Cartilage tissue engineering in the inflammatory milieu

A thesis

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Abstract

Cartilage tissue engineering aims to repair damaged cartilage tissue in arthritic joints. As arthritic joints have significantly higher levels of pro-inflammatory cytokines (such as IL-1β and TNFα) that cause cartilage destruction, it is critical to engineer stable cartilage in an inflammatory environment. Biomaterial scaffolds constitute an important component of the microenvironment for chondrocytes in engineered cartilage. However, it remains unclear how scaffold material influences the response of chondrocytes seeded in these scaffolds under inflammatory stimuli. In the present study, the effects of different material derived scaffolds (silk, collagen, and poly lactic acid (PLA)), structure of scaffolds (pore size), and fabrication methods of scaffolds (aqueous derived (AQ) and hexafluoroisopropanol derived (HFIP) silk scaffolds) were investigated on bovine articular chondrocyte in response to pro-inflammatory cytokines, IL-1β and TNFα. The data indicate that biochemical response of the chondrocytes in inflammatory milieu can be influenced by biophysical properties of scaffold materials; cytokine release kinetics and water uptake abilities. In addition to scaffold material, the role of Nkx3.2, a biochemical factor involved in cartilage formation during development, was investigated on chondrogenesis of human primary cells; human mesenchymal stem cells (hMSCs) and human normal articular chondrocytes (nHACs) and its role on cell response under inflammatory condition. Surprisingly, contrast to its role in mouse mesenchymal stem cell line, C3H10T1/2 in prior studies, transduction of Nkx3.2 inhibited chondrogenesis and redifferentiation of hMSCs and nHACs, respectively. However, knockdown of Nkx3.2 in hMSCs also results in inhibition of chondrogenesis, suggesting that Nkx3.2 is necessary for chondrogenesis but should be maintained in a desired level. Interestingly,
Nkx3.2 exhibited protective roles on nHACs in response to inflamed condition by IL-1β, suggesting its possible use for cartilage repair and regeneration in inflamed arthritic joints. Studies on optimal microenvironment, created by appropriate biophysical and biochemical cues, for cartilage tissue in inflammatory milieu will not only help develop new strategies to engineer stronger and more stable cartilage but also contribute to future clinical application.
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List of abbreviations

70 kilodalton heat shock proteins – HSP70
A disintegrin and metalloproteinase with thrombospondin motifs 4 – ADAMTS4
Alkaline phosphatase – ALP
Aqueous derived silk scaffolds – AQ silk scaffolds
B-cell lymphoma 2 – bcl-2
Bone morphogenetic protein – BMP
Bovine articular chondrocytes – BACs
Collagen type II – Col II
Collagen type IX – Col IX
Collagen type X – Col X
Control - Ctrl
Fibroblast growth factor – FGF
GFP infected hMSCs – GFP-hMSCs
GFP infected nHACs – GFP-nHACs
Glycosaminoglycan – GAG
Hexafluoroisopropanol derived silk scaffolds – HFIP silk scaffolds
Human mesenchymal stem cells – hMSCs
Hypoxia-inducible factor 1-alpha – HIF1α
Hypoxia-inducible factor 2-alpha – HIF2α
Insulin-like growth factor – IGF
Interleukin 6 – IL-6
Interleukin-1 beta – IL-1β
Matrix metallopeptidase – MMP
Mesenchymal stem cells – MSCs
Nk3 homebox2 – Nkx3.2
Nkx3.2 infected hMSCs – Nkx3.2-hMSCs
Nkx3.2 infected nHACs – Nkx3.2-nHACs
Normal human articular chondrocytes - nHACs
Osteoarthritis – OA
Poly lactic acid – PLA
Runt-related transcription factor 2 – Runx2
Scanning electron microscopy (SEM)
Scramble infected hMSCs – scramble-hMSCs
shRNA-Nkx3.2 infected hMSCs – shNkx3.2-hMSCs
SRY (sex determining region Y)-box9 – Sox9
Three dimension – 3D
Transforming growth factor beta – TGFβ
Transforming necrosis factor alpha – TNFα
Two dimension – 2D
1. Introduction

Arthritis is the most common debilitating disease that has been predicted to affect over 67 million people in the US by 2030 [1]. It is a progressive disease that is characterized by the destruction of joint cartilage [2]. Altered biomechanical properties of cartilage and changes in cellular responses to growth factors and cytokines are associated with aging and injury, leading to tissue softening and ultimately degradation of cartilage [3]. Without cartilage serving as a cushion, frictions of juxtaposing bones during joint movement cause severe pain and immobility. Due to limited capacity of cartilage to repair itself, either surgical or pharmacological treatments have been applied to patients to restore damaged joint function and relief pain [4, 5]. However, these treatments are ineffective in improving cartilage lesions and give poor long-term outcomes [4, 5]. Despite the prevalence of this disease, effective therapeutic options for arthritis still remains limited. Thus, appropriate and effective treatments are crucially needed.

Cartilage tissue engineering: promise and limitation

Cartilage tissue engineering has emerged as a new treatment option for cartilage repair and regeneration. Cartilage tissues can generally be reconstructed by seeding cartilage cells in three-dimensional (3D) natural or synthetic scaffolds in the presence of biochemical and biomechanical stimuli [6, 7]. A variety of cell sources, scaffolding materials, and culture conditions have been introduced to engineer cartilage tissue and investigated for their potential use in clinical application [6].

As promising cell sources, articular chondrocytes, adult stem cells, embryonic stem cells, and induced pluripotent stem cells have been investigated for cartilage repair and regeneration [8]. Not only human derived cells, but also other species such as murine,
bovine, porcine cells have been utilized for studies in cartilage tissue engineering both in vitro and in vivo as abundant cell amount can be obtained from large animals, providing less donor variability [9]. A variety of studies have demonstrated in vivo transplantation of cartilage tissue, consisted of chondrocytes embedded within scaffolding materials using animal models [10-12]. Although transplanted cartilage constructs provided improvement in cartilage defect, the engineered tissue contained fibrocartilage like tissue with little portion of hyaline cartilage tissue generated and its long-term stability has not been fully understood [10-12].

In a clinical setting, human articular chondrocytes (i.e. autologous articular chondrocytes) have been utilized for the regeneration of articular cartilage tissue. Articular chondrocytes can be obtained from the same donor thus provide less immunogenicity. However, due to the limited availability of donor tissue, it is difficult to obtain the required amount of autologous chondrocytes for clinical use [13]. Although in vitro expansion has been attempted to obtain the necessary amount of cell number, expanded human articular chondrocytes are likely to de-differentiate, characterized by changes in cell morphology, gene expression, and extracellular matrix synthesis, and lose their chondrogenic capacities to recreate stable hyaline like tissue [14]. Furthermore, transplantation of chondrocytes into cartilage defect in vivo failed to produce hyaline cartilage, followed by cells undergoing hypertrophic differentiation and cell death [9, 15]. To overcome this limitation, there have been studies on chondrocytes culture methodologies to improve proliferative capacity and enhance chondrogenic phenotype in articular chondrocytes [8, 16]. In addition, Implantation of biodegradable scaffold seeded with chondrocytes (i.e. porcine type I/III collagen bilayer seeded with articular
chondrocytes) has resulted in improved regeneration of hyaline like cartilage tissues in biopsies from patients [17, 18]. Moreover, it is considered that treatment using autologous articular chondrocytes are more effective than other techniques, such as mosaicplasty and arthroscopic drilling in terms of pain score and radiological and histological analyses [19]. However, outcomes of these clinical studies carried out after six months to one year, thus further long term studies are needed for evaluation of the tissue stability.

As an alternative cell source, mesenchymal stem cells (MSCs) have widely been demonstrated for cartilage repair and regeneration. MSCs have been found in a variety of adult tissues such as the bone marrow, fat tissue, and skeletal muscle [20]. Due to their abundant proliferative capacity and differentiation potential, they have been considered as a strong potential source for cartilage repair. MSCs are capable of differentiating into multiple lineages such as bone, cartilage, ligament, fat, and muscle under appropriate culture conditions [21]. Chondrogenic medium containing growth factors such as TGF-βs, BMPs, FGFs, and IGFs has extensively been studied on promoting chondrogenic differentiation of MSCs in vitro [22-25]. However, chondrogenic differentiation of MSCs with these growth factors is frequently accompanied by hypertrophic differentiation by producing hypertrophic markers such as collagen X, MMP13, and alkaline phosphatase (ALP) [26, 27].

Apart from in vitro studies, in vivo studies by transplantation of engineered cartilage into cartilage defects using animal models have been demonstrated in past years. However, although generating hyaline like cartilage tissue by MSC-derived chondrocytes
has been observed after weeks in vivo, the tissue formed after longer time points has been found to be the inferior fibrocartilage like tissue [28, 29].

Human MSCs (hMSCs) has been applied for articular cartilage repair in patients with cartilage defects and they have been considered as effective cell source as chondrocytes for clinical use. Previous studies have demonstrated that implantation of MSC in cartilage defect exhibited improved cartilage repair [30]. Articular cartilage repair by microfracture procedure also involves MSCs [31, 32]. Although short-term studies have shown successful clinical outcome, fibrocartilage like tissue and poor mechanical properties have been observed in long-term studies [31, 33].

Major reason of these unsuccessful outcomes involves the instability of implanted engineered cartilage tissues, followed by loss of regenerative cells through cell dedifferentiation or cell death upon transplantation and poor integration due to insufficient matrix protein secreted by implanted cells and matrix degradation [13, 34].

Therefore, despite significant attempts to engineer cartilage tissues, more comprehensive approaches for improved function in engineered cartilage are crucial for their use in clinical applications. Controlling appropriate cell differentiation, cell death along with synthesis of matrix proteins is important aspects in order to improve outcomes of cartilage repair and regeneration and ultimately to engineer strong and stable cartilage.

Human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs) are another attractive cell sources for cartilage tissue engineering [35-37]. hESCs have immunosuppressive properties thus protect them from the host immune response and have been considered as an “off-the-shelf” product, though there are yet ethical issues remained [8, 38]. iPSCs possess great differentiation capability and can be obtained from
patient, providing less risk of rejection and disease transmission [39, 40]. However, studies on cartilage repair and regeneration using these cells are yet limited thus more comprehensive studies on hESCs and iPSCs need to be addressed before clinical use [40].

**Inflammatory environment in osteoarthritic joint**

Osteoarthritis (OA) is a disease that involves inflammatory mediators released by joint tissues such as cartilage, subchondral bone, synovium, muscle, tendon, ligament, and fat pad [41]. Inflammation triggered by these tissues play a major role in the pathogenesis of cartilage destruction and OA progression [41].

Pro-inflammatory cytokines, mainly interleukin-1 β (IL-1β) and transforming necrosis factor-α (TNFα), are present in osteoarthritic joint and have been shown to be involved in cell death and cartilage destruction. Nuclear factor kappa B (NFκB) is a main transcription factor that mediate pro-inflammatory cytokine signals and leads to induction of nitric oxide (NO), cyclooxygenase 2 (Cox2), and prostaglandin E2 (PGE2). This process results in downregulation of cartilage matrix proteins including Sox9 and type collagen II, and activation of cartilage degrading enzymes including aggregcanases and proteases such as metalloproteinase 13 (MMP13) and MMP3 [13, 42, 43].

As discussed earlier, failure of generating hyaline cartilage tissue and the long-term instability of the cartilage tissue formed in vivo are major obstacles to overcome in current cartilage tissue engineering. Inflammation, present in arthritic joint, is regarded as a major factor that causes instability of the cartilage tissue. In cartilage defect, transplanted chondrogenic cells can be lost due to leakage of the cell suspension, and apoptosis and necrosis induced by pro-inflammatory cytokines, NO, and mechanical
stress present in OA environment, resulting in decreased viability of implanted cells [13, 34]. Furthermore, pro-inflammatory cytokines can cause dedifferentiation or incomplete differentiation of transplanted cells, and matrix degradation, resulting in insufficient matrix production and poor integration with the host [13, 34].

Previous studies have shown that the presence of pro-inflammatory cytokines, IL-1β and TNFα, as well as osteoarthritis-conditioned medium inhibited chondrogenesis in human MSCs [44, 45]. In addition, primary bovine articular chondrocytes cultured with pro-inflammatory cytokines, IL-1α, TNFα, and IFNγ decreased their viability and proliferation and enhanced NO production [46]. Moreover, another study has demonstrated that matured tissue engineered cartilage constructs subjected to inflammatory condition produced significantly increased amount of aggrecanase enzyme compared to the constructs during chondrogenic differentiation [47]. These data suggest that providing appropriate environment for engineered cartilage tissue to be resistant to and be stable against pro-inflammatory cytokines is critical and the potential effect of inflammation in arthritic joint has to be taken into consideration when engineering cartilage tissue.

3D scaffold material for cartilage tissue engineering: biochemical and biophysical properties

Scaffolding material, one of important components of cartilage tissue engineering, provides a 3D environment for cells and improves cellular functions and behaviors. A variety of natural and synthetic materials has been introduced for cartilage tissue engineering and used for the 3D culture and implantation of chondrogenic cells or stem
cells. Multiple studies have shown that scaffolds made from different materials have differential surface features and physical characteristics that affect cell growth, cell attachment, and matrix production [48-50].

A number of studies have demonstrated the comparison between different materials in cartilage matrix production. In a recent comparison between silk and agarose hydrogels, Chao et al. found that cartilage constructs derived from these two materials yielded similar biochemical and biomechanical properties [51]. Work from Erickson et al. indicated that bovine articular chondrocytes (BACs) seeded in agarose gels had a higher level of GAG/DNA ratio than in scaffolds derived from hyaluronic acid (HA) and self-assembly peptides [52]. Consistently, Mouw et al. also reported that agarose gels supported a higher GAG/DNA ratio in BACs than alginate, collagen, fibrin or polyglycolic acid (PGA) [53]. In contrast, Hu et al. showed that PGA cartilage constructs contained more collagen than agarose constructs [54]. Another study compared polycaprolactone (PCL), poly-glycerol sebacate (PGS) and poly (1,8 octanediol-co-citrate) (POC) scaffolds, and found that POC supported the highest collagen II/collagen I ratio and higher aggrecan expression from porcine chondrocytes [55]. In addition to studying scaffolds of different materials, multiple groups also compared the properties of composite scaffolds that were derived from the same material, but with different modifications. It was found that various modifications of PCL and polyethylene glycol (PEG) scaffolds supported different GAG/DNA and collagen II levels [56, 57]. Together, these thorough studies indicated that scaffolding properties have significant impacts on cartilage gene expression. It is likely that electric charge, porosity and surface chemistry of the scaffolds all influence the cellular function of chondrocytes [52].
Aside from biochemical properties by different materials and compositions, structural properties of biomaterials surrounding cells can also affect cell proliferation and matrix production [58]. For example, large pore-sized gelatin scaffolds supported higher levels of cartilage matrix marker collagen II, but also higher levels dedifferentiation marker collagen I and hypertrophic marker collagen X than smaller pore-sized scaffolds for rat chondrocytes [58]. Wang et al. also reported that small pore size increased cell attachment but had limits on cell proliferation and differentiation while larger pore size exhibited increased cell proliferation and advanced chondrocyte differentiation in rat chondrocytes [59]. Another study has shown that pore size of scaffold influenced redifferentiation potential in human articular chondrocytes where smaller pore sized scaffolds supported more hyaline-like extracellular matrix than scaffolds with larger pore size [60]. Furthermore, studies have demonstrated that different cell types have differential preference to scaffold pore sizes [61, 62]. These previous studies strongly suggest that structural properties of biomaterials surrounding cells are also one of important factors that influence cell behavior and cell differentiation [63].

Silk as biomaterials for cartilage tissue engineering

Silk from the silkworm, *Bombyx mori*, widely used as materials for textile and biomedical sutures [64], provides good biocompatibility, slow degradation rates, and impressive mechanical properties which can offer significant advantages for tissue engineering as scaffolding material [64, 65]. Silk-based biomaterials with different
physical forms such as gels and porous sponges have previously been demonstrated their exceptional benefits in cartilage regeneration [66-68].

Silk can be processed with different fabrication methods, which result in scaffolds of different surface chemistry, degradation profiles and mechanical properties. The two most established ways are aqueous - and the organic solvent-based fabrication methods. With respect to the aqueous method (AQ), a water based-silk fibroin solution is used for scaffold processing. With respect to the organic solvent method, lyophilized silk fibroin is first dissolved in the organic solvent, hexafluoroisopropanol (HFIP) and further processed with methanol which generates optimized water solubility and stability in silk structure [69, 70]. The resulting HFIP- derived silk scaffolds exhibit a lower degradation profile than aqueous silk scaffolds based on multiple in vivo studies [71, 72]. For both AQ and HFIP-methods, porous scaffolds of different pore sizes can be generated. This is achieved by incubating the silk solution with NaCl particles of different sizes, thus allowing the growth of tissues of different cell sizes and spatial cell organizations. Previous studies have investigated the advantages for the use of HFIP derived and aqueous derived (AQ) silk scaffolds in cartilage tissue engineering as well as other tissues such as bone, ligament, and tooth [71, 73-75].

