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Journal of Cellular Physiology

Hypoxia Promotes Chondrogenesis in Rat Mesenchymal Stem Cells: A Role for AKT and Hypoxia-Inducible Factor (HIF)-1α

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Mesenchymal stem cells (MSCs) are multipotent cells capable of developing along the chondrogenic, osteogenic and adipogenic lineages. As such, they have received interest as a potential cell source for tissue engineering strategies. Cartilage is an avascular tissue and thus resides in a microenvironment with reduced oxygen tension. The aim of this study was to examine the effect of a low oxygen environment on MSC differentiation along the chondrogenic route. In MSCs exposed to chondrogenic growth factors, transforming growth factor-β and dexamethasone, in a hypoxic environment (2% oxygen), the induction of collagen II expression and proteoglygan deposition was significantly greater than that observed when cells were exposed to the chondrogenic growth factors under normoxic (20% oxygen) conditions. The transcription factor, hypoxia-inducible factor-1α (HIF-1α), is a crucial mediator of the cellular response to hypoxia. Following exposure of MSCs to hypoxia (2% oxygen), HIF-1α translocated from the cytosol to the nucleus and bound to its target DNA consensus sequence. Similarly, hypoxia evoked an increase in phosphorylation of both AKT and p38 mitogen activated protein kinase, upstream of HIF-1α activation. Furthermore, the PI3 kinase/AKT inhibitor, LY294002, and p38 inhibitor, SB 203580, prevented the hypoxia-mediated stabilisation of HIF-1α. To assess the role of HIF-1α in the hypoxia-induced increase in chondrogenesis, we employed an siRNA knockdown approach. In cells exposed to HIF-1α siRNA, the hypoxia-induced enhancement of chondrogenesis, as evidenced by upregulation of collagen II, sox-9 and proteoglycan deposition, was absent. This provides evidence for HIF-1α being a key mediator of the beneficial effect of a low oxygen environment on chondrogenesis.

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Adult mesenchymal stem cells (MSCs) are multipotent cells present in the bone marrow which, under appropriate conditions, can differentiate into mesenchyme-derived cell types such as chondrocytes, osteoblasts and adipocytes (Pittenger et al., 1999). The manipulation of these cells in vitro has received significant attention since this represents a source of cells for developing novel tissue engineered constructs for use in the treatment of musculoskeletal defects. MSCs have been isolated from a number of species and have been shown to retain their osteo-, chondro- and adipogenic potential in vitro (Kadiyala et al., 1997; Pittenger et al., 1999; Da Silva Meirelles and Nardi, 2003). We, and others, have previously demonstrated that the chondrogenic differentiation of these cells may be induced by specific cytokines and growth factors (Jaiswal et al., 2000) biophysical stimulation (Chen et al., 2003) and provision of a suitable 3-dimensional (3-D) environment (Farrell et al., 2006).

Cartilage tissue has a poor intrinsic repair capability due, in part, to its avascular nature. Tissue engineering of cartilage has potential to treat cartilage-related diseases such as arthritis (Guilak et al., 2004) and it is considered to offer advantages over autologous chondrocyte transplantation which is hampered by high donor site morbidity (Brittberg et al., 2001). The proclivity of adult MSCs to differentiate into cartilage lends support for their use in cartilage repair, however, the factors that control optimal MSC chondrogenesis remain to be fully resolved. In vivo, cartilage resides in a low oxygen microenvironment and is exposed to hypoxic conditions of as low as 1% oxygen (Haselgrove et al., 1993). Most in vitro tissue engineering strategies are performed within a 20% oxygen environment, but it is becoming clear that changes in ambient oxygen levels has a profound influence on osteogenesis (Lennon et al., 2001; Salim et al., 2004). While rat marrow-derived MSCs exhibit enhanced osteogenesis when maintained in 5% oxygen (Lennon et al., 2001), adipose-derived MSCs display reduced osteo- and chondrogenesis following exposure to hypoxia (Malladi et al., 2006).

The ability of cells to sense and respond to changes in oxygen tension is vital for many physiological events (Brahimi-Horn and Pouysségur, 2007). Hypoxia-inducible factor (HIF-1) is a key transcriptional regulator of cellular responses to low ambient oxygen levels. HIF-1 is an oxygen-sensitive, dimeric complex composed of HIF-1α and HIF-β subunits. During normoxia HIF-1α resides in the cytosol but is rapidly degraded by the ubiquitin-proteosome pathway. A reduced oxygen environment stabilises HIF-1α allowing HIF-1α to subsequently translocate to the nucleus and, in concert with HIF-1β, initiate the transcription of hypoxia-related genes such as vascular endothelial growth factor (VEGF; Semenza, 2001). In addition

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to hypoxia, certain growth factors and pro-inflammatory cytokines, as well as ultrasound, are potent regulators of HIF-1α stabilisation, translocation and activation (Hellwig-Burgel et al., 1999; Haddad and Land, 2001; Tang et al., 2007). Several protein kinases, including c-jun N-terminal kinase (Comerford et al., 2004) and p38 mitogen-activated protein kinase (Xu et al., 2004) have been implicated as regulators of HIF-1α stabilisation. Furthermore, in fibroblasts the PI-3 kinase (PI3K)/AKT pathway has been demonstrated to be pertinent in the regulation of HIF-1α accumulation and activation (Zhang et al., 2006), and in osteoblasts AKT phosphorylation is stimulated by ultrasound leading to downstream HIF-1α activation (Tang et al., 2007). Consequences of HIF-1α activation include induction of transcriptional targets such as VEGF and erythropoietin (Sharp and Bernaudin, 2004). The chondrogenic transcription factor, SOX-9, is also under transcriptional control by HIF-1α and the elegant studies by Robins et al. (2005) have demonstrated that a hypoxic challenge stimulates chondrogenesis in a HIF-1-dependent manner in mouse ST2 stromal cells.

