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Physiological normoxia and chondrogenic potential of chondrocytes

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TABLE OF CONTENTS

1. ABSTRACT

Cartilage is poorly vascularised with a limited capacity for repair following damage. The poor vascularisation results in cartilage tissue having a low normoxic value. This study examined and compared the effects of physiological cartilage normoxia (2% O_2), hypoxia (0.2% O_2), and hyperoxia (21% O_2) on human articular chondrocytes (hAC) during similar time courses to those prior to transplant in cell therapy procedures. hAC were isolated and maintained at 0.2% O_2, 2% O_2, or 21% O_2. Population doublings (PDs), cell surface area, chondrogenic differentiation potential, RT-PCR, quantitative RT-PCR and immunohistochemistry (Collagen Type II) were used to confirm chondrogenic differentiation of micromass pellets in different O_2. Isolation and maintenance of hAC at less than 2% O_2 resulted in significant alterations in surface area (smaller), rate of proliferation (reduced), and chondrogenic differentiation potential (enhanced). Chondrogenic gene expression appeared largely insensitive to O_2 concentration. A relationship was apparent between collagen type II protein presence and O_2 concentration. Oxygen concentrations of 2% O_2 or less promoted retention of a dedifferentiated hAC phenotype and enhanced stability of hAC chondrogenesis.

2. INTRODUCTION

Damaged cartilage has a limited capacity for repair from even minor injury due to the lack of apparatus required to mount a normal wound-healing response (1). In some individuals cartilage injury does not repair but becomes symptomatic. Debridement or microfractures are current techniques that can fail within a few years. Autologous Chondrocyte Implantation (ACI) is the only commonly used cell-based therapeutic technique currently available for repair to damaged cartilage (2). It is ideally suited for the repair of small localized chondral defects at first presentation and also for larger, chronic lesions where sub-chondral damage has not occurred (3). Clinical success of the ACI procedure is based upon the symptomatic relief that occurs in sufferers post surgery. A positive outcome is represented by a prolonged absence of pain and increased function following the transplant procedure. In practice, successful tissue generation of solely hyaline type cartilage occurs in 22% of ACI procedures, with a further 48% developing a functional but suboptimal fibrocartilage tissue and the balance producing a fibroblastic material that fails to function as cartilage substitute after an averaged 15 months (4). The reasoning for the production of mixed fibrocartilage remains unclear though *in vitro* de-

differentiation of chondrocytes may be a contributing factor (5).

Cartilage itself is a relatively avascular tissue (1). A consequence of this is that oxygen and nutrient supply occurs largely through intracellular diffusion (6, 7). Arterial blood contains approximately 10% O_2 while venous blood levels are reduced to 5% O_2 which also represents the mean oxygen level in human tissue (8). The physiological normoxic value of cartilage is predicted to be approximately 2% O_2 although it is almost certainly lower than this in some zones (9, 10). The importance of this for appropriate cartilage tissue function is illustrated by the association of elevated *in vivo* oxygen levels with the pathology of both osteoarthritic and rheumatoid diseases (9, 11).

Chondrocytes are described as having an enhanced proliferative capacity and an improved retention of differentiation capacity in reduced oxygen tensions when compared to 21% O_2 cultured counterparts (6, 12). The mechanisms underwriting these descriptions are hinted at by the role of hypoxia-signaling transcription factors, HIF1 and HIF2, in the promotion of expression of key chondrogenic genes such as COL2A1, ACAN, and SOX9 in articular chondrocytes (13-15). Taken together these collected observations support the use of reduced oxygen conditions for recovery of cells for use in procedures such as ACI. However it remains unclear what effect reduced oxygen tensions would have on human articular chondrocytes (hAC) cell numbers, growth rates, and subsequent differentiation capacities during the critical hAC recovery phase prior to autologous transplantation.

In this study we have examined the effect of physiological normoxia (2% O_2) and virtual anoxia (0.2% O_2) on size, growth rate, differentiation capacity, and chondrogenic gene expression during chondrogenesis of hAC as compared to physiological hyperoxic (21% O_2) conditions. This analysis was performed solely on the therapeutically relevant recovery and expansion phase of hAC.

3. MATERIALS AND METHODS

3.1. Chondrocytes isolation and culture methodology

hAC were isolated with donor consent from female patients aged between 60 and 75 undergoing total knee replacement surgery for osteoarthritis at the Robert Jones and Agnes Hunt Hospital. In each instance a cartilage biopsy was taken from grossly normal tissue and dissected into three identical fragments. Individual fragments were labelled as 21% O_2, 2% O_2, and 0.2% O_2 and all incubations and subsequent routine culture and differentiation assays were maintained in that O_2 concentration thereafter. Samples were digested with Collagenase Type II (CLS-2, Worthington) with 10% DNase (Sigma, UK). We included a commercially sourced Passage 0 hAC (Promocell HCH-10 Cat.: C12710) in our analysis. These chondrocytes were obtained through biopsy from grossly normal tissue from a 71 year old female patient undergoing knee replacement. These were thawed and cultured at 21% O_2 to 80% confluence and split at a 1:4 ratio to provide 2 flasks in 21% O_2, and one each in 2% O_2 and 0.2% O_2. Oxygen control was provided by either controlled N_2 purging of standard tri-gas incubators (RS Biotech, Irvine, UK) using a N_2 generator (Peak Scientific, UK) or by following the modular methodology described elsewhere (16, 17). All hAC were cultured in DMEM/F12 (Lonza) supplemented with 10% fetal bovine serum and media was refreshed twice weekly. Media was pre-treated at the appropriate oxygen level for a minimum of 3 hours prior to exposure to cells following previously described methodology (18). Cells were passaged at ~80% confluence and re-plated at 1:4 split ratios. Population doublings (PDs) were determined by the formula (log (output cell count) – log (input cell count))*3.32.

3.2. Image analysis

Image analysis was performed using the Image J (NIH) platform. Images of early passage chondrocytes were recorded 48 hrs after plating under a 10X objective on a Nikon Eclipse TS100 with a Canon EOS 400D. Image files were opened and individual cells were traced using the freehand selection tool before being measured. Pixel dimensions were normalized to the scale bar and the surface area of individual chondrocytes measured. Measurements were recorded across three independent images for each O_2 concentration where the surface area of a minimum of 150 individual chondrocytes was recorded.

