EFFECTS OF OXYGEN AND CULTURE SYSTEM ON *IN VITRO* PROPAGATION AND REDIFFERENTIATION OF OSTEOARTHRITIC HUMAN ARTICULAR CHONDROCYTES

Karsten Schrobback, PhD, (corresponding author)

Institute of Health and Biomedical Innovation, Queensland University of Technology (QUT), 60 Musk Ave, Kelvin Grove, Australia 4059

Email: k.schrobback@qut.edu.au

Travis Jacob Klein, PhD

Institute of Health and Biomedical Innovation, Queensland University of Technology, 60 Musk Ave, Kelvin Grove, Australia 4059

Email: t2.klein@qut.edu.au

Ross Crawford, MD, PhD

Institute of Health and Biomedical Innovation, Queensland University of Technology, 60 Musk Ave, Kelvin Grove, Australia 4059

Email: r.crawford@qut.edu.au

Zee Upton, PhD

Institute of Health and Biomedical Innovation, Queensland University of Technology, 60 Musk Ave, Kelvin Grove, Australia 4059

Email: z.upton@qut.edu.au

Jos Malda, MSc, PhD

Department of Orthopaedics, University Medical Center Utrecht

PO Box 85500, 3508 GA, Utrecht, The Netherlands

Email: j.malda@umcutrecht.nl

and

Institute of Health and Biomedical Innovation, Queensland University of Technology,

60 Musk Ave, Kelvin Grove, Australia 4059.

David Ian Leavesley, PhD

Institute of Health and Biomedical Innovation, Queensland University of Technology,

60 Musk Ave, Kelvin Grove, Australia 4059

Email: d.leavesley@qut.edu.au

Abstract

Regenerative medicine-based approaches for the repair of damaged cartilage rely on the ability to propagate cells while promoting their chondrogenic potential. Thus, conditions for cell expansion should be optimized through careful control of environmental conditions. In particular, appropriate oxygen tension and cell expansion substrates as well as controllable bioreactor systems are likely critical for expansion and subsequent tissue formation during chondrogenic differentiation. We, therefore, evaluated the effects of oxygen and microcarrier culture on the expansion and subsequent differentiation of human osteoarthritic chondrocytes. Freshly isolated chondrocytes were expanded on tissue culture plastic or CultiSpher-G microcarriers under hypoxic (5% pO_2) or normoxic (20% pO_2) conditions followed by cell phenotype analysis using flow cytometry. Cells were redifferentiated in micromass pellet cultures over 4 weeks, either under hypoxia or normoxia. Chondrocytes cultured on tissue culture plastic proliferated faster, expressed higher levels of cell surface markers CD44 and CD105 and demonstrated stronger staining for proteoglycans and collagen type II in pellet cultures compared to microcarrier-cultivated cells. Pellet wet weight, GAG content and expression of chondrogenic genes were significantly increased in cells differentiated under hypoxia. In addition, HIF-3α mRNA was up-regulated in these cultures in response to the low oxygen tension. These data confirm the beneficial influence of reduced oxygen on *ex vivo* chondrogenesis. However, hypoxia during cell expansion and microcarrier bioreactor culture did not enhance their intrinsic chondrogenic potential. Therefore, further improvements in cell culture conditions are required before chondrocytes from osteoarthritic and aged patients can become a useful cell source for cartilage regeneration.

Key words: Cartilage tissue engineering, chondrocyte, microcarrier, hypoxia, differentiation

Introduction

Articular cartilage exhibits a limited intrinsic capacity to regenerate. Focal cartilage defects can lead to the development of osteoarthritis, a painful and debilitating disease. Given the limited treatment options that are currently available to treat such tissue defects, new therapeutic approaches are being investigated, including tissue-engineered cartilage, with the aim of restoring function to damaged tissue (Nesic et al. 2006, Hutmacher et al. 2003). Although techniques such as autologous chondrocyte transplantation are already being applied in the clinic for the treatment of acute injuries, they are not yet suitable for patients suffering from osteoarthritis (Sittinger and Burmester 2006). Among the clinical challenge of restoring damaged cartilage is obtaining sufficiently large cell numbers that are suitable for transplant. Osteoarthritic chondrocytes have been tested for their suitability for cartilage regeneration with varying success. Some investigators have reported reduced synthesis of cartilage matrix (Dorotka et al. 2005) and collagen (Tallheden et al. 2005) by osteoarthritic cells compared to chondrocytes from non-osteoarthritic tissue after *in vitro* differentiation. Others found comparable levels of cartilage matrix proteins secreted by chondrocytes from both sources (Dehne et al. 2009, Stoop et al. 2007).

The regenerative behaviour of chondrocytes is regulated by a complex array of biochemical and biophysical factors, including mechanical stimulation and oxygen tension (Vinatier et al. 2009). Biomechanical stimulation of chondrocytes in bioreactors can enhance matrix synthesis and improve biochemical composition of cartilaginous constructs (Martin et al. 2004, Vunjak-Novakovic et al. 1996). Stirred bioreactors in combination with microcarriers can also offer flexible and passage-free culture systems (Malda and Frondoza 2006). The cultivation of human and bovine

chondrocytes on gelatine microcarriers in stirred bioreactors is reported to yield high proliferation rates and enhanced chondrogenic potential compared to conventional monolayer culture (Malda et al. 2003a, Melero-Martin et al. 2006, Frondoza et al. 1996). However, using non-osteoarthritic human articular chondrocytes, we found similar redifferentiation capacity between microcarrier- and monolayer-propagated cells, suggesting the need for further optimization of culture conditions (Schrobback et al. 2011).

Oxygen is also an important factor for chondrocyte homeostasis. The oxygen partial pressure (pO_2) of articular cartilage *in situ* is estimated to range between ~7-10% at the superficial interface and ~1% near the subchondral bone (Silver 1975, Malda et al. 2003b). However, the *in vitro* effects of oxygen on the proliferation of chondroprogenitors is controversial. Bone marrow-derived mesenchymal stem cells exhibit higher *in vitro* proliferative activity under moderate hypoxia (3-8% pO_2) compared to ambient oxygen (D'Ippolito et al. 2006, Lennon et al. 2001, Ren et al. 2006). In contrast, oxygen tensions of 1.5% and below inhibited the growth of chondrocytes from rabbit (Marcus 1973) and bovine cartilage (Egli et al. 2008) while moderate hypoxia was observed to have a positive effect on proliferation of animal-derived articular chondrocytes (Hansen et al. 2001, Henderson et al. 2010). Conversely, other investigators have reported that there is no influence on the growth rates of bovine chondrocytes (Malda et al. 2004a, Grimshaw and Mason 2000).

Less controversial is the influence of low oxygen on the *in vitro* differentiation of chondrocytes. Human articular chondrocytes obtained from healthy (Murphy and Polak 2004), as well as osteoarthritic tissues (Yudoh et al. 2005, Katopodi et al. 2009), increase the expression of chondrogenic markers under low oxygen

concentrations. In this respect, the transcription factors of the hypoxia-inducible factor (HIF) family have been implicated in regulating the chondrocyte responses to oxygen deprivation (Coimbra et al. 2004). Of the three known HIF species, only the roles of HIF-1α and HIF-2α have been described in cartilage (Yudoh et al. 2005, Gelse et al. 2008, Lafont et al. 2007). Despite experimental evidence supporting the beneficial effects of low oxygen for the chondrogenic phenotype, articular chondrocytes continue to be cultivated *in vitro* under ambient oxygen.

We, therefore, hypothesised that reduced oxygen conditions improve the proliferation and redifferentiation capacity of human osteoarthritic articular chondrocytes cultivated on microcarriers (CultiSpher G) in a stirring bioreactor compared to conventional monolayer culture technique at ambient oxygen tension. We further evaluated the immunophenotype of *ex vivo* expanded articular chondrocytes and their redifferentiation potential in micromass cultures in normoxic (20% pO_2) and hypoxic (5% pO_2) conditions and investigated whether members of the HIF family were regulated by oxygen tension and correlated with chondrogenic potential.

