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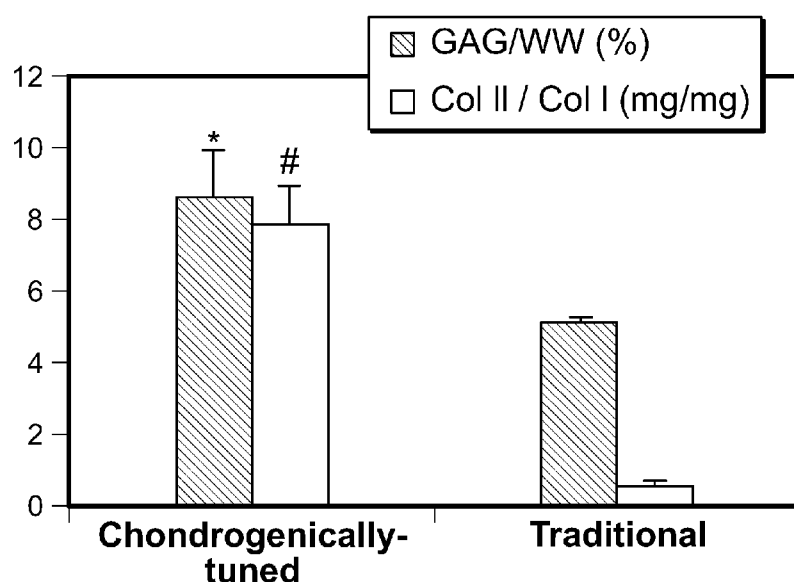


FIG. 1

(57) **Abstract:** The present disclosure provides compositions and methods for engineering cartilage of clinically-relevant geometry and biomechanical properties. In particular, the present disclosure provides processes involving expansion, redifferentiation and construct formation to provide neocartilage, which resembles native cartilage.

COMPOSITIONS AND METHODS FOR BIOENGINEERING CARTILAGE

CROSS-REFERENCE TO RELATED APPLICATION(S)

[0001] This application claims the benefit under 35 USC 119(e) of U.S. Provisional Application No. 61/675,318, filed July 24, 2012, which is hereby incorporated by reference in its entirety.

FIELD

[0002] The present disclosure generally relates to the field of tissue engineering. More specifically, the present disclosure relates to methods and compositions for the production of neocartilage, which resembles native cartilage.

BACKGROUND

[0003] Due to its complex nature and the large forces that the knee joint is responsible for transmitting, injuries to the knee joint are common in sports and in age-related tissue degeneration. The two major cartilaginous structures of this joint, the knee meniscus and articular cartilage, are particularly prone to injury because of their role in force distribution and transmission. Due to the lack of blood supply to the inner portion of the meniscus and articular cartilage, the likelihood of sufficient intrinsic repair following injury is very low and permanent degenerative changes in these structures frequently occurs (Buckwalter, J Orthop Sports Phys Ther, 28:192, 1998; and Athanasiou and Sanchez-Adams, Synthesis Lectures on Tissue Engineering, 1:1, 2009).

[0004] U.S. Food and Drug Administration approved cell-based treatments for damaged cartilage rely on adult chondrocyte implantation techniques. This technology, however, requires autologous donor source tissue obtained by creation of a defect in healthy donor tissue and risk of complications related to donor site morbidity. In an effort to reduce the size of the biopsy, cells can be multiplied or expanded to obtain a greater number of cells from the same size biopsy.

[0005] Bioengineering offers the potential to replace damaged cartilage and mitigate long-term debilitating changes to joints by expanding a small population of primary cells to sufficient numbers for use in cartilage regeneration techniques. Unfortunately, the expansion of primary cells to increase their numbers often results in loss of desired native phenotype.

Additionally, increasing the number of passages has been found to decrease the chondrogenic potential of chondrocytes (Darling and Athanasiou, J Orthop Res, 23:425-432, 2005). Specifically, as primary articular chondrocytes (ACs) and meniscus cells (MCs) expand in cell culture, they flatten and change their metabolic emphasis from matrix synthesis to cell proliferation, and thus lose the ability to synthesize cartilaginous matrix components (e.g. collagen type II and aggrecan) while taking on a more fibrous phenotype (Gunja and Athanasiou, Arthritis Res Ther, 9:R93, 2007). Thus, phenotypic changes induced during expansion of chondrocytes using published methods present significant challenges to the use of engineered cartilaginous tissue.

[0006] Accordingly, there exists a need for engineered cartilage constructs with improved biochemical and biomechanical properties, which can be generated from primary chondrocytes or other cells with chondrogenic potential.

SUMMARY

[0007] The present disclosure provides compositions and methods for engineering cartilage of clinically-relevant geometry and biomechanical properties. In particular, the present disclosure provides processes involving expansion, redifferentiation and construct formation to provide neocartilage, which resembles native cartilage.

[0008] Specifically, the present disclosure provides methods of producing cartilage, comprising: a) passaging chondrogenic cells at a cell seeding density of over 12,500 cells/cm² in culture medium on an adherent cell culture surface until over 90% confluence is reached (under conditions effective) to produce an expanded population of chondrogenic cells; b) redifferentiating the expanded population of chondrogenic cells in culture medium on a non-adherent cell culture surface (under conditions effective) to produce a chondrogenic cell aggregate; c) treating the chondrogenic cell aggregate with a solution comprising collagenase (under conditions effective) to produce a chondrogenic cell suspension; and d) culturing the chondrogenic cell suspension in culture medium comprising a matrix degrading enzyme and a cytoskeletal modifying agent (under conditions effective) to produce cartilage. In some embodiments, the cytoskeletal modifying agent comprises an inhibitor of actin polymerization (e.g., cytochalasin, latrunculin, etc.). In some embodiments, the cytoskeletal modifying agent comprises a protein kinase inhibitor (e.g., staurosporine, Rho kinase (ROCK) inhibitor such as Y-27632, etc.). In some preferred embodiments, the cytoskeletal

modifying agent comprises cytochalasin D. In some embodiments, the matrix-degrading enzyme comprises one or more of the group consisting of a hyaluronidase, a chondroitinase, a heparinase and a keratanase. In some preferred embodiments, the matrix-degrading enzyme comprises a hyaluronidase. In some methods, the chondrogenic cell suspension in step d) is cultured using a construct formation technique selected from the group consisting of self-assembly, centrifugation, scaffold seeding and hydrogel encapsulation. In some preferred embodiments, the chondrogenic cell suspension in step d) is cultured using a self-assembly construct formation technique. In some embodiments, the chondrogenic cells in step a) are primary chondrocytes isolated from one or more of the group consisting of articular cartilage, fibrocartilage, elastic cartilage, and hyaline cartilage. In some embodiments, the chondrogenic cells in step a) comprise mesenchymal stem cells isolated from a source selected from the group consisting bone marrow, adipose tissue, synovium, periosteum, dermis, umbilical cord blood, synovial fluid, muscle, and tendon. In some embodiments, the chondrogenic cells in step a) are pluripotent stem cells selected from the group consisting of embryonic stem cells and induced-pluripotent stem cells. In some preferred embodiments, the chondrogenic cells in step a) express one or more of the transcription factors selected from the group consisting of SOX-5, SOX-6, and SOX-9. In some preferred embodiments, the cells in step a) are primary chondrocytes isolated from articular cartilage. In some preferred methods, step a) is repeated from two to six times before redifferentiation in step b) such that the expanded population of chondrogenic cells are passage 2, passage 3, passage 4, passage 5, or passage 6 cells. In some methods, the cell seeding density of step a) is from 15,000 and 30,000 cells/cm². In some methods, over 90% confluence is from about 93% to over-confluence (e.g., at least 93%, 94%, 95%, 95%, 97%, 98%, 99%, 100%, or over-confluence). In some embodiments, over-confluence comprises culturing cells for 1, 2, 3, 4, 5, 6, or 7 days after confluence is reached. In some embodiments, the expanded population of chondrogenic cells of step a) is cryopreserved before redifferentiation in step b). In some methods, the culture medium is a serum-free medium. In some embodiments, the culturing step is done in the absence of exogenous transforming growth factor-beta (TGF-beta).

[0009] Additionally, the present disclosure provides methods of producing cartilage, comprising: culturing a chondrogenic cell suspension in culture medium comprising a matrix degrading enzyme and a cytoskeletal modifying agent (under conditions effective) to produce cartilage. The present disclosure further provides methods of producing cartilage,

comprising: culturing a chondrogenic cell suspension in culture medium comprising a matrix degrading enzyme (under conditions effective) to produce cartilage, with the proviso that the matrix degrading enzyme is not or does not comprise a chondroitinase (e.g., chondroitinase-ABC). Moreover the present disclosure provides methods of producing cartilage, comprising: culturing a chondrogenic cell suspension in culture medium comprising a cytoskeletal modifying agent (under conditions effective) to produce cartilage, with the proviso that the cytoskeletal modifying agent is not or does not comprise a kinase inhibitor (e.g., a Rho-associated kinase (ROCK) inhibitor and/or staurosporine. In some embodiments, the methods further comprise a prior step of: treating a chondrogenic cell aggregate with a solution comprising collagenase (under conditions effective) to produce the chondrogenic cell suspension. In some embodiments, the methods further comprise a prior step of: redifferentiating an expanded population of chondrogenic cells in culture medium on a non-adherent cell culture surface (under conditions effective) to produce the chondrogenic cell aggregate. In some embodiments, the cytoskeletal modifying agent comprises an inhibitor of actin polymerization (e.g., cytochalasin, latrunculin, etc.). In some preferred embodiments, the cytoskeletal modifying agent comprises cytochalasin D. In some embodiments, the matrix-degrading enzyme comprises one or more of the group consisting of a hyaluronidase, a heparinase and a keratanase. In some preferred embodiments, the matrix-degrading enzyme comprises a hyaluronidase. In some methods, the chondrogenic cell suspension in step d) is cultured using a construct formation technique selected from the group consisting of self-assembly, centrifugation, scaffold seeding and hydrogel encapsulation. In some preferred embodiments, the chondrogenic cell suspension in step d) is cultured using a self-assembly construct formation technique. In some embodiments, the chondrogenic cells in step a) are primary chondrocytes isolated from one or more of the group consisting of articular cartilage, fibrocartilage, elastic cartilage, and hyaline cartilage. In some embodiments, the chondrogenic cells in step a) comprise mesenchymal stem cells isolated from a source selected from the group consisting bone marrow, adipose tissue, synovium, periosteum, dermis, umbilical cord blood, synovial fluid, muscle, and tendon. In some embodiments, the chondrogenic cells in step a) are pluripotent stem cells selected from the group consisting of embryonic stem cells and induced-pluripotent stem cells. In some preferred embodiments, the chondrogenic cells in step a) express one or more of the transcription factors selected from the group consisting of SOX-5, SOX-6, and SOX-9. In some preferred embodiments, the cells in step a) are primary chondrocytes isolated from articular cartilage. In some

preferred methods, step a) is repeated from two to six times before redifferentiation in step b) such that the expanded population of chondrogenic cells are passage 2, passage 3, passage 4, passage 5, or passage 6 cells. In some methods, the cell seeding density of step a) is from 15,000 and 30,000 cells/cm². In some methods, over 90% confluence is from about 93% to over-confluence (e.g., at least 93%, 94%, 95%, 95%, 97%, 98%, 99%, 100%, or over-confluence). In some embodiments, over-confluence comprises culturing cells for 1, 2, 3, 4, 5, 6, or 7 days after confluence is reached. In some embodiments, the expanded population of chondrogenic cells of step a) is cryopreserved before redifferentiation in step b). In some methods, the culture medium is a serum-free medium. In some embodiments, the culturing step is done in the absence of exogenous transforming growth factor-beta (TGF-beta).

[0010] Also provided by the present disclosure are compositions comprising the cartilage prepared by a method of either of the two preceding paragraphs. In some embodiments, the cartilage has a glycosaminoglycan per wet weight percentage of over 6%, 7%, 8% or 9%. In some embodiments, the cartilage has a collagen II to collagen I (w/w) ratio of greater than 2, 3, 4, 5, 6, 7 or 8. In some embodiments, the cartilage has a reduced acellular and a proteinaceous central region (AACR) as compared to control cartilage produced using a standard method. In some embodiments, the cartilage expresses SOX9 at an elevated level as compared to control cartilage produced using a standard method. In some embodiments, the cartilage has one or more of the following properties selected from the group consisting of an elevated compressive relaxation modulus, an elevated compressive instantaneous modulus, and an elevated tensile Young's modulus, as compared to control cartilage produced using a standard method. In some embodiments, the cartilage comprises a higher number of cells as compared to control cartilage produced using a standard method. In some instances, the control cartilage is produced using a standard method directly from primary chondrocytes.

BRIEF DESCRIPTION OF THE DRAWINGS

[0011] Figure 1 shows total GAG normalized to wet weight (black bars) and collagen II normalized to collagen I (gray bars) of chondrogenically-tuned and traditionally cultured constructs. Data derived from n= 6 samples per group are displayed as mean $\pm$ SD. Constructs formed with cells expanded with the chondrogenically-tuned protocol had significantly higher GAG/WW and Col II/Col I than constructs formed with cells expanded with the traditional protocol (denoted by asterisk and pound symbol, respectively). Differences were considered significant if $p < 0.05$.

[0012] Figure 2 shows total GAG normalized to wet weight (black bars) and collagen II normalized to collagen I (gray bars). Data derived from $n = 6$ samples per group are displayed as mean $\pm$ SD. Significant differences for GAG/WW and Col II / Col I are denoted with capital and lower case letters, respectively. The horizontal axis corresponds to the primary to passaged cell ratio. Significant differences ($p < 0.05$) exist between groups not sharing the same letter. All groups containing P3 cells had significantly higher GAG/WW than P0 constructs.

[0013] Figure 3 shows the relaxation moduli (black bars) and instantaneous moduli (gray bars) of various P0:P3 constructs at 20% strain. Data derived from $n = 6$ samples per group are displayed as mean $\pm$ SD. Significant differences for relaxation and instantaneous modulus values are denoted with capital and lower case letters, respectively. The horizontal axis corresponds to the primary to passaged cell ratio. Significant differences ($p < 0.05$) exist between groups not sharing the same letter. For both metrics the 50:50 group significantly outperformed all other groups. The 100:0 group had a significantly lower relaxation modulus than all other groups.

