

Promotion of Chondrocyte-specific Function by Inhibition of HIF-targeting Hydroxylases

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A thesis submitted for the degree of Doctor of Philosophy

June 2011

The Kennedy Institute of Rheumatology

Faculty of Medicine

Imperial College London

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ABSTRACT

Human articular cartilage is an avascular tissue, and therefore functions in a hypoxic environment. Cartilage cells, the chondrocytes, have adapted to this and actually use hypoxia to drive tissue-specific functions. Previous work in our laboratory has shown that human chondrocytes enhance cartilage matrix synthesis in response to hypoxia specifically through Hypoxia Inducible Factor 2 α (HIF-2 α) mediated upregulation of master regulator transcription factor SOX9, which in turn drives expression of the main cartilage-specific extracellular matrix genes.

HIF- α isoforms are themselves regulated by specific prolyl hydroxylases (PHDs) which target them for proteosomal degradation. In fact, the PHDs are the true direct oxygen sensors since they require molecular oxygen as a co-substrate. Here we show that PHD2 is the dominant isoenzyme regulating HIF-2 α (and HIF-1 α) stability in human chondrocytes. Moreover, specific inhibition of PHD2 using RNAi-mediated depletion caused an upregulation of SOX9 and enhanced extracellular matrix protein production. Depletion of PHD2 resulted in greater HIF-2 α levels, and therefore enhanced SOX9-induced matrix cartilage matrix production, even when performed in hypoxia (1% oxygen) implying that PHD2 inhibition offers a novel means to enhance cartilage repair. The need for HIF-specific hydroxylase inhibitors was highlighted as treatment with the oxoglutarate analogue dimethyloxalylglycine (which also inhibits the collagen prolyl hydroxylases) resulted in reduced secretion of type II collagen, a critical matrix component.

Complimentary to these anabolic effects, hypoxia was found to mediate anti-catabolic effects, suppressing aggrecan degradation in human cartilage explants. In isolated chondrocytes hypoxia downregulates key matrix metalloproteinases (*ADAMTS5* and *MMP13*) and upregulates metalloproteinase inhibitor (*TIMP3*). Unlike the anabolic effects (HIF-2 α -dependent), these hypoxia induced anti-catabolic chondrocyte responses were predominantly HIF-1 α dependent.

Both anabolic and anti-catabolic hypoxia-induced responses were further enhanced by depletion of PHD2, whose inhibition thus offers a novel means to induce cartilage repair.

Acknowledgements

I would like to express my deep gratitude to my supervisors Dr Chris Murphy and Professor Jeremy Saklatvala for their counsel and encouragement during my project. In particular I would like to thank Dr Chris Murphy for his guidance and unwavering patience in continually striving to make me a better scientist.

Many thanks to my teachers Dr Jerome Lafont, Dr Aida Martinez-Sanchez and Katarzyna Dudek to whom I am very much indebted.

I would like to thank all of my friends from Cell Signalling, Matrix and Cytokine labs, both past and present members for making my studies at the Kennedy Institute of Rheumatology so much fun. In particular, Eugenie Joanny, Kawing Chong, Francesco Marchese, Bryony Brennan, Fiona Watt, Michele Pelosi, Lesley Rawlinson, Jon Crowe, Stefano Lazzarano, Annika Burleigh and Thanos Didangelos.

A special acknowledgement to my family, Elaine, Peter and David for their unconditional love and support during all of my studies.

Finally, I would like to thank my partner Terhi for putting up with the early mornings, late nights and always being so excited to listen about my adventures with cartilage.

Statement of Originality

I declare that the work in this thesis was carried out in accordance with the regulations of Imperial College London. The experimental work presented herein is my own, except where it has otherwise been indicated by reference in the text. Any collaboration and/or assistance of methods or relevant prior experimental findings by others have been acknowledged in the relevant sections. The work has been carried out at the Kennedy Institute of Rheumatology, Imperial College London. No part of this thesis has been submitted for any other degree or examination in the United Kingdom or overseas.

Brendan Thoms

June, 2011

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Abbreviations

2ME2	2-methoxestradiol
2-OG	2-oxoglutarate
Ad	Adenovirus
Ad-Cre	Adenovirus expressing Cre recombinase
Ad-GFP	Adenovirus expressing Green Fluorescent Protein
ADAMTS	A disintegrin and metalloproteinase with thrombospondin motif
ADM	Adrenomedullin
ADRP	Adipose differentiation related protein
APS	Ammonium persulphate
ARE	Adenosine- and uridine-rich elements
ASP	Asporin
Asn	Asparagine
ATF	Activating transcription factor
ATP	Adenosine triphosphate
bHLH	basic helix-loop-helix
BMP	Bone morphogenetic protein
BNIP3	BCL2/adenovirus E1B 19 kDa protein-interacting protein 3
C-ODDD	C-terminal oxygen dependent degradation domain
C-P4H	Collagen prolyl-4-hydroxylases
C-TAD	C-terminal transactivation domain
CD	Cluster of differentiation
cDNA	Complementary DNA
ChIP	Chromatin immunoprecipitation
COMP	Cartilage oligomeric matrix protein
Co ²⁺	Cobalt ion
CO ₂	Carbon Dioxide
COX	Cyclooxygenase
CTF	Cyclic tension force
dd	Double-distilled
DFO	Desferrioxamine
DMEM	Dulbecco's modified Eagle's medium
DMM	Destabilised medial meniscus
DMOG	Dimethyloxalylglycine
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DOC	Deoxycholic acid
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
EMSA	Electrophoretic mobility shift assay
EPO	Erythropoietin
ERT	(o)Estrogen replacement therapy
FBS	Foetal bovine serum
Fe ²⁺	Iron ion
FGF	Fibroblast growth factor
FIH-1	Factor Inhibiting HIF-1 α
FRZB	Frizzled related protein
GAG	Glycosaminoglycan
GLUT1	Glucose transporter 1

Gly	Glycine
HAC	Human Articular Chondrocyte
HANES	Health and nutrition examination survey
HCl	Hydrochloric acid
HCT116	Human colon epithelial carcinoma 116 cell line
HEK293	Human embryonic kidney 293 cell line
HEP3B	Human Hepatoma 3B cell line
HIF	Hypoxia Inducible Factor
HRE	Hypoxia response element
HRP	Horseradish peroxidase
Ig	Immunoglobulin
IHH	Indian hedgehog
IL	Interleukin
IL4R	IL-4 Receptor α -chain gene
iNOS	Inducible nitric oxide synthase
ITS	Insulin transferrin selenium
JNK	c jun N-terminal kinase
LOXL3	Lysyl oxidase homolog 3
Luc	Luciferase
MAPK	Mitogen activated protein kinase
MATN	Matrillin
MCF7	Michigan Cancer Foundation 7 cell line
MCP	Metacarpophalangeal
MEK	MAPK/ERK kinase
MMP	Metalloproteinase
mRNA	Messenger ribonucleic acid
MT-MMP	Membrane bound MMP
N-ODDD	N-terminal oxygen dependent degradation domain
N-TAD	N-terminal transactivation domain
Ni ²⁺	Nickle ion
NICE	National institute for healthy and clinical excellence
NSAID	Non-steroidal anti-inflammatory drug
O ₂	Oxygen
OA	Osteoarthritis
OSM	Oncostatin M
PAGE	Polyacrylamide gel electrophoresis
PAS	PER-ARNT-SIM domain
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline tween
PCR	Polymerase chain reaction
PG	Prostaglandin
PHD	Proyl-hydroxylase domain
PLC/PRF/5	The Alexander hepatoma cell line
PLOD2	Procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2
Pro	Proline
PTHRP	Parathyroid hormone 1 receptor
PVDF	Polyvinylidene difluoride
pVHL	Von Hippel-Lindau disease protein
r.p.m	Revolutions per minute
RIPA	Radio-immunoprecipitation assay buffer

RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase polymerase chain reaction
RUNX2	Runt-related transcriptional factor 2
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
siRNA	small interfering RNA
SOX9	SRY (sex determining region Y)-box 9
TCA	Trichloroacetic acid
TEMED	N'N'N'N'- Tetraethyl methylene diamine
TGF β	Transforming growth factor beta
TIMP	Tissue inhibitor of metalloproteinases
TNF	Tumour necrosis factor
TTP	Tristetraporolin
UV	Ultraviolet
VEGF	Vascular endothelial growth factor

Chapter 1

Introduction

1.1 Background

Oxygen is essential for metabolism - being necessary for the generation of adenosine 5'-triphosphate (ATP) via oxidative phosphorylation, and used as a substrate in many enzymatic reactions in the body (Mitchell, 1961). As organisms evolved from single cellular to multi-cellular it became necessary to develop delivery vessels in order to maintain oxygen homeostasis and facilitate oxygen supply to cells at the core of tissues. In humans, the dissolved levels of oxygen in arterial blood range between 10-14% (pO_2 75-100 mmHg), however, in the event of reduced partial pressure of atmospheric oxygen (e.g.: high altitudes), occlusion of blood vessels supplying oxygen to a tissue (e.g.: myocardial infarction), rapid cell growth (e.g., solid tumor) or cells being located sufficiently far away from vessels that they are beyond the diffusion distance of oxygen ($\sim 100\text{-}300\mu\text{m}$) the tissue will be exposed to significantly reduced oxygen tensions or hypoxia (Torres Filho et al., 1994). Here we define hypoxia as a level of molecular oxygen low enough to stabilise HIF- α isoforms ($\leq 5\%$ O_2) (Schofield and Ratcliffe, 2004). Conversely, we define normoxia as level of oxygen under normal atmospheric conditions (20% O_2). We acknowledge that these are not levels of oxygen normally experienced *in vivo* (10-14% O_2) and may be considered artificially high or supra-physiological. We will elaborate on this issue in our further work (Chapter 6).

A sudden decrease in oxygen tension is associated with many conditions including stroke, myocardial infarction, angina, and peripheral arterial disease and can lead to massive cell death (Fraisl et al., 2009). Cells can alter gene expression and adapt cellular processes in order to survive hypoxic environments. For example, ATP generation shifts from an aerobic to anaerobic state increasing glucose intake, increasing expression of enzymes involved in the glycolysis pathway and decreasing the enzymes leading to the TCA cycle enabling ATP generation to continue in the absence of molecular oxygen. The key mediators of cellular responses to hypoxia are the Hypoxia-inducible factors (HIF-1 α and HIF-2 α). In addition to regulating gene expression responsible for glucose metabolism, HIF-1 α and HIF-2 α control genes responsible for ventilation, angiogenesis, pH regulation, erythropoiesis, cell proliferation, differentiation and apoptosis (Gaber et al., 2005; Gendron et al., 2004; Semenza, 2001).

Interestingly, cartilage is a tissue which has had to adapt to a chronic condition of hypoxia. Indeed, it appears that it can utilise the chronic hypoxic signal of its environment to promote its phenotype or tissue-specific function. For example, hypoxia regulates the production of SRY (sex determining region Y)-box 9 (SOX9), a tissue-specific transcription factor essential for cartilage development and responsible for regulating key extracellular matrix genes including Type II Collagen, Type IX Collagen, Type XI Collagen and Aggrecan (Bi et al., 1999; Domm et al., 2002; Lafont et al., 2008; Lafont et al., 2007; Murphy and Polak, 2004; Murphy and Sambanis, 2001; Tew et al., 2007). Work in our laboratory has further demonstrated that hypoxia inducible factor 2 α (HIF-2 α), not hypoxia inducible-factor 1 α (HIF-1 α), is integral for hypoxic induction of SOX9 and the subsequently enhanced cartilage matrix production (Lafont et al., 2007). Prolyl- and asparaginyl- hydroxylases are members of an oxoglutarate-dependant dioxygenase family responsible for inhibiting HIF in the presence of sufficient oxygen. We hypothesised that antagonising specific hydroxylases may (selectively) stabilise HIF-2 α , promote the human articular chondrocyte phenotype and enhance cartilage deposition without inhibiting essential collagen prolyl- and lysyl-hydroxylases which could interfere with collagen fibre processing.

To introduce the work, I will describe the anatomy, physiology and function of articular cartilage and summarise the knowledge of its extracellular matrix and its turnover. I will review the hypoxia signalling pathway and how it is regulated by HIF-targeting hydroxylases and discuss current views of the effect of hypoxia on the chondrocytes phenotype. Finally I will discuss the pathogenesis of osteoarthritis (OA) and how the inhibition of HIF-targeting hydroxylases may provide a novel therapeutic approach to treat this chronic and disabling disease.

1.2 Cartilage Structure and Function

A diarthrodial or synovial joint is comprised of two bone ends with a smooth layer of hyaline (articular) cartilage coating each epiphysis; these articular surfaces are encased within a capsule which is lined by the synovial membrane. The synovial membrane or synovium lines the inside of the joint capsule and is responsible for producing synovial fluid and providing immunologic protection. The membrane acts like a molecular sieve allowing

water, small molecules and nutrients to pass from the vascular system into the joint cavity but restricts the movement of larger molecules. Type B synoviocytes are resident cells within the synovial membrane and help to control the protein concentration of the synovial fluid. They actively secrete proteins such as collagen, fibronectin and hyaluronan (which confers viscosity and elasticity) into the joint cavity. During movement the synovial fluid is propelled across the articular surface serving as both lubrication and a source of nutrients for the articular cartilage.

Articular cartilage is a dense, avascular, aneural and hydrated tissue. In addition to providing a near frictionless surface for articulation articular cartilage has two main biomechanical properties essential to its function; tensile strength and ability to withstand compressive forces (Buckwalter and Mankin, 1998a; Poole et al., 2002).

Articular cartilage is a specialized connective tissue with an extensive extracellular matrix. Its two primary mechanical properties are mediated by collagen fibrils (predominantly type II) present in the matrix which confer tensile strength to articular cartilage and proteoglycans (mainly aggrecan), these molecules are water-saturated and the resulting swelling pressure creates a gel-like stiffness allowing the tissue to resist deformation and giving cartilage its ability to absorb shocks (Buckwalter and Mankin, 1998a; Muir, 1995; Poole et al., 2002). Articular cartilage is a hydrated tissue with approximately 75-80% of the matrix comprised of water. Of its dry weight, 70% is collagen, 20% is proteoglycan and 10% is composed of other matrix proteins (Eyre, 2002).

1.2.1 The Chondrocyte

Articular cartilage consists of a single cell type, the chondrocyte which is solely responsible for the synthesis, secretion and maintenance of the extracellular matrix (Goldring and Goldring, 2006). Consistent with its function and hypoxic microenvironment, the chondrocyte cytoplasm has a large number of Golgi apparatus and rough endoplasmic reticulum with few mitochondria. Stockwell reported that the number of mitochondria per chondrocyte was significantly reduced in the deeper zones of articular cartilage relative to the upper zones (Stockwell, 1991). As will be discussed (Chapter 1.5), chondrocytes exist under chronic hypoxic conditions and have adapted to this condition, with a strong dependence on anaerobic metabolism for ATP generation (Gibson et al., 2008). Molecular oxygen

consumption in the tissue is very low, approximately 2-5% compared with that of a liver or kidney cell (Archer and Francis-West, 2003; Gibson et al., 2008; Stockwell et al., 1983).

Chondrocytes are characterised by the expression of specific genes such as collagen type II and the SRY (sex determining region Y)-box 9 (SOX9) (Tew et al., 2007). SOX9 is a master transcription factor responsible for the regulation (in concert with L-Sox5 and Sox6) of a number of cartilage extracellular matrix genes including *COL2A1*, *COL9A1*, *COL11A2* and *AGGRECAN* (Bridgewater et al., 1998; Kou and Ikegawa, 2004; Sekiya et al., 2002; Tew et al., 2007; Zhang et al., 2003; Zhao et al., 1997). Sox9 has been demonstrated to be essential for the development and maintenance of cartilage in mice (Bi et al., 1999). Mutations to the *SOX9* gene result in haploinsufficiency of SOX9 resulting in a severe skeletal disease called compomelic dysplasia, characterized by short limbs, bowed legs, narrow gests and dwarfism (Meyer et al., 1997; Wagner et al., 1994). Finally, Sox9 is downregulated in chondrocytes as they enter hypertrophy during endochondral ossification (Zhao et al., 1997).

1.3 The extracellular matrix of cartilage

Human chondrocytes are embedded in articular cartilage at a very low density ($14.1 \times 10^3/\text{mm}^3$), with the bulk of the tissue (dry weight) (~90-95%) comprising extracellular matrix (Stockwell, 1971). Articular cartilage can be divided into four zones: the superficial zone, the transitional zone, the radial zone and the calcified cartilage zone (cartilage interacts with subchondral bone). These zones are each characterised by different amounts and organisation of matrix macromolecules (Fig. 1.1).

The articular surface is comprised of a thin layer of densely packed, fine collagen fibres running parallel to the surface. The fibrils have a periodic banding pattern and many form bundles. Chondrocytes in the superficial zone are flattened and elliptical in shape, displaying cell-surface polarity (aligning parallel to the surface of the tissue). The superficial layer contains high levels of collagen and low levels of proteoglycan (relative to other layers). The layer has a high mechanical strength against the shear and tensile stress of the articular surface. The chondrocytes within the transitional and radial zones are more spherical in shape and are arranged vertically into columnar clusters. Similarly, collagen

fibres are arranged perpendicular to the articular surface and have a larger diameter (Hwang et al., 1992). There are abundant levels of proteoglycans in these layers compared with the superficial layer. Finally, the calcified cartilage zone separates hyaline cartilage from subchondral bone. It contains a tidemark layer (basophilic boundary between calcified and uncalcified cartilage) and large amounts of type X collagen (Broom and Poole, 1982; Havelka et al., 1984; Redler et al., 1975).

Components of articular cartilage are:

1.3.1 Collagen:

Collagen fibrils are present in all layers of articular cartilage and provide structural strength to the tissue (Eyre, 2002). Collagen contains a tripeptide -X-Proline-Glycine motif (where X represents any amino acid). Translated as procollagen, the non-helical N- and C-terminal ends of the molecule are cleaved allowing 3 polypeptide α -helices to interact, polymerise and form the collagen fibril. These fibrils are secreted into the extracellular matrix where they polymerise to form the primary component of the large cross banded fibrils (van der Rest and Garrone, 1991).

Type II collagen (COL2A1) is the most predominant macromolecule in articular cartilage (~90% of the cartilage collagen) and is a homotrimer of three α (II) chains. Type II collagen acts as a structural scaffold conferring tensile strength and creating a meshwork within which other components can be organised. Type II collagen has a half life of approximately 100 years and therefore it is believed that the synthesis of type II collagen in articular cartilage is down-regulated after skeletal maturity with little synthesized during adulthood, making articular cartilage vulnerable to catabolic effects of matrix metalloproteinases (although type II collagen synthesis is increased in osteoarthritic cartilage) (DeGroot et al., 2001; Hermansson et al., 2004; Maroudas et al., 1998; Maroudas et al., 1992). However, we have previously demonstrated type II collagen expression in isolated healthy human articular chondrocytes and that levels of expression can be further enhanced by hypoxia (Lafont et al., 2007; Thoms and Murphy, 2010).

Cartilage also contains other distinct collagen types (Type VI, IX, X and XI collagen). Together, they consist ~10% of total collagen. Type IX and XI collagen are shorter,

heterotrimeric, minor fibrillar collagens (Buckwalter and Mankin, 1998a; Muir, 1995; Poole et al., 2002). Type XI collagen is thought to reside in the central portion of the collagen fibril, acting as a filamentous core template which regulates fibril diameter through its retained N-propeptide domains (Mendler et al., 1989). Type IX collagen localizes to the superficial layer of the fibrils and projects out into the matrix (Poole et al., 1988) (Fig. 1.2). Type VI collagen has been identified in the pericellular matrix (the matrix surrounding the chondrocyte) of articular cartilage, its role is unknown (Soder et al., 2002). Type X collagen is a homotrimer that is highly expressed by chondrocytes within the hypertrophic zone of the epiphyseal growth plate. In mature articular cartilage the expression of type X collagen is restricted to the calcified cartilage (Gannon et al., 1991).

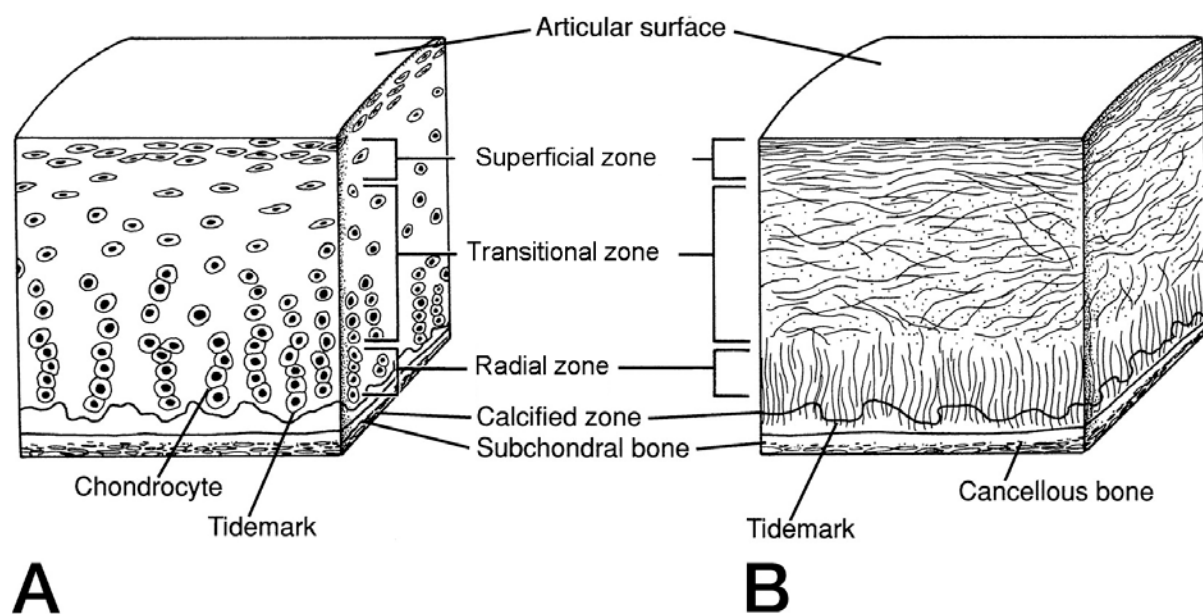


FIGURE 1.1. **Structure of articular cartilage.** A, schematic diagram of the cellular organisation in the four zones of articular cartilage: the superficial, transitional, radial and calcified zone. B, diagram of the collagen fibre architecture through the depth of articular cartilage. (Newman, 1998)

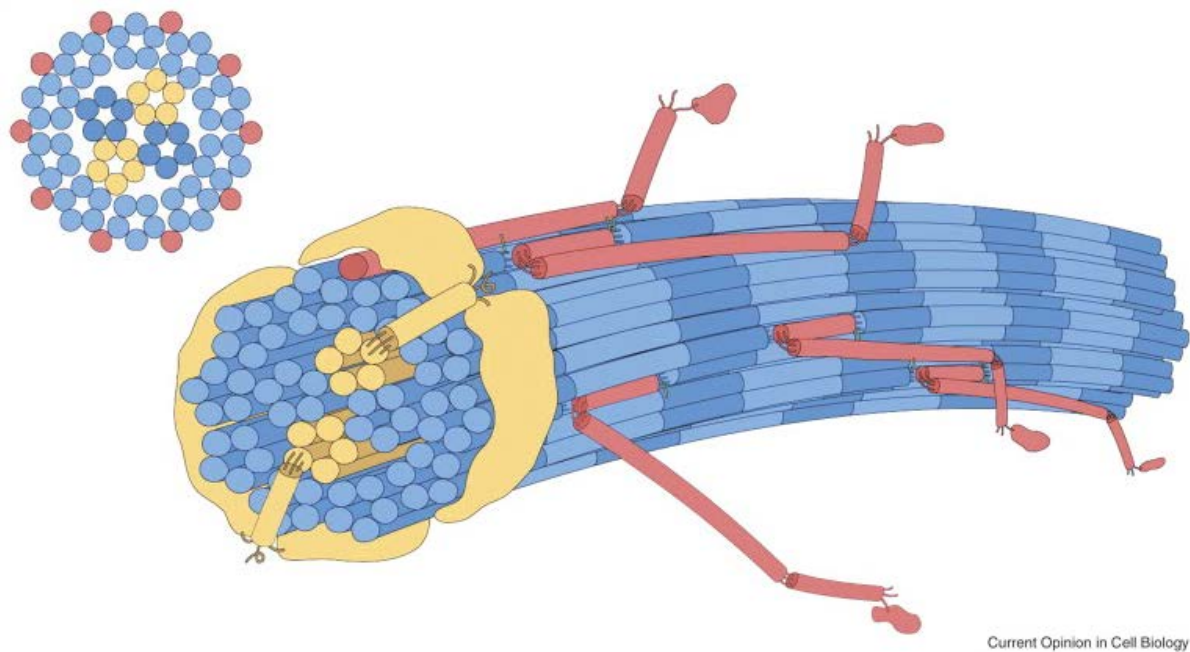
1.3.2 Proteoglycan:

Proteoglycans consist of a core protein covalently attached to one or more glycosaminoglycan (GAG) side chains. These GAG side chains are long unbranched polysaccharides consisting of a repeating disaccharide that contains an amino sugar. Glycosaminoglycans are negatively charged and strongly hydrophilic, this is essential for their function. Water-saturated aggrecan molecules are held within scaffold of collagen fibres creating a swelling pressure and stiffness allowing articular cartilage to resist deformation.

Aggrecan is the main proteoglycan in cartilage and contributes 90% of the total proteoglycan content. In the cartilage extracellular matrix aggrecan is bound to a backbone of hyaluronan (hyaluonic acid) and forms huge aggregates filling the interfibrillar space of the cartilage matrix (Hardingham, 1979; Roughley, 2006). Up to 200 aggrecan molecules bind to each hyaluronan backbone. The hyaluronan backbone is itself anchored to chondrocytes via CD44, a cell associated heperin sulphate proteoglycan.

Each aggrecan molecule consists of a protein core, with three globular domains (G1, G2 and G3) and two interglobular domains. Covalently attached to the core protein are numerous sulphated glycosaminoglycan chains of keratan sulphate (KS) and chondroitin sulphate (CS). The negatively charged and hydrophilic nature of these GAG side chains repel each other and attract water molecules (75% of cartilage, by weight is water). The hydrated swelling pressure of water-saturated aggrecan molecules enables cartilage to resist compression during joint loading (Roughley, 2006). Unlike type II collagen, aggrecan has a significantly shorter half life (~3.4 years) and is therefore synthesised and turned over in adult cartilage (DeGroot et al., 2001; Maroudas et al., 1998; Maroudas et al., 1992).

Other less abundant small proteoglycans found in the matrix include decorin, biglycan, lumican and fibromodulin which are thought to be involved in fibril assembly, organisation and stabilisation of collagen matrix structure within the cartilage extracellular matrix (Hedbom and Heinegard, 1993; Roughley, 2006).



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FIGURE 1.2. Interactions between type II collagen, type IX collagen and type XI collagen molecules. Type II collagen is the most abundant collagen in articular cartilage and confers tensile strength to the tissue. Type XI (blue) and type IX (yellow) collagen are cross-linked to the type II collagen fibre. Type XI collagen is thought to reside in the central portion of the collagen fibril, acting as a filamentous core template which regulates fibril diameter through its retained N-propeptide domains. Type IX (red) collagen localises to the superficial layer of the fibrils and projects out into the matrix (Kadler et al., 2008).

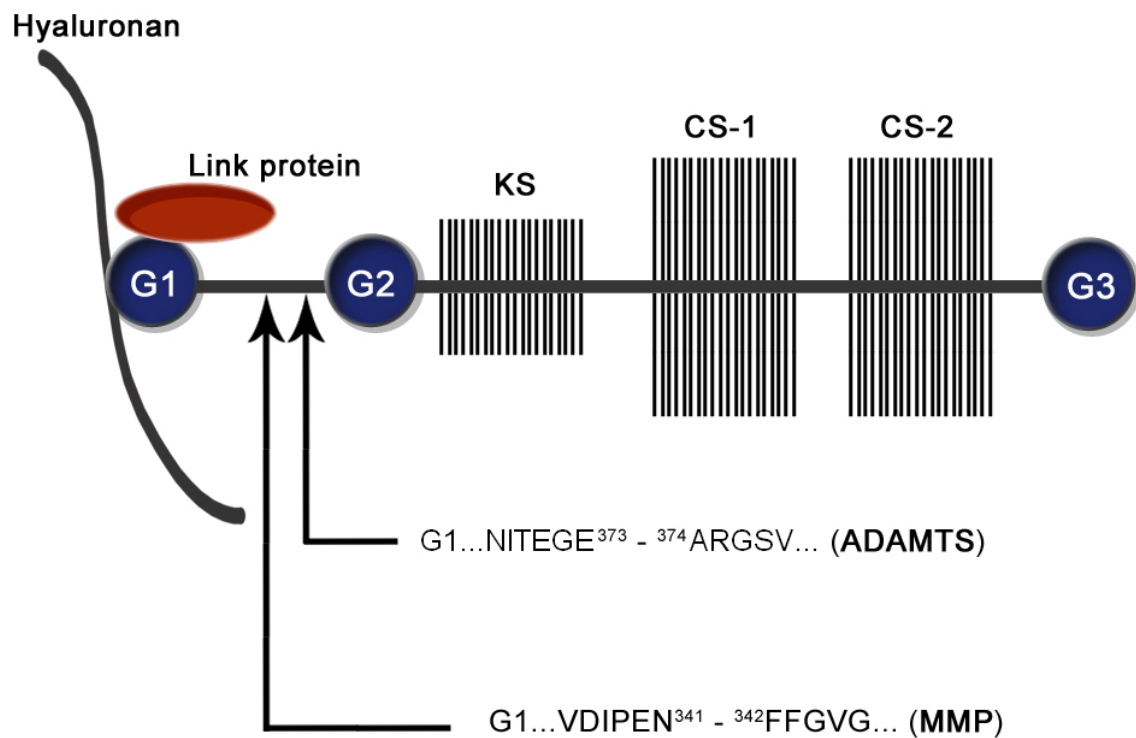


FIGURE 1.3. Domain structure of Aggrecan. Aggrecan is the main proteoglycan in cartilage and contributes 90% of the total proteoglycan content. It has a molecular mass >2,500 kDa. The core protein has 100-150 glycosaminoglycan (GAG) chains attached to it. Each aggrecan molecule consists of a protein core, with three globular domains (G1, G2 and G3), two interglobular domains, all bound to a backbone of hyaluronan (hyaluonic acid). Covalently attached to the core protein are numerous sulphated glycosaminoglycan chains of keratan sulphate (KS) and chondroitin sulphate (CS). Both MMPs and ADAMTSs have been shown to cleave aggrecan at multiple sites, however the two major cleavage sites are within the first interglobular domain (between G1 and G2). Proteolysis occurs predominantly at Glu³⁷³ – Ala³⁷⁴ and at Asn³⁴¹ – Phe³⁴² bonds. The Glu³⁷³ – Ala³⁷⁴ bond is susceptible to ADAMTS cleavage resulting in the N-terminal neopeptide ARGSV. The Asn³⁴¹ – Phe³⁴² bond is cleaved by all known MMPs resulting in the N-terminal neopeptide FFGV (Image provided by Terhi Raukko).

1.3.3 Other matrix molecules

Other proteins in the cartilage matrix include the cartilage oligomeric matrix protein (COMP), fibronectin and chondromodulin-1. COMP is an acidic glycoprotein with 5 identical subunits and is thought to be involved in collagen cross linking and is therefore important for the assembly and stabilisation of the extracellular matrix (Posey and Hecht, 2008; Shen et al., 1995). Fibronectin is encoded by a single gene, but alternative splicing of its pre-mRNA leads to up to 20 different isoforms. Fibronectin forms a dimer, consisting of two monomers linked by a pair of disulphide bonds and regulates a number of cellular processes including cell migration, adhesion and cell cycle progression (Pankov and Yamada, 2002). Chondromodulin-1 is a glycoprotein which strongly inhibits angiogenesis (vascular invasion of cartilage) and has recently been shown to induce the chondrocyte phenotype (Hiraki et al., 1997; Hiraki et al., 1991; Klinger et al., 2011).

1.4 Cartilage extracellular matrix metabolism

Chondrocytes slowly turn over cartilage matrix molecules. Ideally there is an equilibrium between anabolism (matrix synthesis) and catabolism (matrix degradation), and cartilage matrix integrity is maintained. The exact mechanism of cartilage matrix turnover is poorly understood and it is well established that once damaged, cartilage has a limited intrinsic matrix remodelling and repair capacity.

Chondrocytes produce two important classes of enzymes involved in cartilage matrix degradation: ADAMTSs (A Disintegrin And Metalloproteinase with Thrombospondin type 1 Motif) or aggrecanases and MMPs (Matrix-Metalloproteinases).

1.4.1 Aggrecanases (ADAMTSs):

The ADAMTSs are a family of 19 secreted metalloproteinases which contain a zinc atom at their catalytic site and have sequence homology to ADAM proteases (Kuno et al., 1997). ADAMTSs are all initially translated as inactive pre-proenzymes and unlike ADAMs, which are transmembrane proteins (with the exception of ADAM12 and ADAM28), the

ADAMTSs are secreted proteins with at least one thrombospondin type 1 sequence motif (TSR) capable of binding the extracellular matrix (Kuno and Matsushima, 1998; Tang, 2001). All ADAMTS enzymes have a reprotolysin-type zinc-binding motif, HEXXHXXG/N/SXXHD (where X represents any amino acid) at their catalytic site (Porter et al., 2005).

Both MMPs and ADAMTSs have been shown to cleave aggrecan at multiple sites, however the two major cleavage sites are within the first interglobular domain (between G1 and G2). Proteolysis occurs predominantly at Glu³⁷³ – Ala³⁷⁴ and at Asn³⁴¹ – Phe³⁴² bonds. The Glu³⁷³ – Ala³⁷⁴ bond is susceptible to ADAMTS activity resulting in the C-terminal neoepitope NITEGE (bound to the hyaluronan backbone) and the N-terminal neoepitope ARGSV (released) (Nagase and Kashiwagi, 2003; Sandy et al., 1992). ADAMTSs are not active to the Asn³⁴¹ – Phe³⁴² bond, this is cleaved by all known MMPs resulting in C-terminal neoepitope DIPEN and N-terminal neoepitope FFGV (Fig. 1.3). MMP and ADAMTS generated neoepitope aggrecan fragments have been identified in culture media of cartilage explants *in vitro* as well as human synovial fluid *in vivo* (Sandy et al., 1992) (Fosang et al., 1996; Lark et al., 1997; Little et al., 2002; Lohmander et al., 1993; Sandy and Verscharen, 2001).

ADAMTS1,4,5,8,9,15,16 and 18 can all cause aggrecan cleavage at the Glu³⁷³ – Ala³⁷⁴ bond however, ADAMTS4 and ADAMTS5 are the most active aggrecanases under physiological conditions (Abbaszade et al., 1999; Fushimi et al., 2008). Furthermore, although MMPs can cleave aggrecan at the Asn³⁴¹ – Phe³⁴² bond it is believed that aggrecanases are predominantly responsible for aggrecan degradation *in vivo* and have been shown to contribute 80% of the aggrecan fragments detected in osteoarthritic synovial fluid (Struglics et al., 2006).

1.4.2 Matrix Metalloproteinases (MMPs)

MMPs are a family of over 28 multi-domain endopeptidases (Nagase et al., 2006). The MMPs are responsible for matrix turnover and can collectively degrade all cartilage matrix macromolecules. Like ADAMTSs, the MMP family are initially translated as inactive zymogens (proMMPs) and activated by proteolytic cleavage. Similarly, MMPs are all zinc-dependent enzymes, containing a zinc atom at the catalytic site. MMPs can be further subdivided according to substrate and structure. There are five main groups: collagenases

(MMP1, -8, -13 and -18), gelatinases (MMP2 and 9), stromelysins (MMP3 and 10), matrilysins (MMP7 and 26) and the membrane-type MT-MMPs (MMP14, -15, -16, -17 and -24) (Egeblad and Werb, 2002; Murphy et al., 2002).

Type II collagen is cleaved by collagenases (MMP1, -8, -13 and -18) and MMP13 has been identified as having the highest activity against type II collagen (Minond et al., 2006; Tetlow et al., 2001). Collagenases unwind the collagen triple helix and cleave at a single point three quarters along the length away from the N-terminal (Knauper et al., 1996). The neoepitopes formed by this cleavage can be detected by antibodies (Billinghurst et al., 1997; Hollander et al., 1994). This cleavage generates characteristic one-quarter and three-quarter fragments, which are further degraded by gelatinases (MMP2 and MMP9).

Matrix metalloproteinase-3 (MMP-3) or Stromelysin-1 is a key member of the MMP family and has a broad substrate specificity including proteoglycans, gelatin, laminin, vitronectin, fibronectin, type IV collagen. Furthermore, it is believed to control the activation of proMMP zymogens. For example, MMP3 activates proMMP1 and proMMP13 (Knauper et al., 1996; Visse and Nagase, 2003).

1.4.3 Tissue inhibitors of the matrix metalloproteinases (TIMPs)

TIMPs play an important role as the main regulators of matrix metalloproteinases (MMPs) and aggrecanases (ADAMTSs) and are important for extracellular matrix homeostasis. Cartilage homeostasis is a delicate balance between proteases and TIMP activity and a net imbalance has been implicated in cartilage loss.

Four TIMPs have been identified (TIMP1, 2, 3, 4) - they are synthesised by chondrocytes and are endogenous inhibitors of MMP and to a lesser extent ADAMTS activity. TIMP1, -2, -3 and -4 broadly inhibit all MMPs, with different effectiveness (Baker *et al.*, 2002). TIMP3 has been reported to inhibit all MMPs, in addition to ADAM-17, ADAMTS4 and ADAMTS5. The K_i of TIMP3 for ADAMTS4 and ADAMTS5 is in the subnanomolar range (Hashimoto et al., 2001; Kashiwagi et al., 2001). In addition TIMP3 is able to bind to polyanionic matrix components allowing for localisation in the cartilage extracellular matrix. It has been reported that TIMP3 inhibits aggrecanase activity *in vitro* when added exogenously to interleukin-1 α (IL-1 α) or retinoic acid stimulated bovine and

porcine cartilage explants suppressing aggrecan neoepitope release (Gendron et al., 2003). Finally, *Timp3*^{-/-} mice have increased proteinase activity and spontaneously development cartilage degradation illustrating the important role of TIMP3 in regulating extracellular matrix homeostasis (Sahebjam et al., 2007).

1.5 Cartilage and hypoxia

Chondrocytes encounter two forms of permanent stress, hypoxia and mechanical loading. Articular chondrocytes within synovial joints survive in a challenging microenvironment and are dependent upon the diffusion of oxygen from the underlying subchondral bone and the synovial fluid which bathes the articular surface and is propelled by joint movement (Lund-Olesen, 1970; Ogata et al., 1978). For human articular chondrocytes within the core of the larger joints such as the knee, the cells may be 2-3mm distance from an arterial vessel (Haselgrove et al., 1993).

Brighton and Heppenstall investigated the oxygen tension of epiphyseal cartilage in rats and rabbits using a platinum-iridium microelectrode (10µm in diameter). Costochondral cartilage explants from 21 day old Sprague-Dawley male rats were excised and cultured. The oxygen tension was found to gradually decrease from 97.3 mmHg (12.8% O₂) at the subchondral bone to 12.1mmHg (1.5% O₂) in the hyaline cartilage (Brighton and Heppenstall, 1971). Subsequent work was carried out *in vivo* using a platinum-iridium microelectrode to measure oxygen tensions of the proximal tibial epiphyseal plates in six-week-old male New Zealand white rabbits. Similarly *in vitro* oxygen tensions were reported to decrease from the subchondral bone to the hyaline cartilage (108.7 mmHg / 14.3% O₂ to 20.5 mmHg / 2.7% O₂ respectively) (Brighton and Heppenstall, 1971). Lund-Olesen studied the oxygen tension in 103 synovial fluids from knee joints. The mean oxygen tension in synovial fluid from patients with osteoarthritis was 42.92 mmHg, and 63 mmHg in joints injured by trauma (Lund-Olesen, 1970). Therefore, we can assume that chondrocytes near the surface of the tissue are exposed to an oxygen tension approximately 5.72 O₂ – 8.35% O₂ (Lund-Olesen, 1970). Finally, work by Silver in rabbit elastic cartilage supports previous work. Oxygen tension in cartilage 0.5mm cubes was measured using microelectrodes and a

steep oxygen gradient was detected the surface of the tissue (80-90 mmHg / 11-11.6% O₂) to the centre of the cubes (5-8 mmHg / 0.6-1% O₂) (Silver, 1975).

Under normoxic conditions HIF- α isoforms are continuously hydroxylated, ubiquitinated and degraded by the 26S proteasome (Cockman et al., 2000). HIF- α isoforms become stabilised and detectable in nuclear extracts < 3-5% O₂ (Distler et al., 2004). Our group has previously reported the presence of HIF-1 α and HIF-2 α in passaged human articular chondrocytes (Lafont et al., 2007). Furthermore, immunohistochemistry of healthy human articular cartilage confirms the presence of HIF-1 α and HIF-2 α protein (Bohensky et al., 2009; Coimbra et al., 2004; Yudoh et al., 2005). Stewart and colleagues demonstrated that HIF-2 α is expressed in hypertrophic chondrocytes of the mouse growth plate, murine chondrocyte cell line ATDC5 cells and that chick chondrocytes up-regulate HIF-2 α expression during chondrocyte differentiation (Stewart et al., 2006). Immunohistochemical detection of HIF-1 α has been reported in mouse knee cartilage BALB/C mice (Gelse et al., 2008).

In summary, human articular cartilage appears to function in a chronically hypoxic environment throughout life. Depending on their position in the tissue, chondrocytes are exposed to approximately between 1-6% O₂ – oxygen tensions at which HIFs become stabilised. Thus reduced levels of molecular oxygen inhibit HIF-targeting hydroxylases and result in the stabilisation of HIF-1 α and HIF-2 α protein in human articular cartilage. The effects of this on the chondrocyte phenotype and function will be discussed below (Chapter 1.10).

1.5.1 Mechanical Loading

Articular cartilage is subjected to chronic, cyclical stress of various magnitudes. Moderate mechanical loading is an important stimulus for articular cartilage and essential for maintaining extracellular matrix integrity (Urban, 1994). Cyclical mechanical loading has been shown to stimulate proteoglycan synthesis in cartilage explants and monolayer chondrocytes (Korver et al., 1992; Larsson et al., 1991; Veldhuijzen et al., 1979) (Steinmeyer and Knue, 1997; Zhou et al., 2007). Aggrecan, Fibronectin and COMP levels have all been shown to increase transiently at the mRNA level in response to cyclical loading in cartilage explants (Valhmu et al., 1998; Wong et al., 1999). Conversely, immobilisation of normal,

healthy joints in paralysed patients following spinal cord injury results in a decrease in cartilage volume (Vanwanseele et al., 2003; Vanwanseele et al., 2002a; Vanwanseele et al., 2002b).

However, high frequency loading or a single impact loading can lead to cartilage matrix degradation. It has been previously reported that exposure of chondrocytes to high frequency cyclic tension force (CTF) ($>15\text{kPa}$) inhibits proteoglycan and collagen synthesis and induced the expression of proinflammatory interleukin-1 (IL-1) and catabolic matrix degrading proteases MMP2 and MMP9 mRNA. High-velocity loading of canine cartilage explants (5 MPa, 0.3Hz) induces cell death by apoptosis (Chen et al., 2001). Further work by Jeffrey and colleagues demonstrated that in bovine cartilage subjected to a single impact load, cell death and was associated with suppression in proteoglycan and protein synthesis (Jeffrey et al., 1997).

1.6 Osteoarthritis

Osteoarthritis (OA) is the most common joint disease (arthropathy) and is characterised by loss of articular cartilage, subchondral bone thickening and new bone formation in a synovial joint (typically knee, hip, hand, shoulder, spine or foot). Osteoarthritis affects approximately 15% of the total population and greater than 50% of the population over 60 years of age (Poole et al., 2002). In the United Kingdom an estimated 36 million working days are lost each year to osteoarthritis-related disability and in the United States an estimated \$3 billion is spent annually on osteoarthritis (March and Bachmeier, 1997; Woolf, 2003).

1.6.1 Clinical Aspects of Osteoarthritis

Osteoarthritis is a chronic disease causing the progressive loss of articular cartilage and resulting in joint pain, loss of function over time and reduced quality of life. Osteoarthritis may be defined by symptoms (pain, stiffness, crepitus, decreased mobility and deformity) or radiographic criteria (joint space narrowing) or both. Because cartilage is aneural structural changes such as joint space narrowing can often occur without painful

symptoms and symptoms often do not present until late stage disease. Population surveys illustrate that 50% of subjects with radiographic knee osteoarthritis have no pain and 50% of subjects who identified knee pain have no radiographic knee osteoarthritis (McAlindon et al., 1992).

Osteoarthritis can be subdivided into mono- or poly-articular and be further categorised as either idiopathic (of an unknown cause) or secondary (caused by another disease or condition) (Kellgren, 1952; Kellgren and Lawrence, 1957; Kellgren et al., 1963). Physical symptoms upon examination of osteoarthritic joints include: pain, decreased range of motion, crepitus, effusions and tenderness on palpation with the joint line. Patients may describe localised pain on exercise (which is relieved by rest) and stiffness during periods of inactivity. Osteoarthritis is also associated with elevated incidence of depression and a reduced quality of life (Axford et al., 2008; Penninx et al., 1996). Plain radiographs are the gold standard for diagnosis and assessment of progression. Radiographic criteria include: joint space narrowing, osteophyte formation, subchondral sclerosis and bone cysts. Biomarkers such as elevated C-reactive protein (CRP) and COMP may also be indicative of disease progression (Posey and Hecht, 2008; Punzi et al., 2005).

Pharmacological therapy for osteoarthritis is far behind other skeletal diseases such as rheumatoid arthritis and osteoporosis (Conaghan et al., 2008). In the United Kingdom, national institute for clinical excellence (NICE) guidelines layout a core treatment of local muscle strengthening supplemented by pain relief using paracetamol, topical/oral non-steroidal anti-inflammatory drugs (NSAIDs), cyclo-oxygenase 2 (COX-2) inhibitors and opioids. Surgical intervention using arthroscopic lavage and tissue debridement is used in the event of mechanical locking, giving way or X-ray evidence of loose bodies. Finally, osteotomy or joint replacement is used in advanced stages of OA (Conaghan et al., 2008). There are no currently available disease-modifying osteoarthritis drugs. Trials to regulate endogenous catabolic factors by administering protease inhibitors against MMP13 and ADAMTS5 targets have been unsuccessful because of insufficient efficiency and adverse events (Burrage and Brinckerhoff, 2007; Chuma et al., 2004; Flannery, 2010; Fosang and Little, 2008; Little et al., 2007; Nagase et al., 2006; Troeberg et al., 2008).

1.6.2 Aetiology of Osteoarthritis

There are multiple risk factors pertaining to the development and progression of osteoarthritis including; age, gender, heredity and mechanical damage (Buckwalter and Mankin, 1998b; Felson, 2004, 2005; Felson and Chaisson, 1997; Felson et al., 2004; Felson et al., 2000).

Age is consistently identified as a risk factor for osteoarthritis. OA is rare in adults under 40 and prevalence rates of radiographic OA rise steeply after the age of 50 in men and age 40 in women (Felson et al., 1995; van Saase et al., 1989). In addition, gender is a well-recognised risk factor in osteoarthritis. OA is more common in women (especially, especially after enter menopause) than men. Hand and knee OA, in particular has been reported to be more prevalent among women (Hochberg, 1991; Oliveria et al., 1995). Oestrogen is believed to have a chondroprotective role against OA, a study of cartilage volume in postmenopausal women reported use of long term oestrogen replacement therapy (ERT) had significantly greater cartilage volume than controls and ERT may prevent knee articular cartilage degradation (Wluka et al., 2001).

A family history of the disease is often present in patients presenting with OA. Twin-pair and sibling-risk and segregation studies highlighted that there may be a genetic component to osteoarthritis. Studies suggested that OA might be transmitted as a complex, multifactorial trait rather than a single-gene disorder (Loughlin, 2001, 2003, 2005; Loughlin et al., 2002; Spector and MacGregor, 2004). Genome wide linkage scans were performed and several genomic candidates identified linked to OA susceptibility. These genes include: interleukin 1 gene cluster 2q11.2-q13 (IL-1), the matrilin 3 gene (MATN3) 2p24.1, the IL-4 receptor α -chain gene (IL4R) 16p12.1, the secreted frizzled-related protein 3 gene (FRZB) 2q32.1, the metalloproteinase gene ADAM12 10q26.2 and the asporin gene (ASPN) in hip OA 9q22.31 (Kizawa et al., 2005; Loughlin et al., 2004; Loughlin et al., 2002; Smith et al., 2004; Stefansson et al., 2003; Valdes et al., 2006).

The most important risk factor for the development and progression of osteoarthritis is mechanical damage. This includes; occupation-related repetitive injury, obesity, joint deformity, joint misalignment and physical trauma.

It has been previously reported that occupations requiring repetitive bending and physical exertion have a higher prevalence of osteoarthritis (43.4% vs. 26.8%) (Felson et al., 1991). These occupations include: ballet dancers (feet and ankles), farmers (hips), coal miners (knees) and cotton mill workers (hands). Work by Felson and colleagues using the Framingham database in elderly adults found an association between high levels of physical activity and incident of knee OA (Felson et al., 1997). However, a link between low-impact recreational exercise and OA is controversial. 45 long distance runners and 53 controls were studied by Chakravarty and colleagues from 1987 to 2002 and monitored with serial knee radiographs. Although OA was initially more prevalent in runners compared with the control group (6.7% vs. 0%) by the end of the study there was no greater prevalence (20% vs. 32%) or severity (2.2% vs. 9.4% severe OA) of OA in runners versus the control group. It was concluded that long distance running in healthy older individuals was not associated with accelerated radiographic OA (Chakravarty et al., 2008).

Population based studies have shown that obesity increases susceptibility to developing OA (Creamer and Hochberg, 1997). The first National Health and Nutrition Examination Survey (HANES I) reported in a study of 5193 participants (aged 35-74) that there were significant associations of knee osteoarthritis with obesity (Anderson and Felson, 1988). Obese (body mass index between 30-35) women had ~4 fold greater risk of knee OA as compared with non-obese control group and obese men had ~5 fold increased risk of knee OA compared with non-obese control group. The mechanisms for how obesity influences OA is unclear (Anderson and Felson, 1988). Logically, increased body mass increases mechanical stress placed on joints. It is estimated that stresses of between 3-6 times one's body weight are applied across the knee joint during movement (Felson et al., 1995). However, obesity is also associated with an increased risk of hand OA suggesting a possible link between increased adipose tissue and a circulating factor (Carman et al., 1994). Two adipokines (leptin and apelin) have been reported in synovial fluid of OA patients and Bao and colleagues recently reported that recombinant rat leptin (100 µg) is a catabolic factor when injected into knee joints of rats compared to saline controls (Bao et al., 2010; Dumond et al., 2003; Hu et al., 2010a; Hu et al., 2010b). Addition of leptin significantly upregulated catabolic factors MMP2, MMP9, ADAMTS4, ADAMTS5 and cathepsin D mRNA and was

associated with histological depletion of proteoglycan in rat cartilage whilst downregulating anabolic factor bFGF at the mRNA level (Bao et al., 2010).

Finally, physical trauma to the cartilage or indirect damage (to ligaments) leading to joint misalignment is a strong risk factor for OA and may cause as many as 10% cases of OA (Furman et al., 2006; Gelber et al., 2000). Partial thickness cartilage defects in asymptomatic individuals are also likely to result in OA (Cicuttini et al., 2005). Anterior cruciate ligament (ACL) tears and either damage or tears to meniscal tissue result in increased risk of developing OA (Englund and Lohmander, 2004; Lohmander and Felson, 2004; Roos et al., 1995; Roos and Karlsson, 1998; Segawa et al., 2001; Sharma et al., 2008).

In summary, excessive wear and tear through joint overuse, obesity, joint instability and finally gross damage to the articular cartilage surface are all strongly associated with the development of osteoarthritis (OA).

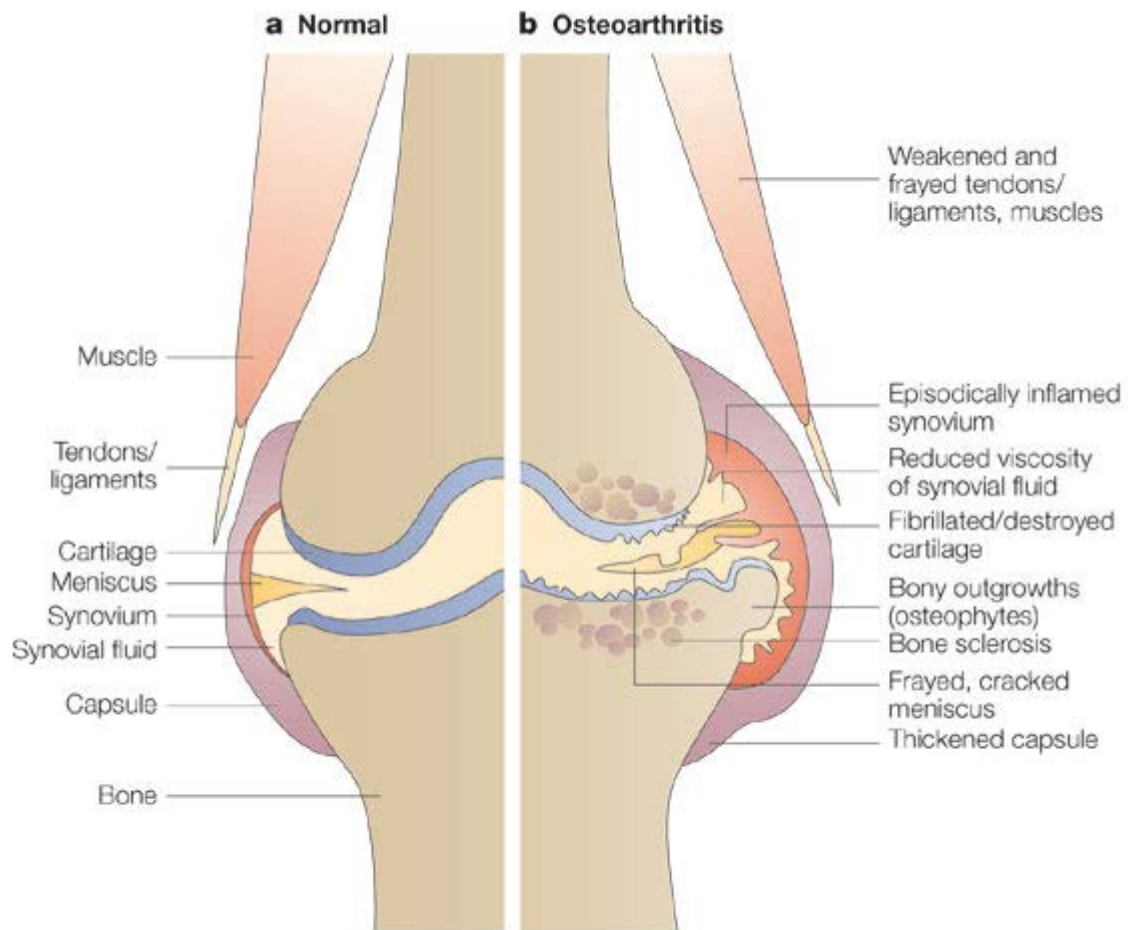


FIGURE 1.4. Articular structures affected in osteoarthritis. A Healthy joint is shown: normal cartilage with a smooth articulating surface, normal underlying bone structure, no signs of synovial inflammation or of meniscal changes. B Osteoarthritic joint: degenerate lesions and 'fibrillated' cartilage, bone remodelling (subchondral sclerosis and osteophytes), episodically inflamed synovium and frayed meniscus (Wieland et al., 2005).

1.6.3 Histological changes in osteoarthritis

Histological changes of osteoarthritis are well characterised and have a number of stages (Buckwalter and Mankin, 1998b; Collins and Mc, 1960; Meachim et al., 1965). Initially there is an progressive loss of proteoglycan, the articular cartilage surface loses its smooth appearance with early roughening and fibrillations becoming visible (Meachim et al., 1965). Microtracks deepen, fibrillations start as horizontal splits following the orientations of collagen fibres and then extend to form deep vertical fissures and clefts (McElligott and Collins, 1960). Clusters of chondrocytes form at these fissures and at the surface of the tissue suggesting chondrocyte activation, proliferation and an attempted repair mechanism (Meachim et al., 1965; Meachim and Sheffield, 1969). Later there are clear/empty lacunae suggesting cell death. Fissures eventually become full thickness cartilage defects.

In addition there are changes to the subchondral bone including; bone sclerosis, bone cysts and osteophyte formation. There may also be episodic inflammation of the synovial membrane although it is unclear if these changes initiate OA or are secondary events (Fig. 1.4).

1.7 Molecular changes in osteoarthritis

Osteoarthritis is traditionally thought of as a mechanical process, a consequence of “wear and tear” on a synovial joint. However, a more accurate description would be to describe an imbalance between net anabolic (matrix synthesis) and net catabolic (protease activity) activity. Articular cartilage destruction can be initiated by cartilage injury, overloading or problems in joint biomechanics. Numerous factors including age, obesity, metabolism and response to growth factors may lead to an imbalance between anabolism and catabolism and hence to a disruption in cartilage homeostasis.

1.7.1 Proteases and Osteoarthritis

In osteoarthritis it is believed that there is an increase in matrix degradation through increased production of proteases (ADAMTSs, MMPs), increased protease activity

(increased cleavage of pro-enzymes) or decreased activity of catabolic regulators (TIMPs). It has been previously suggested that one of the earliest events associated with OA is the loss of aggrecan core protein from the cartilage extracellular matrix and that this is a prerequisite for the subsequent proteolysis of collagen fibres. Because chondrocytes continuously resynthesise aggrecan, this first step is potentially reversible. The second step is considered permanent because, despite repair attempts, the original fibrous collagen network cannot be restored (Mankin and Lippiello, 1970; Mort and Billington, 2001).

Work by two groups monitored mRNA levels in human cartilage samples from early degenerative and late-stage OA donors (Aigner and Stove, 2003; Aigner et al., 2003; Bau et al., 2002). They reported a strong upregulation of MMP3 in early degenerative cartilage and a decrease in late-stage OA cartilage and an increased expression of MMP1, MMP2, MMP, MMP13, ADAMTS4 and ADAMTS5 in late stage OA cartilage (Aigner et al., 2003; Bau et al., 2002).

MMP and ADAMTS generated neoepitope aggrecan fragments have been identified in culture media of cartilage explants *in vitro* as well as human synovial fluid *in vivo* suggesting elevated aggrecanase/MMP levels or activity (Fosang et al., 1996; Lark et al., 1997; Little et al., 2002; Lohmander et al., 1993; Sandy et al., 1992; Sandy and Verscharen, 2001). Little and colleagues generated a line of aggrecan knockin mice with a mutation between the G1 and G2 domains which rendered aggrecan resistant to aggrecanase activity. It was reported that this knockin mutation significantly inhibited proteoglycan loss, diminished cartilage degradation and promoted cartilage repair (Little et al., 2007). Adamts5 knockout mice are significantly resistant to IL-1 α and retinoic acid induced aggrecan cleavage as well as surgically induced cartilage destruction, compared with wild-type mice (Glasson et al., 2005). No resistance to the progression of osteoarthritis was reported in Adamts4 knockout mice after surgical induction of joint instability and therefore Adamts5 is believed to be the more important aggrecanase in mouse cartilage (Glasson et al., 2004). However Song and colleagues demonstrated that the depletion of either ADAMTS5 or ADAMTS4 using siRNA in human cartilage explants inhibits proteoglycan release when explants were stimulated with tumor necrosis factor α (TNF α) and oncostatin M (OSM). This suggests that despite the dominant role of Adamts5 in mouse both ADAMTS4 and

ADAMTS5 may contribute towards cartilage destruction in human osteoarthritis (Song et al., 2007).

MMP13 is a matrix metalloproteinase thought to play a critical role in cartilage destruction. MMP13 is more active against type II collagen than other collagenases and chondrocyte expression of MMP13 mRNA is increased in late-stage human OA cartilage in association with cartilage erosion (Aigner et al., 2001; Minond et al., 2006). Neuhold and colleagues used a tetracycline-induced cartilage specific promoter to constitutively overexpress active human MMP13 in hyaline cartilage of transgenic mice. Overexpression resulted in focal OA cartilage pathology at load bearing sites similar to human OA including proteoglycan and type II collagen degradation (Neuhold et al., 2001). Little and colleagues demonstrated that the cartilage of *Mmp13^{-/-}* mice is protected from structural cartilage damage in a surgically induced model of OA, however there was no significant reduction in aggrecanolytic activity, osteophyte development or chondrocyte hypertrophy (Little et al., 2009). *MMP13^{-/-}* mice were able to inhibit cartilage degradation in the presence of aggrecan loss; this may suggest that aggrecan depletion on its own does not drive cartilage degradation. Proteases are clearly involved in the subsequent degradation of articular cartilage but the mechanism of initiation remains unclear.

Destruction of articular cartilage and joint space narrowing is a clinical presentation of radiographic osteoarthritis, however it is important to note osteoarthritis is not limited to the articular cartilage and that other components of the joint may be involved. Osteoarthritis is associated with subchondral bone remodeling, osteophyte formation, weakness in joint ligaments, loss of synovial fluid volume and episodically inflammation of the synovial membrane. Increased MMP13 and TIMP1 expression was detected in hyperplastic synovial tissue and subchondral bone of osteoarthritic mice suggesting that other tissues may significantly participate in joint destruction and subsequent remodeling (Salminen et al., 2002).

