Effect of Hypoxia on Transforming Growth Factor-β signaling Pathway in Osteoarthritic Human Cartilage

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ABBREVIATIONS

AGC1	Aggrecan
ALK	Activin-like Kinase
BGCAN	Betaglycan
BMP	Bone Morphogenic Protein
BMPR-II	Bone Morphogenic Protein type II receptor
cDNA	Complementary DNA
COX2	Cyclooxygenase 2
ECM	Extracellular Matrix
ENG	Endoglin
GDF10	Growth Differentiation Factor 10
HAS2	Hyaluronan Synthase 2
HIF	Hypoxia Inducible Factor
NOV	Nephroblastoma Overexpressed
OA	Osteoarthritis
OAHAC	Osteoarthric Human Articular Cartilage
PAI-1	Plasminogen Activator Inhibitor-1
PCR	Polymerase Chain Reaction
PHD3	Prolyl Hydroxylase 3
pO_2	Oxygen Tension
RUNX2	Runt related transcription factor 2
SMAD	Regulatory Mothers Against Decapentaplegic homolog
TGFBR-II	Transforming Growth Factor-β type II receptor
TGF-β	Transforming Growth Factor-β
VEGF	Vascular Endothelial Growth Factor

Aim and Outline of this thesis

Tissue engineering of cartilage aims to improve regeneration of damaged cartilage by dealing with cell or tissue based cultures. Since cartilage has a poor ability to regenerate itself, to achieve a proper repair of damaged cartilage is quite challenging. Altering the physical environment in tissue or cell culture of cartilage in order to closely mimic its native environment showed promising outcomes in the studies on articular cartilage. Culture designs are another criteria to develop better cartilage tissue engineering strategies. Integration of suitable low oxygen tension, hypoxia, with proper experimental design might be one of the best strategy in order to generate a cell based culture which resembles nature of healthy cartilage.

Firstly, a comprehensive introduction of cartilage tissue and literature survey on effect of hypoxia on articular cartilage was considered in **Section 1**.

In **Section 2**, transforming growth factor-beta (TGF-β) signaling pathway and its function in the case of osteoarthritis was covered.

The main aim of this thesis study is to investigate the effect of hypoxia on transforming growth factor-beta (TGF-β) signaling pathway in human osteoarthritic cartilage. An article format of the thesis was presented in the **Section 3** of this report. In this study we used three dimensional culture of osteoarthritic human chondrocytes. Cells were cultured in a novel bioreactor device for two days at 1% oxygen tension vs 20% oxygen tension. We focused on the effect of hypoxia on the major components of transforming growth factor-beta signaling pathway.

The results obtained during the thesis period but not shown in the Section 3 was presented as **Appendix** in this report in order to account for the readers interest.

CONTENTS

Section 1

Effect of Hypoxia on Articular Cartilage **1-12**

1.1 Introduction 1

1.2 The composition and function of chondrocytes within the articular cartilage tissue 2

1.3 Role of oxygen in articular cartilage 2

1.4 Chondrocyte adaptation to hypoxic environment 4

1.5 Hypoxia effect in Osteoarthritis 6

1.6 *Hypoxia effect on chondrocyte function* 7

Section 2

Transforming Growth Factor-Beta Signaling Pathway **13-15**

2.1 *TGF-β signaling* 13

2.2 *Role of TGF-β in cartilage* 14

Section 3

Effect of TGF-β signaling pathway in Osteoarthritic Human Cartilage **16-29**

3.1 Introduction 17

3.2 Methods 19

3.3 Results 22

3.4 Discussion 24

3.5 Conclusion 28

Appendix

A. Effect of Hypoxia on TGF-β Signaling molecules 30

B. Effect of ALK5 inhibition 32

References 35

Section1

Effect of Hypoxia on Articular Cartilage

1.1 Introduction

Articular cartilage is highly organized avascular tissue. It has main function of protecting the underlying bone by withstanding loading, and providing smooth surfaces close to friction-free movement. The only resident cell type of the articular cartilage is chondrocytes which are responsible for the synthesis and maintenance of extracellular matrix (ECM) whose primary constituents are aggrecans and type II collagen. (Khan *et al*., 2008; Lafont *et al*., 2008). Therefore, the metabolic and structural changes of articular chondrocytes and synthesis and maintenance of ECM play a significant role in the initiation and progression of cartilage pathologies such as osteoarthritis (OA) (Seol *et al*., 2009).

Articular cartilage experiences relatively low level of oxygen than other tissues. Oxygen level within articular cartilage is ranging from 6-10% at the articular surface to less than 1% in the deep zone (Zhou *et al*., 2004; Yodmuang *et al*., 2012). How chondrocytes receive the required amount of oxygen and nutrient for their metabolism depends on diffusion from the synovial fluid that flows through the cartilage tissue during the joint movement. In order to survive in a hypoxic environment, chondrocytes develop mechanisms to sense oxygen availability and adjust the cellular metabolism to consume less oxygen at lower oxygen concentrations (Yodmuang *et al*., 2012).

Studies on chondrocytes in cell or tissue culture have been frequently carried out under atmospheric oxygen tension (21%) which is much higher than the oxygen level in native joints (Grimshaw and Mason *et al*., 2000; Yodmuang *et al*., 2012). Culturing of chondrogenic cells under hypoxia is a recent attempt to understand how chondrocytes behave in different oxygen tensions and to assess how their function may change *in vivo* throughout the depth of the cartilage and in the case of cartilage pathologies (Grimshaw and Mason *et al*., 2000). This section aims to give some insights into not only chondrocyte functions under different hypoxic oxygen tensions, but also chondrocyte adaptation mechanism to low oxygen tension by the help of the previously published literature.

1.2 The composition and function of chondrocytes within the articular cartilage tissue

To know the composition and function of healthy cartilage will help to relate and understand what happens in molecular level and in the case of cartilage pathologies which were mentioned in the represented review.

Articular cartilage is a soft tissue which contains 60-80% water (Getgood *et al*., 2009). The primary constituents of articular cartilage other than water are chondrocytes, and ECM (Khan *et al*., 2008; Getgood *et al*., 2009). Cartilage extracellular matrix is arranged as a network of collagen fibers which provide tensile strength to the tissue and proteoglycans that are able to withstand compressive forces (Muir *et al*., 1995; Poole *et al*., 1997; Gibson *et al*., 2008).

Articular cartilage is a relatively thick tissue with a thickness 2.3 ± 0.5 mm in human femoral condylar cartilage (Stockwell *et al*., 1971; Hunziker *et al*., 2002). The zonal structure of articular cartilage is divided into three unmineralised layers; the superficial zone, the transitional/middle zone and the deep zone (Khan *et al*., 2008).

Cartilage is a heterogeneous tissue which has a depth dependent composition and structure, and it shows anisotropic and nonlinear mechanical properties within the cross section of the tissue (Boshetti *et al*., 2004). Each zone of cartilage posesses different predominat mechanical function and cellular orientation. In superficial zone, chondrocytes are small, flattened and at a high density. Collagen fibrils in that zone are oriented parallel to the articular surface and possess high tensile strength to withstand the tensile stresses during the joint loading (Klein *et al*., 2009). In addition to this, the combination of horizontal cellular orientation together with collagen fibers in superficial zone, provide resistance to shear forces (Getgood *et al*., 2009). In the transient zone, cells are more spherical and randomly oriented as collagen fiber orientation in that zone. In the deep zone, cellular distribution is vertical and chondrocytes are larger compared to the other zones. Collagen fibers are perpendicular in that zone (Klein *et al*., 2009; Klein and Chaudhry *et al*., 2009).

1.3 Role of oxygen in articular cartilage

Oxygen is an important molecule for all higher organisms. It is essential for the oxidative phosphorylation which requires molecular oxygen as an electron acceptor in cellular energy production. Oxygen molecule is not only used for an electron acceptor but also used as a

substrate for the enzymatic reactions (Mitchell *et al*., 1961). Therefore, oxygen homeostasis is required for the maintenance of the cells in the higher organism (Murphy *et al*., 2009).

Articular cartilage experience relatively low levels of oxygen than the other tissues. Oxygen level of articular cartilage is ranging from 6-10% at the articular surface to around 1% in the deep zone (Milner *et al*., 2012). Changes in oxygen tensions, even as little as 1-2%pO_2 difference, can alter the chondrocyte metabolism and function (Fermor *et al*., 2007; Coyle *et al*., 2009). Although 5% oxygen tension seems to be optimal for chondrocyte maintenance, both excessively high and low O_2 tensions can be deleterious for chondrocyte survival and maintaining of a proper metabolic state (Milner *et al*., 2006). Nevetheless, oxygen tensions $\leq$ 5% are often referred as "hypoxia" which is misleading for the reported effects of oxygen tension on cartilage tissue (Fermor *et al.* 2007). Therefore, there is a need to discriminate the oxygen tensions within articular cartilage with respect to its physiological values. Even though there is no clear discrimination of physiological oxygen tensions of cartilage, low O_2 tension (5%), which is considered hypoxic for other tissues, can be considered as normoxic for articular cartilage (Milner *et al*., 2006). Kay and his colleagues introduced physiological cartilage normoxia as 2% O_2 in their study (Kay *et al*., 2011). Based on the literature and oxygen concentrations within the healthy human cartilage depth (Kay *et al*., 2011; Milner *et al*., 2006; 2012; Grimshaw and Mason et al., 2000; Yodmuang *et al*., 2012), physiological cartilage normoxia, hypoxia and hyperoxia can be defined as 2-6% O_2, <2% O_2 and $\geq$ 10% O_2 respectively.

Due to its avascular nature, little oxygen is available for the chondrocyte metabolism. Some of that available oxygen is used for energy production by oxidative phosphorylation. Oxidative phosphorylation takes place in mitochondria (Calderon-Montano *et al*., 2011) thus, occupation of mitochondria in intacellular volume is an important factor for the cells to determine their major energy metabolism. In articular chondrocytes, mitochondria occupies only ~2% of the intracellular volume, compared to 15‑20% intracellular mitochondrial volume of other animal tissues such as liver (Seol *et al*., 2009). Aerobic energy generation is just a minor contributor to the overall energy production by articular chondrocytes due to lack of mitochondria (Schneider *et al*., 2007; Marcus *et al*., 1973). On the other hand, substrate level phosphorylation does not require mitochondria to generate energy; it takes place in cytosol (Calderon-Montano *et al*., 2011). Therefore, up to~95% of chondrocyte energy comes from glycolysis (Heywood *et al*., 2010; Lee and Urban *et al*., 1997; Milner *et al*., 2012).

When there is an excessive amount of oxygen available in cellular environment, to know how chondrocytes consume oxygen would be beneficial in order to understand the role of oxygen for cartilage tissue. It was shown that oxygen consumption rate was independent of oxygen tension when is between 5-21 % and the oxygen consumption rate of bovine chondrocytes slightly increased when oxygen tension was increased from 0% to 5%. Similarly the oxygen consumption rate of porcine articular chondrocytes dropped as oxygen levels fall below 5% (Kuo *et al*., 2011). It can be concluded that oxygen consumption slightly increases when oxygen tension increases from 0 to 5 % and stays stable for the values ≥ 5% pO_2 which suggests 5 % pO_2 as threshold value for the stable oxygen consumption in mammalians.

1.4 Chondrocyte adaptation to hypoxic environment

In order to survive and being able to maintain the cartilage phenotype in hypoxic environment, articular chondrocytes develop highly adaptive mechanisms to sense oxygen availability and to activate a metabolic pathway which requires minimum amount of O_2 (Wang *et al*., 1995; Milner *et al*., 2012; Yodmuang *et al*., 2012). This pathway facilitates adaptation to hypoxia-induced physiological stress by regulating changes in certain gene expressions that are responsible for survival and maintenance of chondrocytes (Bruick and McKnight *et al*., 2002). A family of hypoxia-inducible transcription factors (HIFs) is essential for this adaptive pathway (Bruick and McKnight *et al*., 2002; Pfander *et al*., 2007). Hypoxia-inducible factors (HIFs) also play an important role in cellular adaptation during limb development of mice (Schipani *et al*., 2001; Amarilio *et al*., 2007). Hypoxic induction of hypoxia-inducible factor has first studied on erythropoietin (EPO) more than two decades ago (Semenza and Wang *et al*., 1992). Due to its hypoxic nature, studying HIF mechanism on chondrocytes gained more attention since last decade.

The HIFs are composed of two subunits: oxygen sensing hypoxia-regulated-α subunit (HIF-1α) and the oxygen-insensitive β subunit (HIF-1β) (Bruick and McKnight *et al*., 2002). Under hyperoxia (21%pO_2), HIF-1α protein has a short half-life of less than 5 minutes (Ke and Costa *et al*., 2006; Lafont *et al*., 2010). When the concentration of oxygen is high (< 10%) in the cellular environment, then HIF-1 α is hydroxylated by prolylhydroxylases (PHDs) (Lafont *et al*., 2010). Together with hydroxylated proline Von Hippel- Lindau protein (pVHL), hydroxylated HIF complex is degraded by proteasome. When oxygen level is low (~5–1% pO_2), hydroxylation of HIF-1 α is prevented. Then, HIF-1 α heterodimerizes with HIF-1β and that HIF complex translocate from the cytoplasm to the nucleus. In the nucleus,

the HIF complex binds to specific hypoxia responsive elements (HRE) and induces its specific target gene expressions like glucose transporters (*GLU-1*), and transcription of the specific growth factors such as vascular endothelial growth factor (*VEGF*), insulin-like growth factor-2 (*IGF2*), and transforming growth factor-α (*TGF- α*) (Ke and Costa *et al*., 2006; Lafont *et al*., 2010; Lando *et al*., 2002; Schpani *et al*., 2001).

Under hypoxic conditions, presence of HIF-1 α is a crucial survival factor for the cells of various tissues such as kidney, liver, and heart (Stroka *et al*., 2001; Lafont *et al*., 2010). As described earlier, HIF is has a major effect on adaptation of chondrocytes to the hypoxic conditions (Bruick and McKnight *et al*., 2002). Absence of HIF1- α leads to a loss of energy generation by glycolysis and this causes cell death starting at the deep zone in cartilage (Pfander *et al*., 2007). Furthermore, Zuscik et al., reported that transcription of HIF-1 α in chondrocytes and mesenchymal stem cells inhibited proliferation, but increased ECM production (Zuscik *et al*., 2008). Thus, low oxygen tensions (<5%) is likely to enhance the expression of chondrogenic markers and promote chondrocyte phenotype (Zuscik *et al*., 2008; Milner *et al*., 2006).