*Potential genes involved in chondrogenesis and stability of cartilage tissue*

Gene transfer technology (i.e. the use of genetically modified cells), has been proposed for the treatment of cartilage and its potential use as a means to improve cartilage repair has been demonstrated [76, 77]. Gene based approaches have been applied to inhibit catabolic pathway to prevent matrix degradation (e.g. inhibition of
matrix degrading enzymes (tissue inhibitor of metalloproteinases (TIMP)) and pro-
inflammatory cytokines (IL-1Ra, sTNFR), stimulate anabolic pathways to promote
matrix synthesis (e.g. chondrogenic growth factors (TGFβ, BMPs, IGF-1, FGF-2) and
transcription factors (Sox5, 6, and 9)), and cell protection and proliferation (e.g. growth
factors (IGF-1 and FGF-2) and inhibition of cell apoptosis (bcl-2 and HSP70)) [76].
Transplantation of engineered cartilage derived from genetically modified cells into
cartilage defects has also been described in vivo. Therapeutic target genes such as TGFβ,
BMP2, BMP7, IGF-1, and FGF-2 etc. have been studied and significant improvements in
repairing cartilage defects by genetically modified cells with the target genes has been
observed, suggesting a promising therapeutic option by use of gene transfer approaches
for human articular cartilage defects [78-81].

Several groups have compared delivery of two different target genes on
improving repair of damaged cartilage. Mason et al. compared BMP-7 and sonic
hedgehog (SHH) on cartilage repair by delivering them to MSCs using retrovirus [82]. In
this prior study, they found that both gene products were able to promote cartilage repair
compared to control groups. However, overall cartilage repair by SHH was significantly
improved compared to that by BMP-7 [82]. Another group, Gelse et al. evaluated MSCs
adenovirally transduced with either BMP-2 or IGF-I on the repair of cartilage lesions in
rat femurs [83]. The group found that BMP-2 or IGF transduced MSCs generated hyaline
cartilage like tissue and increased synthesis of collagen type II and proteoglycan.
However, the joints received BMP-2 expressing MSCs exhibited signs of ectopic bone
formation and displayed osteophytes, whereas IGF-1 expressing MSCs did not show any
of these phenomena [83]. These previous studies suggest that the effect of each target
gene may vary in degree of restoring cartilage damage and there is still a need to identify most advantageous genes for supporting chondrogenesis of MSCs and improving cartilage repair.

Not only gene transfer of protein products but also gene transfer of transcription factors has drawn attention on their impact on chondrogenesis and cartilage repair. Viral-mediated transduction of Sox9, a master regulator of chondrogenesis in cartilage development, have been shown to stimulate expression of cartilage matrix proteins, collagen type II and proteoglycans in both human normal and osteoarthritic articular chondrocyts in vitro [84, 85]. Another group has shown that Sox9 transduction in MSCs could promote chondrogenesis in vitro in the absence of exogenous chondrogenic factors but requires mechanical stimulation [86]. In vivo experiment using Sox9 transduced MSCs, cultured under non-chondrogenic condition, has also been demonstrated to repair cartilage defects in rabbit knees with significantly improved cartilage repair [87]. However, in this prior study, they demonstrated that Sox9 transduced MSCs exhibited poor increase in GAG synthesis compared to GFP transduced MSCs in vitro. In addition, Ikeda et al. demonstrated that Sox9 transduction into hMSCs induced chondrogenic differentiation both under non-chondrogenic and chondrogenic conditions [88]. However, successful chondrocyte differentiation was observed only with combination with Sox5 and Sox6 transduction and hypertrophy and osteogenic differentiation was significantly suppressed only when these three Sox genes were transduced into the cells [88].

These previous studies suggest that Sox9 gene can be one of potential regulators for cartilage repair, however, the expression of Sox9 could activate a certain set of genes involved in chondrogenesis and their significant effect on cartilage formation and repair
could be only appeared by combination with other factors, such as mechanical stimuli and biochemical factors [86-88].

*Nkx3.2 as a potential regulator for chondrogenesis*

Nkx3.2 is the vertebrate homologue of Drosophila Bagpipe and induced by Shh in paraxial mesoderm during development. Aside from Sox9, Nkx3.2 is another critical regulator that promotes chondrogenic differentiation of mesenchymal stem cells during cartilage development. Recent study has demonstrated that when Sox9 is infected along with Nkx3.2 in muscle derived mesenchymal stem cells, it promoted chondrogenesis of the cells by enhancing collagen II and aggrecan expression compared to when either Sox9 or Nkx3.2 alone was used [89]. In addition, Nkx3.2 has shown to repress Runx2 in C3H10T1/2 cells; Runx2 is a key transcription factor for osteoblast differentiation and promoting hypertrophic chondrocyte differentiation, suggesting its possible role to prevent MSCs derived chondrocytes from undergoing hypertrophic differentiation [90]. Nkx3.2 has also shown to suppress cell apoptosis by inhibiting NF-κB induced by TNFα in chondrogenic cell line on 2D culture [90, 91]. As previous study has shown that MSC chondrogenesis is prevented by pro-inflammatory cytokines, IL-1β and TNFα through NF-κB pathway [45], Nkx3.2 is a promising transcription factor that could inhibit pro-inflammatory induced cell damage and apoptosis and lead to appropriate chondrogenic differentiation of MSCs, which in turn contribute to reconstruction of stable cartilage in vivo.
2. Significance and objective of the study

Osteoarthritis (OA) is a leading debilitating joint disease that affects people throughout the world. Cartilage tissue engineering has emerged as a potential treatment option for cartilage repair and regeneration. However, despite significant attempts to engineer cartilage tissues, the use of engineered cartilage in clinical application is yet far apart and more comprehensive approaches for improved cartilage function are needed. Inflammation, found to be present in arthritic joint, is involved in inducing cell death and matrix destruction and considered as a major factor that causes instability of implanted engineered cartilage in vivo. Thus, there is a critical need to provide a microenvironment for cartilage tissue to be resistant to and be stable in inflammatory conditions.

Scaffold, one of important components of cartilage tissue engineering, provides a 3D environment for cells and improves cellular functions and behaviors. Although the role of scaffolding material and structure on bioengineered cartilage tissues has extensively been studied, their role on the response of cells to inflammatory stimuli has not thoroughly studied.

Biochemical factors are another important component in engineering cartilage tissue. Growth factors (such as TGFβ, BMPs, and IGFs etc.) have shown to promote chondrogenesis of adult stem cells. However, it has been reported that cells that underwent chondrogenic differentiation are likely to further go through hypertrophy differentiation or differentiate into other lineages such as bone. Furthermore, chondrocytes in OA are likely to exhibit hypertrophic chondrocyte- like phenotype and be involved in OA progression, suggesting that a certain environment may be required for
cells to maintain their appropriate phenotype and also in order to be resistant to inflamed OA conditions.

Nkx3.2 is a transcription factor expressed very early in development during initial cell fate specification of muscle and cartilage. Nkx3.2 is known to inhibit hypertrophy in chondrocytes and differentiation of mesenchymal stem cells into muscle or bone, and also shown to suppress apoptosis in chondrocytes in an inflammatory condition, suggesting its possible role in improving stability and inhibiting inflammation-induced damage in chondrocytes.

The goal of the study is to investigate novel strategies to engineer strong and stable cartilage that is resistant to inflammation and prevent pro-inflammatory cytokine induced damage in engineered cartilage. To achieve the goal, the present study aimed to investigate 1) the role of scaffold material (silk, collagen, and poly-lactic acid (PLA)), structure (different pore sized HFIP silk scaffolds), and fabrication method (AQ and HFIP silk scaffolds) on cell resistance against inflammatory environment and 2) the role of a biochemical factor to enhance stability of cartilage in inflammatory conditions.

In the following chapters, the role of biophysical environment created by different scaffold material, structure, and fabrication on cell response in inflammatory conditions as well as the role of a biochemical factor, Nkx3.2, a transcription factor involved in cartilage development, on chondrogenic differentiation of cells and their stability under inflammatory stimuli were investigated. Studies on biophysical environment and biochemical cues for cartilage tissue to be resistant against inflammatory environment will not only help develop new strategies to engineer stronger and more stable cartilage but also contribute to future clinical application.
3. Materials and Methods

Scaffold preparation

For the study of investigating the role of scaffold materials on cell response to inflammatory conditions, silk, collagen, and PLA scaffolds were evaluated. Three-dimensional (3D) scaffolds derived from collagen (bovine derived type I and III collagens), and polylactic acid (PLA) were purchased from BD Biosciences (San Jose, CA, USA). Average pore sizes of collagen and PLA scaffolds were 100-200 μm and dimensions were 5mm × 3mm (diameter × height), according to manufacturer’s specifications. Silk scaffolds were prepared as previously described [69, 92]. Briefly, cocoons of Bombyx mori were boiled for 30 minutes in an aqueous solution of 0.02M Na₂CO₃ and rinsed with distilled water to eliminate sericin. Purified silk fibroin was solubilized in 9.3M LiBr solution and dialyzed against distilled water. The resulting silk fibroin solution was lyophilized and dissolved in hexafluoroisopropanol (HFIP) to obtain a 10% (w/v) silk solution. To create the desired pore size, 1 mL of the 10% silk-HFIP solution was added to 3.4 g of NaCl with a particle size of 106-212 μm in disk-shaped containers. The containers were tightly covered and left in the fume hood for 1-2 days for the silk-HFIP solution to evenly mix with the salt particles. The solvent was then evaporated for 3 days at room temperature. The scaffolds were treated with methanol for 1-2 days, and then the methanol was evaporated and the scaffolds were immersed in distilled water for additional 2 days to extract the salt particles. Porous silk scaffolds were then cut into disks with the same dimension as collagen and PLA scaffolds (5mm×3mm (diameter× height)) and autoclaved for cell seeding.
For the study of investigating the role of scaffold structure on cell resistance to inflammatory conditions, different pore sized HFIP silk scaffolds were prepared as described above but by adding 4 different sizes of NaCl particles: 106-212μm, 300-425μm, 500-600μm, and 710-850μm. The pore sizes are referred to as 100-200μm, 300-400μm, 500-600μm, and 700-800μm, respectively.

For the study of investigating the role of scaffold fabrication method, AQ- and HFIP derived silk scaffolds generated from a solution of 8w/v% silk were used. AQ silk scaffolds were prepared by adding 4g of NaCl with particle size of 500-600μm into 2mL of the silk solution in disk-shaped containers at room temperature. Twenty-four hours later the containers were immersed in distilled water to extract the salt from the porous scaffolds over 2 days. HFIP silk scaffolds were generated as described above and by adding 3.4g of NaCl with particle size of 500-600μm.

All generated porous silk scaffolds were then cut into small disks (5mm×3mm (diameter× height) and autoclaved for cell seeding.

*Isolation of bovine articular chondrocytes (BACs)*

Bovine articular chondrocytes (BACs) were isolated as previously described [93, 94]. Articular cartilage from all surfaces of a bovine cadaver knee joint (Research 87, Inc. Pleasant Lane, Boylston, MA, USA, (508) 869-0100) was dissected and transferred to a tube containing PBS and 10% penicillin/streptomycin. For dissociation of articular chondrocytes from cartilage matrix, minced cartilage pieces (12-15cm³ total in volume) were treated with 20ml of 1mg/ml hyaluronidase solution (Sigma, St. Louis, MP, USA) for 15min followed by treatments with 20ml of 0.25% trypsin solution (Sigma) for 30min,
and 20ml of 2mg/ml collagenase solution (Sigma) for approximately 15h at 37°C. For removal of any undigested cartilage to obtain a single cell suspension, isolated chondrocytes were passed through a 40μm strainer twice. The single cell population was resuspended in cell freezing medium (90% Fetal bovine serum (FBS) (Thermo Scientific HyClone, New Zealand), 10% DMSO (Sigma)), and stored in liquid nitrogen until experimentation. Cell viability was determined using the standard trypan blue staining protocol, where positive staining indicated cell death when isolated cells were mixed with trypan blue solution (Sigma). At isolation, cell viability was 97.2±2.4%. After freezing and thawing, cell viability was 73.7±4.3%. The viability of thawed cells after three days of culturing was 99.1±4.5%. The purity of the chondrocytes was confirmed by immunocytochemistry for cartilage marker Sox9, which showed 99% of the cells were Sox9-positive. Only passage 0 cells (P0) were used for all experiments.

**BACs seeding and 3D culturing**

To prepare for cell seeding, scaffolds were pre-wetted with sterile DMEM (Gibco, Carlsbad, CA, USA) overnight. Scaffolds were then removed from media and chondrocytes were seeded into scaffolds at a seeding density of 5x10^4 cells/scaffold. This cell density would allow easy access of all chondrocytes to both the scaffolds and pro-inflammatory cytokines. Based on the dimension of the scaffolds, the initial seeding density was to be 3.1x10^3 cells/mm^3. Taken into the consideration of cell proliferation over the culture period, the cell density in the scaffolds should be comparable to the cellularity of the adult native cartilage tissue (15 x10^3 cells/mm^3) [95]. After seeding, cell-loaded scaffolds were placed in a humidified tissue culture incubator at 37°C with 5%
CO₂ for 2 hours to allow for cell attachment. Cell-loaded scaffolds were then cultured in fresh DMEM containing 10% FBS and 1% Antibiotic-antimycotic (Gibco) for 8 days and 16 days on a shaker (5-6rpm, 6hr/day) in a tissue culture incubator. Three experimental groups were included in each independent experiment: control, 10ng/ml of IL-1β, and 10ng/ml of TNFα (Peprotech, Rocky Hill, NJ, USA). Medium was changed every 2-3 days.

**Lentivirus production**

To generate lentivirus encoding GFP or Nkx3.2, 293FT cells in a 10cm dish at 70% confluency were transfected with a total of 12µg of second generation lentiviral vectors; 3µg of transfer vector plasmid (GFP or Nkx3.2), 3µg of pCMV/VSVG, and 6µg of Delta-8.91 using Fugene6. Medium containing lentiviruses were collected in 72hrs and fresh medium was added for the second collection in 48hrs. Harvested medium was stored in -80°C. Collected medium was ultracentrifuged at 22000rpm for 3hrs to concentrate viruses and titration for each virus was determined on 293FT cells. The viruses were aliquoted in a small volume and stored in -80°C.

For lentivirus encoding scramble or shRNA-Nkx3.2, third generation packaging system was used. 293FT cells in a 10cm dish at 70% confluency were transfected with 3µg of transfer vector plasmid (scramble or shRNA-Nkx3.2), 6µg of pMDLg/pRRE, 2µg of pRSV-Rev, and 3µg of pCMV-VSVG. Lentiviruses were obtained and stored as described above.
**Lenti-viral transduction of human mesenchymal stem cells (hMSCs) and 3D culturing**

To investigate the role of Nkx3.2 on chondrogenesis of human mesenchymal stem cells (hMSCs), hMSCs (P2) purchased from Lonza Walkersville, Inc (Walkersville, MD) were plated in 24 well plates and transduced with lenti-virus encoding either GFP or Nkx3.2. To increase transduction rate, cells treated with viruses were centrifuged at 1000rpm for 30min, and the media were changed after 16hrs. Cells were maintained in DMEM containing 10% FBS, 1% Antibiotic-antimycotic, 0.1mM non-essential amino acids (Gibco), and 1ng/ml basic fibroblast growth factor (Pepeotech, Rocky Hill. NJ).

The transduction rate was determined by counting GFP-positive cells under fluorescent microscope. hMSCs infected with GFP (P7) or hMSCs infected with Nkx3.2 (P7) (refer as to GFP-hMSCs and Nkx3.2-hMSCs, respectively) were seeded onto pre-soaked HFIP silk scaffolds with pore size 500-600μm at a seeding density of 0.5 x 10^6 cells/scaffold. After seeding, cell-loaded scaffolds were placed in an incubator at 37°C with 5% CO_2 for 2 hours to allow for cell attachment. Cell-loaded scaffolds were then cultured in chondrogenic medium containing DMEM, 1% Antibiotic-antimycotic, ITS+ supplement (BD Biosciences), 0.1M ascorbic acid 2-phosphate (Sigma Aldrich, St. Louis, MO), 1.25mg/ml human serum albumin (Sigma), 10^-7 M dexamethasone (Sigma), and 10ng/ml transforming growth factor beta 3 (TGFβ3) (R&D Systems) for 21 days on a shaker (5-6rpm, 6hr/day) in a tissue culture incubator. For the role of Nkx3.2 on chondrogenesis of hMSCs induced by BMP2, cell-loaded scaffolds were maintained in chondrogenic medium containing 100ng/ml BMP2 instead of TGFβ3.

To investigate the role of shRNA-Nkx3.2 on chondrogenesis of hMSCs, hMSCs (P2) were transduced with lenti-virus encoding scramble or shRNA-Nkx3.2. hMSCs
infected with scramble (P7) or hMSCs infected with shRNA-Nkx3.2 (P7) (refer to as scramble-hMSCs and shNkx3.2-hMSCs, respectively) were seeded onto pre-soaked HFIP silk scaffolds with pore size 500-600μm at a seeding density of 0.5 x 10⁶ cells/scaffold. Cell-loaded scaffolds were maintained in chondrogenic medium for 21 days on a shaker (5-6rpm, 6hr/day) in a tissue culture incubator.

**Lenti-viral transduction of human articular chondrocytes (nHACs) and 3D culturing**

Normal human articular chondrocytes (nHACs) were purchased from Lonza Walkersville, Inc (Walkersville, MD).

For the study of investigating the role of Nkx3.2 on re-differentiation of nHACs, nHACs (P2) were infected with either GFP or Nkx3.2 virus and cultured in chondrocyte growth medium (Lonza). nHACs infected with GFP (P5) or nHACs infected with Nkx3.2 (P5) (refer to as GFP-nHACs and Nkx3.2-nHACs, respectively) were used for cell seeding on HFIP silk scaffolds with pore size 500-600μm. Cell loaded scaffolds were maintained in chondrocyte differentiation medium (Lonza) for 21 days on a shaker (5-6rpm, 6hr/day) in a tissue culture incubator.

