

# Mechanisms of Chondrocyte Survival and Matrix Synthesis During Hypoxia

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**Abstract:** Growth-plate cartilage and articular cartilage are virtually avascular tissues. Thus, chondrocytes must exist in extreme microenvironmental conditions, most prominently characterized by low oxygen tension. Diffusion distances for oxygen and nutrients between arteries and single chondrocytes range from 50µm to 3mm. Therefore, chondrocytes need specific strategies to adapt to these hostile conditions. Furthermore, they have to synthesize ATP in order to fulfill their main function, i.e. the synthesis of proteoglycans and type II collagen. In recent years increasing evidence for a pivotal role of the transcription factor hypoxia inducible factor-1α (HIF-1α) in cartilaginous tissues has been published. Murine growth-plates with functionally inactivated HIF-1α displayed great defects in their central areas caused by massive cell death. This very important observation points out that HIF-1α is absolutely necessary for chondrocytes to survive developmental hypoxia. Furthermore, it has been shown that HIF-1α has important functions for the regulation of anaerobic energy generation and matrix synthesis. Beside hypoxia, which seems to be more pronounced during osteoarthritis, other factors like pro-inflammatory mediators are able to activate HIF-1α in chondrocytes. Thus, an increasing dependence of OA chondrocytes on the adaptive functions of HIF-1 is reasonable to assume. In this review we will summarize the knowledge about HIF-1α for chondrocyte survival and matrix synthesis of growth-plate and articular cartilage during development and disease.

**Keywords:** Cartilage, OA, HIF-1, hypoxia.

## INTRODUCTION

Oxygen is a necessary prerequisite for oxidative phosphorylation (OXPHOS), which represents the most efficient way to generate ATP. Oxygen levels progressively decrease over distance from blood vessels [1]. This implies that most living cells must possess mechanisms to adapt their energy generation from OXPHOS to anaerobic energy synthesis. Sudden loss of physiological oxygen levels, as in coronary heart disease or brain infarction, leads to massive cell death within these tissues. On the other hand, certain tissues and cells are able to survive in hypoxic or nearly anoxic environments. The most impressive cells in this regard are human articular and growth-plate chondrocytes which are embedded in an avascular extracellular matrix with extremely long diffusion distances from the nourishing arteries [2-5].

It is widely assumed that a distance of 200µm from the closest vessel renders the ATP-synthesis by OXPHOS insufficient due to a lack of oxygen [6]. This implies that living in these environmental conditions requires the existence of specific factors controlling genes necessary for glucose metabolism, energy metabolism, pH regulation and numerous

other cellular hypoxia responsive genes that allow anaerobic energy generation and ultimately cellular survival [7]. The most important member of these factors is the hypoxia-inducible-factor-1 (HIF-1), a heterodimeric transcription factor [7,8]. HIF-1 belongs to the family of basic helix-loop-helix and PER-ARNT-SIM (PAS) domain transcription factors [9]. The α-subunit confers oxygen responsiveness of HIF-1, whereas the β-subunit, also called aryl hydrocarbon receptor nuclear translocator (ARNT), is constitutively expressed. Besides HIF-1α two further α-subunits are known, which are considered to play an inferior role in the cellular response to low oxygen environments. Under normoxic conditions HIF-1α is undetectable in nuclear extracts of most cells, whereas oxygen levels lower than 3% lead to an increased nuclear accumulation of HIF-1α and expression of HIF-1 target genes [10]. Accumulation of HIF-1α and HIF-1 target gene expression is tightly regulated by three iron-dependent prolyl-4-hydroxylases that initiate its degradation through the proteasome after coupling to the von Hippel-Lindau protein (VHL) [11,12].

The importance of HIF-1 in the hypoxic response is underlined by the fact that more than 50 genes are upregulated more than 3-fold by activity of the transcription factor [13]. These hypoxia inducible genes are involved in glucose uptake, glucose metabolism, pH regulation, angiogenesis, vascular tonus, cell growth, apoptosis, cytoskeletal structure, iron metabolism and matrix synthesis [10,14]. Beside low

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oxygen levels, pro-inflammatory cytokines and mechanical stress are potent stimuli to stabilize HIF-1 $\alpha$  and enhance DNA-binding of the transcription factor [15,16]. Several studies have clearly shown that HIF-1 is of pivotal importance in promoting tumour progression, inflammation processes and in the immunological defence against bacterial infections [17-19].

