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DESCRIPTION

FIELD OF THE INVENTION

[0001] This invention relates to ceramidase and cell differentiation.

BACKGROUND OF THE INVENTION

[0002] Cell-based therapy for cartilage repair has gained increasing popularity since the first reports of successful autologous chondrocyte implantation (ACI) over 10 years ago (Minas et al., "Chondrocyte Implantation in the Repair of Chondral Lesions of the Knee: Economics and Quality of Life," *Am. J. Orthop.* 27(11):739-44 (1998)). In ACI, primary chondrocytes are obtained from small biopsies of healthy articular cartilage, expanded, and then placed onto three-dimensional scaffolds for subsequent use in cartilage repair surgery (see Vavken et al., "Effectiveness of Autologous Chondrocyte Implantation in Cartilage Repair of the Knee: A Systematic Review of Controlled Trials," *Osteoarthritis Cartilage* 18(6):857-63 (2010)). Currently, ACI is used in approximately 10% of all cartilage repair procedures performed worldwide where the lesions are less than 2-4 cm² (Cole et al., "Outcomes After a Single-Stage Procedure for Cell-based Cartilage Repair: A Prospective Clinical Safety Trial With 2-year Follow-up," *Am. J. Sports Med.* 39(6):1170-79 (2011)). ACI has also been used in veterinary medicine to improve the outcome of cartilage repair surgery in large (equine) and small (dog) animals (Breinan et al., "Autologous Chondrocyte Implantation in a Canine Model: Change in Composition of Reparative Tissue With Time," *J. Orthop. Res.* 19(3):482-92 (2001); Frisbie et al., "Evaluation of Autologous Chondrocyte Transplantation Via a Collagen Membrane in Equine Articular Defects: Results at 12 and 18 Months," *Osteoarthritis Cartilage* 16(6):667-79 (2008)). There have been many reports documenting the improved clinical effectiveness of ACI as compared to other cartilage repair procedures, and several large, multi-site clinical studies are currently underway (Ebert et al., "Clinical and Magnetic Resonance Imaging-based Outcomes to 5 Years After Matrix-induced Autologous Chondrocyte Implantation to Address Articular Cartilage Defects in the Knee," *Am. J. Sports Med.* 39(4):753-63 (2011)). An important limitation of this procedure, however, is the requirement of two invasive surgeries, the first of which requires extraction of cells from healthy cartilage tissue, and the second to implant the cells that have been expanded *ex vivo*. Recent research has therefore focused on the use of alternative chondrocyte sources where the cells can be obtained less invasively (e.g., nasoseptal (Bichara et al., "Porouspoly(Vinyl Alcohol)-alginate Gel Hybrid Construct for Neocartilage Formation Using Human Nasoseptal Cells," *J. Surg. Res.* 163(2):331-6 (2010))), the generation of chondrocytes from adult stem cells (e.g., mesenchymal stem cells (MSCs) from the bone marrow or adipose tissue), and/or the use of MSCs directly for transplantation (Augello et al., "Mesenchymal Stem Cells: A Perspective From In Vitro Cultures to In Vivo Migration and Niches," *Eur. Cell Mater.* 20:121-33 (2010); Chanda et al., "Therapeutic Potential of Adult Bone Marrow-derived Mesenchymal Stem Cells in Diseases of the Skeleton," *J. Cell*

Biochem. 111(2):249-57 (2010); Hildner et al., "State of the Art and Future Perspectives of Articular Cartilage Regeneration: A Focus on Adipose-derived Stem Cells and Platelet-derived Products," J. Tissue Eng. Regen. Med. 5(4):e36-51 (2011)).

[0003] A key factor in the development of any cell-based therapy is to find safe and effective methods to rapidly expand autologous cells in a manner that retains their phenotype and *in vivo* repair potential. For ACI, research has concentrated on defining the culture media and growth factors used for articular chondrocyte expansion, as well as the improved design and formulation of scaffolds used to adhere the cells and prepare them for surgical re-implantation. Currently, most culture medias used to expand primary articular chondrocytes contain serum supplemented with growth factors, including members of the transforming growth factor (TGF) (β 1, β 2, and β 3) and bone morphogenic families (BMP) (2,4,6,12,13), insulin growth factor 1 (IGF1), fibroblast growth factor 2 (FGF2) and others (see Umlauf et al., "Cartilage Biology, Pathology, and Repair," Cell Mol. Life Sci. 67(24):4197-211 (2010)). Similarly, numerous transcription factors influence chondrogenesis, including Sox9, β -catenin, Smads, and others, resulting in optimal expression of chondrocyte-specific markers. Sox9 in particular is required for pre-cartilage condensation and differentiation of chondroprogenitor cells into chondroblasts (Lee et al., "Sox9 Function in Craniofacial Development and Disease," Genesis 49(4):200-8 (2011)).

[0004] Evaluation of chondrocyte quality from these various cell culture procedures generally relies on documenting the expression of chondrocyte-specific markers, including various collagens (e.g., I and II), extracellular matrix components (e.g., aggrecan), and growth and transcription factors known to influence chondrogenesis. Unfortunately, although many different culture systems have been used to evaluate chondrogenesis *in vitro*, no consensus method exists and current procedures are not very effective at maintaining the chondrogenic phenotype during the expansion period.

[0005] Similarly, many different methods have been used to expand and differentiate MSCs into chondrocytes. Since MSCs represent a very small fraction of the total bone marrow (BM) cell population, they must be enriched by techniques such as flow cytometry, or expanded in culture to obtain enough cells for transplantation. These procedures increase the risk of transformation and/or contamination of the stem cell population. In addition, following the initial expansion they must undergo an *in vitro* differentiation period of several additional weeks, and the chondrogenic potential of these "induced" chondrocytes remains in question (Dashtdar et al., "Preliminary Study Comparing the Use of Allogenic Chondrogenic Pre-differentiated and Undifferentiated Mesenchymal Stem Cells for the Repair of Full Thickness Articular Cartilage Defects in Rabbits," J. Orthop. Res. 29(9): 1336-42 (2011)).

[0006] The present invention is directed to overcoming these and other deficiencies in the art.

SUMMARY OF THE INVENTION

[0007] A first aspect of the present invention relates to an *in vitro* method of producing chondrocytes. This method involves selecting a population of mammalian cells having the potential to differentiate into chondrocytes selected from bone marrow cells, fibroblasts, and mesenchymal stem cells, and treating the selected cell population with a ceramidase protein *in vitro* to transform one or more of the cells in the selected population into chondrocytes.

[0008] A second aspect of the present invention relates to an *in vitro* method of improving the phenotype of a chondrocyte population. This method includes selecting a population of mammalian chondrocytes having a poor chondrocytic phenotype characterized by low expression of collagen 2A1, Sox9, and aggrecan; elevated expression of collagen 10; and/or low staining for proteoglycans with Alcian Blue or Safranin O, and treating the selected cell population with a ceramidase protein *in vitro*, wherein, after said treating, the selected population is characterized by elevated expression of collagen 2A1, Sox9, and aggrecan; reduced expression of collagen 10; and/or enhanced staining for proteoglycans with Alcian Blue or Safranin O.

[0009] A method of promoting chondrogenesis is also disclosed. This method includes selecting a population of bone marrow cells in need of differentiation into chondrocytes, first treating the population of bone marrow cells with a ceramidase to enrich mesenchymal stem cells within the bone marrow cell population, and second treating the population of enriched mesenchymal stem cells with a ceramidase, or a ceramidase and TGF β , to promote differentiation of mesenchymal stem cells into chondrocytes.

[0010] A method of maintaining a cell population in a differentiated state or increasing the number of cells of a population in a differentiated state is also disclosed. This method includes selecting a cell population in need of being maintained in a differentiated state or a cell population in need of an increased number of cells in a differentiated state, and treating the selected cell population with a ceramidase to maintain or increase the number of differentiated cells in the selected cell population.

[0011] A fifth aspect of the present invention relates to an *in vitro* method of producing a population of chondrocyte cells. This method includes selecting a population of stem cells capable of differentiating into chondrocyte cells, selecting a differentiation medium capable of stimulating differentiation into the desired differentiated cells, and *in vitro* culturing the population of stem cells in the differentiation medium and ceramidase protein to stimulate differentiation into the population of chondrocyte cells.

[0012] Research has been focused on enzymes involved in the metabolism of sphingolipids, a group of over 100 lipids that share a common sphingoid base backbone (sphingosine) and have important effects on cell survival and differentiation (e.g., Nikolova-Karakashian et al., "Sphingolipid Metabolism, Oxidant Signaling, and Contractile Function of Skeletal Muscle," *Antioxid. Redox Signal* 15(9):2501-17 (2011)). For example, ceramide is a potent pro-apoptotic lipid that disrupts and reorganizes membrane-embedded signaling platforms, while sphingosine-1-phosphate (S1P) is a proliferative lipid that can be secreted from cells and has

potent mitogenic activities. An intermediate in the conversion of ceramide to SIP is sphingosine itself, another important signaling lipid. Ceramidases are a class of enzymes that hydrolyze ceramide into sphingosine, which is subsequently converted into SIP (Mao et al., "Ceramidases: Regulators of Cellular Responses Mediated by Ceramide, Sphingosine, and Sphingosine-1-phosphate," *Biochem. Biophys. Acta* 1781(9):424-34 (2008)). Importantly, the only way that cells can generate sphingosine is by ceramide hydrolysis via ceramidases (Okino et al., "The Reverse Activity of Human Acid Ceramidase," *J. Biol. Chem.* 278(32):29948-53 (2003)). Thus, these enzymes play critical roles in regulating these lipid signaling pathways.

[0013] Acid ceramidase (AC, EC# 3.5.1.23) is one of five known mammalian ceramidases, each of which has been defined by their unique pH optima and/or intracellular locations. AC has historically been the most extensively studied ceramidase due to its involvement in the human genetic disorder that results from its deficiency, Farber Lipogranulomatosis (FD, MIM #228000). FD is an extremely rare disorder, and surviving patients often present with juvenile arthritis and degenerating joint disease associated with the accumulation of inflammatory nodules. Tracheal abnormalities also have been described, suggesting an important role for AC in hyaline cartilage development.

[0014] Several other studies have demonstrated the importance of sphingolipid signaling on cartilage homeostasis. For example, an early report (Sabatini et al., "Effects of Ceramide on Apoptosis, Proteoglycan Degradation, and Matrix Metalloproteinase Expression in Rabbit Articular Cartilage," *Biochem. Biophys. Res. Commun.* 267(1):438-444 (2000)) showed that a synthetic ceramide derivative (C2 ceramide) stimulated the expression of MMP-1, 3, and 13 in rabbit articular chondrocytes, and induced chondrocyte apoptosis. Gilbert et al. also showed that treatment of bovine articular chondrocytes with sphingomyelinase, an enzyme that produces ceramide by sphingomyelin hydrolysis, decreased expression of collagen II (Gilbert et al., "Sphingomyelinase Decreases Type II Collagen Expression in Bovine Articular Cartilage Chondrocytes Via the ERK Signaling Pathway," *Arthritis Rheum.* 58(1):209-220 (2008)). Elevated ceramide has also been documented in patients with rheumatoid and osteoarthritis (Ciurtin et al., "Correlation Between Different Components of Synovial Fluid and Pathogenesis of Rheumatic Diseases," *Rom. J. Intern. Med.* 44(2): 171-81 (2006)), and inhibition of SIP production in induced rodent models of arthritis has led to beneficial clinical results (Fitzpatrick et al., "Attenuation of Arthritis in Rodents by a Novel Orally-available Inhibitor of Sphingosine Kinase," *Inflammopharmacology* 19(2):75-87 (2011)).

[0015] In addition, it has been found that animals with genetic deficiencies of enzymes involved in glycosaminoglycan (GAG) degradation (i.e., the mucopolysaccharidoses, MPS), have numerous abnormalities in sphingolipid metabolism in their connective tissues. For example, chondrocyte apoptosis and cartilage degradation in the MPS animals is associated with elevated ceramide, while synovial hyperplasia is associated with elevated SIP (Simonaro et al., "Mechanism of Glycosaminoglycan-mediated Joint and Bone Disease: Implications for the Mucopolysaccharidoses and Other Connective Tissue Diseases," *Am. J. Path.* 172:112-122 (2008)). Elevated AC activity can also be detected in serum and synovial fluid from the MPS animals, likely a response to the elevated ceramide.

[0016] To further study the role of AC in cell signaling and disease, a complete AC knockout mouse was constructed several years ago and it was found that embryos lacking AC could not survive beyond the 4-cell stage and underwent apoptosis (Eliyahu et al., "Acid Ceramidase is a Novel Factor Required for Early Embryo Survival," *FASEB J.* 21(7): 1403-9 (2007)). This led to evaluation of the effects of recombinant AC (rAC) on oocyte and embryo survival *in vitro*, revealing that supplementation of media with this enzyme could slow apoptosis and increase cell and/or embryo survival (Eliyahu et al., "Acid Ceramidase Improves the Quality of Oocytes and Embryos and the Outcome of In Vitro Fertilization," *FASEB J.* 24(4):1229-38 (2010)). Subsequent studies have evaluated the anti-apoptosis effects of rAC on other primary cells, including hippocampal neurons treated with the A β protein (He et al., "Deregulation of Sphingolipid Metabolism in Alzheimer's Disease," *Neurobiol. Aging* 31(3):398-408 (2010)) and mouse embryonic stem cells.

[0017] Ceramidases regulate the metabolism of several important bioactive lipids, including ceramide, sphingosine, and sphingosine-1-phosphate. The present invention shows that addition of recombinant rAC as a media supplement markedly improved the chondrogenic phenotype of expanded rat and horse articular chondrocytes, as well as human chondrocytes obtained from aged patients with osteoarthritis. The chondrocyte phenotype was assessed by a combination of histochemical staining (Alcian Blue and Safranin O), western blotting (e.g., Sox9, aggrecan, collagen IIA1, TGF β 1, FGF2, etc.), and/or qPCR. The effects of rAC were evident in monolayer and three-dimensional cultures, including cells grown on collagen scaffolds and fibrin gels. rAC also reduced the number of apoptotic cells in some culture conditions, contributing to overall improved cell quality. It is demonstrated herein that rat, equine, and human articular chondrocytes treated once with rAC in either monolayer cultures or three-dimensional scaffold systems had an improved chondrogenic phenotype after 2-3 week cell expansion, including enhanced expression of Sox9, FGF2, collagen II, aggrecan and other chondrogenic markers.

[0018] In addition to these effects on primary chondrocytes, rAC had a significant impact on bone marrow-derived mesenchymal stem cells (MSCs). Here, it is shown that rat, feline, and equine bone marrow cells grown for only one week in the presence of rAC yield about two-fold more MSCs, and that rAC led to enhanced chondrogenesis of MSCs. When rAC was added to freshly harvested bone marrow cultures, an approximate two-fold enrichment of MSCs was observed by one-week. rAC also improved the chondrogenic differentiation of MSCs, as revealed by histochemical and immunostaining. These effects were observed using both rat and horse cells, and were synergistic with TGF β 1. These results demonstrate that rAC is an important supplement that could be used to improve the quality of cells used for cell-based cartilage repair, and can be useful *in vivo* to induce endogenous cartilage repair in combination with other techniques. Thus, rAC can be included in media used to culture primary chondrocytes and/or MSC chondrocyte differentiation media to improve the production and chondrogenic potential of these cells for cartilage repair. rAC also may added to three-dimensional scaffolds to induce and/or enhance chondrogenesis of cells *in vivo*. It is expected that other ceramidases may also be used for these purposes.

