Hypoxia-induced collagen crosslinking as a mechanism for enhancing mechanical properties of engineered articular cartilage

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SUMMARY

Objective: The focus of tissue engineering of neocartilage has traditionally been on enhancing extracellular matrix and thus biomechanical properties. Emphasis has been placed on the enhancement of collagen type and quantity, and, concomitantly, tensile properties. The objective of this study was to improve crosslinking of the collagen network by testing the hypothesis that hypoxia could promote pyridinoline (PYR) crosslinks and, thus, improve neocartilage's tensile properties.
Methods: Chondrocyte expression of lysyl oxidase (LOX), an enzyme responsible for the formation of collagen PYR crosslinks, was first assessed pre- and post- hypoxia application. Then, the mechanical properties of self-assembled neocartilage constructs were measured, after 4 weeks of culture, for groups exposed to 4% O_2 at different initiation times and durations, i.e., during the 1st and 3rd weeks, 3rd and 4th weeks, 4th week only, continuously after cell seeding, or never.
Results: Results showed that LOX gene expression was upregulated ∼20-fold in chondrocytes in response to hypoxia. Hypoxia applied during the 3rd and 4th weeks significantly increased PYR crosslinks without affecting collagen content. Excitingly, neocartilage tensile properties were increased ∼2-fold. It should be noted that these properties exhibited a distinct temporal dependence to hypoxia exposure, since upregulation of these properties was due to hypoxia applied only during the 3rd and 4th weeks.
Conclusion: These data elucidate the role of hypoxia-mediated upregulation of LOX and subsequent increases in PYR crosslinks in engineered cartilage. These results hold promise toward applying hypoxia at precise time points to promote tensile integrity and direct construct maturation.

Introduction

Articular cartilage (AC) pathologies, resulting from injury/trauma or age-related degeneration, are major health problems in the developed world. Osteoarthritis (OA) is the most frequent chronic musculoskeletal disease, limiting daily activities of the elderly[1]. Over the past two decades, progress has been made on the development of therapeutic approaches for the treatment of early stage cartilaginous defects, thus slowing down their progression to OA. However, current therapeutic procedures including washing, shaving and debridement, stem cell stimulation-based procedures, and explant grafts have thus far proved to be incapable to effect long-term repair[2]. Tissue engineering has the potential to generate tissue with biomechanically competent extracellular matrix (ECM) in vitro[3]. However, limitations in the development of neotissues that mimic the structural composition and, hence, biomechanical behavior of native tissues present a great challenge[4]. To generate neotissues capable of bearing physiological loads, various exogenous stimuli during neocartilage culture have been examined.

In engineering cartilage, hypoxia has been proposed for both stem cells and differentiated chondrocytes. For stem cells, hypoxia has been reported to promote chondro-differentiation[5–7] and to impede hypertrophy[8]. Hypoxia inducible factor 1 (HIF-1) has been hypothesized as critical for hypoxic induction of chondrogenesis[8]. For engineering AC, however, hypoxia has shown mixed results. For instance, some studies have suggested that Sox-9 expression is largely independent of hypoxia[9], while others have shown that this transcription factor increases with reduced oxygen levels[10]. Likewise, ECM components (e.g., aggrecan, collagen type II, proteoglycan 4) have been shown to be upregulated with hypoxia in certain cases[10,11], while others have shown that cartilaginous ECM production is indifferent to hypoxia[12] or even suppressed by it in certain instances[13,14]. In brief, though the majority of evidence

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supports hypoxia as an effective method for enhancing engineered cartilage constructs, mechanisms behind such improvements have largely remained elusive. For hypoxia to be used efficiently and reliably in cartilage tissue engineering, a robust mechanistic connection between hypoxia and mechanical properties must be determined.

In the human body, normal oxygen tension, or normoxia, is between 5% and 13%[15]. Oxygen levels lower than 5% are considered hypoxic[16], while the atmospheric oxygen tension of 21% should be considered hyperoxic[17]. For differentiated chondrocytes, oxygen tension can be as low as 1–2.5%, thus qualifying cartilage as functioning under a hypoxic environment[14]. Thus, oxygen concentration during in vitro culture likely needs to be below 5% to induce significant HIF-1α stabilization and its subsequent upregulation of various matrix-associated genes.

