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Original Article

Development of Serum-Free, Chemically Defined Conditions for Human Embryonic Stem Cell–Derived Fibrochondrogenesis

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This study established serum-free, chemically defined conditions to generate fibrocartilage with human embryonic stem cells (hESCs). Three sequential experimental phases were performed to eliminate serum because of its variability and antigenic potential and characterize the performance of hESCs in serum-free and serum-based conditions. Each phase used a two-stage modular experiment: chondrogenic differentiation followed by scaffold-less tissue engineering, called self-assembly. Phase I studied serum effects, and showed that a 1% serum chondrogenic medium (CM) during differentiation resulted in uniform constructs, whereas a 20% serum CM did not. Furthermore, a no-serum CM during self-assembly led to a collagen content 50% to 200% greater than a 1% serum CM. Thus, a "serum standard" of 1% serum during differentiation and no serum during self-assembly was carried forward. Phase II compared this with serum-free formulations, using 5% knock-out serum replacer or 1-ng/mL transforming growth factor beta 1 (TGF-β1). The TGF-β1 group was chosen as a "serum-free standard" because it performed similarly to the serum standard in terms of morphological, biochemical, and biomechanical properties. In Phase III, the serum-free standard had significantly more collagen (100%) and greater tensile (~150%) and compressive properties (~80%) than the serum standard with TGF-β1 treatment during self-assembly. These advances are important to the understanding of mechanisms of chondrogenesis and creating clinically relevant stem cell therapies.

Introduction

The development of tissue engineering strategies for musculoskeletal cartilage is of interest because of the major social and economic burdens caused by cartilage afflictions such as osteoarthritis and temporomandibular joint (TMJ) disc disorders. Currently, a significant area of investigation involves identifying a useful cell source for cartilage tissue engineering. Cells from adipose,[1] skin,[2] bone marrow,[3,4] umbilical cord blood,[5] and embryonic sources[6–11] have demonstrated a capacity to become cartilage-like cells, but the development of these cell technologies is at different stages, particularly concerning the demonstration of the functional ability to produce cartilage constructs. Human embryonic stem cells (hESCs), for example, are only beginning to be studied for cartilage applications, for example, fibrocartilage such as the TMJ disc, knee meniscus, and hyaline articular cartilage.

The pluripotent nature of hESCs makes it conceivable that these different cartilage applications can be achieved with this single cell source while they are also attractive models of study for tissue morphogenesis because of their primordial state. Furthermore, the advent of new technologies such as induced pluripotent stem (iPS) cells[12–14] emphasizes the need to characterize and investigate the directed differentiation of hESCs for comparative studies with iPS cells and to assess the potential therapeutic uses of each cell source. Using hESCs or other stem cells for human cartilage therapy faces important challenges. For example, a strategy to produce cartilage-like cells must be established. In this regard, several methods have emerged, including the directed chondrogenic differentiation of hESCs[6,15,16] and the generation of mesenchymal precursors from hESCs that can be subsequently chondrogenically differentiated.[8,17,18] In each case, it has been shown that the cells generated in these ways can produce cartilage proteins or have some chondrocyte-like phenotype, but because not all of the approaches have been evaluated for the functional capacity to produce cartilage tissue, it is still unclear what the best strategy to generate chondrocyte-like cells will be.

Another challenge is determining the stimuli sufficient for hESC-derived chondrogenesis. A variety of stimuli, including growth factors,[6,8,19] hypoxia,[11] and mechanical stimulation,[20] have been evaluated, and it appears that the pathways to the

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chondrogenic lineage can be achieved with a variety of agents. Establishing basal conditions for chondrogenic differentiation and subsequent tissue engineering of cartilage may have important implications for understanding these differentiation pathways and would help bioengineers develop well-defined, reproducible methods of producing cartilage constructs.[21] Related to this challenge is the need to eliminate xenogenic products from the culture. Important progress has been made in this area, with serum-free conditions having been used with hESCs for chondrogenic differentiation.[8,15–17] However, these serum-free conditions have not been applied throughout the entire process of chondrogenic differentiation and tissue engineering to generate macroscopic tissue with hESCs.

Toward addressing the aforementioned hurdles, this study systematically evaluated different formulations of chondrogenic media (CM), with the overall goal of identifying serum-free conditions that are sufficient for the chondrogenic differentiation and tissue engineering of fibrocartilage with hESCs. We have previously engineered robust fibrocartilage using distinct growth factor combinations[6] and differentiation timelines[10] but relied on serum-based methods in each of these studies. A secondary objective was to understand how a possible serum-free methodology performed in comparison with a serum-based approach, which has not been done previously. The study was conducted in three phases. Each phase comprised chondrogenic differentiation of hESCs in embryoid body (EB) form followed by a scaffold-less engineering strategy called self-assembly,[22] which has previously been shown to result in fibrocartilage-like constructs (high collagen I, high tensile properties, and low sulfated glycosaminoglycans) that can be evaluated for quantitative biochemistry and biomechanics.[6] In the first phase of the study, the use of serum was evaluated during EB differentiation and self-assembly to establish a "serum standard." In the second phase, two chemically defined, serum-free formulations of CM were compared with the serum standard and with each other to establish a "serum-free standard." Finally, in the third phase, transforming growth factor beta 1 (TGF-β1) and insulin-like growth factor (IGF)-I were used during self-assembly of the serum standard and serum-free standard to ascertain whether these two populations of cartilage-producing cells responded differently to these important cartilage stimuli.

