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(71) Applicant: THE JOHNS HOPKINS UNIVERSITY
[US/US]; Technology Ventures, 100 N. Charles Street, 5th
Floor, Baltimore, MD 21201 (US).(72) Inventors: KUMAR, Anand; 102 Thicket Road, Bal-
timore, MD 21212 (US). LOUGH, Denver; 106 S.
Gilmore Street, Baltimore, MD 21223 (US). MADSEN,
Chris; 204 Fountain Street, Baltimore, MD 21231 (US).
GUO, Qiongyu; 5007 Paducah Road, College Park, MD
20740 (US).(74) Agents: CORLESS, Peter F; et al.; Mintz Levin Cohn
Ferris Glovsky and Popeo, P.C., One Financial Center, Bos-
ton, MA 02111 (US).

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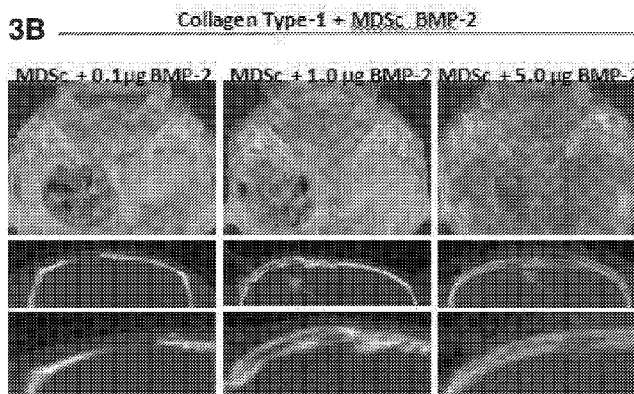
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Figure 3



(57) Abstract: A bone healing, angiogenesis promoting and/or vasculogenesis performing system and methods of use thereof are provided, that employ a unique three-principled regenerative healing strategy of muscle-derived cells (e.g., fibroblast-depleted muscle derived stem cells (MDScs)), a bone induction agent (e.g., including *ex vivo* progenitor cell expansion with early osteo-induction), and a structurally robust scaffold (e.g., a collagen scaffold) support to achieve bone regeneration, angiogenesis promotion and/or vasculogenesis performance, thereby overcoming previous limitations in regenerative healing.

BONE HEALING, ANGIOGENESIS-PROMOTING AND VASCULOGENESIS-PRODUCING SYSTEM

CROSS-REFERENCE TO RELATED APPLICATION

This application is an International Patent Application which claims the benefit of priority under 35 U.S.C. § 119(e) to U.S. Provisional Application No. 62/149,578, filed April 18, 2015 and entitled, “Bone Healing, Angiogenesis-Promoting And Vasculogenesis-Producing System”, the entire content of which is incorporated herein by reference.

FIELD OF THE INVENTION

This invention relates generally to methods and compositions for treating and/or mending bone defects and/or injuries, or for treating damaged, diseased or otherwise aberrant tissue in need of angiogenesis and/or vasculogenesis.

INTRODUCTION

Heterotopic ossification (HO) is a naturally occurring pathologic process of mature bone formation in muscle tissues that occurs *via* an endochondral ossification pathway, and is a robust, naturally occurring process of *de novo* bone formation. Muscle-derived stem cells (MDSCs) are the putative source of heterotopic ossification. A lack of a translational bone regeneration system that employs endochondral ossification has to date prevented deployment of MDSC-involving regenerative cranial human trials.

Research in cranial bone defect healing has been performed previously in both human and animal modeling systems, each applying a range of enhancement elements such as rhBMP2, MSCs or scaffolding (Uchida 2009; Manassero 2013). However, such approaches have essentially involved applying biologic filler, where a wound is left with a milieu of disconnected signal pathways, complex lineage potential and ample extracellular matrices that promote the migration, proliferation and differentiation of the body's most reliable and dynamic cell—the fibroblast that inevitably leads to healing by fibrosis. Whether limited by the therapeutic dosing, form of scaffold delivery, stem cell migration, proliferation and/or differentiation kinetics, such studies have failed to employ the intrinsic capacity of the MDSCs to induce natural tissue polarization when approximated near a growth factor enriched scaffolding unit (Liu 2009; Proceeding 2009).

SUMMARY OF THE INVENTION

The invention is based, at least in part, upon the identification of a system that includes muscle-derived cells (MDCs, *e.g.*, muscle-derived stem cells (MDScs)), a bone induction agent (*e.g.*, a bone morphogenetic protein, *i.e.*, BMP2) and a scaffold (*i.e.*, a collagen scaffold), as a system that effects surprisingly enhanced levels of bone defect healing and also imparts dramatic angiogenesis and/or vasculogenesis at the site of application. Effectively harnessing the potential of MDScs to heal critical-sized bone defects – as the instant invention achieves – is expected to provide a major advance in bone tissue engineering. In addition, the ability to use MDScs to promote angiogenesis and/or vasculogenesis at the site of application of a scaffold (*e.g.*, a collagen disk) is an appealing approach for treatment of diseases and/or disorders characterized by blood vessel blockage, degeneration and/or loss (*e.g.*, joint treatments, muscle-rebuilding treatments and cardiac treatments, *e.g.*, for effecting angiogenesis and/or vasculogenesis at the site of a coronary blockage, are among those specifically contemplated).

In one aspect, the invention provides a method for treating a bone defect in a subject, the method involving obtaining a muscle-derived cell (MDC) population; contacting the MDC population with a bone induction agent; applying the MDC population to a scaffold, thereby forming a MDC-scaffold composition; and contacting the MDC-scaffold composition to a bone of a subject, wherein the bone possesses a bone defect, thereby treating the bone defect in the subject.

In one embodiment, the MDC population is enriched for muscle-derived stem cells (MDScs). In another embodiment, the MDC population is derived from the subject having the bone defect that is treated.

In one embodiment, the MDC population is isolated from a preplate derived by the method of Gharaibeh, B. *et al. Nature Protocols* 3: 1501-1509, and optionally is a preplate 1 (PP1) to preplate 6 (PP6) cellular population. In a related embodiment, the MDC population is isolated from preplate 2 (PP2) or preplate 3 (PP3).

In certain embodiments, the MDC population is an *ex vivo* expanded MDC population. Optionally, the MDC population is fibroblast-depleted, as compared to an unexpanded control MDC population.

In one embodiment, the bone induction agent is a bone morphogenetic protein, optionally BMP2, *e.g.*, rhBMP2, or a fragment thereof.

In certain embodiments, the bone induction agent is present at a concentration sufficient to osteo-induce the MDC population.

In one embodiment, 1-10 μg of the bone induction agent is present in the MDC-scaffold composition, optionally 5 μg of the bone induction agent is present in the MDC-scaffold composition, and/or optionally the bone induction agent is present in the MDC-scaffold composition at a concentration of at least 10 ng/ml, at least 100 ng/ml, and/or at least 1 mg/ml.

In certain embodiments, the scaffold is a Collagen I scaffold, optionally a Collagen I scaffold disk, optionally a Collagen I scaffold disk of 1 to 10 mm diameter, optionally of 5 mm diameter.

In one embodiment, the bone defect is a defect of 1 to 10 mm diameter, optionally of 5 mm diameter.

In another embodiment, the MDC population includes at least 1×10^4 cells, optionally at least 1×10^5 cells, optionally at least 1×10^6 cells, optionally about 2×10^6 cells.

In a further embodiment, the bone defect is significantly reduced in size and/or is healed within eight weeks of contacting the MDC-scaffold composition to the bone of the subject, optionally within four weeks of contacting the MDC-scaffold composition to the bone of the subject, optionally within three weeks of contacting the MDC-scaffold composition to the bone of the subject.

In certain embodiments, contacting the MDC-scaffold composition to a bone of the subject results in functionally polarized healing of the bone defect of the subject.

In one embodiment, the subject is a mammalian subject, optionally a human.

Another aspect of the invention provides a method for promoting angiogenesis and/or producing vasculogenesis in a subject, the method involving obtaining a muscle-derived cell (MDC) population; contacting the MDC population with a bone induction agent; applying the MDC population to a scaffold, thereby forming a MDC-scaffold composition; and contacting the MDC-scaffold composition to a tissue of a subject, where the tissue possesses a region of injury, disease, disorder and/or lack of blood vessels in need of angiogenesis and/or vasculogenesis, thereby promoting angiogenesis and/or vasculogenesis in the subject.

In one embodiment, the tissue is a cardiac tissue, a bone tissue, a muscle tissue, a wounded tissue and/or a joint tissue, optionally a knee joint tissue.

A further aspect of the invention provides a composition for treating a bone defect in a subject, promoting angiogenesis and/or producing vasculogenesis in a tissue of a subject that includes a muscle-derived cell (MDC) population; a bone induction agent; and a scaffold, where the composition is capable of treating a bone defect, promoting angiogenesis and/or

producing vasculogenesis in a subject when applied to a bone or other tissue of the subject, as compared to an appropriate control composition.

Another aspect of the invention provides a kit for treating a subject having a bone defect, where the kit includes a muscle-derived cell (MDC) population, a scaffold and instructions for its use.

In certain embodiments, the kit also includes a bone induction agent, optionally BMP2, optionally rhBMP2 or a fragment thereof.

In another embodiment, the kit includes a muscle-derived cell population is enriched for muscle-derived stem cells (MDScs).

In an additional embodiment, the scaffold of the kit is a Collagen I scaffold, optionally a Collagen I scaffold disk, optionally a Collagen I scaffold disk of 1 to 10 mm diameter, optionally of 5 mm diameter.

An additional aspect of the invention provides a method for treating a bone defect in a subject, the method involving identifying a bone defect in the subject; and using a kit of the invention to treat the bone defect in the subject.

Other aspects of the invention are described in, or are obvious from, the following disclosure, and are within the ambit of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A to 1D demonstrate regeneration of polarized bone using MDScs in osteo-enriched collagen scaffold *in vivo*. The addition of type-1 collagen and BMP2 in MDScs promoted formation of apparent cortical and cancellous bone elements in 2D co-culture (Figures 1A and 1B) and 3D culture (Figures 1C and 1D). Alizarin red staining was applied in Figure 1B. Scale: 200 μ m.

Figures 2A and 2B show that a three-principled regenerative healing strategy of the invention (here involving MDScs + BMP2 + collagen I scaffold) enhanced bone regeneration in critically-sized cranial defects in a murine model. Figure 2A shows micro CT images of an osteo-induced MDSc construct implanted for 3 weeks (right) vs. empty control (left). Figure 2B shows retention of eGFP-MDScs during bone healing confirmed by fluorescent imaging.

Figures 3A and 3B show nascent bone formation within a murine cranial defect wound bed model at 8 weeks. Figure 3A shows a result where 5 mm full-thickness cranial bone defects in murine skull were treated with a BMP2 bound collagen I scaffold implant, in the absence

of MDScs. Figure 3B shows a result where 5 mm full-thickness cranial bone defects in murine skull were treated with a BMP2 bound collagen 1 scaffold seeded with MDSc (2×10^6). Upper panels (transverse view) show a CT reconstruction of murine skull at 8 weeks following creation of 5 mm bone defect, while the white solid arrow marks ingrowth of petri-defect native bone elements. Middle panels (coronal view) show CT reconstruction through a central defect (white dotted line). The solid arrow in the middle panels indicates neo-diploic space formation following implant of MDSc seeded graft after 8 weeks. The lower panel (coronal view) shows a 4x magnification of the middle panel region of interest ("ROI"; white dotted box).

Figures 4A to 4C show the MDSc enrichment process and images of cellular structures resulting upon treatment with various components of the system of the instant invention. Figure 4A shows a drawing of the MDSc enrichment process, showing an expansion/passage process, while Figures 4B and 4C show additional images of polarity establishment and structures formed upon combination of collagen 1 scaffold + BMP2 + MDSc population.

Figure 5 shows images of the testing process, including cranial drilling used to create consistently sized cranial defects for test treatments.

Figures 6A to 6C show cellular images and markers of cell cycle progression in a MDSc population *in vitro*, showing that increasing levels of BMP2 accelerated cell cycle progression. Specifically, as shown in Figures 6B and 6C, BMP2 + collagen I scaffold MDScs exhibited greater multiplication and proliferation.

Figures 7A and 7B show that MDScs subjected to both BMP2 and collagen I contact *in vitro* were multipotent, with Figure 7A showing FACs analysis for cell surface markers of differentiation and Figure 7B showing cellular images of differentiated cells.

Figure 8 shows both scaffold and migratory kinetics of MDScs of the system of the invention, which established that MDScs of the invention could migrate into bone, performing repair of bone defects in a functionally polarized manner capable of complete repair of such defects.

Figures 9A to 9E show that the combination of BMP2 and type 1 collagen acted as a mitogen in MDSc enriched populations, leading to polarized bone formation. Figure 9A shows DIC and fluorescence channels monitoring the real-time proliferation index of MDSc enriched populations plated at 10^6 over 18 hours using live confocal imaging. Populations were treated with a spectrum of BMP2 as indicated on either plastic or type I collagen. Red

fluorescence indicated presence of Cdt1 or the G1 phase of the cell cycle. Green fluorescence indicated the presence of geminin or the G2 phase of the cell cycle. Yellow indicated the overlap of red and green fluorescence or the presence of both Cdt1 and geminin during S-phase. Figure 9B shows exemplary images used to determine the migration index and volume index of confocal acquired image files. Figure 9C shows the correlative migration index and cell volume index for populations in the presence or absence of type 1 collagen and a spectrum of BMP2 concentrations. Figure 9D shows relative quantification of cells within a defined cell cycle parameter (G1, S and G2 phases) at 18 hours in the presence or absence of type I collagen and a spectrum of BMP2 concentrations. Figure 9E shows MDSc population topography and linear tag tracking over time while in the presence of type 1 collagen and BMP2. Black arrow indicates region of mitosis. White arrow indicates limited radial migration. Dotted lines indicate repeated radial migratory vectors by MDSc population undergoing bone formation.

Figure 10 shows that following optimization of migratory kinetics and lineage induction, MDSc were applied to a spectrum of engineered scaffolds for traceable, real-time *in vivo* studies within defect models.

Figures 11A and 11B show that the process of *in vitro* and organotypic optimization permitted the application of such findings to real-time regenerative living studies, which showed efficacy in augmenting the healing of bone defects with functional polarized bone.

Figures 12A and 12B show that MDScs augmented polarized bone healing and diploic space formation in a treated subject, with *in vivo* cortical and cancellous bone formation following delivery of MDSc seeded scaffolds at week three in an explant specifically observed. Figure 12A shows murine eGFP expressing MDSc (2×10^6) seeded onto BMP2 bound Col-1 (collagen I) scaffolds produced a polarized bone construct (cortical and cancellous bone architecture) within 14-21 days following implantation into 5 mm diameter full-thickness skull defects. White hollow arrow indicates cortical bone, while white solid arrow indicates cancellous bone. Scale in μm . Figure 12B shows a Z-stack through the nascent diploic space with explanted MDSc collagen scaffold at eight weeks.

Figure 13 shows that MDSc compositions of the invention (implants comprising collagen I scaffold-MDSc and BMP2) were identified to augment both localized angiogenesis and localized vasculogenesis *in vivo*.

Figures 14A and 14B show that MDSc populations possess multi-lineage cellular potency. Figure 14A shows that MDSc populations harvested from C57BL/6 skeletal muscle maintained the capacity to undergo myocyte differentiation following primary culture. Linear fused pre-myocytic cellular entities were distinguishable at 24 hours, and were able to beat/pulse within 48 hours. Full myocyte phenotype and motion was maintained even at confluence. Fewer linear fused pre-myocytes were detectable under non-myogenic (MSC basal media) conditions. Figure 14B shows that MDSc populations harvested from skeletal muscle displayed a multi-lineage capacity of differentiation when induced in basic adipogenic, chondrogenic and osteogenic media elements. Characterization of tri-lineage cellular potency was conducted using Oil Red, Alcian Blue and Alizarin Red staining solutions to indicate adipogenesis, chondrogenesis and osteogenesis, respectively, and imaged at 10x on an inverted phase filtered epifluorescent Zeiss Axio X10 microscope.

Figures 15A and 15B show that MDSc populations maintained stem cell expansion foci which possessed the intrinsic capacity to form corticocancellous bone while within a closed, 2D *in vitro* culture system. Figure 15A shows plating and growth/differentiation characteristics of adipose-derived stem cell (ADSC), bone marrow-derived mesenchymal stem cell (BM-MSC) and MDSc populations surgically harvested from C57BL/6 murine tissues and cultured separately on type-1 collagen coated plates in MSC basal media conditions. Adherent stem cell expansion focal aggregates were quantified and measured at 24 and 48 hours. ADSC, BM-MSC and MDSc populations all displayed mitogenic activity within the aggregates and subsequent migration of cells away from the centralized foci. However, MDSc populations also displayed forms of multi-cellular structures which organized into non-random linear bridges (black arrows) which joined the stem cell proliferative focal aggregates and permitted other cells to adhere to and migrate vectorally along the cell-based scaffold. Figure 15B shows plating and growth/differentiation characteristics of ADSC, BM-MSC and MDSc populations surgically harvested from C57BL/6 murine tissues and cultured separately on type-1 collagen-coated plates in osteoinductive media conditions. Alizarin Red staining solution was used to indicate osteogenic differentiation. ADSC populations exhibited a typical dispersed form of micro bone aggregates, while BM-MSC populations demonstrated satellite forms of micro bone aggregates surrounding larger centralized ossified foci. Alternatively, the MDSc population was capable of forming non-random corticocancellous ultrastructures, which readily bound Alizarin Red staining solution. Cortical/dense bone is indicated by a black pentagon.

Cancellous/trabecular bone elements are indicated by the black, dot-ended arrows. Images were collected at 10x on an inverted phase filtered epifluorescent Zeiss Axio X10 microscope.

Figures 16A to 16C show that MDSc populations were capable of generating 3D organized bone on deployable implant constructs, for delivery into *in vivo* systems. Figure 16A shows results obtained when 2×10^6 MDScs were seeded onto 1 cm x 1 cm type-1 collagen constructs and cultured under osteoinductive media conditions. At 72 hours, stem cell focal aggregates were observed that were capable of multi-dimensional organization, with MDSc structures growing directly away from the collagen and into the media (white arrow), acquiring a form of intrinsic rigidity. At 7 days, the MDSc-derived structures were capable of spanning the aqueous media environment to adhere to and join separate collagen constructs (black arrow) while also binding the plastic culture vessel (black ball tip arrow). At 14 days, the culture vessel containing the collagen construct(s) harbored 3D corticocancellous bone. Figure 16B shows results utilizing tissues harvested from C57BL/6-Tg(CAG-EGFP)1OsB cells, which intrinsically express eGFP. 2×10^6 ADSCs^{eGFP}, BM-MSCs^{eGFP} or MDScs^{eGFP} were separately seeded onto 1 cm x 1 cm type-1 collagen constructs and cultured under osteoinductive media conditions for 7 days in order to compare population phenotype tendencies through induction while in a 3D culture arrangement, as well as deliverability into living systems. Evaluation on day 7 indicated that ADSCs^{eGFP} remained well dispersed throughout the collagen construct, while BM-MSCs^{eGFP} tended to form multicellular proliferative aggregates. MDScs^{eGFP} however displayed nearly identical non-random organization as seen within prior 2D and 3D *in vitro* systems. Figure 16C shows that full-thickness 5 mm diameter defects were created in the left parietal skulls of C57BL/6 mice using a powered hollow-bore drill bit, and 5 mm collagen constructs containing 2×10^6 ADSCs^{eGFP}, BM-MSCs^{eGFP} or MDScs^{eGFP} were implanted within the void. Cellular eGFP emission was monitored and compared with acellular control constructs. Bio-fluorescent image of mouse depicted an example of the left parietal defect containing a MDScs^{eGFP} construct, while the right side of the dotted line contains a simple acellular construct at 1 week post implant.

Figures 17A and 17B show that osseous defects that received MDSc biologic implants were capable of regenerating vascularized corticocancellous bone with structure comparable to native architecture, including the cranial diploic space found within living systems. Figure

17A shows results for 5mm defects of the C57BL/6 mice which received either implant control or implants containing 2×10^6 of ADSCs^{eGFP}, BM-MSCs^{eGFP} or MDSCs^{eGFP} and were assessed for relative healing, bone formation and vascular ingrowth at 8 weeks. Implant controls contained non-rigid and sunken soft scar tissue over the defect (white pentagon). ADSCs^{eGFP}-containing implants developed fibrotic rims around the defect with particles of osseous aggregate intermixed with scar tissue. BM-MSCs^{eGFP} also developed fibrotic rims around the defect with larger particles of osseous aggregates intermixed within scar tissue; however, the population also notably developed a higher grade blood vessel ingrowth which resulted in more hemorrhagic tissues. MDSCs^{eGFP} populations showed significantly less fibrotic material at the rim of the defect and significantly more bone regeneration. The bone developed within the MDSCs^{eGFP} had more organized blood vessel formation and a less irregularity at the construct-native bone interface. Figure 17B shows that laser scanning multi-photon confocal microscopy of *in vivo* constructs prior to explant revealed significant vascular ingrowth (white arrows) of vessels into construct still containing green fluorescent MDSCs^{eGFP}. Corticocancellous ultrastructure was also appreciated in defects which received MDSCs^{eGFP} while no other implants provided similar cellular organization. Cancellous bone is shown as the white open arrow, while cortical bone region is indicated by a white bracket. Z scanning of the construct within the skull exhibited a diploic space (dual headed arrows) comparable to native bone tissues in only those defects receiving MDSCs^{eGFP}-enriched constructs. The formation of diploic space and relative bone formation was further investigated and validated using a miniCT scan imaging system. The white arrow indicates a lower cortical plate of diploic space on coronal cross-section and correlative location on the sagittal slice.

Figure 18 shows that a relative reduction of the fibroblast population led to an increase in CD34-expressing cellular aggregates. Specifically, the results of pre-plating of muscle-derived cellular suspension following 72 hours of contact is shown. Labels: vimentin/red, CD34/green and DNA/blue. PP1, PP2 and PP3 showed progressive reduction in fibroblasts expressing vimentin, while inversely increasing the relative number of CD34-expressing cells and CD34 cellular aggregates. PP3 (solid white arrows) showed radial outgrowth from CD34-expressing aggregates (white hollow arrows).

Figures 19A and 19B show that osteogenic induction of MDSCs resulted in the formation of bone ultrastructure.

Figures 20A and 20B show results for treatment of MDSCs with collagen + bmp2 only, where blue staining in the middle of the image is DAPI (sub-optimal). The right side image shows angiogenesis into a bone construct, where actin is red, while also showing GFP staining (from stem cells), which indicated that true vasculogenesis was occurring. These data showed the muscle-forming properties of these compositions, for generating vascularized muscle and/or cardiac cells with myotubes, projected as therapeutically useful for conditions of muscle degeneration, whether peripheral or cardiac.

Figure 21 shows explant confocal imaging of the cranium, which demonstrated localized angiogenesis and vasculogenesis following administration of implants containing MDSCs and rhBMP2.

Figures 22A and 22B show that increased concentrations of VEGF enhanced MDSC migration. Figure 22A shows a wound healing assay of GFP-expressing MDSCs treated with various concentrations of VEGF (0 - 250ng/ml). Wounds were created *via* removal of ibidi culture inserts when cells reached confluence. Migration was assessed every 6 hours, and yellow outline indicated the wound edge. Figure 22B shows results for quantification of wound closure. Percent reduction from initial wound area was calculated at each time point using ImageJ. 50 and 250 ng/ml of VEGF led to significantly higher migration rates at 6 and 12 hours, as compared to the control group. Each point refers to mean $\pm$ SEM. Two-way ANOVA was used to evaluate statistical differences among groups (* indicates $p < 0.05$).

Figures 23A and 23B show the upregulation of endothelial marker expression in MDSCs following VEGF stimulation. Figure 23A shows representative immunocytochemistry images of CD31 expression in such cells. HUVECs were used as a positive control. Control MDSC and MDSC stimulated with 250ng/ml of VEGF were cultured for 7 days prior to staining with DAPI (blue) and CD31 (red). Control MDSCs demonstrated a basal level of CD31 expression, which was significantly upregulated following VEGF stimulation. Scale bar = 50 μ m. Figure 23B shows quantification of the percent of cells that exhibited positive CD31 expression. VEGF stimulation of MDSCs led to a significantly higher percent of CD31 expressing cells. Values represent the average of two independent experiments performed in triplicate, and Student's t-test was performed to compare VEGF-stimulated MDSC with control MDSCs (* indicates $p < 0.05$).

Figures 24A and 24B show that VEGF stimulation of MDSc promoted capillary tube formation. Figure 24A shows fluorescent images of capillary tube formation. HUVECs were used as a positive control. Control MDSc and MDSc stimulated with 250 ng/ml of VEGF were cultured for 7 days prior to seeding on matrigel-coated plates overnight. Cells were then stained with Calcein AM and imaged. MDScs stimulated with VEGF demonstrated the ability to form capillary tubes *in vitro*. Scale bar = 200 μ m. Figure 24B shows quantification of capillary tube formation. VEGF-stimulated MDScs exhibited a significantly greater number of branches/mm², junctions/mm², and total length, as compared to control MDScs. A student's t-test was performed for statistical analysis (* indicated p<0.05).

Figures 25A and 25B show that MDSc myogenesis was unimpaired following VEGF stimulation. Figure 25A shows representative images of myotube formation by MDSc stimulated with various concentrations of VEGF (0-250 ng/ml). MDScs were cultured to confluence and allowed to form myotubes for 5 days in culture prior to staining for MyHC (green) and DAPI (blue). Scale bar = 100 μ m. Figure 25B shows quantification of myotube formation. Fusion index was calculated as [(# myotube nuclei/# of total nuclei) x 100]. No significant differences were noted between control MDSc and MDSc stimulated with various concentrations of VEGF. Two-way ANOVA was performed for statistical analysis.

Figure 26 shows compression moduli of regenerated bones as compared with normal bone and collagen showing that both Bone2M5B (MDSC with BMP Group) and Bone-Collagen (Collagen-only group) were stronger than normal bones. Bone 346.9 $\pm$ 33.6 kPa, Bone2M5B 606.4 kPa, Bone-Collagen 512.9 kPa, and Collagen 1 kPa.

DETAILED DESCRIPTION

The present invention relates, at least in part, to the observation that a combined system comprising muscle derived cells (MDCs, *e.g.*, muscle derived stem cells (MDScs)), bone induction agent(s) and a scaffold(s) produced both remarkable localized bone healing and angiogenic/vasculogenic effects when the combined system was employed to contact a region of bone defect in a mammalian subject. In certain aspects, the invention relates to harnessing heterotrophic ossification by employing the newly-identified bone healing system described herein. Generally speaking, the capacity of an MDSc population to form operatively useful and deployable bone substrate *in vitro* for translational regenerative

medicine applications and the utility of such an implant to perform within an *in vivo* model is disclosed herein.

In particular embodiments, relying upon early cell fate decision and dose dependent BMP2 induced mitogenic expansion foci within collagen scaffolds has produced remarkable results of healing critical sized bone defects in a murine animal model. Complete regenerative healing of a 5 mm cranial defect has now been demonstrated, using a collagen disk (5 mm diameter) with 5 µg rhBMP2 and 2×10^6 MDSs, by 8 weeks. In contrast, an empty defect of 5 mm diameter was found to have no appreciable healing at up to 8 weeks; and the same cranial defects treated with 5 µg rhBMP2 and collagen only (no MDCs/MDSs) did not heal and demonstrated abnormal sclerotic bone, which, without wishing to be bound by theory, supported the working hypothesis that muscle stem cell guided normal bone regeneration.

