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RESEARCH ARTICLE

O-Phenanthroline as modulator of the hypoxic and catabolic response in cartilage tissue-engineering models

Nicole Georgi[1†], Ellie B.M. Landman[1†], Travis J. Klein[2], Clemens A. van Blitterswijk[3] and Marcel Karperien[1*]
[1]*Department of Developmental BioEngineering, MIRA Institute for Biomedical Technology and Technical Medicine, University of Twente, Enschede, The Netherlands*
[2]*Cartilage Regeneration Laboratory, Institute of Health and Biomedical Innovation, Queensland University of Technology, Kelvin Grove, Australia*
[3]*Department of Tissue Regeneration, MIRA Institute for Biomedical Technology and Technical Medicine, University of Twente, Enschede, The Netherlands*

Abstract

Hypoxia has been shown to be important for maintaining cartilage homeostasis as well as for inducing chondrogenic differentiation. Ensuring low oxygen levels during *in vitro* culture is difficult, therefore we assessed the chondro-inductive capabilities of the hypoxia-mimicking agent *O*-phenanthroline, which is also known as a non-specific matrix metalloproteinase (MMP) inhibitor. We found that *O*-phenanthroline reduced the expression of *MMP3* and *MMP13* mRNA levels during chondrogenic differentiation of human chondrocytes (hChs), as well as after TNFα/IL-1β exposure in an explant model. Interestingly, *O*-phenanthroline significantly inhibited matrix degradation in a TNFα/IL-1β-dependent model of cartilage degeneration when compared to control and natural hypoxia (2.5% O_2). *O*-Phenanthroline had limited ability to improve the chondrogenic differentiation or matrix deposition in the chondrogenic pellet model. Additionally, *O*-phenanthroline alleviated MMP-induced cartilage degradation without affecting chondrogenesis in the explant culture. The data presented in this study indicate that the inhibitory effect of *O*-phenanthroline on MMP expression is dominant over the hypoxia-mimicking effect. Copyright © 2014 John Wiley & Sons, Ltd.

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1. Introduction

Articular cartilage is an avascular tissue with a limited oxygen supply. The oxygen tension within articular cartilage ranges from 6% at the joint surface to 0.5% in the deeper zones (Lund-Olesen, 1970; Brighton and Heppenstall, 1971). As chondrocytes have an adaptive mechanism, they are able to survive in this hypoxic environment (Pfander and Gelse, 2007a; Murphy *et al.*, 2009). The gradient of oxygenation in cartilage is strongly linked to hypoxia-inducible factor (HIF)1α expression as well as to the level of chondrocytic cell survival growth arrest, cell size and the delay in hypertrophic differentiation (Schipani *et al.*, 2001).

In the presence of oxygen, HIF1 is modified post-translationally by iron-containing prolyl hydroxylases (PHDs), rendering the protein for proteasomal degradation (Maxwell and Salnikow, 2004). Under low oxygen tension, HIF1α is stabilized and subsequently forms a complex with the constitutively expressed HIF1β subunit. This complex translocates to the nucleus, where it binds hypoxia-responsive elements (HREs) in the promoters of target genes, initiating the transcription of genes involved in metabolism, angiogenesis and apoptosis. In a similar manner, the activity of HIF2α, which is a homologue of HIF1α, is regulated via oxygen-dependent degradation (Yang *et al.*, 2010). HIF stabilization and translocation

*Correspondence to: M. Karperien, Department of Developmental BioEngineering, MIRA Institute for Biomedical Technology and Technical Medicine, University of Twente, PO Box 217, 7500AE Enschede, The Netherlands. E-mail: h.b.j.karperien@utwente.nl; Website: www.utwente.nl/tnw/dbe
†These authors contributed equally to this study.

can also be induced by hypoxia-mimicking compounds, which exert their effect by targeting PHD activity (Maxwell and Salnikow, 2004).

Several indications have been found for the involvement of hypoxia in maintaining the chondrogenic phenotype as well as promoting the chondrogenic differentiation of progenitor cells (Muller *et al.*, 2011; Robins *et al.*, 2005; Lafont *et al.*, 2008). The differentiated phenotype of chondrocytes can be disturbed by, for example, exposure to inflammatory factors, such as interleukin (IL)-1β, in the osteoarthritic joint. This process is marked by a change in the chondrocyte phenotype, leading to a major reduction in the synthesis of extracellular matrix molecules such as collagen type II (COL2A1), ultimately resulting in cartilage matrix degradation (Hwang *et al.*, 2005). Interestingly, disturbance of HIF2α expression was found in osteoarthritic cartilage, implying the involvement of this protein in the pathogenic mechanism of cartilage degeneration (Saito *et al.*, 2010). The involvement of HIF2α in cartilage degradation is supported by the findings that overexpression of HIF2α elicits cartilage destruction, whereas genetic depletion of HIF2α inhibits experimentally induced cartilage destruction (Yang *et al.*, 2010). Since both loss of the chondrocyte phenotype and hypertrophic differentiation are involved in osteoarthritis (Pfander and Gelse, 2007b), the role of hypoxia in both processes might indicate that disturbance of hypoxic conditions is involved in the pathogenesis of cartilage degradation. The *in vitro* response of chondrocytes to IL-1β appears to be more pronounced in a hypoxic environment (Martin *et al.*, 2004), whereas IL-1β-induced downregulation of *SOX9*, *ACAN* and *COL2A1* in chondrogenically differentiated human MSCs was partially alleviated by a low oxygen concentration. In contrast, the expression of cartilage-degrading matrix metalloproteinases *MMP1* and *MMP3* was lowered. Therefore, the beneficial effects of hypoxic culture conditions appear to be mainly due to anti-catabolic effects on matrix-degrading proteins and the preservation of *SOX9* expression (Felka *et al.*, 2009).

