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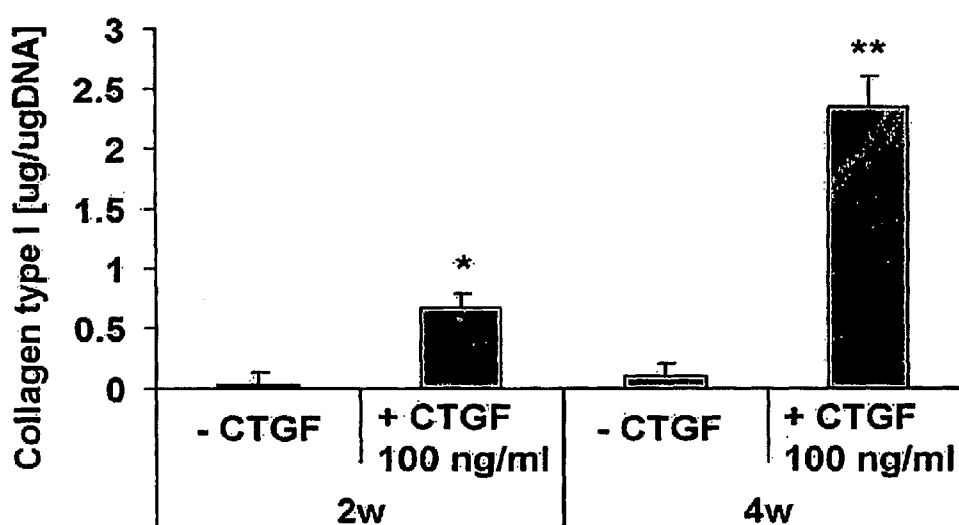
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[Continued on next page]

(54) Title: DIFFERENTIATION OF PROGENITOR CELLS INTO FIBROBLASTS



(57) Abstract: Disclosed herein is a new approach towards differentiation of progenitor cells, for example human mesenchymal cells (hMSCs), into fibroblasts using chemical factors, such as by the treatment of recombinant human connective tissue growth factor (CTGF) and ascorbic acid. One aspect of the invention provides an ex vivo culturing protocol for fibroblastic differentiation of tissue progenitor cells (e.g., human mesenchymal stem cells (hMSCs)) using bioactive factors such as connective tissue growth factor (CTGF). Approaches described herein can provide for significant increases in collagen production from cultured fibroblast progenitor cells.

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DIFFERENTIATION OF PROGENITOR CELLS INTO FIBROBLASTS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional Application Serial No. 60/823,250 filed August 22, 2006, which is hereby incorporated by reference in its entirety.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

[0002] This invention was made in part with Government support under National Institutes of Health Grant Nos. R01DE15291 and R01EB02332. The Government has certain rights in the invention.

INCORPORATION-BY-REFERENCE OF MATERIAL SUBMITTED ON A COMPACT DISC

[0003] Not Applicable.

FIELD OF THE INVENTION

[0004] The present invention generally relates differentiation of progenitor cells into fibroblasts.

BACKGROUND

[0005] Currently, there exists no reliable and convenient way to differentiate progenitor cells or stems cells into fibroblasts. One prior study shows the use of cyclic mechanical stimulus to induce fibroblastic differentiation. Thus, there exists the need for a non-mechanical protocol for the differentiation of tissue progenitor cells to fibroblasts.

SUMMARY

[0006] Disclosed herein is a new approach towards differentiation of progenitor cells, for example human mesenchymal cells (hMSCs), into fibroblasts using chemical factors, such as by the treatment of recombinant human connective tissue growth factor (CTGF).

[0007] One aspect of the invention provides a method of inducing fibroblast differentiation. Such method includes the steps of providing a fibroblast progenitor cell; contacting the fibroblast progenitor cell with a recombinant human connective tissue growth factor (CTGF); and culturing the fibroblast progenitor cell under conditions allowing differentiation to a fibroblast cell.

