Redifferentiated Human Osteoarthritic Chondrocytes for Articular Cartilage Tissue Engineering and Repair

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Institute of Biomaterials and Biomedical Engineering
University of Toronto

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Abstract

When chondrocytes are passaged, they lose their phenotype and the ability to form hyaline articular cartilage (AC) in a process termed dedifferentiation, limiting their clinical applications. Transforming growth factor beta (TGFβ) is known to differentiate human progenitor cells to chondrocytes by upregulating chondrogenic genes. Therefore, we tested if TGFβ could redifferentiate passaged human chondrocytes and restore their ability to form AC. Here we show for the first time that when passaged chondrocytes are cultured in the presence of TGFβ3, hyaline- like cartilaginous tissues self-assemble and develop a superficial zone. In addition, we demonstrate that chondrocytes isolated from AC with advanced osteoarthritis (OA) can form cartilaginous tissues of similar quality to that of chondrocytes derived from moderate OA. This is significant as literature suggests that chondrocytes from advanced OA may have an impaired capacity to form tissues. We further demonstrated that high levels of endoglin (a TGFβ co-receptor known to enhance signaling) may be required for chondrogenesis by human chondrocytes. This is a novel finding and suggests that for clinical applications cells expressing high levels of endoglin may enhance cartilage repair. Finally, redifferentiated chondrocytes were tested for their capacity to form hyaline-like cartilage in vivo in a rabbit osteochondral defect model; however, the use of these cells resulted in collagen type 2-containing granulation tissue
and did not enhance repair when compared to dedifferentiated chondrocytes. Implanting an osteochondral-like tissue construct which resemble native AC may be a better alternative. Studies are ongoing to elucidate methods to generate tissue constructs containing hyaline cartilage, mineralized cartilage, and bone.
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Chapter 1
Introduction and Literature Review

1.1 Cartilage

1.1.1 Articular Cartilage Structure and Function

Articular cartilage (AC) is hyaline cartilage which lines the articulating surfaces of the bones in synovial joints. It is a highly specialized connective tissue that is smooth, lubricated, and reversibly compressible allowing for smooth joint movement. Additionally, it functions to transmit loads and biomechanical signals to the underlying bone and protect the joint from damage during loading. AC is predominately composed of tissue fluid and extracellular matrix (ECM) of which the major constituents are water, collagen type II (COL2) and the large proteoglycan aggrecan (ACAN) [1]. Other minor collagens including types IV, VI, IX, X, XI, XII, XIII, and XIV are present in AC and have been shown to play important roles in development, matrix organization, homeostasis and disease [2]. Two smaller proteoglycans biglycan and decorin are also found in AC at a much lower abundance and localize to the pericellular matrix (PCM) and interterritorial matrix respectively [3],[4]. Along with fibromodulin, lumican, epiphycan, and perlecan these proteoglycans account for only a small fraction of the AC matrix. Only 1-2% of the tissue is cellular and composed of chondrocytes; the only cell type of AC. The PCM is the matrix immediately surrounding the chondrocytes and may function to provide biomechanical and biochemical signals to the cell. The PCM is distinct from the surrounding ECM due to the accumulation of proteins found exclusively (or in a higher concentration), most notably type VI collagen, perlecan, aggrecan monomers and small aggregates, hyaluronan, biglycan, and type IX collagen [5]. Overall AC is divided into four distinct zones, the superficial zone, middle zone, deep zone and the zone of calcified cartilage (Figure 1-1) each characterized by differences in their respective matrix composition and organization.

The superficial zone (SZ) makes up 10-20% of the AC. It has the lowest proteoglycan content of all the zones and contains thin cartilage fibrils that align parallel to the articulating surface. The chondrocytes in this zone have an elongated morphology and are relatively high in number. Proteoglycan 4 (PRG4, also known as Lubricin) and Clusterin have been shown to be
expressed exclusively in the SZ [6]–[8]. As this zone is in direct contact with the adjacent articulating surface it has the highest tensile properties [9] allowing it to withstand forces during articulation. The middle zone (MZ) immediately follows the SZ and makes up 40-60% of the AC volume. This zone specifically expresses cartilage intermediate layer protein [10], is richer in proteoglycan than the SZ and has larger collagen fibrils arranged more randomly. The chondrocytes in this zone have a rounded morphology and are fewer in number than the SZ. Below the MZ is the deep zone (DZ) encompassing 30-40% of AC. This zone contains the highest proteoglycan content and the collagen fibrils are of the largest diameter. They are oriented perpendicular to the articulating surface providing the greatest resistance to compressive forces and anchoring the AC to the zone of calcified cartilage (ZCC). Furthermore, the high proteoglycan content plays an essential role in the compressive properties of the AC, as charged hydrophilic GAG chains found on the proteoglycans provide the tissue with compressive resilience through their ability to entrap water. The collagen constrains the swelling that occurs with the accumulation of water.

The ZCC is situated directly below the DZ and functions to maintain structural integrity of the osteochondral unit during joint movement by dispersing forces laterally. It anchors the cartilage by interdigitating with the subchondral bone. This zone is calcified (contains hydroxyapatite mineral), and is of an intermediate stiffness between the softer viscoelastic AC and subchondral bone [11]. Within the ZCC the perpendicular collagen type II fibers become structurally cemented to collagen type I osteoid deposited by osteoblasts, anchoring the AC to the bone [12]. The chondrocytes in this zone have a hypertrophic phenotype defined as having an enlarged cell size, synthesis of type 10 collagen (ColX) and alkaline phosphatase (ALP), and the ability to calcify the surrounding ECM [13]. This zone is distinct from bone as it is avascular, contains proteoglycans and has smaller disorganized hydroxyapatite crystals with a low concentration of carbonate ions compared with bone mineral [14], [15]. A tidemark separates the calcified matrix from the uncalcified AC.
Figure 1-1: **Structure of healthy human articular cartilage.**Insets depict collagen fiber orientation.

### 1.1.2 The Formation of Articular Cartilage during Embryogenesis

During embryogenesis, early limb buds are composed of undifferentiated mesenchymal cells, and form the template of bone. The mesenchymal cells proliferate and the bud extends distally. Initially the mesenchymal cells will condense [16] to form transient cartilage which serves as the anlagen for bone formation. Cells in this zone differentiate into chondrocytes by expression of the transcription factor Sox9 [17] and the cartilage matrix molecules Col2 and ACAN to form the cartilage anlagen. Bone formation occurs as this tissue remodels. The chondrocytes lose expression of cartilage matrix genes, begin to express coll1, and mineralize the newly deposited matrix. The proximal region of the mesenchymal condensations give rise to the humerus or femur while the more distal regions go on to form the radius and ulna or tibia and fibula as well as the digits [18].
Stable articular cartilage forms following mesenchymal condensation as a secondary remodeling event, wherein the cells at the presumptive joint location elongate perpendicular to the length of the limb, lose expression of Col2 and begin to express collagen type 1 (Col1) and growth and differentiation factor 5 (GDF5) [19]. These cells which become non-chondrogenic and bind to each other via gap junctions form the “interzone”. From the interzone the process of cavitation occurs wherein the cartilaginous elements separate to form two distinct articulating joint surfaces. This process occurs through forces which are generated by hypertrophy and matrix secretion (substantial upregulation of hyaluronan) [18] and is dependent on movement of the embryo [20]. The cells which go on to become articular chondrocytes and form cartilage, as well as the cells which form the synovial tissues and ligaments are derived from the interzone or the superficial zone cells once the joint surface has formed [21], [22].

Interzone cell differentiation to form articular cartilage has been suggested to occur via differential expression of GDF5 and Wingless-Type MMTV Integration Site Family, Member 9A (Wnt9a). Koyama E. et al. have demonstrated in in vitro studies of isolated interzone that treatment with GDF5 enhances chondrogenesis while overexpression of Wnt9a maintains the interzone cell phenotype [22] and prevents chondrogenic differentiation. The effect of Wnt9a overexpression was further shown to be counteracted via treatment with exogenous GDF5 suggesting that this growth factor may act as a chondrogenic switch when expressed, pushing the cells of the interzone to differentiate to chondrocytes and form the articular cartilage. Signalling via Indian hedgehog (IHH) has been shown to play a critical role in the development of the interzone, as knockout studies have illustrated that in the absence of IHH the interzone fails to form, the cartilaginous digit anlagen remains fused, and GDF5 positive cell distribution is abnormal [23]. Overall this suggests IHH signalling may directly regulate joint formation. Other signalling pathways including those belonging to bone morphogenetic protein (BMP) as well as transforming growth factor beta (TGFβ) have also been shown to be required for the establishment of functional synovial joints [23].

1.1.3 Cartilage Injury and Osteoarthritis

AC has a limited ability to repair when damaged. In part this is due to the low proliferation rate of adult chondrocytes (which have been shown to senesce with aging) [24], the dense ECM,
and the avascular, aneural and alymphatic nature of AC, which altogether may hinder cell migration to the damaged area preventing repair. AC may be damaged through injury or disease. Osteoarthritis (OA) is a degenerative disease of the joint which results in the destruction and progressive loss of the AC tissue, and is accompanied by alterations to the subchondral bone, ligaments, capsule and synovial membrane [25]. Overall these changes create an environment in which the joint is no longer protected against compressive and shearing forces, and results in painful moment and debilitation. Once initiated, OA continues to progress.

There are two classes of OA; primary (or idiopathic) and secondary. The exact initiator of primary OA is unknown, but it is believed to result from a combination of risk factors including increasing age, obesity, biological sex (assigned female at birth), and joint malalignment causing increased biomechanical loading [25]. In addition, numerous studies have looked at the role genetic factors may play in the development of OA and have shown that a variety of genes, polymorphisms, non-coding RNAs and DNA modifications have an association with OA [26]–[30]. Contrary to this a recent meta-analysis assessing 199 candidate genes found only 2 had an association with hip OA, while none were significantly associated with knee OA [31]. Thus, the role of genetic influences on the development of primary OA remain controversial or have yet to be fully elucidated. Secondary OA on the other hand is initiated by causative factors such as trauma, surgery on the joint structures and abnormal joints at birth. Hip dysplasia, a condition where the femoral head is insufficiently covered by the acetabulum, and femoroacetabular impingement, a condition of abnormal contact between the proximal femur and rim of the acetabulum, are examples of structural abnormalities which are risk factors for secondary and early onset (younger than 40) OA [32].

Homeostasis of the AC ECM, which is regulated by the chondrocytes, is imperative to maintaining healthy and biomechanically stable tissue. In OA there is an imbalance in this homeostasis which leads to increased catabolism and the destruction of the cartilage tissue. Elevated activity of catalytic enzymes like matrix metalloproteinases (MMP) and aggrecanases results in the loss of proteoglycans, which begins in the superficial region of the tissue and progress downwards [33]. Increased levels of circulating proinflammatory cytokines have been found in patients with cartilage loss [34] and are likely involved in upregulating catabolic enzyme secretion by the chondrocytes. Studies have suggested that the collagen content remains
intact in the early stages of OA however the fibers become disorganized in the SZ and are significantly less parallel to the surface [35], [36]. Ultimately these factors along with altered biomechanical loading and stresses lead to discontinuity in the SZ. As the disease progresses deep fibrillations form, there is a loss of matrix (thinning of the tissue), chondrocyte cloning, mineralization and advancement of the tidemark [37] which eventually results in complete erosion of the tissue down to the bone.

1.1.4 Clinical Therapies for the Repair of Damaged and Diseased Articular Cartilage

There are a variety of biological therapies used clinically which aim to repair AC defects. Autologous chondrocyte implantation (ACI) is one technique used clinically. In this procedure chondrocytes are isolated from an undamaged region of the patient’s AC via a small biopsy, cells are isolated and cell number expanded in vitro and then re-implanted into the defect area under a periosteal flap (1st generation), a collagen membrane (2nd generation) or on/in a scaffold (3rd generation), with the hope that they will form hyaline AC [38], [39]. Unfortunately in most cases fibrocartilage, a type of repair tissue high in collagen type 1 (Col 1) which lacks the durability and the mechanical properties of hyaline cartilage [40] is formed. In a study where full depth biopsies were taken from 58 patients at early time points (8-60 months) following ACI surgery, only 15 percent were hyaline cartilage, 65 percent were fibrocartilage and the remaining were of a mixed morphology [41]. Studies which have assessed the maturation of this tissue at longer follow ups have demonstrated some maturation of the fibrocartilage to hyaline cartilage with time [41], [42]. ACI is still a recommended treatment for the repair of articular cartilage defects of the knee over 2cm² as long as the joint shows minimal osteoarthritic damage (National Institute for Health and Care Excellence Guidance TA477). This may be because despite the prevalence of fibrocartilage repair many patients still report pain reduction and overall satisfaction with ACI. Most recently Ogura T el al. investigated the clinical outcome of ACI in a 20-year follow up. They found that 79 percent of the patients who received ACI had their native knee (no joint replacement) 20 years later and were satisfied with their outcome [43]. However, in many cases ACI is not a successful treatment option and the formation of fibrocartilage is not
optimal for proper joint function. Therefore therapies to enhance hyaline cartilage formation need to be investigated.

Microfracture is another technique used clinically to repair AC defects. In this therapy small holes are drilled into the subchondral bone in the area of the defect to allow progenitor cells from the bone marrow to enter the defect area and generate repair tissue [44]. As with ACI, this procedure results in the formation of fibrocartilaginous repair tissues [45]. Microfracture has been shown to be effective in reducing pain and improving patient reported clinical outcomes for young patients and for small defects, however; for larger defects (greater than 4 cm²) in older patients this technique is not effective [46]. For larger defects in which ACI and microfracture would have poor outcomes, autologous osteochondral mosaicplasty is commonly used. This procedure provides “hyaline-like repair” as cylindrical osteochondral plugs containing bone and articular cartilage are removed from areas of minimal load bearing and implanted into the defect site. Although the process has been shown to result in plug viability and result in a more durable surface than that produced by fibrocartilage repair tissue, this procedure involves generating defects in the articular cartilage and bone in otherwise healthy areas of the joint and can result in donor-site morbidity, inflammation and hemarthrosis (bleeding into the joint) [47]. Furthermore, integration of the donor plugs with the surrounding cartilage is poor, and chondrocyte cell death is prevalent on the surfaces of the plug [48].

In joints with widespread damage to the AC, in cases such as advanced OA, management consists of lifestyle interventions and pharmacological treatments aimed solely at symptom relief. There are no available disease-modifying drugs which slow down the progression of OA. The only therapy is total joint replacement with a prosthetic (“arthroplasty”). Prosthetic joints are completely composed of synthetic materials (metal, organic polymers, ceramic, or a combination of these materials) and can be secured in place with bone cement. Unlike the other treatments discussed, arthroplasty is a major surgical procedure with a much longer recovery time and requiring rehabilitation. Patient satisfaction with arthroplasty has been reported to be quite high [49], although for knee replacement at least 25% of patients complain of persistent pain after joint replacement [50], [51]. In addition prosthetic joints have a limited lifespan and one or multiple revision surgeries are often required suggesting it is not an optimal treatment for young people. Aseptic loosening, instability, infection, polyethylene wear, arthrofibrosis and
malalignment are the predominate causes of arthroplasty failure. Revision surgeries for hip and knee arthroplasty are projected to grow by 137% and 601%, respectively, between 2005 and 2030 [52]. The higher success rates (fewer revisions) of hip arthroplasty may be because the ball and socket design of a hip joint is easier to replicate with metallic implants as compared to a more complicated hinge joint such as the knee [49].

Altogether current therapies possess significant limitations and are insufficient for long term repair of AC and the restoration of functional healthy joints. Bioengineering an AC construct that mimics native cartilage in composition and organization, and can remodel under stress, would overcome the challenges associates with current therapies and may be more suitable to use to resurface the joint and/or repair focal defects. This approach may have the capability to last a lifetime and restore proper joint function; however there are still a number of significant limitations. The availability and suitability of a cell source, dedifferentiation of chondrocytes with cell number expansion in monolayer culture, and the need to recapitulate the zonal structure of native cartilage for proper joint mechanics, are all challenges that need to be overcome with this approach. These will be discussed along with the current methods being investigated to overcome these limitations in subsequent paragraphs.

1.2 Bioengineering Articular Cartilage; Approaches, Challenges and Limitations

1.2.1 Cell Sources and Limitations

A variety of cell sources exist for use in biological therapies, each with their own challenges and limitations. Human embryonic stem cells (ESC) are one potential source and are derived from the inner cell mass of a blastocyst (fertilized embryo). These cells have the potential to differentiate into all the tissues of the body; however research has been hindered as few cell lines are available, due to ethical concerns regarding the use of human embryos. Additionally studies which have explored the use of ESCs in vivo have shown them to have tumorigenic potential forming teratomas [53], [54]. Methods to stably differentiate ECSs to the desired cell lineage need to be developed in order to mitigate this risk. Recently Craft et al.
demonstrated that chondrocytes capable of forming cartilage tissue could be generated from human ESCs which had been pre-differentiated to a paraxial mesoderm fate, via activation of the TGFβ pathway [55]. These chondrocytes could further be differentiated to a hypertrophic phenotype capable of mineralization through supplementation with BMP4. When implanted subcutaneously into nude mice, these tissues maintained a stable differentiated phenotype for up to 8 weeks. Although these results are promising, ESCs are allogeneic in nature their clinical use has the potential to lead to immunological responses or rejection, and will require immunosuppression therapy which presents its own set of risks to the patient.

Induced pluripotent stem cells (iPSC) on the other hand can be generated from autologous adult somatic cells that have been reprogrammed to a pluripotent state via retroviral delivery of four transcription factors (Oct4, Sox2, Klf4, and c-Myc) as first described by Yamanaka et al [56]. iPSCs therefore do not face the ethical stigma associated with ESCs, however they still have tumorigenic potential and require ample genetic manipulation [57], with the most efficient reprogramming strategies resulting in the integration of viral DNA into the chromosome. To date, a standardized method for the safe generation of iPSCs, and their stable differentiation to chondrocytes has yet to be determined [58]. Advances in this area have been made however, and chondrogenic differentiation of human iPSCs pre-differentiated to a mesenchymal progenitor state has been reported to occur with a 70% efficiency [59]. When these iPSCs were compared to ESCs cultured under the same conditions, ESCs were shown to differentiate faster and more efficiently to chondrocytes, indicating that despite being ECS-like, iPSCs do not possess the same level of plasticity.

Direct induction of chondrocyte phenotype by transdifferentiation of human dermal fibroblasts, without pre-differentiation to mesenchymal progenitors, has been reported by Outani H. et al via induction with only 2 of the iPSCs genes, c-Myc and klf4, as well as Sox9 (a transcription factor known to play a role in chondrogenic differentiation during embryogenesis) [59]. Despite this advancement, the incorporation of the oncogenes c-Myc, and Klf4 used to produce iPSCs limits their clinical translatability as they have been shown to increase the likelihood of tumor formation in vivo [60], [61]. Subsequent studies have found that c-Myc and Klf4 can be replaced by Nanog and Lin28 [62] mitigating the risks associated with oncogene incorporation, and enhancing the suitability of these cells for future clinical use. Methods of chondrogenic differentiation by iPSCs induced without the use of known oncogenes has yet to be
determined. However, a study by Boreström et al. demonstrated chondrocyte differentiation and cartilage matrix accumulation by iPSCs that had been generated via non-integrating mRNA transfection of Oct4, Sox2, Klf4, Lin28 and c-Myc, advancing the potential of iPSCs to be used clinically [63]. They further addressed whether the inherent “epigenetic memory” of the starting cell phenotype affects ability of the cells to differentiate. They showed that iPSCs generated from human chondrocytes formed better cartilage tissue than iPSCs generated from human fibroblasts, suggesting a differentiation bias toward the somatic cell origin. Although iPSCs have potential as a cell source which can be used to bioengineer cartilage tissue, the use of oncogenes to induce these cells inhibits their clinical translatability.

Multipotent stem cells such as mesenchymal stromal cells (MSC) derived from adipose tissue or bone marrow, are another potential cell source for cartilage bioengineering. With this approach there is the possibility of using autologous cells, however isolation of tissues or bone marrow from which these cells are derived can be invasive. The main difficulty with the use of these cells for cartilage bioengineering is that they have been shown to terminally differentiate to a hypertrophic chondrocyte phenotype and calcify when implanted in vivo [64]. Methods to stably differentiate MSCs to chondrocytes have yet to be elucidated, however advances have been made. Culture of MSCs in articular chondrocyte conditioned media, or co-culture with articular chondrocytes (1:1) was shown to reduce the frequency of in vitro formed cartilaginous tissues to calcify in vivo [65]. The main limitation of this approach is the need for a large number of primary human articular chondrocytes which are difficult to acquire due to the inherently low number of cells in AC tissue. Alternatively supplementation with parathyroid hormone-related protein (PTHrP) has been shown to suppress hypertrophy of MSCs derived from bone marrow and adipose tissue and may be a more suitable option than co-culture with primary chondrocytes [65]–[67].

1.2.2 Passaged Chondrocytes as a Cell Source

Alternatively, chondrocytes (either autologous or allogeneic) may be a more optimal cell source for cartilage repair therapies as they do not possess the limitations associated with the use of stem cells. In a study comparing the chondrogenic potential of chondrocytes to MSCs,
hypertrophy-associated genes were not induced in chondrocytes and furthermore they resisted mineralization and vascular invasion when implanted in vivo whereas MSCs did not [68]. In addition studies have shown that chondrocytes synthesize more collagen and proteoglycan than stem cells [69] and form tissues with greater mechanical properties [70] suggesting they may be ideal for a tissue engineering approach to cartilage repair. Furthermore, using autologous chondrocytes reduces the possibility for an immunological response against allogeneic cells [71], [72]. Unlike other cell sources, autologous chondrocytes can be obtained easily in a minimally invasive arthroscopic surgery, and are currently being used clinically for cartilage repair therapies [41], [73]–[75], as discussed above for ACI.

1.2.3 Dedifferentiation of Chondrocytes when Serially Passaged

The main limitation with the use of chondrocytes is the inherent low cellularity of AC. Isolated chondrocytes must be serially passaged in monolayer culture to increase cell numbers available for bioengineering applications. It is well documented that chondrocyte expansion in monolayer culture is accompanied by dedifferentiation, a process whereby cultured chondrocytes lose their chondrogenic phenotype and adopt a phenotype more closely resembling that of myofibroblasts [76]–[78]. This process is characterized by the loss of Col 2 and ACAN synthesis with an increase in collagen Col 1 synthesis. Changes in cell morphology are also observed. Within the first two passages, the polygonal phenotype typically observed in chondrocytes is lost and an elongated fibroblastic morphology is gained. Our group has shown this to be associated with changes in actin polymerization (from globular to filamentous actin) and MRTF localization (from cytoplasmic to nuclear) [79]. Dedifferentiated chondrocytes ultimately produce highly inferior tissue that does not resemble native cartilage both in vitro [78], [80] and when used clinically [41], [73]. To bioengineer AC, dedifferentiated chondrocytes need to be redifferentiated to restore the chondrogenic phenotype and produce an ECM resembling that of native cartilage prior to implantation.
1.2.4 Bioengineering AC with Passaged Chondrocytes: Current Methods

Currently, approaches for cartilage bioengineering with passaged chondrocytes center around the use of growth factors, scaffolds, hydrogels, cell density, mechanical stimulation, oxygen concentration and substrate stiffness; many of which have been used in combination. These will be discussed in detail in the subsequent paragraphs.

Exposure of chondrocytes to specific growth factors, many of which are members of the transforming growth factor beta (TGFβ) superfamily, can modify gene expression profiles and direct the cells to synthesize AC ECM. Growth and differentiation factor 5 (GDF5), a prominent marker of cartilage interzone cells [81], [82] and member of the TGFβ superfamily, has been shown to improve the formation of cartilaginous constructs cultured in combination with insulin [83]. Bone morphogenetic proteins (BMP), also part of the TGFβ family, have been most widely studied as chondrogenic differentiation factors in stem cells, with BMP-2, BMP-4, BMP-6 and BMP-7 seemingly the most optimal of the BMPs for chondrogenesis [84]–[88]; however a number of studies with opposing results also exist and suggest that BMP proteins push stem cells to form bone [88]–[90]. This variability may be due to the cell type and culture conditions being used but suggests that standardized methods for reliable differentiation have yet to be determined. Only a few studies have shown positive effects of BMPs on chondrogenesis by cultured chondrocytes [91]–[93], with majority of the literature suggesting that BMPs induce hypertrophy and terminal differentiation [94], [95]. TGFβ on the other hand has been shown to induce chondrogenesis [96]–[98] and inhibit hypertrophy in stem cells. Few groups have looked at the effect of culture with TGFβ during redifferentiation to promote AC formation in human chondrocytes. Results from non-diseased human chondrocytes indicate that it can promote AC tissue formation [99]. Studies are still required to assess the effect of TGFβ on chondrocytes derived from OA cartilage.

Scaffolds and hydrogels are commonly used in tissue engineering as they have the advantage of being engineered to provide a 3D environment, mimic native tissue, offer structural support, and be cross linked with growth factors making them biologically active. New scaffolds are constantly being designed to facilitate the attachment, proliferation, and differentiation of embedded cells. Natural scaffolds composed of collagen [100], agarose [101], fibrin [102] or
hyaluronic acid [103], as well as decellularized tissue matrices [104] have all been utilized with moderate success. Their disadvantage lies in the fact that these materials are difficult to engineer with designer characteristics, such as pore size, and difficulty achieving an even distribution of viable cells throughout the scaffold. Synthetic scaffolds such as those containing poly(ethylene glycol) [105] have also been tried, and although they possess more flexibility when selecting designer properties they still face the same challenges of natural scaffolds. Furthermore scaffolds are more prone to eliciting macrophage infiltration and vascularization [106], foreign body reactions [107] and fibrosis [108], [109] in vivo due to their degradation products.