Materials and Methods

Cell isolation

Articular cartilage was obtained with institutional ethics approval from consenting patients (3 female donors, age 65±6 years) undergoing joint arthroplasty. Cartilage slices were harvested from visually normal tissue (International Cartilage Repair Society anatomical grade 0-1 (Aroen et al. 2004)), minced, washed in phosphate

buffered saline (PBS) (Invitrogen, Carlsbad, CA) and digested overnight with 0.15% *w/v* collagenase type 2 (Worthington, Lakewood, NJ) in serum-free, low-D-glucose (1 g/L) basal medium (Dulbecco's Modified Eagle Medium (DMEM) supplemented with 4 mM L-alanyl-L-glutamine, 1 mM sodium pyruvate, 10 mM N-(2-hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid) (HEPES), 0.1 mM non-essential amino acids, 50 U/mL penicillin, 50 µg/mL streptomycin (all from Invitrogen) and 0.4 mM L-proline (Sigma, St. Louis, MO).

Cell expansion

Freshly isolated human chondrocytes were propagated on tissue culture plastic (TCP) or on porcine gelatine microcarriers (MC) under ambient (20% pO_2) or reduced (5% pO_2) oxygen concentration. Cells were cultivated in low-glucose basal medium supplemented with 0.1 mM L-ascorbic acid (WAKO, Osaka, Japan) and 10% foetal bovine serum (FBS) (Hyclone, Logan, UT) at 37˚C in a humidified 5% CO_2/95% air incubator. Medium was refreshed twice per week in a conventional Class II biosafety cabinet. Cells cultivated under reduced oxygen tension were maintained in a ProOx C-Chamber (Biospherix, Redfield, NY) placed inside a cell culture incubator. The ProOx chamber was dynamically adjusted to 5% pO_2 via an automatic gas-exchange regulator connected to a reservoir containing 5% CO_2/95% N_2.

For monolayer expansion, chondrocytes were seeded at 3,000 cells per cm^2 in tissue culture flasks (Nunc, Roskilde, Denmark) and cultured up to passage 3 over a total of four weeks. Subconfluent cells were released from TCP by washing twice with PBS and incubating in 0.25% trypsin with 1 mM ethylenediamine tetraacetic acid (trypsin/EDTA) (Invitrogen) at 37˚C for 5 min.

For microcarrier expansion, macroporous CultiSpher G microcarrier beads (Percell, Åstorp, Sweden) with a nominal size range of 130-380 µm were used Due to the highly tortuous nature of these microcarriers, reliable determination of available surface area is not feasible (Schrobback et al. 2011, Borg et al. 2009). Chondrocytes were seeded at a density of 10^4 cells per mg per mL, as described elsewhere (Malda et al. 2003a, Schrobback et al. 2011). Briefly, cells were cultivated in spinner flasks (Bellco Glass, Vineland, NJ) using the Variomag BIOSYSTEM 4B (H+P Labortechnik/Thermo Fisher, Waltham, MA). Agitation was initially set to intermittent stirring at 20 revolutions per minute (rpm) for 30 s every 30 min for the first 2 days and thereafter changed to continuous stirring at 50-65 rpm for the remainder of the experiment, keeping the stirring speed as low as possible for the microcarrier aggregates to remain afloat. Cells were harvested from microcarriers by washing twice in PBS and incubating in 0.25% trypsin/EDTA (Invitrogen) at 37˚C for 10-20 min, until the microcarriers were digested. Coarse microcarrier debris was removed by passage through a cell strainer (100 µm pore, BD Biosciences).

Harvested cells were counted with a NucleoCounter (Chemometec, Allerød, Denmark) which confirmed similar levels of viability in cells isolated from monolayer and microcarrier cultures. Population doubling times and total population doublings were calculated and compared between groups. Chondrocytes used in differentiation experiments and for phenotypic analysis were propagated for four weeks either on TCP (3 passages) or on microcarriers. Cells freshly isolated from monolayers or microcarriers were also kept for biochemical assays and RNA isolation to establish DNA, glycosaminoglycan and gene expression levels before redifferentiation (day 0).

Immunophenotypic analysis

Cells were harvested after each different expansion regimen and incubated at room temperature for 30 min in PBS with 3% *m/v* bovine serum albumin (Sigma) (3%BSA/PBS) and one of the following monoclonal antibodies: CD45 (1:20, H5A5, Developmental Studies Hybridoma Bank (DSHB), Iowa City, IA), CD44 (1:20, H4C4, DSHB), CD105 (1:20, P4A4, DSHB) or IgG isotype control (1:50, Invitrogen). Cells were washed twice with cold PBS and then incubated for 1 hr on ice in 3%BSA/PBS containing a secondary, fluorescein isothiocyanate-labelled goat anti-mouse IgG antibody (1:80, CALTAG, Burlingame, CA). Finally, samples were washed again twice with cold PBS and resuspended in 3%BSA/PBS and immediately analysed on a FC500 flow cytometer (Beckman Coulter, Fullerton, CA). Ten thousand events were collected for each cell surface antigen and the parameters analyzed using CXP software (Beckman Coulter). The percentage of positive cells was calculated using the Overton method (Overton 1988).

Chondrogenic differentiation

The differentiation potential of expanded chondrocytes was evaluated using micromass cell pellets in serum-free chondrogenic media (basal medium supplemented with 1.25 mg/mL BSA, 10^{-7} M dexamethasone, 1% *v/v* ITS+1 (all from Sigma), 0.1 mM L-ascorbic acid (WAKO) and 10 ng/mL transforming growth factor type beta 1 (TGF-β1) (Chemicon/MILLIPORE, Billerica, MA)). Pellets were formed by centrifugation of $2.5x10^5$ cells per well in microplates (Axygen Scientific, Union City, CA) at 600 x g and transferred into 24-well plates after day 3. Micromass pellets were cultured either at 20% or 5% pO_2 for up to 28 days, as described under cell

expansion. Media were refreshed twice per week. Pellet samples were taken after 7, 14 and 28 days of culture and weighed.

Quantification of DNA and glycosaminoglycans (GAG)

The DNA content in cell samples was quantitated using the Quant-iT™ PicoGreen® dsDNA assay (Invitrogen). Briefly, cells were incubated overnight at 60˚C with 0.5 mg/mL proteinase K (Invitrogen) in 20 mM Na_2HPO_4, 30 mM $NaH_2PO_4.H_2O$, 5 mM EDTA, pH 7.1. Samples were mixed 1 in 1 with dye solution in 96-well plates (Nunc) and sample fluorescence was measured using a micro-plate reader (BMG Labtech, Offenburg, Germany) at 485 nm excitation and 520 nm emission. DNA content was calculated from λ phage DNA standards.

The GAG content of each proteinase K-digested samples was quantified using the 1,9-dimethylmethylene blue (DMMB, Sigma) assay (Farndale et al. 1982, Farndale et al. 1986). Absorbance at 525 nm was measured with a microplate spectrophotometer (Bio-Rad, Hercules, CA). The quantity of GAG was calculated from standards of chondroitin sulphate (Sigma).

Total messenger ribonucleic acid (mRNA) extraction and qRT-PCR

Total RNA was isolated from cell samples using TRIzol® Reagent (Invitrogen) following the manufacturer's protocol with modifications for high proteoglycan content (Chomczynski and Mackey 1995). Briefly, after chloroform-guided phase separation, RNA was precipitated from the aqueous layer with 0.5 volumes of 2-propanol, 0.5 volumes of acidified salt (1.2 M NaCl, 0.8 M sodium citrate) and 16 µg/mL linear polyacrylamide (Ambion, Austin, TX) at -80°C overnight. RNA was

pelleted and washed in 70% ethanol and resuspended in RNAase-free water. Total RNA was quantified using UV spectroscopy. Only RNA samples with an A_{260}/A_{280} ratio of greater than 1.8 and acceptable agarose tris hydroxymethylaminoethane-acetate-EDTA gel analysis were used in subsequent polymerase chain reaction (PCR) analyses.

