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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :		(11) International Publication Number:	WO 99/26644
A61K 38/08, C07K 7/14, C12N 5/06	A1	(43) International Publication Date:	3 June 1999 (03.06.99)

(21) International Application Number: PCT/US98/25390

(22) International Filing Date: 24 November 1998 (24.11.98)

(30) Priority Data:
60/066,593 26 November 1997 (26.11.97) US

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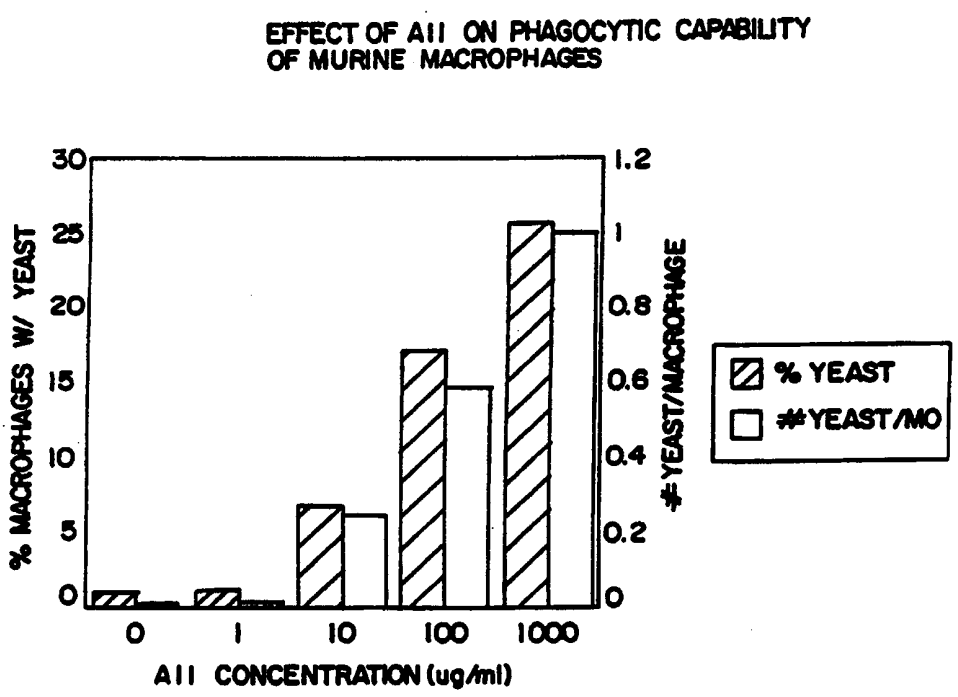
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(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: METHOD FOR PROMOTING HEMATOPOIETIC AND MESENCHYMAL CELL PROLIFERATION AND DIFFERENTIATION



(57) Abstract

The present invention fulfills a need in the art for methods that promote hematopoietic and mesenchymal stem and lineage–specific cell proliferation and differentiation by growth in the presence of angiotensinogen, angiotensin I (AI), AI analogues, AI fragments and analogues thereof, angiotensin II (AII), AII analogues, AII fragments and analogues thereof and AII AT_2 type 2 receptor agonists.

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METHOD FOR PROMOTING HEMATOPOIETIC AND MESENCHYMAL CELL PROLIFERATION AND DIFFERENTIATION

Cross Reference

This application claims priority from U.S. Provisional Application No. 60/066,593 filed November 26, 1997.

Field of the Invention

This present invention relates to methods for use in accelerating the proliferation and differentiation of hematopoietic and mesenchymal cells.

Background of the Invention

Bone marrow contains pluripotent stem cells that are capable of reconstituting either the hematopoietic system or a wide range of mesenchymal tissues. The mechanisms by which hematopoietic and mesenchymal stem cells produce a range of progenitor cell types are quite dissimilar.

The hematopoietic system is composed of a multitude of cell generations ranging from the terminally differentiated to very primitive hematopoietic progenitor cells (HPC). (Traycoff, et al., Experimental Hematology 24:299-306, 1996). HPC are pluripotent progenitor cells that possess the ability to terminally differentiate into hematopoietic lineage-specific progenitor cells (HLSPC). Hematopoiesis is an ongoing process, and therefore HPC must provide a continuous source of HLSPC, which in turn can differentiate into red cells, platelets, monocytes, granulocytes and lymphocytes. (Prockop, Science 276:71-74, 1997). HPC proliferate either by "self-

renewal", to produce HPC-type progeny cells, or with accompanying differentiation, to produce HLSPC. (Traycoff, et al., *supra*).

HPC transplantation therapy has been successful for a variety of malignant and inherited diseases and also provides myelopoietic support for patients undergoing high-dose chemotherapy or radiotherapy. (Emerson, Blood 87:3082-3088, 1996). However, stem cell transplantation has been limited by several features. First, acquiring a sufficient quantity of stem cells to achieve benefit after transfusion requires either extensive, operative bone marrow harvests or extensive pheresis procedures. (Emerson, *supra*). Next, even under these circumstances, only a limited number of useful cells is obtained. Finally, mature blood cell regeneration after transfusion is slow, so that little direct therapeutic benefit is seen for periods of 1 to 3 weeks. (Emerson, *supra*).

The development of *in vitro* culture techniques for hematopoietic cells combined with technologies for isolating relatively pure populations of HPC and HLSPC has made possible their *ex vivo* expansion. (Alcorn and Holyoake, Blood Reviews 10:167-176, 1996, which is incorporated by reference herein). Successful *ex vivo* expansion of HPC, both by self-renewal and proliferation with differentiation, promises many clinical benefits, such as reduction of the number and duration of leucapheresis procedures required for autologous transplantation, thus reducing the risk of disease contamination in the apheresis products. (Alcorn and Holyoake, supra). Furthermore, *ex vivo* expansion may render inadequate HPC populations in peripheral blood and umbilical cord blood sufficient for autologous transplantation and adult allogeneic transplantation respectively. Finally, *ex vivo* expansion of HPC will greatly increase their utility as gene therapy vehicles. (Alcorn and Holyoake,

supra). Similarly, *ex vivo* expansion of HLSPC promises substantial clinical benefits, such as re-infusion of expanded populations of myeloid precursor cells to reduce the period of obligate neutropenia following autologous transplantation, the generation of natural killer cells for use in adoptive immunotherapy protocols, generation of megakaryocyte precursors to alleviate post-transplant-associated thrombocytopenia and more efficient generation of delivery systems for gene therapy. (Alcorn and Holyoake, *supra*).

Human bone marrow, umbilical cord blood, and peripheral blood progenitor cells mobilized by chemotherapy and/or cytokine treatment have been shown to be effective sources of HPC for transplantation following the administration of high-dose therapy to treat malignancy. (Holyoake, et al., Blood 87:4589-4595, 1996). Whatever the source of hematopoietic cells, most studies have used cultured cell populations selected on the basis of HPC-specific surface antigens, such as CD34. These cells can be readily obtained by a number of techniques. (Alcorn and Holyoake, *supra*). The results of several clinical trials using *ex vivo* expanded hematopoietic cells suggests that a fairly small number of HPC cultured *ex vivo* under appropriate conditions can initiate hematologic reconstitution. (Emerson, *supra*).

Survival and proliferation of HPC in *ex vivo* culture requires a combination of synergizing growth factors; the choice of cytokine/growth factor combination and culture system used will largely determine the fate of cells used to initiate the culture. (Alcorn and Holyoake, supra). *In vivo*, blood cell production is thought to be regulated locally by interactions of hematopoietic stem cells with a variety of cell-bound and secreted factors produced by adjacent bone marrow stromal cells. (Alcorn and Holyoake, *supra*). The addition of growth factors and cytokines to the culture

medium is intended to compensate for the absence of stroma-associated activities. Growth factors and cytokines that have been shown to increase production of HPC (in various combinations) include granulocyte colony-stimulating factor (G-CSF), granulocyte/macrophage colony stimulating factor (GM-CSF), stem cell factor (SCF), macrophage colony-stimulating factor (M-CSF), and interleukins 1, 3, 6, and 11 (Reviewed in Takaku, J. Cancer Res. Clin. Oncol. 121:701-709, 1995; Holyoake, et al., *supra*). Conversely, inclusion of macrophage inhibitory protein-1α (MIP-1α), tumor necrosis factor α (TNF-α) or transforming growth factor β (TGFβ) in most expansion cultures reported to date results in decreased HPC and HLSPC yields. (Emerson, *supra*).

A great deal of effort has gone into defining the optimal conditions for *ex vivo* culture of hematopoietic cells. Methods that increase the *ex vivo* proliferation of HPC will greatly increase the clinical benefits of HPC transplantation. This is true both for increased "self-renewal", which will provide a larger supply of HPC capable of reconstituting the entire hematopoietic system, and for proliferation with differentiation, which will provide a larger supply of lineage-specific progenitor cells. Similarly, methods that increase *in vivo* proliferation of HPC will enhance the utility of HPC transplantation therapy by rapidly increasing local concentrations of HPC (and HLSPC) in the bone marrow. Furthermore, methods that result in the differentiation of HPC and HLSPC are useful in providing populations of specific cell types for use in cell therapy.

Transfection of mammalian HPC has been accomplished. (Larochelle, et al., Nature Medicine 2:1329-1337, 1996). Thus, methods that increase the proliferation

of HPC and HLSPC are also useful in rapidly providing a large population of transfected cells for use in gene therapy.

Mesenchymal stem cells (MSC) are pluripotent progenitor cells that possess the ability to differentiate into a variety of mesenchymal tissue, including bone, cartilage, tendon, muscle, marrow stroma, fat and dermis as demonstrated in a number of organisms, including humans (Bruder, et al., J. Cellul. Biochem. 56:283-294 (1994). The formation of mesenchymal tissues is known as the mesengenic process, which continues throughout life, but proceeds much more slowly in the adult than in the embryo (Caplan, Clinics in Plastic Surgery 21:429-435 (1994). The mesengenic process in the adult is a repair process but involves the same cellular events that occur during embryonic development (Reviewed in Caplan, 1994, *supra*). During repair processes, chemoattraction brings MSC to the site of repair where they proliferate into a mass of cells that spans the break. These cells then undergo commitment and enter into a specific lineage pathway (differentiation), where they remain capable of proliferating. Eventually, the cells in the different pathways terminally differentiate (and are no longer able to proliferate) and combine to form the appropriate skeletal tissue, in a process controlled by the local concentration of tissue-specific cytokines and growth factors (Caplan, 1994, *supra*).

Recently, it has been hypothesized that the limiting factor for MSC-based repair processes is the lack of adequate numbers of responsive MSC at the repair site (Caplan, 1994, *supra*). Thus, it has been suggested that by supplying a sufficient number of MSC to a specific tissue site the repair process can be controlled, since the repair site will supply the appropriate exposure to lineage-specific growth factors and differentiation molecules (Caplan, 1994, *supra*). Towards this end, several animal

studies have demonstrated the feasibility of using autologous MSC for repair of various defects associated with mesenchymal tissue. (For review, see Caplan, et al., in The Anterior Cruciate Ligament: Current and Future Concepts, ed. D.W. Jackson, Raven Press, Ltd. NY pp.405-417 (1993). Recent work has demonstrated the feasibility of collection, *ex vivo* expansion in culture, and intravenous infusion of MSC in humans (Lazarus, et al., Bone Marrow Transplantation 16:557-564 (1995); Caplan and Haynesworth, U.S. Patent No. 5,486,359). Further, MSC of animal origin have been transfected with retroviruses and have achieved high level gene expression both *in vitro* and *in vivo* (Allay, et al., Blood 82:477A (1993). Thus, the manipulation of MSC via such techniques seems a promising tool for reconstructive therapies and may be useful for gene therapy.

MSC therapy can serve as a means to deliver high densities of repair-competent cells to a defect site when adequate numbers of MSC and MSC lineage-specific cells are not present *in vivo*, especially in older and/or diseased patients. In order to efficiently deliver high densities of MSC to a defect site, methods for rapidly producing large numbers of MSC are necessary. While MSC have been exposed to a number of growth factors *in vitro*, only platelet-derived growth factor (PDGF) showed mitotic activity (Caplan et al., 1994, *supra*), while none have been demonstrated to independently induce differentiation. Methods that increase the *ex vivo* proliferation and differentiation of MSC will greatly increase the utility of MSC therapy. Similarly, methods that increase *in vivo* proliferation and differentiation of MSC will enhance the utility of MSC therapy by rapidly increasing local concentrations of MSC at the repair site.

Methods that allow for *ex vivo* differentiation of MSC would also provide an important tool for cell therapy. MSCs from various species have been differentiated in vitro into colonies of osteoblasts, chondrocytes, and adipocytes in response to dexamethasone, 1,25 dihydroxyvitamin D_3, or BMP-2. (Prockop, Science 276:71-74, 1997) For example, *ex vivo* culturing of MSC to produce chondrocytes can be used to resurface joint cartilage in patients with degenerative arthritis or in reconstructive plastic surgery in patients with osteoarthritis. Similarly, treatment of MSC to differentiate into osteoclasts can be used for implantation into poorly healing bone.

Furthermore, methods that enhance the proliferation of lineage-specific descendants of MSC, including but not limited to bone marrow stromal cells, osteoclasts, chondrocytes, and adipocytes, will enhance the therapeutic utility of MSC therapy by increasing the concentration of lineage-specific cell types at appropriate repair sites.

Thus, there exists a need in the art for methods that increase the proliferation and differentiation of hematopoietic and mesenchymal pluripotent and lineage-specific cells that are useful in rapidly providing a large population of such cells for use in cell therapy and for making a large population of transfected cells for use in gene therapy.

Summary of the Invention

The present invention fulfills a need in the art for methods that promote hematopoietic and mesenchymal stem and lineage-specific cell proliferation and differentiation by growth in the presence of angiotensinogen, angiotensin I (AI), AI

analogues, AI fragments and analogues thereof, angiotensin II (AII), AII analogues, AII fragments and analogues thereof and AII AT_2 type 2 receptor agonists.

Brief Description of the Drawings

Figure 1 is a graph showing the effect of AII on the phagocytic capability of murine macrophages.

Figure 2 is a graph showing the effect of AII on the phagocytic capability of rat macrophages.

Figure 3 is a graph showing the effect of AII on respiratory burst function in rat peritoneal macrophages.

Figure 4 is a graph showing the effect of AII on respiratory burst function in human PBMC.

Figure 5 is a graph showing the effect of AII(1-7) (SEQ. ID. NO:4) on respiratory burst function in rat peritoneal macrophages.

Figure 6 is a graph showing the effect of GSD 24B (SEQ ID NO:31) on respiratory burst function in rat peritoneal macrophages.

Figure 7 is a graph showing the effect of GSD 22A (SEQ ID NO:18) on respiratory burst function in rat peritoneal macrophages.

Figure 8 is a graph showing the effect of GSD 28 (SEQ ID NO:37) on respiratory burst function in rat peritoneal macrophages.

Figure 9 is a graph showing the effect of AII on proliferation in response to pokeweed mitogen.

Figure 10 is a graph showing the effect of AII on rat bone marrow cultures.

Figure 11 is a graph showing the effect of AII on rat bone marrow cultures.

Figure 12 is a graph showing the effect of AII on murine HSC cultures.

Figure 13 is a graph showing the effect of AII on murine HSC cultures.

Figure 14 is a graph showing the effect of AII on murine HSC cultures.

Figure 15 is a graph showing the effect of AII on murine HSC cultures.

Figure 16 is a graph showing the effect of AII on murine HSC cultures.

Figure 17 is a graph showing the effect of AII on murine HSC cultures.

Figure 18 is a graph showing the effect of AII(1-7) (SEQ ID NO:4) on MSC proliferation.

Figure 19 is a graph showing the effect of GSD 22A (SEQ ID NO:18) on MSC proliferation.

Figure 20 is a graph showing the effect of GSD 24B (SEQ ID NO:31) on MSC proliferation.

Figure 21 is a graph showing the effect of GSD 28 (SEQ ID NO:37) on MSC proliferation.

Figure 22 is a graph showing the effect of AII on alkaline phosphatase expression by MSC.

Figure 23 is a graph showing the effect of AII on GM-CSF secretion by mouse mesenchymal stem cells.

Detailed Description Of The Invention

Within this application, unless otherwise stated, the techniques utilized may be found in any of several well-known references such as: *Molecular Cloning: A Laboratory Manual* (Sambrook, et al., 1989, Cold Spring Harbor Laboratory Press), *Gene Expression Technology* (Methods in Enzymology, Vol. 185, edited by D.

Goeddel, 1991. Academic Press, San Diego, CA), "Guide to Protein Purification" in *Methods in Enzymology* (M.P. Deutshcer, ed., (1990) Academic Press, Inc.); *PCR Protocols: A Guide to Methods and Applications* (Innis, et al. 1990. Academic Press, San Diego, CA), *Culture of Animal Cells: A Manual of Basic Technique, 2nd Ed.* (R.I. Freshney. 1987. Liss, Inc. New York, NY), and *Gene Transfer and Expression Protocols*, pp. 109-128, ed. E.J. Murray, The Humana Press Inc., Clifton, N.J.).

As defined herein, the term "HPC" refers to any hematopoietic pluripotent progenitor cells capable of giving rise to a wide variety of differentiated hematopoietic cell types. Among cell types included within this definition are $CD34^+$ bone marrow mononuclear cells (BMMC) (Berardi, et al., Blood 86:2123-2129, 1995), PBSC (Fritsch, et al., Bone Marrow Transplantation 17:169-178, 1996), cobblestone area forming cells (CAFC) (Lemieux, et al., Blood 86:1339-1347, 1995) and 5-FU BM cells (Alcorn and Holyoake, Blood Reviews 10:167-176, 1996). As defined herein, the term "HLSPC" refers to hematopoietic lineage-specific progenitor cells, and includes the progeny of HPC that are committed to a cell-specific differentiation path. As defined herein, mesenchymal stem cells (MSC) are pluripotent progenitor cells that possess the ability to differentiate into a variety of mesenchymal tissue, including bone, cartilage, tendon, muscle, marrow stroma, fat and dermis. As defined herein, "proliferation" encompasses both cell self renewal and cellular proliferation with accompanying differentiation. As defined herein "differentiation" refers to any cellular processes that distinguish a non-committed cell type from a more lineage committed cell type.

U.S. Patent No. 5,015,629 to DiZerega (the entire disclosure of which is hereby incorporated by reference) describes a method for increasing the rate of

healing of wound tissue, comprising the application to such tissue of angiotensin II (AII) in an amount which is sufficient for said increase. The application of AII to wound tissue significantly increases the rate of wound healing, leading to a more rapid re-epithelialization and tissue repair. The term AII refers to an octapeptide present in humans and other species having the sequence Asp-Arg-Val-Tyr-Ile-His-Pro-Phe [SEQ ID NO:1]. The biological formation of angiotensin is initiated by the action of renin on the plasma substrate angiotensinogen. The substance so formed is a decapeptide called angiotensin I (AI) which is converted to AII by the converting enzyme angiotensinase which removes the C-terminal His-Leu residues from AI. AII is a known pressor agent and is commercially available.

