Low Oxygen Tension During Incubation Periods of Chondrocyte Expansion Is Sufficient to Enhance Postexpansion Chondrogenesis

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To determine whether low oxygen (O_2) tension during expansion affects the matrix density, as well as quantity, of cartilage formed, and to determine whether application of low O_2 tension during incubation periods alone is sufficient to modulate chondrogenic expression, rabbit chondrocytes expanded at either 21% O_2 or 5% O_2 were analyzed for glycosaminoglycan (GAG) and DNA content, total collagen, and gene expression during expansion and postexpansion aggregate cultures. When cultured as aggregates at 21% O_2 , chondrocytes expanded at 5% O_2 produced cartilage aggregates that contained more total GAG, GAG per wet weight, GAG per DNA, and total collagen than chondrocytes expanded at 21% O_2 . Less of an effect on GAG and collagen content was observed when aggregate culture was performed at 5% O_2 . Real-time polymerase chain reaction analysis of *COL2A1* expression showed upregulated levels of type IIA (an early marker) and IIB (a late marker) during expansion and elevated levels of type IIB during aggregate culture in chondrocytes expanded in low O_2 . The application of low O_2 tension during incubation periods of chondrocyte expansion enhances the ultimate cartilage matrix density and quantity, and this enhancement can be achieved through the use of an O_2 control incubator.

Introduction

CARTILAGE TISSUE ENGINEERING offers the promise of improved treatment of articular cartilage damaged by trauma or arthritis and related diseases.¹ Chondrocyte-based cartilage tissue engineering strategies, such as autologous chondrocyte transplantation and matrix-assisted chondrocyte implantation, generally employ *in vitro* expansion of chondrocytes before postexpansion generation of tissue-engineered constructs.^{2,3} Some expansion conditions, such as the inclusion of specific growth factors in the medium, have been shown to significantly enhance postexpansion chondrogenesis.^{4–6} Expansion conditions that enhance postexpansion chondrogenesis are of great interest for their potential to improve cartilage tissue engineering strategies.

Recent work suggests that low oxygen (O_2) tension is an expansion condition capable of enhancing postexpansion chondrogenesis.⁷ This work showed that, during chondrocyte expansion, collagen type II mRNA was 2.9-fold higher in cells expanded at 1.5% as compared with expansion at 21% O_2 , and that, after expansion, aggregate cultures grown at 21% O_2 showed an increase of between 17% and 107% in

the total tissue content of glycosaminoglycans (GAGs) when formed with cells expanded at 1.5% instead of 21% O_2 . These previous findings in bovine chondrocytes suggest that application of low O_2 in the expansion phase of chondrocyte-based cartilage tissue engineering strategies may produce engineered cartilage of increased, and therefore more physiological, GAG content. The current study was conducted to determine the extent to which low O_2 conditions may apply to rabbit chondrocyte expansion and differentiation as a way to optimize conditions for rabbit tissue engineering studies conducted in this laboratory. In addition, it is not known whether low O_2 during expansion can affect the matrix density of the cartilage formed, or simply the quantity, as the previous work in this area reported total GAG content, and did not report GAG content normalized to wet weight, dry weight, or DNA content.⁷ Another question relates to the potential for expansion-phase low O_2 to find widespread use in chondrocyte-based cartilage tissue engineering strategies. The previous study made use of a hypoxic workstation to control ambient O_2 tension during all stages of expansion and aggregate culture, including incubation, medium changes, subculture, and aggregate preparation.⁷ Although

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hypoxic workstations provide rigorous control of ambient O₂ tension when compared to O₂ control incubators, hypoxic workstations are significantly more expensive to purchase and operate, require a glove-box interface that is more difficult for lab personnel to learn and use, and require significantly more space when added to an existing cell culture facility. Therefore, another objective of this study was to determine whether application of low O₂ during incubation periods of chondrocyte expansion is sufficient to produce engineered cartilage of increased GAG content. Another question was whether the previous studies using bovine articular chondrocytes would be comparable to rabbit-derived articular chondrocytes. To achieve these objectives, we compared chondrocytes expanded at 21% O₂ and at 5% O₂ by analyzing molecular markers of the chondrocyte phenotype during expansion and by analyzing molecular markers of chondrogenesis during postexpansion cartilage formation in aggregate cultures. To assess whether low O₂ during expansion can affect the matrix density of the cartilage formed, or simply the quantity, two molecular markers, GAG and total collagen, were assessed at the protein level and normalized to aggregate wet weight.