BRIEF DESCRIPTION OF THE DRAWINGS

[0019]

Figures 1A-1C are graphs illustrating the effect of rAC on AC (Figure 1A), ceramide (Figure 1B), and sphingosine (Figure 1C) activity of primary rat articular chondrocytes. Data were collected at 12 hours, 24 hours, and 48 hours. Samples with rAC (+rAC) are indicated with gray bars and samples without rAC (-rAC) are indicated with black bars. Primary rat articular chondrocytes were isolated and grown in monolayer cultures for 48 hours with or without rAC (rAC, 200 U/ml) added to the media. At the indicated times, proteins and lipids were extracted from the cells, and the AC activity (Figure 1A), ceramide (Figure 1B), and sphingosine (Figure 1C) levels were determined. The addition of rAC to the culture media led to markedly increased AC activity that was sustained for at least 48 hours. In addition, by 12 hours ceramide levels were elevated, but significantly less in cells treated with rAC (i.e., by 48 hours cells treated with rAC had no detectable ceramide elevation). Finally, the levels of sphingosine, the product of ceramide hydrolysis by AC, was elevated in rAC-treated cells by 48 hours.

Figures 2A-2C show chondrogenic marker expression in rat articular chondrocytes. Figure 2A illustrates total cell counts for cultures of rat articular chondrocytes with and without recombinant human AC (rAC) (left panel), and western blot results for Bax, PARP, and GAPDH in cultures of rat articular chondrocytes with and without rAC (right panel). Rat articular chondrocytes were obtained from femurs and grown for three weeks in monolayer cultures using standard culture medium with or without recombinant human AC (rAC 200 U/ml). rAC was added once at the initiation of the cultures. At the end of the three-week expansion period, the cells were harvested and analyzed. Total cell counts revealed no significant differences in the presence of rAC. Western blotting for two apoptosis markers (Bax and PARP) similarly revealed no differences. Figure 2B shows western blots of expanded chondrocytes for chondrogenic markers. The expanded chondrocytes were analyzed by western blot for several important chondrogenic markers, including collagens 1A2 and 2A1, aggrecan, Sox9, FGF-2, and TGF β 1. In all cases, these chondrocyte markers were elevated in the cells treated with rAC. Collagen X is expressed at high levels in fibroblasts, but not in chondrocytes (i.e., a "reverse" marker of chondrogenesis). In contrast to collagens 2A1 and 1A2, collagen X expression was not significantly altered by rAC treatment. Figure 2C shows western blots of horse articular chondrocytes for chondrogenic markers including Aggrecan, Sox9, Collagen II, Bax, and Actin, with and without rAC. Horse articular chondrocytes were obtained surgically from femoral heads and frozen. The frozen cells were then recovered and grown in monolayer cultures for three weeks without rAC. At three weeks, the cells were passaged and re-plated at a density of 1×10^6 , and then grown for an additional one week with or without rAC. At the end of this one-week growth period (P1), the cells were analyzed by western blot, revealing that the expression of two chondrogenic markers, aggrecan and Sox9, were highly elevated in the rAC-treated horse cells. Bax expression also was reduced in the rAC cells, suggesting a reduction in apoptosis by rAC treatment. All experiments were repeated at least three times. Images are

representative from individual experiments.

Figures 3A-3B relate to the expression of collagen 2A1 in rat chondrocytes, with and without rAC. Figure 3A shows confocal immunohistochemistry of collagen 2A1 in chondrocytes with and without rAC (left panel), as well as a graphical representation of collagen 2A1 percentage in chondrocytes with and without rAC (right panel), confirming the high level of expression of collagen 2A1 in rat chondrocytes treated with rAC. Figure 3B shows confocal immunostaining of Sox9 with and without rAC treatment, showing different intracellular distribution of Sox9 two weeks after AC treatment. Blue (DAPI) indicates nuclei, and red indicates collagen 2A1 and Sox9, respectively. Experiments were repeated at least three times. Images are representative from individual experiments. Scale bars: 50µm. * = $p < 0.05$.

Figures 4A-4B relate to chondrogenic markers in primary human chondrocytes in two patients with osteoarthritis. Figure 4A is a graph comparing various chondrogenic markers (collagen 2A1 (Col2a1), TGFβ, Sox9, aggrecan (ACAN), and collagen X (Col10a1)) in patient 1, with and without rAC treatment (left). Primary human chondrocytes obtained from the femoral head of a 74-year-old woman with osteoarthritis (OA) were expanded for three weeks in monolayer culture with or without rAC added to the culture media (DMEM + 10% FBS, rAC added once at the initiation of the culture; 170 U/mlAC). mRNA expression of chondrogenic markers Col2a1, TGFβ1, Sox9, and ACAN were analyzed by RT-PCR. Note the positive influence of rAC on the expression of these markers. The expression of Col10a1 was unchanged in these cultures. The same experiment was repeated on a second set of cells from a patient with osteoarthritis (Patient 2). Figure 4B depicts a graph comparing those same chondrogenic markers in patient 2, with and without rAC. RT-PCR analysis was performed three times on cells grown in individual wells. Consistent with the results obtained with rat and equine chondrocytes (Figures 2A-2C), no significant differences in the number of cells could be found (right panels). * = $p < 0.05$.

Figures 5A-5C relate to chondrocytes grown on three-dimensional collagen scaffolds. Figure 5A shows images of a three-dimensional collagen scaffold on which primary rat chondrocytes were grown. Primary rat chondrocytes were seeded into three-dimensional collagen scaffolds and grown for 7 or 14 days with or without rAC (DMEM containing 10% serum). They were then analyzed for morphology and proteoglycan production by Safranin O staining. The cells grown with rAC were larger and maintained a round phenotype that stained positive with Safranin O (arrows). Shown are representative images from experiments performed three times. Figure 5B depicts a graphical representation comparing cell shrinkage based on Safranin O and H&E staining at 7 and 14 days, both with and without rAC. The cell shrinkage was evaluated based on Safranin O and H&E staining using the following equation: DNA unit size = $\pi (\text{fiber segment diameter}/2)^2 \times (\text{myofiber segment length})/\text{myofiber nuclei}$. * = $p < 0.05$. Figure 5C shows primary rat chondrocytes grown in biodegradable fibrin gels with and without rAC. Primary rat chondrocytes were grown for 2 weeks in biodegradable fibrin gels with or without rAC in the culture media. Alcian Blue staining, another marker of proteoglycan expression, indicated enhanced chondrogenesis (more intense blue color). Experiment was performed two times. * = $p < 0.05$.

Figures 6A-D relate to mesenchymal stem cells (MSCs) cultured in the presence and absence of rAC. In Figure 6A, the number of colony forming units in rat bone marrow, both with and without rAC, are shown. Rat bone marrow cells were isolated and grown for one week with or without rAC in standard culture media. rAC was added to the culture media once at the initial plating (5×10^6 cells/cm²). The number of MSCs in the cultures at one week was determined by two assays: the number of fibroblast-like colony forming units (CFU-F) and by flow cytometry. In Figure 6B, flow cytometry (CD90+/CD45-) in MSCs of rat bone marrow, with rAC and without rAC are shown. An approximate two-three fold increase in the number of rat MSCs was observed using rAC. Figure 6C shows the number of colony forming units in feline cells (plated at a density of $1 - 5 \times 10^6$ cells/cm²), with rAC and without rAC, while Figure 6D shows the number of colony forming units in equine cells (plated at 3×10^8 cells/cm²), with and without rAC. * = $p < 0.05$, ** = $p < 0.005$.

Figures 7A-D relate to isolated rat bone marrow cells used to prepare homogeneous populations of MSCs that were placed into chondrocyte differentiation media, with or without rAC. In Figure 7A, results of rat bone marrow cells in culture media, either with or without rAC, and with or without TGF β 1, are shown (left panel). Figure 7A also illustrates a graphical comparison of the pellet area in the presence or absence of rAC with or without TGF β 1 (right panel). Rat bone marrow cells were isolated and expanded for three weeks in standard culture media (without rAC) to prepare homogenous populations of MSCs. They were then placed into chondrocyte differentiation media (Stem Cell Technology) with or without TGF β 1 and/or rAC. Pellet cultures were grown for three weeks to prepare chondrocytes, and then fixed and analyzed by Alcian Blue and Safranin O staining, markers of chondrogenesis. Small and poorly formed pellets in the absence of TGF β 1 and rAC are evident (upper left). TGF β 1 is a standard supplement used to induce the differentiation of bone marrow MSCs to chondrocytes. Addition of TGF β 1 or rAC to the cultures independently had only a modest effect on the pellet size and staining (upper right and lower left). However, inclusion of both proteins in the culture media had a much more significant effect (lower right), both on the size of the pellets and staining intensity. Pellet size is an indicator of the number of chondrocytes, and staining intensity is a measure of proteoglycan deposition. Figures 7B-D show immunostaining of rat bone marrow cells against Sox9, aggrecan, and collagen 2A1, respectively. Experiments were performed with three independent rats. Representative images are shown from one experiment. Blue (DAPI) indicates nuclei, and red indicates Sox9, Aggrecan, or Collagen IIA1, respectively. Merged images are to the right. Scale bars: 50 μ m. * = $p < 0.05$, ** = $p < 0.005$, *** = $p < 0.001$.

Figures 8A-E show results of isolated horse bone marrow cells used to prepare homogeneous populations of MSCs that were placed into chondrocyte differentiation media, with or without rAC. In Figure 8A, horse bone marrow cells were isolated and expanded for three weeks in standard culture media (without rAC) to prepare homogenous populations of MSCs. They were then placed into chondrocyte differentiation media containing TGF β 1, but with or without rAC. Pellet cultures were grown for three weeks to prepare chondrocytes, and then fixed and analyzed by Alcian Blue and Safranin O staining. Smaller, more diffuse pellets in the absence of rAC are evident. Pellets were also submitted to immunostaining against Sox9 (Figure 8B), Aggrecan (Figure 8C), or Collagen IIA1 (Figure 8D). The higher expression intensity of Sox9,

Aggrecan, and Collagen IIA1 in the pellets is notable. In Figure 8E, pellets were also subjected to an immunostaining against Collagen X. Diminished expression of Collagen X in pellets exposed to rAC can be seen. Blue (DAPI) indicates nuclei, and red indicates Sox9, Aggrecan, Collagen IIA1, or Collagen X, respectively. Merged images are to the right. Scale bars: 50µm. *=p<0.05, **= p<0.005, ***= p<0.001.

DETAILED DESCRIPTION OF THE INVENTION

[0020] A first aspect of the present invention relates to an *in vitro* method of producing chondrocytes. This method involves selecting a population of mammalian cells having the potential to differentiate into chondrocytes selected from bone marrow cells, fibroblasts, and mesenchymal stem cells, and treating the selected cell population with a ceramidase protein *in vitro* to transform one or more of the cells in the selected population into chondrocytes.

[0021] Cells having the potential to differentiate into chondrocytes include bone marrow cells, fibroblasts, and mesenchymal stem cells (see Mizushima et al., "Ceramide, a Mediator of Interleukin 1, Tumour Necrosis Factor α , as Well as Fas Receptor Signalling, Induces Apoptosis of Rheumatoid Arthritis Synovial Cells," Ann. Rheum. Dis. 57:495-9 (1998)).

[0022] Suitable cells according to this and all other aspects of the present invention include mammalian cells, *e.g.*, human cells, equine cells, porcine cells, feline cells, and/or canine cells. Human cells are particularly preferred.

[0023] In this and all aspects of the present invention involving cell populations, embodiments in which the cells are all of one type, as well as embodiments in which the population is a mixture of two or more cell types, are both contemplated.

[0024] Ceramidases hydrolyze the amide linkage of ceramides to generate free fatty acids and sphingoid bases (Nikolova-Karakashian et al., Methods Enzymol. 311:194-201 (2000), Hassler et al., Adv. Lipid Res. 26:49-57 (1993)). There are three types of ceramidases described to date (Nikolova-Karakashian et al., Methods Enzymol. 311:194-201 (2000)). These are classified as acid, neutral, and alkaline ceramidases according to their pH optimum of enzymatic activity.

[0025] Acid ceramidases have optimal enzymatic activity at a pH of 1-5. The murine acid ceramidase was the first ceramidase to be cloned (Koch et al., J. Biol. Chem. 271:33110-33115 (1996)). It is localized in the lysosome and is mainly responsible for the catabolism of ceramide. Dysfunction of this enzyme because of a genetic defect leads to a sphingolipidosis disease called Farber disease (Koch et al., J. Biol. Chem. 271:33110-33115 (1996)).

[0026] The neutral ceramidases have been purified from rat brain (El Bawab et al., J. Biol.

Chem 274:27948-27955 (1999)) and mouse liver (Tani et al., J. Biol. Chem 275:3462-3468 (2000)), and were cloned from *Pseudomonas* (Okino et al., J. Biol. Chem. 274:36616-36622 (1999)), mycobacterium (Okino et al., J. Biol. Chem. 274:36616-36622 (1999)), mouse (Tani et al., J. Biol. Chem 275:11229-11234 (2000)), and human (El Bawab et al., J. Biol. Chem. 275:21508-21513 (2000)). These ceramidases share significant homology, and this homology extends to putative proteins deduced from expressed sequence tag (EST) sequences of *Dictyostelium discoideum* and *Arabidopsis thaliana* (Okino et al., J. Biol. Chem. 274:36616-36622 (1999), El Bawab et al., J. Biol. Chem. 275:21508-21513 (2000)). These ceramidases have a broad pH optimum ranging from 5 to 9 for their activity (Tani et al., J. Biol. Chem 275:11229-11234 (2000), El Bawab et al., J. Biol. Chem. 275:21508-21513 (2000)). They appear to hydrolyze unsaturated ceramide preferentially, saturated ceramide (dihydroceramide) slightly, and hardly hydrolyze phytoceramide (Tani et al., J. Biol. Chem 275:11229-11234 (2000)). The *Pseudomonas*, mouse, and human neutral ceramidases have a reverse ceramidase activity of catalyzing the formation of ceramide from sphingosine and a fatty acid (Okino et al., J. Biol. Chem. 274:36616-36622 (1999), Tani et al., J. Biol. Chem 275:11229-11234 (2000), Kita et al., Biochim. Biophys. Acta 1485:111-120 (2000)). El Bawab et al. (El Bawab et al., J. Biol. Chem. 275:21508-21513 (2000)) have shown previously that the human neutral ceramidase is localized in the mitochondria.