1.7.2 Chondrocyte Phenotype

In response to cartilage matrix degradation OA chondrocytes may revert to a more active/juvenile phenotype and attempt to initiate extracellular matrix repair by proliferating and upregulating collagens, glycoproteins, and small proteoglycans (Lippiello et al., 1977;

Nelson et al., 1998; Pfander et al., 2004a; Rizkalla et al., 1992; Swoboda et al., 1998). Type II collagen was found to be upregulated between 4-7 fold in OA chondrocytes, with particularly high expression in the radial zone (Aigner et al., 1999; Aigner et al., 2001; Nelson et al., 1998; von der Mark et al., 1992). However these molecules are often damaged, have inferior mechanical properties, cannot overcome their catabolic environment and therefore cannot regenerate the normal cartilage matrix architecture (Rizkalla et al., 1992).

Other data suggests that in addition to increased degradation of extracellular matrix proteins, chondrocytes undergo a phenotypic change, downregulating collagen type II, upregulating collagen X expression and becoming hypertrophic (Aigner et al., 2007; Aigner et al., 1999). These hypertrophic cells eventually undergo apoptosis, and the reasons for this are unknown (Hashimoto et al., 1998; Poole et al., 2001).

1.7.3 Osteoarthritis and Hypoxia

It is logical to assume the progressive loss of articular cartilage in osteoarthritis reduces the diffusion distance for oxygen and increases the oxygen tension in oosteoarthritic cartilage compared with healthy tissue. However, experimental evidence suggests that the low partial pressure of molecular oxygen in synovial fluid is further decreased in osteoarthritic joints, and this is associated with increased HIF-1 α activity (Grimmer et al., 2006; Lund-Olesen, 1970; Schneider et al., 1996). It has been suggested that chondrocyte metabolic activity increased in an attempt to repair damaged extracellular matrix. This would consume molecular oxygen, decreasing the partial pressure of molecular oxygen in synovial fluid leading to increased HIF-1 α activity. It has also been suggested that HIF-1 α isoforms are stabilised in HACs in response to cell stress (mechanical or inflammatory), this stabilisation promotes cell survival and is indepdent of oxygen tension (Coimbra et al., 2004).

1.7.4 Regulation of matrix turnover, cytokines and other inflammatory genes

The role of inflammatory cytokines in osteoarthritis *in vivo* is unclear, however they are useful tools to study cartilage degradation *in vitro*.

Interleukin 1 (IL-1) and Tumor necrosis factor α (TNF- α). Proinflammatory cytokine interleukin 1 (IL-1) is a cytokine mainly secreted by macrophages. IL-1 has two forms, α and β , both have similar effects on cartilage. IL-1 has been shown to upregulate catabolic factors (MMPs and ADAMTSs), increase inducible nitric oxide synthase (iNOS) expression and suppress the synthesis of aggrecan and type II collagen in chondrocytes (Bocquet et al., 1986; Kobayashi et al., 2005; Pasternak et al., 1986). The stimulation of cartilage explants with IL-1 induces the rapid depletion of proteoglycans in explants cultures (Flannery et al., 1999; Gebauer et al., 2005; Saklatvala, 1987; Vincenti and Brinckerhoff, 2002). The induction of human IL-1 β in temporomandibular joints of transgenic mice led to degeneration, dysfunction, and related pain in the joint (Lai et al., 2006). However, IL-1 β -knockout mice also develop OA lesions in cartilage of unoperated limbs (Clements et al., 2003). IL-1 is mainly produced by macrophages and although it has been reported that chondrocytes have the capacity to synthesis IL-1 it remains controversial whether it can be produced in amounts necessary for pathogenesis (Gruber et al., 2004). Similarly to IL-1, tumor necrosis factor α (TNF- α) induces the rapid depletion of proteoglycans in explants cultures (Saklatvala, 1987).

Transforming growth factor-beta (TGF- β). The TGF- β superfamily consists of 35 members, including the bone morphogenetic proteins (BMPs) and activins (de Caestecker, 2004). TGF- β has been shown to be an anabolic factor in bovine chondrocytes increasing synthesis of glycosaminoglycan and collagen type II (Davies et al., 2008). In addition it inhibits the cytokine induced cartilage matrix degradation by upregulating matrix components, downregulating MMPs and upregulating TIMPs (Andrews et al., 1989; Hui et al., 2000; Su et al., 1999).

Insulin-like growth factor-1 (IGF-1). Insulin-like growth factor-1 promotes proteoglycan synthesis, even in the presence of IL-1 and TNF- α (Loeser et al., 2005). Treatment of cartilage explants with IGF-1 is chondroprotective, inhibiting cartilage degradation in response to cytokine stimulation (Tyler, 1989). This chondroprotective effect may be mediated by inhibiting MMP activity (Hui et al., 2001).

In summary, articular cartilage is a tissue whose function is dependent upon the integrity and composition of a specialised extracellular matrix which enables the tissue to absorb and resist compression and allow articulation. Although the mechanisms of initiation

are unknown it is believed that during osteoarthritis, an imbalance between net anabolic (matrix synthesis) and net catabolic (protease activity) activity leading to a disruption of cartilage homeostasis and an increase in the degradation of extracellular matrix components. Increased protease (ADAMTSs and MMPs) activity results in loss of the water binding proteoglycans and the collagen network. These changes in cartilage matrix structure are responsible for a net reduction in cartilage tensile strength and a loss of cartilage's shock absorbing properties.

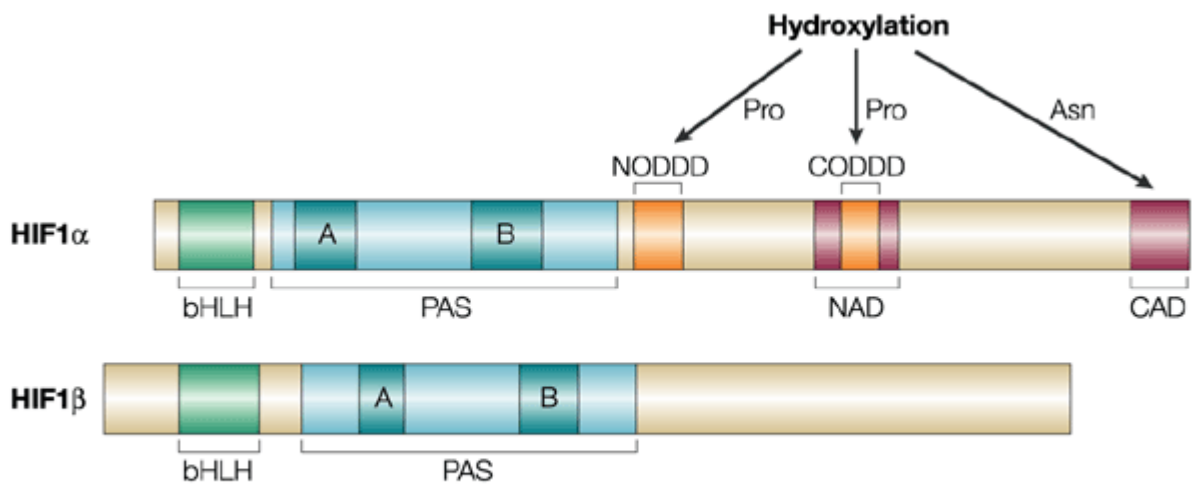
1.8 Hypoxia Inducible Factors (HIFs)

In 1991 Semenza and colleagues analysed the hypoxia induced upregulation of erythropoietin (EPO) in liver cells and using DNase analysis identified the binding of transcription factors to the EPO gene enhancer (Semenza et al., 1991; Semenza and Wang, 1992). Later work illustrated that the upregulation of EPO by hypoxia was present in all mammalian cell lines and therefore this oxygen-sensing transcription factor was not restricted to EPO producing cells (kidney or liver cells) (Maxwell et al., 1993). In 1993 this oxygen sensing transcription factor was identified and named "Hypoxia-Inducible Factor 1" or "HIF-1" (Wang and Semenza, 1993). The protein was eventually purified by ion-exchange DNA-affinity chromatography and its structure deduced (Wang et al., 1995; Wang and Semenza, 1995).

HIF-1 is a hetero-dimer, consisting of an α and β subunit and belongs to the family of basic helix-loop-helix and PER-ARNT-SIM domain containing transcription factors (Distler et al., 2004). These subunits are 120kDa (826aa) and 91-94kDa (789aa) in size respectively (Wang et al., 1995). Both subunits of HIF are constitutively expressed at the mRNA level; however, the α subunit is regulated post-translationally by oxygen levels.

1.8.1 HIF signalling pathway

In well-oxygenated cells HIF-1 α is regulated via two mechanisms. Firstly, the α -subunit is hydroxylated on specific proline residues within two oxygen-dependent-



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FIGURE 1.5. The domain structure of HIF-1 α and HIF-1 β . Both HIF-1 α and HIF-1 β have N-terminal basic helix-loop-helix (bHLH) domains and an intermediate PAS domain or PER-ARNT-SIM domain. HIF-1 α has the following domains; the N-terminal oxygen dependent degradation domain (NODDD); the C-terminal oxygen dependent degradation domain (CODDD); the N-terminal activation domain (NAD) and C-terminal activation domain (CAD). The CODDD and NAD overlap. The hypoxic regulation of HIF-1 α is conveyed by residues within the NODDD (-Pro), CODDD (-Pro) and CAD (-Asn) domains. (Schofield and Ratcliffe, 2004).

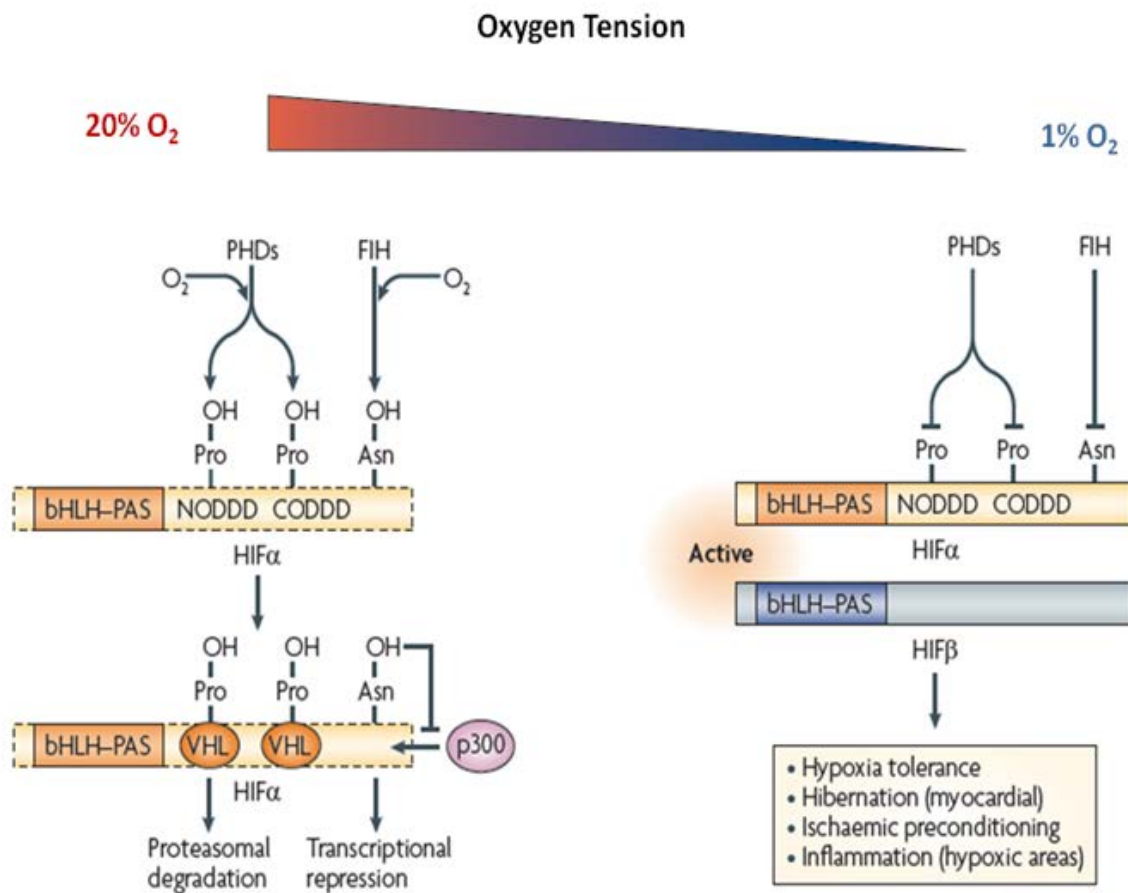


FIGURE 1.6. Regulation of HIF- α isoforms by the direct oxygen sensors, PHDs / FIH-1 enzymes. Decreases in molecular oxygen ($\leq 5\%$ O₂) inhibits two independent hydroxylation pathways involved in the regulation of HIF- α isoform activity: Proyl-hydroxylase domain (PHD) enzymes and Factor Inhibiting HIF-1 α (FIH-1) enzymes. The absence of molecular oxygen inhibits HIF- α isoform hydroxylation allows heterodimerisation with HIF-1 β and the binding of CBP/p300 co-activator. Active heterodimer migrates to the nucleus and there the transcription factor binds to specific hypoxia response elements or HREs (-5'RCGTG-3') in responsive target genes. HIF- α subunits have the following domains: bHLH-PAS - basic helix-loop-helix domain and PER-ARNT-SIM domain, NODD- N-terminal oxygen dependent degradation domain, CODD - the C-terminal oxygen dependent degradation domain. The hypoxic regulation of HIF- α subunits by PHD enzymes is conveyed by residues within the NODD (-Pro) and CODD (-Pro) domains (Fraisl et al., 2009).

degradation domains (NODDD and CODDD) by HIF-targeting prolyl-hydroxylase enzymes (PHD1, PHD2, PHD3 and PHD4) (Fig. 1.5) (Ivan et al., 2001). The PHD enzymes are a subfamily of dioxygenases that use molecular oxygen and 2-oxoglutarate (2-OG) as co-substrates to add hydroxyl groups to specific proline residues on the α subunit (Schofield and Ratcliffe, 2004). The hydroxylated proline residues are recognised by von Hippel–Lindau (VHL) protein of the E3 ubiquitin ligase complex, ubiquitinated and subsequently degraded by the 26S proteasome (Cockman et al., 2000). Therefore, under normoxic conditions HIF- α protein has a very short half life (~5 minutes) (Thoms and Murphy, 2010).

Secondly, another hydroxylase enzyme “factor inhibiting HIF-1” or “FIH-1” can also regulate the activity of HIF- α isoforms (Lando et al., 2002; Mahon et al., 2001). Similarly to the PHD enzymes FIH-1 uses molecular oxygen and 2-oxoglutarate (2-OG) as co-substrates to add hydroxyl groups to a specific asparaginyl residue (Asn803 on HIF-1 α and Asn851 on HIF-2 α) present within the C-terminal transactivation domain of the α subunit (Freedman et al., 2002). The hydroxylation of this residue is believed to disrupt the hydrophobic interactions between the C-TAD alpha helix and CH-1 domain of the CBP/p300 co-activator and so inhibits HIF transcriptional activity (Freedman et al., 2002).

Conversely, at low oxygen tensions (less than 5% O₂) limiting levels of molecular oxygen in the cell inhibits the PHD and FIH enzymes and they are unable to add hydroxyl groups to proline or asparaginyl residues of the α -subunit. As a result, the α -subunit is not targeted by pVHL and not degraded by the 26S proteasome. The α -subunit dimerises with the β subunit, migrates to the nucleus and there the transcription factor binds to specific hypoxia response elements or HREs (-5'RCGTG-3'), which are located in the major groove of DNA typically within the promoter region (Kinoshita et al., 2004; Wang and Semenza, 1993, 1995). In addition, the CBP/p300 co-activator is allowed to bind the C-TAD via its CH-1 domain. The binding to HREs activates gene expression; HIF is known to regulate more than 100 genes via these HRE's (Fig. 1.6) (Schofield and Ratcliffe, 2004).

Because PHD enzymes and FIH-1 use molecular oxygen as substrates, they are, in fact, the direct oxygen sensors.

1.8.2 HIF-1 α , HIF-2 α and HIF-3 α

HIF-2 α (EPAS1) is another HIF- α isoform closely related to HIF-1 α with similar protein organisation and structure (Ema et al., 1997; O'Rourke et al., 1999; Tian et al., 1997). Both have an N-terminal basic helix-loop-helix (bHLH) domain, an intermediate PAS domain or PER-ARNT-SIM domain and an N- and C-terminal transactivation domain (N-TAD / C-TAD). The bHLH domain is found in many transcription factors and mediates DNA binding (Massari and Murre, 2000). The PAS domain is necessary for dimerisation with HIF- β (Moglich et al., 2009). The N-TAD and C-TAD are necessary for the regulation of HIF- α isoform stability and activity. The N-TAD overlaps with the CODDD and is therefore involved in the regulation of α subunit stability. The C-TAD is not directly involved in proteolytic regulation but regulates HIF transcriptional activity via FIH-1.

HIF-2 α shares a 48% amino acid homology to HIF-1 α , with particularly high similarity in key functional domains: there is 83% sequence identity between bHLH domains and 70% sequence identity between PAS domains (Ema et al., 1997). There are also high levels of conservation (70% sequence identity) surrounding the N- and C-terminal oxygen dependent degradation domains (Ema et al., 1997).

HIF-2 α is regulated via the same mechanism as HIF-1 α and has the ability to activate HRE-containing target genes. It has been previously shown that HIF-1 α and HIF-2 α are able to regulate common and also unique targets in hypoxia. Genes such as vascular endothelial growth factor (*VEGF*), adrenomedullin (*ADM*) and adipose differentiation-related protein (*ADRP*) are regulated by both HIF-1 α and HIF-2 α (Hu et al., 2006; Hu et al., 2003). Conversely, Raval and colleagues demonstrated that in VHL-defective renal carcinoma (RCC) cells HIF-1 α specifically regulates pro-apoptotic gene *BNIP3* (Raval et al., 2005). Work by Sowter and colleagues demonstrated, using siRNA, that the knockdown of either HIF-1 α or HIF-2 α resulted in markedly different effects on gene expression and cell migration depending on the cell types (Sowter et al., 2003). Similarly, work by Rankin and colleagues in mouse hepatocytes also demonstrated that HIF-1 α and HIF-2 α have different transcriptional targets (phosphoglycerate kinase 1 and erythropoietin respectively) and therefore distinct roles in regulation of hypoxia-inducible genes (Rankin et al., 2007).

Finally, we have previously demonstrated that the hypoxic induction of SOX9 in human articular chondrocytes is HIF-2 α and not HIF-1 α dependent (Lafont et al., 2007).

Hu and colleagues carried out domain swapping experiments on HIF-1 α and HIF-2 α and found that the replacement of HIF-2 α N-terminal transactivation domain (but not the DNA binding bHLH domain, dimerization PAS domain or C-terminal transactivation domain) was sufficient to convert HIF-2 α into a protein with HIF-1 α functional specificity suggesting that target gene specificity is conferred by the N-terminal transactivation domain (Hu et al., 2007).

In 1998, Gu and colleagues identified a third HIF- α isoform, HIF-3 α . HIF-3 α is similar in structure and organisation to HIF-1 α and HIF-2 α however, HIF-3 α lacks a C-terminal transactivation domain (Gu et al., 1998). HIF-3 α may act as a dominant negative of other HIF- α isoforms, binding to HIF-1 α or HIF-2 α and forming transcriptionally inactive heterodimers. Six different splice variants exist and variant HIF-3 α 4 has been demonstrated to bind to HIF-2 α and attenuate its ability to bind to the HRE of hypoxia inducible genes in CC-RCC (clear-cell renal cell carcinoma) cells (Maynard et al., 2007; Maynard et al., 2003).

In addition to proline and asparaginyl hydroxylation HIF-1 α has been reported to be phosphorylated and acetylated. Richard and colleagues report strong phosphorylation of HIF-1 α by p42/p44 mitogen-activated protein kinases (MAPKs) but not by p38 MAPKs or c-Jun N-terminal kinase (c-JNK). This phosphorylation increases HIF-1 α transcriptional activity and is abrogated in the presence of MEK inhibitor PD98059 (Richard et al., 1999). In addition Jeong and colleagues have shown that the acetylation of HIF-1 α (on Lys⁵³²) by ARD1 enhances ubiquitinated HIF-1 α 's interaction with pVHL increasing its destabilisation (Jeong et al., 2002).

1.9 Proyl-hydroxylase domain enzymes

In 2001 the hydroxylation of two proline residues present on the HIF- α subunit was found to be essential for HIF- α to interact with pVHL and be subsequently degraded (Ivan et al., 2001; Jaakkola et al., 2001; Masson et al., 2001). Subsequent genetic work in

Caenorhabditis elegans and *Drosophila melanogaster* identified a single HIF-prolyl-hydroxylase enzyme which regulated the HIF- α subunit in each organism, named EGL-9 and dHPH respectively (Bruick and McKnight, 2001; Epstein et al., 2001). EGL-9 and dHPH belong to a subfamily of dioxygenases which use oxygen and 2-oxoglutarate to hydroxylate proline residues within the HIF- α oxygen dependent degradation domain (ODDD). Using the amino acid sequence of EGL-9 and dHPH four mammalian prolyl-hydroxylase domain enzymes have been identified: PHD1, PHD2, PHD3 and PHD4. These enzymes are 407 amino acids, 426 amino acids, 239 amino acids and 502 amino acids in size respectively (Bruick and McKnight, 2001; Epstein et al., 2001; Koivunen et al., 2007).

The PHD enzymes are hydroxylases which use molecular oxygen as a substrate. Baculovirus produced PHD enzymes have K_m values for oxygen between 230 - 250 μM (23-25% O_2), which is slightly higher than the concentration of dissolved oxygen under atmospheric conditions ($\sim 200 \mu\text{M}$ or 20% O_2), therefore PHD enzymes can be thought of as direct oxygen sensors as small changes in oxygen tension will effect enzyme activity (Hirsila et al., 2003). Under normoxic conditions one oxygen atom is added to a peptidyl-proline residue in the ODDD to form hydroxyproline and the other oxygen atom is used in an oxidative decarboxylation reaction to convert 2-oxoglutarate to succinate and CO_2 (Berra et al., 2006). The PHD enzymes do not hydroxylase free proline residues within the HIF- α subunit and recognise a specific conserved motif within the N-ODDD and C-ODDD: LXXLAP (where X represents any amino acid).

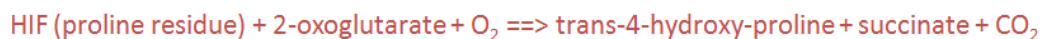
PHDs use ascorbate and Fe^{2+} as cofactors, and this explains why PHD and FIH enzymes are suppressed in the presence of iron chelators such as desferrioxamine (DFO). Cobalt (Co^{2+}) and nickel (Ni^{2+}) salts act as iron antagonists, substituting Fe^{2+} in the PHD enzymes and suppressing oxygen binding activity (Semenza, 1999).

The CLUSTALX multiple sequence alignment shown below illustrates that PHD1 and PHD2 are well-conserved in their C-terminal end (55% identity) and possess less similarity at their N-terminal halves (Epstein et al., 2001). PHD3 is the shortest of the PHD enzymes and aligns mostly with the C-terminal end of PHD1 and PHD2. All PHD enzymes possess a conserved pair of histidine residues (**H**) and one carboxylate motif (**D**); together these form the facial triad which coordinates an Fe^{2+} ion at the catalytic site (**HXD/E...H**

A



Mechanism:



B

CLUSTAL (1.0) multiple sequence alignment

```

PHD1_HUMAN  MANDSGGPGGSPSPSERDRQYCELCGPMENLLRCSCRCSSTFYCKEHQRQDWNKHKLVCCG 60
PHD2_HUMAN  ---WDSFQQPQLS--QALPQLPGSSSEPLEPEPGARMGVESYLPCLLPSTHCPGVP 54
PHD3_HUMAN  -----

PHD1_HUMAN  SEGALGHGVGPHQHSGPAPPAVPPPRAGAREPRKAAARRDNASGDAANGKVMKPPADP 120
PHD2_HUMAN  SEASAGSGT-PRATATSTTASPLDGFPGQDGGELRPLQSEGAALVTWGQQLRAAQGAR 113
PHD3_HUMAN  -----

PHD1_HUMAN  AAAASPCRAAGGQGSAAVAAREAPGKEEPPARSSLFQEKANLYPPENTPGDALSPGGGLR 180
PHD2_HUMAN  EAPFKRWAE DGGDAFSPSKRFWARQENQEA-----ERE GGMSCSCSSGSEASAG--- 164
PHD3_HUMAN  -----M--- 1

PHD1_HUMAN  PNGQTKEPLFALKLALFYIVPCMNWHGICVWDDFLGKETGQQIGDEVRAALHDTGKFTDQGL 240
PHD2_HUMAN  LMEZALPSPAPERLALDYIVPCMRYYGICVKDSPLGAALGGKVLAEVEALKRGGRLRDGQL 224
PHD3_HUMAN  PLGHIMRLDLKIALFYIVPCLHEVGFCYLDNPLGEVVGDCVLERVKQLHCTGALRDGQL 61
      .      ::**::*****:.. *:* *::*** * : . * . * : * : ***

PHD1_HUMAN  VSQWS-DSKDIRGDKITWIEGKEPGCEITGLMSMDLIRHCNGKLGSYKINGRTKAM 299
PHD2_HUMAN  VSQRA-IPPRISIRGDQIAWVEGHEPGCRSIGALMAHVDAVIRHCAGRLGSYVINGRTKAM 283
PHD3_HUMAN  AGPRAGVSFKHLRGDQITWIGNVEGCEAISFLLSLIDRLVLYCGSRLGKYYVMEKSKAM 121
      . . : : : : ***::** *:* *::*** * : : * : : * : : * : : ***

PHD1_HUMAN  VACYPGNGTGYYRVHVDNPNNGDGRCVTCIYYLNKNDKAVSGGILRIFPEGKAQFADIEPK 359
PHD2_HUMAN  VACYPGNGLGYYRVHVDNPHGDSRCITCIYYLNQNWVVKVHGGLLQIFPEGKPVVANI EPL 343
PHD3_HUMAN  VACYPGNGTGYYRVHVDNPNNGDGRCVTCIYYLNKNDKAVHGGILRIFPEGKSFIA DVEPI 181
      ***** *::**::*****:.. *:* *::*** * : : * : : * : : * : : ***
      ↑↑

PHD1_HUMAN  FDRLLFFWSDRRNPFHEVQPAYATRYAITVWYFDADERARAKVKY--LTGEKGVVVELNKP 417
PHD2_HUMAN  FDRLLIFWSDRRNPFHEVQPAYATRYAITVWYFDAKERAAAKDKYQLASGQKGVQVFSQP 403
PHD3_HUMAN  FDRLLFFWSDRRNPFHEVQPSYATRYAMTUVYFDAEERAEAKKKFRNLTKKTESALTED-- 239
      ***** *::**::*****:.. *:* *::*** * : : * : : * : : * : : ***
      ↑↑

PHD1_HUMAN  SDSVGKDV 426
PHD2_HUMAN  PTPT----- 407
PHD3_HUMAN  -----

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FIGURE 1.7. **Proyl-hydroxylase domain (PHD) enzymes.** A PHD1/2/3/4 molecular weights, cellular localisation and enzymatic reactions are shown. B Multiple sequence alignment of PHD1/PHD2/PHD3 using CLUSTALX.

motif). A positively charged Arginine (**R**) residue is used to bind the C-5 carboxyl group of 2-oxoglutarate (Schofield and Ratcliffe, 2004). These 4 residues are essential for the PHD enzymes' function (marked with red arrows on the multiple sequence alignment (Fig. 1.7). PHD1, PHD2 and PHD3 are structurally similar; each has 8 antiparallel β -strands which are linked with short turns and folded into a "jelly-roll" motif (Schofield and Zhang, 1999). The pair of histidine residues (**H**) and one carboxylate motif (**D**) which together form the facial triad necessary to accommodate an Fe^{2+} ion at the catalytic site are found on or close to the 2nd and 7th β -strands (Aravind and Koonin, 2001; Myllyharju and Kivirikko, 1997; Schofield and Zhang, 1999).

PHD4 is a putative HIF-targeting hydroxylase, which has only been recently characterized and in contrast to PHD1, PHD2 and PHD3 it is structurally similar to collagen prolyl 4-hydroxylases (Koivunen et al., 2007). PHD4 is an endoplasmic reticulum (ER) transmembrane protein with its catalytic domain within the lumen of the ER rather than the cytosol. It aligns poorly with the other PHD enzymes but does have identical residues indicating the presence of a facial triad (two histidines and 1 one carboxylate motif). In PHD4, the positively charged lysine is used to bind the C-5 carbosyl group of 2-oxoglutarate rather than arginine (Koivunen et al., 2007). PHD4 has been shown to hydroxylate HIF- α -ODDD reporter constructs and the knockdown of PHD4 using siRNA was shown to increase HIF-1 α levels, albeit by a modest amount (~2 fold) (Koivunen et al., 2007).

The PHD enzymes can be thought of as being cellular oxygen sensors. They are detectable and functional under normoxic conditions. However, PHD2 and PHD3 are themselves up-regulated by hypoxia. Hypoxia response elements are present upstream of PHD2 and PHD3 transcription start sites and electrophoretic mobility-shift assays demonstrate that HIF-1 α binds to these sites (Metzen et al., 2003; Pescador et al., 2005). It is hypothesised that these PHD enzymes are up-regulated under hypoxia in order to rapidly degrade HIF- α isoforms and down-regulate hypoxia responsive genes once the cells are returned to normoxic conditions. PHD1 has not been reported to be induced by hypoxia. Work by Koivunen and colleagues has demonstrated that PHD4 is up-regulated at the protein level in response to hypoxia in cultured human adult fibroblasts and embryonic kidney (HEK293), hepatoma (Hep3B) and fibrosarcoma (HT1080) cells (Koivunen et al., 2007).

Tissue distribution of the PHD enzymes has been investigated at the mRNA level in rats. The mRNAs of PHD1/2/3 were detectable in the heart, liver, kidney, brain, testis and lung (Willam et al., 2006). PHD2 was found to be ubiquitously expressed and has also been shown to be the most abundant PHD enzyme in several cell lines (Appelhoff et al., 2004; Berra et al., 2003; Willam et al., 2006). PHD1 expression was highest in the testes and PHD3 mRNA expression was highest in the heart suggesting particular roles for the proteins in these organs (Willam et al., 2006). Koivunen and colleagues also investigated the distribution of PHD4 in adult human tissue. Northern blotting revealed that PHD4 was highly expressed at the mRNA level in the heart, brain and pancreas. Additional experiments using PCR identified low levels of PHD4 expression in epiphyseal cartilage (Koivunen et al., 2007).

PHD1, PHD2, PHD3 have been detected at the protein level within the maturing zone of the human growth plate and in the mouse chondrocyte cell line N1511 (Terkhorn et al., 2007). PHD1/2/3/4 have been reported at the mRNA in human cartilage and at the PHD1/2/3 have been reported at the protein level (Hirsila, et al, 2003; Koivunen *et al.*, 2007; Terkhorn *et al.*, 2007). Recent microarray experiments in our laboratory on human articular chondrocytes found that while PHD2 mRNA is the most abundant PHD mRNA present under normoxic and hypoxic conditions, PHD3 message was the most highly induced by hypoxia (Murphy et al., 2009).

1.9.1 Enzyme Specificity

Under normoxic conditions two specific, conserved proline residues in the HIF- α subunit are hydroxylated by the PHD enzymes. However, different PHD isoforms show a preference for HIF-1 α 's N-ODDD (P402) or C-ODDD (P564) (Hirsila et al., 2003; Koivunen et al., 2006; Li et al., 2004). All PHD enzymes are more active against C-ODDD than N-ODDD substrates *in vitro* (Koivunen et al., 2006). PHD3 is virtually inactive against N-ODDD substrates (Hirsila et al., 2003; Koivunen et al., 2006; Li et al., 2004).

In 2004, Appelhoff and colleagues used siRNA against the three HIF prolyl-hydroxylases (PHD1, PHD2, PHD3) in a range of cell lines. It was found that all three PHD isoforms contribute towards the regulation of HIF-1 α and HIF-2 α . However, some isoforms show selectivity between HIF-1 α and HIF-2 α (Appelhoff et al., 2004). In MCF7 cells, the knockdown of PHD3 stabilised HIF-2 α at higher levels than when control cells were exposed

to hypoxia and substantially delayed HIF-2 α degradation following re-oxygenation (Appelhoff et al., 2004). The knockdown of PHD3 had minimal effect on HIF-1 α . In contrast, the suppression of PHD2 led to the stabilisation of HIF-1 α (Appelhoff et al., 2004). This work has been supported by other cell line studies suggesting that PHD enzymes have different selectivity against different HIF isoforms, with PHD1 and PHD3 being more active on HIF-2 α and PHD2 preferentially hydroxylating HIF-1 α (Berra et al., 2003; Ginouves et al., 2008).

1.9.2 PHD knockout mice

Work by Takeda and colleagues in 2006 investigated the roles of the PHD enzymes in mice. *Phd1*^{-/-} or *Phd3*^{-/-} mice are apparently normal with no defects, however, *Phd2*^{-/-} mice die mid-gestation between embryonic days 12.5 and 14.5 as a result of placental and heart defects (Takeda et al., 2006). *Phd2*^{-/-} knockouts led to a stabilisation and accumulation of both HIF-1 α and HIF-2 α in the placenta and embryo proper, however, most surprisingly, there was no specific HIF- α increase in the heart (Takeda et al., 2006). The absence of HIF- α stabilisation in the heart is surprising as *Phd2* was found to be the most highly expressed Phd enzyme at the mRNA level (*Phd2*>*Phd1*>*Phd3*), in both the placental and heart tissue. Mice have severe heart defects including underdeveloped myocardium and trabeculae, and there are two possible explanations for these reported heart defects in the absence of elevated HIF- α isoforms levels - either *Phd2* depletion in the embryo has a severe effect on circulatory system (e.g., raising blood pressure) and this indirectly damages the heart or, perhaps more likely, PHD2 regulates a HIF-independent target vital for heart development. In addition, *Phd2*^{-/-} increased HIF-1 α and HIF-2 α levels in the placenta of knockdown mice and was associated with severe defects including significantly reduced labyrinthine branching and widespread penetration of the labyrinth by spongiotrophoblasts and giant cells. In summary it was demonstrated that Phd2 plays an essential role for mouse embryogenesis.

Post-natal tamoxifen-induced, Cre-loxP-mediated *Phd2* knockout in mice leads to increased angiogenesis (vascular density), angiectasia (increased blood vessel dilation) and recruitment of vascular smooth muscle cells in multiple organs in adult mice (Takeda et al., 2007). Six weeks after treatment with tamoxifen major vascular branches were significantly dilated and reddened, although there was no sign of oedema, inflammation or ulceration. *Phd2* knockout led to increased vascular growth in the liver, heart, kidney, lungs and brain

although this effect did not necessarily correlate with local Phd2 depletion (there were very low levels of Phd2 depletion in the brain following tamoxifen treatment). Consistent with work in cell lines, *Phd2*^{-/-} led to the stabilisation and accumulation of HIF-1 α but not HIF-2 α in mouse liver and kidney tissue. Angiogenic factors such as *Vegf-a* and *Epo* were found at increased levels in serum and EPO was overexpressed by 230-fold leading to severe polycythaemia (Takeda et al., 2007). Overall this suggests that Phd2 is a major negative regulator of angiogenesis and erythropoietin (Takeda et al., 2007).

Although *Phd1*^{-/-} or *Phd3*^{-/-} mice are apparently normal with no defects, adult *Phd1*^{-/-} and *Phd3*^{-/-} double knockouts develop moderate erythrocytosis although these levels were not as high as *Phd2*^{-/-} deficient mice (Takeda et al., 2008). Consistent with work in cell lines *Phd1*^{-/-}/*Phd3*^{-/-} double knockout led to preferential HIF-2 α stabilisation and not HIF-1 α in the liver (Takeda et al., 2008).

Takeda and colleagues reported in 2006 that *Phd1*^{-/-} mice are viable and apparently normal. However, subsequent work by Aragones and colleagues suggests that PHD1 may play a role in muscle function and metabolism in mice. Aragones reported that the loss of Phd1 reprograms myofiber glucose metabolism switching from oxidative, aerobic to more anaerobic ATP generation. *Phd1*^{-/-} mice have a decreased exercise tolerance and lower O₂ consumption in skeletal muscle. Surprisingly the loss of Phd1 makes the skeletal muscle more resistant to ischaemic necrosis but the loss of Phd2 (*Phd2*^{+/-}) or Phd3 (*Phd3*^{-/-}) did not provide protection against ischemic cell death (Aragones et al., 2008). This hypoxia tolerance was not due to HIF-dependent angiogenesis, erythropoiesis or vasodilation. *Phd1*^{-/-} had elevated levels of HIF-2 α stabilisation in their myofibers compared to wild-type and by contrast HIF-1 α levels were either low or undetectable in both genotypes. The loss of HIF-2 α in *Phd1*^{-/-} deficient mice abrogated hypoxia tolerance. This study suggests a role for Phd1 in hypoxia tolerance and glucose metabolism, and provides further evidence of PHD selectivity for HIF- α isoforms (Aragones et al., 2008).

In summary, the PHD enzymes possess species, tissue and cellular expression differences. The current literature proposes that while all of the PHD enzymes can hydroxylase HIF-1 α and HIF-2 α , PHD2 is more active against HIF-1 α and PHD1/PHD3 are more active against HIF-2 α , although species and tissue differences are may occur.

1.10 Hypoxia and HIF stabilisation in human articular cartilage

As mentioned above articular cartilage functions in a chronically hypoxic environment throughout life. Depending of tissue depth, chondrocytes are exposed to approximately between 1-6% O₂. Reduced levels of molecular oxygen inhibit HIF-targeting hydroxylases and result in the stabilisation of HIF-1 α and HIF-2 α protein in human articular cartilage. Immunohistochemistry of human articular cartilage confirms the presence of HIF-1 α and HIF-2 α protein (Bohensky et al., 2009; Coimbra et al., 2004; Yudoh et al., 2005). Levels of HIF-1 α stabilisation correspond with the logical pattern of oxygen gradient, increasing in intensity away from the surface of the tissue (Brucker et al., 2005).

1.10.1 HIF stabilisation promotes differentiated chondrocyte phenotype

Experimental evidence suggests that the low partial pressure of molecular oxygen in synovial fluid is further decreased in osteoarthritic joints, and that this is associated with increased HIF-1 α activity (Grimmer et al., 2006; Lund-Olesen, 1970; Schneider et al., 1996). Yudoh and colleagues investigated human articular cartilage of osteoarthritis (OA) patients and reported high expression of HIF-1 α in degenerated regions compared with macroscopically intact regions. The role of HIF-1 α was further investigated by treating chondrocytes with interleukin-1 β under normoxic and hypoxic conditions. Treatment of chondrocytes with interleukin-1 β stabilised HIF-1 α and depletion of HIF-1 α using small interfering RNA significantly increased the number of apoptotic chondrocytes. Similarly, an inflammatory signal (TNF- α) in human chondrocytes and mechanical stress (overloading) in bovine cartilage discs have been shown to stabilise HIF-1 α (Coimbra et al., 2004). It has been suggested that HIF-1 α is anti-apoptotic and protects articular chondrocytes from cell death induced by catabolic stress (Yudoh et al., 2005).

Conversely, Bohensky *et al.*, reported decreased levels of HIF-2 α in osteoarthritic human articular cartilage. The decrease in HIF-2 α was linked with increased cell death in chondrocytes and it was proposed that HIF-2 α is a modulator of autophagy and protects the cells from apoptosis (Bohensky et al., 2009).

Hif-1 α ^{-/-} mice die *in utero* as early as E8.5 exhibiting multiple morphological defects demonstrating that HIF-1 α is essential for embryonic development (Compennolle et al., 2003;

Iyer et al., 1998; Ryan et al., 1998). Similarly, HIF- $\beta^{-/-}$ mice die *in utero* between 9.5 and 10.5 days gestation from primarily from defects in placenta angiogenesis (Kozak et al., 1997; Maltepe et al., 1997). Schipani and colleagues demonstrated that the mouse developmental growth plate is hypoxic and that HIF-1 α is essential for proliferation and survival of murine growth plate chondrocytes *in vivo*. Cartilage-specific HIF-1 α depletion led to cell death within articular and growth plate chondrocytes resulting in long bone, rib and vertebral body defects coupled with decreased expression of the CDK inhibitor p57 (Schipani et al., 2001). It was concluded that HIF-1 α is essential for growth plate development and chondrocyte survival in response to hypoxic conditions.

Scortegagna and colleagues demonstrated that *hif-2 $\alpha^{-/-}$* mice survive but have substantially shorter lifespan, lower body weight, overt cardiac hypertrophy, mitochondrial dysfunction, skeletal myopathy and hepatosteatosis providing evidence that while HIF-2 α is not embryonic lethal, it plays a fundamentally important functional role and is not redundant (Scortegagna et al., 2003). Similarly, homozygous knockout of HIF-2 α in the limb bud mesenchyme caused a modest delay in endochondral bone development at E17.5 (Araldi et al., 2011). Others have shown that homozygous deficient *hif-2 $\alpha^{-/-}$* mice die at the early embryonic stage (Saito et al., 2010; Tian et al., 1998; Yang et al., 2010). A recent publication in Nature Medicine reported that heterozygous deficient *hif-2 $\alpha^{+/-}$* mice grew without abnormalities of major organs but showed mild dwarfism compared to wild-type littermates (Saito et al., 2010). Heterozygous deficient *hif-2 $\alpha^{+/-}$* mice have reduced limb length (8-16% shorter), increased hypertrophic and proliferation zones suggesting a role for *hif-2 α* in endochondral ossification and bone growth in mouse (Saito et al., 2010).

Robins and colleagues identified putative Hypoxia Responsive Elements (HREs) within the promoter of tissue specific transcription factor *Sox9*, in mouse pluripotent mesenchymal stromal (ST2) cells. Hypoxia increased expression of *Sox9* and caused ST2 cells to differentiate along a chondrocyte pathway (Robins et al., 2005). However this hypoxic induction of *Sox9* was not confirmed at the protein level. The hypoxic upregulation of *Sox9* was abolished when these putative HRE sites were mutated (Robins et al., 2005). This hypoxia induced chondrogenesis was further investigated by Kanichai and colleagues. Mouse MSCs were exposed to hypoxia and chondrogenesis observed by measuring collagen II, *Sox9* expression and proteoglycan deposition (Kanichai et al., 2008). When siRNA was

used to knockdown HIF-1 α , the hypoxia-induced chondrogenesis was absent. The authors concluded that HIF-1 α provided a beneficial effect to cartilage and was a key mediator of chondrogenesis (Kanichai et al., 2008). However, Kanichai and colleagues only focused on HIF-1 α and failed to investigate HIF-2 α function.

1.10.2 HIF stabilisation increases cartilage matrix synthesis

Hypoxia not only promotes chondrocyte differentiation but also promotes chondrocyte function. Hypoxia has been shown to increase cartilage matrix deposition in murine epiphyseal chondrocytes. The deletion of the pVHL (which would stabilise both HIF-1 α and HIF-2 α) results in significantly reduced chondrocyte proliferation rate, ~1.4 fold increase in *Col2a1* mRNA expression and the presence of atypical large cells within the resting zone (Pfander et al., 2004b). Pfander and colleagues also isolated and cultured epiphyseal chondrocytes from wild-type and *Hif-1 α ^{-/-}* mice. They reported that the exposure of wild-type cells to 0.5% oxygen increased *Col2a1* protein levels ~2 fold and this hypoxic induction was lost in *Hif-1 α ^{-/-}* cells (Pfander et al., 2003). *Aggrecan* mRNA and protein levels were unchanged upon exposure to hypoxia (Pfander et al., 2003).

Ren and colleagues isolated mouse chondrocytes from the hips and knees of 3- or 4-day old C56BL/6 mice. All mice possessed homologous loxP sites flanking the HIF-1 α locus. Mouse chondrocytes were exposed to hypoxic conditions and the upregulation of *Col2a1* (~1.5 fold) and *Aggrecan* (~1.4 fold) observed via real-time PCR (Ren et al., 2008). When the chondrocytes were infected with an adenovirus containing Cre recombinase (creating *Hif-1 α ^{-/-}* cells) this hypoxia induced extracellular matrix synthesis was lost. Ren and colleagues concluded that the hypoxia induced cartilage matrix synthesis was HIF-1 α dependent (Ren et al., 2008).

Work by Amarilio and colleagues examined the expression of Sox9 under hypoxic conditions in mouse mesenchymal cells derived from E11.5 *Hif1 α* floxed mouse embryos infected with AdCre or AdGfp. Low oxygen tensions increased Sox9 expression (~2 fold) in control cells and this hypoxic induction of Sox9 was lost in *Hif-1 α* depleted cells. This hypoxic induction of Sox9 was not confirmed at the protein level. Conversely, the overexpression of *Hif-1 α* induced an increase in Sox9 expression (Amarilio et al., 2007). Chromatin immunoprecipitation provided evidence for direct recruitment of HIF-1 α to the

Sox9 promoter (Amarilio et al., 2007). Hypoxia upregulated *Col2a1* (~1.7 fold) and *Aggrecan* (~2.3 fold) at the mRNA level in mouse mesenchymal cells and this hypoxic induction is lost in *Hif-1 α* depleted cells.

Saito and colleagues recently overexpressed HIF-2 α in a mouse chondrogenic cell line (ATDC5 cells) treated with normoxia and found overexpression of HIF-2 α significantly increased mRNA levels of *Col2a1* (~2 fold) and *Aggrecan* (~2 fold). ATDC5 cells transfected with siRNA against Hif-2 α did not show a significance decrease in *Col2a1* or *Aggrecan* mRNA levels compared to control (Saito et al., 2010). However this is expected as experiments were performed under normoxic conditions and HIF-2 α would not be stabilised at 20% oxygen due to hydroxylation of its proline residues by the PHD enzymes, and subsequent ubiquitination and proteosomal degradation.

There is significant evidence that hypoxia upregulates expression of cartilage matrix genes in bovine, porcine and human chondrocytes and has similar effects in human meniscal cells (Adesida et al., 2006; Domm et al., 2002; Lafont et al., 2007; Murphy and Polak, 2004; Murphy and Sambanis, 2001; Strobel et al., 2010; Zhang et al., 2011). We have previously demonstrated in healthy human articular chondrocytes that key cartilage matrix genes *COL2A1*, *COL9A1*, *COL11A2* and *AGGRECAN*, whilst upregulated by hypoxia, are not direct HIF-target genes and this hypoxic upregulation is mediated by key cartilage transcription factor SOX9. Furthermore, the hypoxic upregulation of SOX9 was found to be HIF-2 α , and not HIF-1 α dependent.

Although hypoxia has been shown to increase cartilage matrix deposition in murine chondrocytes, this reported hypoxic induction is weaker than that reported in human (and other large animal) articular chondrocytes. Isolated and cultured mouse chondrocytes exposed to hypoxia have typically been shown to upregulate *Col2a1* mRNA 1.5 – 2 fold (Amarilio et al., 2007; Pfander et al., 2003; Pfander et al., 2004b; Ren et al., 2008; Saito et al., 2010). *Aggrecan* mRNA levels have been reported unchanged upon exposure to hypoxia or have been upregulated very modestly (Amarilio et al., 2007; Pfander et al., 2003; Pfander et al., 2004b; Ren et al., 2008; Saito et al., 2010). In contrast HACs treated with hypoxia upregulate *Col2a1* 4 - 12 fold and *Aggrecan* 2 – 5 fold at the mRNA level (Lafont et al.,

2007; Murphy and Polak, 2004; Thoms and Murphy, 2010). Possible reasons for this will be elaborated on in discussion sections of Chapter 4 and Chapter 5.

Collagen prolyl-4-hydroxylases (C-P4Hs) are tetramers (consisting of 2 α subunits and 2 β units) responsible for catalysing the formation of 4-hydroxyproline by hydroxylation of –X-Pro-Gly– triplicates within the pro- α collagen chains. Like HIF-targeting hydroxylases collagen prolyl-4-hydroxylases use 2-oxoglutarate and oxygen as substrates (iron and ascorbate as cofactors) to hydroxylate proline residues and this hydroxylation is essential for the formation of intramolecular hydrogen bonds and subsequent stable triple-helical formation (Jimenez et al., 1973; Kukkola et al., 2003; Myllyharju, 2003; Van Den Diepstraten et al., 2003). Unlike the PHD1/2/3, however, C-P4H enzymes have a low K_m for oxygen (40 μ M vs. 230-250 μ M) suggesting that C-P4H enzymes remain active even under low oxygen tensions (Hirsila et al., 2003). This is logical given the roles of the HIF- and Collagen- Prolyl hydroxylases. PHD1/2/3 act as cellular oxygen sensors and therefore even a small change in oxygen tension can influence their activity. In contrast, C-P4Hs must show high activity even in challenging hypoxic micro-environments (e.g., cartilage extracellular matrix synthesis or wound repair in a tissue with poor vascularisation). Posttranslational modification of type II procollagens are essential steps for formation of the triple helices and their subsequent secretion. It has been demonstrated that hypoxia increases expression of collagen prolyl-4-hydroxylase α and β subunits in primary human articular chondrocytes and this upregulation was abolished by treatment with 2-Methoxestradiol (2ME2) (Grimmer et al., 2006; Hofbauer et al., 2003). 2ME2 has been shown to decrease levels of HIF- α isoforms in chondrocytes through an unknown mechanism of inhibition. Therefore hypoxia has two mechanisms of promoting type II collagen secretion, upregulating COL2A1 gene expression and increasing expression of collagen prolyl-4-hydroxylases (C-P4Hs) necessary for collagen post-translational processing.

In summary, the stabilisation of HIF- α isoforms promotes the chondrocyte survival and phenotype. In addition hypoxia has a strong net anabolic effect upregulating key matrix genes *COL2A1*, *COL9A1*, *COL11A2*, and *AGGRECAN* in human articular chondrocytes (HACs). We have previously demonstrated that the hypoxic upregulation of SOX9 was found to be HIF-2 α , and not HIF-1 α dependent. Finally, hypoxia has been reported to increase levels of collagen prolyl 4-hydroxylase levels suggesting that hypoxia upregulates

not only *COL2A1* gene transcription but also regulates the post-translational processing of type II collagen fibres and hence their secretion. This opens novel, exciting possibilities of manipulating the endogenous oxygen sensing pathways, for example, inhibiting PHD enzymes in order to selectively stabilise HIF-2 α and hence promote articular chondrocyte function, promote cartilage synthesis and to stimulate repair. We performed our research using healthy human articular cartilage and human articular chondrocytes. This provides important clinically relevant information as the data generated can be extrapolated to what may be happening to human articular cartilage *in vivo*.

Interestingly two recent publications in *Nature Medicine* have suggested that HIF-2 α is a transactivator of many catabolic factors and is implicated in experimentally induced mouse models of osteoarthritis (Saito et al., 2010; Yang et al., 2010). We will further discuss this topic in Chapter 4.

1.11 Summary and aims of the project

In summary, the stabilisation of HIF- α isoforms promotes chondrocyte survival and maintenance of the differentiated phenotype, the latter through upregulation of cartilage master regulator, transcription factor SOX9. In addition hypoxia has a strong net anabolic effect upregulating key matrix genes *COL2A1*, *COL9A1*, *COL11A2*, and *AGGRECAN* in human articular chondrocytes (HACs) (Bi et al., 1999; Domm et al., 2002; Murphy and Polak, 2004; Murphy and Sambanis, 2001). Previous work in our laboratory has demonstrated that hypoxia inducible factor 2 α (HIF-2 α), not HIF-1 α , is integral for hypoxia-induced SOX9 expression and the subsequently enhanced cartilage matrix production (Lafont et al., 2007). Prolyl- and asparaginyl- hydroxylases are members of an oxoglutarate-dependant dioxygenase family responsible for inhibiting HIF in the presence of oxygen. We hypothesise that antagonising specific hydroxylases may (selectively) stabilise HIF-2 α , promote the human articular chondrocyte phenotype and enhance cartilage deposition without inhibiting prolyl and lysyl hydroxylases essential for collagen processing (Fig. 1.8).

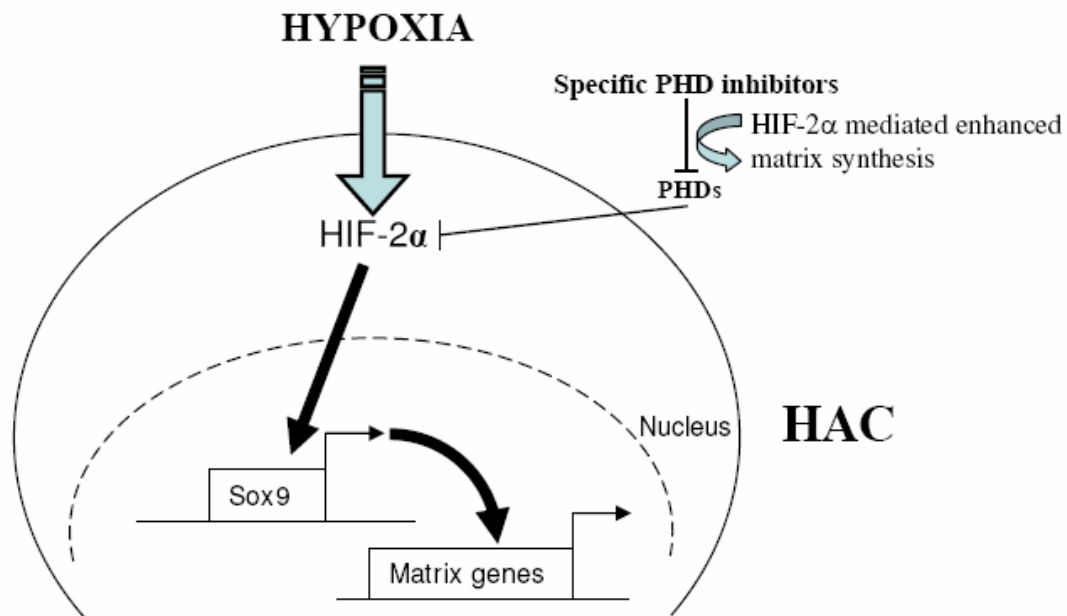


FIGURE 1.8. **Summary and hypothesis.** HIF-2 α mediated induction of cartilage matrix synthesis by human articular chondrocytes (HACs) may be possible through the inhibition of specific HIF-2 α targeting prolyl hydroxylase (PHD) enzymes. Image from (Murphy et al., 2009).

1.11.1 Aims of the Project

1. To firstly investigate the regulation of HIF- α isoforms in human articular chondrocytes by HIF-specific hydroxylases (PHD1, PHD2 and PHD3) and determine (using RNAi) which enzymes are critical (and potentially specific) for HIF-2 α regulation.
2. To discover if inhibition of such potentially HIF-2 α -specific hydroxylases leads to enhanced SOX9 expression and extracellular matrix synthesis in human articular chondrocytes.
3. To compare general hydroxylase inhibition (e.g., DMOG) to specific inhibition of HIF targeting enzymes, with regard to extracellular matrix deposition.
4. To investigate the regulation of key catabolic factors responsible for extracellular matrix degradation by hypoxia in human articular chondrocytes.
5. Finally, to investigate the observed hypoxic regulation of human articular chondrocyte function in other model species, namely pigs and mice. This will provide preliminary results for future possible animal studies.

Chapter 2

Materials and Methods

2.1 Reagents

Unless otherwise stated, all chemicals were supplied by Sigma-Aldrich (Dorset, UK) or BDH Chemicals (Dorset, UK) and are of the highest quality. Bovine serum albumin, electrophoresis grade (BSA) was from PAA Laboratories (Pasching, Austria). Phosphate buffered saline (PBS) was obtained as a 10x sterile solution (VWR, Lutterworth, UK). A 1xPBS solution was generated by diluting the 10x stock with ultrapure ddH₂O.

2.1.1 General buffers and reagents

10xPBS: 43mM Na₂HPO₄, 14mM KH₂PO₄, 14mM KCL and 1.37M NaCl at pH 7.3.

Radio-immunoprecipitation assay (RIPA) lysis buffer: 100mM Trizma base pH7.4, 300mM NaCl, 20mM NaF, 2mM EDTA, 0.2mM β glycerophosphate, 2%(w/v) Na vanadate, 2% deoxycholate, 0.5% SDS and 1% NP-40.

Urea/SDS lysis Buffer: 8 M urea, 10% glycerol, 1% SDS, 5 mM DTT, 10 mM Tris-HCl.

Bradford reagent: 0.01% (w/v) Coomassie Brilliant Blue G-250, 4.7% (v/v) ethanol and 8.5% (v/v) H₃PO₄.

SDS sample buffer: 2% SDS, 50 mM Tris, 10% glycerol, 0.1% bromophenol blue, 5% 2- β -mercaptoethanol.

Transfer buffer: 25mM Trizma Base, 190mM glycine, 20% methanol).

PBST: 1XPBS, 0.1% Tween 20.

Membrane Blocking Solution 1: 5% non-fat dried milk, 0.1% Tween 20 in 1xPBS.

ECL Solution 1: 9ml ddH₂O, 1 ml 1M Tris-HCl, 45 μ l Coumaric acid stock, 100 μ l Luminol stock [pH 8.5]. Add Tris-HCl first and mix. Add Coumaric acid and mix. Then add the Luminol and mix. Store in the dark at 4°C.

ECL Solution 2: 9ml ddH₂O, 1 ml 1M Tris-HCl, 6µl Hydrogen Peroxide, 30% stock [pH8.5]. Store in the dark at 4°C.

Running buffer: 25mM Tris-HCL (pH 8.3), 200mM Glycine, 0.1% SDS).

Deglycosylation Buffer: 50 mM Tris HCl (pH 7.5), 50 mM sodium acetate, and 10 mM EDTA.

4xSDS sample buffer: 2% SDS, 50 mM Tris, 10% glycerol, 0.1% bromophenol blue, 5% 2-β-mercaptoethanol.

2.1.2 Cytokines

Porcine and human cartilage explants, as well as passaged human articular chondrocytes (HACs) were stimulated with IL-1α. Details are described below.

2.1.3 Inhibitors

Mouse and human cartilage explants, as well as human passaged articular chondrocytes (HACs) were treated with pharmacological inhibitors. Details are described below.

2.1.4 Antibodies

Primary and secondary antibodies for western blotting used in experiments are listed in table 2.3 below.

Table. 2.1 Cytokines

Cytokine	Species	Source	Vehicle
Interleukin-1 α	Human	Professor J. Saklatvala	Serum Free DMEM

Table. 2.2 Inhibitors

Inhibitor	Target	Source	Concentration	Vehicle
SB202190	p38 MAP kinase inhibitor	Calbiochem (559388)	2 μ M	DMSO
Desferrioxamine (DFO)	Iron chelator		10mM	PBS
Dimethyloxaloylglycine (DMOG)	2-oxoglutarate dependent hydroxylase family.	Biomol international, LP (E1-347)	2mM	PBS

Table. 2.3 Primary and Secondary antibodies.

Antibody specificity	Antibody Type	Dilution	Source
SOX9 (ab5535)	Rabbit, polyclonal	1:1000	Chemicon, Southampton, UK
PHD2 (ab4561)	Rabbit, polyclonal	1:1000	Abcam, Cambridge, UK
PHD3 (Clone 188e)	Mouse, monoclonal	1:20	Oxygen Sensing Group, Oxford,
FIH-1 (NB100-428SS)	Rabbit, polyclonal	1:1000	Novus Biologicals, Littleton, CO.
HIF-1α (Clone 54)	Mouse, monoclonal	1:250	BD Transduction Laboratories, Lexington, KY
HIF-2α (Sc-13596)	Mouse, monoclonal	1:250	Santa Cruz Biotechnology, Santa Cruz, CA
Col2α1 (MAb8887)	Mouse, monoclonal	1:1000	Chemicon, Southampton, UK
TIMP1	Sheep, polyclonal	1:500	Antibody was provided by Hideaki Nagase (Kennedy Institute, London, UK)
TIMP3 (ab39184)	Rabbit, polyclonal	1:1000	Abcam, Cambridge, UK
Aggrecan ARGSV [BC-3] (ab3773)	Mouse, monoclonal	1:100	Abcam, Cambridge, UK
α-Tubulin (ab7291)	Mouse, monoclonal	1:5000	Sigma Aldrich company ltd, dorset, UK
Rabbit Ig HRP (P0217)	Swine, Ig	1:2000	DAKO Cytomation, A/S, Denmark
Mouse Ig HRP (P0218)	Rabbit, Ig	1:2000	DAKO Cytomation, A/S, Denmark

2.2 Cell and tissue culture

Dulbecco's modified Eagle's medium (DMEM) was obtained as a 1x sterile and endotoxin-free solution from PAA (Pasching, Austria). Note: Unless otherwise stated this was always supplemented with 10% (v/v) foetal bovine serum (FBS) and 1% penicillin/streptomycin (Biosera, East Sussex, UK).

2.2.1 Isolation of human cartilage explants

Healthy human articular cartilage was obtained from patients after they provided informed consent, and following local ethics committee guidelines. Samples of cartilage were obtained from 37 patients (29 male, 8 female; age 8-55, mean age 24.8 years) who received amputations due to osteosarcomas or soft tissue sarcomas. Samples were not taken from amputations where the sarcoma was present in the joint. The cartilage was harvested from the femoral condyle and tibial plateau (on the day of surgery) using aseptic technique, diced into small pieces (3 mm²) and cultured in DMEM.

For most cartilage explant experiments ~60mg cartilage (20 explants) was cultured in a 6cm dish in 5ml of DMEM media and incubated in either 20% oxygen tension or 1% oxygen tension at 37°C in a Galaxy Triple gas incubator (CRS Biotech, Ayrshire, Scotland, UK) supplemented with 5% CO₂.

2.2.2 Isolation of human articular chondrocytes

The cartilage was harvested from the femoral condyle and tibial plateau using aseptic technique, diced into small pieces (3 mm²). This diced cartilage was incubated overnight in DMEM containing 0.5mg/ml collagenase type 2 from *Clostridium histolyticum* (Worthington, Freehold, NH) and incubated at 37°C overnight with constant shaking (~50 rpm).

Following overnight digestion, cells were isolated by passing the digested suspension through a cell strainer (BD Falcon, BD Biosciences, Bedford, USA) to remove any larger undigested material. The cell suspension was centrifuged for 5 minutes at 1500 r.p.m and the cell pellet washed twice with medium. Cells were counted using a standard haemocytometer

and initially seeded at a density of 8×10^3 cells/cm² in DMEM. Cultures were incubated at 37°C, 20% oxygen in a Galaxy triple gas incubator (CRS Biotech, Ayrshire, Scotland, UK). Chondrocytes were passaged at the time of confluency (typically after 7 to 14 days) and re-seeded in 15cm dishes (in 25ml of medium) at a density of 5×10^3 cells/cm².

2.2.3 Isolation of mouse cartilage explants

Mouse hip explants were prepared from the hips of 6-week-old male C57Bl/6 mice. The skin and soft tissue was removed from the hind legs. The hip joints were dislocated and the proximal femoral heads isolated using aseptic technique under an anatomical microscope. Femoral heads were cultured using the same protocol as human explants (5 hips per 6cm dish in 5ml DMEM).

2.2.4 Isolation of mouse chondrocytes

Mouse femoral head explants were incubated overnight in DMEM containing 0.5mg/ml collagenase type 2 from *Clostridium histolyticum* (Worthington, Freehold, NH) and incubated at 37°C overnight with constant shaking (~50 rpm).

Following overnight digestion, cells were isolated by passing the digested suspension through a cell strainer (BD Falcon, BD Biosciences, Bedford, USA) to remove any larger undigested material. The cell suspension was centrifuged for 5 minutes at 1500 r.p.m and the cell pellet washed twice with medium. Cells were counted using a standard haemocytometer and initially seeded at a density of 8×10^3 cells/cm² in DMEM. Cultures were incubated at 37°C, 20% oxygen in a Galaxy triple gas incubator (CRS Biotech, Ayrshire, Scotland, UK). Chondrocytes were passaged at the time of confluency (typically after 7 to 14 days) and re-seeded in 15cm dishes (in 25ml of medium) at a density of 5×10^3 cells/cm².

2.2.5 Porcine cartilage explant isolation and culture:

Porcine articular cartilage was taken from the 2nd-3rd metacarpophalangeal (MCP) joints of 3 to 6 month old (male and female) pigs obtained from a local slaughterhouse within 16 hours of slaughter. Before use porcine trotters were soaked in 1% virkon solution and then washed with 70% ethanol (Dupont Relyon Prevention Solutions, Sudbury, UK).

After skinning the trotter, the MCP joint was opened in a biological safety cabinet. Cartilage explants from 1 joint were dissected using aseptic technique from the articular surface directly into DMEM and cut into cubes (3 mm^3). 10 explants of cartilage (~30 mg wet weight) per well were placed in a 48-well plate with 300 μ l of DMEM media. Following dissection explants were rested in the incubator in 20% oxygen conditions for 24 hours.

2.4 Small interfering RNA (siRNA) transfection:

Primary (P0) or 1st to 3rd passage (P1-P3) human articular chondrocytes (HACs) were seeded at 5×10^3 cells/cm² in 6cm tissue culture dishes (in 5ml of medium). Cultures were incubated at 37°C, 20% oxygen and 5% CO₂ until 50% confluent (typically 1 day). Lipofectamine 2000 (Invitrogen, San Diego, CA) was used to transfect cells with small interfering RNA (siRNA) at a final concentration of 10 nM in serum-free OptiMEM I. The *PHD1*, *PHD2* and *PHD3* targeting siRNA (Applied Biosystems, Foster City, CA) which were used are listed on Table 2.4. siRNA oligos against HIF-1 α and HIF-2 α were used as previously described (Lafont *et al.*, 2007). As a non-targeting control, siRNA against luciferase (Dharmacon, Lafayette, CO) were transfected in parallel. Four hours after transfection the OptiMEM was removed and replaced with DMEM (containing 10% FCS and 1% penicillin/streptomycin) pre-equilibrated at the appropriate oxygen tension (20% or 1%). Cells were subsequently incubated in each oxygen tension for 3 days.

Passaged (P1-P3) mouse articular chondrocytes were transfected with siRNA against Phd2 using the same protocol.

Table. 2.4 Oligonucleotide sequence of siRNA*

Species	siRNA	Sequence
<i>Homo sapiens</i>	siPHD1 #1	5'- CAUGCAGGGCACGAUUAUAGUC -3'
<i>Homo sapiens</i>	siPHD1 #2	5'- GCAUCACCUGUAUCUAUUA -3'
<i>Homo sapiens</i>	siPHD2 #1	5'- GAAGCUGGGCAGCUACAAAAT -3'
<i>Homo sapiens</i>	siPHD2 #2	5'- CAAAUGGAGAUGGAAGAUGUG -3'
<i>Homo sapiens</i>	siPHD3 #1	5'- GGAGAGGUCUAAGGCAAUGTT -3'
<i>Homo sapiens</i>	siPHD3 #2	5'- AGAGCUAGGUCUACUGAUATT -3'
<i>Homo sapiens</i>	siFIH-1 #1	5' - GGUUUUAAACUGGAACUGGATT - 3'
<i>Homo sapiens</i>	siHIF1 α	5'- AGCAGUAGGAAUUGGAACATT -3'
<i>Homo sapiens</i>	siHIF2 α	5'- GCGACAGCUGGAGUAUGAATT -3'
<i>Mus musculus</i>	siPhd2	5'- CAAGGUACGCAUAACUGUTT -3'

*siRNA = small interfering RNA

2.5 Reverse Transcriptase-PCR from cartilage

2.5.1 RNA extraction from cartilage explants

RNA was isolated from ~60mg diced cartilage explants (approximately 20 pieces of 3mm³ cartilage cubes). Culture media was removed and human cartilage explants were transferred to an eppendorf containing 600µl TRIzol (Invitrogen, Paisly, UK). Explants were shaken vigorously at 4°C for 4 hours, before centrifugation at 13000 rpm for 10 minutes at 4°C. The supernatant was taken and RNA was isolated using the RNeasy Mini Kit (Qiagen, Crawley, UK) following manufacturer's guidelines and finally from the QIAamp spin columns eluted in 30µl of RNAase free water. RNA quality and quantity was analysed using a digital spectrophotometer (Cecil Instruments, Cambridge, UK), samples with UV absorbance A_{260}/A_{280} ratio < 1.8 were rejected and RNA was stored at -80°C until use.

2.5.2 RNA isolation from primary and passaged chondrocytes

RNA from primary or passaged chondrocytes was extracted using 600µl RLT buffer supplemented with 1:100 (2-β-mercaptoethanol). RNA was isolated using the RNeasy Mini Kit (Qiagen, Crawley, UK) following manufacturer's guidelines and finally from the QIAamp spin columns eluted in 30µl of RNAase free water. RNA presence, quality and quantity was confirmed using a digital spectrophotometer (Cecil Instruments, Cambridge, UK), samples with UV absorbance A_{260}/A_{280} ratio < 1.8 were rejected and RNA was stored at -80°C until use.

2.5.3 Reverse Transcription of RNA

0.5µg of total RNA in a total volume of 10µl RNAase free water was incubated at 70°C for 10 minutes. Complementary DNA (cDNA) was generated using a reverse transcription kit (Promega, Southampton, England, UK) from 0.5µg of total RNA using manufacturers guidelines. Volumes of reagents used were:

RT Mix per sample	μl
10x Reverse Transcriptase buffer	2
dNTP Mix	2
MgCl ₂ 25mM	4
Random Primers	1
AMV Reverse Transcriptase (25 μl)	0.5
RNase Inhibitor (40u/ μl)	0.5
Total master mix volume	10

2.5.4 Realtime polymerase chain reaction (PCR):

Newly synthesised cDNA was diluted in 80 μl DNase free water and stored in two 50 μl aliquots to reduce future freeze/thawing cycles. Four percent (4 μl) of this cDNA (reverse transcribed from 0.5 μg of initial RNA) was then used for real-time PCR assays using TaqMan technology and a PCR Corbett Research thermocycler (Corbett Research, Australia). The delta delta threshold cycle ($\Delta\Delta\text{Ct}$) method of relative quantitation was used to calculate relative mRNA levels for each transcript examined. The ribosomal protein gene (RPLP0) was used to normalise the data as it was not regulated by hypoxia in our system. Pre-developed primer/probe sets for the following genes were purchased from Applied Biosystems (Foster City, CA): *PHD1*, *PHD2*, *PHD3*, *FIH-1*, *COL2A1*, *COL9A1*, *COL11A2*, *AGGRECAN*, *SOX9*, *VEGF*, *PLOD2*, *LOXL3*, *HIF-1A*, *HIF-2A* and *RPLP0*.

RT-PCR primers (MWG Biotech, Ebersberg, Germany) for the following genes were also used: *ADAMTS4*, *ADAMTS5*, *MMP1*, *MMP3*, *MMP13*, *TIMP1*, *TIMP2*, *TIMP3*. Sequence details are listed on table 2.3

Table 2.5 RT-PCR Primers

Species	Name	Sequence
<i>Homo sapiens</i>	Sense ADAMTS4	5'- ATGGCTATGGGCACTGTCTCTT -3'
<i>Homo sapiens</i>	Anti-sense ADAMTS4	5'- GGAAAGTCACAGGCAGATGCA -3'
<i>Homo sapiens</i>	Sense ADAMTS5	5'- GGCCATGGTAACTGTTTGCT -3'
<i>Homo sapiens</i>	Anti-sense ADAMTS5	5'- AATGTCAGGTTGCAGTG -3'
<i>Homo sapiens</i>	Sense MMP1	5'- GGACCAACAATTTTCAGAGAGTACAAC -3'
<i>Homo sapiens</i>	Anti-sense MMP1	5'- CCAAGAGAATGGCCGAGTTC -3'
<i>Homo sapiens</i>	Sense MMP3	Primers provided by Yasu Sawaji (Kennedy Institute, London, UK)
<i>Homo sapiens</i>	Anti-sense MMP3	
<i>Homo sapiens</i>	Sense MMP13	5'- TTACCAGACTTCACGATGGCATT -3'
<i>Homo sapiens</i>	Anti-sense MMP13	5'- TCGCCATGCTCCTTAATTCC -3'
<i>Homo sapiens</i>	Sense TIMP1	5'- ACCGCAGCGAGGAGTTTCT -3'
<i>Homo sapiens</i>	Anti-sense TIMP1	5'- CTGCAGGTAGTGATGTGCAAGAG -3'
<i>Homo sapiens</i>	Sense TIMP2	5'- ATGTAGTGATCAGGGCCAAAGC -3'
<i>Homo sapiens</i>	Anti-sense TIMP2	5'- AGGGTTGCCATAAATGTCGTTT -3'
<i>Homo sapiens</i>	Sense TIMP3	5'- CCTGCTACTACCTGCCTTGC -3'
<i>Homo sapiens</i>	Anti-sense TIMP3	5'- GCGTAGTGTTTGGACTGGT -3'
<i>Homo sapiens</i>	Sense RPLP0	5'- CCATTGAAATCCTGAGTGATGTG -3'
<i>Homo sapiens</i>	Anti-sense RPLP0	5'- CTTGCTGGCTCCCACTTT -3'
<i>Sus scrofa</i>	Sense ADAMTS5	5'- TGATGGTCATGGTAACTGTTTGCT -3'
<i>Sus scrofa</i>	Anti-sense ADAMTS5	5'- GTAGGTCTGTCCCGGGAGTTC -3'
<i>Sus scrofa</i>	Sense MMP13	5'- CATGAGTTTGGCCATTCCTT -3'
<i>Sus scrofa</i>	Anti-sense MMP13	5'- GTTGGGGTCTTCATCTCCTG -3'
<i>Sus scrofa</i>	Sense TIMP3	5'- CCTGCTACTACCTGCCTTGC -3'
<i>Sus scrofa</i>	Anti-sense TIMP3	5'- GCGTAGTGTTTGGACTGGT -3'
<i>Sus scrofa</i>	Sense SOX9	5'- AGTACCCGCACCTGCACAAC -3'
<i>Sus scrofa</i>	Anti-sense SOX9	5'- TTCAGCAGTCTCCAGAGTTTGC -3'
<i>Sus scrofa</i>	Sense COL2A1	5'- GTCCTCTGCGACGACATAATCTG -3'
<i>Sus scrofa</i>	Anti-sense COL2A1	5'- TCTGTCCCTTTGGTCCCACTT -3'
<i>Sus scrofa</i>	Sense AGGRECAN	5'- AGGCAGAGGTGGCTTCAGTC -3'
<i>Sus scrofa</i>	Anti-sense AGGRECAN	5'- CCAGAATCTAGCAGGGAGTCATC -3'
<i>Sus scrofa</i>	Sense RPLP0	5'- CCTCCTTCTTCCAGGCTTTAGG -3'
<i>Sus scrofa</i>	Anti-sense RPLP0	5'- CACATCACTCAGGATTTCAATGG -3'

2.6 Preparation of cell and tissue lysates

2.6.1 Chondrocytes

6cm wells containing primary (P0) and passaged HACs were washed twice with 1xPBS and lysed with 150µl of either SDS/urea or RIPA lysis buffer supplemented with the P8340 protease inhibitor cocktail (1:100) (Sigma Aldrich company ltd, Dorset, UK). This cocktail is a mixture of protease inhibitors (AEBSF, Aprotinin, Bestatin hydrochloride, E64, Leupeptin hemisulfate salt, Pepstatin A) with a broad specificity for serine, aminopeptidases, cysteine and aspartic proteases.