Methods

The experimental design of this study is depicted in Figure 1. All three phases involved the use of a basal CM composed of high-glucose Dulbecco's modified Eagle medium (DMEM; Invitrogen, Carlsbad, CA), 10^{-7} M dexamethasone, ITS + Premix (6.25 ng/mL insulin, 6.25 mg transferrin, 6.25 ng/mL selenious acid, 1.25 mg/mL bovine serum albumin, and 5.35 mg/mL linoleic acid; Collaborative Biomedical, San Jose, CA), 40 μg/mL L-proline, 50 μg/mL ascorbic acid, and 100 μg/mL sodium pyruvate. Other components were added as indicated in Figure 1, with fetal bovine serum (FBS) obtained from Gemini Bio-Products (West Sacramento, CA), knock out serum replacer (KOSR) from Invitrogen, and growth factors from Peprotech (Rocky Hill, NJ). The general methods for hESC culture, EB formation, EB differentiation, self-assembly, and analysis were the same as performed previously[6] and are briefly detailed below. In choosing "winners" from each phase of the experiment, morphological, biochemical, and biomechanical data were considered.

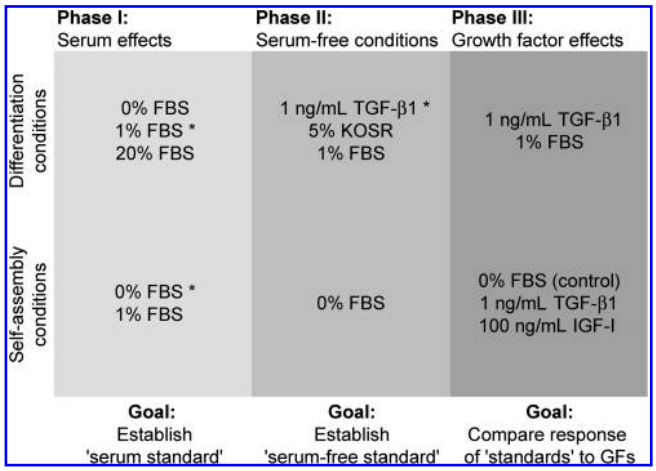

FIG. 1. Experimental plan. This study systematically investigated different formulations of chondrogenic media with the objective of developing serum-free conditions for the chondrogenic differentiation of human embryonic stem cells and tissue engineering of cartilage with these cells. The figure shows the specific media components that were used in each phase of the experiment. An asterisk indicates the winner of a particular phase (please see Results). FBS, fetal bovine serum; TGF-β1, transforming growth factor beta 1; KOSR, knock out serum replacer; IGF, insulin-like growth factor, GF, growth factor.

Culture conditions

hESC expansion. The National Institutes of Health (NIH)-approved BG01V line (American Type Culture Collection, Manassas, VA) was cultured according to recommended protocols, as before.[6] Frozen hESCs at passage 16 (p16) were thawed according to standard protocol and subcultured. The hESCs were passaged with collagenase IV (Invitrogen) every 4 to −5 days and used for the experiment at p22 for Phase I, p20 for Phase II, and p21 for Phase II.

Embryoid body formation and differentiation conditions. Dispase solution (0.1% w/v) was applied for 10 to 15 min to colonies of undifferentiated hESCs in monolayer when the colonies reached 70% to 80% confluence, as previously performed.[6] After two washes and centrifugations with DMEM/F12, the EBs were suspended in a CM according to the experimental plan (Fig. 1) and then distributed into bacteriological petri dishes (Fisher, Hampton, NH). For the entire experiment, medium was completely changed every 48 h. EBs were used for self-assembly or for analysis at 3 weeks.

Self-assembly of chondrogenically differentiated hESCs. EBs were digested in 0.05% trypsin–ethylenediaminetetraacetic acid (Invitrogen) for 1 h, followed by up to 2 h of 0.15% collagenase II (Worthington Biochemical Corp., Lakewood, NJ) digestion. Cells from each digest were counted with a hemocytometer, washed with DMEM, centrifuged at 200× g, and resuspended at a concentration of 5.0×10^5 cells per 20 μL in the appropriate CM (Fig. 1). Constructs were made by seeding the dissociated cell suspension into 3-mm wells of 2% agarose (5.0×10^5 cells/well).