Therefore an important role of HIF-1 in chondrocyte biology is reasonable to assume. In this review we will summarize the knowledge about HIF-1 in chondrocyte survival and matrix synthesis of growth-plate and articular cartilage during development and disease.

### **ROLE OF HYPOXIA AND HIF-1 IN CELL SURVIVAL AND MATRIX SYNTHESIS OF GROWTH-PLATE CHONDROCYTES**

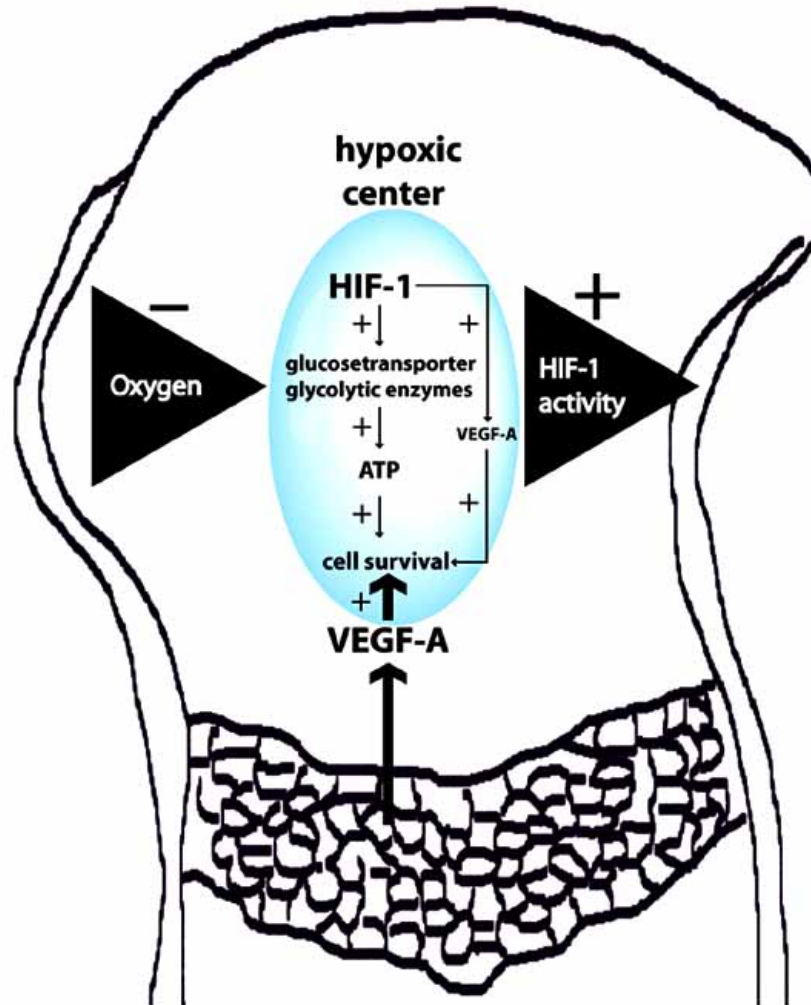
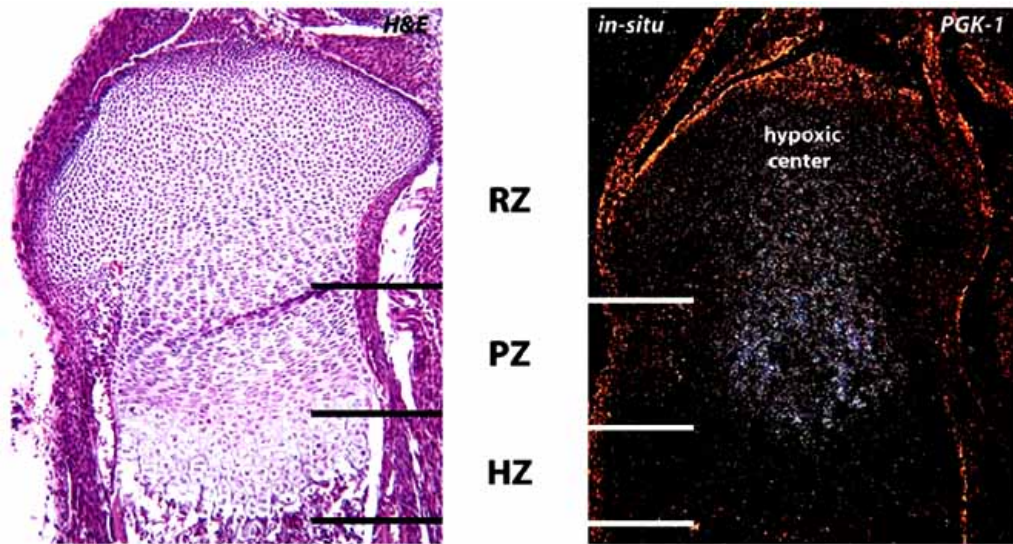
In order to study the physiological function of HIF-1 $\alpha$  in development and tissue growth, several HIF-1 knock out (KO) models were analyzed. Unfortunately, mice with functional inactivation of both HIF-1 $\alpha$  alleles died at mid gestation and revealed a strong retardation of vascularization [20,21]. Thus tissue specific HIF-1 $\alpha$  KO models were introduced to further explore the functional activities of HIF-1.