[0008] In various embodiments, the fibroblast progenitor cell is a mesenchymal stem cell (e.g., a human mesenchymal stem cell). In various embodiments, the CTGF is present at about 1 to about 1000 ng/ml (e.g, about 100 ng/ml). In various embodiments, culturing can occur for about 2 to about 4 weeks. In various embodiments, the cultured fibroblast progenitor cell contacted with CTGF produce substantially more collagen than a cultured fibroblast progenitor cell not contacted with CTGF and ascorbic acid.

[0009] Other objects and features will be in part apparent and in part pointed out hereinafter.

BRIEF DESCRIPTION OF THE DRAWINGS

[0010] Those of skill in the art will understand that the drawings, described below, are for illustrative purposes only. The drawings are not intended to limit the scope of the present teachings in any way.

[0011] Figure 1 is a series of images depicting trichrome staining of tissue sections. The trichrome staining shows marked collagen synthesis upon CTGF for 4 wks. Treatment with 100 ng/ml of CTGF induced an increase in collagen deposition (scale=50 μ m). Further details regarding methodology are presented in Example 2.

[0012] Figure 2 is a bar graph depicting collagen type I contents with or without CTGF treatment. Type I collagen contents in monolayer cultured hMSCs were significantly increased by the treatment with 100 ng/ml CTGF and 50 μ g/ml ascorbic acid by 2 & 4 wks (n=3, *: p<0.01, **: p<0.001). Further details regarding methodology are presented in Example 2.

[0013] Figure 3 is a series of images depicting Tn-C contents with CTGF treatment. Tn-C contents, a marker for ligament fibroblasts, were significantly increased by the treatment with 100 ng/ml CTGF and 50 μ g/ml ascorbic acid by 2 &

4wks (n=3, *: p<0.01, **: p<0.001). Further details regarding methodology are presented in Example 2.

[0014] Figure 4 is a series of images depicting morphology of hMSCs with or without CTGF treatment. Both hMSCs and CTGF-treated hMSCs showed fibroblast-like spindle shape. However, there were no significant differences in cellular morphology caused by CTGF-treatment by 2 & 4 wks (scale = 200 μ m). Further details regarding methodology are presented in Example 2.

[0015] Figure 5 is a series of images depicting safranin O staining of hMSCs treated without CTGF (Figure 5A), with CTGF (Figure 5B), and with chondrogenic medium (FIGURE 5C) for 4 wks (scale = 100 μ m). Further details regarding methodology are presented in Example 3.

[0016] Figure 6 is a series of images depicting von Kossa staining of hMSCs treated without CTGF (Figure 6A), with CTGF (Figure 6B), and with chondrogenic medium (FIGURE 6C) for 4 wks (scale = 100 μ m). Further details regarding methodology are presented in Example 3.

[0017] Figure 7 is a series of bar graphs depicting GAG (Figure 7A) and calcium deposition (Figure 7B) in hMSCs monolayer treated with CTGF or corresponding differentiation medium. GAG content and calcium deposition were not affected by the treatment with 100 ng/ml of CTGF by 2 & 4 wks (n=3, *: p<0.01, **: p<0.001). Further details regarding methodology are presented in Example 3.

DETAILED DESCRIPTION OF THE INVENTION

[0018] Disclosed herein is a new approach towards differentiation of progenitor cells, for example human mesenchymal cells (hMSCs), into fibroblasts using chemical factors, such as by the treatment of recombinant human connective tissue growth factor (CTGF) and ascorbic acid. As demonstrated herein, monolayer-cultured hMSCs treated with CTGF cocktail showed significant increases in markers for fibroblastic differentiation (see Example 2). The inducement of ex vivo differentiation of hMSCs into fibroblasts, as described herein, can be applied to tissue engineering and tissue regeneration of interstitial fibrous tissue, tendons, ligaments, cranial sutures, fascia, periosteum, and any internal organs with interstitial fibrous tissue.