3.3. *In vitro* chondrogenic differentiation

Chondrogenic differentiation was induced by establishing micromass pellets at a density of 2.5 x 10^5 cells/pellet using centrifugation at 150g for 5 minutes before overnight incubation at 37°C. Micromass pellets were established at the earliest timepoint possible in all O_2 conditions tested. Due to variances in doubling times between O_2-levels micromass pellets were established on different days in different conditions. The following day micromass pellets were detached from the microcentrifuge tube wall and either chondrogenic or chondrocyte medium added. Chondrogenic induction (CI) medium comprised 1% ITS+3 Supplement, 0.1µM dexamethasone, 50µg/ml Ascorbic Acid, 40µg/ml Proline, 1% non-essential amino acids, 1% Sodium pyruvate, 1% L-glutamine, and 10ng/ml TGF-ß3 (all obtained from Sigma, UK). All media was O_2-preconditioned to the required level by exposing for a minimum of 3 hours as indicated above. Microcentrifuge tubes with sealed lids were then incubated 37°C/7% CO_2 in the appropriate O_2-environment. At subsequent timepoints (days 1, 5, 10, 20 and 40) pellets were either fixed or snap frozen for subsequent analysis. Medium was changed on all remaining pellets twice per week.

3.4. Histochemistry

Micromass pellets were first rinsed well with PBS prior to fixation in 95% methanol for 15 minutes at room temperature before removal and replacement of methanol with PBS. Samples were stored at <5°C prior to paraffin embedding. Fixed micromass pellets were dehydrated for 1 hr in isopropanol at 50%, 75%, 90% and 100% graded concentrations. Following dehydration pellets were transferred to 100% molten wax for 15 hours at 60°C

before being placed into labeled cassettes, cooled to room temperature, transferred to a cold plate for 2 hours, and stored at <4°C prior to sectioning. Sections from the central region of pellets were selected for subsequent staining. For Alcian Blue staining sections were deparaffinised in xylene before being rehydrated through exposure to isopropanol for 2 minutes each at concentrations of 100%, 90%, 75% and 50%. Slides were then washed in distilled water and transferred into 0.1% Alcian Blue and incubated overnight at room temperature. Stained slides were then dehydrated through isopropanl and xylene series before being air dried and mounted using Eukitt mounting medium (Sigma, UK) and microscopically visualized. For Safranin O stain sections were first prepared as described above. Slides were then counterstained with 0.02% Fast Green for 5 minutes , cleared with 1% Acetic Acid for 30 seconds, and exposed to 0.1% Safranin O for 15 minutes. Slides were then dehydrated through isopropanol and xylene series before being air dried and mounted with X mounting medium.

3.5. Cartilage-linked gene expression

RNA extraction was performed using the RNeasy RNA Extraction Kit (QIAGEN, UK) following manufacturer's instructions. RNA concentration and purity were determined with the Nanodrop-1000 and ND-1000 V3.3 software. Target gene amplification and primer validation was performed using SuperScript III One-Step RT-PCR System with Platinum *Taq* High Fidelity (Invitrogen, UK) following manufacturors guidelines. All primers were designed using Primer3 and NCBI gene sequences (19). All primers were designed to span exons giving a product length of approximately 250bp. Gene products were visualised with 2% agarose gel electrophoresis and UV-based visualisation of ethidium bromide incorporation. Primers were designed against SOX9 (5'-CCGAGCTCAGCAAGACG-3' (Forward), 5'-CTTGAAGATGGCGTTGG-3' (Reverse)), COL1A2 (5'-GACTTTGTTGCTGCTTGC-3' (Forward), 5'-CAAGTCCAACTCCTTTTCC-3' (Reverse)), COL2A1 (5'-TGTCCTTCGGTGTCAGG-3' (Forward), 5'-GTCAGTTGGGCAGATGG-3' (Reverse)), COL10A1 (5'-TTCATGGAGTGTTTTACGC-3' (Forward), 5'-GTCCAGGACTTCCGTAGC-3' (Reverse)), COMP (5'-GGGAACTGCAGGAAACC-3' (Forward), 5'-GCTCTCCGTCTGGATGC-3' (Reverse)), and ACAN (5'-TCTGAGGGTCATCACTGC-3' (Forward), 5'-CTTCTCCTTGGACACACG-3' (Reverse)). ACTB primers were included for control purposes (20). Quantification of gene expression was performed on a Stratagene Mx 3005P (Agilent Technologies, UK) using the Superscript III Platinum SYBR Green One-Step qRT-PCR kit (Invitrogen, UK). COL2A1, COL10A1, COMP and ACAN Ct values were normalized against control Ct values at each timepoint, condition and oxygen concentration tested. GAPDH was used as an expression control during quantification (5'-GACTTCAACAGCGACACC-3' (Forward), 5'-GCAAGAGCACAAGAGGAA-3' (Reverse)). Reduced RNA yields from non-CI micromass pellets prevented quantitative analysis at later timepoints.

3.6. Immunohistochemistry

Cell sections on glass slides were first deparaffinised in xylene using three changes for five minutes each, hydrated through graded alcohols using two changes of ethanol for ten minutes each at 100%, 90% and 75% followed by 1 minute in ddH_2O. Slides were then treated for ten minutes with 0.3% H_2O_2 solution to quench endogenous peroxidase and washed well with PBS. Slides were incubated at room temperature for one hour in 1.5% blocking serum (Goat Serum, G9023, Sigma Aldrich), before incubation with 1:100 dilution anti-COL2A1 (sc-59958; Santa Cruz Biotechnology) in blocking serum for thirty minutes at room temperature. Following incubation, sections were washed with PBS before incubation with the biotinylated secondary antibody according to manufacturer's instructions. Immunoperoxidase staining was conducted using the mouse ABC Staining System (sc-2017; Santa Cruz Biotechnology). After staining slides were dehydrated across an ethanol gradient (75%, 90% and 100% ethanol) followed by three washes in xylene. Excess xylene was removed and the slides mounted in Eukitt mounting medium. Sections were immediately examined and imaged.

3.7. Statistical analysis

Numerical analysis took the form of either Students T-Test or 2-way ANOVA as indicated.

4. RESULTS

4.1. Influence of oxygen on chondrocyte cell size

To ascertain if there was any immediate impact of reduced oxygen tensions on chondrocytes we adopted a simple measure, cell surface area. Analysis of hAC at first or second passage revealed that cells in ≤2% O_2 had a 50% (0.2% O_2) – 62.5% (2% O_2) smaller surface area than those observed in 21% O_2 (Students T-Test, $p \leq 4.3X10^{-14}$) (Figure 1A). hAC derived and maintained in 2% O_2 exhibited low standard deviations and were relatively homogenous in surface area as compared to both 0.2% O_2 (Students T-Test, $p=0.002$) and 21% O_2 (Students T-Test, $p=2.1X10^{-24}$) (Figure 1B).