Unlike scaffolds, hydrogels are 3D networks which are characterized by their ability to absorb large amounts of water and swell readily without dissolving, thus mimicking the properties of living tissues more so than synthetic scaffolds [110]. These qualities allow for nutrients, oxygen, and metabolic products to diffuse easily into their matrices. Furthermore, due to their fluid nature hydrogels can be injectable and can entrap cells prior to being shaped allowing for a uniform distribution through their matrix. Often times hydrogels are designed in combination with natural [100], [102] and synthetic polymers [111], [112] to increase their mechanical properties and make them more durable. Thus, the use of hydrogels is often limited to non-load bearing tissues due to their poor mechanical properties. To overcome this limitation mechanical loading of in vitro formed cartilage tissues is one method that has been shown to increase ECM accumulation and thus the mechanical properties of tissues formed with and without the use of scaffolds/hydrogels [111]–[114].

Low oxygen concentration is another method that has been shown to improve cartilage tissue formation in vitro by passaged chondrocytes [115], [116], as well as enhance chondrogenic differentiation of stem cells (MSC) [117], [118]. This effect has been shown to occur via hypoxia inducible factor 2α activation of both Sox9 dependent and independent pathways [118], [119]. These pathways ultimately increase expression of chondrogenic genes and proteins such as Col 2. The use of soft substrates to prevent dedifferentiation of chondrocytes is another technique that has been explored [120]. Although the softer substrate better maintained the chondrocyte phenotype, proliferation was significantly decreased which may prove problematic for clinical translation in which a large number of cells would be
required. The effect of stiffness has been suggested to be a result of lower cell adhesion [121] and enhanced sensitivity to TGFβ signalling on soft substrates [122].

Finally, high density culture has also been used to redifferentiate chondrocytes and form cartilage tissue without the use of a scaffold, in a self-assembly approach. The self-assembly approach differs from approaches which use scaffolds or hydrogels as it relies on the cells ability to synthesize its own ECM and organize itself to form tissues [123]. Ahmed et al showed that passaged bovine chondrocytes cultured at high density on Col 2 coated membranes have the ability to redifferentiate and self-assemble into cartilaginous tissues which resembled native AC in composition and ECM organization [124]. This method required the use of a chemically defined serum-free media containing insulin, dexamethasone, and high glucose content, all of which were shown to be required for this to occur. No growth factors or serum were needed for the cells to self-assemble and form cartilaginous tissues resembling native cartilage. To the best of our knowledge it has not been established that human passaged chondrocytes can self-assemble and form cartilage tissue under the same conditions. This method of scaffold-free self-assembly shows promise for bioengineering human AC and may be advantageous as it would not possess the same limitations as scaffolds and hydrogels in vivo (ie. biological responses to implantation of synthetic materials).

1.2.5 Forming Constructs Which Recapitulate Native Zonal Joint Structure

One of the disadvantages of the self-assembly approach discussed above is that it can be challenging to bioengineer complex tissues with multiple cells types and architecture. For AC this means engineering a tissue comprised of hyaline cartilage, with a zone of calcified cartilage. As discussed, AC has a complex zonal architecture which provides this tissue with its high load bearing capabilities. Furthermore, this tissue has a calcified cartilage zone which is further anchored to bone. In order to generate a mechanically stable construct that can withstand load bearing upon implantation, tissue engineering approaches need to focus on the development of a construct which incorporates all of these aspects. The majority of studies which aim to generate zonally organized cartilage and bone constructs utilize scaffolds or hydrogels, and may also use
multiple cell types; however, a few studies have utilized self-assembly. These techniques will be discussed in the subsequent paragraphs.

The use of scaffolds to tackle the challenge of recreating a native joint can be useful as they can provide structural support, help guide differentiation, and offer an organizational framework that can be built upon. Nguyen et al. demonstrated that specific combinations of natural and synthetic biomaterials could be used to guide differentiation of MSCs into zone specific chondrocytes [125]. This method utilized a polyethylene glycol (PEG)-based hydrogel crosslinked with chondroitin sulphated (CS) and MMP-protein (to guide differentiation to superficial zone cell phenotype), CS alone (to guide differentiation to middle/transitional zone cell phenotype) or with hyaluronic acid alone (to guide differentiation to deep zone cell phenotype). The authors further showed that by layering these hydrogels they could generate a cartilage construct which mimicked native cartilage in composition and in a gradient of increased compressive modulus from the superficial to the deep zone [126]. Although this study demonstrated that a zonally organized construct could be generated by selecting for properties that would direct stromal cell differentiation, the construct lacked a mineralized layer of cartilage and was not attached to a bone interface. Other groups have achieved this using different biomaterials and cell types. Jiang et al demonstrated that it was possible to generate a construct with three distinct regions of cartilage, calcified cartilage and bone-like matrices using a ceramic-hydrogel scaffold combined with region-specific co-culture of chondrocytes and osteoblasts [127]. One of the limitations of this study was that the materials used in this scaffold are not biodegradable which may limit the clinical translatability of this construct. In a more natural approach utilizing Col 1 microspheres, Cheng et al demonstrated that encapsulated MSCs which has been differentiated to osteogenic or chondrogenic phenotypes could be combined with additional Col 1 and would spontaneously form a layer of calcified cartilage [128]. Although this study achieved the desired outcome, the use of Col 1 as a scaffold may be problematic as it is not found at such high abundance in hyaline cartilage. Studies investigating the use and maturation of this construct in vivo still need to be determined. Aside from these approaches, other scaffolds have also been designed and tested to spatially differentiate cells into cartilage and bone lineages; however these methods have failed to generate a ZCC between the two layers [129]–[131].
To create AC-like tissue with a mineralized zone without the use of a scaffold, Ng et al used a dual-compartment culture approach, in which MSCs were seeded on a porous membrane and the basal compartment received hypertrophic stimuli while the apical compartment received chondrogenic stimuli [132]. As with the previously discussed approaches, this method does not integrate the ZCC with a bone substrate, and therefore a method to integrate this construct with the native bone needs to be determined. To overcome this limitation, bone-like substrates may be used in combination with self-assembly approaches to bioengineer osteochondral-like constructs. Calcium polyphosphate (CPP) [133], [134] is one example of a bone-mimicking biomaterial which has been used for this purpose. In vivo studies of CPP have shown that it is biocompatible and allows for ingrowth of the native bone [133], [134]. Furthermore it has been demonstrated that when bovine chondrocytes are grown on this substrate they self-assemble and form hyaline-like cartilaginous tissues in vitro [135]. Forming a zone of calcified cartilage adjacent to the CPP using deep zone chondrocytes was achievable on this substrate and was shown to increase the load-bearing properties and interfacial shear strength of the tissue [136], [137]. More recently methods to differentiate sheep bone marrow derived MSCs into chondrocytes to form hyaline cartilaginous tissue [138], as well as differentiated to a hypertrophic phenotype to form a ZCC has been established, and used in combination with CPP to form a multiphasic osteochondral-like construct [138]. For this approach, two-fold cell seeding onto the CPP was used, first to form a layer of calcified cartilage, then subsequently to form a layer of hyaline cartilage. Human chondrocytes or MSCs have yet to be used in combination with CCP for this purpose as methods for differentiation to both hyaline cartilage and calcified cartilage still need to be optimized for 3D culture. In order to restore proper joint mechanics in patients with cartilage and bone damage, methods to generate clinically translatable and autologous constructs containing a ZCC need to be elucidated.
1.3 Transforming Growth Factor Beta (TGFβ)

1.3.1 TGFβ Signalling in Cartilage

TGFβ is a secreted protein that is critical for mammalian development, growth, and homeostasis and has a role in cartilage development. Three isoforms have been identified in mammals, termed TGFβ1, TGFβ2, and TGFβ3. TGFβ will initiate cellular responses by binding to and assembling of heterotetrameric receptor complexes on the cell surface (Figure 1-2). When TGFβ binds the type II receptors, it becomes activated and transphosphorylates the type I receptors known as activin receptor-like kinases (ALK). In healthy articular cartilage TGFβ signalling occurs predominately through ALK5, which upon being activated proceeds to activate a cascade of intracellular signals that results in phosphorylation of Smads 2 and 3 [139]. Following phosphorylation Smads 2/3 form a complex with Smad 4 and are translocated into the nucleus where they can bind Sox9 and activate genes involved in chondrogenesis such as COL2 and promote formation of AC [140], [141]. TGFβ has also been shown to bind ALK1 and facilitate intracellular signalling through phosphorylation of Smads 1/5/8 to promote a hypertrophic phenotype [142]. In addition to the type I and type II receptors, three TGFβ coreceptors have been shown to be present in chondrocytes. The first two, Endoglin and Betaglycan [143] (also known as CD105 and TGFβ Receptor 3 (TGFβRIII) respectively), have been shown to increase signalling by enhancing TGF-beta ligand binding to the signalling receptor complex in other cell types [144], [145]. Furthermore, these co-receptors have contrasting affinities for the three TGFβ ligands, with endoglin binding TGFβ 1 and 3 exclusively, while betaglycan has the highest affinity for TGFβ 2 [145], [146]. CD109 is the third co-receptor and is known to play a role in facilitating internalization and cellular degradation of ALK5 [147], [148]. Currently there is little known about the role of these co-receptors in articular chondrocytes and in cells affected by OA and is thus an area of research which needs to be explored.

Besides the Smad dependent canonical signalling pathways mentioned above, TGFβ has also been shown in chondrocytes to promote expression of hyaline matrix by signalling through non-canonical pathways, the most extensively studied being Tak1 (TGFβ activating kinase 1) [149], [150]. In cartilage development, Tak1 has been shown to be essential for proper joint formation and for stimulating proliferation and differentiation of growth plate chondrocytes.
P38, JNK, and ERK are pathways downstream of Tak1 that can be activated by TGFβ and have been associated with chondrogenesis and cartilage homeostasis [153]–[156]. P38 has also been shown in chondrocytes to interact with the canonical pathway and signal via the formation of a phosphorylated complex with Smad3 [156]. Loss of P38 pathway signalling in chondrocytes was demonstrated to result in a hypertrophic phenotype and OA-like changes in vivo [156], implicating this pathway as an important mediator of the chondrocyte phenotype and cartilage homeostasis.

Figure 1-2: TGFβ signalling in chondrocytes.

TGFβs are known to play critical roles in regulating chondrocyte differentiation and in the maintenance of AC tissue. All three isoforms (TGFβ1, TGFβ2, and TGFβ3) are present in AC [157]. Many groups have investigated the role of TGFβ in cartilage and joint formation using
animal models. In 1987 Hiene et al showed that in the developing mouse embryo, TGFβ has a specific pattern of histochemical staining correlating with morphogenetic and histogenetic events involving cells and tissues of mesenchymal or mesodermal origin [158]. This prompted studies in which embryonic mesenchymal cells were exposed to TGFβ and analyzed for the ability to differentiate and secrete cartilage ECM molecules. Several groups have shown that TGFβ can effectively differentiate chick limb mesenchymal cells [159], [160] into cartilage, identifying a role for this growth factor in the differentiation of chondrocytes in vivo. Furthermore, human bone marrow derived MSCs have also been shown to differentiate into chondrocytes and produce AC-like tissue through supplementation with TGFβ in vitro [161] confirming the importance of TGFβ in chondrogenic differentiation in human cells.

1.3.2 TGFβ Signalling in OA

A few studies have looked at the role TGFβ signalling may play in the development of OA. In animal models, knockouts of ALK5 and TGFβ receptor 2 have been shown to result in the development of OA [162], [163]. In human cartilage immunohistochemical staining has shown decreased TGF-beta1 production and downregulation of TGFβ receptor 2 in fibrillated cartilage compared to normal [157]. Ultimately this may play a role in the irreversible nature of OA due to decreased TGFβ signalling in damaged areas of the cartilage which may hinder repair. Furthermore, research has suggested that with aging and in OA the ratio of the type 1 TGFβ receptors expressed on the cell surface changes. Specifically, mouse models of OA have shown that the ratio of ALK1: ALK5 increases [164]. As discussed above, ALK5 signalling via the canonical Smad2/3 pathway and the non-canonical pathways leads to chondrogenesis and AC ECM protein expression [140], [141]. ALK1 signalling on the other hand has been shown to result in hypertrophy [142], [164]. The increased ratio of ALK1: ALK5 observed in animal models of OA has been suggested to contribute to the development and progression of the disease [165]. Studies have yet to be conducted to address if this change occurs in human OA.

Downstream of the membranous receptor ligand signalling complexes, studies have suggested that changes in intracellular TGFβ activated signalling proteins can play a role in the development of OA. In a study by Li et al. knockout of Smad3 in mice was shown to lead to the
development of chondrocyte hypertrophy and OA [166]. This has further been assessed in humans in which studies have found genetic mutations of Smad3 in some adults with primary OA and in individuals with Aneurysm-Osteoarthritis Syndrome [167]–[170]. Loss of functional P38 MAP kinase, a component of the noncanonical TGFβ signalling pathway, has also been shown to lead to a worsened OA phenotype in mice expressing a dominant-negative P38 transgene [171] suggesting a role for P38 signalling in cartilage maintenance. On the other hand P38 signalling has also been shown to be activated via the inflammatory cytokines interleukin 1 and tumor necrosis factor alpha and may also play a role in cartilage destruction in an OA environment [172]. TGFβ activated kinase 1 (Tak1) is upstream of P38 and has been shown to induce OA when upregulated via intra-articular injection of Tak1-encoding adenovirus in rats. This methodology however is not specific to targeting the chondrocytes and effects of Tak1 on the other tissues within the joint may have influenced the observed outcome. This study still suggests however that overactivation of Tak1 and its downstream pathways may influence the development of OA. To the best of our knowledge overactivation of Tak1 has not been assessed in OA in humans, but as with P38, other pathways downstream of Tak1 have been shown to be regulated by inflammatory cytokines in human chondrocytes in vitro and lead to upregulation of catabolic enzymes [173]. In contrast to these studies, van Caam et al have shown that Tak1 crosstalks with the TGFβ activated Smad2/3 and Smad1/5/8 pathways and enhance signalling in bovine chondrocytes suggesting a role for Tak1 in cartilage anabolism [174]. Despite opposing studies with respect to the role of the noncanonical signalling pathways, studies of TGFβ signalling via the canonical ALK5-Smad2/3 pathway support the role of TGFβ in hyaline cartilage anabolism.

1.3.3 TGFβ for Redifferentiation of Passaged Chondrocytes

Few groups have looked at the effect of culture with TGFβ to facilitate redifferentiation of human passaged chondrocytes. One study by Goldberg et al showed that pellet cultures of expanded human chondrocytes which were grown in a serum-free media supplemented with TGFβ1 accumulated more Col 2 than pellets that were cultured in media supplemented with only fetal bovine serum (FBS) [99]. This suggests that TGFβ may promote a hyaline cartilage phenotype in passaged human chondrocytes, however further studies are needed to fully
characterize this beyond accumulation of Col2. In addition to this study, a study by Tekari et al assessed the use of TGFβ1 to promote AC formation by bovine passaged chondrocytes and showed that media supplementation with TGFβ restored the cartilage forming capabilities of cells which prior to passaging had the ability to form hyaline-like cartilage, but lost these capabilities due to extensive passaging in vitro [175]. This study did not assess whether these findings translated to human passaged chondrocytes nor did it address the influence an OA phenotype. As discussed, chondrocytes from fibrillated cartilage show loss of TGFβ1 ligand expression and TGFβ receptor 2 expression compared to undamaged cartilage [157] and this may affect the ability of chondrocytes derived from OA donors to redifferentiate when supplemented with TGFβ. Overall these studies suggest that TGFβ can promote redifferentiation and AC tissue formation, and therefore needs to be investigated further for passaged dedifferentiated human chondrocytes.

1.4 Summary of Introduction

AC is a complex tissue with an inherently limited ability to regenerate when damaged from injury or disease. OA is a disease which leads to the progressive degradation of AC tissue. Current cell based therapies used clinically for the repair of damaged AC do not result in the formation of tissues which recapitulate the zonal structure or the load bearing capacity of the native cartilage. Methods to bioengineer AC that mimics native tissue using a variety of cell sources is under active investigation, and involves both scaffold-free and scaffold based approaches. Autologous chondrocytes may be an ideal cell source for bioengineering as they do not possess the limitations associated with the use of stem cells and are easily attainable via arthroscopy which is minimally invasive. Furthermore chondrocytes can easily be passaged in monolayer culture making it possible to generate a large number of cells. The limitation with the use of passaged chondrocytes is that they dedifferentiate and lose the ability to form cartilage. TGFβ is a promising candidate as a growth factor which may be used to redifferentiate human passaged chondrocytes and promote hyaline cartilage tissue formation in vitro.
1.5 Hypothesis and Objectives

1.5.1 Hypothesis

Human passaged osteoarthritic chondrocytes can be induced to regain a chondrogenic phenotype when cultured with a chemically defined serum-free culture media supplemented with TGFβ to produce hyaline cartilage tissue that compositionally resembles native human cartilage.

1.5.2 Objectives

**Objective 1:** Determine if human passaged osteoarthritic chondrocytes can be redifferentiated and produce hyaline cartilage-like tissue in vitro that compositionally resembles native AC.

**Objective 2:** Assess if chondrocytes isolated from severely osteoarthritic tissues are suitable to use for bioengineering cartilage.

**Objective 3:** Determine if redifferentiated chondrocytes enhance the formation of hyaline cartilage repair when implanted into osteochondral defects (rabbit model).

**Objective 4:** Generate a multiphasic “osteoochondral-like” construct composed of hyaline cartilage and calcified cartilage on a bone substitute material.
1.6 References


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Chapter 2
Formation of Hyaline Cartilage Tissue by Passaged Human Osteoarthritic Chondrocytes


2.1 Abstract

Objective

When serially passaged in standard monolayer culture to expand cell number, articular chondrocytes lose their phenotype. This results in the formation of fibrocartilage when they are used clinically, thus limiting their use for cartilage repair therapies. Identifying a way to redifferentiate these cells in vitro is critical if they are to be used successfully. Transforming growth factor beta (TGFβ) family members are known to be crucial for regulating differentiation of fetal limb mesenchymal cells and mesenchymal stromal cells to chondrocytes. As passaged chondrocytes acquire a progenitor-like phenotype the hypothesis of this study was that TGFβ supplementation will stimulate chondrocyte redifferentiation in vitro in serum-free 3D culture.

Methods

Human articular chondrocytes were serially passaged twice (P2) in monolayer culture. P2 cells were then placed in high density (3D) culture on top of membranes (Millipore) and cultured for up to 6 weeks in a chemically defined serum-free redifferentiation media (SFRM) in the presence or absence of active TGFβ. The tissues were evaluated histologically, biochemically, by immunohistochemical staining, and biomechanically.

Results

Passaged human chondrocytes cultured in SFRM supplemented with 10ng/ml TGFβ3 consistently formed a continuous layer of articular-like cartilage tissue rich in collagen type 2 and aggrecan and lacking collagen type 1 and X in the absence of a scaffold. The tissue
developed a superficial zone characterized by expression of lubricin and clusterin with horizontally aligned collagen fibers.

Conclusions

This study suggests that passaged human chondrocytes can be used to bioengineer a continuous layer of articular cartilage-like tissue in vitro scaffold-free. Further study is required to evaluate its ability to repair cartilage defects in vivo.

2.2 Introduction

Articular cartilage is primarily composed of collagen type 2 (Col 2) and the highly sulphated proteoglycan aggrecan (Acan) in a 3:1 ratio. The tissue however is not homogenous and contains 4 distinct zones, each with unique extracellular matrix (ECM) composition. Current cell based therapies used to repair damaged cartilage, such as autologous chondrocyte implantation and microfracture do not recreate this complex architecture [1, 2]. Therefore there is a need for the development of new biological therapies that can regenerate hyaline cartilage. Bioengineering articular cartilage tissue in vitro that can be implanted into a defect or used to completely resurface the joint is one such approach. One factor that has limited clinical translation is an inability to identify a source of cells that will form articular cartilage with the correct organization and ECM composition, which is essential for proper function and long term tissue survival. Chondrocytes, the cell of articular cartilage, may be the optimal cell source for this purpose as unlike mesenchymal stromal cells (MSC) they do not progress to hypertrophic differentiation in vitro [3, 4], and have been shown to maintain a stable phenotype and resist terminal differentiation when implanted in vivo [4]. Furthermore, in a comparative study human chondrocyte cultures were shown to accumulate significantly more cartilage specific extracellular matrix than chondrocytes derived from adipose derived stromal cells [5]. Autologous chondrocytes can be easily obtained via enzymatic digestion of a small biopsy of articular cartilage; however their number is limited and they must be serially passaged to obtain sufficient cells. During this process they dedifferentiate and lose their phenotype [6], and are subsequently unable to form articular cartilage tissue. A number of investigators have attempted to identify conditions that would prevent dedifferentiation. For example, Martin et al.
supplemented the culture media with fibroblastic growth factor 2 (FGF2) during serial passaging of bovine chondrocytes in an attempt to prevent dedifferentiation [7]. This approach was moderately successful at maintaining the hyaline cartilage phenotype of the passaged cells; however tissues formed by passaged cells did not accumulate as much collagen as the tissues formed by primary cells. Thus the use of articular chondrocytes for bioengineering has been limited as the conditions necessary to prevent dedifferentiation while favouring cell proliferation have not been identified to date.

An alternative approach to this issue of dedifferentiation would be to redifferentiate the passaged cells. Several methods have been developed to do this but they have their limitations. Co-culture of passaged chondrocytes with primary chondrocytes has been shown to redifferentiate passaged chondrocytes and result in formation of stable articular cartilage in vitro [8, 9]. This method however requires a large number of primary chondrocytes, which are difficult to acquire if the tissue is to be autologous, making this technique problematic to use for cartilage formation on a larger scale. Others have attempted to induce redifferentiation using 3-dimensional (3D) culture, such as pellet cultures [10, 11], which in a comparative study was shown to promote chondrocyte phenotype more effectively than culture in monolayer [12]. A third approach has been to use porous 3D scaffolds designed to favour chondrogenesis and promote cartilage specific ECM deposition but this has had varying success [13-15]. In a previous study we showed that passaged bovine chondrocytes could be induced to redifferentiate and form hyaline cartilage scaffold-free after serial passage, using a chemically defined, serum-free chondrogenic redifferentiation media (SFRM) [16]. High glucose concentration, insulin, and dexamethasone, were identified as factors essential for this to occur. Unfortunately, preliminary studies suggested that passaged human chondrocytes would not redifferentiate under these conditions, suggesting that additional factor(s) are required for human cells.

Although many different growth factors could be examined for their ability to induce redifferentiation in human cells, one particular candidate, transforming growth factor beta (TGFβ) appears the most promising. Members of the TGFβ superfamily play vital roles in development and homeostasis of articular cartilage. Three isoforms of TGFβ exist in mammals (TGFβ1, TGFβ2, and TGFβ3) and have been shown to be essential in chondrogenic differentiation of fetal limb mesenchymal cells [17, 18] and MSCs [19] and in regulating proteoglycan synthesis by primary chondrocytes [20, 21]. Furthermore, a recent study has
demonstrated that the TGFβ signalling pathway is the most highly upregulated gene network during the spontaneous repair of articular cartilage defects in rat [22]. Altogether these studies indicate that TGFβ signalling plays an important role in both cell differentiation and repair of articular cartilage. Interestingly in osteoarthritis (OA), a disease of progressive cartilage loss, both TGFβ levels and TGFβ receptors are decreased [23] further supporting the involvement of this growth factor in regulating cartilage homeostasis. We hypothesize that human passaged chondrocytes will require TGFβ in combination with SFRM to promote redifferentiation and articular cartilage tissue formation. Other groups have shown increased accumulation of Col2 and sulphated proteoglycans by human passaged chondrocytes when grown in the presence of TGFβ in pellet cultures [24, 25]. However, tissues formed by pellet culture often have non-uniform matrix deposition, with the outer regions of the pellets accumulating more matrix than the center. Furthermore, tissues formed by chondrocyte pellets generally contain collagen type 1 suggesting that the passaged cells have not fully redifferentiated. To the best of our knowledge this is the first report of the formation of a continuous layer of articular cartilage-like tissue, with a superficial zone formed by passaged human chondrocytes under scaffold-free conditions.

2.3 Materials and Methods

2.3.1 Chondrocyte Isolation and Culture

Human articular cartilage was obtained from femoral condyles resected during joint arthroplasty surgery for osteoarthritis following informed patient consent and with Research Ethics Board (Mt. Sinai Hospital) approval. A total of 26 patient samples were used for this study, 20 females and 6 males with an average age of 62 ± 10 years). Tissue was taken from areas which macroscopically appeared undamaged. Chondrocytes were isolated by enzymatic digestion of the tissue in 0.5% protease (Sigma-Aldrich) for 1 hour at 37°C followed by an overnight digestion in 0.1% collagenase A (Roche) at 37°C. Cells from each patient were cultured separately for experiments (cells from multiple patients were not pooled). Non-passaged primary (P0) cells were seeded directly onto filter membranes after enzymatic isolation from the native cartilage tissue. Alternatively, freshly harvested chondrocytes from the same donor were placed in monolayer at 2000 cells/cm² in DMEM supplemented with 20% fetal bovine serum
(FBS). Chondrocytes were passaged twice (P2) to allow for approximately 400-fold increase in cell number (up to a total of 9 population doublings). P2 \(2 \times 10^6\) cells were seeded onto type II collagen coated Millicell® membrane culture inserts (12mm diameter; EMD Millipore) and cultured for up to 6 weeks in serum-free redifferentiation media (SFRM) consisting of high glucose Dulbecco’s modified Eagle’s medium (DMEM; 4.5 g/L), 1% ITS+ (BD Bioscience, MA), L-proline (40 ug/mL), pyruvate (110 ug/mL), dexamethasone (0.1μM), and L-ascorbic acid (100 ug/mL) in the presence or absence (control) of 10 ng/ml active human recombinant TGFβ3 (R&D Systems). If cultured for longer than 3 weeks the tissues were either “transiently” supplemented with TGFβ3 (first 3 weeks only) or “continuously” supplemented (throughout the entire 6 weeks). In selected cultures, TGFβ was removed after 1 week and β-glycerophosphate (10mM) was added in combination with triiodothyronine (30 nM) or L-thyroxine (50nM) or retinoic acid (100 nM) for an additional 2 weeks of culture to evaluate the ability of the tissues to mineralize.