Next to the role of HIFs in cartilage maintenance, indications have been found for a beneficial effect of hypoxia on chondrogenesis. The performance of expanded human primary chondrocytes (hChs) is often hampered by their limited chondrogenic potential, as well as by their preference for hypertrophic differentiation, which is associated with cartilage calcification. In hypoxic conditions, the chondrogenic potential of hChs as well as MSCs is enhanced in a HIF1α-dependent manner (Muller *et al.*, 2011; Kanichai *et al.*, 2008). Transcriptional activation of *SOX9* has been shown to have a major role in this beneficial effect (Robins *et al.*, 2005). Furthermore, hypertrophic differentiation is inhibited by hypoxic culture conditions *in vitro* (Leijten *et al.*, 2012; Gawlitta *et al.*, 2012).

Since hypoxia is involved in both cartilage homeostasis and chondrogenic performance, modulating hypoxia-induced signalling might be beneficial in preventing cartilage degradation as well as inducing chondrogenesis. Identification and use of compounds that are able to induce HIF1α activity offer a strategy to improve cartilage matrix formation. In a screen of 1408 compounds, *O*-phenanthroline was identified as a potent HIF1α-inducing agent under normoxic conditions (Xia *et al.*, 2009) and outperformed the known hypoxia-mimicking agents cobalt sulphate ($CoSO_4$) and cobalt chloride ($CoCl_2$). *O*-Phenanthroline is a strong chelator of metal ions and has been identified previously as an inhibitor of matrix metalloproteinases (MMP) by chelating Zn^{2+} ions, which are required for MMP activity. Similarly, *O*-phenanthroline blocks the activity of dioxygenases, such as PHDs, by chelating essential free ferrous ions, and thereby activates the HIF pathway (Maxwell and Salnikow, 2004). Both inhibition of MMPs and activation of the HIF pathway might be beneficial for cartilage homeostasis, indicating that *O*-phenanthroline is a potential candidate for the treatment of cartilage degradation.

In this study, we assessed the potential of *O*-phenanthroline to improve chondrogenic performance in a hCh pellet culture model. Furthermore, we tested *O*-phenanthroline's efficiency to reduce matrix catabolic processes in an explant model for cartilage degradation.

2. Materials and methods

2.1. Cell culture

The use of human knee biopsies was approved by a local Medical Ethics Committee in Enschede and Almelo (METC TWENTE). Human primary chondrocytes (hChs) for HIF1α staining were obtained from full-thickness cartilage dissected from knee biopsies of a patient (male, age 56 years, with osteoarthritis) undergoing total knee replacement, as published previously (Hendriks *et al.*, 2006). In short, the harvested cartilage was digested overnight in 0.15% collagenase type 2 solution. After digestion, hChs were washed and cultured up to passage 1 on tissue-culture plastic in chondrocyte proliferation medium [Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% non-essential amino acids, 0.2 mM ascorbic acid 2-phosphate, 0.4 mM proline, 100 U/ml penicillin and 100 mg/ml streptomycin)]at a density of 3000 cells/cm^2 under standard culture conditions (21% O_2, 5% CO_2, 37°C). The chemicals were purchased from Sigma-Aldrich.

2.2. HIF1α immunofluorescence staining

hChs (3000 cells/cm^2) were seeded on coverslips and cultured for 48 h before adding the respective compounds [*O*-phenanthroline 1–25 μM, positive control: cobalt chloride ($CoCl_2$) 25 μM] or transfer of the cultures to a hypoxia incubator. The cells were exposed to the small molecules for 18 and 24 h. In a number of experiments, cells were cultured for 24 and 48 h after an 18 h compound exposure to assess cell growth after compound removal. The samples were washed and fixed [4% paraformaldehyde (PFA), 15

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min] at the indicated time points and stained for HIF1α. In short, cells were blocked [2% bovine serum albumin (BSA), 0.1% Triton X-100 in phosphate-buffered saline (PBS), 20 min] and the monoclonal mouse anti-HIF1α antibody (1:100, Clone H1α67; Novus Biologicals, Littleton, USA) or mouse IgG isotype control antibody (Invitrogen) were incubated overnight. The cells were rinsed with PBS (5 min) before adding AlexaFluor® 488-labelled goat anti-mouse antibody (1:400; Invitrogen) and DAPI (5 μg/ml) in 2% BSA/PBS. The cells were rinsed with PBS (5 min) and covered with ProLong Gold (Invitrogen). For total cell counts, cell numbers were counted using ImageJ software; in short, the images were thresholded to only allow quantification of positive DAPI staining, and the nuclei were then counted using the 'count particles' tool.

2.3. Pellet culture

For the chondrogenesis study, micromass pellets of 0.25×10^6 hChs were seeded in a round-bottomed well plate and centrifuged (5 min, 500 g). Pellets were cultured for 10 days in chondrogenic differentiation medium (DMEM, 1% ITS, 0.2 mM ascorbic acid, 100 U/ml penicillin, 100 mg/ml streptomycin, 100 mg/ml sodium pyruvate, 10 ng/ml TGFβ3, 10^{-7} M dexamethasone) before transferring the pellets to hypoxia (2.5% O_2) or incubation with *O*-phenanthroline (5.0 μM), respectively, for 1 week. The respective *O*-phenanthroline concentration was chosen above the optimal levels in 2D cultures, since diffusion effects in 3D will decrease the cellular concentration.

