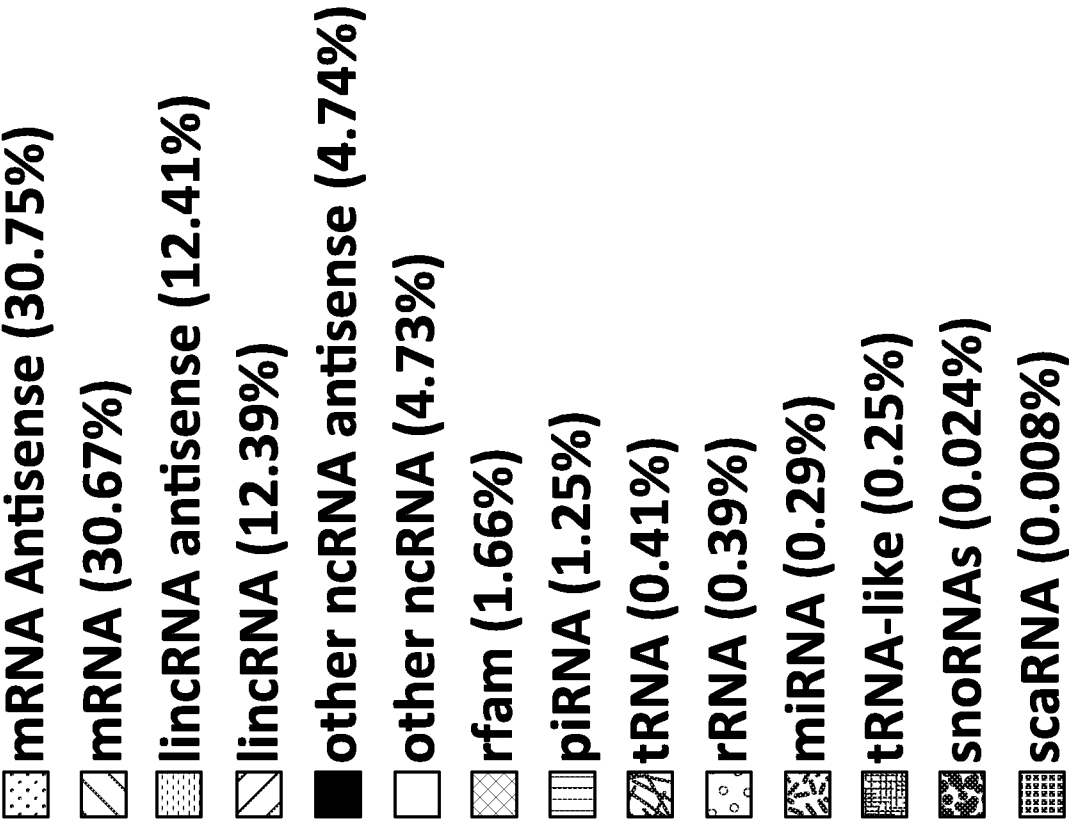
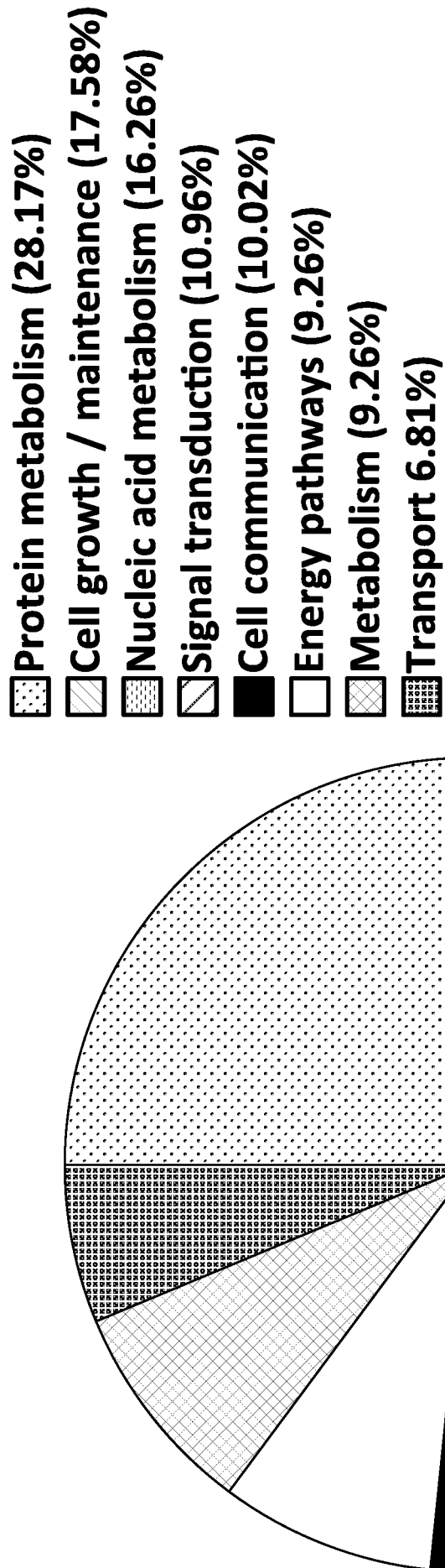
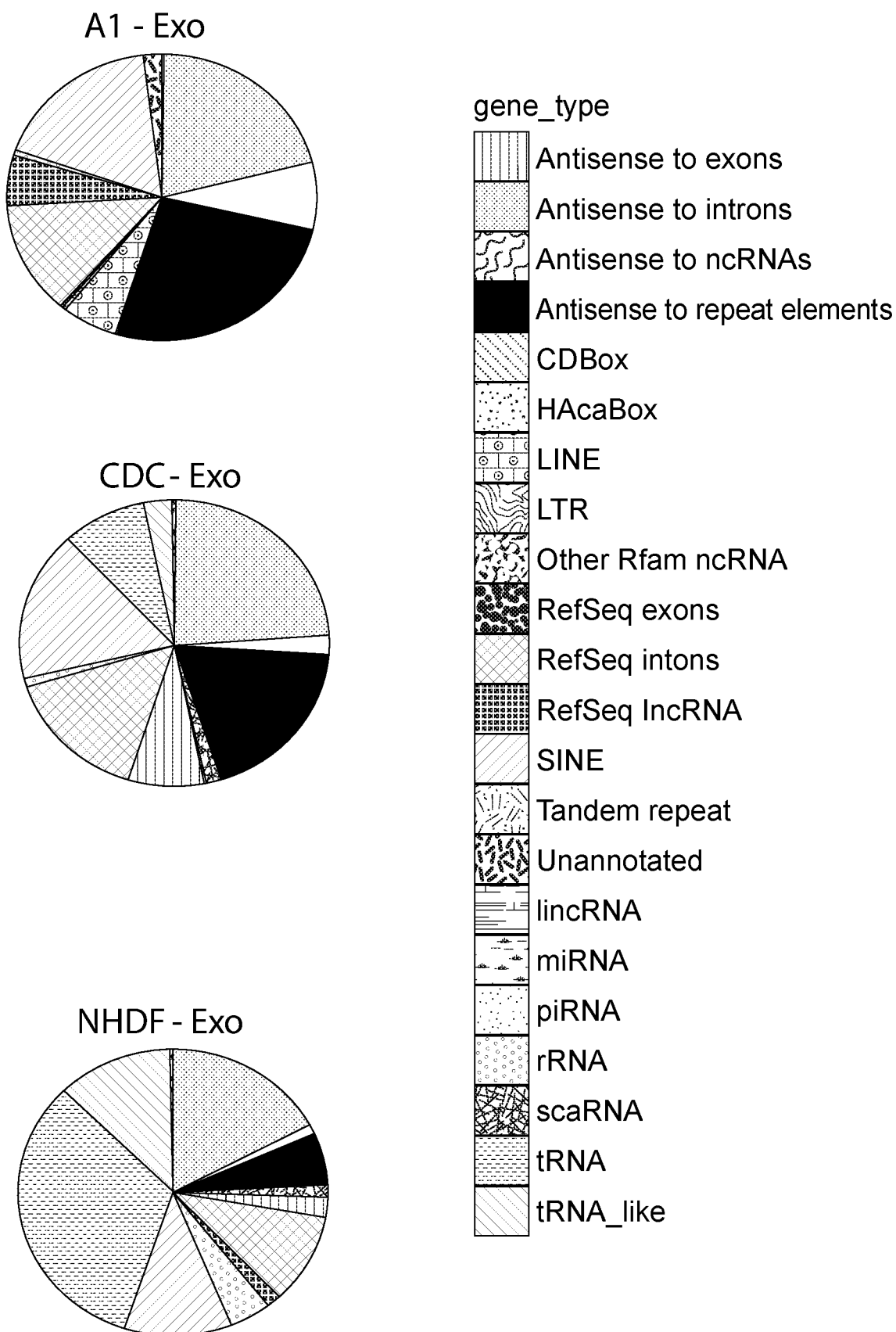


