Author Correction

Pfander, D., Cramer, T., Schipani, E. and Johnson, R. S. (2003). HIF-1α controls extracellular matrix synthesis by epiphyseal chondrocytes. *J. Cell Sci.* **116**, 1819-1826.

There were four errors in both the online and the print versions of this paper. In the paragraph 'Extracellular matrix synthesis is controlled by HIF-1α under hypoxia' in Results on p. 1823, line 10, it should read 'Significantly less extractable proteoglycans were detected in HIF-1α-null than in wild-type cultures after 44 hours exposure to 0.5% oxygen (Fig. 5B)'.

On p. 1824, the legend to Fig. 5, lane 3, should read '(A) Analysis of aggrecan gene expression: Bars represent -fold changes of aggrecan mRNA levels in HIF-1α-null cells compared with wild-type levels (normoxic and hypoxic wild-type expression taken as 1.0, respectively)'.

On p. 1824, the legend to Fig. 6, lanes 12-13, should read '(normoxic and hypoxic wild-type expression taken as 1.0, respectively)'.

On p. 1825, the legend to Fig. 7, lane 7 should read '(untreated normoxic and hypoxic expression taken as 1.0, respectively)'.

We apologize for any inconvenience caused.

HIF-1α controls extracellular matrix synthesis by epiphyseal chondrocytes

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Summary

The transcription factor HIF-1α plays a crucial role in modifying gene expression during low oxygen tension. In a previous study, we demonstrated that HIF-1α is essential for chondrocyte growth arrest and survival in vivo. To explore further the role of HIF-1α in cartilage biology, we undertook studies with primary epiphyseal chondrocytes with a targeted deletion of HIF-1α. In this study, we show that HIF-1α is necessary for regulating glycolysis under aerobic and anaerobic conditions. HIF-1α-null chondrocytes were unable to maintain ATP levels in hypoxic microenvironments, indicating a fundamental requirement for this factor for the regulation of chondrocyte metabolism. Synthesis of the angiogenic factor vascular endothelial growth factor was also significantly induced by hypoxia, and this increase is lost in HIF-1α-null mutant cells. Under hypoxic conditions, aggrecan mRNA and protein levels were significantly reduced in chondrocytes lacking the HIF-1α transcription factor. Interestingly, strongly increased type-II collagen protein levels were detected in wild-type cells after 44 hours of hypoxia. In addition, type-II collagen mRNA and protein levels were strongly decreased under low oxygen in chondrocytes lacking HIF-1α. In summary, our results clearly demonstrate the importance of HIF-1α in maintenance of anaerobic glycolysis, and thereby extracellular matrix synthesis, of epiphyseal chondrocytes.

Key words: Chondrocytes, Hypoxia, HIF-1α, Extracellular matrix

Introduction

Epiphyseal chondrocytes must exist in demanding microenvironmental conditions, facing continuous low oxygen tensions and high lactic acid concentrations. This requires chondrocytes to generate their energy to a significant degree through anaerobic glycolysis. In this pathway, one molecule of glucose is metabolized into two molecules of pyruvate and two molecules of ATP. Under normoxic conditions, the oxidative decarboxylation of pyruvate to acetyl-CoA in the presence of ATP initiates the final common pathway of oxidation. During hypoxia, pyruvate does not enter the citric acid cycle and is finally reduced to lactate (the Pasteur effect). These mechanisms are of pivotal importance in cells and tissues lacking sufficient oxygen. Much effort has been devoted to characterizing the adaptation of mammalian cells to low oxygen levels. Recently, it has been established that hypoxia-inducible factor 1 (HIF-1) plays a central role in mediating the transcriptional response to hypoxia, leading to increased expression of several genes important for survival in hypoxia, including angiogenic factors, glycolytic enzymes and erythropoietin (Semenza, 1998; Semenza, 2000). HIF-1 belongs to the basic helix-loop-helix PAS transcription factor family and consists of two subunits. The α subunit (HIF-1α) confers oxygen responsiveness and is degraded rapidly under normoxic conditions by the ubiquitin-proteasome pathway (Huang et al., 1998; Salceda and Caro, 1997). Under hypoxic conditions HIF-1α rapidly translocates into the nucleus and binds to its partner, HIF-1β [also called aryl hydrocarbon nuclear translocator (ARNT)] to form a heterodimer. This heterodimer, called HIF-1, binds to *cis*-acting promoter regions [hypoxia responsive elements (HREs)] of hypoxia-responsive genes, thereby upregulating their expression (Hofer et al., 2001).

The morphogenesis of long bones begins with the condensation of embryonic mesenchymal cells (Grigoriadis et al., 1988). After initial condensation, the mesenchymal cells differentiate into chondrocytes. At the embryonic growth plate, these chondrocytes proliferate and synthesize type-II collagen, aggrecan, small proteoglycans and glycoproteins (Scott-Savage and Hall, 1979). During this differentiation process the chondrocytic protein expression shows a strict temporal and spatial distribution pattern (Kirsch et al., 1997). When the differentiation process continues, the chondrocytes become hypertrophic and express type-X collagen (Buckwalter et al., 1987b; Kirsch and von der Mark, 1991). Finally, growth-plate chondrocytes mineralize and undergo terminal differentiation followed by apoptotic cell death (Buckwalter et al., 1987a; Gerstenfeld and Shapiro, 1996; Hatori et al., 1995; Kirsch et al., 2000). We and other groups have provided evidence that central areas of the embryonic growth plate have diminished oxygen levels (Rajpurohit et al., 1996; Schipani et al., 2001). We have recently shown that HIF-1 is involved in the regulation of growth arrest and survival of embryonic growth plate chondrocytes in vivo (Schipani et al., 2001). The loss of HIF-