2.3.2 Tissue Content Analysis

In vitro formed tissues were digested with papain (40μg/mL; Sigma-Aldrich) in digestion buffer containing 20 mmol/L ammonium acetate, 1 mmol/L-EDTA and 2 mmol DTT at pH 6.2 for 48 hours at 65°C. DNA content was quantified by the Hoechst 33258 dye binding assay and fluorometry (excitation λ= 365 nm, emission λ= 458 nm). The DNA standard curve is generated using calf thymus DNA (Sigma-Aldrich). Hydroxyproline content was quantified following HCl hydrolysis of the papain digest using the chloramine-T/Ehrlich’s reagent assay and spectrophotometry (λ= 560 nm). L-hydroxyproline (Sigma-Aldrich) was used to generate the standard curve. Sulfated glycosaminoglycan content of papain digested samples was quantified by dimethylmethylene blue dye binding assay and spectrophotometry (λ= 525 nm). Chondroitin sulphate (Sigma-Aldrich) was used to generate the standard curve.

2.3.3 Histology and Immunohistochemistry

Tissues were fixed in 10% PBS buffered formalin (pH 7.4) for 4 hours, placed in a 30% sucrose/PBS solution overnight at 4°C and then snap-frozen in Tissue-Tek OCT frozen
compound (Sakura Finetek). Blocks were sectioned at 7 μm and tissue sections were stained with haematoxylin and eosin, toluidine blue, or picrosirius red, mounted with Micromount (Leica Biosystems) and coverslipped.

For visualization of alkaline phosphatase (ALP) activity, tissues were fixed in 10% neutral buffered formalin for 30 minutes, then embedded and sectioned as described above. Sections were incubated in azo dye (Naphthol AS-MX phosphate and Fast Blue BB salt, both Sigma–Aldrich) for 10 min according to the manufacturer’s protocol, counterstained with eosin, mounted with Micromount (Leica Biosystems) and coverslipped.

For immunohistochemistry, sections were digested with 0.4% pepsin, blocked with 20% goat serum and then incubated overnight at 4°C with antibody reactive with either type I collagen (1:250 CL-50111AP-1 Cedarlane), type II collagen (1:100, MAB8887, EMD Millipore), Type X collagen (1:1500 C7974, Sigma-Aldrich), Lubricin (1:250, MAB401, EMD Millipore) or Clusterin (1:250 NBP1-19637, Novus Biologicals). For aggrecan no antigen retrieval was used (1:500 AHP0022, ThermoFisher Scientific). To visualize reactivity the tissues were incubated with either Alexa-594 goat anti-rabbit IgG (1:1000 Life Technologies), Alexa-488 goat anti-mouse IgG (1:1000, Life Technologies), Alexa-594 goat anti-mouse IgM (1:1500, Life Technologies) or Alexa-488 goat anti-rabbit IgG secondary antibody depending on the species and isotype of the primary antibody. Tissues were counterstained with DAPI (1:2000). Negative controls consisted of replacing the primary antibody with isoform and species matched antibody at the same concentration.

2.3.4 Mechanical Testing

Mechanical testing was performed with a Mach-1 micromechanical tester (Biomomentum, Laval, QC) equipped with a 1 kg load cell (0.05 g resolution, 2.5 kHz sample rate). Thicknesses of the constructs were determined through the needle probe method [26] and was measured twice at different locations near the center of the construct and averaged. Construct mechanical properties (bulk modulus, Young’s modulus, and Poisson’s ratio) were determined through Hayes’s double indentation method [27, 28] using two plane-ended indenters
(2 and 4 mm), indenting the samples to 10% strain at a rate of 10%/s. Constructs were allowed to equilibrate approximately 20 minutes between indentations.

2.3.5 Statistics

Three independent experiments were performed and all conditions were done in triplicate. The data was pooled, expressed as mean ± 95% confidence interval and analyzed using ANOVA with Tukey’s Post Hoc. Significance was assigned at p< 0.05.

2.4 Results

2.4.1 TGFβ is required for in vitro articular cartilage tissue formation by passaged human chondrocytes

To determine if human passaged chondrocytes could be redifferentiated and form articular cartilage, P2 cells were grown in SFRM in the presence or absence of TGFβ. Preliminary studies indicated that both TGFβ1 and TGFβ3 had similar efficacy in stimulating tissue formation at the same concentration, therefore we chose to use TGFβ3 for the remainder of the study. When a concentration of 1ng/ml TGFβ3 was utilized, patient specific differences in the amount of ECM accumulated was observed; however when 10ng/ml TGFβ3 was used consistent ECM was accumulated despite potential patient variability (data not shown). Therefore 10ng/ml was used for the remainder of the study for consistent tissue formation. When P2 chondrocyte cultures were supplemented with 10ng/ml TGFβ3, a continuous layer of robust cartilage tissue formed (Figure 2-1 A, B). In contrast, in the absence of TGFβ the tissue was fragile and could not be easily handled. After 3 weeks of in vitro culture, treated tissues had accumulated significantly more sulphated glycosaminoglycan (Figure 2-1 C) and collagen (Figure 2-1 D) than untreated controls. Histological evaluation shows that the tissues cultured with TGFβ3 form a continuous layer of hyaline cartilage-like tissue with ovoid shaped cells and rich in proteoglycans when stained with toluidine blue (Figure 2-1 F, H). In contrast the cells
cultured in the absence of TGFβ3 were elongated and there was little accumulation of proteoglycans. (Figure 2-1 E, G).

Figure 2-1. TGFB3 is required for cartilaginous tissue formation by passaged human chondrocytes. P2 chondrocytes were cultured on membrane inserts for 3 weeks in SFRM with 10ng/ml TGFβ3. (A) Gross morphology of tissues from control (left) and TGFβ3 supplemented (right) cultures. (B) Image of tissue from TGFβ3 supplemented culture showing thickness of cartilage formed in presence of TGFβ3. (C) Sulphated glycosaminoglycan (GAG) and (D) Hydroxyproline accumulation. Data was normalized to DNA content and expressed as mean ± 95% CI. Haematoxylin and eosin (E, F) and toluidine blue (G, H) staining of histological sections of formalin fixed tissues from control (E, G) and TGFβ3 supplemented (F, H) cultures. N=3 (9 filters). m = membrane. *= p< 0.05. Scale bar = 200μm.
2.4.2 TGFβ supplementation must be maintained for two weeks to allow for significant collagen accumulation by passaged human chondrocytes in the in vitro formed tissues.

The time in culture with TGFβ required for significant ECM accumulation was determined. Although sulphated glycosaminoglycan content was significantly greater than untreated controls by week one (Figure 2-2 A), hydroxyproline content was not significantly greater until the second week of culture (Figure 2-2 B) indicating that continuous treatment with TGFβ for at least two weeks is necessary. Histological examination of tissues harvested at weeks 1, 2 and 3 (Figure 2-2 C) appeared to increase in thickness with time in culture compared to control tissues cultured for 3 weeks without TGFβ. Immunohistochemical studies showed that tissues cultured with TGFβ for longer periods of time appeared to stain more strongly for Acan and for Col 2. Of note, no collagen type 1 (Col 1) was detected in any of the in vitro formed tissues. The cells showed no evidence of hypertrophy, as the tissues were negative for alkaline phosphatase activity (Figure 2-3 B) and collagen type X (Figure 2-3 D). Furthermore, tissues did not mineralize when TGFβ was removed and cultures were supplemented with β-glycerophosphate alone or in combination with triiodothyronine or thyroxine or retinoic acid (data not shown), known inducers of mineralization by growth plate chondrocytes and MSC derived chondrocytes [19, 29-31].
Figure 2-2. A minimum of 2 weeks of culture with TGFβ3 is required for significant accumulation of collagen and proteoglycan. In vitro formed tissues were treated continuously with 10ng/ml TGFβ3 unless otherwise stated and harvested at 1, 2 or 3 weeks of culture. (A) Sulphated GAG and (B) hydroxyproline accumulation. (C) Histological appearance and immunohistochemical staining for collagen type 2 (Col2), aggrecan (ACAN), and collagen type 1 (COL1) of in vitro formed tissues. Human native articular cartilage was used as a positive control. DAPI counterstain. Results are expressed as mean ± 95% CI. * = p< 0.05. m = membrane. Scale bar = 200μm.
Figure 2-3. Hypertrophic markers, alkaline phosphatase (ALP) and collagen type 10 (Col X), are not expressed in 3 week cultured in vitro-formed cartilage tissue. (A) Native articular cartilage has ALP activity (blue) in the deep zone. (B) In vitro formed tissue after 3 weeks in culture with 10ng/ml TGFβ3 shows no ALP activity (blue; eosin counterstain). (C) Immunohistochemistry of native articular cartilage shows Col X (red) staining in the deep zone whereas 3 week in vitro formed tissue (D) is negative. Nuclei were visualized by DAPI (blue). N=3. m= membrane. Scale bar = 200μm.

2.4.3 Passaged chondrocytes accumulate more cartilage ECM than primary chondrocytes.

To determine if the effect of TGFβ3 was specific to passaged chondrocytes, ECM accumulation by human passaged (P2) chondrocytes and primary (P0) chondrocytes (never passaged) was quantified. The passaged chondrocytes formed more robust tissue after 3 weeks of culture with TGFβ, accumulating significantly more collagen (Figure 2-4 A) than primary...
chondrocytes. Both passaged and primary cells accumulated similar amounts of sulphated proteoglycan when cultures were supplemented with TGFβ3 (Figure 2-4 B). Similar to passaged chondrocytes, primary chondrocytes did not form cartilage tissue in the absence of TGFβ3 (SFRM alone).

![Graph](image)

**Figure 2-4.** Passaged chondrocytes accumulate significantly more collagen in response to TGFβ3 than primary chondrocytes. Chondrocytes were seeded directly onto membranes (P0) or after serially passaging twice (P2) and grown for 3 weeks in SFRM with or without 10ng/ml TGFβ3 as stated. (A) Hydroxyproline accumulation. (B) Sulphated GAG accumulation. N=3 (9 samples). Results are expressed as mean ± 95% CI. * = p< 0.05.

2.4.4 In vitro formed cartilage maintains the ability to accumulate significant amounts of collagen after TGFβ3 supplementation is stopped.

If used clinically for cartilage repair, following implantation the in vitro formed tissue would no longer be exposed to exogenous TGFβ. Therefore we investigated whether the chondrocytes would continue to accumulate ECM when supplementation of active TGFβ was discontinued. Cultures in which TGFβ3 was discontinued after week 3, but grown for an additional 3 weeks (termed “transient TGFβ3”), the sulphated glycosaminoglycan content
decreased (Figure 2-5 A). This loss was not significant compared to 3 week old cultures but was significantly reduced when compared to tissues cultured continuously for 6 weeks in the presence of TGFβ3. In contrast, significant amounts of collagen continued to accumulate in the absence of TGFβ3 (Figure 2-5 B). Immunohistochemical studies revealed that it was Col 2 and not Col 1 that accumulated after media supplementation with TGFβ3 ceased (Figure 2-5 C).

Figure 2-5. In vitro formed tissues continue to accumulate significant amounts of collagen type 2 if treatment with exogenous TGFβ3 is discontinued. Passaged (P2) chondrocytes were cultured for 3 weeks in SFRM with 10ng/ml TGFβ3 to allow for tissue formation, then cultured
for an additional 3 weeks in the absence (transient TGFβ3) or presence (continuous TGFβ3) of TGFβ3 treatment. Controls were grown in the absence of TGFβ3 for 6 weeks. (A) Sulphated GAG accumulation. (B) Hydroxyproline accumulation. (C) Immunohistochemistry for type 2 collagen (Col 2) or type 1 collagen (Col 1) with nuclei stained with DAPI. Human native articular cartilage was used as a positive control. Results are expressed as mean ± 95% CI. Scale bar = 200μm.

2.4.5 Mechanical Properties of in vitro Formed Cartilage.

No significant differences in the mechanical properties of the in vitro formed tissues was observed with longer culture (3 vs 6 weeks) or continued compared to transient TGFβ3 supplementation (Table 2-1). Although Poisson’s ratio increased with sustained culture with TGFβ3 for 6 weeks, this was not a significant change. It was not possible to assess the mechanical properties of tissues formed in the absence of TGFβ as there was insufficient tissue for testing.

<table>
<thead>
<tr>
<th></th>
<th>Poisson’s Ratio</th>
<th>Bulk Modulus (kPa)</th>
<th>Young’s Modulus (kPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 Week Culture Sustained TGFβ3</td>
<td>0.297 ± 0.02</td>
<td>20.48 ± 5.94</td>
<td>52.86 ± 2.28</td>
</tr>
<tr>
<td>6 Week Culture Transient TGFβ3</td>
<td>0.305 ± 0.025</td>
<td>19.87 ± 4.93</td>
<td>51.14 ± 12.40</td>
</tr>
<tr>
<td>6 Week Culture Sustained TGFβ3</td>
<td>0.365 ± 0.029</td>
<td>18.87 ± 3.92</td>
<td>50.01 ± 9.89</td>
</tr>
</tbody>
</table>

Table 2-1. Mechanical properties of 3 and 6 week formed cartilage tissues. Passaged (P2) chondrocytes were cultured for 3 weeks in SFRM with 10ng/ml TGFβ3 to allow for tissue formation, then cultured for an additional 3 weeks in the absence (transient TGFβ3) or presence (continuous TGFβ3) of further TGFβ3 treatment. The mechanical properties of the tissues were measured for each condition. Data represented as mean ± SEM.
2.4.6  Tissues cultured for 6 weeks show collagen fiber orientation and protein expression consistent with superficial zone cartilage.

To determine if the in vitro formed tissues showed evidence of zonal organization with the development of a superficial zone, polarized light microscopy was used to assess collagen fiber orientation in the formed tissues. Cells cultured for 3 or 6 weeks with TGFβ3 showed collagen fiber orientation parallel to the surface (Figure 2-6). Immunohistochemical studies demonstrated that at 3 weeks the expression of lubricin, also known as proteoglycan 4 (PRG4) was variable, with some tissues showing no expression and others showing discontinuous expression along the surface (Figure 2-6 white arrow). No clusterin expression was detected at 3 weeks. Tissues grown for 6 weeks either continuously in the presence of TGFβ3 or transiently (for the first 3 weeks only) showed both clusterin and PRG4 at the surface of the cartilage tissue indicative of the presence of a superficial zone. Some PRG4 expression was present at the base of 6 week formed tissues cultured continuously with TGFβ for 6 weeks (Figure 6 white arrowhead).
Figure 2-6: In vitro formed tissues show collagen fiber alignment and protein expression consistent with superficial zone of articular cartilage. (Upper Row). Polarized light microscopy images of histological sections stained with picrosirius red. (Middle Row). Immunohistochemical staining for PRG4. DAPI counterstain. (Lower Row) Immunohistochemical staining for clusterin. DAPI counterstain. White arrow shows area of discontinuous PRG4 expression in 3 week formed tissues. White arrowhead points to area of PRG4 expression at the bottom of the construct. m = membrane. Scale bar = 100um.

2.5 Discussion

In this study we showed that human passaged chondrocytes could form a continuous layer of articular cartilage tissue in vitro when cultured in high density (3D) on top of a
membrane in SFRM supplemented with 10ng/ml TGFβ3 for a minimum of 2 weeks. The tissue developed a superficial zone and was rich in Col 2 and Acan. Importantly the tissue did not contain Col 1 and the cells did not acquire a hypertrophic phenotype as they did not express alkaline phosphatase activity or accumulate Col X, nor did it show any potential to mineralize. To the best of our knowledge this is the first study demonstrating this ability by passaged human chondrocytes to form cartilage tissue in the absence of scaffold and with only one growth factor; TGFβ3. The mechanism by which TGFβ3 promotes redifferentiation has not been elucidated; however TGFβ3 is crucial for promoting chondrogenesis and cartilage tissue formation by MSC in vitro [19, 32]. Interestingly, it has been shown that when chondrocytes are serially passaged, they acquire plasticity resembling that of MSC [33, 34] characterized by spindled cell morphology and the ability to differentiate into all three of the mesenchymal lineages (adipose, chondrogenic and osteogenic); a phenotype not found in primary cells Therefore TGFβ3 may be acting in a similar manner as it does in MSC chondrogenesis to promote articular cartilage tissue formation by the passaged human cells in our system. In support of this, we observed that primary chondrocytes (P0) were unable to accumulate significant amounts of collagen, when cultured under the same conditions as P2 chondrocytes.

Cartilage formation by P2 human chondrocytes required exogenous TGFβ, in contrast to bovine P2 chondrocytes which we showed in previous studies did not require the addition of any growth factors [16]. Interestingly, it has been shown recently that passaged bovine chondrocytes do require exogenous TGFβ for cartilage tissue formation in vitro, but only after passage number 3 (P3) [35] at which point these cells showed significant decreases in mRNA levels of TGFβ2 and both TGFβ receptor types 1 and 2. It is known that chondrocytes from human OA cartilage synthesize less TGFβ and possess lower levels of TGFβ receptors than chondrocytes from non-diseased cartilage [23, 36, 37]. Therefore, TGFβ may be required for human cells as they already have impaired endogenous TGFβ signalling, which may be further affected with serial passaging.

Use of these tissues for biological repair requires that tissue will not degrade in the absence of exogenous growth factor supplementation, as intra-articular injections of TGFβ can induce adverse effects in joint and synovial tissue [38, 39]. Although not entirely similar to the in vivo situation, the in vitro formed cartilage continued to accumulate significant amounts of collagen, which was predominately collagen type 2, when TGFβ was discontinued after 3 weeks of treatment. This suggests that the redifferentiated phenotype may be stable, and if implanted in
vivo the cartilage tissue may continue to mature in the absence of growth factor. Of note, proteoglycan content did not continue to increase in our culture system when TGFβ was discontinued. The reason for this is not known, but importantly this did not affect the mechanical properties of the formed tissues. It is possible that the formed tissues may simply be behaving like native articular cartilage explant cultures, which lose proteoglycan when placed in in vitro culture [40]. Alternatively, the absence of mechanical loading in the in vitro environment may contribute to the inability of the tissue to further accumulate sulphated proteoglycan. Previous studies have demonstrated the role of biomechanics on promoting proteoglycan accumulation [41]. Furthermore, shear forces have been shown to activate latent TGFβ produced by cells in synovial joints [42]. It may be that once the cartilage tissue is implanted in vivo, mechanical stimulation from joint movements and weight bearing may act to maintain the TGFβ and proteoglycan content and allow for further maturation and ECM accumulation. Further study is required to confirm this.

Interestingly, more than 3 weeks of culture was required for the tissues to develop a continuous superficial zone characterized by parallel collagen alignment and expression of PRG4 and clusterin. Continuous 6 week exposure to TGFβ3 was not required for this to occur. TGFβ has been shown to enhance PRG4 expression and accumulation by superficial zone chondrocytes in vitro [43, 44]. There are several potential reasons this occurred. Recently, Albro et al. showed that in bovine chondrocyte tissue constructs, media supplementation with exogenous active TGFβ1 resulted in high affinity binding and internalization by cells in the construct periphery (first 1-2 mm) only [45]. This has also shown to occur in the superficial zone of native bovine cartilage explant cultures [46]. This gradient of exposure to exogenous active TGFβ may be the reason the tissues in our system developed a superficial zone and may explain why PRG4 is most highly expressed and localized to the surfaces of our tissues. Although the effect of TGFβ3 on clusterin expression by chondrocytes is not known, it has been shown to upregulate clusterin mRNA and protein expression in other cell types such as lung epithelial cells [47, 48] and may act similarly through a TGFβ mediated pathway in chondrocytes. In our system, 6 weeks of culture was needed for the development of a superficial zone. It may be that 6 weeks is required for sufficient amounts of PRG4 and clusterin to be accumulated so they can be detected immunohistochemically and for collagen to be detected by polarized light microscopy. Alternatively, it may be a maturation process that, similar to development, requires time. Gannon
et al. demonstrated that it was not until 4 weeks of age that porcine cartilage had developed collagen fibers aligned parallel to the articulating surface [49]. Furthermore, it was not until skeletal maturity was reached that the fibers in the deep zone thickened and became aligned perpendicular to the articulating surface.

As chondrocytes passaged in the presence of serum containing media have been FDA approved for use in other cartilage repair procedures, and are readily obtainable using an arthroscopic approach, they would seem to be the optimal source of cells for cartilage repair. In this study we describe a method to reverse the dedifferentiation that occurs during cell number expansion in monolayer culture and to re-establish the ability of these cells to form a continuous layer of articular cartilage-like tissue in the absence of a scaffold when grown in 3D culture in serum-free media supplemented with TGFβ3. Further study is required to determine whether the in vitro formed cartilage tissue will be suitable to repair focal joint surface defects. Developing a method to expand cell number in the absence of serum supplementation may enhance clinical translation.

2.6 Author Contributions

Vanessa J. Bianchi contributed to study design, the acquisition of the human tissue samples, cell isolation and in vitro cell culture, biochemical assays, immunohistochemistry and histology, fluorescence and light microscopy imaging, data analysis, figure preparation, writing and editing of the manuscript.

Joanna F. Weber performed mechanical testing and analysis on the in vitro formed tissues in this study as well as writing and editing of the manuscript.

Stephen D. Waldman contributed to mechanical testing and analysis, and editing of the manuscript.

David Backstein contributed to the acquisition of the human tissue samples used in this study and editing of the manuscript.

Rita Kandel contributed to study design, data analysis, writing and editing of the manuscript.
2.7 References


media-supplemented active TGF-beta and is ameliorated by the alternative supplementation of latent TGF-beta. Biomaterials 77, 2016.


Chapter 3
Expression of Endoglin, a TGFβ co-receptor, not ALK5 correlates with the Ability of Passaged Human Articular Chondrocytes to form Cartilage Tissue in vitro.

3.1 Abstract

Objective

Chondrocytes are a suitable cell source for bioengineering articular cartilage as cell number can be easily increased in vitro with serial passaging, and passaged cells will reliably form hyaline-like cartilage when cultured with transforming growth factor beta (TGFβ) in 3D culture. However, accessibility to healthy cartilage is limited so cells would have to be obtained from osteoarthritic cartilage (OAC). Analysis of chondrocytes obtained from OAC with severe changes show differentially regulated gene and protein networks compared to chondrocytes obtained from OAC with minimal changes. Additionally, studies in animal models have demonstrated loss of the primary TGFβ receptor ALK5 in severe OA, although it is not known whether the expression levels of ALK5, and its co-receptors endoglin (ENG) and TGFβ receptor 3 (TGFβ RIII) change in human OAC in a grade dependent manner. However, it has been shown that passaged chondrocytes acquire a progenitor phenotype which may potentially eliminate these differences. Thus, the purpose of this study was to assess the effect of OAC grade on chondrogenesis. We hypothesize that chondrocytes obtained from either minimal or severe OAC, following passaging in monolayer culture, will form hyaline cartilage tissue in vitro and show similar profiles of TGFβ signaling molecules.

Methods

OAC was harvested from joints resected for arthroplasty, from areas showing either minimal or severe osteoarthritic (OA) changes and were processed separately. Representative pieces were graded histologically using the OARSI scale and the remainder underwent enzymatic digestion to isolate chondrocytes. Primary cells (P0) were either directly analyzed or seeded into monolayer culture and passaged twice (P2; ~9 doublings). To determine expression of TGFβ signaling molecules P0 and P2 cells were evaluated by image flow cytometry using antibodies reactive with ALK5, TGFβ RIII or ENG. Fluorescent activated cell sorting (FACS)
was used to separate P0 ENG+ and ENG- subpopulations. To assess cartilage tissue forming capability, sorted and unsorted P0 or P2 cells were placed in 3D culture on membrane inserts (2x10^6 cells) and grown for 3 weeks in serum-free high glucose (4.5g/L) DMEM supplemented with 1% ITS and TGFβ3 (10ng/ml). Tissues were evaluated histologically, immunohistochemically (IHC), or biochemically. Experiments were repeated 3 times from separate specimens. Results are expressed as mean ±SEM and analyzed using T-test or ANOVA. Significance was assigned at p<0.05.

Results

OARSI grading of OAC confirmed a significant difference between tissues with minimal and severe changes. Image flow cytometry showed that P0 and P2 cells obtained from low and high grade OAC showed similar numbers of cells that expressed ALK5. There was no significant difference in the amount of ALK5 receptor/cell in these different groups. Few P0 cells, from either low or high grade, expressed TGFβ RIII (<2%). This did not change with passaging. There was no significant difference in the number of low and high grade P0 cells that expressed ENG (Low OA 37.4% ±19.8 vs High OA 34.6% ±10.7). However, more cells expressed ENG with passaging to P2 (Low OA 86.3% ±4.6 vs High OA 89.7% ±3.3) and the level of ENG receptor/cell was significantly greater than P0 cells for both groups. FACS sorting was used to separate P0 cells based on ENG expression. With passaging to P2 ENG- acquired expression of ENG in similar numbers to P2 cells obtained from the unsorted (mixed ENG+/− population) and ENG+ sorted cells. Expression of ENG receptor/cell was higher for P2 cells than for P0 cells with no significant differences between sorted and unsorted groups after passaging. From low or high grade OAC did not form cartilage tissue in the presence of TGFβ3. In contrast, after passaging these cells were able to form cartilage tissues that were similar in amount as there were no significant differences in the proteoglycan or collagen contents accumulated. IHC showed that the tissues that developed were hyaline-like as they contained aggrecan and type II collagen and showed no type I or X collagens.