Components of the system of the instant invention are considered in additional detail below.

Currently employed methods and compositions for treatment of bone defects

Complex injuries resulting in the loss of a significant and functional segment of bone within the craniofacial skeleton pose a significant challenge for reconstructive surgeons (D'Amico RA *et al.*, *Plast Reconstr Surg.* 131: 393-9; Susarla SM *et al.*, *Ann Plast Surg.* 67:655- 61, and Smith DM *et al.* *J Craniofac Surg.* 19: 1244-59). Even in today's age of translational regenerative medicine and biomedical engineering, current reconstructive efforts are often directed to salvage and non-regenerative procedures, due to a loss of tissue factors, regional progenitor cells and/or overwhelming fibroblast incursion in areas of significant tissue destruction. One such reason for this continued difficulty is that many of the contemporary reconstructive techniques are limited by available autologous materials, leaving reconstructive algorithms lacking for want of suitable tissue replacements. Yet another limitation, which is extrinsic to patients and planned procedures, remains embedded within the assortment of "regenerative technologies" marketed by industry. Many of these products, whether a synthetic device or a biologic material, act mainly as a fibrous forming "filler" following application into an organic defect, and therefore remain incapable of delivering the necessary functional, self-propagating and autologous tissue elements that define regeneration (D'Amico RA *et al.* *Plast Reconstr Surg.* 131: 393-91; D'Amico RA and Rubin JP. *Plast Reconstr Surg.* 133: 1511-2; Lough D *et al.* *Plast Reconstr Surg.* 136: 36; Banyard DA *et al.* *Plast Reconstr Surg.* 135: 1740-8; and Lough DM *et al.* *Plast Reconstr*

Surg. 133: 579-90). Unfortunately, the placement of foreign acellular matrices, growth factors, or a combination of such elements only adds irregularity to the evolved extracellular and intracellular signaling pathways which regulate both a cell's inflammatory status and lineage potential. Such signaling distortion promotes the migration, proliferation and differentiation of the body's most reliable and dynamic cell: the scar forming fibroblast.

After defining basic pathologic wound-healing forces, it can be appreciated how complex craniomaxillofacial injuries that suffer the obliteration of local stem cell populations, become increasingly complicated to reconstruct as they reach a critical size. Currently, critically-sized bone and soft tissue defects are reconstructed using a variety of autogenous bone, alloplastic implants, composite free flaps and rigid fixation devices (Susarla SM, et al., *Ann Plast Surg.* 2011 Dec;67(6):655-61, and Valerio IL, et al., *Ann Plast Surg.* 2014 May;72 Suppl 1:S38-45). These materials often fail secondary to infection, extrusion, microsurgical complication, immune reactivity, device fatigue and/or migration events, resulting in removal of such compositions and return to a deteriorating wound bed. Although vascularized composite allo-transplantation (VCA) has gained momentum clinically, a lifelong postoperative course of immune suppression has been associated with significant morbidity and mortality. Presently, there remains limited translational research focused on the *ex vivo* development of regenerative autologous tissues in critically sized craniomaxillofacial defects. This appears to be due to a lack of adequate constructs which are capable of practical deployment, as well as subsequent inability to cover critically-sized voids (Manassero M, et al., *Tissue Eng Part A.* 2013 Jul;19(13-14):1554-63).

With the continued growth of cell therapy applications being utilized in clinical medicine as well as within operative plastic and reconstructive surgery, there has been ongoing interest in utilizing a variety of easily accessible stem cell populations for expanding regenerative medicine efforts (Hu MS, et al., *Plast Surg Int.* 2015;2015: 383581). While the bone marrow-derived mesenchymal stem cell (BM-MSC) and adipose-derived stem cell (ADSC) populations have persisted at the forefront in translational MSC-based cell therapy applications, other MSC families have been identified. The muscle-derived stem cell (MDSc) employed herein, which is a precursor to the well-defined satellite cell (committed to the regeneration of skeletal muscle), is distinct in that it may not be restricted to the development of myogenic or even mesenchymal tissue lineages (Jankowski RJ, et al., *Gene Ther.* 2002 May;9(10):642-7, Cooper RN, et al., *Curr Opin Pharmacol.* 2006 Jun;6(3):295-300, Wada MR, et al., *Development.* 2002 Jun;129(12):2987-95, Usas A, et al., *Biomaterials.*

2007 Dec;28(36):5401-6, Deasy BM, et al., *Blood Cells Mol Dis*. 2001 Sep-Oct;27(5):924-33, Cao B, et al., *Nat Cell Biol*. 2003 Jul;5(7):640-6, Asakura A, et al., *Differentiation*. 2001 Oct;68 (4-5):245-53, and Wada MR, et al., *Development*. 2002 Jun;129(12):2987-95). As the literature provides greater understanding of how MDSCs relate to pathological forms of ectopic osteogenesis, fibrogenesis, and adipogenesis, researchers have become more aware of the MDSC's potency, as well as optimized methods for their isolation and differentiation.

Following loss of critical craniomaxillofacial structures, many patients face few options for the return to a seemingly normal life. This struggle is subsequent to the loss of native functional tissues, the obliteration of the associated stem cells niche, as well as resultant gross asymmetry, scarring, and the likely need to return for additional procedures or interventions.

Muscle-Derived Cells and Compositions

The system of the instant invention includes muscle-derived cells (MDCs). In certain aspects, the MDCs of the invention include early progenitor cells (also termed muscle-derived progenitor cells or muscle-derived stem cells (MDSCs) herein) that show long-term survival rates following transplantation into body tissues, *e.g.*, soft tissues and/or bone, either alone or as a component of the system described herein. To obtain the MDCs of this invention, a muscle explant, preferably skeletal muscle, is obtained from an animal donor, preferably from a mammal, including rats, dogs and humans. This explant serves as a structural and functional syncytium including "rests" of muscle precursor cells (T. A. Partridge et al., 1978, *Nature* 73:306-8; B. H. Lipton et al., 1979, *Science* 205:12924).

Cells isolated from primary muscle tissue contain a mixture of fibroblasts, myoblasts, adipocytes, hematopoietic, and muscle-derived stem cells (MDSCs). The stem cells of a muscle-derived population can be enriched using differential adherence characteristics of primary muscle cells on collagen coated tissue flasks, such as described in U.S. Pat. No. 6,866,842 of Chancellor *et al.* Cells that are slow to adhere tend to be morphologically round, express high levels of desmin, and have the ability to fuse and differentiate into multinucleated myotubes (U.S. Pat. No. 6,866,842 of Chancellor et al.). A subpopulation of these cells was shown to respond to recombinant human bone morphogenic protein 2 (rhBMP2) *in vitro* by expressing increased levels of alkaline phosphatase, parathyroid hormone dependent 3',5'-cAMP, and osteogenic lineage and myogenic lineages (U.S. Pat. No. 6,866,842 of Chancellor et al.; T. Katagiri et al., 1994, *J. Cell Biol.*, 127:1755-1766).

Preplating

In certain embodiments, a preplating procedure can be used to differentiate rapidly adhering cells from slowly adhering cells (MDCs). The preplating technique can be used to differentiate populations of isolated muscle-derived cells based on their adhesion characteristics. In exemplified embodiments of the system of the present invention, populations of rapidly adhering cells (PP1-4, or, in certain instances, only PP1, optionally including in certain embodiments PP2), intermediate adhering populations (*e.g.*, PP2 and/or PP3), and slowly adhering, round MDCs (PP6) were isolated and enriched from skeletal muscle explants and tested for the expression of various markers using immunohistochemistry to determine the presence of pluripotent cells among the slowly adhering cells (see patent application U.S. Ser. No. 09/302,896 of Chancellor et al.).

The PP6 cells expressed myogenic markers, including desmin, MyoD, and Myogenin. The PP6 cells also expressed c-met and MNF, two genes which are expressed at an early stage of myogenesis (J. B. Miller et al., 1999, *Curr. Top. Dev. Biol.* 43:191-219). The PP6 showed a lower percentage of cells expressing M-cadherin, a satellite cell-specific marker (A. Irintchev et al., 1994, *Development Dynamics* 199:326-337), but a higher percentage of cells expressing Bcl-2, a marker limited to cells in the early stages of myogenesis (J. A. Dominov et al., 1998, *J. Cell Biol.* 142:537-544). The PP6 cells also expressed CD34, a marker identified with human hematopoietic stem cells, as well as stromal cell precursors in bone marrow (R. G. Andrews et al., 1986, *Blood* 67:842-845; C. I. Civin et al., 1984, *J. Immunol.* 133:157-165; L. Fina et al., 1990, *Blood* 75:2417-2426; P. J. Simmons et al., 1991, *Blood* 78:2848-2853). The PP6 cells also expressed Flk-1, a mouse homologue of human KDR gene which was recently identified as a marker of hematopoietic cells with stem cell-like characteristics (B. L. Ziegler et al., 1999, *Science* 285:1553-1558). Similarly, the PP6 cells expressed Sca-1, a marker present in hematopoietic cells with stem cell-like characteristics (M. van de Rijn et al., 1989, *Proc. Natl. Acad. Sci. USA* 86:4634-8; M. Osawa et al., 1996, *J. Immunol.* 156:3207-14). However, the PP6 cells did not express the CD45 or c-Kit hematopoietic stem cell markers (reviewed in L. K. Ashman, 1999, *Int. J. Biochem. Cell. Biol.* 31:1037-51; G. A. Koretzky, 1993, *FASEB J.* 7:420-426).

PP6 cells can be isolated by previously described techniques (see, *e.g.*, US Patent No. 8,105,834) to obtain a population of muscle-derived stem cells that have long-term survivability following transplantation. The PP6 muscle-derived stem cell population

comprises a significant percentage of cells that express stem cell markers such as desmin, CD34, and Bcl-2. In addition, PP6 cells express the Flk-1 and Sca-1 cell markers, but do not express the CD45 or c-Kit markers. In certain embodiments, greater than 95% of the PP6 cells express the desmin, Sca-1, and Flk-1 markers, but do not express the CD45 or c-Kit markers. The PP6 cells are utilized within about 1 day or about 24 hours after the last plating.

Upon further optimization of cell populations for use in the bone healing system of the present invention, adhering populations of preplate 2 and preplate 3 (PP2 and PP3) (alternatively, such fractions are also termed herein the “intermediate adherence population” or “intermediate adherence cells”) were isolated and enriched from muscle explants, and tested for the expression of various markers. The “muscle-derived aggregate colonies” (MDAC) resulting from the preplating technique at PP2 and PP3, for example, can exhibit characteristic profiles presenting different morphology, marker profiles and possessing superior regenerative capabilities, as compared with rapidly adhering cells (or fast adhering cell populations), or also as compared with slow-adhering cells. If the intermediate adherence population contains a high amount of fibroblast-like cells, (*i.e.*, large, flat cells versus small, refractive cells), the plates can be further replated (and, in some embodiments, the replating can optionally be repeated several times). Such intermediate adherence cells were also identified as characterized by CD markers (*e.g.*, Sca-1, CD29, CD105, CD73 and CD34 expression and low CD31, CD56, Cd144, and CD146 expression).

Intermediate adherence cells can be isolated by previously described techniques (*see, e.g.*, US Patent No. 8,105,834) to obtain a population of muscle-derived stem cells that possess long-term survivability following transplantation. The intermediate adherence muscle-derived stem cell population comprises a significant percentage of cells that express stem cell markers such as desmin, CD34, and Bcl-2. Intermediate adherence cells can be utilized within about 1 day – *i.e.*, about 24 hours after the last plating.

In certain embodiments, the rapidly adhering cells, the intermediate adherence cells and slowly adhering cells (MDCs) are separated from each other using a single plating technique. First, cells are provided from a skeletal muscle biopsy. The biopsy need only contain about 100 mg of cells. Biopsies ranging in size from about 50 mg to about 500 mg can be used according to both pre-plating and single plating methods of obtaining and expanding MDCs (*e.g.*, MDCs). Further biopsies of 50, 100, 110, 120, 130, 140, 150, 200,

250, 300, 400 and 500 mg can be used according to both pre-plating and single plating methods.

In one embodiment of the invention, the tissue from the biopsy is then stored for 1 to 7 days. This storage is at a temperature from about room temperature to about 4°C. This waiting period causes the biopsied skeletal muscle tissue to undergo stress. While this stress is not necessary for the isolation of MDCs using a single plate technique, using a wait period can result in a greater yield of MDCs.

Tissue from the biopsies is minced and centrifuged. The pellet is resuspended and digested using a digestion enzyme. Enzymes that may be used include collagenase, dispase or combinations of these enzymes. After digestion, the enzyme is washed off of the cells. The cells are transferred to a flask in culture media for the isolation of the rapidly adhering cells. Many culture media may be used. Exemplary culture-media include those that are designed for culture of endothelial cells including Cambrex Endothelial Growth Medium. This medium may be supplemented with other components including fetal bovine serum, IGF-1, bFGF, VEGF, EGF, hydrocortisone, heparin, and/or ascorbic acid. Other media that may be used in a single plating technique include InCell M310F medium. This medium may be supplemented as described above, or used unsupplemented.

The step for isolation of the rapidly adhering cells may require culture in flask for a period of time from about 30 to about 120 minutes. The rapidly adhering cells adhere to the flask in 30, 40, 50, 60, 70, 80, 90, 100, 110 or 120 minutes. After they adhere, the slowly adhering cells are separated from the rapidly adhering cells by removing the culture media from the flask to which the rapidly adhering cells are attached.

The culture medium removed from this flask is then transferred to a second flask. The cells may be centrifuged and resuspended in culture medium before being transferred to the second flask. The cells are cultured in this second flask for between 1 and 3 days. Optionally, the cells are cultured for two days. During this period of time, the slowly adhering cells (MDCs) adhere to the flask. After the MDCs have adhered, the culture media is removed and new culture media is added so that the MDCs can be expanded in number. The MDCs may be expanded in number by culturing them for from about 10 to about 20 days. The MDCs may be expanded in number by culturing them for 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 days. Optionally, the MDCs are subject to expansion culture for 17 days.

As an alternative to the pre-plating and single plating methods, the MDCs of the present invention can be isolated by fluorescence-activated cell sorting (FACS) analysis using labeled antibodies against one or more of the cell surface markers expressed by the MDCs (C. Webster et al., 1988, *Exp. Cell. Res.* 174:252-65; J. R. Blanton et al., 1999, *Muscle Nerve* 22:43-50). For example, FACS analysis can be performed using labeled antibodies directed to CD34, Flk-1, Sca-1, and/or other cell-surface markers to select a population of PP6-like cells that exhibit long-term survivability when introduced into a host tissue. One or more fluorescence-detection labels, for example, fluorescein or rhodamine, can also be used for antibody detection of different cell marker proteins.

Using any of the MDC isolation methods described above or that have been previously described, MDCs that are to be transported, or are not going to be used for a period of time may be preserved using methods known in the art. More specifically, the isolated MDCs may be frozen to a temperature ranging from about -25 to about -90°C . Optionally, the MDCs are frozen at about -80°C ., on dry ice for delayed use or transport. The freezing may be done with any cryopreservation medium known in the art.

Muscle-Derived Cell-Based Treatments

In one embodiment of the present invention, the MDCs are isolated from a skeletal muscle source and, using the MDC-bone induction agent-scaffold system of the invention, introduced or transplanted into bone structures or into a muscle or non-muscle soft tissue site of interest. Advantageously, the MDCs of the present invention are isolated and enriched to contain a large number of stem cells showing long-term survival following transplantation. In addition, the muscle-derived stem cells used in the invention can express a number of characteristic cell markers, such as desmin, CD34, and Bcl-2. Furthermore, in certain embodiments, the muscle-derived stem cells of the invention express the Sca-1, and Flk-1 cell markers, but do not express the CD45 or c-Kit cell markers.

MDCs and compositions comprising MDCs of the present invention can be used to repair, treat, or ameliorate various aesthetic or functional conditions (e.g. defects) through the augmentation of muscle, non-muscle soft tissues and/or bone. In particular, such compositions can be used for the treatment of bone defects and/or injury and/or muscle or soft tissue weakness, disease, injury, or dysfunction. In addition, such MDCs and compositions thereof can be used for promoting angiogenesis and/or producing

vasculogenesis in the location at which a system of the invention is contacted to a subject, tissue, bone and/or organ. Specifically contemplated angiogenesis-promoting and/or vasculogenesis-producing applications of the instant invention include joint repair (*e.g.*, use in joint repair surgeries (*e.g.*, ACL repair) and/or treatment of osteoarthritic joints), rebuilding of muscle cells and/or use in cardiac surgery, *e.g.*, *via* placement at locations of coronary artery blockage and/or regions of damage. The systems of the invention can also be used to augment soft tissue not associated with injury by adding bulk to a soft tissue area, opening, depression, or void in the absence of disease or trauma, such as for “smoothing”. Multiple and successive applications of MDCs/MDSs within the scaffold-bone induction agent system of the invention are also contemplated, as are use of the system in combination with other therapeutic agents, and/or use of the MDCs/MDSs of the invention not only as an agent for angiogenesis promotion, vasculogenesis production and/or repair of local damage *via* promotion of growth of functionally polarized bone and/or cartilage within areas of critical size bone defects (defects of such size that they would not heal spontaneously within the lifetime of a subject), but also as a possible vector for gene therapy *via*, *e.g.*, lentiviral transformation of MDCs/MDSs with constructs comprising therapeutic genetic agents, as has been described previously in the art.

For MDC-based treatments, a skeletal muscle explant is optionally obtained from an autologous or heterologous human or animal source. An autologous animal or human source is advantageous as the least likely to produce a deleterious immune reaction upon (re)introduction to a subject. MDC compositions are then prepared and isolated as described herein or as known in the art. To introduce or transplant the MDCs and/or compositions comprising the MDCs according to the present invention into a human or animal recipient, a suspension of mononucleated muscle cells can optionally first be prepared. Such suspensions contain concentrations of the muscle-derived stem cells of the invention in a physiologically-acceptable carrier, excipient, or diluent. For example, suspensions of MDCs for administering to a subject can comprise 10^8 to 10^9 cells/ml in a sterile solution of complete medium modified to contain the subject's serum, as an alternative to fetal bovine serum. Alternatively, MDC suspensions can be in serum-free, sterile solutions, such as cryopreservation solutions (Celox Laboratories, St. Paul, Minn.). The MDC suspensions can then be introduced into the scaffold that is introduced into a subject, with additional scaffold details described below.

The described cells can be administered as a pharmaceutically or physiologically acceptable preparation or composition containing a physiologically acceptable carrier, excipient, or diluent, used to contact and/or impregnate the scaffold of the system of the invention, and then used to contact the tissues (e.g., bone and/or soft tissues or muscles) of the recipient organism of interest, including humans and non-human animals. The MDC-containing component of the system composition can be prepared by resuspending the cells in a suitable liquid or solution such as sterile physiological saline or other physiologically acceptable aqueous liquids, suitable for use in a scaffold system that is introduced into a subject for therapeutic purpose. The amounts of the components to be used in such compositions can be routinely determined by those having skill in the art.

The MDCs or compositions thereof can be administered by placement of the MDC suspensions onto absorbent or adherent material, i.e., a collagen sponge matrix, and insertion of the MDC-containing material into or onto the site of interest. Notably, the MDC/MDSc systems of the instant invention also include a bone inducing agent, and it is the combination of all three components of the system, i.e., (1) MDC/MDSc, (2) bone inducing agent (e.g., BMP2) and (3) scaffold (e.g., collagen I scaffold/disk) that have herein been identified to produce surprising bone healing and angiogenesis/vasculogenesis results, thereby distinguishing the MDC/MDSc-collagen matrix compositions of the instant invention from previously contemplated MDC-collagen matrix compositions. In one embodiment of the present invention, administration of the MDCs + bone inducing agent within a scaffold can be mediated by endoscopic surgery.

Sterile solutions or suspensions of MDCs can be prepared during preparation of the system compositions of the invention. Such MDC solutions or suspensions can include pharmaceutically- and physiologically-acceptable aqueous or oleaginous vehicles, which may contain preservatives, stabilizers, and material for rendering the solution or suspension isotonic with body fluids (i.e. blood) of the recipient. Non-limiting examples of excipients suitable for use include water, phosphate buffered saline, pH 7.4, 0.15 M aqueous sodium chloride solution, dextrose, glycerol, dilute ethanol, and the like, and mixtures thereof. Illustrative stabilizers are polyethylene glycol, proteins, saccharides, amino acids, inorganic acids, and organic acids, which may be used either on their own or as admixtures. The amounts or quantities, as well as the sites targeted, are determined on an individual basis, and

correspond to the amounts used in similar types of applications or indications known to those of skill in the art.

To optimize transplant success, the closest possible immunological match between donor and recipient is desired. If an autologous source is not available, donor and recipient Class I and Class II histocompatibility antigens can be analyzed to determine the closest match available. This minimizes or eliminates immune rejection and reduces the need for immunosuppressive or immunomodulatory therapy. If required, immunosuppressive or immunomodulatory therapy can be started before, during, and/or after the transplant procedure. For example, cyclosporin A or other immunosuppressive drugs can be administered to the transplant recipient. Immunological tolerance may also be induced prior to transplantation by alternative methods known in the art (D. J. Watt et al., 1984, *Clin. Exp. Immunol.* 55:419; D. Faustman et al., 1991, *Science* 252:1701).

Consistent with the present invention, the MDC-bone induction agent-scaffold system of the invention can be administered to body tissues, including not only bone tissue but also epithelial tissue (i.e., skin, lumen, etc.) muscle tissue (i.e. smooth muscle), and various organ or joint tissues.

The number of cells in an MDC/MDSc suspension/composition and the mode of administration may vary depending on the site and condition being treated. As non-limiting examples, in accordance with the present invention, about 2×10^6 MDCs are used within a collagen disk/collagen scaffold of an exemplary system of the invention for the treatment of a critical size bone defect of a subject. Consistent with the Examples disclosed herein, a skilled practitioner can modulate the amounts and methods of MDC-bone inducing agent-scaffold system treatments according to requirements, limitations, and/or optimizations determined for each case.

In certain embodiments, the MDCs and compositions thereof according to the present invention have utility as treatments for conditions of the lumen and/or of voids or defects in an animal or mammal subject, including humans. Specifically, the muscle-derived stem cells are used for completely or partially blocking, enhancing, enlarging, sealing, repairing, bulking, or filling various biological voids within the body. Voids may include, without limitation, various tissue wounds (i.e., loss of muscle and soft tissue bulk due to trauma; destruction of soft tissue due to penetrating projectiles such as a stab wound or bullet wound;

loss of soft tissue from disease or tissue death due to surgical removal of the tissue including loss of breast tissue following a mastectomy for breast cancer or loss of muscle tissue following surgery to treat sarcoma, etc.), lesions, fissures, diverticulae, cysts, fistulae, aneurysms, and other undesirable or unwanted depressions or openings that may exist within the body of an animal or mammal, including humans. For the treatment of conditions of the lumen and of voids, the MDCs are prepared in association with a bone induction agent and a scaffold as disclosed herein and then localized *via* transplant to the luminal tissue to fill or repair the void. The number of MDCs introduced is modulated to repair large or small voids in a soft tissue environment or in bone, cartilage or other tissue, as required. Remarkable to the instant invention is the surprising efficacy of the currently described system to repair critical size tissue defects, *e.g.*, critical size bone defects (those too large to heal spontaneously in a subject during their lifetime).

In addition, the MDCs and compositions thereof can be used to affect contractility and/or repair muscle tissues, including, *e.g.*, smooth muscle tissue. Thus, the present invention also embraces the use of the MDC system of the invention in restoring muscle contraction, and/or ameliorating or overcoming smooth muscle contractility problems.

Optionally, the MDCs of the invention may be genetically engineered by a variety of molecular techniques and methods known to those having skill in the art, for example, transfection, infection, or transduction. Transduction as used herein commonly refers to cells that have been genetically engineered to contain a foreign or heterologous gene via the introduction of a viral or non-viral vector into the cells. Transfection more commonly refers to cells that have been genetically engineered to contain a foreign gene harbored in a plasmid, or non-viral vector. MDCs can be transfected or transduced by different vectors and thus can serve as gene delivery vehicles to transfer the expressed products into muscle, bone or other treated tissues (further augmenting the therapeutic role of MDCs/MDScs of the instant system of the invention. Viral vectors can be used; however, those having skill in the art will appreciate that the genetic engineering of cells to contain nucleic acid sequences encoding desired proteins or polypeptides, cytokines, and the like, may be carried out by methods known in the art, for example, as described in U.S. Pat. No. 5,538,722, including fusion, transfection, lipofection mediated by the use of liposomes, electroporation, precipitation with DEAE-Dextran or calcium phosphate, particle bombardment (biolistics) with nucleic acid-coated particles (*e.g.*, gold particles), microinjection, and the like.

Exemplary dosing of MDCs/MDSs within the system of the invention includes, e.g., use of about 10^3 to about 10^8 cells per cm^3 of tissue to be treated, optionally about 10^5 to 10^7 cells per cm^3 of tissue to be treated, within the scaffold/bone inducing agent composition of the system of the invention.

Cell count and viability for MDCs and/or enriched MDSs can be measured using a Guava flow cytometer and Viacount assay kit (Guava). Cellular markers can be measured by flow cytometry (Guava) and conjugated anti-marker antibodies. Fluorescent labeling can be performed using conjugated anti-mouse IgG antibodies and other methods.

Myoblasts, the precursors of muscle fibers, are mononucleated muscle cells which differ in many ways from other types of cells. Myoblasts naturally fuse to form post-mitotic multinucleated myotubes which result in the long-term expression and delivery of bioactive proteins (T. A. Partridge and K. E. Davies, 1995, *Brit. Med. Bulletin*, 51:123-137; J. Dhawan et al., 1992, *Science*, 254: 1509-1512; A. D. Grinnell, 1994, In: *Myology*. Ed 2, Ed. Engel A G and Armstrong C F, McGraw-Hill, Inc, 303-304; S. Jiao and J. A. Wolff, 1992, *Brain Research*, 575:143-147; H. Vandenberg, 1996, *Human Gene Therapy*, 7:2195-2200). Myoblasts have been used for gene delivery to muscle for muscle-related diseases, such as Duchenne muscular dystrophy (E. Gussoni et al., 1992, *Nature*, 356:435-438; J. Huard et al., 1992, *Muscle & Nerve*, 15:550-560; G. Karpati et al., 1993, *Ann. Neurol.*, 34:8-17; J. P. Tremblay et al., 1993, *Cell Transplantation*, 2:99-112), as well as for non-muscle-related diseases, e.g., gene delivery of human adenosine deaminase for the adenosine deaminase deficiency syndrome (C. M. Lynch et al., 1992, *Proc. Natl. Acad. Sci. USA*, 89:1138-1142); gene transfer of human proinsulin for diabetes mellitus (G. D. Simonson et al., 1996, *Human Gene Therapy*, 7:71-78); gene transfer for expression of tyrosine hydroxylase for Parkinson's disease (S. Jiao et al., 1993, *Nature*, 362:450); transfer and expression of Factor IX for hemophilia B (Y. Dai et al., 1995, *Proc. Natl. Acad. Sci. USA*, 89:10892), delivery of human growth hormone for growth retardation (J. Dhawan et al., 1992, *Science*, 254:1509-1512).

