

Hypoxia Related Function in Human Osteoarthritic Cartilage



Leo William Edward Hipkiss
Kennedy Institute of Rheumatology
Worcester College
University of Oxford

A thesis submitted for the degree of

Doctor of Philosophy

Michaelmas 2018

This thesis is dedicated to
My brothers old and new
You can do anything you put your minds to

Acknowledgements

Firstly, I would like to sincerely thank my supervisor Dr Chris Murphy, the help and guidance you have provided me, both professionally and personally, has been essential to my successes these past four years.

Additionally, I would like to thank Dr Christine Seidl for having the patience to guide me through learning new techniques and troubleshooting with me at times when I felt truly stuck. Your patience when I ask you the same question for the fifth or sixth time is truly impressive.

Special thanks go to the friends who have suffered through me talking about hypoxia and chondrocytes endlessly these past four years. In particular Joe, for providing me with the distractions that have kept me sane and for being a person I can truly rely on.

I would especially like to acknowledge my family. My parents, Barry and Rachel, your guidance, love and unending support truly mean the world to me, thank you for making me who I am. To my brothers, Sam, Josh and Jack, thanks to each of you for giving me things other than my studies to worry about. A special acknowledgement to my grandparents, Pat and Roland, my grandfather is my personal hero and I will always be grateful to him for inspiring my love of science and discovery. Of course to my grandmother Eileen thank you for always putting up with my cheekiness and sharing your birthday with me for so many years, I'll always miss our birthday morning phone calls.

Finally, I would like to thank my partner Lizzie. You have swiftly become the never ending light in my life and this last year a future with you has become one of my greatest motivators. Thank you for your encouragement, support and being miraculously able to inspire me to continue when my motivation waned.

Abstract

Articular cartilage is an avascular tissue, and therefore, at least in large animals, functions in a hypoxic environment. In healthy human cartilage, chondrocytes exhibit a well-adapted hypoxic response. Previous work in our laboratory has shown that hypoxia in healthy human articular chondrocytes (HACs) increases anabolic function (SOX9, COL2A1, ACAN) via HIF-2 α and decreases catabolism (reducing MMP13 and ADAMTS5) predominantly via HIF-1 α . Inhibition of HIF-targeting hydroxylase PHD2 was also identified as a potential means to induce cartilage repair by enhancing HIF-2 α levels subsequently increasing anabolism via SOX9.

In contrast, evidence from murine studies has suggested HIF-2 α as a catabolic regulator in the progression of OA, targeting key catabolic enzymes MMP13 and ADAMTS5, thus indicating HIF-2 α as a potentially relevant therapeutic target in OA. The role of hypoxia and HIF activity in human OA cartilage and chondrocytes is less clearly understood, and hence was the focus of the current study.

In this study the general response of key cartilage genes, in primary OA HACs, to hypoxia are shown to be beneficial to the chondrocyte phenotype (as it is in healthy HACs). Hypoxia in OA HACs increases levels of SOX9 by 125% and subsequently increases the downstream cartilage matrix gene *COL2A1* by 215% compared to levels observed in normoxia. Additionally, it is reported that the hypoxia-mediated downregulation of *MMP13* and *ADAMTS5* by 32% and 45% respectively. However, it is noted that *TIMP3* is not induced by hypoxia in OA HACs indicating a potentially reduced protective capacity compared to healthy cartilage. Interestingly, it is observed that HIF-1 α is the predominant isoform responsible for the induction of SOX9 in OA HACs (as opposed to HIF-2 α in healthy HACs) noting the depletion of HIF-1 α reduces SOX9 by 54% and 61% in normoxia and hypoxia respectively. Importantly, results represent no evidence of a HIF-2 α dependent induction of catabolic factors in OA HACs, but rather a potentially anti-catabolic role reducing *ADAMTS5* and *MMP13* levels by 111% and 78% respectively in hypoxia. Furthermore, the results shows that, whilst PHD2 depletion on its own is insufficient, combined HIF-targeting hydroxylase inhibition (via DMOG) can enhance the beneficial effects of hypoxia observed in OA HACs (increasing SOX9 levels 2.36 fold above typically observed by hypoxia alone). Therefore, a combined inhibition of the PHDs represents a potential means of increasing cartilage matrix production in an OA setting.

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Chapter 1

Introduction

1.1 Background

Past research, into the effects of hypoxia in healthy human articular cartilage has revealed that cartilage is a tissue which is well adapted to a hypoxic setting. Hypoxia has been shown to promote the chondrocyte phenotype through the promotion of anabolic and anti-catabolic responses via the activity of hypoxia inducible factors (HIF). Our group has reported that, key to the beneficial nature of the hypoxia response is the HIF-2 α dependent induction of SRY (Sex-Determining Region Y)-Box 9 Protein (SOX9) pathways (Lafont et al., 2008, Thoms, 2011, Thoms et al., 2013). SOX9 is considered a master regulator of the cartilage phenotype and has been shown to regulate many key cartilage extracellular matrix genes including Type II, Type IX and Type XI Collagen and Aggrecan (Bell et al., 1997, Sekiya et al., 2000, Mendler et al., 1989). Additionally, we have shown that in healthy cartilage hypoxia induces an anti-catabolic response and inhibits the destruction of healthy articular cartilage - an effect, likely caused by the reduction of key catabolic enzymes and induction of inhibitor of matrix metalloproteinases TIMP3 via the activity of HIF-1 α (Thoms, 2011, Thoms et al., 2013). Crucially, our group has previously shown that the effects of hypoxia in healthy human cartilage mediated by HIF can be enhanced by the depletion of PHD2. PHD2 is a HIF targeting prolyl-hydroxylases belonging to a family of oxyglutarate-dependent dioxygenases which cause the proteolytic degradation of HIF in the presence of oxygen (Schofield and Ratcliffe, 2004). The absence of PHD2 has been shown to enhance the effects of hypoxia we observed in healthy human cartilage, increases SOX9 above levels typically observed in hypoxia and reducing catabolic enzymes further than by hypoxia alone. Therefore, it was suggested that PHD2 depletion may be a relevant target for cartilage repair via an increase in cartilage production and decrease in destruction in healthy human cartilage (Thoms, 2011, Thoms et al., 2013, Thoms and Murphy, 2010).

However, in contrast to our group's previous findings in healthy human cartilage, recent studies conducted in the murine model of osteoarthritis (OA) have indicated HIF-2 α as a mediator of OA. In these studies, HIF-2 α is shown to directly activate catabolic enzymes - MMP13 and ADAMTS5, as well as COL10A1, a marker of hypertrophy (Ryu et al., 2012, Saito et al., 2010, Saito and Kawaguchi, 2010, Yang et al., 2010). As a result of these murine model studies of OA it has been suggested that HIF-2 α may be a suitable therapeutic target in OA. More recent studies using human OA cartilage have provided conflicting results to murine model studies, finding that hypoxia reduces the expression of MMP13 in the human OA setting (Alvarez K, 2014, Markway

et al., 2013). Therefore, I hypothesise that these conflicting reports for the hypoxic responses, crucially the activity of HIF-2 α , are likely due to species differences between murine and human cartilage and the responses of each to hypoxia (McCoy, 2015, Thoms et al., 2013). Thus, in this study I concentrated on elucidating the hypoxic responses and activity of HIF in human OA cartilage and chondrocytes.

In order to introduce this body of work the anatomy and function of articular cartilage will be described. In addition to this the hypoxia signalling pathway, HIF and the regulation of HIF by HIF-targeting hydroxylases are described. I will review the findings concerning the effects of hypoxia in healthy cartilage and its importance to the chondrocyte phenotype. Importantly, I will describe the pathogenesis of OA and finally, I will describe the current research into the hypoxic response of OA human cartilage.

1.2 The Synovial Joint and Cartilage Structure

The diarthrodial or synovial joint is responsible for the smooth, pain free movement of articular bones in the musculoskeletal system. The synovial joint is the most common joint in the human body examples of which include; the femoral joint allowing articulation of the pelvis with the femur; the phalangeal joint aiding flexion of the fingers, and the tibiofemoral synovial joint which articulates between the femoral and tibial condyles responsible for flexion of the knee (Pacifici et al., 2006). The synovial joint is comprised of two bone ends, the epiphysis of which is covered with a smooth layer of articular (hyaline) cartilage. The articular surfaces of the bones are encapsulated by the synovial membrane (synovium), this membrane is responsible for the production of the synovial fluid which lubricates the articular cartilage surface. Additionally, the synovial membrane restricts the movement of large molecules into the joint cavity acting like a molecular sieve allowing only water, small molecules and nutrients to pass freely from the vascular system into the joint cavity (Sophia Fox et al., 2009). Another important contribution of the synovial membrane is the presence of the resident cells of the membrane the synoviocytes, Type a (macrophages) and Type B (fibroblast-like cells). Type A synoviocytes are a ‘non-fixed cell’ that are immunoreactive with an antigen presenting activity and can phagocytose cellular debris and waste inside the joint cavity. Type B synoviocytes control the protein concentration in the synovial fluid by actively producing and secreting, into the joint cavity, proteins such as fibronectin, collagens and hyaluronan (Iwanaga et al., 2000). During articulation of the synovial

joint the synovial fluid serves as both a lubricant and a source of nutrients across the articular cartilage surface.

Articular cartilage itself is a highly specialised connective tissue unique to the synovial joint. Articular cartilage is a thin, avascular, aneural, hydrated layer of hyaline cartilage. Being avascular, articular cartilage has a limited supply of oxygen via diffusion from vasculature of the subchondral bone or the synovial fluid, therefore articular cartilage is a hypoxic tissue believed to experience between 1-7% oxygen (O₂) throughout the tissue (Silver, 1975, Milner et al., 2006, Brighton and Heppenstall, 1971, Zhou et al., 2004). The principle functions of articular cartilage are to provide a smooth, near frictionless surface for articulation which can absorb and dissipate compressive loads (Poole et al., 2001). Integral to these functions is articular cartilage's dense mechanically competent extracellular matrix (ECM). The tensile strength of articular cartilage is mediated by collagen fibrils in the ECM, the most prevalent of which is Type II collagen, the collagens in the ECM also provide a network to which large macromolecules such as proteoglycans like aggrecan are connected. Articular cartilage is a hydrated tissue with water accounting for approximately 65-80% of the wet weight of the matrix. This large proportion of water in the matrix is drawn into the tissue by the negatively charged proteoglycans in the ECM, the water-saturated molecules provide a gel-like stiffness which is the mechanism by which articular cartilage is able to resist compressive loads. Thus, the ability to absorb compressive loads is mediated by proteoglycans, the most predominant of which in the ECM is aggrecan (Poole et al., 2001, Sophia Fox et al., 2009). Whilst, water accounts for the majority of the total wet weight of articular cartilage, its dry weight is comprised of 70% collagen, 20% proteoglycan and the remaining 10% is comprised of other matrix components (Eyre, 2002, Sophia Fox et al., 2009).

1.2.1 Chondrocytes

The ECM of articular cartilage is synthesised, secreted and maintained by its sole resident cell type unique to the tissue, chondrocytes. The chondrocytes represent between 5-10% of the articular cartilage volume and are located within cavities of the ECM known as lacunae (Lin et al., 2006). The chondrocyte cytoplasm consists of a large number of Golgi apparatus, rough endoplasmic reticulum, a small number of elongated mitochondria and variable amounts of glycogen (Lin et al., 2006, Stockwell, 1991). Chondrocytes exist in a physiologically chronic hypoxic environment (1-7% O₂) within the articular cartilage ECM and thus in an adaption to this

environment are highly dependent on anaerobic, glycolytic metabolism for the production of adenosine triphosphate (ATP) (Gibson et al., 2008).

Articular chondrocytes are typically characterised by the abundant transcription of Type II collagen and the transcription factor SRY(Sex determining region Y)-box 9 (SOX9) (Zhao et al., 1997). SOX9 is a DNA-binding protein which drives the expression of key cartilage matrix genes such as; *COL2A1*, *COL9A1*, *COL11A2* and Aggrecan (*ACAN*) (Bell et al., 1997, Bi et al., 1999, Healy, 1999, Sekiya et al., 2000, Tew et al., 2007, Zhao et al., 1997, Bridgewater et al., 1997). SOX9 expression is required for chondrocyte differentiation, being shown to be a differentiate switch in the differentiation of mesenchymal stem cells (MSCs), a population of multipotent cells that can differentiate into different cell lineages, such as chondrocytes (Wang et al., 2014, Bi et al., 1999, Lefebvre and Crombrughe, 1998). Additionally, SOX9 is downregulated in hypertrophic chondrocytes undergoing endochondral ossification (Zhao et al., 1997). SOX9 is known to be essential for the production and maintenance of cartilage in mice (murine) cartilage (Bi et al., 1999, Henry et al., 2012). The importance of SOX9 is further reported in humans as, a mutation in the gene encoding for SOX9 results in a skeletal disorder known as campomelic dysplasia, a severe dwarf syndrome, characterised by short limbs and bowed legs, which affects all cartilage-derived skeletal features (Lefebvre and Crombrughe, 1998).

1.2.2 The Extracellular Matrix (ECM)

The articular cartilage ECM is often described as comprising of four distinct zones; the superficial zone, the transitional (middle) zone, the deep zone and the calcified zone (the site of articular cartilage and subchondral bone interaction) (**Figure 1.1**). The classification of these zones is based upon the composition of the ECM and the organisation of the chondrocytes and collagen fibrils.

The superficial zone is at the immediate surface of the cartilage and serves the main function of protecting the deeper layers of the cartilage from sheer force and pressure. The superficial zone consists of a high number of flattened chondrocytes which are tightly packed at the surface of the cartilage. The zone also consists of Type II and Type IX collagen fibres which are parallel to the surface and contains a relatively low number of proteoglycans (compared to other zones). This zone is in direct contact with the synovial fluid present in the joint and, as a

result of its arrangement of chondrocytes and collagen, provides most of the tensile properties of cartilage resisting sheer and tensile stresses to the cartilage surface (Sophia Fox et al., 2009).

The transitional and deep zones are responsible for the primary resistance to mechanical forces acting on the cartilage. In contrast to the superficial zone these layers contain abundant levels of proteoglycans as well as thicker collagen fibrils. The chondrocytes are less dense in these zones and are spherical as opposed to flat (present in the superficial zone) arranged vertically into columnar clusters in parallel with the collagen fibrils. The collagen fibres in the transitional zone are organised into radial bundles in this layer and are parallel to the articular surface with their orientation beginning to change towards the deep zone where they are perpendicular to the articular surface and larger in diameter (Sophia Fox et al., 2009). The deep zone is distinguished from the calcified zone by the tidemark (a basophilic boundary between calcified and the uncalcified zones above) (Broom and Poole, 1982). Finally, the calcified zone, this zone serves the role securing the cartilage to the bone through the use of collagen fibrils. The collagen fibrils from the deep zone penetrate into the calcified zone anchoring the cartilage to the subchondral bone (Liu et al., 2011).

1.3 Key Components of Articular Cartilage

As described in section 1.2 the ECM of articular cartilage is, in regards to dry weight, comprised of 70% collagen, 20% proteoglycan and the remaining 10% is comprised of other matrix components. In this section the structure and function of collagen and proteoglycans will now be described in more detail.

1.3.1 Collagens

As previously stated, collagen fibrils are disturbed throughout the ECM of articular cartilage providing the tissue with its tensile strength. All collagen contains a tripeptide –X-Proline-Glycine- motif, where X represents any amino acid. Translated procollagen will contain three polypeptide chains known as α -chains which form a triple helix, these α -helices are able to interact and polymerise to form collagen fibrils. These collagen fibrils are then secreted into the ECM polymerise to form large cross banded fibrils (Ricard-Blum, 2011).

In regards to articular cartilage, Type II collagen is the most abundant macromolecule comprising around 90% of the collagen content of cartilage. Type II collagen is formed of three identical α -chains forming a homotrimer. The principle function of Type II collagen in articular cartilage is to act as a structural scaffold/meshwork in which other components of the ECM, such as aggrecan, can be organised (Eyre, 2002, Sophia Fox et al., 2009). Additionally, articular cartilage is known to contain; Types III, VI, IX, X and XI collagen which represent the remaining 10% of the collagen content of cartilage. Unlike Type II collagen, Type IX and XI collagen helices are shorter fibres, formed of different α -chains forming heterotrimer (Ricard-Blum, 2011). Type X collagen is a homotrimer which is expressed in the hypertrophic zone of epiphyseal growth plates by chondrocytes, in healthy mature articular cartilage the expression of Type X collagen is only observed in calcified cartilage and is considered a marker of chondrocyte hypertrophy (Gannon et al., 1991).

In articular cartilage collagen fibrils are comprised of Type II, XI and IX collagens (**Figure 1.3**). Type XI collagen resides in the centre of the collagen fibril and is believed to regulate the fibril diameter by acting as a filamentous core template. Type XI collagen is also responsible for the accretion of Type II collagen to the fibril. Type IX collagen is localised to the surface of the collagen fibrils where it projects out into the matrix (Poole et al., 1988). Six crosslinking sites have been identified on Type IX collagen which are able to form telopeptides to adjacent Type II and XI collagen (Eyre, 2004, Kadler et al., 2008).

1.3.2 Proteoglycans

Proteoglycans (PGs) are defined by their structure, containing a core (monomer) protein with covalently attached glycosaminoglycan (GAG) side chains. PGs are polysaccharide molecules which consist of a repeating disaccharide with an amino acid core. They are negatively charged and hydrophilic which is crucial for their function in articular cartilage (Gandhi and Mancera, 2008). Proteoglycans present in cartilage include; aggrecan, decorin, biglycan, fibrodulin and lumican. Aggrecan is the most abundant proteoglycan in articular cartilage, constituting 90% of the tissue's proteoglycan content, and it is the most crucial for cartilage function.

In the ECM, aggrecan binds to a backbone of hyaluronic acid (hyaluronan) via link proteins, to form large aggregates which fill the interfibrillar space of the matrix (Roughley and Mort, 2014, Wantanabe et al., 1998). A single central filament of hyaluronan can bind with up to 200 aggrecan molecules, being stabilised by the link protein interaction in the ECM. The hyaluronan backbone itself is bound to the chondrocyte via CD44 a heparin sulphate proteoglycan (Aguilar et al., 1999). The resulting large size of the aggregate allows for a high concentration of aggrecan to be retained in the ECM (**Figure 1.3**).

The core protein of aggrecan is multimodular in structure and is formed of three globular domains (G1, G2 and G3). The G1 and G2 domains are separated by a short interglobular domain and the G2 and G3 domains are separated by a longer GAG attachment region, in which around 100 chondroitin sulfate (CS) chains and 60 keratan sulfate (KS) chains covalently attach to the protein core (**Figure 1.3**) (Roughley and Mort, 2014). These negatively charged, hydrophilic GAG side chains attached to the aggrecan core will repel each other and attract water molecules. Water-saturated aggrecan molecules are held in a scaffold of collagen fibrils, forming a hydrated viscous gel which provides a swelling pressure and stiffness enabling the resistance of compressing forces which articular cartilage experiences during joint loading (Wantanabe et al., 1998).

Cartilage also contains smaller non-aggregating proteoglycans known as small leucine-rich repeat proteoglycans (SLRP) which are common to all connective tissues. These SLRPs (decorin, biglycan, fibrodulin and lumican) are smaller in size than aggrecan but are considered to be present in similar molar concentrations. SLRPs differ in structure to aggrecan, this can be summarised by their GAG composition, possessing low amounts of dermatan sulfate chains and keratan sulfate chains. The SLRPs are structured into a series of 10 adjacent domains, which each contain a common leucine-rich motif, with GAG chains attaching between each domain. SLRPs are believed to be involved in the fibril assembly and organisation of the collagen matrix structure within the ECM (Roughley, 2006, Sophia Fox et al., 2009).

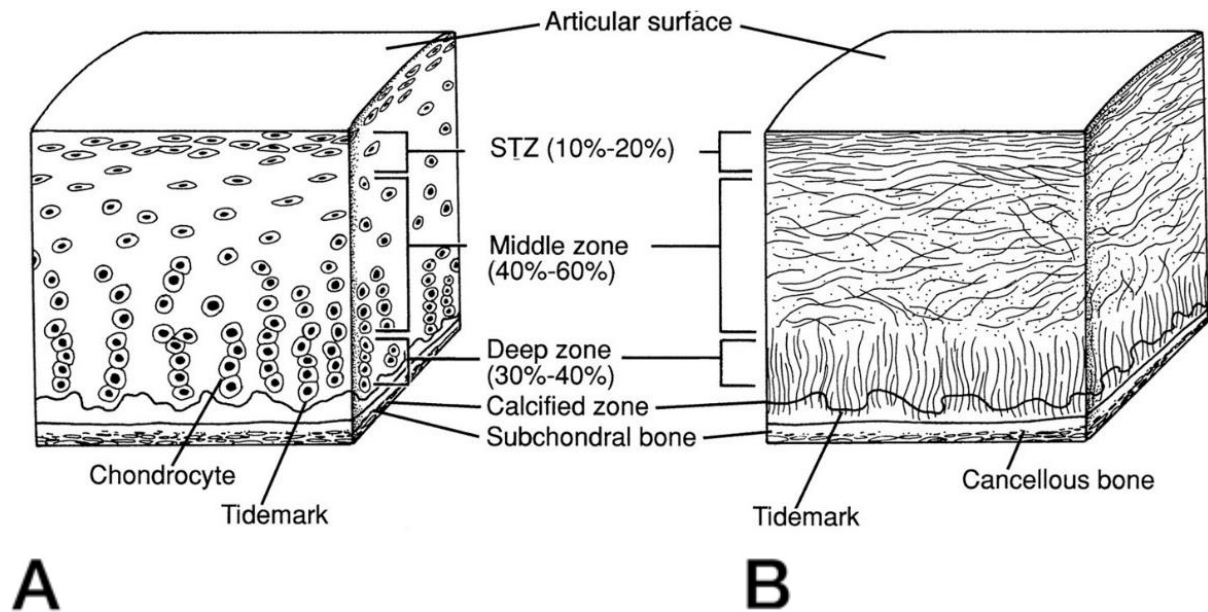
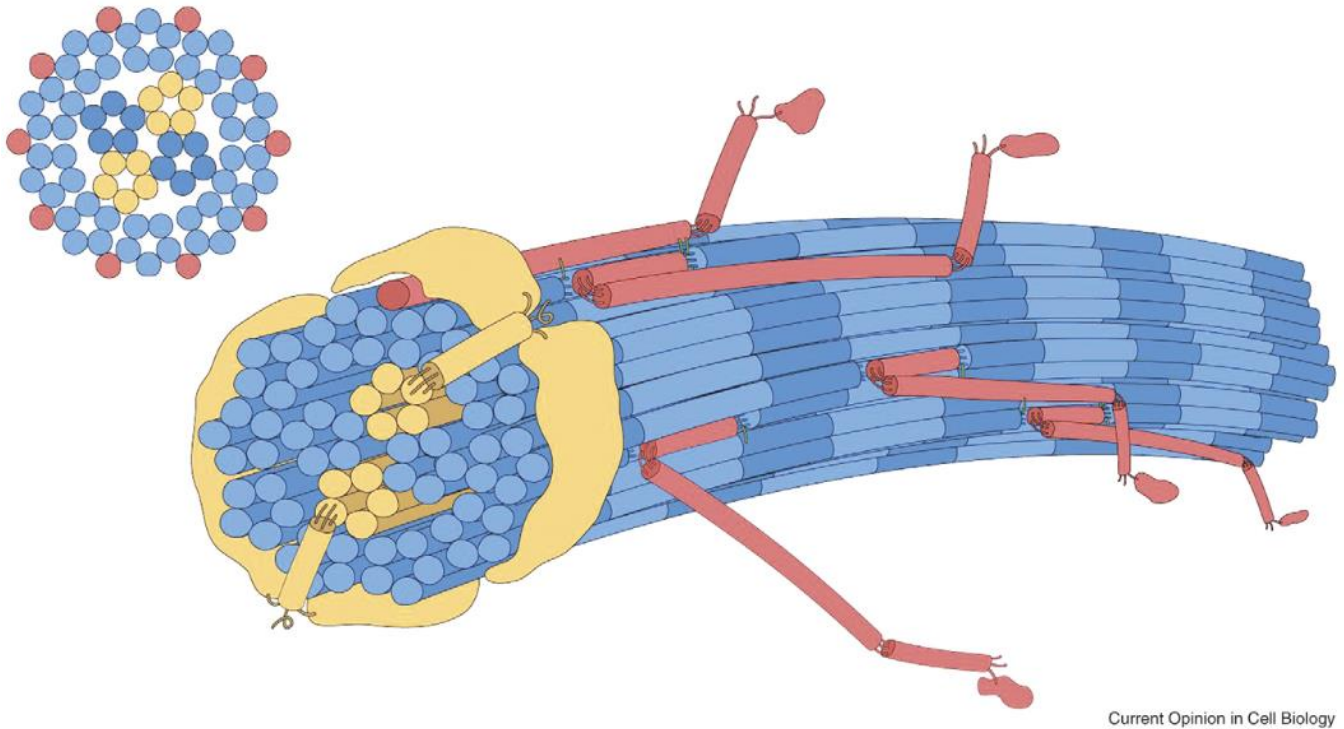


Figure 1.1 Structure of Articular Cartilage. **A** schematic diagram representing the cellular organisation in each of the four zones of articular cartilage; STZ: superficial transition zone, middle zone, deep zone and calcified zone. **B** diagram representing the collagen fibre architecture through the articular cartilage zones. Adapted from (Buckwalter et al., 1994).



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Figure 1.2 Structure of a Thin Cartilage Collagen Fibril. Thin cartilage collagen fibrils are heterotypic and are comprised of type II (blue), type IX (red) and type XI (yellow) collagens. Type IX and XI collagen being crosslinked to the Type II collagen. Type II collagen is the most abundant collagen in articular cartilage and is responsible for its tensile strength. Type IX collagens contain crosslinking sites for Types II and XI collagen, Type IX localise to the surface of the collagen fibrils and project out into the matrix. Type XI collagen extends from the core, where it is responsible for the accretion of Type II collagen, to the fibril surface where it stabilises the fibril diameter through its N-propeptide domains. Obtained from (Kadler et al., 2008).

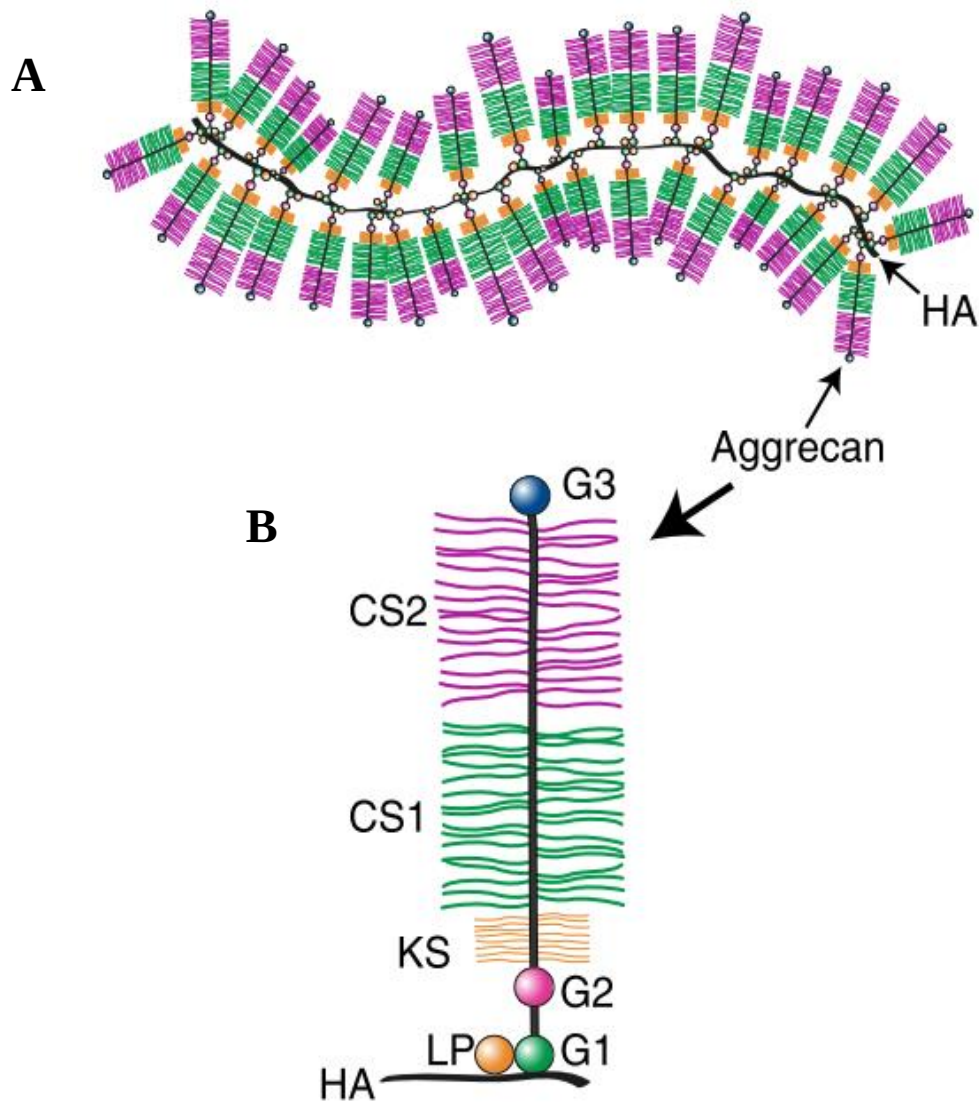


Figure 1.3 Structure of Proteoglycan Aggregates and Aggrecan. **A** Proteoglycan aggregates are formed of a central hyaluronan (HA) filament backbone with aggrecan and link proteins (LP) attached to it. **B** Aggrecan is comprised of; a core protein with 100-150 glycosaminoglycans (GAGs) chains covalently attached to it, three globular domains (G1, G2 and G3) and two interglobular domains between G1 and G2 and between G2 and G3. Covalently bound to the protein core are sulphated GAG chains of keratan sulphate (KS) and chondroitin sulphate (CS). Obtained from (Roughley and Mort, 2014)

1.4 Cartilage Extracellular Matrix Turnover

As previously stated, chondrocytes are responsible for the maintenance of the cartilage matrix. In order to maintain the cartilage matrix there is a balance between anabolism, the production of the matrix (via chondrocyte production of collagens and proteoglycans described in section 1.2-3) and catabolism, the degradation of the matrix. However, chondrocytes are known to have a limited capacity for replication, a feature which results in a limited capacity for repair of the cartilage following damage which disrupts the equilibrium between anabolism and catabolism.

Catabolism or matrix degradation is caused by two key classes of proteases produced by chondrocytes; aggrecanases notably ADAMTSs (A disintegrin and metalloproteinase with thrombospondin type 1 motif) and matrix metalloproteinases (MMPs). Firstly, ADAMTS are a family of 19 secreted extracellular metalloproteinases of the metzincin protease superfamily which have a zinc ion dependent active site. All ADAMTSs have at least one thrombospondin type 1 motif which provides ADAMTS with the capability of binding to the extracellular matrix (Kelwick et al., 2015, Tang, 2001). ADAMTSs are initially secreted as inactive larger precursor proteins which require post-translational processing to become active, for instance it has been shown that activity of ADAMTS5 requires extracellular processing of its proADAMTS5 form by proprotein convertases (specifically furin) to become catalytically active (Longpre et al., 2009). The ADAMTS family can be sub grouped according to their substrates, such as aggrecanases and proteoglycanases (ADAMTS1, 4, 5, 8, 9, 15 and 20), procollagen N-propeptidases (ADAMTS2, 3 and 14) and cartilage oligomeric matrix protein-cleaving enzymes (ADAMTS7 and 12) (Kelwick et al., 2015).

Secondly, in humans MMPs are a group of 23 matrixins which are considered to be the major enzymes responsible for the turnover of the ECM and are able to degrade a multitude of cartilage matrix molecules. Similar to the ADAMTSs, MMPs have a zinc ion dependent active site and are initially translated as inactive proMMPs which are subsequently activated via proteolytic cleavage (Nagase et al., 2006, Murphy et al., 2002, Troeberg and Nagase, 2012). Additionally, MMPs can also be sub grouped according to their substrates examples including collagenases (MMP1, 8, 13 and 14), gelatinases (MMP2 and 9) and stromelysins (MMP3 and 10) (Murphy et al., 2002). In regards to the collagenase activity of MMPs, MMPs 1, 8, 13 and 14 have been shown to be able to cleave Type II collagen, the primary collagen of the ECM. MMP13

specifically is thought to be the primary collagenase with the highest reported affinity for Type II collagen (Mitchell et al., 1996, Welgus et al., 1983, Tetlow et al., 2001).

In regards to their aggrecanase activity both MMPs and ADAMTSs have been shown to be able to degrade aggrecan via cleavage sites within the interglobular domain between G1 and G2 (**Figure 1.3**). This cleavage can occur between the Glu³⁷³-Ala³⁷⁴ and Asn³⁴¹-Phe³⁴² amino acid bond. ADAMTSs (predominantly ADAMTS5) will cleave aggrecan molecules at the site of the Glu³⁷³-Ala³⁷⁴ bond resulting in the production of a C-terminal fragment NITEGE and N-terminal neoepitope ARGSV. NITEGE remains bound to the hyaluronan backbone whilst ARGSV is released from the protein core. MMPs on the other hand cleave aggrecan at the Asn³⁴¹-Phe³⁴² resulting in the production of a C-terminal fragment DIPEN and N-terminal neoepitope FFGV. Whilst the cleavage of aggrecan by MMPs was originally reported to be due to MMP3 it has since been shown that MMP-1, 2, 7, 8, 9 and 13 can cleave aggrecan at the Asn³⁴¹-Phe³⁴² bond site (Tortorella et al., 2000, Song et al., 2007, Troeberg and Nagase, 2012). The neoepitopes produced by cleavage of aggrecan by MMPs and ADAMTSs are able to be detected in both cell culture media and clinically within the synovial fluid of the joint (Larsson et al., 2009, Lark et al., 1997, Sandy and Verscharen, 2001).

Tissue inhibitor of matrix metalloproteinases (TIMPs) are regulators of ADAMTS and MMP activity produced by chondrocytes. TIMPs play a crucial role for the maintenance of articular cartilage matrix homeostasis balancing protease activity. There are four TIMP isoforms that have currently been identified (TIMP1, 2, 3 and 4) (Baker et al., 2002). It has been reported that all TIMPs are able to inhibit all known MMPs although, different effectiveness of the TIMPs is reported with TIMP1 being described as a poor inhibitor for MMP14 and 19 (Nagase et al., 2006). As well as the broad ability of TIMPs to inhibit MMPs, TIMP3 has additionally, been shown to be a strong inhibitor of ADAMTS1, 4 and 5 with K_i for the aggrecanases reported to be in the subnanomolar range (Kashiwagi et al., 2001). In addition to this, Gendron et al. (2003) have shown that TIMP3 is able to inhibit aggrecanase in vitro (bovine and porcine articular cartilage explants) following stimulation with interleukin-1 (IL-1) or retinoic acid. The inhibition of aggrecanase activity was measured via aggrecan neoepitope release into the culture media, following stimulation with catabolic factors ARGSV release was suppressed in the presence of TIMP3 but not TIMP1 or TIMP2. It is due to this activity that the augmentation of TIMP3 is currently investigated as a novel therapeutic treatment to inhibit cartilage degradation in osteoarthritis (Chanalaris et al., 2017).

1.5 Osteoarthritis (OA)

Osteoarthritis (OA) is the most common form of arthritis and has the clinical symptoms of joint pain, joint stiffness and functional impairment. It is described as the degenerative breakdown of cartilage, featuring; loss of articular cartilage, synovitis and changes to the surrounding bone (including subchondral bone thickening and osteophyte formation) and muscle of the synovial joint (**Figure 1.4**) (Wieland et al., 2005). It is thought that during OA (possibly at the site of tissue injury/lesions) the resident cell type (chondrocytes) undergoes a phenotypic change leading to the production of catabolic factors such as aggrecanases and matrix metalloproteinases. This leads to the loss of proteoglycans, such as aggrecan, and damage to the integral collagen network. The end result of these processes is the fibrillation and degradation of the cartilage with eventual need for joint replacement (Goldring, 2000).

In 2010 OA was estimated to affect 3.8% of the global population (4.8% of females, 2.8% of males) with OA of the knee or hip believed to affect 10-20% of over 65s (Cross et al., 2014, Scott et al., 1998). In the United Kingdom alone the prevalence of the disease creates a significant economic burden with an estimated cost of over £852 million for joint replacement in 2010 (Chen et al., 2012).

The characteristic structural changes of OA (which include cartilage loss, calcification of cartilage, vascular invasion of cartilage and bone remodelling) contribute to the development of a biomechanical environment which enhances the progression of the disease (Goldring, 2006, Fuerst et al., 2009). There is significant difficulty involved in the diagnosis of OA given the aneural nature of articular cartilage which delays pain responses during the disease until bone to bone surface interaction occurs. Many structural changes which occur as the disease progresses can occur without pain, as such many symptoms (such as patient reported symptoms like localised pain or joint stiffness and radiographically detected joint space narrowing) are often undetected until the late stages of the disease (Kellgren and Lawrence, 1957, Sinusas, 2012).

1.5.1 Clinical Treatment of OA

Current treatment for OA is typically split into three main approaches, non-pharmacological, pharmacological and surgical. Firstly, non-pharmacological therapies, studies have shown that exercise programmes focussed on muscle strengthening and increasing range of motion significantly improve patient reported OA symptom scores (Thomas et al., 2002). Such exercises are recommended as part of best clinical practice guidelines for OA (Scott et al., 1998). Non-pharmacological treatment is often supplemented by pharmacological therapies in order to provide pain relief for painful symptoms, typical pain relief includes paracetamol, non-steroidal anti-inflammatory drugs (NSAIDs), cyclo-oxygenase (COX-2) inhibitors and opioids. Additionally, intra-articular injections of hyaluronic acid have been shown to provide improvements in pain and function for patients, which last up to four months (Towheed et al., 2006, Sinusas, 2012, Bellamy et al., 2006). In regards to surgical interventions, interventions such as arthroscopic lavage or debridement are used in cases where mechanical locking of the joint is apparent. However, randomised trials have found that such surgical interventions provide no additional patient benefit than a combined exercise and pharmacological therapy approach (Kirkley et al., 2008). Finally, total joint replacements are used in the advanced/end stages of OA, in such cases joint prostheses are used and are the most effective surgical treatment in regards to patient outcomes (Sinusas, 2012, Wieland et al., 2005).

The pathogenesis of OA is often attributed to either an imbalance of repair processes leading to cartilage destruction in which inflammation is a secondary factor, or attributed to synovial inflammation driving OA pain and progression (Berenbaum, 2013). The suggestion that OA is a primarily inflammation driven disease has led to trials of disease-modifying anti-rheumatic drugs (DMARDs) which are commonly used for the treatment of inflammatory forms of arthritis (such as rheumatoid arthritis). Interestingly, results of a recent meta-analysis by Persson et al. (2018) has shown that DMARDs are ineffective in providing pain relief to patients with symptomatic OA when compared to placebo. As a result of this study Persson and colleagues conclude that whilst inflammation may play a role in OA it is likely not the major risk factor of OA, indicating the necessity of specific disease-modifying treatments for osteoarthritis (DMOADs).

However, to date there are no proven effective DMOADs. Multiple disease modifying therapies investigated have currently unfortunately been unsuccessful either due to adverse side effects or poor clinical efficiency. Trials include the use of inhibitors of catabolic factors thought to be critical in the progression of OA such as MMPs and ADAMTSs. Although, the search for new therapeutic targets is ongoing (Krzieski et al., 2007, Karsdal et al., 2016, Fosang and Little, 2008, Troeberg et al., 2008, Goldring and Berenbaum, 2015, Larkin et al., 2015, Wang et al., 2013).

1.5.2 Molecular Changes in OA

There are believed to be multiple risk factors involved in the development of OA including; age, genetic predisposition, gender and cartilage injury through physical trauma. Such risk factors are thought to result in a disruption to the homeostasis of articular cartilage, due to an imbalance between anabolic and catabolic factors (Chen et al., 2017, Goldring, 2006, Li et al., 2013, Tsezou, 2014). Such imbalance between anabolism and catabolism resulting in cartilage degradation in OA is believed to be related to increased expression of inflammation related genes (and a subsequent increase in proteases and protease activity (MMPs and ADAMTSs) and/or a decrease in activity of catabolic regulators such as TIMPs (Goldring et al., 2011, Murphy et al., 2002). Two key events are generally associated with the degeneration of cartilage in OA. Firstly, proteoglycan degradation which is believed to be an early event that is potentially reversible due to the ability of chondrocytes to resynthesise proteoglycans such as aggrecan. Secondly, the breakdown of the collagen network, which once degraded cannot be returned to its original state (Mort and Billington, 2001, Goldring, 2012).

The above indicates a requirement to understand the contribution of proteases (ADAMTS5 and MMP13) to OA progression. Importantly, it has been shown that mRNA levels for key MMPs and ADAMTSs increase in human OA cartilage during the progression of the disease. In early OA, groups have shown that messenger RNA (mRNA) levels of MMP3 increase, whereas, in late stage human OA cartilage an increase in expression of MMP1, 2, 13 and ADAMTS4 and 5 is reported (Bau et al., 2002).

In regards to ADAMTSs, whilst multiple ADAMTSs act as aggrecanases, it is ADAMTS5 which is believed to be the primary aggrecanase responsible for aggrecan degradation in human OA. Initially, Glasson et al. (2005) demonstrated a significant reduction in the severity of cartilage destruction, following surgically induced joint instability, in ADAMTS5 knockout mice, by measuring the production of aggrecan cleavage fragments, compared to wild-type mice. Additionally, Glasson and colleagues show that exposure to proinflammatory cytokine interleukin-1 (IL-1 α) resulted in a significant increase in aggrecan degradation in wild-type mice, whilst ADAMTS5 knockout mice were resistant to IL-1 α induced aggrecan cleavage. Importantly, it was also shown that ADAMTS4 knockout mice had no resistance to the progression of OA following surgical induced joint instability, indicating that ADAMTS5 is the primary aggrecanase in OA in the murine model (Stanton et al., 2005). Subsequent to the findings in the murine model, ADAMTS5 has also been implicated as the major aggrecanase involved in cartilage degradation in human OA cartilage.

Although, it should be noted that it has been shown that inhibition (by siRNA) of both ADAMTS4 and ADAMTS5 in human cartilage explants is sufficient to inhibit degradation/release of proteoglycan fragments when explants were stimulated with tumour necrosis factor alpha (TNF α) (known to induce the depletion of proteoglycans in cartilage explants (Saklatvala, 1987)) indicating a role of both ADAMTS4 and ADAMTS5 in human OA (Song et al., 2007). More recently however, Larkin et al. (2015) investigated the use of generated selective monoclonal antibodies (mAbs) for both ADAMTS4 and ADAMTS5 as DMOADs. Larkin and colleagues found that in human OA cartilage explants the ADAMTS5 mAbs had the greatest effect in suppressing ARGS neoepitope release when compared to ADAMTS4 mAbs treatment. Interestingly, following single treatment with ADMATS5 mAbs ARGS neoepitope release was shown to be suppressed for several weeks. Thus, a dominant role of ADAMTS5 toward cartilage destruction is indicated in human OA.

In regards to the contribution of MMPs to cartilage destruction, it is MMP13 that is believed to play a pivotal role. As previously stated MMP13 has been shown to be the MMP with the highest affinity for type II collagen, in human cartilage. Additionally, increased mRNA expression of MMP13 by chondrocytes in late stage human OA cartilage has also been shown (Bau et al., 2002, Mitchell et al., 1996, Wang et al., 2013). Little et al. (2009) were able to show that the loss of MMP13 protects against cartilage destruction in mice. They generated a MMP13

knockout mouse which, following surgically induced OA (DMM), was resistant to structural cartilage changes when compared to wild-type mice, indicating that structural cartilage damage is dependent on MMP13 in the mouse. However, Little and colleagues note that MMP13 knockout mice exhibited no reduction in chondrocyte hypertrophy, osteophyte development or aggrecan loss. The resistance of MMP13 knockout mice to cartilage erosion with no reduction in aggrecan loss supports the suggestion that aggrecan loss on its own may not drive cartilage degradation therefore indicating the importance of MMP13 in cartilage degradation and as a therapeutic target (Little et al., 2009). As such, MMP13 specific inhibitors, developed as potential DMOADs, have been tested in murine cartilage resulting in increased Type II collagen, increased proteoglycan content and reduced chondrocyte apoptosis (Wang et al., 2013). Finally, Piecha et al. (2010) report that novel selective MMP13 inhibitors were able to reduce Type II collagen degradation in IL-1 α stimulated human OA cartilage by up to 80% when compared to untreated controls.

In summary, whilst the mechanism of initiation of cartilage degradation in OA is unclear, it seems likely that proteases are involved with both ADAMTS5 and MMP13 implicated as the primary proteases. Such increased protease activity in OA would lead to the degradation of key ECM components including the loss of water binding proteoglycans and the integral collagen network. It is these changes to the cartilage matrix structure which are responsible for the loss of the functional properties of articular cartilage. Thus, as detailed above the ability to target and inhibit protease activity is crucial to the development of therapeutic disease modifying treatments.

1.5.3 OA Chondrocyte Phenotype

The resident cell type of articular cartilage, the chondrocyte, has been described as undergoing a phenotypic change in response to/contributing to the cartilage matrix degradation that occurs in OA. Notably, in early OA chondrocytes have been described as attempting matrix repair by; proliferating, increasing synthesis of the cartilage matrix (upregulating proteoglycans, collagens and glycoproteins), and an increase in synthesis of catabolic cytokines and matrix degrading proteases (such as MMPs and ADAMTSs) (Goldring, 2000). Interestingly, whilst an increase in Type II collagen and proteoglycan expression is reported in OA chondrocytes it has been shown that this does not result in the repair of the cartilage matrix (Aigner et al., 1997, Goldring, 2000, Goldring, 2012, Rizkalla, 1992). The lack of repair despite increased collagen synthesis may also be attributed to the shift in the pattern of collagen expression by chondrocytes.

For example, the chondroprogenitor splice variant of Type II collagen, TypeIIA typically absent in normal adult articular cartilage, is re-expressed by mature chondrocytes in OA (Aigner et al., 1999). Additionally, it has been shown that chondrocytes begin to downregulate *COL2A1*, following increased matrix degradation, and instead upregulate *COL10A1*. Type X collagen is a marker of hypertrophic chondrocytes, which will eventually undergo apoptosis (Girkontaite et al., 1996, Poole et al., 2001).

It has also been shown that a role of pro-inflammatory cytokines exists in OA which are derived from the synovium and have also been shown to be produced by the OA chondrocyte (Goldring, 2000, Gruber et al., 2004). Key among these pro-inflammatory cytokines is IL-1, a cytokine which is typically produced by macrophages. In chondrocytes IL-1 β has been shown to upregulate MMPs and ADAMTSs and suppress the synthesis of both Type II collagen and aggrecan (Kobayashi et al., 2005, Flannery et al., 1999). Additionally, exposure of cartilage explants to IL-1 (isoform not specified) has been reported to increase the depletion of proteoglycan (Saklatvala, 1987). Interestingly, Martel-Pelletier et al. (1992) found an increased expression of the IL-1 receptor (IL-1R) in OA chondrocytes in association with an increased production of MMP13, this increase in IL-1R indicates that OA chondrocytes will have a higher sensitivity to IL-1 than normal chondrocytes. In fact Martel-Pelletier and colleagues were able to show that in OA chondrocytes IL-1 (IL-1 α and IL-1 β) concentration required for a half-maximal MMP stimulation were 3-4 times less than normal chondrocytes indicating that the presence of IL-1 at low concentrations could have a drastic effect on cartilage degradation via OA chondrocytes. Furthermore, in human OA synovial fluid an increase in both isoforms of IL-1 was found to be correlated with radiographically symptomatic knee OA (McNulty et al., 2013). However, whilst both in vitro and in vivo evidence suggest a role of IL-1 in cartilage degradation that occurs during OA it should be noted that recent studies in the murine model of OA have found that the depletion or inhibition of both IL-1 α and IL-1 β does not significantly affect either cartilage degradation or protease activity. Such results indicate that, in murine models at least, IL-1 may not be the key mediator of OA (Nasi et al., 2017, van Dalen et al., 2017).

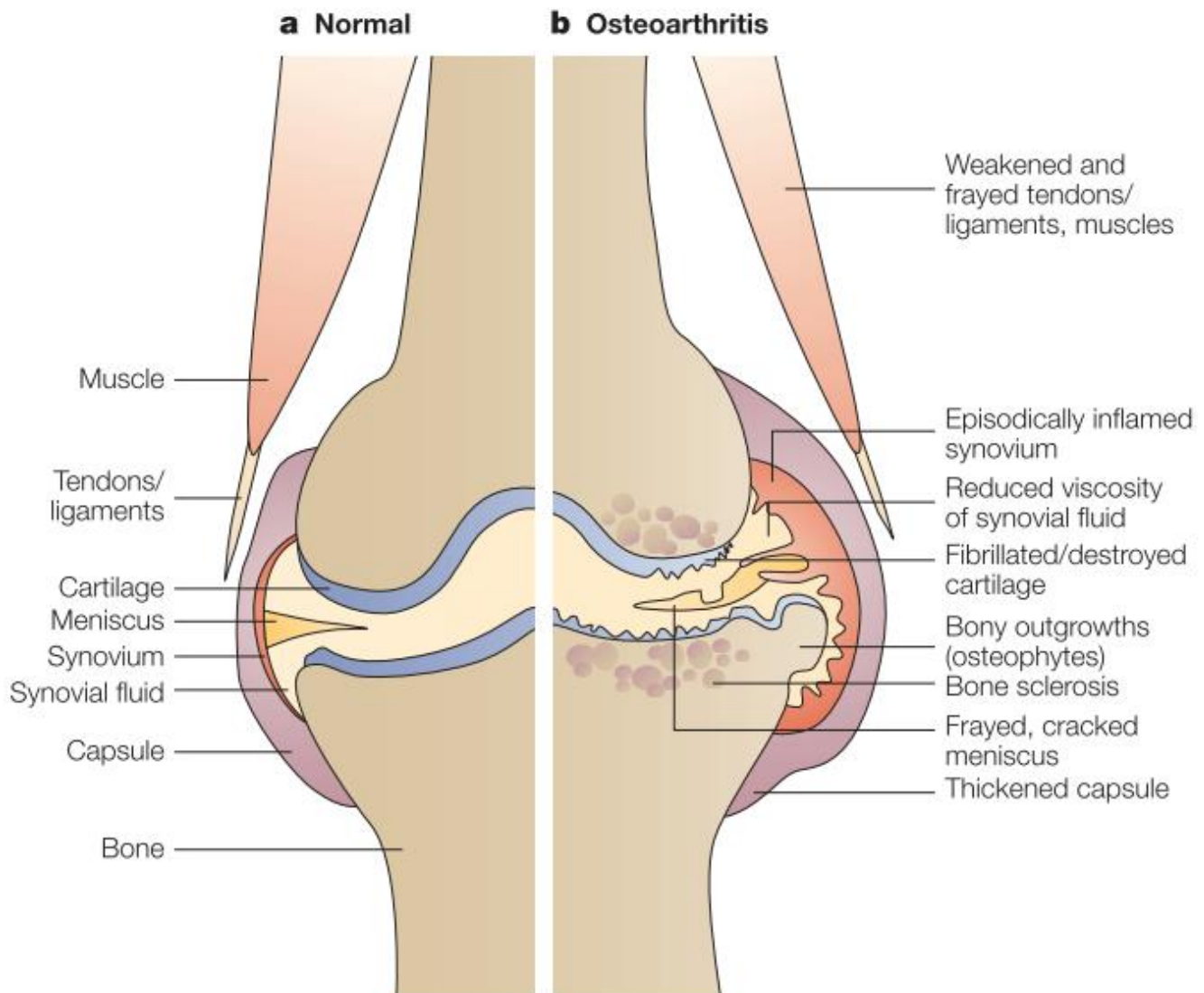


Figure 1.4 Changes to Articular Structures in Osteoarthritis. **A** Represents a normal (healthy) joint; normal cartilage with a smooth articular surface without any fissures, no signs of synovial inflammation and an intact meniscus. **B** Represents typical observations of an osteoarthritic joint; local degenerate lesions, fibrillated cartilage, osteophyte formation on the subchondral bone, bone remodelling (bone sclerosis), synovial inflammation and frayed meniscus. Obtained from (Wieland et al., 2005).

1.6 Cellular Hypoxic Response and the HIF Signalling Pathway

Given the avascular and physiologically chronic hypoxic environment of articular cartilage, the general cellular response to hypoxia and hypoxic signalling is described here. Hypoxia inducible factors (HIFs) are responsible for the mediation of the cellular response to hypoxia. HIFs are transcription factors/DNA-binding proteins that, in combination with co-activators, can transactivate the expression of genes containing oxygen-responsive transcriptional enhancer regions known as hypoxia responsive elements (HREs).

