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Effect of Chondrocyte- Stem Cell interactions on Chondrogenesis of Mesenchymal Stem Cells

A thesis submitted in partial satisfaction of the
requirements for the degree Master of Science

in

Bioengineering

by

Gunjan Gupta

Committee in charge:

Professor Shyni Varghese, Chair
Professor Koichi Masuda
Professor Robert L. Sah

2009

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Chair

University of California, San Diego

2009

DEDICATION

For my amazing parents… Without their love, support and guidance I would not have the dreams I strive to fulfill.

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ACKNOWLEDGEMENTS

I would like to express my gratitude to all those who made it possible for me to complete this thesis. I am deeply indebted to my advisor Dr. Shyni Varghese for her faith in my ability, and whose help, stimulating suggestions and encouragement helped me all the time in my research and in writing the thesis. She was always there to guide me and made research smooth and rewarding.

Besides my advisor I am grateful to my other committee members Dr. Robert L. Sah and Dr. Koichi Masuda for their valuable time, encouragement and insightful comments. I also want to thank Dr. Sah for providing the Osteoarthritic cartilage without which this study would not have been possible.

I would also like to thank all my lab members who made it a convivial place to work and learn. In particular, I would like to thank Allison Finger for her patience, mentoring and advice throughout this study but especially when I was new in the lab. My special thanks to Susan Lin and Aereus Aung for their invaluable help in doing the experiments; Dr. Ramses Ayala for the brainstorming sessions; and Dr. Chao Zhang for knowing everything in the lab.

I cannot finish without saying how grateful I am to my family for giving me their unconditional love and support. Particular thanks, of course, to my parents my role models, and to Prasoon and Madhup, my brothers and best friends. Lastly, and most importantly, I wish to thank my loving husband, Yuvraj who has always been a pillar of support, encouraged me to do my best and just for always being with me.

ABSTRACT OF THE THESIS

Effect of Chondrocyte- Stem Cell interactions on Chondrogenesis of Mesenchymal Stem Cells

by

Gunjan Gupta

Master of Science in Bioengineering

University of California, San Diego, 2009

Professor Shyni Varghese, Chair

Mesenchymal stem cells (MSCs) have been considered as a promising cell source for cartilage tissue regeneration owing to their intrinsic musculoskeletal differentiation potential. A successful MSC-derived hyaline cartilage tissue should be structurally and functionally similar to native cartilage. During embryonic development morphogens have been shown to play a critical role in cartilage tissue formation. In this study we thus investigated the effect of chondrocyte-MSC interactions in the form of soluble morphogens on chondrogenic differentiation of MSCs using a co-culture system, wherein the hMSCs-laden hydrogels and chondrocytes were not in physical contact but can still exchange cell secreted factors. Healthy chondrocytes and osteoarthritic (OA) chondrocytes were able to induce chondrogenesis of hMSCs as analyzed by biochemical assays, gene analysis, and histology. We further explored the role of PTHrP and TGF-β1 on chondrogenesis of hMSCs and found that in their

combined presence, hMSCs had an upregulated expression of cartilage specific markers such as collagen type II and aggrecan and enhanced extracellular matrix production. Even in the absence of TGF-β1 co-culture could induce chondrogenesis. A detailed analysis indicated that TGF-β1 promotes proliferation and chondrogenic differentiation of MSCs, while PTHrP regulates hypertrophy. Mass spectrometry analysis underlined the crucial role of cross-talk on cell secreted morphogens. Extracellular histones inducing growth hormone production formed a major part of the morphogens unique to a co-culture system which is known to promote chondrogenesis. Our experimental observations along with numerical analysis indicated the crucial role of diffusion-mediated morphogenic concentration on chondrogenesis of MSCs.

Chapter 1: INTRODUCTION

Hyaline/Articular cartilage is a highly specialized tissue with excellent load bearing properties and low coefficient of friction allowing smooth joint functions. The specialized three-dimensional (3D) architecture of hyaline cartilage in conjunction with limited self-repairing ability and high functional demands placed upon the tissue make treating cartilage lesions a clinical challenge. The repair capacity of articular cartilage is very limited because of its avascular nature and low mitotic activity of articular chondrocytes embedded within the dense cartilage extracellular matrix. Thus, injuries of the articular cartilage often progress into degradation of the articular surface, leading to osteoarthritis (OA) which might eventually require total joint replacement. OA is a disease of joints in which articular cartilage undergoes dramatic changes due to chondrocyte death and matrix degradation, leading to total tissue breakdown. Age also plays an important role in development of arthritis, which in some form or the other currently affects more than 40 million people in America. Spontaneous repair of untreated articular cartilage injuries typically displays fibro-cartilagineous tissue formation, with reduced biochemical, structural and biomechanical qualities compared to hyaline cartilage.

Most of the current treatments are often limited to anti-inflammatory and pain relieving drugs, dietary supplements, and artificial implants with repetitive surgical interventions. In contrast, cell-based cartilage tissue regeneration therapy promises biologically and functionally active hyaline cartilage tissue replacements. Within the last decade, cell-based treatment strategies such as the transplantation of *in vitro* expanded autologous chondrocytes (ACT) have been explored for surgical intervention

to treat focal articular cartilage injuries. While chondrocytes can contribute to regeneration of functional hyaline cartilage tissue, acquiring clinically required number of chondrocytes from a small biopsy specimen (which themselves are likely diseased) poses a major challenge, and is exacerbated by their deleterious phenotypic changes upon *in vitro* expansion. To this end, MSCs have widely been investigated as an alternative cell source for treating cartilage defects. The advantages of MSCs are that they can be easily isolated and efficiently expanded without altering their phenotype and differentiation ability. Emerging studies also suggests an allogenic use of MSCs due to their immunopriviledged and immunosuppressive qualities, combating the problem of autologous cell transplantation with aged or diseased cells. Recent studies have also highlighted how MSCs secrete numerous trophic factors that enhance host tissue repair by rejuvenating the hostile environment of the damaged site.

Chondrogenic differentiation of MSCs *in vitro* is often achieved by culturing them in a three-dimensional format in presence of TGF-β superfamily growth factors. Differentiation of MSC into chondrocytes is characterized by upregulation of cartilage specific genes, collagen type II, IX, aggrecan, and biosynthesis of matrix components such as collagen type II and proteoglycans. Despite their promising *in vitro* results, successful *in vivo* transplantation of MSCs towards hyaline cartilage tissue regeneration has yet to be achieved. This could be attributed to inefficient chondrogenic differentiation, loss of transplanted cells, and *in vivo* integration failures. Furthermore, several studies investigating the chondrogenic differentiation of MSCs have demonstrated that adult bone marrow derived MSCs undergo an endochondral sequence of differentiation (downstream differentiation of MSC-derived chondrocytes into

hypertrophic chondrocytes followed by mineralization) forming heterotypic osseous tissue instead of stable hyaline cartilage tissues.

There is clearly a need to better understand the environmental cues that control chondrogenic differentiation of MSCs during development process and harnessing these principles to develop a biomimetic approach to regenerate MSC-based cartilage tissues for long lasting articular cartilage tissue repair. During development chondrogenic commitment of mesenchymal cells is largely regulated by their local environment characterized by cell-matrix interaction, soluble factors, and cell-cell interactions. The cell-cell interactions could be either direct contact or in the form of cell secreted soluble factors (morphogens) devoid of direct contacts. Morphogens not only play an important role in differentiation commitment of progenitor cells but also play an important role in defining the size and shape of the developing organs/tissues. It is not just during development that the morphogens act as regulators, the morphogenic factors arising from the diseased/damaged tissue also play a critical role in repair process, because they can modulate the engraftment, differentiation, and contribution (to repair) of adult stem cells those are migrated to the defect responding to the injury signals.

This thesis is focused on understanding the effect of chondrocyte secreted morphogens and their gradients on chondrogenic differentiation of MSCs with the long-term goal of developing MSC-based clinically viable therapeutic strategies for treating chondral cartilage defects. Our studies hinge on the central hypothesis that the differentiation commitment and morphogenesis of MSCs can be regulated using a microenvironment mediated by morphogens and their gradients.