Studies have shown that AII increases mitogenesis and chemotaxis in cultured cells that are involved in wound repair, and also increases their release of growth factors and extracellular matrices (diZerega, U.S. Patent No. 5,015,629; Dzau et. al., J. Mol. Cell. Cardiol. 21:S7 (Supp III) 1989; Berk et. al., Hypertension 13:305-14 (1989); Kawahara, et al., BBRC 150:52-9 (1988); Naftilan, et al., J. Clin. Invest. 83:1419-23 (1989); Taubman et al., J. Biol. Chem 264:526-530 (1989); Nakahara, et al., BBRC 184:811-8 (1992); Stouffer and Owens, Circ. Res. 70:820 (1992); Wolf, et al., Am. J. Pathol. 140:95-107 (1992); Bell and Madri, Am. J. Pathol. 137:7-12 (1990). In addition, AII was shown to be angiogenic in rabbit corneal eye and chick chorioallantoic membrane models (Fernandez, et al., J. Lab. Clin. Med. 105:141 (1985); LeNoble, et al., Eur. J. Pharmacol. 195:305-6 (1991). Therefore, AII may accelerate wound repair through increased neovascularization, growth factor release, reepithelialization and/or production of extracellular matrix.

A peptide agonist selective for the AT2 receptor (AII has 100 times higher affinity for AT2 than AT1) has been identified. This peptide is p-aminophenylalanine6-AII ["(p-NH_2-Phe)6-AII)"], Asp-Arg-Val-Tyr-Ile-Xaa-Pro-Phe [SEQ ID NO.36] wherein Xaa is p-NH_2-Phe (Speth and Kim, BBRC 169:997-1006 (1990). This peptide gave binding characteristics comparable to AT2 antagonists in the experimental models tested (Catalioto, et al., Eur. J. Pharmacol. 256:93-97 (1994); Bryson, et al., Eur. J. Pharmacol. 225:119-127 (1992).

The effects of AII receptor and AII receptor antagonists have been examined in two experimental models of vascular injury and repair which suggest that both AII receptor subtypes (AT1 and AT2) play a role in wound healing (Janiak et al., Hypertension 20:737-45 (1992); Prescott, et al., Am. J. Pathol. 139:1291-1296 (1991); Kauffman, et al., Life Sci. 49:223-228 (1991); Viswanathan, et al., Peptides 13:783-786 (1992); Kimura, et al., BBRC 187:1083-1090 (1992).

Many studies have focused upon AII(1-7) (AII residues 1-7) or other fragments of AII to evaluate their activity. AII(1-7) elicits some, but not the full range of effects elicited by AII. Pfeilschifter, et al., Eur. J. Pharmacol. 225:57-62 (1992); Jaiswal, et al., Hypertension 19(Supp. II):II-49-II-55 (1992); Edwards and Stack, J. Pharmacol. Exper. Ther. 266:506-510 (1993); Jaiswal, et al., J. Pharmacol. Exper. Ther. 265:664-673 (1991); Jaiswal, et al., Hypertension 17:1115-1120 (1991); Portsi, et a., Br. J. Pharmacol. 111:652-654 (1994).

As hereinafter defined, a preferred class of AT2 agonists for use in accordance with the present invention comprises AII, AII analogues or active fragments thereof having p-NH-Phe in a position corresponding to a position 6 of AII. In addition to peptide agents, various nonpeptidic agents (e.g., peptidomimetics) having the

requisite AT2 agonist activity are further contemplated for use in accordance with the present invention.

The active AII analogues, fragments of AII and analogues thereof of particular interest in accordance with the present invention are characterized as comprising a sequence consisting of at least three contiguous amino acids of groups R^1-R^8 in the sequence of general

formula I

$$R^1\text{-}R^2\text{-}R^3\text{-}R^4\text{-}R^5\text{-}R^6\text{-}R^7\text{-}R^8$$

in which R^1 and R^2 together form a group of formula

$$X\text{-}R^A\text{-}R^B\text{-},$$

wherein X is H or a one to three peptide group and a peptide bond between R^A and R^B is labile to aminopeptidase A cleavage;

R^3 is selected from the group consisting of Val, Ala, Leu, norLeu, Ile, Gly, Pro, Aib, Acpc and Tyr;

R^4 is selected from the group consisting of Tyr, Tyr$(PO_3)_2$, Thr, Ser, homoSer and azaTyr;

R^5 is selected from the group consisting of Ile, Ala, Leu, norLeu, Val and Gly;

R^6 is His, Arg or 6-NH_2-Phe;

R^7 is Pro or Ala; and

R^8 is selected from the group consisting of Phe, Phe(Br), Ile and Tyr, excluding sequences including R^4 as a terminal Tyr group.

Compounds falling within the category of AT2 agonists useful in the practice of the invention include the AII analogues set forth above subject to the restriction that R^6 is p-NH_2-Phe.

In one class of preferred embodiments, R^A is suitably selected from Asp, Glu, Asn, Acpc (1-aminocyclopentane carboxylic acid), Ala, Me^2Gly, Pro, Bet, Glu(NH_2), Gly, Asp(NH_2) and Suc. R^B is suitably selected from Arg, Lys, Ala, Orn, Ser(Ac), Sar, D-Arg and D-Lys. Particularly preferred combinations for R^A and R^B are Asp-Arg, Asp-Lys, Glu-Arg and Glu-Lys. Particularly preferred embodiments of this class include the following: AII, AIII or AII(2-8), Arg-Val-Tyr-Ile-His-Pro-Phe [SEQ ID NO:2]; AII(3-8), also known as des1-AIII or AIV, Val-Tyr-Ile-His-Pro-Phe [SEQ ID NO:3]; AII(1-7), Asp-Arg-Val-Tyr-Ile-His-Pro {SEQ ID NO:4]; AII(2-7). Arg-Val-Tyr-Ile-His-Pro [SEQ ID NO:5]; AII(3-7), Val-Tyr-Ile-His-Pro [SEQ ID NO:6]; AII(5-8), Ile-His-Pro-Phe [SEQ ID NO:7]; AII(1-6), Asp-Arg-Val-Tyr-Ile-His [SEQ ID NO:8]; AII(1-5), Asp-Arg-Val-Tyr-Ile [SEQ ID NO:9]; AII(1-4), Asp-Arg-Val-Tyr [SEQ ID NO:10]; and AII(1-3), Asp-Arg-Val [SEQ ID NO:11]. Other preferred embodiments include: Arg-norLeu-Tyr-Ile-His-Pro-Phe [SEQ ID NO:12] and Arg-Val-Tyr-norLeu-His-Pro-Phe [SEQ ID NO:13]. Still another preferred embodiment encompassed within the scope of the invention is a peptide having the sequence Asp-Arg-Pro-Tyr-Ile-His-Pro-Phe [SEQ ID NO:31]. AII(6-8), His-Pro-Phe [SEQ ID NO:14] and AII(4-8), Tyr-Ile-His-Pro-Phe [SEQ ID NO:15] were also tested and found not to be effective.

Another class of compounds of particular interest in accordance with the present invention are those of the general formula II

R^2-R^3-R^4-R^5-R^6-R^7-R^8

in which R^2 is selected from the group consisting of H, Arg, Lys, Ala, Orn, Ser(Ac), Sar, D-Arg and D-Lys;

R^3 is selected from the group consisting of Val, Ala, Leu, norLeu, Ile, Gly, Pro, Aib, Acpc and Tyr;

R^4 is selected from the group consisting of Tyr, Tyr$(PO_3)_2$, Thr, Ser, homoSer and azaTyr;

R^5 is selected from the group consisting of Ile, Ala, Leu, norLeu, Val and Gly;

R^6 is His, Arg or 6-NH_2-Phe;

R^7 is Pro or Ala; and

R^8 is selected from the group consisting of Phe, Phe(Br), Ile and Tyr.

A particularly preferred subclass of the compounds of general formula II has the formula

R^2-R^3-Tyr-R^5-His-Pro-Phe [SEQ ID NO:16]

wherein R^2, R^3 and R^5 are as previously defined. Particularly preferred is angiotensin III of the formula Arg-Val-Tyr-Ile-His-Pro-Phe [SEQ ID NO:2]. Other preferred compounds include peptides having the structures Arg-Val-Tyr-Gly-His-Pro-Phe [SEQ ID NO:17] and Arg-Val-Tyr-Ala-His-Pro-Phe [SEQ ID NO:18]. The fragment AII(4-8) was ineffective in repeated tests; this is believed to be due to the exposed tyrosine on the N-terminus.

In the above formulas, the standard three-letter abbreviations for amino acid residues are employed. In the absence of an indication to the contrary, the L-form of the amino acid is intended. Other residues are abbreviated as follows:

TABLE 1

Abbreviation for Amino Acids

Me^2Gly	N,N-dimethylglycyl
Bet	1-carboxy-N,N,N-trimethylmethanaminium hydroxide inner salt (betaine)
Suc	Succinyl
Phe(Br)	p-bromo-L-phenylalanyl
azaTyr	aza-α'-homo-L-tyrosyl
Acpc	1-aminocyclopentane carboxylic acid
Aib	2-aminoisobutyric acid
Sar	N-methylglycyl (sarcosine)

It has been suggested that AII and its analogues adopt either a *gamma* or a *beta* turn (Regoli, et al., Pharmacological Reviews 26:69 (1974). In general, it is believed that neutral side chains in position R^3, R^5 and R^7 may be involved in maintaining the appropriate distance between active groups in positions R^4, R^6 and R^8 primarily responsible for binding to receptors and/or intrinsic activity. Hydrophobic side chains in positions R^3, R^5 and R^8 may also play an important role in the whole conformation of the peptide and/or contribute to the formation of a hypothetical hydrophobic pocket.

Appropriate side chains on the amino acid in position R^2 may contribute to affinity of the compounds for target receptors and/or play an important role in the

conformation of the peptide. For this reason, Arg and Lys are particularly preferred as R^2.

For purposes of the present invention, it is believed that R^3 may be involved in the formation of linear or nonlinear hydrogen bonds with R^5 (in the gamma turn model) or R^6 (in the beta turn model). R^3 would also participate in the first turn in a beta antiparallel structure (which has also been proposed as a possible structure). In contrast to other positions in general formula I, it appears that beta and gamma branching are equally effective in this position. Moreover, a single hydrogen bond may be sufficient to maintain a relatively stable conformation. Accordingly, R^3 may suitably be selected from Val, Ala, Leu, norLeu, Ile, Gly, Pro, Aib, Acpc and Tyr.

With respect to R^4, conformational analyses have suggested that the side chain in this position (as well as in R^3 and R^5) contribute to a hydrophobic cluster believed to be essential for occupation and stimulation of receptors. Thus, R^4 is preferably selected from Tyr, Thr, Tyr $(PO_3)_2$, homoSer, Ser and azaTyr. In this position, Tyr is particularly preferred as it may form a hydrogen bond with the receptor site capable of accepting a hydrogen from the phenolic hydroxyl (Regoli, et al. (1974), *supra*).

In position R^5, an amino acid with a β aliphatic or alicyclic chain is particularly desirable. Therefore, while Gly is suitable in position R^5, it is preferred that the amino acid in this position be selected from Ile, Ala, Leu, norLeu, Gly and Val.

In the AII analogues, fragments and analogues of fragments of particular interest in accordance with the present invention, R^6 is His, Arg or 6-NH_2-Phe. The unique properties of the imidazole ring of histidine (e.g., ionization at physiological pH, ability to act as proton donor or acceptor, aromatic character) are believed to

contribute to its particular utility as R^6. For example, conformational models suggest that His may participate in hydrogen bond formation (in the *beta* model) or in the second turn of the antiparallel structure by influencing the orientation of R^7. Similarly, it is presently considered that R^7 should be Pro in order to provide the most desirable orientation of R^8. In position R^8, both a hydrophobic ring and an anionic carboxyl terminal appear to be particularly useful in binding of the analogues of interest to receptors; therefore, Tyr and especially Phe are preferred for purposes of the present invention.

Analogues of particular interest include the following:

TABLE 2

Angiotensin II Analogues

AII Analogue Name	Amino Acid Sequence	Sequence Identifier
Analogue 1	Asp-Arg-Val-Tyr-Val-His-Pro-Phe	SEQ ID NO: 19
Analogue 2	Asn-Arg-Val-Tyr-Val-His-Pro-Phe	SEQ ID NO: 20
Analogue 3	Ala-Pro-Gly-Asp-Arg-Ile-Tyr-Val-His-Pro-Phe	SEQ ID NO: 21
Analogue 4	Glu-Arg-Val-Tyr-Ile-His-Pro-Phe	SEQ ID NO: 22
Analogue 5	Asp-Lys-Val-Tyr-Ile-His-Pro-Phe	SEQ ID NO: 23
Analogue 6	Asp-Arg-Ala-Tyr-Ile-His-Pro-Phe	SEQ ID NO: 24
Analogue 7	Asp-Arg-Val-Thr-Ile-His-Pro-Phe	SEQ ID NO: 25
Analogue 8	Asp-Arg-Val-Tyr-Leu-His-Pro-Phe	SEQ ID NO: 26
Analogue 9	Asp-Arg-Val-Tyr-Ile-Arg-Pro-Phe	SEQ ID NO: 27
Analogue 10	Asp-Arg-Val-Tyr-Ile-His-Ala-Phe	SEQ ID NO: 28
Analogue 11	Asp-Arg-Val-Tyr-Ile-His-Pro-Tyr	SEQ ID NO: 29
Analogue 12	Pro-Arg-Val-Tyr-Ile-His-Pro-Phe	SEQ ID NO: 30
Analogue 13	Asp-Arg-Pro-Tyr-Ile-His-Pro-Phe	SEQ ID NO: 31
Analogue 14	Asp-Arg-Val-Tyr(Po_3)$_2$-Ile-His-Pro-Phe	SEQ ID NO: 32
Analogue 15	Asp-Arg-norLeu-Tyr-Ile-His-Pro-Phe	SEQ ID NO: 33
Analogue 16	Asp-Arg-Val-Tyr-norLeu-His-Pro-Phe	SEQ ID NO: 34
Analogue 17	Asp-Arg-Val-homoSer-Tyr-Ile-His-Pro-Phe	SEQ ID NO: 35
Analogue 18	Asp-Arg-Val-Tyr-Ile-His-Pro-Ile	SEQ ID N0: 37

The polypeptides of the instant invention may be synthesized by any conventional method, including, but not limited to, those set forth in J. M. Stewart and J. D. Young, *Solid Phase Peptide Synthesis*, 2nd ed., Pierce Chemical Co., Rockford, Ill. (1984) and J. Meienhofer, *Hormonal Proteins and Peptides*, Vol. 2, Academic Press, New York, (1973) for solid phase synthesis and E. Schroder and K. Lubke, *The Peptides*, Vol. 1, Academic Press, New York, (1965) for solution synthesis. The disclosures of the foregoing treatises are incorporated by reference herein.

In general, these methods involve the sequential addition of protected amino acids to a growing peptide chain (U.S. Patent No. 5,693,616, herein incorporated by reference in its entirety). Normally, either the amino or carboxyl group of the first amino acid and any reactive side chain group are protected. This protected amino acid is then either attached to an inert solid support, or utilized in solution, and the next amino acid in the sequence, also suitably protected, is added under conditions amenable to formation of the amide linkage. After all the desired amino acids have been linked in the proper sequence, protecting groups and any solid support are removed to afford the crude polypeptide. The polypeptide is desalted and purified, preferably chromatographically, to yield the final product.

Preferably, peptides are synthesized according to standard solid-phase methodologies, such as may be performed on an Applied Biosystems Model 430A peptide synthesizer (Applied Biosystems, Foster City, Calif.), according to manufacturer's instructions. Other methods of synthesizing peptides or peptidomimetics, either by solid phase methodologies or in liquid phase, are well known to those skilled in the art.

Alternatively, the peptides can be produced via recombinant DNA technologies. Techniques for recombinant production of the peptides of the invention are well known in the art and can be found in references such as *Gene Expression Technology* (Methods in Enzymology, Vol. 185, edited by D. Goeddel, 1991. Academic Press, San Diego, CA), "Guide to Protein Purification" in *Methods in Enzymology* (M.P. Deutshcer, ed., (1990) Academic Press, Inc.); *PCR Protocols: A Guide to Methods and Applications* (Innis, et al. 1990. Academic Press, San Diego, CA),

Although AII has been shown to increase the proliferation of a number of cell types *in vitro*, it does not necessarily increase the proliferation of all cell types. Studies have shown that AII accelerates cellular proliferation through the production of transforming growth factor β (TGFβ) (Gibbons et al., J. Clin. Invest. 90:456-461 (1992). Thus, since only PDGF is known to be mitogenic for MSC, an ability of AII to effect MSC proliferation would be unexpected. Furthermore, as Emerson (*supra*) has shown that inclusion of TGF-β in most expansion cultures resulted in a decreased HPC and HLSPC yield, it is unexpected that AII, through the action of TGF-β, would be of benefit in such situations. No studies have reported that AII has an effect on the differentiation of either HPC or MSC.

In one aspect of the present invention, a method of increasing *ex vivo* HPC and HLSPC proliferation by exposure to angiotensinogen, angiotensin I (AI), AI analogues, AI fragments and analogues thereof, angiotensin II (AII), AII analogues, AII fragments and analogues thereof and AII AT_2 type 2 receptor agonists is disclosed. Experimental conditions for the isolation, purification, *ex vivo* growth and *in vivo* mobilization of HPC and HLSPC have been reported (Berardi, et al., Blood

86(6):2123-2129, 1995; Fritsch, et al., Bone Marrow Transplantation 17:169-178, 1996; LaRochelle, et al., Nature Medicine 12:1329-1337, 1996; Traycoff, et al., Experimental Hematology 24:299-306, 1996; Holyoake, et al., Blood 87:4589-4595, 1996; Lemieux, et al., Blood 86:1339-1347, 1995 ; Talmadge, et al., Bone Marrow transplantation 17:101-109, 1996; Bodine, et al., Blood 88:89-97, 1996; all references hereby incorporated by reference herein.)

In one embodiment of the invention, HPC are isolated from bone marrow, peripheral blood or umbilical cord blood. HPC is then selected for in these samples. HPC-enriched samples are cultured under appropriate growth conditions, in the presence of angiotensin II (AII), AII analogues, AII fragments and analogues thereof and/or AII AT_2 type 2 receptor agonists. HPC proliferation is assessed at various time points during culture.