The anisotropic, structural and mechanical properties of AC, including its well-developed, collagen fiber architecture, determine tissue functionality and mechanical integrity[18]. Apart from collagen content and organization, the load-bearing capacity of collagen is related to other ECM components. Two features that directly influence native AC stiffness and determine collagen maturity are the type and quantity of collagen crosslinks[19,20]. Thus far, these critical features have not been sufficiently mimicked in the ECM of engineered tissues; lack of progress on this front may be a factor for the current limitations in tissue modulus and strength[19,21]. An opportunity exists in the modulation of collagen crosslinking in engineered neocartilage to enhance tissue structural and functional integrity. While hypoxia has been associated with collagen crosslinking in several other tissues[22–24], its use for inducing crosslinks in engineered cartilage remains unexplored.

In this study, engineered cartilage is formed using a scaffoldless, self-assembling process that removes the potentially confounding effects of scaffolds. The objectives of this study are to examine two hypotheses: (1) that hypoxia (4% oxygen) will enhance the mechanical properties of engineered AC by mediating collagen crosslinking via lysyl oxidase (LOX), and (2) this specific response to hypoxia is dependent on application time.

Methods

Chondrocyte isolation

AC was sterilely harvested from the distal femur of knee joints obtained from 1-week-old calves (Research 87, Boston, MA, USA). Tissue was minced into 1 mm pieces and digested in 0.2% collagenase type II (Worthington, Lakewood, NJ, USA) for 18 h in cell culture medium (Dulbecco's modified Eagle's medium (DMEM) with low glucose (1 g/L)) (Life Technologies corp., Carlsbad, CA, USA), 10% fetal bovine serum (FBS) (Atlanta Biologicals), 1% non-essential amino acids (NEAA) (Life Technologies corp., Carlsbad, CA, USA), 25 mg of L-ascorbic acid (Sigma–Aldrich, St. Louis, MO, USA), and 1% penicillin/streptomycin/fungizone (PSF) (BioWhittaker Inc., Walkersville, MD, USA). After digestion, articular chondrocytes were washed three times in PBS with centrifugation and filtered through a 70-μm filter. Cells then were counted and were frozen at $-80°$C in culture medium supplemented with 10% FBS and 10% dimethyl sulfoxide (Fischer Scientific, Pittsburgh, PA, USA). Cells were stored in liquid nitrogen until used.

Construct formation via the self-assembling process

Constructs were generated by seeding articular chondrocytes into cylindrical, non-adherent wells using a technique adapted from previous work[3,25]. A stainless steel mold consisting of 5 mm diameter cylindrical prongs was placed into molten 2% agarose (Life Technologies corp., Carlsbad, CA, USA) in a 48-well plate. The agarose solidified at room temperature, and the mold was removed. Two changes of control medium (DMEM with GlutaMAX (Life Technologies corp., Carlsbad, CA, USA), 100 nM dexamethasone (Sigma–Aldrich, St. Louis, MO, USA), 1% NEAA, 1% PSF, 1% ITS+ premix (BD Scientific, Franklin Lakes, NJ, USA), 50 mg/mL ascorbate-2-phosphate (Sigma–Aldrich, St. Louis, MO, USA), 40 mg/mL L-proline (Sigma–Aldrich, St. Louis, MO, USA), and 100 mg/mL sodium pyruvate (Fischer Scientific, Pittsburgh, PA, USA)) were used to saturate the agarose before cell seeding. Following isolation, articular chondrocytes were thawed within 5 days of freezing. Viability assessed using trypan blue was >90%. To create each construct, 5.5 million cells in 100 μl control medium were seeded into each gelled, cylindrical agarose well, followed by addition of 400 μl control medium after 4 h. Cells coalesced into free-floating, disc-shaped constructs upon the non-adhesive agarose; $t = 1$ day was defined as 24 h after seeding. Constructs were cultured in the agarose wells until $t = 10$ days, at which point they were unconfined transferred to 48-well plates where they were unrestricted by circumferential confinement. Constructs received 500 μl medium change every 24 h and remained in culture until $t = 28$ days.