Fig. 6A

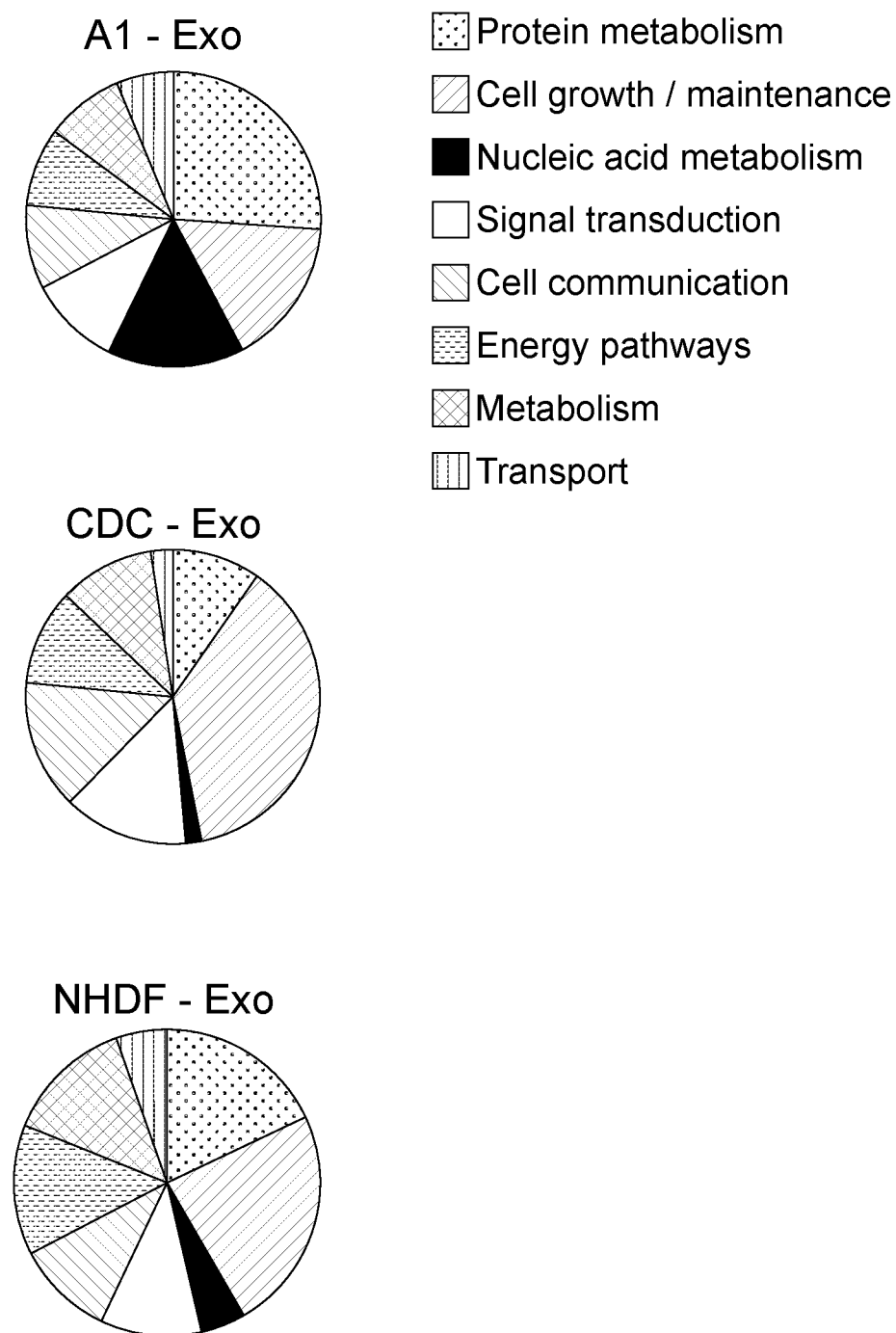
8/37

Fig. 6B

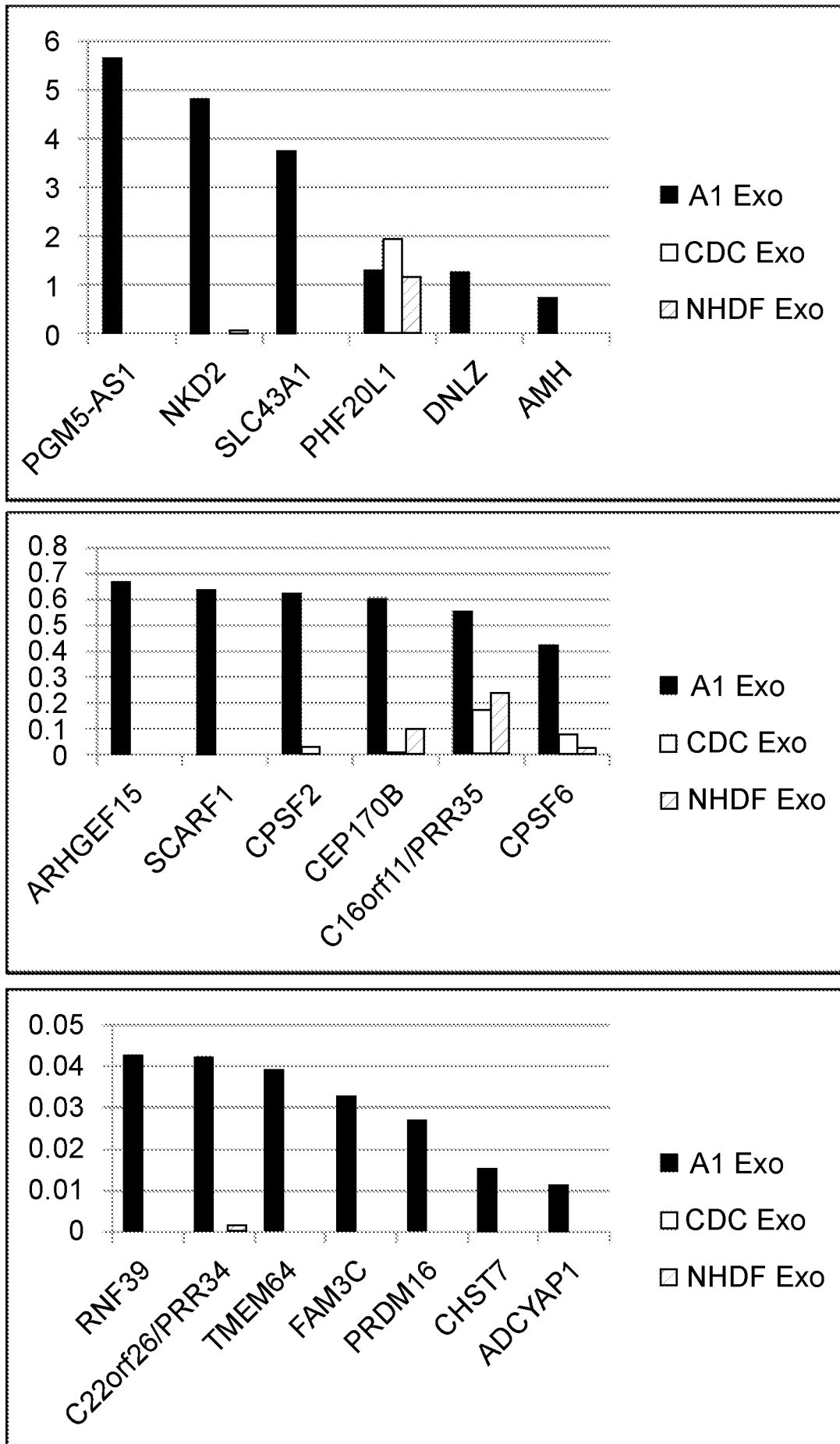
9/37

Fig. 7A

10/37

Fig. 7B

11/37

Fig. 8

12/37

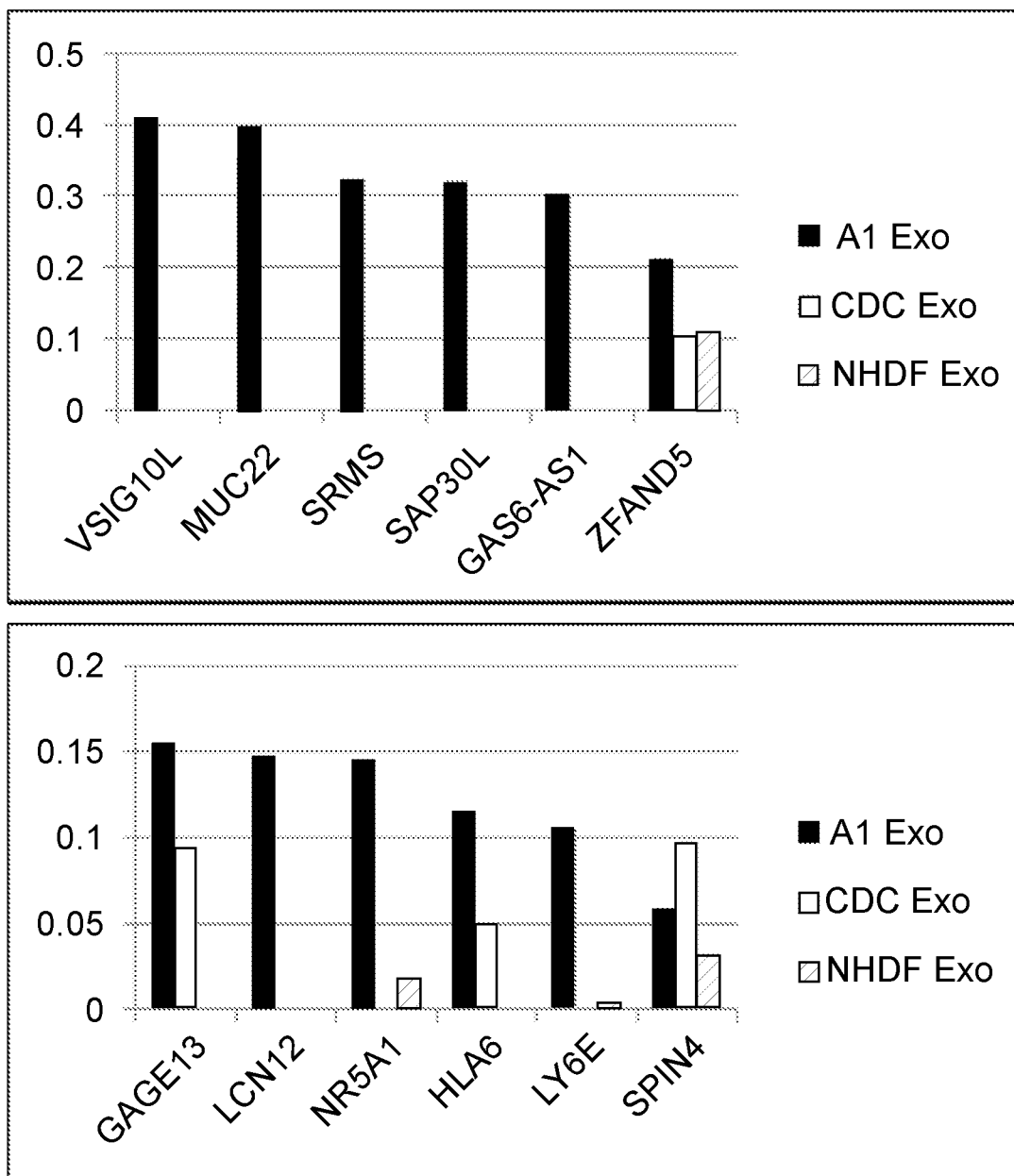
Fig. 8

Fig. 9A

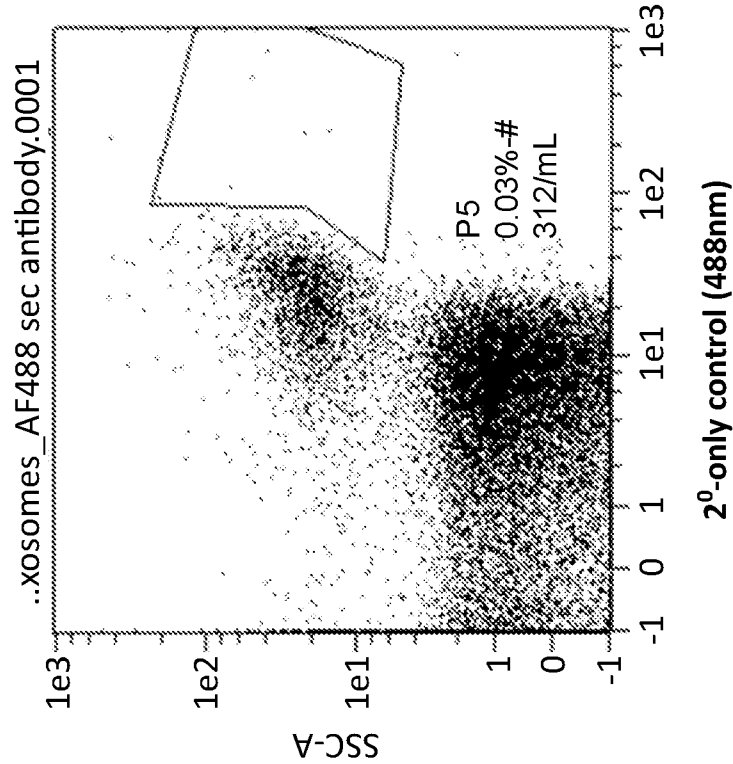


Fig. 9B

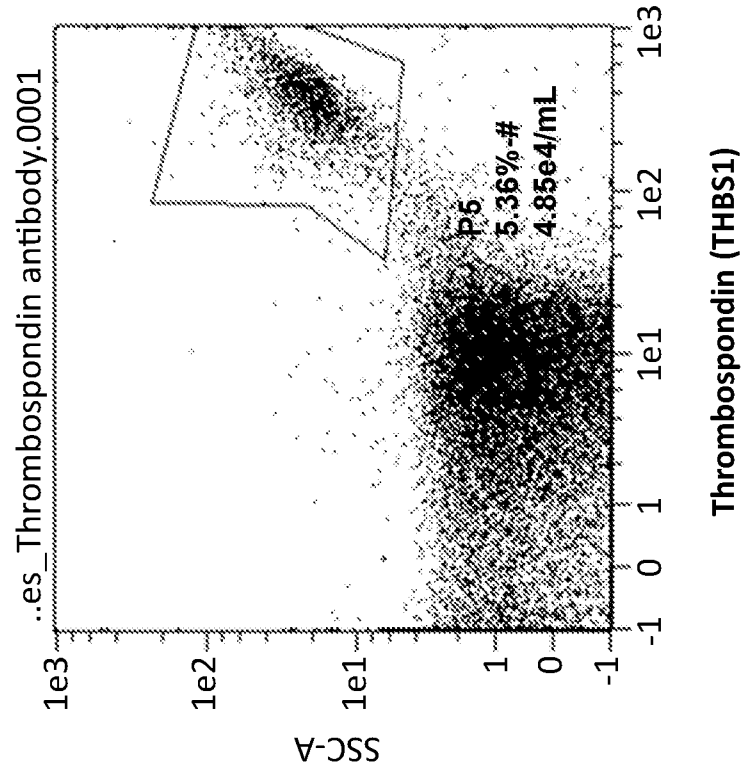