In a preferred embodiment, HPC and HLSPC are isolated from bone marrow aspirates from the posterior iliac crest. $CD34^+$ HPC are isolated from the aspirate by attaching a biotinylated monoclonal antibody specific for CD34 (available from Becton Dickinson, Sunnyvale, CA, USA) to a streptavidin affinity column (Ceprate SC; CellPro, Bothell, WA, USA) and passing the aspirate through the column, followed by appropriate column washing and stripping, according to standard techniques in the art. $CD34^+$ HPC are suspended in culture medium and incubated in the presence of between 0.1 ng/ml and 1 mg/ml angiotensin II (AII), AII analogues, AII fragments and analogues thereof and/or AII AT_2 type 2 receptor agonists. The cells are expanded for a period of between 8 and 21 days and cellular proliferation with accompanying differentiation is assessed via phase microscopy following standard methylcellulose colony formation assays (Berardi, et al., *supra*) at various

points during this time period. Similarly, "self-renewal" of HPC is assessed periodically by reactivity to an antibody directed against $CD34^+$.

In a further preferred embodiment, HPC that have been cultured in the presence of angiotensin II (AII), AII analogues, AII fragments and analogues thereof and/or AII AT_2 type 2 receptor agonists are used for autologous transplantation, to reconstitute a depleted hematopoietic system. Prior to transplantation, the cells are rinsed to remove all traces of culture fluid, resuspended in an appropriate medium and then pelleted and rinsed several times. After the final rinse, the cells are resuspended at between 0.7×10^6 and 50×10^6 cells per ml in an appropriate medium and reinfused into a subject through intravenous infusions. Following transplantation, subject peripheral blood samples are evaluated for increases in the number of HPC, HLSPC, and more mature blood cells at various time points by standard flow cytometry and cell sorting techniques. (Talmadge, et al., *supra*).

In another aspect of the present invention, a method of increasing *ex vivo* MSC and lineage-specific mesenchymal cell proliferation by exposure to angiotensinogen, AI, AI analogues, AI fragments and analogues thereof, AII, AII analogues, AII fragments and analogues thereof and AII AT_2 type 2 receptor agonists is disclosed. Experimental conditions for the isolation, purification and *in vitro* growth of lineage-specific mesenchymal cells, such as bone-marrow derived stromal cells, have been reported (Johnson and Dorshkind, Blood 68(6):1348-1354 (1986); hereby incorporated by reference in its entirety). Other reports describe different conditions for culturing lineage-specific mesenchymal cells *in vitro* (Bab, et al., J. Cell Sci. 84:139-151 (1986); Benayahu, et al., J. Cellular Physiology 140:1-7 (1989); both references hereby incorporated by reference in their entirety).

In one embodiment of the present invention, MSC are isolated from bone marrow aspirates from the posterior iliac crest and/or femoral head cancellous bone, purified, resuspended in appropriate growth medium, counted and diluted to an appropriate concentration to seed in tissue culture plates. Purified MSC are cultured in an appropriate growth medium and growth conditions in a humidified atmosphere. The cells are allowed sufficient time to attach to the tissue culture dish, whereupon non-attached cells are discarded. Adherent cells are placed in growth medium at 37° C in a humidified atmosphere in the presence of between 0.1 ng/ml and 1 mg/ml angiotensinogen, AI, AI analogues, AI fragments and analogues thereof, AII, AII analogues, AII fragments and analogues thereof and/or AII AT_2 type 2 receptor agonists. The cells are expanded for a period of between 2 and 21 days and cellular proliferation is assessed at various time points during this time period. Subsequent medium changes are performed as needed. When the primary cultures are nearly confluent, the cells are harvested for reinfusion into a subject. Cells are examined microscopically to verify the absence of contamination. The cells are rinsed to remove all traces of culture fluid, resuspended in an appropriate medium and then pelleted and rinsed several times. After the final rinse, the cells are resuspended at between 0.7×10^6 and 50×10^6 cells per ml in an appropriate medium and reinfused into a subject through intravenous infusions. Subjects are evaluated for MSC proliferation *in vivo* by means of a repeat diagnostic bone marrow aspirate and biopsy to be compared with the original aspirate and biopsy. In a preferred embodiment, *in vivo* proliferation is assessed by reactivity to an antibody directed against a protein known to be present in higher concentrations in proliferating cells than in non-proliferating cells, such as proliferating cell nuclear antigen (PCNA, or cyclin). Such

antibodies are commercially available from a number of sources, including Zymed Laboratories (South San Francisco, California).

In a preferred embodiment, isolated MSC are placed into Dulbecco's medium MEM (DMEM-LG) (Gibco, Grand Island, NY, USA). The cells are purified by a series of steps. Initially, the cells are pelleted and resuspended in complete medium. The cells are centrifuged through a 70% Percoll (Sigma, St. Louis, MO, USA) gradient at 460X g for 15 minutes, the top 25% of the gradients are transferred to a tube containing 30 ml of complete medium and centrifuged to pellet the cells, which will then be resuspended in complete medium, counted and diluted to seed in 100-mm plates at 50 x 10^6 nucleated cells per plate.

In a further preferred embodiment, purified MSC are cultured in complete medium at 37 °C in a humidified atmosphere containing 95% air and 5% CO_2 and the cells are allowed to attach for 3 days, whereupon non-adherent cells are removed by changing the culture medium. Cellular proliferation of adherent cells and the presence of normal MSC morphology are assessed by phase microscopy at various time points during the subsequent growth period. Subsequent medium changes are performed every four days. When the primary cultures are nearly confluent, the cells are detached with 0.25% trypsin containing 0.1 mM EDTA (Gibco) and either diluted and replated as second passage cells, or used for reinfusion into a subject. Preferably, cells are rinsed free of culture fluid using Tyrode's solution (Gibco). After the final rinse, cells are placed in Tyrode's solution and placed in an incubator at 37 ° C for one hour in order to shed serum proteins. The Tyrode's solution is removed and the cells are preferably placed into TC199 medium (Gibco) supplemented with 1% serum albumin. The cells are rinsed a number of times with this medium and after the final

rinse MSC are resuspended in TC199 plus 1% serum albumin. Subsequently, MSC are injected slowly intravenously over 15 minutes. Evaluation of subsequent bone marrow aspirates are conducted up to 8 weeks after injection.

In a preferred embodiment, assessment of the *in vivo* proliferative effect of angiotensinogen, AI, AI analogues, AI fragments and analogues thereof, AII, AII analogues, AII fragments and analogues thereof and AII AT_2 type 2 receptor agonists on MSC and mesenchymal lineage-specific cells is done by histochemical evaluations of various tissues. In a preferred embodiment, *in vivo* proliferation of MSC and mesenchymal lineage-specific cells is assessed by reactivity to an antibody directed against a protein known to be present in higher concentrations in proliferating cells than in non-proliferating cells, such as proliferating cell nuclear antigen (PCNA, or cyclin).

In a further aspect of the present invention, the effect of the angiotensinogen, AI, AI analogues, AI fragments and analogues thereof, AII, AII analogues, AII fragments and analogues thereof and AII AT_2 type 2 receptor agonists on HPC, HLSPC, MSC and mesenchymal lineage-specific cell differentiation are assessed by examination of changes in gene expression, phenotype, morphology, or any other method that distinguishes a cell undergoing differentiation from a progenitor cell. In one embodiment, MSC are incubated with angiotensinogen, AI, AI analogues, AI fragments and analogues thereof, AII, AII analogues, AII fragments and analogues thereof or AII AT_2 type 2 receptor agonists as described above. MSC are then tested for the production of colony stimulating factors into the culture supernatant as a demonstration of MSC differentiation. In a preferred embodiment, the colony stimulating factor tested for is granulocyte-macrophage colony stimulating factor.

In another preferred embodiment, macrophage differentiation to an elicited or activated state is assessed after exposure to angiotensinogen, AI, AI analogues, AI fragments and analogues thereof, AII, AII analogues, AII fragments and analogues thereof or AII AT_2 type 2 receptor agonists, as described above. The macrophages are assessed for phagocytotic ability by any of the well known art methods, including but not limited to determination of the number of macrophages that have ingested opsonized yeast particles, and the number of yeast per macrophage ingested.

In another preferred embodiment, the respiratory burst activity of leukocytes is assessed after exposure to angiotensinogen, AI, AI analogues, AI fragments and analogues thereof, AII, AII analogues, AII fragments and analogues thereof or AII AT_2 type 2 receptor agonists, as described above. The leukocytes are assessed for respiratory burst activity by any method known in the art, including but not limited to the ability to generate hydrogen peroxide via the respiratory burst system.

In another aspect of the present invention, angiotensinogen, AI, AI analogues, AI fragments and analogues thereof, AII, AII analogues, AII fragments and analogues thereof or AII AT_2 type 2 receptor agonists are used to increase *in vivo* HPC, HLSPC, MSC and mesenchymal lineage-specific cell proliferation. For use in increasing proliferation of HPC, HLSCP, MSC and mesenchymal lineage-specific cells, AII, AII analogues, AII fragments and analogues thereof and AII AT_2 type 2 receptor agonists may be administered orally, parentally, by inhalation spray, rectally, or topically in dosage unit formulations containing conventional pharmaceutically acceptable carriers, adjuvants, and vehicles. The term parenteral as used herein includes, subcutaneous, intravenous, intramuscular, intrasternal, intratendinous, intraspinal, intracranial, intrathoracic, infusion techniques or intraperitoneally.

In a further embodiment of the present invention, a method of increasing *in vivo* HPC, HLSPC, MSC and lineage-specific mesenchymal cell proliferation by exposure to angiotensinogen, AI, AI analogues, AI fragments and analogues thereof, AII, AII analogues, AII fragments and analogues thereof and AII AT_2 type 2 receptor agonists is disclosed, either in the presence or absence of other growth factors and cytokines.

The dosage regimen for increasing *in vivo* proliferation or differentiation of HPC, HLSCP, MSC and lineage-specific mesenchymal cell with angiotensinogen, AI, AI analogues, AI fragments and analogues thereof, AII, AII analogues, AII fragments and analogues thereof and AII AT_2 type 2 receptor agonists is based on a variety of factors, including the type of injury, the age, weight, sex, medical condition of the individual, the severity of the condition, the route of administration, and the particular compound employed. Thus, the dosage regimen may vary widely, but can be determined routinely using standard methods. Dosage levels of the order of between 0.1 ng/ml and 100 mg/ml angiotensinogen, AI, AI analogues, AI fragments and analogues thereof, angiotensin II (AII), AII analogues, AII fragments and analogues thereof and/or AII AT_2 type 2 receptor agonists are useful for all methods of use disclosed herein.

The angiotensinogen, AI, AI analogues, AI fragments and analogues thereof, AII, AII analogues, AII fragments and analogues thereof and AII AT_2 type 2 receptor agonists may be made up in a solid form (including granules, powders or suppositories) or in a liquid form (*e.g.*, solutions, suspensions, or emulsions). The angiotensinogen, AI, AI analogues, AI fragments and analogues thereof, AII, AII analogues, AII fragments and analogues thereof and AII AT_2 type 2 receptor agonists

may be subjected to conventional pharmaceutical operations such as sterilization and/or may contain conventional adjuvants, such as preservatives, stabilizers, wetting agents, emulsifiers, buffers etc.

While angiotensinogen, AI, AI analogues, AI fragments and analogues thereof, AII, AII analogues, AII fragments and analogues thereof and AII AT_2 type 2 receptor agonists can be administered as the sole active pharmaceutical agent, they can also be used in combination with one or more other agents. When administered as a combination, the therapeutic agents can be formulated as separate compositions that are given at the same time or different times, or the therapeutic agents can be given as a single composition.

For administration, the angiotensinogen, AI, AI analogues, AI fragments and analogues thereof, AII, AII analogues, AII fragments and analogues thereof and AII AT_2 type 2 receptor agonists are ordinarily combined with one or more adjuvants appropriate for the indicated route of administration. The compounds may be admixed with lactose, sucrose, starch powder, cellulose esters of alkanoic acids, stearic acid, talc, magnesium stearate, magnesium oxide, sodium and calcium salts of phosphoric and sulphuric acids, acacia, gelatin, sodium alginate, polyvinylpyrrolidine, and/or polyvinyl alcohol, and tableted or encapsulated for conventional administration. Alternatively, the compounds of this invention may be dissolved in saline, water, polyethylene glycol, propylene glycol, carboxymethyl cellulose colloidal solutions, ethanol, corn oil, peanut oil, cottonseed oil, sesame oil, tragacanth gum, and/or various buffers. Other adjuvants and modes of administration are well known in the pharmaceutical art. The carrier or diluent may include time delay

material, such as glyceryl monostearate or glyceryl distearate alone or with a wax, or other materials well known in the art.

In a preferred embodiment of the present invention, the angiotensinogen, AI, AI analogues, AI fragments and analogues thereof, AII, AII analogues, AII fragments and analogues thereof and AII AT_2 type 2 receptor agonist is administered topically. A suitable topical dose of active ingredient of angiotensinogen, AI, AI analogues, AI fragments and analogues thereof, AII, AII analogues, AII fragments and analogues thereof and/or AII AT_2 type 2 receptor agonists is 1 mg/ml administered twice daily. For topical administration, the active ingredient may comprise from 0.001% to 10% w/w, *e.g.*, from 1% to 2% by weight of the formulation, although it may comprise as much as 10% w/w, but preferably not more than 5% w/w, and more preferably from 0.1% to 1% of the formulation.

Formulations suitable for topical administration include liquid or semi-liquid preparations suitable for penetration through the skin (*e.g.*, liniments, lotions, ointments, creams, or pastes) and drops suitable for administration to the eye, ear, or nose.

The present invention, by providing a method for enhanced proliferation of HPC and HLSPC, will greatly increase the clinical benefits of HPC transplantation. This is true both for increased "self-renewal", which will provide a larger supply of HPC capable of reconstituting the entire hematopoietic system, and for proliferation with differentiation, which will provide a larger supply of lineage-specific progenitor cells, for more rapid reconstitution of mature, functioning blood cells. Similarly, methods that increase *in vivo* proliferation of HPC will enhance the utility of HPC transplantation therapy by rapidly increasing local concentrations of HPC (and

HLSPC) in the bone marrow, and thereby more rapidly producing functioning blood cells.

Similarly, methods that increase the proliferation of MSC and mesenchymal lineage-specific cells, will greatly increase the utility of MSC therapy in the repair of skeletal tissues such as bone, cartilage, tendon and ligament. More rapid production of large numbers of MSC and mesenchymal lineage-specific cells will permit more efficient delivery of high densities of these cells to a defect site and more rapid *in vivo* amplification in the local concentration of stem and lineage-specific cells at an appropriate repair site.

The method of the present invention also increases the potential utility of HPC and HLSPC as vehicles for gene therapy in hematopoietic disorders, as well as MSC and mesenchymal lineage-specific cells as vehicles for gene therapy in skeletal disorders by more efficiently providing a large number of such cells for transfection, and also by providing a more efficient means to rapidly expand transfected HPC, HLSPC, MSC and mesenchymal lineage-specific cells.

The present invention may be better understood with reference to the accompanying examples that are intended for purposes of illustration only and should not be construed to limit the scope of the invention, as defined by the claims appended hereto.

Example 1. Macrophage Differentiation: Phagocytosis

Resident peritoneal macrophages have very little phagocytic activity. Exposure of macrophages to inflammatory or activating agents will increase this macrophage function. Resident peritoneal macrophages were harvested from C57B1/6

mice or Sprague Dawley rats and resuspended at a concentration of 1 x 10^6 cells/ml in phosphate buffered saline (PBS). Five separate 0.5 ml cell aliquots were placed on a glass coverslip in a 35 mm petri dish. Prior to incubation, either 0.5 ml of PBS, AII, or AII analogues or fragments at between 1-1000 ug/ml final concentration was added to the individual cover slips. The dishes containing the cover slips were then incubated at 37°C for 4 hours, after which the cover slips were washed 3 to 6 times with PBS. Opsonized yeast particles (Sigma Chemical Co.) (yeast opsonized with adult serum from the same species as that under study) were added to the cover slips and incubated for 2 hours, after which the cover slips were again washed with PBS and inverted onto a glass slide. The number of macrophages that ingested yeast and the number of yeast per macrophage ingested was then determined microscopically. At least 100 macrophages per coverslip were counted. The data from this study are summarized in Tables 3 and 4 and Figures 1 and 2. Table 5 describes the AII analogues and fragments used in these studies.

Table 3. Effect of AII on the Phagocytic Capability of Murine Peritoneal Macrophages

	Concentration of AII (μg/ml)				
	0	1	10	100	1000
	# Macrophages with Yeast				
Yeast #					
0	100	100	100	100	100
1-3	1	1	3	10	20
4-6	0	0	4	10	20
7-9	0	0	0	0	0
% Ingesting	1	1	6.5	16.7	25.6
# Yeast/MO	0.01	0.01	0.24	0.58	1.00

Table 4. Effect of AII on the Phagocytic Capability of Rat Peritoneal Macrophages

(Peptide) µg/ml	Number of Macrophages with Yeast (Yeast Number) 0	1-3	4-6	≥7	% w/Yeast	#/Cell
AII-0	88	2	0	0	2.2	0.04
100	90	30	20	0	35.7	1.14
1000	50	54	40	25	70.4	2.86
AII(1-7)-0	90	3	0	0	3.2	0.06
100	100	21	5	0	20.6	0.53
1000	90	30	10	0	30.8	0.85
GSD22A-0	90	1	0	0	1.1	0.02
100	100	26	5	0	23.7	0.59
1000	100	22	1	0	18.7	0.40
GSD 24-B-0	100	2	0	0	2.0	0.04
100	100	25	5	0	23.1	0.58
1000	100	10	0	0	9.1	0.18
GSD28-0	100	1	0	0	1.0	0.02
100	100	1	0	0	1.0	0.02
1000	100	1	0	0	1.0	0.02

Table 5. Designation for Analogues

Name	Abbreviation	Sequence	SEQ ID NO:
GSD 28	Ile^8-AII	DRVYIHPI	SEQ ID NO:37
GSD 24B	Pro^3-AII	DRPYIHPF	SEQ ID NO:31
GSD 22A	Ala^4-AIII	RVYAHPF	SEQ ID NO:18
AII(1-7)		DRVYIHP	SEQ ID NO:4

Exposure to 10 µg/ml or greater AII tremendously increased the phagocytic capability of peritoneal macrophages. Less than 1% of cells were phagocytic in the resident population (0.01 yeast per cell observed). After exposure to AII this increased to over 25% phagocytic at the highest concentration with on average 1 yeast observed per macrophage (25 fold increase in the number of macrophages able to phagocytose and a 100-fold increase in the number of particles phagocytized).