The specific goals of this study can be summarized as:

1) Evaluation of the effect of primary bovine chondrocyte secreted morphogens on chondrogenic differentiation of MSCs encapsulated in PEGDA hydrogel.
2) Characterization of human osteoarthritic chondrocytes (OA chondrocytes) and evaluating the effect of OA chondrocyte secreted morphogens on MSCs.
3) Evaluation of the effect of *in vitro* expansion of healthy bovine and human OA chondrocytes on secreted morphogens.
4) Evaluation of the role of PTHrP and TGF-β1 signaling pathways in chondrogenic differentiation of MSCs.
5) Identification of the role of diffusion in morphogen-mediated stem cell differentiation.
6) Identification of OA chondrocyte secreted factors that influence chondrogenesis.
7) Engineering matrix interfacial properties to regulate chondrocytes phenotype during *in vitro* expansion.

To this end, the following experiments were designed and analyzed in this thesis:

MSCs encapsulated on poly(ethylene glycol) (PEG) hydrogels (constructs) were co-cultured with, (i) isolated primary bovine chondrocytes (BCC), (ii) primary OA chondrocytes, and (iii) *in vitro* expanded chondrocytes (both bovine and OA). Chondrocytes with various phenotypes were chosen as chondrocyte-secreted morphogenetic factors can differ extensively based on their phenotype. The difference in morphogenetic signals will in turn govern their effect on chondrogenic differentiation of MSCs. We first evaluated the effect of healthy chondrocytes, using bovine chondrocytes (2-4 weeks old calf), on chondrogenic differentiation of human

MSCs. In order to examine the effect of *in vitro* expanded chondrocytes, we have used passage three (P3) chondrocytes. In the case of OA chondrocytes, we used primary OA chondrocytes (grade A) and P1 OA (passaged once) chondrocytes. Chondrogenic differentiation of MSCs was analyzed for morphology, tissue organization, matrix accumulation, and gene expression profile *in vitro* after 21 days of culture. Results were compared against MSCs-laden PEG hydrogels cultured in chondrocyte-conditioned medium containing chondrogenic inducing factors, and non co-cultured MSCs (control) grown in medium containing chondrogenic inducing factors but not chondrocyte secreted factors.

Based on the finding from the above-mentioned experiments, we then investigated the role of signaling molecules TGF-β1 in inducing chondrogenesis and then PTHrP for preventing hypertrophy in hMSC derived cartilage cells and using OA chondrocytes. Additionally, OA chondrocyte secreted factors were identified using a combinatorial mass spectrometry proteomic analysis. For mass spectrometry analysis, supernatants from the following three systems were used: (i) MSC chondrocytes co-culture systems, (ii) MSCs alone (cultured in both growth and differentiating medium) and (iii) chondrocytes alone (for both P0 and P1 OA). A Venn diagram was drawn to then identify the morphogens unique to a co-culture system in anticipation that these cell secreted factors might be contributing to the chondrogenesis of MSCs.

It was observed that the morphogenetic factors mediating chondrogenic differentiation of MSCs depend strongly on the position relative to the chondrocytes and time as evident from the histological sections where the outer surface of the tissue sections

exhibiting cartilage tissue specific staining that progressively reduced from outside to inside.

In order to further verify that indeed the differentiation of MSCs is strongly dependent upon the concentrations of morphogens, we have used computational finite difference scheme to solve the 3D concentration profiles of the morphogenic factors within the cell-laden cylindrical hydrogels.

One of the characteristics of chondrocytes is that they de-differentiate (i.e. lose their chondrocyte phenotype) during their two-dimensional (2D) *in vitro* expansion. Various reports have demonstrated that the de-differentiation of chondrocytes can be limited by providing an appropriate growing environment involving geometrical cues, matrix components, and/or growth factors. In this thesis, we also evaluated the role of cell-matrix interactions in terms of matrix hydrophilic-hydrophobic balance on modulating the chondrocyte phenotype during their 2D *in vitro* expansion. Different hydrogel surfaces were tested for *in vitro* chondrocyte expansion till passage P2 and compared against chondrocytes cultured on standard tissue culture plates (control). The analysis was based on cell adhesion, cell morphology, population doubling, gene expression, and staining.

A quick summary of my findings:

1) hMSC-laden PEG hydrogels co-cultured with primary BCCs exhibited enhanced chondrogenic differentiation .
2) Passaged P3 BCCs did not have a significant effect on chondrogenic differentiation of hMSCs

3) OA chondrocytes were found to express ALP and collagen type X along with other normal chondrogenic markers such as aggrecan and collagen type II. They took a long time (about a week) to attach to the tissue culture plate and proliferate.
4) OA cells de-differentiated after one passage (monolayer culture on standard culture dish). P1 OA chondrocytes were adhered to the culture plate within the first 24 hours unlike the P0 OA cells
5) P0 OA chondrocytes induced chondrogenesis in hMSCs when they were co-cultured. But P0 OA conditioned medium was not effective in inducing chondrogenic differentiations.
6) Co-culture with passaged P1 OA did not induce any chondrogenesis.
7) Chondrogenesis of hMSCs in co-culture can be induced even in the absence of TGF-β1 unlike the control, but the cell proliferation decreases significantly in its absence.
8) hMSCs cultured in conditioned medium exhibited higher levels of collagen type I and type X.
9) Signaling molecule, PTHrP was found to be able to prevent hypertrophic differentiation of hMSC-derived chondrocyte, while promoting their chondrogenic differentiation. Blocking the PTHrP pathway induced the hMSCs-derived chondrocytes to undergo terminal differentiation into hypertrophic chondrocytes. hMSC co-cultured in the presence of both TGF-β1 and PTHrP exhibited maximum chondrogenesis.
10) Identification of chondrocyte secreted factors showed a number of soluble factors in chondrocytes-hMSCs co-culture those were not secreted by hMSC alone or

chondrocytes alone supporting our hypothesis that the cross-talk between the cells plays an important role in cell secreted factors.

11) Hisological analysis in conjunction with modeling suggests that the differentiation pattern of hMSCs is strongly dependent upon the concentration gradient of morphogens. It was observed that MSC-derived chondrocytes undergo hypertrophic differentiation below a certain critical concentration and in our system it was diffusion limited.

12) Hydrogels having a balance of hydrophilic and hydrophobic interactions were tested as an alternative cell culture surface to expand chondrocytes *in vitro* in 2D. The primary results appeared to be highly encouraging; however detailed analysis is needed to confirm the initial findings.

Chapter 2: LITERATURE REVIEW:

2.1) Cartilage

Cartilage is a type of dense connective tissue found in many areas in the body including the articular surface of the bones, the rib cage, the ear, the nose, the bronchial tubes and the intervertebral discs. Its mechanical properties are intermediate between bone and dense connective tissue like tendon and is composed of specialized cells called chondrocytes that produce a large amount of extracellular gel like matrix rich in water, proteoglycans, and collagen fibers [3]. There are mainly 3 types of cartilage: articular cartilage, fibrocartilage and elastic cartilage where in articular cartilage lines the joints between bones and allows for smooth movement, weight bearing and shock absorption [4].

2.2) Articular Cartilage arrangement

Articular cartilage is a hierarchically organized tissue, which enables smooth function of joints by providing a low-friction and a load bearing surface. Articular cartilage can be divided into three zones: the tangential (superficial) zone (SZ), the transitional (middle) zone (MZ) and the radial (deep) zone (DZ), with each region exhibiting characteristic cellular phenotype and matrix properties and composition [3, 5-7]. SZ forms the smooth surface of the joint with densely packed collagen fibers parallel to articular surface. MZ on the other hand has randomly oriented collagen fibers. The collagen fibers in DZ are the largest and are primarily perpendicular to the joint surface. The deepest layer of articular cartilage (tidemark region) is the zone of calcified cartilage, which separates the articular cartilage from the subchondral bone.

The collagen fibers of the radial zone are anchored in the tidemark region [8]. The tidemark and the calcified cartilage collectively constitute the osteochondral interface, which functions as a physical barrier for vascularization and facilitate the pressurization and physiological loading of articular cartilage. The differences in zonal phenotype can be attributed to the different functional roles of the layers like load bearing and mechanical forces they are subjected to. For example, chondrocytes from DZ produce more proteoglycan due to their compressive stress resistance role as compared to SZ chondrocytes that produce more collagen to resist tension. [9]. The three layers of cartilage also interact and play a very important role in chondrocyte mineralization regulated by local paracrine factors such as PTHrP and TGF-β1. DZ chondrocytes become hypertrophic and move down to the endochondral layer to form osteoblasts, the precursors of bone in a feedback loop mediated by PTHrP and Indian Hedgehog [10] [11, 12] (Figure 4). Fig. 1 is adapted from (Adapted from The American journal of sports).