[0027] Alkaline ceramidases have optimal activity at a pH of 9-14. Two alkaline ceramidases were purified from Guinea pig skin epidermis. These two enzymes were membrane bound, and their estimated molecular masses on SDS-PAGE were 60 and 148 kDa, respectively (Yada et al., "Purification and Biochemical Characterization of Membrane-Bound Epidermal Ceramidases from Guinea Pig Skin," J. Biol. Chem. 270:12677-12684 (1995)). No other studies followed on these two proteins. Two yeast (*S. cerevisiae*) alkaline ceramidases, phytoceramidase (YPC1p) and dihydroceramidase (YDC1p), were also cloned and partially characterized (Mao et al., "Cloning of an Alkaline Ceramidase from *Saccharomyces Cerevisiae*. An Enzyme with Reverse (CoA-independent) Ceramide Synthase Activity," J. Biol. Chem. 275:6876-6884 (2000); Mao et al., "Cloning and Characterization of a *Saccharomyces Cerevisiae* Alkaline Ceramidase with Specificity for Dihydroceramide," J. Biol. Chem. 275:31369-31378 (2000)). YPC1p was cloned as a high copy suppressor of the growth inhibition of FB1 as it has fumonisin resistant ceramide synthase activity. The second alkaline ceramidase, YDC1p was identified by sequence homology to YPC1p. A database search reveals that YPC1p and YDC1p are not homologous to any proteins with known functions, but are homologous to putative proteins from *Arabidopsis*, *C. elegans*, peptides deduced from EST sequences of human, mouse, pig, zebra fish, and human genomic sequences. A human homologue has been identified and its cDNA has been cloned. Preliminary results show that this human homologue is also an alkaline ceramidase that selectively hydrolyzes phytoCer.

[0028] Ceramidases suitable for use in this and all aspects of the present invention include acid ceramidase (AC), neutral ceramidase, alkali ceramidase, and other ceramidases. In all aspects of the present invention, the ceramidase can be homologous (*i.e.*, derived from the same species) or heterologous (*i.e.*, derived from a different species) to the tissue, cells, and/or subject being treated. Ceramidase (*e.g.*, AC) precursor proteins undergo

autoproteolytic cleavage into the active form (composed of α - and β -subunits). This is promoted by the intracellular environment, and, based on highly conserved sequences at the cleavage site of ceramidase precursor proteins across species, is expected to occur in most, if not all, cell types. Thus, ceramidase as used herein includes both active ceramidases and ceramidase precursor proteins, which are then converted into active ceramidase protein. Embodiments in which the precursor protein is taken up by the cell of interest and converted into active ceramidase thereby, as well as embodiments in which the precursor protein is converted into active ceramidase by a different cell or agent (present, for example, in a culture medium), are both contemplated.

[0029] Preferably, the ceramidase is an AC (*N*-acylsphingosine deacylase, I.U.B.M.B. Enzyme No. EC 3.5.1.23). AC protein has been purified from several sources, and the human and mouse cDNAs and genes have been obtained. See Bernardo et al., "Purification, Characterization, and Biosynthesis of Human Acid Ceramidase," J. Biol. Chem. 270:11098-102 (1995); Koch et al., "Molecular Cloning and Characterization of a Full-length Complementary DNA Encoding Human Acid Ceramidase. Identification of the First Molecular Lesion Causing Farber Disease," J. Biol. Chem. 271:33110-5 (1996); Li et al., "Cloning and Characterization of the Full-length cDNA and Genomic Sequences Encoding Murine Acid Ceramidase," Genomics 50:267-74 (1998); Li et al., "The Human Acid Ceramidase Gene (ASAH): Chromosomal Location, Mutation Analysis, and Expression," Genomics 62:223-31 (1999). It is produced through cleavage of the AC precursor protein (see Ferlinz et al., "Human Acid Ceramidase: Processing, Glycosylation, and Lysosomal Targeting," J. Biol. Chem. 276(38):35352-60 (2001)), which is the product of the *Asah1* gene (NCBI UniGene GeneID No. 427). ACs and AC precursor proteins that can be used in this and all aspects of the present invention include, without limitation, those set forth in Table 1 below.

Table 1. Exemplary Acid Ceramidase Family Members

<i>Homo sapiens</i>		<i>Caenorhabditis elegans</i>	
UniProt	Q13510, Q9H715, Q96AS2	UniProt	O45686
OMIM	228000	IntAct	O45686
NCBI Gene	427	NCBI Gene	173120
NCBI RefSeq	NP_808592, NP_004306	NCBI RefSeq	NP_493173
NCBI RefSeq	NM_177924, NM_004315	NCBI RefSeq	NM_060772
NCBI UniGene	427	NCBI UniGene	173120
NCBI Accession	Q13510, AAC73009	NCBI Accession	O45686, CAB05556

<i>Mus musculus</i>		<i>Danio rerio</i>	
UniProt	Q9WV54, Q3U8A7, Q78P93	UniProt	Q5XJR7
NCBI Gene	11886	NCBI Gene	450068
NCBI RefSeq	NP_062708	NCBI RefSeq	NP_001006088
NCBI RefSeq	NM_019734	NCBI RefSeq	NM_001006088
NCBI UniGene	11886	NCBI UniGene	450068
NCBI Accession	AK151208, AK034204	NCBI Accession	AAH83231, CB360968
<i>Gallus gallus</i>		<i>Rattus norvegicus</i>	
UniProt	Q5ZK58	UniProt	Q6P7S1, Q9EQJ6
NCBI Gene	422727	NCBI Gene	84431
NCBI RefSeq	NP_001006453	NCBI RefSeq	NP_445859
NCBI RefSeq	NM_001006453	NCBI RefSeq	NM_053407
NCBI UniGene	422727	NCBI UniGene	84431
NCBI Accession	CAG31885, AJ720226	NCBI Accession	AAH61540, AF214647
<i>Pan troglodytes</i>			
NCBI Gene	464022		
NCBI RefSeq	XP_519629		
NCBI RefSeq	XM_519629		
NCBI UniGene	464022		

[0030] Treating according to this aspect of the present invention is carried out by contacting the population of cells with the ceramidase (e.g., AC), using methods that will be apparent to the skilled artisan.

[0031] In some embodiments, treating is carried out by introducing into the cells of the population a ceramidase protein. An approach for delivery of proteins or polypeptide agents (e.g., active ceramidase, ceramidase precursor proteins) involves the conjugation of the desired protein or polypeptide to a polymer that is stabilized to avoid enzymatic degradation of the conjugated protein or polypeptide. Conjugated proteins or polypeptides of this type are described in U.S. Patent No. 5,681,811 to Ekwuribe.

[0032] Yet another approach for delivery of proteins or polypeptide agents involves preparation of chimeric proteins according to U.S. Patent No. 5,817,789 to Heartlein et al. The chimeric

protein can include a ligand domain and the polypeptide agent (e.g., acid ceramidase, other ceramidase, acid ceramidase precursor protein, other ceramidase precursor proteins). The ligand domain is specific for receptors located on a target cell. Thus, when the chimeric protein is delivered to the cell, the chimeric protein will adsorb to the targeted cell, and the targeted cell will internalize the chimeric protein.

[0033] In some embodiments, the ceramidase (e.g., AC) may be administered by introducing into the cell a nucleic acid molecule that encodes the ceramidase (either active ceramidase or ceramidase precursor protein, as described above) (JOSEPH SAMBROOK & DAVID W. RUSSELL, 1-3 MOLECULAR CLONING: A LABORATORY MANUAL (3d ed. 2001); SHORT PROTOCOLS IN MOLECULAR BIOLOGY (Frederick M. Ausubel et al. eds., 1999); U.S. Patent No. 4,237,224 to Cohen & Boyer). Suitable nucleic acid molecules include, but are not limited to, those set forth in Table 1, *supra*.

[0034] Nucleic acid agents for use in the methods of the present invention can be delivered to a cell in a number of ways known in the art. For example, the nucleic acid can be contained within a vector, e.g., a vector that can be transferred to the cell(s) and provide for expression of the nucleic acid therein. Such vectors include chromosomal vectors (e.g., artificial chromosomes), non-chromosomal vectors, and synthetic nucleic acids. Vectors include plasmids, viruses, and phages, such as retroviral vectors, lentiviral vectors, adenoviral vectors, and adeno-associated vectors.

[0035] Nucleic acid agents can be transferred into the cell(s) using *ex vivo* methods, as will be apparent to the skilled artisan. For example, nucleic acids and vectors can be delivered to cells by physical means, e.g., by electroporation, lipids, cationic lipids, liposomes, DNA gun, calcium phosphate precipitation, injection, or delivery of naked nucleic acid.

[0036] As an alternative to non-infective delivery of nucleic acids as described above, naked DNA or infective transformation vectors can be used for delivery, whereby the naked DNA or infective transformation vector contains a recombinant gene that encodes the acid ceramidase/acid ceramidase precursor protein. The nucleic acid molecule is then expressed in the transformed cell.

[0037] The recombinant gene includes, operatively coupled to one another, an upstream promoter operable in the cell in which the gene is to be expressed and optionally other suitable regulatory elements (*i.e.*, enhancer or inducer elements), a coding sequence that encodes the nucleic acid, and a downstream transcription termination region. Any suitable constitutive promoter or inducible promoter can be used to regulate transcription of the recombinant gene, and one of skill in the art can readily select and utilize such promoters, whether now known or hereafter developed. The promoter can also be specific for expression in the cell(s) whose survival is to be promoted. Tissue specific promoters can also be made inducible/repressible using, e.g., a TetO response element. Other inducible elements can also be used. Known recombinant techniques can be utilized to prepare the recombinant gene, transfer it into the expression vector (if used), and administer the vector or naked DNA to the cell. Exemplary

procedures are described in SAMBROOK & RUSSELL, 1-3 MOLECULAR CLONING: A LABORATORY MANUAL (3d ed. 2001). One of skill in the art can readily modify these procedures, as desired, using known variations of the procedures described therein.

[0038] Any suitable viral or infective transformation vector can be used. Exemplary viral vectors include, without limitation, adenovirus, adeno-associated virus, and retroviral vectors (including lentiviral vectors).

[0039] Adenovirus gene delivery vehicles can be readily prepared and utilized given the disclosure provided in Berkner, "Development of Adenovirus Vectors for the Expression of Heterologous Genes," Biotechniques 6:616-29 (1988); Rosenfeld et al., "Adenovirus-mediated Transfer of a Recombinant α 1-antitrypsin Gene to the Lung Epithelium in Vivo," Science 252:431-434 (1991); PCT Publication No. WO/1993/007283 to Curiel et al.; PCT Publication No. WO/1993/006223 to Perricaudet et al.; and PCT Publication No. WO/1993/007282 to Curiel et al. Additional types of adenovirus vectors are described in U.S. Patent No. 6,057,155 to Wickham et al.; U.S. Patent No. 6,033,908 to Bout & Hoeben; U.S. Patent No. 6,001,557 to Wilson et al.; U.S. Patent No. 5,994,132 to Chamberlain & Kumar-Singh; U.S. Patent No. 5,981,225 to Kochanek & Schniedner; U.S. Patent No. 5,885,808 to Spooner & Epenetos; and U.S. Patent No. 5,871,727 to Curiel.

[0040] Adeno-associated viral gene delivery vehicles can be constructed and used to deliver into cells a recombinant gene encoding a desired nucleic acid. The use of adeno-associated viral gene delivery vehicles *in vitro* is described in Chatterjee et al., "Dual-target Inhibition of HIV-1 in Vitro by Means of an Adeno-associated Virus Antisense Vector," Science 258:1485-8 (1992); Walsh et al., "Regulated High Level Expression of a Human γ -Globin Gene Introduced into Erythroid Cells by an Adeno-associated Virus Vector," Proc. Nat'l Acad. Sci. USA 89:7257-61 (1992); Walsh et al., "Phenotypic Correction of Fanconi Anemia in Human Hematopoietic Cells with a Recombinant Adeno-associated Virus Vector," J. Clin. Invest. 94:1440-8 (1994); Flotte et al., "Expression of the Cystic Fibrosis Transmembrane Conductance Regulator from a Novel Adeno-associated Virus Promoter," J. Biol. Chem. 268:3781-90 (1993); Ponnazhagan et al., "Suppression of Human α -Globin Gene Expression Mediated by the Recombinant Adeno-associated Virus 2-based Antisense Vectors," J. Exp. Med. 179:733-8 (1994); Miller et al., "Recombinant Adeno-associated Virus (rAAV)-mediated Expression of a Human γ -Globin Gene in Human Progenitor-derived Erythroid Cells," Proc. Nat'l Acad. Sci. USA 91:10183-7 (1994); Einerhand et al., "Regulated High-level Human β -Globin Gene Expression in Erythroid Cells Following Recombinant Adeno-associated Virus-mediated Gene Transfer," Gene Ther. 2:336-43 (1995); Luo et al., "Adeno-associated Virus 2-mediated Gene Transfer and Functional Expression of the Human Granulocyte-macrophage Colony-stimulating Factor," Exp. Hematol. 23:1261-7 (1995); and Zhou et al., "Adeno-associated Virus 2-mediated Transduction and Erythroid Cell-specific Expression of a Human β -Globin Gene," Gene Ther. 3:223-9 (1996).

[0041] Retroviral vectors which have been modified to form infective transformation systems can also be used to deliver a recombinant gene encoding a desired nucleic acid product into a target cell. One such type of retroviral vector is disclosed in U.S. Patent No. 5,849,586 to

Kriegler & Perez. Lentivirus vectors can also be utilized, including those described in U.S. Patent No. 6,790,657 to Arya, and U.S. Patent Application Publication No. 2004/0170962 to Kafri et al. and U.S. Patent Application Publication No. 2004/0147026 to Arya.

[0042] The amount of ceramidase to be used will, of course, vary depending upon the particular conditions. Generally, the ceramidase is used in an amount sufficient to produce chondrocytes. The amount required to obtain the desired effect may vary depending on the cell type, culture conditions, and duration for which it is desired that chondrocytes be produced. Effective amounts can be determined empirically by those of skill in the art. For example, this may involve assays in which varying amounts of ceramidase are administered to cells in culture and the concentration effective for obtaining the desired result is calculated.

[0043] Ceramidase (e.g., AC) treatment can be carried out as frequently as required and for a duration that is suitable to promote differentiation into chondrocytes. For example, treatment can be carried out once, or multiple times.

[0044] In at least one embodiment, the method further includes treating the selected cell population with one or more transforming growth factors beta (TGF β) together with ceramidase. TGF β proteins regulate various aspects of embryonic development and are expressed in the environment of sympathoadrenal progenitor cells (Wall et al., "TGF-beta Related Genes in Development," Curr. Opin. Genet. Dev. 4:517 (1994)). TGF β proteins are recognizable by C-terminus polypeptide homology and their signaling via the Similar to Mothers Against Decapentaplegic (SMAD) proteins after binding TGF β receptors. Examples include TGF β 1-3, bone morphogenetic proteins (BMPs), etc. In some systems, TGF β regulates expression of parathyroid hormone-related protein (PTHrP) (Pateder et al., "PTHrP Expression in Chick Sternal Chondrocytes is Regulated by TGF-beta Through Smad-mediated Signaling," J. Cell Physiol. 188:343 (2001)). Bone morphogenic proteins (BMP) and growth and differentiation factors (GDFs) are believed to play a central role during skeletogenesis, including joint formation (Francis-West et al., "BMP/GDF-signalling Interactions During Synovial Joint Development," Cell Tissue Res. 296(1):111-119 (1999); see U.S. Patent Pub. No. 2011/0129867 to Thies).