In this study we sought to examine the influence of hypoxia on the chondrogenic differentiation of adult rat MSCs. The results demonstrate that the chondrogenesis evoked by chondrogenic growth factors is further enhanced by hypoxia (2% oxygen). Hypoxia also increased Akt phosphorylation and this was associated with a downstream increase in nuclear translocation and transactivation of HIF-1α. Furthermore, the hypoxia-induced enhancement of chondrogenesis was abolished by siRNA-mediated knockdown of HIF-1α. Thus, in rat MSCs, oxygen tension and engagement of the AKT/HIF-1α pathway is a critical determinant of chondrogenic differentiation.

Materials and Methods

Culture of rat MSCs

Three-month old Wistar rats (250–350 g) were obtained from the Bioresources Unit, Trinity College Dublin. Animals were sacrificed by CO_2 asphyxiation in accordance with local guidelines. The femur was dislocated from the tibia and placed in sterile prewarmed Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, Dorset, England) supplemented with 10% foetal bovine serum; 100 U/ml penicillin/streptomycin; 2 mM Glutamax; 1 mM L-glutamine and 1% non-essential amino acids. The femur and tibia were cut at both epiphyses and marrow was flushed into a 50 ml tube using 5 ml supplemented DMEM and a 25-gauge needle. The suspension was centrifuged (650g, 5 min, 20°C), resuspended in 10 ml of DMEM and passed sequentially through a 16-gauge, 18-gauge and 20-gauge needle. The suspension was passed through a 40 μm nylon mesh and incubated for 30 min in a tissue culture flask in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. The supernatant was removed and incubated in a culture flask at a cell density of 5×10^7 nucleated cells; following a 24 h period the culture medium was replaced to remove non-adherent cells. Upon reaching 80–90% confluence the cells were passaged and replated onto tissue culture flasks. The medium was replaced every 3 days and cells were maintained in a humidified atmosphere of 95% air/5%CO_2 at 37°C until required (normoxia). To determine the purity of the cells, we used fluorescence-associated cell sorting (FACS) and found that 96.9 ± 0.4% of the cells were positive for CD90, a putative MSC marker while expression of the haematopoietic stem cell marker, CD45, was negligible (less than 3%). To induce chondrogenesis the cells were treated with DMEM supplemented with TGFβ (10 ng/ml), ascorbic acid 2-phosphate (0.05 mM) and dexamethasone (100 nM). To provide a hypoxic environment, cells were maintained in a hypoxia chamber (Nuaire US Autoflow) and a gas mixture consisting of 95% N_2, 5% CO_2 was used to calibrate the intra-chamber environment to 2% O_2 (hypoxia).

Chemicals

In experiments designed to inhibit Akt, the cells were incubated with PI_3K-pathway inhibitor, LY294002 2-(4-Morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (Calbiochem, Darmstadt, Germany; 2 μM). In experiments designed to inhibit p38 MAP kinase, the cells were incubated SB 203580 (4-(4-Fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl) 1H-imidazole; Calbiochem, 3 μM).

HIF-1α, p-AKT, p-38, Sox-9 and collagen II immunocytochemistry

MSCs were fixed in 4% paraformaldehyde, permeabilised in tris-buffered saline (TBS) containing 0.1% Triton X-100 for 10 min and refixed with 4% paraformaldehyde for 10 min. Non-reactive sites were blocked using 20% horse serum/2% bovine serum albumin (2 h at room temperature) and cells were incubated overnight with the primary antibody. The antibodies used were anti-collagen II antibody purified from goat serum (Santa-Cruz Biotechnology, Inc., Santa Cruz, CA; 1:1,000 dilution), anti-HIF-1 mouse monoclonal antibody (Novus Biologicals, Littleton, CO), a rabbit polyclonal phospho-Akt (Thr308) antibody (Santa-Cruz Biotechnology, Inc.), a rabbit monoclonal phospho-p38 MAPK (Thr180/Tyr182) (3D7) antibody (Cell Signalling Technology, Inc., Danvers, MA) or goat polyclonal Sox-9 (Santa-Cruz Biotechnology, Inc.). Cells were then incubated with a biotinylated anti-goat IgG (for collagen II and sox-9), biotinylated anti-mouse IgG (for HIF-1) or biotinylated anti-rabbit IgG (for p-Akt, p-p38), followed by incubation in ExtrAvidin-FITC (1:50 dilution; 1 h at room temperature) or Alexa 543 (for sox-9). Confocal fluorescence microscopy imaging was performed (LSM 510 META).

