Osteoarthritis and Cartilage

International Cartilage Repair Society

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Hypoxic chondrogenic differentiation of human embryonic stem cells enhances cartilage protein synthesis and biomechanical functionality

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Summary

Background: Engineering musculoskeletal cartilages with stem cells remains a challenge because researchers must control many factors, including differentiation and cartilage matrix synthesis, particularly collagen II production. Hypoxia has effects on many cellular processes, though few investigations with hypoxia provide quantitative functional data on engineered cartilage.

Objective: This study investigated the effects of hypoxia on chondrogenesis with human embryonic stem cells (hESCs).

Methods: The experiment comprised two phases, embryoid body (EB) differentiation for 3 wks followed by a scaffold-less tissue engineering strategy called self-assembly for 4 wks. During each phase, hypoxic conditions (2% O_2) or normoxic conditions (20% O_2) were applied, and engineered constructs were analyzed for cellular, morphological, biochemical, and biomechanical properties.

Results: Hypoxic conditions significantly altered the chondrogenic differentiation process, whereby cells cultured in these conditions had an enhanced ability to produce collagen II (up to 3.4-times), collagen I (up to 2.9-times), and glycosaminoglycans (GAGs) (up to 1.9-times), resulting in better biomechanical functionality (up to three times in tensile modulus and up to four times in compressive properties). Hypoxic cells had a different expression profile than normoxic cells for cluster of differentiation (CD)44, CD105, and platelet derived growth factor receptor (PDGFR)α, further emphasizing that hypoxia altered hESC differentiation and suggesting that these markers may be used to predict an hESC-derived cell population's chondrogenic potential. Also, normoxic self-assembly outperformed hypoxic self-assembly in tensile and compressive biomechanical characteristics.

Conclusions: These results show that oxygen availability has dramatic effects on the differentiation and synthetic potentials of hESCs and may have important implications for the development of strategies to engineer cartilage.
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Key words: Hypoxia, Human embryonic stem cells, Chondrogenesis, Cartilage, Tissue engineering.

Introduction

Injuries and diseases of articular cartilages such as the temporomandibular joint (TMJ) disc, knee meniscus, and hyaline cartilage can render these tissues functionally deficient, causing significant economic and social burdens for afflicted individuals and society as a whole. Tissue engineering efforts seek to address this problem through the generation of cartilage replacements that can be implanted to replace defunct tissue. Identifying an appropriate cell source for this application is one challenge among many for the field. A variety of progenitor and stem cells have been investigated, and these reports demonstrate that chondrogenesis can be achieved through many manipulations, including, but not limited to, growth factors[1−6], biomechanical stimulation[7,8], growth on scaffolds[9−14], and oxygen availability[15−17].

Each of these manipulations is partly derived from and motivated by knowledge of the development, growth conditions, and physiology of native cartilages. For example, cartilages are mostly avascular tissues, with synovial fluid providing oxygen and nutrients that diffuse through the extracellular matrix. The lack of blood supply creates a hypoxic environment, with reports of oxygen levels that range from 1 to 8%, depending on the location of the tissue and depth inside[18−20]. Consequently, *in vitro* engineering efforts have attempted to mimic this environment, generally showing enhancement of chondrogenesis[15,16,21−24], though not all hypoxic effects are beneficial[25,26]. Nevertheless, it is well accepted that the transcription factor Hypoxia Inducible Factor-1 (HIF-1) has an oxygen sensitive region, HIF-1α[27], and in chondrocytes, this transcription factor appears to control many essential cellular activities, including cell survival[28,29], proliferation[30], differentiation[31], and matrix synthesis[32,33].

Considering the mounting evidence that hypoxic conditions may benefit the engineering of cartilage *in vitro*, we investigated the effects of hypoxia on human embryonic stem cells (hESCs). The experimental design comprised two continuous phases, chondrogenic differentiation in embryoid body (EB) form and tissue engineering of the cells derived from the EBs. We have previously employed a scaffold-less engineering strategy using hESCs to produce relatively large amounts of cartilage matrix that can be evaluated for quantitative biochemistry [i.e., collagen and glycosaminoglycan (GAG) contents] and biomechanics[1]. In this study, hypoxic or normoxic conditions were applied during the

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differentiation and self-assembly of the cells to obtain an understanding of how oxygen tension affects the chondrogenic potential of hESCs. We specifically hypothesized that hypoxic conditions during both differentiation and self-assembly would enhance the ability of hESCs to produce cartilage matrix and thereby improve their functional biomechanical properties. In particular, collagen II content was of interest due to the challenge of producing copious amounts of this important structural protein.