Glucose is another essential molecule for cartilage metabolism. Changes in concentration of either oxygen or glucose in the environment cause to an adaptation mechanism in the chondrocytes. For example, inhibition of glycolysis by glucose deprivation enhances oxygen consumption of chondrocytes (Lee and Urban *et al*., 1997; Heywood *et al*., 2006; 2010). As a survival mechanism chondrocytes initiate an oxidative energy metabolism to obtain basal ATP requirements when glucose supply is limited (Heywood *et al*. 2006; Otto *et al*., 1991) .Conversely, oxygen consumption within articular cartilage is inhibited by high level of glucose in the environment (Heywood *et al*., 2006). This adaptation was decribed as "Crabtree effect" which is also called as "negative Pasteur effect" (Heywood *et al*., 2006). In the case of hypoxia, regarding to Crabtree effect it is expected to see more ATP production by glycolysis than the condition of 1% oxygen tension. Yudoh and his collogues studied on the relation between glucose and oxygen at 1% pO_2 in osteoarthritic human chondrocytes. They showed that significant increases of lactic acid and glucose transporter-1 mRNA levels under hypoxic culture conditions compared with normoxic culture conditions (Yudoh *et al*., 2005). In contrast, HIF- 1α-deficient chondrocytes showed a complete loss of the induced increases in glycolytic activities even under hypoxic culture conditions (Yudoh *et al*., 2005). *In vitro* data of this study clearly indicated that expression of HIF-1α is responsible for the energy generation of hypoxic chondrocytes. It can be concluded that HIF-1α activity is essential for

regulation of glycolysis which is also important for cellular matbolism and energy generation in articular chondrocytes under 1% pO_2.

1.5 Hypoxia effect in Osteoarthritis (OA)

Osteoarthritis is a heterogeneous degenerative joint disease which mostly occurs in older people (Khan *et al*., 2008). During the daily activities, mobilization of healthy articular joints increases the oxygenation of the joints (Fermor *et al*., 2007). On the other hand, inflammatory mediator molecules decrease the oxygen tension in arthritic joints (Stevens *et al*., 1991; Fermor *et al*., 2007). Therefore, it is likely that rheumatoid arthritic (RA) and osteoarthritic joints are more hypoxic than healthy cartilage (James *et al*., 1990; Fermor *et al*., 2007).

Depending on the osteoarthritic stage, chondrocytes can be considered as still being metabolically active. While osteoarthritic chondrocytes have increased synthesis of matrix-destructive enzymes, they also show enhanced gene expression of type II collagen and several other matrix components like aggrecan and biglycan in deep zone to compensate the loss of these components in the superficial zone in early osteoarthritis (Aigner *et al*., 2001; Pfander *et al*., 2007; Poole *et al*., 2006). This activity of osteoarthritic chondrocytes is generally appreciated as a response to ECM repair (Pfander *et al*., 2007). In the study by Pfander *et al.*, the synthesis of type II collagen and HIF-1α were found to be increased significantly when subjected to hypoxia during osteoarthritis (Pfander *et al*., 2007).

Synovial fluids from osteoarthritic joints contain less oxygen than synovial fluids from healthy joints (Pfander and Gelse *et al*., 2007). This hypoxia effect results high expression levels of HIF-1α and HIF-1α target genes such as vascular endothelial growth factor (*VEGF-A*), *GLUT-1* and phosphoglycerate kinase-1 (*PGK1*) in both synovial lining cells and chondrocytes (Pfander and Gelse *et al*., 2007; Pfander *et al*., 2005). Another study reported that HIF-1α transcriptional activity increases paralell to the progression of OA by demonstrating increased VEGF-A synthesis in late stages of OA (Pfander *et al*., 2001; 2006). Grimmer and her collagues studied hypoxia effect in ostearthritic human articular cartilage (OAHAC) both *in vivo* and *in vitro* by applying 1% O_2. Both in vivo and in vitro conditions showed increased synthesis and accumulation of type II collagen (Grimmer *et al*., 2006). Similarly, osteoarthritic human articular chondrocytes maintained in Hyalografts for 21 days at 5%pO_2 showed enhanced expression of the *COL2A1*, while levels of *COL1A1* which is a differentiation marker for chondrocytes were significantly suppressed compared to 21% pO_2

condition (Katopodi and Hardingham *et al*., 2005). On the other hand, *SOX9* expression levels were not altered under any of the conditions (Katopodi and Hardingham *et al*., 2005).

1.6 Hypoxia effect on chondrocyte function

1.6.1 Hypoxia effect on chondrocyte metabolism and energy production

Maintaining the cellular metabolism is essential for all the cell types. Cells need to obtain their nutrients to generate energy for the cellular functions. Regarding to studies (Domm *et al*., 2002; Merceron *et al*., 2010; Malladi *et al*., 2006), culturing of chondrocytes or stem cells under 5% O_2 tension for short durations is good for chondrogenic characteristic. On the other hand, the effect of reduced oxygen tension (less than 5%) is still not clear. Difference in the levels of oxygen tension within the physiological range of articular cartilage can exhibit different inferences about the influence of oxygen on chondrocyte metabolism.

Hypoxia is known to regulate the expression of chondrogenic marker genes, but little is known about chondrocyte survival and proliferation in hypoxic conditions. Seol and his colleagues exhibited that 1% pO_2 inhibited the apoptosis of primary cultured healthy canine chondrocytes induced by proteasome inhibitor and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) treatment compared to standard oxygen tension (~20%) and chondrocyte survival was enhanced by 25% under 1% pO_2 compared to the survival of cells under 20% pO_2 (Seol *et al*., 2009). Another study reported that at the end of 5 days alginate bead culturing, 60% of equine chondrocytes survived at 1% oxygen tension even in the glucose free experimental conditions (Schneider *et al*., 2007). For *in vitro* tissue engineering strategies, to achieve high survival rates is fundamental to obtain enough number of cells to do down stream analysis. Regarding to aforementioned studies on chondrocyte survival rate, 1% pO_2 seems like a good culturing condition that chondrocytes survive and adapt to the condition easily. This might be because of the hypoxic nature of the chondrocytes in the body.

Imposition of hypoxia on chondrocyte culture enhanced glucose utilization due to increase in the rate of energy production by glycolysis in chondrocyte metabolism. Chondrocytes in low oxygen tensions were found being capable of maintaning the cellular energy charge (Rajpurohit *et al*. 1996). Furthermore, Rajpurohit and his coworkers noted that energy conservation and expression of HIF and activator protein-1 (AP-1), which controls cellular processes including proliferation, differentiation and apoptosis, appeared to be near-maximum at low oxygen tensions (Rajpurohit *et al*. 1996).

The study of Yudoh *et al.* reported that HIF-1α is necessary for anaerobic energy generation based on the upregulation of glycolytic enzymes and glucose transporters. Human osteoarthritic articular chondrocytes cultured under hypoxia (1% pO_2) exhibited significant increase of lactic acid concentration and glucose transporter-1 (*GLU-1*) mRNA expression compared to 20% pO_2 culture condition. Additionally, the deficiency HIF-1α led to a significant loss in glycolytic activities under hypoxic culture conditions which demonstrated the necessity of HIF-1 α for energy generation in chondrocytes under 1 % pO_2 (Yudoh *et al*., 2005). Hypoxia enhanced gene expression of glucose transporters. This allowed chondrocytes to enhance their glucose intake and glycolytic rate, thereby providing sufficient amounts of ATP which is the fundamental prerequisite for cell survival (Pfander *et al*., 2007).

On the other hand, Berniakovich and coworkers studied the proliferation rate of mouse bone marrow derived mesenchymal stem cells (BMSC). They showed that the proliferation rate of BMSC was higher in 3% O_2 than in 21% O_2. Furthermore, culturing BMSCs in 3% O_2 for 4 days as an initial phase of cell propagation, and switching to 21% pO_2 for 3 further days exhibited enhanced differentiation of mouse BMSCs (Berniakovich *et al*., 2013). This study concluded that 3%pO_2 increased cell proliferation initially and the differentiation process was inhibited in 3% pO_2 but enhanced when 21%pO_2 was applied (Berniakovich *et al*., 2013). Differentiation of stem cells to chondrocytes is essential for developing stem cell based cartilage tissue engineering researches, regarding to this study reoxygenation is necessary after sufficient cell proliferation is achieved under hypoxia.

1.6.2 Hypoxia effect on cartilage biomechanics

Studying biomechanics of cartilage might help to assess the responses of cartilage to different oxygen tensions like %5 and 20% pO_2. According to Boschetti *et al.*, biomechanical parameters like Young's modulus (E_y) and aggregate modulus (H_A) which is a measure of the stiffness of the tissue determined by confined compression test at equilibrium when all fluid flow has ceased, increased through surface to deep zone in healthy human articular cartilage (Boschetti *et al*., 2004). As previously mentioned, oxygen tension also varies within the cartilage depth therefore oxygen tension might have an effect to cartilage mechanical properties. Removal of articular cartilage from the joint and culturing the cartilage under standard culture conditions (20% O_2) for 6 days led to a significant reduction in the H_A (Fermor *et al*., 2007). As another experimental condition, after 3 days of incubation at 20% O_2, the cartilage explants were swtiched to 5% O_2 condition for further 3 days. The results of this study showed that after further 3 days of incubation at 5% O_2 recovered cartilage from H_A

decrease which 3 days of preincubation at 20% O_2 caused and it increased aggregate modulus to a level similar to freshly joint. On the other hand, culturing chondrocytes at 1% pO_2 for a further 3 days did not show a significant effect as 5% pO_2 did, it almost maintained the value of H_A after 3 days culturing at 20% pO_2 (Fermor *et al*., 2007). In a study by *Yodmuang et al.*, the Young's modulus (E_y) of cartilage was tested in 3 different culturing conditions which are 21% pO_2 for 28 days, 5% pO_2 for 28 days and transient hypoxia—reoxygenation (5% pO_2 for 7 days and 21% pO_2 for 21 days). In the reoxygenation and 21% pO_2 group, at the day 28, E_y was significantly higher than the Young's modulus of the group cultured at 5%pO_2. In the reoxygenation condition Young's modulus has the highest value among the other groups in the experiments (Yodmuang *et al*., 2012). Histology analysis of collagen type II content of chondrocytes under these three different conditions also showed the similar effects as Young's modulus tests showed. This supports the relation of collagen type II with biomechanics of cartilage since one of the important function of collagen type II is to provide tensile strenght to the tissue. Based on those two studies (Fermor *et al*., 2007; Yodmuang *et al*., 2012), it is possible to have an idea about hypoxia effect on mechanical parameters; for the short-term (a few days) exposure of 5% pO_2 on healthy cartilage leads to increase the stiffness of articular cartilage. Long term culturing might require a switch from hypoxia to an elevated oxygen tension in order to maintain stiffness of cartilage in a certain range. Since hypoxia effect on mechanical parameters is not clear due to lack of studies on biomechanics parameters of chondrocytes there is a need to have more studies on mechanical parameter when articular cartilage tissue is treated with low oxygen tensions.

1.6.3 Hypoxia effect on chondrogenic markers

The main aim of tissue engineering based studies is to achive proper functioning cells and tissues which mimic the native cartilage. Studying chondrocyte specific marker expression is the essential goal to validate *in vitro* results with native chondrocyte.

As cartilage has different chondrocyte phenotypes within cartilage depth, expression of the chondrogenic markers also differs within cartilage. In the superficial zone, healthy cartilage expresses proteoglycan 4 (*PRG4*), whereas transient zone markers are *SOX9*, collagen type II, and aggrecan (Zuscik et al., 2008). On the other hand, in the deep layer of cartilage which is close to subchondral bone, expression of collagen type X is dominant (Zuscik *et al*., 2008; Schneider *et al*., 2007).

Only a small portion of *in vitro* studies has been performed with zonal chondrocytes (Schuurman *et al*., 2012; Schuurman *et al*., 2009; Malda *et al.* 2010; Stockwell *et al*., 1971;

Hunziker *et al*., 2002). Those studies reported encouraging outcomes to make zonal harvesting and culturing chondrocytes more prevalent. For instance, equine chondrocytes isolated from 3 zones and cultured in alginate beads showed that cartilage can restore zone specific matrix expression (Schuurman *et al*., 2009). There is only one recent study (Schrobback *et al.* 2012) which has been performed to evaluate zonal marker and hypoxia related gene expressions of human articular chondrocytes which were harvested and cultured at 5% and 20% oxygen tensions by taken into account of zonal discriminations. Chondrocytes isolated from full-thickness, superficial, and middle/deep cartilage obtained from knee replacement surgeries were expanded and redifferentiated under 5% pO_2 and 20% pO_2 separately. Differentiation under 5% pO_2 increased expression of HIF-1α and HIF-2α in middle/deep chondrocytes (Pfander et al., 2007; Schrobback *et al.*, 2012). It is known that chondrocytes in deep layer experience lower oxygen tension (Zhou *et al.*, 2004) than chondrocytes in superficial layer and gene expression of HIF-1α is more when oxygen tension are low in the environment. In addition to this, favored expression of proteoglycan 4 (*Prg4*) by superficial chondrocytes compared to chondrocytes in transient and deep layers was reported (Schrobback *et al.*, 2012). Although studying zonal organization of cartilage *in vitro* is challenging due to the lack of specific zonal cell sorting protocols and limited amounts of cells harvested per zone (Schuurman *et al.*, 2012; Malda *et al.* 2010), reported results of Schrobback et al. gave a huge hope that mimicking the zonal organization of articular cartilage most likely to enhance regenerative approaches for the treatment of cartilage defects.

Aggrecan is one of the most abundant component of ECM of cartilage tissue. Blakeney and his collugues reported ~5-fold increased aggrecan (*ACAN*) gene expression in osteoarthritic human cartilage maintained for ten days in micromass culture under 5% oxygen tension compared to 20% pO_2 culture. This study also showed 4-fold increase in *ACAN* expression when normoxic-cultured cells were transfered to 5% O_2 conditions (Blakeney *et al*., 2011).

It is known that cartilage tissue engineering suffers some intrinsic problems during chondrocyte culturing period. Chondrocyte-specific phenotype is lost during monolayer expansion, while cell proliferation is essential to gain sufficient number of cells for tissue engineering (Schulze-Tanzil *et al*., 2009 ; Qusous and Kerrigan *et al*., 2012). Dedifferentiated chondrocytes can be redifferentiated using three dimensional cultures while cell proliferation is then suppressed (Schulze-Tanzil *et al*., 2009). To make cartilage tissue renowned its function, a number of researchers have been trying to solve the intrinsic problems in chondrocyte tissues by using the tissue engineering techniques (Hunziker *et al*., 2001;

Hunziker *et al*., 2002). Studying both dedifferentiation and re-differentiation steps under hypoxia is a good attempt to elucidate hypoxia effects on chondrocytes.

Murphy and Sambanis investigated the effect of oxygen tension on bovine articular chondrocytes during 20 day monolayer culture period under 1%, 5% and 20 % oxygen tensions. The amount of glycosaminoglycan (GAG) accumulation in the matrix was lower at 1% and 5% pO_2 compared to 20 % pO_2 (Murphy and Sambanis *et al*., 2001). In a different study, human adipose derived stems cells are cultured as pellets in the presence of chondrogenic medium for 4 weeks (Merceron et al., 2010). This study showed no significant difference between 5% and 20% oxygen tensions when the pellet sections were stained with Alcian blue which a staining for determination of presence of GAG in the samples (Merceron et al., 2010). In contrast, type II collagen immunostaining appeared highly positive for the pellets cultured at 5% pO_2. Furthermore, 5% oxygen tension induced gene expression of type II collagen one week earlier than 20% oxygen tension (Merceron et al., 2010). Yodmuang and his group studied gene expressions of *COL2A1*, *ACAN* and *SOX9* of bovine articular chondrocytes seeded in 3D mhydrogels in three different experimental conditions which are at hypoxia (5% pO_2 for 4 weeks), normoxia (21% pO_2 for 4 weeks) and reoxygenation (5% pO_2 for one week and 21% pO_2 for 3 weeks) (Yodmuang et al., 2012). Reoxygenation condition showed increased proteoglycan and collagen type II gene expressions compared to either 21% or 5% pO_2 group (Yodmuang et al., 2012). Similarly, 3D micromass culture model of adipose-derived stem cells showed a markedly decrease immunostaining of type II collagen and extracellular matrix proteoglycan under 2% oxygen tension at the end of day 12 (Malladi et al., 2006).