2.4. Mouse fetal metatarsals

Animal experiments were approved by the ethical committee of the University Medical Centre Utrecht. Mouse fetal metatarsals were isolated from FVB mouse embryos (time-paired, Harlan) at day 17.5 of gestation (van Beek *et al.*, 1995; Haaijman *et al.*, 1999). After isolation, the metatarsals were individually cultured in 24-well plates in 200 μl/well minimal essential medium (MEM)α, supplemented with 10% FBS, 100 U/ml penicillin, 100 mg/ml streptomycin and 1% Glutamax (Invitrogen) for 48 h. After this equilibration period, the metatarsals were cultured in hypoxia or in the presence of 5.0 μM *O*-phenanthroline, either alone or in combination with 10 ng/ml TNFα and IL-1β (R&D Systems), for 4 days for gene expression analysis or for 7 days for histological analysis. The respective *O*-phenanthroline concentration was chosen above the optimal levels in 2D cultures, since diffusion effects in 3D will decrease the cellular concentration.

2.5. Histological analysis

For histological examination, specimens were fixed in 10% buffered formalin and dehydrated in an ethanol series before embedding in paraffin; 5 μm sections were then cut using a rotary microtome (HM355S Microm International). Sections were stained for glycosaminoglycans, using 0.5% Alcian blue (Sigma Aldrich) in water (pH set to 1 using HCl) for 30 min and counterstained in 1% nuclear fast red in 5% aluminium sulphate (Sigma Aldrich) for 5 min.

2.6. Gene expression analysis

Metatarsals (five metatarsals/condition) and pellets (one pellet/condition) were crushed and lysed in Trizol (Invitrogen) for RNA isolation and processed using the Nucleospin RNA II Kit (Bioke) according to the manufacturer's protocol. Subsequently, cDNA was synthesized using the iScript cDNA synthesis kit (Bio-Rad). Quantitative polymerase chain reaction (qPCR) was performed using iQ SYBR Green Supermix (Bio-Rad) on a MyiQ2 Two-Color Real-Time PCR Detection System (Bio-Rad). Gene expression was normalized for B2M expression and expressed as fold induction compared to controls. Primer sequences are listed in Table 1.

3. Results

3.1. Dose–response effect of *O*-phenanthroline on HIF1α expression and cell count

We first tested the *O*-phenanthroline dose–response effect on HIF1α expression in chondrocytes using immunofluorescence staining of HIF1α. We found the highest upregulation of HIF1α expression in cells treated with 2.5 or 5.0 μM *O*-phenanthroline for 18 h (Figure 1A). At concentrations >5.0 μM, the effect of *O*-phenanthroline on HIF1α expression decreased.

In addition, we tested whether *O*-phenanthroline affected cell proliferation by counting total cell numbers. After 18 h, *O*-phenanthroline caused a slight increase in cell counts at concentrations up to 2.5 μM, whereas concentrations >2.5 μM significantly reduced cell counts below the control level (Figure 1B). Since 2.5 and 5.0 μM *O*-phenanthroline effectively induced HIF1α expression, 5.0 μM *O*-phenanthroline was used in subsequent experiments. Since we have shown previously that chondrocytes in a three-dimensional (3D) environment are less sensitive to chemical compounds than cells cultured in monolayer, we used the highest non-toxic concentration of *O*-phenanthroline (Miclea *et al.*, 2011a).

3.2. *O*-Phenanthroline induces HIF1α expression less effectively than hypoxia or $CoCl_2$

The effects of 5.0 μM *O*-phenanthroline, hypoxic culture conditions (2.5% O_2) and the known hypoxia-mimicking compound $CoCl_2$ on HIF1α expression were studied in human chondrocytes. Cells exposed to 5.0 μM *O*-phenanthroline or hypoxia (2.5% O_2) were compared and the effect of either

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Table 1. Primer sequences for qPCR analysis

Gene symbol		Sequence	Annealing temperature (°C)	Product size (bp)
Hum *COL2A1*	For	5′-CGTCCAGATGACCTTCCTACG-3′	60	122
	Rev	5′-TGAGCAGGGCCTTCTTGAG-3′		
Hum *SOX9*	For	5′-TGGGCAAGCTCTGGAGACTTC-3′	60	98
	Rev	5′-ATCCGGGTGGTCCTTCTTGTG-3′		
Hum *MMP9*	For	5′-GTGATTGACGACGCCTTTGC-3′	60	115
	Rev	5′-CGCGACACCAAACTGGATGAC-3′		
Hum *MMP13*	For	5′-AAGGAGCATGGCGACTTCT-3′	60	72
	Rev	5′-TGGCCCAGGAGGAAAAGC-3′		
Hum *B2M*	For	5′-GACTTGTCTTTCAGCAAGGA-3′	60	106
	Rev	5′-ACAAAGTCACATGGTTCACA-3′		
Mus *Acan*	For	5′-AGGCAGCGTGATCCTTACC-3′	60	136
	Rev	5′-GGCCTCTCCAGTCTCATTCTC-3′		
Mus *Col2a1*	For	5′-CGTCCAGATGACCTTCCTACG-3′	60	122
	Rev	5′-TGAGCAGGGCCTTCTTGAG-3′		
Mus *Sox9*	For	5′-TGGGAGCGACAACTTTACCA-3′	60	88
	Rev	5′-AACAGAGAACGAAACCGGGG-3′		
Mus *Mmp9*	For	5′-GGTGATTGACGACGCCTTTGC-3′	60	115
	Rev	5′-CGCGACACCAAACTGGATGAC-3′		
Mus *Mmp13*	For	5′-AAGGAGCATGGCGACTTCT-3′	60	72
	Rev	5′-TGGCCCAGGAGGAAAAGC-3′		
Mus *B2m*	For	5′-GACTTGTCTTTCAGCAAGGA-3′	60	106
	Rev	5′-ACAAAGTCACATGGTTCACA-3′		

treatment on HIF1α expression was assessed. Compared to hypoxia and $CoCl_2$, *O*-phenanthroline less effectively induced HIF1α expression in primary chondrocyte cultures. Low levels of HIF1α expression were detected in hChs under normoxic culture conditions, as reported previously (Coimbra *et al.*, 2004) (Figure 2A). Exposure of hChs for 18 h to hypoxia, defined as 2.5% oxygen, increased HIF1α expression. HIF1α expression was also increased by 25 μM $CoCl_2$ and 5.0 μM *O*-phenanthroline. Both hypoxia-mimicking compounds were less effective than hypoxic culture conditions.