[0014] Figure 4 shows relative SOX9 gene expression in primary and passaged chondrocytes. Primary (black bars) and passaged (gray bars) chondrocytes were assessed for the SOX9 gene expression relative to β -actin and normalized to the relative SOX9 gene expression of primary chondrocytes at day 0 (prior to self-assembly). Gene expression was assessed at day 0, day 2, and day 7 of self-assembly culture. Data derived from $n = 4$ samples per group are presented as mean $\pm$ SD. A two way ANOVA with Tukey's post hoc test, when warranted, was performed. Significant differences ($p < 0.05$) related between time points are denoted with capital letters (A and B), between passage number are denoted with Greek characters (α and β), and between all groups are denoted with lower case letters (a, b, and c).

[0015] Figure 5 provides an overview of an exemplary embodiment of the cartilage bioengineering methods of the present disclosure. Articular chondrocytes (ACs) and meniscus cells (MCs) were isolated from tissue of the rabbit knee joint. Cells were expanded with a chondrogenically-tuned procedure to either passage 3 or 4. These cells were either cryopreserved or used immediately in a redifferentiation culture modality: i) self-assembled without redifferentiation; ii) redifferentiated in aggregate culture for one week then self-assembled; or iii) redifferentiated in pellet culture for one week then self-assembled.

[0016] Figure 6 depicts the morphology of two different constructs. These images taken of bisected constructs illustrate that increasing cryopreservation and redifferentiation reduced the size of the acellular and aprotaceous central region. For morphological images the scale bar indicates 3 mm. Histological pictures were taken at 10X.

[0017] Figure 7A shows normalized collagen and Figure 7B shows normalized GAG levels of various constructs. Separate one-way ANOVAs were performed to determine statistical differences among constructs formed with ACs or MCs. Significant differences ($p < 0.05$) exist between groups that do not contain the same letter.

[0018] Figure 8 shows the biomechanical properties of various constructs. Figure 8A shows the relaxation moduli, Figure 8B shows the compressive instantaneous moduli, and Figure 8C shows the tensile Young's moduli (C) of the indicated constructs. Separate one-way ANOVAs were performed to determine statistical differences among constructs formed with ACs or MCs. Significant differences ($p < 0.05$) exist between groups that do not contain the same letter.

[0019] Figure 9 shows that treatment with cytochalasin-D and hyaluronidase resulted in constructs that possessed the biomechanical and biochemical properties desired in cartilage for clinical use. Figure 9A shows the relaxation moduli, Figure 9B shows the instantaneous moduli, Figure 9C shows the coefficient of viscosity, Figure 9D shows the tensile moduli, and Figure 9E shows the ultimate tensile strength of the indicated constructs. Additionally, Figure 9F shows collagen normalized to wet weight, and Figure 9G shows total GAG normalized to wet weight of the indicated constructs. Collagen I was not detected in chondrogenically-tuned, redifferentiated cells.

DETAILED DESCRIPTION

[0020] The present disclosure provides compositions and methods for engineering cartilage of clinically-relevant geometry and biomechanical properties. In particular, the present disclosure provides processes involving expansion, redifferentiation and construct formation to provide neocartilage, which resembles native cartilage.

Processes for Bioengineering Cartilage Tissue

[0021] Briefly, the first stage involves cell expansion employing a combination of serum-free chondrogenic medium and high seeding and passaging densities. The second stage involves aggregate redifferentiation. The third stage involves construct formation using the

redifferentiated cells and a combination of a matrix degrading enzyme and a cytoskeletal modifying agent. The processes of the present disclosure enhance the mechanical properties and beneficially modulate the resulting construct geometry. In some embodiments, the methods further comprise isolation of primary chondrocytes from donor cartilage to provide suitable cells for the expansion stage. In other embodiments, the methods further comprise culturing pluripotent stem cells to provide suitable cells for the expansion stage.

Chondrocyte Isolation

[0022] First, a suspension of cells that are capable of forming cartilage (e.g., having chondrogenic potential) are obtained. As used herein, the term “chondrogenic cell” refers to cells capable of forming cartilage. Chondrogenic cells include chondrocytes and bone marrow. Other chondrogenic cells include but are not limited to adipose-derived, skin-derived (see, e.g., US 2009/015533), synovium-derived, periosteum-derived, induced pluripotent, and embryonic stem cells (see, e.g., US 2009/0136559). All cell types that are capable of producing matrix specific to cartilage (e.g., collagen type II, aggrecan, glycosaminoglycans, cartilage oligomeric protein, and superficial zone protein) are chondrogenic cells suitable for use in the compositions and methods of the present disclosure. Thus the cells and cell samples used in conjunction with the methods of the present disclosure may comprise chondrocytes, chondro-differentiated cells, fibrochondrocytes, fibrochondro- differentiated cells, and combinations thereof (referred to herein as chondrocytes).

[0023] In some embodiments, the chondrogenic cells may comprise articular chondrocytes. Generally, the articular chondrocytes may be from a bovine or porcine source, or another animal source. Alternatively if the construct is to be used for in vivo tissue replacement, the source of articular chondrocytes may be autologous cartilage from a small biopsy of the patient's own tissue, provided that the patient has healthy articular cartilage that may be used as the start of in vitro expansion. In other embodiments, the chondrogenic cells may comprise allogenic chondrocytes, such as those from histocompatible cartilage tissue obtained from a donor or cell line. Other chondrocytes and chondrogenic cell types will be apparent to those skilled in the art and can be used in the methods of the present disclosure. In certain embodiments, the chondrogenic cells used in conjunction with the methods of the present disclosure may be derived from mesenchymal, embryonic, induced pluripotent stem cells, skin cells, or other stem cells.

[0024] The cells and cell samples used in the methods of the present disclosure may be obtained by any means suitable for obtaining a cell sample comprising chondrogenic cells. In certain embodiments, such a means may comprise enzymatic digestion of native tissue. Suitable enzymes for such an enzymatic digestion include, but are not limited to, one or more collagenases.

[0025] In some embodiments, chondrogenic cells are selected based on their ability to express one or more of the major transcription factors associated with cartilage formation (e.g., SOX-5, SOX-6, SOX-9, etc.). In some embodiments, chondrogenic cells are selected in part based on expression of one or more of CD44, CD90, CD105, CD271, and STRO-1.

[0026] In an exemplary embodiment, chondrocytes are isolated from the tibial and/or femoral articular cartilage surfaces of skeletally-mature donors using 0.2% collagenase type II (Worthington) in chemically-defined culture medium (CM) (DMEM with 4.5 g/L-glucose and GlutaMAX (Invitrogen), 100 nM dexamethasone (Sigma), 1% fungizone, 1% penicillin / streptomycin (BD Biosciences), 1% insulin transferrin selenium premix (ITS+) (BD), 50 mg/mL ascorbate-2-phosphate (Sigma), 40 mg/mL L-proline (Sigma), and 100 mg/mL sodium pyruvate (Fisher Scientific)). After overnight digestion, chondrocytes are frozen at -80°C in culture medium supplemented with 20% fetal bovine serum (FBS) (Gemini Bio-Products) and 10% DMSO (Sigma). Prior to cryopreservation, the chondrocyte viability is determined by trypan blue exclusion. After freezing at -80°C, cells are placed in liquid nitrogen cryo-storage until needed for expansion. Prior to expansion through passage, cells are designated as “primary chondrocytes.”

Chondrocyte Expansion

[0027] In certain embodiments, chondrogenic cells are expanded and reseeded. In order to adhere cells to culture plates, plates are coated with fibronectin (or similar cell-adhesion supporting molecule). Alternatively, the cells are seeded in the presence of 1-10% serum for the first 24 hours. Exemplary culture medium comprises a basal medium (e.g., DMEM, F12, etc.), ITS (insulin, transferrin, selenium), albumin, non-essential amino acids, penicillin, streptomycin, fungizone, dexamethasone, ascorbate-2-phosphate, L-proline, sodium pyruvate, and growth factors (e.g., TGFβ1, PDGF, and/or basic-FGF). Cells are seeded at a density of between 15,000 to 30,000 cells/cm² and allowed to expand in number until 4 days following 95% confluence elapses. Media is refreshed every other day. At this point cells are released

from the cell culture dish using trypsin (0.25% w/v) and EDTA (0.05% w/v). Tryptic activity is halted with a trypsin inhibitor and the remaining cell clumps are treated with collagenase until a single cell suspension is obtained. Cells are then reseeded as described above until a sufficient number of passages is reached (e.g., typically 3, 4, 5 or 6 passages).

[0028] In an exemplary embodiment, primary chondrocytes are rapidly thawed and seeded in flasks for expansion. Following thawing of cryopreserved chondrocytes, cell viability is preferably at least 85%. Chondrocyte expansion methods employ culture medium containing ITS plus dexamethasone, supplemented with 5 ng/mL basic fibroblast growth factor. A seeding density of 2.5×10^4 cells/cm² is used and monolayers are passaged four days after 95% confluence is reached. In contrast, the standard protocol employs culture medium with 10% FBS, a seeding density of 1.1×10^4 cells/cm², and passaging at 90% confluence. To allow adequate cell adhesion for chondrogenically-tuned passaging, 10% FBS is added for the first 24 hours of monolayer seeding. Passaging is performed using 0.25% (w/v) trypsin/0.05% (w/v) EDTA (GIBCO) at 37°C. Since both trypsin digestion and seeding can alter cellular characteristics, passage number as used herein refers to the number of trypsin/EDTA exposures (i.e., cells expanded to passage 3 under these conditions are from cultures subjected to three rounds of trypsin/EDTA treatments). In order to count cells, cell clumps resulting from high density monolayer culture are treated with 0.2% collagenase type II for about 15 min and filtered through a 70 µm cell filter to obtain a single cell suspension.

Aggregate Redifferentiation

[0029] In certain embodiments, expanded chondrogenic cells are aggregated and redifferentiated. A single cell suspension of expanded chondrocytes is placed onto a non-adherent surface resulting in cell aggregation. The cell seeding density is approximately 250,000 cells/cm² and 1,000,000 cells/mL. The medium formulation comprises basal medium, ITS, albumin, non-essential amino acids, penicillin, streptomycin, fungizone, dexamethasone, ascorbate-2-phosphate, L-proline, sodium pyruvate, and growth factors (e.g., TGFβ1, BMP, and/or IGF). Media is refreshed every other day for the duration of the 1-3 week redifferentiation period. At the end of this period, the aggregates are digested using a combination of enzymes (e.g., collagenase and trypsin).

[0030] In an exemplary embodiment, a single-cell suspension of chondrocytes expanded to passage 3 (P3) using the chondrogenically-tuned procedure, is placed onto 2% agarose -

coated 100mm petri dishes at 250,000 cells/cm² and 1 million cells/mL of CM supplemented with 10 ng/mL TGF- β 1. Media is refreshed every other day for the duration of the 1 week redifferentiation period. Following redifferentiation culture, the aggregates are digested using 0.25% (w/v) trypsin/0.05% (w/v) EDTA for 1 hour, then exposed to 0.2% collagenase type II for 1 hour before being filtered through a 70 μ m mesh to obtain a single cell suspension.

Construct Formation

[0031] Cartilage constructs can be formed using the expanded and redifferentiated chondrogenic cells via a number of methods including but not limited to self-assembly, centrifugation, scaffold-seeding and hydrogel encapsulation. During the early phase of construct formation, a combination of a cytoskeletal modifying agent and a matrix degrading enzyme are applied to the developing constructs to achieve improved geometric, biomechanical and biochemical properties.

[0032] In some embodiments, cartilage constructs may be formed using the expanded and redifferentiated chondrogenic cells via self-assembly (see, e.g., US 2009/0142307 and US 2010/0303765). Briefly, the self-assembly process is a process of producing a cartilage construct which comprises culturing an aggregate of chondrogenic cells on a material that is not conducive to cell attachment (e.g., hydrogel such as agarose, alginate, 2-hydroxyethyl methacrylate polymer, etc.).

[0033] In some embodiments, cartilage constructs may be formed using the expanded and redifferentiated chondrogenic cells via centrifugation. The centrifugation method comprises the use of “pellet culture,” in which isolated chondrocytes are first centrifuged into pellets inside centrifuge tubes. After several days of culture in the centrifuge tubes (to allow the mass of cells to aggregate), the pellets are then transferred onto various surfaces, including hydrogels, or left in the centrifuge tubes, either of which allows for the formation of constructs.

[0034] In some embodiments, cartilage constructs may be formed using the expanded and redifferentiated chondrogenic cells via scaffold-seeding. The scaffold-seeding process comprises forming cartilage constructs in which chondrocytes are attached to a scaffold or other surface to promote cell attachment. Methods of achieving cell attachment to a scaffold

or other surface include but are not limited to a cross-linking mechanism comprising polymerization activators and ultraviolet (UV) radiation.

[0035] In some embodiments, cartilage constructs may be formed using the expanded and redifferentiated chondrogenic cells via hydrogel encapsulation. Hydrogel encapsulation comprises mixing chondrocytes with molten hydrogel to form aggregated chondrocytes surrounded by a hydrogel coat. Alternative biomaterials to hydrogel can also be used to form the aggregation coat, such as agarose or alginate.

[0036] In certain embodiments, cartilage constructs may be formed using the expanded and redifferentiated chondrogenic cells via a process comprising sedimentation of the chondrocytes onto a hydrogel coated culture vessel, onto a shaped hydrogel negative mold.

[0037] In an exemplary embodiment, self-assembly wells are created by filling wells of a 48-well plate with 1 mL molten 2% agarose and suspending a 5mm diameter stainless steel rod within the agarose. After 15 minutes the agarose gels and the stainless steel rods are removed leaving 5mm diameter negative impressions. These negative impressions are the self-assembly wells. The self-assembly wells are then infiltrated with culture medium (CM) for 1 week prior to cell seeding. Single-cell suspensions of chondrocytes expanded to passage three or beyond and redifferentiated for one week in aggregate culture are placed in CM containing 200 U/mL hyaluronidase (Sigma) and 2 μ M cytochalasin D (Enzo Life Sciences). A 100 μ L aliquot of this cell suspension is seeded into each self-assembly well to form a construct containing two million chondrocytes. Four hours following seeding, 400 μ L of CM containing 2 μ M cytochalasin D is gently added to each well. After 24 hours, a complete media change is made by replenishment with 500 μ L CM containing 2 μ M cytochalasin D. After a further 24 hours, a complete media change is made by replenishment of 500 μ L of CM without cytochalasin D. Every 24 hours for the remainder of the culture period a complete media change with CM is made. Figure 9 illustrates that treatment with cytochalasin-D and hyaluronidase resulted in the production of cartilage constructs with superior biomechanical and biochemical properties.