Moreover, at the end of 20-day culture period at 1% pO_2, decreased hyaluronan synthesis on bovine articular chondrocytes compared to 20% pO_2 was reported (Murphy and Sambanis *et al*., 2001). Similarly, other researchers found a huge decrease in hyaluronan synthesis after 2D culturing bovive articular chondrocytes for 12 hours at 1% pO_2 compared to 5% pO_2 and 20% pO_2. On the other hand, they exhibited a highly significant increase in hyaluronan synthesis under 5% pO_2 (Hashimoto *et al*., 2006).

Domm and his collogues worked on both monolayer and alginate bead culture of bovine articular chondrocytes which were subjected to either 21% or 5% O_2 for two weeks (Domm *et al*., 2002). They showed that protein expression of collagen type II (COL2) which is the best accepted chondrogenic marker (Lu *et al*., 2010) was not produced by either dedifferentiated monolayer chondrocytes under 5% or 21% oxygen or under 21% oxygen in 3D alginate bead culture. However, differentiated cells in alginate beads subjected to 5% oxygen tension

exhibited a strong COL2 expression (Domm et al., 2002). Additionally, other ECM markers like collagen type IX and GAGs are also found to be higher for alginate beads at 5%pO_2 condition (Domm et al., 2002). This study suggests oxygen is an important parameter in cartilage tissue engineering and post-culturing of dedifferentiated bovine articular chondrocytes in alginate beads under 5% oxygen supports redifferentiation and matrix-production by cartilage cells (Domm et al., 2002). Those results indicates increased expressions of ECM components and chondrogenic characteristic by performing 3D culturing under hypoxia rather than 2D culture.

Foldager et al. also compared monolayer and 3D hypoxic cultures; quantitative RT-PCR results showed an increased expression of cartilage-specific genes such as Sox9, collagen type II, and aggrecan in 3D culture. Das et al. reported that after a 5-day alginate bead cultured human osteoarthritic chondrocytes showed a slightly increased expression of chondrogenic marker genes such as aggrecan, collagen type II, Sox9 and VEGF under 5% pO_2 compared to 20% pO_2 (Das et al., 2010). The essential role of Sox9 in hypoxic induction of the differentiated chondrocyte phenotype was investigated by revealing a Sox9 dependency of HIF-2α expression (Lafont et al., 2007).

Almost all of the studies mentioned above make the same conclusion; applying %5pO_2 and culturing chondrocytes in 3D together up-regulates most of the ECM macromolecules and chondrocyte specific markers. While 20% oxygen tension can increase GAGs for long term culturing like 3-4 weeks, 5% pO_2 upregulates type II collagen in matrix. However effects of oxygen tensions less than 5% is still not clear. Studies performed to compare effects of hypoxia within the range of 0-5% pO_2 is required to obtain best conditions for culturing to improve the cell/tissue based tissue engineering cartilage repair strategies.

In the course of cell-based repair strategies of cartilage this common conclusion helps achieving the closest mimicking the native chondrocyte.

Section 2

Transforming Growth Factor-Beta (TGF-β) Signaling Pathway

2.1 TGF-β Signaling

Transforming growth factor-β (TGF-β) is a pleiotropic cytokine that is expressed by all cells and tissues in all higher organisms (Jenkins *et al*., 2008). When TGF-β is synthesized, it is in an inactive form which is called as latent TGF-β. The latent form composes of TGF-β pro-protein itself and its propeptide which is know as latency associated peptide (LAP). Further association of latent TGF-β binding protein (LTBP) with TGF-β-LAP complex by formation of disulfate bonds gives rise to a large latent complex which becomes covalently attached to ECM proteins (Finnson *et al*., 2012). Inactive TGF-β is unable to carry out its signaling function. Therefore, latent TGF-β needs to be activated in order to involved in physiological functions such as healing, development, growth and maintenance of various cells and tissues (Jenkins *et al*., 2008; Finnson *et al*., 2012). The activation of TGF-β is a complex and tightly regulated process (Jenkins *et al*., 2008). Latent TGF-β can be activated by a number of physical processes including reactive oxygen species, and biological processes such as proteolysis (Jenkins *et al*., 2008; Annes *et al*., 2003). In a study by Annes and his coworkers (Annes *et al*., 2003), activation of TGF-β was conceptualized as a sensor model. In this model, latent TGF-β was considered as an ECM-localized sensor and it comprises LTBP as a localizer, LAP as a detector and TGF-β as an effector. They stated that failure to localize latent TGF-β complex resulted in altered activity of TGF-β. The primary role of TGF-β is to 'report' an alteration of the ECM. Therefore, latent TGF-β as a sensor responds to ECM damage or perturbations in ECM. The responds are mostly activation of TGF-β by activator molecules to start TGF-β signaling in order to regulate mechanisms in cellular milieu (Annes *et al*., 2003).

Transforming growth factor (TGF)–β and related factors of it have attracted more attention since last decade because of their functions as regulation of proliferation, differentiation, adhesion and apoptosis of the cells in a wide range of organisms (Derynck et al., 1998; Messagué and Chen et al.,2000; Miyazono et al., 2000). Members of transforming growth factor beta family include TGF-βs, activins and inhibins, nodal, myostatin, bone morphogenetic proteins (BMPs), growth/differentiation factors (GDFs), and anti-Müllerian hormone (AMH) (Miyazawa *et al*., 2002). Signal transduction by these factors involves four classes of molecules: a family of membrane receptor serine/threonine kinases (type I and type II TGF-beta receptors), auxiliary membrane receptors (endoglin and betaglycan), a family of

cytoplasmic proteins (the Smad family) that serves as substrates for these receptors, and nuclear DNA-binding factors that associate with Smads forming transcriptional complexes (Chen *et al*., 1998; Finnson *et al*., 2010). Signaling is initiated by specific binding of cytokines of the TGF-β superfamily to a specific pair of type II receptor kinases. This complex induces the phosphorylation and activation of type I receptor kinase (Chen *et al*., 1998). The activated type I receptor phosphorylates a specific regatulatory Smad (R-Smads). Type I receptors for TGF-β ligand such as ALK5 and ALK1 recognize Smad2 and Smad3, whereas type I receptors for BMP ligand such as ALK3 and ALK6 recognize Smad1, Smad5, and Smad8 (Massague *et al*., 2000; Miyazawa *et al*., 2002). Then R-Smads associate with the related protein Smad4 (Co-Smad) and move into the nucleus. (Chen *et al*., 1998). Smad complexes accumulate in the nucleus, where they regulate gene transcriptions by recruiting transcriptional coactivators or inhibitors to DNA (Zhang *et al*., 2003). Because of the diversity of processes controlled by different TGF-β family members, this signaling pathway is largely responsible for context-specific effects of the TGF-β family of proteins (Zhang *et al*., 2003; Chen *et al*., 1998).

2.2 Role of TGF-β in cartilage

Transforming growth factor-beta (TGF-β) plays a significant role in promoting chondrocyte anabolism *in vitro* and *in vivo* (Grimaud *et al*., 2002). Transforming growth factor-beta is a multifunctional regulator of cartilage repair and homeostasis with its critical roles in chondrocyte proliferation, differentiation and extracellular matrix (ECM) production. (Finnson *et al*., 2010). TGF-β also inhibits terminal differentiation. Therefore, it blocks endochondral bone formation (van der Kraan *et al*., 2010; van der Kraan *et al*., 2009). It has been reported that TGF-β signaling not only stimulated production of type II collagen and aggrecan, but also blocked degradation of ECM proteins by increasing production of protease inhibitors (van der Kraan *et al*., 2010; Finnson *et al*., 2012). These actions of TGF-β suppress the effect of OA. Therefore, TGF-β may potentially promote cartilage integrity and repair (Blom *et al*., 2007). Deficient TGF-β signaling has been implicated in a number of cartilage-related disorders including rheumatoid arthritis and osteoarthritis (OA). (Finnson *et al*., 2012). It has been shown that disruption of TGF-β signalling led to the development of degenerative joint disease resembling human osteoarthritis (OA) in mice (Serra *et al*., 1997). However, positive regulation of TGF-β is not valid in late stages of OA (van der Kraan *et al*., 2009).

It has been reported that OA caused deregulation of TGF-β signaling pathway by altering function or expression of TGF-β signaling molecules (Finnson *et al*., 2012). Van der Kraan et

al., explained the dual effects of TGFβ on chondrocytes by the fact that TGFβ can signal via different receptors and related Smad signaling routes (van der Kraan *et al*., 2010). In this study, it has been also exhibited that signaling via ALK5 (Smad2/3 route) resulted in markedly different chondrocyte responses than ALK1 signaling (Smad1/5/8) (van der Kraan *et al*., 2010). Furthermore, ALK1 mRNA expression showed highly correlation with levels of matrix metalloproteinase 13 (MMP-13) which is an enzyme responsible for breakdown of extracellular matrix and highly expressed in the case of OA whereas ALK5 mRNA levels correlated with aggrecan and collagen type II level. (Finnson *et al*., 2012). In another study, it has been found that not only progression of osteoarthritis but also ageing was correlated with loss of ALK5 and Smad 3 expression in murine articular cartilage (Blaney Davidson *et al*., 2009). Conversely, expression of ALK1 did not decrease with ageing as ALK5 in this study. They also reported the increased ratio of ALK1/ALK5 in OA articular chondrocytes (Blaney Davidson *et al*., 2009). These articles mutually stated that loss of the Smad2/3 signaling and relatively enhanced Smad1/5/8 signaling can be the root of the OA process and the ratio of ALK1/ALK5 is an important criteria to determine the overall effect of TGF-β in chondrocytes (van der Kraan *et al*., 2010; Blaney Davidson *et al*., 2009). In addition, inhibitory Smads (Smad6 and Smad7) have crucial role in negative regulation of TGF-β signaling. Smad7 inhibits both signaling routes, whereas Smad6 only inhibits Smad1/5/8 signaling (van der Kraan *et al*., 2009; Li *et al*., 2003).

Like type I receptors and Smad molecules, function and expression of type II and type III receptors are also altered in OA. TGF-β type II receptor (TβRII) levels were shown to be decreased in human OA cartilage (Verdier *et al*., 2005). It has been found that deficient TβRII led to terminal chondrocyte differentiation and the development of OA-like features in mice skeletal tissue (Serra *et al*., 1997). On the other hand, it has been reported that endoglin protein levels were enhanced in human OA cartilage as compared to normal cartilage (Finnson *et al*., 2010). Expression and function of betaglycan in the case of ostearthritis is poorly understood. However, in a rat model of OA, it has been reported that betaglycan levels did not change (Appleton *et al*., 2007; Finnson *et al*., 2012).

It can be concluded from the studies that targeting the specific components of TGF-β signaling may potentially promote therapeutic strategies in order to inhibit the progression of the disease in articular cartilage.

Section 3

Effect of Hypoxia on Transforming Growth Factor-β Signaling Pathway in Osteoarthritic Human Cartilage

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Abstract

Objective: Due to avascularity, chondrocytes have poor ability to regenerate themselves which is challanging for tissue engineering to achieve full functioning cartilage. Culturing chondrocytes or cartilage tissue has been frequently perfomed by using atmospheric oxygen tensions which is hyperoxic compared to nature of healthy cartilage. Implementing hypoxia as culturing condition closely mimics the environment of native cartilage. Not only culturing conditions, but also the cellular mechanisms such as TGF-β signaling is essential to study the regulations of cellular activities of chondrocytes. The purpose of the present study was to investigate the effect of hypoxia on both chondrogenic markers and TGF-β signaling in human osteoarthritic chondrocytes.

Methods: Osteoarthritic human articular chondrocytes were cultured in alginate beads in a microbioreactor for 48 hours. Hypoxic oxygen tension (1% pO_2) and 20% pO_2 (control) was used as experimental conditions. Gene expression levels of hypoxia targets, chondrogenic markers, and TGF-beta signaling were determined by quantitative RT-PCR.

Results: Chondrocytes cultured under hypoxic condition showed upregulation of both chondrogenic markers and hypoxia targets which is an indication for good adaptation of chondrocytes to hypoxia. Gene expressions of crucial molecules of TGF-beta signaling was enhanced by hypoxia.

Conclusion: Hypoxia enhances chondrogenic markers and expression of major components of TGF-β signaling in 3D culture of osteoarthritic human chondrocytes. Targeting enhancement specific components of TGF-β signaling by implementing proper experimental conditions like hypoxia may represent a suitable therapeutic strategy for the treatment of osteoarthritis.

Keywords: Chondrocyte, Hypoxia, TGF-β, Osteoarthritis

3.1 Introduction

Articular cartilage is highly organized avascular tissue which protects the underlying bone by withstanding to loading and provides close to friction free movement. There is no other cell type than chondrocytes in cartilage tissue for the repair mechanism in the case of cartilage damage or disease (Khan et al., 2008; Lafont et al., 2008). Due to avascularity, chondrocytes have a poor ability to regenerate themselves and it is challenging to restore full tissue function when it is damaged. The field of cartilage tissue engineering aims to repair or regenerate damaged tissue and thereby halt disease progression and restore functionality for a prolonged time. This field has attacted great interest for improving articular cartilage therapy (Zhang *et al*., 2009; Getgood *et al*., 2009). Therefore, chances of mimicking the cartilage native environment is needed to restore the functionality of the tissue.

Due to its avascular nature, chondrocytes receive the required amount of oxygen and nutrients for their metabolism by diffusion from the synovial fluid that flows through the cartilage tissue during the joint movement. Therefore cartilage tissue experiences relatively lower level of oxygen than other tissues (Milner *et al*., 2012; Yodmuang *et al*., 2012). Oxygen tension within articular cartilage is ranging from 6-10% at the articular surface to around 1% in the deep zone (Grimshaw and Mason *et al*., 2000; Zhou *et al*., 2004). In order to survive and being able to maintain the cartilage phenotype in hypoxic environment, articular chondrocytes develop highly adaptive mechanisms to sense oxygen availability and to activate a metabolic pathway which requires a minimum amount of oxygen (Wang *et al*., 1995; Milner *et al*., 2012; Yodmuang *et al*., 2012). Up to ~95% of energy generation from glycolysis which is anaerobic energy generation is considered one of the fundamental adaptations to the low oxygen status of cartilage to minimize oxygen consumption by leading lack of mitochondria in chondrocytes (Heywood *et al*., 2010; Lee and Urban *et al*., 1997; Milner *et al*., 2012). However, cell or tissue culture of cartilage have been frequently carried out under atmospheric oxygen tensions (21% pO_2) which is hyperoxic compared to that of physiological oxygen level of articular cartilage (Berniakovich *et al*., 2013; Yodmuang et al., 2012).