4.2. Oxygen effects on chondrocyte growth rate

We next examined the proliferative potential of hAC, from three independent sources, over the ACI-relevant first 50 days of *in vitro* culture using PDs as the unit of measurement. During the ACI procedure chondrocytes are recovered from an enzymatically digested patient biopsy over a 3-5 week *in vitro* culture period (2). We therefore focused our analysis on this period. hAC established and maintained in 21% O_2 displayed increased cumulative PDs when compared to cells established and maintained in ≤2% O_2 (2% O_2, $p<0.05$ after day 36; 0.2% O_2, $p<0.05$ after day 38; both Students T-Test) (Figure 2). We did not observe any significant differences between the growth rates of the in-house derived chondrocytes and the commercially sourced 'established' cells. Population doubling rates of hAC cultured in 21% O_2, 2% O_2 and 0.2% O_2 had normalized to near identical rates by day 50 (Figure 2B).

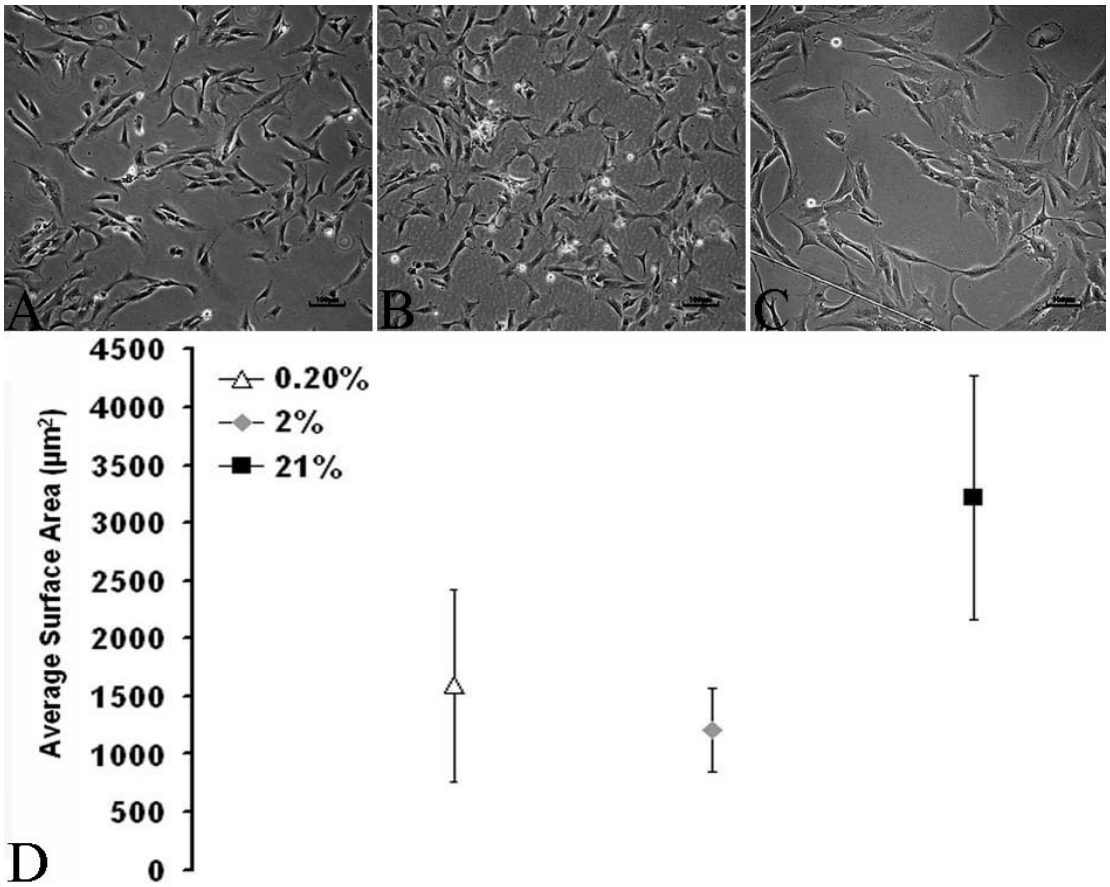

Figure 1. Reduced oxygen levels promote a reduction in chondrocyte cell surface area. Representative images of early passage chondrocytes (PD 3 after approximately 20 days in culture) in 0.2% O_2 (A), 2% O_2 (B), and 21% O_2 (C). Scale bar indicates 100μm. D. Quantitative determination of chondrocyte surface area. Y-axis indicates average surface area in μm^2. Error bars indicate +/- one Standard Deviation. 0.2% O_2 values represented by white triangles, 2% O_2 values indicated by grey diamonds, and 21% O_2 values are indicated by black squares.

4.3. Histological determination of chondrogenic rate

Chondrogenic induction was initiated in the O_2 concentration which was used for the previous culture and maintenance of hAC. Chondrogenic induction was initiated at or around PD10 after approximately 50 days culture in an oxygen-dependant manner (Figure 2). Three micromass pellets were established for each scheduled analysis timepoint (days 1, 5, 10, 20, and 40) in each oxygen concentration for each chondrocyte sample for histological analysis. After fixation and sectioning chondrogenic induction was established by the presence/absence of positive stain after batch processing for both Alcian Blue and Safranin O. In this semi-quantitative analysis a positively stained section (irrespective of homogeneity of stain or percentage of section positively stained) was scored as 1 and a negatively stained section was scored as 0. Representative images for each timepoint and O_2 concentration are shown in Figure 3A. As anticipated chondrogenic induction was observed irrespective of the O_2 concentration. However, significant O_2-dependent differences were noted (Figure 3B). At day 5 in the presence of CI 0.2% O_2 pellets displayed a more rapid onset than in all other conditions (2-way ANOVA, $p<0.005$) and by day 20 CI pellets in $\leq$2% O_2 produced significantly more histological-stain positive sections than in 21% O_2 (2-way ANOVA, $p<0.01$). Comparisons between CI and non-CI samples demonstrated significantly more cartilage formation at day 10 (Student's T-Test, $p<0.05$) and 20 (Student's T-Test, $p<0.01$) in 21% O_2, and day 20 ($p<0.001$) and 40 ($p<0.05$) in 0.2% O_2. Direct comparison between CI pellets illustrated that in 2% O_2 significantly greater cartilage formation over 21% O_2 was observed at day 40 (Student's T-Test, $p<0.05$). No significant differences between pellets maintained in non-CI conditions, irrespective of oxygen conditions, were observed. Pellet size varied considerably across the experimental range without the emergence of a clear relationship between size and O_2. Non-CI pellets had a substantially reduced stability at sectioning irrespective of O_2 condition used throughout.