1α in growth-plate chondrocytes induced apoptotic cell death (Schipani et al., 2001). Apoptotic chondrocytes, normally restricted to the zone of terminal differentiation, were apparent specifically in central areas of the growth plate lacking oxygen. Our experimental mouse model suggested that HIF-1 might act as a survival factor for chondrocytes by regulating the expression of glycolytic enzymes and cell-cycle regulators.

To test our hypothesis that HIF-1α-mediated alterations in energy generation of chondrocytes might lead to an insufficient expression of type-II collagen and aggrecan, we cultured murine growth-plate chondrocytes and deleted their HIF-1α gene. In addition, we determined the role of HIF-1α in energy production and cell growth under normoxic and hypoxic conditions in vitro. We found that HIF-1 has an essential and unexpected role in extracellular matrix synthesis. This, coupled to its role in chondrocyte survival, makes it a factor of central importance in cartilage morphogenesis.

Materials and Methods

Chondrocyte isolation and culture

Chondrocytes were isolated from newborn mice as described by Shakibaei (Shakibaei, 1995). In brief, forelimbs and hindlimbs were dissected and hand, skin and particularly muscles removed. The epiphyses were microdissected and placed in HBSS (Gibco BRL). Epiphyses were digested in 0.25% trypsin containing EDTA for 30 minutes at 37°C (without Ca^{2+} and Mg^{2+}) and 195 U ml^{-1} collagenase in HBSS (Worthington). Chondrocytes were plated at a density of 4×10^5 cells per well of six-well-plates and grown in monolayer cultures in high glucose DMEM (Gibco BRL) supplemented with 5% fetal calf serum (FCS) (Hyclone) and 1% penicillin/streptomycin. Cells were cultured either with 0.5% or 20% oxygen, balanced with N_2 in a 3-Gas incubator (Sanyo) in a humidified atmosphere. Growth curves were determined by counting the chondrocytes with a haemocytometer (normoxia only) days 2, 4, 6 and 8 after plating. Cells were harvested after trypsinization with 0.1% trypsin containing EDTA (Gibco BRL). During all experiments, medium was changed daily (except during experimental exposure to hypoxia or normoxia).

Determination of deletion frequency by quantitative PCR

All murine newborns used displayed homozygous flanking of the *HIF-1α* locus by *loxP* sites as described in detail previously (Ryan et al., 1998; Sauer, 1998). At day 1 post-plating, adherent chondrocytes were infected with adenovirus containing either β-galactosidase or Cre recombinase (generously supplied by Frank J. Giordano, Yale University, New Haven, CT) to create wild-type chondrocytes (+/+) or HIF-1α-deleted cells (–/–), respectively. Cells were incubated with adenovirus containing medium for 24 hours, fresh medium was added and cells were allowed to recover for at least 24 hours before starting the experiments. Genomic DNA was prepared by digestion in 10 mM Tris-HCl pH 7.5, 100 mM NaCl, 10 mM EDTA, 0.5% SDS with 0.4 μg μl^{-1} proteinase K (Roche) overnight at 65°C. DNA was extracted with phenol-chloroform-isoamylaclohol (25:24:1, pH 8.2) and precipitated with three volumes of ethanol and half a volume of 6 M ammonium acetate. DNA was resuspended in 10 mM Tris, 1 mM EDTA, pH 8.0. Primer express software (Applied Biosystems) was used to design forward, reverse and fluorescein-dye-tagged oligonucleotides (Operon) for use in real-time PCR (Table 1). Loss of the conditional *HIF-1α* alleles was measured using 0.9 μM each forward and reverse primers, 0.25 μM fluorescein-dye-tagged oligonucleotide and TaqMan Universal Master Mix (Roche). Reaction conditions were: 95°C for 10 minutes, and 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The degree of excision was calculated by comparison of intact *HIF-1α* DNA relative to an unexcised gene, in this case c-*Jun*. Deletion efficiency was determined at 60, 80, 100 and 200 viral particles per cell [multiplicity of infection (MOI)]. By this approach, a MOI of 100 was found to result in deletion frequencies of 74.1% and therefore all further experiments were conducted using MOI 100. Cultures infected with adenovirus containing β-galactosidase (MOI 100) served as wild-type controls.

Lactic acid measurement

Supernatants from different chondrocyte cultures were collected after 22 hours under normoxic or hypoxic (0.5% oxygen) conditions. Lactic acid was determined by a colorimetric assay (Sigma) at 540 nm according to the manufacturer's instructions. Lactic acid levels were normalized to total protein content using the Bradford assay (Biorad).

