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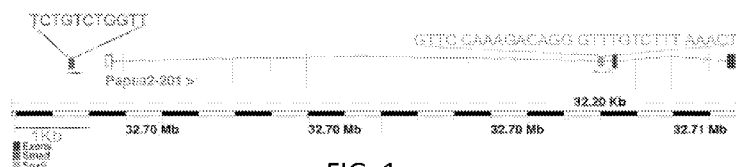


FIG. 1

(57) Abstract: Disclosed are methods for screening potential osteoarthritis therapeutics. The methods of screening can comprise growing a modified chondrocyte comprising an altered TGF β signaling pathway gene in the presence of a compound suspected of being an osteoarthritis therapeutic; growing said modified chondrocyte in the absence of the compound; determining an osteoarthritis characteristic in the modified chondrocyte in the presence of the compound and in the absence of the compound; and comparing the osteoarthritis characteristic of the modified chondrocytes, wherein a rescue of the osteoarthritis characteristic in the presence of the compound is indicative of a osteoarthritis therapeutic.

IN VITRO TISSUE-ENGINEERED CARTILAGE MODELS FOR OSTEOARTHRITIS DRUG TESTING

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims benefit of U.S. Provisional Application No. 62/032,064, filed August 1, 2014, which is hereby incorporated herein by reference in its entirety.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

[0002] This invention was made with government support under RO1 AR062507 awarded by the National Institute of Health/National Institute of Arthritis Musculoskeletal and Skin disease. The government has certain rights in the invention.

BACKGROUND

[0003] Osteoarthritis (OA) is a leading cause of disability in the industrialized world but little is known about mechanisms of cartilage destruction associated with osteoarthritis. Chondrocytes and the extracellular matrix they produce are essential to maintain the normal structure and biomechanical properties of articular cartilage. Articular cartilage has a limited intrinsic capacity for repair placing major obstacles in treatment strategies for OA. Nevertheless, surprisingly little is known about the factors or downstream targets that regulate maintenance of articular cartilage, information that would be the foundation of any prevention or treatment strategies.

[0004] Transforming Growth Factor-beta (TGF- β) signaling is critical for maintenance of articular cartilage. Members of the TGF- β family are secreted signaling molecules that regulate many aspects of growth and differentiation. TGF- β has many biological effects on multiple tissues making it difficult to use TGF- β ligand directly as a therapy for cartilage repair or regeneration. Furthermore, changes in TGF- β signaling as cartilage ages complicate the use of TGF- β ligand for OA. Thus, finding therapeutics that alter the TGF- β pathway can provide a useful therapy for OA.

BRIEF SUMMARY

[0005] Disclosed are methods for screening potential osteoarthritis therapeutics comprising growing a modified chondrocyte comprising an altered TGF β signaling pathway gene in the presence of a compound suspected of being an osteoarthritis therapeutic; growing said modified chondrocyte in the absence of the compound; determining an osteoarthritic characteristic in the modified chondrocyte in the presence of the compound and in the absence of the compound; and comparing the osteoarthritic characteristic of the modified chondrocytes, wherein a reversal of the osteoarthritic characteristic in the presence of the compound is indicative of a osteoarthritis

therapeutic. In some instances, the modified chondrocyte is genetically modified. In some instances, the modified chondrocyte is a mammalian chondrocyte. For example, in some instances, the mammalian chondrocyte is a human chondrocyte

[0006] Disclosed are methods for screening potential osteoarthritis therapeutics comprising growing a modified chondrocyte comprising an altered TGF β signaling pathway gene in the presence of a compound suspected of being an osteoarthritis therapeutic; growing said modified chondrocyte in the absence of the compound; determining an osteoarthritic characteristic in the modified chondrocyte in the presence of the compound and in the absence of the compound; and comparing the osteoarthritic characteristic of the modified chondrocytes, wherein a reversal of the osteoarthritic characteristic in the presence of the compound is indicative of a osteoarthritis therapeutic, wherein the altered TGF β signaling pathway gene is an altered TGF β R gene. In some instances, the TGF β R gene is TGF β R type II. In some instances, the altered TGF β R gene comprises a dominant negative mutation in the TGF β R gene. In some instances, the dominant negative mutation is a truncation of the TGF β R gene. For example, in some instances, the truncation of the TGF β R gene is the deletion of the kinase domain.

[0007] Disclosed are methods for screening potential osteoarthritis therapeutics comprising growing a modified chondrocyte comprising an altered TGF β signaling pathway gene in the presence of a compound suspected of being an osteoarthritis therapeutic; growing said modified chondrocyte in the absence of the compound; determining an osteoarthritic characteristic in the modified chondrocyte in the presence of the compound and in the absence of the compound; and comparing the osteoarthritic characteristic of the modified chondrocytes, wherein a reversal of the osteoarthritic characteristic in the presence of the compound is indicative of a osteoarthritis therapeutic, wherein growing the modified chondrocytes comprises culturing the modified chondrocytes on a cell culture dish, multiwell plate, or flask. In some instances, the growing of the modified chondrocytes comprises culturing the modified chondrocytes on a scaffold. In some instances, the scaffold is a polylactic acid composition.

[0008] Disclosed are methods for screening potential osteoarthritis therapeutics comprising growing a modified chondrocyte comprising an altered TGF β signaling pathway gene in the presence of a compound suspected of being an osteoarthritis therapeutic; growing said modified chondrocyte in the absence of the compound; determining an osteoarthritic characteristic in the modified chondrocyte in the presence of the compound and in the absence of the compound; and comparing the osteoarthritic characteristic of the modified chondrocytes, wherein a reversal of the osteoarthritic characteristic in the presence of the compound is indicative of a osteoarthritis

therapeutic, wherein the growing of the modified chondrocytes comprises culturing the modified chondrocytes in a bioreactor.

[0009] Disclosed are methods for screening potential osteoarthritis therapeutics comprising growing a modified chondrocyte comprising an altered TGF β signaling pathway gene in the presence of a compound suspected of being an osteoarthritis therapeutic; growing said modified chondrocyte in the absence of the compound; determining an osteoarthritic characteristic in the modified chondrocyte in the presence of the compound and in the absence of the compound; and comparing the osteoarthritic characteristic of the modified chondrocytes, wherein a reversal of the osteoarthritic characteristic in the presence of the compound is indicative of a osteoarthritis therapeutic, wherein growing the modified chondrocytes results in formation of engineered cartilage tissue.

[0010] Disclosed are methods for screening potential osteoarthritis therapeutics comprising growing a modified chondrocyte comprising an altered TGF β signaling pathway gene in the presence of a compound suspected of being an osteoarthritis therapeutic; growing said modified chondrocyte in the absence of the compound; determining an osteoarthritic characteristic in the modified chondrocyte in the presence of the compound and in the absence of the compound; and comparing the osteoarthritic characteristic of the modified chondrocytes, wherein a reversal of the osteoarthritic characteristic in the presence of the compound is indicative of a osteoarthritis therapeutic, wherein the osteoarthritic characteristic is a loss of proteoglycans, hypertrophic differentiation of the modified chondrocytes, a decrease in stiffness of the engineered cartilage tissue, or fibrillation of the matrix, clustering of the chondrocytes, or size of the chondrocytes.

[0011] In some instances, hypertrophic modified chondrocytes are determined by detecting the presence of a hypertrophy biomarker.

[0012] In some instances, the loss of proteoglycans is determined using alcian blue staining or aggrecan staining.

[0013] Disclosed are methods for screening potential osteoarthritis therapeutics comprising growing a modified chondrocyte comprising an altered TGF β signaling pathway gene in the presence of a compound suspected of being an osteoarthritis therapeutic; growing said modified chondrocyte in the absence of the compound; determining an osteoarthritic characteristic in the modified chondrocyte in the presence of the compound and in the absence of the compound; and comparing the osteoarthritic characteristic of the modified chondrocytes, wherein a reversal of the osteoarthritic characteristic in the presence of the compound is indicative of an osteoarthritis therapeutic, wherein the reversal of the osteoarthritic characteristic comprises an increase of proteoglycans, a decrease in the hypertrophy biomarker, a return of the stiffness of the modified

chondrocytes or a change in histology of the modified chondrocytes or engineered cartilage tissue.

[0014] Disclosed are methods for screening potential osteoarthritis therapeutics comprising growing a modified chondrocyte comprising an altered TGF β signaling pathway gene in the presence of a compound suspected of being an osteoarthritis therapeutic; growing said modified chondrocyte in the absence of the compound; determining an osteoarthritic characteristic in the modified chondrocyte in the presence of the compound and in the absence of the compound; and comparing the osteoarthritic characteristic of the modified chondrocytes, wherein a reversal of the osteoarthritic characteristic in the presence of the compound is indicative of a osteoarthritis therapeutic, wherein the compound is suspected of being an osteoarthritis therapeutic that alters the TGF- β pathway. In some instances, altering the TGF- β pathway comprises increasing sumoylation of SOX9 or increasing the activity of PAPS synthetase 2 (PAPSS2).

[0015] Additional advantages of the disclosed method and compositions will be set forth in part in the description which follows, and in part will be understood from the description, or may be learned by practice of the disclosed method and compositions. The advantages of the disclosed method and compositions will be realized and attained by means of the elements and combinations particularly pointed out in the appended claims. It is to be understood that both the foregoing general description and the following detailed description are exemplary and explanatory only and are not restrictive of the invention as claimed.

BRIEF DESCRIPTION OF THE DRAWINGS

[0016] The accompanying drawings, which are incorporated in and constitute a part of this specification, illustrate several embodiments of the disclosed method and compositions and together with the description, serve to explain the principles of the disclosed method and compositions.

[0017] Figure 1 is a schematic of Papss2 gene and regulatory elements. Smad3 binding site (SEQ ID NO:1) is the sequence shown on the far left and paired Sox9 (SEQ ID NO:2) is the sequence shown on the far right. Arrows indicate direction of binding sequence.

[0018] Figure 2 shows that TGF- β regulates Sox9 protein. (A) Sox9 and β -tubulin proteins were detected in lysates from rib costal chondrocytes treated with TGF- β 1 (5ng/ml) or vehicle (4mM-HCl with 0.1% BSA) for 2h and 5h. Treatment with TGF- β resulted in increased levels of Sox9 protein. (B) Sox9 and 18S mRNA were examined by RT-PCR of RNA isolated from rib chondrocytes left untreated or treated with TGF- β 1 (5ng/ml) for 2h and 4h. mRNA levels were not affected by TGF- β . Similar results were seen with limb mesenchyme, ATDC5 cells, and bovine articular chondrocytes. (C) Bovine cells were treated with cyclohexamide with or

without TGF- β treatment. Loss of Sox9 protein was measured over time by western blot. Reduced Sox9 levels reached a plateau after 3 hours in cells not treated with TGF- β . In contrast, Sox9 levels were not reduced until 6 hours in the presence of TGF- β suggesting TGF- β promotes Sox9 protein stability. (D) C3H10T1/2 cells were transfected with WT FLAG-Sox9 and treated with and without TGF- β 1 for 5h, lysates were subjected to Western blot for Sox9 and β -tubulin, and to IP of FLAG followed by Western blot for SUMO1. Treatment with TGF- β resulted in increased sumoylation of Sox9. (E) Bovine chondrocytes were infected with Adeno FLAG WT Sox9 for 48h then treated with TGF- β for 24h followed by IP for FLAG and western for SUMO1 and FLAG. Western for tubulin (no IP) was used as a control. Treatment with TGF- β resulted in increased SUMO-Sox9.

[0019] Figure 3 shows an overall model as related to specific aims in the proposal.

[0020] Figure 4. (A) Location of Sox9 sumoylatable lysine (K) residues and phosphorylatable serine (S) residues. (B) Mapping of Sox9 sumoylation mutants.

[0021] Figure 5 shows a modified form of Sox9 is detectable in the nucleus in chondrocytes. (A) Primary bovine articular chondrocytes were plated for 24h and cellular fractionation was performed. A high MW form of Sox9 was detected in the nucleus by western blot (B) Bovine chondrocytes were treated with (+) or without (-) 5ng TGF- β 1/ml. Endogenous Sox9 was IPed from the nuclear lysates. Western blot using anti SUMO antibody confirmed the high MW weight Sox9 band was the sumoylated form. The arrow designates the specific band. (C) Nuclear lysates from TGF- β 1 +/- cells were used in western blot for Sox9. Treatment with TGF- β increased the level of the high MW form of Sox9 in the nucleus. Western blot of β -tubulin and CREB were used as controls for cytosolic and nuclear fractions, respectively

[0022] Figure 6 shows an association of PIAS1 and Sox9 in response to TGF- β . (A) Primary bovine chondrocytes were infected with Ad FLAG Sox9. Cells were untreated (-) or treated (+; duplicate samples shown) with TGF- β . IP was done using anti-FLAG to bring down Sox9 and associated proteins. Western blot was done with anti-PIAS1. There was increased association of FLAG-Sox9 and endogenous PIAS1 in response to TGF- β . (Sorry about the bubbles lane 3 is better.) (B) ATDC5 cells were transfected with FLAG-PIAS1 and then left untreated (-) or treated (+) with TGF- β . IP was done using anti-FLAG to bring down PIAS1. Western blot was done with anti- Sox9 to detect associated endogenous Sox9. There was an increase in association of PIAS1 and SUMO-Sox9 with TGF β treatment.

[0023] Figure 7. (A) Bovine chondrocytes were infected with Ad-GFP or Ad-FLAGSox9. Western blot confirms expression of FLAG Sox9. (B) Ad-GFP cells were treated with TGF- β . RNA was used in real time RT-PCR to confirm up-regulation of PRG4 and PAPSS2 in virus

infected cells. (C) Real time RT-PCR shows up regulation of PAPSS2 by FLAG Sox9. (D) There was no additional increase in Papss2 expression in Ad-Sox9 infected cells treated with TGF- β .

[0024] Figure 8 shows a concentric cylinder bioreactor schematic diagram depicting bioreactor configuration and construct placement on inner bob.

[0025] Figures 9 is a graph showing mechanical properties in engineered cartilage (35 days in bioreactor). Shear modulus was measured using a Bohlin CVO rehometer. Low oxygen content lead to constructs with native material properties. * $p < 0.01$.

[0026] Figures 10A and 10B show knee joint histology in wild-type mice showed cartilage with smooth articular surfaces (A), whereas knee joint histology in DNIIR mice showed fibrillated articular surfaces (B) and other OA symptoms.

[0027] Figures 11A-11D show that TGF- β upregulates Papss2 and Prg4 (A and B), maintains type II collagen (C), and blocks type X collagen (D).

[0028] Figures 12A, 12B, and 12C show chondrocytes were successfully transduced with eGFP and DNIIR adenoviruses (A and B), and the DNIIR adenovirus reduced Papss2 and Prg4 expression as was observed in DNIIR mice (C and D).

[0029] Figures 13A-13H show PLLA scaffolds (A) were seeded with chondrocytes and cultivated in a bioreactor (B) for 4, 12, and 20 days (C-E respectively), resulting in tissue formation after 20 days. Histology showed the outermost layer of cells aligned with the tissue surface (F), toluidine blue staining identified proteoglycans in the extracellular matrix (G), and live-dead staining showed the majority of cells were living.

DETAILED DESCRIPTION

[0030] The disclosed method and compositions may be understood more readily by reference to the following detailed description of particular embodiments and the Example included therein and to the Figures and their previous and following description.

[0031] It is to be understood that the disclosed method and compositions are not limited to specific synthetic methods, specific analytical techniques, or to particular reagents unless otherwise specified, and, as such, may vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only and is not intended to be limiting.

A. Definitions

[0032] It is understood that the disclosed method and compositions are not limited to the particular methodology, protocols, and reagents described as these may vary. It is also to be

understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention, which will be limited only by the appended claims.