Materials and Methods

Preparation

Articular cartilage was harvested from six skeletally mature male New Zealand White rabbits (12–16 months old) during nonsurvival surgeries in accordance with the guidelines of the Animal Care and Use Committee of Case Western Reserve University. For each rabbit, cartilage was harvested from both humeral heads and pooled. The six cartilage samples were cleaned, manually diced, and digested sequentially in testicular hyaluronidase (Sigma Chemical Co, St. Louis, MO), trypsin–ethylenediaminetetraacetic acid (Invitrogen, Carlsbad, CA), and collagenase Type II (Wor-

thington, Lakewood, NJ), as previously described.⁸ After digestion, calf serum was added, the cell suspension was passed through a 70 µm nitex filter to remove acellular contaminants, and the resulting isolated chondrocytes were collected. The six cartilage samples, and the resulting groups of chondrocytes, were always treated independently.

Study design

Chondrocytes were split from each of six rabbits into two equal groups and expanded under 21% or 5% O₂ (Fig. 1). During expansion, gene expression was analyzed by real-time polymerase chain reaction (PCR). After expansion (end of P⁰), the chondrocytes were cultured as aggregates to promote chondrogenesis. Aggregate culture was performed at both 21% and 5% O₂. During and after aggregate culture, gene expression was analyzed by real-time PCR, wet weights were taken for all aggregates, and the cartilage formed in aggregates was analyzed by GAG, DNA, and collagen assays, and by histological and immunohistochemical analyses.

Chondrocyte expansion

Chondrocytes from each rabbit were divided into two equal groups, with each group plated onto one or two T-75 flasks to achieve an initial seeding density of 3000–7000 cells/cm², depending on cell availability. One group was incubated at 21% O₂ and the other group was incubated at 5% O₂, with both groups at 37°C and 5% CO₂. The 21% O₂ condition was simply that of standard (37°C and 5% CO₂) cell culture using a standard CO₂-control cell culture incubator (MCO-18AIC; Sanyo, Tokyo, Japan). The 5% O₂ condition was achieved using an O₂/CO₂-control cell culture incubator (MCO-18M; Sanyo), which controls O₂ indirectly by injecting N₂ into the incubator to dilute the ambient O₂ to produce low-O₂ conditions, whereas CO₂ levels are maintained as in a standard CO₂-control cell culture incubator.

FIG. 1. Study design.

The low O₂ condition used in this study, 5% O₂, was selected based on both the predicted physiological O₂ tension present in articular cartilage—approximately 1% to 8% O₂^{9,10}—and on previous studies on the effect of O₂ on chondrogenesis—1% to 10% O₂.¹¹ The expansion medium (Dulbecco's modified Eagle's medium with 1 g/L glucose supplemented with 10% fetal bovine serum [Invitrogen; lot #1256415]) was changed two times per week. All cell culture work, including medium changes, passaging, and aggregate preparation, was performed under atmospheric O₂ conditions (approximately 21% O₂), meaning that groups incubated at 5% O₂ were intermittently exposed to 21% O₂. Flasks were passaged at confluence (end of P⁰ culture). For each group, aliquots of 250,000 cells were stored in RNeasy lysis buffer (Qiagen, Valencia, CA) for subsequent RNA extraction, and 500,000 cells were divided into two equal groups and replated onto two T-175 flasks. Flasks were reincubated at the O₂ tension at which the respective chondrocytes had been cultured during P⁰. Medium was changed two times per week until aggregate preparation. Cell counts performed at the end of P⁰ and P¹ were used to determine proliferation rate in terms of the number of cell doublings per day during expansion, as previously described.⁸ At the end of P¹ culture (confluence), the chondrocytes were trypsinized, and chondrocytes from each group (two flasks per group) were either saved in RNeasy lysis buffer for subsequent RNA extraction (two aliquots of 250,000 cells each) or used to generate aggregates, as described below.

