MOLECULAR MECHANISMS MODULATING CHONDROGENESIS

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MOLECULAR MECHANISMS MODULATING CHONDROGENESIS

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Summary

In the last decade various tissue engineering strategies have emerged for articular cartilage repair. MSCs are currently being used in various clinical trials to exploit the multilineage capacity and differentiation potential of these cells. Although promising clinical results have been seen, controlling the commitment and differentiation of the cells to the expected pathway remains challenging. Our area of investigation will focus its efforts in achieving the understanding of proper molecular mechanisms that could control the commitment and differentiation of the cells whilst avoiding a hypertrophic or fibrous phenotype. The first and second chapters will provide a background, significance, and an overview on the use of environmental conditions, such as hypoxia, for cartilage repair. Chapter three focuses on validating chondrogenesis of adipose derived stem cells (aMSCs) in low oxygen cultures. Cell type specific effects of low oxygen and 3D environments indicated that genetic programming of aMSCs to a chondrocytic phenotype is effective under hypoxic conditions, as evidenced by increased expression of cartilage-related biomarkers and biosynthesis of a glycosaminoglycan positive matrix. Chapter four and five draw major attention to the molecular mechanisms by which miRNAs could direct chondrogenesis during the hypoxic response of MSCs and explores the potential of microRNA-210 (miR-210) to enhance *in vitro* chondrogenic differentiation of stem cells. Hypoxic regulated miR-210 was found to be essential in the regulation of genes in charge of several functions crucial for cartilage development, chondrogenic differentiation and the oxidative stress response. Exogenous miR-210 expression can potentially be utilized instead of inducing chondrogenic differentiation using TGFβ1 in a three dimensional culture under low oxygen, to promote chondrogenesis of MSCs while inhibiting their hypertrophic differentiation. Chondrogenesis improvement was evidenced by increased expression of cartilaginous markers, proteoglycan deposition and collagen II protein content.

Chapter six will reveal the effects of synovial fluid on *in vitro* models of primary chondrocytes and mesenchymal stem cells. Metabolic activity assays on primary chondrocytes and aMSCs showed both cell types survived and proliferated during culture with synovial fluid (SF). Moreover, synovial fluid seems to be permissive for chondrogenic differentiation of aMSCs in the presence of chondrogenic cocktail, which was confirmed by positive type II collagen immunohistochemistry. Our results serve as an initial screening of the possibilities of SF to replace fetal bovine serum as a culture supplement for *in vitro* expansion of human primary chondrocytes and aMSCs. Chapter seven explores the role of ZNF648, a cartilage specific transcription factor, expressed in immature cartilage and growth plate, to understand its role during cartilage development and to provide a molecular mechanism to create a competent tissue for cartilage repair. In the growth plate, this ZNF648 protein seems to maintain a chondrocytic phenotype of immature cells, whereas in later stages of cartilage maturation its expression is reduced. An enhanced expression of zinc finger 648 (ZNF648) increased collagen type II (COL2A1) expression in growth plate derived chondrocytes while reducing its expression in articular chondrocytes, which suggested an important regulatory mechanism during early development of the chondrocytes in the growth plate but not in terminally differentiated chondrocytes such as the ones in articular cartilage. Thus, development of novel approaches using ZNF648 to improve and maintain cartilage homeostasis is thought to play an essential role in future clinical therapies. Ultimately, chapter eight will provide a general discussion of the results presented in this thesis and presents future perspectives for the use of stem cells in cartilage regeneration therapies.

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CHAPTER 1

1.General introduction and thesis outline

1.1 BACKGROUND AND SIGNIFICANCE

In the last decade various tissue engineering strategies have emerged for articular cartilage repair. MSCs are currently being used in various clinical trials with promising clinical results [1]. Two different notions about MSC therapies have been described. The first notion proposes the term of medicinal signaling cells (MSCs) and relies in the trophic functions of MSCs to support tissue regeneration [2]. Caplan described them as “sentinels” that survey the damage, isolate foreign components, stabilize the injured tissues, provide antibiotics and encysting protection before a medicinal sequence can be initiated to regenerate the damaged tissue [3]. In the second notion, MSCs are describes as stem cells and exploits the multilineage capacity and differentiation potential of these cells. For the development of this thesis, the latter notion, related to the differentiation capacity of these stem cells into chondrocytes, was studied.

Numerous studies have focused on implantation of primary cells or stem cells with a combination of growth factors and biomaterials with the goal of becoming the gold standard for cartilage repair [4]. However, controlling the commitment and differentiation of the cells to the expected pathway remains challenging [5]. The use of stem cells and in particular mesenchymal stem cells (MSCs) from adult sources has emerged as a viable solution to overcome limitations of current cartilage restoration procedures. Multipotent adult MSCs reside in several tissues including bone marrow, skeletal muscle, neural tissue, adipose tissue, and synovium. Currently, most studies for cartilage repair have focused on bone marrow-derived MSCs. As an alternative, human adipose tissue-derived mesenchymal stem cells (aMSCs) are an attractive cellular therapeutic due to the minimally invasive tissue harvest, high abundancy of cells, and rapid expansion *ex vivo* [4, 6]. Moreover, these cells are multipotent, can produce musculoskeletal extracellular matrix proteins once confluent, and are able to differentiate into osteogenic and chondrogenic cell lineages [7]. For cartilage tissue homeostasis

and regeneration, an adequate production of extra cellular matrix (ECM) is necessary to provide the cells with a three dimensional scaffold and to regulate angiogenesis and inflammation processes [8]. MSCs differentiated into chondrocytes are marked by production of sex determining region Y box 9 (*SOX9*), aggrecan (*ACAN*), collagen type II (*COL2A1*), transforming growth factor- β (*TGF β*), fibronectin (*FN1*), neural cell adhesion molecule (*N-CAM*) and N-cadherin (*CDH2*) [1, 9-11]. However, during chondrogenesis of MSCs the expression of cartilage hypertrophy markers, such as collagen type X (*COL10A1*) and metaloproteinases (*MMPs*), is also observed [12]. Prevention of hypertrophic differentiation and posterior ossification is important for clinical application of MSCs in cartilage tissue engineering [13].

To maintain the cartilage homeostasis, researchers have used extracellular matrix proteins and combinations of growth factors to promote stem cell attachment, proliferation and initial chondrogenic differentiation [14, 15]. In addition, the role of these factors is to prevent the formation of fibrous cartilage by decreasing collagen type I (*COL1A1*) expression but maintaining collagen type II (*COL2A1*) deposition during *in vitro* chondrogenesis. For cartilage repair strategies, the most used biomolecules are those who act to initiate signaling molecules cascades during chondrogenesis [15]. TGF β , BMP and IGF families are the most extensively studied developmental morphogens to stimulate chondrogenic differentiation of stem cells *in vitro* [10, 16, 17]. In addition, WNT, NOTCH and FGF are interesting candidates based on their *in vivo* roles during cartilage homeostasis [18, 19]. Various members of these families have been tested *in vitro* as chondrogenic inducers in combination with three-dimensional culture environments, mechanical loading and/or genetic approaches. Consequently, stem cells and chondrocytes could closely interact with the biochemical and environmental stimuli during chondrogenesis to maintain a normal chondrocyte phenotype.

A small number of transcription factors has been described over the years as key factors during cartilage development. These factors play an essential role in regulating standard cartilage markers during chondrogenesis of stem cells, including *COL2A1*, *COL9A1*, *COL10A1*, *COL11A1*, *ACAN* and hyaluronan protein link 1 (*HAPLN1*) [20-24]. Yet the master transcription factor that regulates chondrogenic differentiation is *SOX9* [25]. This gene is expressed early during chondrogenesis [26] and its central role is to regulate the expression of main chondrocytic genes, such as *COL2A1* and *ACAN* [20, 27]. Moreover, several studies have revealed the importance of *SOX9* in decreasing runt-related transcription factor 2 (*RUNX2*), osterix (*SP7*), *COL10A1* and *COL1A1* expression, thereby inhibiting early and late osteogenesis [21, 22, 27, 28]. Besides *SOX9*, *SOX5* and *SOX6* are also prochondrogenic factors that are essential in the regulation of *COL2A1* and other cartilage related genes such as *ACAN* [29].

Other transcription factors have been identified as important for chondrogenesis. Various studies have revealed that down-regulation or inhibition of forkhead box O3 (*FOXO3*) [30], zinc finger protein 415 (*ZNF145*) [31, 32], homeobox D10, D11 and D13 (*HOXD10*, *HOXD11* and *HOXD13*) [33], decreased the expression of important chondrocyte specific markers during chondrogenic differentiation of MSCs, whereas overexpression of factors such as homeobox A2 and D9 (*HOXA2*, and *HOXD9*) increases differentiation of MSCs into chondrocytes [33]. In addition SMADs have been described as important to regulate chondrogenic differentiation of MSCs. Furumatsu et al., demonstrated that SMAD family member 3 (*SMAD3*) binds to transcription factor *SOX9*, stimulating chondrogenic differentiation [34], whereas *SMAD2* and *SMAD3* expression depends on TGFβ1 levels during early stages of chondrogenesis [35]. Interestingly, transcription factors *SMAD3* and *HOXA2* have an inhibitory effect on MSC chondrogenic differentiation by interacting with the *SOX9* transcription factor [36].

Furthermore, Lui and colleagues showed that overexpression of WNT family member 11 (*WNT11*) increased chondrogenic differentiation of MSCs by promoting expression of chondrocytic genes [18].

Nongenetic approaches, such as using microenvironmental factors (e.g oxygen conditions), are an attractive alternative due to the safety and easiness to use. Numerous studies have indicated that hypoxia promotes chondrocyte proliferation, survival, and stimulates the expression of gene transcripts to promote cartilage specific ECM formation, suggesting an important role of low oxygen in earlier chondrogenesis, cartilage maintenance and degradation [4, 37, 38]. Previous studies described *SOX9* as partly controlled by hypoxia inducible factor (*HIF1 α*) during skeletal formation [19], whilst an increased expression of *SOX9* has been observed in human articular chondrocytes during low oxygen cultures [39]. Hirao et al. demonstrated that hypoxic conditions also promoted a chondrocyte commitment of cells in the mesenchymal lineage while inhibiting terminal differentiation by activating the transcription of *SOX9* in both cells and organ cultures [40]. Moreover, hypoxia inducible transcription factors *HIF1 α* and *HIF2 α* were found to be increased during chondrogenic differentiation of MSCs [39, 41, 42], and were shown to potentiate chondrogenic differentiation by maintaining the chondrocytic phenotype and cell function, while inhibiting hypertrophy and subsequently endochondral ossification *in vitro* [39]. Hence, hypoxia may be a key factor in tissue engineering for cartilage repair. However, the mechanisms by which articular chondrocytes regulate genes that are sensitive to oxygen levels under physiological conditions remain poorly understood.

During the past decade, multiple studies have demonstrated that miRNAs are involved in the hypoxic response and contribute to the repression of specific genes under low oxygen conditions [45]. MicroRNAs are small non-coding RNA molecules of about 22 nucleotides which principal function is RNA silencing and

post-transcriptional regulation of gene expression. They frequently repress target genes by reducing the half-life time of their mRNA and/or by inhibiting protein translation. A single miRNA can regulate hundreds of distinct mRNAs simultaneously and a single mRNA can also be regulated by several distinct miRNAs. They contain hypoxia responsive elements (HREs) in their promoter region and are regulated by HIF transcription factors in response to low oxygen [46]. Although *HIF1 α* is known as a transcriptional activator, it also functions as a transcriptional repressor via induction of miRNAs. Expression of *miR-155*, *miR-424*, *miR-17-92*, *miR-21*, *miR-23*, *miR-24*, *miR-26*, *miR-103*, *miR-107*, *miR-181* and *miR-210* [47-49] were found to be induced under hypoxic conditions and are potential targets of *HIF1 α* . In addition, some of these miRNAs have been implicated in regulating pre and post-natal chondrogenesis. Georgi et al., [50] analyzed miRNA expression in MSCs after 1 week of chondrogenic differentiation and identified *miR-210* and *miR-630* as positive regulators of chondrogenesis. These two miRNAs were remarkably expressed at higher levels in donors with high chondrogenic potential. In contrast, *miR-181* and *miR-34a* were considered as negative regulators of chondrogenesis and their expression was increased in MSCs donors with a low chondrogenic potential [50]. Some other miRNAs that were found to be induced during hypoxia include *miR-181a*, which is expressed in chondrosarcoma and enhances *VEGF* expression [51], *miR-146a* that promotes chondrocytes autophagy and induces cartilage degeneration during osteoarthritis [52], and *miR-138* which inhibition helps to maintain a chondrocytic phenotype and reduces the progression of human articular chondrocytes dedifferentiation [53].

In particular *miR-210* stands out in several studies as the only hypoxia inducible miRNA [44, 46, 47] and was found to be essential in the regulation of several biological processes, including cell cycle regulation, cell survival, differentiation, angiogenesis and metabolism, and functions, such as oxidative stress response

during low oxygen [50, 54, 55]. *MiR-210* expression during hypoxic preconditioning of stem cells caused a cytoprotective effect, helping to maintain cell stemness and supporting cell differentiation [56]. This regulation happened in concert with the transcription factor *HIF1 α* [43].

Despite the efforts, current cell-based repair strategies have been proven unsuccessful for treating large cartilage defects and osteoarthritic lesions. Therefore, proper regeneration of this tissue remains an unresolved question. Our area of investigation will focus its efforts in achieving the understating of proper tissue engineering strategies that could control the commitment and differentiation of the cells while avoiding a hypertrophic phenotype. We will investigate the application of microenviromental conditions to mimic the physiological environment of the cells. Among them, low oxygen tension, which emulates the hypoxic environment of the cells, and the presence of different serum types, such as amongst others synovial fluid, will provide a new insight into the field. Furthermore, the study of a cartilage specific transcription factor will help understanding the specific parameters to create a competent tissue for cartilage repair.

1.2 AIMS AND OUTLINE OF THE THESIS

This thesis aims to elucidate the molecular and environmental mechanisms by which we could enhance chondrogenic differentiation of adult mesenchymal stem cells in combination with existing tissue engineering strategies for cartilage regeneration and repair. A better understanding of the use of micro-environmental conditions, such as hypoxia, during differentiation might lead to improvements in differentiation protocols for future stem cell therapies.

Chapter two will provide an overview on the field of cartilage tissue engineering and the use of environmental conditions, such as hypoxia, for cartilage repair and regeneration, including cell sources, growth factors, genetic and mechanical

stimulation. This chapter will focus as well on the challenges that current strategies present.

Chapter three focuses on validating the molecular gene expression of adipose derived stem cells (aMSCs) during chondrogenic differentiation in low oxygen cultures. It describes aMSC's chondrogenic potential and performance whilst showing their hypertrophic potential when cultured in various 3D- environments and hypoxia.

Chapter four and five draw major attention to the molecular mechanisms by which miRNAs could direct chondrogenesis during the hypoxic response of MSCs in culture conditions. Chapter four draws special attention to the regulation and role of *microRNA-210* and its target genes during cartilage development and for the maintenance of healthy cartilage, whereas chapter five explores the potential of *microRNA-210* to enhance *in vitro* chondrogenic differentiation of stem cells.

Chapter six will reveal the effects of synovial fluid on *in vitro* models of primary chondrocytes and mesenchymal stem cells. Since some concerns have been raised regarding the safety of fetal bovine serum (FBS), the right choice of serum is crucial for cell culture. Particularly with regard to clinical application, human alternatives for FBS are clearly to be preferred. Synovial fluid is commonly present in diseased joints and its interaction with stem cells has not been described. In this chapter, we showed the potential of synovial fluid as a promising novel culture supplement for chondrocyte and aMSCs expansion and chondrogenic differentiation and due to its non-zoonotic (animal free) nature is therefore potentially better for clinical applications in cartilage regeneration.

Chapter seven will explore the role of *ZNF648*, a cartilage specific transcription factor identified by RNAseq analysis, which is expressed in immature cartilage and growth plate. *In vitro* cell cultures, functional analysis and a conditional knockout mouse helped us in the understanding of the role of *ZNF648* during

cartilage development. Ultimately, **chapter eight** will provide a general discussion and overall conclusion of the project.

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CHAPTER 2

2. Hypoxia regulatory mechanisms during cartilage development and cartilage-tissue engineering.

2.1 INTRODUCTION

Articular cartilage (AC) is the connective tissue found at the ends of diarthrodial joints and is adapted to provide lubrication during motion [1]. During early stages of limb development, the vasculature is subjected to an extensive remodeling process leaving the prechondrogenic condensation avascular [2]. Due to this avascular environment, AC is formed and maintained in a hypoxic environment during development into adulthood [3], and functions from this stage onwards at an oxygen tension that is lower than other tissues. It has been determined that the oxygen supply at the articular surface is approximately 6 to 10%, whereas in the deepest layers of AC oxygen supply is no more than 1 to 6% of oxygen [4]. Cramer et al. provided evidence that the growth plate is also characterized by a hypoxic microenvironment during fetal development [5]. The upper hypertrophic zone and the central portion of the proliferative zone in the growth plate are exposed to low oxygen tensions [6], suggesting that in physiological conditions chondrocytes depend on diffusion from the epiphyseal and subchondral bone to rely on nutrient supply [7].

Chondrocytes are responsible for the synthesis and maintenance of its extra cellular matrix, which gives the articular cartilage its mechanical integrity for the daily load-bearing conditions [8]. Chondrocytes are able to survive in a sustained low oxygen environment. Thus, it has been hypothesized that hypoxia is an important factor in regulating growth and survival of chondrocytes, while it has been suggested as a key factor to conserve cartilage integrity [9]. A number of pathophysiological findings propose that a correlation does exist between hypoxia and chondrogenesis [10]. Additionally, several studies sustain that a low oxygen tension environment may be beneficial for the maintenance of the chondrocyte phenotype *in vitro*, avoiding potential dedifferentiation during cell culture and expansion [11]. However, the exact mechanism by which chondrocytes are regulated by oxygen tension under normal conditions remains

poorly understood. In this chapter we will present an overview of the hypoxia regulatory mechanisms and their role during cartilage development and cartilage tissue engineering.

2.2 THE HYPOXIC PATHWAY

The process of oxygen regulation is mostly coordinated by the HIF (Hypoxia-Inducible Factor) pathway. HIF is a basic helix-loop-helix transcription factor that has two main roles. The first role is to transactivate gene-encoding proteins that participate in homeostatic responses. The second role involves the modulation of multiple key metabolic pathways for the maintenance of oxygen homeostasis [12]. HIF is a heterodimer of bHLH-PAS proteins and consists of an unstable alpha subunit and a stable beta subunit. These bind DNA to the hypoxia response elements (HREs), which are present in the promoter region of target genes to activate their expression [13]. Three HIF isoforms are known: HIF1 α , HIF2 α and HIF3 α [14]. These isoforms have structural similarities but distinct roles. This conservation of the HIF genes across different species suggests that they perform essential non-overlapping functions during the hypoxic response [6] in both humans and animals [15].

In mammals, the three genes that encode HIF α subunits appear to be regulated in a similar manner [16]. The HIF1 α protein is ubiquitously expressed [17], whereas its homologs, HIF2 α /Endothelial PAS domain protein (EPAS) [16] and HIF3 α [18], have more restricted expression patterns. HIF β is generally found to be constitutively expressed and insensitive to changes in oxygen availability [19]. Although there is evidence for hypoxic induction of *HIF α* mRNA levels in some cell types [20], the predominant modes of HIF α regulation occur post-translationally. Under normal oxygen conditions, hypoxia inducible factor α subunits HIF1 α and HIF2 α are hydroxylated by prolyl hydroxylase domain (PHD) enzymes at specific proline residues (**Figure 2.1.A**), then they are ubiquitinated through interaction with the von Hippel-Lindau tumor suppressor protein

(pVHL) (**Figure 2.1.B**), and subsequently degraded by the 26S proteasomal pathway in the cytosol, resulting in minimal transcriptional activity (**Figure 2.1.C**). Under hypoxia, the activity of the HIF-targeting prolyl hydroxylase enzymes is inhibited, allowing HIF1 α /2 α to rapidly accumulate in the cytoplasm (**Figure 2.1.D**). Then, HIF1 α /2 α gets phosphorylated, translocated into the nucleus (**Figure 2.1.E and F**), and dimerized with HIF1 β in order to activate transcription of its target genes.

Low oxygen has the capability of influence cell signals and functions [21]. Many of the factors that increase during hypoxia are regulated by HIF activation. Transcriptional selectivity studies have defined exclusive targets for each of the isoforms while defining genes that are responsive to some of them [22]. Around 100 HIF targets have been described in the literature [23]. HIF-dependent transcription activates genes involved in cell autonomous development pathways including hematopoietic, endothelial, myocyte, adipocyte, chondrocyte, trophoblast, and neuronal differentiation programs, and various physiological activities such as erythropoiesis, angiogenesis, autophagy and energy metabolism [12, 24-26].

It is believed that HIF factors are involved in chondrocyte development and in the regulation of cartilage homeostasis [17, 18, 20]. *HIF1 α* and *HIF2 α* have been the most extensively studied. Both genes have shown to be involved in promoting chondrogenesis and to play an active role in chondrocyte development [15]. Both *HIF1 α* and *HIF2 α* play an important role in the activation of hypoxia responsive genes and could activate important pathways for cartilage homeostasis and repair. Given the importance of HIF in maintaining oxygen homeostasis and essential functions in humans, it is expected that HIF perturbation would disrupt the most basic developmental processes. Lyer et al. observed that inactivation of HIF causes severe developmental abnormalities and impairment of joint development [27].

Figure 2.1 Hypoxia pathway regulation in normoxia and hypoxia. **(A)** In normoxia, hypoxia inducible factor α subunits HIF1 α and HIF2 α are hydroxylated by PHD, **(B)** then it is ubiquitinated through interaction with pVHL, and **(C)** subsequently degraded by the 26S proteasomal pathway in the cytosol resulting in minimal transcriptional activity. **(D)** In hypoxia, the activity of the HIF-targeting prolyl hydroxylase enzymes is inhibited allowing HIF1 α /2 α to rapidly accumulate in the cytoplasm. (e and f) Then, HIF1 α /2 α gets phosphorylated, translocated into the nucleus and dimerized with HIF1 β in order to activate transcription of its target genes.

2.2.1 HIF1 α

HIF1 α is the most important factor involved in the cellular response to low oxygen and is known as the master regulator of oxygen homeostasis [28]. HIF1 α acts as a transcription factor and activates the transcription of genes (**Figure 2.1**). Interestingly, *HIF1 α* has been identified as a key mediator for chondrocytes to respond to fluctuations of oxygen availability during cartilage development and repair, and may serve as a target for modulating chondrocyte functions [29].

Fernández-Torres et al. have shown that *HIF1α* gene plays a protective role against joint damage and might have a beneficial effect in maintaining cartilage homeostasis [30]. Schipani et al. revealed that cartilage structures in the limbs of mouse embryos are highly hypoxic and that *HIF1α* is essential for accurate growth-plate and joint formation [6]. Moreover, Mackie et al. revealed that *HIF1α* could act synergistically with *BMP2* to promote the expansion and proliferation of chondrocytes while inhibiting hypertrophic differentiation and subsequently endochondral ossification [14]. A need of HIF1α transcription factor for proper chondrocyte function and cartilage homeostasis was clear in mice with an inactivation of HIF1α in cartilaginous structures. A decreased in collagen type II and aggrecan, and cartilage degeneration was observed in Hif1α KO mouse when compared to wild type [31]. Nevertheless, no studies have established the necessity of HIF1α during cartilage formation.

2.2.2 HIF2α

Hypoxia-inducible factor 2 (HIF2) is a heterodimer of two proteins, HIF2α and HIF1β [32]. HIF2α is regulated via oxygen-dependent post-translational degradation and involved in controlling hypoxic responses through activation of target genes [8] (**Figure 2.1**). Although HIF1α and HIF2α show different sensitivity to oxygen tension and display distinct cellular activities, HIF2α helps HIF1α to mediate the response of cells to hypoxia [10]. In recent studies, HIF2α has emerged as the primary HIF involved in chondrocyte differentiation and its importance in articular cartilage homeostasis has been reported [32]. Lafont et al. provided evidence that HIF2α is essential for hypoxic induction of the human articular chondrocyte phenotype and promotes cartilage-matrix synthesis through induction of key cartilage genes [8]. Since it is highly expressed in degenerated cartilage diseases such as osteoarthritis (OA) and rheumatoid arthritis (RA) in humans and mice, it also appears to be implicated in catabolic mechanisms leading to cartilage breakdown and endochondral bone formation

[33]. Furthermore, a *Hif2α*-deficient mouse showed a multi organ failure and oxidative damage, which demonstrated the importance of this protein in tissue development and oxygen homeostasis [28].

2.2.3 HIF3α

Hypoxia Inducible Factor 3 Alpha Subunit (HIF3α) is a protein-coding gene that acts as a regulator of hypoxia-inducible gene expression [34]. Although HIF3α functions remain largely unexplored, available data on HIF3α indicate that this hypoxia inducible factor functionality differs from HIF1α and HIF2α [12]. HIF3α is induced at the protein level by hypoxia and regulated by HIF1α and HIF2α at the transcriptional level [35]. Yet, HIF3α could act as negative regulator of HIF1α and HIF2α [19]. Recent studies have described that *HIF3α* gene expression was lower in OA chondrocytes than in healthy chondrocytes. A lower *HIF3α* gene expression was found in the hypertrophic zone of human embryonic epiphyseal tissue, suggesting that *HIF3α* is a negative regulator of hypertrophic differentiation [35]. However, *HIF3α*'s role in chondrocyte development and phenotype has not been studied in much detail. Overall, the previous data indicate manipulation of the HIF pathway during chondrogenic differentiation in culture could promote cartilage regeneration and repair [6].

2.3 HYPOXIA DURING SKELETAL FORMATION IN MAMMALS

Chondrogenesis and endochondral ossification are cartilage differentiation processes that lead to skeletal formation and growth. Both processes are instrumental in skeletal repair after trauma [32]. In endochondral ossification, chondrocytes undergo well-ordered and controlled phases of proliferation, hypertrophic differentiation, mineralization of the surrounding matrix, death, blood vessel invasion and finally replacement of cartilaginous matrix with bone [6]. This process of chondrocytic matrix replacement by bone tissue is a well-defined temporal and spatial sequence of events and it is impacted by a number of different types of matrix molecules, growth factors, microenvironmental

conditions and stress [37]. As a principal factor during this process of endochondral ossification, vascular endothelial growth factor (VEGF), an angiogenic promoter protein, acts as a coordinator of chondrocyte death and extracellular matrix remodeling by functional activation of chondrocytes, chondroclasts and osteoblasts and attracting the ingrowth of blood vessel [5]. Moreover, hypertrophic chondrocytes synthesize type X collagen and mineralize their surrounding matrix before being replaced by bone tissue [37].

Due to the cartilage avascular nature, it relies on diffusion to obtain critical nutrients, such as oxygen and glucose. Oxygen distributes from the surrounding synovial fluid into the tissue, creating an oxygen gradient in the cartilage [38]. Zhou et al. created a mathematical model to calculate oxygen levels in human articular cartilage. This model proposed that oxygen levels may range from 5% on the articular surface to 1% in the deep zone [39]. This oxygen gradient depends on several factors, including oxygen concentration in synovial fluid, cartilage thickness and cell density, with oxygen tension decreasing with distance from the cartilage surface. *In vivo* measurements exhibited that oxygen tension in human articular cartilage ranges from 7% (53 mm Hg) in the superficial layer to less than 1% (7.6 mm Hg) in the deep zone [4]. Moreover, calculated oxygen tensions fell with increasing distance from the synovial surface to ~2% in the deep zone of bovine knee cartilage and to 3.6% for bovine ankle cartilage and dog knee, but remained above 5% for rabbit knee cartilage [39]. Furthermore, Brighton et al. described oxygen measurements on *in vivo* preparations of tibial epiphyses of rabbits and rats [3]. The proximal tibial epiphyses exhibited a low oxygen tension of approximately 19.5 millimeters of mercury in the resting zone whilst increasing progressively to a high tension of approximately 95.2 millimeters of mercury in the metaphyseal bone. It is clear that oxygen tension within healthy cartilage will differ from joint to joint and from species to species.

However, in all species oxygen tension decreases from the cartilage surface to deeper zones to an estimate of around 1% of oxygen [39].

It has been suggested that low oxygen maintains cartilage homeostasis by inhibiting angiogenesis and hypertrophic differentiation by reducing *VEGF* and *COL10A1* expression while inducing several genes required for matrix production such as glycosaminoglycans [40] and *COL2A1* during chondrocyte development [41]. Oxygen tension can have significant effects on the metabolism of articular cartilage, including changes in proteoglycan synthesis [42, 43]. Kuiper et al. suggested that chondrocytes synthesize more sulphated glycosaminoglycans (GAGs) when deeper in the tissue in conditions of chronic hypoxia [44]. Culturing cartilage at 5% O₂ significantly increased proteoglycan and collagen synthesis compared with 20% O₂ [42]. However, cartilage cultured at 1% O₂ caused a significant decrease in proteoglycan and collagen synthesis compared with 20% O₂. Also, an increased in hyaluronan synthesis occurred after cells were culture for 12 hours at 5% O₂ compared to 20% O₂, but decreased at 1% O₂ compared with 20% O₂ [45]. An adequate balance in oxygen tension is still necessary to achieve proper chondrogenesis for cartilage regeneration strategies.

2.4 CROSSTALK BETWEEN HYPOXIA AND CARTILAGE RELATED PATHWAYS

Gene expression and translation of hundreds of genes and proteins have been described to be oxygen dependent and to respond in helping chondrocytes during various physiological processes such as proliferation, cell fate, senescence and apoptosis [15]. Several studies have unravelled a complex molecular network that is involved in regulating the hypoxic response in physiological conditions [46]. These include various signaling pathways, fibroblast growth factors (FGFs), transforming growth factor β (TGF β), bone morphogenic proteins (BMPs), WNT, β -catenin, Hedgehog (HH), and Notch signaling pathways.

In native cartilage, growth factors stimulate cell growth, proliferation, differentiation and apoptosis [47]. Moreover, these specific biomolecules are strongly related to tissue damaged repair. Thus, growth factors are largely used in tissue regeneration approaches. For cartilage repair strategies, the most used biomolecules are those who act to initiate or suppress signaling molecules cascades during chondrogenesis. TGF β , BMP and IGF families are the most extensively studied developmental morphogens to stimulate chondrogenic differentiation of stem cells *in vitro* [48]. In addition, WNT, NOTCH and FGF are interesting candidates based on their *in vivo* roles during cartilage homeostasis [49]. Various members of these families have been tested *in vitro* as chondrogenic inducers and in combination with tri-dimensional environments, mechanical loading, genetic approaches and hypoxia [50]. Stem cells and chondrocytes closely interact with the biochemical and environmental stimuli during chondrogenesis to maintain a normal chondrocyte phenotype and avoid hypertrophic differentiation. Moreover, these biomolecules closely interact with the hypoxia pathway to activate lineage specific transcription factors such as *SOX9*. *SOX9* signaling helps to maintain the chondrocytic phenotype by inhibiting *RUNX2* expression preventing hypertrophy and posterior endochondral ossification [14]. In this section we will define how these signals, in combination with low oxygen tension, enhance *in vitro* chondrogenic differentiation of stem cell cultures for cartilage tissue engineering.

2.4.1 WNT signaling

WNT signaling has been previously described as a key factor during cartilage homeostasis. It participates in a tightly regulated and highly specific regulatory process of chondrogenic differentiation in progenitor cells [15]. Specifically, WNT activity is required for proliferation and maintenance of the chondrocytic phenotype [14]. In the case of *WNT3a* and *WNT5b* they promote chondrogenesis while delaying hypertrophy. *WNT11* overexpression in MSCs during

chondrogenic differentiation promotes a chondrocytic phenotype but also up-regulates *RUNX2* and indian hedgehog (*IHH*) expression, two genes involved in hypertrophic differentiation [15]. *WNT1*, *WNT4*, *WNT7a*, *WNT8* and *WNT9* have shown to inhibit chondrocyte differentiation and were found increased during OA progression [52, 53].

Overexpression of WNT signalling can increase chondrocyte hypertrophy and expression of degrading metalloproteinases in cartilage. Unmarino et al. mentioned that new research links the degradation of articular cartilage and OA progression to the interaction of *HIF1 α* with the WNT signaling pathway [54]. Interestingly, *HIF1 α* increases the expression of WNT signaling inhibitors Dickkopf (DKK1), frizzled B (FRZB) and Gremlin 1 (GREM1), which decreased the expression of hypertrophic markers during chondrogenesis of MSCs and prevented cartilage loss [87, 133, 134]. Moreover, the presence of WNT signaling inhibitors increased the expression of collagen type II and aggrecan. Therefore, regulation of the WNT signaling pathway by hypoxia and the HIF signaling pathway is fundamental to control both cartilage maintenance and prevention of OA progression.

2.4.1.1. Wnt inducible signaling proteins (WISPs)

WNT inducible signaling proteins (WISPs) are highly expressed in skeletal tissue and osteoprogenitor cells [55]. These proteins are known to participate in the intention to restore damaged articular cartilage during subchondral bone development. They have an important role in the formation, growth, differentiation and maintenance of both bone and cartilage [29]. An up-regulation of WNT signaling pathway and *WISP1* during OA progression increased the expression of matrix metalloproteinases (MMPs), interleukins and aggrecanases. Therefore, inhibition of *WISP1* signaling is essential for cartilage preservation. In **Chapter 4** using various target prediction software's, we show that *WISP1* is a potential target of miR-210 during the hypoxia response.

2.4.1.2. β -catenin

Regulation of β -catenin levels is important for the homeostasis of articular chondrocytes. Excessive or insufficient *β -catenin* will enhance pathological maturation at the periphery of the cartilage and early apoptosis at the center. The interaction between *HIF1 α* and *β -catenin* has been described as a negative regulator of WNT signaling and promotes a good balance in cartilage homeostasis and chondrocyte stability [55]. In contrast, *HIF2 α* positively regulates WNT/ β -catenin and interacts with NF- κ B pathways to promote chondrocyte hypertrophy, apoptosis and endochondral ossification [33].

2.4.2 Transforming growth factor beta (TGF β)

Members of the TGF β superfamily are growth factors known to be essential for multiple stages of embryonic chondrogenesis [15]. They participate in stimulating differentiation of chondroprogenitor cells into chondrocytes while inhibiting chondrocyte hypertrophy and maturation. TGF β family members, such as TGF β 1, 2, 3, and BMPs, are known as major regulators in chondrogenesis and osteogenesis [14]. These factors serve as stimuli to promote growth and *in vitro* chondrogenic differentiation of MSCs [6]. However, *in vitro* administration of TGF β 1 has been shown to redirect human articular chondrocytes towards hypertrophy [15]. Several pro-chondrogenic genes that are regulated by TGF β require also *HIF1 α* expression. Ueno et al. demonstrated that silencing *HIF1 α* could attenuate TGF β 1 induced chondrogenic gene expression, whereas Sanchez-Elsner et al., confirmed that low oxygen mediates an interaction between *HIF1 α* and TGF β via SMAD3/4 signaling pathway [56, 57]. Hence, stimulation of chondrocytes with TGF β could require HIF1 α protein accumulation.

2.4.3 Bone morphogenetic protein (BMP)

Bone Morphogenetic proteins (BMPs) are cytokines that belong to the transforming growth factor- β (TGF β) superfamily [15]. They are essential for mesenchymal condensastion and chondrogenic differentiation of mesenchymal

stem cells (MSCs) [14]. BMP2 has shown to stimulate *COL2A1* gene expression in human articular chondrocytes. Moreover, BMP2 and BMP4 have been shown to induce hypertrophy in progenitor cells, whereas BMP7 maintains a chondrogenic potential while preventing chondrocyte hypertrophy [8] and terminal differentiation [58].

2.4.4 Insulin Like Growth Factor (IGF)

IGFs are single-chain polypeptides that have similar function and structure to insulin. These hormones are involved in mediating growth and development. IGF1 is a small protein that regulates growth in adults and plays a main role in DNA synthesis in multiple cell types, including chondrocytes. It penetrates into articular cartilage through the synovial fluid to stimulate chondrocyte proliferation and proteoglycan homeostasis [59]. In recent years, addition of IGF1 has shown to stimulate the chondrogenic potential of stem cells. As observed, IGF1 stimulates proteoglycan production while promoting the maintenance of the chondrogenic phenotype of articular chondrocytes. In addition, it has been demonstrated that a combination of IGF1 with either BMP7 or TGF β 1 results in greater repair potential when compared with either factor alone [60].

2.4.5 Fibroblast Growth Factor (FGF)

FGFs are known to be involved during cartilage development [15]. Progression of chondrocyte differentiation and maturation has been associated with *FGF2*, *FGF9*, and *FGF18* expression [6]. *FGF2* has the function of priming cells for chondrocytic differentiation whilst enhancing chondrocyte proliferation [6, 60]. Previous studies have shown that interaction of FGF2 with TGF β and WNTs enhances chondrogenesis [15]. In addition, FGF9 and FGF18 are known regulators of early chondrogenic differentiation and are involved in augmenting ECM production as a result of gene expression up-regulation of pro-chondrogenic proteins such as SOX9, IHH and COL2A1. In a rat model of osteoarthritis, Fgf18 acted as a pro-

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chondrogenic factor and was shown to stimulate repair of damaged cartilage [15]. Interestingly, the interaction of HIF1 α and FGF correlated to early chondrogenesis and chondrogenic differentiation [135].

2.4.6 Hedgehog signaling

Sonic hedgehog (SHH) signaling is critically important during early chondrogenesis due to its role in controlling cell fate and its synergism with other pro-chondrogenic factors. It acts synergistically with BMP signaling to increase *SOX9* expression while interacting with the FGF family to enhance chondrogenesis in the earliest stages of endochondral ossification [15]. IHH is synthesized during pre-hypertrophic and hypertrophic stages of chondrocytes in the growth plate. It has shown to stimulate chondrocyte proliferation and hypertrophy, while inducing differentiation of perichondrial progenitor cells into osteoblasts to form the collarbone [62]. Although IHH is not considered one of the master regulators of chondrogenesis, previous studies showed that IHH could be as potent as TGF β 1 and BMP2 in chondrogenic induction of primary MSCs [136]. Precise homeostatic regulation of *IHH* is crucial for promoting chondrogenesis over osteogenesis. Bijlsma et al. demonstrated that hypoxia is able to induce a hedgehog response in adult mice [63, 64]. *In vitro* models of adult stem cells showed that accumulation of HIF1 α was sufficient to secrete SHH and hedgehog signaling. In contrast, pharmacological inhibition and knockdown of HIF1 α in ischaemic adult tissue inhibited SHH and hedgehog pathway activation. Thus, induction of hedgehog signaling can be controlled through HIF1 α accumulation after exposure to low oxygen [65]. Altogether, hedgehog factors are important pro-chondrogenic signaling factors that could have significant impact in cartilage tissue engineering.

2.5 HYPOXIA AND CARTILAGE DESTRUCTION

Since oxygen tension is altered in arthritic joints [65], the oxygen conditions during OA development have been largely studied. A depletion of oxygen in

patients with OA has been reported to be due to alterations in blood flow, gas exchange and inflammatory factors in the synovial membrane and subchondral bone [6]. Several groups have reported an increase in HIF1 α and HIF2 α transcriptional activity in OA cartilage and OA chondrocytes, particularly in the late stages of disease [33]. *HIF1 α* is up-regulated by other factors, such as inflammatory cytokines, mechanical loading and reactive oxygen species, which are key factors for the regulation of cartilage degradation. In addition, Houard et al. found that *HIF2 α* is a modulator of matrix degradation in articular cartilage during OA progression. They indicated that *HIF2 α* could potentiate *IL6* and *TNF α* signaling in diseased chondrocytes [68]. Moreover, a number of catabolic enzymes associated with OA progression have been identified as direct *HIF2 α* targets [69]. Although, maintaining a hypoxic environment is essential for cartilage homeostasis, the imbalance in oxygen tension during cartilage degeneration in osteoarthritis (OA) and rheumatoid arthritis (RA) progression increases cartilage degradation, increasing inflammation and metalloproteinase expression, while decreasing proteoglycan deposition.

Osteoarthritis is a degenerative joint disease that predominantly induces mechanical stress changes in cartilage and bone [29]. OA constitutes a major health problem that is responsible for chronic disability and impacts the quality of life of millions of people worldwide [39]. Several cytokines and inflammatory markers have been found elevated during OA progression, including *RUNX2*, *COL10A1*, *IHH*, alkaline phosphatase (*ALPL*), *MMP13*, interleukin 1 β (*IL1 β*) and tumor necrosis factor alpha (*TNF α*), which are all closely associated with cartilage degradation [39, 66]. *IL1 β* and *TNF α* have been largely measured in synovial fluid during cartilage OA and their concentrations are highly dependent on the stage of disease [68].

Rheumatoid arthritis is a polyarticular chronic inflammatory autoimmune disease that largely affects the joints and is associated with cartilage destruction

and functional disability. During the development of RA, cartilage is destroyed by pro-inflammatory cytokines, such as TNF α with additional roles from IL6 and IL1 β . These cytokines are mainly derived from the synovial membrane or cartilage and accumulate in the synovial fluid. They are known to contribute to joint inflammation and cartilage destruction by increasing expression of matrix degrading enzymes [39].

Degeneration of articular cartilage has been associated to several factors that predispose the tissue towards disease. Among these factors are aging, injuries, joint instability, mechanical stress, inflammation and metabolic disorders [39, 66]. The stress generated by these factors often leads to a disruption in the balance of cartilage anabolic and catabolic processes, which can result in chondrocytes phenotypic change, cell death and an increase in inflammatory genes [65]. Ultimately, all these changes will lead to deterioration of structural and functional properties of the cartilage and joint destruction [66]. Although there is a broad range of stress factors that contribute to the development of arthritis, the exact mechanism of its pathogenesis is still unknown [137, 138].