[0033] It must be noted that as used herein and in the appended claims, the singular forms "a", "an", and "the" include plural reference unless the context clearly dictates otherwise. Thus, for example, reference to "a modified chondrocyte" includes a plurality of such modified chondrocytes, reference to "the modified chondrocyte" is a reference to one or more modified chondrocytes and equivalents thereof known to those skilled in the art, and so forth.

[0034] "Optional" or "optionally" means that the subsequently described event, circumstance, or material may or may not occur or be present, and that the description includes instances where the event, circumstance, or material occurs or is present and instances where it does not occur or is not present.

[0035] Ranges may be expressed herein as from "about" one particular value, and/or to "about" another particular value. When such a range is expressed, also specifically contemplated and considered disclosed is the range from the one particular value and/or to the other particular value unless the context specifically indicates otherwise. Similarly, when values are expressed as approximations, by use of the antecedent "about," it will be understood that the particular value forms another, specifically contemplated embodiment that should be considered disclosed unless the context specifically indicates otherwise. It will be further understood that the endpoints of each of the ranges are significant both in relation to the other endpoint, and independently of the other endpoint unless the context specifically indicates otherwise. Finally, it should be understood that all of the individual values and sub-ranges of values contained within an explicitly disclosed range are also specifically contemplated and should be considered disclosed unless the context specifically indicates otherwise. The foregoing applies regardless of whether in particular cases some or all of these embodiments are explicitly disclosed.

[0036] Throughout the description and claims of this specification, the word "comprise" and variations of the word, such as "comprising" and "comprises," means "including but not limited to," and is not intended to exclude, for example, other additives, components, integers or steps. In particular, in methods stated as comprising one or more steps or operations it is specifically contemplated that each step comprises what is listed (unless that step includes a limiting term such as "consisting of"), meaning that each step is not intended to exclude, for example, other additives, components, integers or steps that are not listed in the step.

[0037] The term "polylactic acid composition" can be used for a composition made by dissolving polylactic acid in methylene chloride and adding NaCl to form a paste that is dried. In

some instances, the polylactic acid can be poly-D-lactic acid (PDLA), poly-L-lactic acid (PLLA), or a racemic mixture of the two.

[0038] The term “modified chondrocyte” refers to any chondrocyte that has been altered from its native state. In some instances, a modified chondrocyte has an altered TGF β signaling pathway gene.

[0039] The term “osteoarthritic characteristic” refers to any characteristic that is used to define or diagnose osteoarthritis. For example, an osteoarthritic characteristic can be a description of the state of osteoarthritic tissue or cells. Examples of osteoarthritic characteristics are a loss of proteoglycans, hypertrophic differentiation of the modified chondrocytes, a decrease in stiffness of the engineered cartilage tissue, and fibrillation of the matrix, clustering of the chondrocytes, or size of the chondrocytes

[0040] “Altering the TGF- β pathway” or “alters the TGF- β pathway” refers to changing the TGF- β pathway from what is considered normal or what is present in healthy cells or tissue. Altering can comprise an increase or decrease in the amount of a TGF- β pathway gene or an increase or decrease in the activity of a protein encoded from a TGF- β pathway gene.

[0041] Unless defined otherwise, all technical and scientific terms used herein have the same meanings as commonly understood by one of skill in the art to which the disclosed method and compositions belong. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present method and compositions, the particularly useful methods, devices, and materials are as described. Publications cited herein and the material for which they are cited are hereby specifically incorporated by reference. Nothing herein is to be construed as an admission that the present invention is not entitled to antedate such disclosure by virtue of prior invention. No admission is made that any reference constitutes prior art. The discussion of references states what their authors assert, and applicants reserve the right to challenge the accuracy and pertinency of the cited documents. It will be clearly understood that, although a number of publications are referred to herein, such reference does not constitute an admission that any of these documents forms part of the common general knowledge in the art.

[0042] Disclosed are materials, compositions, and components that can be used for, can be used in conjunction with, can be used in preparation for, or are products of the disclosed method and compositions. These and other materials are disclosed herein, and it is understood that when combinations, subsets, interactions, groups, etc. of these materials are disclosed that while specific reference of each various individual and collective combinations and permutation of these compounds may not be explicitly disclosed, each is specifically contemplated and

described herein. If a class of molecules A, B, and C are disclosed as well as a class of molecules D, E, and F and an example of a combination molecule, A-D is disclosed, then even if each is not individually recited, each is individually and collectively contemplated. Thus, in this example, each of the combinations A-E, A-F, B-D, B-E, B-F, C-D, C-E, and C-F are specifically contemplated and should be considered disclosed from disclosure of A, B, and C; D, E, and F; and the example combination A-D. Likewise, any subset or combination of these is also specifically contemplated and disclosed. Thus, for example, the sub-group of A-E, B-F, and C-E are specifically contemplated and should be considered disclosed from disclosure of A, B, and C; D, E, and F; and the example combination A-D. This concept applies to all aspects of this application including, but not limited to, steps in methods of making and using the disclosed compositions. Thus, if there are a variety of additional steps that can be performed it is understood that each of these additional steps can be performed with any specific embodiment or combination of embodiments of the disclosed methods, and that each such combination is specifically contemplated and should be considered disclosed.

B. Methods of Screening

[0043] Disclosed are methods for screening potential osteoarthritis therapeutics comprising a) growing a modified chondrocyte comprising an altered TGF β signaling pathway gene in the presence of a compound suspected of being an osteoarthritis therapeutic; b) growing said modified chondrocyte in the absence of the compound; c) determining an osteoarthritic characteristic in the modified chondrocyte in the presence of the compound and in the absence of the compound; and d) comparing the osteoarthritic characteristic of the modified chondrocytes, wherein a reversal of the osteoarthritic characteristic in the presence of the compound is indicative of a osteoarthritis therapeutic.

1. Modified Chondrocytes

[0044] In some instances of the disclosed methods, the modified chondrocyte can be a mammalian cell. In some instances the mammalian cell can be a human cell. In some instances, the modified chondrocytes can be chondrocytes that are harvested from a mammal and then modified. For example, the modified chondrocytes can be human chondrocytes.

[0045] In some instances, the modified chondrocytes can be multipotent stem cells (MSCs), induced pluripotent stem cells (iPSCs) or any pluripotent cell source.

[0046] In some instances of the disclosed methods, the modified chondrocyte can be genetically modified. In some instances, the genetic modification can be a mutation in a TGF β signaling pathway gene. For example, the genetic modification can be in the TGF β R gene or Smad3 gene.

[0047] Disclosed are methods for screening potential osteoarthritis therapeutics comprising a) growing a modified chondrocyte comprising an altered TGF β signaling pathway gene in the presence of a compound suspected of being an osteoarthritis therapeutic; b) growing said modified chondrocyte in the absence of the compound; c) determining an osteoarthritic characteristic in the modified chondrocyte in the presence of the compound and in the absence of the compound; and d) comparing the osteoarthritic characteristic of the modified chondrocytes, wherein a reversal of the osteoarthritic characteristic in the presence of the compound is indicative of a osteoarthritis therapeutic; wherein the altered TGF β signaling pathway gene can be an altered TGF β R gene. In some instances, the TGF β R gene can be TGF β R type II. In some instances, the altered TGF β R gene comprises a dominant negative mutation in the TGF β R gene. For example, the dominant negative mutation can be a truncation of the TGF β R gene. TGF β R contains a ligand binding domain, transmembrane domain and juxtamembrane domain and a kinase domain. In some instances, the truncation of the TGF β R gene can be the deletion of the kinase domain. The deletion can be a deletion of the entire domain or a deletion of a part of the kinase domain, which results in the kinase domain being non-functional.

2. Growing modified chondrocytes

[0048] Disclosed are methods for screening potential osteoarthritis therapeutics comprising a) growing a modified chondrocyte comprising an altered TGF β signaling pathway gene in the presence of a compound suspected of being an osteoarthritis therapeutic; b) growing said modified chondrocyte in the absence of the compound; c) determining an osteoarthritic characteristic in the modified chondrocyte in the presence of the compound and in the absence of the compound; and d) comparing the osteoarthritic characteristic of the modified chondrocytes, wherein a reversal of the osteoarthritic characteristic in the presence of the compound is indicative of a osteoarthritis therapeutic; wherein growing the modified chondrocytes comprises culturing the modified chondrocytes on a cell culture dish, multiwell plate, or flask. Any known cell culture apparatus can be used for culturing the modified chondrocytes. In some instances the cell culture apparatus can be a bioreactor. Thus, the disclosed methods can comprise the growing of the modified chondrocytes comprising culturing the modified chondrocytes in a bioreactor. In some instances, the growing of the modified chondrocytes comprises culturing the modified chondrocytes on a scaffold. In some instances, the scaffold can be a polylactic acid composition. Examples of polylactic acid can be poly-D-lactic acid (PDLA), poly-L-lactic acid (PLLA) or a racemic mixture of the two. In some instances, any known cell culturing scaffold can be used.

[0049] Disclosed are methods for screening potential osteoarthritis therapeutics comprising a) growing a modified chondrocyte comprising an altered TGF β signaling pathway gene in the presence of a compound suspected of being an osteoarthritis therapeutic; b) growing said modified chondrocyte in the absence of the compound; c) determining an osteoarthritic characteristic in the modified chondrocyte in the presence of the compound and in the absence of the compound; and d) comparing the osteoarthritic characteristic of the modified chondrocytes, wherein a reversal of the osteoarthritic characteristic in the presence of the compound is indicative of a osteoarthritis therapeutic; wherein growing the modified chondrocytes results in formation of engineered cartilage tissue. Engineered cartilage tissue comprises cells and a matrix. The engineered cartilage tissue can have the same properties of natural cartilage tissue. For example, engineered cartilage tissue comprises the natural architecture and extracellular matrix present in native cartilage tissue. Engineered cartilage tissue can comprise type II collagen and aggrecan.

3. Osteoarthritic characteristics

[0050] Disclosed are methods for screening potential osteoarthritis therapeutics comprising a) growing a modified chondrocyte comprising an altered TGF β signaling pathway gene in the presence of a compound suspected of being an osteoarthritis therapeutic; b) growing said modified chondrocyte in the absence of the compound; c) determining an osteoarthritic characteristic in the modified chondrocyte in the presence of the compound and in the absence of the compound; and d) comparing the osteoarthritic characteristic of the modified chondrocytes, wherein a reversal of the osteoarthritic characteristic in the presence of the compound is indicative of a osteoarthritis therapeutic; wherein the osteoarthritic characteristic is a loss of proteoglycans. The loss of proteoglycans can be determined using alcian blue staining or aggrecan staining. Assays used to determine loss of proteoglycans are well known in the art.

[0051] Disclosed are methods for screening potential osteoarthritis therapeutics comprising a) growing a modified chondrocyte comprising an altered TGF β signaling pathway gene in the presence of a compound suspected of being an osteoarthritis therapeutic; b) growing said modified chondrocyte in the absence of the compound; c) determining an osteoarthritic characteristic in the modified chondrocyte in the presence of the compound and in the absence of the compound; and d) comparing the osteoarthritic characteristic of the modified chondrocytes, wherein a reversal of the osteoarthritic characteristic in the presence of the compound is indicative of a osteoarthritis therapeutic; wherein the reversal of the osteoarthritic characteristic comprises an increase of proteoglycans. An increase of proteoglycans can be

determined using alcian blue staining or aggrecan staining. Assays used to determine the presence or increase of proteoglycans are well known in the art.

[0052] Disclosed are methods for screening potential osteoarthritis therapeutics comprising a) growing a modified chondrocyte comprising an altered TGF β signaling pathway gene in the presence of a compound suspected of being an osteoarthritis therapeutic; b) growing said modified chondrocyte in the absence of the compound; c) determining an osteoarthritic characteristic in the modified chondrocyte in the presence of the compound and in the absence of the compound; and d) comparing the osteoarthritic characteristic of the modified chondrocytes, wherein a reversal of the osteoarthritic characteristic in the presence of the compound is indicative of a osteoarthritis therapeutic; wherein the osteoarthritic characteristic is hypertrophic differentiation of the modified chondrocytes. In some instances, hypertrophic modified chondrocytes can be determined by detecting the presence of a hypertrophy biomarker. Hypertrophy biomarkers can be type X collagen or MMP13. In some instances, hypertrophic modified chondrocytes can be determined

[0053] Disclosed are methods for screening potential osteoarthritis therapeutics comprising a) growing a modified chondrocyte comprising an altered TGF β signaling pathway gene in the presence of a compound suspected of being an osteoarthritis therapeutic; b) growing said modified chondrocyte in the absence of the compound; c) determining an osteoarthritic characteristic in the modified chondrocyte in the presence of the compound and in the absence of the compound; and d) comparing the osteoarthritic characteristic of the modified chondrocytes, wherein a reversal of the osteoarthritic characteristic in the presence of the compound is indicative of a osteoarthritis therapeutic; wherein the reversal of the osteoarthritic characteristic comprises a decrease in the hypertrophy biomarker. Hypertrophy biomarkers can be type X collagen or MMP13.

[0054] Disclosed are methods for screening potential osteoarthritis therapeutics comprising a) growing a modified chondrocyte comprising an altered TGF β signaling pathway gene in the presence of a compound suspected of being an osteoarthritis therapeutic; b) growing said modified chondrocyte in the absence of the compound; c) determining an osteoarthritic characteristic in the modified chondrocyte in the presence of the compound and in the absence of the compound; and d) comparing the osteoarthritic characteristic of the modified chondrocytes, wherein a reversal of the osteoarthritic characteristic in the presence of the compound is indicative of a osteoarthritis therapeutic; wherein the osteoarthritic characteristic is a decrease in the stiffness of the engineered cartilage tissue. The decrease in stiffness of the engineered cartilage tissue is compared to the stiffness of healthy cartilage tissue whether

engineered or native. Thus, an osteoarthritic characteristic would be a decrease in the stiffness compared to normal or healthy cartilage tissue. The term “stiffness” refers to the Young’s elastic modulus which measures the force per unit area that is required to compress a sample.

[0055] Disclosed are methods for screening potential osteoarthritis therapeutics comprising a) growing a modified chondrocyte comprising an altered TGF β signaling pathway gene in the presence of a compound suspected of being an osteoarthritis therapeutic; b) growing said modified chondrocyte in the absence of the compound; c) determining an osteoarthritic characteristic in the modified chondrocyte in the presence of the compound and in the absence of the compound; and d) comparing the osteoarthritic characteristic of the modified chondrocytes, wherein a reversal of the osteoarthritic characteristic in the presence of the compound is indicative of a osteoarthritis therapeutic; wherein the reversal of the osteoarthritic characteristic comprises a return of the stiffness of the modified chondrocytes.

[0056] Disclosed are methods for screening potential osteoarthritis therapeutics comprising a) growing a modified chondrocyte comprising an altered TGF β signaling pathway gene in the presence of a compound suspected of being an osteoarthritis therapeutic; b) growing said modified chondrocyte in the absence of the compound; c) determining an osteoarthritic characteristic in the modified chondrocyte in the presence of the compound and in the absence of the compound; and d) comparing the osteoarthritic characteristic of the modified chondrocytes, wherein a reversal of the osteoarthritic characteristic in the presence of the compound is indicative of a osteoarthritis therapeutic; wherein the osteoarthritic characteristic is alterations in normal histology of the cartilage tissue including loss of zonal structure of the tissue, fibrillation of the matrix, clustering of the chondrocytes, or an increase in size of the chondrocytes. Fibrillation of the matrix can be determined by the edges of the tissue not being smooth. Clustering of the chondrocytes can refer to the chondrocytes not having matrix in between them.