Hypoxia application

Following seeding, constructs were divided into five treatment groups. Constructs in the first group (control) were incubated continuously at 37°C, 5% CO_2, and 21% O_2. Constructs from the other groups were incubated under the same conditions but also treated with 4% O_2 (hypoxia) during the following culture periods: $t = 1$–7 days and 15–21 days (1st and 3rd weeks), $t = 15$–28 days (3rd and 4th weeks), $t = 22$–28 days (4th week), and $t = 1$–28 days (continuous hypoxia). Every other day, 500 μl of medium was changed. After 4 weeks of culture, constructs were divided into parts for histological, biochemical, and biomechanical assessments.

Histology

Samples of the neotissue were cryoembedded in Histoprep (Fisher Chemical, Vernon Hills, IL, USA) and sectioned at 14 μm with orientation from top to the bottom of the construct, in all groups. Following sectioning, samples were fixed in formalin for 15 min. Qualitative evaluation of collagen and glycosaminoglycan (GAG) content of the samples was performed by using Safranin-O/fast green and Picrosirius red stains as previously described[26]. Phenotype maintenance of articular chondrocytes was evaluated with immunohistochemistry (IHC). Briefly, samples were fixed in chilled (4°C) acetone, rehydrated, and stained for collagen type I and II by following protocols provided by Chondrex and Vectastain as previously described[26]. Native bovine AC and patellar tendon were used as positive type II and type I controls, respectively.

Quantitative biochemistry

Biochemical samples were lyophilized, and dry weight was recorded. Subsequently, samples were digested using a pepsin-elastase protocol as previously described[26]. Collagen content in the engineered tissue was evaluated using a colorimetric hydroxyproline assay[27]. A Blyscan Glycosaminoglycan Assay kit (Biocolor, Newtownabbey, Northern Ireland) was used for sulfated GAG content quantification[28,29]. Cell content in the engineered tissue was approximated using a Picogreen dsDNA reagent (Molecular Probes, Eugene, OR, USA) for DNA quantification and a conversion factor of 7.7 pg DNA/cell.

High-performance liquid chromatography (HPLC)

The abundance of pyridinoline (PYR) crosslinks in the engineered tissue was quantified using HPLC. Portions of the constructs were weighed wet, digested in 400 μl 6 N HCl, and dried in a vacuum concentrator. 50 μL of an aqueous solution containing 10 nmol pyridoxine/mL and 2.4 μmol homoarginine/mL was used for sample re-suspension and then diluted 5-fold with an aqueous solution of 0.5% heptafluorobutyric acid (HFBA) in 10% acetonitrile. Following this, 50 μL of each sample was injected into a 25 mm C18 column (Shimadzu, Columbia, MD, USA) and eluted using a solvent profile as previously described[30]. A calibration curve was performed using PYR standards (Quidel, San Diego, CA, USA) for crosslink quantification.

Compression testing

The compressive properties of the constructs were evaluated with creep indentation testing as previously described[3]. A 0.7 g (0.007 N) mass was applied through a 0.45 radius flat, porous indenter tip on each sample stabilized to a stainless steel surface. Compressive properties of the neocartilage, represented by aggregate modulus, permeability and Poisson's ratio were calculated by adapting a semi-analytic, semi-numeric, linear biphasic model[31].

Tensile testing

A uniaxial material testing apparatus (Instron, model 5565; Canton, MA, USA) (Instron Model 5565) was used to quantify the tensile properties of the constructs as previously described[26]. Each specimen was prepared by cutting it into a dog-bone shape with a 1-mm-long gauge length. Photographs were taken of each specimen and thickness and width were determined using ImageJ software. Uniaxial tension until failure within the gauge length at a strain rate of 1% of the gauge length per second was performed using a 5 kN load cell. Force-displacement curves were generated, and stress–strain curves were calculated by normalizing data to specimen dimension. The apparent Young's modulus, a measure of specimen tensile stiffness, was determined by least squares fitting of the linear region of the stress–strain curve. The ultimate tensile strength (UTS) was determined as the maximum stress reached during a test.

