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## TGF- $\beta$ 1, GDF-5, and BMP-2 Stimulation Induces Chondrogenesis in Expanded Human Articular Chondrocytes and Marrow-Derived Stromal Cells

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**Key Words.** Mesenchymal stem cells • Arthritis • Differentiation • Chondrogenesis • Tissue regeneration • Adult stem cells

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### ABSTRACT

Replacement of degenerated cartilage with cell-based cartilage products may offer a long-term solution to halt arthritis' degenerative progression. Chondrocytes are frequently used in cell-based FDA-approved cartilage products; yet human marrow-derived stromal cells (hMSCs) show significant translational potential, reducing donor site morbidity and maintaining their undifferentiated phenotype with expansion. This study sought to investigate the effects of transforming growth factor  $\beta$ 1 (TGF- $\beta$ 1), growth/differentiation factor 5 (GDF-5), and bone morphogenetic protein 2 (BMP-2) during postexpansion chondrogenesis in human articular chondrocytes (hACs) and to compare chondrogenesis in passaged hACs with that of passaged hMSCs. Through serial expansion, chondrocytes dedifferentiated, decreasing expression of chondrogenic genes while increasing expression of fibroblastic genes. However, following expansion, 10 ng/mL TGF- $\beta$ 1, 100 ng/mL GDF-5, or 100 ng/mL BMP-2 supplementation during three-dimensional aggregate culture each upregulated one or more markers of chondrogenic gene expression in both hACs and hMSCs. Additionally, in both cell types, the combination of TGF- $\beta$ 1, GDF-5, and BMP-2 induced the greatest upregulation of chondrogenic genes, that is, Col2A1, Col2A1/Col1A1 ratio, SOX9, and ACAN, and synthesis of cartilage-specific matrix, that is, glycosaminoglycans (GAGs) and ratio of collagen II/I. Finally, TGF- $\beta$ 1, GDF-5, and BMP-2 stimulation yielded mechanically robust cartilage rich in collagen II and GAGs in both cell types, following 4 weeks maturation. This study illustrates notable success in using the self-assembling method to generate robust, scaffold-free neocartilage constructs using expanded hACs and hMSCs. STEM CELLS 2015;33:762–773

### INTRODUCTION

Arthritis is a degenerative pathology affecting one in five adults in the United States [1] and often results in debilitating pain and loss of function. One in 12 adults in the United States reports activity limitations resulting from doctor-diagnosed arthritis [1]. Moreover, age-adjusted prevalence of arthritis is greater in women, compared with men (24.3%, [23.8–24.8] vs. 18.3% [17.7–18.7]) [1]. Current standards of care often result in inferior fibrocartilage formation, warranting multiple revision surgeries and failing to halt the degenerative progression of arthritis [2]. In contrast, replacement of degenerated cartilage with cell-based products would inhibit arthritis' progression and offer patients a long-term solution [2].

Current FDA-approved cell-based cartilage products use the patient's own cells to replace regions of degeneration. In the case of autologous chondrocyte implantation (ACI), cartilage is obtained from the patient during a prelimi-

nary arthroscopic surgery. In ACI procedures, cartilage slices are obtained arthroscopically from a "minor load-bearing area," for example, the upper medial femoral condyle [3, 4]. In the case of allografts, cartilage is frequently obtained from juvenile donors due to the increased cell density and synthetic potential of younger chondrocytes [5–7]. While ACI has demonstrated a positive outcome at 5 years post-op [8], there remain the challenges of donor site morbidity, reduced synthetic potential of chondrocytes with age, and cost and pain associated with multiple surgeries. Nonetheless, all cell-based cartilage products currently use chondrocytes or, to a lesser extent, marrow-derived stromal cells (MSCs). As such, this study sought to address two objectives: (a) to investigate a growth factor regimen to further enhance post-expansion chondrogenesis in human articular chondrocytes and (b) to compare chondrogenesis in expanded human articular chondrocytes (hACs) with human MSCs (hMSCs).

Toward the first objective, ACs may be expanded *in vitro* to increase the donor cell population, although efforts are needed to fully recover the chondrogenic phenotype postexpansion. It is well-documented that chondrocytes demonstrate a shift in phenotype during monolayer expansion. The shift is associated with increased proliferation and decreased chondrogenic matrix secretion, termed dedifferentiation [9–11]. Alterations in cell surface markers coinciding with hAC dedifferentiation in monolayer have been characterized by increases in CD10+, CD105+, CD166+, CD73+, and CD90+ cells [12–14]. This pattern of surface marker expression, inclusive of archetypal markers of MSCs, suggests a more primitive, undifferentiated state [11, 12]. However, the metabolic differences for these two cell types, especially the mechanical and biochemical properties of the resulting extracellular matrix types have yet to be explored. While the increased proliferation occurring during this phenotypic shift enhances the translational potential of the cell source, it is essential to recover the chondrogenic phenotype prior to engineering cartilage.

Toward the second objective, MSCs may be isolated from bone marrow aspirates, indicating their potential in autologous therapies and furthermore suggesting their use in off-the-shelf allogeneic products. Allogeneic MSCs have been shown to persist *in vivo* several months postimplantation suggesting lack of immune recognition and/or cell clearance [15]. Additionally, the immunomodulatory effects of MSCs further enhance the translational potential of the cell source [15, 16]. Efforts have been made toward inducing chondrogenic differentiation of MSCs. However, the field has yet to establish a consistent method for differentiating MSCs toward the generation of mechanically robust cartilage rich in collagen II and glycosaminoglycans (GAGs), paralleling native articular cartilage [17]. As such, MSCs offer a promising cell source for cartilage tissue engineering, but further refinement is needed to determine suitable culture conditions prior to translation to the clinic.

In both dedifferentiated expanded ACs, and in undifferentiated MSCs, efforts have focused on inducing a chondrogenic phenotype postexpansion through medium formulations, three-dimensional culture conditions, and growth factor regimens. Growth factors may be used during monolayer expansion to prime cells, toward modulation of postexpansion cell phenotype [18, 19]. Additionally, postexpansion, three-dimensional culture conditions such as pellet cultures, polymer gels, and rotational cultures have previously resulted in increases in cartilage-specific matrix expression [10, 18–23]. In the case of aggregate culture, a single-cell suspension is seeded in a nonadherent dish, in rotation, allowing the cells to form suspended aggregates. Aggregate culture of fourth passage chondrocytes for 1 week has significantly increased collagen and GAG content, and neocartilage tensile properties, compared with no aggregation and with pellet cultured cells [22]. Aggregate culture may offer a promising strategy for recovering the chondrocyte phenotype and for differentiating MSCs toward the chondrogenic lineage. Additionally, aggregate culture may be combined with growth factors to induce robust chondrogenesis *in vitro*.

