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REVIEW ARTICLE

Lack of oxygen in articular cartilage: consequences for chondrocyte biology

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Summary

Controlling the chondrocytes phenotype remains a major issue for cartilage repair strategies. These cells are crucial for the biomechanical properties and cartilage integrity because they are responsible of the secretion of a specific matrix. But chondrocyte dedifferentiation is frequently observed in cartilage pathology as well as in tissue culture, making their study more difficult. Given that normal articular cartilage is hypoxic, chondrocytes have a specific and adapted response to low oxygen environment. While huge progress has been performed on deciphering intracellular hypoxia signalling the last few years, nothing was known about the particular case of the chondrocyte biology in response to hypoxia. Recent findings in this growing field showed crucial influence of the hypoxia signalling on chondrocytes physiology and raised new potential targets to repair cartilage and maintain tissue integrity. This review will thus focus on describing hypoxia-mediated chondrocyte function in the native articular cartilage.

Keywords
cartilage repair, chondrocytes, hypoxia

Introduction

Chondrocytes are the only resident cell type of the articular cartilage [with exception of a probable stem cell niche (Dowthwaite *et al.* 2004)]. Their essential known function consists in secreting a large and very specific extracellular matrix. Although the exact composition of the matrix is not entirely known, it is established that type II Collagen and Aggrecan are the most abundant matrix macromolecules, which confer the two main biomechanical properties to that tissue: tension resistance and ability to withstand compression forces respectively (Muir 1995; Buckwalter & Mankin 1998). Other collagens are also part of the cartilage matrix: collagen type 6 located in the pericellular matrix (Söder *et al.* 2002), collagen type 9 and 11, and, COMP (Hedbom *et al.* 1992), leucin-rich proteins PRELP (Bengtsson *et al.* 2002) and other related molecules.

Chondrocytes are very difficult to study. One reason is because normal human cells and tissue are very difficult to obtain. Another major obstacle comes from the chondrocyte

instability when these cells are cultured *in vitro* (Von der Mark *et al.* 1977; Benya *et al.* 1978). Whereas they almost do not proliferate in cartilage, monolayer cultured primary chondrocytes start proliferating and de-differentiate with passage: they lose their round shape for a fibroblast-like shape and the main chondrocyte markers such as type II collagen (Col-2), Aggrecan or Sox9 rapidly drop (Glowacki *et al.* 1983). To date, Sox9 is considered as the key transcription factor controlling (in concert with L-Sox5 and Sox6) Col-2 and Aggrecan expression (Lefebvre *et al.* 1997, 1998). However, little is known about extracellular matrix gene regulation (Okazaki & Sandell 2004), and it is still unclear what controls the chondrocyte phenotype and what maintains a chondrocyte functional in adult cartilage. Answering these questions is thus crucial to understand patho-physiological situations where dedifferentiation occurs (such as OA) or to develop cartilage repair strategies based on autologous transplantation, where dedifferentiation also occurs.

Brief overview of what controls cartilage integrity

The list of extracellular signals controlling adult cartilage integrity has grown recently: Growth factors [anabolic factors such as TGF-beta, BMP… (Majumdar *et al.* 2001), or catabolic factors such as IL-1… (Kaiser *et al.* 2004)], were the most described. But crucial enzymes for cartilage degradation have been identified and also belong to these soluble factors such as metallo-proteinases [MMP-13 which degrades preferentially type II collagen (Knauper *et al.* 1996)] and aggrecanases [ADAMTS-4 and 5 which are the major cartilage degradation in OA (Tetlow *et al.* 2001; Glasson *et al.* 2005)]. It is accepted for long that the imbalance between these factors is the determinant of the cartilage integrity.

However, more recently, the role of other extracellular signals was shown: the 3D structure of the surrounding matrix [modulating chondrocyte cytoskeleton (Blain 2009)], and the matrix composition itself [modulating matrix assembly or the inflammatory response (Sjoberg *et al.* 2005; Halasz *et al.* 2007)]. Finally, it is now accepted that mechanical loading and hypoxia are two permanent stresses that impact dramatically the adult articular cartilage. Indeed, compression applied on cartilage is a potent regulator of matrix genes and chondrocyte physiology through mechano-transduction pathways. Exploration of hypoxia effect has only emerged recently with description of hypoxia intracellular signalling, although it was known for a long time that oxygen level was low in adult cartilage compared to other tissues (0.5–5% depending on depth) (Lund-Olesen 1970; Brighton & Heppenstall 1971). In fact, it now clear that hypoxia is a strong promoter of matrix deposition by the chondrocytes (Murphy & Sambanis 2001).