ATP measurement

To measure ATP, chondrocytes were cultured 22 hours either with (20%) or without (0.5%) oxygen. The ATP Bioluminescence Assay Kit CLS II (Roche) was used. The assay is based on the light-emitting oxidation of luciferin by luciferase in the presence of extremely low levels of ATP. After collecting the chondrocytes by scraping, cells were centrifuged for 10 minutes at 500 ***g*** in the cold. Chondrocyte pellets were resolved in boiling 100 mM Tris buffer containing 4 mM EDTA. Boiling was continued for another 2 minutes in order to inactivate NTPases. Cell remnants were removed by a further centrifugation step at 1000 ***g***. Supernatants were separated and placed on ice. Determination of free ATP concentrations was as outlined in the manufacturer's protocol. Light emission was measured at 562 nm using a luminometer (Berthold). ATP levels were normalized to protein content using the Bradford assay (Biorad).

RNA isolation and RT-PCR

Chondrocytes were lysed by addition of RNA-Bee (Tel-Test, Friendswood, TX) directly to the six-well plates. Cell lysate was scraped and carefully pipetted into a tube. After supplementation with bromochloropropane, tubes were centrifuged at 14,000 ***g*** for 10 minutes at 4°C. After carefully pipetting the aqueous phase, RNA was precipitated with isopropanol. After a 13,000 ***g*** spin, the RNA pellet was washed in 70% ethanol. RNA yield was spectrophotometrically determined. A digestion step with DNase I (Gibco BRL) was introduced to avoid interference of genomic DNA with the PCR reactions. For reverse transcription, the Superscript First-Strand Synthesis System for reverse-transcription polymerase chain reaction

Table 1. Sequences of primers and probes

	Forward	Reverse	Probe
Aggrecan	TGAGAGAGGCGAATGGAACG	GCCCGAGGGTTCTAGCATG	AAAAAGGGCACCGTGGCCTGTGGAGACCCC
c-Jun	TGCATGCTATCATTGGCTCATAC	CACACCATCTTCTGGTGTACAGTCT	CCCGGCAACACACA-MGB
Type-II collagen	ACTTGCCAAGACCTGAAACTCTG	AAACTTTCATGGCGTCCAAGG	CACCCCGAGTGGAAGAGCGGAGACTACTGG
Glut-1	GGGCATGTGCTTCCAGTATGT	ACGAGGAGCACCGTGAAGAT	CAACTGTGCGGCCCCTACGTCTTC
HIF-1α	CTATGGAGGCCAGAAGAGGGTAT	CCCACATCAGGTGGCTCATAA	AGATCCCTTGAAGCTAG-MGB
PGK-1	CTGTGGTACTGAGAGCAGCAAGA	CAGGACCATTCCAAACAATCTG	TAGCTCGACCCACAGCCTCGGCATAT
VEGF	AGTCCCATGAAGTGATCAAGTTCA	ATCCGCATGATCTGCATGG	TGCCCACGTCAGAGAGCAACATCAC

(RT-PCR) (Gibco BRL) with random hexamer primers was used according to the manufacturer's instructions.

Real-time PCR

For PCR analyses, cDNAs from triplicate wells of three independent experiments (22 hours hypoxia or normoxia) were diluted to a final concentration of 10 ng μl^{-1}. For PCR reactions TaqMan Universal Mastermix (Applied Biosytems) was used. 50 ng cDNA was used as template to determine the relative amounts of mRNA by real-time PCR (ABI Prism 7700 sequence detection system) using specific primers and probes; *HIF* and *Jun* probes contained Minor-Groove-Binding elements (MGBs) (ABI Systems) for further thermal stabilization. The reaction was conducted for as follows: 95°C for 4 minutes, and 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. 18S rRNA was amplified as an internal control. Cycle threshold (Ct) values were measured and calculated by the Sequence detector software. Relative amounts of mRNA were normalized to 18S rRNA (Applied Biosystems) and calculated with the software program Microsoft Excel. Relative mRNA contents were calculated as $x=2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct=\Delta E - \Delta C$ and $\Delta E=Ct_{sample} - Ct_{18S}$ and $\Delta C=Ct_{control} - Ct_{18S}$.

Quantification of soluble VEGF isoforms in conditioned medium

Soluble vascular endothelial growth factor (VEGF) isoforms were determined by using the DuoSet ELISA development KIT for mouse VEGF (R&D Systems). Cell culture supernatants (wild-type and HIF-1α-null cells) from triplicates of three different experiments were harvested after exposure to either 22 hours of normoxia or hypoxia, centrifuged at 2000 ***g*** and stored at –20°C. Further VEGF ELISA was conducted according to the manufacturer's instructions. VEGF concentrations were normalized to protein content (Biorad).

Type-II collagen ELISA

For type-II collagen quantification the native type-II collagen detection kit was used (Chondrex). Wild-type and HIF-1α-null chondrocytes were exposed to 44 hours of hypoxia or normoxia. After removing the media, chondrocyte layer was washed with PBS twice. 0.5 ml 0.05 M acetic acid was added directly to the cell layer. Cells were harvested by scraping. Cells were transferred to a microcentrifuge tube and 50 μl 1% pepsin solution (in 0.05 M acetic acid) was added. Suspension was digested on a rotator overnight at 4°C. 50 μl of TSB (1 M Tris, 2 M NaCl, 50 mM $CaCl_2$, pH 7.8) was added and the pH was adjusted to 8.0. Following, suspension was incubated with 50 μl of 0.1% pancreatic elastase (Sigma) in TSB for 30 minutes at 37°C. Suspension was centrifuged at 10,000 ***g*** for 5 minutes. Supernatant was diluted 1:10 with sample dilution buffer solution. The further type-II collagen ELISA was conducted as recommended in the manufacturer's instructions. Type-II collagen concentrations were normalized to protein content (Biorad).