Quantitative reverse transcribed-PCR (qTR-PCR) was applied to determine the presence and copy numbers of specific mRNA species in chondrocyte micromass cultures. Oligonucleotide primers were designed with Primer Express (Applied Biosystems) or primer sequences reported in the literature (Table 1). Samples containing 300 ng of total RNA were treated with DNase I (Invitrogen) and reverse transcribed into complementary DNA (cDNA) with SuperScript™ III first-strand synthesis supermix (Invitrogen) following the manufacturer's protocols. Absolute quantification was performed using standard curves (Leong et al. 2007). All reactions were performed in duplicate in 7.5 µL volumes in 384-well plates using Express SYBR® GreenER™ qPCR supermix universal (Invitrogen) and the 7900HT fast real-time PCR system (Applied Biosystems, Foster City, CA). Each reaction contained 1 X qPCR mix, 132 nM forward and reverse primers and 0.2 µL undiluted cDNA. PCR amplification followed a three step cycling protocol of an initial 5 min incubation at 50ºC, 2 min denaturation at 95ºC and 40 cycles of 95ºC for 15 s and 60ºC for 30 s. All reactions included a post-amplification melt curve analysis to validate amplification of the correct sequence. Amplification plots were analysed with ABI Sequence Detection System software version 2.3 (Applied Biosystems). The cDNA copy number of genes of interest was calculated by direct comparison to the known standards and normalised to the housekeeping gene 18S rRNA. 18S rRNA has been reported to be amongst the most stable housekeeping genes in samples from human

OA cartilage tissue (Pombo-Suarez et al. 2008), chondrocytes stimulated with various cytokines (McAlinden et al. 2004) and in monolayer culture under hypoxia (Foldager et al. 2009), and we found it to be stably expressed under our culture conditions.(.

Safranin O staining

Cell pellet cultures were probed for GAG with safranin O. Briefly, fresh frozen micromass pellet samples embedded in Tissue-Tek™ O.C.T. compound (Sakura Finetek, Tokyo, Japan) were cut to yield ~5 μm thick cross-sections. Sections were fixed in 100% ice-cold acetone and stained using haematoxylin, fast green FCF (0.001% *w/v*) and safranin O (0.1% *w/v*) (all from Sigma) (Lillie 1965). Representative images were captured with a digital camera (QImaging, Surrey, Canada) mounted on a microscope (Olympus, Tokyo, Japan).

Immunohistochemical analysis on frozen sections

To determine the protein expression of collagen type I and type II in chondrocyte pellets, fresh-frozen sections were fixed in 100% ice-cold acetone for 15 min, then treated with 0.1% hyaluronidase (Sigma) in PBS for 20 min at 37°C and blocked with 2.5% normal horse serum (Vector laboratories, Burlingame, CA) for 20 min. Samples were subsequently incubated with monoclonal antibodies specific for collagen type I (1:300, I-8H5, MP Biomedicals, Solon, OH), collagen type II (1:100, II-II6B3, DSHB), or an isotype-matched control antibody (1:1000, Sapphire Bioscience, Redfern, Australia), in a humidified chamber overnight at 4°C. A 5 min wash in PBS was performed after each step. Subsequently, biotinylated secondary horse anti-

rabbit/mouse IgG antibody (Vector) was applied to the sections for 30 min, followed by incubation with the avidin and biotinylated horseradish peroxidase complex reagent (Vector) for 30 min in a humidified chamber. Samples were then incubated with ImmPACT™ DAB Substrate (Vector) for 5 min, followed by rinsing with tap water and counterstaining in Mayer's haematoxylin (Amber Scientific, Midvale, Australia). Images of samples were captured with a digital camera (QImaging, Surrey, Canada) mounted on a microscope (Olympus).

Statistical analysis

Statistical analyses were performed using Minitab 15 (Minitab, Inc., State College, PA). Analysis of variance was determined using a general linear model in which the donor was considered a random effect. To test the effect of oxygen concentration during cell differentiation, data from day 0 were excluded. Effects of cultivation time, culture system or oxygen levels during expansion were tested separately for each differentiation condition including day 0 data. In addition, data sets from each individual time point were independently tested for statistical significance. In cases where a significant difference was detected between conditions, Tukey's post-hoc test for pair-wise comparisons was performed. Correlations between the expression of the different genes from chondrocyte pellets during differentiation were analyzed by linear regression. Statistical significance was accepted to be present at $p<0.05$.

Results

Expansion of chondrocytes with hypoxic and normoxic conditions using different culture systems

The time required for the population of freshly isolated osteoarthritic chondrocytes to double over four weeks of *ex vivo* propagation was shorter on tissue culture plastic than in the microcarrier cultures ($p<0.001$) (Fig. 1 a). Consequently, the number of population doublings of chondrocytes in the monolayer cultures was significantly higher than the number of population doublings observed in microcarrier cultures after an equivalent period ($p<0.001$) (Fig. 1 b). However, no significant effects of oxygen level on cell growth were observed, regardless of the culture system.

Immunophenotype of cultivated chondrocytes

The hematopoietic cell marker CD45 was not detected in any of the cultured populations, as determined by flow cytometry (Fig. 2). Subpopulations expressing the putative stem cell surface markers CD105 and CD44 (Grogan et al. 2007, Pittenger et al. 1999) were more numerous among monolayer cultures than among populations cultured on microcarriers. In both culture systems, expansion under hypoxic conditions resulted in slightly higher percentages of cells positive for CD44 and CD105 compared to chondrocytes expanded under normoxic conditions.

Redifferentiation of cultivated chondrocytes (biochemical assays)

Wet weight, and DNA and GAG content were determined in micromass pellet cultures maintained under either normoxic (20% pO_2) or hypoxic (5% pO_2) conditions over four weeks.

Varying pO_2 tensions during expansion or differentiation had no effect on the DNA content of cell pellets (both p=0.1) (Fig. 3 a,b). However, cells propagated on microcarriers showed overall lower DNA amounts than monolayer-expanded groups ($p<0.001$). DNA content decreased over the first 7 days of culture ($p<0.05$) and correlated with microscopic observations indicating that some cells from the initial cell suspension failed to remain aggregated as a mass; particularly, in microcarrier-cultured samples (data not shown).

Exposing chondrocyte micromass pellets to hypoxic conditions during differentiation resulted in a significant increase in wet weight ($p<0.001$) compared to micromass pellets differentiated under normoxic conditions (Fig. 3 c,d). Pellets formed from monolayer-expanded cells produced more tissue than pellets formed from microcarrier-expanded chondrocytes when differentiated under normoxic ($p<0.001$) or hypoxic ($p<0.01$) conditions. Pellets differentiated under normoxic conditions declined in wet weight from day 7 to day 28 of culture ($p<0.05$).

The GAG content of micromass tissue pellets was greater when chondrocytes were differentiated under hypoxic conditions compared to those differentiated under normoxic conditions ($p<0.01$) (Fig. 3 e,f). Cells cultivated as monolayers produced more GAG than cells cultivated on microcarriers ($p<0.05$). The GAG data have not been normalised to DNA content, as the decrease in DNA content over time results in misleading overestimation of synthesised GAGs at later time points.

Gene expression during differentiation (qRT-PCR)

The presence and copy number of cartilage-specific mRNA species, aggrecan and collagen type II (COL2A1) were determined by qRT-PCR. Aggrecan mRNA levels were higher when chondrocytes were cultivated in monolayers, rather than on

microcarriers ($p<0.001$) (Fig. 4 a d0). Only cells redifferentiated under hypoxic conditions increased their overall expression of aggrecan ($p<0.01$). While aggrecan mRNA transcripts significantly decreased with extended culture in micromass pellets under normoxic conditions ($p<0.05$) (Fig. 4 a), aggrecan mRNA expression under hypoxic conditions peaked after 7 days ($p<0.05$) and declined only at the end of the culture period ($p<0.01$). Transcripts encoding COL2A1 were undetectable after the initial *ex vivo* expansion of isolated cells (Fig. 4 b d0), but were re-expressed during micromass pellet culture in hypoxic conditions ($p<0.05$) (Fig. 4 b).