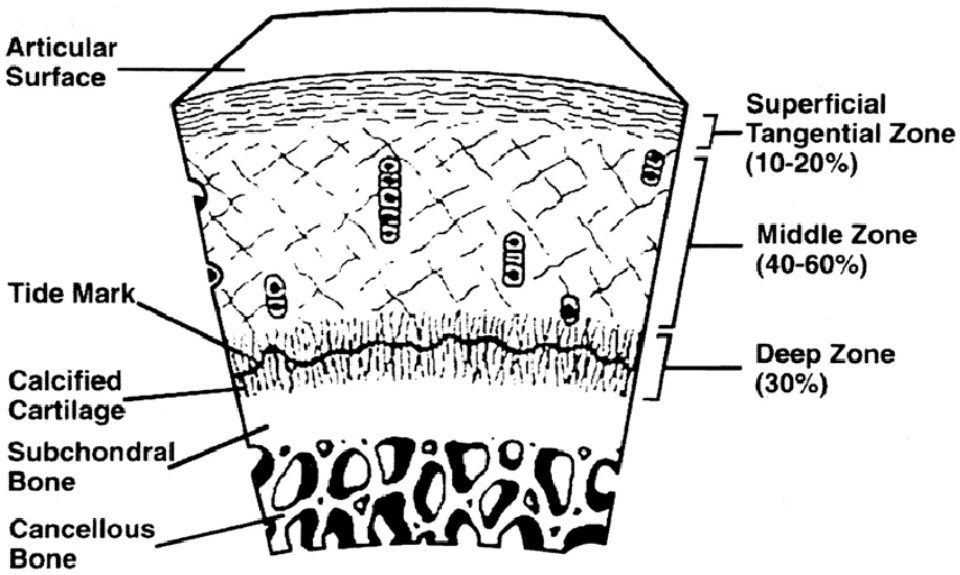


Figure 1: Different layers of articular cartilage

One of the characteristics of articular cartilage is that they are avascular, which drastically reduces self repair capacity of the articular cartilage. Degradation, injury, wear and tear of articular cartilage results in pain and restriction of joint movement. Since articular cartilage shows little or no intrinsic capacity for repair in response to injury or disease, it often leads to progressive damage and joint degeneration such as Osteoarthritis (OA). It is not just the cartilage injuries that lead to OA development. OA is also a degenerative process mostly related to aging leading to slow degradation and loss of cartilage tissue. During OA, the homeostatic balance between degradation and synthesis of cartilage tissue is disturbed and cartilage loses its structural and functional integrity leading to fibrillation and formation of fissures. According to Gerstenfeld et al. [13] it currently affects over 40 million people in the United States and this number would increase to 60 million by 2020. A variety of treatment strategies have been used to treat cartilage lesions which includes transplantation of autograft and allograft plugs, microfracture, drilling of the subchondral bone, autologus chondrocyte implantation, and material based implants, and none of these have been entirely successful in creating cartilage tissues which are structurally and functionally amenable to native tissue.

2.3) Current therapeutic options and cell sources

Due to its limited capacity for regeneration and self-repair as well as the scarcity of available therapeutic options, lesions of articular cartilage can have severe consequences [14]. The current treatments are based on 2 fundamentally different strategies to repair damaged articular cartilage: 1) stimulation of articular cartilage

repair, or 2) transplantation of articular cartilage. Table 1, adapted from Reference [14] lists some of the current treatments.

Table 1: Current treatments.

Method	Technique
Penetration of subchondral bone	Debride damaged or degenerated articular cartilage and penetrate subchondral bone by drilling, microfracture, or abrasion.
Osteotomy	Alter joint alignment to decrease contact stresses on the regions of damaged articular cartilage.
Joint distraction	Temporarily decrease joint contact stresses by use of an external fixator.
Soft tissue grafts-periosteum or perichondrium	Replace damaged or lost articular cartilage with soft tissue grafts that can form cartilage.
Cell transplantation-chondrocytes and MSCs	Harvest cells that can form cartilage, grow them in culture, and implant them in chondral or osteochondral defects
Growth factors	Apply or implant growth factors that stimulate cartilage formation in chondral or osteochondral defects.
Artificial matrices and biomaterials	Implant artificial matrices/biomaterials that allow or promote cartilage formation in chondral and osteochondral defects (these matrices may be combined with growth factors or cell transplants).

Methods of stimulating repair include penetration of subchondral bone, reduction of articular surface contact stress by osteotomy or joint distraction, and transplantation methods include implantation of soft tissue grafts, chondrocytes, mesenchymal stem cells, growth factors, or artificial matrices. Size-limited injuries specifically can be

treated by autologous chondrocyte transplantation (ACT). But none of these treatments so far have proved to be functionally efficient because of several reasons like being too invasive, inability to restore biological function and strength, long term stability, formation of fibrous cartilage, shortage of autografts/allografts, and *in vivo* integration failures [15-17]. Many of these treatments reduce the symptoms and pain but do not restore normal function of hyaline cartilage tissue. The age of the patient may also influence the outcome of any corrective therapy.

Although the developments in materials sciences and surgical strategies have evolved to offer better outcomes, regaining the efficient functionality of articular cartilage (joint) relies on replacement of the lost tissue with biological replacements, which structurally and functionally mimic the native tissue. This concept drives the development of cell based therapies such as ACT for cartilage repair. Indeed chondrocyte-based cartilage tissue repair is the best option because they are already committed to form cartilage tissue. However, one of the limitations of chondrocyte based repair strategies is the small number of chondrocytes that can be extracted, induced cartilage lesion (on the non-load bearing surface) associated with biospsy, and the difficulty in *in vitro* expansion of chondrocytes in monolayer wherein they lose their phenotype upon expansion. Various studies have demonstrated strategies, mainly involving 3D cultures and exogenous growth factors which can retard the de-differentiation of chondrocytes upon expansion or re-differentiate chondrocytes that had de-differentiated [18-20]. It has also been reported that proliferative capacity of chondrocytes decreases with the person's age [2]. Furthermore, ACT-based strategies may not be applicable for treatments associated with OA because of their diseased state

[20, 21]. To this end, adult mesenchymal stem cells (MSCs) exhibiting inherent chondrogenic differentiation potential have been touted as an alternative cell source of cartilage tissue regeneration. In fact, surgical treatments involving bleeding of subchondral bone harness the chondrogenic differentiation potential of MSCs to repair cartilage lesions. However, the disadvantages of these procedures are the transient clinical improvements, and fibrocartilage formation with poor durability [13]. This warrants the need for developing strategies that enhance contribution of MSC into hyaline cartilage formation [22].

2.4) Human Mesenchymal Stem cells as a possible cell source

MSC-based cartilage tissue regeneration is an attractive proposition because of their easy isolation, immuosuppressive properties, and ability to proliferate and differentiate into chondrocytes given the right cues [11, 12]. However, chondrogenic differentiation of MSCs often results in heterotypic osseous tissue mimicking the endochondral ossification (long bone formation) of mesenchymal cells during development [11, 23]. During development, the endochondral ossification involves condensation of mesenchymal cells followed by their differentiation into chondrocytes and cartilage tissue formation. Some of these chondrocytes (chondrocytes in the centre of the cartilage mold) stop proliferating and undergo hypertrophic differentiation. These hypertrophic chondrocytes promote calcification of the matrix and recruit blood vessels through secretion of various angiogenic factors such as vascular endothelial growth factor. The hypertrophic chodnrocytes later undergo apoptosis and the cartilage matrix

left behind serves as a scaffold for bone formation [24]. A schematic of the endochondral ossification process is depicted in Fig. 2.