Assessments

Analysis of differentiated EBs. At 3 weeks, EBs were cryo-sectioned and stained for collagens using picrosirius red, for glycosaminoglycans (GAGs) using Alcian blue, and for collagen I and collagen II using immunohistochemistry.[22] Stains for mesodermal tissue markers were used to detect unwanted differentiation, including hematoxylin and eosin (muscle and general morphology), von Kossa (calcified tissues), and Oil red O (adipose). Standard protocols were followed for each of these stains. During Phase II, cells from the enzymatic digest were used for flow cytometry analysis to ascertain whether mesodermal markers were present by blocking with goat serum for 1 h and adding 10 μg/mL of immunoglobulin G_1 isotype control (BD Biosciences, Franklin Lakes, NJ), mouse anti-human CD44 (an adhesion protein and matrix receptor,[23] Sigma-Aldrich, St. Louis, MO), mouse anti-human CD105 (a TGF-β receptor,[24] Invitrogen), or mouse anti-human platelet-derived growth factor receptor alpha (PDGFRα; a receptor involved in mesodermal development,[25] R&D Systems, Minneapolis, MN) for 30 min. Cells were washed in phosphate buffered saline, and then the Alexa Fluor 488 goat anti-mouse antibody (Molecular Probes, Carlsbad, CA) was applied to the different groups for 30 min. Samples were fixed in 0.5% paraformaldehyde and stored at 4°C until analysis with a BD FACSCalibur system (BD Biosciences). At least 10,000 events were recorded with each sample, and analysis of whole cells was performed using appropriate scatter gates to avoid debris and aggregates.

Analysis of self-assembled constructs. At the 7-week time point (after 4 weeks of self-assembly), each construct was measured for wet weight after excess water was carefully blotted. Diameter and thickness measurements were made using digital calipers with an accuracy of 0.01 mm (Mitutoyo, Aurora, IL). Constructs were used for histology, biochemical assays, or biomechanical testing. Histological assessments for self-assembled constructs included picrosirius red, Alcian blue, immunohistochemistry for collagen I and collagen II, von Kossa, hematoxylin and eosin, and Oil red O. Biomechanical testing included tensile testing using an Instron 5565 (Instron, Norwood, MA) and unconfined compression using a modified creep indentation apparatus.[6,26]

Previously described protocols were used for PicoGreen, dimethylmethylene blue, and hydroxyproline tests, and one set of samples was used for these assays.[22] For collagen I and II, a second set of samples was used with previously described protocols for sandwich enzyme-linked immunosorbent assays.[6]

Statistics

Data were analyzed with appropriate analysis of variance models using Tukey's post hoc test when applicable and a significance value of $p \leq 0.05$. At least three samples were analyzed for biochemical assays and biomechanical tests. All data are reported as means ± standard deviations.

Results

Phase I: Serum effects

The two experimental factors during this phase are referred to as EB%, denoting the FBS level during EB differentiation, and SA%, denoting the FBS level during self-assembly (SA). Groups are identified according to their EB% first and then SA% (i.e., FBS 0-0, FBS 0-1, FBS 1-0, FBS 1-1, FBS 20-0, and FBS 20-1). Phase I results are shown in Figure 2, including gross morphology (Fig. 2A), cellular content (Fig. 2B), and collagen content (Fig. 2C). These results are described below. Please also see Supplemental Fig. 1 and 2 (available online at www.liebertonline.com/ten).

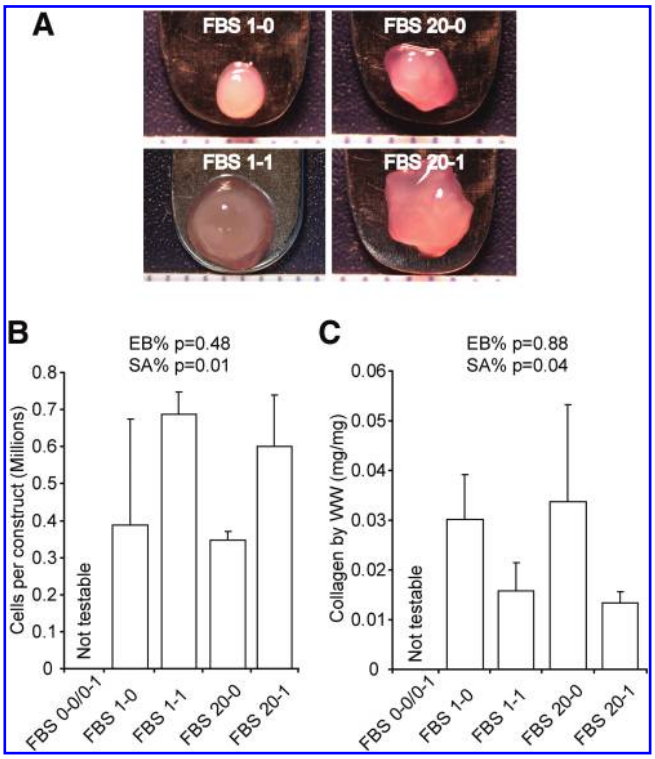

FIG. 2. Phase I results. Two experimental factors were evaluated in Phase I: the fetal bovine serum (FBS) percentage during differentiation (EB%) and during self-assembly (SA%). Four of six treatments in Phase I yielded testable constructs for analysis, explained in Results and Discussion. When high serum levels were present during differentiation, the morphology of the constructs was not uniform (**A**, markings are in mm). When serum was present during self-assembly, the constructs had significantly higher cellularity (**B**) but lower collagen content (**C**). $p < 0.05$ indicates that the experimental factor was significant for the respective assay. Color images available online at www.liebertonline.com/ten.