Aggregate preparation, culture, and harvest

Twenty-four aggregates, with 250,000 chondrocytes per aggregate, were generated from each expansion group, with 12 aggregates from each group cultured at 21% O₂ and 12 at 5% O₂. Therefore, for each of the six rabbits included in the study, there were four groups of aggregates, with 12 aggregates per group: two groups in which chondrocytes expanded under 21% O₂ were split and then cultured as aggregates at 21% and 5% O₂, and two groups in which chondrocytes expanded under 5% were also split and then cultured as aggregates at 21% and 5% O₂. Aggregates were prepared and cultured as previously described.^{8,12} Briefly, aliquots of 250,000 chondrocytes in defined medium were pipetted into the wells of an autoclave-sterilized 96-well, V Bottom, 300- μ L polypropylene microplate (Phenix, Hayward, CA). A polypropylene lid (Phenix) was placed on the plate, and the plate centrifuged for 5 min at 500 g and then incubated at 37°C. To ensure that the aggregates could float freely, 24 h after seeding 100 μ L of medium from each well was aspirated and then gently released back into the well to release the aggregate from the bottom of the well. The only important modification of this previously published protocol^{8,12} was that transforming growth factor-beta (TGF- β) was not added to the medium. Although there is extensive literature on the beneficial effects of TGF- β supplementation during chondrogenic culture of human chondrocytes and mesenchymal stem cells, TGF- β was not added to the medium because the results of a preliminary experiment indicated that addition of TGF- β 1 or TGF- β 3 during aggregate culture of rabbit articular chondrocytes had little or no effect on GAG or DNA content (when compared to basal conditions; data not shown). The medium was changed three times per week. Four aggregates from each of the four groups were

collected on day 3 and again on day 7 and stored in RNeasy lysis buffer for subsequent RNA extraction. In addition, on day 7, one aggregate from each group was collected and processed for collagen assay. At 3 weeks of aggregate culture, the remaining three aggregates from each of the four groups were collected and processed for histology (one aggregate from each group) and for combined GAG and DNA analysis (two aggregates from each group). Wet weights of all aggregates were taken after blotting on filter paper.

Methods of measurement

Gene expression analysis. Total RNA was extracted from chondrocytes released from monolayer using the TRIzol Plus Purification Kit (Invitrogen). Total RNA was extracted from aggregates using the RNeasy Mini Kit (Qiagen, Valencia, CA), with aggregate homogenization performed using the kit's lysis buffer and a FastPrep System (Qbiogene, Morgan Irvine, CA). Aggregates collected at day 3 of aggregate culture did not yield sufficient RNA for real-time PCR and were excluded from gene expression analysis. Reverse transcription reaction was performed per manufacturer's protocol (Applied Biosystems, Foster City, CA). For real-time PCR, five genes of interest were examined: type IIA (*COL2A1* type IIA) and type IIB (*COL2A1* type IIB) collagen, the alternatively spliced isoforms of collagen II; aggrecan (*AGC1*); alpha 2 type I collagen (*COL1A2*); and transcription factor sox-9 (*SOX9*). For *COL1A2*, previously published primers and TaqMan probes were used.¹³ For the other four genes of interest, primers and TaqMan probes were designed using available rabbit mRNA in GenBank (Table 1). The probe for each gene was designed to span one exon–exon boundary, to prevent amplification of genomic DNA. Reaction products were analyzed using a 7500 Real-Time PCR System (Applied Biosystems). Expression levels of all genes were normalized to 18S rRNA levels (Eukaryotic 18S rRNA Endogenous Control [FAM MGB Probe]; Applied Biosystems).

Histological and immunohistochemical analyses. Aggregates were dehydrated through a graded series of alcohol washes and embedded in paraffin, and 5 μ m sections were cut. For histochemical staining of GAGs, representative sections were stained for 6 min in 0.1% safranin-O containing 1% acetic acid and counterstained for 2 min in 1% Fast Green containing 7% acetic acid. For immunohistochemical analysis, sections were rehydrated and stained with antibodies to type I and II collagen and elastin, with sources, dilutions for the primary antibodies, and the staining protocol as previously described.⁸

GAG and DNA analyses. Aggregates were enzymatically digested, and the total GAG content was assayed colorimetrically by a safranin-O assay as previously described.^{14,15} DNA content was measured on the same aggregates using Hoechst dye 33258 (General Electric, Piscataway, NJ), as previously described.⁸