4.4. Gene expression during chondrogenesis

Chondrogenesis is reliant on the expression of a specific subset of genes which are responsible for the formation of the ultimate species of cartilage. COL2A1 expression is commonly associated with hyaline cartilage

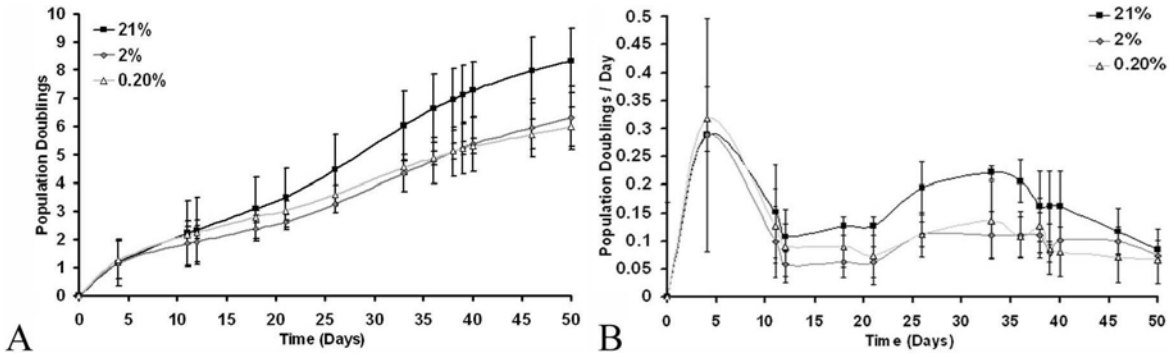

Figure 2. Reduced oxygen levels promote reduced growth rates in *in vitro* cultured chondrocytes. Averaged and smoothed growth curves of three subject-independent (n=1) chondrocyte cultures maintained under varying O_2 concentrations over the initial 50 day period. y-axis indicates cumulative population doublings. 21% O_2 values are indicated by black squares, 2% O_2 values indicated by grey diamonds, and 0.2% O_2 values represented by white triangles.X-axis indicates Time (days). Error bars represent +/- one Standard Deviation throughout.

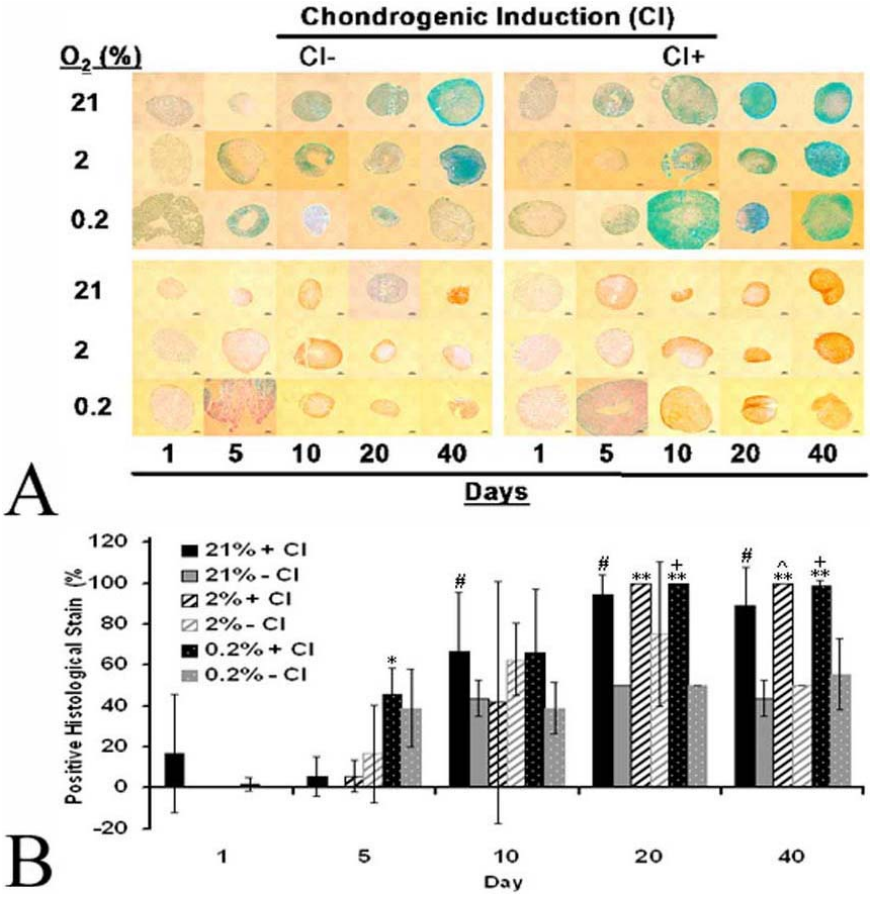

Figure 3. Chondrogenic induction under reduced oxygen conditions. (A) Histologically stained sections from chondrogenically-induced (CI+) or non-induced (CI-) sectioned micromass pellets. Representative images for Alcian Blue (top panel) and Safranin O (bottom panel) stained samples are shown for each O_2 concentation over a 40 day timecourse. (B) Combined scoring methodology of histologically stained micromass pellet sections. X-axis indicates Days, y-axis indicates Positive Histological Stain per sample group micromass pellet analysed. Error bars indicate +/- one Standard Deviation. * indicates $p<0.005$ vs 21% CI +and 2% CI + at day 5. ** indicates $p<0.01$ vs 21% CI + at days 20 and 40. # indicates $p<0.05$ vs 21% CI – at days 10, 20, and 40. + indicates $p<0.05$ vs 0.2% CI – at days 20 and 40. ^ indicates $p<0.05$ vs 21% CI+ at day 40. Data represent a minimum of n=3 for each chondrocyte population in each oxygen concentration used.