[0057] Disclosed are methods for screening potential osteoarthritis therapeutics comprising a) growing a modified chondrocyte comprising an altered TGF β signaling pathway gene in the presence of a compound suspected of being an osteoarthritis therapeutic; b) growing said modified chondrocyte in the absence of the compound; c) determining an osteoarthritic characteristic in the modified chondrocyte in the presence of the compound and in the absence of the compound; and d) comparing the osteoarthritic characteristic of the modified chondrocytes, wherein a reversal of the osteoarthritic characteristic in the presence of the compound is indicative of a osteoarthritis therapeutic; wherein the reversal of the osteoarthritic characteristic comprises a change in histology of the modified chondrocytes. A change in

histology of the modified chondrocytes can be a restoration of the zonal structure of the cartilage tissue, reduced fibrillation of the matrix, reduced clustering of the chondrocytes, or reduced size (hypertrophy) of chondrocytes. Fibrillation of the matrix can be determined by the edges of the tissue not being smooth. Clustering of the chondrocytes can refer to the chondrocytes not having matrix in between them.

4. Compounds suspected of being osteoarthritis therapeutics

[0058] Disclosed are methods for screening potential osteoarthritis therapeutics comprising a) growing a modified chondrocyte comprising an altered TGF β signaling pathway gene in the presence of a compound suspected of being an osteoarthritis therapeutic; b) growing said modified chondrocyte in the absence of the compound; c) determining an osteoarthritic characteristic in the modified chondrocyte in the presence of the compound and in the absence of the compound; and d) comparing the osteoarthritic characteristic of the modified chondrocytes, wherein a reversal of the osteoarthritic characteristic in the presence of the compound is indicative of a osteoarthritis therapeutic; wherein the compound is suspected of being an osteoarthritis therapeutic that alters the TGF- β pathway. In some instances, altering the TGF- β pathway comprises altering sumoylation of SOX9. For example, altering sumoylation of SOX9 can include increasing sumoylation of SOX9. In some instances, altering the TGF- β pathway comprises altering protein levels of SOX9. For example, altering protein levels of SOX9 can include increasing protein levels of SOX9. In some instances, increasing sumoylation of SOX9 comprises increase mRNA levels of SOX9.

[0059] Disclosed are methods for screening potential osteoarthritis therapeutics comprising a) growing a modified chondrocyte comprising an altered TGF β signaling pathway gene in the presence of a compound suspected of being an osteoarthritis therapeutic; b) growing said modified chondrocyte in the absence of the compound; c) determining an osteoarthritic characteristic in the modified chondrocyte in the presence of the compound and in the absence of the compound; and d) comparing the osteoarthritic characteristic of the modified chondrocytes, wherein a reversal of the osteoarthritic characteristic in the presence of the compound is indicative of a osteoarthritis therapeutic; wherein the compound is suspected of being an osteoarthritis therapeutic that alters the TGF- β pathway, wherein altering the TGF- β pathway comprises altering the activity of PAPS synthetase 2 (PAPSS2). For example, altering the activity of PAPSS2 can be increasing or decreasing the activity of PAPSS2. In some instances, the activity of PAPSS2 comprises generating PAPS. PAPSS2 activity can be measured with alcian blue staining. mRNA or protein levels of PAPSS2 can also be measured. In some instances, Paps can be measured.

C. Kits

[0060] The materials described above as well as other materials can be packaged together in any suitable combination as a kit useful for performing, or aiding in the performance of, the disclosed method. It is useful if the kit components in a given kit are designed and adapted for use together in the disclosed method. For example disclosed are kits for screening potential osteoarthritis therapeutics, the kit comprising a compound suspected of being an osteoarthritis therapeutic. Also disclosed are kits for screening potential osteoarthritis therapeutics, the kit comprising modified chondrocytes. The kits also can contain cell culture media or a cell culture apparatus such as a flask, cell culture dish, multiwall plate, or bioreactor.

Examples

[0061] Osteoarthritis is a leading cause of disability in the industrialized world but little is known about mechanisms of cartilage destruction associated with osteoarthritis. Recent advances in the genetic manipulation of mice have led to new animal models and new concepts that are relevant to understanding osteoarthritis in humans. These studies help understand the factors that mediate the development, persistence, and repair of articular cartilage and to identify specific targets for prevention and treatment strategies for osteoarthritis. TGF- β is a multifunctional peptide that has been shown to regulate cellular differentiation and tissue-specific gene expression. Previously, transgenic mice were generated that express a dominant-negative mutation of the TGF- β type II receptor (Tgfr2) in articular cartilage. Altered responsiveness to TGF- β resulted in a progressive skeletal disease that resembled osteoarthritis in humans. Several down-stream targets of TGF- β have been identified that regulate post-translational processing of the major extracellular matrix proteins in articular cartilage. One in particular, 3-Prime-Phosphoadenosine 5-Prime-Phosphosulfate Synthase 2 (Papss2), has been associated with Spondyloepimetaphyseal Dysplasias in humans and is required for proper sulfation of proteoglycans in cartilage. In addition to TGF- β , the transcription factor Sox9 is also associated with the maintenance of mature articular cartilage. TGF- β enhances the level of Sox9 protein in chondrocytes independently of changes in mRNA. The data indicate that treatment with TGF- β results in sumoylation of Sox9. Sumoylation has been shown to regulate protein stability, activity and cellular localization of proteins. This study is directed to: 1a) determining which sites on Sox9 are sumoylated in response to TGF- β and determining the role of sumoylation in TGF- β -mediated Sox9 levels, localization, and activity; 1b) determining the mechanism of TGF- β -mediated sumoylation of Sox9; 2) determining the mechanism of TGF- β mediated expression of Papss2 and 3) determining if Papss2 activity can alleviate cartilage

degeneration when TGF- β signaling is disrupted and determining if activation of TGF- β 's chondroprotective signals can restore biochemical and biomechanical properties to OA cartilage. These studies can identify mechanisms of chondroprotection that can be used as targets for therapies in osteoarthritis.

[0062] According to the Centers for Disease Control, Osteoarthritis (OA) is the most common form of arthritis and the primary cause of disability in the US. OA is characterized by erosion of articular cartilage leading to joint pain and eventual loss of joint function. The loss of articular cartilage during OA is mainly due to changes in chondrocyte function. The mechanisms involved in these changes remain unclear. Transforming growth factor-beta (TGF- β) is a multifunctional peptide that regulates chondrocyte differentiation and tissue-specific gene expression. Previously, transgenic mice that express a dominant-negative mutation of the TGF- β type II receptor (DNRIIR) in cartilage were generated resulting in a progressive skeletal disease that resembles OA in humans. The results indicated that TGF- β normally prevents joint degeneration, however, the downstream effectors of this chondroprotective effect are not known. Alterations in chondrocyte function and diminished extracellular matrix deposition, leading to cartilage degeneration, have also been associated with a decrease in the expression of the SRY (sex-determining region Y) box 9 (Sox9) transcription factor. Nevertheless, up-stream regulators of Sox9 activity have not been very well characterized. Several down-stream targets of TGF- β have been identified that regulate post-translational processing of the major extracellular matrix proteins in articular cartilage. One in particular, 3-Prime-Phosphoadenosine 5-Prime-Phosphosulfate Synthase 2 (Papss2), has been associated with Spondyloepimetaphyseal Dysplasias and OA in humans and is required for proper sulfation of proteoglycans in cartilage. A putative paired Sox9 binding site was identified in the first intron of the Papss2 gene and these studies indicate that Sox9 regulates Papss2 expression. Furthermore, treatment with TGF- β enhances sumoylation of Sox9 protein in chondrocytes, which may alter the stability or activity of the protein. This study is designed to determine the mechanism of TGF- β 's chondroprotective actions. TGF- β can maintain the differentiated chondrocyte phenotype in permanent cartilages, like articular cartilage, by regulating Sox9 levels and activity via protein sumoylation. Sox9 in turn regulates the expression of specific down-stream targets of TGF- β that have global effects on the extracellular matrix including Papss2. The chondroprotective effects of TGF- β also have reparative functions. Since TGF- β has broad biological activity in many tissues, these studies can elucidate the cartilage specific targets of TGF- β for investigation into new therapies to repair damage to articular cartilage.

[0063] Sumoylation can affect Sox9 levels, localization, and activity in response to TGF- β . It has been shown that there are three lysine residues in the Sox9 protein that can be sumoylated. The results indicate that TGF- β increases sumoylation of Sox9. Sumoylation has been shown to regulate protein stability, activity and cellular localization. Therefore, FLAG-tagged Sox9-encoding plasmids were generated in which combinations of the three sumoylatable lysine residues were converted to arginine. These mutants as well as wild-type Sox9 can be used to determine the role of sumoylation on TGF- β mediated Sox9 levels, localization, and transcriptional activation.

[0064] PIAS1 can regulate sumoylation of Sox9 by TGF- β . It has been shown that TGF- β can mediate sumoylation of various target proteins through PIAS1. It can be determined if treatment with TGF- β results in activation of PIAS1 and increased association of PIAS1 with Sox9. RNA interference as well as a dominant-negative PIAS1 expression vectors will then be used to examine the requirement of PIAS1 for Sox9 sumoylation in response to TGF- β .

[0065] Sox9 can be required for TGF- β mediated expression of Papss2. TGF- β regulates the expression of Papss2 mRNA in bovine articular chondrocytes and Papss2 mRNA and staining for sulfated proteoglycans is down-regulated in DNIIR mice. This study can determine if Sox9 is sufficient to stimulate expression of Papss2 and is necessary for TGF- β mediated regulation. It can also be determined if sumoylation is also required for TGF- β mediated expression of Papss2.

[0066] Papss2 activity can alleviate cartilage degeneration when TGF- β signaling is disrupted. Both engineered bovine articular chondrocyte constructs and in vivo transgenic mouse models can be used to test this hypothesis. Bovine cartilage infected with control and DNIIR adenovirus with or without a Papss2 expressing virus can be grown in a bioreactor. Cartilage can be tested to quantify the effect of TGF- β and Papss2 activity in regulating cartilage biochemical biomechanical properties. Next, mice expressing Papss2 under the control of the Col2a promoter can be generated and crossed to DNIIR mice. Biomechanical and biochemical properties can be compared in control and double transgenic mice. In addition, human OA cartilage grown in bioreactors can be used to show that stimulation of TGF- β 's chondroprotective functions has some reparative effects on OA cartilage.

A. Significance

[0067] OA is a leading cause of disability in the industrialized world but little is known about mechanisms of cartilage destruction associated with osteoarthritis. Chondrocytes and the extracellular matrix they produce are essential to maintain the normal structure and biomechanical properties of articular cartilage. Articular cartilage has a limited intrinsic capacity for repair placing major obstacles in treatment strategies for OA. Nevertheless, surprisingly little

is known about the factors or downstream targets that regulate maintenance of articular cartilage, information that would be the foundation of any prevention or treatment strategies.

[0068] Genetic manipulation of the mouse has yielded new concepts that are relevant to OA and indicate that TGF- β signaling is critical for maintenance of articular cartilage. Members of the Transforming Growth Factor-beta (TGF- β) family are secreted signaling molecules that regulate many aspects of growth and differentiation. TGF- β s signal through heteromeric serine/threonine kinase receptors. The current model is that TGF- β ligand binds to the TGF- β type II receptor (Tgfr2) on the cell surface. Tgfr2 is then able to recruit the Type I receptor (Tgfr1 (ALK5)) to form a heterotetrameric complex of two type I and two type II receptors. Tgfr2, which is a constitutively active kinase, phosphorylates the GS domain of Tgfr1, activating the type I serine/threonine kinase. Downstream targets of the Tgfr1 kinase then transduce the signal to the nucleus. The Smad family of proteins has been identified as important transducers of TGF- β signaling. Smads are directly phosphorylated by the type I receptor, translocate to the nucleus and act as transcription factors. Receptor associated Smads can be separated into two broad classes: those generally activated by TGF- β / Activin signaling (Smad 2/3) and those generally activated by BMP signaling (Smad1/5/8). Smads have been shown to bind DNA directly or in some cases cooperate with transcription co-factors or co-repressors. Smad independent, non-canonical signaling pathways have also been identified. These pathways include signaling through Erk, JNK and p38 MAPK pathways, as well as the PI3K pathway, and Rho-like GTPases. Previously, it was shown that dominant-negative interference of Tgfr2 (DNIIR) in mice results in OA-like symptoms in the peripheral joints indicating TGF- β has chondroprotective functions. Similarly, mice deleted for Smad3 or Latent TGF- β Binding Protein 3 (LTBP3), an extracellular mediator of TGF- β signaling demonstrated OA symptoms. Furthermore, degradation of phosphorylated Smad3 by over-expression of Smurf2, an E3 ubiquitin ligase, resulted in OA in transgenic mice. Later, it was demonstrated that genetic variations in the human SMAD3 gene are associated with early onset hip and knee OA. More recently, age related changes in the way TGF- β signals were associated with OA. In young healthy chondrocytes, TGF- β primarily signals through Tgfr1 to activate Smad2/3 signaling and provide chondroprotective signals. In aged mice (> 1yr) and OA cartilage, the ratio of an alternate type I receptor, Acvr1 (ALK1), which signals through Smad1/5/8, to normal Tgfr1 was increased. This alternative signaling pathway was associated with hypertrophic differentiation and expression of catabolic factors like MMP-13. Together, the results indicate that TGF- β signaling has a critical role in cartilage homeostasis yet the mechanisms of TGF- β action are not known. This proposal focuses on TGF- β 's chondroprotective effects.

[0069] TGF- β has many biological effects on multiple tissues making it difficult to use TGF- β ligand directly as a therapy for cartilage repair or regeneration. Furthermore, changes in TGF- β signaling as cartilage ages complicate the use of TGF- β ligand for OA. Cartilage specific downstream effectors of TGF- β 's chondroprotective functions would be good targets for therapy but the molecular targets of TGF- β signaling in normal articular cartilage are largely unknown. To begin to address this issue an affymetrix based microarray was performed to find genes in bovine articular chondrocytes grown in micromass culture that were regulated by treatment with TGF- β (GEO accession # GSE29233). RNA was isolated from three independently derived cultures. RNA was labeled and hybridized to Affymetrix Bovine GeneChips containing 23,000 transcripts according to manufactures instructions. Data was analyzed using GeneSprings software generating a list of genes that were regulated at least 2-fold with a T-test $p < 0.05$. Sixty-one genes were down-regulated by treatment with TGF- β and 65 were found to be up-regulated. Known TGF- β regulated genes were identified on the array including: Transforming growth factor-beta induced protein IG-H3 precursor (β IG-H3), Proteoglycan 4/ Lubricin, and Parathyroid hormone-like hormone. Several genes that are known to globally regulate post-translational modifications of ECM proteins were also identified as being up-regulated by TGF- β including PAPS synthetase 2 (PAPSS2), which is associated with Pakistani type spondyloepimetaphyseal dysplasia (OMIM 612847). Papss2 was also identified as a TGF- β responsive gene in a microarray study focused on gene expression in the developing axial skeleton. Regulation of selected genes, including PAPSS2, were verified in bovine cells and in articular cartilage from DNIIR mice using real time RT-PCR.

[0070] Mutations in human PAPSS2 cause the Pakistani type of spondyloepimetaphyseal dysplasia and a spontaneous mutation in mice, brachymorphic (bm), results from an autosomal recessive mutation in the Papss2 gene. Skeletal alterations in mice and humans with deficiency in PAPSS2 have similarities to those of mice with deficiency in TGF- β signaling including kyphosis, post-natal short stature, and progressive degeneration of articular cartilage. Papss2 is a bifunctional enzyme whose main function is to generate PAPS, the sulfate donor for most sulfotransferase reactions. PAPS is created from ATP and inorganic sulfate in a two-step process that involves either Papss1 or Papss2 enzymes. Generation of PAPS is the rate-limiting step for most sulfation reactions. Sulfation is a common modification of proteins and carbohydrates but it is especially important in articular cartilage where the large amount of sulfation on the glycosamino glycans linked to the core proteins of proteoglycans provide the necessary biomechanical properties for cartilage to function. Papss2 is highly expressed in cartilage and the importance of this enzyme in maintaining the cartilage phenotype is clear from the largely

cartilage specific phenotypes associated with mutations in this gene, nevertheless, almost nothing is known about how enzyme levels are regulated.