RT-PCR

Real-time PCR analysis was performed to investigate hypoxia-mediated gene expression of the target genes. Dynamic changes in LOX expression can occur during the first 18 h of a culture's introduction to hypoxia[17,32]. In order to observe LOX expression at steady state, measurements within the short time period immediately after hypoxia exposure were avoided. On the other hand, since LOX-induced crosslink formation can require weeks to mature, it was desirable to assess for LOX expression as early as possible. Thus, hypoxia was applied in 3 week-engineered

neocartilage for 2 days and the gene expression of LOX was quantified as previously described[17]. S18 (housekeeping gene) and LOX primers were purchased from US Biological. RT was performed by incubating 500 ng of RNA with SuperScript III (Life Technologies corp., Carlsbad, CA, USA) as recommended by the manufacturer. Real-time PCR was done using SYBR Green mastermix and 1 μm primers on a Rotor-gene 3000 real-time PCR machine (Corbett Research, Bath, UK). A 10 min denaturing step was employed, followed by 45 cycles of 95°C (15 s) and 60°C (60 s). The take-off cycle (C_T) for each gene of interest (GOI) was compared to the house-keeping gene GAPDH. Relative gene expressions were calculated using the $2^{-\Delta\Delta CT}$ method.

Statistical analysis

All biochemical and mechanical assessments in this study were performed using $n = 5–8$ samples per group. Numerical data are represented as mean ± 95% confidence interval for the mean (95% CI). The presence of outliers was determined using either Grubb's test and/or by noting testing abnormalities (e.g., failure at the grips during tensile testing). Prior to performing statistical tests, the data were checked for both normality and equal-variance using Levene's test. To compare among treatment groups, one-way analysis of variance (ANOVA) was performed using StatView software (StaView inc Cary, Nesbit, MS, USA). If significance was identified, Fisher's post hoc testing was applied. Unequal sample sizes were addressed by non-parametric all-pairs multiple comparisons based on pairwise rankings in the one-way design with the Steel–Dwass procedure. P-values less than 0.05 were considered significant.

Results

Gross morphology and histology

At the end of the culture period the morphological properties and composition of the engineered constructs were assessed via gross inspection and histological evaluation. All constructs presented with similar flat surfaces without abnormalities and no significant differences were detected in morphology among the groups. Table I describes morphological characteristics (diameter, thickness, and wet weight (WW)) of the constructs. Histology showed that all constructs stained uniformly positive for both GAG and collagen content (Fig. 1). Additionally, IHC showed that neocartilage from all groups stained positive for collagen type II and negatively for collagen type I proving normal AC phenotype maintenance in all groups (Fig. 1).

Biochemical properties

Growth of the constructs was evaluated through assessment of the amount of collagen, GAG, DNA, and PYR of neotissue at the end of the culture period. Table II describes the biochemical

Table I
Growth metrics and biochemical content of 4 weeks neocartilage constructs*

Groups	Diameter (mm) ($P = 0.4645$)	Thickness (mm) ($P = 0.2384$)	Wet weight (mg) ($P = 0.4566$)	Water content (%) ($P = 0.7472$)	Cell number (millions) ($P = 0.2669$)
Control	5.21 ± 0.22	1.53 ± 0.11	29.87 ± 4.93	88.75 ± 13.23	3.01 ± 2.86
1st & 3rd weeks	5.17 ± 0.33	1.35 ± 0.25	29.65 ± 8.80	90.50 ± 12.11	4.03 ± 3.26
3rd & 4th weeks	5.11 ± 0.23	1.40 ± 0.22	26.45 ± 2.06	87.46 ± 7.93	4.24 ± 5.22
4th week	5.09 ± 0.33	1.48 ± 0.42	28.30 ± 7.42	87.81 ± 9.52	4.46 ± 4.39
Continuous	5.20 ± 0.24	1.45 ± 0.35	28.09 ± 10.95	88.19 ± 5.43	2.64 ± 2.22