Chondrocyte dedifferentiation markers, collagen type I (COL1A1) and versican (Benya and Shaffer 1982, Binette et al. 1998), were strongly expressed at levels equivalent to, or greater than, aggrecan and COL2A1 (Fig. 4 a-d). COL1A1 expression levels were higher in cells cultured on tissue culture plastic than in those from microcarrier cultivation at day 0; however, this effect was reversed after one week of culture under hypoxic conditions (Fig. 4 c). Cells expanded on tissue culture plastic also expressed higher levels of versican after cell propagation than cells from microcarrier cultures ($p<0.01$) (Fig. 4 c d0), which remained evident under normoxic conditions at day 7 ($p<0.01$) (Fig. 4 c). Under normoxic differentiation conditions, transcript levels of COL1A1 and versican were up-regulated after 7 days ($p<0.05$) (Fig. 4 c,d). In contrast, hypoxic differentiation conditions attenuated changes in expression for several days. However, we did not detect any significant influence of oxygen on these genes following 28 days of differentiation culture (VCAN: $p=0.1$, COL1A1: $p=0.4$) (Fig. 4 c,d).

Transcripts encoding the hypertrophy-associated gene, collagen type X (COL10A1), were barely detectable following expansion in all conditions (Fig. 4 e d0). While

expression of this gene increased with subsequent cultivation, this was independent of normoxic ($p<0.001$) and hypoxic conditions ($p<0.01$).

We also examined gene products known to regulate cellular responses to reduced oxygen, the hypoxia inducible factors (HIFs). Surprisingly, cells that were initially established *ex vivo* in normoxic conditions expressed significantly more transcripts encoding HIF-1α and HIF-2α than chondrocytes initially established in hypoxic conditions, ($p<0.05$) (Fig. 5 a,b d0). Messenger RNA synthesis of HIF-1α and HIF-2α declined over the course of redifferentiation independently of atmospheric oxygen tension. However, the expression of HIF-1α was significantly elevated in hypoxic conditions ($p<0.05$). When exposed to normoxic differentiation conditions, chondrocytes expanded as monolayers expressed measurably more transcripts encoding HIF-1α ($p<0.01$) and HIF-2α ($p<0.05$) than chondrocytes initially expanded on microcarriers. We also probed for the expression of HIF-3α and found that initially it was barely detectable after cell expansion (Fig. 5 c). However, thereafter, cells cultivated in micromass pellets under normoxic ($p<0.05$) and hypoxic ($p<0.001$) conditions were found to express HIF-3α.

We also examined the expression of the HIF regulator, egl nine 1 homolog (EGLN1). The expression of EGLN1 was striking and paralleled the expression of HIF-1α ($R^2=0.61$; $p<0.001$) and HIF-2α ($R^2=0.68$; $p<0.001$). However, there was no correlation with the expression of HIF-3α, or with other chondrocyte-specific genes we examined (Fig. 5 d). Predictably, EGLN1 mRNA synthesis was down-regulated with extended culture ($p<0.001$) and significantly greater in cell pellets cultured under hypoxic conditions than in cell pellets cultured under normoxia ($p<0.05$).

Matrix synthesis during differentiation (histology / IHC)

To determine the *de novo* synthesis of cartilaginous matrix, micromass pellet samples were stained with hematoxylin, Fast Green FCF and safranin O (for GAGs) and with antibodies specific for collagen type II and I.

Tissue from chondrocyte micromass cultures expanded on tissue culture plastic exhibited greater intensity staining for GAGs (Fig. 6 a-i) and immuno-reactivity with collagen type II antibodies than micromass cultures from microcarrier-expanded chondrocytes (Fig. 6 j-o). Cells located near the center of pellets formed from monolayer cultures were surrounded by extracellular matrix and assumed a rounded morphology (Fig. 6 b,g). Micromass pellets formed from chondrocytes expanded in microcarrier cultures were more densely packed with minimal extracellular matrix (Fig. 6 j,m). This extracellular matrix stained only faintly for GAGs and was poorly immuno-reactive for collagen type II (Fig. 6 k,n). In contrast immuno-reactivity for collagen type I was found to be uniformly intense and well distributed in all micromass pellets formed from microcarrier-expanded cells (Fig. 6 l,o). Pellets from all groups were found to have a common superficial rim formed from multiple layers of flattened, elongated cells (Fig. 6). These superficial layers did not stain with safranin O and showed only localised immuno-reactivity for collagens, in particular type I.

Different oxygen tensions during cell expansion on microcarriers had no recognisable influence on the cell phenotype and the quality of matrix in the pellets (Fig. 6). However, among the monolayer expansion groups, pellets with cells redifferentiated under hypoxic conditions exhibited more intense staining for GAG and higher immuno-reactivity for collagen type II (Fig. 6 b,d) than those under

normoxic conditions (Fig. 6 a,c). Nonetheless, all monolayer-expanded groups were also highly positive for collagen type I (Fig. 6 e,f,i).

Discussion

The aim of this study was to evaluate the effect of culture systems and different oxygen tensions on the propagation and the redifferentiation capacity of human osteoarthritic chondrocytes. The chondrogenic potential of osteoarthritic cells was determined *in vitro* in micromass pellets exposed to normoxic (20% pO_2) or hypoxic conditions (5% pO_2).

Exposing the chondrocytes to different oxygen tensions during cell propagation failed to confer any measurable effect on proliferation or the chondrogenic potential of human osteoarthritic chondrocytes. This is consistent with the findings of a similar study, in which moderate hypoxia during microcarrier expansion of bovine articular chondrocytes did not improve cell growth or subsequent differentiation compared to ambient oxygen concentrations (Malda et al. 2004a). In contrast, other recent studies which examined rabbit and bovine chondrocytes isolated from the humeral head found that monolayer expansion under 5% and 1.5% oxygen, respectively, enhanced their redifferentiation capacity (Egli et al. 2008, Henderson et al. 2010). These studies also found that low oxygen tension had no effect on the redifferentiation of the chondrocytes in micromass pellets.

We observed that the synthesis of ECM, including GAG and collagen type II, as well as mRNA encoding chondrogenic markers were enhanced when cell pellets were cultured under hypoxic conditions. There is, indeed, growing support in the literature for this (Murphy and Polak 2004, Yudoh et al. 2005, Katopodi et al. 2009, Malda et al. 2004b, Khan et al. 2007, Koay and Athanasiou 2008). Some reports also claim

that the high expression of proteins up-regulated during dedifferentiation, such as collagen type I and versican, is sustained. We made similar observations at the mRNA and protein level. The transcription of COL1A1 and VCAN genes was diminished only slightly at early time points under hypoxic conditions, suggesting that a majority of cells fail to fully redifferentiate to the chondrogenic phenotype. In addition, the expression of these genes at high levels and the detection of the hypertrophy marker, COL10A1, may be associated with osteoarthritis. Osteoarthritic chondrocytes are reported to also change from a chondrocytic to a dedifferentiated and hypertrophic phenotype (Sandell and Aigner 2001). Given the appearance of COL10A1 as a marker of terminal differentiation and the high cell loss observed in the micromass pellets towards the end of the redifferentiation period, it is also possible that some chondrocytes did not respond well to the four weeks in this simplified *in vitro* environment, irrespective of the oxygen environment.