[0071] To begin to formulate a model of how TGF- β regulates Papss2, a search for transcription factor binding sites in the mouse Papss2 gene was performed. The DialignTx program (Subramanian et al., 2008) was used to compare mouse and human DNA sequences 2Kb upstream of the Papss2 start site and the large first intron of the Papss2 gene to find putative phylogenetically conserved regulatory regions. Next, the Genomatix software suite and their nucleotide distribution matrices were used to identify potential transcription factor binding sites in the conserved upstream and first intron sequences. The program returns results based on an optimized matrix threshold, which minimizes the number of false positives. A putative Smad3 binding site was identified 1Kb upstream of the mouse Papss2 start site (Figure 1). Several individual putative Sox9 and Sox5 binding sites were also observed. The Sox9 transcription factor, along with Sox5 and Sox6, play an important role in the commitment of mesenchymal cells toward the chondrocyte lineage. A deficiency in Sox9 in humans is associated with Campomelic Dysplasia, a skeletal malformation characterized by generalized hypoplasia in endochondral bones. Sox9 is also expressed in mature articular chondrocytes and high expression levels of Sox9 are associated with enhanced levels of aggrecan and type II collagen and maintenance of articular cartilage. Sox9 usually binds as dimer to paired Sox9 sites to mediate expression of chondrocyte specific genes. Papss2 and Col2a1 have a similar genomic structure in that they both have a small first exon and very large first intron with many regulatory elements. A strong chondrocyte specific binding site for Sox9 is found in the first intron of the Col2a1 gene. Likewise, a putative paired Sox9 site was observed in the large first intron of Papss2 approximately 400bp from the start of exon 2 (Figure 1). It was previously shown that Papss2 and Sox9 are co-expressed during skeletal development also supporting a potential role for Sox9 in mediating Papss2 expression. The observations indicate a role for Smad3 and Sox9 in regulation of Papss2 expression.

[0072] Sox9 is post-translationally modified by phosphorylation, ubiquitination, and sumoylation. Sumoylation is a post-translational modification somewhat similar to ubiquitination. It involves the attachment of SUMO, a small peptide, to lysine residues on proteins. Unlike ubiquitination, sumoylation does not necessarily target a protein for degradation. Sumoylation has varying effects on protein function including altering stability, subcellular localization, or protein-protein interactions. Sumoylation has recently been implicated in altering protein functions during arthritis. Similar to ubiquitination, sumoylation occurs through the activities of E1, E2, and E3 sumoyl ligases. Protein Inhibitor of Activated

STAT1 (PIAS1) is an E3 sumoyl ligase. PIAS1 interacts directly with Sox9 leading to its stabilization. It was proposed that sumoylation of Sox9 blocked ubiquitination of the protein protecting it from proteosomal degradation. Furthermore, PIAS1 and SUMO1 altered the subnuclear localization of Sox9 when co-transfected into COS1 cells. Nevertheless, the role of sumoylation in regulation of Sox9 activity is controversial and there are results that indicate that PIAS1 can have indirect effects on Sox9 activity. Recent evidence has suggested a role for TGF- β in mediating sumoylation of various target transcription factors including including Smad4, SnoN, and KLF4 via PIAS1. It has been shown that TGF- β treatment results in up regulation of Sox9 protein without alterations in Sox9 mRNA levels in mouse limb micromass, rib chondrocytes, and bovine articular chondrocytes in culture as well as in cells of the ATDC5 chondrogenic cell line (Figure 2A, B). The lack of regulation at the mRNA level was confirmed with microarray data. Likewise, it was recently shown that Sox9 mRNA levels are repressed in mesenchymal cells by transcriptional repressors of TGF- β signaling, including TGIF and SnoN. The loss of Sox9 protein was delayed in cultures after treatment with cyclohexamide in the presence of TGF- β relative to cultures grown in the absence of TGF- β indicating TGF- β regulates the stability of Sox9 protein (Figure 2C). When C3H10T1/2 cells were transfected with FLAG-tagged wild type Sox9 and then treated with TGF- β , there was an increase in sumoylated Sox9 relative to untreated controls as measured by IP-western blot (Figure 2D). Similar results were seen in cultures of bovine articular chondrocytes (Figure 2E) TGF- β can regulate stability and function of Sox9 through protein sumoylation (Aim #1).

[0073] TGF- β , acting through PIAS1 mediated sumoylation of Sox9, can act as a chondroprotective factor by regulating the expression of enzymes that globally regulate the biochemical properties of the extracellular matrix, including Papss2 (Figure 3).

[0074] Although OA is one of the most common forms of musculoskeletal disability in the world, treatment options are limited. One of the main functions of articular cartilage in diarthrodial joints is load bearing and the cartilage matrix has to be finely tuned to have the proper biomechanical properties to deal with various types of mechanical load. Surprisingly little is known about how cartilage is maintained even though this information would form the basis of any new prevention or treatment strategies. TGF- β has been shown to have chondroprotective functions in cartilage but the mechanisms are not known. Cartilage specific downstream effectors of TGF- β 's chondroprotective signals would make good targets for prevention or treatment of osteoarthritis. Two targets for TGF- β 's action in cartilage have been identified: regulation of Papss2 expression and regulation of Sox9 activity through sumoylation. TGF- β , acting through sumoylation and Sox9 activity can regulate the expression of an

important enzyme, Papss2, required for maintaining structural integrity in cartilage. For example, if sumoylation through TGF- β in general is part of the mechanism through which TGF- β regulates cartilage homeostasis, methods to promote sumoylation could be investigated as treatment strategies, even if the exact targets remain elusive. Another important aspect of the proposed experiments is the innovative use of bioreactors to generate cartilage tissue for biochemical and biomechanical analysis. To test factors that can prevent cartilage degeneration in the presence of altered TGF- β signaling, genetically engineered cartilage tissue can be generated in bioreactors. This tissue can then be used in biomechanical and biochemical assays. The method can be scaled up for treatment screening and is more efficient than generating transgenic mice for experimentation.

B. TGF- β regulates Sox9 function through PIAS1 mediated sumoylation.

1. Rationale

[0075] Treatment with TGF- β results in stabilization of Sox9 protein without alterations in mRNA levels and that treatment with TGF- β results in increased sumoylation of FLAG-tagged wild type Sox9 protein (Figure 2). Sumoylation was previously shown to regulate stability, localization, and activity of a wide variety of proteins. This study determines the role of sumoylation on the stability, localization, and activity of Sox9 in response to TGF- β . Since it is known that TGF- β can activate PIAS1 in other cell types and PIAS1 interacts directly with Sox9 this study can determine the part of TGF- β 's chondroprotective activity is mediated through the regulation of Sox9 levels, localization, and activity via sumoylation by PIAS1. This would represent a novel mechanism of TGF- β action in cartilage.

2. Methods

i. Determining if sumoylation affects Sox9 levels, localization, and activity in response to TGF- β .

a. Experimental Plan:

[0076] The experiments described herein can use bovine articular chondrocytes grown in micromass culture as the model system although mouse costal chondrocytes, limb mesenchyme, C3H10T1/2, or ATDC5 cells can be used as needed. Costal chondrocytes represent a transient cartilage and limb mesenchyme represents an early stage of differentiation. C3H10T1/2 and ATDC5 cells are pluripotent mesenchymal cell lines that can be induced to form cartilage. One of the advantages of bovine articular chondrocytes for these studies is that they represent a mature permanent type of cartilage. In addition, unlike human or mouse tissue, a large number of cells can be obtained for molecular and biochemical experiments on a regular basis. Cultures are set up using cartilage from bovine metacarpal-phalangeal joints according to known

protocols. These cultures can mimic what has been observed in DNIIR mice. Alcian blue staining was used as a measure of proteoglycan content and alkaline phosphatase staining was used as a measure of late hypertrophic differentiation. Alcian blue staining was quantified as the absorbance at 620nm of the guanidine extracted stain over the quantity of DNA measured by the fluorescence of Hoechst dye. Alkaline phosphatase activity was quantified as nanoMolar p-Nitrophenol released per hour over the amount of DNA. Treatment with TGF- β over seven days resulted in increased alcian blue staining (0.25 vs. 0.55 A₆₂₀/μgDNA, $p < 0.0001$) and decreased alkaline phosphatase activity (0.29 vs. 0.16 mMol pNp/hr/μg/DNA, $p < 0.002$) confirming that TGF- β promotes the accumulation of cartilage matrix and prevents hypertrophic differentiation. To determine the effects of dominant-negative interference of TGF- β signaling on the micromass cultures, cells were infected with either Ad- β -gal or an adenovirus containing the dominant-negative TGF- β receptor (Ad-DNIIR) and placed into micromass culture. Several adenoviruses have been used and generated in the past to study various aspect of skeletal development. Cells were infected with approximately 10^9 pfu of virus for two hours while they were in suspension. Cells were then placed into micromass culture. Viral infection was detected using X-gal staining 48 hours after infection. More recently Ad-GFP has been used as a control. Ad- β -gal and Ad-DNIIR infected cells were stained for Alcian blue and Alkaline Phosphatase as described above Alcian blue staining was reduced in Ad-DNIIR infected cultures when compared to Ad- β -gal infected cultures whereas Alkaline phosphatase staining was increased in Ad-DNIIR infected cultures. The results are in agreement with the phenotype observed in the DNIIR mice. Together the results validate the use of bovine cells and adenovirus vectors to study the molecular mechanisms mediating TGF- β action in articular cartilage.

[0077] Mouse Sox9 has three sumoylation-targeted lysine residues (K61, K253 and K396). In order to examine these post-translational modifications in Sox9 protein, lysine to arginine mutations have been generated in all three sumoylation sites alone (3xKR) or in combination (seven mutants total, see Figure 4), through site-directed mutagenesis, and placed them into expression vectors. Each mutant as well as the WT Sox9 control is FLAG-tagged on the N-terminal for easy identification. Expression plasmids will initially be transfected into C3H10T1/2 or ATDC5 cells, where transfection efficiencies can be between 30% and 70%, and sumoylation can be measured in response to TGF- β treatment by immunoprecipitation using FLAG antibody followed by western blot with SUMO1 antibody. Which or if all of the potential sites are sumoylated in response to TGF- β can be determined by comparing sumoylation in all of the mutants. Since primary chondrocytes generally have lower transfection efficiency (1 to 10%)

than the cell lines, all of the mutants as well as WT FLAG-Sox9 are placed into adenovirus vectors and adenoviral transduction can be used for subsequent experiments.

[0078] Next, if sumoylation is required for the increase in Sox9 stability observed after treatment with TGF- β can be determined. Relative protein half-life of the appropriate FLAG tagged mutants and WT FLAG-Sox9 can be determined in the presence and absence of TGF- β using two techniques; pulse-chase labeling using [35S]-methionine or treatment with cyclohexamide followed by determining the time course of the disappearance of the tagged proteins using western blot. Based on previous experiments, the FLAG tagged sumoylation mutants can exhibit reduced stability relative to the tagged wild type controls. Furthermore, if TGF- β mediated protein stability requires sumoylation, addition of TGF- β cannot affect stability of the tagged mutant Sox9 proteins.

[0079] Subcellular localization of FLAG-Sox9 proteins can be determined using immunofluorescence and confocal microscopy with specific antibodies against FLAG, and also by cellular fractionation. Data indicates that TGF- β can mediate the localization of Sox9. When limb mesenchyme is treated with TGF- β there is an increase in nuclear localization of Sox9 as seen by immunofluorescent staining. Sox9 is located primarily in the cytoplasm of mesenchymal cells before the formation of chondrogenic nodules and later in the cells between the nodules. Treatment with TGF- β resulted in nuclear localization of Sox9 in the internodule regions. Mice with alterations in TGF- β signaling (DNIR and Col2aCre;Tgfr2lox/lox) appear to have overall decreased levels of Sox9 staining in cartilage making it difficult to determine if there is a specific effect on localization using staining of the endogenous protein. Sumoylation is not an absolute requirement for nuclear translocation (Figure 5A); however sumoylation can regulate the overall levels of Sox9 in the nucleus or the activity. To determine if sumoylation plays a role in TGF- β mediated localization of Sox9, mutant, and WT FLAG-Sox9 can be transduced into cells that are either untreated or treated with TGF- β . Localization of FLAG-Sox9 can be determined by confocal microscopy and immunostaining to the FLAG tag. If sumoylation is involved in TGF- β mediated nuclear localization, localization of the mutant Sox9 proteins would not be altered by treatment with TGF- β . It is difficult to quantify changes in nuclear localization using immunofluorescent localization. Therefore nuclear fractionation studies can be used. Cell fractionation was used to show the existence of a 100kDa MW Sox9 protein in the nuclear fraction of cells isolated from bovine articular chondrocytes (Figure 5A). Primary bovine articular chondrocytes. were cultured in monolayer for 24h and nuclear fractionation was performed followed by western blot. Sox9 was detected in both nuclear and cytosolic fractions using Sox9-specific antibodies. CREB and β -tubulin antibodies were used to

determine the purity of the nuclear and cytosolic fractions respectively. In the nuclear fraction two Sox9 bands were detected: one at the same molecular weight as the cytosolic Sox9 and one with higher molecular weight. IP of endogenous nuclear Sox9 and western blot with anti-SUMO Abs was used to confirm that the 100kD MW weight band in the nucleus was SUMO-Sox9 (Figure 5B). This higher MW band was increased in the nucleus after treatment with TGF- β (Figure 5C). Next, virus transduced cells can be treated with or without TGF- β , fractionated, and WT and mutant FLAG-Sox9 can be immunoprecipitated using antibodies to FLAG. Sumoylated protein can be identified by western blot with SUMO1 antibodies. Total Sox9 protein levels can be determined by western blot with Sox9 antibodies. The ratio of sumoylated WT and mutant Sox9 protein in nuclear and cytosolic fractions can be compared in samples from untreated and TGF- β treated cells. Treatment with TGF- β can result in an increase in sumoylated WT FLAG-Sox9 in the nuclear fraction; sumoylation mutants of Sox9 can have altered nuclear levels relative to WT Sox9; and treatment with TGF- β would not affect nuclear levels of the sumoylation mutants.