* The effects of hypoxia as a function of application time during the self-assembly of AC (control, 1st & 3rd weeks, 3rd & 4th weeks, 4th week, and continuous hypoxia groups) were examined on gross morphology (diameter, thickness, and WW) and biochemical properties (water content and cell number) after 4 weeks of culture. Values are mean ± 95% CI. No significant differences were detected among controls and hypoxia-treated groups. See Results section for details. Col = collagen.
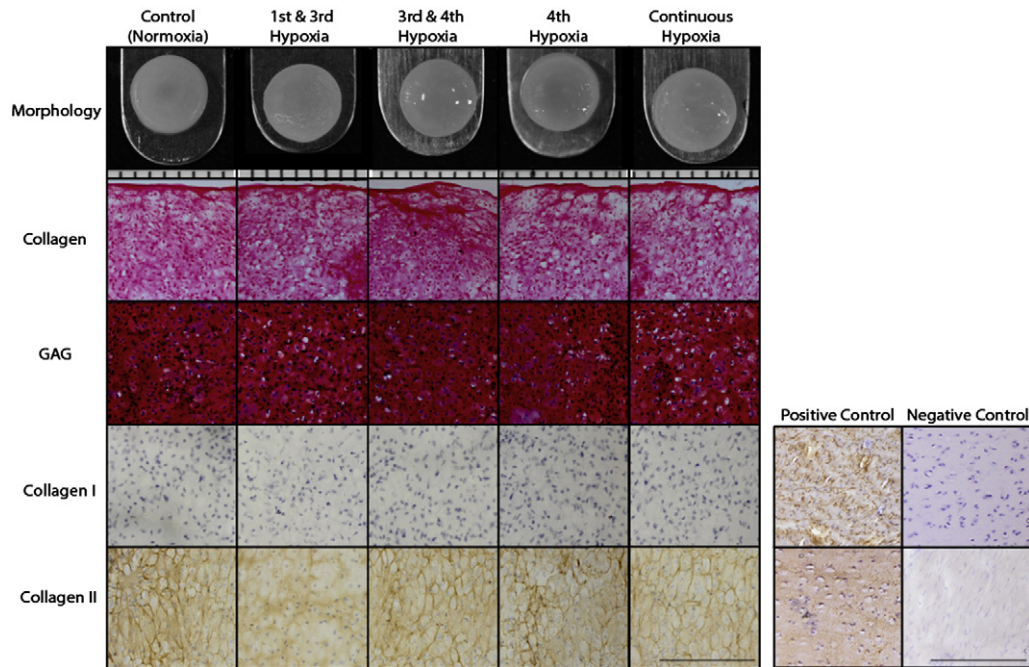
Fig. 1. Gross morphology, histology, and IHC of self-assembled neocartilage. Both controls and hypoxia-treated constructs formed uniform neocartilage constructs with similar flat surfaces without physical abnormalities. The orientation of cryosectioning was from top to bottom in all groups (14 μm sections). Safranin-O/fast green staining for GAGs and Picrosirius red staining for collagen showed that constructs produced these matrix components uniformly for all groups. IHC illustrated that all groups were positive stained for collagen type II but not for collagen type I, suggesting AC phenotype. For gross morphology (top row), each notch represent 1 mm. For histology, bar represents 200 μm.

characteristics of the constructs. No significant differences were detected in GAG per wet weight (GAG/WW) among the groups ($P = 0.718$). For collagen per wet weight (Col/WW), no significant differences were detected among control, and late hypoxia application groups (3rd and 4th weeks, and 4th week group) ($P = 0.2716$ and $P = 0.9919$, respectively). Interestingly, constructs treated with hypoxia at an early stage (1st and 3rd weeks and all weeks groups) presented with significantly lower amount of collagen over control and late treated groups ($P = 0.004$ and $P = 0.004$).

Hypoxia applied during 3rd and 4th weeks significantly increased the amount of PYR/WW over controls (34% increase) ($P = 0.003$), while no significant effects were detected on the other applied groups over controls ($P = 0.4160$, $P = 0.9753$, and $P = 0.9991$ for 1st and 3rd weeks, 4th week and continuous groups, respectively) (Table II). In contrast, when PYR content was normalized to collagen content (PYR/Col), early application of hypoxia (1st and 3rd weeks, continuous groups) exhibited the highest amount of collagen crosslinks ($P < 0.001$ and $P = 0.001$, respectively) (Table II). For the 3rd and 4th weeks group, the amount of PYR/Col was also significantly higher over controls ($P = 0.0499$), while in the 4th week group no significant differences were detected ($P = 0.4583$).