Methods

CULTURE CONDITIONS

hESC expansion

The National Institutes of Health (NIH)-approved H9 line (WiCell, Madison, WI, USA) was cultured according to standard protocols using a defined medium (www.wicell.org) and a gamma-irradiated CF-1 (Charles River Laboratories, Wilmington, MA, USA) mouse embryonic fibroblast (MEF) feeder layer on T75 culture plates (Nunc, Rochester, NY, USA). Frozen hESCs at passage 33 (p33) were thawed according to standard protocol and sub-cultured. The hESCs were passaged with collagenase IV (Invitrogen, Carlsbad, CA, USA) every 5–6 days and were used for the experiment at p38.

EB formation and differentiation conditions

Dispase solution [0.1% w/v in Dulbecco's Modified Eagle Medium/F12 (DMEM/F-12)] was applied for 10–15 min to colonies of undifferentiated hESCs in monolayer when the colonies reached 70–80% confluence, as previously performed[1]. After two washes and centrifugations with DMEM/F-12, the EBs were suspended in chondrogenic medium (CM) consisting of high-glucose DMEM (Invitrogen), 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, USA), 40-μg/ml L-proline, 50-μg/ml ascorbic acid, 100-μg/ml sodium pyruvate, and 1% fetal bovine serum (FBS, Gemini Bio-Products, West Sacramento, CA, USA). A ratio of 6-ml CM per T75 culture plate was used. The EBs were then distributed into sterile polystyrene petri dishes (12-ml CM per dish, 100 mm × 15 mm, Fisher, Hampton, NH, USA). A Billups-Rothenberg modular incubator chamber (Del Mar, CA, USA) was used according to manufacturer recommendations for hypoxic culture in a custom gas mixture of 2% O_2, 5% CO_2, and balance N_2 (Matheson TriGas, Houston, TX, USA). The incubator chamber was flushed with the custom gas mixture every 24 h at 20 l/min for 5 min. The incubator chamber was humidified by sterile deionized water, and the entire enclosure was placed in a 37°C incubator (5% CO_2, Thermo Fisher Scientific, Waltham, MA, USA). This incubator also housed the normoxic control EBs. EBs were used for self-assembly or for analysis at $t = 3$ wks. For the entire experiment, medium was completely changed every 48 h. During medium changes, the modular incubator chamber was open to normoxic conditions for less than 10 min. The O_2 and CO_2 levels were independently verified using a Kitagawa Gas Detection System (Matheson TriGas).

Self-assembly of chondrogenically differentiated hESCs

After 21 days of differentiation ($t = 3$ wks), hypoxic and normoxic EBs were digested in 0.05% trypsin−EDTA (Invitrogen) for 1 h followed by up to 2 h of 0.15% collagenase II (Worthington Biochemical Corp., Lakewood, NJ, USA) digestion, similar to previous studies[1,34]. Cells from each digest were counted with a hemocytometer, washed with DMEM containing 1% FBS, centrifuged at 200× g, and resuspended at a concentration of 5.0×10^5 cells per 20 μl in CM. Constructs were made by seeding the dissociated cell suspension into 3-mm wells of 2% agarose (5.0×10^5 cells per well, 300 μl of media per construct). We have previously used agarose in a similar manner to produce cartilage constructs with bovine articular chondrocytes and postulate that uniform constructs form largely due to the nonadhesive nature of agarose, which encourages cell−cell interactions during the initial stages of self-assembly[35]. Constructs from both differentiation conditions were then cultured in hypoxic or normoxic conditions, giving four total treatments: normoxic → normoxic (NN), normoxic → hypoxic (NH), hypoxic → normoxic (HN), and hypoxic → hypoxic (HH). All constructs were cultured with the same CM described above, except no serum was used during self-assembly.