[0080] Finally, transcriptional activity can be determined using co-transfection or co-viral transduction with a luciferase reporter plasmid that contains the Sox9 elements from the type II collagen promoter (Col2A1). These experiments can be done first with the ATDC5 cells since a high level of cotransfection can be achieved in these cells. Cells can be cotransfected with the Sox9 firefly luciferase reporter plasmid, a control renilla plasmid in which renilla luciferase is under the control of the CMV promoter, and WT or mutant FLAG-Sox9 expression plasmids. After transfection and recovery, cells can be left untreated or treated with TGF- β and cell lysates can be analyzed for activity. The Dual Luciferase assay from Promega in a 98-well format using a luminometer with the dual injection format can be used. The experiment can be repeated using bovine articular chondrocytes transduced with the appropriate adenoviruses. Cotransfection with WT FLAG-Sox9 can result in increased activity from the Sox9 reporter. Treatment with TGF- β can also result in an increase in activity from the Sox9 reporter and perhaps further increases in the presence of exogenous WT FLAG-Sox9. If sumoylation regulates transcriptional activity of Sox9, alterations in activity in the presence of mutant FLAG-Sox9 relative to WT FLAG-Sox9 can be seen. If sumoylation is required for TGF- β mediated Sox9 activity, regulation in samples cotransfected with the mutant forms of FLAG-Sox9 would not be seen.

ii. Determining if PIAS1 regulates sumoylation of Sox9 by TGF- β .

a. Experimental Plan:

[0081] Sumoylation occurs through the activities of E1, E2, and E3 sumoyl ligases. PIAS1 is an E3 sumoyl ligase. PIAS1 interacts directly with Sox9 leading to its stabilization and it has

been shown that TGF- β acts through PIAS1 to mediate sumoylation of a variety of transcription factors. First, IP-Western will be used to determine if treatment with TGF- β enhances the interaction of tagged PIAS1 and Sox9 in chondrocytes. Results (Figure 6) indicated that treatment with TGF- β results in increased association of PIAS1 and Sox9 in bovine articular chondrocytes and ATDC5 cells. Next, to determine if PIAS1 is required for TGF- β -mediated sumoylation of Sox9, PIAS1 activity can be blocked using two methods: shRNA can be used to block PIAS1 protein expression, and a dominant-negative form of PIAS1 that lacks the ring finger domain can be used to block activity. ShRNA sets with homology to human, mouse and rat genes for PIAS1 are available in expression vectors and lentiviral particle from Open Biosystems and other vendors. Several shRNAs come in each set. The efficiency of PIAS1 knock down can be determined by transfecting or transducing cells with shRNA or a scrambled vector control. Next, PIAS1 levels can be determined using western blot. If none of the shRNAs work in bovine cells either new shRNA sequences can be generated using the published sequence for bovine PIAS1 or experiments in mouse costal chondrocytes or ATDC5 or C3H10T1/2 cells can be performed. Likewise, the dominant-negative activity of the Ring Finger mutant can be tested using a functional assay for PIAS1 sumoylation activity. Next, IP-Western can be used to detect native or WT FLAG- Sox9 sumoylation with and without PIAS inhibition in the presence and absence of TGF- β . Finally, the effects of PIAS inhibition can be tested on Sox9 stability, localization, and activity in the presence and absence of TGF- β . If PIAS1 is the enzyme responsible for TGF- β -mediated sumoylation of Sox9, blocking PIAS1 activity can prevent sumoylation of Sox9 in response to TGF- β treatment and potentially affect stability, localization, and activity of Sox9 depending on the degree of PIAS inhibition achieved with the selected tools.

[0082] The role of sumoylation in cartilage homeostasis is not known. Since TGF- β regulates sumoylation of Sox9 it is likely that TGF- β mediated sumoylation also regulates the chondrocyte phenotype. It has been shown that primary articular chondrocytes in culture can become hypertrophic after extended time in culture, similar to phenotypic changes seen in OA. TGF- β can delay hypertrophic differentiation in articular chondrocytes and promote deposition of proteoglycans as measured by Alcian Blue staining. Knock down of PIAS1 or sumoylation in general can lead to accelerated hypertrophy in bovine articular chondrocytes and prevent TGF- β mediated protection of the chondrocyte phenotype. For this set of experiments, cells can be infected with the PIAS1 ShRNA or the ring finger mutants and untreated or treated with TGF- β described above. Alternatively cells can be treated with a general antagonist of sumoylation, ginkgolic acid. Cells can be stained with Alcian Blue and for Alkaline Phosphatase activity

after seven days in culture. In addition, RNA can be isolated from cells at various times after infection and treatment to detect the expression of collagen II, X, and I by RT-PCR. Staining with BrdU can be used to assess cellular proliferation and TUNEL staining can be used to assess apoptosis. Altered sumoylation and specifically PIAS1 activity can mimic the effects of DNIIR on the cartilage phenotype and prevent TGF- β mediated stimulation of Alcian Blue and decrease of Alkaline Phosphatase staining.

[0083] There are advantages and disadvantages to using the bovine model. The advantages are that a lot of cells can be obtained on a regular basis. Bovine articular cartilage is biochemically and mechanically very similar to human cartilage, more so than mouse cartilage. The disadvantages include that primary cells in general are difficult to transfect; however, adenoviruses can be used to transduce genes of interest into cells. More than one Adenovirus vector can infect a cell, but it is important to make sure that the titers of the viruses are the same in any co-transduction experiments so that you get equal amounts of transduction. It is also possible to make adenoviruses that express two different products using a double expression cassette. Another disadvantage of the bovine system is that there are not as many reagents available as there are for human or mouse. For example, most commercially tested ShRNAs are directed to human or mouse transcripts. The bovine genome has been sequenced and is fairly well annotated at this time so it is possible to generate our own molecular reagents for bovine tissue. Furthermore, most of the Antibodies used that recognize mouse proteins also recognize bovine proteins (for example see Figure 2C with Sox9 antibody). Additional model systems can be used: ATDC5 or C2H10T1/2 cells, mouse costal chondrocytes, and mouse limb mesenchyme. Treatment with TGF- β results in stabilization of Sox9 protein in all of these systems.

[0084] In addition to sumoylation, it is established that phosphorylation of serines on the Sox9 protein can change its activity. Phosphorylation of Sox9 in response to TGF- β can affect this parameter as well. There is close proximity between sumoylated K64 and phosphorylated S61. The sumoylation or phosphorylation of K64 or S61, respectively, can alter the binding of kinases or SUMO ligases to the neighboring residue. Expression vectors with all three phosphorylatable serine residues mutated to alanines (3xSA) in WT-Sox9 and in triple lysine to arginine (3xKR) Sox9 have been generated.

[0085] Although PIAS1 is known to be activated by TGF- β and has been documented to mediate sumoylation of Sox9, other sumoylating enzymes exist. If PIAS1 is not the sumoylating enzyme responsible for Sox9 post-translational SUMO-modification in response to TGF- β , other members of the PIAS family of SUMO ligases can be used. For example, PIASy has been

shown to mediate sumoylation by TGF- β in some cases. In addition, the E2 sumoylation ligase Ubc9 is also involved in TGF- β mediated sumoylation.

[0086]

C. Determining if Sox9 is required for TGF- β mediated expression of Papss2.

1. Rationale

[0087] Papss2 was identified in a microarray screen as a potential mediator of TGF- β 's chondroprotective effects. TGF- β stimulated Papss2 mRNA expression in bovine cells and reduced Papss2 mRNA expression was observed in cartilage from DNIIR mice. Papss2 is a bifunctional enzyme whose main function is to generate PAPS, the sulfate donor for most sulfotransferase reactions. Humans and mice with deficiency in Papss2 exhibit osteoarthritis as well as other skeletal abnormalities. Very little is known about how the levels of this enzyme are regulated even though matrix sulfation is essential for the maintenance of articular cartilage. The Papss2 gene contains a paired Sox9 binding site in the first intron and a Smad3 binding site upstream of the start site. Since TGF- β regulates Papss2 mRNA expression as well as levels and sumoylation of Sox9 protein, TGF- β can act through sumoylation and Sox9 to regulate expression of Papss2 mRNA. Smad3 can also be involved.

2. Experimental Plan

[0088] First, the time course of Papss2 expression can be determined and whether or not regulation by TGF- β is direct or requires new protein synthesis. Cells in the presence or absence of cyclohexamide can be untreated or treated with TGF- β for varying times between 0.5 and 8 hours. RNA can be isolated from cells and Papss2 mRNA can be measured by real time RT-PCR. TGF- β stimulates Papss2 mRNA levels in bovine chondrocytes and in ATDC5 cells. If Papss2 mRNA is still up regulated by TGF- β in the presence of cyclohexamide, this would indicate that regulation is direct and does not require new protein synthesis. If cyclohexamide blocks up-regulation, then new protein synthesis is required for this response.

[0089] Next, it can be determined if Sox9 is sufficient to regulate PAPSS2 expression. PAPSS2 expression was measured by real time RT-PCR in bovine chondrocytes infected with Ad-FLAGSox9 or Ad-GFP virus with or without TGF- β 1 (Figure 7). Results were analyzed using REST software. In control cells infected with Ad-GFP, treatment with TGF- β resulted in up regulation of PRG4 and PAPSS2. When gene expression was compared in untreated cells infected with Ad-GFP or Ad-FLAGSox9, PAPSS2 was up regulated in Ad-FLAGSox9 expressing cells. In contrast, PRG4 was not up regulated with expression of Sox9. Addition of TGF- β to the Ad-Sox9 expressing cells did not result in further induction of PAPSS2 although PRG4 was up-regulated. Similar results were observed in ATDC5 cells. The results indicate

Sox9 regulates PAPSS2 expression in chondrocytes. Next, cells can be infected with adenovirus containing WT, dominant negative, or sumoylation mutant FLAG-Sox9 and Papss2 mRNA levels can be determined by RT-PCR. This can also be done in the presence and absence of TGF- β . A SUMO-Sox9 expression vector has been generated. SUMO is fused to the N-terminus of Sox9. This strategy has been used in the past to mimic sumoylation on other Sox family proteins. If sumoylation has a role, SUMO-Sox9 should be able to enhance Papss2 mRNA expression over the level of WT FLAG-Sox9 alone. The dominant-negative mutant can result in reduced Papss2 expression and/or attenuate TGF- β mediated Papss2 expression. In addition, the sumoylation mutants of Sox9 would not be able to cause up-regulation of Papss2 mRNA. It can also be determine whether sumoylation in general or PIAS1 specifically is required for TGF- β mediated regulation of Papss2. Here cells can be treated with an inhibitor of sumoylation, ginkgolic acid, or infected with ShRNA to PIAS1 or the ring finger mutants described above (Aim #1B). Cells can be left untreated or treated with TGF- β and the levels of Papss2 mRNA can be determined by real time RT-PCR. If sumoylation by PIAS1 is required then the loss of PIAS1 function can prevent regulation of Papss2 by TGF- β .

[0090] In a similar manner it can be determined if Smad is necessary and/or sufficient to regulate Papss2 expression. A Smad3 binding site was identified in a conserved region 1Kb upstream of the Papss2 start site. Smad3 is part of the canonical TGF- β signaling cascade. It is directly phosphorylated by the TGF- β type I receptor and translocates to the nucleus to regulate gene transcription. WT and dominant-negative forms of Smad2 and Smad3 can be infected into cells and the levels of Papss2 mRNA can be measured in the presence or absence of TGF- β . If Smad3 is involved, exogenous WT Smad3 should increase Papss2 expression and the dominant-negative forms should reduce expression and block up-regulation in the presence of TGF- β . Smad and Sox9 can also cooperate in regulation of Papss2. WT Sox9 and Smad3 as well as the mutants described above can be ectopically expressed in cells together with and without TGF- β and Papss2 mRNA levels can be measured.

[0091] As indicated above, analysis of the Papss2 promoter sequence indicates that there is a strong Smad3 site and a strong Sox9 site in the Papss2 gene (Figure 1). These DNA elements will be PCR cloned into the promoterless luciferase vector, pGL2 Basic (Promega, Madison, WI). The reporter construct can be introduced into ATDC5 cells using Fugene 6 (Roche), then cells can be left untreated or treated with 5ng/ml of pTGF- β 1 for 8 hours. Luciferase assays can be performed using the dual- luciferase reporter system from Promega. The promoterless pGL2 Basic can serve as a control for TGF- β effects on the plasmid backbone, if any. If transcriptional regulation is mediated through the Smad3 or Sox9 DNA regulatory elements in the promoter,

more luciferase activity can be seen in TGF- β 1 treated cells relative to the untreated cells. Confirmation can include mutation of the specific binding site and demonstration of loss of activity. It was previously shown that Smad3 can interact with Sox9 and p300 on the Sox9 DNA binding element to regulate chromatin structure and Col2a1 gene expression. In this case, it does not appear that Smad3 binds to the DNA directly. If TGF- β mediates Papss2 expression through the Sox9 site, experiments can include gel shift and super shift, and chromatin IP (ChIp) assays to determine if Sox9 and Smad3 interact on the Papss2 regulatory elements.

[0092]

D. Determining if Papss2 activity can alleviate cartilage degeneration when TGF- β signaling is disrupted.

1. Rationale

[0093] Mice that express a dominant negative mutation in the TGF- β type II receptor have been generated. The mice developed a condition similar to human osteoarthritis characterized by degeneration of the cartilage. Significant reduction in mechanical properties of DNIIR cartilage has been observed as demonstrated by micro-indentation. Indentation tests were performed on a Bose electroforce system. Using an impervious plane-ended cylindrical indenter 178 μ m in diameter, tests were comprised of a four-step stress-relaxation protocol, with each step of 5 μ m displacement separated by 200s relaxation. A 1 μ m tip needle was then pushed through the cartilage surface and thickness was measured from the load-displacement curve. Slopes of stress-strain plots during loading and relaxation steps were calculated as instantaneous and equilibrium stiffness respectively. In the DNIIR mutants, both instantaneous and equilibrium stiffness were reduced at 20 weeks as compared to control littermates.

[0094] To begin to determine the mechanism of how TGF- β regulates the biomechanical properties of the cartilage, a microarray screen was performed comparing untreated and TGF- β treated bovine cartilage. PAPSS2 was identified in this screen and verified that Papss2 mRNA and protein levels were down regulated in articular cartilage from DNIIR mice. Papss2 is required for sulfation of glycosaminoglycans on cartilage matrix proteoglycans. Staining with Alcian blue and antibodies specific to unsulfated and sulfated chondroitin indicated the matrix in DNIIR cartilage was hyposulfated. Sulfation provides for many of the biomechanical properties found in cartilage because the sulfate groups attract water to the matrix. Alterations in water content or movement would affect stiffness of the cartilage matrix. Some of the chondroprotective effects of TGF- β are mediated through its regulation of Papss2 and sulfation of the matrix.

2. Experimental Plan

[0095] A concentric cylinder bioreactor has been developed and validated for the production of tissue-engineered cartilage. To determine if PAPSS2 can restore the biomechanical properties of cartilage with disrupted TGF- β signaling, bovine cartilage expressing genes of interest generated in the bioreactor can be used. First, it can be determine if cartilage engineered to express DNIIR recapitulates the phenotype seen in mice and in bovine chondrocyte micromass cultures. Bovine articular chondrocytes can be isolated and infected with adenovirus containing DNIIR (Ad-DNIIR) or a control GFP containing adenovirus (Ad-GFP). Each bioreactor can be seeded with 80 million chondrocytes and 16 poly-L-lactic acid (PLLA) cylindrical scaffolds (Figure 8; 1cm in diameter, 2 mm thick, 85-95% porous with pore sizes ranging from 150-300 μ m). Typically, 260 million cells can be obtained per isolation using four metacarpal phalangeal joints. This allows two types of samples (ie DNIIR and control) to be generated (each in a separate bioreactor) with 16 constructs generated for each condition. Cartilage constructs are generated in media with 10% FBS under low oxygen conditions (5% O₂) as described in (Saini and Wick, 2004). Constructs can be grown for 28 to 35 days at which time they will be removed for analysis. RNA can be isolated from four separate constructs from each condition using the Trizol procedure. DNIIR expression can be confirmed using real time RT-PCR. Regulation of Papss2 in control and DNIIR cartilage can also be compared. Alternatively, protein can be isolated from the cartilage constructs and western blot can be used to confirm DNIIR (via FLAG tag) and Papss2 (Sigma WH0009060M7) expression. Next, four constructs from each condition can be frozen, dried and protease digested to yield four separate lysates from each condition that can be used to determine DNA, GAG, and hydroxyproline content by standard methods. The lysates can also be used for FACE analysis (flourophore assisted charbohydrate electrophoresis) to determine the relative levels of chondroitin sulfate versus non-sulfated chondroitins in DNIIR and control samples. An alternative to the FACE analysis is to measure sulfate incorporation by labeling constructs for the last 24 hours with radioactive [³⁵S]H₂SO₄ following standard protocols. Another set of 4 constructs can be used for histological analysis. Samples can be fixed and embedded in paraffin and cut in cross section. Sections can be stained with hematoxylin and eosin to observe general structure. Sections can also be stained with Alcian blue as described above to observed relative levels of sulfated proteoglycans. Immunostaining can be used to localize chondrotin 4-sulfate and unsulfated chondroitin using specific antibodies obtained from Seikagaku (clone 2-B-6 and 1-B-5 respectively). The last set of four control and DNIIR constucts can be used for microindentation analysis as described above. The engineered constructs are larger and thicker than mouse cartilage. Flow dependent behavior of DNIIR and control tissue engineered cartilage

can also be measured under confined compression using a Bose ELeCtroForce ELF (ELF 3300, EnduraTEC, Minnetonka, MN) testing apparatus. Briefly, a 4-mm diameter sample can be cut from the tissue-engineered cartilage, placed in the ELF apparatus, covered with a porous platen. The sample is kept in a bath of PBE with protease inhibitor during the testing. After preloading at 0.01N, stress relaxation testing is initiated at strains of 5, 10, 15, 20% with the stress allowed to reach equilibrium for 15 minutes between strains. The cartilage aggregate modulus is obtained from the slope of the by equilibrium stress versus strain curve. The construct shear modulus can also be measured using a Bohlin CVO® rheometer. Four mm diameter biopsy punches from the tissue-engineered cartilage are placed on the rheometer base plate with a small amount of PBE with protease inhibitors to maintain tissue hydration. The sample is preloaded using an impermeable platen with the rheometer gap set to 90% of the sample thickness. After the sample has equilibrated, the oscillation test is performed with a frequency sweep in the range of 0.01-1.0 Hz with logarithmic increase in the frequency. A typical test requires 15-20 minutes. This testing protocol causes very little change in the fluid volume of the construct to allow measurement of the flow independent viscoelastic behavior of cartilage. This method has been used to show that low oxygen tension results in engineered cartilage with native material properties (Figure 9). If the engineered cartilage mimics what is seen in mouse articular cartilage alterations in Papss2 expression, hyposulfation in the matrix, and reduced stiffness as well as decreased shear modulus and lower aggregate modulus in the DNIIR samples relative to controls can be seen.