Conclusions

This study demonstrated that passaged cells obtained from either low or high grade OAC can be used to bioengineer hyaline-like cartilage. It is not clear why nonpassaged (P0) cells are unable to form cartilage in vitro even though they express similar levels of ALK5 as P2 cells, the
TGFβ receptor considered to regulate chondrogenesis. Clearly additional co-receptors and/or factors are needed as with passage these cells change to predominantly ENG expressing cells which also have greater endoglin receptor/cell than P0 cells. Further studies are required to determine if high levels of ENG are required for tissue formation by P2 cells.

3.2 Introduction

Osteoarthritis (OA) is a chronic disease affecting millions of people globally [1]. The cause is believed to be multifactorial and ultimately results in an imbalance in the catabolic and anabolic processes within the articular cartilage (AC) leading to progressive destruction of the tissue. Biological approaches to repairing damaged cartilage tissue are currently being investigated as an alternative to total joint replacement; the gold standard treatment for patients with advanced OA. Chondrocytes are a good cell source for these approaches as they are easily attainable from the individual via arthroscopy. The cells however are acquired from tissues affected by OA, and a multitude of studies have demonstrated that OA chondrocytes have an altered phenotype. They have been shown to have increased expression of cytokine receptors [2] and production of catabolic enzymes [3], [4]. In addition, studies have demonstrated loss of expression of hyaline cartilage matrix genes in chondrocytes derived from diseased tissues compared to normal [5], and in comparison to cartilage from the same joint with less advanced OA [6].

More recently studies using microarray analysis or RNA sequencing comparing chondrocytes obtained from tissue with minimal OA to those cells derived from tissues with severe OA from the same joint [7]–[9] have identified a multitude of differentially regulated gene and protein networks. Altered gene networks include those linked to ECM organization, mitotic cell cycle, skeletal development, transcriptional regulation, apoptosis and Wnt signaling [9]. These observations suggest that despite the fact that cells from the same joint share systemic and locally generated factors, chondrocytes in sites of advanced OA are phenotypically distinct from chondrocytes in sites which appear less or un-affected by OA. What remains unclear is whether these differences influence the ability of the cells to form new cartilage and ultimately be used for cell therapies.
Passaging human chondrocytes in monolayer culture is often required to increase cell numbers and this results in dedifferentiation, a process whereby chondrocytes lose their phenotype [10]. This has long been a limitation to the use of these cells for bioengineering cartilage. However, in a previous study we showed that growing these passaged cells in 3D culture in the presence of transforming growth factor beta (TGFβ) could re-establish an articular chondrocyte phenotype and result in hyaline cartilage matrix production and accumulation in vitro [11]. In the absence of exogenous TGFβ supplementation, the cells did not self-assemble into hyaline-like tissues. The importance of TGFβ for neocartilage formation by passaged bovine chondrocytes [12] and human mesenchymal stromal cells (MSCs) [13] have also been demonstrated validating the importance of this growth factor in early in vitro chondrogenesis.

Studies in both animal models and in human OA have suggested that TGFβ signaling pathways may be altered in chondrocytes derived from severe OA, and this has been suggested to play a role in the pathogenesis of the disease. In mouse models, expression of the primary TGFβ receptor ALK5 was shown to be decreased with increased severity of OA [14]. Activation of ALK5 by TGFβ binding results in downstream signaling involving the phosphorylated SMAD-2/3 complex which has been shown to promote expression of cartilage matrix genes [15]. In addition to ALK5, two TGFβ co-receptors have also been identified in human chondrocytes, TGFβ receptor 3 (TGFBRIII, also known as betaglycan) and endoglin (ENG, also known as CD105) [16], and have been shown in other cell types to enhance TGFβ signaling [17]. Few studies have investigated the role TGFBRIII may play in in vitro chondrogenesis, but it has been shown that TGFBRIII is essential for signaling by TGFβ2 which has a low affinity for TGFβ receptors [18], [19]. To the best of our knowledge no studies have investigated the role of ENG expression in neocartilage formation by chondrocytes. Studies in adult stem cells which have been sorted for ENG positive cells have demonstrated that ENG confers chondrogenic potential [20]–[23], with one study demonstrating this was facilitated via SMAD2 signaling [21]. The role these co-receptors may play in in vitro chondrogenesis by articular chondrocytes is still undetermined. Furthermore, it is unclear whether the altered phenotype of chondrocytes derived from severe OA, which may influence the ability of the cells to form neocartilage in response to TGFβ supplementation, is maintained with passaging.

To better understand these questions, the cells and the tissues formed by primary and passaged cells obtained from AC showing severe OA (termed ‘High OA”) and from
macroscopically undamaged regions of AC (termed “Low OA”) from the same joint compartment were compared. Furthermore, the effect of dedifferentiation on the expression of key TGFβ receptors and co-receptors was examined. The hypothesis of this study was that following serial passaging in monolayer culture, chondrocytes obtained from Low OA and High OA will form hyaline cartilage tissue in vitro and show similar profiles of TGFβ receptor and co-receptor molecules.

3.3 Materials and Methods

3.3.1 Isolation of Chondrocytes from Low OA and High OA Grade Cartilage

Human articular cartilage was obtained from femoral condyles resected during joint arthroplasty surgery for osteoarthritis following informed patient consent and with Research Ethics Board (Mt. Sinai Hospital) approval. Tissue was either taken from areas which macroscopically appeared undamaged and termed “Low OA” or from areas which showed signs of severe OA (cartilage erosion) and termed “High OA”. Representative pieces of tissue from both groups were processed for histological grading. Chondrocytes were isolated by enzymatic digestion from the remainder of the tissue. This consisted of incubation in 0.5% protease (Sigma-Aldrich) for 1 hour at 37°C followed by an overnight digestion in 0.2% collagenase A (Roche) at 37°C. Cells from each patient and each experimental group (Low OA or High OA) were cultured separately (cells were not pooled). The cells were either placed in 3D culture directly or in monolayer culture at 2000 cells/cm² in high glucose Dulbecco’s modified Eagle’s media (DMEM) supplemented with 20% fetal bovine serum (FBS). Chondrocytes were passaged twice (P2) to allow for approximately 9 population doublings as described previously and then placed in 3D culture [11].

3.3.2 3D Membrane Culture

Membrane inserts (12 mm diameter; EMD Millipore™) were coated with collagen type 2 (Col 2; Sigma-Aldrich, c9301) prior to use. 100µl of Col 2 solution (0.05 mg/ml Col 2 dissolved in 0.01% Acetic Acid) was used to coat each membrane and dried overnight under sterile
conditions. They were washed with PBS Mg and Ca -/- immediately before use. Primary (P0) or passaged (P2 cells, 2 x 106) were placed on the membranes and were cultured in serum-free media (SFM) consisting of DMEM (Wisent Bioproducts, 319-016-CL), 1% ITS+ (Wisent Bioproducts, 315-080-ZL), L-proline (40 ug/mL, Sigma-Aldrich, P5607), sodium pyruvate (110 ug/mL, ThermoFisher, 11360070), dexamethasone (0.1μM, Sigma-Aldrich, D4902), and L-ascorbic acid (100 ug/mL, Sigma-Aldrich, A4403). 10 ng/ml human recombinant TGFβ3 (R&D Systems, 243-B3) was added after 24h. Unless otherwise stated cells were cultured for 3 weeks and the media was completely changed three times per week.

3.3.3 Tissue Content Analysis

In vitro formed tissues were digested with papain (40 µg/mL; Sigma-Aldrich) in buffer containing 20 mmol/L ammonium acetate, 1 mmol/L EDTA and 2 mmol DTT at pH 6.2 for 48 hours at 65°C. DNA content was quantified by the Hoechst 33258 dye binding assay and fluorometry (excitation λ= 365 nm, emission λ= 458 nm) [24]. The DNA standard curve was generated using calf thymus DNA (Sigma-Aldrich). Hydroxyproline content was quantified following HCl hydrolysis of the papain digest using the chloramine-T/Ehrlich’s reagent assay and spectrophotometry (λ= 560 nm) [25]. L-hydroxyproline (Sigma-Aldrich) was used to generate the standard curve. Sulfated glycosaminoglycan content of papain digested samples was quantified by dimethylmethylene blue dye binding assay and spectrophotometry (λ= 525 nm) [26]. Chondroitin sulphate (Sigma-Aldrich) was used to generate the standard curve.

3.3.4 Histology and Immunohistochemistry

Representative tissues were fixed in 10% PBS buffered formalin (pH 7.4) overnight then placed in a 30% sucrose/PBS solution overnight at 4oC. Tissues were snap-frozen in Tissue-Tek OCT (Sakura Finetek) to generate blocks for cryosectioning. Seven μm sections were cut and either stained with haematoxylin and eosin, or toluidine blue and mounted with Micromount (Leica Biosystems) and glass coverslips. The tissues were graded using the OARSI grading scale [27]. Three representative tissue samples isolated from regions designated “Low OA” or “High OA” from 3 separate patients were graded.
For immunohistochemistry, sections were digested with 0.4% pepsin, blocked with 20% goat serum (in 0.1% Triton solution) and then incubated overnight at 4°C with antibody reactive with either type I collagen (COL 1, 1:250 Cedarlane, CL-50111AP-1), Col 2 (1:100, EMD Millipore, MAB8887), type X collagen (Col X, 1:1500 Abcam, ab49945), or PRG4 (1:250, EMD Millipore, MAB401). For ACAN no antigen retrieval was used (1:500, ThermoFisher Scientific, AHP0022). Tissues were subsequently incubated with either Alexa-594 goat anti-rabbit IgG (1:1000 Life Technologies, A11012), Alexa-488 goat anti-mouse IgG (1:1000, Life Technologies, A-11008), or Alexa-594 goat anti-mouse IgM (1:1500, Life Technologies, A-21044) depending on the primary antibody for 1 hour at room temperature. Tissues were counterstained with a DAPI solution (0.5 mg/ml). Negative controls consisted of replacing the primary antibody with isoform and species matched IgG or IgM antibody at the same protein concentration. Tissues were washed 3 times with PBS --/-- between incubations.

3.3.5 Flow Cytometry

Cells were isolated from articular cartilage and either serially passaged to P2 as described above or plated as P0 cells at high density (60,000 cells/ cm2) in monolayer culture overnight in DMEM with 20% FBS to allow the cells to recover from the digestion process. The attached chondrocytes maintained a rounded/polygonal shape within this time frame and were termed “P0”. In a previous study we showed that bovine P0 cells did not start to dedifferentiate until day 3 of culture [28]. P2 cells were utilized upon confluency. Flasks containing P0 or P2 cells were washed 3 times with PBS --/-- and undiluted Accutase (Sigma-Aldrich, A6964) was used to detach cells for flow cytometry analysis with the Amnis ImageStream imaging flow cytometer.

In general, stained cells were resuspended in a volume of 50 μl PBS--/-- 1% FBS in a 1.5ml low retention microfuge tube (Sigma-cat#T4816). Samples were then acquired on a 5 laser 12 channel ImageStream MKII imaging flow cytometer at 60 X magnification following ASSIST calibration (Amnis Corporation). Appropriate collection channels corresponding to FITC/AF488, DAPI/Hoechst, APC1 and PECF594 were used for acquisition along with lasers 405nm (100mw), 488nm (150mw), 561nm (200mw), 592nm (300mw) and 642nm (150mw) for excitation. A bright-field (BF) area lower limit of 50 μm2 was used to eliminate debris and speed beads during sample acquisition while samples were collected in a series of 20-30X103 event
raw image files (.rif). For single stained compensation controls bright field (BF) illumination was
turned off and approximately 2000 events within the positive signal fraction were acquired in all
channels. An initial compensation matrix was generated by loading the single stained raw image
files into the IDEAS compensation wizard (IDEAS version 6.2) with further refinements to the
compensation matrix made as necessary through manual adjustment [29] (see supplementary
note). Once generated the compensation matrix could then be applied to the sample raw image
files to create compensated image files (.cif) which could then be analyzed.

ENG and TGFBRIII: Staining was performed on live cells. P0 or P2 cells were
suspended in a staining solution of PBS -/- with 2% FBS and 1 million cells/ 200 µL. Cells were
co-stained with 5 uL of ENG APC-conjugated antibody (BD Biosciences, 562408) and 10 µL
TGFBRIII FITC conjugated antibody (R&D Systems, FAB242F) in 200 µL for 1h on ice.
Samples were gently vortexed every 15 min. DAPI (0.02 µg/ mL) was added to assess viability.
Unstained cells, APC and FITC single stained cells and cells permeabilized with 0.01% Triton
and stained with DAPI were used compensation controls. Data was analyzed using the IDEAS
6.2 software.

ALK5 Staining: For ALK5 staining (Abcam, ab31013) cells were fixed and
permeabilized (Fix & PermTM Cell Permeabilization Kit, ThermoFisher Scientific, GAS003) as
the antibody is reactive for the intracellular portion of the receptor as per the manufacturer’s
instructions. Briefly, cells were fixed with Medium A (1 million cells/ 100uL) for 15 min and
subsequently permeabilized with Medium B (1 million cells/ 100uL). Cells were then
resuspended in a blocking and staining media consisting of PBS-/- with 10% goat serum and
20% FcR Blocking Reagent (Miltenyi Biotec, 130-059-901) at 1 million cells in 200 µL for 10
min. Primary antibody was added directly (1:100) and incubated for 30min at RT. Cells were
washed with PBS -/- to remove unbound primary antibody, resuspended in PBS/-/- with 10% goat
serum and then incubated for 30 min with secondary antibody (4ug/ml, Alexa 488; Invitrogen
A11008). Samples were gently vortexed every 15 min during both primary and secondary
antibody incubation. Cells were washed and resuspended in PBS/-/- for analysis. Hoechst dye
was then added (2ug/ml) and flow cytometry was performed. Unstained cells and cells stained
with Alexa 488 secondary antibody only (no primary) were used as compensation controls. Data
was analyzed using the IDEAS 6.2 software.
3.3.6 Fluorescence Activated Cell Sorting (FACS) for ENG

Following enzymatic harvest from the cartilage P0 cells were seeded overnight as described above and detached with undiluted Accutase (Sigma-Aldrich, A6964). Cells were resuspended in a solution of PBS +/- with 5mM MgCl2 (M2393, Sigma-Aldrich) and treated with 100µg/mL DNAse I (5 million cells/ mL, Roche 04536282001) for 20 min. Cells were pelleted at 500 rcf for 5 min and washed 1X with PBS +/- with 5mM MgCl2 solution. Cells were filtered (100 µm filter) and resuspended in PBS +/- with 2% FBS at 10million cells/ 200µL for staining. 5 uL of ENG APC-conjugated antibody (BD Biosciences, 562408) was added per 200µL and incubated for 30 min at RT. Cells were washed once with PBS/-/ and resuspended at 5million cells/ mL in a solution of PBS-/- with 25mM HEPES and 1% heat inactivated FBS. Cells were sorted using the ASTRIOS EQ cell sorter (Beckman Coulter, USA). P0 chondrocytes were sorted into three populations, ENG-, ENG+DIM, or ENG+BRIGHT based on fluorescent intensity. Unstained cells were used as controls for gating.

3.3.7 Population Doubling Time Analysis

P0 cells FACS sorted as either ENG-, ENG+DIM, or ENG+BRIGHT were seeded at 2000 cells/cm2 in 6 well plates and cultured in DMEM with 20% FBS for 1, 4, 7 or 14 days. At each time point cells were harvested with 2X Trypsin (Wisent Bioproducts, 325-044-EL) and counted using a haemocytometer. Cells from two separate wells were counted for each time point. Population doubling time was calculated using the following formula; DT= duration*log2/log(final concentration)-log(initial concentration).

3.3.8 Statistical Analysis

Unless otherwise stated three independent experiments were performed for each condition tested using cells from 3 different patient samples (N=3). The data was pooled, expressed as mean ± 95% SEM and analysed using a paired t-test or one-way ANOVA with Tukey’s Post Hoc. Significance was assigned at p< 0.05.
3.4 Results

3.4.1 Passaging enables chondrocytes to form hyaline-like cartilaginous tissues with similar amounts of matrix regardless of whether cells were obtained from High OA or Low OA grade tissues.

Histological sections of AC, representative of those tissues from which the chondrocyte populations were obtained, were graded using the OARSI grading scale [27] to assess if macroscopic examination was sufficient to differentiate minimally altered (Low OA) from severely degraded (High OA) cartilage (Figure 3-1). Macroscopically Low OA areas were intact while High OA areas were uneven in texture and dull (Figure 3-1 E). Histologically tissues derived from High OA regions of the joint showed deep fibrillations, tissue erosion, loss of proteoglycans, and chondrocyte cloning (Figure 3-1 B and D) indicative of advanced OA. In contrast Low OA cartilage showed only minor surface discontinuity and maintained proteoglycan content throughout (Figure 3-1 A and C). OA grades were significantly different between Low OA and High OA tissues (Figure 3-1 F).

Figure 3-1: Minimally affected (Low OA) and severely degraded (High OA) osteoarthritic human cartilage can be correctly identified and separated by macroscopic inspection. (A-D) Formalin fixed histological sections of cartilage tissue dissected from Low OA (A, C) and High OA (B, D) areas. (A-B) Haematoxylin and eosin or (C-D) toluidine blue staining of tissue sections. (E) Gross morphology of human cartilage tissue illustrating areas of Low OA and High OA. (F) OARSI grading of histological sections of cartilage derived from Low OA and High OA regions. N=3.
Previous work showed that primary chondrocytes derived from Low OA cartilage did not form cartilaginous tissues when cultured in SFM in the presence of TGFβ3 [11]. We confirmed that this was also true for High OA chondrocytes. There were no significant differences between the two cell populations with respect to sulphated glycosaminoglycan (GAG) accumulation (Figure 2 A) or collagen accumulation (Figure 3-2 B). Histologically, the tissues are thin (Figure 2 C, D) and accumulate little proteoglycan as shown by toluidine blue staining (Figure 3-2 E, F). After serial passaging however, P2 chondrocytes cultured under the same conditions accumulate approximately 3 times more proteoglycan (Figure 3-2A and Figure 3-3A) and 12 times more collagen (Figure 3-2B and Figure 3-3B) than P0 cells which were not passaged. Histological evaluation (Figure 3-3 C, D) and immunohistochemical studies (Figure 3-3 E, F) show no differences between tissues formed by Low OA and High OA. Both groups accumulate collagen type 2 (Col2) and aggrecan (ACAN) throughout the tissue and show little proteoglycan-4 (PRG-4) expression at 3 weeks. Collagen type 1 (Col1) and collagen type X (ColX) were not detected by immunohistochemistry in either group at 3 weeks.

Figure 3-2: **Primary chondrocytes do not form cartilaginous tissues in vitro regardless of the grade of OA.** Primary chondrocytes were cultured in 3D culture on membranes in SFM supplemented with 10ng/ml TGFβ3 for 3 weeks. (A) Sulphated glycosaminoglycan (GAG) and (B) hydroxyproline content of the tissues. (C-F) Histological sections of 3 week old tissue stained with haematoxylin and eosin (C,D) or toluidine blue (E, F). Size bar = 100μm. m = membrane.
Figure 3-3: **Passaged (P2) chondrocytes form cartilaginous tissues with similar amounts of matrix regardless of whether cells were obtained from High or Low OA tissues.** P2 chondrocytes were cultured in 3D culture in SFM supplemented with 10ng/ml TGFβ3 for 3 weeks. (A) Sulphated glycosaminoglycan (GAG) and (B) hydroxyproline contents of 3 week formed tissues. (C,D) Histological sections of in vitro formed tissues stained with haematoxylin and eosin show a continuous layer of cartilage-like tissue. (E-F) Immunohistochemically stained sections of 3 week formed tissues with antibodies reactive with Aggrecan (ACAN, green), Collagen type 2 (Col2, green), Lubricin (PRG4, green), Collagen type 1 (Col1, red), or Collagen type 10 (ColX, red). DAPI (blue) counterstain. Native cartilage was used as positive controls.
3.4.2 Cells from Low OA and High OA show no grade specific differences and maintain expression of ALK5 with serial passaging, but increase expression of ENG.

To further investigate why P2 chondrocytes form cartilaginous tissues in response to culture with TGFβ3 while P0 chondrocytes do not, image flow cytometry was used to assess expression of the TGFβ receptor ALK5, and its potential co-receptors ENG and TGFβRIII in P0 and P2 chondrocytes derived from High and Low OA (Figure 3-4). The number of cells expressing ENG as well as the density of ENG (assessed by median fluorescent intensity value) increased significantly with passaging to P2 for both Low OA and High OA (Figure 3-4 A). No significant difference between the two grades was detected in either the P0 or P2 cells. TGFβRIII was scarcely expressed (< 5%) in both P0 and P2 cells and receptor density decreased after passaging for both Low OA and High OA (Figure 3-4 B). ALK5 was expressed in the majority of P0 cells (< 95%), and this expression was maintained in P2 cells with no significant change in the density of ALK5 (Figure 3-4 C). Overall no grade specific differences in the expression of these receptors were identified, with Low OA and High OA populations showing similar levels of expressions for these receptors in P0 and P2 cells. In addition to showing similar receptor expression profiles when passaged, cell size increased with passaging and the population appeared more homogeneous when brightfield images of individual cells were compared (Supplementary Figure 3-1 A and B).
Figure 3-4: Image flow cytometry labelling for Endoglin, TGFBRIII and ALK5 shows no grade specific differences. P0 or P2 cells were assessed using image flow cytometry and stained with antibodies reactive for (A) ENG, (B) TGFBRIII or (C) ALK5. Percent gated and median pixel fluorescent intensity values (indicative of receptor density) are graphed for each receptor. Grade specific differences were not detected. Image galleries to the right of the graphs show staining of representative cells from each group. Brightfield (gray), TGFBRIII (green), ENG (Red), and ALK5 (green). N=3
3.4.3 ENG expression is acquired with serial passaging in monolayer.

When passaged, the number of the cells expressing ENG as well as the density of ENG/cell increased significantly (Figure 3-4 A). To determine if this phenotype was acquired with passaging in monolayer culture, or whether the ENG+ population predominated as a result of being more proliferative, fluorescence activated cells sorting was used to isolate P0 chondrocytes into three populations, ENG-, ENG+DIM, or ENG+BRIGHT. These were then cultured separately in monolayer. To assess proliferation, growth curves were generated and doubling time was calculated (Supplementary Figure 3-2). Analysis of doubling time revealed that the ENG+BRIGHT proliferated the fastest of the 3 groups (ENG- 5.4 days, ENG+DIM 4.7 days, and ENG+BRIGHT 4.3 days) however; as the ENG- population also proliferated it appeared unlikely that ENG+ population solely contributed to the primarily ENG+ phenotype observed in P2 cells. To confirm this, the P0 ENG- population was passaged to P2 and flow cytometry was used to determine if the cells became ENG+. All cells became ENG+ after passaging (Figure 3-5 A). Furthermore, P2 cells that were either sorted at P0 as ENG+DIM or ENG+BRIGHT were analyzed they were observed to maintain expression of ENG. There were no significant differences in median fluorescent intensity at P2 between sorted and unsorted populations indicating that the density of ENG/cell was the same between groups after passaging (data not shown). Similarly, unsorted cells which were used as controls became primarily ENG+. When P2 cells obtained from sorted or unsorted P0 cells were seeded separately in 3D culture on membranes in the presence of TGFβ3 for 3 weeks, no significant differences in the amount of collagen or proteoglycan accumulated could be detected (Figure 3-5 B). Immunohistochemistry showed no differences between the groups with respect to expression of Col2, Col1, ColX and ACAN (Figure 3-5 C).
Figure 3-5: **P0 chondrocytes become ENG+ after serial passaging.** (A) P0 chondrocytes were sorted as either ENG-, ENG+DIM, or ENG+BRIGHT and passaged separately to P2. At P2 flow cytometry was used to assess expression of ENG in cells from each group. Cells that were ENG- became ENG+ after passaging in similar amounts to cells sorted as ENG+DIM, or ENG+BRIGHT, or unsorted cells. N=2 (B) Sulphated GAG and hydroxyproline content of 3 weeks formed tissues by P2 cells cultured on membranes with TGFβ3. Immunohistochemically...
stained sections of 3 week tissues with antibodies reactive with Aggrecan (ACAN, green), Collagen type 2 (Col2, green), Collagen type 1 (Col1, red), or ColX, (red). DAPI (blue) counterstain. Native cartilage was used as positive controls (top row). N=3

3.5 Discussion

In this study we demonstrated that following serial passage in monolayer culture, chondrocytes acquired from cartilage with high grade OA were able to form cartilage-like tissues similar to that developed by cells obtained from tissues with low grade OA changes. P0 cells from either grade OA were unable to form tissue in the presence of TGFβ3, and cell passaging was required for chondrocytes to be able to form tissue. This is unexpected as ALK5, which has been implicated in regulating chondrogenesis by chondrocytes, was present in the vast majority (< 95%) of P0 cells. Furthermore, the percentage of cells expressing ALK5 and the density of ALK5/cell did not significantly change after passaging, suggesting that additional factors or co-factors may be influencing the ability of P2 cells to form cartilaginous tissues in response to TGFβ. Evaluation of the co-receptors ENG and TGFβRIII showed that with cell passaging the cells all expressed ENG and that the level of expression per cell increased. In contrast TGFβRIII showed no change. This suggests that ENG may play an important role in regulating chondrocyte redifferentiation and acquisition of the ability to form cartilage tissue in the presence of TGFβ3.