HIFs were originally identified when analysis of the upregulation of erythropoietin (EPO) in liver cells, in a low oxygen environment (hypoxia, $\leq 5\%$ O₂), identified binding of oxygen sensing transcription factors to an EPO gene enhancer (Semenza et al., 1991). Further analysis, including the purification of the proteins identified resulted in the naming and deduction of the structure of HIF-1 (Semenza and Wang, 1992). HIF is a heterodimer comprised of two subunits, a subunit post translationally regulated dependent on oxygen levels known as HIF- α and a constitutively expressed subunit aryl-hydrocarbon receptor nuclear transporter (ARNT) or HIF- β (Semenza, 1998). The HIF complex belongs to the family of basic-helix-loop-helix (bHLH) and PER-ARNT-SIM (PAS) domain containing transcription factors (Distler et al., 2004, Semenza and Wang, 1992).

Following the discovery of HIF-1 α , additional genes encoding for different HIF- α isoforms have been identified, namely HIF-2 α and HIF-3 α (Hara et al., 2001, Tian et al., 1997). To date HIF-1 α and HIF-2 α are the most studied and well understood of the isoforms and are known to be similar in regards to their protein organisation and structure. Both HIF-1 α and HIF-2 α have; N-terminal bHLH domains, PAS domains (which is required for the dimerization to HIF- β) and N-/C- terminal transactivation domains (N-TAD and C-TAD respectively) (Fernandez-Torres, 2017, Schofield and Ratcliffe, 2004) (**Figure 1.5**). Additionally, both HIF-1 α and HIF-2 α have two oxygen dependent degradation domains (NODDD and CODDD).

In well-oxygenated (normoxia) cells the HIF- α subunits are regulated by two hydroxylation dependent mechanisms. Firstly, HIF- α subunits can be hydroxylated at specific proline residues in the N-TAD, which is contained within the CODDD (**Figure 1.5-6**), by HIF-targeting prolyl hydroxylase domain enzymes (PHDs). PHD enzymes belong a family of Fe (II) and 2-oxyglutarate (2-OG) dependent oxygenases whose activity is highly oxygen dependent.

PHDs will use molecular oxygen and 2-OG as a co-substrate to hydroxylate the aforementioned proline residues on the HIF- α subunit (Schofield and Ratcliffe, 2004). The hydroxylation of the proline residues creates two binding sites recognised by von-Hippel-Lindau protein (pVHL), a component of the E3 ubiquitin ligase complex, which subsequently results in the ubiquitination and proteasomal degradation (by the 26S proteasome) of the HIF- α subunit (Bruick, 2001, Kaelin and Ratcliffe, 2008, Schofield and Ratcliffe, 2004, Webb et al., 2009). It has been shown that due to the activity of the PHDs, in normoxic conditions the HIF- α subunit is quickly degraded with a half-life of under 5 minutes (Thoms and Murphy, 2010).

The second mechanism by which HIF- α subunits are regulated by hydroxylation is by the hydroxylase enzyme known as factor inhibiting HIF-1 (FIH). Similar to the PHDs, FIH is a 2-OG dependent oxygenase. FIH uses molecular oxygen and 2-OG as co-substrates to hydroxylate specific a conserved asparagine residue present in the C-TAD of the HIF- α subunits (**Figure 1.5-6**). This hydroxylation prevents the interaction of HIF- α to co-activators CBP/p300 thus inhibiting the transcriptional activity of HIF (Elkins et al., 2003, Freedman, 2002, Hewitson et al., 2002, Lando et al., 2002).

In hypoxia the availability of molecular oxygen is limited inhibiting the activity of both PHD and FIH (due to their aforementioned requirement of molecular oxygen). In fact, as the activity of PHDs and FIH is dependent on oxygen levels the hydroxylases are typically considered the true oxygen sensors of the cell. In the absence of hydroxylation the HIF- α sub unit is stable, not targeted for proteasomal degradation by the E3 ubiquitin ligase complex, and is able to recruit it's co-factors to dimerise with HIF- β (co-activators p300 and CREB binding protein (CBP)). Following heterodimerisation active HIF translocates to the nucleus where it binds to the aforementioned HREs characterised by the presence of the sequence -5'RCGTG3'- (Semenza et al., 1996) (**Figure 1.6**). It has been shown that HIF is able to target and regulate a multitude of genes, related to; transcription, cell migration, energy metabolism and other cellular responses via the described HREs (Schofield and Ratcliffe, 2004).

1.6.1 HIF Isoforms; HIF-1 α , HIF-2 α and HIF-3 α

As previously mentioned, following the discovery of HIF-1 α by Semenza et al. (1992), additional HIF- α isoforms were discovered. Firstly, a second HIF- α isoform, EPAS1 (encoding for HIF-2 α) was discovered and originally believed to be an endothelial cell specific isoform, however it has since been determined that HIF-2 α is expressed in multiple cell types, co-expressed with HIF-1 α (Tian et al., 1996, Wiesener et al., 2002). As previously mentioned, HIF-1 α and HIF-2 α share a similar organisation of their protein structure, containing an N-terminal bHLH domains, a PAS domain and two transactivation domains (C-TAD and N-TAD). Additionally, both HIF-1 α and HIF-2 α are post transcriptionally regulated in the same manor via hydroxylation of specific proline (PHDs) or asparginyl residues (FIH) in their ODDD by 2-OG dependent oxygenases (Schofield and Ratcliffe, 2004). The proline residues hydroxylated by PHDs reside in the NTAD of HIF-1 α (Pro402 and Pro564) and HIF-2 α (Pro405 and Pro531), whereas the asparginyl residue reside in the CTAD of HIF-1 α (Asn803) and HIF-2 α (Asn851) (Fernandez-Torres, 2017) (**Figure 1.5**). Both of these isoforms also share high amino acid homology (48%), the individual functional components of HIF-1 α and HIF-2 α also share high levels of homology in regards to the bHLH (83.9%) and PAS (66.5%) motifs, as well as their ODDD (70%) and the C-TAD (67%) (Ema et al., 1997, Flamme et al., 1997, Hu et al., 2007).

Like HIF-1 α , HIF-2 α also heterodimerises with HIF- β in hypoxia, ultimately targeting genes containing HREs in their 3' promoter region. However, despite the high homology, inactivation of each HIF- α isoform originally revealed different phenotypes. HIF-1 α null mice have been shown to be embryonically lethal due to resultant defective cardiac morphogenesis (Ryan et al., 1998), whereas, inactivation of HIF-2 α results in varied phenotypes dependent on genetic background with mitochondrial dysfunction, respiratory distress leading to death (Compernelle et al., 2002, Scortegagna et al., 2003). Furthermore, it has been shown that both HIF- α isoforms have both common and unique targets. Common gene targets shared between HIF-1 α and HIF-2 α have been reported to include vascular endothelial growth factor (*VEGF*), adipose differentiation related protein (*ADRP*), glucose transporter 1 (*GLUT-1*) and filaggrin (*FLG*) (Hu et al., 2003, Keith et al., 2011). In regards to unique targets HIF-1 α has been shown to be target genes involved in glycolysis such as; phosphoglycerate kinase 1 (*PGK-1*), lactate dehydrogenase A (*LDHA*), hexokinase-2 (*HK-2*) and phosphofructokinase (*PFK*) (Hu et al., 2003). HIF-2 α has been shown to have gene targeting specificity for genes such as; transforming

growth factor alpha (*TGF α*), erythropoietin (*EPO*) and cyclin D1 (*CCND1*) (Hu et al., 2003, Keith et al., 2011, Raval et al., 2005).

Interestingly, however these differences in target genes are believed to be conferred by the activity of the transactivation domains of each isoform (Lau et al., 2007). In fact Hu et al. (2007) have shown that replacement of the N-TAD of HIF-2 α with the analogous N-TAD region of HIF-1 α is sufficient to convert HIF-2 α into a protein with the functional specificity of HIF-1 α . Importantly Hu and colleagues noted that only replacement of N-TAD (and not C-TAD or bHLH and PAS motifs) results in this change in target gene specificity of the subunit. Therefore, the group report that N-TAD is likely responsible for conferring gene specificity to the HIF- α isoforms.

Finally, a third HIF- α isoform, HIF-3 α has also been described (Gu et al., 1998). HIF-3 α has a similar organisation of its protein structure to HIF-1 α and HIF-2 α , containing a bHLH and PAS motif as well an N-TAD domain however, HIF-3 α does not contain a C-TAD domain (required for binding to co-activator p300) (**Figure 1.4**) (Fernandez-Torres, 2017, Gu et al., 1998, Heikkila et al., 2011). The function of HIF-3 α is less clearly understood than other HIF- α isoforms. It has been reported that 10 splice variants of HIF-3 α exist. HIF-3 α may act as a dominant negative of other HIF- α isoforms, this is as some HIF-3 α variants are able to bind to HIF- β potentially limiting the activity of other HIF- α isoforms where HIF- β is a limiting factor (Hara et al., 2001, Makino et al., 2002). Additionally, in mice, Makino et al. (2002) have shown that inhibitory PAS domain protein (IPAS) is a splicing variant of the HIF-3 α locus. IPAS is described as a dominant negative of HIF- α , this is as IPAS is able to bind to other HIF- α subunits inhibiting the interaction between HIF and HREs of target genes. In humans Maynard et al. (2005) have discovered that a HIF-3 α splice variant (HIF-3 α 4) exists which is similar for the variant encoding for IPAS in mouse. HIF-3 α 4 has been shown to form complexes with HIF-1 α and HIF- β acting as a dominant negative to HIF-1 α inhibiting its activity. Importantly, it is noted that each of the HIF- α isoforms have been shown to be expressed in human articular cartilage by chondrocytes (Coimbra et al., 2004, Markway et al., 2015). The functional role of HIFs in the hypoxic response of both normal and osteoarthritic cartilage will be discussed further in section 1.7 and 1.9 respectively.

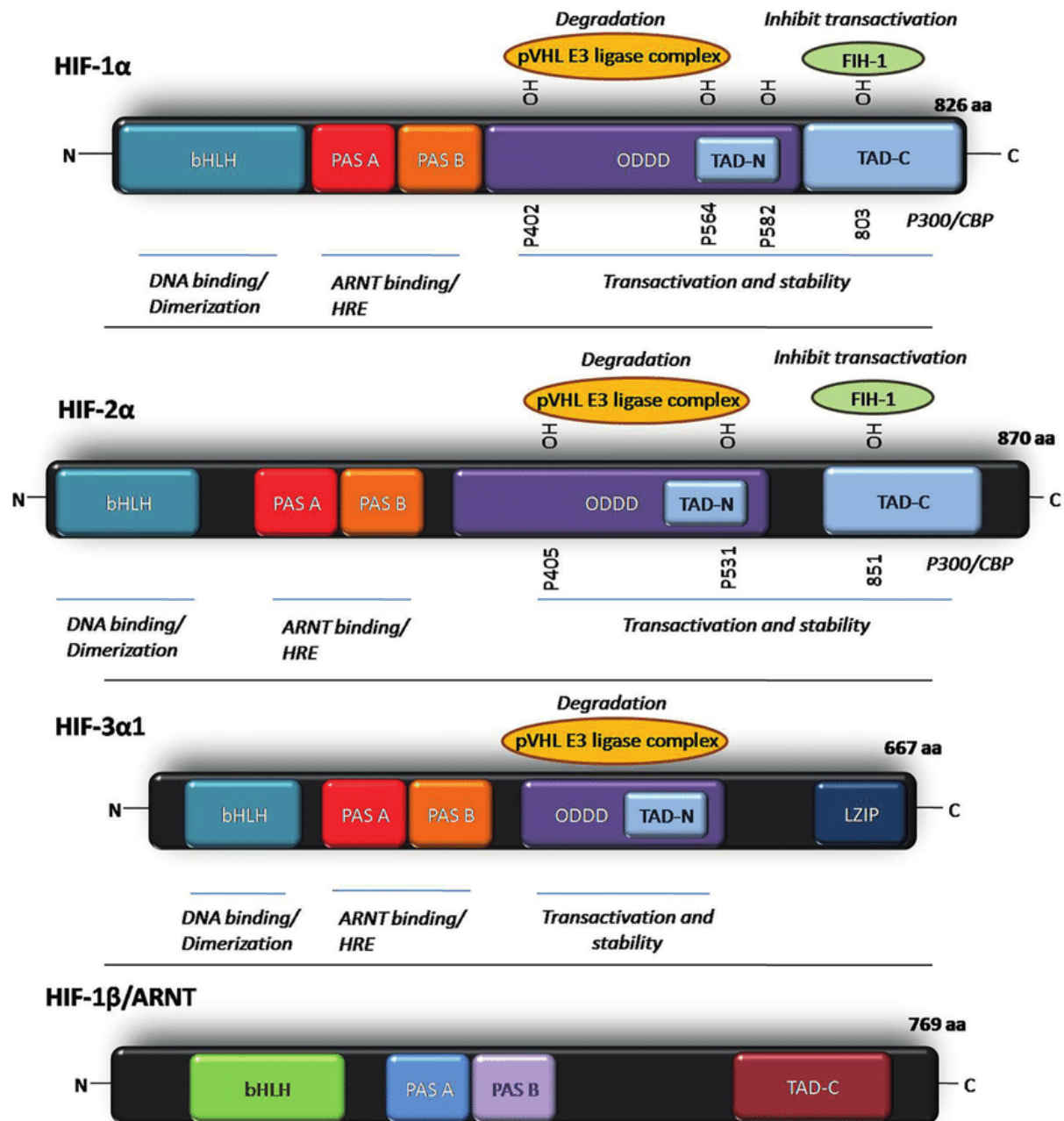


Figure 1.5 The Domain Structure of HIF Members. The structural domains of HIF-1α, HIF-2α and HIF-3α and the transcriptional binding partner HIF-1β/ARNT which form transcriptionally active HIF complexes. Both HIF-α and HIF-β have N-terminal basic helix-loop-helix (bHLH) domains and Per-ARNT-Sim (PAS) domains which are required for heterodimerisation and DNA binding to hypoxia responsive elements (HREs) on target genes. HIF-α subunits contain two transactivation domains, C terminal activation domain (TAD-C) and N terminal transactivation domain (TAD-N) with the exception of HIF-3α which lacks TAD-C. The hypoxic regulation of HIF-α subunits is conveyed by proline residues within the oxygen dependent degradation domains (ODDD). Inhibition of HIF-1α and HIF-2α transactivation by FIH-1 is conveyed by asparagine residue in TAD-C. Only HIF-3α contains a leucine zipper domain (LZIP) in the C-terminal. Obtained from (Fernandez-Torres, 2017)

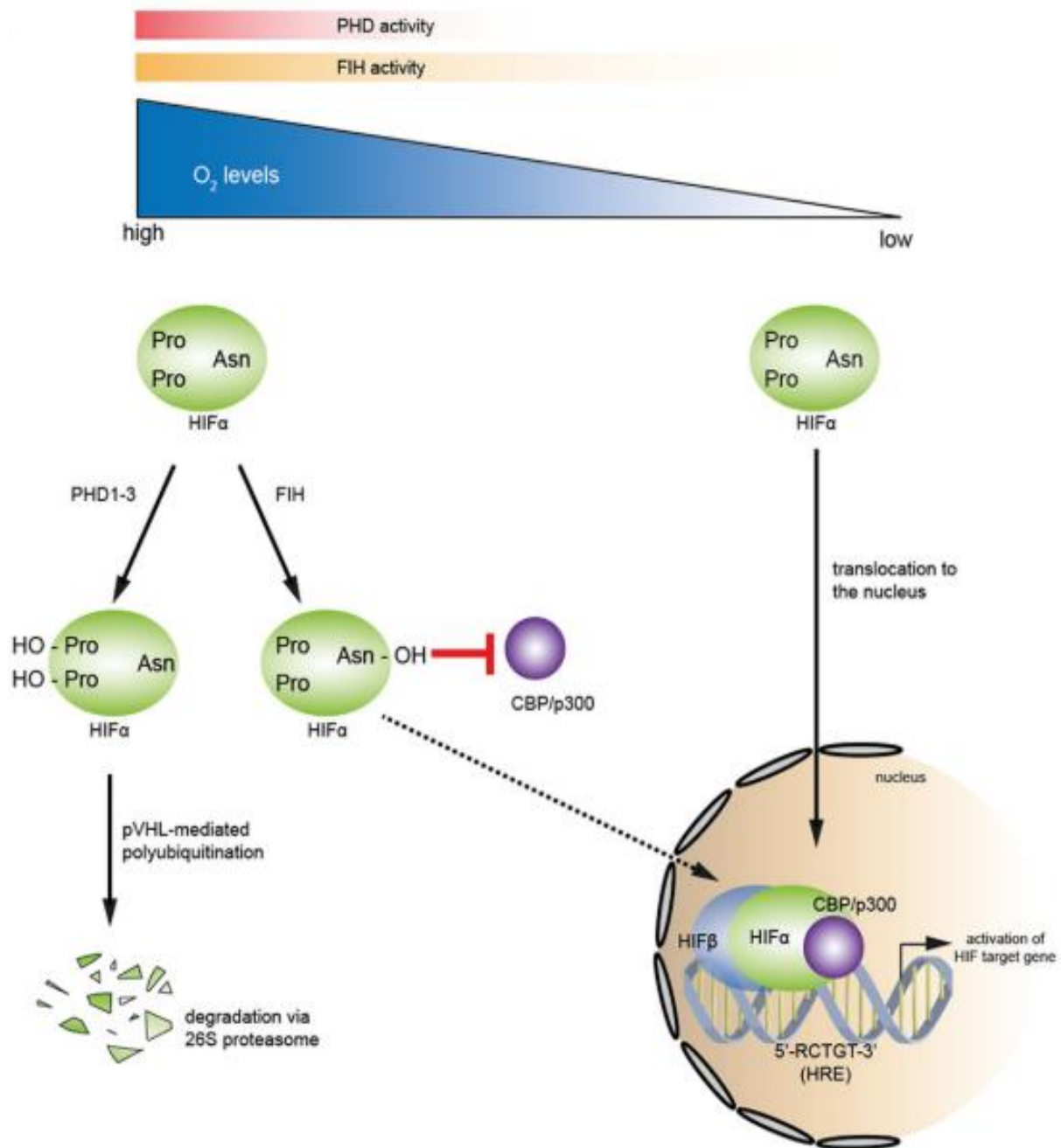


Figure 1.6 Hypoxia HIF Sensing Pathway. The activity of HIF- α subunits is regulated in an oxygen dependent manner by two independent hydroxylation pathways; HIF prolyl hydroxylases (PHDs) and asparaginyl hydroxylase factor inhibiting HIF-1 α (FIH). In the presence of oxygen hydroxylation of prolyl residues by PHDs on the oxygen degradation domains (ODDD) of HIF- α subunit results in the Von Hippel Lindau (pVHL) mediated polyubiquitination and the subsequent degradation of HIF- α . In the presence of oxygen hydroxylation of the asparagine residue on the HIF- α subunit inhibits the binding of CBP/p300 co-activator repressing HIF- α transcriptional activity. However, in reduced oxygen tensions ($\leq 5\%$ O₂) oxygen dependent hydroxylase activity is inhibited/reduced. Thus HIF- α heterodimerises with HIF- β and co-activator CBP/p300. Transcriptionally active HIF migrates the nucleus and binds to specific hypoxia responsive elements (HREs) -5'-RCTGT3'- on targets genes activating their transcription. Obtained from (Chan et al., 2016).

1.7 HIF Stabilisation in Human Articular Chondrocytes

As previously mentioned (section 1.2) due to the structure of articular cartilage it functions in a chronically hypoxic environment. Through the depth of the tissue it is predicted that the ECM and chondrocytes are exposed to a range of 1-7% O₂ (Silver, 1975, Milner et al., 2006, Brighton and Heppenstall, 1971, Zhou et al., 2004). Due to this reduction in availability of molecular oxygen, the activity of HIF-targeting hydroxylases (PHDs and FIH) is reduced, leading to the subsequent stabilisation of HIF-1 α and HIF-2 α in human articular cartilage. As such the presence of both HIF- α isoforms has been confirmed in human articular normal and OA cartilage via immunohistochemistry (Bohensky et al., 2009, Coimbra et al., 2004, Yudoh et al., 2005). Additionally, the suggestion has been made that reduced partial pressure of molecular oxygen in the synovial fluid of OA joints is associated with an increase in the activity of HIF-1 α (Grimmer et al., 2006, Lund-Olsen, 1970).

In regards to HIF stabilisation, it has been shown that factors other than hypoxia are able to stabilise the HIF- α subunits. Firstly, it has been reported, in the small animal model, that HIF-2 α expression can be regulated by hydrostatic pressure changes in small animal model (rabbit) chondrocytes. Inoue et al. (2015) were able to show that gene expression of HIF-2 α is significantly increased following the application of 5 megapascal (mPA) or pressure although the mechanism contributing to this regulation remains unclear. In addition to the regulation of HIF-2 α by pressure, Inoue and colleagues also show that inflammation stress is able to regulate HIF-2 α expression. Stimulation of chondrocytes with pro-inflammatory cytokine IL-1 β resulting in a significant 3.7 fold increase in HIF-2 α gene expression (Inoue et al., 2015).

The role of inflammatory stress and the ability of pro-inflammatory cytokines to regulate HIF is of particular interest in OA cartilage. An example of this effect is shown in experimental evidence reported by Yudoh et al. (2005), indicating increased gene expression of HIF-1 α in OA cartilage in correlation with progression of cartilage degradation. Yudoh and colleagues further investigated the role of HIF-1 α in OA by analysing the effect inflammatory stress (in the form of IL-1 β) in hypoxia in human articular chondrocytes (HACs). Yudoh and colleagues found that stimulating HACs with IL-1 β enhanced HIF-1 α gene expression and increased HIF-1 α protein expression above levels typically observed by hypoxia alone. The regulation of HIF-1 α by IL-1 β here is reported to be phosphatidylinositol 3-kinase and p38 a mitogen-activated protein kinase (MAPK) dependent, as inhibitors for each caused a decrease in IL-1 β induced HIF-1 α (Yudoh et

al., 2005). In addition to this, Coimbra et al. (2004) have reported that HIF-1 α can also be induced by TNF- α and that this induction can also be reduced by the inhibition of p38 MAPK. Thus, whilst the mechanism of pro-inflammatory cytokine induction of HIF is unclear it has been shown that the mechanism is, to some degree, p38 MAPK dependent. This upregulation of HIF-1 α in response to catabolic factors in OA HACs has been reported by other groups and is believed to act as a chondroprotective factor in the presence of increased catabolic activity (such as proteases) (Sartori-Cintra et al., 2012, Yudoh et al., 2005, Schipani et al., 2001, Coimbra et al., 2004).

However, the levels of HIF-2 α have been shown to decrease in OA cartilage, with the reduction of HIF-2 α linked to increased cell death. Thus, suggesting a role of HIF-2 α in the modulation of autophagy, preventing apoptosis (Bohensky et al., 2009). Interestingly, research in the murine model of OA has shown an increase in HIF-2 α expression in OA cartilage when compared to normal articular cartilage. Additionally research has shown the induction of HIF-2 α by pro-inflammatory cytokines (including IL-1 β), in mice, results in the subsequent transactivation of catabolic factors, implicating HIF-2 α as a regulator of OA (Ryu et al., 2012, Yang et al., 2010). The implication of the relationship between HIF, inflammation and the progression OA in articular cartilage will be described further (Section 1.7.3).

1.7.1 HIF and the Chondrocyte Phenotype

The importance of HIF in regards to general development and specifically to chondrogenesis has been shown via the production of murine knockouts of both HIF-1 α and HIF-2 α . HIF-1 α ^{-/-} and HIF- β ^{-/-} mice are embryonic lethal, exhibiting multiple morphological abnormalities, highlighting the importance of the HIF pathway in embryonic development and vascularisation (Ryan et al., 1998, Compennolle et al., 2003, Kozak et al., 1997).

In addition to this, cartilage specific HIF-1 α deletion in mice has been reported to result in cell death of articular and growth plate chondrocytes in developing mice, resulting in multiple vertebral body defects. The effects of HIF-1 α depletion in this case has been linked to decreased expression of the Cyclin-Dependent Kinase (CDK) inhibitor p57 in the differentiating cells (Schipani et al., 2001). Additionally, murine data has indicated that HIF-1 α is crucial in the synthesis of ECM components. Pfander et al. (2003) assessed the hypoxic response of HIF-1 α ^{-/-} murine chondrocytes in comparison to wild-type murine chondrocytes. Pfander and colleagues noted that HIF-1 α ^{-/-} murine chondrocytes were unable to maintain ATP levels indicating the

requirement of HIF-1 α in chondrocyte metabolism. The group also note that the hypoxic induction of; vascular endothelial growth factor (VEGF), aggrecan mRNA and protein, and Type II collagen mRNA and protein observed in wild-type murine chondrocytes was lost in the HIF1- $\alpha^{-/-}$ murine chondrocyte. In regards to HIF-2 α , HIF-2- $\alpha^{-/-}$ mice are, unlike HIF-1 $\alpha^{-/-}$, not embryonic lethal, however the lifespan of HIF-2 $\alpha^{-/-}$ is significantly shorter compared to wild-type mice. Scortegagna et al. (2003) report that HIF-2 $\alpha^{-/-}$ mice have a syndrome of multiple-organ pathology including; cardiac hypertrophy, skeletal myopathy, mitochondrial dysfunction and hypocellular bone marrow, indicating an important functional role of HIF-2 α .

Finally, in relation to HIF and chondrogenesis, Robins et al. (2005) investigated the hypothesis that oxygen tension (hypoxia) was able to modulate the fate of bone mesenchymal cells (ST2 cells) via the expression of genes that promote chondrogenesis. In this study Robins and colleagues show the activation of genes known to be expressed by chondrocytes in ST2 cells exposed to hypoxia. Notably, a hypoxic dependent increase in the expression of key cartilage transcription factor SOX9 caused ST2 cells to differentiate toward a chondrocyte pathway. Additionally, Robins and colleagues identified a HRE on the promoter region of SOX9 which the group suggested is activated by HIF-1 α noting a nuclear accumulation of HIF-1 α . The hypoxia dependent upregulation of SOX9 was confirmed by putative mutations of the HRE site on the SOX9 promoter which abolished the observed mRNA level increase in hypoxia, however direct binding of HIF-1 α to the SOX9 promoter was not investigated (Robins et al., 2005). Hypoxia induced chondrogenesis was further investigated by Kanichai et al. (2008) by exposing mouse mesenchymal cells to hypoxia and measuring chondrogenesis via Type II collagen and proteoglycan deposition, as well as the expression levels of SOX9. Crucially, Kanichai and colleagues were able to show that hypoxia induced chondrogenesis is related to the activity of HIF-1 α . By using siRNA targeting HIF-1 α , the group were able to show that hypoxia induced chondrogenesis in the mouse mesenchymal cells was absent without the presence of HIF-1 α . It is important to note however that the contribution of HIF-2 α was not investigated in this study (Kanichai et al., 2008).

In summary, these studies show the importance of HIF in the development, the differentiation and the survival of articular chondrocytes.

1.7.2 Hypoxic Responses of Normal Cartilage

In normal or healthy articular cartilage hypoxia and the activity of HIF-1 α and HIF-2 α have been documented as promoting the chondrocyte function. In addition to the effects of HIF-1 α in murine mesenchymal cells described above, Amarilio et al. (2007) also show that murine mesenchymal cells produce a 2 fold increase in the expression of *SOX9* when exposed to hypoxia, an increase which is lost in HIF-1 α depleted cells. Subsequently, *COL2A1* and *ACAN* mRNA expression (targets of *SOX9*) show a 1.7 fold and a 2.3 fold upregulation in hypoxia which is also lost in HIF-1 α depleted cells. Importantly, Amarilio and colleagues use chromatin immunoprecipitation (ChIP) to show direct binding of HIF-1 α to the *SOX9* promoter in murine mesenchymal cells (Amarilio et al., 2007). HIF-1 α has also been shown to be responsible for the hypoxic induction of collagen prolyl-4-hydroxylases (C-P4Hs) in murine epiphyseal growth plates, key enzymes in collagen synthesis responsible for post-translational modification of collagen that infers stability of the collagen molecules. Thus, the activity of HIF-1 α increases collagen secretion via increased gene expression (by *SOX9* upregulation) and enhanced synthesis (via C-P4H upregulation) in the developing murine chondrocyte (Aro et al., 2012).

Importantly, hypoxic upregulation of *SOX9* and subsequent upregulation of key cartilage matrix genes (such as *COL2A1* and *ACAN*), as well as a downregulation of catabolic factors, has also been observed in larger animal models such as porcine and bovine chondrocytes, as well as in human chondrocytes (Lafont et al., 2008, Murphy and Polak, 2004, Zhang et al., 2011, Strobel et al., 2010, Thoms et al., 2013, Grimmer et al., 2006). Similar to the murine data HIF-1 α has been shown to enhance collagen mRNA expression and synthesis (via C-P4Hs) in HACs, via *SOX9*, demonstrating the importance of HIF-1 α to the cartilage matrix (Grimmer et al., 2006). In contrast to data from the murine model, HIF-2 α , not HIF-1 α , has been described as being the HIF isoform responsible for the hypoxic upregulation, of gene expression and protein levels, of *SOX9*, Type II Collagen and *ACAN*. Using siRNA targeting *HIF-1 α* and *HIF-2 α* Lafont et al. (2007) show that HIF-1 α depletion had no effect on the upregulation of key matrix genes in hypoxia, whereas, HIF-2 α depletion abolished the effects of hypoxia reducing gene and protein expression levels within range of levels observed in normoxia. Further to this ChIP assay evidence has shown that HIF-2 α binds directly to a HRE on the *SOX9* promoter in the normal HACs (Thoms et al., 2013).

Work conducted previously in our group, Thoms et al. (2013), reports distinct roles for HIF-1 α and HIF-2 α that promote the function of HACs (**Figure 1.7**). As mentioned HIF-2 α appears to have a role in the anabolism of the HAC directly binding to and upregulating the expression of SOX9, subsequently upregulating key matrix components (COL2A1 and ACAN). Interestingly, HIF-1 α is shown to have a chondroprotective (anti-catabolic) effect, when using siRNA targeting HIF-1 α the hypoxic downregulation of MMP13 and ADAMTS5 was lost, alongside a loss of the hypoxic induction of TIMP3. The HIF-1 α dependent upregulation of metalloproteinases inhibitor TIMP3 and the subsequent downregulation of catabolic factors indicates a predominant role of HIF-1 α in anti-catabolism. The effectiveness of this hypoxia dependent anti-catabolism was explored using human cartilage explants stimulated with IL-1 α . In culture media from IL-1 α stimulated explants in normoxia the group detected increased ARGS neopeptide compared to control, however, in hypoxia the spontaneous and IL-1 α incited release of ARGS was inhibited. This inhibition of ARGS released in hypoxia represents the chondroprotective effects of hypoxia in human cartilage. In summary, this data indicates that hypoxia is a beneficial environment promoting the function of HACs.

The chondroprotective effects of hypoxia, in regards to the hypoxic downregulation of MMP13 in HACs have also been reported by Strobel et al. (2010). Strobel and colleagues describe a reduced expression of MMP13 mRNA and protein expression in HACs cultured in 5% O₂. This downregulation was confirmed to be due to the involvement of HIFs by the addition of cadmium chloride (a HIF- α inhibitor). When HIFs were inhibited, the hypoxic downregulation of MMPs was absent in HACs cultured in hypoxia.

Crucially, the beneficial effects of hypoxia and the activity of HIF can be enhanced in HACs through the inhibition of a specific PHD. PHD2 has been shown to be the dominant PHD in regards to HIF stabilisation in HACs (Thoms and Murphy, 2010, Appelhoff et al., 2004). As such, previous work in our group has shown that the depletion of PHD2 (by siRNA) in hypoxia increases levels of HIF- α protein above levels observed in hypoxia alone. This increase in HIF- α isoforms also has a functional effect on the expression of known HIF targets in HACs. The increased levels of HIF-2 α in hypoxia in PHD2 depleted HACs are reported to increase SOX9 expression and gene expression of cartilage collagens (including COL2A1) above levels observed by hypoxia alone. In addition to this, the hypoxic downregulation of catabolic factors MMP13 and ADAMTS5 has also been shown to be enhanced by PHD2 depletion in HACs (Thoms et al., 2013, Thoms and Murphy, 2010). Interestingly, it should be noted the reduced levels of

ADAMTS5 are indicated to be an effect of PHD2 depletion that is independent of HIF (**Figure 1.7**). The summary of these results indicates that the depletion of PHD2 is able to enhance the positive hypoxia-induced effects on HAC function, and thus represents a potential therapeutic target in OA.

1.7.3 HIF Responses of Osteoarthritic Cartilage

Past work by our group suggest that the hypoxic response and the activity of HIF could be modified via hydroxylase activity to provide therapeutic benefit based on evidence around the hypoxic response of normal healthy HACs. However, the activity of HIFs in human OA cartilage and chondrocytes is less clearly understood, and requires further investigation. Interestingly, studies investigating the activity of HIFs in OA models in mice have indicated a potential species-difference in the previously described role of HIFs in human cartilage, specifically in regards to the activity of HIF-2 α .

Firstly, as previously mentioned, it has been reported that increased levels of HIF-1 α and HIF-2 α are detected in both human and murine OA cartilage (Pfander et al., 2005, Saito et al., 2010, Giatromanolaki et al., 2003, Yang et al., 2010). In OA cartilage, HIF-1 α is believed retain its chondroprotective properties, maintaining the ATP levels and matrix synthesis of OA chondrocytes via the upregulation of genes involved in glycolysis such as *GLUT-1* and *PGK-1*. Thus, the increased level of HIF-1 α in OA cartilage is believed to be in response to catabolic factors in an attempt to protect and maintain the tissue (Grimmer et al., 2007, Pfander et al., 2005, Zhang et al., 2015a, Yudoh et al., 2005, Fernandez-Torres, 2017).

In regards to HIF-2 α however, in murine models of OA, it is more commonly believed that HIF-2 α is a regulator of cartilage degradation upregulated in response to inflammation during the progression of OA. As such it is often suggested that HIF-2 α may prove a potential therapeutic target for the treatment of the disease (Fernandez-Torres, 2017, Husa et al., 2010, Koh and Powis, 2012, Lee et al., 2015, Saito and Kawaguchi, 2010, Thysen et al., 2015, Zhang et al., 2015a, Cheng et al., 2015). The most compelling evidence suggesting that HIF-2 α is a transactivator of catabolic enzymes playing a role in OA comes from two independent murine model studies published in *Nature Medicine*.

Firstly, Yang et al. (2010) identified HIF-2 α as a gene upregulated in murine articular chondrocytes stimulated with IL-1 β as a result of screening the UniGeneLibrary for genes which may regulate cartilage destruction. Subsequently the group show that under pathological conditions, specifically IL-1 β stimulation, HIF-2 α expression was increased in murine chondrocytes. The group then investigated whether the enhanced expression of catabolic enzymes by IL-1 β (NOS2, PTGS2, MMP9, 12, 13 and ADAMTS4, 5) was mediated by HIF-2 α . Yang and colleagues found that overexpression of HIF-2 α (via EPAS-1 encoding adenovirus) did indeed result in the upregulation of catabolic factors including MMP13 and ADAMTS5, additionally, siRNA targeting *HIF-2 α* blocked the upregulation of these genes in IL-1 β stimulated murine chondrocytes. Furthermore, the group noted that both EPAS-1 transgenic mice and overexpression of HIF-2 α in wild-type mouse joints resulted in accelerated joint destruction. Whereas, mice with the deletion of a single *HIF-2 α* allele (HIF-2 α ^{+/-}) with a surgical model of OA (destabilised medial meniscus/DMM) were reported to exhibit increased resistance to cartilage destruction.

Importantly, Yang et al. (2010) investigated whether HIF-2 α regulated the expression of MMPs and ADAMTSs directly by analysing potential binding of HIF-2 α to their human and murine promoter regions (each of which containing a HIF-2 α binding motif). The results of this investigation indicated that HIF-2 α can directly target the human promoter regions for *MMP1*, 3, 12 and 13 (*MMP9*, *ADAMTS4*, *NOS2* and *PTGS2* only show evidence of direct binding on the mouse promoter). Finally, despite the presence of a HIF-2 α binding motif *MMP2*, 14, 15 and *ADAMTS5* were not affected by HIF-2 α .

A second murine study by (Saito et al., 2010) conducted a screen of transcription factors which regulated Type X collagen (*COL10A1*), a marker of hypertrophy, in a murine chondrogenic cell line (ADCT5) and in a non-chondrogenic human cell line (HeLa). Saito and colleagues reported HIF-2 α as a transactivator of *COL10A1*; additionally, the group identified HIF-2 α also regulated expression of MMP13 and VEGF in ADCT5 and HeLa cell lines. The regulation of MMP13, *COL10A1* and VEGF was described as being due to direct binding of HIF-2 α to HREs identified on the promoter region of the genes which was confirmed by ChIP and electrophoretic mobility shift assays. Similar to the research by Yang and colleagues, the group supported their findings using mice with a genetic deletion for one HIF-2 α allele. In this study HIF-2 α ^{+/-} mice with surgically induced OA were reported to be more resistant to cartilage degradation than wild-type mice. Furthermore, it is reported that levels of *COL10A1*, MMP13

and VEGF were increased in the OA joint of wild-type mice whilst their expression was repressed in HIF-2 α ^{+/-} mice severely delaying endochondral development in juvenile mice. However, it has also been shown that conditionally inactivated HIF-2 α , within the limb bud mesenchyme of juvenile mice, causes only a modest delay to endochondral development (Araldi et al., 2011).

In regards to the effect of HIF-2 α in humans Saito et al. (2010) report a relationship between a single nucleotide polymorphism (SNP) in the human *HIF-2 α* gene and knee OA in a Japanese population. However, Nakajima et al. (2012) performed a large-scale replication study with over 8000 subjects (comprised of cohorts from Japan, China, Australia and Europe) investigating the association between the *HIF-2 α* SNP and knee OA. The results from this large-scale meta-analysis found no statistically significant association between the *HIF-2 α* SNP and knee OA. The group notes that the significance of HIF-2 α in regards to human OA should be evaluated carefully before associations are made with the experimental evidence in the murine model.

Jian-Lin et al. (2016) have reported a correlation between increased *HIF-2 α* mRNA and protein levels in OA human cartilage explants, with an increased OA severity determined by radiographic severity (severity determined by Kellgren and Lawrence grading) prior to sample collection. Due to this correlation the group suggest that HIF-2 α may serve as a biochemical marker and a potential therapeutic target in human knee OA (Jian-Lin et al., 2016). However, this study does not investigate the activity HIF-2 α in human cartilage or chondrocytes and this suggestion is made based on evidence for the catabolic activity of HIF-2 α in the murine model (Saito et al., 2010, Yang et al., 2010). Additionally, it has been reported that in IL-1 β stimulated human meniscal cells, HIF-2 α is responsible for the induction of MMP13 and COL10A1 however this effect was not investigated in human chondrocytes (Ishizuka et al., 2016).

Interestingly, while evidence concerning the activity of HIF-2 α appears to suggest a catabolic role in murine models of OA, this is in stark contrast to previous findings in normal human articular chondrocytes reporting anabolic properties of HIF-2 α activity (Thoms et al., 2013). Furthermore, current findings in relation to the hypoxic response of OA human articular chondrocyte are in conflict with the findings in the murine OA model. Similar to the findings in normal HACs several groups have found that some catabolic factors are reduced by hypoxia in OA HACs. For examples, in regards to MMP13 (evidence from Yang and colleagues suggesting

direct binding of HIF-2 α on both murine and human gene promoter region), two groups report a decreased MMP13 expression by hypoxia in OA HACs (Markway et al., 2013, Alvarez K, 2014).

To summarise, HIF-2 α is described as a catabolic regulator of OA as a result of evidence reported in murine models of OA. However, experimental evidence concerning hypoxia in human OA chondrocytes does not appear to support this description. Current studies in regards to the hypoxic response in human OA HACs do not investigate the specific roles of the HIF- α isoforms. Therefore, there is a current requirement of further investigation into the hypoxic response and the role of HIF in a human OA setting in order to correctly identify potential therapeutic targets. Finally, the conflicting reports between the effects of hypoxia in OA may be due to species related differences between humans and mice - a subject which will be discussed in Chapter 6.

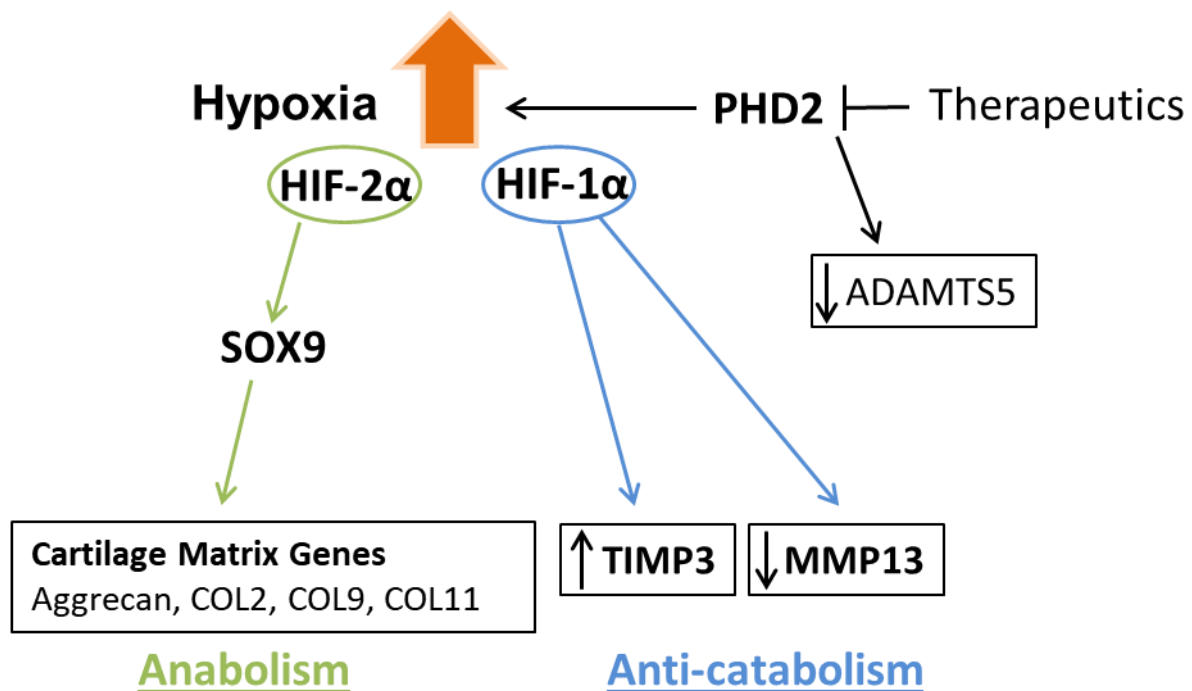


Figure 1.7 Summary of Healthy Human Cartilage Regulation by Hypoxia. Hypoxia increases the production of cartilage via direct binding of HIF-2 α to SOX9 resulting in the upregulation of key cartilage matrix genes in healthy human cartilage. Hypoxia inhibits the destruction of the tissue through HIF-1 α mediated reduction in collagenase MMP13 and simultaneous increase of matrix metalloproteinase inhibitor TIMP3. Inhibition of HIF targeting hydroxylase PHD2 reduces the levels of aggrecanase ADAMTS5 independent of HIF. Crucially the inhibition of PHD2 increases levels of HIF-1 α and HIF-2 α , and their subsequent effects, above levels observed by hypoxia alone. It is believed that PHD2 may represent a therapeutic target for cartilage repair due to the enhanced beneficial hypoxic response observed in its absence. Adapted from (Thoms et al., 2013)

1.8 Summary and Aims of the Project

In summary, the hypoxia signalling pathway and the activity of HIFs are crucial to chondrocyte survival and the maintenance of the phenotype through the hypoxic upregulation of the master regulator SOX9, ultimately supporting the structure and function of articular cartilage. In addition, hypoxia is a beneficial microenvironment for the normal healthy human articular chondrocyte, coordinating anabolism through the upregulation of key matrix genes such as ACAN and COL2A1 of which post-translational modification is also enhanced by hypoxic induction of C-P4Hs (Aro et al., 2012, Murphy and Polak, 2004, Bi et al., 1999). Hypoxia also provides chondroprotective effects through the upregulation of TIMP3, whilst also resulting in the downregulation of key catabolic enzymes ADAMTS5 and MMP13. Previous work in our laboratory has shown that in normal HACs these effects can be attributed to individual HIF isoforms; HIF-2 α binding directly to SOX9 inducing SOX9 protein expression and increasing matrix production and HIF-1 α the predominant isoform responsible for TIMP3 induction and catabolic factor downregulation. Additionally, it has been shown that the beneficial hypoxic response of HACs can be enhanced via depletion of PHD2 resulting in an increase of SOX9 levels beyond observed by hypoxia alone. Thus, suggesting PHD2 as a promising target to enhance the effects of hypoxia with potential for cartilage repair (Lafont et al., 2007, Thoms et al., 2013, Thoms and Murphy, 2010).

However, murine data suggest a very different picture with regard to HIF function in OA. It has been suggested that HIF-2 α plays a role as a catabolic regulator of cartilage degradation; evidence indicating the ability of HIF-2 α to directly target HREs on genes expressing catabolic factors such as MMP13 (Brennan et al., 2009, Ryu et al., 2012, Saito et al., 2010, Saito and Kawaguchi, 2010, Yang et al., 2010). Such evidence suggests that HIF-2 α inhibition may reduce catabolic factors in OA and therefore could itself be targeted for therapeutic benefit.

Given the seemingly conflicting reports concerning the role of HIFs and the hypoxic response in both normal and OA chondrocyte settings, this thesis sought to specifically investigate the hypoxic response, with the aim to elucidate the role of HIF- α isoforms, in the human OA cartilage/chondrocyte setting. Additionally, this work aimed to identify whether depletion or inhibition of HIF-targeting hydroxylases could manipulate the hypoxic response in OA HACs to potentially infer therapeutic benefit as previously hypothesised when considering the benefits of hypoxia in normal HACs.

1.8.1 Hypothesis

It is hypothesised that culture within a hypoxic environment will remain beneficial to the human chondrocyte/cartilage phenotype in an osteoarthritis setting, similar to effects of hypoxia reported in healthy HACs within the current literature. Additionally, it is hypothesised that HIF-2 α targets will remain anabolic in nature in OA HACs in contrast to data reported from murine OA models which suggest a catabolic role for HIF-2 α in OA.

1.8.2 Specific Aims

- 1) Uncover the effects of hypoxia on anabolic and catabolic responses in human OA chondrocytes and cartilage**
- 2) Elucidate the role of individual HIF- α isoforms in the above responses**
- 3) Identify whether the hypoxia pathway and subsequent response of OA chondrocytes can be manipulated via depletion of HIF-targeting hydroxylases**
- 4) Identify whether hypoxia protects against aggrecan degradation in the human OA cartilage explant**

Chapter 2

Materials and Methods

2.1 Reagents

Chemicals used to produce the buffers and reagents described below were purchased from Sigma-Aldrich (Dorset, UK), VWR Chemicals (Bedfordshire, UK) or MP Biochemicals (Leicester, UK). Phosphate-buffered saline (PBS) was purchased as a 10x sterile solution (VWR Chemicals) and diluted to a working 1x solution using double distilled water (ddH₂O).

2.1.1 Buffers and Reagents

PBST: 1x PBS, 0.1%(w/v) TWEEN 20.

2x sodium dodecyl sulphate (SDS) Sample Buffer: 100mM Tris-CL (pH 6.8), 200mM DTT, 4%(w/v) SDS.

4x SDS Loading Buffer: 200mM Tris-CL (pH6.8), 400mM DTT, 8%(w/v) SDS 0.4%(w/v) Bromophenol Blue and 40%(v/v) Glycerol.

Transfer Buffer: 25mM Tris, 190mM Glycine and 20%(v/v) Methanol.

Polyvinylidene difluoride (PVDF) Membrane Blocking Buffer: 5%(w/v) Dried Milk in 1xPBST.

Coomassie Stain: 0.1%(w/v) Coomassie R250, 10%(v/v) acetic acid and 40%(v/v) methanol.

Coomassie Destain: 20%(v/v) methanol and 10%(v/v) acetic acid.

Ponceau Red Stain: 0.1%(w/v) Ponceau S, 1%(v/v) Acetic acid.

Ponceau Red Destain: 5%(v/v) acetic acid

Deglycosylation Buffer: 20mM Tris (pH 7.5), 10mM EDTA and 20mM Sodium Acetate.

Tris/Borate/EDTA (TBE) Buffer: 89 mM Tris, 89mM Boric Acid and 2mM EDTA.

2.1.2 Cytokines

Human articular cartilage explants were stimulated with Interlukin-1 β (IL-1 β).

Table 2.1 Cytokines

Cytokine	Species	Vehicle	Source
IL-1 β	Human	Serum Free DMEM	R&D Systems (Minneapolis, USA)

2.1.3 Inhibitors

Passaged human articular chondrocytes (HACs) were cultured in the presence of Dimethyloxaloylglycine (DMOG)

Table 2.2 Inhibitors

Inhibitor	Target	Concentration	Vehicle	Source
DMOG	2-oxoglutarate dependent hydroxylase family	1mM	PBS	Sigma-Aldrich (Dorset, UK)

2.1.4 Antibodies

Primary antibodies and their relevant secondary antibodies used for western blotting are detailed in the tables below.

2.1.5 Trialled HIF-1 α and HIF-2 α Antibodies

Primary antibodies for HIF-1 α and HIF-2 α which were trialled and found not to be optimal for use with OA HACs are detailed in the table below. Antibodies were trialled by comparing visualisation of hypoxic stabilisation of HIF-1 α and HIF-2 α protein via western blot. Antibodies that were not used in the dataset either produced no specific bands or were suboptimal in regards to quality and reproducibility of western blots.

Table 2.3 Primary Antibodies

Antibody Specificity	Antibody Type	Dilution	Source
α-Tubulin (ab7291)	Mouse, monoclonal	1:5000	Sigma Aldrich company ltd, Dorset, UK
Anti-Aggrecan ARGxx [BC-3] (ab3773)	Mouse, monoclonal	1:100	Abcam, Cambridge, UK
SOX9 (ab5535)	Rabbit, polyclonal	1:1000	Chemicon, Southampton, UK
HIF-1α (NB100-105)	Mouse, monoclonal	1:500	Novus Biologicals, Littleton, CO, USA
HIF-1α (Clone 54)	Mouse, monoclonal	1:200	BD Transduction Laboratories, Lexington, KY, USA
HIF-2α (sc-46691)	Mouse, monoclonal	1:500	Santa Cruz Biotechnology, Santa Cruz, CA, USA
PHD1 (A300-326A)	Rabbit, polyclonal	1:1000	Bethyl Laboratories, Montgomery, TX, USA
PHD2 (NB100-137)	Rabbit, polyclonal	1:1000	Novus Biologicals, Littleton, CO, USA
PHD3 (ab30782)	Rabbit, polyclonal	1:250	Sigma Aldrich company ltd, Dorset, UK

Table 2.4 Secondary Antibodies

Antibody Specificity	Antibody Type	Dilution	Source
Rabbit Ig HRP (P0218)	Swine, Ig	1:2000	DAKO Cytomation A/S, Denmark
Mouse Ig HRP B(P0218)	Rabbit, Ig	1:2000	DAKO Cytomation A/S, Denmark

Table 2.5 Trialled HIF-1 α and HIF-2 α Antibodies Unused Within Thesis Data Set

Antibody Specificity	Antibody Type	Source
HIF-1α (NB100-134)	Rabbit, polyclonal	Novus Biologicals, Littleton, CO, USA
HIF-1α (sc13515)	Mouse, monoclonal	Santa Cruz Biotechnology, Santa Cruz, CA, USA
HIF-2α (Ab199)	Rabbit, polyclonal	Abcam, Cambridge, UK
HIF-2α (D9E3)	Rabbit, monoclonal	Cell Signalling Technology, Danvers, MA, USA
HIF-2α (ab8365)	Mouse, monoclonal	Abcam, Cambridge, UK
HIF-2α (NB100-122)	Rabbit, polyclonal	Novus Biologicals, Littleton, CO, USA
HIF-2α (sc13596)	Mouse, monoclonal	Santa Cruz Biotechnology, Santa Cruz, CA, USA

2.2 Cell and Tissue Culture

Osteoarthritic (OA) human articular cartilage samples were obtained from patients diagnosed with end stage/advanced OA and recovered following total knee replacement (TKR). Prior to obtaining samples, following local ethics committee guidelines, patients provided informed consent to the Oxford Musculoskeletal Biobank (OMB). All samples were received from patients of the Nuffield Orthopaedic Centre (Oxford, UK). A total of 30 OA patients cartilage samples obtained are used in this body of work (14 male, 16 female, mean age 69.2 years, age range 41-88 years).

Dulbecco's Modified Eagle Medium 1x (DMEM) was obtained, sterile, for the purposes of cell and tissue culture from Gibco (Basingstoke, UK). Unless otherwise stated DMEM was supplemented with 10% fetal bovine serum (FBS) filter sterile and 1% Penicillin/Streptomycin also obtained from Gibco (Basingstoke, UK).

2.2.1 Human Cartilage Explant Isolation and Culture

OA cartilage samples were harvested from patient's femoral condyle and tibial plateau on the day of Total Knee Arthroplasty (TKA) surgery. For cartilage explant experiments a single piece of full thickness cartilage was cut into 12 equal cuboid explants (~60mg wet weight each).