During endochondral ossification growth-plate chondrocytes undergo well characterized steps of proliferation and differentiation. Round and proliferative chondrocytes synthesize type II collagen and subsequently differentiate into hypertrophic cells, which predominantly express type X collagen [22,23]. Finally, post-hypertrophic terminal differentiated chondrocytes localized next to the chondro-osseous border release matrix vesicles, mineralize their surrounding matrix and eventually undergo apoptosis [24]. This spatially and temporally controlled process is followed by vascularization and replacement of the mineralized cartilage matrix by trabecular bone [22]. Whereas the growth-plate is an avascular tissue, the surrounding perichondrium possesses a high amount of vessels. These perichondrial vessels are the source of oxygen, which diffuses into the growth plate driven by the oxygen gradient. In a landmark study Schipani and colleagues demonstrated for the first time the importance of the transcription factor HIF-1 $\alpha$  in growth plates of mice [25]. In the first set of experiments they visualized hypoxic areas in the center of the resting and proliferating zone by nitroimidazole (EF-5) immunostaining, a marker of bio-reductive activity. They provided clear experimental evidence that the growth-plate is hypoxic with an outside-inside gradient of oxygenation (Fig. 1). Mice with functional inactivation of HIF-1 $\alpha$  in all cartilaginous elements died immediately after birth. These mice displayed great defects in epiphyses of long bones, ribs and vertebral bodies, resulting from massive cell death [25]. Obviously, epiphyseal chondrocytes are not able to survive this unique developmental hypoxia in the absence of HIF-1 (Fig. 1). In addition, the typical zonal architecture of growth plates was disturbed and gene expression of type II collagen was greatly reduced. However, chondrocytes around these defects displayed an increased number of actively proliferating cells. Based on *in situ* hybridization analyses Schipani and colleagues hypothesized that down-regulation of the *p57<sup>kip2</sup>* gene, a key regulator of chondrocyte cell growth arrest, might be responsible

