Biochemical and Biophysical Research Communications 417 (2012) 305–310

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

# Oxygen tension regulates the osteogenic, chondrogenic and endochondral phenotype of bone marrow derived mesenchymal stem cells

Eamon J. Sheehy, Conor T. Buckley, Daniel J. Kelly *

*Trinity Centre for Bioengineering, School of Engineering, Trinity College Dublin, Dublin 2, Ireland*

ARTICLE INFO

*Article history:*
Received 8 November 2011
Available online 1 December 2011

*Keywords:*
Oxygen
MSC
Chondrogenesis
Osteogenesis
Hypertrophy
Endochondral

ABSTRACT

The local oxygen tension is a key regulator of the fate of mesenchymal stem cells (MSCs). The objective of this study was to investigate the effect of a low oxygen tension during expansion and differentiation on the proliferation kinetics as well as the subsequent osteogenic and chondrogenic potential of MSCs. We first hypothesised that expansion in a low oxygen tension (5% $pO_2$) would improve both the subsequent osteogenic and chondrogenic potential of MSCs compared to expansion in a normoxic environment (20% $pO_2$). Furthermore, we hypothesised that chondrogenic differentiation in a low oxygen environment would suppress hypertrophy of MSCs cultured in both pellets and hydrogels used in tissue engineering strategies. MSCs expanded at 5% $pO_2$ proliferated faster forming larger colonies, resulting in higher cell yields. Expansion at 5% $pO_2$ also enhanced subsequent osteogenesis of MSCs, whereas differentiation at 5% $pO_2$ was found to be a more potent promoter of chondrogenesis than expansion at 5% $pO_2$. Greater collagen accumulation, and more intense staining for collagen types I and X, was observed in pellets maintained at 20% $pO_2$ compared to 5% $pO_2$. Both pellets and hydrogels stained more intensely for type II collagen when undergoing chondrogenesis in a low oxygen environment. Differentiation at 5% $pO_2$ also appeared to inhibit hypertrophy in both pellets and hydrogels, as demonstrated by reduced collagen type X and Alizarin Red staining and alkaline phosphatase activity. This study demonstrates that the local oxygen environment can be manipulated *in vitro* to either stabilise a chondrogenic phenotype for use in cartilage repair therapies or to promote hypertrophy of cartilaginous grafts for endochondral bone repair strategies.



## 1. Introduction

Understanding how environmental factors regulate MSC fate during proliferation and differentiation is critical for developing new therapies for tissue repair. The local oxygen environment has been shown to be a key modulator of MSC phenotype [1]. Motivated by the fact that bone is vascularised and cartilage is not, oxygen tension has been proposed as a regulatory factor in determining osteogenic or chondrogenic differentiation [2]. In current tissue engineering or regenerative medicine strategies MSCs are typically expanded and differentiated in 'normoxic' conditions (20% $pO_2$). *In vivo*, however, bone marrow derived MSCs reside in stem cell niches of lower oxygen levels (1–7% $pO_2$) [3–5] and therefore the normoxic conditions applied during *in vitro* expansion and differentiation place MSCs in a non-physiological hyperoxygenated state [6]. Physiological oxygen tensions in bone have been shown to be between 5% and 12.5% $pO_2$ but can reduce to 1% $pO_2$ in a fracture haematoma [7–10], whereas physiological oxygen levels in articular cartilage are between 1% and 5% $pO_2$ [10]. Previous studies have demonstrated that expansion of bone marrow derived MSCs at 5% $pO_2$ enhances subsequent osteogenesis [11] and chondrogenesis [12]. Furthermore, differentiation at 5% $pO_2$ has been shown to enhance chondrogenesis of MSCs [13,14] and the functional properties of cartilaginous tissues engineered using infrapatellar fat pad [15] and bone marrow [16] derived MSCs.

A major challenge in cartilage tissue engineering using MSCs is the prevention of hypertrophy and terminal differentiation [17]. When implanted subcutaneously in nude mice chondrogenically primed bone marrow derived MSCs fail to produce stable cartilage resistant to vascularisation and calcification [18]. Recently, this obstacle in cartilage tissue engineering has been realised as a potential benefit in bone tissue engineering with chondrogenically primed bone marrow derived MSCs being used to produce bone *in vivo* via endochondral ossification [19,20]. Therefore regulation of the hypertrophic phenotype of MSCs is critical when attempting to engineer either cartilage or endochondral bone. The embryonic endochondral ossification process is characterised by blood vessels infiltrating cartilaginous matrix thus increasing the oxygen levels in the tissue [21] and a low oxygen tension has previously been demonstrated to suppress hypertrophy of adipose tissue derived MSCs [22] and of the mesenchymal stem cell line C3H101/2 [23].

* Corresponding author. Fax: +353 1 679 5554.
*E-mail address:* kellyd9@tcd.ie (D.J. Kelly).

0006-291X/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2011.11.105

Clearly oxygen tension is a key regulator of stem cell fate, playing a key role not only in determining the initial differentiation pathway of the cell, but possibly also its terminal phenotype. In spite of the growing body of work demonstrating the importance of this cue in regulating stem cell differentiation, it is still unclear if the local oxygen tension regulates plasticity during stem cell expansion and whether specific oxygen tensions preferentially support certain phenotypes during differentiation. The objective of this study is to explore the role of oxygen tension during both expansion and differentiation on the proliferation kinetics and the subsequent osteogenic and chondrogenic potential of bone marrow derived MSCs. It is first hypothesised that expanding MSCs in a physiological or low oxygen tension (5% $pO_2$) would improve their subsequent potential to differentiate along either the osteogenic or chondrogenic route compared to expansion in normoxic conditions (20% $pO_2$). It is further hypothesised that chondrogenic differentiation in a low oxygen environment would suppress hypertrophy of MSCs. As part of this study, MSCs will be directed along the chondrogenic pathway in both traditional pellet culture system and in agarose hydrogels to investigate if the extracellular environment influences the response of MSCs to different external oxygen tensions. If the local oxygen environment can be manipulated to both enhance chondrogenesis and ultimately suppress hypertrophy within the MSC seeded scaffolds or hydrogels, it will facilitate engineering functional cartilaginous grafts for clinical applications.