Quantification of alcian-blue stainable proteoglycans

Chondrocytes were cultured either with (20%) or without (0.5%) oxygen over 44 hours. Quantification of proteoglycans was conducted as described by Kitaoka et al. (Kitaoka et al., 2001). In brief, cells were fixed with 10% neutral buffered formalin for 10 minutes at room temperature. After washing the cells twice with PBS, cells were incubated with 3% acetic acid for 10 minutes. Proteoglycans were stained with 1% alcian blue in 3% acetic acid (pH 2.5) for 30 minutes at room temperature. After washing the cell layer twice, alcian blue was extracted with 500 μl dimethyl sulfoxide. Absorbance was measured at 650 nm.

Statistical analysis

Data are given as mean ± standard deviation. For inference, the statistical analysis software program StatView was used. Statistical differences were identified using the unpaired Student's *t* test. $*P \leq 0.05$, $**P \leq 0.01$.

Results

Epiphyseal chondrocytes lacking HIF-1α are growth retarded

Initially, we determined the growth rate of wild-type cells compared with chondrocytes with cre/*loxP*-mediated deletion of HIF-1α. Wild-type chondrocytes revealed a higher proliferation rate than HIF-1α-null chondrocytes, with decreases in cell number during the exponential growth phase (Fig. 1A). Next, we analysed cell shape at 20% oxygen. Between days 1 and 4 of chondrocyte culture, we detected more polygonal and, in particular, bipolar stretched chondrocytes in HIF-1α-null cultures (Fig. 1B,C). After day 5 post-plating, wild-type and HIF-1α-null cells were mostly confluent and the chondrocytes appeared to be round or polygonal in wild-type and mutant cultures, respectively (Fig. 1D,E).

HIF-1α is required for energy generation by epiphyseal chondrocytes

To determine whether chondrocytes lacking HIF-1α display deficient energy generation, we measured free ATP levels in cells exposed to different oxygen levels. We have recently shown that primary wild-type fibroblasts generate increased ATP levels under hypoxia than under normoxia. Additionally, HIF-1α-null fibroblasts showed a reduction of ~50% of free ATP (Seagroves et al., 2001). Interestingly, primary chondrocytes displayed a slight increase in free ATP in hypoxia compared with cells cultured at 20% oxygen (Fig. 2A). In primary chondrocytes lacking HIF-1α and exposed to 22 hours of hypoxia, a significant reduction of free ATP was seen.

We then measured lactic acid, the end product of anaerobic glycolysis. A previous study of cartilage explants suggested the existence of a negative Pasteur effect under hypoxic and anoxic conditions (Lee and Urban, 1997). However, our experiments did not confirm these results. Indeed, by contrast, we found a significant increase in lactic acid under hypoxic conditions, paralleling rising levels of free ATP and suggesting an enhanced glucose use by anaerobic glycolysis (Fig. 2B). In primary chondrocytes with cre/*loxP*-mediated deletion of HIF-1α, we detected a significant reduction of lactic acid under hypoxia compared with wild-type cells. This suggests that the increased glycolytic rate seen in wild-type cells is mediated by HIF-1α. In addition, HIF-1α-null chondrocytes exposed to 20% oxygen revealed a moderate decrease in lactic acid concentration, indicating that glycolysis is also contributing to energy generation in epiphyseal chondrocytes under aerobic conditions.

HIF-1α increases expression of Glut-1, PGK-1 and VEGF

To help to determine whether increased levels of ATP in wild-type chondrocytes under 0.5% oxygen are generated mainly by anaerobic glycolysis, real-time PCR analyses of phosphoglycerate kinase-1 (PGK-1) and glucose transporter 1

(Glut-1) transcripts were conducted. As demonstrated in Fig. 3A, we detected a significant increase in PGK-1 mRNA levels under hypoxia compared to normoxia. HIF-1α-null chondrocytes showed a complete loss of the induced increase in PGK-1 message. Mutant chondrocytes also had significantly reduced PGK mRNA under normoxic conditions. Similar results were obtained for Glut-1 transcripts (Fig. 3B), supporting the hypothesis that HIF-1α is responsible for sustaining glucose use and glucose uptake in low oxygen tension conditions. It has been previously shown that VEGF is a target gene of HIF-1α-mediated hypoxic response (Semenza, 1998; Semenza, 2000). In addition, VEGF is known to be expressed by chondrocytes during normal development and pathological conditions such as osteoarthritis (Gerber et al., 1999; Pfander et al., 2001). Previous studies have clearly established the importance of VEGF for metaphyseal angiogenesis, thus contributing to the maintenance of oxygen levels in growth plate and surrounding tissues (Carlevaro et al., 2000; Gerber et al., 1999). We detected a strong induction of VEGF mRNA levels in normal chondrocytes exposed to 0.5% oxygen (Fig. 3C). The increase in VEGF transcript levels was lost in HIF-1α-mutant cells. Furthermore, even chondrocytes with HIF-1α deletion cultured under normoxic conditions showed reduced VEGF mRNA. Similar results were obtained examining protein by determination of soluble VEGF concentrations in conditioned medium using a VEGF-ELISA (Fig. 4).