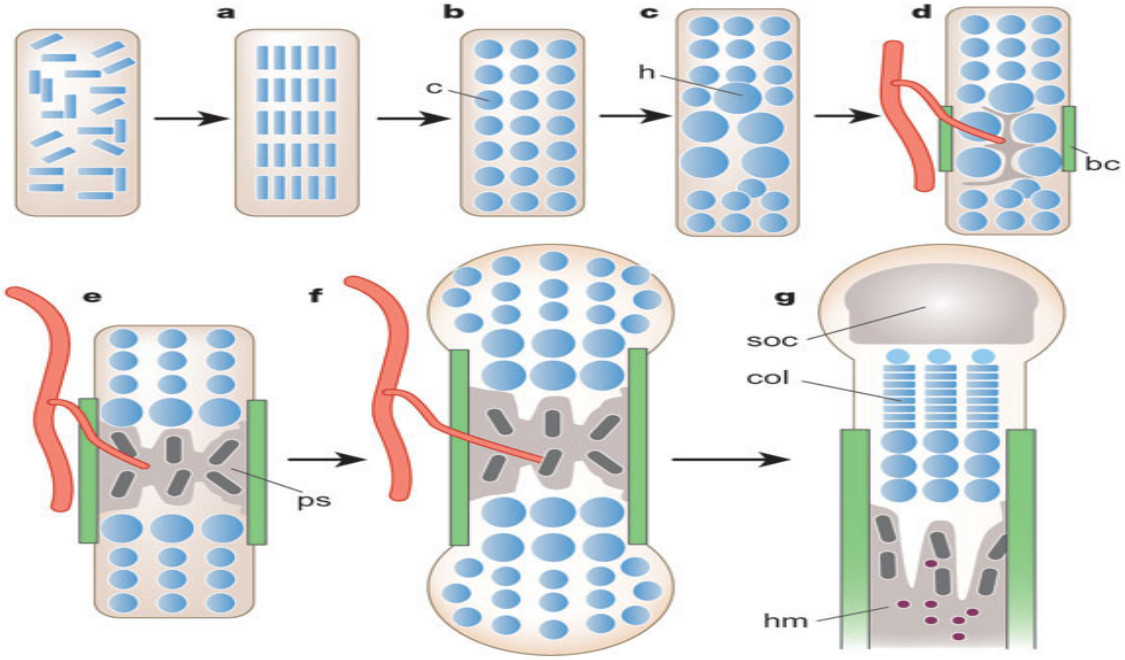


Figure 2: Endochondral ossification
Adapted from [29]: a, Mesenchymal cells condense. b, Cells of condensations become chondrocytes (c). c, Chondrocytes at the centre of condensation stop proliferating and become hypertrophic (h). d, Perichondrial cells adjacent to hypertrophic chondrocytes become osteoblasts, forming bone collar (bc). Hypertrophic chondrocytes direct the formation of mineralized matrix, attract blood vessels, and undergo apoptosis. e, Osteoblasts of primary spongiosa accompany vascular invasion, forming the primary spongiosa (ps). f, Chondrocytes continue to proliferate, lengthening the bone. Osteoblasts of primary spongiosa are precursors of eventual trabecular bone; osteoblasts of bone collar become cortical bone. g, At the end of the bone, the secondary ossification centre (soc) forms through cycles of chondrocyte hypertrophy, vascular invasion and osteoblast activity. The growth plate below the secondary centre of ossification forms orderly columns of proliferating chondrocytes (col). Haematopoietic marrow (hm) expands in marrow space along with stromal cells.

Insights from the endochondral ossification process suggest that during development, mesenchymal cells give rise to two types of cartilage: permanent and transient. The hyaline cartilage is found mainly in articular cartilage, while the transient cartilage is found in the growth plate. The chondrocytes of transient cartilage undergo

terminal differentiation into hypertrophic chondrocytes, followed by apoptosis and calcification leading to long bone formation as discussed earlier.

It is not only that the differentiating MSCs in chondrogenic cultures, express genes or presence of protein corresponding to hypertrophic chondrocytes, but also undergo mineralization in vivo [13, 22, 25, 26]. These findings are a severe concern for the clinical applications of MSCs for cartilage repair. Additionally, in vivo transplantation of MSCs results in their apoptosis and it is strongly dependent upon their pre-differentiation (or committed) state [27]. Studies by Mauck et al. [28] have showed that while MSCs undergo chondrogenic differentiation of MSCs, the mechanical properties of the engineered tissue and the amount of extracellular matrix formed are lower than that of chondrocytes under identical conditions . Therefore, a successful MSC-mediated hyaline cartilage tissue repair requires enhanced *in vivo* cell survival, effective and efficient chondrogenic differentiation of MSCs, and formation of functionally and phenotypically stable hyaline cartilage tissue (i.e., maintaining MSC-derived chondrocytes in their pre-hypertrophic state by preventing terminal differentiation of MSC-derived chondrocytes into hypertrophic chondrocytes followed by matrix mineralization). This requires an understanding of developmental cues and factors, which promote chondrogenic differentiation of MSCs into permanent hyaline cartilage by preventing their terminal differentiation into hypertrophic chondrocytes and incorporating these insights into tissue engineering strategies to regenerate MSC-based hyaline cartilage.

Skeletogenesis is an orchestrated multi-step process involving mesenchymal cell recruitment, migration, proliferation, and *in vivo* condensation. This complex process is

controlled by cellular interactions with the surrounding matrix, growth and differentiation factors, and other environmental factors that initiate or suppress cellular signaling pathways and transcription of specific genes in a temporal-spatial manner. Various approaches involving biomaterials (3D structural support), exogenous growth factors, bioreactor for dynamic mechanical cues, chondrocyte secreted factors, and genetic manipulations have been used in the past to promote chondrogenic differentiation of stem cells [14, 20, 29-32]. While these approaches have been showed to be effective in chondrogenic differentiation of MSCs, they were not effective in preventing terminal differentiation of MSC-derived chondrocytes into hypertrophic chondrocytes [22, 33]. Furthermore, current tissue engineering approaches based on engineering MSC-based cartilage tissues with structural and functional properties amenable to native cartilage tissue need to integrate developmental cues. Hence the development of effective methods to maintain an articular cartilage phenotype without hypertrophy, ossification or fibrogenesis, and a delivery system to localize the cells within a lesion without compromising their chondrogenic differentiation or the integrity of the repair tissue are additional requirements for the use of MSCs in articular cartilage regeneration.

Interestingly, it has been shown that the chondrogenic differentiation of MSCs can be regulated by preserving the *in vivo* conditions and spatial geometry which induces a much more stable chondrogenesis compared to *in vitro* models [34]. There are also studies showing a dependency between *in vivo* mineralization of transplanted MSCs and their pre-differentiation state [17, 35].

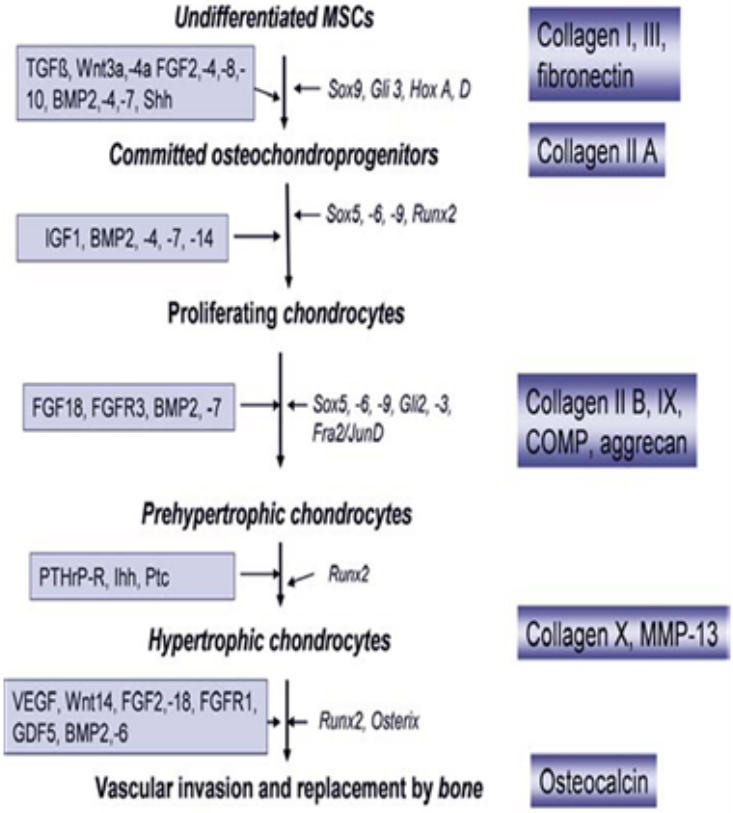


Figure 3: Chondrogenic differentiation cascade.
Mesenchymal stem cells undergo chondrogenic differentiation during embryonic development and produce cartilaginous Anlagen for bone development. The entire cascade is governed by an interconnected web of growth factors, transcription factors, matrix molecules etc. The highest profile among them has transcription factor Sox9 whose expression is necessary for the initiation of chondrogenic differentiation while at hypertrophic stages it is suppressed. This figure shows different stages of chondrogenesis along with the stage-specific ECM markers. Signaling and growth factors are shown in light grey boxes and matrix molecules are depicted in two-colored grey boxes. **[41]**

2.5) Role of different signaling factors

2.5.1) Sox-9

SOX9 is essential for the differentiation of condensed mesenchymal cells into chondrocytes and acts further at every stage of chondrocyte differentiation. For instance, SOX9 is expressed in mesenchymal cells undergoing condensations and in proliferating chondrocytes, but not in hypertrophic chondrocytes. In cultured cells, it

stimulates transcription of a number of cartilage specific matrix genes, including Col2 (a1), Col1 (a2) and aggrecan [36, 37].