For the study of investigating the role of Nkx3.2 on the response of redifferentiated nHACs to inflammatory conditions, nHACs (P4) were seeded onto HFIP silk scaffolds with pore size 500-600μm and redifferentiated for 3-4 weeks in chondrocyte differentiation medium. Cells were then retrieved from scaffolds by trypsinizing and centrifuged at 1500rpm for 5min. Cell pellets were infected with either GFP or Nkx3.2 virus, incubated for 1.5-2hrs, and plated onto tissue culture plates. After 2 days of culture, GFP-nHACs and Nkx3.2-nHACs were passaged to 24 well plates at a seeding density of
2.5 x 10^4 cells/well and maintained in the differentiation medium for 3 days in the absence or presence of 5 ng/ml of IL-1β.

**Lenti-viral transduction of C3H10T1/2 cells**

C3H10T1/2 cells were infected with either GFP or Nkx3.2 (refer to as GFP-10T1/2 and Nkx3.2-10T1/2, respectively) and sorted for GFP positive cells by FACS. Sorted cells were maintained in DMEM containing 10% FBS and 1% Antibiotic-antimycotic. For chondrogenic differentiation, GFP-10T1/2 and Nkx3.2-10T1/2 were plated onto 24 well plates for 2D culture or in high-density micromass cultures (1x10^5 cells in 10μl of medium) and cultured for 3 and 7 days in chondrogenic medium containing 300 ng/ml of BMP2.

**Bright field microscopy and scanning electron microscopy (SEM)**

Bright field images of scaffolds were taken using Leica MZ 16F microscope and Olympus DP70 digital camera. Top and side views of scaffolds were captured. For SEM analysis, cell-loaded scaffolds were fixed in 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer (pH=7.4) at 4°C overnight. Samples were then treated with 1% osmium tetroxide for 1 hr, dehydrated in ethanol, and subsequently dried on an Edwards Auto 306 Vacuum Evaporator. The samples were then cross-sectioned and sputter coated with palladium-gold. Chondrocytes grown inside the scaffolds were observed using an ISI DS130 scanning electron microscope at the Tufts Imaging Facility.
**Histological staining of scaffolds**

Cell-loaded scaffolds were fixed in 10% neutral-buffered formalin for histological evaluation. Silk and collagen scaffolds were embedded in paraffin while PLA scaffolds were embedded in OCT for frozen sectioning as PLA melts in xylene during the paraffinization process. The embedded samples were sectioned at 10µm thickness and were subsequently processed and stained with hematoxylin and eosin (H&E) and toluidine blue using standard protocols. For studies on the roles of different pore sized HFIP silk scaffolds and AQ and HFIP silk scaffolds, samples were embedded in paraffin and sectioned at 5µm thickness. For studies on the role of Nkx3.2 on human primary cells, samples were embedded in paraffin and sectioned at 5µm thickness. Histological evaluation for H&E and toluidine blue on these sections were performed following standard protocols. For Col II staining, Diaminobenzidine (DAB) immunocytochemistry was performed. Primary antibody for type II collagen (Abcam, Cambridge, MA) were added on sections and development of staining was carried out using DAB kit.

Image J software was used to quantify chondrocyte dimensions from images of H&E staining as well as the intensities of toluidine blue in toluidine blue-stained slides using established protocols [96].

**RNA isolation and real-time RT-PCR**

Total RNA from cell-loaded scaffolds was obtained using the RNeasy mini kit (Qiagen, Hilden, Germany), according to the manufacturer’s protocol. For each experiment, at least three independent samples per treatment per time point were used for RT-PCR analysis. The RNA was reverse transcribed into cDNA using the M-MLV
reverse transcriptase kit (Invitrogen), random primers (Invitrogen) and dNTPs (New England BioLabs, MA, USA). All cDNA was stored at -20°C for later analyses. For each RT-PCR reaction, 5~10 ng of cDNA was mixed with gene specific primers and SYBR® green SuperMix (Quanta Bioscience, Inc., Gaithersburg, MD, USA) and loaded on the iQ5 Real time PCR Thermocycler and Detection system (BioRad, Hercules, CA, USA) and analyzed by iQ5 optical system software. All transcript levels were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) level.

*Analysis of cytokine release from the scaffolds into the medium*

0.1ng, 1ng, or 10ng of pro-inflammatory cytokines, IL-1β or TNFα (Peprotech) were loaded onto pre-wetted scaffolds in a volume of 10μl. For each material, 3-6 scaffolds per treatment per time point were used. Loaded scaffolds were incubated for 6 hr at room temperature, immersed into 1ml of culture medium, and then placed in a humidified tissue culture incubator at 37°C with 5% CO2. The scaffolds were removed and transferred into fresh medium at each time point (t=10min, 1hr, 1d, 3d, and 5d). At every time point, medium conditioned by the scaffolds was collected and stored in -80°C for later analysis. The initial loading amount and concentrations of pro-inflammatory cytokines at all time points present in the collected media samples were quantified using ELISA (Quantikine; R&D Systems, Minneapolis, MN, USA). Percent release was calculated as the ratio of the amount of cytokines in the medium to the initial amount of cytokines loaded onto the scaffolds. Percent cumulative release was calculated as the ratio of cumulative amount of cytokines in the medium at each time point (i.e. sum
cytokine amount at each time point and all prior time points) to the initial amount of cytokines loaded onto the scaffolds.

**Analysis of water uptake abilities of the scaffolds**

The water uptake abilities of scaffolds were determined using a previously established protocol [70]. For each material, 3-6 scaffolds/treatment were used. Scaffolds were immersed in distilled water for 24 hours at room temperature. The wet weight of the scaffolds \( W_{\text{wet}} \) was measured after removing excess water from the scaffolds. The scaffolds were then dried in an oven at 65°C overnight and the weight of dried scaffolds \( W_{\text{dry}} \) was then measured. The water uptake (%) values were obtained using the following formula:

\[
\text{Water uptake} \ (\%) = \left( \frac{W_{\text{wet}} - W_{\text{dry}}}{W_{\text{wet}}} \right) \times 100
\]

**Statistical analysis**

All data were presented as mean ± SD (standard deviation) with a minimum of \( n=3 \). One-way ANOVA with post-hoc Tukey test (GraphPad Prism; http://www.graphpad.com) or two-way ANOVA with Bonferroni post test was used for determination of statistical differences. \( p < 0.05 \) was considered statistically significant.
Chapter 1

The influence of scaffold material on chondrocytes

in inflammatory conditions
Rationale

In selecting suitable scaffolding material for cartilage constructs, it is critical to consider the biocompatibility and mechanical strength of the material, as well as its ability to support maximum cartilage matrix production. As bioengineered cartilage constructs will be eventually transplanted into arthritic joints that have elevated levels of pro-inflammatory cytokines that destroy cartilage, it is especially important to select scaffolds that support the stability of bioengineered cartilage in an inflammatory environment.

Multiple studies have shown that scaffolds made from various biomaterials have different surface features and physical characteristics that affect cell growth, cell attachment and matrix production. However, little is known about how scaffold material may influence the homeostasis of the chondrocytes seeded within the scaffolds under inflammatory stimuli.

In this chapter, we compared different material based scaffolds; silk, collagen, poly lactic acid (PLA) scaffolds on cell response in the presence of pro-inflammatory cytokines. Biophysical properties of the scaffolds in terms of their abilities to release pro-inflammatory cytokines and uptake water were investigated to correlate with biochemical response of cells in inflammatory milieu.

Experimental approach

To investigate the role of scaffold material on cells response under inflammatory conditions, we examined the following experiment.
1. The role of scaffold material on cell response under inflammatory conditions:

Bovine articular chondrocytes (BACs) were seeded onto silk, collagen, and PLA scaffolds and cultured in the absence or presence of pro-inflammatory cytokines, IL-1β and TNFα. Biochemical response of cells (gene and protein expression) were correlated with biophysical properties of scaffolds (cytokine release kinetics and water uptake ability)
**Results**

Silk, collagen and PLA scaffolds support different chondrocyte morphologies under IL-1β and TNFα treatments

To evaluate the effect of scaffolding material on the morphology of chondrocytes under inflammatory stimuli, we performed scanning electron microscopy (SEM) analysis. PLA scaffolds had the smoothest surfaces, while collagen scaffolds had the roughest (Fig.1A). Although all scaffolds had the same pore sizes, pores in the PLA and silk scaffolds were more homogeneously distributed than those in the collagen scaffolds, which are lined with collagen fibrils (Fig.1A). Furthermore, the shapes of the pores differed slightly for each scaffold. Pores of silk and PLA scaffolds were round, while those of collagen scaffolds were sometimes more oval-shaped (Fig.1A). After culturing for 16 days in these scaffolds, regardless of the material, chondrocytes were attached to the surfaces of the scaffolds and evenly distributed (Fig.1B and 1C).
Figure 1. Morphological characterization of scaffolds and chondrocytes by scanning electron microscopy (SEM). (A) SEM micrographs of cell-free silk, collagen (COL), and polylactic-acid (PLA) scaffolds. Top panels: low magnification, scale bar: 200μm. Bottom panel, high magnification, scale bar: 50μm. (B) Low magnification images of chondrocytes inside the scaffolds after 16 days of culture. Scale bar: 200μm. (C) High magnification images of chondrocytes inside the scaffolds after 16 days of culture (see arrows). Scale bar: 25μm. The treatments are: Ctrl (no cytokines added), IL-1β (10ng/ml), and TNFα (10ng/ml).
Silk, collagen and PLA scaffolds support different cartilage gene expression in chondrocytes under IL-1β and TNFα treatments

*Cartilage matrix deposition*

To investigate the effect of scaffold material on articular chondrocyte homeostasis under inflammatory conditions, we evaluated cartilage matrix production using histological analysis. H&E staining on sections of cell-loaded silk, collagen and PLA scaffolds shows that chondrocytes grown in silk and collagen scaffolds tended to be more compact, while those grown in PLA scaffolds appeared larger (Fig. 2A). To provide a more quantitative analysis on cell sizes, we calculated the areas of chondrocytes shown in these sections. Cells in PLA scaffolds indeed had larger surface areas than those in silk and collagen scaffolds (Fig. 2B). Histological analysis using toluidine blue indicates that chondrocytes cultured in silk and collagen scaffolds had more intense toluidine blue staining than those cultured in PLA-scaffolds, suggesting the presence of a higher level of total glycosaminoglycans (GAGs) in the silk and collagen scaffolds (Fig. 2A) [97]. In the presence of IL-1β and TNFα, staining surrounding the chondrocytes cultured in silk and collagen scaffolds became less intense, while those in the PLA scaffolds remained at lower levels (Fig. 2A). Quantification of staining intensities using the Image J software indicated that PLA scaffolds supported lower cartilage matrix production in control or IL-1β and TNFα treatment conditions than silk and collagen scaffolds (Fig. 2D), which is consistent with the flatter cell morphology of the chondrocytes grown in PLA scaffolds and supports the notion that flatter cell morphology is correlated with less cartilage matrix production [98].
Figure 2. Histological analyses of chondrocytes grown in silk, collagen and PLA scaffolds.  
A. H&E staining images.  
B. Average area (µm²) occupied by individual chondrocytes was quantified by using Image J.  
C. Toluidine blue staining images.  
D. Average toluidine blue staining per cell, as quantified using Image J analysis. Scale bars: 25µm. Data present mean ± SD. *p<0.05.
Cartilage matrix-related genes

To determine whether the differences in cartilage matrix production in chondrocytes grown in silk, collagen and PLA scaffolds were due to differed transcriptional levels of genes controlling cartilage matrix production, degradation and cell survival, we performed qRT-PCR analysis on cultures of day 8 and day 16. First, the expression of cartilage matrix genes collagen II, collagen IX and aggrecan were evaluated. Collagen II and collagen IX are the signature collagens uniquely expressed in the cartilage tissues, while aggrecan is the major proteoglycan component in the extracellular matrix (ECM), and consists of the aggrecan core protein and the GAGs that bind to it. Thus aggrecan expression is expected to reflect the overall GAG content [99, 100]. On the other hand, Sox9 is a transcription factor that serves as a master regulator of chondrogenesis by directly binding to the collagen II and aggrecan promoters [101, 102].

At day 8, there were no significant differences in Sox9 expression in chondrocytes grown in the three types of scaffolds under normal conditions (Fig. 3A). However, the expression of collagen II, collagen IX and aggrecan was already significantly higher in chondrocytes seeded in silk scaffolds as compared to collagen and PLA in control samples (Fig. 3A). In the presence of IL-1β, chondrocytes grown in silk scaffolds expressed significantly higher levels of Sox9, collagen II and collagen IX than those in PLA scaffolds at day 8 (Fig. 3A). Upon TNFα treatment, silk scaffolds still supported a higher level of collagen IX expression than PLA scaffolds at day 8 (Fig. 3A). By 16 days of culture, all cartilage matrix-related genes were further downregulated by pro-inflammatory cytokines, however, chondrocytes seeded in silk and collagen scaffolds continued to show an overall higher expression in cartilage matrix genes than those in PLA scaffolds (Fig. 3B). A time
course longer than day 16 was not performed, as cartilage matrix gene expression was already reduced to a minimum level.

**A. Day 8**

**Sox 9**

**Col II**

**Col IX**

**Aggrecan**
B. Day 16

**Figure 3.** mRNA analysis of genes associated with cartilage matrix production in chondrocytes cultured in silk, collagen and PLA scaffolds. qRT-PCR analysis of Sox9, collagen (Col II), collagen IX (Col IX), and aggrecan in control (Ctrl), IL-1β treated (10ng/ml) or TNFα treated (10ng/ml) samples. For each treatment, results from three independent samples are shown. (A) Gene expression from Day 8 cultures. (B) Gene expression from Day 16 cultures. All gene expression levels were normalized to GAPDH. Data present mean ± SD. *p<0.05.

**Chondrocyte hypertrophy and dedifferentiation genes**

The expression of chondrocyte hypertrophy markers collagen X and alkaline phosphatase (ALP) were evaluated (Fig. 4). In addition, collagen I expression, which should be upregulated if the chondrocytes are de-differentiated, was assayed (Fig. 4). The
levels of collagen X and collagen I were similar in different scaffolds, in the absence or presence of IL-1β and TNFα at both day 8 and day 16. However, ALP was generally strongly induced by TNFα but not IL-1β (Fig. 4). These data suggest that scaffold material did not affect the status of hypertrophy and de-differentiation in these articular chondrocytes.

A. Day 8

![Gene expression analysis of chondrocyte hypertrophy and dedifferentiation markers in chondrocytes cultured in silk, collagen and PLA scaffolds. The expression of hypertrophy markers collagen X (Col X) and alkaline phosphatase (ALP), and dedifferentiation marker collagen I (Col I) in Ctrl (no cytokine treatment) or IL-1β (10ng/ml) and TNFα (10ng/ml) treated samples were evaluated. For each treatment, results from three independent samples are shown. (A) Gene expression from Day 8 cultures. (B) Gene expression from Day 16 cultures. All gene expression levels were normalized to GAPDH. Data present mean ± SD. *p<0.05.]

Figure 4.
**Chondrocyte-degradation-related genes**

It is well established that metalloproteinases that degrade collagens and aggrecan, including MMP3, MMP13 and ADAMTS4, are induced by pro-inflammatory cytokines [103]. The results indicated that scaffolds of different materials elicited different responses with respect to the various matrix-degrading enzymes and at various time points to IL-1β and TNFα. At day 8, significantly higher levels of MMP3, MMP13 and ADAMTS4 were induced by IL-1β in chondrocytes grown in silk scaffolds than in those grown in collagen and PLA scaffolds, while there was no difference in TNFα treated samples (Fig. 5A). Interestingly however, after 16 days of culture, while there were no differences in between scaffolds under control conditions, MMP13 expression was significantly lower in chondrocytes cultured in silk and collagen scaffolds than those in PLA scaffolds under IL-1β or TNFα treatment (Fig. 5B).
A. Day 8

**Figure 5.** Gene expression analysis of cartilage degradation enzymes in chondrocytes cultured in silk, collagen and PLA scaffolds. The expression of cartilage-degrading enzymes MMP3, MMP13, and ADAMTS4 in Ctrl (no cytokine treatment) or IL-1β (10ng/ml) and TNFα (10ng/ml) treated samples were evaluated. For each treatment, results from three independent samples are shown. (A) Gene expression from Day 8 cultures. (B) Gene expression from Day 16 cultures. All gene expression levels were normalized to GAPDH. Data present mean ± SD. *p<0.05.
**Cell adhesion-related genes**

Different scaffolding materials have different surface chemistry properties and rigidity, which may affect cell adhesion and may in turn influence gene expression [48-50]. Thus, the expression of integrins and cadherins, which are mediators of cell adhesion were evaluated [104-106]. Both α1 and β1 integrins are highly expressed in the chondrocytes and bind to collagens in cartilage ECM [104]. On the other hand, N-cadherin, which normally mediates condensation during chondrocyte differentiation, exhibits diminished expression in differentiated chondrocytes [107-112]. We found that chondrocytes grown in PLA scaffolds expressed significantly higher levels of α1-integrin than those in silk scaffolds at day 8 upon IL-1β treatment (Fig. 6A) and at day 16 in all conditions (Fig. 6B). In addition, N-cadherin was much more strongly induced in chondrocytes by TNFα than IL-1β (Fig. 6A and 6B). Given that chondrocytes in PLA scaffolds exhibited a more flattened morphology (Fig. 2), this result is consistent with another study that showed that increased expression of α1-integrin is associated with an elongated rather than round chondrocyte cell shape [98].
Figure 6. Gene expression analysis of cell adhesion molecules in chondrocytes cultured in silk, collagen and PLA scaffolds. The expression of α1 integrin, β1 integrin, and N-cadherin in Ctrl (no cytokine treatment) or IL-1β (10ng/ml) and TNFα (10ng/ml) treated samples were evaluated. For each treatment, results from three independent samples are shown. (A) Gene expression from Day 8 cultures. (B) Gene expression from Day 16 cultures. All gene expression levels were normalized to GAPDH. Data present mean ± SD. *p<0.05.
Cell death-related genes

Next, the expression of caspases and inducible nitric oxide synthase (iNOS), which are mediators of apoptosis and induced by pro-inflammatory cytokines were evaluated [113]. Caspase 3 was more strongly induced by IL-1β and TNFα in chondrocytes grown in collagen and PLA scaffolds than in silk scaffolds at day 16 (Fig. 7). In contrast, there were no significant differences in caspase 8 expression in chondrocytes grown in scaffolds of different materials throughout the culture periods (Fig. 7). On the other hand, iNOS was expressed at higher levels in chondrocytes grown in silk scaffolds than in collagen and PLA scaffolds (Fig. 7). Additionally, while TNFα strongly induced caspase 8 expression, IL-1β preferentially induced iNOS expression, thereby suggesting that IL-1β and TNFα have different effects on these genes (Fig. 7).
Figure 7. Gene expression analysis of apoptosis-related factors in chondrocytes cultured in silk, collagen and PLA scaffolds. The expression of caspase 3, caspase 8, and iNOS in Ctrl (no cytokine treatment) or IL-1β (10ng/ml) and TNFα (10ng/ml) treated samples were evaluated. For each treatment, results from three independent samples are shown. (A) Results from Day 8 cultures. (B) Results from Day 16 cultures. All gene expression levels were normalized to GAPDH. Data present mean ± SD. *p<0.05.
Silk, collagen and PLA scaffolds have different cytokine release properties and water uptake capacities

To understand the possible underlying mechanisms that cause the differential responses to pro-inflammatory cytokines, we evaluated the following biophysical properties of the three different types of scaffolds.