for the increase of mitotic cells. Furthermore, in cultured growth-plate chondrocytes functional inactivation of HIF-1 $\alpha$  resulted in a significantly hampered energy generation [26]. It was clearly shown that growth plate chondrocytes exposed to 1% oxygen switch their ATP-generation from OXPHOS to anaerobic energy generation by increasing synthesis of glucose transporters and key glycolytic enzymes, necessary for anaerobic ATP-synthesis. Interestingly, chondrocytes are able to slightly increase free ATP levels under hypoxic conditions compared to ambient conditions in the presence of high levels of glucose. Deletion of HIF-1 $\alpha$  led to a significant reduction of ATP levels and a complete loss of the hypoxia-elucidated gene expression, suggesting that HIF-1 displays a fundamental prerequisite for chondrocytic energy generation in low oxygen environments [26]. Unexpectedly, deletion of HIF-1 $\alpha$  significantly reduced gene expression of glucose transporter and glycolytic enzymes already under normoxic conditions, suggesting that HIF-1 is also involved in the cellular control of glucose uptake and utilization in chondrocytes at ambient conditions. Furthermore, synthesis of main matrix components depended on the presence of HIF-1. Proteoglycan synthesis remained stable in growth-plate chondrocytes exposed to 1% oxygen, but deposition of native type II collagen was significantly enhanced. Hypoxic chondrocyte cultures with functionally inactivated HIF-1 $\alpha$  displayed a significant reduction of proteoglycans and type II collagen. However, in the absence of functioning HIF-1 $\alpha$  type II collagen levels displayed a much more substantial reduction when compared to amounts of extractable proteoglycans [26].

In addition, hypoxic expression of vascular endothelial growth factor-A (VEGF-A), the most potent pro-angiogenic factor characterized to date, is mainly controlled *via* HIF-1. In the fetal growth plate VEGF-A synthesis is detectable predominantly in the hypertrophic zone (Fig. 1) and to a lesser extent in the proliferating and pre-hypertrophic zone [27,28]. Murine growth-plate chondrocytes express three VEGF-A isoforms, which result from alternative splicing of the single gene product. VEGF<sub>188</sub> interacts with heparin sulphate at the cell surface, whereas the soluble isoforms VEGF<sub>164</sub> and VEGF<sub>120</sub> diffuse freely through the matrix and are therefore able to deliver proangiogenic signals to target structures [29]. It has been shown that functional inactivation of HIF-1 leads to significantly decreased gene expression of VEGF-A in hypoxic environments [29]. Interestingly, even growth plates lacking the soluble VEGF isoforms display defects in the hypoxic center caused by cell death [30,31]. However, epiphyseal defects in these mice were smaller compared to central death areas of HIF-1 $\alpha$ -null growth-plates. *In vitro*, the phenomenon of central cell death in the HIF-1 $\alpha$ -null epiphysis could be partially rescued by supplementation of recombinant VEGF<sub>164</sub> [31]. Taken together these data point out that soluble VEGF-A isoforms are necessary for survival of central growth plate chondrocytes (Fig. 1). The underlying mechanisms of VEGF-A's role as survival factor have yet to be elucidated.

To further analyze the function of HIF-1 $\alpha$  we functionally inactivated the von Hippel-Lindau protein gene (*Vhlh*) in all cartilaginous elements of mice [32]. The newborn mice were viable at birth and displayed a severe dwarfism. Morphologically, *Vhlh* null growth-plates displayed the presence



**Fig. (1).** Role of HIF-1 $\alpha$  in energy generation and cell survival of growth-plate chondrocytes. Left upper panel demonstrates the unique zonal architecture of a murine growth plate with resting (RZ), proliferating (PZ) and hypertrophic (HZ) zone (stained with hematoxylin/eosin). Right upper panel reveals an *in situ*-hybridization for cDNAs of phosphoglyceratkinase-1 (PGK-1). Signals are restricted to the postulated hypoxic center of the murine growth-plate. The lower panel is demonstrating the role of HIF-1 $\alpha$  in controlling anaerobic energy generation and thereby cell survival within the center of the growth-plate. Beside HIF-1 dependent ATP generation, VEGF-A-synthesis by central and hypertrophic chondrocytes seems to be an important prerequisite for chondrocyte survival.

of enlarged and atypical chondrocytes in the resting and proliferating zone, a strongly reduced cell number and the disturbance of the typical zonal architecture [32]. By *in situ* hybridization analyses and histochemical staining we excluded that these atypical enlarged chondrocytes resulted from a premature differentiation process. As expected, *Vhlh* null growth-plates showed an accumulation of HIF-1 $\alpha$  as well as increased mRNA expression of HIF-1 target genes and type II collagen (Col2a). Even growth-plate chondrocytes with a deletion of VHL displayed slightly increased mRNA-levels of Col2a compared to wild-type cells [32]. To further analyze whether all observed alterations in the *Vhlh* null epiphysis resulted from increased transcriptional activity of HIF-1 $\alpha$ , *HIF-1 $\alpha$ /Vhlh*-null mice were created. These mice displayed a similar phenotype as the straight HIF-1 $\alpha$  null mice, proving the hypothesis that all seen effects in *Vhlh* null growth plates results from an increased transcriptional activity of HIF-1.