As modulators of cartilage morphogenesis *in vivo*, the transforming growth factor  $\beta$  (TGF- $\beta$ )/bone morphogenetic protein (BMP) superfamily of growth factors offers substantial promise in modulating chondrogenic differentiation of MSCs and redifferentiation of expanded hACs. The TGF- $\beta$ /BMP super-

family alters the chondrogenic potential of marrow-derived mesenchymal progenitor cells, as well as the proliferation, differentiation, and extracellular matrix synthesis of ACs [24]. Members of the [TGF- $\beta$ /BMP] superfamily, such as TGF- $\beta$ 1, growth/differentiation factor 5 (GDF-5), and BMP-2, play critical roles in cartilage morphogenesis [25]. GDF-5, also known as cartilage-derived morphogenetic protein 1, has been shown to induce mesenchyme condensation and chondrogenesis in rat limb bud cells [26]. Additionally, GDF-5 expression in human articular cartilage has been shown to inhibit catabolic matrix metalloproteinase-13 (MMP13) expression while stimulating anabolic SOX9 and ACAN expression in a dose dependent manner. Specifically, 100 ng/mL GDF-5 robustly increased ACAN expression and GAG production, compared with 10 ng/mL [27]. TGF- $\beta$ 1 has been shown to increase chondrogenic differentiation of MSCs in a dose-dependent manner [28], and this effect is heightened in three-dimensional culture conditions [29]. Both GDF-5 and TGF- $\beta$ 1 stimulate cartilage formation *in vitro*, including upregulation of aggrecan synthesis [26, 30]. BMP-2 is natively expressed during limb bud formation [31]. *In vitro*, BMP-2, particularly in combination with TGF- $\beta$ , has been shown to induce expression of genes critical to cartilage formation in hMSCs [32]. Specifically, in bovine synovium-derived mesenchymal stem cells, 10 ng/mL TGF- $\beta$ 1 enhanced 200 ng/mL BMP-2-induced GAG production and arrested the downstream differentiation of cells toward generating a mature, articular type of cartilage [33]. TGF- $\beta$ 1, GDF-5, and BMP-2 demonstrate promise in upregulating postexpansion chondrogenesis in both hMSCs and dedifferentiated hACs. These factors modulate cartilage formation *in vivo* via interrelated signaling pathways, and as such, the interactions between the factors are of significant interest to *in vitro* neocartilage formation.

This study sought to investigate and compare the chondrogenic potential of the hACs and hMSCs in response to TGF- $\beta$ 1, GDF-5, and BMP-2 stimulation during aggregate culture, in a full factorial design, toward the generation of mechanically robust, self-assembled neocartilage. Chondroinduction was assessed immediately following aggregate culture (gene expression) and 2 weeks following neocartilage self-assembly (matrix synthesis). Carrying forward the superior biochemical stimulation regimen, neocartilage was matured for 4 weeks for each cell type and characterized biochemically and mechanically. It was first hypothesized that second passage chondrocytes would demonstrate evidence of dedifferentiation through serial monolayer expansion, characterized by decreased chondrogenic matrix synthesis. Second, it was hypothesized that for hACs and hMSCs, TGF- $\beta$ 1, GDF-5, and BMP-2 would each independently enhance chondrogenic gene expression and matrix synthesis. Furthermore, it was expected that a combined TGF- $\beta$ 1, GDF-5, and BMP-2 treatment would lead to the greatest upregulation of chondrogenic genes and protein synthesis after 2 weeks. Finally, carrying forward the superior growth factor regimen, it was hypothesized that both cell types would similarly generate mechanically robust cartilage rich in collagen II and GAGs.

## MATERIALS AND METHODS

### Chondrocyte Isolation and Expansion

Human articular cartilage was obtained within 48 hours post mortem (28 years of age, male) (Musculoskeletal Transplant

Foundation, Kansas City, MO, <http://www.mtf.org>). Cartilage was minced and digested in 0.2% collagenase type II (Worthington, Lakewood, NJ, <http://www.worthington-biochem.com>) with 3% fetal bovine serum (FBS, Atlanta Biologicals, Lawrenceville, GA, <https://www.atlantabio.com>) for 18 hours at 37°C in chemically defined chondrogenic culture medium (CHG) composed of Dulbecco's modified Eagle's medium (DMEM), high glucose, GlutaMAX Supplement (Gibco, Grand Island, NY, <http://www.invitrogen.com>), 1% penicillin-streptomycin-fungizone (BD Biosciences, Bedford, MA, <http://www.bdbiosciences.com>), 1% insulin, transferrin, and selenous acid (ITS+) premix (BD Biosciences), 1% nonessential amino acids (Gibco), 100 nM dexamethasone, 50 µg/mL ascorbate-2-phosphate, 40 µg/mL L-proline, and 100 µg/mL sodium pyruvate [34]. Cells were filtered (70 µm filter, BD Biosciences), counted, and stored frozen in liquid nitrogen until use. Chondrocytes were expanded to second passage (P2) in CHG supplemented with 2% FBS, 1 ng/mL TGF-β1 (Peprotech, Rocky Hills, NJ, <http://www.peprotech.com>), 10 ng/mL platelet-derived growth factor (PDGF, Peprotech), and 5 ng/mL basic fibroblastic growth factor (bFGF, Peprotech). The growth factor cocktail of TGF-β1, bFGF, and PDGF-bb has been shown to increase proliferation and postexpansion chondrogenic potential in osteoarthritic and nondiseased hACs as well as ear and nasal chondrocytes [19, 35–37]. At 80%–90% confluence, cells were passaged twice using 0.5% Trypsin-EDTA (Gibco) for 7 minutes, followed by 0.2% collagenase type II solution (as described above) for 20 minutes.

First passage adult human marrow stromal cells (28 years of age, male) were obtained from the Texas A&M Health Science Center College of Medicine Institute for Regenerative Medicine (Temple, TX, <http://medicine.tamhsc.edu/irm/>). Cells were expanded to third passage (P3) as described above in 60:40 DMEM, low glucose, GlutaMAX Supplement (Gibco): Ham's F-12 Nutrient Mix, GlutaMAX Supplement, 2% FBS, 1% penicillin-streptomycin-fungizone (BD Biosciences), 1% ITS+ premix (BD Biosciences), 1 nM dexamethasone, 29 µg/mL ascorbate-2-phosphate, supplemented with 10 ng/mL TGF-β1 and 20 ng/mL bFGF [38].

From primary to P1, hACs experienced four population doublings, and, from P1 to P2, 4.4 population doublings occurred. hMSCs were obtained at P1, with an average six to seven population doublings reported from primary to P1. From P1 to P2, hMSCs experienced 4.8 population doublings, and, from P2 to P3, hMSCs experienced 3.5 population doublings. hACs experienced a total of 8.4 population doublings, while hMSCs experienced a total of approximately 14.7 population doublings.

### Postexpansion Chondrogenic Differentiation

After passaging, hACs and hMSCs were independently maintained in aggregate culture for 7 days. During aggregate culture, cells were maintained on agarose-coated plates (1% agarose in phosphate buffered saline (PBS), Fisher Scientific, Fair Lawn, NJ, <http://www.fishersci.com>) at 750,000 cells per mL in CHG. In a full-factorial design, cells were maintained in CHG medium alone (CM) or CHG was supplemented with (a) 10 ng/mL TGF-β1, (b) 100 ng/mL GDF-5 (Shenandoah Biotechnology, Warwick, PA, <http://www.shenandoah-bt.com>), or (c) 100 ng/mL BMP-2A (Shenandoah Biotechnology), or all resulting combinations.

After 7 days, aggregates were digested for 45 minutes in 0.5% Trypsin-EDTA, followed by 30 minutes in 0.2% collagenase type II solution (as described above). Digestion resulted in a single-cell suspension. A subset of cells were analyzed for gene expression, and the remainder were used in neocartilage formation.