The use of myoblasts to treat muscle degeneration, to repair tissue damage or treat disease is disclosed in U.S. Pat. Nos. 5,130,141 and 5,538,722. Also, myoblast transplantation has been employed for the repair of myocardial dysfunction (S. W. Robinson et al., 1995, *Cell Transplantation*, 5:77-91; C. E. Murry et al., 1996, *J. Clin. Invest.*, 98:2512-2523; S. Gojo et al., 1996, *Cell Transplantation*, 5:581-584; A. Zibaitis et al., 1994, *Transplantation Proceedings*, 26:3294).

In accordance with the present invention, muscle-derived cells, including MDSCs, may be primary cells, cultured cells, or cloned. They may be histocompatible (autologous) or nonhistocompatible (allogeneic) to the recipient, including humans.

Optionally, the MDCs are myoblasts and muscle-derived stem cells, optionally autologous myoblasts and muscle-derived stem cells which will not be recognized as foreign to the recipient. In this regard, the MDCs/myoblasts/MDSCs used for the compositions of the invention will desirably be matched vis-à-vis the major histocompatibility locus (MHC or HLA in humans). Such MHC or HLA-matched cells may be autologous. Alternatively, the cells may be from a person having the same or a similar MHC or HLA antigen profile. The patient may also be tolerized to the allogeneic MHC antigens. The present invention also encompasses the use of cells lacking MHC Class I and/or II antigens, such as described in U.S. Pat. No. 5,538,722.

In accordance with the present invention, muscle-derived cells, including MDSCs, may be manipulated and/or expanded by a variety of techniques and methods known to those having skill in the art.

Bone Induction Agents

Exemplary bone induction agents of the invention include bone morphogenetic proteins, particularly BMP2, BMP4 and BMP9, though also including BMP1, BMP3, BMP5, BMP6, BMP7, BMP8a, BMP8b, BMP10 and/or BMP15. Other art-recognized cytokines/growth factors are also contemplated for inclusion within the systems of the invention.

By way of example, a human BMP2 preprotein sequence of accession number NP_001191.1 is:

MVAGTRCLLALLLPQVLLGGAAGLVPELGRRKFAAASSGRPSSQPSDEVLSEFELRL
LSMFGLKQRPTPSRDAVPPYMLDLYRRHSGQPGSPAPDHLERAASRANTVRSFH
HEESLEELPETSGKTTRRFFNLSSIPTEEFITSAELQVFREQMQDALGNSSFHHRINI
YEIIKPATANSKFPVTRLDDTRLVNQNASRWESFDVTPAVMRWTAQGHANHGFEVVE
VAHLEEKQGVSKRHVRISRLHQDEHSWSQIRPLLVTFGHDGKGHPLHKREKRQAK
HKQRKRLKSSCKRHPLYVDFSDVGWNDWIVAPPGYHAFYCHGECFPPLADHLNSTN
HAIVQTLVNSVNSKIPKACCVPTELSAISMLYLDENEKVVVLKNYQDMVVEGCGCR
(SEQ ID NO: 1). Recombinant human forms of BMP2 ("rhBMP2") are commercially available (*e.g.*, LifeTechTM BMP2 Recombinant Human Protein). Without being bound by

theory, in certain embodiments, BMP2 and/or rhBMP2 are used in an amount sufficient to osteo-induce MDC and/or MDSc cells. In such embodiments, use of active regions and/or fragments of the BMP2 polypeptide is also contemplated, as is use of variant and/or mutated forms of the BMP2 or rhBMP2 polypeptide, provided that osteo-induction of contacted MDC and/or MDSc cells is maintained.

Other contemplated bone induction agents of the present invention include compounds such as cholesterol derivatives (*e.g.*, oxysterol), and certain polypeptide growth factors, such as, osteogenin, Insulin-like Growth Factor (IGF)-1, IGF-II, TGF- β 1, TGF- β 2, TGF- β 3, TGF- β 4, TGF- β 5, osteoinductive factor (OIF), basic Fibroblast Growth Factor (bFGF), acidic Fibroblast Growth Factor (aFGF), Platelet-Derived Growth Factor (PDGF), vascular endothelial growth factor (VEGF), Growth Hormone (GH), growth and differentiation factors (GDF)-5 through 9, as well as proteins including, osteopontin, osteonectin, osterix, and Runx-2.

Scaffold

Exemplary scaffolds of the invention include collagen scaffolds, *e.g.*, a collagen I scaffold, such as a collagen I disk. Collagen is commercially available, and exemplary methods for synthesis of a collagen scaffold (*e.g.*, a collagen I sponge) are both known in the art and/or described herein.

Without wishing to be bound by theory, it is believed that the rigidity of a collagen scaffold is advantageous for use in the systems of the invention in supporting/promoting functionally polarized bone cell growth. However, other forms of scaffold are alternatively or additionally contemplated for use in the instant invention, including, *e.g.*, matrigel, hydrogel, gelatin sponge, calcium phosphate and/or calcium hydroxyapatite scaffolds, among other art-recognized scaffold materials. Combinations of different scaffold materials within a single scaffold are also contemplated.

Other exemplary scaffolds contemplated for the invention include, for example, ceramic scaffolds (*e.g.*, hydroxyapatite and tri-calcium phosphate), synthetic polymers (*e.g.*, poly-L-lactic acid (PLLA), polyglycolic acid (PGA), and poly-DL-lactic-co-glycolic acid (PLGA)), decellularized tissue and/or decalcified bone, allograft bone, biological materials (*e.g.*, proteoglycans, alginate-based substrates, and chitosan), and a hybrid composition using any or all of the prior listed scaffold materials, among other art-recognized scaffold and/or biocompatible support compositions.

Definitions

As used herein, the term "scaffold" refers to a solid support capable of containing and/or otherwise supporting cells and compounds of the invention. As described above, a scaffold of the invention can comprise, *e.g.*, collagen, gelatin, matrigel, hydrogel, calcium phosphate, calcium hydroxyapatite, etc., as well as combinations thereof.

As used herein, a "critical size bone defect" is a bone defect (*e.g.*, void, fracture, etc.) that will not heal spontaneously within the lifetime of a subject).

Sizes of bone and/or tissue defects, voids, fractures, etc. contemplated as treatable with the compositions of the invention include defects, voids, fractures, etc. of less than 2 mm in any relevant dimension, at least 1 mm in a relevant dimension (optionally, diameter), at least 2 mm in a relevant dimension, at least 3 mm in a relevant dimension, at least 4 mm in a relevant dimension, at least 5 mm in a relevant dimension, at least 6 mm in a relevant dimension, at least 7 mm in a relevant dimension, at least 8 mm in a relevant dimension, at least 9 mm in a relevant dimension, at least 10 mm in a relevant dimension, at least 20 mm in a relevant dimension, at least 30 mm in a relevant dimension, at least 40 mm in a relevant dimension, at least 50 mm or more in a relevant dimension; 10 cm or less in a relevant dimension, 9 cm or less in a relevant dimension, 8 cm or less in a relevant dimension, 7 cm or less in a relevant dimension, 6 cm or less in a relevant dimension, 5 cm or less in a relevant dimension, 4 cm or less in a relevant dimension, 3 cm or less in a relevant dimension, 2 cm or less in a relevant dimension, 1 cm or less in a relevant dimension, and 5 mm or less in a relevant dimension.

As used herein, the term "bone morphogenic protein," or "BMP" generally refers to a group of polypeptide growth factors belonging to the TGF- β superfamily. BMPs are widely expressed in many tissues, though many function, at least in part, by influencing the formation, maintenance, structure or remodeling of bone or other calcified tissues. Members of the BMP family are potentially useful as therapeutics. For example, BMP-2 has been shown in clinical studies to be of use in the treatment of a variety of bone-related conditions.

The term "bone induction agent" and "osteogenic" as used herein refers to a material that stimulates growth of new bone tissue.

The term, "preplate" or "preplating" refers to a technique used to isolate cells (*e.g.*, stem cells or progenitor cells) from skeletal muscle, based on the ability of such cells to adhere to collagen-coated tissue flasks. The preplating technique can be used to differentiate rapidly adhering cells from slowly adhering cells, or to identify an "intermediate adherence

population" (alternatively referred to as an "intermediate adhering cell population"), based on differentiable surface markers and/or stem-like properties. The preplate technique involves culturing digested muscle tissue for a set period of time to allow the fibroblastic cell fraction to attach, while transferring the supernatant containing the myogenic fraction into a new plate, thereby enriching for the desired cells (Gharaibeh, B. *et al. Nature Protocols* 3: 1501-1509, incorporated herein by reference).

The term, "intermediate adherence population" or "muscle-derived aggregate colonies (MDACs)" as used herein refers to a population of cells, for example, of preplate 2 or preplate 3 (PP2 or PP3), which are characterized by their "intermediate" adhesion characteristics (thus adhering at the PP2 or PP3 stage, rather than at, *e.g.*, PP1 ("rapidly" or "early" adhering cells) or PP6 ("slowly" or "late" adhering cells) and expression of CD markers, including, for example, Sca-1, CD29, Cd105, CD73, CD31, and CD34 expression, and low CD45, CD56, CD144, and CD146 expression. The MDAC populations resulting from the preplating technique, for example, can have characteristic profiles that exhibit different morphology, marker profiles and possess superior regenerative capabilities, as compared with rapidly adhering cells or fast adhering cell populations, or as compared with slowly adhering cells or late adhering populations. If the MDAC population contains a high amount of fibroblast-like cells (*i.e.*, large, flat cells versus small, refractive cells), the plates can be replated, effectively allowing for propagation of this select cell/aggregate population.

The term, "rapidly adhering cells" or "fast adhering cell populations," as used herein refers to the first cells to adhere during the early stages of the preplating technique (*i.e.*, within minutes to hours of seeding, preplate 1 or, in certain embodiments, preplate 2 (PP1 or in certain embodiments PP2, respectively)). In some embodiments, these rapidly adhering cells may be comprised of mostly fibroblastic-like and myoblast cells.

The term, "slow adhering population," "slow adhering cells," or "muscle-derived aggregate colonies (MDACs)" as used herein refers to a population of cells, for example, of preplate 4, preplate 5, or preplate 6 (PP4, PP5, or PP6), that are characterized by their delayed adhesion characteristics during the preplating process, and expression of CD markers.

The term "effective amount" includes an amount effective, at dosages and for periods of time necessary, to achieve the desired result, *e.g.*, sufficient to produce significant reduction and/or healing of a bone defect and/or promote angiogenesis/produce vasculogenesis in a bone or other tissue of a subject. An effective amount of a system composition of the invention may vary according to factors such as the disease/injury state, age, and weight of the subject, and the ability of the MDC-bone inducing agent-scaffold

composition to elicit a desired response in the subject. Administration regimens may be adjusted to provide the optimum therapeutic response. An effective amount is also one in which any toxic or detrimental effects (*e.g.*, side effects) of a system composition of the invention are outweighed by the therapeutically beneficial effects. The term "effective amount" can also be used in reference to, *e.g.*, a bone inducing agent of the invention, *e.g.*, an effective amount of BMP2 within a composition of the invention might be determined either in advance or empirically and might be confirmed via assessment of a phenotypic output/endpoint of administration of a system composition of the invention (*e.g.*, the extent of bone healing and/or angiogenesis and/or vascularization observed in the subject administered the system of the invention).

"Ameliorate," "amelioration," "improvement" or the like refers to, for example, a detectable improvement or a detectable change consistent with improvement that occurs in a subject or in at least a minority of subjects, *e.g.*, in at least about 2%, 5%, 10%, 15%, 20%, 25%, 30%, 40%, 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 98%, 100% or in a range between any two of these values. Such improvement or change may be observed in treated subjects as compared to subjects not treated with a scaffold-bone induction agent-MDC composition of the invention, where the untreated subjects have, or are subject to developing, the same or similar injury/condition, disease, symptom or the like. Amelioration of an injury/condition, disease, symptom or assay parameter may be determined subjectively or objectively, *e.g.*, *via* self-assessment by a subject(s), by a clinician's assessment or by conducting an appropriate assay or measurement, including, *e.g.*, a quality of life assessment, a slowed progression of a disease(s) or condition(s), a reduced severity of a disease(s) or condition(s), or a suitable assay(s) for the level or activity(ies) of a biomolecule(s), cell(s), by detection of respiratory or inflammatory disorders in a subject, and/or by modalities such as, but not limited to photographs, video, digital imaging and pulmonary function tests. Amelioration may be transient, prolonged or permanent, or it may be variable at relevant times during or after a scaffold-bone induction agent-MDC composition is applied to a subject or is used in an assay or other method described herein or a cited reference, *e.g.*, within timeframes described *infra*, or about 12 hours to 24 or 48 hours after the contacting or use of a scaffold-bone induction agent-MDC composition to about 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 21, 28 days, 5 weeks, 6 weeks, 7 weeks, 8 weeks, 3, 6, 9 months or more after a subject(s) has received such treatment.

The "modulation" of, *e.g.*, a symptom, level or biological activity of a molecule, or the like, refers, for example, to the symptom or activity, or the like that is detectably

increased or decreased. Such increase or decrease may be observed in treated subjects as compared to subjects not treated with a MDC-bone inducing agent-scaffold composition, where the untreated subjects have, or are subject to developing, the same or similar disease, condition, symptom or the like. Such increases or decreases may be at least about 2%, 5%, 10%, 15%, 20%, 25%, 30%, 40%, 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 98%, 100%, 150%, 200%, 250%, 300%, 400%, 500%, 1000% or more or within any range between any two of these values. Modulation may be determined subjectively or objectively, *e.g.*, by the subject's self-assessment, by a clinician's assessment or by conducting an appropriate assay or measurement, including, *e.g.*, quality of life assessments, suitable assays for the level or activity of molecules, cells or cell migration within a subject and/or by modalities such as, but not limited to photographs, video, digital imaging and pulmonary function tests. Modulation may be transient, prolonged or permanent or it may be variable at relevant times during or after a MDC-bone inducing agent-scaffold composition is administered to a subject or is used in an assay or other method described herein or a cited reference, *e.g.*, within times described *infra*, or about 12 hours to 24 or 48 hours after the contacting or use of a scaffold-bone induction agent-MDC composition to about 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 21, 28 days, 5 weeks, 6 weeks, 7 weeks, 8 weeks, 3, 6, 9 months or more after a subject(s) has received such treatment.

By "fragment" is meant a portion of a polypeptide or nucleic acid molecule. This portion contains at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90% of the entire length of the reference nucleic acid molecule or polypeptide. A fragment may contain 10, 20, 30, 40, 50, 60, 70, 80, 90, or 100, 200, 300, 400, 500, 600, 700, 800, 900, or 1000 nucleotides or amino acids.

As used herein, the term "polypeptide", and the terms "protein" and "peptide" which are used interchangeably herein, refers to a polymer of amino acids. Exemplary polypeptides include gene products, naturally-occurring proteins, homologs, orthologs, paralogs, fragments, and other equivalents, variants and analogs of the foregoing.

As used herein, "subject" includes organisms which are capable of suffering from a defect, injury, disease and/or disorder treatable by a MDC-bone inducing agent-scaffold composition or who could otherwise benefit from the administration of a MDC-bone inducing agent-scaffold composition as described herein, such as human and non-human animals. Preferred human animals include human subjects. The term "non-human animals" includes all vertebrates, *e.g.*, mammals, *e.g.*, rodents, *e.g.*, mice, and non-mammals, such as non-human primates, *e.g.*, sheep, dog, cow, chickens, amphibians, reptiles, etc.

Ranges provided herein are understood to be shorthand for all of the values within the range. For example, a range of 1 to 50 is understood to include any number, combination of numbers, or sub-range from the group consisting 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50.

Unless specifically stated or obvious from context, as used herein, the term "or" is understood to be inclusive.

Unless specifically stated or obvious from context, as used herein, the terms "a", "an", and "the" are understood to be singular or plural.

Any compositions or methods provided herein can be combined with one or more of any of the other compositions and methods provided herein.

Various methodologies of the instant invention include a step that involves comparing a value, level, feature, characteristic, property, etc. to a "suitable control", referred to interchangeably herein as an "appropriate control". A "suitable control" or "appropriate control" is a control or standard familiar to one of ordinary skill in the art useful for comparison purposes. In one embodiment, a "suitable control" or "appropriate control" is a value, level, feature, characteristic, property, etc. determined prior to performing a methodology of the instant invention, as described herein. For example, a level or manner of healing of a critical size bone defect typical to an untreated or control-treated subject can be determined prior to or concurrent with contacting a composition of the invention to a tissue or organism/subject. In another embodiment, a "suitable control" or "appropriate control" is a value, level, feature, characteristic, property, etc. determined in a cell or organism, *e.g.*, a control or normal cell or organism, exhibiting, for example, normal traits. In yet another embodiment, a "suitable control" or "appropriate control" is a predefined value, level, feature, characteristic, property, etc.

The terms "identical" or "percent identity" in the context of two or more polypeptides or nucleic acids, refer to two or more sequences or subsequences that are the same or have a specified percentage of nucleotides or amino acid residues that are the same, when compared and aligned (introducing gaps, if necessary) for maximum correspondence, not considering any conservative amino acid substitutions as part of the sequence identity. The percent identity may be measured using sequence comparison software or algorithms or by visual inspection. Various algorithms and software are known in the art that may be used to obtain alignments of amino acid or nucleotide sequences. One such non-limiting example of a

sequence alignment algorithm is the algorithm described in Karlin *et al.*, *Proc. Natl. Acad. Sci.*, 87:2264-2268 (1990), as modified in Karlin *et al.*, *Proc. Natl. Acad. Sci.*, 90:5873-5877 (1993), and incorporated into the NBLAST and XBLAST programs (Altschul *et al.*, *Nucleic Acids Res.*, 25:3389-3402 (1991)). In certain embodiments, Gapped BLAST may be used as described in Altschul *et al.*, *Nucleic Acids Res.* 25:3389-3402 (1997). BLAST-2, WU-BLAST-2 (Altschul *et al.*, *Methods in Enzymology*, 266:460-480 (1996)), ALIGN, ALIGN-2 (Genentech, South San Francisco, California) or Megalign (DNASTAR) are additional publicly available software programs that can be used to align sequences. In certain embodiments, the percent identity between two nucleotide sequences is determined using the GAP program in GCG software (e.g., using a NWSgapdna.CMP matrix and a gap weight of 40, 50, 60, 70, or 90 and a length weight of 1, 2, 3, 4, 5, or 6). In certain embodiments, the GAP program in the GCG software package, which incorporates the algorithm of Needleman and Wunsch (*J. Mol. Biol.* (48):444-453 (1970)) may be used to determine the percent identity between two amino acid sequences (e.g., using either a Blossum 62 matrix or a PAM250 matrix, and a gap weight of 16, 14, 12, 10, 8, 6, or 4 and a length weight of 1, 2, 3, 4, 5). In certain embodiments, the percent identity between nucleotide or amino acid sequences is determined using the algorithm of Myers and Miller (CABIOS, 4:11-17 (1989)). For example, the percent identity may be determined using the ALIGN program (version 2.0) and using a PAM120 with residue table, a gap length penalty of 12 and a gap penalty of 4. Appropriate parameters for maximal alignment by particular alignment software can be determined by one skilled in the art. In certain embodiments, the default parameters of the alignment software are used. In certain embodiments, the percentage identity "X" of a first amino acid sequence to a second sequence amino acid is calculated as $100 \times (Y/Z)$, where Y is the number of amino acid residues scored as identical matches in the alignment of the first and second sequences (as aligned by visual inspection or a particular sequence alignment program) and Z is the total number of residues in the second sequence. If the length of a first sequence is longer than the second sequence, the percent identity of the first sequence to the second sequence will be longer than the percent identity of the second sequence to the first sequence.

As a non-limiting example, whether any particular polynucleotide has a certain percentage sequence identity (e.g., is at least 80% identical, at least 85% identical, at least 90% identical, and in some embodiments, at least 95%, 96%, 97%, 98%, or 99% identical) to a reference sequence can, in certain embodiments, be determined using the Bestfit program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group,

University Research Park, 575 Science Drive, Madison, WI 53711). Bestfit uses the local homology algorithm of Smith and Waterman, *Advances in Applied Mathematics* 2: 482-489 (1981), to find the best segment of homology between two sequences. When using Bestfit or any other sequence alignment program to determine whether a particular sequence is, for instance, 95% identical to a reference sequence according to the present invention, the parameters are set such that the percentage of identity is calculated over the full length of the reference nucleotide sequence and that gaps in homology of up to 5% of the total number of nucleotides in the reference sequence are allowed.

The polypeptides of the present invention can be recombinant polypeptides, natural polypeptides, or synthetic polypeptides. It will be recognized in the art that some amino acid sequences of the invention can be varied without significant effect of the structure or function of the protein. Such mutants include deletions, insertions, inversions, repeats, and type substitutions.

The polypeptides and analogs can be further modified to contain additional chemical moieties not normally part of the protein. Those derivatized moieties can improve the solubility, the biological half-life or absorption of the protein. The moieties can also reduce or eliminate any desirable side effects of the proteins and the like. An overview for those moieties can be found in Remington's *Pharmaceutical Sciences*, 20th ed., Mack Publishing Co., Easton, PA (2000).

The isolated polypeptides described herein can be produced by any suitable method known in the art. Such methods range from direct protein synthetic methods to constructing a DNA sequence encoding isolated polypeptide sequences and expressing those sequences in a suitable transformed host. In some embodiments, a DNA sequence is constructed using recombinant technology by isolating or synthesizing a DNA sequence encoding a wild-type protein of interest (BMP2). Optionally, the sequence can be mutagenized by site-specific mutagenesis to provide functional analogs thereof. See, e.g. Zoeller et al., *Proc. Nat'l. Acad. Sci. USA* 81:5662-5066 (1984) and U.S. Pat. No. 4,588,585.

The data obtained from cell culture assays and animal studies of the MDC-bone inducing agent-scaffold system of the invention can be used in formulating a range of dosage/relative concentrations of components of a scaffold-bone induction agent-MDC composition for use in humans. The dosages of MDC/MDS and/or BMP2 ranges to include within the scaffold system compositions of the invention can be determined based upon efficacy and/or toxicity profiling, as would be known within the art.

Therapeutically effective amounts of bone inducing agent are contemplated to include, e.g., in some embodiments, 10, 30, 100, or 1000 pg, or 10, 30, 100, or 1000 ng, or 10, 30, 100, or 1000 µg, or 10, 30, 100, or 1000 mg, which may be included within a system composition of the invention.

A therapeutically effective amount of a compound of the compositions of the present invention can be determined by methods known in the art.

Kits

The therapeutic compositions of the invention can be included in a kit, container, pack, or dispenser together with instructions for administration. The kits may include a muscle-derived cell (MDC) population of the invention, optionally also including a bone induction agent (*e.g.*, BMP2, rhBMP2 or fragment thereof, or other induction agent as set forth elsewhere herein or known in the art), a scaffold (*e.g.*, collagen I), and instructions for its use. In certain kit embodiments, the muscle-derived cell population is optionally contacted with a bone induction agent and applied to a scaffold to form a MDC-scaffold composition. The MDC-scaffold composition is then optionally contacted to a bone defect. The MDC population, optionally combined and/or packaged with a bone induction agent and/or scaffold, can be packaged in a suitable container.

Methods of Treatment

The present invention provides for both prophylactic and therapeutic methods of treating a subject at risk of (or susceptible to) a bone injury and/or a cardiac or other vascular injury, disease or disorder.

"Treatment", or "treating" as used herein, is defined as the application or administration of a system of the invention (*e.g.*, a MDSc + BMP2 + collagen disk composition as described herein) to a patient, or application or administration of such a therapeutic agent to an isolated tissue or cell line from a patient who has the injury, disease and/or disorder and/or application or administration of such a therapeutic agent to an organ or tissue grown *in vitro* that is optionally derived from a patient who has the injury, disease and/or disorder (or, where an isolated tissue, cell line and/or organ or tissue grown *in vitro* is used, optionally from a subject not having the injury, *e.g.*, for transfer to isolated tissue, cell or supernatant to a patient having the injury, disease or disorder) for a symptom of the injury, disease or disorder, with the purpose to cure, heal, alleviate, relieve, alter, remedy,

ameliorate, improve or affect the injury, disease or disorder and/or the symptoms of the injury, disease or disorder.

Exemplary Conditions Treatable with (Scaffold-MDC-Bone Induction Agent)

Compositions

The following is provided as exemplary, specifically contemplated therapeutic uses of the compositions of the invention, and is not intended to be limiting in any manner.

Bone Healing

Multiple surgical specialties, including orthopaedic, plastic, and maxillofacial, are concerned with bone healing augmentation. Physicians in these disciplines rely on bone augmentation techniques to improve healing of fracture non-unions, oncologic and traumatic bone defect reconstructions, joint and spine fusions, and artificial implant stabilizations. Unfortunately, current techniques of autograft, allograft, and electrical stimulation are often suboptimal. Therefore, tissue engineering approaches toward bone formation have immense implications.

Intramuscular bone formation is a poorly understood phenomenon. It can be present in the clinically pathologic states of heterotopic ossification, myositis ossificans, fibrodysplasia ossificans progressiva and osteosarcoma. Radiation therapy and the anti-inflammatory drug, indomethacin, can suppress myositis ossificans. However, neither the mechanism of formation nor suppression of ectopic bone is clearly understood. A growing family of bone morphogenetic proteins (BMPs), members of the transforming growth factor β (TGF- β) superfamily, are recognized as being capable of stimulating intramuscular bone. Human BMP2 in recombinant form (rhBMP2) and BMP-encoding cDNA contained in a plasmid construct induce bone formation when injected into skeletal muscle (E. A. Wang et al., 1990, *Proc. Natl. Acad. Sci. USA*, 87:2220-2224; J. Fang et al, 1996, *Proc. Natl. Acad. Sci. USA*, 93:5753-5758). Current applications focus on injecting rhBMP2 directly into non-unions and bone defects.

However, the identification herein of a remarkably effective approach to treatment of bone defects using MDC-bone induction agent (i.e., BMP2)-scaffold compositions of the invention hold enormous promise in the arena of bone healing and may also shed light on the physiologic mechanism of ectopic bone formation. The results described herein are

particularly compelling in their identification of functionally polarized repair of bone defects using the system compositions of the instant invention, advantageously distinguishing such results from previous attempts at bone repair, some of which have relied upon administration of, e.g., BMP2 to bone, but which have not achieved complete repair of bone defects (including restoration of functionally polarized cells in regrowth of a region of bone defect).

Cardiac Disorders

The MDC-bone induction agent (i.e., BMP2)-scaffold compositions of the invention were also identified as remarkably proficient in both promoting angiogenesis (inducing blood vessel growth in a treated tissue) and performing vasculogenesis (e.g., where the MDCs/MDSs of a composition of the invention actually grow new blood vessels). Use of such compositions to repair regions of cardiac damage, e.g., application of the compositions of the invention to regions of the heart that have incurred damage related to, coronary artery disease, myocardial infarction, etc., is therefore contemplated as providing a new and effective therapeutic.

Intraarticular Diseases and Disorders

Degenerative and traumatic joint disorders are encountered frequently as the population becomes more active and lives longer. These disorders include arthritis of various etiologies, ligament disruptions, meniscal tears, and osteochondral injuries. Currently, the clinician's tools consist primarily of surgical procedures aimed at biomechanically altering the joint, such as anterior cruciate ligament (ACL) reconstructions, total knee replacement, meniscal repair or excision, cartilage debridement, etc. Use of the tissue engineering compositions of the invention to these intraarticular disease states theoretically offers a more biologic and less disruptive reparative process.

Intraarticular administration of the MDC-bone induction agent (i.e., BMP2)-scaffold compositions of the invention are contemplated for treatment of such conditions, noting both the remarkable efficacy of such compositions in promoting angiogenesis and performing vasculogenesis, as well as the potential for such compositions to repair cartilage injury/defects.