Morphology of EBs (3 weeks) and self-assembled constructs (7 weeks). EBs from the 1% FBS and 20% FBS groups demonstrated cartilaginous proteins such as collagen I and collagen II, similar to previous results.[6] The EBs in the no-serum group (0% FBS) did not grow, nor did they yield testable self-assembled constructs. At 7 weeks, the 20% FBS differentiation groups produced constructs that were not homogeneous in appearance, whereas the 1% FBS differentiation groups appeared uniform (Fig. 2A). The SA% was a significant factor for the diameter of the constructs, with serum exposure during self-assembly leading to larger constructs ($p = 0.01$, 1.9 ± 0.1 mm for FBS 1-0, 4.0 ± 0.4 mm for FBS 1-1, 2.3 ± 0.01 mm for FBS 20-0, and 2.4 ± 0.2 mm for FBS 20-1). The EB% was not a significant factor affecting the diameter of the

constructs ($p = 0.13$). The constructs exhibited collagen I and II (Supplemental Fig. 1), as seen previously.[6] They did not show any unwanted differentiation according to histology (Supplemental Fig. 2).

Biochemistry. EB% was not a significant factor for any biochemical characteristics. On the other hand, the presence of serum during self-assembly (i.e., SA%) was a significant factor for the final cell numbers and total collagen content ($p < 0.05$, Fig. 2B and C). In terms of specific collagens, the FBS 1-0 group had significantly more collagen II by wet weight (WW, in μg/mg) than the other groups (2.3 ± 0.7 for FBS 1-0, 0.4 ± 0.1 for FBS 1-1, 0.6 ± 0.1 for FBS 20-0, and 0.5 ± 0.3 for FBS 20-1). The collagen I by WW (in μg/mg) was similar in all groups (9.8 ± 3.2 for FBS 1-0, 7.4 ± 2.6 for FBS 1-1, 8.9 ± 1.1 for FBS 20-0, and 5.6 ± 2.5 for FBS 20-1). The GAG content by WW (in μg/mg) was also similar in all groups (8.4 ± 2.8 for FBS 1-0, 4.9 ± 0.7 for FBS 1-1, 5.4 ± 0.9 for FBS 20-0, and 6.1 ± 1.3 for FBS 20-1).

Biomechanics. EB% did not have a significant effect on any of the biomechanical properties of the constructs. SA% was a significant factor ($p < 0.05$) for the relaxed compressive modulus (2.0 ± 0.7 kPa for FBS 1-0, 1.6 ± 0.5 kPa for FBS 1-1, 2.5 ± 0.5 kPa for FBS 20-0, and 1.2 ± 0.5 kPa for FBS 20-1). The instantaneous compressive modulus was similar ($p > 0.05$) in all groups (15.8 ± 5.8 kPa for FBS 1-0, 17.0 ± 8.1 kPa for FBS 1-1, 14.9 ± 2.6 kPa for FBS 20-0, and 8.3 ± 1.8 kPa for FBS 20-1). There was no significant difference in tensile properties. The tensile modulus was 2.2 ± 1.0 MPa for FBS 1-0, 1.0 ± 0.8 MPa for FBS 1-1, 2.2 ± 1.3 MPa for FBS 20-0, and 2.2 ± 1.3 MPa for FBS 20-1. The ultimate tensile strength was 1.8 ± 1.2 MPa for FBS 1-0, 0.5 ± 0.2 MPa for FBS 1-1, 1.5 ± 0.9 MPa for FBS 20-0, and 1.7 ± 1.1 MPa for FBS 20-1.

Phase I summary

The results of Phase I demonstrated that a minimal amount of serum (1% during chondrogenic differentiation and none during self-assembly) was sufficient to produce uniform constructs with biochemical and biomechanical properties on par with or better than the other groups. Thus, the FBS 1-0 group was chosen as the serum standard.

Phase II: Serum-free conditions

The three experimental groups during this phase are named according to the main medium component: FBS (the serum standard), KOSR, and TGF-β1.

Morphology of EBs (3 weeks) and self-assembled constructs (weeks). EBs from all three groups grew and displayed typical morphological findings at 3 weeks, as before.[6] At 7 weeks, the three groups exhibited similar morphological characteristics, except that the KOSR group had a smaller diameter ($p < 0.05$, 2.4 ± 0.1 mm for FBS, 1.9 ± 0.1 mm for KOSR, and 2.3 ± 0.1 mm for TGF-β1). Otherwise, all treatments appeared homogeneous and smooth (Fig. 3A). The constructs exhibited collagen I (Fig. 3A) and collagen II (Supplemental Fig. 3, available online at www.liebertonline.com/ten). They did not show any unwanted differentiation according to histology (Supplemental Fig. 4, available online at www.liebertonline.com/ten). There were differences between the cells from each treatment in terms of cell surface markers (Table 1), particularly in the proportion of cells positive for CD44 and PDGFRα in the TGF-β1 group compared with the other groups.