[0038] In other embodiments, the cartilage construct (e.g., neocartilage or bio-engineered cartilage) is subject to further treatments. For instance in some embodiments, the cartilage construct is subjected to one or more of chondroitinase-ABC digestion, hydrostatic pressure and direct compression as previously described (US 2011/0053262). Alternatively or

additionally when the chondrogenic cells are obtained from a xenogenic or allogenic donor, the cartilage constructs are treated with a decellularization agent to remove the donor cells (see, e.g., US 2011/0212894) to produce a substantially acellular cartilage construct. Suitable decellularization agents include but are not limited to SDS, tributyl phosphate, triton-X, hypotonic solutions (10 mM or less salt) and hypertonic solutions (50 mM or more salt).

Analysis of the Constructs

[0039] The properties of cartilage constructs may be tested using any number of criteria including, but not limited to, morphological, biochemical, and biomechanical properties, which also may be compared to native tissue levels. Morphological examination includes but is not limited to histology analysis using safranin- O and fast green staining for proteoglycan and GAG content, as well as picro-sirius red staining for total collagen, immunohistochemistry for collagens I and II, and confocal and scanning electron microscopies for assessing cell-matrix interactions. Biochemical assessments includes picogreen for quantifying DNA content, DMMB for quantifying GAG content, hydroxyproline assay for quantifying total collagen content, ELISA for quantifying amounts of specific collagens (I and II), and RT-PCR for analysis of mRNA expression of proteins associated with the extracellular matrix (e.g. collagen and aggrecan). Constructs may be evaluated on the basis of changes to the acellular and aprotinaceous central region (AACR) as compared to control cartilage.

[0040] Constructs also may be evaluated using one or more of incremental tensile stress relaxation, incremental compressive stress relaxation, and biphasic creep indentation testing to obtain moduli, strengths, and viscoelastic properties of the constructs. Incremental compressive testing under stress relaxation conditions may be used to measure a construct's compressive strength and stiffness. Incremental tensile stress relaxation testing may be used to measure a construct's tensile strength and stiffness. Additionally, indentation testing under creep conditions may be used to measure a construct's modulus, Poisson's ratio, and permeability.

Neocartilage

[0041] The cartilage produced using the methods of the present disclosure more closely resembles native cartilage than that produced using standard methods. For instance, the cartilage produced using the methods of the present disclosure possesses one or both of an

elevated glycosaminoglycan per wet weight percentage and an elevated collagen II to collagen I (w/w) ratio as compared to control cartilage produced using a standard method. In some embodiments, the cartilage has a reduced acellular and aprotaceous central region (AACR) as compared to control cartilage. In some embodiments, the cartilage has an elevated level of SOX9 as compared to control cartilage. In some embodiments, the cartilage has one or more of the following properties selected from the group consisting of an elevated compressive relaxation modulus, an elevated compressive instantaneous modulus, and an elevated tensile Young's modulus, as compared to control cartilage. In some embodiments, the cartilage comprises a higher number of cells as compared to control cartilage. For comparison purposes, control cartilage is cartilage produced directly from primary chondrocytes or after passaging the primary chondrocytes once or twice at a cell seeding density equal to or less than 11,000 cells/cm² until 90% or less confluence is reached.

Definitions

[0042] Unless defined otherwise, all scientific and technical terms are understood to have the same meaning as commonly used in the art to which they pertain. For the purpose of the present disclosure, the following terms are defined.

[0043] The term "construct" or "cartilage construct" as used herein refers to a three-dimensional mass having length, width, and thickness, and which comprises living mammalian tissue produced in vitro.

[0044] The terms "self-assemble" or "self-assembly" as used herein refer to a process in which specific local interactions and constraints between a set of components cause the components to autonomously assemble, without external assistance, into the final desired structure.

[0045] As used herein, the term "cell aggregate" refers to a cluster of cells. In contrast, the term "cell suspension" refers to a dispersion of cells in a liquid.

[0046] As used herein, the term "cartilage" refers to an avascular protective tissue in the form of a matrix comprising collagen, proteoglycans and elastin.

[0047] As used herein, the singular form "a", "an", and "the" includes plural references unless indicated otherwise. For example, "an" excipient includes one or more excipients.

[0048] The phrase “comprising” as used herein is open-ended, indicating that such embodiments may include additional elements. In contrast, the phrase “consisting of” is closed, indicating that such embodiments do not include additional elements (except for trace impurities). The phrase “consisting essentially of” is partially closed, indicating that such embodiments may further comprise elements that do not materially change the basic characteristics of such embodiments. It is understood that aspects and embodiments described herein as “comprising” include “consisting” and/or “consisting essentially of” aspects and embodiments.

[0049] The term “about,” as used herein, generally refers to an approximate amount. For instance, the term “about #” refers to an amount that is 90-110% of # (e.g., about 100 grams refers to an amount between 90 to 110 grams).

EXAMPLES

[0050] Abbreviations: ACI (autologous chondrocyte implantation); AACR (acellular and aprotinaceous central region); ACs (articular chondrocytes); bFGF (basic fibroblast growth factor); CM (culture medium); col I (collagen type I); col II (collagen type II); ECM (extracellular matrix); GAG (glycosaminoglycan); ITS (insulin, transferrin, selenium); MCs (meniscus cells); P3 (passage 3); P4 (passage 4); TGF- β 1 (transforming growth factor β 1); and WW (wet weight).

EXAMPLE 1

Chondrogenically-Tuned Expansion Enhances the Cartilaginous Matrix Forming Capabilities of Primary Adult Chondrocytes

[0051] This example compares the results of a standard chondrocyte expansion protocol and an exemplary chondrogenically-tuned expansion protocol of the present disclosure. .

Materials and Methods

[0052] *Chondrocyte Isolation.* New Zealand White Rabbits (Heaton Rabbitry) were obtained within 8 hours following sacrifice. Chondrocytes were isolated from both the tibial and femoral articular cartilage surfaces of skeletally-mature (18-24 month old) rabbits using 0.2% collagenase type II (Worthington) in chemically defined culture medium (CM) (DMEM with 4.5 g/L-glucose and GlutaMAX (Invitrogen), 100 nM dexamethasone (Sigma), 1% fungizone, 1% penicillin/streptomycin (BD Biosciences), 1% insulin transferrin selenium premix (ITS+) (BD), 50 mg/mL ascorbate-2-phosphate (Sigma), 40 mg/mL L-proline

(Sigma), and 100 mg/mL sodium pyruvate (Fisher Scientific)). After overnight digestion, chondrocytes from ten rabbits were pooled and frozen at -80°C in culture medium supplemented with 20% fetal bovine serum (FBS) (Gemini Bio-Products) and 10% DMSO (Sigma). Prior to cryopreservation, the chondrocyte viability as determined by trypan blue exclusion was $>95\%$. After freezing at -80°C , cells were placed in liquid nitrogen cryo-storage until they were needed for expansion. Prior to expansion through passage, cells were designated as “primary” chondrocytes.

[0053] *Chondrocyte Expansion.* Primary chondrocytes were rapidly thawed and seeded on T-225 flasks for expansion. Following thawing of cryopreserved chondrocytes, cell viability was approximately 85%. A preliminary study compared the expansion characteristics and resultant construct properties following chondrogenically-tuned expansion or the standard protocol for chondrocyte expansion. The standard protocol employed CM media with 10% FBS instead of ITS+ and dexamethasone, a seeding density of 1.1×10^4 cells/cm², and passaging at 90% confluence. The chondrogenically-tuned protocol employed CM medium supplemented with 5 ng/mL basic fibroblast growth factor (bFGF) (Peprotech), a seeding density of 2.5×10^4 cells/cm², and passaging 4 days after 95% confluence was met. To allow adequate cell adhesion for chondrogenically-tuned passaging, it was necessary to add 10% FBS for the first 24 hours of monolayer seeding. Passaging was performed using 0.25% (w/v) trypsin/0.05% (w/v) EDTA (GIBCO) at 37°C . Since both trypsin digestion and seeding can alter cellular characteristics, passage number in this study refers to the number of trypsin/EDTA exposures. For instance, cells expanded to passage 3 (P3) under these conditions experienced three trypsin/EDTA treatments. Primary chondrocytes are also referred to as P0 chondrocytes.

[0054] *Construct Seeding.* Self-assembly wells were created by filling a well of a 6-well plate with approximately 17 mL molten 2% agarose (Fisher Scientific) and placing a silicone cap on top of the well. The silicone cap was cylindrical with a diameter of 35mm and height of 10mm and had 15 silicone posts (3mm diameter and 10mm long) projecting from its underside into the agarose. After the agarose had set, the silicone cap was removed leaving negative impressions of the 3mm diameter and 10mm long posts in the hardened agarose. These negative impressions were the self-assembly wells. The agarose cylinders containing the self-assembly wells were removed from the 6-well plate, placed into a 100mm petri dish with 15 mL CM, and allowed to become infiltrated with CM for 1 week prior to seeding.

Assembly wells were then placed into CM medium for 1 week prior to seeding. Suspensions of primary and expanded cells were combined to obtain a range of primary to passaged chondrocyte (P0:P3) ratios (100:0, 50:50, 25:75, 10:90, 2:98, 0:100). A 50 μ L aliquot of a 40×10^6 cells per mL solution was seeded into a self-assembly well to form a construct containing 2×10^6 chondrocytes. Time of seeding was designated as $t = 0$. Constructs remained in the 3mm diameter agarose wells until four days after they grew to come in contact with the well edges, at which time they were transferred to 5mm diameter agarose wells. Throughout the study, all constructs were cultured in the same volume of media and received media changes every other day for four weeks of 3D culture. At this time, constructs were subjected to gross morphological, histological, and biochemical assessments.

[0055] *Construct Weight and Gross Morphology.* Prior to destructive assays, six constructs from each experimental group were blotted dry, weighed for wet weight, and photographed for gross morphology. Construct dimensions (diameter and height) were measured via image analysis with Image J. Constructs were then divided into sections for histological, biochemical, and biomechanical testing.

[0056] *Histology.* Two samples from each treatment were frozen and sectioned at 14 μ m and fixed in 10% neutral buffered formalin. Safranin-O/Fast Green staining was used to examine glycosaminoglycan (GAG) distribution. Picrosirius Red staining was used for qualitative examination of collagen content and distribution.

[0057] *Quantitative Biochemistry.* Biochemical analysis included Blyscan GAG assay (Biocolor) based on dimethylmethylene blue (DMMB) binding, a modified colorimetric hydroxyproline assay (Woessner, Arch Biochem Biophys, 93:440-447, 1961), PicoGreen (Invitrogen) for DNA content, and enzyme-linked immunosorbent assays (ELISAs) for collagens I and II. Samples were lyophilized for 48 hours then digested in pepsin for 4 days at 4°C followed by a 1-day elastase digest. For collagen II ELISA, Chondrex reagents and protocols were used. For collagen I ELISA, a similar protocol was employed with antibodies from US Biological. Both ELISAs employed bovine collagen standards that have been previously validated to accurately quantify rabbit collagen types I and II. DNA content data from the PicoGreen assay was converted to cell number using a conversion factor of 7.7 pg DNA per cell (Kim et al., Anal Biochem, 174:168-176, 1988). Six constructs per group were digested independently and used for biochemical testing.

[0058] *Unconfined Compression.* A 2mm diameter punch was taken from each construct for analysis of compressive properties. Calipers were used to measure of the diameter of the compression sample to verify a 2mm diameter sample was consistently obtained. The platens of an Instron 5565 were placed into contact and the platen-to-platen displacement was zeroed. Compression samples were loaded to 0.2 N to determine sample height and compressed such that deformation occurred along the height of the cylinder, at 10% strain per second, to 10%, 20% and 30% strain. Testing was conducted in a PBS bath (pre-load 0.2% strain, increments held for 10 min). The curve fitting tools in Matlab were used to determine viscoelastic compressive properties (relaxation modulus, instantaneous modulus, coefficient of viscosity) as previously described by fitting the experimental data curves to the theoretical solution of the viscoelastic Kelvin model (Adkisson et al., Am J Sports Med, 38:1324-1333, 2010). Biomechanical properties were assessed using six constructs from each group.

[0059] *Real Time Polymerase Chain Reaction.* Total RNA was extracted from primary and passaged chondrocytes after expansion and prior to self-assembly (i.e., before $t = 0$). RNA was extracted using the protocol associated with the RNAqueous kit (Ambion). RNA concentration was determined using a NanodropTM spectrophotometer, and a consistent amount of RNA across all samples was reverse-transcribed using the Super Script III kit (Invitrogen) to obtain cDNA. Real-time polymerase chain reaction (PCR) was performed on the cDNA using a FAM-conjugated primer and probe mixture from Applied Biosystems for β -actin (assay ID: Oc03824857_g1) and SOX9 (assay ID: Oc04096872_m) on a Rotor-gene system (Corbett Research), following the protocol provided by Applied Biosystems. Three independent samples from each group were analyzed in triplicate. Data from the PCR assessment were analyzed using the delta-delta Ct (take-off cycle) method (Livak and Schmittgen, Methods, 25:402-408, 2001). Four samples per group were analyzed using RT-PCR.

[0060] *Statistics.* The study was performed three times (triplicates) with cells from three different 10-rabbit harvests. A single factor ANOVA was used to examine the results obtained from biochemical and biomechanical testing. A two factor ANOVA was used to analyze the real-time PCR data. Tukey's post-hoc test was used, when warranted, to determine significance among groups. Significance for all tests was defined as $p < 0.05$. In all figures, significance between experimental groups or levels of particular factors exist by groups not sharing the same letter or symbol.

Results

[0061] Expansion. The preliminary study comparing standard and chondrogenically-tuned expansion protocols found similar time durations to reach P3 (20 vs. 16 days respectively), and an approximately 64-fold increase in cell number at P3. Additionally, as shown in Figure 1 significant increases in the glycosaminoglycan (GAG) per wet weight (WW) and the collagen II to collagen I ratio were observed when chondrogenically-tuned expansion methods of the present disclosure were employed. Cellular morphological changes were noted throughout the expansion procedure. Regardless of the expansion protocol employed, shifts from a rounded cell shape to a more elongated shape with greater numbers of cellular projections were observed.