## 2. Methods

### 2.1. Cell isolation and expansion

Bone marrow derived MSCs were isolated aseptically from the femoral shafts of four pigs and expanded according to a modified method developed for human MSCs [24] in DMEM GlutaMAX supplemented with 10% v/v foetal bovine serum (FBS) and 100 U/ml penicillin–100 μg/ml streptomycin (expansion medium) (all Gibco, Biosciences, Dublin, Ireland) at either 20% or 5% $pO_2$. The media was replaced twice weekly. Following colony formation MSCs were trypsinised, counted, seeded at a density of $5 \times 10^3$ cells/cm$^2$ and expanded to passage two (P2) at either 20% or 5% $pO_2$. Cells from each donor were kept separate for all experiments.

### 2.2. CFU-f assay and proliferation kinetics

Freshly isolated porcine MSCs were seeded in 100 mm diameter petri dishes at a density of $50 \times 10^3$ mononuclear cells (MNCs)/cm$^2$ and maintained in expansion medium at either 20% or 5% $pO_2$. After 10 days expanded cells were fixed with 2% paraformaldehyde and stained with crystal violet. The number of colonies from each dish were counted in order to calculate the colony forming unit efficiency. In addition the diameter of the 10 largest colonies from each dish was calculated using ImageJ software (Rasband, W.S., Image J, US National Institutes of Health, Bethesda, USA, http://www.imagej.nih.gov/ij/, 1997–2011).

To examine proliferation kinetics, freshly isolated MSCs were seeded in T-25 flasks at a density of $50 \times 10^3$ MNCs/cm$^2$ and expanded at either 20% or 5% $pO_2$. At days 7, 9, 12 and 14 of expansion, cells were trypsinised and counted using a hemacytometer and 0.4% trypan blue. After colony formation (P0), cells were replated at a density of $5 \times 10^3$ MSCs/cm$^2$, expanded at either 20% or 5% $pO_2$ and counted at days 2, 4 and 7 of expansion.

### 2.3. Osteogenesis

Passage 2 BM-MSCs were seeded in six well plates at a density of $3 \times 10^3$ MSCs/cm$^2$ and maintained in expansion medium at either 20% or 5% $pO_2$. Cells were allowed to adhere for 24 h, after which they were supplemented with β-glycerophosphate (20 μg/ml), dexamethasone (100 nM) and L-ascorbic acid-2-phosphate. Negative controls were also maintained in parallel. The culture medium was replaced twice a week for a period of 14 days.

### 2.4. Chondrogenesis in pellets

MSCs ($250 \times 10^3$, P2) were pelleted by centrifugation at 650 g. Pellets were maintained in a chondrogenic medium consisting of hgDMEM GlutaMax supplemented with penicillin (100 U/ml)–streptomycin (100 μg/ml) (both Gibco), 100 μg/ml sodium pyruvate, 40 μg/ml L-proline, 50 μg/ml L-ascorbic acid-2-phosphate, 1.5 mg/ml bovine serum albumin, 1 × insulin–transferrin–selenium, 100 nM dexamethasone, 2.5 μg/ml amphotericin B (all from Sigma–Aldrich, Dublin, Ireland) and 10 ng/ml recombinant human transforming growth factor-β3 (Prospec-Tany TechnoGene Ltd., Israel) at either 20% or 5% $pO_2$ for a period of 21 days. The culture medium was replaced twice weekly with discarded media stored at −80 °C for further analysis.

### 2.5. Chondrogenesis in agarose hydrogels

MSCs (P2) were suspended in 2% agarose (type VII; Sigma–Aldrich) at a density of $15 \times 10^6$ MSCs/ml. The agarose cell suspension was cast in a stainless steel mould and cored using a biopsy punch to produce construct cylinders (Ø5 × 3 mm). Constructs were maintained in chondrogenic medium at either 20% or 5% $pO_2$ for a period of 42 days. The culture medium was replaced twice weekly with discarded media stored at −80 °C for further analysis.

### 2.6. Biochemical analysis

Deposited matrix from osteogenically treated wells was digested in 1 M hydrochloric acid at 60 °C and 10 rpm for 18 h. The calcium content was determined using a Sentinel Calcium kit (Alpha Laboratories Ltd., UK). Pellets and agarose constructs were digested with papain (125 μg/ml) in 0.1 M sodium acetate, 5 mM L-cysteine–HCl, 0.05 M EDTA, pH 6.0 (all from Sigma–Aldrich) at 60 °C and 10 rpm for 18 h. DNA content was quantified using the Hoechst Bisbenzimide 33258 dye assay, with a calf thymus DNA standard. Sulphated glycosaminoglycan (sGAG) content was quantified using the dimethylmethylene blue dye-binding assay (Blyscan, Biocolor Ltd., Northern Ireland), with a chondroitin sulphate standard. Total collagen content was determined by measuring the hydroxyproline content, using a hydroxyproline-to-collagen ratio of 1:7.69 [25,26]. Alkaline phosphatase (ALP) activity in the media was measured using a Sensolyte pNPP Alkaline Phosphatase assay kit (Cambridge Biosciences, UK) with a calf intestine ALP standard.