Fig. 9C

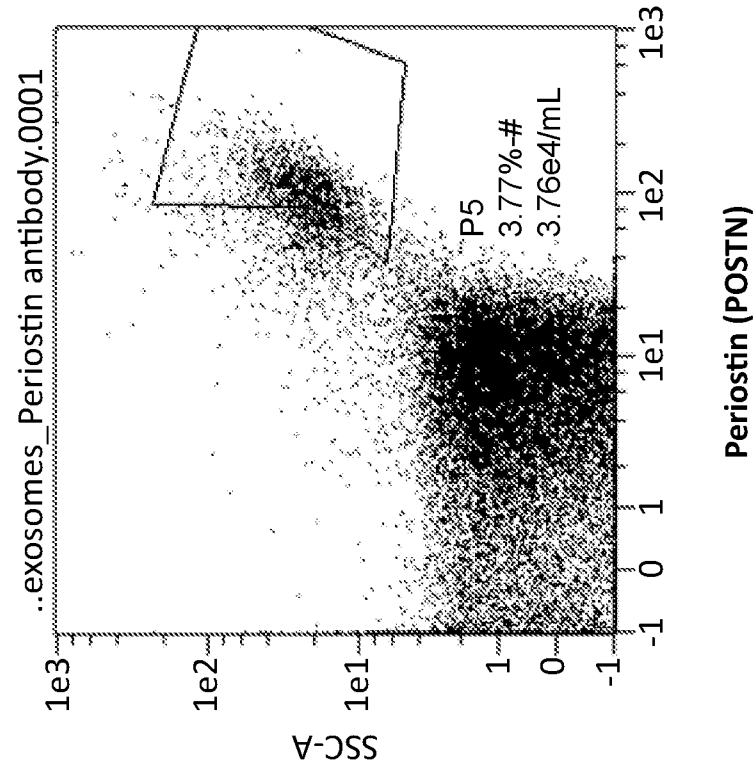


Fig. 9D

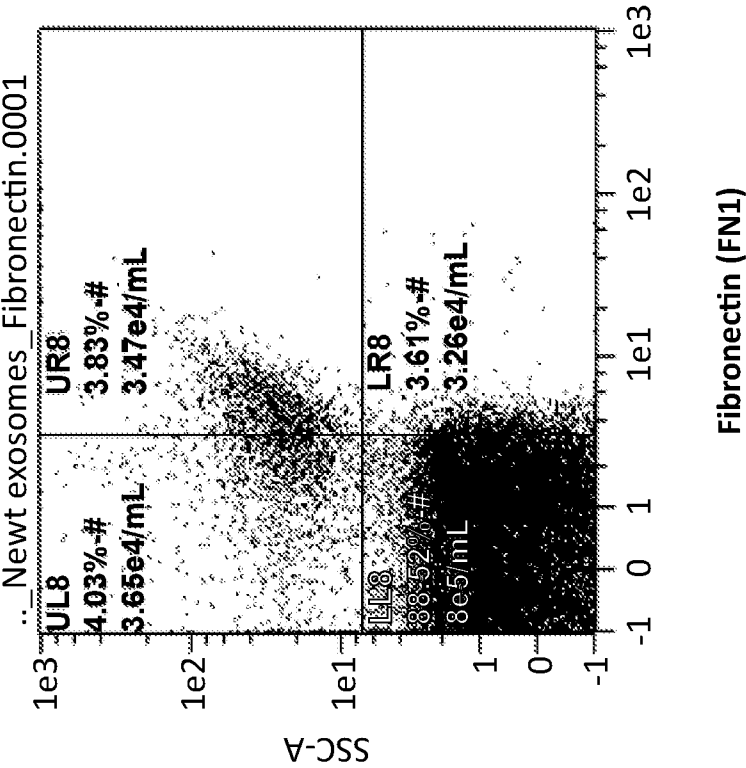


Fig. 9E

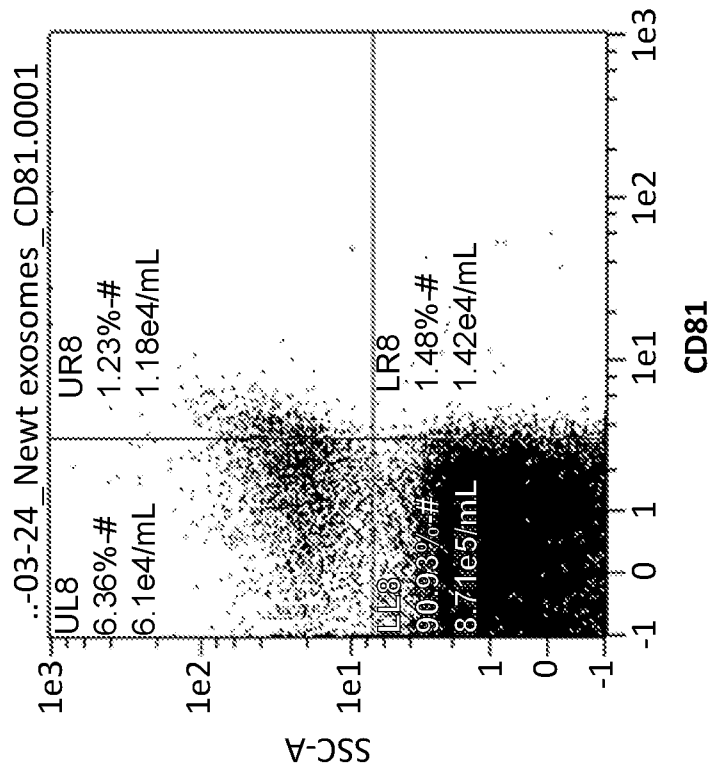


Fig. 9F

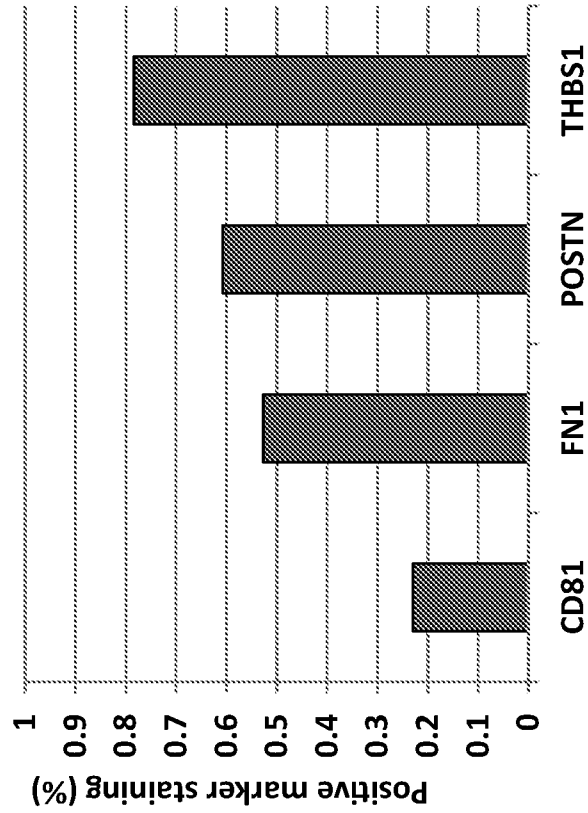


Fig. 10B

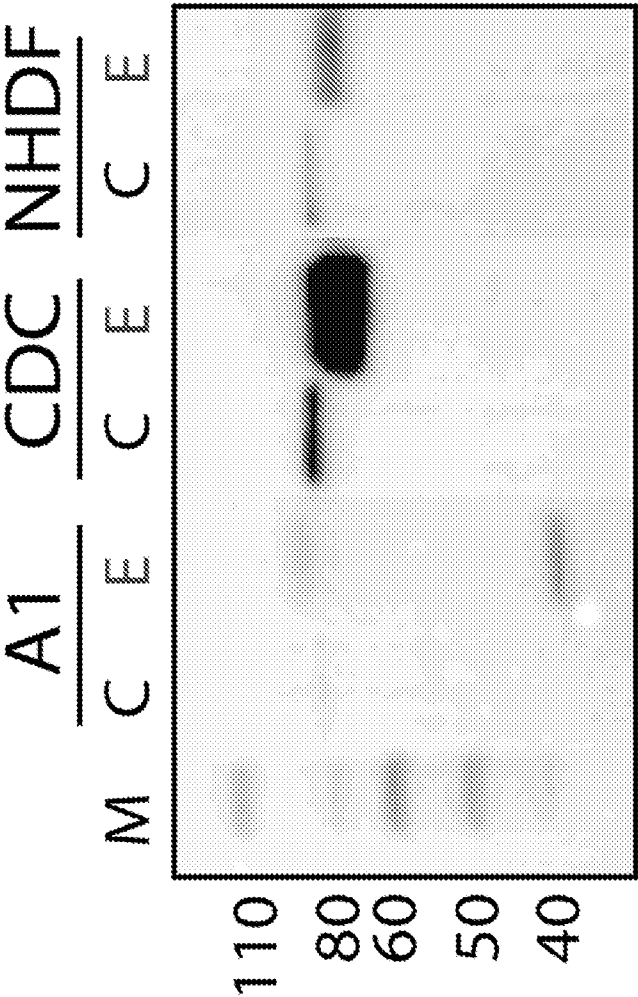


Fig. 10A