[0062] Gross Morphology and Histology. Morphological differences were noted among the six experimental groups (P0:P3 chondrocytes, 100:0, 50:50, 25:75, 10:90, 2:98, 0:100) within the first week following seeding. The diameter and the growth kinetics of the constructs both varied directly with respect to the ratio of expanded chondrocytes. That is, the higher the percentage of expanded chondrocytes, the faster the construct grew and reached the well edge compared to those with lower percentages of expanded cells. Table 1-1 lists the numerical differences in the gross morphological properties of constructs at $t = 4$ weeks of culture.

Table 1-1 Biochemical Results After Four Weeks of Culture

	100:0	50:50	25:75	10:90	2:98	0:100
Diameter (mm)	2.6 ± 0.1^a	2.8 ± 0.1^d	3.1 ± 0.1^c	3.3 ± 0.1^{ab}	3.3 ± 0.1^{ab}	3.4 ± 0.1^a
Thickness (mm)	1.6 ± 0.1^d	2.2 ± 0.1^c	2.5 ± 0.1^b	2.5 ± 0.1^b	2.6 ± 0.1^b	2.7 ± 0.1^a
Wet Weight (mg)	7.3 ± 0.3^e	12.2 ± 0.6^d	15.0 ± 0.7^c	17.6 ± 0.6^b	19.4 ± 1.2^a	20.4 ± 0.6^a
Collagen (mg)	0.7 ± 0.1^b	0.9 ± 0.1^{ab}	0.95 ± 0.1^a	0.8 ± 0.2^{ab}	1.0 ± 0.1^a	0.9 ± 0.1^{ab}
GAG (mg)	0.3 ± 0.1^c	1.0 ± 0.2^b	1.3 ± 0.2^{ab}	1.5 ± 0.3^a	1.4 ± 0.2^a	1.6 ± 0.2^a
Col 1 (μg)	57 ± 14^a	50 ± 12^{ab}	42 ± 12^{ab}	36 ± 10^b	46 ± 13^{ab}	33 ± 6^b
Col 2 (μg)	195 ± 29^b	290 ± 40^a	283 ± 46^a	291 ± 26^a	329 ± 67^a	292 ± 31^a

Values shown as mean $\pm$ SD. Data denoted by different letters indicate significant differences ($p < 0.05$)

[0063] Histological examination with Picrosirius Red showed uniform collagen staining throughout all constructs and a small recess at the center of the construct. Safranin-O/Fast Green staining to identify GAGs revealed similar, intense staining of all groups except the 100:0 group, which stained faintly only at the center of the constructs. Safranin-O/Fast Green showed that incorporation of any amount of expanded cells increased staining intensity. There was an increasing trend in construct diameter and thickness when increasing the amount of P3 cells in the construct.

[0064] *Quantitative Biochemistry.* Quantitative biochemical values for collagen, GAG, and collagen types I and II are shown in Table 1-1. Collagen content ranged between 0.7 and 1.0 mg per construct. The 100:0 group was shown to contain significantly less collagen than the 2:98 and 25:75 groups. GAG content ranged from 0.3 to 1.6 mg per construct. All groups containing expanded cells had significantly higher GAG content than the pure primary cell group. As shown in Figure 2, GAG content increased 2-fold from pure primary cell to pure expanded cell constructs (black bars). Collagen type I and type II content per construct ranged between 33-57 μ g and 195-329 μ g, respectively (Table 1-1). Notably, collagen type I content for pure primary cells was approximately two times greater than the pure passaged cell group. All groups containing passaged cells possessed a significantly greater amount of collagen type II than the pure primary 100:0 group. Specifically, a 50% increase was observed when comparing the 0:100 to the 100:0 group. In Figure 2, a trend of increasing collagen type II to collagen type I ratio was observed with an increased amount of passaged cells per construct (gray bars). The PicoGreen assay showed significant differences in construct cellularity among groups, with a range of 1.0-1.5 million cells. The 100:0 group possessed a significantly lower number of cells than all other groups.

[0065] *Unconfined Compression.* At the 20% strain level, significant differences were observed in all three independent parameters of the viscoelastic model. As shown in Figure 3, the relaxation moduli ranged from 193 ± 37 to 443 ± 68 kPa with significantly higher values associated with all groups containing passaged cells (black bars). Figure 3 also shows that instantaneous moduli varied from 801 ± 55 to 1157 ± 128 kPa with the 50:50 group having a significantly greater value than all other groups (gray bars). Finally, the coefficient of viscosity ranged from 14 ± 2 to 26 ± 5 MPa with a decreasing trend associated with a decrease in the amount of primary cells. Similar trends were obtained at the 10% and 30% strain levels.

[0066] *Real-time PCR.* Prior to self-assembly, primary and P3 chondrogenically-tuned chondrocytes showed no significant differences in terms of SOX9 expression. The levels of SOX9 expression did not significantly increase by $t = 2$ days. However, by $t = 7$ days, SOX9 expression had significantly increased in both primary and P3 chondrocytes compared to prior time points and was significantly higher in passaged cells compared to primary cells as shown in Figure 4. Compared to primary chondrocytes before seeding, primary chondrocytes at $t = 7$ days had approximately twice the SOX9 expression level, while passaged

chondrocytes at $t = 7$ days had approximately three-times the SOX9 expression level. Additionally, the two-factor ANOVA showed that overall P3 chondrocytes had higher SOX9 expression than primary chondrocytes.

[0067] *Conclusion.* When expanded through passage, chondrocytes are known to lose their ability to produce high-quality cartilaginous matrix. The present disclosure provides improved cartilage constructs formed with expanded chondrocytes through alterations in the expansion protocol and the ratio of primary to expanded chondrocytes used to form the constructs. The exemplary chondrogenically-tuned expansion protocol of the present disclosure resulted in similar monolayer growth rates as those obtained using traditional serum-containing medium for production of constructs with enhanced cartilaginous properties. Various ratios of primary to chondrogenically-expanded chondrocytes were then self-assembled to form neocartilage. Biochemical analysis showed that constructs formed with only expanded cells had twice the GAG per wet weight and collagen II/collagen I ratio compared to constructs formed with primary chondrocytes. Biomechanically, compressive properties of constructs formed with only passaged cells matched the instantaneous modulus and exceeded the relaxation modulus of constructs formed with only primary cells. These counterintuitive results show that by applying proper expansion and three-dimensional culture techniques the cartilage forming potential of adult chondrocytes expanded through passaging can be enhanced over that of primary cells.

EXAMPLE 2

Self-Assembled Meniscus Cells and Articular Chondrocytes

[0068] This example describes the impact of varying passage number, cryopreservation and redifferentiation on the biomechanical properties of meniscus cells (MCs) and articular chondrocytes (ACs) in tissue construct form. Figure 5 provides an overview of exemplary methods utilized to generate tissue constructs from primary cells.

Materials and Methods

[0069] *Chondrocyte Isolation and Expansion.* Knee joints of skeletally-mature New Zealand White Rabbits (Heaton Rabbitry) were obtained within 8 hours of sacrifice. Articular cartilage from both tibial and femoral surfaces and meniscal fibrocartilage were sterilely dissected. These tissues were minced into ~1 mm pieces and digested in 0.2% collagenase

type II (Worthington) in chemically defined chondrogenic culture medium (CM = DMEM with 4.5 g/L-glucose and GlutaMAX (Invitrogen), 100 nM dexamethasone, 1% fungizone, 1% penicillin/streptomycin (BD Biosciences), 1% ITS+ premix (BD), 50 mg/mL ascorbate-2-phosphate, 40 mg/mL L-proline, and 100 mg/mL sodium pyruvate (Fisher Scientific)). Following overnight digestion, cells were isolated via sequential centrifugation and PBS dilution and resuspended in freezing media (CHG with 20% fetal bovine serum (FBS) (Gemini Bio-Products). Vials containing cells were frozen at a controlled rate to -80°C and then placed into liquid nitrogen cryo-storage.

[0070] The protocols employed to expand both articular chondrocytes (ACs) and meniscus cells (MCs) are based on a previous work (Example 1). Briefly, primary ACs and MCs were thawed and expanded on T-225 flasks at an initial density of 2.5×10^4 cells/cm² in CM media supplemented with 5 ng/mL basic fibroblast growth factor (bFGF). During the first 24 hours following a seeding or passaging event it was necessary to add 10% FBS to allow adequate cell adhesion. Besides this initial 24 hour period, all cells were expanded in CM media with bFGF, which was changed every other day. Both ACs and MCs were maintained in culture until 4 days after confluence. Cells were expanded to passage 3 (P3) or passage 4 (P4) under these conditions and then either frozen for 4 weeks before use or used immediately for construct formation with or without redifferentiation.

[0071] *AC and MC Redifferentiation.* Two types of cellular redifferentiation were employed: pellet culture or aggregate culture for the P3 and P4 groups of AC and MC cells. Cells from each of the groups were either subjected to one of the two redifferentiation cultures or immediately used for construct formation, making 8 distinct experimental groups. A “no-pellet” experimental control was included for both P3 and P4 groups, bringing the total to 12 distinct experimental groups. Aggregate culture involved seeding 700,000 cells/cm² onto a 2% agarose (Fisher Scientific) coated petri dish in CHG media with 10 ng/mL transforming growth factor β 1 (TGF- β 1) (Peprotech). Pellet culture involved spinning down 250,000 cells in each well of a V-bottom 96 well plate and culturing in CHG media with 10 ng/mL TGF- β 1. The duration of redifferentiation culture was 1 week for both of the techniques and media was changed every other day. Following redifferentiation culture, aggregates and pellets were digested for 1 hour in trypsin followed by 1 hour in 0.2% collagenase and filtration through a 70 μ m mesh to obtain a cell solution. The process was repeated for all 12 groups with an additional freezing step after collagenase treatment and

filtration. Thus, in the end, 24 distinct experimental groups and respective conditions were tested. Table 2-1 shows the distinct experimental groups and their abbreviations (in reference to differentiation, N=none; A=aggregate; P=pellet). For example, 3ANN group refers to passage 3, articular construct, not frozen, not redifferentiated, while 4MYP group refers to passage 4, meniscus construct, frozen, redifferentiated in a pellet.

Table 2-1. Construct Group Naming Abbreviations

Frozen	Passage	Redifferentiation	Articular Constructs	Meniscus Constructs
No	3	None	3ANN	3MNN
No	3	Aggregate	3ANA	3MNA
No	3	Pellet	3ANP	3MNP
No	4	None	4ANN	4MNN
No	4	Aggregate	4ANA	4MNA
No	4	Pellet	4ANP	4MNP
Yes	3	None	3AYN	3MYN
Yes	3	Aggregate	3AYA	3MYA
Yes	3	Pellet	3AYP	3MYP
Yes	4	None	4AYN	4MYN
Yes	4	Aggregate	4AYA	4MYA
Yes	4	Pellet	4AYP	4MYP
Yes	0	None	OA	OM

[0072] *Construct Seeding.* Cells from the 24 distinct groups plus control primary ACs and MCs were used to create tissue constructs by placing 2 million cells into a 3 mm diameter agarose well. To create agarose wells, 2% molten agarose was placed into a well of a 6-well plate and a positive die consisting of multiple 3 mm diameter silicone posts was plunged into the agarose. After an hour, when the agarose had set, the positive die was removed and the agarose block containing multiple 3 mm diameter wells was allowed to equilibrate in CHG media for 1 week prior to construct seeding. Media was changed every other day for the duration of the 4-week 3D-culture period and constructs remained confined in the agarose wells for the duration of the study. After 4 weeks, constructs were removed from culture to perform gross morphological, histological, biochemical, and biomechanical assessments.

[0073] *Gross Morphology and Histology.* After removal from culture, constructs were photographed to determine diameter and height. Constructs were then bisected at the midpoint of the construct's height. Any liquid or paste-like material present within the AACR was removed and the halves were photographed again to enable quantification of the percent

of the cross sectional area occupied by the AACR. Then both halves were weighted to obtain a measurement of wet weight. If available, two entire constructs were used for histological examination but when the total number of constructs in a group precluded this, portions of at least two constructs were used. Histological samples were snap-frozen in HistoPrep (Fisher Scientific) and sectioned to 14 μm . Safranin-O/Fast Green staining allowed the visualization of glycosaminoglycan (GAG) distribution. Picrosirius Red staining allowed the visualization of collagen distribution.

[0074] *Quantitative Biochemistry.* The wet weight and dry weight (following lyophilization) of biochemical samples were determined. Collagen, GAG, DNA, collagen I, and collagen II content was quantitatively measured through a variety of assays. First, constructs ($n=6$) were solubilized by a 4 day digestion in a 1.1 mg/mL pepsin (Sigma) solution at 4°C and a subsequent 2 day digestion in a 100 $\mu\text{g/mL}$ elastase solution. Following digestion, the Blyscan GAG assay (Biocolor), a modified colorimetric hydroxyproline assay for collagen content (36) the PicoGreen (Invitrogen) assay for DNA content, and enzyme-linked immunosorbent assays (ELISAs) for collagens I and II were performed. For collagen I and II determination, a sandwich ELISA technique was used. For collagen II, Chondrex antibodies and standards were used. For collagen I, US Biological antibodies and Chondrex standards were used. DNA content data from the PicoGreen assay was converted to cell number using a conversion factor of 7.7 pg DNA per cell (Kim et al., Anal Biochem, 174:168, 1988).

[0075] *Unconfined Compression Testing.* Images of cylindrical construct portions designated for compression testing were taken to accurately determine the diameter of the test specimen. The unconfined compression test employed for this study has been described previously (Allen and Athanasiou, J Biochem, 39:312, 2006). Briefly, test specimens were placed into a PBS bath and compressed to 10% and 20% of their initial height using an Instron 5565 and each strain increment was held for 10 minutes. In conjunction with a custom program, the curve fitting tools on Matlab (Mathworks) were used to determined viscoelastic compressive properties (relaxation modulus, instantaneous modulus, coefficient of viscosity) as previously described (Allen and Athanasiou, supra). Portions of six constructs from each group were assessed for biomechanical properties.