[0096] The next step can be to see if ectopic expression of Papss2 can prevent alterations observed in DNIIR cartilage. For this experiment the number of chondrocytes isolated can be scaled up and three bioreactors used for three conditions: control Ad-GFP infected, Ad-DNIIR-Ad-GFP double infected, and Ad-GFP-Ad-Papss2 double infected. Analysis can proceed as described above: verification of DNIIR and Papss2 expression, DNA, GAG, FACE, and hydroxproline assays, histology and immunostaining, as well as microindentation assays. Ectopic expression of Papss2 under the control of the constitutive CMV promoter can prevent alterations in matrix sulfation and stiffness observed in DNIIR cartilage.

[0097] To determine if the bioreactor cartilage behaves similarly to cartilage in vivo, results obtained from the engineered tissue can be compared to that of mouse cartilage in vivo. Transgenic mice that overexpress Papss2 under the control of the Col2a promoter can be generated. After the mice are made and characterized, they can be crossed to DNIIR mice to determine if expression of Papss2 can alleviate joint degeneration observed in these mice.

Characterization can include gross, histological, biochemical, and biomechanical characterization as described above.

[0098] The next step can be to determine the effects of Tgfb's chondroprotective signaling and down-stream targets (i.e. PAPSS2) on samples of human OA cartilage. Restoring chondroprotective signals can have some reparative effects on OA cartilage. Cells from normal and OA cartilage grown and maintained in the bioreactors described above can be used. Normal and OA cartilage can be obtained from the National Disease Research Interchange. Tissue can be obtained from a combination of cadaver and surgical discards (for example from joint replacement or amputation surgery). Chondrocytes can be isolated and seeded on PLLA scaffolds and analyzed as described above. In the first set of experiments, normal and OA cartilage can be compared for: 1- DNA, GAG, SO₄, and hydroxyproline content; 2- histology; 3- mechanical properties; and 4- gene and protein expression, specifically, the expression of Tgfb signaling molecules and known down-stream targets (including Papss2). OA cartilage can have reduced GAG and SO₄ content and the mechanical properties of the OA cartilage can be altered relative to the control cartilage. Next, human OA chondrocytes can be isolated and infected with adenovirus containing an activated TGF- β Type I receptor (Ad-Tgfr1T202D) or a control GFP containing adenovirus (Ad-GFP). Tgfr1 signaling through Smad2/3 is dominant in healthy cartilage and is thought to promote Tgfb's chondroprotective effects. Cells can be seeded in the bioreactor and analyzed as described above. In addition, expression of the activated receptor, which contains a FLAG tag can be confirmed by western blot and activity of the transduced receptor can be determined by phospho Smad 3 expression. If Tgfr1 activity results in increased GAG and SO₄ content then in addition to being chondroprotective, signaling through Tgfr1/ Smad3 can be reparative. Expression of the activated receptor can result in up-regulation of known targets of Tgfb including Papss2.

[0099] Adenovirus vectors can be used to transduce genes of interest into bovine articular chondrocytes. The advantages of the adenovirus as a vector for this application include its high level of infectivity and high level of gene expression in chondrocytes.

[00100] It is unlikely that one downstream target mediates all of TGF- β s effects on cartilage homeostasis. The bioreactor system allows many genes, combinations of genes, or drugs for chondroprotective activity to be tested. For example, it was found that PLOD2, an enzyme involved in hydroxylation of lysine on collagen, is also regulated by TGF- β . Hydroxylation of lysine is involved in cross-linking of collagen and is important in maintaining the structural integrity of the matrix. Mutations in PLOD2 are associated with Bruck syndrome 2 (OMIN # 609220), which is characterized by skeletal defects including multiple fractures and scoliosis.

Unlike mutations in PAPSS2, mutations in PLOD2 appear to affect bone structure more than that of cartilage although a role in TGF- β mediated chondroprotection is still possible.

E. Role of TGF- β in Chondroprotection

[00101] Osteoarthritis and TGF- β . Osteoarthritis (OA) is characterized by the erosion of articular cartilage. Transforming growth factor- β (TGF- β) is a peptide that regulates chondrocyte differentiation and gene expression, and previous work has shown that mice with a dominant-negative mutation of the TGF- β type II receptor (DNIIR) exhibit OA-like symptoms and a reduction in the expression of genes that may help maintain healthy cartilage (Figure 10A and 10B).

[00102] Phosphoadenosine phosphosynthetase 2 (Papss2) is a downstream target of TGF- β and is required for proper sulfation of chondroitin glycosaminoglycans (GAGs) on cartilage matrix proteoglycans. Sulfation provides many of the biomechanical properties found in cartilage: sulfate groups attract water to cartilage matrix, and alterations in water content and movement affect the stiffness of cartilage matrix. In articular cartilage of DNIIR mice, Papss2 mRNA and protein levels are down-regulated, matrix is hyposulfated, and stiffness is reduced.

[00103] Proteoglycan 4 (Prg4) is a downstream target of TGF- β and encodes for lubricin, a protein secreted in joints that helps lubricate the articulating surfaces of cartilage. Overexpression of Prg4 has been shown to have a protective effect on the cartilage of mice with age-related OA. In the articular cartilage of DNIIR mice, Prg4 mRNA levels are down-regulated.

[00104] In clinical OA and DNIIR-induced OA, a treatment involving increased expression of Papss2 can result in increased cartilage matrix sulfation and increased cartilage stiffness, possibly rescuing the OA phenotype. Other downstream targets of TGF- β can also play roles in modulating biochemical and biomechanical properties of OA cartilage. The goal of this research is to determine whether Papss2 and other targets could be used to rescue the OA phenotype. In vitro engineered OA cartilage can be used as a model to identify potential targets because the model is faster and less expensive than using mice and can still be biochemically and mechanically tested.

1. Methods and Results

[00105] Micromasses and TGF- β . Bovine chondrocytes were plated in micromass, TGF- β was added to the cells, and the cells were cultured for up to 7 days after TGF- β treatment. mRNA was harvested after 5 hours, 24 hours, 3 days, and 7 days of TGF- β treatment, and quantitative real-time RT-PCR (qPCR) was used to determine relative mRNA levels of Papss2, Prg4, type II collagen, and type X collagen (Figure 11).

[00106] DNIIR-infected Micromasses. Bovine chondrocytes were transduced with an adenovirus encoding for either eGFP or DNIIR and then plated in micromass. TGF- β was added to the cells, and mRNA was harvested after 24 hours of TGF- β treatment. qPCR was used to determine relative mRNA levels for Papss2 and Prg4 (Figure 12).

[00107] In Vitro Tissue-Engineered Cartilage. Bovine chondrocytes were suspended in Dulbecco's Modified Eagle Medium containing 10% fetal bovine serum, L-ascorbic acid, non-essential amino acids, L-proline, penicillin/streptomycin, HEPES buffer, and fungizone. The cells were then dynamically seeded onto 16 poly-L-lactic acid (PLLA) scaffolds in a bioreactor (Figure 13 A and B) and cultivated in the bioreactor for up to 20 days. Constructs were observed at 4-, 12-, and 20-day time points (Figure 13 C-H).

2. Conclusions

[00108] TGF- β maintains cartilage-specific gene expression over time, and DNIIR adenovirus can reduce Papss2 and Prg4 gene expression in chondrocytes in vitro as in the DNIIR mice. Also, a bioreactor can be used to engineer in vitro 3D cartilaginous tissue.

[00109] 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 method and compositions described herein. Such equivalents are intended to be encompassed by the following claims.

CLAIMS

We claim:

1. A method for screening potential osteoarthritis therapeutics comprising
 - a) growing a modified chondrocyte comprising an altered TGF β signaling pathway gene in the presence of a compound suspected of being an osteoarthritis therapeutic;
 - b) growing said modified chondrocyte in the absence of the compound;
 - c) determining an osteoarthritic characteristic in the modified chondrocyte in the presence of the compound and in the absence of the compound; and
 - d) comparing the osteoarthritic characteristic of the modified chondrocytes, wherein a reversal of the osteoarthritic characteristic in the presence of the compound is indicative of a osteoarthritis therapeutic.
2. The method of claim 1, wherein the modified chondrocyte is genetically modified.
3. The method of any of claims 1-2, wherein the altered TGF β signaling pathway gene is an altered TGF β R gene.
4. The method of any of claims 3, wherein the TGF β R gene is TGF β R type II.
5. The method of claim 3, wherein the altered TGF β R gene comprises a dominant negative mutation in the TGF β R gene.
6. The method of claim 5, wherein the dominant negative mutation is a truncation of the TGF β R gene.
7. The method of claim 6, wherein the truncation of the TGF β R gene is the deletion of the kinase domain.
8. The method of any of claims 1-7, wherein the modified chondrocyte is a mammalian chondrocyte.
9. The method of claim 8, wherein the mammalian chondrocyte is a human chondrocyte.
10. The method of any of claims 1-5, wherein growing the modified chondrocytes comprises culturing the modified chondrocytes on a cell culture dish, multiwell plate, or flask.
11. The method of any of claims 1-10, wherein the growing of the modified chondrocytes comprises culturing the modified chondrocytes on a scaffold.
12. The method of claim 11, wherein the scaffold is a polylactic acid composition.
13. The method of any of claims 1-12, wherein the growing of the modified chondrocytes comprises culturing the modified chondrocytes in a bioreactor.

14. The method of any of claims 1-13, wherein growing the modified chondrocytes results in formation of engineered cartilage tissue.
15. The method of any of claims 1-14, wherein the osteoarthritic characteristic is a loss of proteoglycans.
16. The method of any of claim 1-14, wherein the osteoarthritic characteristic is hypertrophic differentiation of the modified chondrocytes.
17. The method of any of claims 14, wherein the osteoarthritic characteristic is a decrease in stiffness of the engineered cartilage tissue.
18. The method of any of claims 1-14, wherein the osteoarthritic characteristic is fibrillation of the matrix, clustering of the chondrocytes, or size of the chondrocytes.
19. The method of claims 16, wherein hypertrophic modified chondrocytes are determined by detecting the presence of a hypertrophy biomarker.
20. The method of claim 15, wherein the loss of proteoglycans is determined using alcian blue staining or aggrecan staining.
21. The method of any of claims 1-15, wherein the reversal of the osteoarthritic characteristic comprises an increase of proteoglycans.
22. The method of any of claims 1-14 and 16, wherein the reversal of the osteoarthritic characteristic comprises a decrease in the hypertrophy biomarker.
23. The method of any of claims 1-14 and 17, wherein the reversal of the osteoarthritic characteristic comprises a return of the stiffness of the modified chondrocytes.
24. The method of any of claims 1-14 and 18, wherein the reversal of the osteoarthritic characteristic comprises a change in histology of the modified chondrocytes.
25. The method of any of claims 1-24, wherein the compound is suspected of being an osteoarthritis therapeutic that alters the TGF- β pathway.
26. The method of claim 25, wherein altering the TGF- β pathway comprises increasing sumoylation of SOX9.
27. The method of claim 25, wherein altering the TGF- β pathway comprises increasing protein levels of SOX9.
28. The method of claim 25, wherein altering the TGF- β pathway comprises increasing the activity of PAPS synthetase 2 (PAPSS2).
29. The method of claim 28, wherein the activity of PAPSS2 comprises generating PAPS.

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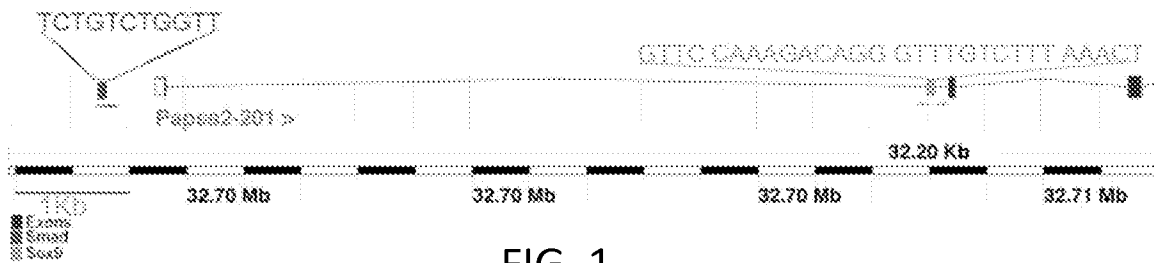