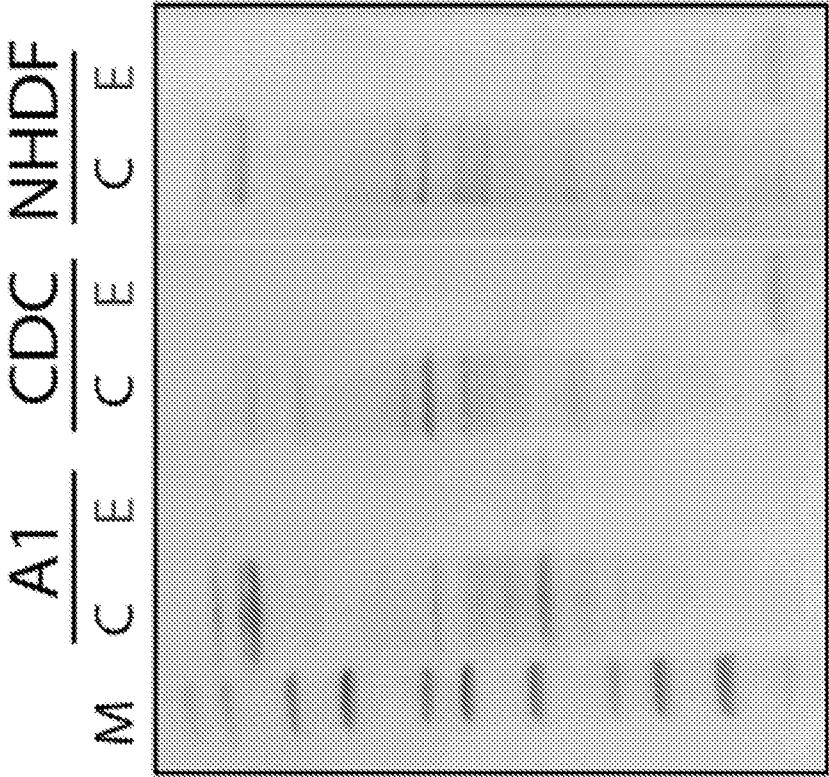


Fig. 11

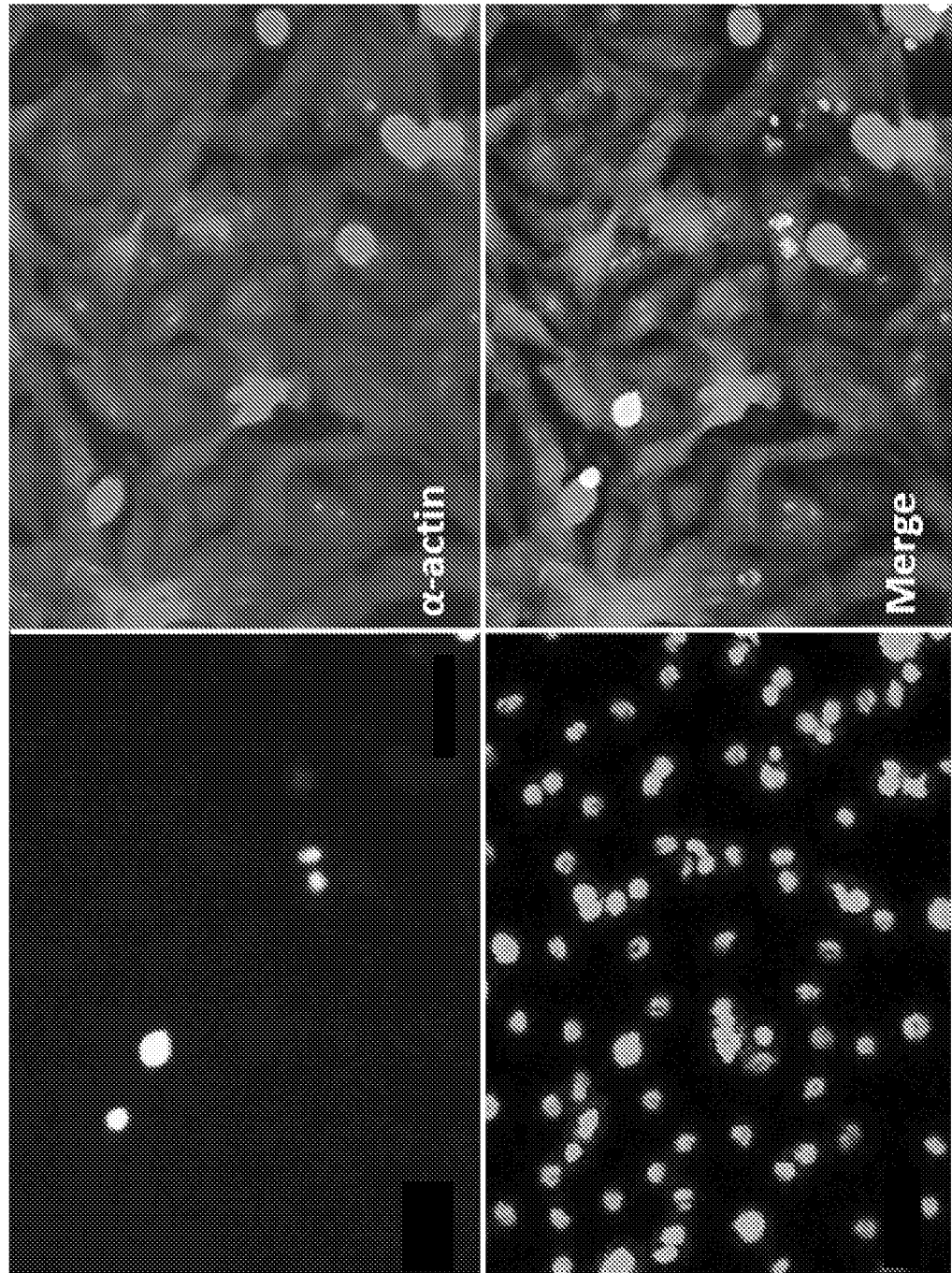


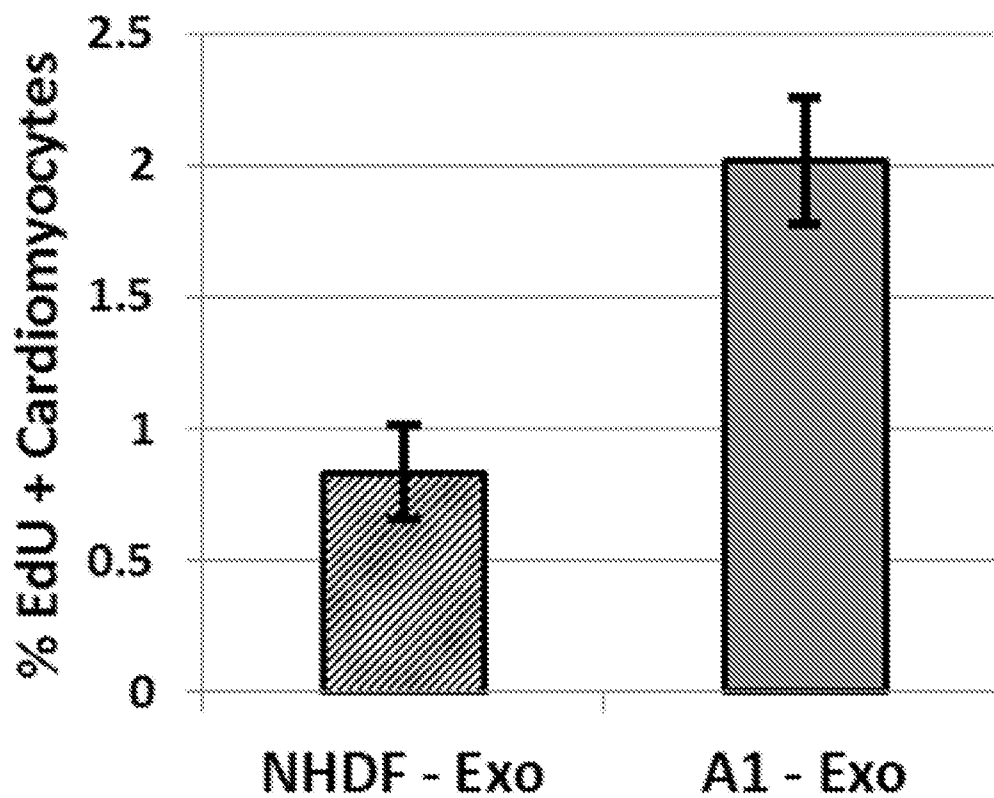
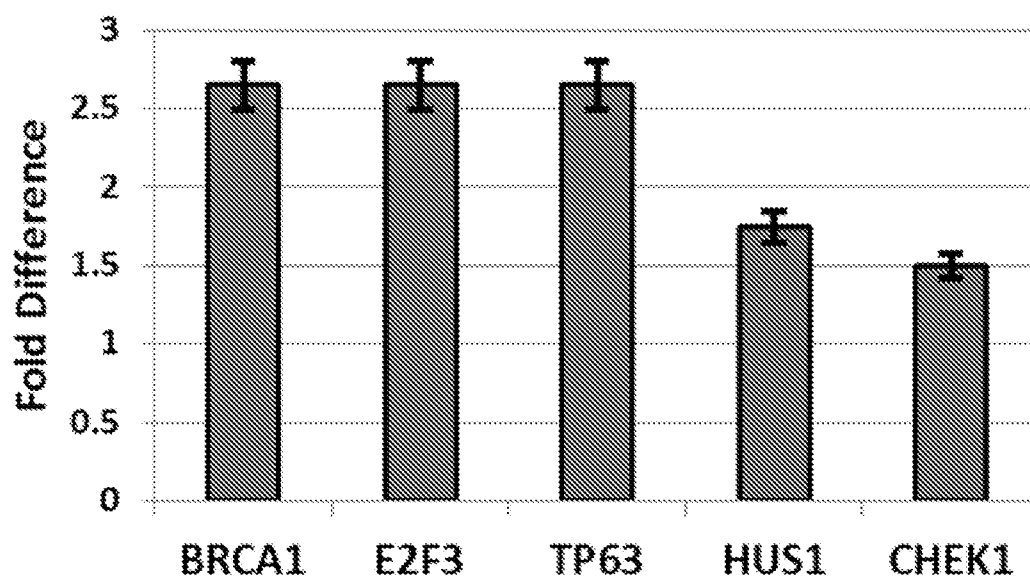
Fig. 11B**Fig. 11C**

Fig. 12B

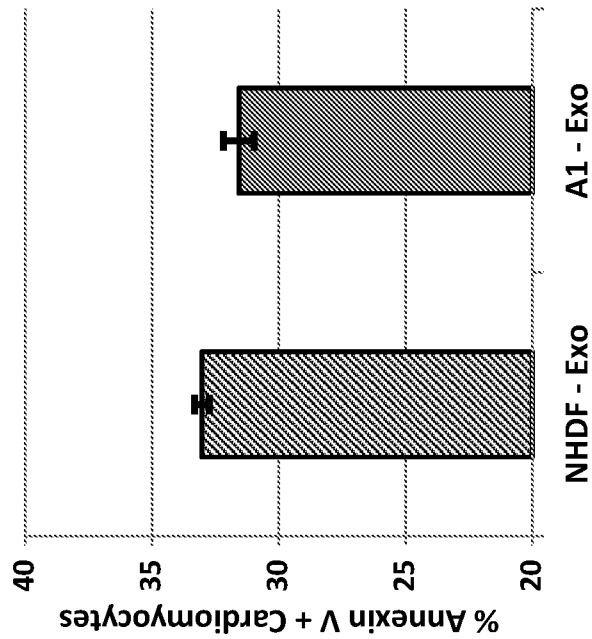
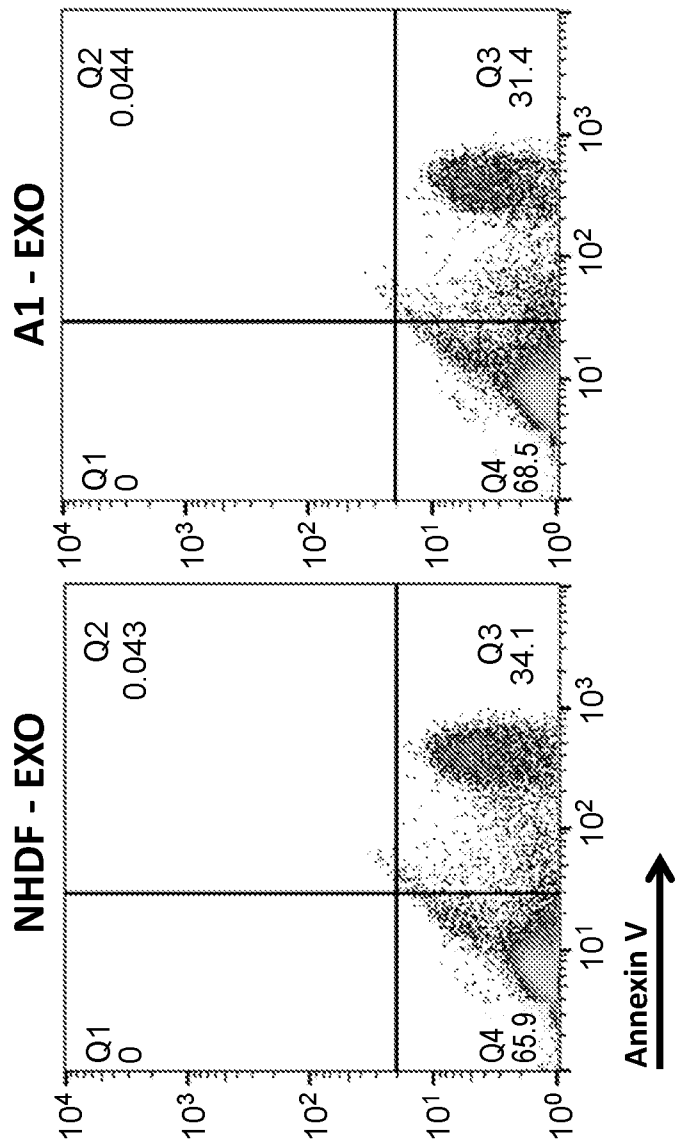