### 2.7. Histology and immunohistochemistry

At the final time point of experiments, samples were fixed in 4% paraformaldehyde overnight, dehydrated in a graded series of ethanols, embedded in paraffin wax, sectioned at 5 μm and affixed to microscope slides. The sections were stained with 1% Alcian Blue 8GX in 0.1 M HCl to assess sGAG content, Picro-Sirius Red to assess collagen distribution and 1% Alizarin Red to assess calcium distribution (all Sigma–Aldrich). Collagen types I, II and X were evaluated using a standard immunohistochemistry technique as previously described [27]. Positive and negative controls were included in the immunohistochemistry staining for each batch.

### 2.8. Statistical analysis

All statistical analyses were carried out using Minitab 15.1. Results are reported as mean ± standard deviation. Groups were analysed by a general linear model for analysis of variance with groups of factors. Tukey's test was used to compare conditions. Significance was accepted at a level of $p < 0.05$.

## 3. Results

### 3.1. Oxygen tension regulates the proliferation kinetics of MSCs

No statistical differences were found in the CFU-f efficiency of cells expanded at 5% or 20% $pO_2$, see Fig. 1A and B. However, expansion at 5% $pO_2$ significantly increased the colony diameter compared to expansion at 20% $pO_2$ (4.69 ± 0.59 vs. 3.3 ± 0.46 mm; $p < 0.001$), see Fig. 1A and C. Similar results were obtained in CFU-f assays replicated using MSCs from a second donor (data not shown). Expansion at 5% $pO_2$ also significantly increased the cell yield obtained at days 7, 9 and 14, culminating in a total cell yield at day 14 of 2.05 ± 0.62 ($\times 10^6$) compared to 0.86 ± 0.36 ($\times 10^6$) cells for the 20% $pO_2$ expansion group; $p < 0.05$, see Fig. 1D. When cells were replated after the first passage no significant differences were found in cell yields between different oxygen tensions, Fig. 1E.

### 3.2. Expansion in a low oxygen environment enhances the osteogenic potential of MSCs

For MSCs expanded at 20% $pO_2$ calcium accumulation was higher for cells subsequently differentiated at 5% $pO_2$ compared to 20% $pO_2$, see Fig. 2A. Calcium accumulation was further increased if MSCs were both expanded and differentiated at 5% $pO_2$. Of all groups, MSCs expanded and differentiated at 20% $pO_2$ accumulated the least amount of calcium and stained weakest with Alizarin Red, see Fig. 2B.

### 3.3. Differentiation in a low oxygen environment enhances glycosaminoglycan synthesis during chondrogenesis in pellets and hydrogels

For MSCs expanded at either 5% $pO_2$ or 20% $pO_2$, subsequent differentiation at 5% $pO_2$ significantly enhanced sGAG accumulation in both pellets (Fig. 3A) and hydrogels (Fig. 3C). In pellet culture, collagen accumulation following differentiation at 20% $pO_2$ was significantly higher than differentiation at 5% $pO_2$, see Fig. 3B. Expansion at a low oxygen tension had no significant effect on the biochemical content of pellets. Pellets differentiated at 5% $pO_2$ appeared larger and stained strongly for Alcian Blue, whereas differentiation at 20% $pO_2$ resulted in smaller pellets that stained strongly for Picro-Sirius Red, see Fig. 3E.

By day 42 in hydrogel culture, collagen accumulation following differentiation at 5% $pO_2$ was significantly higher than differentiation at 20% $pO_2$, see Fig. 3D. Expansion at a low oxygen tension had no significant effect on collagen accumulation, with the exception of day 42, where expansion at 5% $pO_2$ significantly increased collagen accumulation of constructs subsequently differentiated at 20% $pO_2$. Constructs differentiated at 5% $pO_2$ demonstrated increased Alcian Blue and Picro-Sirius Red staining compared to constructs differentiated at 20% $pO_2$, see Fig. 3F. Differentiation at 5% $pO_2$ also resulted in a more homogenous sGAG distribution.

### 3.4. Oxygen tension regulates the hypertrophic phenotype of chondrogenically primed MSCs

MSCs undergoing chondrogenic differentiation in pellets maintained at 5% $pO_2$ demonstrated reduced Alizarin Red, collagens I and X staining compared to maintenance at 20% $pO_2$, see Fig. 4A. Increased collagen II staining was observed in pellets maintained at 5% $pO_2$. MSC-seeded agarose hydrogels maintained at 20% $pO_2$ stained homogenously for Alizarin Red (calcium), while staining was confined to the periphery of constructs maintained at 5%