[0019] One aspect of the invention provides an ex vivo culturing protocol for fibroblastic differentiation of tissue progenitor cells (e.g., human mesenchymal stem cells (hMSCs)) using bioactive factors such as connective tissue growth factor (CTGF). Fibroblastic differentiation from stem cells can be employed in engineering of tissues including, but not limited to, tendons, ligaments, periodontal ligament, cranial sutures and as interstitial filler of all organs. As demonstrated herein, the treatment with recombinant human CTGF on cultured hMSCs showed significant increases in type I collagen and tenascin-C (Tn-C) contents by 2 and 4 wks (see e.g., Example 2). In addition, CTGF-treated hMSCs failed to show osteogenic or chondrogenic differentiation (see e.g., Example 3). Thus is demonstrated that CTGF is an effective induction factor for fibroblastic differentiation of hMSCs.

[0020] Generally, the tissue progenitor cell is a precursor to tissue of interest and differentiates in the presence of CTGF. Such cells can be isolated, purified, and/or cultured by a variety of means known to the art. Methods for the isolation and culture of tissue progenitor cells are discussed in, for example, Vunjak-Novakovic and Freshney (2006) Culture of Cells for Tissue Engineering, Wiley-Liss, ISBN 0471629359. The tissue progenitor cells can be derived from the same or different species as the transplant recipient. For example, the progenitor cells can be derived from an animal, including, but not limited to, mammals, reptiles, and avians, more preferably horses, cows, dogs, cats, sheep, pigs, and chickens, and most preferably human.

[0021] Connective tissue growth factor is available from a variety of commercial sources (e.g., BioVendor Laboratory Medicine, Inc., Candler, NC). Preferably, the connective tissue growth factor is preferably human connective tissue growth factor (e.g., Accession No. NP_001892). Connective tissue growth factor can be supplied at, for example, a concentration of about 0 to 1000 ng/mL. For example, the growth factor can be present at a concentration of about 50, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 700, 750, 800, 850, 900, 950, or 1000 ng/mL.

[0022] The fibroblast progenitor cell contacted with the connective tissue growth factor is incubated under conditions allowing differentiation to a fibroblast cell. Methods of culturing progenitor cells are generally known in the art and such methods can be adapted so as to provide optimal conditions for differentiation of progenitor cells contacted with connective tissue growth factor.

[0023] Having described the invention in detail, it will be apparent that modifications, variations, and equivalent embodiments are possible without departing the scope of the invention defined in the appended claims. Furthermore, it should be appreciated that all examples in the present disclosure are provided as non-limiting examples.

EXAMPLES

[0024] The following non-limiting examples are provided to further illustrate the present invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples that follow represent approaches the inventors have found function well in the practice of the invention, and thus can be considered to constitute examples of modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments that are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention. It shall be understood that any method described in an example may or may not have been actually performed, or any composition described in an example may or may not have been actually been formed, regardless of verb tense used.

Example 1: Isolation and Culture-expansion of hMSCs

[0025] Human bone marrow samples taken from healthy donors ranging from 18 to 45 years of age were purchased from AllCells (Berkeley, CA). The bone marrows were prepared to isolate hMSCs per previous methods (Alhadlaq and Mao (2004) Stem Cells Dev. 13, 436-448; Marion ET AL. (2005) Mech. Adv. Mat. Struct. 12, 1-8). Briefly, the whole bone marrow sample was incubated with mesenchymal cell enrichment cocktail (RosetteSep™; StemCell Technologies, Inc., Vancouver, Canada). After incubation, the marrow sample was diluted with PBS containing 2% FBS and 1 mM EDTA. Diluted sample was then layered on top of a density gradient solution (Ficoll-Paque®; StemCell Technologies, Inc., Vancouver, Canada). Following centrifugation, enriched cells were removed and resuspended in cell culture media consisting of 89% DMEM, 10% FBS, and 1% penicillin–streptomycin (basal cell culture media). Cells were passaged up to two times each time upon confluency. Upon the second passage, the isolated hMSCs were grown in

monolayer (5,000 cells/well) in 12-well culture plates in basal cell culture media, with fresh medium change every 3-4 days.