ASSESSMENTS

Analysis of differentiated EBs

At $t = 3$ wks, EBs from both differentiation conditions were collected for analysis. Cells were digested as above with trypsin followed by collagenase

II and analyzed with flow cytometry to ascertain whether mesodermal markers were present by blocking with goat serum for 1 h, and adding 10 μg/ml of either immunoglobulin (Ig)G_1 isotype control (BD Biosciences, Franklin Lakes, NJ, USA), mouse anti-human CD44 (an adhesion protein and matrix receptor[36], Sigma-Aldrich, St. Louis, MO, USA), mouse anti-human CD105 (a transforming growth factor (TGF)-β receptor[37], Invitrogen), or mouse anti-human PDGFRα (a receptor involved in mesodermal development[38], R&D Systems, Minneapolis, MN, USA) for 30 min. Cells were washed in phosphate buffered saline (PBS), and then the Alexa Fluor® 488 goat anti-mouse antibody (Molecular Probes, Carlsbad, CA, USA) 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. This analysis was done once for each treatment.

EBs were also cryo-sectioned and stained for collagens using picrosirius red, GAGs using Alcian blue, and collagen I and collagen II using immunohistochemistry (IHC)[35]. Histology was used to detect unwanted differentiation, including hematoxylin and eosin (general morphology), von Kossa (calcified tissues such as bone), and Oil red O (adipose). Standard protocols were followed for each of these stains.

Analysis of self-assembled constructs

At the $t = 7$ wks time point (after 4 wks of self-assembly), each construct was measured for wet weight (WW) after carefully blotting excess water. Diameter and thickness measurements were made using digital calipers with an accuracy of 0.01 mm (Mitutoyo, Aurora, IL, USA). Constructs were either used for histology, biochemical assays, or biomechanical testing. Histological assessments for self-assembled constructs included picrosirius red, Alcian blue, IHC for collagen I and collagen II, von Kossa, Masson's trichrome, and Oil red O. Biomechanical testing included tensile testing using an Instron 5565 (Instron, Norwood, MA, USA) and unconfined compression using a modified creep indentation apparatus that used a flat platen rather than an indenting tip to apply a step load to the sample and record its deformation, as before[1,39].

Previously described protocols were used for picogreen, dimethyl-methylene blue (DMMB), and hydroxyproline tests, and one set of samples was used for these assays[35]. For collagens I and II, a second set of samples was used with previously described protocols for sandwich enzyme-linked immunosorbent assays (ELISAs)[1]. Flow cytometry was performed on cells enzymatically isolated from the self-assembled constructs once per treatment. The isolation of cells and protocols for flow cytometry were performed as before at $t = 3$ wks, using the same reagents, cell numbers, and appropriate gating.

Statistics

Data were analyzed with a two factor analysis of variance (ANOVA), using Tukey's *post hoc* test when applicable and a significance value of $P < 0.05$. A single experiment was conducted, and three to four samples were analyzed for biochemical assays and biomechanical tests. All data are reported as mean ± standard deviation.

Results

EB ANALYSIS ($T = 3$ WKS)

Over the 3 wks course of differentiation in EB form, EBs in hypoxic and normoxic conditions noticeably grew in size. The EBs appeared as highly hydrated structures with loosely connected matrix at $t = 3$ wks (Supplements 1−3), similar to previous work[1]. Typical EB size was approximately 300−500 μm in diameter. Sequential dissociation of the EBs with trypsin and collagenase released the cells from this matrix within 3 h. Flow cytometry analysis of the cells (Table I) demonstrated differential expression of mesodermal markers between the hypoxic and normoxic differentiation conditions.