2.6 HYPOXIA AND TISSUE ENGINEERING

Over the past decade, tissue engineering has become the prospective solution for defects resulting from severe trauma, osteodegenerative diseases and mechanical stress. Significant research on this field has been made by engineers and physicians [15], which have described different methods to restore joint function and prevent joint degeneration. To achieve a proper tissue engineering strategy for regeneration of cartilage, several characteristics are needed: 1) an appropriate cell source with the ability to proliferate, differentiate and maintain a chondrocytic phenotype over a long time period, 2) a proper biological factor that facilitates the differentiation of chondroprogenitor cells, and 3) a biocompatible and mechanically suitable biomaterial scaffold that resembles the cartilaginous matrix providing a 3D architecture for the cells [9, 48, 51]. Tissue

engineering requires the use of different cell types, scaffolds, and genetic stimuli to repair or reconstitute articular cartilage. Therefore, we will describe some of the methods utilized in cartilage tissue engineering strategies and the use of low oxygen conditions (e.g. hypoxia) as an enhancer of chondrogenic differentiation.

2.6.1 Cell sources

2.6.1.1 Chondrocytes

Cartilage is composed of an abundant extracellular matrix consisting of proteoglycans and collagens type II, IX, and XI which are synthesized by chondrocytes. Brittberg et al. were the first to isolate articular chondrocytes for cartilage repair and developed a technique known as autologous chondrocyte implantation (ACI) [139]. They considered chondrocytes as the ideal cell source for engineering cartilage implants since they retain a chondrogenic phenotype and could synthesize a hyaline articular cartilage extracellular matrix. Although short-term clinical results were good, several disadvantages of the technique were also reported [139]. Among its limitations is the reduced supply of chondrocytes, which requires a long expansion time to obtain sufficient cells for transplantation, increasing the potential to de-differentiate, and promote a fibrocartilage formation instead of a hyaline cartilage repair [65]. Considering the aforementioned problems associated with the use of autologous articular chondrocytes, the control of oxygen tension in cell cultures has become an important parameter for tissue engineering [71]. Several studies have described how *in vitro* low oxygen cultures are beneficial for the maintenance of a chondrocytic phenotype (**Table 2.1**). Despite of the culture system, hypoxic culture delays the de-differentiation of murine, bovine and human chondrocytes. Under hypoxia, chondrocytes showed an increase in the synthesis of cartilage extracellular matrix proteins including aggrecan, collagen type II, collagen type IX and collagen type XI [7]. Moreover, low oxygen suppressed hypertrophic differentiation and subsequently terminal differentiation by inhibiting matrix

degrading enzymes *MMP3* and *MMP13* [72] and decreasing *COL1A1* gene expression [74]. Furthermore, most of the existing literature uses bovine chondrocytes instead of human-isolated chondrocytes. These bovine chondrocytes are obtained from healthy cartilage and have not been exposed to any cartilage degradation. In contrast, the human chondrocytes obtained for the ACI technique, are surrounding by OA synovial fluid and cartilage degrading enzymes. However, how these data can be extrapolated to the human situation remains to be seen.

Previous publications showed an increase in chondrocytes proliferation and cell number under lower oxygen concentrations [75]. Robins et al. also observed that in low oxygen cultures not only *COL2A1* was expressed, but also vascular endothelial growth factor (*VEGF*) was up-regulated [76]. The increase in expression of *VEGF* correlates with the onset of the healing process found after osteochondral fractures. Hypoxia inducible factor (HIF) regulates *VEGF*, which participates in angiogenesis and nitric oxide generation, is essential for vasodilation, and causes an increase in blood flow to ischemic tissues [77]. *VEGF* factors are required during development of collateral vessel growth, blood flow, tissue perfusion, extravasation of inflammatory cells, tissue remodeling and repair [78]. Moreover, local production of *VEGF* has shown to limit ischemia induced tissue damage and inflammation during chronic hypoxia. *VEGFA* is a critical mediator of vasculogenesis during development, has neuroprotective effects in ischemic brain [79], and has a positive influence on inflammatory response in the lung. Recent studies suggest that *VEGFB* has a major role in regulating fatty acid and electron transport [80, 81], whereas *VEGFC* knockout showed embryonically lethality and leads to fluid accumulation due to deficiency of lymphatic drainage [77, 78].

Table 2.1 Chondrocytes and low oxygen for cartilage repair strategies					
Species	Oxygen tension	Culture Method	Culture time	Results	References
Bovine	5, 21%	Agarose	48 hours	↑GAGs, Mmps, <i>Adamts5</i> , <i>Mmp13</i>	111
	5, 21%	Alginate beads	3 weeks	↑ <i>Col2a1</i> , [(3)H] proline and [(35)] sulfate incorporation	75
	5, 21%	Monolayer, Alginate beads	2-3 weeks	↑ cell number, <i>Col2a1</i> , <i>Col9a1</i> , GAGs, proline incorporation	144
	5, 21%	Alginate beads	2 weeks	↑ <i>Col2a1</i> , proteoglycan synthesis, ↓ <i>Col1a1</i> , proline incorporation	143
	5, 21%	Alginate beads	3 weeks	↑cell number, <i>Col2a1</i> , <i>Col10a1</i> , GAG synthesis	76
	2, 20%	Alginate beads	2 weeks	↑ <i>Col2a1</i> , <i>Acan</i> , GAG synthesis, ↓ <i>Mmp3</i> , <i>Mmp13</i>	72
Human	1, 21%	Cell suspension	16 hours	↑ <i>COL1A1</i> , <i>HIF1A</i> , <i>HIF2A</i> , ↓ <i>COL2A1</i>	11
	<1, 2, 5, 21%	Alginate beads	96 hours	↓ Cell viability, ↑ GAGs	141
	2, 20%	Pellets, PCL scaffold	4 weeks	↑ <i>COL2A1</i> , <i>HIST2H4</i> , <i>HIF1A</i> , <i>SOX9</i> , ↓ <i>ACAN</i> , <i>COL1A1</i>	74
	1, 20%	Alginate beads	48 hours	↑ <i>GDF1</i> , <i>PHD3</i> , <i>VEGF</i> , <i>SOX9</i> , <i>COL1A1</i> , <i>MMP13</i> , <i>TGFB2</i> , <i>SOX9</i>	142
Murine	0.5, 20%	Monolayer	1 week	↑ <i>Vegf</i> , <i>Vegf</i> , <i>Hif1a</i>	5
	2, 21%	Monolayer	3 days	↑ <i>Acan</i> , <i>Col2a1</i> , <i>Sox9</i> , <i>Hif1a</i> , ↓ <i>Vegfa</i> , <i>Bmp4</i> , <i>Tgfb</i>	140

2.6.1.2 Mesenchymal stem cells

Mesenchymal stem cells (MSCs) are multipotent progenitor cells that possess the ability to be expanded for up to 40 generations without losing their stemness [73]. They are capable of undergoing differentiation into chondrogenic, osteogenic and adipogenic lineages. These cells are promising candidates for tissue engineering due to the variety of sources (e.g adipose tissue, bone marrow, muscle, periodontal ligament, lung, liver, spleen, thymus, amnion, placenta, umbilical cord blood, synovial membrane and cartilage) and the ease of isolation [12]. The safety of these stem cells has been demonstrated in phase III clinical studies. Currently, 52 clinical studies involving MSCs for osteoarthritis (OA) therapy are referenced at ClinicalTrials.gov [7].

Previous data confirmed that the *in vivo* implantation of MSCs into cartilage defects induces desirable healing outcomes and improved clinical signs in treated patients [15, 61]. Riester et al. showed that injection of AMSCs in early stages of OA inhibited synovial thickening and cartilage loss [82]. There is evidence that

suggests that under more physiological oxygen tension (1-7% oxygen) MSCs increase their chondrogenic potential, while decreasing hypertrophic differentiation, apoptosis, cellular senescence and endochondral ossification [74]. In hypoxic conditions an elevated glycosaminoglycan production, up-regulation of several chondrogenic genes, collagen cross-linking and enhanced mechanical strength has been observed. *In vitro* experiments have shown *HIF1 α* is able to potentiate chondrogenesis of MSCs, while inhibiting hypertrophy and subsequently endochondral ossification [40]. In addition, *HIF2 α* was found to increase the synthesis and cartilaginous matrix production during chondrogenic differentiation of stem cells [83]. Thus, the hypoxia pathway seems crucial for completing chondrogenesis on MSCs.

2.6.1.2.1 Bone marrow derived stem cells (bMSCs)

Bone marrow derived mesenchymal stem cells are the most studied and used stem cells for cartilage engineering due to their efficiency in differentiating into the chondrocytic lineage. Among their advantages, the ability to harvest cells from many different bone marrow locations, high proliferation rate, their differentiation capacity and the ability to maintain a relative stable phenotype *in vitro* endorse them as a good cell source for cartilage repair [84].

In recent years, the use of oxygen levels to control MSC proliferation and differentiation has received major interest. As shown in **Table 2.2**, several studies have shown that bMSCs cultured under low oxygen tension (1-5% oxygen) during chondrogenic induction are able to differentiate into hyaline cartilage that better resembles the permanent features of articular cartilage [85]. Human bMSCs treated with TGF β 1, 2 or 3 that were cultured in monolayer and high density pellets under low oxygen conditions, expressed typical articular cartilage biomarkers and inhibitors of hypertrophic differentiation such as *COL2A1*, *SOX9*, *ACAN*, Gremlin 1 (*GREM1*), Frizzled-related protein (*FRZB*) and Dickkopf WNT signaling inhibitor 1 (*DKK1*) [86].

Species	Oxygen tension	Culture Method	Chondrogenic Differentiation	Other Differentiation	Culture time	Results	References
Bovine	5, 20%	PCL scaffold	TGFβ3	-	6 weeks	↑GAGs synthesis, <i>Col10a1</i> , <i>Alpl</i> , calcium accumulation, <i>Col2a1/Col1a1</i> ratio, ↓cellularity	155
Human	2%	Monolayer	TGFβ3	Adipo, Osteo, Neural, Endodermal	3 weeks	↑self renewal, cell doubling times, <i>SOX2</i> , <i>NANOG</i> , <i>OCT4</i> , <i>REST</i> , <i>HIF1A</i> , <i>RE1</i> , Wnt pathway, PI3K/Akt pathway, ↓ <i>AIF</i> , <i>BAK</i> , <i>p53</i> , β-galactosidase activity	156
	2, 20%	Monolayer	TGFβ3	Adipo, Osteo, Neural, Endodermal	2 weeks	↑proliferation, <i>OCT4</i> , <i>HIF2A</i> , connexin-43, cell density, neural and endodermal differentiation	157
	2.5, 21%	High-density pellets	TGFβ3	-	5 weeks	↑GAGs synthesis, <i>SOX9</i> , <i>COL2A1</i> , <i>ACAN</i> , <i>GREM1</i> , <i>FRZB</i> , <i>DKK1</i> , ↓ <i>COL10A1</i> , <i>MMP13</i> , <i>PANX3</i>	85, 86
	<1, 21%	High-density Monolayer	TGFβ1	Adipo/Osteogenic	48 hours	↑ <i>OPN</i> , <i>VEGF</i> , ↓ <i>RUNX2</i> , <i>SP7</i> , <i>COL1A1</i>	158
	5, 21%	High-density pellets	TGFβ3	Adipo/Osteogenic	4 weeks	↑colony formation, proliferation, metabolic activity, osteogenic genes, alkaline phosphatase activity, ↓adipogenesis, chondrogenesis	146
	1.5, 21%	Monolayer, Micromass	Chondrocyte differentiation media (Cambrex)	Adipo/Osteogenic	3 weeks	↑cell proliferation, <i>HIF1A</i> , <i>COL2A1</i> , not changes in adipo/osteogenesis	147
	2, 20%	Monolayer, High-density pellets	-	Adipo/Osteogenic	2 weeks	↑proliferation, cell number, ↑ <i>OCT4</i> , <i>HIF2A</i> , connexin-43, fibronectin	104
	5, 20%	High-density pellets	TGFβ2	Hypertrophic	4 weeks	↑proteoglycan deposition, <i>COL2A1</i> , ↓ <i>COL10A1</i> , <i>VEGF</i> , <i>ALPL</i> , <i>MMP13</i>	154
	2, 20%	Monolayer, pellet culture	TGFβ1	-	2 weeks	↑sulfated GAGs production, <i>COL2A1</i> , <i>ACAN</i> , ↓ <i>ADAMTS5</i> , <i>MMP2</i> , <i>MMP13</i>	40
	3, 21%	High-density pellets	TGFβ1	-	3 weeks	↑colony formation, GAGs, <i>AGG</i> , <i>COL1A2</i> , <i>COL2A1</i> , <i>COL10A1</i> , <i>COMP</i> , <i>SOX9</i> , <i>HIF1A</i> , <i>HIF2A</i> , <i>TGFB</i>	92
	2, 20%	High-density pellets	-	Adipo/Osteogenic	2 weeks	↑colony formation, <i>OCT4</i> , formation of embryoid bodies, <i>REX1</i> , ↓fibronectin	104
Ovine	5, 20%	High-density pellets	-	-	2 weeks	↑population growth, sulphated GAGs, <i>Col2a1</i> , ↓osteogenesis	145
Porcine	5, 20%	High-density pellets, Agarose hydrogels	TGFβ3	Osteogenic	3 weeks	↑cell colonies, <i>Col2a1</i> , ↓collagen accumulation, alizarin red staining, alkaline phosphatase activity	84
Murine	5, 20%	Monolayer	-	Osteogenic	2 weeks	↑ <i>Col2a1</i> , chondroitin sulfate, colony formation, proliferation rate, osteoblast differentiation, alkaline phosphatase activity, calcium content, von Kossa staining, protein synthesis, total collagen accumulation	159
	2, 20%	Monolayer	TGFβ	-	3 weeks	↑ <i>Col2a1</i> , proteoglycan deposition, <i>Hif1a</i> , <i>Akt</i> , <i>p38</i>	151

In addition, GAG synthesis and proteoglycan deposition were also increased at lower oxygen levels. The increased expression of hyaline cartilage markers was found after exposing bMSCs to hypoxia for 48 hours and were still present after 5 weeks of culture. In contrast, hypertrophic markers and terminal differentiation decreased under low oxygen tension, as demonstrated by down-regulation of *COL10A1*, *MMP13*, *RUNX2*, *SP7*, *COL1A1*, *ALPL*, reduced Alizarin Red staining and

alkaline phosphatase activity [87]. Hence, bMSCs are an attractive alternative to chondrocytes for therapeutic approaches in cartilage tissue engineering.

2.6.1.2.2 Adipose Derived Stem Cells (aMSCs)

In light of the limitations of bMSCs (e.g. donor site morbidity and relatively low yield of cells), adipose tissue derived MSCs (aMSCs) were studied as an attractive stem cell population to use in cartilage repair. Interestingly, the adipose tissue stromal vascular fraction contains 10 to 100-fold more clonogenic cells than bone marrow [87], and large quantities can be easily obtained through liposuction. Recent studies indicated that aMSCs are a promising cell source for tissue regeneration and their effects are mostly mediated by their ability to produce various bioactive molecules such as growth factors, cytokines and extracellular vesicles. Despite the large body of evidence indicating the beneficial effects of aMSCs on cartilage regeneration, the efficiency of the therapies are donor specific and change according to patients' age, sex and existent disease [88]. However, low oxygen tension seems to stimulate chondrogenic commitment while inhibiting osteogenic and adipogenic differentiation of aMSCs, as has been reported for bMSCs [73].

Table 2.3 provides a summary of studies that have examined the effect of oxygen tension during chondrogenic differentiation of aMSCs cultured in alginate beads, micromass, high-density pellets, monolayer and PCL scaffolds. Previous results revealed that hypoxia notably enhances chondrogenesis by increasing cell proliferation as well as the expression of chondrogenic markers, *ACAN*, *COL2A1*, *SOX9*, *HAPLN1* and *COMP*. Collectively, published data revealed that low oxygen is an efficient and reliable tool for augmenting chondrogenic differentiation of aMSCs. However, the influence of hypoxia is strongly dependent of experimental settings, culture methods, donor characteristics and oxygen tension. Yet, it is still not clear if hypoxia could maintain a stable chondrogenic phenotype of aMSCs while prevent hypertrophic differentiation *in vivo*, as has

been published for bMSCs. Future studies could address this issue in adapted animal models for cartilage repair.

Table 2.3 aMSCs and low oxygen for cartilage repair strategies							
Species	Oxygen tension	Culture Method	Chondrogenic Differentiation	Other Differentiation	Culture time	Results	References
Human	5, 20%	Alginate beads	TGFβ1	-	2 weeks	↑protein synthesis, total collagen synthesis, GAGs synthesis, COL2A1, chondroitin 4 sulfate, ↓proliferation rate	150
	2, 21%	High-density pellets, Monolayer	TGFβ1	Adipogenic, Osteogenic	3 weeks	↑REX1, SOX2, OCT4, NANOG, HIF1A, proliferation rate, cell number, chondrogenesis, ↓adipogenesis,	105
	5, 21%	High-density pellets	TGFβ1	-	4 weeks	↑SOX9, ACAN, COL2A1, MMP13, COL10A1	122
	5, 20%	High-density pellets	TGFβ3	-	2 weeks	↑HIF2A, GAGs synthesis, SOX5, SOX6, SOX9, ACAN, versican, COL2A1, COL9A1, COL11A1,	149
	2.5, 21%	High-density pellets, PCL scaffold	TGFβ1	-	4 weeks	↑SOX9, HAPLN1, COMP, HIST2H4, HIF1A, COL10A1, GLI1, IHH, ↓COL1A1	74
Murine	2, 21%	Micromass, Monolayer	TGFβ1	Osteogenic	1 week	↑Cell number, DNA synthesis, proliferation, chondrogenesis, ↓Mmp2, Mmp3, Mmp8, Mmp13, osteogenesis, Runx2, Opa	106
	2, 21%	Alginate beads	BMP6	Adipogenic, Osteogenic	2 weeks	↑proliferation rate, Pona, Col2a1, adipogenesis	153
	2, 21%	Micromass	TGFβ1	Osteogenic	2 weeks	↑proliferation rate, ↓chondrogenesis, osteogenesis	152
Rabbit	5, 21%	Monolayer	TGFβ1	-	4 weeks	↑Acan, Col2a1, ↓Runx2	82

2.6.1.2.3 Other Stem Cells

Other stem cells sources which are currently reviewed for cartilage tissue engineering are shown in **Table 2.4**. Synovium derived stem cells (sMSCs) are currently studied as a novel and promising stem cell source for cartilage repair. In physiological conditions sMSCs migrate into articular cartilage lesions where they differentiate into chondrogenic progenitor cells [89]. Several *in vitro* and animal studies have shown high chondrogenic potential when compared to bMSCs. The advantage of sMSCs cells over other stem cell sources is the ability to grow fast and the less invasive transplantation for the patient. However, their harvesting process is more invasive than for bMSCs. These cells have not been used clinically.

Embryonic stem cells (ESCs) are derived from the inner cell mass of a blastocyst, an early stage pre-implantation embryo. Recent data confirmed that human ESCs

can be induced *in vitro* into the chondrogenic lineage using certain microenvironmental conditions [90]. Low oxygen tension has been the microenvironmental condition selected to minimize the spontaneous differentiation of ESCs, while improving their chondrogenic potential even during prolonged cultures (18 months). Despite extensive data looking at the effect of hypoxia effects on ESCs, the effect of low oxygen during chondrogenic differentiation of ESCs has not been studied in great detail.

Articular cartilage-derived stem cells (CSCs) are believed to be more committed to the chondrogenic lineage when compared to mesenchymal stem cells that are harvested from outside the joint. Cartilage-derived stem cells are mostly harvested from the superficial zone of adult articular cartilage. In addition, these cells can also be harvested from osteoarthritic (OA) cartilage, which contains larger pools of progenitor cells. CSCs are believed to be primed to differentiate into hyaline cartilage instead of fibrous or hypertrophic tissue making them ideal for cartilage repair [90]. Jayasuriya et al. observed the high chondrogenic potential of these cells, and used them to they successfully repair cartilage defects in 15 patients [91]. In addition, McCarthy et al. found higher expression of *COL2A1* in CSCs when compared to articular chondrocytes when implanted in goat chondral defects [129]. These results strongly suggested that cartilage derived progenitor cells have the potential to create hyaline cartilage instead of hypertrophic cartilage once implanted. However, the major limitation of their use in clinical studies is that they account for less than 1% of all cells found in articular cartilage.

Dermis isolated stem cells (DIAS) are cells isolated from the dermis and have been able to differentiate into the chondrogenic lineage in the presence of cartilage extra cellular matrix. Kaplakci et al. [93] showed that in goat DIAS cultured in micromasses, chondrogenically induced with TGFβ1 and hypoxia (5% oxygen) during 2 weeks, the production of type II collagen and

glycosaminoglycan content was increased. However, low oxygen culture resulted in a significant decrease in cell isolation and proliferation. The use of these cells is, however, promising in the field of tissue engineering due to their availability and negligible damage to the donor site.

Table 2.4 Other stem cells and low oxygen for cartilage repair strategies								
Cell type	Species	Oxygen tension	Culture Method	Chondrogenic Differentiation	Other Differentiation	Culture time	Results	References
SMSCs	Human	2, 5, 21%	Layered chondrocytes sheets	-	-	2 weeks	↑cell metabolic activity, proliferation, proteoglycans, COL2A1, ↓MMP3, MMP13	131
	Human	5, 21%	High-density pellets	TGFβ3, BMP7	-	2 weeks	↑colony number, cell number, proliferation, chondrogenic potential	88
	Porcine	5, 21%	High-density pellets	TGFβ3	-	2 weeks	↑proliferation, Col2a1, GAGs synthesis, ↓Col1a1	148
ESCs	Human	2, 21%	Monolayer	-	-	3 weeks	↑FGFR1, FGFR2, FRAT2, p-SMAD2/3, embryoid body formation, ↓SOX17, desmin, GATA4, brachyury, CDX2	160
	Human	3, 12, 21%	Monolayer	-	-	3 weeks	↑OCT4/NANOG, FOXA2, SOX17, AFP, GATA4, ↓reduced BrdU incorporation, aneuploidy	161
	Human	5, 20%	Monolayer	-	-	18 months	↑OCT4, NANOG, telomerase activity	162
	Porcine	3, 5, 21%	Monolayer	-	-	2 weeks	↑proliferation, formation of embryoid bodies, Oct4, embryonic antigen 4, human chorionic gonadotropin, progesterone	89
CSCs	Bovine	2, 20%	Micromass	BMP4, BMP7, TGFβ1	-	2 weeks	↑proteoglycan deposition, Szp, Col2a1, matrix synthesis	90
	Human	2, 20%	High-density pellets	TGFβ1	-	2 weeks	↑matrix synthesis, COL2A1, ↓COL1A1, COL10A1	91
DIASs	Goat	5, 20%	Micromass	TGFβ1	-	2 weeks	↑Col2a1, GAGs synthesis, ↓proliferation	93
PSCs	Mouse	1, 21%	Micromass	TGFβ1	Osteogenic, Adipogenic	1 week	↑alcian blue stain, Col2a1, Acan, Vegf, Glut1, Ldja	94
	Rat	5, 20%	Monolayer	-	Osteogenic	1 week	↑Hif1a, Vegf, Runx2, Bmp2, Alpl, Bsp, Ocn, periostin	163
IFPSCs	Porcine	5, 20%	Monolayer, hydrogel	TGFβ3	-	6 weeks	↑ECM production, mechanical functionality	50

Another progenitor cell source that is believed suitable for chondroprogenitor cells is the periosteum. Periosteum-derived cells (PSCs) exhibit high self-renewal capacity, stable phenotype throughout expansion and are inducible for chondrogenesis under the correct culture condition. Van Gastel et al. [94] observed that mouse PSCs chondrogenically induced with TGFβ1 and cultured in a micromass system under hypoxic conditions, significantly increased cartilage formation. These results suggested that under hypoxic conditions PSCs could be

differentiated into an osteochondroprogenitor cell and be potentially used for osteochondral defects.

Moreover, non-cartilaginous knee joint tissues such as infrapatellar fat pad (IFP) have a significant chondrogenic potential. These tissue could be more available and clinically feasible for collection of chondroprogenitor cells. IFP stem cells cultured in a low oxygen tension of 5% and in the presence of TGF β 3, enhanced chondrogenesis and improved their mechanical functionality [50].

Despite the increase in the amount of cartilage repair related research in the last decade, the exact mechanism by which stem cells differentiate into permanent hyaline cartilage is still unknown. However, therapeutic approaches that include the use of stem cells in combination with environmental regulation like oxygen tension, have a potential in optimizing chondrogenesis protocols for proper regeneration of articular cartilage. Further investigation including animal models and clinical trials is still required.

2.6.2 Genetic Manipulation

There is currently a great need to develop regulatory strategies that can be used in conjunction with stem cells to promote the most desirable repair response. Such strategies include the use of recombinant growth factors, microRNAs or small interfering RNAs (siRNAs) to attenuate further damage. This genetic manipulation could be performed during cell expansion as a strategy to precondition the cells before implantation. In this section, some data will be summarized regarding the use of siRNAs, microRNAs and knockout strategies in combination with hypoxia inducible factors and/or oxygen tension for cartilage repair.

Overexpression of HIF1 α using recombinant adenoviruses in MSCs, enhanced proliferation, migration, cell survival and expression of pro-angiogenic genes. Chromatin immunoprecipitation (ChIP) assays provided evidence for direct

interaction of HIF1 α with the *SOX9* promoter, thus supporting HIF1 α as a key component in the mechanism that regulates chondrogenesis by regulating *SOX9* expression [96]. Furthermore, HIF1 α expression can be increased using Cobalt chloride (CoCl₂), which inhibits prolylhydroxylases and stimulates HIF1 α breakdown. Even under normoxic conditions, CoCl₂ can be used to increase expression of ECM genes and cell viability, and protects chondrocytes from apoptosis in an HIF1 α dependent manner.

In contrast, knockdown and knockout experiments have been performed to study the critical role of hypoxia inducible factor HIF1 α during chondrogenesis and cartilage development. Schipani et al. examined a *HIF1 α* deletion using siRNAs in *COL2A1* expressing chondrocytes. *HIF1 α* knockdown accelerated catabolic stress-induced apoptosis and inhibited chondrogenesis [6]. Similarly, mouse articular chondrocytes with an Hif1 α deletion exhibited lower chondrogenic capacity [97]. Thus demonstrating that HIF1 α is critical for the survival of hypoxic chondrocytes and negatively regulates chondrocyte proliferation.

Preliminary data obtained in an conditional knockout model of Hif1 α in limb buds using a *Prrx1* promoter, suggested that hypoxia and Hif1 α may play a role in early stages of chondrocyte differentiation [98]. In addition, a dramatic reduction in *Sox9* expression followed by differentiation arrest, resulted in severe skeletal malformations. Mice lacking Hif1 α exhibited shorter and deformed bones whereas their growth plates were wider [99]. Chondrocytes in the interior of the developing growth plate displayed increased premature programmed cell death. Furthermore, chondrocytes exposed to hypoxia, but lacking *HIF1 α* , had markedly decreased expression of *COL2A1* and *ACAN*, suggesting the importance of this pathway in chondrocyte lineage progression [100]. In recent years, HIF2 α participation in chondrogenesis has also been studied. *HIF2 α* knockdown experiments resulted in a marked decrease in expression of chondrogenic genes

SOX9 and *COL2A1* [83]. Moreover, a conditional KO of Hif2 α in cartilage tissue, inhibited chemokine production and cartilage destruction. Therefore, the effects of hypoxia on chondrogenesis involves both HIF1 α and HIF2 α dependent and independent mechanisms, which rely on cell type and oxygen tension.

Furthermore, hypoxia triggers the expression of microRNAs. MicroRNAs are small non-coding RNA molecules of about 22 nucleotides which principal function is RNA silencing and post-transcriptional regulation of gene expression. Several studies have implicated a large number of microRNAs in regulating pre- and post-natal chondrogenesis. During the past decade multiple studies have demonstrated that miRNAs are involved in the hypoxic response and contribute to the repression of specific genes under low oxygen conditions [101]. These microRNAs contain HREs in their promoter region and are regulated by HIF transcription factors in response to low oxygen [102]. Although, HIF1 α is known as transcriptional activator, it also functions as transcriptional repressor via induction of miRNAs. Expression of miR-155, miR-424, miR-17-92, miR-21, miR-23, miR-24, miR-26, miR-103, miR-107, miR-181 and miR-210 [103-105], were found to be induced under hypoxic conditions and are potential targets of HIF1 α . In particular miR-210 was induced during hypoxic preconditioning of stem cells. This causes a cytoprotective effect and helps to maintain cell stemness but also supported cell differentiation [106].

2.6.3 Mechanical Stimulation

Because articular cartilage is a tissue subjected to various mechanical stimuli, including hydrostatic pressure and compressive or shear stress, the role of mechanical stimuli in combination with low oxygen tension has been considered to potentiate cartilage repair. Previously it has been described how MSCs are able to sense and discern their mechanical environment [107]. Accordingly, it has been reported that mechanical stimuli can be used for generating cartilage-engineered constructs with collagen and glycosaminoglycan content closely

resembling that of native cartilage [108]. In addition, mechanical stimuli applied to chondrocytes has been found essential for maintaining cartilage integrity. Porcine MSCs embedded in agarose hydrogels supplemented with TGF β 3 under low oxygen conditions and dynamic compression reduced GAG accumulation compared to controls. This study concluded that continuous exposure to low oxygen tension was a more potent pro-chondrogenic stimulus than 1 h/day of dynamic compression. In addition, dynamic compression has been shown to regulate chondrogenesis of bone marrow derived MSCs encapsulated in an agarose gel under low oxygen tension [109]. By preventing neo-vascularization and promoting a local hypoxic environment, mechanical loading could indirectly promote chondrogenesis of MSCs. However, *in vitro* experiments demonstrated that scaffolds under microenvironmental conditions (low glucose media and low oxygen concentration) with TGF β 3 stimulation could also enhance secretion of sulfated glycosaminoglycan and collagen type I [110].

Hydrostatic pressure has shown to promote and increase access to nutrients or from the mechanical stimulation of fluid flow. Elder et al. described how 5% oxygen in combination with intermittent hydrostatic pressure promoted chondrogenic gene expression in cells [112]. Bovine articular chondrocytes seeded on porous polyurethane scaffolds and cultured under normoxic (21% O₂) or hypoxic (5% O₂) conditions were subjected to a cyclic axial compression (10-20%; 0.5 Hz) applied for 1 h daily using a bioreactor. Cells cultured under reduced oxygen tension resulted in enhanced gene expression of *Col2a1*, *Acan*, and higher glycosaminoglycan content, whereas the expression of *Col1a1* was down-regulated in standard oxygen cultures (21% O₂). Moreover, histological analysis showed more intense Col2a1 and weaker Col1a1 staining in hypoxic than in normoxic cultures [113]. Therefore, the combination of mechanical stimuli and a hypoxic environment seems to enhance cartilage repair and

chondrogenesis of cells better than mechanical stimuli alone, by contributing to the stabilization of the chondrocytic phenotype and matrix synthesis.

2.6.4 Scaffold free cultures and biomaterials for cartilage tissue engineering

The function of a tissue engineering scaffold is to provide a temporary structure for the cells, which combined with biomechanical or biochemical signals could better fill the defect areas, enhance chondrogenic phenotype of the cells, and promote chondrogenesis on *in vivo* and/or *ex vivo* cultures [64]. Because cartilage production varies with scaffold architecture, the importance of choosing the appropriate scaffold for cartilage tissue engineering is vital.

Alternatively, scaffold free culture systems using high density pellets and micromass culture systems are used to stimulate cartilage formation. These culture systems have been proven to increase GAG accumulation under hypoxia conditions [50, 73, 74, 85, 93]. These cellular aggregates place cells in an environment that closely mimics the cell condensation observed during *in vivo* embryonic development of cartilage [15]. High-density pellets in combination with addition of members of the TGF β superfamily were found beneficial for complete *in vitro* chondrogenesis of MSCs [73]. Ovine MSCs expanded at 5% O₂ revealed a higher proliferation potential and an enhanced chondrogenic differentiation in both pellet culture and in a collagen type I hydrogel compared to those expanded at 20% O₂ [114]. Sheehy et al. demonstrated that MSCs expanded at 5% O₂ proliferated faster resulting in higher cell yields whereas differentiation at 5% O₂ was found to be a more potent promoter of chondrogenesis [84]. Higher COL1A1 and COL10A1 deposition was observed in pellets maintained at normoxia when compared to hypoxia. In a low oxygen environment, pellets demonstrated higher COL2A1 synthesis, a reduced COL10A1 and Alizarin Red staining and alkaline phosphatase activity [84]. Moreover, previous studies showed that implanting co-cultured pellets of stem

cells and chondrocytes in cartilage knee defects stimulates cartilage formation by increasing ECM synthesis [115].

Despite the good results observed with scaffold-free techniques, tissue engineering also studies the use of scaffolds whose primary objective is to replicate the characteristics of the target-tissue ECM. The scaffold should allow cells to adopt their native morphology and functionality, facilitating the encapsulation of cells and supporting cell growth, proliferation, and differentiation [116, 117]. Decellularized extra cellular matrices have been used with the idea of preserving the natural architecture of the tissue. Gilpin et al. confirmed that decellularized osteochondral scaffolds can successfully induce primary canine bMSCs to produce repair tissue with a glycosaminoglycan content and stiffness comparable to that of native cartilage under hypoxia [118, 119].

Hydrogels are versatile and appealing biomaterials for tissue engineering and cell therapy applications, due to their unique combination of properties similar to natural ECMs, such as mechanical stiffness, elasticity, water content, and degradation [120]. Various types of hydrogels derived from different natural or synthetic polymers have been used for reconstruction of articular cartilage tissues. Lynch et al. observed that after 10 days in culture at 1% oxygen, collagen production on PVCL-g-HA hydrogels was 10-fold increased compared to meHA controls [121], whereas Portron et al. observed the formation of a cartilaginous tissue when combining preconditioned rabbit or human adipose stromal cells (ASC) in chondrogenic medium containing TGF β and cultured within a Si-HPMC hydrogel [122]. Similarly, infrapatellar pad derived stem cells (IFPSCs) differentiated at 5% oxygen tension and encapsulated in either agarose or fibrin hydrogels exhibited significant GAGs accumulation after 42 days in culture [123]. A low oxygen tension enhanced chondrogenesis of IFPSCs constructs in the presence of TGF β 3 [49]. Rat bMSCs cultured on collagen-chitosan microbeads under 5% O₂ (hypoxia), demonstrated that low oxygen enhances initial

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progenitor cell survival. However, chondrogenic differentiation was not strongly supported in any of the microbead formulations [124]. In addition, IFPSCs displayed a diminished chondrogenic potential upon encapsulation in a three-dimensional hydrogel compared with pellet culture, synthesizing significantly lower levels of glycosaminoglycan and collagen on a per cell basis [125]. Hence, low oxygen conditions in combination with the appropriate scaffold could help in preprogramming the cells into a chondrogenic lineage while supporting cell proliferation and extra cellular matrix synthesis.

2.7 CHALLENGES, PROSPECTIVES AND FUTURE DIRECTIONS

From a surgical perspective, an estimated of 250,000 articular cartilage repair procedures are performed annually in the U.S [126]. However, these cartilage repair therapies do not consistently produce hyaline repair tissue, fill the entirety of the defect or integrate the repair tissue with the adjacent native tissue. The physiological conditions of repeated high-loading biomechanical motion and a low oxygen atmosphere makes the mechanisms of articular cartilage formation and maintenance complex [127]. Given the complexity of articular cartilage in composition, structure, and function, there are still some key challenges to be addressed for cartilage and osteochondral tissue regeneration. Consequently, a succesful cartilage tissue engineering strategy requires the maintainance or increase in COL2A1 matrix deposition while preventing the formation of fibrous cartilage by inhibiting COL1A1 expression and secretion once the cells are implanted *in vivo*.

Currently, non-genetic approaches are becoming attractive due to their ease of manipulation [28]. Overall, low oxygen tension seems to improve the chondrocytic phenotype of animal and human articular chondrocytes [12] while increasing chondrogenesis on stem cells derived from different sources (e.g fat pad, adipose, bone marrow, cartilage end plate, etc.) as previously described in this chapter [28, 94, 128, 129]. Moreover, the combination of low oxygen tension

(1 to 5% oxygen) under the adequate biochemical and/or biomechanical induction maintains a chondrocytic phenotype throughout the culture time. Furthermore, the possibility of plating chondrocytic cells on different scaffolds provides an adaptable structure for cell proliferation, differentiation, and enhances ECM synthesis. Altogether, the combination of these approaches could significantly improve the chance for success during *in vivo* implantation.

Although a large number of studies exhibited an improvement in chondrogenesis, there are still some reports that differ from the positive effects of low oxygen for GAG accumulation and collagen type II synthesis [28, 94, 122, 128, 129]. The variance could be related to differences in culture conditions and the factors used during chondrogenic induction. Moreover, the population characteristics of chondrocytes and stem cells used is also important to achieve good results. However, due to the small number of donors assessed on *in vitro* studies, conclusions regarding donor to donor variability cannot be stated with confidence.

An adequate balance in low oxygen tension is still required for full cartilage repair. Oxygen tension control will depend on cell population and characteristics and is involved in the delicate balance of stemness, chondrogenic differentiation, and terminal differentiation. Certain hypoxic levels could enhance chondrogenesis of chondroprogenitor cells while a different oxygen level could help increase OA progression. Moreover, prolonged hypoxia could lead to situations in which the intrinsic healing capacity of the tissue stalls. For *in vitro* cartilage engineering strategies, a delicate balance must be reached between the stimulatory and inhibitory effects of the low supply of oxygen. Further studies are required to elucidate the appropriate oxygen tension for each stage of chondrogenesis and the appropriate time of culture to create *in vivo* cartilage repair.

The need to recreate the native cartilage microenvironment at different stages of development will be beneficial to effectively treat the cartilage defect according to the patients needs. Lenas et al. advocate that a methodology to design a tissue *in vitro* needs to resemble articular cartilage's developmental process and should enable the control of each sequential differentiation step and should block terminal differentiation [130]. Optimization of protocols will indeed be essential to readily generate cartilage implants suitable for clinical use. A preconditioning stage in which the culture conditions mimic the different settings of the native environment, including not only oxygen tension but also mechanical stimulation will be also beneficial. Recent studies used static culture for the preconditioning stage of the cells when using scaffolds. This caused a heterogeneous distribution of the oxygen tension throughout the tissue. Cells in the core of the scaffold will be exposed to lower levels of oxygen to those cells in the outer parts of the scaffold. This heterogeneity could create differences in differentiation, proliferation and ultimately tissue organization [113, 131]. A bioreactor culture is currently under study to allow a more homogeneous tissue formation *in vitro* and could represent a potential solution to this limitation [132].

Lastly, maintenance of a hypoxia environment *in vitro* requires large and expensive equipment. Recent findings in this growing field showed crucial influence of hypoxia signalling on chondrocyte maintenance and raised new potential targets to repair cartilage and maintain tissue integrity. Future studies would need to determine an exact mechanism by which low oxygen tension could be firmly maintained during long-term cultures. This thesis focused its efforts in evaluating different molecular mechanisms to mimic a hypoxic environment without the need of a hypoxia chamber and in investigating molecular mechanisms that could support the chondrogenesis generated during low oxygen cultures.

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CHAPTER 3

3. Hypoxia promotes in vitro chondrogenic differentiation of adipose derived stem cells (aMSCs) in Three-Dimensional cultures.

ABSTRACT

To optimize human adipose-derived mesenchymal stem cells (aMSCs) and primary chondrocytes for therapeutic applications in cartilage regeneration, we investigated the role of low oxygen culture on chondrogenic differentiation. Cells were cultured for 21 days in chondrogenic media under normoxic (20% oxygen) or hypoxic (2% oxygen) conditions using two distinct 3-dimensional (3D) culture methods (high-density pellets and cells seeded in poly- ϵ -caprolactone scaffolds).

Gene expression of chondrocyte related genes and histological analysis was monitored during differentiation. The expression of a panel of cartilage markers including *COL2A1*, *ACAN* and *DCN*, was up regulated during chondrogenic differentiation in both 3D-culture systems and both aMSCs and human chondrocytes. Expression of several cartilage-related genes was modulated independent of oxygen levels, but we observed cell type-specific responses that depend on low oxygen or the type of 3D-culture system. For example, expression of *ACAN* and *HAPLN1* was more robust in pellet cultures when compared to scaffold cultures and hypoxia enhanced the expression of chondrogenic genes in scaffold cultures. Histological analysis revealed that both 3D-culture methods support formation of a glycosaminoglycan and COL2A1 positive matrix.

Cell type specific effects of low oxygen and 3D environments indicate that mesenchymal cell fate and differentiation potential is remarkably sensitive to oxygen. Genetic programming of aMSCs to a chondrocytic phenotype is effective under hypoxic conditions as evidenced by increased expression of cartilage-related biomarkers and biosynthesis of a glycosaminoglycan positive matrix. Furthermore, under low oxygen conditions 3D-culture environments improve the chondrogenic potential of aMSCs even further.

3.1 INTRODUCTION

Articular cartilage is a well-organized tissue that is adapted for friction-free synovial joint movements. Focal cartilage defects in articular cartilage caused by trauma or osteochondritis dissecans are leading causes of osteoarthritis (OA) in young patients [1]. When these focal lesions are loaded, increased stress on the surrounding cartilage leads to expansion of the defect size and further cartilage breakdown. Articular cartilage has reduced healing capacity that has been attributed to avascularity, reduced cellularity and low cell turnover [2]. In particular, the poor vascularity may impair normal tissue repair by humoral factors and stem/progenitor cells [3]. Due to the limited healing capacity, the regeneration of articular cartilage surface is critical in order to prevent future progression of degenerative arthritis and OA.

Treatments for symptomatic relief using injections of cortisone and hyaluronic acid, and cartilage repair stimulation procedures such as microfracture, subchondral drilling, abrasion, arthroplasty, and osteochondral graft transfers, which attempt to restore normal joint kinematics, have been performed for many years with some clinical success [4]. Currently, cell-based therapies such as autologous chondrocyte implantation (ACI) have become the gold standard for cartilage repair and regeneration. ACI is widely used for functional restoration of focal injuries (2-4 cm²) [5, 6] and has demonstrated promising clinical results in younger patients [7]. However, repair of more diffuse articular cartilage lesions is poor, especially in diffuse osteoarthritis in older patients. The ACI procedure has a number of limitations [8-10], including donor site morbidity, limited source of cells, limited expansion in vitro and production of fibrocartilage. In addition, the major drawback of ACI is the dedifferentiation of chondrocytes resulting from the expansion of the chondrocytes for multiple passages in monolayer in order to acquire a workable amount of cells for downstream implantation [11]. Furthermore, this dedifferentiation is defined by the gradual loss of chondrocyte phenotype.