HIF-1α transactivation assay

The Panomics TransBinding HIF1-α assay kit (Panomics, Inc., 6539, Fermont, CA) was used to quantify HIF1-α activation. Briefly, MSCs were exposed to 2% O_2 and nuclear extracts were prepared according to manufacturers instructions and stored at −80°C. Triplicate nuclear extracts were mixed with the biotinylated HIF-1 probe and the resulting HIF1-α/HIF-1 probe complex was conjugated to the streptavidin-coated assay plate. The HIF1-α/HIF-1 probe complexes were detected using an HIF1-α specific antibody, followed by binding of the HRP-conjugated secondary antibody. The resulting colorimetric reaction was quantified by Genesis Lite Software (Version 3.03, Life Sciences, Basingstoke, UK), at 450 nm in a fluorometer (Labsystems Multiskan RC, MTX Labsystems, Inc., Vienna, VA).

RNA interference

Custom ON-TARGET Plus Smart pool small interfering RNA (siRNA) containing a mixture of 4 SMART selection designed siRNAs targeting rat HIF1-α (Gen Bank™ accession number NM_024359; HIF1-α siRNA) was purchased from Dharmacon (Lafayette, CO). MSCs were transfected with HIF1-α siRNA (100 nM) using Dharmacon transfection lipid number 4, for a period of 1 week under 5% O_2, with chondrogenic factors (100 nM dexamethasone, 0.05 mM ascorbic acid-2-phosphate and 5 ng/ml transforming growth factor-α). A control siRNA duplex containing at least four mismatches to any rat gene (ON-TARGET Plus siControl Non-Targeting siRNA; Con siRNA) was used in parallel experiments. Optimal transfection efficiency and conditions were determined by using FAM labelled non-specific siRNA (SiGlo green; Dharmacon). Effective HIF1-α knockdown

using the selected siRNA sequence was validated using immunocytochemistry.

Histological staining for proteoglygan deposition

Paraformaldehyde-fixed MSCs were rinsed in distilled water. Cover slips were placed in 250 μl Safranin-O (1% in dH_2O; Sigma-Aldrich) for 20 min and then washed five times in distilled water and dehydrated through 70% alcohol, spirit, absolute alcohol and xylene. DPX (Distrene-80 plasticizer xylene, BDH Laboratory Supplied, Poole, England) was placed onto each coverslip and samples were mounted, cell side down, onto a glass slide. The slides were examined at 20X using phase contrast microscopy. Images were acquired by a ColorView camera. Each image was imported into UTHSCSA ImageTool (The University of Texas Health Science Centre, San Antonio, TX), threshold value set, and converted to a binary image. The percentage of black pixels to white was then calculated for each image. Average values were calculated, giving the percentage of proteoglycan at 3 weeks of treatment. Graphs and statistics of results were plotted and calculated in Prism software (GraphPad Software, Inc., San Diego, CA).

Statistics

Statistical analysis was carried out using one-way analysis of variance (ANOVA) followed by the post-hoc Student–Newman–Keuls test when significance (at the 0.05 level) was indicated. When comparisons were being made between two treatments, a paired Student's *t*-test was performed and $P < 0.05$ or $P < 0.01$ were considered significant.

Results

2% oxygen increases chondrogenesis evoked by chondrogenic growth factors

Exposure of MSCs to chondrogenic growth factors (CFs) for 3 weeks in normoxia evoked a significant increase in proteoglycan deposition and collagen II expression as assessed by Safranin-O staining and immunocytochemistry, respectively (Fig. 1). However, when MSCs were exposed to CFs to 2 weeks in normoxia followed by 1 week in hypoxia (2% oxygen), proteoglycan deposition and collagen II expression was significantly enhanced ($P < 0.01$, ANOVA, compared to cells maintained in normoxia, $n = 5$), demonstrating that hypoxia favours chondrogenesis in rat MSCs.

Nuclear expression and activity of HIF-1α is increased by 2% oxygen

Exposure to a hypoxic environment (2% oxygen) evoked a rapid (within 1 h) translocation of HIF-1α to the nucleus (Fig. 2A) and this was associated with a significant increase in HIF-1α binding to its DNA consensus sequence within nuclear extracts ($P < 0.01$, Student's *t*-test, $n = 5$; Fig. 2B).

A role for AKT in stabilisation of HIF-1α

In response to hypoxia (2% oxygen, 30 min) the expression of phospho-Akt (p-Akt) within MSCs is significantly enhanced (Fig. 3A) within the cytosol and nucleus (Fig. 3B). To determine whether Akt plays a part in stabilizing HIF-1α, MSCs were