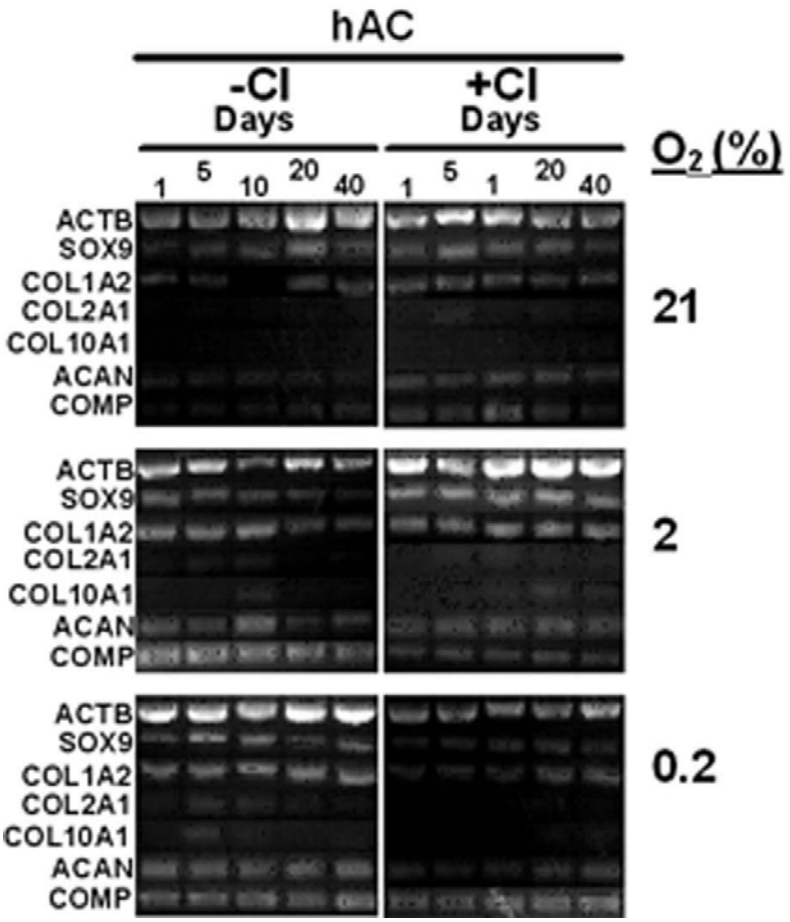

Figure 4. Chondrogenic gene expression in micromass pellets. ACTB, SOX9, COL1A2, COL2A1, COL10A1, ACAN, and COMP gene expression was determined in micromass pellets both with (+CI) and without (-CI) chondrogenic induction. Analysis was performed over a 40 day timecourse in 21% O_2, 2% O_2, and 0.2% O_2 conditions.

while COL10A1 is associated with hypertrophic cartilage (21, 22). We sought to confirm the expression of chondrogenic genes and to determine if a clear relationship between chondrogenic stage, gene expression, and oxygen tension existed. With two exceptions all transcripts examined were detected in differentiating hAC to varying degrees (Figure 4). The exceptions were COL2A1 and COL10A1 in both 21% O_2 non-CI and 0.2% O_2 CI.

Gel based interpretation of RT-PCR products does not provide a reliable quantification methodology. To explore gene expression changes further we performed real-time RT-PCR on COL2A1, COL10A1, ACAN, and COMP using GAPDH as an expression control (Figure 5). A compounding factor during this analysis was reduced RNA yields from non-CI pellets at later timepoints with the exception of 0.2% O_2 samples. This indicates that CI is crucial for maintenance of cell viability except in the most limiting of environments. A solitary significant alteration in gene expression level was noted for COL2A1 in 21% O_2 CI cultured samples (Day 20 vs. Day 1) while significant upregulation (Day 40) and downregulation (Day 20) were both noted in non-CI samples (Figure 5A). A more complex picture emerged in 2% O_2 non-CI where significant upregulation of COL2A1 was observed at all timepoints, compared to Day 1 levels, except Day 20 where significant downregulation was noted against all other timepoints. The 2% O_2 CI micromass pellets exhibited significant upregulation of COl2A1 against all earlier timepoints for Days 10 and 20 only. Consistent significant upregulation of COL2A1 was observed in 0.2% O_2 non-CI at all timepoints vs. Day 1 while CI supplementation induced significant upregulation at Day 40 against all other only timepoints while day 20 was significant only vs. Days 1 and 5. Similar to COL2A1 no alterations in expression levels were noted in COL10A1 in 21% non-CI pellets while CI stimulated significant upregulation at all timepoints after Day 5 (Figure 5B). Significant upregulation of COL10A1 was noted at all timepoints after Day 20 in less than 2% O_2 CI pellets. All 2% O_2 or less non-CI samples excluded definitive characterization due to scarcity of sample (2% O_2 non-CI) or inconsistency of expression profile (0.2% O_2 non-CI).

Individual significant alterations in ACAN expression were noted in non-CI 21% O_2 samples but again scarcity of sample and low RNA yields prevented extensive analysis (Figure 5C). In the 0.2% O_2 non-CI pellets