Biomechanical properties

To quantify the influence of low oxygen tension applied at different time periods on the biomechanics of self-assembled neocartilage, compressive properties represented by the aggregate modulus, permeability, and poison's ratio, and tensile properties represented by Young's modulus (E_Y) and UTS and were determined. No significant difference was detected for compressive properties among groups ($P = 0.4017$, $P = 0.1101$, and $P = 0.3124$ for aggregate modulus, permeability, and Poisson's ratio, respectively) (Table III). Low oxygen tension applied during 3rd and 4th weeks of the self-assembling process significantly increased the tensile stiffness of the constructs over controls ($\sim 80\%$ increase) ($P < 0.0001$). Similar trends were observed concerning the UTS of the constructs (Table III). Enhanced mechanical properties reflected the observed changes in the PYR content.

RT-PCR

Real-time PCR was employed to investigate the effects of hypoxia in the engineered neocartilage using the self-assembly method. Application of hypoxia in the neotissue promoted an

Table II
Biochemical properties of self-assembled neocartilage*

Groups	Collagen/WW % ($P < 0.0001$)	GAG/WW % ($P = 0.718$)	PYR/WW (nmol/g) ($P = 0.0036$)	PYR/Col (nmol/mg) ($P < 0.0001$)
Control	3.03 ± 0.61^A	4.39 ± 2.10	4.99 ± 1.86^B	0.16 ± 0.030^D
1st & 3rd weeks	1.93 ± 1.27^B	3.84 ± 3.85	$5.68 \pm 1.00^{A,B}$	0.30 ± 0.169^A
3rd & 4th weeks	3.33 ± 1.23^A	4.96 ± 3.44	6.63 ± 1.90^A	0.20 ± 0.034^C
4th week	3.03 ± 1.50^A	4.91 ± 3.50	5.19 ± 1.06^B	$0.17 \pm 0.105^{C,D}$
Continuous	1.91 ± 0.44^B	4.44 ± 2.96	5.08 ± 1.88^B	0.26 ± 0.063^B

* Biochemical properties of the engineered neocartilage as represented by Col/WW, GAG/WW, PYR/WW and PYR/Col. Values are mean ± 95% CI. Fisher's *post hoc* testing was applied if $P < 0.05$. Groups not connected by the same letter are significantly different. See Results section for details. Col = collagen.

Table III
Biomechanical properties of the engineered neocartilage*

Group	Aggregate modulus (MPa) ($P = 0.4017$)	Permeability (10^{-15} m^4/N's) ($P = 0.1101$)	Poisson's ratio ($P = 0.0773$)	Young's modulus (MPa) ($P < 0.0001$)	UTS (MPa) ($P = 0.4600$)
Control	0.11 ± 0.06	18.50 ± 15.69	0.09 ± 0.22	$0.45 \pm 0.23^{B,C}$	0.07 ± 0.04
1st & 3rd weeks	0.10 ± 0.06	16.85 ± 16.89	0.08 ± 0.02	0.19 ± 0.23^{C}	0.11 ± 0.07
3rd & 4th weeks	0.12 ± 0.05	11.62 ± 9.68	0.05 ± 0.11	0.82 ± 0.53^{A}	0.06 ± 0.09
4th week	0.13 ± 0.04	16.04 ± 8.70	0.01 ± 0.02	$0.53 \pm 0.54^{A,B}$	0.11 ± 0.11
Continuous	0.11 ± 0.06	24.98 ± 28.31	0.06 ± 0.12	$0.25 \pm 0.19^{B,C}$	0.18 ± 0.18

* Compression properties of tissue engineered neocartilage constructs represented by aggregate modulus, permeability, and Poisson's ratio, and tensile properties represented by Young's modulus and UTS. Values are mean ± 95% CI. Fisher's *post hoc* testing was applied if $P < 0.05$. Groups not connected by the same letter are significantly different. See Results section for details.

18-fold increase in the gene expression of LOX over controls cultured under normoxic conditions.

Discussion

This is the first study to demonstrate that hypoxia's effect on enhancing engineered cartilage's tensile properties is through collagen crosslinking. Experimental data proved both hypotheses motivating the study, showing (1) hypoxia is a viable method for promoting collagen crosslinking in engineered tissue through LOX gene expression and (2) distinctly different responses can be elicited by this stimulus by manipulating application time and duration. Hypoxia can be restricted to influence only the tensile properties of the constructs, while other neocartilage properties such as gross morphology, collagen and GAG biochemistry, and compressive stiffness remain unaltered. By determining a method wherein hypoxia can be isolated to act only through collagen crosslinks, this study provides a robust method for improving the tensile properties of engineered cartilage that has the potential to be extended to other engineered tissues.