2.5.2) Transforming Growth Factors

Transforming growth factors have been shown to play an important role in chondrogenic differentiation of MSCs [34, 38] *in vitro.* TGF-β1 induced chondrogenesis is a well-established standard procedure in high-density cultures resulting in the appearance of a chondrocyte-like phenotype [38]. Experimental evidence also indicates that cartilage tissue contains large amounts of TGF-β1 and surface molecules for TGF-β1 activity and it plays an important role in proliferation and differentiation of chondrocytes embedded in cartilage matrices. There is a marked increase in the proliferation capacity of hMSCs and differentiated chondrocytes in the presence of TGF-β1 [34]. In addition, TGF-β1 has been proved to play an important role in early stages of chondrogenesis as well by stimulating the production of type 2 collagen and proteoglycans [30, 39, 40]. Chondrogenic differentiation of MSCs is characterized by upregulation of cartilage-specific molecules such as collagen type II and aggrecan. However, during long term culture, the MSC-derived chondrocytes undergo further differentiation characterized by upregulation of collagen X and ALP (Alkaline Phosphatase). This is predominant *in vivo* where MSC-derived chondrocytes undergo calcification. Previous studies have also demonstrated the potential role of TGF-β/Smad signaling pathways in repressing chondrocyte hypertrophy differentiation and maintaining articular cartilage phenotype.

Thus, although TGF-β1 is good for induction of chondrogenic differentiation of MSCs it is not solely enough to prevent terminal differentiation MSC-derived chondrocytes into hypertrophic chondrocytes [41]. Several culture systems have been devised and optimized for the induction of hMSCs into chondrocytes using other growth factors along with TGF-β1 like IGF, dexamethasone etc. in pellet culture [31, 42]. However, various studies have reported the increased expression of collagen type X and ALP activity in most of these *in-vitro* chondrogenesis of hMSCs.

Potential of hMSC chondrogenesis has also been explored in a 3D environment like PEGDA, PLGA and other hydrogels to try and provide the native environment and minimally invasive surgical procedures [43, 44].

2.5.3) PTHrP

Advancement of the calcified region and tidemark duplication are observed with age and progressive OA. Paracrine regulators such as Parathyroid hormone related protein (PTHrP) have been shown to play an important role in chondrocyte proliferation and differentiation during endochondral bone formation [12]. A recent study by Jiang et al. also indicates the role of PTHrP on mineralization of chondrocytes/cartilage tissue [7]. PTHrP is a protein secreted during fetal life by perichondrial cells at the ends of cartilage moulds and by early proliferative chondrocytes. PTHrP acts on the same G-protein-coupled receptor used by parathyroid hormone, the calcium-regulating hormone. These PTH/PTHrP receptors (PPRs) are expressed at low levels by proliferating chondrocytes and at high levels by pre-hypertrophic/early hypertrophic chondrocytes. After the permanent and transient cartilage is formed in fetal life, they

synthesize PTHrP. This ligand then acts on PTH/PTHrP receptors on chondrocytes; its action keeps chondrocytes proliferating and delays their further differentiation. Indian hedgehog (Ihh) is synthesized by chondrocytes that have just stopped proliferating and is required for synthesis of PTHrP. Ihh stimulates chondrocyte proliferation directly and, through stimulation of PTHrP synthesis, determines the distance from the end of the bone at which chondrocytes stop proliferating and undergo hypertrophic differentiation. The feedback loop between PTHrP and Ihh serves to regulate the pace of chondrocyte differentiation and the sites at which perichondrial cells first differentiate into osteoblasts. PTHrP acts by phosphorylation of transcription factor Sox 9 and suppression of synthesis of mRNA coding for Runx2. It has been shown that treatment with PTHrP increases chondrogenesis as well [45].

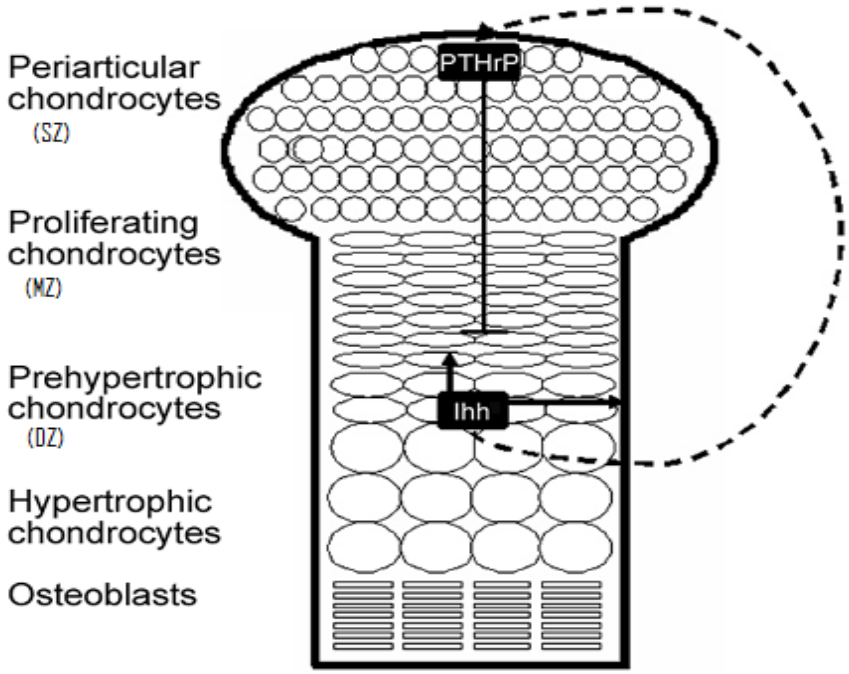


Figure 4: Ihh/PTHrP feedback loop [46]

2.6) Effect of Microenvironment on chondrogenic differentiation of MSCs

In native environment, the lineage/tissue specific differentiation of stem cells are regulated by their local microenvironment comprising of extracellular matrices, growth factors and cytokines, paracrine regulators, and cell secreted morphogens. The cellular events of chondrogenesis are strictly regulated by cues from their local environment. Regulatory genes for chondrogenesis are controlled by these environmental factors and cellular interactions that are a part of complex signaling processes involving oxygen levels, mechanical stress, cellular contact etc. that in turn maintain homeostasis and the chondrogenic phenotype. It has been recently proven that extra cellular matrix of cartilage creates a specific niche for chondrogenic differentiation of hMSCs which is in turn directed by the cross talk between the stem cells and the micro-environment [2, 47]. In articular cartilage, chondrocytes are surrounded by an abundant ECM, which is composed of a highly hydrated complex network of molecules. 3D environment is a pivotal factor that has a significant role in supporting or in restoring the chondrocytic phenotype. Therefore, 3D environments have been used for chondrogenic differentiation of MSCs using pellet [48] or micromass [49] culture systems, or biomaterial support. The former two culture conditions are known to promote cell-cell aggregations, while the latter provides a 3D environment. Both in pellet and micromass culture, MSCs are packed or seeded in high cell density to mimic the mesenchymal condensation observed during embryologic chondrogenesis and to promote cell–cell contact. Additionally, various studies have investigated the effect of cell-matrix

interactions, mechanical cues, and growth factors mimicking the developmental approaches to regulate chondrogenic differentiation of MSCs.

In addition to these environmental factors, cell secreted morphogens have also been utilized to modulate differentiation signaling pathways leading to commitment and tissue formation. Many of these studies have implicated that mechanisms of endochondral ossification of condensing mesenchyme can be regulated by cell-secreted morphogenetic factors. Chondrocyte secreted factors have been in the past shown to regulate both chondrogenic and osteogenic differentiation of MSCs [25, 26, 50-52]. In addition to chondrocytes, embryonic calvarial [53], embryonic limb bud [54], and articular perichondrial cells [55] have also been reported to have stimulatory effects on chondrogenesis. Studies have also investigated the effect of chondrocyte secreted factors on chondrogenic differentiation of stem cells/progenitor cells [32, 56]. Co-culture systems are extensively employed to study cell-cell interactions and the role of paracrine factors. Figure 5 shows the schematics of how co-culture and cell secreted factors direct stem cell differentiation.