To investigate the mechanisms underlying the differences observed at varying oxygen levels, we measured the mRNA levels encoding different factors involved in the cellular response to hypoxia. We found that HIF-1α and HIF-2α were constitutively transcribed in our differentiation cultures, thus were unresponsive to varying oxygen tensions at mRNA levels. HIFα subunits are ubiquitinated under normoxia by the von Hippel-Lindau complex (pVHL) for subsequent proteasomal degradation (Maxwell et al. 1999, Maynard et al. 2003). The binding of pVHL is mediated through hydroxylation of conserved proline residues in the HIF-α protein in an iron- and oxygen-dependent manner by HIF prolyl hydroxylases (Jaakkola et al. 2001, Ivan et al. 2001). In our study, EGLN1 (HIF prolyl hydroxylase 2), which has also been found to be expressed in growth plate chondrocytes (Terkhorn et al. 2007), paralleled the expression pattern of HIF-1α and HIF-2α. Interestingly, all three

genes showed higher transcript levels during expansion, particularly under normoxic conditions, than during redifferentiation. However, it remains to be investigated if the basal levels of these transcripts in dedifferentiated and redifferentiated chondrocytes have any functional relevance. We also report, to our knowledge for the first time, transcription of the HIF-3α gene in redifferentiated chondrocytes. Although mRNA transcript levels were extremely low, potentially indicative of expression by a minority of chondrocytes, transcription was dependent on low oxygen and, surprisingly, seemed to follow the expression profiles of other chondrogenic marker genes during differentiation. HIF-3α is thought to be a competitive inhibitor of HIF-1/2α action (Maynard et al. 2005). Its regulation at the transcriptional level under hypoxia appears to be unique for this member of the HIF family (Heidbreder et al. 2003). Moreover, HIF-1α can directly up-regulate HIF-3α splice variants, thereby, providing a further level of negative feedback to the HIF system (Makino et al. 2007). However, the regulation of the cellular response to hypoxia is very complex and a specific physiological role for HIF-3α during chondrogenesis requires clarification.

When comparing the influence of the different cultivation systems on the propagation of human articular chondrocytes, we observed shorter doubling times for cell populations cultivated in monolayer up to passage 3, than for cell populations maintained in microcarrier culture. Since the latter underwent fewer cell divisions, these chondrocytes would be expected to be less dedifferentiated (Mandl et al. 2004). We were, therefore, surprised to find that expansion on microcarriers did not improve cell viability, GAG synthesis and mRNA expression of chondrogenic proteins of osteoarthritic chondrocytes, when subsequently redifferentiated, compared to the conventional monolayer system. This contrasts with the findings of other studies which report beneficial effects of the gelatine microcarrier culture

system for the redifferentiation potential of monolayer-propagated human osteoarthritic chondrocytes, nasal chondrocytes and immature bovine chondrocytes (Malda et al. 2003a, Melero-Martin et al. 2006, Frondoza et al. 1996). A major difference in our study is the use of freshly isolated osteoarthritic chondrocytes. This raises the general question of how and why the chondrogenic potential of these cells may have been restricted through microcarrier propagation.

An unexpected finding from our studies was the phenotypic differences of our *in vitro* cultured chondrocytes. Chondrocytes harvested from microcarriers expressed reproducibly lower levels of the surface markers CD44 and CD105, than cells expanded in monolayer culture (Fig. 2). CD44, the major hyaluronan receptor, and CD105, an accessory receptor for members of the TGF-β superfamily, are up-regulated in human chondrocytes with increased passage *in vitro* (Diaz-Romero et al. 2008, Diaz-Romero et al. 2005). In fact, subclones of chondrocytes with higher chondrogenic capacity showed higher levels of CD44 (Grogan et al. 2007) and CD105 has been utilised to identify multipotent mesenchymal stem cells (Pittenger et al. 1999). It is, therefore, possible that monolayer cultivation, unlike the microcarrier system, selects for a subpopulation of chondrocyte progenitors with higher CD44 and CD105 levels and higher differentiation potential.

The differences in the surface marker expression of cells from monolayer and microcarrier cultures, in particular for CD105, could also be explained by the slightly prolonged trypsin treatment used to harvest chondrocytes from microcarriers. However, we found that cells released from microcarriers by the use of collagenase also expressed lower CD44 and CD105 levels than monolayer-expanded cells (data not shown). The cell release process may still have had various effects on microcarrier-propagated cells. Firstly, the prolonged enzymatic treatment of cells on

microcarriers could have influenced their gene expression. Secondly, despite subsequent washing and sieving of the cell suspension, chondrocytes released from gelatine microcarriers will be exposed to large amounts of collagen fragments. Fragments of extracellular matrix proteins, particularly at high concentrations, are known to alter catabolic and anabolic pathways in cartilage (Homandberg and Hui 1994, Fichter et al. 2006). It is, therefore, possible that the large quantities of degradation products released from gelatine microcarriers during the harvest had a deleterious impact on subsequent chondrocyte redifferentiation, particularly, as cells had already been affected by osteoarthritis. An alternative explanation is that by subjecting the heterogeneous osteoarthritic chondrocytes (Tallheden et al. 2005, Katopodi et al. 2009, Barbero et al. 2004, Martin and Buckwalter 2003) to multiple passages in monolayer culture, compared to only one in microcarrier culture, one may specifically select for healthier, more rapidly growing cells from a population with variable chondrogenic potential; this phenomenon widely described, for example, in the case of the cross-contamination of cell lines by HeLa cells (Markovic and Markovic 1998). Moreover, culturing chondrocytes on a material derived from collagen type I could have generally altered their gene expression profile compared to cells propagated on TCP. Therefore, the choice of carrier material could also be critical for successful cultivation of osteoarthritic chondrocytes on microcarriers.

Conclusions

Our data suggest that porous gelatine microcarrier expansion offers few benefits over conventional monolayer propagation when applied to freshly isolated articular chondrocytes from osteoarthritic tissue. Furthermore, oxygen concentration has no influence on the chondrogenic potential of osteoarthritic chondrocytes when applied

during cellular expansion. However, exposure to mild hypoxia can improve cell viability and the expression of chondrogenic markers during redifferentiation. Moderate hypoxia enhances the redifferentiation of cells with existing differentiation capacity. Nevertheless, it may not be possible to restore the cellular function in all propagated chondrocytes, particularly those impaired by disease. The high expression levels of dedifferentiation markers, such as collagen type I and versican, in cartilaginous tissue constructs from osteoarthritic chondrocytes are of major concern for potential clinical applications. An improved understanding of mechanisms underlying chondrocyte senescence and strategies to modify, and potentially reverse this process, are required to enable chondrocytes from osteoarthritic and aged patients to become a useful cell source for cartilage tissue engineering.

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Figures

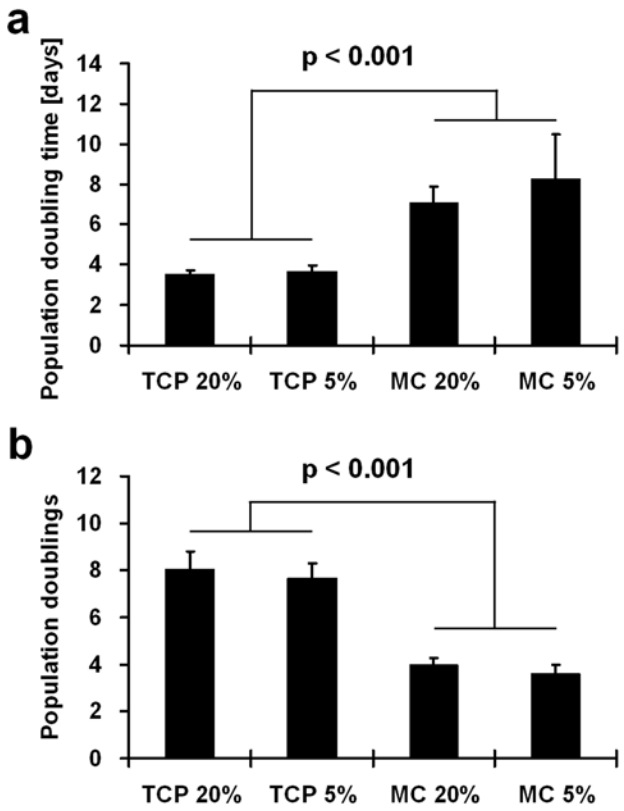

Fig. 1 Effect of oxygen and culture system on chondrocyte proliferation. Freshly isolated human articular chondrocytes from osteoarthritic cartilage were seeded either on tissue culture plastic (TCP) at 3,000 cells/cm^2 or on gelatine microcarriers (MC) at 1x10^4 cells per mg microcarrier per mL culture medium in a stirring bioreactor (at 50-65 rpm). Cells were cultured under 5% or 20% pO_2 for four weeks and media (containing 10% FBS) were refreshed twice per week. Cells on TCP were passaged twice during culture before reaching confluency. Cells from the different expansion cultures were counted after harvest. (a) population doubling times and (b) population doublings during expansion are presented as mean + standard deviation (n = 3 donors).