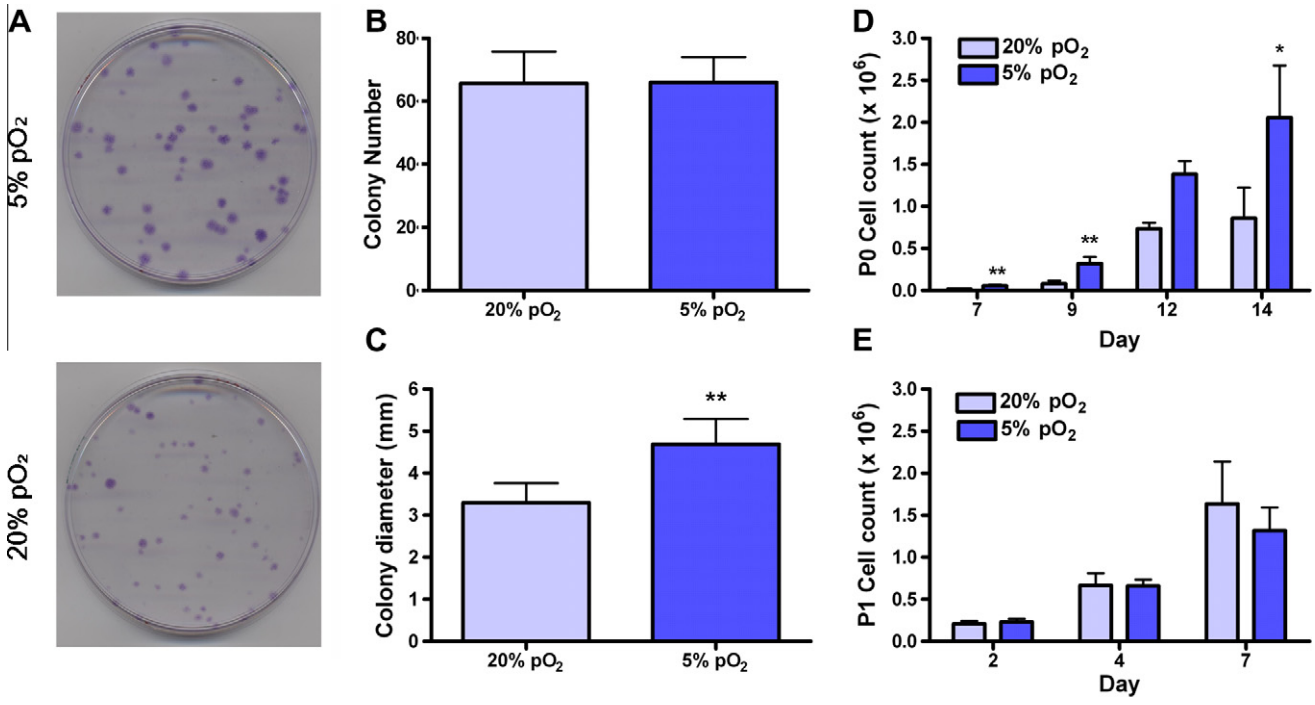


**Fig. 1.** Oxygen tension regulates the proliferation of MSCs. (A) CFU-f image of MSCs expanded at 20% or 5% $pO_2$ ($n = 3$). (B) Colony number of MSCs expanded at 20% or 5% $pO_2$. (C) Colony diameter of MSCs expanded at 20% or 5% $pO_2$. (D) Cell count of MSCs expanded at 20% or 5% $pO_2$ at P0 ($n = 3$–4). (E) Cell count of MSCs expanded at 20% or 5% $pO_2$ at P1 ($n = 3$–4). $^*p < 0.05$, $^{**}p < 0.001$, vs. expansion at 20% $pO_2$.

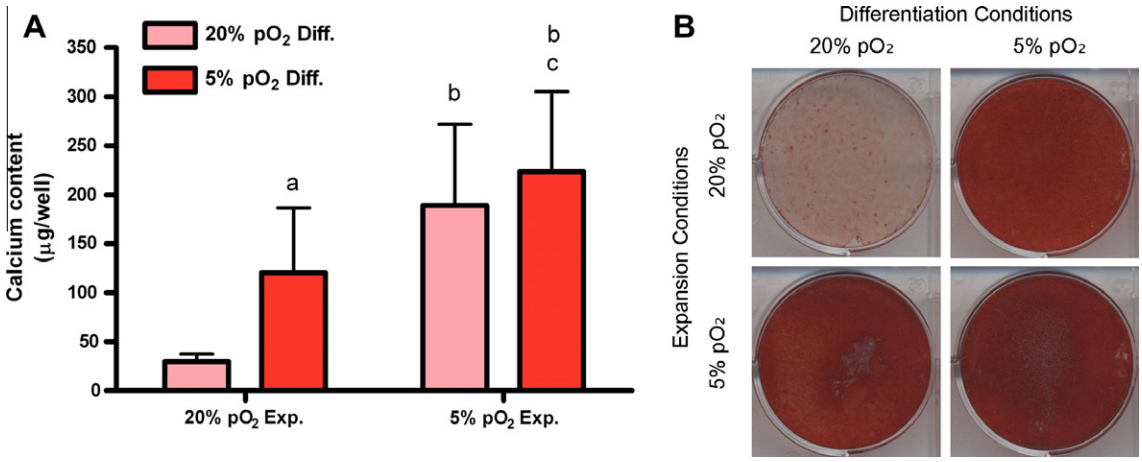


**Fig. 2.** Expansion in a low oxygen environment enhances the osteogenic potential of MSCs. (A) Calcium accumulation of MSCs expanded (Exp.) at 20% or 5% $pO_2$ and subsequently differentiated (Diff.) at 20% or 5% $pO_2$ (two donors, $n = 3$ per donor). (B) Alizarin Red staining. $p < 0.05$, (a) vs. opposite differentiation condition, (b) vs. opposite expansion condition, (c) vs. opposite differentiation and expansion condition.