[0076] *Tensile Testing.* Tensile samples were cut into a dog-bone shape and photographed to ensure the accurate determination of geometric properties. Immediately prior to testing,

samples were glued onto a strip of paper spanning a precisely measured gap. The strip of paper was grasped in the clamps of an Instron 5565 and then the strip was cut to allow the load to only act on the sample. Specimens were elongated until failure at 1% of the gauge length per second. Geometric, load, and elongation data was processed using a custom program to isolate the linear region and thus determine the Young's modulus of the material.

[0077] *Statistics.* A four factor ANOVA was used to examine the results obtained from biochemical and biomechanical testing with $n=6$. The four factors and corresponding levels follow: Cell type (AC, MC), passage number (P3, P4), cryo-storage after expansion (yes, no), and redifferentiation before self-assembly (none, pellet, aggregate). Tukey's post-hoc test was used, when needed (main effects test: $p < 0.05$), to determine significant differences among levels of a factor and among all groups ($p < 0.05$).

Results

[0078] *Gross Morphology and Histology.* The group abbreviations used for this study are shown in Table 2-1 (reference to differentiation, N=none; A=aggregate; P=pellet). Gross morphological images were taken. Mean values for geometric properties, wet weight (WW) and percent hydration can be found in Table 2-2, and the statistical effects of the various treatments on these properties can be found in Table 2-3. Upon bisection and histological examination, a majority of the constructs exhibited an acellular and aprotinaceous central region (AACR). However, in the 4AYA group (passage 4, articular, frozen, aggregate group), this structure was not present and in 4MYA (passage 4, meniscus, frozen, aggregate group) group the size of the AACR was significantly reduced. The percentage of the construct cross section that was occupied by the AACR is shown in Table 2-2 and was observed to decrease with freezing and redifferentiation as shown in Figure 6.

[0079] In constructs that did not undergo a redifferentiation step, the substance within the AACR was beige in color, had a paste like texture, and upon trypan blue staining, was shown to be heavily populated with dead cells. When redifferentiation was applied, the AACR was smaller, filled with a clear fluid and very few dead cells were observed after trypan blue staining. AACR percentage ranged from 0% (4AYA, 3AYN) to 38.5% (4ANN) for AC constructs and 13.8% (4MYA) to 40.8% (4MNN). Although, constructs were confined in 3 mm wells the 4ANA and the 4MNA groups reached 3.7 mm in diameter by radially deforming the agarose wells, which was the maximum for AC and MC constructs,

respectively. For AC constructs, height ranged from 1.4 mm (4AYN) to 5.5 mm (3ANP) and, for MC constructs, height ranged from 0.5 mm (4MYN) to 6.1 mm (0M). Constructs hydration varied from 81.1% (4AYA) to 87.7% (3ANP, 4ANP) for AC constructs and 82.3% (3MYN) to 91.7% (0M) for MC constructs.

[0080] Histological staining for collagen and GAG through Picosirius Red and Safranin O dyes revealed nothing remarkable regarding protein distributions. Differences in staining intensity followed the trends apparent in the quantitative biochemical analysis.

Table 2-2. Morphological and Biochemical Properties of Constructs

	3ANN	3ANA	3ANP	4ANN	4ANA	4ANP	3AYN	3AYA	3AYP	4AYN	4AYA	4AYP	0A
Diameter (mm)	3.6±0.1 abc	3.3±0.2 e	3.6±0.1 a	3.4±0.1 de	3.7±0.1 a	3.6±0.1 a	3.1±0.1 fg	3.6±0.1 ab	3.5±0.1 bcd	1.8±0.1 h	3.2±0.1 f	3.5±0.1 cd	3.0±0.2 g
Height (mm)	3.1±0.2 e	4.2±0.5 cd	5.5±0.3 a	3.0±0.1 o	4.1±0.3 cd	5.0±0.1 b	1.9±0.1 f	4.0±0.3 cd	4.3±0.3 c	1.8±0.1 f	1.4±0.1 g	4.1±0.2 cd	3.9±0.4 d
AACR %	34.9±2.5 b	19.8±1.8 ef	21.3±1.3 de	38.5±4.3 a	22.2±1.8 d	21.4±1.7 de	0±0 u	21.7±2.3 de	25.8±1.4 d	20.1±2.4 u	0±0 u	17.6±1.9 f	37.5±1.3 a
Cell (10 ⁶)	1.6±0.5 g	3.4±0.7 bc	3.5±0.7 abc	1.8±0.2 g	3.3±0.9 cd	4.0±0.5 a	2.2±0.4 fg	3.9±0.2 ab	3.9±0.4 ab	0.5±0.1 h	1.7±0.1 g	2.7±0.4 de	2.5±0.7 ef
Hydration (%)	87.6±1.4 a	85.2±1.7 bc	87.7±0.3 a	85.6±0.9 b	85.2±0.8 bc	87.7±0.7 a	85.4±0.8 b	85.2±0.5 bc	86.4±1.3 ab	83.8±2.0 c	81.1±2.3 d	86.1±1.3 b	86.1±0.8 b
Col2/Col1 (g/g)	7.8±2.1 cde	12.3±3.0 a	9.3±2.4 abcd	6.8±3.3 de	9.8±2.6 abcd	9.0±3.2 bcd	7.1±1.4 cde	11.7±3.3 ab	9.7±0.9 abcd	10.0±2.8 abc	9.1±2.5 bcd	8.5±3.0 cd	5.3±2.3 e
	3MNN	3MNA	3MNP	4MNN	4MNA	4MNP	3MYN	3MYA	3MYP	4MYN	4MYA	4MYP	0M
Diameter (mm)	2.7±0.1 e	3.4±0.1 d	3.6±0.1 a	2.1±0.1 g	3.7±0.1 a	3.5±0.1 bc	2.2±0.1 f	3.4±0.1 cd	3.6±0.1 ab	0.9±0.1 j	1.3±0.1 i	1.6±0.1 h	2.0±0.1 g
Height (mm)	2.3±0.2 f	2.9±0.2 d	3.5±0.5 c	1.7±0.1 g	4.3±0.2 b	2.7±0.5 de	1.7±0.1 g	2.5±0.2 ef	4.1±0.2 b	0.5±0.1 j	0.6±0.2 hi	1.0±0.3 h	6.1±0.4 a
AACR %	28.6±3.4 o	20.9±1.4 e	27.9±2.9 d	40.8±2.3 a	25.9±2.0 d	26.1±2.0 d	35.4±3.6 b	20.2±1.8 e	32.2±2.6 c	18.5±3.0 e	13.8±2.8 f	15.2±3.3 f	36.5±1.8 b
Cell (10 ⁶)	1.3±0.3 e	2.6±0.5 cd	3.3±0.6 ab	0.4±0.2 f	2.9±0.7 bc	3.2±0.4 ab	0.8±0.2 f	2.3±0.5 d	3.6±0.5 a	0.1±0.1 i	0.1±0.1 i	0.4±0.1 g	2.1±0.3 d
Hydration (%)	89.0±2.4 b	84.9±0.9 de	85.6±1.0 cde	84.6±1.4 def	85.0±3.9 de	86.7±1.1 bcd	82.3±4.2 f	83.6±0.9 ef	87.7±1.0 bc	N.T.	N.T.	N.T.	91.7±1.4 a
Col2/Col1 (g/g)	4.5±1.3 de	10.1±1.8 a	6.2±1.2 bcd	4.0±2.5 de	6.1±3.4 bcd	5.2±3.2 cd	4.8±2.5 de	8.3±2.9 ab	7.8±3.1 abc	N.T.	N.T.	N.T.	2.0±1.3 e

Values shown as mean ± SD. Data denoted by different letters indicate significant differences (p<0.05)

Table 2-3. Effects of Three Factors on Morphological, Biochemical and Biomechanical Properties of Articular and Meniscus Constructs

AC Constructs							
Factor Level	Frozen		Passage		Rediff.		
	No	Yes	3	4	N	A	P
Diameter	A	B	A	B	B	A	A
Height	A	B	A	B	C	B	A
Cyst %	A	B	A	A	A	B	A
Cell	A	B	A	B	C	B	A
Hydration	A	B	A	B	B	C	A
Col2/Col1	A	A	A	A	B	A	B
GAG/WW	B	A	B	A	B	A	C
Col/WW	B	A	A	A	B	A	B
E _R	B	A	B	A	B	A	B
E _∞	B	A	B	A	B	A	B
Tensile E _Y	B	A	A	A	B	A	B

MC Constructs							
Factor Level	Frozen		Passage		Rediff.		
	No	Yes	3	4	N	A	P
Diameter	A	B	A	B	B	A	A
Height	A	A	A	A	B	A	A
Cyst %	A	B	A	B	A	C	B
Cell	A	A	A	A	C	B	A
Hydration	A	B	A	A	AB	B	A
Col2/Col1	A	A	A	B	C	A	B
GAG/WW	B	A	B	A	B	A	B
Col/WW	B	A	A	A	A	A	B
E _R	B	A	A	A	B	A	C
E _∞	B	A	A	A	C	A	B
Tensile E _Y	B	A	A	A	A	A	B

[0081] *Biochemistry.* Results of the biochemical assessments for collagen and GAG normalized to wet weight are shown in Figure 7. Results for cell number and collagen 2 normalized to collagen 1 are shown in Table 2-2. The effects of the levels of the various factors are shown in Table 2-3, significant differences ($p < 0.05$) exist between group that do not contain the same letter. For AC constructs, collagen per wet weight ranged from 3.5% (3ANP) to 12.1% (4AYA). For MC constructs, collagen per wet weight ranged from 4.9% (4MNP) to 14.6% (3MYA). GAG per wet weight varied from 1.7% (0A) to 8.3% (4AYA) for AC constructs and 0.3% (0M) to 5.9% (3MYA) for MC constructs. Although all constructs were seeded with 2 million cells, final cell numbers ranged from 0.5 million (4AYN) to 4

million (4ANP) for AC constructs and 0.1 million (4MYN, 4MYA) to 3.6 million (4MNP). Collagen 2 was more abundant than collagen 1 in all constructs as illustrated by the collagen 2/collagen 1 ratio that ranged from 5.3 (0A) to 12.3 (3ANP) for AC constructs and 2.0 (0M) to 10.1 (3MNA) for MC constructs.

[0082] *Biomechanics.* The compressive relaxation modulus, compressive instantaneous modulus, and the tensile Young's modulus were determined and are displayed in Figure 8. The effects of the levels of the various factors on these properties are shown in Table 2-3. The relaxation modulus varied from 88 kPa (0A) to 358 kPa (4AYA) for AC constructs and 37 kPa (0M) to 330 kPa (3MYA) for MC constructs. The instantaneous modulus ranged from 649 kPa (3ANN) to 2057 kPa (4AYA) for AC constructs and 143 kPa (0M) to 1969 kPa (3MYA) for MC constructs. The tensile modulus varied from 164 kPa (0A) to 907 kPa (4AYA) for AC constructs and 106 kPa (0M) to 1271 kPa (3MYA) for MC constructs.

[0083] *Conclusion.* Articular cartilage and meniscus of the knee joint lack intrinsic repair capacity and thus, injuries to these tissues result in eventual osteoarthritic changes to the joint. Tissue engineering offers the potential to replace damaged cartilage and mitigate long-term debilitating changes to the joint. In order to enhance the ability of adult articular chondrocytes (ACs) and meniscus cells (MCs) to produce robust scaffoldless neocartilage, the effects of passage number, cryopreservation, and redifferentiation prior to construct formation were studied during development of the present disclosure. By increasing passage number more cells were obtained from a tissue biopsy and no detrimental effects were observed when employing passage 4 cells versus passage 3 cells. Cryopreservation of cells enables the generation of a cell bank, which reduces lead time and enhances consistency of cell-based therapies. Surprisingly, cryopreservation was shown to enhance the biomechanical properties of the resultant self-assembled constructs. With regards to redifferentiation prior to construct formation, aggregate redifferentiation was shown to enhance the biochemical and biomechanical properties of self-assembled constructs. In conclusion, the utility of ACs and MCs were enhanced by increasing passaging number, cryopreserving cells, and applying aggregate redifferentiation prior to neotissue formation.

CLAIMS

We claim:

1. A method of producing cartilage, comprising:
 - a) passaging chondrogenic cells at a cell seeding density of over 12,500 cells/cm² in culture medium on an adherent cell culture surface until over 90% confluence is reached to produce an expanded population of chondrogenic cells;
 - b) redifferentiating the expanded population of chondrogenic cells in culture medium on a non-adherent cell culture surface to produce a chondrogenic cell aggregate;
 - c) treating the chondrogenic cell aggregate with a solution comprising collagenase to produce a chondrogenic cell suspension; and
 - d) culturing the chondrogenic cell suspension in culture medium comprising a matrix degrading enzyme and a cytoskeletal modifying agent to produce cartilage.
2. The method of claim 1, wherein the cytoskeletal modifying agent is an inhibitor of actin polymerization.
3. The method of claim 1, wherein the cytoskeletal modifying agent is cytochalasin D.
4. The method of claim 1, wherein the matrix-degrading enzyme is selected from the group consisting of hyaluronidase, chondroitinase, heparinase and keratanase.
5. The method of claim 1, wherein the matrix-degrading enzyme is hyaluronidase.
6. The method of claim 1, wherein the chondrogenic cell suspension in step d) is cultured using a construct formation technique selected from the group consisting of self-assembly, centrifugation, scaffold seeding and hydrogel encapsulation.
7. The method of claim 1, wherein the chondrogenic cell suspension in step d) is cultured using a self-assembly construct formation technique.
8. The method of any one of claims 1-7, wherein the chondrogenic cells in step a) are primary chondrocytes isolated from one or more of the group consisting of articular cartilage, fibrocartilage, elastic cartilage, and hyaline cartilage.

9. The method of any one of claims 1-7, wherein the chondrogenic cells in step a) comprise mesenchymal stem cells isolated from a source selected from the group consisting bone marrow, adipose tissue, synovium, periosteum, dermis, umbilical cord blood, synovial fluid, muscle, and tendon.

10. The method of any one of claims 1-7, wherein the chondrogenic cells in step a) are pluripotent stem cells selected from the group consisting of embryonic stem cells and induced-pluripotent stem cells.

11. The method of any one of claims 1-7, wherein the chondrogenic cells in step a) express one or more of the transcription factors selected from the group consisting of SOX-5, SOX-6, and SOX-9.

12. The method of any one of claims 1-7, wherein the cells in step a) are primary chondrocytes isolated from articular cartilage.