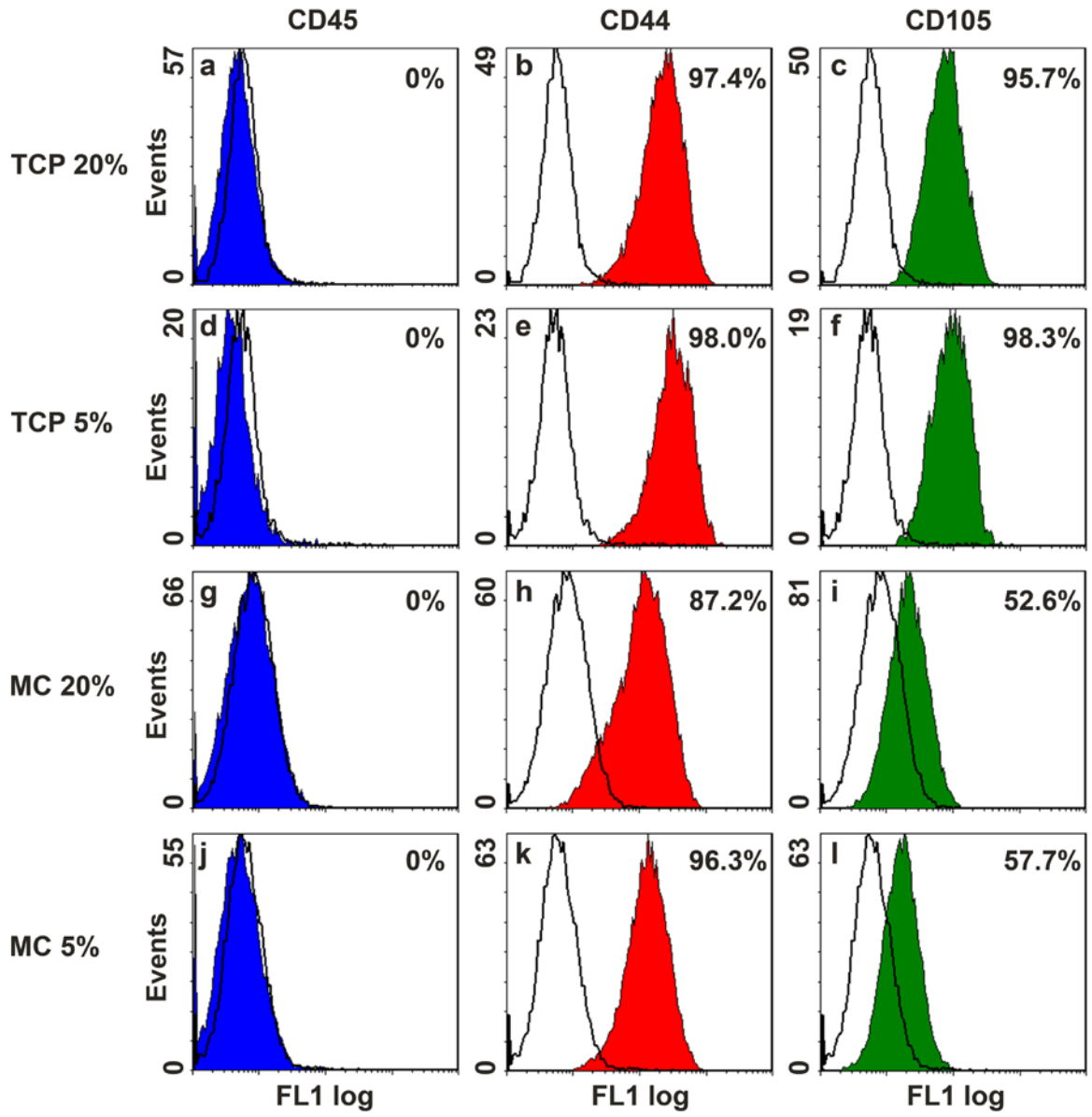

Fig. 2 Effect of oxygen and culture system on immunophenotype of expanded chondrocytes. Human osteoarthritic chondrocytes were expanded either on (a-f) tissue culture plastic (TCP) up to passage 3 or (g-l) on gelatine microcarriers (MC) for four weeks under either (a,b,c,g,h,i) 20% or (d,e,f,j,k,l) 5% pO_2. Cells were probed with antibodies against CD45, CD44 or CD105 or an IgG isotype control antibody and labelled with a FITC-conjugated secondary antibody, then analysed by flow cytometry. Histograms of representative expression patterns for one donor are shown with the surface markers (a,d,g,j) CD45, (b,e,h,k) CD44 and (c,f,i,l) CD105 plotted against a matched IgG control (no color) along with the percentage of positive cells, calculated using the Overton method.

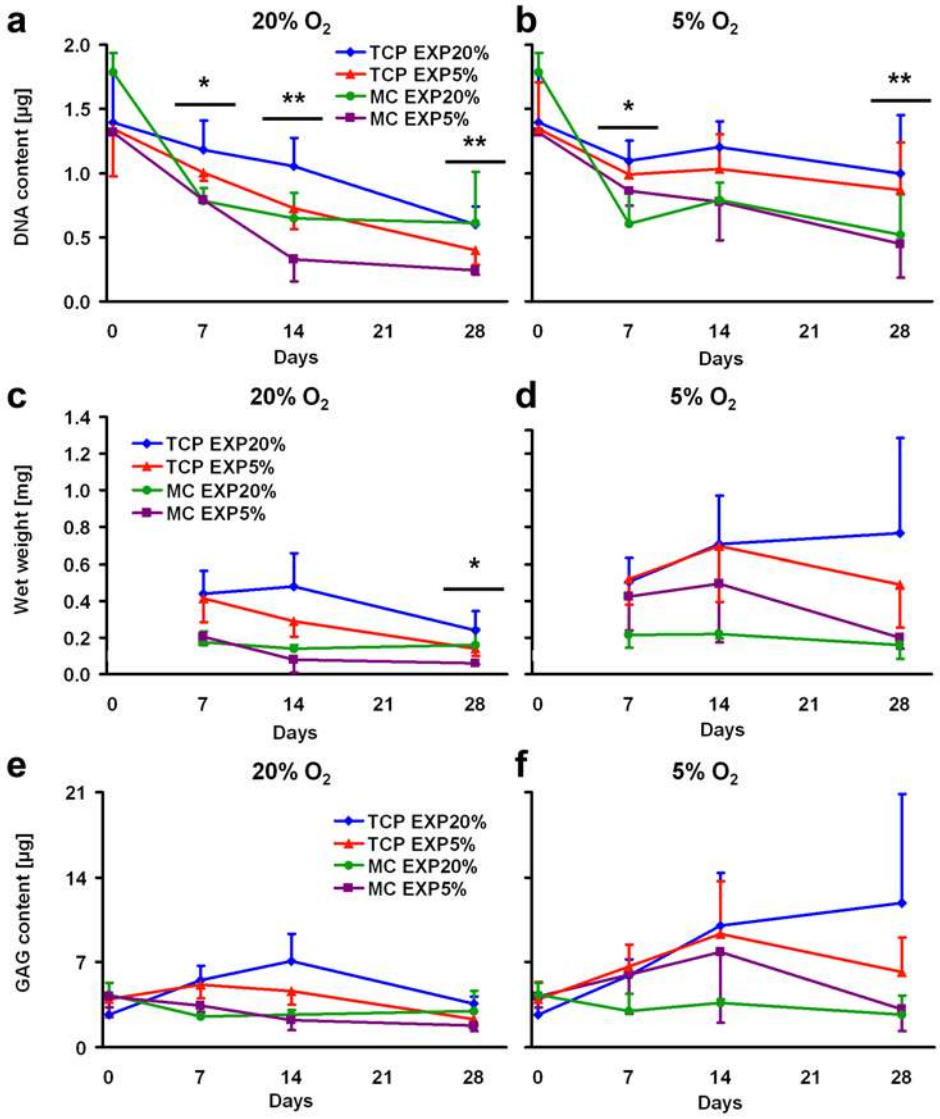

Fig. 3 (a,b) DNA content, (c,d) wet weight and (e,f) GAG content during pellet redifferentiation culture. Following cultivation either on tissue culture plastic (TCP) or on microcarriers (MC) under 5% or 20% pO_2, respectively, cells ($2.5x10^5$ cells per pellet) were pelleted and cultured either under (a,c,e) 20% or (b,d,f) 5% pO_2 in serum-free chondrogenic media for up to 28 days. Results for DNA content, wet weight and GAG content are expressed as mean ± standard error (n = 3 donors). Significant differences between day 0 (day 7 for wet weight) and later time points are indicated by * ($p<0.05$) or ** ($p<0.01$). Main effects were detected as follows: DNA content: TCP vs. MC = $p<0.001$; wet weight: differentiation under 20% pO_2 vs. 5% pO_2 = $p<0.001$, TCP vs. MC = $p<0.01$; GAG content: differentiation under 20% pO_2 vs. 5% pO_2 = $p<0.01$, TCP vs. MC = $p<0.01$.