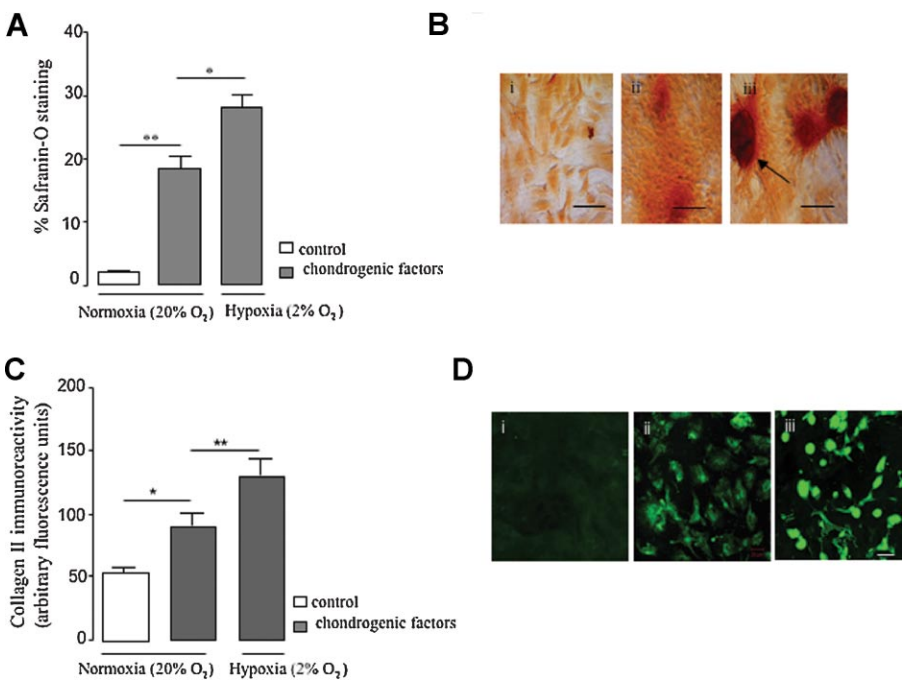


Fig. 1. Hypoxia enhances growth factor-induced chondrogenesis. A: MSCs maintained at 20% O_2 for 21 days in DMEM supplemented with chondrogenic factors (CF: 100 nM dexamethasone, 0.05 mM ascorbic acid-2-phosphate and 5 ng/ml transforming growth factor-β) display a significant increase in Safranin O staining. In cells exposed to CFs in normoxia for 14 days followed by 7 days in a hypoxic (2% O_2) environment, the Safranin O deposition was further significantly increased. Results are expressed as means ± SEM for five individual experiments, $^{***}P < 0.001$. B: Representative Safranin O staining in (i) MSCs in normoxia, (ii) MSCs exposed to CFs in normoxia, (iii) MSCs in hypoxia and (iv) MSCs exposed to CFs in hypoxia. Scale bar is 40 μm. C: MSCs maintained at 20% O_2 for 21 days in DMEM supplemented with chondrogenic factors (CF: 100 nM dexamethasone, 0.05 mM ascorbic acid-2-phosphate and 5 ng/ml transforming growth factor-β) display a significant increase in collagen II immunoreactivity. In cells exposed to CFs in normoxia for 14 days followed by 7 days in a hypoxic (2% O_2) environment, collagen II immunoreactivity was further significantly increased. Results are expressed as means ± SEM for five individual experiments, $^{***}P < 0.001$. D: Representative collagen II immunoreactivity in (i) MSCs in normoxia, (ii) MSCs exposed to CFs in normoxia, (iii) MSCs in hypoxia and (iv) MSCs exposed to CFs in hypoxia. Scale bar is 20 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

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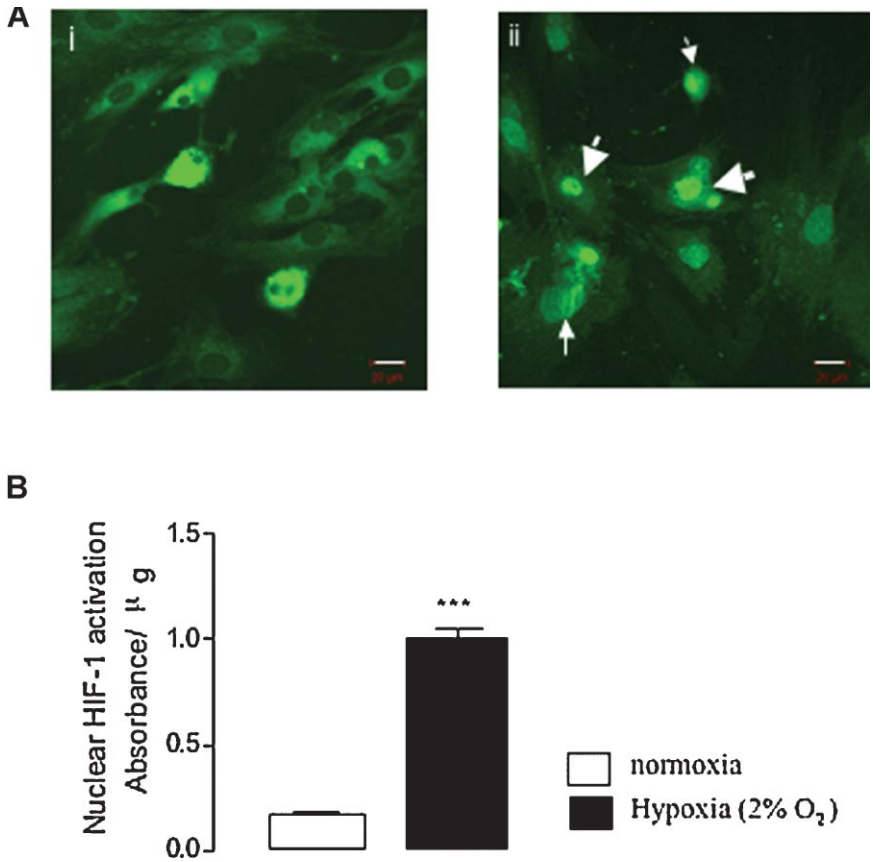