As shown in Table 4, both concentrations of the peptides tested (with the exception of GSD 28) elevated that phagocytic capability of rat macrophages. However, none of the analogues resulted in the magnitude of an effect observed with AII. This suggests that AII and, to a lesser extent, AII analogues will stimulate macrophage differentiation to an elicited or activated state, which leads to the ingestion and clearance of bacteria and cellular debris.

Example 2 Leukocyte Differentiation: Respiratory Burst

The respiratory burst of leukocytes (macrophages and polymorphonuclear neutrophils) is one component of the mediator system used to kill bacteria. As with phagocytosis, the level of this respiratory burst activity in resident macrophages is low. With differentiation, either to an elicited (inflammatory) or activated state, this functional activity is significantly elevated. Studies were conducted to assess the effect of in vitro exposure of murine or rat peritoneal macrophages and human peripheral blood mononuclear cells (PBMC) to various concentrations of AII on the capacity to generate hydrogen peroxide via the respiratory burst system. For the human studies, five different donors were examined.

The murine or rat peritoneal cells were harvested by lavage with 5-15 ml of cold PBS with 0.5% bovine serum albumin. The human PBMC were harvested by venipuncture from normal human volunteers and isolated from peripheral blood by Ficoll Hypaque density centrifugation. After isolation, the cells were resuspended at 1 x 10^6 cells/ml and placed at 100 µl per well into 96 well plates. The cells were incubated with various concentrations of AII or AII analogues and fragments for 4 hours at 37°C. The cells were then preloaded with a fluorescent probe for hydrogen

peroxide, 2,7 dichlorofluorescein acetate, which is nonfluorescent in the absence of hydrogen peroxide. Fifteen minutes later, 10 ng/ml of phorbol myristate acetate (+PMA) or PBS (-PMA) was added to stimulate the production of hydrogen peroxide. One hour after stimulation the level of fluorescence produced was measured on a Cytofluor 2350 multiwell fluorometer. Representative results from this study are shown in Figures 3-8.

In the absence of PMA or peptide, no hydrogen peroxide production is observed. Some variability in the response to AII was seen (i.e. the concentration of AII necessary to increase the level of this function); however, in all studies AII increased the ability of leukocytes to generate hydrogen peroxide both alone and in response to stimulation with PMA. Further, the effect of pre-exposure to analogues and fragments of AII (GSD 22A, GSD 24B, GSD 28 and AII(1-7)) on the respiratory burst activity of PBMC was assessed (Figures 5-8). For all analogues and fragments, a much higher concentration of the peptide was needed to increase the respiratory burst activity. Up to 100 times more of these analogues were necessary; however, an increase in the respiratory burst capacity was observed for all analogues tested. The analogues were able to stimulate that function both in the presence and absence of PMA. These data indicate that AII was able to stimulate the differentiation of monocytes/macrophages from three species.

Example 3. Proliferative response of human lymphocytes

Upon stimulation of lymphocytes with mitogen or antigen, these cells undergo blastogenesis and proliferation. In the absence of such stimuli, proliferation is seldom observed. One method to measure cellular proliferation in a short term assay is via

measurement of the amount of the nucleotide thymidine that is incorporated into newly synthesized DNA. The effect of AII on the proliferation of human PBMC in the presence and absence of pokeweed mitogen ("PWM") was assessed.

Human PBMC were collected from normal volunteers and isolated via Ficoll Hypaque (Sigma Chemical, St. Louis) density centrifugation. After isolation of the buffy coat, the cells were washed 3X to remove the Ficoll Hypaque, counted in trypan blue (0.01%) and resuspended at a concentration of 1 x 10^6 cells/ml in RPMI 1640 containing 10% fetal calf serum and antibiotics. A 100 µl aliquot of cells was added to each well. Thereafter, various concentrations (0.1 to 1000 µg/ml final concentration) of AII in RPMI 1640 containing 10% FCS and antibiotics were added to various wells in triplicate. To the appropriate wells, PWM (20 µg/ml final concentration) was added. These plates were incubated at 37°C in 5% CO_2 for 48 hours. Subsequently, 0.5 µCi of ^{3}H-thymidine was added to each well, which were incubated at 37°C for an additional 24 hours prior to harvest by a multiwell automated sample harvester (Skatron) onto glass fiber filters. These filters were dried, placed in scintillation fluid and the amount of thymidine incorporated was determined by beta counting. The results are shown in Figure 9.

In the absence of mitogen, no increase in thymidine incorporation was observed after exposure to AII. However, in two separate experiments (cells from 2 different donors) AII was shown to increase the amount of thymidine incorporated 50% to 100% in response to PWM. These data show that AII is able to increase the proliferation of cells from the hematopoietic lineage (e.g. lymphocytes).

Example 4 Rat Mesenchymal Stem Cell Proliferation

These studies were conducted to determine the effect that AII would have on the proliferation of MSC. Bone marrow cells were harvested from the femur and tibia of female Sprague Dawley rats by flushing the bones with Dulbecco's Minimal Essential Medium-High Glucose ("DMEM-HG") with a syringe and an 18 gauge needle. These cells were cultured in 24 well plates at 5 x 10^3 cells/mm^2 in DMEM-HG containing selected lots of fetal calf serum (FCS) and antibiotics (complete medium) at 37°C incubator containing 5% CO_2 in air. Twenty-four hours after the initiation of the cultures the medium and nonadherent cells were aspirated and fresh medium was added. To each of these several wells, complete medium with (1 to 100 μg/ml) or without AII was added. The migration of cells from the clones and their proliferation was followed by microscopic examination. Every 4 days the old medium was removed and fresh medium was added to the cultures.

The data from these experiments are shown in Figures 10 and 11. Addition of AII to the cultures significantly increased the number of sites from which the MSC were migrating (CFU) and the size (number of cells) of the colonies formed. This occurred only in the presence of serum that in itself would support MSC growth, albeit to a lesser extent. As can be seen, AII caused an increase in the number of MSC in a concentration dependent manner at all time points examined in the presence of two different serum lots. These data support the hypothesis that AII can increase the proliferation of rat MSC.

Example 5 Effect of Angiotensin II on Murine Progenitor Cells

HPC were harvested from C57B1/6 mice by immunomagnetic affinity chromatography and placed in semi-solid cultures with optimal growth medium. At various times after initiation of culture, the formation of colonies and the size of the colonies (number of cells/colony) were assessed microscopically.

Female C57B1/6 mice were purchased from Simonson and used as a source of bone marrow cells in this study. The bone marrow was harvested, either from untreated mice or from mice injected with 5-fluorouracil (5-FU) (3 mg/mouse; approximately 150 mg/kg) in the tail vein 48 hours before harvest, from the femur and tibia of mice by flushing with phosphate buffered saline (PBS), pH 7.4, containing 2% fetal bovine serum (FBS) with a 21-gauge needle. The eluant from the flushing was centrifuged and the pellet was resuspended at 4 x 10^7 nucleated cells/ml in PBS containing PBS containing 2% FBS and 5% normal rat serum.

The reagents for immunomagnetic labeling were purchased from Stem Cell Technologies, Inc. (Vancouver, BC). Biotin-labeled monoclonal antibodies to the following murine lineage-specific cell surface antigens were included in a cocktail for HPC enrichment and used according to the manufacturer's instructions: CD5 (Ly-1), CD45-R (B220), CD11b (Mac-1), Myeloid Differentiation Antigen (Gr-1) and Erythroid Cells (TER 119) Ten µl of antibody cocktail was added for each of the 2 sets of bone marrow (normal and 5-FU-treated), mixed and allowed to incubate at 4°C for 15 minutes. The cells were then resuspended at 4 x 10^7 cells/ml in PBS containing 2% FBS. The antibody cocktail was then washed out and 100 µl anti-biotin tetramer was added for each ml of cells. The suspension was mixed and incubated at 4°C for

15 minutes. Sixty µl of magnetic colloid was then added for each ml of cells, the combination was mixed and incubated at 4°C for 15 minutes to yield the immunomagnetically-labeled bone marrow cells.

A column containing a stainless steel matrix was prepared by washing the matrix with PBS followed by washing with PBS containing 2% protein. The immunomagnetically-labeled bone marrow cells were loaded onto the column and unlabeled cell-containing medium (enriched HPC) was collected in the flow through fraction at a flow rate of 0.2 ml/minute. Medium was added to the top of the column so that it did not run dry until 8 to 10 ml of enriched HPC were harvested. Approximately 2% of the cells loaded onto the column were isolated in the enriched HPC fractions.

The enriched HPC cell fractions were diluted into a semi-solid medium containing 0.9% methylcellulose in alpha minimal essential medium (alpha MEM), 30% fetal calf serum, 1% bovine serum albumin, 10^{-4} M 2-mercaptoethanol, 2 mM L-glutamine, and 2% conditioned medium containing colony stimulating factors. The conditioned medium was supernatant from splenocyte cultures (1 x 10^6 cells/ml) incubated for 48 hours in RPMI 1640 containing 10 µg/ml pokeweed mitogen (PWM), 10% FCS, and antibiotics. Various concentrations of AII, between 0 and 100 µg/ml were added in a small volume to the wells of microtiter dishes, to which between 2 x 10^4 cells/ml for the normal and 2.5 x 10^4 cells/ml for the 5-FU treated cells. The cells were incubated at 37°C and 5% CO_2 for 14 days. At day 14 only, macroscopic cell colonies were observed in the wells containing enriched HPC from untreated (normal) mice treated with 10 µg/ml (18 macroscopic colonies) and 100

μg/ml AII (100 macroscopic colonies). Microscopic evaluation of the cells was performed at various days after initiation of incubation, and the results are summarized in Figures 12-17.

Figures 12-14 and 16 represent the number of colonies containing more than a certain number of cells/colony as a function of the duration and concentration of AII exposure (Figures 12-14 for normal cells; Figure 16 for 5-FU treated cells.) Figures 15 and 17 represent the number of cells per colony seen after incubation of from normal (Figure 15) or 5-FU treated (Figure 17) mice with various concentrations of AII as a function of time. The results clearly demonstrate that HPC colony size increases proportionately with exposure to increased concentrations of AII, and thus that AII increases HPC proliferation.

Example 6. Effect of AII Analogues and Fragments on Rat Mesenchymal Stem Cell Proliferation

These studies were conducted to determine the effect that inclusion of AII analogues and fragments in the cell culture of MSC would have on the proliferation of these cells. Bone marrow cells were harvested from the femur and tibia of female Sprague Dawley rats by flushing the bones with Dulbecco's Minimal Essential Medium-High Glucose ("DMEM-HG") with a syringe and an 18 gauge needle. These cells were cultured in 24 well plates at 5 x 10^3 cells/mm^2 in DMEM-HG containing selected lots of fetal calf serum (FCS) and antibiotics (complete medium) at 37°C incubator containing 5% CO_2 in air. Twenty-four hours after the initiation of the cultures the medium and nonadherent cells were aspirated and fresh medium was added. To each of these several wells, complete medium with (1 to 100 μg/ml) or

without AII analogues and fragments (see Table 5) was added. The migration of cells from the clones and their proliferation was followed by microscopic examination. Every 4 days the old medium was removed and fresh medium was added to the cultures.

Addition of AII analogues or AII fragments to these cultures had a profound effect on the number of sites from which MSC were migrating (CFU) and the size (number of cells) of the colonies formed. The results from these studies can be seen in Figures 18-21. As can be seen, AII analogues and fragments caused an increase in the number of MSC at all time points examined. These data indicate that AII analogues and fragments can increase the proliferation of rat MSC.

Example 7. Differentiation of MSC that have undergone proliferation in the presence of AII.

Mesenchymal stem cells isolated from bone marrow and grown under appropriate conditions can express characteristics of multiple cell types, including cells involved in the generation of bone, cartilage, muscle and tendons. Osteogenic cells (cells that can form bone tissue) express the enzyme alkaline phosphatase when cultured in medium that drives them toward their osteogenic differentiation.

Bone marrow from female Sprague Dawley rats were harvested by flushing the femur with medium. The cells were placed in culture dishes 9 cm^2 in diameter, allowed to adhere overnight, and then placed in DMEM-LG medium containing antibiotics and 10% fetal calf serum together with varying concentrations of AII. At various times after culture initiation, the cells were washed with Tyrode's buffer and placed in osteogenic medium (DMEM-LG containing 10% fetal calf serum, 100 nM

dexamethasone and 0.05 mM ascorbic acid) for 4 days prior to assessment of the level of alkaline phosphatase activity per well. Briefly, the wells were washed with Tyrode's buffer and 1 ml alkaline phosphatase substrate solution (50 mM glycine, pH 10.5, containing 1 mM magnesium chloride and 2.5 mM p-nitrophenyl phosphate) to each well. Fifteen minutes after addition of this aqueous substrate, the buffer was removed from the culture and mixed with 1 ml of 1N sodium hydroxide to stop the reaction. The absorbance of the resultant mixture at 405 nm was then determined via spectroscopy. The level of alkaline phosphatase activity is expressed as the level of absorbance per culture dish. These data are shown in Figure 22 and demonstrate that AII can accelerate the proliferation of cells that express alkaline phosphatase when placed in medium appropriate to induce osteogenic differentiation.

Example 8. Induction of mesenchymal stem cell differentiation by AII

Studies have also shown that exposure of murine bone marrow stem cells to AII increases the formation of colonies in the absence of exogenous colony stimulating factors. As these factors are necessary for the formation of colonies, these data suggested that AII may have stimulated non-hematopoietic cells (i.e.: mesenchymal cells) to release the necessary colony stimulating factors. This was further confirmed by data from cultures of further purified hematopoietic progenitor cells. After further purification of lineage-negative bone marrow cells to those that express the protein Sca1, which eliminates mesenchymal cells from the culture, AII increased the number and size of colonies formed only in the presence of exogenous colony stimulating factors (U.S. Application Serial No. 09/012,400, hereby incorporated by reference in its entirety).

One factor whose production is induced during differentiation of mesenchymal stromal cells is granulocyte-macrophage-colony stimulating factor (GM-CSF). Therefore, the effect of AII on the differentiation of mesenchymal cells was assessed by the production of colony stimulating factors, specifically GM-CSF, into the culture supernatant.

Bone marrow cells were harvested from the femur and tibia of C57B1/6 mice (Battin and Kingdom, Gilroy, CA) with saline containing 2% fetal calf serum. Thereafter, the red blood cells were lysed by brief exposure to a hypotonic ammonium chloride solution. The nucleated cells were then resuspended in 3 ml of 5X phosphate buffered saline (PBS; pH 7.2) and rapidly layered over 7 ml of Percoll (70% Percoll gradient separation). This combination of nucleated bone marrow cells and Percoll were centrifuged at 1800 rpm for 20 minutes. After centrifugation, the cells in the upper 25% of the Percoll gradient were harvested and washed 3 times with PBS to remove excess Percoll. The cells were then resuspended at 4 x 10^5 cells/ml in alpha minimal essential medium containing 10% horse serum, 10% fetal calf serum and 5 x 10^{-5} M 2-mercaptoethanol. These cells were cultured overnight in 24-well plates to allow adherence to the tissue culture plastic. Thereafter, the non-adherent cells were removed by washing with PBS and the medium was replaced with fresh medium containing various concentrations of angiotensin II (AII) (0.01- 100 μg/ml). Twenty four, 48, or 72 hours after addition of AII, supernatants were harvested and frozen until the time of assay.

After all samples were collected, the level of GM-CSF in the supernatant was measured by Quantikine ELISA (R & D Systems, Minneapolis, MN) according to the manufacturer's instructions. As shown by the data shown in Figure 23, exposure to

AII at concentrations ranging from 0.01 to 100 μg/ml induced an increase in the production of GM-CSF by murine mesenchymal stem cells. These data demonstrate that AII can induce mesenchymal stem cell differentiation.

We claim:

1. A method of accelerating the differentiation of mesenchymal stem and lineage-specific cells comprising contacting the mesenchymal stem and lineage-specific cells with an amount effective to accelerate differentiation of mesenchymal stem and lineage-specific cells of at least one active agent comprising a sequence consisting of at least three contiguous amino acids of groups R^1-R^8 in the sequence of general formula I

$$R^1\text{-}R^2\text{-}R^3\text{-}R^4\text{-}R^5\text{-}R^6\text{-}R^7\text{-}R^8$$

in which R^1 and R^2 together form a group of formula

$$X\text{-}R^A\text{-}R^B\text{-},$$

wherein X is H or a one to three peptide group and a peptide bond between R^A and R^B is labile to aminopeptidase A cleavage;

R^3 is selected from the group consisting of Val, Ala, Leu, norLeu, Ile, Gly, Pro, Aib, Acpc and Tyr;

R^4 is selected from the group consisting of Tyr, Tyr$(PO_3)_2$, Thr, Ser, homoSer and azaTyr;

R^5 is selected from the group consisting of Ile, Ala, Leu, norLeu, Val and Gly;

R^6 is His, Arg or 6-NH_2-Phe;

R^7 is Pro or Ala; and

R^8 is selected from the group consisting of Phe, Phe(Br), Ile and Tyr, excluding sequences including R^4 as a terminal Tyr group.

2. The method of claim 1 wherein the active agent is selected from the group consisting of AII, AIII, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:31, and SEQ ID NO: 37.

3. The method of claim 1 wherein the concentration of active agent is between about 0.01 ng/kg and about 10.0 mg/kg.

4. An improved cell culture medium for growth of mesenchymal stem and lineage-specific cells, wherein the improvement comprises addition to the cell culture medium of an amount effective to accelerate differentiation of mesenchymal stem and lineage-specific cells of at least one active agent comprising a sequence consisting of at least three contiguous amino acids of groups R^1-R^8 in the sequence of general formula I

$$R^1\text{-}R^2\text{-}R^3\text{-}R^4\text{-}R^5\text{-}R^6\text{-}R^7\text{-}R^8$$

in which R^1 and R^2 together form a group of formula

$$X\text{-}R^A\text{-}R^B\text{-},$$

wherein X is H or a one to three peptide group and a peptide bond between R^A and R^B is labile to aminopeptidase A cleavage;

R^3 is selected from the group consisting of Val, Ala, Leu, norLeu, Ile, Gly, Pro, Aib, Acpc and Tyr;

R^4 is selected from the group consisting of Tyr, Tyr$(PO_3)_2$, Thr, Ser, homoSer and azaTyr;

R^5 is selected from the group consisting of Ile, Ala, Leu, norLeu, Val and Gly;

R^6 is His, Arg or 6-NH_2-Phe;

R^7 is Pro or Ala; and

R^8 is selected from the group consisting of Phe, Phe(Br), Ile and Tyr, excluding sequences including R^4 as a terminal Tyr group.

5. The improved cell culture medium of claim 5 wherein the active agent is selected from the group consisting of AII, AIII, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:31, and SEQ ID NO: 37.