As dedifferentiation progresses, molecular markers associated with chondrocytes and cartilage formation, such as aggrecan (*ACAN*), type II collagen (*COL2A1*), decorin (*DCN*), cartilage oligomeric matrix protein (*COMP*) and SRY (sex determining region Y)-box 9 (*SOX9*), start to decrease, whereas the expression of fibroblastic and osteogenic markers increase [12]. Moreover, spontaneous articular cartilage healing typically occurs via the development of fibrocartilaginous scar tissue that exhibits inferior biomechanical properties to hyaline articular cartilage and which may interfere with currently available clinical strategies for cartilage repair [13]. Fibrocartilaginous healing also fails to restore friction-free motion and is unable to withstand normal loads and compression forces, thus placing the joint at risk for progressive degeneration [14]. Promoting effective articular cartilage regeneration remains a clinical challenge.

Use of stem cells, and in particular mesenchymal stem cells (MSCs) from adult sources, has emerged as a viable solution to overcome limitations of current cartilage restoration procedures. Multipotent adult MSCs reside in several tissues including bone marrow, skeletal muscle, neural tissue, adipose tissue and synovium. MSCs are currently being used in various clinical trials with promising clinical results [15, 16]. However, promoting chondrogenic differentiation of MSCs can be challenging. Currently, most studies for cartilage repair have focused on bone marrow derived MSCs. As an alternative, adipose-tissue derived human mesenchymal stem cells (aMSCs) are an attractive cellular therapeutic due to minimally invasive tissue harvest, high abundance of cells and rapid expansion *ex vivo* [17]. Moreover, these cells are multipotent and can produce musculoskeletal extracellular matrix proteins once confluent and are able to differentiate into osteogenic and chondrogenic lineages [18, 19].

While there are many studies on chondrogenic differentiation of bone marrow derived MSCs, our studies address a major gap in our knowledge by investigating the chondrogenic potential of aMSCs under multiple culture conditions relevant

to cartilage tissue engineering, including hypoxia and propagation on 3D culture environment. The function of a tissue engineering scaffold to provide a temporary structure for the cells is evaluated [19]. Electrospun polymeric nanofibers emerged as potential scaffold for cartilage repair due to their capacity of mimicking the natural extracellular matrix [19, 33]. These scaffolds combined with the appropriate biochemical signals could better fill the defect areas, enhance chondrogenic phenotype of the cells, and promote chondrogenesis on *in vivo* and/or *ex vivo* cultures [33]. Moreover, a side-by-side comparison between aMSC and primary chondrocyte gene expression and histological analysis provides a new insight in the field.

3.2 MATERIALS AND METHODS

3.2.1 Cell harvest and monolayer culture.

Human adipose tissue derived mesenchymal stromal cells (aMSCs) were derived from lipo-aspirates obtained from consenting healthy donors. Human primary chondrocytes were obtained from the calcaneal apophysis of consenting donors. Studies with both cell types were approved by the Mayo Clinic Institutional Review Board (IRB). Maintenance media for aMSCs was comprised of advanced MEM (Gibco/Thermo Fisher Scientific, Waltham, MA) supplemented with 5% human platelet lysate (PL-Max, MillCreek Life Sciences, Rochester, MN), 1% penicillin/streptomycin, 1% Glutamax (Gibco/Thermo Fisher Scientific) and 0.2% Heparin (Baxter, Deerfield, IL). Human primary chondrocytes were cultured in maintenance medium that contained advanced MEM (Gibco/Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Atlanta, GA, Atlanta, GA) and 1% penicillin/streptomycin (Gibco/Thermo Fisher Scientific). Cells were cultured using standard procedures in T-175 cm² flasks at 37 °C, 95% humidity and 5% CO₂ until they reached 80% confluence.

Prior to each experiment, cells were detached from T175 flasks by trypsinization using TrypLE Express (Gibco/Thermo Fisher Scientific). For monolayer culture, aMSCs were plated in 6 well-plates at a density of 3,000 cells/cm² and maintained for 21 days in chondrogenic media which consisted of serum- or platelet lysate-free culture media supplemented with 40 mg/ml of L-proline (Sigma-Aldrich, Saint-Louis, MO), 50 mg/ml Insulin Transferrin Selenium-premix (Gibco/Thermo Fisher Scientific), 50 mg/ml of ascorbic acid (Sigma-Aldrich), 10 ng/ml of TGF- β 1 and 0.1 μ M dexamethasone (Sigma-Aldrich). Cell culture medium was replaced every three days.

3.2.2 Fabrication of nanofibrous poly- ϵ -caprolactone (PCL) scaffolds

Scaffolds were fabricated using an electrospinning technique described previously [20]. Scaffolds were composed of 9.5 wt% homogeneous solution of poly- ϵ -caprolactone. This solution was prepared by dissolving 1.05 g of high molecular weight (80,000) poly- ϵ -caprolactone (Sigma-Aldrich) in 0.8 g N, N-dimethylformamide (Thermo Fisher Scientific), 6.0 chloroform (Thermo Fisher Scientific) and 3.2 mL acetone (Thermo Fisher Scientific). The solution was stirred for 4 hours. The polymer solution was subsequently placed in a 20 mL glass syringe with a 20-G needle. The solution was dispensed at a rate of 3.3 mL/h while applying a 30 kV electrical field. All procedures were performed in a chemical hood. A glass plate mounted on an aluminum block placed 15 cm away from the needle collected the fibers. This procedure was followed until the sheets reached a thickness of 1 mm. Discs of 10 mm in diameter were cut from the sheets using a dermal punch. Both sides of the scaffold were sterilized by ultraviolet irradiation in a laminar flow hood for 30 min. To provide a hydrophilic surface for efficient cell attachment, scaffolds were pre-wetted by immersion in different ethanol concentrations for 30 minutes each (95%, 50%

and 25% ethanol), 30 minutes in distilled water and finally overnight in HBSS buffer in the 37°C incubator.

3.2.3 Chondrogenic differentiation

Induction of chondrocyte differentiation in high-density cellular aggregates was performed using pellet cultures in accordance with a protocol published previously [21]. To induce chondrogenic differentiation, 250,000 cells (aMSCs or human chondrocytes) were seeded in low attachment 96 round-bottom well plates (Corning). Pellets were formed by centrifugation and maintained for 24 hours in 37 °C, 95% humidity and 5% CO₂ until cells coalesced. Following pellet formation, maintenance media was exchanged with chondrogenic differentiation media, which was replaced every three days.

For PCL scaffolds, aMSCs and human primary chondrocytes grown in T175 cell culture flasks were trypsinized, counted, and plated at a density of 400,000 cells/scaffold. Scaffolds were placed in 24-well culture plates pre-coated with 0.3% poly-(hydroxyethyl-methacrylate) to prevent cell attachment to the tissue-culture plate surface. Scaffolds were incubated at 37°C for 4 hours to allow correct migration of the cells through the nanofibers. During the first 4 hours, 30 µl of complete media was added every 30 minutes to each scaffold to prevent desiccation of the fibers. After 4 hours of incubation 2 ml of complete media was added. Cell viability was monitored using a Live/Dead staining kit (Life Technologies, Carlsbad, CA) at Days 1, 3 and 7 in culture to visualize the live cells attached to the nanofibrous PCL scaffold. Fluorescence observations were performed using a semi-automated ZEISS Axio inverted light microscope with appropriate filters (Zeiss, Oberkochen, Germany). Cell culture medium was replaced every three days.

3.2.4 Normoxia and hypoxia incubation

To evaluate the response to oxygen tension of aMSCs and human primary chondrocytes, both cell types cultured in monolayer, pellets and scaffolds were

allowed to differentiate for up to 21 days in chondrogenic differentiation media under either normoxic (21% oxygen) or hypoxic (2.1% oxygen) conditions using an integrated system with a cell culture hood and incubator accessible through a gas-lock (I-Glove, BioSpherix, New York, NY).

3.2.5 Gene expression analysis

Total RNA was isolated using the miRNeasy Micro Kit (Qiagen, Hilden, Germany) following the instructions of the manufacturer. For pellet cultures, at least five pellets were pooled from each condition and lysed using an 18-gauge needle and Qiazol lysis buffer (Qiagen, Hilden, Germany) at each time point. Scaffolds were lysed using the same syringe-needle homogenization method. Lysates of cells used for plating the cells on scaffolds or pellet culture were obtained on day 0 (D0), and at D1, D7 and D14 after culture under various conditions. Biological triplicates were used for analysis. RNA concentrations and purity levels were measured using a NanoDrop (Thermo Fisher Scientific) and isolated RNA was reverse transcribed into cDNA using the SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA). Gene expression was quantified using quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR) with primers for representative chondrogenic genes (Table S1). Real-time qPCR reactions were performed with 10 ng cDNA per 10 μ L with QuantiTect SYBR Green PCR Kit (Qiagen) and the CFX384 Real-Time System (BioRad, Hercules, CA). Gene expression levels were quantified using the $2^{-\Delta\Delta C_t}$ method. Various housekeeping genes were tested for low variation across our samples including *GAPDH*, *ACTB* and *AKT1*. Because the housekeeping gene *AKT1* was found to be more stably expressed than *GAPDH*, we normalized all data relative to *AKT1* (mean $\pm$ standard error of the mean, n=3).

Table S1. List of forward and reverse primer sequences used for gene expression analysis.

Official Gene Symbols	Forward primer sequence	Reverse primer sequence
AKT1	GTGCCTATCAGGACAAGGTCT	GATGCCTTTCACCACGACTTC
COL10A1	AAGAATGGCACCCCTGTAATGT	ACTCCCTGAAGCCTGATCCA
COL1A1	GCTACCCAACCTTGCCTTCATG	TGCAGTGGTAGGTGATGTTCTGA
COL2A1	TGAAGGTTTTCTGCAACATGGA	TTGGGAACGTTTGCTGGATT
COMP	AAGAACGACGACCAAAAGGAC	CATCCCTATACCATCGCCA
DCN	ATGAAGGCCACTATCATCCTCC	GTGCGGTCATCAGGAACCTT
GLI1	AACGCTATACAGATCCTAGCTCG	GTGCCGTTTGGTCACATGG
HAPLN1	TCTGGTGCTGATTCAATCTGC	TGCTTGGATGTGAATAGCTCTG
HIF1A	TTCCTTCTCTTCCGCGTG	ACTTATCTTTTCTTGCTGTCGC
HISTH4	AGCTGTCTATCGGGCTCCAG	CCTTTGCCTAAGCCTTTTCC
IHH	AACTCGCTGGCTATCTCGGT	GCCCTCATAATGCAGGGACT
SOX9	TGTATCACTGAGTCATTTCAGTGT	AAGGTCTGTCAGTGGGCTGAT

Note: All primer sequences are indicated from 5'- end to 3' end (5' → 3').

3.2.6 Histological analysis

Cell pellets and scaffolds were fixed overnight in 10% neutral buffered formalin. Samples were then washed and dehydrated in graded series of ethanol (70%-100%) and processed with Xylene (50%-100%) prior to paraffin embedding. Paraffin blocks were cut into consecutive sections of 5 µm thickness using a microtome and placed onto charged microscope glass slides for histological staining. Following deparaffinization, sections were stained for glycosaminoglycan content using 0.5% (wt/vol) Alcian Blue 8GX dye (Sigma-Aldrich) and counterstained with Nuclear Fast Red (Sigma-Aldrich).

The presence of COL2A1 was localized by immunohistochemistry (IHC). In brief, after removal of paraffin and rehydration, sections were washed with distilled water. Pepsin antigen retrieval was performed using 10 mg/mL pepsin for 10 minutes at 37°C in a humidified chamber. The mouse and rabbit specific HRP (ABC) Detection IHC Kit (Abcam, Cambridge, UK) was used according to the manufacturer's instructions. Briefly, protein block was applied after which slides were incubated overnight with 1 µg/mL of anti-Collagen Type II Antibody (Millipore, Darmstadt, Germany) or mouse IgG1 Isotype Control (Biosciences, Osage, IA) at 4°C in a humidified chamber. Slides were sequentially incubated with biotinylated polyvalent secondary antibody and streptavidin peroxidase plus (supplied in the kit) and were then treated with 3, 3'-diaminobenzidine

(DAB) enhanced liquid substrate system for IHC (Sigma-Aldrich) diluted 1:1 with TBS for 10 minutes until color development. Stained sections were analyzed using a ZEISS Axio inverted light microscope as described above.

3.2.7 Statistical analysis

Quantitative data for gene expression profiles have been presented as mean $\pm$ standard deviation from 3 independent donors for aMSCs and one donor for human primary chondrocytes for each experimental condition and time point. The experimental factors analyzed for their association with gene expression values included oxygen level (2 levels: normoxia, hypoxia), culture condition (3 levels: 2D monolayer, 3D high-density pellet, 3D PCL scaffold), time (5 levels: baseline and days 1, 3, 7, and 14), and cell type (2 levels: aMSCs, chondrocytes). Each combination of the experimental conditions was performed in triplicate. Separate analyses were performed for each of the 12-targeted genes. The Mayo Clinic Biostatistics Core completed all statistical analysis. The analyses were performed using generalized linear models utilizing generalized estimating equations (GEE) to account for the within-donor correlation among the subsamples from each donor. Significant main effects with more than 2 levels were analyzed further by generating pairwise contrasts to identify levels that are significantly different from each other. In order to protect against an increased type I error rate associated with multiple comparisons, the reported *P* values were adjusted using the Benjamini-Hochberg method to control the false discovery rate. All analyses were conducted in SAS version 9.4 (SAS Institute Inc., Cary, NC). Statistical analysis is represented as * for $p < 0.05$ and as ** for $p < 0.0001$.

3.3 RESULTS

3.3.1 aMSCs undergo chondrogenic differentiation and adopt a phenotype resembling growth plate cartilage

Expression of chondrogenic markers in differentiated aMSCs cultured in monolayer cultures was compared to primary human chondrocytes to determine whether a chondrocytic phenotype was achieved. aMSCs and chondrocytes exhibited increased expression of several chondrogenic genes over time. Chondrocytes significantly up-regulated *ACAN*, *HAPLN1* and *SOX9* over the time course compared to aMSCs (**Figure 3.1.A**). Surprisingly, differentiating aMSCs exhibited higher levels of gene expression for *COL2A1*, *DCN* and *COMP* and also expression of hypertrophic markers including *IHH*, *GLI1* and *COL10A* than chondrocytes (**Figure 3.1.B and C**). These results suggest that the mechanisms utilized by aMSCs undergoing chondrogenic differentiation are different to those used by primary human chondrocytes, perhaps reflecting adoption of phenotypes resembling growth plate cartilage rather than articular cartilage.

3.3.2 Culture on PCL-scaffolds supports cartilage-specific extracellular matrix formation by both aMSCs and chondrocytes

To aid in the clinical translation of aMSCs for the treatment of cartilage defects, we investigated whether a 3D nanofibrous PCL scaffolds could support chondrogenic differentiation of aMSCs. Cells seeded on scaffolds were compared to those seeded on high-density pellet cultures. After fabrication, scaffolds were visualized by light microscopy and were observed to be homogenous in size and shape (**Figure 3.2.A**). aMSCs were seeded onto the scaffold and live/dead staining revealed that the cells attached and proliferated within the scaffold after 1, 3, 7 and 14 days. At all-time points, the majority of cells were viable (green) with few non-viable cells (red). Moreover, fluorescence microscopy at day 14 revealed cells were tightly packed indicating cells reached a confluent state (**Figure 3.2.B**).

Figure 3.1. Gene expression profiles of aMSCs and human primary chondrocytes in monolayer cultures during chondrogenic differentiation. **(A)** Earlier and robust expression of ECM proteins *ACAN* and *HAPLN1*, and transcription factor *SOX9* was observed in human primary chondrocytes over the chondrogenic time course. **(B)** aMSCs showed an increased expression of *COL2A1* while up-regulating ECM proteins *DCN* and *COMP* when compared to human primary chondrocytes. **(C)** Hypertrophic phenotype is induced in aMSCs over time, which is evidenced by the up-regulation of *IHH*, *GLI1*, and *COL10A1* over time in aMSCs. Data are presented as mean $\pm$ standard error of the mean, $n=3$. For RT-qPCR three technical replicates were used. Statistical analysis is represented as * for $p<0.05$ and as ** for $p<0.0001$.

Gene expression analysis revealed differences between the pellet culture and scaffold system. Compared to pellet cultures, 3D-scaffolds did not promote expression of chondrogenic ECM markers including *HAPLN1*, *COMP*, *ACAN*,
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COL2A1 and the transcription factor *SOX9* (**Figure 3.2.C, D, E, F and G**). Also, scaffold cultures did not enhance expression of *HIST2H4* and *HIF1A*, which was evident in pellet cultures (**Figure 3.2.H and I**). Significant up-regulation of *HIF1A* in pellet cultures suggests a hypoxic microenvironment within the pellet even when cultured under regular oxygen conditions. This upregulation was not seen in cells cultured in scaffolds. Moreover, expression of hypertrophic markers *IHH* and *COL10A1* were down regulated in scaffold cultures throughout the time course (**Figure 3.2.J and K**). *DCN* protein was the only chondrogenic marker to show a similar expression in both pellet and scaffold cultures (**Figure 3.2.L**). In contrast, osteogenic/fibroblastic marker *COL1A1* and hypertrophic marker *GLI1* were increased in scaffolds over pellets by 2.9-and 1.09 fold respectively (**Figure 3.2.M and N**).

Histological analysis of aMSCs cultured in scaffolds and pellets at day 21 of chondrogenic differentiation indicated that both cultures permit development of an Alcian blue positive matrix (**Figure 3.2.O, a and d**). However, the matrix was denser in pellet cultures (**Figure 3.2.O, b and c**). In scaffolds, cells were located at the periphery indicating poor cell migration towards the scaffold center (**Figure 3.2.O, e and f**). To validate the expression of *COL2A1* was detected by IHC in pellets at day 21 of culture (**Figure 3.2.P**). In summary, scaffold increased hypertrophic marker expression and osteogenic potential of the aMSCs when compared to pellet cultures. Chondrogenic differentiation of aMSCs in pellet cultures promoted the expression of cartilage-like ECM production, proliferation and a hypoxic microenvironment. Chondrogenic differentiation in either pellets or on scaffolds using human primary chondrocytes was also examined. Chondrogenic markers *COL2A1* and *ACAN*, hypertrophic markers *IHH* and *GLI1*, and osteogenic marker *COL1A1* gene expression were similar in both 3D culture conditions (**Figure 3.3**). However, chondrocytes ECM proteins *HAPLN1* and *COMP* were enhanced in 3D-scaffolds when compared to pellets (**Figure 3.3.A and B**).

Figure 3.2 Gene expression and histological analysis differences between PCL-scaffold and high-density pellets cultures using AMSCs. **(B)** Live(green)/dead(red) analysis showing aMSCs attached and proliferated on the scaffold. **(C to N)** Gene expression analysis of chondrogenic, proliferative and fibroblastic markers. Data is presented as mean $\pm$ standard error of the mean, n=3. For RT-qPCR three technical replicates were used. Statistical analysis is represented as * for $p < 0.05$ and ** for $p < 0.0001$. **(O)** Alcian blue staining of aMSCs in either PCL-scaffolds or high-density pellet cultures at day 21 of chondrogenic differentiation. (Scale bar = 100 μ m). **(P)** COL2A1 detected by immunohistochemistry in pellets at day 21 of culture, shown at 10x and 40x magnification.

Scaffold cultures down-regulated expression of *SOX9*, *DCN*, *COL10A1*, *HIF1A* and *HIST2H4* when compared to high-density pellets (**Figure 3.3.E to J**). Histological analysis showed alcian blue staining in chondrocytes when cultured in both 3D conditions at day 21 (**Figure 3.3.M**). Within pellets, cells were located in both the periphery and the center and a dense matrix was observed throughout the pellet (**Figure 3.3.M, b and c**). In scaffolds, poor migration of the cells within the scaffold was observed with aMSCs (**Figure 3.3.M, e and f**). Chondrocytes did not show any specific orientation or arrangement within the pellet or scaffold. A light expression of *COL2A1* was detected by IHC in pellets at day 21 of culture (**Figure 3.3.N**), indicating a chondrogenic matrix. Together, these results suggest that both high-density pellets and PCL-scaffolds cultures are conducive to the production of a glycosaminoglycan positive matrix, and the cartilage-like gene expression profile indicates that the chondrocytic phenotype was preserved. Gene expression analysis revealed similarities between aMSCs and chondrocytes under both 3D culture conditions. Scaffold cultures did not support the expression of chondrogenic transcription factor *SOX9*, hypertrophic marker *COL10A1*, hypoxia inducible factor *HIF1A* and *HIST2H4* in both aMSCs and chondrocytes. Also, ECM protein *DCN* mRNA levels were decreased in scaffolds when compared to pellets at day 1 in both AMSCs and chondrocytes. However, chondrogenic markers *COL2A1* and *ACAN* were different in both cell cultures. Although the majority of the gene expression patterns look similar, the levels of expression varied between cells. Chondrocyte cultures expressed higher mRNA levels of *SOX9*, *ACAN*, *COMP* and *HAPLN1* whereas aMSCS expressed higher levels of *COL2A1* and hypertrophic genes.

Figure 3.3. Analysis of human primary chondrocytes differentiated in 3D-scaffold cultures and high-density pellets. **(A to L)** Gene expression analysis of chondrogenic, proliferative and fibroblastic markers. Data is presented as mean $\pm$ standard error of the mean, $n=3$. For RT-qPCR three technical replicates were used. Statistical analysis is represented as * for $p<0.05$ and ** for $p<0.0001$. **(M)** Alcian blue staining of primary chondrocytes cultured in 3D-scaffolds cultures and high-density pellets at day 21 of chondrogenic differentiation (Scale bar = 100 μ m). **(N)** COL2A1 detected by IHC in pellets at day 21 of culture, shown at 10x and 40x magnification.

3.3.3 Hypoxia promotes cartilage-specific gene expression in aMSCs in both pellets and PCL-scaffolds

To determine if low oxygen tension enhances chondrogenic differentiation, we compared pellet cultures of both aMSCs and chondrocytes in normoxia (21% O₂) or hypoxia (2.1% O₂). Gene expression analysis revealed that while the expression of a number of cartilage related genes was generally independent of oxygen levels, several cell type-specific responses to hypoxia were observed. In aMSCs, normoxia stimulated the expression of ECM proteins *ACAN*, *COL2A1* (**Figure 3.4.A and B**) and *DCN* (data not shown) after one week of culture. Conversely, hypoxia stimulated the preferential expression of the proliferation marker *HIST2H4* as well as *HIF1A*, which is the master regulator of the cellular response to hypoxia [22, 23] (**Figure 3.4.C and D**).

Low oxygen conditions also increased levels of hypertrophic mRNA markers *COL10A1* (**Figure 3.4.E**), as well as *GLI1* and *IHH* (data not shown). A reduced oxygen environment accelerated down-regulation of *COL1A1*, thus decreasing the osteogenic and/or fibroblastic potential of chondrocytes (**Figure 3.4.F**). Moreover, hypoxia increased the expression of the chondrogenic transcription factor *SOX9* (1.6 fold, day 3), *HAPLN1* (2.1 fold, day 3) (**Figure 3.4.G and H**) and *COMP* (1.8 fold, day 1, data not shown) compared to normoxia in aMSCs. Our studies also revealed that despite phenotypic differences between aMSCs and chondrocytes, hypoxia enhanced expression of *HIST2H4* and a slight up-regulation of *HIF1A* on day 1 in both cells. The latter finding suggests that low oxygen and *HIF1A* protein may play an important role in regulating cell proliferation and survival earlier during differentiation.

We also evaluated whether oxygen tension modulated chondrogenic differentiation of aMSCs and chondrocytes cultured on scaffolds. Gene expression analysis revealed similarities between aMSCs and chondrocytes in both low and high oxygen cultures, although cell type specific differences were observed.

Figure 3.4. The effect of oxygen tension on differentiation potential of aMSCs and chondrocytes cultured in high-density pellets. aMSCs and chondrocytes were placed in pellet cultures and were exposed to normoxic (20%O₂) or hypoxic conditions (2.1%O₂) for 14 days. Gene expression analysis of chondrocyte specific markers, hypoxia related factor protein *HIF1A* and hypertrophic markers was performed at days 0, 1, 3, 7 and 14. Data is presented as mean $\pm$ standard error of the mean, n=3. For RT-qPCR three technical replicates were used. Statistical analysis is represented as * for $p < 0.05$ and ** for $p < 0.0001$.

In aMSCs differentiated on scaffolds, hypoxia increased the expression of chondrogenic markers *ACAN* (**Figure 3.5.A**), *SOX9* (**Figure 3.5.G**) and *HAPLN1* (**Figure 3.5.H**) whereas normoxia up-regulated osteogenic/fibroblastic marker *COL1A1* (**Figure 3.5.B**) and hypertrophic markers *COL10A1* (**Figure 3.5.E**), *GLI1* and *IHH* (data not shown). Normoxia also increased mRNA levels of *COL2A1* at day 7 (**Figure 3.5.F**). In chondrocytes, hypoxia up-regulated the expression of transcription factor *SOX9* (**Figure 3.5.G**), *HIST2H4* and *HIF1A* (**Figure 3.5.C and D**), while normoxia increased *COL1A1* (**Figure 3.5.B**). Interestingly, low oxygen tension enhanced expression of chondrogenic genes in chondrocytes that were repressed in aMSCs, including *COL2A1* (**Figure 3.5.F**), *DCN* and *GLI1* and *IHH* (data not shown). Conversely, chondrocytes on scaffolds cultured under normoxic conditions up-regulated several chondrogenic genes including *ACAN* (**Figure 3.5.A**) and *HAPLN1* (**Figure 3.5.H**). The hypertrophic marker *COL10A1* was the only gene that was not expressed by chondrocytes grown on scaffolds (**Figure 3.5.E**). In summary, low oxygen conditions enhance the expression of several chondrogenic markers of aMSCs cultured on scaffolds, but chondrocytes were largely unaffected by differences in oxygen tension levels.

3.4 DISCUSSION

Tissue engineering offers possibilities for optimization of cartilage repair by combining different cell types, biomaterials, and growth factors for the support of cartilage regeneration [24]. Although several procedures have been developed and studied, no standardized clinical protocol has yet been established [25]. The use of stem cells and in particular mesenchymal stem cells from adult sources has emerged as a viable solution to overcome limitations of current cartilage repair procedures.

The chondrogenic potential of clinical-grade and human platelet lysate-expanded human aMSCs under multiple culture conditions relevant to cartilage tissue engineering, including hypoxia and propagation in a 3D culture environment.

Figure 3.5. The effect of oxygen tension on differentiation potential of aMSCs and primary human chondrocytes cultured in PCL-scaffolds. PCL-scaffolds were seeded with either aMSCs or chondrocytes and placed under hypoxic or normoxic conditions in the presence of chondrogenic differentiation medium for 14 days. Gene expression analysis was performed at days 0, 1, 3, 7 and 14. Data is presented as mean $\pm$ standard error of the mean, $n=3$. For RT-qPCR three technical replicates were used. Statistical analysis is represented as * for $p<0.05$ and ** for $p<0.0001$.

While there are many studies on chondrogenic differentiation of bone marrow derived MSCs, our studies address a major gap in our knowledge by investigating Most of the previous studies have been performed using animal serum (FBS),

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which is not applicable for clinical studies. In our case, human platelet lysate presents a non-xenogeneic option for cell expansion and differentiation of aMSCs for translational studies. This study provides a detailed side-by-side comparison of aMSCs and human primary chondrocytes during chondrogenic differentiation, which was not previously available in the literature. Furthermore, current protocols for chondrogenic differentiation efficiently create hypertrophic cartilage which upon implantation undergoes endochondral ossification and mineralization [26]. Several studies have attempted to understand the mechanisms by which MSCs can be programmed for chondrogenic differentiation while avoiding further progression into hypertrophic cartilage [27-29]. Mimicking the natural hypoxic environment of cartilage development under *in vitro* conditions is beneficial because it mediates metabolic programming of the chondrogenic fate of MSCs into different subtypes of hyaline cartilage. The results presented in this study validate the biological properties of self-renewing and differentiation potential of aMSCs. However, a need for better chondrogenic differentiation strategies that avoid a hypertrophic phenotype of the cells is still required.

The comparison between normoxia and hypoxia of aMSCs and chondrocytes cultured in pellets revealed an enhanced expression of chondrogenic markers that were expressed in both oxygen conditions. Previous studies have described neo-hyaline cartilage tissue formation demonstrated by increased *COL2A1* expression and higher glycosaminoglycan content [30]. Furthermore, extracellular matrix proteins (e.g., *ACAN*, *DCN*, *COMP* and *HAPLN1*) are highly expressed in human cartilage and human articular chondrocytes [17, 31, 32]. In our studies, both cell types respond to hypoxia by enhancing the expression of *HIST2H4*, *HIF1A* and *COL2A1*, suggesting that *HIF1A* plays an important role in regulation of earlier cell proliferation and the expression of *COL2A1* under low oxygen conditions. As a consequence of continuous hypoxia under chondrogenic induction, stem cells start to produce hyaline cartilage that is resistant to

hypertrophic differentiation, whereas incubation under normoxia conditions results in hypertrophic cartilage that resembles epiphyseal cartilage [31, 33]. Our data indicates that under chondrogenic induction aMSCs develop a chondrocyte-like phenotype, but may have a propensity to exhibit a hypertrophic response despite being exposed to a hypoxic environment. In contrast, Leijten et al. showed that hypoxia cultures of bone marrow derived MSCs increased chondrogenic differentiation while avoiding hypertrophic differentiation of the cells [21]. Furthermore, our study suggests that the molecular response observed in aMSCs undergoing chondrogenic differentiation is different from the molecular response by primary human chondrocytes, in particular because aMSCs may adopt a phenotype resembling growth plate cartilage rather than articular cartilage.

Oxygen responsiveness and chondrogenic potential also appear to be affected by the choice of cell culture model. Several cell type independent effects in gene expression in high-density pellets versus scaffolds and cell type specific expression were observed. In the current investigation, we found that changing the cell environment from a two-dimensional culture to a 3D culture model (i.e., high density pellets or PCL-scaffold), improved the chondrogenic potential of aMSCs and primary chondrocytes by increasing cell-cell interactions and generating a low oxygen environment within cellular aggregates. We observed that conventional monolayer culture did not support the chondrogenic differentiation of aMSCs, whereas both high-density pellets and scaffolds cultures are conducive to the production of a glycosaminoglycan positive matrix. A cartilage-like gene expression profile indicates that aMSCs acquire a chondrocytic phenotype but is not stable. Moreover, pellet culture promoted proliferation and a hypoxic microenvironment, whereas the scaffold increased hypertrophic marker expression and osteogenic potential of the aMSCs. Our results using nanofibrous *PCL* scaffolds corroborate previous studies and

support the chondrogenic differentiation potential of MSCs when cultured under appropriate inductive conditions [34].

The potential use of scaffolds for implantation relies on their flexible designs, excellent biomechanical properties and slow degradation rate. There is evidence that MSCs favor nanotopographical geometry and porous materials over plastic for growth and differentiation [24, 34]. Scaffolds have been largely used to provide cells with structural support for attachment, proliferation, and differentiation in a 3D environment. Moreover, scaffolds facilitate optimal filling of the entire defect and provide mechanical support for the healing tissue, potentially reducing the rehabilitation time for the patient [35, 36]. A combination of a biodegradable scaffold with stem cells would better resemble the native tissue composition and mechanical properties and could potentially regenerate hyaline cartilage tissue. For articular cartilage surface regeneration, several scaffolds have been used including nanomaterials, functional mechano-compatible scaffolds, multi-layered scaffolds, and ECM scaffolds [34]. Some of these scaffolds can cover the entire chondral defect but their use is typically accompanied by production of a COL1A1 rich ECM, thus producing a biomechanical inferior and non-durable matrix that does not resemble native cartilage [37, 38]. In our studies, low oxygen cultures repressed the expression of fibroblastic/osteogenic marker *COL1A1* in both aMSCs and chondrocytes on both 3D-cultures. Taken together, the results presented here support the important role of oxygen regulation in chondrogenic differentiation of aMSCs for clinical applications.

The cell type specific role of low oxygen in differentiation of aMSCs indicates that mesenchymal cell fate and differentiation potential is remarkably sensitive to oxygen. The oxygen status is a dominant environmental parameter for directing chondrogenic differentiation of aMSCs and reduces their tendency to generate a fibroblastic ECM, while helping chondrocytes to maintain their phenotype by enhancing *COL2A1* expression and inhibiting additional matrix production.

Hence, hypoxia is more effective in promoting a chondrocytic phenotype at earlier stages of differentiation. Our findings indicate that genetic programming of aMSCs to a chondrocytic phenotype is effective in cellular aggregates (pellet culture) under hypoxic conditions as reflected by increased expression of cartilage-related biomarkers and biosynthesis of a glycosaminoglycan positive matrix. Although expression of cartilage-related genes is more robust in pellets than scaffolds, hypoxia enhances the low levels observed in scaffold cultures. Our results provide a new starting point for optimization of aMSCs in cartilage engineering strategies that leverage the chondrogenic effects of hypoxia in the 3D environment of an implantable scaffold for treatment of focal cartilage defects.

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CHAPTER 4

4. Integrated analysis of miRNA-seq and mRNA-seq on hypoxic mesenchymal stem cells reveals the role of hypoxia-regulated miRNAs for promoting chondrogenesis of MSCs.

ABSTRACT

An optimal tissue engineering strategy that regenerates the natural cartilage structure is required for a successful cartilage tissue repair. Hypoxia, a common environmental condition, influences cell signals and functions [1], and has been suggested to maintain cartilage homeostasis by inhibiting angiogenesis and hypertrophic differentiation, while inducing several genes required for matrix production such as glycosaminoglycans [2] and *COL2A1* during chondrocyte development [3]. Several studies have unraveled a complex molecular network that is involved in regulating the hypoxic response in physiological conditions [4]. Although it is believed that under hypoxic conditions an appropriate stimulus could drive chondrogenesis of MSCs, the exact mechanisms are not known. During the past decade, multiple studies have demonstrated that miRNAs are involved in the hypoxic response and contribute to the repression of specific genes under low oxygen conditions [5]. Moreover, several studies have implicated a large number of microRNAs in regulating pre- and post-natal chondrogenesis. The goal of this study is to find the regulatory mechanisms of hypoxia-induced microRNAs and its gene targets, which could improve chondrogenesis of stem cells. To achieve this, a meta-analysis of microRNA and mRNAseq of adipose tissue derived (aMSCs) and bone marrow derived (bMSCs) stem cell cultures in hypoxia was completed. Despite that several microRNAs have been described to participate during chondrogenesis of cells, we found hypoxic regulated miR-210 to be essential in the regulation of genes in charge of several functions necessary for cartilage development, chondrogenic differentiation and oxidative stress response. miR-210 also regulates gene expression in concert with the transcription factor HIF1. Several hypoxia responsive genes were regulated in both MSCs. In both MSCs genes that participate in similar biological processes such as hypoxia response, cell metabolism, apoptosis and cell cycle were up-regulated; while genes increased in

bMSCs were involved in cartilage development and maintenance, and genes increased in aMSCs were involved in bone extracellular matrix synthesis and osteoarthritis development. Remarkably, miR-210 seems to play a role in regulating several cellular processes and functions that could improve chondrogenic differentiation of stem cells for cartilage regeneration therapies.

4.1 INTRODUCTION

Articular cartilage (AC) is a form of hyaline cartilage that is present at the articular surface of bones in the joints. It provides a smooth, lubricated, low-friction and wear-resistant surface for articulation, translation and rotation between bones. Once damaged, cartilage has a limited capacity to heal and repair. Therefore, preservation of the cartilage tissue is vital for joint health [6]. This preservation depends dominantly on the articular cartilage biochemical and mechanical characteristics. If these characteristics change over time, cartilage degeneration starts [7]. The limited capacity of cartilage to repair damage has led to the development of several cartilage engineering strategies. Cell therapies entail tissue extraction from a healthy donor site and *in vitro* culture of the cells for several passages until a significant amount of cell is acquired [8]. Slow proliferation rates, extended culture time and dedifferentiation of the cells have been often observed before cells are re-implanted in the patient, leading to poor chondrogenic response and often donor site morbidity. Because healthy chondrocytes reside and survive in a sustained low oxygen environment [8], several authors have hypothesized that hypoxia is an important factor in regulating the growth and survival of chondrocytes [9]. A number of pathophysiological findings propose that a correlation does exist between hypoxia and chondrogenesis [8]. Additionally, several studies have shown that a low oxygen tension environment may be beneficial to preserve the chondrocytic phenotype of the cells during *in vitro* cultures, avoiding the potential dedifferentiation of the cells during expansion. However, the exact mechanism by which chondrocytes are regulated by oxygen tension remains poorly understood.

During the past decade multiple studies have demonstrated that miRNAs are involved in the hypoxic response and can contribute to the repression of specific genes under low oxygen conditions [10, 11]. MicroRNAs (miRNAS) are small noncoding RNA molecules of about 20 nucleotides in length and control the

expression of at least 60% of protein coding genes. miRNAs participate in post-transcriptional regulation of gene expression, and are involved in RNA silencing. They play a role in the pathogenesis of several diseases including cancer [12]. MiRNAs frequently repress target genes by reducing the half-life time of their mRNA and/or inhibiting protein translation. To do so, miRNAs are incorporated in the RNA-induced silencing complex (RISC) and target the 3' untranslated region (UTR) of mRNAs, leading to translation inhibition or degradation of the mRNA. A single miRNA can regulate hundreds of distinct mRNAs simultaneously and a single mRNA can also be regulated by several distinct miRNAs [13]. In this study we seek to identify oxygen-regulated miRNAs and respective gene targets that regulate cartilage development and maintenance. Therefore, this will provide an adequate mechanism to improve *in vitro* chondrogenesis of mesenchymal stem cells for cartilage tissue engineering applications.

4.2 MATERIALS AND METHODS

4.2.1 Cell harvest and expansion

Two different sources of MSCs were used for these studies. Bone marrow stem cells (bMSCs) were derived from bone marrow aspirates approved by the local ethical committee of the Medisch Spectrum Twente. Informed written consent was obtained for all samples. Human bMSCs were isolated from fresh bone marrow samples of three donors (age average: 52 years old, 25% male, 75% female) and cultured as described previously by Georgi and colleagues [9]. Adipose derived mesenchymal stem cells (aMSCs) were obtained from three representative donors (age average: 42 years old, 75% male, 25% female) that were previously validated for tri-lineage differentiation potential [14]. aMSCs were obtained with approval from the Mayo Clinic Institutional Review Board (IRB) as described by Dudakovic et al. [15]. Both MSCs were expanded in maintenance media. For bMSCs this media consisted of DMEM with 4.5 g/L glucose and sodium pyruvate (Corning, Corning NY), supplemented with 10%

fetal bovine serum (Atlanta Biologicals, Atlanta, GA) and 1% penicillin/streptomycin (Gibco/Thermo Fisher Scientific). Maintenance media for aMSCs was composed of Advanced MEM (Gibco/Thermo Fisher Scientific, Waltham, MA) supplemented with 5% human platelet lysate (PL-Max, MillCreek Life Sciences, Rochester, MN), 1% penicillin/streptomycin, 1% Glutamax (Gibco/Thermo Fisher Scientific), and 0.2% heparin (Baxter, Deerfield, IL). Cells were cultured using standard techniques in T-175 cm² flasks at 37°C, 95% humidity, and 5% CO₂ until they reached 80% confluence. Prior to each experiment, cells were detached from T175 flasks by trypsinization using TrypLE Express (Gibco/Thermo Fisher Scientific).

4.2.2 Cell culture under Normoxia and Hypoxia conditions

To evaluate miRNA and mRNA expression of both aMSCs and bMSCs in response to oxygen tension, both cells were plated in 6-well plates at a density of 3,000 cells/cm² and maintained for 7 days in maintenance media under either normoxic (20% oxygen) or hypoxic (2.1% oxygen) conditions using an integrated system with a cell culture hood and an incubator accessible through a gas-lock (I-Glove, BioSpherix, New York, NY). After 7 days in culture, total RNA was isolated using the miRNeasy Micro Kit (Qiagen, Hilden, Germany) following the instructions of the manufacturer. Three different biological replicates for each experimental condition (e.g., 3 samples for normoxia and 3 samples for hypoxia) were used concurrently for RNA isolation and subsequent expression analysis to ensure consistency in RNA quality and expression values. RNA concentrations and purity levels were measured using a NanoDrop (Thermo Fisher Scientific).

4.2.3 RNA extraction from tissues

Cartilage tissue specimens were collected from patients undergoing tissue removal as part of planned surgical procedures. The specimens used in this investigation were collected under Institutional Review Board (IRB). Written informed consent was obtained for all collected tissues. From the 10 samples

obtained, 6 samples were collected from 3 different donors from whom we obtained both ankle and knee tissues. Each of the other 4 samples was collected from a different donor. There was an overall predominance of males (90%) individuals between the ages of 34 and 71 (average was 63 years old). After collection, articular cartilage specimens were classified according to radiological findings using the Collins grading scheme as described previously [16]. For this study, joints were specifically classified using the following criteria: G0 joints displayed normal articular cartilage throughout (n=5), G1 joints had some destruction of superficial cartilage and swelling and/or softening (n=7), G2 joints exhibited extensive superficial damage to articular cartilage but not significant loss of cartilage and no damage to bone (n=6), G3 joints had areas with total articular cartilage loss with observable penetration of defects to the subchondral bone plate (n=4), and a G4 joint was identified as having complete loss of cartilage and full thickness exposure to bone along the articular surface of the joint. Half of the donors were classified as G0 and half as G3/4.

Before RNA extraction, samples were snap frozen in liquid nitrogen and stored at -80°C after surgical harvest. Then, frozen tissues were grounded into a powder using a mortar and pestle and homogenized in Qiazol reagent (Qiagen, Hilden, Germany). Total RNA was extracted using the RNeasy minikit (Qiagen, Hilden, Germany) and quantified using a NanoDrop 2000 spectrophotometer (Thermo Fischer Scientific, Wilmington, Delaware).

4.2.4 Next generation RNA sequencing

For samples selected for next generation sequencing, RNA integrity was assessed using the Agilent Bioanalyzer DNA 1000 chip (Invitrogen, Carlsbad, CA). Only samples with an RNA Integrity Number (RIN) and DV200 score greater than our Sequencing Core's minimum cutoff (RIN >6 and DV200 > 50%) were used for sequencing. RNA sequencing and subsequent bioinformatics analysis were

performed in collaboration with the Mayo Clinic RNA sequencing and bioinformatics cores as previously described [15].