Fig. 2. Oxygen (2%) promotes translocation of HIF-1α to the nucleus and increases HIF-1α transactivation. A: MSCs were exposed to (i) 20% O_2 (normoxia) or (ii) 2% O_2 (hypoxia) for 1 h. Hypoxia increases HIF-1α immunoreactivity within the nucleus (indicated by arrows). Scale bar is 20 μm. B: Nuclear extracts isolated from MSCs and exposed to hypoxia (2% O_2) for 1 h, HIF-1α transactivation was significantly increased compared to MSCs maintained under normoxic conditions. Results are expressed as mean ± SEM for six independent observations, $^{**}P < 0.01$. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

pretreated with the Akt inhibitor, LY294002 (2 μM), prior to exposing to hypoxia. Under hypoxia, HIF-1α translocates to the nucleus (Fig. 3Ci), however, in the presence of LY294002, the hypoxic stimulus does not induce the accumulation of HIF-1α within the nucleus and HIF-1α immunoreactivity displays a diffuse pattern of staining. This result demonstrates that hypoxia induces phosphorylation of Akt and that Akt is an upstream signalling molecule involved in the hypoxia-mediated stabilisation and nuclear translocation of HIF-1α.

Similarly, in response to hypoxia (2% oxygen, 30 min) an increase in phosphorylation of p38 was observed (Fig. 4A) and the p38 inhibitor, SB 203580, prevented the hypoxia-mediated stabilisation of HIF-1α (Fig. 4B). Thus, the stabilisation of HIF-1α by hypoxia involves both AKT and p38.

The hypoxia-induced increase in collagen II expression and proteoglycan deposition is abrogated by HIF-α siRNA

Figure 5A demonstrates that the hypoxia-induced increase in collagen II expression is prevented in cells in which HIF-1α knockdown has been achieved by HIF-1α siRNA ($P < 0.01$, AVOVA, compared to cells exposed to control non-targeting siRNA, $n = 5$).

Similarly, Figure 5B demonstrates that the hypoxia-induced increase in proteoglygan deposition is prevented in cells exposed to HIF-1α siRNA ($P < 0.01$, AVOVA, compared to cells exposed to control non-targeting siRNA, $n = 5$). The stimulatory effect of hypoxia on sox-9 expression is also prevented in cells in which HIF-1α has been knocked down (Fig. 5C) and this is likely to contribute to the downstream inhibition of chondrogenesis given the pertinent role of sox-9 in activation of the chondrogenic programme.

Discussion

The aim of this study was to examine the influence of hypoxia on the chondrogenic differentiation of rat MSCs. The results demonstrate that rat MSCs undergo chondrogenesis, as evidence by enhanced collagen II expression and proteoglycan deposition, when exposed to TGFβ and dexamethasone in a normoxic environment. When the MSCs were exposed the chondrogenic factors for 2 weeks in normoxia, followed by 1 week in hypoxia (2% oxygen), chondrogenesis was significantly enhanced, demonstrating that a reduced oxygen tension favours differentiation along the chondrogenic route. The hypoxic environment increased HIF-1α nuclear accumulation and its transactivation in an Akt- and p38-dependent manner. Furthermore, siRNA-mediated knockdown of HIF-1α prevented the hypoxia-induced increase in chondrogenesis providing evidence of a role for HIF-1α in the

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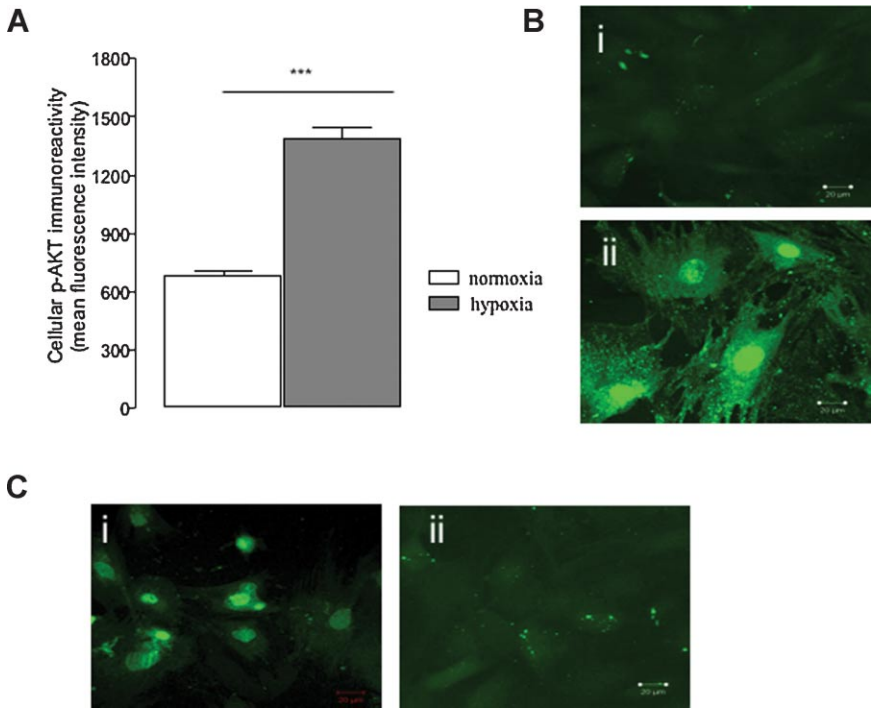