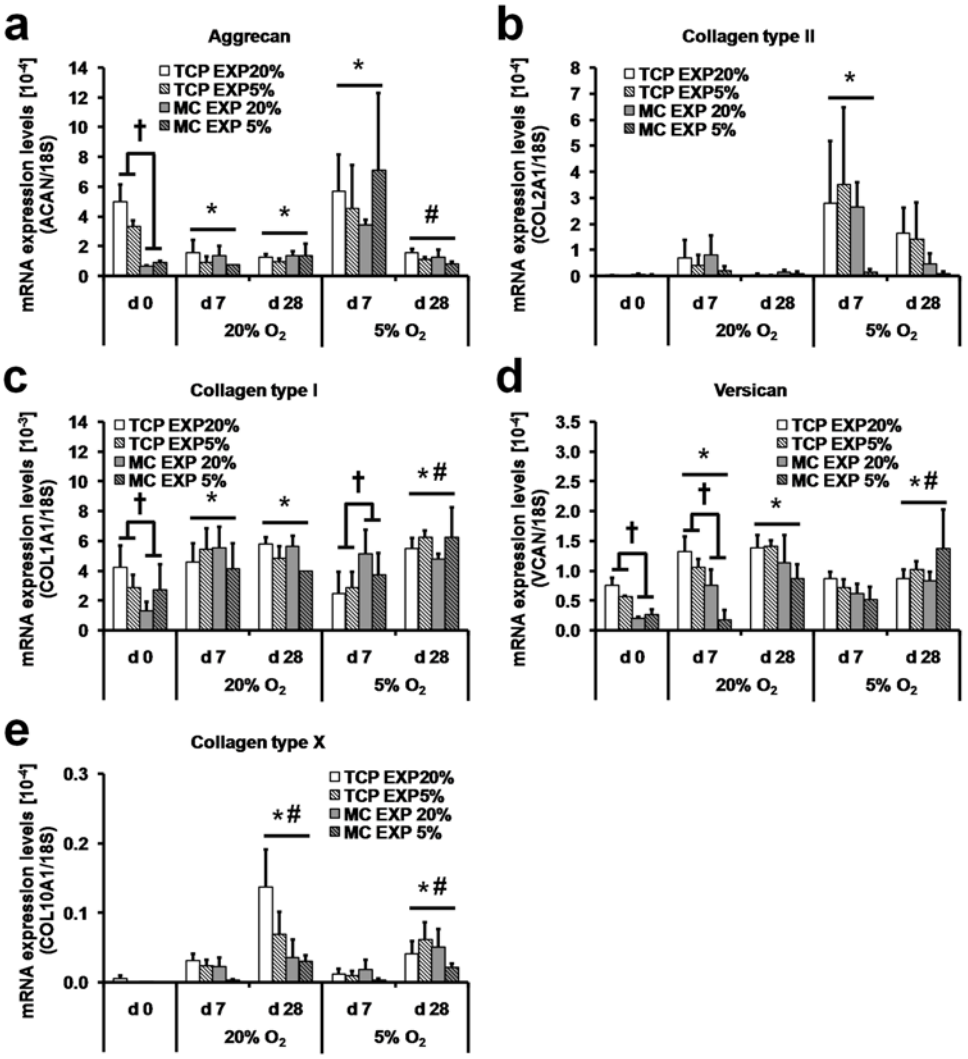

Fig. 4 mRNA expression of chondrogenic, dedifferentiation and hypertrophic markers in pellet redifferentiation culture. Following cultivation either on tissue culture plastic (TCP) or on microcarriers (MC) under 5% or 20% oxygen tension, respectively, pellets ($2.5x10^5$ cells) were formed and cultured either under 5% or 20% oxygen tension in serum-free chondrogenic media for up to 28 days. The levels of mRNA expression for (a) aggrecan, (b) collagen type II, (c) collagen type I (d) versican, (e) collagen type X were determined by quantitative real-time RT-PCR calculated with reference to a specific gene standard. Results from each treatment were expressed as the copy number of the target gene divided by the copy number of the 18S ribosomal gene. Data are represented as mean + standard error (n = 3 donors). Significant differences between culture conditions at a particular time point are indicated by † ($p<0.05$). Significant differences between day 0 and day 7 or later time point(s) are indicated by * ($p<0.05$) or # ($p<0.05$), respectively. Main effects of oxygen tension during differentiation culture were detected for the expression of aggrecan ($20\%O_2$ vs. $5\%O_2$ = $p<0.01$) and collagen type II ($20\%O_2$ vs. $5\%O_2$ = $p<0.05$).