Collagen content. Total collagen content was assayed to quantify collagen content of the matrix present in each sample. The individual samples were dried in a Savant concentrator and then weighed. The weighed tissues were

TABLE 1. CHARACTERISTICS OF THE NOVEL RABBIT-SPECIFIC OLIGONUCLEOTIDES USED FOR REAL-TIME POLYMERASE CHAIN REACTION

Target gene	Accession no.	Amplicon size (bp)	Forward primer sequence	Reverse primer sequence	Probe sequence
COL2A1 type IIA	D83229	63	GCTGCCCTTCGGTGTC	CTTCCCTCTGCACACA	CCTCCTGGACATCCTG
COL2A1 type IIB	D83229	61	GCTGCCCTTCGGTGTC	CTCCTTCTGCCCTTTGGT	CAGGATGTCGGAACCC
AGC1	L38480	65	GAGACCTATGACGTACTGCTT	GGCGACGTTGCGTAAAGA	CCTCACCTCCATCTCC
SOX9	AY598935	68	CAAGACCCTCGGGAAGCT	CCGCCCTCTCCACGAA	CTCGTTCAGCAGTCTCC

hydrolyzed in 6M HCl at 108°C for 18h. The hydrolysates were neutralized by dilution in water and dried on a speed Vac concentrator (Savants Instruments, Farmingdale, NY), and the samples were then reconstituted in 1% *n*-heptafluorobutyric acid. Aliquots of the samples were taken for hydroxyproline determination to quantify the collagen content. The hydroxyproline content and collagen content determination was carried out using the methods of Woessner.¹⁶

Analysis of data

Data are summarized as mean $\pm$ standard deviation. Differences between groups were assessed by paired *t*-tests for doubling rate, gene expression, total GAG, GAG per wet weight, GAG per DNA, total collagen, and collagen per wet weight data and by two-factor analysis of variance for aggregate wet weight data. The sample size for each statistical comparison was six rabbits, unless, as indicated, an insufficient number of chondrocytes or aggregates led to smaller samples size. Values of $p < 0.05$ were considered to indicate statistically significant differences.

Results

Proliferation rate

During P⁰ culture, the proliferation rate of chondrocytes expanded at 5% O₂ was 15% higher than that of chondrocytes expanded at 21% O₂, but the difference was not statistically significant, but did show a strong trend ($p = 0.07$) for more rapid proliferation in the 5% O₂ group (0.38 ± 0.17 vs. 0.33 ± 0.20 doublings/day in four rabbits). During first passage, the proliferation rate of chondrocytes expanded at 5% O₂ was 12% higher than that of chondrocytes expanded at 21% O₂, and the difference was statistically significant (0.58 ± 0.15 vs. 0.52 ± 0.14 doublings/day in six rabbits, $p = 0.03$).

Gene expression

When compared with chondrocytes expanded at 21% O₂, chondrocytes expanded at 5% O₂ expressed significantly higher levels of COL2A1 type IIA, during P⁰ and P¹, and COL2A1 type IIB, during P⁰ and aggregate culture at either 21% or 5% O₂ (Fig. 2). Type IIA and type IIB are two alternatively spliced isoforms of COL2A1 that have been shown to be expressed early (IIA) and late (IIB) during chondrogenesis.^{17,18} Expression of aggrecan message (AGC1) was low during expansion and was then upregulated (two- to sixfold) during aggregate culture, regardless of whether chondrocytes were expanded at 21% O₂ or 5% O₂. Conversely, expression of COL1A2, an indicator of chondrocyte dedifferentiation, was higher during expansion than during aggregate culture (7- to 24-fold), regardless of whether chondrocytes were expanded at 21% O₂ or 5% O₂ (data not shown). The expression levels of SOX9, a key regulator of COL2A1 expression,^{19,20} showed a less than twofold increase from expansion to aggregate culture, and the increase was not affected by expansion condition (data not shown).