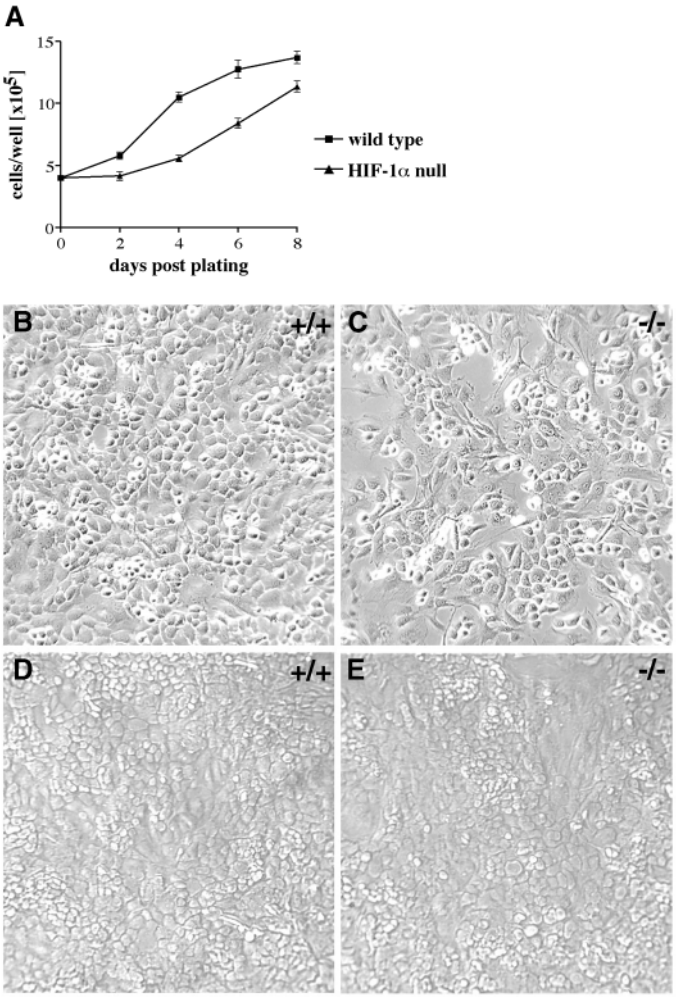

Fig. 1. Growth and morphology of wild-type and HIF-1α-null epiphyseal chondrocytes. (A) The growth curves of wild-type chondrocytes compared with cells with Cre-recombinase-induced deletion of HIF-1α. The increase of HIF-1α-null chondrocytes (exponential phase) is strongly retarded compared with wild-type cells. All data points are representing means ± standard deviations of duplicates from two independent experiments. All cells were infected with MOI 100 of adenovirus containing either Cre recombinase or β-galactosidase. In the lower panel, wild-type chondrocytes (+/+) on days 3 (B) and 5 (D) post-plating are compared with mutant chondrocytes lacking HIF-1α (–/–) (C,E). For the first few days in mutant cultures, single chondrocytes showed a bipolar or stretched cell shape. At day 5 post-plating, chondrocytes from both cultures were mostly confluent and appeared round or polygonal (D,E).

Extracellular matrix synthesis is controlled by HIF-1α under hypoxia

Finally, to test whether HIF-1α-mediated alterations in energy production affect the expression of the main cartilage matrix

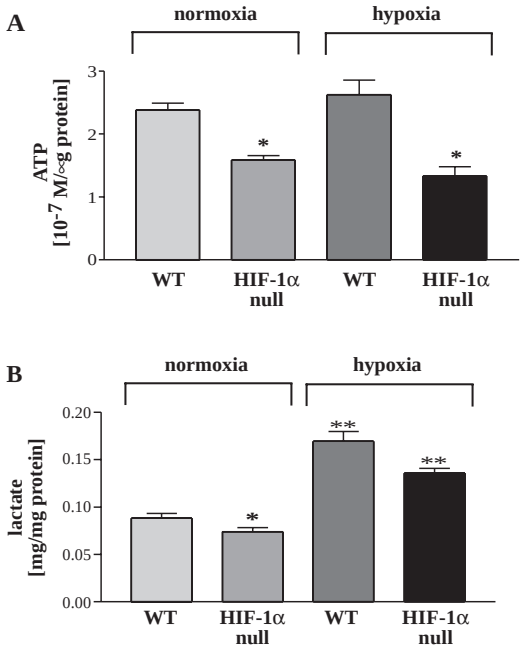

Fig. 2. Free ATP and lactate levels of wild-type and mutant chondrocytes cultured under normoxia and 22 hours hypoxia. (A) Bars representing means ± standard deviations of free ATP-levels measured in three independent experiments, normalized to protein content. Free ATP levels were determined in mutant and wild-type cultures under normoxia and hypoxia. (B) Lactate levels in supernatants of wild-type chondrocytes cultured at 0.5% oxygen were significantly increased compared with normoxic levels. Reduced lactate concentrations were detected in supernatants of HIF-1α-null chondrocytes under normoxia and hypoxia. Bars are representing the means ± standard deviations of measured triplicates from three independent experiments, normalized to protein concentrations. Statistical differences were calculated as: wild-type normoxia vs HIF-1α-null normoxia; wild-type hypoxia vs HIF-1α-null hypoxia; wild-type normoxia vs wild-type hypoxia. *$P \leq 0.05$; **$P \leq 0.01$.