[0045] In at least one embodiment, treating involves culturing the cell population in a culture medium containing the ceramidase, a nucleic acid molecule encoding the ceramidase, and/or a cell that contains (and expresses) the nucleic acid molecule and which secretes the ceramidase into the culture medium.

[0046] Treating according to this and all aspects of the present invention are carried out *in vitro*. *In vivo* treatments include, for example, embodiments in which the population of cells is present in a mammalian subject. In such embodiments the population of cells can be either autologous (produced by the subject), homologous, or heterologous. Suitable subjects according to these embodiments include mammals, e.g., human subjects, equine subjects, porcine subjects, feline subjects, and canine subjects.

[0047] In at least one embodiment, treating is carried out on a solid support. Suitable solid supports include, without limitation, monolayer culture supports, three-dimensional tissue scaffolds, hydrogels, and foamed scaffolds made of resorbable biomaterials. A three-dimensional cell scaffold should possess sufficient mechanical strength to maintain its form and structure in response to the pressure exerted by the surrounding tissue upon implantation in situ and to the strain exerted on the scaffold by cells in the interior of the scaffold. The aim of the scaffold structure and cells in the interior of the scaffold or native cells of the surrounding tissue is to perform the function of the native cells or tissue that they are meant to supplement or replace. Consequently, an environment which allows for growth and differentiation of the cells of the scaffold into tissue should be provided by the three-dimensional scaffold. A number of scaffolds for implantation into a recipient are well-known (see, e.g., U.S. Patent Pub. Nos. 2004/0126405 to Sahatjian et al., 2005/0107868 to Nakayama et al., 2006/0039947 to Schmidmaier et al., and PCT Pub. No. WO/2008/003320 to Ulrich-Vinther et al.). The treating may also be carried out on gel matrix, such as a hydrogel. Examples of gel matrices that may be used include, but are not limited to, collagen gel, fibrin glue, polyglycolic acid, polylactic acid, polyethylene oxide gel, alginate or calcium alginate gel, poly-(2-hydroxyethyl methacrylate) (i.e., a hydrogel), polyorthoester, hyaluronic acid, polyanhydride, chitosan, gelatin, agarose, and other bioresorbable and biocompatible materials such as those described in EP 0705878 A2 to Burns et al. and U.S. Patent No. 8153117 to Binette et al. To promote chondrocyte proliferation and function, the biological gel can additionally contain appropriate nutrients (e.g., serum, salts such as calcium chloride, ascorbic acid, and amino acids) and growth factors (e.g., somatomedin, basic fibroblast growth factor, transforming growth factor- β (as described *supra*), cartilage growth factor, bone-derived growth factor, or a combination thereof).

[0048] Another aspect of the present invention relates to an *in vitro* method of improving the phenotype of a chondrocyte population. This method includes selecting a population of chondrocytes having a poor chondrocytic phenotype characterized by low expression of collagen 2A1, Sox9, and aggrecan; elevated expression of collagen 10; and/or low staining for proteoglycans with Alcian Blue or Safranin O, and treating the selected cell population with a ceramidase protein *in vitro*, wherein, after said treating, the selected population is characterized by elevated expression of collagen 2A1, Sox9, and aggrecan; reduced expression of collagen 10; and/or enhanced staining for proteoglycans with Alcian Blue or Safranin O.

[0049] Suitable chondrocytes according to this aspect of the present invention include, without limitation, articular chondrocytes, nasal chondrocytes, tracheal chondrocytes, meniscal chondrocytes, and aural chondrocytes. These include, for example, mammalian chondrocytes, e.g., human chondrocytes, equine chondrocytes, porcine chondrocytes, feline chondrocytes, and canine chondrocytes. Preferably, the chondrocytes are primary chondrocytes.

[0050] The chondrocytic phenotype is determined by evaluating one or more chondrocyte markers, i.e., a substance whose localization or expression in a chondrocyte aids in the identification of the chondrocyte. These include, for example, collagen 2A1, collagen 10, type

IX collagen, type XI collagen, Sox9, aggrecan, GAPDH, TGF β 1, FGF-2, cartilage proteoglycan (aglycan) or components thereof, hyaluronic acid, and chondromodulin.

[0051] A poor chondrogenic phenotype is characterized by low expression of collagen 2A1, Sox9, and aggrecan; elevated expression of collagen 10; low staining for proteoglycans with Alcian Blue or Safranin O; and/or low expression of other chondrocyte-specific growth factors. In at least one embodiment, an improved chondrogenic phenotype is characterized by elevated expression of collagen 2A1, Sox9, and aggrecan; low expression of collagen 10; enhanced staining for proteoglycans with Alcian Blue or Safranin O; and/or elevated expression of other chondrocyte-specific growth factors.

[0052] Suitable ceramidases and methods of treating the population of cells with ceramidase include all those set forth *supra*.

[0053] A method of promoting chondrogenesis is also disclosed. This method includes selecting a population of bone marrow cells in need of differentiation into chondrocytes, treating the population of bone marrow cells with a ceramidase to enrich mesenchymal stem cells within the bone marrow cell population, and treating the population of enriched mesenchymal stem cells with a ceramidase, or a ceramidase and TGF β , to promote differentiation of mesenchymal stem cells into chondrocytes.

[0054] Suitable bone marrow cells include mammalian bone marrow cells, *e.g.*, human bone marrow cells, equine bone marrow cells, porcine bone marrow cells, feline bone marrow cells, and canine bone marrow cells. Human bone marrow cells are particularly preferred.

[0055] In at least one embodiment, the method further includes treating the chondrocytes with a ceramidase to improve the phenotype of the chondrocytes, as discussed *supra*.

[0056] Suitable ceramidases and methods of treating the populations of cells with ceramidase include all those set forth *supra*.

[0057] A method of maintaining a cell population in a differentiated state or increasing the number of cells of a population in a differentiated state is also disclosed. This method includes selecting a cell population in need of being maintained in a differentiated state or a cell population in need of an increased number of cells in a differentiated state and treating the selected cell population with a ceramidase to maintain or increase the number of differentiated cells in the selected cell population.

[0058] Suitable cells populations include mammalian cells populations, *e.g.*, human cells populations, equine cells populations, porcine cells populations, feline cells populations, and/or canine cells populations. Human cells populations are particularly preferred.

[0059] In at least one embodiment, the differentiated cells are primary cells. Suitable differentiated cells include, without limitation, chondrocytes, neurons, hepatocytes, bone cells,

lung cells, and cardiac cells. In a preferred embodiment, the cells are chondrocytes. Suitable chondrocytes include those described *supra*.

[0060] In at least one embodiment, the number of differentiated cells in the cell population is maintained. In at least one embodiment, the number of differentiated cells in the cell population is increased. As will be apparent to the skilled artisan, maintaining or increasing the overall number of differentiated cells in the population can be achieved by decreasing or preventing de-differentiation of cells in the population that are already differentiated, by stimulating the differentiation of undifferentiated cells in the population, or both.

[0061] Suitable ceramidases and methods of treating the populations of cells with ceramidase include all those set forth *supra*.

[0062] Another aspect of the present invention relates to an *in vitro* method of producing a population of chondrocyte cells. This method includes selecting a population of stem cells capable of differentiating into chondrocyte cells, selecting a differentiation medium capable of stimulating differentiation into the desired differentiated cells, and *in vitro* culturing the population of stem cells in the differentiation medium and ceramidase protein to stimulate differentiation into the population of chondrocyte cells.

[0063] Suitable stem cells according to this aspect of the present invention include, without limitation, embryonic stem cells, somatic stem cells, induced pluripotent stem cells, totipotent stem cells, pluripotent stem cells, and multipotent stem cells. Exemplary stem cells include, for example, hematopoietic stem cells, mesenchymal stem cells, neural stem cells, endothelial progenitor cells, epithelial stem cells, epidermal stem cells, and cardiac stem cells.

[0064] The differentiated cells of this aspect of the present invention are chondrocytes. The selection of suitable stem cells and suitable differentiation media to produce the desired differentiated cells will be apparent to the skilled artisan.

[0065] Suitable ceramidases include all those set forth *supra*.

[0066] Culturing according to this aspect of the present invention may be carried out as described above.

[0067] Culturing carried out *in vivo*, e.g., in the body of a mammalian subject is also disclosed. Suitable mammalian subjects include human subjects, equine subjects, porcine subjects, feline subjects, and canine subjects. The population of stem cells can be produced by the subject or, alternatively, the population of stem cells (which can be either homologous or heterologous) can be artificially introduced into the subject. The differentiation medium can be a commercial differentiation medium or a natural differentiation medium produced by the subject. In addition to the culturing methods described *supra*, culturing according to these embodiments can include, for example: implanting in the subject a solid support as described above (e.g., a tissue scaffold) having the ceramidase disposed thereon and/or therein, administering the

ceramidase to the subject at a tissue site containing the population of stem cells, and/or selectively damaging a tissue of the subject to stimulate production of the differentiation medium.

[0068] In all embodiments that involve administering ceramidase to a subject, an active ceramidase, ceramidase precursor protein, and/or nucleic acid encoding ceramidase/ceramidase precursor protein can be administered. Administration can be accomplished either *via* systemic administration to the subject or *via* targeted administration to affected tissues, organs, and/or cells. The ceramidase may be administered to a non-targeted area along with one or more agents that facilitate migration of the ceramidase to (and/or uptake by) a targeted tissue, organ, or cell. Additionally and/or alternatively, the ceramidase itself can be modified to facilitate its transport to (and uptake by) the desired tissue, organ, or cell, as will be apparent to one of ordinary skill in the art.

[0069] Typically, ceramidase will be administered to a subject in a vehicle that delivers the ceramidase to the target cell, tissue, or organ. Exemplary routes of administration include, without limitation, by intratracheal inoculation, aspiration, airway instillation, aerosolization, nebulization, intranasal instillation, oral or nasogastric instillation, intraperitoneal injection, intravascular injection, topically, transdermally, parenterally, subcutaneously, intravenous injection, intra-arterial injection (such as *via* the pulmonary artery), intramuscular injection, intrapleural instillation, intraventricularly, intralesionally, by application to mucous membranes (such as that of the nose, throat, bronchial tubes, genitals, and/or anus), or implantation of a sustained release vehicle.

[0070] In some embodiments, ceramidase is administered orally, topically, intranasally, intraperitoneally, intravenously, subcutaneously, or by aerosol inhalation. In some embodiments, ceramidase is administered *via* aerosol inhalation. In some embodiments, ceramidase can be incorporated into pharmaceutical compositions suitable for administration, as described herein.

[0071] The ceramidase may be orally administered, for example, with an inert diluent, or with an assimilable edible carrier, or they may be enclosed in hard or soft shell capsules, or they may be compressed into tablets, or may be incorporated directly with the food of the diet. For oral therapeutic administration, ceramidase may be incorporated with excipients and used in the form of tablets, capsules, elixirs, suspensions, syrups, and the like. Such compositions and preparations should contain at least 0.1% of ceramidase. The percentage of ceramidase in these compositions may, of course, be varied and may conveniently be between about 2% to about 60% of the weight of the unit. The amount of ceramidase in such therapeutically useful compositions is such that a suitable dosage will be obtained.

[0072] The tablets, capsules, and the like may also contain a binder such as gum tragacanth, acacia, corn starch, or gelatin; excipients such as dicalcium phosphate; a disintegrating agent such as corn starch, potato starch, or alginic acid; a lubricant such as magnesium stearate; and a sweetening agent such as sucrose, lactose, or saccharin. When the dosage unit form is

a capsule, it may contain, in addition to materials of the above type, a liquid carrier, such as a fatty oil.

[0073] The ceramidase may also be administered parenterally. Solutions or suspensions of ceramidase can be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof in oils. Illustrative oils are those of petroleum, animal, vegetable, or synthetic origin, for example, peanut oil, soybean oil, or mineral oil. In general, water, saline, aqueous dextrose and related sugar solutions, and glycols such as propylene glycol or polyethylene glycol, are preferred liquid carriers, particularly for injectable solutions. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

[0074] The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases, the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (e.g., glycerol, propylene glycol, and liquid polyethylene glycol), suitable mixtures thereof, and vegetable oils.

[0075] Ceramidase may also be administered directly to the airways in the form of an aerosol. For use as aerosols, ceramidase in solution or suspension may be packaged in a pressurized aerosol container together with suitable propellants, for example, hydrocarbon propellants like propane, butane, or isobutane with conventional adjuvants. Ceramidase may also be administered in a non-pressurized form.

[0076] Exemplary delivery devices include, without limitation, nebulizers, atomizers, liposomes (including both active and passive drug delivery techniques) (Wang & Huang, "pH-Sensitive Immunoliposomes Mediate Target-cell-specific Delivery and Controlled Expression of a Foreign Gene in Mouse," *Proc. Nat'l Acad. Sci. USA* 84:7851-5 (1987); Bangham et al., "Diffusion of Univalent Ions Across the Lamellae of Swollen Phospholipids," *J. Mol. Biol.* 13:238-52 (1965); U.S. Patent No. 5,653,996 to Hsu; U.S. Patent No. 5,643,599 to Lee et al.; U.S. Patent No. 5,885,613 to Holland et al.; U.S. Patent No. 5,631,237 to Dzau & Kaneda; and U.S. Patent No. 5,059,421 to Loughrey et al.; Wolff et al., "The Use of Monoclonal Anti-Thyl IgG1 for the Targeting of Liposomes to AKR-A Cells in Vitro and in Vivo," *Biochim. Biophys. Acta* 802:259-73 (1984)), transdermal patches, implants, implantable or injectable protein depot compositions, and syringes. Other delivery systems which are known to those of skill in the art can also be employed to achieve the desired delivery of ceramidase to the desired organ, tissue, or cells.

[0077] Administration can be carried out as frequently as required and for a duration that is suitable to provide effective treatment. For example, administration can be carried out with a single sustained-release dosage formulation or with multiple daily doses.

[0078] The amount to be administered will, of course, vary depending upon the treatment regimen. The dose required to obtain an effective amount may vary depending on the agent, formulation, and individual to whom the agent is administered.

[0079] Typically, ceramidase will be administered as a pharmaceutical formulation that includes ceramidase and any pharmaceutically acceptable adjuvants, carriers, excipients, and/or stabilizers, and can be in solid or liquid form, such as tablets, capsules, powders, solutions, suspensions, or emulsions. The compositions preferably contain from about 0.01 to about 99 weight percent, more preferably from about 2 to about 60 weight percent, of ceramidase together with the adjuvants, carriers and/or excipients. In some embodiments, an effective amount ranges from about 0.001 mg/kg to about 500 mg/kg body weight of the subject. In some embodiments, the effective amount of the agent ranges from about 0.05 mg/kg to about 30 mg/kg, from about 0.1 mg/kg to about 30 mg/kg, from about 1 mg/kg to about 25 mg/kg, from about 1 mg/kg to about 20 mg/kg, or from about 1 or 2 mg/kg to about 15 mg/kg.