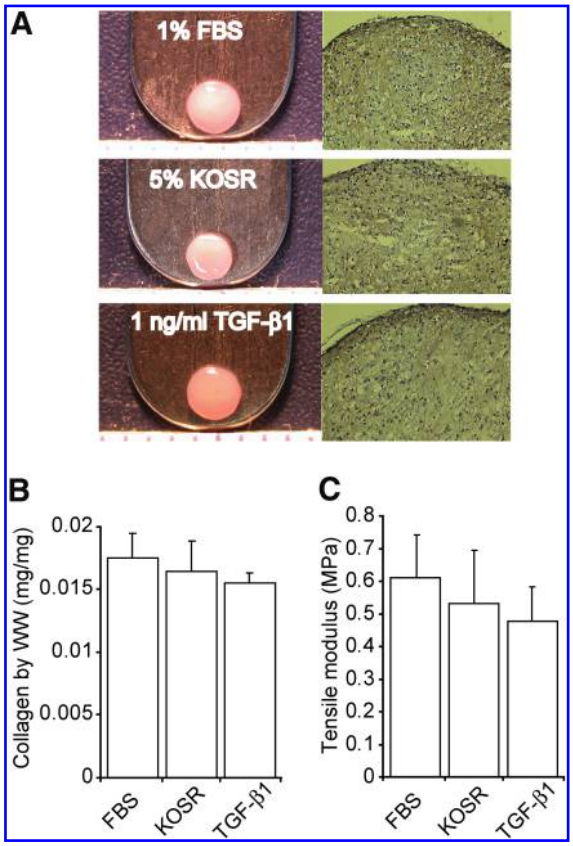

FIG. 3. Phase II results. The two serum-free methods performed similarly in most respects to the serum standard, appearing uniform (markings are in mm) and composed mostly of collagen I (20×) (**A**), having similar total collagen content (**B**), and exhibiting no differences in biomechanical properties (**C**). The transforming growth factor beta 1 (TGF-β1) group was chosen as the winner of Phase II because it performed on par with the serum standard and did not contract as the knock out serum replacer (KOSR) group did. It was also appealing because of its simplicity. FBS, fetal bovine serum. Color images available online at www.liebertonline.com/ten.

Biochemistry. The composition of the constructs in each group was similar, in terms of cell numbers, GAG content, total collagen content (Fig. 3B), and specific collagen contents. Cell numbers were $2.2 \pm 0.1 \times 10^5$ for FBS, $2.3 \pm 0.2 \times 10^5$ for KOSR, and $2.2 \pm 0.3 \times 10^5$ for TGF-β1. GAG content by WW was 0.9 ± 0.1 μg/mg for FBS, 1.0 ± 0.1 μg/mg for KOSR, and 1.1 ± 0.1 μg/mg for TGF-β1. The collagen I content by WW was 15.6 ± 1.5 μg/mg for FBS, 15.4 ± 1.8 μg/mg for KOSR, and $12.9 \pm .8$ μg/mg for TGF-β1. The collagen II content by WW was 0.9 ± 0.2 μg/mg for FBS, 1.5 ± 0.5 μg/mg for KOSR, and 0.7 ± 0.1 μg/mg for TGF-β1.

Biomechanics. The KOSR group had a higher relaxed compressive modulus than the TGF-β1 group ($p < 0.05$): 2.6 ± 0.4 kPa for FBS, 3.0 ± 0.3 kPa for KOSR, and 1.9 ± 0.4 kPa for TGF-β1. Otherwise, the biomechanics of the treatment groups were not statistically different. The instantaneous

Table 1. Mesodermal Surface Markers for Phase II Cells (3 Weeks)

Treatment	Isotype control %	CD44	CD105	PDGFRα
FBS	2.4	44.9	12.1	13.0
Knock out serum replacer	1.4	34.4	8.5	11.3
TGF-β1	2.8	69.1	13.0	21.1

Shown are the percentage of cells from each treatment that had FLH1 $> 10^3$ after appropriate gating (see Methods). Each treatment from Phase II yielded positive cells for CD44, CD105, and platelet-derived growth factor receptor alpha (PDGFRα). There was a difference in the level of signal between each group, however. Most notably, the transforming growth factor beta 1 (TGF-β1) group had a higher proportion of cells positive for CD44 and PDGFRα than the fetal bovine serum (FBS) group.

compressive modulus was 25.4 ± 10.3 kPa for FBS, 17.9 ± 2.7 kPa for KOSR, and 18.2 ± 2.8 kPa for TGF-β1. The tensile modulus is shown in Figure 3C. The ultimate tensile strength was 0.4 ± 0.1 MPa for FBS, 0.7 ± 0.1 MPa for KOSR, and 0.5 ± 0.1 MPa for TGF-β1.

Phase II summary

The serum-free groups performed similarly in most respects to each other and to the serum standard. Although the KOSR group had a slightly higher relaxed modulus, it was smaller in diameter and otherwise similar to the TGF-β1 group. The appealing aspect about the TGF-β1 group was that the differentiation conditions were defined and known; on the other hand, KOSR, although chemically defined, is a commercial product whose contents are not published. Thus, the TGF-β1 group was chosen as the serum-free standard for the final phase of the experiment.