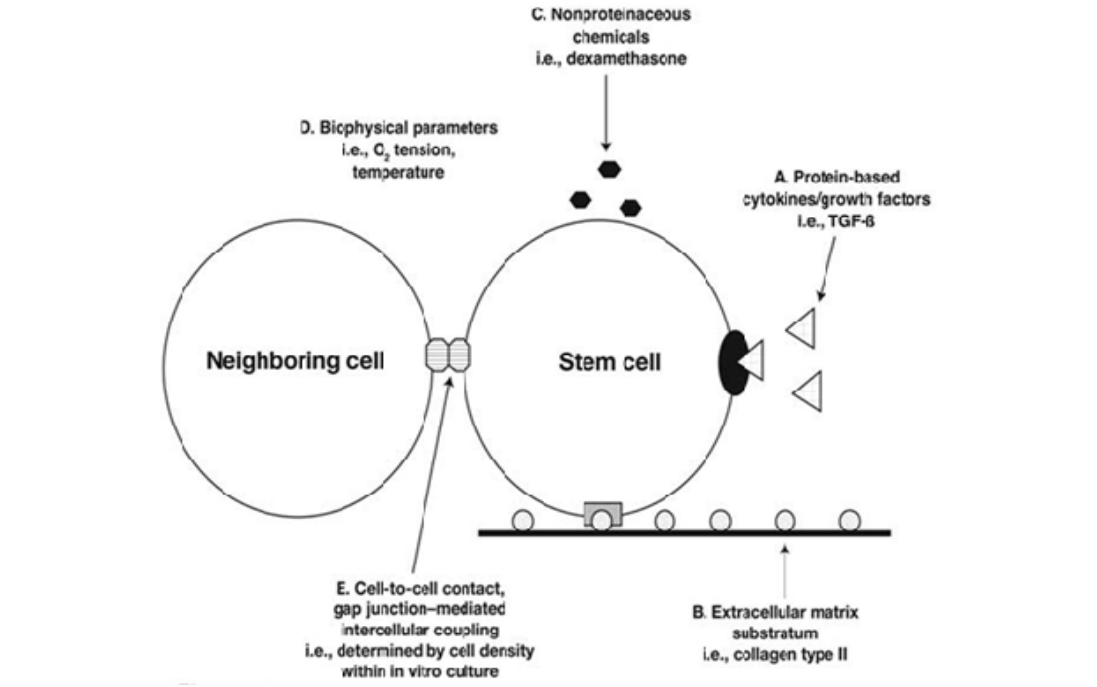


Figure 5: Schematics of directing stem cell differentiation.
Schematic diagram representing the major components of a defined culture milieu for directing the differentiation of stem cells into the chondrogenic lineage. **(A):** Protein-based cytokines and growth factors; **(B):** extracellular matrix; **(C):** nonproteinaceous chemicals; **(D):** biophysical parameters such as O2 tension and temperature; **(E):** cell density, which determines the degree of cell-to-cell contact and gap junction–mediated intercellular coupling. [2]

In choosing a model system for *in vitro* experiments, there has to be a balance between being able to observe measure or manipulate cell behavior while mimicking the native environment. Most tissues in the body consist of more than one cell type and matrix which are continuously interacting to maintain normal function and repair. In a co-culture system, two or more cell types brought together in the same culture environment very likely interact and communicate and thus copy normal development and homeostasis [1, 41]. Given the layered spatial arrangement of chondrocytes in the cartilage, it is believed that the differentiation pathway of hMSCs from stem cells to chondrocytes to osteoblasts is governed by precise cellular interactions *in vivo*.

Homologous and heterologous intercellular communication is critically involved in the development of cartilage during differentiation.

A study of these cellular interactions using a co-culture model is an essential step towards inhibition of terminal differentiation of chondrocytes in vitro. It is very unlikely that complete cartilage regeneration is initiated by the delivery of one growth factor applied at a certain dose at one time point or during a specific duration of time, or that delivery of one (or two) growth factors to a defect can trigger the whole cascade of cellular events necessary for regeneration into normally shaped and functional cartilage tissue. A whole range of factors exchanged during co-culture of two or more cell types might be involved in tissue regeneration.

It has been shown that co-culture of articular cartilage in a transwell system (without cell-cell contact) with immature growth plate cartilage was able to inhibit/delay terminal differentiation [57]. One study also explored the mixed culture of hMSCs and chondrocytes and showed a paracrine effect/ cell-cell interaction effect to improve cartilage phenotype and matrix production [58]. Coculturing of a human ESC line maintained in suspension with human chondrocytes in the presence of 10% FBS resulted in induction of collagen II and Sox9 protein expression by paracrine factors derived from the chondrocytes [51].

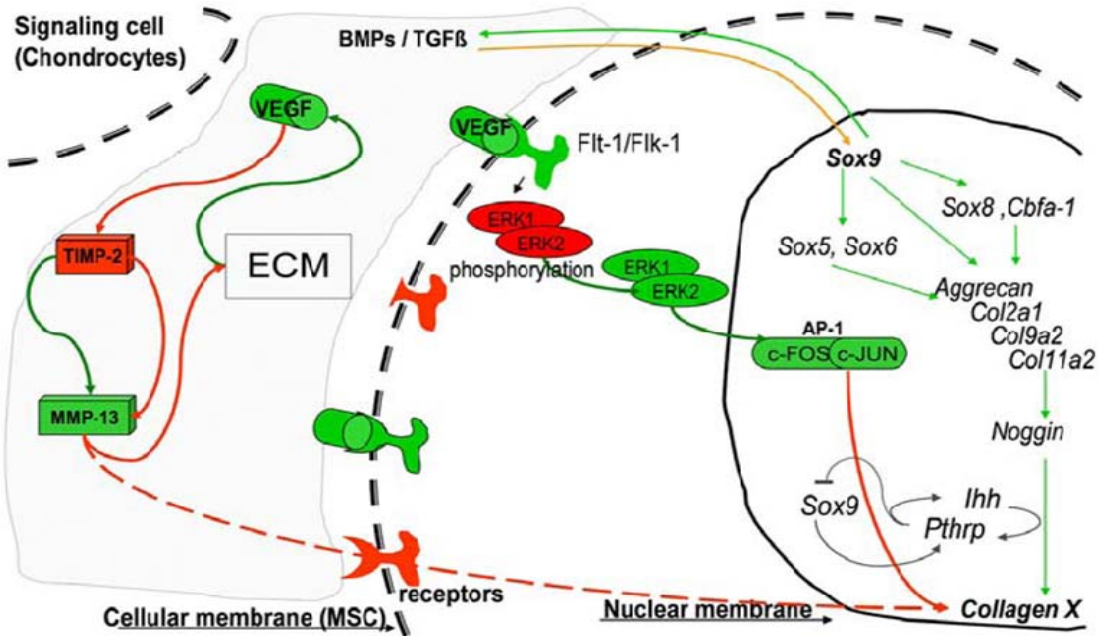


Figure 6: Co culture effect: Molecular events during chondrogenesis.
Schematic representation of known and putative molecular control of chondrogenic differentiation by selected signaling molecules. The effect can be pre-transcriptional by suppressing gene expression, post-transcriptional on protein biosynthesis or post-translational by matrix modulation via proteases and their inhibitors. Green arrows indicate upregulation of gene and protein expression, yellow arrows depict a positive effect to maintain the expression and red arrows mean inhibition of the gene and protein expression. (Adapted from [1])

Studies on paracrine signals from the microenvironment and their effect on MSC commitment and differentiation have demonstrated that the chondrogenic differentiation pathway of bone marrow derived MSCs is strongly influenced by the representative microenvironment. The important observation that cartilage tissue derived factors can suppress collagen X expression is a foundation for future studies on soluble factors to discover a direct correlation between the effect and the putative effectors and to identify the signaling molecules and cascades involved. Recent studies [50, 59] identify molecular markers and delineate the different stages in chondrogenesis along with describing a co culture system for hMSCs and human chondrocytes to obtain

neocartilage by closely emulating the natural conditions. The effect can be explained by some examples like it is known that mature chondrocytes secret TGF-β and extracellular matrixes, such as type II collagen, which are both strong chondrogenic inductive potential.