The ACL is the second most frequently injured knee ligament. Unfortunately, the ACL has a low healing capacity, in part because of the lack of blood flow to the knee joint and possibly secondary to its encompassing synovial sheath or the surrounding synovial fluid.

Because complete tears of the ACL are incapable of spontaneous healing, current treatment options are limited to surgical reconstruction using autograft or allograft. The replacement graft, often either patella ligament or hamstring tendon in origin, undergoes ligamentization with eventual collagen remodeling (S. P. Arnoszczy et al., 1982, *Am. J. Sports Med.*, 10:90-95). Therefore, augmentation of this ligamentization process using the compositions of the invention presents an additional and compelling therapeutic application of the compositions of the invention.

Summary

Tremendous effort has gone into developing translational applications and materials for the repair and/or regeneration of bone. Despite advances in stem cell biology, material sciences, as well as growth factor development and refinement, autogenous bone grafting remains the gold standard for the repair of bone defects (Deschaseaux F and Pontikoglou C, Sensébé L. *J Cell Mol Med.* 2010 Jan;14(1-2):103-15, and Tessier P, et al., *Plast Reconstr Surg.* 2005 Oct;116(5 Suppl):6S-24S; discussion 92S-94S). At this time, the continued preference and superiority attributed to autogenous bone appears to relate more to its enduring regenerative capability, and related adaptability within a defect, rather than initial structural support. Engineered materials possess distinct clinical advantages, in that they offer immediate rigid application, but at the cost of non-integration and ultimate biologic failure. In essence, autogenous bone appears to offer a biologic warranty.

Despite understanding that harvested bone autograft heals a defect primarily through osteogenesis with supplemental osteoconduction and osteoinduction, contemporary industry-derived product alternatives for bone focus mainly upon scaffold or drug independently. To recapitulate autograft healing, an underappreciated mesenchymal stem cell population, the MDSc was used. Scaffold-based devices or biologics primarily employ cellular migratory mechanisms and rely upon cells from peri-wound bed tissues to enter the wound and form primitive bone matrix at an augmented rate (osteoconduction). Locally deployable drugs or growth factors are formulated with the intent of driving cellular precursors down an osteoinductive pathway, leading to an increased rate of primitive bone matrix deposition. Yet the most critical sized bone voids are associated with local tissue destruction and loss of native progenitor populations, and are mainly rich in fibroblasts and their precursors. To overcome this limitation, the addition of fresh cellular elements to the wound is necessary, as has been demonstrated by the success of autografts.

As methods of harvest, purification and delivery of potent cellular suspension(s) continue to evolve, emerging opportunities to deploy a variety of stem cell and/or progenitor populations will become available (*e.g.*, as provided herein) as means of a novel effort to advance the fields of plastic and reconstructive surgery and improve patient outcomes. While access to surgical applications utilizing stem cell therapies (where the stem cells are immediately available) increase the options for practicing real-time regenerative medicine, those employing such therapies will also need to remain aware of complexities surrounding “the unknown” that is harbored within deployable potent cell populations.

Thus, evidence has been provided herein that skeletal muscle-derived cells possessed a subgroup of MDSCs which were capable of multi-lineage differentiation, including, but not limited to myogenic, adipogenic, chondrogenic and osteogenic forms. The regenerative potential of heterogeneous cell populations derived from fat, bone marrow, and muscle, where each demonstrated vastly different migratory and proliferative indices as noted in Figures 15A and 15B, were also characterized. Additionally, the receptor-mediated signaling pathway (BMP-2) was identified not to have induced the same regenerative process in stem cells derived from different mesodermal sources. Furthermore, these cells possessed the ability to self-assemble into corticocancellous bone-resembling structures in both 2D and 3D settings, when in the presence of both type-1 collagen and BMP-2. While ADSC and BM-MSC also maintained the capacity to undergo osteogenic differentiation, the resulting ultrastructures did not exhibit organized bone. Without wishing to be bound by theory, BMP-2 appears to have acted as a mitogen on MDSC populations, inducing the aggregation of proliferative focal expansion centers where cells divide and migrate radially, while a simple collagen scaffold provided the minimal necessary extracellular niche to eventually form a complex tissue that resembled functionally polarized bone. These findings were consistent in *in vitro* studies and were further validated within an *in vivo* translational murine model, which utilized an *in vitro* scaffold as a deployable implant for placement into critical sized skull defects. While biomedical engineering, material science and industry continue to produce exciting new technologies and inventive applications, it is the practical application of these products by plastic and reconstructive surgeons, as well their relative comfort level, intuition and clinical outcomes that will define the future of the field in bone regeneration.

Thus, the results set forth herein showed that MDSC populations containing progenitor/stem cell aggregates (optionally of a specific pre-plate fraction) in combination with other necessary entities (collagen scaffold and BMP-2) underwent early fate decisions that ultimately allowed for formation of a basic *ex-vivo* bone construct. Such a construct was

then delivered to a critical bone defect and remarkably underwent further propagation toward true organized bone formation and regenerative healing. The simplicity of this autogenous MDSc-derived construct allowed for a streamlined harvest, processing and implantation into bone defects that required enhanced healing and/or neo-genesis of vascularized bone and provides a composition and means of overcoming the previously mentioned limitations in tissue regeneration.

The practice of the present invention employs, unless otherwise indicated, conventional techniques of chemistry, molecular biology, microbiology, recombinant DNA, genetics, immunology, cell biology, cell culture and transgenic biology, which are within the skill of the art. See, e.g., Maniatis et al., 1982, *Molecular Cloning* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.); Sambrook et al., 1989, *Molecular Cloning*, 2nd Ed. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.); Sambrook and Russell, 2001, *Molecular Cloning*, 3rd Ed. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.); Ausubel et al., 1992, *Current Protocols in Molecular Biology* (John Wiley & Sons, including periodic updates); Glover, 1985, *DNA Cloning* (IRL Press, Oxford); Anand, 1992; Guthrie and Fink, 1991; Harlow and Lane, 1988, *Antibodies*, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.); Jakoby and Pastan, 1979; *Nucleic Acid Hybridization* (B. D. Hames & S. J. Higgins eds. 1984); *Transcription And Translation* (B. D. Hames & S. J. Higgins eds. 1984); *Culture Of Animal Cells* (R. I. Freshney, Alan R. Liss, Inc., 1987); *Immobilized Cells And Enzymes* (IRL Press, 1986); B. Perbal, *A Practical Guide To Molecular Cloning* (1984); the treatise, *Methods In Enzymology* (Academic Press, Inc., N.Y.); *Gene Transfer Vectors For Mammalian Cells* (J. H. Miller and M. P. Calos eds., 1987, Cold Spring Harbor Laboratory); *Methods In Enzymology*, Vols. 154 and 155 (Wu et al. eds.), *Immunochemical Methods In Cell And Molecular Biology* (Mayer and Walker, eds., Academic Press, London, 1987); *Handbook Of Experimental Immunology*, Volumes I-IV (D. M. Weir and C. C. Blackwell, eds., 1986); Riott, *Essential Immunology*, 6th Edition, Blackwell Scientific Publications, Oxford, 1988; Hogan et al., *Manipulating the Mouse Embryo*, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1986); Westerfield, M., *The zebrafish book. A guide for the laboratory use of zebrafish (Danio rerio)*, (4th Ed., Univ. of Oregon Press, Eugene, 2000).

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

Incorporation by Reference

Each of the applications and patents cited in this text, as well as each document or reference cited in each of the applications and patents (including during the prosecution of each issued patent; "application cited documents"), and each of the PCT and foreign applications or patents corresponding to and/or claiming priority from any of these applications and patents, and each of the documents cited or referenced in each of the application cited documents, are hereby expressly incorporated herein by reference. More generally, documents or references are cited in this text, either in a Reference List before the claims, or in the text itself; and, each of these documents or references ("herein-cited references"), as well as each document or reference cited in each of the herein-cited references (including any manufacturer's specifications, instructions, etc.), is hereby expressly incorporated herein by reference.

The invention is further described for illustrative purposes, in the following specific, non-limiting Examples.

EXAMPLES

Example 1: MDC Isolation

Enrichment and isolation of MDCs was performed by the following method, adapted from, *e.g.*, U.S. Pat. No. 6,866,842 of Chancellor *et al.*

Muscle explants were obtained from the hind limbs of mice. Mice were sacrificed and sprayed with 70% ethanol to completely saturate the lower body. Skin/connective tissue was then removed from muscle, beginning at the ankle and moving up the leg. Muscle explants were placed in sterile PBS on ice while remaining mice were dissected (though explants were performed on no more than two mice at a time).

Tissue explants were washed with HBSS in a 50 mL conical tube to remove any fur that continued to be associated with the explants. The explant tissue was then transferred to a sterile tissue culture dish and a few drops of sterile PBS were added (to keep tissue moist). Tissue was minced with fine, sharp sterile scissors. Mincing proceeded for a few minutes, until tissue was a slurry. 1-2 mL of a collagenase/dispase/CaCl₂ solution (freshly mixed 50 μ L CaCl₂ + 10 mL dispase + 10 mL collagenase, comprising 2.5 mM CaCl₂) were then added (2 mL per gram of tissue). Mincing was continued for several minutes more, and minced tissue was then transferred to a sterile 15 mL tube. The tube was incubated on a shaker for 20 min at 37 °C. The suspension was then centrifuged for 5 min at 350 x g, pelleting cells. Supernatant was removed, and the cell pellet was re-suspended in 5 mL proliferative media (PM: DMEM, supplemented with 10% (vol/vol) FBS (Fetal Bovine Serum), 10% (vol/vol) HS (Horse Serum), 0.5% (vol/vol) CEE (Chick Embryo Extract) and 100 U ml⁻¹ penicillin/streptomycin, sterile-filtered by passing through 0.22 micron filter).

Cells were then plated into a collagen-coated T25 flask, which was incubated at 37 °C in a humidified, 5% CO₂ incubator for 2 h. Cells were then examined under a microscope, to confirm that the isolated cell suspension contained large numbers of refractive nuclei, as well as other debris. Two hours after plating, early adhering cells were noted to attach to the flask; and this first population of adherent cells was labelled as PP1. Supernatant containing non-adherent cells was transferred into a collagen-coated T-25 flask, labelled PP2 (preplate 2). 5 ml of PM was then added to the plate containing PP1, and flasks were returned to the incubator.

Passage: After 24 h, the supernatant of the PP2 flask was transferred into a 15-ml tube and centrifuged at 930 x g at 4 °C for 5 min. Supernatant was disposed, and the resulting cell pellet was resuspended in 5 ml of PM. The cell suspension was then transferred into a new

T-25 flask, labeled PP3. 5 ml of PM were added to the PP2 flask, which was returned to the incubator.

The above passage process was repeated every 24 h until population PP6 was created. This last cell suspension was maintained in the T-25 flask, and was examined under an inverted microscope. Slowly adhering cells were confirmed to have attached by PP6. At this stage, cells appeared small, round, refractive and sparse.

MDScs obtained by this process were then expanded in the following manner: the adherent cells were washed with 3 ml of PBS, 2 ml pre-warmed 0.1% (wt/vol) trypsin-EDTA solution was added, and the mixture was incubated at 37 °C for 2–3 min and subsequently examined under the microscope to ensure that all of the cells were detached. The reaction was stopped by adding 3 ml of PM, and the mixture was centrifuged at 930 x g at 4 °C for 5 min. Count the cells in the pellet were counted and replated at a density of 225–250 cells/cm². Maintain the Cells were maintained at a low cell density (50% confluence), thereby avoiding the appearance of differentiation involving forming multinucleated myotubes and colonies of flat and spindle-shaped cells that would have been observable under the microscope. Cell culture involved changing media every other day with PM, as noted above. Cells became visible at 5-6 days post-isolation, and passage was performed normally when cells were 50% confluent.

Cells obtained by the above procedure were used to create the scaffold-bone induction agent-BMP2 compositions of the system of the invention by associating them with a collagen I scaffold and BMP2 of specific embodiments of the invention. The lineage characterization of the slowly adhering cell populations obtained from MDCs via the above-described expansion protocol can optionally be characterized for Myogenic, Endothelial, Hematopoietic, and Fibroblast components.

Example 2: Cell harvest and culture

MDSc were harvested from murine skeletal muscle from the hind limb of C57BL/6 or C57BL/6-Tg(CAG-EGFP)10sb, depending on the need for fluorescent emission under asptic conditions and were minced using sterile forceps and scalpel and further processed for culture on collagen coated plates as previously described by Lavasani M, *et al.*, *Methods Mol Biol.* 976: 53-65. Identity of the MDSc population was characterized by flow cytometry directed at the following CD markers: Sca-1, CD29, CD105, CD73 and CD34 expression and low CD31, CD 56, CD144 and CD146. Additionally, immunofluorescent imaging was used

to validate the finds in flow cytometry using the following CD markers and Alkaline Phosphatase expression. To further corroborate the protein directed identification platforms, a subset of the population which underwent RNA insolation and was assayed using a high throughput Mesenchymal Stem Cells PCR Array (SABiosciences®). Cells were cultured in basal MSC proliferative media (BMPM) [392.5 mL Dulbecco's Modified Eagle Medium (DMEM, high glucose; Invitrogen, Cat. #11995-073), 50 mL FBS (10%), 50 mL HS (10%), 5 mL Penicillin/Streptomycin (1%) and 2.5 mL Chick Embryo Extract (CEE; Accurate Chemical Co. Cat. #CE650T-10)] until reaching 70% confluence. Cells were then collected with 0.1% (wt/vol) Trypsin-EDTA (Life Technologies. #15400-054), washed with BMPM and spun at $1,932 \times g$ for 5 min to achieve a soft pellet. Supernatant was aspirated and the cell pellet was washed with HBSS (Invitrogen, Cat. #24020-117).

Cells undergoing *in vitro* tri-lineage differentiation were treated with the StemPro® Adipogenesis, Chondrogenesis or Osteogenesis Differentiation Kits (Life Technologies) per the manufacture's provided manual and protocols. Cells utilized for collagen implant studies were seeded onto 1 cm x 1 cm x 0.2 cm type-1 collagen scaffolds under the same basal MSC proliferative media conditions as those plated on collagen coated culture dishes. Experimental osteogenic induction studies performed on 2D and 3D cultured systems employed basal MSC proliferative media supplemented with 1.0 µg/ml of Bone Morphogenetic Protein-2 Human Recombinant ProSpec™ Bio Protein-Specialists.

ADSCs were harvested using previously published methods by Lough DM, et al., *Plast Reconstr Surg.* 2014 Mar;133(3):579-90. Briefly mice were shaved, cleansed with 70% ethanol and HBSS. Circumferential full thickness skin was harvested and underlying subcutaneous inguinal fat pads were collected. Fat was washed 3x in HBSS containing Penicillin/Streptomycin (1%) for 5 minutes each and then SVF isolation was carried out following the Pittsburg Protocol.

The bone marrow MSC were then harvested. Following dissection of skeletal muscle from disarticulated lower limb for MDSc isolation, a 30-gauge needle on a 10 cc syringe containing BMPM was used to flush the bone marrow cells from both ends of the bone shafts into a 50 ml conical top tube fitted with a 100 µm filter (BD Falcon). Further cellular isolation utilized a previously published methods by Soleimani M and Nadri S. *Nat Protoc.* 2009;4(1):102-6.

Example 3: Animal model and studies

All animal studies presented in this article were evaluated and approved by the Johns Hopkins University Animal Care and Use Committee (ACUC) under protocol no. MO14166 and complied with the Recognition and Alleviation of Distress in Laboratory Animals Committee on Recognition and Alleviation of Distress in Laboratory Animals, National Research Council (2008).

Cranial defect studies: using a C57BL/6 murine model (n=30) 5 mm full-thickness cranial defects (left parietal skull) were operatively created in C57BL/6 (n=30) using a powered 5 mm hollow core bur hole bit. Defects then received either 1.) no implant 2.) an acellular collagen-only implant or 3.) a cellular enriched implant containing either ADSC^{eGFP}, BM-MSC^{eGFP} or MDSc^{eGFP}. Additionally, a subgroup was used to compare fluorescent emission of eGFP-expressing cells to contralateral intact skulls and acellular implants, in order to determine a comparative baseline for cell tracing and viability studies. All animals received post-operative subcutaneous normal saline fluid resuscitation and buprenorphine pain control. Fluorescent emission was monitored using a UVP iBox® Spectra™ In Vivo Imaging System at weeks 1, 4 and 8 to monitor viability of ADSC^{eGFP}, BM-MSC^{eGFP} or MDSc^{eGFP} enriched implants. At 8 weeks, animals were imaged using a Carbon nanotube (CNT) based microCT imaging system and Zeiss AxioExaminer with 710NLO-Meta multiphoton and confocal modules to determine the resultant ultrastructure of subsequent bone formation and correlative location and/or organization of eGFP expressing cellular elements. Additional explant imaging studies utilized a Zeiss 510 Meta laser scanning microscope to determine vascular ingrowth of implants and relative quantity and location of ADSC^{eGFP}, BM-MSC^{eGFP} or MDSc^{eGFP} populations within Z planar stack cross sections.

Example 4: Type-1 collagen and Bone Morphogenic Protein-2 (BMP2) augmented the rate of polarized bone formation from cellular expansion foci

Co-culture of osteo-induced populations (MDSc + rhBMP2 + Collagen I) with non-induced MDSc populations following 48 hours of barrier separation permitted defined cortical bone and cancellous bone elements following the allowance of cell to cell contact (Figures 1A and 1B). The osteo-induced population underwent cortical neo-genesis while the non-induced population subsequently developed cancellous or trabecular elements within fourteen days. Furthermore, the MDSc enriched populations formed both expansions foci

and polarized bone elements in 3D collagen scaffolds following BMP2 treatment (Figures 1C and 1D).

Example 5: MDSCs were viable throughout the healing process and mediated bone regeneration

Significant healing of a 5 mm cranial defect using a collagen disk (Dia. 5 mm) with 5 μ g rhBMP2 and 2×10^6 MDSCs by 3 weeks was demonstrated (Figure 2A). In contrast, an empty defect of 5 mm diameter was found to have no appreciable healing up to 8 weeks. Transplanted murine eGFP-MDSCs within the biological scaffolds at 1, 2, and 3 weeks after implementation with *in vivo* epifluorescence using the Xenogen IVIS system, were detected (Figure 2B).

Example 6: Defects treated with collagen scaffold only, and increasing BMP doses did not heal, whereas treatment with MDSCs mediated more normal and robust bone formation

Defects treated with collagen scaffold only and collagen with BMP and increasing doses of 1 μ g and 5 μ g did not heal at 8 weeks (Figure 3A). In contrast, defects treated with 2×10^6 MDSCs seeded on a collagen scaffold demonstrated dose dependent rhBMP2-mediated healing with more normal and robust bone formation by increasing doses of rhBMP2 from 0.1 μ g to 1 μ g and finally to 5 μ g of rhBMP2 (Figure 3B).

Example 7: MDSc-BMP2-Collagen I Scaffold Compositions Effected Functionally Polarized Bone Repair, Promoted Angiogenesis and Performed Vasculogenesis in Treated Mice

The enrichment process used to obtain MDSc enrichment for the currently exemplified MDSc-BMP2-Collagen I scaffold compositions is depicted in the drawing of Figure 4A, particularly noting the expansion/passage process employed. As shown in Figures 4B and 4C, polarity establishment and structures relevant to healing of bone defects were identified as formed upon formation and use of a collagen 1 scaffold + BMP2 + MDSc population composition of the invention. As shown in Figure 5, which shows elements of the model system testing process, a drill was used to produce uniform 5 mm diameter cranial defects in treated mice of the above and instant example.

The BMP2 + collagen I scaffold MDSCs were confirmed to have produced cell cycle progression, as well as enhanced multiplication and proliferation of treated cells, as compared

to non-treated cells (Figures. 6A to 6C). Notably, increasing doses of BMP2 in such compositions showed progressively increased cell cycle progression response (Figures. 6B and 6C).

To confirm the multipotency of the MDSCs isolated and used in the invention, MDSCs were demonstrated as capable of the following forms of differentiation: reversion to muscle cells (if left untreated, data not shown), adipogenesis, chondrogenesis and osteogenesis (Figure 7B). Thus, MDSCs employed in the invention were multipotent.

One remarkable finding for the tested MDSC-BMP2-Collagen I scaffold compositions of the invention was the ability of such a system to produce functionally polarized repair of bone defects. Scaffold and migratory kinetics of the MDSCs of the compositions of the invention were examined, and such studies helped confirm that appropriately functionally polarized healing was occurring in target bone defects treated with the compositions of the invention – i.e., both scaffold and migratory kinetics of MDSCs of the invention were established to migrate into bone, performing repair of bone defects in a functionally polarized manner capable of complete repair of such defects (Figure 8).

Polarized bone formation *via* use of the compositions of the invention was further documented. Specifically, the combination of BMP2 and type 1 collagen was further established to have acted as a mitogen in MDSC enriched populations, leading to polarized bone formation. The real-time proliferation index and volume index, as well as mitosis and cell cycle progression of MDSC enriched populations plated at 10^6 over 18 hours was found to increase upon treatment with both BMP2 and Collagen I scaffold, with results obtained using live confocal imaging (Figures 9A to 9E).

Following optimization of migratory kinetics and lineage induction, MDSC were applied to a spectrum of engineered scaffolds for traceable, real-time *in vivo* studies within defect models (Fig. 10).

The process of *in vitro* and organotypic optimization permitted the application of such findings to real-time regenerative living studies, which showed remarkable efficacy in augmenting the healing of bone defects with functional polarized bone (Figures 11A and 11B).

MDSC-BMP2-collagen I scaffold composition treatments were specifically observed to have dramatically augmented polarized bone healing and diploic space formation in a treated subject, with *in vivo* cortical and cancellous bone formation observed following

delivery of such MDSc seeded scaffolds at week three in an explant. Murine eGFP expressing MDSc (2×10^6) seeded onto BMP2 bound Col-1 (collagen I) scaffolds produced a polarized bone construct (cortical and cancellous bone architecture) within 14-21 days following implantation into 5 mm diameter full-thickness skull defects (Figure 12A). A Z-stack through the nascent diploic space (which was remarkably re-established in cranial defects upon treatment with the exemplary compositions of the invention) was observed for explanted MDSc collagen scaffold at eight weeks.

Not only did the collagen I scaffold-MDSc and BMP2 compositions of the invention achieve remarkable repair of bone defects, in a functionally polarized manner consistent with native bone morphology, the collagen I scaffold-MDSc and BMP2 compositions of the invention also both promoted angiogenesis and produced localized vasculogenesis *in vivo* (Figure 13). Observation of MDSc cells of the transplanted scaffold-MDSc-BMP2 compositions forming new blood vessels at the site of therapy was particularly surprising (Figure 13, top right images), and indicated that the collagen I scaffold-MDSc and BMP2 compositions of the invention could possess high value for promotion of angiogenesis and production of vasculogenesis in a localized manner for a variety of conditions in which such processes would be beneficial (*e.g.*, not only bone healing, but also coronary/vascular induction (*e.g.*, cardiac treatment), muscle and/or joint/cartilage repair, etc.). Thus, the compositions of the invention can be broadly and advantageously applied to a number of conditions, diseases and/or disorders, to achieve positive therapeutic outcomes.

Example 8: MDScs generated myogenic cellular entities while maintaining a multi-lineage potency comparable to other MSC populations

Skeletal muscle has remained a primary source of satellite and progenitor cells, which are primarily responsible for muscle regeneration following injury. Recently, however, muscle tissue has also been identified as a valuable source of adult MDScs, which appear to be distinct from satellite cells in that they intrinsically possess the ability to undergo multi-lineage differentiation (Usas A, et al., *Biomaterials*. 2007 Dec;28(36):5401-6, Deasy BM, et al., *Blood Cells Mol Dis*. 2001 Sep-Oct;27(5):924-33, and Cao B, et al., *Nat Cell Biol*. 2003 Jul;5(7):640-6). While MDScs possess a high myogenic capacity, which has been shown to effectively regenerate both skeletal and cardiac muscle, this unique population can also undergo adipogenic, chondrogenic and osteogenic differentiation (Asakura A, et al.,

Differentiation. 2001 Oct;68 (4-5):245-53, and Wada MR, et al., *Development*. 2002 Jun;129(12):2987-95).

To define the cellular capacity of the MDSc population to undergo multi-lineage differentiation, C57BL/6 hind limb skeletal muscle, inguinal fat pads and bone marrow contents were harvested separately, thereby allowing filtered cell suspensions of muscle, fat and bone marrow to be cultured in distinct media solutions (including, for example, basal MSC proliferative media, as well as separate myogenic, adipogenic, chondrogenic and osteogenic induction media) (Figures 14A and 14B). Within 24 hours, replicate cultures containing muscle-derived cell suspensions exhibited multinucleated linear cell structures, which correlated to myogenic precursors (Figures 14A and 14B). Cultures that continued to 100% confluence in myogenic media sustained these myogenic precursors until they matured into beating myocytes surrounded by alternative stromal cell populations. Such multinucleated linear precursor cells were less evident in muscle-derived cell suspensions that were cultured in basal MSC proliferative media, confirming an apparent myogenic signal induction requirement for myocyte formation.

While fat and bone marrow-derived cell suspensions did not readily exhibit myogenic cell differentiation, all cell suspensions were able to undergo tri-lineage differentiation following adipogenic, chondrogenic and osteogenic induction (Figures 14A and 14B). Such results indicated that cell suspensions derived from fat, bone marrow and muscle contained populations of ADSCs, BM-MSCs and MDSCs, respectively. Further, while ADSCs, BM-MSCs and MDSCs maintained classic tri-lineage cellular potency, MDSCs also harbored an intrinsic capacity to undergo immediate myogenic induction.

Example 9: MDSCs generated self-organizing corticocancellous bone ultrastructure while expanding in culture

A variety of MSC populations that harbor the capacity to undergo osteogenic induction have previously been utilized to generate bone within closed culture systems and animal models (Alghazali KM, et al., *Drug Metab Rev*. 2015 Dec 10:1-24, and Smith DM, et al., *Plast Reconstr Surg*. 2011 Nov;128(5):1053-60). Despite continued success and a variety of applications among extensively studied unique stem cell populations, such as ADSC, BM-MSC, as well as the more recently discovered Leucine-rich repeat-containing G-protein coupled receptor 5 and 6 (LGR5 and 6) epithelial stem cell and induced pluripotent stem cell (iPS) entities, none have been able to regenerate a form of self-organizing osseous substrate that accurately embodies the native appearance of bone. Reasons for such limitations reveal

the complexity of proper phenotype development and how, despite harboring the potential of multi-lineage differentiation within a transcriptome, cells require specific environments, growth factors and cell-to-cell interactions to truly act in a functional regenerative manner (Suenaga H, et al., *J Mater Sci Mater Med.* 2015 Nov;26(11):254, Seebach C, et al., *Tissue Eng Part A.* 2015 May;21(9-10):1565-78, and Seebach C, et al., *Cell Transplant.* 2012;21(8):1667-77). In essence, without the proper niche interface and subsequently related commanding factors, potent cells either achieve some limited form of differentiation or undergo cellular senescence, neither of which results in practical bone formation.