SELF-ASSEMBLED CONSTRUCT MORPHOLOGY ($T = 7$ WKS)

The cells from the sequential digestion were used to make at least 12 constructs for each group. After the seeding of dissociated cells into the 3-mm agarose wells, cells in all four groups filled the entire well initially and, within 24 h, coalesced with noticeable contraction away from the outer edges

Table I

Mesodermal surface markers expressed at t = 3 wks *and* t = 7 wks. *The cells from chondrogenically differentiated EBs obtained at* t = 3 wks *as well as cells digested from self-assembled constructs at* t = 7 wks *were examined for CD44, CD105, and PDGFRα. Shown are the percentage of cells from each treatment that had fluorescence intensity (measured on channel FLH1) > 10^3 after appropriate gating (see* Methods). *Positive (+) expression is defined as greater than 1% over the treatment's isotype control. Any other result was considered negative (−). The results indicate that the expression of these markers changes depending on oxygen tension and stage of chondrogenesis (EB at* t = 3 wks *or self-assembly at* t = 7 wks)

Treatment	Isotype control (%)	CD44 (%)	CD105 (%)	PDGFRα (%)
Normoxic EBs (t = 3 wks)	4.5	29.7 (+)	12.4 (+)	8.9 (+)
Hypoxic EBs (t = 3 wks)	2.7	4.1 (+)	19.1 (+)	3.4 (−)
N → N (t = 7 wks)	2.9	38.5 (+)	34.8 (+)	7.4 (+)
N → H (t = 7 wks)	2.2	38.3 (+)	17.6 (+)	2.7 (−)
H → N (t = 7 wks)	2.7	5.1 (+)	17.0 (+)	1.1 (−)
H → H (t = 7 wks)	2.4	6.7 (+)	11.4 (+)	4.7 (+)

of the well. This contraction depended on both differentiation and self-assembly conditions. At *t* = 7 wks, cells differentiated under hypoxic conditions produced constructs with significantly smaller diameter ($P < 0.0001$) but greater thickness ($P < 0.0001$) than cells differentiated under normoxic conditions (Fig. 1). Conversely, there was a significant decrease ($P = 0.007$) in thickness for the constructs self-assembled under hypoxic conditions (HH and NH) compared to those in normoxic conditions (HN and NN). The self-assembly condition was also a significant factor on the WWs of the constructs, with hypoxic self-assembled constructs being lower compared to normoxic ($P < 0.0001$). The differentiation condition was not a significant factor on WW ($P = 0.96$, Fig. 1).

The cells differentiated under normoxic conditions (NH and NN) made constructs that were not as matrix rich as compared to the cells differentiated under hypoxic conditions (HH and HN), which produced dense, collagen-rich constructs (Fig. 1, Supplements 4−5). Other mesodermal tissues were not seen with standard histological stains for skeletal muscle, calcified tissue, or adipose (Supplements 1−3). Flow cytometry (Table I) on cells isolated from self-assembled constructs demonstrated differential expression of cell surface markers between hypoxic and normoxic self-assembly conditions, and the pattern of expression of the cell surface markers appeared to change between the end of EB differentiation (*t* = 3 wks) and the end of self-assembly (*t* = 7 wks).

BIOCHEMICAL ANALYSIS (T = 7 WKS)

Hypoxic chondrogenic differentiation was a significant factor ($P < 0.05$) for sulfated GAG content (Fig. 2), total collagen [Fig. 3(A)], collagen I [Fig. 3(B)], and collagen II contents [Fig. 3(C)]. In the two groups composed of hypoxic chondrogenically differentiated cells, collagen I and collagen II accounted for a greater proportion of the total collagen. Specifically, collagens I and II comprised 74% of HH constructs and 82% of HN constructs, while the proportions were 49% and 50% for NH and NN, respectively [Fig. 3(A)]. It also appeared that any time hypoxic conditions were used, the proportion of collagen II to total collagen increased about twofold, with HH and HN having about 10%, NH having 9%, and NN with 4% [Fig. 3(A)].

The self-assembly oxygen tension significantly affected the final number of cells in the constructs at *t* = 7 wks, with HH and NH constructs having fewer cells ($P = 0.0004$, in million cells, 0.36 ± 0.04 for NN, 0.19 ± 0.03 for NH, 0.31 ± 0.09 for HN, and 0.17 ± 0.07 for HH). The EB differentiation condition was not a significant factor for cell number.