[0080] Methods of treating a certain disease or disorder are also disclosed. These methods involve selecting a mammalian subject having the disease or disorder and administering a population of certain cells to the subject to treat the disease or disorder.

[0081] The population of cells is produced by selecting a population of stem cells capable of differentiating into a desired population of differentiated cells, selecting a differentiation medium capable of stimulating differentiation into the desired differentiated cells, and culturing the population of stem cells in the differentiation medium and ceramidase to stimulate differentiation into the population of differentiated cells, as described *supra*.

[0082] In at least one embodiment, culturing is carried out *in vitro*. In these embodiments, the population of stem cells can be taken from the subject or from a second subject before culturing, culturing carried out *in vitro* to produce differentiated cells, and the differentiated cells then administered to the first subject (e.g., by injecting the differentiated cells into the first subject).

[0083] In at least one embodiment, culturing is carried out *in vivo*. In these embodiments, the population of stem cells can be produced by the subject or by a second subject. Culturing can be carried out, for example, in the first subject or the second subject, using the *in vivo* culturing methods described above. In at least one embodiment, the stem cells are produced by the subject and culturing includes administering the ceramidase to the subject at a tissue site containing the population of stem cells. In at least one embodiment, the stem cells are produced by a second subject and culturing includes administering the ceramidase to the second subject at a tissue site containing the population of stem cells. Ceramidase can be administered to the first or second subject using the methods described above.

[0084] Mammalian subjects include, for example, human subjects, equine subjects, porcine subjects, feline subjects, and canine subjects. Human subjects are particularly preferred.

[0085] In one aspect, the disease or disorder is a joint disease or disorder and a population of chondrocytes produced according to the methods of the present invention is administered. Exemplary types of joint disease or disorders include, without limitation, osteoarthritis, Rheumatoid arthritis, mucopolysaccharidosis, degenerative joint disease, and joint injury.

[0086] In another aspect, the disease or disorder is a neurodegenerative disease or disorder and a population of neurons is administered. Exemplary types of neurodegenerative diseases or disorders include, without limitation, Alzheimer's disease, Frontotemporal Dementia, Dementia with Lewy Bodies, Prion disease, Parkinson's disease, Huntington's disease, Progressive Supranuclear Palsy, Corticobasal Degeneration, Multiple System Atrophy, amyotrophic lateral sclerosis, inclusion body myositis, degenerative myopathy, spinocerebellar atrophy, metabolic neuropathy, diabetic neuropathy, endocrine neuropathy, orthostatic hypotension, brain injury, spinal cord injury, and stroke.

[0087] In another aspect, the disease or disorder is a cardiac disease or disorder and a population of cardiac cells is administered. Exemplary types of cardiac diseases or disorders include, without limitation, heart disease, cardiac injury, hypercardia, heart infarction, mitral regurgitation, aortic regurgitation, septal defect, and tachycardia-bradycardia syndrome.

[0088] In another aspect, the disease or disorder is diabetes and a population of pancreatic cells is administered.

[0089] Any suitable approach for administering the population of cells to the subject can be utilized to practice these aspects, as will be apparent to the skilled artisan. This may include, for example, transplantation to a particular site in the body, such as a particular tissue or organ. Systemic infusion of cells may also be performed.

[0090] Therapeutic agents to treat the disease or disorder can also be administered in addition to administering the population of cells, as will be apparent to the skilled artisan.

[0091] Dosage, toxicity, and therapeutic efficacy of therapeutic agents and/or cells can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., for determining the LD₅₀ (the dose lethal to 50% of the population) and the ED₅₀ (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD₅₀/ED₅₀. Agents/cells which exhibit high therapeutic indices may be desirable. While agents/cells that exhibit toxic side effects may be used, care should be taken to design a delivery system that targets such agents/cells to the site of affected tissue in order to minimize potential damage to uninfected cells and, thereby, reduce side effects.

[0092] Therapeutic agents and/or cells are administered to a subject in an amount to treat the disease or disorder. The amount is a quantity sufficient to achieve a desired therapeutic and/or prophylactic effect, e.g., an amount which results in the prevention of, or a decrease in, the

cause or symptoms associated with the disease or disorder that is being treated.

[0093] The effective amount of a therapeutic agent/cell population administered to the subject will depend on the type and severity of the disease or disorder and on the characteristics of the individual, such as general health, age, sex, body weight, and tolerance to drugs. It will also depend on the degree, severity, and type of disease or disorder. The skilled artisan will be able to determine appropriate dosages depending on these and other factors.

[0094] It is to be appreciated that certain aspects, modes, embodiments, variations and features of the present invention are described in various levels of detail in order to provide a substantial understanding of the present technology. The definitions of certain terms as used in this specification are also provided. Unless defined otherwise, all technical and scientific terms used herein generally have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs.

EXAMPLES

[0095] The following examples are provided to illustrate embodiments of the present invention but are by no means intended to limit its scope.

Example 1-Materials and Methods

[0096] *Animals* - Animals were raised under National Institutes of Health and USDA guidelines for the care and use of animals in research. Normal rats were derived from the MPS VI breeding colony (Kunieda et al., "Mucopolysaccharidosis Type VI in Rats: Isolation of cDNAs Encoding Arylsulfatase B, Chromosomal Localization of the Gene, and Identification of the Mutation," *Genomics* 29(3):582-7 (1995)) maintained at the Mount Sinai School of Medicine, while the larger animals (cats and horses) were maintained at the University of Pennsylvania School of Veterinary Medicine. The animals were housed with *ad libitum* food and water. Euthanasia was performed on cats using 80 mg/kg of sodium pentobarbital (Veterinary Laboratories, Lenexa, KS) in accordance with the American Veterinary Medical Association guidelines. Euthanasia of rats was performed using carbon dioxide inhalation. Equine cells were obtained during routine surgical procedures.

[0097] *Primary chondrocyte isolation and culture* - Methods for rat articular chondrocyte isolation and culture have been published elsewhere (Simonaro et al., "Mechanism of Glycosaminoglycan-mediated Joint and Bone Disease: Implications for the Mucopolysaccharidoses and Other Connective Tissue Diseases," *Am. J. Path.* 172:112-122 (2008)). Briefly, to establish rat primary chondrocyte cultures, femoral caps and meniscus were collected and washed three times in 0.9% NaCl supplemented with 5% penicillin/streptomycin (v/v) and fungizone 0.1% (v/v). The cartilage was digested with 0.5 mg/ml pronase prepared in

0.9% NaCl (v/v) + 5% penicillin/streptomycin (v/v) + 0.1% fungizone for 2 hours at 37°C, and with 1 mg/ml collagenase (Sigma Chemical Co.) prepared in DMEM containing 10% FBS (v/v), 1% penicillin/streptomycin (v/v), and 0.1% fungizone overnight at 37°C. Cells were then cleaned by filtrating the resulting media through a 40 µm cell strainer, and isolated by centrifugation (1200 rpm for 8 minutes). They were further washed twice in DMEM supplemented with 10% FBS (v/v), 1% Pen Strep (v/v), and fungizone 0.1%, and plated in 75 cm² flasks at a density of 7000 cells per cm² in DMEM containing 10% FBS (v/v), 1% penicillin/streptomycin (v/v), and fungizone 0.1%. The freshly plated cells were considered passage 0 (P0). When reaching subconfluency, cells were trypsinized and passaged (P1) for further expansion. For subsequent passages, the cells were grown for seven days, trypsinized, and then reseeded at the same cell density. In some experiments rAC was added to the media at P0 (200 U/ml final concentration). Unless otherwise noted, rAC was added to the cells only once, and subsequent media changes did not include the enzyme.

[0098] To grow rat chondrocytes in three-dimensional cultures, 5x5 mm collagen sponges (CollaCote, Zimmer Dental) were used. The sponges were pre-wet in DMEM containing 10% FBS (v/v) and antibiotics, and the cells, which had been previously isolated as described above, were suspended in media and pipetted onto the sponge. To distribute the chondrocytes throughout the sponge, it was compressed slightly to circulate the media and distribute the cells. The sponges were then placed in culture dishes and grown in DMEM containing 10% FBS and antibiotics with and without rAC. The media was changed every three days with fresh media lacking rAC.

[0099] To grow rat chondrocytes in fibrin gels, 7.5×10^6 cells were seeded in 200 µl of TISSEEL® (Baxter Healthcare, Deerfield, IL) gel in 96 well plates and cultured in standard media (DMEM containing 10% FBS) with or without rAC (200 U/ml). Media was changed every four days with fresh rAC included each media change. Cells were maintained for two weeks prior to analysis.

[0100] To obtain equine articular chondrocytes, cartilage explant tissue was recovered from the articular surfaces of the stifle joints of geldings (age 3-12 years) by use of a 6-mm biopsy punch. The punch was pushed into the cartilage perpendicular to the articular surface until contact was made with the surface of the bone. The punch was then rotated and removed and the resultant explant was removed from the bone by use of a scalpel blade via dissection parallel to the articular surface. Cartilage explants were trimmed to a thickness of approximately 100 mm to eliminate any mineralized tissue that might have affected subsequent culture or analysis procedures. Chondrocytes were isolated from explant tissue as described elsewhere (Novotny et al., "Biomechanical and Magnetic Resonance Characteristics of a Cartilage-like Equivalent Generated in a Suspension Culture," *Tissue Eng* 12:2755-2764 (2006)), and either frozen (P0) or used for cell expansion in DMEM/F12 media containing 50µg of ascorbate-2-phosphate/ml (v/v), 1% (v/v) penicillin/streptomycin, 1% (v/v) glutamate, and 0.1% (v/v) fungizone.

[0101] Human articular cartilage was collected from patients undergoing total knee

replacement surgery with written consent in accordance with an IRB-approved protocol (#09-0248). Cartilage slices were collected into DMEM/F12 media without FBS (containing 1% (v/v) penicillin/streptomycin, 1% (v/v) glutamate, and 0.1% (v/v) fungizone), and then incubated with Pronase (1mg/ml) in DMEM/F12 containing 10% FBS (v/v) for 30 minutes at 37°C in a shaker. Following centrifugation at 200xg, the cartilage was washed twice with PBS and then incubated with collagenase P (1mg/ml) in DMEM/F12 containing 10% FBS (v/v) overnight at 37°C in a shaker. The cartilage was then filtered using a 40-70 µm filter, centrifuged, and the supernatant was discarded. The cells were washed once with PBS, resuspended in DMEM/F12 media containing 10% FBS (v/v), and then plated at a density of $1 \times 10^5/\text{cm}^2$. In some experiments, rAC was added to the culture media immediately at the time of cell plating (200 U/ml final concentration).

[0102] *Acid ceramidase, ceramide, and sphingosine measurements* - Rat chondrocytes were grown in 12-well plates with or without rAC (200 U/ml), and AC activity, ceramide, and sphingosine levels were determined in cell lysates at 12, 24, and 48 hours. Cells were harvested and total proteins were extracted using Cell Lytic™ Cell Lysis Reagent (Sigma, Saint Louis, MO, USA). Proteins were quantified (Bio-Rad, Hercules, CA, USA), and the lysates were subjected to AC enzymatic activity measurement (He et al., "A Fluorescence-based High-performance Liquid Chromatographic Assay to Determine Acid Ceramidase Activity," Anal. Biochem. 274(2):264-9 (1999)). Lipids also were extracted from the cell lysates, and ceramide and sphingosine levels were quantified as already published (He et al., "Deregulation of Sphingolipid Metabolism in Alzheimer's Disease," Neurobiol. Aging 31(3):398-408 (2010)).

[0103] *Mesenchymal stem cell isolation and culture* - Rat MSCs were obtained from the femoral and tibia cavities of adult rats. Femurs and tibias were isolated, the extremities were removed, and the bone marrow was flushed out using PBS. After two washings in PBS, bone marrow cells were counted and plated at a density of 5×10^6 cells/cm² in Alpha MEM supplemented with 20% (v/v) FBS, 1% (v/v) penicillin/streptomycin, 1% (v/v) glutamate, and 0.1% (v/v) fungizone. Bone marrow cells from cats were collected during surgical procedures and shipped on ice and in PBS overnight.

[0104] For horses, bone marrow was collected aseptically from the sternum by use of an 11-gauge bone marrow biopsy needle and a 60-ml sterile Luer-tip syringe that contained 10 ml of sterile acid-citrate-dextrose solution. After aspiration of the bone marrow, the aspirate was briefly mixed with the acid-citrate-dextrose solution, and the syringe then was placed on ice and transported to the laboratory for isolation of MSCs. Once received, cells were washed twice with PBS and plated at a density between $1-5 \times 10^6$ cells/cm² (feline cells) and 3×10^8 cells/cm² (equine cells) in Alpha MEM supplemented with 20% (v/v) FBS, 1% (v/v) penicillin/streptomycin, 1% (v/v) glutamate, and 0.1% (v/v) fungizone. The freshly plated bone marrow cells were treated with or without rAC at day 0 (200 U/ml final concentration). At day three, medium was removed and replaced by fresh medium lacking rAC. Medium was subsequently changed twice a week, and colonies were passaged when they reached subconfluency.

[0105] In some experiments, the cells were stained after one week of culture with crystal violet (0.5% [Sigma] w/v in methanol) to count the number of colonies. For these assays (CFU-F), cells were incubated 30 minutes at room temperature and rinsed four times with PBS, before a final washing in water. Only colonies with a diameter greater than 1 mm were counted.

[0106] In other experiments, flow cytometric analysis was performed to assess the number of MSCs. For these analyses, MSCs were collected, washed twice in PBS supplemented with 2% FBS and marked for CD90-FITC and CD45-PE (Cat# 11-0900-81 and Cat#554878, respectively, BD Biosciences Pharmingen, San Diego, CA, USA), diluted in PBS + FBS 2% for 15 minutes, on ice and in the dark. After subsequent washings, cells were analyzed in a LSR II flow cytometer (BD Biosciences, San Jose, CA, USA). Flow cytometry analysis could not be performed on the cat and horse cells due to the lack of suitable antibody reagents.

[0107] To test the effect of rAC on the chondrogenic potential of BM-MSCs, bone marrow cells were amplified for 3 weeks in Alpha MEM supplemented with 20% (v/v) FBS, 1% (v/v) penicillin/streptomycin, 1% (v/v) glutamate, and 0.1% (v/v) fungizone. During the three weeks of culture, the cells were passaged when they reached subconfluency. At the end of three weeks they were trypsinized, counted, and 5×10^5 cells were differentiated in pellet cultures using conical culture tubes. Chondrogenic differentiation was performed in DMEM high glucose media containing 6.25 µg/ml insulin, 6.25 µg/ml transferin, 1.25 mg/ml bovine serum albumin, dexamethasone 100 nM, ascorbate-2-phosphate 50 µM, 5.33 µg/ml linoleic acid, and 10 ng/ml TGFβ1, as described previously (Anjos-Afonso et al., "Isolation, Culture and Differentiation Potential of Mouse Marrow Stromal Cells," CURR. PROTOC. STEM CELL BIOL. Chapter 2, Unite 2B.3 (2008)). To evaluate the effect of rAC on the chondrogenic differentiation, in some experiments rAC was added into the chondrogenic medium (200 U/ml final concentration). Medium was changed every three days, with or without rAC. Cells were maintained in a humidified incubator, at 37°C under 5% CO₂.