Explants were pre-incubated in serum free DMEM in either normoxia or hypoxia for 24 hours prior to use, unless otherwise stated. Explants were typically cultured in 48 well plates, two explants per well, in 500µl of serum free DMEM. Explants were incubated in either 'normoxia' or 'hypoxia'. Normoxia being 20% O₂, 5% CO₂ at 37°C in a Galaxy 170s Incubator (CRS Biotech, Ayrshire, Scotland, UK) and hypoxia being 5% O₂, 5% CO₂ at 37°C in an InvivoO₂ 400 workstation (Ruskin Technology, Bridgend, UK).

2.2.2 Human Articular Chondrocyte Isolation

Cartilage from the femoral condyle and tibial plateau of the OA sample was harvested and diced into small cubes (1-2mm). The cartilage was digested by incubation in DMEM containing 2mg/ml collagenase A from *Clostridium histolyticum* (Sigma Aldrich, Dorset, UK), 2.5µg/ml amphotericin B (Gibco, Basingstoke, UK) and 1% Penicillin/Streptomycin which was filter sterilised with a 0.2µm filter (Sartorius stedim, Epsom, UK). Incubation occurred at 37°C with constant shaking (100rpm) for 20 hours.

Following incubation the digested material was passed through a 70µm nylon mesh cell strainer (BD Falcon, BD Biosciences, Bedford, USA) to remove any undigested material from the cell suspension. The strained suspension was placed in an equal volume of DMEM and centrifuged at 1000rpm for 5 minutes, to form a cell pellet. The cell pellet was then washed twice with DMEM. Cells were counted using a haemocytometer, using Trypan blue (Sigma, Dorset, UK) to assess cell viability, and seeded at a density of 2.2×10^4 cells/cm² in DMEM. Cells to be passaged were cultured until confluency (10 to 14 days) and re-seeded in 10cm dishes (12ml DMEM). Where primary (P0) cells were required they were seeded onto wells of a 6 well plate (2.5ml DMEM) immediately following isolation.

2.2.3 Total Knee Arthroplasty (TKA) Cartilage Use Compared to Unicompartamental Knee Arthroplasty (UKA) Cartilage

Human cartilage samples used in this study were obtained solely from TKA surgeries as opposed to UKA. The decision to use cartilage exclusively sourced from TKA surgeries was made due to a myriad of benefits when using the tissue to the use UKA OA cartilage samples, which had been collected in the initial stages of research.

Firstly, TKA OA cartilage samples had a greater amount of OA cartilage available for harvest than UKA. Additionally, TKA samples contained cartilage ranging from diseased near full thickness to heavily degraded thinner cartilage sections whereas UKA cartilage samples contained consistently thin and heavily degraded cartilage. This nature of UKA samples meant that the samples did not contain cartilage viable for explant culture which was required by this study, meaning that TKA were the appropriate sample source in this regard.

Secondly, during preliminary experiments conducted in this study (**Appendix: A.1**) UKA samples were collected and harvested for freshly isolated OA chondrocytes for comparison of their hypoxic response to freshly isolated healthy chondrocyte. During these preliminary experiments considerable debris was noted in the cultures of OA chondrocytes isolated from UKA samples. Initially, a protocol was designed which consisted of additional washes of contaminated OA chondrocytes in Trypsin containing EDTA to remove debris before continuing with cell culture. However, later comparisons of the debris present in HACs from UKA samples, to images in published materials determined that this debris was most likely to be calcium containing crystals (Liu et al., 2009). Calcium containing crystals are believed to contribute to the progression of subsets of OA, eliciting inflammatory responses in chondrocytes, including an increase in the matrix metalloproteinase MMP13 (Liu et al., 2009, Rosenthal, 2011, Sallis, 2006, Nguyen et al., 2013). Therefore the potential presence of calcium containing crystals in UKA samples could directly affect the experimental results of this study. Thus, it was decided that data would only be collected from cartilage samples sourced from TKAs as these calcium crystal debris were not observed in OA cartilage/HACs obtained from TKA samples.

2.3 Small Interfering RNA (siRNA) Transfection of Chondrocytes

Human articular chondrocytes (HACs) at 2nd passage (P2) were seeded at 2.2×10^4 cells/cm² in 6 well plates (2.5ml DMEM). Cells were incubated in 20% O₂, 5% CO₂ at 37°C until they reached 60% confluency. To prepare for transfection the DMEM was removed and optiMEM (Gibco, Basignstoke, UK) was added (600µl). A complex of Lipofectamine 2000 (Invitrogen, San Diego, CA, USA) and siRNA (10nM final concentration) in serum free optiMEM (400µl) was used to transfect the cells. Two hours after transfection DMEM was added to the cells (1.5ml). Transfected cells were then cultured in either 20% or 1% oxygen for 48 hours. Small interfering RNA oligonucleotides (siRNA oligos) targeting *PHD1* (EGLN2), *PHD2* (EGLN1), *PHD3* (EGLN3), *HIF-1α* (EPAS2) and *HIF-2α* (EPAS1) which were used for transfection are described in the table below. As a non-targeting control a negative control siRNA (Qiagen, Cambridge, MA, USA) was transfected in parallel.

2.3.1 Assessment of siRNA Transfection Efficiency

To assess the efficiency of target gene knockdown, three siRNAs targeting either *PHD1*, *PHD2* or *PHD3* were tested. For each gene of interest the siRNA selected were required to have target sites on different positions on the target gene. In order to test the efficiency of the selected siRNA, each of the three siRNA per target gene were transfected into HACs individually and their effectiveness was assessed by the percentage knockdown of the target gene in regards to mRNA levels. Subsequently the least effective siRNA, in terms of knockdown at the mRNA level, was discarded (**Table 2.7**) and the two siRNA which had the most effective target knockdown were transfected simultaneously (**Table 2.6**). The most effective siRNA were coupled and transfected at a final concentration of 10nM, their effectiveness at this stage was confirmed at the mRNA and protein level. This combined siRNA approach is used within the dataset observed in **Chapter 4**. In regards to *HIF-1α* and *HIF-2α* targeting siRNA, the siRNA used in this thesis were previously optimisation within the group and are known to be selective for the HIF-α subunits of choice (i.e. HIF-1α siRNA does not directly affect the expression of HIF-2α) (Lafont *et al.* 2007).

In addition to this, siRNAs were also tested in primary (P0) and second passage (P2) HACs. Ultimately, second passage HACs were selected due to the poor rate of successful transfection observed in primary HACs. However, the limited successful primary HAC transfection data is included in **Appendix: A2**.

Table 2.6 Human siRNA Sequences

siRNA	Sequence	Source
PHD1#1	5'-CCG GCC CGT GGT AGC CAA CAT-3'	Qiagen, Cambridge, MA
PHD1#2	5'-CCG CAT GGC AGA CAG CTT AAA-3'	Qiagen, Cambridge, MA
PHD2#1	5'-AGG AGG AAG ATA AGC TAC TAA-3'	Qiagen, Cambridge, MA
PHD2#2	5'-TAG CGG GTG CAT GGC GCA GTA-3'	Qiagen, Cambridge, MA
PHD3#1	5'-CTG CAT CTA TCT ATC GAA CAA-3'	Qiagen, Cambridge, MA
PHD3#2	5'-TTC CTT TGT TAT GTA CGG ATA-3'	Qiagen, Cambridge, MA
HIF-1α	5'- AGC AGU AGG AAU UGG AAC ATT -3'	Lafont <i>et al.</i> 2007
HIF-2α	5'- GCG ACA GCU GGA GUA UGA ATT -3'	Lafont <i>et al.</i> 2007

Table 2.7 Trialled Human siRNA Sequences Unused within Thesis Data Set

siRNA	Sequence	Source
PHD1#3	5'-CAU GCA GGG CAC GAU AUA GUC-3'	Applied Biosystems, Foster City, CA
PHD2#3	5'-GAA GCU GGG CAG CUA CAA AAT-3'	Applied Biosystems, Foster City, CA
PHD3#3	5'-GGA GAG GUC UAA GGC AAU GUU-3'	Applied Biosystems, Foster City, CA

2.4 Isolation of RNA and Protein from Cell Lysates

Six-well plates containing primary (P0) and passaged (P2) OA HACs were washed with 1000 μ l 1x PBS (pre-equilibrated to incubation conditions) and lysed with 600 μ l Trizol® reagent (Invitrogen, San Diego, CA). The lysate was homogenized according to manufacturer's protocol and transferred to a 1.5ml Eppendorf tube, which was immediately placed on ice. Samples were then either used immediately for RNA and protein isolation procedure or stored at -20°C (short term - <1 month) or -80°C for at least one month.

2.4.1 Isolation of RNA from Chondrocytes

RNA was isolated from the HAC lysate using Trizol and according to manufacturer's guidelines (the only deviation from this protocol was the use of two 75% ethanol washes instead of the single wash suggested). The resulting RNA pellet was re-suspended in 12µl of RNase-free water. RNA quantity and quality was assessed using a digital spectrophotometer (Nanodrop 1000, ThermoFisher, Massachusetts, USA). RNA quantity was recorded in ng/µl and quality assessed by UV absorbance A260/A280 and A260/A230 ratios with values <1.8 being rejected (RNA either re-precipitated to improve absorbance ratios or discarded) RNA was then either used immediately or stored at -80°C.

To evaluate the integrity of the RNA, samples were separated by electrophoresis on an agarose gel (1% agarose in TBE buffer) stained with SYBR safe DNA gel stain (Invitrogen, San Diego, CA, USA). RNA samples (100ng) with RNA gel loading dye blue (BioLabs, New England, USA) were loaded into the gel and run at 100V for 30 minutes in TBE Buffer. Once the RNA was separated in the gel the fragments were visualised in UV light using a gel doc imaging system (BioRad, Hertfordshire, UK). Intact total RNA was determined by analysing the 28S and 18S ribosomal RNA (rRNA) bands, where clear defined bands and a 2:1 ratio of the bands (28S:18S) indicates intact RNA (**Figure 2.1**).

2.4.2 Isolation of Protein from Chondrocytes

Protein was isolated from the HAC Trizol lysate according to the manufacturer's guidelines. The resulting protein pellet was then re-suspended in 80µl 2x SDS-PAGE suspension buffer supplemented with a protease inhibitor cocktail (cOmplete tablets, Roche, Hertfordshire, UK). Re-suspension was aided by incubation at 55°C with constant shaking (1000rpm) for 30 minutes. Protein was then stored at -20°C until use.

As a result of the 0.3M Guanidine HCL washes used during isolation of protein from Trizol lysates, common protein quantification methods could not be used. Therefore an equal volume of protein containing lysis buffer (from culture dishes seeded at equal cell density) was loaded into each lane when performing SDS-PAGE/Western blot with the quantity loaded per lane being confirmed by a loading control (α -tubulin).

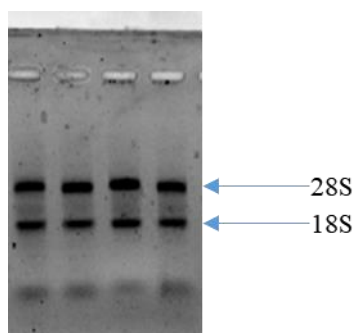


Figure 2.1 Agarose gel with intact RNA
100ng RNA showing clearly visible 28S and 18S
rRNA bands at a 2:1 ratio

2.5 Reverse Transcription and qPCR of RNA

2.5.1 Reverse Transcription of RNA

Prior to reverse transcription (RT), 500ng of RNA in a total volume of 10µl RNase free water was treated with Turbo™ DNase (ThermoFisher, MA, USA) to remove any contaminating DNA from the sample according to the manufacturer's guidelines (the removal reagents supplied also remove the subsequent DNase and divalent cations from the RNA sample). Complementary DNA (cDNA) was then generated from the treated RNA (500ng in 10µl RNase free water) using a high capacity reverse transcription kit (Applied Biosystems, Massachusetts, USA) following the manufacturer's guidelines (reagent volumes given below) using a C1000 touch thermocycler (BioRad, Hertfordshire, UK). Synthesised cDNA (20µl) was diluted in 120µl DNase free water resulting in a total 140µl of sample which is either used immediately or frozen at -20°C until use.

Table 2.8 RT Kit Mix Volumes per Reaction

Component	Volume/Reaction (µl)
10x RT Buffer	2
25x dNTP Mix	0.8
10x Random Primers	2
MultiScribe Reverse Transcriptase	1
RNase Inhibitor	1
Nuclease free H ₂ O	3.2
Total Per Reaction	10

2.5.2 Quantitative Polymerase Chain Reaction (qPCR)

Of the initial reverse transcribed 500ng sample RNA, 4.4µl cDNA (3.14% of 500ng) was used per reaction for PCR assays. Primers, detailed in **Table 2.7**, were designed for use with SYBR green master mix (fast SYBR green master mix (Applied Biosystems)) and a ViiA™ 7 Real-Time PCR System (ThermoFisher Massachusetts, USA). In regards to quality control steps taken, each sample was run in triplicate on a 384 well qPCR plate allowing for the evaluation of variation within samples via the standard deviations between replicates. Secondly, each 384 qPCR plate included a no template control in order to identify any DNA contamination or primer dimer formation. Also, following a completed qPCR run the melt curves for each primer set were assessed, to ensure that no non-specific PCR reactions had occurred such as the formation of primer dimers (a clean run indicated by a single sharp peak). Finally, data was normalised against the ribosomal protein gene (RPLP0), a housekeeping gene which previous research has indicated RPLP0 is not regulated by hypoxia in chondrocytes.

A relative standard curve was created using five serial dilutions ($\frac{1}{4}$) of combined mRNA from untreated HACs cultured in normoxia and hypoxia. The relative standard curve was used to calculate relative mRNA levels as target values were identified using the curve, this value was then normalised by dividing by the mRNA values for the housekeeping gene. Normalised target values were then compared to determine fold change. This data is presented in the form of a log scale graph where, individual points indicate normalised values for a single biological replicate (i.e. 7 points is equal to 7 unique patient samples) and the mean and standard deviations are represented by a single line and two error bars respectively.

Table 2.9 SYBR Green qPCR Primers

Name	Sequence
RPLP0 Forward RPLP0 Reverse	5'-CCATTGAAATCCTGAGTGATGTG-3' 5'-CTTCGCTGGCTCCCACCTT-3'
SOX9 Forward SOX9 Reverse	5'-CCCACCTTGTGGCCAATCAG-3' 5'-TTGATTTTCGCTGCTCCATTAG-3'
AGGRECAN Forward AGGRECAN Reverse	5'-CGTGCGCCCATCAACA-3' 5'-ACACCTCCCCCTCAAGTCTGT-3'
COL2A1 Forward COL2A1 Reverse	5'-GGAAGAGTGGAGACTACTGGATTGAC-3' 5'-TCCATGTTGCAGAAAACCTTCA-3'
COL10A1 Forward COL10A1 Reverse	5'-CACGCAGAATCCATCTGAGAATAT-3' 5'-GTTTCAGCGTAAACACTCCATGAA-3'
HIF-1 α Forward HIF-1 α Reverse	5'-TCTGAACGTCGAAAAGAAAAGTC-3' 5'-GGCAACTGATGAGCAAGCT-3'
HIF-2 α Forward HIF-2 α Reverse	5'-AGATGGCGACATGATCTTTCTGT-3' 5'-CCTGTTAGCTCCACCTGTGTAAGT-3'
PHD1 Forward PHD1 Reverse	5'-AAC ATC GAG CCA CTC TTT GAC-3' 5'-TCC TTG GCA TCA AAA TAC CAG-3'
PHD2 Forward PHD2 Reverse	5'-TACGCCACTGTAACGGGAAG-3' 5'-CGTTCCATTGCCCCGGATAAC-3'
PHD3 Forward PHD3 Reverse	5'-ACG AAG TGC AGC CCT CTT AC-3' 5'-TCA GCA TCA AAG TAC CAG ACA GT-3'
ADAMTS5 Forward ADAMTS5 Reverse	5'-GGC CAT GGT AAC TGT TTG CT-3' 5'-AAT GTC AGG TTG CAC TG-3'
MMP13 Forward MMP13 Reverse	5'-TTA CCA GAC TTC ACG ATG GCA TT-3' 5'-TCG CCA TGC TCC TTA ATT CC-3'
TIMP3 Forward TIMP3 Reverse	5'-CCT GCT ACT ACC TGC CTT GC-3' 5'-GGC CTA GTG TTT GGA CTG GT-5'

2.6 SDS-PAGE Gel Electrophoresis and Western Blotting

2.6.1 SDS-PAGE

Protein samples in 2x SDS-Sample buffer (containing protease inhibitors) were prepared for gel electrophoresis by the addition of a 4x Loading buffer (1:4 loading buffer/sample buffer). The protein was boiled (100°C) for 5 minutes in this buffer and then briefly centrifuged to collect the sample.

The protein was then loaded into either a 4-12% or 8% NuPAGE Bis-Tris gels purchased from Invitrogen (San Diego, CA, USA). Electrophoresis occurred using a Mini Gel Tank (ThermoFisher, Massachusetts, USA) and NuPAGE MOPS SDS running buffer (Invitrogen, San Diego, CA, USA) which is optimised for use with NuPAGE Bis-Tris gels. According to manufacturer instructions electrophoresis occurred at 200V for between 30-60 minutes depending on the desired separation of proteins. A lane in each gel contained 5µl of colour protein standard, broad range (Biolabs, New England, USA) to monitor progression of electrophoresis and estimate protein sizes.

2.6.2 Western Blotting

Western blot was used to detect specific proteins from cell lysates that had previously been resolved in a SDS-PAGE gel via electrophoresis. Prior to western blotting the resolved proteins in the Bis-Tris gel were transferred to PVDF membranes (Millipore, Bedford, USA) PVDF membranes were activated in 100% methanol for 30 seconds. To complete the transfer the membrane and Bis-Tris gel were sandwiched together between four sheets of 3mm chromatography paper and two sponges, all of which were equilibrated in transfer buffer. The sandwich is then sealed into a cassette and placed into a wet/tank blotting system (BioRad, Hertfordshire, UK) containing transfer buffer, the transfer is then carried out at a constant voltage (110V) for 90 minutes. The system was kept cool by adding an ice block to the transfer tank.

The efficiency of the transfer was assessed by two methods, Ponceau red stained the membrane to visualise protein and Coomassie blue stained the Bis-Tris gel to visualise remaining protein in the gel. To use Ponceau red staining the PVDF membrane was gently

agitated in a Ponceau red solution for 5 minutes (or until bands were visible), the presence of even red bands indicating an efficient transfer. The membrane was then washed twice (5 minutes per wash) in a Ponceau destain solution until the Ponceau red stain was removed.

For Coomassie stain the gel was immersed in Coomassie solution immediately after transfer and agitated on a gyrating rocker table (ThermoFisher, Massachusetts, USA) for 60 minutes. Once the gel was stained the Coomassie solution was replaced with a Coomassie destain for a further 60 minutes. Following destain the bands representing the proteins remaining in the gel became visible. The intensity of these bands was used as an indicator of the efficiency of the protein transfer.

After staining, the membrane was submerged (protein side up) in PVDF membrane blocking buffer for 60 minutes followed by overnight incubation at 4°C in a primary antibody (all antibodies were diluted in blocking solution). After primary antibody incubation the membrane was washed, whilst agitated, three times (10 minutes per wash) in 1x PBST, which was followed by 60 minutes incubation, with gentle agitation, in the relevant horseradish peroxidase conjugated secondary antibody. Following secondary antibody incubation the membrane was washed three times in 1x PBST. After washes the membrane was placed, protein side up, into an X-ray cassette and excess 1x PBST was drained off the membrane. An ECL plus solution (BioRad, Hertfordshire, UK) was then mixed, according to manufacturer instruction, and pipetted on to the membrane, after one minute this solution was removed. The membrane was then sealed in saran wrap (protein side up) and transported to a dark room where the HRP-bound proteins visualized using blue-sensitive film (Fujifilm Super RX, Fujifilm, Bedford, UK).

In cases where the PVDF membrane was re-probed with other antibodies the membrane was incubated for 15 minutes, at room temperature, in a 1x Re-Blot Plus Strong Solution (Chemicon, Temecula, USA). Following the stripping buffer the membrane was then washed three times in 1xPBST, incubated in blocking solution for 30 minutes and finally, incubated in the subsequent antibodies as detailed above. Any given membrane was re-probed a maximum of three times.

2.6.3 Absent Protein Data

Protein level analysis of cell secreted proteins Type II collagen, MMP13, ADAMTS5 and TIMP3 are absent from this body of work. Whilst the analysis of the expression of these proteins was originally planned for experiments in this study, technical issues and resulting time constraints have resulted in their exclusion. Technical issues encountered in this study affected both cell culture and western blotting (in regards to protein transfer to PVDF membrane). In terms of cell culture, lack of adherence of freshly isolated cells, cell death and contamination meant that RNA and non-secreted protein analysis was prioritised and cells were not cultured separately in serum free media. Additionally, technical issues affecting the efficiency of protein transfer during western blotting resulted in time constraints which led to the exclusion of cell secreted protein analysis from this study. Potential implications of the absence of this data are discussed in **Chapter 6**.

In regards to the absence of PHD1 protein expression level analysis, PHD1 protein data was collected using the antibody described in **Table 2.3**, however the specificity of the bands detected by this antibody could not be confirmed following knockdown of PHD1 (via siRNA). As the specificity of the detected bands could not be confirmed, PHD1 protein data was excluded from this study.

2.6.4 Absence of Western Blot Quantification

Western blot data presented in the study represent a qualitative analysis of protein levels. The decision not to quantify western blot bands was made in some cases due to the presence of oversaturated bands within the region of the specific bands of interest. Additionally, basal levels of protein between donor samples were observed to vary affecting the intensity of the detected specific protein band. As such, several blots represent different protein levels between donors on the same film, leading to bands which may not fall into the linear range of the immunosignal. Taken together, these technical problems therefore precluded absolute quantification of western blot data.

2.7 Cartilage Explant Culture and ARGS Detection

OA human articular cartilage was cut in equal size sections from the same area of tissue as previously described (**section 2.2.1**). Explants were cultured in 500µl serum free DMEM containing 1%(v/v) ITS (Sigma Aldrich, Dorset, UK) to ‘rest’ for 24 hours in 20% Oxygen. After 24 hours the rest media was collected and stored at -20°C. Explants were then incubated with serum free DMEM and either stimulated with IL-1 β (1ng/ml and 10ng/ml concentrations) or used as control (serum-free DMEM only). Explants, both with and without IL-1 β (10ng/ml), were then cultured at 20% and 1% oxygen for 24 hours after which the media was collected and stored at -20°C.

A 4x Deglycosylation buffer (147µl) containing Chondrotinase (1.5µl) and β -galactosidase (1.5µl) was added to the media collected from explants. The mixture was then vortexed for 15 seconds and incubated over night at 37°C. Following incubation samples were again vortexed and centrifuged briefly in preparation for acetone precipitation. Once vortexed ice-cold acetone was added to each sample (1:5 ratio of sample to acetone), the samples were then vortexed for 20 seconds before being incubated overnight at -20°C. Samples were then vortexed and centrifuged (13,000rpm, 10 minutes) producing a colourless pellet, the supernatant was then aspirated and the pellets were dried in a vacuum centrifuge. Once dry the pellets were resuspended in 2x SDS buffer (50µl). Samples were loaded onto an 8% Bis-Tris gel and blotted as previously described (**Section 2.6**) using an anti-ARGSV antibody (listed in **Table 2.3**).

2.7.1 Generation of Decellularised Cartilage Explants

Dead explants/explants with a decellularised matrix were generated by freezing and thawing OA cartilage explants (Gilpin and Yang, 2017, Hubbard et al., 1988, Jahn et al., 2010, Kheir et al., 2011). Explants were placed into an Eppendorf tube and frozen at -70°C by submersion into a dry ice/isopropanol slurry for 30 seconds. Once frozen the explants were thawed by submersion in serum free DMEM warmed to 37°C for one minute. This cycle of freezing/thawing was repeated three times. Following freeze/thaw cycles cartilage explants were cultured as described in section 2.7.

2.8 Histology of Cartilage Explants

At the end of a treatment period both explants (every pair of explants treated as a technical replicate for their relevant experimental condition) from each well were immediately fixed for 48 hours in a 10% neutral formalin solution. Once fixed the explants were imbedded in paraffin wax blocks and 4-5µm thick sections of the explant were made using a Accu-Cut 200 rotary microtome (Sakura, Thatcham, UK) and placed on to glass slides (Superfrost Plus, Thermo Scientific, MA, USA) and baked overnight at 37°C. The sections were then dewaxed in xylene and stained with Hematoxylin and Eosin (H&E) as well as Safranin O according to a pre-programmed protocol on a Tissue-Tek DRS 2000 Multiple Slide Stainer (Sakura, Thatcham, UK). H&E was used to stain the cytoplasm and nucleus of cells within the explant and Safranin O staining the proteoglycans. Stained explants were then covered with a glass coverslip before microscopic imaging. Images of the sections were taken at 4x magnification with an Olympus BX51 microscope (Olympus, Tokyo, Japan). In addition to Safranin O staining, immunohistochemical staining of explant sections was planned (**Appendix: A3**) however due to the issues concerning the use of OA explants as discussed in **Chapter 5** these experiments were not believed to have the potential to add additional value to the data set.

2.9 Statistical Analysis

Statistical analysis was performed using GraphPad Prism 7 (GraphPad Software Inc. SanDiego, CA, USA). Data were analysed using an un-paired t-test where a *P* value below 0.05 being deemed statistically significant. Where represented on a graph results are shown with a log scale and data is represented as the mean with standard deviations, each data point represents a unique experiment/patient sample (biological replicate).

Chapter 3

Hypoxic Response of OA HACs

3.1 Introduction

It has previously been shown that healthy human articular chondrocytes (HACs) are well adapted to hypoxia and that hypoxia is particularly relevant for the promotion of the chondrocyte phenotype (Lafont et al., 2008, Murphy and Polak, 2004). This beneficial hypoxic response in HACs is mediated by the activity of two HIF isoforms; HIF-1 α and HIF-2 α . This promotion of the chondrocyte phenotype has been described in healthy HACs as the promotion of cartilage production and the inhibition of its destruction. Cartilage production is promoted in hypoxia via HIF-2 α which upregulates, via direct binding, the key cartilage transcription factor SOX9, a transcription factor responsible for the upregulation of key cartilage matrix genes. Inhibition of cartilage destruction in hypoxia is mediated by anti-catabolic effects of HIF-1 α such as the inhibition of MMPs and the induction of TIMP3 (Lafont et al., 2007, Thoms, 2011, Thoms et al., 2013, Schipani et al., 2001, Zhang et al., 2011).

It is thought that during osteoarthritis (OA), the chondrocyte undergoes a phenotypic change leading to the increased production of catabolic matrix metalloproteinases (MMPs) and aggrecanases (ADAMTSs). This leads to the loss of proteoglycans, such as aggrecan, causing a degradation of the cartilage matrix. The collagen network is also damaged resulting in the loss of the load bearing capabilities of cartilage resulting in further damage to the tissue. The end result of these processes, due to the chondrocytes limited capacity for repair, is the fibrillation and degradation of the cartilage (Goldring, 2000, Goldring, 2012, Pearle et al., 2005, Wieland et al., 2005). Furthermore, it has been suggested in a murine model of OA that the hypoxic response of chondrocytes is involved in the increased production of catabolic enzymes in OA via a shift in the activity of HIF-2 α . In murine OA HIF-2 α is shown to upregulate key matrix metalloproteinases (i.e. MMP13, ADAMTS5) (Ryu et al., 2012, Saito et al., 2010, Saito and Kawaguchi, 2010, Yang et al., 2010).

3.1.2 Chapter 3 Specific Aims and Hypothesis

- 1) **Identify whether key cartilage genes known to be affected by hypoxia in healthy HACs are differentially affected in OA HACs.**
- 2) **Identify targets of HIF-1 α and HIF-2 α and the individual and joint contributions of the HIF isoforms to the hypoxic response of OA HACs.**

In this chapter the hypoxic responses of OA HACs and the specific roles of HIF- α isoforms are investigated. It is hypothesised that a hypoxia will remain beneficial to the chondrocyte phenotype in an OA setting, increasing anabolism via SOX9 and decreasing the production of catabolic factors. Additionally, it is hypothesis that HIF-2 α will retain an anabolic role, as previously observed in healthy HACs.

3.2 General Response to Hypoxia in Primary OA HACs

Osteoarthritic (OA) human articular chondrocytes (HACs) were cultured in either normoxia (20% oxygen) or hypoxia (1% oxygen) for 48 hours. Primary chondrocytes were used to investigate the response to hypoxia. OA cartilage was obtained from 5 male and 6 female donors; age range 51-88 years, mean age 71.6 years. The messenger RNA (mRNA) levels of genes of interest were normalised to a housekeeping gene, ribosomal protein RPLP0. It has been previously demonstrated within our group that RPLP0 is not regulated by hypoxia and is used as a standard housekeeping gene for hypoxic experiments (Lafont et al., 2008, Thoms et al., 2013). Once normalised, the mRNA levels were analysed to observe potential responses to hypoxia. In addition to mRNA, protein from OA HACs cultured as previously described was also analysed by western blot to identify responses to hypoxia at the protein level. As previously explained western blots here are not quantified and represent a qualitative analysis of protein levels.

The mRNA levels of hypoxia inducible factor (HIF) genes - *EPAS2* (encoding for HIF-1 α protein) and *EPAS1* (encoding for HIF-2 α protein), are on average lower in hypoxia than normoxia with a reduction of 25% and 22% respectively (**Figure 3.1**) although this change is not statistically significant in primary OA chondrocytes ($P>0.05$). Whilst the mRNA levels of HIF are not significantly affected by hypoxia western blots reveal that the protein levels of

both HIF-1 α and HIF-2 α are increased in response to a hypoxic environment in OA HACs (**Figure 3.2**). In these blots for HIF-1 α and HIF-2 α the upper bands indicate the protein of interest. The specificity of these bands was assessed by their predicted molecular weight, the response of the bands to hypoxia, hydroxylase inhibition and particularly to siRNA targeting each protein.

Next the hypoxic response of the genes encoding for the prolyl hydroxylase domain (PHD) enzymes, involved in the post translational regulation (via hydroxylation) of HIF, were analysed. *PHD1* mRNA levels were not significantly affected by hypoxia with a reduction of 19% when compared to normoxia ($P>0.05$). Whilst *PHD2* mRNA levels were on average higher in hypoxia, with a 22% increase, this increase is not statistically significant ($P>0.05$). However, mRNA levels of *PHD3* are significantly increased by 357% in hypoxia when compared to normoxia ($P<0.01$) (**Figure 3.3**). When investigated at the protein level it can be observed that the PHD2 and PHD3 protein levels are increased in primary chondrocytes when cultured in a 1% Oxygen (O₂) environment (**Figure 3.4**).

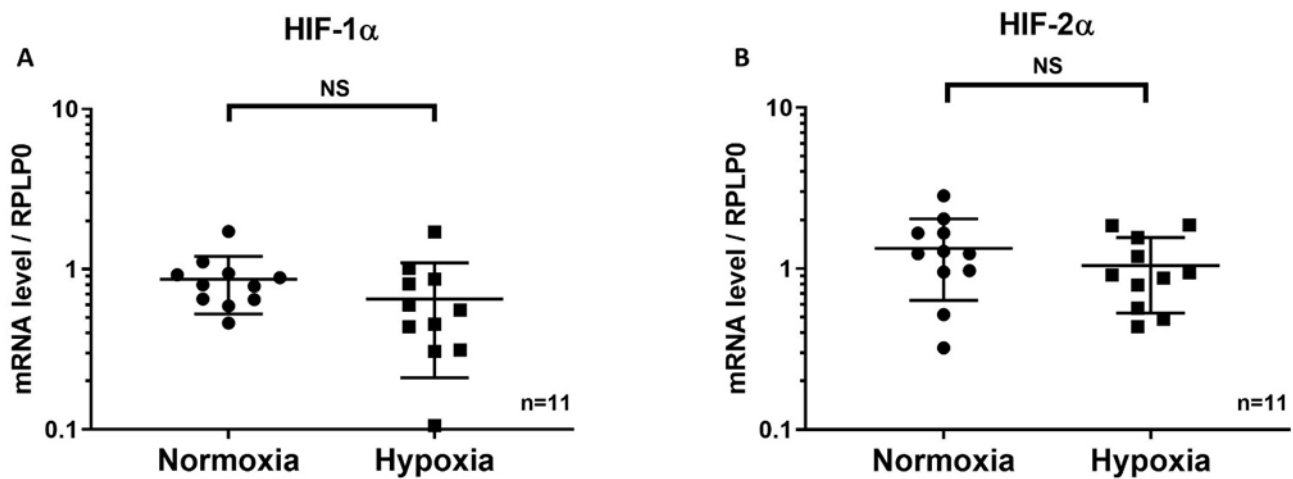


Figure 3.1 Effect of hypoxia on HIF gene expression in OA HACs. Data were obtained from primary (P0) OA HACs following 48hrs of culture in either 20% oxygen (Normoxia) or 1% oxygen (Hypoxia). Real Time PCR was performed using Sybr Green. Data show mRNA levels (normalised using RPLP0 housekeeping gene) for HIF-1 α (A) and HIF-2 α (B). Data plot represented as mean $\pm$ standard deviations. Note: NS – not significant.

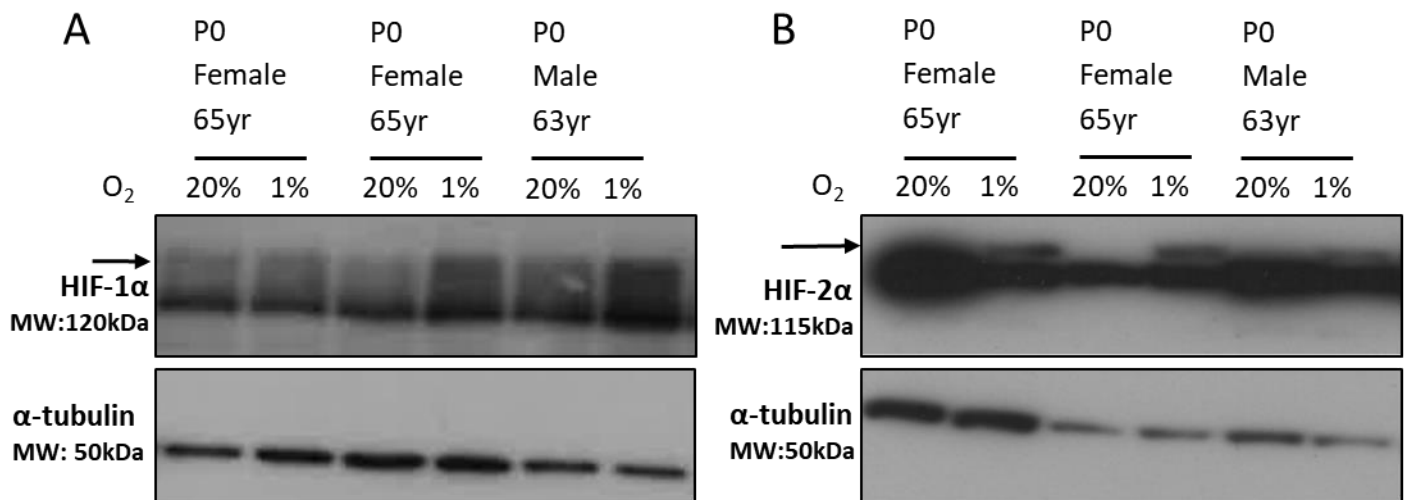


Figure 3.2 Effect of hypoxia on HIF protein levels in OA HACs. Data are from primary (P0) OA HAC cultures from 3 unique donors. Chondrocytes were cultured in either 20%O₂ or 1%O₂ for 48 hours. Western blotting shows a marked increase in the levels of HIF-1 α (A) and HIF-2 α (B) protein under hypoxic conditions. α -tubulin was used as a loading control. Specific bands are indicated with a black arrow (upper bands) Western blots representative of n=5 Note: yr – years old MW – molecular weight.

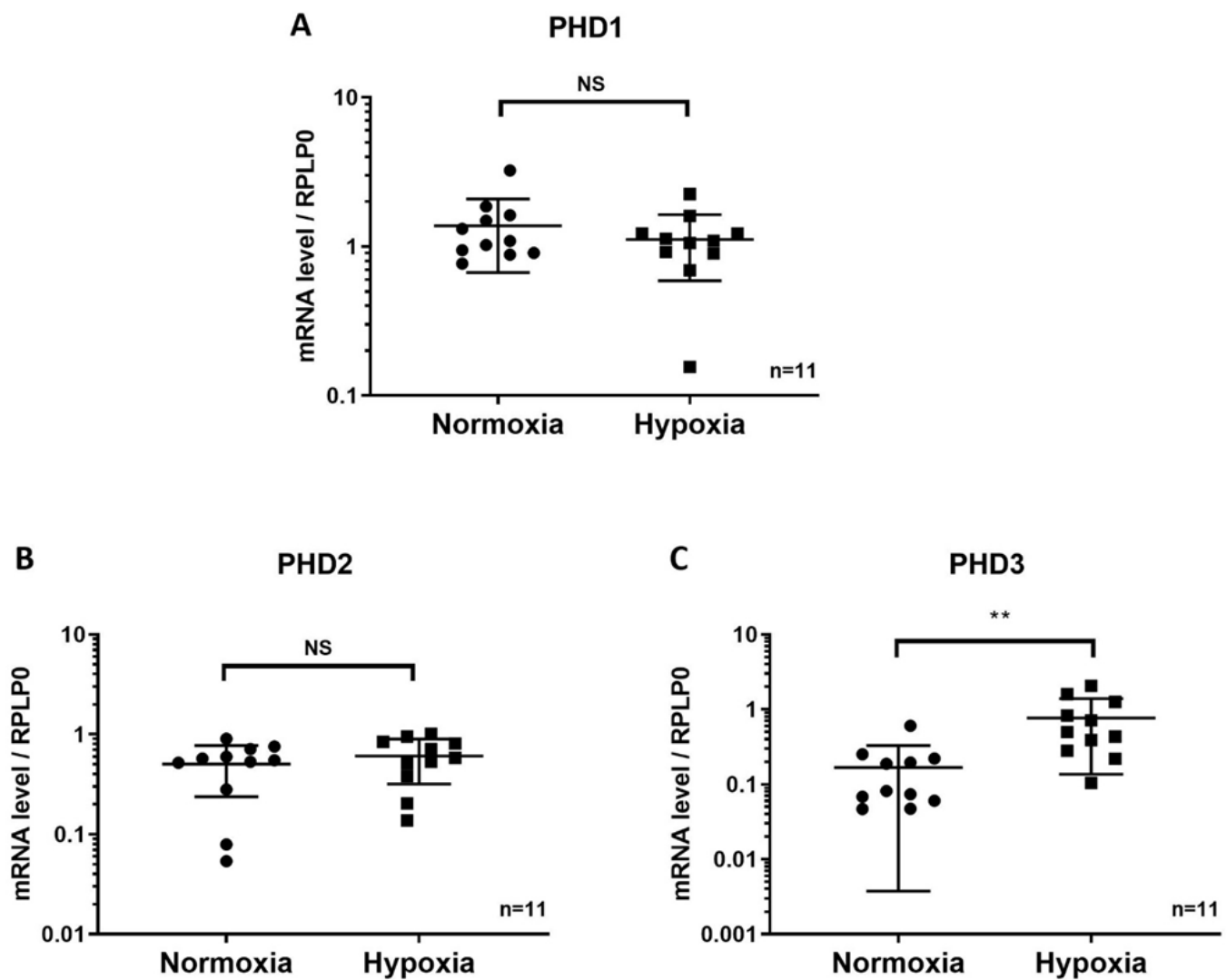


Figure 3.3 Effect of hypoxia on PHD gene expression in OA HACs. Data were obtained from primary (P0) OA HACs following 48hrs of culture in either 20% oxygen (Normoxia) or 1% oxygen (Hypoxia). Real Time PCR was performed using Sybr Green. Data show mRNA levels (normalised using RPLP0 housekeeping gene) for PHD1 (**A**) and PHD2 (**B**) and PHD3 (**C**). Data plot represented as mean $\pm$ standard deviations.

Note: NS – not significant, $**P \leq 0.01$.

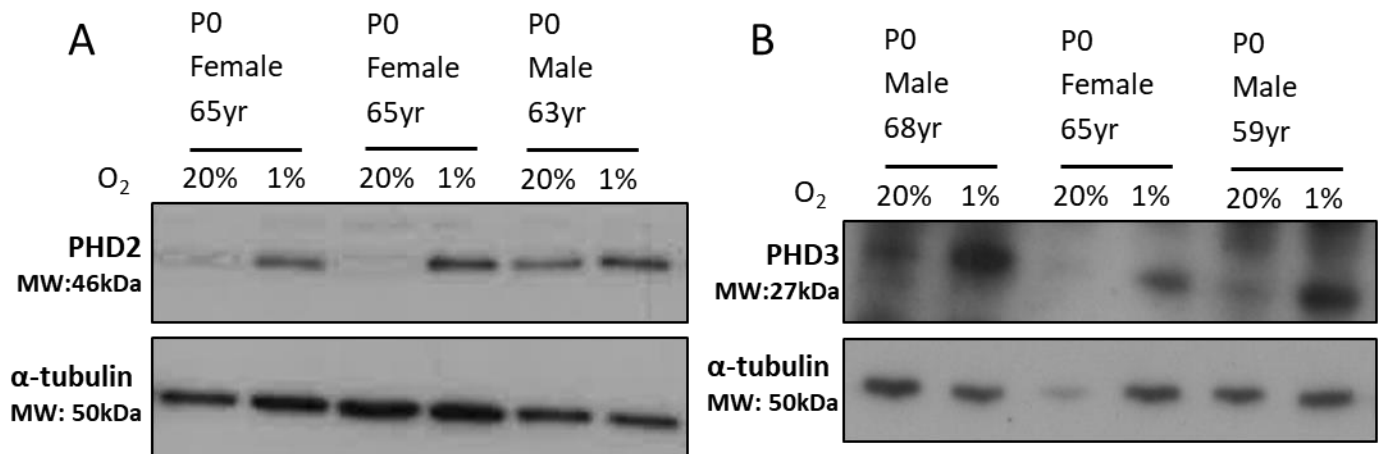


Figure 3.4 Effect of hypoxia on PHD2 and PHD3 protein levels in OA HACs. Data are from primary (P0) OA HAC cultures from 3 unique donors. Chondrocytes were cultured in either 20%O₂ or 1%O₂ for 48 hours. Western blotting shows an increase in the levels of PHD2 (**A**) and PHD3 (**B**) protein under hypoxic conditions. α-tubulin was used as a loading control. Western blots representative of n=5 Note: yr – years old MW – molecular weight.

The mRNA levels of cartilage transcription factor *SOX9* and key cartilage matrix genes, *COL2A1* and *ACAN*, were also analysed in response to hypoxia in primary OA HACs. Firstly, it was observed that the key cartilage transcription factor *SOX9* had a significant induction when cultured in hypoxia of 125% ($P < 0.05$). Gene expression levels of *COL2A1* were also significantly increased 215% when comparing levels in normoxia to hypoxia ($P < 0.05$). Finally, *ACAN* had on average higher gene expression levels in hypoxia (15% increase) however this increase was not found to be statistically significant ($P > 0.05$) (**Figure 3.5**). The hypoxic upregulation of *SOX9* was further confirmed at the protein level by western blot. It can be observed that the bands representing levels of *SOX9* protein are of greater intensity when primary OA HACs are cultured in 1% O_2 (hypoxia) as opposed to 20% O_2 (normoxia) (**Figure 3.6**).

Finally, the mRNA levels of; key catabolic enzymes *ADAMTS5* and *MMP13*, hypertrophy marker *COL10A1* and anti-catabolic enzyme inhibitor *TIMP3* were analysed to determine their responses to hypoxia. Firstly, the aggrecanase *ADAMTS5* - following hypoxic incubation mRNA levels are significantly reduced by 45% compared to normoxia ($P < 0.005$). Whilst the collagenase *MMP13* is not significantly reduced at the mRNA level when incubated in hypoxia there is a general trend indicating a reduction of *MMP13* expression in hypoxia with an average of a 32% reduction in mRNA levels in hypoxia when compared to normoxia ($P > 0.05$). Marker of hypertrophy *COL10A1* has on average higher mRNA levels in hypoxia compared to normoxia with a 33% increase; however, this is statistically not significant ($P > 0.05$). Finally, inhibitor of matrix metalloproteinases *TIMP3* was not significantly affected by hypoxia in primary OA HACs with a decrease of 18% when compared to normoxia ($P > 0.05$) (**Figure 3.7**). The reduction in mRNA levels of *ADAMTS5* and *MMP13* under hypoxic conditions suggests an anti-catabolic effect of hypoxia is present in the OA HACs.

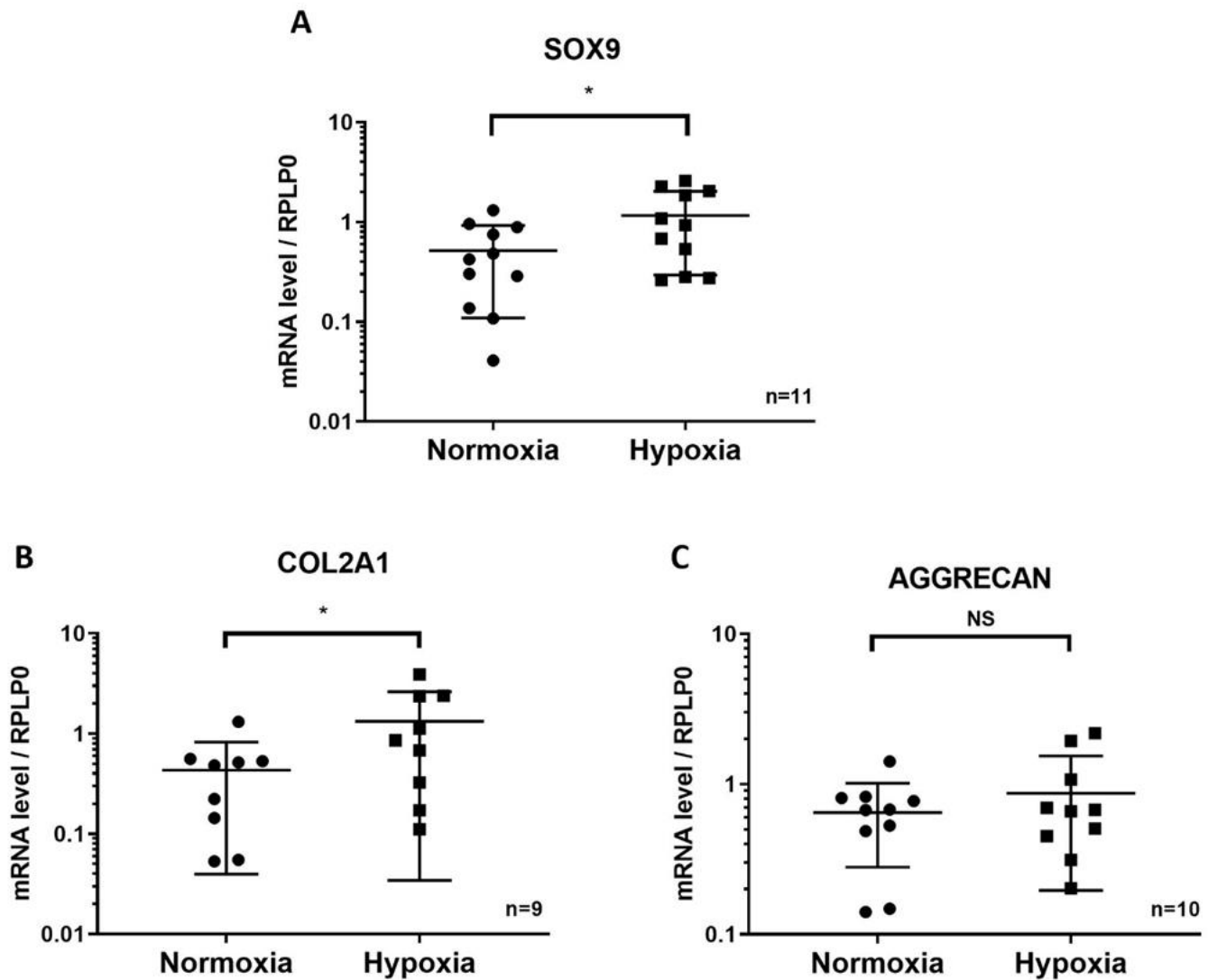


Figure 3.5 Effect of hypoxia on cartilage marker gene expression in OA HACs. Data were obtained from primary (P0) OA HACs following 48hrs of culture in either 20% oxygen (Normoxia) or 1% oxygen (Hypoxia). Real Time PCR was performed using Sybr Green. Data show mRNA levels (normalised using RPLP0 housekeeping gene) for SOX9(A) and COL2A1(B) and AGGRECAN(C). Data plot represented as mean +/- standard deviations.

Note: NS – not significant, $*P \leq 0.05$.

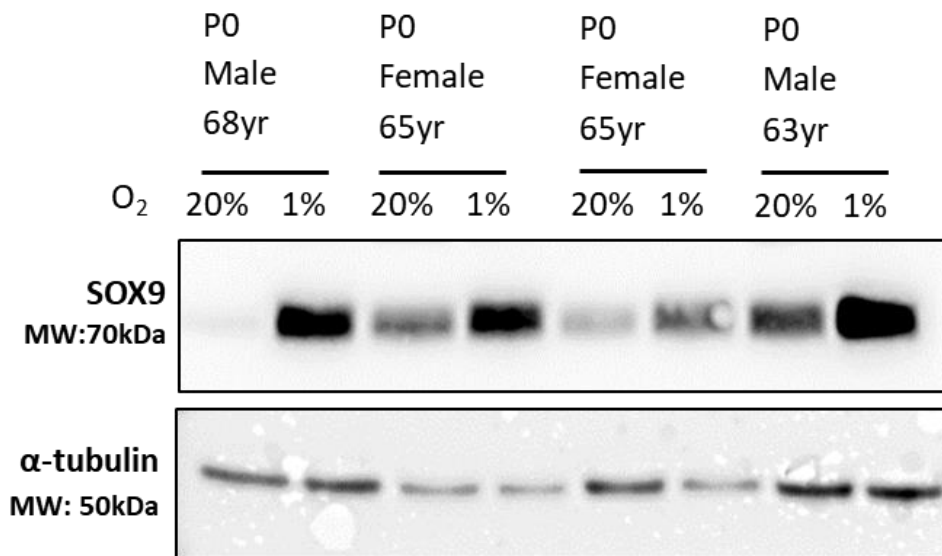


Figure 3.6 Effect of hypoxia on SOX9 protein levels in OA HACs. Data are from primary (P0) OA HAC cultures from 4 unique donors. Chondrocytes were cultured in either 20%O₂ or 1%O₂ for 48 hours. Western blotting shows a marked increase in the level of SOX9 protein under hypoxic conditions. α -tubulin was used as a loading control.

Western blots representative of n=6 Note: yr – years old MW – molecular weight.