The observation of an increased collagen type II synthesis strengthened the idea that, besides its effects on energy generation, cell survival and matrix synthesis, HIF-1 $\alpha$  stabilizes the unique phenotype of chondrocytes. This hypothesis is supported by a recent publication, showing that hypoxia initiates a differentiation process towards a chondrocytic phenotype in pluripotential mesenchymal stromal cells [33]. Furthermore, articular chondrocytes exposed to low oxygen levels increased gene expression of the transcription factor SOX-9, a specific marker of chondrocytic differentiation (own unpublished data).

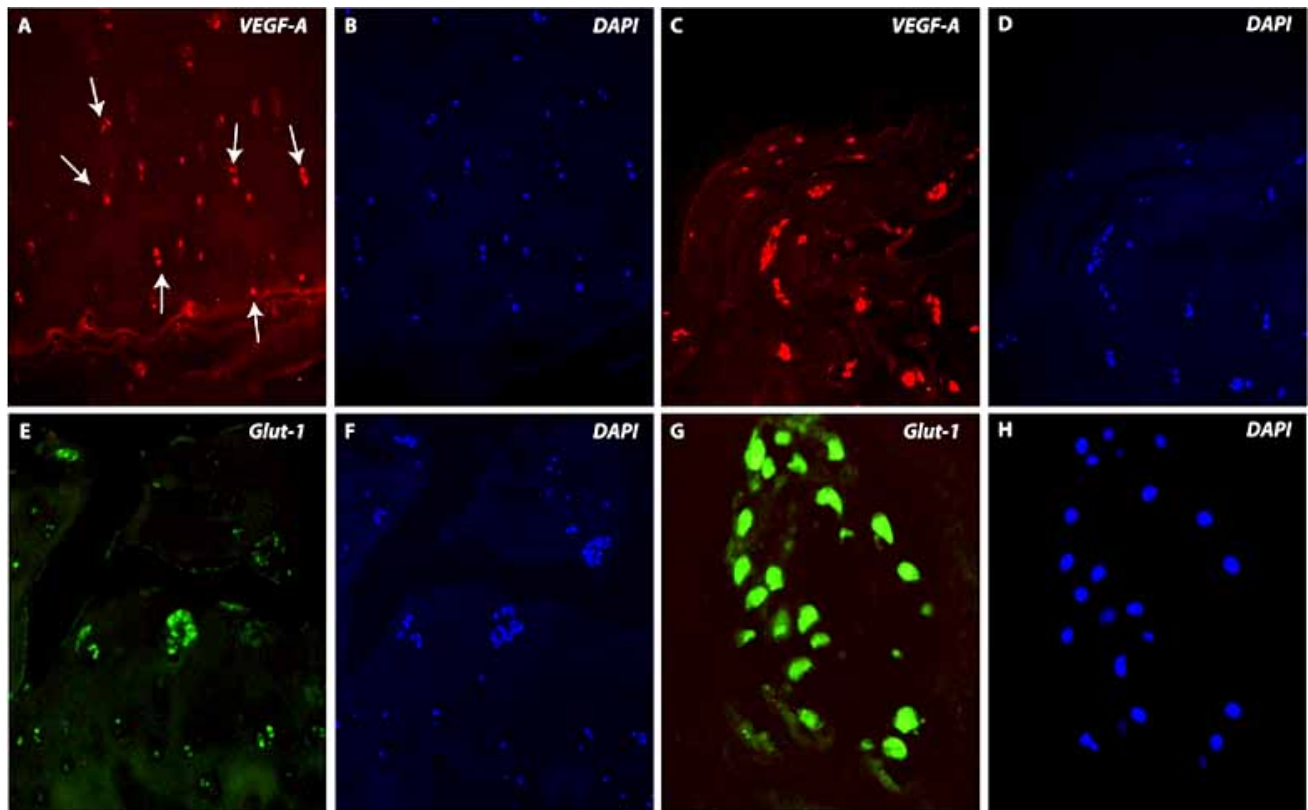
## ROLE OF HYPOXIA AND HIF-1 IN HEALTHY AND OA CARTILAGE

Articular chondrocytes have to exist in extremely demanding microenvironmental conditions [3,34]. They are the only living elements within an extensive extracellular matrix of cartilage. This highly specialized matrix consists mainly of proteoglycans, glycoproteins and a complex collagen network [35-38]. Articular cartilage is repeatedly deformed during joint movement and then able to recover from this deformation [39]. Articular chondrocytes receive nutrients and oxygen through their contact with synovial fluid, which washes across the cartilage surface propelled by the movement of the respective joint [40]. Unfortunately, the partial pressure of oxygen within this synovial fluid is very low and further decreased under pathological conditions, such as osteoarthritis (OA) or rheumatoid arthritis [41-44]. Thus, oxygen supply to articular chondrocytes is very limited. It has been calculated by Silver and colleagues that chondrocytes in the upper zones of healthy cartilage live in an environment of 6-10 % oxygen and this oxygen content drops to 1% next to the calcified cartilage [3]. In addition to that it has been calculated that oxygen concentrations are almost zero at a depth of 0.096 cm from the surface in compressed cartilage [45]. Animal experiments have further proven that the chondro-osseous junction is not permeable for gases [46]. This fact might explain the gradient of oxygenation from the surface to the calcified cartilage. Further studies have shown that oxygen consumption of articular chondrocytes under ambient conditions is only 2-5% of that of kidney or liver cells, suggesting that these cells are well adapted to very low oxygen levels [2].