Culturing chondrocytes under 5% pO_2 that more closely resembles their *in vivo* environment has been shown to have positive effects on cell survival and synthesis of extracellular matrix components such as collagen type II, aggrecan and glycosaminoglycan (Domm *et al*., 2002; Katopodi *et al*., 2005; Hashimoto et al., 2006; Schrobback *et al*., 2012).

Transforming growth factor-beta (TGF-β) plays a significant role in promoting chondrocyte anabolism *in vitro* and *in vivo* (Grimaud *et al*., 2002). TGF-β is a multifunctional regulator of cartilage repair and homeostasis with its critical roles in chondrocyte proliferation, differentiation and extracellular matrix (ECM) production. (Finnson *et al*., 2010). It has been shown that disruption of TGF-β signalling led to the development of degenerative joint disease resembling human osteoarthritis (OA) in mice (Serra *et al*., 1997). Therefore, application of TGF-β may potentially promote cartilage integrity and repair (Blom *et al*., 2007).

Cytokines of the TGF-β superfamily include nearly 30 proteins in mammals, e.g., TGF-βs activins and inhibins, nodal, myostatin, bone morphogenetic proteins (BMPs), growth/differentiation factors (GDFs), and anti-Müllerian hormone (AMH) (Miyazawa *et al*., 2002). Among these cytokines TGF-βs and BMPs were identified as more related with cartilage tissue *in vivo*. Therefore, TGF-β signaling pathway upon TGF-β or BMP ligand binding is essential for cartilage tissue (Wozney and Rosen et al., 1998; Miyazawa *et al*., 2002). A simplified representation of TGF-β signaling is shown in the **Fig. 1**. Both type II and type I receptors are required for signal transduction. In addition, type III receptors (betaglycan, and endoglin) act as co-receptor for certain members of the TGF-β superfamily (Mokrosinski and Krajewska *et al*., 2008; Miyazawa *et al*., 2002). TGF-β binds to type II receptor; this binding might be enhanced by the presence of a type III receptor (Hui and Friedman *et al*., 2003; Pinzani and Marra *et al*., 2001). Upon the ligand binding, type II receptor (TGFBR-II or BMPR-II) activates type I receptor kinases (activin like kinases-ALKs) through phosphorylation of type I receptor.(Pinzani and Marra et al., 2001). Then activated type I receptor leads to phosphorylation of intracellular regulatory Smad (R-SMAD) proteins (Miyazono *et al*., 2000). Type I receptors for TGF-β ligand such as ALK5 and ALK1 recognize Smad2 and Smad3, whereas the type I receptors for BMP ligand such as ALK3 and ALK6 recognize Smad1, Smad5, and Smad8 (Massague *et al*., 2000; Miyazawa *et al*., 2002). Activated R-SMADs form a complex with Co-SMAD (Smad4) and translocate to the nucleus where they regulate transcription of target genes which play fundamental roles in cellular regulations (Hui and Friedman *et al*., 2003; Miyazono *et al*., 2000).

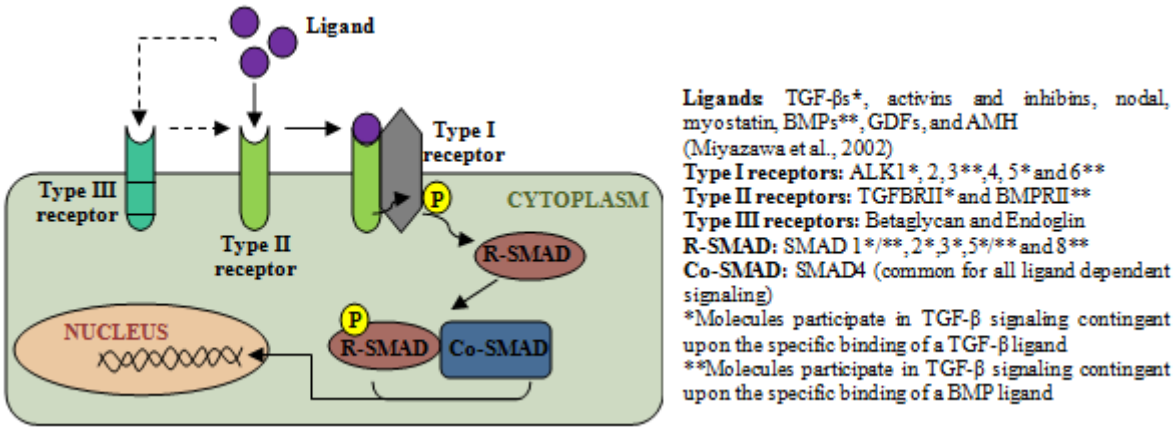

Fig. 1. Simplified representation of TGF-β signaling pathway

Multiple micro-environmental factors regulate chondrocyte function and phenotype (Djouad *et al*., 2007). Extracellular parameters like hypoxia might affect TGF- β signaling of chondrocytes. There are three mammalian isoforms of TGF- β (1,2 and 3) and each of them shows different reactions to hypoxia depending on the cell type. In human dermal fibroblasts hypoxia regulates the synthesis of TGF- β1 (Falanga *et al*., 1991), while it has been shown the upregulation of TGF- β2 in human endothelial cells (Akman *et al*., 2001). Martin and his colleagues reported the upregulation of TGF- β1 and type I receptor of TGF- β signaling and downregulation of chondrogenic markers like aggrecan and collagen type II under the effect of 5%pO_2 for 1.5 hours (Martin *et al*., 2004). There is no study which has been performed with human osteoarthritic chondrocytes to investigate longer term effect of hypoxia on TGF-β signaling. The purpose of the present study was to investigate the effect of hypoxia on both chondrogenic markers and TGF-β signaling molecules in human osteoarthritic chondrocytes.

3.2 Methods

Culturing, RNA isolation and cDNA synthesis were performed by Ruud H.J. Das. These methods were also included in this paper. Moreover, a novel culturing system, µ24 bioreactor, was introduced in the methods part.

3.2.1 Cell Culture

Full thickness cartilage was harvested from patients undergoing total knee replacement surgery. Harvested cartilage tissue was treated with 0.2% protease in physiological saline solution (Sigma-Aldrich) for 90 minutes and put into basal medium (DMEM, 4.5 g/L glucose with 10% Fetal Calf Serum (FCS), 50µg/ml gentamicine and 1.5 µg/ml fungizone (all

Invitrogen)) supplemented with 0.15% collagenase B (Roche Diagnostic) for overnight digestion. The following day, harvested cell number was determined using a haemocytometer. Chondrocytes were then seeded at a density of 7,500 cell/cm^2 in T175 culture flasks for expansion of culture. Alginate beads were created with second passage chondrocytes in 1.2% low viscosity alginate (Keltone LV) by dripping a 1.2% low viscosity alginate suspension with 4 million cells/ml into a $CaCl_2$ solution with help of 23" gauge needle. Afterwards the beads were collected from $CaCl_2$ solution and washed twice with physiological saline and once with basal medium and transferred a T175 culture flask. Alginate beads were transferred to μ24 bioreactor (Applicon Biotechnology) after five days of pre-culture.

3.2.2 ALK5 inhibition

A specific ALK5 inhibitor (SB-525334, Sigma) was used to inhibit ALK5 which is a type I TGF-β receptor. Two hours before the induction of hypoxia (1% pO_2) as bioreactor culturing setting, 10μM of the inhibitor was added to alginate bead culture.

3.2.3 Bioreactor

3.2.3.1 Bioreactor System

The microbioreactor is (Applikon B.V., Schiedam, The Netherlands) a recently developed cell culture system which includes a specialized single use 24 well-reactor cassette for culturing [Fig. 2 (A)]. It has an advantage over culturing in incubator by offering independent control of temperature, pH and dissolved oxygen (DO) in each single well of bioreactor cassette. The microbioreactor also provides continuous monitoring of experimental parameters like oxygen and pH for every individual well and saves the data for a user defined data log time. Therefore this culturing system provides testing multiple conditions in an experiment. In the MicroReactor system a proportional-integral-derivative (PID) tuning is fundamental. PID settings have been optimized to minimaze the deviations from setpoint (Applikon Biotechnology).

Oxygen tension and pH are measured through two sensor patches at the bottom of every single well [**Fig. 2** (B)]. Hypoxic conditions are achieved using air or pure oxygen as counter gas with infusion of inert nitrogen (N_2) as purging gas. pH is controlled by infusion of CO_2 (to lower pH) or NH_3 (to raise pH). Individual well temperature is controlled by the use of a heater and temperature sensor, situated outside of the bioreactor. An orbital shaker system ensures homogeneity of the medium through agitation (Das et al., 2010).

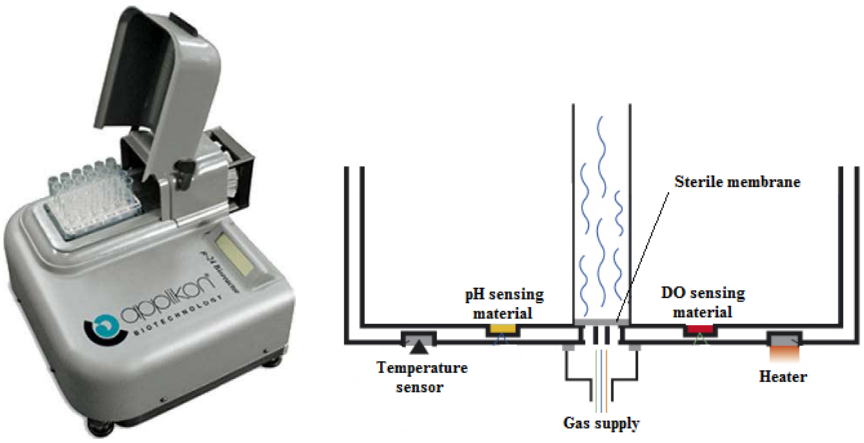

Fig. 2. The µ-24 bioreactor (A) (Das et al., 2008), simplified representation of one well of the 24 well cassette of µ-24 bioreactor (B) (Modified from Pall Corporation)

3.2.3.2 Bioreactor Culture

Thirty alginate beads/well were transferred to a specific 24 well cassette, designed for use with µ24 bioreactor.Four milliliter of redifferentiation medium (DMEM, 4.5 g/L glucose with 1:100 ITS+premix, 10ng/ml TGF-β2, 10 ng/ml IGF-1, 25 µg/ml L-ascorbic acid and 50 µg/ml gentamicin and 1.5 µg/ml fungizone) was added to every well. Oxygen tension was lowered by continious sparging with inert nitrogen. Oxygen was maintained at the desired set point by using air as counter gas. Medium pH was maintained at 7.2 using CO_2. Beads were harvested for RNA isolation after 48 hours duration of bioreactor culturing.

3.2.4 RNA isolation, RNA purification and cDNA synthesis

Alginate beads were harvested and dissolved in 150 µl/bead 55mM sodium citric acid and spun down for 8 minutes at 1600 rpm. In order to isolate RNA, cell pellets occurred after centrifuging step were resuspended in 350 µl RNABee (TEL-TEST Inc.). After addition of 60 µl chloroform, samples were centrifuged for 20 minutes at 13,000xg. Total RNA was purified using the Qiagen RNA Micro kit according to manufacturer's instructions (Qiagen GmbH) and nucleic acid content was determined spectrophotometrically (NanoDrop ND1000, Isogen Life Science). Each sample was reverse transcribed into complementary DNA (cDNA) using RevertAid First Strand cDNA Synthesis Kit (MBI Fermentas).

3.2.5 Gene Expression Analysis

For gene expression analysis quantitative reverse-transcriptase polymerase chain reaction (RT-PCR) was performed by using CFX96 Touch™ Real-Time PCR Detection System (Bio-

Rad Laboratories, Inc.). Gene expression analysis of chondrogenic marker (aggrecan (AGC1)), hypoxia targets (vascular endothelial growth factor (VEGF), prolyl hydroxylase 3 (PHD3), growth differentiation factor 10 (GDF10), hyaluronan synthase 2 (HAS2) and cyclooxygenase 2 (COX2)), TGF-β targets (plasminogen activator inhibitor-1 (PAI-1), and nephroblastoma overexpressed (NOV)), BMP target (Runt related transcription factor 2 (RUNX2)) and type III receptors of TGF-β signaling (betaglycan (BGCAN) and endoglin (ENG)) was performed. In order to normalize the data to an internal control (housekeeper gene), $2^{-\Delta Ct}$ method where ΔCt = (Ct gene of interest- Ct internal control) was used. Geometric means of housekeeper expressions; Beta-2 microglobulin (B2M), ubiquitin (UBC), beta-actin (ACTB), hypoxanthine phosphoribosyltransferase 1 (HPRT1) and 18SrRNA was calculated and used as a housekeeper index for internal control in $2^{-\Delta Ct}$ method.

3.2.6 Statistical Analysis

Statistical analysis was performed using SPSS 21.0 software. Four donors for some of the hypoxia targets (VEGF, PHD3 and GDF10) and two donors for the rest of gene of interests were used. Every condition was performed in duplicates (for the aforementioned three hypoxia target genes n=8 per condition, for the rest n=4 per condition). The effect of experimental conditions was tested using a mixed-effect linear regression model. Therefore the correlation in the data that exists within each donor was accounted. In the software "donor" was incorporated as a random effect to correct for basal differences in specific expression between patients. Experimental conditions were introduced as "fixed" effect to to software. *P* values less than 0.05 were considered significant.

3.3 Results

3.3.1 Hypoxia Targets and Chondrogenic Markers

Osteoarthritic chondrocytes redifferentiated in alginate beads showed a good adaptation to hypoxia. All of the hypoxia targets that were tested in this study showed upregulation for 1% pO_2 condition compared to control condition (20% pO_2). Upregulation of the specific hypoxia targets; vascular endothelial growth factor (VEGF), prolyl hydroxylase 3 (PHD3), growth differentiation factor 10 (GDF10) and cyclooxygenase 2 (COX2) was significant for 1% pO_2 [**Fig. 3**]; especially expression of VEGF was 25 times more than in the case normoxia and it exhibited the most significant upregulation among the other hypoxia targets. Slightly increased expression of aggrecan (AGC1) under hypoxia effect was not significant to claim

that reduced oxygen tension alters expression of this chondrogenic marker. On the other hand, the most relevant chondrogenic marker, collagen type II expression was significantly higher under hypoxia (data for COL2 was provided by Ruud H.J. Das).

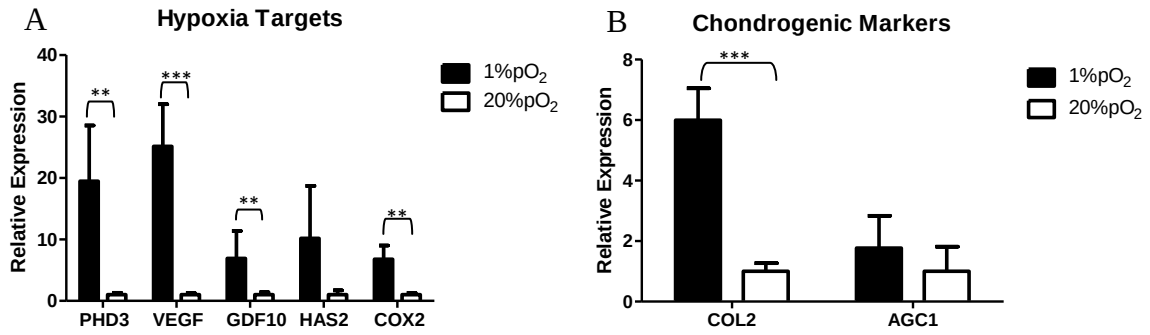

Fig. 3 Relative gene expression of hypaxia targets (A) and chondrogenic markers (B) under hypoxia and normoxia.*P<0.05, **P<0.01, ***P<0.001

3.3.2 Effect of hypoxia on TGF-β ligands

As TGF-β signaling ligands, gene expression of three isoforms of TGF-β (1,2 and 3) was tested in the presence of hypoxia effect and results were compared with 20% pO2 condition. After 2 days of bioreactor culture, only TGF-β2 was significantly up-regulated under 1% pO2 while expression of the other isoforms were not significantly altered (data was shown in **Appendix A.1**).