High-throughput next generation microRNA sequencing was performed using the NEBNext Small RNA library prep kit on an Illumina HiSeq 2000 as described previously by Riester et al. [17]. MicroRNAs sequences were trimmed off adapters using Cutadapt [18] and alignment of these sequences (greater than 17 nucleotides in length) was performed against the reference genome using Bowtie [19]. Then, microRNAs quantification was obtained using miRDeep2 [20]. All other analyses were performed on robustly expressed microRNAs with at least 1 normalized read per million in either of the conditions (e.g. hypoxia or normoxia).

For mRNA sequencing, library preparation was performed using the TruSeq RNA library preparation kit (Illumina, San Diego, CA). Poly-A mRNA was purified using oligo dT magnetic beads. Then, cDNA synthesis was performed on purified mRNA using SuperScript III reverse transcriptase and random primers (Invitrogen). DNA fragments were enriched by PCR using primers included in the Illumina Sample Prep Kit. Sample concentration and size distribution were determined on an Agilent Bioanalyzer DNA 1000 chip and Qubit fluorometry (Invitrogen, Carlsbad, CA). RNA-Seq data was analyzed using the MAPRSeq v.1.2.1 system including alignment with TopHat 2.0.6 [21] and HTSeq software [22]. Expression values for each gene were normalized to 1 million reads and corrected for gene length (reads per kilobase pair per million mapped reads, RPKM).

4.2.5 Computational analysis and statistics

Normalized read counts obtained from the RNA sequencing data were analyzed to assess differential gene expression between our different conditions. Soft filtering using RPKM values above 0.1, fold changes of 1.4-fold and a *p-value*

below 0.05 were performed to all our data sets. Statistical analysis was performed using JMP® Pro v10.0.0 (SAS Institute), and *p* values were determined with two-tailed paired Student T-test.

Computational analysis was achieved using various online software tools. For visualization, Venn diagrams were generated using Venny online tool [23] to compare different conditions. Unsupervised hierarchical clustering was performed using the Pearson correlation method on the MORPHEUS matrix visualization and analysis software [24]. For microRNAs robustly expressed in hypoxia (RPKM>1), a combinatorial target prediction analysis was performed using TargetHub, TargetScan and TargetMiner online software. Gene ontology analysis and functional gene annotation clustering were performed on genes differentially expressed using GO [25, 26] and Database for Annotation and Visualization and Integrated Discovery v6.8 database (DAVID 6.8). In addition, gene-gene interaction network and gene function were analyzed using the Search Tool for the Retrieval of Interacting Gene/Protein (STRING) version 10.5 [27] and GeneMANIA 2018 [28].

4.3 RESULTS

4.3.1 microRNAseq analysis reveals a unique hypoxia induced miR-210 and a clear miRNA footprint of bMSCs on different oxygen conditions.

To evaluate and identify potential miRNAs that are regulated in hypoxia, three different bMSCs donors were cultured for one week in monolayer cultures and different oxygen environments: normoxia (20% oxygen) or hypoxia (2.1% oxygen). An unsupervised hierarchical clustering using the Pearson correlation method was performed and identified microRNAs that were significantly regulated (RPKM>1 and a *p-value* < 0.05). As shown in **Figure 4.1.A**, the clustering dendrogram shows independent clustering of normoxia and hypoxia cultured samples, suggesting a distinctive miRNA response on each oxygen

condition. No distinction between cell passage 2 (P2) and passage 6 (P6) was observed among the three donors. Fold change between hypoxia and normoxia resulted in 14 hypoxia up-regulated and 2 hypoxia down-regulated microRNAS (*Figure 4.1.B*).

Figure 4.1 MicroRNAseq analysis was performed on three bMSCs donors cultured in normoxia (20% oxygen) and hypoxia (2.1% oxygen) conditions over one week in monolayer cultures. **(A)** Unsupervised hierarchical clustering using the Pearson correlation method shows independent clustering of normoxia and hypoxia cultured samples. Passage 2 (P2) and passage 6 (P6) were compared. **(B)** Fold change between hypoxia and normoxia resulted in 16 regulated miRNAs from which hsa-miR-210 was induced by 50-fold (black bar). A second group of modestly regulated microRNAs with fold change between 1.4 and 4 was also observed (grey bars). A third group consisted of downregulated miRNAs (blue bars). Expression levels are represented as Log2 of RPKM values between hypoxia and normoxia cultures ± standard error of the mean. Three biological replicates (n=3) and one technical replicate were used.

We observed that hypoxia regulated miRNAs can be distributed over three groups based on their fold change. The first group is represented by hsa-miR-210 which was induced 50-fold in the low oxygen condition (black bar). The second group consists of miRNAs that are only modestly increased (1.4 to 4 fold) in low oxygen conditions (grey bars), which includes hsa-miR-155-5p, hsa-miR-195-5p, hsa-miR-584-5p, hsa-miR-130-3p, hsa-miR-296-3p, hsa-miR-32-3p, hsa-miR-92b-5p, hsa-miR-31-5p, hsa-miR-221-3p, hsa-miR-542-3p, hsa-miR-26b-5p, hsa-miR-671-5p and hsa-miR-106b-5p. Lastly, 2 miRNAs (hsa-miR-126-3p and hsa-miR-16-1-3p) were significantly down-regulated in hypoxia (blue bars). These results proposed miR-210 as the unique hypoxia inducible microRNA.

4.3.2 Modestly induced miRNAs during low oxygen culture do not participate in a specific molecular program.

To identify genes that may be regulated by the modestly induced microRNAs in hypoxia, a target prediction analysis was performed. TargetScan software was used on 4 microRNAs (e.g. hsa-miR-221-3p, hsa-miR-155-5p, hsa-miR-26b-5p and hsa-miR-31-5p). More than 2000 genes were predicted targets in any of the 4 hypoxia-controlled miRNAs, whereas only 38 were predicted for all of them. Common targets were represented and compared using a Venn diagram as shown in **Figure 4.2.A**. 294 genes were targeted by at least 2 miRNAs whereas 32 genes were targeted by 3 or 4 miRNAs. Gene ontology analysis showed there was neither correlation nor interaction between these 32 common genes. In addition, DAVID bioinformatics resources 6.8 demonstrated an absence of specifically enriched pathways.

Then, an unbiased analysis was performed by comparing the 294 predicted targets found on TargetScan against our bMSCs mRNAseq dataset. mRNAseq analysis show only 27 genes were significantly regulated in low oxygen cultures. A comparison of these 27 genes and the 294 miRNA-31/221/155/26b gene targets revealed 11 targets in common (**Figure 4.2.B**). Log2 fold change between

hypoxia and normoxia showed only 4 gene targets were significantly regulated. *BDNF* and *PRELP* were up regulated more than 1.4 times in hypoxia (black bars) whilst *CDKN1C* and *ZNF704* were down regulated more than 1.4 times (blue bars). Based on our target prediction analysis it is concluded that no overall specific molecular program was targeted by the modestly induced microRNAs in hypoxia.

Figure 4.2 TargetScan was used to predict the targets of each of the 4 most abundant modestly induced microRNAs in low oxygen: hsa-miR-221-3p, hsa-miR-155-5p, hsa-miR-26b-5p and hsa-miR-31-5p. **(A)** Although, more than 2000 genes were predicted targets of any of the 4 hypoxia-controlled microRNAs, only 294 genes were targeted by at least 2 of them. **(B)** Unbiased analysis of all targets was performed by comparing the 294 gene targets found on TargetScan against the 27 significantly regulated genes from our bMSCs mRNAseq dataset. Log2 fold change between hypoxia and normoxia showed only 4 from the 11 genes in common were significantly regulated in hypoxia: *BDNF* and *PRELP* (1.4 fold change in hypoxia, black bars) and *CDKN1C* and *ZNF704* (0.7 fold change in hypoxia, blue bars). Expression levels are represented as Log2 of RPKM values between hypoxia and normoxia cultures $\pm$ standard error of the mean. Three biological replicates (n=3) and one technical replicate were used.

4.3.3 Hypoxia regulated miR-210 gene targets are mostly involved in stress response but also in cartilage regulatory programs.

Similarly, a target prediction analysis was performed on miR-210 using three different algorithms: TargetHub, TargetScan and TargetMiner. The analysis indicated 1210 genes that were predicted using TargetHub, 4099 using TargetScan and 4525 using TargetMiner. Venn diagram comparison using Venny revealed a total of 2429 genes that were predicted by at least two algorithms (**Figure 4.3.A**) and these genes were considered potential miR-210 targets. From these, a total of 182 genes were predicted by three algorithms and were considered bona fide target genes of miR-210.

An integrated analysis of miR-210 potential and bona fide targets and bMSCs mRNAseq was performed to expose miR-210 gene targets regulated in hypoxia. First, a comparison between the 2429 potential miR-210 gene targets and the mRNAseq dataset was done revealing 191 down-regulated genes in hypoxia and 294 up-regulated. Since miRNAs function is to down-regulate target genes, further analysis was performed only in down-regulated genes. Gene ontology analysis was completed and exhibited involvement of these genes in several cellular processes and functions. Among the cellular functions we observed cellular response to chemical stimulus (n=8, *p-value* 0.0327), positive regulation of signal transduction (n=7, *p-value* 0.0285), positive regulation of cell signaling (n=7, *p-value* 0.0276), positive regulation of cell communication (n=7, *p-value* 0.0151), positive regulation of cell differentiation (n=6, *p-value* 0.0138), regulation of signaling receptor activity (n=5, *p-value* 0.0334) and skeletal system development (n=5, *p-value* 0.0147) (**Figure 4.3.B**). Molecular function regulation (n=7, *p-value* 0.0142), receptor regulator activity (n=5, *p-value* 0.00701) and receptor ligand activity (n=5, *p-value* 0.00431) are among the cellular functions found (**Figure 4.3.C**). Results were shown as fold enrichment, number of genes (n=# of genes) and Bonferroni-corrected for $P < 0.05$. Moreover, five different pathways were found for these genes: apoptosis (*BCL2*), hypoxia

inducible pathway (*EPO*), cell cycle regulation (*HOXB9*, *IGF1* and *SFN*), TGFβ signaling pathway (*BMP8B*) and WNT signaling pathway (*ENT11*, *WNT7B* and *WNT9A*). Therefore, miR-210 potential gene targets participate in cell differentiation, skeletal system development and cellular stress response.

Figure 4.3. A target prediction analysis was performed to investigate miR-210 targets that are involved in the response of bMSCs to low oxygen. **(A)** Venn diagram comparison revealed a total of 2429 genes that were predicted by at least two algorithms whereas 182 genes were predicted by three algorithms. **(B-C)** Gene ontology analysis exposed participation of potential miR-210 gene targets down-regulated in hypoxia in different cellular processes **(B)** and different functions **(C)**.

Further analysis was performed on the 182 miR-210 bona fide gene targets. A comparison against the down-regulated genes during the hypoxic response in the bMSCs mRNAseq data was completed. The comparison between these 103 hypoxia down-regulated genes and the 182 miR-210 predicted targets showed 5 genes in common: *CENPP*, *BACH2*, *PELI2*, *WISP1* and *WNT11*. Gene functional analysis revealed centromere protein P (*CENPP*) is involved in cell cycle, BTB and

Physical interactions of the genes are represented with red lines. Moreover, three different pathways were observed with this analysis (blue lines). *WNT11* participates in the WNT and hedgehog signaling pathways, *PELI2* participates in the NF-KappaB pathway Toll-like receptor-signaling pathway whereas *CENPP* was found to interact in cell cycle and mitosis of the cells. Gene functional analysis revealed these genes are involved in cellular response (n=3, *p-value* 0.0716), developmental processes (n=2, *p-value* 0.0465), metabolism (n=1, *p-value* 0.028), cell adhesion (n=1, *p-value* 3.56E-11) and regulation (n=3, *p-value* 1.17E-13) (**Figure 4.4.B**).

Functional protein association network analysis (STRING) depicted an interaction of *WNT11* with several other factors of the WNT/Frizzled signaling pathway such as *WIF1*, *FZD4*, *FZD2*, *SFRP1*, *SFRP2* and *FZD6* (**Figure 4.5.A**). STRING network analysis shows a correlation between *WISP1* and several proteoglycan proteins decorin (*DCN*) and biglycan (*BGN*) while it interacts with metalloproteinases *MMP2* and *MMP13* (**Figure 4.5.B**). It also revealed an interaction of *BACH2* with *PAX* and *MAF* genes, which are involved in oxidative stress response (**Figure 4.5.C**). *CENPP* was found to participate in assembly of kinetochore proteins and mitotic progression (**Figure 4.5.D and E**) whereas *PELI2* is involved in lipopolysaccharide signalling.

To summarize our findings, we observed that miR-210 under low oxygen tension targets specific genes that are involved in cartilage development and oxidative stress response.

4.3.4 Common gene targets of hypoxia inducible factor HIF1 and miRNA-210 in bMSCs play essential roles in tissue development, oxidative stress response and cell cycle regulation.

We next complemented the list of potential miR-210 targets with a literature survey. **Table 4.1** describes 86 genes identified as miR-210 targets in the literature and their functional classification. The genes are involved in various cellular processes such as: cell cycle, apoptosis, angiogenesis, DNA damage, mitochondrial metabolism, development and differentiation, DNA binding, chromosome structure, membrane trafficking, migration and adhesion, translation and transcription, and protein modification and transport [44-54]. Interestingly, the majority of these genes participate in cell cycle regulation for growth survival, and development and differentiation during adipogenesis and osteogenesis. From those 86 genes only 56 have been experimentally validated.

Table 4.1 Table lists 86 miR-210 gene targets described in the literature

Cell cycle	Angiogenesis	Apoptosis	DNA damage	Mitochondrial metabolism	Development Differentiation	DNA binding	Chromosome structure/ Inactivation	Membrane Trafficking	Migration/ Adhesion	Translation/ Transcription	Protein modification/ Transport
ACVR1B APC BUB1B CCNF CDC25B CDK10 CLASP2 DAPK E2F3 ELK3 FAM83D FGFR1.1 HOXA1 HOXA9 MINT NIPBL PIK1 SEH1L SERTAD2 TCF7L2	EFNA3 ELK3 PTP1B	AIFM3 BCL2 CASP8AP2 DAPK E2F3 MINT PTP1B RAD52 UBQLN1	RAD52	ATP11C COX10 DDAH1 ELK3 GPD1L HYPB=SETD2 ISCU1 ISCU2 NDUFA4 SDHD1	ACVR1B APC EDNF CTGF EFNA3 ELK3 HECTD1 HOXA1 HOXA3 HOXA9 MEI1 MINT NIPBL NIPX1 TCF7L2	CEB1 CHD9 CPEB2 RAD52	CEB1 CHD9 NIPBL SMCHD1 XIST	ABCB9 TNPO1	APC CLASP2 EFNA3 HOXA3 MDGA1 MID1IP1 NCAM1 P4HB PTEN1	CELF2 CPEB2 HYH3 KCMF1 UBQLN1	PIAR1 TNPO1

Note: Genes listed in this table were summarized from references 44 to 54.

Literature review was also performed in order to obtain a list of hypoxia inducible factor 1 (HIF1) targets. Around 233 of them have been described in the literature [55-57] and are shown in **Table 4.2**. These genes participate in several molecular programs such as oxygen delivery, cell survival, glucose and energy metabolism, cell migration and adhesion, cell proliferation and viability, angiogenesis, apoptosis and gene transcription [55-59]. GO analyses on HIF1

targets revealed different functions including blood vessel development, positive regulation of MAP kinase activity, cell-cell signaling, positive regulation of muscle contraction, regulation of cell development, apoptosis regulation and most importantly genes involved in skeletal system development (*TIPARP*, *EDN*, *PTGS2*), oxidative stress response (*STC2*).

Since miR-210 is the only miRNA that is robustly regulated in hypoxic cultures of bMSCs we examined a potential overlap and/or co-regulation of gene expression between miR-210 and HIF1. Comparison showed that 71 of the 233 genes regulated by HIF1 (27%) are also potential target genes of miR-210. STRING network and GO analysis was performed on the common genes. Interestingly, several functions and pathways were revealed: bone remodeling (*FOSL2*), hypoxia inducible factors (*EPO*), cell cycle arrest and apoptosis (*FOXO3*, *EIF2S1* and *PIK3R3*), inhibition of matrix metalloproteinases (*MMP14*) and cytokines and inflammatory response (*PDGFA*, *BBID*, *LEP* and *EPO*).

A comparison of these common targets and our mRNAseq data shows 7 HIF1/miR-210 common targets were found up-regulated in hypoxia, whilst 1 gene was found down-regulated. The hypoxic up-regulated genes included *STC2*, *IGF2*, *CHAC1* and *CDC42EP4*. GO analysis was performed on these genes which revealed participation in several functions including apoptosis (*CHAC1*), response to endoplasmic reticulum stress (*CHAC1*), Notch signaling pathway (*CHAC1*), skeletal system development (*IGF2*), response to oxidative stress and hypoxia (*STC2*) and organization of actin cytoskeleton (*CDC42EP4*). In contrast, the down-regulated gene *PIM1* has been described to participate in apoptosis and cell cycle regulation. Thus, hypoxia inducible factor and miR-210 regulated genes share common functionality. They both participate in the regulation of genes essential for oxidative stress response and cell cycle and tissue development. For further experimental analyses, **Chapter 5** will focus on hypoxia down-regulated target genes with a role in tissue development and oxygen response.

Table 4.2 HIF1 gene targets described in the literature.						
Oxygen Delivery/ Cell survival	Glucose/ Energy Metabolism	Migration/ Adhesion	Cell proliferation/ Viability	Angiogenesis	Apoptosis	Transcription
ADM ADRP EPO FLT1 HMOX1 IGF2 IGFBP1 IGFBP2 IGFBP3 NOS2 SLC2A1 TF TFRC TGFA VEGF	AK3 ALDOA ALDOC AMF/GPI ANO1 CATHD COL5A1 CP ENO1 FN1 GAPDH HK1 HK2 IGFBP1 IGFBP2 IGFBP3 LDH LDHA LEP MMP2 NT5E P4HB PAI1 PDHK PFKBF3 PFKL PGK1 PKM SLC2A1 SLC2A3 TF TFRC TGM2 TNF TP11 UPAR	ANF CMET CXCR4 GPI ITGB2 KRT14 KRT18 KRT19 LRP1 MIC2 SDF-1 TGFA VIM	CCNG2 CDK5R1 CDKN1A EPO IGF2 IGFBP1 IGFBP2 IGFBP3 TGFA TGFB3 WAF1	ANG4 EGVEGF ENG LEP LRP1 PDGD TGFB3 VEGF	NIP3 NIX RTP801	DEC1 DEC2 ETS1 NUR77

Note: Genes listed in this table were summarized from references 55 to 59.

4.3.5 Targets genes of hypoxia inducible factor HIF1 and microRNA-210 in aMSCs play essential roles in oxidative stress response, tissue homeostasis and inflammation.

To test whether our observations in bMSCs can be extrapolated to other mesenchymal stem cell sources, a microRNAseq was performed on aMSCs cultured in monolayer during 14 days under hypoxia or normoxia conditions. Analysis using a soft filter of fold change (hypoxia/normoxia) above 1.4 and a *p*-

value below 0.05 (paired, T-test) exhibited 13 up-regulated miRNAs and 182 down-regulated miRNAs in low oxygen cultures. For our analyses we only focused on microRNAs increased in hypoxia. Among the 13 up-regulated miRNAs, the most abundant were miR-210 with more than 25-fold change and miR-151a-3p, miR-423-5p, miR-181a-2-3p, miR-195-3p all increased by 1.4 to 5-fold (**Figure 4.6.A**). Comparison of all miRNAs regulated in both MSCs showed that miR-210 was the only up-regulated miRNA in common whereas miR-378-a-3p was the only miRNA down-regulated in both cells. Hence, in both bMSCs and aMSCs miR-210 was the only strongly up-regulated miRNA under hypoxic conditions.

To identify potential miR-210 targets that regulate the hypoxic response in aMSCs, an mRNAseq analysis was performed. Soft filtering of $FC > 1.4$, RPKM > 0.01 and *p-value* < 0.05 (paired, t-test) was applied to all dataset values. Comparison against the 182 miR-210 genes predicted during target prediction analysis (shown on **Figure 4.3**) revealed only 15 potential miR-210 targets regulated in aMSCs under low oxygen tension. 2 miR-210 gene targets were found significantly increased in hypoxia (e.g. *EFNA3* and *CELFB*) whereas 13 targets were down-regulated (e.g. *FGFR1*, *CDC25B*, *CCNF*, *BUB1B*, *FAM83D*, *SEH1L*, *CLASP2*, *CASP8AP2*, *COX10*, *GPD1L*, *TNPO1*, *SMCHD1* and *PTPN1*) (**Figure 4.6.B**). A cell type specific regulation was observed for *FGFR1*; in bMSCs this gene was down-regulated whereas it increased 1.67 times in aMSCs.

STRING gene/gene interaction analysis showed co-expression of several of these hypoxia-regulated genes and co-expression with genes involved in cell cycle such as *CCNB2*, *CCNA*, *CENPE* and *MKI67* (purple line). Involvement in Cadherin and Heme signaling pathways by most genes is represented with blue lines (**Figure 4.7.A**). GO analysis shows up-regulated genes *CELFB* and *EFNA3* are related to development of skeletal muscle and, vascular and epithelial development. In

contrast, GO analysis on down-regulated genes revealed 2 of these genes have been previously implicated in oxygen response and musculoskeletal development (e.g. *FGFRL1* and *COX10*).

Figure 4.6 MicroRNA and mRNA combinatorial analysis were performed on aMSCs. **(A)** Most abundant microRNAs up-regulated in aMSCs. Expression levels are represented as fold change of RPKM values $\pm$ standard error of the mean. **(B)** 15 miR-210 gene targets regulated in hypoxia: 2 up-regulated genes while 13 were down-regulated in hypoxia. Expression levels are represented as mean RPKM value $\pm$ standard error of the mean. **(C)** Log2 fold change of miR210/HIF1 common targets regulated in hypoxic aMSCs. 6 common gene targets were found up-regulated in hypoxia while 10 targets decreased. Expression levels are represented as Log2 of RPKM values between hypoxia and normoxia cultures $\pm$ standard error of the mean. Three biological replicates (n=3) and one technical replicate were used.

GO classification was represented in a pie chart showing that 35% of regulated genes are involved in cellular processes, 28% in cell metabolism, 13% in biological regulation, 8% in cellular organization and biogenesis whereas a small percent is involved in localization, reproduction and stimulus response (**Figure 4.7.B**).

Additionally, identification of HIF1/miR-210 common targets regulated during aMSCs hypoxic response was completed. As shown in **Figure 4.6.C**, 6 common gene targets were found up-regulated in hypoxia (e.g. *STC2*, *PDK1*, *CHAC1*, *NDRG1*, *PLOD1* and *ELL2*). Remarkably, oxidative stress responsive gene *STC2* was significantly up-regulated in both MSCs. GO analysis revealed involvement of these genes in functions including hypoxia response (*PLOD1*, *NDRG1*, and *PDK1*), response to endoplasmic reticulum stress (*CHAC1*), oxidative stress response (*STC2*) and regulation of transcription (*ELL2*). In contrast, 10 gene targets were decreased in hypoxia (e.g. *ISCU*, *PPP5C*, *SOX4*, *CDC42EP4*, *ARHGEF9*, *ACACA*, *FGD6*, *ILF3*, *GMPPB* and *NUP93*). These genes are involved in regulation of apoptosis (*ARHGEF9*), tissue homeostasis (*ACACA*, *FGF6* and *ISCU*), response to hydrogen peroxide (*NUP93* and *PPP5C*), gene expression (*ILF3*), organization of actin cytoskeleton (*CDC42EP4*) and skeletal tissue development (*SOX4*). Participation in 4 different signaling pathways, HIF1A signaling pathway (*PDK1*), Notch signaling pathway (*CHAC1*), SMAD signaling pathway (*NUP93*) and MAPK signaling pathway (*PPP5C*) was observed.

Furthermore, target prediction analysis was performed for the 4 most abundant modestly induced miRNAs (e.g. miR-151a-3p, miR-195-3p, miR-493-5p and miR-181a-2a-3p) in aMSCs. 12 gene targets were predicted for all 4, *SOCS5*, *RYBP*, *ATP2A2*, *PKIA*, *GABRB1*, *AGO2*, *ZFAND5*, *PURB*, *ZNF704*, *TANC2*, *RPS6KA3* and *FTO*. PANTHER pathway analysis on the genes indicated several pathways related to these genes, including CCKR signaling, inflammation mediated by chemokines,

A

In summary, miR-210 targets regulated in hypoxic aMSCs are involved in musculoskeletal tissue development and stress response of the cells to low oxygen whereas miR-210/HIF1 targets are involved mostly in stress response and tissue homeostasis. Likewise, modestly induced miRNAs seem to participate in the inflammatory response of the cells to low oxygen.

4.3.6 MiR-210 may play an important role in the inflammatory response and cartilage homeostasis in healthy cartilage tissues.

To categorize mRNAs that are regulated in healthy cartilage, mRNAseq analysis was performed on healthy (no OA) and diseased cartilage (G3/4). The top 20 of genes up-regulated in G0 vs G3-G4 cartilage (Fold change G0/G3-G4 >1.4 and *p-value* below 0.05) are shown in **Figure 4.8.A**. GO analysis showed most of the genes were involved in skeletal system development, ECM organization and cartilage development. Among their different functions we found TGFβ production, glycosaminoglycan metabolic process, chondroitin sulfate biosynthetic process, catabolic process, and wound healing. Moreover, some of these genes (e.g. *TNFRSF11B*, *FRZB*, *LUM*, *CTGF* and *DCN*) were also involved in various signaling pathways including TNFα, WNT, TGFβ and VEGF. Interestingly, genes involved in anoxia, inflammatory response, oxygen homeostasis and cellular response to hypoxia such as *CTGF*, *CDO1*, *EPAS1/HIF2A* and *HIF1A* were also up-regulated in healthy cartilage compared to diseased tissues. Some other genes regulated in healthy cartilage included *ZFP36*, *ID2* and *SLC38A2*, which are linked to cellular response to stimulus, regulation of metabolic process and cellular response to mechanical stimulus.

Figure 4.8.B displays miR-210 expression during osteoarthritis progression. MiR-210 expression values increased in the first stage of osteoarthritis (G1 stage) by 104.9-fold (*p-value*=0.032) compared to G0 while increasing 21 to 8-fold on chronic stages G2 (*p-value* =0.045) and G3/4 (*p-value*=0.048) respectively, compared to healthy cartilage (no OA, G0). This result suggests a

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potential role of miR-210 in early osteoarthritis while its importance may decrease during the progression of disease.

Figure 4.8 mRNAs that are regulated in healthy cartilage (no OA, G0) against diseased cartilage (G3/4). **(A)** Top 20 of up-regulated genes in healthy cartilage. **(B)** MiR-210 RPKM values during osteoarthritis progression. Expression levels are represented as RPKM values $\pm$ standard error of the mean. Three biological replicates (n=3) and one technical replicate were used. Statistical analysis is represented as * for $p<0.05$.

To define miR-210 potential gene targets in healthy cartilage (G0), an analysis of genes regulated in healthy cartilage (G0, no OA), genes regulated in severe OA cartilage (G3-4) and miR-210 predicted targets was performed. 764 genes were up regulated whilst 247 genes were down regulated in healthy cartilage (no OA, G0) when compared to severe OA samples (G3-4). From those, 80 miR-210 gene targets were up regulated in healthy cartilage (no OA). Among these genes: *FRZB*, *PRKCE*, *PRKCQ*, *EP300* and *ACVR1B*, have been previously associated to cartilage development related pathways such as WNT and BMP signaling pathways. The list of the 80 genes is listed in **Table 4.3A**. From the 243 genes down-regulated in G0 vs G3-G4 cartilage, 29 genes were also miR-210 targets and are listed in **Table 4.3.B**. Functional analysis showed some of these genes are involved in cell response during hypoxia (e.g. *STC2*) and cartilage degradation (e.g. *MMP14* and

ADAMTS10). Hence, healthy (no OA, G0) cartilage presents specific gene expression of genes involved in cartilage homeostasis and cartilage development.

Table 4.3 List of regulated genes in healthy cartilage (no OA, G0) when compared to diseased cartilage (severe OA, G3-4) that are also miR-210 targets.

A

Up-regulated mi-R210 targets					
FRZB	WHSC1L1	FITM2	MFHAS1	PRKCE	GRAMD1C
TMOD1	EP300	VPS13D	ESR1	ZDBF2	NLRC5
FOSL2	ASH1L	PRKACB	PRKCQ	MKL2	PHF16
TMTC1	RAPH1	MFAP3L	CLOCK	MOCOS	GRAMD2
ANTXR1	ZNF385B	SLC30A2	ATP11A	KLF12	KCND3
B4GALT5	TMEM133	NCAPD3	TMED8	RALGPS1	TANC2
HIP1	TUBGCP6	ADARB1	HMBBOX1	EMILIN3	GABRB1
CLIP2	MLL2	FOXI2	SLC37A1	RIC3	HIST1H2AK
MGLL	CDC42BPA	EGFR	HS3ST3B1	PPM1L	SAMD12
CACNA1C	MYO18A	ACVR1B	BAZ2B	GABRR2	TIMD4
SLC12A7	RAPGEF5	SETBP1	EPB41L5	STC1	PRLR
ACACA	PTPRD	HDAC4	SPEG	PAIP2B	FSD1L
CDK18	TEAD3	DNAJC18	TET2	PDE3B	MDGA1
CLLU1	FGF17				

B

Down-regulated miR-210 targets					
LAMC2	TMEM45A	NDUFB5	CLSTN1	PDLIM7	COL6A1
SPATA20	ADAMTS10	SPNS1	SNX8	TMEM101	COTL1
PKP1	CSMD2	QSOX1	PGAP3	STC2	PHLDA3
HSPA5	PLCXD1	HM13	STK24	MMP14	NT5M
SLC16A3	COX6B2	EHD2	RASA3	TCN2	

4.3.7 Similar regulatory mechanisms enhance chondrogenic differentiation of MSCs during the hypoxic response.

To define the microRNAs and gene targets involved in the regulation and response of MSCs (both bMSCs and aMSCs) to low oxygen, combinatorial analysis of both microRNAseq and mRNAseq datasets was completed. Previous microRNAseq analysis revealed 17 microRNAs up-regulated in bMSCs (e.g. miR-210, miR-221, miR-155, miR-26b, miR-31 and miR-134), whereas 18 microRNAs were up regulated in aMSCs (e.g. miR-210, miR-195, miR-181a, miR-151a, miR-423 and miR-493). MiR-210 and miR-195 were the common microRNAs regulated in both MSCs. In contrast, 2 common microRNAs were found down regulated in both MSCs: miR-378c and miR-16-1-3p.

To describe the genes important for healthy cartilage maintenance that can be regulated by low oxygen in both MSCs, a comparison of healthy cartilage, and MSCs cultured in hypoxia was performed. First, all up-regulated genes in hypoxia (FC hypoxia/normoxia >1.4) in both MSCs were compared to healthy cartilage

(Fold change healthy cartilage/disease cartilage > 1.4). No genes were found in common between both MSC cell sources and healthy cartilage. However, some cell type specific genes were found in common between cartilage and aMSCs. Common genes between cartilage and aMSCs included *HLA-C*, *KIAA1191*, *SLC6A9*, *AOX1*, *RHOU*, *HOXD4*, *KCNA6*, *TMEM92*, *GJA3*, *FGFR2*, *MKX*, *SCNN1A* and *VWDE*. Functional analysis on these genes showed *FGFR2* is involved in the FGF and WNT signaling pathways, *HOXD4* is linked to transcription regulation, stem cell differentiation and embryonic skeletal formation, whereas *KIAA1191* participates in the regulation of neuronal survival, differentiation and axonal outgrowth. In addition, genes in common between cartilage and bMSCs included *TNFRSF11B*, *ERRFI1*, *SNX25*, *TIPARP*, *DDR1*, *ZNF773*, *ANGPT2*, *C7*, *SLC25A21* and *LOC100507086*. GO analyses revealed involvement in TNF signaling pathway, apoptotic signaling pathway (*TNFRSF11B*), SMAD and TGF β signaling pathway (*SNX5*).

Common up-regulated genes between aMSCs and bMSCs included *TNFRSF10D*, *STC2*, *COX7A1*, *NOL3-ARC*, *HSPB2-C11orf52* and *APOA1*. Functional analysis revealed involvement in the TNF mediated signaling pathway (*TNFRSF10D*, *APOA1* and *NOL3*) and TGF β signaling pathway (*APOA1*). Moreover, 2 of these genes are also targets of miR-210: *STC2* and *TNFRSF10D*. Comparison of down-regulated genes was also completed. No genes were regulated by both MSCs and healthy cartilage whereas 50 genes were found in common between cartilage and aMSCs (e.g. *BHLHE40*, *TUBB3*, *NQO1*, *ITPRIP*, *IRF2BP2*, *RGS2*, *FRMD4A*, *MCM2*, *LPCAT4*, *RIN1*, *ETFB*, *UBA7*, *FN3KRP*, *LHFPL2*, *ACOT7*, *SARS2*, *GAMT*, *NCAPH*, *C6orf1*, *NTHL1*, *CHCHD10*, *LETMD1*, *NR4A3*, *IFIT3*, *PRSS3*, *FAM102B*, *MLKL*, *FOXRED2*, *PGAP2*, *C14orf159*, *ETV4*, *PARPBP*, *FANCD2*, *GALNT12*, *TFAP4*, *CCDC15*, *ANKRD39*, *LRRN4CL*, *MIR2116*, *FN3K*, *KRT17*, *OAS2*, *CCDC150*, *RNFT2*, *FCRLA*, *SEMA4A*, *AKR7A3*, *WDR88*, *SULT1A1* and *TTLL13*) and 1 gene was common between cartilage and bMSCs (e.g. *IGDCC4*). Gene ontology analysis exhibited that

these genes participate in CCKR signaling, cytoskeletal regulation, insulin/IGF pathway and MAP kinase cascade whereas *IGDCC4* is involved in neural crest differentiation. Additionally, 10 genes were found down regulated in both aMSCs and bMSCs: *SLFN11*, *PRICKLE1*, *SLC35F2*, *TRIM59*, *AMPH*, *FAM46B*, *SHMT1*, *PDGFD*, *ARHGAP11B* and *BACH2*. GO analysis revealed participation of these genes in different pathways such as PDGF, MAPK, TGF β signaling pathway (*PDGFD*) and WNT signaling pathway (*PRICKLE1*). Transcriptional regulatory activity was described for *BACH2*. 3 of these genes were also miR-210 gene targets: *SHMT1*, *SLC35F2* and *BACH2*. Although low oxygen tension does not seem to regulate the same genes in both MSCs, it appears to coordinate specific regulatory mechanisms that are essential for chondrogenic differentiation for both aMSCs and bMSCs. In addition, a common regulatory mechanism in both MSCs controls the oxidative stress response under hypoxia.

Analysis of the top genes regulated in hypoxia was completed to obtain the hypoxic signature for each cell type. First, soft filtering was applied and genes with more than 1.4-fold change, RPKM value above 0.01 and *p-value* below 0.05 were chosen. Then, two different sets of genes were analyzed: the top 20 most expressed genes in hypoxia (according to RPKM values) and the top 20 genes with highest fold change Hypoxia/Normoxia genes. **Figure 4.9.A** shows the top 20 genes with the highest RPKM values in hypoxic aMSCs. Several of them have been linked to oxygen response (e.g. *LDHA*, *LOXL2*, *LOX*, *PLOD2*, *PKM*, *THBS1*, *ENO1* and *P4HA1*), whereas some are related to bone extracellular matrix and osteoarthritis development (e.g. *COL1A1*, *COL1A2*, *COL5A1*, *GREM1* and *TGFB*). Other genes participate in various cell processes such as cell metabolism, apoptosis and cell cycle (e.g. *MIF*, *ITGA5*, *ALDOA*, *CCDC80*, *GAPDH*, *PGK1* and *TPI1*). Moreover, several genes positioned in the top 20 genes of highest fold change between hypoxia and normoxia (**Figure 4.9.B**) have been associated with different cell processes (e.g. *GPR146*, *PPFIA4*, *FGF11*, *SPAG4*, *VLDLR*, *PFKFB4*,

ALDOC, *CCDC80*, *BNIP3*, *BNIP3L*, *ICAM5*, *RAB20*, *FBX016*, *FER1L4*, *RPL17P50* and *ZNF395*) whereas only a small part was linked to oxygen response (e.g. *NDUFA4L2*, *CA9*, *HIF3A*, *STC2* and *ENO2*). From these genes, only *HIF3A* is also a miR-210 target.

Figure 4.9. (A and C) Top 20 genes with highest RPKM values and (B and D) top20 of highest fold change between hypoxia and normoxia in hypoxic aMSCs and bMSCs.

The same analysis was completed for bMSCs (**Figure 4.9.C**). In this case, the 20 genes with highest RPKM in hypoxia included several genes linked to oxygen response (e.g. *TIMP1*, *HSPB7*, *MFGE8*, *ERRF1*, *STC2* and *CDH13*) and cartilage development/maintenance (e.g. *ADAMTS1*, *TIMP1*, *DDK1*, *ID3*, *ACAN*, *TNFRSF11B*, *SMAD3* and *HAPLN3*), whereas the rest were linked to cell cycle (e.g. *CCND1* and *ID3*) and other cell processes such as cell differentiation, cell proliferation, apoptosis, calcium regulation and cell transformation (e.g. *FOXO2*, *GPR1*, *ID2*, *CACNA2D1*, *TMEM200A* and *FOSL1*). Some of these genes were also found in the top 20 of highest fold change between hypoxia and normoxia (e.g. *ACAN*, *ADAMTS1*, *CACNA2D1*, *ERRF1*, *HAPLN3*, *MFGE8*, *STC2* and *TNFRSF11B*); genes

that mostly participate in oxygen response and cartilage development (**Figure 4.9.D**). The rest of the genes participate in stress response, immune response, cancer, membrane trafficking, cell adhesion and migration, cell cycle and cytoskeletal binding (e.g. *BDNF*, *EPHB6*, *CPA4*, *FCRLB*, *CPNE7*, *MYPN*, *MALL*, *PALMD*, *CD14*, *LRRN3*, *RHCE* and *TNFAIP3*).

Interestingly, genes up regulated in each MSC depict significant differences between aMSCs and bMSCs during hypoxic response. Both MSCs up-regulate genes that participate in similar biological processes, such as hypoxia response, cell metabolism, apoptosis and cell cycle. In addition, genes increased in bMSCs are involved in cartilage development and maintenance whereas genes increased in aMSCs are involved in bone extracellular matrix synthesis and osteoarthritis development.

4.4 DISCUSSION

The importance of signalling molecules such as miRNAs for cartilage development, maintenance, and destruction [71-74] has been extensively studied. Some have been found induced under hypoxic conditions including miR-155, miR-424, miR-17-92, miR-21, miR-23, miR-24, miR-26, miR-103 and miR-107; with miR-210 standing out in several studies as the only highly inducible miRNA under low oxygen [75, 76]. A number of pathophysiological findings propose that a correlation does exist between hypoxia and chondrogenesis [8]. Additionally, several studies sustain that a low oxygen tension environment may be beneficial to preserve the chondrocyte phenotype of the cells during *in vitro* cultures, avoiding the potential de-differentiation of the cells during expansion [8, 77, 78]. However, the molecular mechanisms by which chondrocytes are regulated by oxygen tension remains poorly understood.

In this study, we have performed an integrated analysis of miRNA-seq and mRNA-seq on hypoxic mesenchymal stem cells and cartilage tissue to identify

hypoxia-regulated miRNAs and their gene targets. First, we examined the hypoxic microRNA footprint on both aMSCs and bMSCs and concluded which miRNAs were significantly regulated in both MSCs. Then, prediction analysis was utilized to list potential and bona fide miR-210 targets in aMSCs and bMSCs. Gene ontology analysis examined the functions and regulatory mechanisms of the predicted genes. We have identified important regulatory mechanisms that control the stress response and skeletal development of the cells during low oxygen cultures. HIF1 gene targets were also analyzed against our mRNAseq data to examine which genes could be regulated by both HIF1 and miR-210 under low oxygen tension. This analysis was performed on bMSCs and also aMSCs. Subsequently, investigation of common target genes and regulatory mechanisms was performed on both MSCs. Lastly, analysis of a healthy cartilage mRNA signature was compared against genes regulated under low oxygen tension in both aMSCs and bMSCs to obtain a list of genes that could potentially enhance chondrogenic differentiation on each cell type. Some regulatory mechanisms were cell type specific whereas others were shared between both MSCs.

Thus, we have reported on various findings: i) miR-210 is the unique hypoxia induced miRNA in both bMSCs and aMSCs, ii) modestly induced miRNAs in hypoxia vary with cell type and do not participate in a specific molecular program, iii) hypoxia regulated miR-210 gene targets are involved in stress response and in cartilage development, iv) common gene targets of HIF1 and microRNA-210 play an essential roles in oxidative stress response, cell cycle regulation and skeletal development and it appears that HIF1 and miR-210 act in concert in the regulation of many of these genes, v) miR-210 may play an important role in the inflammatory response and cartilage homeostasis in healthy cartilage tissues, and vi) aMSCs and bMSCS have a different microRNA and mRNA footprint under low oxygen.

miR-210 was the dominant miRNA involved in regulation of gene expression responses in both aMSCs and bMSCs, at least part of its effects on gene expression are regulated in concert with the transcription factor HIF1. Although, low amounts of genes were shown to overlap in both MSCs, an important overlap in cellular processes and functions was found. This was achieved by the interaction with other genes that may have redundant functions but are expressed in a cell type specific manner. Hypoxia regulates two important cellular functions, skeletal development and oxidative stress response in MSCs. The first set of genes is involved in preventing hypertrophic and ossification by increasing cartilage ECM protein synthesis (e.g. *COL2A1* and *ACAN*) [37, 79] through regulatory pathways, such as WNT and TGF β [39, 80]. The remaining genes are involved in the cell response to oxidative stress [41] mediated by increasing anti-inflammatory response [81] and cell cycle regulation [82] to prevent apoptosis and DNA damage. Increased expression of genes involved in WNT, FGF and TGF β signaling pathways [66] might be relevant for chondrogenic differentiation. These signaling pathways have all been implemented in maintenance of healthy cartilage and/or regulation of chondrogenic differentiation (**Chapter 2**). Thus, the beneficial effects of hypoxia on chondrogenic differentiation can be at least in part attributed to the regulation of these signaling pathways.