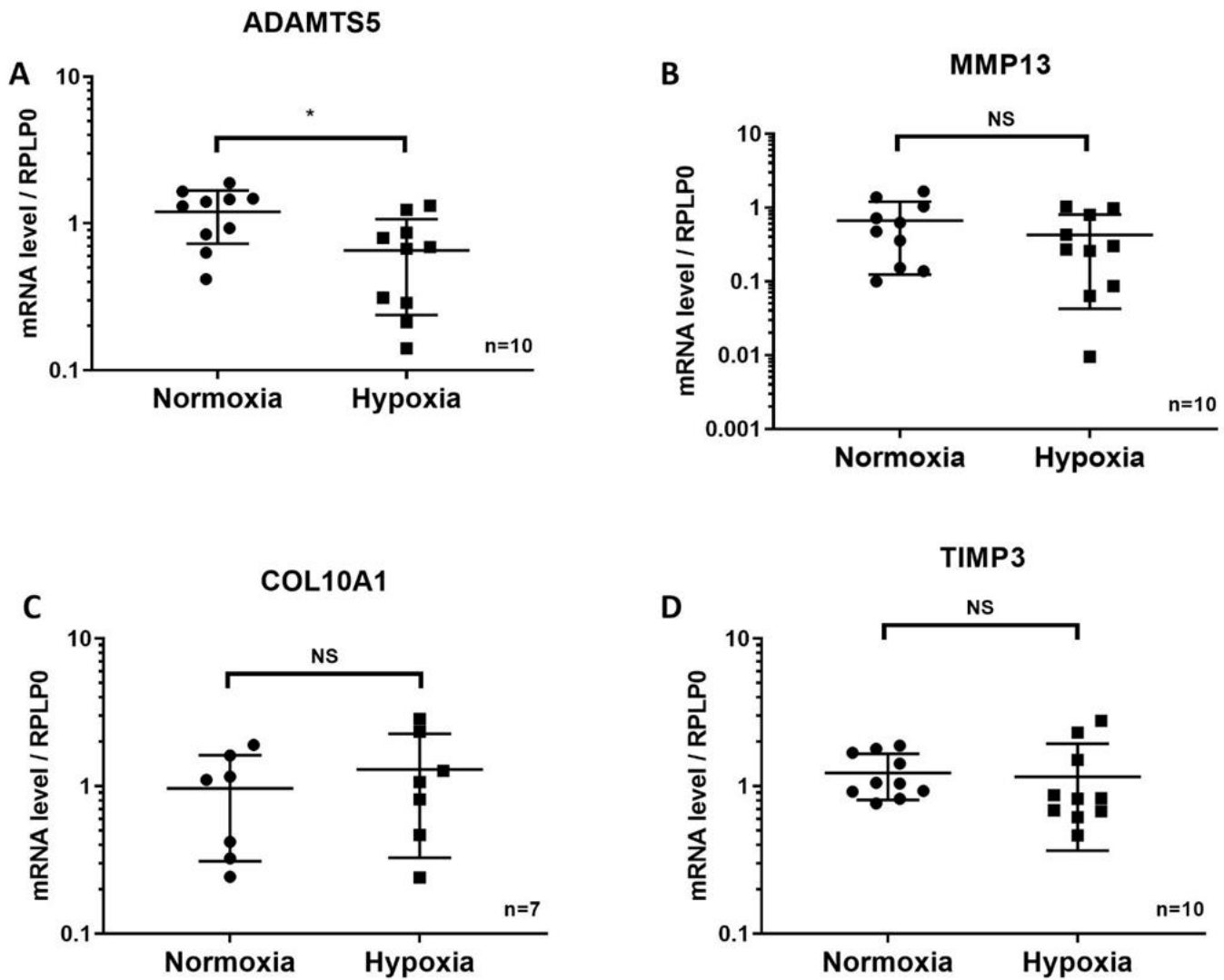


Figure 3.7 Effect of hypoxia on ADAMTS5, MMP13, COL10A1 and TIMP3 gene expression in OA HACs. Data were obtained from primary (P0) OA HACs following 48hrs of culture in either 20% oxygen (Normoxia) or 1% oxygen (Hypoxia). Real Time PCR was performed using Sybr Green. Data show mRNA levels (normalised using RPLP0 housekeeping gene) for ADAMTS5(A) and MMP13(B), COL10A1(C) and TIMP3(D). Data plot represented as mean +/- standard deviations. Note: NS – not significant, * $P \leq 0.05$.

3.3 Individual and combined Knockdown of HIF-1 α and HIF-2 α in OA HACs

The effects of HIF1 α and HIF2 α in OA HACs were investigated using second passage (P2) OA HACs, OA cartilage was obtained from 2 female and 3 male donors; age range 41-74 years, mean age 60.6 years. HACs were transfected with small interfering RNA (siRNA) targeting either HIF-1 α , HIF-2 α or combined siRNA to simultaneously deplete HIF-1 α +HIF-2 α (non-targeting siRNA used as control). Once transfected HACs were cultured for 48 hours in either normoxia (20% oxygen) or hypoxia (1% oxygen), following culture mRNA and protein levels were analysed as previously described. Statistics stated for mRNA levels are compared to control in the relevant oxygen tension unless otherwise stated.

Following transfection, the mRNA levels of *HIF-1 α* were reduced by an average of 67% in normoxia ($P<0.05$) and 62% in hypoxia ($P<0.01$) when *HIF-1 α* was targeted individually and reduced by 65% in normoxia ($P<0.05$) and 59% in hypoxia ($P<0.01$) as a result of combined HIF-1 α /HIF-2 α siRNA (**Figure 3.8A**). The depletion of HIF-1 α was then confirmed at the protein level by western blot, as reduced band intensity in normoxia and hypoxia compared to control was observed following both individual HIF-1 α and combined HIF-1 α /HIF-2 α targeting. This reduced band intensity indicates lower levels of HIF-1 α protein present following transfection thus confirming depletion. Interestingly, also observed here is a potential increase in HIF-1 α protein in normoxia where HIF-2 α is targeted (**Figure 3.8C**).

The mRNA levels of *HIF-2 α* were also reduced by an average reduction of 72% in normoxia ($P<0.001$) and by 80% in hypoxia when targeted individually ($P<0.0001$) and reduced by 65% in normoxia ($P<0.01$) and 70% in hypoxia ($P<0.001$) when targeted in combination with HIF-1 α siRNA (**Figure 3.8B**). The depletion of HIF-2 α was then confirmed at the protein level in hypoxia (bands undetectable in normoxic conditions) with a decrease in band intensity compared to control in both individual HIF-2 α and combined HIF-1 α /HIF-2 α siRNA lanes. Interestingly, a band of much higher intensity than control can be observed following HIF-1 α siRNA suggesting that higher levels of HIF-2 α protein are present when HIF-1 α levels are reduced perhaps as a compensatory increase due to the reduction of HIF-1 α protein present (**Figure 3.8D**).

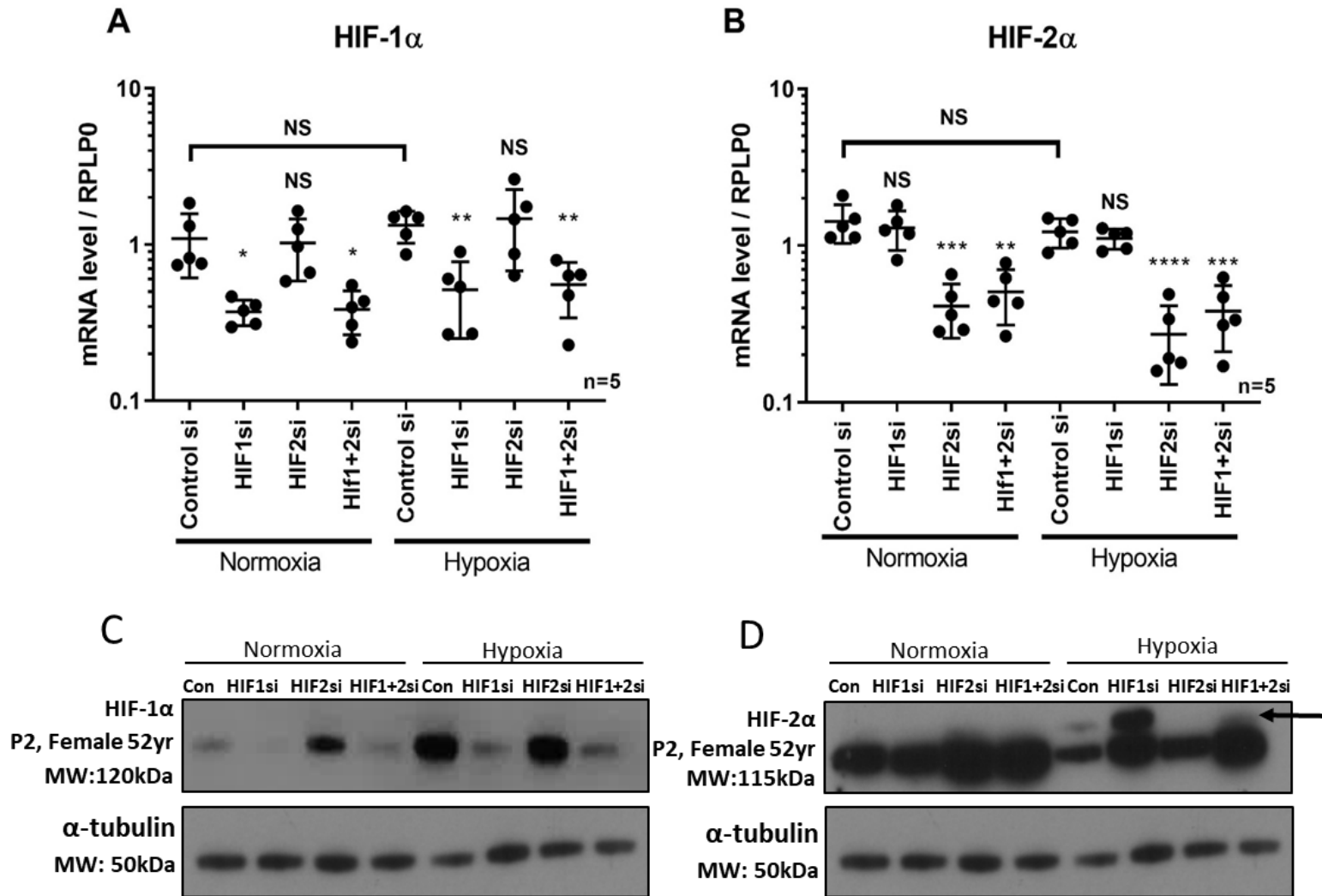


Figure 3.8 Knockdown of HIF-1 α and HIF-2 α in OA HACs. Data were obtained from second passage (P2) OA HACs following 48hrs of culture post siRNA transfection in either 20% oxygen (Normoxia) or 1% oxygen (Hypoxia). Chondrocytes were transfected with 10nM siRNA targeting HIF-1 α (HIF1si), HIF-2 α (HIF2si), both HIF-1 α and HIF-2 α simultaneously (HIF1+2si), or a non-targeting control (Control si). Real Time PCR was performed using Sybr Green. Data show mRNA levels (normalised using RPLP0 housekeeping gene) for HIF-1 α (A) and HIF-2 α (B). Data plot represented as mean $\pm$ standard deviations. Western blots confirming knockdown of HIF-1 α (C) and HIF-2 α (D) protein. Specific HIF-2 α band indicated by black arrow. Note: NS – not significant, * $P \leq 0.05$ ** $P \leq 0.01$ *** $P \leq 0.001$ **** $P \leq 0.0001$ compared to Control si in normoxia or hypoxia. Western blots representative of n=4. yr – years old. MW – molecular weight.

Once HIF1 α /HIF2 α knockdown was confirmed by both protein and mRNA the effect of the knockdown on the PHDs was then assessed. *PHD1* mRNA levels were not significantly affected by either HIF-1 α , HIF-2 α or combined HIF1 α /HIF2 α depletion ($P>0.05$) (**Figure 3.9A**). The mRNA levels of *PHD2* showed an average 46% and 58% reduction, in normoxia ($P<0.01$) and hypoxia ($P<0.05$) respectively, following HIF-1 α depletion. Similar reductions in *PHD2* mRNA were observed following combined HIF-1 α /HIF-2 α depletion with a 45% and 36% reduction in normoxia and hypoxia respectively ($P<0.05$). While no significant change was observed following individual HIF-2 α depletion ($P>0.05$) therefore suggesting a HIF-1 α dependent effect on PHD mRNA levels (**Figure 3.9B**). Finally, the mRNA levels of *PHD3* were also reduced following individual HIF-1 α depletion by an average of 67% in normoxia ($P<0.05$) and 73% in hypoxia ($P<0.01$) a stronger reduction than observed in *PHD2*. Combined HIF-1 α /HIF-2 α depletion also reduced *PHD3* mRNA by 71% in normoxia ($P<0.05$) and 79% in hypoxia ($P<0.01$). Similar to effects observed on *PHD2*, the depletion of HIF-2 α had no significant effect on the mRNA levels ($P>0.05$) again suggesting a HIF-1 α effect (**Figure 3.9C**).

It is worth noting that *PHD3* mRNA levels in hypoxia, following HIF-1 α depletion, are not significantly different to control in normoxia ($P>0.05$). This indicates that the hypoxic induction of *PHD3* has been abolished following the knockdown of HIF-1 α suggesting the induction is a HIF-1 α dependent effect. It can also be observed that the depletion of HIF-1 α in normoxia reduces the mRNA levels of *PHD2* and *PHD3* below levels typically observed in normoxia ($P<0.05$) suggesting activity of HIF-1 α under normoxic conditions (**Figure 3.9**).

Next, the effects of HIF1 α /HIF2 α knockdown on cartilage transcription factor *SOX9* and key cartilage matrix genes; *COL2A1* and *ACAN* were analysed. Firstly, *SOX9* mRNA, in normoxia mRNA levels were reduced following; HIF-1 α depletion by 54% ($P<0.0001$), HIF-2 α depletion by 13% ($P<0.05$) and combined HIF1 α /HIF2 α depletion reducing *SOX9* mRNA by 52% ($P<0.0001$). However, in hypoxia *SOX9* levels were not significantly affected by HIF-2 α depletion ($P>0.05$) only being significantly reduced by HIF-1 α depletion by 43% ($P<0.05$) and combined HIF1 α /HIF2 α depletion reducing levels by 50% ($P<0.05$) (**Figure 3.10A**). These results suggest that both HIF-1 α and HIF-2 α have an effect on *SOX9* mRNA in normoxia, while only HIF-1 α has an obvious effect in hypoxia.

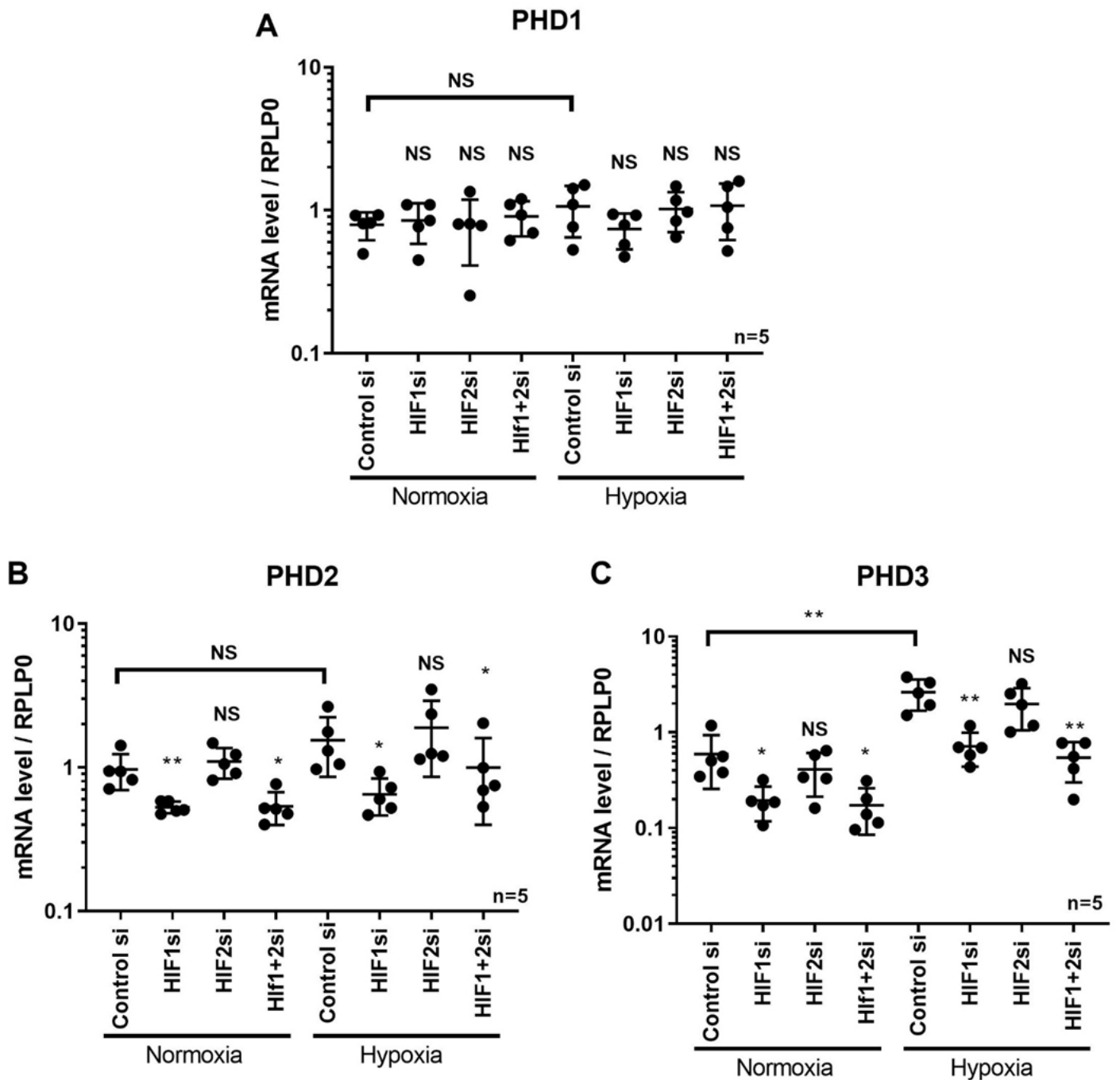


Figure 3.9 Effect of HIF knockdown on PHD gene expression levels in OA HACs. Data were obtained from second passage (P2) OA HACs following 48hrs of culture post siRNA transfection in either 20% oxygen (Normoxia) or 1% oxygen (Hypoxia). Chondrocytes were transfected with 10nM siRNA targeting HIF-1 α (HIF1si), HIF-2 α (HIF2si), both HIF-1 α and HIF-2 α simultaneously (HIF1+2si), or a non-targeting control (Control si). Real Time PCR was performed using Sybr Green. Data show mRNA levels (normalised using RPLP0 housekeeping gene) for PHD1(A) , PHD2(B) and PHD3(C). Data plot represented as mean $\pm$ standard deviations.

Note: NS – not significant, * $P \leq 0.05$ ** $P \leq 0.01$ compared to Control si in normoxia or hypoxia.

The effect of HIF-1 α /HIF-2 α depletion of *COL2A1* was similar to that observed in *SOX9* appearing to be predominantly affected by the loss of HIF-1 α . The results show that a 54% reduction in normoxia ($P<0.05$) and a 61% reduction in hypoxia ($P<0.05$) following siRNA directed reduction of HIF-1 α . Combined HIF-1 α /HIF-2 α depletion also reduced *COL2A1* mRNA levels by 47% in normoxia ($P<0.05$) and by 71% in hypoxia ($P<0.05$). These reductions following HIF-1 α depletion suggest a HIF-1 α dependent effect on *COL2A1* mRNA (**Figure 3.10B**). Finally, with regards to the effect of HIF-1 α /HIF-2 α knockdown on *ACAN* mRNA, in accordance with *SOX9* and *COL2A1* data the loss of HIF-1 α has a significant effect. HIF-1 α depletion reduces *ACAN* mRNA by 46% in normoxia ($P<0.05$) and by 54% in hypoxia ($P<0.05$) whilst combined HIF-1 α /HIF-2 α results in a similar, although non-significant, reduction of 41% in normoxia ($P>0.05$) and a significant reduction of 61% in hypoxia ($P<0.05$). However, HIF-2 α depletion does not appear to have any significant effect on *ACAN* mRNA levels ($P>0.05$) (**Figure 3.10C**).

In *SOX9*, *COL2A1* and *ACAN* the reduction in mRNA levels observed following HIF-1 α depletion in hypoxia reduced to be not significantly different to those observed in normoxia (**Figure 3.10**). This is similar to observations made in *PHD3* (**Figure 3.9**), indicates that likely HIF-1 α is responsible for the hypoxic induction observed in these genes. Whilst these effects observed in *SOX9* mRNA appear HIF-1 α dependent it is worth considering that the mRNA levels following HIF-2 α depletion are scattered with two patients' data indicating a similar effect to HIF-1 α depletion in hypoxia. HIF-2 α depletion in the same two patients also express mRNA levels similar to HIF-1 α depletion in *COL2A1* and *ACAN*. When reviewing the patient information these two patients (41 and 45 years old) are much younger than the rest of the patients in the data set (70+ years old) whilst not conclusive this observation may indicate an age-related effect on HIF-2 α activity (**Figure 3.10**).

Following analysis of *SOX9* mRNA levels, the effect of HIF-1 α /HIF-2 α knockdown on *SOX9* protein levels was investigated. In normoxia the reduced band intensity following HIF-1 α depletion compared to control indicates that less *SOX9* protein is present, suggesting a HIF-1 α dependent effect. The combined HIF-1 α /HIF-2 α knockdown also appears to reduce the levels of *SOX9* in normoxia compared to control whilst the depletion of HIF-2 α doesn't appear to have an effect. Interestingly in hypoxia the level of *SOX9* protein appears to indicate a different effect following HIF-1 α /HIF-2 α knockdown. In hypoxia when comparing band intensity to control, combined HIF-1 α /HIF-2 α depletion causes the greatest reduction in *SOX9*

protein (comparable to control in normoxia), followed by the slight reduction seen as a result of HIF-2 α depletion. These results suggesting a potential effect of HIF-2 α on SOX9 at the protein level in hypoxia. However, the effect of HIF-1 α depletion on SOX9 protein levels, previously observed in normoxia, is less apparent in hypoxia (visible in a single blot) (**Figure 3.11**).

Finally, the effects of HIF1 α /HIF2 α knockdown on the mRNA levels of key catabolic enzymes, *ADAMTS5* and *MMP13* and anti-catabolic enzyme inhibitor *TIMP3* were assessed. Firstly, with regard to *ADAMTS5* mRNA levels, individual and combined HIF1 α /HIF2 α knockdown had no observable significant effect ($P>0.05$) in normoxia. In hypoxia however, HIF-2 α depletion and combined HIF1 α /HIF2 α depletion significantly increased *ADAMTS5* mRNA by 111% ($P<0.05$) and by 93% ($P<0.01$) respectively whilst HIF-1 α depletion had no effect ($P>0.05$). The depletion of HIF-2 α increased the mRNA levels of *ADAMTS5* in hypoxia, to the level observed in control in normoxia, suggesting that the suppression of *ADAMTS5* observed in hypoxia in OA HACs may be a HIF-2 α dependent effect (**Figure 3.12A**).

The mRNA levels of *MMP13* in normoxia are not significantly affected by HIF-2 α depletion ($P>0.05$) and while there is a slight increase on average in combined HIF1 α /HIF2 α depletion (18% increase) this is not significant ($P>0.05$). Whereas HIF-1 α depletion in normoxia increases *MMP13* mRNA levels by 25% ($P<0.05$) suggesting that the level of HIF-1 α in normoxia is sufficiently active to have a suppressing effect on *MMP13*. In hypoxia however, HIF-1 α depletion on average indicates an increase in *MMP13* mRNA (59% increase) although this is not significant ($P>0.05$). Instead HIF-2 α depletion results in a 78% increase in *MMP13* ($P<0.05$) and an increase of 217% in combined HIF1 α /HIF2 α knockdown ($P<0.01$) (**Figure 3.12B**). Although the results in hypoxia appear to suggest a HIF-2 α dependent effect in hypoxia on *MMP13*, at odds with the effect of HIF-2 α on *MMP13* in normoxia, the previously described compensatory increase of HIF-1 α when HIF-2 α is reduced (**Figure 3.8D**) may have decreased levels of *MMP13* mRNA when HIF-2 α is depleted in hypoxia masking the effect of HIF-2 α . This can be further supported by the greater fold increase of *MMP13* mRNA in OA HACs that have combined HIF1 α /HIF2 α knockdown.

TIMP3 mRNA levels in normoxia are reduced by 70% as a result of HIF-1 α knockdown ($P<0.05$) and are also reduced in combined HIF1 α /HIF2 α knockdown by 59% ($P<0.05$) with no significant effect of HIF-2 α observed. These results in normoxia suggest a

HIF-1 α dependent effect on *TIMP3* mRNA levels. Similar to *MMP13* results this would suggest that HIF-1 α is active in normoxia to a significant degree. In hypoxia HIF-1 α knockdown also results in a significant 68% reduction in *TIMP3* mRNA compared to control ($P<0.05$) however while the combined HIF1 α /HIF2 α knockdown reduces *TIMP3* mRNA levels to lower control (42% reduction) it is not statistically significant ($P>0.05$). This could potentially be due to the effect of HIF-2 α knockdown in hypoxia, although this effect is not significant on average the reduction of HIF-2 α protein increases *TIMP3* by 41% ($P>0.05$) (**Figure 3.12D**). This effect of HIF-2 α , acts in opposition to the observed HIF-1 α effect potentially explaining the non-significant effect of combined HIF1 α /HIF2 α knockdown.

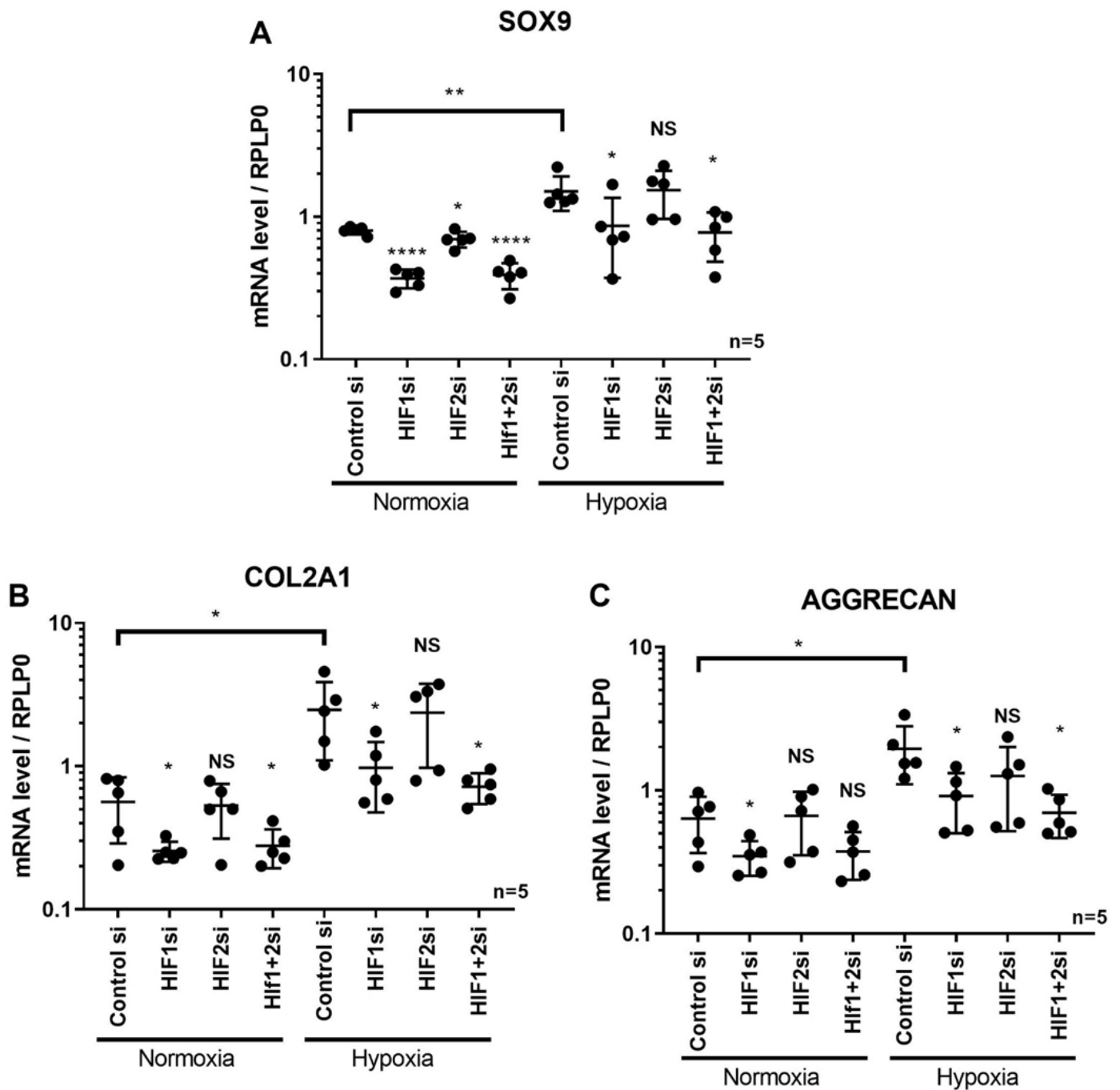


Figure 3.10 Effect of HIF depletion on SOX9, COL2A1 and AGGRECAN gene expression levels in OA HACs. Data were obtained from second passage (P2) OA HACs following 48hrs of culture post siRNA transfection in either 20% oxygen (Normoxia) or 1% oxygen (Hypoxia). Chondrocytes were transfected with 10nM siRNA targeting HIF-1 α (HIF1si), HIF-2 α (HIF2si), both HIF-1 α and HIF-2 α simultaneously (HIF1+2si), or a non-targeting control (Control si). Real Time PCR was performed using Sybr Green. Data show mRNA levels (normalised using RPLP0 housekeeping gene) for SOX9(A), COL2A1(B) and AGGRECAN(C). Data plot represented as mean +/- standard deviations. Note: NS – not significant, * $P \leq 0.05$ ** $P \leq 0.01$ *** $P \leq 0.001$ **** $P \leq 0.0001$ compared to Control si in normoxia or hypoxia.

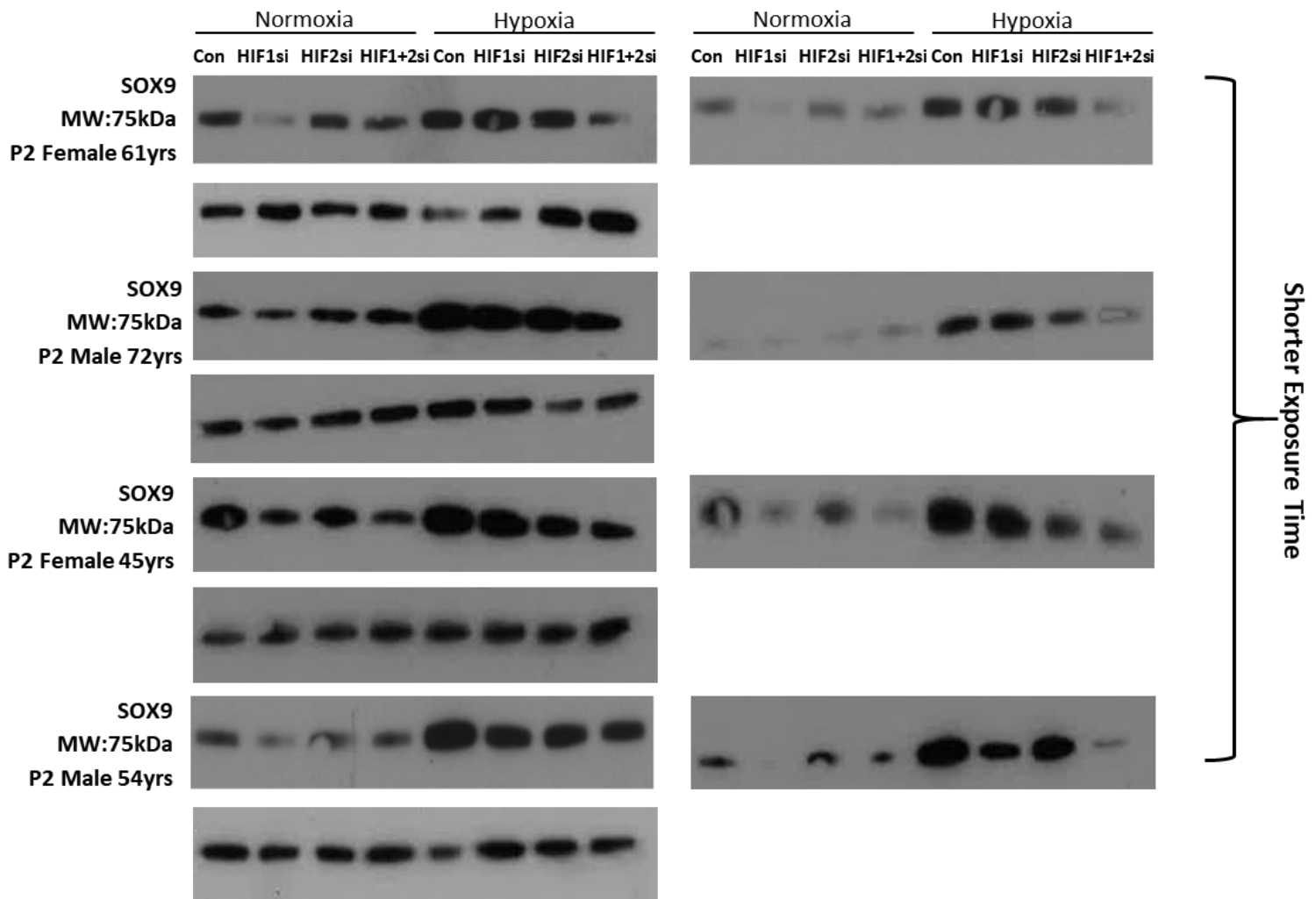


Figure 3.11 Effect of HIF depletion on SOX9 protein levels in OA HACs. Data were obtained from second passage (P2) OA HACs following 48hrs of culture post siRNA transfection in either 20% oxygen (Normoxia) or 1% oxygen (Hypoxia). Chondrocytes were transfected with 10nM siRNA targeting HIF-1 α (HIF1si), HIF-2 α (HIF2si), both HIF-1 α and HIF-2 α simultaneously (HIF1+2si), or a non-targeting control (Con). Western blotting shows the levels of SOX9 protein in both normoxia and hypoxia following HIF siRNA transfection. α -tubulin was used as a loading control. Shorter exposure times for SOX9 blots displayed to the right. Western blots representative of n=4 Note: yr – years old MW – molecular weight.

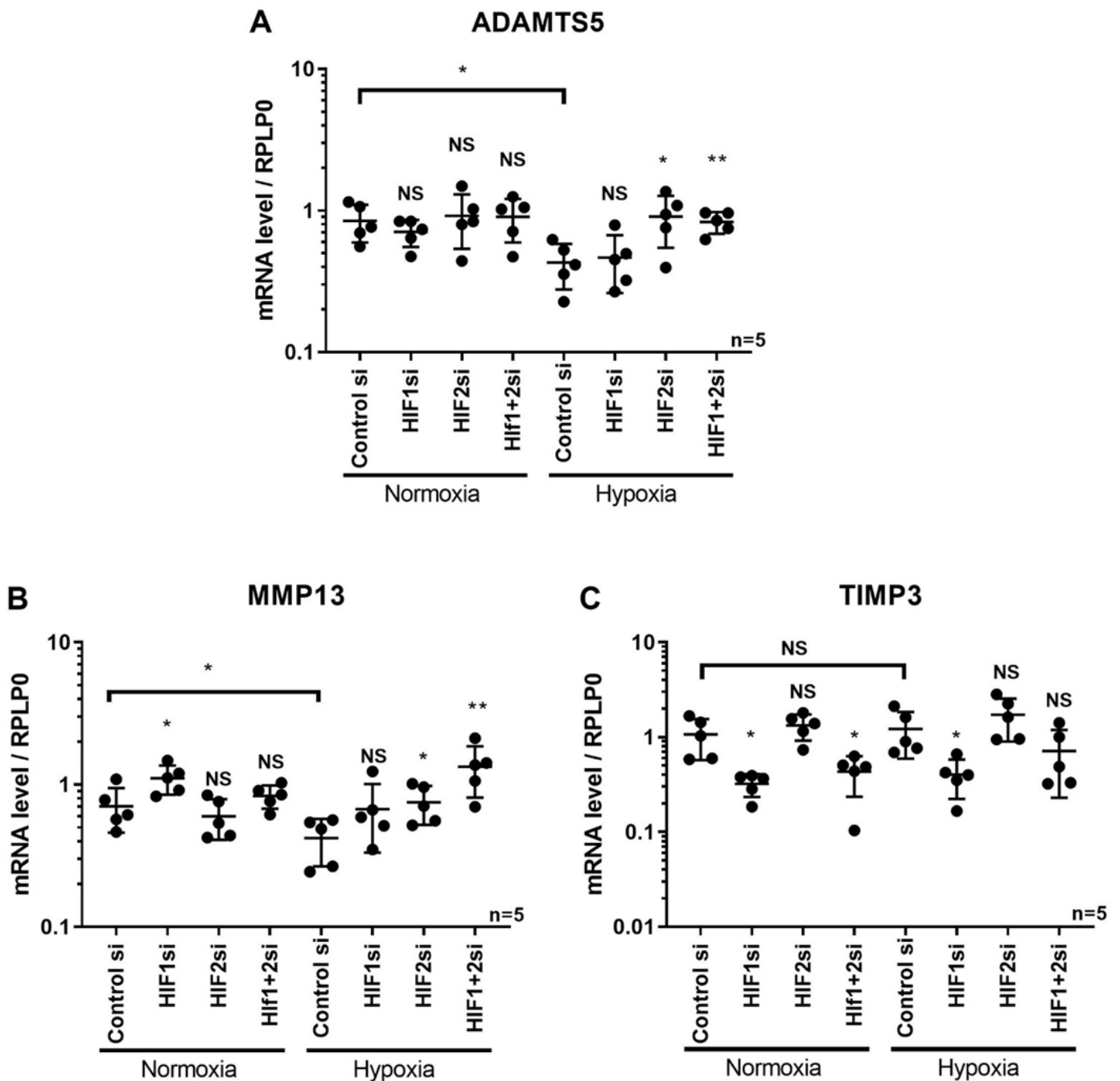


Figure 3.12 Effect of HIF depletion on ADAMTS5, MMP13 and TIMP3 gene expression levels in OA HACs. Data were obtained from second passage (P2) OA HACs following 48hrs of culture post siRNA transfection in either 20% oxygen (Normoxia) or 1% oxygen (Hypoxia). Chondrocytes were transfected with 10nM siRNA targeting HIF-1 α (HIF1si), HIF-2 α (HIF2si), both HIF-1 α and HIF-2 α simultaneously (HIF1+2si), or a non-targeting control (Control si). Real Time PCR was performed using Sybr Green. Data show mRNA levels (normalised using RPLP0 housekeeping gene) for ADAMTS5(A), MMP13(B) and TIMP3(C). Data plot represented as mean $\pm$ standard deviations. Note: NS – not significant, * $P \leq 0.05$ ** $P \leq 0.01$ compared to Control si in normoxia or hypoxia.

3.4 Discussion

The results in this chapter indicate that the beneficial adaption of human chondrocytes to hypoxia, in terms of expression of key anabolic and catabolic genes previously described in our group (Lafont et al., 2008, Thoms et al., 2013) is retained to a certain extent in the primary OA HAC. These similarities include the promotion of key cartilage transcription factor SOX9, which has been shown to drive matrix gene expression in human chondrocytes (Lafont et al., 2008), and the anti-catabolic effect of hypoxia reducing gene expression of key catabolic enzymes (Thoms et al., 2013). Although, nuanced variations of the response to hypoxia detailed in healthy HACs are observed in the OA setting that may provide insight into a potentially blunted hypoxic effect. Results here are also supported by previously described effects of hypoxia in OA HACs although with some variation in catabolic gene expression reported between groups (Markway et al., 2013, Alvarez K, 2014). In the case of MMP13 both groups report a decrease in response to hypoxia in OA HACs, however Markway and colleagues find that ADAMTS5 is not significantly reduced by hypoxia in OA HACs. However, it is noted that there is a large degree of inter-donor variance caused by limited clinical samples sizes used in these studies (N=5) (Markway et al., 2013, Alvarez K, 2014)

Previously our group have described clearly defined independent roles of the HIF- α subunits in healthy HACs. In healthy HACs, HIF-1 α was described as predominantly mediating the anti-catabolic observed in hypoxia, whilst HIF-2 α was shown to be responsible for the induction of the chondrocyte phenotype in hypoxia through direct binding to SOX9 (Lafont et al., 2007, Thoms et al., 2013). Whilst the role of HIF-2 α was shown to be beneficial in healthy HACs, the role of HIF-2 α in OA is debated within the community as it has been described as a catabolic regulator of OA in the murine model (Yang et al., 2010, Saito et al., 2010, Ryu et al., 2012). Additionally, hypertrophy marker COL10A1 and collagenase MMP13 have been shown to be induced via HIF-2 α in mice (Ishizuka et al., 2016). Binding motifs of HIF-2 α were also identified on the hypoxia responsive elements (HREs) on the human promoter region of multiple collagenases and aggrecanase (Yang et al., 2010).

Therefore, in the present study the individual roles of HIF-1 α and HIF-2 α were investigated in the human OA chondrocytes via RNA interference. Here it is reported that distinct differences in the roles of the HIF- α subunit were present in the OA HAC. Results indicted HIF-1 α was the HIF- α subunit primarily responsible for the induction of SOX9 and

therefore the promotion of cartilage production, with a reduced effect of HIF-2 α observed. Whilst the role of HIF-2 α was less clearly defined, results indicated an increase in catabolic genes following HIF-2 α depletion in hypoxia, suggesting a potentially anti-catabolic role. Results here therefore do suggest a shift in HIF- α activity in human OA. However, these results are in conflict with the suggestion that HIF-2 α is a catabolic transcription factor in the OA process.

3.4.1 Response to Hypoxia in Primary OA HACs Remains Benificial to Chondrocyte Phenotype

In this section the response of OA HACs to hypoxia is reported. Understanding the activity of OA chondrocytes in hypoxia is key as cartilage is an avascular tissue, which is physiologically chronically hypoxic with oxygen tensions that have been reported to be in the region of 1%-7% O₂ throughout the thickness of the tissue (Silver, 1975, Milner et al., 2006, Brighton and Heppenstall, 1971, Zhou et al., 2004). Additionally, the comparison between effects reported here and those previously described in healthy primary HACs could begin to develop a profile of potential OA related changes in hypoxic response.

Firstly, results show that, as expected, both HIF-1 α and HIF-2 α are stabilised at the protein level in hypoxia (**Figure 3.2**). This is to be expected as the activity of HIF-targeting prolyl hydroxylase enzymes (PHDs) is suppressed in low oxygen due to their requirement of molecular oxygen. Therefore, as previously described in healthy HACs, hypoxia also results in stabilised and active HIF in OA HACs.

The PHDs, specifically PHD2 and PHD3 were themselves found to be induced by hypoxia in OA HACs at both the mRNA and protein level (**Figure 3.3, 3.4**). This effect is consistent with previous observations made in our group and also correlates with the hypoxic induction of PHDs detailed in other cell types (Fujita et al., 2012, Pescador et al., 2005, Metzen, 2005).

Importantly, results here show that gene expression levels of key cartilage transcription factor SOX9 and gene expression levels of cartilage extracellular matrix proteins COL2A1 are increased in response to hypoxia in primary OA HACs (**Figure 3.5, 3.6**). This upregulation of SOX9 in hypoxia is also clearly visible at the protein level, this protein upregulation appears

to be greater than the hypoxic induction of *SOX9* mRNA, although this is not confirmed as western blots represented here provide qualitative data only. The hypoxic induction of *SOX9* here is consistent with previous group findings indicating a similar induction in healthy cartilage (Thoms et al., 2013, Lafont et al., 2007). However, with regards to *ACAN* gene expression a significant induction in response to hypoxia was not observed in the primary OA HAC with a reduced response to hypoxia compared to *COL2A1* (**Figure 3.5C**). This represents a potential difference to the hypoxic response of healthy HACs as a significant induction was previously reported in our group. Although, this difference can potentially be explained by the duration of cell culture in hypoxia, culture here occurred for 48 hours whereas a significant induction of *ACAN* was previously reported in chondrocytes following 7 days of culture. Whilst, the hypoxic induction of *ACAN* in healthy HACs was significant it was similar to results shown here with a weaker induction in hypoxia when compared to the hypoxic induction of *COL2A1* (Lafont et al., 2007).

The previously described anti-catabolic effects of hypoxia in healthy HACs were also assessed here in the primary OA HAC. Firstly, with regards to *ADAMTS5* mRNA levels which showed a significant reduction following culture in hypoxia compared to normoxia (**Figure 3.7A**). This reduction of *ADAMTS5* is a similar reduction to that observed previously in healthy cartilage (Thoms et al., 2013). However, others have reported that aggrecanase expression (*ADAMTS4* and *ADAMTS5*) was only reduced by hypoxia in healthy HAC and not the OA HAC (Markway et al., 2013). This difference can potentially be explained by Markway and colleague's use of 14 day pellet culture to redifferentiated chondrocytes where results reported within this chapter are from 48 hour monolayer culture.

Next the *MMP13* mRNA levels were shown to not significantly be affected by hypoxia in the primary OA HAC, although a general trend indicating a decrease in *MMP13* mRNA expression was observed (**Figure 3.7B**). Similar to *ADAMTS5*, the reduction of *MMP13* gene expression in hypoxia was described as an anti-catabolic effect in healthy HACs. The hypoxic reduction of *ADAMTS5*, and to a lesser extent *MMP13*, gene expression in primary OA HACs therefore suggest that an anti-catabolic mechanism in response to hypoxia is still present to some extent in OA. These results also go some way towards indicating that HIF-2 α may not have a catabolic effect in OA in hypoxia, this will be discussed further below.

An interesting difference between the response of primary OA HAC and the previously described response of healthy HAC in hypoxia is reported here in the gene expression of *TIMP3*. In the primary OA HAC our results show that hypoxia has no significant effect of the gene expression of *TIMP3* (**Figure 3.7D**), data showing on average a reduced expression, whereas healthy chondrocytes have been shown to have a near 3 fold increase in *TIMP3* mRNA in response to hypoxia (Thoms et al., 2013). The lack of response to hypoxia here of *TIMP3*, *TIMP3* being an anti-catabolic enzyme inhibitor which can inhibit both collagenases (*MMP13*) and aggrecanases (*ADAMTS5*) (Baker et al., 2002), could potentially indicate a reduced anti-catabolic effect of hypoxia in OA.

Finally, the gene expression of *COL10A1*, a marker of hypertrophy, was on average slightly increased although not significantly affected by hypoxia in OA HACs (**Figure 3.7C**). This result suggests hypoxia may not affect hypertrophy in the OA HAC. However, hypoxia has previously been shown to decrease *COL10A1* expression in redifferentiated OA HACs (Markway et al., 2013). Also, similar to the comment made on the expression of *ADAMTS5*, expression reducing in hypoxia, these results conflict with the catabolic role of HIF-2 α , that has been described in the literature, suggesting that HIF-2 α induces the expression of *COL10A1* and *ADAMTS5* (Ishizuka et al., 2016).

In summary these results show that the OA HACs response to hypoxia remains beneficial to the chondrocyte phenotype resulting in a similar gene expression profile as healthy chondrocytes. The described beneficial response to hypoxia is a result of the observed increase in cartilage production via *SOX9* hypoxic induction, as well as, the hypoxic reduction of the expression of key matrix metalloproteinases in OA HACs. However, it should be noted that there is potentially a reduced protective effect of hypoxia in OA HACs, this is indicated by change in response to hypoxia of two genes. Firstly, the lack of hypoxic induction of *TIMP3*, whereas a hypoxic induction of *TIMP3* is commonly observed in healthy HACs. Secondly, whilst hypoxia reduces *MMP13* mRNA in OA HACs this effect is no statistically significant as previously reported in healthy HACs.

3.4.2 Different Roles of HIF-1 α and HIF-2 α in OA HACs

The knockdown of both HIF-1 α and HIF-2 α was confirmed by western blot when comparing the intensity of specific bands following siRNA transfection to control. Interestingly, following the depletion of each HIF- α sub unit a compensation effect was observed. In normoxia but not hypoxia, following the knockdown of HIF-2 α , the protein levels of HIF-1 α were noticeably increased above levels observed in control. This compensatory increase in HIF-1 α protein resulted in levels in normoxia comparable those observed in hypoxia (**Figure 3.8C**). Similarly, in hypoxia, following the knockdown of HIF-1 α , the protein levels of HIF-2 α were increased above levels observed in control (**Figure 3.8D**). Therefore, these results indicate a compensatory increase in the protein levels of a HIF- α subunit following the depletion of the other subunit, which should be considered when evaluating the effect of each HIF- α knockdown.

Compensatory upregulation of one HIF- α subunit, following the depletion of the other HIF- α subunits, has previously been described in chondrocytes (Bohensky et al., 2009) as well as other cell types (Menrad et al., 2010). Schulz et al. (2012) described a mechanism in which compensatory upregulation of the HIF- α subunits occurred via enhanced translation. Compensatory upregulation of HIF- α was also shown not to be related to either increased mRNA expression or alterations in protein stability. Using HIF-2 α knockdown in HepG2 cells, an enhanced translational efficiency of HIF-1 α was identified by polysomal fractionation (Schulz et al., 2012). The RNA binding protein human antigen R (HuR) was investigated as a potential mediator of the response due to previous descriptions of HuR promoting HIF-1 α translation in response to the hypoxia mimic CoCl₂ (Galban et al., 2008). When HuR was initially investigated an increase in levels of HuR in the HIF-2 α knockdown cells was noted. Schulz et al. (2012) therefore generated a HuR knockdown cell in which HIF-2 α was also depleted; here they noted that in the absence of HuR the previously observed compensatory increase of HIF-1 α was no longer present and notably also attenuated the induction of known HIF-1 α target BNIP3. As a result, Schulz and colleagues report a role of HuR in the compensatory upregulation of HIF-1 α via enhanced translation. Importantly it is also noted that in HuR knockdown cells levels of HIF-2 α are reduced indicating that HIF-2 α is also a HuR target. The experiment described by Schulz and colleagues therefore provides a potential

explanation for the compensatory increase of HIF- α subunits observed in OA HACs (**Figure 3.8C-D**).

Following HIF-1 α knockdown the response of both *PHD2* and *PHD3* mRNA levels indicate that basal levels of *PHD2* and the hypoxic induction of *PHD3* are HIF-1 α dependent (**Figure 3.9B-C**). While the effects of individual HIF- α subunits of the PHDs was not previously reported by our group in healthy HACs, these effects are consistent with the identification of HREs that have been shown to bind with HIF-1 α on both *PHD2* and *PHD3* genes in the literature (Metzen, 2005, Pescador et al., 2005). This hypoxic up-regulation of *PHD2* and *PHD3* has been hypothesised to act as a negative feedback mechanism in cells in order to rapidly halt hypoxic responses during reoxygenation of a cell by the hydroxylation and subsequent degradation HIF- α (D'Angelo et al., 2003).

The depletion of HIF- α subunits in OA HACs indicate that the increase in mRNA levels of *SOX9* (and subsequently cartilage matrix genes *COL2A1* and *ACAN*) observed in hypoxia is predominantly a HIF-1 α dependent effect with a reduced effect of HIF-2 α observed (**Figure 3.10**). A reduced effect of HIF-2 α on *SOX9* is described here as a significant reduction of *SOX9* was observed in normoxia following HIF-2 α depletion. It was also noted that in hypoxia data from two independent donors consistently exhibited a reduction in *SOX9*, *COL2A1* and *ACAN* following HIF-2 α depletion. Interestingly, these donors are considerably younger than the rest of the data set. This observation could indicate an age-related change of the activity of HIF-2 α as opposed to a disease related change. However, due to the small sample sizes and the unavailability of younger OA donors/older healthy donors, such an effect cannot be reported conclusively by this data and instead indicates an avenue for future investigation as discussed in **Chapter 6.2.1**.

SOX9 protein levels also indicate a HIF-1 α dependent response in normoxia, indicating activity of HIF-1 α is present even under normoxic conditions. However, the effect of HIF-1 α depletion on *SOX9* was less obvious in hypoxia, where a slight reduction of *SOX9* following HIF-2 α depletion was observed. Instead, in hypoxia, combined HIF-1 α /HIF-2 α depletion resulted in the greatest reduction of *SOX9* protein (**Figure 3.11**). A potential explanation for this difference in the effect of HIF-1 α depletion on *SOX9* protein is the previously described compensation of HIF-2 α protein as a result of reduced HIF-1 α levels (**Figure 3.8D**). With the indication of a HIF-2 α related effect on *SOX9* protein in hypoxia, the increased levels of HIF-

2 α observed following HIF-1 α depletion could offset any effect the loss of HIF-1 α may have on SOX9 production in hypoxia by inducing SOX9 in absence of HIF-1 α .

Surprisingly, the predominately HIF-1 α dependent regulation of SOX9 reported here in OA HACs is a striking difference to the HIF-2 α dependent effect previously reported by our group in healthy HACs (Thoms et al., 2013). As previously hypothesised this change could be age dependent as opposed to disease dependent – the average age of healthy donors used to detail the effect of HIF on SOX9 was 33 years old whereas the average age of OA cartilage donors used here was 60 years old. It is important to note also that whilst the mechanism of SOX9 induction appears to be altered in the OA HAC the overall anabolic effect of hypoxia elevating SOX9 mRNA and protein levels remains consistent. Additionally, SOX9 has indeed previously been described as a HIF-1 α target in a cartilage setting (Amarilio et al., 2007, Duval et al., 2009, Zhang et al., 2011).

Interestingly, the hypoxia-induced reduction of *ADAMTS5* mRNA is reported here as a HIF-2 α dependent effect (**Figure 3.12A**) since the depletion of HIF-2 α reversed the hypoxic suppression typically observed in OA HACs. The reduction of *ADAMTS5* in hypoxia had previously been reported to be independent of either HIF-1 α or HIF-2 α in healthy HACs and instead as PHD2 dependent (Thoms et al., 2013).

Results also indicated that both HIF-1 α and HIF-2 α are important for the hypoxic suppression of *MMP13* mRNA levels. A significant increase in *MMP13* mRNA was observed following HIF-1 α depletion in normoxia, and in hypoxia following reduced HIF-2 α . The importance of both HIF- α subunits in the hypoxic suppression of *MMP13* was supported by the observation that depletion of both HIF- α subunits resulted in the greatest average increase of *MMP13* mRNA levels. This may indicate a combined suppressing effect of HIF-1 α and HIF-2 α on the gene expression levels of *MMP13* in hypoxia (**Figure 3.12B**).