13. The method of any one of claims 1-7, wherein step a) is repeated from two to six times before redifferentiation in step b) such that the expanded population of chondrogenic cells are passage 2, passage 3, passage 4, passage 5, or passage 6 cells.

14. The method of any one of claims 1-7, wherein the cell seeding density of step a) is from 15,000 and 30,000 cells/cm².

15. The method of any one of claims 1-7, wherein over 90% confluence is from about 93% to over-confluence.

16. The method of any one of claims 1-7, wherein the expanded population of chondrogenic cells of step a) is cryopreserved before redifferentiation in step b).

17. The method of any one of claims 1-7, wherein the culture medium is a serum-free medium.

18. A composition comprising the cartilage prepared by the method of any one of claims 1-7.

19. The composition of claim 18, wherein the cartilage has a glycosaminoglycan per wet weight percentage of over 6%.

20. The composition of claim 18, wherein the cartilage has a collagen II to collagen I (w/w) ratio of greater than 2.
21. The composition of claim 18, wherein the cartilage has a reduced acellular and a proteinaceous central region (AACR) as compared to control cartilage produced using a standard method.
22. The composition of claim 18, wherein the cartilage expresses SOX9 at an elevated level as compared to control cartilage produced using a standard method.
23. The composition of claim 18, wherein the cartilage has one or more of the following properties selected from the group consisting of an elevated compressive relaxation modulus, an elevated compressive instantaneous modulus, and an elevated tensile Young's modulus, as compared to control cartilage produced using a standard method.
24. The composition of claim 18, wherein the cartilage comprises a higher number of cells as compared to control cartilage produced using a standard method.
25. A method of producing cartilage, comprising:
culturing a chondrogenic cell suspension in culture medium comprising a matrix degrading enzyme and a cytoskeletal modifying agent to produce cartilage.
26. A method of producing cartilage, comprising:
culturing a chondrogenic cell suspension in culture medium comprising a matrix degrading enzyme to produce cartilage, with the proviso that said matrix degrading enzyme does not comprise chondroitinase-ABC.
27. A method of producing cartilage, comprising:
culturing a chondrogenic cell suspension in culture medium comprising a cytoskeletal modifying agent to produce cartilage, with the proviso that said cytoskeletal modifying agent does not comprise a kinase inhibitor.
28. The method of any one of claims 25-27, further comprising a prior step of:
treating a chondrogenic cell aggregate with a solution comprising collagenase to produce the chondrogenic cell suspension.

29. The method of claim 28, further comprising a prior step of:
redifferentiating an expanded population of chondrogenic cells in culture medium on a non-adherent cell culture surface to produce the chondrogenic cell aggregate.
30. The method of claim 29, wherein the culturing step is done in the absence of exogenous transforming growth factor-beta (TGF-beta).