6. The improved cell culture medium of claim 4 wherein the concentration of active agent is between about 0.01 ng/ml and about 10.0 mg/ml.

7. A kit for the differentiation of mesenchymal stem and lineage-specific cells, comprising:

(a) an amount effective to accelerate differentiation of mesenchymal stem and lineage-specific cells of at least one active agent comprising a sequence consisting of at least three contiguous amino acids of groups R^1-R^8 in the sequence of general formula I:

$$R^1\text{-}R^2\text{-}R^3\text{-}R^4\text{-}R^5\text{-}R^6\text{-}R^7\text{-}R^8$$

in which R^1 and R^2 together form a group of formula

$$X\text{-}R^A\text{-}R^B\text{-},$$

wherein X is H or a one to three peptide group and a peptide bond between R^A and R^B is labile to aminopeptidase A cleavage;

R^3 is selected from the group consisting of Val, Ala, Leu, norLeu, Ile, Gly, Pro, Aib, Acpc and Tyr;

R^4 is selected from the group consisting of Tyr, $Tyr(PO_3)_2$, Thr, Ser, homoSer and azaTyr;

R^5 is selected from the group consisting of Ile, Ala, Leu, norLeu, Val and Gly;

R^6 is His, Arg or 6-NH_2-Phe;

R^7 is Pro or Ala; and

R^8 is selected from the group consisting of Phe, Phe(Br), Ile and Tyr, excluding sequences including R^4 as a terminal Tyr group; and

(b) instructions for using the amount effective of active agent as a cell culture medium supplement.

8. The kit of claim 7 further comprising cell growth medium.

9. The kit of claim 7 further comprising a sterile container.

10. The kit of claim 7 wherein the active agent is selected from the group consisting of AII, AIII, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:31, and SEQ ID NO: 37.

11. The kit of claim 7 wherein the concentration of active agent is between about 0.01 ng/ml and about 10.0 mg/ml.

12. A method of accelerating the differentiation of mesenchymal stem and lineage-specific cells comprising contacting the mesenchymal stem and lineage-specific cells with an amount effective to accelerate differentiation of mesenchymal stem and lineage-specific cells of at least one active agent comprising a sequence

consisting of at least three contiguous amino acids of groups R^2-R^8 in the sequence of general formula II

$$R^2\text{-}R^3\text{-}R^4\text{-}R^5\text{-}R^6\text{-}R^7\text{-}R^8$$

in which R^2 is selected from the group consisting of H, Arg, Lys, Ala, Orn, Ser(Ac), Sar, D-Arg and D-Lys;

R^3 is selected from the group consisting of Val, Ala, Leu, norLeu, Ile, Gly, Pro, Aib, Acpc and Tyr;

R^4 is selected from the group consisting of Tyr, $Tyr(PO_3)_2$, Thr, Ser, homoSer and azaTyr;

R^5 is selected from the group consisting of Ile, Ala, Leu, norLeu, Val and Gly;

R^6 is His, Arg or 6-NH_2-Phe;

R^7 is Pro or Ala; and

R^8 is selected from the group consisting of Phe, Phe(Br), Ile and Tyr.

13. The method of claim 12 wherein the active agent is selected from the group consisting of SEQ ID NO:2, SEQ ID NO:17, and SEQ ID NO:18.

14. The method of claim 12 wherein the concentration of active agent is between about 0.01 ng/ml and about 10.0 mg/ml.

15. An improved cell culture medium for growth of mesenchymal stem and lineage-specific cells, wherein the improvement comprises addition to the cell culture medium of an amount effective to accelerate differentiation of mesenchymal stem and lineage-specific cells of at least one active agent comprising a sequence consisting of

at least three contiguous amino acids of groups R^2-R^8 in the sequence of general formula II

$$R^2\text{-}R^3\text{-}R^4\text{-}R^5\text{-}R^6\text{-}R^7\text{-}R^8$$

in which R^2 is selected from the group consisting of H, Arg, Lys, Ala, Orn, Ser(Ac), Sar, D-Arg and D-Lys;

R^3 is selected from the group consisting of Val, Ala, Leu, norLeu, Ile, Gly, Pro, Aib, Acpc and Tyr;

R^4 is selected from the group consisting of Tyr, $Tyr(PO_3)_2$, Thr, Ser, homoSer and azaTyr;

R^5 is selected from the group consisting of Ile, Ala, Leu, norLeu, Val and Gly;

R^6 is His, Arg or 6-NH_2-Phe;

R^7 is Pro or Ala; and

R^8 is selected from the group consisting of Phe, Phe(Br), Ile and Tyr.

16. The improved cell culture medium of claim 15 wherein the active agent is selected from the group consisting of SEQ ID NO:2, SEQ ID NO:17, and SEQ ID NO:18.

17. The improved cell culture medium of claim 15 wherein the concentration of active agent is between about 0.01 ng/ml and about 10.0 mg/ml.

18. A kit for the differentiation of mesenchymal stem and lineage-specific cells, comprising:

(a) an amount effective to accelerate differentiation of mesenchymal stem and lineage-specific cells of at least one active agent comprising a sequence

consisting of at least three contiguous amino acids of groups R^2-R^8 in the sequence of general formula II

$$R^2\text{-}R^3\text{-}R^4\text{-}R^5\text{-}R^6\text{-}R^7\text{-}R^8$$

in which R^2 is selected from the group consisting of H, Arg, Lys, Ala, Orn, Ser(Ac), Sar, D-Arg and D-Lys;

R^3 is selected from the group consisting of Val, Ala, Leu, norLeu, Ile, Gly, Pro, Aib, Acpc and Tyr;

R^4 is selected from the group consisting of Tyr, $Tyr(PO_3)_2$, Thr, Ser, homoSer and azaTyr;

R^5 is selected from the group consisting of Ile, Ala, Leu, norLeu, Val and Gly;

R^6 is His, Arg or 6-NH_2-Phe;

R^7 is Pro or Ala; and

R^8 is selected from the group consisting of Phe, Phe(Br), Ile and Tyr; and

(b) instructions for using the amount effective of active agent as a cell culture medium supplement.

19. The kit of claim 18 further comprising cell growth medium.

20. The kit of claim 18 further comprising a sterile container.

21. The kit of claim 18 wherein the active agent is selected from the group consisting of SEQ ID NO:2, SEQ ID NO:17, and SEQ ID NO:18.