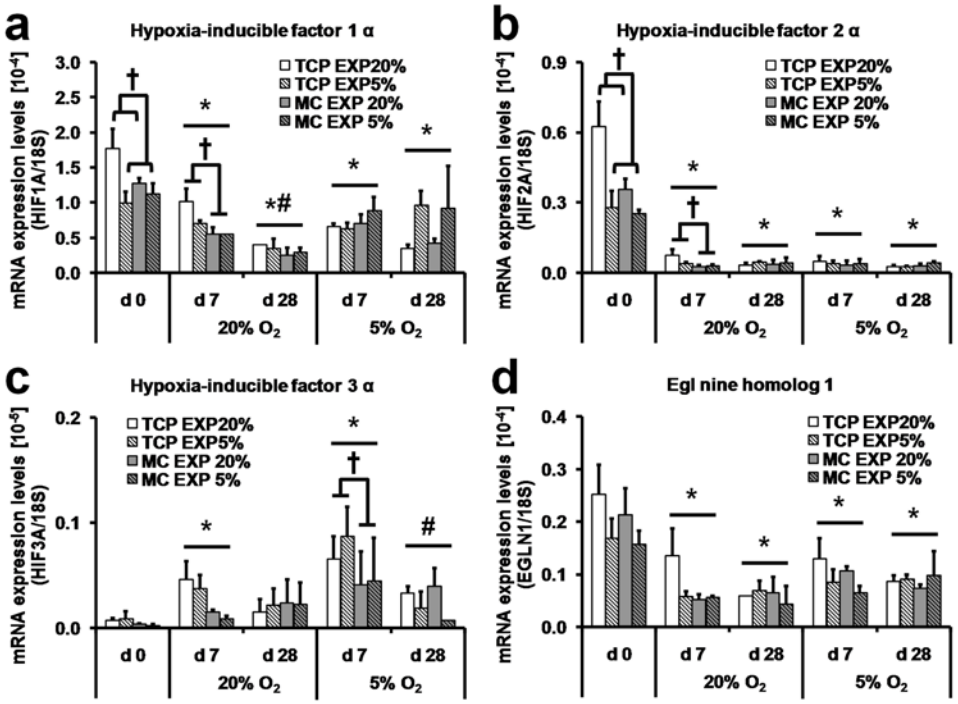

Fig. 5 mRNA expression of HIF family members in pellet redifferentiation culture. Following cultivation either on tissue culture plastic (TCP) or on microcarriers (MC) under 5% or 20% pO_2, respectively, pellets ($2.5x10^5$ cells) were formed and cultured either under 5% or 20% pO_2 in serum-free chondrogenic media for up to 28 days. The levels of mRNA expression for (a) hypoxia-inducible factor (HIF) 1α, (b) HIF-2α, (c) HIF-3α and (d) egl nine homolog 1 (HIF prolyl hydroxylase 2) was determined by quantitative real-time RT-PCR calculated with reference to a specific gene standard. Results from each treatment were expressed as the copy number of the target gene divided by the copy number of the 18S ribosomal gene. Data are represented as mean + standard error (n = 3 donors). Significant differences between culture conditions at a particular time point are indicated by † ($p<0.05$). Significant differences between day 0 and day 7 or later time point(s) are indicated by * ($p<0.05$) or # ($p<0.05$), respectively. Main effects of oxygen tension during differentiation culture were detected for the expression of HIF-1α ($20\%O_2$ vs. $5\%O_2$ = $p<0.05$), HIF-3α ($20\%O_2$ vs. $5\%O_2$ = $p<0.05$) and egl nine homolog 1 ($20\%O_2$ vs. $5\%O_2$ = $p<0.05$).

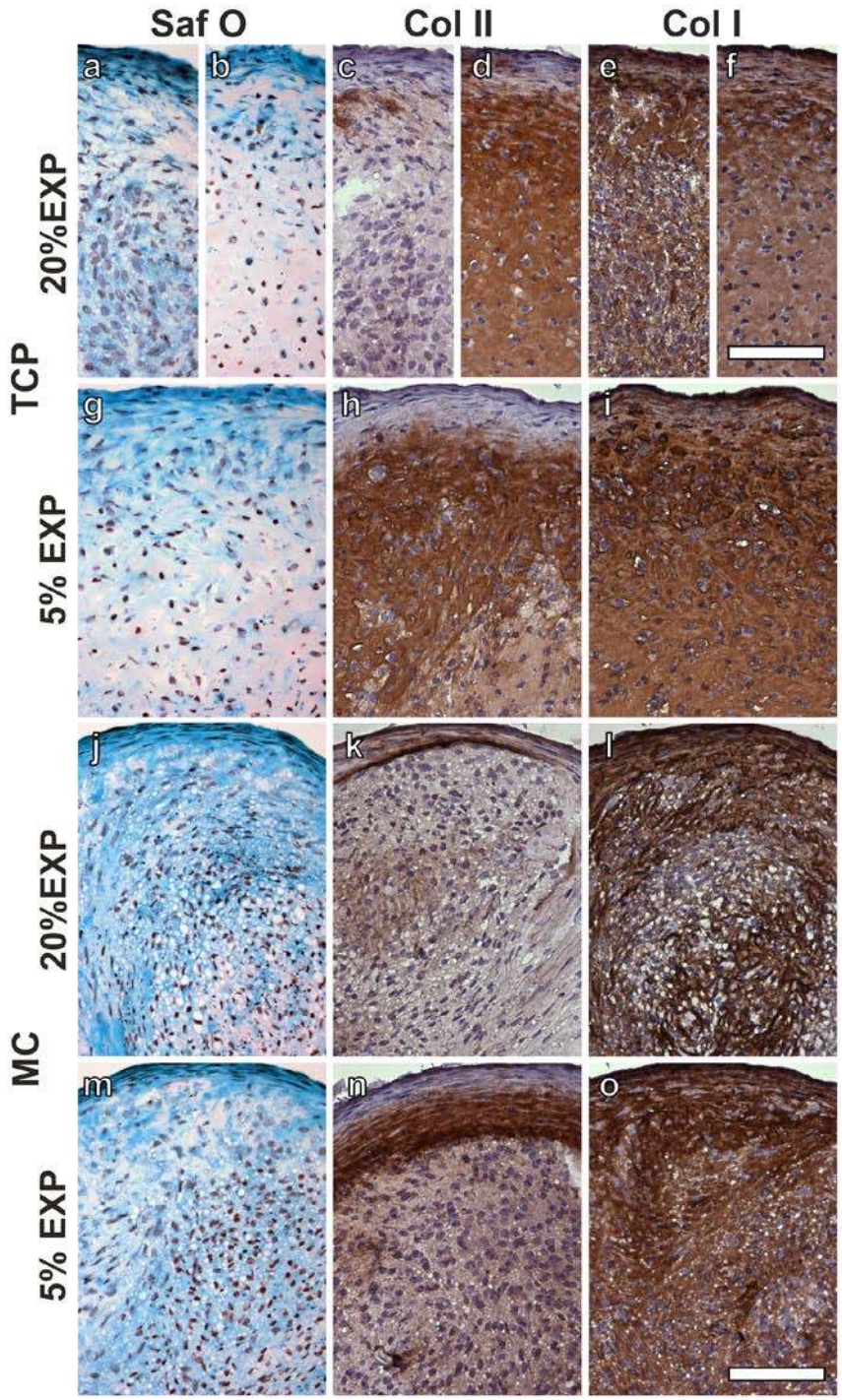

Fig. 6 Histology and immunohistochemistry on pellet cultures from human osteoarthritic chondrocytes. Following cultivation either (a-i) on tissue culture plastic (TCP) or (j-o) on microcarriers (MC) under (a-f,j-l) 20% (20% EXP) or (g-i,m-o) 5% (5% EXP) pO_2, respectively, cells ($2.5x10^5$ cells per pellet) were pelleted and cultured either under (a,c,e) 20% or (b,d,f,g-o) 5% pO_2 in serum-free chondrogenic media for 28 days. Sections were stained with (a,b,g,j,m) safranin O and Fast Green or probed with antibodies against (c,d,h,k,n) collagen type II or (e,f,i,l,o) type I and counterstained with haematoxylin. Positive staining appears red for GAG (safranin O), cyan for cell matrix (Fast Green), brown for collagens (DAB) and purple for nuclei (haematoxylin). Scale bars represent 100 µm.

Tables

Table 1: Oligonucleotide primers used for qRT-PCR

Gene symbol (primer source)	**Sequence (5'→3')**	**GenBank accession #**	**Amplicon position**
COL1A1 (Martin et al. 2001)	F: CAGCCGCTTCACCTACAGC R: TTTTGTATTCAATCACTGTCTTGCC	NM_000088	4335-4417
COL2A1 (Martin et al. 2001)	F: GGCAATAGCAGGTTCACGTACA R: CGATAACAGTCTTGCCCCACTT	NM_001844	4454-4532
COL10A1 (Daouti et al. 2005)	F: ACCCAACACCAAGACACAGTTCT R: TCTTACTGCTATACCTTTACTCTTTA TGGTGTA	NM_000493	201-264
ACAN (Primer-BLAST)	F: GCCTGCGCTCCAATGACT R: TAATGGAACACGATGCCTTTCA	NM_001135	739-844
VCAN (Primer-BLAST)	F: CTGGATGGTGATGTGTTCCAC R: CCTGGTTTTCACACTCTTTTGC	NM_004385	1061-1139
HIF1A (Primer-BLAST)	F: GGGTTGAAACTCAAGCAACTGTC R: GTGCTGAATAATACCACTCACAACG	NM_001530	1351-1448
EPAS1 (HIF2A) (Primer-BLAST)	F: CGCACAGAGTTCTTGGGAGC R: TGCAGACCTTGTCTTGAAGGTG	NM_001430	2460-2540
HIF3A (Li et al. 2006)	F: TGGAGCTGCTGGGAGTGAGA R: GGGCTCATTCAGGTTCAGGAGT	NM_152794	1794-1951
EGLN1 (PHD2) (Gelse et al. 2008)	F: TCAATGGCCGGACGAAAG R: CATTTGGATTATCAACATGACGTACA	NM_022051	4031-4111
18S rRNA (Primer-BLAST)	F: GATCCATTGGAGGGCAAGTCT R: CCAAGATCCAACTACGAGCTTTTT	NR_003286	589-691