General overview of the HIF signalling pathway

In 1995, Semenza *et al.* discovered cells can sense surrounding oxygen level through Hypoxia–Inducible transcription Factors (initially named HIF1-α) whose expression and function are mainly post-translationally regulated by hydroxylation reactions (Semenza & Wang 1992; Semenza *et al.* 1994). Under high oxygen environment, these proteins have a very short half-life (<5 min). This is due to specific hydroxylated proline that are recognized by the Von Hippel-Lindau protein (pVHL-containing E3 ubiquitin ligase complex) and that targets them to degradation via the proteasome (Semenza 2000). Conversely, when oxygen level is low (5–1%), hydroxylation decreases and HIF-1α is prevented from a rapid degradation. Then HIF1-α heterodimerizes with the constitutively expressed HIF-1β (also called Aryl hydrocarbon Nuclear Translocator ARNT), translocates into the nucleus, and binds specific consensus sequences (-RCGTG-) on gene promoters (Figure 1).

More recently, *HIFs* family enlarged with the discovery of *Hif-2α*, whose product is highly similar to the other (~50% homology) (Ema *et al.* 1997). A higher complexity came with *Hif-3α* identification because this gene was shown to

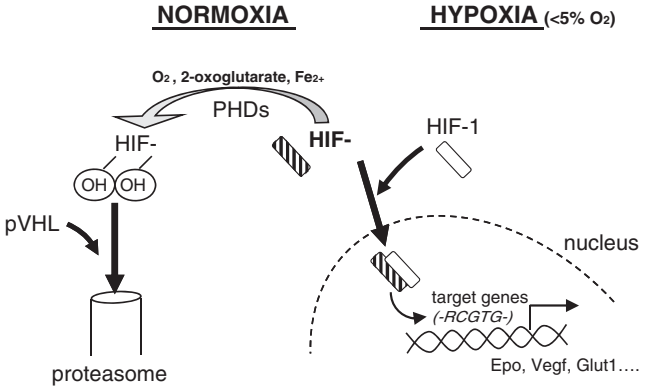

Figure 1 HIFs regulation and signalling. Hydroxylation of HIF-α is inhibited in low oxygen environment (less than 5%) because prolylhydroxylases (PHDs) activity is dependent on oxygen. Then non-hydroxylated HIF-α can heterodimerize with HIF1-β, and translocate into the nucleus, where it binds to consensus sequences.

produce at least six different proteins following alternative splicing (Maynard *et al.* 2003).

Hif-1α and *-2α* genes share the same organization, and thus give the same protein structure: a bHLH domain on the amino-terminal, an intermediate PAS domain (PER-ARNT-SIM) and a transactivation domain (TAD) (Sowter *et al.* 2003). The TAD contains one N-terminal TAD (NTAD) and one C-terminal domain (CTAD) (Yan *et al.* 2007). Unlike the others, HIF-3α lacks the TAD, suggesting it could act as a dominant negative of HIF-1α and -2α (Maynard *et al.* 2005).

But the 'real' oxygen sensors are the hydroxylases targeting the proline residues of HIFs (prolyl-hydroxylases, PHD-1 to 3), since they use oxygen as a co-factor and thus directly rely on oxygen level within the cell. *In vitro* experiments have shown that proline hydroxylation occurs on the NTAD (on the Pro^{402} and Pro^{564} of the human HIF-1α) in the oxygen degradation domain (ODD) (Jaakkola *et al.* 2001). Recently, it has been described that an asparagine residue located in the CTAD (on the Asn^{803} of the human HIF-1α) is also hydroxylated by an asparaginyl hydroxylase (Factor inhibiting HIF, FIH) inactivating HIF (Mahon *et al.* 2001; Lando *et al.* 2002). The recruitment of co-activator such as p300/CBP has been shown to be essential for its transcriptional activity (Lando *et al.* 2002).