FIG. 1

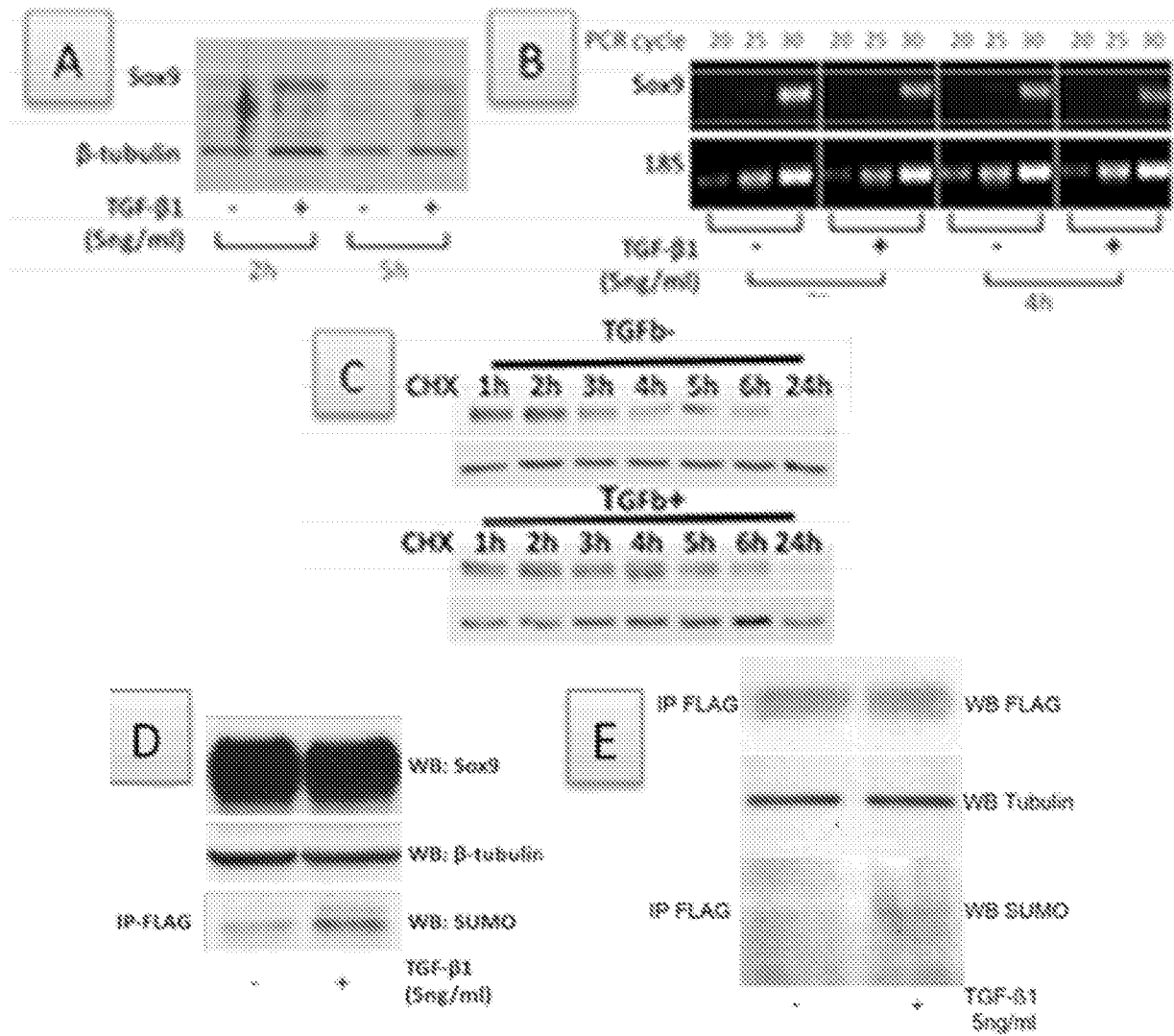


FIG. 2A, FIG. 2B, FIG. 2C, FIG. 2D, FIG. 2E

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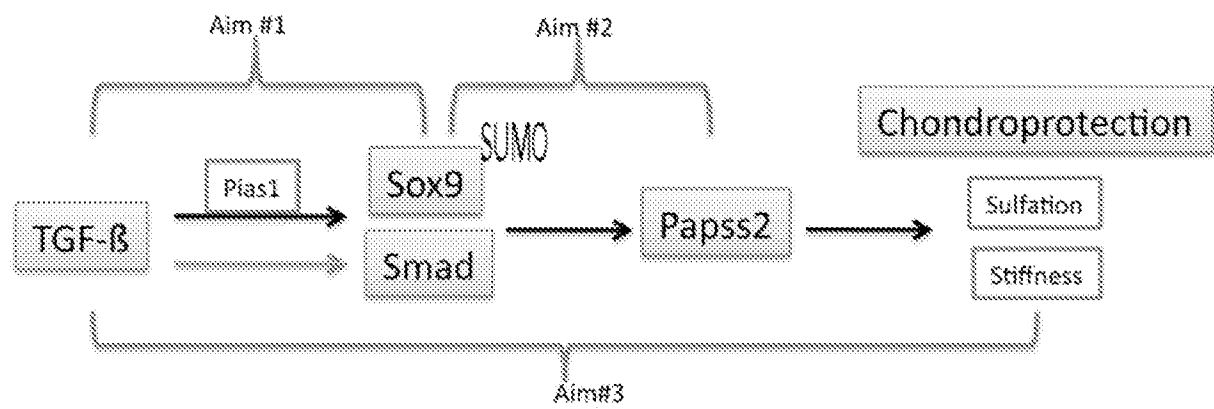


FIG. 3

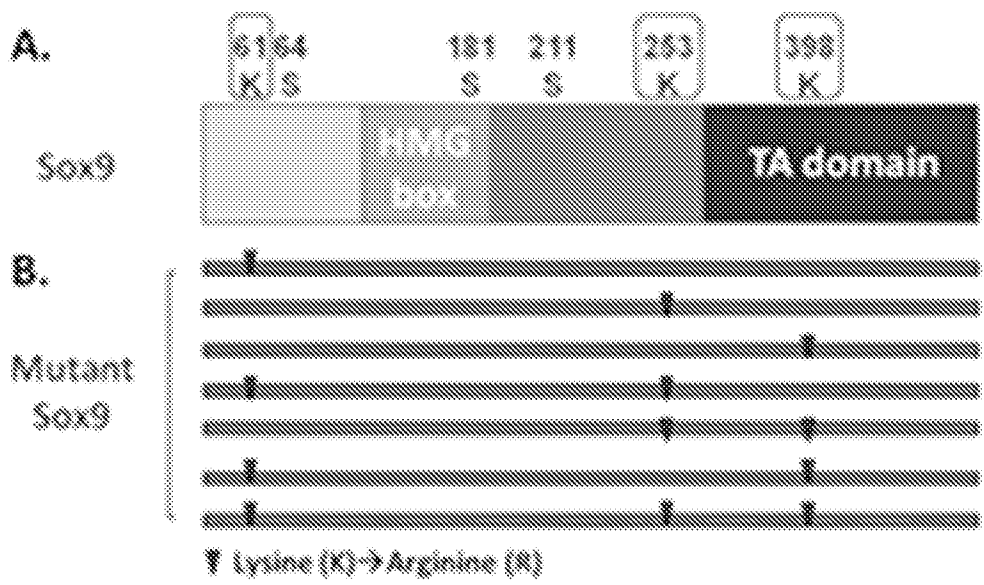


FIG. 4

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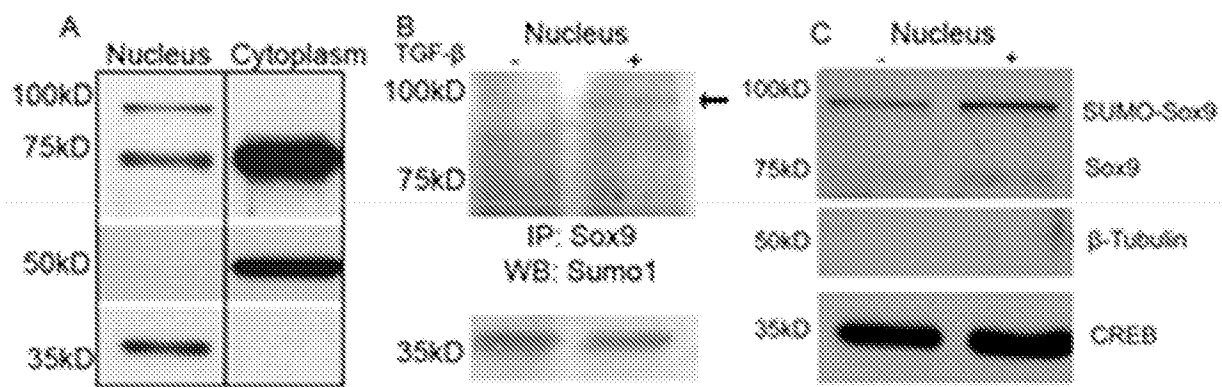


FIG. 5A, FIG. 5B, FIG. 5C

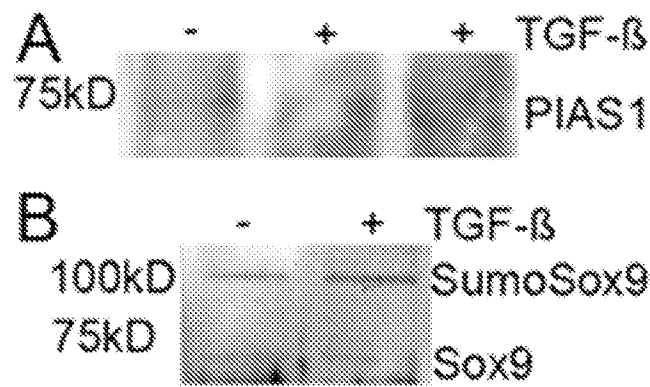


FIG. 6A and FIG. 6B

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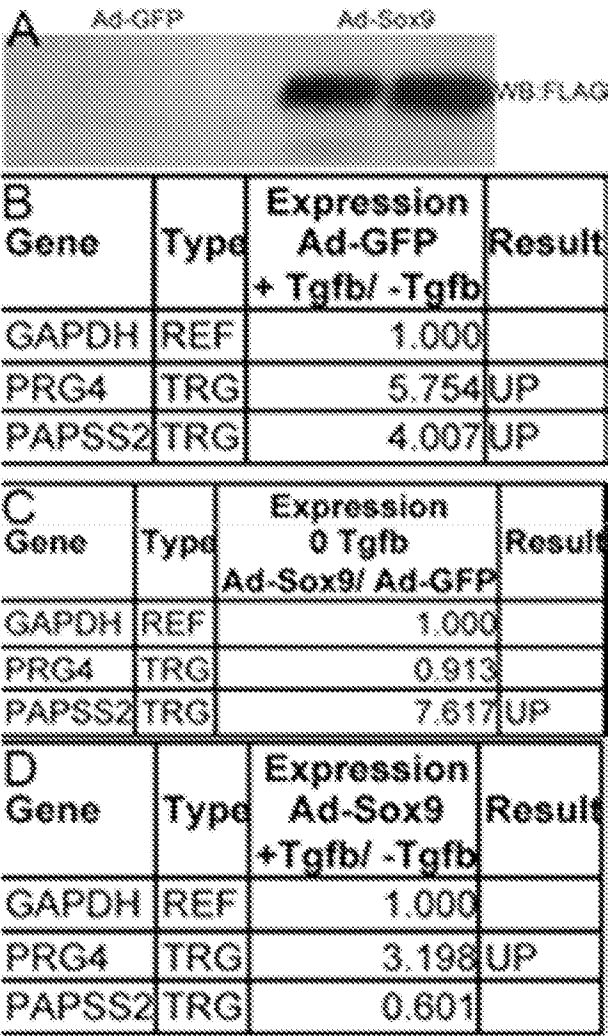
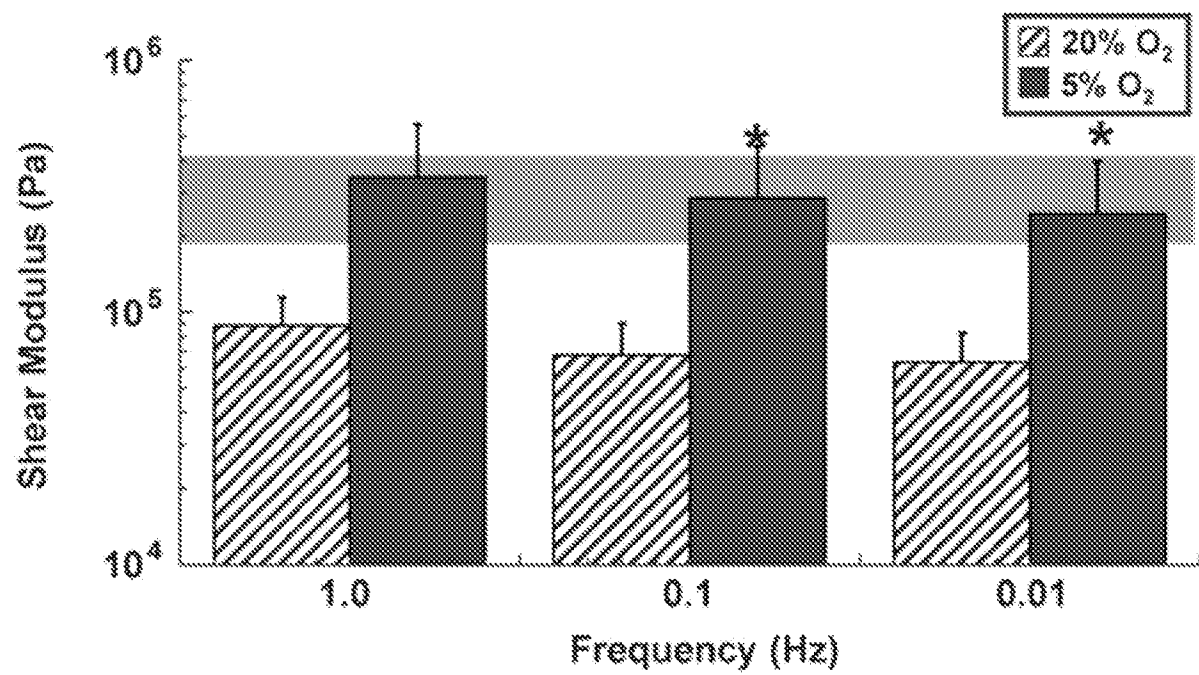


FIG. 7A, FIG. 7B, FIG. 7C, FIG. 7D

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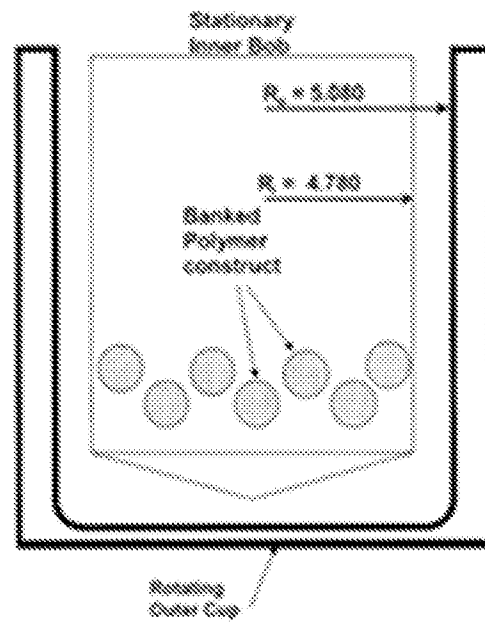