2.7) Osteoarthritic (OA) cells v/s normal cells

As we have discussed, during development stem cell commitment is largely regulated by their local environment, mainly characterized by cell secreted paracrine factors (morphogens). It is not just during development that the morphogens act as a regulators, the morphogenic factors arising from the diseased/damaged tissue also play a critical role in repair process, because they can modulate the engraftment, differentiation, and contribution (to repair) of adult stem cells that migrate to the defect responding to the injury signals. Interestingly some studies have indicated an increased number of MSCs and chondro-progenitor cells in OA cells as compared to normal cells [60]. A different study has compared the chondrogenic potential of normal versus osteoarthritic cartilage [61]. It shows a clear difference between chondrogenic potential and cartilage patterns of normal and OA tissue. Except the hypertrophic markers, little significant difference was observed when gene expression profiles of OA chondrocytes were compared to that of normal [35]. Suggesting that the biosynthetic profile of chondrocytes would depend on the in vitro culture conditions rather than on the diseased state of the chondrocytes. Given that most of the autologous chondrocyte source for an osteoarthritic patient is degraded tissue, we concentrated on investigating

the effect of OA chondrocytes on chondrogenic differentiation of MSCs developing an optimal co-culture system to study different pathways involved like TGF-β and PTHrP.

Chapter 3: MATERIALS & METHODS

3.1.) Cell culture:

3.1.1) Human Mesenchymal stem cells:

p7043L hMSC cell line was obtained from Tulane University as P0. Cells were cultured at 37 ºC and 5% CO_2 in T-175 flasks at the seeding density of ~4000 cells/cm^2 in MSC growth medium (MSC-GM; 500 ml high glucose DMEM supplemented with 20% Premium Select FBS, 1% L-Glutamine and 1% Pen/Strep) till they became confluent and were then subsequently passaged. Medium was changed twice a week. Cells were passaged with 0.025% trypsin–EDTA for 5 min at 37°C and replated in T-175 flasks at 4000 cells/cm^2. MSCs were frozen in liquid nitrogen in freezing medium (1 million cells/ml) until needed. Freezing medium for hMSCs was made up of: 65 ml αMEM (with 2mM L-glutamine) + 30 ml premium select FBS + 5ml DMSO + 1 ml Pen/strep. Cell viability after thawing was consistently above 92%. When needed, the frozen cells were thawed, plated in 175-cm^2 flasks in MSC-GM and grown until confluent. Passage 4 cells were trypsinized, centrifuged at 1500 rpm for 10 mins, and resuspended in hydrogel solution as described below in Sec. 3.1.2.

3.1.2) **Encapsulation of hMSCs using photopolymerization:**

Hydrogel solution was prepared by mixing 10% (w/v) poly(ethylene glycol)-diacrylate (Nectar) in sterile phosphate-buffered saline (PBS) with penicillin (100 µg/ml) and streptomycin (100 *m*g/mL). The photoinitiator, Igracure D 2959 (10% w/v in 70%EtOH), was added to the PEGDA solution and mixed thoroughly to make a final concentration of 0.05% (w/v). Immediately before photo-encapsulation, MSCs were

resuspended in the polymer solution to make a concentration of 20 million cells/mL and gently mixed to make a homogeneous suspension. 65 µl of cell–polymer–photoinitiator suspension was transferred into cylindrical molds (made of sterilized caps of 0.5 ml micro centrifuge tubes) with a ~6-mm internal diameter and exposed for 5 min to long-wave, 365-nm ultraviolet (UV) light at 4 mW/cm^2. The hydrogels (pore size ~10 nm) were then removed from their molds and transferred to the trans-wells (pore size 0.4 µm) in the 24 well plate of the co-culture system [43].

3.1.3) Isolation and Culture of Osteoarthritic cartilage:

OA tissue chunks were obtained from patients undergoing knee transplant at UCSD. The two samples we got were both Grade A: a) 1.97g from a 62 year old female, and b) 1.3 g from a 73 year old female.

Chondrocytes were extracted using the following protocol-

1) A 10 cm culture dish was used to dice the cartilage chunks into ~1 mm^3 pieces using a scalpel blade.
2) Cartilage pieces were washed with PBS+pen/strep a total of 3 times and transferred to 50 ml conical tubes.

   Collagenase solution was added (For 3 g of tissue: 0.045g collagenase type II, dissolved in 30 ml DMEM and filter sterilized to which 1.8 ml Atlanta Biologicals Advantage FBS is added) and the conical tube was put on an orbital shaker at 120 rpm inside the incubator (37 ºC and 5% CO_2) to digest the tissue for 14-16 hours.
3) After 14 hours, a 10 ml pipette was used to mix the cartilage pieces to aid in digestion and if clumps were present, they were incubated for 15 minutes intervals.

4) The cell suspension was filtered through a 70 μm cell filter (VWR) and centrifuged for 10 min at 1100 rpm. Supernatant was aspirated and pellet resuspended in 40 ml PBS+Pen/Strep. The step was repeated 2 more times till supernatant had no yellow color.
5) Finally the pellet was suspended in 40 ml warm chondrocyte culture medium (CCM; 500 ml high glucose DMEM + 5 ml HEPES stock + 5 ml non-essential amino acids + 2 ml 0.1 M proline stock + 2.5 ml 10 mg/ml Vit. C + 5 ml 100x Pen/Strep + 50 ml FBS Atlanta advantage + 5 ml Sodium pyruvate).
6) Cells were counted using a hemacytometer and then a coulter counter.
7) If not used immediately, they were frozen in freezing medium (50% DMEM + 40% FBS + 10% DMSO) 2 million cells/ml.

Cell viability on thawing was approximately 80-85% consistently. OA cells were seeded in monolayer at ~7500 cells/cm^2 for all purposes and cultured in CCM for 7 days at 37 ºC in 5% CO_2 before doing the first medium change following which medium change was done twice a week till they became confluent and were subsequently passaged.

3.1.4) Isolation and Culture of Bovine Chondrocytes (BCC):

Bovine chondrocytes were also isolated using the same protocol (Section 3.1.3) and cells were seeded in monolayer at ~5000 cells/cm^2 and cultured in CCM at 37 ºC in 5% CO_2. Medium change was done twice a week till they became confluent and were subsequently passaged**.**

3.1.5) Population doubling:

OA cells were cultured in a 24 well plate as described above till confluency at 37°C and 5% CO2. After they reached the logarithmic growth phase, at a specific time each day, cells from three wells were trypsinized and counted using a Z2 Coulter Particle Counter. It usually took 3 days for cells to start proliferating logarithmically, but sometimes (especially at passage 0) it took 6-7 days. The number was taken as mean and standard deviation (n=3). Given two measurements of growing cells, q_1 at time t_1 and q_2 at time t_2, and assuming a constant growth rate, doubling time can be calculated as:

$$T_d = (t_2 - t_1) * \frac{\log(2)}{\log(\frac{q_2}{q_1})}.$$

The equations were calculated using Ref 30.

3.2) Experimental design for evaluating the effect of chodnrocyte secreted factors on chondrogenic differentiation of hMSCs:

Three experimental groups were used:

(i) Co-culture studies were done using P0 OA, P1 OA, P0 BCC and P3 BCC,

(ii) chondrocyte conditioned medium containing chondrogenesis inducing factors, and

(iii) chondrogenic medium without chondrocyte secreted factors (control)

Co-culture: OA cells were plated in a 24 well culture plate at ~10,000 cells/cm^2 and allowed to attach and expand for 7 days in CCM before starting the co-culture. P1 OA, P0 BCC and P3 BCC on the other hand were plated 2 days before the start of co-culture at ~5000cell/cm^2. hMSCs-laden PEG (Mn=3400) hydrogels placed in transwells

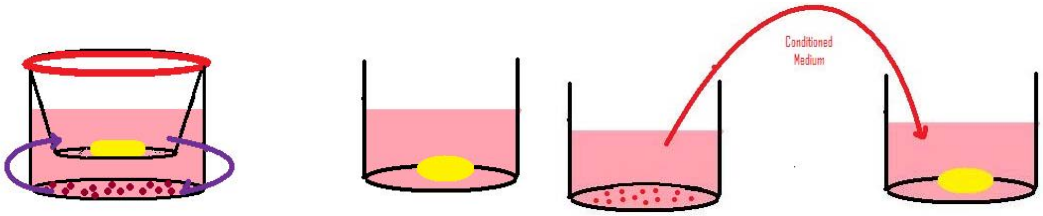
which were slotted into the 24 wells plates having the chondrocyte monolayer. CCM was replaced with chondrogenic medium (CGM; 500ml high glucose DMEM + 5ml pen/strep + 5ml ITS + premix + 1 ml Dexamethasone + 1 ml proline + 1 ml ascorbic acid-2-phosphate + 4.5ml sodium pyruvate + 5ml TGF-β_1).