Aggregate wet weight and morphology

For aggregate culture at 21% O₂, chondrocytes expanded at 5% O₂ produced aggregates that were 69%, 34%, and 13%

FIG. 2. Gene expression during expansion and at 1 week of aggregate culture. Expression levels of all genes of interest were normalized to the expression levels of 18S rRNA. Expansion condition (21% or 5% O₂) did not produce a significant difference in expression levels of COL1A2 or SOX9 at any of the time points and are not shown ($n = 4$ rabbits).

larger by wet weight than those produced by chondrocytes expanded at 21% O₂ after days 3, 7, and 21, respectively (1.40 ± 0.14 vs. 0.83 ± 0.45 , 2.33 ± 0.46 vs. 1.74 ± 0.65 , and 3.05 ± 0.27 vs. 2.70 ± 0.80 mg; Fig. 3); these differences were statistically significant ($p < 0.01$). Similarly, for aggregate culture at 5% O₂, chondrocytes expanded at 5% O₂ produced aggregates that were 86%, 45%, and 37% larger by wet weight than those produced by chondrocytes expanded at 21% O₂ after days 3, 7, and 21, respectively (1.23 ± 0.19 vs. 0.66 ± 0.40 , 2.53 ± 0.24 vs. 1.75 ± 0.44 , and 3.65 ± 0.37 vs. 2.67 ± 0.66 mg; Fig. 3); these differences were also statistically significant ($p < 0.0001$). For aggregate culture at either 21% O₂ or 5% O₂, there was not a significant interaction between expansion O₂ conditions and time in aggregate culture as determined by two-factor analysis of variance ($p = 0.82$ for 21% O₂ aggregate culture and $p = 0.52$ for 5% O₂ aggregate culture); that is, for both aggregate culture conditions, the

significant difference in wet weight, described above, was relatively unchanged over time.

GAG and collagen content

Expansion conditions were found to have more effect on GAG and collagen content when aggregate culture was performed at 21% O₂ than at 5% O₂. For aggregate culture at 5% O₂, chondrocytes expanded at 5% O₂ produced aggregates that contained, on average, 40% more total collagen ($p = 0.007$, with the 5% O₂ expansion group having more total collagen in samples from six of six rabbits; data not shown) than those produced by chondrocytes expanded at 21% O₂. There was no significant difference in total GAG, GAG per wet weight, GAG per DNA, or collagen per wet weight (data not shown). In contrast to the modest effect observed during aggregate culture at 5% O₂, for aggregate

FIG. 3. Aggregate wet weight and morphology as a function of time in aggregate culture. (A) Aggregate wet weight. $n = 5$, 5, and 6 rabbits for days 3, 7, and 21, respectively. (B) Images of aggregate gross morphology. Because a reduction in aggregate size was associated with a change from a more spherical morphology to a more discoidal or bowl-like morphology, the apparent diameter of the aggregates was a poor indicator of wet weight. Scale bar = 1 mm.

culture performed at 21% O₂, chondrocytes expanded at 5% O₂ produced aggregates that contained, on average, 37% more total GAG ($p = 0.026$, with the 5% O₂ expansion group having more total GAG in samples from five of six rabbits), 23% more GAG per wet weight ($p = 0.012$, six of six rabbits), 32% more GAG per DNA ($p = 0.011$, six of six rabbits), and 116% more total collagen ($p = 0.014$, six of six rabbits) than those produced by chondrocytes expanded at 21% O₂ (Fig. 4). There was no significant difference in collagen per wet weight, although the results and p -value indicate a strong trend ($p = 0.070$; Fig. 4).

Histological and immunohistochemical staining

Aggregates were examined by histology after histochemical staining with safranin-O to detect GAGs and after immunohistochemical staining for collagen type II. Compared

with chondrocytes expanded at 21% O₂, chondrocytes expanded at 5% O₂ produced aggregates that stained as intensely or more intensely for GAGs and collagen II (Fig. 5). The aggregate cultures were pleomorphic, but were generally homogeneously stained throughout the matrix with both safranin-O and type II collagen. Immunostaining for collagen I was very faint or undetectable in all aggregates (data not shown).