### Gene Expression Following Chondrogenic Differentiation

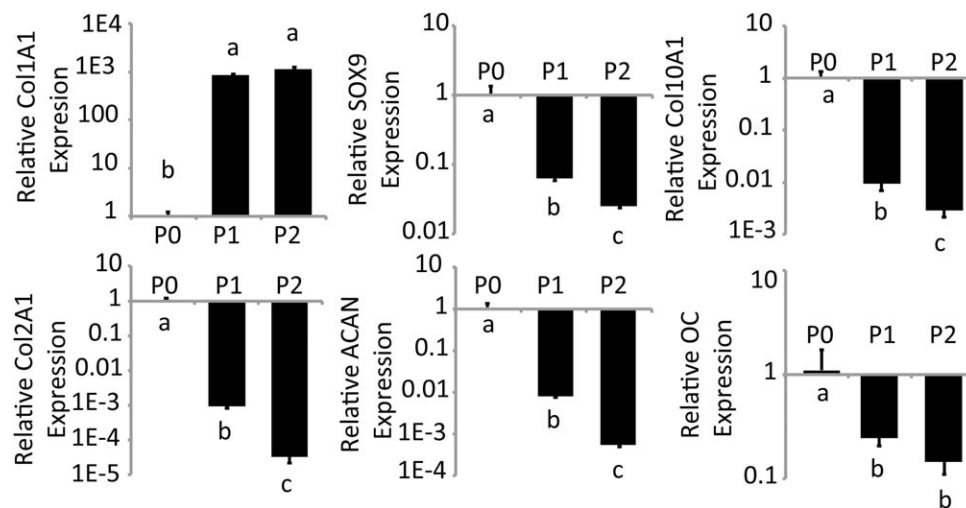
Following aggregate digestion, cells from each treatment were stored in lysis buffer (Invitrogen, Carlsbad, CA, <http://www.invitrogen.com>) ( $1 \times 10^6$  cells per mL) at  $-80^\circ\text{C}$  until use. Primary ACs were similarly stored in lysis buffer following cell isolation as described above. RNA extraction was performed using RNAqueous-micro kit (Invitrogen). Reverse transcription was then performed to generate cDNA using Superscript III First Strand Synthesis Supermix (Invitrogen) for quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). qRT-PCR was carried out using PCR Mastermix (TaqMan Gene Expression Assays, Applied Biosystems, Carlsbad, CA, <http://www.appliedbiosystems.com>) in a Corbett thermocycler (Corbett Research, Redwood City, CA, <http://www.corbettlifescience.com>) for the following genes: Col1A1 (Hs01076777\_m1), Col2A1 (Hs00264051\_m1), ACAN (Hs00153936\_m1), SOX9 (Hs00165814\_m1), Col10A1 (Hs00166657\_m1), OC (Hs01587814\_g1), and EF1a (Hs00265885\_g1, housekeeping gene). Col1A1 is a marker of a fibroblastic phenotype while Col2A1 and ACAN are markers of chondrogenic phenotype. SOX9 is a transcription factor modulating chondrogenic differentiation and matrix synthesis. Col10A1 is a marker of a hypertrophic phenotype and OC, also known as BGLAP or osteocalcin, is a marker of osteoblastic differentiation. The  $\Delta\Delta C_t$  method was used to analyze the relative changes in gene expression, as detailed in legends of Figures 1–3 [39].

### Neocartilage Self-Assembly

Neocartilage was formed following the self-assembling process in nonadherent agarose wells [40]. A custom stainless steel mold was constructed with 5 mm diameter cylindrical prongs. The mold was sterilized and immersed in 900 µL of sterile molten 2% molecular biology grade agarose (Fisher Scientific) in PBS in each well of a 48-well plate. Agarose solidified at room temperature for 17 minutes and four exchanges of CHG were used to saturate the wells with medium. For both hAC and hMSC neocartilage formation,  $2 \times 10^6$  cells were added to each well in 100 µL of CHG (cell suspension:  $2 \times 10^7$  cells per mL) supplemented with 200 units/mL hyaluronidase type I-S from bovine testes (Sigma Aldrich, St. Louis, MO, <https://www.sigmaaldrich.com>) and 2 µM cytochalasin D (Enzo Life Sciences, Farmingdale NY). Medium was added to each well 4 hours after seeding and subsequently was exchanged every 24 hours. For the first 72 hours, CHG was supplemented with 2 µM cytochalasin D.

### Histological Evaluation

After 2 weeks in culture, hAC and hMSC neocartilage was evaluated histologically. Samples were frozen in HistoPrep Frozen Tissue Embedding Media (Fisher Scientific) and cryosectioned to 14 µm. Samples were stained with Safranin-O/fast-green for GAGs, picosirius red for collagen, and alizarin red to detect calcification. The presence of collagens I, II, and X were detected immunohistochemically as previously described [40]. Rabbit



**Figure 1.** Articular chondrocyte dedifferentiation in monolayer. Gene expression in serial passages is presented relative to expression in primary chondrocytes. Passage 1 (P1) and passage 2 (P2) cells demonstrate progressive loss in chondrogenic gene expression (Col2A1, SOX9, and ACAN) and an increase in fibroblastic gene expression (Col1A1). Hypertrophic genes (Col10A1, OC) decrease with passage. Data are presented mean  $\pm$  SD. All groups not connected by a common letter are significantly different.

anti-type I collagen polyclonal antibody at 1:200 dilution (Fitzgerald Industries International, Acton, MA, [www.fitzgerald-fii.com](http://www.fitzgerald-fii.com)) was used to detect collagen I. Rabbit anti-human type II collagen polyclonal antibody at 1:200 dilution (Fitzgerald Industries International) was used to detect collagen II. Mouse monoclonal anti-type X collagen antibody at 1:300 dilution (Abcam, Cambridge, MA, <http://www.abcam.com>) was used to detect collagen X. In all histological analyses, human bone and articular cartilage were used as staining controls.

### Mechanical and Biochemical Construct Evaluation

Based on gene expression and histological analysis at 2 weeks, the aggregate culture stimulation regimen demonstrating the greatest increase in markers of chondrogenic gene expression and matrix synthesis was identified. Carrying forward this regimen, hAC and MSC constructs were self-assembled and maintained in culture for 4 weeks and evaluated mechanically and biochemically.

Mechanical properties of constructs were assessed in tension and compression. Tensile testing was conducted using a Test Resources 840L. Samples were strained at a constant strain rate of 1% per second. Young's modulus ( $E_y$ ) and ultimate tensile strength were evaluated using a custom MATLAB program. Compressive testing was performed using an Instron 5565. Samples were preconditioned with 15 cycles of 5% compressive strain and then tested in 10% and 20% sequential stress-relaxation. A Kelvin solid viscoelastic model was fit to the data to establish the following compressive material properties at each strain level: instantaneous modulus ( $E_i$ ), relaxation modulus ( $E_r$ ), and coefficient of viscosity ( $\mu$ ) [41]; viscoelastic models may be used to characterize the time-dependent mechanical properties of articular cartilage. A stress-relaxation modality under unconfined compression [42–44] allows for the extraction of compressive instantaneous and relaxation moduli as well as the coefficient of viscosity. In a recent study by our group [45] we demonstrate the equivalence of a Kelvin solid viscoelastic model to a linear biphasic model within the strain ranges used here. In this

model, a combination of elastic and viscous elements is used to characterize both the solid and fluid fractions of the matrix and the time-dependence of the load response.

Tissues were evaluated biochemically for collagen content, GAG content, and cellularity. Samples were weighed before and after lyophilization and digested in 125  $\mu$ g/mL papain (Sigma) in 50 mM phosphate buffer (pH 6.5) containing 2 mM *N*-acetyl cysteine (Sigma) and 2 mM EDTA for 18 hours at 60°C. Collagen content was quantified using a modified chloramine-T hydroxyproline assay and Sircol type I collagen standard. Samples were hydrolyzed with 2 N NaOH for 20 minutes at 110°C and neutralized with 2 N HCl. Reaction with chloramine-T for 20 minutes at room temperature was followed by reaction with Ehrlich's aldehyde solution at 65°C for 20 minutes [46]. GAG content was measured using Blyscan GAG assay (Bicolor, Westbury, NY) based on 1,9-dimethylmethylene blue binding to the sulfated polysaccharide component of proteoglycans.