Cell numbers significantly increased between 18 and 42 h in both control and hypoxia (Figure 2B); interestingly, the cell numbers increased more in cultures exposed to hypoxia than in the control (Figure 2B). In cultures exposed to *O*-phenanthroline, proliferation was inhibited, while no difference in cell count was observed in cultures exposed to $CoCl_2$ (Figure 2B). Some cell nuclei showed a slight increase in size after *O*-phenanthroline incubation (not shown).

To study whether the effects on cell proliferation were reversible, we next examined the influence of the compounds on cell numbers after a pulse stimulation of 18 h, followed by a recovery period of 48 h. As expected, the cell population cultured under control conditions continued to increase over time. In addition, the inhibitory effect of $CoCl_2$ on cell numbers was alleviated; however, cell numbers in cultures treated with *O*-phenanthroline continued to decrease (Figure 2C).

3.3. *O*-Phenanthroline increased *SOX9* mRNA expression in chondrogenic pellets without affecting cartilage matrix deposition

The effect of *O*-phenanthroline on the chondrogenesis of hChs was investigated in 3D micromass pellets. In line with previous observations, chondrogenic performance in hCh pellets was increased when the cells were cultured in hypoxia, as assessed using Alcian blue staining (Figure 3A). No difference in the intensity of Alcian blue staining was observed in pellets treated with *O*-phenanthroline compared to control (Figure 3A). Gene expression analysis showed increased mRNA expression of the cartilage transcription factor *SOX9* when cells were cultured in the presence of 5.0 μM *O*-phenanthroline. *COL2A1* mRNA expression was significantly upregulated by hypoxic culture conditions, whereas *O*-phenanthroline did not significantly affect *COL2A1* mRNA expression. The mRNA expression of the cartilage matrix-degrading enzyme *MMP13* was significantly decreased by both hypoxia and *O*-phenanthroline, whereas *MMP9* expression was not significantly affected by hypoxia or *O*-phenanthroline (Figure 3B).

3.4. *O*-Phenanthroline blocks IL-1β/TNFα-induced cartilage degradation

As shown previously, treatment with IL-1β and TNFα for 7 days potently induced cartilage matrix degradation in mouse fetal metatarsals (Landman *et al.*, 2013). When cultured in hypoxic conditions, IL-1β- and TNFα-induced cartilage degradation was reduced, as observed by light microscopy. In addition, Alcian blue staining of mouse fetal metatarsals showed that the glycosaminoglycan content of the extracellular matrix was better preserved in hypoxia. Interestingly, 5.0 μM *O*-phenanthroline was more effective in preserving the original shape of the metatarsals, as well as the glycosaminoglycan content of the extracellular matrix, when compared to hypoxia treatment (Figure 4A).

Measurements of mouse metatarsals revealed a decrease in total length as well as the size of the primary

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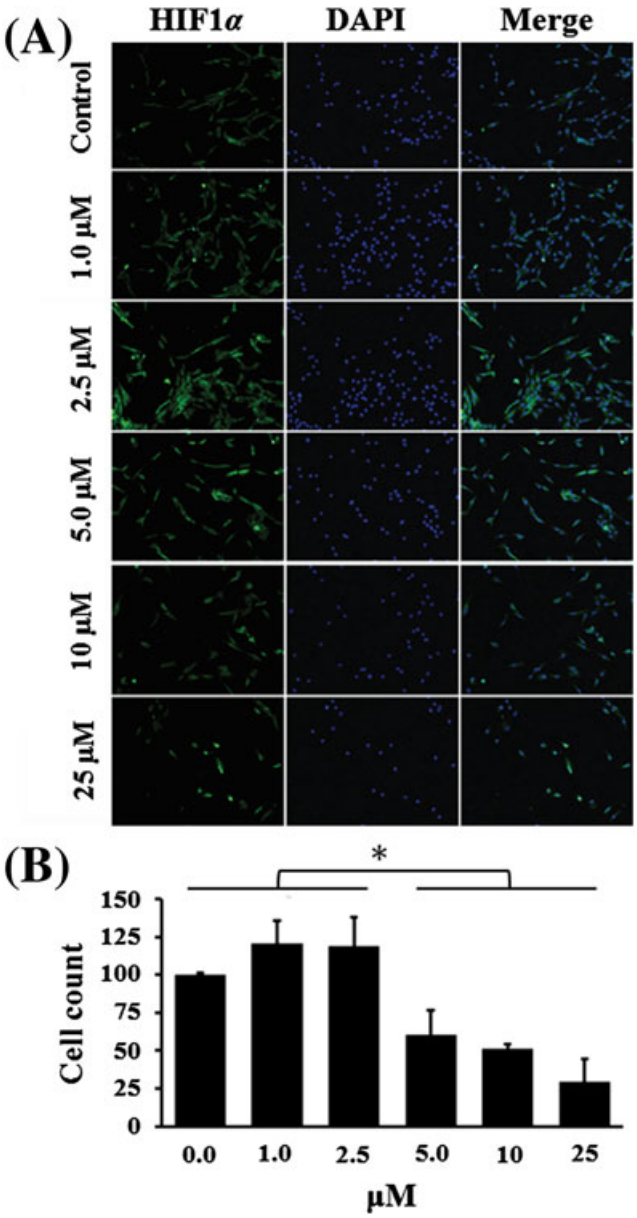

Figure 1. *O*-Phenanthroline dose-dependently affects HIFα expression and cell numbers. (A) hChs were exposed for 18 h to concentrations of 1.0–25 μM *O*-phenanthroline and the effect on HIF1α expression was studied using immunofluorescence. HIF1α is shown in green (Alexa488) and nuclei are in blue (DAPI); a representative picture out of two experiments is shown. (B) hCh counts of cells exposed to different concentrations of *O*-phenanthroline, indicating a toxic influence of the compound, starting from 5.0 μM onwards; data represent the mean confidence intervals (α =0.05) counts in three different wells. [Colour figure can be viewed at wileyonlinelibrary.com]

ossification centre after treatment with IL-1β and TNFα. *O*-Phenanthroline partially blocked the decrease in size of the metatarsals upon exposure to IL-1β and TNFα, but led to a significant increase in the size of the primary ossification centre. In contrast, the growth of the primary ossification centre was impaired when metatarsals were cultured in hypoxia (Figure 4B).