Example 2: Treatment with Connective Tissue Growth Factor

[0026] Upon near confluence, hMSCs were treated with DMEM supplemented with 0 and 100 ng/ml of recombinant human CTGF (BioVendor, Candler, NC) and 50 µg/ml ascorbic acid, with conditioned medium change every third day. Two to 4 weeks following CTGF and ascorbic acid treatment, the following molecular markers were assayed by ELISA: collagen type I (Chondrex, Redmond, WA) and tenascin-C (Tn-C; IBL-America, Minneapolis, MN), selected markers for ligament fibroblasts (Altman et al. (2002) FASEB J. 16, 270-272). The lysis of the selected extracellular matrix was performed using 0.5 M acetic acid solution. Collagen deposition was visualized using Trichrome staining.

[0027] Results showed that collagen and tenascin-C synthesis increases when hMSCs are treated with CTGF. Exposure of 100 ng/ml recombinant human CTGF and 50 µg/ml ascorbic acid to hMSCs induced remarkable increases in collagen deposition in 4 wks as revealed by Trichrome straining (see e.g., Figure 1B), in comparison with hMSCs without CTGF and ascorbic acid supplements. These qualitative data were substantiated by quantitative analysis of collagen content. By 2 wks, collagen content of hMSCs treated with 100 ng/ml of CTGF increased significantly by approx. 4.5 fold in comparison with hMSCs without CTGF ($p < 0.01$). By 4 wks, this increase was approx. 9.5 fold ($p < 0.001$) (see e.g., Figure 2). Tn-C content, another indication of fibroblastic differentiation, in hMSCs treated with CTGF was approx. 2 folds higher than hMSCs without CTGF by 2 wks (Fig. 3). By 4 wks of CTGF treatment, Tn-C content of hMSCs treated with CTGF remained approx. 1.8 folds higher than hMSCs without CTGF.

[0028] Thus, the parallel increases in type I collagen and Tn-C contents suggest that hMSCs treated by CTGF have differentiated into cells that synthesize abundant type I collagen even though there were no significant changes in cellular morphology (see e.g., Figure 4). However, one must at least rule out the possibility of osteogenic differentiation of these CTGF-treated hMSCs, given that osteoblasts also synthesize abundant type I collagen (see Example 3).

Example 3: Osteogenic and Chondrogenic Differentiation

[0029] hMSCs were separately induced to differentiate into osteoblasts and chondrocytes to serve as controls for the above-described fibroblastic differentiation. Chondrogenic medium contained a supplement of 10 ng/ml TGF- β 3 (R&D Systems Inc., Minneapolis, MN), whereas osteogenic medium contained 100 nM dexamethasone, 10 mM β -glycerophosphate, and 0.05 mM ascorbic acid-2-phosphate (Sigma-Aldrich, St. Louis, MO) per prior methods with osteogenic or chondrogenic supplemented medium (Alhadlaq and Mao (2004) Stem Cells Dev. 13, 436-448; Marion et al. (2005) Mech. Adv. Mat. Struct. 12, 1-8). At Days 14 and 28, von Kossa staining and calcium content assay (Calcium reagents, Raichem, Columbia, MD) were performed to evaluate osteogenic differentiation, whereas safranin O staining and glycosaminoglycan (GAG) assay were performed to evaluate chondrogenic differentiation (BlyscanTM, Biocolor, UK).

[0030] Results showed that CTGF-treated hMSCs fail to show osteogenic or chondrogenic differentiation. hMSCs cultured in chondrogenic or osteogenic supplemented medium showed corresponding chondrogenic or osteogenic differentiation (see e.g., Figure 5C and Figure 6C, respectively). However, neither hMSCs nor hMSCs treated with 100 ng/ml of CTGF showed any positive label of chondrogenic or osteogenic differentiation (see e.g., Figure 5A; Figure 5B; Figure 6A; Figure 6B). GAG content of hMSC-derived chondrocytes was significantly higher than hMSCs with or without CTGF (Fig. 7A). Calcium content of hMSC-derived osteoblasts was significantly higher than hMSCs with or without CTGF (Fig. 7B).