3.3.3 Effect of hypoxia and ALK5 inhibition on ligand specific target genes

PAI-1 and NOV was considered as specific genes expressed upon binding of TGF-β ligand (TGF-β target) whereas RUNX2 was accounted as BMP target which is gene expressed upon BMP ligand binding to specific TGF-β receptors. TGF-β targets showed a slight upregulation under the effect of 1% pO_2 in osteoarthritic chondrocytes [Fig. 4]. Conversely, BMP target (RUNX2) exhibited a significant down regulation in the case of hypoxia.

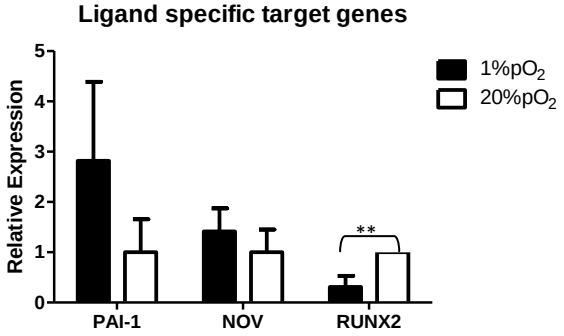

Fig.4. Relative expression of TGF-β targets and BMP target (RUNX2) under hypoxia and normoxia. **P<0.01

ALK5 inhibitor (SB-525334) was tested for of TGF-β and BMP targets under the effect of hypoxia (1% pO_2). Samples without ALK5 inhibitor and with 10mM inhibitor was compared [**Fig.5**]. ALK5 inhibition slightly suppressed the expression of PAI-1 in hypoxic samples. On the other hand, expression of NOV and RUNX2 were not altered.

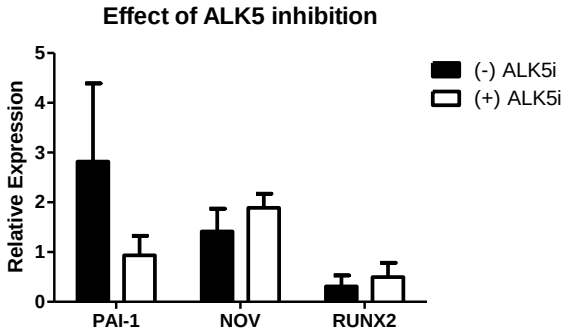

Fig.5. Effect of ALK5 inhibition on expression of TGF-β targets and BMP target (RUNX2) under hypoxia

3.3.4 Effect of hypoxia on TGF-β receptors

Imposing 1% pO_2 to 3D-OAHAC culture led to downregulation of TGF-β type III receptors (BGCAN and ENG) which are co-receptors in TGF-β signaling pathway. (The results are not significant) [**Fig.6**]. On the other hand, type I receptors (ALK1 and ALK5) showed a significant upregulation while expression of type II receptors was unaffected by hypoxia (data was shown in **Appendix A.2**).

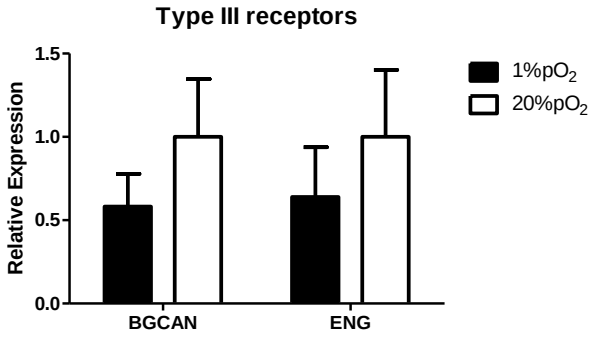

Fig.6. Relative expression of TGF-β type III receptors (BGCAN and ENG) under hypoxia and normoxia.

3.4 Discussion

In this paper, the effect of reduced oxygen tension on TGF-β signaling pathway was studied in alginate bead microbioreactor culture of osteoarthritic human chondrocytes. As a first criteria, chondrogenic marker and hypoxia target expressions were considered in order to

investigate the effect of imposed culturing conditions to OA chondrocytes. Then consequences of hypoxia on major TGF-β signaling molecules were taken into account.

In order to survive and be able to maintain the cartilage phenotype in hypoxic environment, articular chondrocytes develop a highly adaptive mechanism through the prevalent hypoxia-inducible factor-1α (HIF-1α) which requires minimum amount of oxygen in the cellular environment for its activity otherwise it is degraded by prolyl hydroxylases (PHDs) which are real sensors due to their use of oxygen as co-factor (Wang *et al*., 1995; Lafont *et al*., 2010). It was found that mRNA of PHD2 and PHD3 were induced during a hypoxia challenge in human chondrocytes (Ke and Costa *et al*., 2006; McMahon *et al*., 2006). Fujita and his coworkers reported that the induction of PHD3 mRNA expression in human nucleus pulposus cells was about 50-fold at 72 hours in 1% pO2 (Fujita *et al*., 2012). Our results were showed the similar outcomes for PHD3 expression. At the end of 48 hour 3D bioreactor culture, PHD3 was significantly upregulated in redifferentiated OA human chondrocytes at 1% pO_2. In addition to PHD3 upregulation, one of the growth factor that is induced by the activation of HIF-1α is VEGF (Lafont *et al*., 2010). Pfander et al. (Pfander *et al*., 2007) stated that hypoxia led to high level expressions of HIF-1α and HIF-1α target genes such as VEGF. It is known that production of VEGF can be induced in cells that are not receiving enough oxygen (De Bond *et al*., 2002). Therefore this highly supports, the significant upregulation of VEGF in our study. Furthermore, GDF10 was reported as hypoxia-inducible gene by Lafont et al,. (Lafont *et al*., 2008). This study was performed with 2D culture of healthy human chondrocytes and they showed 6 times higher GDF10 mRNA expression under 1% pO_2 compared to normoxia at the end of 4 days. Following 48 hours of hypoxia, we found 7 times higher expression of GDF10 in 3D osteoarthritic human chondrocyte culture compared to 20% pO_2 control condition. Refering to our results, we suggest PHD3, VEGF and GDF10 as hypoxia targets for 3D culturing of human chondrocytes.

A family of enzymes responsible for the synthesis of hyaluronan (HA), designated HAS for HA synthase, has been identified (Hashimoto *et al*., 2006) In a study by Hashimoto and his group, they reported enhanced mRNA expression of HAS2 in bovine articular chondrocytes which were cultured in 2D under 5% pO_2 (Hashimoto *et al*., 2006). In the same study they showed a huge decrease in HA synthesis at 1% pO_2 compared to 5%, 10% and 20% pO_2. Similarly another study showed decreased synthesis of HA synthesis in bovine articular chondrocytes cultured in 2D under 1% pO_2 (Murphy *et al*., 2001).Conversely, our results exhibited upregulation of HAS2 under 1% pO_2 in human OA chondrocytes cultured in

alginate beads. This indicated that HAS2 is not only hypoxia target but also redifferentiation marker in 3D culture system. However, there was a huge variation in expression level of HAS2 between donors in the presented study.

Cyclooxygenase-2 was also reported as HIF-1α target gene (Xue and Shah *et al.*, 2013; Lee *et al.*, 2010). It has been shown the upregulation of COX2 under 1% pO_2 in monolayer culture of normal human esophageal cells (Lee *et al.*, 2010). Similarly, our results exhibited hypoxia induced COX2 expression in human OA chondrocytes.

Studying the expressions of chondrogenic markers is the most important critaria to evaluate the behaviour of chondrocytes in the applied culturing conditions. Downregulation of chondrogenic markers implies the dedifferation of the chondrocytes while upregulation of these markers refers redifferentiation. Our results showed upregulation of one of the key chondrogenic markers, collagen type II in the case of hypoxia. It has been found that 1% pO_2 exhibited an increased expression of aggrecan and collagen type II in healthy human articular chondrocytes at the end of 6 day in 3D culturing (Foldager *et al*., 2011). Blakeney and his colleagues reported 5 fold increased mRNA aggrecan expression in OAHAC maintained for ten days at 5% oxygen tension (Blakeney *et al*., 2011). Our outcomes for aggrecan gene expression was not significant to claim that reduced oxygen tension alters expression of this chondrogenic marker.

Three mammalian TGF-β isoforms (TGF-β1, TGF-β2 and TGF-β3) are encoded by structurally nearly identical genes and synthesized by a wide variety of cell types (Javelaud and Mauviel *et al*., 2004). Gene expressions of these TGF-β isoforms especially in human chondrocytes under the hypoxia effect are poorly understood. Our study showed that oxygen deprivation (hypoxia) significantly upregulated expression of TGF-β2 while expressions of the other isoforms are not significantly altered; TGF-β1 was even slightly downregulated. Therefore, alginate bead culture of OAHAC which was treated with 1% pO_2 selectively regulated TGF-β2.

There was a slight increase in the expression of PAI-1 when 3D chondrocyte culture was exposed to 1% pO_2 for 2 days in our study. In another study, Zhu and his collagues found the similar effect for 2% pO_2 on mRNA expression of PAI-1 in chondrocytes from rat knee joint (Zhu *et al*., 2009). In Finnson et al., the expression of PAI-1 and type II collagen was studied as in relation of type I receptors of TGF-β (ALK1 and ALK5) in OAHAC (Finnson *et al*., 2008). They reported that ALK1 inhibited, whereas ALK5 promoted, TGF-β1–induced transcriptional activity and mRNA expression of PAI-1 and type II collagen. In our study, inhibition of ALK5 suppressed the expression of PAI-1 for chondroytes treated with 1% pO_2.

Therefore, our results showed that expression of PAI-1 which is one of the TGF-β target gene, depends on TGF-β signaling through ALK5. On the other hand, expression of PAI-1 was inversely related to the level of endoglin and betaglycan observed in osteoarthritic human chondrocytes. Furthemore, we found correlation between PAI-1 and TGF-β2. Enhanced expression of PAI-1 by ALK5 might be led by increased endogenous TGF-β2 expression. As another TGF-β target gene, we tested the expression of NOV under the effect of hypoxia and ALK5 inhibition. We showed that expression of NOV did not alter by either the effect of hypoxia or the effect of ALK5 inhibition. Conversely, in a study it was reported that NOV gene expression was regulated by HIF-1α and TGF-β3 in 2D culturing of human trophoblast cell line JEG3 at 1% pO_2 for 1 day (Wolf *et al*., 2010). On the other hand, Lafont and his coworkers reported the correlation of NOV expression with TGF-β2 mRNA expression in a monolayer culture of ATDC5 cells and primary chondrocytes during 3 week culture period (Lafont *et al*., 2006). NOV expression was highest for ATDC5 cells at day 8. They also showed that NOV acted with TGF-β1 in a cascade of gene regulation. Redifferentiation and short duration of our experiments compared to Lafont et al., (Lafont *et al*., 2006) in our 3D culture might be a reason for unaltered regulation of NOV expression for our experimental cases.

Hirao and his coworkers studied expression of RUNX2 in the pluripotent mesenchymal cell line and mice embryo forelimb which were cultured under 5% pO_2 (Hirao et al., 2006). They reported that hypoxia suppressed the hypertrophy of chondrocytes by down-regulating RUNX2 gene expression (Hirao *et al*., 2006). Similarly, in another study it has been reported that osteoblast like cells which were exposed to hypoxia showed decreased mRNA expression of RUNX2 in a time dependent manner to 4 days (Park *et al*., 2002). Our data also exhibited that hypoxia significantly downregulated RUNX2 expression. In our study we considered RUNX2 as target gene expressed upon the BMP ligand binding in TGF-β signaling pathway. We found thar expression of RUNX2 is related to the presence of high oxygen levels in the cellular environment in order to signaling through the BMP ligand binding in TGF-β signaling pathway. Interestingly, expression of RUNX2 was sligthly enhanced when ALK5 function was inhibited. Therefore we showed that activity of ALK5 suppressed the expression of RUNX2 meaning that ALK5 was not a favorable TGF-β type I receptor for the expression of RUNX2 upon the BMP ligand binding under hypoxia.

Endoglin is a TGF-β co-receptor that has been shown to modulate TGF-β signalling in endothelial cells (Bernabeu *et al*., 2009; Finnson *et al*., 2010). Endoglin displayed high affinity for TGF-β1, intermediate affinity for TGF-β3, and no affinity for TGF-β2 in cell lines

of human chondrocytes. (Parker *et al*., 2003; Mokrosinski and Krajewska *et al*., 2008). Our results inhibited downregulation of endoglin expression under the influence of hypoxia. Our results exhibited that hypoxia suppressed the expression of endoglin due to decreased expression of TGF-β1 under hypoxia. Refering to our results for endoglin, we suggest there is a correlation between the expressions endoglin and TGF-β1. On the other hand, since hypoxia effect in our 3D culture increased the gene expression of TGF-β2 which does not to bind endoglin, co-receptor endoglin was not favorably used in our hypoxic cell culture in TGF-β signaling pathway. Thus, under hypoxia, cellular mechanisms might downregulate the expression of endoglin. Finnson et al. (Finnson *et al*., 2010) suggested endoglin as a potential differentiation marker for human osteoarthritic chondrocytes under standard oxygen tension (20% pO_2). This might endorse that hypoxia led to better redifferentiation of our 3D culture by downregulating the expression of endoglin for human OA chondrocytes under hypoxia. Similar to endoglin expression in this study, we found decreased expression of betaglycan for the samples imposed to hypoxia condition. We report that hypoxia downregulated the expressions of type III receptors in OAHAC during bioreactor culturing in alginate beads at 1% oxygen tension. Presence of high oxygen tensions in cellular environment might induce expression of type III receptors.

Differences in design and duration of the experiments, state of cell culture, and the experimental parameters participate to the variation of the results between studies. On the other hand, tight control of extracellular parameters during the culture period plays an important role to eliminate variations in the results and to adjust the suitable conditions for the cells. Novel μ24 bioreactor is a convient device to control extracellular parameter during the culturing period.

3.5 Conclusion

Our findings for hypoxia target genes indicated a good adaptation of OAHAC cultured as alginate beads in microbioreactor to hypoxic culturing conditions. This suggests implementing hypoxia effect to *in vitro* cell culture mimiced the native environment of cartilage and helped to maintain its fundamental mechanisms like HIF pathway *in vitro.* Our data showed upregulation of major elements of TGF-β signaling such as TGF-β2, type I receptors, and TGF-β target genes, in alginate bead culture of osteoarthritic human chondrocytes for 2 days at 1% pO_2. Although it was hard to make clear explanations for the expression of some gene of interests due to high standard deviation.