Although all these genes have been described in the literature, none were previously linked to miR-210. The present work suggested a link of *WNT11*, *WISP1* and *BACH2*, found by target prediction analysis, and *SHMT1* and *SLCC3F2*, found by mRNAseq, as bona fide miR-210 targets essential for cartilage development and oxidative stress response regulation. This regulatory mechanism may promote cartilage development through releasing a brake on ECM protein synthesis, proteoglycan deposition, aggrecan and collagen type II synthesis. Although *BACH2* has not been described as a participant in any

pathway involved in cartilage development, its inhibition by miR-210 during hypoxia may help in inducing an anti-inflammatory response by inhibiting oxidative stress induced apoptosis and by decreasing *HMOX1* gene expression. Moreover, the difference in gene expression and pathways regulated during low oxygen response suggested a difference in the chondrogenic potential of aMSCs and bMSCs.

Though the identity of genes shows little overlap between MSCs, the biological processes and functions regulated by both cells in low oxygen are very similar. Suggesting the participation of several genes, not detected in this study, in similar functions as those previously described. Furthermore, common genes between MSCs and cartilage diverged in both oxygen conditions. Genes increased in hypoxia are mostly involved in cartilage regulatory mechanisms, while genes decreased in hypoxia participate in several biological processes not related to cartilage (e.g. glucose and intestinal mucosa regulation, and neural crest differentiation).

Figure 4.10 Identification of genes that can be modulated by miR-210 expression. To summarize our findings a model was created using the information obtained along the study.

Nevertheless, genes that are commonly regulated in both MSCs indicated that during the hypoxic response they are involved in preventing premature endochondral ossification of the cells whereas up-regulated genes are involved in cartilage maintenance of healthy cartilage. Our results suggest that some matrix degradation genes are direct targets of miR-210, suggesting that miR-210 can be potentially used to target these genes avoiding progression of disease. Interestingly, high induction of miR-210 could cause an inflammatory response on the cells and negative regulation of cartilage development.

One of the potential limitations of our study is the small sample number (n=3) used. Although RNAseq is expensive; a larger sample number would increase reliability of the results. Moreover, only one culture condition (monolayer) was examined in this study. Further analysis on different cell culture conditions (e.g. chondrogenic differentiation, 3D scaffolds, etc) and oxygen levels will help to validate the results observed in this study.

The results from this combinatorial analysis of microRNAseq and mRNAseq may be a useful tool to control identified potential gene targets to enhance chondrogenic differentiation of MSCs in different cultures or help in promoting *in vitro* cartilage regeneration. Several of the genes found in this analysis can be used for pharmacological targeting or genetic approaches in order to promote a stable and better chondrogenic phenotype of mesenchymal stem cells.

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CHAPTER 5

5. miRNA-210 promotes chondrogenesis of MSCs

ABSTRACT

Articular Cartilage has limited capacity to respond to damage limiting the overall capacity to heal and repair. Although current techniques have shown interesting results, they still present major weaknesses. Thus, an optimal tissue engineering strategy that regenerates the natural cartilage structure is required for a successful cartilage tissue repair. Hypoxia has been described as a key factor to preserve cartilage integrity whilst a large number of microRNAs have been implicated in regulating chondrogenesis. Previously, we identified miR-210 as the unique hypoxia induced miRNA in adipose and bone marrow derived Mesenchymal Stem Cells (MSCs), which gene targets are mostly involved in the regulation of cartilage homeostasis and oxidative stress responses. Its expression is strongly upregulated during chondrogenic differentiation of MSCs. Based on these observations; we hypothesize that preconditioning of MSCs with miR-210 before chondrogenic exposure may accelerate early stages of chondrogenesis and avoid hypertrophic differentiation of the cells. Interestingly, under low oxygen cultures overexpression of miR-210 enhanced chondrogenesis of MSCs as good as TGF β 1. Chondrogenesis improvement was evidenced by increased expression of cartilaginous markers, proteoglycan deposition and collagen II protein content. In both oxygen conditions, the combined stimulation of MSCs with TGF β 1 and miR-210 decreased the synthesis and deposition of important chondrogenic matrix proteins such as collagen II and aggrecan while increasing the expression of the fibroblastic marker collagen I. Functional experiments revealed miR-210 importance for early chondrogenesis of MSCs during *in vitro* cultures. Hence, exogenous miR-210 expression can be used instead of chondrogenic differentiation cocktail in standard or low oxygen cultures to promote chondrogenesis of MSCs while inhibiting their hypertrophic differentiation. This potential treatment can help in developing improved protocols for the formation of hyaline cartilage using MSCs.

5.1 INTRODUCTION

Articular Cartilage is composed of a dense extracellular matrix (ECM) made of 80% water, 18% type II collagen and proteoglycans, and a small portion of other proteins. Chondrocytes, specialized cells which role is to develop, maintain and repair the ECM, encompass only 2-3% of the articular cartilage volume [1]. However, chondrocytes have limited potential for replication and limited capacity to respond to damage [2], negatively impacting the overall capacity of articular cartilage to heal and repair. Although current techniques including osteochondral grafting and cellular therapies such as autologous chondrocyte implantation have shown promising results, they still present major weaknesses. Thus, an optimal tissue engineering strategy that regenerates the natural cartilage structure is required for successful cartilage tissue repair. Much attention has been paid to the use of mesenchymal Stem Cells (MSCs) as an alternative cell source for engineering new cartilage [3]. This has started the exploration of environmental factors like oxygen tension and/or additives like signaling molecules to boost chondrogenic differentiation of mesenchymal stem cells [4]. One of these environmental factors is oxygen tension. Oxygen tension has been shown to stimulate cartilage formation [1] by primary chondrocytes as well as MSCs. The exact mechanism is however still largely unknown. Several studies have demonstrated that miRNAs are involved in the hypoxic response and can contribute to the repression of specific genes under low oxygen conditions [5]. Also, hypoxia has been described as a key factor to preserve cartilage integrity whilst a large number of microRNAs have been implicated in regulating pre and post-natal chondrogenesis [6].

We have identified miR-210 as the unique hypoxia induced miRNA in both bMSCs and aMSCs (**Chapter 4**). Its gene targets are mostly involved in the regulation of a stress response, in preventing hypertrophy, degradation and ossification of cartilage. Nevertheless, during the hypoxic response, direct gene targets of miR-

210 are involved in avoiding progression of disease and preventing earlier endochondral ossification of the cells. Despite several microRNAs have been described to participate during chondrogenesis of cells [7], we found hypoxic regulated miR-210 to be essential in the regulation of genes in charge of several functions necessary for *in vitro* cartilage development. Our goal is to precondition MSCs using miR-210 before chondrogenic exposure to accelerate early stages of chondrogenesis and avoid hypertrophic differentiation of the cells. Inhibitory experiments will also be conducted to study the effect and the relevance of miR-210 during chondrogenesis of MSCs.

5.2 MATERIALS AND METHODS

5.2.1 Monolayer and high-density pellet cultures

For cell harvest and expansion, same methods and protocols described in section 4.2.1 (**Chapter 4**) were used. Both MSCs were expanded in growth media. For bMSCs this media consisted of DMEM with 4.5 g/L glucose and sodium pyruvate (Corning, Corning NY), supplemented with 10% fetal bovine serum (Atlanta Biologicals, Atlanta, GA) and 1% penicillin/streptomycin (Gibco/Thermo Fisher Scientific). Maintenance media for aMSCs was composed of advanced MEM (Gibco/Thermo Fisher Scientific, Waltham, MA) supplemented with 5% human platelet lysate (PL-Max, MillCreek Life Sciences, Rochester, MN), 1% penicillin/streptomycin, 1% Glutamax (Gibco/Thermo Fisher Scientific), and 0.2% heparin (Baxter, Deerfield, IL). For monolayer culture, bMSCs were plated in 6-well plates at a density of 3,000 cells/cm². Cells were cultured using standard techniques in T-175 cm² flasks at 37°C, 95% humidity, and 5% CO₂ until they reached 80% confluence. Prior to each experiment, cells were detached from T175 flasks by trypsinization using TrypLE Express (Gibco/Thermo Fisher Scientific). High-density cellular aggregates were performed using pellet cultures in accordance with a protocol published previously [6]. To form the pellets, 250,000 cells were seeded in 96 round-bottom well plates followed by a

centrifugation step to initiate pellet formation. Pellets were maintained at 37°C, 95% humidity, and 5% CO₂ until cells coalesced. Both cultures were kept under standard culture conditions. Media was replaced every 3 to 4 days.

5.2.2 Cell culture under Normoxia and Hypoxia conditions

To evaluate expression of bMSCs in response to oxygen tension, both cells were plated in monolayer or in high-density pellet cultures and maintained for up to 21 days in maintenance media under either normoxic (20% oxygen) or hypoxic (2% oxygen) conditions using an integrated system with a cell culture hood and an incubator accessible through a gas-lock (I-Glove, BioSpherix, New York, NY).

5.2.3 Chondrogenesis of bMSCs.

Chondrocyte differentiation was induced in high-density pellets. Following pellet formation, maintenance media was exchanged with chondrogenic differentiation media which consisted of culture media supplemented with 40 mg/mL of L-proline, 50 mg/mL Insulin Transferrin Selenium-premix, 50 mg/mL of ascorbic acid, 10 ng/mL of TGF- β 1, and 0.1 μ M dexamethasone. MSCs were allowed to differentiate up to 21 days. Medium was refreshed every 3 to 4 days. Total RNA was isolated using the miRNeasy Micro Kit following the instructions of the manufacturer.

5.2.4 Functional analysis of miR-210.

Gain and loss of function experiments were performed in bMSCs. Before transfection, all cells were seeded in monolayer culture and maintained in culture media until 80% confluency was reached. Due to the absence of endogenous miR-210 in normoxia, this condition was used for gain of function experiments, whilst low oxygen conditions under which miR-210 is induced and abundantly expressed, were used for loss of function experiments. First, a dose response experiment was completed to examine toxicity of the miRNA transfection on the MSCs. Different concentrations: 5, 10, 20 and 50 μ M of MISSION hsa-miR-210 mimic (HMI0373, Sigma Aldrich, Saint Louis, MN, USA),

MISSION hsa-miR-210 inhibitor (HSTUD0373, Sigma Aldrich) and MISSION Negative control miRNA (HMC0002, Sigma Aldrich) were used. Mature sequence used in both miR-210 mimic and inhibitor was CUGUGCGUGUGACAGCGGCUGA whereas negative control miRNA mature sequence was GGUUCGUACGUACACUGUUCA. Transfections were performed using Lipofectamine RNAiMAX (Thermo Fisher, Waltham, MA, USA) following manufacturer's instructions and using a 2 to 1 Lipofectamine siRNA ratio. Optimal transfection dose was calculated from these experiments. A stimulatory dose of 20 μ M of miR-210 mimic was used for gain of function experiments whereas 10 μ M of miR-210 inhibitor was selected for loss of function experiments. In both experiments gene expression, protein analysis and histological analysis were performed. An outline for the experimental set-up of this chapter is included in **Figure S5.1**.

Figure S5.1. Experimental outline.

5.2.5 Gene expression analysis

For pellet cultures, at least 4 pellets were pooled from each condition to obtain sufficient RNA. Pooled pellets from each time point were lysed using an 18-gauge needle and Qiazol lysis buffer (Qiagen, Hilden, Germany) to yield an RNA sample for one biological replicate. For monolayer cultures, one well from the 6-well plate was collected as one biological replicate. Three different biological replicates for each experimental condition (e.g., 3 samples for normoxia and 3 samples for hypoxia) were used. Total RNA was isolated using the miRNeasy Micro Kit (Qiagen, Hilden, Germany) following the instructions of the manufacturer. RNA concentrations and purity levels were measured using a NanoDrop (Thermo Fisher Scientific).

Table 5.1 miRNA Primers sequences used for gene expression analysis.

Gene ID	Forward Primer Sequence	Reverse Primer Sequence
miR-191-5p	CAACGGAATCCCAAAAGCAGCTG	U6 Reverse Primer - TaKaRa
miR-210-3p	CTGTGCGTGTGACAGCGGCTGA	U6 Reverse Primer - TaKaRa
miR-21-5p	TAGCTTATCAGACTGATGTTGA	U6 Reverse Primer - TaKaRa

Note: All primer sequences are listed as 5' to 3'.

miRNA-210 expression analysis was performed in monolayer and high-density pellets during low oxygen cultures in chondrogenic differentiation and functional experiments. For baseline gene expression values, lysates were obtained from expanded cells in monolayer immediately prior to plating cells in pellet culture on day 0 (D0: plating day). Subsequent samples were harvested at D1, D3, and D7 e. Isolated RNA was reverse transcribed into cDNA using MiR-X miRNA First-Strand Synthesis (TaKaRa, Shiga, Japan). Real-time qPCR was performed using a CFX384 Real-Time System (BioRad, Hercules, CA). miR-21-5p and miR-191-5p were included as reference genes. MiR-210-5p was normalized to miR-21-5p because it showed the lowest variability. Primer sequences are listed in **Table 5.1**.

Table 5.2 Primers sequences used for gene expression analysis.

Gene ID	Forward Primer Sequence	Reverse Primer Sequence
ACAN	GTGCCTATCAGGACAAGGTCT	GATGCCTTTACCACGACTTC
ACTB	AGCACAGAGCCTCGCCTTT	CGGCGATATCATCATCCAT
AKT1	ATGGCGCTGAGATTGTGTC	CCCGGTACACCAGTTCTTC
CCNB2	CCGACGGTGTCCAGTGATT	TGTTGTTTTGGTGGGTTGA
COL10A1	CCAGCACGCAGAATCCATC	ATGCCTGTGGGCATTTGGTA
COL1A1	GTAACAGCGGTGAACCTGG	CCTCGCTTTCCTCCTCTCC
COL2A1	GGGATCGTGGTGACAAAGGT	CTGGGCAGCAAAGTTCCAC
GAPDH	ATGTTTCGTATGGGTGTGAA	TGTGGTCATGAGTCCTTCCA
HAPLN1	TCTGGTGCTGATTCAATCTGC	TGCTTGGATGTGAATAGCTCTG
HIF1A	TTCTTCTCTTCTCCGCGTG	ACTTATCTTTTCTTGTCTCGC
MKI67	ACGCCTGGTTACTATCAAAAGG	CAGACCCATTACTTGTGTTGGA

Note: All primer sequences are listed as 5' to 3'.

mRNA gene expression analysis was performed at distinct time points during chondrogenic differentiation. RNA lysates were obtained prior to plating cells in high-density pellet culture on day 0 and after 7 days of culture in high-density pellet cultures. 1µg of isolated RNA was used as a template for reverse transcription using M-MLV Reverse Transcriptase (Promega, Madison, USA). Gene expression was quantified using quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR) with 10 ng cDNA per 10 µL with QuantiTect SYBR Green PCR Kit (Qiagen) and the CFX384 Real-Time System (BioRad, Hercules, CA). Relative transcript abundance of genes of interest was normalized to AKT1 expression using the $2^{-\Delta\Delta Ct}$ method. Representative chondrogenic genes (e.g. *COL2A1*, *COL1A1*, *HAPLN1*, *ACAN* and *COL10A1*), proliferative markers (e.g. *CYCLIN2A* and *HIST2H4*), hypoxia inducible genes (e.g. *HIF1A* and *HIF3A*) and housekeeping genes (e.g. *GAPDH*, *ACTB* and *AKT1*) described in **Table 5.2** were used. All values are represented as relative transcript compared to AKT1 (housekeeping gene) $\pm$ standard error of the mean. Three biological replicates (n=3) and three technical replicate was used.

5.2.7 Western Blotting

Total protein was harvested from all different cells 72 hours after transfection. Five cell pellets were pooled from each condition to obtain sufficient protein

yield. Pooled pellets from each time point were washed twice with ice cold PBS and mechanically lysed using an 18-gauge needle in the presence of radioimmunoprecipitation buffer (150mM NaCl, 50mM Tris pH 7.4, 1% w/v sodium deoxycholate, 0.1% w/v sodium dodecyl sulfate and 1% v/v Triton X-100) supplemented with protease inhibitor cocktail (Sigma) and PMSF (Sigma), and stored at -80°C until quantification. Protein was quantified using the *DC*TM protein assay (Bio-Rad) according to the manufacturer protocol. Western blotting and membrane development was performed as previously described [8] and the following primary antibodies were used to detect proteins: mouse monoclonal anti-HIF1A (1:500; 610959, BD Biosciences), mouse monoclonal anti-COL2A1 (1:500; MAB8887; Millipore) and rabbit monoclonal anti-GAPDH (1:1000; D16H11; Cell Signaling).

5.2.8 Histological Analysis

Cell pellets were collected after 21 days of culture and fixed overnight in 10% neutral buffered formalin. Samples were then washed and dehydrated in graded series of ethanol (70% to 100%) and processed with xylene (50% to 100%) prior to paraffin embedding. Paraffin blocks were cut into consecutive sections of 5 µm thickness using a microtome and placed onto charged microscope glass slides for histological staining. Following deparaffinization, sections were stained for glycosaminoglycan content using 0.5% (wt/vol) Alcian Blue 8GX dye (Sigma-Aldrich) and counterstained with Nuclear Fast Red (Sigma-Aldrich). Collagen type I and collagen type II immunohistochemistry (IHC) was also performed. After removal of paraffin and rehydration, sections were washed with distilled water. Antigen retrieval was performed using 1 mg/mL pronase for 30 minutes at 37°C followed by 10 mg/mL hyaluronidase for 30 minutes at 37°C, both in a humidified chamber. Briefly, protein block was applied using 5% Bovine serum albumin (BSA) in PBS after which slides were incubated overnight with 1 µg/mL of anti-COL2A1 mouse monoclonal Antibody (Millipore, Darmstadt, Germany),

anti-HIF1A mouse monoclonal Antibody (BD Biosciences, Osage, IA) or anti-mouse IgG monoclonal Antibody (Abcam) as a control at 4°C in a humidified chamber. Slides were sequentially incubated with biotinylated goat anti-mouse IgG HRP secondary antibody and were then treated with 3,3'-diaminobenzidine (DAB) enhanced liquid substrate system for IHC (Sigma-Aldrich) for 10 minutes until color development. Sections were subsequently analyzed using a ZEISS Axio inverted light microscope.

5.2.9 Statistical Analysis

Quantitative data for gene expression profiles have been presented as mean $\pm$ standard deviation from 3 independent MSCs donors for each experimental condition and time point. Statistical analysis was performed using JMP® Pro v10.0.0 (SAS Institute), and *p*-values were determined using Tukey HSD test. Significance is noted in the figures when applicable as * for *p* < 0.05 and ** for *p* < 0.01.

5.3 RESULTS

5.3.1 miR-210 is the distinctive hypoxia induced microRNA in MSCs.

To evaluate and identify potential miRNAs that are regulated in hypoxia, three different bMSCs and three different aMSCs donors were cultured for one week in monolayer cultures and different oxygen environments: normoxia (20% oxygen) or hypoxia (2% oxygen). MicroRNAseq analysis was previously performed on the samples (**Chapter 4**). From section 4.3.7 only 2 microRNAs were commonly regulated in both MSCs: miR-210 and miR-195. However, the only robustly induced microRNA was miR-210. In bMSCs, miR-210 was induced more than 50-fold in hypoxia when compared to normoxia (**Figure 4.1.B**), whereas in aMSCs fold induction was around 25-fold (**Figure 4.6.A**). Subsequently, both cells were cultured in monolayer or high-density pellets for 7 days in normoxia or hypoxia. After this time, RNA was extracted and gene expression was analyzed using RT-qPCR. As shown in **Figure 5.1.A**, miR-210 is induced by hypoxia in both cultures.

Fold-induction for both cells was significantly higher for high-density cultures than in monolayer cultures possibly due to an exaggerated hypoxic effect in the pellet cultures.

Figure 5.1 (A) miR-210 is highly induced in hypoxia in both MSCs during pellet or monolayer cultures. **(B)** High-density pellets were used to estimate chondrogenic potential of both MSCs after 7 days in monolayer cultures. Values are represented as relative transcript compared to *AKT1* (housekeeping gene) ± standard error of the mean. Three biological replicates (n=3) and three technical replicate were used. Statistical analysis is represented as * for $p < 0.05$, ** for $p < 0.01$ and *** for $p < 0.001$.

High-density cultures were used to estimate the chondrogenic potential of both types of MSCs. After 7 days of culture, gene expression of chondrogenic markers (e.g. *ACAN*, *COL2A1*, *DCN*, etc), proliferation markers (e.g. *CYCLINB2* and *HIST2H4*) and hypoxia induced *HIF1A* were examined (**Figure 5.1.B**). Chondrogenic markers *COL2A1* and *ACAN* were found to be up-regulated in hypoxia when compared to normoxia in bMSCs. However, expression levels in aMSCs of both chondrogenic markers were unnoticeable. Interestingly, in both MSCs the relative expression of master chondrogenic transcription factor *SOX9* was increased in hypoxia. Yet, the expression was significantly up-regulated in bMSCs whereas in aMSCs the expression was low. In contrast, the induction of

expression of various proteoglycan markers such as *DCN* and *COMP* was not cell-type dependent. However, chondrogenic marker *HAPLN1* was only expressed in both normoxia and hypoxia in bMSCs. Moreover, proliferation marker *HIST2H4* was increased when cells were cultured in hypoxia compared to normoxia. Lastly, hypoxia inducible factor *HIF1A* regulation was similarly regulated in both MSCs. Thus, hypoxia enhances expression of miR-210 irrespective of cell type and upregulates the expression of various chondrogenic markers. The latter clearly showed some cell type specific regulation particularly in the fold change of the response.

Figure 5.2. (A) RT-qPCR analysis of bMSCs cultured in normoxia and hypoxia. Day 7 chondrogenic differentiation and growth media cultured cells. Values are represented as relative transcript compared to AKT1 (housekeeping gene) $\pm$ standard error of the mean. Three biological replicates (n=3) and three technical replicates were used. Statistical analysis is represented as * for $p < 0.05$, ** for $p < 0.01$ and *** for $p < 0.001$. **(B)** Histological analysis of MSC grown in maintenance or chondrogenic media and subjected to normoxia and hypoxia conditions. Alcian Blue and Nuclear fast red was used for glycosaminoglycan staining whereas immunohistochemistry was used to obtain specific collagen II staining. (Scale bar = 100 μ m).

5.3.2 Hypoxia promotes chondrogenic potential of bMSCs and endogenous expression of miR-210.

We next determined whether miR-210 induction by hypoxia was affected by culturing cells in growth or differentiation media. As observed in **Figure 5.2.A** endogenous miR-210 expression is induced in a similar manner in both growth and differentiation media. Chondrogenic markers *COL2A1* and *SOX9* were also induced in both growth and differentiation media but only in hypoxic conditions. Also, proliferation marker *MKI67* was significantly induced in hypoxia. However, *ACAN* was only induced in chondrogenic differentiation media and enhanced in low oxygen cultures. In contrast, collagen type I (*COL1A1*) was significantly down regulated in hypoxia when cultured in either medium. **Figure 5.2.B** shows histological staining of bMSCs cultured during 21 days. Alcian blue and *COL2A1* specific staining displayed that chondrogenic media and hypoxic cultures increase the chondrogenic potential of bMSCs. Hence, the most abundant proteoglycan deposition and cartilaginous marker expression was found in cells cultured in the chondrogenic differentiation cocktail in low oxygen conditions.

Figure S.5.2. RT-qPCR analysis depicts miR-210 expression of bMSCs transfected with miR-210 mimic **(A)** or inhibitor **(B)** at day 3 after transfection. Values are represented as relative transcript compared to *AKT1* (housekeeping gene) $\pm$ standard error of the mean. Three biological replicates (n=3) and three technical replicates were used. Statistical analysis is represented as * for $p < 0.05$, ** for $p < 0.01$ and *** for $p < 0.001$.

5.3.3 miR-210 overexpression enhances expression of chondrogenic markers in a normoxic environment.

To assess the role of miR-210 in chondrogenic differentiation of bMSCs, a gain of function analysis was performed in cells cultured in normoxia. First, a dose response experiment was performed to identify the correct stimulatory dose of the miR-210 mimic. Different doses from 5 μ M to 50 μ M were utilized and compared (**Figure 5.5.2**). Functional experiments were performed using 20 μ M of miR-210 mimic and 10 μ M of miR-210 inhibitor. 24 hours after transfection bMSCs were cultured in high-density pellets for up to 21 days. Gene expression analysis was completed during day 1, day 3 and day 7 after transfection for miRNA analysis and day 7 for mRNA analysis.

As observed in **Figure 5.3.A**, miR-210 expression was highly induced by the mimic when compared to negative control during all time-course. A 100-fold up-regulation on day 1 and day 3 was observed. Moreover, miR-210 mimic enhanced standard cartilaginous makers at day 7. *COL2A1*, *ACAN* and *HAPLN1* increased following the induction of miR-210 while *COL1A1* expression was down-regulated (**Figure 5.3.B**). *COL2A1/COL1A1* ratio suggested the importance of miR-210 and the presence for collagen type II expression (**Figure 5.3.C**). Additionally, histological analysis performed at day 21 after transfection, shows an increase in proteoglycan deposition observed by Alcian blue staining in **Figure 5.3.D**, and collagen type II enhanced staining in cells transfected with the miR-210 mimic whereas collagen type I expression was decreased (**Figure 5.3.E**). Moreover, a regulatory feedback loop was observed between HIF1A and miR-210. An enhanced expression of miR-210 promoted the expression of hypoxia inducible factor HIF1A at the mRNA and protein level (**Figure 5.3.F**). Thus, miR-210 enhances standard cartilaginous markers in bMSCs cultured under regular oxygen conditions and positively regulated HIF1A expression.

Figure 5.3. Gain of function analysis was performed on bMSCs at day 7. **(A)** miR-210 was up-regulated throughout the time course of the experiment. **(B)** Chondrogenic markers were enhanced by miR-210 exogenous expression whereas *COL1A1* decreased. **(C)** *COL2A1/COL1A1* ratio demonstrated the importance of miR-210 for promoting *COL2A1* expression. Values are represented as relative transcript compared to *AKT1* (housekeeping gene) $\pm$ standard error of the mean. Three biological replicates ($n=3$) and three technical replicate were used. Statistical analysis is represented as * for $p<0.05$, ** for $p<0.01$ and *** for $p<0.001$. **(D and E)** Histological analysis revealed enhanced proteoglycan deposition and collagen type II synthesis in cells transfected with miR-210 whilst collagen type I synthesis decreased. (Scale bar = 100 μ m). **(F)** Hypoxia inducible factor HIF1A was also induced in a miR-210 dependent manner at the mRNA and protein level.

5.3.4 miR-210 inhibition decreases chondrogenic differentiation of MSCs.

We next performed loss of function experiments of miR 210 in bMSCs cultured in hypoxia. After dose response experiments, 10 μ M of miR-210 inhibitor was selected as the maximal stimulatory dose (see **Figure S.5.2.B**). Loss of function experiments was performed by transfecting cells in monolayer cultures after hypoxic exposure. After 24 hours, high-density pellets were formed as previously described. MiRNA gene expression analysis was completed on day 1, day 3 and day 7 after transfection whereas mRNA expression analysis was performed at day 7 after transfection. **Figure 5.4.A** shows miR-210 expression increased throughout the time course in cells transfected with the negative control. In contrast, cells transfected with the miR-210 inhibitor had significantly lower expression of miR-210 (17-fold decrease at day 7). After miR-210 inhibition was confirmed, expression of chondrogenic markers was evaluated at day 7. As observed in **Figure 5.4.B**, chondrogenic markers *COL2A1*, *ACAN* and *HAPLN1* decreased in cells transfected with miR-210 inhibitor, suggesting a potentiating role of miR-210 on the expression of these genes.

Contrarily, *COL1A1* expression was enhanced 4-fold in cells transfected with inhibitor compared to negative control miRNA dramatically impacting the *COL2A1*/*COL1A1* ratio (**Figure 5.4.C**). Histological analysis shows that inhibition of endogenous miR-210 expression prevents proteoglycan deposition and collagen types II and I protein synthesis (**Figure 5.4.D and E**) pointing to discordance between the inhibitory effect on *COL1A1* gene expression and protein expression. In addition, HIF1A was slightly increased at the mRNA and protein level when miR-210 activity was blocked, suggesting a negative regulatory loop between HIF1A and miR-210 (**Figure 5.4.F**). Consequently, gain and loss of functions experiments demonstrated the relevance of miR-210 activity for chondrogenic potential of MSCs.

Figure 5.4. Loss of function experiments were performed by transfecting cells in monolayer cultures after hypoxic exposure. **(A)** miR-210 expression increased throughout the time course in cells transfected with the negative control miRNA whereas cells transfected with the miR-210 inhibitor had significantly lower expression of miR-210. **(B and C)** Chondrogenic markers *COL2A1*, *ACAN* and *HAPLN1* decreased in cells transfected with miR-210 inhibitor whilst *COL1A1* expression was up-regulated at day 7. Values are represented as relative transcript compared to *AKT1* (housekeeping gene) $\pm$ standard error of the mean. Three biological replicates ($n=3$) and three technical replicates were used. Statistical analysis is represented as * for $p < 0.05$, ** for $p < 0.01$ and *** for $p < 0.001$. **(D and E)** Histological analysis shows that inhibition of endogenous miR-210 expression prevents proteoglycan deposition and collagen types II and I synthesis. (Scale bar = 100 μ m). **(F)** HIF1A was slightly increased at the mRNA and protein levels when miR-210 activity was blocked.

Previous results suggested that miR-210 is an important hypoxia regulatory factor promoting chondrogenesis of bMSCs in culture by increasing the

COL2A1/COL1A1 mRNA ratio and proteoglycan deposition. Interestingly, upregulation of miR-210 appears sufficient to stimulate the expression of certain cartilage markers, such as *COL2A1* and *ACAN*.

5.3.5 miR-210 and TGFβ1 interaction restricts chondrogenic differentiation of bMSCs.

Previously, we have speculated that the TGFβ signaling pathway is one of the likely targets of miR-210 (**Chapter 4**). Since TGFβ is an essential component in the chondrogenic cocktail for induction of chondrocyte differentiation in bMSC cultures, we therefore set out to explore the interaction between miR-210 and TGFβ1 in more detail. Transfection of cells using miR-210 mimic or inhibitor was applied in monolayer culture, after which high-density pellets were formed. Throughout the time course cells were maintained in chondrogenic differentiation media containing TGFβ1. For miR-210 overexpression experiments cells were cultured in normoxia while for loss of function cells were cultured in hypoxia. **Figure 5.5.A** shows miR-210 expression along the time course of the experiments. In normoxia cultures, miR-210 was significantly enhanced in cells transfected with the miR-210 mimic over a one-week time span compared to the negative control miRNA. In addition, hypoxic cultures showed a decrease in miR-210 expression in cells transfected with the inhibitor compared to negative control miRNA. Chondrogenic differentiation potential was also analyzed. Both *COL2A1* and *ACAN* expression followed miR-210 activity.

In cells transfected with the mimic, both chondrogenic markers increased more than 2-fold whereas in miR-210 inhibited cells they decreased (**Figure 5.5.B**). In contrast, *COL1A1* gene expression increased in the absence of miR-210. Chondrogenically differentiated cells cultured in hypoxia significantly increased the *COL2A1/COL1A1* ratio. Remarkably, histological analysis at day 21 suggested a clear interaction of TGFβ1 and miR-210. The inhibition of miR-210 in hypoxic cultures decreased both *COL2A1* and *COL1A1* expression (**Figure 5.5.C**). In

addition, protein analysis using western blotting at day 3 shows *COL2A1* expression follows miR-210 induction or inhibition (**Figure 5.6.B and C**).

Figure 5.5. (A) miR-210 endogenous expression was overexpressed or inhibited in the presence of chondrogenic differentiation medium containing TGFβ1. (B) RT-qPCR analysis showed chondrogenic markers *COL2A1* and *ACAN* followed the expression of miR-210 at day 7. In contrast, *COL1A1* expression opposed miR-210 activity. *COL2A1/COL1A1* ratio revealed a delayed in chondrogenesis in miR-210 overexpressed MSCs whilst best chondrogenesis was observed in cells cultured in hypoxia and induced with TGFβ1. Values are represented as relative transcript compared to *AKT1* (housekeeping gene) ± standard error of the mean. Three biological replicates (n=3) and three technical replicates were used. Statistical analysis is represented as * for $p<0.05$, ** for $p<0.01$ and *** for $p<0.001$. (C) Histological analysis exposed a delay in chondrogenesis of MSCs when they are exposed to both miR-210 mimic and TGFβ1. (Scale bar = 100 μm).

Moreover, HIF1A expression was induced by miR-210 overexpression and blocked during miR-210 inhibition at the mRNA and protein level (**Figure 5.6**). Comparison between cells cultured in growth media (**Figure 5.3.B and C**) and cells in chondrogenic media (**Figure 5.5.B**) showed a better potential of miR-210 mimic to enhance *COL2A1*/*COL1A1* ratio, and proteoglycan and collagen type II deposition in cells expanded in growth media and overexpressing miR-210. For chondrogenesis purposes, hypoxia and miR-210 might be a better regulatory mechanism to enhance cartilaginous markers whilst preventing hypertrophic differentiation on the cells than TGF β 1.

Figure 5.6. (A) RT-qPCR gene expression analysis of *HIF1A* at day 7. Values are represented as relative transcript compared to *AKT1* (housekeeping gene) $\pm$ standard error of the mean. Three biological replicates ($n=3$) and three technical replicate were used. Statistical analysis is represented as * for $p<0.05$, ** for $p<0.01$ and *** for $p<0.001$. **(B and C)** HIF1A protein levels were induced or inhibited following miR-210 expression.

5.3.6 miR-210 promotes chondrogenesis of bMSCs similarly to TGFβ1 in hypoxic conditions, but interaction of both factors increases hypertrophic differentiation of the cells.

Transfection of miR-210 mimic further increased miR-210 expression in cells cultured in growth media under hypoxic conditions (**Figure 5.7.A**). Gene expression analysis was performed on standard cartilage markers *COL2A1*, *ACAN* and *HAPLN1* to analyze chondrogenesis on the cells. Both *HAPLN1* and *ACAN* expression increased in cells transfected with the miR-210 mimic, whereas a slight decrease in *COL2A1* was observed (**Figure 5.7.B**). In addition, miR-210 mimic without the addition of chondrogenic media, significantly increased *COL2A1* protein expression at day 3. (**Figure 5.7.D**). *COL1A1* expression decreased in cells transfected with miRNA-210 mimic while it increased in cells containing both mimic and TGFβ1. The change in *COL2A1*/*COL1A1* ratio exposed how overexpression of miR-210 in cells can promote chondrogenesis of MSCs similarly to the chondrogenic differentiation cocktail. However, miR-210 overexpression seems to enhance collagen type X expression. *HIF1A* mRNA levels were not regulated by the miR-210 mimic nor by TGFβ1 induction. In contrast, the addition of chondrogenic media increased HIF1A protein content but this expression decreased when miR-210 mimic was added (**Figure 5.7.C and D**). Therefore, miR-210 promotes chondrogenesis on bMSCs in a similar way as cells cultured in chondrogenic differentiation cocktail and hypoxia. The addition of chondrogenic cocktail to cells overexpressing miR-210 decreases the synthesis and deposition of important chondrogenic proteins such as *ACAN* and *HAPLN1* while increasing fibroblastic marker collagen type I, suggesting that the beneficial effects of miR-210 are dose-dependent.

Figure 5.7. Gene expression analysis was performed 7 days after transfection. **(A)** miR-210 expression was overexpressed in the presence of growth media or chondrogenic differentiation media containing TGF β 1 in hypoxia. **(B)** Both *HAPLN1* and *ACAN* decreased in cells transfected with miR-210 mimic whereas a slight decrease in *COL2A1* was observed in chondrogenic-differentiated cells. Interestingly, *COL1A1* expression decreased in chondrogenic differentiated cells transfected with negative control miRNA while it increased in cells containing both mimic and TGF β 1. Collagen type X increased with the addition of miR-210 mimic. *COL2A1/COL1A1* ratio exposed how overexpression of miR-210 in cells can promote chondrogenesis of MSCs similarly to the chondrogenic differentiation cocktail. Values are represented as relative transcript compared to *AKT1* (housekeeping gene) $\pm$ standard error of the mean. Three biological replicates (n=3) and three technical replicates were used. Statistical analysis is represented as * for $p < 0.05$, ** for $p < 0.01$ and *** for $p < 0.001$. **(C and D)** *HIF1A* mRNA levels were not regulated by the miR-210 mimic nor TGF β 1 induction. In contrast, the addition of chondrogenic media increased *HIF1A* protein content but this expression decreased when miR-210 exogenous expression increased.

5.4 DISCUSSION

Formation of cartilaginous tissues with sufficient collagen type II and proteoglycans is essential to reconstruct cartilage with restored functions. However, this is still difficult to accomplish with current tissue engineering methods. Engineered cartilage using MSCs may become hypertrophic resulting in the production of bone-like or fibrotic tissues once implanted [9]. The physiological conditions in the articular cartilage are very complex, consisting of low oxygen environment and repeated mechanical loading. Pre-programming the cells in the cartilage native physiological conditions may be helpful for enhancing the chondrogenic phenotype while preparing the cells for implantation. Not a lot of literature was found on tissue engineering applications using low oxygen tension. Although some biomaterials used in cartilage regeneration strategies mimic a hypoxic microenvironment for the cells, the cells are still subjected to normal oxygen levels in culture. The equipment required to maintain the cells in a hypoxic microenvironment is often expensive and space restrictive. As a consequence, it would be interesting to perform *in vitro* chondrogenic differentiation of MSCs using a regulatory mechanism that would mimic the hypoxic response on the cells without the need of low oxygen cultures.

miRNAs are involved in the hypoxic response and contribute to the repression of specific genes under low oxygen conditions [10-13]. Several miRNAs are induced under hypoxic conditions (e.g. miR-210, -21, -23, -24, -26, -103/107, and -181) [14] and have been implicated in various cellular and molecular processes, such as cell growth arrest, stem cell survival, mitochondrial metabolism, DNA repair, angiogenesis and osteoblastic and adipogenic differentiation [15]. MicroRNAseq analysis performed on bMSCs and aMSCs cultured over one week in monolayer and exposed to normoxia or hypoxia conditions, revealed miR-210 as the only robustly inducible miRNA in hypoxia with a fold change of at least 25. miR-210 has been established as the only hypoxic induced miRNA agreed on by all the studies to date [12]. Yet, miR-210 regulation of MSCs chondrogenesis has not

been proven. When compared directly, the absolute levels of miR-210 induced by hypoxia are largely similar across most cells [7]. However, in our studies, a two-fold change difference of miR-210 expression in bMSCs compared to miR-210 expression in aMSCs suggested why bMSCs could be better at promoting chondrogenesis than aMSCs. Remarkably, exposure of cultured cells to low pH, oxidant stress, growth factor deprivation, and osmotic stress to mimic hypoxia-associated processes does not induce miR-210 expression [16]. To describe the regulatory mechanism of miR-210 during chondrogenesis of bMSCs, its expression was regulated using a miR-210 specific mimic or an inhibitor. miR-210 mimic increased expression up to 100-fold and inhibitor decreased it by 17-fold. This exogenous expression of miR-210 was conserved in culture for at least 1 week. Functional experiments revealed miR-210 importance for early chondrogenesis of MSCs during *in vitro* cultures. Interestingly, exogenous induction of miR-210 enhanced chondrogenesis of bMSCs as good as TGFβ1 induction under low oxygen cultures based on the level of expression of various chondrogenic marker genes and (immuno)histological analysis. However, the addition of chondrogenic cocktail to cells overexpressing miR-210 decreases the synthesis and deposition of important chondrogenic proteins, such as *ACAN* and *HAPLN1*, while increasing fibroblastic marker collagen type I. A compensatory mechanism seems to keep collagen type I expression in bMSCs in cells containing chondrogenic cocktail, despite the changes in miR-210 and collagen type II levels. In cartilage, TGFβ1 controls the production of extracellular matrix (ECM) by increasing the synthesis of proteoglycans, collagens and fibronectin. However, it also exerts inflammatory responses and osteophyte formation if present in the joint for too long. Despite the fact that TGFβ1 increases cellular proliferation and causes earlier and extensive deposition of collagen type II and greater accumulation of proteoglycans, it also induces gene expression of degrading enzymes such as MMPs and collagen type X. An increase in angiogenic markers expression could start a pro-inflammatory response in the cells promoting the

up-regulation of hypertrophic and early mineralization markers over cartilaginous genes [17].

Figure 5.8. Mechanistic model of chondrogenic differentiation via miR-210 regulation in bMSCs. **(A)** TGFβ1 induces hypertrophic cartilage formation of MSCs in normoxia by increasing standard cartilaginous markers but also promoting hypertrophic markers. **(B)** Hypoxia induction of endogenous miR-210 programs MSCs to express cartilaginous markers *COL2A1* and *ACAN* but also increases *COL1A1*, which could result in fibrocartilage formation. **(C)** Exogenous induction of miR210 using miRNA mimic induces standard cartilaginous markers whilst inhibiting hypertrophic/fibroblastic markers promoting MSCs to form hyaline cartilage. In contrast, induction of exogenous levels of miR-210 in the presence of TGFβ1 decreases chondrogenesis of MSCs reducing expression of cartilage markers and increasing *COL1A1* expression. Up-regulated genes are indicated with red whereas down-regulated genes are indicated in blue.

Additionally, multiple groups have found that miR-210 is specifically induced by HIF1A [16]. Although *HIF1A* gene expression is primarily triggered by changes in oxygen concentration, it is also modulated by several other pathways, including reactive oxygen species and the phosphatidylinositol 3-kinase (PI3K)/Akt

pathway [18]. Conclusively, **Figure 5.8** describes the mechanistic model of miR-210 regulation of chondrogenesis in MSCs. Chondrogenic differentiation cocktail containing TGFβ1 in standard oxygen cultures enhances chondrogenesis of MSCs evidenced by increased proteoglycan deposition and cartilaginous markers expression. However, it also promotes hypertrophic markers (**Figure 5.8.A**). Hypoxia induction of endogenous miR-210 programs bMSCs to express cartilaginous markers *COL2A1* and *ACAN* but increases *COL1A1*, which could result in fibrocartilage formation (**Figure 5.8.B**). Exogenous induction of miR-210 using miRNA mimic induces standard cartilaginous markers whilst inhibiting hypertrophic/fibroblastic markers promoting bMSCs to form hyaline cartilage. In contrast, induction of exogenous levels of miR-210 in the presence of TGFβ1 decreases chondrogenesis of bMSCs reducing expression of cartilage markers and increasing *COL1A1* expression (**Figure 5.8.C**).