Fig. 12A



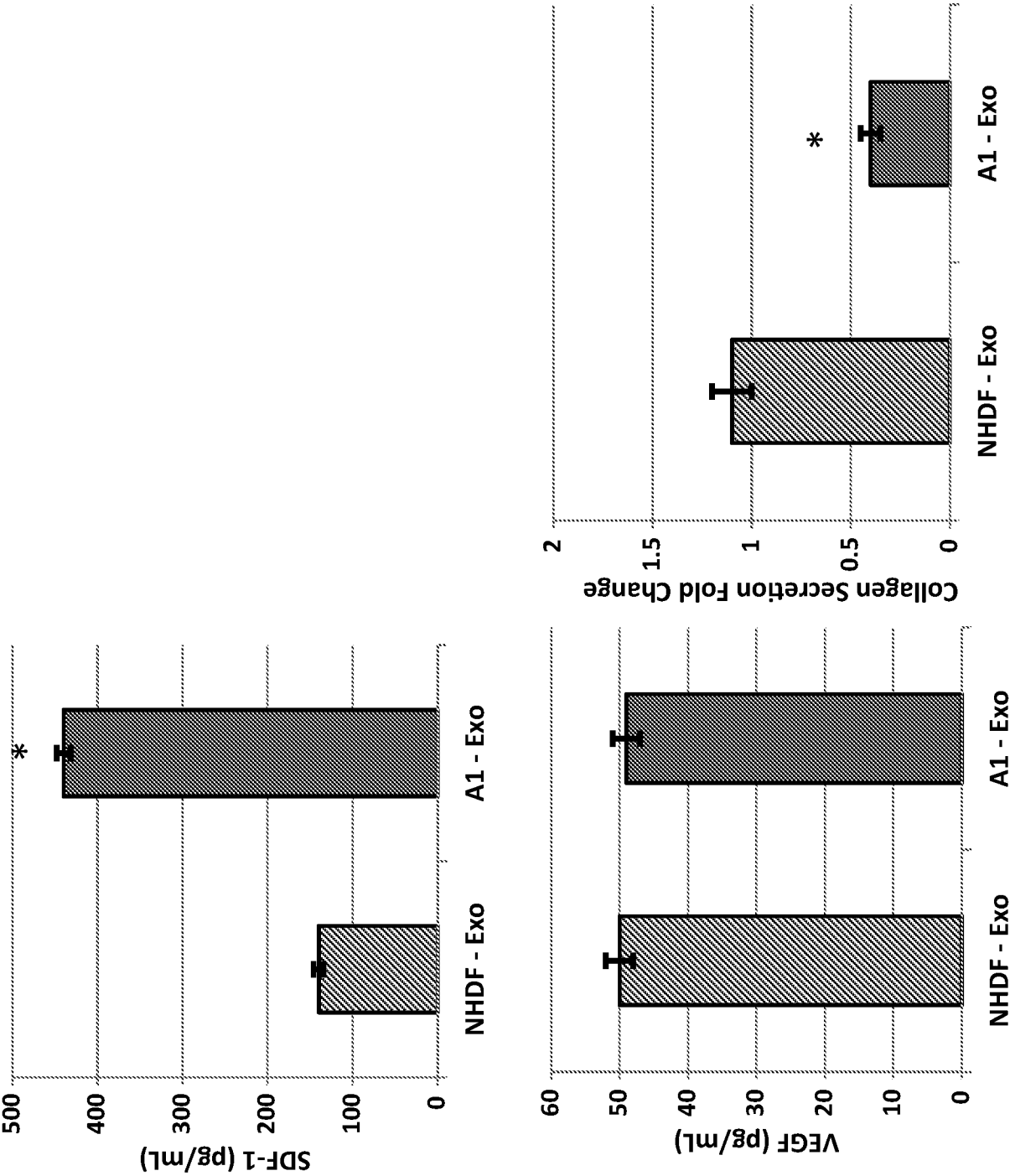


Fig. 13A

Fig. 13B

Fig. 13C

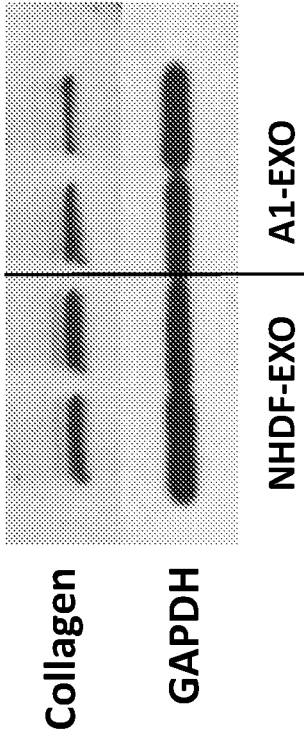


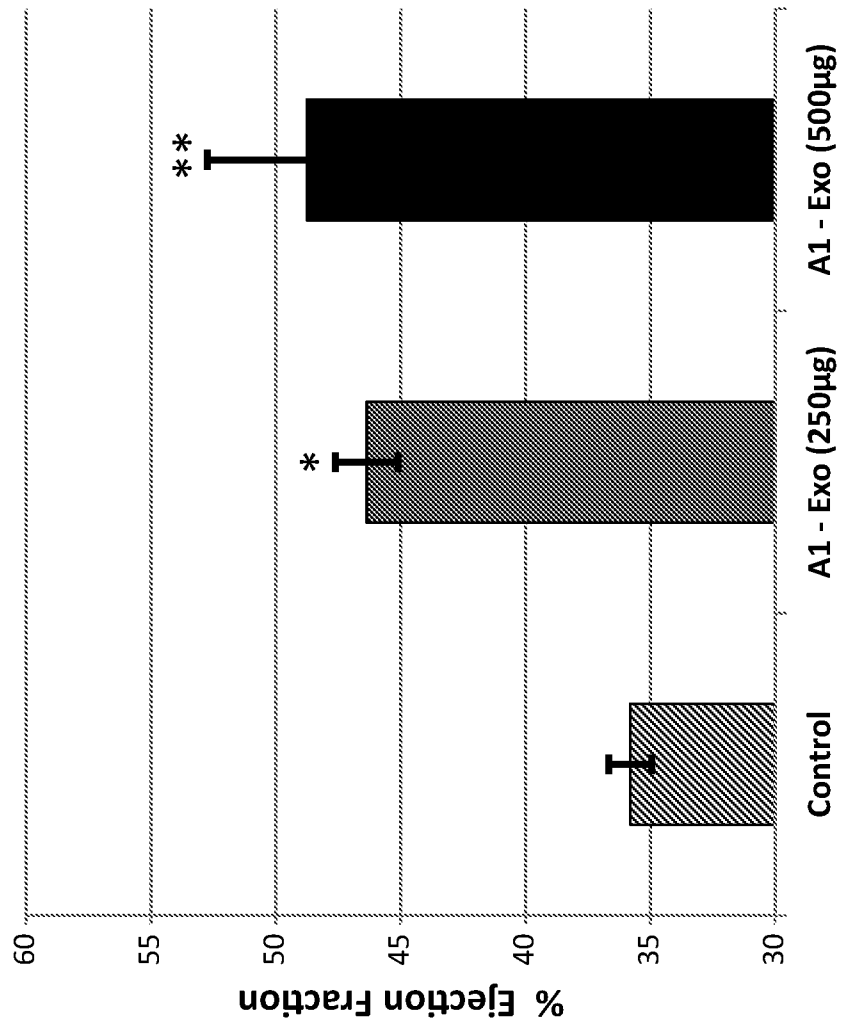
Fig. 14

Fig. 15B

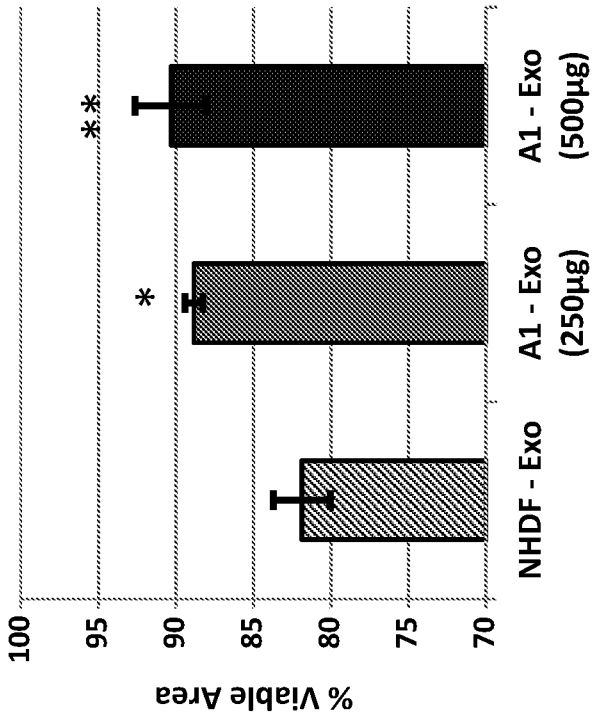


Fig. 15A

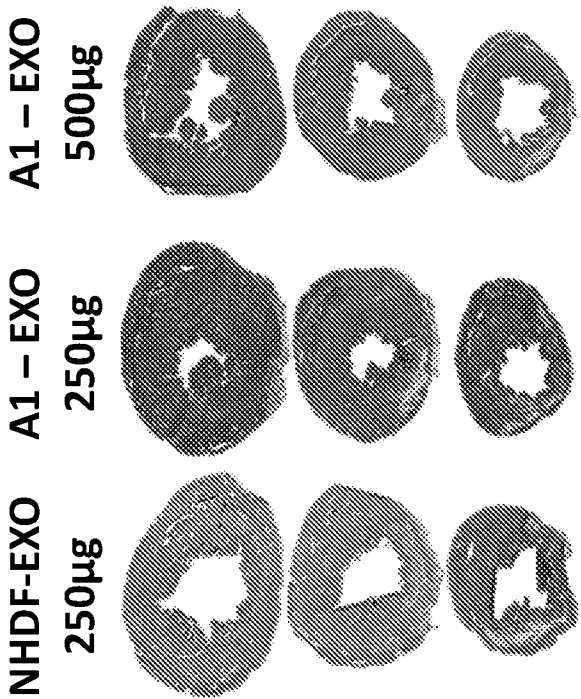


Fig. 15D

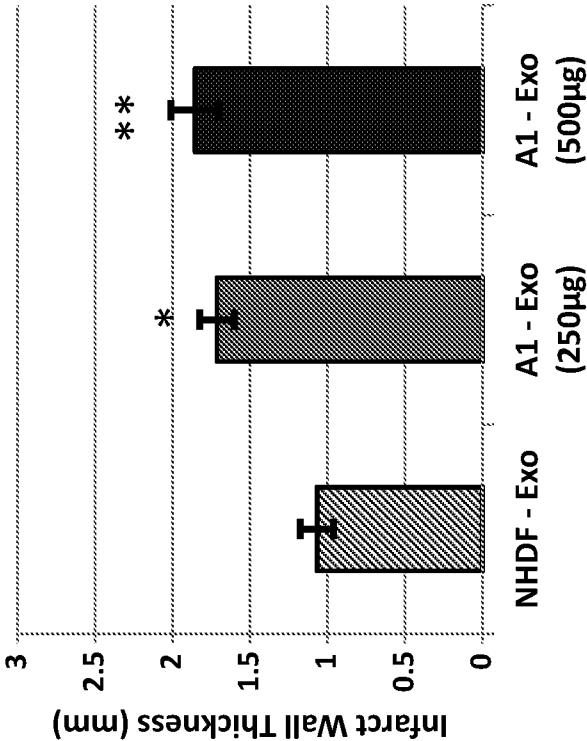


Fig. 15C

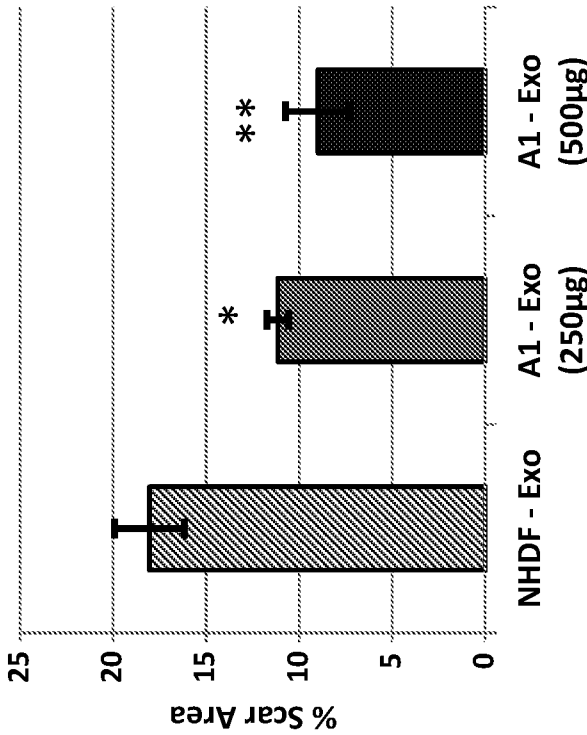


Fig. 16A

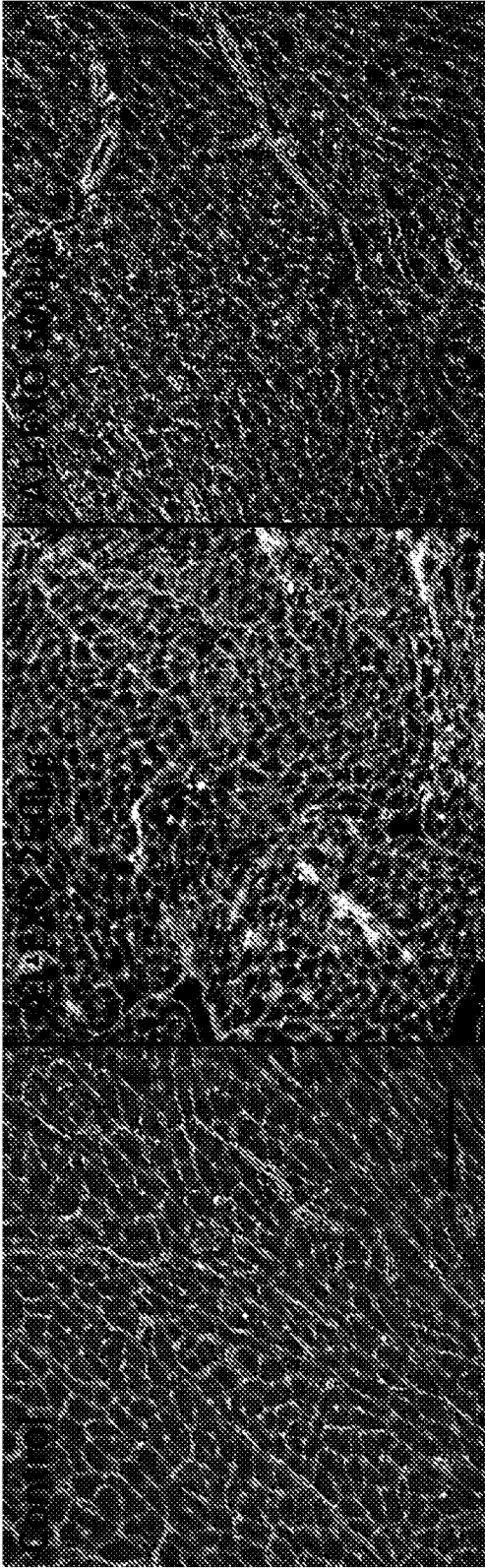


Fig. 16B

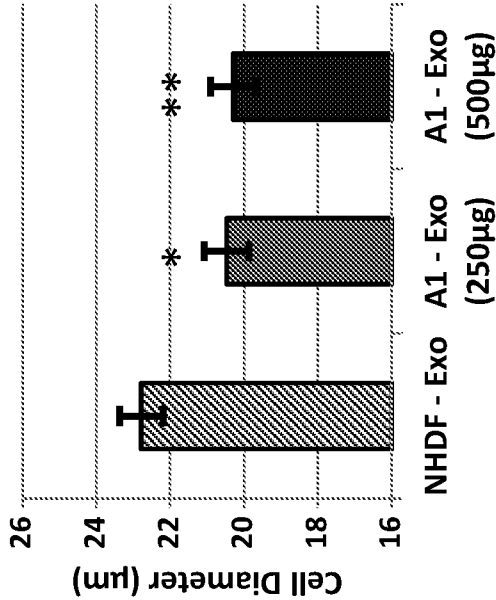
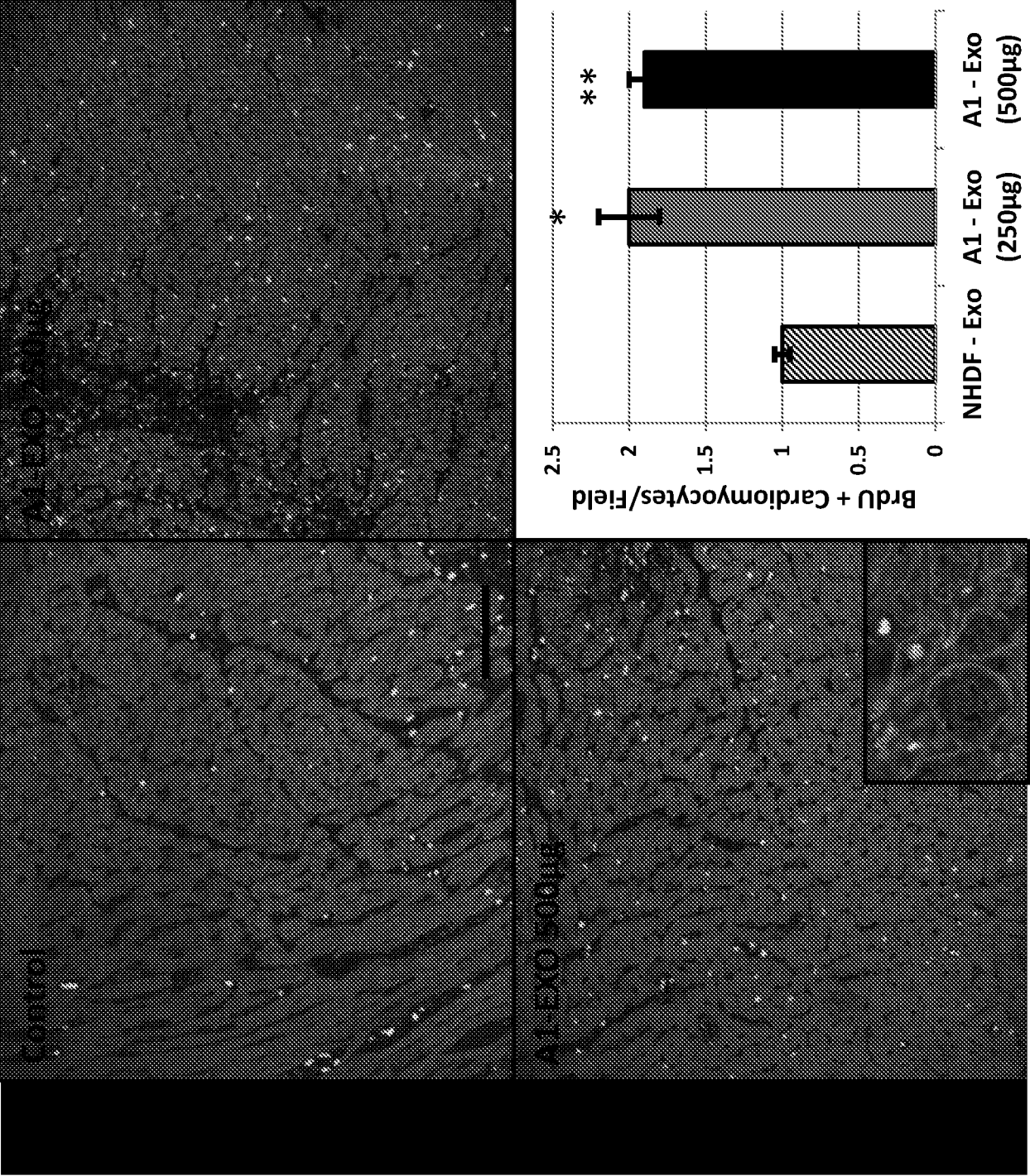