[0108] *Recombinant AC production and purification* - Human recombinant AC (rAC) was produced as described in He et al., "Purification and Characterization of Recombinant, Human Acid Ceramidase. Catalytic Reactions and Interactions With Acid Sphingomyelinase," J. Biol. Chem. 278(35):32978-86 (2003). Briefly, rAC overexpressing CHO cells were grown to confluency in DMEM supplemented with 10% v/v FBS and 1% penicillin/streptomycin. The conditioned medium was collected, and rAC was concentrated by pressure filtration (cut off 30kDa, Amicon, Billerica, MA, USA) and purified using a fast protein liquid chromatography (FPLC) system (Amersham Biosciences, Piscataway, NJ, USA). The amount of rAC was quantified by enzyme activity measurement and western blot analysis (Eliyahu et al., "Acid Ceramidase is a Novel Factor Required for Early Embryo Survival," FASEB J. 21(7):1403-9 (2007)).

[0109] *Processing of the differentiated mesenchymal stem cell pellet cultures* - Once BM-MSCs were differentiated in the chondrogenic media with or without rAC, pellets were removed from the incubator, washed twice, and fixed in paraformaldehyde 4% for 15 minutes at room

temperature. They were then dehydrated by successive incubations in 70% ethanol (30 minutes at room temperature), 95% ethanol (2x 30 minutes at room temperature), and 100% ethanol (2x 30 minutes at room temperature). Pellets were then cleared in two successive baths of xylenes (2x 30 minutes at room temperature), and paraffin embedded and microtome processed to create 6 μ m slices on polyprep slides (Sigma).

[0110] For histochemical staining, pellets were deparaffinized in xylenes, hydrated to distilled water, and stained with either Alcian Blue 8GX 1% (w/v), pH 2.5 (Sigma) for 30 minutes at room temperature or Safranin O (Sigma) 0.1 % (w/v) for 5 minutes at room temperature. Slides were washed, dehydrated, and cleared with xylenes before covering with mounting medium.

[0111] *Immunohistochemistry* - For immunohistochemical analysis, pellets were deparaffinized in xylenes, hydrated, and washed three times in PBS. If needed (i.e. for Sox9 immunostaining), cells were permeabilized in 0.2% (v/v) Triton X-100 made in PBS (pH 7.4) for five minutes at room temperature, post-fixed in paraformaldehyde, and washed in PBS several times. Slides were then blocked two hours at room temperature in PBS supplemented with tween 0.1% (v/v) and 10% (v/v) FBS. After blocking, slides were incubated with primary antibody diluted in PBS containing 0.1% Tween 0.1 and 5% FBS overnight at 4°C. The following primary antibodies from Santa Cruz Biotechnology, Santa Cruz, CA, were used for immunostaining: Rabbit anti-Sox 9 (H-90, sc-20095), rabbit anti-collagen IIA1 (H-300, sc-28887), goat anti-collagen XA1 (E-14, sc-323750), and goat anti-aggrecan (D-20, sc-16492). After exposure to the primary antibodies, slides were then incubated one hour at room temperature with the corresponding secondary antibody conjugated with Cy3, diluted in PBS / tween 0.1% / 10% FBS. Finally, the slides were washed several times in PBS, and mounted with a DAPI containing medium (Vector Laboratories, Burlingame, CA, USA). Localization of the primary antibodies was visualized using the fluorescent Cy-3 second antibody and laser-scanning confocal microscopy (Zeiss LSM510).

[0112] For immunohistochemistry of the primary chondrocyte cultures, P3 cells were plated on chamber slides (5000 cells/chamber, lab-tek2 chamber slide, Thermo Fisher Scientific) and grown until subconfluency. Cells were then washed twice in PBS, and fixed in paraformaldehyde 4% for 15 minutes at room temperature, and immunostained as described above.

[0113] *Western blot analysis* - Primary chondrocytes were harvested by trypsinization and washed, and the proteins were extracted in Cell Lytic™ M Cell Lysis Reagent (Sigma, Saint Louis, MO, USA). Proteins were quantified (Bio-Rad, Hercules, CA, USA) and analyzed by western blotting (Novex Protein Analysis Solutions, Invitrogen, Carlsbad, CA, USA). The following primary antibodies were used for western blot analysis from Santa Cruz Biotechnology, CA, USA. Rabbit anti-Sox 9 (H-90, sc-20095), goat anti-collagen 1A2 (M-19, sc-8788), rabbit anti-collagen 2A1 (H-300, sc-28887), goat anti-aggrecan (D-20, sc-16492), mouse anti-FGF2 (sc-135905), rabbit anti TGF β 1 (sc-146), mouse anti-Bax (2D2) (sc-20067), rabbit anti-PARP-1/2 (H-250) (sc-7150), rabbit anti-GAPDH (FL-335) (sc-25778), and goat anti-actin (C-11) (sc-1616). Rabbit anti-collagen X (ab58632) from Abcam (Cambridge, MA, USA)

was also used.

[0114] *RNA isolation and quantitative RT-PCR* - For RNA isolation, cells were trypsinized, washed twice in PBS, and resuspended in Trizol /chloroform (5/1 (v/v)) (Life Technologies). After centrifugation, the aqueous upper phase was saved and purified through an affinity column (RNeasy Mini Kit, Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. RNA was resuspended in RNase-free water and quantified, and 1 µg of RNA was subjected to reverse transcription.

[0115] For qPCR analysis of the human articular chondrocyte cultures, cDNA was synthesized with Superscript VILO (Invitrogen). SYBR green qPCR (Invitrogen Platinum Taq) was performed and gene expression was normalized to GAPDH (2^{-ddCt} method).

[0116] *Statistical analysis* - Where appropriate, statistical analysis was carried out using a standard student's t-test analysis, one-way analysis of variance (ANOVA) with the variable group, multivariate analyses of variance (MANOVAs) followed by post hoc Bonferroni adjustments. The results were considered significant at P<0.05. Statistics were performed using Sigma Stat 3.1 (Systat Software).

Example 2-Changes in Sphingolipid Metabolism Following rAC Treatment of Primary Articular Chondrocytes

[0117] Primary rat articular chondrocytes were isolated and grown in monolayer cultures as described in Example 1 using DMEM containing 10% FBS with or without rAC. For the baseline timepoint, cells were collected from 6 rats and pooled without culture. The acid ceramidase, ceramide, and sphingosine levels in these cell lysates were compared to cells grown for 12, 24, and 48 hours with or without rAC. The data is summarized in Figures 1A-1C.

[0118] As expected, cells grown in media supplemented with rAC exhibited markedly increased AC activity by 12 hours, and this was sustained through 48 hours. Ceramide levels were also increased compared to baseline by 12 hours, but less in cells exposed to rAC. In fact, by 48 hours the ceramide levels in the rAC-treated cells had returned to baseline, while in those without rAC treatment remained significantly elevated. Also, by 48 hours significant elevations of sphingosine, the product of AC activity, was increased in the rAC-treated cells. By 7 days AC, ceramide, and sphingosine levels had returned to baseline regardless of rAC treatment.

[0119] Overall, these results demonstrated that rAC was taken up by rat chondrocytes and retained biological activity by hydrolyzing ceramide and producing sphingosine. The fact that the AC levels were returned to baseline by 7 days was consistent with its expected intracellular half-life of 48-72 hours (Bernardo et al., "Purification, Characterization and Biosynthesis of Human Acid Ceramidase," J. Biol. Chem. 270(19):11098-102 (1995)).

Example 3-Effects of rAC Supplementation on Expanded Monolayer Cultures of Primary

Articular Chondrocytes

[0120] The effects of rAC supplementation on the phenotype of articular chondrocytes after cell expansion were next evaluated. For these experiments, rat chondrocytes were grown for three weeks with and without rAC in DMEM containing 10% FBS. rAC was added once at the time of the initial cell plating (P0). All analyses were performed at the end of the three-week expansion period unless otherwise mentioned.

[0121] No effect of rAC treatment on the total number of cells was observed (Figure 2A), consistent with the fact that the levels of two apoptotic markers, Bax and PARP, were also very similar with and without rAC treatment.

[0122] However, despite obtaining the same number of cells, there was a marked effect of rAC treatment on cell quality at the end of the 3-week expansion period, as determined by the expression of various chondrocyte markers. As shown in Figure 2B, western blot analysis revealed that the expression of collagen 2A1, collagen 1A2, Aggrecan, Sox9, TGF β 1, and FGF2 each was enhanced in cells treated with rAC. Similar effects were evident in cells grown using a different culture media, RPMI containing 10% FBS. Notably, expression of collagen X, a marker of chondrocyte hypertrophy and dedifferentiation (Nadzir et al., "Comprehension of Terminal Differentiation and Dedifferentiation of Chondrocytes During Passage Cultures," J. Biosci. Bioeng. 112(4):395-401 (2011)), was reduced in these cultures.

[0123] In order to confirm that the effects of rAC were not rat-specific, equine articular chondrocytes that were provided frozen at P0 were next studied. The frozen cells were thawed and expanded for three weeks without rAC, and then re-plated with or without rAC in the culture media. They were then grown for one additional week and then analyzed. As shown in Figure 2C, the expression of three important chondrocyte markers, aggrecan, Sox9, and collagen II, were markedly enhanced in the rAC-treated cells, consistent with what was observed in the rat. The lack of other equine-specific antibodies precluded the analysis of additional chondrocyte markers in these cultures. Of interest, a reduction in the expression of the pro-apoptotic Bax protein in the rAC-treated equine cells was observed, more than that seen in the rat cells. This could be due to the fact that the equine cells were provided to researchers frozen, eliciting additional stress-related cell death during the culture period that was reduced by rAC.

[0124] Next, the rAC-treated and untreated rat chondrocytes were evaluated by immunohistochemistry and confocal microscopy. These analyses confirmed that after three week expansion the number of cells expressing collagen 2A1 in these cultures was increased approximately 40% following rAC treatment (Figure 3A). In addition, the intracellular distribution of Sox9 appeared different, with more of the transcription factor localizing to the nucleus in the cells after exposure to rAC (Figure 3B).

[0125] Osteoarthritis (OA) is a common, age-related disorder that results in cartilage degradation, and patients with OA frequently undergo surgical procedures to repair their defective joints. Articular cartilage was obtained from two aged OA patients and the cells expanded in monolayer culture for three weeks with or without rAC in the media (added once at the initial cell plating). In Figure 4A, PCR analysis showed that cells from patient 1 exhibited significantly elevated expression of Sox9, collagen 2A1, aggrecan, and TNF β 1 mRNA in response to treatment. In cells from patient 2 (Figure 4B), Sox9 expression was significantly elevated, and there was a trend towards elevated collagen IIA1 and aggrecan expression as well. As was observed with the rat and horse cells, the total number of chondrocytes obtained after the expansion period was similar, despite this differential gene expression pattern. It is again notable that no elevation of collagen X expression was observed in the rAC treated cells from either patient.

[0126] Overall, these results indicate that supplementing chondrocyte media with rAC once at the time of initial cell plating had a significant, positive effect on the chondrogenic phenotype after three weeks of expansion.

Example 4-Effects of rAC Supplementation on Three-Dimensional Culture of Primary Articular Chondrocytes

[0127] To achieve effective implantation of expanded chondrocytes in patients, after the initial monolayer expansion period the cells are generally seeded onto three-dimensional scaffolds for subsequent growth and transplantation. Therefore, the effects of rAC treatment on primary rat chondrocytes grown on collagen-coated scaffolds were evaluated (Figure 5A). For this experiment, the cells were seeded directly onto the scaffolds bathed in DMEM containing 10% FBS serum with or without rAC. They were then grown for 7 or 14 days and analyzed. As shown in the representative images, cells grown in the presence of rAC were larger and maintained a rounder phenotype than those without rAC. Importantly, these cells also stained positive for Safranin O, a commonly used marker of proteoglycan expression. The degree of cell shrinkage was further quantified and significantly less shrinkage was observed in rAC treated cells (Figure 5B).

[0128] In addition to collagen-coated scaffolds, biodegradable fibrin gels are also frequently used for implantation of the cells into the damaged cartilage site. As shown in Figure 5C, rAC treatment of rat chondrocytes grown in such gels for two weeks exhibited enhanced staining for Alcian Blue, an important marker of proteoglycan deposition.

Example 5-Effects of rAC Supplementation on the Yield of Bone Marrow-derived Mesenchymal Stem Cells

[0129] As noted above, recent interest in cell-based cartilage repair has focused on the use of

MSCs, which can be readily obtained from adult bone marrow, adipose tissue, or other autologous sources, and may be induced *in vitro* or *in vivo* to form chondrocytes and other mesenchymal cell lineages. Therefore, the effects of rAC on adult rat bone marrow-derived MSC before and after their differentiation into chondrocytes was next evaluated. Addition of rAC to the culture media at the time of initial plating of the rat bone marrow cells led to an approximate two to three-fold increase in the number of MSCs obtained at day 5, as judged by the number of colony forming fibroblast units (CFU-F) or by flow cytometry (Figure 6A). To confirm that these findings were not rat-specific, the effect of rAC supplementation on bone marrow MSCs obtained from cats and horses was also analyzed (Figures 6B, 6C, 6D). Due to the lack of suitable antibody reagents for flow cytometry, the number of MSCs in these species were quantified by CFU-F only, revealing increases similar to those observed with the rat cells.

Example 6-Effects of rAC Supplementation on the Chondrogenic Differentiation of Bone Marrow Mesenchymal Stem Cells

[0130] The effects of including rAC on the chondrogenic differentiation media of MSCs was also evaluated. For these studies differentiation was carried out in high-density pellet cultures using standard media with or without TGF β 1 (see Example 1, *supra*). Rat bone marrow cells were grown for three weeks in the absence of rAC to obtain a population of adherent MSCs that was approximately 90% pure (CD90+/CD40-). They were then grown in high-density pellet cultures for an additional three weeks in chondrocyte differentiation media. As shown in Figure 7A, rat pellets grown in the presence of rAC were significantly larger (>2-fold) and stained more intensely for proteoglycans using Alcian Blue and Safranin O. The effects of rAC were independent of TGF β 1, and the two factors worked synergistically to yield optimal results. The rat pellets also were analyzed by immunohistochemistry and confocal microscopy for Sox9, aggrecan, and collagen II expression. As illustrated by the images in Figures 7B-7D, the expression of these three chondrogenic markers were significantly elevated in cells supplemented with rAC. To confirm these findings, the same studies were performed using equine cells, and essentially the same results were obtained (Figures 8A-8D).

[0131] As described earlier for the chondrocyte expansion studies, no change or a reduction was observed in the levels of collagen X, a marker of chondrocyte hypertrophy and dedifferentiation, in contrast to collagen II, which was elevated. To examine this further, the differentiated equine chondrocytes for collagen X were also stained, and it was found that the levels were decreased in cells treated with rAC (Figure 8E). Taken together, the elevated expression of collagen II, aggrecan, and Sox9 along with low collagen X expression suggests that the treated cells have a more chondrogenic phenotype conducive to cartilage repair.