SDS/urea lysis buffer was used to lyse cultures for subsequent detection of HIF- α isoforms. RIPA lysis buffer was used all other cellular proteins were being analysed.

Wells were scraped with a cell scraper and cell lysates were transferred to an eppendorf, sonicated using a probe (Ultrasonic, sonicator frequency 4.5) for 5 seconds and centrifuged at 13,000 RPM for 20 minutes at 4°C in order to remove any cell debris. 150 µl supernatant was removed and assayed for protein concentration using the Bradford assay (see section 2.6.4).

2.6.2 Secreted proteins

HACs were cultured in a 5×10^3 cells/cm² in 6cm tissue culture dishes. HACs were transfected using protocol described above (2.4) and cultured for 24 hours in DMEM (containing 10% FCS and antibiotics) at the appropriate oxygen tension (20% or 1%). After 24 hours medium was removed and HACs were washed twice in serum free media. HACs were then cultured in 2200µl serum free DMEM supplemented with 1% penicillin/streptomycin and 1% Insulin, transferrin, and selenium (ITS) (Sigma Aldrich company ltd, dorset, UK). HACs were serum starved for 2 days. 1ml of conditioned medium was treated with 100µl of deoxycloic acid (0.15%), left at room temperature for 10 minutes and then precipitated by addition of 110µl trichloroacetic acid (72%). Samples were centrifuged at 12000RPM for 10 minutes. The supernatant was aspirated and the protein pellet washed with 200µl ice cold acetone. Samples were air dried and the pellet resuspended in 4x SDS sample buffer.

For the detection of secreted TIMP1 and TIMP3 HACs were cultured using the protocol above, except for the detection of TIMP3 cell culture medium was supplemented with 200ug/ml Heparin. After 2 days of serum starvation 600µl of media was precipitated with 1ml of ice cold acetone, vortexed and left at -20°C for 20 minutes. Samples were centrifuged at 13,000 RPM for 10 minutes at 4°C, air dried and resuspended in 4xSDS sample buffer.

2.6.3 Cartilage explants

10 Human articular cartilage explants or 5 mouse hip explants were washed twice with 1xPBS and lysed in 150µl of RIPA lysis buffer supplemented with the P8340 protease inhibitor cocktail (1:100) (Sigma Aldrich company ltd, dorset, UK). Samples were shaken vigorously at 4°C for 4 hours. Finally, samples were centrifuged at 13000RPM for 20 minutes and the supernatant was aspirated.

2.6.4 Determination of protein concentration by Bradford Assay

The protein concentration of each extract was determined using the Bradford assay. Cell lysates were diluted 1:100 in Bradford reagent and the A_{595nm} was measured using a digital spectrophotometer (Cecil Instruments, Cambridge, UK). A_{595nm} was measured three times for each extract, an average value taken of the three readings and the protein concentration (µg/ml) was calculated using the digital spectrophotometer (Cecil Instruments, Cambridge, UK).

The presence of the highly abundant and charged cartilage matrix components precluded use of this method to accurately determine protein concentrations from cartilage explant lysates. Therefore an equal volume of lysis buffer (from an equal number of similarly sized explants) was loaded into each lane when performing SDS-PAGE and the protein loading confirmed by analysing the loading control (α -tubulin) levels.

It was not possible to accurately determine cellular protein concentrations of passaged HACs lysed in SDS/urea lysis buffer. Therefore in these experiments parallel dishes were lysed using RIPA in order to estimate concentration.

For the normalisation of secreted proteins the volume of media used was adjusted after standardisation according to total protein measurement using the Bradford assay from the cell layer.

2.7 SDS-PAGE:

Resolving buffer	380mM Tris/HCl pH 8.8 6%, 8%, 10%, 12% or 15% (w/v) acrylamide 0.1% (w/v) SDS 0.07% (w/v) ammonium persulphate 0.07% (v/v) TEMED
Stacking buffer	125mM Tris/HCl pH6.8 3.9% (w/v) acrylamide 0.1% SDS 0.1% (w/v) ammonium persulphate 0.1% (v/v) TEMED

The electrophoretic separation of proteins was carried out using a Mighty Small II vertical electrophoresis system (Hoefer scientific instruments) and separated according to molecular weight using standard protocol (Laemmli, 1970; Schagger and von Jagow, 1987).

Resolving and stacking gels were prepared by mixing 30% acrylamid:bis-acrylamide (37.5:1) with the associated resolving (350mM Tris-HCL pH 8.8), and 0.1% SDS) and stacking (125mM Tris-HCL (pH 6.8), and 0.1% SDS) gel buffers. The acrylamide concentration ranged from 6% - 15% for resolving gels and was fixed at 3.9% for stacking gels. Gels were polymerised by adding ammonium persulphate (APS) to 0.07% (w/v) and N,N,N',N'-tetramethylethylenediamine (TEMED) to 0.07% (v/v).

Protein samples were boiled for 5 minutes in 4x SDS-Sample buffer, loaded onto gels and electrophoresed in running buffer (National diagnostics) at a constant voltage (100V) for 1 ½ hours. SeeBlue molecular weight markers (Invitrogen) were loaded onto each gel in order to monitor electrophoresis and estimate the size of proteins.

2.8 Western blotting and immunodetection

Western blot was used to detect specific proteins in cellular lysates or secreted proteins which had been resolved by SDS-PAGE electrophoresis. PVDF-transfer membranes (Millipore, Bedford, USA) were pre-hydrated using 100% methanol for 10 seconds. The PVDF membrane and SDS-PAGE gel were then equilibrated in transfer buffer, placed on top of one another and sandwiched between four sheets of 3mm chromatography paper and two sponges in a gel holder cassette. The cassette was then placed in a Trans-blot chamber (Biorad, Hercules, CA) filled with transfer buffer. The transfer of proteins from SDS-PAGE gel to PVDF membrane was carried out at a constant voltage (100V) for 1 ¼ hours. The transfer buffer was kept at 4°C by a water-circulating cooling pump.

Following transfer PVDF membranes were pre-hydrated using 100% methanol for 10 seconds and placed protein side up on a clean non-porous surface for 10 minutes. The membranes were gently agitated in blocking solution for 1 hour (5% Tesco low fat dried milk powder in 1xPBST) and then incubated with the primary antibody overnight at 4°C. A list of primary antibodies used for immunoblotting can be found in table 2.3.

Following overnight incubation membranes were washed 3 times, for 10 minutes each wash with PBST. Appropriate anti-mouse or anti-rabbit horseradish peroxidase-conjugated secondary antibodies were diluted in membrane blocking solution and applied to the membrane for 1 hour at room temperature with gentle agitation. Following incubation membranes were washed four times, 10 minutes each wash, with PBST.

Excess PBST was drained off the membrane and the membrane placed protein side up on a clean non-porous surface (eg: a gel staining box). Equal volumes of ECL Solution 1 and ECL Solution 2 (GE healthcare) were mixed and pipetted onto the membrane. After one minute the excess detection reagents were drained off and the PVDF placed protein side down on a saran wrap. The saran wrapped membranes were placed in an X-ray cassette and the HRP-bound proteins visualized using blue-sensitive film (Fujifilm Super RX, Fujifilm, Bedford, UK).

In order to detect PHD3 and HIF-2 α 40 μ g of protein was used. Otherwise 20 μ g of protein was resolved by SDS-PAGE electrophoresis.

To re-probe membranes with further antibodies, membranes were incubated with 1x stripping buffer (Re-Blot Plus Strong Solution, Chemicon, Temecula, USA) for 20 minutes at room temperature with gentle agitation. Membranes were washed twice with PBST for 10 minutes, treated with blocking solution for 30 minutes and subsequently incubated with another antibody.

2.9 Articular cartilage explant culture and ARGSV-Neoepitope detection

Human or porcine articular cartilage was cut into cubes (3 mm³) and 10 explants (~30 mg wet weight) per well were placed in a 48-well plate with 300 μ l of media. Explants were rested in 20% oxygen for 24 hours, washed with serum-free DMEM and preincubated for 48 hours at either 20% oxygen tension or 1% oxygen tension. Explants were stimulated with IL-1 α or vehicle (serum free media) and then further cultured for 24 hours at either 20% oxygen tension or 1% oxygen tensions.

300 μ l of media was collected, deglycosylated overnight at 37°C with 1 μ l chondroitinase ABC and 1 μ l keratinase (Seikagaku Kogyo, Tokyo, Japan) in 98 μ l deglycosylation buffer. Acetone precipitation was carried out as previously described (2.6.3). Samples were loaded onto a 6% PAGE gel and blotted with anti-ARGSV neoepitope antibody (ab3773, 1:100).

2.10 Statistical analysis

Data were compared using either a paired t-test or one-way analysis of variance with Bonferroni post-test. The software GraphPad Prism 4 (GraphPad Software, San Diego, CA) was used to perform statistical analysis. Results are expressed as a mean +/- the SEM from

independent experiments (i.e., tissue or cells obtained from individual donors). A *P* value less than 0.05 was considered significant.

Chapter 3

Promotion of the Human Articular Chondrocyte Phenotype by Inhibition of HIF-specific Hydroxylases

3.1 Introduction

Articular cartilage consists of a single cell type, the chondrocyte, which is solely responsible for the synthesis and maintenance of the extracellular matrix (Poole et al., 2002). The rigid nature of type II collagen fibrils present in the matrix confers tensile strength to the tissue and the swelling pressure caused by water-saturated aggrecan molecules creates a compressive gel-like stiffness allowing the tissue to resist deformation and giving cartilage its ability to absorb shocks (Buckwalter and Mankin, 1998a, b; Poole et al., 2002). Due to its shock-absorbing and articulating functions, articular cartilage cannot afford a blood or nerve supply. The tissue is therefore dependent on diffusion of oxygen from the synovial fluid on one side and the vascularised underlying bone on the other. As a result, articular cartilage (at least in larger animals and man) is maintained in a low oxygen environment (1-6% oxygen tension) throughout life (Brighton and Heppenstall, 1971; Milner et al., 2006; Silver, 1975). There is now significant evidence that hypoxia is a critical parameter in promoting the chondrocyte phenotype. It has been shown that hypoxia upregulates key cartilage transcription factor SOX9 and increases expression of the main extracellular matrix genes in bovine and human chondrocytes (Domm et al., 2002; Katopodi et al., 2009; Lafont et al., 2007; Murphy and Polak, 2004; Murphy and Sambanis, 2001).

The response of cells to hypoxia is mediated by HIFs, hetero-dimeric transcription factors consisting of an α and β subunit (Semenza, 2000). Both subunits of HIF are constitutively expressed at the mRNA level, however, the α -subunit is regulated post-translationally by oxygen levels. In well-oxygenated cells, the α -subunit is hydroxylated on specific, conserved proline residues by HIF-targeting prolyl hydroxylase domain enzymes (PHD1, PHD2 and PHD3) (Ivan et al., 2001; Jaakkola et al., 2001; Yu et al., 2001). The PHD enzymes are a subfamily of dioxygenases that use molecular oxygen and 2-oxoglutarate (2-OG) as co-substrates to add hydroxyl groups to specific proline residues on the α subunit (Bruick and McKnight, 2001; Epstein et al., 2001; Ivan et al., 2001). The hydroxylated proline residues on HIF- α are recognised by von Hippel–Lindau (VHL) protein of the E3 ubiquitin ligase complex, ubiquitinated and subsequently degraded by the 26S proteasome (Cockman et al., 2000). As intracellular oxygen concentrations are reduced, the activity of the prolyl hydroxylase enzymes is suppressed, HIF- α isoforms are not hydroxylated and therefore escape proteosomal degradation and so can heterodimerize with HIF-1 β , translocate to the

nucleus and bind to *cis*-regulatory hypoxia response elements (HRE's) located in target genes. Previous work in our laboratory has demonstrated that hypoxic induction of the key cartilage matrix genes in human chondrocytes is mediated specifically by the HIF-2 α isoform which upregulates SOX9, leading to enhanced expression of the matrix genes (Lafont et al., 2007).

Suppression of PHD enzymes leads to stabilisation of HIFs and offers a potential treatment option for many ischaemic disorders, such as peripheral artery occlusive disease, myocardial infarction and stroke (Fraisl et al., 2009). We hypothesise that antagonising specific PHDs could be a novel way of promoting cartilage matrix deposition from chondrocytes by enhancing HIF-2 α levels, upregulating key transcription factor SOX9 and increasing extracellular matrix synthesis.

In this chapter we report that PHD2 is the dominant isoenzyme regulating HIF-2 α stability in human chondrocytes. Moreover, specific inhibition of PHD2 using RNAi-mediated depletion caused an upregulation of SOX9 and enhanced extracellular matrix protein production. Most importantly, depletion of PHD2 resulted in greater HIF-2 α levels, and subsequently enhanced SOX9-induced cartilage matrix production, compared to the levels normally found in hypoxia (1% oxygen). This suggests the exciting possibility that PHD2 inhibition offers a novel means to enhanced cartilage matrix synthesis above normal levels in the joint. In addition PHD2 inhibition upregulated key pro-Lysyl hydroxylases involved in collagen formation implying that PHD2 inhibition offers a novel means to enhance cartilage repair. The need for HIF-specific hydroxylase inhibitors was highlighted as treatment with the 2-oxoglutarate analogue dimethyloxalylglycine (which also inhibits the collagen prolyl hydroxylases) resulted in reduced secretion of type II collagen, a critical matrix component.

3.2 Up-regulation of SOX9 and key extracellular proteins in human cartilage explants under hypoxic conditions

Human cartilage explants from a range of patients (3 male and 2 female donors; age 14-38 years; mean age 25 years) were incubated for 3 days under normoxic (20% oxygen) or

hypoxic (1% oxygen) conditions. Messenger RNA (mRNA) levels of cartilage-specific transcription factor *SOX9* as well as key cartilage matrix proteins were analysed after normalising the data using the housekeeping gene *RPLP0*. As we have previously demonstrated ribosomal protein (RPLP0) is not regulated by hypoxia (Lafont et al., 2008; Lafont et al., 2007). Consistent with our previous work in passaged human articular chondrocytes, hypoxia significantly upregulates cartilage specific transcription factor *SOX9* as well as of key cartilage extracellular matrix genes *COL2A1*, *COL9A1*, *COL11a2*, *AGGRECAN* at the mRNA level (Fig. 3.1) (Lafont et al., 2007). Exposure to 1% oxygen tension increases *SOX9* expression levels 5.8 fold, *COL2A1* expression 29.9 fold, *COL9A1* 18.7 fold, *COL11a2* 14.7 fold and *AGGRECAN* 16.9 fold (compared to control levels in normoxia).

In order to confirm the hypoxic upregulation of *SOX9* in human cartilage explants at the protein level cartilage explants from a range of patients (3 male and 1 female donors; age 10-54 years [mean age 35.5 years]) were cultured at various oxygen tensions (20%, 3% and 1%) or treated with hypoxia mimetics: the 2-oxoglutarate analogue DMOG or an iron chelating agent, DFO for 3 days. DFO and DMOG inhibit all members of the iron, 2-oxoglutarate dependent hydroxylase family, which include the HIF-targeting hydroxylases. *SOX9* protein levels appear to be upregulated in human cartilage explants in response to hypoxia and these levels of upregulation correspond with decreasing levels of molecular oxygen, i.e., 1%>3%>20% (Fig. 3.2 A). *SOX9* was also appeared to be upregulated at the protein level in response to prolyl-hydroxylase domain enzyme inhibitors DMOG and DFO (Fig. 3.2 B, C, D). Lack of protein quantification in these (and other western blots) mean that we cannot confirm the hypoxic upregulation of *SOX9* at the protein level, we will discuss this Chapter 6. We attempted to investigate the levels of HIF- α isoform stabilisation in human cartilage however we encountered difficulty lysing cartilage using urea lysis buffer and we were not successful.

In summary, prolyl-hydroxylase domain protein inhibition using either hypoxia, DMOG or DFO was found to induce *SOX9* at the protein level in human cartilage explants and this is consistent with the hypoxic induction of *SOX9* reported at the mRNA level (Figure 3.1).

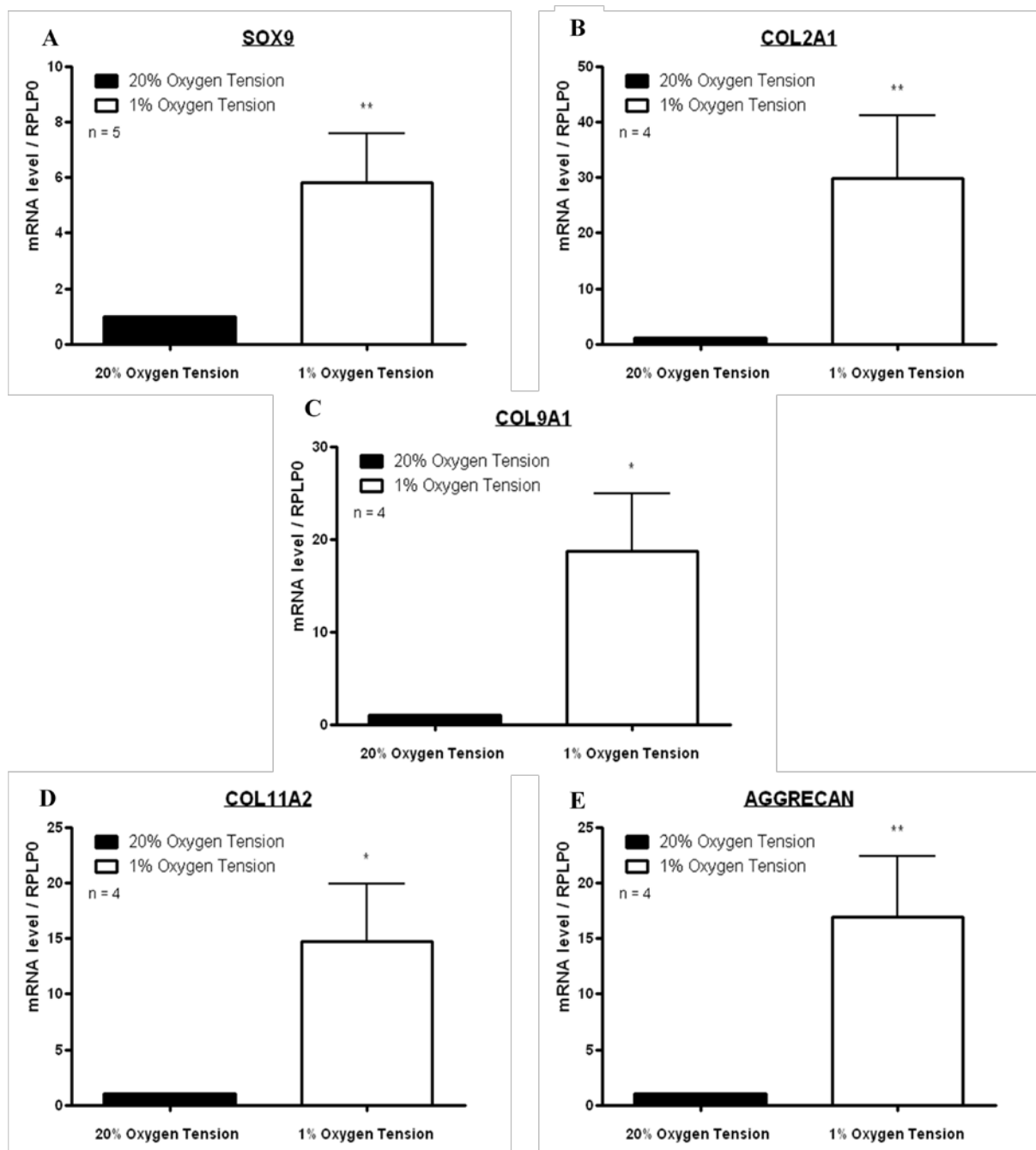


FIGURE 3.1. Hypoxia upregulates cartilage specific transcription factor SOX9 and main cartilage matrix proteins in human cartilage explants. Data are based on human cartilage explants. TaqMan PCR data showing relative levels of mRNA for SOX9, COL2A1, COL9A1, COL11A2 and AGGRECAN (A – E, respectively) after culture for 3 days in 20% and 1% oxygen. All values were normalised using the *RPLP0* housekeeping gene and represent mean and SEM results of 4-5 independent experiments (i.e., cells from 4-5 different donors). * $P < 0.05$; ** $P < 0.01$ (versus 20% oxygen).

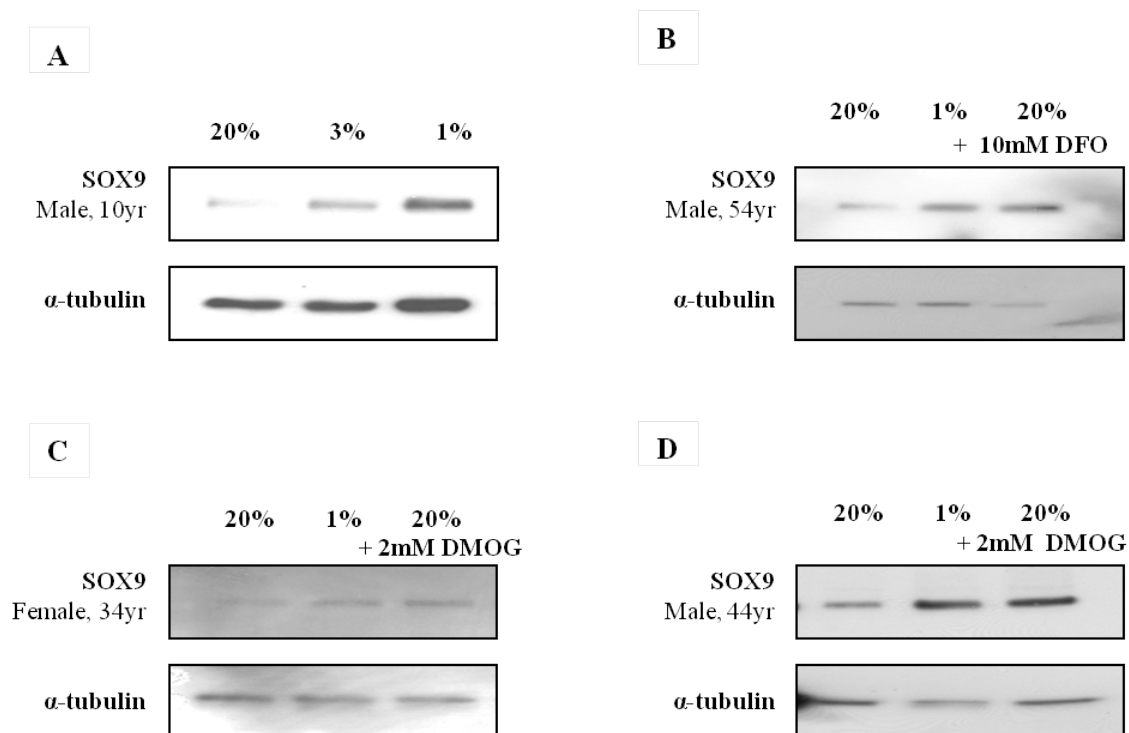


FIGURE 3.2. Hypoxia appears to increase SOX9 protein levels in human cartilage explants. Data are based on human cartilage explants from 4 different donors (A-D). The western blots show increasing SOX9 levels at decreasing oxygen tensions (20%, 3% and 1%) or after treatment with hypoxia mimics: 2-oxoglutarate analogue DMOG or iron chelating DFO for 3 days.

3.3 HIF-targeting hydroxylases - PHD2 is the Most Abundant, and PHD2 and -3 are Hypoxia Inducible in HACs

Our initial experiments in human articular chondrocytes were to assess relative abundance of the proyl-hydroxylase domain (PHD) enzymes in human articular chondrocytes (HACS) and determine the presence/absence of a hypoxic induction as their relative levels might suggest which PHD enzyme was dominant (although different specificities may also play a part).

HACs from 7 patients (5 male and 2 female donors; age 11-44 years [mean age 16 years]) were cultured for 3 days under normoxic (20% oxygen) or hypoxic conditions. The mRNA levels of *PHD1*, *PHD2* and *PHD3* were analysed after normalising the data using the housekeeping gene *RPLP0*. The relative abundance of each gene was calculated after setting the expression of *PHD3* at 20% oxygen to a value of 1. This method of calculating the relative abundance of the three PHD isoenzymes at the mRNA level makes the assumption that the efficiency of amplification between the different PCR primers is equal (in practice, all amplification efficiencies were similar, being over 90%).

PHD2 was the most abundant transcript of the three isoenzymes in both 20% and 1% oxygen. Interestingly, although *PHD3* had the most pronounced hypoxic induction (average 19.2 fold induction) it was the least abundant PHD enzyme at the mRNA level in both 20% and 1% oxygen (Fig. 3.3 A). This is consistent with data at the protein level. *PHD2* and *PHD3* proteins were found to be induced by hypoxia (Fig. 3.3 B & C). In addition 20µg of *PHD2* was visualised using enhanced chemiluminescence after 3 minutes exposure to X-ray film whereas 40µg *PHD3* required 45 minutes exposure to detect any protein.

Our previous microarray study found that *PHD2* was the most abundant enzyme in both normoxia and hypoxia (Murphy et al., 2009). *PHD3* mRNA levels were the lowest in normoxia but under hypoxia *PHD3* mRNA levels were greater than *PHD1* (20% - *PHD2*>*PHD1*>*PHD3*. 1% - *PHD2*>*PHD3*>*PHD1*). This slight discrepancy between the work shown in Figure 3.3 and our previous microarray study could be due to the use of passaged HACs in this study while primary HACs were used by Murphy and colleagues

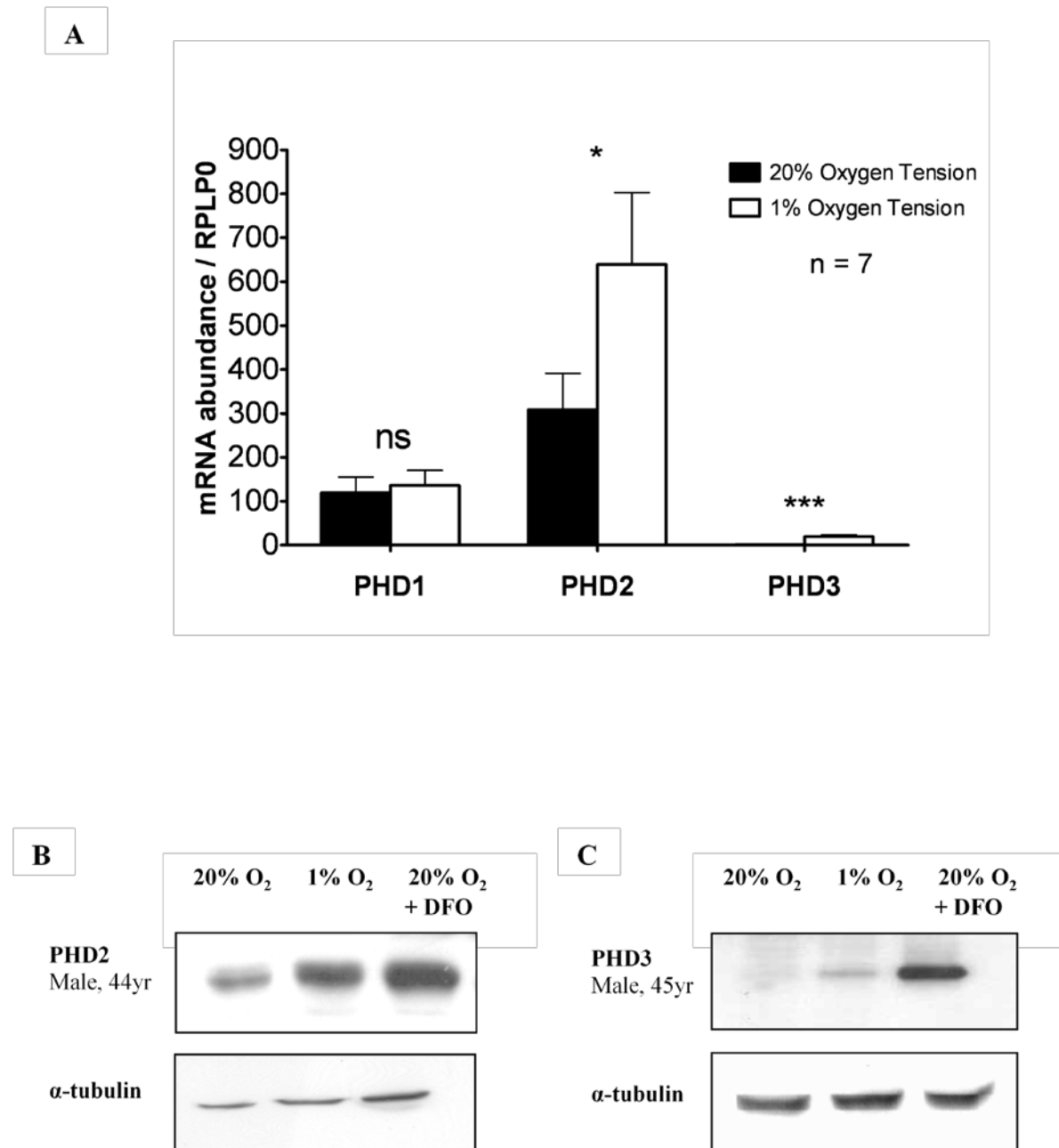


FIGURE 3.3. PHD2 is the most abundant HIF-targeting hydroxylase in HACs, while PHD2 and PHD3 are both hypoxia inducible. Data are from passaged (P1-P3) HACs after culture for 3 days at 20% oxygen, 1% oxygen or treated with 10mM of the hypoxia mimetic desferrioxamine (DFO). A, TaqMan-based data showing the relative mRNA abundance in cultured HACs of the PHD enzymes at the mRNA level in normoxic or hypoxic conditions (PHD3 mRNA levels in 20% oxygen were assigned a value of 1). Values are the mean and S.E.M. of 7 independent experiments (i.e., HACs from 7 different donors); ns = not significant; * $P < 0.05$; *** $P < 0.001$. B, Western blots showing increased PHD2 levels in hypoxia (1% oxygen), and after treatment with 10mM DFO. C, PHD3 levels, like PHD2 were increased by hypoxia and DFO, but were undetectable in 20% oxygen.

Thus PHD2 and PHD3 are induced by hypoxia. PHD2 is the most abundant PHD enzyme in human articular chondrocytes. PHD3, although expressed at relatively low levels, is the most highly induced by hypoxia.

3.4 Knockdown of PHD1/2/3 in passaged human articular chondrocytes using siRNA

In order to determine the role of the different PHD enzymes in the regulation of HIF-1 α / HIF-2 α in HACs we generated a range of siRNA oligonucleotides against each PHD isoform. PHD1, PHD2 and PHD3 were specifically depleted in HACs using small interfering RNA (siRNA) and lysed 3 days after transfection (Fig. 3.4 A, B, C). 10nM of a single siRNA oligonucleotide was used in each transfection and two different oligonucleotides were used for each gene (for sequences see Table 2.4). SiRNA against luciferase (siLuc) was used as a non-targeting control. Messenger RNA analysis by real-time PCR revealed that PHD1 was depleted 72% (siPHD1 #1) and 95% (siPHD1 #2), on average, compared to siLuc levels. PHD2 was depleted 82% (siPHD #1) and 86% (siPHD2 #2), on average, while PHD3 was depleted by 87% (siPHD3 #1) and 84% (siPHD3 #2).

Western blotting confirmed the depletion of PHD2 (Fig. 3.4 D) and PHD3 (Fig. 3.4 E) at the protein level. PHD1 was not assessed as no specific antibody was available. Knockdown of PHD3 was performed in the presence of the iron chelator DFO as PHD3 levels were more easily detectable in DFO. The oligonucleotides which produced the greatest knockdown at the mRNA level were used in subsequent experiments.

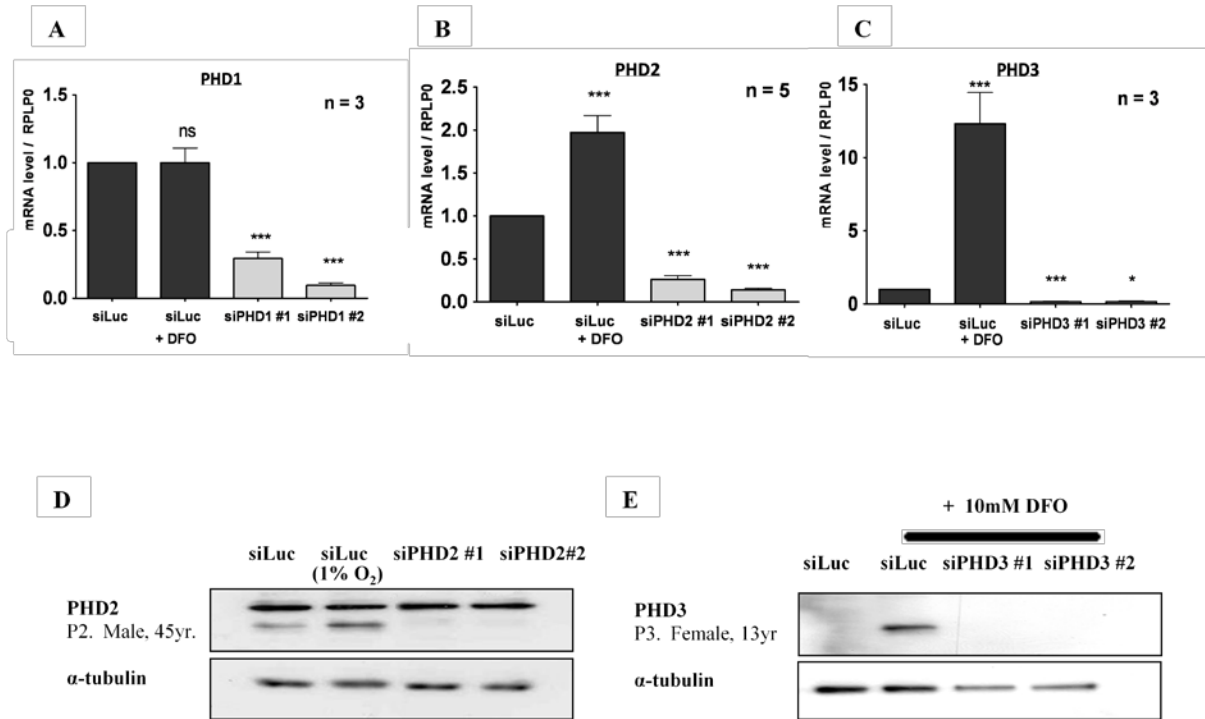


FIGURE 3.4. RNAi-mediated depletion of PHD1/2/3 in HACs. TaqMan real-time PCR data showing mRNA depletion of PHD1 (A), PHD2 (B) and PHD3 (C) in passaged HACs (P2-P3) after 3 days in culture. Cells were transfected with 10nM siRNA directed against each PHD and an siRNA targeting luciferase (siLuc) was used as a control. Values represent the mean and S.E.M. from 3-5 independent experiments (i.e., HACs from 3-5 different donors). Note: ns - not significant. * $P < 0.05$; *** $P < 0.001$ versus 20% oxygen siLuc. D, Western blot showing RNAi-mediated depletion of PHD2 protein in HACs (in 20% oxygen). E, RNAi-mediated PHD3 protein depletion is shown in the presence of 10mM DFO, as PHD3 levels in normoxia were undetectable. In all cases, HACs were cultured for 3 days before termination of experiment.

3.5 Depletion of PHD2 in Normoxia Stabilises HIF- α Isoforms in HACs, and Upregulates SOX9

In order to assess the functional effect of the knockdowns on HIF- α isoform stabilisation, HACs were transfected with a single oligonucleotide siRNA against PHD1, PHD2, PHD3 or luciferase (as a non-targeting control). Following transfection HACs were cultured under normoxic conditions for 3 days before lysis.

Under normoxic conditions HIF-1 α / HIF-2 α are hydroxylated by the PHD enzymes and are rapidly degraded. The knockdown of PHD2 in HACs significantly stabilised both HIF-1 α and HIF-2 α in 20% oxygen (Fig. 3.5 A). The knockdown of PHD1 also contributed to the stabilisation of both HIF-1 α and HIF-2 α in normoxia, but to a much lesser extent than PHD2 depletion. Despite its pronounced hypoxic induction, depletion of lowly expressed PHD3 had no detectable effect on levels of either HIF- α isoform in normoxia.

Since depletion of PHD2 had the dominant effect with regard to HIF- α stabilisation in normoxia, we performed reoxygenation experiments to further assess its role in HIF- α regulation. In these reoxygenation experiments, HACs were transfected with siRNA against PHD2 and cultured for 3 days in hypoxia before being suddenly switched to 20% oxygen. Cells were lysed 0-, 5-, 10-, 20-, 40- and 60- minutes after exposure to normoxia (20% oxygen). In parallel cultures, siRNA against luciferase (siLuc) was used as a control in order to provide the optimal control for accurate comparison with PHD2 depletion. We found that, in agreement to previous reports (Appelhoff et al., 2004), upon exposure to normoxia HIF- α subunits have a very short half-life (approximately 5 minutes). If the knockdown of individual PHD enzymes significantly reduces the rate of HIF- α isoform decline upon reoxygenation this would provide further evidence of the PHD enzyme's contribution to HIF- α protein regulation. Here we demonstrate that the knockdown of PHD2 greatly slows the degradation of HIF-1 α and HIF-2 α (Fig. 3.5 B, C) thus confirming its key role in regulation of the protein stability of both HIF- α isoforms.

In summary, PHD2 (the most abundant PHD enzyme) was found to be the dominant proyl-hydroxylase domain (PHD) enzyme responsible for the regulation of both HIF-1 α and HIF-2 α . To further investigate how specific PHD enzyme depletion may affect the

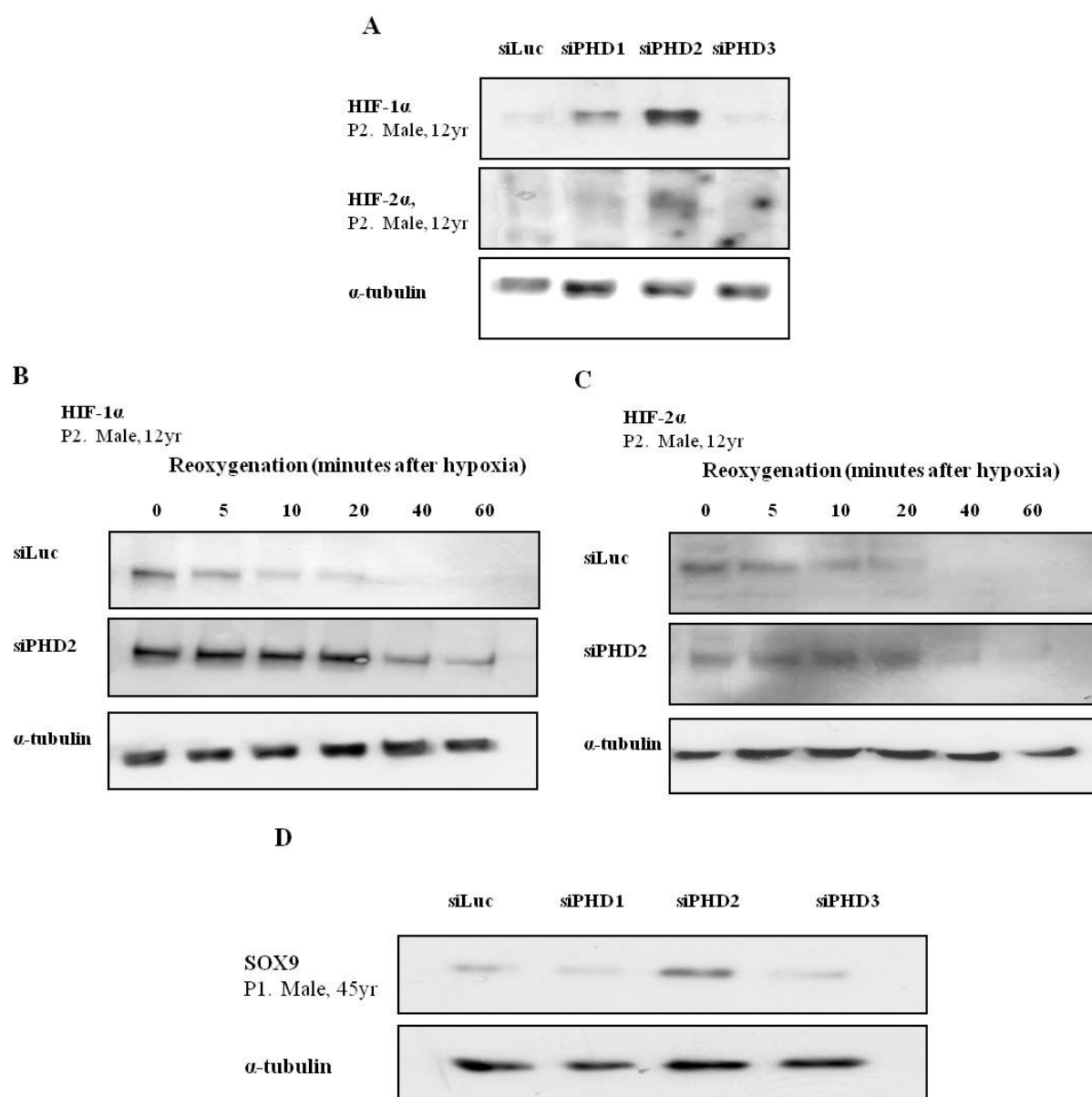


FIGURE 3.5. PHD2 depletion in normoxia stabilises HIFs and upregulates SOX9 in HACs. *A*, Unlike PHD1 or PHD3, depletion of PHD2 resulted in significant stabilisation of both HIF-1α and HIF-2α protein in HACs following 3 days culture in 20% oxygen. Control (siLuc) shows that neither HIF-α isoform is normally detectable in the presence of 20% oxygen. Depletion of PHD2 significantly slows the rate of HIF-1α (*B*) and HIF-2α (*C*) degradation upon reoxygenation, i.e., switching the cells from 1% to 20% oxygen. Following siRNA transfection HACs were incubated for 3 days in 1% oxygen before being exposed to 20% oxygen for the indicated time (in minutes) prior to lysing. Control siRNA (siLuc) transfections were carried out in parallel as a control. Note: for simplicity α-tubulin loading controls from a representative experiment are shown in each case. *D*, Depletion of PHD2, but not PHD1 or PHD3, resulted in increased SOX9 protein levels in HACs after 3 days in culture in 20% oxygen.

chondrocyte phenotype PHD1, PHD2 and PHD3 were depleted in HACs under normoxic conditions and levels of key cartilage specific transcription factor SOX9 assessed at the protein level. Our previous work has demonstrated that hypoxic induction of SOX9 is HIF-2 α dependent (Lafont et al., 2007). Only depletion of PHD2 resulted in enhanced SOX9 protein levels compared to luciferase controls (Fig. 3.5 D) in agreement with our previous work that SOX9 is upregulated in response to HIF- α isoform stabilisation.

3.6 PHD2 Depletion Enhances Expression of Cartilage Matrix by HACs in Both Normoxia and Hypoxia

In order to examine the effect of PHD2 knockdown on the main markers of the chondrocyte phenotype, primary and passaged HACs were cultured for 3 days at 20% and 1% oxygen and transfected with either siRNA against PHD2 or luciferase as a non-targeting control. The knockdown of PHD2 was confirmed at the mRNA level (Fig. 3.8 A) and protein level (Fig. 3.6 A). The effect of PHD2 depletion on HIF- α isoforms was investigated (Fig. 3.6 B and C). Markers of the chondrocyte phenotype (SOX9) and essential matrix components of the cartilage extracellular matrix (COL2A1, COL9A1, AGGRECAN) were examined at the mRNA level (Fig 3.8 B – F) and protein level (Fig 3.7 D and E). Finally two enzymes involved in the first steps of collagen cross linking: Lysyl oxidase homolog 3 (LOXL3) and procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2 (PLOD2) were examined (Fig 3.9 A and B) as well as the message levels of mRNA levels of *HIF1 α* and *HIF2 α* (Fig. 3.10 A and B)

There was no detectable difference in HIF-1 α levels between PHD2 depleted cultures in 20% and 1% oxygen, both being greater than that achieved by hypoxia alone (siLuc) (Fig. 3.6 B). In contrast the knockdown of PHD2 at 20% oxygen stabilises HIF-2 α to levels approximately equal to that in hypoxia alone (siLuc). However, depletion of PHD2 in 1% oxygen further enhanced HIF-2 α levels (Fig. 3.6 C) suggesting that PHD2 is not the only factor regulating HIF-2 α levels in hypoxia.

Levels of SOX9 and key cartilage matrix protein COL2A1 were analysed in PHD2-depleted HAC cultures from a range of patients. Both SOX9 and secreted type II collagen

levels were upregulated by PHD2 depletion in normoxia to levels roughly equal with (or slightly greater than) hypoxia. PHD2 depletion under hypoxic conditions, however, increased protein levels above hypoxia alone. The enhancement of SOX9 and COL2A1 expression in response to PHD2 depletion correlates with HIF-2 α stabilisation, i.e., with greatest levels achieved by PHD2 depletion in 1% oxygen (Fig. 3.7A, B). SOX9 mRNA was also significantly upregulated in response to PHD2 depletion in a manner similar to the response at the protein level (Fig. 3.8 A). In fact, all the key cartilage extracellular matrix genes were upregulated in response to PHD2 inhibition. The knockdown of PHD2 under 1% oxygen tension (approximate physiological oxygen tension at the core of cartilage) significantly increases SOX9 expression 6 fold, COL2A1 expression 38.5 fold, COL9A1 34.5 fold, COL11 α 2 58.9 fold and AGGRECAN 6.5 fold (compared to control levels in normoxia) (Fig. 3.8 C to F respectively). This is important as it demonstrates that PHD2 depletion can increase the matrix production levels over than occurring in physiological conditions (1% oxygen).

Our group has previously demonstrated that two enzymes involved in the first steps of collagen cross linking Lysyl oxidase homolog 3 (LOXL3) and procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2 (PLOD2) are upregulated by hypoxia (Lafont et al., 2008). Here we confirm this hypoxic upregulation in HACs (Fig 3.9 A and B). Depletion of PHD2 in 1% oxygen tension upregulates PLOD2 mRNA expression 16.1 fold and LOXL3 mRNA expression 5.35 fold (compared to control levels in normoxia) exceeding levels in hypoxic alone. It is a further benefit that PHD2 inhibition not only upregulates chondrocyte gene expression of extracellular matrix collagens but also key enzymes necessary post-translationally for collagen formation and stabilisation. As expected, expression of a non-hypoxia regulated genes prolyl-hydroxylases 1 (PHD1) and Factor Inhibiting HIF (FIH-1) were unaltered in response to PHD2 depletion (FIG. 3.9 C, D).

Interestingly hypoxia significantly downregulated the mRNA levels of HIF2 α and in particular, HIF1 α at the mRNA level. Depletion of PHD2 significantly decreases HIF1 α mRNA levels in normoxia (Fig. 3.10 A). Suppression of PHD2 had no effect on HIF2 α mRNA expression in hypoxia or normoxia (Fig. 3.10 B). This data is consistent with work previously described by Kim and colleagues and will be discussed further in the subsequent discussion section (Chapter 4).

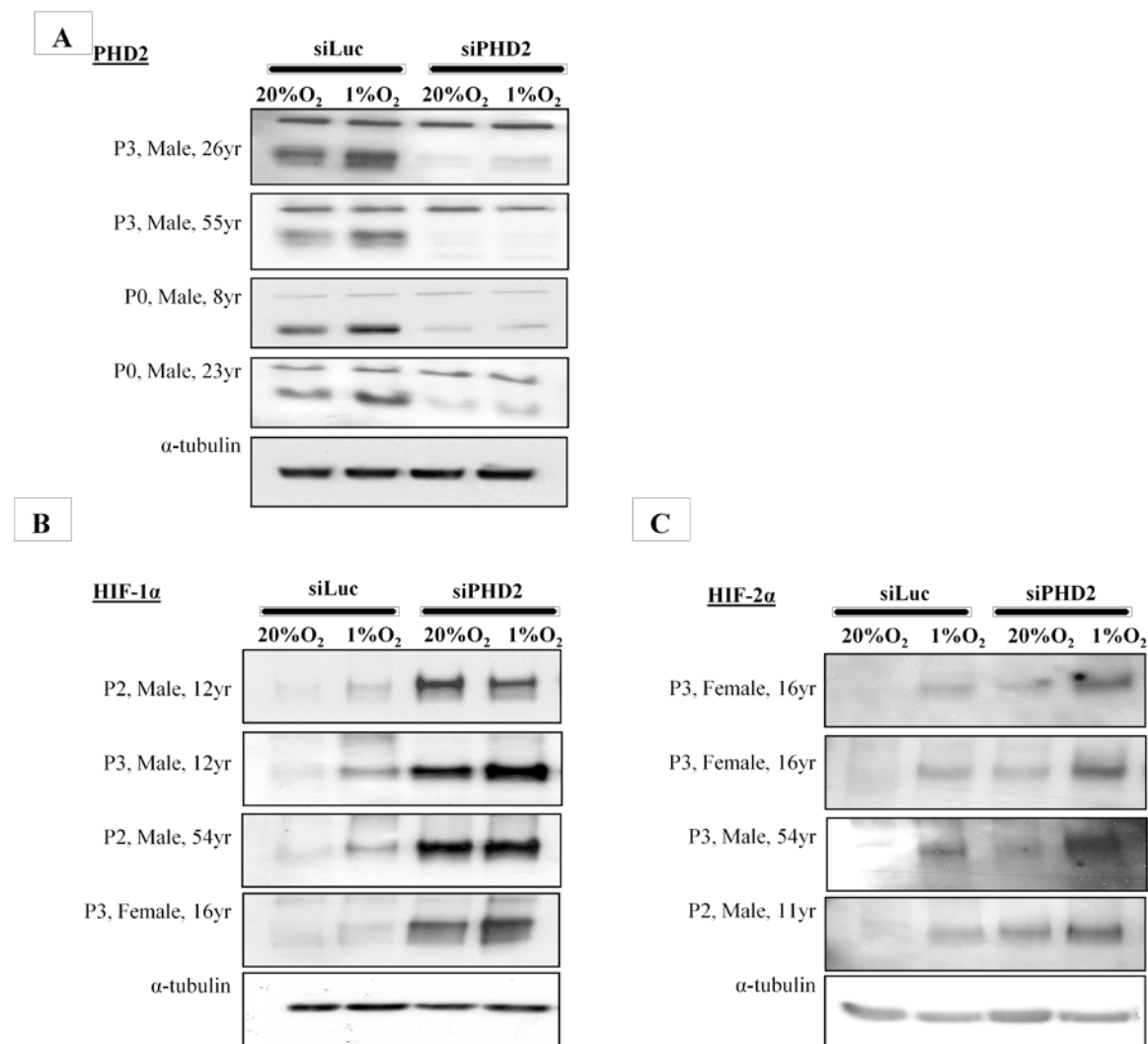


FIGURE 3.6. PHD2 depletion in HACs stabilises HIF-1 α and HIF-2 α in normoxic conditions and further enhances HIF-2 α stabilisation in hypoxia. A, PHD2 was depleted in HACs in both 20% and 1% oxygen cultures. B, HIF-1 α levels were enhanced in PHD2 depleted HACs; while no difference was detectable between PHD2 depleted cultures in 1% or 20% oxygen. C, HIF-2 α levels were enhanced following PHD2 depletion in both normoxia and in hypoxia. Note: All data in the figure were obtained from primary and passaged HACs after 3 days in culture. Cells were transfected with 10nM siRNA against PHD2 (siPHD2), or, as a control against luciferase (siLuc). For simplicity only α -tubulin loading controls from representative experiments are shown.

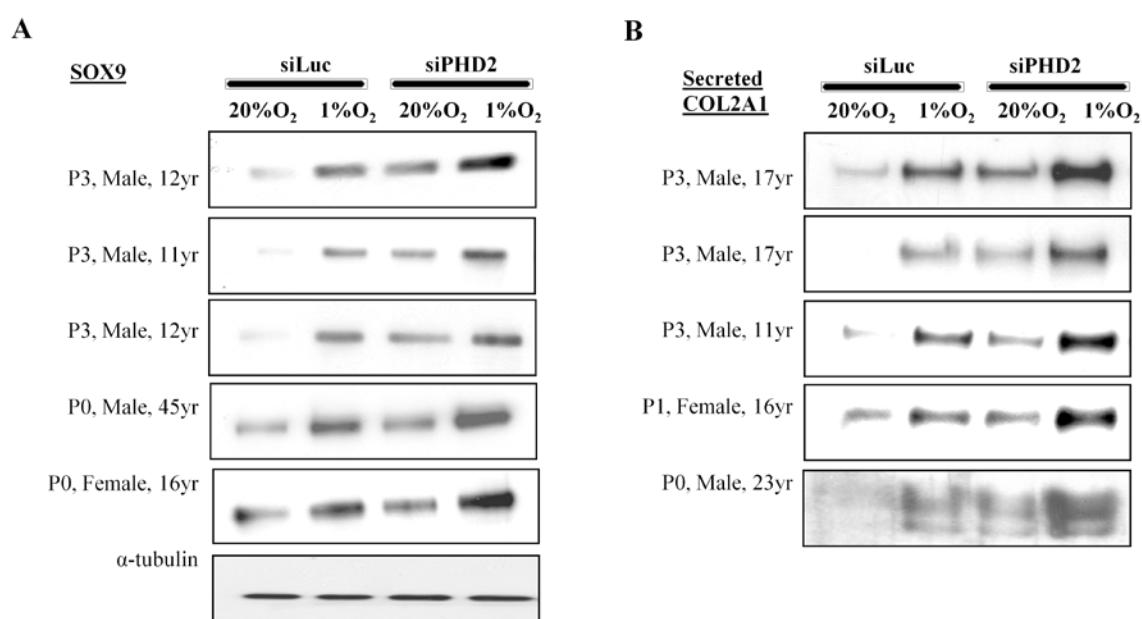


FIGURE 3.7. PHD2 depletion in HACs upregulates SOX9 and secreted type-II collagen protein levels both in normoxia and in hypoxia. PHD2 was depleted in HACs in both 20% and 1% oxygen cultures. **A**, SOX9 protein levels were enhanced following PHD2 depletion in both normoxia and in hypoxia with greatest levels occurring in hypoxia. **E**, Secreted type-II collagen was detected following TCA precipitation of culture medium. Data were normalised based on Bradford measurements of protein content in the cell layers. Secreted type-II collagen protein levels followed a similar pattern to SOX9, being highest in PHD2 depleted HACs cultured in hypoxia. Note: All data in the figure were obtained from primary and passaged HACs after 3 days in culture. Cells were transfected with 10nM siRNA against PHD2 (siPHD2), or, as a control against luciferase (siLuc). For simplicity only α -tubulin loading controls from representative experiments are shown.

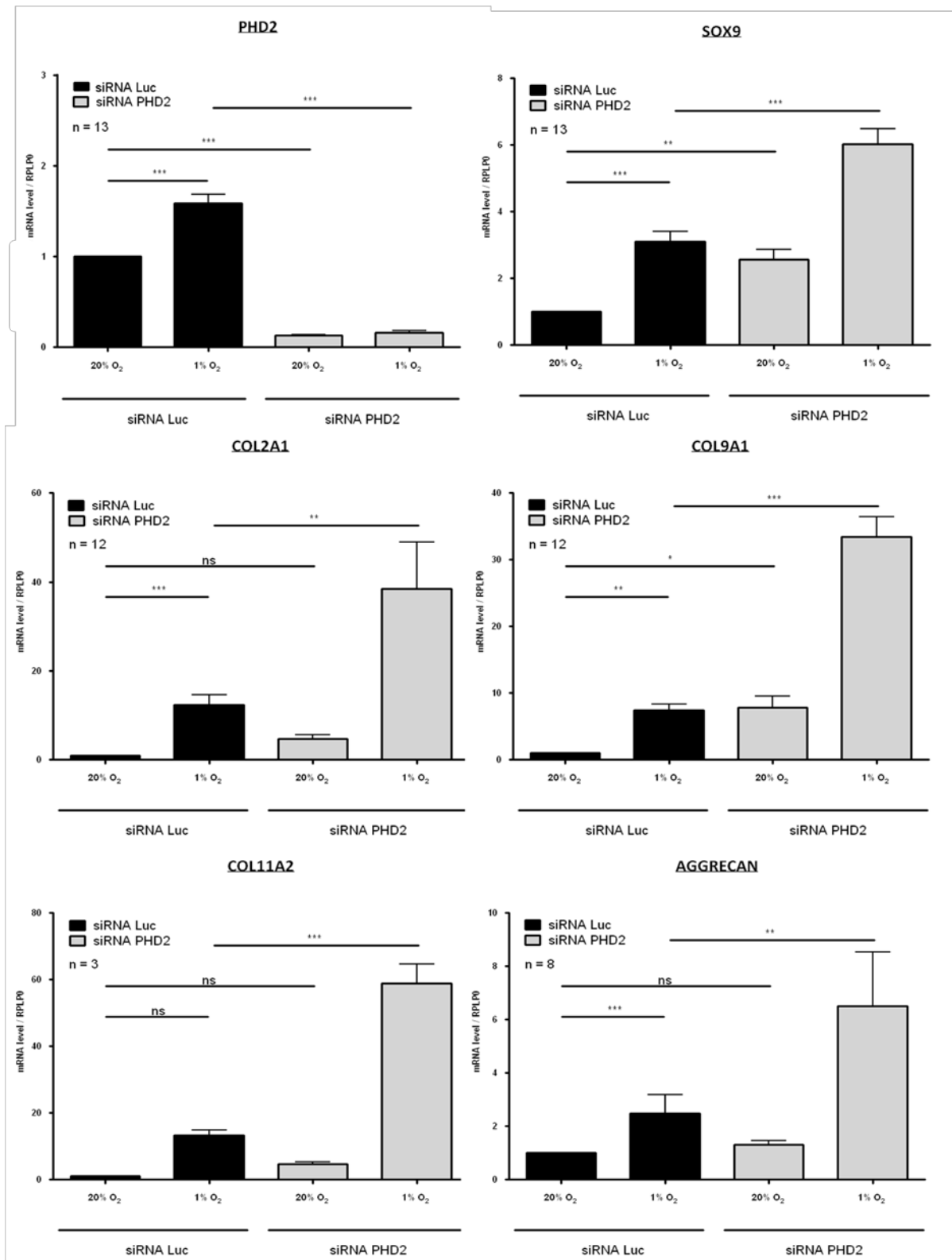


FIGURE 3.8. PHD2 depletion in HACs enhances expression of the key differentiated chondrocyte markers. All data in the figure were obtained from passaged HACs after 3 days in culture at either 20% or 1% oxygen. Cells were transfected with 10nM siRNA against PHD2 (siPHD2), or, as a control against luciferase (siLuc). TaqMan real-time PCR data showing mRNA expression PHD2 (A) and of key chondrocyte markers *SOX9*, *COL2A1*, *COL9A1*, *COL11A2*, *AGGRECAN* (B – F, respectively) are upregulated by hypoxia and further enhanced by PHD2 depletion. Note: ns – not significant; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

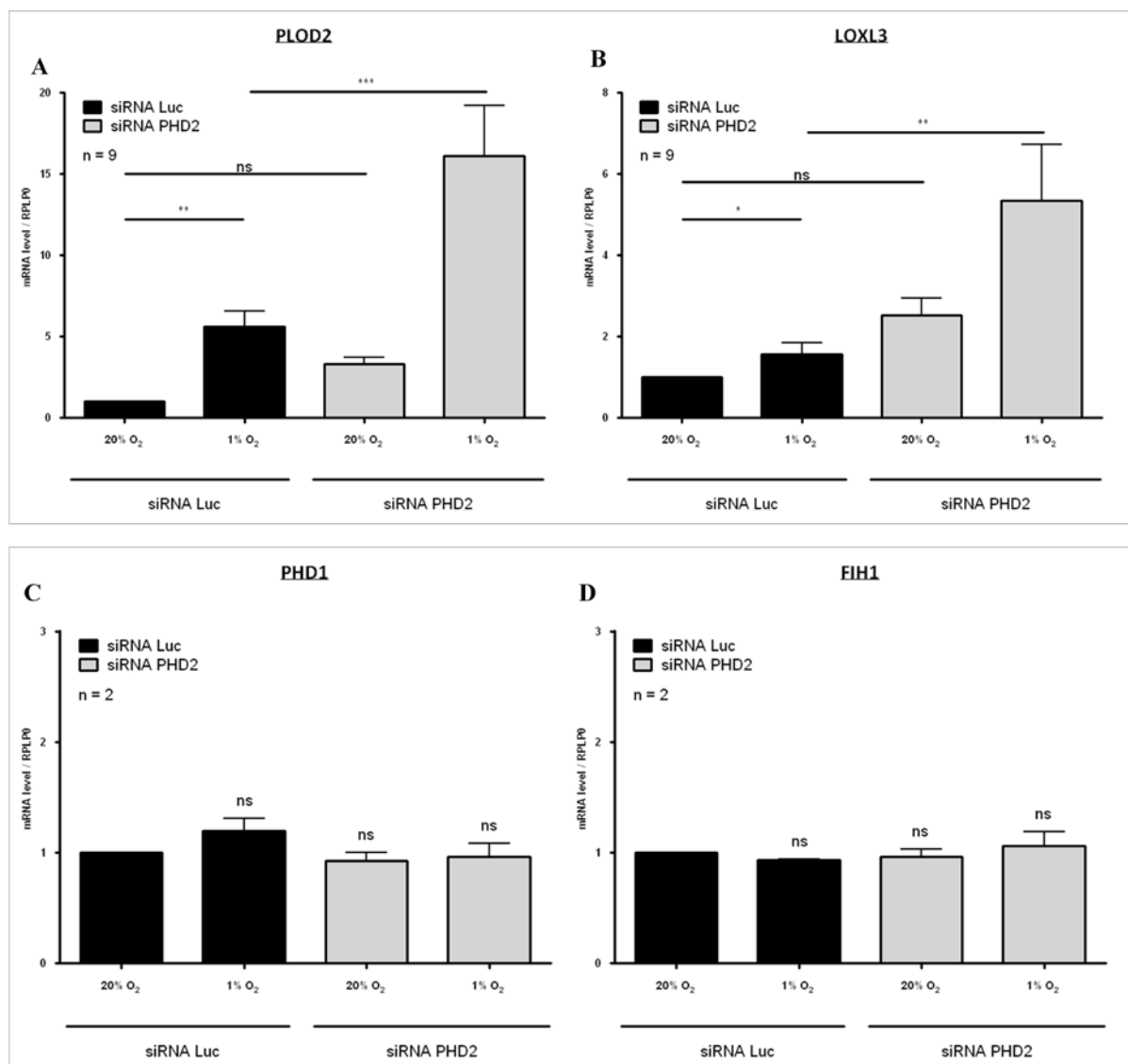


FIGURE 3.9. PHD2 depletion in HACs enhances expression of the key collagen processing enzymes. All data in the figure were obtained from passaged HACs after 3 days in culture at either 20% or 1% oxygen. Cells were transfected with 10nM siRNA against PHD2 (siPHD2), or, as a control against luciferase (siLuc). TaqMan real-time PCR data showing mRNA expression key collagen processing genes *PLOD2* and *LOXL3*. *PLOD2* and *LOXL3* upregulated by hypoxia and further enhanced by PHD2 depletion. (A and B respectively). *PHD1* and *FIH1*, are two known non-hypoxia inducible genes (C and D respectively). These show no change in response to PHD2 depletion. Note: ns – not significant; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

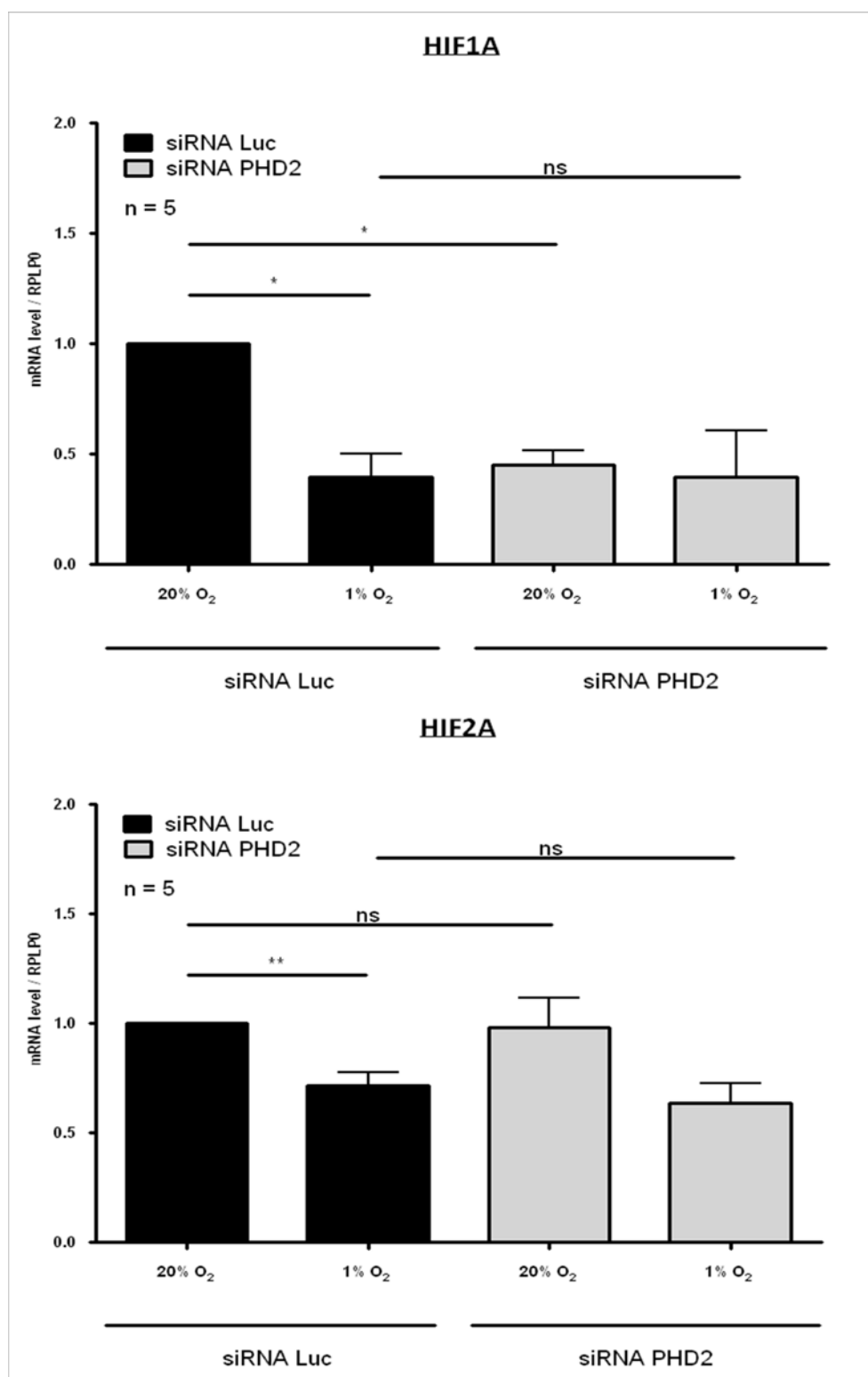


FIGURE 3.10. Hypoxia downregulates expression of HIF-1 α and HIF-2 α at the mRNA level. All data in the figure were obtained from passaged HACs after 3 days in culture at either 20% or 1% oxygen. Cells were transfected with 10nM siRNA against PHD2 (siPHD2), or, as a control against luciferase (siLuc). TaqMan real-time PCR data showing mRNA expression hypoxia significantly downregulated the mRNA levels of HIF1 α and HIF2 α at the mRNA level (A and B respectively). Note: ns – not significant; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

3.7 Factor inhibiting HIF (FIH-1) knockdown does not increase SOX9 levels when combined with PHD2 depletion

The asparaginyl hydroxylase Factor Inhibiting HIF or FIH-1 regulates the transcriptional activity of HIF- α . FIH-1 hydroxylates a specific asparaginyl residue within the c-terminal transactivation domain of the α -subunit. This hydroxylation disrupts the binding of the CBP/p300 coactivator and inhibits HIF transcriptional activity.

In order to investigate the regulation of FIH-1 by hypoxia, HACs were cultured for 3 days under normoxic conditions, hypoxic conditions or in the presence of hypoxia mimetic, iron chelator DFO. Consistent with our previous work FIH-1 was found not to be regulated by hypoxia (Fig. 3.11 A).

In order to investigate the role of FIH-1 in regulating HIF- α in HACs, FIH-1 was depleted using small interfering RNA (siRNA). Passaged chondrocytes were transfected with 10nM of a single siRNA and lysed 3 days after transfection. siRNA against luciferase was used as a non-targeting control. Messenger RNA analysis by real-time PCR revealed the effectiveness of the knockdown to be 97% knockdown (compared with siLuc 20% oxygen) (Fig. 3.11 B). This knockdown was confirmed at the protein level (Fig. 3.11 C).

In order to determine if we could create an additive effect by increasing both HIF-2 α stabilisation and activity HACs were transfected with siRNA against both PHD2 and FIH-1. Passaged human articular chondrocytes were transfected with siRNA against PHD2 plus FIH-1, PHD2 alone or luciferase alone as a non-targeting control and cultured for 3 days at 20% or 1% oxygen. Protein levels of key cartilage-specific transcription factor SOX9 were used as a functional readout. PHD2 + FIH-1 knockdown did not have an additive effect and did not result in levels of SOX9 greater than PHD2 depletion alone. Possible reasons for this will be examined in our discussion.

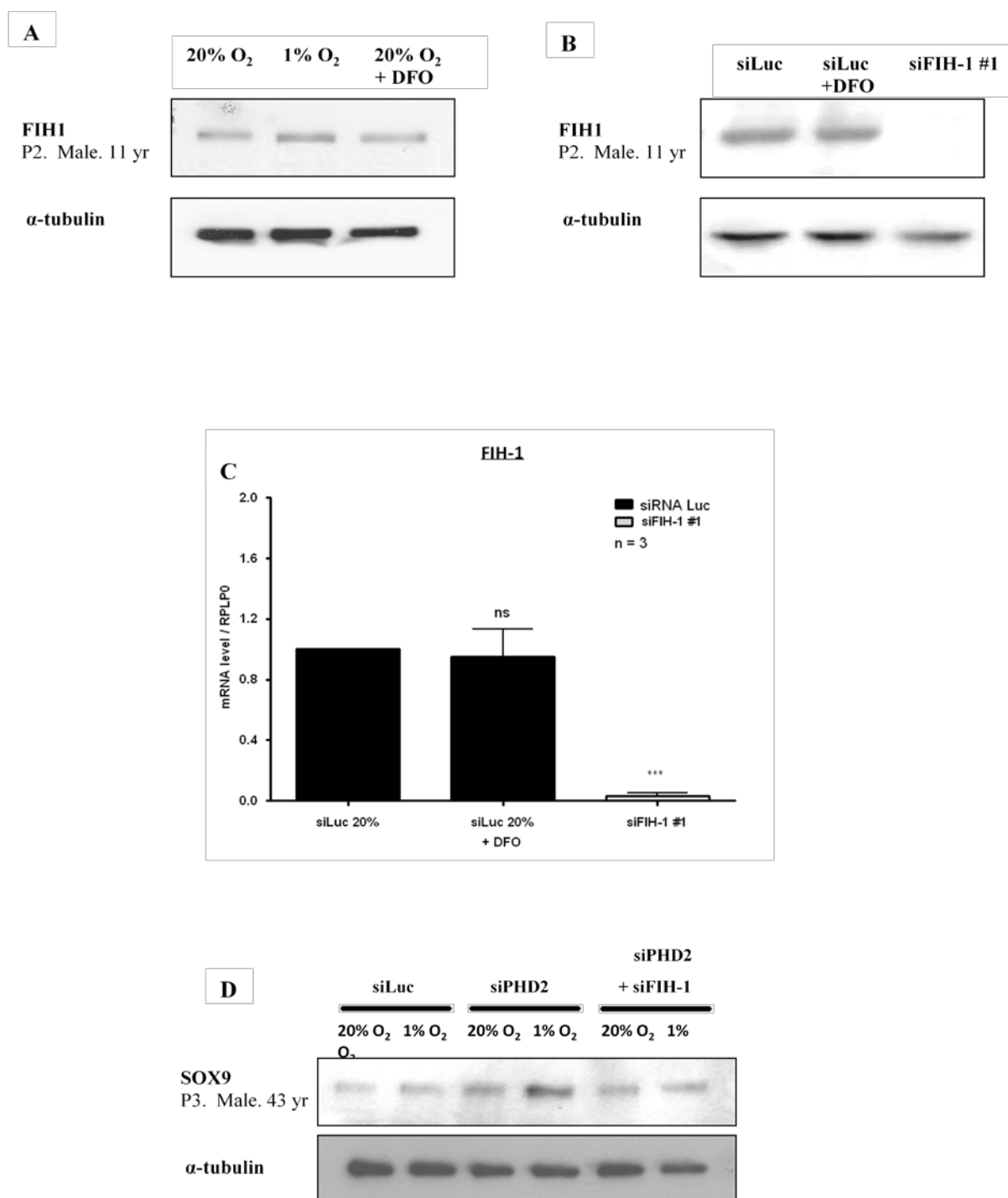


FIGURE 3.11. RNAi-mediated depletion of FIH-1 in HACs has no significant effect on SOX9 expression when combined with PHD2 knockdown. Passaged HACs (P2) cultured for 3 days at 20% oxygen, 1% oxygen or treated with 10mM of the hypoxia mimetic desferrioxamine (DFO). **A**, western blot showing no significant increase in FIH-1 levels in hypoxia (1% oxygen), and after treatment with 10mM DFO. Passaged (P2) HACs were then transfected with 10nM siRNA directed against FIH-1 and an siRNA targeting luciferase (siLuc) was used as a control. **B**, Western blot showing RNAi-mediated depletion of PHD2 protein in HACs (in 20% oxygen). Confirmed using Taqman real-time PCR, **C**. Note: ns - not significant. *** $P < 0.001$ versus 20% oxygen siLuc. Finally, passaged HACs (P3) were cultured for 3 days at either 20% or 1% oxygen. Cells were transfected with 10nM siRNA against PHD2 (siPHD2), both PHD2/FIH-1 (siPHD2+siFIH-1) or against luciferase as a control (siLuc). **D**, western blot shows FIH-1 depletion has no significant increase in SOX9 expression when combined with PHD2 knockdown.

3.8 General Hydroxylase Inhibition Upregulates SOX9 but Inhibits Expression and Secretion of the Cartilage Collagens

Finally we investigated the possible therapeutic role of broad (i.e., non-specific) 2-oxoglutarate-dependent hydroxylase inhibition. The 2-oxoglutarate analogue DMOG inhibits all members of the 2-oxoglutarate dependent hydroxylase family, which includes not only the HIF-targeting hydroxylases (PHD1, PHD2, PHD3, FIH-1), but also the collagen prolyl-4-hydroxylases. 2mM DMOG treatment increased expression of SOX9 at the mRNA (Fig. 3.12 C) and protein level (Fig. 3.12 A, D). Mitogen inducible gene-6 (*MIG-6*), a previously identified HIF-2 α target gene was also shown to be upregulated by DMOG at the protein level (Fig. 3.12 B). Similarly vascular endothelial growth factor (*VEGF*), a classic HIF-target gene was likewise upregulated by DMOG (Fig. 3.12 E).

However, DMOG-induced upregulation of SOX9 didn't result in upregulation of the cartilage-specific collagens *COL9A2*, *COL11a2*, and *COL2A1* (Fig. 3.13 A, B, C and D). Western blot analysis demonstrates that, in fact, the addition of 2mM DMOG inhibits secretion of type II collagen protein into the culture medium (Fig. 3.13. D). Therefore, DMOG, like hypoxia and PHD2 depletion, upregulates SOX9. However, it has completely the opposite effect of hypoxia and PHD2 depletion with regard to expression of the cartilage collagens – decreasing their levels.

The significance of this for a collagen-rich like cartilage will be detailed in our discussion.

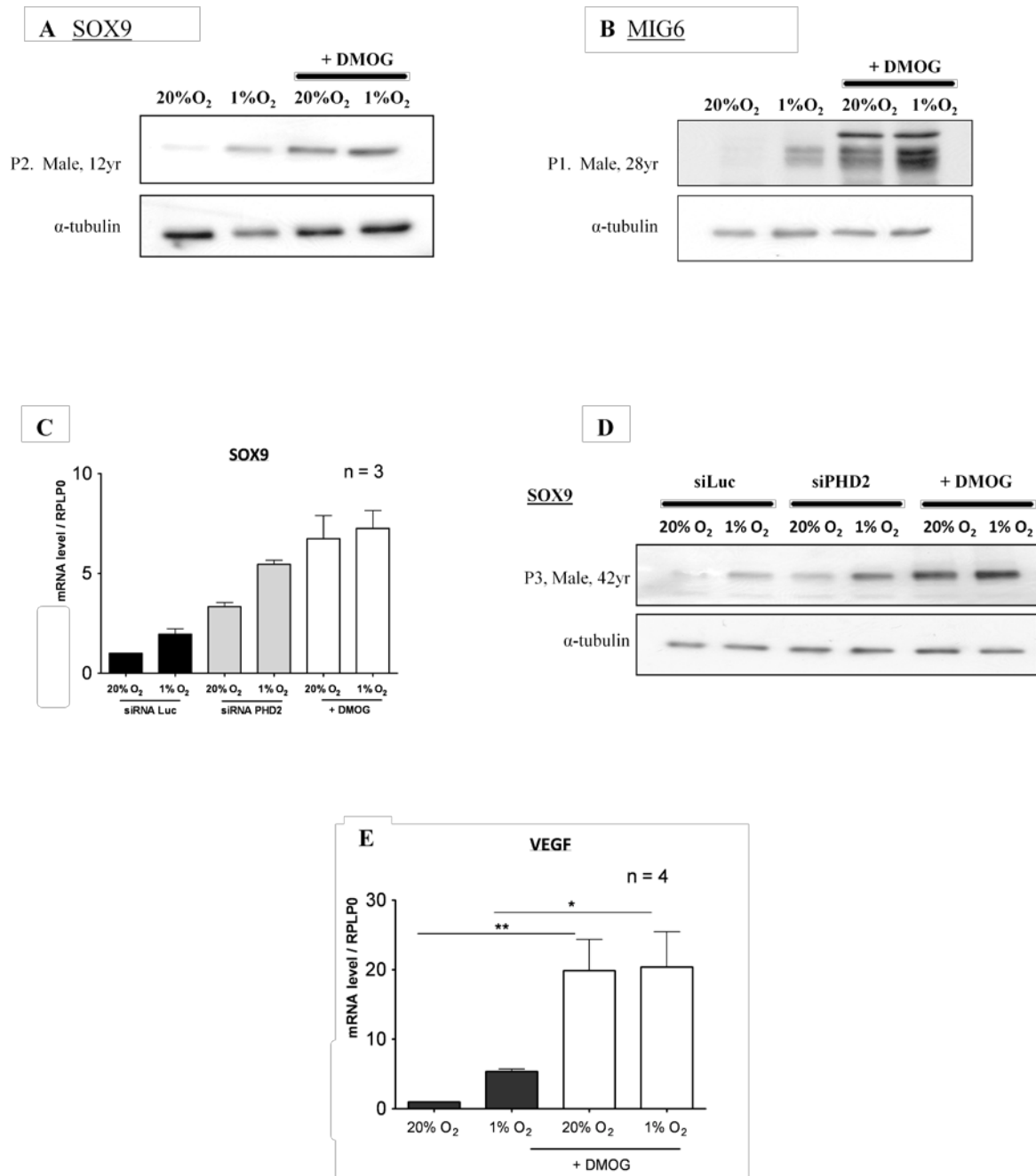


FIGURE 3.12. General hydroxylase inhibition upregulates hypoxia dependent genes SOX9, MIG6 and VEGF. Passaged HACs were cultured for 3 days under normoxic (20%) or hypoxic (1%) conditions, in the presence/absence of 2mM dimethyloxaloylglycine (DMOG) - a general hydroxylase inhibitor. Addition of DMOG enhanced SOX9 protein (A and D) and mRNA levels (C). MIG6, a previously identified HIF-2 α target gene was upregulated in response to DMOG at the protein level (B). E, Expression of classic HIF target gene VEGF was significantly enhanced by DMOG.

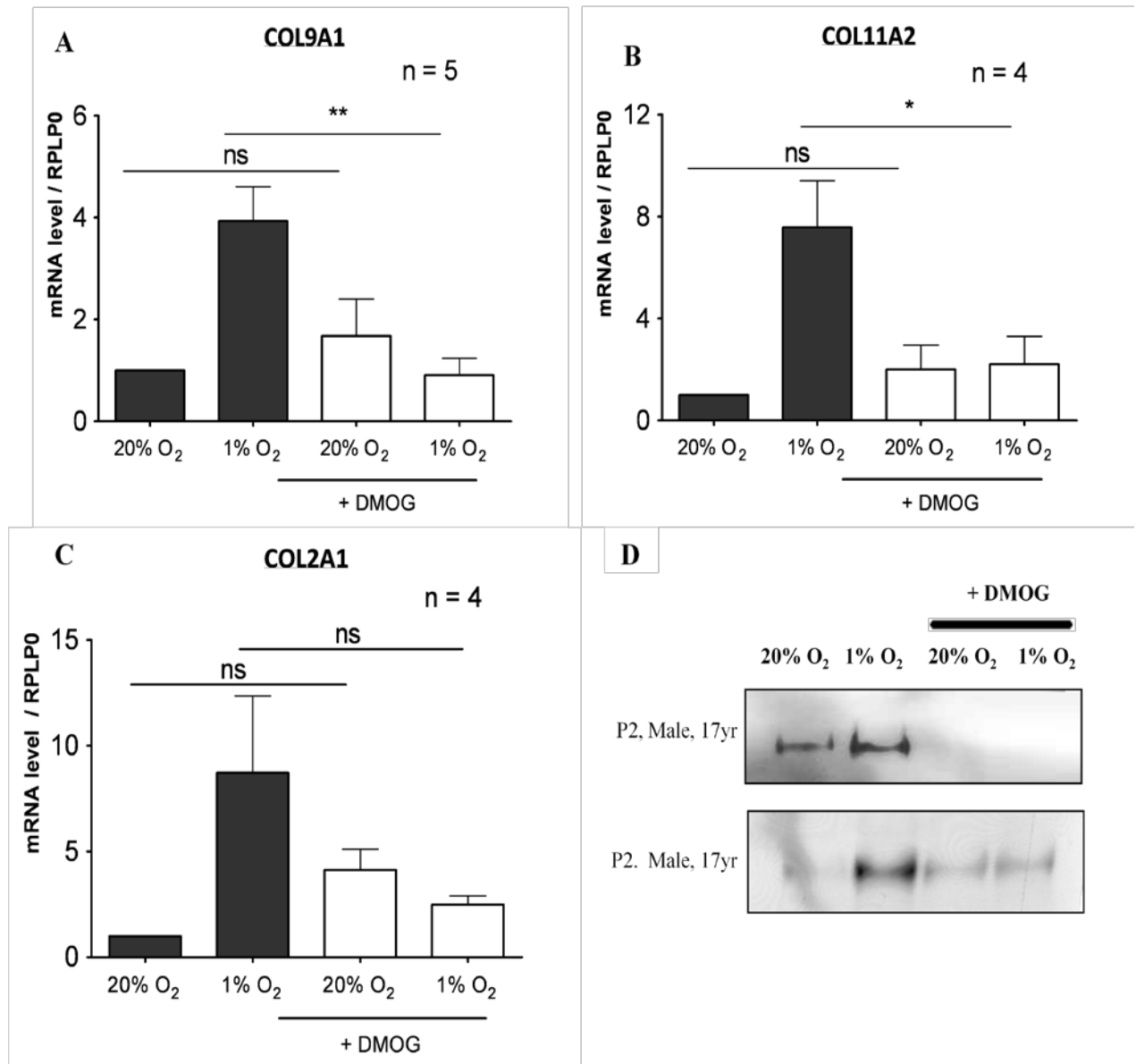


FIGURE 3.13. General hydroxylase inhibition inhibits expression and secretion of the cartilage collagens. Passaged HACs were cultured for 3 days under normoxic (20%) or hypoxic (1%) conditions, in the presence/absence of 2mM dimethyloxaloylglycine (DMOG) - a general hydroxylase inhibitor. DMOG was found to decrease the expression of the cartilage-specific collagens *COL9A1* (A), *COL11A2* (B) and *COL2A1* (C) mRNA levels. D, DMOG treatment inhibited secretion of type-II collagen in HACs. Note: ns – not significant, * $P < 0.05$; ** $P < 0.01$.

3.9 Discussion

We have previously shown that HIF-2 α (not HIF-1 α) is essential for hypoxic induction of cartilage matrix gene expression in HACs (Lafont et al., 2007). Furthermore, it achieves this predominantly through upregulation of transcription factor SOX9, which drives matrix gene expression (Lafont et al., 2008). Since HIFs themselves are regulated by specific prolyl hydroxylases (PHDs), we investigated which of these enzymes targets HIF-2 α . With the aim of promoting the chondrocyte phenotype and enhancing cartilage deposition without inhibiting the prolyl- and lysyl- hydroxylases essential for collagen processing. Using an RNAi approach we identified PHD2 as the key hydroxylase in HACs responsible for regulating HIF-2 α . Crucially, we provide clear evidence that such specific inhibition of PHD2 promotes the differentiated phenotype and enhances cartilage matrix production by HACs both in normoxia and hypoxia. This opens up the exciting possibility of PHD2-specific inhibition as a means to stimulate cartilage repair.

3.9.1 Hypoxia upregulates SOX9 and key extracellular matrix proteins in human cartilage explants

Cartilage functions in a chronically hypoxic environment throughout life. Depending on depth chondrocytes are exposed to varying oxygen tensions; 6% O₂ near the surface and approximately 1% O₂ at the core of the tissue.

Here we report that SOX9 and cartilage extracellular matrix proteins (COL2A1, COL9A1, COL11A2 and AGGRECAN) are upregulated in response to hypoxia in healthy human cartilage explants. There is a clear upregulation of SOX9 at the protein level in human articular cartilage explants this hypoxic upregulation correlates with decreasing levels of molecular oxygen (1%>3%>20%).

Assuming that chondrocytes experience physiological oxygen tensions ~6-1% O₂ it might be more appropriate to report that the exposure of human cartilage explants to supra-physiological oxygen tension (20% O₂) reduces the expression of SOX9 and key cartilage matrix genes COL2A1, COL9A1, COL11A2 and AGGRECAN. Chondrocytes near the surface of the tissue experience oxygen tension ~6% O₂, here we demonstrate that these levels of SOX9 can be increased by inhibition of PHD enzymes (limiting molecular

oxygen) and opens opportunities to further enhance the endogenous chondrocytes phenotype by PHD enzyme inhibition.

This work in human explants is consistent with our previous work in human articular chondrocytes and further evidence that hypoxia is a critical parameter in promoting the chondrocyte phenotype and increasing the synthesis of extracellular matrix proteins (Lafont et al., 2007; Murphy and Polak, 2004; Murphy and Sambanis, 2001). Healthy human cartilage explants are a rare and clinically valuable tissue. Unlike human osteoarthritic cartilage (which has undergone advanced degenerative changes and has a altered phenotype), murine cartilage or a chondrocyte cell line we believe that our data may be directly extrapolated to adult human cartilage and provides important, clinically valuable information.

3.9.2 PHD2 is the most abundant, but PHD3 the most hypoxia-inducible, HIF targeting hydroxylase in human articular chondrocytes

The relative abundance of the various PHD enzymes was measured at the mRNA level under normoxic and hypoxic conditions. PHD1/2/3 were all detectable in human articular chondrocytes, with PHD2 being the most abundant. This is consistent with existing literature. These hydroxylases have been previously reported in human cartilage at the mRNA (PHD1/2/3/4) and protein (PHD1/2/3) level (Hirsila et al., 2003; Koivunen et al., 2007; Terkhorn et al., 2007). In addition, mRNA studies in rats report that in all organs examined (heart, liver, kidney, brain, testis and lung) PHD2 was reported to be the most ubiquitously expressed (Koivunen et al., 2007; Terkhorn et al., 2007; Willam et al., 2006).