The described HIF-2 α dependent hypoxic reduction of *ADAMTS5* and the effect of combined HIF- α depletion on *MMP13* in OA HACs are in conflict with the suggested catabolic role of HIF-2 α presently described within the literature. *COL10A1*, *MMP13* and *ADAMTS5* have all previously been described as targets induced by HIF-2 α in mice with a strong suggestion that HIF-2 α is a mediator of OA (Jian-Lin et al., 2016, Nakajima et al., 2012, Ryu et al., 2012, Saito et al., 2010, Yang et al., 2010, Zhang et al., 2015a). However, when

investigated in OA HACs the proposed catabolic effects of HIF-2 α were not observed, rather when assessing results in OA HACs the suggestion that HIF-2 α may provide an anti-catabolic response to hypoxia could be made.

In regards to proposed HIF-2 α mediated MMP13 expression, Saito et al, (2010) have shown binding of HIF-2 α to the identified hypoxia responsive binding sites (HREs) on the promoter of the MMP13 human gene via chromatin immunoprecipitation (ChIP) using cell lysates of a human chondrosarcoma cell line (SW1353). More recently Hashimoto et al. (2013) have shown that HIF-2 α transactivation of MMP13 is dependent on a single CPG site (-110-bp) on the promoter of the human gene. Additionally, Hashimoto and colleagues have shown that the methylation of this specific CPG site reduces the transactivation of MMP13 by HIF-2 α in HACs indicating the demethylation of the CPG is essential for HIF-2 α mediated MMP13 expression. Interestingly, Hashimoto and colleagues also note that the CPG site, containing HRE core sequence on the MMP13 promoter, is the most demethylated site when comparing OA to healthy HACs. These results therefore indicate that a HIF-2 α dependent induction of MMP13 would be observed here in OA HACs. However more recently Alvarez K (2014) have shown that when culturing OA HACs in 2% oxygen (hypoxia) MMP13 expression is decreased with no correlation with methylation status of CPG sites reported.

Finally, with regards to *TIMP3* mRNA levels, although elevated levels were not observed under hypoxic conditions the basal levels of *TIMP3* are shown to be dependent on the activity of HIF-1 α (**Figure 3.12C**). Whilst, the absence of a hypoxic induction of *TIMP3* mRNA is an interesting difference between the previously reported hypoxic induction in healthy HACs, both in previous studies in healthy HACs (Thoms et al., 2013) and the present study in OA HACs, a HIF-1 α dependence on the levels of *TIMP3* mRNA levels is observed.

In summary the results of HIF-1 α and HIF-2 α knockdown in OA HACs indicate a shift in HIF- α subunit activity compared to previous observations made in healthy HACs. The role of the HIF- α subunits here are in contrast to the previously described anabolic role of HIF-2 α and anti-catabolic role of HIF-1 α in healthy HACs (Thoms et al., 2013). In terms of the hypoxic induction of cartilage matrix production (via SOX9), in OA HACs, HIF-1 α appears to play the most significant role. Whilst, HIF-2 α has previously been described as being essential for SOX9 induction in healthy HACs this activity appears to be greatly reduced in OA. Instead, HIF-2 α appears to be involved in the anti-catabolic effects of hypoxia, results indicating that

HIF-2 α is, at least, partially responsible for the hypoxic reduction of key catabolic genes in OA HACs (Table 3.1).

Table 3.1 Summary of Chapter 3

HIF- α Isoform	HIF-1 α	HIF-2 α	HIF-1 α /HIF-2 α
Effect of HIF- α Isoform Depletion by siRNA on Targets	PHD2 ↓	<i>SOX9</i> mRNA (Normoxia Only) ↓	PHD2 ↓
	PHD3 ↓	<i>MMP13</i> (Hypoxia Only) ↑	PHD3 ↓
	<i>SOX9</i> (No Effect On Protein in Hypoxia) ↓	<i>ADAMTS5</i> (Hypoxia Only) ↑	<i>SOX9</i> ↓
	<i>COL2A1</i> ↓	HIF-1 α (Protein Only) ↑	<i>COL2A1</i> ↓
	<i>ACAN</i> ↓		<i>ACAN</i> (Hypoxia Only) ↓
	<i>MMP13</i> (Normoxia Only) ↑		<i>MMP13</i> (Hypoxia Only) ↑
	<i>TIMP3</i> ↓		<i>ADAMTS5</i> (Hypoxia Only) ↑
			<i>TIMP3</i> (Normoxia Only) ↓

Chapter 4

Effects of PHD Depletion in OA HACs

4.1 Introduction

It has previously been suggested that the hypoxic responses of human articular chondrocytes (HACs) and their beneficial effects could be tuned to potentially provide a therapeutic benefit in osteoarthritis (OA) by the inhibition of HIF-targeting prolyl-hydroxylase domain enzymes (PHDs). Thoms et al. (2013) have shown that PHD2 is the dominant isoenzyme regulating the stability of HIF-1 α and HIF-2 α in healthy HACs. When PHD2 is depleted in hypoxia in healthy HACs the levels of HIF-1 α and HIF-2 α are increased to levels greater than hypoxia alone. This increased stabilisation of HIF-2 α due to PHD2 depletion has been shown, in healthy HACs, to result in enhanced levels of SOX9 and thus increase cartilage matrix production to a greater extent than observed typically in hypoxia. Additionally, PHD2 depletion resulted in a greater reduction of ADAMTS5 and MMP13 than typically observed in hypoxia. Interestingly, Thoms and colleagues note that the reduction of ADAMTS5 following PHD2 depletion is independent of HIF. Therefore the suggestion is made that PHD2 inhibition enhances the anabolic and the anti-catabolic effects of hypoxia in healthy HACs and thus may represent a promising therapeutic target for cartilage repair.

In OA it is typically reported that there is increased production of metalloproteinases (i.e. ADAMTSs, MMPs) by chondrocytes. Studies have shown that this increase in ADAMTSs and MMPs plays a key role in the pathogenesis of OA by degrading the cartilage matrix and key matrix substrates such as aggrecan (Goldring, 2012, Bau et al., 2002, Jackson et al., 2014, Song et al., 2007). Additionally, a decrease in production of SOX9 and subsequently type II collagen and aggrecan is observed in degenerated zones of cartilage during the progression of OA in HACs (Zhang et al., 2015b, Fukui et al., 2008). Combined, this leads to a shift in the balance of anabolism and catabolism in cartilage leading to the progressive loss of cartilage observed in OA due to the loss of structural integrity of the extracellular matrix (Grimmer et al., 2006). Therefore, the relevance of tuning the transcriptional responses to hypoxia for therapeutic benefit cannot be over looked in OA. In **Chapter 3** it is reported that the response of OA HACs to hypoxia, similar to hypoxic response of healthy HACs (Thoms et al., 2013), is beneficial to cartilage by promoting matrix production via SOX9 and reducing gene expression levels of key catabolic enzymes; *MMP13* and *ADAMTS5* therefore indicating the potential benefit in manipulating hypoxic response.

4.1.2 Chapter 4 Specific Aims and Hypothesis

- 1) **Uncover the roles of individual PHD isoforms in the regulation of genes affected by a hypoxic environment in OA HACs**
- 2) **Identify the effect of combined PHD isoform inhibition via general hydroxylase inhibition**
- 3) **Explore whether beneficial hypoxic responses can be further enhanced via depletion of the PHDs**

In this chapter the potential to enhance the beneficial effects of hypoxia observed in OA HACs, through individual depletion of PHDs is investigated. It is hypothesised that depletion of individual PHD isoforms or a combined inhibition of all PHD isoforms will enhance the beneficial responses of OA HACs to hypoxia providing an avenue for potential therapeutic benefit.

4.2 Effect of PHD Knockdown on OA HACs

The effects the prolyl hydroxylase domain (PHD) enzymes (PHD1, PHD2 and PHD3) were investigated using second passage (P2) osteoarthritic (OA) human articular chondrocytes (HACs). OA cartilage was obtained from 5 female and 2 male donors; age range 57-86 years, mean age 68.1 years. HACs were transfected with small interfering RNA (siRNA) targeting; PHD1, PHD2 and PHD3. Once transfected HACs were cultured for 48 hours in either normoxia (20% oxygen) or hypoxia (1% oxygen), following cell culture messenger RNA (mRNA) and protein levels were analysed as previously described. Changes in mRNA levels stated are compared to control in the relevant oxygen tension unless otherwise stated.

Following the transfection of siRNA targeting PHD1 qPCR data showed that, compared to control, mRNA levels of *PHD1* were reduced on average by 62% in normoxia ($P < 0.05$) and by 68% in hypoxia ($P < 0.01$). No significant effect of *PHD2* or *PHD3* siRNA was observed in either normoxia or hypoxia ($P > 0.05$) (**Figure 4.1A**). These qPCR data indicate a significant reduction in *PHD1* mRNA indicating a successful knockdown. As previously described (**Chapter 2.6.3**) PHD1 protein data is not represented as western blot analysis of PHD1 protein levels was found to be unreliable.

The mRNA levels of *PHD2* also show a significant reduction of 67%, on average, in normoxia ($P<0.001$) and a 75% reduction in hypoxia ($P<0.0001$) when compared to controls. No significant effect of *PHD1* or *PHD3* siRNA on *PHD2* mRNA levels was observed in either normoxia or hypoxia ($P>0.05$) (**Figure 4.1B**). Depletion of PHD2 protein levels was confirmed by western blot. A pronounced reduction in band intensity compared to control in both normoxia and hypoxia indicates lower levels of PHD2 protein following siRNA transfection confirming knockdown of PHD2. No effects following PHD1 or PHD3 siRNA were observed on PHD2 protein levels (**Figure 4.1D**).

The knockdown of PHD3 following siRNA transfection was assessed, mRNA levels of *PHD3* compared to controls were reduced by 73% in normoxia ($P<0.0001$) and by 79% in hypoxia ($P<0.001$). On average PHD2 siRNA increased *PHD3* mRNA levels by 14% in both normoxia and hypoxia however this effect was not found to be statistically significant (**Figure 4.1B**). Depletion of PHD3 protein levels was confirmed by western blot in hypoxia (PHD3 does not typically present bands in normoxia). Band intensity compared to control is reduced following PHD3 siRNA indicating a reduction in the amount of PHD3 protein present confirming knockdown of PHD3 (**Figure 4.1E**). However, it is worth noting that band intensity following PHD2 siRNA is increased compared to control suggests a compensatory increase in PHD3 protein. Interestingly, this indicates that higher levels of PHD3 protein are present following the depletion of PHD2 protein levels (**Figure 4.1E**).

As PHDs are known to be responsible for the regulation of hypoxia inducible factors (HIF- α), once knockdown of the PHDs was confirmed, the effects of PHD depletion on HIF-1 α and HIF-2 α were assessed. Firstly, the mRNA levels of the HIF- α isoforms were analysed. Interestingly PHD isoform knockdown had no significant effect on *HIF-1 α* mRNA levels, although, it is noted that, following the knockdown of PHD1 and PHD2 in normoxia *HIF-1 α* mRNA levels were reduced by 28% and 34% respectively ($P>0.05$). However, in hypoxia no effect on *HIF-1 α* mRNA levels was observed following individual knockdown of the three PHD isoforms ($P>0.05$) (**Figure 4.2A**). The mRNA levels of *HIF-2 α* were not changed significantly in either normoxia or hypoxia following PHD knockdown ($P>0.05$) (**Figure 4.2B**).

Next potential effects on the protein levels of HIF-1 α and HIF-2 α following PHD knockdown were investigated. Firstly, with regards to HIF-1 α protein levels in normoxia, when comparing band intensities, HIF-1 α is observed to be stabilised above levels observed following control in PHD2 knockdown. This stabilisation of HIF-1 α protein, due to PHD2 depletion in normoxia, results in protein levels comparable to hypoxic induction alone. However, in hypoxia PHD2 knockdown does not appear to affect the levels of HIF-1 α when compared to control (**Figure 4.3**), this could be a potential outcome of the PHD3 compensation previously described (**Figure 4.1E**). Interestingly in several western blots PHD1 and PHD3 knockdown appear to reduce the level of HIF-1 α protein compared to control in both normoxia and hypoxia (**Figure 4.3A-C**).

When comparing band intensity following PHD knockdown to control in hypoxia the level of HIF-2 α protein does not appear to be affected by the depletion of any of the three PHDs. This observation is unexpected, given previous findings in our group indicating a stabilisation of HIF-2 α when PHD2 is depleted in healthy HACs (Thoms and Murphy, 2010). However, similar to effect of PHD depletion on HIF-1 α in hypoxia, the lack of an observable effect on HIF-2 α protein levels could also be a potential outcome of the PHD3 compensation previously described (**Figure 4.1E**).

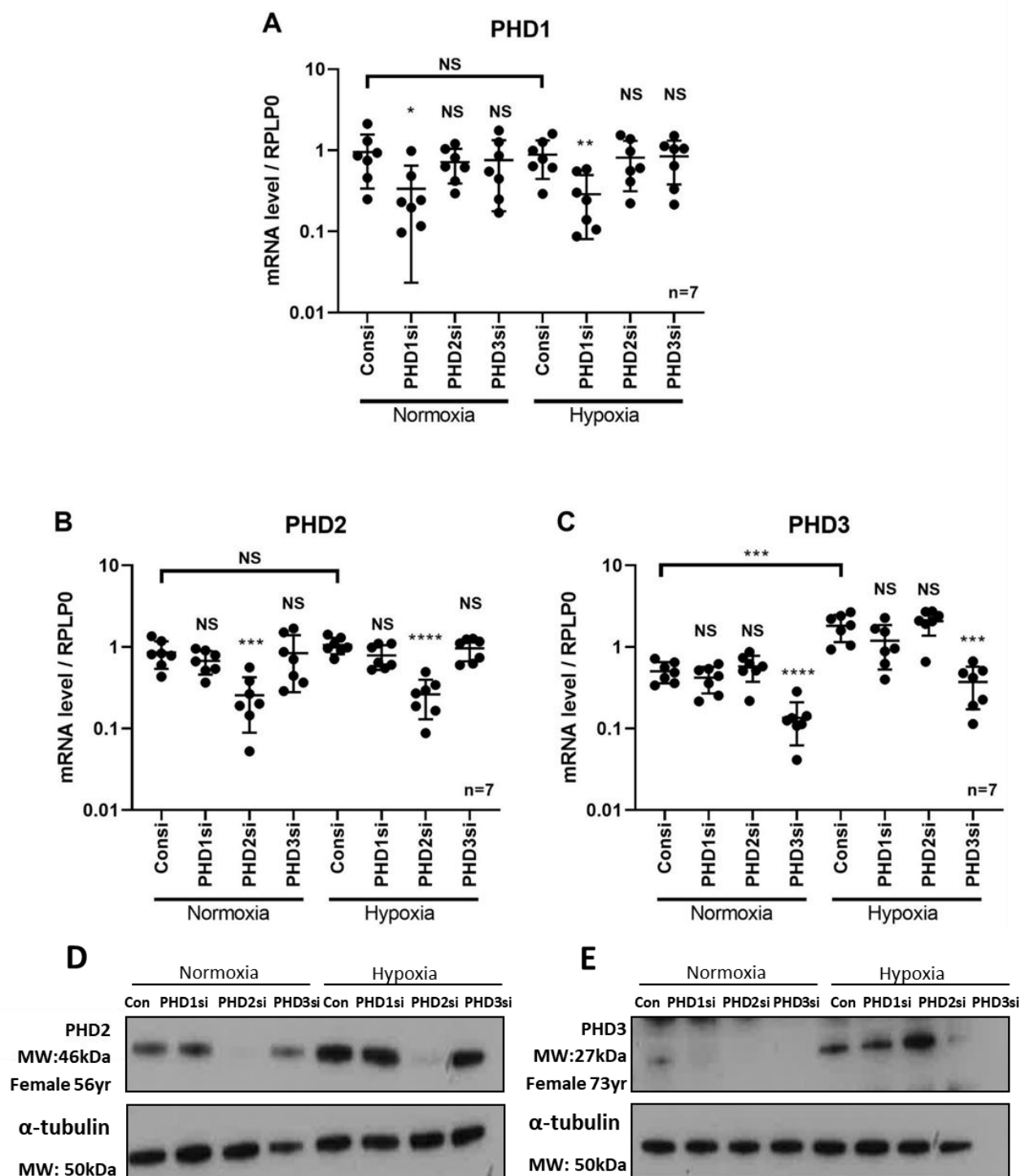


Figure 4.1 Knockdown of PHDs in OA HACs. Data were obtained from second passage (P2) OA HACs following 48hrs of culture post siRNA transfection in either 20% oxygen (Normoxia) or 1% oxygen (Hypoxia). Chondrocytes were transfected with 10nM siRNA targeting PHD1 (PHD1si), PHD2 (PHD2si) and PHD3 (PHD3si) or a non-targeting control (Consi). Real Time PCR was performed using Sybr Green. Data show mRNA levels (normalised using RPLP0 housekeeping gene) for PHD1(A), PHD2(B) and PHD3(C) Western blots confirming knockdown of PHD2 (D) and PHD3 (E). Data plot represented as mean $\pm$ standard deviations.

Note: NS – not significant, $*P \leq 0.05$ $**P \leq 0.01$ $***P \leq 0.001$ $****P \leq 0.0001$ compared to Consi in normoxia or hypoxia. Western blots representative of n=4. yr – years old MW – molecular weight.

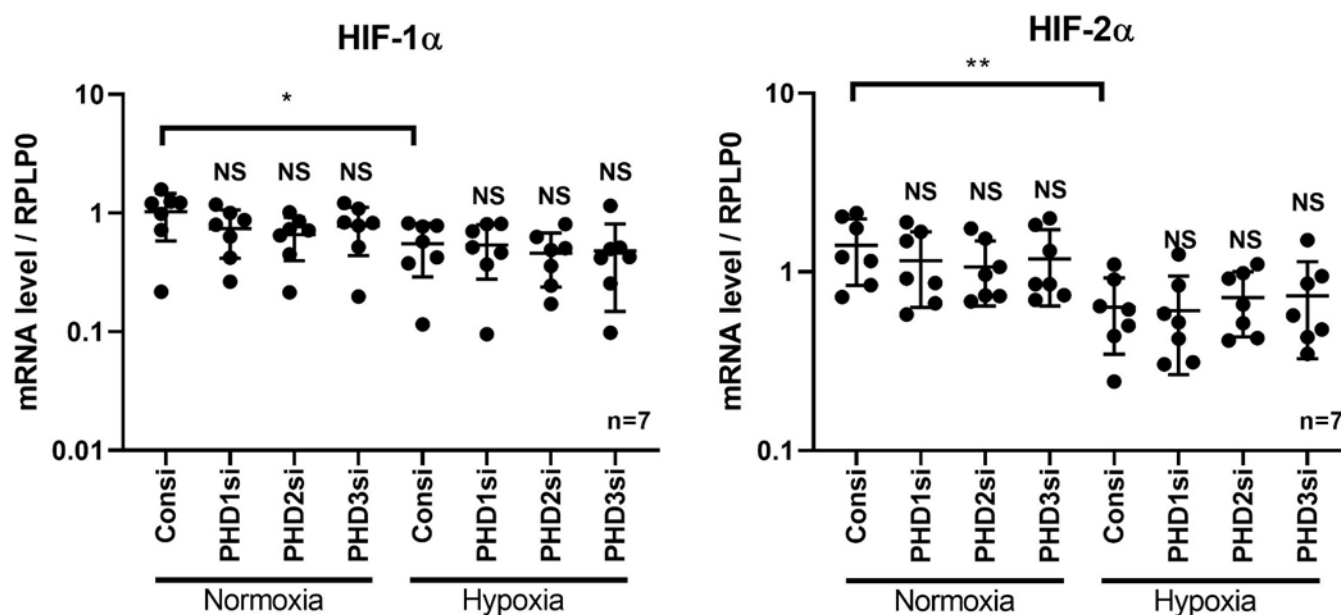


Figure 4.2 Effect of PHD depletion on HIF gene expression levels in OA HACs. Data were obtained from second passage (P2) OA HACs following 48hrs of culture post siRNA transfection in either 20% oxygen (Normoxia) or 1% oxygen (Hypoxia). Chondrocytes were transfected with 10nM siRNA targeting PHD1 (PHD1si), PHD2 (PHD2si) and PHD3 (PHD3si) or a non-targeting control (Consi). Real Time PCR was performed using Sybr Green. Data show mRNA levels (normalised using RPLP0 housekeeping gene) for HIF-1α (A) and HIF-2α (B). Data plot represented as mean +/- standard deviations. Note: NS – not significant, $*P \leq 0.05$ $**P \leq 0.01$ compared to Consi in normoxia or hypoxia.

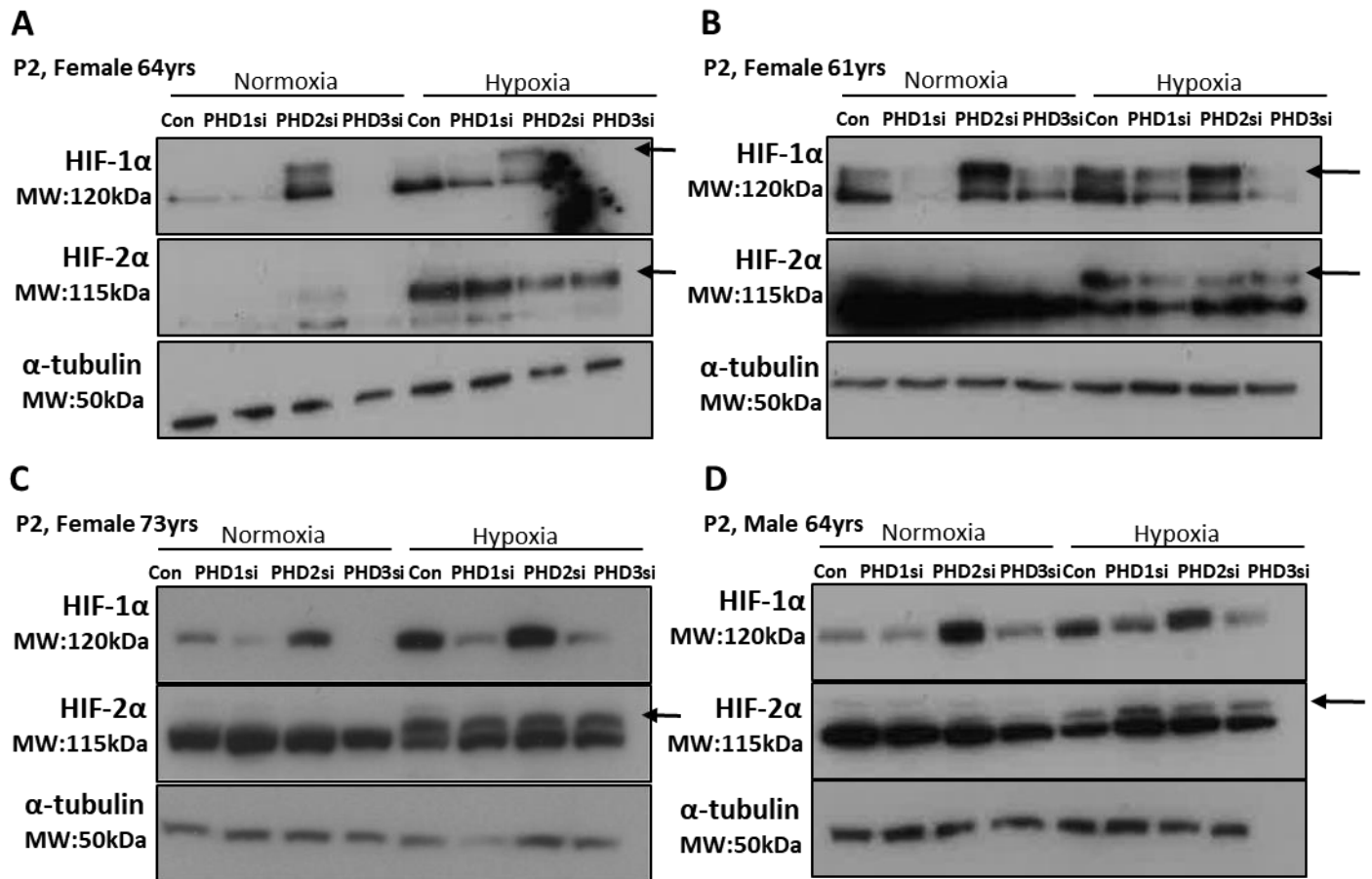


Figure 4.3 Effect of PHD depletion of HIF protein levels in OA HACs. Data were obtained from second passage (P2) OA HACs following 48hrs of culture post siRNA transfection in either 20% oxygen (Normoxia) or 1% oxygen (Hypoxia). Chondrocytes were transfected with 10nM siRNA targeting PHD1 (PHD1si), PHD2 (PHD2si) and PHD3 (PHD3si) or a non-targeting control (Con). Western blotting shows the levels of HIF-1α and HIF-2α protein in normoxia and hypoxia following PHD siRNA transfection. A black arrow indicates the specific band for each blot. Novus Biologicals antibody (NB100-105) for HIF-1α is used in blots (A) and (B). BD Transduction Laboratories antibody (Clone 54) for HIF-1α is used in blots (C) and (D). α-tubulin was used as a loading control. Western blots representative of n=5 Note: yr – years old MW – molecular weight.

Once the effects of PHD knockdown on the HIF- α isoforms had been analysed, the subsequent effects on cartilage transcription factor SOX9, and key cartilage matrix genes *COL2A1* and *ACAN* were also assessed. Firstly, with regards to SOX9 mRNA, individual PHD isoform knockdown does not have a significant effect on mRNA levels in both normoxia and hypoxia ($P>0.05$) (**Figure 4.4A**). This lack of effect following PHD2 depletion is interesting given the previously described stabilisation of HIF-1 α in normoxia to levels similar to hypoxia (**Figure 4.3**). The lack of effect on SOX9 following knockdown of the PHDs can also be observed at the protein level. Whilst the protein levels of SOX9 are increased following hypoxic induction, no additional effect of PHD knockdown can be observed. Most interestingly, when comparing band intensity of PHD2 knockdown in normoxia (where increased HIF-1 α is present) to control, the levels of SOX9 protein appear unaffected (**Figure 4.5**). These results suggest that the increased levels of HIF-1 α in normoxia, following PHD2 knockdown, do not result in an increase in levels of SOX9. Thus whilst levels of HIF-1 α protein were increased it appears the activity of HIF-1 α was not increased above levels normally observed in normoxia. The lack of increased HIF-1 α in hypoxia is likely due to the increased levels PHD3 protein following PHD2 depletion.

The mRNA levels of *COL2A1* were not significantly affected by the depletion of PHD2 and PHD3 isoforms in normoxia or hypoxia ($P>0.05$). However it is noted that *COL2A1* mRNA levels were on significantly reduced by PHD1 knockdown in normoxia. PHD1 in normoxia, reducing *COL2A1* gene expression levels by 39% in normoxia ($P<0.05$). (**Figure 4.3B**). *ACAN* mRNA was also not significantly affected by either PHD2 and PHD3 depletion or PHD1 depletion in hypoxia ($P>0.05$) However, a similar result can be observed in regards to the gene expression levels of *ACAN* following PHD1 knockdown. Although, not statistically significant, similar to the effects on *COL2A1* PHD1 depletion on average reduces *ACAN* mRNA by 29% in normoxia ($P>0.05$). (**Figure 4.3C**). As SOX9 targets, the non-significant response of *COL2A1* and *ACAN* when PHD2 is depleted is consistent with the observations of no significant change in SOX9 mRNA and protein levels. However, the effect of PHD1 depletion significantly reducing mRNA levels of *COL2A1* ($P<0.05$) and on average reducing gene expression of *ACAN* in normoxia is an interesting effect to note due to the consistency of the effect on both genes in contrast to the results of the other PHD isoforms.

Finally, with regards to the effects of PHD knockdown on the mRNA levels of key catabolic enzymes, *ADAMTS5* and *MMP13* and anti-catabolic enzyme inhibitor *TIMP3*. The mRNA levels of *ADAMTS5* show an average increase of 61% following PHD2 depletion in hypoxia although this effect is not significant ($P>0.05$). The depletion of PHD1 in normoxia, whilst not significant, is also worth noting reducing *ADAMTS5* mRNA by 43% ($P>0.05$). Although these effects are worth noting *ADAMTS5* mRNA was not significantly affected by PHD depletion in normoxia or hypoxia ($P>0.05$) (**Figure 4.6A**).

MMP13 mRNA levels were significantly reduced in both normoxia and hypoxia following PHD1 depletion by 59% and 45% respectively ($P<0.05$). Notably, PHD 2 depletion in normoxia reduced *MMP13* mRNA by 48% however, this effect is not statistically significant ($P>0.05$). Interestingly, no notable effect of PHD2 depletion was observed in hypoxia ($P>0.05$). PHD3 depletion had no effect of the levels of *MMP13* mRNA in both normoxia and hypoxia ($P>0.05$) (**Figure 4.6B**). The reduction of *MMP13* mRNA, following PHD1 depletion in normoxia, is in line with the reduction observed by hypoxia alone. Therefore these results suggest that there is some level of dependency of *MMP13* mRNA on the activity of PHD1 in OA HACs.

TIMP3 mRNA levels are on average reduced, although not significantly, following PHD1 and PHD2 depletion in normoxia by 15% and 16% respectively ($P>0.05$). Additionally, in hypoxia PHD1 knockdown reduces the levels of *TIMP3* mRNA by 30% ($P>0.05$) with PHD2 also reducing mRNA levels by 20% ($P>0.05$). Whilst no significant effect of PHD3 depletion is observed, there is a reduction of *TIMP3* mRNA expression in hypoxia by 24% following PHD3 depletion ($P>0.05$) (**Figure 4.6C**). Whilst these results indicate no significant effect on *TIMP3* mRNA levels the relatively high average reduction of *TIMP3* mRNA following individual knockdown of PHD isoforms may suggest that each of the isoforms may contribute to the regulation of *TIMP3* to some degree. These results could therefore potentially suggest a combined effect of the PHDs on the levels of *TIMP3* mRNA which could be dependent on a combined PHD isoform knockdown.

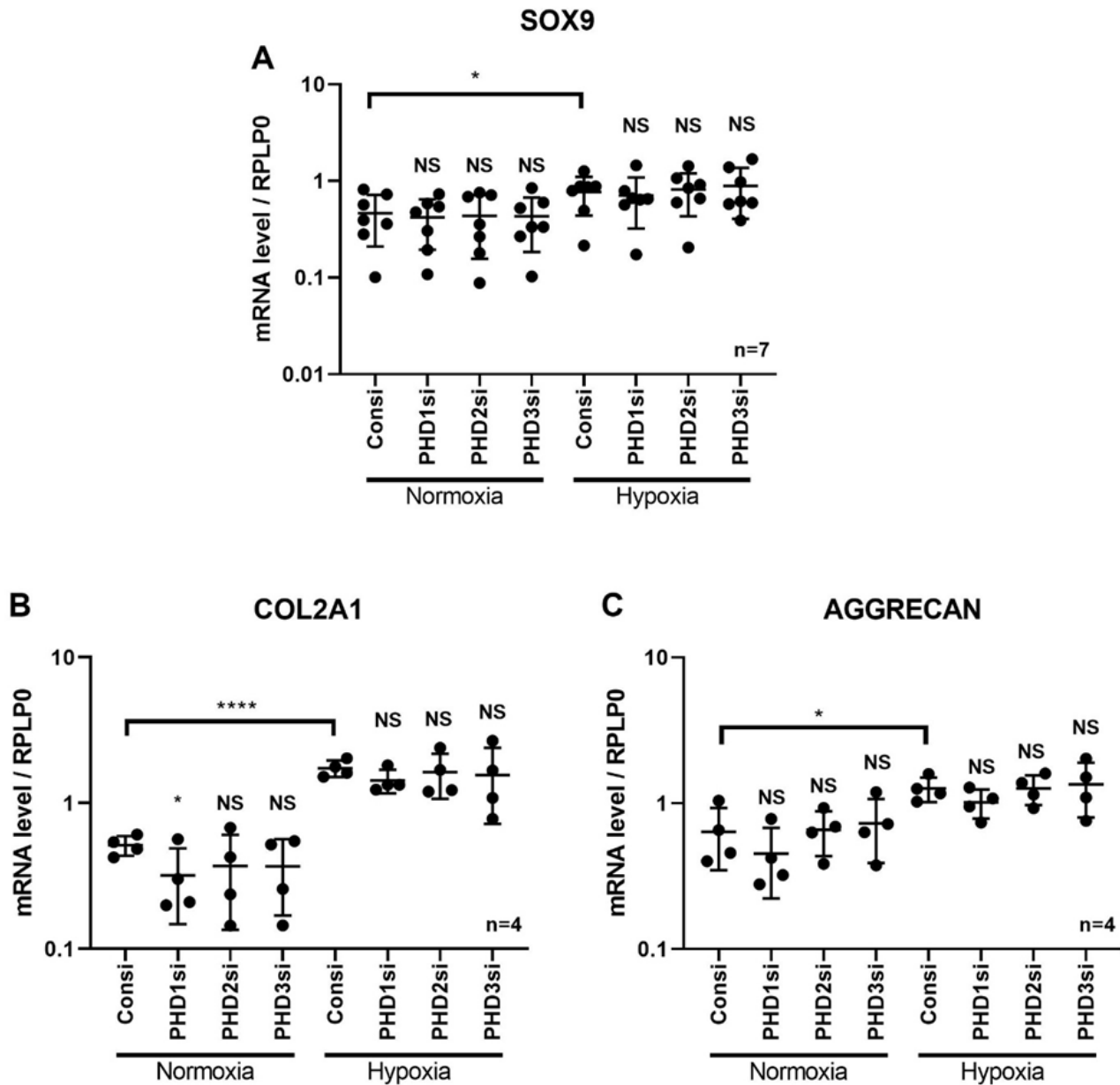


Figure 4.4 Effect of PHD depletion on cartilage marker gene expression in OA HACs. Data were obtained from second passage (P2) OA HACs following 48hrs of culture post siRNA transfection in either 20% oxygen (Normoxia) or 1% oxygen (Hypoxia). Chondrocytes were transfected with 10nM siRNA targeting PHD1 (PHD1si), PHD2 (PHD2si) and PHD3 (PHD3si) or a non-targeting control (Consi). Real Time PCR was performed using Sybr Green. Data show mRNA levels (normalised using RPLP0 housekeeping gene) for SOX9(A), COL2A1(B) and AGGRECAN(C). Data plot represented as mean +/- standard deviations. Note: NS – not significant, $*P \leq 0.05$ $****P \leq 0.0001$ compared to Consi in normoxia or hypoxia.

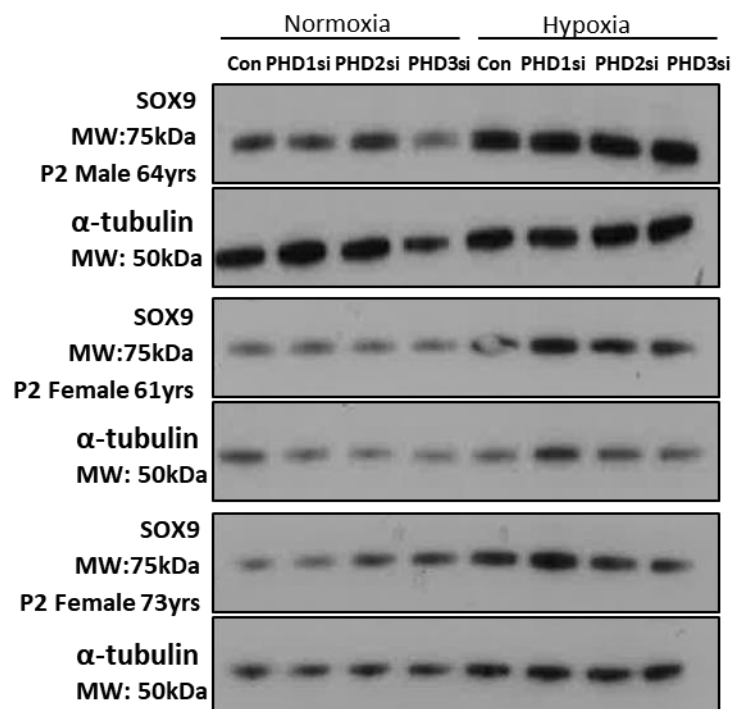


Figure 4.5 Effect of PHD depletion on SOX9 protein levels in OA HACs.

Data were obtained from second passage (P2) OA HACs following 48hrs of culture post siRNA transfection in either 20% oxygen (Normoxia) or 1% oxygen (Hypoxia). Chondrocytes were transfected with 10nM siRNA targeting PHD1 (PHD1si), PHD2 (PHD2si) and PHD3 (PHD3si) or a non-targeting control (Con). Western blotting shows the levels of SOX9 protein in both normoxia and hypoxia following PHD siRNA transfection. α -tubulin was used as a loading control.

Western blots representative of n=4 Note: yr – years old MW – molecular weight.

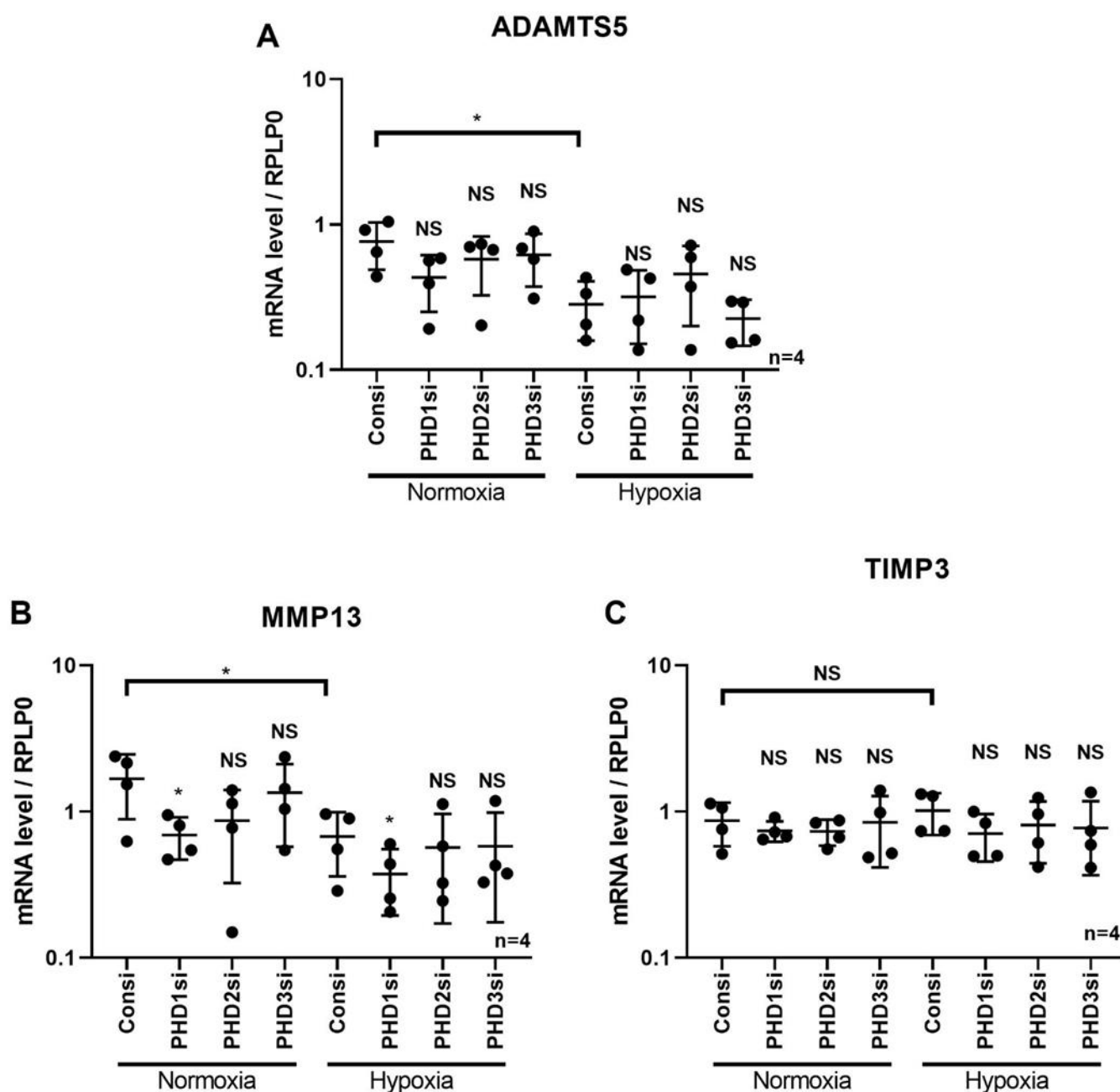


Figure 4.6 Effect of PHD depletion on ADAMTS5, MMP13 and TIMP3 gene expression levels in OA HACs. Data were obtained from second passage (P2) OA HACs following 48hrs of culture post siRNA transfection in either 20% oxygen (Normoxia) or 1% oxygen (Hypoxia). Chondrocytes were transfected with 10nM siRNA targeting PHD1 (PHD1si), PHD2 (PHD2si) and PHD3 (PHD3si) or a non-targeting control (Consi). Real Time PCR was performed using Sybr Green. Data show mRNA levels (normalised using RPLP0 housekeeping gene) for ADAMTS5(A), MMP13(B) and TIMP3(C). Data plot represented as mean +/- standard deviations.

Note: NS – not significant, * $P \leq 0.05$ compared to Consi in normoxia or hypoxia.

4.3 Effect of General Hydroxylase Inhibition via DMOG in OA HACs

As a potential compensation between PHDs following knockdown was observed (**Figure 4.1D**) the effect of combined PHD and general hydroxylase inhibition on OA HACs was investigated. Second passage OA HACs were used obtained from 2 male and 1 female donor, age range 41-83 years, mean age 66.6 years. To investigate the effect of general hydroxylase inhibition OA HACs were cultured with or without 1mM Dimethyloxaloylglycine (DMOG) for 48 hours in either normoxia or hypoxia. Following culture mRNA and protein levels were analysed as previously described. The mRNA data here is given as fold change compared to normoxia, this is as the low sample size and spread in baseline values of gene expression between donor samples affected the interpretation of results however, the effect of DMOG was consistent between donors in terms of fold change.

Firstly, the effect of DMOG hydroxylase inhibition on HIF-1 α and HIF-2 α was assessed. As expected, DMOG did not significantly affect mRNA level expression of either HIF- α subunit when compared to normoxia ($P>0.05$). Interestingly DMOG did not result in further reduce *HIF- α* mRNA levels beyond that which is observed by hypoxia alone suggesting that this effect is independent of the activity of hydroxylases (**Figure 4.7**). At the protein level an increase in both HIF-1 α and HIF-2 α can be observed when cultured with DMOG, as expected due to the combined inhibition of the PHDs. These results show that DMOG can increase the levels of HIF above levels that would normally be observed by hypoxia alone, indicating that there the PHDs remain active to some extent in 1% oxygen (**Figure 4.8**).

Next the effect of DMOG on the PHDs at the mRNA level was assessed. The mRNA levels of *PHD1* were not significantly affected by culture with DMOG ($P>0.05$) (**Figure 4.9A**). With the addition of DMOG *PHD2* mRNA were increased an average of 2.2 fold in normoxia ($P>0.05$) and 2.18 fold in hypoxia ($P<0.05$) however, this increase was in line with the induction of *PHD2* observed by hypoxia alone (**Figure 4.9B**). The addition of DMOG significantly increased the mRNA of *PHD3* with an average fold increase of 43.1 in normoxia ($P<0.01$) and 49.5 in hypoxia ($P<0.01$). The effect of DMOG on *PHD3* was a significant increase on the levels of *PHD3* mRNA observed by hypoxia alone ($P<0.01$) (**Figure 4.9C**). Whilst DMOG inhibits the activity of the PHD protein, the mRNA levels can indicate the activity of increased HIF-1 α protein as the induction of *PHD2* and *PHD3* have been shown to be HIF-1 α dependent (**Figure 3.9**).

Next the subsequent effects of general hydroxylase inhibition, on cartilage transcription factor SOX9 and key cartilage matrix genes; *COL2A1* and *ACAN* were assessed. Firstly, with regards to SOX9 mRNA, DMOG increased mRNA levels further than hypoxia alone (average 2.36 fold increase $P<0.05$) with an average 4.29 fold increase in normoxia ($P<0.01$) and a 5.71 fold increase in hypoxia ($P<0.05$) (**Figure 4.10A**). Western blots of SOX9 protein also indicate that DMOG results in a stronger induction of SOX9 than by hypoxia alone. When comparing band intensity of 1% oxygen to 1% oxygen with DMOG it can be seen that the level of SOX9 is increased (**Figure 4.11**). These results indicate that DMOG enhances the hypoxic induction of SOX9. This is most likely due to the increased stabilisation of HIF-1 α and HIF-2 α protein following hydroxylase inhibition (**Figure 4.8**) as the induction of SOX9 has been previously shown to be HIF- α dependent (**Figure 3.10 and 3.11**).

Whilst DMOG enhances the hypoxic induction of SOX9 the mRNA levels of *COL2A1* show no significant changes following culture with DMOG ($P>0.05$) (**Figure 4.10B**). The same observation is made when assessing the effect of DMOG on *ACAN* mRNA levels. Whilst hypoxia results in an average 1.83 fold increase ($P<0.05$) the addition of DMOG does not significantly changes *ACAN* mRNA levels ($P>0.05$) (**Figure 4.10C**). As DMOG significantly enhances the induction of SOX9 mRNA and protein, above levels typically observed in hypoxia, a correlated increase in both *COL2A1* and *ACAN* would be expected as previously observed by hypoxia alone. These results therefore suggest a DMOG dependent effect of the mRNA levels of both *COL2A1* and *ACAN* resulting in a lack of induction in the presence of increased SOX9 protein.

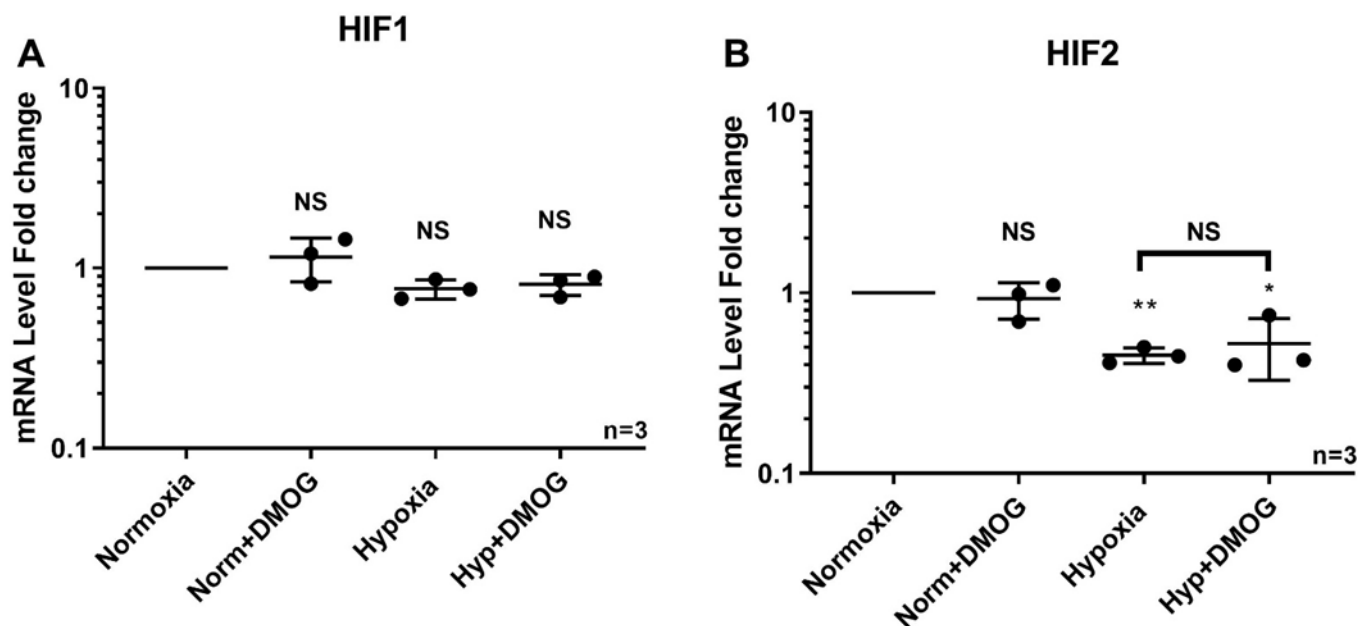


Figure 4.7 Effect of DMOG on HIF gene expression in OA HACs. Data were obtained from second passage (P2) OA HACs following 48hrs of culture (with and without 1mM DMOG) in either 20% oxygen (Normoxia) or 1% oxygen (Hypoxia). Real Time PCR was performed using Sybr Green. Data show fold change in mRNA levels (normalised using RPLP0 housekeeping gene) for HIF-1 α (A) and HIF-2 α (B) compared to normoxia. Data plot represented as mean +/- standard deviations.
Note: NS – not significant, * $P \leq 0.05$, ** $P \leq 0.01$

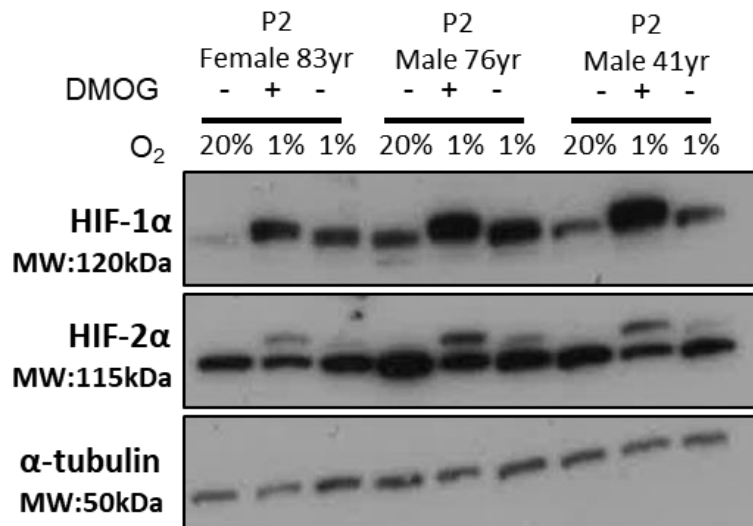


Figure 4.8 Effect of DMOG on HIF protein levels in OA HACs. Data were obtained from second passage (P2) OA HACs following 48hrs of culture (with and without 1mM DMOG) in either 20% oxygen (Normoxia) or 1% oxygen (Hypoxia). Data show the protein levels for HIF-1α and HIF-2α. α-tubulin was used as a loading control. Western blots representative of n=3 Note: yr – years old MW – molecular weight.

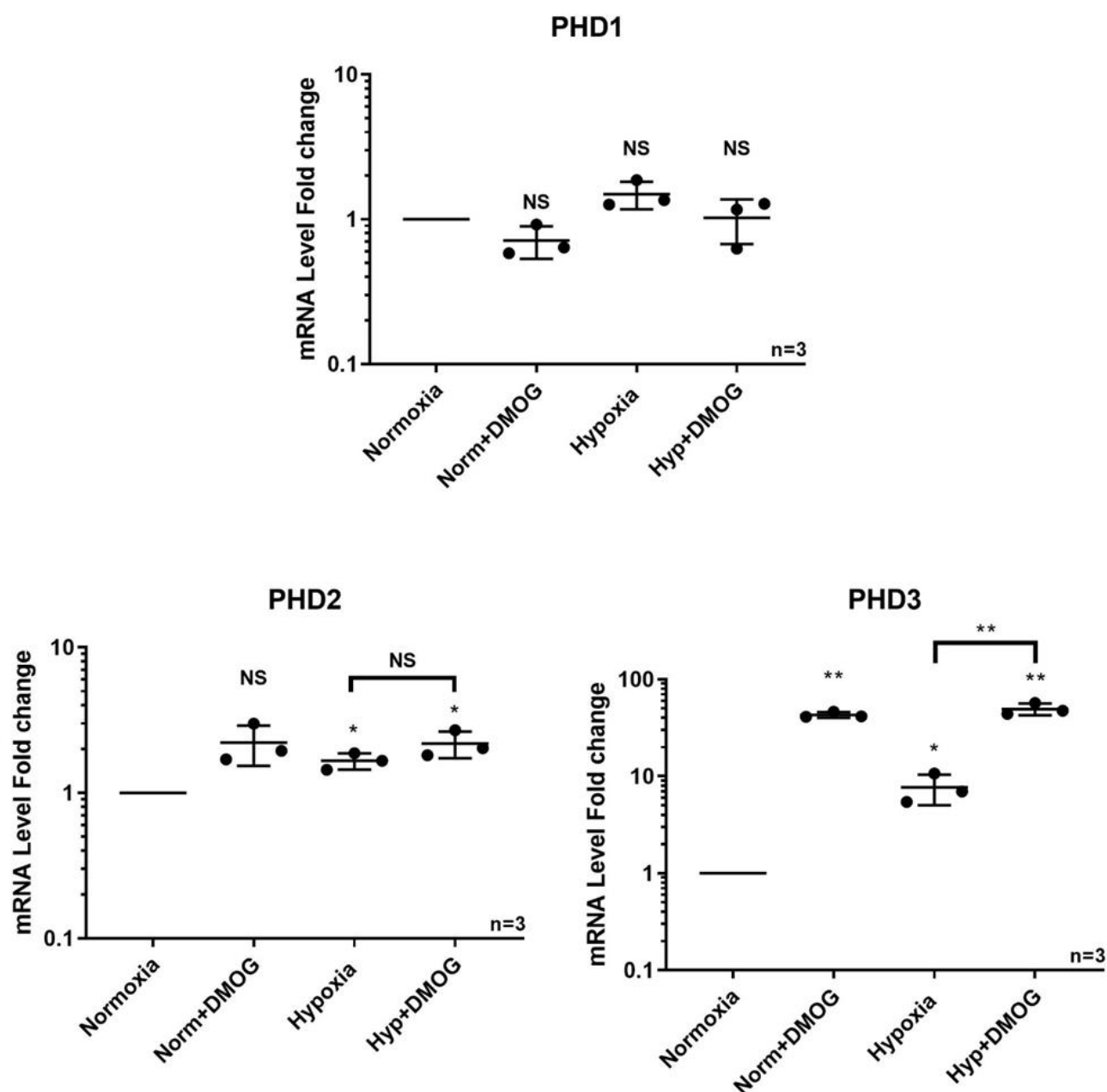


Figure 4.9 Effect of DMOG on PHD gene expression in OA HACs. Data were obtained from passaged (P2) OA HACs following 48hrs of culture (with and without 1mM DMOG) in either 20% oxygen (Normoxia) or 1% oxygen (Hypoxia). Real Time PCR was performed using Sybr Green. Data show fold change in mRNA levels (normalised using RPLP0 housekeeping gene) for PHD1(A) , PHD2(B) and PHD3(C) compared to normoxia. Data plot represented as mean $\pm$ standard deviations. Note: NS – not significant, * $P \leq 0.05$ ** $P \leq 0.01$.