This idea is further supported by a recent publication by Brucker and colleagues showing that HIF-1 $\alpha$  maintains tonic activity in deeper layers of intact cartilage [47]. In addition, HIF-1 $\alpha$  was perinuclearly stored and not completely degraded after removal of the top layers during normoxic conditions. Immediately after exposing cartilage explants to hypoxia a restoration of the nuclear HIF-1 $\alpha$  accumulation was detectable. These results provide convincing evidence that HIF-1 $\alpha$ -dependent gene expression is highly conserved in articular cartilage, specifically in deep zone chondrocytes. By real-time PCR analyses Yudoh *et al.* have demonstrated that HIF-1 $\alpha$  transcripts are increased in degenerated cartilage compared to macroscopically intact cartilage within one joint [48]. In addition, it has been shown by cDNA array technology that HIF-1 $\alpha$  transcripts are increased in mRNA extracts from OA compared to normal knee joints [49]. These novel and important findings are confirming our own studies and those of other groups on the presence and distribution of HIF-1 $\alpha$  and its target genes PGK-1, Glut-1 and VEGF-A, showing an increased number of chondrocytes stainable for the transcription factor and its target genes during OA (Fig. 2), where lowered oxygen levels are reasonable to assume [50-52].

But even high levels of pro-inflammatory cytokines and changes in mechanical loading, which are known to influence the initiation and progression of OA, are candidates to activate HIF-1-dependent gene expression [15,16]. As shown by Coimbra and colleagues, pro-inflammatory TNF- $\alpha$  leads to a substantially accumulation of HIF-1 $\alpha$  in chondrocyte cultures [53]. By inhibition of the transcription factor, NF- $\kappa$ B stabilization of HIF-1 $\alpha$  was abrogated, suggesting a potential link between inflammatory and hypoxic pathways [15,53]. Further analyses were conducted to clarify the impact of HIF-1 in promoting energy generation, matrix synthesis and cell survival of articular chondrocytes. During hypoxic conditions functional inactivation of HIF-1 $\alpha$  using RNA-interference in the presence of interleukin-1 $\beta$  resulted in a significantly increased number of apoptotic chondrocytes [48]. In addition, it has been proven that HIF-1 $\alpha$  is necessary for anaerobic energy generation and proteoglycan synthesis by articular chondrocytes, an observation that confirms our own data in growth-plate chondrocytes [26,48].