The major limitation of the present study is the use of serum containing media (growth media) versus the serum-free media with TGFβ1 (chondrogenic media). Previously, some studies have described the benefits of the presence of FBS for chondrogenesis of bMSCs [19]. However, this chondrogenic potential was inconsistent and varied from batch to batch. In contrast, several studies have described the benefits of serum-free media conditions to enhance and promote higher expression of glycosaminoglycans and collagen type II [20] when compared to serum based cultures. In the present study, we observed how MSCs in the presence of serum (e.g FBS or PL) and under hypoxia expressed *SOX9*, *ACAN* and *COL2A1*. Remarkably, histological analysis showed that a proper chondrogenic induction and collagen type II deposition in MSCs requires the presence of TGFβ1 and hypoxia. Furthermore, the high chondrogenic potential observed under this culture condition (e.g. hypoxia + TGFβ1) was also achieved by overexpression of miR-210 even in cultures containing FBS or platelet lysate (data not shown) and under standard oxygen conditions (e.g. normoxia). Future studies will elucidate if the use of serum-free conditions and miR-210 will further

induce chondrogenic differentiation of bMSCs more abundantly than under serum containing cultures.

Therefore, miR-210 exogenous induction can be used instead of chondrogenic differentiation cocktail in standard or low oxygen cultures to promote chondrogenesis of bMSCs while inhibiting their hypertrophic differentiation. In this study, we propose that hypoxia stimulated chondrogenesis of MSCs is mainly driven by upregulation of miR-210. From our results, we conclude that miR-210 in combination with hypoxic conditions enhanced *in vitro* chondrogenesis of bMSCs in comparison with chondrogenic differentiation cocktail containing TGFβ1 in the cells. Both standard cartilage markers and the *COL2A1*/*COL1A1* ratio was increased when miR-210 mimic was added to the cells. Remarkably, the use of miR-210 mimic does not present negative side effects, such as increase in *COL1A1* expression and induction of hypertrophic markers, as compared to TGFβ1. This potential treatment can help in regeneration of hyaline cartilage preventing formation of fibrocartilage *in vivo*.

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CHAPTER 6

6. Human synovial fluid assists human primary chondrocytes and mesenchymal stem cells chondrogenic differentiation during culture expansion.

ABSTRACT

Synovial fluid (SF) is a biological fluid that covers the synovial joints and provides metabolic supply to the chondrocytes in the native cartilage tissue. Moreover, it maintains lubrication and shock absorption properties due to its mechanical properties. The lubrication and elasticity of this tissue is derived from its rich composition in hyaluronan and lubricin. This fluid is highly examined to study pathophysiological changes during osteoarthritis. Our purpose was to determine the usability of synovial fluid as a human based product for *in vitro* expansion, culture and chondrogenic re-differentiation of human chondrocytes and stem cells for clinical applications in cartilage regeneration. Metabolic activity assays on primary chondrocytes and adipose-derived stromal stem cells (aMSCs), showed both cell types survived and proliferated during culture with synovial fluid. However, different effects were observed. aMSC preferred SF samples with higher protein content whereas primary chondrocyte viability was increased with lower protein content samples. Primary chondrocyte proteoglycan deposition was maintained in cell cultures containing synovial fluid. Synovial fluid seems to promote chondrogenic differentiation of aMSC in the presence of chondrogenic differentiation medium, which was confirmed by positive collagen type II immunohistochemistry. Our results serve as an initial screening of the possibilities of SF to replace fetal bovine serum as a culture supplement for *in vitro* expansion of human primary chondrocytes and aMSCs. Thus, synovial fluid is a promising non-zoonotic and animal free alternative for culture expansion of chondrocytes and stem cells for cartilage repair strategies.

6.1 INTRODUCTION

The development of cell-based therapies to improve cartilage repair has received considerable interest in recent years. Cells need exposure to a wide variety of stimuli to mimic *in vivo* conditions. Attempts to identify the required stimuli to drive the formation of permanent articular cartilage have been largely unsuccessful to date. Synovial fluid (SF) is a viscous, mucinous biological fluid that covers the synovial joints and it's located between the articular cartilage and the synovial membrane [1]. Due to the avascular nature of cartilage, this tissue does not obtain its metabolic supply from the blood vessels but from the synovial fluid. Thus, its principal function is to provide enough metabolic supply (e.g. oxygen and nutrients) to the chondrocytes in the cartilage. In physiological conditions during movement, synovial fluid maintains the lubrication of the cartilage surface in articular joints, allowing bones to freely articulate by friction reduction and shock absorption [2]. The mechanical properties of the synovial fluid are derived from the actively secreted substances: hyaluronic acid, lubricin, proteinases, collagenases and prostaglandins that lubricate and increase the elasticity in the joint. Although chondrocytes in cartilage constantly produce hyaluronan and proteoglycans, they produce it at a low level [3]. Hyaluronan is one of the principal components of SF [4, 5].

Currently, various clinical trials inject stem cells into the knee joint with the hope of promoting healing of osteochondral defects and osteoarthritis [6]. For stem cell therapies, the study of the interaction of stem cells with the joint microenvironment is essential to understand cell viability and differentiation potential in physiological conditions. Moreover, is important to determine if synovial fluid exerts negative or positive effects on these cells. Due to its location and the ease of collection [7], synovial fluid has been examined to study changes in the joint microenvironment [8]. However, there are no studies about the effects of the synovial fluid to the injected stem cells. The purpose of this study aimed to determine the usability of synovial fluid as a human derived product for

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in vitro expansion, culture and differentiation of human chondrocytes and human adipose-derived stem cells for clinical applications.

6.2 MATERIALS AND METHODS

6.2.1 Collection of fluid.

Synovial fluid specimens were obtained from patients undergoing knee replacement surgery or knee drainage as therapy for flare-ups of joint disease, with approval from the Mayo Clinic Institutional Review Board (IRB) (IRB #14-000440). Synovial fluid was collected using knee aspiration. In total synovial fluid of 54 patients was collected and analyzed in this study. For all donors, the stage of disease was classified in accordance with the Kellgren and Lawrence classification system of knee osteoarthritis. Grading was determined by visible cartilage lesions seen with radiographic imaging and was grouped into healthy (KL 0), mild (KL grade 1-2), moderate (KL grade 3) and severe (KL grade 4). After biological samples were collected in the procedure room, they were centrifuged at 12000 rpm for 10 minutes and the supernatant was aliquoted into sterile tubes. All samples were snap frozen and stored at -80°C until analysis.

6.2.2 Specimen analysis.

Physical and chemical evaluation was performed on the samples including the recording of the aspirated volume, the color and clarity. Synovial fluid samples were classified as previously described based on their appearance (color and clarity) [9]. For chemical examination protein concentration was determined using DC™ Protein Assay (Biorad, Hercules, USA) following the manufacturer's instructions.

6.2.3 Cell harvest and monolayer culture.

Two different cell types were used for these studies. Adipose derived mesenchymal stem cells (aMSCs) were obtained from lipo-aspirates from three representative healthy donors as described by [10]. Human primary

chondrocytes were obtained from the calcaneal apophysis of consenting donors. Studies with both cell types were approved by the Mayo Clinic Institutional Review Board (IRB). Both cell types were expanded in growth media. Growth media for aMSCs was composed of Advanced MEM (Gibco/Thermo Fisher Scientific, Waltham, MA) supplemented with 5% human platelet lysate (PL-Max, MillCreek Life Sciences, Rochester, MN), 1% penicillin/streptomycin, 1% Glutamax (Gibco/Thermo Fisher Scientific) and 0.2% heparin (Baxter, Deerfield, IL). Passage 4 human primary chondrocytes were cultured in maintenance medium that contained advanced MEM (Gibco/Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS, Atlanta Biologicals, Atlanta, GA, Atlanta, GA) and 1% penicillin/streptomycin (Gibco/Thermo Fisher Scientific). Cells were cultured using standard procedures in T-175 cm² flasks at 37 °C, 95% humidity and 5% CO₂ until they reached 80% confluence. Prior to each experiment, cells were detached from T175 flasks by trypsinization using TrypLE Express (Gibco/Thermo Fisher Scientific).

6.2.4 Proliferation and viability assays

For viability assays, both aMSCs and chondrocytes were cultured in 96 well-plates at a density of 3,000 cells/cm². Proliferating cells were used for analysis. Synovial fluid samples with a protein content of 10µg/µL were used as stock solution for further dilutions. Cells were grown in a volume per volume or two-fold dilutions series of the synovial fluid stock sample diluted in culture media. The dilutions used were: 10 µg/µl, 5 µg/µl, 2.5 µg/µl, 1.25 µg/µl, 0.63 µg/µl, 0.3 µg/µl and 0.15 µg/µl or volume per volume dilutions of 1/2, 1/5, 1/10, 1/20, 1/40, 1/80 and 1/160. Fetal bovine serum (FBS) or platelet lysate were used as a control. Growth media was replaced with advanced MEM media containing different dilutions of SF, 5% platelet lysate (PL) or 10% FBS, and cultured for 24hrs. To examine if the PBS content in the diluted samples affects the metabolic activity of chondrocytes, proliferative and confluent cells were analyzed using

two-fold dilutions of synovial fluid as described before. Metabolic activity was determined using the CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (MTS) (Promega, Madison, USA). Optimal culture concentration of synovial fluid for each cell type was deducted from these experiments.

For proliferation rate and growth curve analysis, cells were seeded in 6 well-plates at a density of 5,000 cells/cm². For this set of experiments, the 10 most stimulatory and the 10 most inhibitory synovial fluid samples for each cell type were used. Growth media was replaced with advanced MEM media containing 5% SF, 5% PL or 10% FBS, and cultured for 7 days. For cell quantification, live staining was performed using LIVE/DEAD viability/cytotoxicity kit for mammalian cells (ThermoFisher Scientific, Waltham MA, USA). Cell counting was performed each day from day 1 until day 7. Pictures were taken from 3 different parts of each well and representative images were examined.

6.2.5 Chondrogenic differentiation potential

High-density cellular aggregates were generated using pellet culture in accordance with a protocol published previously [11]. To form the pellets, 250,000 cells were seeded in 96 round-bottom well plates and then pellet formation was induced by centrifugation. Pellets were cultured at 37°C, 95% humidity, and 5% CO₂ until cells coalesced. After 1 day, pellets were either maintained in growth media or chondrogenic media supplemented with 0.5µg/µL of SF from samples of either the low, medium or high protein group, or 10% FBS or 5% PL as a control. Chondrogenic media consisted of advanced MEM containing 40 mg/ml of L-proline (Sigma-Aldrich, Saint-Louis, MO), 50 mg/ml Insulin Transferrin Selenium-premix (Gibco/Thermo Fisher Scientific), 50 mg/ml of ascorbic acid (Sigma-Aldrich), 10 ng/ml of TGF-β1 and 0.1 µM dexamethasone (Sigma-Aldrich). Pellets were maintained for up to 21 days in hypoxic (2% oxygen) conditions using an integrated system with a cell culture hood and an

incubator accessible through a gas-lock (I-Glove, BioSpherix, New York, NY). Cell culture medium was replaced every three days.

6.2.6 Gene expression analysis

For pellet culture, at least 4 pellets were pooled from each condition to obtain sufficient RNA. Pooled pellets from each time point were lysed using an 18-gauge needle and Qiazol lysis buffer (Qiagen, Hilden, Germany) to yield an RNA sample for one biological replicate. Three different biological replicates for each experimental condition were used concurrently for RNA isolation. Total RNA was isolated using the miRNeasy Micro Kit (Qiagen, Hilden, Germany) following the instructions of the manufacturer. RNA concentrations and purity levels were measured using a NanoDrop (Thermo Fisher Scientific). For baseline gene expression values, lysates were obtained prior to plating cells in pellet culture on day 0 (D0: plating day) and 7 days after culture in high-density pellets. 1 µg of isolated RNA was used as a template for reverse transcription using M-MLV Reverse Transcriptase (Promega, Madison, USA). Gene expression was quantified using quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR) with 10 ng cDNA per 10 µL with QuantiTect SYBR Green PCR Kit (Qiagen) and the CFX384 Real-Time System (BioRad, Hercules, CA). Relative transcript abundance of genes of interest was normalized to *AKT1* expression using the $2^{-\Delta\Delta Ct}$ method. Representative chondrogenic genes (e.g *COL2A1*, *ACAN* and *SOX9*) and proliferative marker *MKI67* were used.

6.2.7 Histological Analysis

Cell pellets were collected after 21 days in culture and fixed overnight in 10% neutral buffered formalin. Samples were then washed and dehydrated in graded series of ethanol (70% to 100%) and processed with xylene (50% to 100%) prior to paraffin embedding. Paraffin blocks were cut into consecutive sections of 5 µm thickness using a microtome and placed onto charged microscope glass slides for histological staining. Following deparaffinization, sections were stained for

glycosaminoglycan content using 0.5% (wt/vol) Alcian Blue 8GX dye (Sigma-Aldrich) and counterstained with Nuclear Fast Red (Sigma-Aldrich). Collagen type II immunohistochemistry (IHC) was also performed. After removal of paraffin and rehydration, sections were washed with distilled water. Antigen retrieval was performed using 1 mg/mL pronase for 20 minutes at 37°C followed by 1 mg/mL collagenase for 60 minutes at 37°C, both in a humidified chamber. Briefly, protein block was applied using 1x TBS 0.1% Tween-20 buffer after which slides were incubated overnight with 1 µg/mL of anti-Collagen Type II mouse monoclonal Antibody (Millipore, Darmstadt, Germany) or anti-IgG mouse monoclonal Antibody (Biosciences, Osage, IA) as a control at 4°C in a humidified chamber. Slides were sequentially incubated with biotinylated anti-goat IgG secondary antibody and were then treated with 3,3'-diaminobenzidine (DAB) enhanced liquid substrate system for IHC (Sigma-Aldrich) for 10 minutes until color development. Stained sections were analyzed using a ZEISS Axio inverted light microscope.

6.2.8 Cytokine analysis.

Human interleukin 10 (IL-10), interleukin 6 (IL-6), interleukin 1-β (IL-1β) and tumor necrosis factor alpha (TNFα) concentrations were measured with a human enzyme-linked immunosorbent assay (ELISA) kits (R&D, Minneapolis, MN, USA), according to the manufacturer's instructions. Synovial fluid samples were diluted according to the manufacturers' instructions. The electrochemiluminescence was measured using a Nanospectrometer. Given the nonparametric nature of the data, the cytokines concentrations were Log2 transformed.

6.2.9 Statistical analysis.

Quantitative data for gene expression profiles were presented as mean ± standard deviation from 3 independent donors for each experimental condition and time point. Statistical analysis was performed using JMP® Pro v10.0.0 (SAS Institute), and *p* values were determined with the non-parametric comparison

for all pairs using Steel-Dwass Method. Significance is noted in the figures when applicable as * for $p\text{-value} < 0.05$ and ** for $p\text{-value} < 0.01$.

6.3 RESULTS

6.3.1 Patient cohort

The current analysis included synovial fluid samples from knees from 54 patients undergoing knee replacement surgery or knee drainage to treat flare-ups of joint disease. **Table 6.1** summarized all analysis conducted on the 54 collected samples. In **Table 6.2** the samples from this cohort were categorized according to their OA grade, 2 patients (3.70%) had no visible radiological features of OA (G0), 14 patients (25.92%) had mild OA (G1/2), 18 patients (33.33%) had moderate OA (G3) and 20 patients (37.04%) had severe OA (G4). Age and gender were described for each group. Thirty (56.00%) women and twenty-four (44.00%) men were included in the study. The mean $\pm$ SD age of patients was 62.3 ± 13.6 years (range, 21-87 years). Mean age was observed to increase with KL score. In general, no predominant knee was found in patients, 25 samples were collected from the right knee whereas 25 were collected from the left knee.

6.3.2 Sample characterization

Physical examination was performed on the samples. The mean volume was 22 mL with a range from 4 to 115 mL. Color and clarity of each sample was observed and recorded. Samples were categorized in 4 groups; transparent samples were categorized as normal, light yellow samples as non-inflammatory, dark yellow and cloudy samples as inflammatory whereas red/brown samples as hemorrhagic. None of the samples were colorless and clear or classified as normal SF, 31 samples were light yellow and clear and classified as non-inflammatory OA, 20 samples were dark yellow and cloudy and classified as inflammatory OA and 2 samples were red/brown and classified as septic or hemorrhagic samples (**Figure 6.1**).

Table 6.1 Summarized samples characteristics

Donor characteristics				Clinical Parameters			Biochemical Parameters			
Sample ID	Gender	Age (years)	Anatomical Side	Fluid Vol (mL)	Appearance	OA grade	Protein content (ug/μL)	Chondrocytes Viability (abs)	AMSCs Viability (abs)	Average Viability (abs)
1	M	76	R	8	Non-inflammatory	G4	26.91	1.14	1.03	1.08
2	M	76	L	5	Inflammatory	G4	30.57	0.86	0.82	0.84
3	M	77	L	20	Non-inflammatory	G3	41.52	1.22	1.09	1.15
5	F	47	L	5	Inflammatory	G2	70.86	1.01	1.04	1.02
6	M	40	L	16	Inflammatory	G2	22.20	1.11	0.95	1.03
7	F	45	R	12	Inflammatory	G0	75.24	1.07	0.99	1.03
8	M	65	L	16	Non-inflammatory	G3-4	20.58	1.04	0.88	0.96
9	M	55	L	27	Non-inflammatory	G2	27.74	0.93	0.83	0.88
10	M	52	L	8	Non-inflammatory	G2-3	83.76	1.09	1.02	1.06
11	M	52	R	17	Non-inflammatory	G3	46.86	1.01	0.96	0.98
12	F	57	R	4	Non-inflammatory	G3	19.48	1.18	1.03	1.10
13	M	61	L	24	Inflammatory	G4	22.76	1.27	0.94	1.10
14	F	65	L	12	Non-inflammatory	G4	55.16	1.19	0.84	1.02
15	F	78	R	12	Non-inflammatory	G4	56.81	1.19	0.81	1.00
16	F	57	R	12	Non-inflammatory	G3	83.85	1.08	0.91	0.99
17	M	21	R	4	Non-inflammatory	G0	37.91	NA	NA	NA
18	F	80	L	13	Non-inflammatory	G4	48.74	1.38	0.89	1.13
19	M	51	L	9	Inflammatory	G2	59.41	1.06	0.90	0.98
20	M	57	L	48	Inflammatory	G3	40.50	1.22	0.99	1.10
21	M	57	R	50	Inflammatory	G3	46.83	1.05	0.94	0.99
22	F	68	R	11	Non-inflammatory	G2	71.65	1.11	0.90	1.01
23	F	53	L	5	Non-inflammatory	G3	30.60	0.96	0.79	0.88
24	F	85	R	5	Inflammatory	G4	NA	NA	NA	NA
25	F	57	L	40	Inflammatory	G3	104.63	0.96	0.83	0.90
26	F	76	R	5	Non-inflammatory	G2	22.89	0.87	0.71	0.79
27	F	79	L	12	Inflammatory	G4	NA	NA	NA	NA
28	F	64	R	110	Non-inflammatory	G3	41.78	0.85	1.07	0.96
29	F	56	R	28	Inflammatory	G4	27.02	NA	NA	NA
30	F	74	L	14	Non-inflammatory	G4	25.01	1.20	1.03	1.12
31	F	71	R	5	Non-inflammatory	G3	23.78	1.24	0.93	1.09
32	M	64	R	7	Non-inflammatory	G3	26.77	0.83	0.89	0.86
33	M	62	R	34	Inflammatory	G2	34.18	0.83	0.89	0.86
34	M	46	R	8	Inflammatory	G2	26.58	1.02	0.85	0.93
35	M	62	R	58	Inflammatory	G3	44.90	0.70	0.99	0.85
36L	M	77	L	70	Non-inflammatory	G4	63.68	1.08	1.01	1.05
37	M	77	R	115	Non-inflammatory	G4	18.23	0.90	1.13	1.02
38	F	79	L	21	Non-inflammatory	G4	18.53	0.95	1.04	0.99
39	F	79	R	51	Non-inflammatory	G4	22.83	1.21	0.88	1.04
40	F	53	L	32	Non-inflammatory	G1	129.18	0.76	0.96	0.86
41	M	67	L	5	Inflammatory	G4	17.57	0.72	0.77	0.75
42	M	55	R	17	Inflammatory	G4	21.91	0.85	0.86	0.86
43	F	54	L	10	Hemorrhagic	G1	37.96	1.04	1.13	1.08
44	F	87	L	24	Non-inflammatory	G3	26.85	1.19	1.12	1.15
45	F	51	R	10	Non-inflammatory	G2	35.89	0.88	1.15	1.01
46	M	30	L	8	Non-inflammatory	G1	69.20	0.74	1.12	0.93
47	F	67	L	23	Inflammatory	G2	35.00	0.67	1.19	0.93
48	F	72	L	6	Non-inflammatory	G4	78.83	0.87	1.04	0.95
49	F	69	R	32	Non-inflammatory	G4	75.79	1.00	1.00	1.00
50	F	61	R	11	Inflammatory	G2-G3	17.44	0.85	0.99	0.92
51	M	81	L	37	Inflammatory	G4	34.77	1.01	0.93	0.97
52	F	58	R	13	Hemorrhagic	G2	24.60	1.12	1.02	1.07
53	M	56	NA	25	Non-inflammatory	G3	12.05	0.96	1.56	1.26
54	F	51	NA	10	Non-inflammatory	G3	10.78	0.93	1.59	1.26

Note: Viability assays values are presented as absorbance at 490nm relative to media alone.

Also, chemical examination was performed on the samples. An initial quantification of undiluted samples was performed. Protein concentration value mean was 43.39 ± 25.08 with a range from 17.44 to 129.18 $\mu\text{g}/\mu\text{L}$. Accuracy of

quantification was checked by performing a dilution series of each sample. Protein content was determined when linearity of the measurements was observed.

Table 6.2 Patient demographics.

Grade OA	No OA (G0)	Mild OA (G1-2)	Moderate OA (G3)	Severe OA (G4)
#Samples	2	15	17	21
Anatomy (Right, Left)	Knee (2 R, 0 L)	Knee (6 R, 9 L)	Knee (10 R, 7 L)	Knee (8 R, 13 L)
Age, mean (range)	33 (21-45)	54 (30-76)	63 (52-87)	74 (56-85)
Gender (% Female)	60%	57%	54%	61%

After protein content was quantified, SF samples were ranked based on real value protein concentration and categorized according to OA grade. As shown in **Figure 6.1.A** SF protein content was higher in patients with no or mild OA, while samples with a lower protein content were collected from patients with moderate and/or severe OA grades. This suggests that protein content might be associated with disease state with concentration decreasing along OA development. Comparison of fluid volume and samples clarity against OA grade was also performed. Contrarily to protein content, fluid volume of samples increased in samples during OA development **Figure 6.1.B**, whereas samples clarity showed clear differences between the samples. Moderate and severe SF samples presented more non-inflammatory aspect of the fluid; whereas mild OA samples appeared to be more inflammatory and hemorrhagic **Figure 6.1.C**. Samples were normalized to an equal protein concentration using PBS and used for further experimental analysis.

6.3.3 Sample stratification.

Stratification methods were examined to determine a better categorization for further experimentation using MSC and human chondrocyte cultures. Donor characteristics and chemical analysis of the samples were compared among the OA grades. From **Table 6.3**, we observed that slightly more severe OA samples and females were included in the study. Age and volume aspirated increased with OA progression. However, protein concentration decreased with OA progression. The effects of disease state of the synovial fluid samples on the viability of aMSC and chondrocyte were negligible. Protein yield quantification also suggested that protein content increases in OA SF samples compared to no OA samples.

Figure 6.1. Categorization of SF samples according to OA grade to examine protein content **(A)**, fluid volume of samples **(B)** and fluid clarity **(C)**. Results shown in panel A and C are represented as a mosaic plot, results in panel B are represented as median and 95% confidence intervals (CI).

Table 6.3 Samples stratification according to OA classification.

	No OA (G0)	Mild OA (G1-2)	Moderate OA (G3)	Severe OA (G4)
#Samples	2	14	18	20
Age (mean $\pm$ stdev)	33 $\pm$ 16.97	54.14 $\pm$ 11.88	61.87 $\pm$ 9.80	73.83 $\pm$ 7.69
Gender (% Females)	50%	57%	56%	55%
Volume aspirated (mean $\pm$ stdev)	8 $\pm$ 5.7	15.07 $\pm$ 9.86	25.78 $\pm$ 27.05	25.15 $\pm$ 26.93
Protein concentration (mean $\pm$ stdev)	56.57 $\pm$ 26.39	47.67 $\pm$ 29.83	45.31 $\pm$ 25.83	39.14 $\pm$ 20.83
Protein Yield (mg)	527	773	1160	948
Chondro MTS assay (mean $\pm$ stdev)	1.07 $\pm$ 0.11	0.94 $\pm$ 0.15	1.02 $\pm$ 0.17	1.05 $\pm$ 0.15
AMSCs MTS assay (mean $\pm$ stdev)	0.99 $\pm$ 0.21	0.97 $\pm$ 0.14	1.04 $\pm$ 0.09	0.94 $\pm$ 0.09

SF samples were divided in to low (<30 μ g/ μ L), medium (30 to 60 μ g/ μ L) and high (>60 μ g/ μ L) protein groups for future cell culture experiments. Protein content was presented in a Log2 scale (**Figure 6.2**). Fetal bovine serum (FBS) and human platelet lysate (PL) that were used for experimental controls had a protein content of 35 μ g/ μ L and 17.93 μ g/ μ L, respectively. 90% of the samples in the whole set had a protein content higher than PL. Samples with high protein content were around 46% of the whole set (24 samples), medium protein group were 44% (23 samples) and 9.60% (5 samples) in the low protein group. The synovial fluid samples that presented better viability in aMSCs had more protein content and its protein values were divided across the three protein groups (6 to low, 5 to medium and 7 to high protein content group), whereas chondrocytes were more viable in samples with lower protein content (11 samples in low group, 10 in medium group and 7 in high protein content group).

Moreover, synovial fluid protein quantification revealed variability in protein content among the samples per group of 29.20 ± 11.90 , 57.30 ± 9.50 and 91.10 ± 6.30 μ g/ μ L, for the low, medium and high protein group respectively. Although this stratification gives a good amount of information about OA role in SF characterization, the cell's viability difference was not clear. For this, a

stratification of the samples according to MTS viability assay was suggested. MTS results from each cell type were examined in **Table 6.4**.

In **Table 6.4.A** and **Figure 6.3.A** we show that human chondrocytes preferred SF samples with a lower protein yield and advanced OA grades. Interestingly, 50% of the samples improved the viability of the cells compared to media alone. No statistical difference in age or OA grade was found. As observed previously, chondrocytes were more active when protein yield was low. Also, **Table 6.4.B** and **Figure 6.3.B** show aMSCs stratification according to their MTS viability assays. Only 35% of the samples provide better viability than media alone. As validated before, aMSCS preferred samples with higher protein yield. Age at aspiration did not have a role in aMSCs or chondrocytes viability.

Figure 6.2. Protein content division in three different groups: High, medium and low. All SF samples are represented in gray bars whereas control serums, FBS and PL, are represented in black bars.

Table 6.4 Samples stratification according to MTs viability assays.

A Chondrocytes	Viability	<u><0.7</u>	<u>0.7-1.0</u>	<u>1.0-1.4</u>
	#Samples	2	20	27
	Age (mean $\pm$ stdev)	64.5 $\pm$ 3.54	61.4 $\pm$ 11.85	63.41 $\pm$ 13.13
	Gender (%Females)	50%	55%	52%
	Grade OA	Mild-Moderate	Moderate-Severe	Moderate-Severe
	Protein Yield (mg)	1705	1134	837
	Protein content (mean $\pm$ stdev)	44.76 $\pm$ 27.48	42.24 $\pm$ 22.24	11.41 $\pm$ 0.90

B AMSCs	Viability	<u>0.7-1.0</u>	<u>1.0-1.4</u>	<u>>1.4</u>
	#Samples	30	17	2
	Age (mean $\pm$ stdev)	62.1 $\pm$ 10.90	64.64 $\pm$ 14.82	53.5 $\pm$ 3.54
	Gender	47%	65%	50%
	Grade OA	Moderate-Severe	Mild-Moderate	Mild
	Protein Yield (mg)	1027	1034	1560
	Protein content (mean $\pm$ stdev)	35 $\pm$ 0	41.41 $\pm$ 32.02	43.67 $\pm$ 20.83

Note: Values are presented as fold change of viability of SF samples over media alone. <0.7 were inhibitory samples, 0.7-1.0 were neutral samples and >1.0 were stimulatory samples for each cell type.

However, OA grade seems to affect viability of the cells. aMSC were slightly more viable in mild OA samples whereas chondrocytes grew better in moderate and severe OA samples. This suggested that aMSC are more affected by the content of synovial fluid and the OA grade. Further comparison of the similarities and differences in the response of aMSCs and chondrocytes to similar SF samples was performed. 27 samples elicited a different response in aMSCs and chondrocytes whereas 28 samples resulted in a similar response according to MTS cell viability as a read out (samples with MTS viability of more than 1 in both cells, see **Table 6.1**). Also, we found that 16% of all SF samples behaved in a similar manner in both cell types. In this case, samples that provided viability better than media alone for both cells were categorized in the medium and high protein content groups.

6.3.4 Viability of mesenchymal stem cells and chondrocytes in synovial fluid

To investigate if synovial fluid maintains metabolic activity of mesenchymal stem cells and human chondrocytes viability assays and growth curves were performed. These assays were used to determine the optimal synovial fluid concentration for culturing each cell type *in vitro*. For cell culture experiments, all synovial fluid samples were normalized to a protein concentration of 10 µg/µL in PBS. Metabolic activity of human mesenchymal stem cells culture in SF revealed differences between the low, medium and high protein content groups. Cells were grown in a volume per volume (v per v) or two-fold dilutions series of SF samples (**Figure 6.4.A**). Volume per volume was diluted starting from 1/5 dilution until 1/160 in media. Dilutions of 1/2 were added due to the significance in a clinical setting, since stem cells injections for knee therapy are diluted in a 1/2 concentration. Two-fold dilutions were performed starting from the normalized samples with 10 µg/µL concentration and diluted up to 0.3µg/µL in media.

Figure 6.3 Mosaic plot of MTS viability assays of (A) aMSCs and (B) chondrocytes compared to OA grade.

Figure 6.4. Cell viability assays in SF serum of three different groups compared to FBS and PL. **(A)** Volume per volume (black) dilutions and normalized two-fold dilutions (blue). Viability assays on human AMSCS **(B and C)** and human primary chondrocytes **(D and E)**. Data represented as mean $\pm$ STDEV. Three biological replicates (n=3) and three technical replicates were used.

All dilutions were performed using normalized samples to the same concentration to avoid variability due to protein content. Relative metabolic activity to media alone in proliferative aMSC revealed that both two-fold dilutions and v per v had a similar metabolic activity. FBS was the serum that induced more metabolic activity whereas high protein group had an enhanced metabolic activity across all dilutions when compared to medium and low content group (**Figure 6.4.B and C**). Despite the normalization of the samples to

the same protein content, samples from each group interacted in a different manner with aMSC, which suggest the content (e.g. proteins, cytokines, etc) of each group can affect the behavior of the cells. In lower dilutions SF groups were more similar, but differed significantly from FBS and PL. However, in higher dilutions (e.g. $\frac{1}{2}$ and $\frac{1}{5}$) the difference between PL, FBS and SF samples is reduced. In some dilutions, 1 in 10, 1 in 5 and 1 in 2 and $1.25 \mu\text{g}/\mu\text{L}$ and $2.5 \mu\text{g}/\mu\text{L}$, an increase in metabolic activity of the cells in SF compared to PL was observed. By comparing the clinical relevant dilution (e.g. $\frac{1}{2}$) we observed that the majority of samples had a metabolic activity higher than 0.7. Yet, a slight increase in high protein SF compared to other SF groups, FBS and PL was detected.

Metabolic activity of human chondrocyte culture in SF revealed differences between the low, medium and high protein content groups when cultured in volume per volume dilutions (**Figure 6.4.D**). Chondrocytes grew in synovial fluid derived from patients with low and medium protein concentrations showed an enhanced metabolic activity compared to those grown in synovial fluid from patients with high protein concentrations. Along all dilutions, SF with low protein content and FBS were the ones with better metabolic activity whereas PL was the serum with lower metabolic activity. Dilutions between $\frac{1}{20}$ and $\frac{1}{5}$ seemed better to culture chondrocytes. In these dilutions medium and low protein SF samples were better than PL and slightly similar to FBS. By comparing the $\frac{1}{2}$ dilution that is commonly used for stem cell delivery, we observed all SF samples decreased compared to PL and FBS. This can be due to a serum shock when protein volume is too high. Contrarily, in lower dilutions (e.g. $\frac{1}{40}$, $\frac{1}{80}$ and $\frac{1}{160}$) metabolic activity increased in synovial fluid samples compared to FBS and PL.

A comparison between the two-fold diluted normalized samples showed no significant variation between dilutions in low, medium and high protein content

groups (**Figure 6.4.E**). All SF samples showed an enhanced metabolic activity compared to FBS and PL, except in the stock solution (10 $\mu\text{g}/\mu\text{L}$) where FBS was significantly enhanced. Overall, in culture using non-normalized samples, FBS demonstrated efficient cell proliferation and cell viability compared to other serums. In aMSC, both FBS and PL showed a good viability compared to SF samples. For human chondrocyte, PL serum significantly decreased the viability of the cells whereas SF samples did almost as good as FBS. In contrast to aMSC, chondrocyte prefer to be seeded in low protein content SF. In culture with normalized serums, both cells exhibited different behavior. aMSC preferred FBS, PL and high protein content SF samples, whereas chondrocyte showed higher viability in all SF samples compared to FBS and PL. Synovial fluid from patients with more severe OA typically had a higher protein concentration due to increased inflammatory proteins, and may therefore be less suitable for promoting chondrocyte growth and viability. For cell culture purposes, either normalized dilutions or volume per volume dilutions can be used because a similar behavior was observed. The ideal SF concentrations identified in these experiments were: for 1) volume per volume dilutions: 1/20, 1/10, 1/5 and 1/2 and 2) Two-fold normalized dilutions 1.25, 284.50 or 5 $\mu\text{g}/\mu\text{L}$. Lastly, both cell types preferred more serum content over diluted serum in their growth media.

6.3.5 Glucose importance for human chondrocytes cultured in synovial fluid.

Since the normalization of SF samples was performed using PBS, we examined if this addition could affect the metabolic activity of chondrocytes. For this, chondrocytes were grown in different dilutions of the 10 $\mu\text{g}/\mu\text{L}$ normalized samples. Two-fold dilutions were prepared in media or PBS and all data is represented as relative mean to PBS or media alone.

As observed in **Figure 6.5.A**, most of the SF samples diluted in media showed higher proliferation than samples diluted in PBS. However, in the 10 $\mu\text{g}/\mu\text{L}$

dilution both dilutions are similar. Also, a slight increase of viability was observed in cells cultured in low protein containing SF. In confluent cells, the difference between cells cultured in diluted SF samples in media or PBS was more notorious. Chondrocytes were more sensitive when confluent to dilution in PBS (**Figure 6.5.B**). Metabolic activity of 100% samples was higher than PBS alone, showing no cell viability at all. Thus, the significant down-regulation of chondrocytes viability in samples diluted in PBS suggested that although SF can be used as a tissue culture agent for chondrocytes it requires supplements present in media to maintain the cells proliferation and survival. Presence of glycosaminoglycans in SF does not provide enough nutrients to the cells as opposed to the high glucose content of cell culture media.

Figure 6.5. Cell viability assays of human primary chondrocytes cultured in SF serum of low, medium and high protein content. Proliferative (**A**) and confluent human primary chondrocytes (**B**) were compared. SF samples diluted in media are represented in blue whereas SF samples diluted in PBS are represented in red. Data represented as relative absorbance to media or PBS alone $\pm$ STDEV. Three biological replicates (n=3) and three technical replicates were used.

6.3.6 Growth curve assays.

From the $\frac{1}{2}$ dilution used in the metabolic activity assays, the 4 most stimulatory and 4 most inhibitory samples were chosen for each cell type. The most

stimulatory samples in aMSC showed higher protein yield and mild OA classification. Stimulatory samples in aMSC were SF45, SF47, SF28, SF44 and SF3 whereas inhibitory samples were SF41, SF26, SF34, SF15 and SF23. At day 0 (plating day) around 50,000 cells were seeded per well. At day 1 there is an indication that cells have a stress response and started to die, after this, cells started proliferating over time (**Figure 6.6.A**). This initial shock due to serum change was stronger in some samples than others. PL and FBS were the serums with better proliferation capacity whereas some SF samples had better proliferation potential than others. Although the majority of the samples showed better proliferation over time, some samples did not recover enough after 7 days in culture. They were considered not suited for cell culture. SF45 and SF47 were on the top 10 of SFs stimulating proliferation in the aMSC MTS assay. In contrast, SF23 and SF44 had the lowest proliferation capacity in aMSCS. On average stimulatory samples have higher cell numbers along the time course compared to inhibitory samples. The morphology of the cells and cell density was observed by live/dead staining at days 1 and 7 (**Figure 6.6.B**). FBS, PL and stimulatory SFs show a high proliferation capacity at day 7 when compared to day 1. However, inhibitory SFs show slow proliferation and low cell number at day 7. Although stimulatory SFs increased the proliferation of aMSC, FBS and PL seemed to perform better for these cells. Also, the cells in SF look more fibroblastic than in FBS or PL. Similarly, the most stimulatory samples in chondrocytes showed lower protein yield and were linked to higher OA grades. From day 1 all SF samples, FBS and PL showed an increase in proliferation of chondrocytes along the time course (**Figure 6.6.C**). Some samples had better proliferation potential than others.

Figure 6.6. Growth curve assays in human aMSCs (**A and B**) and human primary chondrocytes (**C and D**). Data represented as mean $\pm$ STDEV. Three biological replicates ($n=3$) and two technical replicates were used. (Scale bar = 100 μ m).

Although FBS and PL showed good proliferation, three SF samples had better proliferative capacity (e.g. SF32, SF28 and SF42), and had better or similar proliferation capacity than FBS over the 7 days in culture, reaching almost a 10-fold increase in cell number. In contrast, SF3, SF31, SF50 and SF18 were the SF samples with less proliferation capacity. On average, stimulatory samples had higher cell numbers along the time course compared to inhibitory samples. Only one stimulatory sample did not perform as expected (e.g. SF15). Morphology of the cells in the 4 serums did not show any significant difference (**Figure 6.6.D**). Still, the inhibitory SF compared to other serums significantly decreased cell density. Moreover, PL and stimulatory SF showed a similar trend in cell density at day 7.

6.3.7 Chondrogenic differentiation.

6.3.7.1. Synovial fluid seems to maintain chondrocytic potential of primary chondrocytes in culture.

To determine how synovial fluid affects differentiation of chondrocytes, an *in vitro* differentiation assay was performed using passage 4 human primary chondrocytes that were allowed to differentiate up to 21 days in three different synovial fluid samples. Samples from each group were used (e.g. low, medium and high protein content). Different serums were added in growth media or in growth media with chondrogenic cocktail. The different serums used were 10% FBS, 5% SF and 5% PL. Chondrocytes were seeded in high-density pellets and cultured under hypoxic conditions (2.1% oxygen). Gene expression was examined after one week in culture. An overall comparison between SF and FBS showed some similarities between both serums. Growth media cultures containing SF and FBS up-regulated cartilage markers *DCN*, *SOX6*, *SOX9* (**Figure 6.7.A**) and *COMP* (data not shown). However, *COL2A1*, *COL1A1* and *ACAN* were differentially regulated. These three chondrocytic markers were higher in FBS than in SF. Moreover, a comparison between both serums with chondrogenic

differentiation shows that SF with chondrogenic cocktail increased the expression of all chondrogenic markers compared to FBS. Proliferation marker *HIST2H4* showed that SF promotes proliferation of chondrocytes when cultured in a high-density pellet under hypoxia conditions.

Figure 6.7. Chondrocyte culture with FBS or SF from each protein content group. **(A)** Gene expression analysis of cartilage specific markers and proliferation marker *HIST2H4*. Data represented as mean ± STDEV. Statistical differences were noted using * if *p-value*<0.05 or ** if *p-value*<0.01. Three biological replicates (n=3) and three technical replicates were used. **(B)** Alcian blue staining of high-density pellets, **(C)** Collagen type II immunohistochemistry of the cultures. (Scale bar = 100 μm).

Gene expression analysis also suggested that chondrocytes were proliferating after 21 days. Histological analysis of cell cultures using alcian blue staining

revealed no differences between the SF samples and FBS when cells were grown in growth media (**Figure 6.7.B**). However, under chondrogenic induction, FBS and high protein content samples enhanced proteoglycan deposition compared to medium and low protein content samples which were faintly stained by alcian blue. Furthermore, analysis of collagen type II specific deposition revealed that all SF samples produced COL2A1 without the addition of chondrogenic media (**Figure 6.7.C**). Interestingly when chondrogenic cocktail was added to the SF samples neither of them stained slightly positive for collagen type II. However, chondrogenic cocktail did increase collagen type II production in cells plated with FBS.

6.3.7.2. Synovial fluid preserves proteoglycan deposition of chondrocytes in culture and promotes chondrogenic differentiation of aMSCs in the presence of chondrogenic cocktail.

To determine how synovial fluid affects differentiation of chondrocytes and aMSC, an *in vitro* chondrogenic differentiation experiment was performed. For each cell type, the 10 most stimulatory and 10 most inhibitory SF samples in terms of viability were used. From **Table 6.4.A** samples with viability above 1.4 for chondrocytes MTS assay were used to culture chondrocytes. From **Table 6.4.B** samples with viability above 1.4 for aMSC MTS assay were used for aMSC culture. 10% FBS, 5%PL and 5%SF were used with growth or chondrogenic media. Cells were cultured in high-density pellet and incubated under hypoxia (2.1% oxygen) conditions.