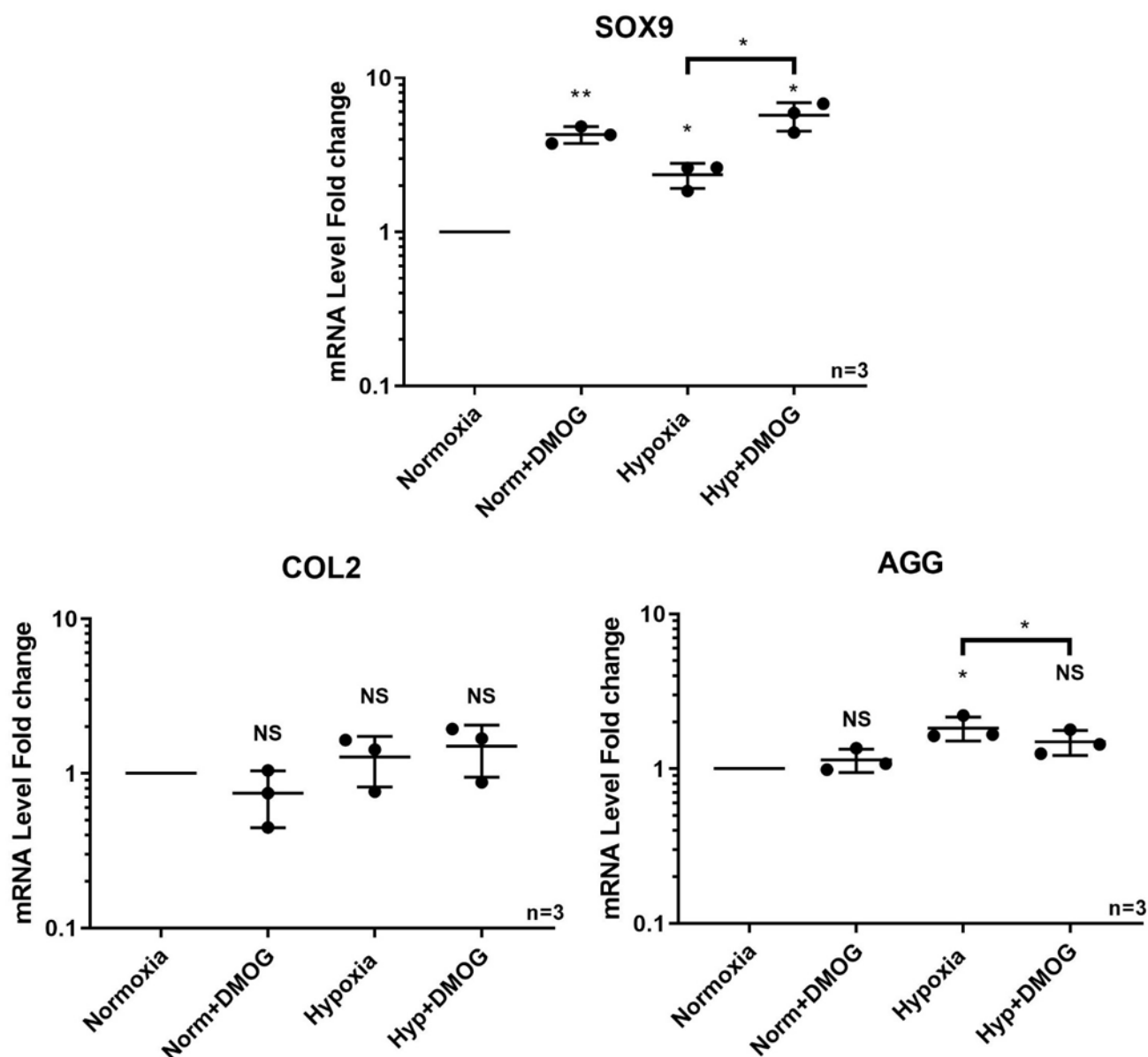


Figure 4.10 Effect of DMOG on cartilage marker gene expression in OA HACs. Data were obtained from passaged (P2) OA HACs following 48hrs of culture (with and without 1mM DMOG) in either 20% oxygen (Normoxia) or 1% oxygen (Hypoxia). Real Time PCR was performed using Sybr Green. Data show fold change in mRNA levels (normalised using RPLP0 housekeeping gene) for SOX9(A), COL2A1(B) and AGGRECAN(C) compared to normoxia. Data plot represented as mean $\pm$ standard deviations. Note: NS – not significant, $*P \leq 0.05$ $**P \leq 0.01$.

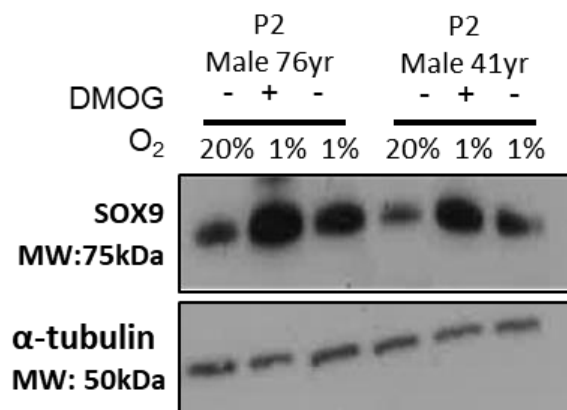


Figure 4.11 Effect of DMOG on SOX9 protein levels in OA HACs. Data were obtained from second passage (P2) OA HACs following 48hrs of culture (with and without 1mM DMOG) in either 20% oxygen (Normoxia) or 1% oxygen (Hypoxia). Data show the protein levels for SOX9. α-tubulin was used as a loading control. Western blots representative of n=2 Note: yr – years old MW – molecular weight.

Finally, with regards to the effects of hydroxylase inhibition via culture with DMOG on the mRNA levels of key catabolic enzymes, *ADAMTS5* and *MMP13*, and anti-catabolic enzyme inhibitor *TIMP3*. On average DMOG reduced *ADAMTS5* mRNA levels below reductions normally observed in hypoxia (0.52 fold average reduction), with an average fold reduction of 0.31 fold in normoxia with DMOG and 0.28 fold in hypoxia with DMOG although this greater reduction was not statistically significant ($P>0.05$) (**Figure 4.12A**).

The mRNA levels of *MMP13* following hydroxylase inhibition show a greater reduction than by hypoxia alone. Hypoxia reduces *MMP13* with an average fold reduction of 0.67 fold ($P>0.05$) whereas culture with DMOG in normoxia showed an average 0.19 fold reduction ($P<0.01$) and in hypoxia a 0.16 fold reduction ($P<0.01$) (**Figure 4.12B**). Interestingly *MMP13* mRNA is greatly reduced following culture with DMOG, which inhibits all PHDs, this observation therefore supports the previous suggestion of a PHD1/PHD2 dependent effect on *MMP13* mRNA levels (**Figure 4.6B**).

The mRNA levels of *TIMP3* are not significantly affected by hypoxia as previously observed ($P>0.05$) and no significant effect of culture with DMOG is observed in hypoxia ($P>0.05$). However, whilst these effects are not significant it should be noted that culture with DMOG does reduce *TIMP3* mRNA levels on average. Whereas, in normoxia DMOG on average causes a 0.67 fold reduction compared to normoxia alone ($P<0.05$) and in hypoxia DMOG reduces the induction observed by hypoxia alone from 1.47 fold to 1.06 fold ($P>0.05$) (**Figure 4.12C**). Although only statistically significant in a normoxic setting, these observations could support the previous suggestion of a PHD isoform dependent effect on the mRNA levels of *TIMP3* given the consistent average reduction when cultured with DMOG.

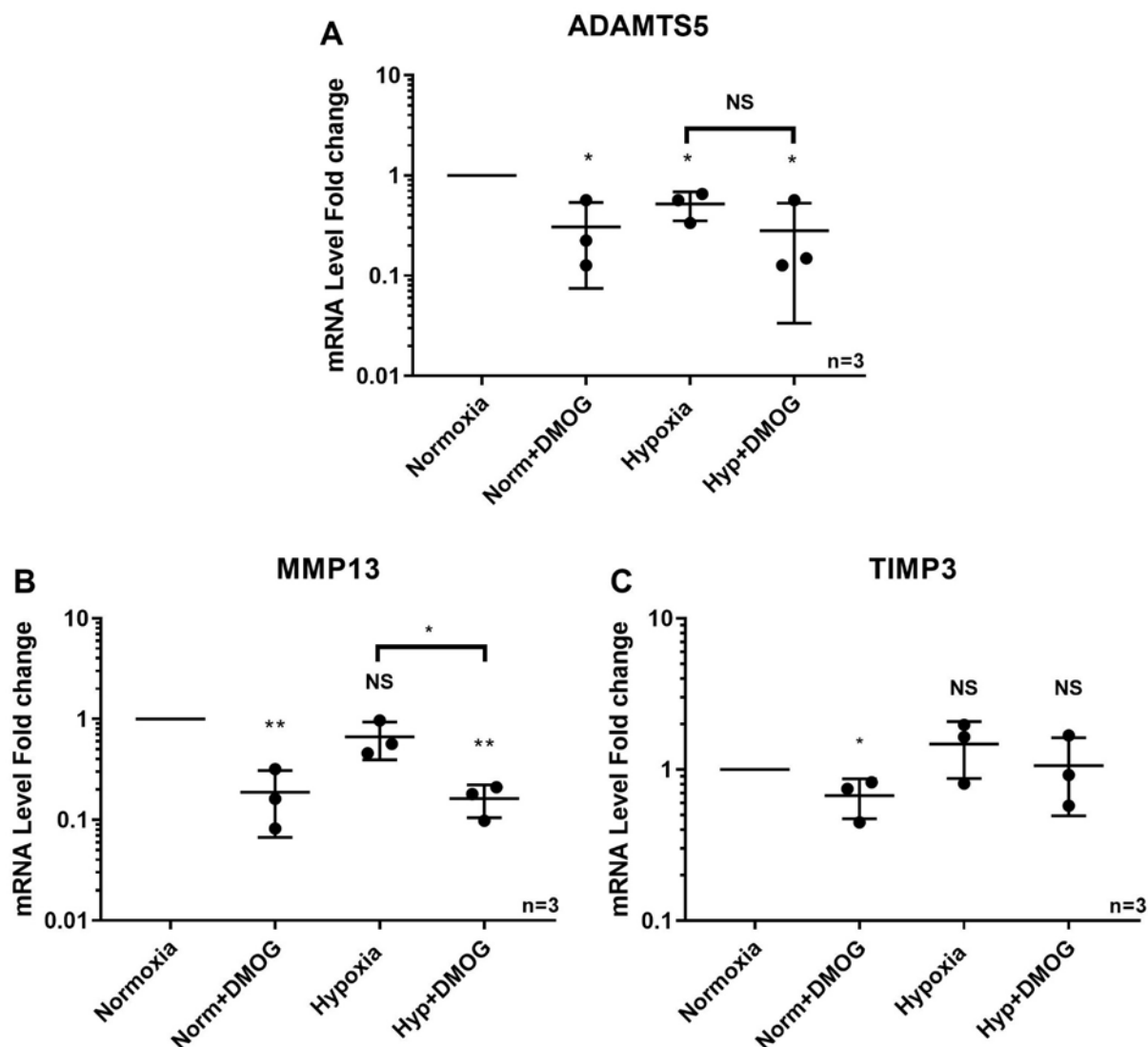


Figure 4.12 Effect of DMOG on ADAMTS5, MMP13 and TIMP3 gene expression in OA HACs. Data were obtained from passaged (P2) OA HACs following 48hrs of culture (with and without 1mM DMOG) in either 20% oxygen (Normoxia) or 1% oxygen (Hypoxia). Real Time PCR was performed using Sybr Green. Data show fold change in mRNA levels (normalised using RPLP0 housekeeping gene) for ADAMTS5(A), MMP13(B) and TIMP3(C) compared to normoxia. Data plot represented as mean +/- standard deviations.

Note: NS – not significant, $*P \leq 0.05$ $**P \leq 0.01$.

4.4 Discussion

As previously discussed, the hypoxic responses of OA HACs appear to remain beneficial to the cell phenotype via increased cartilage production (SOX9 and matrix genes) and the reduced expression of key catabolic genes. These effects were determined to be dependent of the activity of HIF- α subunits. Therefore, as HIFs are regulated by PHDs the effect of the PHD enzymes on both HIF and their targets was investigated by siRNA transfection in OA HACs. Additionally, in healthy HACs, Thoms and Murphy (2010) have previously described enhanced matrix synthesis as a result of PHD2 inhibition via increased stabilisation of HIF-2 α in hypoxia. Thoms and Murphy also noted that the hypoxic reduction in ADAMTS5 mRNA was a result of PHD2 inhibition and was independent of the HIF- α subunits.

The effects of PHD inhibition in OA HACs observed in the present study somewhat differ from the effects described in healthy HACs (Thoms et al., 2013). Following siRNA transfection for individual PHDs, similar to healthy HACs, results here show that only PHD2 inhibition had an effect on the stabilisation of HIF. However, PHD2 inhibition only resulted in increased levels of HIF-1 α protein in normoxia with no significant effect on HIF-1 α or HIF-2 α protein in hypoxia. Importantly, a compensatory increase of PHD3 protein, when PHD2 was reduced, is observed in OA HACs. Such a compensatory increase could potentially offset the effects of an individual PHD2 knockdown providing a potential explanation for the lack of effect of PHD2 depletion of HIF- α levels in hypoxia.

Additionally, increased levels of HIF-1 α protein in normoxia following PHD2 inhibition did not result in an increase of SOX9, where an increase would be predicted as HIF-1 α siRNA data indicated SOX9 was dependent on HIF-1 α (**Figure 3.10-11**). This, therefore suggests possible post-translation regulation inhibiting the activity of HIF-1 α in normoxia. These results (specifically the absence of SOX9 induction) suggest that specific inhibition of PHD2 in OA HACs may not provide the therapeutic benefit previously hoped for following the results of PHD inhibition in healthy HACs (Thoms et al., 2013).

In order to investigate the effect of general 2-oxoglutarate dependent hydroxylase inhibition, OA HACs were cultured with 2-oxoglutarate analogue DMOG. Culture with DMOG results in the inhibition of the PHDs, as well as factor inhibiting HIF-1 α (FIH-1).

Following general hydroxylase inhibition, both HIF- α subunits were increased to levels above those typically observed in hypoxia. Likely as a result of increased HIF- α protein levels, the mRNA and protein levels of SOX9 were also enhanced above typical hypoxic induction. Although DMOG results in reduced collagen protein secretion (due to inhibition of collagen prolyl-4-hydroxylases) these results indicate that combined inhibition of hydroxylases (PHDs, FIH-1) can enhance the induction of SOX9 to a greater extent than by hypoxia alone.

In summary, results in this chapter indicate that individual inhibition of PHDs is not adequate to enhance the beneficial transcriptional responses to hypoxia in OA HACs probably due to compensatory effects in PHD expression. However, results following culture with DMOG show that there is potential to manipulate the response by combined inhibition of HIF targeting hydroxylases. Such a manipulation of the hypoxic response could potentially increase cartilage matrix production.

4.4.1 Individual PHD Knockdown Has no Effect on Cartilage Production

The knockdown of *PHD1*, *PHD2* and *PHD3* was confirmed by qPCR at the mRNA and at the protein level for PHD2 and PHD3 (the absence of PHD1 westerns blot data explained previously in **Chapter 2.6.3**) by western blot when comparing band intensity to control. Importantly, in hypoxia, following the depletion of PHD2, PHD3 protein levels are increased above levels normally observed in hypoxia alone (**Figure 4.1**). It is important to note that the increase in PHD3 following PHD2 depletion was not previously observed by our group in the healthy HACs (Thoms and Murphy, 2010).

An induction and compensatory role of PHD3 following the loss of PHD2 has previously been observed in mouse embryonic fibroblasts (MEF) (Minamishima et al., 2009). In a previous study by Minamishima et al. (2008) comparing mice lacking PHD2 and mice lacking von Hippel-Lindau protein (pVHL), they found that some HIF target genes were induced in mice lacking pVHL that were not induced in mice lacking PHD2. These differences suggested residual hydroxylation of HIF by PHD1 or PHD3. To investigate the effect further Minamishima and colleagues used PHD2^{-/-} MEF and pVHL ^{Δ/Δ} MEF to compare the effects on HIF and HIF target genes and additionally, to further elucidate a potential role of PHD3. Interestingly, they found that PHD2^{-/-} MEFs had lower levels of HIF-1 α than pVHL ^{Δ/Δ} ; however, HIF-1 α could be further induced in PHD2^{-/-} following DMOG but not in pVHL ^{Δ/Δ}

MEFs. As PHD2^{-/-} MEFs had higher levels of PHD3 mRNA and protein than PHD2^{+/+} MEFs, PHD3 was investigated as a compensator for PHD2 loss. By creating PHD2^{f/f} PHD3^{-/-} MEFs Minamishima and colleagues were able to show that combined loss of PHD2 and PHD3 had a greater induction of HIF-1 α than the loss of PHD2 alone. Interestingly, the group also show that PHD2 inactivation in PHD3^{+/+} MEFs resulted in increased HIF-1 α , HIF-1 α target GLUT1 and PHD3 which reduced to baseline after 12 days, whereas PHD2 inactivation in PHD3^{-/-} MEFs resulted in increased HIF levels that remained elevated even after 12 days. Finally, Minamishima et al. (2009) suggest that PHD3 regulates the intensity and duration of the HIF response following the loss of PHD2. The results of this study therefore indicate the relevance of considering the impact of the compensatory increase of PHD3, following PHD2 depletion observed here in OA HACs, on the subsequent stabilisation of HIF.

Each PHD isoform has been shown to be able to hydroxylate HIF- α (Bruick, 2001, Epstein, 2001). PHDs are also shown to have similar K_m values for recombinant oxygen dependent degradation domains of both HIF-1 α and HIF-2 α , PHD1 has slightly lower values whilst PHD2 and PHD3 K_m values are equal (Koivunen et al., 2006). The PHDs also have similar K_m values for O₂ at slightly above the concentrations of dissolved O₂ in air (Hirsila et al., 2003) suggesting that activity of the PHDs should not differ based on O₂ availability. It has been suggested that differential function of the PHD isoforms in the regulation of HIF- α subunits is heavily dependent on the abundance of the enzymes (Appelhoff et al., 2004). Interestingly, Appelhoff et al. (2004) have also shown that loss of PHD2 when PHD3 is abundant can be sufficient to see effects of PHD3 on HIF-1 α where such effects are not typically observed. This could therefore indicate increased levels of PHD3 due to compensatory increase in protein following loss of PHD2 could affect HIF stabilisation in OA HACs. Therefore, careful consideration of the potential effects of this PHD3 compensation, following the loss of PHD2, on HIF stability and activity should be made.

Individual knockdown of PHD isoforms did not have a statistically significant effect on HIF- α mRNA levels although HIF-1 α was on average slightly reduced in normoxia by PHD1 and PHD2 depletion (**Figure 4.2A**). Importantly though, it is observed that there is a reduction of HIF- α mRNA in hypoxia (where PHDs are reduced) in the passaged OA HACs. Kim et al. (2010) provide a potential explanation for the reduced levels of HIF- α mRNA when PHDs levels are reduced. Kim and colleagues have shown in various cell lines that there is an induction of tristetraprolin (TTP) in hypoxia. TTP is a regulator of mRNAs containing

adenosine/uridine-rich elements (AREs), TTP controls the stability of target genes via binding to the three prime untranslated region (3'UTR) containing ARE motifs leading to the recruitment of factors which mediate mRNA destruction (Lai et al., 1999). Kim and colleagues were able to identify TTP binding motifs on 3'UTR of HIF-1 α and successfully demonstrated binding of TTP to HIF-1 α binding sites they described. TTP binding to HIF-1 α was observed to result in reduced HIF-1 α mRNA and protein stability (Kim et al., 2010). Further to this, reduced levels of TTP have been suggested to result in increased HIF-1 α in several cancers (Brennan et al., 2009, Kim et al., 2010). However, it should be noted that more recently a multilevel regulation of HIF-1 α by TTP has been described in a macrophage differentiation model, in which the mode of TTPs HIF-1 α regulation is changed upon phosphorylation stabilising HIF-1 α mRNA rather than destabilising (Fahling et al., 2012). In summary, TTP provides a potential explanation of the reduced steady state HIF-1 α mRNA levels following the inhibition of the PHDs. PHD depletion could result in an increase in levels of TTP described in hypoxia and in turn decreased levels of HIF-1 α mRNA. However, future work is needed to address this issue.

The regulation of HIF-2 α mRNA by hypoxia is less well described. A suggestion has been made that levels of HIF-2 α protein are maintained in hypoxia through the inhibition of iron regulatory protein (IRP1) dependent mRNA translation repression. (Zimmer et al., 2008). Thus, the reduction of HIF-2 α at the mRNA level by hypoxia observed in passaged OA HACs (**Figure 4.2B**) provides an avenue for future investigation. If HIF-2 α mRNA could be increased in hypoxia, potentially further increasing HIF-2 α protein levels, then beneficial effects of HIF-2 α observed here in OA HACs may be increased.

At the protein level only, HIF-1 α appeared to be affected by PHD knockdown in OA HACs, as PHD2 depletion increased levels of HIF-1 α in normoxia to approximately the levels observed in hypoxia alone (**Figure 4.3B**). However, PHD2 depletion does not appear to affect HIF-1 α protein levels in hypoxia. This result is in contrast to previous observations made in our group in healthy HACs in which PHD2 increased levels of HIF-1 α and HIF-2 α , most notably increasing protein levels of HIF-2 α in hypoxia above those typically observed in hypoxia alone (Thoms et al., 2013). These differences between healthy and OA HACs are likely due to a compensatory increase in PHD3 following PHD2 observed here in OA HACs which has not previously been observed in healthy HACs.

As previously discussed, the compensation of PHD3 in hypoxia following PHD2 depletion could offset the effects of PHD2 knockdown. It has been previously shown that PHD2 most prominently (compared to PHD1 and 3) regulates HIF-1 α (Appelhoff et al., 2004). Additionally, PHD2 depletion in adult mice leads to stabilisation of HIF-1 α (Takeda et al., 2007). Therefore, it is possible that PHD3 is regulating the levels of HIF-1 α in hypoxia such that a further increase due to the loss of PHD2 cannot be observed. Such effects have been previously described by Appelhoff et al. (2004) who found that individual PHD inhibition did not increase HIF-1 α protein levels in hypoxia, whereas, combined inhibition of the PHD isoforms resulted in increased HIF-1 α in hypoxia. Furthermore, the increased levels of PHD3 following PHD2 depletion in OA HACs could explain the difference in HIF-2 α stabilisation when compared to the effects observed in healthy HACs (Thoms et al., 2013).

Therefore, the lack of increased stabilisation of both HIF-1 α and HIF-2 α in PHD2 depleted OA HACs in hypoxia could potentially be caused by the compensation of PHD3 in following a reduction in PHD2 expression. This compensation was not previously observed in healthy HACs (Thoms et al., 2013) and could explain the differences in HIF levels following PHD2 knockdown observed between OA and healthy HACs.

In PHD2 depleted OA HACs HIF-1 α protein levels were stabilised in normoxia to levels comparable to control in hypoxia (**Figure 4.3**). However, no enhanced effect on the previously described HIF-1 α dependent increase in SOX9 in OA HACs, was observed at either the mRNA or protein level, in normoxia (**Figures 4.4-5**). Subsequently downstream targets of SOX9 (COL2A1 and AGGRECAN) are also not significantly affected by PHD knockdown. Results discussed in **Chapter 3** would predict that levels of SOX9 would increase with increased HIF-1 α levels. Given that the levels of HIF-1 α in normoxia, following the loss of PHD2, are similar to hypoxia it could be anticipated that SOX9 would also increase to levels similar to those observed in hypoxia. These results imply decreased HIF-1 α transcriptional activity in normoxia. The mechanism remains unclear, however, post-translational regulation of HIF-1 α is one possibility. Therefore, further consideration of post-translational regulation of HIF- α is given below.

As the potential regulation of enhanced HIF-1 α protein levels following PHD2 loss observed in OA HACs occurs in normoxia, it is important to consider the activity of factor inhibiting HIF-1 α (FIH). Similar to PHDs, FIH-1 is a 2-oxoglutarate dependent oxygenase that

is able to hydroxylate HIF- α subunits. FIH is able to use molecular oxygen available in normoxia to hydroxylate a conserved asparagine residue on the C-terminal transactivation domain (C-TAD) of HIF- α preventing binding to co-factor p300 thus inhibiting HIF- α transcriptional activity (Elkins et al., 2003, Freedman, 2002, Hewitson et al., 2002, Lando et al., 2002). Such an inhibition of transcriptional activity could explain the lack of SOX9 induction following increased HIF-1 α levels as a result of PHD2 loss in normoxia.

Interestingly, FIH has been shown to inhibit the activation of HIF-1 α target genes (Khan et al., 2011). Further to this Dayan et al. (2006) have shown that (in HeLa and LS174Tr cell lines) silencing either PHD2 or FIH led to the partial induction of hypoxic genes (such as PHD3), whilst combined inhibition of PHD2 and FIH resulted in a full hypoxic response. Dayan and colleagues hypothesise that this combined inhibition is required as HIF-1 α has two transcriptional activation sites N-terminal (NTAD) and C-terminal, of which FIH inhibits only C-TAD. Therefore, the suggestion is made that HIF-1 α can differentially express genes by either the C-TAD or N-TAD in response to hypoxia. Such a differential transcriptional activation could explain the lack of increased SOX9 response following a HIF-1 α increase in normoxia, where FIH is active and able to hydroxylate C-TAD. The effect of combined FIH and PHD2 depletion was previously investigated in our group in healthy HACs. Here it was observed that FIH depletion had no effect on SOX9 even when combined with PHD2 depletion (Thoms, 2011). However, a future investigation into the effect of FIH inhibition may be relevant in OA HACs given the differences in response to PHD depletion between healthy and OA HACs.

It has also been shown that HIF-1 α lacking C-TAD is able to activate transcription targets via interaction between N-TAD and the cysteine/histidine-rich region 3 (CH3) of co-factor p300 - such activation is described as an explanation for the transcriptional activity of HIF-1 α in normoxia (Ruas et al., 2010). Activity of HIF-1 α in normoxia is observed here in OA HACs by the reduction of HIF-1 α targets, such as SOX9, in normoxia following HIF-1 α siRNA transfection (**Figure 3.10-11**). A requirement of both N-TAD and C-TAD to maximally induce transcription targets could explain the lack of SOX9 induction following increased HIF-1 α when PHD2 is depleted, as enhanced HIF-1 α is only observed in normoxia where FIH will hydroxylate C-TAD.

The above indicates the need for future studies investigating the activity of FIH in OA HACs, the potential impact of C-TAD hydroxylation on HIF-1 α targets in normoxia and the effects of combined FIH/PHD depletion.

Additionally, in regards to post-transcriptional regulation of HIF- α , HIF-1 α can be regulated by RNA-binding proteins (RBP) such as tristetraprolin (TTP), which has been shown to be able to regulate HIF-1 α mRNA and thus protein (Kim et al., 2010). Furthermore, it has been shown that TTP is able to bind to the AREs in the 3'UTR of HIF-1 α mRNA in normoxia, affecting both mRNA stability and protein translocation (Fahling et al., 2012). Such reports indicate the importance of understanding the basal levels of TTP in normoxia as well as the described increase in hypoxia in both healthy and OA HACs. Another RBP of interest, as previously discussed, is human antigen R (HuR), which has been shown to affect the translation of HIF-1 α (Schulz et al., 2012, Galban et al., 2008). HuR is described as being able to stabilise HIF-1 α mRNA and protein and therefore, provides another example of the impact RBPs can have on the hypoxic response (Gorospe et al., 2011). Thus, a deeper understanding of the activity of RBPs in HACs could potentially allow for fine tuning of the HIF response through adaption of their activity to potential therapeutic benefit.

Following assessment of key cartilage genes after PHD depletion, potential effects of PHD1 (independent of HIF- α as PHD1 depletion did not affect HIF levels) have been identified. When PHD1 is reduced the mRNA levels of *COL2A1* and *ACAN* were notably reduced in normoxia (**Figure 4.4B-C**), whilst the mRNA level of *MMP13* is reduced following the knockdown of PHD1 in both normoxia and hypoxia in OA HACs.

The depletion of PHD2 has a notable increase on the mRNA levels of *MMP13* in normoxia. This is most likely due to the increased level of HIF-1 α in normoxia, following the loss of PHD2, as previous data in **Chapter 3** have shown that the reduction of *MM13* mRNA observed in hypoxia is dependent on HIF-1 α (**Figure 3.12B**). Importantly, no significant effect of PHD2 depletion on the mRNA level of *ADAMTS5* was observed in OA HACs. As PHD2 depletion has previously been described in our group as reducing *ADAMTS5* in healthy HACs (Thoms et al., 2013), this observation indicates another difference between the healthy and OA HACs.

Interestingly, individual depletion of all three PHD isoforms reduces the mRNA levels of *TIMP3* in hypoxia in OA HACs (although the effect was not statistically significant) (**Figure 4.6C**). Thus, suggesting that the PHDs are potentially able to regulate the levels of *TIMP3*. This data could therefore suggest a combined effect of HIF-1 α and the PHDs on the level of *TIMP3* mRNA. The loss of *TIMP3* mRNA following the depletion of HIF-1 α (**Figure 3.12C**) could be a combined effect of HIF-1 α loss and the reduction in PHD2 and PHD3 that also occurs as a result of reduced HIF-1 α (**Figure 3.9B-C**). This result could therefore suggest that combined the PHDs may be able to regulate *TIMP3* independently of HIF-1 α /HIF-2 α . This suggestion is further supported by the observation that the protein levels of HIF- α are unaffected by PHD inhibition in hypoxia, indicating that effects observed on *TIMP3* mRNA are not due to change in the level of HIF- α protein.

A possible explanation for these effects of PHD depletion (seemingly independent of HIF- α activity) is the potential ability of PHDs to regulate non-HIF targets. There is currently a suggestion in the community of novel functions of the PHDs either by hydroxylating non-HIF targets or a hypothesised non-hydroxylation related effect. In some cases, the HIF-independent effects of PHDs have been described as specific to an individual isoform (Cummins et al., 2006, Wong et al., 2013, Duran et al., 2013, Yang et al., 2014). Given the developing understanding of HIF-independent PHD effects and the indication of PHD dependent activity in the OA HAC, described in this discussion, future research into the potential PHD dependent targets may provide a greater understanding of differences observed between the hypoxic response of healthy and OA HACs.

In summary, individual depletion of the PHDs does not appear to be sufficient to enhance the typically observed hypoxic response in OA HACs. This inability of PHD isoform knockdown to enhance the hypoxic response (specifically SOX9 hypoxic induction) is a clear difference between the healthy and OA HACs. This difference being potentially influenced by RBPs, FIH or by variations in PHD independent of HIF in OA. The data here therefore indicate a potential requirement of combined hydroxylase inhibition in order to enhance the levels of HIF in OA. There is also an interesting avenue for future investigation exploring potential HIF-independent activity of PHDs in HACs.

4.4.2 General Hydroxylase Inhibition Enchases OA HACs Hypoxic Responses

DMOG acts as a general hydroxylase inhibitor which when used during OA HAC culture allows for the assessment of combined PHD and FIH inhibition. Assessment of HIF-1 α and HIF-2 α mRNA following the addition of DMOG revealed that general hydroxylase inhibition did not significantly change the mRNA levels in either normoxia or hypoxia (**Figure 4.7A-B**). This suggests that average reduction of HIF- α mRNA seen in passaged OA HACs is an effect independent of hydroxylase activity and most likely as a result of the previously discussed activity of RBPs such as TTP. However, individual depletion of PHD1 and PHD2 in normoxia was observed to reduce HIF-1 α mRNA levels. Compensatory activity of PHD isoforms, similar to those described by Minamishima et al. (2009), following the loss of either PHD1 or PHD2 regulating HIF- α mRNA stability in normoxia could potentially explain this effect.

HIF- α protein levels are enhanced above levels typically observed in hypoxia when cultured with 1mM DMOG in hypoxia in OA HACs (**Figure 4.8**). Notably, bands representing both HIF-1 α and HIF-2 α in samples treated with DMOG appear to be of a slightly higher molecular weight than those untreated in normoxia and hypoxia. The predicted molecular weight for HIF-1 α is 93kDa however, HIF-1 α and HIF-2 α western blot bands are routinely observed within the 100-120kDa range, as it is post translationally modified HIF un-degraded typically detected at 110kDa. The bands may therefore vary in molecular weight due to either; hydroxylation/ubiquitination or degradation of the protein (degradation resulting in a reduced molecular weight between 40-60kDa). In the case of the bands observed in **Figure 4.8**, the application of DMOG results in the inhibition of the activity of all hydroxylases (inclusive of the PHDs) and as a result HIF is likely to be detected in a non-hydroxylated/non-ubiquitinated form. It is this change which is likely to have slightly altered the travel time during gel electrophoresis/molecular weight of the protein detected in samples treated with DMOG. Additionally, the typically observed non-specific band present below HIF-2 α (**Figures 3.2, 3.8, 4.3 and 4.8**) does not appear to be linked to the concentration of HIF-2 α itself, unspecific bands can be observed to have high intensity in the absence of a specific band as well as when a strong specific signal is detected (**Figure 4.8**), suggesting that the variation of the unspecific band is unrelated to the level of HIF-2 α detected.

In regards to HIF- α targets, DMOG on average, enhances the typically observed hypoxic response of known targets such as *PHD3* (**Figure 4.9**), *ADAMTS5* (although not statistically significant **Figure 4.11A**), *MMP13* (**Figure 4.11B**) and importantly *SOX9* mRNA and protein (**Figure 4.10-11**). This enhancement of *SOX9* levels above that typically observed following hypoxia alone is most likely due to the increased stabilisation of HIF- α as a result of DMOG. As previously stated in **Chapter 3**, results here indicate that the increase in *SOX9* in hypoxia in OA HACs is primarily HIF-1 α dependent, with the suggestion of a potential contribution of HIF-2 α although with a potentially reduced effect. It is important to note that no significant effect of DMOG on the downstream targets of *SOX9*, *COL2A1* and *ACAN* is observed (**Figure 4.10B-C**) - such an effect would typically be expected given the enhanced levels of *SOX9*.

The results discussed here are similar to previous observations made by our group when culturing healthy HACs with DMOG. Culture with DMOG resulted in enhanced levels of *SOX9* in hypoxia in healthy HACs. It was also noted that DMOG-induced upregulation of *SOX9* did not result in the increase of cartilage-specific collagens such as *COL2A1* in healthy HACs. The effect of DMOG was investigated further and our group was able to show that culture with DMOG inhibited the secretion of type II collagen into the culture media (Thoms and Murphy, 2010).

The lack of increased *COL2A1* and the previously described inhibition of type II collagen secretion in healthy HACs (Thoms and Murphy, 2010) is likely due to the general hydroxylase inhibition caused by DMOG. DMOG, as previously described, is a 2-oxoglutarate analogue which will inhibit all members of iron, 2-oxoglutarate dependent hydroxylases. Included in this family of hydroxylases inhibited by DMOG are the collagen prolyl-4-hydroxylases (C-P4Hs). C-P4Hs are responsible for post translational processing of collagens. C-P4Hs catalyse the formation of 4-hydroxyproline by hydroxylation of prolines in the -X-Pro-Gly- sequences in collagens. 4-hydroxyproline residues are essential for the formation of the collagen triple helix and furthermore the subsequent secretion of collagens (Myllyharju, 2003). As a result of C-P4H inhibition, DMOG results in unhydroxylated procollagen chains which are retained by cells for intracellular degradation (Berg et al., 1983). Similar observations to those made here, concerning the effect of DMOG on *COL2A1*, in OA and previously in healthy HACs, have been made in mice. Gelse et al. (2008) found that murine chondrocytes cultured with DMOG showed enhanced levels of *SOX9* whilst having no

induction of *COL2A1* mRNA. Further to the described effects of DMOG on C-P4Hs above, Gelse and colleagues also reported an intracellular accumulation of type II collagen which they describe as most likely being due to impaired collagen processing interfering with collagen secretion. Therefore, whilst DMOG provides a crude method to assess the effect of general hydroxylase inhibition a more selective approach, such as specific PHD inhibitors, will be required to fine tune the hypoxic response in OA HACs.

The increased stabilisation of both HIF- α subunits and HIF- α target SOX9 following culture with DMOG indicates an ability to potentially enhance cartilage production (through the activity of SOX9) via inhibition of hydroxylase activity. The potential implied benefit of enhanced SOX9 is further supported by the findings of Zhang et al. (2015b). Zhang and colleagues have shown that elevated levels of SOX9 can mediate the suppression of ADAMTS5 in early OA, suggesting both an anabolic and anti-catabolic effect of enhanced SOX9 is possible in OA. However, it is important to consider here that the results following culture of OA HACs with DMOG represent the inhibition of all PHD isoforms and FIH. Whilst it has been suggested that the activity of both the PHDs and FIH should be considered for the therapeutic regulation of HIF target genes (Chan et al., 2016) it will first be important to understand the effects of combined PHD inhibition and the inhibition of FIH individually. Manipulating the hypoxic response and the activity of HIF to produce an enhanced chondrocyte phenotype remains possible in OA HACs as indicated by the effects of DMOG. However, to achieve this could potentially require the inactivation of FIH alone, the inhibition of select PHDs via specific inhibitors or a combined FIH and select PHD inactivation. Therefore, further research in OA HACs into the activity of the PHDs and FIH should be conducted to understand the HIF- α independent and HIF- α dependent contributions of these hydroxylases. Such an understanding could provide insight into appropriate targets to enhance the beneficial hypoxic response of healthy and OA HACs to potential therapeutic benefit.

Interestingly, *TIMP3* mRNA levels are on average reduced (although significantly only in normoxia) when cultured with DMOG (in both normoxia and hypoxia) in OA HACs (**Figure 4.12C**). This data following general hydroxylase inhibition with DMOG correlates with the previously described effect of PHD depletion on mRNA levels of *TIMP3* (**Figure 4.6C**), suggesting a partially PHD dependent regulation of *TIMP3*. Future research into the expression of *TIMP3* protein in OA HACs following both HIF-1 α and hydroxylase inhibition should be conducted to further explore this proposed PHD dependent effect on *TIMP3* in OA HACs.

In summary DMOG indicates combined hydroxylase inhibition can produce an enhanced hypoxic response in OA HACs. Results indicate that such an enhancement could increase cartilage production (via SOX9) and increase the protection provided by hypoxia (through reduction of metalloproteinases *MMP13* and *ADAMTS5* (**Table 4.1**)). However, as has been previously suggested by our group due to the general (non-specific) hydroxylase inhibitory activity of DMOG, inhibitors specific to HIF targeting hydroxylases will be necessary in order to carefully modulate HIF levels. Therefore, future investigation into the effects of PHDs, such as those potentially independent of HIF α described in this chapter, will likely be essential. Whilst results of culture with DMOG suggest a potentially beneficial effect of combined inhibition of the PHDs, in terms of HIF response, inhibition of the PHDs could produce unintended effects as a result of previously unknown HIF- α independent activity of the PHDs in OA HACs.

Table 4.1 Summary of Chapter 4

PHD Isoform	PHD1	PHD2	PHD3	PHD1/2/3 (DMOG)
Effect of PHD Isoform Depletion	<i>MMP13</i> ↓	PHD3 (Protein Only) Compensatory Increase See Chapter 4.4.1 ↑	N/A	HIF-1 α (Protein) ↑
	<i>COL2A1</i> (Normoxia Only) ↓	HIF-1 α (Protein Only) (Normoxia Only) ↑		HIF-2 α (Protein) ↑
				SOX9 (mRNA + Protein) ↑
				<i>ACAN</i> ↑
				<i>MMP13</i> ↓
				<i>TIMP3</i> ↓

Chapter 5

End Stage OA Explant Experiments

5.1 Introduction

It has previously been shown in healthy human cartilage explants that hypoxia reduces the levels of key catabolic enzymes including ADAMTS5 (Thoms et al., 2013). By assessing the release of aggrecan fragments into explant culture media, Thoms and colleagues have shown that the anti-catabolic response of hypoxia in healthy human cartilage is able to inhibit the spontaneous and IL-1 α induced degradation of aggrecan. Therefore indicating that, the anti-catabolic effect of hypoxia in healthy human cartilage is able to reduce levels of cartilage matrix destruction.

As the results of **Chapter 3** indicate an anti-catabolic capability of osteoarthritic (OA) human articular chondrocytes (HACs), in this chapter the potential of hypoxia to inhibit spontaneous and IL-1 β induced aggrecan degradation in OA cartilage explants is investigated. Aggrecan degradation in human OA cartilage explants can be measured by the release of degraded aggrecan fragments (ARGSV neoepitope) into explant culture media and by visualising proteoglycan content remaining in the explant through histological staining (Safranin O).

The degradation of aggrecan is caused by the cleavage of the core protein by aggrecanases (ADAMTSs such as ADAMTS5). Cleavage of aggrecan by aggrecanases occurs within the first interglobular domain between the Glu³⁷³-Ala³⁷⁴ amino acid bond. This cleavage results in the production of the N-terminal neoepitope ARGSV (Tortorella et al., 2000, Song et al., 2007, Troeberg and Nagase, 2012). The N-terminal fragment or ARGSV is released from the cartilage explant following aggrecan cleavage and can then be detected in the culture medium by antibodies which recognise ARGSV neoepitope via western blotting. The amount of detected ARGSV in OA explant culture medium is used to measure the relative amount of aggrecan degradation (Larsson et al., 2009).

In this chapter it is reported that the results of investigation into the potential inhibition of aggrecan degradation via the hypoxic response of OA HACs are inconclusive. The contents of this chapter will identify and discuss the key issues encountered when assessing the proteoglycan content of end stage OA human cartilage.

5.1.2 Intended Chapter 5 Specific Aims and Hypothesis

- 1) Identify whether hypoxia protects OA cartilage from spontaneous aggrecan degradation**
- 2) Identify if hypoxia can protect against aggrecan degradation with OA cartilage is challenged with IL-1 β**

In this chapter the effects of a hypoxic culture and challenge with IL-1 β on OA cartilage explants are investigated. It is hypothesised that a hypoxic setting will protect OA cartilage from spontaneous and IL-1 β driven aggrecan degradation, although to a lesser degree than previously observed in healthy cartilage explants.

5.2 The Effect of Hypoxia and IL-1 β Stimulation on Human OA Cartilage Explants

In this chapter the effect of hypoxia on aggrecan degradation is investigated. Initially to investigate the effects of hypoxia in human osteoarthritic (OA) articular cartilage, explants were cultured in 20% oxygen (normoxia) and 5% oxygen (hypoxia) stimulated with IL-1 β (1ng/ml or 10ng/ml) or unstimulated. Explants were cultured for a rest period (unstimulated) of 24 hours in 20% oxygen and 5% oxygen followed by 24 hours with stimulation (including control) in the same oxygen tension as the rest period. Explants were then fixed and culture media collected for further use.

Aggrecan content was measured using two methods – firstly, using histology to stain explants with safranin O visualising the proteoglycan content within the explant, where a red stain represents the presence of proteoglycan and the absence of staining indicating a potential loss of proteoglycan content. Secondly, western blot was used to detect aggrecan fragments released from explants using an antibody which recognise ARGSV neoepitope. OA cartilage explants were obtained from 8 OA patients (donors include 3 male and 5 female; age range 57-78 years, mean age 69.1 years) with each set of explants taken from the same larger piece of cartilage.

Initial histological staining appeared to show no noticeable differences in safranin O staining between oxygen tensions in multiple biological replicates. In the first set of explants less staining could be observed at the articular surface of the explants in each IL-1 β stimulated condition compared to control, reduced staining here suggests a potential loss of proteoglycans following IL-1 β stimulation. When comparing between oxygen tensions the amount of staining appeared to be similar indicating no effect of explant culture in hypoxia (**Figure 5.1**). In another experiment a similar result was observed, with decrease in staining between control and both IL-1 β concentrations. There was potentially a slight decrease in the presence of staining in control at 5% oxygen compared to 20% otherwise the level of staining was again comparable at the different oxygen tensions (**Figure 5.2**). Therefore the staining of these initial experiments suggest that hypoxic culture has no effect on the loss proteoglycan content in human OA explants.

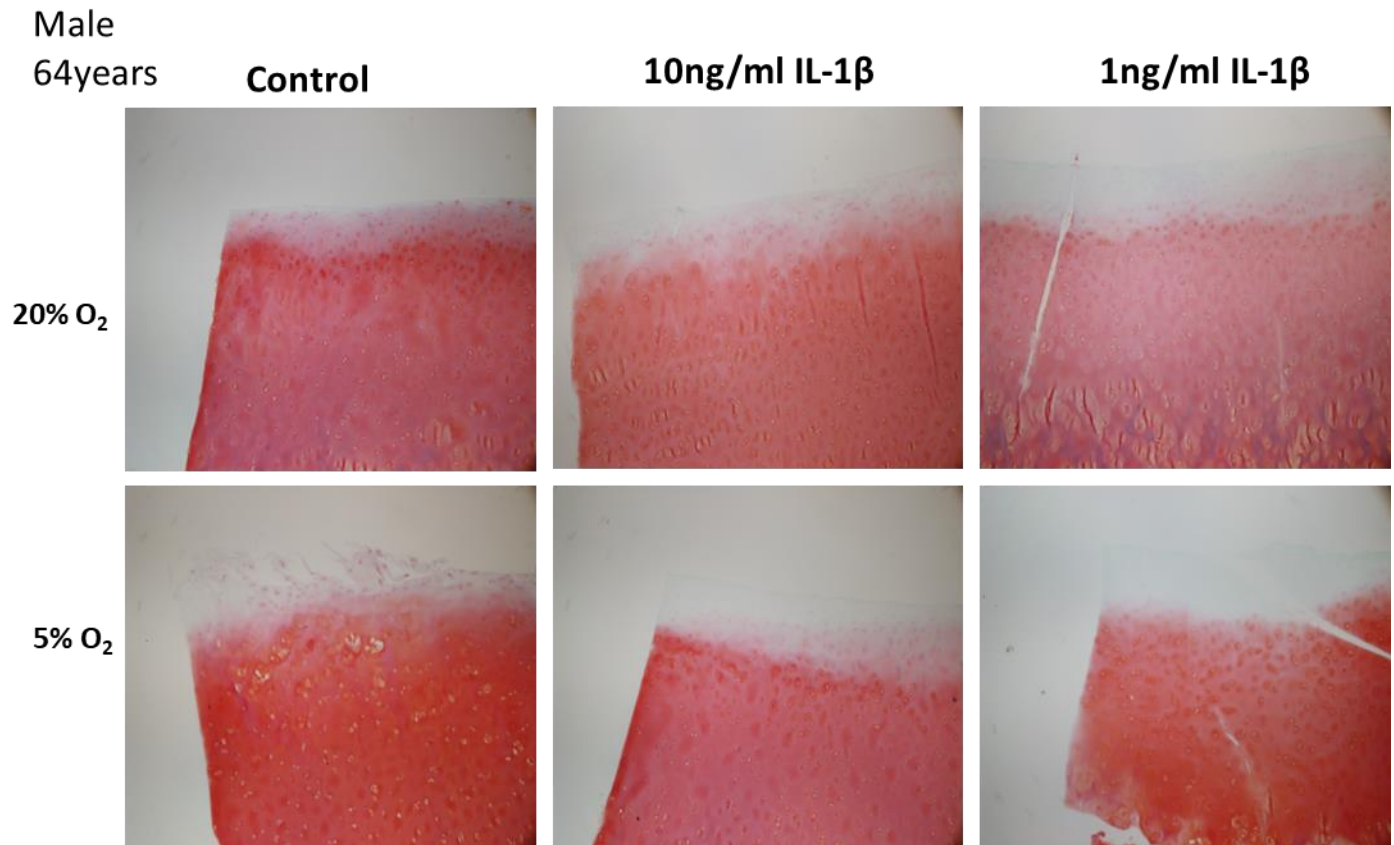


Figure 5.1 IL-1 β induced loss of proteoglycan is comparable between high and low oxygen tensions. Human OA articular cartilage explants were cultured in serum-free DMEM for a 24 hour rest period followed by 24hours with or without (control) IL-1 β . Explants were cultured at either 20% O₂ (Oxygen) or 5% O₂. Explants were then fixed and stained with Safranin O to visualise proteoglycans (red staining).

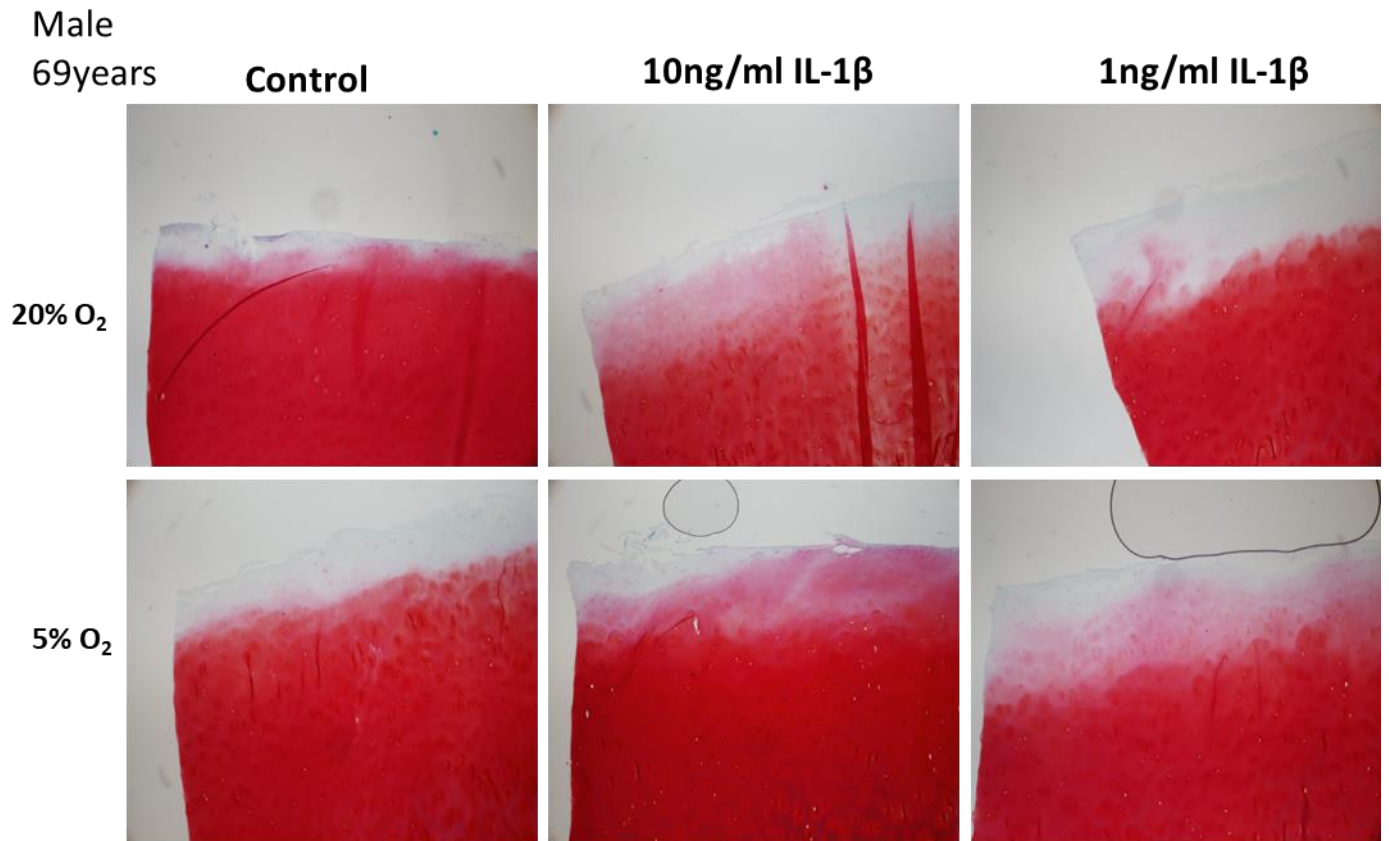


Figure 5.2 IL-1 β induced loss of proteoglycan is comparable between high and low oxygen tensions. Human OA articular cartilage explants were cultured in serum-free DMEM for a 24 hour rest period followed by 24hours with or without (control) IL-1 β . Explants were cultured at either 20% O₂ (Oxygen) or 5% O₂. Explants were then fixed and stained with Safranin O to visualise proteoglycans (red staining).