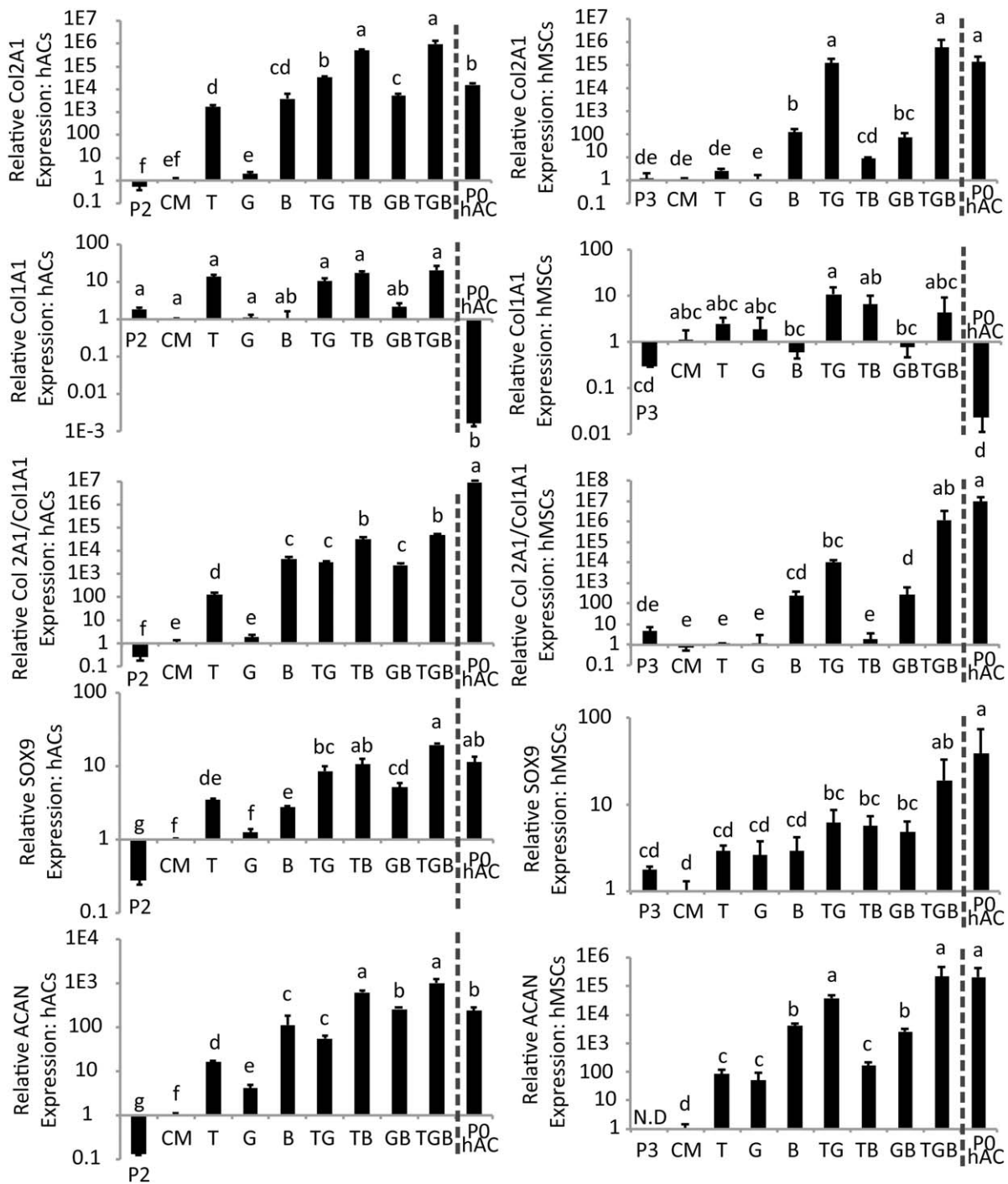
### Statistical Analysis

Gene expression, biochemical tissue content, and mechanical properties were presented as mean  $\pm$  SD. Gene expression was analyzed in a one-way ANOVA, followed by Tukey's post-hoc test where warranted ( $p < .05$ ). Due to the magnitude of differences in mRNA levels between experimental groups, statistical significance was determined using the logarithm of the values.

## RESULTS

### AC Dedifferentiation in Monolayer

hAC gene expression was assessed in primary and first and second passage cells (Fig. 1). With increasing passage number, ACs demonstrated decreased chondrogenic gene expression. Normalized to mRNA expression in primary chondrocytes, first and second passage cells demonstrated progressive, significant decreases in Col2A1, SOX9, and ACAN expression. On the contrary, Col1A1 expression significantly increased in first and second passage cells.

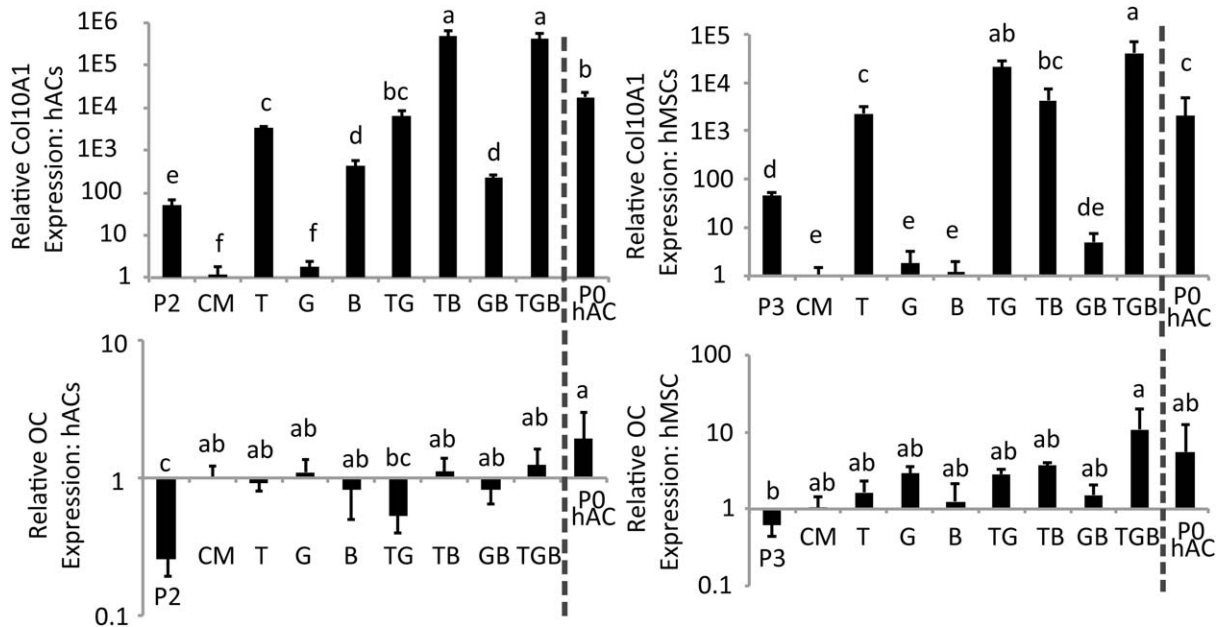


**Figure 2.** Chondrogenic gene expression. hACs and hMSCs passaged prior to aggregate culture (P2/P3, respectively) and following aggregate culture in the absence of growth factors (CM) or in the presence of TGF- $\beta$ 1 (T), GDF-5 (G), or BMP-2 (B), and all resulting combinations. Primary hAC (PO hAC) is presented as a control. Gene expression is presented relative to aggregate culture in the absence of growth factors (CM). As single factors, BMP-2 led to the greatest upregulation of chondrogenic gene expression in both cell types. Additionally, TGF- $\beta$ 1 significantly increased chondrogenic gene expression in hACs, while its effects were less robust in hMSCs. In both cell types, TGF- $\beta$ 1/GDF-5/BMP-2 combined treatment led to the greatest increase in chondrogenic gene expression. Data are presented mean  $\pm$  SD. All groups not connected by a common letter are significantly different ( $p < .05$ ). Abbreviations: hACs, human articular chondrocytes; hMSCs, human marrow-derived stromal cells.