BIOMECHANICAL EVALUATION (T = 7 WKS)

Hypoxic chondrogenic differentiation was a significant factor for the tensile modulus [Fig. 4(A)]. For example, HN and HH constructs had higher tensile moduli than NH constructs (3.3-fold and 2.6-fold, respectively). The ultimate tensile strength was not altered by differentiation or self-assembly conditions ($P > 0.05$, in MPa, 0.88 ± 0.39 for NN, 0.36 ± 0.14 for NH, 0.66 ± 0.23 for HN, and 0.66 ± 0.20 for HH).

The compressive properties of the constructs were also higher when hypoxic chondrogenic differentiation was involved [Fig. 4(B)]. For example, HH constructs had an instantaneous modulus (E_0) that was at least two times that of both NN and NH constructs, while HN constructs were almost four times higher than these same groups. The
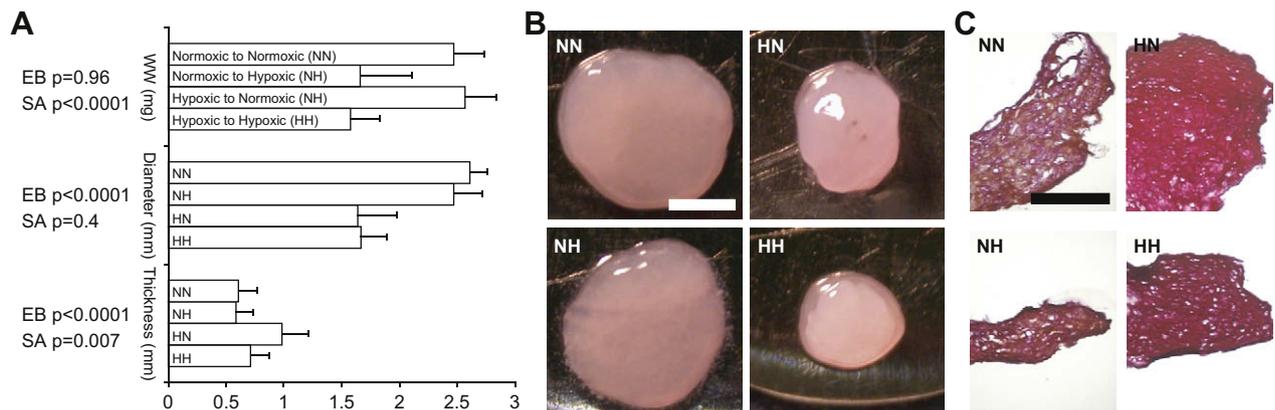

Fig. 1. Morphology of self-assembled constructs altered by hypoxia. Morphological measurements (A) demonstrate that the EB differentiation conditions had no effect on the WWs of the constructs, though the self-assembly conditions (SA) did, with hypoxic self-assembly conditions resulting in lower WW. The hypoxic EB differentiation conditions resulted in opposite effects on construct diameter (smaller, seen in A, B) and thickness (larger, seen in A, C), while the self-assembly conditions significantly affected only the thickness of the constructs (A, C), with hypoxic self-assembly constructs being thinner. The scale bar in (B) is 1 mm (200×). The scale bar in (C) is 500 μm (200×).

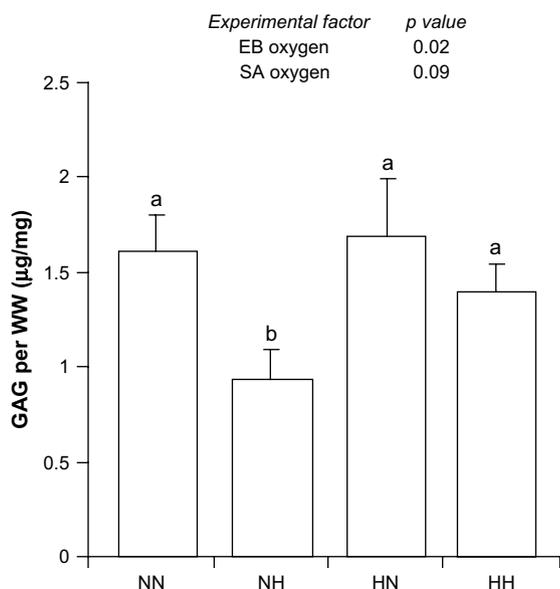| Experimental factor	p value
EB oxygen	0.02
SA oxygen	0.09