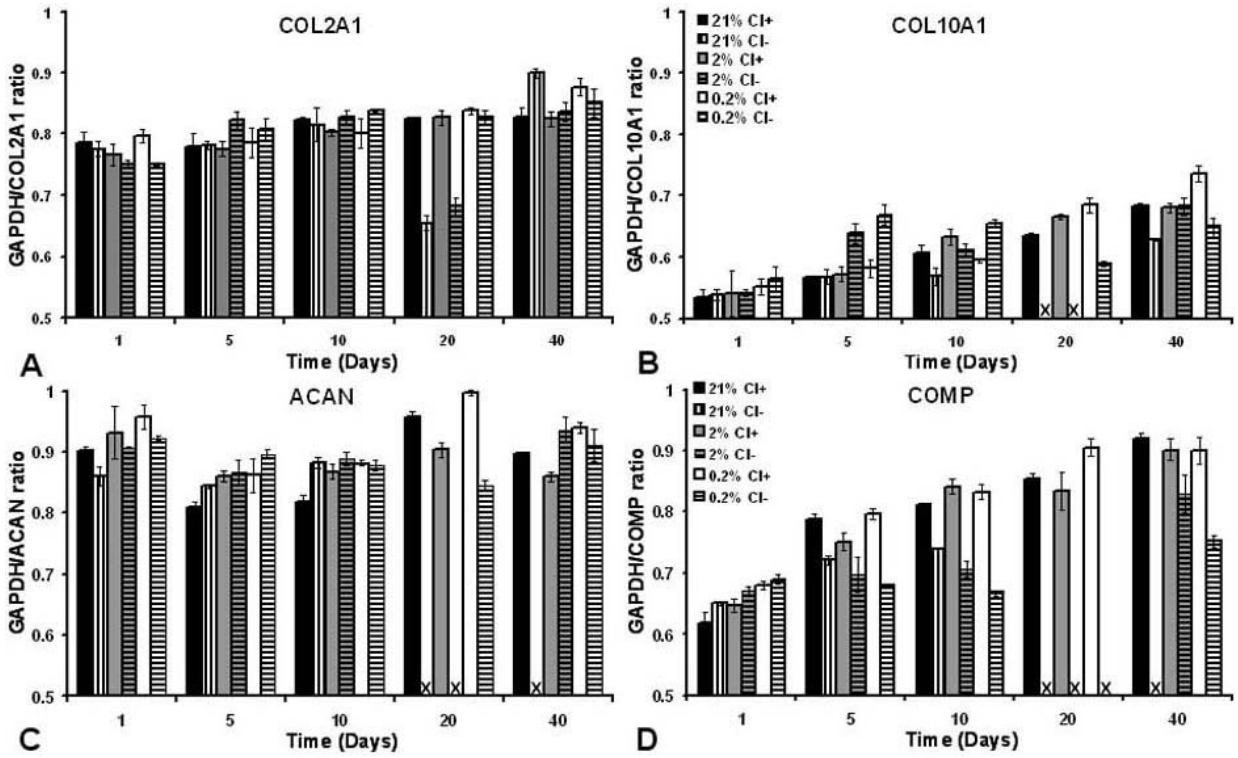

Figure 5. Chondrogenic gene expression quantification in micromass pellets. Normalised gene expression levels were determined for COL2A1 (A), COL10A1 (B), ACAN (C), and COMP (D) in micromass pellets both with (CI+) and without (CI-) chondrogenic induction. Analysis was performed over a 40 day timecourse in 21% O_2, 2% O_2, and 0.2% O_2 conditions. For clarity the legends presented in (B) and (D) are consistent for (A) and (C). Target gene expression is presented as a ratio of GAPDH expression where increased target abundance results in ratios closer to 1. Data are presented as an average of three independent replicates where error bars indicate standard deviation. Negative normalised Ct values indicate gene upregulation while positive normalised Ct values indicate gene downregulation. X indicates sample unavailability.

significant downregulation in expression was noted at Day 5 and Day 10 vs. Day 1 only. Upregulation of ACAN was noted only in CI pellets at 21% O_2 (Day 20) and 0.2% O_2 (Day 20) while downregulation was recorded at Day 5 (21% O_2, 2% O_2, 0.2% O_2), Day 10 (21% O_2, 2% O_2, 0.2% O_2), Day 20 (2% O_2), and Day 40 (2% O_2). COMP expression on the other hand underwent widespread upregulation across timepoints and in all oxygen concentrations with CI (Figure 5D). In the non-CI samples COMP was upregulated at Day 5 (21% O_2), Day 10 (2% O_2), and Day 40 (0.2% O_2) whereas downregulation was noted for 0.2% O_2 only (Day 10).

We next sought to determine if O_2 played in role in the regulation of target gene expression. Significant alterations in COL2A1, COL10A1, ACAN, and COMP expression were noted but these were without obvious pattern in response to varying O_2. Similarly no overall consistent trends were apparent with COL2A1, COL10A1, ACAN or COMP transcript abundance and presence/absence of CI (Figure 5).

4.5. Detection of type II collagen

Finally we sought to demonstrate the translation of COL2A1 into matrix-integrated type II collagen. Histological analysis had demonstrated that maximal glycan detection was achieved at Day 20 irrespective of O_2 concentration used. We therefore examined pellet sections from that timepoint across all conditions. The strongest type II collagen expression was detected in the non-CI 2% O_2 samples though evidence of staining was seen throughout with the 21% O_2 non-CI and CI samples providing the weakest signal (Figure 6).

5. DISCUSSION

Cell based therapeutics for the repair of cartilage are now well into their second decade of use (23). Though evidently highly successful there remain some elements concerning return to functionality which require further clarification (4). We hypothesized that the derivation and maintenance of chondrocytes in *in vivo* normoxic levels would slow the process of dedifferentiation and promote subsequent chondrogenic redifferentiation.

In this study we have described that the derivation and maintenance of hAC in less than or equal to 2% O_2 results in a smaller, less rapidly cycling cell, with increased stability of chondrogenic differentiation at later timepoints. The lower oxygen concentration (0.2% O_2) was included to evaluate chondrocyte response in a virtually oxygen free environment such as would be encountered

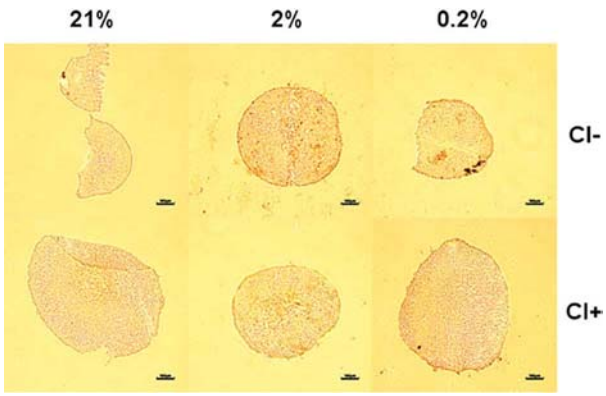

Figure 6. Type II collagen immunohistochemistry in micromass pellets. Type II collagen presence was determined in sections from micromass pellets both with (+CI) and without (-CI) chondrogenic induction. Analysis was performed on day 20 sections from 21% O_2, 2% O_2, and 0.2% O_2 conditions.

where the cell was situated greater than 150µm from vascular supply (24). As cartilage has poor vascular supply it is possible that such regions exist in the middle, transitional and superficial zones of the tissue. The reduced surface area of 2% O_2 or less cultured hAC is maintained for around 100 days in culture and after a further 400 days occupied the same area as the early passage 21% O_2 cells (data not shown). Over the same time period 21% O_2 hAC displayed an approximate 300% increase in surface area. This strongly supports the original hypothesis of slowed dedifferentiation. The reduced growth rate observed in 2% O_2 or less hAC was unanticipated. It should be noted that this observation relates to the first 50 days of culture period only and not beyond. In our experimentation irrespective of O_2 concentration no obvious difference was noted in the maximal PD of hAC cultures. It is also worth noting that the age range of our donors was outside that of the normal ACI range. Previous studies have identified age-related differences in human articular chondrocyte behavior. These differences include increased decorin expression and reductions in a number of parameters (cell yields, proliferative potential, biglycan expression, and aggrecan synthesis) with increasing age (25-28). These age-related changes do not become apparent until well after skeletal maturity has passed and remain unchanged thereafter (25, 26, 28, 29). These reports support our assertion that the age of the chondrocyte donors will not have impacted on our reported findings and further that this remains relevant for ACI. The reduced growth rate of hAC in 2% O_2 or less is likely to be a response to a phased switch from a glycolytic to an oxidative phosphorylation means of ATP generation as described elsewhere (30). The reported switch from glycolytic to oxidative phosphorylation even in reduced O_2 conditions implies strongly that further refinement of the hAC *in vitro* culture milieu is required before the development of accurate models can proceed. Chondrogenic potential was increased in hAC by day 20 of micromass pellet-based chondrogenesis in 2% O_2 or less vs. 21% O_2. In addition we observed increased uniformity of cartilage formation across all samples in 2% O_2 or less as indicated by the overall reduction in standard deviations (Figure 3B). This supports our original hypothesis of *in vivo* normoxic levels promoting chondrogenic redifferentiation.