The ability to release pro-inflammatory cytokines

The rationale was that higher affinity of the scaffolds for pro-inflammatory cytokines would lead to higher local concentrations of these factors within the cartilage construct, and differences of the cytokine levels in the scaffold may in turn affect cartilage gene expression. Therefore, to investigate how the scaffolds release or retain IL-1β and TNFα, we applied equal amounts of these cytokines to silk, collagen and PLA scaffolds, and then evaluated the amount of cytokines that leached out into the medium. As it was uncertain of the capacity of the scaffolds to adsorb IL-1β or TNFα, three different amounts (0.1ng, 1ng and 10ng) were applied to the scaffolds, the scaffolds were placed in the medium, and assayed by ELISA for the amount of cytokines leached into the medium at different time points (Fig. 8 and Fig. 9). Regardless of loading levels, the amount of IL-1β released from silk scaffolds was significantly higher than that from collagen and PLA scaffolds at the initial time point of 10min, as well as at the end of the study (cumulative release) (Fig. 8A and 8B). In contrast, collagen scaffolds maintained a steady release over the first day of the study and had the lowest cumulative release of IL-1β at early time points (Fig. 8A and 8B). The release of TNFα from silk, collagen and PLA scaffolds exhibited a similar trend to that of IL-1β, with silk scaffolds releasing the
highest amount of TNFα than other scaffolds (Fig. 8C and 8D). This finding suggests that silk scaffolds may support a microenvironment where there are lower levels of IL-1β and TNFα, a result that may help to explain the higher levels of cartilage matrix gene expression in these scaffolds as compared to PLA scaffolds.
Figure 8. Evaluation of cytokine release kinetics of silk, collagen and PLA scaffolds. Three different amounts of pro-inflammatory cytokines IL-1β or TNFα (0.1, 1 and 10ng) were loaded onto empty scaffolds of silk, collagen (COL), polylactic-acid (PLA). ELISA was used to verify the initial loading amount and to evaluate the amount of cytokines leached into the medium at 5 different time points: 10min, 1hr, 1 day, 3 days and 5 days. (A) Percent release of IL-1β from scaffolds at each time point. (B) Analysis of IL-1β cumulative release from the scaffolds. (C) Percent release of TNFα from scaffolds at each time point. (D) Analysis of TNFα cumulative release from scaffolds. Data present mean ± SD. Statistical analysis of the data was determined by two-way ANOVA. *p<0.05.
Figure 9. Statistical analysis on cytokine release kinetics of the scaffolds, showing detailed statistical comparisons. Linear graphs in cytokine release kinetics of different materials in Figure 8 were formatted into bar graphs to show detailed statistical comparisons. (A) Percent release of IL-1β from scaffolds at each time point. (B) Cumulative release analysis of IL-1β from the scaffolds. (C) Percent release of TNFα from scaffolds at each time point. (D) Cumulative release analysis of TNFα from scaffolds. Data present mean ± SD. Statistical analysis of the data was determined by two-way ANOVA. *p<0.05.
**Water uptake ability of the scaffolds**

Water uptake ability is known to reflect the hydrophilicity property of the scaffolds [114-116]. The rationale was that this property could affect the effective cytokine concentrations in the scaffold microenvironment of the chondrocytes and in turn chondrocyte gene expression. Using established protocols [70, 117], it was found that silk and collagen scaffolds have a significantly higher water uptake capacity than PLA scaffolds, suggesting that silk and collagen scaffolds created a more hydrated microenvironment for the chondrocytes (Fig. 10).

![Figure 10](image.png)

**Figure 10.** Analysis of water uptake properties of silk, collagen and PLA scaffolds. Percentage of water uptake in the scaffolds was determined. Statistical analysis of the data was determined by one-way ANOVA. Data present mean ± SD. *p<0.05.
Discussion

A major goal of cartilage tissue engineering is to repair damaged cartilage tissues caused by mechanical stress and high levels of pro-inflammatory cytokines in arthritic joints. It is clear that the stability of bioengineered cartilage can be compromised by pro-inflammatory cytokine-induced cartilage matrix degradation [118, 119]. Several biochemical factors or reagents, such as IGF-I, PDGF, and Cox-2 inhibitor celecoxib, were shown to possess inhibitory activities to IL-1β-induced matrix reduction in chondrocytes in 2D cultures [120, 121]. In 3D cultures, dexamethasone and MMP inhibitor TIMP-1 demonstrated a protective effect against IL-1α [118, 122]. Interestingly, genipin, a cross-linking reagent, was found to inhibit IL-1α-induced GAG reduction when administered in the culture medium, possibly by stabilizing the ECM [123]. On the other hand, dynamic loading did not alleviate the catabolic effect of IL-1α and IL-1β in the cartilage construct [124]. Since dynamic loading has been widely regarded to enhance cartilage matrix production, this study suggests that matrix production under normal conditions and matrix maintenance under inflammatory conditions are two related but not identical issues [124]. Apart from biochemical factors, very little is known about the contribution of other components in a 3D cartilage toward inflammatory response.

Scaffolds constitute an important component of the microenvironment for chondrocytes in bioengineered cartilage. Selecting the optimal scaffolds, together with supplementing the optimal biochemical factors in the culture medium, will lead to the enhancement of the stability of bioengineered cartilage. However, it is still unclear how scaffold material influences the response of chondrocytes grown in the scaffolds to exogenous inflammatory stimuli, which may not be revealed by only studying
chondrocyte growth under normal conditions. In this investigation, the differences in gene expression in 3D cultured chondrocytes under the treatment of pro-inflammatory cytokines, IL-1β and TNFα were analyzed. Chondrocytes cultured in scaffolds of different materials (silk, collagen and PLA) showed varying responses in gene expression to pro-inflammatory cytokines, including matrix production and degradation, cell adhesion and cell death. Overall, silk and collagen scaffolds supported higher levels of cartilage matrix gene expression than PLA under IL-1β and TNFα treatments, which correlated with toluidine blue stainings that reflected the level of GAGs. Additionally, chondrocyte gene expression was compared with cell morphology and the biophysical properties of the scaffolds, such as release profiles of IL-1β and TNFα and water uptake abilities. Together, these data strongly suggest that scaffolding is an important component of the microenvironment for chondrocytes and plays a significant role in chondrocyte homeostasis in an inflammatory environment.

Here, the effect of scaffoldings in the context of pro-inflammatory cytokine treatment was investigated, and porous scaffolds of silk, collagen and PLA, which were not directly compared in previous studies, were evaluated. Under control conditions, silk and collagen scaffolds supported higher levels of cartilage matrix deposition and expression of cartilage matrix genes collagen II, collagen IX and aggrecan than PLA scaffolds. In the presence of IL-1β and TNFα, while all cartilage gene expression was significantly reduced, silk and collagen scaffolds still supported higher cartilage matrix levels.

Importantly however, in other instances, chondrocytes’ responses to inflammatory stimuli in different scaffolding materials may not be predicted from studying only non-
inflammatory conditions. For example, in the case of MMP13 expression at day 16, while there was no difference among chondrocytes in different materials under control conditions, there was significantly higher MMP13 expression in the chondrocytes grown in PLA scaffolds than silk and collagen scaffolds in the presence of IL-1β and TNFα (Fig. 5). This result suggests that different scaffolding materials can elicit different responses to pro-inflammatory cytokines in the chondrocytes and that such effects can only be revealed when the cells are challenged with inflammatory stimuli. A recent investigation indicated that although there was no difference in cartilage matrix production in different PEG-modified scaffolds under static conditions, significant differences were observed when scaffolds were cultured under dynamic stimulation [57]. Thus, this investigation is consistent with the notion that scaffolding material influences the response to exogenous stimuli, including biochemical and biomechanical signals.

It was also observed that chondrocytes responded differently to the two different cytokines IL-1β and TNFα. In terms of ADAMTS4 expression, chondrocytes grown in silk scaffolds showed a significantly stronger response at day 8 to IL-1β than TNFα, while those grown in collagen and PLA scaffolds responded to IL-1β and TNFα equally. Furthermore, IL-1β is much more potent than TNFα in inducing MMP3 (day 16), MMP13 (day 8) and iNOS expression (day 16), while TNFα induces the expression of hypertrophic marker alkaline phosphatase (day 8) and cell death indicator caspase 8 (day 8 and day 16) more readily, suggesting that these two cytokines can activate different signaling pathways. Another group also noted the differential responses chondrocytes to IL-1β and TNFα when the cells were cultured as monolayers for up to three days, suggesting that TNFα was a stronger inducer of cell death as it activated the expression of
both caspase 3 and 8 more strongly [125, 126]. This is consistent with the observation of caspase 8 expression in the study; however, IL-1β and TNFα similarly induced caspase 3 expression, a discrepancy possibly due to the differences between 2D and 3D culture systems. In addition to the differences in the activities of TNFα and IL-1β, differential gene expression with respect to culture time was also observed. For example, although there was no difference in aggrecan expression in chondrocytes cultured in different scaffolds upon IL-1β and TNFα treatments at day 8, there were significant differences in the expression by day 16 (Fig. 2), thereby suggesting that different scaffolding materials may exhibit different kinetic profiles in regulating cartilage gene expression.

Biomaterials have different physical and chemical characteristics such as surface roughness and material hydrophobicity that can affect cell attachment, cell shape and chondrocyte gene expression [49, 127]. The histological and SEM analyses showed that chondrocytes in PLA scaffolds had a more spread-out, sheet-like structure. Correspondingly, cells grown in PLA scaffolds had significantly higher expression of cell adhesion molecule α1 integrin and the lowest amount of cartilage matrix deposition and gene expression than those grown in silk and collagen scaffolds. This is consistent with a report by Ronziere et al, in which inhibition of α1 integrin resulted in a more rounded cell morphology and enhanced collagen II expression in chondrocytes embedded in collagen gels [98].

To understand the mechanisms by which scaffolds influence the behavior of chondrocytes under inflammatory conditions, we investigated cytokine release kinetics of scaffolds with a rationale that the rate of scaffolds to release cytokines would impact the local environment of the chondrocytes. Prior studies, many aiming at targeted protein
release, have analyzed the release profiles of BMP2, VEGF and IGF-I proteins from silk, collagen and PLA scaffolds [128-134]. However, the release of pro-inflammatory cytokines has not been extensively studied. Here, silk, collagen and PLA scaffolds were found to have different kinetics in releasing IL-1β and TNFα, thus proposing that different scaffolding materials may support different levels of pro-inflammatory cytokines in the chondrocytes' microenvironment. Regardless of the amount of cytokine loaded, silk scaffolds released IL-1β and TNFα at a much faster rate than collagen and PLA scaffolds. This suggests that silk material may not adsorb as much cytokines as collagen and PLA-based scaffolds do, which may provide chondrocytes with a more optimal microenvironment. Indeed, the faster cytokine release rate by silk is correlated with a higher level of cartilage matrix production in chondrocytes grown in silk scaffolds. Interestingly, while collagen scaffolds consistently released cytokines more slowly than PLA scaffolds, collagen scaffolds supported higher levels of matrix gene expression, suggesting that other factors are also involved. It is possible that collagen provides an additional biochemical regulation on chondrocyte behavior through its binding to integrins [135, 136].

Hydrophilicity of the scaffolding material might be an additional property that influences cartilage gene expression and matrix deposition. Previous studies have shown that the morphology and bulk hydrophilic/hydrophobic qualities of the scaffolds influence the rate of water uptake of the scaffolds and may affect the subsequent cell reaction to inflammatory stimuli [114-116]. Our data showed that silk and collagen scaffolds have higher water uptake abilities than PLA scaffolds, which is correlated with higher levels of cartilage matrix gene expression in chondrocytes cultured in silk and
collagen scaffolds. Furthermore, chondrocytes in PLA scaffolds had a more flattened morphology and elevated α1 integrin expression, which may also be correlated with material hydrophobicity. The result with these porous scaffolds is consistent with other analyses using PEG-based hydrogels as cartilage constructs, where swelling ratio positively correlated with collagen II and aggrecan expression [57, 137, 138]. Therefore, scaffold swelling ratio and water uptake property can be an additional physical property of the scaffolds that regulate cartilage gene expression under normal and inflammatory conditions.

In summary, this study constitutes one of the first steps toward understanding the contribution of scaffold material to inflammatory response. It clearly shows that scaffolding, as an important component of the chondrocyte microenvironment, plays a critical role in matrix production and destruction as well as cell death, especially under inflammatory conditions. These analyses prompted us to conclude that silk and collagen scaffolds are the most optimal scaffolds for supporting stable cartilage matrix production than PLA scaffolds, based on the following criteria: 1) higher level of cartilage matrix gene expression and matrix deposition; 2) lower levels of cartilage degradation enzymes; 3) cell morphology that resembles native cartilage cells; 4) lower retention of inflammatory cytokines; and 5) higher water uptake ability. It will be interesting to determine how the other properties of scaffolding materials, such as porosity, rigidity, degradation rate, adhesion domains, local stiffness and surface chemistry, can regulate the response of chondrocytes to inflammatory stimuli [138-141]. In particular, determining whether seeding density could alter the influence of scaffolding material on chondrocyte gene expression can be studied, as a higher seeding density would be needed.
to generate cartilage constructs for clinical applications. It is also likely that other cell sources or cell types, such as mesenchymal stem cells, may exhibit differential responses to inflammatory cytokines when grown in scaffolds of different materials. Further biochemical and biophysical studies of the cartilage constructs will help us to understand the interaction of stem cells or chondrocytes with its niche or microenvironment. It is conceivable that selecting the proper scaffolding material and optimizing its biophysical properties will aid in the engineering of more stable cartilage tissues.
Chapter 2

Scaffold structure and fabrication method affect pro-inflammatory milieu in 3D-cultured chondrocytes
Rationale

As demonstrated in Chapter 1, different material derived scaffolds can lead to differential response of cells to pro-inflammatory cytokines, IL-1β and TNFα. Aside from different scaffolding materials, structure properties of scaffolds have also been shown to affect cartilage gene expression. In addition, scaffolds derived from different fabrication method (i.e. AQ-derived and HFIP derived silk scaffolds) have shown to have different biophysical properties of scaffolds such as surface roughness, swelling ratio, and degradation profile which all can influence cell behavior and function. However, it is unclear whether the structure and fabrication method of the scaffold would also influence the seeded chondrocytes in terms of their homeostasis in an inflammatory microenvironment.

To understand the effect of structure and fabrication method on the gene expression of bioengineered cartilage in inflammatory conditions, we compared different pore sized scaffolds, ranged from 100µm to 800µm, and silk scaffolds derived from two different fabrication methods, AQ and HFIP derived silk scaffolds, on cell response in the presence of pro-inflammatory cytokines.

Experimental approach

To investigate the role of structure and fabrication method of scaffolds on cell response under inflammatory conditions, we examined the following experiments.
1. **The role of fabrication methods of scaffolds on cell response under inflammatory conditions:** Bovine articular chondrocytes (BACs) were seeded onto AQ and HFIP silk scaffolds and cultured in the absence or presence of pro-inflammatory cytokines, IL-1β and TNFα. Biochemical response of cells (gene and protein expression) were correlated with biophysical properties of scaffolds (cytokine release kinetics and water uptake ability).