### hAC Chondrogenic Redifferentiation Following Aggregate Culture

Gene expression was measured in primary hACs, in hACs following monolayer expansion (P2), and in hACs following aggregate culture in the absence (CM) or presence of exoge-

nous factors. mRNA expression is presented in Figure 2, normalized to CM. Compared with P2 cells, CM significantly increased relative Col2A1/Col1A1, SOX9, and ACAN expression. Chondrogenic gene expression was further enhanced in the presence of exogenous factors. Col2A1, Col2A1/Col1A1 ratio, SOX9, and ACAN expression were significantly increased in the



**Figure 3.** Hypertrophic gene expression. hACs and hMSCs passaged prior to aggregate culture (P2/P3, respectively) and following aggregate culture in the absence of growth factors (CM) or in the presence of TGF- $\beta$ 1 (T), GDF-5 (G), or BMP-2 (B), and all resulting combinations. Primary hAC (P0 hAC) is presented as a control. Gene expression is presented relative to aggregate culture in the absence of growth factors (CM). As a single factor, TGF- $\beta$ 1 led to the greatest upregulation of Col10A1 expression in both cell types. OC expression was not significantly altered in the presence of growth factors. Data are presented mean  $\pm$  SD. All groups not connected by a common letter are significantly different ( $p < .05$ ). Abbreviations: hACs, human articular chondrocytes; hMSCs, human marrow-derived stromal cells.

presence of TGF- $\beta$ 1 and BMP-2 single treatments. GDF-5 alone significantly increased only ACAN expression. Combined TGF- $\beta$ 1/BMP-2 and TGF- $\beta$ 1/BMP-2/GDF-5 significantly increased chondrogenic gene expression (Col2A1, Col2A1/Col1A1 ratio, SOX9, and ACAN expression) over single treatments, respectively. No significant differences were observed between TGF- $\beta$ 1/BMP-2 and TGF- $\beta$ 1/GDF-5/BMP-2 combinatorial treatments. Notably, TGF- $\beta$ 1/GDF-5/BMP-2 treatment led to significantly greater Col2A1 and ACAN expression compared with primary hACs.

### MSC Chondrogenesis Following Aggregate Culture

Gene expression in hMSCs was quantified following monolayer expansion (P3) and aggregate culture, in the absence (CM) or presence of exogenous factors and compared with primary hACs. mRNA expression was normalized to CM (Fig. 2). No significant differences were observed between P3 and CM, with the exception of ACAN expression, which was not detected in P3 cells. However, BMP-2 significantly increased Col2A1, Col2A1/Col1A1 ratio, and ACAN expression. Combined TGF- $\beta$ 1/GDF-5 led to a significant interaction, significantly increasing Col2A1, Col2A1/Col1A1 ratio, SOX9, and ACAN expression over CM. The greatest increase in Col2A1, Col2A1/Col1A1 ratio, SOX9, and ACAN gene expression was observed in the presence of TGF- $\beta$ 1/GDF-5/BMP-2. Most notably, no significant differences were observed between TGF- $\beta$ 1/GDF-5/BMP-2 and primary ACs in relative expression of chondrogenic markers: Col2A1, Col2A1/Col1A1 ratio, SOX9, and ACAN.

### hAC and MSC Hypertrophic Gene Expression

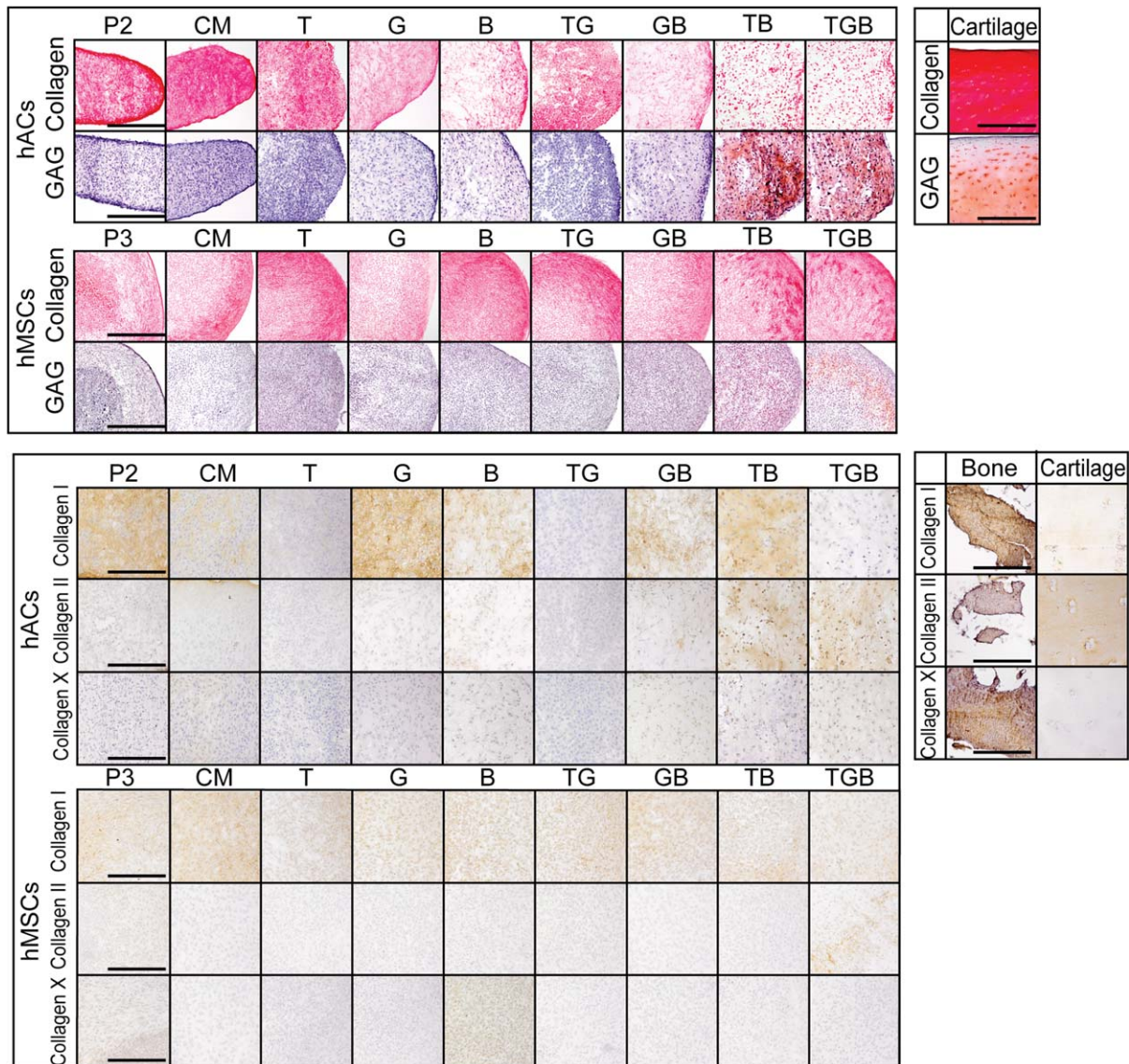
Relative expression of Col10A1 and OC in hACs and hMSCs is presented in Figure 3, normalized to expression in CM, for the respective cell types. In hACs, TGF- $\beta$ 1 and BMP-2 signifi-

cantly upregulated Col10A1 expression, although the expression level remained significantly less than that of primary ACs. Combined TGF- $\beta$ 1/BMP-2 and TGF- $\beta$ 1/GDF-5/BMP-2 led to the greatest increase in Col10A1. In hACs OC expression was not altered in the presence of exogenous factors, compared with chondrogenic medium alone, and relative expression was not significantly different from primary ACs, excluding the TGF- $\beta$ 1/GDF-5 combinatorial treatment. In hMSCs, TGF- $\beta$ 1 was the most potent single factor stimulus of Col10A1 expression. OC expression in hMSCs was not significantly different in the presence of exogenous factors, compared with CM.