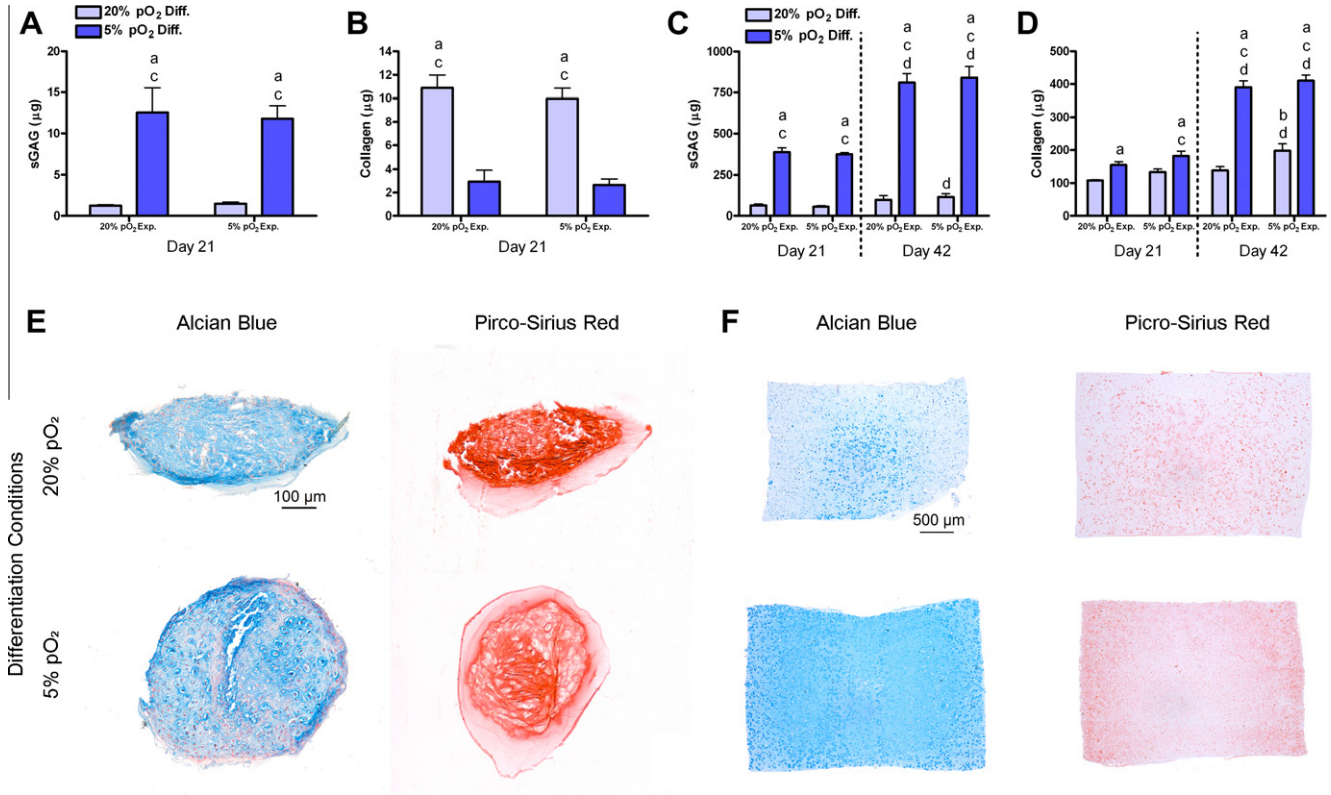


**Fig. 3.** Oxygen tension differentially regulates collagen synthesis of MSCs during chondrogenesis in pellets and hydrogels. (A–D) sGAG and collagen accumulation in pellets (A and B) and hydrogels (C and D) for MSCs expanded (Exp.) at 20% or 5% $pO_2$ and subsequently differentiated (Diff.) at 20% or 5% $pO_2$. (E and F) Alcian Blue and Picro-Sirius Red staining for MSC pellets (E) and hydrogels (F) differentiated at 20% or 5% $pO_2$ following expansion at 5% $pO_2$. $n = 3$–4 for biochemical analysis, $n = 1$–2 for histology. $p < 0.05$, (a) vs. opposite differentiation condition, (b) vs. opposite expansion condition, (c) vs. opposite differentiation and expansion condition, (d) vs. day 21.

$pO_2$, see Fig. 4B. Collagen types I and X staining was generally pericellular at both oxygen tensions, but appeared to co-localise to regions of greater Alizarin Red staining (i.e. confined to the periphery of constructs maintained at 5% $pO_2$). Hydrogels maintained at 5% $pO_2$ also demonstrated increased collagen II staining.

When MSCs were expanded at 20% $pO_2$, alkaline phosphatase activity in the media during the first 3 weeks of culture was significantly higher for pellets (300.07 ± 16.1 vs. 65.68 ± 7.25 ng; $p < 0.001$) and cell seeded hydrogels (817.48 ± 14.35 vs. 683.77 ± 18.81 ng; $p < 0.01$) maintained at 20% $pO_2$ compared to 5% $pO_2$. Similar results were obtained when MSCs were expanded at 5% $pO_2$ (data not shown).