2. **The role of pore size of scaffolds on cells response under inflammatory conditions:** BACs were seeded onto 4 different pore sized silk scaffolds and cultured in the absence or presence of pro-inflammatory cytokines, IL-1β and TNFα. Biochemical response of cells (gene and protein expression) were correlated with biophysical properties of scaffolds (cytokine release kinetics and water uptake ability).
**Results**

HFIP silk scaffolds support a higher level of cartilage matrix production and a lower level of matrix degradation in 3D cultured chondrocytes.

To determine whether silk scaffolds generated by different fabrication methods affect the ability to support chondrocyte gene expression under inflammatory conditions, we analyzed primary bovine articular chondrocytes grown in porous silk scaffolds generated by the AQ or HFIP methods.

Scanning electron microscopy (SEM) analysis showed that while these scaffolds have the same pore size (500-600µm), the pores of cell-free AQ silk scaffolds had rougher surfaces than those of HFIP silk scaffolds (Fig. 11B). Interestingly, chondrocytes in AQ silk scaffolds seemed to be flatter than those in the HFIP scaffolds (Fig. 11C-11E).

We performed toluidine blue staining to evaluate cartilage matrix deposition. Toluidine blue is a basic dye that exhibits a color shift toward the violet-purple range (i.e. metachromasia) when the level of glycosaminoglycan (GAG) is very high. Significantly, a much stronger and metachromatic toluidine blue staining was observed in chondrocytes grown in the HFIP silk scaffolds than in AQ silk scaffolds under the control condition, suggesting of a higher level of matrix deposition in HFIP scaffolds (Fig. 11F). While IL-1β and TNFα treatments reduced the intensity of toluidine blue staining in all scaffolds, the staining is still stronger around chondrocytes grown in HFIP scaffolds than in AQ scaffolds (Fig. 11F).
Figure 11. Morphological characterization of scaffolds and chondrocytes by scanning electron microscopy (SEM) and histological evaluation. (A) Bright field images of cell-free AQ and HFIP silk scaffolds. Scale bar: 2mm. (B) SEM micrographs of cell-free AQ and HFIP silk scaffolds. Top panels: low magnification, scale bar: 200μm. Bottom panel, high magnification, scale bar: 40μm. (C) Low magnification images of chondrocytes inside the scaffolds after 16 days of culture. Scale bar: 200μm. (D) High magnification images of chondrocytes inside the scaffolds after 16 days of culture. Scale bar: 50μm. (E) H&E staining images of chondrocytes grown in AQ and HFIP silk scaffolds. (F) Toluidine blue staining images of chondrocytes grown in AQ and HFIP silk scaffolds. The treatments are: Ctrl (no cytokines added), IL-1β (10ng/ml), and TNFα (10ng/ml).
To provide a more quantitative measure for such differences, and to determine whether the difference in matrix production is caused by a difference in gene expression, we performed real time-RT-PCR to assess cartilage matrix and degradation-related genes. We examined the expression of collagen II (Col II), collagen IX (Col IX) and Aggrecan as cartilage matrix genes. We also examined the expression of pro-inflammatory cytokines IL-1β and IL-6, which are known to promote the expression of cartilage degradation genes such as MMP3, MMP13 and ADAMTSs [142-144].

In the absence of pro-inflammatory cytokines at day 8, chondrocytes in AQ and HFIP silk scaffolds expressed similar levels of cartilage matrix genes collagen II (Col II), collagen IX (Col IX) and Aggrecan, with minimal expression of cartilage degradation genes. In the presence of IL-1β and TNFα, the level of matrix-related genes was significantly reduced, although the expression of Col II and Aggrecan were slightly higher in chondrocytes grown in HFIP-silk scaffolds under IL-1β treatment (Fig.12). Chondrocytes grown in HFIP silk scaffolds also showed a significantly higher level of endogenous IL-1β, MMP13 and ADAMTS4 than those in AQ-silk scaffold, suggesting that chondrocytes grown in HFIP silk scaffold may be more metabolically active under IL-1β treatment [145], but no differences were observed in control and TNFα-treated samples (Fig.12). HIF1α and HIF2α are hypoxia-induced factors that play important roles in regulating cartilage gene expression, and thereby, is beneficial to cartilage, while HIF2α promotes the expression of matrix degradation genes and induces OA, thus it is detrimental to cartilage [146-148]. In the presence of pro-inflammatory cytokines, HIF2α was induced, however, HIF1α and HIF2α expression was not significantly different between the scaffolds at day 8.
At day 16, the difference in cartilage matrix gene expression between chondrocytes cultured in AQ and HFIP silk scaffolds became more pronounced, as chondrocytes grown in HFIP silk scaffolds showed two times the levels of cartilage matrix genes than those in AQ silk scaffold (Fig.13). Furthermore, the endogenous levels of IL-1β, IL-6, MMP3, MMP13, and HIF2α also exhibited lower in HFIP silk scaffolds in control condition. In the presence of IL-1β or TNFα, expression levels of IL-1β, IL-6, MMP3, and HIF2α were significantly lower in HFIP silk scaffolds. Since we did not observe any differences in the expression of HIF1α at any time point, it suggests that there is no difference in oxygen levels in AQ and HFIP scaffolds. Although we observed lower levels of IL-1β and IL-6 in HFIP scaffolds under TNFα treatment, we did not detect significant levels of TNFα expression itself in any conditions, suggesting that TNFα does not induce its own expression in our system (data not shown). Overall, our gene expression analysis is consistent with the observed higher level of matrix deposition in HFIP silk scaffolds, suggesting that HFIP scaffold is more optimal in maintaining cartilage integrity.
Figure 12. mRNA analysis of genes in chondrocytes cultured in AQ and HFIP silk scaffolds at day 8. All gene expression levels were normalized to GAPDH. Data present mean ± SD. *p<0.05.

Figure 13. mRNA analysis of genes in chondrocytes cultured in AQ and HFIP silk scaffolds at day 16. All gene expression levels were normalized to GAPDH. Data present mean ± SD. *p<0.05.
Scaffold pore-size influences the pro-inflammatory microenvironment in 3D cultured chondrocytes

We next investigated whether scaffold pore size influences cartilage matrix production and gene expression under control and inflammatory conditions. Based on the data shown earlier, we chose to use HFIP silk scaffolds as they supported better cartilage matrix production in chondrocytes. In addition to the 500-600µm pore size which we used earlier in comparison with AQ and HFIP silk scaffolds, we also included three other pore sizes: 100-200, 300-400, and 700-800µm (Fig.14A and B). SEM confirmed the pore sizes of these scaffolds, but did not reveal any significant differences in cell morphology among cells grown in the scaffolds (Fig.14C and D). H&E analysis from day 16 cultures showed that chondrocytes in different scaffolds exhibited similar cell shapes (Fig.14E).
Figure 14. Morphological characterization of scaffolds and chondrocytes by scanning electron microscopy (SEM) and histological evaluation. (A) Bright field images of cell-free HFIP silk scaffolds with different pore sizes. Scale bar: 2 mm. (B) SEM micrographs of cell-free HFIP silk scaffolds with different pore sizes. Top panels: low magnification, scale bar: 200 μm. Bottom panel, high magnification, scale bar: 40 μm. (C) Low magnification images of chondrocytes inside the scaffolds after 16 days of culture. Scale bar: 200 μm. (D) High magnification images of chondrocytes inside the scaffolds after 16 days of culture. Scale bar: 40 μm. (E) H&E staining images of chondrocytes grown in HFIP silk scaffolds with different pore sizes. (F) Toluidine blue staining images of chondrocytes grown in HFIP silk scaffolds with different pore sizes. The treatments are: Ctrl (no cytokines added), IL-1β (10 ng/ml), and TNFα (10 ng/ml).
Gene expression analysis from 8 day of culture indicated that while there was no major difference in the expression of cartilage matrix genes Col II, Col IX, and Aggrecan, there was significant difference in the expression of cartilage degradation-related genes in cells grown in these different pore sized scaffolds (Fig.15). At 16 days of culture, Col II and Col IX expression were significantly higher in cells in larger pore sized scaffolds in control condition while no major differences in the expression were observed in cells under cytokine treatment (Fig.16). However, no major differences in intensity in toluidine blue staining were found among the scaffolds both in the absence or presence of pro-inflammatory cytokines (Fig.14).

At both time points, in the absence of pro-inflammatory cytokine treatments, larger pore-sized scaffolds supported low levels of endogenous pro-inflammatory cytokines IL-1β and IL-6 expression. Correspondingly, we observed that lower levels of MMP3, MMP13, ADAMTS4 as well as HIF2α in larger pore sized scaffolds under control conditions and the trend was maintained under IL-1β and TNFα treatment (Fig.15 and 16). In overall, cartilage degrading enzyme expression were expressed at higher level in chondrocytes grown in smaller pore sized scaffolds in the presence of pro-inflammatory cytokines, suggesting that larger pore size support less cartilage degrading enzyme expression than smaller pore sized scaffolds. Interestingly, we did observe a higher level of HIF1α in the smallest pore size scaffolds than other larger pore sized scaffolds at day 16, which may indicate that as chondrocytes grew, the microenvironment in smaller pore sized scaffolds might have become more hypoxic.
Figure 15. mRNA analysis of genes in chondrocytes cultured in HFIP silk scaffolds with different pore sizes at day 8. All gene expression levels were normalized to GAPDH. Data present mean ± SD. *p<0.05.

Figure 16. mRNA analysis of genes in chondrocytes cultured in HFIP silk scaffolds with different pore sizes at day 16. All gene expression levels were normalized to GAPDH. Data present mean ± SD. *p<0.05.
Different silk scaffolds have different profiles of pro-inflammatory cytokine release.

To investigate why scaffolds fabricated using different fabrication methods and with different structures would result in differential endogenous IL-1β levels or cartilage matrix and cartilage degrading enzyme gene expression upon IL-1β and TNFα treatment, we examined the ability of the scaffolds to adsorb and release pro-inflammatory cytokines. We reasoned that the difference in scaffold structures might cause a difference in the diffusion of the cytokines from inside to outside of the scaffolds, which would lead to a difference in the inflammatory microenvironment for the chondrocytes [149]. We thus applied equal amounts of these cytokines to AQ, HFIP, and HFIP with different pore sizes and then evaluated the amount of cytokines that leached out into the medium. As we were uncertain of the capacity of the scaffolds to adsorb IL-1β or TNFα, we applied two different amounts (1 ng and 10 ng) to the scaffolds, and assayed the medium at different time points using ELISA (Fig. 17). We found that IL-1β and TNFα were released from HFIP silk scaffolds at a faster rate than AQ silk scaffolds (Fig. 17A and B). At the early time point of 10 mins, 70% of IL-1β and 60% of TNFα were released by HFIP silk scaffolds, while only 40% of IL-1β or TNFα were released by AQ silk scaffolds, suggesting that HFIP scaffolds retained less IL-1β and TNFα than AQ silk scaffolds did (Fig. 17A and B). With respect to scaffolds of different pore sizes, we found that larger pore-sized scaffolds released more IL-1β and TNFα than smaller pore-sized scaffolds did, which may be due to the less physical barrier present in larger pore sized scaffolds (Fig. 17C and D). This result is consistent with the fact that HFIP silk scaffolds with larger pore sizes supported a lower level of endogenous IL-1β and IL-6 even in the
absence of exogenously administered pro-inflammatory cytokines which correlates with lower levels of matrix degradation-associated genes in larger pore-sized scaffolds.

Another scaffold property that may affect the microenvironment of the chondrocytes is the ability to uptake water, which is correlated with the hydrophilicity of the biomaterials. The degree of hydration of the microenvironment could determine local concentration of nutrient or pro-inflammatory cytokines, which in turn affect chondrocyte gene expression. We found that HFIP silk scaffolds had a higher ability to retain water than AQ silk scaffolds, suggesting that HFIP silk scaffolds may create a more hydrated microenvironment for the chondrocytes (Fig.17E). Expectedly, HFIP silk scaffolds of different pore size did not have differential water uptake abilities, as they are derived from same scaffold material using the same preparation method (Fig.17F).
A

B

C
Figure 17. Evaluation of cytokine release kinetics of silk scaffolds. Two different amounts of pro-inflammatory cytokines IL-1β or TNFα (1 and 10ng) were loaded onto empty scaffolds of AQ, HFIP, and HFIP with different pore sizes. ELISA was used to verify the initial loading amount and to evaluate the amount of cytokines leached into the medium at 5 different time points: 10min, 1hr, 1 day, and 3 days. (A) Analysis of IL-1β cumulative release from AQ and HFIP silk scaffolds. (B) Analysis of TNFα cumulative release from AQ and HFIP silk scaffolds. (C) Analysis of IL-1β cumulative release from HFIP silk scaffolds with different pore sizes. (D) Analysis of TNFα cumulative release from HFIP silk scaffolds with different pore sizes. (E and F) Analysis of water uptake properties of AQ, HFIP, and HFIP silk scaffolds with different pore sizes. Statistical analysis of the data for cytokine release was determined by two-way ANOVA. Statistical analysis of the water uptake for AQ and HFIP and for HFIP scaffolds with different pore sizes was determined by student t-test and one-way ANOVA, respectively. Data present mean ± SD. *p<0.05.
Discussion

Scaffolding constitutes an integral component of the microenvironment architecture for the chondrocytes in the 3D bioengineered cartilage constructs. A hallmark of arthritis is the elevated inflammation levels in the joints. However, the function of scaffolding on cartilage gene expression in inflammatory environment still remains largely elusive. In the present study, we demonstrated that scaffold fabrication method and scaffold pore size influence the expression of endogenous pro-inflammatory cytokines and chondrocyte gene expression under both control and IL-1β and TNFα treatment.

Biomaterials with different chemical and physical properties have been shown to affect cell attachment, cell shape and gene expression, leading to differential gene expression levels [49]. Previous studies have demonstrated that different cell types exhibited varied differentiation potential in AQ or HFIP silk scaffolds. AQ silk scaffolds supported more optimal osteogenic differentiation from human mesenchymal stem cells (hMSCs), while HFIP silk scaffolds supported better soft dental pulp formation from human dental pulp progenitor cells [71, 92]. Another study revealed that elastic cartilage cells from rabbit ears expressed a higher overall matrix deposition in sucrose/HFIP scaffolds than in AQ silk scaffolds [150]. However, none of these studies examined the performance of AQ and HFIP scaffolds in terms of inflammatory microenvironment. Here, we found that AQ and HFIP scaffolds supported different levels of pro-inflammatory cytokine expression expressed by the chondrocytes even under control conditions. Furthermore, HFIP scaffolds supported much lower endogenous pro-
inflammatory cytokine IL-1β and IL-6 expression under TNFα treatment, which corresponds to the lower levels of MMP3 and HIF2α.

The alteration of chondrocyte gene expression by different types of scaffolds is likely due to the unique chemical and physical characteristics of scaffolds resulted from different fabrication methods. HFIP silk scaffolds showed to have smoother surfaces than AQ silk scaffolds. The pores of HFIP scaffolds are slightly angular in shape, while those of AQ silk scaffolds are round, which is consistent with prior studies [70, 150]. In addition, the data suggests that HFIP silk scaffolds have a higher hydration capacity and exhibit faster cytokine release rate. It is also established that AQ silk scaffolds are more rigid with faster degradation rates, while HFIP silk scaffolds are more elastic and deformable, with lower degradation rates [70, 71, 150]. Therefore, different hydrophobicity, cytokine release kinetic, surface roughness and rigidity resulted from different fabrication methods of the same silk scaffold material can all contribute to the biochemical outcome of the chondrocytes.

Biomaterials with various pore sizes ranged from small (~50μm) to large pore sizes (~500μm) have been examined for cartilage tissue regeneration and redifferentiation potential of chondrocytes in previous studies [60, 68, 151]. Moreover, this structural property of the biomaterial surrounding cells can alter cell behavior and cell fate. It has been shown that altering the dimensions of titanium nanotubes allowed not only enhanced adhesion of but also osteogenic differentiation hMSCs in the absence of osteogenic inducing medium [152]. Another study has demonstrated that different pore sizes affected cell attachment and cell viability of mouse osteogenic cells in collagen-GAG scaffolds [63]. These previous studies strongly suggest that the pore size of
biomaterials surrounding cells play important roles in cell behavior and cell
differentiation.

In the present study, we evaluated chondrocyte gene expression upon pore size
variations of HFIP scaffolds, which varied the structure properties of scaffolds, while
maintaining the material properties of the silk scaffolds. Pore size variation largely
affected mRNA expression of cartilage degrading enzymes in the absence or presence of
pro-inflammatory cytokines over time. We found that larger pore sized scaffolds
supported a higher level of cartilage matrix gene expression and a lower level of cartilage
degrading enzyme expression than smaller pore sized scaffolds. Furthermore, we also
found that pore size of scaffolds influences endogenous expression of cartilage degrading
enzymes.

We had investigated the property of scaffolds in retaining and releasing pro-
inflammatory cytokines. We had reasoned that when equal amount of pro-inflammatory
cytokines were applied to the scaffold, the faster cytokines leached into the medium, the
less cytokines would be available for the chondrocytes within the scaffolds to encounter.
While the protein release profile from the scaffolds has been studied for other proteins
such as BMP-2, VEGF, and IGF-1, it has rarely been attempted for pro-inflammatory
cytokines [129-133]. In our prior analysis on different scaffolding materials, faster
leaching of the pro-inflammatory cytokine was found to be correlated with higher level of
cartilage matrix production [153].