Phase III: Growth factor effects on serum and serum-free standards

The growth factors TGF-β1 and IGF-I were used during the self-assembly of the serum and serum-free standards. (The same basal CM was used during self-assembly of all treatments, and exogenous growth factors were added as appropriate. No serum was used during self-assembly of any treatment during Phase III.) Including the no-growth-factor controls, this yielded six treatments. These are referred to by the differentiation condition first (F for FBS and T for TGF-β1), followed by the self-assembly condition (C for control, T for TGF-β1, and I for IGF-I). Therefore, the six groups were F-C, F-T, F-I, T-C, T-T, and T-I.

Morphology of EBs (3 weeks) and self-assembled constructs (7 weeks). EBs again displayed typical morphological findings at 3 weeks, similar to Phases I and II. At 7 weeks (Fig. 4A), constructs treated with TGF-β1 during self-assembly (F-T and T-T) were smaller in diameter than the other groups ($p < 0.05$, 2.7 ± 0.1 mm for T-C, 2.2 ± 0.2 mm for T-T, 2.5 ± 0.1 mm for T-I, 2.5 ± 0.1 mm for F-C, 2.1 ± 0.1 mm for F-T, and 2.3 ± 0.2 mm for F-I). The constructs exhibited collagen I and II and did not show any unwanted differentiation according to histology (Supplemental Figs. 5 and 6, available online at www.liebertonline.com/ten).

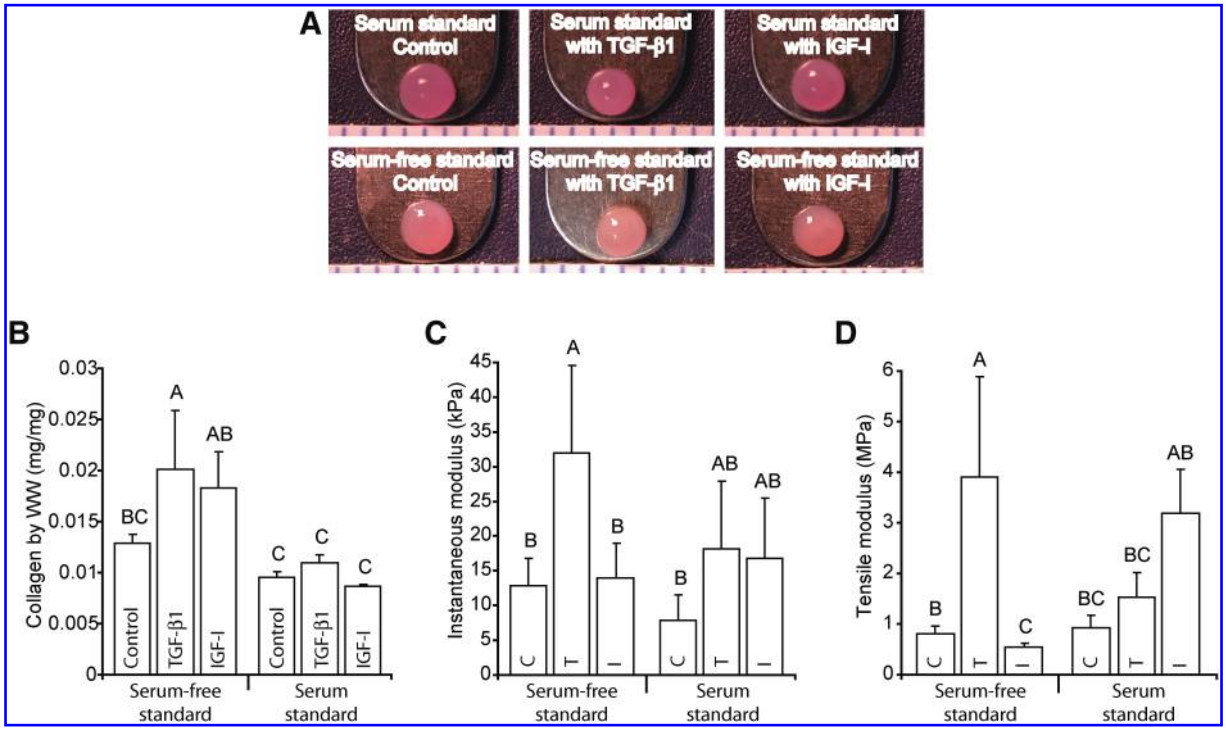

FIG. 4. Phase III results. Uniform constructs were obtained with all six treatments (**A**). Only the serum-free standard had a significant response to transforming growth factor beta 1 (TGF-β1) over its control in terms of collagen production (**B**), compressive properties (**C**), and tensile properties (**D**). The distance between markers in (**A**) is 1 mm. IGF, insulin-like growth factor. Color images available online at www.liebertonline.com/ten.