proteins, we determined mRNA levels of type-II collagen and aggrecan. The gene expression profile of aggrecan was closely related to chondrocytic energy generation (Fig. 5A). In HIF-1α-null chondrocytes, aggrecan mRNA levels were 51% of wild type under hypoxia, whereas only an 18% reduction was detected under ambient conditions. To test further whether decreased transcript levels of aggrecan affected overall proteoglycan synthesis, we measured the amounts of extractable proteoglycans produced by cultured chondrocytes. Significantly more extractable proteoglycans were detected in HIF-1α-null than in wild-type cultures after 44 hours exposure to 0.5% oxygen (Fig. 5B). Type-II collagen mRNA levels were also diminished in mutant cells under hypoxia, to ~76% of wild-type levels (Fig. 6A). Moreover, in accordance with a previous study on articular chondrocytes (Hansen et al., 2001), we measured strongly increased concentrations of type-II collagen in wild-type cultures under hypoxia compared with normoxia (Fig. 6B). To determine whether the reduced expression of extracellular matrix components in HIF-1α-mutant cells under hypoxic conditions was due to a failure in activating glycolysis, we treated chondrocytes with 2-deoxyglucose (2-DG), a well characterized chemical inhibitor of glycolysis. Incubation with 2-DG resulted in a highly significant reduction of type-II collagen expression under both normoxic and hypoxic conditions (Fig. 7), further supporting the central importance of glycolytic energy production for matrix synthesis.

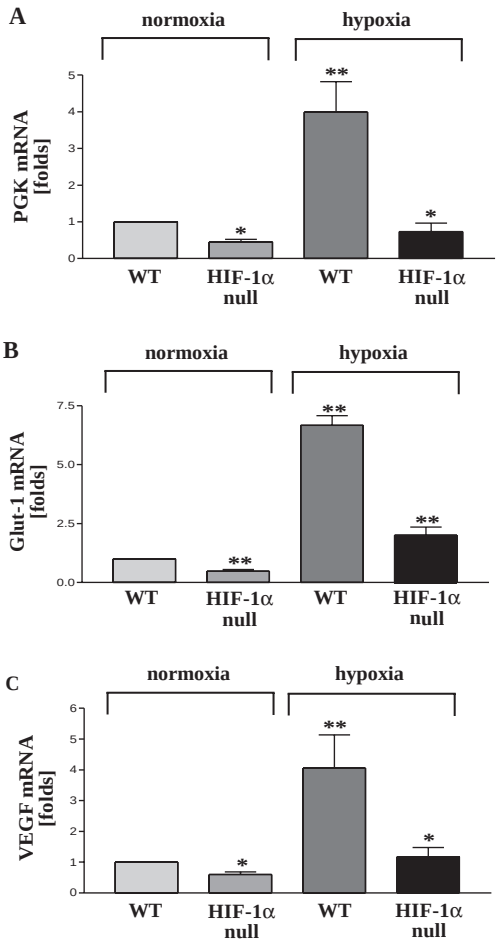

Fig. 3. Quantification of phosphoglycerate kinase-1, glucose transporter-1 and VEGF mRNA levels in wild-type and HIF-1α-null chondrocytes. Chondrocytes were exposed to either 0.5% or 20% oxygen for 22 hours. (A) Bars represent the severalfold changes in PGK-1 mRNA levels compared with wild-type chondrocytes under normoxia (onefold expression). (B) Bars represent the severalfold changes in Glut-1 mRNA levels compared with wild-type chondrocytes under normoxia (normoxic expression taken as 1.0). (C) Bars represent the severalfold changes in VEGF mRNA levels compared with wild-type chondrocytes under normoxia (onefold expression). Data are given as means ± standard deviations (triplicates from three independent experiments). Statistical differences were calculated as: wild-type normoxia vs HIF-1α-null normoxia; wild-type hypoxia vs HIF-1α-null hypoxia; wild-type normoxia vs wild-type hypoxia. *$P \leq 0.05$; **$P \leq 0.01$.

Discussion

Virtually all animal cells and tissues shift to some extent from mitochondrial respiration to anaerobic glycolysis for energy production when exposed to low oxygen levels. In previous studies, we and other groups have provided evidence that the embryonic growth plate of developing long bones is hypoxic (Rajpurohit et al., 1996; Schipani et al., 2001). Even chondrocytes in the deep zone of articular cartilage are in a hypoxic microenvironment, suggesting that chondrocytes require extensive adaptive mechanisms for low oxygen levels (Marcus, 1973; Silver, 1975).

In a previous study, we found that deletion of HIF-1α in the cartilage led to spatially unrestricted cell division and ultimately necrosis in the embryonic growth plate (Schipani et al., 2001). To analyse the role of HIF-1α in cartilage biology in an in vitro setting, we isolated chondrocytes from murine neonatal tissue. This allowed a more controlled evaluation of the role of HIF-1α in the growth and survival of chondrocytes under conditions of differential oxygenation.