Figure 17



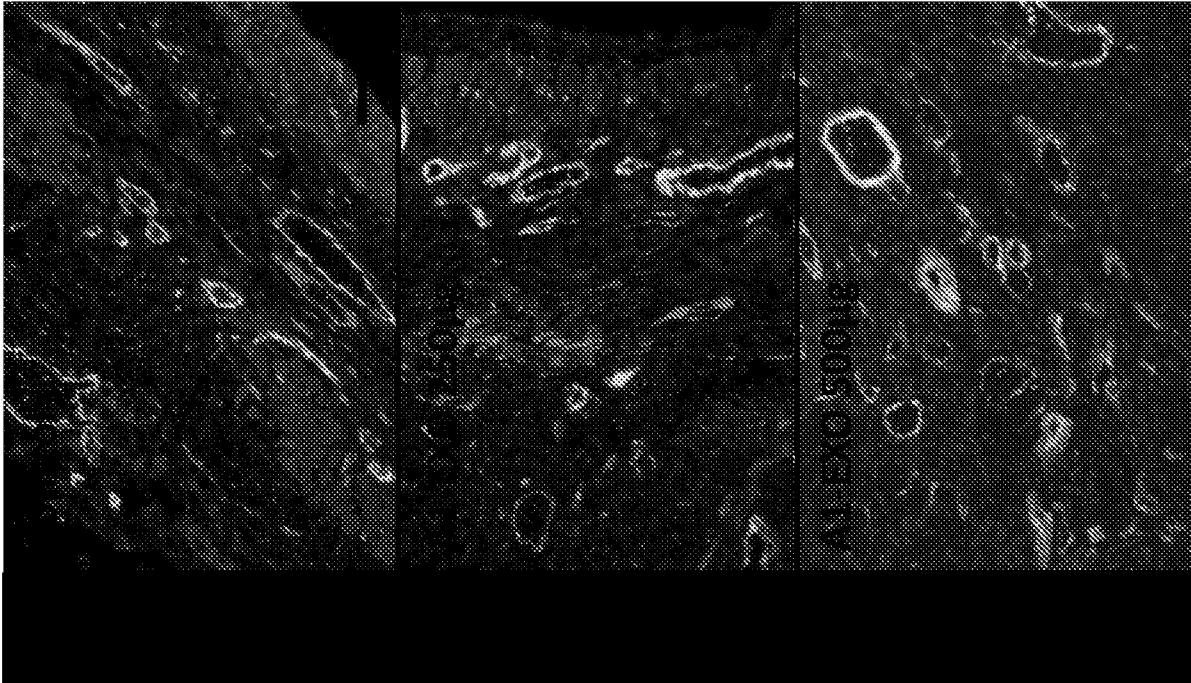
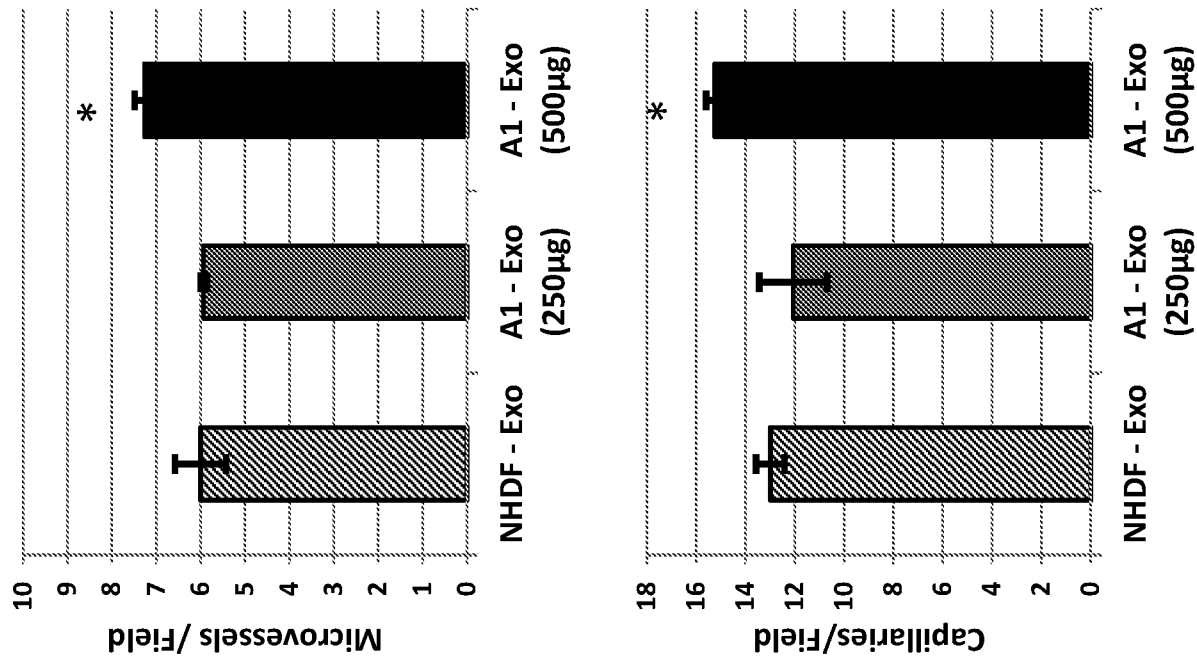