Others post-translational modifications such as phosphorylation (by erk, AMP-activated Kinase) or acetylation (on the Lys^{532} of the human HIF-1α) were shown to increase its transcriptional activity (Richard *et al.* 1999) or to enhance its destabilization (Jeong *et al.* 2002) respectively but are still controversial.

HIF signalling applied to articular cartilage: role in chondrocyte differentiation and matrix deposition

Detection of the HIF protein in human articular cartilage is challenging, but some important findings describing the role of HIF-1α during mouse cartilage development have been performed. The first one came with Schipani *et al.* who demonstrated that the developmental growth plate was hypoxic: a cartilage specific depletion of HIF-1α was realized in mouse and showed an increase of chondrocyte death coupled with decreased expression of the CDK inhibitor *p57*, strongly suggesting HIF-1α was essential for chondrocyte growth arrest and survival (Schipani *et al.* 2001). Using epiphyseal chondrocytes from newborn mice lacking HIF-1α, it was proposed that VEGF is secreted within the growth plate, and thereby triggers survival signals (Cramer *et al.* 2004). Provot *et al.* recently detailed the role of HIF-1α during chondrogenesis in growth plate. Using a conditional knock-out, they showed early chondrogenesis (formation of cartilaginous primordiae) and joint formation were impaired (Provot *et al.* 2007). Because it was more recently discovered, HIF-2α is less documented, but it was shown to be elevated during chondrocyte differentiation accompanied by VEGF increase, suggesting a role in the metabolic shift in the growth plate (Stewart *et al.* 2006).

Interestingly, experiments on mice also suggested that HIFs not only participate to the differentiation of chondrocytes but also to the function of chondrocytes. Indeed, it was shown that HIF-1α increases matrix deposition such as type II collagen (Pfander *et al.* 2003). Moreover, deletion of pVHL in chondrocytes (which results in HIF-1α and -2α overexpression) increases matrix deposition during growth-plate development (Pfander *et al.* 2004).

In vitro studies demonstrated that hypoxia promotes articular cartilage function by increasing expression of cartilage matrix genes in bovine and human articular chondrocytes (HACs) (Murphy & Sambanis 2001; Domm *et al.* 2002), with similar results in human meniscal cells (Adesida *et al.* 2006). Hypoxia was shown to increase type II collagen and Aggrecan and the key transcription factor Sox9 (Murphy & Polak 2004). Identification of the mechanism was performed using siRNA in HACs. By selective HIF-1α and HIF-2α depletion in HACs placed in prolonged hypoxia, we have shown that HIF-2α and not HIF-1α is critical for Sox9 induction and, as a consequence for Collagen 2 expression (Lafont *et al.* 2007).

Role of PHDs in cartilage

As PHDs are the true oxygen sensors of hypoxia, these enzymes may be critical in controlling the chondrocyte phenotype.

All the PHDs are expressed in maturing zone of the mouse growth plate (Terkhorn *et al.* 2007). We observed PHD2 was the most expressed compared to PHD3 and 1, in HACs *in vitro* (Lafont *et al.* 2008; Murphy *et al.* 2009). Interestingly, PHD2 was shown by Pouysségur *et al.* to be the main hydroxylase regulating HIF-1α (Berra *et al.* 2003). Selective PHD activity for HIF-1α or HIF-2α has been already shown by Ratcliffe and Gleadle (Appelhoff *et al.* 2004), suggesting that PHD could have a tissue specific selective activity.

Because we have shown HIF-2α and not HIF-1α was involved in the control of the human chondrocyte phenotype (Lafont *et al.* 2007), it is now tempting to find out which PHD selectively controls HIF-2α expression in HACs.

Inhibiting one specific PHD in cartilage could thus lead, through the specific HIF-2α increase, to a better control of the chondrocyte phenotype without HIF-1α overexpression [which promotes angiogenic phenotype, favouring tumourigenesis (Fang *et al.* 2001), or induces catabolic cytokines such as Interleukin-1 (Zhang *et al.* 2006)]. *In vivo* approaches targeting HIFs proteins have already been performed: inhibition of HIF-1α expression has been obtained in mouse knee after intra-articular injection of the anti-angiogenic compound 2-methoxyestradiol (through inhibition of HIFs) (Gelse *et al.* 2008). As a result, authors described a progression of OA (osteophytes, signs of degeneration, loss of superficial matrix), showing that *in vivo* inhibition of HIFs signalling impairs cartilage integrity and validating the approach targeting HIF *in vivo* to control cartilage integrity.