Example 7-Discussion of Examples 1-9

[0132] The dedifferentiation of chondrocytes remains one of the main barriers in ACI (Schulze-

Tanzil, G., "Activation and Dedifferentiation of Chondrocytes: Implications in Cartilage Injury and Repair," *Ann. Anat.* 191(4):325-38 (2009)). Indeed, chondrocytes, responsible for the production of a highly abundant extracellular matrix, are very sensitive to mechanical and biochemical stresses. During the amplification process it takes only a few days for the cells to change their shape from rounded to fibroblastic, and to start displaying abnormal features. For example, the cytoskeleton becomes abnormal with the expression of F-actin stress-fibers, the extracellular matrix proteins are abnormally expressed (e.g., decrease of Col 2A, proteoglycans, glycoprotein), proteases (e.g., MMPs, ADAM-TS) are activated, and signaling proteins are abnormally expressed (loss of Sox9). Moreover, although dedifferentiated cells can be re-differentiated when implanted in three-dimensional culture systems such as alginate, agarose, or fibrin systems, irreversible de-differentiation is usually observed in preparations of primary chondrocytes. Hence it is of primary interest to maintain primary chondrocytes as close as possible to their initial chondrogenic profile and/or to improve their capacity to re-differentiate prior to re-implantation.

[0133] This invention shows that a single treatment of primary chondrocytes with rAC, although not improving the yield of primary chondrocytes, greatly improves their chondrogenic phenotype after 2-3 week expansion. In particular, the expression of collagen 2A1, aggrecan, FGF2, and Sox9 was markedly increased in rat cells at the end of the 3-week expansion period (Figures 2A-2B and 3A-3B). In equine and human cells, the expression of these chondrogenic markers was similarly increased (Figures 2A-2C and 4A-4B). This improvement of the phenotype may be explained by the better subcellular localization of the transcription factor Sox9 in the nucleus of cells treated with rAC (Figure 3A-3B), although how a single treatment with rAC influences the nuclear localization of Sox9 over several weeks remains yet unclear. Finally, when grown in three-dimensional culture systems, i.e. collagen sponges or fibrin gels, addition of rAC improved the differentiation of the cells, as shown by H&E and safranin O staining (Figures 5A-5C). Cells were bigger, and the better differentiation was more and more evident with time (Figures 5A and 5B). In addition, the expression of proteoglycans was improved in cells treated with rAC (Figure 5C). Overall, the present Examples demonstrate that a single treatment with rAC improves the phenotype of primary chondrocytes after expansion. It is speculated that these changes are due to the short-term changes in sphingolipid metabolism induced by rAC (Figures 1A-1C), presumably leading to transcriptional and post-translational changes resulting in these downstream effects.

[0134] The main limitations in the use of MSCs for cell-based cartilage repair are that (i) MSCs lose their ability to differentiate into chondrocytes very early (3 or 4 passages), shortening the time for amplification prior to differentiation, and that (ii) proper *ex vivo* and *in vivo* protocols to differentiate MSCs into chondrocytes that are fully functional and do not undergo hypertrophy are still lacking. Numerous articles have been published describing the use of new agents for the differentiation of MSCs toward chondrocytes. Among the candidates, proteins of the TGF β family (TGF β 1, TGF β 3), but also bone morphogenic proteins (BMPs), are the most described. Another strategy relies on the engineering of three-dimensional scaffolds releasing growth factors, anti-apoptotic factors, or differentiation factors, in which the MSCs can be seeded at high density to mimic the natural conditions of differentiation.

[0135] In the present Examples, it is shown that (i) a single addition of rAC into the culture media of rat bone marrow increases the yield of MSCs at one week (Figures 6A-6D), as seen by CFU-F assay and flow cytometry analyses, and (ii) repeated treatment with rAC during the chondrogenic differentiation process improves the quality of the resulting MSC-derived chondrocytes, as seen by alcian blue staining, safranin O staining, and immunostaining (Figures 7A-7D and 8A-8E). Notably, the levels of Sox9, collagen IIA1, and aggrecan were more elevated in cells differentiated in the presence of rAC, but the level of collagen X was also diminished, revealing a partial blockage of hypertrophy in cells differentiated in presence of rAC.

[0136] Thus, two positive influences of rAC treatment were observed with MSCs, although the mechanisms behind these observations might be different. For example, while the treatment at P0 was probably beneficial because the rAC was reducing the stress response induced by the extraction of the cells from their natural environment and their plating in an artificial medium (yielding more viable cells), the benefit of the treatment during the differentiation process may be due to (i) rearrangement of the lipid raft structures at the cell surface and better signaling through the TGF β pathway, (ii) a decrease in ceramide and an increase of sphingosine or SIP, known to play a role in chondrogenesis and cartilage homeostasis, and/or (iii) reduction of the stress response by itself, in particular since the differentiation medium is devoid of serum and hence a very potent stress inducer.

[0137] Overall, the present Examples for the first time show that the addition of rAC to culture media has a positive influence on the chondrogenic phenotype of expanded primary and MSC-derived chondrocytes, likely through alterations of the sphingolipid signaling pathway. These findings could have an important impact on cell-based cartilage repair by providing higher quality cells for transplantation, and/or by including the enzyme directly in three-dimensional scaffolds to improve chondrogenesis *in vivo*.

REFERENCES CITED IN THE DESCRIPTION

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PATENTKRAV

1. Fremgangsmåde *in vitro* til forbedring af fænotypen af en chondrocytpopulation, hvilken fremgangsmåde omfatter:

udvælgelse af en population af chondrocytter fra pattedyr med en mangelfuld chondrocytisk fænotype kendetegnet ved lav ekspresion af collagen 2A1, Sox9, og aggrecan; forhøjet ekspresion af collagen 10; og/eller lav farvning for proteoglycaner med Alcian Blue eller Safranin O; og

behandling af den udvalgte cellepopulation med et ceramidaseprotein *in vitro*, hvor, efter behandlingen, den udvalgte population er kendetegnet ved forhøjet ekspresion af collagen 2A1, Sox9, og aggrecan; reduceret ekspresion af collagen 10; og/eller forbedret farvning for proteoglycaner med Alcian Blue eller Safranin O.

2. Fremgangsmåde *in vitro* ifølge krav 1, hvor chondrocytterne er primære chondrocytter, artikulære chondrocytter, nasale chondrocytter, tracheale chondrocytter, meniscale chondrocytter eller aurale chondrocytter.

3. Fremgangsmåde *in vitro* ifølge krav 1 eller krav 2, hvor chondrocytterne er humane chondrocytter.

4. Fremgangsmåde *in vitro* til fremstilling af chondrocytter, hvilken fremgangsmåde omfatter:

udvælgelse af en population af pattedyreceller med potentiale til at differentiere til chondrocytter udvalgt fra knoglemarvsceller, fibroblaster og mesenchymale stamceller, og

behandling af den udvalgte cellepopulation med et ceramidaseprotein *in vitro* for at transformere én eller flere af cellerne i den udvalgte population til chondrocytter.

5. Fremgangsmåde *in vitro* ifølge krav 4, hvor cellerne er humane celler.

6. Fremgangsmåde *in vitro* ifølge krav 4, hvor den udvalgte cellepopulation omfatter knoglemarvsceller eller mesenchymale stamceller eller består af mesenchymale stamceller.

7. Fremgangsmåde *in vitro* ifølge krav 1 eller krav 4, hvor behandlingen omfatter: dyrkning af den udvalgte cellepopulation i et dyrkningsmedium indeholdende ceramidaseproteinet.

8. Fremgangsmåde *in vitro* ifølge krav 1 eller krav 4, hvor behandlingen udføres på en fast bærer.

9. Fremgangsmåde *in vitro* ifølge krav 4, der endvidere omfatter: behandling af cellerne med TGF β .

10. Fremgangsmåde *in vitro* ifølge et hvilket som helst af de foregående krav, hvor ceramidaseproteinet er udvalgt fra sur ceramidase, neutral ceramidase og alkalisk ceramidase.

11. Fremgangsmåde *in vitro* ifølge krav 10, hvor ceramidasen er et protein, er aktiv ceramidase eller et ceramidaseforløberprotein.

5 12. Fremgangsmåde *in vitro* til fremstilling af en population af chondrocytter, hvilken fremgangsmåde omfatter:

udvælgelse af en population af stamceller, der kan differentiere til chondrocytter

udvælgelse af et differentieringsmedium, der kan stimulere differentiering til de ønskede differentierede celler, og

10 dyrkning *in vitro* af populationen af stamceller i differentieringsmediet og ceramidase protein for at stimulere differentiering til populationen af chondrocytter.

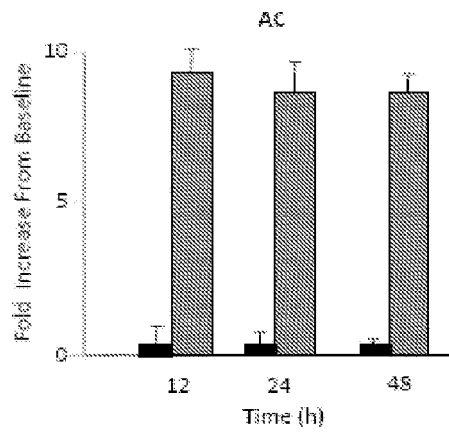
13. Fremgangsmåde *in vitro* ifølge krav 12, hvor stamcellerne er udvalgt fra gruppen bestående af embryostamceller, somatiske stamceller, inducerede pluripotente stamceller, totipotente stamceller, pluripotente stamceller og multipotente stamceller.

15 14. Fremgangsmåde *in vitro* ifølge krav 12 eller krav 13, hvor ceramidaseproteinet er udvalgt fra sur ceramidase, neutral ceramidase og alkalisk ceramidase.

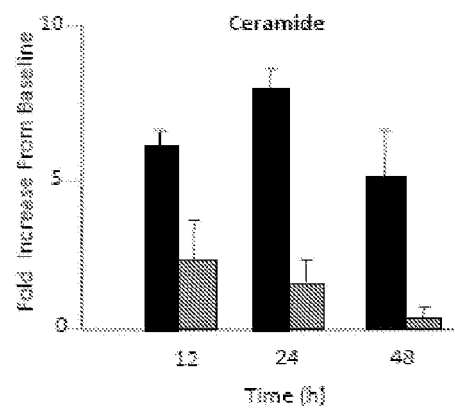
15. Fremgangsmåde *in vitro* ifølge et hvilket som helst af kravene 12 til krav 14, hvor ceramidasen er et protein, er aktiv ceramidase eller et ceramidaseforløberprotein.

DRAWINGS

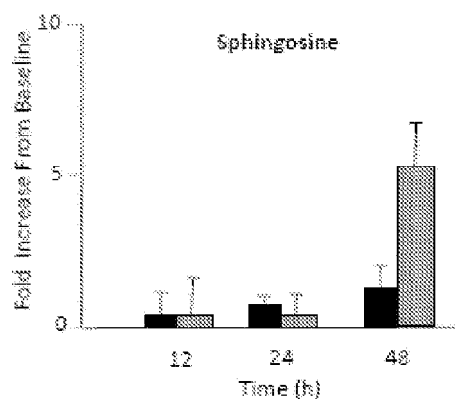
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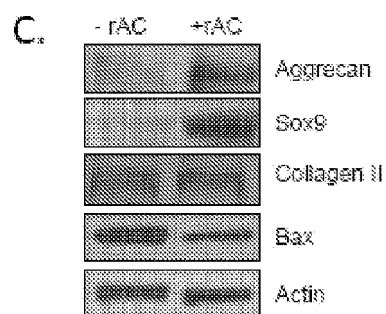
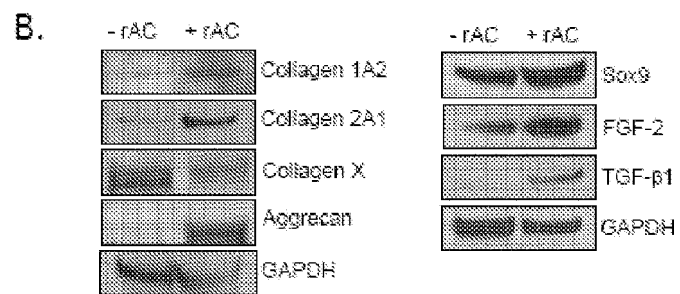
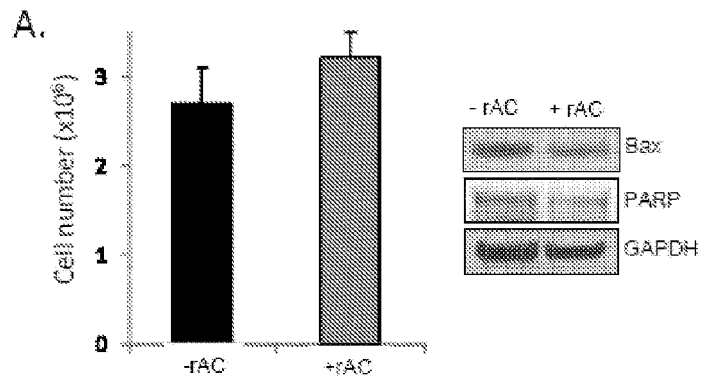
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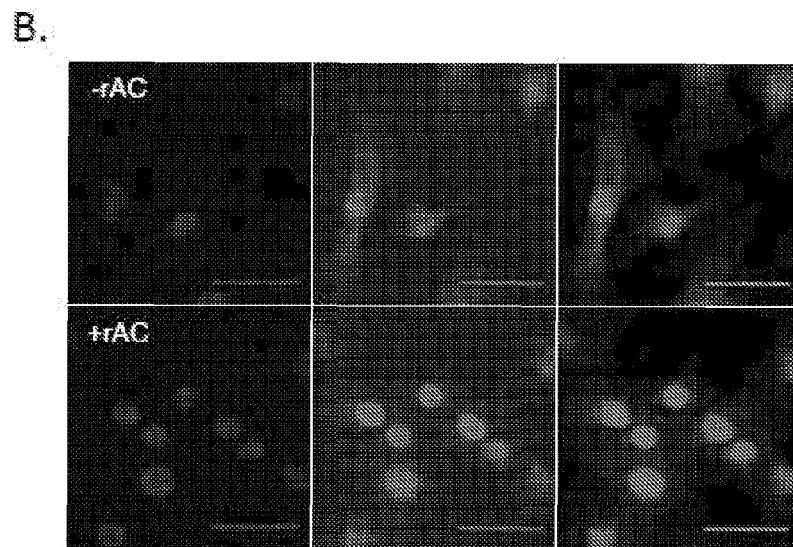
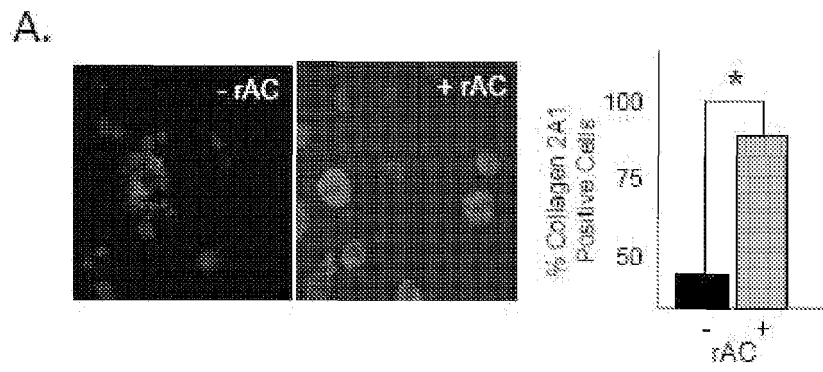
C.