### Matrix Synthesis Following Aggregate Culture

hAC and hMSC constructs were self-assembled following aggregate culture and evaluated histologically following 2 weeks in vitro culture (Fig. 4). All hAC constructs stained positively for collagen. In hAC constructs, notable increases in GAG staining were present in TGF- $\beta$ 1/BMP-2 and TGF- $\beta$ 1/GDF-5/BMP-2 treatments. With increased GAG in these two treatments, collagen staining appeared more diffuse. In hACs, collagen I staining was greatest following aggregate culture in chondrogenic medium alone. Collagen II staining was most intense in TGF- $\beta$ 1/BMP-2 and TGF- $\beta$ 1/GDF-5/BMP-2 combinatorial treatments. Collagen X staining appeared negligible in all groups.

hMSC constructs stained intensely for collagen. GAG staining was notably increased in TGF- $\beta$ 1/GDF-5/BMP-2 combinatorial treatment. Constructs stained positively for collagen I, independent of treatment. Collagen II staining was diffusely present in TGF- $\beta$ 1/BMP-2 combined treatment and most intense in TGF- $\beta$ 1/GDF-5/BMP-2 treatment. Collagen X



**Figure 4.** Histological evaluation of constructs at 2 weeks. hACs and hMSCs passaged prior to aggregate culture (P2/P3, respectively) and following aggregate culture in the absence of growth factors (CM) or in the presence of TGF- $\beta$ 1 (T), GDF-5 (G), or BMP-2 (B), and all resulting combinations. Picrosirius red was used to detect collagen, and Safranin-O/fast green was used to detect GAG. Scale bar = 500  $\mu$ m. Immunohistochemistry was used to detect collagen I, II, and X. Scale bar = 200  $\mu$ m. In hAC constructs, TGF- $\beta$ 1/BMP-2 and TGF- $\beta$ 1/GDF-5/BMP-2 led to the greatest increase in GAG and collagen II staining while TGF- $\beta$ 1/GDF-5/BMP-2 led to the greatest increases in hMSCs. Abbreviations: GAG, glycosaminoglycan; hACs, human articular chondrocytes; hMSCs, human marrow-derived stromal cells.

collagen was negligible in all treatments. However, staining was more intense in BMP-2 treatment.

#### hAC and MSC Neocartilage Maturation

Based upon increased chondrogenic gene expression and matrix production over single treatments and dual treatments, full TGF- $\beta$ 1/GDF-5/BMP-2 treatment was carried forward in both hACs and hMSCs and constructs were matured for 4 weeks.

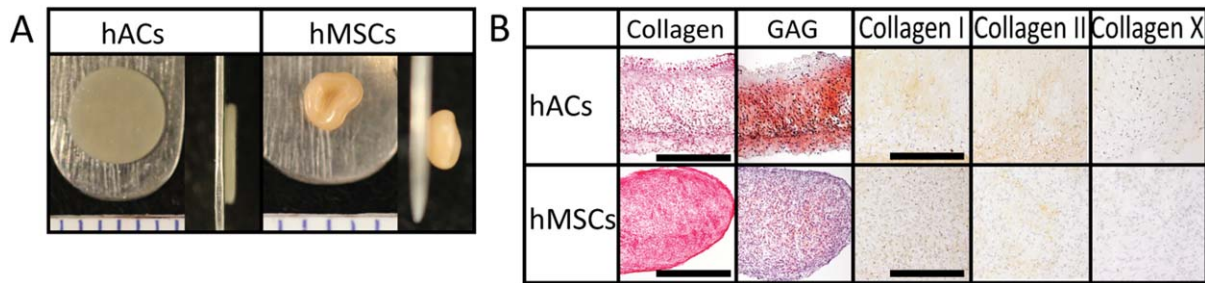
hACs and hMSCs generated robust cartilaginous tissues (Fig. 5) after 4 weeks. hAC constructs were flat, cylindrical tissues. Initially seeded in 5 mm diameter wells, the final diameter of hAC constructs was  $5.2 \pm 0.8$  mm, with a measured circumference of  $16.3 \pm 0.3$ . In contrast, throughout the 4 weeks culture, hMSC constructs contracted notably. Matrix

remained homogeneous throughout the tissue thickness. While the hMSC construct diameter was inconsistent, the circumference was measured to be  $7.5 \pm 0.2$  mm.

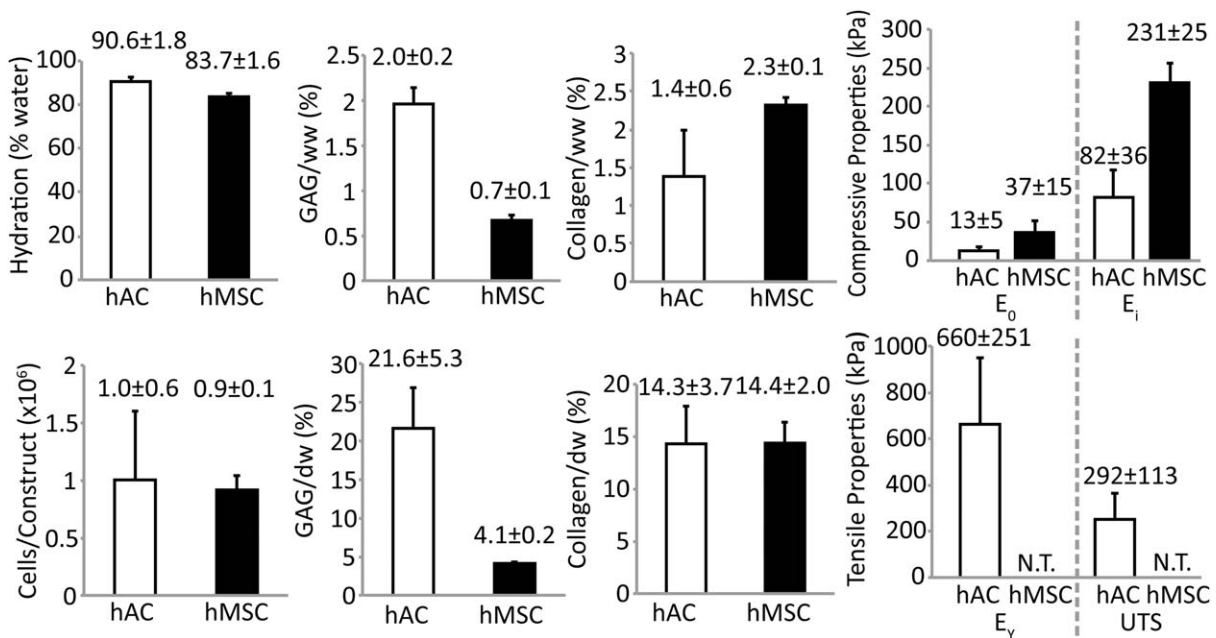
Constructs demonstrated collagen II and GAG, independent of cell type (Fig. 5). Picrosirius red and Safranin-O revealed the presence of collagen and GAG, respectively. hAC constructs demonstrated intense GAG staining, while hMSC constructs demonstrated intense collagen staining. Both cell types stained positively for collagens I and II. Collagen X was minimally apparent in hAC constructs and not detectable in hMSC constructs.