The mechanisms underlying the reduction in cell size and reduced proliferation rate remain unclear at this time though similar observations have been reported in other cell types (16). Reduced oxygen levels are associated with HIF family member stabilization in chondrocytes and induction of increased expression of SOX9, COL2A1, ACAN and correlate with reductions in expression of COL1A1, COL1A2, and COL3A1 (13-15, 31). We initially performed gel-based inspection of transcript abundance following RT-PCR which suggested increased levels of all transcripts in 2% O_2 or less including the reportedly hypoxia repressed COL1A2.

Closer inspection of the expression levels of the chondrogenesis-linked genes; COL2A1, COL10A1, ACAN, and COMP, revealed significant expression changes occurring throughout the duration of *in vitro* pellet culture irrespective of oxygen or CI. Instability and reduced RNA yields prevented complete timecourse analysis of non-CI pellets in all instances. This presents a slight paradox as only in 0.2% O_2 non-CI pellets was robust hyaline cartilage-linked COL2A1 gene upregulation observed. This argues that both virtual anoxia and potentially glucose are the critical components for sustained hAC culture and differentiation as previously described for cells extracted from other similar tissues (32). Further experimentation is required to determine if reduced surface area, reduced proliferative rate, stabilized differentiation, and gene expression changes observed with hAC are linked to glycolytic respiration *per se* or to an *in vivo* approximation of O_2 levels.

The enhancement of chondrogenic potential fits well within the existing literature. In hAC for instance, previous reports have described an increase in GAG production after micromass pellet-based chondrogenic differentiation in reduced oxygen (12). Reports have also detailed increased glycosylaminoglycan production, collagen secretions and hyaluronic acid synthesis for bovine AC in reduced oxygen tensions (33, 34). Similar

observations have been noted for chondrocytes from human nasal passages and from bovine AC illustrating uniform chondrocyte relevance and pan-species implications of these findings (33-35). It is also important to note that reports have also described little alteration in matrix gene synthesis and differentiation potential of chondrocytes under reduced oxygen tensions though the reason for this disparity is unclear (36, 37).

This study has clear implications for hAC and their use in cell-based therapeutics such as ACI. The increase in cell size, proliferative rate, and decreased chondrogenic potential all argue against the use of 21% O_2 as a standard condition for expansion of hAC prior to transplantation. Clinical studies incorporating the use of 2% O_2 or less in hAC expansion have yet to emerge but it could be predicted from our results that improved histological score/pain/mobility correlations could emerge as a consequence.

6. ACKNOWLEDGMENTS

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7. REFERENCES

1. A. Lindahl, M. Brittberg and L. Peterson, Cartilage repair with chondrocytes: clinical and cellular aspects. *Novartis Found Symp* 249, 175-186 (2003)

2. M. Brittberg, A. Lindahl, A. Nilsson, C. Ohlsson, O. Isaksson and L. Peterson, Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation. *N Engl J Med* 331, 889-895 (1994)

3. J. Wasiak, C. Clar and E. Villanueva, Autologous cartilage implantation for full thickness articular cartilage defects of the knee. *Cochrane Database Syst Rev* 3, CD003323 (2006)

4. S. Roberts, I. W. McCall, A. J. Darby, J. Menage, H. Evans, P. E. Harrison and J. B. Richardson, Autologous chondrocyte implantation for cartilage repair: monitoring its success by magnetic resonance imaging and histology. *Arthritis Res Ther* 5, R60-R73 (2003)

5. H. A. Breinan, T. Minas, H. P. Hsu, S. Nehrer, C. B. Sledge and M. Spector, Effect of cultured autologous chondrocytes on repair of chondral defects in a canine model. *J Bone Joint Surg Am* 79, 1439-1451 (1997)

6. J. A. Martin, A. J. Klingelhutz, F. Moussavi-Harami and J. A. Buckwalter, Effects of oxidative damage and telomerase activity on human articular cartilage chondrocyte senescence. *J Gerontol A Biol Sci Med Sci* 59, 324-337 (2004)

7. F. Moussavi-Harami, Y. Duwayri, J. A. Martin, F. Moussavi-Harami and J. A. Buckwalter, Oxygen effects on senescence in chondrocytes and mesenchymal stem cells: consequences for tissue engineering. *Iowa Orthop* J 24, 15-20 (2004)

8. G. d'Ippolito, S. Diabira, G. A. Howard, P. Menei, B. A. Roos and P. C. Schiller, Marrow-isolated adult multilineage inducible (MIAMI) cells, a unique population of postnatal young and old human cells with extensive expansion and differentiation potential. *J Cell Sci* 117, 2971-2981 (2004)

9. S. Peansukmanee, A. Vaughan-Thomas, S. D. Carter, P. D. Clegg, S. Taylor, C. Redmond and A. Mobasheri, Effects of hypoxia on glucose transport in primary equine chondrocytes *in vitro* and evidence of reduced GLUT1 gene expression in pathologic cartilage *in vivo*. *J Orthop Res* 27, 529-535 (2009)

10. I. A. Silver, Measurement of pH and ionic composition of pericellular sites. *Philos Trans R Soc Lond B Biol Sci* 271, 261-272 (1975)

11. O. A. Savage and H. J. Taylor, Preliminary Observations on the Oxygen and Carbon Dioxide Gas Tensions in the Knee-Joint in Normal and Pathological Conditions. *Ann Rheum Dis* 1, 134-140 (1939)

12. S. Stroebel, M. Loparic, D. Wendt, A. D. Schenk, C. Candrian, R. L. P. Linberg, F. Moldovan, A. Barbero and I. Martin. Anabolic and catabolic responses of human articular chondrocytes to varying oxygen percentages. *Arthritis Research & Therapy* 12. 2010.