Fig. 3. Hypoxia increases p-AKT upstream of HIF-1α stabilisation. A: Exposure of MSCs to hypoxia (2% O_2) for 30 min evoked a significant increase in phospho-Akt immunoreactivity. Results are expressed as mean ± SEM for five independent observations, $^{**}P < 0.01$. B: MSCs were exposed to (i) 20% O_2 (normoxia) or (ii) 2% O_2 (hypoxia) for 30 min. Hypoxia increases phospho-Akt immunoreactivity within the cytosol and nucleus (indicated by arrows). Scale bar is 20 μm. C: MSCs exposed to (i) 2 O_2 (hypoxia) for 1 h an increase in HIF-1α immunoreactivity within the nucleus was observed. When MSCs were exposed to hypoxia (2% O_2 1 h) in the presence of LY294002 (ii, 2 μM), the hypoxia-induced mobilisation of HIF-1α was prevented. Images are representative of n = 5 independent experiments. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

enhancement of cartilage formation within a low oxygen microenvironment.

In ATDC5 chondroprogenitor, hypoxia alone favours chondrogenesis, whilst insulin-mediated chondrogenesis is inhibited by hypoxia (Chen et al., 2006) and in mouse ST2 stromal cells, exposure to 1% oxygen increases the expression of a number of genes associated with chondrocyte differentiation and promotes a chondrocyte phenotype (Robins et al., 2005). In contrast, in adipose-derived mesenchymal cells grown in micromass culture, hypoxia has an inhibitory effect on both chondrogenesis and osteogenesis (Malladi et al., 2006). Thus, it is becoming clear that the ambient oxygen tension has an impact on cartilage and bone formation, and this has physiological implications since endochondral bone is formed under avascular conditions and chondrocytes in the developing growth plate are hypoxic (Schipani et al., 2001).

The transcription factor, HIF-1α, is a component of the cell's intrinsic ability to respond to a low oxygen environment. HIF-1α is expressed by human chondrocytes (Coimbra et al., 2004) and a number of cartilage-related genes are under transcriptional control by HIF-1α (Robins et al., 2005). Given that cartilage tissue resides in a low oxygen environment, HIF-1α is thought to play a key role in maintaining the chondrocyte phenotype under this condition. Recently, Provot et al. (2007) have demonstrated that the mesenchymal condensations that give origin to endochondral bones are hypoxic during foetal development and that HIF-1α regulates early chondrogenesis and joint development in vivo, since conditional knockout of HIF-1α impairs the formation of the cartilaginous primordia. Also, in adipose-derived stromal cells HIF-1α expression controls the balance between adipogenic and chondrogenic differentiation (Malladi et al., 2007). In this study we examined the role of HIF-1α in the chondrogenesis of MSCs maintained in hypoxia. Under normoxic conditions HIF-1α immunoreactivity was detected in the cytosol of MSCs. Usually cytosolic levels of HIF-1α are low under normoxia due to hydroxylation of HIF-1α by oxygen-sensing prolyl hydroxylase domain (PHD) proteins which target HIF-1 for degradation at the proteosome (Brahimi-Horn and Pouysségur, 2007). Our observation that HIF-1α is stabilised within the cytosol of normoxic MSCs may reflect an ability of these cells to rapidly engage the transcriptional activity of HIF-1α in the face of a hypoxic challenge and such a capability would make these cells ideally primed for rapid adaptation to hypoxia. Alternatively, the non-hypoxic stabilisation of HIF-1α in MSCs may represent an, as yet uncharacterised, biological function for HIF-1α that requires its activation under normoxia. Indeed, there is emerging evidence demonstrating the accumulation of HIF-1α within a non-hypoxic environment (Déry et al., 2005) primarily through increased translation of the HIF-1α protein. The non-hypoxic stimulators of HIF-1 include growth factors, cytokines (Déry et al., 2005) and adipogenic differentiation (Floyd et al., 2007), although the factors that are responsible for the cytosolic accumulation of HIF-1α in normoxic MSCs, and the role of HIF-1 therewith, are interesting questions that require further investigation. When the MSCs were exposed to 2% oxygen, HIF-1α translocated to the nucleus and this nuclear localisation was concomitant with an increase in HIF-1α transactivation, as assessed by HIF-1α interaction with its DNA binding site. This result demonstrates that rat MSCs engage the HIF-1α pathway in response to a hypoxic challenge in a similar manner to that previously reported for micromass cultures of