Figures 1A–C



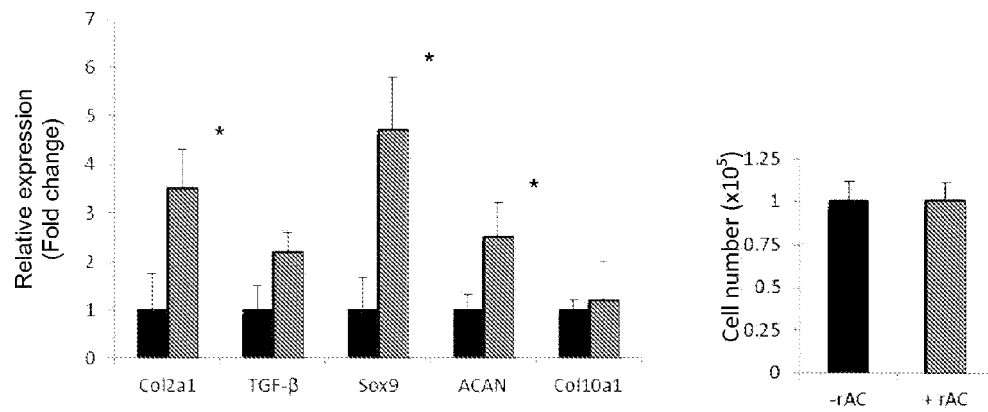
Figures 2A–C



Figures 3A-B

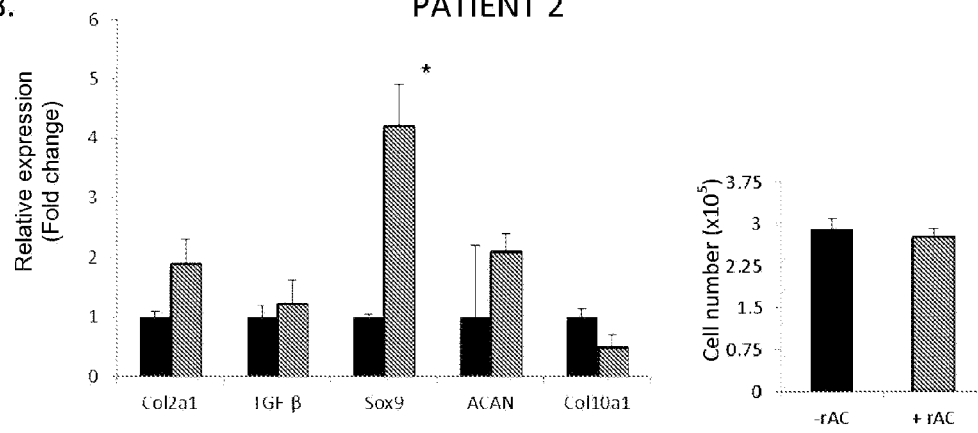
A.

PATIENT 1

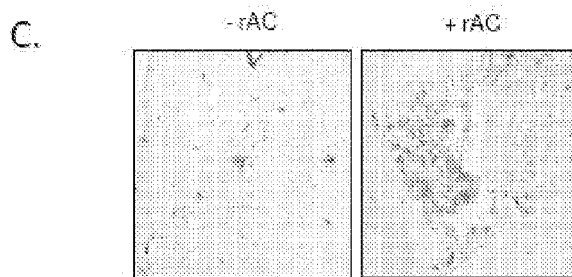
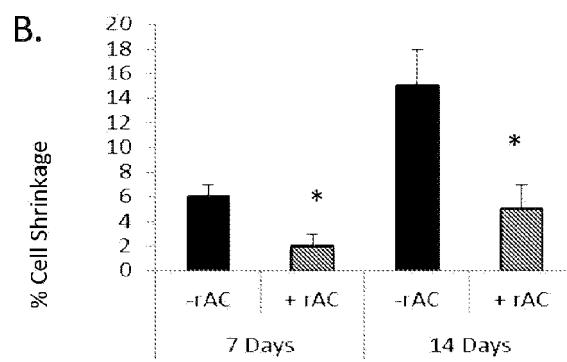
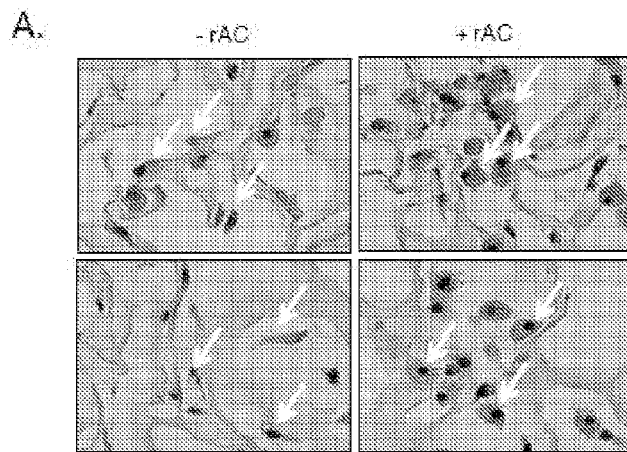


B.

PATIENT 2

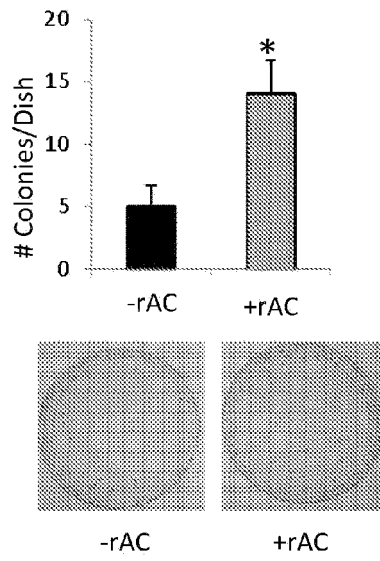


Figures 4A-B

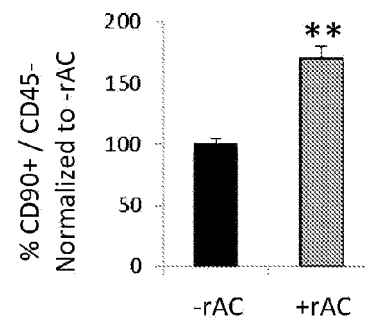


Figures 5A-C

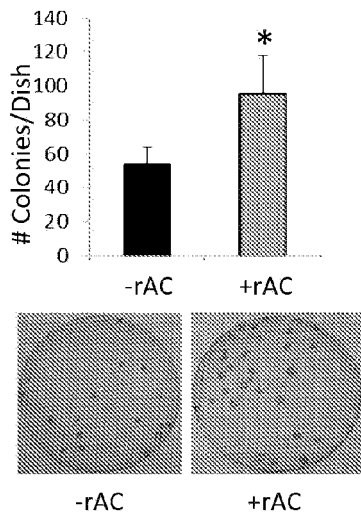
A.



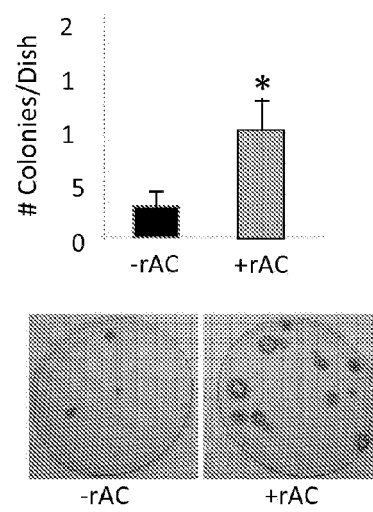
B.



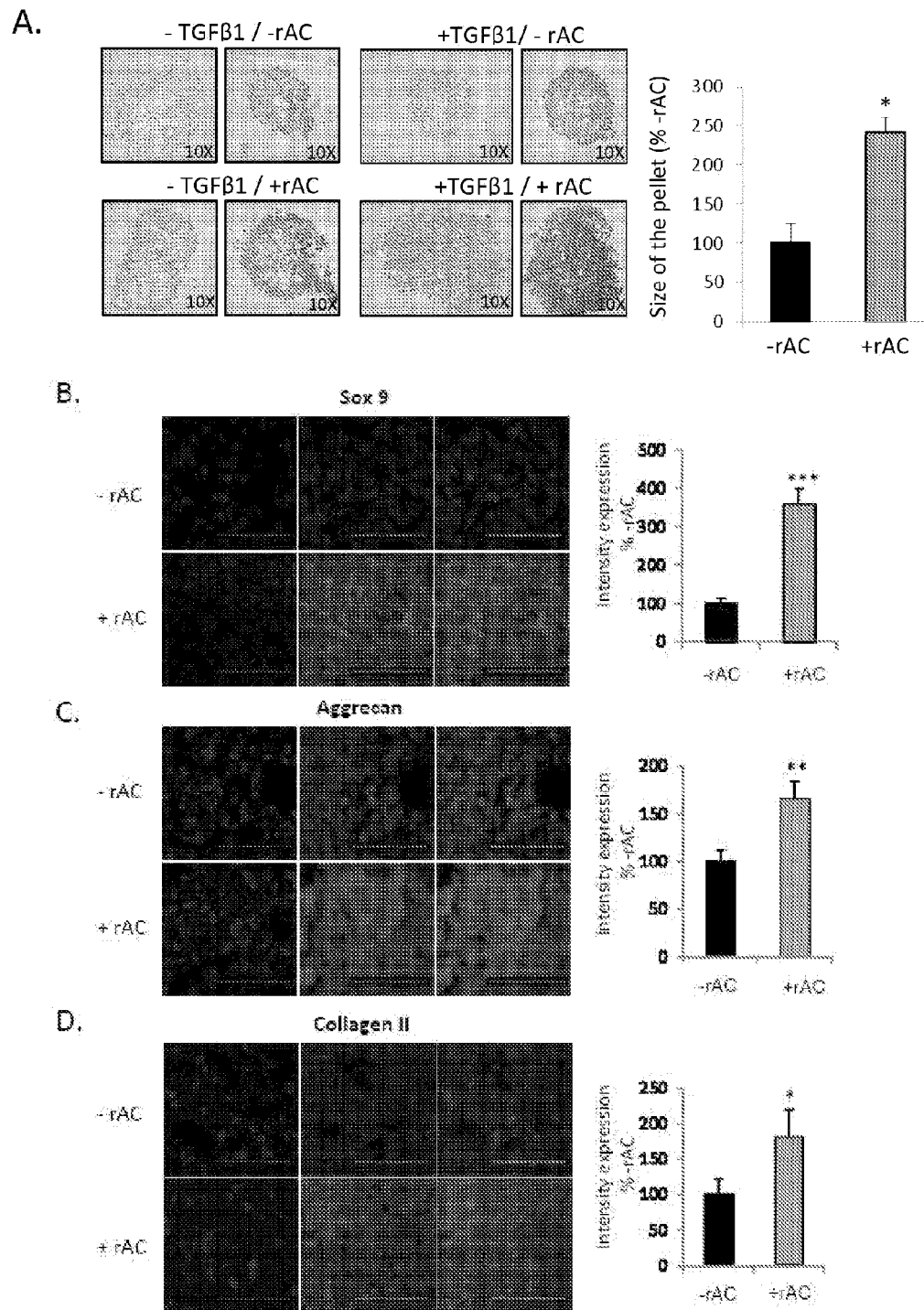
C.



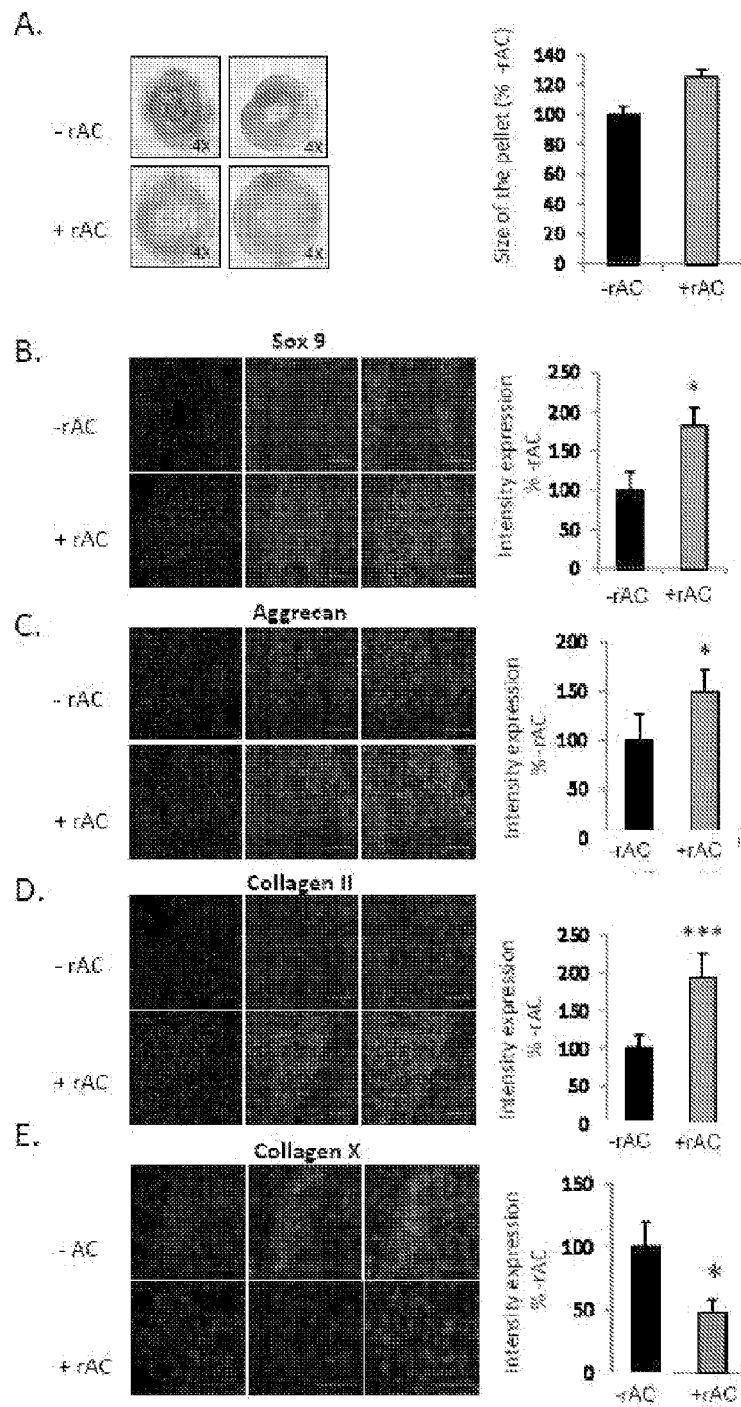
D.



Figures 6A–D



Figures 7A–D



Figures 8A-E