Here, we found HFIP scaffolds (vs. AQ scaffolds) and larger pore sized HFIP
scaffolds (vs. smaller pore sized scaffolds) released IL-1β and TNFα at a faster rate, and
supported higher levels of cartilage matrix. It is not clear why HFIP-silk scaffolds leach
the cytokines faster than AQ-silk scaffolds. It could be related to the smoother surface characteristics of the HFIP scaffolds or their surface chemistry. With respect to pore size, it can be surmised that larger pore size would allow pro-inflammatory cytokines to pass more freely, which would result in faster leaching of the cytokines into the medium.

Interestingly, even in the absence of exogenous IL-1β and TNFα, there was a difference in chondrocyte gene expression. This may be related to the level of endogenous IL-1β expression, which was lowest in HFIP scaffolds (vs. AQ) and those with larger pore sizes (vs. smaller pore sizes). As IL-1β is able to induce its own expression, as well as other pro-inflammatory cytokines (such as IL-6) and cartilage degradation related genes MMPs and HIF2α [142, 147, 154], faster release of IL-1β from the scaffold to the medium, be it endogenous or exogenous IL-1β, may be beneficial to cartilage matrix maintenance. Thus, this property may be an important component of the scaffold as the chondrocyte niche. Certainly, other properties of scaffolding material, including rigidity or degradation rate may also contribute to the chondrocyte niche in regulating the response of chondrocytes to inflammatory stimuli.

The relationship between inflammation and regeneration is intriguing. For some tissues and processes, such as during zebrafish neuron regeneration or fracture healing of the bone, the response to inflammatory stimuli is essential [155, 156]. However, prolonged inflammation could be damaging to the tissues [157, 158]. Unlike many vascularized tissues, the avascular cartilage has poor regeneration ability. Cartilage injury is accompanied by high levels of inflammation, which although subsides over time, may have a profound effect on chondrocyte homeostasis [159]. Many of cartilage injuries (such as ACL or meniscus tear) lead to osteoarthritis (OA) years later. Significantly
higher levels of pro-inflammatory cytokines IL-1β and TNFα were found in early OA joints [160]. However, higher levels of other pro-inflammatory mediators such as IL-6 and leptin were reported to be predominant in end stage OA synovial fluid [161]. Such localized inflammation will compromise the stability of the cartilage. Our study demonstrates that scaffold structure can be an important component to consider when engineering cartilage for use under inflammatory conditions, and thus provides insight into the endeavor of enhancing cartilage regeneration.
Chapter 3

The role of Nkx3.2 on chondrogenesis of hMSCs
Rationale

Nkx3.2, a molecule involved in cartilage development, provides as a potential gene for promoting chondrogenic differentiation and preventing hypertrophy in mouse mesenchymal stem cell lines as previously reported. However, its role in human primary cells has not been thoroughly studied. Human bone marrow derived mesenchymal stem cells (hMSCs) exhibits abundant proliferation ability and can be obtained from various tissues. Chondrogenic differentiation of hMSCs in vitro is often accompanied by hypertrophic differentiation, resulting in undesired cell phenotype for cartilage repair.

In this chapter, we investigated the role of Nkx3.2 on chondrogenesis of hMSCs through gain of function and loss of function approaches. We examined whether Nkx3.2 play a role on promoting chondrogenesis of hMSCs and preventing hMSCs from undergoing hypertrophy, as seen in the mouse mesenchymal stem cell line.

Experimental approaches

To investigate the role of Nkx3.2 on chondrogenesis of hMSCs, we examined the following experiments.

1. The role of Nkx3.2 on chondrogenesis of hMSCs under non-chondrogenic or chondrogenic condition (TGFβ3+dexamethasone) : hMSCs were infected with either lenti-GFP or lenti-Nkx3.2 and exposed to chondrogenic medium containing TGFβ3 (10ng/ml) and dexamethasone for 21days
2. The role of Nkx3.2 on chondrogenesis of hMSCs under chondrogenic condition (BMP2+dexamethasone): hMSCs were infected with either lenti-GFP or lenti-Nkx3.2 and exposed to chondrogenic medium containing BMP2 (100ng/ml) and dexamethasone for 21 days.

3. The role of Nkx3.2 on chondrogenesis of C3H10T1/2 cells: FACS sorted either GFP or Nkx3.2 expressing C3H10T1/2 cells were cultured in chondrogenic medium containing BMP2 (300ng/ml).

4. The role of shRNA-Nkx3.2 on chondrogenesis of hMSCs under chondrogenic condition (TGFβ3+dexamethasone): hMSCs were infected with either lenti-scramble or lenti-shRNA-Nkx3.2 and exposed to chondrogenic medium containing TGFβ3 (10ng/ml) and dexamethasone for 21 days.
Results

Nkx3.2 is induced during chondrogenic differentiation in hMSCs

When hMSCs were cultured for 21 days under chondrogenic condition, containing chondrogenic factors, TGFβ3 and dexamethasone, Nkx3.2 was significantly expressed in hMSCs compared to the expression in cells under non-chondrogenic conditions, suggesting that Nkx3.2 is induced during chondrogenic differentiation and may be involved in the process of chondrogenesis in hMSCs (Fig.18).

To investigate the role of Nkx3.2 during chondrogenic differentiation in hMSCs, we infected hMSCs with either lentiviral constructs encoding for either GFP or GFP-Nkx3.2. Transduction rate of GFP and Nkx3.2 in hMSCs was 75.9±8.7% and 87.3±5.6%, respectively (Fig.19). To confirm chondrogenic differentiation of GFP-hMSCs, used as control in the study, we performed RT-PCR for chondrogenic genes, Sox9, Nkx3.2, collagen II (Col II), and Aggrecan. As expected, Sox9, Nkx3.2, Col II, and Aggrecan expression in GFP-hMSCs were induced over the time course of 21 days under chondrogenic condition (Fig.20).

Figure 18. Gene expression Nkx3.2 in hMSCs after 21 days of culture under Ctrl: growth medium and chondrogenic medium. Chondrogenic medium contains TGFβ3 and dexamethasone. Gene expression level was normalized to GAPDH level.
Figure 19. Transduction efficiency of lentivirus-GFP and Nkx3.2-GFP on hMSCs. (Top) Bright field and fluorescence images of GFP- and Nkx3.2-GFP infected hMSCs. (Bottom) Transduction rate determined by counting GFP-positive cells in GFP- and Nkx3.2-infected hMSCs.
Figure 20. Gene expression analysis of chondrogenic markers in GFP-hMSCs under chondrogenic condition induced by TGFβ3. All gene expression levels were normalized to GAPDH. Data present mean±SD. *p<0.05.

Nkx3.2 inhibits cartilage matrix protein synthesis in hMSCs under chondrogenic condition

To look at the effect of Nkx3.2 on chondrogenesis of hMSCs, we compared expression of Sox9, Nkx3.2, Col II, and Aggrecan between GFP-hMSCs and Nkx3.2-hMSCs under chondrogenic condition (Fig.21). While GFP-hMSCs showed increased expression of Sox9 over the time course, Nkx3.2-hMSCs expressed significant decrease at day 21 compared to GFP-hMSCs. As expected, Nkx3.2 expression was significantly highly expressed in Nkx3.2-hMSCs than GFP-hMSCs. GFP-hMSCs expressed significantly higher levels of Col II and Aggrecan expression under chondrogenic
condition than Nkx3.2-hMSCs. Consistently, as shown in analysis of GAG content, determined by DMMB dye, GFP-hMSCs produced significantly higher GAG than Nkx3.2-hMSCs (Fig.22).

**Figure 21.** Gene expression analysis of chondrogenic markers in GFP-hMSCs and Nkx3.2-hMSCs under chondrogenic condition induced by TGFβ3. All gene expression levels were normalized to GAPDH. Data present mean±SD. *p<0.05 when the expression in GFP-hMSCs significantly higher than that in Nkx3.2-hMSCs. # p<0.05 when the expression in GFP-hMSCs significantly lower than that in Nkx3.2-hMSCs.
Under non-chondrogenic condition, both GFP- and Nkx3.2-hMSCs exhibited extremely low level of GAG content compared to those in chondrogenic medium (Fig.22). This data suggest that Nkx3.2 alone does not promote or inhibit chondrogenic differentiation of hMSCs under non-chondrogenic condition, and may need another factor to regulate chondrogenesis. Consistent with gene expression and GAG analyses, while GFP-hMSCs exhibited significant amount of proteoglycan and Col II, determined by histological staining for Toluidine Blue and Col II, Nkx3.2-hMSCs expressed very low amounts of proteoglycan and Col II (Fig.23).

The inhibition of chondrogenesis in the presence of chondrogenic factors suggests that Nkx3.2 may prevent hMSCs from undergoing chondrogenesis possibly by disrupting TGFβ induced signals to produce cartilage matrix protein for chondrogenesis.

**Figure 22.** Ratio of Glycosaminoglycan (GAG)/DNA in GFP-hMSCs and Nkx3.2-hMSCs under non-chondrogenic or chondrogenic condition at day 21. Chondrogenic medium contains TGFβ3 and dexamethasone. Data present mean±SD. *p<0.05.
Figure 23. Histological evaluation of GFP-hMSCs and Nkx3.2-hMSCs grown in silk scaffolds under chondrogenic condition induced by TGFβ3. (Top) H&E staining images, (middle) Toluidine Blue staining images, and (Bottom) Col II staining images. Scale bar: 100µm.
To confirm this observation, we repeated the experiment with an earlier time point, day 8. Consistent to the results from the first experiment, Nkx3.2 inhibited chondrogenic differentiation in hMSCs at all time points as confirmed by mRNA expression of Col II and Aggrecan and GAG content (Fig.24-26).

**Figure 24.** Gene expression analysis of chondrogenic markers in GFP-hMSCs from repeated experiment under chondrogenic condition induced by TGFβ3. The repeated experiment for the role of Nkx3.2 on hMSCs in the presence of TGFβ3 was carried out including earlier time point, day8. All gene expression levels were normalized to GAPDH. Data present mean±SD. *p<0.05.

**Figure 25.** Gene expression analysis of chondrogenic markers in GFP-hMSCs and Nkx3.2-hMSCs from repeated experiment under chondrogenic condition induced by TGFβ3. The repeated experiment for the role of Nkx3.2 on hMSCs in the presence of TGFβ3 was carried out including earlier time point, day8. All gene expression levels were normalized to GAPDH. Data present mean±SD. *p<0.05 when the expression in GFP-hMSCs significantly higher than that in Nkx3.2-hMSCs. *p<0.05 when the expression in GFP-hMSCs significantly lower than that in Nkx3.2-hMSCs.
Figure 26. Ratio of GAG/DNA in GFP-hMSCs and Nkx3.2-hMSCs from repeated experiment under chondrogenic condition at day 21. Data present mean±SD. *p<0.05.

Nkx3.2 inhibits chondrogenesis in hMSCs from a different donor

To examine whether inhibition of chondrogenesis by Nkx3.2 can be observed in hMSCs from a different donor, we infected hMSCs from another donor with either GFP or Nkx3.2 and evaluated for chondrogenic differentiation. As shown in Figure 27, GFP-hMSCs showed significantly increased Col II expression compared to Nkx3.2-hMSCs at day21. Although Aggrecan did not reveal a statistically significant difference in the expression, the mean value was relatively higher in GFP-hMSCs than Nkx3.2-hMSCs, suggesting the potential role of Nkx3.2 in inhibiting chondrogenesis in hMSCs when overexpressed.
Figure 27. Gene expression analysis of chondrogenic markers in GFP-hMSCs and Nkx3.2-hMSCs from a different donor under chondrogenic condition induced by TGFβ3. All gene expression levels were normalized to GAPDH. Data present mean±SD. *p<0.05 when the expression in GFP-hMSCs significantly higher than that in Nkx3.2-hMSCs. #p<0.05 when the expression in GFP-hMSCs significantly lower than that in Nkx3.2-hMSCs.

Nkx3.2 can promote chondrogenesis in C3H10T1/2 cells

Our data suggest that human Nkx3.2 inhibits chondrogenesis in hMSCs. This result seems to contradict previous results by Kawato et al. where transient expression of mouse Nkx3.2 promoted chondrogenesis in mouse C3H10T1/2 cell progenitor population [162]. To determine whether the result is caused by a difference between human and mouse cells in response to Nkx3.2, or it is due to a difference in Nkx3.2 type (human vs. mouse), we infected C3H10T1/2 cells with either GFP or Nkx3.2 lenti-virus, which used in the experiments in the present study, to examine whether Nkx3.2 could promote chondrogenesis in C3H10T1/2 cells. The infected cells were sorted for GFP positive cells by FACS to acquire pure population of GFP or Nkx3.2 expressing cells.
Figure 28. Gene expression analysis of Col II and Aggrecan in GFP-10T1/2 and Nkx3.2-10T1/2 cultured on 2D under chondrogenic condition induced by BMP2 (300ng/ml). All gene expression levels were normalized to GAPDH. Data present mean±SD. *p<0.05 when the expression in GFP-hMSCs significantly higher than that in Nkx3.2-hMSCs. #p<0.05 when the expression in GFP-hMSCs significantly lower than that in Nkx3.2-hMSCs.

Figure 29. Gene expression analysis of Col II, Col IX, and Aggrecan in GFP-10T1/2 and Nkx3.2-10T1/2 cultured in 3D micromass under chondrogenic condition induced by BMP2 (300ng/ml). All gene expression levels were normalized to GAPDH. Data present mean±SD. *p<0.05 when the expression in GFP-hMSCs significantly higher than that in Nkx3.2-hMSCs. #p<0.05 when the expression in GFP-hMSCs significantly lower than that in Nkx3.2-hMSCs.
Sorted C3H10T1/2 cells were cultured on tissue culture plate (2D) and in micromass (3D). Cells were maintained in chondrogenic medium containing a chondrogenic factor, BMP2 (300ng/ml) for 3 and 7 days, as reported by Kawato et al., and analyzed for chondrogenic markers, Col II, Col IX and Aggrecan. As shown in Figure 28 and 29, consistent with the report from Kawato et al., Nkx3.2 infected C3H10T1/2 cells exhibited significantly higher expression of Col II, Col IX, and Aggrecan. This suggests that human Nkx3.2 indeed promote chondrogenesis in the mouse cells, even though it has an opposite role on human MSCs. Thus, the role of Nkx3.2 varies depending on cell type, and Nkx3.2 may act in a different way on human and mouse.

**Nkx3.2 inhibits chondrogenesis of hMSCs in the presence of BMP2**

TGFβ induced chondrogenesis of hMSCs has widely been used in many studies as well as other chondrogenic growth factors such as BMPs and IGFs. It has been shown that chondrogenesis of MSCs was more enhanced by TGFβ1 than by BMP2 [163, 164]. However, in another study, chondrogenesis of MSCs induced by TGFβ was promoted with addition of BMPs in obtaining large amounts of cartilage rich in proteoglycans [165], suggesting TGFβ and BMPs may interplay to support the chondrogenesis of MSCs.

It is possible that Nkx3.2 does not cooperate with TGFβ signaling pathway to promote chondrogenesis in hMSCs but needs signaling pathway activated by other factors to activate chondrogenic differentiation in hMSCs.

Indeed, molecular analysis has shown that Nkx3.2 cooperate with BMPs to promote chondrogenesis. During cartilage development, Nkx3.2 plays an important role to promote chondrogenic differentiation of mesenchymal stem cells. It has been shown
that chondrogenesis by Nkx3.2 requires BMP signals to be present to undergo chondrogenic differentiation. Forced expression of Nkx3.2 promoted chondrogenesis and induced Sox9 expression in chick embryo explants in the presence of BMP signals, suggesting the importance of BMP signals present for Nkx3.2 to induce chondrogenesis [166, 167]. Moreover, given that previous studies demonstrating the role of Nkx3.2 on collagen II expression during chondrogenic differentiation and preventing hypertrophic differentiation in C3H10T1/2 cells were performed in the presence of BMP2, the role of Nkx3.2 in chondrogenesis in hMSCs may be appeared with BMP signals, not with TGFβ3 signals. BMP and TGFβ activate different Smad proteins; Smad1/5 and Smad2/3, respectively, which act as transcription factors in the nucleus to activate chondrogenic genes [168, 169]. Nkx3.2 has been shown to primarily bind to Smad1, but lesser amount of Smad2 [170]. Thus, it is possible that Nkx3.2 cooperate with BMP2 signaling pathway rather than TGFβ signaling pathway to induce chondrogenesis.

To look at the effect of Nkx3.2 on chondrogenesis of hMSCs in the presence of BMP2, we cultured GFP infected or Nkx3.2 infected hMSCs in chondrogenic medium containing BMP2. As shown in Figure 30, GFP-hMSCs underwent chondrogenesis with increased expression of Nkx3.2, Col II and Aggrecan under chondrogenic condition induced by BMP2. However, Nkx3.2-hMSCs inhibited their expression as seen in the experiment with TGFβ3 (Fig.31). To confirm the observation, we repeated the experiment with BMP2 and consistent to data from the first experiment, Nkx3.2 inhibited chondrogenic differentiation in hMSCs (Fig. 34-35).

In analysis of GAG content, after 21 days of culture, although GAG content was similar between GFP-hMSCs and Nkx3.2-hMSCs, due to lower amount of DNA content
in Nkx3.2-hMSCs, total GAG content obtained by ratio of GAG to DNA appeared to be higher in Nkx3.2-hMSCs (Fig.32). Given that GAG contents in both GFP- and Nkx3.2-hMSCs induced by BMP-2 (5~6μg) are significantly lower amount than that by TGFβ3 (40~50μg) and that there was no significant difference in Toluidine Blue staining (Fig.33), GAG contents between the groups may not be largely different. This was also confirmed by repeated experiments (Fig.36).