Our results also showed that PHD2 and PHD3 are up-regulated at the mRNA level in response to hypoxia in human articular chondrocytes. This hypoxic induction was confirmed at the protein level by western blot for PHD2 and PHD3. The hypoxic induction of PHD2 and PHD3 at the protein level has been reported elsewhere in cell lines (Appelhoff et al., 2004). Electrophoretic mobility-shift assays have demonstrated the binding of HIF-1 α to HREs upstream of PHD2 and HREs have been identified (using bioinformatics) 40kb upstream of PHD3 (Metzen et al., 2003; Pescador et al., 2005).

However, it is not clear why PHD2 and PHD3 are up-regulated in response to hypoxia. It has been hypothesised that this occurs in a negative feedback mechanism

ensuring the rapid degradation of HIF- α isoforms and downregulation of hypoxia responsive genes once the cells encounter increased oxygen levels (D'Angelo et al., 2003). Although we observed PHD3 to be the most highly hypoxia-inducible hydroxylase (17.8 fold), the levels of PHD3 were relatively low and it did not appear to significantly contribute towards HIF- α stabilisation in HACs.

It is illogical that PHD3 is so strongly regulated by hypoxia but has no function in chondrocytes therefore our data implies that either PHD3 does regulate HIF- α but upon depletion levels of stabilised HIF-1 α / HIF-2 α protein levels are too low to detect or PHD3 may have a role outside of targeting HIF- α isoforms in HAC. Interestingly, Köditz and colleagues recently demonstrated that PHD3 interacts with activating transcription factor-4 (ATF-4). ATF-4 is induced by hypoxia/DMOG in a pVHL independent manner and siRNA-mediated down-regulation of PHD3, but not of PHD2, was sufficient to stabilise ATF-4 under normoxic conditions (Koditz et al., 2007).

3.9.3 The regulation of HIF- α in human articular chondrocytes by PHD1, PHD2 and PHD3

Previous work has demonstrated that PHD1, PHD2 and PHD3 have the ability to hydroxylate both HIF-1 α and HIF-2 α . Work in a range of cell lines has suggested differential function of the PHD enzymes in regulation of HIF- α isoforms (Appelhoff et al., 2004). For example, in MCF7 cells knockdown of PHD3 stabilised HIF-2 α to higher levels compared with hypoxic control, and substantially delayed HIF-2 α degradation following re-oxygenation (Appelhoff et al., 2004).

Further work using knockout mice (see Chapter 1) suggests that PHD2 is more active against HIF-1 α and PHD1/PHD3 are more active against HIF-2 α . Although these studies indicate that PHDs may show differential regulation of HIF- α isoforms, at least some of the observed differences are most likely due to the relative abundance of each hydroxylase (which is dependent on cell and tissue type). In fact, the K_m values of all three PHD isoenzymes for recombinant HIF-1 α and HIF-2 α oxygen degradation domains (ODDDs) are quite similar, with PHD1 having the lowest K_m value for both recombinant HIF- α ODDDs (Koivunen et al., 2006). All three PHD isoenzymes also have virtually identical K_m values for

oxygen (Hirsila et al., 2003) implying that changes in oxygen tension will not affect the relative contribution of the three enzymes to HIF- α stabilisation.

In our experiments with HACs, only depletion of the most abundant hydroxylase (PHD2) significantly stabilised both HIF-1 α and HIF-2 α in 20% oxygen and slowed degradation of each HIF- α isoform in reoxygenation experiments. PHD3 depletion had no detectable effect on either HIF α isoform stabilisation. Our data also suggests that PHD1 slightly contribute towards the regulation of HIF-1 α and HIF-2 α isoforms.

Interestingly, however, there was a difference between HIF-1 α and HIF-2 α in regards to their stabilisation by PHD2 depletion. No further increase in HIF-1 α stabilisation was detectable in PHD2 depleted HACs when cultured in 1% oxygen compared to that seen in 20% levels. In contrast, HIF-2 α levels were further increased when PHD2 depleted cells were cultured in hypoxia implying that other factors in addition to PHD2 regulate HIF-2 α in hypoxia. PHD1 may be one such factor since we observed some HIF-2 α stabilisation following depletion of PHD1 in normoxia (although less than that caused by PHD2 knockdown).

Factor inhibiting HIF-1 α (FIH-1), is an iron-dependent dioxygenase capable of regulating HIF- α isoform activity (Lando et al., 2002; Mahon et al., 2001), we found the knockdown of FIH-1 had no significant effect on SOX9 expression even when this knockdown was combined with a PHD2 knockdown. This may be because the increase in HIF-2 α activity has no effect on SOX9 expression or because use of two different siRNA oligonucleotides simultaneously decreases the knockdown efficiency and therefore does not stabilize HIF-2 α to levels equal with PHD2 depletion alone.

Surprisingly, we report that hypoxia and the stabilisation of HIF- α isoforms by inhibition of PHD2 appears to downregulate the expression HIF-1 α and HIF-2 α at the mRNA level. This will be further discussed in Chapter 4 in the context of further data.

It has been previously demonstrated *in vitro* using recombinant PHD enzymes expressed in insect cells that PHD1/2/3 have essentially identical K_m values for oxygen, approximately 25% O₂ (Hirsila et al., 2003). This K_m value is higher than the expected

oxygen tension encountered *in vivo* (6%-1% O₂) and is consistent with PHD2 enzyme function (small change in oxygen tension can influence their activity). More recently it has been shown *in vitro* using recombinant PHD2 and peptides containing HIF-1 α and HIF-2 α ODDD's that PHD2 has a lower K_m for oxygen, approximately 10% O₂ and has some functional activity even at 1% O₂ (~400 d.p.m.). Here we demonstrate that PHD2 still displays significant activity in 1% oxygen, since depletion of PHD2 under hypoxic conditions resulted in greatly increased levels of both HIF- α isoforms and further enhanced SOX9 expression. As described above chondrocytes experience physiological oxygen tensions ~6-1% O₂ *in vivo* therefore, 20% oxygen tension is artificially higher than *in vivo* and 1% oxygen tension represents the most extreme microenvironment (the core of the cartilage). It might be more appropriate to report that the exposure of human articular chondrocytes to supra-physiological oxygen tension (20% O₂) represses the chondrocytes phenotype, reducing the expression of SOX9 and key cartilage matrix genes COL2A1, COL9A1, COL11A2 and AGGRECAN.

By inhibiting PHD2 we are able to upregulate HIF-1 α / HIF-2 α (and subsequently increase SOX9 and cartilage matrix gene expression) to levels in excess maximum physiological conditions. We are able to exploit endogenous oxygen sensing pathways to promote articular chondrocyte function, fully “turning on” the HIF-signalling pathway and do better than nature. To be able to enhance HIF-2 α levels above those typically found in hypoxia is potentially of great importance since we have previously shown that HIF-2 α upregulates cartilage-specific matrix expression through induction of SOX9 (Lafont et al., 2008; Lafont et al., 2007). Furthermore, our results were generated using freshly isolated (P0) and passaged (P1-P3) HACs. Healthy human articular chondrocytes are rare and clinically valuable cells. Unlike human osteoarthritic chondrocytes, murine cartilage or a chondrocyte cell line our data can be directly extrapolated to adult human cartilage and provides important, clinically valuable information.

3.9.4 Therapeutic applications of work

Experiments with the general hydroxylase inhibitor dimethyloxalylglycine (DMOG) highlight the importance of development of HIF-specific hydroxylase inhibitors with regard to the collagen rich tissue cartilage. Addition of 2mM DMOG enhanced SOX9 levels in HACs, and we have previously shown that this is dependent (like hypoxia) specifically on

HIF-2 α (Murphy et al., 2009). Addition of DMOG upregulated SOX9 however, unlike the case of hypoxia or PHD2 depletion, DMOG did not result in upregulation of the cartilage-specific collagen genes: *COL2A1*, *COL9A1* and *COL11A2*. Most importantly, secretion of type II collagen was inhibited by DMOG. This is most likely due to the fact that DMOG also inhibits the collagen prolyl 4-hydroxylases (C-P4Hs).

Collagen prolyl-4-hydroxylases (C-P4Hs) are tetramers (consisting of 2 α subunits and 2 β subunits) responsible for catalysing the formation of 4-hydroxyproline by hydroxylation of –X-Pro-Gly- residues within the pro- α collagen chains. Like HIF-targeting hydroxylases collagen prolyl-4-hydroxylases use 2-oxoglutarate and oxygen as substrates (and iron and ascorbate as cofactors) to hydroxylate proline residues and this hydroxylation is essential for the formation of intramolecular hydrogen bonds and subsequent stable triple-helical formation (Jimenez et al., 1973; Kukkola et al., 2003; Myllyharju, 2003; Van Den Diepstraten et al., 2003). Unlike the PHD1/2/3 C-P4H enzymes have a low K_m for oxygen (40 μ M vs. 230-250 μ M) suggesting that C-P4H enzymes can remain active even under low oxygen tensions (Hirsila et al., 2003). This makes sense given the roles of the HIF- and Collagen- Prolyl hydroxylases. PHD1/2/3 act as cellular oxygen sensors therefore even a small change in oxygen tension can influence their activity. In contrast, C-P4Hs must be able to act in stressful micro-environments (eg: cartilage extracellular matrix synthesis or wound repair in a tissue with poor vascularisation). Posttranslational modification of type II procollagens are essential steps for formation of the triple helices and subsequent secretion. Inhibition of C-P4Hs by DMOG leads to misfolded, unhydroxylated procollagen chains, these are retained by the cell and targeted for intracellular degradation (Berg et al., 1983).

Similarly to our work Gelse and colleagues recently carried out a study injecting the joints of STR/ORT mice with the non-specific hydroxylase inhibitor dimethyloxallylglycine (DMOG). STR/ORT mice spontaneously develop osteoarthritis and injection of DMOG was unable to prevent the formation of severe arthritis. Although Sox9 was up-regulated in response to DMOG injection, immunohistochemistry and immunofluorescence revealed incomplete post-translational processing and intracellular accumulation of type II collagen in chondrocytes. It was concluded that this impaired secretion of collagen was due to inhibition of collagen prolyl-4-hydroxylases by the non-specific hydroxylase inhibitor DMOG (Gelse et al., 2008).

This stresses the importance of developing PHD2- specific inhibitors (or at least HIF-specific prolyl-4 hydroxylases) which do not inhibit key collagen prolyl-4-hydroxylases which are essential for correct collagen posttranslational modification. Furthermore, Grimmer and colleagues have previously demonstrated that the collagen prolyl-4-hydroxylases α and β subunits were significantly enhanced in response to hypoxia in HACs (Grimmer et al., 2006; Hofbauer et al., 2003). We aim to investigate this in our further work and demonstrate that collagen prolyl 4-hydroxylase levels can be further enhanced by PHD2 depletion. Complementary with these findings here we report that two further enzymes involved in collagen fibril formation, *LOXL3* and *PLOD2* are unregulated in response to hypoxia and further enhanced by PHD2 inhibition (Lafont et al., 2008). This suggests that hypoxia upregulates not only *COL2A1* transcription but also accelerates the post-translational processing of type II collagen fibres and their secretion. Furthermore, these two effects may be enhanced by PHD2 enzyme inhibition.

This adverse effect of general hydroxylase inhibition on collagen processing is particularly relevant to cartilage as it is a highly collagen rich tissue. For this reason the use of DMOG clearly has no therapeutic application in the treatment of arthritis.

In summary, here we demonstrate that consistent with our previous work in human articular chondrocytes hypoxia promotes chondrocyte function in human cartilage explants. Moreover, work in human articular chondrocytes demonstrated that the specific depletion of HIF-2 α targeting hydroxylase PHD2 significantly upregulated the cartilage master regulator, transcription factor SOX9, and key matrix genes *COL2A1*, *COL9A1*, *COL11A2*, and *AGGRECAN*. We believe that the use of healthy human articular cartilage and human articular chondrocytes provides important clinically relevant data that may be directly extended to human articular cartilage *in vivo*.

Crucially, here we demonstrate that PHD2 displays significant activity even at 1% oxygen, since depletion of PHD2 under hypoxic conditions resulted in greatly increased levels of both HIF- α isoforms and further enhanced SOX9 expression. 1% oxygen tension represents the most extreme microenvironment (the core of the cartilage). By inhibiting PHD2 we are able to upregulate HIF-1 α / HIF-2 α (and subsequently increase SOX9 and

cartilage matrix gene expression) to levels in excess of those achieved by physiological conditions. This opens novel, exciting possibilities of manipulating the endogenous oxygen sensing pathways in order to promote articular chondrocyte function, promote cartilage synthesis and to stimulate repair. In addition, PHD2 inhibition upregulated key pro-Lysyl hydroxylases involved in collagen formation implying that PHD2 inhibition offers a novel means to enhance cartilage repair upregulating SOX9 and key cartilage matrix proteins with inhibiting the enzymes involved in pro-collagen fibril formation.

Chapter 4

Hypoxia is an Anti-catabolic Regulator of Human Articular Cartilage

4.1 Introduction

Osteoarthritis is traditionally thought of as a mechanical process, a consequence of “wear and tear” on a synovial joint. However, a more accurate description would be to describe an imbalance between net anabolic (matrix synthesis) and net catabolic (protease) activity (Cawston et al., 1999; Grimmer et al., 2006; Nelson et al., 1998; Rizkalla et al., 1992).

In osteoarthritis it is believed that there is an increase in cartilage matrix degradation through an increased production of proteases (ADAMTSs, MMPs), an increase in protease activity (increased cleavage of pro-enzymes) or a decrease in catabolic regulators (TIMPs). Increased mRNA expression of catabolic matrix metalloproteinases and aggrecanases, e.g., MMPs-1,-3 and -13, and ADAMTS-4 and -5 has been found in human osteoarthritic cartilage and are associated with increased matrix loss (Bau et al., 2002; Song et al., 2007; Stanton et al., 2005; Vincenti and Brinckerhoff, 2002). In addition chondrocytes appear to undergo phenotypic changes, upregulating collagen X expression and thus adopting somewhat of a hypertrophic phenotype (Aigner et al., 2007). Increased net catabolism compromises the structural integrity of cartilage extracellular matrix, the tissue loses its ability to absorb and respond to mechanical stress (Grimmer et al., 2006). Destruction of the cartilage extracellular matrix leads to joint pain, loss of function over time and reduced quality of life.

Here we report the benefit of hypoxia, namely the stabilisation of HIF- α isoforms inhibits IL-1 α induced aggrecan cleavage in human cartilage explants. Furthermore, hypoxia suppresses the endogenous release of aggrecan fragments in unstimulated cartilage suggesting that hypoxia has an anti-catabolic effect in cartilage is able to reduce matrix degradation. In order to investigate the anticatabolic effect we monitored expression of a number of genes and found that the stabilisation of HIF- α isoforms in human articular cartilage explants has an anti-catabolic effect downregulating mRNA expression of several catabolic proteases associated with cartilage matrix degradation and upregulating anti-catabolic enzyme tissue inhibitor of metalloproteinases 3 (TIMP3). This result in human tissue (cartilage explants) was replicated in HACs (monlayer cultures) and using an RNAi approach we provide clear evidence that hypoxia and specifically the stabilisation HIF-1 α (not HIF-2 α) has an anti-catabolic effect in human articular cartilage. Furthermore, we show

that depletion of PHD2 enhances this anti-catabolic effect in both normoxia and hypoxia. Finally, stimulation with IL-1 α stabilises HIF-1 α but decreases HIF-2 α in HACs. Data suggests that this may be partly mediated at the transcriptional level and would seem to represent a different mechanism than hypoxia of HIF regulation.

4.2 Hypoxia inhibits aggrecan degradation in human articular cartilage explants

In order to study the effect of hypoxia on aggrecan degradation, we stimulated human articular cartilage explants with proinflammatory cytokine interleukin 1 α (IL-1 α). Stimulation with IL-1 α causes the cleavage of the aggrecan core protein by aggrecanases within the first interglobular domain (between G1 and G2) (Flannery et al., 1999; Gebauer et al., 2005; Saklatvala, 1987; Vincenti and Brinckerhoff, 2002). Proteolysis occurs at the Glu³⁷³ – Ala³⁷⁴ bond resulting in the C-terminal neoepitope NITEGE (bound to the hyaluronan backbone) and the N-terminal neoepitope ARGSV (Nagase and Kashiwagi, 2003; Sandy et al., 1992). This C-terminal aggrecan fragment can be detected by western blotting cartilage explant culture medium with antibodies which recognise the ARGSV neoepitope. In human articular cartilage explants anti-ARGSV neoepitope antibodies recognise a dominant 150 kDa band and a fainter 250 kDa fragment. Release of ARGSV neoepitope was detected 24 hours after stimulation with IL-1 α and even present in unstimulated cartilage.

Freshly dissected healthy human cartilage explants from 4 donors were pre-incubated in normoxic or hypoxic conditions for 48 hours before treatment, stimulated with either IL-1 α or vehicle (serum free media) and returned to original oxygen tensions. In human cartilage explants hypoxia inhibited the release of ARGSV neoepitope when explants were stimulated with IL-1 α (Fig 4.1). Generally, this inhibition of ARGSV neoepitope release was IL-1 α dose dependent and long exposure of X-ray film revealed that hypoxia also inhibited ARGSV neoepitope release in unstimulated cartilage explants suggesting that hypoxia may be able to inhibit endogenous matrix turnover (Fig 4.1).

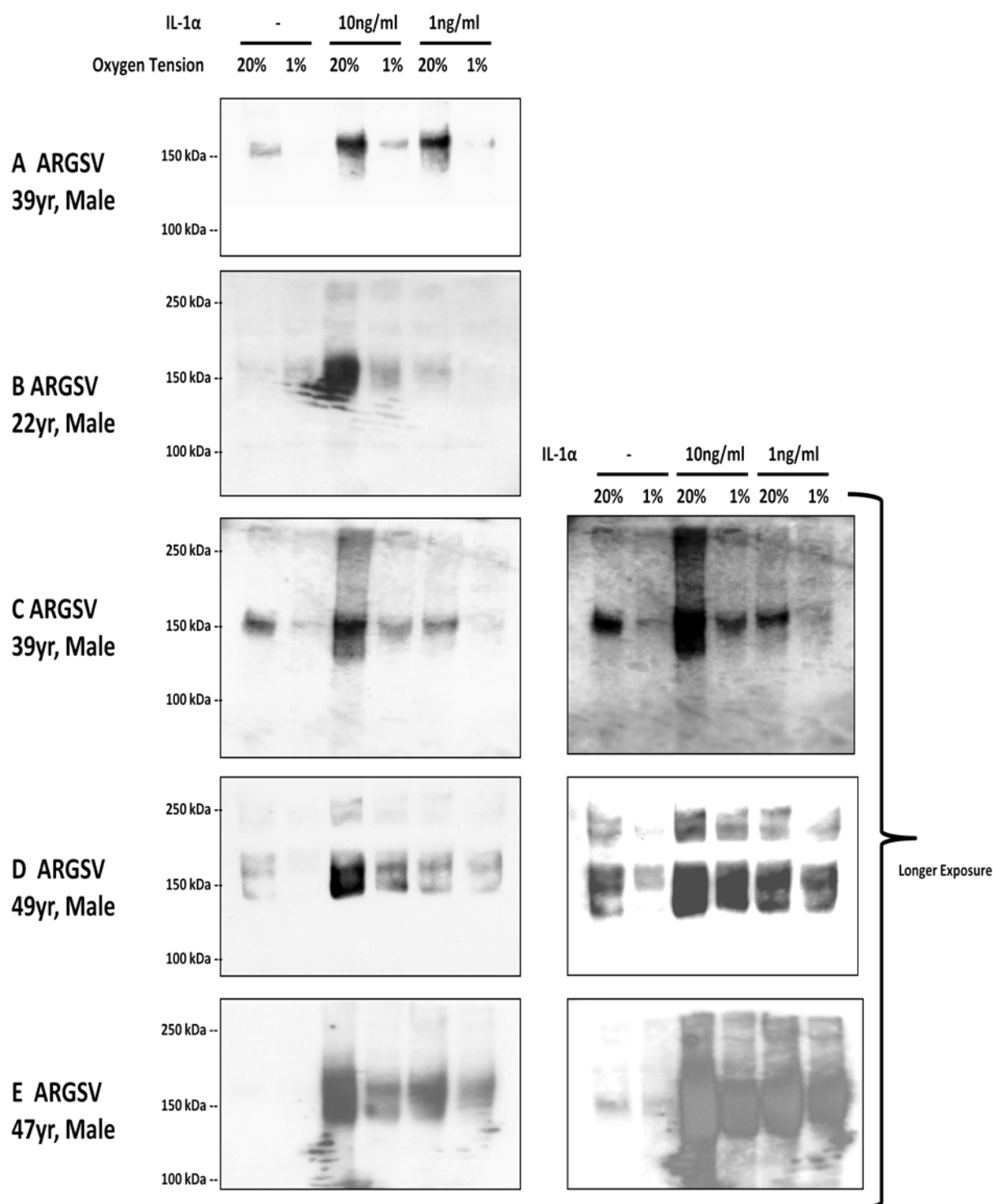


FIGURE 4.1. Hypoxia inhibits aggrecan degradation in human articular cartilage explants. Healthy human cartilage explants from 4 donors were pre-incubated in normoxic or hypoxic conditions for 48 hours before stimulation with either IL-1 α (10ng/ml or 1ng/ml) or vehicle (serum free media) and returned to their original oxygen tensions. Degraded aggrecan fragments released into the medium were detected by western blotting using an anti-ARGSV neopeptide antibody. Culture medium was precipitated using acetone. Note: longer exposure blots are also shown to highlight neopeptide release under control (non-IL-1 α) conditions.

However, not all patients followed this trend: Figure 4.1 A, there appears to be no dose dependent release of ARGSV when cartilage explants treated with 20% oxygen are stimulation with 10ng/ml or 1ng/ml of IL-1 α . Figure 4.1 C, there is a higher level of ARGSV release in unstimulated 20% oxygen control compared with 20% oxygen 1ng/ml IL1 α stimulated explants. Figure 4.1 E, the hypoxic inhibition of ARGSV release in unstimulated cartilage explants is very subtle. These differences may be due to patient variability or due to gel loading error.

In summary, treatment with hypoxia inhibits aggrecan degradation in both unstimulated and IL-1 α stimulated in healthy human cartilage explants.

4.3 Down-regulation of catabolic and upregulation of anti-catabolic enzymes in human cartilage explants under hypoxic conditions

Human cartilage explants from a range of patients (3 male and 2 female donors; age 14-46 years [mean age 26.6 years]) were incubated for 3 days under normoxic (20% oxygen) or hypoxic (1% oxygen) conditions. The messenger RNA levels (mRNA) of catabolic enzymes *ADAMTS4*, *ADAMTS5*, *MMP1*, *MMP3*, *MMP13* as well as anti-catabolic inhibitors *TIMP1*, *TIMP2* and *TIMP3* were analysed after normalising the data using the housekeeping gene *RPLP0* (Fig. 4.2 A to H). Aggrecanases *ADAMTS4* and *ADAMTS5* cleave aggrecan at a well-defined site. Similarly, the matrix metalloproteinases *MMP1*, *MMP3* and *MMP13* have the ability to cleave aggrecan but they are also implicated in the catabolism of collagen fibers and other matrix components following loss of aggrecan from cartilage.

Here we report that hypoxia significantly downregulates key aggrecanase *ADAMTS5* expression (1.75 fold reduction) at the mRNA level (Fig. 4.2 B). Hypoxia had also significantly downregulating collagenase *MMP13* mRNA expression 3 fold (Fig. 4.2 E). *TIMP3* was upregulated at the mRNA level (8.48 fold) in response to hypoxia (Fig. 4.2 H). This data shows a predominantly anticatabolic response to hypoxia, with downregulation of key catabolic enzymes and upregulation of essential catabolic inhibitor *TIMP3* which inhibits a broad spectrum of MMPs, ADAM-17, *ADAMTS4* and *ADAMTS5*.

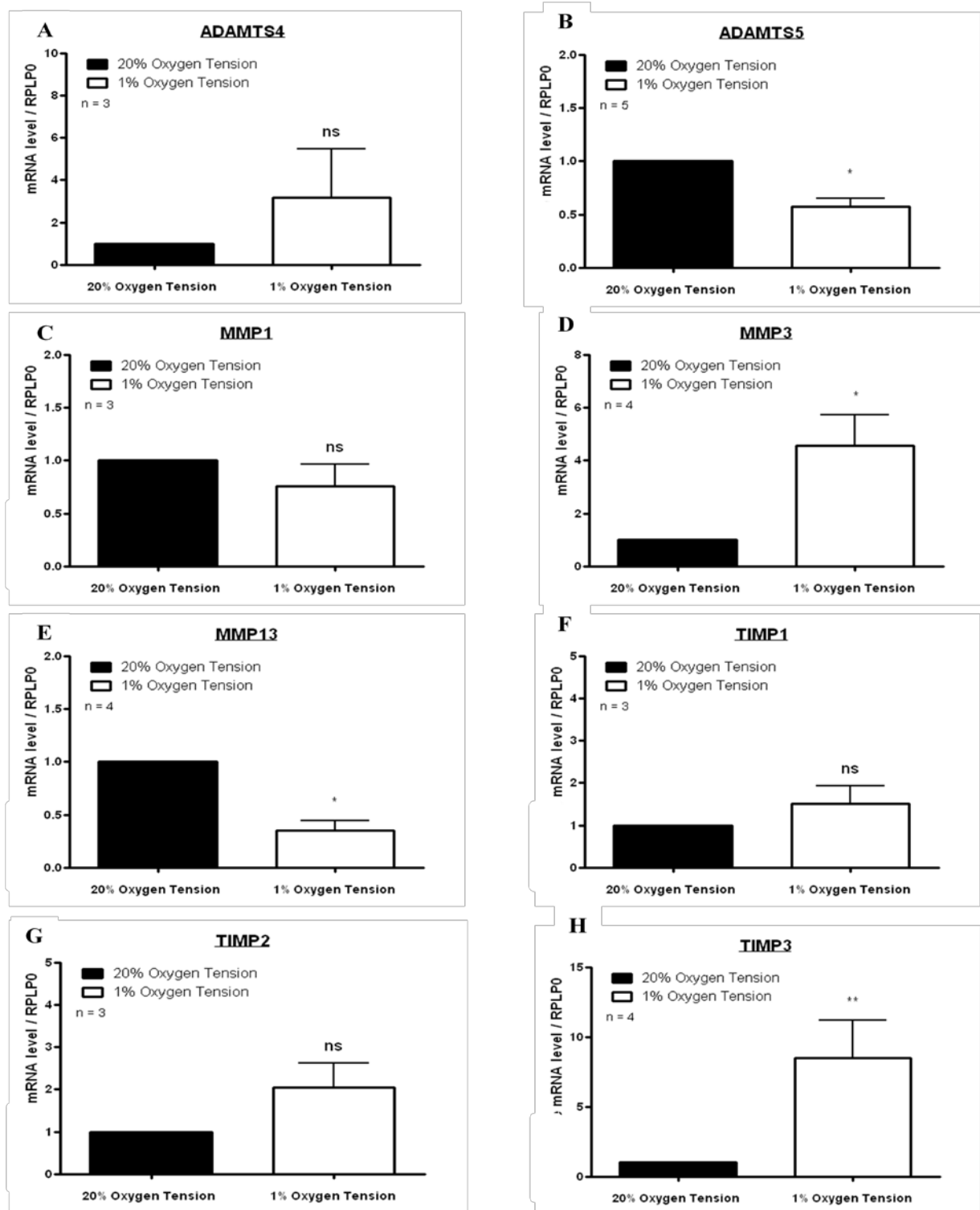


FIGURE 4.2. Hypoxia downregulates key cartilage catabolic enzymes ADAMTS5 and MMP13, upregulates matrix metalloproteinase MMP3 and anti-catabolic factor TIMP3 in human cartilage explants. Data are based on human cartilage explants from 4-5 different donors. TaqMan PCR data showing relative levels of mRNA for *ADAMTS4*, *ADAMTS5*, *MMP1*, *MMP3*, *MMP13*, *TIMP1*, *TIMP2* and *TIMP3* (A to H, respectively) after culture for 3 days at 20% and 1% oxygen. All values were normalised using the housekeeping gene *RPLP0* and represent mean and SEM results of 3-5 independent experiments (i.e., cells from 4-5 different donors). Note: ns - not significant. * $P < 0.05$; ** $P < 0.01$ (versus 20% oxygen)

4.4 Anti-catabolic effects of hypoxia are HIF-1 α and not HIF-2 α dependent

In order to determine whether the anti-catabolic effects were mediated by either HIF-1 α or HIF-2 α , we depleted each isoform in human articular chondrocytes (HACs) using siRNA. HACs were transfected with siRNA against HIF-1 α , HIF-2 α , HIF-1 α + HIF-2 α or luciferase (as a non-targeting control), cultured for 3 days at either 20% oxygen or 1% oxygen before lysis. Real-time PCR revealed that HIF-1 α mRNA was depleted by 89%, on average and HIF-2 α message was depleted 96% compared to 1% oxygen siLuc levels (Fig. 4.3 A and B). Western blotting confirmed depletion of HIF- α isoforms at the protein level (Fig. 4.3 C). Faintly detectable levels of HIF-1 α protein were present in the normoxic control (siLuc 20%) and longer exposure of the blots revealed that the knockdown of HIF-1 α using siRNA was able to reduce protein levels below this normoxic control (Fig. 4.3 C)

Following specific depletion of each HIF- α protein, we assessed the functional effects of the knockdowns on levels of *ADAMTS5*, *MMP13* and *TIMP3* mRNA levels after normalising the data using the housekeeping gene *RPLP0*. *ADAMTS5* and *MMP13* were significantly downregulated by hypoxia compared with siLuc in 20% oxygen (1.63 and 1.49 fold downregulated respectively) (Fig. 4.4 A and B). The hypoxic downregulation of *MMP13* was significantly reversed by HIF-1 α depletion, resulting in *MMP13* mRNA levels greater than those present under normoxic conditions (Fig. 4.4 A). Most interestingly, this suggests that even under normoxic conditions the low level of HIF-1 α protein may be functional in HACs and this has an anti-catabolic effect suppressing *MMP13* expression. Longer exposure revealed detectable levels of HIF-1 α protein (and faintly detectable levels of HIF-2 α protein) even under normoxic conditions. The depletion of HIF-2 α had no significant effect on the hypoxic downregulation of *MMP13* and the depletion of HIF-1 α + HIF-2 α significantly reverses *MMP13* downregulation in a manner similar to HIF-1 α depletion alone.

Curiously, unlike *MMP13*, the hypoxic suppression of *ADAMTS5* mRNA levels could not be reversed using either HIF-1 α or HIF-2 α depletion suggesting a different, HIF-independent mechanism of regulation (Fig. 4.4 B).

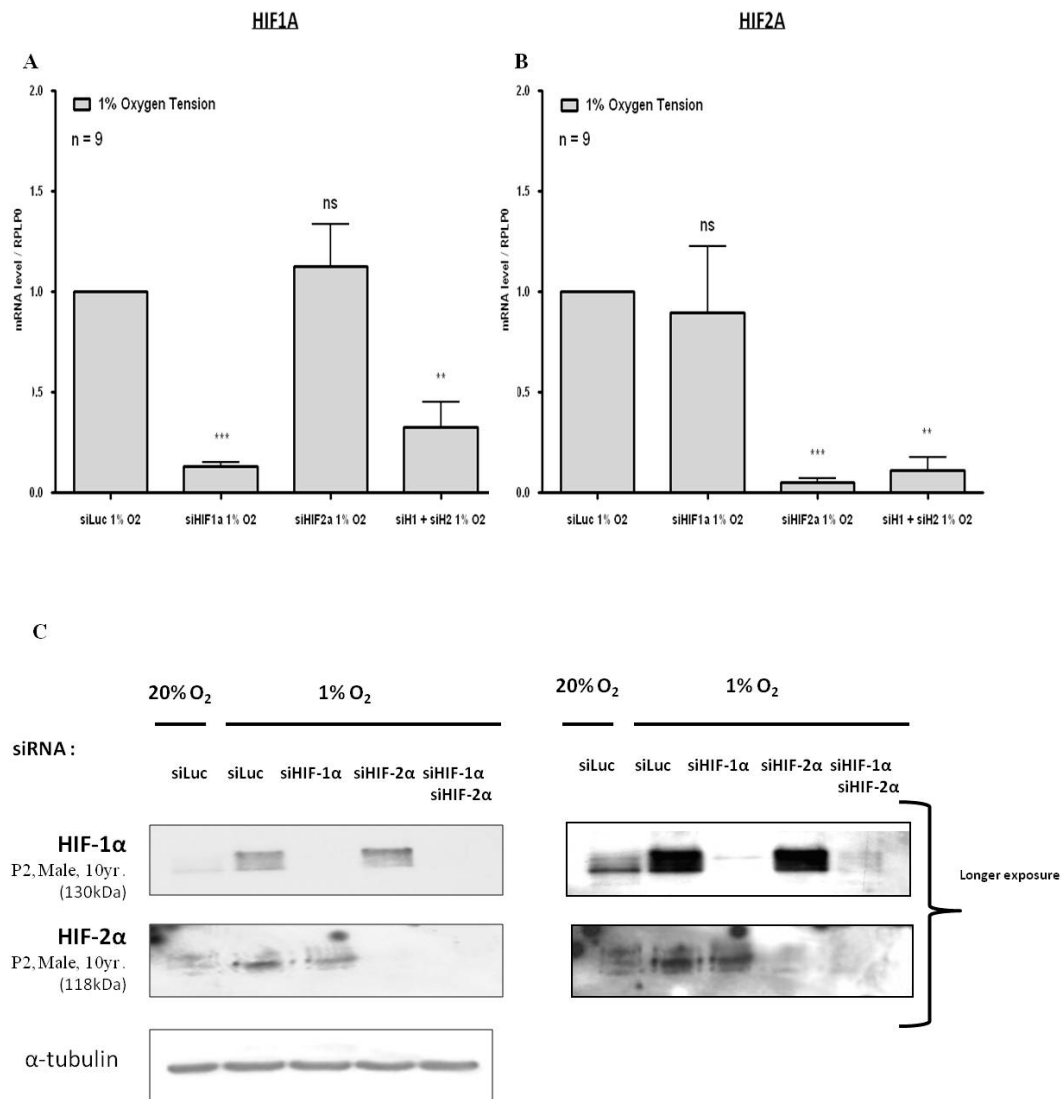


FIGURE 4.3. Knockdown of HIF-1 α and HIF-2 α in passaged HACs. All data in the figure were obtained from passaged (P1-P3) HACs after 3 days in culture at either 20% or 1% oxygen. Cells were transfected 10nM siRNA against HIF-1 α (siHIF-1 α), HIF-2 α (siHIF-2 α) or both at the same time (siH1 + siH2). siRNA against luciferase was used as a control (siLuc). TaqMan Real-time PCR data showing relative mRNA levels of *HIF-1 α* (A), *HIF-2 α* (B) versus hypoxic control (siLuc 1%). Western blot confirming knockdown of HIF-1 α and HIF-2 α in HACs (C).

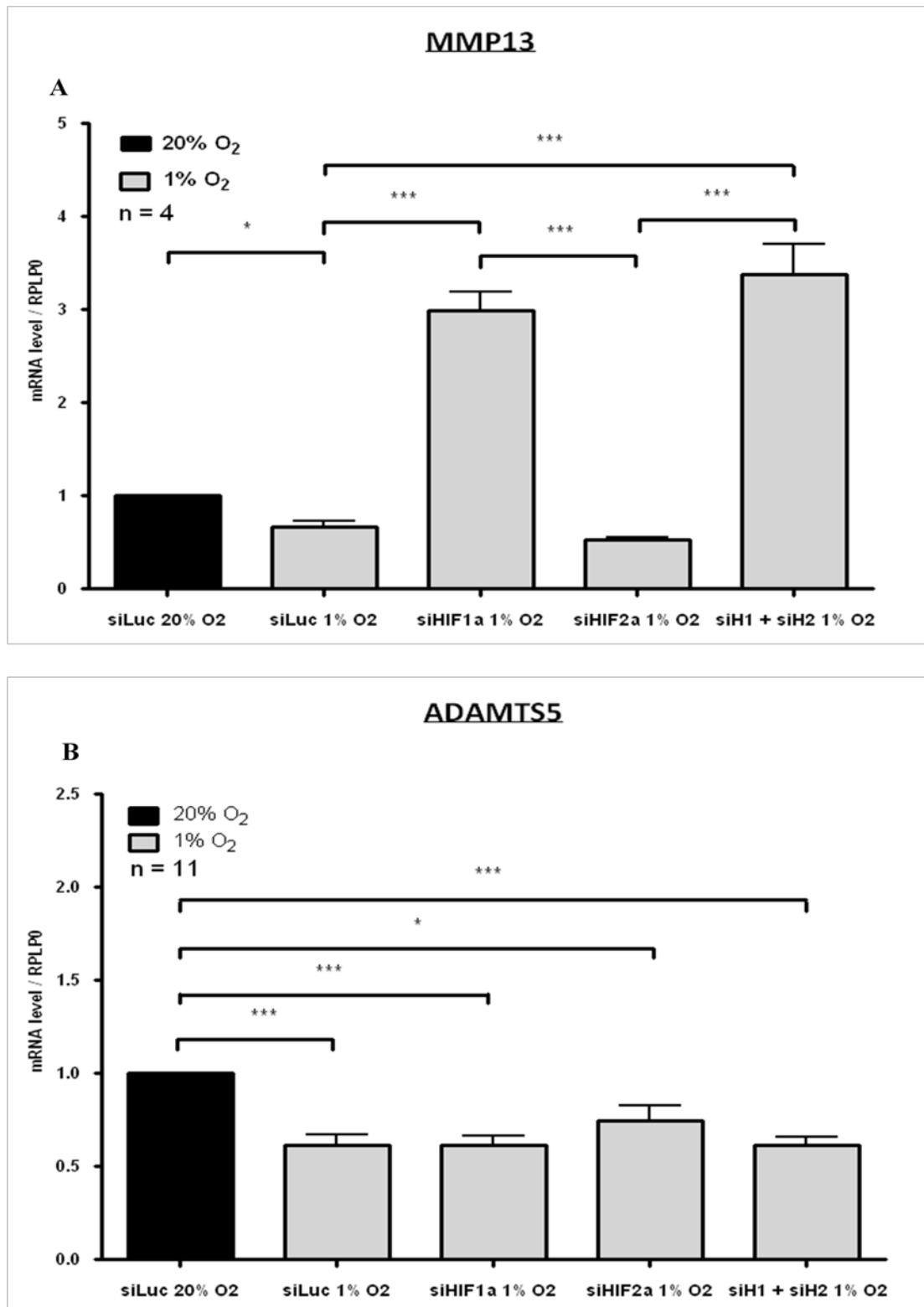


FIGURE 4.4. Role of HIF-1 α , but not HIF-2 α in hypoxic downregulation of MMP13. All data in the figure were obtained from passaged (P1-P3) HACs after 3 days in culture at either 20% or 1% oxygen. Cells were transfected 10nM siRNA against HIF-1 α (siHIF-1 α), HIF-2 α (siHIF-2 α) or both at the same time (siH1 + siH2). siRNA against luciferase was used as a control (siLuc). TaqMan Real-time PCR data showing the effect of HIF-1 α and HIF-2 α depletion on relative mRNA levels of *MMP13* (A) and *ADAMTS5* (B) under hypoxic conditions. Hypoxia induced downregulation of *MMP13* is HIF-1 α and not HIF-2 α dependent, while *ADAMTS5* downregulation was unaffected by depletion of either HIF isoform. Note: * $P < 0.05$; *** $P < 0.001$.

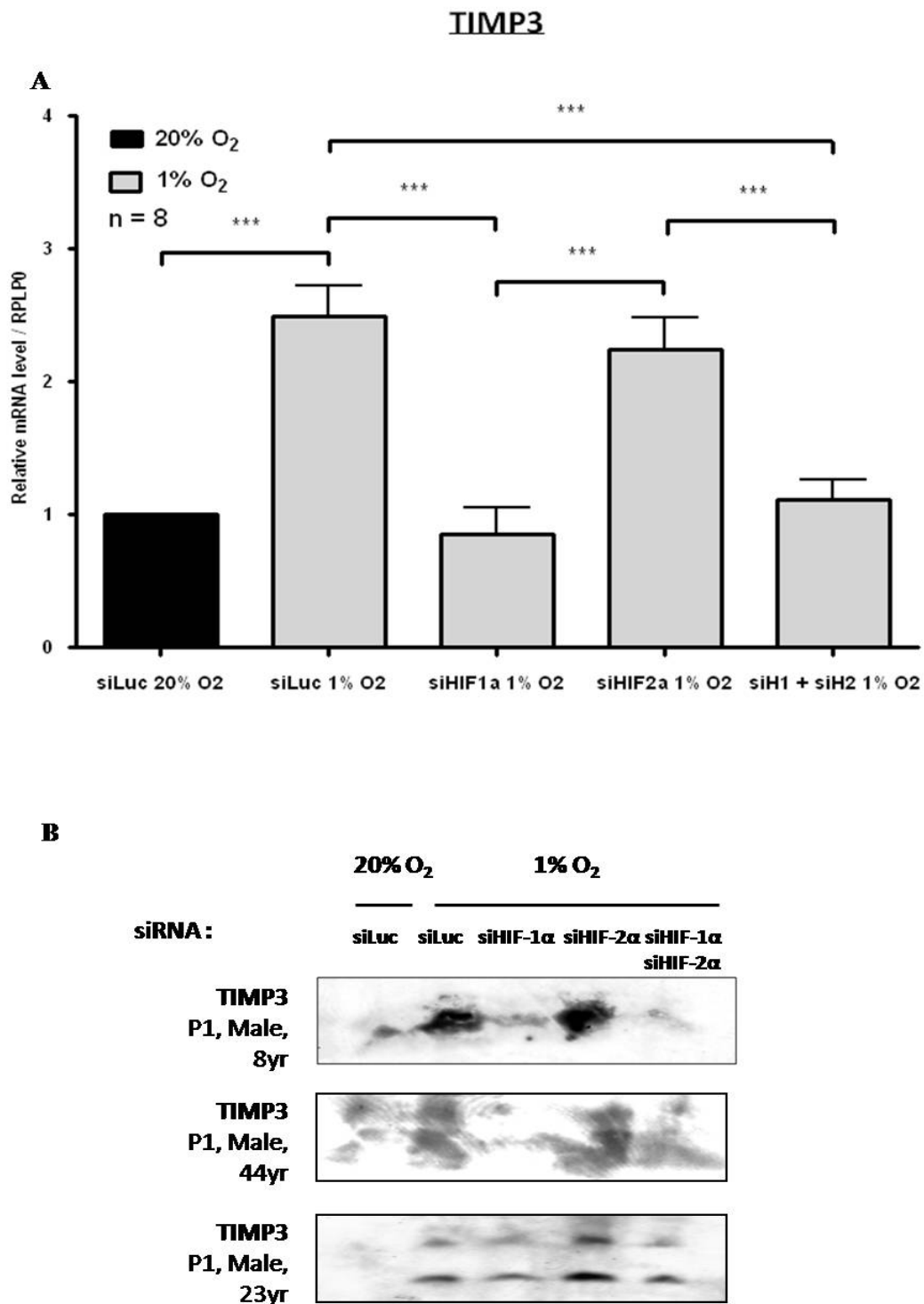
Figure 4.5

FIGURE 4.5. Hypoxic induction of TIMP3 is HIF-1 α , but not HIF-2 α dependent. All data in the figure were obtained from passaged (P1-P3) HACs after 3 days in culture at either 20% or 1% oxygen. Cells were transfected 10nM siRNA against HIF-1 α (siHIF-1 α), HIF-2 α (siHIF-2 α) or both at the same time (siH1 + siH2). siRNA against luciferase was used as a control (siLuc). TaqMan real-time PCR data showing the effect of HIF-1 α and HIF-2 α depletion under hypoxic conditions on mRNA levels of *TIMP3* (A) compared with normoxic control (siLuc 20%). Hypoxic upregulation of TIMP3 is HIF-1 α , and not HIF-2 α dependent. Western blotting of secreted TIMP3 confirmed mRNA data at the protein level (B).

TIMP3 mRNA expression was significantly increased in response to hypoxia (Fig. 4.5 A). This hypoxic induction was confirmed at the mRNA (Fig. 4.5 A) and protein level (Fig. 4.5 B). The hypoxic mRNA induction was not significantly affected by HIF-2 α depletion but abolished by HIF-1 α depletion (Fig. 4.5 A). This data was supported by western blot analysis and confirmed at the protein level from culture media of passaged HACs demonstrating that these anti-catabolic effects of hypoxia are HIF-1 α , and not HIF-2 α dependent (Fig. 4.5 B). Detection of *TIMP3* following HIF- α isoforms depletion was challenging and often *TIMP3* did not produce a clean band. However when considered with mRNA data this hints at a HIF-1 α dependence.

In summary, *MMP13* mRNA was downregulated and *TIMP3* upregulated by hypoxia in a HIF-1 α (not HIF-2 α) dependent manner. In fact, HIF-1 α depletion reversed the hypoxic downregulation of *MMP13* mRNA to levels exceeding normoxia suggesting functional HIF-1 α activity even in normoxic conditions. Surprisingly, *ADAMTS5* mRNA downregulation in response to hypoxia was independent of the HIF- α isoforms.

4.5 Regulation of catabolic mediators in human articular chondrocytes by hypoxia

In order to further investigate a possible anti-catabolic role of the HIF-signalling pathway, passaged HACs were transfected with siRNA against PHD2 or luciferase as a non-targeting control. Following transfection, passaged HACs were cultured for 3 days under normoxic (20% oxygen) or hypoxic (1% oxygen) conditions. The mRNA levels of catabolic enzymes *ADAMTS4*, *ADAMTS5*, *MMP1*, *MMP3*, *MMP13* as well as anti-catabolic enzymes *TIMP1*, *TIMP2* and *TIMP3* and a marker of chondrocyte hypertrophy *COL10A1* were analysed after normalising the data using the housekeeping gene *RPLP0* (Fig. 4.6. Fig. 4.7. Fig. 4.8). Consistent with our work in human cartilage explants we observed an anti-catabolic effect of hypoxia in isolated HACs. Hypoxia downregulated aggrecanase *ADAMTS5* expression (2 fold reduction) and collagenase *MMP13* expression (1.56 fold reduction) (Fig. 4.6 B. Fig. 4.7 C). Unlike in explants, *MMP3* was not found to be regulated by hypoxia (Fig. 4.7 B).

Depletion of PHD2 using siRNA enhanced the downregulation of *ADAMTS5* and *MMP13* mRNA expression. This pattern of hypoxic downregulation correlated with the pattern of HIF- α isoform stability. The knockdown of PHD2 under 20% oxygen downregulates *ADAMTS5* by 2.5 fold and *MMP13* by 2 fold (compared with control levels in normoxia). The knockdown of PHD2 in 1% oxygen tension enhances downregulation beyond hypoxic levels alone for *ADAMTS5* expression (downregulated 3.22 fold) and *MMP13* (3.22 fold) compared with control levels in normoxia (Fig. 4.6 B. Fig. 4.7 C). Depletion of PHD2 in 20% and 1% oxygen tension had no significant effect on *MMP3* mRNA expression (Fig. 4.7 B).

Consistent with our work in human cartilage explants catabolic regulators *TIMP1* and *TIMP2* were found not to be regulated by hypoxia (Fig. 4.8 A and B). This lack of a hypoxic induction of *TIMP1* was confirmed at the protein level by western blotting the culture medium (following acetone precipitation) (Fig. 4.8 A). *TIMP3*, a key regulator of matrix metalloproteinases was upregulated 2.5 fold at the mRNA level in response to hypoxia (Fig. 4.8 C). *TIMP3* was not detectable in HAC culture medium following acetone precipitation and western blotting. However, following treatment of HAC cultures with 200 μ g/ml heparin, hypoxic upregulation of *TIMP3* was confirmed at the protein level. Depletion of PHD2 in HACs upregulated *TIMP3* at the mRNA level, however this trend was found to be not statistically significant. Messenger RNA levels of a key marker of chondrocyte hypertrophy, *COL10A1* were not regulated by hypoxia and depletion of PHD2 did not have a significant effect on *COL10A1* mRNA levels (Fig. 4.8 D).

In summary, our work in HACs reinforces our findings in human cartilage explants, that is hypoxia has an anticatabolic effect, downregulating expression of key catabolic enzymes (*ADAMTS5* and *MMP13*) and upregulating essential catabolic inhibitor *TIMP3* which inhibits a broad spectrum of MMPs in addition to the aggrecanases *ADAMTS4* and *ADAMTS5*. The hypoxic upregulation of *TIMP3* was confirmed at both mRNA and protein level. The marker of chondrocyte hypertrophy *COL10A1* was found to be not regulated by hypoxia. The hypoxia-induced downregulation of key catabolic enzymes can be further enhanced by PHD2 enzyme depletion representing a further benefit of PHD2-specific inhibition with regard to cartilage maintenance and potentially repair.

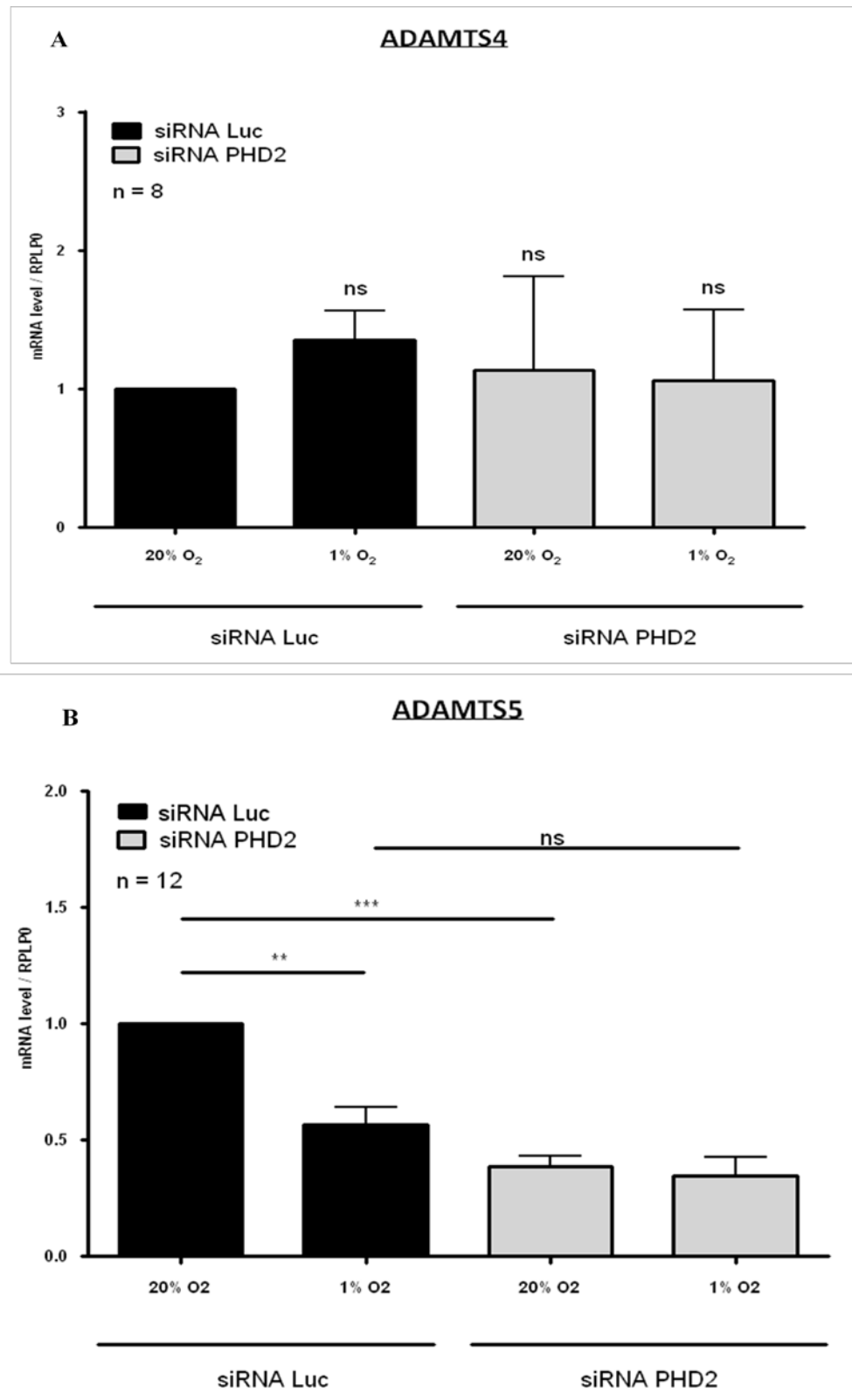


FIGURE 4.6. PHD2 depletion in HACs enhances hypoxic downregulation of aggrecanase ADAMTS5 at the mRNA level. All data in the figure were obtained from passaged HACs after 3 days in culture at either 20% or 1% oxygen. Cells were transfected with 10nM siRNA against PHD2 (siPHD2), or, as a control against luciferase (siLuc). TaqMan real-time PCR data showing mRNA expression of key aggrecanase enzymes ADAMTS4 (A) and ADAMTS5 (B). ADAMTS5 mRNA is downregulated in response to hypoxia and this hypoxic downregulation is further enhanced by PHD2 depletion. Note: ns – not significant; ** $P < 0.01$; *** $P < 0.001$.

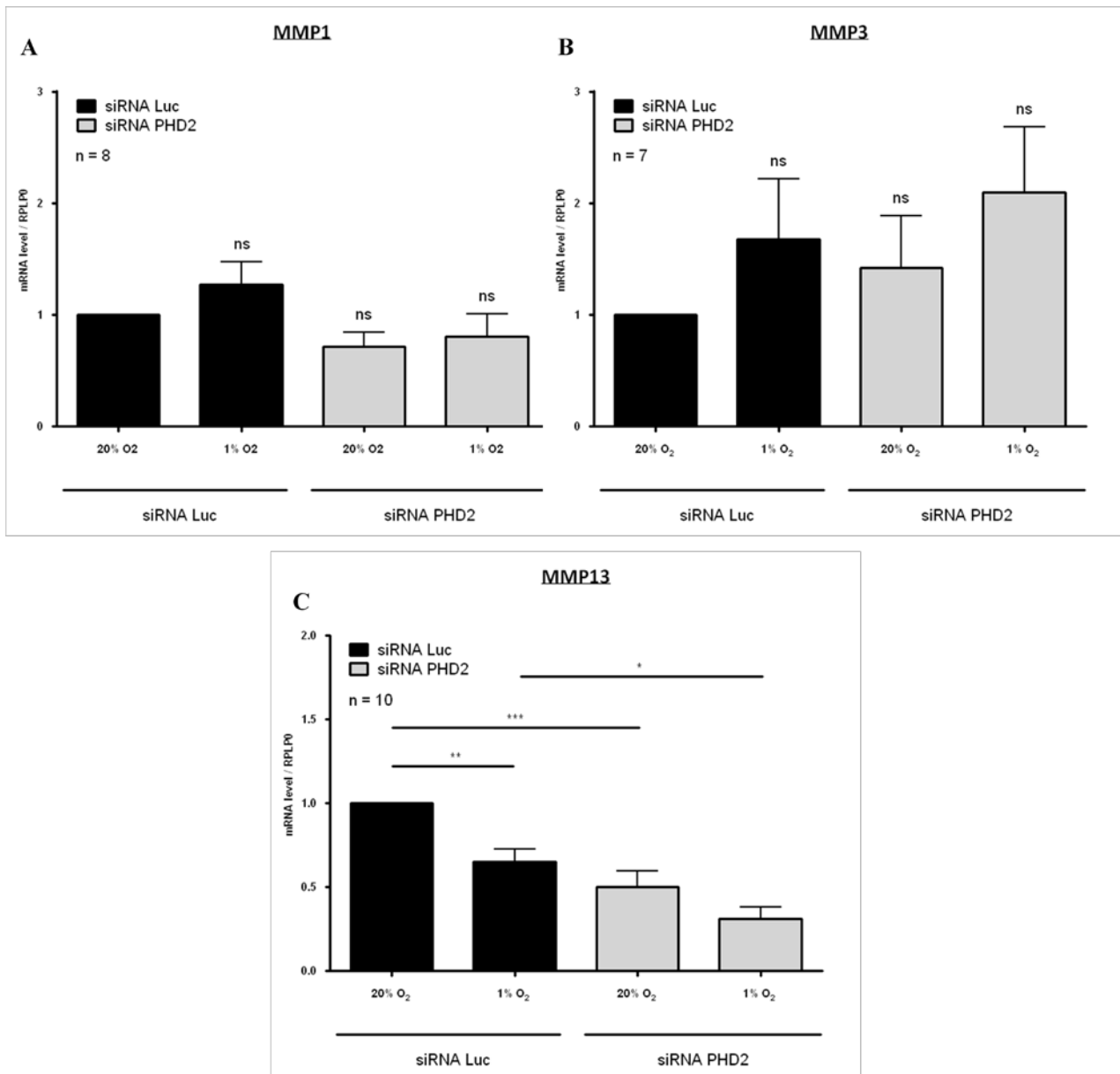


FIGURE 4.7. PHD2 depletion in HACs enhances hypoxic downregulation of key catabolic enzyme MMP13 at the mRNA level. All data in the figure were obtained from passaged HACs after 3 days in culture at either 20% or 1% oxygen. Cells were transfected with 10nM siRNA against PHD2 (siPHD2), or, as a control against luciferase (siLuc). TaqMan real-time PCR data showing mRNA expression of matrix metalloproteinase enzymes *MMP1*, *MMP3* and *MMP13* (A, B and C, respectively). *MMP13* mRNA levels are downregulated in response to hypoxia and this hypoxic downregulation is further enhanced by PHD2 depletion. Note: ns – not significant; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

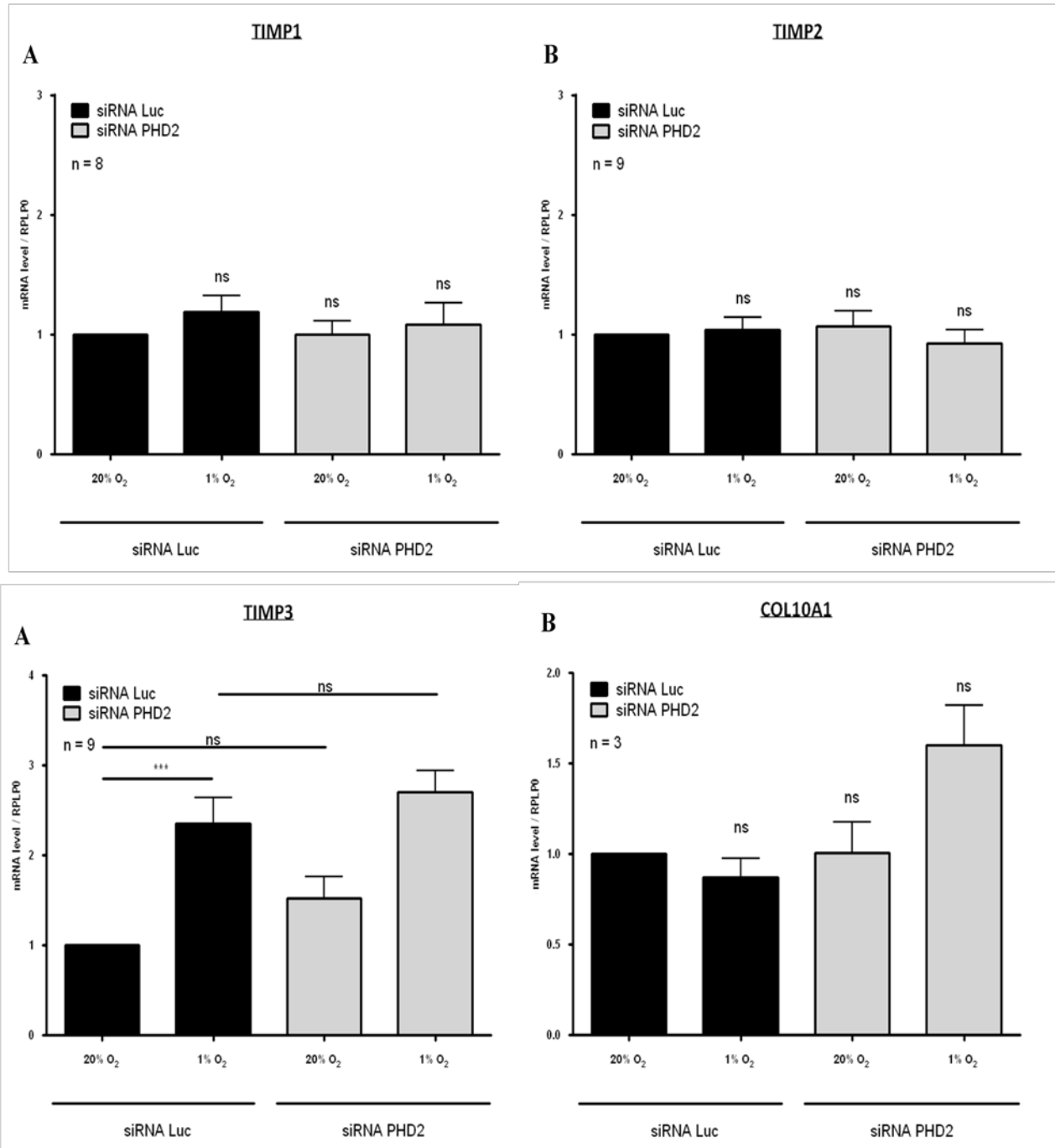


FIGURE 4.8. Hypoxia upregulates catabolic regulator TIMP3 and does not regulate hypertrophy marker COL10A1 at the mRNA level. All data in the figure were obtained from passaged HACs after 3 days in culture at either 20% or 1% oxygen. Cells were transfected with 10nM siRNA against PHD2 (siPHD2), or, as a control against luciferase (siLuc). TaqMan real-time PCR data showing relative mRNA levels of catabolic regulators *TIMP1*, *TIMP2*, *TIMP3* (A, B and C, respectively) and marker of chondrocyte hypertrophy *COL10A1* (D). Hypoxia significantly increases mRNA levels of TIMP3, although PHD2 depletion did not have an effect. Note: ns – not significant; *** $P < 0.001$.

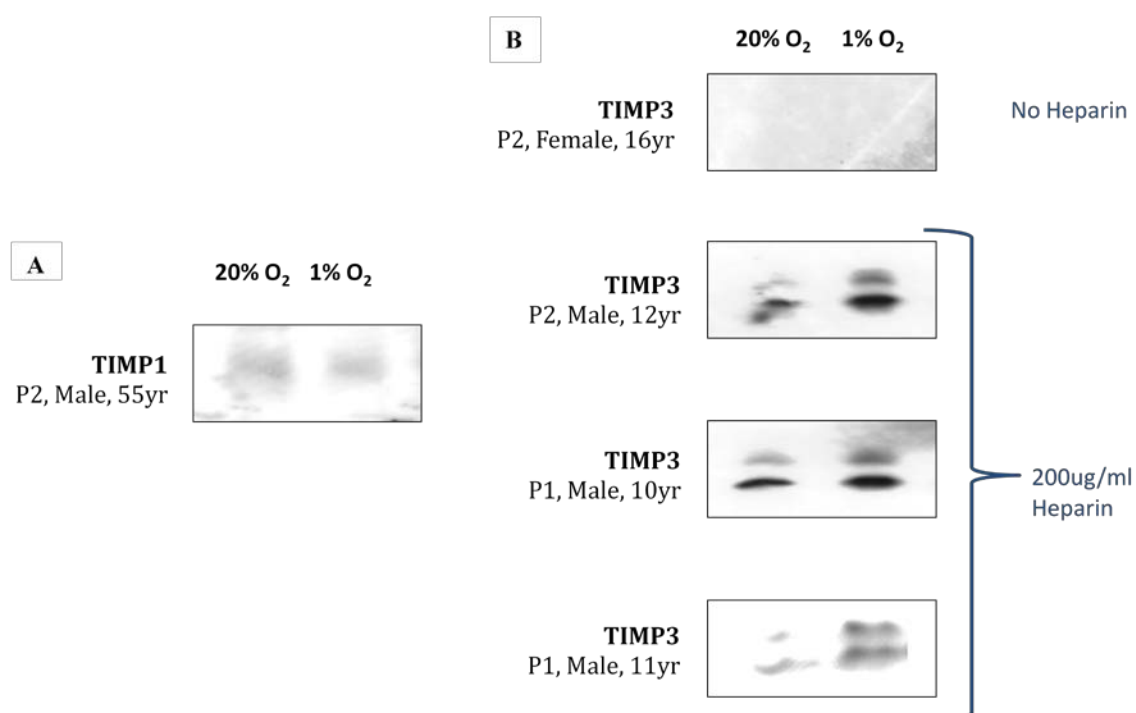


FIGURE 4.9. **Hypoxia enhances TIMP3 secretion in HACs.** Data are from passaged (P1-P3) HACs after culture for 3 days at 20% oxygen or 1% oxygen. Secreted TIMP1 (A) and TIMP3 (B) was detected following acetone precipitation of culture media. Cell culture medium was supplemented with 200µg/ml heparin in order to detect TIMP3. Data were normalised based on Bradford measurements of protein content of cell layers.

4.6 IL-1 α stimulation stabilises HIF-1 α but not HIF-2 α in human articular chondrocytes

Passaged human articular chondrocytes from a range of patients were treated with 10ng/ml IL-1 α or vehicle (serum free media) and cultured under normoxic (20% oxygen) or hypoxic (1% oxygen) conditions for 24 hours. In HACs cultured under normoxic conditions IL-1 α stimulation resulted in HIF-1 α protein stabilisation. There was some variability between patients, but generally, levels of HIF-1 α protein were greater than or roughly equal with unstimulated hypoxic levels (1% oxygen). HACs cultured at 1% oxygen and stimulated with IL-1 α could achieve levels of HIF-1 α above those in hypoxic alone (Fig. 4.10). This suggests IL-1 α stimulation may be able to stabilise HIF-1 α isoforms using a mechanism independent of inhibiting HIF- α -prolyl hydroxylation. In stark contrast, IL-1 α stimulation of HACs cultured under normoxia did not result in HIF-2 α stabilisation and IL-1 α stimulation under hypoxia actually decreased HIF-2 α protein levels compared to hypoxia alone (Fig 4.11).

In a series of timecourse experiments HACs were cultured under normoxia for 2 days before being exposed to 10ng/ml IL-1 α and lysed at 0-, 1-, 3-, 6-, 12- and 24- hours. The rate of HIF-1 α stabilisation varied between patients to some extent but in all cases IL-1 α stimulation resulted in a transient HIF-1 α stabilisation peaking between 6 to 12 hours and significantly reduced at 24 hours (the final timepoint of the experiment) (Fig 4.12. A. Fig. 4.13. A). In contrast, IL-1 α stimulation in HACs cultured under normoxia did not result in HIF-2 α stabilisation (Fig. 4.12. B. Fig. 4.13. B). The mRNA levels of *HIF-1 α* and *HIF-2 α* were also analysed after normalising the data using the housekeeping gene *RPLP0*. We observed that IL-1 α stimulation resulted in increased levels of *HIF-1 α* mRNA, although the time-course was different to the HIF-1 α protein response, since mRNA levels continued to rise throughout the experimental period. Again the HIF-2 α response was very different to HIF-1 α since HIF-2 α mRNA levels were completely unaffected by IL-1 α stimulation (Fig. 4.14 + Fig. 4.15).

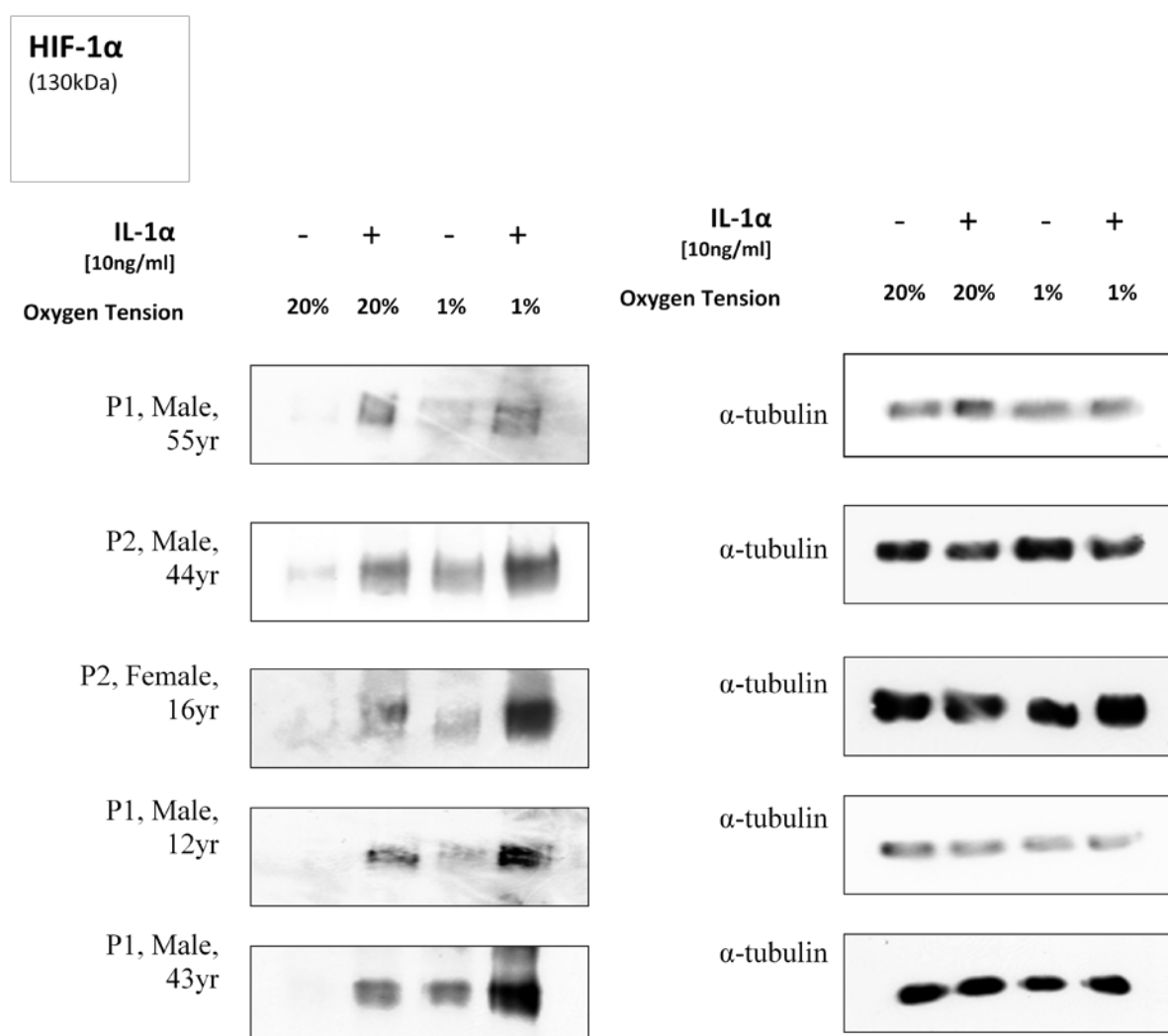


FIGURE 4.10. IL-1 α stimulation stabilises HIF-1 α in human articular chondrocytes. Passaged (P1-P2) HACs from 5 different donors were stimulated with either IL-1 α [10ng/ml] or vehicle (serum free media) and incubated at either 20% or 1% oxygen tension for 24 hours. IL-1 α stabilises HIF-1 α protein even under normoxic conditions to levels greater than or roughly equal with unstimulated hypoxic control (1% oxygen). HACs cultured at 1% oxygen and stimulated with IL-1 α has higher levels of HIF-1 α than hypoxia alone.

HIF-2 α

(118kDa)

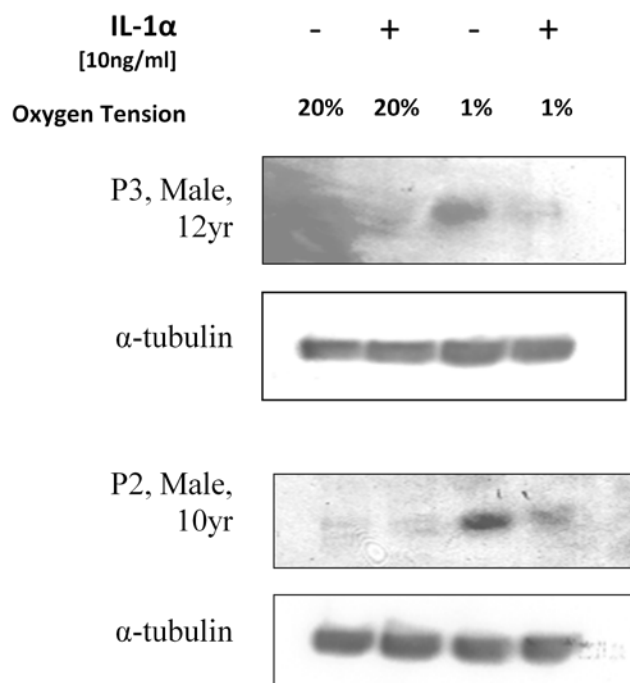


FIGURE 4.11. IL-1 α stimulation does not stabilise HIF-2 α in human articular chondrocytes under normoxic conditions and may decrease protein levels in hypoxia. Passaged (P2-P3) HACs from 2 different donors were stimulated with either IL-1 α [10ng/ml] or vehicle (serum free media) and incubated at either 20% or 1% oxygen tension for 24 hours. IL-1 α stimulation of HACs cultured under normoxia did not result in HIF-2 α stabilisation and IL-1 α treatment under hypoxia appeared to result in reduced HIF-2 α levels.

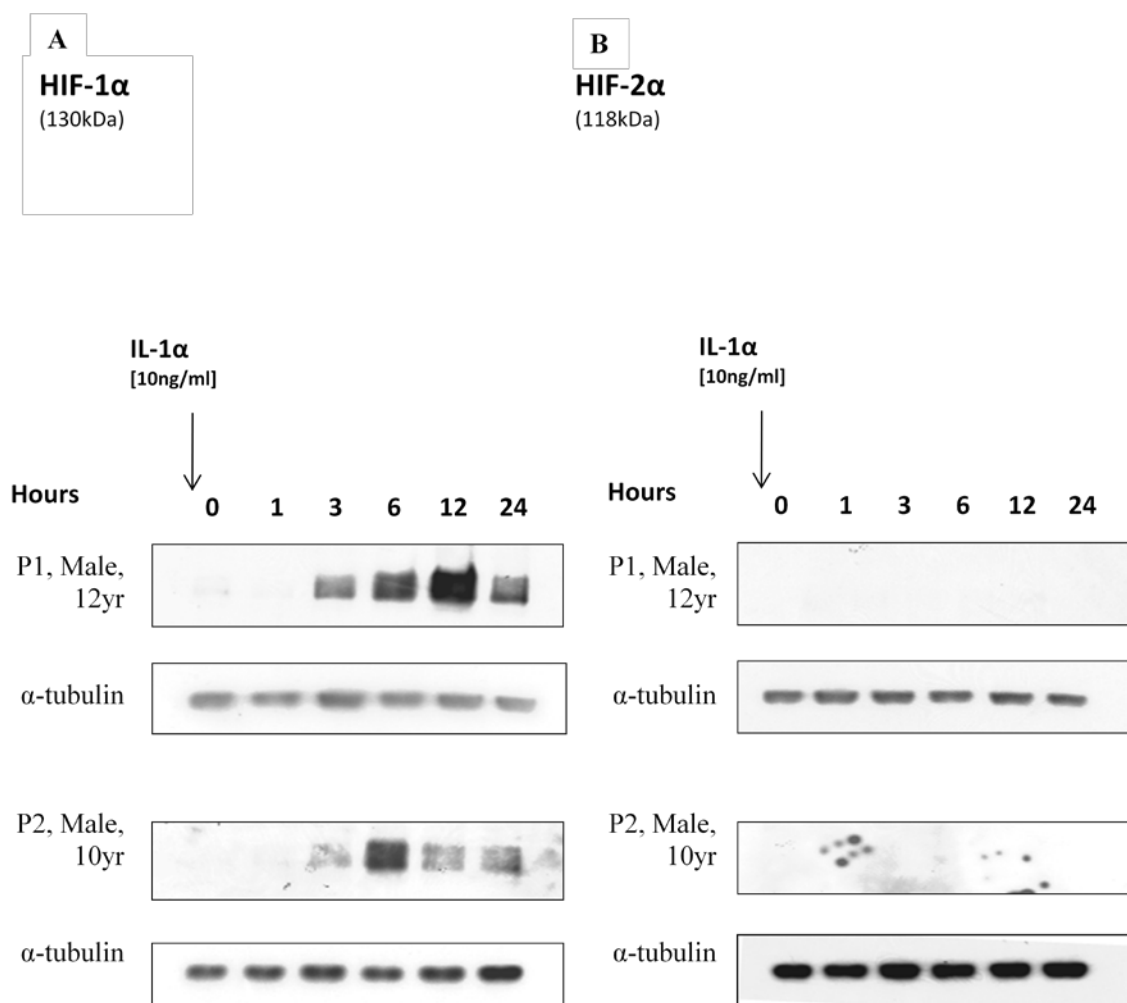


FIGURE 4.12. IL-1α stimulation stabilises HIF-1α in human articular chondrocytes peaking at between 6 and 12 hours but does not affect HIF-2α levels. In a timecourse experiment passaged (P1-P2) HACs were cultured under normoxia for 2 days before being exposed to 10ng/ml IL-1α and lysed at 0, 1, 3, 6, 12 and 24 hours thereafter. The timepoint of peak HIF-1α levels varied between donors but in all cases IL-1α stimulation resulted in HIF-1α stabilisation peaking between 6-12 hours (A). In contrast IL-1α stimulation in HACs cultured under normoxia did not result in any detectable HIF-2α stabilisation (B).

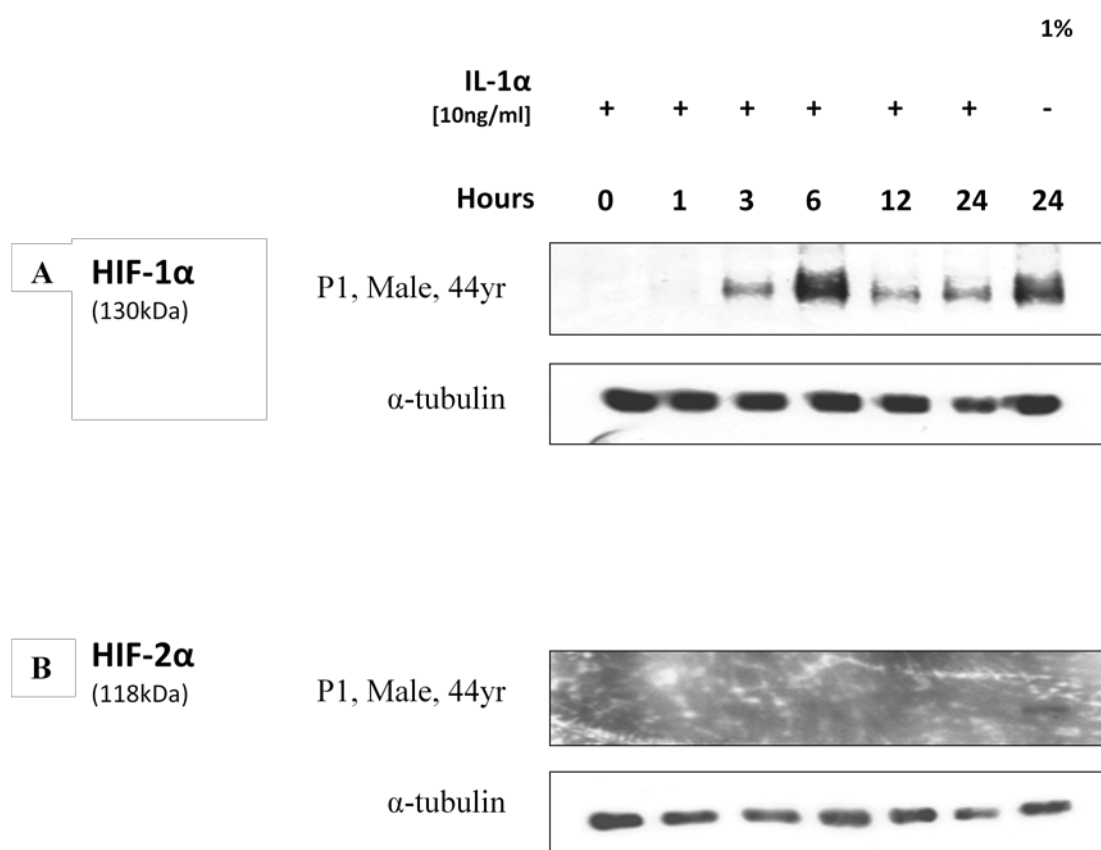


FIGURE 4.13. IL-1 α treatment (in normoxia) stabilises HIF-1 α , but not HIF-2 α in human articular chondrocytes. In a timecourse experiment passaged HACs were cultured under normoxia for 2 days before being exposed to 10ng/ml IL-1 α and lysed at 0, 1, 3, 6, 12 and 24 hours thereafter. A 24 hour hypoxia control was added to this experiment. Consistent with previous experiments IL-1 α stimulation resulted in HIF-1 α stabilisation peaking at 6 hours. This peak in HIF-1 α levels was higher than that achieved after 24 hours in hypoxia (last lane) (A). In contrast, unlike hypoxia treatment, IL-1 α stimulation in HACs cultured under normoxia did not result in HIF-2 α stabilisation (B).

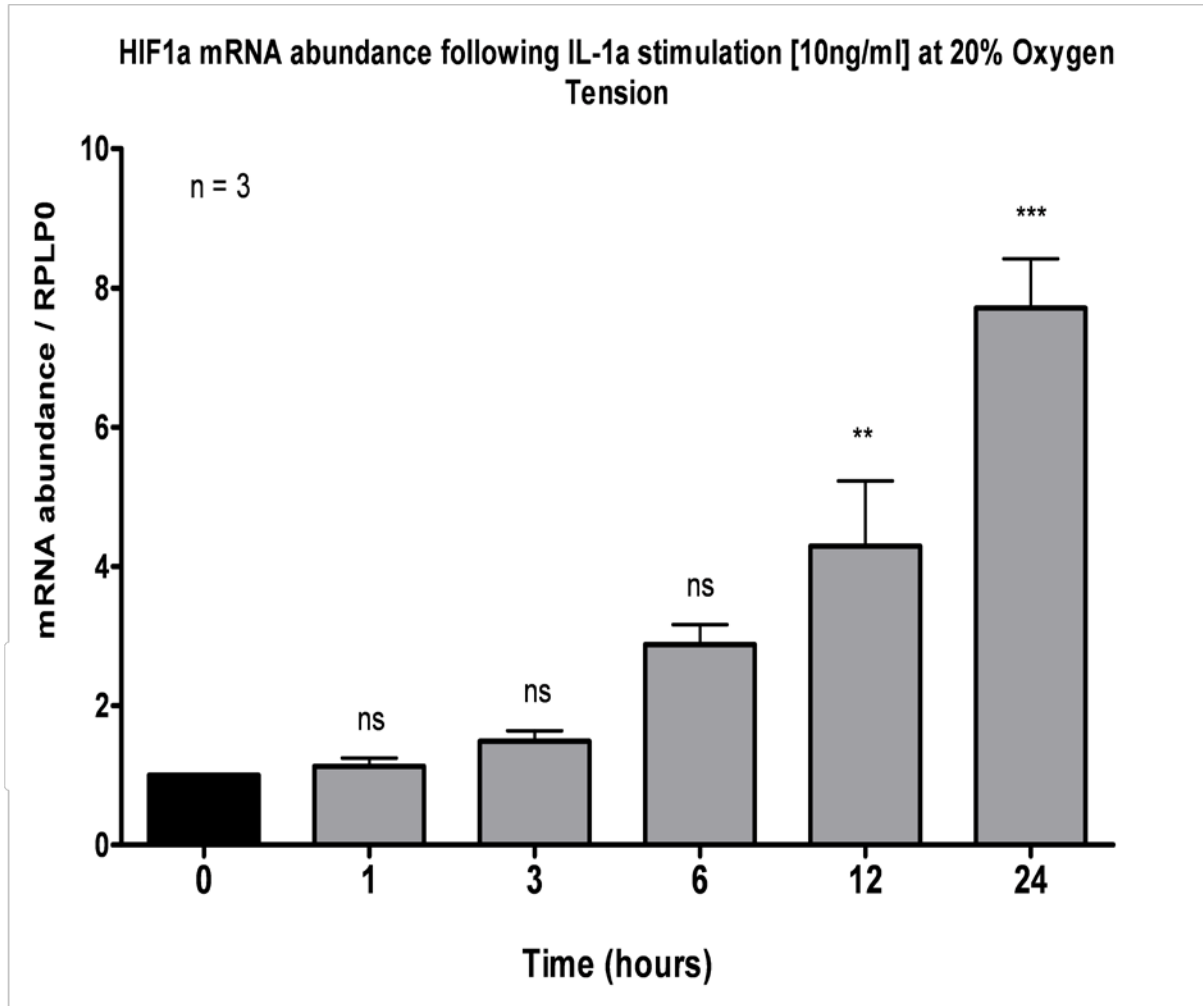


FIGURE 4.14. IL-1 α stimulation increases HIF-1 α mRNA levels in human articular chondrocytes. In a timecourse experiment passaged (P2-P3) HACs were cultured under normoxia for 2 days before being exposed to 10ng/ml IL-1 α and lysed at 0, 1, 3, 6, 12 and 24 hours thereafter. TaqMan PCR data showing relative levels of mRNA for *HIF-1 α* over the timecourse. All values were normalised using housekeeping gene *RPLP0* and represent mean and SEM results of 3 independent experiments (i.e., cells from 3 different donors). IL-1 α stimulation in HACs increased *HIF-1 α* steady state mRNA levels over the 24 hour period. Note: ns – not significant; ** P < 0.01; *** P < 0.001.

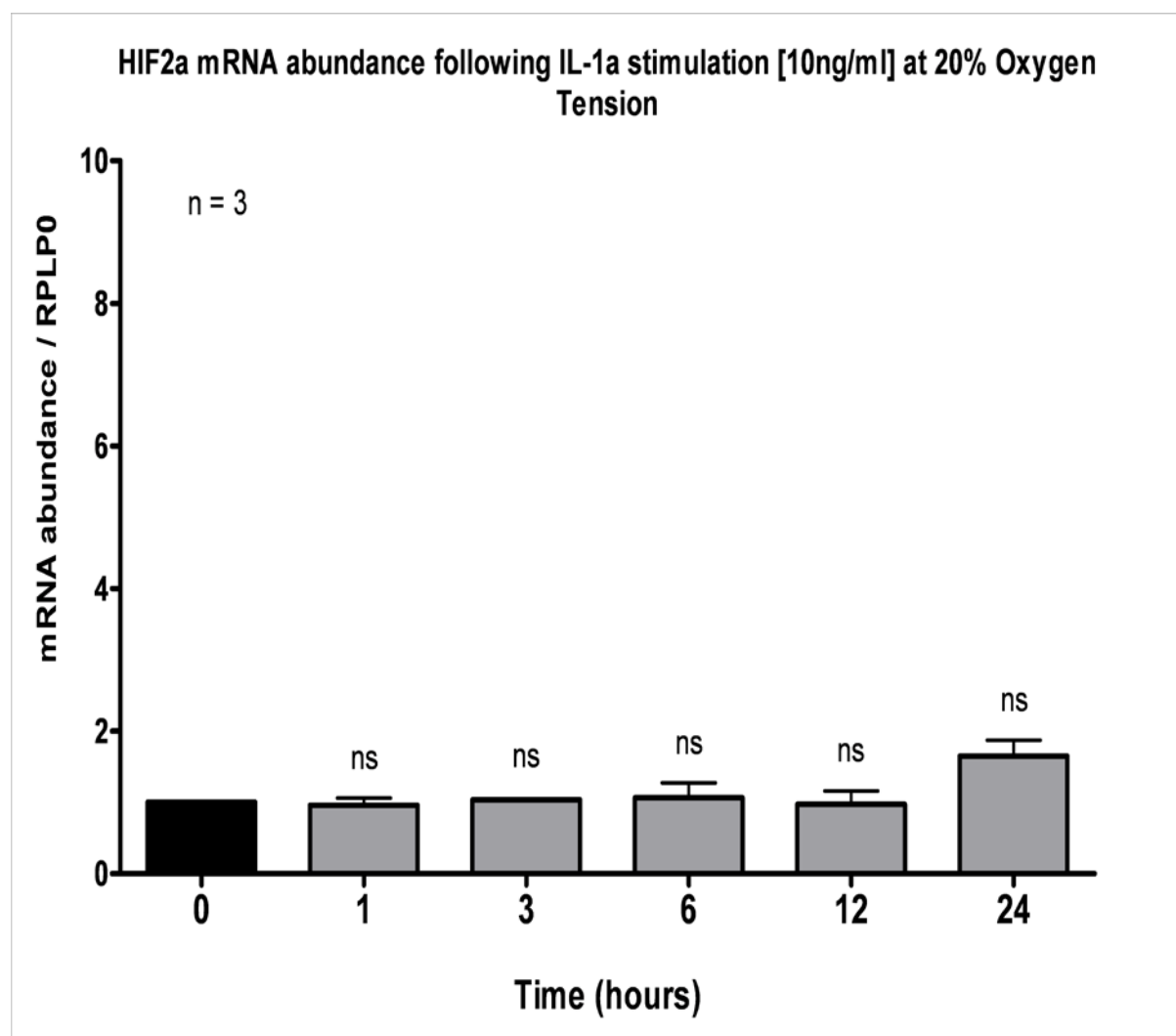


FIGURE 4.15. IL-1 α treatment has no effect on HIF-2 α mRNA steady state levels in human articular chondrocytes. In a timecourse experiment passaged (P2-P3) HACs were cultured under normoxia for 2 days before being exposed to 10ng/ml IL-1 α and lysed at 0, 1, 3, 6, 12 and 24 hours thereafter. TaqMan PCR data showing relative levels of mRNA for *HIF-2 α* over the timecourse. All values were normalised using those obtained from the *RPLP0* housekeeping gene and represent mean and SEM results of 3 independent experiments (i.e., cells from 3 different donors). Note: ns, not significant.

In order to further probe how HIF-1 α might be regulated by IL-1 α we monitored levels of HIF-targeting PHD2 protein (Fig. 4.16), which we previously identified as the dominant regulator of HIF- α isoforms in HACs. IL-1 α stimulation of HACs had no effect on the levels of PHD2 protein and although we haven't directly assessed PHD activity it seems likely that an alternative mechanism of HIF-1 α stabilisation independent of the PHDs is responsible for the IL-1 α response. Since there was a difference in the steady state mRNA levels of HIF-1 α in response to IL-1 α there may be transcriptional or mRNA stability regulation of HIF-1 α by IL-1 α .

It has been previously demonstrated in mouse macrophages that the p38 MAP kinase pathway regulates mRNA stability via both the expression and post-translational modification of Tristetraporolin (TTP) (Mahtani et al., 2001). TTP is a well known regulator of adenosine/uridine-rich element (ARE) containing mRNAs and controls target gene stability by binding to the 3'-untranslated region (3'-UTR) containing ARE motifs and recruiting factors that mediate decapping, deadenylations and mRNA destruction. Importantly, TTP is in an active dephosphorylated state in unstimulated HAC. However, in response to IL-1 α stimulation TTP is phosphorylated and inactivated (serines 52 and 178) through the p38 pathway and MAPK-activated protein kinase 2 (MK2) allowing the accumulation of ARE-containing mRNAs.

In order to further investigate these findings HACs from a range of patients were pre-incubated with either p38 MAP kinase inhibitor SB202190 or vehicle (DMSO) 1 hour before being exposed to 10ng/ml IL-1 α and lysed after 6 hours. IL-1 α stimulation upregulates *HIF-1 α* mRNA 2.41 fold (Fig. 4.17 A), this value is consistent with levels from earlier timepoint experiments (Fig. 4.15) and HIF-1 α stabilisation is detectable at the protein level. Treatment with 2 μ M SB202190 inhibits IL-1 α induced increase in *HIF-1 α* gene expression and reduces HIF-1 α stabilisation at the protein level (although does not totally abolish it) (Fig. 4.17 A and B). This data suggests that the activation of the p38 pathway is necessary for the stabilisation of HIF-1 α , however because we did not check the stability of HIF-1 α mRNA we cannot confirm that this effect is mediated by TTP inactivation and promoting HIF-1 α mRNA stability and not p38 MAP kinase directly/indirectly increasing HIF-1 α transcription.

This implications of these findings will be further discussed in the subsequent section.

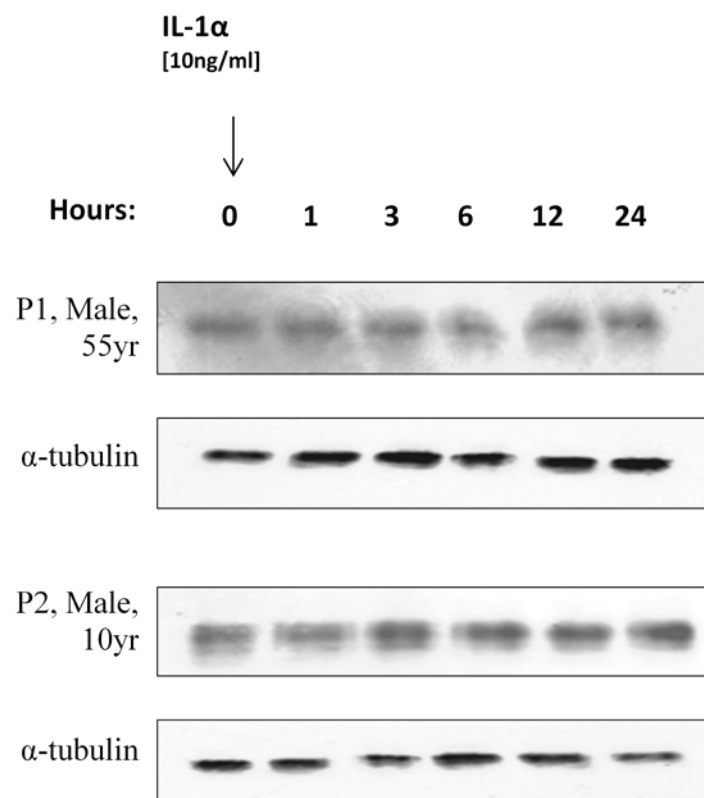
PHD2
(46kDa)

FIGURE 4.16. IL-1 α stimulation does not alter protein levels of PHD2, the dominant HIF- α regulator in human articular cartilage. In a timecourse experiment passaged (P1-P2) HACs from 2 donors were cultured in 20% oxygen for 2 days before being exposed to 10ng/ml IL-1 α and lysed at 0, 1, 3, 6, 12 and 24 hours thereafter. Western blotting was performed to study PHD2 protein levels and there appears to be no significant affect on PHD2 levels in response to IL-1 α .

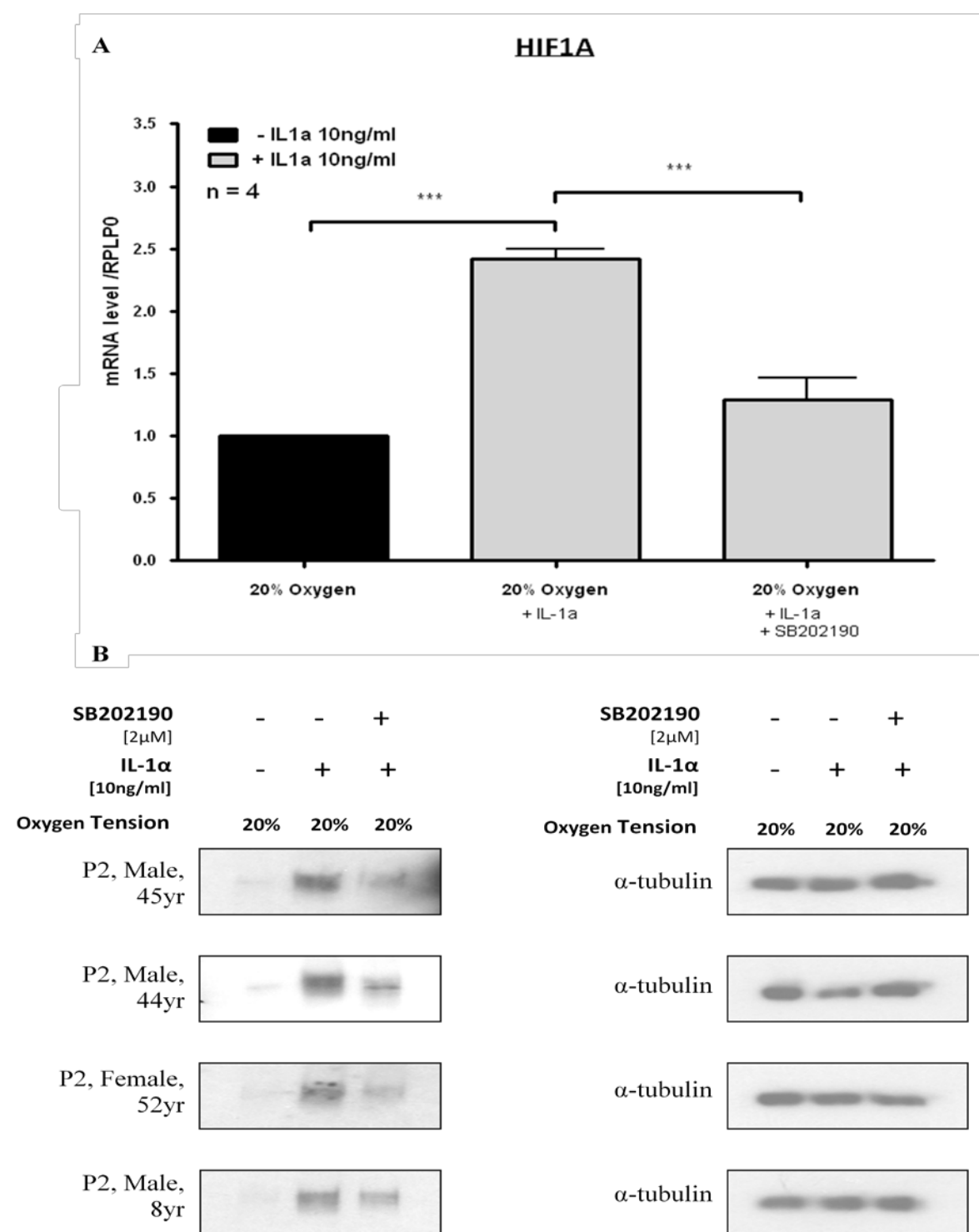


FIGURE 4.17. IL-1 α induced increase in HIF-1 α mRNA and protein levels are partially p38 MAP kinase dependent. Passaged (P2) HACs from 4 different donors were pre-incubated with either 2 μ M p38 MAP kinase inhibitor SB202190 or vehicle (0.1% DMSO) 1 hour before being exposed to 10ng/ml IL-1 α and lysed after 6 hours. TaqMan PCR data showing relative levels of mRNA for *HIF-1 α* 6 hours after IL-1 α treatment (A). Preincubation with 2 μ M SB202190 partially inhibits the IL-1 α induced increase in *HIF-1 α* expression. This was confirmed at the protein level by western blotting (B). Note: ***P < 0.001.

4.7 Discussion

We have identified PHD2 as the key hydroxylase in HACs responsible for regulating HIF-1 α and HIF-2 α . We have shown that specific inhibition of PHD2 promotes the differentiated phenotype and enhances cartilage matrix production by HACs both in normoxia and hypoxia.

Here we report the benefit of hypoxia, namely the stabilisation of HIF- α isoforms inhibits IL-1 α induced aggrecan cleavage in human cartilage explants. Furthermore, hypoxia suppresses the endogenous release of aggrecan fragments in unstimulated cartilage suggesting that hypoxia has an anti-catabolic effect in cartilage is able to reduce matrix degradation. In order to investigate the anticatabolic effect we monitored expression of a number of genes and found that the stabilisation of HIF- α isoforms in human articular cartilage explants has an anti-catabolic effect downregulating mRNA expression of several catabolic proteases associated with cartilage matrix degradation and upregulating anti-catabolic inhibitor tissue inhibitor of metalloproteinases 3 (TIMP3). This result in human tissue (cartilage explants) was replicated in HACs (monolayer cultures) and using an RNAi approach we provide clear evidence that hypoxia and specifically the stabilisation HIF-1 α (not HIF-2 α) has an anti-catabolic effect in human articular cartilage. Furthermore, we show that depletion of PHD2 enhances this anti-catabolic effect in both normoxia and hypoxia. Finally, stimulation with IL-1 α stabilises HIF-1 α but decreases HIF-2 α in HACs. Data suggests that this may be partly mediated at the transcriptional level and would seem to represent a different mechanism than hypoxia of HIF regulation.