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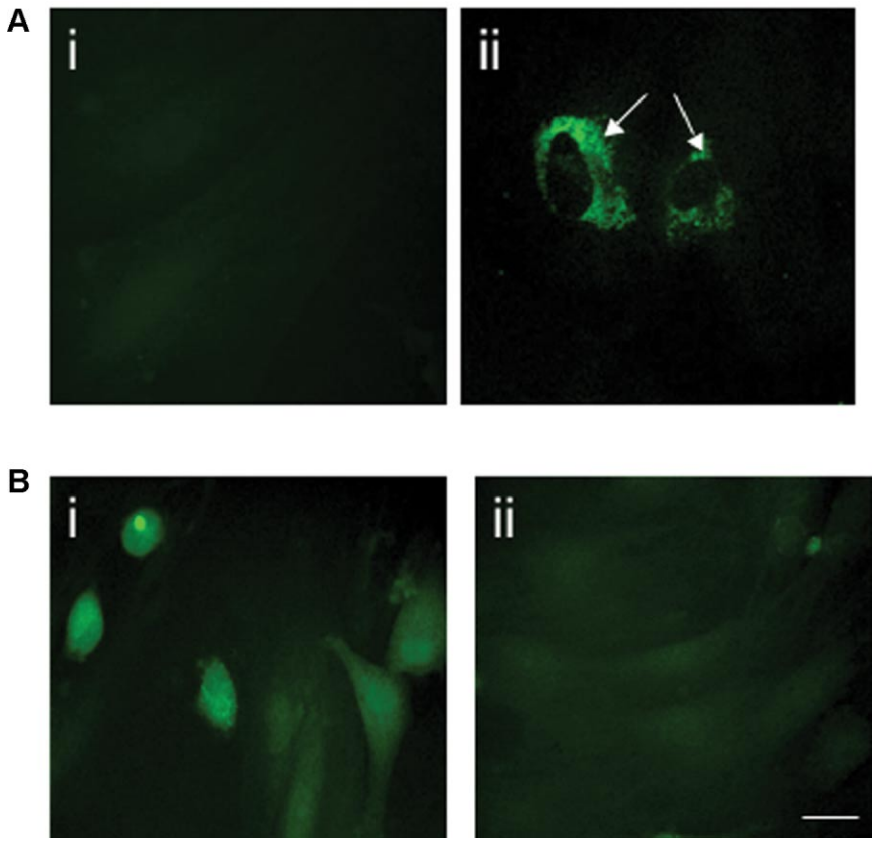


Fig. 4. Hypoxia increases p-p38 upstream of HIF-1α stabilisation. A: MSCs were exposed to (i) 20% O_2 (normoxia) or (ii) 2% O_2 (hypoxia) for 30 min. Hypoxia increases phospho-p38 immunoreactivity within the perinuclear area (indicated by arrows). Scale bar is 20 μm. B: MSCs exposed to (i) 2% O_2 (hypoxia) for 1 h intense HIF-1α immunoreactivity was observed. When MSCs were exposed to hypoxia (2% O_2 1 h) in the presence of SB 203580 (ii, 3 μM), the hypoxia-induced stabilisation of HIF-1α was prevented. Images are representative of n = 5 independent experiments. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

adipose-derived stromal cells (Malladi et al., 2007). In our study, the hypoxic environment also increased the expression of the phosphorylated forms of AKT and p38, and the PI3K/AKT inhibitor, LY294002 and p38 inhibitor, SB 203580, both prevented the hypoxia-induced accumulation and nuclear translocation of HIF-1α. This lends support of a role for AKT and p38 in regulating HIF-1α stabilisation in rat MSCs. An involvement for MAP kinases in HIF-1α stabilisation has previously been reported in other cells types (Comerford et al., 2004; Xu et al., 2004), and the AKT pathway regulates HIF-1α expression and activity in fibroblasts (Zhang et al., 2006) and osteoblasts (Tang et al., 2007). These regulatory effects may be due to a direct influence on the function of the HIF-1α transactivation domain or through modification of HIF-1α protein expression (Semenza, 2002). In chondrocytes, hypoxia favours chondrocytic commitment, as opposed to osteoblastic differentiation, via p38 MAP kinase signalling (Hirao et al., 2006) and our findings are consistent with a link between p38 and the cellular adaptations to hypoxia that govern chondrogenesis.

To investigate the role of HIF-1α in the hypoxia-mediated enhancement of chondrogenic differentiation we employed an siRNA knockdown approach. In MSCs lacking HIF-1α the induction of collagen II and proteoglycan deposition evoked by chondrogenic factors was comparable in cells grown in normoxic and hypoxic condition, thereby indicating that the hypoxia-mediated increase in chondrogenesis was mediated via HIF-1α. The HIF-1α knockdown also prevented the hypoxia-mediated induction of sox-9 which, given that sox-9 is a pertinent transcriptional regulator of the chondrogenic programme indicates that the regulatory role of HIF-1α in chondrogenesis is linked to the induction of the sox-9 targets, aggrecan and collagen II. Indeed, Robins et al. (2005) have demonstrated that the ability of hypoxia to transactivate the sox-9 promoter was almost completely abolished by deletion of HIF-1α consensus sites within the Sox-9 proximal promoter. The approach used in the current study demonstrates that MSCs devoid of HIF-1α are unable to support the enhancement of chondrogenesis within the hypoxic environment and lend support for HIF-1α being a critical mediator of MSC responsiveness to hypoxia. Recent evidence supports the involvement of a HIF-1α-mediated regulation of sox-9 in the differentiation of prechondrogenic cells to chondrocytes under hypoxic conditions in vivo during early skeletogenesis (Amarilio et al., 2007).