Figure 18

Fig. 19

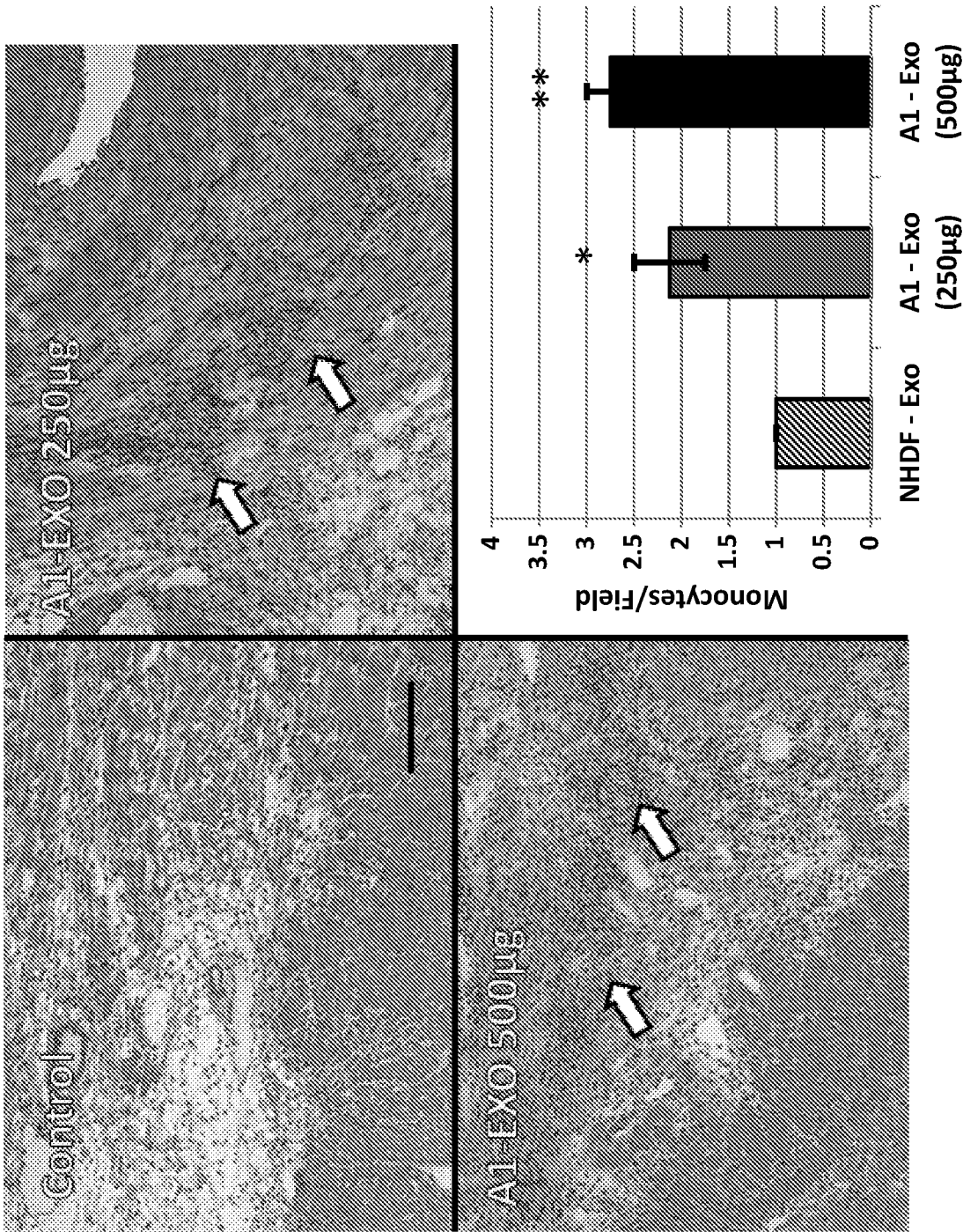


Fig. 20

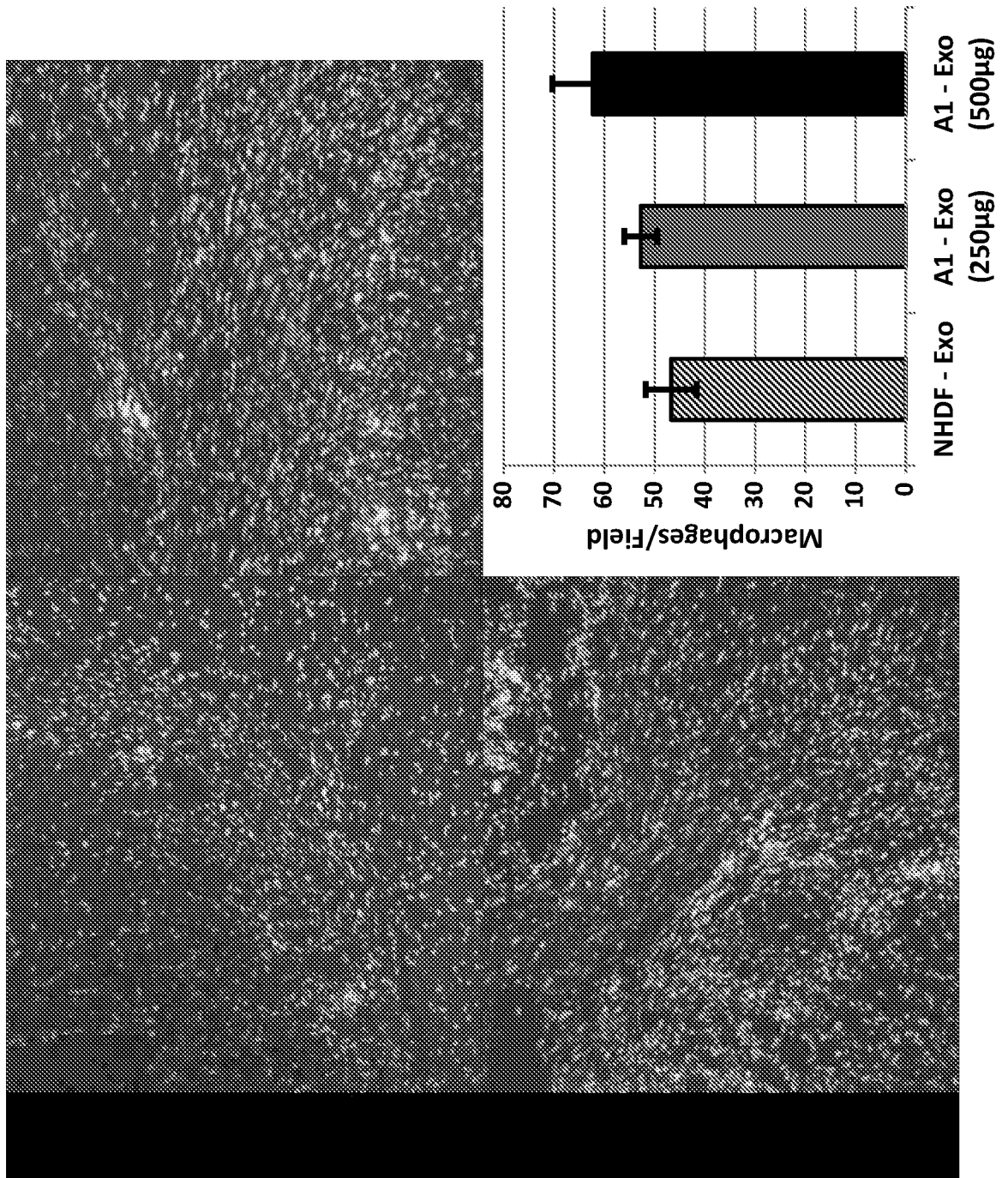


Figure 21

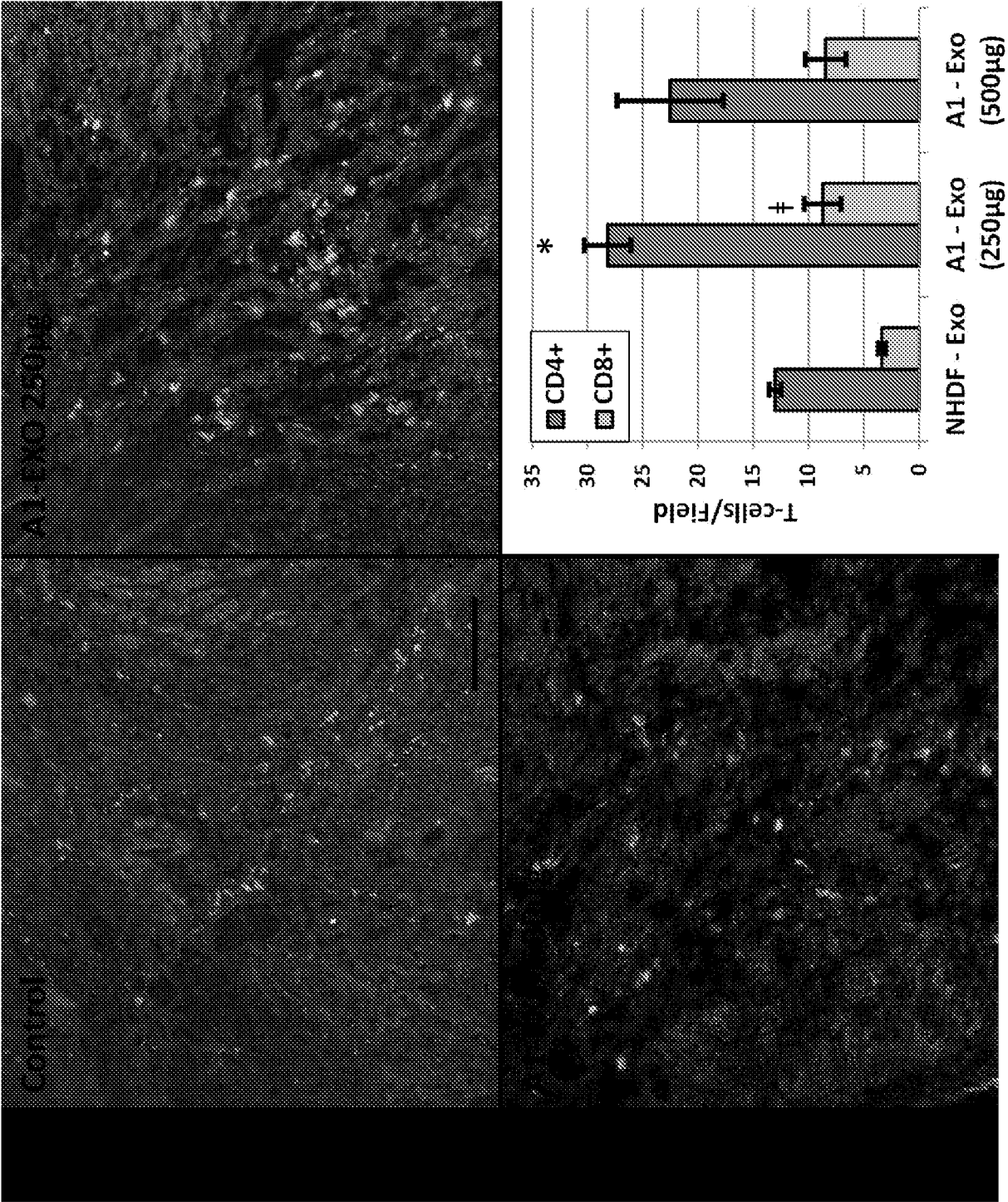


Fig. 22A

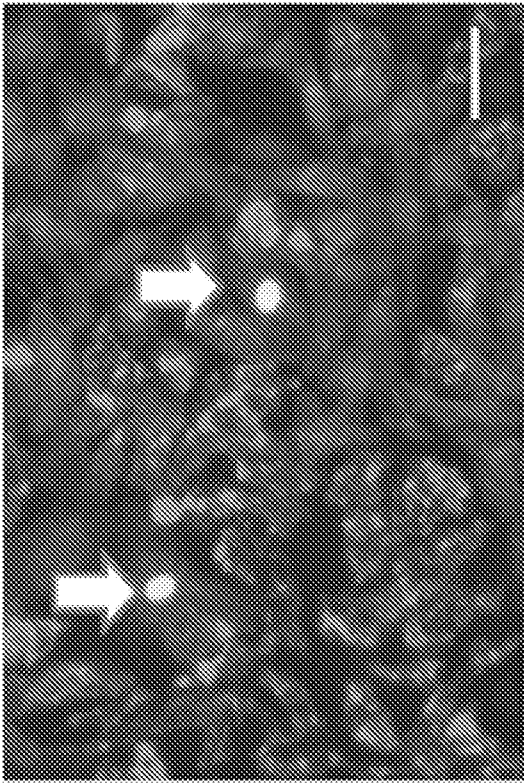


Fig. 22B

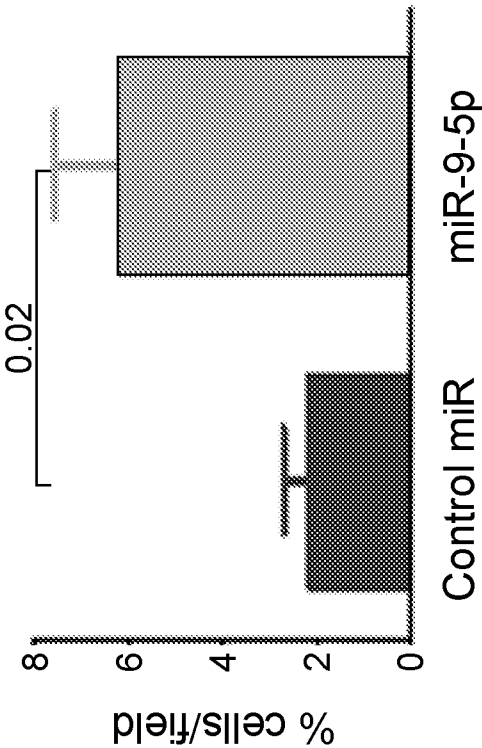


Fig. 22C

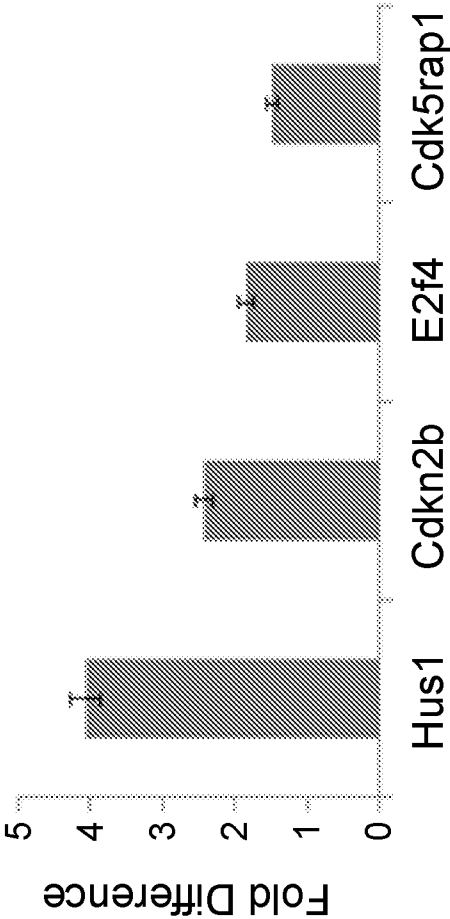


Fig. 23B

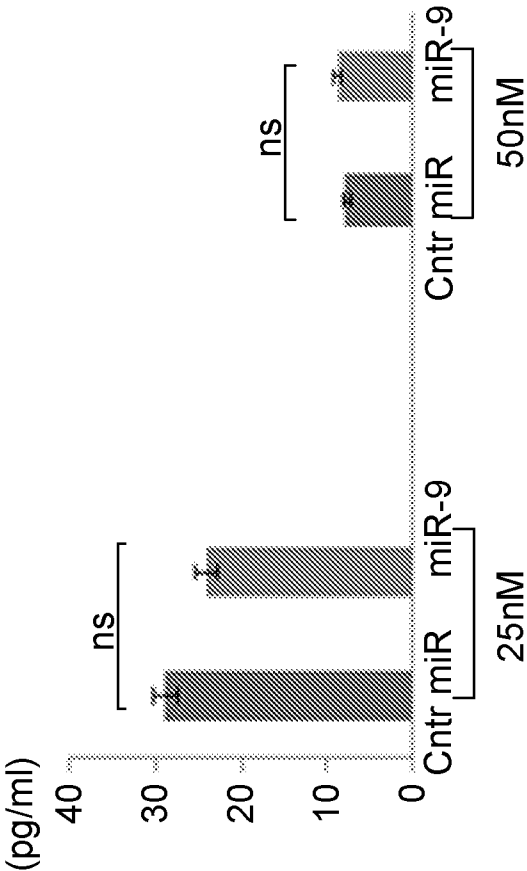


Fig. 23A

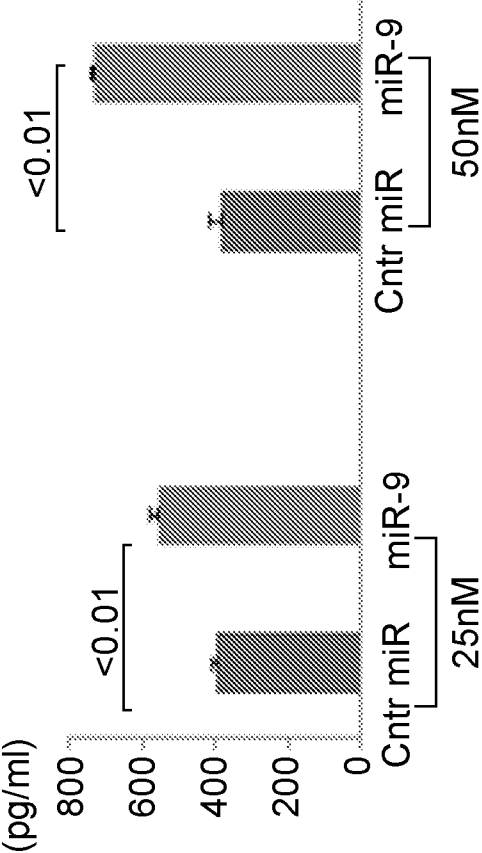


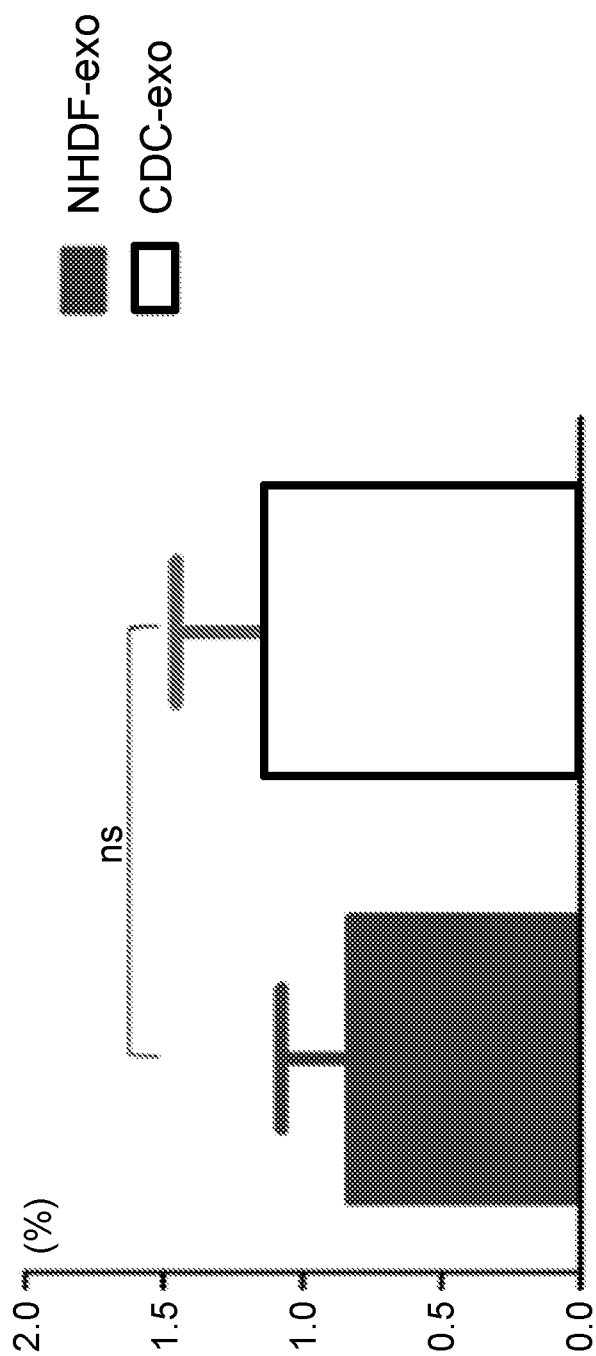
Fig. 24

Figure 25

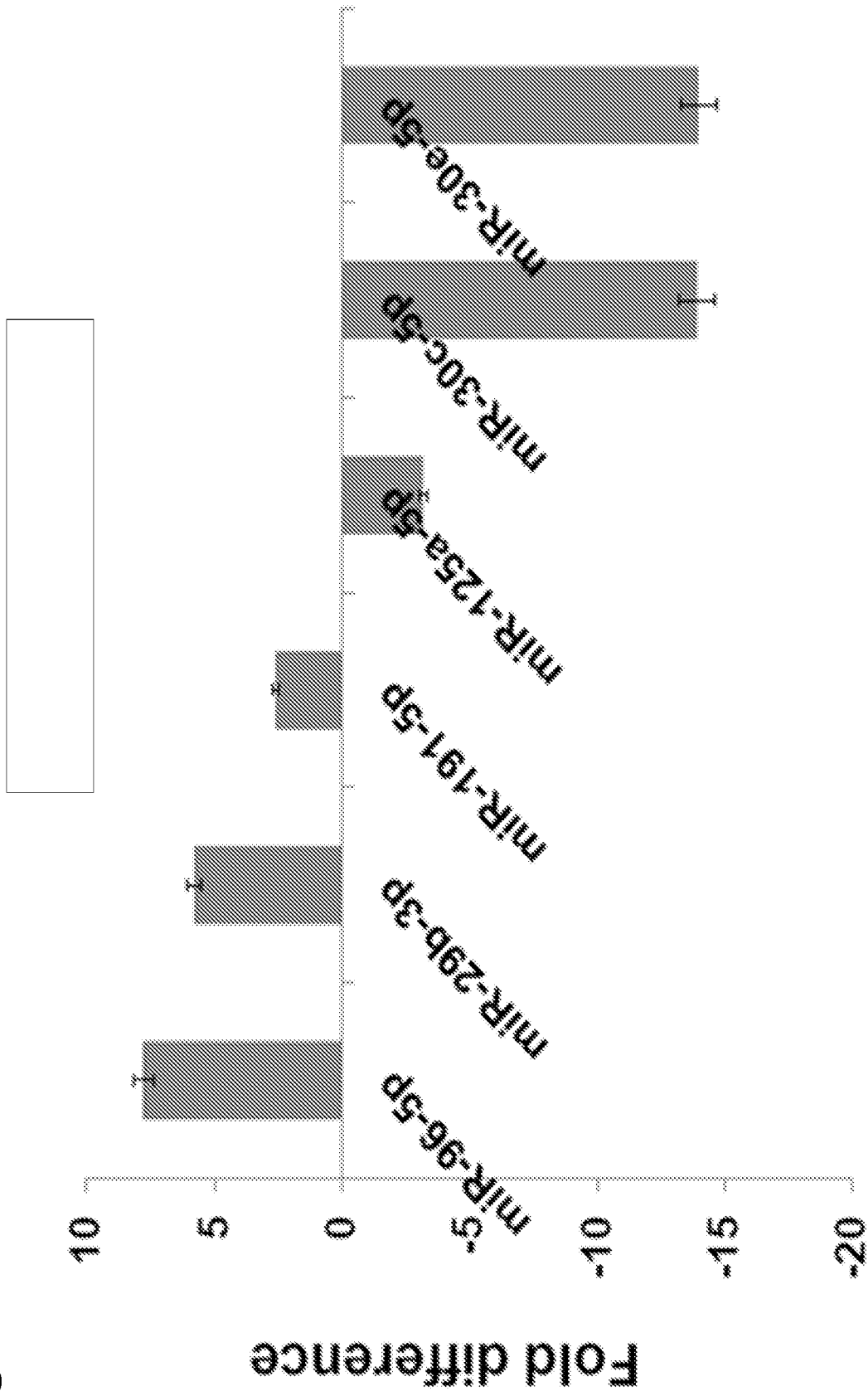


Fig. 26A

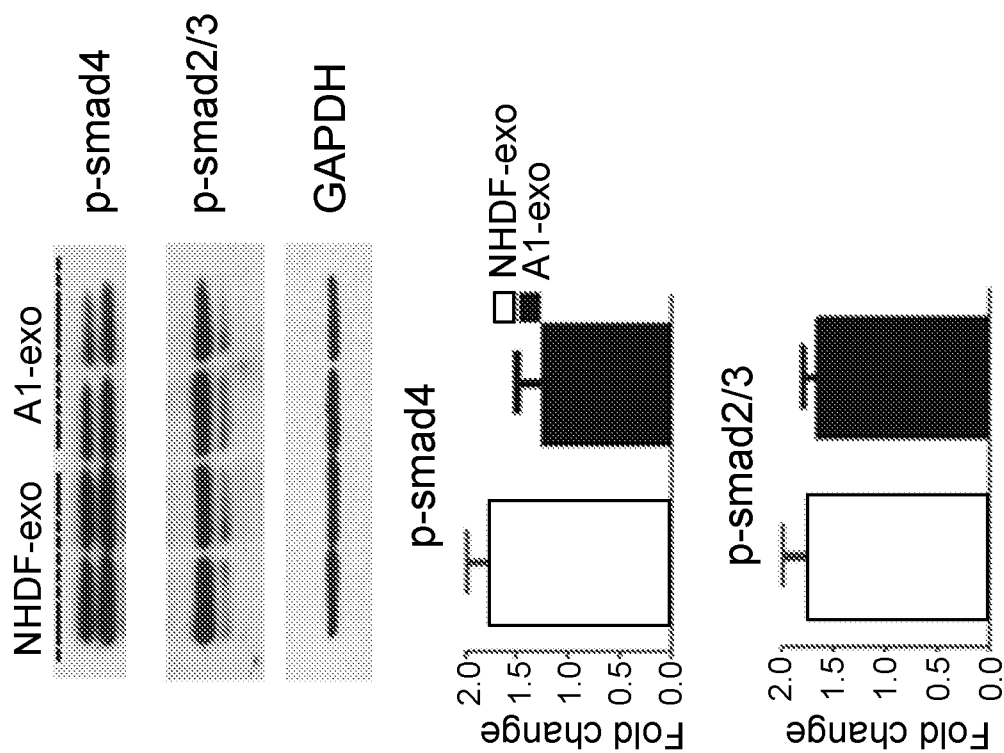


Fig. 26B

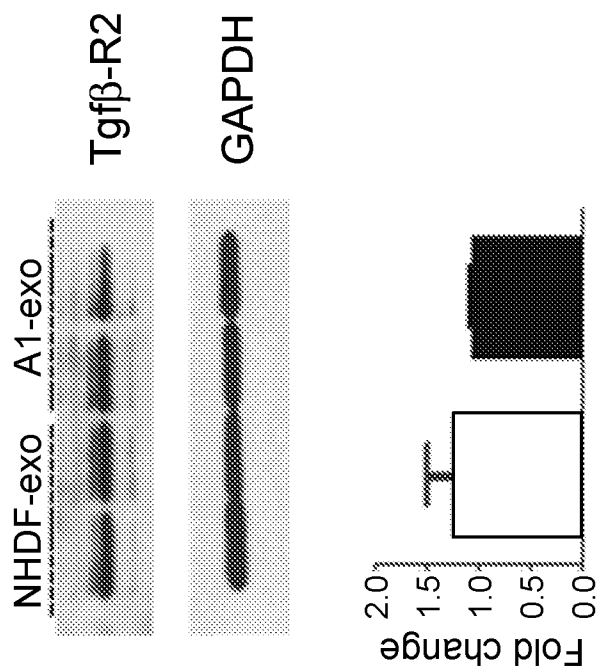


Fig. 27A

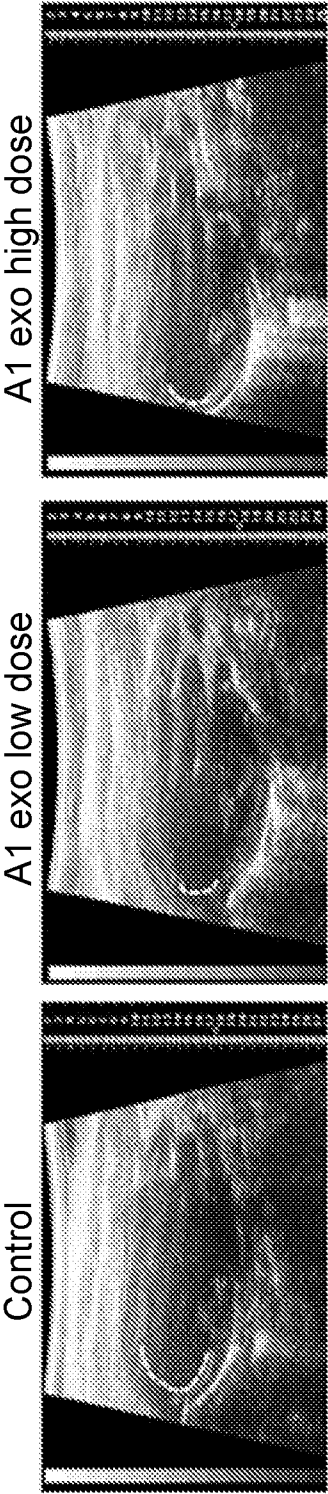


Fig. 27B

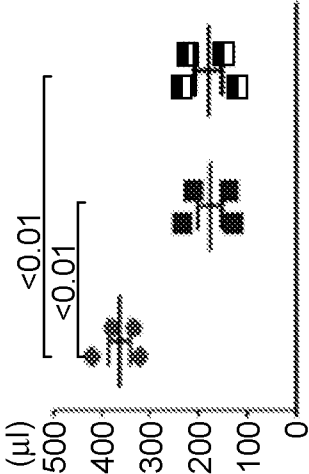


Fig. 27C

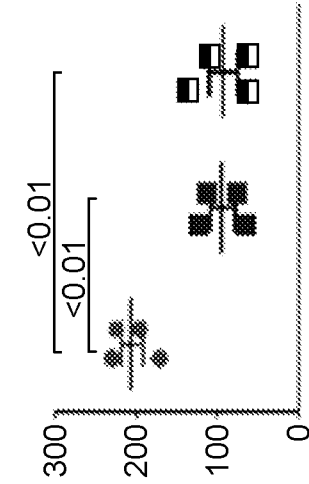


Fig. 27D

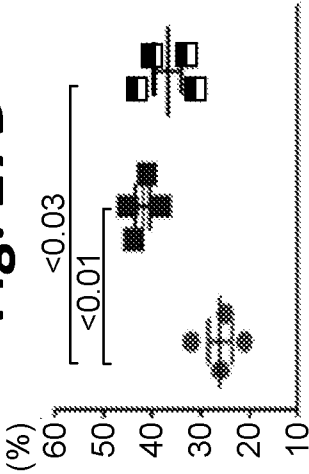
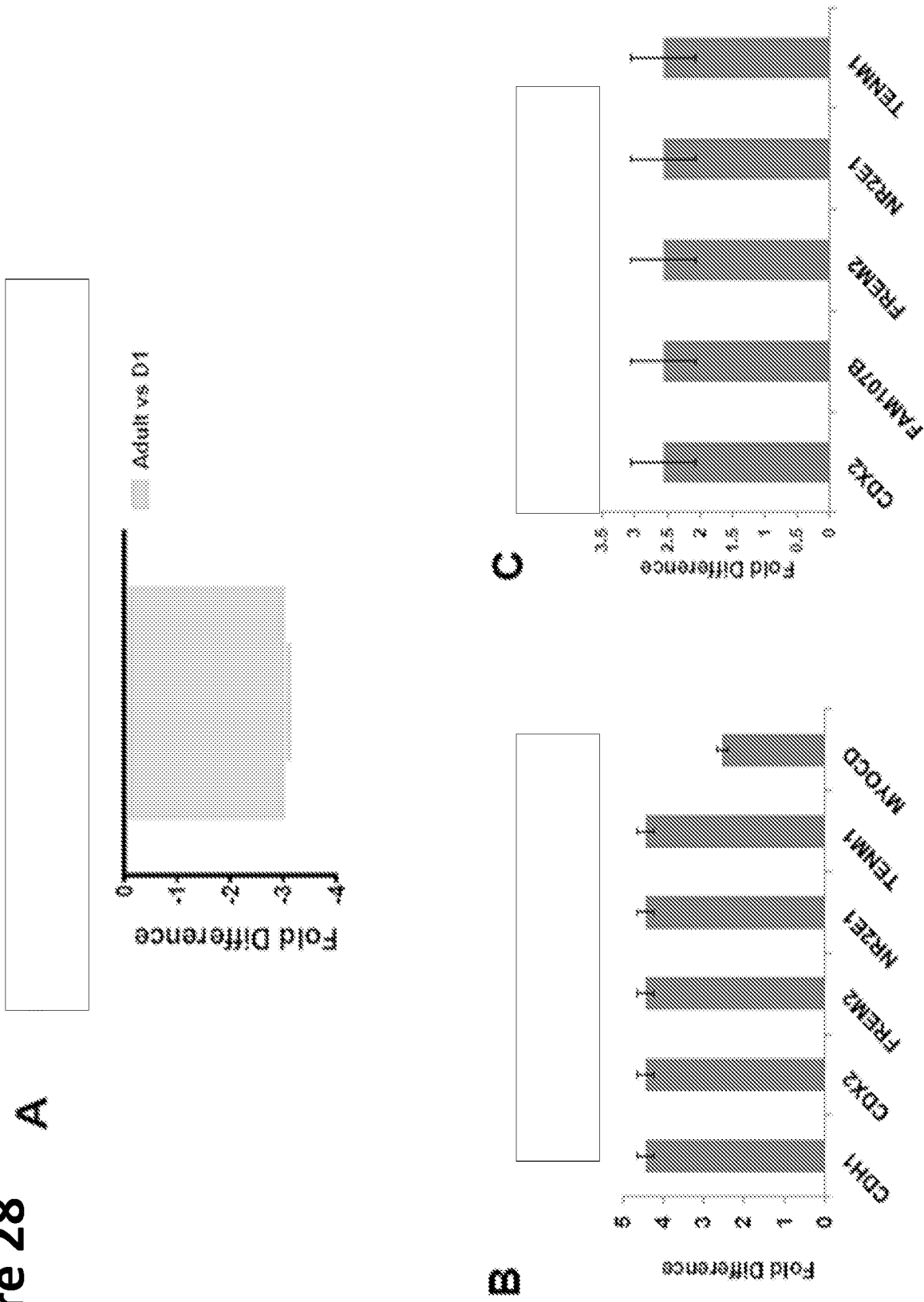


Figure 28



INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2016/035561

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61K 9/00; A61K 9/10; A61K 35/00; A61K 35/12; A61K 47/46; A61P 9/00 (2016.01)

CPC - A61K 35/12; A61K 47/46; A61L 2300/626; A61L 2300/64; C12N 2502/1329 (2016.08)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC - A61K 9/00; A61K 9/10; A61K 35/00; A61K 35/12; A61K 47/46; A61P 9/00

CPC - A61K 35/12; A61K 47/46; A61L 2300/626; A61L 2300/64; C12N 2502/1329