ENG and TGFβRIII have both been shown to function to enhance TGFβ mediated signaling [30], [31]. In chondrocytes, Parker et al suggested that they may interact by forming a heteromeric complex [16]. Unlike ENG which binds TGFβ1 and TGFβ3 exclusively, TGFβRIII binds all 3 isoforms of TGFβ with highest affinity for TGFβ2 [18], [30]. TGFβ2 signals poorly in the absence of TGFβRIII due to its low affinity for the receptor complex, and thus the function of TGFβRIII has been shown to have the most pronounced effects for TGFβ2 mediated signaling [31]. In this study we showed that both primary and passaged chondrocytes had very few cells expressing TGFβRIII which is not surprising given that low amounts of TGFβ2 are present in adult normal and OA cartilage [32]. However, contrary to our findings, Parker et al. using the immortalized chondrocyte cell lines C-28/I2 and tsT/AC62 show abundant TGFβRIII expression by immunoprecipitation and SDS-PAGE [16]. It may be that the process of immortalization affects TGFβRIII expression levels. It is unclear why our results for TGFβRIII expression in P0
cells differ from those by Parker et al. Interestingly a recent study by Zheng et al. which used RNA interference to downregulate TGFβRIII in human MSCs showed that this significantly enhanced TGF-β3-induced chondrogenic differentiation [33]. Low levels of this co-receptor, as illustrated in our system, may also support chondrogenesis by passaged chondrocytes.

ENG, in contrast, significantly increased with serial passaging of chondrocytes, and this corresponded with an enhanced ability to form neocartilage in vitro in response to 3D culture in the presence of TGFβ3. Passaged chondrocytes have been shown to express ENG by others [34]–[36]. The ENG extracellular domain expresses the RGD integrin binding motif [37] and has been shown to play a role in cell adhesion, morphology and migration [38], [39] which may explain the increased expression of ENG with serial passaging in monolayer culture. To the best of our knowledge no study has been performed examining the regulatory role of ENG in modulating chondrogenesis by passaged articular chondrocytes. Numerous studies have demonstrated an enhanced chondrogenic potential for adipose and synovium derived stem cells enriched for ENG, more commonly referred to as CD105 in the stem cell literature [20], [21], [40]. In a study by Cleary et al. who were able to sort out CD105- passaged human MSCs from two donors, they demonstrated that these cells had a diminished capacity for attachment and proliferation and lacked the ability to form pellets during chondrogenic differentiation studies [41]. This would support our hypothesis that CD105 expression is important for chondrogenesis. Although their study suggested that the level of CD105 was not important, this was based on bulk analysis for CD105 positivity which could mask variable levels of expression per cell. Interestingly passaged chondrocytes in our study showed increased CD105 density/individual cell. Alternatively, it may not be that CD105 quantity alone does predicts chondrogenic potential but that the presence of ALK5, as we hypothesize for our system, is also needed to mediate chondrogenesis.

ENG enhances signaling by TGFβ mediated pathways by interacting with the TGFβ receptors ALK5 and ALK1 which signal via SMAD2/3 or SMAD1/5/8 respectively. TGFβ signaling through ALK5-SMAD2/3 in chondrocytes has been shown to be responsible for cartilage anabolic effect, while TGFβ signaling via ALK1-SMAD1/5/8 is associated with catabolism [42]. It should be noted that most of the P0 and P2 cells expressed ALK5. Evaluation of ALK1 was attempted but was not successful so no characterization could be performed (data not shown). Interestingly for primary chondrocytes, Finnson et al. demonstrated that ENG acted
to enhance signaling by ALK1 and SMAD1/5/8 and inhibited signaling via the ALK5 and SMAD2/3 pathway [36]. They also demonstrated that ENG increased with passaging, consistent with our findings, however they did not examine whether ENG still acted to differentially regulate ALK1 pathway signaling in after passaging.

When passaged the chondrocyte phenotype is altered and has been suggested to become more MSC-like, as shown by their ability to differentiate into adipocytes, osteocytes and chondrocytes [43], [44]. This may explain why only P2 chondrocytes formed hyaline-like cartilage tissues when treated with TGFβ3, as this growth factor is commonly used for chondrogenic differentiation of human MSCs [13], [45], [46] which express ENG and have been shown to differentiate via SMAD2/3 signaling [47]–[50]. Studies in other cell types have also shown ENG can act via ALK5-SMAD2 signaling [17] and can differentially regulate signaling in favor of ALK5 [51]. ENG may be acting in the P2 cells in our system in a similar manner to enhance signaling via ALK5-SMAD2/3 pathways which mediates hyaline cartilaginous tissue formation. This may also explain why grade specific differences in tissue formation were not seen, as both High OA and Low OA cells acquired a primarily ENG+ phenotype when passaged and contained similar levels of ALK5. Overall there are numerous studies to suggest ENG can enhance TGFβ signaling by both the ALK5 [17], [51] and ALK1 [36], [52], [53] receptors. It is probable that ENG plays multiple roles and acts in a context and tissue-specific manner, and the mechanism by which it may be acting in our system requires further investigation.

As passaged chondrocytes are FDA approved for use clinically, it is important to understand whether the OA phenotype of the tissue from which the cells are derived can influence the ability of the cells to form new cartilage. In this study we showed that the grade of OA did not affect the quality of hyaline cartilaginous tissues formed in vitro. Furthermore, we showed that the presence of ALK5 alone does not predict the ability of chondrocytes to form tissue in response to culture with TGFβ3, as both P0 and P2 cells contained ALK5 in equivalent abundance, however only P2 cells formed hyaline-like neocartilage. This may be due to upregulation of ENG with serial passaging, which has been shown by others to enhance TGFβ mediated signaling by ALK5 [17], [51]. Future studies are required to assess how increased expression of ENG as a result of serial passaging may influence tissue formation by human articular chondrocytes in response to culture with TGFβ.
Supplementary Figure 3-1: When passaged to P2 chondrocytes become larger and appear more homogeneous. (A) Brightfield images of 8 randomly selected chondrocytes acquired in flow from P0 and P2 cultures of Low OA and High OA. (B) Representative histograms of cell area show an increase in cell size when cells are passaged. N=3.
Supplementary Figure 3-2: **ENG+ sorted P0 chondrocytes proliferate more rapidly than ENG- sorted chondrocytes.** (A) Growth curve depicting cell counts at days 0, 1, 4, 7 and 14. (B) Table showing average values for cells counts ± SD (C) Brightfield images of cells on monolayer prior to counting. N=2

### 3.7 Supplementary Note

Unlike conventional flow cytometry, the ImageStreamX® platform and its attendant image analysis software (IDEAS®) allow complex image acquisition of large numbers of suspended objects “in flow” and their subsequent analysis. Multispectral/multimodal images are collected using a high-speed CCD camera with time delay integration to enhance sensitivity [ruben 23-25]. Each image pixel is assigned Cartesian coordinates and a grey-scale intensity value. IDEAS® houses a diverse selection of “features” which are mathematical expressions that can be applied to pixel intensity values within the mask definition. Organized into eight morphometric/photometric parameters (size, shape, texture, intensity, signal strength, comparison, system and location), the masks and features can either be implemented individually or in Boolean combinations to affect complex image analysis. Generated numerical values for
features can be plotted for all images to produce statistics within histograms or two-feature bivariate scatter plots. The IDEAS® software also links each data point on the graph to its corresponding image, allowing the user to visually inspect each cell to aid in the setting of regions of interest for generating statistics and gates for subset analysis.

The compensation matrix is a table of coefficients normalized to 1. Each coefficient represents the normalized amount of leakage of the fluorochrome from primary to secondary channels. The process of applying the compensation matrix to the raw image file creates a “compensated image file” to which downstream analysis can be performed (IDEAS reference manual).
3.8 References


Chapter 4
Re-differentiated Chondrocytes in Fibrin Gel for the Repair of Articular Cartilage Lesions


4.1 Abstract

Background: Autologous chondrocyte implantation (ACI) which uses passaged chondrocytes commonly leads to the formation of fibrocartilage. When chondrocytes are passaged to increase cell numbers, they lose their phenotype and the ability to form hyaline cartilage. The use of transforming growth factor β (TGFβ) to re-differentiate passaged chondrocytes has been validated in vitro, however it has not been established if re-differentiated chondrocytes will enhance defect repair when implanted in vivo. Furthermore, fibrin gel has shown potential in orthopaedic surgery as a fixative and scaffold and could be an appropriate carrier to enhance retention of cells in the repair site.

Purpose: To investigate if re-differentiated chondrocytes in fibrin gel will form cartilage tissue in vitro and if these re-differentiated cells will enhance the formation of hyaline cartilage when implanted into critical size osteochondral defects.

Study Design: Controlled Laboratory Study.

Methods: Rabbit and human chondrocytes were serially passaged twice (P2) in monolayer culture. P2 cells were used directly (de-differentiated) or re-differentiated in high density culture with TGFβ3. De-differentiated or re-differentiated cells were mixed with fibrin gel to form fibrin clots which were cultured in vitro to assess the use of fibrin gel as a scaffold, or implanted in vivo in a critical size osteochondral defect in NZW rabbit knee joints. Rabbits were sacrificed 6 weeks post-implantation and tissues were assessed histologically and by immunohistochemistry.
Results: TGFβ improved the formation of cartilaginous tissues by passaged chondrocytes in vitro, and culture in fibrin gel did not affect the cell phenotype. Implantation of de-differentiated cells in vivo resulted in fibrocartilaginous repair tissues. Re-differentiated chondrocyte implants resulted in granulation tissues containing the hyaline cartilage marker, collagen type 2.

Conclusions: Re-differentiated chondrocytes maintain their chondrogenic differentiation in fibrin clots. Although re-differentiated chondrocytes show a different reparative response than de-differentiated chondrocytes they do not appear to enhance repair at an early time point. Another study of longer duration is required to assess tissue maturation.

Clinical Relevance: Re-differentiation of passaged chondrocytes with TGFβ3 prior to implantation does not improve defect repair in the first 6 weeks.

4.2 Introduction

Articular cartilage (AC) has a very limited capacity for repair when damaged. Current therapies used clinically, include autologous chondrocyte implantation (ACI) and microfracture (MF). Clinical outcomes of ACI and MF show that in the majority of cases, fibrocartilage tissue is formed [1], [2]. This tissue contains a mixture of collagens type 1 (Col 1) and type 2 (Col 2) and is mechanically inferior to native AC rendering it more susceptible to wear and degradation, and ultimately leading to failure [3]. A recent meta-analysis study by DiBartola et al. which compared clinical and histological outcomes of MF to ACI demonstrated that only 8.2% of tissues assessed histologically from MF patients were hyaline cartilage, compared to 30.9% of tissues from ACI patients (generation 1, periosteal flap) [4]. This correlated with MF having worse clinical outcomes and unlike ACI, the repair tissues formed by MF showed no evidence of maturation to hyaline cartilage with time. Other studies have also demonstrated better clinical outcomes of ACI compared to MF [5]–[7] suggesting that repair strategies which utilize chondrocytes may be more effective than those which utilize mesenchymal stromal cells (MSCs) from the bone marrow. In support of this a study by Pelttari et al. demonstrated enhanced hyaline matrix accumulation and resistance to hypertrophy in cartilaginous tissue formed by human chondrocytes compared to human MSCs [8], and phenotype was maintained after in vivo transplantation. Attempts have been made to increase the success of ACI by using a collagen
type I/III membrane as a patch, or through matrix-induced autologous chondrocyte implantation (MACI), in which the cells are seeded on a collagen scaffold. However, studies comparing these techniques have not found a significantly increased repair tissue quality [4], [9]. Therefore, alternative strategies to increase the success of cartilage repair therapies remain to be determined.

One reason for the failure of current biological therapies is the limited differentiation of the cells within the defect [10]. In MF, MSCs from the bone marrow, which have not been differentiated and have multi-lineage potential, are assumed to be responsible for affecting this repair. In ACI chondrocytes are used, however due to the inherent low cellularity of AC autologous chondrocytes must be serially passaged to acquire enough cells. This process leads to de-differentiation and the cells lose the ability to form cartilage tissue [11]–[14]. In both cases, pre-differentiation to chondrocytes which have the ability to form cartilage may be an essential step to increasing the success of these therapies. One approach to inducing re-differentiation is to co-culture MSCs or de-differentiated chondrocytes with primary chondrocytes. This has been shown to successfully differentiate these cells to chondrocytes [15]–[17], however when it comes to clinical translation this is not a viable method as procuring a large number of healthy primary chondrocytes is not feasible.

Recently it has been shown that passaged human chondrocytes can be re-differentiated by culturing the cells at high density in a chemically defined serum-free media (SFM) supplemented with transforming-growth factor beta 3 (TGFβ3) [18]. Under these conditions, the cells formed a continuous layer of hyaline cartilage-like tissue which showed evidence of zonal organization in as little as 3 weeks. The tissues formed by TGFβ3 treated cells did not express markers of hypertrophy, unlike what has been shown for chondrocytes treated with other growth factors such as bone morphogenetic proteins [19], [20]. Furthermore, it was demonstrated that the tissues continue to accumulate significant amounts of Col2 without the need for continuous (more than 3 weeks of culture) treatment with TGFβ3. This information suggested that the re-differentiated phenotype of the cells was stable.

Thus, the goal of this study was to determine if re-differentiated chondrocytes grown in fibrin gel retain this phenotype in the absence of TGFβ and if these cells enhance early cartilage repair in a focal defect model. To this end we first demonstrated that both rabbit and human re-differentiated chondrocytes will form hyaline cartilage tissue in fibrin gel in vitro. Their efficacy
was then evaluated in a small cohort pilot study using New Zealand White (NZW) rabbits. These methods were utilized for three reasons. Firstly, critical sized osteochondral defects in the knee can be generated in these animals [21]. Secondly, Kang et al. demonstrated that chondrocytes from NZW rabbits de-differentiate when serially passaged in monolayer [13]. Thirdly, the first ACI study was performed in NZW rabbits prior to being translated clinically [22]. Fibrin gel was utilized as it has been shown by others to successfully maintain cells in full thickness chondral and osteochondral defects [23]–[26] and it has been used clinically for ACI [27].

4.3 Materials and Methods

4.3.1 Cell Isolation and Passaging: Rabbit

For in vitro experiments rabbit articular cartilage was isolated from the hind legs of 5 NZW rabbits which ranged in age from 9 months to 1 year. The cartilage was digested in 0.5% protease (Sigma, Canada, P5147) for 30 min and then 0.5% collagenase A (Sigma, Canada, 11088793001) was added to the protease containing solution (ratio of 1:3). Enzymatic solutions were made in Hams F12 media (Wisent Bioproducts, Canada, 318-011-CL). Tissues were incubated at 37°C and were agitated every 30 min until completely digested. The mixture was passed through a 100 µm cell filter, centrifuged at 600 rcf for 6 minutes and resuspended in Hams F12 media supplemented with 5% FBS. Cells were counted using a hemocytometer and plated at 2000 cells/cm² in monolayer culture. The cells were serially passaged to P2 (~9 population doublings). 1X Trypsin (Thermo-Fisher, Canada, 15090046) was used to detach cells at each passage.

4.3.2 Cell Isolation and Passaging: Human

Human articular cartilage was obtained from femoral condyles resected during knee arthroplasty surgery following informed patient consent and with approval from the Research Ethics Board. Three patient samples were used for this study, 2 females (age 65 and 82) and 1 male (age 64). Cartilage was taken from areas of osteoarthritic tissue which showed minimal
osteoarthritic changes macroscopically. Chondrocytes were isolated by enzymatic digestion and passaged in monolayer culture to P2 (9 population doublings) as described previously[18].

4.3.3 3D Membrane Culture for Re-differentiation

P2 de-differentiated cells (2 x 10^6) were seeded onto type II collagen coated Millicell® membrane culture inserts (12mm diameter; EMD Millipore, United States, PICM01250) and cultured in serum-free re-differentiation media (SFM) consisting of high glucose Dulbecco’s modified Eagle’s medium (DMEM; Wisent Bioproducts, Canada, 319-016-CL), 1% ITS+ (Wisent Bioproducts, Canada, 315-080-ZL), L-proline (40 µg/mL), sodium pyruvate (110 µg/mL), dexamethasone (0.1µM), and L-ascorbic acid (100 µg/mL). The media was supplemented with 10 ng/ml human recombinant TGFβ3 (R&D Systems, United States, 243-B3) after 24h unless otherwise specified.

4.3.4 In vitro fibrin clot formation and culture

Agarose wells were created by placing 3ml of autoclave sterilized molten agarose (4% in Ham’s F12; Sigma–Aldrich, United States) in a 12 well tissue culture dish. After the agarose had cooled and solidified a 4mm skin biopsy punch was used to generate a well. The wells were washed with PBS-/-/ prior to use.

P2 de-differentiated cells were seeded on membranes and treated with TGFβ3 for 3 weeks in SFM. Tissues were digested enzymatically to isolate the chondrocytes (now termed “re-differentiated”). Briefly, the in vitro formed tissues were digested with 0.5% protease for 30 min after which 0.5% collagenase was added to the solution (1:3 ratio). After complete digestion the mixture was passed through a 100 µm cell filter and centrifuged at 600 rcf for 6 min. Cells were counted using a haemocytometer. P2 de-differentiated cells were used directly following serial passaging in monolayer as controls. To encapsulate the cells in fibrin gel (Artiss; Baxter, Canada) and form a “fibrin clot”, 1.4 X 10^6 cells were resuspended in 10µl of the thrombin component and then mixed with 10µl of the fibrin component. The mixture was quickly pipetted into the 4mm diameter pre-generated agarose well and placed at 37°C for 3 min to facilitate clot
formation. Media was added to the cultures immediately after clotting. Fibrin clots were cultured for 3 weeks in SFM (without TGFβ). Media was changed 3 times per week.

4.3.5 Surgical Methods

Animal surgeries were performed with REB approval from the Animal Care Committee. Two surgeries were performed on the animals, the first to collect cartilage for isolation of autologous chondrocytes, and the second for implantation of autologous cells into generated defects. Animals were 4 months of age at the time of the first surgery. Three animals received P2 (de-differentiated) chondrocytes and three received re-differentiated chondrocytes in fibrin gel. One defect was created per animal at the time of the second surgery in the opposite femoral condyle to that from which the chondrocytes were harvested.

Surgery 1: Isolation of Autologous Chondrocytes: The knee was prepared by shaving the skin around the joint and disinfecting with two successive applications of ethanol followed by betadine. Rabbits were induced for anesthesia using ketamine (35 mg/kg) and xylazine (5 mg/kg) administered intramuscularly. Anesthesia was maintained with isoflurane. Anesthetized animals were placed in the supine position on the operating table. The patella was visualized via a medial longitudinal parapatellar incision, and then everted to expose the articular cartilage in the trochlear groove. A 4mm biopsy punch was used to create one defect in the articular cartilage which was removed from the bone with a scalpel blade. The excised articular cartilage was placed in F12 cell culture media until further processing. The patella was then reduced and closed with 5-0 vicryl interrupted sutures. Animals were allowed full ambulation.

Surgery 2: Implantation of Autologous P2 or Re-differentiated Chondrocytes: At the time of the second surgery animals were between 5-6 months of age. Histologically it has been shown that growth plate closure in the NZW rabbit distal femur occurs between 19-23 weeks of age[28] suggesting the rabbits used in this study had reached skeletal maturity. Fibrin clots containing either re-differentiated or de-differentiated P2 cells (1.4 X 10^6 cells/ clot) were formed as described above approximately 1 hour prior to surgery and maintained in F12 media until implanted. The animals were prepared for surgery and the trochlear groove was exposed as described above. A 4mm diameter biopsy punch was used to create a chondral defect in the
trochlear groove. Cartilage was removed with a scalpel blade and a hand drill with a 1.5 mm diameter burr was used to create an osteochondral defect 4mm wide and 1.5mm deep. By moving the burr laterally, a 4mm diameter defect was created. One fibrin clot was implanted per defect and additional fibrin gel (10 µl) was placed above and below the implant to facilitate fixation to the bone and adjacent native cartilage. Gentle pressure was applied to the implant during fixation. The joint was sutured as described above. Animals were sacrificed after 6 weeks.

4.3.6 Histology and Immunohistochemistry

In vitro formed tissues were fixed in 10% PBS buffered formalin (pH 7.4) overnight, and placed in a 30% sucrose/PBS solution overnight at 4°C. Tissues were then frozen in Tissue-Tek OCT frozen compound (Sakura Finetek, USA, 4583). Samples harvested from the rabbits and consisting of in vivo formed tissues and surrounding cartilage and bone were fixed in 10% PBS buffered formalin (pH 7.4) for 7 days then decalcified in a 0.5M EDTA pH 7.2 solution for 2 weeks. Solutions were changed 3 times per week. After decalcification, tissues were placed in a 30% sucrose/PBS solution overnight at 4°C and subsequently snap-frozen in Tissue-Tek OCT. Blocks were sectioned at 7 μm and tape-stabilized cryosectioning was used to help adhere the sections to the slides[29]. Tissue sections were stained with haematoxylin and eosin or toluidine blue, and mounted with Micromount (Leica Biosystems, Canada, 3801731) and coverslipped. One representative section of tissue from each defect was scored using the ICRS II scoring system [30] and the total score for each of the parameters for 3 defects was averaged.

For immunohistochemistry, sections were digested with 2.5mg/ml pepsin, followed by 2.5 mg/ml trypsin and 25 mg/ml hyaluronidase and then blocked with 20% goat serum. Sections were incubated overnight at 4°C with antibody reactive with either type I collagen (Rabbit, 1:500, Ab90395 Abcam, Canada; Human, 1:250 CL-50111AP-1 Cedarlane, Canada), or type II collagen (Rabbit and Human, 1:500, MAB8887, EMD Millipore, Canada). For aggrecan staining no antigen retrieval was used (Rabbit and Human, 1:500 AHP0022, ThermoFisher Scientific, Canada). To visualize immunoreactivity the tissues were incubated with either Alexa-594 goat anti-rabbit IgG (1:1000, A-11012), Alexa-488 goat anti-mouse IgG (1:1000, A-11001), or Alexa-594 goat anti-mouse IgG (1:1000, A-11005 all from Life Technologies, Canada) secondary antibody. Tissues were counterstained with DAPI (1μg/ml). Negative controls consisted of
replacing the primary antibody with isoform and species matched antibody at the same concentration. Positive controls consisted of native cartilage or bone.

4.3.7 Fluorescence Quantification

Fluorescence intensity was quantified using FIJI (ImageJ) software. Multi-coloured RGB images were separated into single channels and converted to grayscale. A rectangular region of interest (ROI) was created and all images from the same experiment were analyzed using the same size ROI. The total ROI pixel intensity was measured and was divided by the total number of pixels in the ROI to obtain average pixel intensity and graphed as arbitrary units (A.U.). For analysis of the repair tissues formed in vivo, 3 images from 3 different histological sections were used per defect. For analysis of in vitro formed tissues 1 image from 3 different histological sections was used.

4.3.8 RNA extraction and RT-PCR

Total RNA was extracted from native rabbit articular cartilage, passaged (P2) or re-differentiated cells using TRIzol reagent (Thermo Fisher Scientific, Canada, 15596026) and reverse transcribed to cDNA using Superscript II Reverse Transcriptase (Invitrogen, Canada, 18064014) according to the manufacturer's instruction. Relative real-time PCR based on SYBR Green (Invitrogen) was performed using primers specific for 18S rRNA, ACAN, COL1, COL2, PRG4, COLX, and RUNX2 (Supplementary Table 1). Each reaction contained 40 ng of cDNA, 6 μL of SYBR Green master mix, and 1 μL each of forward and reverse primers (10 mM). Absolute quantification was performed using the Light Cycler 96 SW 1.1 software and mRNA levels were determined from Cq values according to Pfaffl’s mathematical model for relative real-time PCR[28]. 18S rRNA was used for normalization.
4.3.9 Tissue Content Analysis

In vitro formed tissues were digested with papain (40 µg/mL; Sigma-Aldrich) in digestion buffer containing 20 mmol/L ammonium acetate, 1 mmol/L-EDTA and 2 mmol DTT at pH 6.2 for 48 hours at 65°C. Hydroxyproline content was quantified using the chloramine-T/Ehrlich’s reagent assay [31] and spectrophotometry ($\lambda= 560$ nm) upon HCl hydrolysis of the papain digest. L-hydroxyproline (Sigma-Aldrich) was used to generate the standard curve. Sulfated glycosaminoglycan content of papain digested samples was quantified by dimethylmethylene blue dye binding assay [32] and spectrophotometry ($\lambda= 525$ nm). Chondroitin sulphate (Sigma-Aldrich) was used to generate the standard curve. DNA content was quantified by the Hoechst 33258 dye binding assay [33] and fluorometry (excitation $\lambda= 365$ nm, emission $\lambda= 458$ nm). The DNA standard curve is generated using calf thymus DNA (Sigma-Aldrich).

4.3.10 Statistics

Three independent experiments were performed using cells from 3 different animals (N=3) and all in vitro conditions were done in triplicate (n=9). The data was pooled, expressed as mean ± SEM and analyzed using a paired t-test, Mann-Whitney U, or one-way ANOVA with Tukey’s Post Hoc. Significance was assigned at p< 0.05.

4.4 Results

4.4.1 TGFβ3 promotes re-differentiation and enhances hyaline cartilage tissue formation by rabbit passaged chondrocytes in vitro.