Fig. 2. GAG content affected by differentiation conditions. The two experimental factors (EB oxygen and self-assembly, or SA, oxygen) appeared to affect the GAG contents, with hypoxic differentiation and normoxic self-assembly higher. Only the EB oxygen conditions were significant, however. Note: groups not connected by the same letter are significantly different ($P < 0.05$).

relaxed moduli exhibited a similar pattern ($P < 0.05$, in kPa, 1.49 ± 0.29 for NN, 1.67 ± 0.35 for NH, 4.05 ± 1.09 for HN, and 2.49 ± 0.45 for HH).

Discussion

Understanding the factors and conditions that modulate chondrogenesis is of great importance to the development of cartilage tissue engineering strategies. This study demonstrates that hypoxic differentiation conditions enhance the chondrogenic potential of hESCs. Particularly exciting are the unique findings that (1) collagen II protein synthesis can be substantially increased in these stem cells and (2) the ratio of collagens I and II can be altered (Fig. 3). Furthermore, in several important metrics, normoxic self-assembly outperforms hypoxic self-assembly. The dramatic effects of oxygen availability were also manifested at the cellular level, where differences in expression patterns for CD44, CD105 and PDGFRα were observed. Together, these results demonstrate that oxygen availability has potent effects on the differentiation and synthetic potentials of hESCs.

Collagen II is the most abundant protein in hyaline articular cartilage, but producing this protein in large amounts remains elusive with engineered tissues. Previous studies on the effects of hypoxia on chondrocytes have shown that collagen II production can be increased in monolayer culture[24,32]. Investigations on the effects of hypoxia on adult cell systems, including tissue-derived stem cells and dermal fibroblasts, have demonstrated marked effects on cellular properties, such as surface markers and gene expression, and/or protein production[16,17,40,41], though none have specifically quantified collagen II or biomechanics. This study extends the important findings with chondrocytes and other cells to hESCs using a scaffold-less engineering strategy[1,35,42], demonstrating collagen II, collagen I, total collagen, and GAG contents are increased by hypoxic differentiation conditions (Figs. 2 and 3). This overall enhancement of chondrogenic potential was manifested in higher tensile and compressive stiffness properties (Fig. 4). Characterizing the makeup of engineered cartilages, especially the collagen matrix, is essential to making functional replacements, as the biomechanics of native cartilages are strongly tied to their structural composition[43]. Collagen I and/or collagen II dominate the extracellular matrix of native cartilages. Figure 3 demonstrates that the hypoxic differentiated cells produce a collagen matrix that has a higher proportion of these important collagens. Modulating the type of collagens that stem cells produce may be critical in determining their cartilage applicability.
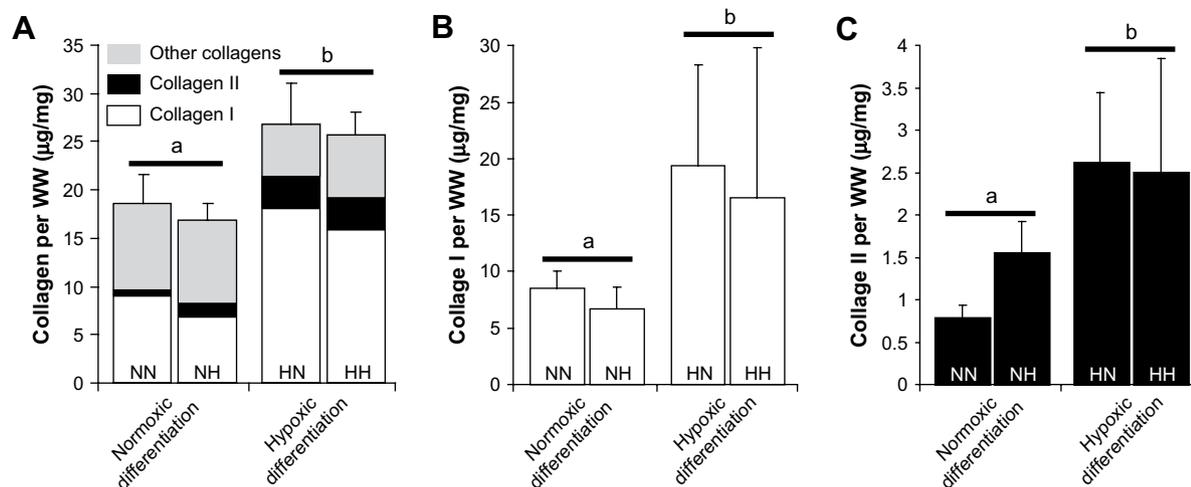