22. The kit of claim 18 wherein the concentration of active agent is between about 0.01 ng/ml and about 10.0 mg/ml.

FIG. 1 EFFECT OF AII ON PHAGOCYTIC CAPABILITY OF MURINE MACROPHAGES

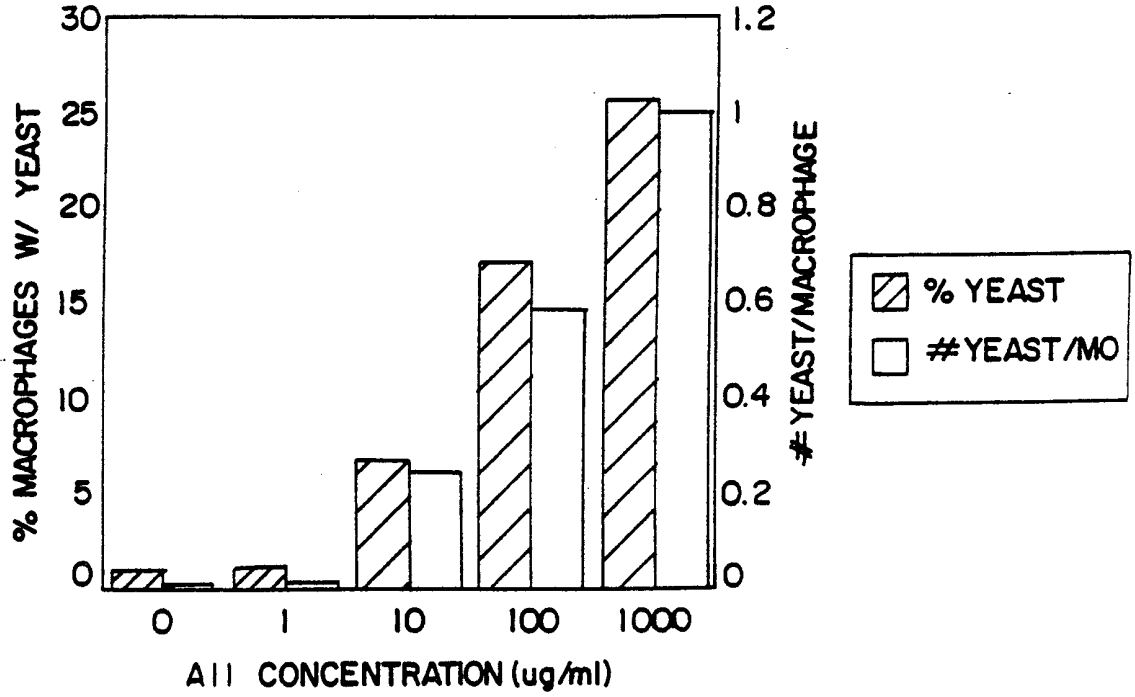


FIG. 2 EFFECT OF AII ON PHAGOCYTIC CAPABILITY OF RAT MACROPHAGES

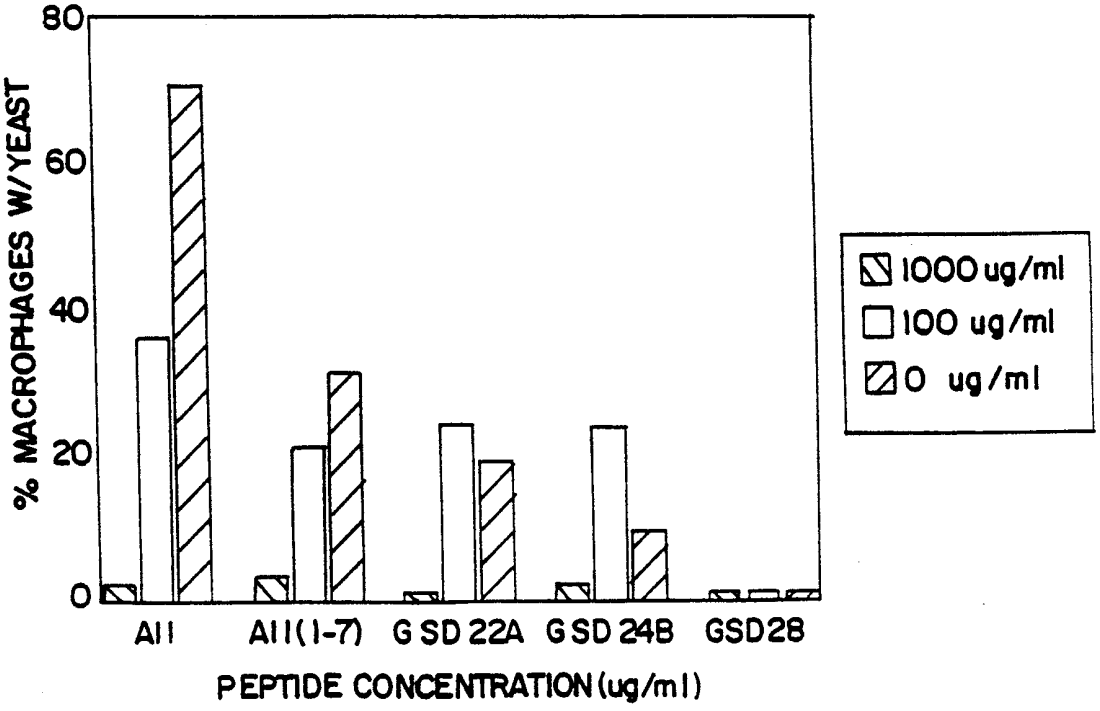


FIG. 3 EFFECT OF ANGIOTENSIN II ON RESPIRATORY BURST FUNCTION IN RAT PERITONEAL MACROPHAGES

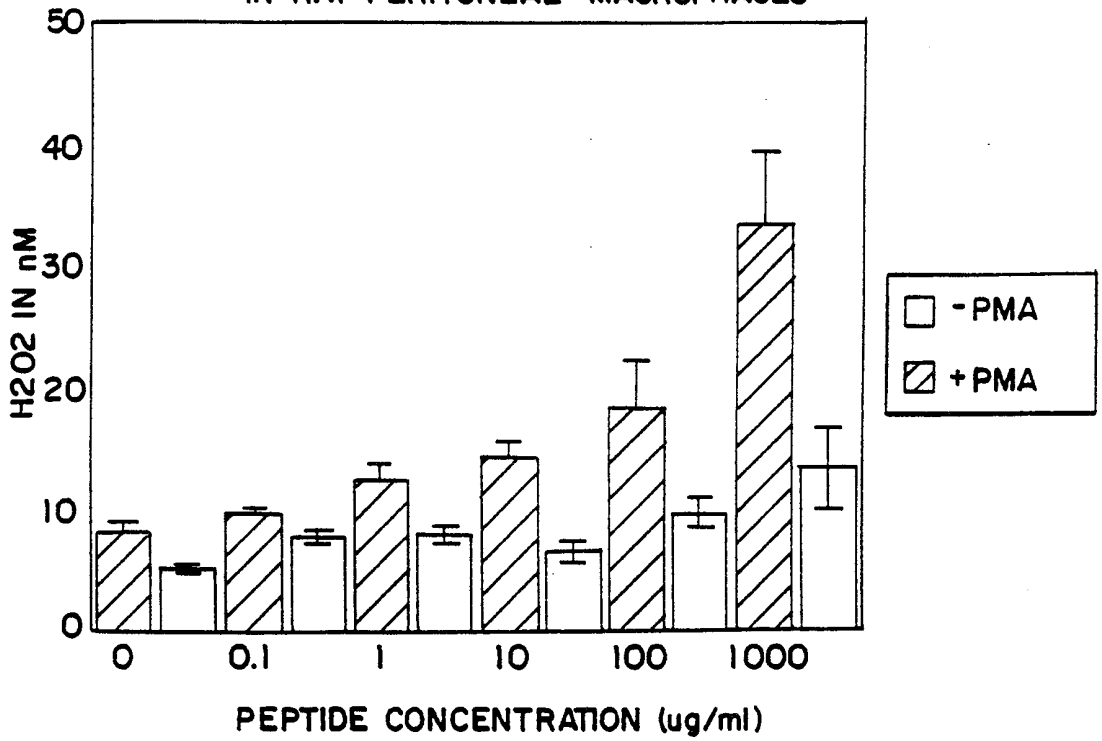


FIG. 4 EFFECT OF ANGIOTENSIN ON RESPIRATORY BURST FUNCTION IN HUMAN PBMC

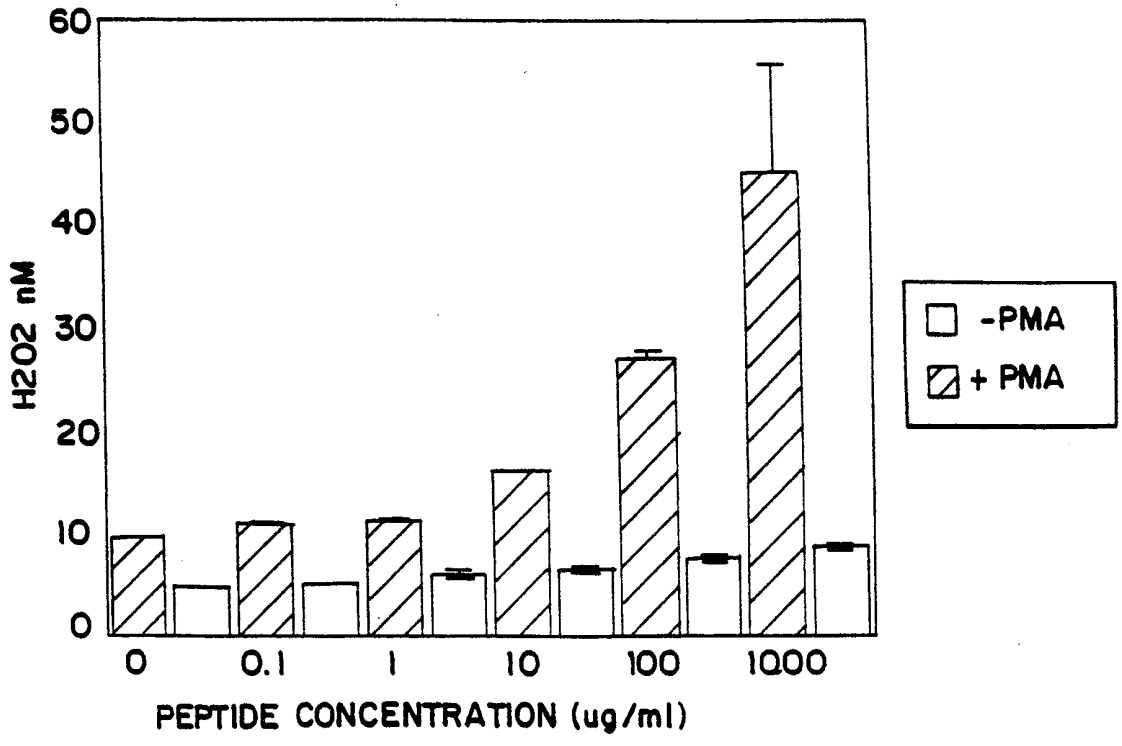


FIG. 5 EFFECT OF ANGIOTENSIN 1-7 ON RESPIRATORY BURST FUNCTION IN RAT PERITONEAL MACROPHAGE

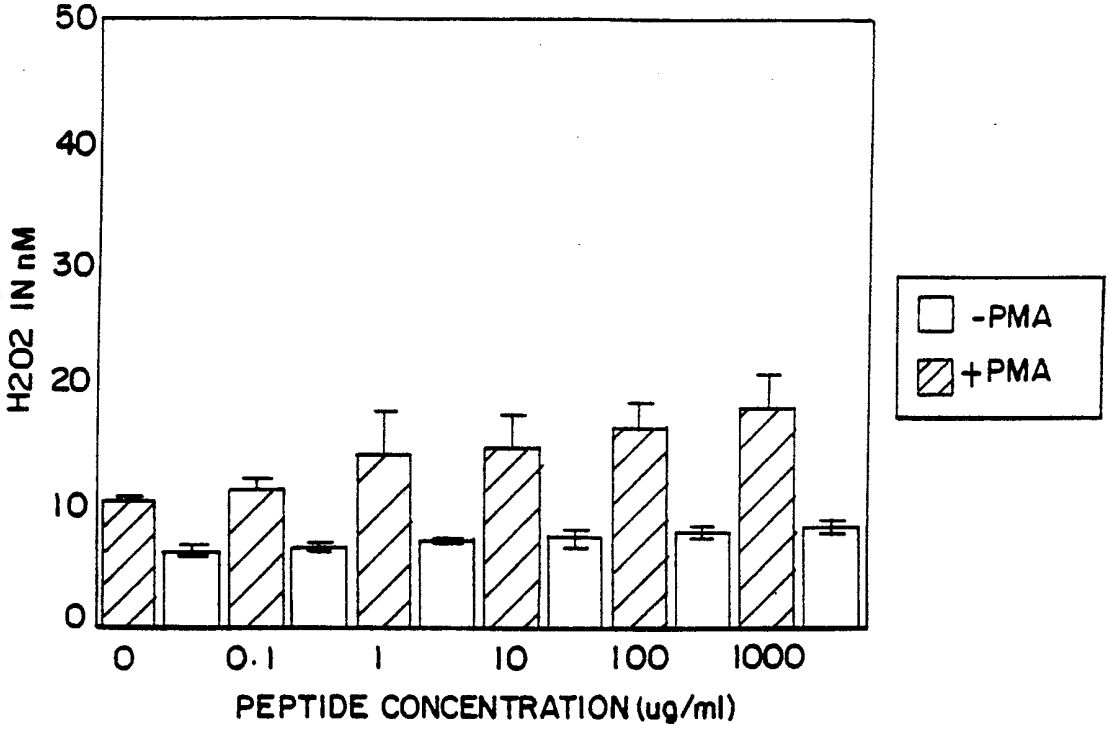


FIG. 6 EFFECT OF GSD 248 ON RESPIRATORY BURST FUNCTION IN RAT PERITONEAL MACROPHAGE

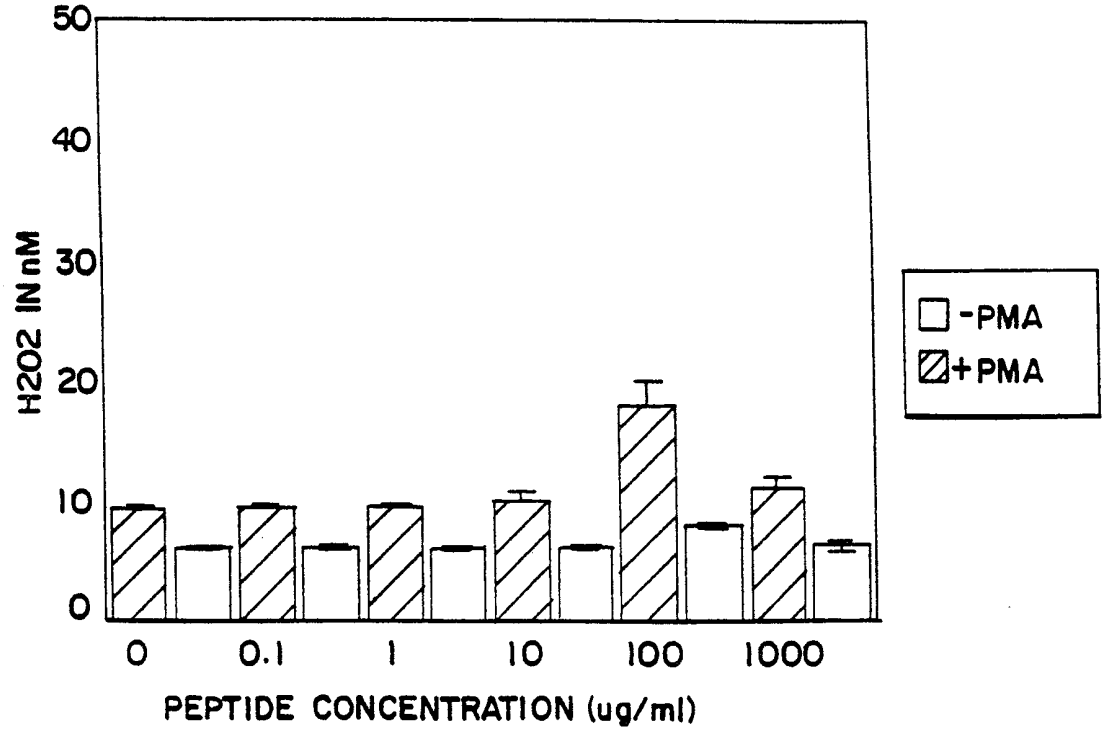


FIG. 7 EFFECT OF GSD 22A ON RESPIRATORY BURST FUNCTION IN RAT PERITONEAL MACROPHAGES

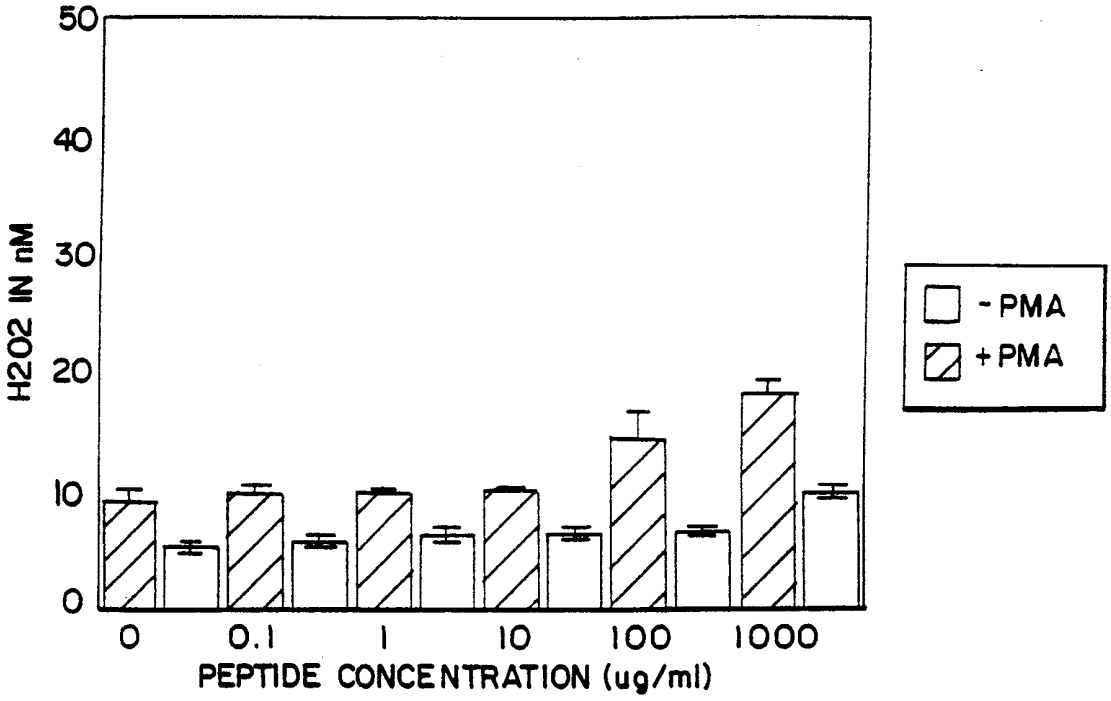


FIG. 8 EFFECT OF GSD 28 ON RESPIRATORY BURST FUNCTION IN RAT PERITIONEAL MACROPHAGE

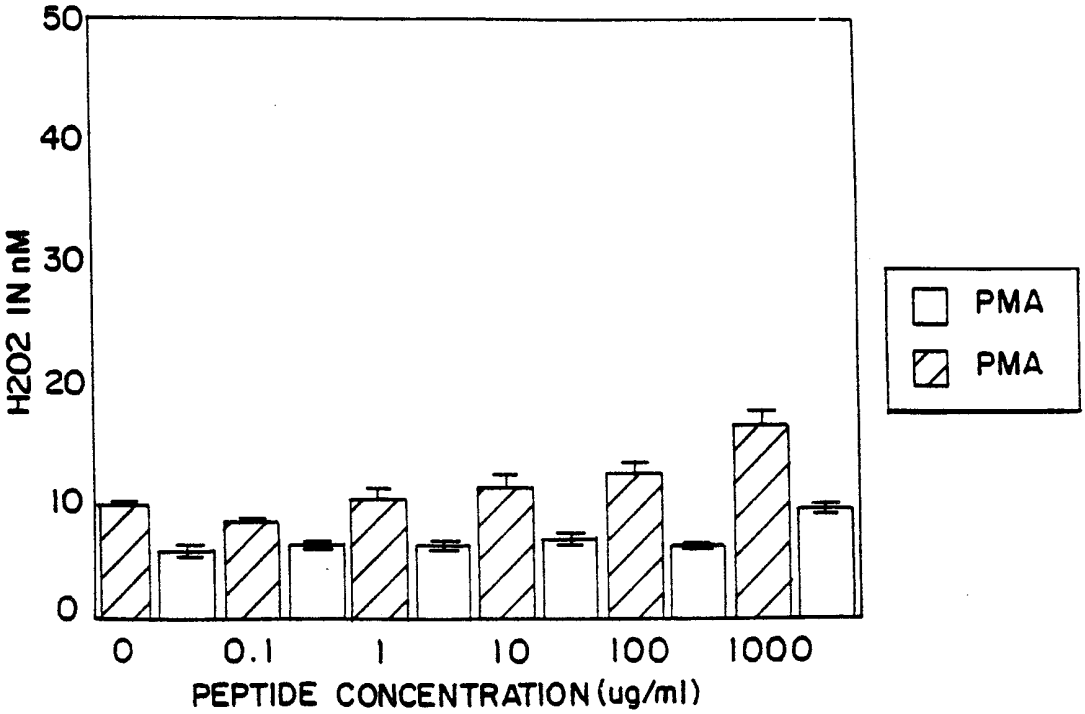


FIG. 9

EFFECT OF AII ON PROLIFERATION IN RESPONSE TO POKEWEED MITOGEN

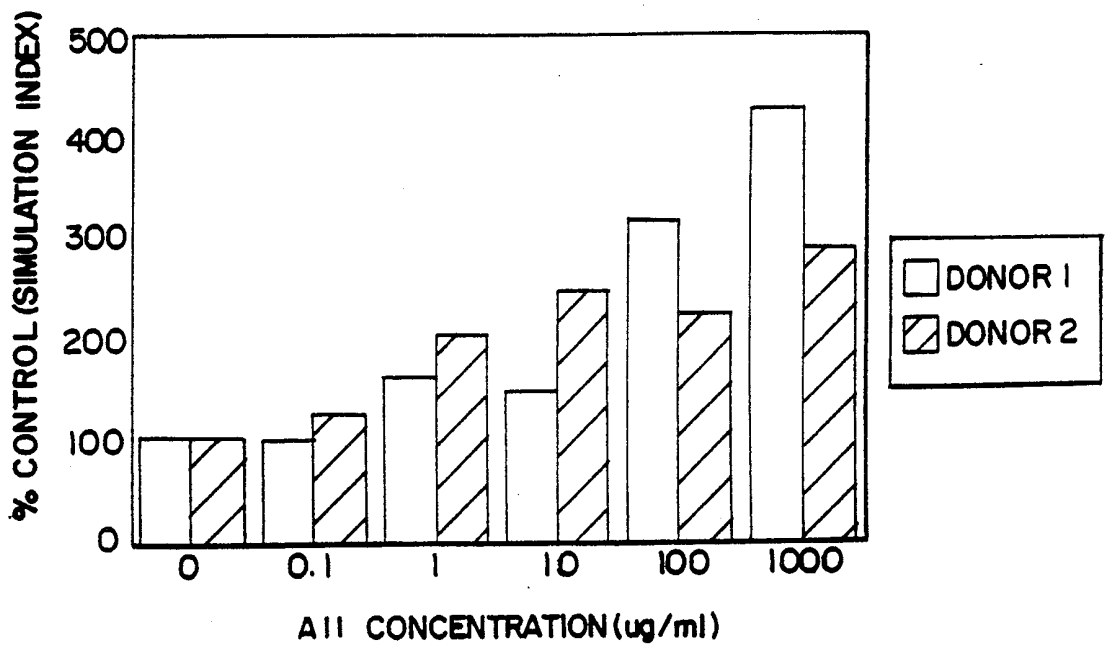


FIG. 10

EFFECT OF AII ON RAT BONE MARROW CULTURES SERUM 3

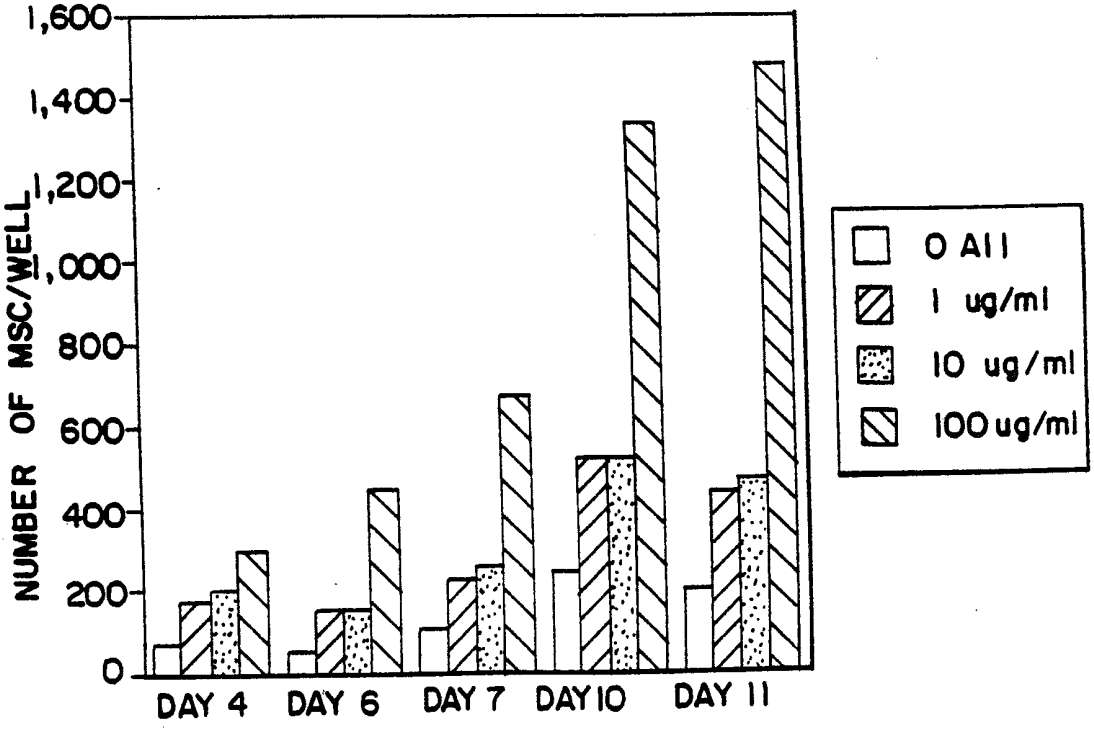


FIG. 11

EFFECT OF AII ON RAT BONE MARROW CULTURES
SERUM 3 9/5/97 CULTURE

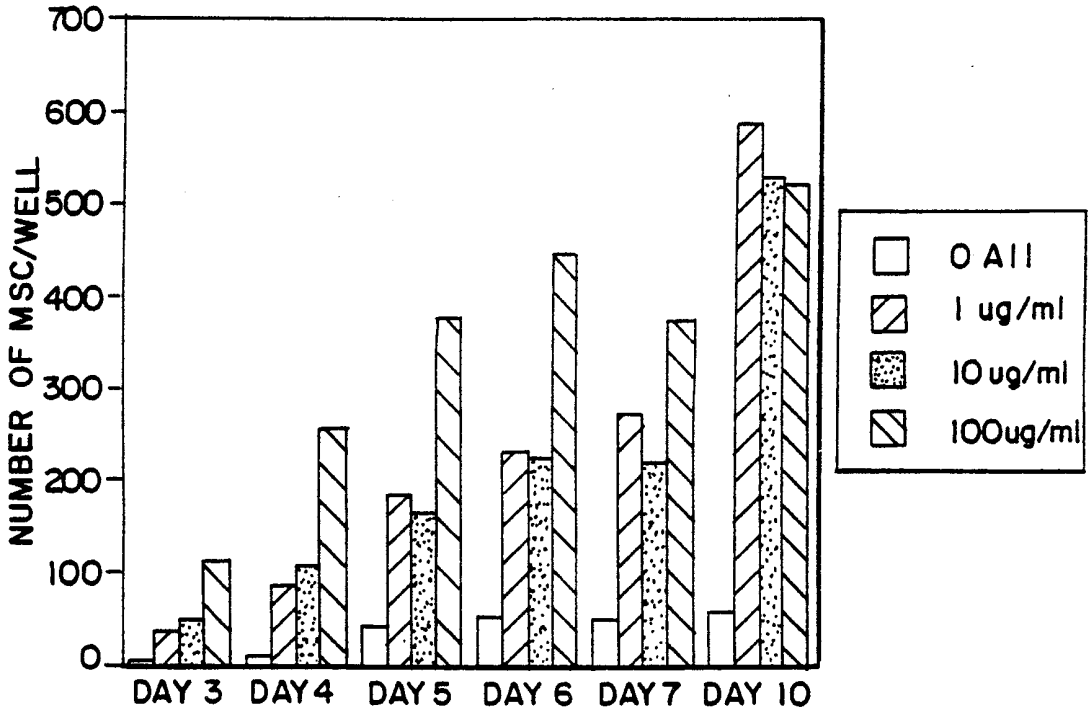
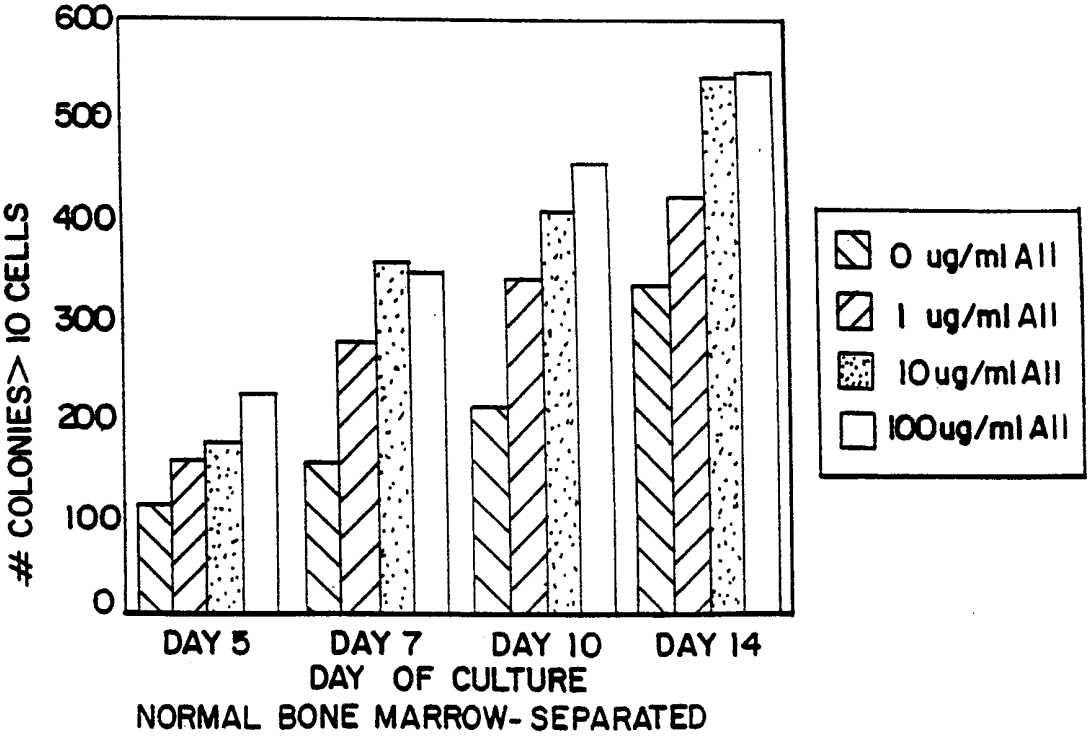