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

USPC - 424/400; 435/325; 435/377 (keyword delimited)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

PatBase, Google Patents, Google Scholar, PubMed

Search terms used: exosome newt salamander urodele notophthalmus A1

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2014/028493 A2 (CEDARS-SINAI MEDICAL CENTER) 20 February 2014 (20.02.2014) entire document	1-20
A	WO 2013/048734 A1 (TUFTS MEDICAL CENTER, INC.) 04 April 2013 (04.04.2013) entire document	1-20
A	US 2014/0031256 A1 (AGENCY SCIENCE TECHNOLOGY AND RESEARCH) 30 January 2014 (30.01.2014) entire document	1-20
A	IBRAHIM et al. "Exosomes as critical agents of cardiac regeneration triggered by cell therapy," Stem Cell Reports, 08 May 2014 (08.05.2014), Vol. 2, Pgs. 606-19. entire document	1-20
P, X	TSELIU et al. "Abstract 15925: Newt Exosomes are Bioactive on Mammalian Heart, Enhancing Proliferation of Rat Cardiomyocytes and Improving Recovery After Myocardial Infarction," Circulation, 10 November 2015 (10.11.2015), Vol. 132, Iss. 3, Pg. 1 of 1. entire document	1-20

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

12 August 2016

Date of mailing of the international search report

05 OCT 2016

Name and mailing address of the ISA/

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