Biochemistry. There were several significant differences between the serum and serum-free standards when exposed to the growth factors. The serum-based groups had more cells than the serum-free treatments ($p < 0.001$, $1.3 \pm 0.1 \times 10^5$ for T-C, $2.0 \pm 0.3 \times 10^5$ for T-T, $1.4 \pm 0.1 \times 10^5$ for T-I, $3.3 \pm 0.1 \times 10^5$ for F-C, $3.9 \pm 0.6 \times 10^5$ for F-T, and $3.7 \pm 0.4 \times 10^5$ for F-I). The serum-free differentiation condition was a significant factor for GAG content by WW ($p < 0.01$, 0.9 ± 0.1 μg/mg for T-C, 1.1 ± 0.2 μg/mg for T-T, 1.0 ± 0.2 μg/mg for T-I, 0.8 ± 0.01 μg/mg for F-C, 0.9 ± 0.04 μg/mg for F-T, and 0.8 ± 0.1 μg/mg for F-I) and total collagen content by WW ($p < 0.001$, Fig. 4B). Application of TGF-β1 during self-assembly of the serum-free standard was the only treatment to have significantly higher total collagen than control (Fig. 4B). There were no significant differences between the groups in terms of their specific collagen content. The collagen I content by WW was 5.9 ± 2.0 μg/mg for T-C, 7.2 ± 2.1 μg/mg for T-T, 7.4 ± 2.1 μg/mg for T-I, 8.7 ± 1.1 μg/mg for F-C, 9.1 ± 2.9 μg/mg for F-T, and 10.2 ± 1.6 μg/mg for F-I. The collagen II content by WW was 2.3 ± 0.7 μg/mg for T-C, 2.0 ± 0.8 μg/mg for T-T, 2.5 ± 0.6 μg/mg for T-I, 1.4 ± 0.2 μg/mg for F-C, 1.9 ± 0.8 μg/mg for F-T, and 2.2 ± 0.1 μg/mg for F-I.

Biomechanics. Treatment with TGF-β1 during self-assembly of the serum-free standard resulted in significantly greater compressive and tensile properties than in all other groups (Fig. 4C, D). Although there was a trend toward better mechanical properties in the serum standard when growth factors were applied (F-T and F-I), there was no significant difference from control for those groups (Fig. 4C, D).

Phase III summary

The T-T group performed best of all the other treatments in terms of cartilage protein composition and biomechanics.

Discussion

This study systematically evaluated different formulations of CM and established serum-free, chemically defined methods for the engineering of fibrocartilage with hESCs. This is the first demonstration that serum-free methods can be used during chondrogenic differentiation and cartilage tissue engineering with hESCs. Furthermore, this study demonstrates significant effects of TGF-β1 on the self-assembly of the serum-free standard (1 ng/mL of TGF-β1 in CM during differentiation) while also showing that cells differentiated under the serum-free and serum-based methods have a different response to growth factors during self-assembly. Overall, in demonstrating these results, this study opens the door to well-controlled, systematic studies of hESC-derived chondrogenesis by eliminating the variation of serum from one batch to another; increases the translatability of this technology because xenogenic products such as serum are not necessary; and demonstrates that the chondrogenic potential of hESCs is not fixed after differentiation but can be further enhanced during self-assembly. These three points are vital to the future goal of engineering functional cartilage for human cartilage applications.

This study was designed to develop new principles for the development of serum-free, chemically defined methods for hESC-derived fibrochondrogenesis. In Phase I, the key differences were in the morphology of the constructs and their biochemical properties. Specifically, the results showed that at least a small amount of exogenous stimulus was necessary for the survival of the EBs through 3 weeks of differentiation, because the 0% group did not yield any usable cells. It is well documented that serum contains growth factors and cytokines that are important to cell survival and proliferation.[27,28] Thus, it was not surprising that the 0% group did not perform well.

This left a comparison of low (1%) and high (20%) serum levels during differentiation and their response to the self-assembly conditions (no serum or 1% FBS). This comparison showed major differences in the morphologies of the resulting self-assembled constructs (Fig. 2), suggesting varying capacities of the cells from each differentiation condition to engineer cartilage. Indeed, we have shown previously that hESCs are sensitive to chondrogenic differentiation conditions with distinct growth factor regimens[6] and hypoxia.[11] Specifically, it appears that the differentiated cells have unique expression of cell surface markers with each distinct differentiation condition, including adhesion proteins and growth factor receptors.[11] This may result in the observed differences in tissue characteristics in each of the phases of this experiment as well as in our previous work,[6,11] because these proteins are known to play important roles in chondrogenesis.[29–33] The expression of these surface markers is also interesting in light of the response of the cells to growth factors (discussed in detail later). It is possible that the high level of serum produced higher variation in surface marker expression in the cell population, causing the constructs to grow in odd shapes. The process of cellular aggregation based on cellular adhesion proteins has been elegantly described.[34,35] This previous work and our findings suggest that cell purification between the steps of differentiation and self-assembly may further benefit our process.