Figure 30. Gene expression analysis of chondrogenic markers in GFP-hMSCs under chondrogenic condition induced by BMP2. All gene expression levels were normalized to GAPDH. Data present mean±SD. *p<0.05.

Figure 31. Gene expression analysis of chondrogenic markers in GFP-hMSCs and Nkx3.2-hMSCs under chondrogenic condition induced by BMP2. All gene expression levels were normalized to GAPDH. Data present mean±SD. *p<0.05 when the expression in GFP-hMSCs significantly higher than that in Nkx3.2-hMSCs. #p<0.05 when the expression in GFP-hMSCs significantly lower than that in Nkx3.2-hMSCs.
Figure 32. Ratio of GAG/DNA in GFP-hMSCs and Nkx3.2-hMSCs under chondrogenic condition induced by BMP2. Data present mean±SD. *p<0.05.

Figure 33. Toluidine blue staining on sections from scaffolds with either GFP-hMSCs or Nkx3.2-hMSCs under chondrogenic condition induced by BMP-2.
Figure 34. Gene expression analysis of chondrogenic markers in GFP-hMSCs from repeated experiment under chondrogenic condition induced by BMP2. All gene expression levels were normalized to GAPDH. Data present mean±SD. *p<0.05.

Figure 35. Gene expression analysis of chondrogenic markers in GFP-hMSCs and Nkx3.2-hMSCs from repeated experiment under chondrogenic condition induced by BMP2. All gene expression levels were normalized to GAPDH. Data present mean±SD. *p<0.05 when the expression in GFP-hMSCs significantly higher than that in Nkx3.2-hMSCs. #p<0.05 when the expression in GFP-hMSCs significantly lower than that in Nkx3.2-hMSCs.
As shown in the results, Nkx3.2 exhibited a similar effect on chondrogenesis in hMSCs in the presence of BMP2 as compared to TGFβ. Nkx3.2-hMSCs exhibited poor expression of Col II and Aggrecan over the time course in the presence of chondrogenic medium containing TGFβ3 or BMP2. These data suggest that Nkx3.2 acts as a repressor during chondrogenic differentiation in hMSCs when overexpressed.
Nkx3.2 is necessary during chondrogenesis of hMSCs

To further investigate the role of Nkx3.2 during the chondrogenesis, whether it is necessary during the process of chondrogenic differentiation in hMSCs, we have knockdowned Nkx3.2 expression in hMSCs using lenti-virus encoding for shRNA against Nkx3.2. Nkx3.2 expression was upregulated in scramble-hMSCs over time and was significantly expressed at low level in shRNA-Nkx3.2 treated hMSCs though the expression in both cells was expressed at similar level at day 21. Although Col II and Aggrecan showed increased expression in scramble-hMSCs over time, shRNA-Nkx3.2 treated hMSCs expressed relatively lower expression of Col II and significantly low expression of Aggrecan (Fig.37). These data suggest that Nkx3.2 is necessary during chondrogenic differentiation, but may be required only at certain levels to facilitate chondrogenic differentiation in hMSCs.

**Figure 37.** Gene expression analysis of chondrogenic markers in scramble-hMSCs and shRNA-Nkx3.2 cells under chondrogenic condition induced by TGFβ3. All gene expression levels were normalized to GAPDH. Data present mean±SD. *p<0.05 when the expression in GFP-hMSCs significantly higher than that in Nkx3.2-hMSCs. #p<0.05 when the expression in GFP-hMSCs significantly lower than that in Nkx3.2-hMSCs.
Nkx3.2 exhibits increased cell adhesion markers in hMSCs

GFP-hMSCs and Nkx3.2-hMSCs exhibited differences in cell size and cell morphology. This difference was observed when repeated experiment for the role of Nkx3.2 on hMSCs’s chondrogenesis by TGFβ3 was carried out. As shown in Figure 38, Nkx3.2-hMSCs appear to be more spread out and bigger in cell size compared to GFP-hMSCs. Moreover, Nkx3.2-hMSCs had vacuole like structure in cytoplasm. Interestingly, when evaluated for cell adhesion markers, integrin α1, α2, α5, and β1 and N-cadherin, Nkx3.2-hMSCs showed significantly increased expression in these markers compared to GFP-hMSCs (Fig.39).

Figure 38. Cell size comparison between GFP-hMSCs and Nkx3.2-hMSCs at passage number 6. The images are taken before cells were trypsinized for seeding onto scaffolds. The cells were used for the repeated experiment; the role of Nkx3.2 on chondrogenesis of hMSCs under chondrogenic condition induced by TGFβ3.
Figure 39. Gene expression analysis of cell adhesion markers in GFP-hMSCs and Nkx3.2-hMSCs from repeated experiment under chondrogenic condition induced by TGFβ3. All gene expression levels were normalized to GAPDH. Data present mean±SD. *p<0.05 when the expression in GFP-hMSCs significantly higher than that in Nkx3.2-hMSCs. # p<0.05 when the expression in GFP-hMSCs significantly lower than that in Nkx3.2-hMSCs.

To confirm this observation, we examined these cell adhesion markers in samples from the initial experiment (Fig. 40). When compared to GFP-hMSCs, as observed in the repeated experiment, Nkx3.2-hMSCs showed significantly higher cell adhesion marker expressions.
Figure 40. Gene expression analysis of cell adhesion markers in GFP-hMSCs and Nkx3.2-hMSCs from the initial experiment under chondrogenic condition induced by TGFβ3. All gene expression levels were normalized to GAPDH. Data present mean±SD. *p<0.05 when the expression in GFP-hMSCs significantly higher than that in Nkx3.2-hMSCs. #p<0.05 when the expression in GFP-hMSCs significantly lower than that in Nkx3.2-hMSCs.

This difference in cell adhesion markers were revealed in hMSCs from a different donor (Fig. 41). These data suggest that Nkx3.2 may be involved in determining cell size and cell morphology and that this activity of Nkx3.2 may have prevented cells from undergoing proper chondrogenic differentiation in hMSCs.
Figure 41. Gene expression analysis of cell adhesion markers in GFP-hMSCs and Nkx3.2-hMSCs from a different donor under chondrogenic condition induced by TGFβ3. All gene expression levels were normalized to GAPDH. Data present mean±SD. *p<0.05 when the expression in GFP-hMSCs significantly higher than that in Nkx3.2-hMSCs. #p<0.05 when the expression in GFP-hMSCs significantly lower than that in Nkx3.2-hMSCs.

Discussion

Nkx3.2 is a molecule involved in cartilage formation during development and its important role has been demonstrated in many studies. Recent reports demonstrate the role of Nkx3.2 on promoting chondrogenic differentiation and inhibiting hypertrophy markers in the mouse mesenchymal cell lines, C3H10T1/2 cells. These reports thus brought potential possibility of utilizing Nkx3.2 to improve cartilage differentiation. Sox9, a critical transcription factor involved in cartilage formation and a master regulator of cartilage matrix proteins, has been shown to promote hMSCs’s chondrogenic
differentiation in the absence of chondrogenic factors, indicating possible use of biochemical factors in improving chondrogenic differentiation of human primary cells. Thus in the present study, the role of Nkx3.2 was investigated on human primary cells, hMSCs.

Interestingly, contrary to the observation in the mouse mesenchymal cell line [162], hMSCs expressing ectopic Nkx3.2 exhibited poor expression of Col II and Aggrecan and inhibited chondrogenesis in hMSCs while GFP infected hMSCs underwent chondrogenesis with increased expression of Col II and Aggrecan. Furthermore, Nkx3.2-hMSCs showed significantly lower expression in Col II and Aggrecan than GFP-hMSCs in the presence of BMP2, which is another growth factor known to stimulate chondrogenic differentiation in hMSCs. This was carried out under assumption that Nkx3.2 may interact with BMP2, but not with TGFβ signaling pathway to promote chondrogenic differentiation in hMSCs based on previous studies. However, in both cases, Nkx3.2 inhibited chondrogenesis in hMSCs. These data suggest that overexpression of Nkx3.2 is not desirable for chondrogenesis of hMSCs.

TGFβ has been shown to induce chondrogenesis by stabilizing the Sox9 transcription complex through Smad2/3 [171, 172]. Smad2/3 signaling pathway has been shown to interact with MAPK signaling pathway including p38 and ERK for chondrogenesis in mesenchymal stem cells [173]. Furthermore, BMP2 has been shown to act through the Smad1/5/8 signaling pathway to induce chondrogenesis and also to promote chondrogenesis of MSCs with the presence of TGFβ. Thus, for further investigation on the effect of Nkx3.2 on the inhibition of chondrogenesis in hMSCs, downstream signaling pathways activated by TGFβ and BMP2 can be studied.
It is possible that the level of Nkx3.2 expression may need to be maintained at a certain level for hMSCs to undergo proper chondrogenic differentiation, especially as it is induced in hMSCs during chondrogenic differentiation. Thus, shRNA-against Nkx3.2 was used to examine the role of endogenous Nkx3.2 on chondrogenesis of hMSCs.

Compared to control group, scramble-hMSCs, shRNA infected hMSCs showed overall lower expression of Col II and Aggrecan during chondrogenic differentiation, suggesting that Nkx3.2 is necessary for chondrogenesis of hMSCs.

Interestingly, Nkx3.2-hMSCs exhibited more spread out and larger in cell size than GFP-hMSCs. Gene expression analysis in cell adhesion molecules, integrin α1, α2, α5, β1, and N-cadherin showed that Nkx3.2-hMSCs expressed these markers at higher level than GFP-hMSCs, which was consistently observed in all repeated experiments.

However, the difference in cell morphology between GFP-hMSCs and Nkx3.2-hMSCs was more noticeable in some experiments, and not so obviously found in others, suggesting a possible difference in transduction efficiency and donor variability.

Integrins act as the cell adhesion molecules that mediate cell-matrix and cell-cell interactions. The interactions of cells have been shown to mediate various cellular processes including cell morphology, motility, proliferation, and differentiation. Integrins have shown to play important roles in chondrogenic differentiation of hMSCs. It has been shown that differentiation of human mesenchymal stem cells (hMSCs) into chondrocytes requires cell signaling pathways activated by their binding to fibronectin (α5β1) [174]. hMSCs upregulates fibronectin production upon binding to fibronectin which supports cell condensation, cell signaling at the early stages of chondrogenic differentiation. However, the level of fibronectin has been shown to be downregulated at the later stages
of chondrogenic differentiation of hMSCs [174, 175]. Previous studies have also demonstrated that the presence of fibronectin or its major binding site, RGD (arginine-lysine-aspartic acid) is required to initiate chondrogenic differentiation of hMSCs, but the differentiation of these cells is inhibited when these molecules are persistently present [176, 177]. As Nkx3.2-hMSCs expressed consistently higher levels of integrin α5 and β1 over 21 days of cultures than GFP-hMSCs, this may result in inhibition of chondrogenesis in hMSCs. Not only α5β1 but also integrin α1β1 and α2β1 play important roles in hMSCs during chondrogenic differentiation as they serve as receptors for collagens. However, as shown in the example of α5β1 above, persistently expressed integrins may disrupt proper chondrogenic differentiation in hMSCs. These cell adhesion molecules are also involved in determining cell morphology. Thus it is possible that overexpression of Nkx3.2 in hMSCs may have disrupted the focal adhesion and actin cytoskeleton properties in cells which are under downstream of integrins, resulting in impaired chondrogenic differentiation in hMSCs. For further studies, downstream of integrins such as Rho/Rac family signaling pathways can be investigated on the effect of Nkx3.2 on cell morphology.

Our data suggest that the role of Nkx3.2 varies between human and mouse or primary cells and cell lines. Thus, it is important to evaluate potential molecules for cartilage repair and regeneration ultimately in human primary cells, not just in cells from other animals to understand their effects for therapeutic purposes.
Chapter 4

The role of Nkx3.2 on redifferentiation of nHACs and its effect under inflammatory condition
**Rationale**

Human articular chondrocyte has been utilized for cartilage tissue engineering as it provides native phenotype and less immunogenicity. However, due to small cell number obtained from a donor, cell expansion in vitro is necessary to acquire sufficient amount of cells, resulting in de-differentiation and loss of their chondrogenic capacities to recreate stable hyaline like tissue.

Many transcription factors have differential roles at different development stages. Among them, Sox9 and Nkx3.2 have been shown to interplay to promote chondrogenesis as shown in chick embryo explants and muscle derived stem cells. However, their role in human primary cells is likely different. Although transduction of Sox9 has been shown to be involved in inducing hMSCs’s chondrogenesis, we demonstrated in Chapter 3 that Nkx3.2 inhibited chondrogenesis of hMSCs. Furthermore, it has been shown that transduction of Sox9 enhanced chondrogenic gene expression and cartilage matrix formation in de-differentiated normal and OA articular chondrocytes in the presence of chondrogenic factors, TGFβ3 and IGF1 [84, 178]. However, the effect of Nkx3.2 on de-differentiated articular chondrocytes is not well known. To investigate the role of Nkx3.2, whether it can enable the cells to exhibit chondrogenic phenotype as shown by Sox9, we transduced human articular chondrocytes with Nkx3.2 and examined its effect during chondrogenic redifferentiation process. In addition, the role of Nkx3.2 on redifferentiated articular chondrocytes was also examined to look at whether its role varies depending on the cell state.

We have looked at Nkx3.2 expression in bovine articular chondrocytes in the absence or presence of IL-1β and found that its expression was significantly
downregulated in the cells treated with IL-1β, suggesting that Nkx3.2 may interfere with IL-1β signaling pathway. Thus to investigate the role Nkx3.2 on inflammatory response, we also examined whether transduction of Nkx3.2 influences the response toward pro-inflammatory stimuli in the redifferentiated human articular chondrocytes.

**Experimental approaches**

To investigate the role of Nkx3.2 on human normal articular chondrocytes (nHACs) during redifferentiation and its role on the redifferentiated nHACs under inflammatory condition, we performed the following experiments.

1. **The role of Nkx3.2 on nHACs’s redifferentiation**: nHACs infected with either lenti-GFP or lenti-Nkx3.2 were cultured for 28 days in chondrogenic redifferentiation medium.

2. **The role of Nkx3.2 on redifferentiated nHACs and its role in response to pro-inflammatory cytokine**: redifferentiated nHACs were infected with either lenti-GFP or lenti-Nkx3.2 and exposed to pro-inflammatory cytokine, IL-1β.
**Results**

nHACs can be redifferentiated in 3D porous silk scaffolds

nHACs purchased from Lonza are in dedifferentiated state thus redifferentiation process is essential to acquire normal differentiated articular chondrocytes. According to the convention protocol, nHACs can be differentiated in alginate beads for 3 weeks. However, we performed this experiment using silk scaffolds to be consistent with our prior studies. Thus, to determine whether nHACs can be redifferentiated in 3D silk scaffolds, nHACs were seeded onto HFIP silk scaffolds with a pore size of 500-600µm and cultured for 14 and 21 days in chondrogenic medium. As shown in Figure 42, nHACs were able to redifferentiate in silk scaffolds with increased expression of Sox9 and Col II.

![Figure 42](image-url)

**Figure 42.** Gene expression of Sox9 and Col II in nHACs grown in silk scaffolds. nHACs were seeded onto scaffolds and cultured under redifferentiation chondrogenic medium for 21 days. All gene expression levels were normalized to GAPDH.
Nkx3.2 inhibits cartilage matrix gene expression and promotes hypertrophic gene expression in nHACs during chondrogenic redifferentiation

To investigate the role of Nkx3.2 during chondrogenic differentiation in nHACs, we infected nHACs with either lenti-viral constructs encoding for either GFP or GFP-Nkx3.2. The transduction rates of GFP and Nkx3.2 in nHACs was 67±12.3% and 74.7±11.4%, respectively (Fig.43). Nkx3.2 was significantly induced during redifferentiation in control group, GFP-nHACs (Fig.44). As expected, Nkx3.2-nHACs expressed constitutively higher Nkx3.2 expression than GFP-nHACs during 28days (Fig.44).

Figure 43. Transduction efficiency of lentivirus-GFP and Nkx3.2-GFP on nHACs. Bright field and fluorescence images of GFP- and Nkx3.2-GFP on nHACs
Figure 44. Gene expression of Nkx3.2 in GFP-nHACs and Nkx3.2-nHACs during redifferentiation. Gene expression levels were normalized to GAPDH. Data present mean±SD. *p<0.05 when the expression in GFP-nHACs significantly higher than that in Nkx3.2-nHACs. # p<0.05 when the expression in GFP-nHACs significantly lower than that in Nkx3.2-nHACs.

Figure 45. Gene expression analysis of chondrogenic markers in GFP-nHACs and Nkx3.2-nHACs during redifferentiation. All gene expression levels were normalized to GAPDH. Data present mean±SD. *p<0.05 when the expression in GFP-nHACs significantly higher than that in Nkx3.2-nHACs. # p<0.05 when the expression in GFP-nHACs significantly lower than that in Nkx3.2-nHACs.
Redifferentiation of nHACs was observed over 28 days of culture. As similar to inhibitory role of Nkx3.2 on chondrogenesis of hMSCs, Nkx3.2 inhibited cartilage matrix gene expression Col II and Aggrecan over time, indicating that Nkx3.2 may act as an inhibitory molecule during redifferentiation of nHACs (Fig. 45).

In addition, hypertrophy markers Runx2 and Col X were examined in nHACs over the redifferentiation. Compared to GFP-nHACs, Nkx3.2-nHACs exhibited significantly upregulated Runx2 and Col X expression which gradually increased over time (Fig. 46).