In view of the importance of the proposed niche interface to MSC functional development, the ability of the MDSc of the musculoskeletal system to undergo an alternative form of osteogenic induction when provided with a common biologic binding surface (type-I collagen) and contacted with BMP-2 (Bone Morphogenetic Protein 2) was examined. With the initial intent of determining the comparative effect(s) of the biologic binding surface upon cell populations, discrete ADSC, BM-MSC and MDSc groups were isolated and plated at 2×10^6 cells/mL on collagen-coated plates and cultured in basal MSC proliferative media. At 24 and 48 hour time points, quantification of both number and size of mitotic cellular focal aggregates were compared across ADSC, BM-MSC and MDSc replicates (Figures 15A and 15B). At 24 hours the average relative quantity (n) of distinct ADSC, BM-MSC and MDSc mitotic cellular foci per high powered field were ($0.8 \pm 0.2SD$), ($4.7 \pm 0.7 SD$) and ($6.2 \pm 0.9 SD$), respectively, while the related relative diameter (μm) of each mitotic cellular foci were ($86.3 \pm 12.4 SD$), ($263.6 \pm 27.4 SD$) and ($142.7 \pm 18.2 SD$).

At the 48 hour interval timepoint, differences in ADSC, BM-MSC and MDSc population phenotypes and/or organizational behavior became grossly apparent under live phase contrast microscopy (Figures 15A and 15B). Whereas both the ADSC and BM-MSC group depicted a common behavior of central mitogenic proliferation and circumferential migration from the proliferative foci, the MDSc formed an additional structural element that was distinct from and not identified in any of the ADSC or BM-MSC replicates. Present within all MDSc culture replicates were linear assemblies of cells, which were organized into elevated, bridging support systems (Figure 15A, black arrows). These cell-to-cell interactive structures joined the proliferative focal aggregates and permitted other cells to bind to and migrate vectorially along the self-assembled, cellular-derived scaffold.

Having compared the ADSC, BM-MSC and MDSc expansion foci in the presence of a biologic binding surface, examination of how the addition of BMP-2 further altered the organization of these distinct populations was undertaken. Utilizing the above-established

system, ADSC, BM-MSC and MDSc groups were isolated and plated at 2×10^6 cells/mL on collagen-coated plates cultured in basal MSC proliferative media, and additionally supplemented with 1.0 $\mu\text{g}/\text{ml}$ of BMP-2. At day 7, the cultures were prepared and stained with Alizarin Red solution to determine relative bone formation and generalized architecture of the cellular material generated (Figure 2). ADSC replicates exhibited a typical dispersed form of micro-aggregate bone deposition, while BM-MSC culture replicates demonstrated satellite forms of similar micro-aggregates surrounding larger centralized ossified foci. Alternatively, the MDSc groups were capable of forming organized corticocancellous bone, which readily bound Alizarin Red staining solution throughout the resultant osseous ultrastructure.

These resultant findings indicated that although ADSC, BM-MSC and MDSc all possessed some form of MSC multi-lineage potential, each population retained an intrinsic preferred behavior while in the presence of specific culture conditions, biologic binding surface agents and/or inductive growth factors. While the ADSC, BM-MSC and MDSc all formed an arrangement of bone product, the MDSc was the only MSC subpopulation capable of generating a form of self-organizing tissue that resembled cortical and cancellous bone in a 2D *in vitro* system.

Example 10: MDSCs generated 3D organized bone on deployable biologic implants for delivery into living systems

Noting the importance of the proposed niche interface to MSC functional development, the MDSc population that readily formed corticocancellous bone structure in a 2D system was then evaluated for whether it could undergo similar self-assembling osteogenic differentiation in a 3D system, resulting in a bone construct capable of implantation into living systems.

To test for translational utility, ADSC, BM-MSC and MDSc groups were harvested and seeded at 2×10^6 cells/mL upon 3D collagen scaffolds and cultured in basal MSC proliferative media, supplemented with 1.0 $\mu\text{g}/\text{ml}$ of BMP-2. Groups were monitored over 14 days to compare the above 2D culture findings to cellular activities in a 3D system.

While ADSC, BM-MSC and MDSc populations all exhibited similar osteogenic differentiation and migratory behaviors in 2D culture, the MDSc replicate groups immediately began to exhibit a new and different form of structural organization in the 3D system (Figure 16A). At 72 hours, scaffold-adherent MDSCs began to advance rigid linear structures, which were capable of elevating cell populations directly up and away from the

collagen scaffold at approximately 70° -90° angles (white arrow). Further culture progression revealed that the MDSc-derived structures were capable of spanning inter-scaffold spaces, to adhere to and join separated scaffolds (black arrow) while also binding the plastic culture vessel (black ball tip arrow). Within 14 days, the culture wells holding MDSc-enriched collagen scaffolds(s) contained densely organized bone-appearing 3D corticocancellous tissue, while ADSC and BM-MSC exhibited similar aggregate organization as seen in the above-described 2D system.

To investigate these *in vitro* results for translational and pragmatic clinical application in reconstructive surgical efforts, an MDSc-enriched biologic scaffold was examined for possible function as a form of autologous bio-reactive implant similar to an autograft, within a well-established cranial defect model. Using tissues harvested from C57BL/6-Tg(CAG-EGFP)10sb, which intrinsically express enhanced green fluorescent protein (eGFP), populations of 2×10^6 of ADSC^{eGFP}, BM-MSC^{eGFP} or MDSc^{eGFP} were separately seeded onto collagen scaffolds and cultured under osteoinductive media conditions for 14 days, to define cellular traceability once implanted into a syngeneic C57BL/6 cranial defect, while also comparing the real-time changes observed in the structural phenotype of each population within a 3D *in vivo* osteoinductive environment (Figure 16B). ADSC^{eGFP}, BM-MSC^{eGFP} and MDSc^{eGFP} replicates all displayed similar structural arrangements as those seen in the above 2D and 3D systems for non-fluorescent cell types over the 14 days. Intermediate day 7 was provided as an exemplary depiction of the cell-enriched scaffolds (Figure 16B).

After comparative validation of the ADSC^{eGFP}, BM-MSC^{eGFP} and MDSc^{eGFP} with prior non-fluorescent cell populations, 2×10^6 of ADSC^{eGFP}, BM-MSC^{eGFP} or MDSc^{eGFP} were separately seeded onto collagen scaffolds and allowed to adhere in culture for 2 hours. 5 mm diameter full-thickness cranial defects were operatively created in C57BL/6 (n=30) using a 5 mm hollow core drill bit. Defects then received either 1.) No implant 2.) An acellular collagen implant or 3.) A cell-enriched implant containing either ADSC^{eGFP}, BM-MSC^{eGFP} or MDSc^{eGFP} (Figure 3c.). Fluorescent emissions of ADSC^{eGFP}, BM-MSC^{eGFP} and MDSc^{eGFP} were compared *in vivo* with non-defect skulls and acellular collagen implants, to determine baseline auto-fluorescence and track cellular construct viability post-operatively (Figure 16C).

These results indicated that ADSC^{eGFP}, BM-MSC^{eGFP} and MDSc^{eGFP} were capable of viably seeding collagen-based scaffolds while maintaining the ability to undergo osteogenesis and surgical implantation into a living cranial defect system. However, MDScs seeded onto a 3D collagen scaffold, unlike ADSC and BM-MSC groups, appeared uniquely capable of self-

assembly into a 3D tissue strongly resembling corticocancellous bone through apparent scaffold outgrowth, anchoring and multi-substrate spanning behaviors.

Example 11: Enriched biologic implants were capable of regenerating vascularized corticocancellous bone and even diploic space in a living system

The fields of orthopedic, plastic and craniomaxillofacial surgery are privileged with a wide variety of implant-based devices and/or biologics which can be applied to further reconstructive efforts in clinical medicine. However, despite ever-advancing technologies, none of these implants truly employ an autologous stem cell-enriched construct capable of potentiating neo-osteogenic regeneration within a bone defect. Moreover, without engaging a regenerative mechanism of nascent bone development, native architecture and tissue polarity remains unobtainable, thus leaving many of the constructs currently available simply augmenting fibrous scar for defect coverage.

Because of the essential need for a deployable autologous construct capable of complete and accurate bone regeneration, the ability of an MDSc-enriched implant to regenerate 3D corticocancellous *in vivo* over an 8 week time period within the established cranial defect model was comparatively analyzed. At 8 weeks, cranial defects that received either implant control(s) or cellular-enriched implants (2×10^6 of ADSCs^{eGFP}, BM-MSCs^{eGFP} or MDSCs^{eGFP}) were imaged and harvested, to assess relative healing indices, bone formation and vascular ingrowth (Figures 17A and 17B).

While assessment of acellular implant controls revealed a sunken and non-rigid scar within the defect, ADSC^{eGFP}-enriched implants developed fibrotic rims around the wound with particulate bone aggregates intermixed with scar tissue (white pentagons)(Figure 17A). Defects administered implants containing BM-MSCs^{eGFP} also developed fibrotic rims around the defect, with larger aggregates intermixed within thicker scar tissue. Additionally, BM-MSCs^{eGFP} also notably developed a higher grade of blood vessel ingrowth, resulting in hemorrhagic tissues (white pentagon). Those defects which received implants seeded with MDSCs^{eGFP} showed significantly less fibrotic material at the rim of the defect and significantly more bone formation. Moreover, the intra-defect bone grossly exhibited more planarity as compared to the irregular amorphous bone aggregates seen for ADSC^{eGFP} and BM-MSC^{eGFP} replicates (Figures 17A). The bone developed within the MDSCs^{eGFP} exhibited more organized blood vessel formation and a less irregularity at the construct-native bone interface (white pentagon).

To investigate the extent of cellular integration and regenerative potential further, laser scanning multi-photon confocal microscopy (Figures 17B) was utilized. Imaging of constructs prior to explantation revealed significantly more vascular ingrowth (white arrows indicating RFP labeled blood vessels) into MDSc^{eGFP}-enriched implants, which clearly demonstrated green fluorescence of the MDSc^{eGFP}. Such findings were not present in ADSc^{eGFP} and BM-MSc^{eGFP} replicate groups. Additionally, corticocancellous ultrastructure was also observed in defects administered MDScs^{eGFP}, whereas no other implants examined provided similar cellular organization. Sectional Z-plane scanning of the construct within the skull at 8 weeks revealed a diploic space (dual headed white arrows). This diploic formation was comparable to native bone tissues only in those defects administered MDSc^{eGFP}-enriched implants. Diploic space and relative bone formation was further investigated and validated using a miniCT scan imaging system, which demonstrated comparable bone substrate density (Figures 17B).

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

CLAIMS

We claim:

1. A method for treating a bone defect in a subject, the method comprising:
obtaining a muscle-derived cell (MDC) population;
contacting the MDC population with a bone induction agent;
applying the MDC population to a scaffold, thereby forming a MDC-scaffold composition; and
contacting the MDC-scaffold composition to a bone of a subject, wherein the bone comprises a bone defect,
thereby treating the bone defect in the subject.
2. The method of claim 1, wherein the MDC population is enriched for muscle-derived stem cells (MDScs).
3. The method of claim 2, wherein the MDC population is isolated from a preplate derived by the method of Gharaibeh, B. *et al. Nature Protocols* 3: 1501-1509, and optionally is a preplate 1 (PP1) to preplate 6 (PP6) cellular population.
4. The method of claim 3, wherein the MDC population is isolated from preplate 2 (PP2) or preplate 3 (PP3).
5. The method of claim 1 or claim 2, wherein the MDC population is derived from the subject having the bone defect that is treated.
6. The method of any of the above claims, wherein the MDC population is an *ex vivo* expanded MDC population.
7. The method of claim 6, wherein the MDC population is fibroblast-depleted, as compared to an unexpanded control MDC population.
8. The method of any of the above claims, wherein the bone induction agent is a bone morphogenetic protein.

9. The method of any of the above claims, wherein the bone induction agent is BMP2, optionally rhBMP2 or a fragment thereof.
10. The method of any of the above claims, wherein the bone induction agent is present at a concentration sufficient to osteo-induce the MDC population.
11. The method of any of the above claims, wherein 1-10 μg of the bone induction agent is present in the MDC-scaffold composition, optionally wherein 5 μg of the bone induction agent is present in the MDC-scaffold composition, optionally wherein the bone induction agent is present in the MDC-scaffold composition at a concentration of at least 10 ng/ml, optionally at least 100 ng/ml, optionally at least 1 mg/ml.
12. The method of any of the above claims, wherein the scaffold is a Collagen I scaffold, optionally a Collagen I scaffold disk, optionally a Collagen I scaffold disk of 1 to 10 mm diameter, optionally of 5 mm diameter.
13. The method of any of the above claims, wherein the bone defect is a defect of 1 to 10 mm diameter, optionally of 5 mm diameter.
14. The method of any of the above claims, wherein the MDC population comprises at least 1×10^4 cells, optionally at least 1×10^5 cells, optionally at least 1×10^6 cells, optionally about 2×10^6 cells.
15. The method of any of the above claims, wherein the size of the bone defect is significantly reduced in size or is healed within eight weeks of contacting the MDC-scaffold composition to the bone of the subject, optionally within four weeks of contacting the MDC-scaffold composition to the bone of the subject, optionally within three weeks of contacting the MDC-scaffold composition to the bone of the subject.
16. The method of any of the above claims, wherein contacting the MDC-scaffold composition to a bone of the subject results in functionally polarized healing of the bone defect of the subject.
17. The method of any of the above claims, wherein the subject is a mammalian subject.

18. The method of any of the above claims, wherein the subject is human.
19. A method for promoting angiogenesis in a subject, the method comprising:
 - obtaining a muscle-derived cell (MDC) population;
 - contacting the MDC population with a bone induction agent;
 - applying the MDC population to a scaffold, thereby forming a MDC-scaffold composition; and
 - contacting the MDC-scaffold composition to a tissue of a subject, wherein the tissue comprises a region of injury, disease, disorder and/or lack of blood vessels in need of angiogenesis,thereby promoting angiogenesis in the subject.
20. The method of claim 19, wherein the tissue is selected from the group consisting of a cardiac tissue, a bone tissue, a muscle tissue, a wounded tissue and a joint tissue, optionally wherein the joint tissue is a knee joint tissue.
21. A composition for treating a bone defect in a subject, promoting angiogenesis and/or producing vasculogenesis in a tissue of a subject comprising:
 - a muscle-derived cell (MDC) population;
 - a bone induction agent; and
 - a scaffold,wherein the composition is capable of treating a bone defect, promoting angiogenesis and/or producing vasculogenesis in a subject when applied to a bone or other tissue of the subject, as compared to an appropriate control composition.
22. A kit for treating a subject having a bone defect, the kit comprising a muscle-derived cell (MDC) population, a scaffold and instructions for its use.
23. The kit of claim 22, further comprising a bone induction agent.
24. The kit of claim 22, wherein the muscle-derived cell population is enriched for muscle-derived stem cells (MDScs).

25. The kit of claim 23, wherein the bone induction agent is BMP2, optionally rhBMP2 or a fragment thereof.

25. The kit of claim 22, wherein the scaffold is a Collagen I scaffold, optionally a Collagen I scaffold disk, optionally a Collagen I scaffold disk of 1 to 10 mm diameter, optionally of 5 mm diameter.

26. A method for treating a bone defect in a subject, comprising:

(a) identifying a bone defect in the subject; and

(b) using the kit of claim 22 to treat the bone defect in the subject.

Figure 1

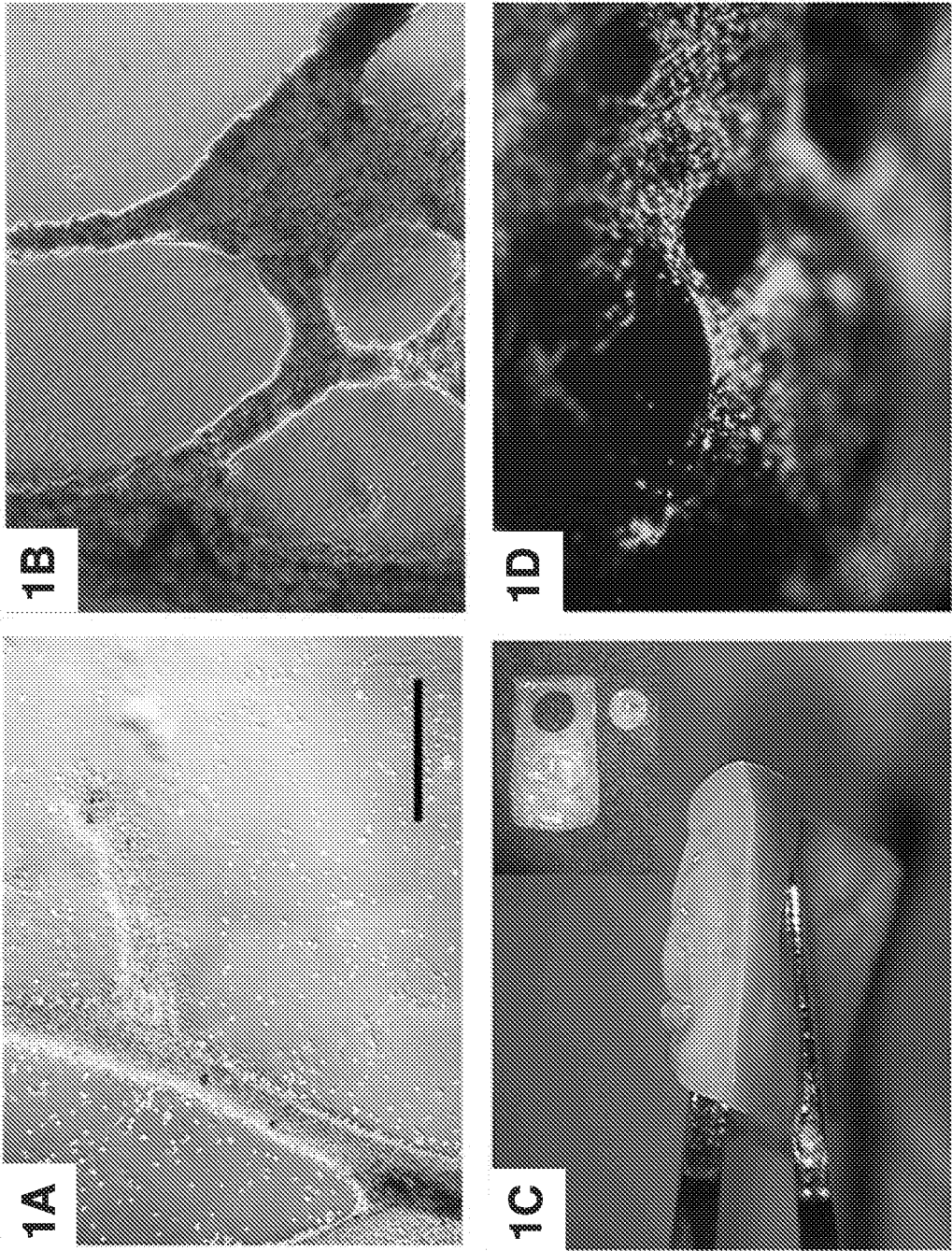


Figure 2

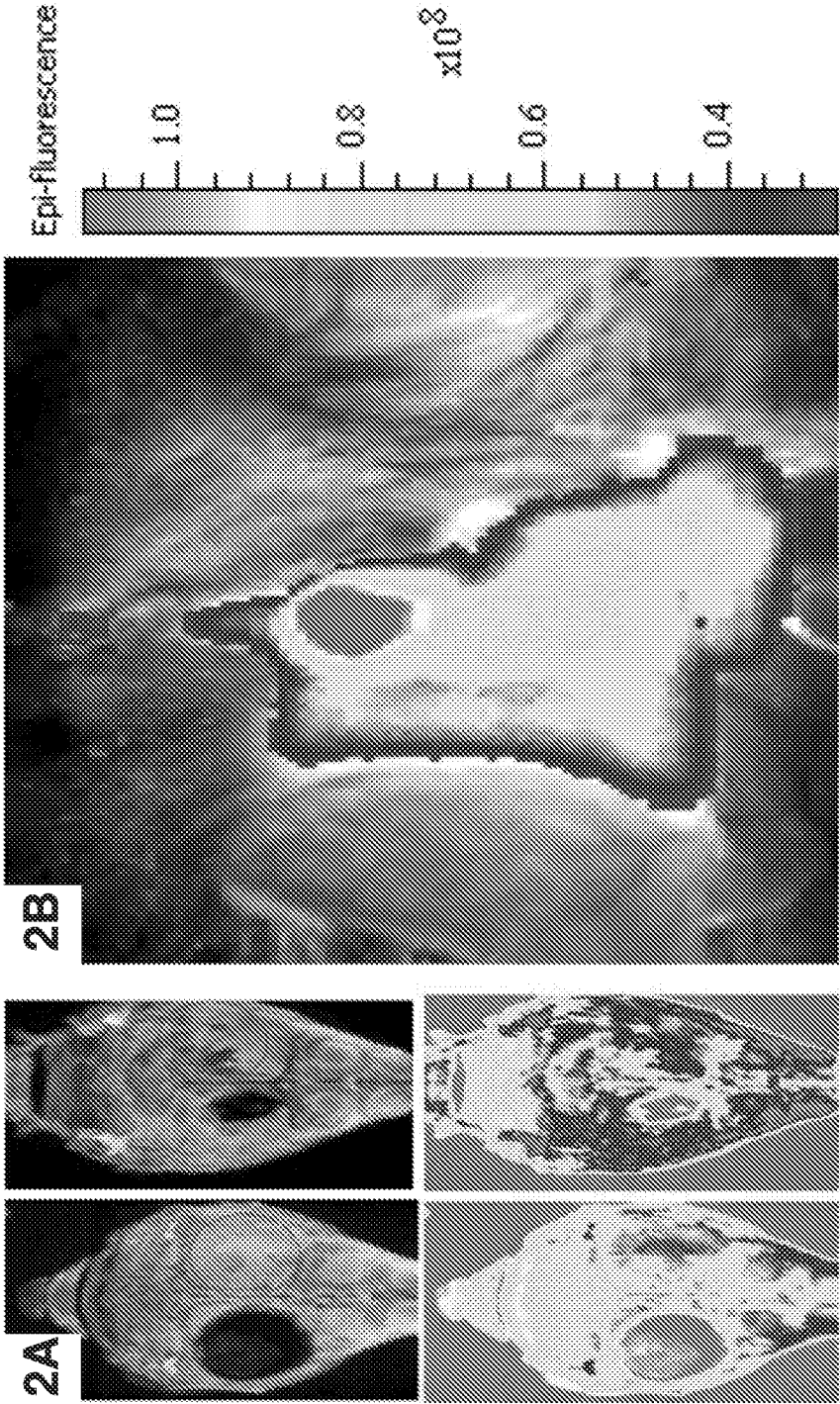


Figure 3

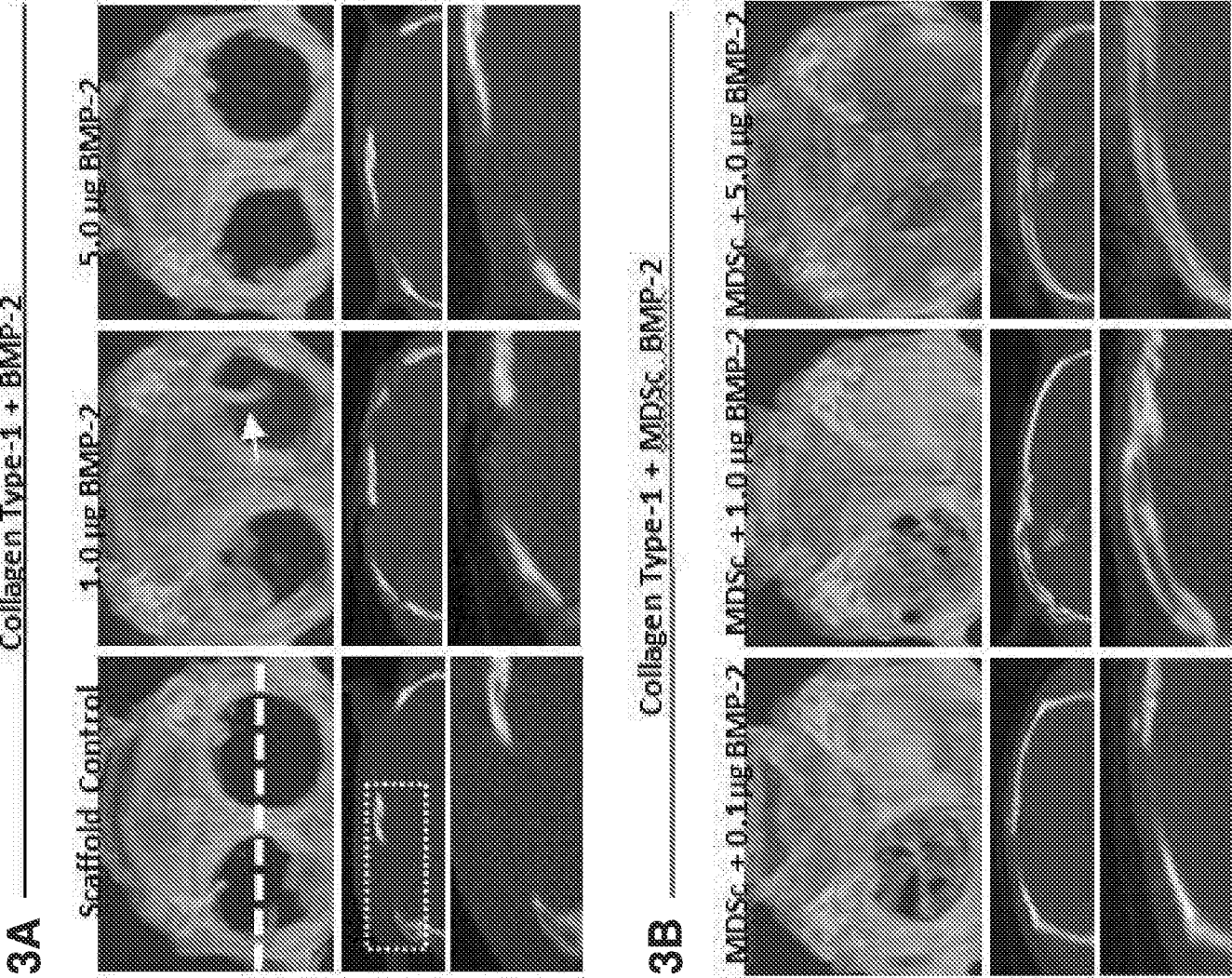


Figure 4

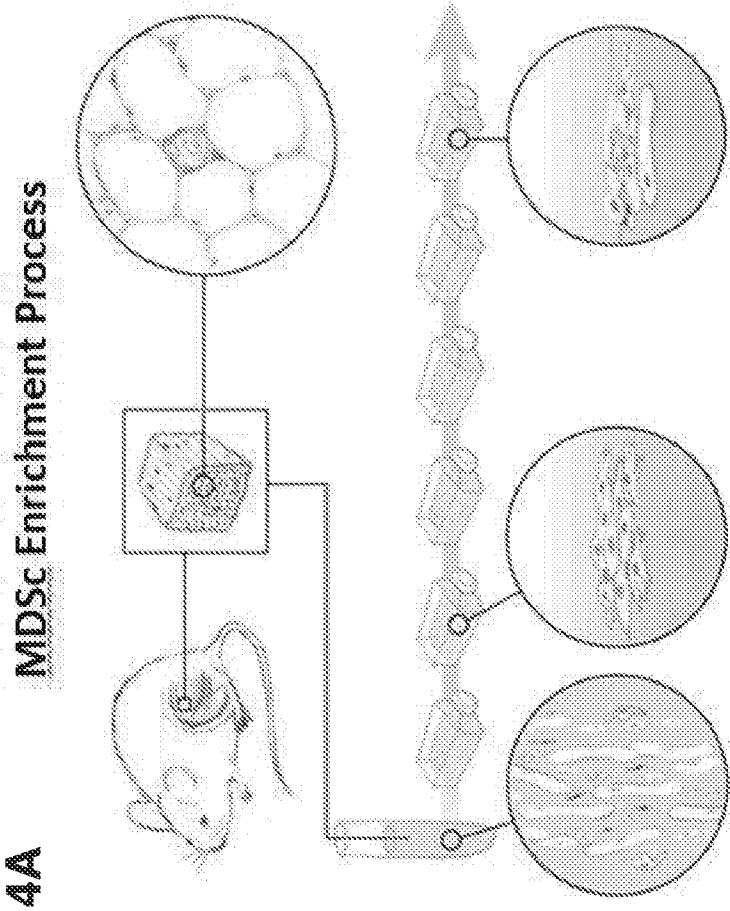
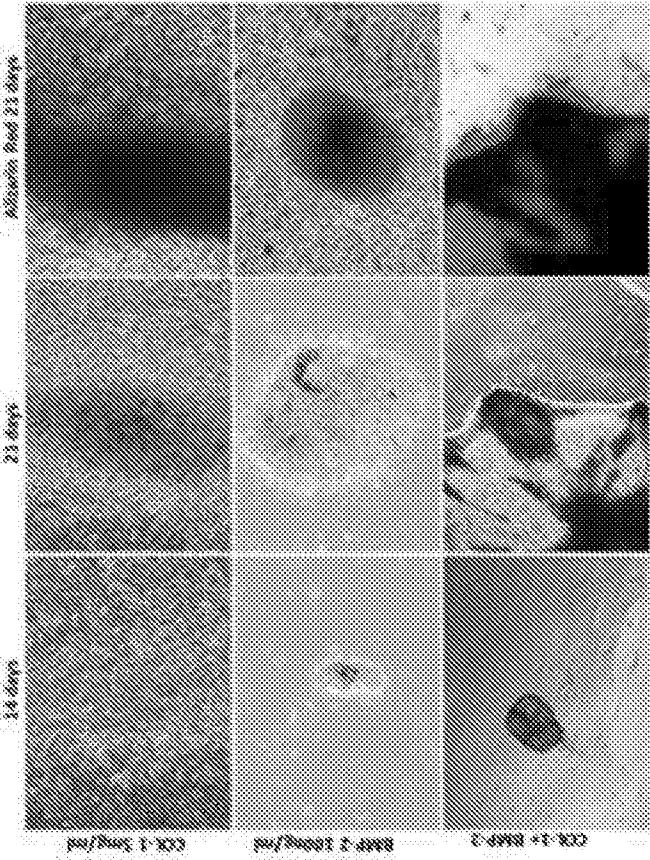