In summary, this study demonstrates that rat MSCs respond to hypoxia by rapidly increasing AKT and p38 phosphorylation and subsequent stabilisation of HIF-1α. The long-term exposure (7 days) to hypoxia enhances chondrogenesis evoked by TNF-β, and HIF-1α is intricately involved in the hypoxia-mediated enhancement of cartilage formation. This molecular pathway is likely to have physiological relevance for cartilage development. Furthermore, when considering the optimal culture conditions for cartilage tissue engineering, a low oxygen

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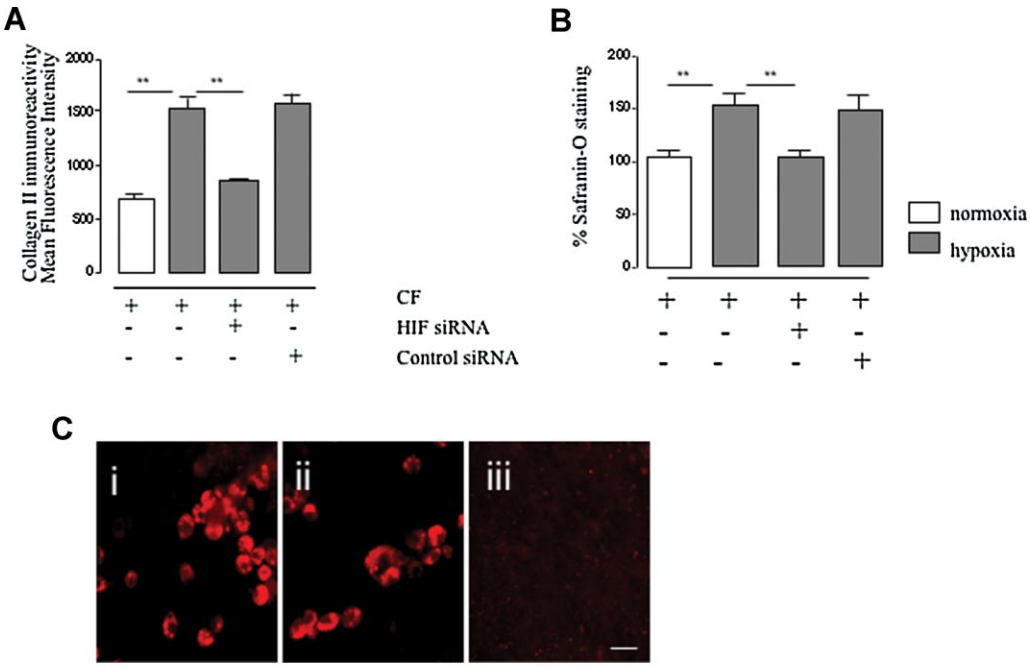


Fig. 5. HIF-1α siRNA prevents the hypoxia-induced enhancement of chondrogenesis. A: Cells exposed to chondrogenic factors (CF: 100 nM dexamethasone, 0.05 mM ascorbic acid-2-phosphate and 5 ng/ml transforming growth factor-β) under hypoxia (2% O_2) a significant increase in collagen II production is observed compared to cells maintained under normoxia. The HIF-1α siRNA abolished the hypoxia-mediated increase in collagen II production. In contrast, when MSCs were exposed to hypoxia in the presence of the control non-targeting siRNA, the hypoxia-mediated increase in collagen II immunoreactivity was retained. Results are expressed as mean ± SEM for five independent observations, $^{**}P < 0.01$. B: Cells exposed to chondrogenic factors (CF: 100 nM dexamethasone, 0.05 mM ascorbic acid-2-phosphate and 5 ng/ml transforming growth factor-β) under hypoxia (2% O_2) a significant increase in proteoglycan deposition is observed compared to cells maintained under normoxia. The HIF-1α siRNA abolished the hypoxia-mediated increase in proteoglycan deposition. In contrast, when MSCs were exposed to hypoxia in the presence of the control non-targeting siRNA, the hypoxia-mediated increase in proteoglycan deposition was retained. Results are expressed as mean ± SEM for five independent observations, $^{**}P < 0.01$. C: Cells exposed to chondrogenic factors (CF: 100 nM dexamethasone, 0.05 mM ascorbic acid-2-phosphate and 5 ng/ml transforming growth factor-β) under hypoxia (2% O_2) Sox-9 expression was induced (i). Also, when MSCs were exposed to hypoxia and CFs in the presence of the control non-targeting siRNA (ii), Sox-9 expression was induced. In contrast, the HIF-1α siRNA abolished the induction of Sox-9 immunoreactivity (iii). Images are representative of 5 independent experiments. Scale bar is 20 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

environment is likely to be of value to stimulate chondrogenesis in vitro for the development of cartilage implants from MSC precursors.

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