Chondrocytes passaged twice (P2) in monolayer culture acquired an elongated morphology, characteristic of a de-differentiated phenotype compared to P0 cells (Figure 4-1 A). To assess whether TGFβ3 stimulates chondrogenic gene expression in passaged rabbit chondrocytes mRNA levels were evaluated in P2 cells grown at high density (3D) on membrane cultures in SFM in the presence of 10ng/ml TGFβ3 and compared to P2 cells prior to seeding in 3D culture and to P0 cells. Gene expression (Figure 4-1 B) demonstrated that TGFβ3
significantly upregulated expression of the hyaline matrix molecules Col 2 and aggrecan (ACAN) compared to P2 and P0 cells. Proteoglycan-4 (PRG4), although more highly expressed in P2+TGFβ3 treated cells compared to P2, did not reach levels of P0 cells. P2 cells showed significantly upregulated expression of Col 1, a marker of de-differentiation, and runt-related transcription factor 2 (RUNX2), a marker of hypertrophy compared to TGFβ3 treated cells. Collagen type 10 (COLX) expression was not significantly different in P2 and P2+TGFβ3, but was significantly lower than those in P0 cells.

To determine whether 3D culture or TGFβ3 was responsible for chondrogenic redifferentiation and cartilage tissue formation, P2 cells were seeded on membranes and cultured in SFM for 3 weeks with (+TGFβ3) or without (control; -TGFβ3) TGFβ3. Cells cultured with TGFβ3 formed a continuous layer of tissue (Figure 4-2 A) which appeared thicker than tissues formed by P2 cells (Figure 4-2 B) and stained strongly for proteoglycans as visualized with Toluidine Blue staining (Figure 4-2 C). Immunohistochemical analysis (Figure 4-2 D) showed that when treated with TGFβ3 tissues stained strongly for Col 2 and ACAN. Neither group accumulated Col 1. Biochemical quantification confirmed that cells grown in the presence of TGFβ3 accumulated significantly more sulphated proteoglycans and collagen compared to cells cultured without the addition of TGFβ3 (Figure 4-2 E). Overall these results demonstrate that with serial passaging, P2 cells acquire a de-differentiated phenotype which was reversed when grown in 3D culture in SFM with TGFβ3.
Figure 4-1: TGFβ3 modulates chondrogenic gene expression. (1A) Phase contrast microscopy of primary (P0) or twice passaged (P2) chondrocytes in monolayer culture. (1B) Gene expression analysis of primary (P0), passaged (P2) or re-differentiated (P2+TGFβ) chondrocytes.
**Figure 4-2: TGFβ3 enhances hyaline-like cartilage tissue formation by passaged rabbit chondrocytes.** P2 chondrocytes were cultured on membrane inserts for 3 weeks in SFM in the presence or absence of TGFβ3. (2A) Gross morphology of tissues. Histological sections of 3 week formed tissues stained with (2B) haematoxylin and eosin, (2C) toluidine blue or (2D) immunohistochemically (ACAN: green. Col2: green. Col1: red) with DAPI (nuclear) counterstain. (2E) Sulphated glycosaminoglycan (GAG) and hydroxyproline contents in 3 week formed tissues. N=3.

**4.4.2 Re-differentiated chondrocytes show enhanced hyaline cartilage-like tissue formation when cultured in fibrin clots in vitro.**

To assess if re-differentiated cells would maintain their phenotype and form hyaline cartilage-like tissue when embedded in fibrin clots, cells re-differentiated for 3 weeks in 3D culture with TGFβ3 and subsequently isolated from the formed tissues were mixed with fibrin gel and placed in a 4mm agarose well (Figure 4-3 A). As a control P2 cells which were not re-differentiated were also mixed with fibrin gel to form clots (de-differentiated). The fibrin clots were cultured for 3 weeks in SFM without TGFβ3. Upon harvest, the macroscopic appearance of the fibrin clots did not show obvious differences between the two groups (Figure 4-3 B) with respect to opacity, therefore tissues were assessed by histology and immunohistochemistry.
Figure 4-3 C and 4-3 D. Histologically, tissues formed in fibrin clots containing re-differentiated chondrocytes appeared thicker and more uniform (Figure 4-3 C) and appeared to stain more strongly for proteoglycans with Toluidine Blue (Figure 4-3 D) than the fibrin clots containing P2 cells. Immunohistochemistry (Figure 4-3 E) showed that tissues formed by re-differentiated chondrocytes have greater accumulation of Col 2 and ACAN than the tissues formed by P2 cells which was confirmed with quantification of fluorescent intensity in 3 biological replicates (Figure 4-3 F). Neither group showed strong immunohistochemical staining for Col 1. The average ratio of Col 2/Col 1 was greater for tissues formed by re-differentiated cells (5.3) compared to de-differentiated cells (2.4).

Figure 4-3: Re-differentiated rabbit chondrocytes maintain their chondrogenic phenotype when cultured in fibrin clots in vitro. P2 de-differentiated or re-differentiated chondrocytes were cultured in fibrin clots in SFM for 3 weeks without TGFβ3. (3A) Appearance of as made agarose well, with or without cell-containing fibrin clot. (3B) Gross morphology of fibrin clots. (3 C-D) Histological sections of the tissues stained with (3C) haematoxylin and eosin, (3D) toluidine blue or (3E) immunohistochemically (ACAN: green. Col2: green. Col1: red.). DAPI
To determine whether re-differentiated human chondrocytes would also form hyaline-like cartilage when grown in fibrin clots in the absence of TGFβ3, the same experiment was conducted using P2 human chondrocytes (Figure 4-4). After 3 weeks of culture in vitro, fibrin clots containing re-differentiated cells appeared larger than fibrin clots containing de-differentiated cells (Figure 4-4 A). This was confirmed in the histological sections of the tissues (Figure 4-4 B and C). When assessed via immunohistochemistry, fibrin clots containing de-differentiated chondrocytes accumulated ACAN and the fibrocartilage marker Col 1 (Figure 4-4 D). In contrast, re-differentiated chondrocytes accumulated ACAN and Col 2, and did not accumulate Col1. Quantification of the fluorescent intensity for histological sections stained for Col 2, Col 1, and ACAN confirmed enhanced hyaline-like cartilaginous tissue formation, as re-differentiated cells accumulated significantly more Col 2, and significantly less Col 1 than fibrin clots formed with de-differentiated cells (Figure 4-4 E). The average ratio of Col 2/Col 1 was greater for re-differentiated cell containing fibrin clots (1.8) compared to de-differentiated (0.6).

**Figure 4-4:** Re-differentiated human chondrocytes accumulate more hyaline-like matrix compared to de-differentiated chondrocytes when cultured in fibrin clots in vitro. P2 de-differentiated or re-differentiated human chondrocytes were cultured in fibrin clots in SFM for 3 weeks without TGFβ3. (4A) Gross morphology of fibrin clots after 3 weeks of culture. (4B-D) Histological sections of the tissues stained with (3B) haematoxylin and eosin, (4C) toluidine blue (nuclear) counterstain. (4F) Average fluorescent intensity for histological sections stained for Col 2, Col 1, and ACAN. Results are expressed as mean ±SEM. N=3.
or (4D) immunohistochemically (ACAN: green. Col2: green. Col1: red.). DAPI (nuclear) counterstain. (4E) Average fluorescent intensity for histological sections stained for Col 2, Col 1, and ACAN expressed as mean ±SEM. *=p < 0.05. N=3.

4.4.3 Distinct tissue and cellular morphology was observed in repair tissues formed by P2 cells as compared to re-differentiated cells at 6 weeks post-implantation in osteochondral defects.

Based on the findings that re-differentiated rabbit and human chondrocytes maintained their hyaline-like chondrogenic phenotype in fibrin clots in the absence of TGFβ, they were evaluated for their ability to repair a cartilage defect in a small pilot study in vivo. In initial experiments the fibrin-cell suspension was injected and clotted in a 4mm diameter full thickness chondral defect. This method proved challenging due to the small volume of gel required to fill the defect and leakage of the fibrin-cell suspension out of the defect prior to clot formation. After 6 weeks in vivo histological assessment demonstrated that no repair tissue was present within the defect and no spontaneous repair occurred (Supplementary Figure 4-1). This confirmed that a critical sized defect had been utilized. As a result of these findings, in a subsequent experiment pre-made cell-containing fibrin clots were implanted in an osteochondral defect (Figure 4-5 A) using a method published previously for fibrin clot implants containing MSCs [24].

Repair tissue filled the defect site in all animals 6 weeks post implantation (Figure 4-5 B). The repair tissues which formed in the de-differentiated cell containing implants appeared fibrocartilaginous and stained strongly for proteoglycans with toluidine blue. In contrast, repair tissues in the re-differentiated cell implants did not stain as strongly for proteoglycans and appeared fibrous in areas.
Figure 4-5: Histology of repair tissues 6 weeks following implantation with pre-formed fibrin clots containing either de-differentiated or re-differentiated autologous rabbit chondrocytes. (5A) Schematic illustrating experimental design. (5B) Toluidine blue staining of histological sections of the repair tissues and subchondral bone (r=repair tissue b= bone). Each image represents a separate defect. Inset shows macroscopic images of the defect after harvest.

Interestingly the morphology of the cells in the repair tissue was strikingly different between the two groups. De-differentiated chondrocyte implants contained cells that were large and hypertrophic-like in morphology, while the cells in the re-differentiated chondrocyte implants contained cells that were smaller and round or elongated (Figure 4-6). Furthermore, repair tissues from the de-differentiated cell implants showed little accumulation of proteoglycan in the upper most regions adjacent to the articulating surface. In the re-differentiated cell...
implants granulation tissues was present in all the animals in the deep aspect of the repair tissues adjacent to the subchondral bone. This was not observed in animals which received de-differentiated cell-containing implants indicating a completely different reparative response occurred between the two groups. Histological sections of repair tissues from each implant were graded using the ICRS-II scale [30] and averaged. Repair tissues from the de-differentiated cell-containing implants had an average score of 41.7± 2.7 while repair tissues from the re-differentiated cell-containing implants had an average score of 29.3 ± 1.9. Scores for each individual parameter for each animal are listed in Supplemental Figure 4-2. A score of 100 indicates normal hyaline cartilage.

Figure 4-6: Repair tissues show different morphologies. Histological sections of formalin fixed and decalcified tissue stained with toluidine blue. Top = adjacent to the articulating surface. Bottom = adjacent to the subchondral bone. De-differentiated cell fibrin clot implants generate fibrocartilage and show little proteoglycan staining at the top of the tissue. Re-differentiated cell fibrin clot implants generate granulation tissue adjacent to the subchondral bone. Each image depicts repair tissue from a separate defect. All images were taken at the same magnification.
Immunohistochemical staining demonstrated that the repair tissues from the de-differentiated cell implants resembled fibrocartilage as they contained both Col 1 and Col 2 as well as ACAN (Figure 4-7 A). Interestingly, despite being vascularized and appearing histologically similar to granulation tissue, repair tissues from the re-differentiated cell implants stained positively for the hyaline markers, Col 2 and ACAN in addition to Col 1. Quantification of fluorescent intensity demonstrated that the fibrocartilage formed by de-differentiated cell-containing implants accumulated significantly more Col 2 and ACAN than the granulation tissues formed by the implants which contained re-differentiated cells. Furthermore, the average ratio of Col 2/Col 1 was greater for tissues formed by de-differentiated cells (2.8) than for tissues formed by re-differentiated cells (2.1).

**Figure 4-7: Immunohistochemical staining of the repair tissues present at 6 weeks post-implantation.** (7A) Histological sections of repair tissues were stained with antibodies reactive with either ACAN (green), Col 2 (green) or Col 1 (red). DAPI (nuclear) counterstain. Each column represents a separate defect. (7B) Average fluorescent intensity for histological sections stained for Col 2, Col 1, and ACAN. * = p< 0.05. Results are expressed as mean ±SEM. N=3.

**4.5 Discussion**

Chondrocytes, when passaged in monolayer culture, lose their phenotype and ability to form hyaline cartilage [11], [12], [14], [34]. In this study we demonstrated that passaged rabbit chondrocytes can be re-differentiated and form cartilaginous tissue when grown in 3D culture in the presence of TGFβ3 for 3 weeks. This phenotype is stable as chondrocytes can be isolated
from this tissue and re-cultured at high cell density in fibrin clots in the absence of TGFβ3 and the re-differentiation was maintained. Passaged human chondrocytes obtained from osteoarthritic cartilage responded similarly suggesting that this approach could be used clinically. Interestingly for human chondrocytes re-differentiation was essential to significantly decrease Col 1 accumulation and significantly increase accumulation of Col 2. Future studies are required to determine if chondrocytes from non-diseased cartilage show the same response. Unfortunately, the use of re-differentiated rabbit cells did not enhance cartilage repair in the short term (6 weeks) following implantation into osteochondral defects in vivo. Interestingly, implantation of de-differentiated cells resulted in fibrocartilaginous repair, while the defects contained granulation tissue when re-differentiated cells were utilized. It is not known why re-differentiated cell-containing fibrin clot implants resulted in granulation tissue repair. TGFβ is known to play a role in promoting angiogenesis in other tissue types [35], [36]. It is possible that this growth factor was being released by these cells as chondrocytes can produce TGFβ [37], or alternatively there may have been release of TGFβ that had stuck non-specifically during re-differentiation culture prior to implantation. As this was a pilot study, a larger study utilizing more animals is required to confirm these findings.

When fibrin clots were formed within a chondral defect at the time of surgery, no repair tissue was present (Supplementary Figure 1). Although only speculative, it is possible that the fibrin clots may not have anchored well to the underlying bone and adjacent native cartilage and dislodged with articulation. As rabbit cartilage is thin, the next experiments were therefore conducted using osteochondral defects which were deeper and allowed for a larger volume of fibrin gel to be used. Additionally, the fibrin clots were formed prior to implantation to prevent leakage of the cell-fibrin suspension out of the defect during clotting. With this strategy repair tissue was present in all the defects. One limitation of this study is that the cells were not pre-labelled prior to implantation, and therefore it is not possible to say with certainty that the repair tissues were formed by the implanted cells. However as the repair tissue that formed in the control implants was fibrocartilage, a common outcome for ACI which uses passaged chondrocytes [1], [38], which would suggest that the cells were retained in the defect.

The presence of Col 2 and ACAN in the granulation tissue was unexpected as this tissue is primarily composed of collagen types 1 and 3 [39]. This raises the possibility that despite the presence of granulation tissue it may have the potential to form hyaline cartilage. Interestingly,
multipotent MSCs are can be found in a perivascular location [40], [41]. A recent study by Dickinson et al. identified a novel population of bone marrow derived MSCs with an enhanced capacity for cartilage tissue formation [42] and demonstrated that these MSCs were present surrounding blood vessels. It is possible that such progenitor cells are present in the vascularized repair tissues may explain why the granulation tissue in our study contained Col 2. Further to this point, one study which examined the early events in cartilage repair after microfracture showed that the initial repair consisted of granulation tissue, which matured into cartilage with time [43]. As only a short term study was performed with the recommendation of the International Cartilage Repair Society regarding an early time point analysis for screening of new cartilage repair therapies in rabbits [44], [45], assessment at a later time is required to determine what happens to the repair tissues over time.

In conclusion re-differentiation of passaged rabbit and human articular chondrocytes, when grown in the presence of TGFβ3, results in the reacquisition of a hyaline cartilage-forming capability. This phenotype was stable as when the cells were isolated from these tissues, embedded in fibrin gel, and cultured in vitro in the absence of TGFβ3 the redifferentiated phenotype was maintained. In a short-term small pilot pre-clinical study, it was observed that the defects which received implants composed of re-differentiated chondrocytes formed granulation tissue containing Col 2 and ACAN 6 weeks post-implantation. In contrast those defects filled with de-differentiated cell implants formed fibrocartilage. Additional studies utilizing a larger number of animals with longer follow up is required to determine whether the use of redifferentiated passaged chondrocytes will lead to hyaline cartilage tissue repair.

4.6 Author Contributions

Vanessa J. Bianchi contributed to study design, the acquisition of both rabbit and human tissue samples, cell isolation and in vitro cell culture, biochemical assays, immunohistochemistry and histology, fluorescence and light microscopy imaging, data analysis, figure preparation, writing and editing of the manuscript.

Adrienne Lee contributed to the design of in vivo experiments, performed surgery on the animals used in this study and contributed to editing of the manuscript.
Jesse Anderson contributed to the design of in vivo experiments, performed surgery on the animals used in this study and contributed to editing of the manuscript.

Justin Parreno contributed to study design, performed quantification analysis on fluorescent images and contributed to writing and editing of the manuscript.

John Theodoropoulos contributed to the design of in vivo experiments and contributed to editing of the manuscript.

David Backstein contributed to the acquisition of the human tissue samples used in this study and editing of the manuscript.

Rita Kandel contributed to study design, data analysis, writing and editing of the manuscript.
4.7 Supplementary Figures

**Supplementary Figure 4-1**: Rabbit defects show no repair 6 weeks following injection of a cell-fibrin gel suspension into the chondral defect. (A) Schematic illustration of methodology used. (B) Macroscopic appearance (inset) and histological sections of formalin fixed and decalcified defects of untreated and cell-fibrin filled defects 6 weeks after surgery stained with (toluidine blue). n=native cartilage, b=bone.
### 4.8 Supplementary Tables

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Reverse (5'-3'): CCATCCAAATCGGTAGTAGCG | 60 | 107.9 |
| RUNX2 | Forward (5'-3'): TCCGAAATGCGCTCTGCTGTATG  
Reverse (5'-3'): CAAGGTGAAACTCTTTTGAGTGT | 60 | 106.2 |
| COL1 | Forward (5'-3'): CGATGGCCCTCGCGTTCACTG  
Reverse (5'-3'): GCTACGCTTTCTGTGCTAGT | 60 | 103.7 |
| PRG4 | Forward (5'-3'): TCGAGACACAGAAACCGTCG  
Reverse (5'-3'): GGCCTGGGGATCACATAAGG | 60 | 102.3 |
| ACAN | Forward (5'-3'): ACTGTGACATAGACCAGGAGGT  
Reverse (5'-3'): GATGCTGCTCAGGTTGAGT | 60 | 104.2 |
| COLX | Forward (5'-3'): CCTAGGGATTTCCACCGAGC  
Reverse (5'-3'): TCTGGGAGGTGAATGGAGT | 60 | 102.9 |
| COL2 | Forward (5'-3'): TGCAGGAGGAGGAGGAGGTAT  
Reverse (5'-3'): GGCAGTCCTTGAGGTGTTTCA | 60 | 124.3 |

Supplementary Table 4-1: Rabbit PCR primers.
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Supplementary Table 4-2: ICRS-II grading of histological sections of repair tissues. Individual scores for each parameter are listed for 3 biological replicates.
4.9 References


5.1 Summary

Objective

Articular cartilage (AC) has a limited capacity to repair when damaged. Current cell-based therapies are unable to fully repair the damaged tissue or replicate the zonal organization of AC, which may be essential for the long-term success of the treatment. Native AC is composed of hyaline cartilage which anchors to the underlying subchondral bone via a zone of calcified cartilage (ZCC). Bioengineering an osteochondral-like construct containing hyaline-like cartilage with a ZCC situated on a bone material is one method which may be used to facilitate repair. Mesenchymal stromal cells (MSCs) derived from bone marrow are one cell source which could be used for this purpose. MSCs can be differentiated to chondrocytes when cultured with transforming growth factor beta (TGFβ), and these cells have been suggested to be prone to hypertrophic differentiation making them suitable for use to form both hyaline-like cartilage and a ZCC. The conditions to induce hypertrophic differentiation and mineralization by chondrogenically differentiated human MSCs have yet to be fully elucidated however; studies have suggested that activation of bone morphogenetic protein (BMP) pathways either through culture with BMP2, thyroid hormones, or the small molecule FK506, can induce this phenotype. Therefore, we hypothesize that biphasic articular cartilage-like constructs can be generated through a twofold cell seeding approach in which chondrogenic differentiation of human MSCs is performed with TGFβ3, and hypertrophic differentiation via activation of BMP signalling pathways.

Methods

MSCs isolated from human bone marrow aspirates were passaged at 5000 cells/cm² and used for experiments between P2-P5. To facilitate chondrogenic differentiation cells were seeded at a density of 1-2 x 10⁶ cells/membrane and cultured in a serum-free chondrogenic media (SFM)
containing 10ng/ml TGFβ3 for 2-3 weeks. To test conditions which may subsequently induce hypertrophic differentiation, media supplementation with TGFβ3 was ceased and SFM was supplemented with either 10mM beta-glycerophosphate (β-GP) alone or in combination with 1μM FK506, 0.1μg/ml BMP-2, 30nM Triiodothyronine (T3) or 50nM L-Thyroxine (T4). To generate biphasic constructs, 1 x 10^6 cells were seeded per membrane, pre-differentiated to chondrocytes for 2 weeks, and subsequently differentiated to hypertrophic chondrocytes via culture with 1μM FK506 for an additional 3 weeks. MSCs from the same joint sample which were not induced to hypertrophy and maintained in chondrogenic differentiation media for 5 weeks were isolated from the formed tissues and seeded on top of the calcified cartilage layer at 2 x 10^6 cells/membrane. Bi-layered constructs were cultured for 2 weeks in SFM with 10mM β-GP. Tissues were assessed with histology and immunohistochemistry.

Results

When cultured under chondrogenic differentiation conditions for 3 weeks, MSCs formed cartilaginous tissues which accumulated the hyaline cartilage extracellular matrix proteins collagen type 2 (COL2) and aggrecan (ACAN). Collagen type 10 (COLX), a marker of hypertrophy located predominantly in the native ZCC, was also accumulated in the in vitro formed tissues. Collagen type 1 (COL1), a component of fibrocartilage, was not accumulated. When the chondrogenically pre-differentiated cells were cultured in hypertrophic differentiation conditions with β-GP alone, the tissues did not mineralize. When cultured with β-GP in addition to T3, T4, BMP-2 or FK506 the cells formed a layer of calcified cartilage however; none of these approaches worked reliably. Bilayered constructs showed a localized ZCC which stained positively for alkaline phosphatase and for mineral deposition. This zone accumulated COL2 and ACAN as well as COL1 and COL10. The uncalcified layer stained for the hyaline cartilage markers COL2 and ACAN. Unlike in the calcified layer, COL1 was not accumulated, nor was COLX as readily accumulated in this hyaline-like zone.

Conclusions

Activation of BMP signalling pathways in chondrogenically pre-differentiated human MSCs can be used to induce hypertrophic differentiation and the formation of mineralized cartilage. Furthermore, human MSCs not further induced to hypertrophy can be seeded on top of this mineralized tissue and be used to form a layer of hyaline-like cartilage. Variability among
human MSC populations poses a limitation to the use of these cells, as we found that reliable hypertrophic differentiation was not achievable with any of the methods tested. Future studies on methods which may be used to promote consistent differentiation need to be further investigated.

5.2 Introduction

Articular cartilage (AC) is inherently limited in its ability to fully repair when damaged. In the majority of cases the cell-based therapies used clinically which aim to repair hyaline cartilage result in the formation of fibrocartilage tissue [1]–[4] which lacks the structure and mechanical properties of AC [5]. Ultimately regardless of whether the AC is damaged via injury or disease, the final course of treatment is joint replacement with a prosthetic. Although prosthetics can be effective at alleviating pain in some patients they have a limited lifespan, with the mean time to failure for knee arthroplasty reported to occur in as little as 5.9 years [6]. Thus prosthetics are not an ideal treatment for young patients as they are more likely to require revision surgery in a lifetime [7]. Bioengineering an AC construct which recapitulates the native composition and architecture, and restores proper joint function, may provide a lifelong solution.

AC is composed only of chondrocytes and is classically divided into 4 zones, the superficial zone (SZ) the middle zone (MZ) the deep zone (DZ) and a zone of calcified cartilage (ZCC). The first 3 zones closest to the articulating surface are composed primarily of collagen type 2 (COL2) and the proteoglycan aggrecan (ACAN), with differential expression of other molecules, for example proteoglycan-4 (PRG4) is expressed predominantly in the SZ [8], [9]. The ZCC is a specialized region adjacent to the bone which contains chondrocytes that express collagen type 10 (COLX) and alkaline phosphatase (ALP) and have the ability to mineralize the surrounding matrix. This zone is of an intermediate stiffness between the AC and bone and acts to anchor these two tissues together and transmit forces and mechanical signals [10]. Studies of bioengineered osteochondral-like constructs have illustrated that incorporating a ZCC increases the compressive properties and interfacial shear strength of in vitro formed cartilage with the underlying substrate [11]–[13]. Designing constructs with this interface may be critical for their clinical success as it could mitigate potential shearing and dislodgement of the implanted tissue when exposed to the biomechanical stresses associated with weight bearing and joint articulation.
Various methods and cell sources have been utilized to bioengineer hyaline cartilage with a mineralized zone. By selectively isolating chondrocytes from the DZ (bottom 30%) of bovine AC, St-Pierre et al. created an osteochondral-like construct with a mineralized zone integrated to the top surface of a bone substitute [11]. Although the use of primary deep zone chondrocytes may be effective at generating mineralized cartilage, when translating these studies clinically the availability of human DZ cells is limited. Monolayer culture which is used to increase the number of cells available is associated with loss of phenotype [14], [15]. Thus the majority of studies have utilized mesenchymal stromal cells (MSCs) as they can be differentiated to hypertrophic chondrocytes [16], and form bone or cartilage tissue. One concern with the use of MSCs for generating an unmineralized layer of hyaline cartilage is their potential to mineralize in vivo [17]. However multiple studies have shown that MSCs can resist hypertrophic differentiation and mineralization long term when implanted in a joint cavity, particularly if pre-differentiated prior to implantation [18]–[20]. Chondrogenic differentiation of MSCs has been shown to be reliably achievable through high density culture in a chemically defined serum free media supplemented with dexamethasone and transforming growth factor beta (TGFβ) [21], [22].