Hypoxia target genes, chondrogenic markers and several components of the TGF-β signaling pathway displayed increased expression in human OA chondrocyte when hypoxia effect was imposed to 3D culture. To obtain good chondrogenic characteristic and growth factor induced cellular regulation is essential for cartilage repair treatment models. Our findings suggest that 3D culture model by integrating hypoxia effect and targeting specific components of the TGF-β pathway may represent a suitable therapeutic strategy for the treatment of OA .

Appendix

A. Effect of Hypoxia on TGF-β Signaling molecules

A.1 Effect of hypoxia on TGF-β isoforms

Relative gene expressions of three mammalian isoforms of TGF-β (1,2 and 3) were tested in the presence of hypoxia effect. Our results showed TGF-β2 was significantly up-regulated under 1% pO2 while expression of the other isoforms were not significantly altered. The results were discussed in **Section3.4** (Data was provided by Ruud H.J. Das).

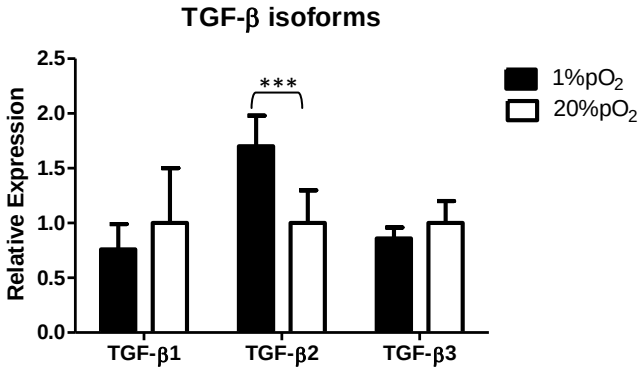

Fig.7. Relative expressions of TGF-β isoforms (1,2 and 3) under hypoxia and normoxia. ****P*<0.001.

A.2 Effect of hypoxia on TGF-β receptors

Hypoxia effect on TGF-β type I, type II and type III (co-receptors) were tested. As type I receptors ALK1 and ALK5, as type II receptor TGFBRII and as type III receptors BGCAN and ENG were tested (Data for type I receptors and type II receptor were provided by Ruud H.J. Das). Hypoxia was significantly upregulated type I receptors which are crutial for signal transduction to Smad molecules.

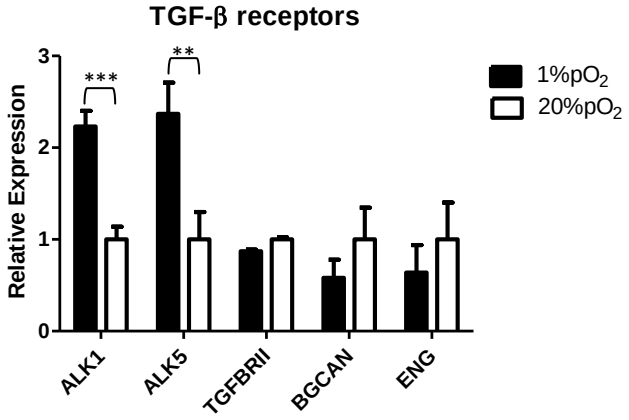

Fig.8. Relative expressions of TGF-β type I receptors (ALK1 and ALK5), type II receptor (TGFBRII) and type III receptors (BGCAN and ENG) under hypoxia and normoxia. **P<0.01, ***P<0.001

A.3 Effect of Hypoxia on TGF-β Targets

Nitric oxide synthase 2 (NOS2) is considered as part of inniate immune response. NOS2 is regulated by cytokines (O'Brien *et al*., 2001). It has been found that TGF-β1 and β2 reduced the destructive potential of NOS2 (O'Brien *et al*., 2001).

TGF-β-associated kinase 1 (TAK1) is a member of the mitogen activated protein kinase kinase kinase (MAPKKK) family (Sorrentino *et al*., 2008). It has been shown that TGF-β isoforms 1 and 2 specifically activated TAK1 through the interaction of type I receptor (ALK5) (Sorrentino *et al*., 2008). In this study, these genes were tested as a trial for only one donor.

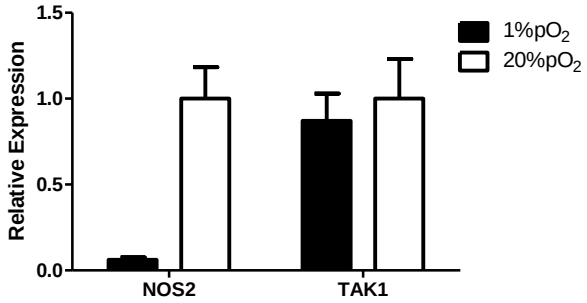

Fig.9. Relative expressions of TGF-β targets under hypoxia and normoxia.

A.4 Effect of hypoxia on Cartilage-specific matrix

Chondromodulin-1 (ChM-1) is a cartilage-specific matrix glycoprotein that stimulates the growth of chondrocytes (Yanagihara *et al*., 2000). It has been shown that ChM-1 protein was restricted to the avascular zone of cartilage (Yanagihara *et al*., 2000). In the presented study, this gene was tested as a trial for only one donor. Expression of this gene was seen almost at the last cycles of amplification for control condition. Therefore, ChM-1 was not tested for other donors.

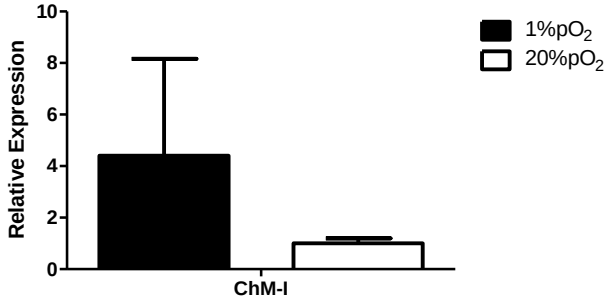

Fig.10. Relative expression of cartilage-specific matrix glycoprotein under hypoxia and normoxia.

B. Effect of ALK5 inhibition

B.1 Effect of ALK5 inhibition on Chondrogenic Markers

ALK5 inhibitor (SB-525334) was tested for chondrogenic markers under the effect of hypoxia (1% pO_2). Samples without ALK5 inhibitor ((-) ALKi) and with 10mM inhibitor ((+) ALKi) was compared. ALK5 inhibition down regulated the upregulated expression [**Fig. 3.A**] of COL2 and AGC1 in hypoxic samples. Therefore, we showed importance of ALK5 in regulation of maintenance and production of ECM (Data for COL2 was provided by Ruud H.J. Das).

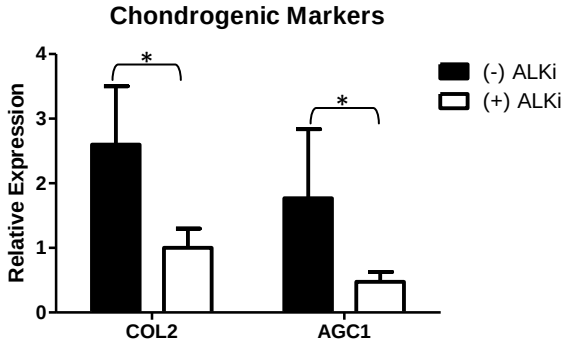

Fig.11. Effect of ALK5 inhibition on expression of chondrogenic markers under hypoxia. *P<0.05

B.2 Effect of ALK5 inhibition on Hypoxia Targets

ALK5 inhibition effect was tested for hypoxia targets under the effect of hypoxia (1% pO_2). Samples without ALK5 inhibitor ((-) ALKi) and with 10mM inhibitor ((+) ALKi) was compared. ALK5 inhibition significantly suppressed the expression of PHD3, VEGF and COX2 in hypoxic samples. We suggest ALK5 route of TGF-β signaling that is essential for cellular regulations and maintenance under hypoxia.

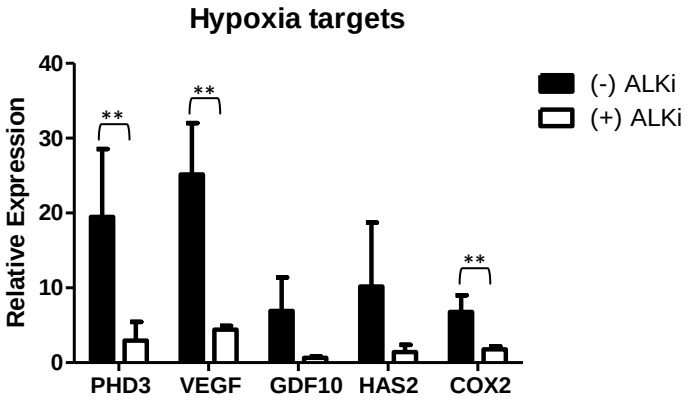

Fig.12. Effect of ALK5 inhibition on expression of hypoxia targets under hypoxia. **P<0.01.

B.3 Effect of ALK5 inhibition on TGF-β Type III Receptors

ALK5 inhibition was not altered the expressions of type III receptors (BGCAN and ENG) in OAHAC at 1% pO_2.

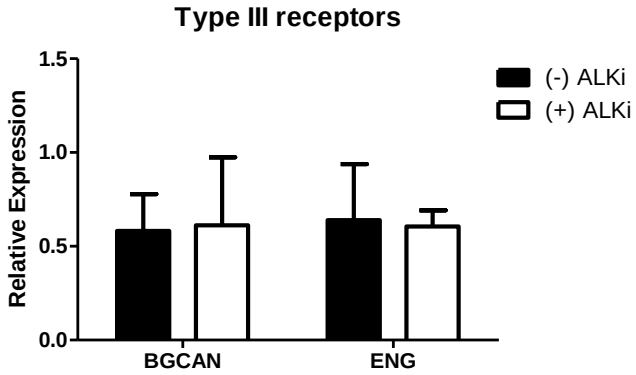

Fig.13. Effect of ALK5 inhibition on expression of type III receptors under hypoxia

B.4 Effect of ALK5 inhibition on TGF-β Type I Receptor Target

We considered DNA-binding protein inhibitor (ID1) as ALK1 target gene. We showed the experimental conditions were not changed the expression of ID1. The results indicated that ALK1 signaling route was not dependent on TGF-β signaling through ALK5.

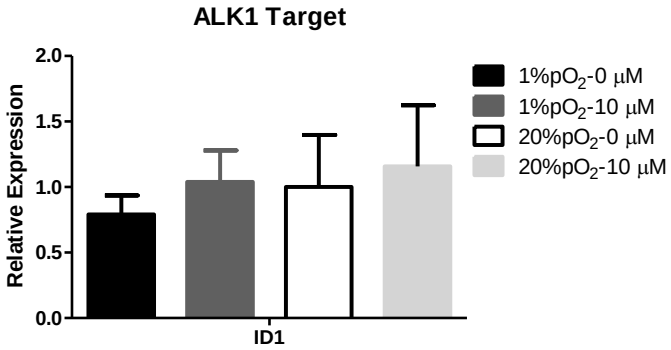

Fig.14. Effect of hypoxia and ALK5 inhibition on expression of ALK1 target gene (ID1)

B.5 Effect of ALK5 inhibition on Inhibitory Smad Molecule

Inhibitory Smads (Smad6 and Smad7) have crucial role in negative regulation of TGF-β signaling. Smad7 inhibits both signaling routes (Smad1/5/8 and Smad 2/3) whereas Smad6 only inhibits Smad1/5/8 signaling, (van der Kraan *et al*., 2009; Li *et al*., 2003). In the presented study we tested the effect of Smad7 for all experimental conditions. Relative expression of Smad7 was not altered depending on either hypoxia or ALK5 inhibition.

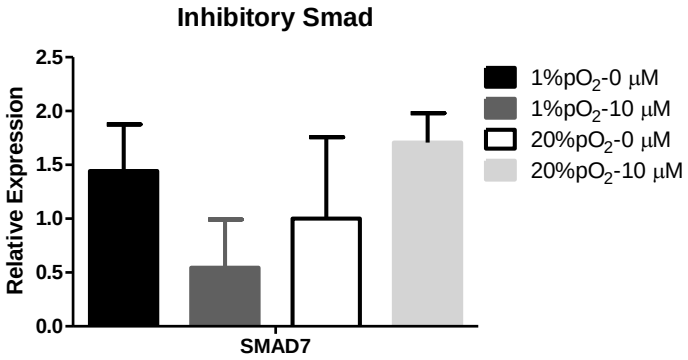

Fig.15. Effect of hypoxia and ALK5 inhibition on expression of inhibitory Smad 7

References

Adesida, Adetola B., Aillette Mullet-Sierra, and Nadr M. Jomha. "Hypoxia Mediated Isolation and Expansion Enhances the Chondrogenic Capacity of Bone Marrow Mesenchymal Stromal Cells." *Stem Cell Researh and Therapy 3:9* (2012): 1-12.

Aigner, T., Zien A., Gehrsitz, A., "Anabolic and catabolic gene expression pattern analysis in normal versus osteoarthritic cartilage using DNA-array technology." *Arthritis Rheum* 44 (2001):2777–2789.

Akman, Hasan O., Hong Zhang, M.A.Q Siddiqui, William Solomon, Eric L.P Smith, and Olcay A. Batuman. "Response to Hypoxia Involves Transforming Growth Factor-b2 and Smad Proteins in Human Endothelial Cells." *Hemostais, Thrombosis and Vascular Biology* 98.12 (2001): 3324-331.

Amarilio, R., Viukov, S.V., Sharir, A. "HIF1alpha regulation of Sox9 is necessary to maintain differentiation of hypoxic prechondrogenic cells during early skeletogenesis." *Development* 134 (2007):3917–3928.

Annes, J.P., J.S. Munger, and D.B. Rifkin. "Making Sense of Latent TGFbeta Activation . "*Journal of Cell Science* 116.2 (2003): 217-24.

Appleton, C. T. G., V. Pitelka, J. Henry, and F. Beier. "Global Analyses of Gene Expression in Early Experimental Osteoarthritis." *Arthritis & Rheumatism* 56.6 (2007): 1854-868.

Applikon Biotechnology " MicroReactor trial/installation guide".

Bernabeu, C., J.M. Lopez-Novoa, and M. Quintanilla. "The Emerging Role of TGF-beta Superfamily Co-receptors in Cancer." *Biochim. Biophys. Acta.* 1792.10 (2009): 954-73.

Berniakovich, Ina, and Marco Giorgio. "Low Oxygen Tension Maintains Multipotency, WhereasNormoxia Increases Differentiation of Mouse Bone Marrow Stromal Cells."*Int. J. Mol. Sci.* 14 (2013): 2119-134.

Blaney Davidson EN, Remst DF, Vitters EL, van Beuningen HM, Blom AB, Goumans MJ, van den Berg WB, van der Kraan PM. "Increase in ALK1/ALK5 ratio as a cause for elevated MMP-13 expression in osteoarthritis in humans and mice. " *J Immunol* 182 (2009):7937-7945.

Blakeney, W., R. Carey-Smith, M. Underhill, B. Short, and D. Wood. "Hypoxic Regulation of Chondrocyte Differentiation and its Application to Cartilage Repair" *J Bone Joint Surg Br* (2011) Vol. 93-B No. SUPP II 97

Blom, Arjen B., Peter M. Van Der Kraan, and Wim B. Van Den Berg. "Cytokine Targeting in Osteoarthritis." *Current Drug Targets* 8.2 (2007): 283-92.