4.7.1 Hypoxia has an anti-catabolic effect and this is enhanced by PHD2 depletion

Proinflammatory cytokine interleukin 1 (IL-1) has been shown to upregulate catabolic enzymes (MMPs and ADAMTSs), increase inducible nitric oxide synthase (iNOS) expression and suppress the synthesis of aggrecan and type II collagen in chondrocytes (Bocquet et al., 1986; Kobayashi et al., 2005; Pasternak et al., 1986). The stimulation of cartilage explants with IL-1 induces the rapid depletion of proteoglycans in cartilage explant cultures and is chondrocyte dependent (Flannery et al., 1999; Gebauer et al., 2005; Saklatvala, 1987; Vincenti and Brinckerhoff, 2002). Here we report that although there was

some variability between patients, human cartilage explants exposed to hypoxia are more resistant to IL-1 α induced aggrecan cleavage than normoxic controls providing evidence of the anti-catabolic and chondroprotective effect of hypoxia. The anti-catabolic effect of hypoxia in response to IL-1 α stimulus is striking, however, IL-1 is mainly produced by macrophages and although it has been reported that chondrocytes have the capacity to synthesis IL-1 it remains controversial whether it can be produced in amounts necessary for pathogenesis (Gruber et al., 2004).

The addition of IL-1 α is a harsh inflammatory stimulus used to trigger aggrecan release and whilst a useful experimental tool it may not be an accurate representation of the physiological environment in cartilage. Therefore unstimulated cartilage was also cultured overnight in either 20% oxygen or 1% oxygen in order to provide data that could more easily be extended to healthy human articular cartilage. Assuming that chondrocytes experience physiological oxygen tensions ~6-1% oxygen, 1% oxygen would be representative of the microenvironment at the core of the tissue. Here we report that hypoxia suppresses the release of endogenous aggrecan fragments in unstimulated cartilage suggesting that hypoxia has an anti-catabolic effect in cartilage is able to reduce matrix degradation even in normal (unstimulated) tissue. It is also possible to suggest that supra-physiological, (20% oxygen), conditions has a catabolic effect on cartilage explants increasing the rate of cartilage degradation. Unlike bone, which undergoes perpetual remodelling, articular cartilage is believed to have a very low rate of matrix turnover. Our work is consistent with this finding as there are very low levels of aggrecan ARGSV neoepitope release in unstimulated 1% oxygen (physiological levels).

We suggest that this anti-catabolic hypoxic effect on human cartilage may be mediated by the downregulation of key catabolic enzymes responsible for matrix turnover (ADAMTS5 and MMP13) and the upregulation of the metalloproteinase inhibitor TIMP3. As we have previously demonstrated in our previous chapter (Chapter 3), the beneficial effects of HIF- α stabilisation in chondrocytes can be enhanced above physiological maximum levels (1% oxygen). This will be further discussed below.

The ADAMTS's (A Disintegrin And Metalloproteinase with Thrombospondin Motifs) are a family of 19 metalloproteinases with sequence homology to ADAM protease

but which lack the transmembrane domain, having at least one thrombospondin type 1 sequence motif (TSR) familiar with extracellular matrix proteins. MMP and ADAMTS generated neopeptide aggrecan fragments have been identified in culture media of cartilage explants *in vitro* as well as human osteoarthritic synovial fluid suggesting elevated aggrecanase/MMP levels or activity *in vivo* (Fosang et al., 1996; Lark et al., 1997; Little et al., 2002; Lohmander et al., 1993; Sandy et al., 1992; Sandy and Verscharen, 2001). Little and colleagues generated a line of aggrecan knockin mice with a mutation between the G1 and G2 globular domains, this mutation rendered aggrecan resistant to aggrecanase activity. It was reported that knockin mutation significantly inhibited proteoglycan loss, diminished cartilage degradation and promoted cartilage repair (Little et al., 2007). Adamts5 knockout mice are significantly resistant to IL-1 α + retinoic acid induced aggrecan cleavage as well as surgically induced cartilage destruction, compared with wild-type mice (Glasson et al., 2005). Adamts5 knockout mice are significantly resistant to IL-1 α + retinoic acid induced aggrecan cleavage as well as surgically induced cartilage destruction, compared with wild-type mice (Glasson et al., 2005). Resistance to the progression of osteoarthritis was not reported in Adamts4 knockout mice after surgical induction of joint instability and therefore Adamts5 is believed to be the main aggrecanase in mouse (Glasson et al., 2004). However Song and colleagues demonstrated that the depletion of either ADAMTS5 or ADAMTS4 using siRNA in human cartilage explants inhibits proteoglycan release when explants were stimulated with tumour necrosis factor α (TNF- α) and oncostatin M (OSM). This suggests that despite the dominant role of Adamts5 in mouse both ADAMTS4 and ADAMTS5 may contribute towards cartilage destruction in human osteoarthritis (Song et al., 2007).

MMP13 is a matrix metalloproteinase thought to play a critical role in cartilage destruction. MMP13 is more active against type II collagen than other collagenases and chondrocytes' expression of MMP13 mRNA is increased in late-stage human osteoarthritis (OA) cartilage in association with cartilage erosion (Aigner et al., 2001; Minond et al., 2006). It has been previously suggested that one of the earliest events associated with OA is the loss of aggrecan core protein from the cartilage extracellular matrix and this is a prerequisite for the subsequent proteolysis of collagen fibres. Because chondrocytes continuously resynthesise aggrecan, this first step is potentially reversible. The second step is considered permanent because, despite repair attempts, the original fibrous collagen network cannot be restored (Mankin and Lippiello, 1970; Mort and Billington, 2001). Neuhold and colleagues

used a tetracyclin-induced cartilage specific promoter to constitutively overexpress active human MMP13 in hyaline cartilage of transgenic mice. Overexpression resulted in focal OA cartilage pathology at load bearing sites similar to human OA including proteoglycan and type II collagen degradation (Neuhold et al., 2001). Little and colleagues demonstrated that the cartilage of *Mmp13*^{-/-} mice is protected from structural cartilage damage in a surgically induced model of OA, however there was no significant reduction in aggrecanolysis, osteophyte development or chondrocyte hypertrophy (Little et al., 2009). *MMP13*^{-/-} mice were able to inhibit cartilage degradation in the presence of aggrecan loss, this may suggest that aggrecan depletion on its own does not drive cartilage degradation.

In summary, ADAMTS5 and MMP13 are catabolic enzymes clearly associated with cartilage destruction and potentially key to the chronic disease of osteoarthritis in humans.

Four TIMPs have been identified (TIMP1, 2, 3, 4), they are synthesised by chondrocytes and are endogenous inhibitors of MMP and to a lesser extent ADAMTS activity. TIMP1, 2, 3 and 4 all broadly inhibit all MMPs, with different effectiveness (Baker et al., 2002). TIMP3 has been reported to inhibit all MMPs, in addition to ADAM-17, ADAMTS4 and ADAMTS5. The K_i of TIMP3 for ADAMTS4 and ADAMTS5 is in the subnanomolar range (Hashimoto et al., 2001; Kashiwagi et al., 2001). In addition TIMP3 is able to bind to polyanionic matrix components allowing for localisation in the cartilage extracellular matrix. It has been reported that TIMP3 inhibits aggrecanase activity *in vitro* when added exogenously to IL-1 α or retinoic acid stimulated bovine and porcine cartilage explants suppressing aggrecan neoepitope release (Gendron et al., 2003). Finally, *Timp3*^{-/-} mice have increased proteinase activity and spontaneously development cartilage degradation illustrating the important role of TIMP3 in regulating extracellular matrix homeostasis (Sahebjam et al., 2007).

In the present study we reported that hypoxia inhibits aggrecan cleavage in IL-1 α stimulated and unstimulated human cartilage explants. In order to further investigate this mechanism we analysed the gene expression of a number of catabolic and anti-catabolic genes. Hypoxia was found to significantly downregulate key catabolic genes *ADAMTS5* and *MMP13* at the mRNA level in cartilage explants and isolated HACs.

This downregulation could be further enhanced by PHD2 depletion in HACs, which stabilises both HIF-1 α and HIF-2 α above hypoxic levels. The knockdown of PHD2 at 20% and 1% oxygen tension in HACs decreased *ADAMTS5* expression 2.5 fold and 3.22 fold respectively and *MMP13* 2.0 fold and 3.22 fold respectively (compared to control levels in normoxia) and this pattern of downregulation corresponded with the pattern HIF- α isoforms stabilisation. Crucially, here we demonstrate that similar to the anabolic effect reported in Chapter 3, PHD2 displays significant activity even at 1% oxygen and the depletion of PHD2 under hypoxic conditions decreases levels of key proteases *ADAMTS5* and *MMP13* compared with hypoxic controls. Assuming 1% oxygen mimics the physiological microenvironment chondrocytes experience at the core of healthy human articular cartilage, the inhibition of PHD2 is able to enhance anti-catabolic effects beyond endogenous levels. This offers exciting opportunities of manipulating the endogenous oxygen sensing pathways and inhibiting cartilage matrix degradation.

TIMP3 mRNA was upregulated in response to hypoxia and this hypoxic induction was confirmed at the protein level. PHD2 knockdown at 20% and 1% in HACs upregulated *TIMP3* 1.5 fold and 2.7 fold respectively (compared to control levels in normoxia) however these upward trends were found to be not statistically significant.

We did not confirm the downregulation of *ADAMTS5* and *MMP13* at the protein level because we did not have access to suitable antibodies. It is possible to suggest that *ADAMTS5* and *MMP13* mRNA levels do not necessarily correspond with secreted protein levels, however results of the aggrecan neoepitope assay suggest a downregulation of these key catabolic enzymes in response to hypoxia. In summary, hypoxia downregulates catabolic genes *ADAMTS5* and *MMP13* and increases expression of catabolic regulator *TIMP3*, this may suggest that the HIF-pathway is anti-catabolic in human cartilage and HACs. This hypoxic downregulation of key catabolic gene *MMP13* can be further enhanced at the mRNA level by PHD2 enzyme depletion representing a further benefit of PHD2-specific inhibition with regard to the repair of cartilage matrix.

Interestingly, HIF-1 α depletion under hypoxic conditions reverses *MMP13* mRNA downregulation to levels exceeding normoxia. This would suggest that even under normoxic conditions there are low levels of HIF-1 α protein present in HACs, and indeed HIF-1 α was

detectable in 20% oxygen, albeit at much lower levels than hypoxia (Fig 4.3). Our results suggest that these low levels of stabilised HIF-1 α have an anti-catabolic effect downregulating MMP13 mRNA. This is interesting and unexpected finding, since HIF- α isoforms are thought to be completely degraded under normoxic conditions. It has been previously demonstrated *in vitro* using recombinant PHD that PHD1/2/3 have essentially identical K_m values for oxygen, approximately 25% O₂, suggesting that the PHD enzymes are fully active in 20% oxygen (Hirsila et al., 2003). Perhaps the PHD enzymes are not 100% efficient at hydroxylating HIF-1 α in 20% oxygen or maybe there are other factors present helping to stabilise HIF-1 α in cartilage. In order to confirm this we intend to carry out further work depleting HIF-1 α and HIF-2 α in HACs under normoxic conditions and measuring MMP13 mRNA using real-time PCR. MMP13 mRNA hypoxic downregulation was found to be HIF-1 α , and not HIF-2 α dependent.

In contrast to MMP13, ADAMTS5 mRNA hypoxic downregulation could not be reversed by HIF-1 α or HIF-2 α depletion suggesting a hypoxia-dependent but HIF1/2 α -independent mechanism. This is very interesting in the context of our PHD2 depletion data and suggests that PHD2 is able to regulate ADAMTS5 through an indirect and unknown mechanism. One possible explanation is that PHD2 hydroxylates or interacts with a non-HIF target responsible for the regulation of ADAMTS5 mRNA expression. PHD2 ability to interact with non-HIF targets has been previously hypothesised as Phd2^{-/-} mice have severe heart defects without the accumulation of either HIF-1 α and HIF-2 α isoforms in heart tissue.

TIMP3, a main regulator of a broad spectrum of MMPs as well as ADAM-17, ADAMTS4 and ADAMTS5 was upregulated at the mRNA and protein level in response to hypoxia and this was also found to HIF-1 α dependent, but not HIF-2 α dependent.

Two recent publications in *Nature Medicine* have suggested that HIF-2 α is a transactivator of many catabolic enzymes and have implicated it in a mouse model of osteoarthritis (Saito et al., 2010; Yang et al., 2010).

Saito and colleagues initially performed a screen of transcription factors that regulate the expression of type X collagen in chondrocytes (COL10A). COL10A1 is a marker of chondrocyte hypertrophy. HIF-2 α was reported to be a strong transactivator of the COL10A1

promoter, and later identified to regulate expression of vascular endothelial growth factor (VEGFA) and matrix metalloproteinase-13 (MMP-13) in a mouse chondrogenic cell line (ATDC5) and human nonchondrogenic (HeLa) cells. A HRE was identified in the COL10A1 promoter via consensus sequence and HIF-2 α was shown to bind to the region via electrophoretic mobility shift assay (EMSA) and chromatin immunoprecipitation (ChIP). Catabolic factors MMP3, MMP9 as well as markers for endochondral ossification associated runt-related transcription factor-2 (RUNX2), Indian hedgehog (IHH) and parathyroid hormone 1 receptor (PTH1R) were also identified as possible transcriptional targets of HIF-2 α . This work was supported by *in vivo* evidence as genetic deletion of one HIF-2 α allele (Epas1^{+/-}) resulted in mice which were more resistant to cartilage degradation and osteophyte formation than their wildtype littermates following surgical induction of osteoarthritis. Consistent with work in cell lines, expression of Col10a1, Vegf and Mmp13 was noticeably suppressed in Epas1^{+/-} mice compared to wildtype littermates. HIF-2 α protein levels were reported to be higher in human osteoarthritic cartilage versus non-diseased cartilage and a functional single nucleotide polymorphism (rs17039192) in human HIF-2 α gene was associated with knee osteoarthritis in a Japanese population.

Complimentary to Saito *et al.* Yang and colleagues found that the overexpression of HIF-2 α (using Epas1 overexpression adenovirus) enhanced expression of catabolic factors Mmp1, Mmp3, Mmp9, Mmp12, Mmp13, Adamts4, Nos2 and Ptgs2 in mouse articular chondrocytes and these levels were suppressed when HIF-2 α was depleted using siRNA. Both Epas1-transgenic mice (under promoter and enhancer regions of Col2a1 gene) and the overexpression of HIF-2 α in wildtype mouse knee joints via intra-articular injection of Ad-Epas1 resulted in joint tissue destruction. Consistent with Saito and colleagues genetic deletion of one HIF-2 α allele (Epas1^{+/-}) conferred more resistance to cartilage destruction than wildtype littermates following surgical induction of osteoarthritis or collagenase injection into knee joints. Similar to Saito and colleagues, HIF-2 α protein levels were reported higher in human osteoarthritic damaged cartilage regions versus non-damaged regions.

Both publications use the DMM (destabilised medial meniscus) osteoarthritis mouse model which has a strong inflammatory component. Furthermore, intra-articular injection of Ad-Epas1 in wildtype mouse knee joints causes complete joint destruction and clear

synovitis. Episodically inflammation of the synovial membrane has been reported in osteoarthritis in humans however it is debatable whether levels of inflammation this intense can be considered true osteoarthritis.

Saito and colleagues reported that a functional single nucleotide polymorphism (SNP) (rs17039192) in human HIF-2 α gene (*EPAS1*) was associated with knee osteoarthritis in a Japanese population. However, a recent large genetic study using ~7000 subjects from four independent populations (Japanese, Chinese, European and Australian) further investigated the associating between the HIF-2 α SNP and knee osteoarthritis. Replication of the work using a larger sample size found no statistical significant association between mutations in the HIF-2 α (*EPAS1* gene) and human knee osteoarthritis (Nakajima et al., 2011).

Gelse and colleagues carried out a study injecting the joints of BALB/C mice with 2-Methoxestradiol (2ME2). 2ME2 has been shown to decrease levels of HIF- α isoforms in chondrocytes through an unknown mechanism of inhibition. Injection of 2ME2 led to osteophyte formation and progressive joint destruction compared to controls, without an accompanying inflammatory response. Immunohistochemistry revealed a decrease in intracellular HIF-1 α levels (and presumably HIF-2 α levels) resulting in increased apoptosis of articular chondrocytes (Gelse et al., 2008). This suggests that HIF- α isoforms in mouse articular cartilage are essential for maintaining cartilage function, at least through prevention of cell death.

Here we demonstrate that in contrast to two recent publications in *Nature Medicine* the stabilisation of HIF-1 α and HIF-2 α (using hypoxia) in human explants and HACs does not increase the expression of catabolic genes: mRNA expression of *MMP1*, *ADAMTS4* and *COL10A1* (a marker of chondrocyte hypertrophy and endochondral ossification) are unaffected by HIF- α isoform stabilisation. Infact HIF- α isoform stabilisation using either hypoxia or PHD2 knockdown downregulated the expression of *ADAMTS5* and *MMP13* at the mRNA level in human cartilage explants and HACs. Furthermore the stabilisation of HIF- α isoforms upregulates metalloproteinase inhibitor TIMP3.

Consistent with work carried out by Yang *et al.*, we report that HIF- α isoform stabilisation in human articular chondrocytes increases mRNA expression of *MMP3* at the

mRNA level. *MMP3* mRNA was increased 4.5 fold in human cartilage explants in response to hypoxia. Similarly *MMP3* mRNA was upregulated 1.67 fold by hypoxia in HACs although this was not found to be significant. Matrix metalloproteinase-3 (MMP-3) or Stromelysin-1 is a key member of the MMP family and has a broad substrate specificity including proteoglycans, gelatine, laminin, vitronectin, fibronectin and type IV collagen. *MMP3* mRNA levels are reported to be strongly upregulated in early degenerated human articular cartilage relative to healthy articular cartilage but then decreased in late stage OA (Aigner et al., 2003; Bau et al., 2002). Furthermore, it is believed to control the activation of proMMP zymogens, for example: *MMP3* activates proMMP1 and proMMP13 (Knauper et al., 2002; Visse and Nagase, 2003). It is associated with growth plate dissolution in rats with collagen-induced arthritis (Takahashi et al., 2002). ~250 fold upregulation of *MMP-3* mRNA and ~40 upregulation of *ADAMTS5* mRNA was reported in bovine cartilage explants after application of a single injurious compression (Jeffrey et al., 1997). It is difficult to explain why hypoxia may have two simultaneous opposing catabolic (upregulating *MMP3*) and anti-catabolic (downregulation *ADAMTS5/MMP13*, upregulating *TIMP3*) effects. However, aggrecan neoepitope experiments reveal that overall treatment with hypoxia in porcine cartilage has an anti-catabolic effect with regard to endogenous matrix degradation (Fig. 4.1).

A recent publication by Ströbel and colleagues observed that in HACs the expression of *MMP1* and *MMP13* was reduced at the mRNA and protein level in response to low oxygen tension (5%) (Strobel et al., 2010). This hypoxia induced downregulation of *MMP13* is consistent with the findings of the present study. *MMP2* protein levels were also slightly reduced by hypoxia but this was not significant. *MMP3* levels were not investigated. Further investigations using immunohistochemistry and scanning electronmicroscopy revealed that hypoxia significantly reduced the amount type II collagen C-telopeptide fragments released, increased collagen fibril length and reduced collagen fibrillation. When cadmium chloride (a HIF- α inhibitor) was added to HACs under hypoxic conditions the hypoxia induced downregulation was abrogated confirming the involvement of the HIF-pathway in this anti-catabolic effect (Strobel et al., 2010). Norman and colleagues report similar findings but in human renal fibroblasts. Fibroblasts cultured under hypoxic conditions downregulate *MMP1* mRNA levels and upregulate *TIMP1* and *TIMP3* leading to decreased extracellular matrix turnover (Norman et al., 2000).

In order to explain the species differences between human and mouse it is necessary to consider if HIF-2 α is detectable in normal undamaged human and mouse articular cartilage. Both publications in *Nature Medicine* report elevated levels of HIF-2 α protein levels in human osteoarthritic cartilage versus non-diseased cartilage. Neither paper considers HIF-1 α stabilisation. Immunohistochemistry of human articular cartilage confirms the presence of HIF-1 α and HIF-2 α protein (Bohensky et al., 2009; Coimbra et al., 2004; Yudoh et al., 2005). Levels of HIF-1 α stabilisation correspond with logical pattern of oxygen gradient, increasing in intensity away from the surface of the tissue (Brucker et al., 2005). Yudoh and colleagues investigated human articular cartilage of osteoarthritis (OA) patients and reported high expression of HIF-1 α in degenerated regions compared with macroscopically intact regions. Inflammatory signals (IL-1 β , TNF- α) in human chondrocytes and mechanical stress (overloading) in bovine cartilage discs have been shown to stabilise HIF-1 α (Coimbra et al., 2004; Pufe et al., 2001; Yudoh et al., 2005). It has been suggested that HIF-1 α is anti-apoptotic and protects articular chondrocytes from cell death induced by catabolic stress (Yudoh et al., 2005). Conversely, Bohensky and colleagues reported that osteoarthritis decreases levels of detectable HIF-2 α in human articular cartilage. The decrease in HIF-2 α was linked with increased cell death in chondrocytes and it was proposed that HIF-2 α module is a modulator of autophagy and protects cells from apoptosis (Bohensky et al., 2009). However, as previously demonstrated in the previous Chapter HIF- α isoforms have a very short half life under normoxic conditions (20% oxygen) and therefore it is challenging to process and fix tissue samples as small differences in processing time can influence HIF- α isoforms stabilisation.

We have previously shown that in HACs hypoxia upregulates key extracellular matrix genes via SOX9 and this is dependent upon HIF-2 α suggesting an anabolic and not catabolic role of HIF-2 α in human articular cartilage (Lafont et al., 2007). This again is in contrast to recent work by Saito *et al.*, and Yang *et al.*, who reported elevated levels of HIF-2 α in cartilage of experimentally induced osteoarthritis in mice and osteoarthritic cartilage in humans.

Yang and colleagues report that an absence of detectable HIF-2 α protein in undamaged articular cartilage of CBA/CaCrI and C57BL/6 mice. Saito *et al.*, report very faintly detectable levels of HIF-2 α protein in undamaged articular cartilage of C56BL/6 mice.

Human cartilage in the major joints is several millimetres thick (2.26mm) whilst mouse cartilage is only a few cell layers thick (0.058mm) (1971, Stockwell). It is logical to assume that human articular cartilage has a much lower oxygen tension and chondrocytes within the tissue are exposed to more severe chronic hypoxia than in mouse. The absence of a persistent hypoxic signal in mouse cartilage and the absence of permanent HIF-1 α and HIF-2 α isoform stabilisation may be the cause of species differences. Perhaps humans adapted this permanent HIF-2 α and HIF-1 α stabilisation as a signal to promote the chondrocyte phenotype, enhance matrix deposition and suppress matrix turnover whereas mouse has adapted HIF-2 α as a catabolic factor which may actually be responsible for promoting osteoarthritis.

In summary, there are two mechanisms of chondroprotection: upregulation of anabolism and suppression of catabolism. Having previously discussed the anabolic benefits of hypoxia here we report that hypoxia and namely the stabilisation of HIF- α isoforms inhibits aggrecan degradation in IL-1 α stimulated and unstimulated human cartilage explants suggesting that hypoxia has an anti-catabolic effect in human cartilage explants is able to repress matrix degradation. We believe that the use of unstimulated healthy human cartilage explants may give our data high clinical relevance and means that it can be easily extrapolated to endogenous human articular cartilage. Further investigation in human tissue (cartilage explants) and HACs suggested that the observed anti-catabolic effects may be mediated by the downregulation of key proteases responsible for matrix turnover (ADAMTS5 and MMP13) and the upregulation of the metalloproteinase inhibitor TIMP3. Moreover, we provide clear evidence that hypoxia and specifically the stabilisation HIF-1 α (not HIF-2 α) has an anti-catabolic effect in human articular cartilage. It is interesting that the two mechanisms of hypoxia-induced chondroprotection are each mediated by a different HIF- α isoform: anabolism, by HIF-2 α and anti-catabolism, by HIF-1 α .

Finally, using an RNAi approach we show that depletion of PHD2 enhances this anti-catabolic effect in both normoxia and hypoxia. Assuming 1% oxygen mimics the physiological microenvironment chondrocytes experience at the core of healthy human articular cartilage, the inhibition of PHD2 is able to significantly enhance anti-catabolic effects above endogenous levels. This offers exciting opportunities of manipulating the chondrocyte oxygen sensing pathways and inhibiting cartilage matrix degradation above

endogenous: Fully turning on the HIF-pathway and doing better than nature. The therapeutic applications of manipulating oxygen sensing pathways to enhance cartilage matrix synthesis, reduce activity of catabolic proteases and enhance chondrocytes phenotype will be further discussed in Chapter 6.

4.7.2 IL-1 α stimulation stabilises HIF-1 α and decreases HIF-2 α in human articular chondrocytes

Proinflammatory cytokine interleukin 1 (IL-1) has two forms, α and β , both have similar effects on cartilage. The stimulation of cartilage explants with IL-1 upregulates catabolic enzymes (MMPs and ADAMTSs) and induces the rapid depletion of proteoglycans in explants cultures (Flannery et al., 1999; Gebauer et al., 2005; Saklatvala, 1987; Vincenti and Brinckerhoff, 2002). Yang and colleagues recently reported that the stimulation of primary mouse chondrocytes with IL-1 β enhanced HIF-2 α mRNA levels and increased protein stabilisation. This IL-1 β induced stabilisation was found to be NF- κ B and c-Jun N-terminal kinase (Jnk) dependent (Yang et al., 2010). Unfortunately, the effect of IL-1 β on HIF-1 α levels was not investigated by the authors.

We report that IL-1 α stimulation of HACs results in an increase in *HIF-1 α* at the mRNA level and HIF-1 α protein stabilisation even under normoxic conditions. Levels and rate of HIF-1 α stabilisation varied between patients, over a timecourse HIF-1 α protein levels peaked between 6 – 12 hours however the mRNA levels continued to increase over a 24 hour period of IL-1 α treatment. Here we report that 24 hours after IL-1 α stimulation in normoxia HIF-1 α levels are approximately greater than or equal with unstimulated hypoxic controls (in 1% oxygen). HACs cultured at 1% oxygen and stimulated with IL-1 α increased levels of HIF-1 α protein above those seen in hypoxia alone. In contrast to Yang and colleagues (who used murine chondrocytes), IL-1 α stimulation in HACs suppressed HIF-2 α levels rather than increased them.

Surprisingly we report that the transient burst of HIF-1 α levels in response to IL-1 α stimulation had no effect on PHD2 levels. Electrophoretic mobility-shift assays have demonstrated the binding of HIF-1 α to HREs upstream of PHD2 and HREs have been identified (using bioinformatics) 40kb upstream of PHD3 (Metzen et al., 2003; Pescador et al., 2005). It is logical to assume that an increase in HIF-1 α levels would also increase PHD2

(and PHD3) levels. Perhaps the transient burst of HIF-1 α was not sufficient to significantly upregulate PHD2 protein levels (PHD3 levels were not monitored).

The mechanism of HIF-1 α stabilisation by IL-1 α remains unclear. HACs cultured at 1% oxygen and stimulated with IL-1 α increased levels of HIF-1 α protein above those seen in hypoxia alone suggesting an additive effect (a mechanism of stabilisation independent of inhibition HIF- α -proline hydroxylation). If the stabilisation of HIF-1 α by IL-1 α was mediated by PHD-inhibition we would expect to see a decrease in PHD enzyme activity or abundance. IL-1 α stimulation did not affect PHD2 abundance levels (the dominant regulator of HIF-1 α in HACs). Moreover, IL-1 α stimulation stabilises HIF-1 α levels, but decreases HIF-2 α under hypoxic conditions. If PHD2 activity was inhibited by IL-1 α stimulation we would expect to see both HIF- α isoforms stabilised (no HIF-1 α specificity) and no a decrease of HIF-2 α under hypoxic conditions.

Perhaps IL-1 α stimulation inhibited PHD1 or PHD3 activity. However, when investigated using RNAi specific depletion PHD1 did not result in HIF-1 α specific stabilisation and PHD3 did not have a detectable effect on levels of either HIF- α isoform in normoxia (Chapter 3). This suggesting that PHD enzyme abundance /activity are not decreased by IL-1 α stimulation and perhaps the mechanism of HIF-1 α stabilisation is mediated by an HIF-1 α mRNA stability / transcriptional effect.

In an effort to further probe the mechanism of HIF-1 α stabilisation further we carried out experiments using the MAPK p38 inhibitor SB202190. These experiments suggested that the IL-1 α induced HIF-1 α stabilisation may be p38 MAP kinase dependent. Pre-incubation with SB202190 abolished IL-1 α induced *HIF-1 α* mRNA upregulation and decreased HIF-1 α protein stabilisation in HACs. The suppression of HIF-1 α protein stabilisation in HACs was not complete and may suggest that the mechanism of HIF-1 α stabilisation by IL-1 α may not be completely p38 MAP kinase dependent. This work would be consistent with previous observations, Yudoh and colleagues report IL-1 β stimulation of HACs results in HIF-1 α stimulation and this stimulation was phosphatidylinositol 3-kinase and p38 MAP kinase dependent (Yudoh *et al.*, 2004). Similarly the stimulation of healthy and osteoarthritic chondrocytes under normoxic conditions with [10ng/ml] TNF- α has been reported to stabilise HIF-1 α protein (Coimbra *et al.*, 2004). This TNF- α induced stabilisation

was partially abrogated by 1 hour pre-incubation of healthy and osteoarthritic chondrocytes with p38 MAP kinase inhibitor (SB203580) or NF- κ B inhibitor pyrrolidine dithiocarbamate (PDTC) and it was concluded that TNF- α induced HIF-1 α stabilisation was partially dependent on the p38 MAP kinase and NF- κ B signalling pathways (Coimbra et al., 2004).

Adenosine/uridine-rich elements (ARE's) are common RNA stability determinants found in the 3'- untranslated region (3'- UTR) of mRNAs characterised by rapid, transient gene expression for example, cytokines, growth factors and proto-oncogenes (Lai et al., 1999; Sawaoka et al., 2003). Tristetraporol (TTP) is a well known regulator of ARE-containing mRNAs and controls target gene stability by binding to the 3'-UTR-containing ARE motifs and recruiting factors that mediate decapping, deadenylations and mRNA destruction. It has been previously demonstrated in mouse macrophages that the p38 pathway regulates both the expression and post-translational modification of TTP (Mahtani et al., 2001). Kim and colleagues recently identified 8 putative TTP-binding motifs in HIF-1 α 3'-UTR, demonstrated that TTP binds directly to HIF-1 α 3'-UTR mRNA and that the overexpression of TTP, reduced not just HIF-1 α steady state levels, but also reduced HIF-1 α protein stabilisation in 1% oxygen (Kim et al., 2010). It may be that IL-1 α acts through the p38 pathway, phosphorylating TTP by MAPK-activated protein kinase 2 (MK2), inhibiting its activity and preventing the mRNA-destabilising function of TTP leading to accumulation of ARE containing mRNAs. This would increase HIF-1 α mRNA stability and potentially lead to greater HIF-1 α protein stabilisation. Addition of the SB202190 inhibitor would prevent TTP phosphorylation and lead to HIF-1 α mRNA destruction.

In chapter 3 we previously reported that hypoxia significantly downregulated the mRNA levels of *HIF2 α* and in particular, *HIF1 α* at the mRNA level. Depletion of PHD2 significantly decreases *HIF1 α* mRNA levels even under normoxic (20% oxygen) conditions (Fig. 3.10 A). Kim and colleagues reported the hypoxic induction of TTP in several cell lines (HEK293T, Hep3B, PLC/PRF/5 and HCT116) (Kim et al., 2010). This seems to be consistent with our results. Inhibition of PHD enzymes (either limiting molecular oxygen or PHD2 depletion) increases levels of TTP and therefore accelerates the rate of HIF-1 α mRNA destruction. In our further work we intend to confirm the hypoxic induction of TTP and its regulation of HIF-1 α mRNA in HACs.

However, stabilised HIF-1 α protein was detectable 3 hours after stimulation with IL-1 α , this correlated with an approximately 2.5 – 3 fold increase in mRNA levels. If IL-1 α 's HIF-1 α stabilising ability was mediated through either increasing the rate of HIF-1 α gene transcription or an increase in HIF-1 α mRNA stability it is difficult to explain how such a modest increase in mRNA level is able to overcome the endogenous cellular turnover of HIF- α isoforms in the cytoplasm under normoxic conditions. Perhaps there is a “lag time” before the PHD enzymes are able to adjust to the new levels of HIF-1 α protein. It would be interesting to see if this “lag time” was more prolonged under hypoxic conditions.

In summary, the addition of IL-1 α stabilises HIF-1 α even under normoxic conditions (albeit in a transient fashion) and IL-1 α decreases HIF-2 α protein levels under hypoxic conditions in HACs. This is in contrast to recent work in mouse chondrocytes which reported that the addition of IL-1 β stabilises HIF-2 α (Yang et al., 2010). The IL-1 α induced stabilisation of HIF-1 α in the present study was partially suppressed by SB202190, an inhibitor p38 MAP kinase and therefore believed to be partially p38 MAP kinase dependent and suggests a transcriptional effect. However, the exact mechanism of stabilisation remains unknown. Our data suggests that the stabilisation of HIF-1 α is, at least partially p38 dependent, however further work is needed to uncover the mechanistic details.

Chapter 5

Effects of Hypoxia on Porcine and Murine Cartilage and Chondrocytes

5.1 Introduction

We previously identified PHD2 as the key hydroxylase in human articular chondrocytes (HACs) responsible for regulating HIF-1 α and HIF-2 α . We have shown that both hypoxia and the specific inhibition of PHD2 promote the differentiated phenotype by upregulating SOX9, enhancing cartilage matrix production by HACs and decreasing the rate of matrix degradation.

HIF-1 α has previously been described as a critical factor for murine chondrocyte development and survival (Schipani et al., 2001). Furthermore, it has been reported that hypoxia (specifically the stabilisation of HIF-1 α) increases cartilage extracellular matrix synthesis in murine chondrocytes and upregulates Sox9 in mouse mesenchymal cells (Amarilio et al., 2007; Pfander et al., 2003). Recently work by Zhang and colleagues reported that the induction of ischemic osteonecrosis in porcine femoral heads stabilised HIF-1 α , upregulated Sox9 and increased cartilage matrix deposition in epiphyseal cartilage (Zhang et al., 2011). Given our previous human data we investigated whether the effect of PHD enzyme inhibition would have similar effects in murine and porcine cartilage.

Here we report that similarly to our work with human explants, hypoxia has a pro-anabolic and an anti-catabolic effect in porcine cartilage. Hypoxia inhibited aggrecan cleavage in unstimulated porcine cartilage explants and even suppresses aggrecan cleavage when explants are stimulated with proinflammatory cytokine interleukin 1 α (IL-1 α). It is believed that this anti-catabolic effect is mediated by the hypoxic downregulation of *Adamts5* at the mRNA level and increasing secreted Timp3 levels. In addition, we demonstrate that hypoxia Sox9 and *Col2a1* mRNA in porcine cartilage explants suggesting that hypoxia promotes the chondrocyte phenotype and increases extracellular matrix deposition. However, mouse articular cartilage data suggests there are key species differences in the way articular chondrocytes respond to hypoxia between man and mouse. Sox9 and cartilage matrix expression (*Col2a1* and *Col11a2*) are not regulated by hypoxia in murine articular cartilage and chondrocytes.

5.2 Hypoxia inhibits IL-1 α induced articular aggrecan degradation in porcine cartilage explants

In order to study the effect of hypoxia on aggrecan degradation, we stimulated porcine articular cartilage explants with proinflammatory cytokine interleukin 1 α (IL-1 α). Stimulation with IL-1 α causes the cleavage of the aggrecan core protein by aggrecanases within the first interglobular domain (between G1 and G2) (Flannery et al., 1999; Gebauer et al., 2005; Saklatvala, 1987; Vincenti and Brinckerhoff, 2002). Proteolysis occurs at the Glu³⁷³ – Ala³⁷⁴ bond resulting in the C-terminal neoepitope NITEGE (bound to the hyaluronan backbone) and the N-terminal neoepitope ARGSV. This C-terminal aggrecan fragment can be detected by western blotting cartilage explant culture medium with antibodies which recognize the ARGSV neoepitope. In contrast to previous work human articular cartilage explants (Chapter 4) in porcine explants anti-ARGSV neoepitope antibodies recognise a dominant >250 kDa band and a fainter 150 kDa fragment. Release of ARGSV neoepitope was detected 24 hours after stimulation with IL-1 α and even present in unstimulated cartilage.

Porcine articular cartilage was taken from the 2nd-3rd metacarpophalangeal (MCP) joints of 3 to 6 month old (male and female) pigs. Cartilage explants from 4 joints were cut into cubes (3 mm³), pre-incubated in normoxic or hypoxic conditions for 48 hours stimulated with either IL-1 α or vehicle (serum free media) and returned to original oxygen tensions. We found that similar to human cartilage explants hypoxia inhibited the release of ARGSV neoepitope when explants were stimulated with IL-1 α (Fig 5.1). Consistent with previous work in human cartilage explants the ARGSV neoepitope release was IL-1 α dose dependent and long exposure of X-ray film revealed that hypoxia also inhibited ARGSV neoepitope release in unstimulated cartilage explants (Fig. 5.1).

In summary, treatment of cartilage explants with hypoxia inhibits IL-1 α induced aggrecan cleavage in both human and porcine human cartilage explants.

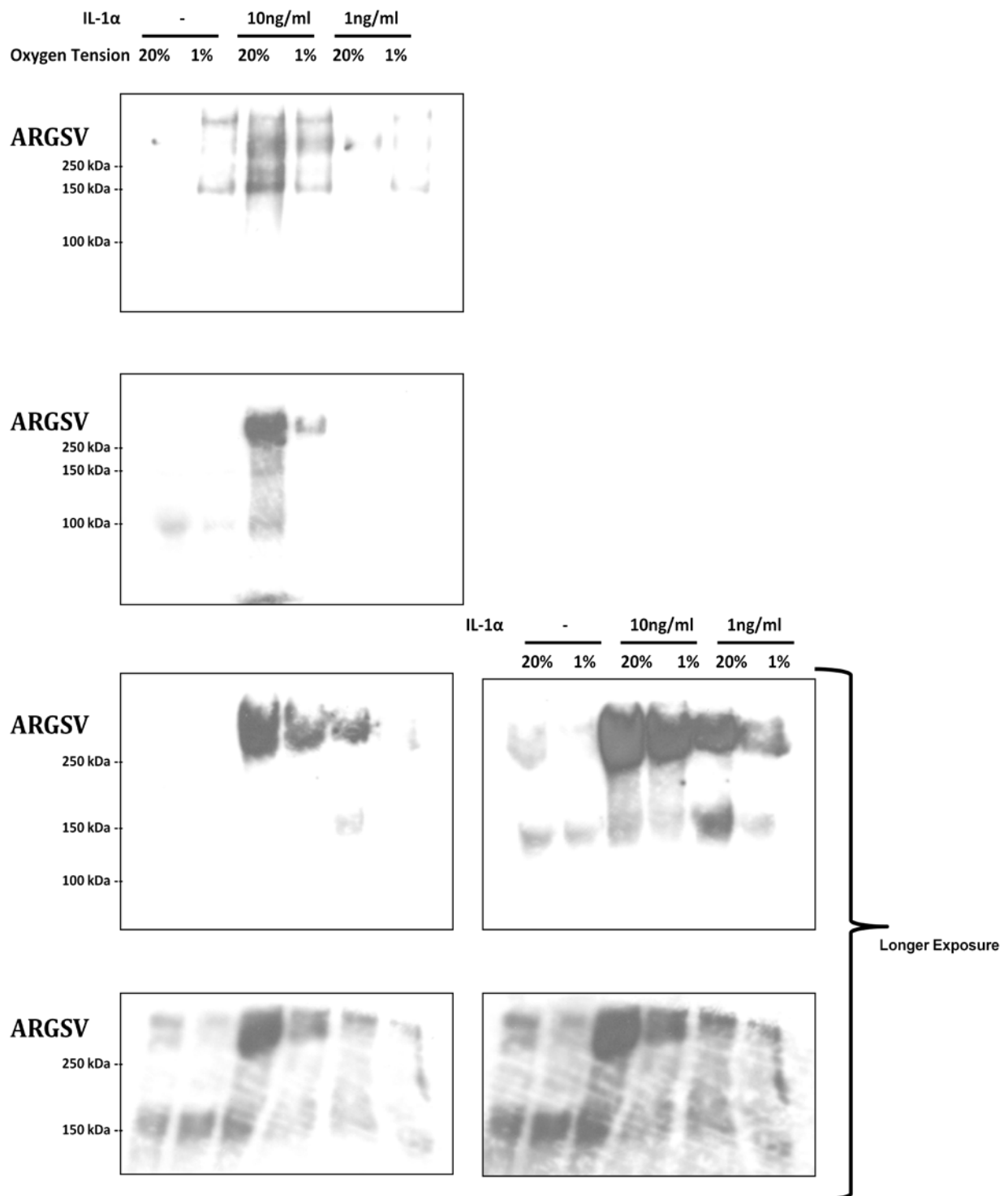


FIGURE 5.1. Hypoxia inhibits aggrecan degradation in porcine articular cartilage explants.

Porcine articular cartilage was taken from the 2nd-3rd metacarpophalangeal (MCP) joints of 3 to 6 month old (male and female) pigs. Cartilage explants from 4 joints were cut into cubes (3 mm³), pre-incubated in normoxic or hypoxic conditions for 48 hours before treatment with either IL-1 α (10ng/ml or 1ng/ml) or vehicle (serum free media) and returned to original oxygen tensions. Aggrecan fragments containing ARGSV neopeptide were released into the medium, precipitated using acetone precipitation and detected via Western blotting using anti-ARGSV neopeptide antibodies.

5.3 Up-regulation of anabolic and anti-catabolic factors in porcine cartilage explants under hypoxic conditions

Porcine articular cartilage was taken from the 2nd-3rd metacarpophalangeal (MCP) joints of 3 to 6 month old (male and female) pigs obtained from a local slaughterhouse within 16 hours of slaughter. After skinning the trotter, the MCP joint was opened in a biological safety cabinet. Cartilage explants from 1 joint were dissected using aseptic technique from the articular surface directly into DMEM and cut into cubes (3 mm³).

20 Porcine cartilage explants (~60mg wet weight) per dish were incubated for 3 days under normoxic (20% oxygen) or hypoxic (1% oxygen) conditions. The messenger RNA (mRNA) levels of master regulator transcription factor *Sox9* as well as key cartilage matrix proteins (*Col2a1*, *Aggrecan*), catabolic enzymes (*Adamts5*, *Mmp13*) and key proteinase inhibitor (*Timp3*) were analysed after normalising the data using the housekeeping gene *RPLP0*.

Similarly to human explants, hypoxia upregulates *Sox9* (1.9 fold) and *Col2a1* (3.1 fold) at the mRNA level (Fig. 5.2 A, B, C). *Aggrecan* mRNA levels are increased by hypoxia 1.53 fold but this trend was not statistically significant (Fig. 5.2 C).

Adamts5 expression was downregulated (1.6 fold) in response to 1% oxygen tension (Fig. 5.2 D). In contrast to human cartilage explants hypoxia increased the average mRNA levels of *Mmp13* by 2.93 fold, however this difference was not significant (Fig. 5.2 E). Finally *Timp3* mRNA was not significantly affected by hypoxia although clearly increased levels of *Timp3* protein were detected in the culture medium by western blot (Fig. 5.2 F, G).

In summary, consistent with human data the treatment of porcine cartilage explants with hypoxia increases *Sox9* expression, upregulates key cartilage gene *Col2a1*, decreases aggrecanase *Adamts5* and upregulates *Timp3* at the protein level.

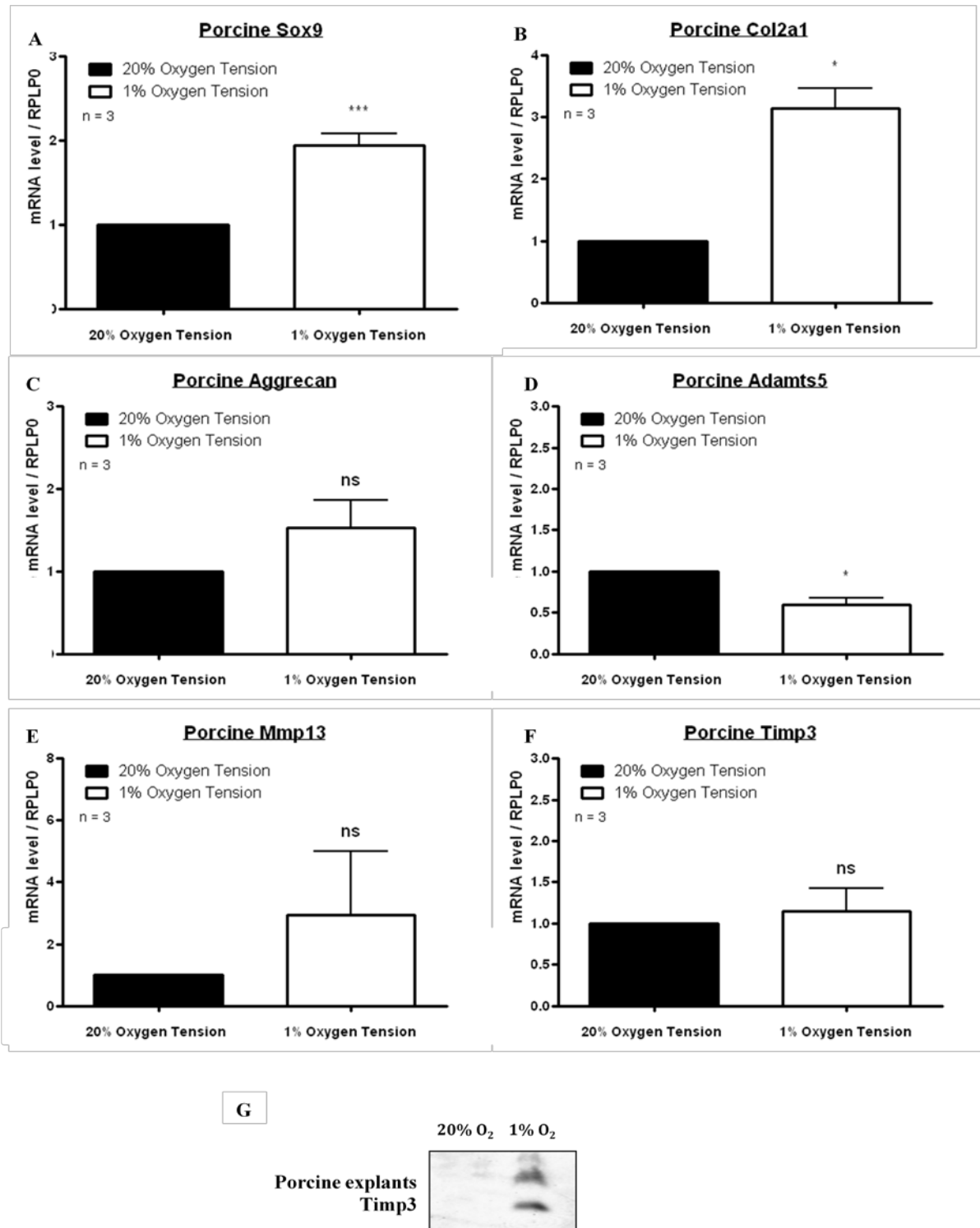


FIGURE 5.2. Hypoxia upregulates cartilage specific transcription factor Sox9, main cartilage matrix protein type II collagen, key catabolic regulator Timp3 and downregulates aggrecanase Adamts5 in porcine articular cartilage explants. TaqMan PCR data showing relative levels of mRNA for *Sox9*, *Col2a1*, *Aggrecan*, *Adamts5*, *Mmp13* and *Timp3* (A – F, respectively) after 20 porcine explants were cultured for 3 days in 20% or 1% oxygen. All values were normalised using the *RPLP0* housekeeping gene and represent mean and SEM results of 3 independent experiments (i.e., explants from 3 different porcine trotters). Note: ns - not significant; * $P < 0.05$; *** $P < 0.001$ (versus 20% oxygen). Secreted TIMP3 (G) was detected in 1% oxygen treated explant cultures following acetone precipitation of culture media. Cell culture medium was supplemented with 200 μ g/ml heparin in order to detect TIMP3.

5.4 No hypoxic regulation of cartilage specific transcription factor Sox9 in murine hip cartilage explants and isolated chondrocytes

Mouse hip explants were prepared from the hips of 6-week-old male C57Bl/6 mice. The skin and soft tissue was removed from the hind legs. The hip joints were dislocated and the proximal femoral heads isolated using aseptic technique under an anatomical microscope. 5 femoral heads were cultured in two different oxygen tensions (20%, 1% oxygen) or treated with 2mM of 2-oxoglutarate analogue DMOG for 3 days. In contrast to our previous findings in human and porcine cartilage explants, hypoxia did not induce Sox9 at the protein level and even DMOG treatment has no significant effect on Sox9 levels in murine cartilage explants (Fig. 5.3. A and B). This experiment was repeated in passaged murine chondrocytes and consistent with femoral head data the inhibition of HIF-hydroxylases using either hypoxia or DMOG did not induce Sox9 expression over control conditions (20% oxygen) (Fig. 5.3. C and D). These experiments suggest that in contrast to human (and porcine) cartilage Sox9 is not regulated by the HIF-pathway in murine articular cartilage.