Fig. 3. Hypoxic conditions control total and specific collagen contents. The total collagen (A), collagen I (B, white bars), and collagen II (C, black bars) contents are shown with the contribution of each specific collagen to the total collagen summarized in A. Hypoxic differentiation consistently resulted in higher collagen production at $t = 7$ wks (A–C). For example, there was a 3.4-fold increase in collagen II and 2.2-fold increase in collagen I between HN and NN constructs. Furthermore, the proportion of collagens I and II was higher in the constructs made with hypoxic differentiated cells, indicating greater maturity of the collagen matrix.
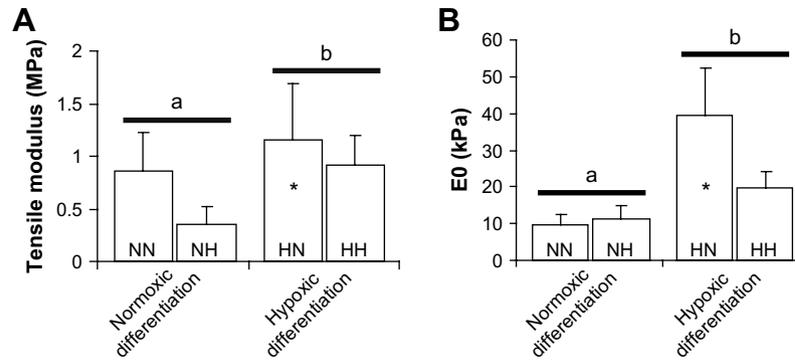
Fig. 4. Biomechanics enhanced with hypoxic differentiation. Constructs assembled with hypoxic differentiated cells had higher tensile (A) and compressive (B) moduli. The HN group in particular performed best amongst all groups [for Fig. 4(A) and (B), * compares highest mean with lowest mean with $P < 0.05$].

At the cellular level, the present study observed differential surface marker expression between conditions (Table I). It is possible that the observed differences may be due to selection of cells (e.g., those having higher proliferative potential or resistance to apoptosis during enzymatic digestion); however, the digestion method was the same for all treatments, regardless of time point, differentiation condition, or self-assembly condition. It is also possible that hypoxia exerted effects on tissue-development patterns, as others have observed[44]. It is interesting to note that the hypoxic cell population at $t = 3$ wks did not express CD44 or PDGFRα to the extent that the normoxic cells did. Hypoxia activates a platelet derived growth factor receptor pathway that can impact cell survival, proliferation, and metabolism[45]. Our results indicate that levels of PDGFRα may not be elevated after hypoxic treatment, which agrees with hypoxia studies in other tissues. No change in PDGFRα was observed in the rat corpus cavernosum after transient *in vivo* hypoxia[46], while up-regulation of PDGFRα was a short-term response that occurred on initial exposure to hypoxic−ischemic injury in the rat neonatal brain[47]. On the other hand, hypoxic down-regulation of CD44 has been observed[48]. The results that the hypoxic differentiated cells had low levels of PDGFRα and CD44 appear to be consistent with the reported effects of hypoxia on these proteins. Furthermore, CD44 gene expression has been observed to decrease as mesenchymal stem cells chondrogenically differentiate[49], and elevated CD44 surface marker expression is associated with dedifferentiation of human articular chondrocytes in monolayer culture[50]. Our results in conjunction with the above reports suggest that PDGFRα and CD44 may not be positively predictive of a chondrogenic phenotype that involves higher production of collagen II. It was also notable that the expression pattern of the surface markers was relatively static for the hypoxic-differentiated cells in either self-assembly condition (hypoxic or normoxic), while this was not true for the normoxic-differentiated cells. It will be of future interest to investigate whether these differences in expression patterns, particularly in the adhesion protein CD44, may be related to the observed morphological differences between conditions, particularly the diameters and heights of the HN and HH constructs vs the NN and NH constructs (Fig. 1). Overall, the tissue characterization and these flow cytometry results illustrate that hypoxic chondrogenic differentiation affects hESCs at many levels.