To push chondrogenically pre-differentiated MSCs to a hypertrophic phenotype a variety of factors have been reported. Media supplementation with the thyroid hormones L-thyroxine (T4) or Triiodothyronine (T3) have been shown to push MSCs to mineralize their matrix [21], [23], [24]. Bone morphogenic proteins (BMP) have also been used to activate hypertrophic pathways in MSCs [25], [26] and may act to induce mineralization through upregulation of alkaline phosphatase (ALP) [27]; an enzyme which acts to make phosphate ions available for biomineralization. The small molecule FK506 (also known as Tacrolimus) has been shown to activate the BMP pathway in MSCs in the absence of BMP protein [28] and may be a safer option than using BMPs for clinical applications [29]. To the best of our knowledge FK506 has not been tested for its ability to induce mineralization by chondrocytes differentiated from MSCs. We hypothesize that hyaline-like cartilage tissues with a ZCC can be generated via a sequential cell seeding approach and treatment of chondrogenically pre-differentiated MSCs with thyroid hormones, BMP-2 or FK506.
5.3 Methods

5.3.1 MSC Isolation and Monolayer Culture

MSCs were isolated from bone marrow aspirates as described by Juneja S et al. [30] and generously donated as cryopreserved cells between passage 1-3. Briefly, mononuclear cells (MNCs) were isolated using Ficoll Paque PLUS medium and centrifugation. MSCs were isolated from the MNC fraction via plastic adherence. Colonies appeared 24 hours after plating. Cells were cultured until flasks were 70-80% confluent at which point cells were either cryopreserved or subsequently passaged. For our experiments, cryopreserved cells were thawed at 37°C and further passaged in monolayer culture (up to a maximum of P5) in alpha-MEM media (with deoxyribonucleosides) supplemented with 10% fetal bovine serum, 1X GlutaMAX-I (Gibco; 35050061), 1 ng/ml fibroblast growth factor-2, 1 ng/ml Heparin (Heparin Leo), and 50 ug/ml L-Ascorbic-Acid. Cells were detached with 10X TrypLE select (Thermofisher Scientific; A1217701), collected via centrifugation at 500g for 5 min, and resuspended in culture media for counting. Cells were seeded at a density of 5000cells/cm² for serial passaging. Media was changed 3 times per week. Cells were utilized at 90-100% confluency for subsequent experiments.

5.3.2 Chondrogenic Differentiation

Prior to use, membranes (12 mm diameter; EMD Millipore) were coated with 100μl of Col2 solution (0.05 mg/ml Col 2 dissolved in 0.01% Acetic Acid) and were dried overnight under sterile conditions. Membranes were washed with PBS -/- immediately before use. MSCs (2 X 10⁶) were seeded onto membranes in alpha-MEM media supplemented with 10% FBS and 10uM rock inhibitor (ROCKi, 688001, EMD Millipore) for the first 24hours then switched to serum free media (SFM) consisting of high glucose Dulbecco’s modified Eagle’s medium (DMEM; 4.5 g/ L), 1% ITS+ (BD Bioscience, MA), L-proline (40 ug/mL), sodium pyruvate (110 ug/mL), dexamethasone (0.1μM), and L-ascorbic acid (100 ug/mL) for the remainder of the culture period. ROCKi was kept in the culture media for the first 72 hours to prevent contraction. For chondrogenic differentiation 10 ng/ml human recombinant TGFβ3 (R&D Systems) was
added to the cultures. Unless otherwise stated cells were cultured for 3 weeks (21 days) and media was changed three times per week.

5.3.3 Hypertrophic Differentiation and Mineralization

Cells were seeded at a density of 1-2 X 10^6 cells per filter as specified and chondrogenically differentiated for 14-21 days prior to hypertrophic differentiation. Mackay et al. demonstrated that 14 days of chondrogenic induction prior to hypertrophic differentiation enhanced the hypertrophic phenotype [21], while Mueller et al. suggested that 14 days was not enough to fully differentiate the cells to a chondrogenic phenotype [16], thus both durations were tested. For hypertrophic induction media supplementation with TGFβ3 was ceased and cells were cultured in SFM supplemented with either 30nM T3 (Sigma, T6397), 50nM T4 (Sigma, T1775) 0.1ug/ml BMP-2 (Abcam, ab50099), 1uM FK506 (Abcam, ab120223), 5% FBS or neither as specified. 10mM beta-glycerophosphate (β-GP) and 100ug/ml L-ascorbic acid were also added to all cultures. Media was changed 3 times per week. For some experiments the chondrogenically pre-differentiated cells were isolated from in vitro formed cartilaginous tissues and subsequently seeded on Col2 coated membranes at a density of 1-2 X 10^6 cells per filter (as specified) prior to hypertrophic differentiation. Pre-differentiated cells were seeded on membranes directly in SFM without ROCKi as we found that chondrogenically pre-differentiated cells did not have a contractile phenotype. This methodology was utilized in a previous study for hypertrophic differentiation of sheep MSCs [13]. Cells were isolated from in vitro formed cartilaginous tissues by digestion in 0.1% collagenase solution for 4 hours at 37°C. The cell suspension was filtered using a 100um filter and cells were counted using a haemocytometer prior to seeding on membranes.

5.3.4 Bi-Layered Construct Formation

For the formation of a mineralized layer cells were seeded at a density of 1 X 10^6 cells per membrane and chondrogenically pre-differentiated for 2 weeks. TGFβ3 was removed from culture and tissues were subsequently cultured under hypertrophic differentiation conditions in
SFM with 1uM FK506 [28] for 3 weeks. MSCs from the same patient sample were seeded at 2 X 10^6 cells per membrane and cultured under chondrogenic differentiation conditions for 5 weeks, in parallel to cultures being utilized for mineralization. The chondrogenically differentiated MSCs were isolated from 5 week formed tissues by digestion in 0.1% collagenase for 4 hours. The cell suspension was filtered using a 100μm filter and cells were counted using a haemocytometer. Cells were seeded on top of mineralized cartilage at 2 X 10^6 cells per membrane in SFM. After allowing 24h for cells to attach, the bi-layered constructs were maintained in SFM supplemented with 10mM β-GP and 100μg/ml L-ascorbic acid for 2 weeks.

5.3.5 Histology and Immunohistochemistry

Tissues were fixed in 10% PBS buffered formalin (pH 7.4) overnight then placed in a 30% sucrose/PBS solution overnight at 4°C. Tissues were snap-frozen in Tissue-Tek OCT frozen compound (Sakura Finetek) to generate blocks for cryosectioning. Blocks were sectioned at a thickness of 7 μm.

For immunohistochemistry, sections were digested with 0.4% pepsin (in 1X TBS pH 2), blocked with 20% goat serum (in 0.1% Triton solution) and then incubated overnight at 4°C with antibody reactive with either type I collagen (COL 1, 1:1000 Abcam, ab90395), Col 2 (1:500, MAB8887, EMD Millipore), Type X collagen (Col X, 1:1500, Abcam, Ab49945), or PRG4 (1:400, MAB401, EMD Millipore). For ACAN no antigen retrieval was used (1:500 AHP0022, ThermoFisher Scientific). Tissues were subsequently incubated with either, Alexa-488 goat anti-mouse IgG (1:1000, Life Technologies, A11001 (secondary for Col 2, ACAN, and PRG4)), Alexa-594 goat anti-mouse IgG (1:2000, Life Technologies, A11032 (secondary for Col1) ) or Alexa-594 goat anti-mouse IgM (1:2000, Life Technologies, A21044 (secondary for Col X)) for 1 hour at room temperature. Tissues were counterstained with DAPI (1μg/ml). Negative controls were performed for all conditions and consisted of replacing the primary antibody with isoform and species matched antibody at the same concentration. Tissues were washed 3 times with PSB-/- between incubations and mounted with PermaFluor™ aqueous mounting medium (Thermofisher Scientific; TA-030-FM) and glass coverslips.
For visualization of alkaline phosphatase (ALP) activity, tissues were fixed for only 30 minutes, then embedded and sectioned as described above. Sections were incubated in azo dye (Naphthol AS-MX phosphate and Fast Blue BB salt, both Sigma–Aldrich) for 30 min according to the manufacturer’s protocol, counterstained with eosin, mounted with Micromount (Leica Biosystems) and coverslipped.

For visualization of mineral Von Kossa staining was utilized. Tissue sections were incubated for 1 hour under UV light with 100 µl of 5% (w/v) silver nitrate solution per section. Unreacted calcium was removed by treating sections with 1% (w/v) sodium thiosulfate for 5 minutes. Sections were then counter stained with 0.1% (w/v) toluidine blue, mounted with Micromount (Leica Biosystems) and coverslipped.

5.4 Results

5.4.1 Cartilaginous Tissue Formation by Chondrogenically Differentiated MSCs

MSCs were cultured in SFM with 10ng/ml TGFβ3 for 3 weeks. Immunohistochemical staining was performed on formalin fixed histological sections of tissue (Figure 5-1). Staining for the hyaline cartilage proteins ACAN, Col2 and PRG4 illustrate that cartilaginous tissues accumulate Col2 and ACAN but not PRG4. Collagen type 1 (Col1) which is expressed in fibrocartilage tissues and bone was not detected. ColX, a marker of hypertrophy, was present in the formed tissues. These tissues, which were harvested after 3 weeks and were not further cultured under hypertrophic differentiation conditions, did not mineralize (data not shown).
Figure 5-1: **Chondrogenic differentiation of MSCs and cartilaginous tissue formation.** Immunohistochemically stained sections of 3 week formed cartilaginous tissues cultured in SFM with 10ng/ml TGFβ3. Antibodies reactive with Aggrecan (ACAN, green) Collagen type 2 (Col2, green) and Lubricin (PRG4, green), Collagen type 1 (Col1, red), and Collagen type 10 (ColX, red). DAPI (blue) counterstain. Native cartilage was used as positive controls. White arrowhead shows region containing subchondral bone staining for Col1. Size bar = 200µm.
5.4.2 Hypertrophic Differentiation by Chondrogenically Pre-differentiated MSCs

Detailed methods of the different conditions tested to induce mineralization of chondrogenically pre-differentiated MSCs are summarized in the following chart.

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<td><strong>Hypertrophic Differentiation</strong>:&lt;br&gt;<em>Duration</em>: 21 days&lt;br&gt;<em>Condition</em>: 10mM β-GP&lt;br&gt;N=3; n=9&lt;br&gt;(0 of 3 patient samples mineralized)</td>
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<td><strong>Method 2</strong>&lt;br&gt;Chondrogenic Differentiation:&lt;br&gt;<em>Duration</em>: 21 days&lt;br&gt;<em>(seeded 2 X 10^6 per membrane)</em>&lt;br&gt;Tissues digested following chondrogenic differentiation and cells seeded 1 X 10^6 per membrane&lt;br&gt;Hypertrophic Differentiation:&lt;br&gt;<em>Duration</em>: 21 days&lt;br&gt;<em>Condition</em>: 0.1ug/mL BMP-2 10mM β-GP&lt;br&gt;N=1; n=2</td>
</tr>
<tr>
<td>Method 3</td>
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</table>
| **Chondrogenic Differentiation:**  
*Duration:* 21 days  
(seeded 2 X 10⁶ per membrane)  
Tissues digested following chondrogenic differentiation and cells seeded 1 X 10⁶ per membrane  
**Hypertrophic Differentiation:**  
*Duration:* 21 days  
*Condition:*  
30nM T3 or 50nM T4  
10mM β-GP  
N=2; n=8  
(1 of 2 patient samples mineralized)  
**Chondrogenic Differentiation:**  
*Duration:* 14 days  
(seeded 1 X 10⁶ per membrane)  
**Hypertrophic Differentiation:**  
*Duration:* 21 days  
*Condition:*  
5% FBS  
10mM β-GP  
N=1; n=3  
**Chondrogenic Differentiation:**  
*Duration:* 14 days  
(seeded 1 X 10⁶ per membrane)  
**Hypertrophic Differentiation:**  
*Duration:* 21 days  
*Condition:*  
1uM FK506 or 0.1ug/ml BMP-2  
10mM β-GP  
N=3; n=16  
(2 of 3 patient samples mineralized)  

Table 5-1: **Methods tested to induce hypertrophic differentiation and mineralization by chondrogenically pre-differentiated MSCs.** Five different methods (designated as Method 1-5)
were tested for their ability to induce mineralization by chondrogenically pre-differentiated MSCs. Patient ID, passage number used for experimentation, age, sex, and experimental method are listed. Red text indicates that the cells from that patient sample mineralized. N=1-3 and is listed under experimental methods.

Various methods as well as biological and chemical factors were tested for their ability to induce hypertrophic differentiation and mineralization by chondrogenically pre-differentiated MSCs (Table 1) cultured in 3D. In Method 1, passaged MSCs were chondrogenically differentiated, isolated from in vitro formed cartilaginous tissues (a methodology previously used by Lee et al. [13]), and then subsequently placed in hypertrophic differentiation conditions, with β-GP as the only additional factor to promote hypertrophy. Our results suggest that culture with dexamethasone, ascorbic acid, and β-GP, commonly used for osteogenic differentiation and referred to as “DAB” [31], was not sufficient to induce mineralization in our 3D culture system by chondrogenically pre-differentiated MSCs. Although histological analysis showed that cells treated with β-GP had ALP expression while controls did not, no mineral was accumulated in the tissues as demonstrated with Von Kossa staining (Figure 5-2). Of note, the process of isolating the pre-differentiated MSCs from in vitro formed cartilaginous tissues resulted in the loss of cells to a great extent (data not shown); therefore, subsequent approaches utilized a lower cell number for hypertrophic differentiation, or skipped the isolation step.
Figure 5-2: **Method 1:** β-GP alone is unable to induce mineralization of chondrogenically pre-differentiated MSCs. Histological sections of formalin fixed tissue were stained for ALP (blue) and counterstained with eosin (top row) or stained with Von Kossa for mineral deposition (black) and counterstained with toluidine blue (bottom row). Native articular cartilage was used as a positive control. N=3.

In Methods 2 and 3, T3, T4, or BMP-2 was added to cultures to promote hypertrophic differentiation. Both methods showed mineral deposition at 3 weeks and showed some ALP expression in the areas adjacent to the mineralized regions (Figures 5-3 and 5-4). For cultures treated with T3 and T4, only 1 of the 2 patient samples tested mineralized. As discussed, loss of cells with isolation was a concern with using methods 1-3, therefore methods that did not utilize an isolation step were also tested. For these methods, in vitro formed cartilaginous tissues were not digested to isolate the cells, but rather the culture media was switched after 14 days to hypertrophic differentiation media. In method 4, SFM was supplemented with 5% FBS. With this method, ALP was expressed regardless of whether β-GP was present or not (Figure 5-5), which may be a result of factors present in the serum. However, only in cultures containing β-GP was mineral accumulated and it was localized to the very edges (top and bottom surfaces) of the construct adjacent to ALP which was expressed throughout the center of the tissues. As this was not the desired outcome, the use of serum was not further investigated.
Figure 5-3: **Method 2: Chondrogenically pre-differentiated MSCs will form a layer of mineralized tissue when cultured with BMP-2.** Histological sections of formalin fixed tissue were stained for ALP (blue) and counterstained with eosin (top row) or stained with Von Kossa for mineral deposition (black) and counterstained with toluidine blue (bottom row). Mineralized in vitro formed cartilage from bovine deep zone chondrocytes was used as a positive control. N=1.

Figure 5-4: **Method 3: Thyroid hormone supplementation induces mineralization of tissues formed by chondrogenically pre-differentiated MSCs.** Histological sections of formalin fixed tissue were stained for ALP (blue) and counterstained with eosin (top row) or stained with Von Kossa for mineral deposition (black) and counterstained with toluidine blue (bottom row). Native articular cartilage was used as a positive control. N=2.
Figure 5-5: Method 4: In serum containing media, tissues formed by chondrogenically pre-differentiated MSCs form a layer of mineralization on the outer edges of the construct. Histological sections of formalin fixed tissue were stained for ALP (blue) and counterstained with eosin (top row) or stained with Von Kossa for mineral deposition (black) and counterstained with toluidine blue (bottom row). Mineralized in vitro formed cartilage from bovine deep zone chondrocytes was used as a positive control. Black arrows point to area positive for mineral. N=1.

Methods 2 and 3 demonstrated that the use of thyroid hormones or BMP-2 had the potential to push chondrogenically pre-differentiated MSCs to mineralize the matrix throughout the tissue, but this approach did not work reliably. One of the ways in which thyroid hormones have been suggested to induce mineralization is by indirectly increasing BMP signalling pathways and upregulating BMP-4 [32], [33]. Therefore in Method 5 we decided to test if in vitro-formed cartilaginous tissues would mineralize if treated with the chemical FK506, which has been shown to activate BMP pathways and induce osteogenesis in MSCs [28]. We found that tissues cultured with either FK506 or BMP-2 mineralized and stained positively for ALP expression. (Figure 5-6). In the absence of β-GP, no mineralization was observed.
Figure 5-6: **Method 5: FK506, a small molecule inducer of the BMP receptor, will promote mineralization of tissues formed by chondrogenically pre-differentiated MSCs, similar to BMP-2.** Histological sections of formalin fixed tissue were stained for ALP (blue) and counterstained with eosin (top row) or stained with Von Kossa for mineral deposition (black) and counterstained with toluidine blue (bottom row). Mineralized in vitro formed cartilage from bovine deep zone chondrocytes was used as a positive control. N=3.

### 5.4.3 Formation of a Bi-layered Construct Containing Hyaline-like Cartilage with a ZCC

Activation of the BMP pathways either with thyroid hormones, BMP-2 or FK506 was demonstrated to induce hypertrophic differentiation and mineralization of *in vitro* formed cartilage. Clinically, FK506 is FDA approved and is used as an immunosuppressant drug, while the use of BMP-2 is accompanied by an FDA issued warning regarding its use due to demonstrated adverse effects [29]. Therefore, FK506 was used to promote mineralization of *in vitro* formed cartilage for the construction of a bi-layered construct. Histological and immunohistochemical staining (Figure 5-7) shows that tissues which were cultured under hypertrophic differentiation conditions (“1-layer”) accumulate mineral and express ALP. Immunohistochemistry demonstrates that the mineralized tissues accumulate COL2 and ACAN as well as COL1 and COLX. Following hypertrophic differentiation, the mineralized tissues were seeded with a second layer of patient matched cells which had been cultured under chondrogenic differentiation conditions for 5 weeks in parallel to mineralizing cultures. The
combined bi-layered constructs ("2-layers") were cultured for an additional 2 weeks. Bi-layered tissue constructs integrated with each other and showed hyaline cartilage matrix accumulation, staining for ACAN and COL2 uniformly throughout the tissue. COL1 was only expressed in at the bottom of the tissue in the mineralized region. COLX was predominantly expressed in the mineralized region however some COLX was present in tissues formed by the second cell layer. Mineral deposition and ALP expression remained localized to the bottom layer of the tissue in bi-layered constructs.

Figure 5-7: **Bi-layered articular cartilage-like constructs with a ZCC.**
Immunohistochemically stained sections of bi-layered (2-layer) or single layer (1-layer) cartilaginous tissues. Antibodies reactive with Aggrecan (ACAN, green) Collagen type 2 (Col2, green), Collagen type 1 (Col1, red), and Collagen type 10 (ColX, red). DAPI (blue) counterstain. Histological sections were stained with Von Kossa to visualize mineral (black) with toluidine blue counterstain, or with naphthol phosphate and fast blue to visualize ALP (blue) with an Eosin counterstain. Native cartilage was used as positive controls. N=1.
5.5 Discussion

This study demonstrated that human bone marrow derived MSCs could be used to form hyaline-like cartilaginous tissues which contained a localized ZCC through a serial differentiation and twofold cell seeding approach. Through the activation of BMP signalling pathways either directly with BMP-2 or FK506, or indirectly by treating cultures with thyroid hormones [32], [33], chondrogenically pre-differentiated MSCs would be induced to mineralize the surrounding matrix, however no method did this reproducibly. A multitude of in vitro and in vivo studies of BMP proteins and the BMP signalling pathway have illustrated that this signalling network plays a pivotal role in skeletal development including osteoblast differentiation and bone formation (extensive review by Wu et al. [34]). Recently it has been shown that BMP signalling is transduced to an osteogenic signal through the formation of RUNX2-SMAD transcriptional regulatory complexes [35]. SMAD proteins are directly phosphorylated upon BMP receptor activation, either by BMP proteins or by FK506 [28] and may act in our system in a similar manner to induce hypertrophic differentiation and mineralization. For in vitro cultures of human MSCs, Osyczka et al. demonstrated that ALP and osteopontin (a marker of new bone formation [36]) were upregulated when cells were cultured in a serum-free media supplemented with BMP-2 [27]. The mechanism by which the factors utilized in our system induce hypertrophy and mineralization need to be further investigated however; the aforementioned studies suggest the mechanism may be a result of SMAD phosphorylation and RUNX2 regulation of osteogenic genes with BMP receptor activation and subsequent downstream signalling.

Of the 5 different methods utilized in our system, none were able to reliably induce mineralization. Patient age, passage number, or gender did not appear to correlate with the potential to mineralize. Although MSCs derived from the bone marrow have been shown to have a better chondrogenic and osteogenic differentiation ability when compared to patient matched stem cells isolated from other sources [37], [38], there is still a multitude of reports of donor to donor variability in the differentiation capacity of MSCs [38]–[40]. Studies have investigated methods to reduce this variability, and include assessment of specific gene expression profiles which may indicate an enhanced capacity to differentiate to a specific lineage [40], or sorting for
surface markers other than those used to classically identify MSCs from the bone marrow, as was recently described for a new population of cells with osteogenic and chondrogenic capabilities termed “human skeletal stem cells” [41]. Furthermore, a study by Janicki et al. demonstrated that proliferation-based potency testing and switching to enriched expansion conditions could enhance the bone-forming ability of inferior MSC populations [39]. For cultures of MSCs which show a poor osteogenic response to BMPs, modulation of the ERK and PI3-kinase pathways by treating cultures with the MEK inhibitor PD98059 was shown to enhance osteogenesis by increasing nuclear accumulation of phosphorylated SMAD proteins [27]. Finally, decreasing the concentration of dexamethasone from 100nM to 10nM [42] or treating cultures transiently with 100nM [43] dexamethasone, are other strategies which have been shown to reduce variability with MSC differentiation, and could be explored further in our system.

Interestingly, immunohistochemistry showed that COL1 and COLX accumulated predominantly in the mineralized layer of the biphasic constructs. As the cells in this layer were treated with FK506 while the cells used to generate the unmineralized hyaline-like layer were not, this phenotype may be due to the treatment. Studies of hypertrophic differentiation or osteogenic induction with FK506 are limited for human MSCs, however a study using rat MSCs showed upregulation of Col1 gene expression in FK506 treated cultures [44]. To the best of our knowledge no studies have investigated the role FK506 may play in upregulating COLX. In our system COLX may be inherently expressed by undifferentiated MSCs and therefore accumulated in the ECM prior to hypertrophic differentiation with FK506. Our results as shown in Figure 1 for cartilaginous tissues formed by MSCs suggest this may be the case. Once the cells were chondrogenically differentiated however, COLX did not readily reaccumulate, as it was not as strongly expressed in the hyaline-like layer of bilayered constructs. Therefore, expression of COL1 in the mineralized tissues may be due to FK506 treatment, as it was not present in tissues which were only cultured under chondrogenic conditions, while expression of COLX was present in tissues prior to hypertrophic differentiation. Further analysis of the mechanism of action of FK506 in our system needs to be conducted.

Future directions include increasing the number of replicates for biphasic constructs formed on membranes and moving towards creating an osteochondral-like construct by forming the bilayered constructs on top of a bone substitute material as described by Lee et al for sheep
MSCs [13]. Furthermore, future studies should include shear testing of the interface strength between constructs containing a ZCC and comparing to those without. Finally, exploring methods which may minimize differentiation variability, as discussed above, should be performed to help mitigate the variability observed between patients. Overall this work provides proof of concept that hyaline-like cartilaginous tissues with a ZCC can be created with human MSCs using a two-step cell seeding approach, and further identifies FK506 as a potential factor for inducing hypertrophic differentiation.
5.6 References


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Chapter 6
Discussion and Conclusions

6.1 Discussion

As discussed in depth in Chapter 1, developing methods to regenerate articular cartilage (AC) are necessary as this tissue is incapable of repairing when damaged. One approach is to bioengineer hyaline cartilage using autologous patient-derived cells. Although a variety of cell sources could be utilized, each with their own set of limitations, the focus of the investigations that are described in this thesis utilized human chondrocytes. The paucity of primary chondrocytes is a limitation to the use of these cells themselves, and further issues arise when these cells are serially passaged in an effort to increase cell number, a process which results in dedifferentiation [1]. Dedifferentiated chondrocytes lose their phenotype and the ability to form hyaline cartilage as demonstrated both in vitro [1]–[3] and in vivo when used clinically for autologous chondrocyte implantation therapy (ACI) [4], [5]. The first part of this thesis (Chapter 2, published in Tissue Engineering Part A, and Chapter 3) focused on the development and characterization of a methodology that results in redifferentiation of passaged human chondrocytes and restoration of their ability to form hyaline-like cartilage in vitro. The second part of this thesis (Chapter 4; under revision for American Journal of Sports Medicine) was devoted to investigating a potential cartilage repair strategy using these redifferentiated chondrocytes for defect repair in vivo. The third part of the thesis (Chapter 5) focussed on developing an “osteochondral-like” construct with a zone of calcified cartilage which entailed using differentiated mesenchymal stromal cells (MSCs).