Engineered constructs were evaluated quantitatively for tissue hydration, cellularity, matrix synthesis, and mechanical properties (Fig. 6). Both cell types generated constructs with collagen content of approximately 14% per tissue dry weight.





**Figure 5.** Gross morphology and histology of constructs at 4 weeks. **(A):** hAC constructs demonstrated a flat, cylindrical morphology. hMSC constructs were misshapen and demonstrated evidence of matrix contraction. Morphology ruler segments: 1 mm. **(B):** Picrosirius red was used to detect collagen, and Safranin-O/fast green was used to detect GAG. Scale bar = 500  $\mu$ m. Immunohistochemistry was used to detect collagens I, II, and X. Scale bar = 200  $\mu$ m. For both cell types, constructs stained positively for GAG and collagens I and II. Collagen X was undetectable in hMSC constructs and minimally detectable in hAC constructs. Abbreviations: GAG, glycosaminoglycan; hACs, human articular chondrocytes; hMSCs, human marrow-derived stromal cells.



**Figure 6.** Matrix content and mechanical properties of constructs at 4 weeks. Data are presented mean  $\pm$  SD. hMSC constructs were not testable (N.T.) in tension due to the gross morphology of the tissue. Abbreviations: hACs, human articular chondrocytes; hMSCs, human marrow-derived stromal cells; UTS, ultimate tensile strength.

The GAG content of hAC constructs was more than two times that of hMSC constructs. hAC constructs were evaluated mechanically in both tension and compression. hMSC constructs were evaluated in only compression, as the morphology did not allow tensile specimens to be obtained. hAC constructs demonstrated an average relaxation modulus of 13 kPa and an average instantaneous modulus of 82 kPa. Tensile stiffness and strength were on average 660 kPa and 292 kPa, respectively. hMSC constructs demonstrated an average relaxation modulus of 37 kPa and an average instantaneous modulus more than 231 kPa.

**DISCUSSION**

This study sought to investigate a regimen of TGF- $\beta$ /BMP superfamily factors to enhance chondrogenic gene expression and matrix synthesis in hACs and hMSCs and to generate mechanically robust neocartilage employing the self-assembling method in both cell types. First, it was confirmed that hACs

dedifferentiated with serial expansion, showing decreased expression of chondrogenic genes and increased expression of fibroblastic genes. Confirming the hypothesis, TGF- $\beta$ 1, GDF-5, and BMP-2 each upregulated one or more markers of chondrogenic gene expression in both hACs and hMSCs. Both cell types responded superiorly to combinatorial TGF- $\beta$ 1/GDF-5/BMP-2 stimulation evidenced by the significant upregulation of chondrogenic genes and cartilage-specific matrix synthesis. Finally, following aggregate culture supplemented with TGF- $\beta$ 1/GDF-5/BMP-2, mechanically robust cartilage demonstrating the presence of collagen II and GAG was generated in both cell types. hMSCs generated constructs with superior compressive properties, yet the gross morphology of hAC constructs was more reminiscent of hyaline articular cartilage. Both hMSCs and hACs offer promising cell sources in cartilage tissue engineering, with chondrogenesis similarly enhanced in the presence of TGF- $\beta$ 1, GDF-5, and BMP-2. This work represents the first time the self-assembling method was used to generate mechanically robust neocartilage, using hACs and hMSCs.

hACs demonstrated evidence of dedifferentiation through serial passages commencing as early as P1 [12, 47]. Specifically, expression of chondrogenic genes Col2A1, SOX9, and ACAN significantly decreased in P1 and P2 cells, progressively, while Col1A1 gene expression significantly increased in P1 and P2 cells. Gene expression in this study parallels prior observations in which chondrocytes demonstrated increased proliferative potential with loss of the differentiated chondrocyte phenotype, including decreased aggrecan synthesis and a switch from collagen II to collagen I synthesis [9–11]. The decrease in Col10A1 and OC gene expression, observed with increasing passage, parallels prior investigations in which chondrocytes demonstrated significantly decreased Col10A1 and OC with increasing duration in monolayer culture [11, 14]. Expanding hACs not only enhances the translational potential of the cell source by eliciting rapid proliferation but also evokes a loss in the differentiated phenotype. This phenotypic shift requires appropriate culture conditions for recovering chondrogenic gene and protein expression.

hMSCs and dedifferentiated hACs both demonstrated enhanced chondrogenic gene expression and matrix synthesis in the presence of TGF- $\beta$ /BMP superfamily proteins. In both cell types, BMP-2 alone significantly increased Col2A1, Col2A1/Col1A1 ratio, SOX9, ACAN expression, and GDF-5 alone significantly increased only ACAN expression. In hACs, TGF- $\beta$ 1 increased Col2A1, relative Col2A1/Col1A1, SOX9, and ACAN expression. However, TGF- $\beta$ 1 had a less robust effect as a single treatment in hMSCs, upregulating only ACAN expression. Of the two factor treatments, in hACs, TGF- $\beta$ 1/BMP-2 was most beneficial in inducing chondrogenic gene expression leading to a 5E5-fold increase in Col2A1, 600-fold increase in ACAN, and 10-fold increase in SOX9 expression, compared to aggregate culture in chondrogenic medium alone. In hMSCs, a significant interaction was observed between TGF- $\beta$ 1 and GDF-5 leading to the greatest increase in Col2A1, Col2A1/Col1A1 ratio, and ACAN expression. In hMSCs, TGF- $\beta$ 1/GDF-5 led to a 1E5-fold increase in Col2A1, 3000-fold increase in ACAN, and sixfold increase in SOX9, compared to aggregate culture in chondrogenic medium alone. In terms of protein expression, 2 weeks following self-assembly, TGF- $\beta$ 1/BMP2 and TGF- $\beta$ 1/GDF-5/BMP-2 treatments demonstrated the greatest GAG and collagen II synthesis in both cell types. These results confirm that both hMSCs and hACs respond beneficially to TGF- $\beta$ /BMP superfamily proteins during aggregate culture toward inducing chondrogenesis.

Ultimately, the combination of all three growth factors was most beneficial in inducing chondrogenic gene expression and matrix synthesis in both hMSCs and hACs. In hACs, TGF- $\beta$ 1/GDF-5/BMP-2 led to a 1E6-fold increase in Col2A1, 1000-fold increase in ACAN, and 20-fold increase in SOX9 expression, over chondrogenic medium alone. In hMSCs, TGF- $\beta$ 1/GDF-5/BMP-2 led to a 6E5-fold increase in Col2A1, 2E5-fold increase in ACAN, and 20-fold increase in SOX9 expression, over chondrogenic medium alone. On the protein level, in hACs, GAG synthesis was not visibly different between TGF- $\beta$ 1/BMP2 and TGF- $\beta$ 1/GDF-5/BMP-2. Importantly however, the relative collagen II/collagen I synthesis was greatest in the presence of all three factors. This result suggests the addition of GDF-5 reduced collagen I synthesis. Previous work on the effects of GDF-5 in articular cartilage ECM maintenance has

demonstrated that GDF-5 expression inhibits catabolic MMP13 expression while stimulating anabolic SOX9 and ACAN expression [27]. This cascade manifests through DKK1 inhibition of Wnt signaling. As such, in this study, it is plausible that interactions between GDF-5, BMP-2, and TGF- $\beta$  yielded decreased catabolic effects and increased anabolic effects resulting in increased chondrogenesis. Future work is suggested to elucidate the role of these signaling cascades in neocartilage formation.