Gene expression analysis showed that *O*-phenanthroline increased the mRNA expression of *SOX9*, whereas mRNA expression of *Acan* and *Col2a1* was decreased in mouse fetal metatarsals. Both hypoxia and *O*-phenanthroline decreased the expression of *Mmp9* without affecting *Mmp13* expression (Figure 4C). In line with previous studies, treatment of the mouse fetal metatarsals with IL-1β and TNFα had a deleterious effect on the expression of *Acan* and *Col2a1*. Interestingly, this effect could be partially alleviated by culturing the metatarsals in hypoxia and was more pronounced in the presence of *O*-phenanthroline (Figure 4C). We found, in accordance with previous studies (Landman *et al.*, 2013), that the expression of matrix-catabolic genes, such as *Mmp13* and *Mmp9*, was upregulated by treatment of the metatarsals with IL-1β and TNFα. Hypoxic culture conditions as well as *O*-phenanthroline downregulated the expression of both catabolic genes (Figure 4C).

4. Discussion

The data presented in this study demonstrate that *O*-phenanthroline can be employed to reduce IL-1β/TNFα-mediated regulation of MMPs and matrix degradation in organ culture, whereas it has limited capability to improve matrix deposition in chondrogenically differentiating hCh pellet cultures. These findings are partially complementary to the findings of Stroebel *et al.* (2010), describing that the HIF inhibitor cadmium chloride diminished the inhibitory effect of low oxygen levels on *MMP1* and *MMP13* expression in chondrocytes. However, we did not find a stimulating effect of *O*-phenanthroline on GAG accumulation and *COL2A1* and *ACAN* expression.

In previous research, *O*-phenanthroline has been shown to induce the HIF1α-based hypoxic response as efficiently as 1.0% O_2 or $CoCl_2$ (Xia *et al.*, 2009). The strong iron chelator *O*-phenanthroline activates the cellular hypoxic response by depriving the HIF hydroxylases of their ferrous ion, whereas $CoCl_2$ makes use of its cobalt ions to compete with iron (Banerji *et al.*, 2005). In immunological staining of chondrocytes for HIF1α, we demonstrated that both *O*-phenanthroline and $CoCl_2$ induced HIF1α stabilization, but less effectively than the natural hypoxic response, based on HIF1α-staining. In addition, exposure of the cells to *O*-phenanthroline or $CoCl_2$ led to growth arrest after 42 h of exposure. The strong chelating capacity of *O*-phenanthroline for a variety of ions might explain side-effects not seen in hypoxic culture conditions. Similar mechanisms might apply for the competing activity for active ion-binding sites of $CoCl_2$. These findings are in line with the results of Felber *et al.* (1962), showing that *O*-phenanthroline can block the activity of DNA polymerase in *Escherichia coli* by the formation of Zn^{2+} complexes. Inhibition of DNA polymerase may well explain *O*-phenanthroline's inhibitory effects on cell proliferation. Additionally, it has been shown that derivatives of *O*-phenanthroline can be targeted to cancer cells to induce cell death (Sergeeva *et al.*, 2011) and that the formed complexes of *O*-phenanthroline can have a pro-apoptotic effect (Zhou *et al.*, 2002).

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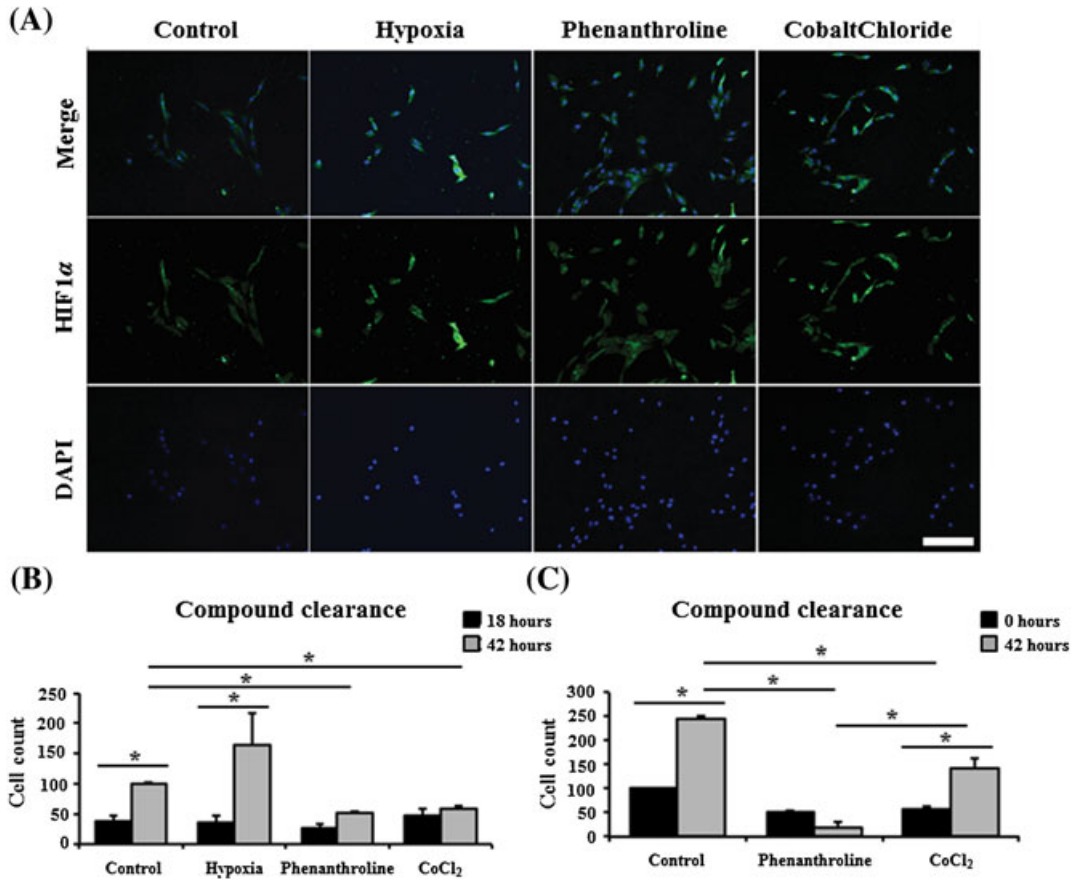