In **Figure 6.8.A**, we show that the potential to sustain cell viability does not correlate with chondrogenic potential in presence of SF samples. SF does not enhance expression of chondrogenic genes in chondrocytes when compared to FBS or PL. Statistical differences were found between stimulatory and inhibitory SF samples and FBS. *ACAN* and *SOX9* increased in all cultures when compared to day 0, whereas none of the serums was capable of preserving the *COL2A1*

expression during culture when compared to day 0. Histological analysis of proteoglycan deposition using alcian blue staining was performed at day 21.

Figure 6.8. Chondrocyte cultured in FBS, PL or SF. Stimulatory or Inhibitory SF samples were used. **(A)** Gene expression analysis of cartilage specific markers and proliferation marker MKI67. Data represented as mean ± STDEV. Statistical differences were noted using * if *p*-value<0.05 or ** if *p*-value<0.01. Three biological replicates (n=3) and three technical replicates were used. **(B)** Alcian blue staining, **(C)** Type II collagen immunohistochemistry of the cultures in growth or chondrogenic media. (Scale bar = 100 μm).

Alcian blue staining revealed clear differences between FBS and SF samples **(Figure 6.8.B)**. In both growth and chondrogenic media, we observed an intense alcian blue staining in stimulatory SF samples when compared to the rest of serums. Similar matrix deposition was observed for FBS, PL and inhibitory SF samples. Comparison between stimulatory SF samples and allograft cartilage

revealed similarities in the intensity of proteoglycan staining. Moreover, collagen type II specific staining revealed that despite that proteoglycan deposition is achieved using any serum, it does not enhanced collagen type II staining (**Figure 6.8.C**). When chondrogenic cocktail was added, only inhibitory SF samples had a slight positive matrix. None of the serums had the capability of conserving the same intensity of the allograft cartilage staining.

Figure 6.9. aMSC cultured in FBS, PL or SF. Stimulatory or Inhibitory SF samples were used. **(A)**

Gene expression analysis of cartilage specific markers and proliferation marker *MKI67*. Data represented as mean ± STDEV. Statistical differences were noted using * if *p-value*<0.05 or ** if *p-value*<0.01. Three biological replicates (n=3) and three technical replicates were used. **(B)** Alcian blue staining, **(C)** Type II collagen immunohistochemistry of the cultures in growth or chondrogenic media. (Scale bar = 100 µm).

Gene expression of aMSCs chondrogenic differentiation was examined after one week of differentiation (**Figure 6.9.A**). In growth media, the control serum (e.g. 194

PL) promotes the expression of *ACAN* and *SOX9*. However, compared to FBS it does not promote *COL2A1* and a lower proliferation was observed compared to FBS. Both SF groups were capable of promoting expression of *ACAN* and collagen type II. But, stimulatory SF samples enhanced the expression of chondrogenic markers *COL2A1* and *ACAN* better than inhibitory samples, FBS or PL. *SOX9* expression was steady among the different serums.

Statistical difference reflected the comparison between PL and the other serums. When chondrogenic media is added, a decreased expression in *ACAN*, *SOX9* and *MKI67* was observed, whereas collagen type II increased in both SF groups and in FBS. Comparison between inhibitory against stimulatory groups of SF samples revealed stimulatory samples enhanced cartilage markers *SOX9* and *COL2A1* in a similar way to PL or FBS. Alcian blue staining in both SF groups showed a similar proteoglycan deposition to FBS and PL when chondrogenic media was included (**Figure 6.9.B**). As observed in the collagen type II immunohistochemistry all SF samples presented low chondrogenic potential whereas FBS shows higher staining of collagen type II when cells were cultured with no chondrogenic cocktail. In the presence of chondrogenic differentiation cocktail both SF groups and FBS showed a similar deposition of collagen type II (**Figure 6.9.C**). To conclude, viability does not correlate with chondrogenic potential of SF samples. For chondrocytes cultures, stimulatory samples seemed to promote the deposition of proteoglycan and glycosaminoglycan in a better way than inhibitory samples and this was increased by the addition of chondrogenic cocktail. However, stimulatory SF samples had lower potential to deposit collagen type II whereas inhibitory samples seemed to promote collagen type II. Despite our best efforts, neither serum can promote chondrogenesis as good as is seen in the allograft cartilage tissue, but SF samples are safe to use to culture primary chondrocyte and seemed to help the cells maintain their phenotype better than FBS or PL. For AMSCs, SF samples can increase expression of *ACAN*, collagen type

II in a similar way that FBS and PL. SF samples from both groups were enough to promote proteoglycan deposition on the aMSC. However, collagen type II matrix produced by these cells was poor. Collagen type II matrix was increased by the addition of chondrogenic media for all serums. SF was capable of producing cartilage matrix with the addition of chondrogenic cocktail similar to no serum conditions with chondrogenic media.

Table 6.5. Literature review of cytokines present during OA progression.

OA Biomarkers	Official name	Reference	Description	OA Progression (H or L)
IL-1α	Interleukin 1 alpha	Tsuchiuda AL et al., 2014, Cleaver CS et al., 2001	Induces collagen release, Role in RA. Involved in various immune responses, inflammatory processes, and hematopoiesis. Released in response to cell injury. Induces apoptosis.	H
IL-1β	Interleukin 1 beta	Tsuchiuda AL et al., 2014	Role in RA. This cytokine is produced by activated macrophages as a propeptide. Its an important mediator of the inflammatory response, and is involved in a variety of cellular activities, including cell proliferation, differentiation, and apoptosis.	H
IL-4	Interleukin 4	Tsuchiuda AL et al., 2014	Participates in at least several B-cell activation processes as well as of other cell types (PubMed:3016727). It is a costimulator of DNA-synthesis.	L
IL-6	Interleukin 6	Tsuchiuda AL et al., 2014, Acosta JC et al., 2008, Kojima H et al., 2012, Gobezie R et al., 2007, Kokebie R et al., 2011, Scanzello CR et al., 2009	Secreted by senescent cells, induces DNA damage and telomere shortening. Not produced by cartilage, high in cartilage defects than in OA chondrocytes. Also role in RA, Levels correlate with radiographic OA, In vitro stimulation increases TIMP1 And COL2. This gene encodes a cytokine that functions in inflammation and the maturation of B cells. The protein is primarily produced at sites of acute and chronic inflammation. The functioning of this gene is implicated in a wide variety of inflammation-associated disease states, including susceptibility to diabetes mellitus and systemic juvenile rheumatoid arthritis.	H
IL-7	Interleukin 7	Rubenhagen R et al., 2012, Tsuchiuda AL et al., 2014	This cytokine and the hepatocyte growth factor (HGF) form a heterodimer that functions as a pre-pro-B cell growth-stimulating factor. Hematopoietic growth factor capable of stimulating the proliferation of lymphoid progenitors. It is important for proliferation during certain stages of B-cell maturation.	L
IL-10	Interleukin 10	Tsuchiuda AL et al., 2014, Papalia R et al., 2016	The protein encoded by this gene is a cytokine produced primarily by monocytes and to a lesser extent by lymphocytes. This cytokine has pleiotropic effects in immunoregulation and inflammation. This cytokine can block NF-kappa B activity, and is involved in the regulation of the JAK-STAT signaling pathway.	H
IL-13	Interleukin 13	Tsuchiuda AL et al., 2014, Van Dyken SJ et al., 2013, LafyatisR et al., 2012.	Higher in cartilage defects, Macrophage activation in tissue homeostasis and repair, Prevents collagen release. This cytokine down-regulates macrophage activity, thereby inhibits the production of pro-inflammatory cytokines and chemokines.	H
IL-17	Interleukin 17	Papalia R et al., 2016	The protein encoded by this gene is a proinflammatory cytokine produced by activated T cells. This cytokine regulates the activities of NF-kappaB and mitogen-activated protein kinases. This cytokine can stimulate the expression of IL6 and cyclooxygenase-2 (PTGS2/COX-2), as well as enhance the production of nitric oxide (NO). High levels of this cytokine are associated with several chronic inflammatory diseases including rheumatoid arthritis, psoriasis and multiple sclerosis.	H
IL-18	interleukin 18	Rubenhagen R et al., 2012	The protein encoded by this gene is a proinflammatory cytokine that augments natural killer cell activity in spleen cells, and stimulates interferon gamma production in T-helper type I cells.	L
IL1Ra	interleukins	Rubenhagen R et al., 2012	This protein inhibits the activities of interleukin 1, alpha (IL1A) and interleukin 1, beta (IL1B), and modulates a variety of interleukin 1 related immune and inflammatory responses.	L
TNFα	Tumor necrosis factor alpha	Tsuchiuda AL et al., 2014, Papalia R et al., 2016, Coimbra JB et al., 2004	Also role in RA, If TNF α is produced also IL8 and IL1 are up regulated. Mainly secreted by macrophages. It can bind to, and thus functions through its receptors TNFRSF1A/TNFR1 and TNFRSF1B/TNFR. involved in the regulation of cell proliferation, differentiation, apoptosis, lipid metabolism, and coagulation. This cytokine has been implicated in a variety of diseases, including autoimmune diseases, insulin resistance, and cancer.	H
IFNγ	Interferon	Tsuchiuda AL et al., 2014	Higher in cartilage defects. Triggers a cellular response to viral and microbial infections. It is a potent activator of macrophages, it has antiproliferative effects on transformed cells and it can potentiate the antiviral and antitumor effects of the type I interferons.	H
OSM	Oncostatin M	Tsuchiuda AL et al., 2014, Cleaver CS et al., 2001	Induces collagen release, Not produced by cartilage. This protein is a secreted cytokine and growth regulator that inhibits the proliferation of a number of tumor cell lines. This protein also regulates the production of other cytokines, including interleukin 6, granulocyte-colony stimulating factor and granulocyte-macrophage colony stimulating factor in endothelial cells.	H

6.3.8 Cytokine analysis.

Analysis of the cytokine composition was performed to optimize samples for culture conditions. First, a literature review was performed to examine a list of cytokines present in cartilage OA compared to healthy cartilage (**Table 6.5**).

Tsuchiuda et al. examined the presence of cytokine in synovial fluid from cartilage defect and OA cartilage and found that IL1 β , IL10, IL6 and TNF α were significantly up-regulated in OA compared to cartilage defects [12]. They found these cytokines to be expressed in expanded OA chondrocyte *in vitro*. A subset of the samples was examined for ELISA analyses of these 4 cytokines. For reliability testing, we analyzed each sample and standard curve controls in duplicate. We observed that all 24 samples were positive for the three of the four cytokines; however, some differences between the samples were also observed (**Figure 6.10**). Moreover, we detected that even in undiluted samples the intrinsic levels of IL1 β were low in comparison to other cytokines (e.g. IL6 and IL10) levels. Therefore, the collected samples may not express IL1 β .

Figure 6.10. Boxplot demonstrating the difference in concentration of synovial fluid proinflammatory cytokines IL1 β , IL10, IL6 and TNF α . Values are presented as median (line), interquartile range (box) and outliers (circles). Statistical differences were noted using * if p -value < 0.05.

A non-parametric comparison for all pairs using Steel-Dwass Method was performed. The median and 95% confidence intervals (CI) for each cytokine were described below. For IL1 β the median was 6.28 $\pm$ 0.86 pg/mL with a lower

CI of 6.65 ± 0.67 and an upper CI of 6.65 ± 1.21 . For IL10 the median was 74.46 ± 93.26 pg/mL with a lower CI of 35.08 ± 72.48 and an upper CI of 113.84 ± 130.81 . For IL6 the median was 143.87 ± 145.94 pg/mL with a lower CI of 82.25 ± 113.42 and an upper CI of 205.49 ± 204.71 . For TNF α the median was 16.75 ± 3.24 pg/mL with a lower CI of 15.38 ± 2.51 and an upper CI of 18.11 ± 4.54 . IL6 was inversely associated with protein content in the samples. In contrast, IL10 was highly increased in samples with higher protein content. IL1 β and TNF α content were similar among the samples. However, a slight increase of both cytokines was found in medium protein content samples. Thus, SF samples with lower protein content possessed more inflammatory cytokines than the other two SF groups. Interestingly, the low protein content samples were previously found to impair cell viability more than SF with higher protein content.

Figure 6.11. Cytokine concentrations categorized according to visual inspection of clarity of SF. Values are presented as mean $\pm$ STDEV. Statistical differences were noted using * if p -value <0.05 .

In **Figure 6.11**, cytokine categorized according to visual inspection of the clarity of the fluid validated previous observations of no variability and low concentrations of IL1 β and TNF α . However, both IL10 and IL6 were found increased in samples that presented a non-inflammatory and inflammatory

aspect. Thus, low protein SF samples presented a visual aspect of OA inflammatory and higher cytokine markers. These samples were shown to improve viability of aMSC, but decreased viability of human chondrocyte.

6.4 DISCUSSION

Fetal bovine serum (FBS) has long been the gold standard medium supplement for laboratory-scale MSC culture and other cells. However, FBS has a poorly characterized composition and poses risk factors, as it may be a source of xenogenic antigens and zoonotic infections [13]. FBS is rich in fetal growth factors and hormones that stimulate cellular proliferation and maintenance; and it facilitates differentiation toward various lineages [14]. The right choice of serum or serum replacement is crucial for cell culture. Particularly with regard to clinical application, human alternatives for fetal bovine serum are clearly to be preferred.

Scientists and users involved in cell therapy or advanced therapy medicinal products know the need for an alternative. Since concerns have been raised regarding the safety of FBS, other serums have been tested and used with some success [15]. Each type of culture supplement has advantages and disadvantages, and they carry severe effects on functionality and viability of the cells. Use of platelet lysate was studied and used to culture a broad range of different cell types. Long-term culture of MSCs in media supplemented either with FBS or pooled PL induced similar gene expression and DNA methylation changes in expanded cells [16]. The use of PL-supplemented medium generates significantly more MSCs than the use of fetal bovine serum. Thus, platelet lysate can be an adequate non-xenogenic alternative for fetal calf serum. However, PL also triggers an inflammatory response in MSCs and induces the secretion of factors maintaining macrophages in a proinflammatory state [17].

Several studies determined that the overall protein content of PL across different batches was highly similar (40–60 g/L) but the growth factor content differed

substantially. These studies also used proteomics to illustrate the impact of different serum compositions during cell expansion. In our study, SF was used as an alternative for both FBS and PL. SF rinses all intrinsic structures of joints. It's used in the evaluation of joint diseases and is been broadly used for the analysis of its constituents to understand OA. Moreover, SF can be a promising growth-stimulating additive for culturing of stem cells and other cell types. For stem cell delivery therapies, the importance of synovial fluid characterization lies in the fact that this fluid interacts with the cells once implanted. Thus, it is important to know if synovial fluid could enhance, limit or reject stem cell or chondrocyte upon implantation.

Our data indicates that synovial fluid can enhance the metabolic activity of chondrocyte and aMSC at a level that is at least equivalent to FBS and better than human alternative PL. Also, this data shows that proliferation seemed to correlate with protein content and pathology of the sample. Synovial fluid with medium protein content may be likewise effective during *in vitro* expansion and in promoting chondrocytic phenotype maintenance in human chondrocyte than fetal bovine serum. Moreover, synovial fluid supplemented with chondrogenic media in aMSC slightly promoted proteoglycan deposition after 21 days in culture. However, due to the differences found between the SF samples during cell culture, further normalization of the samples will be essential to preserve both stem cells and chondrocytes viability and maintenance.

Concentrations of synovial fluid protein are known to vary with the degree of joint inflammation. Comparing the protein profile to determine potential key components that could influence cell culture is important for the normalization of the synovial fluid. Proteomic analysis has been previously used as an analytical tool to detect biomarkers for disease. Also, these cytokine profile are different between synovial fluid, cartilage tissue and cultured chondrocytes [18]. IL6 has been observed to stimulate the expression of MMPs that act to degrade cartilage

[19]. IL1 β and TNF α presence has been shown to inhibit chondrogenic differentiation of stem cells by blocking TGF β signaling and *SOX9* expression[20]. In our dataset, both IL1 β and TNF α expression were low and similar across the samples. However, in the case of IL6 higher expression was found in low protein content samples. Interestingly, these samples affected the viability of both aMSC and human primary chondrocyte. Thus, correlation between viability, protein content and cytokines profile could help for the stratification of samples for future therapeutic use.

Our results serve as an initial screening of the possibilities of SF to replace fetal bovine serum as a culture supplement for *in vitro* expansion of human primary chondrocyte and aMSC. Due to its non-zoonotic (animal free) nature is therefore potentially better for clinical applications. However, compared to FBS and PL, synovial fluid cannot be obtained in large quantities, which would make it difficult to be used.

In conclusion, this study suggested that in clinical therapies where aMSC are injected into a diseased joint, the presence of a synovial fluid rich environment would not affect the viability of the aMSC implanted. Thus, the existence of OA SF does not significantly disrupt cell number and/or proliferation. Increasing the potential to conserve an appropriate cell number to perform the expected function. Future studies will aim to analyze the precise composition of synovial fluid (e.g. growth factors, microRNAs and exosomes) to optimize and define culture conditions.

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CHAPTER 7

7. Novel transcription factor ZNF648 might regulate cartilage development and homeostasis through the modulation of cartilage related signaling pathways.

ABSTRACT

RNA-seq analysis of mesenchymal tissues shows a unique expression of *ZNF648* in all cartilaginous tissues like the growth plate, articular cartilage, chondrosarcoma and intervertebral disc compared to non-cartilaginous musculoskeletal tissues. Primary growth plate cells showed a higher expression of this transcription factor when compared to primary articular chondrocytes. In addition, *ZNF648* expression was also found increased in moderate diseased cartilage tissues. This *ZNF648* protein in the growth plate seems to maintain normal differentiation of pre-committed chondrogenic mesenchymal progenitor cells (e.g., human growth plate and immature mouse chondrocytes) into chondrocytes, whereas in later stages of cartilage maturation *ZNF648* expression is reduced. Although the expression is present in primary tissues, it is significantly reduced when cells are removed from the native tissue environment. A reduction in *ZNF648* expression was found in primary cells passaged two times and on in vitro cell cultures of primary cells. We used in vitro cell culture, gain and loss of function analysis and a *Zfp648* null mouse to understand the role of *ZNF648* during cartilage development. Interestingly, we found that *ZNF648* may regulate different programs in articular and growth plate chondrocytes. An enhanced expression of *ZNF648* increased *COL2A1* expression in growth plate cells while reducing the expression in articular chondrocytes cells. This suggested an important regulatory mechanism during early development of the chondrocytes in the growth plate and not in terminally differentiated chondrocytes such as the ones in articular cartilage. Similarly, knock down experiments validated the regulatory role of *ZNF648* or *Zfp648* in enhancing *collagen type II* and decreasing *collagen type I*. This concept would be ideal for formation of hyaline cartilage over fibrocartilage in tissue regeneration strategies. Lastly, *Zfp648* null mice obtained using CRISPR/Cas9 strategies showed skeletal differences in younger mice (e.g. 2 weeks, 3 weeks) whereas in skeletal mature mice (e.g. 12 weeks of age) no phenotype was observed.

Remarkably, the phenotype obtained from 2-week-old null mouse indicates an important role of this transcription factor in the development of long bones and during endochondral ossification stages. Zfp648 depletion not only affected the onset of the formation of secondary ossification center but also it delayed the formation of normal long bones. In this study we aimed to analyze driving molecular factors such as transcription factor ZNF648 that may directly regulate cartilage development or homeostasis through modulating gene expression and other cartilage-related signaling pathways. Thus, development of novel approaches to improve cartilage regeneration from a mechanistic and molecular point using ZNF648 to maintain cartilage homeostasis is thought to play an essential role in future clinical therapies.

7.1 INTRODUCTION

The growth plate is a complex cartilaginous structure that critically regulates natural growth of long bones and the axial skeleton. It is a highly specialized cartilaginous tissue adjacent to the joint in skeletally immature vertebrates. Genetic disorders, infection, tumor, and traumatic injuries to the growth plate can result in shortening and progressive angular deformity of the affected limb in the growing child [1, 2]. Pediatric limb length discrepancy and angular deformities are clinically challenging problems that cost an estimated \$2.2 billion dollars in treatment each year in the United States [3]. Each year, one in three hundred U.S. children sustains a growth plate fracture, and four percent will sustain permanent damage to the growth plate resulting in deformity or shortening [4]. This damage could impact later function and quality of life.

Without appropriate treatment, growth plate injuries result in lifelong limp, chronic pain, arthritis, and disability. However, there are no pharmacotherapies available for local or selective treatment of growth plate disorders, leaving mechanical treatments as the only available option [5]. Mild deformities may be corrected by surgical tethering [6]. Though, for correcting leg length discrepancy, this often entails performing surgery on the normal leg to destroy the healthy growth plate, resulting in overall decreased stature in adulthood. Unfortunately, these techniques do not work at all if the child is too close to skeletal maturity. For severe deformities, shortening the longer leg is not feasible because it would result in severe secondary deformity and dysfunction. Thus, complex surgical reconstruction is undertaken using external fixation devices or intramedullary lengthening nails [7]. Although the process requires months of rehabilitation, the lengthening still takes place too quickly for the human body to adapt, resulting in various adverse events for the patients. Even after completion of the treatment, fracture is common because the regenerated bone is not robust [8]. Thus, there is a significant need for improved understanding of growth plate signaling

pathways to reduce surgical morbidity and provide local pharmacologic treatment options.

A comprehensive biologic approach to the treatment of the physis is needed in order to restore normal growth, decrease morbidity, and improve patient outcomes. Local pharmacologic treatment to promote physeal growth could provide slow, gradual correction of limb deformities without associated comorbidities and prolonged recovery. Reversible and controllable agents could be used to provide precision correction of an individual's deformity, mitigating the risk of over- or under-correction. Moreover, these interventions could encourage growth in the short limb and suppress growth in the long limb avoiding shortening stature in the adulthood. However, a lack of robust data and scientific knowledge of the physis has delayed the development of local pharmaceuticals to treat growth plate disorders. Hence, a better understanding of the regenerative properties of the physis also holds promise to regenerate not only the growth plate but to treat other disabling problems of the musculoskeletal system. Our overall goal is to elucidate the mechanism of transcription regulation in musculoskeletal tissues. RNA-seq analysis of mesenchymal tissues was examined to identify expression of *ZNF648* in all cartilaginous tissues like growth plate, articular cartilage, chondrosarcoma and intervertebral disc and compared to non-cartilaginous musculoskeletal tissues. Expression of *ZNF648* will be also examined in tissues, primary cell digestions and immortalized cells from human and mouse. Differences in gene expression and protein would be identified. Functional analysis on different cells will be performed with the goal of obtaining information about the mechanistic effects of gain and loss of function of *ZNF648* in cartilage. Moreover, a *Zfp648* null mouse obtained using CRISPR/Cas9 strategies will be studied to observe skeletal phenotypic differences. In this study we aimed to analyze driving molecular factors such as transcription factor *ZNF648* that may directly regulate cartilage development or homeostasis

through modulating gene expression and other cartilage-related signaling pathways.

7.2 MATERIALS AND METHODS

7.2.1 RNA extraction from tissues

More than 1200 tissue specimens were collected from patients undergoing tissue removal as part of planned surgical procedures. The specimens used in this investigation were collected under Institutional Review Board (IRB) of Mayo Clinic. Written informed consent was obtained for all collected tissues. Some cartilage samples were collected from human cadaver donors through the Gift of Hope Organ and Tissue Donor Network (Elmhurst, IL), with approval by the local ethics committee and informed consent from each family (ORA#: L03090306). Before RNA extraction, samples were snap frozen in liquid nitrogen and stored at -80°C after surgical harvest. Then, frozen tissues were grounded into a powder using a mortar and pestle and homogenized in Qiazol reagent (Qiagen, Hilden, Germany). Total RNA was extracted from using the RNeasy minikit (Qiagen, Hilden, Germany) and quantified using a NanoDrop 2000 spectrophotometer (Thermo Fischer Scientific, Wilmington, Delaware). Before RNA extraction, articular cartilage specimens were classified according to radiological findings using the Collins grading scheme as described previously [9]. For this study, joints were specifically classified using the following criteria: G0 joints displayed normal articular cartilage throughout (n=5), G1 joints had some destruction of superficial cartilage and swelling and/or softening (n=7), G2 joints exhibited extensive superficial damage to articular cartilage but not significant loss of cartilage and no damage to bone (n=6), G3 joints had areas with total articular cartilage loss with observable penetration of defects to the subchondral bone plate (n=4), and a G4 joint was identified as having complete loss of cartilage and full thickness exposure to bone along the articular surface of the joint.

7.2.2 Next generation RNA sequencing

RNAseq analysis was performed using the musculoskeletal tissue collection collected in Dr. van Wijnen's laboratory. We analyzed and compare musculoskeletal tissues and cell cultures of cartilage, physis, bone, tendon, muscle and ligament from different states of disease, and healthy adult and pediatric patients. For samples selected for next generation sequencing, RNA integrity was assessed using the Agilent Bioanalyzer DNA 1000 chip (Invitrogen, Carlsbad, CA). Only samples with an RNA Integrity Number (RIN) and DV200 score greater than our Sequencing Core's minimum cutoff (RIN >6 and DV200 > 50%) were used for sequencing. High-throughput RNA sequencing and subsequent bioinformatic analysis were performed in collaboration with the Mayo Clinic RNA sequencing and bioinformatics cores as previously described [10]. Library preparation was performed using the TruSeq RNA library preparation kit (Illumina, San Diego, CA). Poly-A mRNA was purified using oligo dT magnetic beads. Then, cDNA synthesis was performed on purified mRNA using SuperScript III reverse transcriptase and random primers (Invitrogen). DNA fragments were enriched by PCR using primers included in the Illumina Sample Prep Kit. Sample concentration and size distribution was determined on an Agilent Bioanalyzer DNA 1000 chip and Qubit fluorometry (Invitrogen, Carlsbad, CA). RNA-Seq data was analyzed using the MAPRSeq v.1.2.1 system including alignment with TopHat 2.0.6 [11] and HTSeq software [12]. Expression values for each gene were normalized to 1 million reads and corrected for gene length (reads per kilobasepair per million mapped reads, RPKM).

7.2.3 Human cell harvest and expansion

Human primary chondrocyte were obtained from healthy donors undergoing amputation procedures for congenital limb deformity or from donors undergoing total knee replacement surgery. All zones of growth plate and articular cartilage were used. All tissues and cells were collected under protocols approved by the Mayo Clinic Institutional Review Board. After surgical harvest, both articular

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cartilage and growth plate specimens were immediately placed in a solution of 2% Pronase (Sigma Aldrich) in culturing media (DMEM supplemented with 1% anti-mycotic/antibiotic) for 1 hour in a shaking incubator at 37°C. Then, samples were incubated overnight in a 0.36% collagenase-P (Worthington) solution in culturing media in a shaking incubator at 37°C overnight. Undigested tissue was removed by filtering the cell suspension with a cell strainer. Cells were centrifuged and washed in two changes of PBS. Lastly, chondrocytes were re-suspended in maintenance media that contained Advanced MEM (Gibco/Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Atlanta, GA) and 1% penicillin/streptomycin (Gibco/Thermo Fisher Scientific).

A comparison between the passaging of cells was performed. Passage 0 (primary digested cells), passage 1 and passage 2 cells were cultured using standard techniques in 10 cm² dish at 37°C, 95% humidity and 5% CO₂ until they reached 80% confluence. Prior to each passage, cells were detached from the 10-cm² dish by trypsinization using TrypLE Express (Gibco/Thermo Fisher Scientific) and re-plated under the same standard conditions. RNA was extracted from cells at passage 0 (primary digested cells), passage 1 and passage 2 for gene expression analysis.

For time course (day 1, day 3 and day 7) culture experiments, primary articular cartilage chondrocyte from healthy and OA patients, and primary growth plate chondrocyte were plated in high-density cellular aggregates as described previously [13]. Cells were cultured for one week in maintenance media and media was refreshed every 2 to 3 days. RNA was extracted from cells after 1, 3 and 7 days for gene expression analysis.

7.2.4 Isolation, culture and analysis of immature mouse chondrocytes (mACs)

Primary immature mouse articular chondrocytes (mACs) were isolated from post-natal day 7 old mice as previously described by Camilleri et al.

[14]. Freshly isolated mACs were re-suspended in at a concentration of 2×10^7 cells/mL in maintenance media (DMEM supplemented with 2% FBS and 1% anti-mycotic/antibiotic) and plated in micromass culture by pipetting 10 μ L of cell suspension on to a low-adhesion tissue culture dish. Micromasses formed by incubating for 1h at 37°C, 5% CO₂, after which growth medium was carefully added and left undisturbed for 7 days. Before RNA extraction at day 7, micromasses were washed with PBS to remove medium, mechanically disrupted with a scalpel, and 700 μ L of QIAzol was added to lyse cells. After RNA was extracted gene expression analysis was performed.

7.2.5 Cell culture and expansion of immortalized cells lines

Human and mouse immortalized cell lines were used as positive and negative controls in our studies. Two different human cell lines were used, TC28A2 and chondrosarcoma cell line L835, whereas one mouse immortalized cell line ATDC5 was utilized. TC28A2 human chondrocyte cell lines (Sigma) and ATDC5 mouse chondrogenic cell line (Sigma) were both expanded using Advanced MEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin and cultured in standard conditions. Chondrosarcoma cell line L835 was obtained from the University of Leiden and cultured as previously described [15]. HEK293 cells were cultured and expanded using maintenance media, which consisted of DMEM with 10% FBS and 1% anti-mycotic/antibiotic under standard conditions. When 80% confluency was achieved, cells were detached from the flasks and quantified before experimental cultures were performed.

7.2.6 Functional analysis of ZNF648

Gain and loss of function experiments were performed in human and mouse cells. Before transfection, all cells were seeded in 6-well plates with at a density of 10,000 cells per cm². Cells were cultured in maintenance media (specific for each cell) until 80% confluency was reached.

Dose response experiments were performed using HEK293 cells. For this, 2, 4 and 5 µg of an ZNF648 plasmid tagged with a GFP or mCherry protein was used (RFP-ZNF648 plasmid). Transfection was performed using Lipofectamine following the manufacturer's instructions. A negative plasmid only containing GFP or mCherry tag was used as a negative control. After 72 hours of transfection, ZNF648 expression was analyzed using fluorescent microscopy analysis and western blotting. RNA was collected from the cells and gene expression analysis was performed. Metabolic assay and Hoechst staining was performed to analyze the toxicity of the transfected plasmid after 24 and 72 hours of transfection.

Gain and loss of function experiments were performed on primary and immortalized chondrocytes. For gain of function, transfection was performed using the Lipofectamine protocol and adding 5 µg of each plasmid to each well. For loss of function experiments 10nM of *ZNF648* siRNA for human cells or 10nM of *Zfp648* siRNA for mouse cells was used following Lipofectamine manufacturer's instructions. After 72 hours of transfection, gene expression, protein analysis and fluorescent imaging were performed. MTS activity assay was performed after 24 hours of transfection.

7.2.7 RNA isolation, reverse transcription and real time quantitative PCR (RT-qPCR)

For all experiments, total RNA was isolated using the Direct-zol RNA kit (ZYMO Research, Irvine USA) following the instructions of the manufacturer. Three different biological replicates for each experimental condition were used concurrently for RNA isolation and subsequent expression analysis, to ensure consistency in RNA quality and expression values. Then, RNA concentrations and purity levels were measured using a NanoDrop (Thermo Fisher Scientific). For reverse transcription, 1µg of RNA was used as template. The Reverse Transcription System using M-MLV Reverse Transcriptase (Promega, Madison, USA) was used to convert RNA to cDNA. Real-time PCR reactions were composed

of 10 ng of cDNA, gene-specific primers (**Table 7.1**) and 1x QuantiTect SYBR® Green PCR Master Mix (Qiagen) in a final volume of 10µL. RT-qPCR was detected using the CFX384 real time system machine (Bio-Rad). Relative transcript abundance of genes of interest was normalized to *AKT1* expression using the $2^{-\Delta\Delta C_t}$ method. Gene expression analysis of chondrogenic markers (e.g. *COL2A1*, *PRG4*, *ACAN*, *SOX9*, and *SOX6*), proliferative markers (e.g. *HIST2H4*, *MKI67* and *CCNB2*) and *ZNF648* was performed. For mouse cells, *ZNF648* mouse homolog *Zfp648* primer was used.

7.2.8 Western Blotting

Total protein was harvested from all different cells after 72 hours of transfection. Cells were washed twice with ice cold PBS and mechanically disrupted using a cell scraper, in the presence of immunoprecipitation buffer (150mM NaCl, 50mM Tris pH 7.4, 1% w/v sodium deoxycholate, 0.1% w/v sodium dodecyl sulfate and 1% v/v Triton X-100) supplemented with protease inhibitor cocktail (Sigma), and PMSF (Sigma) and stored at -80°C until quantification. Protein content was quantified using the *DCTM* protein assay (Bio-Rad) according to the manufacturer's protocol. Western blotting and membrane development was performed as previously described [16]. The following primary antibodies were used to detect proteins: rabbit polyclonal anti-ZNF648 (1:200; 170269; Abcam), rabbit polyclonal anti-ZNF648 (1:400; AP54705PU-N; Origene), mouse monoclonal anti-COL2A1 (1:500; MAB8887; Millipore) and rabbit monoclonal anti-GAPDH (1:1000; D16H11; Cell Signaling).

7.2.9 MTS activity assay and Hoechst Staining

MTS activity was assayed after 24 and 72 hours according to the manufacturer's protocol (Promega). Absorbance was measured at 490 nm using a SpectraMAX Plus spectrophotometer (Molecular Devices). Then, MTS solution was aspirated from wells, and cells were washed once with PBS. After aspiration of PBS, 500 µl of Hoechst 33258 (Sigma, 94403) (0.5 µl/ml in PBS) was added to each well.

Plates were left to incubate in the dark at room temperature for 20 min. After incubation, fluorescence intensity was measured at a 340-nm excitation wavelength and 460-nm emission wavelength using an F200 Infinite Pro (Tecan) plate reader. Measurements were fit to a standard DNA curve to determine relative DNA content.

7.2.10 Histological analysis of human and mouse tissues

Histological analysis was performed on articular cartilage, growth plate and epiphyseal bone. After surgical harvest, tissues were washed with saline solution and immediately fixed in 10% neutral buffered formalin for one day. Then, samples were dehydrated in graded ethanol and paraffin embedded. Immunohistochemistry (IHC) was performed on serial sections of 5µm thickness using the Mouse and Rabbit Specific HRP (ABC) Detection IHC kit (Abcam) and DAB Enhanced Liquid Substrate System (Sigma-Aldrich) according to instruction manuals. Primary antibodies used were rabbit polyclonal anti-ZNF648 (1:500; 170269; Abcam), mouse monoclonal anti-COL2A1 (1:40; MAB8887; Millipore), rabbit monoclonal anti-COL1A1 (1:100; 138492; Abcam), mouse monoclonal anti-MKI67 (1:100; 238019; Abcam), rabbit monoclonal anti-mouse IgG (1:100; 190475; Abcam) and goat anti-rabbit IgG (1:100; 205718; Abcam).

7.2.11 Immunofluorescence

Cells were cultured in a density of 1×10^4 cells per slide in standard conditions. Before fixation, slides were washed thrice with PBS followed by addition of 4% paraformaldehyde in PBS for 10 minutes at room temperature. Then, glass slides were immersed in PBS containing 0.1% Triton X-100 for 10 minutes at room temperature. For protein block, PBS containing 5% BSA was used and slides were blocked for 2 hours at room temperature. After washing steps with PBS, specific primary antibodies were added and incubated overnight in the cold room. The primary antibodies used included rabbit polyclonal anti-ZNF648 (1:500; 170269; Abcam) and mouse monoclonal anti-COL2A1 (1:500; MAB8887;

Millipore). After washing steps, secondary antibodies were added to the slides. Goat anti-rabbit IgG Alexa Fluor 647 (1:500; abcam; 150083) and goat anti-mouse IgG Alexa Fluor 488 (1:500; abcam; 150113) were incubated for 30 minutes at room temperature in the dark. Glass slides were then mounted into microscope slides using VECTA shield with DAPI (Vector laboratories) mounting media. Images were digitally photographed using the AxioVert A1 (Zeiss) and Zen software package (Zeiss).

7.2.12 Genetic null mutation of ZNF648 in mouse and evaluation of skeletal phenotyping

A Zfp648 global knockout mouse model was generated with the expertise of the Mayo Clinic Transgenic and Knockout Core Facility. The mouse Zfp648 locus was ablated using a Crispr/Cas-9 strategy that targeted the Zfp648 exon using two guide sequences: GGTAGGAAGGGTGGCTCCCC (gRNA1), and CTGAGTGTCAACTGCGAGT (gRNA2) and generated a deletion of 288bp around the transcriptional start site (TSS) (**Figure 7.1.A**). Six generations of the offspring from the founders were paired with C57BL/6 mice in order to eliminate off target effects of the Crispr/Cas-9 gene targeting technique (**Figure 7.1.B**). Gene knockout and germline transmission of the null allele was confirmed by DNA sequencing and PCR with allele specific primers of generation F1 mice (**Figure 7.1.C**). For experimentation, an analysis of all three genotypes: knockout (KO; Zfp648 -/-), Wild Type (WT; Zfp648 +/+) and Heterozygous (HET; Zfp648 +/-) was performed.

Skeletal phenotype of the mouse was studied using microCT, histology and immunohistochemistry of growth plate, cartilage and bone. Radiographic analysis was performed using a Faxitron X-ray imaging cabinet (Faxitron Bioptics, Tucon, AZ). Total body length, tibial length, femur length and growth plate depth were evaluated using NIH Image J software. Average depth, growth plate depth, and hypertrophic zone depths within growth plate were determined.

Figure 7.1. Schematic of the *Zfp648* Crispr/Cas-9 strategy to generate the null mouse (A). Breeding strategy to eliminate off-target effects (B). Gene knockout confirmation using PCR analysis (C).

Comparison of epiphysis and growth plate depth was also compared among the different phenotypes. For histological analyses, mice were sacrificed at post-natal day 7. Right hind limb was fixed in 10% neutral buffered formalin, decalcified with 15% EDTA and embedded in paraffin. Serial sections were taken at 5 μ m thickness and stained with Alcian Blue/Eosin or Safranin O/Fast Green/Hematoxylin. Immunohistochemistry (IHC) was performed as previously described. Primary antibodies used included rabbit polyclonal anti-ZNF648 (1:500; 170269; Abcam), mouse monoclonal anti-COL2A1 (1:40; MAB8887; Millipore), rabbit monoclonal anti-COL1A1 (1:100; 138492; Abcam), mouse monoclonal anti-MKI67 (1:100; 238019; Abcam), rabbit monoclonal anti-mouse IgG (1:100; 190475; Abcam) and goat anti-rabbit IgG (1:100; 205718; Abcam). Images were digitally photographed using the AxioVert A1 (Zeiss) and Zen software package (Zeiss).

7.2.13 Statistical Analysis

Data is presented as the mean $\pm$ standard deviation (STD). Statistical analysis was performed using JMP® Pro v10.0.0 (SAS Institute), and *p* values were

determined using the Student T-test. Significance is noted in the figures when applicable as * if $p<0.05$ and ** if $p<0.01$.

7.3 RESULTS

7.3.1 Identification of *ZNF648* as a novel cartilage specific zinc-finger protein

Gene expression analysis performed on more than 1200 samples including bone, growth plate, and articular cartilage showed zinc finger 648 (*ZNF648*) was highly expressed and specific to cartilaginous tissues including growth plate, articular cartilage, chondrosarcoma and intervertebral disc compared to non-cartilaginous musculoskeletal tissues (**Figure 7.2**). Gene ontology analysis classified *ZNF648* as a regulator of transcription and a nuclear factor.

Figure 7.2 Identification of *ZNF648* as a novel cartilage specific protein. *ZNF648* expression in RNAseq is specific in cartilage when compared to other mesenchymal tissues and/or cells. All data is represented as RPKM.

7.3.2 *ZNF648* and *Zfp648* expression is highly specific to cartilage and not found in bone, following the expression pathways of *COL2A1*.

Gene expression analysis on pediatric articular cartilage (AC), growth plate (GP), and bone was carried out using cartilage related markers collagen type II (*COL2A1*) and aggrecan (*ACAN*) as well as *AKT1* and *GAPDH* as housekeeping genes (**Figure 7.3**). *ZNF648* was highly expressed in pediatric articular cartilage, and expression correlated with that of both *COL2A1* and *ACAN* (**Figure 7.3A**). Pediatric articular cartilage and growth plate had high expression of *ZNF648*, *ACAN* and *COL2A1*, whereas in primary human articular chondrocytes (hACs) obtained from adult patients, expression of *ZNF648* was lost (**Figure 7.3B**). Proteoglycan-4 (*PRG4*) expression was positive only in articular cartilage tissue and cells. Human articular chondrocyte cells did express *HIST2H4*, a marker of proliferation, which was higher than in the native tissues. In addition, histological analysis revealed *ZNF648* was expressed in both growth plate and chondrosarcoma tissues, but was not present in epiphyseal bone, comparable to *COL2A1* (**Figure 7.3C**).

We next sought to determine if the cartilage specific expression of *ZNF648* found in humans held true in mice. Wild type (WT) C57BL/6J mice were sacrificed at 1 week and 12 weeks of age, and qPCR gene expression analysis was performed on multiple tissues (**Figure 7.4A and B**). *Zfp648* the mouse homolog of *ZNF648* was highly specific for articular cartilage in mice and followed a similar expression pattern to *Col2a1* and *Acan*. Interestingly, we observed that expression of *Zfp648* and *Col2a1* decreased in skeletally mature mice (12 weeks old) compared to younger animals (3 weeks old). Further comparison was performed between mouse immature articular chondrocytes (mACs) extracted from 1-week old mice and incubated in culture for 1 week, and ATDC5, a mouse immortalized chondrocyte cell line (**Figure 7.4C**). Robust expression of *Zfp648* was found in immature mouse chondrocytes whereas the expression was low in ATDC5 immortalized cells.

Figure 7.3 (A) Gene expression analysis of cartilage markers and *ZNF648* on bone (blue bars) and cartilage (orange bars) tissues. **(B)** Gene expression analysis performed on pediatric articular cartilage (AC), growth plate (GP), and bone and compared to human articular chondrocytes (hACs). **(C)** Histological analysis of *ZNF648* and *COL2A1* on human GP fibula, chondrosarcoma tissue and epiphyseal bone. All data represented by mean $\pm$ STDEV. Three biological replicates ($n=3$) and three technical replicates were used. Statistical analysis was noted by * if p -value <0.05 and ** if p -value <0.01 .