Figure 4 (Continued)

4B



4C

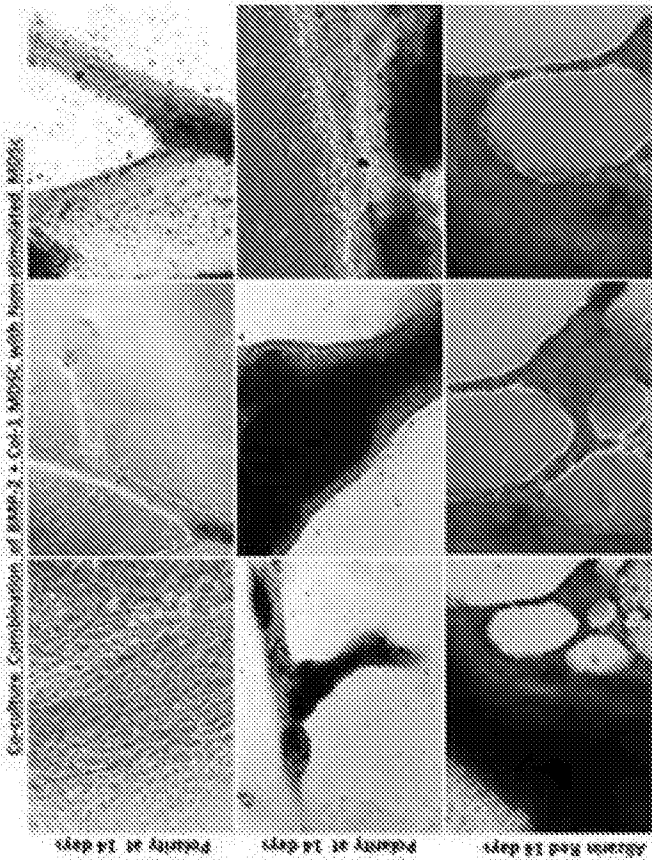




Figure 5

Figure 6

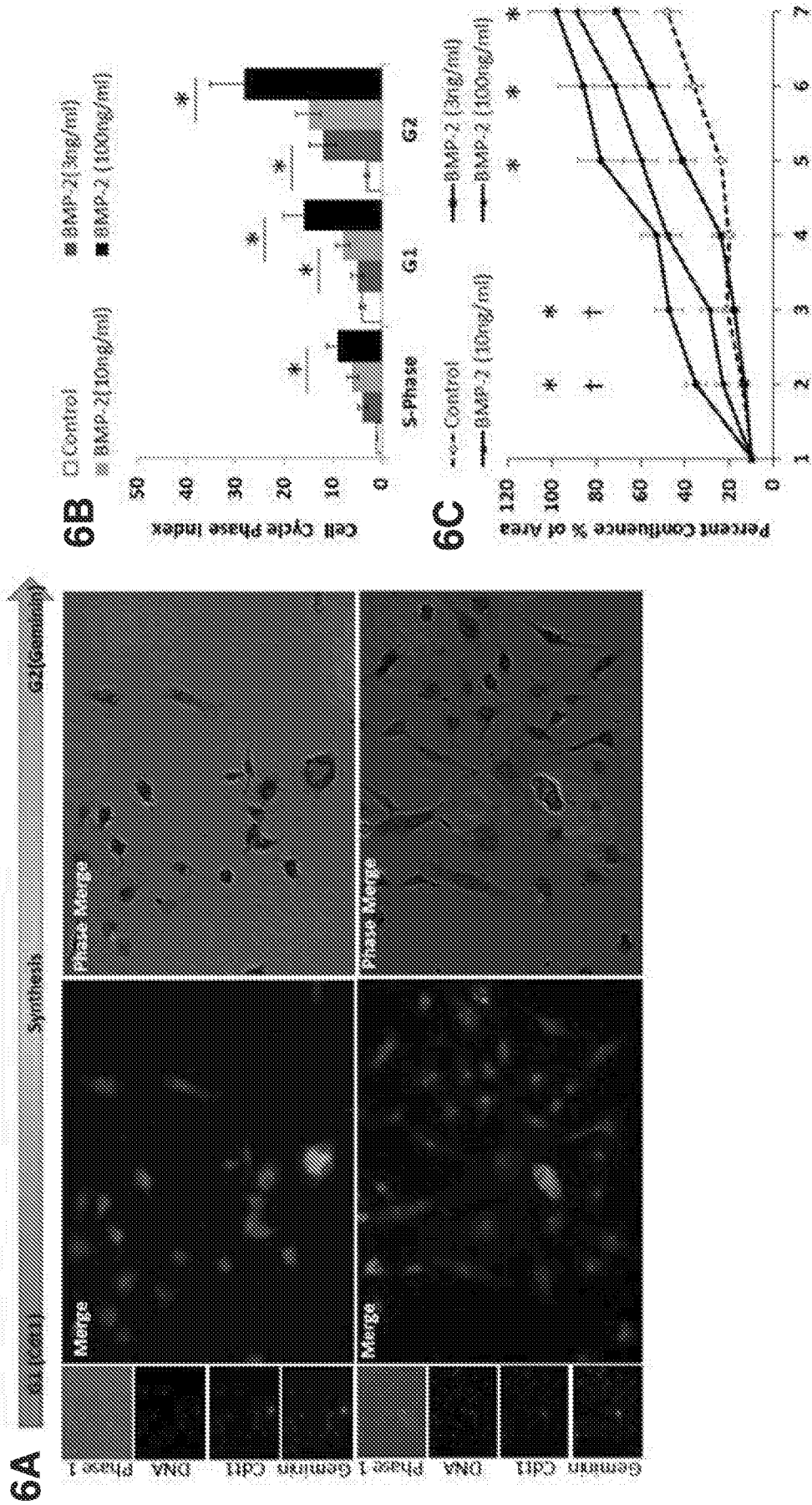


Figure 7

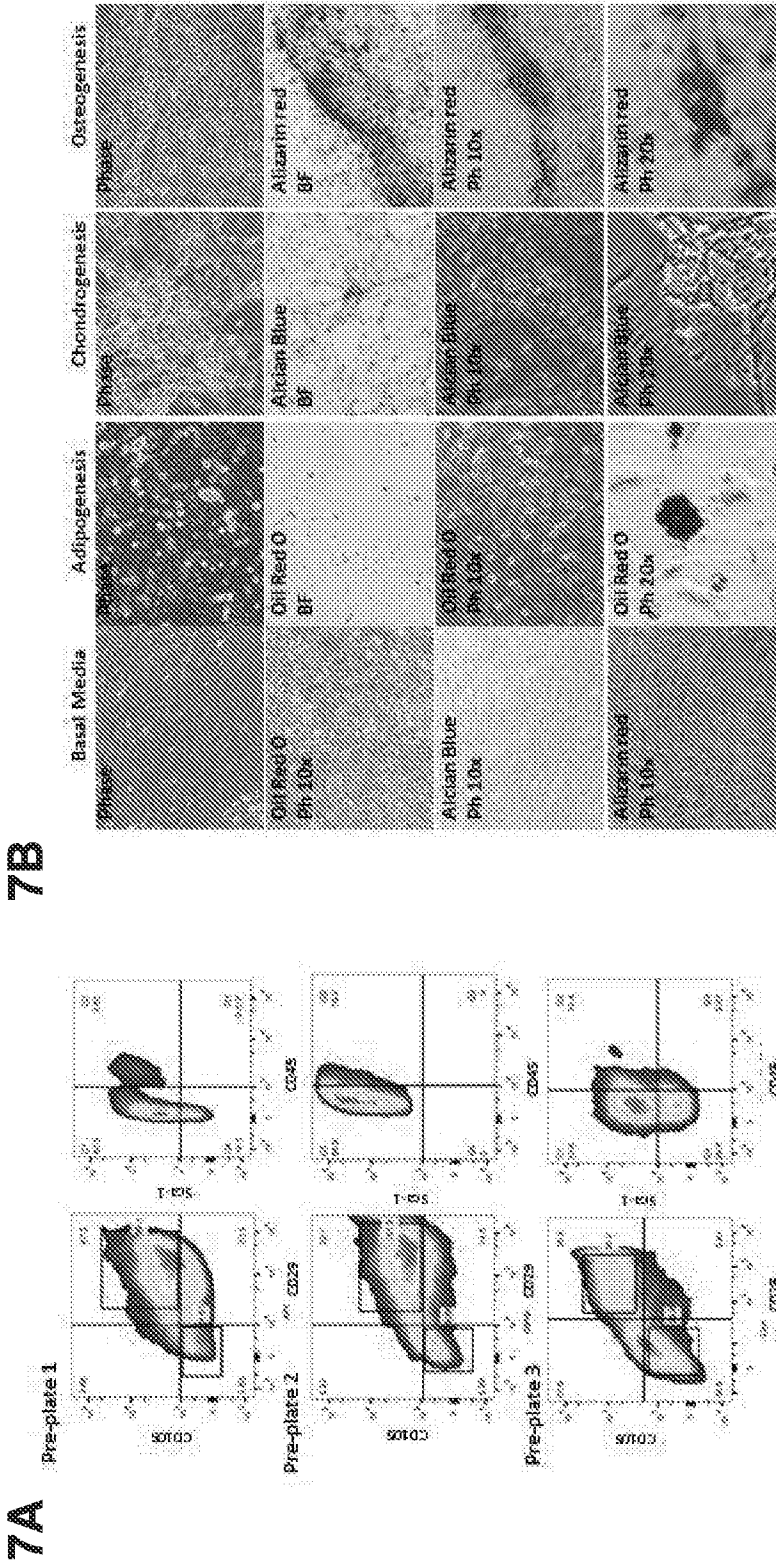


Figure 8 Scaffold Analytics Migratory Kinetics

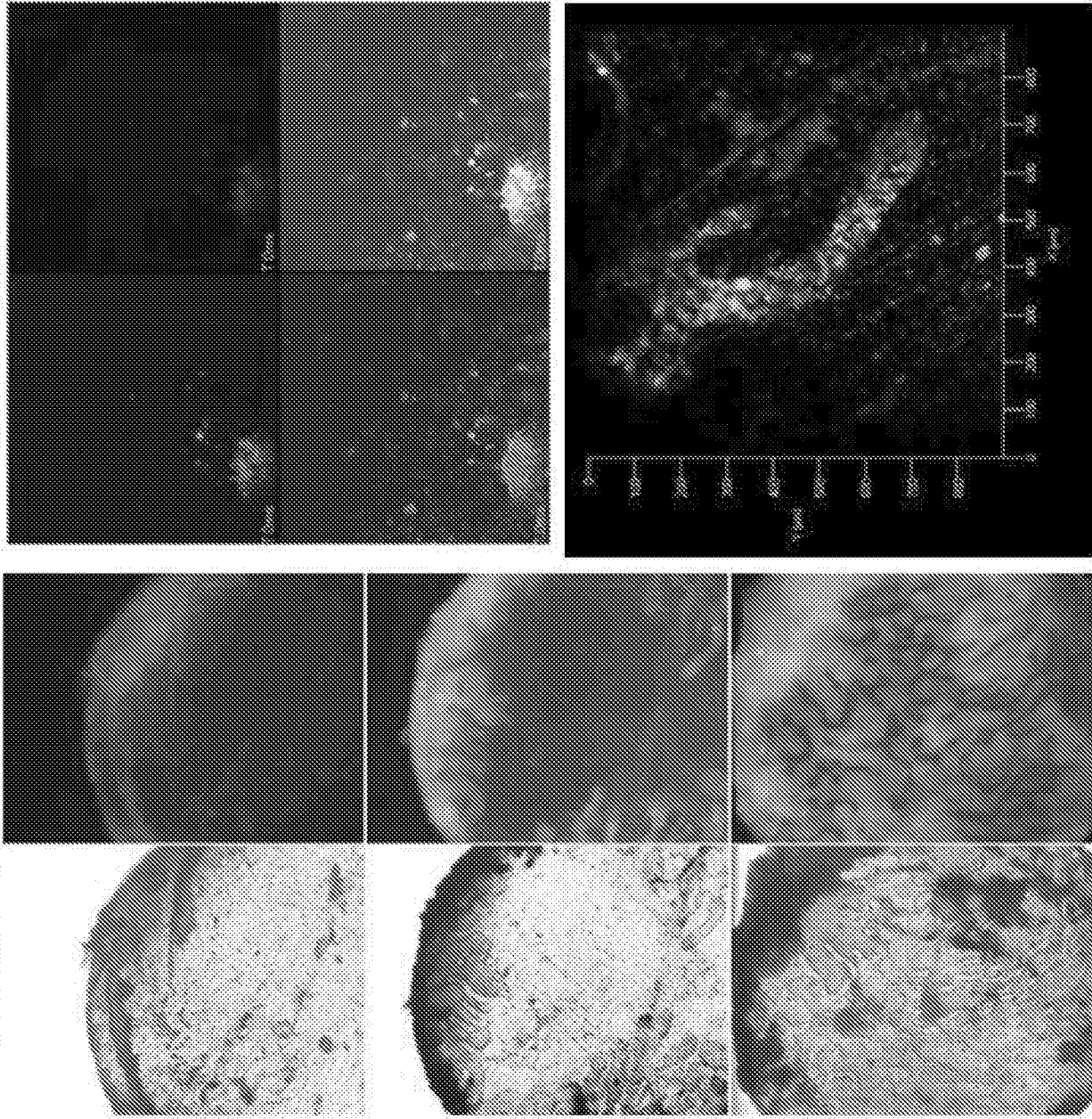


Figure 9A

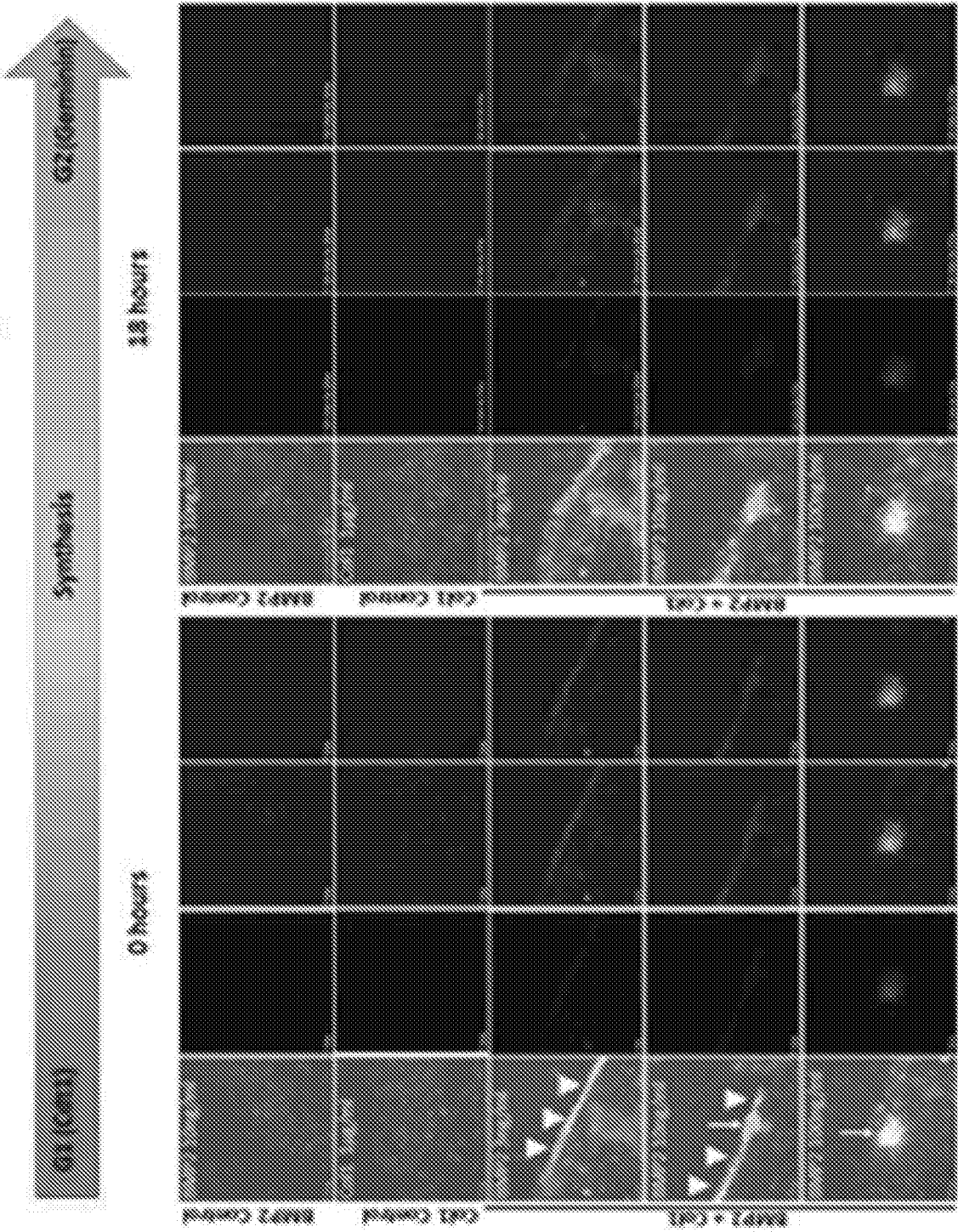




Figure 9B

Figure 9C

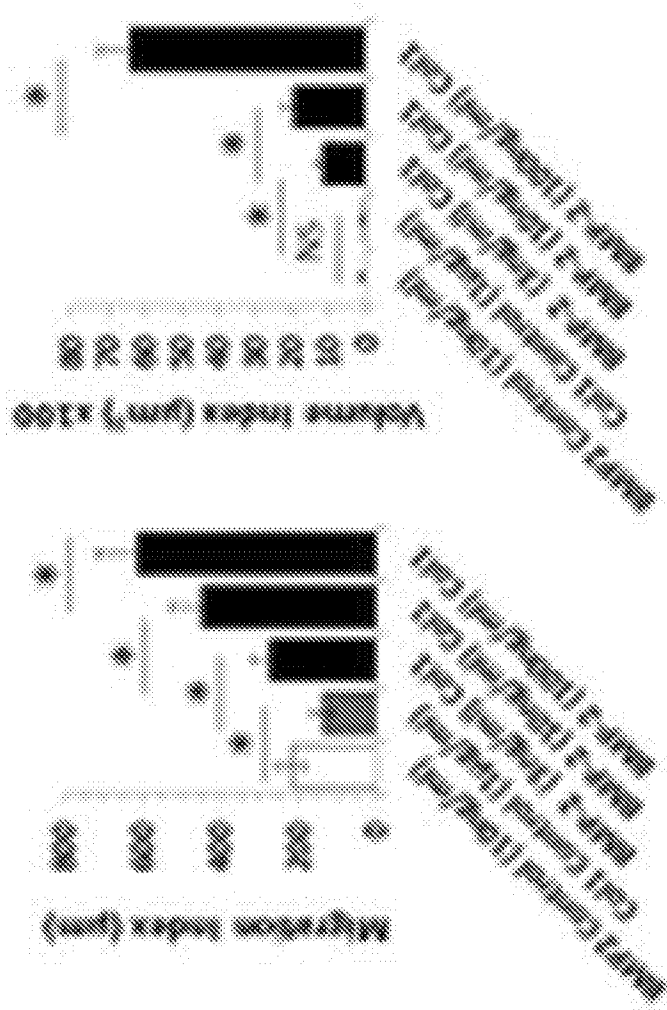


Figure 9D

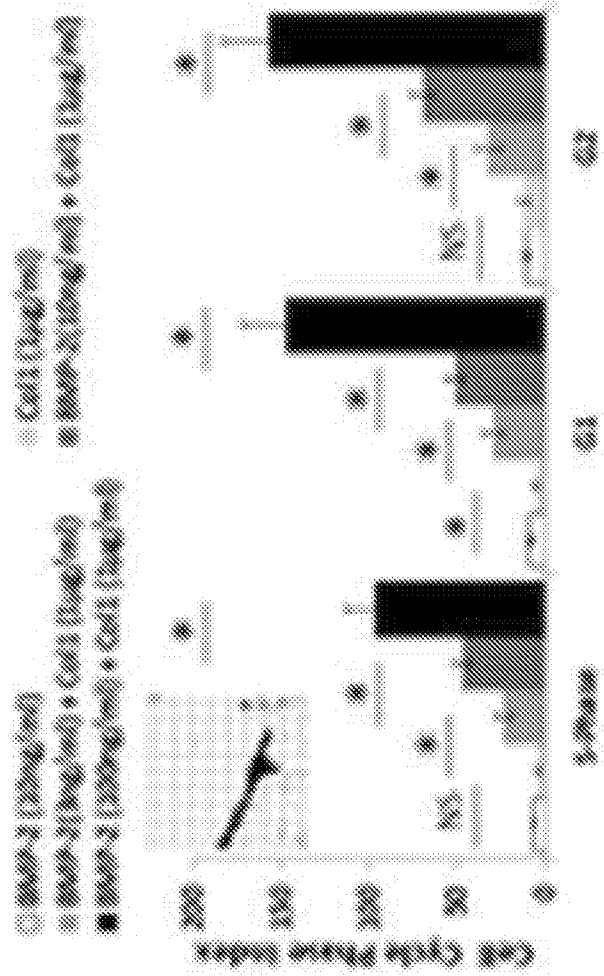
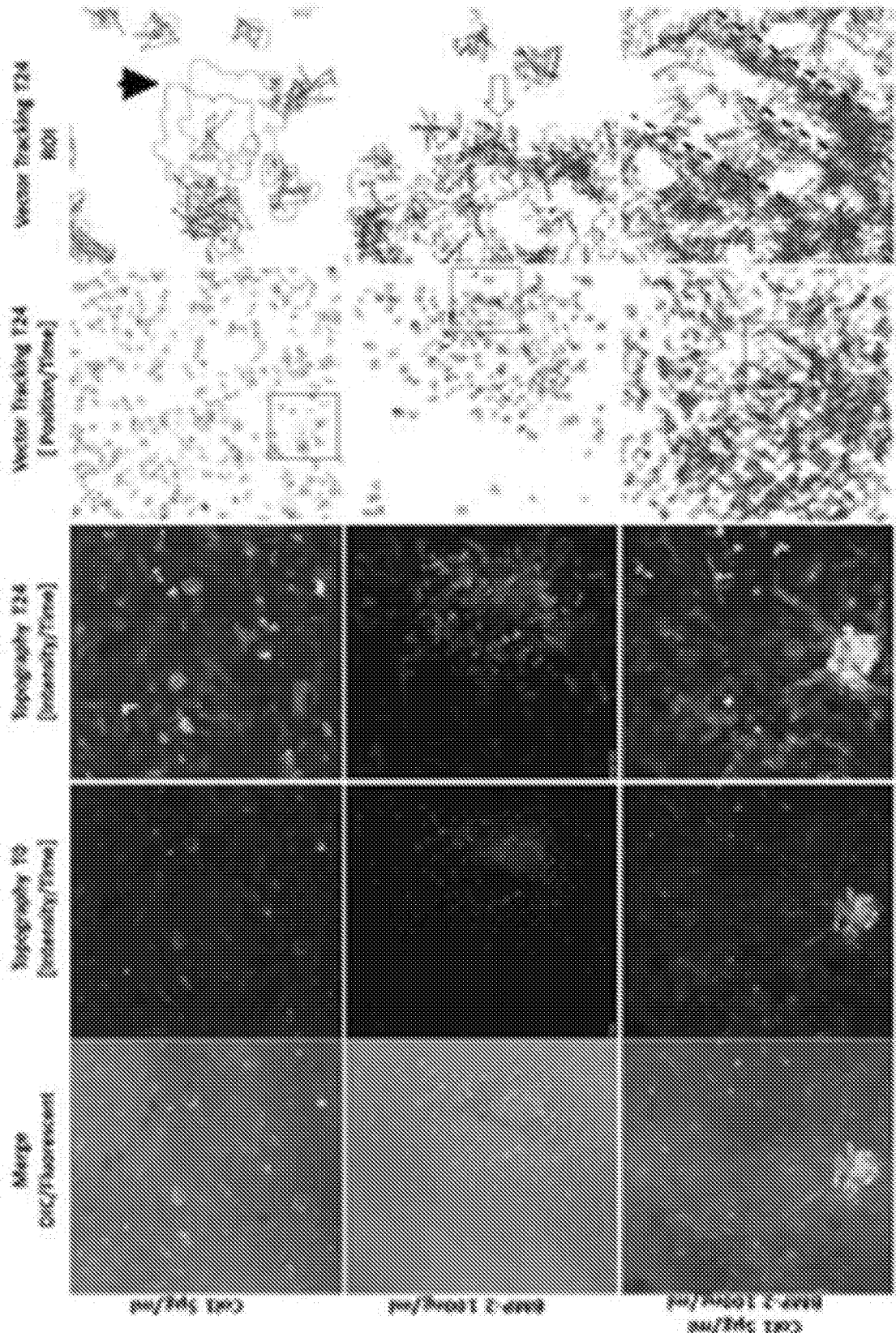