In contrast to our previous study, in which we found uncontrolled cell growth of HIF-1α-null chondrocytes, mutant cells in vitro displayed a retarded exponential growth phase. This discrepancy might be explained by the fact that

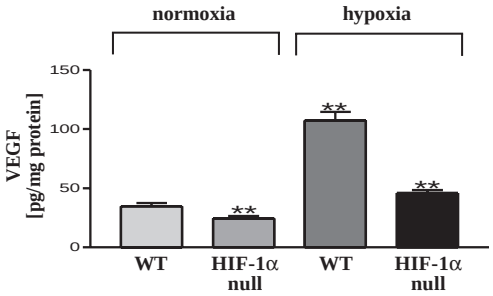

Fig. 4. Quantification of soluble VEGF isoforms. Soluble VEGF isoforms (VEGF 120 and VEGF 164) were determined in conditioned medium after either 22 hours hypoxia or normoxia. Bars represent the means ± standard deviations (triplicates from three independent experiments, normalized to protein concentrations). Statistical differences were calculated as: wild-type normoxia vs HIF-1-null normoxia; wild-type hypoxia vs HIF-1-null hypoxia; wild-type normoxia vs wild-type hypoxia. **$P \leq 0.01$.

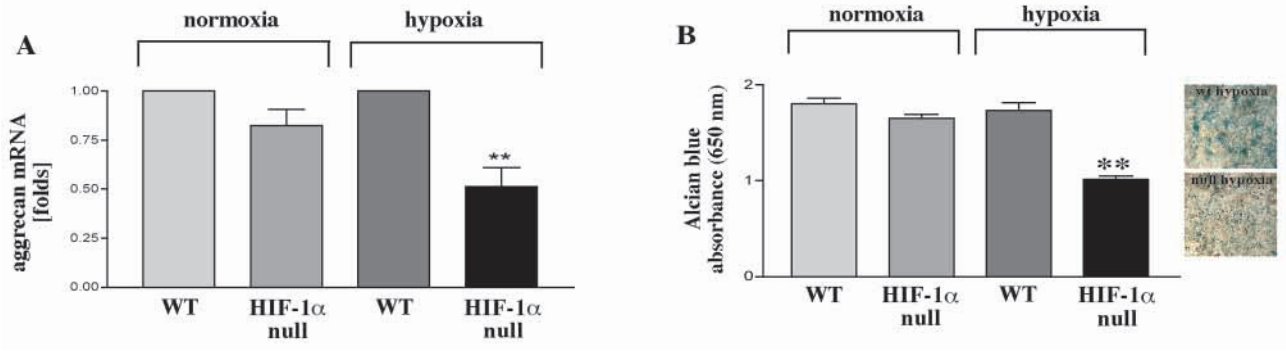

Fig. 5. Quantification of extracellular matrix expression in wild-type and HIF-1α-null chondrocytes. Epiphyseal chondrocytes were cultured under either 0.5% or 20% oxygen for 22 hours. (A) Analysis of aggrecan gene expression: Bars represent -fold changes of aggrecan mRNA levels in HIF-1α-null cells compared with wild-type levels. (B) Alcian-blue staining of extracellular proteoglycans. Bars represent means of alcian-blue absorbance ± standard deviations of wild-type and HIF-1α-null cultures exposed to normoxia or hypoxia (duplicates from three independent cultures). Data are given as mean ± standard deviations (triplicates from three independent experiments) and statistical differences were calculated as: wild-type normoxia vs HIF-1α-null normoxia; wild-type hypoxia vs HIF-1α-null hypoxia; wild-type normoxia vs wild-type hypoxia. ***P*≤0.01.

chondrocytes in vitro are showing the typical cell growth phases, whereas growth plate chondrocytes in vivo undergo strictly temporal and spatial events such as proliferation, differentiation, cell cycle arrest and apoptosis. However, no major changes in chondrocytic cell shape were detected in mutant chondrocyte cultures.

We found that the generation of free ATP in wild-type chondrocytes is slightly increased under hypoxic conditions, suggesting a highly effective adaptation of chondrocytes to hypoxic microenvironments. Support for the hypothesis that chondrocytes are able to exist at low oxygen tensions is provided by Stockwell et al., who showed in a previous study that the per-cell oxygen consumption of chondrocytes is less than 10% of hepatocyte oxygen consumption (Stockwell, 1983).

We found that free ATP levels were significantly reduced in hypoxic chondrocytes lacking HIF-1α, to 51% of that of wild-type cells. Even under normoxic conditions, null chondrocytes showed ~33% reduced ATP levels compared with their wild-type counterparts. In accordance with these findings, articular chondrocytes have been found to have 70-80% reduced ATP levels under normoxia, using antimycin as an inhibitor of mitochondrial oxidative phosphorylation (Johnson et al., 2000). These results provide evidence that HIF-1α might act as an essential element controlling a minor part of aerobic and a major part of anaerobic energy production of epiphyseal chondrocytes. To characterize the metabolic activity of primary chondrocytes further, levels of lactate (the end product of anaerobic glycolysis) were determined. As seen in our previous studies of HIF-1α-null fibroblasts, we here again demonstrated decreased lactic acid levels in mutant cultures compared with wild-type cultures under hypoxic conditions (Seagroves et al., 2001). We further show that hypoxia strongly induces the mRNA expression of PGK-1 and Glut-1 (by 4- and 6.7 times, respectively), and show that this induction is completely dependent on functional HIF-1α. These results clearly indicate that HIF-1α is required for energy production in epiphyseal chondrocytes. In addition, a significant reduction of PGK-1 and Glut-1 mRNA levels was detected under normoxic conditions, suggesting an important role for HIF-1α in controlling basal glucose metabolism.