FIG. 12

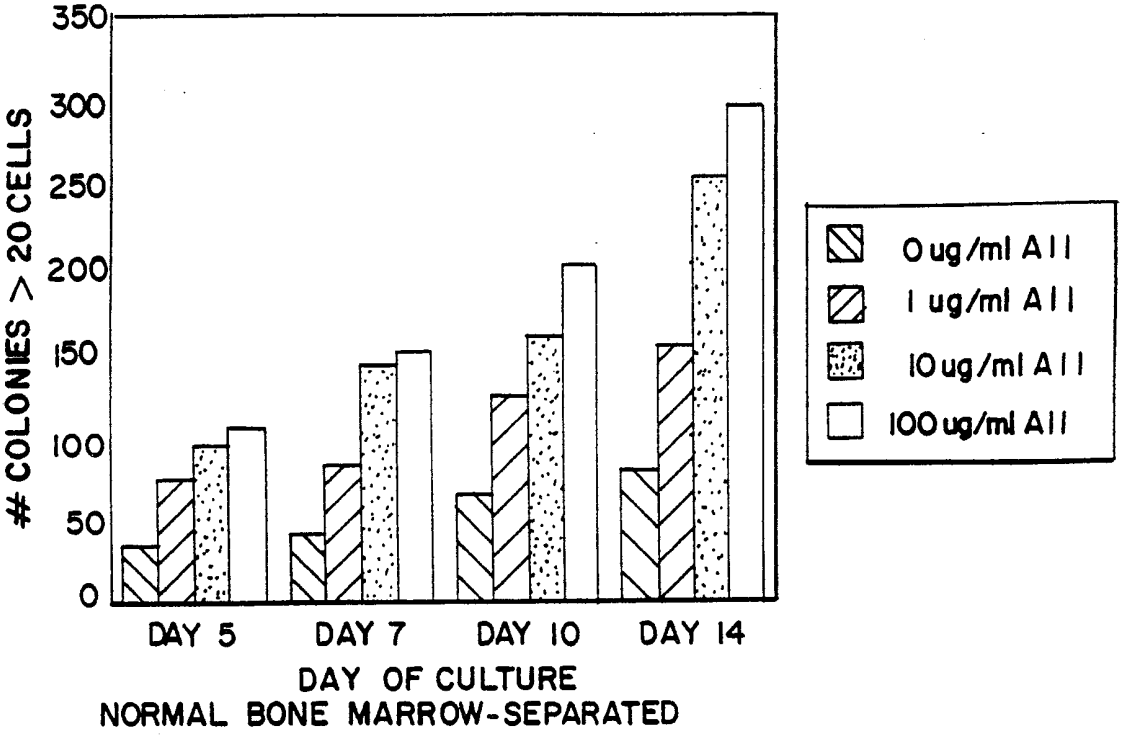
EFFECT OF ANGIOTENSIN II ON MURINE HSC CULTURES



NORMAL BONE MARROW- SEPARATED

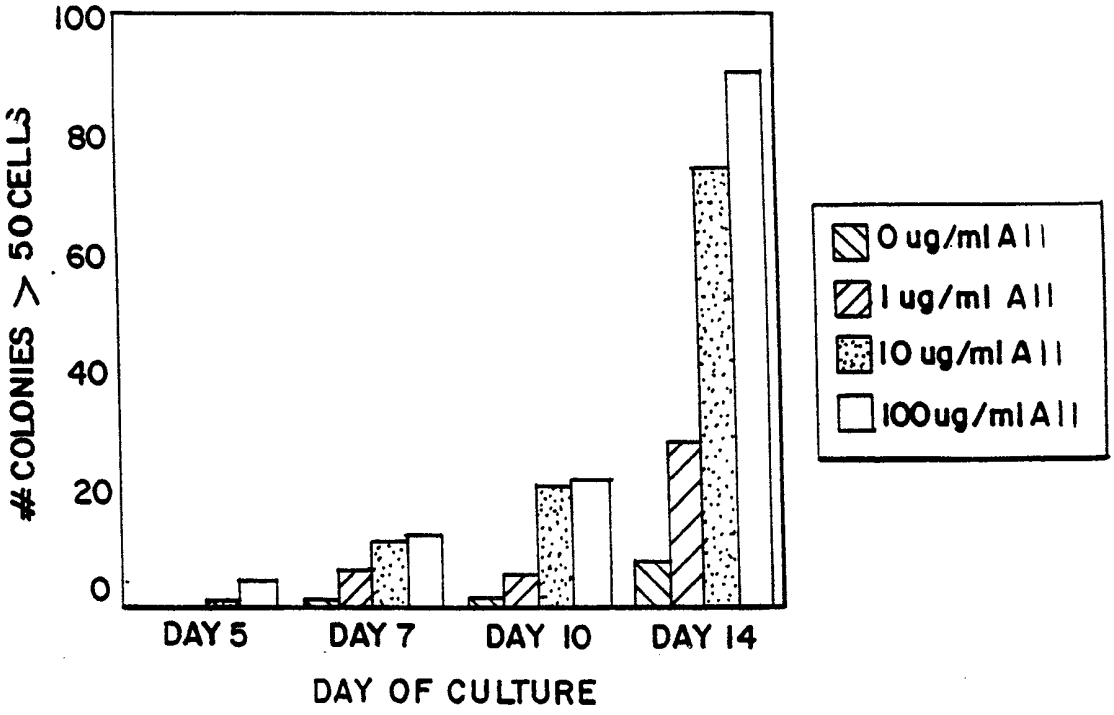
EFFECT OF ANGIOTENSIN II ON MURINE HSC CULTURES

FIG. 13



NORMAL BONE MARROW-SEPARATED

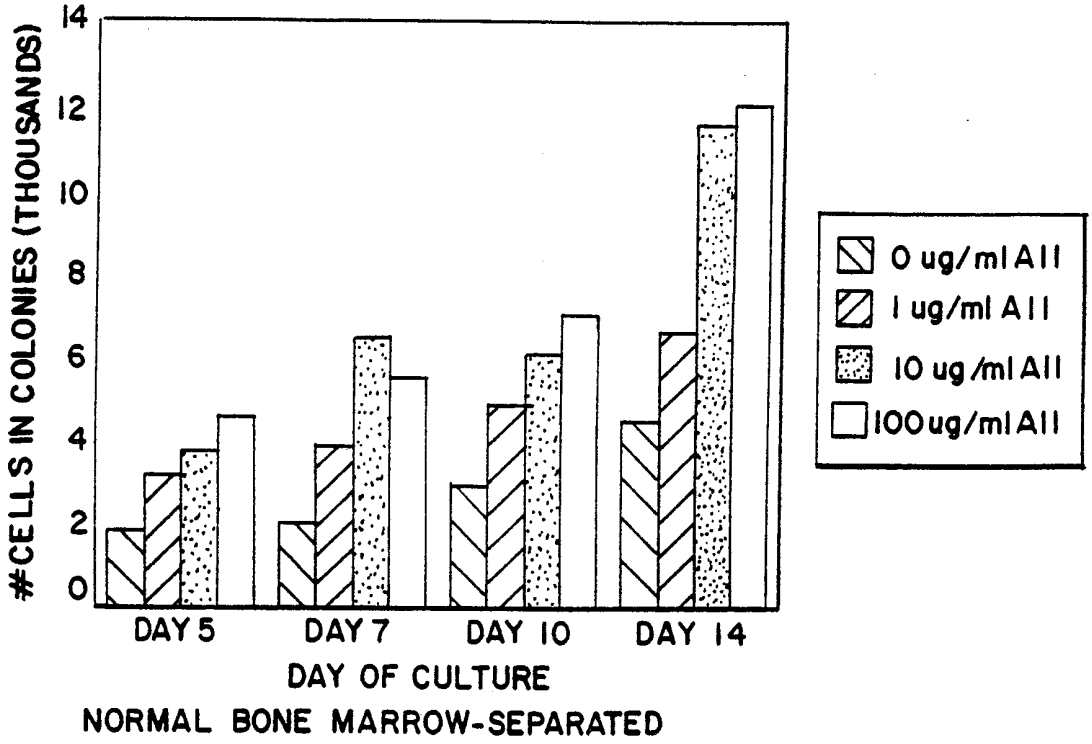
FIG. 14 EFFECT OF ANGIOTENSIN II ON MURINE HSC CULTURES



NORMAL BONE MARROW-SEPARATED

EFFECT OF ANGIOTENSIN II ON MURINE HSC CULTURES

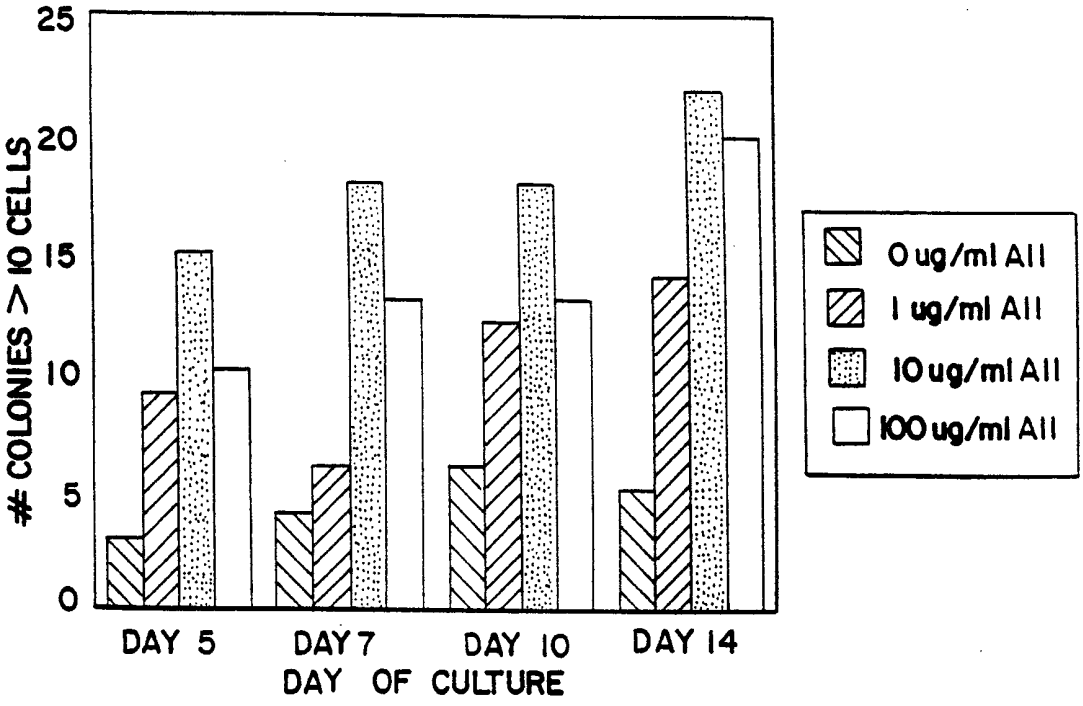
FIG. 15



NORMAL BONE MARROW-SEPARATED

FIG. 16

EFFECT OF ANGIOTENSIN II ON MURINE HSC CULTURES



5-FU BONE MARROW- SEPARATED

FIG. 17

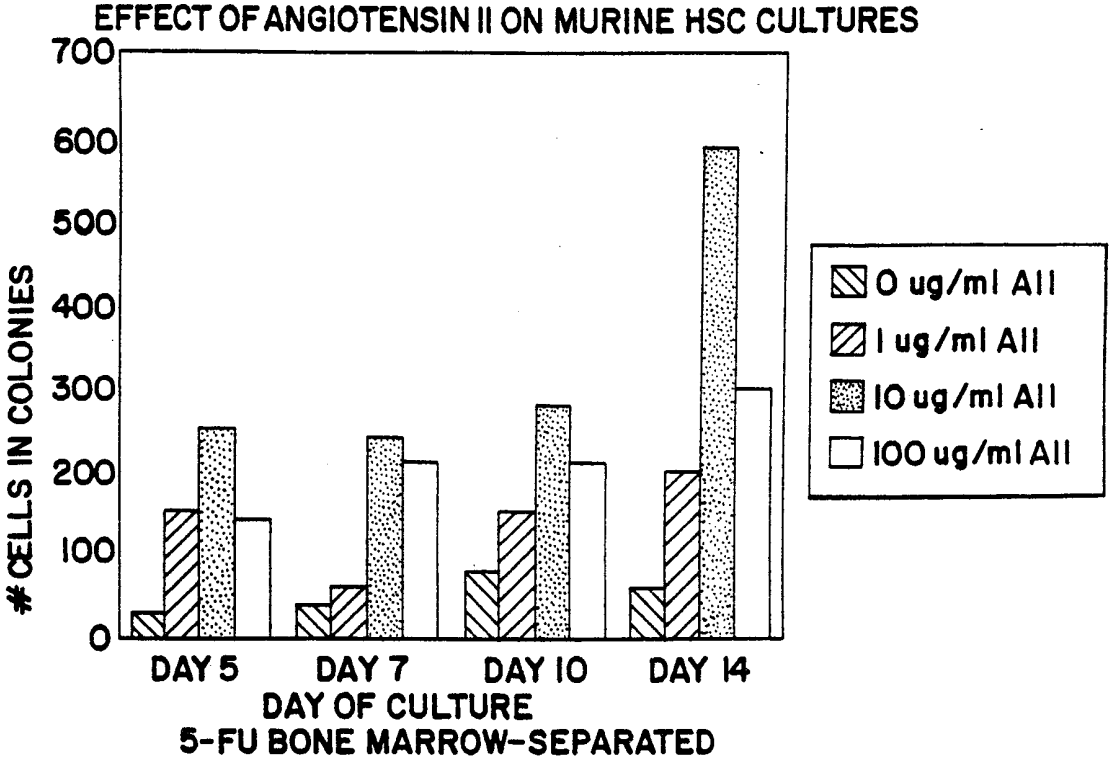

EFFECT OF ANGIOTENSIN II ON MURINE HSC CULTURES
#CELLS IN COLONIES
700
600
500
400
300
200
100
0
DAY 5
DAY 7
DAY 10
DAY 14
DAY OF CULTURE
5-FU BONE MARROW-SEPARATED
0 ug/ml AII
1 ug/ml AII
10 ug/ml AII
100 ug/ml AII

FIG. 18

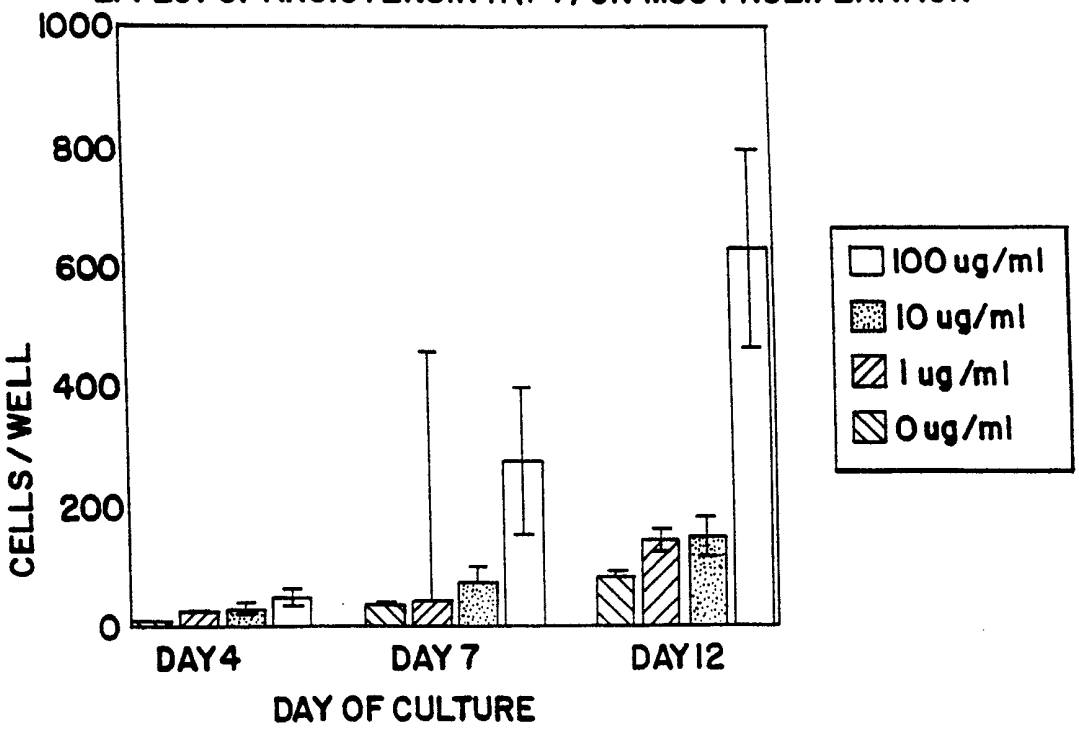

EFFECT OF ANGIOTENSIN II (1-7) ON MSC PROLIFERATION
CELLS/WELL
1000
800
600
400
200
0
DAY4
DAY 7
DAY 12
DAY OF CULTURE
100 ug/ml
10 ug/ml
1 ug/ml
0 ug/ml