Figure 2. HIF1α expression and cell counts after exposure to hypoxia and small-molecule compounds. (A) hChs were exposed to hypoxia, *O*-phenanthroline (5.0 μM) and $CoCl_2$ (25 μM), respectively. After 18 h exposure, HIF1α expression was visualized using immunofluorescence. The most intense HIF1α stain was found after exposure to hypoxia followed by the known hypoxia-mimicking substance $CoCl_2$ and *O*-phenanthroline. HIF1α is shown in green (Alexa488) and nuclei are in blue (DAPI); translocation of HIF1α to the nucleus was not visible; a representative picture out of two replicates is shown; scale bar =100 μm. (B) hCh cell counts 18 and 42 h after exposure to hypoxia or small-molecule hypoxia mimics; data represent the confidence intervals (α =0.05) of three counts in different wells. (C) hCh cell counts after compound removal, indicating the irreversible influence of *O*-phenanthroline on the proliferation potential; hChs exposed to $CoCl_2$ start to proliferate again after compound removal; data represent the mean confidence intervals (α =0.05) counts in three different wells. [Colour figure can be viewed at wileyonlinelibrary.com]

In hCh pellet cultures, *O*-phenanthroline was added after 10 days of redifferentiation culture and removed after 1 week of exposure. *O*-Phenanthroline-treated pellets demonstrated the strongest activation of *SOX9* mRNA expression, as well as downregulation of the mRNA encoding the catabolic genes *MMP9* and *MMP13*. *SOX9*, a major transcription factor regulating chondrogenesis, was reported previously to be strongly associated with HIF1α and to be upregulated after hypoxic exposure in hChs (Robins *et al.*, 2005). Our data show that the primary hypoxic response induced by *O*-phenanthroline more effectively induced *SOX9* mRNA expression as compared to the low oxygen (2.5% O_2) treatment. *COL2A1*, a regulated gene of chondrogenesis downstream of *SOX9*, showed no upregulation upon *O*-phenanthroline exposure but a positive response after hypoxic treatment. Aigner *et al.* (2003) demonstrated previously that *COL2A1* expression is not necessarily correlated to *SOX9* expression and might thereby underline the different genetic response between hypoxia and *O*-phenanthroline treatment in the pellet culture and the explant model. Furthermore, histological staining for glycosaminoglycans indicated a higher amount of matrix synthesis in hypoxic-cultured pellets and emphasizes the findings at the mRNA level. The downregulation of *MMP9* and *MMP13* in hypoxic conditions and *O*-phenanthroline treatment indicate an anticatabolic effect. We confirmed that *O*-phenanthroline can act as potential inhibitor of *MMP* mRNA expression and likely reduces its activity in explant cultures (Maxwell and Salnikow, 2004), as demonstrated by better metatarsal preservation.

The use of *ex vivo* organ cultures in the investigation of the effect of small-molecule compounds can provide information about their effect on various cells, tissues and ultimately on tissue growth. Miclea *et al.* (2011b) showed that metatarsals can be employed to study the onset, as well as possible interventions, of cartilage degradation. It has been shown that osteoarthritic cartilage destruction is marked by an overexpression of HIF2α, leading to upregulation of a pool of MMPs and other catabolic mediators (Yang *et al.*, 2010). *O*-Phenanthroline is known both as an MMP inhibitor (Felber *et al.*, 1962) and an inhibitor of PHD-mediated HIF1α degradation (Maxwell and Salnikow, 2004). Consequently, it might provide an active