CLAIMS

What is claimed is:

1. A method of inducing fibroblast differentiation comprising:
providing a fibroblast progenitor cell;
contacting the fibroblast progenitor cell with a recombinant human connective tissue growth factor (CTGF); and
culturing the fibroblast progenitor cell under conditions allowing differentiation to a fibroblast cell.
2. The method of claim 1 wherein the fibroblast progenitor cell is a mesenchymal stem cell.
3. The method of claim 1 wherein the fibroblast progenitor cell is a human mesenchymal stem cell.
4. The method of any one of claims 1-3, wherein the CTGF is present at about 1 to about 1000 ng/ml.
5. The method of claims 4 wherein the CTGF is present at about 100 ng/ml.
6. The method of any one of claims 1-5, wherein culturing occurs for about 2 to about 4 weeks.
7. The method of any one of claims 1-6, wherein the cultured fibroblast progenitor cell contacted with CTGF produce substantially more collagen than a cultured fibroblast progenitor cell not contacted with CTGF and ascorbic acid.

FIG. 1

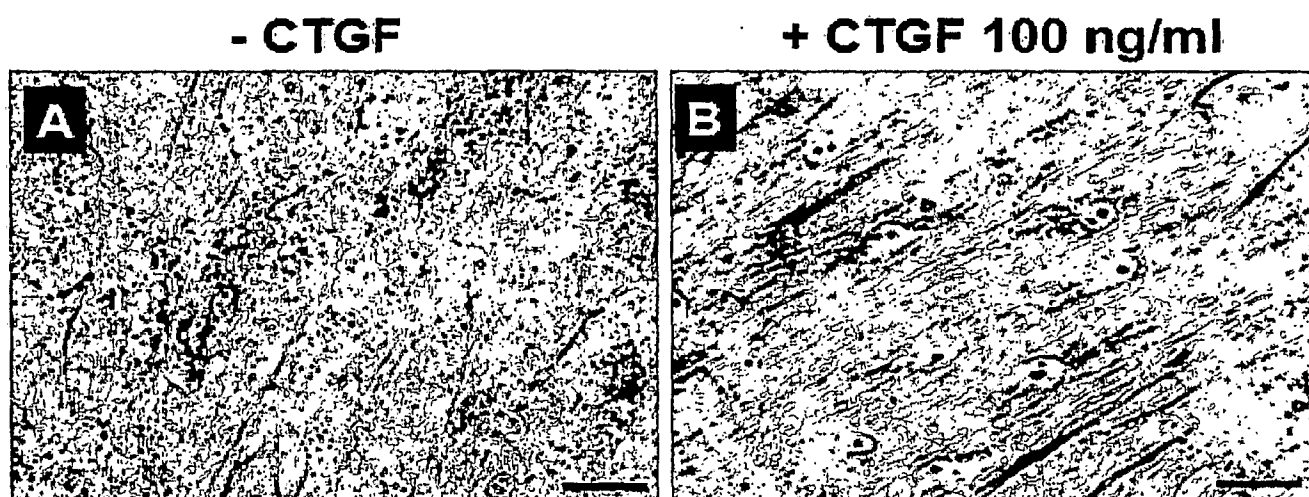


FIG. 2

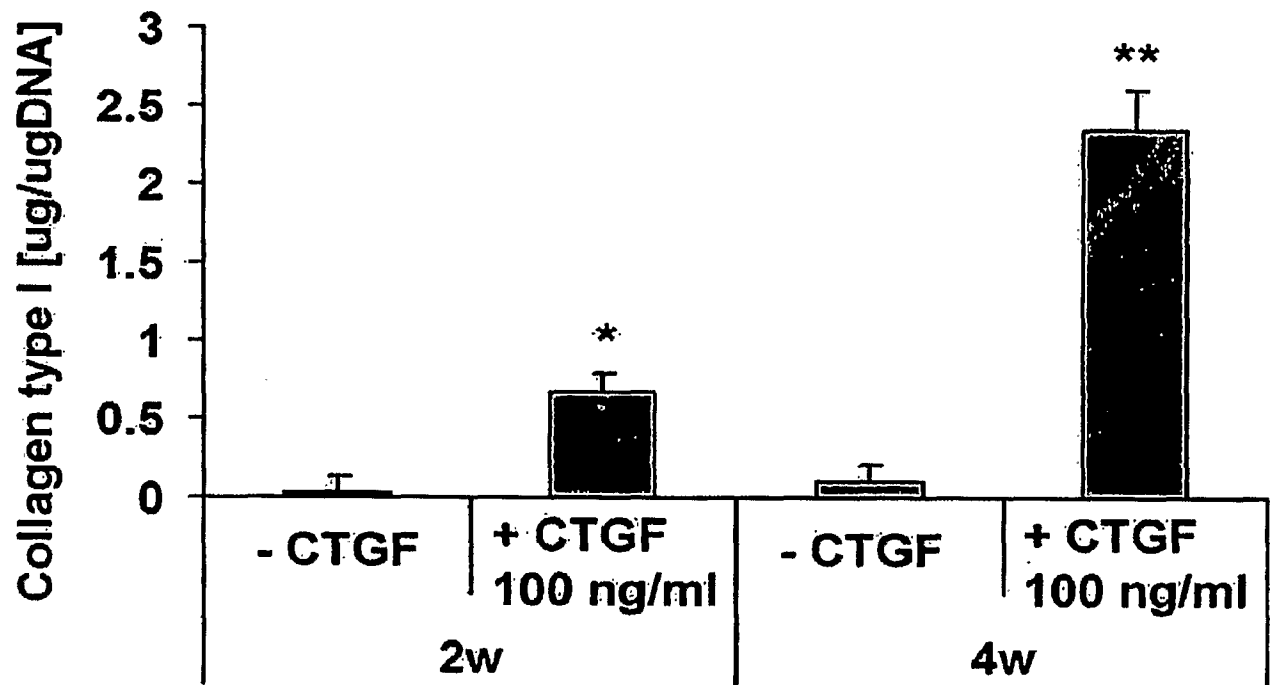


FIG. 3

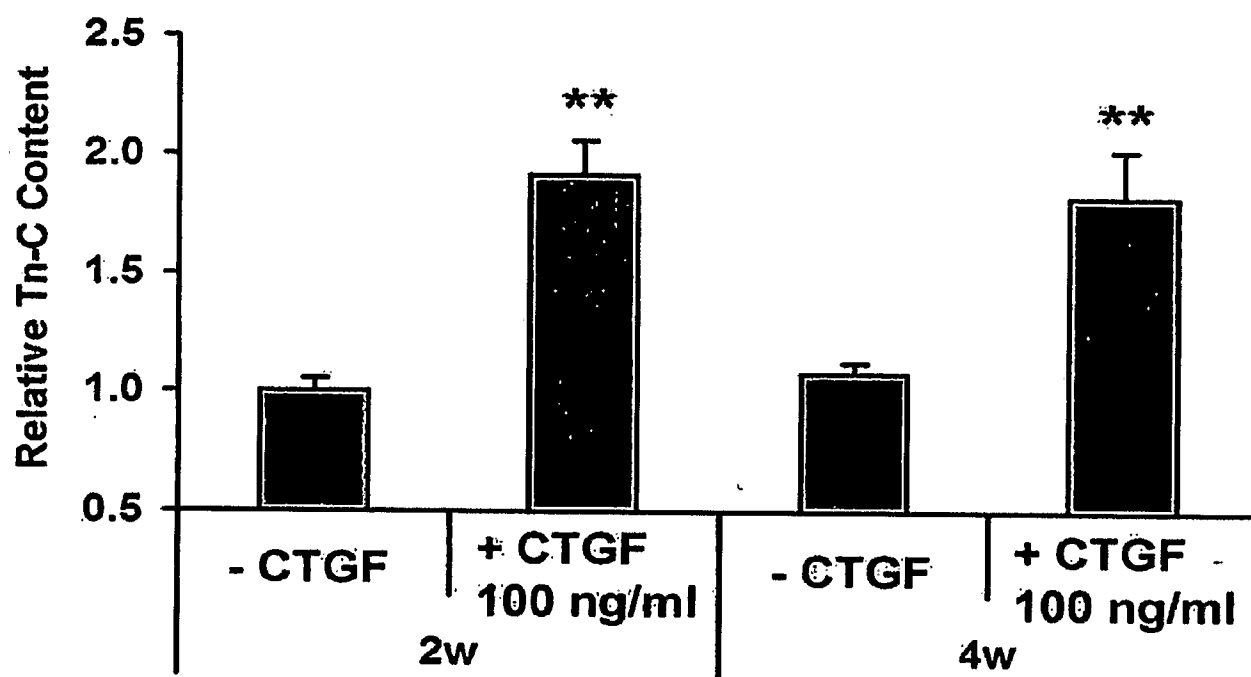


FIG. 4

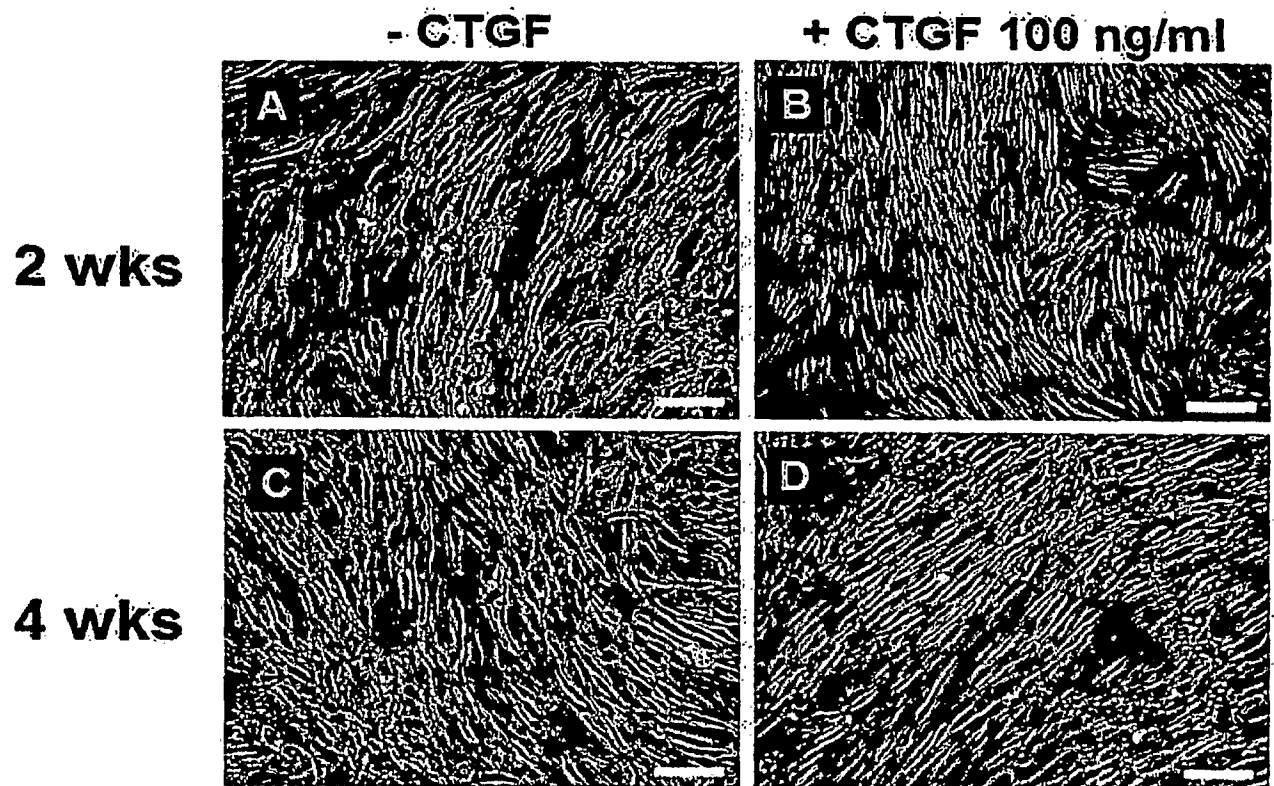


FIG. 5

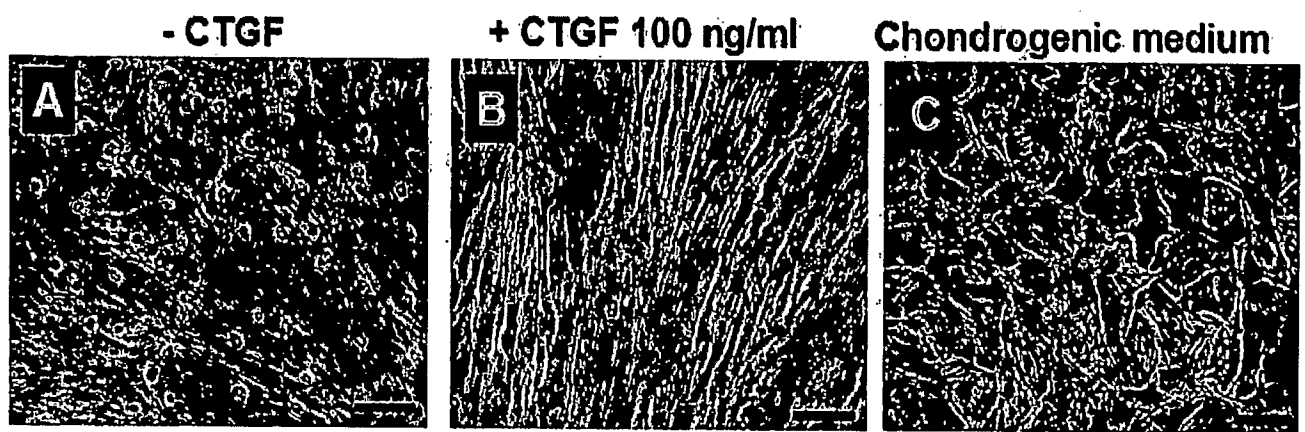


FIG. 6

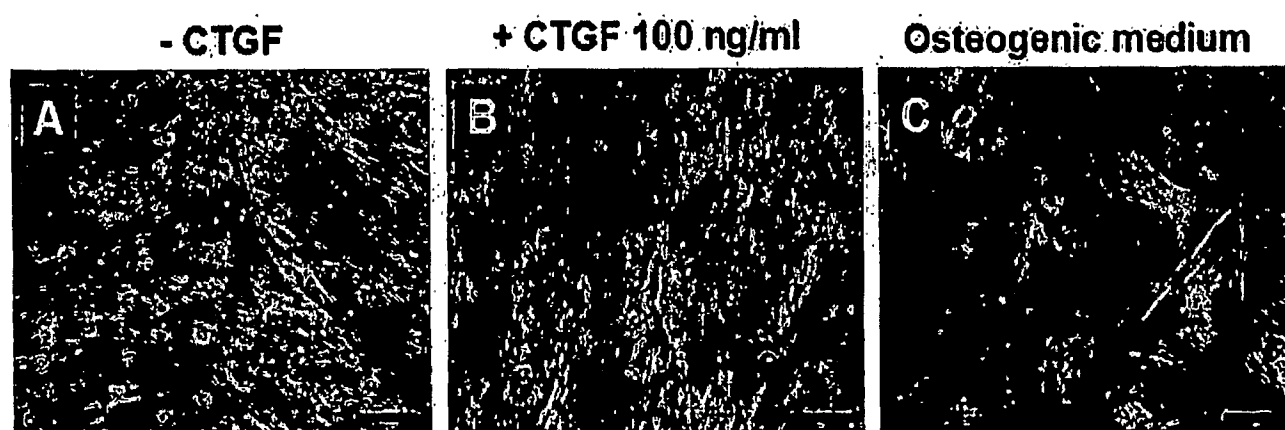


FIG. 7