In hMSCs, GAG, collagen II, and relative collagen II/collagen I synthesis was greatest in the presence of all three factors. It is noteworthy that in the presence of TGF- $\beta$ 1, GDF-5, and BMP-2, in both hACs and hMSCs, relative expression of Col2A1, SOX9, and ACAN gene expression was not significantly less than primary articular chondrocytes. These findings are supported by prior observations suggesting that despite redundancies in the TGF- $\beta$ /BMP superfamily signaling, the expression patterns and functions of each member are essential to skeletal patterning and development [48, 49]. Combined TGF- $\beta$ 1/GDF-5/BMP-2 stimulation *in vitro* superiorly enhances chondrogenesis in both expanded ACs and MSCs toward engineering robust cartilage.

As TGF- $\beta$ /BMP superfamily factors modulate mesenchymal condensation, cell differentiation, and matrix synthesis during cartilage morphogenesis, it is also important to evaluate the effects of these factors in hypertrophy induction/inhibition. OC is a marker of osteoblastic differentiation, and Col10A1 is a marker of hypertrophic chondrocytes. In both hACs and hMSCs no significant differences were observed in OC gene expression in the presence of any single growth factor or combined treatment, over chondrogenic medium alone. Col10A1 gene expression was significantly increased in the presence of TGF- $\beta$ 1 and BMP-2 in hACs. In hMSCs, Col10A1 was increased with TGF- $\beta$ 1 alone. However, in hACs, collagen X protein expression appeared negligible in all treatments. In hMSCs, collagen X was only detected following BMP-2 treatment. This expression was inhibited in combined TGF- $\beta$ 1/BMP-2 and TGF- $\beta$ 1/GDF-5/BMP-2 treatments. In cartilage morphogenesis, TGF- $\beta$  isoforms inhibit hypertrophy and contribute to maintenance of the articular cartilage phenotype [25, 50, 51]. Importantly in TGF- $\beta$ 1/GDF-5/BMP-2 treatment, collagen X was undetectable by immunohistochemistry, further indicating the generation of an articular cartilage phenotype in both cell types. Collagen I was detected in all treatments in both cell types. However, with collagen X minimally detected, or undetectable, it is suggested that the collagen I present was indicative of incomplete chondrogenic differentiation, rather than a hypertrophic phenotype.

Carrying forward the combined TGF- $\beta$ 1, BMP-2, and GDF-5 treatment for each cell type, engineered neocartilage was generated demonstrating the presence of GAG and collagen II. hAC constructs were composed of approximately 14% collagen and more than 20% GAG by dry weight. hMSC constructs were composed of 14% collagen and 4% GAG by dry weight. hMSC constructs were composed of 84% water, and hAC constructs were composed of 91% water. In both cell types, after 4 weeks, constructs stained positively for collagens I and II. Collagen X was minimally detected in hAC constructs and undetectable in hMSC constructs. Employing the self-assembling process following TGF- $\beta$ 1/BMP-2/GDF-5

stimulation during aggregate culture yielded neocartilage constructs in both cell types, demonstrating the presence of type II collagen and GAG.

Notably, this work represents the first time the self-assembling process was used to generate scaffold-free mechanically robust neocartilage composed of type II collagen and GAG, using adult hACs and adult hMSCs. Seeded at high density in nonadherent wells, chondrocyte self-assembly has been shown to be cadherin-mediated. Additionally, subsequent matrix synthesis has been shown to parallel native cartilage development with upregulation of collagen VI within 24 hours, followed by collagen II synthesis initiated by day 4 [52]. Reviewed previously, stem cell populations have been shown to differentiate toward proper phenotypes for cartilage engineering, however rarely is this associated with formation of implantable tissue [53]. Notably, in this study, paralleling native cartilage formation, the self-assembling process was used to generate robust cartilage using hACs and hMSCs. The methods used here, including the differentiation protocol and self-assembling process, will likely have significant potential in chondrogenesis in alternate stem cell populations, including dermis-isolated adult stem cells [54–59].

Toward achieving a suitable morphology for implantation, an enzymatic regimen composed of cytochalasin D and hyaluronidase was applied for the first 72 hours following self-assembly. Cytochalasin D was used to prevent cell contraction through temporary inhibition of actin polymerization [60]. Hyaluronidase was used to facilitate enhanced cell-cell interactions to minimize cell apoptosis during initial cell aggregation [61]. In hACs, a flat, cylindrical construct morphology was achieved. However, in hMSCs, an initial flat, cylindrical morphology was compromised over time as cytoskeletal elements induced misshaping and gross contraction of the tissue. Ultimately, hMSC constructs achieved an average compressive instantaneous modulus of 231 kPa while hAC constructs demonstrated an average compressive instantaneous modulus of 82 kPa. The superior compressive properties in hMSC constructs are reflective of construct misshaping and matrix contraction, apparent histologically and reflected in the tissue hydration (Figs. 5, 6). Previous efforts in our group have consistently faced challenges with gross tissue contraction in the generation of hMSCs-based constructs. Results of this study establish the need for a balance between minimizing tissue contraction and maintaining or improving mechanical properties, such that a tissue of suitable morphology and mechanical properties may be generated for implantation. Robust cartilage was achieved using the self-assembling process for the first time in hACs and hMSCs.

Toward using engineered constructs in joint repair, generating tissue capable of sustaining *in vivo* loads is essential. Mechanical characterization of mature, human patellar cartilage demonstrates an aggregate modulus of  $530 \pm 94$  kPa, which exceeds the mechanical properties achieved in this study [62]. Previous work has demonstrated engineered cartilage continues to mature *in vivo* and further efforts may focus on enhancing both compressive and tensile properties through the application of exogenous biochemical and physi-

cal stimuli [63]. As such, subsequent maturation studies using animal models of joint disease as well as injury are suggested.

This study was limited by failure to optimize growth factor concentrations. The chondrogenesis induced by the factors investigated here has the potential to be enhanced in each cell type upon optimizing growth factor stimulation time, duration, and concentration. Additionally, another limitation of this study was the 4 week endpoint. Future efforts may investigate *in vitro* culture beyond 4 weeks as well as *in vivo* maturation of engineered constructs. Lastly, the osteogenic markers investigated here could be extended to incorporate bone sialoprotein, which would add significant value in investigations at time points beyond those studied here.

## CONCLUSIONS

Clinically motivated, the field strives to move toward the generation of mechanically robust neocartilage using highly translational cell sources. All cell-based cartilage products currently available use chondrocytes or MSCs. This study establishes for the first time the ability to generate scaffold-free neocartilage from both hACs and hMSCs using the self-assembling process. In both cell populations, TGF- $\beta$ 1, GDF-5, and BMP-2 stimulation during postexpansion aggregate culture significantly enhanced chondrogenic gene expression, matrix synthesis, and yielded robust cartilage after 4 weeks maturation, rich in collagen II and GAG. Furthermore, the methods established here may be extended to alternate stem cell populations, including dermis-derived stem cells. This work further enhances the translational potential of both hACs and hMSCs in the generation of scaffold-free, cell-based cartilage products for addressing pathologies of diarthrodial joints.

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## AUTHOR CONTRIBUTIONS

M.K.M. and D.J.H.: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing, and final approval of manuscript; J.H.: conception and design, financial support, provision of study material, data analysis and interpretation, manuscript writing, and final approval of manuscript; K.A.A.: conception and design, financial support, administrative support, provision of study material, data analysis and interpretation, manuscript writing, final approval of manuscript. M.K.M. and D.J.H. contributed equally to this work.

## DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

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