## 4. Discussion

This study demonstrates that the local oxygen tension plays a key role in regulating not only the proliferation kinetics of bone marrow derived MSCs, but also their subsequent osteogenic and chondrogenic potential and the inherent tendency of chondrogeni-

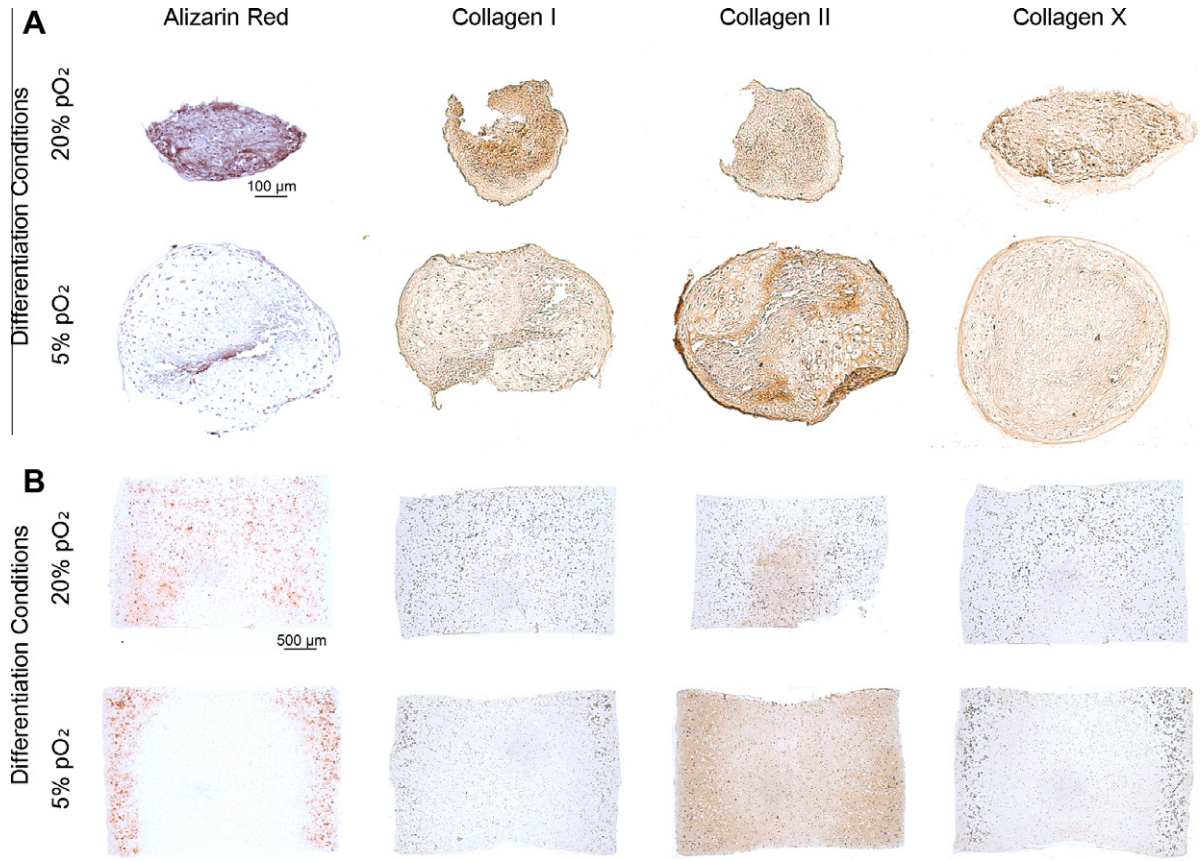


**Fig. 4.** Oxygen tension regulates the hypertrophic phenotype of chondrogenically primed MSCs. Alizarin Red and Collagen types I, II and X staining for MSC pellets (A) and hydrogels (B) differentiated at 20% or 5% $pO_2$ following expansion at 5% $pO_2$.

cally primed MSCs to proceed towards terminal differentiation. Expansion at a low oxygen tension was found to accelerate proliferation of freshly isolated MSCs, as demonstrated by the formation of larger colonies and the attainment of higher cell yields. Expansion at a low oxygen tension also enhanced the osteogenic capacity of MSCs, with cells expanded at 5% $pO_2$ accumulating significantly more calcium compared to cells expanded at 20% $pO_2$ during osteogenesis. Differentiation of MSCs at a low oxygen tension was found to be a more potent regulator of chondrogenesis than expansion at a low oxygen tension. Pellets and cell seeded hydrogels maintained at 5% $pO_2$ accumulated significantly more sGAG, although the influence of oxygen tension on total collagen synthesis was dependant on whether MSCs were maintained in pellets or hydrogels. Differentiation at 5% $pO_2$ suppressed markers of fibroblastic (collagen type I staining) and hypertrophic differentiation (collagen type X staining, ALP activity), reduced calcium accumulation (Alizarin Red staining) in both pellets and hydrogels and supported a more stable cartilaginous phenotype (collagen type II staining).

There are a number of contrasting reports in the literature on the effect of oxygen tension on osteogenesis of MSCs, which can possibly be explained, at least in part, by the varying species, cell types and oxygen levels used in different studies. The results of this study agree with the findings of Lennon et al. [11] that a low oxygen tension (5% $pO_2$) during expansion and differentiation enhances osteogenesis of bone marrow derived MSCs. In previous studies demonstrating that differentiation in a low oxygen environment inhibits osteogenesis of bone marrow derived MSCs [5,28], a lower oxygen tension of 3% $pO_2$ has been used. It has also previously been demonstrated that inhibition of osteogenic differentiation of MSCs occurs at 2% $pO_2$ unless cells are first pre-conditioned to this oxygen level during the expansion phase [29]. Since physiological oxygen levels in bone have been shown to be between 5% and 12.5% $pO_2$, a lower oxygen tension may be unable to facilitate direct osteoblastic differentiation. The hypoxic conditions experienced by MSCs during embryonic long bone development and regenerative events such as fracture repair would appear to promote the formation of bone via endochondral rather that direct intramembranous ossification. The fact that 20% $pO_2$ is also outside the physiological oxygen levels in bone may also explain the reduced osteogenesis at this oxygen tension observed in our study. Of the other studies which have demonstrated reduced osteogenic differentiation at 5% $pO_2$, adipose tissue derived MSCs [2] and C3HT101/2 cells [23] have been used, suggesting that the local oxygen tension has differential effects on MSCs from different sources.