In cartilage, VEGF is mainly expressed by hypertrophic growth-plate chondrocytes and articular chondrocytes (Horner et al., 1999; Pfander et al., 2001). In addition to its well-characterized angiogenic properties, VEGF is known to play a crucial role in long-bone development (Gerber et al., 1999). The factor acts as a paracrine and autocrine mediator, centrally involved in metaphyseal angiogenesis and new bone formation

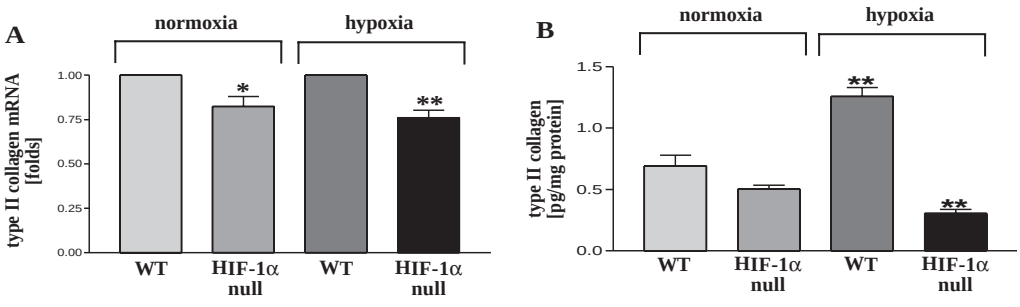

Fig. 6. Analysis of type-II collagen expression in normal and HIF-1α-deficient chondrocytes. Epiphyseal chondrocytes were cultured under normoxic or hypoxic conditions for 22 hours. (A) Analysis of type-II collagen gene expression. Bars represent -fold changes of type-II collagen mRNA levels in HIF-1α-null compared with wild-type chondrocytes (normoxic expression taken as 1.0). Data are given as mean ± standard deviations (triplicates from three independent experiments). (B) Quantification of type-II collagen protein. Bars represent means ± standard deviations (triplicates from three independent experiments, normalized to total protein content). Statistical differences were calculated as: wild-type normoxia vs HIF-1α-null normoxia; wild-type hypoxia vs HIF-1α-null hypoxia; wild-type normoxia vs wild-type hypoxia. **P*≤0.05; ***P*≤0.01.

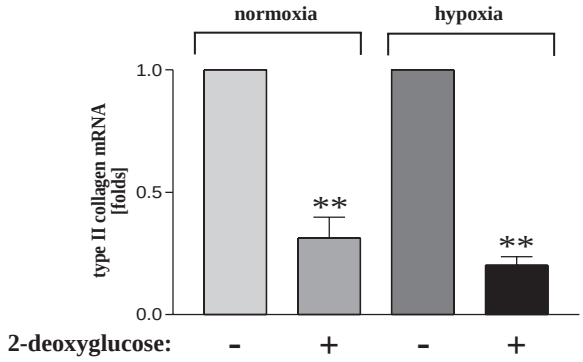

Fig. 7. Effect of chemical glycolysis inhibition on type-II collagen expression. Wild-type epiphyseal chondrocytes were incubated with or without 0.2 M 2-deoxyglucose, cultured under normoxic or hypoxic conditions for 22 hours, and type-II collagen gene expression was analysed by quantitative real-time PCR. Bars represent -fold changes of mRNA levels compared with untreated values (normoxic expression taken as 1.0). Data are given as mean ± standard deviations (triplicates from three independent experiments). $^{**}P \leq 0.01$.

(Carlevaro et al., 2000). In our previous study, we have clearly shown that VEGF expression is markedly but not completely depressed in growth-plate chondrocytes lacking HIF-1α in vivo (Schipani et al., 2001). Interestingly, although wild-type chondrocytes in vitro demonstrate increased VEGF synthesis during hypoxia, this increase is completely lost in HIF-1α-null cells, suggesting that VEGF regulation in vivo is more complex and influenced by additional mechanisms (e.g. cell-cell or cell-matrix interactions).

Finally, in order to determine whether diminished energy production during hypoxia affects gene expression of matrix molecules, we analysed mRNA and protein levels of aggrecan and type-II collagen. In the absence of HIF-1α, aggrecan expression is strongly decreased at the mRNA and protein levels during diminished oxygen delivery. In murine embryos lacking HIF-1α, strongly reduced type-II collagen signals were detected specifically in central hypoxic areas of the growth plate, where energy limitations are thought to be most evident (Schipani et al., 2001). Protein and mRNA expression levels of type-II collagen were strongly reduced in mutant cells exposed to hypoxia; this demonstrates that hypoxia-induced matrix gene expression requires the presence of HIF-1α.

In summary, we have demonstrated the importance of HIF-1α to sustain cell growth, to maintain energy generation and to allow matrix molecule expression in the hypoxic microenvironment. This provides evidence for a novel and exciting role for the transcription factor, in production and maintenance of extracellular matrix in the critically important central region of the growth plate of cartilage. Further study of this phenomenon will allow a better understanding of how cartilage formation occurs in the context of the challenges of hypoxia.

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