Transcriptional regulation of chondrocytes genes by the HIFs

Study of the transcriptional regulation of chondrocyte genes by HIFs has just been started a few years ago. Sox9 is the first one to studied because its promoter contains several putative HIF Responsive Elements (HRE). To do so, mouse stromal cells (ST2) were transfected with a Sox9 promoter construct containing 6.8 kb. Authors showed an up-regulation of the promoter activity under hypoxia. When putative HRE sequences (located within the first 500 bp) were mutated, such an up-regulation was abolished especially with two of them (Robins *et al.* 2005). These results have been confirmed more recently in micromass culture. Amarilio *et al.* showed by chromatin immunoprecipitation, a recruitment of HIF-1α on the Sox9 promoter precisely on the same HRE (Amarilio *et al.* 2007). Whether HIF-2α directly binds to Sox9 promoter in humans remains unknown.

Very little is known about transcriptional mechanisms by which chondrocytes markers are regulated. Because articular cartilage is chronically hypoxic, HIFs proteins are permanently highly expressed within the chondrocytes, which must affect their transcriptional response to others stimulus. As a consequence, studying transcriptional regulation of chondrocytes genes by HIFs is the next crucial step.

How oxygen tension affects the chondrocyte biology

Despite several studies have been performed to understand how hypoxia could affect chondrocyte specific gene expression, none of them tried to describe the overall effect on the chondrocyte transcriptome. To answer this basic question and characterize the gene response of HACs to hypoxia, a large scale assay has been recently performed in our group using a microarray analysis (Lafont *et al.* 2008). A list of 101 chondrocyte specific and hypoxia responsive genes has been identified by comparing HACs in 20% oxygen with 1% oxygen. They were identified as down-regulated by passage (dedifferentiation) and up-regulated (redifferentiation) by hypoxia and thus are considered as possible chondrocytes markers/regulators.

The first finding of this large scale study reveals that all the already established chondrocytes markers (sox9, collagen 2 and aggrecan, all the chondrocyte-specific collagens etc.…) are positively regulated by hypoxia, reinforcing the positive effect of low oxygen on chondrocytes.

Beside these genes, other hypoxia-responsive genes were found of potential functional interest for the chondrocyte phenotype: (i) matrix maturing enzymes such as hydroxylases or proteoglycan synthases (participating to the matrix turnover); (ii) inflammatory mediators; (iii) transcription factors including DEC1, Dlx5 [associated with the chondrocyte differentiation (Hsu *et al.* 2006) but never reported in HACs] or Mef2c [recently identified as involved in the craniofacial development (Arnold *et al.* 2007; Verzi *et al.* 2007)]; (iv) growth factors including the angiogenic VEGF and antiangiogenic ChMI or TGF-beta family members; and (v) receptors such as FGFR-3 or the Collagen receptor DDR1.

A very recent study has shown that HIF-2α, by preventing apoptosis and limiting ROS production, is also of importance in chondrocyte autophagy regulation (Bohensky *et al.* 2009). Human tissue analysis showed that decreased expression of HIF-2α is associated with autophagy in OA tissue and ageing cartilage, suggesting a positive role for HIF-2α in maintaining cartilage integrity. These results again demonstrate the importance of the hypoxic environment for the chondrocyte biology, having a larger effect on the chondrocyte phenotype than originally thought. Because hypoxia triggers essential signals, affecting many aspects of the chondrocyte biology and involved in the chondrocyte phenotype maintenance, hypoxia can be considered as a potent anabolic factor on dedifferentiated chondrocytes.