In order to further investigate this we isolated murine chondrocytes from the femoral heads of 6-week-old male C57Bl/6 mice. Passaged murine chondrocytes were cultured under normoxia, hypoxia or in the presence of 2mM DMOG for 3 days. The mRNA levels of master regulator transcription factor *Sox9* and key cartilage matrix proteins (*Col2a1*, *Col11a2*), as well as known hypoxia responsive genes *Phd2* and *Glut1* were analysed after normalising the data using the housekeeping gene *Rplp0* (Fig. 5.4. A to E). In contrast to HACs we did not observe an induction of genes associated with the chondrocyte phenotype in response to hydroxylase inhibition. *Col2a1* was not upregulated in response to hypoxia (Fig 5.4. C). *Sox9* mRNA levels were not affected by 1% oxygen tension, this lack of SOX9 hypoxic induction is consistent with previous work at the protein level in both mouse chondrocytes and mouse hip cartilage explants (Fig. 5.3).

Due to the limited number of murine chondrocytes we were unable to assess stabilisation of HIF- α isoforms at the protein level in response to PHD enzyme inhibition. However, the upregulation of known hypoxia responsive gene *Phd2* in response to hypoxia and DMOG treatment suggests a functional HIF response to hypoxia in these cells (Fig. 5.4 A).

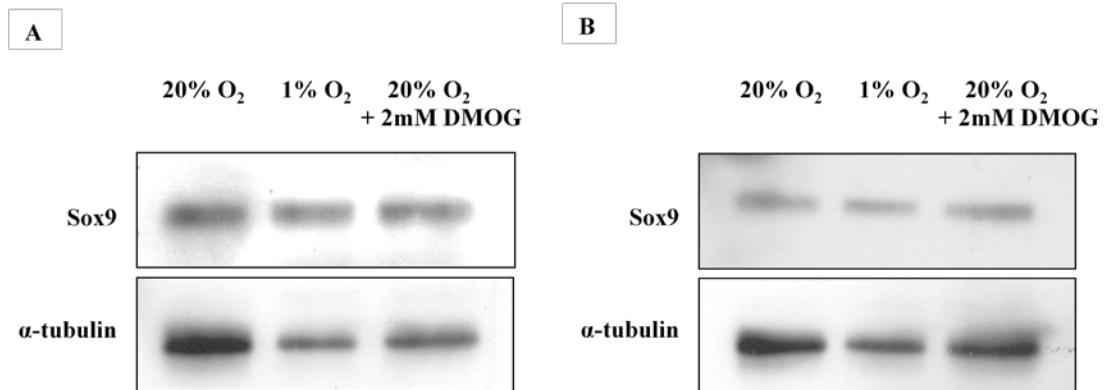
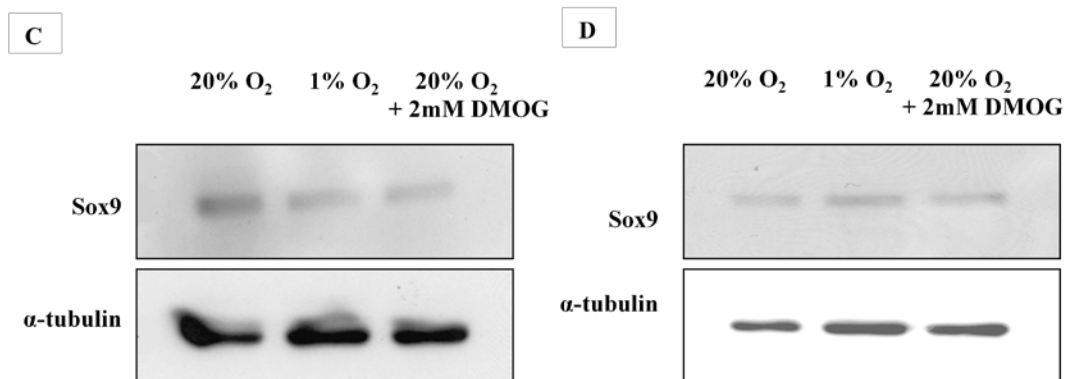
Mouse Hip Explants:**Passaged Mouse Chondrocytes (Passage 2):**

FIGURE 5.3. No hypoxic regulation of Sox9 protein levels in murine hip explants and isolated chondrocytes in response to hypoxia. Data are based on mouse femoral hip explants from 6-week-old male C57Bl/6 mice after culture for 3 days at 20% oxygen, 1% oxygen or treated with 2-oxoglutarate analogue DMOG (5 hip explants per data point). *A* and *B*, western blot showing no changes in SOX9 levels in response to either treatment. Mouse articular chondrocytes (passage 2) were cultured for 3 days at 20% oxygen, 1% oxygen or treated with 2-oxoglutarate analogue DMOG. *C* and *D*, western blot showing change in SOX9 levels in response to either treatment.

Figure 5.4

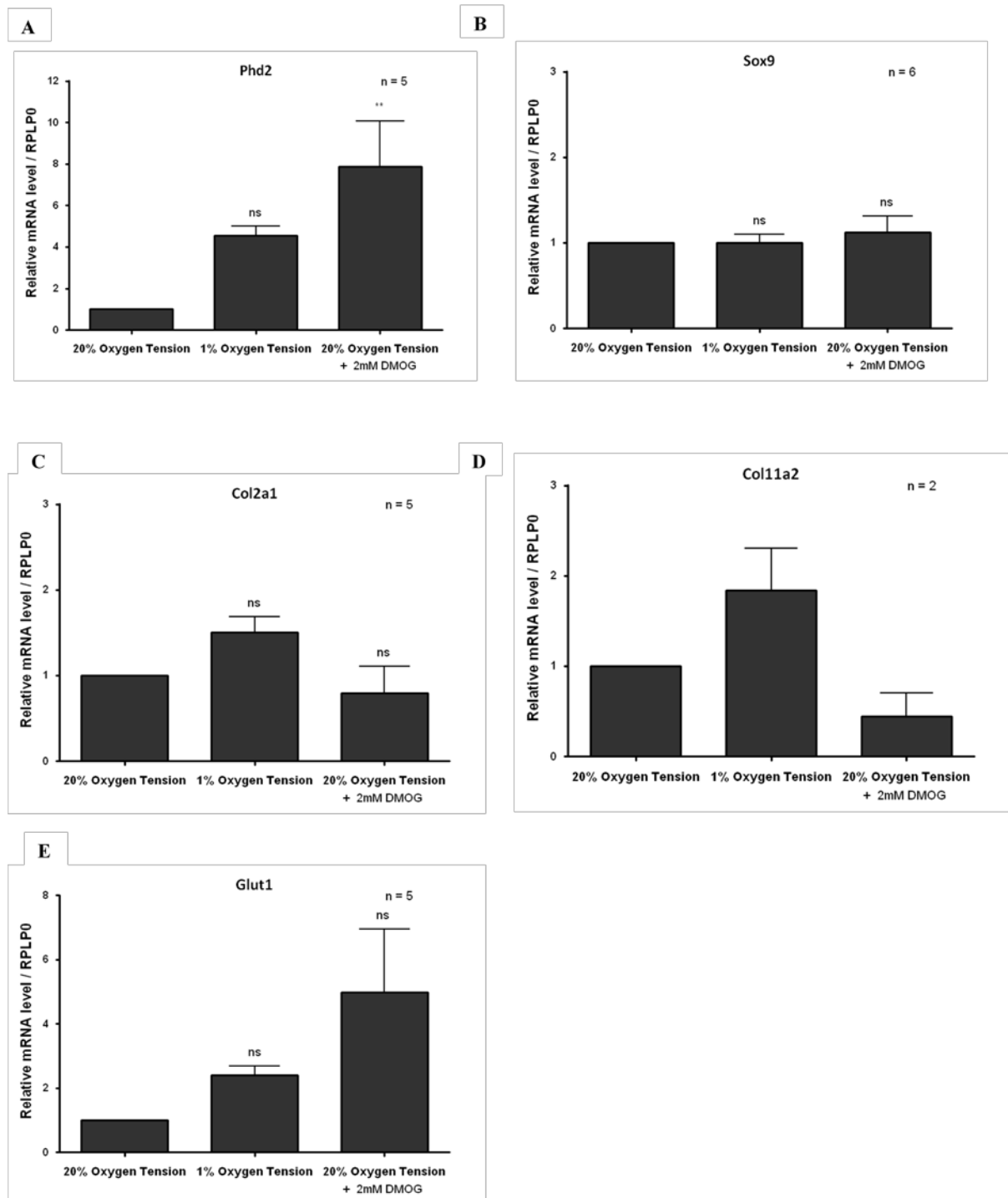


FIGURE 5.4. Hypoxia does not regulate cartilage specific transcription factor SOX9 or key cartilage matrix collagens at the mRNA level. Mouse chondrocytes (passage 2) were cultured for 3 days at 20% oxygen, 1% oxygen or treated with 2-oxoglutarate analogue DMOG. TaqMan PCR data showing relative levels of mRNA of *Phd2* (A), *Glut1* (E) and key chondrocyte markers *Sox9*, *Col2a1*, *Col11a2*, (B – D, respectively) were analysed after normalising the data using *Rplp0*. Key chondrocyte marker levels were unchanged in response to hypoxia or DMOG treatment. Note: ns - not significant; ** $P < 0.01$ (versus 20% oxygen).

We considered that mouse hip explants were not pure populations and may contain a mixture of articular, growth plate and hypertrophic chondrocytes. Using RT-PCR we confirmed that the passaged chondrocytes expressed high levels of key chondrocyte markers *Sox9*, *Col2a*; while the marker of chondrocyte hypertrophy *Col10a1* was only detectable at very low levels (data not shown).

In summary, studies of mouse femoral hip cartilage and isolated mouse chondrocytes suggest that *Sox9* is not regulated by the HIF-pathway at the protein or mRNA level. This work is different to the data found in humans (and pigs) and possible reasons for the species difference will be examined in the discussion.

5.5 Phd2 depletion in mouse chondrocytes does not appear to regulate Sox9 at the protein level

In order to further investigate the role of HIF-pathway on the main markers of the chondrocyte phenotype, we transfected passaged mouse chondrocytes with small interfering RNA (siRNA) against either Phd2 (the main regulator of HIF-1 α and HIF-2 α in HACs) or Luciferase (as a non-targeting control). Following transfection, passaged mouse chondrocytes were cultured for 3 days at either 20% or 1% oxygen and lysed. Real-time PCR was used to confirm Phd2 knockdown, Phd2 mRNA was depleted 81% on average compared with luciferase control (Fig. 5.5. A). There appeared to be no regulation of *Sox9* in mouse chondrocytes at the protein level in response to Phd2 knockdown (Fig. 5.6). Due to a limited number of cells were unable to draw any conclusions of the effect of Phd2 knockdown on mRNA level of *Sox9* and *Col2a1*. Preliminary results suggest that Phd2 depletion may increase expression known hypoxia (Fig. 5.5. B, C, D).

In summary, consistent with our previous work in mouse femoral hip cartilage and passaged mouse chondrocytes, the specific depletion of HIF-targeting hydroxylase PHD2 has appears to no significant effect on *Sox9* levels. This provides further evidence that the HIF-pathway does not regulate the chondrocyte phenotype in murine cartilage. Possible reasons for these species difference will be examined in the discussion.

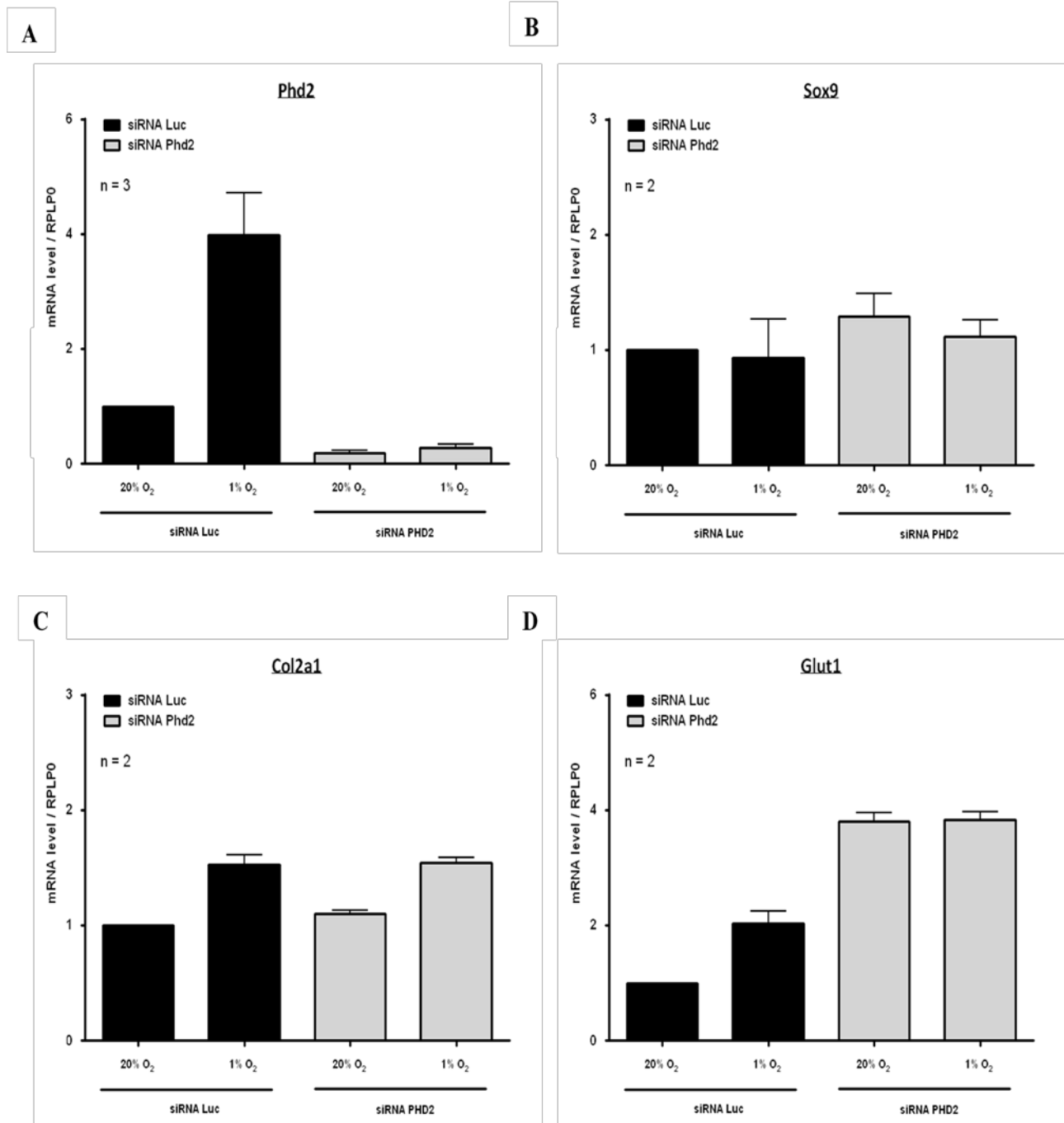


FIGURE 5.5. Phd2 depletion in passaged mouse chondrocytes. All data in the figure were obtained from passaged mouse chondrocytes (P2-P3) after 3 days in culture at either 20% or 1% oxygen. Cells were transfected with 10nM against PHD2 (siPHD2), or, as a control against luciferase (siLuc). TaqMan PCR data showing relative levels of mRNA for *Phd2*, *Sox9*, *Col2a1*, *Glut1* (A – D, respectively) were analysed after normalising the data using the housekeeping gene *Rplp0*. PHD2 depletion increased expression of hypoxia response gene *Glut1* but did not alter mRNA levels of *Sox9* or *Col2a1*.

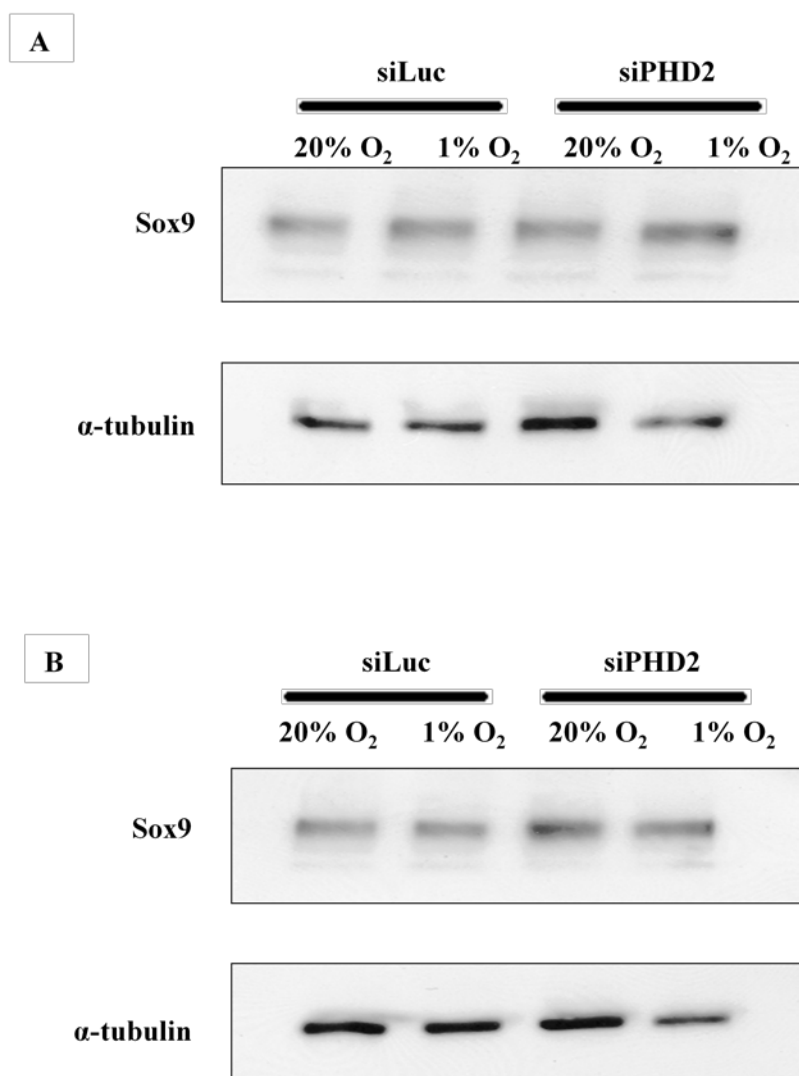
Passaged Mouse Chondrocytes (P4):

FIGURE 5.6. Sox9 protein is not regulated by Phd2 enzyme depletion in passaged mouse chondrocytes under 20% or 1% oxygen tension. All data in the figure were obtained from passaged mouse chondrocytes (P4) after 3 days in culture at either 20% or 1% oxygen. Cells were transfected with 10nM against PHD2 (siPHD2), or, as a control against luciferase (siLuc). A and B, western blots showing no change in SOX9 protein levels in response to PHD2 enzyme depletion.

5.6 Discussion:

We previously identified PHD2 as the key HIF-targeting hydroxylase in human articular chondrocytes (HACs) responsible for regulating HIF-1 α and HIF-2 α . We have shown that hypoxia in HACs promotes the differentiated phenotype, enhances cartilage matrix production and decrease the rate of endogenous matrix degradation. These effects can be further enhanced by specific inhibition of PHD2 in hypoxia which we demonstrated to be the main regulator of HIF-1 α and HIF-2 α in HACs.

Investigating the effect of hypoxia and HIF-targeting hydroxylase inhibition in murine and porcine cartilage we have identified interesting species differences. Here we report that hypoxia has an anabolic and an anti-catabolic effect in porcine cartilage. The stabilisation of HIF- α isoforms inhibits IL-1 α induced aggrecan cleavage in porcine cartilage explants. Furthermore, hypoxia suppresses the endogenous release of aggrecan fragments in unstimulated cartilage suggesting that hypoxia has an anti-catabolic effect in cartilage and is able to reduced matrix degradation. In order to further investigate the similarities with human cartilage we monitored expression of a number of genes and found that the stabilisation of HIF- α isoforms in porcine cartilage explants has anabolic and anti-catabolic effect. Hypoxia upregulated anabolic genes (*Sox9* and *Col2a1*), downregulated key aggrecanase (*Adamts5*) and upregulated anti-catabolic enzyme tissue inhibitor of metalloproteinases 3 (*Timp3*). This is similar to our previous work in human cartilage explants and HACs; hypoxia enhances cartilage matrix synthesis and reduces activity of catabolic proteases.

However, murine cartilage and chondrocytes showed strikingly different results, with *Sox9* and subsequent matrix gene expression being unaffected by hypoxia and PHD inhibition. This may explain some of the previously reported discrepancies between mouse and human (larger animal studies) with respect to HIF regulation of cartilage function.

5.6.1 Hypoxia in cartilage has an anabolic and anti-catabolic effect in porcine cartilage explants

Here we report that, similar to our work in human cartilage, hypoxia has an anti-catabolic effect in porcine cartilage explants. Porcine cartilage explants exposed to hypoxia

are more resistant to IL-1 α induced aggrecan cleavage than normoxic controls providing evidence of the anti-catabolic and chondroprotective effect of hypoxia.

Similarly to our previous work with healthy human articular cartilage (Chapter 4), porcine cartilage was also cultured overnight in either 20% oxygen or 1% oxygen without IL-1 α stimulation in order to more accurately mimic physiological conditions *in vivo*. Assuming that chondrocytes experience physiological oxygen tensions of ~1-6% oxygen, 1% oxygen would be representative of the microenvironment at the core of the of the tissue. Here we report that hypoxia suppresses the release of endogenous aggrecan fragments in unstimulated cartilage suggesting that hypoxia has an anti-catabolic effect in cartilage is able to reduce matrix degradation even in healthy unstimulated tissue. It is also possible to suggest that supra-physiological, (20% oxygen), conditions have a catabolic effect on cartilage explants increasing the rate of cartilage degradation.

In order to further investigate the similarities between human and porcine cartilage porcine cartilage explants were cultured for 3 days under normoxic (20% oxygen) and hypoxic (1% oxygen) conditions. Hypoxia had an anabolic effect in porcine cartilage explants upregulating *Sox9* and *Col2a1* at the mRNA level. We have previously demonstrated that this hypoxic upregulation in HACs was HIF-2 α dependent and it would be interesting to determine if this hypoxic regulation was HIF-1 α or HIF-2 α dependent. We also observed an anti-catabolic effect as hypoxia downregulated key aggrecanase *Adamts5* (responsible for aggrecan core protein cleavage) and upregulated anti-catabolic enzyme tissue inhibitor of metalloproteinases 3 (*Timp3*) (although this was not confirmed at the mRNA level). This anabolic/anti-catabolic effect of hypoxia is consistent with our findings in human cartilage (Chapter 3 and Chapter 4). We suggest that this anti-catabolic hypoxic effect on porcine cartilage may be mediated by the downregulation of key aggrecanase *Adamts5* and the upregulation of the metalloproteinase inhibitor *Timp3*.

However there were some differences between porcine and human cartilage explants. Although we observed, on average, an increase in *Aggrecan* mRNA levels in hypoxia (~1.5 fold) this trend was not statistically significant. In addition we observed greater hypoxic induction of *Sox9* and extracellular matrix genes in human cartilage compared with porcine cartilage, for example: hypoxia upregulates *Sox9* (5.8 fold) and *Col2a1* (29.9 fold) in human

cartilage vs. Sox9 (1.9 fold) and Col2a1 (3.1 fold) in porcine cartilage. Possible reasons for this species difference will be explained below (Chapter 5.5.2). Finally, in contrast to human explants *Mmp13* expression was increased ~2.93 fold in response to hypoxia however this was found to be not statistically significant. MMP13 is a key catabolic enzyme with high activity against type II collagen and thought to play a critical role in cartilage destruction. However aggrecan-derived neopeptide assays reveal that, like for human explants, treatment with hypoxia in porcine cartilage has an anti-catabolic effect with regard to endogenous matrix turnover (Chapter 4).

Recent work by Zhang and colleagues in the porcine model supports our work. Ischemic osteonecrosis was surgically induced in Yorkshire immature pigs aged 5 to 6 weeks old by applying a ligature around the femoral neck. Gross and histological assessment of femoral heads revealed significantly thicker cartilage layer in pigs after two weeks of surgically induced ischaemia compared with control group (1200µm versus 1800µm) (Kim et al., 2009a; Zhang et al., 2011). Messenger RNA was isolated from cartilage 24 hours after ischemic induction and consistent with our work hypoxia was found to significantly upregulate Sox9 (~2.3 fold), Col2a1 (~2.5 fold) and Aggrecan (~1.5 fold) at the mRNA level. Significant increases in HIF-1α and Sox9 levels were confirmed at the protein level in ischemic femoral heads via immunostaining of epiphyseal cartilage 2 weeks after the surgical induction of ischemia. Zhang and colleagues concluded that HIF-1α stabilisation in porcine epiphyseal cartilage upregulates Sox9 levels and enhances cartilage matrix deposition; however they failed to investigate HIF-2α.

In summary, consistent with our previous work in human cartilage explants we report that hypoxia has two mechanisms of chondroprotection in porcine cartilage explants: anabolic / anti-catabolic effects. Stabilisation of HIF-α isoforms increased expression of key cartilage master regulator transcription factor Sox9 and key cartilage matrix gene Col2a1 at the mRNA level. In addition, stabilisation of HIF-α isoforms inhibits aggrecan degradation in IL-1α stimulated and unstimulated porcine cartilage explants suggesting that hypoxia has an anti-catabolic effect in porcine cartilage explants in both normal and inflammatory settings. The use of unstimulated porcine cartilage explants gives our data high clinical relevance and gives a more accurate representation of what may be expected *in vivo*. Finally, analysis of gene expression revealed that Aggrecanase *Adamts5* was downregulated in

response to hypoxia and catabolic-regulator Timp3 was upregulated suggesting a possible mechanism by which hypoxia inhibits aggrecan degradation in porcine articular.

5.6.2 Hypoxia and the mouse chondrocyte phenotype

Here we report that inhibition of mouse HIF-targeting hydroxylase enzymes using hypoxia and dimethyloxaloylglycine (DMOG) did not significantly affect Sox9 expression in either mouse cartilage explants or isolated chondrocytes. Key extracellular matrix protein *Col2a1* was found to be largely unaffected by hypoxia. DMOG induced upregulation of Phd2 mRNA (a hypoxia inducible gene) and this was used as a positive control of HIF- α isoform stabilisation. Specific depletion of Phd2 in passaged mouse chondrocytes using siRNA did not appear to regulate Sox9 protein levels further supporting the lack of hypoxic regulation of the chondrocyte phenotype.

Hif-1 α ^{-/-} mice die *in utero* as early as E8.5 exhibiting multiple morphological defects demonstrating that HIF-1 α is essential for embryonic development (Compennolle et al., 2003; Iyer et al., 1998; Ryan et al., 1998). Similarly, HIF- β ^{-/-} mice die *in utero* between 9.5 and 10.5 days gestation from primarily from defects in placenta angiogenesis (Kozak et al., 1997; Maltepe et al., 1997). Schipani and colleagues demonstrated that the mouse developmental growth plate is hypoxic and that HIF-1 α is essential for proliferation and survival of murine growth plate chondrocytes *in vivo*. Cartilage-specific HIF-1 α depletion led to cell death within articular and growth plate chondrocytes resulting in long bone, rib and vertebral body defects coupled with decreased expression of the CDK inhibitor p57 (Schipani et al., 2001). It was concluded that HIF-1 α is essential for growth plate development and chondrocyte survival in response to hypoxic conditions.

Scortegagna and colleagues demonstrated that *Hif-2 α ^{-/-}* mice survive but have substantially shorter lifespan, lower body weight, overt cardiac hypertrophy, mitochondrial dysfunction, skeletal myopathy and hepatosteatosis compared with *Hif-2 α ^{+/+}* providing evidence that while HIF-2 α is not embryonic lethal, it plays an important functional role and is not redundant (Scortegagna et al., 2003). Similarly, homozygous knockout of HIF-2 α in the limb bud mesenchyme caused a modest delay in endochondral bone development at E17.5 (Araldi et al., 2011). Others have shown that homozygous deficient *Hif-2 α ^{-/-}* mice die at the early embryonic stage (Tian et al., 1998). A recent publication in Nature Medicine

reported that heterozygous deficient *Hif-2 α* ^{+/-} mice grew without abnormalities of major organs but showed mild dwarfism compared to wild-type littermates (Saito et al., 2010). Heterozygous deficient *Hif-2 α* ^{+/-} mice have a reduced limb length (8-16% shorter), increased hypertrophic and proliferation zones suggesting a role of *Hif-2 α* in endochondral ossification and bone growth in mouse (Saito et al., 2010).

Hypoxia has been shown to increase cartilage matrix deposition in murine epiphyseal chondrocytes. The deletion of the pVHL (which would stabilise both HIF-1 α and HIF-2 α) results in significantly reduced chondrocyte proliferation rate, ~1.4 fold increase in *Col2a1* mRNA expression and the presence of atypical large cells within the resting zone (Pfander et al., 2004b). Pfander and colleagues also isolated and cultured epiphyseal chondrocytes from wild-type and *Hif-1 α* ^{-/-} mice. They reported that the exposure of wild-type cells to 0.5% oxygen increased *Col2a1* protein levels ~2 fold and this hypoxic induction was lost in *Hif-1 α* ^{-/-} cells (Pfander et al., 2003). *Aggrecan* mRNA and protein levels were unchanged upon exposure to hypoxia (Pfander et al., 2003).

Ren and colleagues isolated mouse chondrocytes from the hips and knees of 3- or 4-day old C56BL/6 mice. All mice possessed homologous loxP sites flanking the HIF-1 α locus. Mouse chondrocytes were exposed to hypoxic conditions and the upregulation of *Col2a1* (~1.5 fold) and *Aggrecan* (~1.4 fold) observed via real-time PCR (Ren et al., 2008). When the chondrocytes were infected with an adenovirus containing Cre recombinase (creating *Hif-1 α* ^{-/-} cells) this hypoxia induced extracellular matrix synthesis was lost. Ren and colleagues concluded that the hypoxia induced cartilage matrix synthesis was HIF-1 α dependent (Ren et al., 2008)(Ren et al., 2008).

Work by Amarilio and colleagues examined the expression of tissue specific transcription factor Sox9 under hypoxic conditions in mouse mesenchymal cells derived from E11.5 *Hif1 α* floxed mouse embryos infected with AdCre or AdGfp. Low oxygen tensions increased Sox9 expression (~2 fold) in control cells and this hypoxic induction of Sox9 was lost in *Hif-1 α* depleted cells. This hypoxic induction of Sox9 was not confirmed at the protein level. Conversely, the overexpression of *Hif-1 α* induced an increase in Sox9 expression (Amarilio et al., 2007). Chromatin immunoprecipitation provided evidence for direct recruitment of HIF-1 α to the Sox9 promoter (Amarilio et al., 2007). Hypoxia

upregulated *Col2a1* (~1.7 fold) and *Aggrecan* (~2.3 fold) at the mRNA level in mouse mesenchymal cells and this hypoxic induction is lost in *Hif-1α* depleted cells (Amarilio et al., 2007).

Saito and colleagues recently overexpressed HIF-2α in a mouse chondrogenic cell line (ATDC5 cells) treated with normoxia and found overexpression of HIF-2α significantly increased mRNA levels of *Col2a1* (~2 fold) and *Aggrecan* (~2 fold). ATDC5 cells transfected with siRNA against Hif-2α did not show a significance decrease in *Col2a1* or *Aggrecan* mRNA levels compared to control (Saito et al., 2010). However this is expected as experiments were performed under normoxic conditions HIF-2α would not be stabilised at 20% oxygen due to hydroxylation of its proline residues by the PHD enzymes.

Although hypoxia has been shown to increase cartilage matrix deposition in murine chondrocytes this reported hypoxic induction is far weaker than we have reported in HACs. Isolated and cultured mouse chondrocytes exposed to hypoxia typically upregulate *Col2a1* mRNA 1.5 – 2 fold and these values are consistent with our murine data (~1.5 fold) (Amarilio et al., 2007; Pfander et al., 2003; Pfander et al., 2004b; Ren et al., 2008; Saito et al., 2010). *Aggrecan* mRNA levels have been reported unchanged upon exposure to hypoxia or upregulated between 1.4 – 2.3 fold at the mRNA level (Amarilio et al., 2007; Pfander et al., 2003; Pfander et al., 2004b; Ren et al., 2008; Saito et al., 2010). In contrast HACs treated with hypoxia upregulate *Col2a1* 4 - 12 fold and *Aggrecan* 2 – 5 fold at the mRNA level (Lafont et al., 2007; Murphy and Polak, 2004; Thoms and Murphy, 2010).

The reasons for these differences between human and porcine cartilage on the one hand and mouse cartilage on the other may be due to the age of the tissue studied (development vs. adult) and also anatomical differences.

Addressing the first point initially, many studies focus on mouse development, mesenchymal stem cell differentiation and limb bud formation. As embryos grow, cells quickly move beyond the diffusion distance of oxygen; limb bud formation and endochondral ossification occur in an avascular and therefore hypoxic environment. HIF-α isoforms are clearly necessary for embryonic development and Schipani and colleagues demonstrated that HIF-1α is essential for normal growth plate development and chondrocyte survival

(Compernelle et al., 2003; Iyer et al., 1998; Ryan et al., 1998). However, is mouse adult cartilage hypoxic and can mouse developmental data be extrapolated to adult human articular cartilage?

All species' articular cartilage is avascular, however, despite this similarity there are key differences in joint loading, cell density and cartilage thickness. Differences in body weight (72kg human, 90kg porcine and 0.025kg mouse) and stance (biped versus quadruped) play a significant role in joint mechanics and loading (Adams, 2006). Murine cartilage has ~23 fold greater cell density than human cartilage. Finally human and porcine cartilage in the major joints is several millimetres thick (~2.26mm human, ~1.2mm porcine) whilst mouse cartilage is only a few cell layers thick (~0.058mm) (Brighton and Heppenstall, 1971; Lund-Olesen, 1970; Stockwell, 1971; Zhang et al., 2011).

One reason for species differences between cartilage in larger animals (human and porcine) and mouse may be cartilage thickness. It is logical to assume that articular cartilage from larger animals has a much lower oxygen tension and chondrocytes within the tissue are exposed to more severe and chronic hypoxia than in smaller animals. Presumably this is why hypoxic induction of cartilage matrix genes in human, porcine and bovine chondrocytes is much stronger than in mouse chondrocytes (Domm et al., 2002; Lafont et al., 2007; Murphy and Polak, 2004; Murphy and Sambanis, 2001; Thoms and Murphy, 2010).

Immunohistochemistry of healthy human articular cartilage reports detectable levels of HIF-1 α and HIF-2 α protein (Coimbra et al., 2004; Yudoh et al., 2005). Immunohistochemical detection of HIF- α isoforms in human osteoarthritic cartilage suggests a decrease in HIF-2 α and an increase in HIF-1 α (Bohensky et al., 2009; Coimbra et al., 2004; Yudoh et al., 2005). In contrast, Yang and colleagues report that an absence of detectable HIF-2 α protein in undamaged articular cartilage of CBA/CaCrI and C57BL/6 mice. Saito and co-workers report very faintly detectable levels of HIF-2 α protein in undamaged articular cartilage of C56BL/6 mice. HIF-1 α detection was also reported in the undamaged articular cartilage of 12-week old BALB/C mice (Gelse et al., 2008).

The absence of a severe chronic hypoxic signal in mouse cartilage would explain the lack of permanent HIF-2 α isoform stabilisation. Differences between species may be due to

scale. Perhaps larger animals adapted this permanent HIF-2 α isoform stabilisation as a signal to promote the chondrocyte phenotype and enhance matrix deposition whereas mouse did not

In summary, whilst mouse articular cartilage has some similarities to human articular cartilage, there are key species differences in the way chondrocytes respond to hypoxia. Sox9 and key cartilage matrix gene *Col2a1* are not regulated by hypoxia in mouse articular chondrocytes. These species differences between human, porcine and mouse may be due to how larger animals have adapted to take advantage of the chronic hypoxic stimulus present in their thicker articular cartilage. Our data suggests that the inhibition of PHD enzymes to promote the chondrocyte phenotype and enhance matrix deposition is of limited use in mouse models and future pre-clinical work should focus on larger animals (e.g., pigs, cows, sheep).

Chapter 6

Conclusions, Further Work and Directions

6.1 Summary

We report that PHD2 is the dominant PHD enzyme in human articular chondrocytes (HACs) responsible for regulating both HIF-1 α and HIF-2 α . Consistent with what we have previously reported in HACs (Lafont et al., 2007), hypoxia promotes an anabolic response in freshly isolated chondrocytes and human cartilage explants. We also here demonstrate that specific depletion of HIF targeting hydroxylase PHD2 in freshly isolated and passaged HACs significantly upregulates the cartilage master regulator, transcription factor (SOX9) and key matrix genes (*COL2A1*, *COL9A1*, *COL11A2*, and *AGGRECAN*).

The use of non-specific hydroxylase inhibitor dimethyloxallylglycine (DMOG) upregulates SOX9 expression in HACs but inhibits collagen prolyl-4-hydroxylase function leading to suppression of type II collagen secretion. This illustrates the importance of HIF-specific hydroxylase inhibition and demonstrates the adverse effects of non-specific hydroxylase inhibition on collagen processing. Crucially, it is particularly relevant to cartilage as it is a highly collagen rich tissue. Interestingly, we observed that PHD2 inhibition upregulates key pro-Lysyl hydroxylases involved in collagen formation implying that PHD2 inhibition offers a novel means to enhance cartilage repair not just by upregulating SOX9 and key cartilage matrix proteins but through upregulation of enzymes involved in pro-collagen fibril formation.

Here we demonstrate that hypoxia (predominantly through the stabilisation of HIF- α isoforms) inhibits aggrecan degradation in IL-1 α stimulated and unstimulated human cartilage explants suggesting that hypoxia also an anti-catabolic effect in human cartilage explants. Further investigation in human tissue (cartilage explants) and HACs suggested that the observed anti-catabolic effects may be mediated by the downregulation of key proteases responsible for matrix turnover (ADAMTS5 and MMP13) and the upregulation of the metalloproteinase inhibitor TIMP3. Moreover, we provide clear evidence that hypoxia and specifically the stabilisation HIF-1 α (rather than HIF-2 α) has an anti-catabolic effect in human articular cartilage. Therefore hypoxia has dual, complimentary mechanisms of chondroprotection in human articular cartilage: a anabolic effect (mediated predominantly by HIF-2 α) and an anti-catabolic effect (predominantly mediated by HIF-1 α). Using an RNAi

approach we show that depletion of PHD2 enhances this anti-catabolic effect in both normoxia and hypoxia.

Crucially, we report that PHD2 displays significant activity even in 1% oxygen, since depletion of PHD2 under such conditions resulted in greatly increased levels of both HIF- α isoforms and further enhanced anabolic/anti-catabolic gene expression. 1% oxygen tension represents the lower end of the range of oxygen tensions in cartilage. By inhibiting PHD2 we are able to upregulate HIF-1 α /HIF-2 α (and subsequently increase the anabolic/anti-catabolic effect) to levels in excess of those achieved by such physiological hypoxic conditions. This opens novel, exciting possibilities of manipulating the endogenous oxygen sensing pathways in order to promote articular chondrocyte function, inhibit matrix destruction, enhance cartilage synthesis and to stimulate repair. Phrased concisely: Inhibiting PHD2 allows us to enhance HIF-signalling pathway in cartilage above physiological levels allowing us to do better than nature.

We also observed that IL-1 α stabilises HIF-1 α even under normoxic conditions and IL-1 α decreases HIF-2 α protein levels under hypoxic conditions in HACs. The IL-1 α induced stabilisation of HIF-1 α in the present study was partially suppressed by SB202190, an inhibitor p38 MAP kinase and therefore believed to be partially p38 MAPK dependent. However, the exact mechanism of stabilisation remains unknown.

Consistent with our findings in human cartilage explants we report that hypoxia has two mechanisms of chondroprotection in porcine cartilage explants: anabolic and anti-catabolic. Hypoxia-induced stabilisation of HIF- α isoforms increased expression of key cartilage master regulator transcription factor Sox9 and key cartilage matrix gene *Col2a1* at the mRNA level. In addition, stabilisation of HIF- α isoforms inhibits aggrecan degradation in IL-1 α stimulated and unstimulated porcine cartilage explants suggesting that hypoxia has an anti-catabolic effect in porcine cartilage explants. Finally, analysis of gene expression revealed that Aggrecanase *Adamts5* was downregulated in response to hypoxia while the endogenous protease inhibitor *Timp3* was upregulated suggesting a possible mechanism by which hypoxia inhibits aggrecan degradation in porcine cartilage.

Whilst mouse articular cartilage has obvious similarities to human articular cartilage there are key species differences in the way the resident chondrocytes respond to hypoxia. Sox9 protein and *Col2a1* mRNA levels were not found to be regulated by hypoxia. These species differences between human and porcine on the one hand, and mouse on the other, perhaps illustrate how larger animals have adapted to take advantage of chronic the hypoxic stimulus present in their cartilage. These species differences reported in this thesis should inform our approach to future clinically orientated studies.

6.2 Future work

6.2.1 Quantitative data from western blotting

Western blotting is typically used for qualitative purposes. Furthermore, it can be difficult to deduce changes at the protein level when either there is high variability in α -tubulin loading control or there is no loading control at all (ARGSV neoepitope release). In order to confirm our results we intend to provide quantitative data that can be statistically tested. Western blotting films will either be scanned or PVDF blots analysed using a chemiluminescence camera. Quantitative values of protein levels will be determined by dividing the densitometry value of the desired protein by the α -tubulin loading control. This will provide quantitative data which can be statistically analysed to determine if changes in protein amounts are significant. This method assumes that the bands analysed are within the linear range of each immunosignal (not underexposed or oversaturated). This will enable us to more accurately confirm changes at the protein level.

6.2.2 Use of more physiological oxygen tensions

Here we defined hypoxia as the level of molecular oxygen at which HIF- α isoforms become stabilised ($\leq 5\%$ O₂) and defined normoxia as level of oxygen under normal atmospheric conditions (20% O₂). As previously mentioned (see Chapter 1) we acknowledge that these are not levels of oxygen normally experienced *in vivo* as the levels of molecular oxygen in vascularised tissues may vary between 10-14% O₂. This raises interesting questions about the therapeutic applications of our results as normoxic data may be considered artificially high or supra-physiological. We intend to repeat PHD2 knockdown at a range of oxygen tensions 20%, 10%, 5% in order to examine if PHD2 enzyme inhibition

enhances chondrocyte function at these oxygen levels. We may also be interesting to examine the effect of anoxia (0% O₂) on chondrocyte function.

6.2.3 Examining the effect of PHD inhibition in osteoarthritic cartilage

Here we report the beneficial effect of PHD2 inhibition in healthy human articular cartilage and HACs. In order to confirm the therapeutic application of PHD inhibition we intend to repeat our experiments in osteoarthritic cartilage explants and osteoarthritic chondrocytes. In this thesis our donors ranged in age from 8-55 years, in future work it would be particularly interesting to focus on more elderly, osteoarthritic donors (>60 years) as this would more accurately represent our target population.

6.2.4 Use of clinically relevant animal models

We reported in Chapter 5, whilst there are some similarities, mouse articular chondrocytes do not respond the same way to hypoxia as human articular chondrocytes. Specifically, hypoxia does not promote the differentiated chondrocyte phenotype or enhance matrix deposition in mouse articular chondrocytes. Sheep are often used in cartilage repair studies, while cow and pig cartilage shows a similar hypoxia responsiveness to human (Domm et al., 2002; Kim et al., 2009a; Lafont et al., 2007; Thoms and Murphy, 2010; Zhang et al., 2011). Therefore, more suitable animal models to investigate the effectiveness of PHD-specific inhibitors in a preclinical setting would be these larger animals - porcine, bovine or ovine models.

6.2.5 Regulation of collagen prolyl-4-hydroxylases by hypoxia

Collagen prolyl-4-hydroxylases (C-P4Hs) are tetramers (consisting of 2 α subunits and 2 β units) responsible for catalysing the formation of 4-hydroxyproline by hydroxylation of –X-Pro-Gly– triplicates within the pro- α collagen chains. Like HIF-targeting hydroxylases collagen prolyl-4-hydroxylases use 2-oxoglutarate and oxygen as substrates (iron and ascorbate as cofactors) to hydroxylate proline residues and this hydroxylation is essential for the formation of intramolecular hydrogen bonds and subsequent stable triple-helical formation within the endoplasmic reticulum (Jimenez et al., 1973; Kukkola et al., 2003; Myllyharju, 2003; Van Den Diepstraten et al., 2003).

It has been demonstrated that hypoxia increases expression of collagen prolyl-4-hydroxylases α and β subunits in primary human articular chondrocytes and this upregulation was abolished by treatment with 2-Methoxestradiol (2ME2) (Grimmer et al., 2006; Hofbauer et al., 2003). It will be of interest to investigate the hypoxic induction of C-P4H α 1, C-P4H α 2 and C-P4H β in primary and passaged HACs. In addition, we intend to determine if this hypoxic upregulation is HIF-1 α or HIF2 α dependent and investigate if levels of C-P4H α 1, C-P4H α 2 and C-P4H β can be further enhanced by PHD2 depletion. This would provide further evidence for the beneficial effects of PHD2 inhibition; enhancing both COL2A1 mRNA and the cellular machinery necessary for collagen post-translational processing.

6.2.6 Regulation of HIF- α activity by FIH-1

The asparaginyl hydroxylase Factor Inhibiting HIF (FIH-1) regulates the transcriptional activity of HIF- α . FIH-1 hydroxylates a specific asparaginyl residue within the c-terminal transactivation domain of the α -subunit. This hydroxylation disrupts the binding of the CBP/p300 coactivator and inhibits HIF transcriptional activity. We have previously demonstrated we can deplete FIH-1 expression using RNAi in passaged HACs achieving a 97% knockdown compared with luciferase control. In order to investigate the effects in chondrocytes we intend to knockdown the expression of FIH-1 under normoxic (20% oxygen) and hypoxic (1% oxygen) conditions in primary and passaged human articular chondrocytes and monitor the expression of HIF-target genes (e.g., *SOX9*, *MIG6*, *VEGF*, *GLUT1*), in addition to binding of HIFs to isolated HREs.

6.2.7 Regulation of key matrix metalloproteinase ADAMTS5 by PHD2

In the present study we found that hypoxia significantly downregulates key catabolic genes *ADAMTS5* and *MMP13* at the mRNA level in cartilage explants and isolated HACs. This downregulation could be further enhanced by PHD2 depletion in HACs. Interestingly, *ADAMTS5* hypoxic downregulation was found not to be HIF-1 α or HIF-2 α dependent.

We previously mentioned that PHD2's ability to interact with non-HIF targets has been hypothesised. *Phd2*^{-/-} mice have severe heart defects without the accumulation of either HIF-1 α or HIF-2 α isoforms in heart tissue. We intend to investigate this direct or indirect interaction between PHD2 and *ADAMTS5*.

6.2.8 Mechanism of HIF-1 α stabilisation by IL-1 α stimulation

Here we report that treatment of HACs with proinflammatory IL-1 α stabilises HIF-1 α protein even under normoxic conditions. The IL-1 α induced stabilisation of HIF-1 α was partially suppressed by SB202190, an inhibitor p38 MAPK.

Kim and colleagues demonstrated that tristetraprolin (TTP), a regulator of mRNA stability is hypoxia inducible and binds to the 3'-UTR of HIF-1 α via AU-rich elements and downregulates the transcript's stability. It will be of interest to ascertain if the IL-1 α induced stabilisation of HIF-1 α in HACs is due to mRNA stability changes and to elucidate the possible role of TTP in this process.

6.3 Final Discussion and Therapeutic applications

Loss of articular cartilage due to age or focal cartilage defects resulting from sports related trauma are difficult to repair, as once damaged cartilage has a limited intrinsic matrix remodelling and repair capacity. In addition to sports injuries another major cause of cartilage loss is the age-related disease osteoarthritis. Although the mechanisms of initiation are unclear (and probably diverse) osteoarthritis is a chronic disease believed to be caused by an imbalance between net anabolic (matrix synthesis) and net catabolic (matrix degradation) activity. This leads to the progressive loss of articular cartilage over time and finally results in joint pain, loss of function and reduced quality of life.

6.3.1 Current limitations of clinical interventions

Focal cartilage lesions can be managed using a number of procedures including lavage, microfracture (generating fibrocartilage scar tissue plug) and nonbiological implants (metallic or cell-free polymer plugs) (Stroh et al., 2011). However these tissue plugs do not have the same biomechanical properties as normal articular cartilage and may degrade over time.

There is no current effective disease modifying pharmacological therapy for osteoarthritis. Conventional treatments are aimed at treating the symptoms and include physical therapy, suppression of inflammation, analgesia and in advance stages of OA, surgical intervention (joint replacement with a prosthesis) (Conaghan et al., 2008).

Catabolic aggrecanases ADAMTS-4 and ADAMTS-5, and collagenases such as MMP-13 have previously been identified as responsible for joint destruction and identified as promising targets to curtail OA disease pathology. Unfortunately trials of small molecule inhibitors targeting these catabolic factors have produced disappointing results and stress the importance of minimising inhibitor toxicity, limiting side effects of the inhibitor and the need for specificity in targeting individual metalloproteinases responsible for joint destruction. (Burrage and Brinckerhoff, 2007; Chuma et al., 2004; Flannery, 2010; Fosang and Little, 2008; Little et al., 2007; Nagase et al., 2006; Troeberg et al., 2008).

Osteoarthritis is a disease of irreversible articular cartilage degradation and leads to chronic disability. Conventional therapeutics do not inhibit the underlying pathologic tissue catabolism or encourage tissue repair. Indeed, even if effective, aggrecanase and collagenase inhibitors might only solve half the problem. They may have a strong anti-catabolic effect, preventing further cartilage degradation, however, patients often present physical symptoms late in the disease after there has already been significant cartilage destruction. Existing catabolic inhibitors are unable to reverse previous matrix loss and do not have a strong anabolic effect.

6.3.2 Novel dual anabolic and anti-catabolic therapeutic approach

We propose a novel therapeutic approach manipulating the endogenous oxygen sensing pathways in chondrocytes. We aim to inhibit PHD2 and stabilise both HIF-1 α and HIF-2 α . We have demonstrated that hypoxia stabilises HIF-1 α and HIF-2 α in chondrocytes and that each isoform has distinct targets providing two complementary mechanisms of chondroprotection: upregulation of anabolism (predominantly via HIF-2 α) and suppression of catabolism (predominantly via HIF-1 α). Theoretically, inhibition of HIF-targeting PHD2 provides a novel mechanism to promote chondrocyte function *in vivo* by suppressing MMP and ADAMTS mediated matrix degradation and reversing matrix destruction by increasing expression of cartilage extracellular matrix proteins and repairing articular cartilage.

Crucially, through this work we demonstrate that PHD2 displays significant activity even at the low end of the physiological oxygen tension range in human articular cartilage (1% oxygen), since depletion of PHD2 under these conditions resulted in greatly increased levels of both HIF- α isoforms. Thus inhibiting PHD2 can fully activate, in a controlled manner, the HIF-signalling pathway in cartilage allowing us to enhance its effects above physiological conditions. Furthermore, our work was carried out in healthy human cartilage explants and healthy freshly isolated and passaged human articular chondrocytes. Healthy human cartilage is a rare and clinically invaluable tissue. Unfortunately, human osteoarthritic cartilage samples are often unusable due to the advanced, end-stage degradative changes. In addition, we believe our primary human cell/tissue data are more applicable to the clinical setting than, for example, murine cartilage or chondrocyte cell line studies.

6.3.3 PHD enzyme-specific inhibition

Experiments with the general hydroxylase inhibitor dimethyloxallylglycine (DMOG) highlight the importance of development of HIF-specific hydroxylase inhibitors with regard to the collagen rich tissue cartilage. Gelse and colleagues carried out a study injecting the joints of STR/ORT mice with the non-specific hydroxylase inhibitor dimethyloxallylglycine (DMOG). STR/ORT mice spontaneously develop osteoarthritis and injection of DMOG was unable to prevent the formation of severe arthritis. Although Sox9 was up-regulated in response to DMOG injection, immunohistochemistry and immunofluorescence revealed incomplete post-translational processing and intracellular accumulation of type II collagen in chondrocytes. Similarly, our work demonstrates that whilst DMOG upregulates SOX9 in HACs it inhibits type II collagen secretion (Thoms and Murphy, 2010). It was concluded that this impaired secretion of collagen was due to inhibition of collagen prolyl-4-hydroxylases by the non-specific hydroxylase inhibitor DMOG (Berg et al., 1983; Gelse et al., 2008). Presumably, addition of DMOG leads to misfolded, unhydroxylated procollagens that are retained within the cell. This stresses the importance of developing specific PHD inhibitors (or at least HIF-specific prolyl-4 hydroxylases) which do not inhibit key collagen prolyl-4-hydroxylases which are essential for correct collagen posttranslational modification.

Lionard and colleagues recently investigated the role of specific PHD enzyme inhibition *in vivo* in order to stabilise HIF- α isoforms and promote post-ischaemic

neovascularisation. The right femoral artery was ligated in mice in order to create an ischaemic event and the mice injected with plasmids encoding for short hairpin RNA directed against PHD1, PHD2, PHD3 or a control. Transfection was associated with a specific, transient decrease in targeted PHD enzyme mRNA, an increase in HIF-1 α levels and proangiogenic factors (VEGF, eNOS) (Loinard et al., 2009). Microangiography revealed that 14 days after transfection, PHD2 and PHD3 depletion increased capillary density by 1.8- and 2.1-fold, and foot perfusion by 1.2- and 1.4-fold, respectively, compared with controls. HIF-2 α levels were not investigated; however this work suggests exciting possibilities for therapeutic use of specific-PHD enzyme inhibition (Loinard et al., 2009).

The use of shRNA *in vivo* to promote cartilage repair is appealing as it would provide a PHD specific depletion without inhibiting key collagen proyl-4-hydroxylases. Song and colleagues have previously demonstrated that ADAMTS4 and ADAMTS5 mRNA expression could be depleted in human cartilage explants *in vitro* using siRNA (Song et al., 2007). However, the extensive cartilage extracellular matrix make the tissue resistant to transfection requiring large amounts of siRNA (100nM) and results in inefficient knockdowns (Song and colleagues could only achieve a 60-75% depletion at the mRNA level). This level of PHD enzyme depletion may not be sufficient to stabilise HIF- α *in vivo*, the depletion would only be transient and the high amount of siRNA could have adverse side effects.