Discussion

This study in rabbits indicates that the application of low O₂ during incubation periods of chondrocyte expansion is sufficient to produce engineered cartilage of increased GAG content. The data demonstrate that, when cultured as aggregates at 21% O₂, chondrocytes expanded at 5% O₂ produce cartilage aggregates that contain more total GAG, more GAG

FIG. 4. Glycosaminoglycan and collagen content during aggregate culture at 21% O₂. The glycosaminoglycan assay was performed after 3 weeks of aggregate culture, and the collagen assay after 1 week of aggregate culture, as shown in Figure 1. Each data point represents the average of one to two samples from a given rabbit. For each rabbit, a line connects the sample from chondrocytes expanded at 5% O₂ with the sample from chondrocytes expanded at 21% O₂. The *p*-values are for comparison of expansion conditions as assessed by paired *t*-tests. *n* = 6 rabbits for each assay.

per wet weight, more GAG per DNA, and more total collagen than chondrocytes expanded at 21% O₂. The application of low O₂ during incubation periods of chondrocyte expansion was found to have less effect on GAG and collagen content when aggregate culture was performed at 5% O₂.

Previous work by Egli *et al.* established that low O₂ is an expansion condition capable of enhancing postexpansion of bovine chondrocytes,⁷ and the results shown here are in general agreement for rabbit chondrocytes. In contrast to this study, Egli *et al.*⁷ made use of a hypoxic workstation to control ambient O₂ tension during all stages of expansion and aggregate culture. Although hypoxic workstations provide rigorous control of ambient O₂ tension, their potential to find widespread use in chondrocyte-based cartilage tissue engineering strategies may be hampered by their cost, restrictive interface, and size. To address this issue, the present study used O₂ control incubation to examine whether application of low O₂ during incubation periods of chondrocyte expansion is sufficient to produce engineered cartilage of increased GAG content. As with the work by Egli *et al.* and other previous studies in which O₂ tension was controlled in the gas phase rather than the liquid phase, in this study it is likely that O₂ tension at the level of the cells was lower than the level being regulated in the gas phase, as the amount of detectable O₂ at the cell surface during monolayer culture is a function of cell density, the O₂ tension in the incubator, and cellular activity.²¹

Several of the findings of the present work parallel those of the work by Egli *et al.* First, the present data show that chondrocytes expanded at 5% O₂ produced aggregates that contained significantly more total GAG than those produced by chondrocytes expanded at 21% O₂. The 5% O₂ expansion group had more total GAG in samples from five of six rabbits, with differences ranging from 0.49% (no difference) to 200%. Egli *et al.* reported similar finding using bovine cells, with aggregates from a 1.5% O₂ expansion group having more total GAG than a 21% O₂ expansion group in three of three experiments, with differences ranging from 17% to

107%. Second, in this study, the expansion condition was only found to affect GAG content when aggregate culture was performed at 21% O₂. For aggregate culture at 5% O₂, there was no significant difference in total GAG in aggregates produced by chondrocytes expanded at 5% or 21% O₂. Similarly, Egli *et al.* reported that aggregate culture at 1.5% or 5% O₂ did not, or to a lesser extent, support the formation of a cartilage-like matrix, although total GAG content for these samples was not quantified. Last, as with the data reported by Egli *et al.*, in this study we found no substantial difference in the level of the transcripts encoding aggrecan core protein, despite the increase in the content of total GAG. More generally, on the level of transcripts, Egli *et al.* reported that no consistent differences could be observed between aggregates cultured after expansion at 1.5% O₂ or 21% O₂. In this study we observed that, when compared with chondrocytes expanded at 21% O₂, chondrocytes expanded at 5% O₂ expressed significantly higher levels of *COL2A1* type IIA and *COL2A1* type IIB during specific periods of expansion and aggregate culture and produced aggregates of more total collagen protein, yet the expression levels of *SOX9* were not affected by expansion condition. It remains to be determined whether this apparent discrepancy would be resolved by a more detailed analysis of gene expression—gene expression during aggregate culture was assessed at a single time point (7 days of aggregate culture)—or if our findings and the findings of Egli *et al.* are indicative of more complex factors, such as phosphorylation of Sox9²² or a chondrogenic pathway that is not dependent on Sox9 upregulation.²³

Although this study achieved the objective of determining whether application of low O₂ during incubation periods of chondrocyte expansion is sufficient to produce engineered cartilage of increased GAG content, the study was not designed to test whether application of low O₂ during incubation periods of chondrocyte expansion produced the same effect as would be produced in a hypoxic workstation. The parallels described above between our findings and those of the work by Egli *et al.* certainly suggest that the benefits of

FIG. 5. Histology and immunohistochemistry at 3 weeks of aggregate culture. Low and high magnification images of safranin-O/Fast Green–stained and collagen II–immunostained sections of aggregates. Scale bars = 200 μ m. Color images available online at www.liebertonline.com/ten.

low O_2 expansion achieved through the use of an O_2 control incubator may be similar to those produced through the use of a hypoxic workstation, but determination of the functional and biochemical extent of this similarity requires further investigation.