Since the work of Hansen and co-workers it is well established that even in cultured articular chondrocytes hypoxia increases the accumulation of type II collagen [54]. However, beside the synthesis of matrix destructive enzymes osteoarthritic chondrocytes are metabolically activated and increase gene expression of type II collagen and several other matrix components [55-58]. This activity of OA chondrocytes is generally appreciated as an aim to restore the ECM. In different studies with biochemical as well as molecular biology approaches, type II collagen synthesis was found to be increased between 4- and 7-fold during OA [56,59]. Beside type II pro-collagen synthesis post-translational modification processes are essential steps for collagen triple helix formation and secretion. Important members of involved enzymes are collagen prolyl-4-hydroxylases (C-P4H), which catalyze the formation of 4-hydroxyproline [60]. In humans, three isoforms of this  $\alpha_2\beta_2$ -tetrameric enzyme exist, namely prolyl-4-hydroxylase I ( $\alpha(I)_2\beta_2$ ), II ( $\alpha(II)_2\beta_2$ ) and III ( $\alpha(III)_2\beta_2$ ), which consist of different catalytic  $\alpha$ -subunits and identical  $\beta$ -subunits identi-



**Fig. (2).** HIF-1 target gene expression by normal and OA chondrocytes *in vivo*. Panel **A** is demonstrating an immunofluorescence staining of vascular endothelial growth factor-A (VEGF-A) by deep zone chondrocytes (arrowheads indicate positive chondrocytes) of healthy articular cartilage (Cy-3). Panel **B** shows the corresponding DNA-staining of the same slide by DAPI. Panel **C** displays a VEGF-A immunofluorescence staining of a severe OA cartilage sample (Cy-3). Panel **D** reveals the same slide stained for DNA (DAPI). Panel **E** shows an osteoarthritic cartilage slide stained for glucosetransporter-1 (Glut-1) using an immunofluorescence technique (FITC). Panel **F** displays the same slide stained for DNA (DAPI). Panel **G** demonstrates a high-power magnification of a chondrocyte cluster stained for Glut-1 using FITC as dye. Panel **H** displays the corresponding DNA-staining (DAPI).

fied as protein disulfide-isomerase (PDI) [61,62]. Our recently published data showed experimental evidence for enhanced synthesis of collagen prolyl-4-hydroxylases II during the development of OA, suggesting posttranslational modification processes as an additional causal factor of increased type II collagen accumulation in degenerative cartilage diseases [63]. Given the heterogeneous distribution of oxygen in normal articular cartilage and the appearance of extremely hypoxic areas in deep zones of healthy and probably more pronounced in OA cartilage, we analyzed the influence of *in vitro* hypoxia for type II collagen levels, HIF-1 $\alpha$  accumulation and C-P4H-synthesis in primary articular chondrocyte cultures. Increased type II collagen accumulation induced by 1% oxygen was accompanied by stabilization, nuclear translocation and increased activity of HIF-1 $\alpha$ . Furthermore, synthesis of C-P4H II was significantly enhanced under hypoxic conditions and completely abolished by chemical inhibition of HIF-1 [63].

These data suggest that the decrease of oxygen levels in synovial fluids from OA joints might accelerate the post-translational modification of type II collagen, thus contributing to the increased synthesis of collagen type II during OA.

## CONCLUSIONS

The results summarized here clearly show the pivotal role of HIF-1 $\alpha$  in long bone development, cartilage biology

and probably osteoarthritis. Recent publications in the field of developmental biology and cartilage research point out that HIF-1 $\alpha$  is a highly conserved transcription factor in all investigated cartilage tissues. HIF-1 $\alpha$  has key functions in controlling energy generation, cell survival and matrix synthesis by articular and growth-plate chondrocytes. Future studies should aim at outlining whether stabilization of HIF-1 $\alpha$  is a potential tool to increase cell vitality, energy generation and matrix synthesis and thereby cartilage integrity in osteoarthritis.

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