However another set of explants appeared to show decreased safranin O staining in hypoxia compared to normoxia indicating a loss of proteoglycans in hypoxia. Similar to the aforementioned, in these explants less staining can be observed between control and IL-1 β stimulated conditions, with staining further reduced at higher concentration of IL-1 β indicating greater proteoglycan loss. Whereas in contrast to previous experiments it can be observed that these explants show reduced staining in each condition in hypoxia when compared to explants cultured in normoxia (**Figure 5.3**).

Further replicates began to show further inconsistencies both within experimental conditions and between biological replicates. Initially a set of explants appeared to show more staining in hypoxia compared to normoxia suggesting greater proteoglycan content in explants cultured in hypoxia. When comparing control in 20% oxygen to 5% oxygen it could be observed that there was a less staining in 20%, although it was noted that the control explant in 5% had an unusually smooth surface. The IL-1 β stimulated explants also gave a strange result as the lower concentration of IL-1 β have less staining than IL-1 β at higher concentrations. In this case, similar to the control explants, the 10ng/ml IL-1 β explants showed little to no loss of staining whilst having an unusually smooth surface (**Figure 5.4**). Another set of explants had interestingly shown reduced staining in control compared to the IL-1 β challenged condition indicating greater proteoglycan content in the presence of IL-1 β . However explants in this set also had an unusually smooth surface when either no or very little reduction in staining could be observed such as IL-1 β 1ng/ml in 20% oxygen and IL-1 β 10ng/ml in 5% oxygen (**Figure 5.5**).

A final set of IL-1 β stimulated explants provided a potential explanation of inconsistencies within and between biological replicates. In this set there was again an explant with an unusually smooth surface (Control 20% oxygen) and what appears to be a decreased staining in 20% oxygen compared to 5% oxygen. However an interesting observation was made on the control explant at 5% oxygen, here the surface of the explant appeared to show signs of delamination. It appeared that the explant was losing a portion of the surface where little staining was present (**Figure 5.6**). This delamination could potentially explain the smooth explant surfaces which had little to no loss of staining that were observed in previous biological replicates.

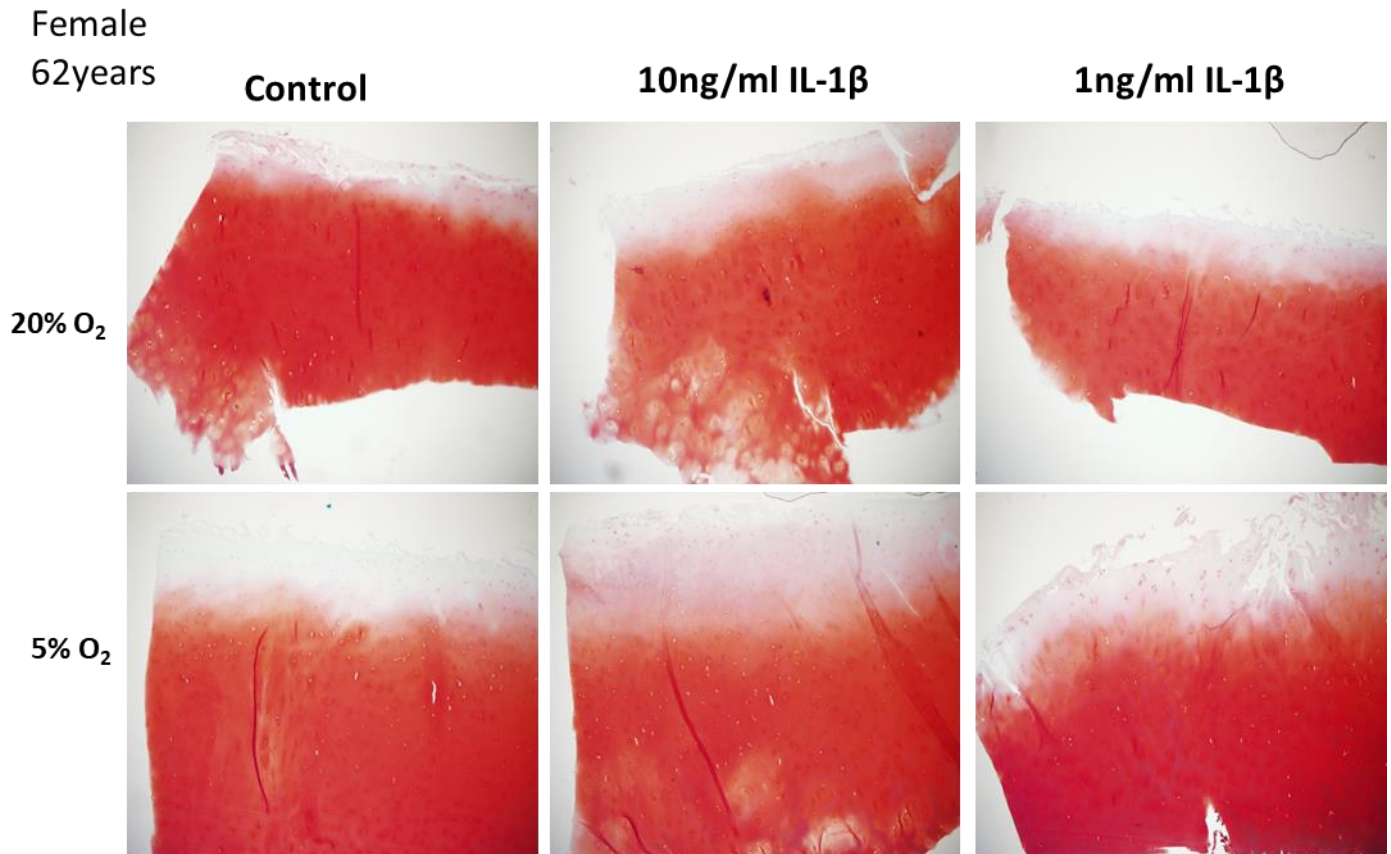


Figure 5.3 Increased loss of proteoglycan in explants at 5% O₂ compared to 20%O₂. Human OA articular cartilage explants were cultured in serum-free DMEM for a 24 hour rest period followed by 24hours with or without (control) IL-1 β . Explants were cultured at either 20% O₂ (Oxygen) or 5% O₂. Explants were then fixed and stained with Safranin O to visualise proteoglycans (red staining).

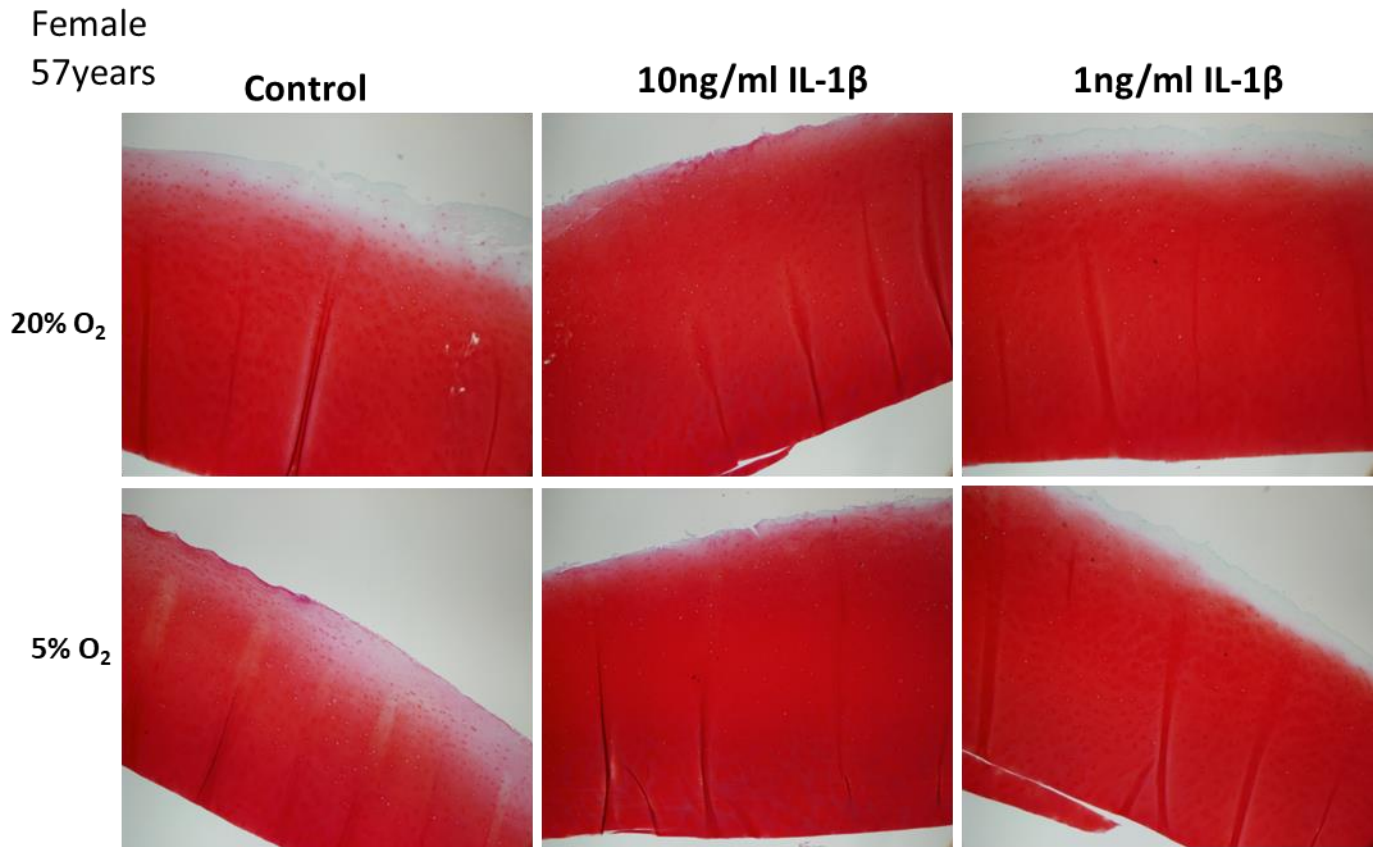


Figure 5.4 Less staining in Control and 1ng/ml IL-1 β at 20% O₂ than in 5%O₂. Human OA articular cartilage explants were cultured in serum-free DMEM for a 24 hour rest period followed by 24hours with or without (control) IL-1 β . Explants were cultured at either 20% O₂ (Oxygen) or 5% O₂. Explants were then fixed and stained with Safranin O to visualise proteoglycans (red staining).

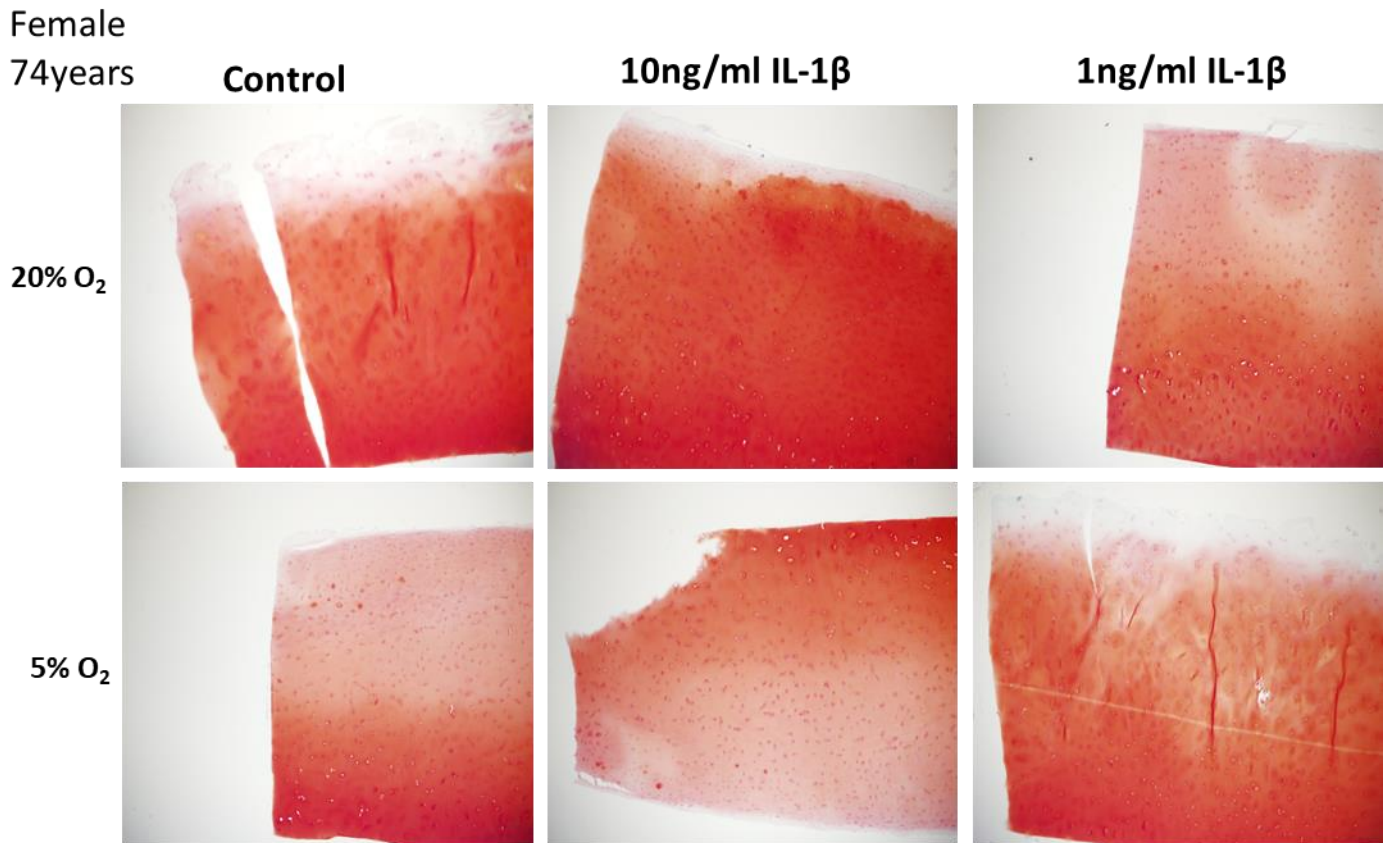


Figure 5.5 OA cartilage surface appears smooth following IL-1 β in 20%O₂ and following 10ng/ml IL-1 β in 5%O₂. Human OA articular cartilage explants were cultured in serum-free DMEM for a 24 hour rest period followed by 24hours with or without (control) IL-1 β . Explants were cultured at either 20% O₂ (Oxygen) or 5% O₂. Explants were then fixed and stained with Safranin O to visualise proteoglycans (red staining).

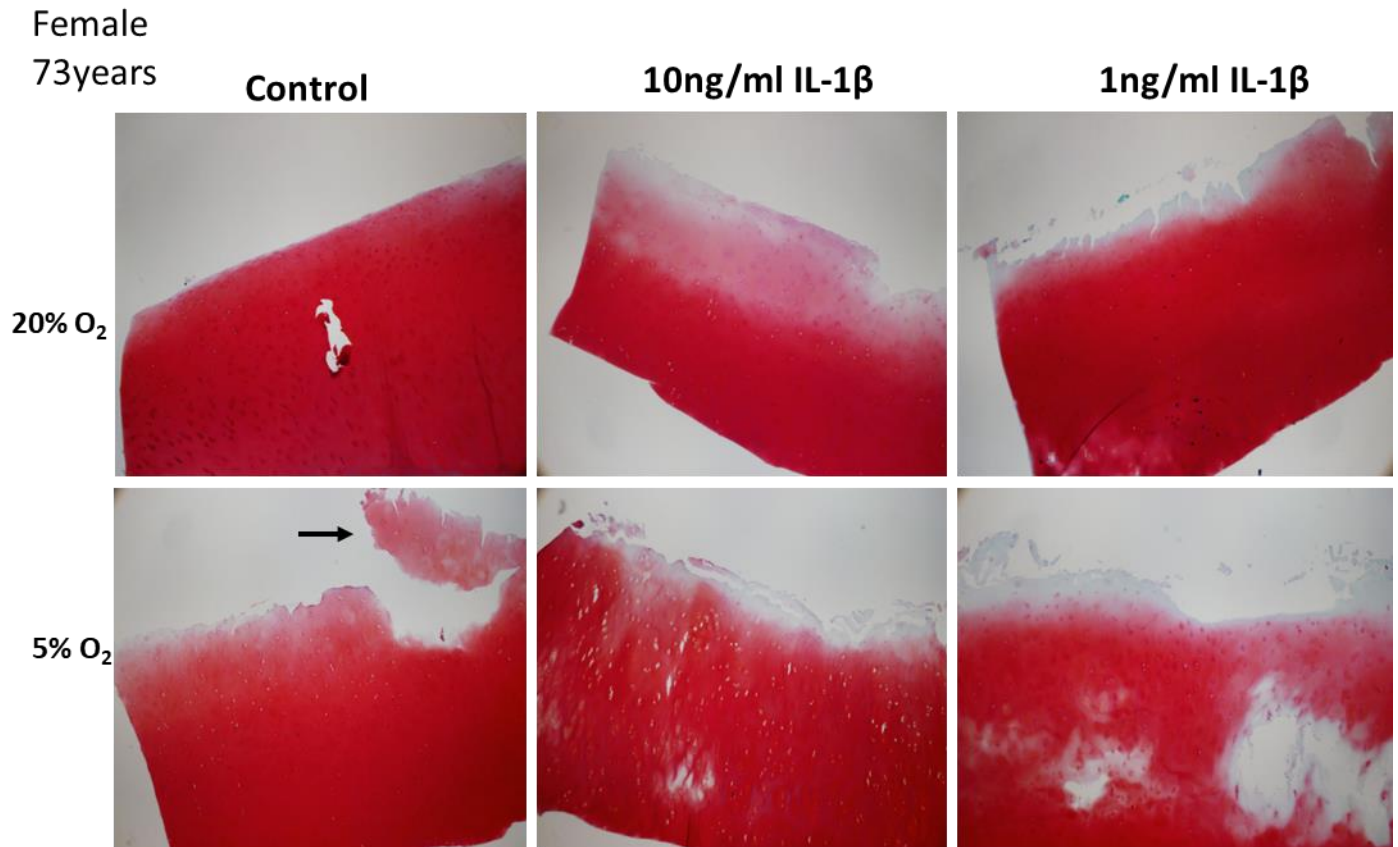


Figure 5.6 Delamination of OA explant surface. Human OA articular cartilage explants were cultured in serum-free DMEM for a 24 hour rest period followed by 24 hours with or without (control) IL-1 β . Explants were cultured at either 20% O₂ (Oxygen) or 5% O₂. Explants were then fixed and stained with Safranin O to visualise proteoglycans (red staining). Delamination is indicated by the black arrow in the bottom left panel.

In order to investigate the possibility that delamination was affecting the interpretation of the histological results, a set of explants were cultured without IL-1 β stimulation. Instead explants were cultured for 24 hours and 48 hours in normoxia and hypoxia (5% and 20% oxygen) with a 0hr explant taken to compare cultured explants surface to a freshly dissected explant.

In these explants reduced staining can be observed at 24 hours compared to 48 hours, in the case of control 48 hours the staining appears to be comparable to the 0hr explant. However the surface of the explants following 48 hours of culture are smooth and do not have the fibrillated appearance at the surface that can be seen on the 0hr explant. Delamination can also be observed in this experiment, on the 24 hour 20% oxygen explant a large section of the surface with little safranin O staining can be seen lifting away from the explant (**Figure 5.7**). This observation supports delamination of the damaged cartilage surface as the potential explanation for inconsistency between biological replicates. Such inconsistency when culturing these explants makes using histology to provide meaningful insight into the effects of hypoxia on proteoglycan content of human OA cartilage extremely difficult.

The western blots show the release of ARGSV (representing amount of aggrecan degradation) into the rest media and conditioned media, rest media meaning media from the first 24 hours of culture where the explants were unstimulated with IL-1 β . It has been noted on the rest media section of the blots which lanes represent the media from explants that would go on to be IL-1 β stimulated for later comparison. The results of the western blots show a considerable amount of inconsistency in regards to the released ARGSV detected (**Figure 5.8**).

For instance **A-B** show the most intense bands indicating increased ARGSV release in 5% oxygen, with more intense bands when comparing unstimulated lanes. Additionally, a greater amount of ARGSV was detected in IL-1 β stimulated lanes (5% O₂) suggesting that no protection is offered by hypoxia. Further to this in blot **A**, a band of high intensity can be observed in the rest media where the explant is not stimulated by IL-1 β therefore band intensity (ARGS release) should be theoretically even. Whereas the intensity of the bands in blot **C** suggest that there is an increased release of ARGSV in 20% oxygen when explants are not stimulated and most notably when stimulated with 1ng/ml IL-1 β . Finally, blots **D-E** band intensity suggest a generally inconsistent release of ARGSV from explants, both indicating increased release from unstimulated explants in 5% oxygen whilst also showing a decrease of

ARGSV release in 10ng/ml IL-1 β when compared to 20% oxygen in the absence of IL-1 β (**Figure 5.8**).

The general inconsistency in the intensity of bands indicating ARGSV release from explants, and therefore aggrecan degradation within explants, make interpreting any effects of hypoxia or IL-1 β stimulation inappropriate in this case and potentially when using human OA cartilage explants in general.

To investigate the potential leaching of proteoglycans and ARGSV fragments pre-existing in the human OA cartilage explants, into the media a set of acellular (Dead) explants were generated. These explants were subjected to five freeze/thaw cycles, alternating the temperature of the explants between -80°C and 37°C. The 'dead' explants were then cultured in varying oxygen tensions (20%, 5% and 1% oxygen) for either 24 hours or 48 hours at 37°C. A 0hr explant was also taken, 0hrs being immediately following the freeze/thaw cycles. Following culture the explants were fixed and histologically stained with safranin O, culture media was also taken for analysis of ARGSV neoepitope release (as previously described).

The histological staining of the explants show a reduction in staining between 24 and 48 hours in both 20% and 5% oxygen. Whilst the explant cultured at 5% oxygen for 24 hours has the same unusually smooth surface (as previously described), the reduced staining may suggest that there is a loss of proteoglycan content from 'dead' OA explants (**Figure 5.9**). The western blot for this experiment shows bands representing ARGSV after 24 and 48 hours culture of the 'dead' OA explant. This ARGSV content released into the media also potentially indicates the leaching of ARGSV fragments, pre-existing in the 'dead' cartilage explant, into the culture media (**Figure 5.10**).

In summary the inconsistencies observed in analysis of human OA cartilage explants by both histology of the explant and analysis of culture media content by western blot make drawing specific conclusions very difficult. The delamination of the explant surface and the potential leaching of proteoglycans from the explant suggest that such end stage human OA explants may not be suitable material to infer information about the effect hypoxia may be having in a human OA setting.

Male
78years

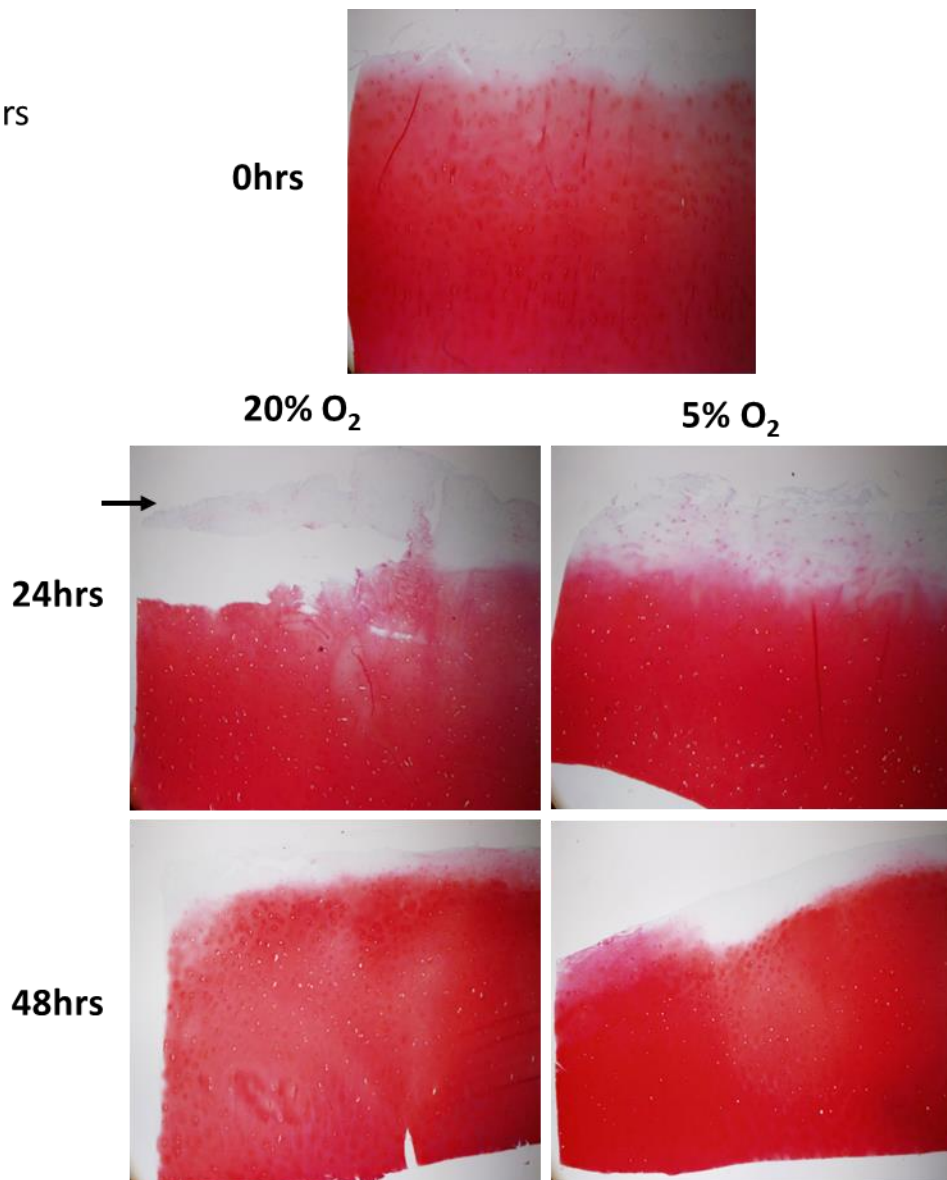


Figure 5.7 Delamination of OA explant surface. Human OA articular cartilage explants were cultured in serum-free DMEM for 24 or 48 hours (0hrs fixed immediately following explantation) at 20% and 5% oxygen (no rest period prior to culture). Explants were then fixed and stained with Safranin O to visualise proteoglycans (red staining). Delamination is indicated by the black arrow in the top left panel.

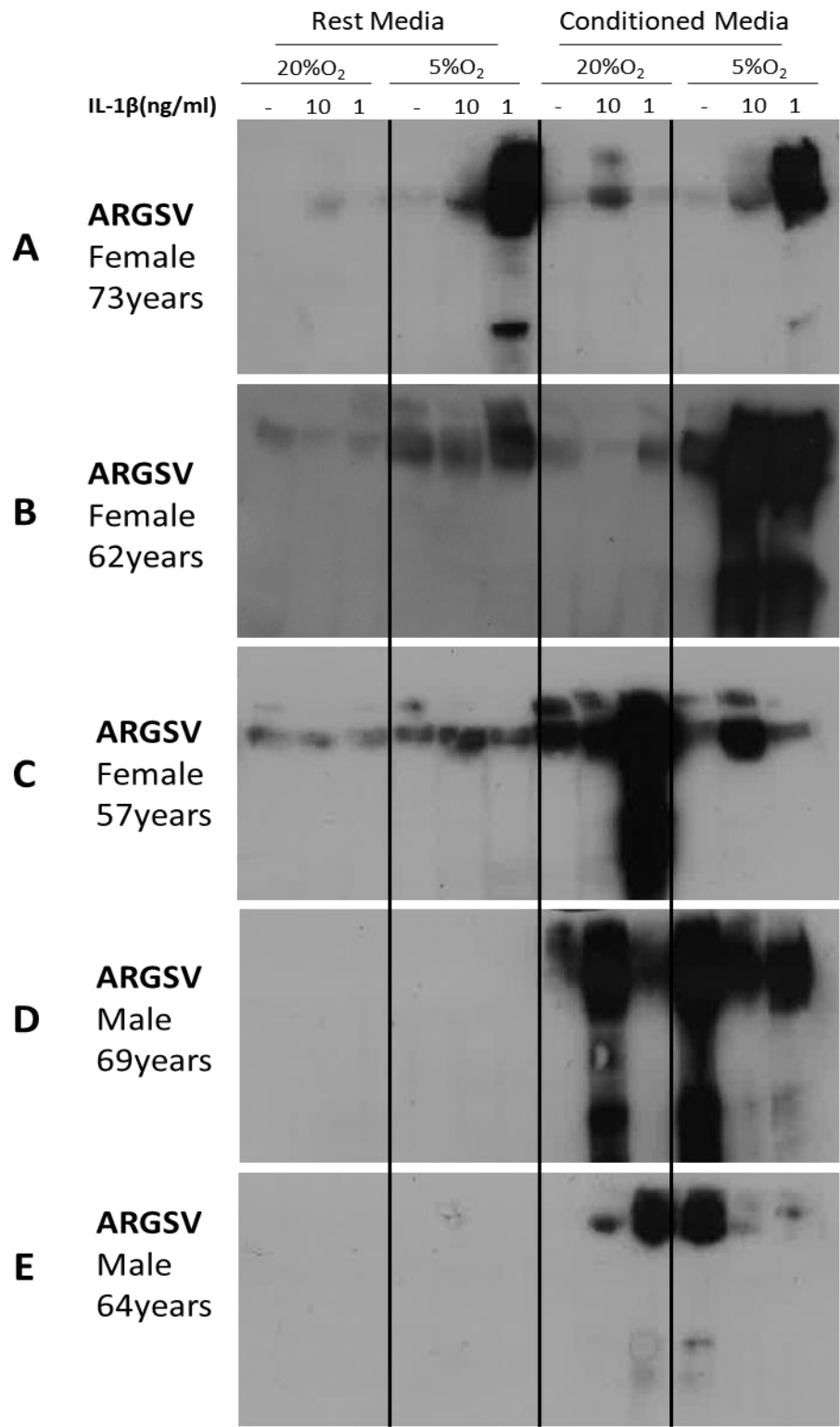


Figure 5.8 Inconsistent ARGSV release across experimental conditions. Media collected from human OA articular cartilage explants cultured in serum-free DMEM for a 24 hour rest period (rest media) followed by 24 hours with or without IL-1 β (1 or 10ng/ml) (conditioned media). Explants were cultured at either 20% O₂ (Oxygen) or 5% O₂. Rest media lanes indicate which explants would go on to be IL-1 β stimulated, no media during the rest period contained IL-1 β . Western blotting shows levels of ARGSV released from explants into the culture media. Western blots representative of n=5 Note: Con – control yr – years old Molecular Weight 50-250kDa

'Dead' Freeze/Thaw
OA Explant
Female 76years

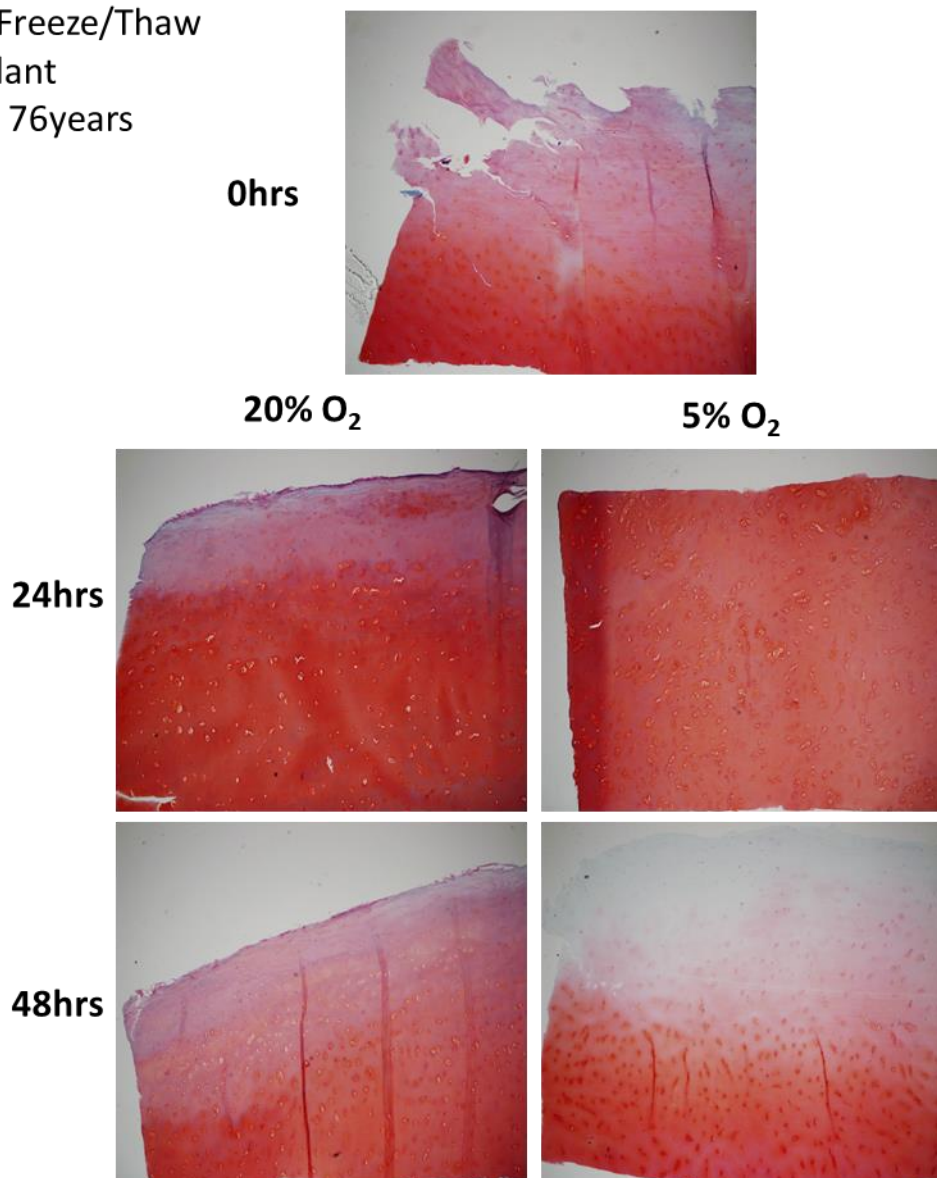


Figure 5.9 Potential leaching of proteoglycans from 'dead' explant. Human OA articular cartilage explants were freeze/thawed and then cultured in serum-free DMEM for 24 or 48 hours (0hrs fixed immediately following explantation) at 20%, 5% or 1% oxygen. Explants were then fixed and stained with Safranin O to visualise proteoglycans (red staining).

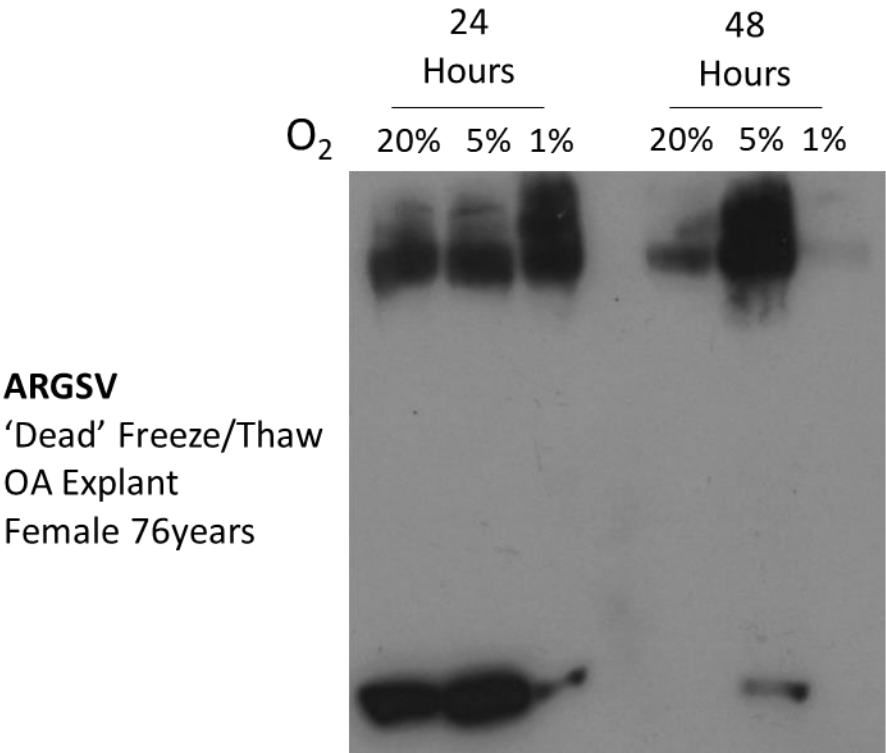


Figure 5.10 Levels of ARGSV released into media from ‘dead’ explant. Media collected from human OA articular cartilage explants Human OA articular cartilage explants were cultured in serum-free DMEM for 24 or 48 hours at 20%, 5% or 1% oxygen. Western blotting shows levels of ARGSV released from explants into the culture media.

5.3 Discussion

The purpose of the work in this chapter was to identify whether hypoxia is able to provide a protective effect on OA cartilage using explants. It has previously been observed in our group that, in healthy human cartilage explants, hypoxia inhibits spontaneous cartilage degradation (assessed by ARGSV release). Crucially it has been described that, in healthy human cartilage, hypoxia is able to inhibit the increased cartilage degradation that is observed with the addition of IL-1. This protective effect of hypoxia was observed in both human and porcine healthy cartilage (Thoms et al., 2013). Therefore, to elucidate potential effects of hypoxia in OA cartilage explants, the proteoglycan content remaining within the OA cartilage (by histology) and the degraded ARGSV fragments released into the media (by western blot) were assessed following culture in either normoxia or hypoxia when challenged/unchallenged by IL-1 β .

However, as described in the results (**Chapter 5.2**), several inconsistencies were observed in both the results of histological analysis of proteoglycan content in OA explants, and the analysis of aggrecan fragments released into the media by OA explants. Such inconsistencies, discussed below, make drawing conclusions on the effect of hypoxia on human OA cartilage inappropriate in these experimental conditions.

5.3.1 Delamination of End Stage OA Cartilage Explants

Histological analysis via Safranin O staining of proteoglycans on human OA explants was used to assess the proteoglycan content of the explant following culture. However, results observed were inconsistent, with levels of safranin O staining varying between donors in both control and experimental conditions. Surprisingly, in multiple OA explant sections, the observation was made that the surface of the explants was smooth and did not have the typical fibrillated/uneven appearance, such as the surface observed in 0 hour OA cartilage explants (**Figure 5.7**). A fibrillated/uneven articular surface is a common feature of OA cartilage (Meachim, 1972, Piperno et al., 1998, Tummala et al., 2011, Stockwell, 1991) and is a criterion for the histopathological grading of OA in cartilage. It is also observed that the cellular organisation of explants with a smooth tissue surface differs from explants with a fibrillated surface. It can be observed that the flattened cell shape, typically observed at the articular

surface (Sophia Fox et al., 2009), is absent in OA cartilage explants with a smooth surface; instead the chondrocyte's appearance is more typical of mid-zone cartilage.

The osteoarthritis research society international (OARSI) histopathology grading system is commonly used to score cartilage for the determination of OA severity. The OARSI score is produced by assessing the severity/progression OA (grade) and the extent of cartilage involvement throughout its depth (score). The OARSI score is therefore a combined assessment of the severity and extent of OA. The grade of OA is described as a key feature with associated criteria. The OARSI grading system describes the observation of surface discontinuity and fibrillation of the cartilage surface as criteria required for the initial grades of OA (Pritzker et al., 2006). These criteria therefore support the suggestion that the smooth cartilage surface observed in the OA cartilage explants used in the above experiments are atypical. The OA cartilage used in this study was not OARSI scored, as cartilage was sourced from donors clinically diagnosed with advanced/end stage OA undergoing joint replacement.

Importantly, it should be noted that explants used in this experiment were taken in parallel from a single piece of full thickness OA cartilage. Therefore, the severity of OA observed should be similar between each explant within an individual donor cartilage sample. Thus, the observation that some OA explants, within an individual donor sample, have a smooth cartilage surface whilst other explants have a typically fibrillated surface indicates a potential loss of the fibrillated tissue surface.

The suggestion is made above that the surface of the end stage OA cartilage has delaminated in several explants. Examples of such delamination in action are noted in the results (**Figure 5.6-7**). It is believed that explants sections which have an atypical smooth surface, also absent typical cell organisation, have lost a surface fragment through delamination. Supporting this suggestion is the OARSI histopathology grading system in which delamination is described a criteria of grade 4 (Pritzker et al., 2006). Pritzker et al. (2006) describe delamination as the loss of a superficial zone fragment being caused by a tissue interface with different mechanical compliance (soft/hard) caused by degraded cartilage matrix adjacent to intact matrix. It is suggested that crack propagation begins and as it expands makes the degraded matrix fragment less mechanically stable generating a matrix reaction at the edge of the interface. This ultimately leads to the extension of the cracks formed dislodging the degraded matrix fragment (delaminating). Finally, Pritzker and colleagues state delamination

is recognised by the relatively smooth cartilage surface with subjacent tissue exhibiting mid zone cartilage organisation. This description of delamination in the OARSI grading system supports the suggestion here that fragments of the OA explant surface were lost through delamination. The delamination of the OA explant surface could have occurred either during culture or following culture during processing for histological staining.

Firstly, addressing the suggestion that delamination of the OA cartilage explant surface could occur during culture. When observing the Safranin O staining of 0hr OA explants, reduced staining and thus, a loss of proteoglycan content at the tissue surface is observed (**Figure 5.7**). Following 24 hours of culture, in several oxygen tensions without the addition of IL-1, the proteoglycan content (indicated by safranin O staining) is reduced further, although a fibrillated surface of the explant is still observed. However, the explant cultured in 20% O₂ appears to be delaminating, losing the cartilage surface which has less positive staining for proteoglycans. Finally, following 48 hours of culture the cartilage explants appear to have greater proteoglycan content than explants cultured for 24 hours as indicated by safranin O staining. However, these explants are observed to have a smooth un-fibrillated surface suggesting the surface is lost between 24 and 48 hours of culture likely due to degradation of the tissue. If this suggestion is accurate it would suggest that the tissue surface could have been lost when OA explants were cultured for a total of 48 hours (rested for 24 hours before 24 hours of culture with or without IL-1 (**Figures 5.1-6**)).

However, if the OA tissue surface delaminated prior to the 24-hour experimental culture period then an effect of IL-1, increasing degradation of the cartilage and loss of proteoglycans (Flannery et al., 1999, Hubbard et al., 1988), on the tissue surface below the delaminated fragment would be expected. In most cases where the explant surface is smooth in IL-1 treated explants there is strong safranin O staining which indicates an absence of activity of IL-1 (**Figure 5.4-6**). Therefore, the suggestion is made that the degraded areas of the OA explant surface delaminated during the fixing and embedding processes in preparation for histology. Delamination of the explant surface occurring after culture would then explain the absence of an IL-1 effect (decreased proteoglycan staining) when a smooth cartilage surface is observed.

The implications of delamination of the OA explant surface are such that it would be inappropriate to attempt to draw conclusions on the effect of IL-1 and hypoxia. As a fragment of the cartilage surface is lost so is a significant amount of information concerning the effect any experimental conditions on the proteoglycan content of the OA explant. The difficulties experienced here in regards to the culture of OA explants are likely caused by the abnormal and degraded matrix of the end stage OA cartilage used (Heinegard and Saxne, 2011). Here it is shown that loss of the cartilage surface can occur between 24-48 hours of culture of OA explants. Healthy cartilage explants however, have been shown to remain stable for long periods of culture, Moo E et al. (2011) have shown that when cultured for 17 days healthy bovine cartilage explants have been shown to remain stable in regards to; wet mass, proteoglycans, glycosaminoglycans (GAG), collagens and DNA contents.

5.3.2 Potential Leaching of Aggrecan Fragments from OA Cartilage Explants

The results of western blots assessing ARGSV release into the media are also inconclusive representing no consistency in results of experimental conditions between individual donors (**Figure 5.8**). Our group had previously shown that ARGSV release from healthy cartilage explants in hypoxia was reduced in both spontaneous and IL-1 induced conditions (compared to normoxia) (Thoms et al., 2013). However, in this study, the inconsistent level of ARGSV detected, notably between explants in rest conditions, was hypothesised to potentially be due to the leaching of degraded aggrecan fragments retained in the OA cartilage (Roughley and Mort, 2014), from the matrix into the media following explantation.

Leaching of proteoglycans fragments (potentially present prior to explantation) was assessed using via freeze/thaw cycles to produce explants with a decellularised matrix (Jahn et al., 2010, Kheir et al., 2011, Hubbard et al., 1988). Results of this experiment showed that over the course of 48 hours of culture there was release of varied levels of ARGSV epitope from the generated acellular OA explants (**Figure 5.9-10**). These results therefore indicate that ARGSV release from OA explants can occur in the absence of the activity of chondrocytes within the tissue. Notably, assessment of western blots indicates that acellular OA explants cultured for 24 hours released higher levels of ARGSV than those cultured for 48 hours. This inconsistency in the release of ARGSV from acellular explants, as well as the potential

leaching of ARGSV fragments from OA cartilage once explanted, can provide an explanation as to the inconsistency observed in ARGSV release in experimental conditions (**Figure 5.8**). Importantly, Moo E et al. (2011) show that proteoglycan content of healthy explants is not significantly reduced due to leakage as a result of a distorted collagen network following explantation.

However, it should also be considered that the act of freeze/thawing to produce acellular explants could also result in damage to proteoglycans contributing to the effect observed. Kheir et al. (2011) describe reduced GAG content following freeze thawing in porcine cartilage however, whether GAGs are continually lost during culture following freeze/thaw cycles was not described. Notably, Kheir and colleagues use an adaptive protocol which includes additional freeze/thaw cycles in a hypotonic buffer followed by incubation in a hypotonic sodium dodecyl sulfate (SDS) containing buffer. It is stated in their discussion that it cannot be determined whether GAG loss was caused by freeze/thaw cycles alone or hypotonic/SDS buffer incubation.

In future work with end stage OA cartilage explant, with the indication of leaching of pre-degraded proteoglycans, an increased resting period of the explant could provide a potential avenue to improve experiments. Investigation into the level of ARGSV release and its duration could provide vital information about the suitability of the cartilage for this form of assay. Such information could also provide a more appropriate starting point for experimental conditions if ARGSV release from OA explants was found to stabilise over time.

In summary, the results of investigation into the effect of hypoxia on the degradation of OA cartilage are inconclusive. It is suggested here that there are inherent difficulties in the use of explants from end stage OA human cartilage. Recently there has been more discussion within the field about the value of negative data in OA where there is a general lack of mechanistic understanding (Vincent and Malfait, 2017). It should therefore be noted that whilst this chapter represents what is typically described as negative data, there are important discussion points worth noting about the specific difficulties involved when using end stage OA human cartilage. Negative data, such as the results described here, could inform and improve the methodologies of future experiments in the field and potentially provide insight into previously undescribed aspects of OA.

Chapter 6

Final Discussion, Future Work and Conclusions

6.1 Summary

Hypoxia has been previously described as being able to enhance the production of the cartilage matrix and inhibit its destruction in healthy human cartilage/chondrocytes via the reported target genes of hypoxia inducible factors (HIF), HIF-1 α and HIF-2 α (Lafont et al., 2008, Lafont et al., 2007, Thoms et al., 2013). This thesis investigated the hypoxic response in an osteoarthritis (OA) setting to assess whether the previously described effects of hypoxia in healthy cartilage were preserved in OA. It has been shown here that the general response of OA human articular chondrocytes (HACs) to hypoxia remains beneficial to the chondrocyte phenotype. Hypoxia results in increased cartilage production, via induction of key cartilage transcription factor SOX9, whilst also resulting in a decrease of key catabolic enzymes (*ADAMTS5*, *MMP13*). Interestingly, it is also observed that, whilst levels of *TIMP3* are shown to be HIF-1 α dependent, hypoxia does not significantly increase *TIMP3* mRNA levels (increased by hypoxia in healthy HACs) indicating a potentially reduced anti-catabolic potential of hypoxia in OA HACs. However, the changes in mRNA levels for key genes described here may not directly correlate to protein expression and future work is required to assess the changes caused by hypoxia in OA HACs at the protein level.

Our group had previously reported that the hypoxic induction of SOX9 in healthy chondrocytes was due to direct binding of HIF-2 α . However, it is demonstrated here via the use of HIF- α targeting small interfering RNAs (siRNAs) that there is a shift in the target genes of the HIF- α subunits in OA HACs. It is observed here that HIF-1 α is the subunit predominantly responsible for the induction of SOX9. Interestingly, it is shown that the depletion of HIF-1 α in normoxia (20% oxygen) resulted in a reduction in SOX9 indicating activity of HIF-1 α in a normoxic setting. An effect of HIF-2 α on the levels of SOX9 mRNA is observed in OA HACs although this effect is greatly reduced compared to previous observations made in healthy HACs. However, a greater effect of HIF-2 α on the mRNA levels of SOX9 in hypoxia is observed in OA HACs from younger donor samples, therefore these differences in the activity of HIF (in regards to SOX9) between OA and healthy HACs could indicate an age related change in activity. This provides an interesting discussion point given the difference in younger average ages (33 years) of donors used for healthy cartilage compared to the generally older age of OA donors (60 years). Therefore, the potential disparity between age related and disease related changes to human cartilage are discussed further below.

Furthermore, in regards to HIF-2 α , it has been shown previously in the murine OA model that HIF-2 α is a catabolic regulator of OA (Saito et al., 2010, Saito and Kawaguchi, 2010, Yang et al., 2010). Crucially however, it is reported here in OA HACs that HIF-2 α contributes to the reduction of mRNA levels of metalloproteinases *ADAMTS5* and *MMP13* in hypoxia, indicating an anti-catabolic role in human OA. These results using OA HACs in an in vitro setting, suggest that the proposed targeted reduction of HIF-2 α may not provide therapeutic benefit in human OA (Saito and Kawaguchi, 2010). These conflicting reports of HIF-2 α activity in OA are potentially explained by species related differences and are discussed further below.

In healthy cartilage individual depletion of prolyl hydroxylases (PHDs) was shown to stabilise HIF- α protein above levels observed in hypoxia alone. Specifically, PHD2 was shown to be the dominant PHD enzyme in healthy HACs regulating HIF-1 α and HIF-2 α . The increased levels of HIF-2 α were shown to result in a greater induction of SOX9 than observed in hypoxia alone indicating PHD2 as a therapeutic target (Thoms et al., 2013, Thoms and Murphy, 2010). Importantly, here it is demonstrated that PHD2 depletion had the most significant effect on HIF- α protein levels, increasing the levels of HIF-1 α in normoxia to levels typically observed in hypoxia. However, increased stabilisation of HIF-1 α in normoxia did not result in further induction of SOX9 suggesting a possible post-transcriptional regulation of HIF-1 α in OA HACs in normoxia. The potential involvement of factor inhibiting HIF (FIH) which regulates the transcriptional activity of HIF- α in normoxia is suggested. Additionally, the ability of RNA binding proteins (RBP) to regulate the stability of HIF- α mRNA and protein is discussed. Therefore, it is suggested that the activity of FIH and post transcriptional regulation of HIF are avenues that warrant future investigation.

The observation is made that a compensatory increase of PHD3 occurs when PHD2 is depleted in OA HACs. It is theorised here that the potential effects of PHD2 depletion in hypoxia, such as the stabilisation of HIF- α protein above levels observed by hypoxia alone, are offset by the compensatory increase of PHD3 in hypoxia in OA HACs. Crucially, the observed compensation of PHD3 suggests that individual depletion of the PHDs is not sufficient to enhance the effects of hypoxia in OA HACs.

It has been previously shown by our group that in healthy HACs depletion of PHD2 reduced levels of *ADAMTS5* independently of HIF- α (Thoms et al., 2013). This effect of PHD2

depletion is not observed here in the healthy HACs. Interestingly however, HIF independent activity of the PHDs is observed; PHD1 depletion resulting in decreased *MMP13* and *COL2A1* and a reduction of *TIMP3* following the individual depletion of each of the three PHD isoforms.