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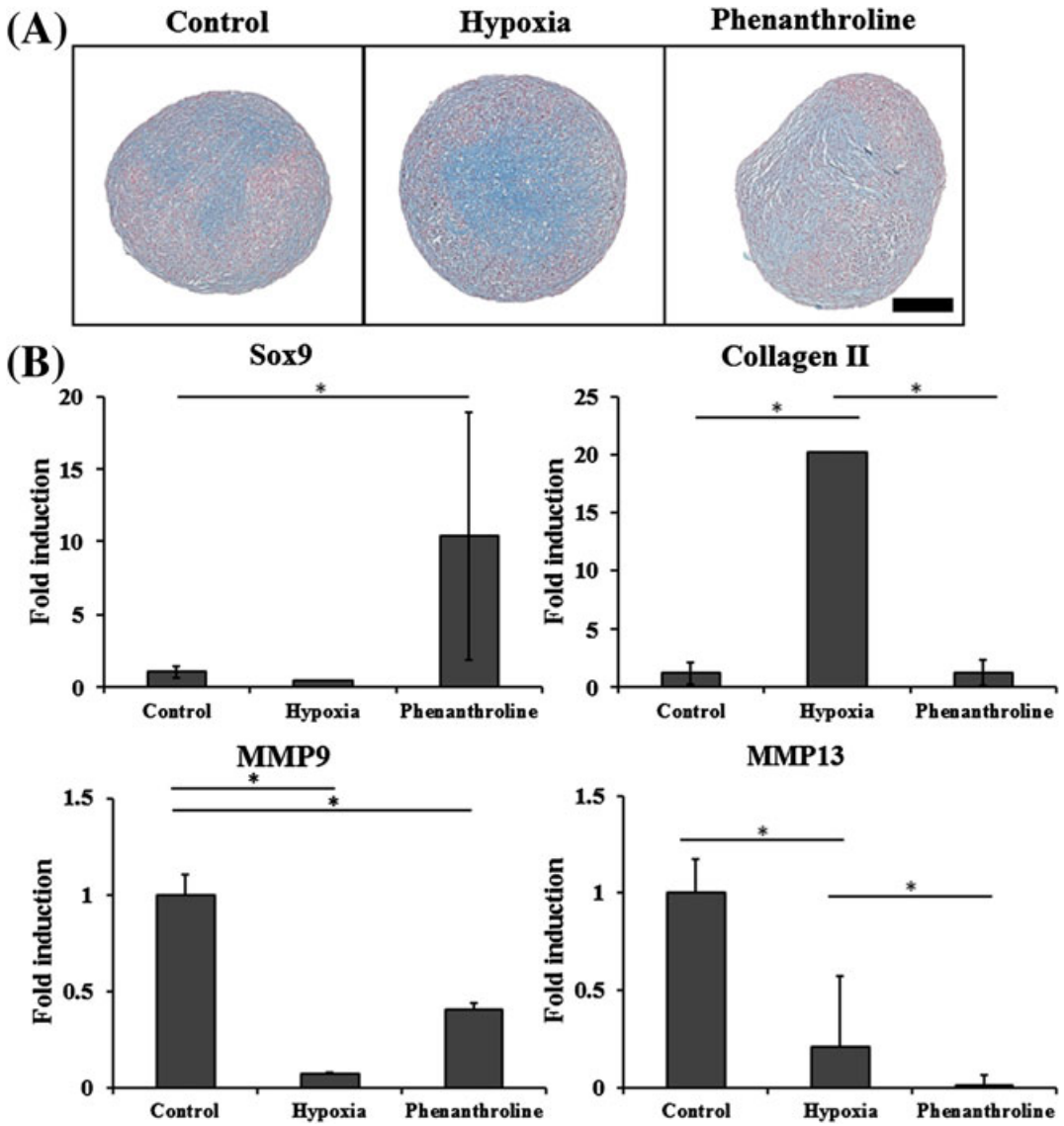

Figure 3. ***O*-Phenanthroline does not induce cartilage matrix deposition, whereas hypoxia increases the cartilage matrix. (A) Alcian blue staining for sulphated GAGs after 7 days of exposure to 5.0 μM *O*-phenanthroline or hypoxia after 10 days of chondrogenic redifferentiation of hChs in pellet culture, indicated no beneficial effect of phenanthroline exposure on matrix formation; a representative picture out of three experiments is shown; scale bar =100 μm. (B) The mRNA expression of the chondrogenic marker *SOX9* was significantly more upregulated by *O*-phenanthroline, compared to hypoxia, whereas *COL2A1* expression was downregulated by *O*-phenanthroline and $CoCl_2$, in contrast to hypoxia. Hypoxia as well as *O*-phenanthroline and $CoCl_2$ downregulated mRNA expression of *MMP9* and *MMP13*; data represent the mean of three pellets ±confidence intervals (α =0.05). [Colour figure can be viewed at wileyonlinelibrary.com]**

compound to overcome an imbalance in cartilage anabolism and catabolism, as occurs in response to pro-inflammatory cytokines. Exposure to IL-1β and TNFα induced total degradation of *ex vivo*-cultured metatarsals, whereas hypoxia and *O*-phenanthroline reduced matrix degradation and maintained the original shape of the metatarsals, as shown by Alcian blue staining and measurements of longitudinal bone length. Furthermore, the growth of the primary ossification centre was decreased when metatarsals were cultured under hypoxia but increased when exposed to *O*-phenanthroline (Figure 4B). These findings suggest an effect on endochondral ossification, which is likely to be independent of the hypoxic response when *O*-phenanthroline is used (Leijten *et al.*, 2012). This regulation might be linked to the proposed role of HIF2α in this process and to the downregulation of matrix-degrading proteins such as MMPs (Yang *et al.*, 2010). The opposite effects of *O*-phenanthroline and hypoxic culture conditions on the primary ossification centre in cartilage explant cultures suggests that both treatments have common, as well as distinct, cellular effects (Hirao *et al.*, 2006).