Previous studies have also shown that maintenance in a low oxygen environment enhances chondrogenesis of bone marrow derived MSCs [13,16], infra-patellar fat pad derived MSCs [15] and adipose tissue derived MSCs [22]. We observed that oxygen tension differentially regulated total collagen synthesis in pellets and hydrogels, but that a low oxygen tension supported increases in type II collagen in both culture systems. This differential effect of an altered oxygen tension on total collagen synthesis may be due to the specific cell morphologies that develop in the two culture environments. MSCs encapsulated in agarose hydrogels, which are known to support a chondrogenic phenotype, demonstrated a spherical morphology at both 20% and 5% $pO_2$. When MSCs were maintained in pellet culture, cells differentiated at 5% $pO_2$ appeared to be surrounded by a more spherical chondron (see Supplementary Fig. S1), while a less spherical, more elongated, cellular morphology was observed in pellets maintained at 20% $pO_2$, suggesting that higher oxygen tensions support a more fibro-

blastic phenotype synthesising higher levels of type I collagen. The mechanism by which a lower oxygen tension ultimately leads to changes in cell shape is presently unclear.

The process of *in vivo* endochondral ossification is characterised initially by MSC condensation and chondrogenic differentiation at a low oxygen tension, followed by hypertrophic differentiation, vascularisation and mineralisation at a higher oxygen tension [21]. In this study we demonstrated that differentiation in a low oxygen environment suppressed ALP activity and collagen X synthesis (both markers of hypertrophy) in pellets and hydrogels. Suppressing hypertrophy is a critical step in the successful development of stem cell based therapies for cartilage repair as maintenance of a chondrogenic phenotype must be achieved in order to ensure long-term *in vivo* stability [18]. Further studies are required to better understand the molecular mechanisms by which a low oxygen microenvironment suppresses terminal differentiation of chondrogenically primed MSCs.

Bone marrow derived MSCs reside in stem cell niches of low oxygen and it has been previously demonstrated that *in vitro* expansion at a low oxygen tension helps maintain their stemness [5,6] and improve their subsequent differentiation potential [11,12]. Here we have demonstrated that through manipulation of the *in vitro* oxygen environment of MSCs it is possible to promote a more stable chondrogenic phenotype for use in cartilage tissue engineering applications, or alternatively to promote a hypertrophic phenotype as a means to repair bone via endochondral ossification [17]. Future studies will explore if the hypertrophic phenotype of cartilaginous templates can be manipulated *in vitro* by controlling the local oxygen tension and then used as engineered constructs *in vivo* for endochondral bone repair.

## Acknowledgments

Funding was provided by Science Foundation Ireland (President of Ireland Young Researcher Award: 08/YI5/B1336) and the European Research Council (StemRepair – Project number 258463).

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.11.105.