**Figure 46.** Gene expression analysis of hypertrophy markers in GFP-nHACs and Nkx3.2-nHACs during redifferentiation. All gene expression levels were normalized to GAPDH. Data present mean±SD. *p<0.05 when the expression in GFP-nHACs significantly higher than that in Nkx3.2-nHACs. #p<0.05 when the expression in GFP-nHACs significantly lower than that in Nkx3.2-nHACs.
This inhibitory effect of Nkx3.2 on nHACs is similar to the inhibition of chondrogenesis in hMSCs. This experiment was repeated with nHACs from a different donor. Consistent to the results from the first experiment, Nkx3.2 inhibited the expression of chondrogenic marker Col II and significantly increased the expression of hypertrophic marker Runx2 (Fig.47).

**Figure 47.** Gene expression analysis of Col II and Runx2 in GFP-nHACs and Nkx3.2-nHACs from a different donor during redifferentiation. All gene expression levels were normalized to GAPDH. Data present mean±SD. *p<0.05 when the expression in GFP-nHACs significantly higher than that in Nkx3.2-nHACs. *p<0.05 when the expression in GFP-nHACs significantly lower than that in Nkx3.2-nHACs.

Nkx3.2 exhibits small and round cell shape in redifferentiated nHACs.

hMSCs and nHACs used in the study are undifferentiated and dedifferentiated state, respectively. Overexpression of Nkx3.2 had inhibitory effects on these cells, preventing chondrogenic differentiation. These data leads to further investigation;
whether Nkx3.2 has same effects on redifferentiated chondrocytes. To investigate whether Nkx3.2’s effects are different depending on cell state, we infected redifferentiated nHACs with either lenti-GFP or lenti-Nkx3.2.

![Figure 48](image)

**Figure 48.** Gene expression of Col II in nHACs after 28 days of culture under chondrogenic redifferentiation medium. Gene expression level was normalized to GAPDH. Data present mean±SD. *p<0.05.

After nHACs were successfully re-differentiated in silk scaffolds for 28 days, confirmed by significantly increased Col II expression (Fig. 48), the redifferentiated nHACs were retrieved from scaffolds. Collected cells were infected with either lenti-GFP or lenti-Nkx3.2 in a pellet and plated on 2D tissue culture plates to acquire evenly distribute cell population. After 2 days of incubation, each cell type was passaged onto 24 well plates and subject to either non- or pro-inflammatory cytokine, IL-1β (5 ng/ml). As generating lenti-virus amount necessary and acquiring sufficient cell number for 3D culture were not feasible, we performed the experiment in 2D culture.
After 3 days of culture, Nkx3.2 infected nHACs expressed significantly higher expression of Nkx3.2 than GFP infected nHACs (Fig. 49).

Interestingly, GFP-nHACs and Nkx3.2-nHACs exhibited distinct cell morphology. While GFP-nHACs were spread out and more likely dedifferentiated chondrocyte phenotype, Nkx3.2-nHACs exhibited smaller and rounder cell morphology like articular chondrocytes (Fig. 50). This morphological difference was also observed in GFP-nHACs and Nkx3.2-nHACs upon IL-1β treatment. No major difference in cell morphology between control and IL-1β treated group was observed.

**Figure 49.** Gene expression of Nkx3.2 in GFP-nHACs and Nkx3.2-nHACs in the absence or presence of IL-1β after 3 days of culture. Gene expression level was normalized to GAPDH. Data present mean±SD. *p<0.05.
Figure 50. Bright field and fluorescence images of GFP-nHACs and Nkx3.2-nHACs after 3 days of culture.

Nkx3.2 expresses higher cartilage matrix proteins and less degrading enzyme in the presence of pro-inflammatory cytokine

Redifferentiated nHACs that were transduced with either lenti-GFP or -Nkx3.2 and cultured in the absence or presence of IL-1β were evaluated for cartilage matrix markers, Col II and Aggrecan and cartilage matrix degrading enzyme, MMP13. In the presence of IL-1β, Aggrecan expression was significantly downregulated in both groups while Col II did not show reduction in the expression, possibly due to the features of de-differentiation of nHACs (Fig.51).
Although Nkx3.2-nHACs did not exhibit major differences in Col II and Aggrecan expression compared to GFP-nHACs, Nkx3.2-nHACs expressed significantly higher Aggrecan expression than GFP-nHACs in the presence of IL-1β (Fig.51).

![Figure 51](image)

**Figure 51.** Gene expression analysis of Col II and Aggrecan in GFP-nHACs and Nkx3.2-nHACs in the absence or presence of IL-1β after 3 days of culture. All gene expression levels were normalized to GAPDH. Data present mean±SD. *p<0.05.

Interestingly, while GFP-nHACs expressed significantly higher MMP13 upon IL-1β treatment, Nkx3.2-nHACs did not exhibit an increase in MMP13 expression under IL-1β treatment, suggesting Nkx3.2 may protect cells from IL-1β induced matrix damage (Fig.52). Furthermore, the expression of MMP13 was significantly lower compared to that in GFP-hMSCs treated with IL-1β (Fig.52).
To confirm this observation, we repeated this experiment with nHACs from a different donor. As shown in Figure 53, cells that were redifferentiated for 24 days showed increased Col II and Aggrecan expression compared to dedifferentiated cells at day 0. Consistent to cell morphology seen in the first experiment, Nkx3.2-nHACs revealed small and round cell shape when compared to GFP-nHACs (Fig. 54). As observed in the results from the first experiment, Col II in nHACs did not show a reduction under IL-1β treatment (Fig. 55). Interestingly, however, Nkx3.2-nHACs exhibited significantly higher Col II expression than GFP-nHACs upon IL-1β treatment. In the presence of IL-1β, Aggrecan expression was significantly higher in Nkx3.2-nHACs than GFP-nHACs and MMP13 expression was also upregulated in GFP-nHACs while Nkx3.2-nHACs had no significant increase in the expression (Fig. 55 and 56).

These data strongly suggest that Nkx3.2 may have a potential role in rescuing cells from pro-inflammatory cytokine IL-1β induced damage.

Figure 52. Gene expression of MMP13 in GFP-nHACs and Nkx3.2-nHACs in the absence or presence of IL-1β after 3 days of culture. Gene expression level was normalized to GAPDH. Data present mean±SD. *p<0.05.
Figure 53. Gene expression analysis of Col II and Aggrecan in nHACs from a different donor after 24 days of culture under chondrogenic redifferentiation medium. All gene expression levels were normalized to GAPDH. Data present mean±SD. *p<0.05.

Figure 54. Bright field and fluorescence images of GFP-nHACs and Nkx3.2-nHACs from a different donor after 3 days of culture.
Figure 55. Gene expression analysis of Col II and Aggrecan in GFP-nHACs and Nkx3.2-nHACs from a different donor in the absence or presence of IL-1β after 3 days of culture. All gene expression levels were normalized to GAPDH. Data present mean±SD. *p<0.05.

Figure 56. Gene expression of MMP13 in GFP-nHACs and Nkx3.2-nHACs from a different donor in the absence or presence of IL-1β after 3 days of culture. Gene expression level was normalized to GAPDH. Data present mean±SD. *p<0.05.
To investigate whether Nkx3.2 expression in redifferentiated nHACs have any effects on hypertrophy markers, we have analyzed mRNA expression of Col X and Runx2 (Fig. 57). Col X and Runx2 expression were upregulated in response to IL-1β in GFP-nHACs. Nkx3.2-nHACs expressed significantly higher Col X expression than GFP-nHACs in the absence of IL-1β while no difference was observed in Runx2 expression between the groups. In the presence of IL-1β, Col X and Runx2 expression was not significantly different between GFP-nHACs and Nkx3.2-nHACs (Fig. 57).

Figure 57. Gene expression analysis of hypertrophy markers in GFP-nHACs and Nkx3.2-nHACs in the absence or presence of IL-1β after 3 days of culture. All gene expression levels were normalized to GAPDH. Data present mean±SD. *p<0.05.
Discussion

To investigate the role of Nkx3.2 on the redifferentiation of nHACs, we infected nHACs with either lenti-GFP or –Nkx3.2 and cultured the cells in chondrogenic redifferentiation medium for 4 weeks. As observed in hMSCs’s chondrogenesis, Nkx3.2 inhibited redifferentiation of nHACs with significantly decreased expression of Col II and Aggrecan compared to GFP-nHACs. While hMSCs can become multiple tissues such as cartilage, bone, and ligament, etc., under appropriate conditions, de-differentiated nHACs can become chondrogenic cells when cultured in 3D and in chondrogenic medium. This redifferentiation process in human articular chondrocytes utilizes part of the signaling processes in the early chondrogenesis in limb bud cells [179]. In chapter 3, the data suggest that Nkx3.2 is necessary for hMSCs to undergo chondrogenesis, but overexpression of Nkx3.2 inhibits hMSCs chondrogenesis. Thus, it is possible that the level of Nkx3.2 needs to be remained at an optimal level for hMSCs’s chondrogenesis. It is not clear whether Nkx3.2 is required in a similar way during chondrocyte redifferentiation. To understand whether or not Nkx3.2 is necessary during the redifferentiation process, one needs to perform an RNAi knock-down of Nkx3.2, and investigate whether this would affect TGFβ-mediated re-differentiation in nHACs.

Previous studies by Kawato et al. and Lengner et al. demonstrated the role of Nkx3.2 on suppressing Runx2 in mouse mesenchymal stem cells, C3H10T1/2 cells to promote chondrogenic differentiation [90, 162]. However, in the present study, nHACs expressing Nkx3.2 exhibited significantly higher expression of Runx2 as well as Col X during redifferentiation. This observation is contradictory to the data from those prior studies with C3H10T1/2 cells. Furthermore, Lengner et al. also demonstrated that Runx2
repression by Nkx3.2 varied between undifferentiated cells and cells committed to the osseous lineage, indicating that its role on the repression differ depending on the cell state; uncommitted and committed [90]. Runx2 expression was highly expressed in Nkx3.2-nHACs compared to GFP-nHACs during redifferentiation. However, redifferentiated nHACs infected with Nkx3.2 did not exhibit major difference in the expression when compared to redifferentiated nHACs infected with GFP in both the absence and presence of IL-1β. Thus, it is possible that the role of Nkx3.2 could vary depending on cell state. As discussed in Chapter 3, Nkx3.2 could exhibit a differential role on human and mouse or primary cells and cell lines. Indeed, prior study by Li et al. demonstrated that chondrogenic and osteogenic differentiation potentials between murine primary bone marrow stromal cells and C3H10T1/2 cells are similar [180]. Thus it is more likely due to differential role of Nkx3.2 on human and mouse. For further studies, the role of Nkx3.2 on chondrogenesis of murine primary cells can be investigated.

Interestingly, in the presence of IL-1β, redifferentiated nHACs infected with Nkx3.2 exhibited higher expression of Aggrecan and lower expression of MMP13 than control cells which are infected with GFP. This protective function of Nkx3.2 on redifferentiated nHACs in the presence of IL-1β is intriguing, suggesting that Nkx3.2 may interfere with IL-1β signaling pathway.

Distinct cell morphology between GFP- and Nkx3.2 infected redifferentiated nHACs suggests possible role of Nkx3.2 on determining cell shape. As cell morphology of chondrocytes is important to maintain their phenotype [14, 98], rounder cell morphology in Nkx3.2 infected nHACs may be more desirable. In contrast to the effect of Nkx3.2 on hMSCs which exhibited flat and spread out cell morphology, Nkx3.2
supported small and round cell morphology on redifferentiated nHACs, suggesting possible differential effect of Nkx3.2 varied on cell state. Thus for further investigation on the role of Nkx3.2 on determining cell shape, signaling pathways involved in cell morphology, downstream of integrins and Rho/Rac family can be investigated in uncommitted and committed cells.

In addition, it has to be in consideration that redifferentiated nHACs begin to start dedifferentiating after they are retrieved from scaffolds and plated on 2D. Even though cells grown on 2D expressed still significantly higher expression of chondrogenic markers compared to that in dedifferentiated cells, it is important to maintain cells in differentiated state for accurate analysis and further investigation for the effect of Nkx3.2. Although Nkx3.2 presented as a potential protective molecule against cytokine induced damage in nHACs, it is essential to study whether this effect can be persisted when incubated for longer time.

In the present study, as preparing virus amounts needed for infecting cells for 3D culture is not feasible, cells were retrieved from scaffolds and only desired amount of cells were infected and grown on 2D. It would be supportive if there are improved experimental systems to investigate potential roles of genes on differentiated primary cells in 3D and also under inflammatory conditions.

Nevertheless, it is very interesting finding that Nkx3.2 has a rescuing effect on cells from cytokine induced damage under inflammatory condition and this protective effect could be further investigated with other pro-inflammatory cytokines (e.g. TNFα) or with other promising genes (e.g. Sox9) for synergistic effects. Another interesting finding was the differential role of Nkx.3.2 on different cell state. As demonstrated in Chapter 3,
Nkx3.2 inhibited chondrogenesis in hMSCs, which are undifferentiated cells. Thus, it would be interesting to look at how Nkx3.2 affects differentiated hMSCs and its role under inflammatory conditions. Although more comprehensive studies on Nkx3.2’s effects on human primary cells are needed, this brings in possible use of molecules derived from its tissue of origin for cartilage repair and regeneration and ultimately contributes to the development of advanced cartilage tissue engineering.
5. Conclusion

Cartilage tissue engineering is an attractive therapeutic option in treating OA as demonstrated by numerous studies. However, the instability of engineered cartilage tissue remains a big challenge to overcome. Although engineering cartilage tissue using a variety of different sources including cell types, scaffolding materials, and culture environment have been extensively studied, engineering cartilage under consideration of a real environment of OA has not been comprehensively studied.

Inflammation present in OA joint is one of major causes for instability of engineered cartilage. Pro-inflammatory cytokines and their mediators have been demonstrated to be involved in the progression of OA. Thus, it is important to consider inflamed joint environment when evaluating the application of engineered tissue for cartilage repair and regeneration.

As one step to improve this hindrance, engineering stable cartilage under inflammatory conditions, induced by pro-inflammatory cytokines, IL-1β and TNFα, were aimed in the present study. As discussed earlier in previous sections, scaffold material, structure, and fabrication play important roles in cell response under inflammatory conditions and thus suggesting that biochemical and biophysical properties of scaffolding materials have to be taken into consideration for engineering stable cartilage in inflammatory environment.

Apart from the effect of scaffolding materials, use of a biochemical factor, Nkx3.2 was also evaluated in its role on cell differentiation and protecting cells from cytokine induced damage in inflammatory conditions. Results indicate its potential use for engineering stable and strong cartilage tissue in inflamed joint. These studies suggest
that studies on biophysical environment and biochemical signals for cartilage tissue to be resistant to inflammatory environment are critical to enhance application of cartilage tissue engineering for OA treatment.

Aside from inflammation, another major concern in cartilage tissue engineering is mechanical stress induced by normal activity and joint movement. Cartilage is exposed to mechanical loading which plays an important role in the maintenance of structure and health of the tissue. Mechanical forces are essential for chondrocyte metabolism, and a balance between anabolic and catabolic signaling is critical in maintaining healthy cartilage [181]. Compression of articular cartilage caused by mechanical loading and deformation of the matrix stimulate chondrocyte metabolism and affect the synthesis and degradation of ECM molecules [182].

Thus evaluation of mechanical properties of scaffolding materials that can recapitulate mechanical properties of native cartilage is crucial. It has been shown that dynamic compression improved chondrogenic marker expression in human articular chondrocytes from osteoarthritis patients. However, the degree of improved expression was different between chondrocytes from different zonal [183, 184]. This study suggests the significant role of mechanical force to restore function of chondrocytes in osteoarthritic environment and that mechanical force exposure may vary by area in articular cartilage. It also implies that mechanical property of scaffolding material is thus important for tissue engineering application for treating OA. In addition to mechanical property, its ability to support ECM deposition and to maintain cell phenotype with appropriate cell differentiation and cell growth under inflammatory conditions should also be in consideration to assess stable cartilage in OA joint.
Recently, studies on cartilage tissue engineering have extensively been progressed with applied scaffolding materials, alternative cell sources, and various kinds of exogenous stimuli (i.e. biochemical and biomechanical factors). Previous works have shown the role of functional biomaterials modified with growth factors on cartilage regeneration, development of biomimetic scaffolds, and design of composite scaffolds for better cartilage behavior and mechanical integrity.

Co-culture strategy has also been introduced and gained substantial attention. Recent studies have shown that articular chondrocytes not only promote chondrogenesis of MSCs in co-culture but also inhibit undesired hypertrophy of differentiating MSCs [185, 186]. Further analyses have suggested that articular chondrocytes secret soluble factors such as TGF-βs, IGF-1, and BMPs, which are differentiation stimulating factors used to induce chondrogenesis of MSCs in vitro, thus may improve chondrogenesis of MSCs [187].

A variety of growth factors has been applied for cartilage tissue engineering to improve cell functions and behaviors. Interestingly, possible application of transcription factors (e.g. Sox9) for cartilage regeneration and repair has been demonstrated and brought an attention.

As described above, new strategies have been introduced as potent means to improve cartilage repair and regeneration. However, despite of these attempts, to date, successful transplantation of engineered cartilage tissue into cartilage defects in vivo or in clinical use is yet achieved. Comprehensive studies on promoting chondrogenesis of cells and regulating cellular responses by environment in OA joint as well as creating appropriate chondrogenic niche in inflamed condition for engineering healthy cartilage...
tissue, provided by optimal scaffolding material, biochemical and biomechanical stimuli are crucial to improve the application of cartilage tissue engineering for clinical use.
6. References