The method to redifferentiate passaged human chondrocytes involved the use of high density (3D) culture, an optimized serum-free media containing insulin, dexamethasone and high glucose content, and was reliant on supplementing the media with transforming growth factor beta 3 (TGFβ3) [6]. We showed that only when cultured with TGFβ3 did the in vitro formed tissues accumulate the major hyaline matrix macromolecules, collagen type 2 (COL2), aggrecan (ACAN), and proteoglycan 4 (PRG4). In the absence of TGFβ3, hyaline-like cartilaginous tissues did not form. This is in contrast to passaged bovine chondrocytes which we have shown
will form cartilaginous tissues when cultured under the same conditions in the absence of treatment with TGFβ [7]. In our study human chondrocytes were isolated from AC derived from joints affected by osteoarthritis (OA), which was shown previously by others to have decreased TGF-β and TGF-β receptor expression compared to cells from normal AC [8]. It is possible that early passage bovine chondrocytes synthesize enough endogenous TGFβ to support the formation of neocartilage, while human chondrocytes do not. In support of this Tekari et al. demonstrated that extensively passaged bovine chondrocytes (passage 3-6) show decreased levels of transcripts coding for TGF-β isoforms and TGF-β receptors and also lose the ability to form cartilaginous tissues which was reversible with exogenous TGFβ supplementation [9].

TGFβ signalling is known to be a critical mediator of chondrogenesis and cartilage homeostasis. When the components of the heterotetrametric TGFβ signalling complex are individually knocked down in AC, animals develop an OA-like phenotype characterized by cartilage loss, dysregulation in synthesis of anabolic versus catabolic factors, osteophyte formation and chondrocyte apoptosis [10], [11]. Furthermore, in experimentally induced OA Blaney-Davidson et al. showed progressive loss of the type 1 TGFβ receptor ALK5, and postulated that this led to a dysregulation in cartilage anabolism, which is mediated by ALK5 signalling, versus catabolism [12]. Aside from changes to the TGFβ signalling pathway, studies of human chondrocytes from OA cartilage have shown increased production of catabolic enzymes [13], loss of expression of hyaline matrix genes [14], [15], and a multitude of differentially regulated gene and protein networks [16]–[18] including those linked to ECM organization, mitotic cell cycle, skeletal development, transcriptional regulation, apoptosis and Wnt signalling. Therefore, in Chapter 3 of this thesis we investigated whether the severity of OA in AC from which the chondrocytes were isolated, influenced the ability of these cells to redifferentiate and form hyaline-like neocartilage in vitro using the methods established in Chapter 2.

When chondrocytes isolated from AC with severe OA changes were compared to chondrocytes isolated from AC with only minimal to mild OA changes, we found that neither of these populations could form hyaline-like cartilage in response to culture with TGFβ unless they were serially passaged before being exposed to exogenous TGFβ. After passaging however, cells from both groups formed cartilaginous tissues and accumulated comparable amounts of hyaline-like extracellular matrix (ECM). It was intriguing that passaged (P2) chondrocytes and not P0
cells would form cartilaginous tissues as these cells have dedifferentiated; a phenotype classically thought to impair the ability of these cells to form cartilage [1]. However, more recent studies have suggested that the dedifferentiated phenotype is progenitor-like resembling that of MSCs [19], [20] which can be differentiated into adipocytes, osteoblasts, and chondrocytes. Differentiation of MSCs to chondrocytes is commonly facilitated in vitro through culture with TGFβ [21]–[23], and thus the acquisition of an MSC-like phenotype may explain why only the passaged chondrocytes in our system redifferentiated and formed cartilage in response to culture with TGFβ3. To further investigate why P2 cells may be more equipped to form neocartilage than primary (P0) cells in response to culture with TGFβ3 we used image flow cytometry to assess expression of the TGFβ receptor ALK5, known to signal via pathways that facilitate cartilage anabolism [24]–[27]. Our results indicated that ALK5 was expressed in the vast majority of both P0 and P2 cells (> 95%), which would suggest that both populations should be capable of forming hyaline-like cartilage in response to TGFβ however; only P2 cells formed tissues suggesting that additional factors may be involved in this signalling. Therefore, we proceeded to investigate expression of the TGFβ co-receptors endoglin (ENG) and TGFβ receptor 3 (TGFβRIII) both of which have been shown to enhance TGFβ signalling through interactions with the receptor complex [28]–[31].

As TGFβRIII was minimally expressed in both primary and passaged chondrocytes we determined that it was unlikely that this co-factor was playing a major role in TGFβ mediated signalling in our cells. ENG expression however significantly increased with passaging resulting in a population of cells in which the majority (>80%) expressed this co-receptor. Furthermore, by assessing the median fluorescent intensity values we determined that the density of ENG/cell also increased significantly with passaging. Ultimately, passaging the chondrocytes resulted in a population of cells which primarily expressed ENG and ALK5, which have been shown in other cell types to work together to facilitate TGFβ mediated signalling [32]–[35]. In passaged endothelial cells, Santibanez et al. showed that the presence of ENG lead to enhanced signalling via the ALK5 mediated SMAD2 pathway [32], the same pathway recognized to facilitate cartilage anabolism in chondrocytes [36]. Furthermore, Fan et al. showed that synovium derived stem cells that were sorted for ENG had an enhanced chondrogenic potential which was mediated by increased phosphorylation of SMAD2 [37]. We hypothesize that for our system, a similar interaction may be occurring allowing the P2 cells to respond to TGFβ3 and form
hyaline-like cartilage. Furthermore, no significant differences in the expression of these receptors was found between chondrocytes isolated from High (severe) OA compared to Low (minimal) OA, which may explain why both populations of cells formed cartilage and accumulated comparable amounts of hyaline-like ECM. Overall this study determined that the severity of OA from the AC which the cells were isolated did not affect the ability of the cells to form neocartilage after passaging, and this may be due to the similar ENG and ALK positive phenotype of the cells after passaging. This is significant as it means that chondrocytes obtained from cartilage with severe OA changes are still suitable to use for tissue engineering purposes, and further indicates that cells from diseased cartilage can be used for cartilage repair and it is not necessary to use healthy cells, which are very limited in number.

In addition to identifying conditions that would facilitate redifferentiation, it was shown in Chapter 2 that the redifferentiated phenotype was stable, as cells that were transiently treated with TGFβ3 (for 3 weeks) continued to accumulate COL2 in the weeks after TGFβ3 was ceased. Chapter 4 describes the studies done to determine if these cells could be used to repair an osteochondral defect in rabbits. First it was shown that passaged rabbit chondrocytes behaved similarly to human chondrocytes and formed stable hyaline cartilage when grown in fibrin gel clots using the same redifferentiation strategy described in Chapter 2. The fibrin gel would serve as the carrier to maintain the cells in the defect and has been used by others successfully [38]–[40]. These cells were then implanted into an osteochondral defect and evaluated 6 weeks later.

P2 (dedifferentiated) chondrocytes were used as controls. Interestingly the repair response differed greatly between these two cell types. When redifferentiated cells were implanted, granulation tissue was present in the defect whereas fibrocartilage repair was seen when dedifferentiated cells were implanted. The development of fibrocartilage repair is the standard outcome for therapies which utilize passaged chondrocytes [4], [5]. However the finding of granulation tissue in defects repaired by redifferentiated cells was completely unexpected. Granulation tissue is typically generated during wound repair in connective tissues and bone [41]–[45]. As the cells were not pre-labelled it was not possible to confirm that the granulation tissue arose from the implanted cells, a definite limitation of this study. It is however considered unlikely as the repair was so different than when dedifferentiated cells were implanted. This raised the question if residual TGFβ remained in the implanted cells as subcutaneous injections of TGFβ into a wound has been shown to rapidly induce granulation
tissue in the skin [46]. Although a similar response did not occur after intra-articular injections of TGFβ in mice joints, the conditions were different as there was osteochondral defect [47]. Interestingly, when assessed via immunohistochemistry the granulation tissues contained COL2, a protein not typically present in this tissue [48], [49]. This raises the possibility that the repair tissue maybe capable of remodeling into hyaline cartilage, In support of this, a study by Frisbie et al. which investigated repair after microfracture in equine models showed that sites of granulation tissues which appeared early in the repair process matured to hyaline-like cartilage when assessed at a later time point [50]. Overall at 6-weeks post implant the results of our study suggest that redifferentiated chondrocytes do not enhance repair. Longer term follow-up studies will be required to determine how the tissues mature.

As the early in vivo results using redifferentiated chondrocytes in fibrin gel were not optimal, we revisited the idea of bioengineering a zonally organized AC tissue construct suitable for implantation and load bearing. In a previous study by our group Lee et al. demonstrated that an osteochondral-like construct, containing hyaline-like cartilage anchored to a bone substitute material by a zone of calcified cartilage, could be generated using sheep MSCs [51]. Chapter 5 describes the studies aimed at developing an osteochondral-like construct by human cells. To achieve this goal both hyaline-like cartilage and a ZCC needed to be generated. As the methods to form hyaline-like cartilage using human passaged chondrocytes were already established, we investigated whether these cells could also be used to form a ZCC. In Chapter 2 we showed that the tissues formed by redifferentiated chondrocytes did not express the hypertrophic chondrocyte markers, collagen type 10 (ColX) or alkaline phosphatase (ALP). When these cells were cultured under known mineralization conditions it was not possible to induce them to form mineralized cartilage (data not shown). Other studies have demonstrated the ability of chondrocytes to resist hypertrophic differentiation and mineralization both in vitro and when implanted in vivo [52], [53]. This may be due to the secretion of parathyroid hormone-related protein (PTHrP) by chondrocytes which has been shown to inhibit mineralization by MSCs, which unlike chondrocytes downregulate PTHrP during in vitro chondrogenesis [53]. Epigenetic differences may also play a role in chondrocyte resistance to hypertrophic differentiation, as the chondrocytes that are found in adult AC that are outside of the ZCC are not hypertrophic.

Although chondrocyte resistance to calcification is advantageous in vivo, generating a ZCC is essential for the integrity of the construct, as studies have shown this increases the
interfacial shear strength between the neocartilage and bone [54], which would help mitigate shearing of the cartilage from the bone substitute material following implantation. Therefore, in Chapter 5 we investigated the use of human bone marrow derived MSCs for the formation of a zone of calcified cartilage (ZCC) as these cells can undergo hypertrophic differentiation. Before proceeding to investigate hypertrophic differentiation strategies, we first assessed the ability of the human MSCs to form hyaline-like cartilage. Using the same methods established in Chapter 2 for redifferentiation of passaged chondrocytes, MSCs were differentiated to chondrocytes and formed hyaline-like cartilage in vitro. Like the tissues formed by chondrocytes this tissue contained the hyaline ECM proteins COL2 and ACAN, however unlike the tissues formed by chondrocytes COLX was also accumulated. Following chondrogenic differentiation, we investigated a number of different strategies and factors that could be added to the culture media, which have been shown to induce hypertrophic differentiation. The thyroid hormones Triiodothyronine [51], [55], [56] and L-Thyroxine [57], bone morphogenic protein 2 [58], and the small molecule known as FK506 [59] have all been shown to induce mineralization by MSCs and were all tested for their ability to induce mineralization in our system. This data is summarized in Table 1 of Chapter 5.

Overall, we found that all of these factors were capable of promoting hypertrophic differentiation and mineralization however; none of these treatments did so reproducibly. Factors such as patient age and cell passage number did not correlate with the potential of the MSCs to mineralize. MSCs are known to show donor to donor variability with respect to differentiation capacity [60]–[62]. Methods to achieve more reliable hypertrophic and osteogenic differentiation have been investigated [61], [63]–[65] and could be explored further in our system in future studies. In cells where mineralization did occur a ZCC was formed, and MSCs that had been differentiated to chondrocytes were seeded on top of the ZCC and cultured together for 2 weeks. The tissue which formed appeared to integrate with the ZCC and was hyaline-like accumulating COL2 and ACAN. Furthermore, the mineralized zone remained isolated to the initially constructed ZCC and did not appear to advance into the hyaline-like cartilage, nor was ALP expressed in the unmineralized areas. Ultimately an in vivo study would be required to assess whether the hyaline-like zone would remain uncalcified long term however; these initial experiments indicated that human bone marrow derived MSCs have the potential for use in our system to form an osteochondral-like construct.
6.2 Future Directions

6.2.1 Assessing the function of ENG in vitro and in vivo

In chapter 3 we found that only after passaging would chondrocytes form cartilaginous tissues when cultured with TGFβ3 despite the presence of ALK5 in both P0 and P2 cells. This correlated with the increased expression of the TGFβ co-receptor ENG and led us to hypothesize that ENG and ALK5 may be working together in passaged cells to enhance TGFβ signalling and facilitate redifferentiation. TGFβ signalling through the interactions of ENG and ALK5 has been demonstrated in other cell types [32]–[35]. To validate our hypothesis studies in which ENG is knocked out or silenced in P2 chondrocytes would be required. Methods such as the use of ENG specific siRNA which would prevent translation of the ENG protein or using CRISPR-Cas9 to completely remove or permanently silence this gene could be utilized in future studies. In these experiments in which ENG is silenced, assessment of the ALK5 downstream signalling pathway should also be investigated to determine how loss of ENG affects TGFβ signalling. To do this western blot analysis or flow cytometry could be utilized with antibodies reactive for the phosphorylated SMAD2 or SMAD3 proteins, as these proteins are known to be phosphorylated as a direct result of TGFβ binding to the ALK5 receptor in chondrocytes [27].

Furthermore, the isoform of ENG that is being upregulated in P2 cells may also play a role in enhancing this signalling and should be investigated. Two variants of ENG have been reported and are known as either Short (S-ENG) or Long (L-ENG), based on the different lengths of the cytoplasmic tail, generated as a result of alternative splicing [66]. Due to the homology of the extracellular domain, both variants bind TGFβ [66]. Interestingly however studies have indicated that these variants may interact with the two Type 1 TGFβ receptors (ALK1 and ALK5) preferentially, thereby differentially modulating signalling via these two pathways. In studies of endothelial cells and myoblasts S-ENG has been shown to favourably interact with ALK5 and enhance signalling via this pathway [33], [35]. In our study, the ENG antibody used binds the extracellular domain and therefore does not differentiate L-ENG from S-ENG. Primers specific for L-ENG or S-ENG could be designed and used for PCR analysis to assess which transcript of ENG is being upregulated in P2 cells. Additionally, antibodies for L-
ENG or S-ENG specific domains could be generated and western blot, immunohistochemistry, or flow cytometry analysis could be used to visualize and quantify the expression of each variant.

Aside from the role ENG may play in TGFβ signalling in P2 cells, there are other factors that could be contributing to the enhanced chondrogenic potential observed in these cells. SMAD6 and SMAD7 proteins are components of the TGFβ signalling pathway which have been shown to be inhibitory [67], and it is possible that in P0 cells higher levels of expression of these proteins contribute to the lack of tissue formation in response to culture with TGFβ. In support of this research by Baugé et al. showed that interleukin-1β (IL-1β), a key cytokine in OA, can upregulate SMAD7 in human chondrocytes [68]. As our chondrocytes are obtained from OA cartilage, it is possible that SMAD7 is upregulated. Immunocytochemistry or western blot analysis could be performed to determine if SMAD7 is playing a role in our system. Furthermore, silencing of SMAD6 or SMAD7 could also be used to test the effects of downregulating these proteins on TGFβ mediated tissue formation by P0 cells. In addition to upregulation of SMAD7 the same group also showed that IL-1β can mediate degradation of TGFβ receptor 2, which may also contribute to the reduced responsiveness of P0 OA chondrocytes to TGFβ [69].

Signalling via the canonical SMAD pathway is only one of multiple pathways that can be activated by TGFβ. Non-canonical signalling pathways such as Tak1 and its downstream P38, JNK and ERK pathways, as well as Wnt3a and Map Kinase pathways have also been shown to be activated by TGFβ and can play a role in chondrogenesis by both MSCs and chondrocytes [24], [70]–[76]. It is possible that different TGFβ signalling pathways are being activated in P0 and P2 cells resulting in enhanced chondrogenesis after passaging. Studies examining which signalling pathways are activated in P0 and P2 cells would be required to determine this and should be explored in future studies.

Numerous studies of cartilage defect repair and osteoarthritis in animal models in vivo have demonstrated that it is the ENG positive cell population which migrates to the defect site and contributes to the repair tissue [77]–[79]. This suggests that ENG may play a role in chondrogenesis in vivo, and that loss of ENG may inhibit neocartilage formation and defect repair. To assess this, ENG haploinsufficient (ENG+-) mice [80] could be used to assess
spontaneous defect repair and compared to mice with both copies of ENG (ENG+/+). In addition, chondrocytes sorted for ENG positivity could be implanted into critical size defects and assessed for their ability to repair the defect and compared to tissues formed by cells sorted as ENG negative. Both of these studies would address the function of ENG in cartilage repair in vivo and should be explored in future studies.

6.2.2 Modifications to our in vivo Study

In Chapter 4 we assessed the use of redifferentiated chondrocytes for osteochondral defect repair in vivo and demonstrated that the use of these cells resulted in the formation of COL2-containing granulation tissue. To the best of our knowledge this is the first study to show such a result. One of the limitations of this study was that the cells were not labelled prior to implantation, and so it is not known with certainty that the cells which generated the repair tissue were the cells that were originally implanted. As the control group formed repair tissues which were fibrocartilage, the standard outcome for therapies which use passaged chondrocytes [5], this would support that the implanted cells contributed to the repair tissue. In future studies labelling of the cells prior to implantation should be performed in order to address this question. Although the results of our study at this early time point indicated that redifferentiated chondrocytes did not enhance repair, a longer time point should be investigated to determine maturation of the granulation tissue, as the presence of COL2 may indicate that the tissue is remodelling.

For our study the cells were implanted into the defects shortly after they were embedded in fibrin gel, using a technique similar to that which has been used clinically [81]. Fibrin gel is well accepted as a biocompatible and natural material that supports cell attachment and can be used as a scaffold suitable for tissue engineering or as a cell carrier (recent reviews [82], [83]). Although this scaffold could support cartilaginous tissue formation in vitro and in vivo, the poor mechanical properties of fibrin gel, and gel shrinkage due to degradation are limitations to use of this material as a scaffold [84]. Although viscoelastic, fibrin gel possesses irreversible deformation [85] which can be a limitation to its use in a load bearing tissue like AC. In future studies, culturing the fibrin clots in vitro for a period before implantation should be investigated as the deposited ECM would increase the integrity and mechanical properties of the implant which may influence the success of the therapy. Other methods that have been used to increase
the mechanical properties of fibrin gel include combining the fibrin gel with natural polymers such as gelatin, hyaluronic acid, and chondroitin-6-sulfate [86]. In future studies, degradation of the fibrin gel, as well as studies of the mechanical properties of the tissues at various time points should be assessed.

One additional factor that could be tested in future studies is the influence of cell density. For our study, only one cell density was used to generate the cell laden fibrin clots. This cell number was acquired from an existing study in which fibrin clots containing MSCs were implanted in rabbit osteochondral defects [87]. However, numerous studies have illustrated how cell density can influence cartilaginous tissue formation by MSCs [88]–[92] and human chondrocytes [93] and should be explored further in future studies.

6.2.3 Bioengineering Zonally Organized AC on a Bone Substitute and in vivo Validation

In chapter 5 we demonstrated that it was feasible to use human bone marrow derived MSCs to engineer hyaline-like cartilage tissue in vitro which contained a localized ZCC. This was achieved using a two-step cell seeding method previously described by Lee et al. for sheep MSCs [51]. In our study these tissues were engineered on membranes, however in order to create an osteochondral-like construct these tissues need to be engineered on bone. In previous studies, the Kandel lab and collaborators identified and validated in vivo a bone substitute material known as calcium polyphosphate (CPP) [94]–[97]. They demonstrated that this material was biocompatible and could support the ingrowth of native bone [96], [98], and showed that CPP could support the formation of cartilage tissue by chondrocytes [99]–[101] and sheep MSCs [51]. In future studies, the biphasic cartilage constructs should be engineered on CPP.

In our study we found that one of the biggest limitations with the use of human MSCs was the variability in differentiation potential observed between patient samples. Methods such as modulation of the ERK and PI3-kinase pathways by treating cultures of MSCs with the MEK inhibitor PD98059 [65], selecting for populations of cells which may have an enhanced osteogenic differentiation potential [62], [102], culture with IL-1β [103], or switching to enriched expansion conditions [61], have all been described to enhance the osteogenic
differentiation potential of MSCs and could be tested in our system. Interestingly, Levi et al. have shown that in adipose derived stem cells, ENG depletion correlated with enhanced osteogenic differentiation [104] and this was due to decreased signalling via the TGFB-SMAD2 pathway. Both passaged chondrocytes and bone marrow derived MSCs have been shown to express ENG [105], which may explain why the P2 chondrocytes used in our study were incapable of mineralizing, and why variability has been overserved for mineralization by cultures of MSCs. Silencing of ENG in chondrogenically differentiated MSCs may be explored in future studies as a method to enhance mineralization in our system.

We showed in chapter 5 that the cartilaginous tissues formed by MSCs contained COLX. In native cartilage COLX is primarily expressed in the ZCC. The presence of COLX throughout the bioengineered cartilage may indicate the cells are hypertrophic and this has been shown to result in calcification of the cartilage when implanted \textit{in vivo} [52]. In the hyaline-like cartilaginous tissues formed by passaged chondrocytes COLX was not accumulated. Biphasic cartilage in which the ZCC is bioengineered using MSCs and the hyaline-like cartilage is bioengineered using chondrocytes may be one method to eliminate COLX accumulation outside of the ZCC. Furthermore, cartilaginous tissues formed by human passaged chondrocytes have been shown to resist calcification when implanted \textit{in vivo} unlike tissues formed by human MSCs [52]. Ultimately \textit{in vivo} studies would be required to assess the stability of the differentiated phenotype, resistance to calcification, and the longevity of the osteochondral-like construct. Studies of osteochondral defect repair using these bioengineered cartilage-CPP constructs should be conducted in future experiments.

6.3 Significance and Conclusions

Chondrocyte dedifferentiation is largely accepted to be a limitation to use of these cells clinically for cartilage repair therapies, due to loss of phenotype and the ability to form hyaline cartilage [7], [106]–[108]. This thesis shows for the first time a method to reliably generate a self-assembling and continuous layer of hyaline-like cartilaginous tissue with human passaged chondrocytes, functionally demonstrating the chondrogenic capacity of dedifferentiated cells. In addition, this method generated tissues expressing markers consistent with the superficial zone of AC on the outer regions of the \textit{in vitro} formed cartilage; a finding which has never been shown
before for tissues formed by human chondrocytes. This method involved the use of a chemically-defined serum free media supplemented with TGFβ3, a growth factor known to have undesirable effects to the surrounding joint tissues when added exogenously [47], [109]. The results presented throughout Chapters 2 and 4 of this thesis demonstrated the stability of the redifferentiated phenotype, as it was shown that collagen type 2 accumulates in the extracellular matrix of previously established and newly forming tissues after only short-term (3 week) culture with TGFβ3 and without the need for continued exogenous supplementation. This finding may circumvent the concern of using TGFβ3 clinically, allowing for future exploration into the development of therapies utilizing tissues formed by redifferentiated cells for cartilage repair therapies.

Furthermore, in Chapter 3 we demonstrated that the severity of OA from which the chondrocytes were derived did not influence the ability of the cells to form cartilaginous tissues; a significant finding as chondrocytes from healthy cartilage are limited and may not be available for clinical therapies. Interestingly we showed that passaged (dedifferentiated) cells had a greater chondrogenic potential than primary (non-passaged) cells, which were unable to form tissue when cultured under the same conditions as passaged cells. Although TGFβ3 was indispensable for cartilaginous tissue formation by passaged cells it was unable to promote neocartilage formation by primary cells. Our results suggest that this may be due to upregulation of ENG with passaging in combination with the presence ALK5, both of which have been demonstrated to work together to facilitate TGFβ signalling via chondrogenic pathways [37]. Future studies are required to assess the role of ENG in neocartilage formation by passaged chondrocytes, a topic which has yet to be explored. These findings may aid in identifying suitable surface markers which can be used to sort for chondrocytes with enhanced chondrogenic potential. If translated clinically, these cell populations may be utilized to increase the success of therapies such as autologous chondrocyte implantation in which hyaline-like cartilage formation is limited.

One of the limitations to the use of redifferentiated chondrocytes is their resistance to hypertrophic differentiation and mineralization [52], [53], [110]. Although beneficial clinically when aiming to form stable hyaline cartilage, this feature limits the use of these cells for generating a ZCC. With bone marrow derived MSCs however, mineralization can be induced [52], [56], [59], [111]–[113] and therefore these cells were used to bioengineer a ZCC in Chapter 5. For the first time this study showed the potential for the use of FK506, a clinically used drug
and chemical activator of BMP signalling, for the induction of mineralization by chondrogenically pre-differentiated MSCs in high density culture. This is significant as the use of BMP2 clinically is advised against due to FDA warnings regarding the potential for adverse side effects. Using a two-fold cell seeding approach previously described by Lee et al. for sheep MSCs [114], the ZCC was layered with chondrogenically pre-differentiated MSCs to form a layer of hyaline-like cartilage. For clinical applications, future studies could explore layering the ZCC with redifferentiated chondrocytes, as these cells are resistant to hypertrophic differentiation and ColX accumulation in vitro and in vivo [53]. As an alternative to implanting bioengineered tissues we proposed the use of redifferentiated cells in fibrin gel; however, our pilot study indicated that redifferentiated chondrocytes did not enhance repair compared to dedifferentiated chondrocytes. Implanting pre-formed tissues may be a more ideal solution and is the recommended approach going forward.
6.4 References:


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