Therefore a PHD inhibitor would preferably be a small molecule inhibitor which is; PHD-enzyme (and preferable PHD2) specific and does not suppress collagen proyl-4-hydroxylase function. In order to investigate the therapeutic applications of PHD2 inhibition the following issues would need to be considered:

6.3.4 Small molecule inhibitors of PHDs

Hydroxylase enzyme small molecule inhibitors are already being developed by several biotech and pharmaceutical companies and offer an exciting therapeutic approach for the treatment of: anaemia, wound healing, thalassemia and inflammatory disorders (Fraisl et al., 2009; Yan et al., 2010). Iron chelator desferoxamine, a non-specific inhibitor of the PHD enzymes has historically been used as a treatment for anaemia. Similarly, HIF- α regulation of vascular endothelial growth factor (VEGF), hypoxia tolerance and ischaemic preconditioning makes PHD enzyme inhibition an attractive target for therapeutic

angiogenesis. It has been suggested that PHD enzyme inhibition could be used in the event of blood vessel occlusion (e.g., stroke and myocardial infarction) to promote the formation of new blood vessel formation (angiogenesis) and reperfusion of the ischaemic tissue or promote hypoxia tolerance and cell survival in cells downstream of the occlusion in order to protect against acute ischaemic damage (Fraisl et al., 2009).

In a recent review of patent applications it was reported that many companies including GlaxoSmithKline, FibroGen, Bayer and Merck & Co have submitted patent applications for PHD inhibitors as HIF-stabilisers (reviewed by (Yan et al., 2010)). FG-2216 and FG-4592 are two examples of oral PHD inhibitors which have completed Phase II clinical trials. Both inhibitors are designed by FibroGen for the treatment of anaemia and reports indicate that inhibitors significantly increase levels in haemoglobin and erythropoietin levels in patients with chronic kidney disease (Urquilla *et al.*, 2004). Other examples of small molecule inhibitors include: ASP-1517 (Astellas), GSK1278863A (GlaxoSmithKline) and AKB-6548 (Akebia Therapeutics) which are all currently in Phase I clinical trials (Yan et al., 2010).

Cartilage is a collagen rich tissue, therefore it is essential that PHD inhibition be achieved without inhibiting 2-oxoglutarate dependent collagen prolyl-4-hydroxylases (C-P4H α 1, C-P4H α 2 and C-P4H β). Amgen claim to have developed several quinolone and azaquinolone based compounds which selectively inhibit PHD2, without significantly affecting collagen prolyl-4-hydroxylase function. Compound 42 is an example of one of these compounds and is reported in a review by Yan and colleagues to have the following selective properties IC₅₀: PHD2 = 0.003 μ M; C-P4H α 1 > 40 μ M; C-P4H α 2 = 34.3 μ M (Yan *et al.*, 2010). No experimental data could be found regarding this compound.

6.3.5 Side effects and complications of PHD inhibition

Concerns of the unwanted side effects of PHD inhibitors can be linked to either the long term stabilisation of HIF- α isoforms outside of articular cartilage or the activation of undesirable genes by HIF- α isoforms in chondrocytes. Addressing the first point initially, aberrant PHD enzyme inhibition and HIF- α isoforms stabilisation in other tissues has been associated with polycythemia, cancer and hypoxic pulmonary hypertension (Yan et al., 2010).

Heterozygous mutation in the *PHD2* gene (H374R) is associated with heritable polycythemia (due to elevated EPO expression) and recurrent paraganglioma (rare neuroendocrine neoplasm) (Ladroue et al., 2008). Patients have very high haemoglobin levels (~23 g/dl vs. 13 g/dl WT) and are at an increased risk of serious thromboembolic events including myocardial infarction and stroke (Unger et al., 2010).

During periods of rapid cell division (eg: solid tumours) cells may outgrow their vascular supply. Cells are located sufficiently far from the blood vessel that they are beyond the diffusion distance of oxygen (~100-300µm) and as a result solid tumours often experience hypoxia. Stabilisation of HIF- α switches cells' to an anaerobic metabolism, stimulates new blood vessel formation and promotes cell survival. Hypoxia is a sign of advanced disease and associated with poor clinical outcome (Kim et al., 2009b; Ruan et al., 2009). Mutations in pVHL are linked with a heritable cancer syndrome predisposes patients to multiple vascularized tumours (Ivan et al., 2001). However, it is unclear whether HIF- α stabilisation itself is responsible for tumourigenesis or HIF- α stabilisation is due to the hypoxia microenvironment of solid tumours).

Recent work in mice and humans suggests that HIF- α stabilisation plays an important role in pulmonary hypertension (tissue remodelling of the vascular lumen leading to right ventricular hypertrophy) (Yildiz, 2009). HIF-1 α ^{+/-} and HIF-2 α ^{+/-} mice are resistant to vascular remodelling, pulmonary hypertension, right ventricular failure (Brusselmans et al., 2003; Yu et al., 1999). Consistent with work in mice, inhibition of PHD enzymes with DFO treatment in humans activates HIF and mildly elevates pulmonary hypertension (Balanos et al., 2002).

These negative side effects are intuitive if HIF- α isoforms are stabilised in vascularised tissues as this is a non-physiological stress. However we would not expect these effects in articular cartilage as hypoxia is a chronic state and inhibiting PHD2 would enhance HIF- α isoforms above current, physiological levels. Therefore, out of concern for the many possible effects described above for other tissues it is important to attempt to restrict HIF- α manipulation to the chondrocytes within the articular cartilage.

Addressing the second concern, HIF- α isoforms are known to regulate more than 100 genes via hypoxia response elements and have a diverse range of transcriptional targets ranging from energy metabolism, to apoptosis and angiogenic signalling (Schofield *et al.*, 2004). The stabilisation of HIF- α isoforms in HACs will promote the chondrocyte phenotype however, it will also upregulate all other HIF-target genes and may have undesirable consequences. It is important to highlight that hypoxia also upregulates many beneficial proteins including chondromodulin-1 (Chm-1) which stabilises the chondrocyte phenotype and has an anti-angiogenic effect in cartilage (Klinger *et al.*, 2011; Lafont *et al.*, 2008; Murphy, 2011).

6.3.6 Administration of PHD inhibitors

Due to possible side effects of systemic HIF stabilisation it is important to stabilise HIF- α isoforms only within articular cartilage. In order to achieve this we would suggest that the compound be administered via an intra-articular injection rather than oral (and systemic HIF- α stabilisation).

However, it is important to consider that an intra-articular injection of the compound would not necessarily be articular cartilage-specific. Logically PHD2 would also be inhibited in meniscal cartilage, synovial fibroblasts and possibly the fat pad and subchondral bone. Adesida and colleagues reported that hypoxia upregulates the expression cartilage matrix genes in human meniscus cells (Adesida *et al.*, 2006). However, hypoxia has been shown to upregulate vascular endothelial growth factor (VEGF) and pro-apoptotic BCL2/adenovirus E1B 19 kDa protein-interacting protein 3 (BNIP3) in osteoarthritic synovial fibroblasts (Barbara Muz, Data not published).

Osteoarthritis may be characterised by symptoms (pain, stiffness, crepitus, decreased mobility and deformity) or radiographic criteria (e.g., joint space narrowing) or both. Because cartilage is aneural symptoms do not often present until late-stage disease and confusingly structural changes such as joint space narrowing can often occur without painful symptoms.

This raises an interesting question, when should a PHD2 inhibitor be administered, and how frequently? Should it be given to healthy non-symptomatic patients to maintain cartilage structural integrity throughout life or only administered immediately after injury? Joint pain is a symptom of end stage disease, by which point has there already been too much articular cartilage destruction to initiate HIF- α mediated repair?

Studies in large animal models are initially needed to help better inform these important issues. Injections of an intra-articular PHD-inhibitor in humans would rely upon early detection using advanced imaging in order to identify early structural changes in a patient's cartilage.

6.3.7 Tissue Engineering

One exciting area of possible focal cartilage defect treatment is cell-based therapies; e.g., repair by autologous chondrocyte implantation. These therapies rely on the culture of human chondrocytes *in vitro* before they are seeded onto a scaffold and implanted. However, the chondrocyte phenotype is unstable *in vitro* and is lost in standard cell culture conditions (Benya et al., 1978; von der Mark et al., 1977). Chondrocytes cultured as a monolayer de-differentiate with passage. They lose their round shape and appear more fibroblastic like. Chondrocyte markers SOX9, type II collagen and aggrecan are downregulated and type I collagen is upregulated (Glowacki et al., 1983). Type I collagen has inferior mechanical properties and is found in fibrocartilage, rather than articular. The differentiated chondrocyte phenotype can be maintained by culturing chondrocytes in type II collagen matrices, 3-dimensional alginate scaffolds (alginate beads) or culturing cells under hypoxic conditions (Murphy and Sambanis, 2001; Shakibaei and De Souza, 1997; Stewart et al., 2007).

A small molecule PHD2 inhibitor would offer a novel way to restore the healthy phenotype of osteoarthritic chondrocytes *in vivo* or restoring the differentiated phenotype to passaged human chondrocytes *in vitro* (in for example, within a cartilage bioreactor). Alternatively, passaged human chondrocytes could be transfected with a construct expressing shRNA against PHD2 under a regulatable promoter (e.g., tamoxifen-inducible) before being implanted into lesions. This would enable regulation of shPHD2 expression and limit HIF- α manipulation to the cartilage lesion.

6.4 Conclusions

This thesis documents our efforts to investigate the regulation of HIF- α isoforms in human articular chondrocytes by prolyl hydroxylases domain enzymes (PHD1, PHD2, PHD3) with the aim of development of a novel therapeutic approach to promote chondrocyte function in cartilage.

Using RNAi we were able to identify PHD2 as the dominant PHD in HACs responsible for regulating both HIF-1 α and HIF-2 α . Knockdown of PHD2 alone was sufficient to stabilise both HIF1- α and HIF-2 α in normoxic conditions, and the levels of HIF-1 α and HIF-2 α in hypoxia could also be further enhanced with PHD2 depletion.

We report that depletion of PHD2 provides two mechanisms of chondroprotection in HACs. Stabilisation of HIF-2 α promotes the chondrocytes phenotype (via SOX9) and cartilage extracellular matrix synthesis (*COL2A1*, *COL9A1*, *COL11A2* and *AGGRECAN*). Complimentary to this anabolic effect, stabilisation of HIF-1 α has an anti-catabolic effect downregulating key matrix metalloproteinases (*ADAMTS5* and *MMP13*), upregulating metalloproteinase inhibitor (TIMP3) and is able to suppress aggrecan degradation in human cartilage explants. These results offer an exciting novel therapy to maintain and repair articular cartilage.

Chapter 7

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Chapter 8

Appendix

Inhibition of Hypoxia-inducible Factor-targeting Prolyl Hydroxylase Domain-containing Protein 2 (PHD2) Enhances Matrix Synthesis by Human Chondrocytes*

Received for publication, February 17, 2010, and in revised form, March 31, 2010 Published, JBC Papers in Press, April 19, 2010, DOI 10.1074/jbc.M110.115238

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Human articular cartilage is an avascular tissue, and therefore it functions in a hypoxic environment. Cartilage cells, the chondrocytes, have adapted to this and actually use hypoxia to drive tissue-specific functions. We have previously shown that human chondrocytes enhance cartilage matrix synthesis in response to hypoxia specifically through hypoxia-inducible factor 2 α (HIF-2 α)-mediated up-regulation of master regulator transcription factor SOX9, which in turn drives expression of the main cartilage-specific extracellular matrix genes. HIF- α isoforms are themselves regulated by specific prolyl hydroxylase domain-containing proteins, which target them for proteosomal degradation. In fact, prolyl hydroxylase domains are the direct oxygen sensors because they require molecular oxygen as a co-substrate. Here, we have identified PHD2 as the dominant isoenzyme regulating HIF-2 α stability in human chondrocytes. Moreover, specific inhibition of PHD2 using RNA interference-mediated depletion caused an up-regulation of SOX9 and enhanced extracellular matrix protein production. Depletion of PHD2 resulted in greater HIF-2 α levels and therefore enhanced SOX9-induced cartilage matrix production compared with the levels normally found in hypoxia (1% oxygen) implying that PHD2 inhibition offers a novel means to enhance cartilage repair *in vivo*. The need for HIF-specific hydroxylase inhibition was highlighted because treatment with the 2-oxoglutarate analogue dimethyloxalylglycine (which also inhibits the collagen prolyl 4-hydroxylases) prevented secretion of type II collagen, a critical cartilage matrix component.

Articular cartilage consists of a single cell type, the chondrocyte, which is solely responsible for the synthesis and maintenance of the extracellular matrix (1). The rigid nature of type II collagen fibrils present in the matrix confers tensile strength to the tissue, and the swelling pressure caused by water-saturated aggrecan molecules creates a compressive gel-like stiffness allowing the tissue to resist deformation and giving cartilage its ability to absorb shocks (1, 2). Due to its shock-absorbing and articulating functions, articular cartilage cannot afford a blood or nerve supply. The tissue is therefore dependent on diffusion of oxygen from the synovial fluid on one side and the vascularized underlying bone on the other. As a result,

articular cartilage (at least in larger animals and man) is maintained in a low oxygen environment (1–5% oxygen tension) throughout life (3–5). There is now significant evidence that hypoxia is a critical parameter in promoting the chondrocyte phenotype. It has been shown that hypoxia up-regulates key cartilage transcription factor SOX9 and increases expression of the main extracellular matrix genes in bovine and human chondrocytes (6–10).

The response of cells to hypoxia is mediated by hypoxia-inducible factors (HIFs),² heterodimeric transcription factors consisting of an α and β subunit (11). Both subunits are constitutively expressed at the mRNA level; however, the α subunit is regulated post-translationally by oxygen levels. In well oxygenated cells, the α subunit is hydroxylated on specific, conserved proline residues by HIF-targeting prolyl hydroxylase domain-containing proteins (PHD1, PHD2, and PHD3) (12–14). The PHD isoenzymes are a subfamily of dioxygenases that use molecular oxygen and 2-oxoglutarate as co-substrates to add hydroxyl groups to specific proline residues on the HIF- α subunit (15–17). The hydroxylated proline residues on HIF- α are recognized by von Hippel-Lindau protein of the E3 ubiquitin ligase complex, ubiquitinated and subsequently degraded by the 26 S proteasome (18). As intracellular oxygen concentrations are reduced, the activity of the PHDs is suppressed, and HIF- α isoforms are not hydroxylated and therefore escape proteosomal degradation and so heterodimerize with HIF-1 β , translocate to the nucleus, and bind to *cis*-regulatory hypoxia-response elements in target genes. Previous work in our laboratory has demonstrated that hypoxic induction of the key cartilage matrix genes in human chondrocytes is mediated specifically by the HIF-2 α isoform that up-regulates SOX9, leading to enhanced expression of the cartilage matrix genes (9).

Suppression of PHD enzymes leads to stabilization of HIFs and offers a potential treatment option for many ischemic disorders, such as peripheral artery occlusive disease, myocardial infarction, and stroke (19). We hypothesized that antagonizing specific PHDs could be a novel way of promoting cartilage matrix deposition from chondrocytes by increasing HIF-2 α stability and up-regulating key transcription factor SOX9. We investigated the role of each PHD isoenzyme in isolated healthy

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² The abbreviations used are: HIF, hypoxia-inducible factor; PHD, prolyl hydroxylase domain; RNAi, RNA interference; HAC, human articular chondrocyte; DMOG, dimethyloxalylglycine; DFO, desferrioxamine; siRNA, small interfering RNA; FIH, factor inhibiting HIF.

human articular chondrocytes adopting an RNAi-mediated approach. We provide strong evidence that specific inhibition of PHD2 enhances cartilage matrix production by chondrocytes, and these results thus identify PHD2-specific inhibition as a novel means to stimulate chondrocyte anabolism and therefore cartilage repair.

EXPERIMENTAL PROCEDURES

Cell Culture—Healthy human articular cartilage was obtained from patients after they provided informed consent and following local ethics committee guidelines. Samples of cartilage were obtained from patients following above knee amputations due to soft tissue sarcomas and osteosarcomas not involving the joint. Tissue was obtained from 21 patients (17 male, 4 female; aged 8–55 years, mean age 26.6 years). Cartilage was digested as described previously (9). Briefly, the cartilage was diced into small pieces and digested in collagenase overnight. Freshly isolated cells were initially seeded at a density of 8×10^3 cells/cm² in Dulbecco's modified Eagle's medium with 10% fetal calf serum. Chondrocytes were passaged at the time of confluency (~7 days) and re-seeded at 5×10^3 cells/cm².

Small Interfering RNA Transfection—Primary (P0) or passaged (P1–P3) human articular chondrocytes (HACs) were seeded at 5×10^3 cells/cm² in 6-cm dishes. Cultures were incubated at 37 °C, 20% oxygen, 5% CO₂ until 50% confluent (typically after 1 day). Lipofectamine 2000 (Invitrogen) was used to transfect cells with small interfering RNA (siRNA) at a final concentration of 10 nM in serum-free Opti-MEM I. *PHD1*, *PHD2*, and *PHD3* targeting siRNAs, which were used, are listed in Table 1. As a control, siRNAs against luciferase (Dharmacon, Lafayette, CO) were transfected in parallel. Four hours after transfection, the Opti-MEM was removed and replaced with Dulbecco's modified Eagle's medium containing 10% fetal calf serum pre-equilibrated at the appropriate oxygen tension (20 or 1%). Cells were subsequently incubated in each oxygen tension for 3 days.

RNA Isolation, Reverse Transcription, and Real Time PCR—RNA was extracted and prepared using the RNeasy mini kit (Qiagen, Crawley, UK) following the manufacturer's guidelines. cDNA was generated using a reverse transcription kit (Promega, Southampton, UK) and random primers from 0.5 μg of total RNA. Newly synthesized cDNA was diluted 5-fold in DNase-free water. Four percent of this cDNA was then used for real time PCR assays using TaqMan technology and a PCR Corbett Research thermocycler (Corbett Research, Australia). The $\Delta\Delta$ threshold cycle ($\Delta\Delta C_t$) method of relative quantitation was used to calculate relative mRNA levels for each transcript examined. The ribosomal protein gene (*RPLP0*) was used to normalize the data as it was not regulated by hypoxia in our system. Pre-developed primer/probe sets for the following genes were purchased from Applied Biosystems (Foster City, CA): *PHD1*, *PHD2*, *PHD3*, *FIH1*, *COL2A1*, *COL9A1*, *COL11A2*, *AGGRECAN*, *SOX9*, *VEGF*, and *RPLP0*.

Protein Extraction and Western Blotting—To detect HIF- α isoforms, cells were lysed immediately after termination of the experiment in urea lysis buffer (8 M urea, 10% glycerol, 1% SDS, 5 mM dithiothreitol, 10 mM Tris-HCl); otherwise, the cells were lysed in radioimmunoprecipitation assay buffer (100 mM

Trizma base, pH 7.4, 300 mM NaCl, 20 mM NaF, 2 mM EDTA, 0.2 mM β -glycerophosphate, 2%(w/v) sodium vanadate, 2% deoxycholate, 0.5% SDS, and 1% Nonidet P-40). A protease inhibitor mixture (Sigma) was added just prior to cell lysis. Cell lysates were sonicated for 5 s and spun at 12,000 rpm for 10 min at 4 °C to remove any cell debris. 20 μg of protein was resolved by SDS-PAGE, transferred onto polyvinylidene difluoride membranes for Western blotting, and visualized using the enhanced chemiluminescence method. To detect PHD3 and HIF-2 α , 40 μg of protein was used. Primary antibodies used were rabbit anti-SOX9 (Chemicon AB5535, 1:1000), rabbit anti-PHD2 (Abcam AB4561, 1:1000), mouse anti-PHD3 (clone 188e, a kind gift from Professor Chris Pugh, Oxford, UK), mouse anti-HIF-1 α (BD Transduction Laboratories, clone 54, 1:250), mouse anti-HIF-2 α (Santa Cruz Biotechnology Sc-13596, 1:250), and mouse anti- α -tubulin monoclonal antibody (Sigma, 1:4000).

For detection of secreted type II collagen, HACs were serum-starved for 2 days, 24 h following transfection. Approximately 1 ml of conditioned medium was precipitated with trichloroacetic acid, loaded on a 6% polyacrylamide gel, and blotted with anti-COL2A1 antibody (Chemicon MAB8887, 1:1000). The exact volume of media used was adjusted after standardization according to total protein content in the cell layer, measured using the Bradford assay.

Statistical Analysis—Data were compared using a one-way analysis of variance with Bonferroni post test. The software GraphPad Prism 4 (GraphPad Software, San Diego) was used to perform statistical analysis. Results are expressed as a mean $\pm$ S.E. Probability values less than 0.05 were considered significant.

RESULTS

PHD2 Is the Most Abundant PHD Isoenzyme, and PHD2 and -3 are Hypoxia-inducible in HACs—The mRNA levels of *PHD1*, *PHD2*, and *PHD3* under normoxic (20% oxygen) and hypoxic (1% oxygen) conditions were analyzed after normalizing the data using the housekeeping gene *RPLP0*. The relative abundance of each gene was calculated after assigning the expression level of *PHD3* at 20% oxygen to a value of 1. This method of calculating the relative abundance of the three PHD isoenzymes at the mRNA level makes the assumption that the efficiency of amplification between the different PCR primers is equal (in practice, all amplification efficiencies were similar, being over 90%). *PHD2* was the most abundant transcript of the three isoenzymes in both 20 and 1% oxygen (Fig. 1A). Interestingly, although *PHD3* had the most pronounced hypoxic induction (average 19.2-fold induction), it was the least abundant PHD enzyme at the mRNA level in both 20 and 1% oxygen (Fig. 1A). This is consistent with data at the protein level. PHD2 and PHD3 proteins were found to be induced by hypoxia (Fig. 1, B and C). In addition, 20 μg of PHD2 was visualized using enhanced chemiluminescence after 3 min of exposure to x-ray film, whereas 40 μg of PHD3 required 45 min of exposure to detect any protein (note, the top band in all PHD2 blots is nonspecific).

Depletion of PHDs in HACs—PHD1, PHD2, and PHD3 were specifically depleted in HACs using siRNA (Fig. 2, A–C, respec-

PHD2 Depletion Promotes Matrix Production by Chondrocytes

tively). Two different oligonucleotides were used for each gene (for sequences see Table 1). siRNA against luciferase (siLuc) was used as a control. Messenger RNA analysis by real time PCR revealed that PHD1 was depleted 72% (siPHD1 number 1) and 95% (siPHD1 number 2) on average compared with siLuc

levels. PHD2 was depleted 82% (siPHD number 1) and 86% (siPHD2 number 2) on average, and PHD3 was depleted by 87% (siPHD3 number 1) and 84% (siPHD3 number 2) on average. Western blot confirmed the depletion of PHD2 (Fig. 2D) and PHD3 (Fig. 2E) at the protein level. Knockdown of PHD3 was performed in the presence of the iron chelator desferrioxamine (DFO) as PHD levels were easily detectable in DFO, but they were relatively low in hypoxia and were undetectable in normoxia. The oligonucleotides that produced the greatest knockdown at the mRNA level were used in further experiments.

Depletion of PHD2 in Normoxia Stabilizes HIF-2 α and Up-regulates Master Chondrocyte Transcription Factor SOX9—In normoxia, HIF- α isoforms are rapidly degraded in the cell as there is sufficient oxygen for the HIF-targeting prolyl hydroxylases to target them for von Hippel-Lindau-mediated proteosomal degradation. However, the knockdown of PHD2 in HACs greatly stabilized both HIF-1 α and HIF-2 α in normoxia (20% oxygen) suggesting that PHD2 significantly contributes to HIF- α regulation (Fig. 3A). The knockdown of PHD1 also contributed to the stabilization of HIF-1 α in normoxia but to a much lesser extent than PHD2 depletion, and it had no detectable effect on HIF-2 α levels. PHD3 suppression had no detectable effect on levels of either HIF- α isoform in normoxia. Because depletion of PHD2 alone had such a pronounced effect on HIF-2 α stabilization, and we have previously shown that HIF-2 α specifically mediates hypoxic induction of the differentiated chondrocyte phenotype through up-regulation of master tissue-specific transcription factor SOX9 (9), we further explored PHD2 regulation of HIF-2 α isoform stability through reoxygenation experiments. PHD2-depleted HACs were cultured in hypoxia for 3 days before being exposed to 20% oxygen for 0, 5, 10, 20, 40, and 60 min prior to lysing. Small interfering

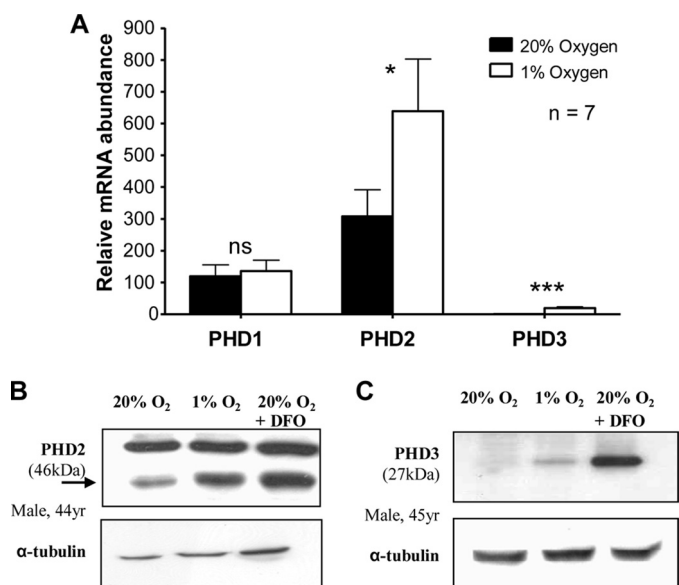


FIGURE 1. PHD2 is the most abundant PHD isoenzyme in HACs, whereas PHD2 and PHD3 are both hypoxia-inducible. Data are from passaged (P1–P3) HACs after culture for 3 days at 20% oxygen, 1% oxygen or treated with 10 nM of the hypoxia mimetic DFO. *A*, TaqMan-based data showing the relative mRNA abundance in cultured HACs of the PHD enzymes at the mRNA level in normoxic or hypoxic conditions (PHD3 mRNA levels in 20% oxygen was assigned a value of 1). Values are the means $\pm$ S.E. of seven independent experiments (i.e. HACs from seven different donors); ns = not significant; *, $p < 0.05$; ***, $p < 0.001$. *B*, Western blot showing increased PHD2 levels (lower band) in hypoxia (1% oxygen), and after treatment with DFO. *C*, PHD3 levels, like PHD2 were increased by hypoxia and DFO.

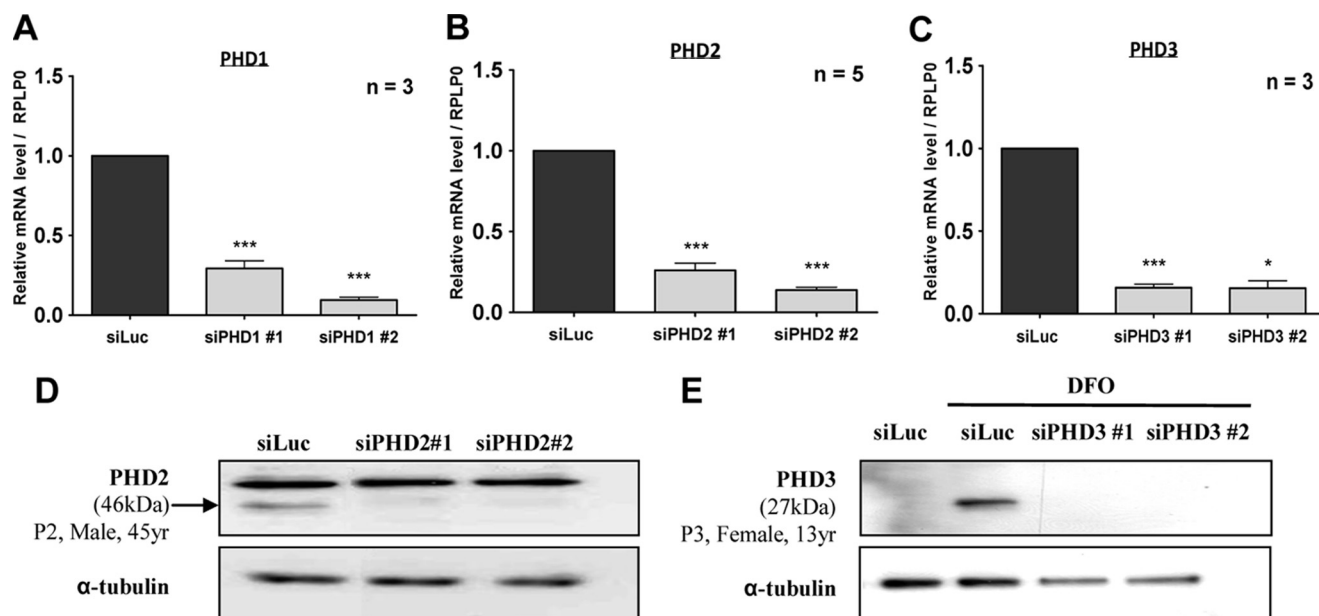


FIGURE 2. RNAi-mediated depletion of PHD1/2/3 in HACs. TaqMan real time PCR data showing mRNA depletion of PHD1 (*A*), PHD2 (*B*), and PHD3 (*C*) in passaged HACs after 3 days in culture. Cells were transfected with 10 nM siRNA directed against each PHD, and an siRNA targeting luciferase (siLuc) was used as a control. Values represent the mean $\pm$ S.E. from three to five independent experiments (i.e. HACs from three to five different donors). ns, not significant; *, $p < 0.05$; ***, $p < 0.001$ versus 20% oxygen siLuc. *D*, Western blot showing RNAi-mediated depletion of PHD2 protein (lower band) in HACs (in 20% oxygen). *E*, RNAi-mediated PHD3 protein depletion is shown in the presence of the hypoxia mimetic DFO, as PHD3 levels in normoxia were undetectable. In all cases, HACs were cultured for 3 days before termination of experiment.

RNA against luciferase (siLuc) was used as a control to provide accurate comparison with PHD depletion. Upon exposure to normoxia, HIF- α subunits have a very short half-life (~ 5 min). However, the knockdown of PHD2 greatly slowed the rate of degradation of both HIF-1 α and HIF-2 α (Fig. 3, B and C, respectively), thus confirming the importance of PHD2 in regulation of HIF-2 α stability.

We next investigated the levels of HIF-2 α -inducible cartilage-specific transcription factor SOX9 in PHD1/2/3 knockdowns in HACs in 20% oxygen. Strikingly, only depletion of PHD2 resulted in enhanced SOX9 protein levels compared with luciferase controls (Fig. 3D). This is critical because SOX9 drives expression of the cartilage-specific extracellular matrix genes that are essential for the formation of a suitable load-bearing tissue, the primary function of articular cartilage.

PHD2 Depletion Promotes the Differentiated HAC Phenotype in Both Normoxia and Hypoxia—We next investigated the effect of PHD2 depletion in hypoxic as well as normoxic conditions in primary and passaged HACs (Fig. 4A). There was no

detectable difference in HIF-1 α levels between PHD2-depleted cultures in 20 and 1% oxygen, both being greater than that achieved by hypoxia alone (siLuc in 1% oxygen) (Fig. 4B). In contrast, the knockdown of PHD2 at 20% oxygen stabilizes HIF-2 α to levels approximately equal to that in hypoxia alone (Fig. 4C). However, depletion of PHD2 in 1% oxygen further enhanced HIF-2 α levels suggesting that PHD2 is not the only factor regulating HIF-2 α levels in hypoxia. The finding that PHD2 depletion in hypoxia resulted in greater stabilization of both HIF- α isoforms compared with control hypoxic conditions indicates that there is still significant PHD enzyme activity in 1% oxygen, which is in the physiological range for human articular cartilage.

Levels of SOX9 were analyzed in PHD2-depleted HAC cultures from a range of patients. SOX9 protein was enhanced by PHD2 depletion in a manner very similar to that of HIF-2 α , *i.e.* with greatest levels achieved in 1% oxygen (Fig. 4D). We next investigated type II collagen (COL2A1) as it is regulated by SOX9, and critically it provides the required tensile strength needed for cartilage to perform its load-bearing and articulating functions. Secreted levels of this matrix protein were also significantly enhanced by PHD2 depletion, with highest levels occurring when PHD2 was depleted in hypoxia (Fig. 4E).

Following PHD2 depletion in HACs (Fig. 5A), SOX9 mRNA was significantly up-regulated (Fig. 5B) in a manner similar to the response observed at the protein level. In fact, the key cartilage extracellular matrix genes (which are regulated by master chondrocyte transcription factor SOX9) were up-regulated in response to PHD2 inhibition. The knockdown of PHD2 in 1% oxygen tension increased COL2A1 expression 38.5-fold, COL9A1 34.5-fold, COL11A2 58.9-fold, and AGGRECAN 6.5-fold (compared with control levels in normoxia) (Fig. 5, C–F, respectively). Expression of a nonhypoxia-regulated gene, factor inhibiting HIF (FIH), was unaltered in response to PHD2 depletion (Fig. 5G).

General 2-Oxoglutarate-dependent Hydroxylase Inhibition Up-regulates SOX9 but Inhibits Secretion of the Cartilage Collagen COL2A1—The 2-oxoglutarate analogue DMOG inhibits all members of the 2-oxoglutarate-dependent hydroxylase family, which includes not only the HIF-targeting hydroxylases, but also the collagen prolyl 4-hydroxylases. DMOG treatment increased expression of SOX9 at the gene (Fig. 6A) and protein level (Fig. 6B). VEGF, a classic HIF target gene was also up-regulated by DMOG (Fig. 6C). However, DMOG-induced up-regulation of SOX9 did not result in induction of the cartilage-specific collagens COL9A2, COL11A2, and COL2A1 (Fig. 6,

TABLE 1

Oligonucleotide sequence of PHD targeting siRNAs

siRNA	Sequence
siPHD1 #1	5'- CAUGCAGGGCAGCAUUAUAGUC -3'
siPHD1 #2	5'- GCAUCACCGUGUAUCUAUUA -3'
siPHD2 #1	5'- GAAGCUGGGCAGCUACAAAAT -3'
siPHD2 #2	5'- CAAAUUGGAGAUGGAAGAUGUG -3'
siPHD3 #1	5'- GGAGAGGUCUAAGGCAAUGTT -3'
siPHD3 #2	5'- CAGGUUAUGUUCGCCACGUGGTT -3'

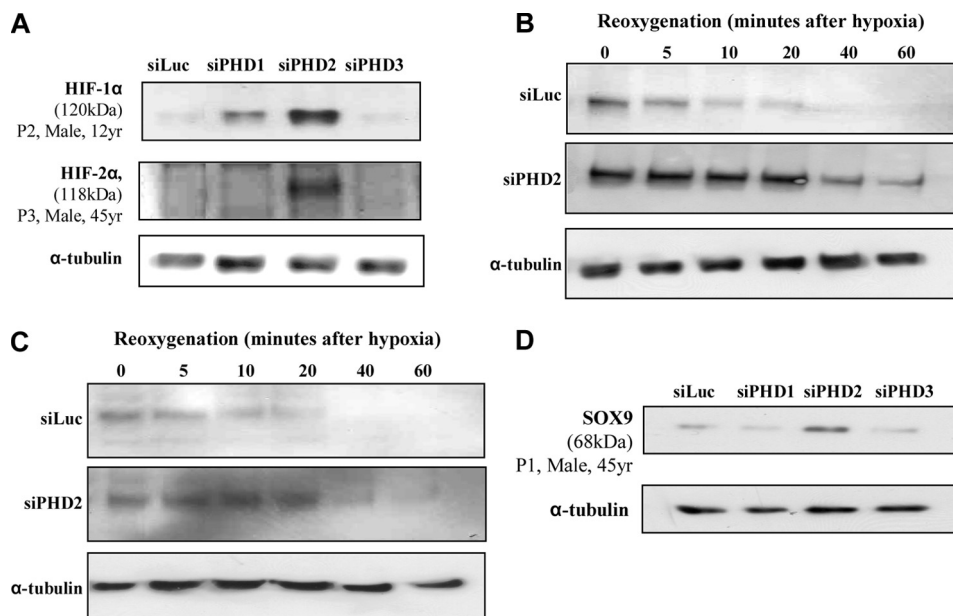


FIGURE 3. PHD2 depletion in normoxia stabilizes HIFs and up-regulates master chondrocyte transcription factor SOX9. A, unlike PHD1 or PHD3, depletion of PHD2 resulted in significant stabilization of both HIF-1 α and HIF-2 α protein in HACs following 3 days of culture in 20% oxygen. Control (siLuc) shows that neither HIF isoform is normally detectable in the presence of 20% oxygen. Depletion of PHD2 significantly slows the rate of HIF-1 α (B) and HIF-2 α (C) degradation upon reoxygenation, *i.e.* switching the cells from 1 to 20% oxygen. Following siRNA transfection, HACs were incubated 3 days in 1% oxygen before being exposed to 20% oxygen for the indicated time (in minutes) prior to lysing. Control siRNA (siLuc) transfections were carried out in parallel. Note, for simplicity α -tubulin loading controls from representative experiments are shown. D, depletion of PHD2, but not PHD1 or PHD3, resulted in increased SOX9 protein in HACs after 3 days in culture in 20% oxygen.

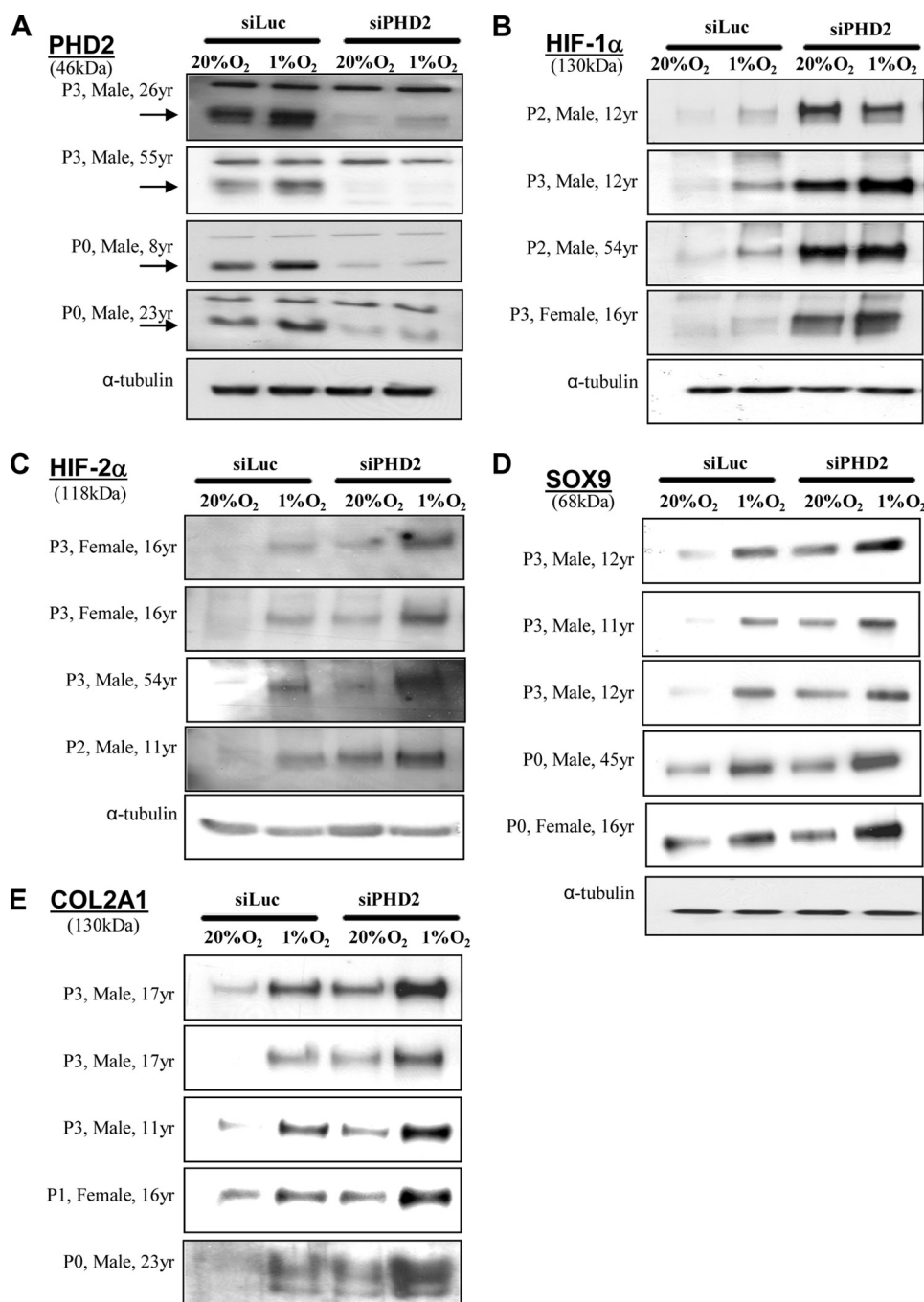


FIGURE 4. PHD2 depletion in HACs up-regulates SOX9 and secreted COL2A1 protein levels both in normoxia and in hypoxia. A, PHD2 was depleted in HACs in both 20 and 1% oxygen cultures. B, HIF-1α levels were enhanced in PHD2-depleted HACs, although no difference was detectable between PHD2-depleted cultures in 1 or 20% oxygen. C, HIF-2α levels were enhanced following PHD2 depletion in both normoxia and in hypoxia. D, SOX9 protein levels, like HIF-2α, were up-regulated by PHD2 depletion, with greatest levels occurring in hypoxia. E, secreted COL2A1 was detected following trichloroacetic acid precipitation of culture medium. Data were normalized to total protein content in the cell layer (as described under "Experimental Procedures"). Secreted COL2A1 protein levels followed a similar pattern to HIF-2α and SOX9, being highest in PHD2-depleted HACs cultured in hypoxia. Note, all data in the figure was obtained from primary and passaged HACs after 3 days in culture. Cells were transfected with 10 nM siRNA against PHD2 (*siPHD2*) or as a control against luciferase (*siLuc*). For simplicity, only α-tubulin loading controls from representative experiments are shown.

D–F, respectively). Western blot analysis demonstrates that the addition of DMOG completely inhibits secretion of type II collagen protein into the culture medium (Fig. 6G). Therefore, DMOG, like hypoxia and PHD2 depletion, up-regulates SOX9. However, crucially, unlike hypoxia or PHD2 depletion, this

does not lead to enhanced cartilage matrix gene expression, and in the case of COL2A1, it completely inhibited its secretion thus preventing elaboration of a functional cartilage matrix.

DISCUSSION

We have previously shown that HIF-2α (not HIF-1α) is essential for hypoxic induction of cartilage matrix gene expression in HACs (9). Furthermore, it achieves this predominantly through up-regulation of tissue-specific transcription factor SOX9, which drives cartilage matrix gene expression (20). Because HIFs themselves are regulated by specific prolyl hydroxylases (PHDs), we investigated which of these enzymes targets HIF-2α and whether their specific inhibition could promote matrix production. Using an RNAi approach, we identified PHD2 as the key hydroxylase in HACs responsible for regulating HIF-2α. Crucially, we provide clear evidence that such specific inhibition of PHD2 promotes the differentiated phenotype and enhances cartilage matrix production by HACs both in normoxia and hypoxia. The latter finding, *i.e.* enhanced HIF-2α stabilization (and subsequent SOX9 induction of the matrix genes) by PHD2 inhibition even in hypoxia, which is the normal condition of cartilage (3–5), suggests that PHD2 still shows significant activity *in vivo*. This opens up the exciting possibility of PHD2-specific inhibition as a means to enhance cartilage matrix synthesis *in vivo*, a fact with obvious relevance to development of novel cartilage repair therapies.

PHD1, -2, and -3 were all detectable in normal HACs, with PHD2 being the most abundant. These hydroxylases have previously been reported in human cartilage at the mRNA and protein level (21). In addition, mRNA studies in rats reported that in all organs examined (heart, liver, kidney, brain, testis and lung) PHD2 was found to be the most commonly expressed (22, 23). Similarly to previous reports in other cell types (24, 25), our results showed that PHD2 and PHD3 are up-regulated in response to hypoxia in HACs. It has been

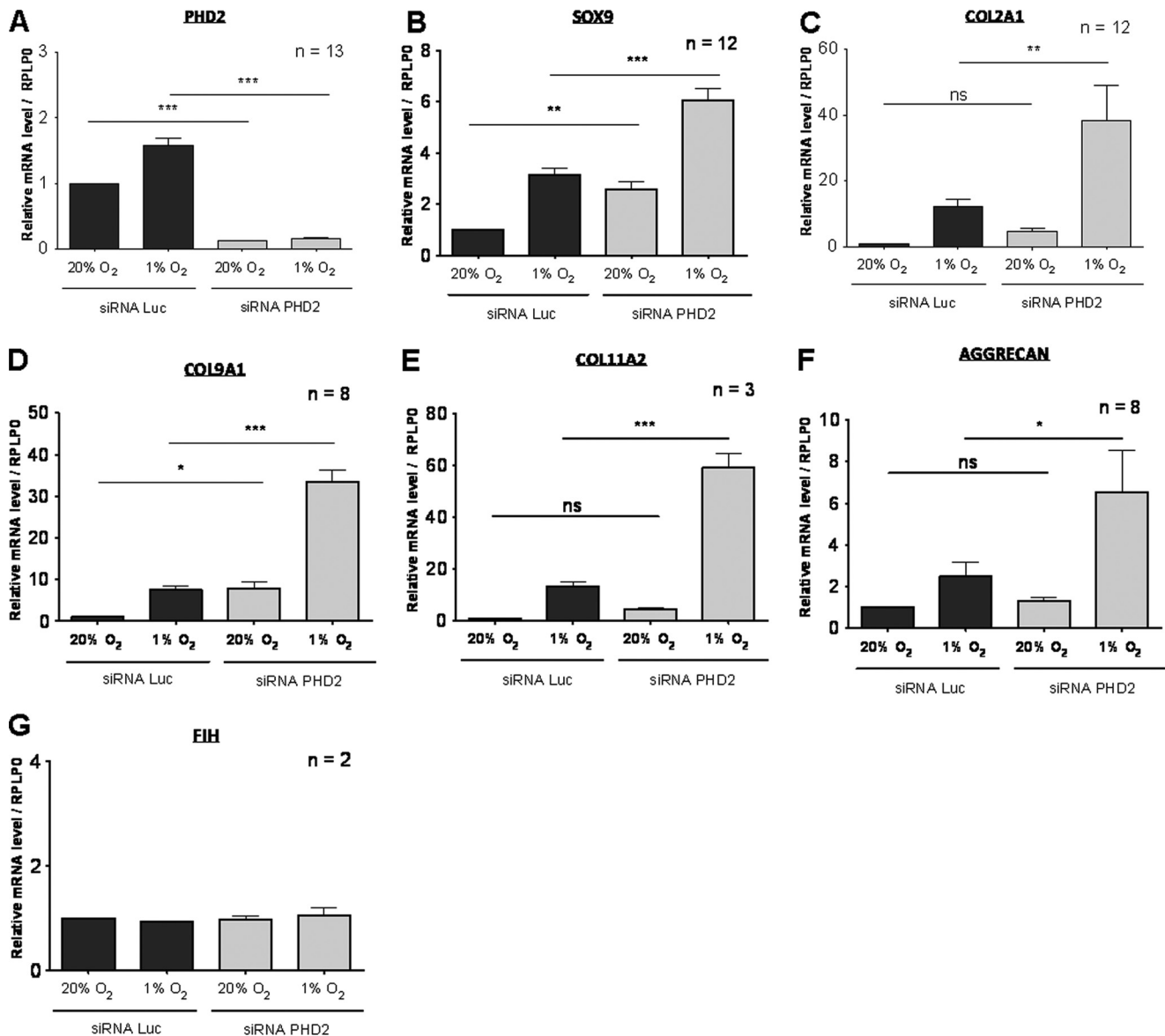


FIGURE 5. **PHD2 depletion in HACs enhances expression of the key differentiated chondrocyte markers.** TaqMan real time pcr data showing relative mRNA levels as follows: A, PHD2; B, SOX9; C, COL2A1; D, COL9A1; E, COL11A2; and F, AGGRECAN in control and PHD2-depleted HAC cultures. G, FIH, a known nonhypoxia inducible gene, showed no change in response to PHD2 depletion. ns, not significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

hypothesized that this occurs in a negative feedback mechanism ensuring the rapid degradation of HIF- α isoforms and down-regulation of hypoxia-responsive genes once the cells encounter increased oxygen levels (26). Although we observed PHD3 to be the most highly hypoxia-inducible hydroxylase (17.8-fold), it levels were relatively low, and it did not appear to significantly contribute toward HIF- α stabilization in HACs. Koditz *et al.* (27) and colleagues recently demonstrated that PHD3 interacts with activating transcription factor-4 (ATF-4) suggesting it may play a role in regulating transcription factors other than HIFs.

Work in a range of cell lines has suggested differential function of the PHD enzymes in regulation of HIF- α isoforms (28). In addition, Phd2 deletion in adult mice leads to accumulation of HIF-1 α but not HIF-2 α in mouse liver and kidney tissue (29). In contrast Phd1/Phd3 double knockouts leads to preferential HIF-2 α stabilization but not HIF-1 α in

the liver (30). Aragones *et al.* (31) reported that Phd1^{-/-} mice had elevated levels of HIF-2 α in their myofibers and that HIF-1 α levels were either low or undetectable. Although these studies indicate that PHDs may show differential regulation of HIF- α isoforms, at least some of the observed differences are most likely due to the relative abundance of each hydroxylase (which is dependent on cell and tissue type). In fact, the K_m values of all three PHD isoenzymes for recombinant HIF-1 α and HIF-2 α oxygen degradation domains are quite similar, with PHD1 having the lowest K_m value for both recombinant HIF- α oxygen degradation domains (32). All three PHD isoenzymes also have virtually identical K_m values for oxygen (21) implying that changes in oxygen tension will not affect the relative contribution of the three enzymes to HIF- α stabilization. In our experiments with HACs, only depletion of the most abundant hydroxylase (PHD2) significantly stabilized both HIF-1 α and HIF-2 α in 20% oxygen

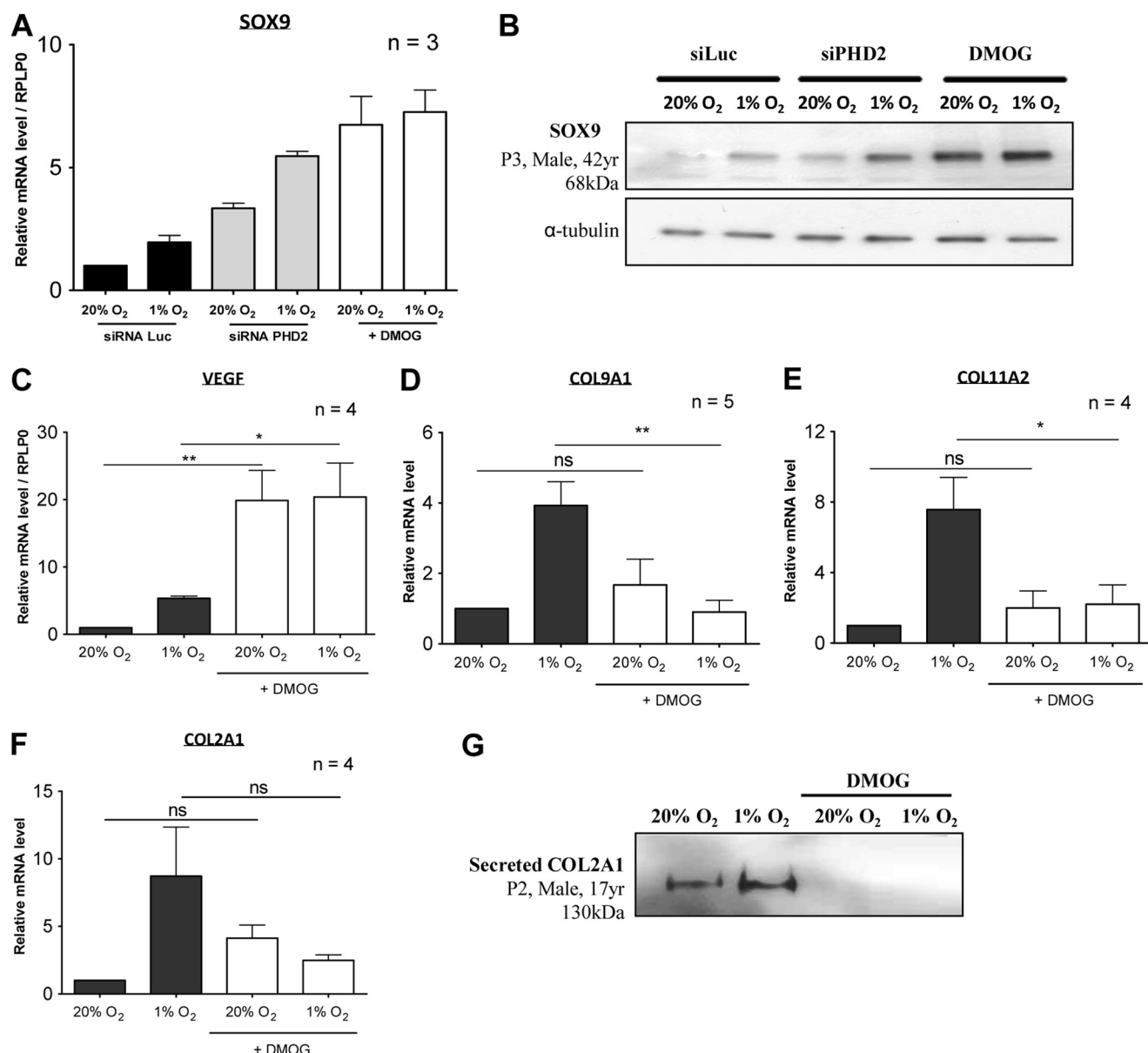


FIGURE 6. General 2-oxoglutarate-dependent hydroxylase inhibition up-regulates SOX9 but inhibits the cartilage collagens. Passaged HACs were cultured for 3 days under normoxic (20%) or hypoxic (1%) conditions, in the presence/absence of 2-oxoglutarate analogue and hydroxylase inhibitor DMOG. Addition of DMOG enhanced SOX9 mRNA (A) and protein levels (B). C, expression of classic HIF target gene VEGF was also significantly enhanced by DMOG; however, expression of the cartilage-specific collagens COL9A1 (D), COL11A2 (E), and COL2A1 (F) was not induced by DMOG. G, DMOG treatment inhibited secretion of COL2A1 protein in HACs. ns, not significant; *, $p < 0.05$; **, $p < 0.01$.

and slowed degradation of each HIF- α isoform in reoxygenation experiments.

Interestingly, however, there were differences between HIF-1 α and HIF-2 α in regard to their stabilization by PHD2 depletion. No further increase in HIF-1 α stabilization was detectable in PHD2-depleted HACs when cultured in 1% oxygen compared with that seen in 20% levels. In contrast, HIF-2 α levels were further increased when PHD2-depleted cells were cultured in hypoxia implying that other factors in addition to PHD2 may contribute to HIF-2 α regulation in hypoxia. Neither PHD1 nor PHD3 appear to be such factors because their depletion had little impact on HIF-2 α levels. Factor inhibiting HIF (FIH) is an iron-dependent dioxygenase capable of repressing HIF- α activity (33, 34). However, depletion of FIH had no effect

on SOX9 expression even when performed in combination with depletion of PHD2 (data not shown).

Despite the fact that K_m values for oxygen are reported to be between 10 and 25% oxygen, *i.e.* higher than that encountered *in vivo* (21, 32), our results indicate that PHDs still display significant activity in 1% oxygen because depletion of PHD2 in hypoxia resulted in greatly increased levels of both HIF- α isoforms. To be able to enhance HIF-2 α levels above those typically found in hypoxia (the physiological condition of cartilage) is potentially of great importance because we have previously shown that HIF-2 α up-regulates cartilage-specific matrix expression through induction of SOX9 (9, 20). Here, we demonstrate that specific depletion of HIF-2 α targeting hydroxylase PHD2 did indeed significantly up-regulate the cartilage

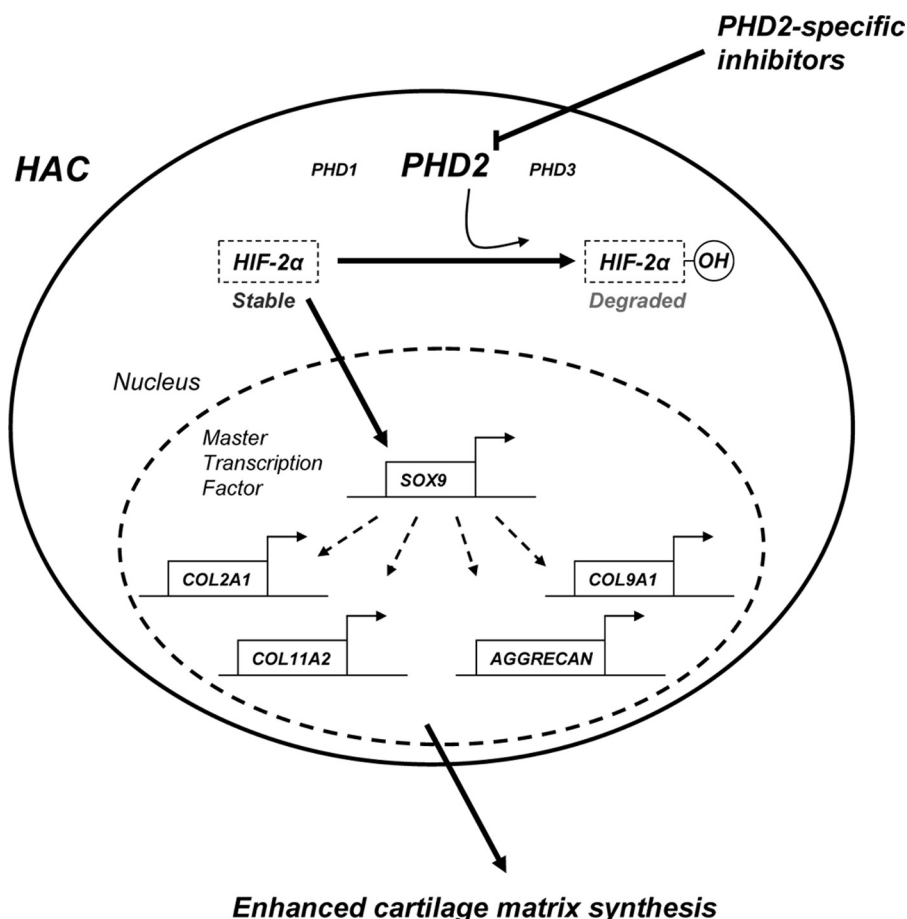


FIGURE 7. Diagrammatic representation detailing the mechanism by which PHD2 inhibition leads to enhanced cartilage matrix production. HAC, human articular chondrocyte.

master regulator, transcription factor SOX9, which subsequently up-regulated expression of key matrix genes *COL2A1*, *COL9A1*, *COL11A2*, and *AGGRECAN*, crucially above the levels normally detected in hypoxia (1% oxygen). In addition, PHD2 depletion enhanced levels of secreted COL2A1 protein in HACs above those naturally occurring in hypoxia. We have thus identified PHD2-specific inhibition as a novel means to enhance cartilage matrix synthesis (pathway detailed in Fig. 7).

Experiments with the general hydroxylase inhibitor DMOG highlight the importance of development of HIF-specific hydroxylase inhibitors with regard to chondrocyte metabolism. Addition of DMOG enhanced SOX9 levels in HACs, and we have previously shown that this is dependent (like hypoxia) specifically on HIF-2α (35). However, unlike the case of hypoxia or PHD2 depletion, DMOG did not result in mRNA induction of the cartilage-specific collagen genes, *COL2A1*, *COL9A1*, and *COL11A2*. Furthermore, secretion of COL2A1 protein was blocked by DMOG. The latter is most likely due to the fact that DMOG also inhibits the collagen prolyl 4-hydroxylases, which are critical for post-translational processing of collagen, in particular triple helical formation (36). Similar results were reported by Gelse *et al.* (37) who carried out a study injecting the joints of STR/ORT mice with DMOG. These mice spontaneously develop osteoarthritis, and injection of DMOG was unable to prevent the formation of

severe arthritis. However, the authors reported intracellular accumulation of type II collagen in chondrocytes presumably due to incomplete post-translational processing. Also similarly to this study, they reported a lack of *Col2a1* mRNA induction by DMOG in mouse chondrocytes, despite enhanced Sox9 levels. They suggested that the increased intracellular accumulation of the collagens caused by DMOG may operate via a negative feedback mechanism to down-regulate gene expression, and counter any of the positive effects of enhanced Sox9 levels. Although this is a plausible explanation, further work is needed to clarify the issue. What these results do clearly illustrate, however, is the need for inhibitors that are specific to the HIF-targeting PHDs.

This adverse effect of general hydroxylase inhibition on collagen processing is particularly relevant to cartilage as it is a most highly collagen-rich tissue. In fact, expression of the collagen prolyl 4-hydroxylases are actually up-regulated by hypoxia (38). In addition, we have previously shown that two further enzymes involved in collagen processing

LOXL3 and *PLOD2* are also hypoxia-inducible (20). Therefore, inhibition of PHD2 can up-regulate not only *COL2A1* transcription but also enzymes necessary for its post-translational processing, thus representing a further benefit of PHD2-specific inhibition with regard to elaboration of a functional cartilage matrix by HACs. For this approach to be applied to animal studies, and eventually clinical studies, inducible cartilage-specific PHD2 knock-out mice would be informative, in addition to development of PHD isoform-specific (or at least HIF-targeting hydroxylase-specific) small molecule inhibitors. This is currently an area of active research.

Acknowledgments—We thank Dr. Jerome Lafont for excellent technical assistance and valuable suggestions and Professor Chris Pugh for gifting us an anti-PHD3 antibody.

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