Boschetti, F., G. Pennati, F. Gervaso, G.M Peretti, and G. Dubini. "Biomechanical Properties of Human Articular Cartilage under Compressive Loads." *Biorheology* 41 (2004): 159-66.

Brighton, Carl T., Toshihiro Kitajima, and Robert M. Hunt. "Zonal Analysis of Cytoplasmic Components of Articular Cartilage Chondrocytes." *Arthritis & Rheumatism* 27.11 (1984): 1290-299.

Bruick, R.K., and S.L. McKnight. "Oxygen Sensing Gets a Second Wind." *Science* 295 (2002): 807-08.

Calderon-Montano J , Burgos-Moron E , Perez-Guerrero C , Salvador J , Robles A ,Lopez-Lazaro M . "Role of the Intracellular pH in the Metabolic Switch between Oxidative Phosphorylation and Aerobic Glycolysis - Relevance to Cancer ". *WebmedCentral CANCER* 2011;2(3):WMC001716.

Caron, M.M.J, P.J Emans, M.M.E Coolsen, L. Voss, D.A.M Surtel, A. Cremers, L.M Van Rhijn, and T.J.M Welting. "Redifferentiation of Dedifferentiated Human Articular Chondrocytes: Comparison of 2D and 3D Cultures." *Osteoarthritis and Cartilage*20 (2012): 1170-178.

Chen, Y.G., A. Hata, R.S. Lo, N. Pavletich, and J. Massague. "Determinants of Specificity in TGF-beta Signal Transduction." *GENES & DEVELOPMENT* 12 (1998): 2144-152

Coyle, Christian H., Nicholas J. Izzo, and Constance R. Chu. "Sustained Hypoxia Enhances Chondrocyte Matrix Synthesis." *Journal of Orthopaedic Research* 27.6 (2009): 793-99.

Das, R.H.J, G.J.V.M Van Osch, M. Kreukniet, J. Oostra, H. Weinans, and H. Jahr. "Effects of Individual Control of PH and Hypoxia in Chondrocyte Culture." *Journal of Orthopaedic Research* (2010): 537-45.

Das, Ruud, Martijn Kreukniet, Harrie Weinans, Gerjo Van Osch, and Holger Jahr. "Control of Oxygen Tension and PH in a Bioreactor for Cartilage Tissue Engineering." *Bio-Medical Materials and Engineering 18* (2008): 279-82.

De Bont, E.S.J.M., V.M.E. Neefjes, W.A. Kamps, S. Rosati, and E. Vellenga. "New Vessel Formation and Aberrant VEGF/VEGFR Signaling in Acute Leukemia: Does It Matter?" *Leukemia & Lymphoma* 43.10 (2002): 1901-909.

Derynck, Rik, Ying Zhang, and Xin-Hua Feng. "Smads: Transcriptional Activators of TGF-β Responses." *Cell* 95 (1998): 737-40.

Djouad, F., B. Delorme, M. Maurice, D. Noel, and C. Jorgensen. "Microenvironmental Changes during Differentiation of Mesenchymal Stem Cells towards Chondrocytes."*Arthritis Research and Therapy* 9.2 (2007): 1-12.

Domm, C., M. Schunke, J. Steinhagen, S. Freitag, and B. Kurz. "Influence of Various Alginate Brands on the Redifferentiation of Dedifferentiated Bovine Articular Chondrocytes in Alginate Bead Culture under High and Low Oxygen Tension." *Tissue Engineering* 10.11-12 (2004): 1796-805.

Domm, C., M. Schünke, K. Christesen, and B. Kurz. "Redifferentiation of Dedifferentiation Bovine Articular Chondrocytes in Alginate Culture under Low Oxygen Tension."*Osteoarthritis and Cartilage* 10 (2002): 13-22.

Falanga, V., V. Su Wen Qian, D. Danielpour, M. H. Katz, A. B. Roberts, and M. B. Sporn. "Hypoxia Upregulates the Synthesis of TGF-beta1 by Human Dermal Fibroblasts."*Journal of Investigative Dermatology* 97.4 (1991): 634-37.

Felson, D.T. "Osteoarthritis: New Insights. Part 1: The Disease and Its Risk Factors." *Ann Intern Med* 133.8 (2000): 635-46.

Fermor, B., S.E Christensen, I. Youn, J.M Cernanec, C.M Davies, and J.B Weinberg. "Oxygen, Nitric Oxide and Articular Cartilage." *European Cells and Materials* 13 (2007): 56-65.

Finnson, K. W., W. L. Parker, P. Ten Dijke, M. Thorikay, and A. Philip. "ALK1 Opposes ALK5/Smad3 Signaling and Expression of Extracellular Matrix Components in Human Chondrocytes." *Journal of Bone and Mineral Research* 23.6 (2008): 896-906.

Finnson, K.W., W.L Parker, Y. Chi, C.D. Hoemann, M.B. Goldring, J. Antoniou, and A. Philip. "Endoglin Differentially Regulates TGF-b-induced Smad2/3 and Smad1/5 Signalling and Its Expression Correlates with Extracellular Matrix Production and Cellular Differentiation State in Human Chondrocytes." *Osteoarthritis and Cartilage*18 (2010): 1518-527.

Finnson, K.W., Y. Chi and A. Philip (2012). TGF-β Action in the Cartilage in Health and Disease, Principles of Osteoarthritis- Its Definition, Character, Derivation and Modality-Related Recognition, Dr. Bruce M. Rothschild (Ed.), ISBN: 978-953-51-0063-8, InTech, DOI: 10.5772/29243.

Foldager, Casper B., Anna B. Nielsen, Samir Munir, Michael Ulrich-Vinther, Kjeld Soballe, Cody Bünger, and Martin Lind. "Combined 3D and Hypoxic Culture Improves Cartilage-specific Gene Expression in Human Chondrocytes." *Acta Orthopaedica* 82 (2) (2011): 234-40.

Fujita, N. "Expression of Prolyl Hydroxylases (PHDs) Is Selectively Controlled by HIF-1alpha and HIF-2alpha Proteins in Nucleus Pulposus Cells of the Intervertebral Disc."*The Journal of Biological Chemistry* 287.20 (2012): 16975-6986.

Getgood, A., T.P.S. Bhullar, and N. Rushton. "Current Concepts in Articular Cartilage Repair." *Orthopaedics and Trauma* 23.3 (2009): 189-200.

Gibson, J. S., P. I. Milner, R. White, T. P. A. Fairfax, and R. J. Wilkins. "Oxygen and Reactive Oxygen Species in Articular Cartilage: Modulators of Ionic Homeostasis."*Pflugers Arch* 455.4 (2008): 563-73.

Grayson, Warren L., Feng Zhao, Bruce Bunnell, and Teng Ma. "Hypoxia Enhances Proliferation and Tissue Formation of Human Mesenchymal Stem Cells." *Biochemical and Biophysical Research Communications* 358.3 (2007): 948-53.

Grimaud, E., D. Heymann, and F. Redini. "Recent Advances in TGF-beta Effects on Chondrocyte Metabolism. Potential Therapeutic Roles of TGF-beta in Cartilage Disorders." *Cytokine Growth Factor Rev.* 13.3 (2002): 241-57.

Grimmer, C., N. Balbus, U. Lang, T. Aigner, T. Cramer, L. Muller, B. Swoboda, and D. Pfander. "Regulation of Type II Collagen Synthesis during Osteoarthritis by Prolyl-4-Hydroxylases: Possible Influence of Low Oxygen Levels." *American Journal Of Pathology* 169.2 (2006): 491-502.

Grimshaw, M., and R.M Mason. "Bovine Articular Chondrocyte Function in Vitro Depends upon Oxygen Tension." *Osteoarthritis and Cartilage* 8.5 (2000): 386-92.

Hashimoto, K, Fukuda, K., Yamazaki, K., Yamamoto, N., Matsushita, T., Hayakawa, S., Munakata, H., Hamanishi, C. "Hypoxia-induced hyaluronan synthesis by articular chondrocytes: the role of nitric oxide."*Inflamm Res* **55** (2006): 72-77.

Heywood, Hannah K., Dan L. Bader, and David A. Lee. "Rate of Oxygen Consumption by Isolated Articular Chondrocytes Is Sensitive to Medium Glucose Concentration."*Journal of Cellular Physiology* 206.2 (2006): 402-10.

Heywood, Hannah K., Martin M. Knight, and David A. Lee. "Both Superficial and Deep Zone Articular Chondrocyte Subpopulations Exhibit the Crabtree Effect but Have Different Basal Oxygen Consumption Rates." *Journal of Cellular Physiology* (2010): 630-39.

Hirao, M., N. Tamai, N. Tsumaki, H. Yoshikawa, and A. Myoui. "Oxygen Tension Regulates Chondrocyte Differentiation and Function during Endochondral Ossification." *Journal of Biological Chemistry* 281.41 (2006): 31079-1092.

Hui, A.Y., and S.L. Friedman. "The Transforming Growth Factor β (TGF-β) Signalling Pathway." *Expert Reviews in Molecular Medicine* 5 (2003)

Hunziker, E. "Growth-factor-induced Healing of Partial-thickness Defects in Adult Articular Cartilage." *Osteoarthritis and Cartilage* 9.1 (2001): 22-32.

Hunziker, E., T.M Quinn, and H.J Hauselmann. "Quantitative Structural Organization of Normal Adult Human Articular Cartilage." *Osteoarthritis and Cartilage* 10.7 (2002): 564-72.

James MJ, Cleland LG, Rofe AM, Leslie AL "Intraarticular pressure and the relationship between synovial perfusion and metabolic demand." *J Rheumatol* 17(1990):521-527.

Javelaud, D., and A. Mauviel. "Mammalian Transforming Growth Factor-βs: Smad Signaling and Physio-pathological Roles." *The International Journal of Biochemistry & Cell Biology* 36.7 (2004): 1161-165.

Jenkins, G. "The Role of Proteases in Transforming Growth Factor-β Activation." *The International Journal of Biochemistry & Cell Biology* 40.6-7 (2008): 1068-078.

Katopodi, Theoni, and Tim Hardingham. "Human Osteoarthritic Chondrocytes Are Capable of Forming Cartilage Matrix on Hyalograft C under Hypoxic Conditions." European Cells and Materials 10.2 (2005) page:24.

Kay, A., J. Richardson, and N.R Forsyth. "Physiological Normoxia and Chondrogenic Potential of Chondrocytes." *Front Biosci.* (2011): 1365-374.

Ke, Q., and M. Costa. "Hypoxia-Inducible Factor-1 (HIF-1)." *Molecular Pharmacology*70.5 (2006): 1469-480.

Khan, I.M, S.J Gilbert, S.K Singhrao, V.C Duance, and C.W Archer. "Cartilage Integration: Evaluation of the Reasons for Failure of Integration during Cartilage Repair: A review." *European Cells and Materials* 16 (2008): 26-39.

Klein, T.J., Chaudhry, M., Bae, W.C., and Sah, R.L. "Depth-dependent biomechanical and biochemical properties of fetal, newborn, and tissue-engineered articular cartilage". *J Biomech* 40 (2009): 182-90.

Koay, E., and K. Athanasiou. "Hypoxic Chondrogenic Differentiation of Human Embryonic Stem Cells Enhances Cartilage Protein Synthesis and Biomechanical Functionality."*Osteoarthritis and Cartilage* 16.12 (2008): 1450-456.

Kuo, Jonathan, Changcheng Shi, Sarah Cisewski, Lixia Zhang, Michael J. Kern, and Hai Yao. "Regional Cell Density Distribution and Oxygen Consumption Rates in Porcine TMJ Discs: An Explant Study." *Osteoarthritis and Cartilage* (2011): 1-21.

Lafont, J., C. Jacques, G. Le Dreau, M. Laurent, and C. Martinerie. "New Target Genes for NOV/CCN3 in Chondrocytes: TGF-beta2 and Type X Collagen." *J Bone Miner Res.* 20.12 (2006): 2213-223

Lafont, Jérôme E. "Lack of Oxygen in Articular Cartilage: Consequences for Chondrocyte Biology." *International Journal of Experimental Pathology* 91.2 (2010): 99-106.

Lafont, Jerome E., Sonia Talma, Christine Hopfgarten, and Christopher L. Murphy. "Hypoxia Promotes the Differentiated Human Articular Chondrocyte Phenotype through SOX9-dependent and -independent Pathways*." *The Journal of Biological Chemistry* 8th ser. 283 (2008): 4778-786.

Lafont, Jerome, Sonia Talma, and Christopher Murphy. "Hypoxia-Inducible Factor 2α Is Essential for Hypoxic Induction of Human Articular Chondrocyte Phenotype."*Arthritis & Rheumatism* 56.10 (2007): 3297-306.

Lando, David; Peet, Daniel J.; Whelan, Dean A.; Gorman, Jeffrey J.;Whitelaw, Murray L. "Asparagine hydroxylation of the HIF transactivation domain: a hypoxic switch." *Science* 295 (2002): 858-861.

Lane, J. M., C. T. Bringhton, and B. J. Menkowitz. "Anaerobic and Aerobic Metabolism in Articular Cartilage." *J. Rheumatol.* (1977): 334-42.

Lee, J. J., M. Natsuizaka, and H. Nakagawa. "Hypoxia Activates the Cyclooxygenase-2-prostaglandin E Synthase Axis." *Carcinogenesis* 31.3 (2010): 427-34

Lee, Robert B., and Jill P.G Urban. "Evidence for a Negative Pasteur Effect in Articular Cartilage Robert B." *Biochem. J.* 321 (1997): 95-102.

Li, X., A. M. Ionescu, E. M. Schwarz, X. Zhang, H. Drissi, J. Edward Puzas, R. N. Rosier, M. J. Zuscik, and R. J. O'Keefe. "Smad6 Is Induced by BMP-2 and Modulates Chondrocyte Differentiation." *Journal of Orthopaedic Research* 21.5 (2003): 908-13.

Lu, ZuFu, Behrouz Zandieh Doulabi, ChunLing Huang, Ruud A. Bank, and Marco N. Helder. "Collagen Type II Enhances Chondrogenesis in Adipose Tissue–Derived Stem Cells by Affecting Cell Shape." *Tissue Engineering Part A* 16.1 (2010): 81-90.

Malda, Jos, Werner Ten Hoope, Wouter Schuurman, Gerjo J.V.M. Van Osch, Paul René Van Weeren, and Wouter J.A. Dhert. "Localization of the Potential Zonal Marker Clusterin in Native Cartilage and in Tissue-Engineered Constructs." *Tissue Engineering Part A* 16.3 (2010): 897-904.

Malladi, P., Y. Xu, M. Chiou, A.J Giaccia, and M.T Longaker. "Effect of Reduced Oxygen Tension on Chondrogenesis and Osteogenesis in Adipose-derived Mesenchymal Cells." *AJP: Cell Physiology* 290.4 (2006): C1139-1146.

Mandelbaum, B., and D. Waddell. "Etiology and Pathophysiology of Osteoarthritis."*Orthopedics* 28.(2Suppl) (2005): 207-14.