FIG.19

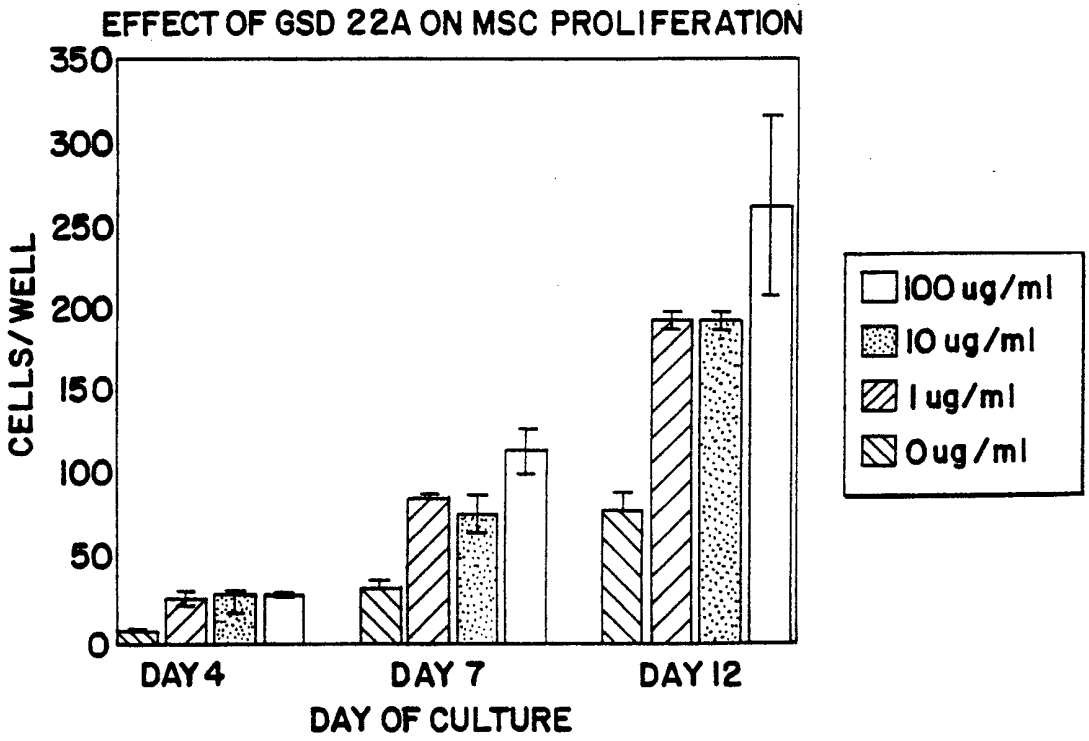

EFFECT OF GSD 22A ON MSC PROLIFERATION
350
300
250
200
150
100
50
0
CELLS/WELL
DAY 4
DAY 7
DAY 12
DAY OF CULTURE
100 ug/ml
10 ug/ml
1 ug/ml
0 ug/ml

FIG.20

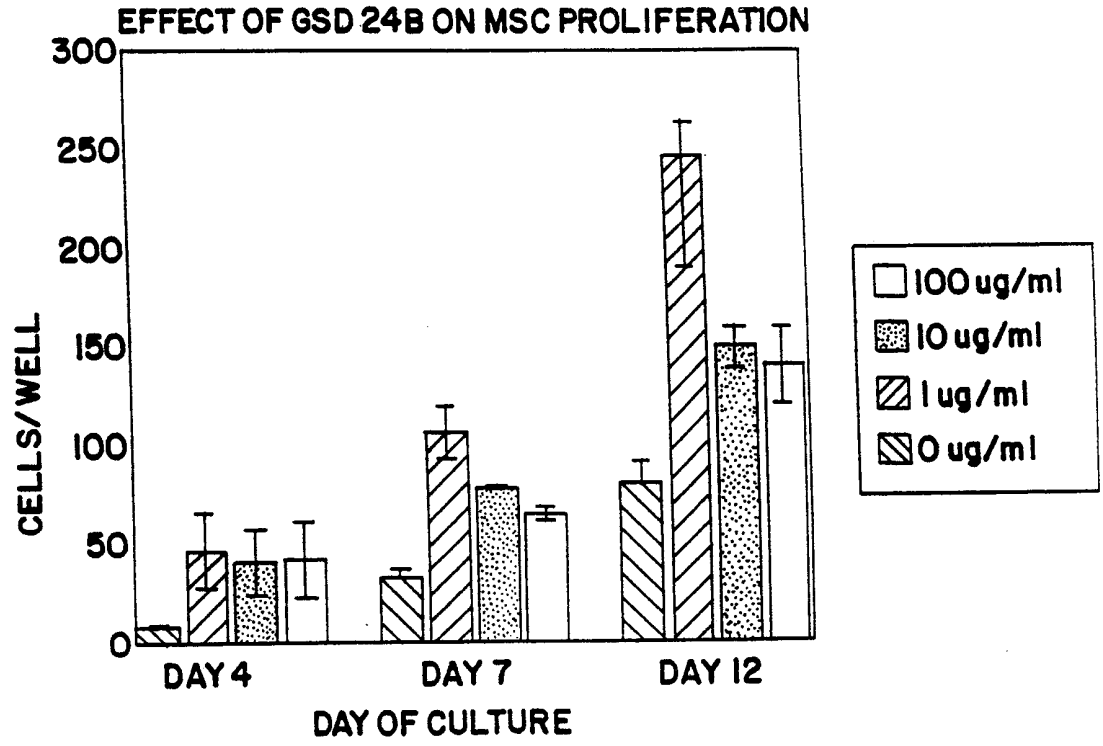

EFFECT OF GSD 24B ON MSC PROLIFERATION
300
250
200
150
100
50
0
CELLS/WELL
DAY 4
DAY 7
DAY 12
DAY OF CULTURE
100 ug/ml
10 ug/ml
1 ug/ml
0 ug/ml

FIG. 21

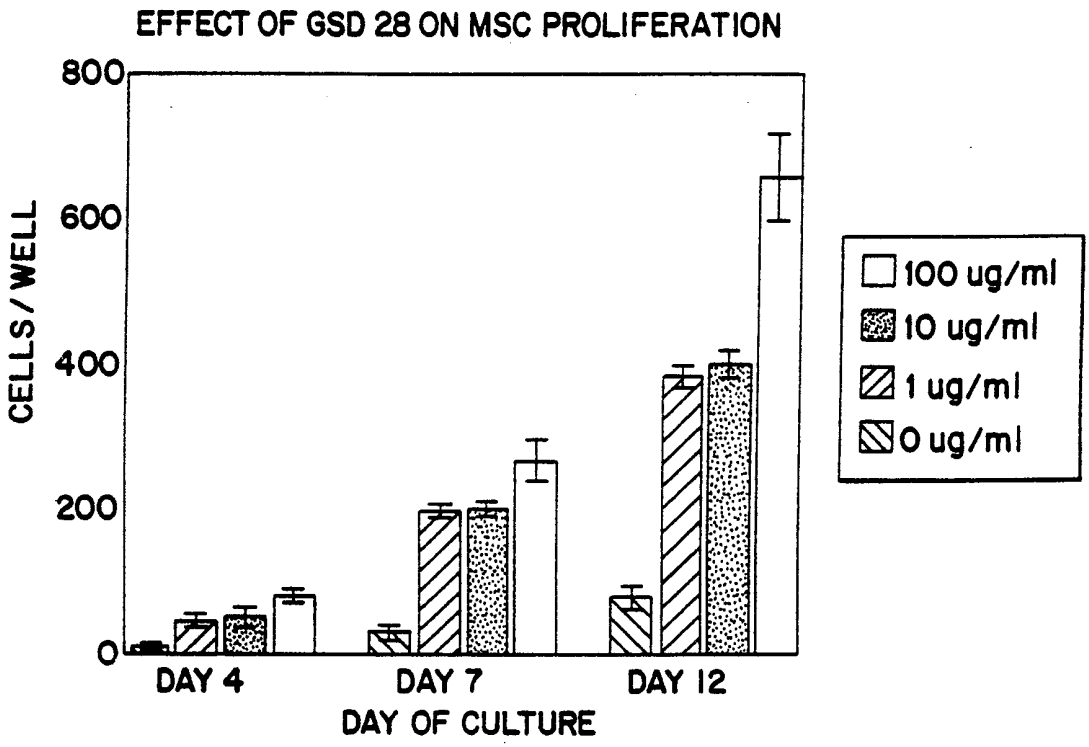

EFFECT OF GSD 28 ON MSC PROLIFERATION
800
600
400
200
0
CELLS / WELL
DAY 4
DAY 7
DAY 12
DAY OF CULTURE
100 ug/ml
10 ug/ml
1 ug/ml
0 ug/ml

FIG. 22

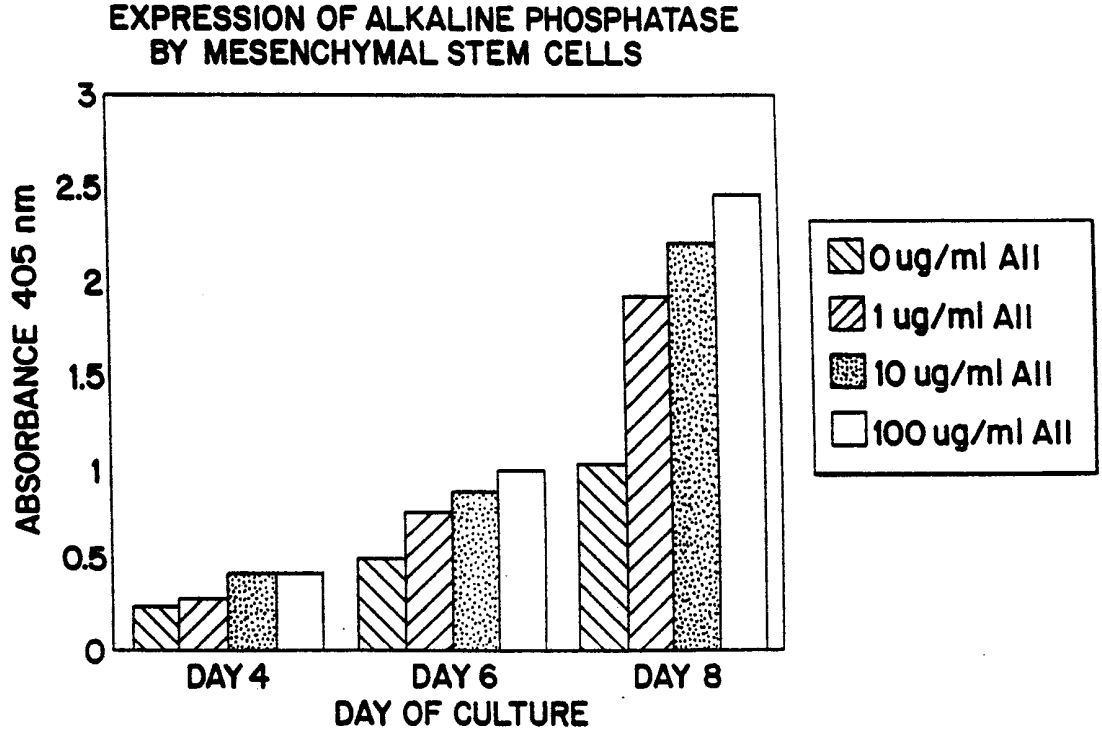
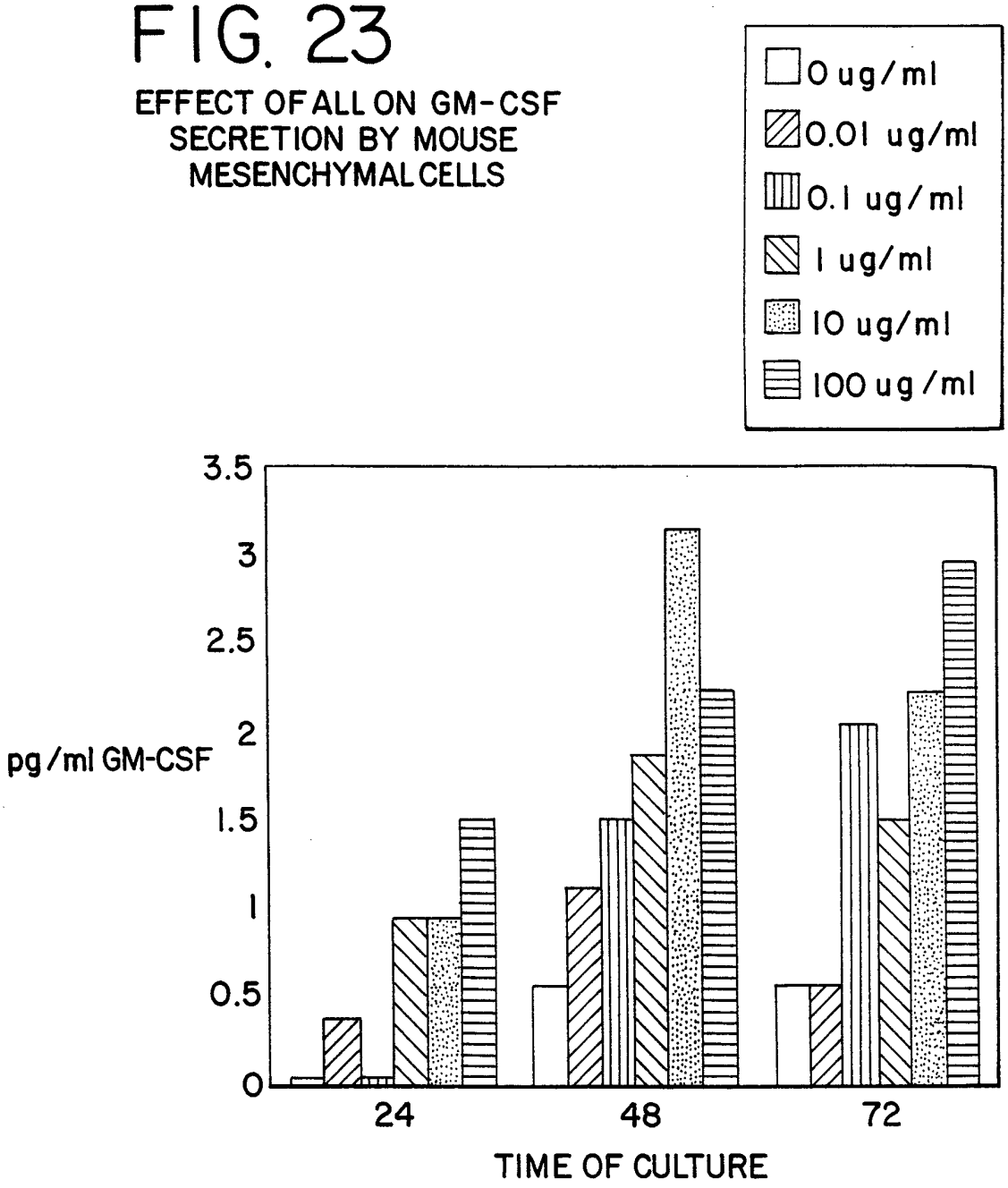

EXPRESSION OF ALKALINE PHOSPHATASE
BY MESENCHYMAL STEM CELLS
3
2.5
2
1.5
1
0.5
0
ABSORBANCE 405 nm
DAY 4
DAY 6
DAY 8
DAY OF CULTURE
0 ug/ml All
1 ug/ml All
10 ug/ml All
100 ug/ml All

FIG. 23

EFFECT OF ALL ON GM-CSF SECRETION BY MOUSE MESENCHYMAL CELLS



SEQUENCE LISTING

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<210> 1

<211> 8

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:angiotensin II

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Asp Arg Val Tyr Ile His Pro Phe

1 5

<210> 2

<211> 7

<212> PRT

<213> Artificial Sequence

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Arg Val Tyr Ile His Pro Phe

1 5

<210> 3

<211> 6

<212> PRT

<213> Artificial Sequence

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Val Tyr Ile His Pro Phe

1 5

<210> 4

<211> 7

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<223> Description of Artificial Sequence:AII(1-7)

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Asp Arg Val Tyr Ile His Pro

1 5

<210> 5

<211> 6

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<223> Description of Artificial Sequence:AII(2-7)

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Arg Val Tyr Ile His Pro

1 5

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Val Tyr Ile His Pro

1 5

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Ile His Pro Phe

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<210> 8

<211> 6

<212> PRT

<213> Artificial Sequence

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Asp Arg Val Tyr Ile His

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<210> 9

<211> 4

<212> PRT

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<210> 10

<211> 4

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<210> 13

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Arg Val Tyr Nle His Pro Phe

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His Pro Phe

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Xaa Xaa Tyr Xaa His Pro Phe

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1 5

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Ala Pro Gly Asp Arg Ile Tyr Val His Pro Phe

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Glu Arg Val Tyr Ile His Pro Phe

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Asp Lys Val Tyr Ile His Pro Phe

1 5

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1 5

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<212> PRT

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<212> PRT

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1 5

<210> 28

<211> 8

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<213> Artificial Sequence

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Asp Arg Val Tyr Ile His Ala Phe

1 5

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<211> 8

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Asp Arg Val Tyr Ile His Pro Tyr

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<210> 30

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<212> PRT

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Pro Arg Val Tyr Ile His Pro Phe

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Asp Arg Val pTyr Ile His Pro Phe

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1 5

<210> 35

<211> 9

<212> PRT

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Asp Arg Val hSer Tyr Ile His Pro Phe

1 5

<210> 36

<211> 8

<212> PRT

<213> Artificial Sequence

<220>

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Sequence:p-aminophenylalanine 6 AII

<400> 36

Asp Arg Val Tyr Ile Xaa Pro Phe

1 5

<210> 37

<211> 10

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:angiotensin I

<400> 37

Asp Arg Val Tyr Ile His Pro Phe His Leu

1 5 10

INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/US 98/25390

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K38/08 C07K7/14 C12N5/06

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 6 A61K C07K C12N

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO 98 32457 A (UNIVERSITY OF SOUTHERN CALIFORNIA, USA) 30 July 1998 see the whole document	1-22
X	US 5 015 629 A (DIZEREGA GERE S) 14 May 1991 cited in the application see the whole document	1-22
X	WO 95 08337 A (UNIV SOUTHERN CALIFORNIA) 30 March 1995 see claims	1-22
X	WO 96 39164 A (UNIV SOUTHERN CALIFORNIA) 12 December 1996 see the whole document	1-22

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"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

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Date of the actual completion of the international search	Date of mailing of the international search report
13 April 1999	21/04/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Chakravarty, A

2

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INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/US 98/25390

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	MRUG M ET AL: "Angiotensin II stimulates proliferation of normal early erythroid progenitors." JOURNAL OF CLINICAL INVESTIGATION, (1997 NOV 1) 100 (9) 2310-4, XP002077069 see abstract -----	1-22

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int[illegible]onal Application No
PCT/US 98/25390

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9832457 A	30-07-1998	AU 6648598 A	18-08-1998
US 5015629 A	14-05-1991	NONE	
WO 9508337 A	30-03-1995	AU 7730294 A	10-04-1995
		CA 2172493 A	30-03-1995
		EP 0730465 A	11-09-1996
		JP 9502977 T	25-03-1997
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		ZA 9407338 A	10-05-1995
WO 9639164 A	12-12-1996	US 5834432 A	10-11-1998
		AU 5990796 A	24-12-1996
		CA 2221730 A	12-12-1996
		EP 0828505 A	18-03-1998