Figure 9E



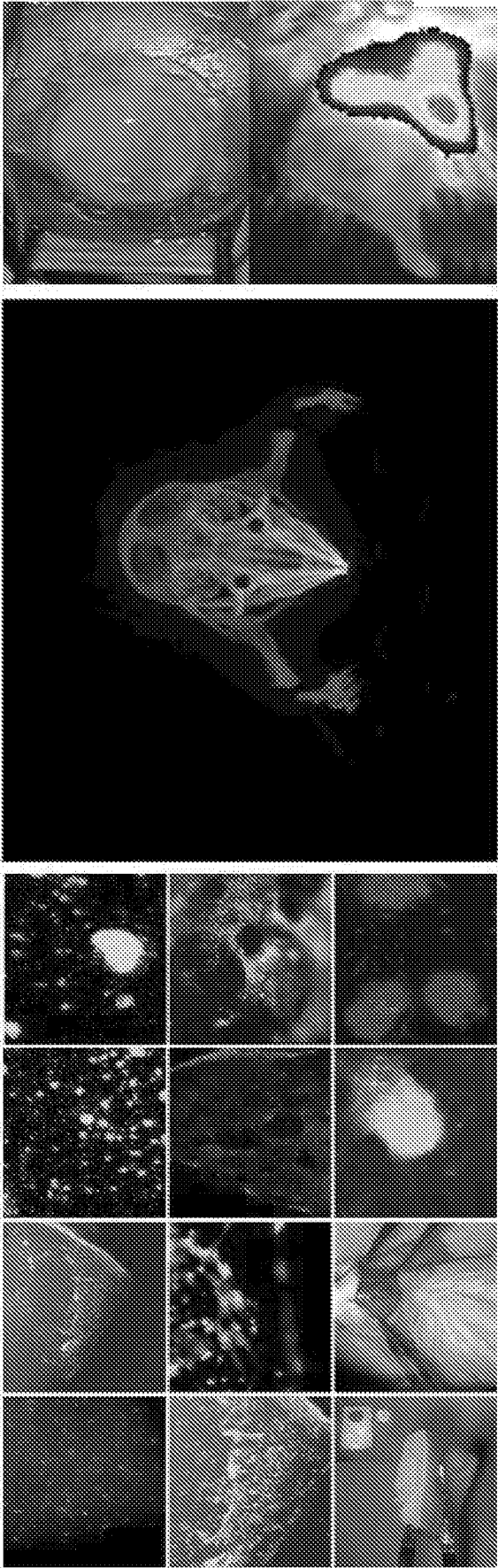
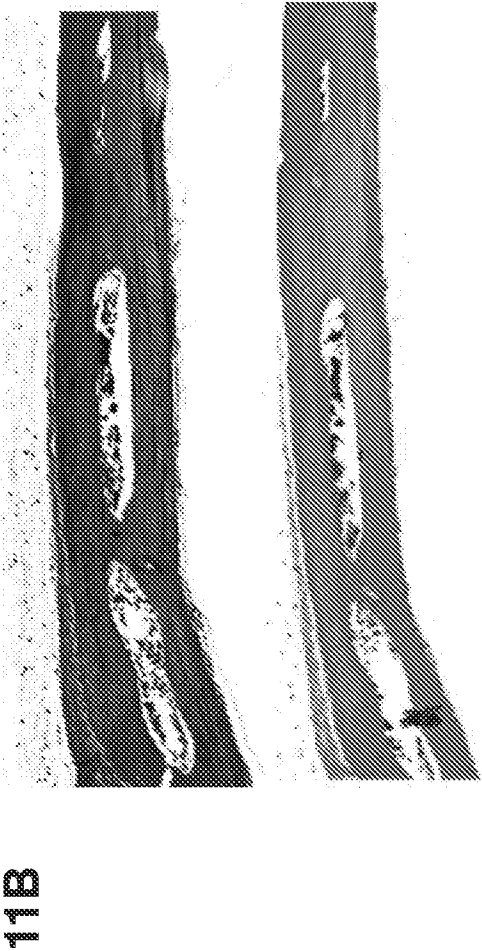
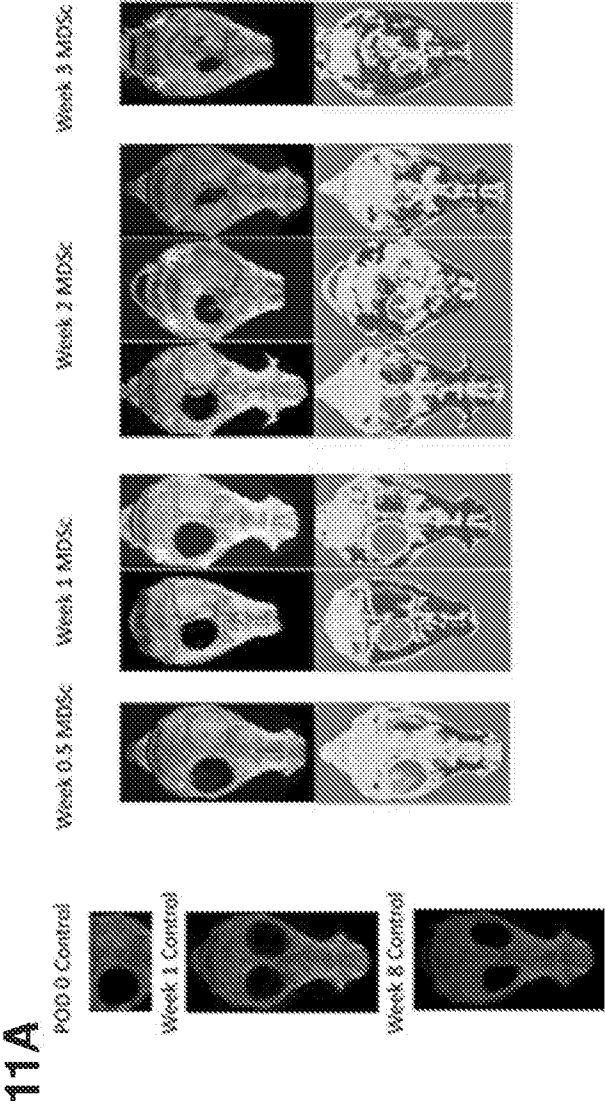


Figure 10

Figure 11



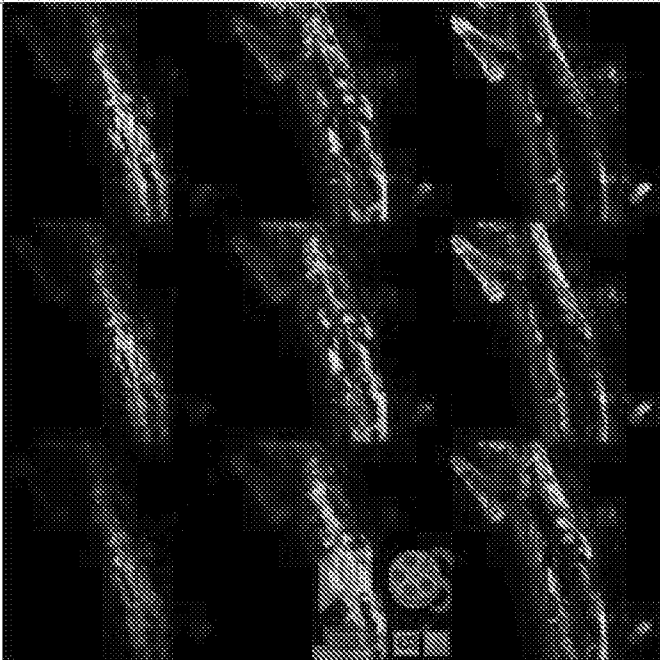
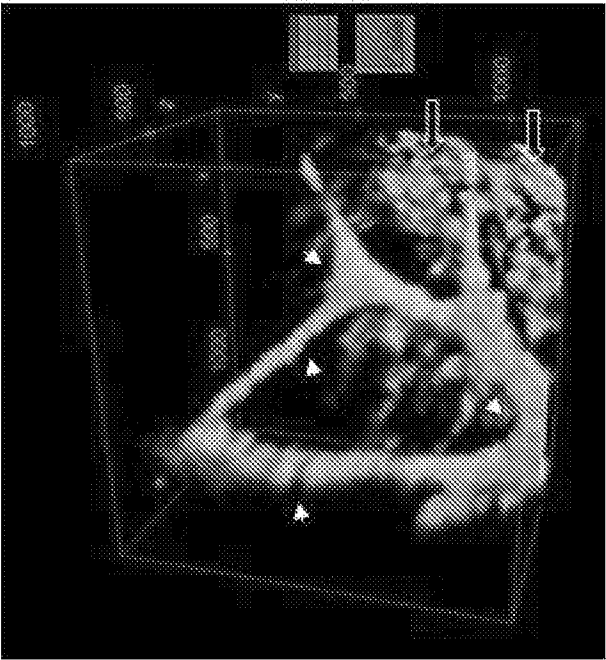