Although 20% FBS has been used for the chondrogenic differentiation of ESCs,[36,37] this study represented the first time that these differentiated cells were used in a tissue engineering strategy. The 1% FBS formulation of CM has been previously used to chondrogenically differentiate hESCs and engineer cartilage that mostly resemble native fibrocartilage.[6] The biochemical results demonstrated that no FBS was necessary for self-assembly and was better than having FBS during self-assembly, because the cartilage protein contents were consistently higher when no serum was present. It has been observed that cells in serum-containing medium do not produce as much extracellular matrix as cells in serum-free cultures,[38] and this may be postulated to be a direct inhibitory effect of serum on matrix production at the cellular level. Other hypotheses can be entertained, including the idea that the effect of serum during self-assembly favors cell proliferation rather than matrix production. Our data lend evidence to this, because the cell numbers were higher, and matrix content was lower when serum was present during self-assembly than under the serum-free condition. Again, this may be a result of serum containing a number of cytokines and growth factors that influence these cellular processes.[27,28] Thus, the pathways of cell proliferation and matrix production may not be simultaneously compatible, and the presence or absence of serum may be used to tip the balance between these processes during self-assembly. Overall, the results of the first phase showed that a small amount of serum (1%) was necessary during the chondrogenic differentiation of hESCs and that no serum was necessary during self-assembly.

Phase II used the self-assembly conditions of Phase I (no serum) to compare the serum standard with serum-free formulations of CM. Similar serum-free methods have been used previously to differentiate stem cells into cartilage-like cells[16,17,39,40] and engineer cartilage,[8] but none of the previous work with hESCs has used serum-free, chemically defined methods throughout chondrogenic differentiation and cartilage tissue engineering. This study demonstrates two methods of achieving this in the KOSR and TGF-β1 groups. These two treatments of Phase II performed similarly in most regards. Although the KOSR group had slightly better compressive properties than the TGF-β1 group, the TGF-β1 group performed on par with the serum standard and did not contract like the KOSR group. It also had the advantage over the KOSR group of being a better-defined system of study. Although KOSR is a chemically defined medium component, its contents are not published.

The differing levels of cell surface markers at 3 weeks between the TGF-β1 and FBS groups were interesting in light of the differential response these groups had to the growth factors during self-assembly, as seen in Phase III. The serum-free standard (1 ng/mL TGF-β1 during chondrogenic differentiation) had a marked response to TGF-β1 during self-assembly, exhibiting higher cartilage protein production and better biomechanical properties than the serum-free control and the corresponding serum-based group (Fig. 4). There was no difference between the cell populations derived from the serum and serum-free methods in CD105 (Table 1), a component of the TGF-β receptor.[41] TGF-β1 regulates cellular processes by binding to a heteromeric complex of type I and type II serine/thionine kinase receptors, and CD105 binds TGF-β1 with high affinity through its association with TGF-β receptor type II.[42] One hypothesis that may address the differential responses to TGF-β1 is that the cells generated using distinct differentiation conditions have varying expression patterns of these specific growth factor receptors. The difference in the number of cells positive for PDGFRα between the serum-free and serum-based methods may also explain the distinct responses to TGF-β1 during self-assembly. For example, TGF-β1 can increase or decrease PDGFRα expression, depending on the cell type.[43–48] Also, PDGF has been shown to be a cofactor in the induction of type I procollagen expression by TGF-β1 in smooth muscle cells.[49] Thus, our results (Fig. 4) and the connections between TGF-β1, PDGF isoforms, and PDGFRs suggest that PDGFRα may be a useful predictor for responsiveness of engineered cells to TGF-β1. Similarly, CD44 may also be a useful marker (Table 1) for this purpose, although the connection between this adhesion protein and TGF-β1 is not as well characterized.

Toward understanding how differentiated hESCs respond to specific stimuli, such as growth factors, and translating stem cell technologies to human therapies, it is important to establish well-defined, reproducible conditions. This work establishes basal conditions for the generation of cartilage-like cells and macroscopic cartilage with hESCs, showing that just a small level of TGF-β1 is sufficient to sustain the survival and chondrogenic differentiation of hESCs. Several challenges remain regarding translation of this technology, however. It is important to indicate that the NIH-sanctioned lines pose a challenge to any therapeutic use because of their history of mouse feeder cell contact and contact with other xenogenic products. To address these issues, mouse feeder cells have been replaced with human cells;[50] alternatively, feeder-free conditions can be used.[51] Although the NIH-sanctioned lines may not be clinically usable, they nevertheless represent useful model systems to address many pertinent questions regarding hESCs for cartilage applications, including how to generate cartilage-producing cells and how to manipulate the characteristics of hESC-derived cartilage. In particular, we have shown that the differentiation of hESCs can be manipulated to generate functionally distinct cartilages under serum-based conditions;[6] now that serum-free conditions have been established in this study, it is conceivable that functionally useful, clinically applicable musculoskeletal cartilages can be generated with hESCs once the NIH approves new lines.

In summary, this study establishes serum-free, chemically defined methods of producing fibrocartilage with hESCs. We demonstrate that at least a small level of exogenous stimulus (i.e., 1% FBS, 1 ng/mL TGF-β1, or 5% KOSR) is necessary for hESC survival during EB differentiation but not during self-assembly. Additionally, we show that hESCs differentiated under serum-free and serum-based conditions respond differentially to growth factors, with a large and significant effect of TGF-β1 on cartilage protein production and biomechanics for the serum-free group but not for the serum-based group. Generating cartilage under defined conditions is important for understanding the mechanisms involved in chondrogenesis, as well as the translation of cell-based technologies to clinical therapies for cartilage afflictions.

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Disclosure Statement

No competing financial interests exist.

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