In addition to the above results that were consistent with the previous findings of Egli *et al.*,⁷ this study also assessed whether low O_2 during expansion affects the matrix density of the cartilage formed, or simply the quantity, as the previous study reported total GAG content, rather than GAG content normalized to wet or dry weight. We found that for aggregate culture performed at 21% O_2 , low O_2 during expansion does affect the matrix density of the cartilage formed, as chondrocytes expanded at 5% O_2 produced aggregates that contained not only more total GAG but also more GAG per wet weight, more GAG per DNA, and more total collagen than those produced by chondrocytes expanded at 21% O_2 , whereas there was no significant difference in collagen per wet weight. In contrast, we found that expansion conditions had no effect on cartilage matrix

density for aggregate culture at 5% O_2 , as chondrocytes expanded at 5% O_2 produced aggregates that contained more total collagen but no significant difference in total GAG, GAG per wet weight, GAG per DNA, or collagen per wet weight. It was also found that chondrocytes expanded at 5% O_2 produced aggregates significantly larger than those produced by chondrocytes expanded at 21% O_2 . In fact, chondrocytes expanded at 5% O_2 generated, in a period of 1 week, cartilage tissue of wet weight comparable to that generated in a period of 3 weeks by chondrocytes expanded at 21% O_2 (Fig. 3). These new observations collectively indicate that application of low O_2 during incubation periods of chondrocyte expansion alone is sufficient to produce engineered cartilage of enhanced matrix density and quantity. However, it is important to note that the biomechanical properties of the neo-cartilage generated during postexpansion chondrogenesis were not assessed in either this study or the previous study by Egli *et al.*,⁷ and it is not known whether chondrocytes expanded at 5% O_2 produced neo-cartilage with biomechanical properties similar to or different than that produced by chondrocytes expanded at 21% O_2 .

As noted, aggregates collected at day 3 of aggregate culture did not yield sufficient RNA for real-time PCR and were excluded from gene expression analysis. This observation raises the possibility of cell death during aggregate culture, particularly during the early phases of aggregate culture. To assess the potential effects of O_2 tension on cell viability, we performed an additional single rabbit experiment using the same experimental groups described above and assaying cell viability (DAPI staining of nuclei and a Hoechst staining of dead cells) and total DNA (as described above) in quadruplicate samples from each experimental group at 1, 3, 5, and 7 days of aggregate culture. The results suggest that cell death is not an overriding factor in the outcome of these experiments. Cell viability was greater than 94% for all experimental groups at 1 day of aggregate culture and increased to greater than 98% viability at days 3, 5, and 7 (data not shown). Total DNA was fairly constant within each experimental group during aggregate culture (data not shown). These results suggest that it is unlikely that cell death was responsible for the low RNA yield in the day 3 aggregates. The same aggregate homogenization and RNA extraction protocol was used for day 3 and day 7 aggregates, and it is possible that optimization of the protocol to each set of aggregates would increase RNA yield for the day 3 aggregates.

In conclusion, this work in rabbits shows that application of low O_2 during incubation periods of chondrocyte expansion is sufficient to produce engineered cartilage of increased GAG content, as has previously been produced using a hypoxic workstation to produce low O_2 during all stages of expansion. In addition, the present results show that low O_2 during expansion enhances the matrix density of the cartilage formed, not simply the quantity. Based on these findings, we suggest that application of low O_2 during incubation periods of chondrocyte expansion may be a useful tool for enhancing chondrocyte-based cartilage tissue engineering strategies, such as autologous chondrocyte transplantation or matrix-assisted chondrocyte implantation. The fact that the benefits of low O_2 expansion can be achieved through the use of an O_2 control incubator provides the potential for expansion-phase low O_2 to find widespread use in chondrocyte-based cartilage tissue engineering strategies.

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Disclosure Statement

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