Similar to observations made in healthy HACs (Thoms and Murphy, 2010), general hydroxylase inhibition by Dimethyloxaloylglycine (DMOG) in OA HACs is able to increase the protein levels of both HIF-1 α and HIF-2 α above levels typically observed in hypoxia. This increase in HIF- α protein results in a greater induction of SOX9 than observed in hypoxia alone. Additionally, it is observed that DMOG reduces levels of key metalloproteinases further than is observed by hypoxia alone. This therefore indicates the therapeutic potential for inducing an enhanced hypoxic response of OA HACs, increasing both cartilage production and protection, via enhanced levels of HIF- α protein. Thus, the suggestion is made here that the effect of combined selective inhibition of the PHDs could enhance levels of HIF- α above those observed physiologically which results indicate could provide a therapeutic benefit in the human OA setting (**Figure 6.1**). Therefore, it is suggested that the effects of combined selective PHD inhibition should be investigated further.

Finally, it has previously been shown by our group that hypoxia is able to inhibit the spontaneous degradation and degradation induced by interleukin-1 (IL-1) in healthy cartilage (Thoms et al., 2013). Whilst results of hypoxic culture of OA HACs suggest a protective effect of hypoxia is retained in OA, via reduction of matrix metalloproteinases mRNA, such an effect could not be conclusively demonstrated using OA cartilage explants. This is due to the inconsistency of results following culture of end stage OA human cartilage explants. Delamination of the OA explant surface is described here which makes the interpretation of proteoglycan levels within the tissue via histological staining very difficult. Additionally, the suggestion is made that leaching of pre-existent aggrecan fragments (ARGSV) from OA cartilage following explantation occurs for at least 48 hours. Leaching of pre-existent ARGSV fragments makes it difficult to interpret the potential effects of hypoxia on the rate of degradation. It is suggested that long term cultures of human OA explants could provide insight into the baseline levels of ARGSV potentially improving similar investigations in the future. Importantly, these results taken together represent the inherent difficulties that should be considered when investigating OA using end stage OA human cartilage.

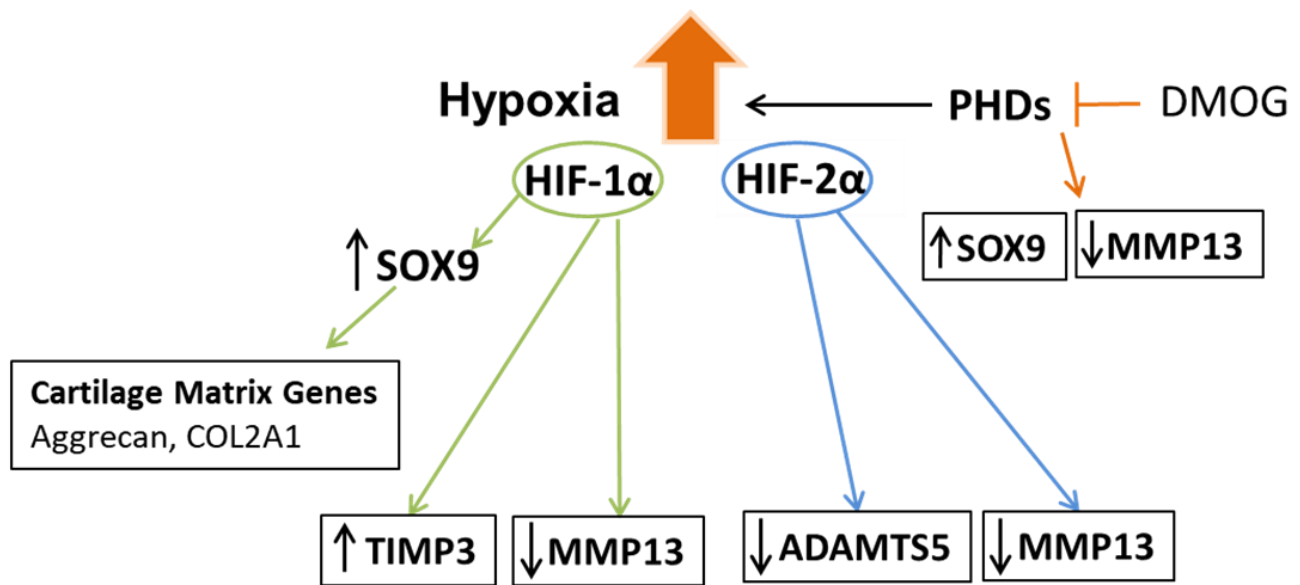


Figure 6.1 Summary of hypoxia related function in OA HACs. Hypoxia increases the production of cartilage via activity of HIF-1 α targeting SOX9 resulting in the upregulation of key cartilage matrix genes in OA HACs. HIF-1 α inhibits the destruction of the tissue through reduction in collagenase MMP13 and simultaneous increase of matrix metalloproteinase inhibitor TIMP3. Crucially the combined inhibition of PHDs increases levels of HIF-1 α and HIF-2 α , and their subsequent effects, above levels observed by hypoxia alone. It is believed further research into the effects of selective combined PHD inhibition will allow for a manipulation of the hypoxia response system (via HIF) providing potential therapeutic benefit in a human OA setting.

6.2 Final Discussion

6.2.1 Aging and OA

As donors of OA tissue used in this study were in general considerably older than donors of healthy cartilage used previously within our group (Thoms et al., 2013) it is important to consider that differences observed between healthy and OA cartilage used in these studies may not solely be disease related and could be contributed to by age related changes in articular cartilage and HACs.

Age has been described as the strongest predictor for the development of OA affecting around 9.6% of men and 18% of women over 60 years of age (Woolf and Pfleger, 2003). However, the relationship between aging and osteoarthritis is poorly defined. The complexity of the relationship between aging and OA is in part due to the many typically described features of OA such as reduced cell density, reduced cartilage thickness, calcification and impaired anabolic and anti-catabolic cell responses, also being observed as age related changes in cartilage (Loeser, 2010, Lotz and Loeser, 2012, Valdes and Stocks, 2017, Loeser et al., 2014).

It is suggested that biological ageing impacts the function of the mitochondria within a given cell, regulating key aspects of aging (Sun et al., 2016). Summarily, it is suggested that the mitochondrion is the principal source of reactive oxygen species (ROS) (Valdes and Stocks, 2017). Increased ROS can create an imbalance of oxidants and antioxidants within the cell leading to oxidative stress causing damage to the DNA within a cell. In terms of cartilage, the chondrocyte has very low proliferative activity which means that damage caused to DNA will accumulate over time affecting cellular function. One such example of damage caused by oxidative stress is mutations in the mitochondrial DNA (mtDNA) which is particularly susceptible to damage as it is not protected by a nuclear membrane (Henrotin et al., 2005). It has been shown that such mitochondrial dysfunction, which is associated with aging, can affect multiple pathways related to cartilage degradation in OA chondrocytes including oxidative stress through increased ROS production (Blanco et al., 2011). The potential for cartilage degradation due to aging, such as the processes described above indicate the importance of unpicking the effect of OA from catabolic changes occurring in cartilage due to aging.

An example of the potential importance of using age matched cartilage samples, when investigating the effects of OA has been further expanded upon by Vidal-Bralo et al. (2016) who have shown that premature biological aging of cartilage, in regards to epigenetic changes, occurs as a component of OA pathogenesis. Vidal-Bralo and colleagues compared biomarkers of epigenetic aging in human cartilage from OA donors and age matched cartilage samples obtained from cadavers with no macroscopic signs of OA. To assess epigenetic aging Vidal and colleagues used DNA methylation age-measures (DmAM) which use the methylation status of CpG sites that have been described as age related (Horvath, 2013). The results of their study found that the differences in DmAM between age matched OA and control donor cartilage samples indicated that OA cartilage was on average 3.7 years older in regards to epigenetic aging (Vidal-Bralo et al., 2016). This increased epigenetic age of OA cartilage could affect transcriptional regulation within cartilage as is observed with aging (Lopez-Otin et al., 2013). Interestingly, in addition to this it has been shown that control over HIF-1 α binding can be influenced by the methylation status of CpG sites with consensus hypoxia responsive elements (HREs) (Watson et al., 2010).

The implication of such research is that effects exclusive to OA are observed in addition to the hallmarks of aging, which often overlap with the OA pathogenesis. In fact Vidal-Bralo et al. (2016) results show that OA exacerbates the catabolic effects observed with aging. In this case the use of age matched samples was imperative to elucidate the effects of OA on biological aging related processes.

Thus, in combination this information shows the importance of using matched cartilage samples to try to distinguish between effects of aging and the effects of OA. This information can be of crucial importance to determine whether the results observed in an OA study are due to the disease or the result of functional changes with age that will not have occurred in the healthy cartilage typically obtained from younger donors.

6.2.2 Species Differences in OA Research

The results within this study indicate in OA HACs a potentially anti-catabolic role of HIF-2 α with some the suggestion of an anabolic response, although blunted compared to healthy HACs (Thoms et al., 2013). However, as previously stated this result is in conflict with the suggestion within the literature that in murine models, HIF-2 α is a catabolic regulator of OA, being shown to enhance key catabolic enzymes *MMP13* and *ADAMTS5* through direct binding to HREs (Saito et al., 2010, Saito and Kawaguchi, 2010, Yang et al., 2010). The results of these studies finding that deletion of a single HIF-2 α allele reduces the OA severity within the murine model. Importantly, in OA HACs the opposite is observed; results indicating an increase of *MMP13* and *ADAMTS5* mRNA following siRNA induced knockdown of HIF-2 α .

Crucially, these findings indicating a catabolic role of HIF-2 α are conducted in the murine model where OA has been experimentally induced. The use of different species when investigating the activity of HIF- α subunits is the most likely cause of these conflicting descriptions of HIF-2 α activity. Firstly, in regards to the dissimilarities in the physiological condition of the cartilage in both species - murine average cartilage thickness is reported as being over 7 times thinner than human cartilage (0.3mm thick and 2.1mm thick respectively) (McCoy, 2015). Whilst cartilage is an avascular tissue, diffusion of oxygen from vessels within the subchondral bone and the synovial fluid will likely result in an oxygen gradient in cartilage (Imhof et al., 2000, Lund-Olsen, 1970, Silver, 1975). Given the much thinner cartilage in mice (compared to humans) it is likely that a greater oxygen tension exists due to greater diffusion of oxygen from the subchondral bone and synovial fluid.

In addition to the likely difference in oxygen tension due to articular cartilage thickness it has also been shown that the hypoxic response of murine chondrocytes can differ from observations made in larger animals. It has previously been described that hypoxic induction of cartilage matrix genes is weaker in murine chondrocytes than previously observed by our group in larger animal models (Lafont et al., 2007, Murphy, 2001). For example, wild type murine chondrocytes are observed as having no hypoxic induction of *COL2A1* and *ACAN* mRNA (Pfander et al., 2003). These differences in species response to hypoxia could be due to the development of mechanisms, in larger animals such as humans, which enhance cartilage matrix production in hypoxic environment. Such a mechanism may have developed in larger

species, which are likely to experience lower cartilage oxygen levels than mice due to greater cartilage thickness (Adams, 2006).

This disparity between human and murine cartilage properties in regards to average cartilage thickness and the general hypoxic response indicates a fundamental difference between the species that can be directly related to the activity of HIF in hypoxia. As a result, careful consideration to species differences should be considered when implicating therapeutic targets for human OA, such as HIF-2 α .

6.3 Future Work

6.3.1 Further Protein Level Assessment by Western Blot

It is important to consider that within this study protein level assessment for many secreted proteins is not assessed such as; Type II collagen, aggrecan and TIMP3. It is acknowledged here that the mRNA levels for the genes encoding for these proteins may not directly relate to the protein levels produced by the chondrocyte. The absence of this data is a limitation of this study. Whilst the implications of protein level following changes in mRNA can be inferred, it will be of crucial importance in future work to assess the levels of these proteins to enhance understanding of the hypoxic response of OA HACs. Assessment of the levels of secreted proteins could provide further insight into the effects described in this thesis, potentially confirming the conclusion that hypoxia remains a beneficial environment for the chondrocyte. Assessment of these protein levels in the future will enable a more accurate confirmation of the hypoxic response in OA HACs.

6.3.2 Elucidating HIF-Independent PHD Activity in Human Cartilage

Results of PHD depletion by RNAi, in both OA and healthy HACs have indicated activity of the PHDs independent of their HIF- α targeting effects. In healthy HACs a significant downregulation of *ADAMTS5* mRNA, beyond that typically observed by hypoxia, was found following the depletion of PHD2. This effect was determined to be independent of HIF- α (Thoms et al., 2013). In **Chapter 4** it is reported that in OA HACs significantly reduced mRNA levels of *MMP13* and *TIMP3* were observed following the depletion of PHD1 and

individual depletion of PHD1/2/3 respectively. PHD1 depletion did not affect proteins levels of HIF- α subunits indicating a HIF-independent effect.

Given growing evidence of HIF-independent effects of PHDs in human cartilage, future investigations will focus on assessing whether these effects are the result of direct interaction between PHDs and the affected genes or if the response is mediated indirectly. Elucidating the mechanism behind these responses may provide insight into the differences in PHD activity observed between healthy and OA HACs.

6.3.3 Regulation of HIF- α subunits by FIH in Human Cartilage

In **Chapter 4** the post translational regulation of HIF-1 α by FIH is described as potentially masking the effects of enhanced HIF-1 α protein levels following PHD2 depletion in normoxia. It was suggested that hydroxylation of specific asparaginyl residues within the C-terminal transcription domain (C-TAD) on the HIF- α subunit by FIH could inhibit the transcriptional activity of HIF-1 α reducing HIF dependent induction of SOX9. Furthermore, it is suggested that the activity of FIH could explain some of the disparity in effects of PHD depletion between healthy and OA HACs.

Thus, to investigate the effect of FIH in both healthy and OA cartilage I suggest the depletion of FIH by RNAi. To determine the effect of FIH, the expression of HIF target genes would be assessed (i.e. SOX9) following depletion of FIH by siRNA transfection. Additionally, the binding of HIFs to isolated HREs of target genes should be compared in control and in FIH depleted HACs.

6.3.4 Effect of Selective Combined Hydroxylase Depletion on Hypoxic Response in OA HACs

Here it is shown that whilst individual depletion of PHDs was not sufficient to enhance the hypoxic response of OA HACs, general hydroxylase inhibition is able to stabilise protein levels of HIF-1 α and HIF-2 α above levels typically observed in hypoxia alone. The increased level of HIF-1 α and HIF-2 α in hypoxia following culture with DMOG increased the mRNA and protein levels of SOX9 beyond typical hypoxic induction. Additionally, general hydroxylase inhibition with DMOG reduced key catabolic enzymes gene expression to below

levels observed by hypoxia alone. However, general hydroxylase inhibition via DMOG is known to disrupt type II collagen secretion (through inhibition of collagen prolyl hydroxylases) which is crucial to the cartilage structure.

Therefore, I suggest that the effect of combined selective depletion of the hydroxylases (i.e. PHDs, FIH) could enhance levels of HIF- α above those observed physiologically. An increase in HIF- α protein levels which could provide a therapeutic benefit in the human OA setting as indicated by the results of general hydroxylase inhibition in chapter 4. For example, the compensation of PHD3 where PHD2 is depleted could offset the effects of PHD2 depletion. Future work using siRNAs targeting both PHD2 and PHD3 would reveal if combined depletion is adequate to observe an enhanced hypoxic response similar to DMOG and observations made by PHD2 depletion alone in healthy HACs.

Increased knowledge of the combined regulation of HIF- α sub units by hydroxylases may indicate particular hydroxylases as appropriate targets to enhance the beneficial properties of the hypoxic response observed in OA HACs.

6.3.5 HIF Independent Effects of PHD Depletion

In this study the effects of PHD depletion seemingly independent of the HIF are described. Interestingly, reduction in mRNA expression of *TIMP3* following the individual depletion of PHD1, PHD2 and PHD3 is observed. Additionally, results show reductions in mRNA expression of *COL2A1*, *ACAN* and *MMP13* in normoxia following the depletion of PHD1. Given the developing understanding of HIF-independent PHD effects and the indication of HIF-independent PHD activity in the OA HAC I suggest future research into the potential HIF-independent PHD targets. Such research could provide insight into non HIF-dependent benefits to PHD depletion in articular cartilage.

6.3.6 Investigating Hypoxic Signalling in Age Matched Human Cartilage

Whilst differences in hypoxia related function are described between the results in OA HACs in this thesis, and previous results in our group in healthy HACs there is a noteworthy difference in the average age of the cartilage donors that is considered. In this thesis OA cartilage donor age range is from 41-88 with an average age of 67.8 years. However, past

research conducted in our group used healthy cartilage from donors with a much younger age range typically between the ages of 8-50 years.

As discussed in **Chapter 6.2.1** significant changes occur with cartilage with aging some of which are linked to the progression of OA. Therefore, it is important to investigate whether the differences in the hypoxia related function observed here are due to OA, as opposed to age related changes in the cartilage.

In order to ensure that the effects of hypoxia related function in OA cartilage are in fact due to the disease, as opposed to change related to aging, age matched healthy cartilage would need to be used. Similar to the methodology used by Vidal-Bralo et al. (2016), healthy cartilage from older donors can be obtained from cadavers with no history of OA. The cartilage from older donors (60+ years) can be examined for macroscopic and microscopic evidence of OA. The absence of OA in cadaveric cartilage samples could be verified through the use of OARSI scoring methodology.

6.4 Conclusions

This thesis documents the investigation into hypoxic related functions in osteoarthritic human cartilage and articular chondrocytes. Here I aimed to identify whether the hypoxia response of healthy human cartilage, previously described as beneficial to chondrocyte function promoting the production and inhibiting the destruction of cartilage, is retained in OA human cartilage and chondrocytes.

It is reported that hypoxia in OA HACs promotes the chondrocyte phenotype (via induction of SOX9) and inhibits cartilage destruction via downregulation of key metalloproteinases *MMP13* (average reduction at P0, significant reduction observed in P2 OA HACs) and *ADAMTS5*. Therefore, indicating that the general response to hypoxia remains beneficial to the cartilage phenotype in OA cartilage. However, a potentially reduced capacity to inhibit metalloproteinases is identified in OA HACs, this is as TIMP3 is not significantly increased in hypoxia as previously observed in healthy HACs.

Interestingly, using RNAi HIF-1 α was identified as the main HIF- α subunit responsible for maintaining levels of SOX9 in normoxia and increasing them in hypoxia in OA HACs. This is a key change to the activity of HIF isoforms described previously in healthy human cartilage where HIF-2 α was shown to bind directly to SOX9. Crucially however, no evidence that HIF-2 α enhances catabolic activity was found in OA (in contrast to results reported in mice), instead identifying the reduction of *ADAMTS5* in hypoxia as HIF-2 α dependent.

Finally, it is reported that in contrast to previous findings in healthy cartilage, individual depletion of PHDs is not sufficient to enhance the levels of HIF-1 α or HIF-2 α in hypoxia and as a result has no effect on the expression of SOX9 in OA HACs. However, general hydroxylase inhibition enhances hypoxic levels of HIF resulting in an enhanced hypoxic response, increasing SOX9 above levels observed in hypoxia alone, as well as further reducing *MMP13* and *ADAMTS5*.

Overall, these results indicate that, despite some notable differences, hypoxia promotes cartilage function in OA human cartilage as in healthy human cartilage. Additionally, in OA cartilage, combined inhibition of hydroxylase activity proves to be a potential target to enhance hypoxia related function for therapeutic benefit.

Chapter 7

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Appendix

A.1 Preliminary UKA OA HAC and Healthy HAC mRNA Comparisons

Osteoarthritic (OA) and human articular chondrocytes (HACs) obtained from Unicompartmental knee arthroplasty (UKA) and healthy HACs were cultured in either normoxia (20% oxygen) or hypoxia (1% oxygen) for 48 hours. Primary chondrocytes were used to investigate the response to hypoxia and compare basal levels of genes between healthy and OA HACs. OA cartilage was obtained from 2 male and 1 female donors; age range 61-75 years, mean age 70.3 years. Healthy cartilage was obtained from 2 male and 1 female donor; age range 25-37 years, mean age 29.6 years. The messenger RNA (mRNA) levels of genes of interest were normalised to the housekeeping gene, ribosomal protein RPLP0. It has been previously demonstrated within our group that RPLP0 is not regulated by hypoxia and is used as a standard housekeeping gene for hypoxic experiments (Lafont et al., 2008, Thoms et al., 2013).

This data represents preliminary experiments which aimed to compare basal levels of genes (**Figures A.1-A.3**) as well identify potential differences in the level of hypoxia induction between primary healthy and OA (UKA sourced) HACs. However, as detailed in **Chapter 2.2.3** chondrocytes isolated from cartilage sourced from UKA donor samples were observed to frequently contain debris which appeared to be calcium containing crystals. It has previously been established within the literature that calcium containing crystals cause the increased production of catabolic and proinflammatory mediators, which include MMP13, in HACs and as such contribute to cartilage degradation (Liu et al., 2009, Rosenthal, 2011, Sallis, 2006, Nguyen et al., 2013). These debris/calcium containing crystals were not present in cases where chondrocytes were isolated from total knee arthroplasty (TKA) samples. Therefore, due to the potential contamination of gene expression results by calcium crystals in UKA samples only data from chondrocytes from TKA samples were assessed and used in the main body of the thesis. Additionally, due to time constraints and issues regarding the sourcing of new healthy cartilage samples, it was not possible to obtain freshly isolated/primary healthy chondrocytes for comparison to primary OA HACs beyond the scope of this preliminary data.

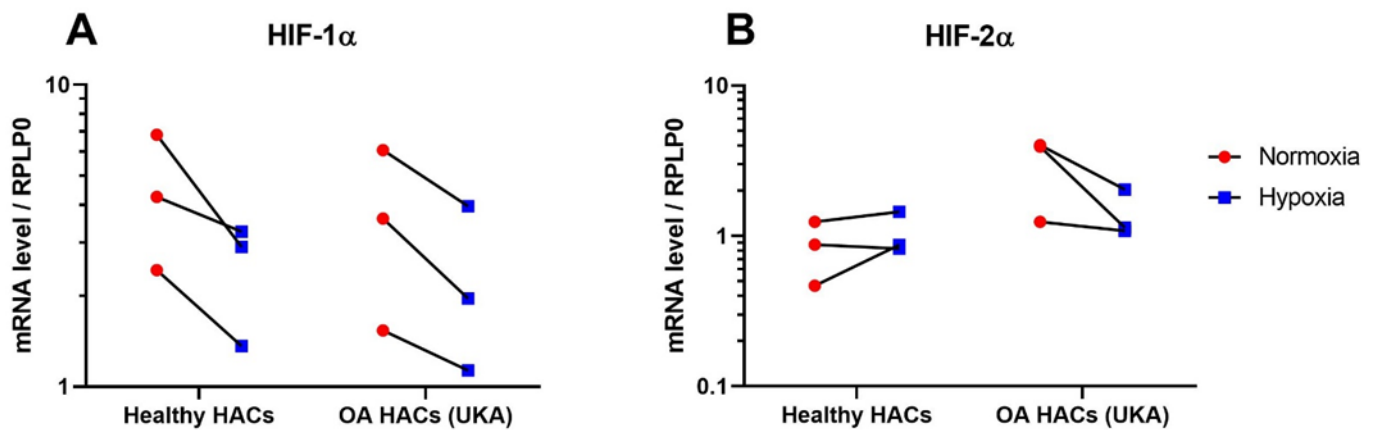


Figure A.1 Comparison between OA and Healthy HAC expression of HIF-1 α and HIF-2 α basal mRNA levels in normoxia and Hypoxia. Data were obtained from freshly isolated(P0) OA HACs and (P0) Healthy HACs following 48hrs of culture in either 20% oxygen (Normoxia) or 1% oxygen (Hypoxia). Real Time PCR was performed using Sybr Green. Data show mRNA levels (normalised using RPLP0 housekeeping gene) for HIF-1 α (A) and HIF-2 α (B). Data plot represented as log scale with lines connecting samples from the same unique patient.

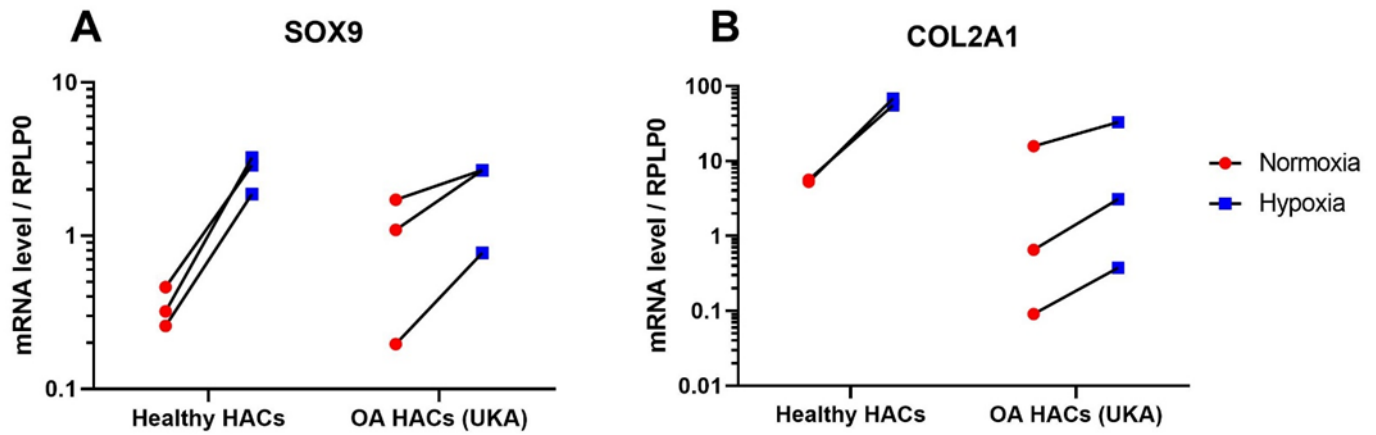


Figure A.2 Comparison between OA and Healthy HAC expression of SOX9 and COL2A1 basal mRNA levels in normoxia and Hypoxia. Data were obtained from freshly isolated(P0) OA HACs and (P0) Healthy HACs following 48hrs of culture in either 20% oxygen (Normoxia) or 1% oxygen (Hypoxia). Real Time PCR was performed using Sybr Green. Data show mRNA levels (normalised using RPLP0 housekeeping gene) for SOX9(**A**) and COL2A1 (**B**). Data plot represented as log scale with lines connecting samples from the same unique patient.

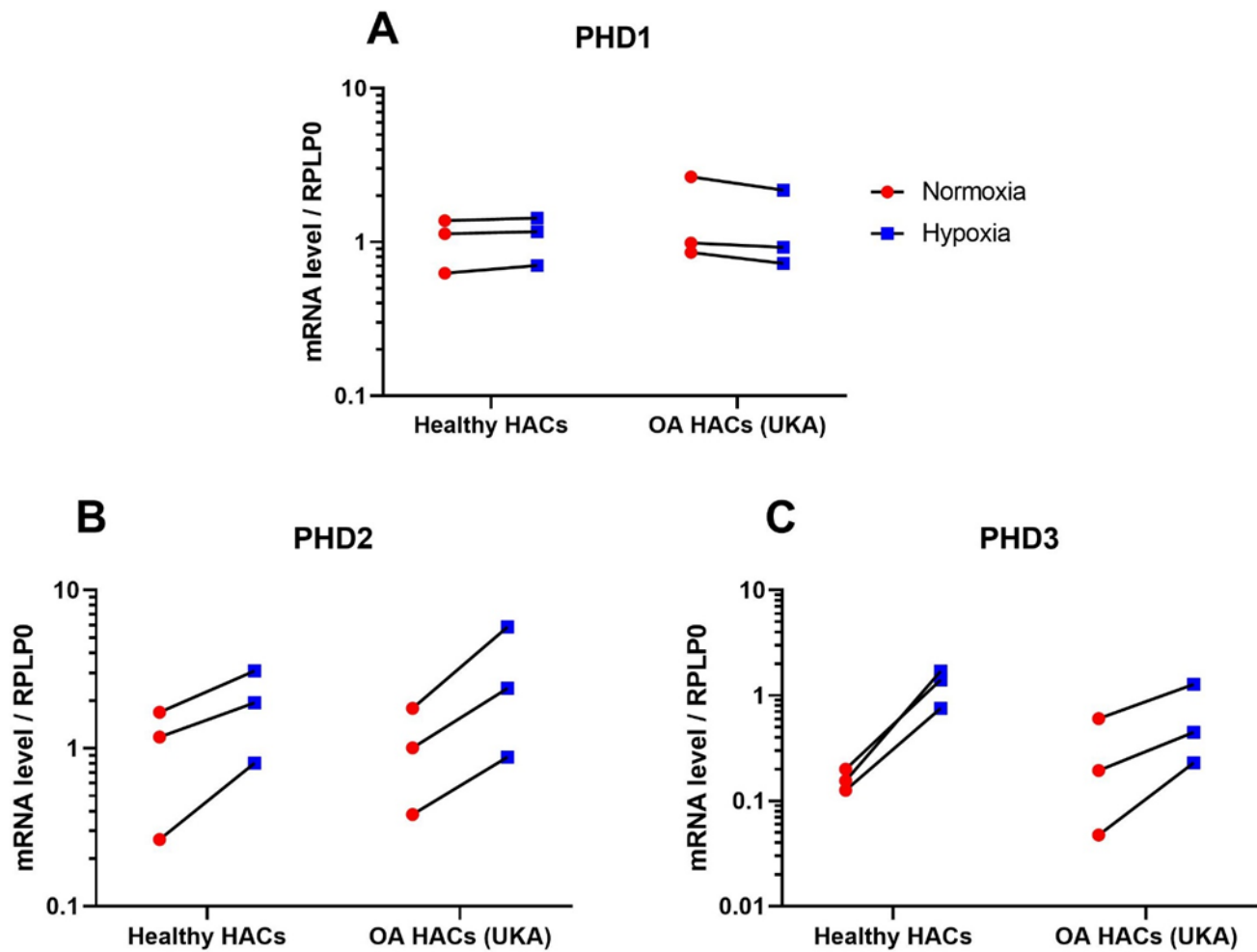


Figure A.3 Comparison between OA and Healthy HAC expression of PHD1, PHD2 and PHD3 basal mRNA levels in normoxia and Hypoxia. Data were obtained from freshly isolated (P0) OA HACs and (P0) Healthy HACs following 48hrs of culture in either 20% oxygen (Normoxia) or 1% oxygen (Hypoxia). Real Time PCR was performed using Sybr Green. Data show mRNA levels (normalised using RPLP0 housekeeping gene) for PHD1 (A), PHD2 (B) and PHD3 (C). Data plot represented as log scale with lines connecting samples from the same unique patient.

A.2 Limited Success with P0 OA Chondrocytes PHD Targeting siRNA Transfection

Investigation into the effects the prolyl hydroxylase domain (PHD) enzymes (*PHD1*, *PHD2* and *PHD3*) was attempted using primary (P0) osteoarthritic (OA) human articular chondrocytes (HACs). OA cartilage was obtained from 2 male donors; aged 63 and 72 years, mean age 67.5 years. HACs were transfected with small interfering RNA (siRNA) targeting; PHD1, PHD2 and PHD3. Once transfected HACs were cultured for 48 hours in either normoxia (20% oxygen) or hypoxia (1% oxygen), following cell culture messenger RNA (mRNA) and protein levels were analysed as previously described. Changes in mRNA levels stated are compared to control in the relevant oxygen tension unless otherwise stated.

This limited data set represents the results of successful transfections using primary (P0) OA HACs. It was initially intended that preliminary siRNA experiments would be conducted in second passage (P2) HACs before producing a larger data set using P0 HACs. However attempts to transfect siRNA into P0 HACs were largely unsuccessful resulting primarily in mRNA data with no indication of a knockdown or in some cases cell death during culture. **Figure A4-7** represents two sample sets where the knockdown of the target genes was deemed to be successful using primary chondrocytes (P0). Whilst the data set is too small to analyse statistically it can be observed that the data obtained from successful P0 HAC transfections follow the same trends as described in **Chapter 4**.

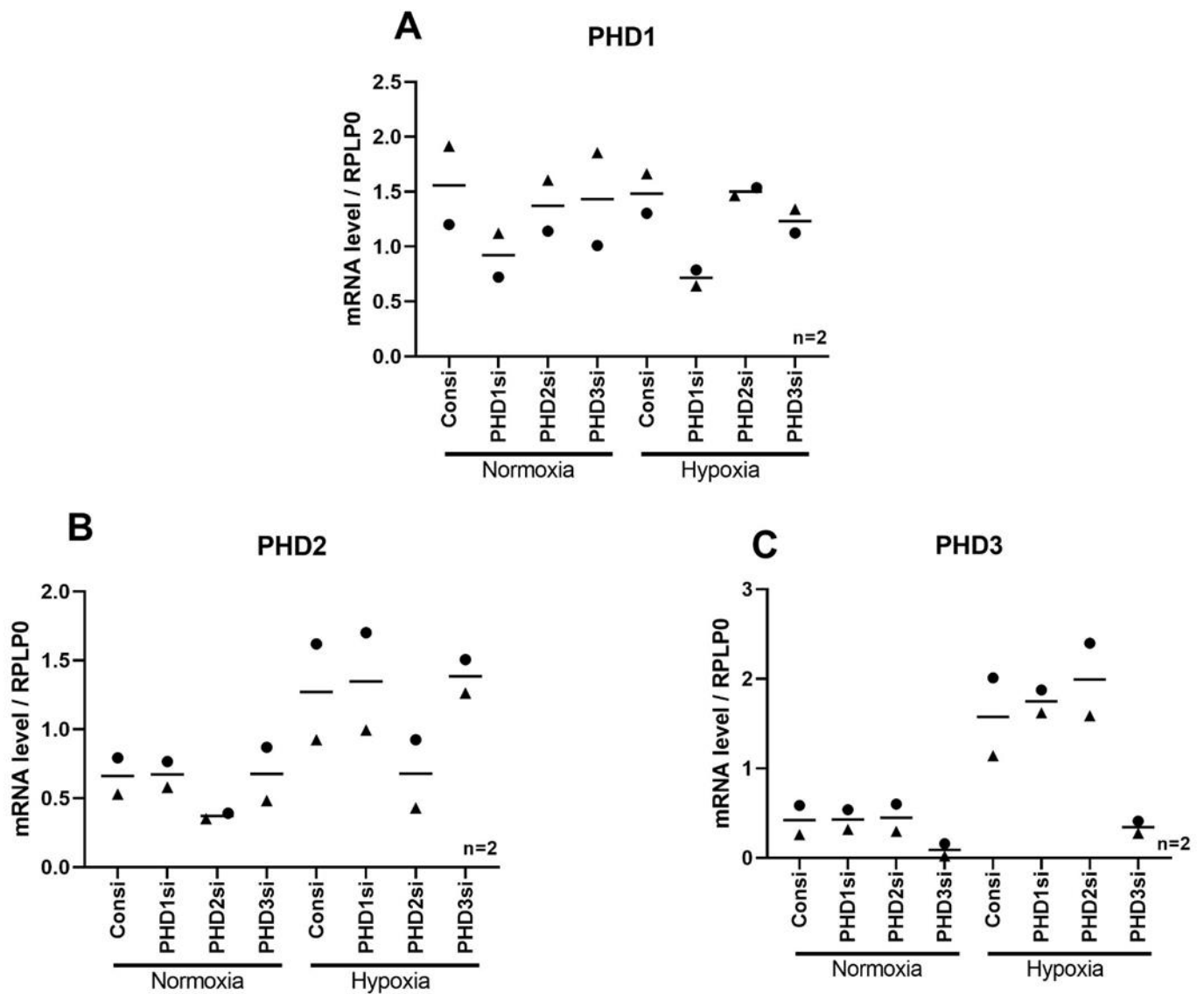


Figure A.4 Knockdown of PHDs in P0 OA HACs. Data were obtained from primary (P0) OA HACs following 48hrs of culture post siRNA transfection in either 20% oxygen (Normoxia) or 1% oxygen (Hypoxia). Chondrocytes were transfected with 10nM siRNA targeting PHD1 (PHD1si), PHD2 (PHD2si) and PHD3 (PHD3si) or a non-targeting control (Consi). Real Time PCR was performed using Sybr Green. Data show mRNA levels (normalised using RPLP0 housekeeping gene) for PHD1(A), PHD2(B) and PHD3(C) Data plot represented individual values and the mean.

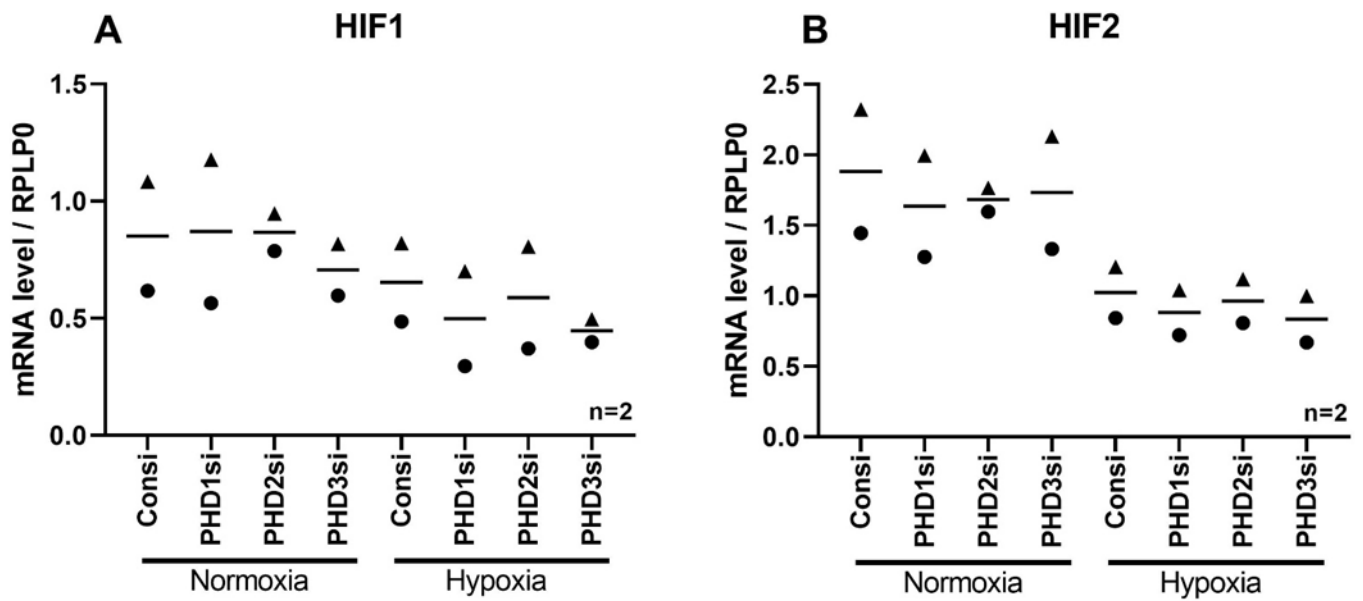


Figure A.5 Effect of PHD depletion on HIF gene expression levels of P0 OA HACs.

Data were obtained from primary (P0) OA HACs following 48hrs of culture post siRNA transfection in either 20% oxygen (Normoxia) or 1% oxygen (Hypoxia). Chondrocytes were transfected with 10nM siRNA targeting PHD1 (PHD1si), PHD2 (PHD2si) and PHD3 (PHD3si) or a non-targeting control (Consi). Real Time PCR was performed using Sybr Green. Data show mRNA levels (normalised using RPLP0 housekeeping gene) for HIF-1 α (A) and HIF-2 α (B). Data plot represented as individual values and the mean.

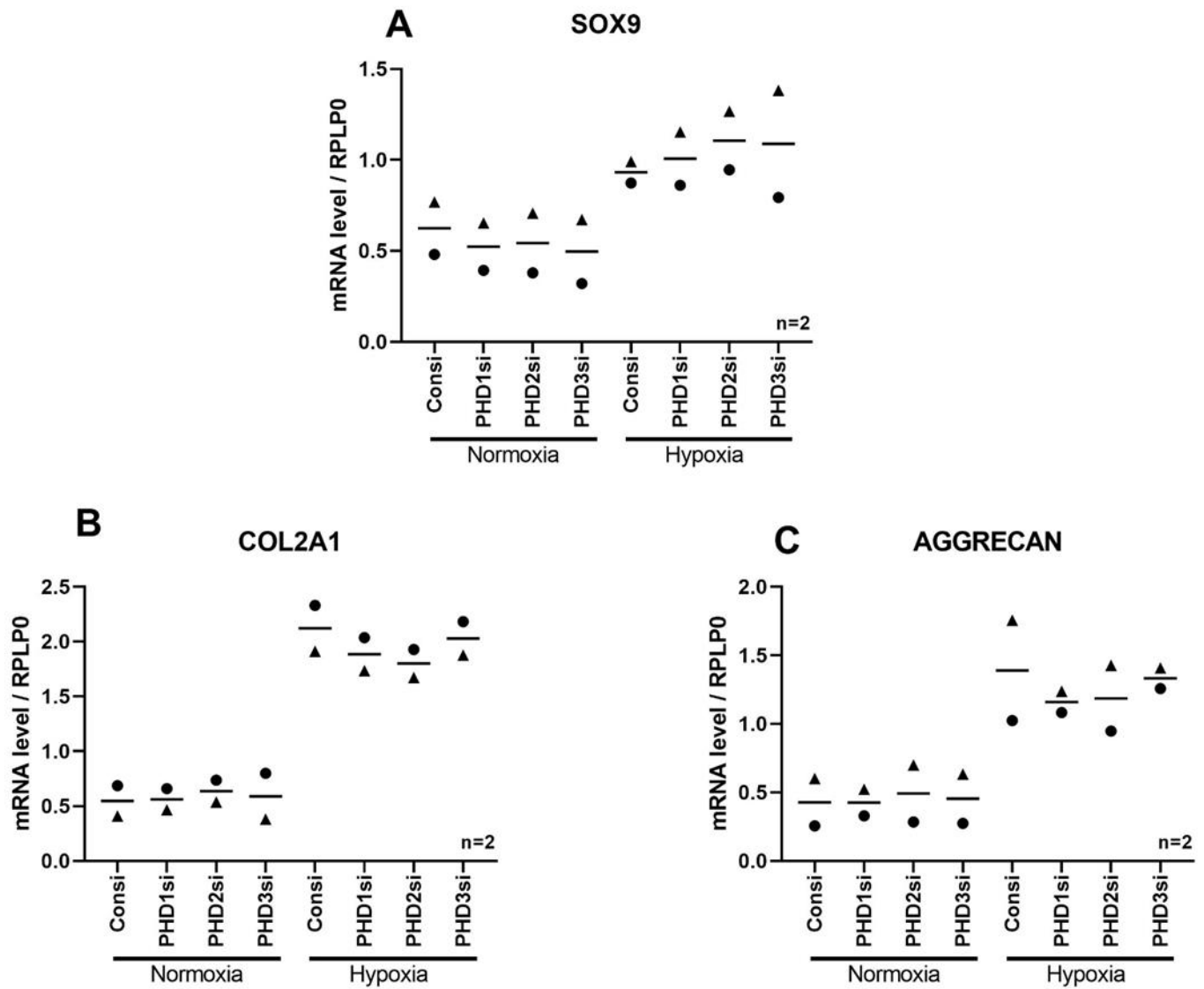


Figure A.6 Effect of PHD depletion on cartilage marker gene expression in P0 OA HACs. Data were obtained from (P0) OA HACs following 48hrs of culture post siRNA transfection in either 20% oxygen (Normoxia) or 1% oxygen (Hypoxia). Chondrocytes were transfected with 10nM siRNA targeting PHD1 (PHD1si), PHD2 (PHD2si) and PHD3 (PHD3si) or a non-targeting control (Consi). Real Time PCR was performed using Sybr Green. Data show mRNA levels (normalised using RPLP0 housekeeping gene) for SOX9(A), COL2A1(B) and AGGRECAN(C). Data plot represented as individual values and the mean.

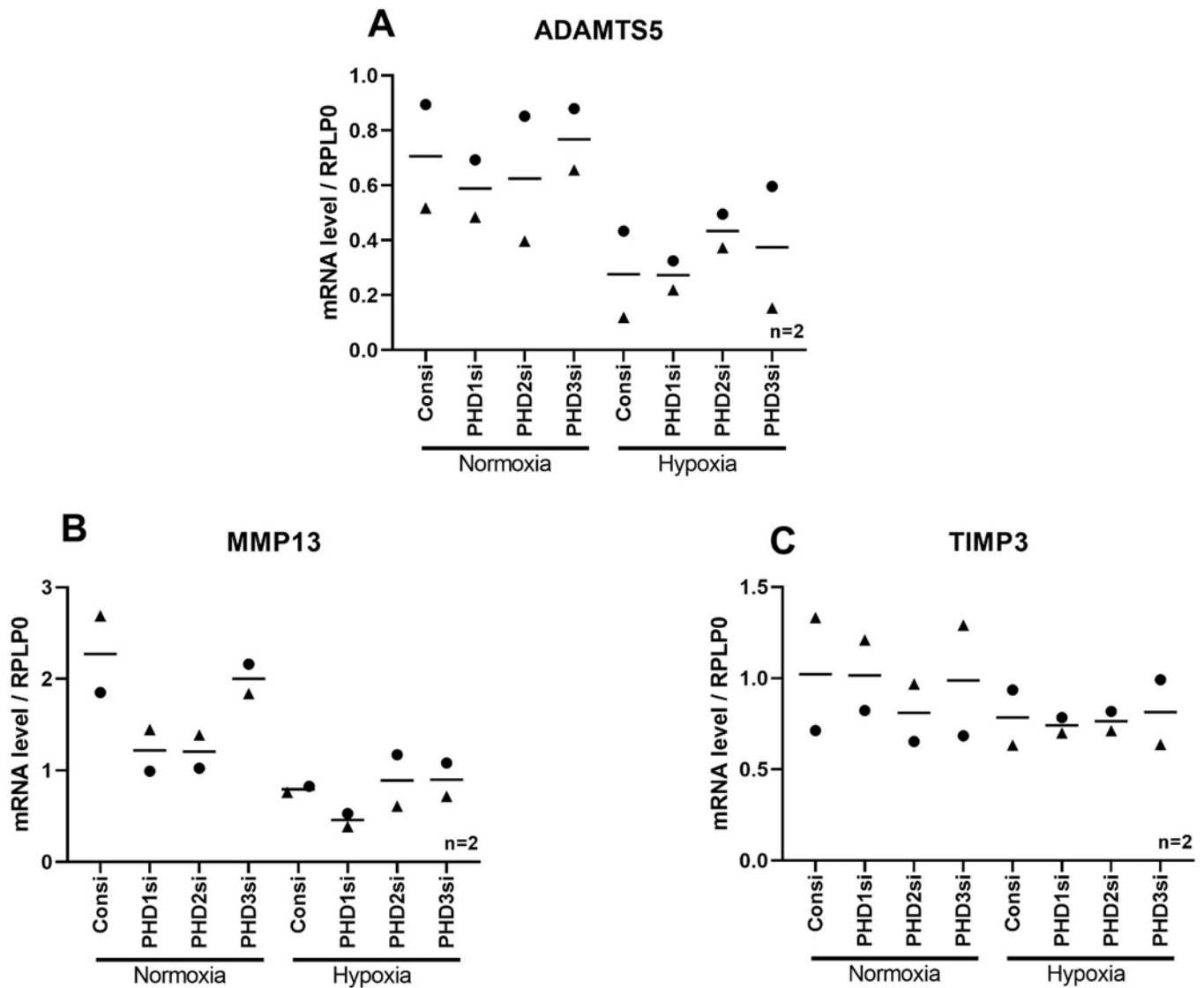


Figure A.7 Effect of PHD depletion on ADAMTS5, MMP13 and TIMP3 gene expression levels in P0 OA HACs. Data were obtained from (P0) OA HACs following 48hrs of culture post siRNA transfection in either 20% oxygen (Normoxia) or 1% oxygen (Hypoxia). Chondrocytes were transfected with 10nM siRNA targeting PHD1 (PHD1si), PHD2 (PHD2si) and PHD3 (PHD3si) or a non-targeting control (Consi). Real Time PCR was performed using Sybr Green. Data show mRNA levels (normalised using RPLP0 housekeeping gene) for ADAMTS5(A), MMP13(B) and TIMP3(C). Data plot represented as individual values and the mean.

A.3 Immunohistochemistry of OA Cartilage Explants

IHC staining was performed by first deparaffinising and rehydrating explant section slides according to a pre-programmed protocol on a Tissue-Tek DRS 2000 Multiple Slide Stainer (Sakura, Thatcham, UK). The slides were then immersed in a citrate buffer (10mM Citric Acid, 0.05% Tween 20, pH 6) at 95°C for 30 minutes, following immersion the slides were cooled for 20 minutes at room temperature (RT). Serial sections were then washed in phosphate buffered saline (PBS) for 10 minutes before being incubated for 30 min at RT in a blocking solution containing 3% normal goat serum (BSA) in PBS and overnight at 4°C with primary antibodies specific for SOX9. Control sections were incubated in the absence of a primary antibody (Negative control). Following primary antibody incubation, sections were rinsed in PBS (2 x 10 min) and incubated for one hour at RT in an appropriate secondary antibody. This was followed by 45 minutes in ABC solution (prepared as directed by manufacturer (Vector Laboratories)). Bound peroxidase was then visualised by applying diaminobenzidine tetrahydrochloride (DAB) solution (Vector Laboratories) for five minutes. The sections were then washed briefly (5 mins) in distilled water before dehydration according to a pre-programmed protocol on a Tissue-Tek DRS 2000 Multiple Slide Stainer (Sakura, Thatcham, UK) and the application of a cover slip.

Immunohistochemical (IHC) staining was originally planned to identification the levels and localisation of SOX9, HIF-1 α , HIF-2 α and catabolic factors such as MMP13. However, as mentioned in **Chapter 2.8** and **Chapter 5** several issues affected the interpretation of results when using OA cartilage explant sections. This issues included the potential delamination of the cartilage surface layer, such delamination would have had obvious effects on the ability to assess and compare the quantity and localisation of proteins between explant samples. As a result, although work had been started toward analysing explant sections using IHC (**Figure A.8**) these experiments were not conducted further as it was decided that the methodology would not be viable to produce reliable data given the difficulties experienced.

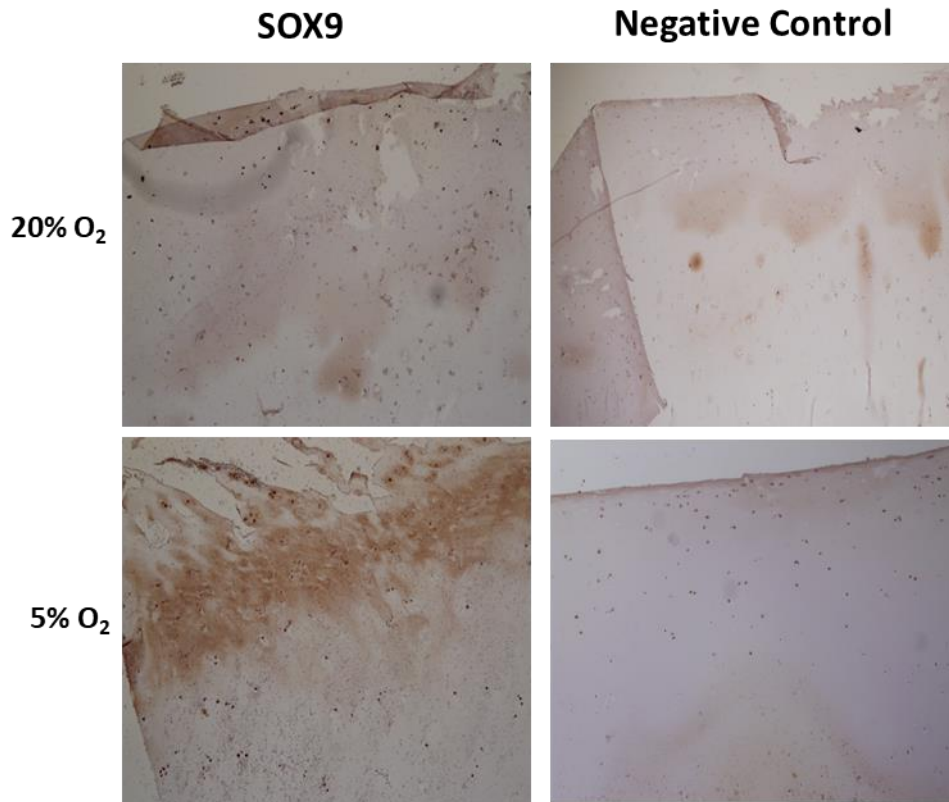


Figure A.8 Preliminary IHC staining for SOX9 in normoxia and Hypoxia using OA cartilage Explants. Human OA articular cartilage explants were cultured in serum-free DMEM for a 24 hour rest period followed by 24hours at either 20% O₂ (Oxygen) or 5% O₂. Explants were then prepared for IHC and incubated with antibodies specific for SOX9 with brown staining indicative of a positive response. Note: No primary antibody used for negative control