In this study, hypoxia was applied in tissue engineered AC to enhance crosslinking by promoting gene expression of LOX. Low oxygen tension (4% O$_2$) applied during the 3rd and 4th weeks of self-assembly of neocartilage significantly increased the amount of PYR molecules (PYR/WW and PYR/Col) over controls and the other treated groups with concomitant increase in the gene expression of LOX (18-fold) over controls. In contrast, no significant differences were observed among the 3rd and 4th weeks, the 4th week and control groups for collagen, GAG and DNA content per WW. Additionally, hypoxia applied during the 3rd and 4th weeks increased the tensile stiffness of neocartilage by approximately 80% over controls ($P < 0.0001$). These results demonstrate that application of hypoxia can be directed to promote collagen crosslinking and enhance the tensile properties in engineered cartilage, independently of other biochemical and biomechanical properties.

In contrast, hypoxia applied during times other than the 3rd and 4th weeks did not result in improved properties. At times, these properties were diminished, shedding light onto the mixed results seen in the cartilage engineering literature that employ hypoxia. Early application of hypoxia (1st and 3rd weeks and continuous application groups) significantly increased the PYR/Col content, but collagen content was diminished simultaneously. Early hypoxia application allowed for a more time for collagen crosslinking to form. For reference, the characteristic time constants for the formation of immature (difunctional) and mature (trifunctional) PYR crosslinks have been reported to be 1–2 and 7–30 days, respectively[33]. However, early hypoxia application also significantly decreased the collagen content of neotissue at these groups. The net effect is that early hypoxia application did not increase PYR/WW, and the mechanical properties of neotissue remained unchanged. Biochemical evaluation further showed no significant differences in terms of the number of cells per construct and GAG/

WW in these groups. Thus, it appears that early hypoxia application is actually detrimental to engineering self-assembled cartilage when it is applied during a time previously identified as the collagen synthesis phase[25].

These results are similar to a previous study investigating the effects of 5% and 20% O$_2$ applied continuously for 2, 4 and 6 weeks in a scaffold-free chondrocyte culture[13]. In this study, engineered cartilage from scaffold-free cultured chondrocytes at 20% O$_2$ produced better ECM than that at 5% O$_2$. Thus, the present study offers an examination on the conflicting results currently seen with hypoxia; hypoxia applied during the collagen synthesis phase of engineered cartilage can diminish collagen production and therefore adversely affect construct biomechanical properties. Use of hypoxia to induce crosslinking should instead be employed after collagen is already present. This time dependence issue should be further investigated in other cartilage engineering approaches.

In three-dimensional cultures or engineered tissues, work on studying the effects of different oxygen concentrations might be impaired by poor oxygen diffusion that creates an oxygen gradient within tissue[34]. Additionally, HIF-1α protein is very unstable at the environmental oxygen level while its mRNA is not affected by hypoxic conditions[16]. This makes it difficult to demonstrate, experimentally, that the HIF-1α protein increases in response to hypoxia. However, the link between hypoxia, LOX gene expression, and LOX-mediated collagen crosslinking has been explored in many other tissues[17,23,25,35]. Specifically, growth of porcine aortic endothelium cells in 0% or 2% oxygen tension resulted in little change in cell numbers or cell protein, but a fall in collagen synthesis and in proline and lysine hydroxylases, as well as a rise in LOX gene expression, were observed[35]. Similarly, the crosslink pattern and the gene expression of lysyl hydroxylase 2 (LH2) were investigated using skin cells from systemic scleroderma, cultured under low oxygen tension conditions. Prolonged hypoxia induced a marked increase of the mRNA level of LH2 in relation to collagen I[22]. Thus, the use of hypoxia to promote LOX- and LH-mediated collagen crosslinking can be considered as a robust mechanism and this experiment showed not only that hypoxia can be used in engineering cartilage, but that its effects can be isolated to tensile properties only.