1/10

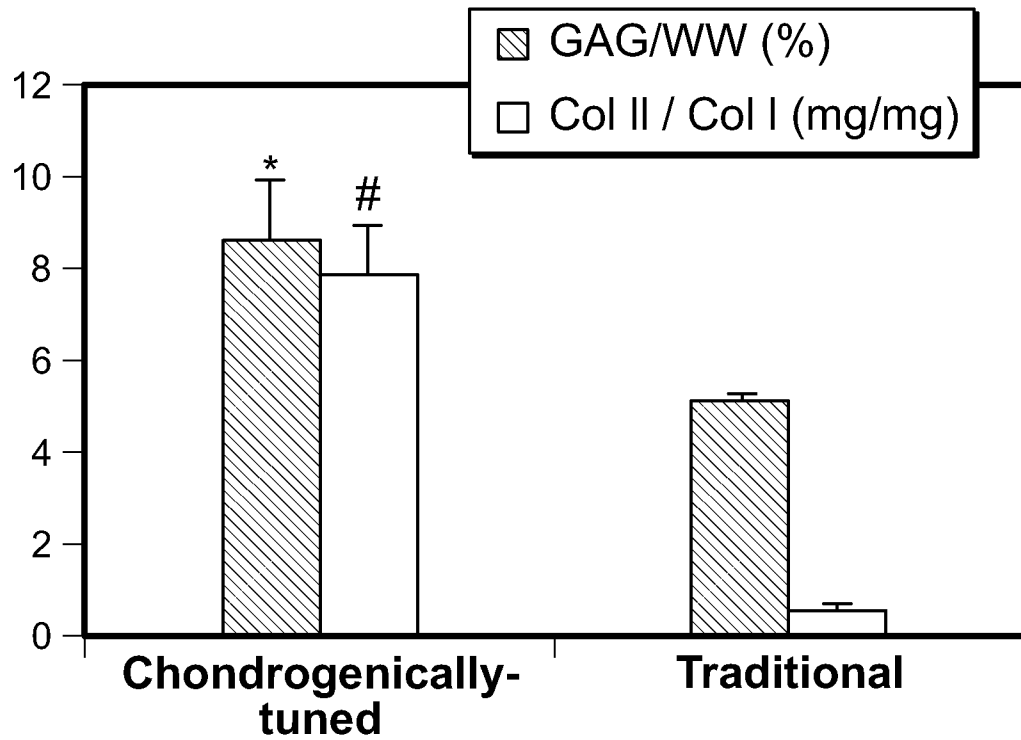


FIG. 1

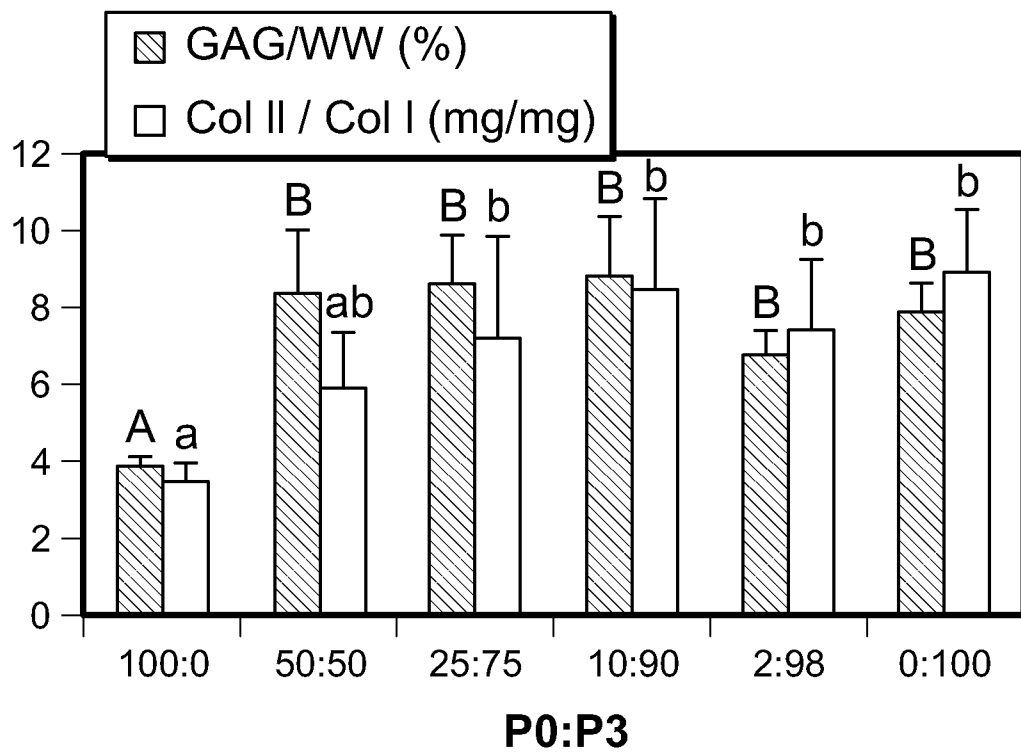


FIG. 2

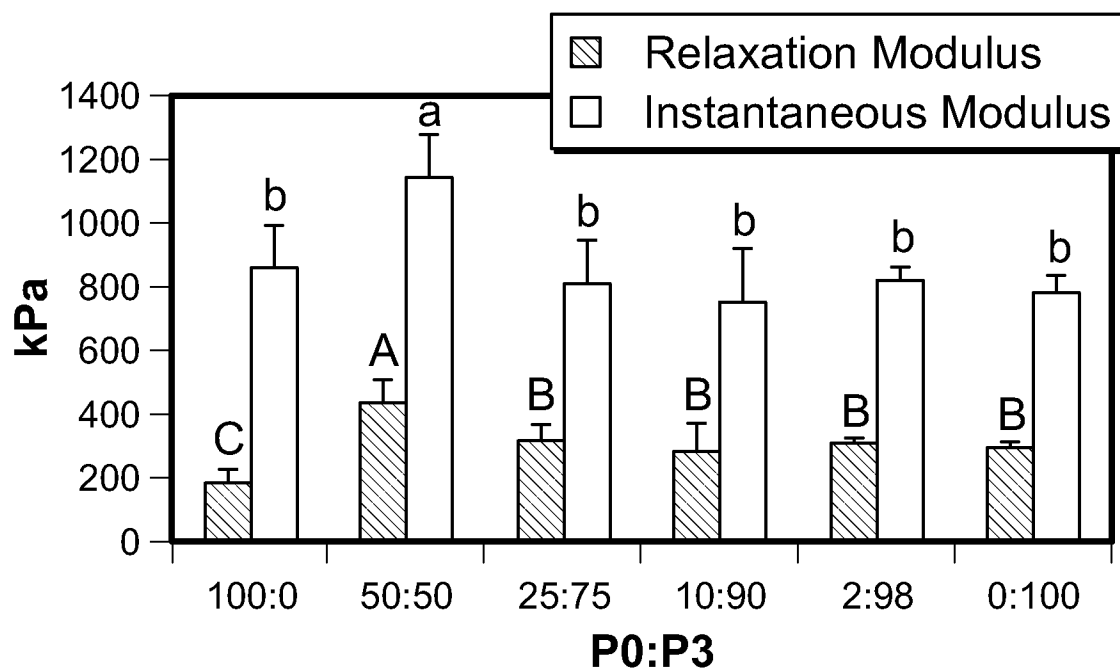


FIG. 3

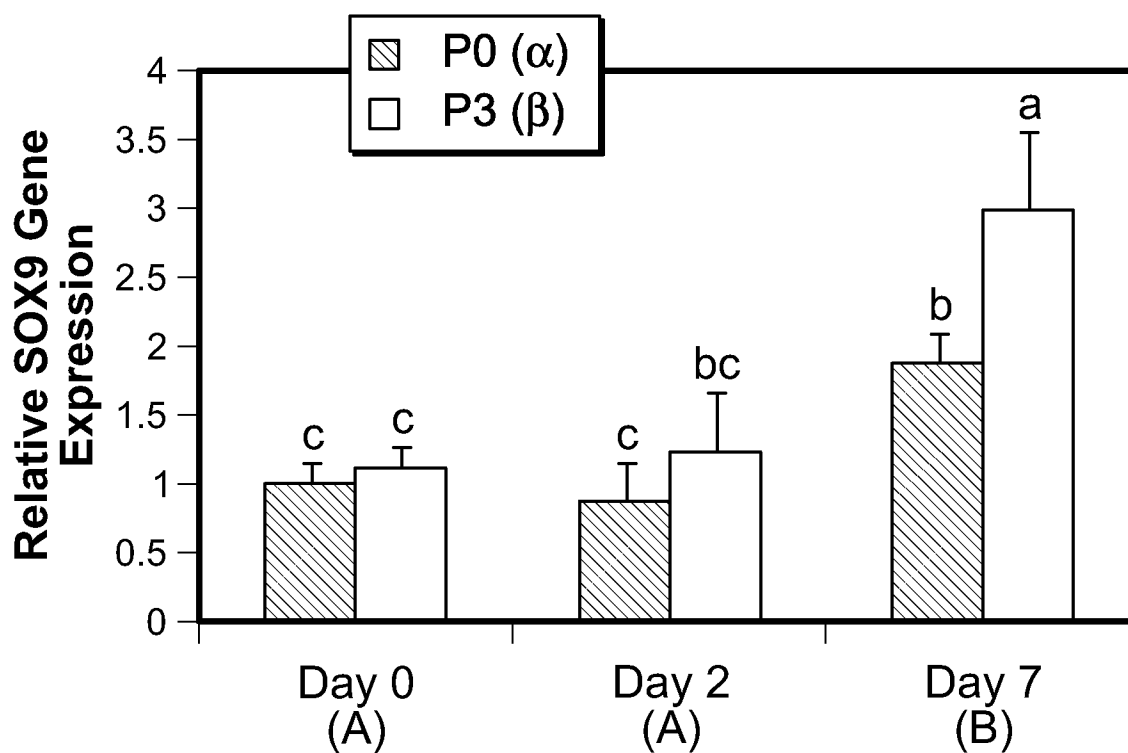


FIG. 4

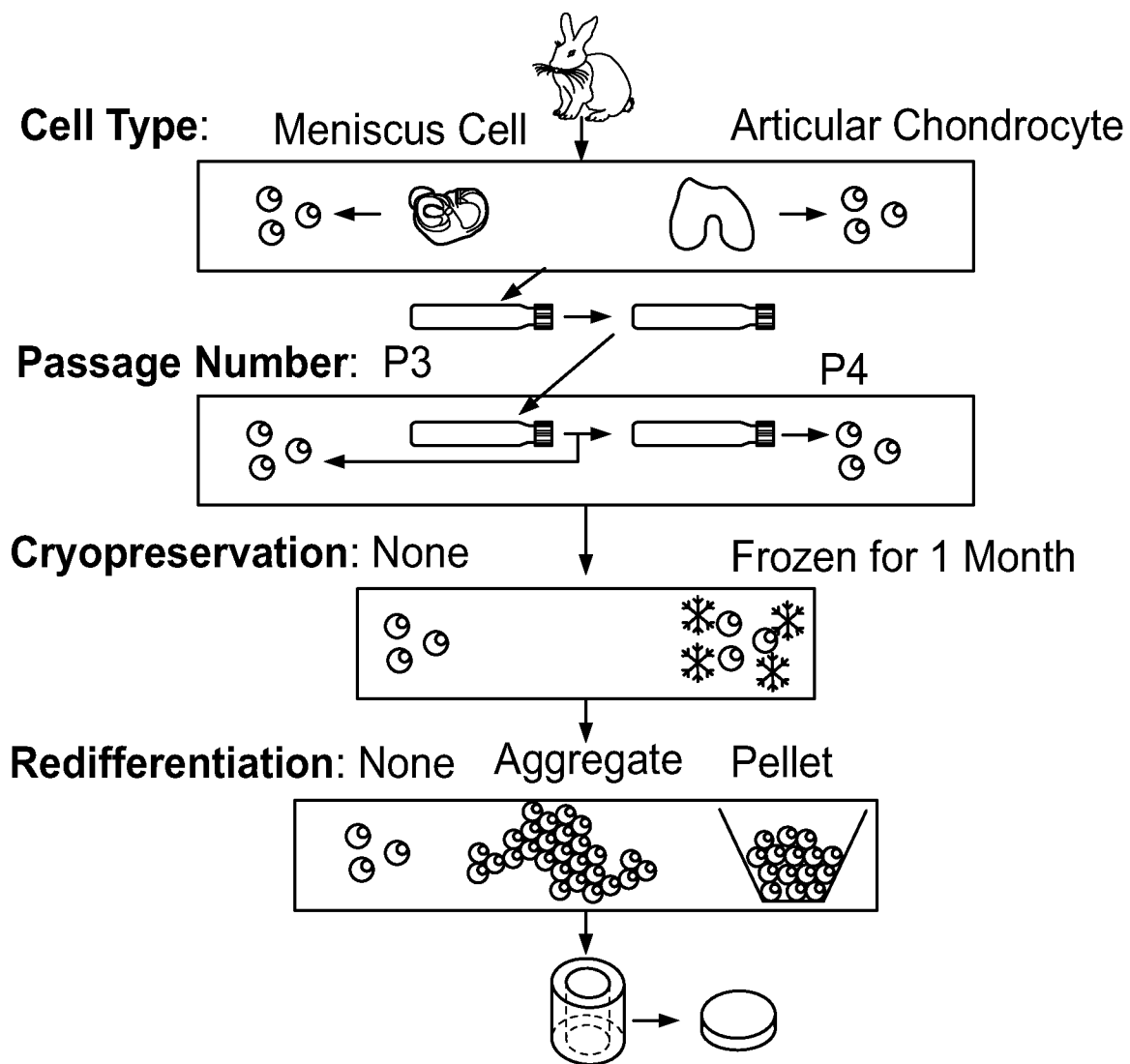


FIG. 5

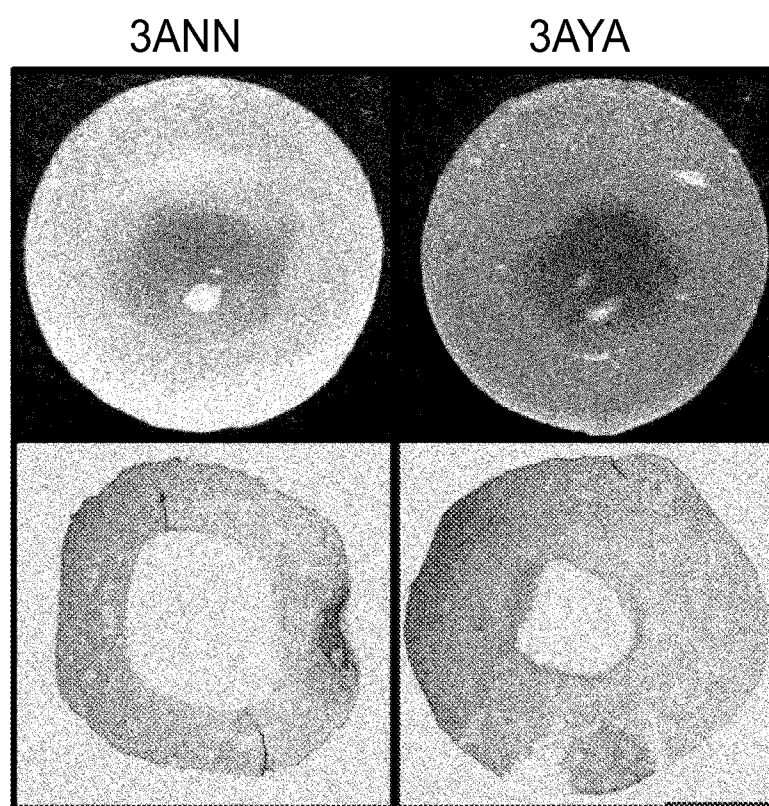


FIG. 6

5/10

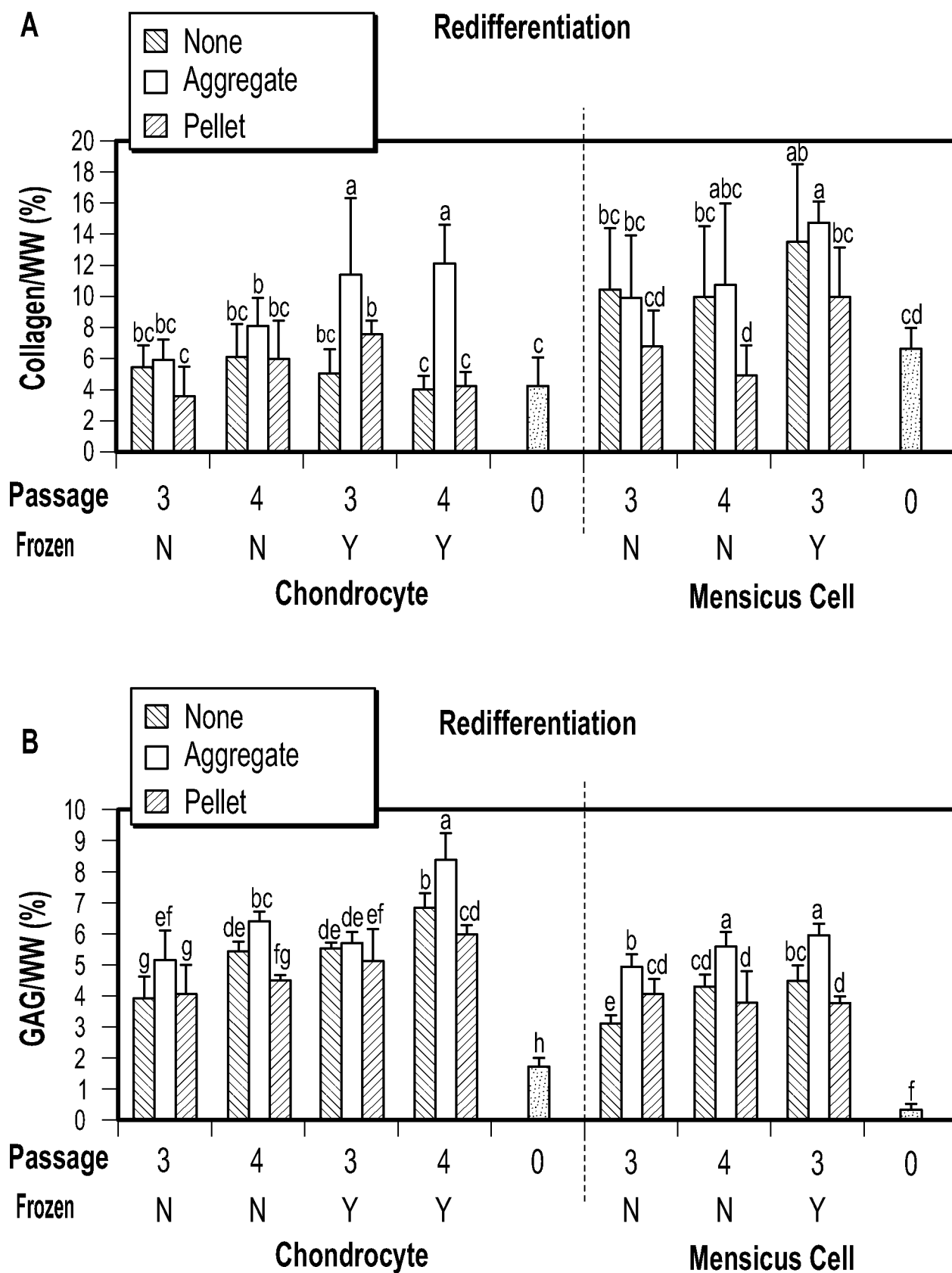


FIG. 7

6/10

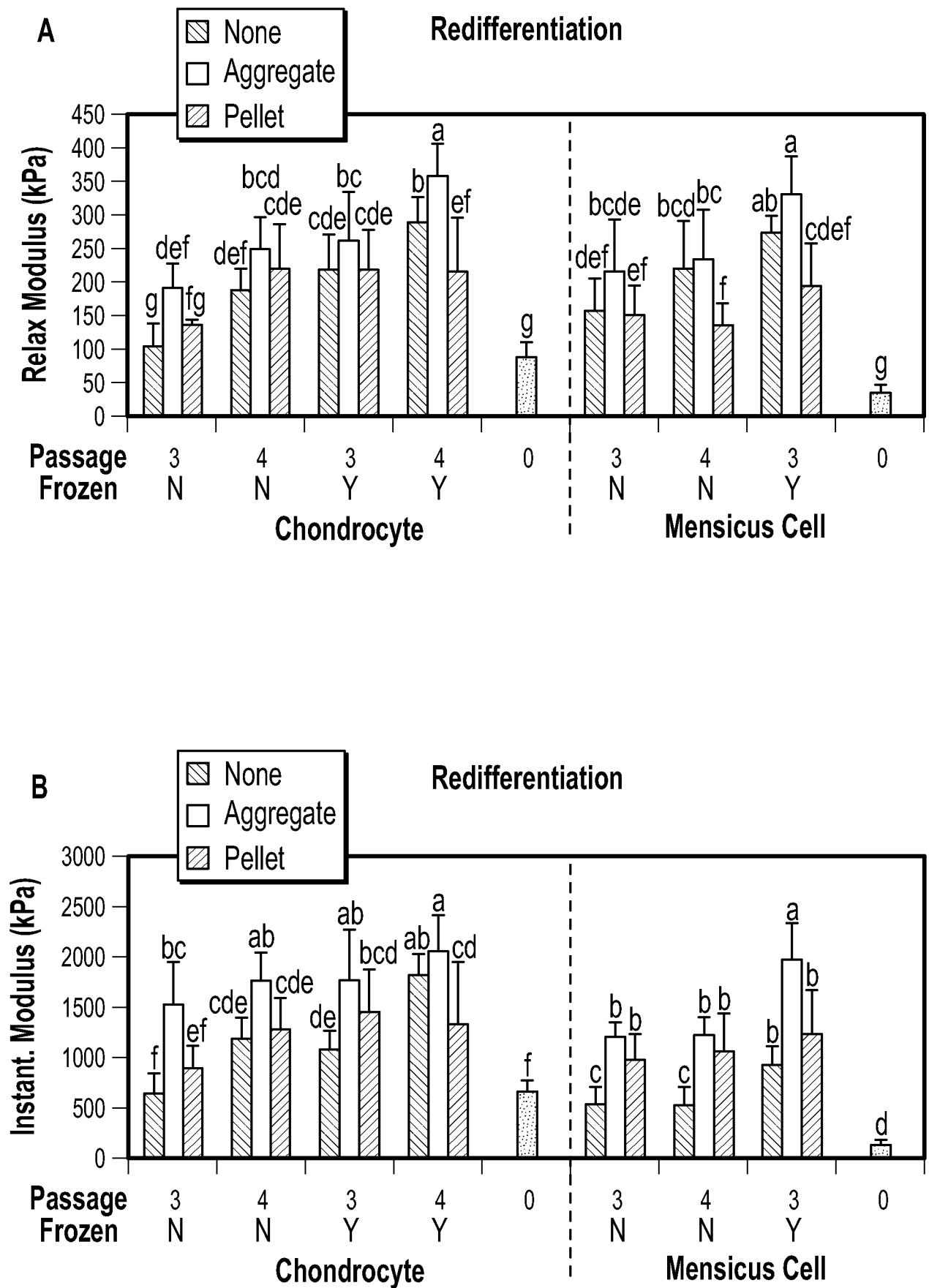


FIG. 8

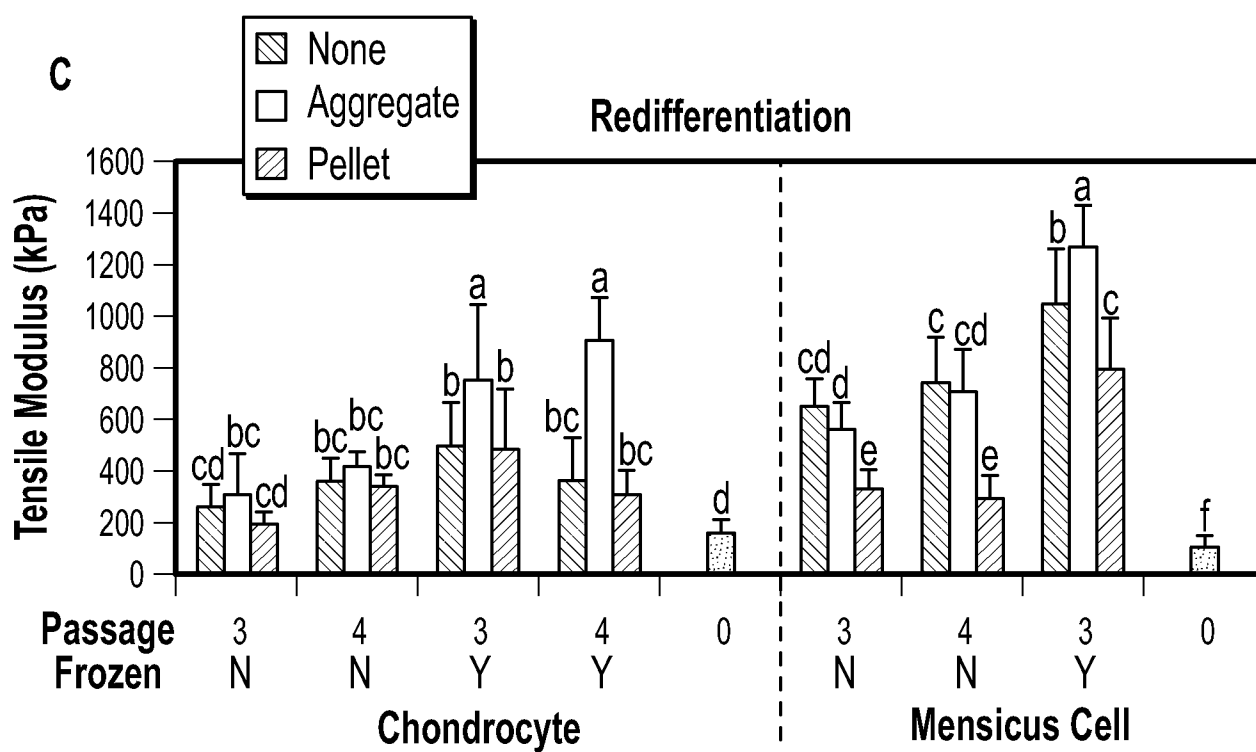
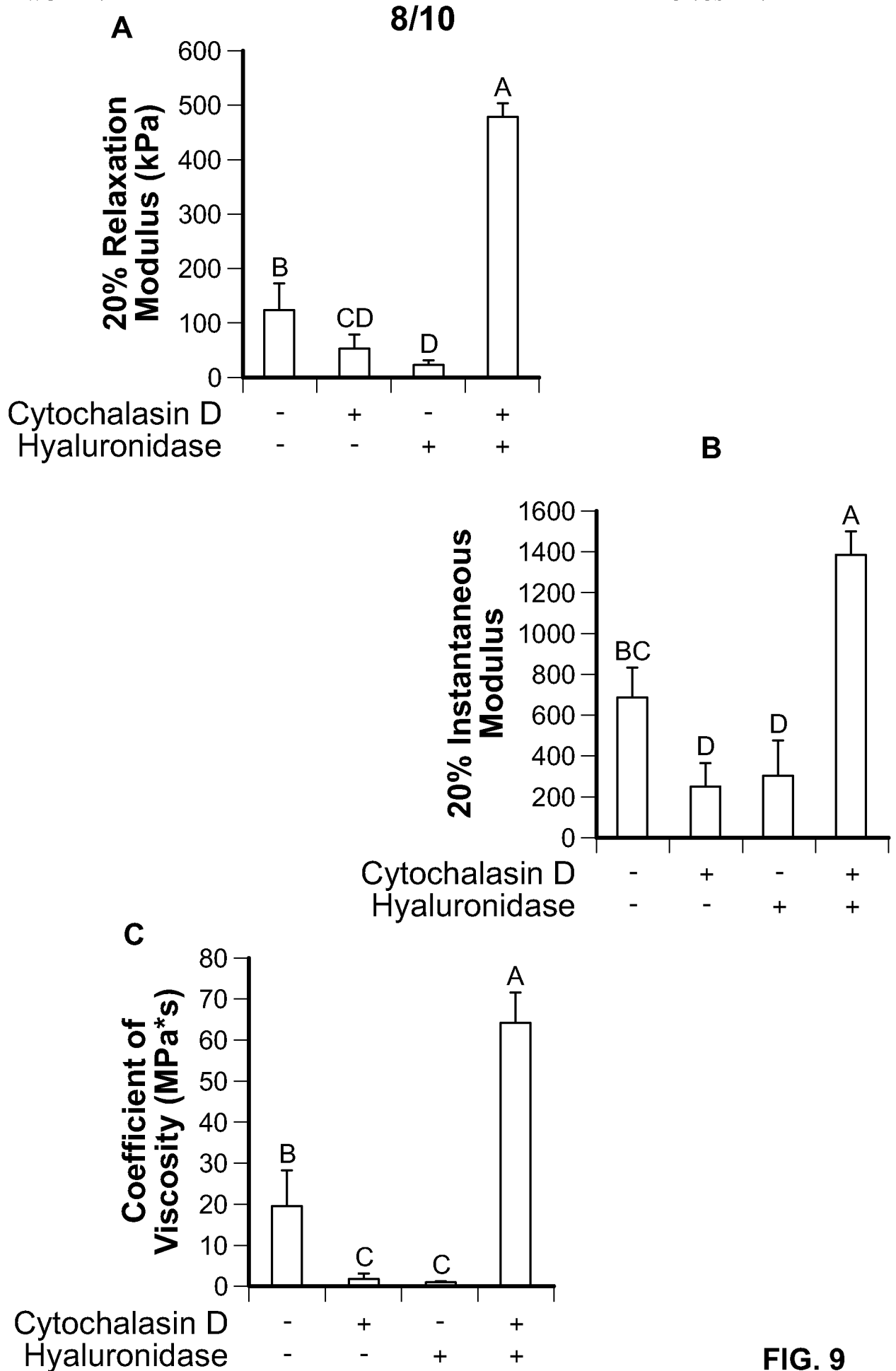
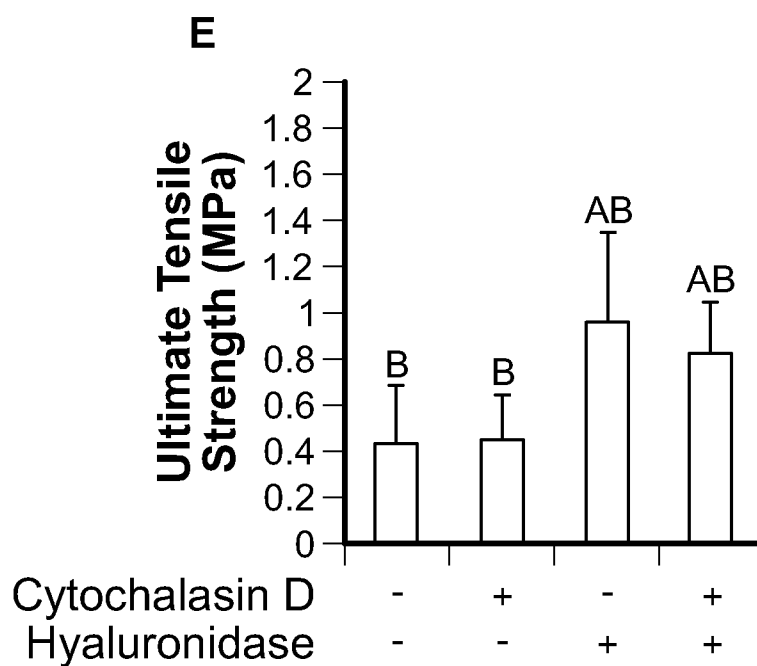
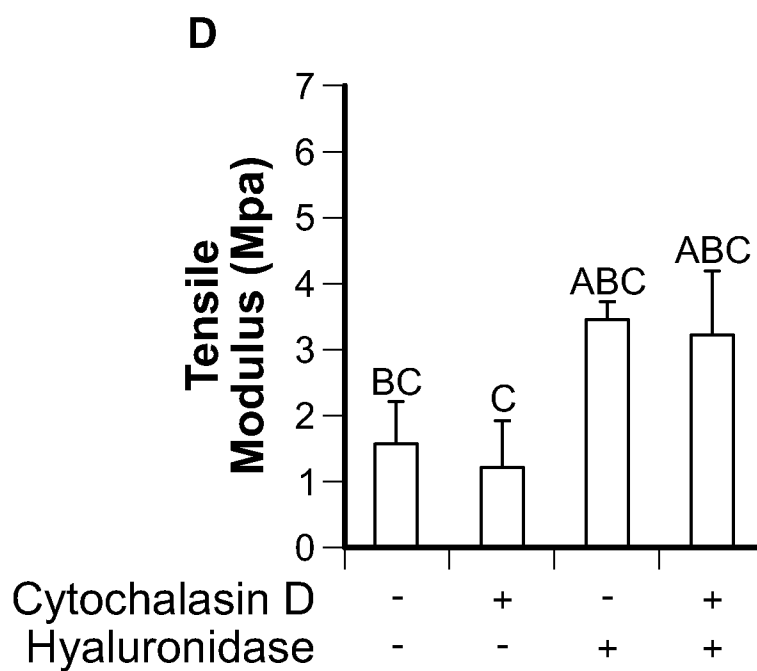


FIG. 8 (Cont.)

**FIG. 9**

**FIG. 9 (Cont.)**

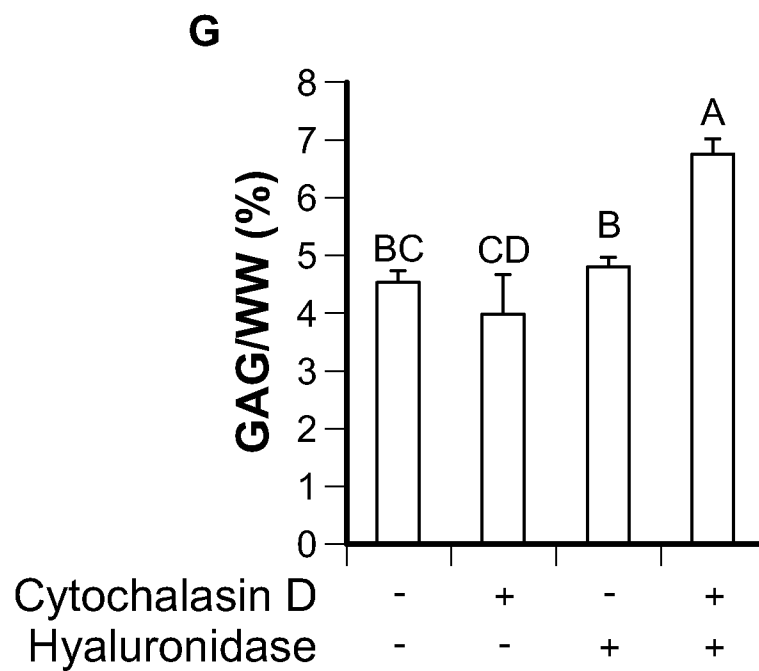
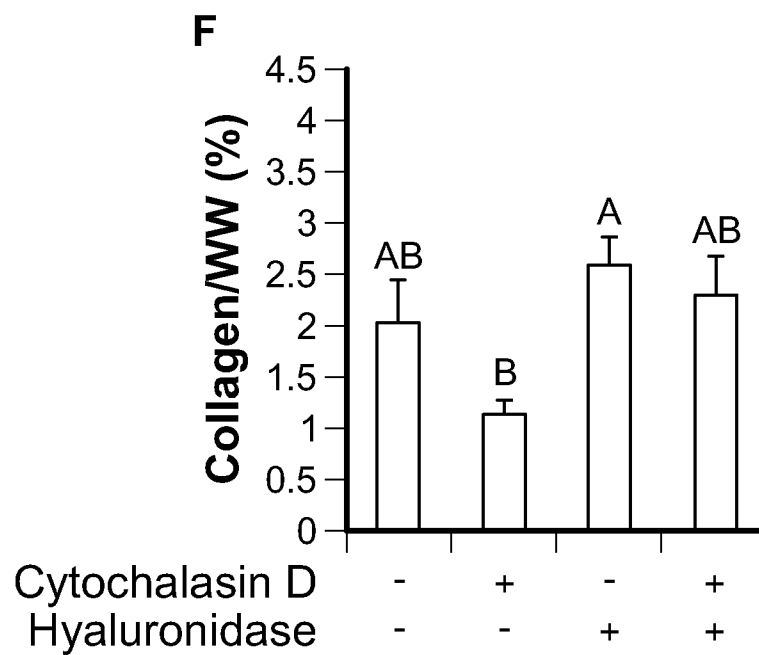


FIG. 9 (Cont.)

A. CLASSIFICATION OF SUBJECT MATTER

C12N 5/077(2010.01)i, C12N 5/07(2010.01)i

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)

C12N 5/077; A61F 2/30; C12N 5/00; C12N 5/02; A61P 19/02; A61K 35/32

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

Korean utility models and applications for utility models

Japanese utility models and applications for utility models

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

eKOMPASS(KIPO internal) & keywords: cartilage, matrix degrading enzyme, cytoskeletal modifying agent, chondrogenic cells, redifferentiation, cytochalasin D

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2011-0053262 A1 (ATHANASIOU, K. A. et al.) 3 March 2011 See paragraphs [0026], [0030] and [0034]; claims 1-3, 8-10 and 15-16.	26,28
Y		25
A		1-24, 27, 29-30
X	ZANETTI, N. C. et al., 'Induction of chondrogenesis in limb mesenchymal cultures by disruption of the actin cytoskeleton', The Journal of Cell Biology, July 1984, Vol. 99, No. 1, pp. 115-123. See abstract; tables 1-2; figures 1-3.	27
Y		25
X	WO 2010-022074 A1 (WILLIAM MARSH RICE UNIVERSITY) 25 February 2010 See abstract; page 5, lines 1-10 and 28-34; claims 1 and 13-14.	26,28