Hypoxia and pathologies of cartilage

Analyses of diseased joints, such as RA joints, showed new blood vessels, which is now recognized as an aspect of the progression of the disease (Etherington *et al.* 2002). High proportion of vessels in RA joint synovium associated with neovascular markers is also detected. Measurement of oxygen in synovial fluid in different pathologies such as RA or

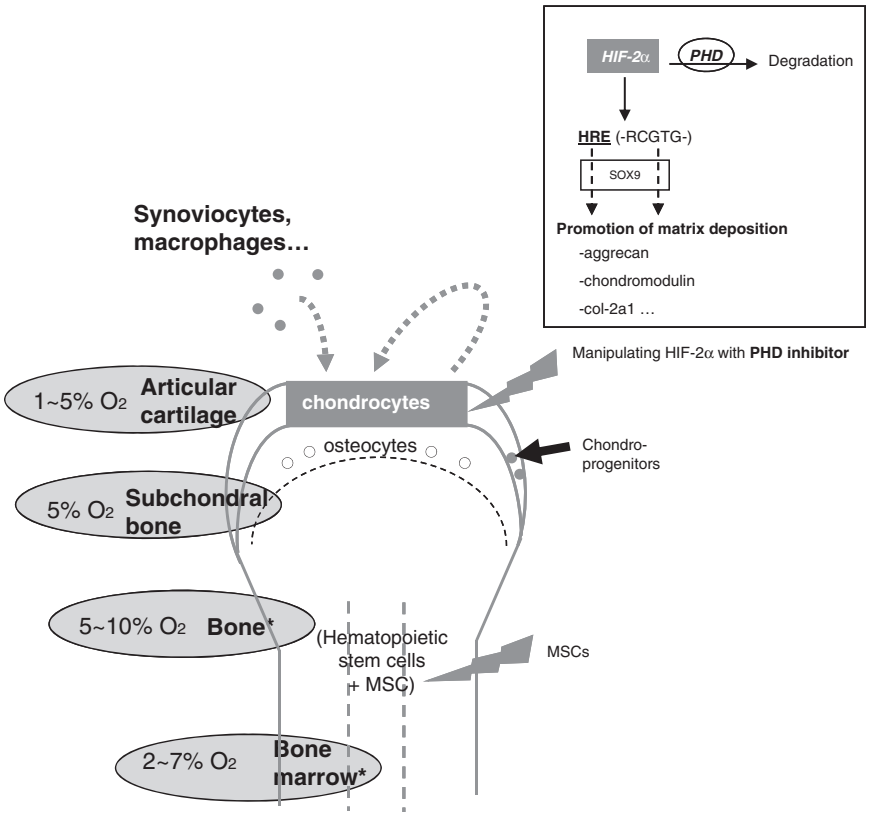

Figure 2 Importance of HIF-2α in cartilage repair strategies. Levels of oxygen are low in the surrounding tissues of joints (Lund-Olesen 1970; Ishikawa & Ito 1988). In articular chondrocytes, it promotes matrix deposition. Manipulating the oxygen-dependent transcription factor HIF-2α could be of help in cartilage repair strategies.

OA revealed many differences (Lund-Olesen 1970, Sivakumar *et al.* 2008), suggesting that oxygen level is essential for normal healing and repair in the joint. Recent data showed HIF-2α was expressed with VEGF in synovial tissue. However it is still unclear how oxygen tension affects development of joint pathologies (Sivakumar *et al.* 2008).

Conclusion and perspectives

Despite its simple cell composition (mainly one resident cell type), cartilage should be considered in its whole complexity: an interface structure between soft tissue and bone tissue, harbouring interactions with different cell type (monocytes, synoviocytes, osteocytes…), being in a permanent stress (hypoxia, pH, mechanical stimulation). We suggested that hypoxia, through the hypoxia inducible transcription factor HIF-2α, is triggering a chondro-anabolic stimulus, enhancing the matrix deposition of dedifferentiated chondrocytes, and thus probably contributing to articular cartilage integrity (Figure 2). It raises the possibility of improving therapies through manipulation of HIF-2α via the inhibition of a specific prolylhydroxylase.

As explained in this review, articular cartilage is in fact integrating permanently and simultaneously several signals. Beside its intrinsic effects through HIF-2α, hypoxia probably affects dramatically other signalling pathways that constantly influence chondrocytes. Thus, future work will certainly have to revisit the effects of some of growth factors by integrating the 'hypoxia' factor in cartilage.

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