Figure 12

12A

12B

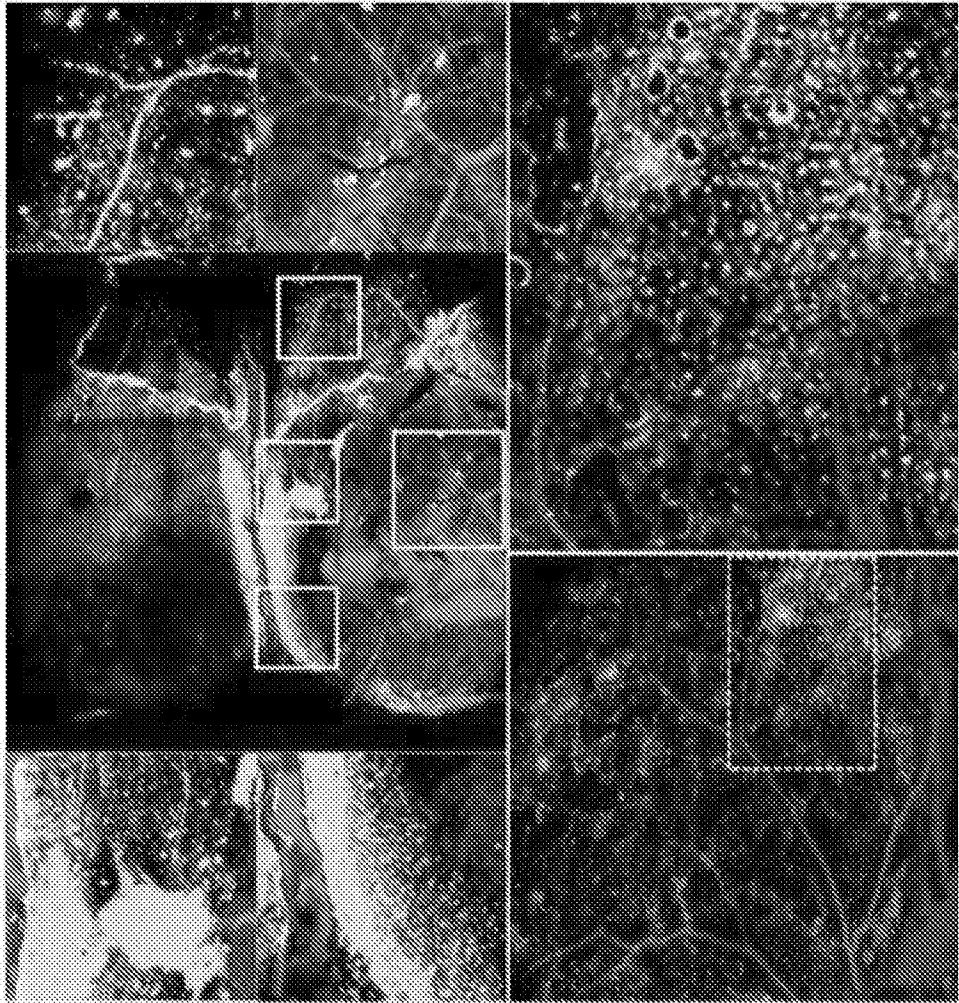


Figure 13

Figure 14A



Figure 14B

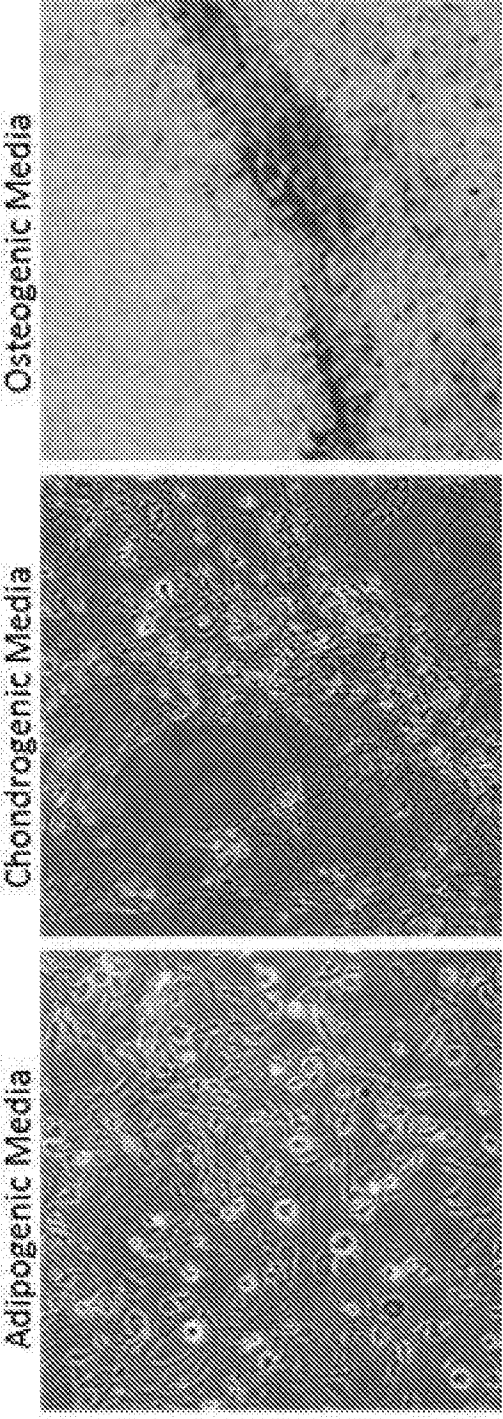


Figure 15A

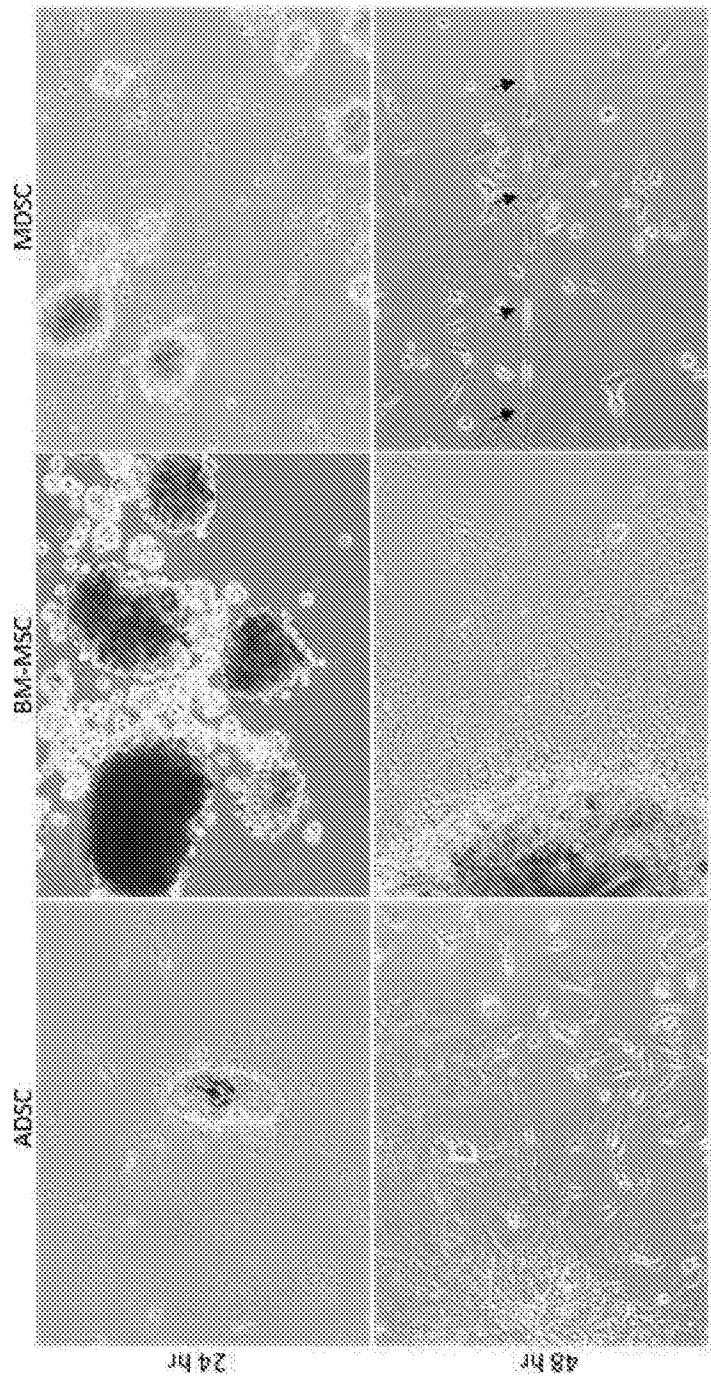


Figure 15B

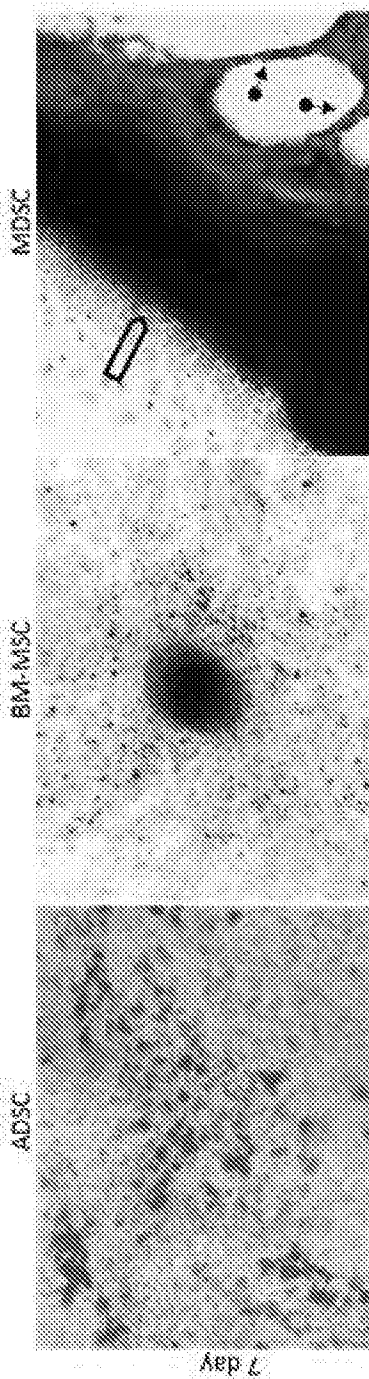


Figure 16A

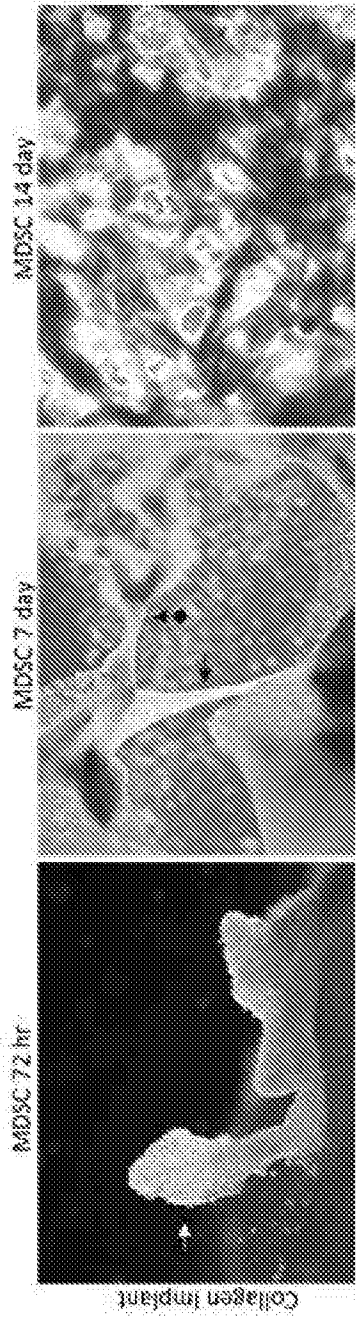


Figure 16B

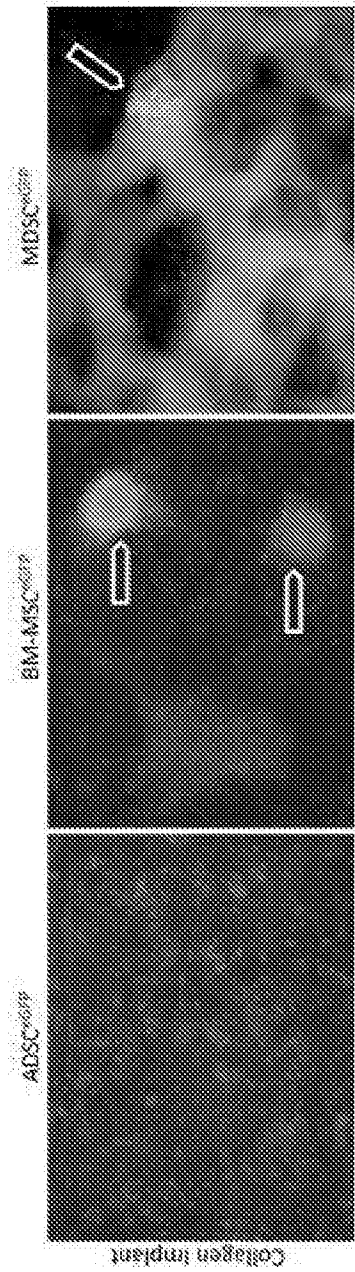


Figure 16C



Figure 17A

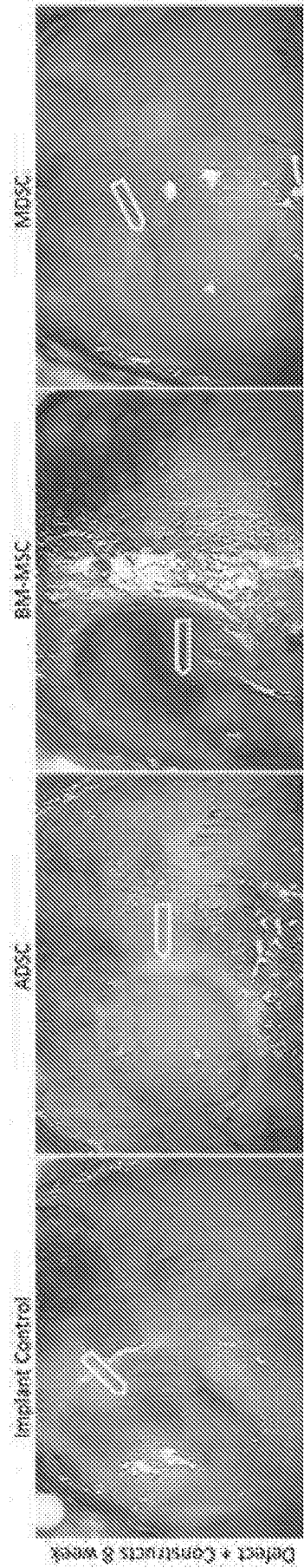


Figure 17B

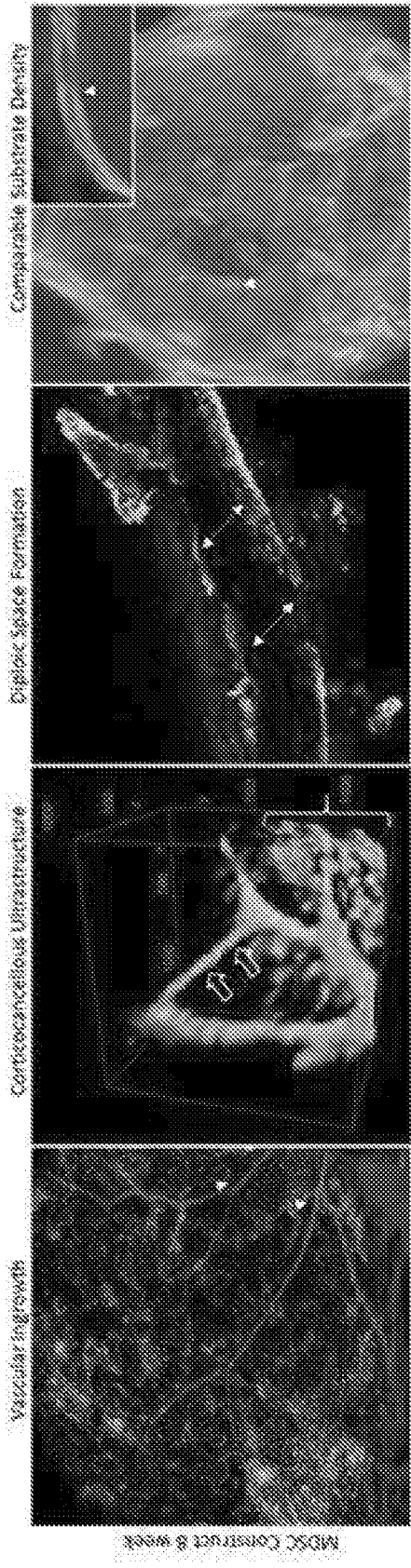


Figure 18

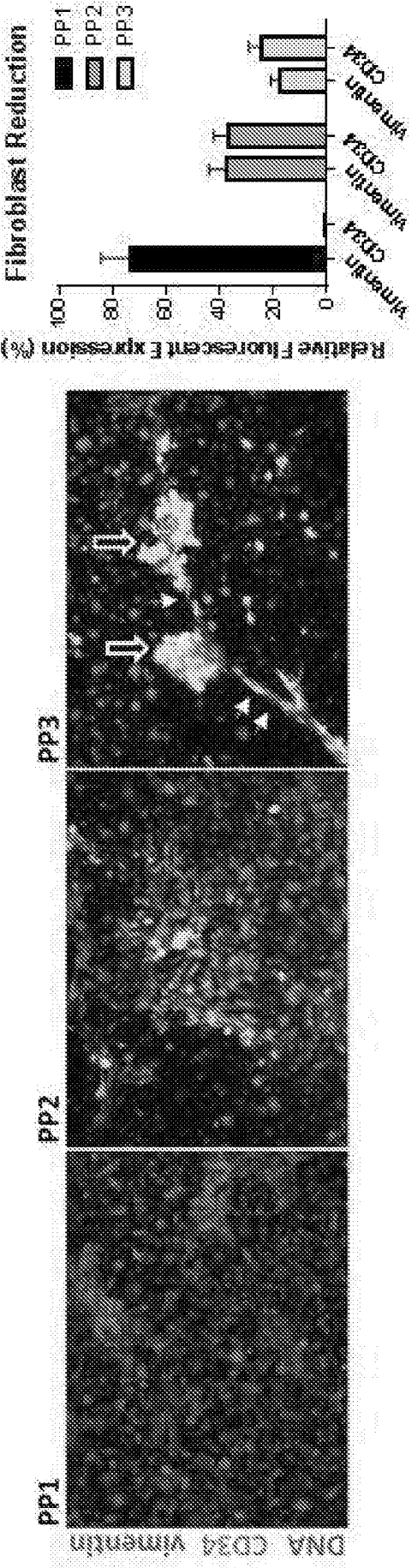


Figure 19A

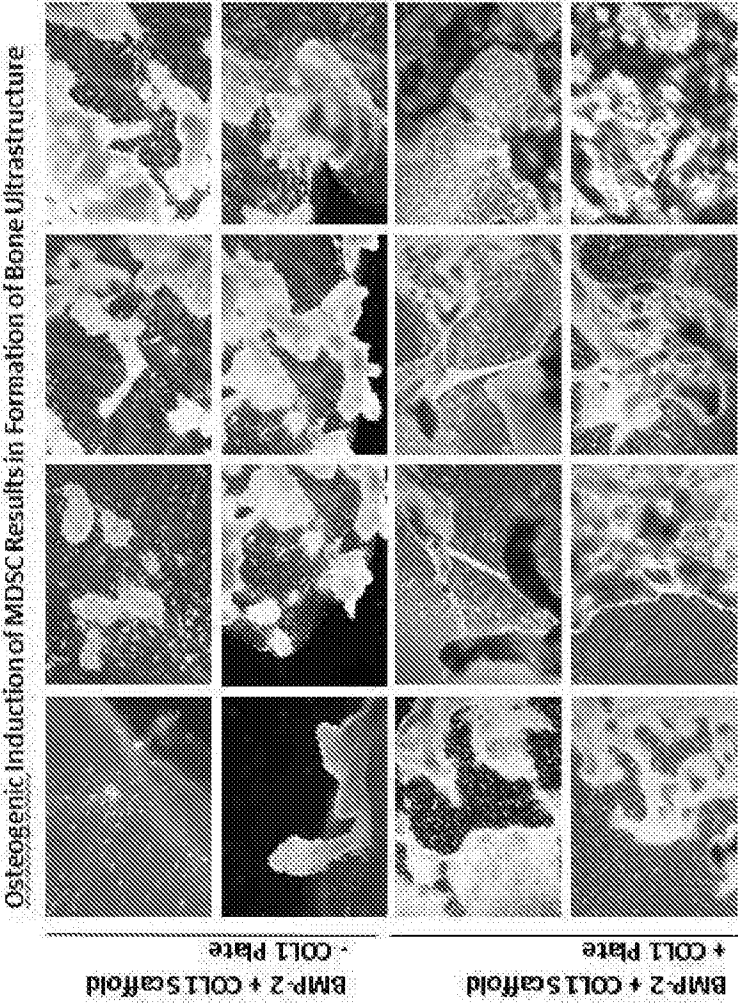


Figure 19B

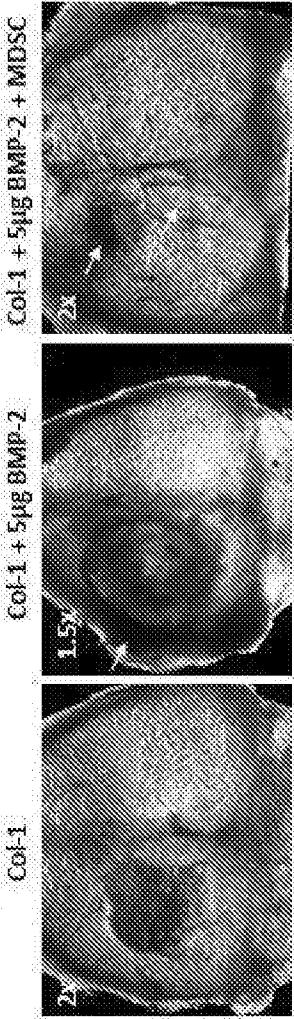


Figure 20B

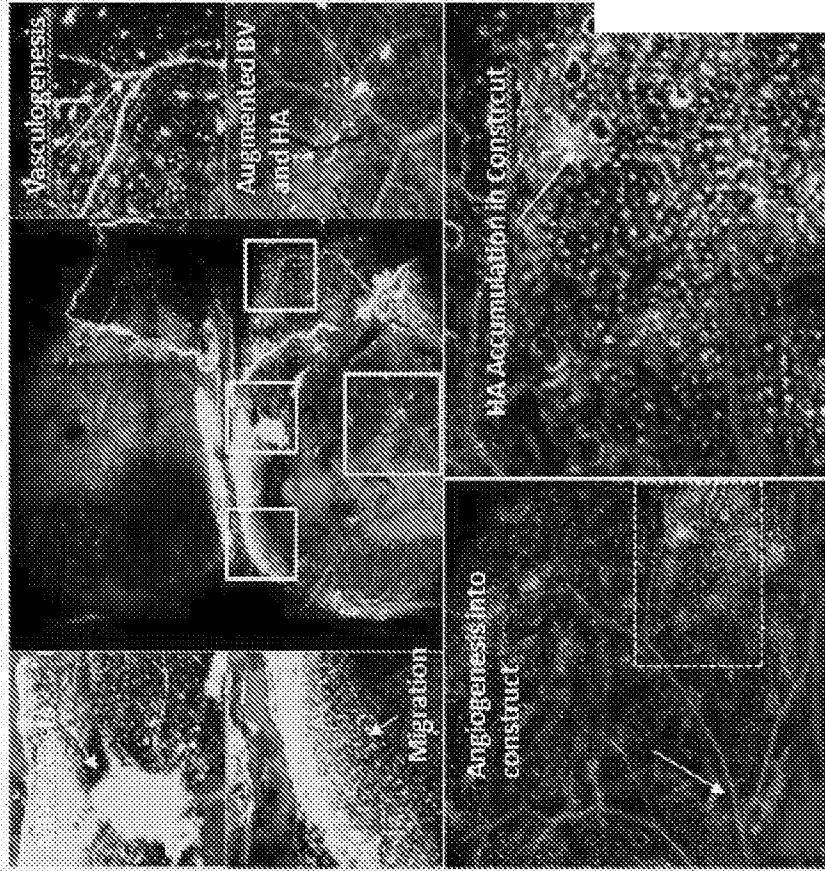
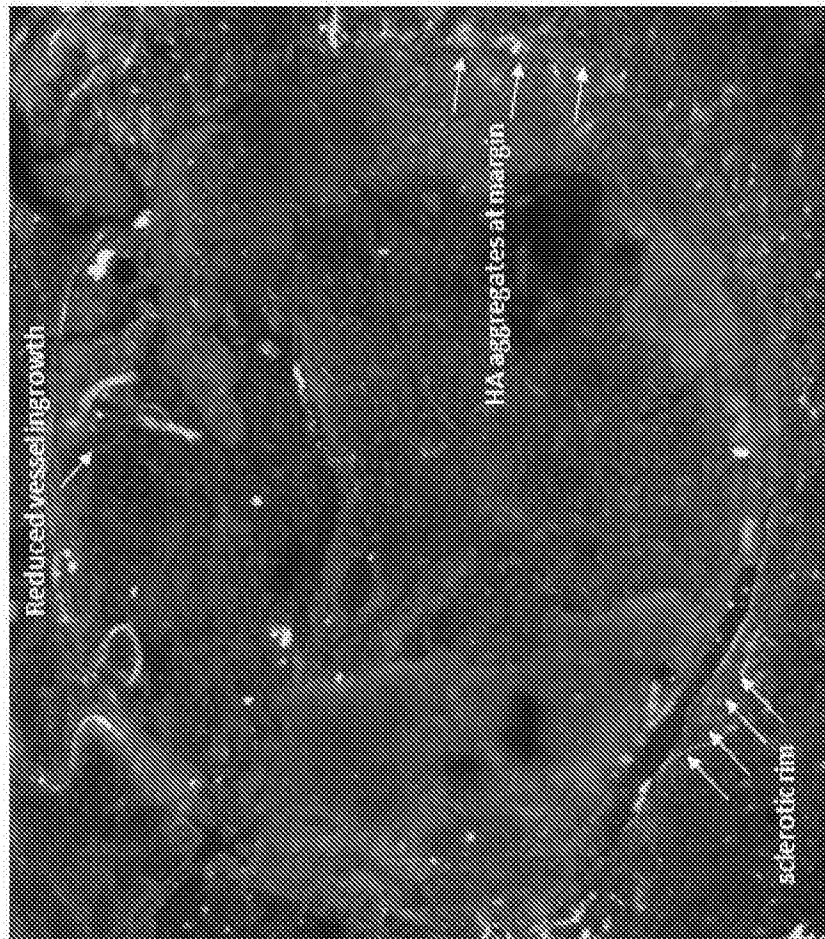


Figure 20A



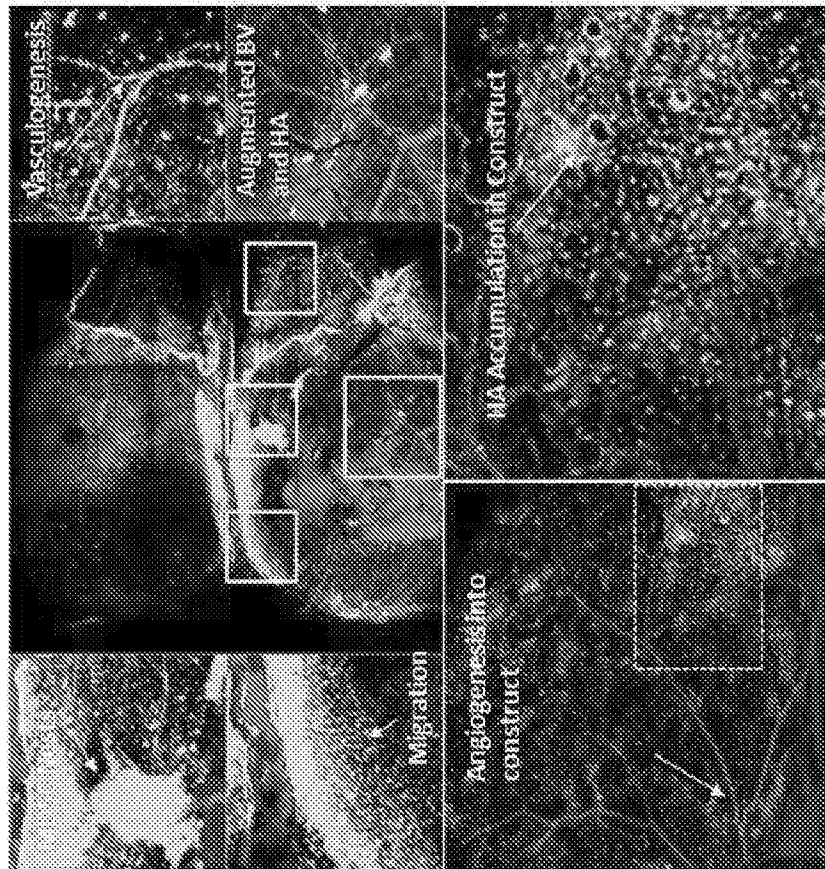


Figure 21

Figure 22A

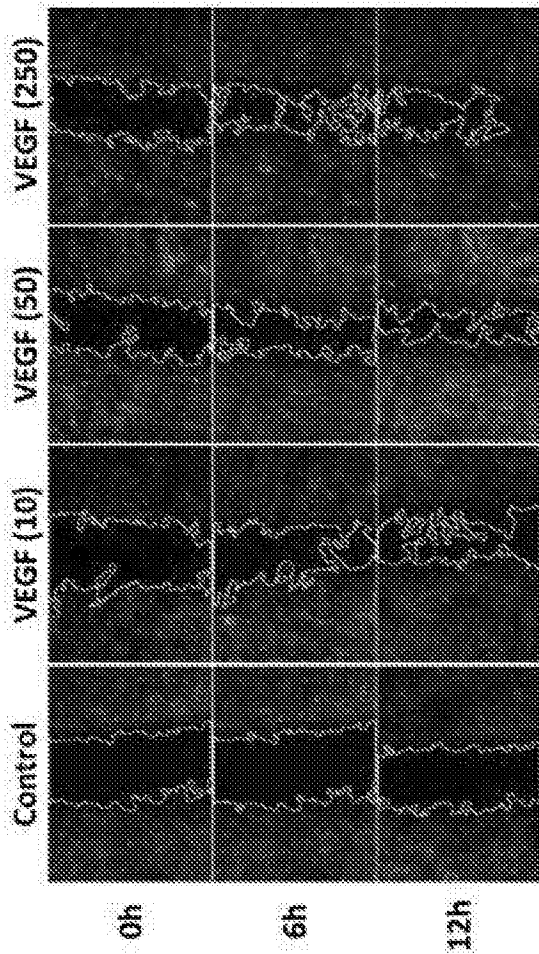


Figure 22B

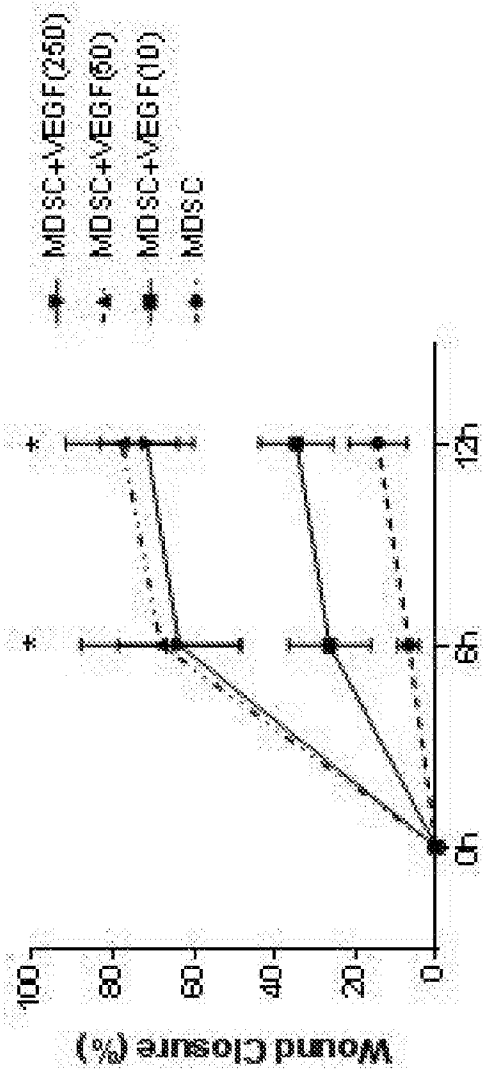


Figure 23A

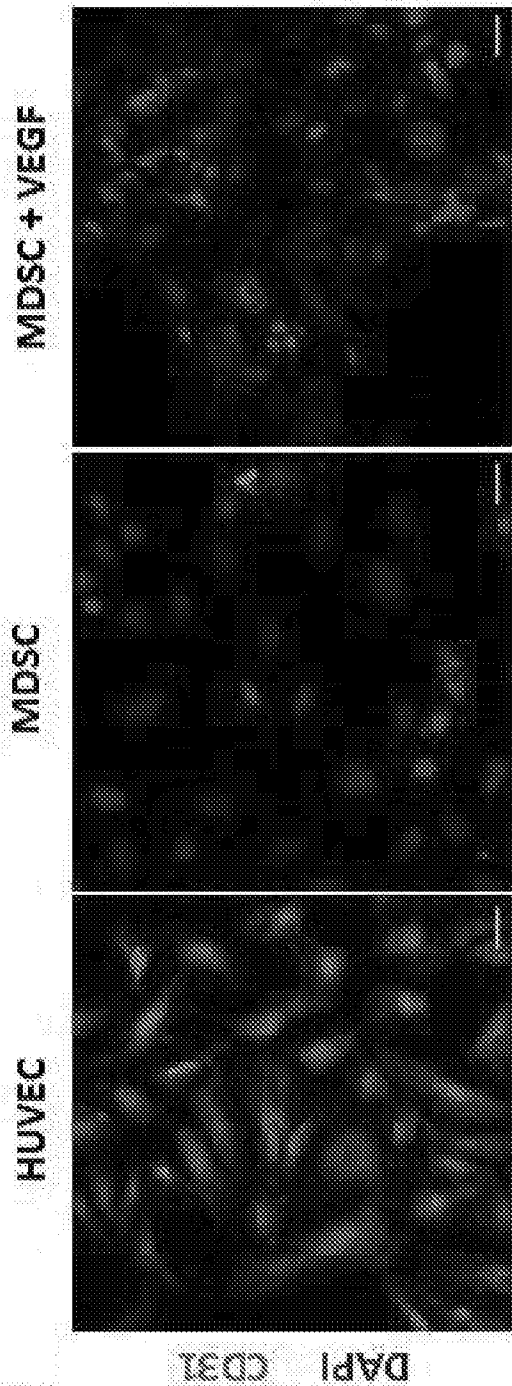


Figure 23B

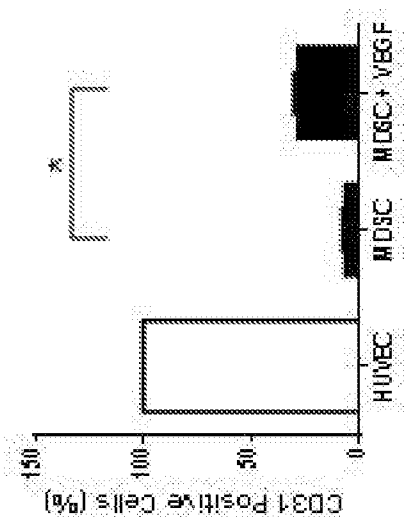


Figure 24A

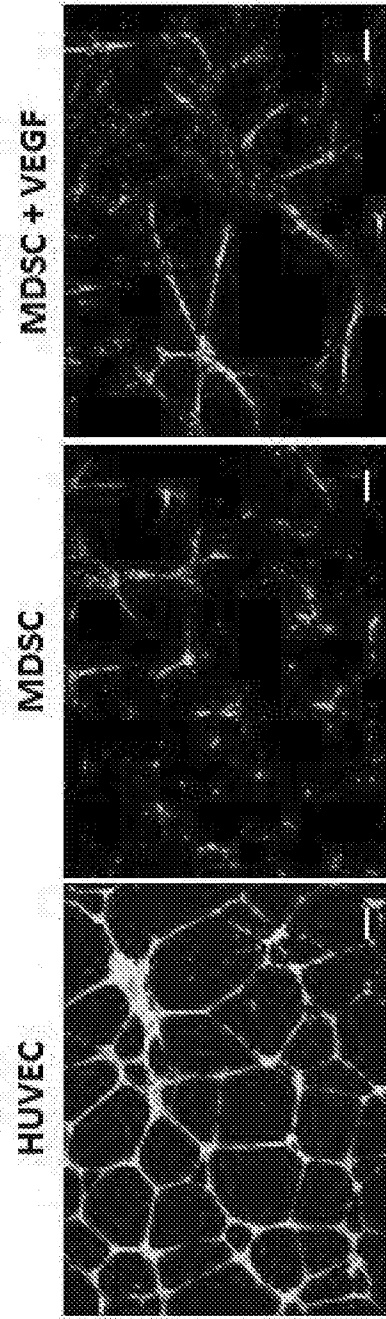


Figure 24B

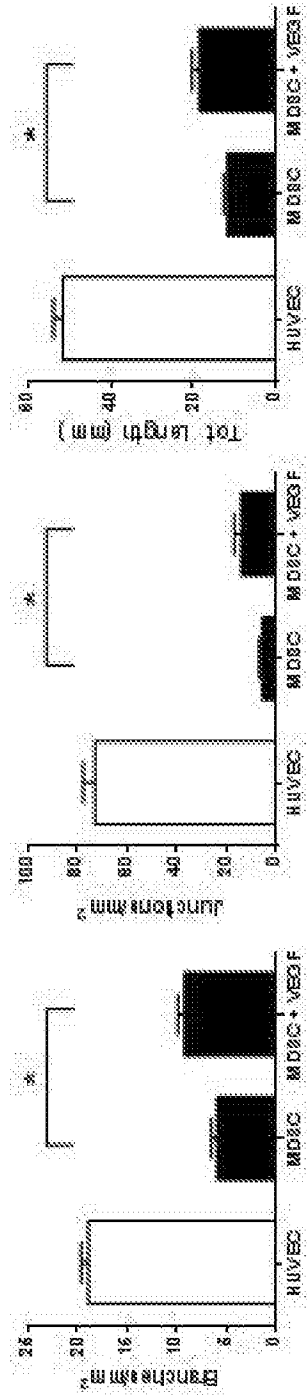


Figure 25A

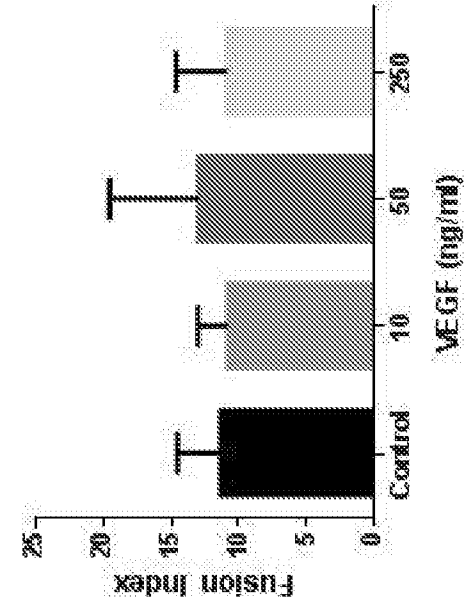
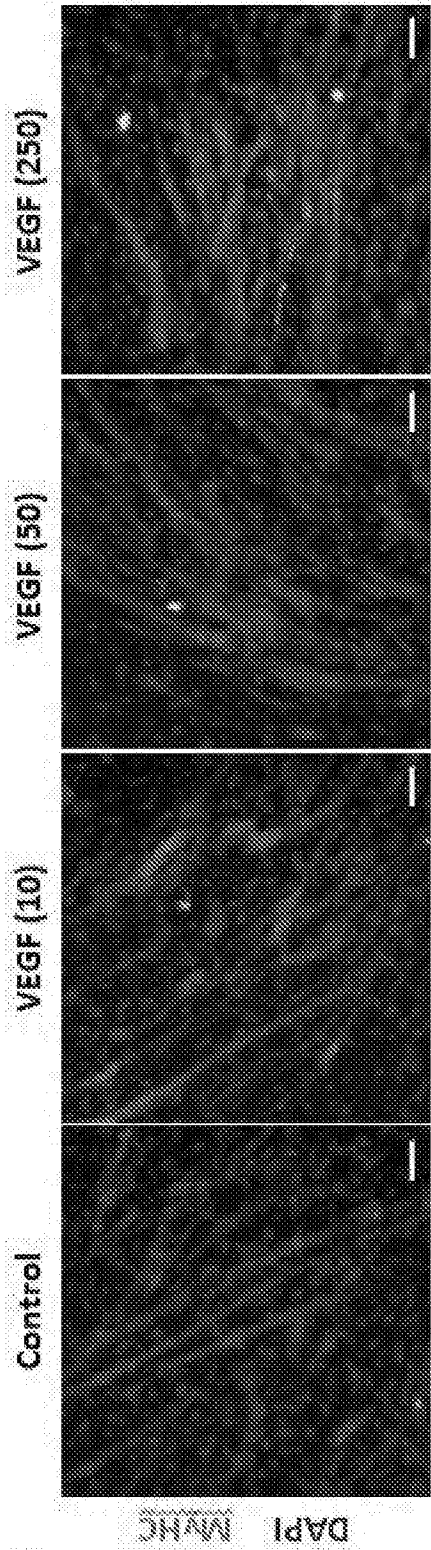


Figure 25B

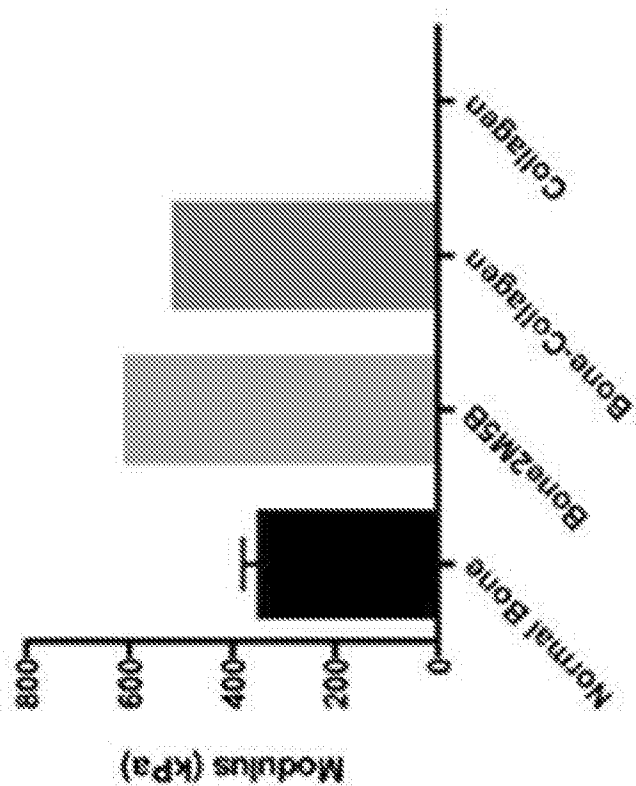


Figure 26

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US16/27729

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

a. ☐ forming part of the international application as filed:

☐ in the form of an Annex C/ST.25 text file.

☐ on paper or in the form of an image file.

b. ☐ furnished together with the international application under PCT Rule 13*ter*. 1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

c. ☒ furnished subsequent to the international filing date for the purposes of international search only:

☒ in the form of an Annex C/ST.25 text file (Rule 13*ter*. 1(a)).

☐ on paper or in the form of an image file (Rule 13*ter*. 1(b) and Administrative Instructions, Section 713).

2. ☒ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US16/27729

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 6-18
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US16/27729

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61K 35/32, 35/34; A61P 19/08; C12N 5/077 (2016.01)

CPC - A61K 35/32, 35/34; C12N 5/0658

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(8): A61K 35/00, 35/32, 35/34; A61P 19/00, 19/04, 19/08; C12N 5/077 (2016.01)

CPC: A61K 35/00, 35/32, 35/34; C12N 5/00 5/0658, 5/0659

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 practicable, search terms used)

PatSeer (US, EP, WO, JP, DE, GB, CN, FR, KR, ES, AU, IN, CA, INPADOC Data); EBSCO Discovery; PubMed; Google; Google Scholar; Google Patents; KEYWORDS: bone, defect, muscle derived cell, BMP2, scaffold

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ----- Y	US 2012/0141428 A1 (CHANCELLOR, M et al.) June 07, 2012; abstract; paragraphs [0038], [0039], [0041], [0044], [0047], [0059], [0060], [0063], [0072], [0126], [0195], [0288], [0310]	1-4, 5/1-2, 19-21 --- 23, 25A, 26
X ----- Y	US 2013/0039896 A1 (SMITH, R et al.) February 14, 2013; abstract; paragraphs [0007], [0018], [0019], [0029]-[0031], [0041]; claim 22, claim 33	22, 24, 25B --- 23, 25A, 26
A	US 2013/0156741 A1 (UNIVERSITY OF PITTSBURGH - OF THE COMMONWEALTH SYSTEM OF HIGHER EDUCATION) June 20, 2013; entire document	1-4, 5/1-2, 19-24, 25A, 25B, 26
A	US 2015/0044185 A1 (UNIVERSITY OF PITTSBURGH - OF THE COMMONWEALTH SYSTEM OF HIGHER EDUCATION) February 12, 2015; entire document	1-4, 5/1-2, 19-24, 25A, 25B, 26

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

* Special categories of cited documents:

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

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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search

22 July 2016 (22.07.2016)

Date of mailing of the international search report

04 OCT 2016

Name and mailing address of the ISA/

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P.O. Box 1450, Alexandria, Virginia 22313-1450

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