Marcus, Ralph E., and Leon Sokoloff. "The Effect of Low Oxygen Concentration on Growth, Glycolysis, and Sulfate Incorporation by Articular Chondrocytes in Monolayer Culture." *Arthritis & Rheumatism* 16.5 (1973): 646-56.

Martin, G., R. Andriamanalijaona, P. Bogdanowicz, and J.P. Pujol. "Effect of Hypoxia and Reoxygenation on Gene Expression and Response to Interleukin-1 in Cultured Articular Chondrocytes." *Arthritis & Rheumatism* 50.11 (2004): 3549-560.

Massagué, Joan, and Ye-Guang Chen. "Controlling TGF-β Signaling." *Genes and Development* 14 (2000): 627-44.

McMahon, S. "Transforming Growth Factor Beta1 Induces Hypoxia-inducible Factor-1 Stabilization through Selective Inhibition of PHD2 Expression." *Journal of Biological Chemistry* 281.34 (2006): 24171-4181

Merceron, C., C. Vinatier, S. Portron, M. Masson, J. Amiaud, L. Guigand, Y. Cherel, P. Weiss, and J. Guicheux. "Differential Effects of Hypoxia on Osteochondrogenic Potential of Human Adipose-derived Stem Cells." *AJP: Cell Physiology* 298.2 (2010): C355-364.

Milner, P.I, P.A Fairfax, J.A Browning, R.J Wilkins, and J.S Gibson. "The Effect of O_2 Tension on PH Homeostasis in Equine Articular Chondrocytes." *Arthritis & Rheumatism* 54.11 (2006): 3523-532.

Milner, Peter I., Wilkins, Robert J. and Gibson, John S. (2012). Cellular Physiology of Articular Cartilage in Health and Disease, Principles of Osteoarthritis- Its Definition, Character, Derivation and Modality-Related Recognition, Dr. Bruce M. Rothschild (Ed.), ISBN: 978-953-51-0063-8, InTech, DOI: 10.5772/28379.

Mitchell, Peter. "Coupling of Phosphorylation to Electron and Hydrogen Transfer by a Chemi-Osmotic Type of Mechanism." *Nature* 191.4784 (1961): 144-48.

Miyazono, Kohei. "Positive and Negative Regulation of TGF-β Signaling." *Journal of Cell Science* 113 (2000): 1101-109.

Mokrosinski, J., and W.M. Krajewska. "TGF Beta Signalling Accessory Receptors." *Postepy Biochem* 54.3 (2008): 264-73.

Muir, Helen. "The Chondrocyte, Architect of Cartilage. Biomechanics, Structure, Function and Molecular Biology of Cartilage Matrix Macromolecules." *BioEssays* 17.12 (1995): 1039-048.

Murphy, C., and Sambanis, A.. "Effect of Oxygen Tension on Chondrocyte Extracellular Matrix Accumulation." *Connective Tissue Research* 42.2 (2001): 87-96.

Murphy, Christopher L., Brendan L. Thoms, Rasilaben J. Vaghjiani, and Jérôme E. Lafont. "HIF-mediated Articular Chondrocyte Function: Prospects for Cartilage Repair."*Arthritis Research & Therapy* 11.1 (2009): 213.

O'Brien, William J., T. Heimann, L. Tsao, B.T. Seet, G. McFadden, and J.L. Taylor. "Regulation of Nitric Oxide Synthase 2 in Rabbit Corneal Cells." *IOVS* 42.3 (2001): 713-19.

Otte, P. "Basic Cell Metabolism of Articular Cartilage. Manometric Studies." *Z Rheumatol*.50.5 (1991): 304-12.

Pall Corporation. "Micro-24 MicroReactor System." *Micro-24 MicroReactor System*.

Park, J.H., B.H. Park, T.S. Park, and H.S. Baek. "Hypoxia Decreases Runx2/Cbfa1 Expression in Human Osteoblast-like Cells." *Mol. Cell Endocrinol.* 192 (2002): 197-203.

Parker, W.L., M.B. Goldring, and A. Philip. "Endoglin Is Expressed on Human Chondrocytes and Forms a Heteromeric Complex With Betaglycan in a Ligand and Type II TGF-Beta Receptor Independent Manner." *Journal of Bone and Mineral Research* 13.2 (2003): 289-302.

Pfander D., Kortje, D., Zimmermann, R.”Vascular endothelial growth factor in articular cartilage of healthy and osteoarthritic human knee joints.” *Ann Rheum Dis* 60.(2001):1070–1073.

Pfander, D., and Gelse, K.. "Hypoxia and Osteoarthritis: How Chondrocytes Survive Hypoxic Environments." *Current Opinion in Rheumatology* 19.5 (2007): 457-62.

Pfander, D., B. Swoboda, and C. Thorsten. "The Role of HIF-1α in Maintaining Cartilage Homeostasis and during the Pathogenesis of Osteoarthritis." *Arthritis Research & Therapy* 8.104 (2006): 1-2.

Pfander, D., C. Thorsten, and B. Swoboda. "Hypoxia and HIF-1α in Osteoarthritis."*International Orthopaedics* 29 (2005): 6-9.

Pinzani, M., and F. Marra. "Cytokine Receptors and Signaling in Hepatic Stellate Cells."*Semin Liver Dis.* 3 (2001): 397-416.

Poole, A.R., M. Kobayashi, T. Yasuda, S. Laverti, and F. Mwale. "Type II Collagen Degradation and Its Regulation in Articular Cartilage in Osteoarthritis." *Ann Rheum Dis* 61 (2006): ii78-ii81.

Poole, C. Anthony. "Review. Articular Cartilage Chondrons: Form, Function and Failure."*Journal of Anatomy* 191.1 (1997): 1-13.

Qusous, Ala, and Mark J.P Kerrigan. "Quantification of Changes in Morphology, Mechanotransduction, and Gene Expression in Bovine Articular Chondrocytes in Response to 2-Dimensional Culture Indicates the Existence of a Novel Phenotype."*Cartilage XX(X)* (2012): 1-13.

Rajpurohit, Ramesh, Cameron J. Koch, Zhuliang Tao, Cristina Maria Teixeira, and Irving M. Shapiro. "Adaptation of Chondrocytes to Low Oxygen Tension: Relationship between Hypoxia and Cellular Metabolism." *Journal of Cellular Physiology* 168.2 (1996): 424-32.

Robins, J., N. Akeno, A. Mukherjee, R. Dalal, B. Aronow, P. Koopman, and T. Clemens. "Hypoxia Induces Chondrocyte-specific Gene Expression in Mesenchymal Cells in Association with Transcriptional Activation of Sox9." *Bone* 37.3 (2005): 313-22.

Schipani, Ernestina, Heather E. Ryan, Susanna Didrickson, Tatsuya Kobayashi, Melissa Knight, and Randall S. Johnson. "Hypoxia in Cartilage: HIF-1alpha Is Essential for Chondrocyte Growth Arrest and Survival." *Genes and Development* (2001): 2865-876.

Schneider, Nicole, Ange Mouithys-Mickalad, Jean-Philippe Lejeune, Claire Duyckaerts, Francis Sluse, Ginette Deby-Dupont, and Didier Serteyn. "Oxygen Consumption of Equine Articular Chondrocytes: Influence of Applied Oxygen Tension and Glucose Concentration during Culture." *Cell Biology International* 31.9 (2007): 878-86.

Schrobback, K., J. Malda, R.W Crawford, Z. Upton, D.I. Leavesley, and T.J. Klein.” Effects of Oxygen on Zonal Marker Expression in Human Articular Chondrocytes” *Tissue Engineering Part A.* 18(9-10), (2012):920-33

Schulze-Tanzil, Gundula. "Activation and Dedifferentiation of Chondrocytes: Implications in Cartilage Injury and Repair." *Annals of Anatomy - Anatomischer Anzeiger* 191.4 (2009): 325-38.

Schuurman, W., D. Gawlitta, T.J. Klein, W. Ten Hoope, M.H. Van Rijen, W.J. Dhert, P.R. Van Weeren, and J. Malda. "Zonal Chondrocyte Subpopulations Reacquire Zone-specific Characteristics during in Vitro Redifferentiation." *Am. J. Sports Med.* Suppl1 (2009): 97S-104S.

Schuurman, W., T.J Klein, WJ Dhert, PR Van Weeren, DW Hutmacher, and J. Malda. "Cartilage Regeneration Using Zonal Chondrocyte Subpopulations: A Promising Approach or an Overcomplicated Strategy." *J Tissue Eng Regen Med.* (2012)

Semenza, Gregg L., and Guang L. Wang. "A Nuclear Factor Induced by Hypoxia via De Novo Protein Synthesis Binds to the Human Erythropoietin Gene Enhancer at a Site

Required for Transcriptional Activation." *Molecular and Cellular Biology* 12.12 (1992): 5447-454.

Seol, J.W, H.B Lee, Y.J Lee, Y.H Lee, H.S Kang, I.S Kim, N.S Kim, and S.Y Parkt. "Hypoxic Resistance to Articular Chondrocyte Apoptosis – a Possible Mechanism of Maintaining Homeostasis of Normal Articular Cartilage." *The FEBS Journal* 276 (2009): 7375-385.

Serra R, Johnson M, Filvaroff EH, LaBorde J, Sheehan DM, Derynck R, et al. "Expression of a truncated, kinase-defective TGF-β type II receptor in mouse skeletal tissue promotes terminal chondrocyte differentiation and osteoarthritis." *J Cell Biol* (1997);139:541-52.

Sorrentino, A., N. Thakur, S. Grimsby, and A. Marcusson. "The Type I TGF-β Receptor Engages TR AF6 to Activate TAK1 in a Receptor Kinase-independent Manner."*Nature Cell Biology* 10.10 (2008): 1199-207.

Stevens, C. R., R. B. Williams, A. J. Farrell, and D. R. Blake. "Hypoxia and Inflammatory Synovitis: Observations and Speculation." *Annals of the Rheumatic Diseases* 50.2 (1991): 124-32.

Stockwell, R. A. "The Interrelationship of Cell Density and Cartilage Thickness in Mammalian Articular Cartilage." *J. Anat* 109, 3 (1971): 411-21

Stroka, D.M., T. Burkhardt, I. Desbaillets, R.H Wenger, D.A Neil, C. Bauer, M. Gassmann, and D. Candinas. "HIF-1 Is Expressed in Normoxic Tissue and Displays an Organ-specific Regulation under Systemic Hypoxia." *FASEB J.* 15.13 (2001): 2445-453.

van der Kraan, P.M., E.N. Blaney Davidson, A. Blom, and W.B. van den Berg. "TGF-beta Signaling in Chondrocyte Terminal Differentiation and OsteoarthritisModulation and Integration of Signaling Pathways through Receptor-Smads." *Osteoarthritis and Cartilage* 17 (2009): 1539-545.

van der Kraan, P.M., E.N. Blaney Davidson, and W.B. van den Berg. "A Role for Age-related Changes in TGFβ Signaling in Aberrant Chondrocyte Diff Erentiation and Osteoarthritis." *Arthritis Research and Therapy* 12.201 (2010): 1-9.

Verdier MP, Seite S, Guntzer K, Pujol JP, Boumediene K. "Immunohistochemical analysis of transforming growth factor β isoforms and their receptors in human cartilage from normal and osteoarthritic femoral heads. " *Rheumatol Int* 25 (2005): 118-124.

Wang, David W., Beverley Fermor, Jeffrey M. Gimble, Hani A. Awad, and Farshid Guilak. "Influence of Oxygen on the Proliferation and Metabolism of Adipose Derived Adult Stem Cells." *Journal of Cellular Physiology* 204.1 (2005): 184-91.

Wang, G. L. "Hypoxia-Inducible Factor 1 Is a Basic-Helix-Loop-Helix-PAS Heterodimer Regulated by Cellular O2 Tension." *Proceedings of the National Academy of Sciences* 92.12 (1995): 5510-514.

Windhaber RA, Wilkin RJ, Meredith D. 2003. "Functional characterisation of glucose transport in bovine articular chondrocytes." *Pflugers Arch* 446(5):572–577.

Wolf, N., W. Yang, C.E. Dunk, E. Winterhager, and A. Gellhaus. "Regulation of the Matricellular Proteins CYR61 (CCN1) and NOV (CCN3) by Hypoxia-Inducible Factor-1." *Endocrinology* 151.6 (2010): 2835-845.

Wozney, John M., and Vicki Rosen. "Bone Morphogenetic Protein and Bone Morphogenetic Protein Gene Family in Bone Formation and Repair." *Clinical Orthopaedics and Related Research* 346 (1998): 26-37.

Xue, X., and Y.M. Shah. "Hypoxia-inducible Factor-2α Is Essential in Activating the COX2/mPGES-1/PGE2 Signaling Axis in Colon Cancer." *Carcinogenesis* (2013): 163-69.

Yanagihara, Itaru, M. Yamagata, N. Sakai, C. Shukunami, H. Kurahashi, M. Yamazaki, T. Michigami, Y. Hiraki, and K. Ozono. "Genomic Organization of the Human Chondromodulin-1 Gene Containing a Promoter Region That Confers the Expression of

Reporter Gene in Chondrogenic ATDC5 Cells." *Journal of Bone and Mineral Research* 15.3 (2000): 421-29.

Yodmuang, Supansa, Ivana Gadjanski, Pen-hsiu G. Chao, and Gordana Vunjak-Novakovic. "Transient Hypoxia Improves Matrix Properties in Tissue Engineered Cartilage."*Journal of Orthopaedic Research* (2012): 1-10.

Yudoh, Kazou, Hiroshi Nakamura, Kayo Masuko-Hongo, Tomohiro Kato, and Kusuki Nishioka. "Catabolic Stress Induces Expression of Hypoxia-inducible Factor (HIF)-1α in Articular Chondrocytes: Involvement of HIF-1α in the Pathogenesis of Osteoarthritis." *Arthritis Research & Therapy* 7.4 (2005): R904-914.

Zhang, H., H.O. Akman, and O.A. Batuman. "Cellular Response to Hypoxia Involves Signaling via Smad Proteins." *Blood* 101.6 (2003): 2253-260.

Zhang, Lijie, Jerry Hu, and Kyriacos A. Athanasiou. "The Role of Tissue Engineering in Articular Cartilage Repair and Regeneration." *Crit Rev Biomed Eng* 37.(1-2) (2009): 1-57.

Zhou, Shengda, Zhanfeng Cui, and Jill P. G. Urban. "Factors Influencing the Oxygen Concentration Gradient from the Synovial Surface of Articular Cartilage to the Cartilage-bone Interface: A Modeling Study." *Arthritis & Rheumatism* 50.12 (2004): 3915-924.

Zhu, G., Y. Tang, X. Liang, M. Zheng, J. Yang, H. Zhou, L. Li, and T. Qin. "Role of Hypoxia-inducible Factor-1 Alpha in the Regulation of Plasminogen Activator Activity in Rat Knee Joint Chondrocytes." *Osteoarthritis and Cartilage* 17.11 (2009): 1494-502.

Zuscik, Michael J., Matthew J. Hilton, Xinping Zhang, Di Chen, and Regis J. O'Keefe. "Regulation of Chondrogenesis and Chondrocyte Differentiation by Stress." *Journal of Clinical Investigation* 118.2 (2008): 429-38.