13. E. Duval, S. Leclercq, J. M. Elissalde, M. Demoor, P. Galera and K. Boumediene, Hypoxia-inducible factor 1alpha inhibits the fibroblast-like markers type I and type III collagen during hypoxia-induced chondrocyte redifferentiation: hypoxia not only induces type II collagen and aggrecan, but it also inhibits type I and type III collagen in the hypoxia-inducible factor 1alpha-dependent redifferentiation of chondrocytes. *Arthritis Rheum* 60, 3038-3048 (2009)

14. J. E. Lafont, S. Talma and C. L. Murphy, Hypoxia-inducible factor 2alpha is essential for hypoxic induction of the human articular chondrocyte phenotype. *Arthritis Rheum* 56, 3297-3306 (2007)

15. C. L. Murphy, B. L. Thoms, R. J. Vaghjiani and J. E. Lafont, Hypoxia. HIF-mediated articular chondrocyte function: prospects for cartilage repair. *Arthritis Res Ther* 11, 213 (2009)

16. N. R. Forsyth, A. Musio, P. Vezzoni, A. H. Simpson, B. S. Noble and J. McWhir, Physiologic oxygen enhances human embryonic stem cell clonal recovery and reduces chromosomal abnormalities. *Cloning Stem Cells* 8, 16-23 (2006)

17. W. E. Wright and J. W. Shay, Inexpensive low-oxygen incubators. *Nat Protoc* 1, 2088-2090 (2006)

18. C. B. Allen, B. K. Schneider and C. W. White, Limitations to oxygen diffusion and equilibration in *in vitro* cell exposure systems in hyperoxia and hypoxia. *Am J Physiol Lung Cell Mol Physiol* 281, L1021-L1027 (2001)

19. S. Rozen and H. Skaletsky, Primer3 on the WWW for general users and for biologist programmers. *Methods Mol Biol* 132, 365-386 (2000)

20. N. R. Forsyth and J. McWhir, Human embryonic stem cell telomere length impacts directly on clonal progenitor isolation frequency. *Rejuvenation Res* 11, 5-17 (2008)

21. M. A. Cremer, E. F. Rosloniec and A. H. Kang, The cartilage collagens: a review of their structure, organization, and role in the pathogenesis of experimental arthritis in animals and in human rheumatic disease. *J Mol Med* 76, 275-288 (1998)

22. K. Pelttari, E. Steck and W. Richter, The use of mesenchymal stem cells for chondrogenesis. *Injury* 39 Suppl 1, S58-S65 (2008)

23. H. Vasiliadis, G. Salanti, A. Georgoulis, A. Lindahl and L. Peterson, Assessment Of Clinical Outcomes Ten To Twenty Years After Autologous Chondrocyte Implantation. *Osteoarthritis Cartilage* (2010)

24. J. Folkman, P. Hahnfeldt and L. Hlatky, Cancer: looking outside the genome. *Nat Rev Mol Cell Biol* 1, 76-79 (2000)

25. A. Barbero, S. Grogan, D. Schafer, M. Heberer, P. Mainil-Varlet and I. Martin, Age related changes in human articular chondrocyte yield, proliferation and post-expansion chondrogenic capacity. *Osteoarthritis Cartilage* 12, 476-484 (2004)

26. P. A. Guerne, F. Blanco, A. Kaelin, A. Desgeorges and M. Lotz, Growth factor responsiveness of human articular chondrocytes in aging and development. *Arthritis Rheum* 38, 960-968 (1995)

27. P. J. Roughley, L. I. Melching and A. D. Recklies, Changes in the expression of decorin and biglycan in human articular cartilage with age and regulation by TGF-beta. *Matrix Biol* 14, 51-59 (1994)

28. G. Verbruggen, M. Cornelissen, K. F. Almqvist, L. Wang, D. Elewaut, C. Broddelez, R. L. de and E. M. Veys, Influence of aging on the synthesis and morphology of the aggrecans synthesized by differentiated human articular chondrocytes. *Osteoarthritis Cartilage* 8, 170-179 (2000)

29. K. Bobacz, L. Erlacher, J. Smolen, A. Soleiman and W. B. Graninger, Chondrocyte number and proteoglycan synthesis in the aging and osteoarthritic human articular cartilage. *Ann Rheum Dis* 63, 1618-1622 (2004)

30. H. K. Heywood and D. A. Lee, Low oxygen reduces the modulation to an oxidative phenotype in monolayer-expanded chondrocytes. J Cell Physiol 222, 248-253 (2010)

31. R. Amarilio, S. V. Viukov, A. Sharir, I. Eshkar-Oren, R. S. Johnson and E. Zelzer, HIF1alpha regulation of Sox9 is necessary to maintain differentiation of hypoxic prechondrogenic cells during early skeletogenesis. *Development* 134, 3917-3928 (2007)

32. S. R. Bibby and J. P. Urban, Effect of nutrient deprivation on the viability of intervertebral disc cells. *Eur Spine J* 13, 695-701 (2004)

33. C. Domm, M. Schunke, K. Christesen and B. Kurz, Redifferentiation of dedifferentiated bovine articular chondrocytes in alginate culture under low oxygen tension. *Osteoarthritis Cartilage* 10, 13-22 (2002)

34. K. Hashimoto, K. Fukuda, K. Yamazaki, N. Yamamoto, T. Matsushita, S. Hayakawa, H. Munakata and C. Hamanishi, Hypoxia-induced hyaluronan synthesis by articular chondrocytes: the role of nitric oxide. *Inflamm Res* 55, 72-77 (2006)

35. J. Malda, C. A. van Blitterswijk, G. M. van, D. E. Martens, J. Tramper and J. Riesle, Low oxygen tension stimulates the redifferentiation of dedifferentiated adult human nasal chondrocytes. *Osteoarthritis Cartilage* 12, 306-313 (2004)

36. A. B. Adesida, L. M. Grady, W. S. Khan, S. J. Millward-Sadler, D. M. Salter and T. E. Hardingham, Human meniscus cells express hypoxia inducible factor-1alpha and increased SOX9 in response to low oxygen tension in cell aggregate culture. *Arthritis Res Ther* 9, R69 (2007)

37. M. J. Grimshaw and R. M. Mason, Bovine articular chondrocyte function *in vitro* depends upon oxygen tension. *Osteoarthritis Cartilage* 8, 386-392 (2000)

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