Interestingly, normoxic self-assembly outperformed hypoxic self-assembly conditions in terms of cell numbers and WWs, while being typically higher in all other quantitative assays (Figs. 2−4). Previous work has shown that cell proliferation of adult stem cells is inhibited by hypoxic conditions[16]. Others have postulated that the cells have a higher energy demand when they must synthesize cartilage matrix *de novo*[25]. This energy demand would be more easily quenched by oxidative phosphorylation as opposed to anaerobic glycolysis. However, hypoxic self-assembly should not be ruled out entirely. Since the collagen II content in the NH constructs was two times that of NN constructs, it seems the synthetic repertoire of chondrogenically differentiated hESCs can still be modulated during self-assembly. The biochemical basis for this may be related to the constitutively expressed protein HIF-1[27], as its oxygen sensitive region has been linked to an essential enzyme involved in collagen synthesis[33]. Balancing the metabolic demands of self-assembling cartilage with the desire to produce more collagen II will be an intriguing future challenge with hESCs, as others have shown that sequential regulation of oxygen conditions can improve chondrogenesis with ATDC5 cells[23].

This study raises new avenues of exploration in other respects, including the need to demonstrate the plasticity of differentiated hESCs. As the evidence mounts regarding their possible utility for therapeutic purposes, it will become increasingly important to establish strict standards of safety for these cells, including the demonstration that the differentiated cells do not form teratomas. To date, our histological results have shown the absence of unwanted differentiation in our cartilage constructs in terms of bone, muscle, and adipose tissues (Supplements 1−3). Another level of safety involves the elimination of xenogenic medium supplements such as serum. This study demonstrates that a minimal amount of serum (1% during differentiation and none during self-assembly) is sufficient, even in hypoxic conditions. Another goal of this laboratory is to establish serum-free conditions for hESC-derived chondrogenesis. Establishing minimal serum conditions for this process is one step toward this goal. An additional challenge in this research will be controlling the hypoxic conditions and limiting the exposure of the cells to normoxia during medium changes.

In our study of hESCs for cartilage tissue engineering we continue to test a working hypothesis that cells can be generated with different functional capacities to produce cartilage constructs depending on their differentiation conditions. The modular experimental design of these studies allows the testing of this important hypothesis by including an EB differentiation phase, where varying conditions can be used (i.e., hypoxic or normoxic), and a self-assembly

phase, where either the same or different self-assembly conditions can be used, so long that a full-factorial design is employed. The evidence to date supports our hypothesis, as differences in the chondrogenic potentials of hESCs in this study were demonstrated due to oxygen availability, as well as previous work that investigated effects of growth factors[1] and differentiation timelines[34]. The ability to assess chondrogenic potential at the tissue level is of great importance toward engineering functional cartilage with stem cells. At the same time, elucidating the basic mechanisms of differentiation and identifying potential cellular markers of chondrogenic cells that can be used for isolation purposes (Table I) remain vital to the pursuit of stem cell therapies.

In summary, this study demonstrates large and significant effects of hypoxia on hESC-derived chondrogenesis. Hypoxic differentiation conditions enhanced the ability of the cells to produce functional cartilage constructs, and this was demonstrated at the protein and biomechanical levels. In particular, the significant increase in collagen II production by hypoxic conditions raises the possibility of generating a spectrum of different cartilages, including TMJ cartilages, knee meniscus, and hyaline articular cartilage, with a single cell source in hESCs. Identifying potent regulators of chondrogenesis and understanding how to use them are important steps toward generating functional cartilage for the replacement of damaged or diseased tissue with stem cells.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

Supplementary data

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.joca.2008.04.007.

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