Further proof of the robustness of the hypoxia-tensile relationship can be seen in engineering other tissues[17,34,36]. It has been reported that 7% O$_2$ applied in human vascular-derived myofibroblasts seeded onto a biodegradable scaffold similarly increases construct properties as seen in this study[36]. In another case, 4% O$_2$ enhanced gene expression of LOX and LH2[17]. It is, thus, conceivable that the use of hypoxia can be translated to other musculoskeletal tissues. The ECM of native ligaments, tendons, bone, and other musculoskeletal tissues is composed of proteoglycans and fibrillar proteins, such as elastin and collagen. The load-bearing capacity of collagen is, apart from collagen content and organization, highly dependent on collagen crosslinking, which stabilizes the collagen fibrils[37]. The *in vitro* formation of these highly important ECM components can be affected by mechanical, biochemical, and

environmental stimuli, such as oxygen concentration[36,38,39]. As with this experiment, hypoxia could potentially improve the mechanical properties of other engineered musculoskeletal tissues through collagen crosslinking enhancement. However, application time for these engineered tissues must be optimized as to restrict its effects on crosslinks only.

Due to HIF-1α's link with other ECM gene expression, this study provides strong support for further development of other hypoxia duty cycles. Specifically, hypoxia may be applied during certain times to encourage proper differentiation, switching to normoxia to induce collagen synthesis, and returning to hypoxia to promote crosslinking. The motivation that underlies such a duty cycle can be found in multiple studies. For stem cells, hypoxia, through HIF-1α overexpression, has been shown to be effective and sufficient in inducing a chondrocytic phenotype on human bone marrow stem cells without use of exogenous growth factors[40]. Similar results have been obtained with adipose-derived stem cells[41]. While hypoxia seems beneficial for chondrodifferentiation, early application during neocartilage tissue engineering does not result in improved properties as demonstrated by this and prior work[13]. Thus, the present study provides strong support for further development of other hypoxia duty cycles, suggesting that, until robust mechanisms can be determined for hypoxia's effects on ECM synthesis, this potent stimulus should only be applied for differentiation and for collagen crosslinking.

Aside from PYR, other cartilage crosslinks such as arginoline and advanced glycation end products (AGEs) might similarly be manipulated to influence neocartilage stiffness. Arginoline makes up for half of the mature crosslinks in native cartilage[42] but cannot be measured *via* HPLC, necessitating future studies on its characterization in engineered tissues. AGEs occur over a time-scale of decades as sugars react with the lysine and arginine residues during aging. Examples of common AGEs include crosslinks, such as pentosidine[43–45], methylglyoxal-lysine dimer (MOLD)[44], and threosidine[46], as well as N^{ε}-(carboxymethyl)lysine (CML)[47] and N^{ε}-(carboxyethyl)lysine (CEL)[48]. Though past work has correlated AGEs with stiffening of the collagen matrix[49], AGEs have also been implicated in the progression of age-related cartilage degeneration such as OA[50]. AGEs may thus be an inferior candidate when compared to PYR and arginoline for enhancing neocartilage mechanical properties.

In this experiment, low oxygen tension ($4\% \ O_2$) was investigated at various application times during the self-assembling process both to identify optimal regimens for hypoxia application and to elucidate a mechanism through which hypoxia-induced increases in cartilage mechanical properties occur. Hypoxia applied during the collagen synthesis phase resulted in decreased collagen content. However, hypoxia applied later in culture served to induce LOX expression, crosslink formation, and increased tensile properties. Strong evidence from both this study and a plethora of prior studies suggest that this occurs through the robust mechanism of HIF-1α-induced upregulation of LOX. The results shown here are promising as they show that hypoxia can be used to tissue engineer neocartilage with robust tensile properties. In the future, while one may consider the possibility of employing hypoxia to achieve similar biomechanical improvements in cartilage just showing signs of mechanical degradation, a different regimen will have to be identified for intra-articular regulation of oxygen tension. Additionally, hypoxia may be beneficial for enhancing the mechanical properties of autografts or allografts.

Author contributions

All authors have contributed to the conception and design of the study, and acquisition, analysis and interpretation of data. The manuscript has been drafted, revised and finally approved by all authors. All authors take responsibility for the integrity of the work.

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