Y		25
A	PIRTTINIEMI, P. et al., 'Effect of cytochalasin D on articular cartilage cell phenotype and shape in long-term organ culture' European Journal of Orthodontics, October 1998, Vol. 20, No. 5, pp. 491-499. See abstract; figures 1-2	1-30
A	US 2010-0303765 A1 (ATHANASIOU, K. A. et al.) 2 December 2010 See abstract; claims 1-4.	1-30



Further documents are listed in the continuation of Box C.



See patent family annex.

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

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

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

"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

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Date of the actual completion of the international search

17 October 2013 (17.10.2013)

Date of mailing of the international search report

17 October 2013 (17.10.2013)

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/US2013/051534

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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WO 2010-022074 A1	25/02/2010	None	
US 2010-0303765 A1	02/12/2010	AT 500318 T AU 2007-234365 A1 AU 2007-234366 A1 AU 2007-254048 A1 CA 2648327 A1 CA 2648648 A1 DE 602005026690 D1 EP 1819290 A2 EP 1819290 A4 EP 1819290 B1 EP 2007312 A2 EP 2007875 A2 EP 2007881 A2 ES 2420304 T3 US 2009-0136559 A1 US 2009-0142307 A1 US 2009-0155333 A1 WO 2006-017176 A2 WO 2006-017176 A3 WO 2007-115336 A2 WO 2007-115336 A3 WO 2007-115337 A2 WO 2007-115337 A3 WO 2007-136936 A2 WO 2007-136936 A3	15/03/2011 11/10/2007 11/10/2007 29/11/2007 11/10/2007 29/11/2007 14/04/2011 22/08/2007 13/08/2008 02/03/2011 31/12/2008 31/12/2008 31/12/2008 23/08/2013 28/05/2009 04/06/2009 18/06/2009 16/02/2006 30/03/2006 11/10/2007 22/05/2008 11/10/2007 03/07/2008 29/11/2007 23/10/2008