7.3.3 Expression of *ZNF648* is abundant in primary chondrocytes whereas its expression decreases during cell culture and passaging *ZNF648* expression in primary cultures of chondrocytes derived from growth plate and articular cartilage was analyzed during cell culture and passaging. We observed that while cells started proliferating more during passaging, they lost the expression of *ZNF648* and chondrogenic marker *COL2A1* (**Figure 7.5A**).

Figure 7.4 (A, B and C) Gene expression analysis of cartilage markers and *Zfp648* was performed on mouse tissues, immortalized chondrocytes (ATDC5) and mouse immature articular chondrocytes (mACs). All data represented by mean ± STDEV. Three biological replicates (n=3) and three technical replicates were used. Statistical analysis was noted by * if *p*-value <0.05 and ** if *p*-value <0.01.

A comparison of allograft healthy cells (**Figure 7.5 B**), OA chondrocytes (**Figure 7.5 C**) and growth plate chondrocytes (**Figure 7.5 D**) revealed that after 1 week in culture, the expression of cartilage markers *COL2A1*, *ACAN* and *SOX9*, and *ZNF648* decreased in all cell cultures. In addition, *ZNF648* mRNA expression was 10 times lower in OA chondrocytes than in healthy chondrocytes. In contrast, chondrocytes from articular cartilage seemed to proliferate more (e.g. *HIST2H4*

expression increases) while a fibroblastic phenotype (e.g. *COL1A1* expression increased) was induced. Growth plate chondrocytes did not proliferate in culture.

Figure 7.5 (A) *ZNF648* expression decreases in passaged cells. **(B, C and D)** Growth plate and articular chondrocytes cultured for one week in high-density pellet. All data represented by mean ± STDEV. Three biological replicates (n=3) and three technical replicates were used. Statistical analysis was noted by * if *p*-value <0.05 and ** if *p*-value <0.01.

7.3.4 *ZNF648*-mCherry plasmid overexpression in HEK293T cells induced *ACAN* expression and cell proliferation.

Overexpression experiments were performed by transfecting HEK293T cells using 2, 4 and 5µg of an RFP-*ZNF648* fusion construct (Origene) (**Figure 7.6.A**). An increased in nuclear localization of the fusion protein was seen while negative

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control showed whole cell staining (**Figure 7.6.B**). Protein lysates from the same experiment were analyzed. Lanes 1 to 3 showed no positive staining of ZNF648-specific antibody in the negative control transfected cells. In contrast, ZNF648-mCherry transfected cells (Lanes 4 to 6) exhibited significant ZNF648 positive staining (**Figure 7.6C**).

Figure 7.6 (A) ZNF648-mCherry plasmid vector used for overexpression experiments. (B) Fluorescent microscopy from dose response experiments showing specific nuclear localization of cells transfected with the ZNF648-mCherry plasmid. (C) A ZNF648-mCherry fused protein was observed at 100kDa in western blotting analysis, and only in cells transfected with the plasmid. (D) MTS assay and Hoechst showed no toxicity from the transfection.

Two specific ZNF648 bands were observed in the blot with two different molecular weights, one band at 72kDa and another band at 100kDa. The ZNF648 protein had a predicted molecular weight of 72kDa. However, ZNF648-mCherry

fusion protein had a predicted molecular weight of about 100 kDa (72kDa of ZNF648 plus 37kDa of mCherry). The faint band observed at about 72 kDa may represent some protein degradation.

Figure 7.7 RT-qPCR showed up-regulation in mRNA expression of *ZNF648* and *ACAN* while down-regulating *COL1A1* expression in transfected cells. Increased expression of *MKI67* suggested an effect on proliferation of *ZNF648* overexpression. The induction of *COL2A1* and *ACAN* expression occurred independent of *SOX9*, which was not induced after overexpression. All data represented by mean ± STDEV. Statistical analysis (*ZNF648* versus control) was noted by * if *p*-value <0.05 and ** if *p*-value <0.01.

GAPDH was observed at 37kDa and was used as a housekeeping control. Cell metabolic activity was evaluated at 24 hours and 72 hours after transfection.

Cellular density was quantified using Hoechst staining. At both time points, no toxicity of ZNF648-mcherry overexpression was observed (**Figure 7.6.D**).

After 72 hours of transfection, a higher mRNA expression of *ZNF648* was observed in cells transfected with the RFP-ZNF648 plasmid. *COL2A1* expression remained steady, but increased significantly with the use of 4µg of RFP-ZNF648 plasmid when compared to the control (**Figure 7.7**). In addition, *ACAN* and *MKI67* showed higher expression in cells transfected with RFP-ZNF648. In contrast, no expression of cartilage transcription factor *SOX9* and a lower expression of *COL1A1* were observed in cells overexpressing *ZNF648*. No changes in proliferation of the cells were suggested by the steady expression of *CCNB2* in both conditions. Thus, overexpression of *ZNF648* was sufficient to induce the up-regulation of *ACAN* expression irrespective of *SOX9* expression whilst decreasing the expression of *COL1A1*, suggesting a down-regulation of a fibroblastic signaling in the cells.

7.3.5 ZNF648 overexpression regulates specific genes in human articular cartilage and growth plate chondrocytes.

To further study the subcellular localization of ZNF648, overexpression experiments were performed by transfecting HEK293T cells for 72 hours using 5µg of ZNF648 tagged with eGFP (Origene) (**Figure 7.8.A**). ZNF648 and housekeeping gene (GAPDH) expression were compared side by side in nuclear extracts, cytoplasmic extracts, and whole cell lysates (**Figure 7.8.B**). Non-transfected cells (Lane 1) and negative control plasmid transfected cells (Lanes 2 to 4) did not stain with a ZNF648 specific antibody. Cells transfected with ZNF648-eGFP showed positive staining in whole cell lysates and nuclear extracts, whereas no staining was observed in the cytoplasmic extracts. Thus, our data demonstrated that chondrocyte specific transcription factor ZNF648 is a 72-kDa protein that localizes in the nucleus. GAPDH was observed at 37kDa and was used as a housekeeping control.

Next, we explored the effect of overexpression of ZNF648 in human primary chondrocytes from articular cartilage and growth plate. Both cells were obtained from healthy pediatric tissue and were from the same donor. In untransfected cells, ZNF648 positive staining was localized in both nucleus and cytoplasm. This was likely due to the endogenous levels of ZNF648 in human primary chondrocytes. Forced overexpression of ZNF648 plasmid reduced COL2A1 expression (green, **Figure 7.8C**) in articular chondrocytes compared to the negative control, whereas forced overexpression in growth plate chondrocytes increased COL2A1 staining (green, **Figure 7.8D**) compared to the negative control.

Figure 7.8 (A) Plasmid vector containing ZNF648 tagged with eGFP used for overexpression experiments. (B) Nuclear localization of ZNF648 protein in HEK293T cells overexpressing ZNF648. (C and D) Overexpression experiments in human articular cartilage and growth plate chondrocytes showing decreased expression of collagen type II in articular chondrocytes and increased expression in growth plate chondrocytes.

7.3.6 Knockdown experiments in human primary chondrocytes imply *ZNF648* regulates *COL2A1* expression.

We performed further work to understand the cellular effects resulting from an absence of *ZNF648*. In articular chondrocytes, knockdown of *ZNF648* was observed and resulted in loss of *COL2A1* and *MKI67* expression. This suggested that *ZNF648* positively regulates expression of key cartilage genes and cell cycle and/or proliferation of the cells (**Figure 7.9.A**).

Figure 7.9 (A) Articular chondrocytes were transfected with a *ZNF648* siRNA. *ZNF648* loss was followed by decrease of *COL2A1* and proliferation marker *MKI67*. In contrast, *COL1A1* and *ACAN* were up regulated. (B) *ZNF648* siRNA transfection in growth plate chondrocytes obtained from human pediatric patients was performed. *ZNF648* loss was followed by a decrease in *COL2A1* expression, whereas *COL1A1* and *MKI67* expression were increased. (C and D) No toxicity was observed from the transfection as assessed using an MTS assay. All data represented by mean $\pm$ STDEV. Statistical analysis was noted by * if p -value <0.05 and ** if p -value <0.01 .

In contrast, an increased expression of *ACAN* and *COL1A1* was observed in the absence of *ZNF648*. Similar results were found for growth plate chondrocytes (**Figure 7.9.B**). Knockdown of *ZNF648* decreased *COL2A1* expression while increasing *COL1A1*. However, in growth plate chondrocytes the absence of *ZNF648* increased the expression of proliferation marker *MKI67* while *ACAN*

expression was steady. Hence, *ZNF648* correlated with *COL2A1*, but not *ACAN* and *MKI67* expression in either chondrocyte cell type. No toxicity was observed from the transfection as assessed using an MTS assay. (Figure 7.9 C and D).

7.3.7 ZNF648 controls COL2A1 induction in human immortalized TC28A2 cells and chondrosarcoma cell line L835.

ZNF648 knock down experiments were also performed in human immortalized TC28A2 cells and human L835 chondrosarcoma cell line. Due to the absence of *ZNF648* in TC28A2, overexpression experiments were conducted.

Figure 7.10 (A) TC28A2 human immortalized chondrocytes transfected with a ZNF648 expression vector showed increased expression of *COL1A1*, *COL2A1* and *MKI67* expression. (B and E) Metabolic activity assay did not show any signs of toxicity on the cells. (C) Increased protein expression of specific ZNF648 antibody was shown in overexpressed TC28A2 cells. (D) Human chondrosarcoma cell line L835 transfected with *ZNF648* siRNA decreased expression of *ZNF648*, followed by a decreased in *COL2A1* and *MKI67*. However, *COL1A1* and *ACAN* expression increased in *ZNF648* depleted cells. All data represented by mean ± STDEV. Statistical analysis was noted by * if *p*-value <0.05 and ** if *p*-value <0.01.

RNA was collected and extracted after 72 hours of transfection. TC28A2 cells showed overexpression of *ZNF648* and simultaneously increased *COL2A1*, *COL1A1* and *MKI67* expression (**Figure 7.10 A**). However, *ACAN* expression decreased with *ZNF648* overexpression. Protein analysis depicts an increase in *ZNF648* expression (Lanes 4 to 6) in TC28A2 cells overexpressing *ZNF648*. Lower expression was detected in non-transfected cells. Collagen type II expression followed the trend of *ZNF648* (**Figure 7.10 C**). GAPDH was used as a sample loading control. In addition, knock down experiments in chondrosarcoma cell line L835, showed a decreased of *ZNF648* gene expression followed by down-regulation of *COL2A1* and *MKI67*. In contrast, *ACAN* and *COL1A1* expression increased in the absence of *ZNF648* (**Figure 7.10.D**). MTS assays showed no toxic effects of the transfection on the cells (**Figure 7.10 B and E**). Immortalized cells showed a similar gene expression trend compared to human primary cells, implying a positive association between *ZNF648* and *COL2A1* expression, and a negative association with *COL1A1* expression. Consequently, *ZNF648* seems to regulate *COL1A1* and *COL2A1* expression in both, primary cells and in immortalized tumor cells.

7.3.8 *ZNF648* mouse homolog *Zfp648* regulates *Col2a1* induction in ATDC5 and in immature mouse articular chondrocytes.

Transfection experiments were performed on immortalized mouse chondrocytes and mouse articular chondrocytes. Due to the absence of *Zfp648* in ATDC5, overexpression experiments were conducted instead. RNA expression analysis conducted using RT-qPCR showed overexpression of *Zfp648* in ATDC5 increased *Col2a1* whilst decreasing expression of *Col1a1* and *Mki67* (**Figure 7.11 A**). Western blot analysis showed *Zfp648* was increased in transfected cells (Lanes 3 to 6) compared to control (Lanes 1 to 3). We did not detect *Col2a1* expression in ATDC5 (**Figure 7.11 C**).

Figure 7.11 (A) ATDC5 mouse immortalized chondrocytes transfected with a Zfp648 expression vector showed increased expression of *Col2a1* while decreasing *Col1a1* and *Mki67*. (B and E) No signs of toxicity on the cells were observed. (C) Increased ZFP648 protein expression was shown in overexpressed cells only. (D) Human chondrosarcoma cell line L835 transfected with ZNF648 siRNA decreased expression of *Zfp648*, which was associated with a decrease in *Col2a1* and *Mki67*. In contrast, *Col1a1* and *Acan* expression increased in *Zfp648* depleted cells. (F) Depletion of Zfp648 and Col2a1 was observed in Zfp648 siRNA transfected cells. All data represented by mean $\pm$ STDEV. Statistical analysis was noted by * if *p*-value <0.05 and ** if *p*-value <0.01.

Likewise, *Zfp648* knockdown in mouse articular chondrocytes decreased *Zfp648* mRNA expression and down-regulated *Col2a1*. In contrast, *Col1a1* and *Mki67* were increased in the absence of *Zfp648* (Figure 7.11 D). Protein analysis showed depletion of Zfp648 and collagen type II expression in Zfp648 siRNA transfected cells (Lanes 3 to 6) compared to control (Lanes 1 to 3) (Figure 7.11 F). No differences in MTS metabolic activity were observed (Figure 7.11 B and E). Thus, both immortalized and mouse primary cells mouse followed the trend previously observed in human TC28A2 and chondrosarcoma cells. *Zfp648* overexpression induced *Col2a1* expression whereas inhibition decreased *Col2a1* expression. An up-regulation of *Mki67* proliferation marker may be an indicative for increased proliferation.

7.3.9 Zfp648 null mice survive beyond birth and do not have overt skeletal defects.

The effect of Zfp648 ablation in mice was determined. Homozygous Zfp648 null mice survived beyond birth. No overt skeletal defects were observed after 12 weeks (**Figure 7.12 A and B**). Weight was obtained at three different time points, no difference among the phenotypes was observed (**Figure 7.12 C**). Zfp648 expression was increased over time in wild type (WT) and was depleted in cartilage of null mice (KO) (**Figure 7.12 D**). In mouse cartilage native tissue an antagonistic effect for *Col2a1* and *Col1a1* was observed.

Figure 7.12 (A and B) Skeletal defects were not observed in null mice. **(C)** Null mice, heterozygous and wild types were weighted after 3, 6 and 12 weeks. No differences in weight among the phenotypes were observed. **(D)** RNA expression of *Zfp648* shows the depletion in null mice. In mouse cartilage native tissue an antagonistic effect for *Col2a1* and *Col1a1* was observed. Data represented by mean $\pm$ STDEV. Statistical analysis was noted by * if p -value <0.05 and ** if p -value <0.01 .

7.3.10 Young Zfp648 null mouse exhibit reduced skeletal size and growth plate width with a delayed secondary ossification center and disorganization of growth plate zones.

To assess the skeletal phenotypic differences of Zfp648 null mice at earlier time points, 2-week-old null and wild type mice were sacrificed. In **Figure 7.13.A** we

observed that RNA expression of *Zfp648* was lost in null mice. A reduced expression of *Col2a1* and *Acan* was also observed. Radiographic analysis on both phenotypes revealed a reduced skeletal size (whole body, femur and tibial length) in *Zfp648* null mice when compared to wild type mice. In contrast, a higher growth plate width was observed in *Zfp648* null mice (**Figure 7.13.B**).

Figure 7.13 (A) RNA expression shows decreased of *Zfp648*, *Col2a1* and *Acan* in 2-week old *Zfp648* null mice. **(B)** Radiographic analysis showed a reduced skeletal size shown by reduced in whole body, femur and tibial length. In addition, growth plate height was significantly increased in null mice. Secondary ossification center measured in the radiographies showed a decrease in area and width in null mice. All data represented by mean ± STDEV. Statistical analysis was noted by * if *p*-value <0.05 and ** if *p*-value <0.01.

Secondary ossification center area and width, were measured from the radiographies, and showed a decreased in null mice compared to wild type.

Histological analysis performed on mice tibias of 2-week-old pups using Safranin O/Fast green/Hematoxylin staining revealed a delay in the onset of secondary ossification center formation in Zfp648 null mice (**Figure 7.14.A**). A lack of mineralized tissue in the location of the secondary ossification center was also observed by Alcian blue/Eosin staining. A cartilage specific staining was observed all over the growth plate zone in null mice, whereas growth plate and articular cartilage were well defined in wild type mice.

Figure 7.14. (A) Safranin O/Fast green/ Hematoxylin staining and alcian blue/eosin showed differences between wild type and null mice at 2 weeks of age. Scale bars (black bars) indicate 100μm. **(B)** Disorganization of growth plate zones and differences in their lengths were observed and measured. Scale bars (white bars) indicate 200μm. Measurements were represented by mean ± STDEV. Statistical analysis was noted by * if p -value <0.05 and ** if p -value <0.01. **(C)** Immunohistochemistry revealed specific expression of cartilage marker Col2a1 overall growth plate zones in null mice. In contrast, Col1a1 expression was absent. Mki67 expression depicted an increase of proliferation in all zones. Scale bars (black bars) indicate 100μm.

In **Figure 7.14.B** we observed the difference in growth plate zonal distribution in null mice. There was no articular cartilage defined and low mineralization was observed in the zone of the secondary ossification center. Two hypertrophic chondrocyte zones were observed in the null mice and were measured. Measurements exposed the increase in length of all growth plate zones in Zfp648 null mice. Immunohistochemistry using specific antibodies for Col2a1, Col1a1 and Mki67 was performed to assess differences in cartilage specific staining (**Figure 7.14.C**). Wild type mice showed specific staining of Col2a1 in the growth plate and articular cartilage; whereas surrounding mineralized tissue was specifically stained with Col1a1. Mki67 was not expressed in the WT mice. Null mice showed an overall staining of growth plate with Col2a1 and no staining with Col1a1. Faint Col1a1 staining was found at the upper part of the tibia, where the articular cartilage should be located. In addition, Mki67 specific staining was found along the growth plate showing the overall increase in proliferation in the growth plate zone on this phenotype.

7.4 DISCUSSION

The clinical need to develop new cartilage repair strategies arises from the fact that cartilage has a low self-repair capacity and is not suitable to respond to traumatic injury. Therefore, these injuries often result in altered biomechanics of the joint and joint instability. The mechanism by which articular cartilage homeostasis is maintained and is able to resist hypertrophic differentiation and subsequent endochondral ossification remains unknown. Thus, the complex molecular events resulting in the induction and maintenance of the chondrogenic phenotype during embryogenesis, may lead to the identification of novel mechanisms involved in cartilage formation. Fundamental knowledge of these mechanisms might prove transcription factors such as Zinc Finger 648 (ZNF648) protein to be crucial for cartilage repair strategies.

RNA-seq analysis revealed a unique expression of *ZNF648* in all cartilaginous tissues. Primary growth plate cells showed a higher expression of this transcription factor when compared to primary articular chondrocytes. Fagerberg L et al. previously published an RNAseq analysis performed on normal tissues derived from 95 human individuals representing 27 different tissues but lacking cartilage tissue [17]. They found *ZNF648* expression in testis and ovaries at substantially lower values of 0.100 and 0.125 RPKM, respectively, compared to values ranging between 0.5 and 3 RPKM in our data set. In liver and skin, even lower levels of *ZNF648* were found. We therefore concluded that *ZNF648* is highly specific for cartilaginous tissues with minor expression in reproductive organs.

ZNF648 belongs to the transcription factor family C2H2-ZNF that is found only in eukaryotic species. Zinc finger proteins (ZNF) are mostly expressed in human and mouse. It has been suggested that these can act alone or partner with other transcription factors (TFs) to create distinct functionalities [18]. These TFs constitute the single largest family of regulators in the human genome (~400 genes). A large part of these TFs have a tissue-specific expression, suggesting that they control processes that are characteristic of individual tissues. For example *ZNF337* is highly expressed in fetal brain, which suggests an involvement in brain development. However, the biological functions of most of these TFs are unknown [19]. In our study, this *ZNF648* protein seems to maintain normal differentiation of pre-committed chondrogenic mesenchymal progenitor cells into chondrocytes in the growth plate, whereas in later stages of cartilage maturation *ZNF648* expression is reduced. The highly expression of *ZNF648* in growth plate suggests an involvement of in cartilage formation.

Although the expression of *ZNF648* is present in primary tissues, it is significantly reduced when cells are removed from the native tissue environment. The depletion of *ZNF648* expression was always associated with a

reduction in *COL2A1* expression. In the case of articular chondrocytes it was interesting to observe that throughout the passaging of these cells, *SOX9* and *ACAN* expression were enhanced. This phenomenon was not observed in cells cultured *in vitro*. Interestingly, an enhanced expression of *ZNF648* increased collagen type II expression while decreasing collagen type I. This concept would be ideal for formation of hyaline cartilage over fibrocartilage in tissue regeneration strategies. Still, it is unclear if *COL2A1* is a direct target of *ZNF648*. Further study will help elucidate *ZNF648* signaling mechanisms for cartilage development. Previously a list of target genes of transcription factors predicted using known transcription factor binding sites by computational association, showed *SOX2*, *SOX5*, *ARNT* (*HIF1B*), *RUNX2*, *SMAD2/3*, *HIF1A*, *POU5F1*, *HAPLN1*, *GLI1*, *GLI2*, *GLI3* and *SOX9*, among the predicted targets of transcription factors *ZNF648* [22-25]. In addition, among the predicted pathways of *ZNF648* they found glycosaminoglycan degradation and biosynthesis, cell cycle, fatty acid biosynthesis, WNT signaling, Hedgehog signaling and signaling pathways regulating pluripotency of stem cells [23]. The differential gene expression observed in our study between growth plate and articular chondrocytes could indicate a regulatory mechanism of *ZNF648* to promote chondrogenesis and cartilage formation; this could occur by regulation of other cartilage specific genes and not necessarily of *COL2A1*, but by interacting with one or various pathways involved in cartilage development, including *SOX9* and *RUNX2*.

Some of the ZNF proteins act also as repressor, depending of the domain on the N-terminal site (e.g *ZNF133* and *ZNF 140*) [20], whereas they are also involved in cell differentiation, cell proliferation, apoptosis, and neoplastic transformation [21]. In addition, a couple of ZNF proteins have been previously linked with chondrogenesis. *ZNF219* and *ZNF449* were both described as implicated in *SOX9* mediated chondrogenesis [30, 31]. Whole mount *in situ* hybridization experiments indicated that *Zfp219* was specifically expressed in developing

limbs where Col2a1 and Sox9 were strongly expressed. Zfp219 markedly enhanced the transcriptional activity of Sox9 on the Col2a1 gene promoter, suggesting Zfp219 as important in the regulation of chondrocyte differentiation as a transcriptional partner of Sox9 [30]. Also, ZNF proteins are thought to integrate numerous signals through the action of microRNAs. Target genes of microRNAs were predicted by searching genes for sites matching conserved and nonconserved miRNA seed regions. They predicted miR-210 as one of the miRNAs involved with ZNF648 [26- 28]. In Chapters 4 and 5, we exhibited miR-210 as essential for chondrogenesis of stem cells. The interaction of miR-210 and ZNF648 could indicate a regulatory mechanism to induced or repress the expression of COL2A1 and ACAN.

Previously, predicted mouse and human phenotype were described in the literature [22-25]. Cartilage related phenotypes were associated with ZNF648 loss of expression. Among them, abnormal nose, ear, trachea and larynx morphology, abnormal embryonic tissue, abnormal embryogenesis phenotype were associated in mouse; whilst polydactyly, abnormal bone, short limb and short stature, and abnormal ear and nose morphology were linked to the human phenotype. Remarkably, the phenotype obtained from 2-week-old null mouse indicates an important role of this transcription factor in the development of long bones and during endochondral ossification stages. A mineralized cartilage instead of bone was observed between articular cartilage and growth plate. Furthermore, overexpression of ZNF648 in cells induces up-regulation of MKI67 that was also observed in Zfp648 null mice. These observations may implicate ZNF648 in the regulation of chondrocyte proliferation.

In this study we aimed to analyze driving molecular factors such as transcription factor ZNF648 that may directly regulate cartilage development or homeostasis through modulating gene expression and other cartilage-related signaling pathways. Thus, development of novel approaches to improve cartilage

regeneration from a mechanistic and molecular point using ZNF648 to maintain cartilage homeostasis is thought to play an essential role in future clinical therapies.

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CHAPTER 8

8. Summary, General Discussion and Outlook

8.1 Mesenchymal Stem Cells versus Medicinal Signaling Cells

Over the past decade, tissue engineering has become the prospective solution from defects resulting from severe trauma, osteodegenerative diseases and mechanical stress [1]. Tissue engineering requires the use of different cell types, scaffolds, growth factors and genetic stimuli to repair or reconstitute articular cartilage [2]. However, choosing an adequate cell type for these strategies is crucial. Two different notions about the use of MSCs have been described. The first notion proposes the term of medicinal signaling cells (MSCs) and relies in the trophic functions of MSCs where these cells survey the damage, isolate foreign components, stabilize the injured tissues, provide antibiotics, anti-inflammatory components and pro-regenerative components and encysting protection before a medicinal sequence can be initiated to regenerate the damaged tissue [3, 4]. In the second notion, the multilineage capacity and differentiation potential of MSCs is exploited to provide new chondrocytes.

Sixteen years after stem cells were first discovered, Urist et al. introduced the term ‘mesenchymal stem cells’ to highlight the multilineage differentiation potential of these cells [5-6]. Collectively, his work showed that these cells could differentiate into cartilage, bone and fat. Since then, several cell therapies, MSC based products and different treatments have been studied. Around 3,000 research articles referring to MSCs have been published every year. However, since 2010, Caplan retracted from his initial label of calling MSCs stem cells and proposed a new name: Medicinal Signaling Cell. This name change was based on the ability of these cells to secrete proteins that modulate the immune response, reduce inflammation, promote wound healing and inhibit cell death [4]. Previous data confirmed that the implantation of MSCs *in vivo* into cartilage defects, induced desirable healing outcomes in treated patients [7]. Riester et al. showed that injection of aMSCs in early stages of OA inhibited synovial thickening and cartilage loss [8].

Although, the notion of these cells to differentiate *in vitro* is still valid, their heterogeneous behavior represents a negative factor for the *in vivo* treatments [1]. In this thesis we used the potential of MSCs to differentiate into chondrocytes and presented different molecular mechanisms to enhance their *in vitro* differentiation potential. The overall goal was to pre-program the cells before implantation to improve *in vivo* results. However, or biggest limitation lies in that no *in vivo* validation of these strategies was performed.

8.2 Hypoxia improves *in vitro* chondrogenic differentiation of MSCs.

The mechanism by which articular cartilage homeostasis is maintained and is able to resist hypertrophic differentiation and subsequent endochondral ossification remains unknown. Thus, the complex molecular events occurring in the induction and maintenance of the chondrogenic phenotype during embryogenesis may lead to the identification of novel mechanisms involved in cartilage formation. The importance of the hypoxic environment for cartilage tissue engineering was highlighted in **Chapter 2**. Pre-programming the cells in the cartilage native physiological conditions may be helpful for enhancing the chondrogenic phenotype while preparing the cells for implantation.

In **Chapter 3**, the chondrogenic differentiation potential of aMSC expanded in human platelet lysate was examined. This study differed from previous aMSCs studies because of the use of a non-xenogeneic culture serum, which is applicable for clinical translation. Our findings indicated that genetic programming of aMSCs to a chondrocytic phenotype is effective in cellular aggregates (pellet culture) under hypoxic conditions as reflected by increased expression of cartilage-related biomarkers and biosynthesis of a glycosaminoglycan positive matrix. These results were also found by Leijten and colleagues during chondrogenic differentiation of bone marrow derived bMSCs [9]. Formation of cartilaginous tissues with sufficient collagen type II and proteoglycans is essential to reconstruct the cartilage with restored functions. The oxygen status

was a dominant environmental parameter for directing chondrogenic differentiation of aMSCs and reduced their tendency to generate a fibroblastic ECM, while helping chondrocytes to maintain their phenotype by enhancing. Hence, hypoxia was more effective in promoting a chondrocytic phenotype at earlier stages of differentiation.

Furthermore, **Chapters 4 and 5** studied the importance of signaling molecules such as miRNAs for cartilage development, maintenance, and destruction [10-13]. We proposed that hypoxia stimulated chondrogenesis of MSCs is mainly driven by upregulation of miR-210. Functional experiments revealed miR-210 importance for early chondrogenesis of MSCs during *in vitro* cultures. Exogenous miR-210 expression can be used instead of chondrogenic differentiation cocktail in standard or low oxygen cultures to promote chondrogenesis of MSCs. Remarkably the use of miR-210 mimic does not present negative side effects such as increased in *COL1A1* expression or induction of hypertrophic markers compared to TGFβ1. This potential treatment can help regenerating hyaline cartilage whilst preventing formation of fibrocartilage *in vivo*. Although positive results were achieved from previous experiments, there still a need to validate that this degree of differentiation achieved *in vitro* is stable for implantation, and will maintain the phenotype and functionality of the cells over time.

8.3 Synovial fluid is an alternative culture serum for *in vitro* expansion of MSCs and chondrocytes for clinical therapies

Fetal bovine serum (FBS) has long been the gold standard medium supplement for laboratory-scale MSC culture and other cells. However, FBS has a poorly characterized composition and poses risk factors, as it may be a source of xenogenic antigens and zoonotic infections [14]. The need for an alternative is known to scientists and users involved in cell therapy or advanced therapy medicinal products.

Since concerns have been raised regarding the safety of FBS other serums have been proven with some success [15]. As an alternative, platelet lysate (PL) can be an adequate non-xenogenic alternative for fetal calf serum. However, PL triggers an inflammatory response in MSCs and induces the secretion of factors maintaining macrophages in a pro-inflammatory state [16]. In **Chapter 6**, SF was used as an alternative for both FBS and PL. We examined if it could enhance, limit or reject stem cells or chondrocytes upon implantation. Our data indicated that synovial fluid enhances the metabolic activity of chondrocytes and aMSCs at a level that is at least equivalent to FBS and better than human alternative PL. Thus, we found synovial fluid to be a promising novel culture supplement for chondrocyte and aMSCs expansion and due to its non-zoonotic (animal free) nature is therefore potentially better for clinical applications in cartilage regeneration. However, the limitations of synovial fluid were highlighted in the chapter. The differences found between the SF samples during cell culture, suggested further normalization of the samples would be essential to preserve both stem cells and chondrocytes viability and maintenance. Because concentrations of synovial fluid protein are known to vary with the degree of joint inflammation, comparing the protein profile to determine potential key components that could influence cell culture is important for the normalization of the synovial fluid. Thus, correlation between viability, protein content and cytokines profile could help for the stratification of samples for future therapeutic use.

8.4 Cartilage specific transcription factors could improve cartilage regeneration and prevent osteoarthritis development.

The complex molecular events occurring in the induction and maintenance of the chondrogenic phenotype during embryogenesis may lead to the identification of novel mechanisms involved in cartilage formation. Fundamental knowledge of

these mechanisms might prove transcription factors such as Zinc Finger 648 (ZNF648) protein to be crucial for cartilage repair strategies (**Chapter 7**).

ZNF648 that belongs to the transcription factor family C2H2-ZNF is part of a large family of TFs, which have tissue-specific expression and are thought to control processes that are characteristic of individual tissues. However, the biological functions of most of these TFs are unknown [19]. In our study, cartilage-specific transcription factor ZNF648 protein seems to maintain normal differentiation of pre-committed chondrogenic mesenchymal progenitor cells into chondrocytes in the growth plate, whereas in later stages of cartilage maturation *ZNF648* expression was reduced.

Further study will help elucidate ZNF648 signaling mechanisms for cartilage development. Previously a list of potential target genes of ZNF648 exhibited several cartilage related genes including SOX2, SOX5, HAPLN1, GLI1, SOX9, RUNX2, while targeting hypoxia related genes ARNT (HIF1B), HIF1A [22-25]. The differential gene expression observed in our study between growth plate and articular chondrocytes, could indicate a regulatory mechanism of *ZNF648* to promote chondrogenesis and cartilage formation; this could occur by regulation of other cartilage specific genes and not necessarily of *COL2A1*, but by interacting with one or various pathways involved in cartilage development, including *SOX9*, *RUNX2* and the hypoxic pathway.

8.5 Concluding remarks and future perspectives.

Tissue engineering offers possibilities for optimization of cartilage repair by combining different cell types, biomaterials, and growth factors for the support of cartilage regeneration [17]. Despite the efforts, current cell-based repair strategies have been proven unsuccessful for treating large cartilage defects and osteoarthritic lesions. Proper regeneration of this tissue remains an unresolved question and no standardized clinical protocol has yet been established.

Engineered cartilage using MSCs may become hypertrophic resulting in the production of bone-like or fibrotic tissues once implanted [18]. Several studies have attempted to understand the mechanisms by which MSCs can be programmed for chondrogenic differentiation while avoiding further progression into hypertrophic cartilage [19, 20]. The physiological conditions in the articular cartilage are very complex consisting of low oxygen environment and repeated mechanical loading. These non-genetic strategies have become more attractive due to their easiness for manipulation. For *in vitro* cartilage engineering strategies, a delicate balance must be reached between the stimulatory and inhibitory effects of the low supply of oxygen. Although some biomaterials used in cartilage regeneration strategies mimic a hypoxic microenvironment for the cells, the cells are still subjected to normal oxygen levels in culture. However the equipment required to maintain the cells in a hypoxic microenvironment is often expensive and space restrictive. As a consequence, it would be interesting to perform *in vitro* chondrogenic differentiation of MSCs using a regulatory mechanism that would mimic the hypoxic response on the cells without the need of low oxygen cultures. This regulatory mechanism would also serve to avoid the dedifferentiation of the cells; enhance chondrogenic differentiation *in vitro* before implantation in the patient and /or to prevent the loss of chondrocyte phenotype during culture expansion.

In this thesis we studied different molecular mechanisms to potentiate chondrogenesis of MSCs during culture by achieving a similar gene expression and protein deposition to human primary chondrocytes. To validate the relevance of these molecular mechanism, their effects on human primary chondrocytes were also studied. Hypoxia, synovial fluid and transcription factor ZNF648 seemed relevant not only for chondrogenesis of MSCs but also to maintain the chondrocyte's phenotype. In **Figure 8.1** a summary of the findings

encountered during the development of this thesis will put in perspective the molecular mechanisms found and its interactions.

Figure 8.1 Molecular mechanisms modulating chondrogenesis. Up-regulated genes (red box), down-regulated genes (blue box) and inconclusive genes (green box). Dotted lines denoted molecular mechanisms suggested in the literature but not validated during the thesis development. For normoxia, addition of TGFβ1 or exogenous miR-210 (eMiR-220) was denoted by a + symbol.

aMSCs differentiated under serum containing media with TGFβ1 and hypoxic conditions was found to increase the expression of *COL2A1* and *ACAN*, but also increased the expression of hypertrophic markers. However, under hypoxic conditions, miR-210 was observed to be the main driver of chondrogenesis of stem cells, and proved that its induction enhances chondrogenic differentiation as strong as TGFβ1 addition in stem cells. Higher COL2A1/COL1A1 ratio on cells

overexpressing miR-210 when compared to TGFβ1 induction, suggested miR-210 could decreased the cells fibroblastic properties.

Althought miR-210 has been largely studied in relation to hypoxia, recently has been also linked to cartilage. Lin L et al. found miR-210 among the highly expressed miRNAs in human primary articular chondrocytes at early passages [22], whereas Zhang Z et al. and Georgi N et al. found miR-210 expressed during chondrogenic differentiation of aMSCs and bMSCs respectively [23, 24]. miR-210 has been related to RUNX2, SMAD 4, SMAS 5 and SOX2, which are all linked to chondrogenesis [27]. *In vivo* administration of miR-210 by intra-articular injection effectively promoted the healing of partly damaged anterior cruciate ligaments (ACLs) [28]. However, the efficacy to restore articular or growth plate cartilage *in vivo* is unknown. Microarray analysis found miR-210 down-regulated in OA cartilage compared with normal cartilage and suggested miR-210 as a negative regulator of the progression of OA, which increases chondrocyte proliferation and prompts extracellular matrix deposition by directly targeting HIF-3α [25, 26]. In our study, we found that exogenous addition of miR-210 to MSCs *in vitro* promoted the expression of COL2A1 and ACAN while inhibiting the expression of COL1A1. However, we did not analyzed any of our cultures after 28 days, nor investigate its potential *in vivo* outcome.

Despite that miR-210 seems like a good candidate to promote chondrogenic differentiation, collagen type II and ACAN levels are still significantly lower than those from human primary chondrocytes. Consequently, the study of a transcription factor (ZNF648) that could regulate early development of chondrocytes would be essential for cartilage repair strategies. As observed in **Chapter 7**, ZNF648 may be an important regulatory mechanism during early development of the chondrocytes. It increased significantly the expression of collagen type II while decreasing expression of collagen type I in cells overexpressing ZNF648. Therefore, both ZNF648 and miR-210 are beneficial for

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earlier chondrogenesis of cells, suggesting a possible interaction between both factors. Both are significantly enhanced in cartilage native tissue, are expressed in low oxygen conditions and promote collagen type II and aggrecan expression. Target genes predicted miR-210 as one of the miRNAs involved with ZNF648 [26- 28]. Possibly, under low hypoxia, transcription factor ZNF648 could participate in promoting a cascade of genes that also regulate miR-210 expression (e.g. HIF1A). The expression of both factors at a certain time seems important during cartilage development and chondrogenesis. ZNF648 up-regulation will induce genes and proteins important for cartilage maintenance (e.g. SOX9, COL2A1, ACAN) while miR-210 will create an equilibrium by inhibiting expression of cartilage degradation proteins (e.g. RUNX2, COL1A1, COL10A1, MMPs) (**Figure 8.1.**).

Although SOX9 is vastly known as the master regulator of cartilage formation and chondrocyte differentiation, ZNF648 null mice indicated a possible role of ZNF648 during cartilage development and chondrocyte proliferation. Akiyama et al. revealed that SOX9 remains a central determinant for the maintenance of the lineage fate and multistep differentiation program of growth plate chondrocytes, whilst preventing osteoblastic differentiation and allowing hypertrophy during the prehypertrophy stage [30,31]. Our results showed that in immature cells, the decreased expression of *ZNF648* was followed by down-regulation of *SOX9*. However, this was not observed for articular chondrocytes, *SOX9* expression was preserved even when *ZNF648* expression was lost. This suggested similar roles of ZNF648 and SOX9 during embryonic cartilage development, but a different role for cartilage homeostasis and maintenance. SOX9 is necessary both to specify and maintain the lineage choice of the cells and to activate stage-specific markers [32, 33]. We therefore propose, that *SOX9* activates the expression of *ZNF648* during cartilage development. Then, while SOX9 maintains its own expression through positive feedback during further cartilage development stages, once no longer

needed, *ZNF648* expression is inhibited by miR-210 mechanisms in a SOX9 dependent manner.

Taking into account the newer notion of the MSCs as trophic cells once implanted *in vivo*, it would be important to combine both the trophic functions and the differentiation potential to derive better MSCs for cell therapies. If these MSCs are better as signaling cells during injury and help to activate the necessary mechanisms of response during this stage, the co-cultures of these MSCs with primary chondrocytes would be an ideal candidate for cartilage repair. The MSCs would secrete the proteins that modulate the immune response, reduce inflammation, promote wound healing and inhibit cell death [21], while chondrocytes would proliferate and induce cartilage specific matrix production to repair the defect. Using the molecular mechanisms described in this thesis (**Figure 8.1.**) we could further enhance the differentiation potential of MSCs, which in combination with their own trophic effects could enhance therapeutic outcome. However, it is important to understand the interaction between these trophic functions, their differentiation potential and the efficacy after implantation. Lastly, to be used in cell therapies, several parameters need to be studied to optimize and augment therapeutic efficacy including optimal route of administration, the correct dose, the best cell populations and timing of cultures.

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Curriculum Vitae

Catalina Galeano Garces was born on the 11th of June 1990 in Medellin, Colombia. In 2008 she started her studies in Biomedical Engineering at EIA University-CES University in Envigado, Colombia. During her studies she participated in several traineeships in biotechnology and biomechanics. In December 2013 she received her bachelors diploma with an

undergraduate honorable mention. During her bachelor's she moved to France to pursue a master degree in engineering at Ecole Supérieur des Arts et Metiers (Arts et Metiers ParisTech). During her master's she participated as a summer intern at the VU medical center in Amsterdam, The Netherlands under the supervision of Dr. Marco Helder in a project titled "Cell-matrix interactions in pelvic organ prolapse". Then she continued with a Master of Sciences in Tissue Engineering and Tissue Biomechanics from the Paris Institute of Technology (ParisTech). In April 2014 she graduated and obtained her MEng and MSc diplomas. Her master Project was focused on Cell adhesion control on implantable biomedical devices and was performed under the supervision of Dr. Vincent Semetey at the Institut Curie in Paris. Since 2013 she has been working under the supervision of Dr. Andre van Wijnen at the Department of Orthopedic Surgery at Mayo Clinic. In 2015, she started her PhD at the department of Developmental BioEngineering, The Netherlands, under the supervision of Prof. Dr. Marcel Karperien and Prof. Dr. Andre van Wijnen. The subject of her research was the identification of molecular mechanisms that could improve chondrogenesis of stem cells for cartilage regeneration therapies.

List of Publications

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Galeano-Garces C, Galeano-Garces D, Larson AN, van Wijnen A, Karperien M. *Integrated analysis of miRNA-seq and mRNA-seq on hypoxic mesenchymal stem cells reveals the role of hypoxia-regulated miRNAs for promoting chondrogenesis of MSCs*. (Chapter 4). In preparation.

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Galeano-Garces C, Galeano-Garces D, Thaler R, Pichurin O, Bouska G, Milbrandt T, Karperien M, van Wijnen AJ, Larson AN. *Novel transcription factor ZNF648 might regulate cartilage development and homeostasis through the modulation of cartilage related signaling pathways*. (Chapter 7). In preparation.

Abstracts selected for poster presentation

Galeano-Garces C, Camilleri ET, Riester S, Dudakovic A, Kakar S, Westendorf JJ, Smith J, Dietz AB, Cool SM, Im HJ, Krych AJ, Karperien M, and van Wijnen AJ. *Molecular validation of chondrogenic differentiation and hypoxia responsiveness of adipose-tissue derived human mesenchymal stem cells*. International Cartilage Repair Society (ICRS) World Congress 2015, Chicago USA.

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