5. Future perspective

Ultimately, we conclude that small-molecule hypoxia-mimicking compounds, such as *O*-phenanthroline or $CoCl_2$, can provide a platform to induce part of a hypoxic response. Off-target effects are likely due to competition with metal ions for active positions in proteins, which is

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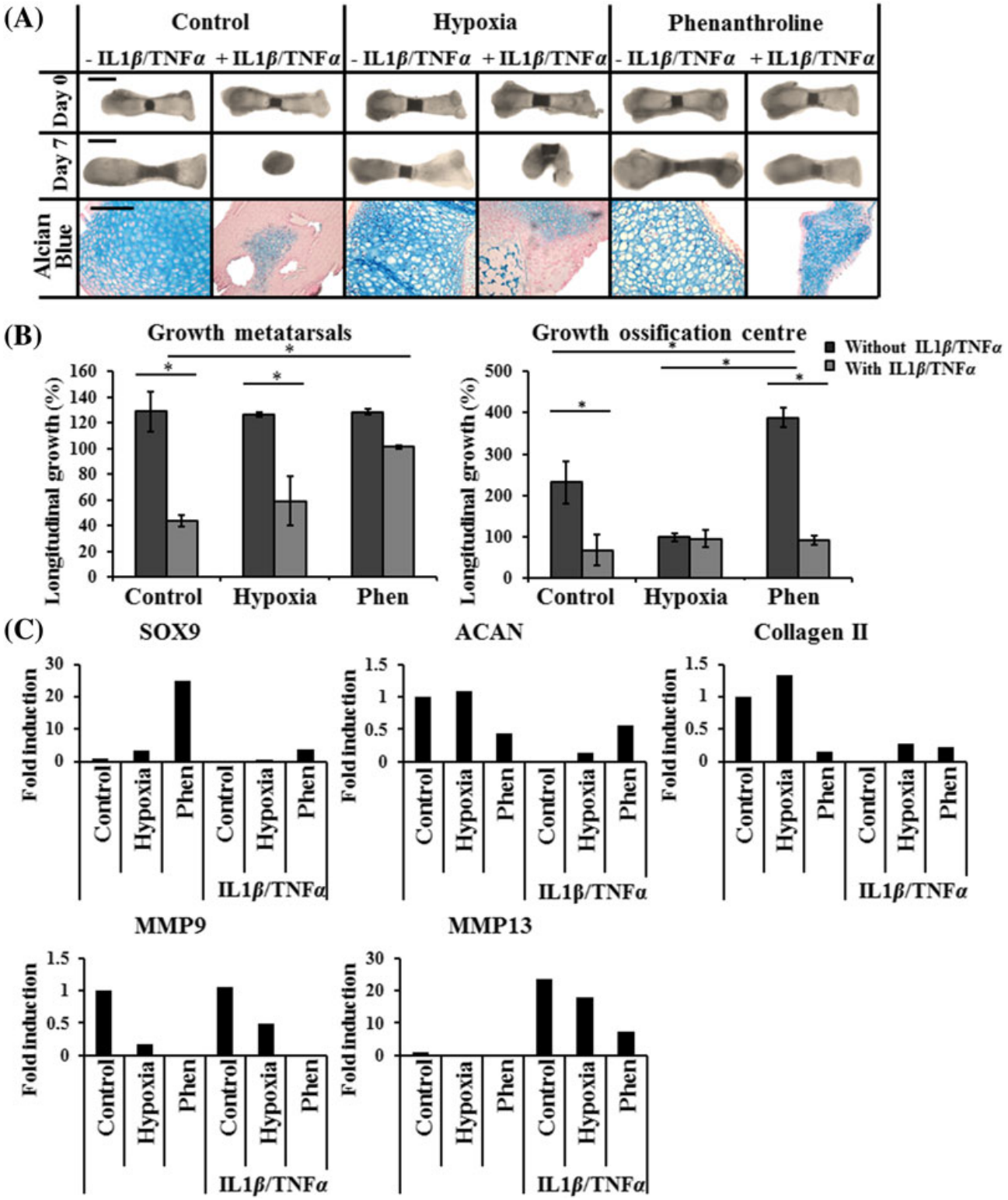

Figure 4. *O*-Phenanthroline blocks IL-1*β*/TNF*α*-induced cartilage matrix degradation in mouse fetal metatarsals. (A) Both light microscopy and Alcian blue staining show a protective effect of *O*-phenanthroline against IL-1*β*- and TNF*α*-induced (10 ng/ml each) cartilage matrix degradation; representative pictures out of three experiments are shown; scale bars =500 μm for the overview pictures and 100 μm for the Alcian blue staining. (B) The longitudinal growth of the metatarsals, as measured along the sagittal axis, was significantly decreased by treatment with IL-1*β* and TNF*α*. *O*-Phenanthroline partially rescued the decrease in size of the metatarsals. The growth of the primary ossification centre was decreased when metatarsals were cultured in hypoxia, whereas treatment with *O*-phenanthroline significantly increased ossification. Treatment with IL-1*β* and TNF*α* decreased the size of the ossification centre in all conditions. Data represent the mean of three metatarsals/condition ±confidence intervals (α =0.05). (C) Gene expression analysis shows that mRNA expression of the chondrogenic marker *Sox9* is induced by *O*-phenanthroline, whereas the cartilage matrix genes *Acan* and *Col2a1* are downregulated. Both *O*-phenanthroline and hypoxic culture conditions partially block IL-1*β*/TNF*α*-induced downregulation of these genes. Hypoxia and *O*-phenanthroline both downregulate *Mmp9* and *Mmp13* mRNA expression, whether or not IL-1*β* and TNF*α* are added. Data represent mRNA analysis of five pooled metatarsals. [Colour figure can be viewed at wileyonlinelibrary.com]

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not limited to the hypoxic pathway but can affect a myriad of cellular responses, leading to genetic modifications, cell arrest and apoptosis. These effects are highly undesired for medical therapy and at present allow for only a limited *ex vivo* applicability of these small-molecule compounds.

However, our findings highlight the important role of HIF signalling in the maintenance of articular cartilage integrity, and we demonstrate the possibility of chemical modulation of the pathway to benefit chondrogenesis and prevent cartilage degradation. The beneficial effect of chemical mimicking of the hypoxic response can be used in the development of putative treatment for cartilage degradation. Nevertheless, for *in vivo* applications in osteoarthritic therapy, intense dose–effect studies need to be performed or more specific small compounds have to be developed to limit negative side-effects.

Conflict of interest

The authors have declared that there is no conflict of interest.

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