## References

[1] A. Mohyeldin, T. Garzón-Muvdi, A. Quiñones-Hinojosa, Oxygen in stem cell biology: a critical component of the stem cell niche, Cell Stem Cell 7 (2010) 150–161.
[2] C. Merceron, C. Vinatier, S. Portron, M. Masson, J. Amiaud, L. Guigand, Y. Chérel, P. Weiss, J. Guicheux, Differential effects of hypoxia on osteochondrogenic potential of human adipose-derived stem cells, American Journal of Physiology – Cell Physiology 298 (2010) C355–C364.
[3] J.L. Grant, B. Smith, Bone marrow gas tensions, bone marrow blood flow, and erythropoiesis in man, Annals of internal medicine 58 (1963) 801–809.
[4] H. Kofoed, E. Sjontoft, S.O. Siemssen, H.P. Olesen, Bone marrow circulation after osteotomy. Blood flow, $pO_2$, $pCO_2$, and pressure studied in dogs, Acta Orthopaedica Scandinavica 56 (1985) 400–403.
[5] G. D'Ippolito, S. Diabira, G.A. Howard, B.A. Roos, P.C. Schiller, Low oxygen tension inhibits osteogenic differentiation and enhances stemness of human MIAMI cells, Bone 39 (2006) 513–522.
[6] W.L. Grayson, F. Zhao, R. Izadpanah, B. Bunnell, T. Ma, Effects of hypoxia on human mesenchymal stem cell expansion and plasticity in 3D constructs, Journal of Cellular Physiology 207 (2006) 331–339.
[7] C.T. Brighton, A.G. Krebs, Oxygen tension of healing fractures in the rabbit, Journal of Bone and Joint Surgery – Series A 54 (1972) 323–332.
[8] R.B. Heppenstall, G. Grislis, T.K. Hunt, Tissue gas tensions and oxygen consumption in healing bone defects, Clinical Orthopedics Number 106 (1975) 357–365.
[9] E. Potier, E. Ferreira, R. Andriamanalijaona, J.P. Pujol, K. Oudina, D. Logeart-Avramoglou, H. Petite, Hypoxia affects mesenchymal stromal cell osteogenic differentiation and angiogenic factor expression, Bone 40 (2007) 1078–1087.
[10] J.E. Lafont, Lack of oxygen in articular cartilage: consequences for chondrocyte biology, International Journal of Experimental Pathology 91 (2010) 99–106.
[11] D.P. Lennon, J.M. Edmison, A.I. Caplan, Cultivation of rat marrow-derived mesenchymal stem cells in reduced oxygen tension: effects on in vitro and in vivo osteochondrogenesis, Journal of Cellular Physiology 187 (2001) 345–355.
[12] M. Zscharnack, C. Poesel, J. Galle, A. Bader, Low oxygen expansion improves subsequent chondrogenesis of ovine bone-marrow-derived mesenchymal stem cells in collagen type I hydrogel, Cells Tissues Organs 190 (2009) 81–93.
[13] M. Kanichai, D. Ferguson, P.J. Prendergast, V.A. Campbell, Hypoxia promotes chondrogenesis in rat mesenchymal stem cells: a role for AKT and hypoxia-inducible factor (HIF)-1α, Journal of Cellular Physiology 216 (2008) 708–715.
[14] J.C. Robins, N. Akeno, A. Mukherjee, R.R. Dalal, B.J. Aronow, P. Koopman, T.L. Clemens, Hypoxia induces chondrocyte-specific gene expression in mesenchymal cells in association with transcriptional activation of Sox9, Bone 37 (2005) 313–322.
[15] C.T. Buckley, T. Vinardell, D.J. Kelly, Oxygen tension differentially regulates the functional properties of cartilaginous tissues engineered from infrapatellar fat pad derived MSCs and articular chondrocytes, Osteoarthritis and Cartilage 18 (2010) 1345–1354.
[16] E.G. Meyer, C.T. Buckley, S.D. Thorpe, D.J. Kelly, Low oxygen tension is a more potent promoter of chondrogenic differentiation than dynamic compression, Journal of Biomechanics 43 (2010) 2516–2523.
[17] D. Gawlitta, E. Farrell, J. Malda, L.B. Creemers, J. Alblas, W.J.A. Dhert, Modulating endochondral ossification of multipotent stromal cells for bone regeneration, Tissue Engineering – Part B: Reviews 16 (2010) 385–395.
[18] K. Pelttari, A. Winter, E. Steck, K. Goetzke, T. Hennig, B.G. Ochs, T. Aigner, W. Richter, Premature induction of hypertrophy during in vitro chondrogenesis of human mesenchymal stem cells correlates with calcification and vascular invasion after ectopic transplantation in SCID mice, Arthritis and Rheumatism 54 (2006) 3254–3266.
[19] E. Farrell, S.K. Both, K.I. Odörfer, W. Koevoet, N. Kops, F.J. O'Brien, R.J.B. De Jong, J.A. Verhaar, V. Cuijpers, J. Jansen, R.G. Erben, G.J.V.M. Van Osch, In-vivo generation of bone via endochondral ossification by in-vitro chondrogenic priming of adult human and rat mesenchymal stem cells, BMC Musculoskeletal Disorders 12 (2011) 1471–2474.
[20] C. Scotti, B. Tonnarelli, A. Papadimitropoulos, A. Scherberich, S. Schaeren, A. Schauerte, J. Lopez-Rios, R. Zeller, A. Barbero, I. Martin, Recapitulation of endochondral bone formation using human adult mesenchymal stem cells as a paradigm for developmental engineering, Proceedings of the National Academy of Sciences of the United States of America 107 (2010) 7251–7256.
[21] T. Ma, W.L. Grayson, M. Fröhlich, G. Vunjak-Novakovic, Hypoxia and stem cell-based engineering of mesenchymal tissues, Biotechnology Progress 25 (2009) 32–42.
[22] M.C. Ronzière, E. Perrier, F. Mallein-Gerin, A.M. Freyria, Chondrogenic potential of bone marrow- and adipose tissue-derived adult human mesenchymal stem cells, Bio-Medical Materials and Engineering 20 (2010) 145–158.
[23] M. Hirao, N. Tamai, N. Tsumaki, H. Yoshikawa, A. Myoui, Oxygen tension regulates chondrocyte differentiation and function during endochondral ossification, Journal of Biological Chemistry 281 (2006) 31079–31092.
[24] D.P. Lennon, A.I. Caplan, Isolation of human marrow-derived mesenchymal stem cells, Experimental Hematology 34 (2006) 1604–1605.
[25] W. Kafienah, T.J. Sims, Biochemical methods for the analysis of tissue-engineered cartilage, Methods in Molecular Biology 238 (2004) 217–230.
[26] N.Y. Ignat'eva, N.A. Danilov, S.V. Averkiev, M.V. Obrezkova, V.V. Lunin, E.N. Sobol, Determination of hydroxyproline in tissues and the evaluation of the collagen content of the tissues, Journal of Analytical Chemistry 62 (2007) 51–57.
[27] T. Vinardell, C.T. Buckley, S.D. Thorpe, D.J. Kelly, Composition-function relations of cartilaginous tissues engineered from chondrocytes and mesenchymal stem cells isolated from bone marrow and infrapatellar fat pad, Journal of Tissue Engineering and Regeneration Medicine 5 (2011) 673–683.
[28] C. Fehrer, R. Brunauer, G. Laschober, H. Unterluggauer, S. Reitinger, F. Kloss, C. Gülly, R. Gaßner, G. Lepperdinger, Reduced oxygen tension attenuates differentiation capacity of human mesenchymal stem cells and prolongs their lifespan, Aging Cell 6 (2007) 745–757.
[29] E. Volkmer, B.C. Kallukalam, J. Maertz, S. Otto, I. Drosse, H. Polzer, W. Bocker, M. Stengele, D. Docheva, W. Mutschler, M. Schieker, Hypoxic preconditioning of human mesenchymal stem cells overcomes hypoxia-induced inhibition of osteogenic differentiation, Tissue Engineering – Part A 16 (2010) 153–164.