FIG. 8

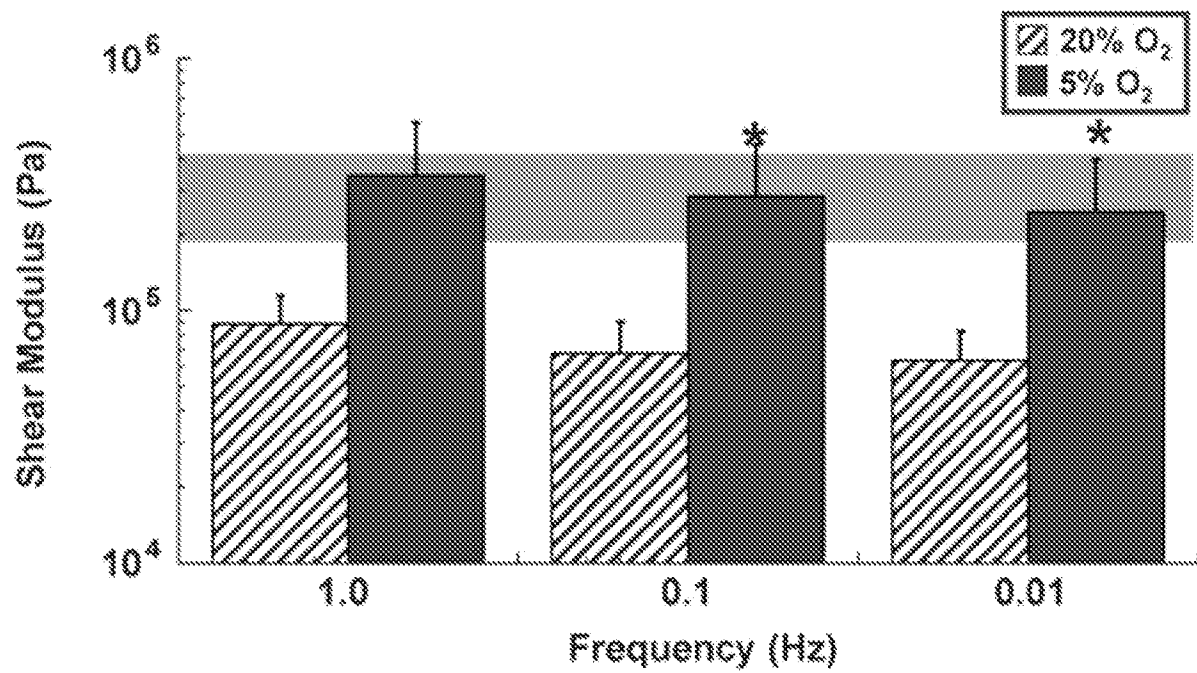


FIG. 9

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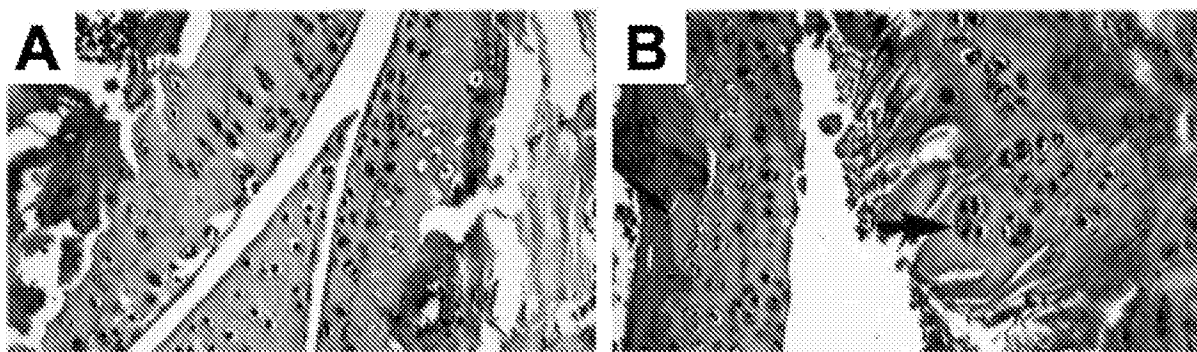


FIG. 10A, FIG. 10B

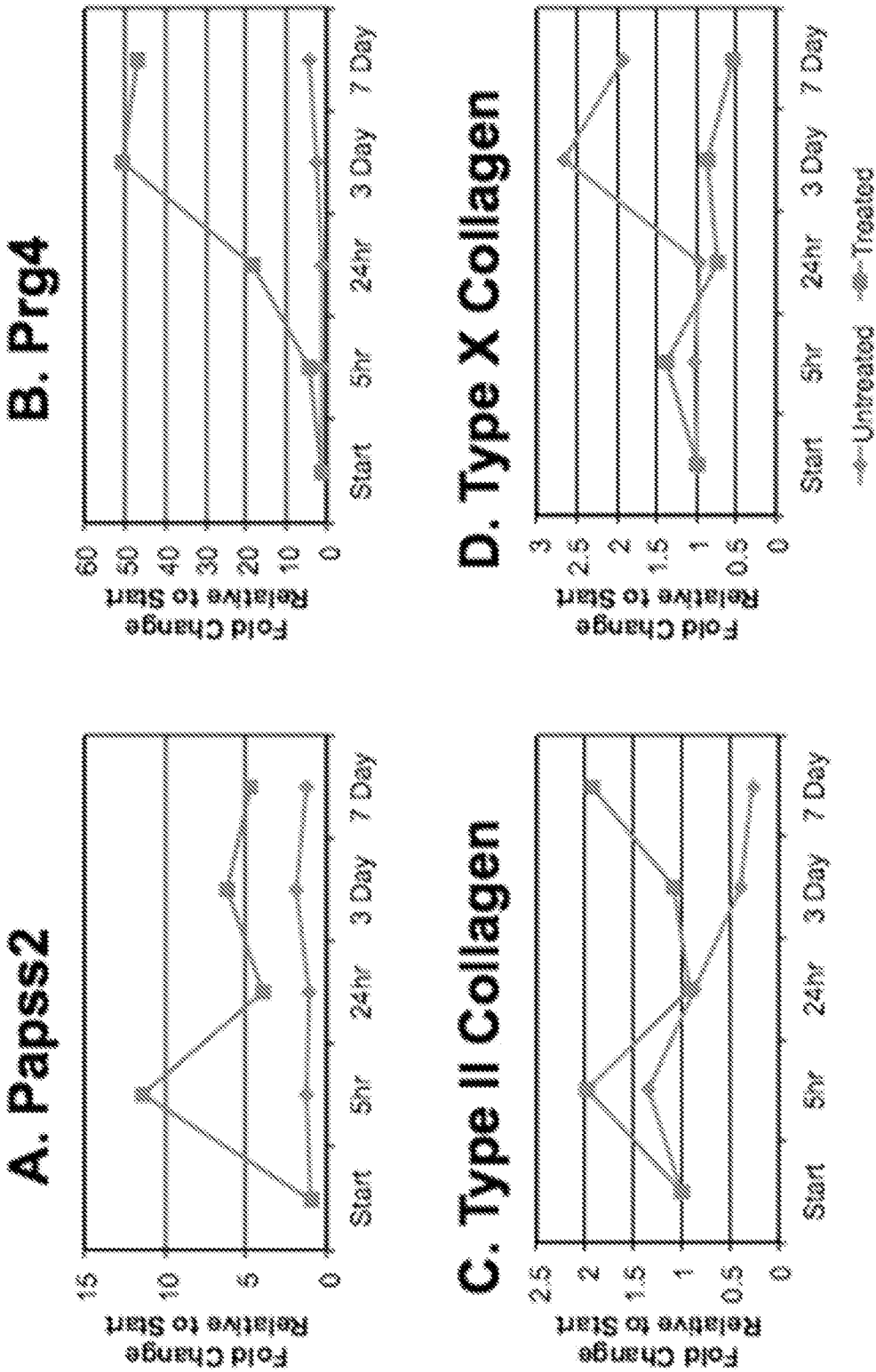


FIG. 11A, FIG. 11B, FIG. 11C, FIG. 11D

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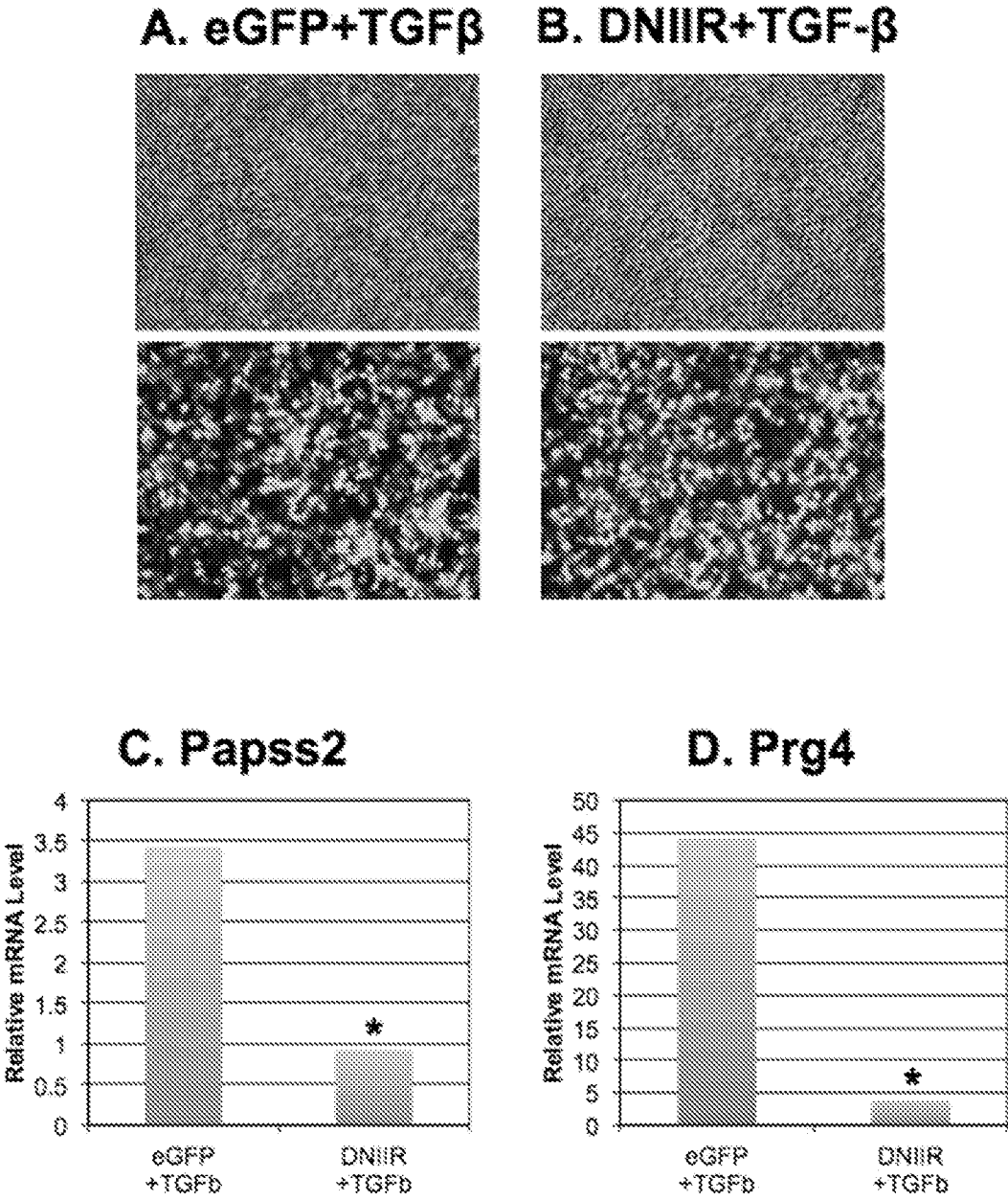


FIG. 12A, FIG. 12B, FIG. 12C

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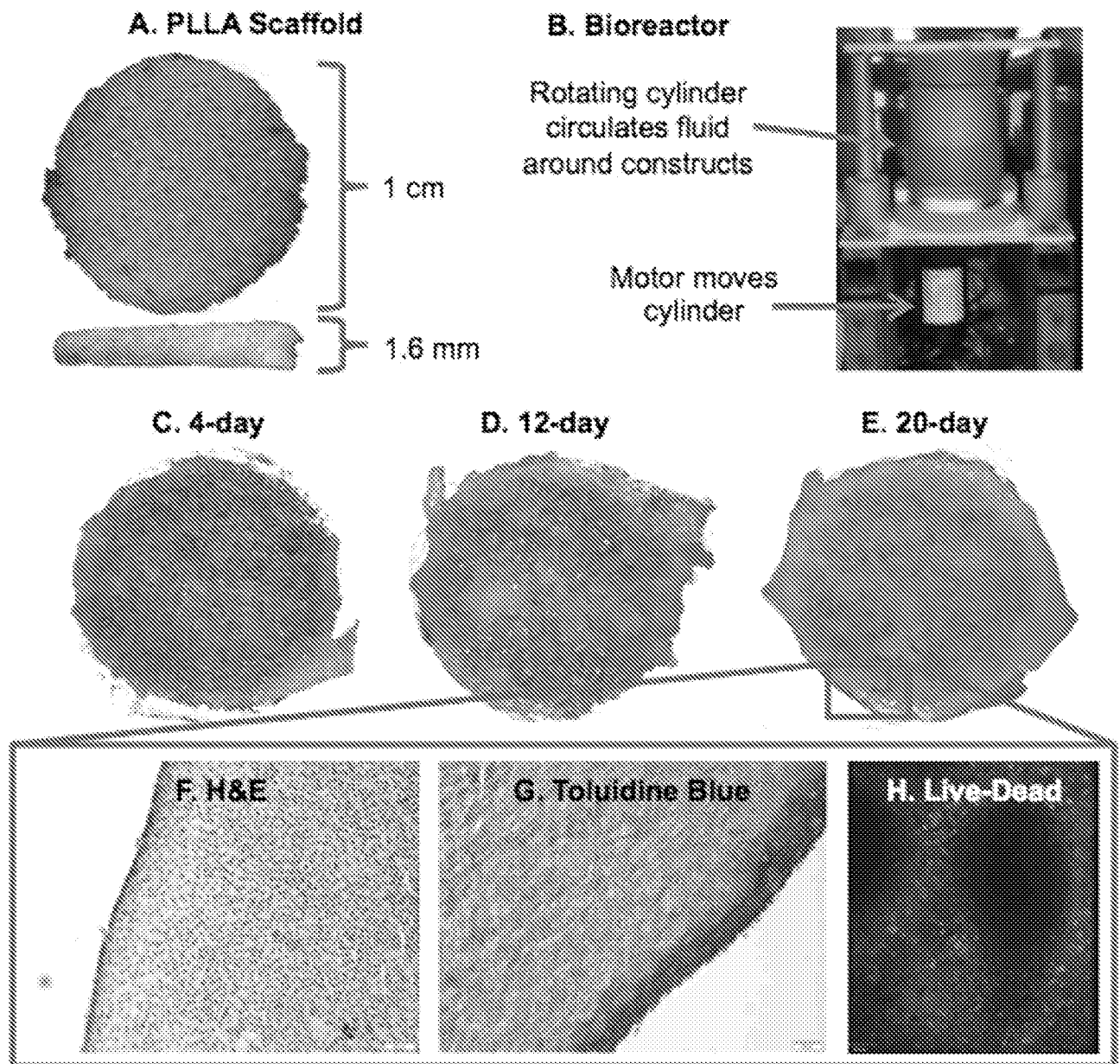


FIG. 13A, FIG. 13B, FIG. 13C, FIG. 13D, FIG. 13E,
FIG. 13F, FIG. 13G, FIG. 13H

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 15/43081

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/US 15/43081

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.: 8-29
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/US 15/43081

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - C12N 5/07, 15/00, 15/11; C07K 14/00 (2015.01)

CPC - C07K 14/705, 14/495; C12N 15/113, 15/00

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): C12N 5/07, 15/00, 15/11; C07K 14/00 (2015.01)

CPC: C07K 14/705, 14/495; C12N 15/113, 15/00

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

CPC: C07K 14/705, 14/495; C12N 15/113, 15/00 (text search)

USPC: 435/361, 320.1; 514/44A; 514/16.8; 530/350 (text search)

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

Electronic data bases: PatBase; Google Patents; Google Scholar

Search terms: Dominant negative TGFbetaRII (DNIIR), chondrocytes, osteoarthritis (OA), drug screen, MMP13, proteoglycan, type X collagen, SOX9, chondroprotective effects,

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SHEN et al. Deletion of the transforming growth factor beta receptor type II gene in articular chondrocytes leads to a progressive osteoarthritis-like phenotype in mice. Arthritis Rheum December 2013 Vol 65 No 12 Pages 3107-3119. Especially abstract, pg 3111 col 2 para 2 continued to pg 3112 col 1 para 1, pg 3116 col 1 para 3	1-7
Y	CHEN et al. Inactivation of the type II receptor reveals two receptor pathways for the diverse TGF-beta activities. Science 28 May 1993 Vol 260 No 5112 Pages 1335-1338. Especially pg 1336 col 1 para 3 continuing to pg 1336 col 2 para 2.	1-7
A	WO 2011/123394 A1 (Nannuru et al.) 06 October 2011 (06.10.2011). Especially pg 2 para 2-4.	1-7
A	PIECHA et al. Novel selective MMP-13 inhibitors reduce collagen degradation in bovine articular and human osteoarthritis cartilage explants. Inflamm Res May 2010 Vo 59 No 5 Pages 379-389. Especially abstract.	1-7

☐ Further documents are listed in the continuation of Box C.

* 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

01 October 2015 (01.10.2015)

Date of mailing of the international search report

09 NOV 2015

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents
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