Control: hMSC constructs were grown in chondrogenic medium (CM) only

Conditioned Medium: Chondrocytes were plated in T-175 flasks and cultured in CCM till confluency. At that point, CCM was replaced with high glucose DMEM without serum and conditioned DMEM was collected after 24 hours. This conditioned DMEM was used to make chondrogenic medium instead of normal DMEM.

Control and co-culture studies were also done in chondrogenic medium without TGF-β_1, to evaluate the effect of chondrocyte secreted factors without TGF-β_1. Additionally, studies were done for co-culture and control with PTHrP protein by supplementing the medium with $3x10^{-7}$ M human 1-34 PTHrP and with PTHrP pathway blocker cyclopamine at 10μM concentration. All medium changes were done every 3 days and only half of the medium was replenished so as not to remove all cell secreted factors.

Experimental time points were Day 0, Day 3, Day 7, Day 14 and Day 21 ($n=3$/group) when hMSC constructs were collected for analyses. Histological, biochemical, and RNA analyses were performed to evaluate both the differentiation of MSCs into a chondrogenic phenotype as well as the accumulation of ECM components.



Co- Culture Control Conditioned Medium

Figure 7: Schematics of Experimental model

Analysis:

1) PCR: To evaluate gene expression (β-actin, Aggrecan, Collagen 1, Collagen 2, Sox 9, ALP, Collagen 10).
2) Weight: Construct wet weights and dry weights were taken and biochemical assays were normalized to dry weight.
3) Biochemical assays: GAG assay, Collagen assay and DNA assay were performed.
4) Histilology and immuno-staining: Live dead assay to check cell viability. Cryosections were stained for Safranin-O, collagen type II and collagen type X.

3.3) Live Dead assay:

Live/Dead viability kit (Moleular probes, Cat # L-3224) was used to perform this assay. The following protocol was used:

1) Constructs were sliced into thin pieces.
2) Slices were rinsed with 1 ml PBS 3 times.

3) Live/Dead assay solution was prepared (0.5 µl of Calcein AM and 2µl of EthD-1 added to 1 ml DMEM).
4) Sufficient solution was added to cover the construct slices and then incubated for 30 minutes at 37ºC.
5) Live/Dead assay solution was aspirated and slices rinsed with PBS.
6) Fluorescence was observed under fluorescent light. (Red cells→ Dead nucleus staining, Green cells→ Live cytoplasm staining).

3.4) Histology and Immunostaining.

PEGDA hydrogels were fixed overnight in 4% paraformaldehyde in PBS (pH 7.4) at room temperature and moved to 70% ethanol at 4 ºC until processing. Constructs were transferred to 20% sucrose solution in PBS overnight and then to 50:50, 20% sucrose:OCT for 2 hours before embedding in OCT (freezing in liq. N_2) for cryosectioning in histo-molds. Samples were stored at -20ºC till they were cryosectioned into 55 µm sections that were again stored at -20 ºC till staining. Images were collected on a Zeiss confocal microscope.

3.4.1) Safranin-O staining protocol:

1. Cryosection slides were rehydrated in distilled water for 5-10 minutes at room temperature and tissue sections were not allowed to dry after rehydration.
2. Staining jars were prepared according to the chart below with each jar holding ~100 ml.

Jar	Contents
1	0.1% Safranin-O in distilled water (0.1 g Safranin-O powder + 100 mL distilled water)
2	Distilled water
3	Distilled water
4	Distilled water
5	95% Ethanol
6	95% Ethanol
7	100% Ethanol
8	100% Ethanol
9	100% Ethanol
10	Xylene
11	Xylene
12	Xylene

3. Slides were placed in the first jar for 5 minutes then transferred to the next jar, and tabular protocol was followed in each jar for 5 minutes.
4. Slides were mounted after last xylene wash using ~150 µl Permount Mounting medium per slide and by placing cover slip on top (bubbles under cover slip were avoided).
5. Mounting medium was allowed to dry overnight and pictures were taken using a light microscope the next day.
6. Mounted slides were stored at -20°C.

3.4.2) Alcian Blue staining:

1. Micromass culture was done for P0 and P1 OA. 300,000 cells in 50 µl CCM were spotted in a culture well. Cells were allowed to attach for 2 hours before medium was added slowly. The micromass was allowed to grow for 7 days (P0) and for 3 days (P1) before staining. Everything was done in triplicates.

2. Micromass culture was fixed in 4% PFA for 15 mins after aspirating medium.

3. 1 ml of 1% w/v Alcian Blue 8X in 0.1NHCl was added and incubated for 30 mins.
4. Excess was wiped using tissue without water.
5. Cultures were dehydrated and washed using 95% EtOH, 100% EtOH and xylene. 2 washes of 2 mins for each.
6. They were observed under microscope and intensity measured at 600 nm after collecting with Guanidine-HCl.

3.4.3) Collagen type II immunofluorescent staining protocol:

1. Slides were allowed to warm to room temperature and a circle was drawn around each section using a PAP pen to keep reagents from running off of the slide.
2. Slides were rehydrated in PBS for 5-10 minutes at room temperature and tissue sections were not allowed to dry after that.
3. Blocking Buffer was prepared for blocking step and diluting antibodies: 3% BSA and 0.1% Triton-X 100 in 1X PBS.
4. Sections were blocked for nonspecific binding by incubating them in Blocking Buffer for 1 hour at room temperature in a humidified chamber without letting the slides touch each other. 50-75 µl of blocking solution was used per section.
5. Type II collagen polyclonal antibody (Fitzgerald 70R-CR008X) was diluted in blocking buffer at 1:300 and sections were incubated for 1 hour at room temperature in a humidified chamber. 50-75 µl of antibody was used per section.

6. Primary antibody was grained from slides and washed for 3 x 10 minutes in 1X PBS at room temperature.
7. Secondary antibody (Alexa Fluor 488 goat anti-rabbit IgG) was diluted at 1:250 in blocking buffer and 50-75 µl of diluted secondary antibody was added to each section and incubated for 1 hour at room temperature in a humidified chamber in dark.
8. Secondary antibody was drained from slides and washed for 3 x 10 minutes in 1X PBS in dark.
9. Slides were mounted with DAPI mounting medium and cover slip edges were sealed with clear fingernail polish. Slides were viewed via fluorescence microscopy as soon as fingernail polish was dry.
10. Mounted slides were stored in the dark at -20°C.

3.4.4) Collagen type X immunofluorescent staining protocol:

1. Slides were allowed to warm to room temperature and a circle was drawn around each section using a PAP pen to keep reagents from running off of the slide.
2. Slides were rehydrated in PBS for 5-10 minutes at room temperature without letting tissue sections dry after rehydration.
3. Blocking Buffer was prepared for blocking step and diluting antibodies: 3% BSA and 0.1% Triton-X 100 in 1X PBS.
4. Slides were pretreated by heating to 41°C (PBS was kept on the slides to prevent the sections from drying out).

5. Slides were blocked for nonspecific binding by incubating them in Blocking Buffer for 1 hour at room temperature in a humidified chamber without letting the slides touch each other. 50-75 µl of blocking solution was used per section.
6. Type X collagen polyclonal antibody (Fitzgerald 20R-CR030) was diluted in blocking buffer at 1:300 and sections were incubated for 1 hour at room temperature in a humidified chamber using 50-75 µl of antibody per section.
7. Primary antibody was drained from slides were washed for 3 x 10 minutes in 1X PBS at room temperature.
8. Secondary antibody (Alexa Fluor 488 goat anti-rabbit IgG) was diluted at 1:250 in blocking buffer and 50-75 µl of diluted secondary antibody was added to each section and incubated for 1 hour at room temperature in a humidified chamber in dark.
9. Secondary antibody was drained from the slides and washed 3 x 10 minutes in 1X PBS in dark**.**
10. Slides were mounted with DAPI mounting medium and cover slip edges were sealed with clear fingernail polish. Slides were viewed via fluorescence microscopy as soon as fingernail polish was dry.
11. Mounted slides were in the dark at -20°C.

3.5) Biochemical Assays.

Constructs were collected at different time points, lyophilized, and digested with papain. The protocol followed for papain digestion is:

1. Wet weight of constructs was taken and they were lyophilized for 18-24 hours.

2. 0.035g of cysteine was dissolved in 20 mL of PBE buffer and inn the hood a syringe and a needle was used to take out 0.10 cc (0.10 mL) of papain and it was mixed with the cysteine solution.
3. 1 mL was added to each tube with lyophilized constructs. Homogenizer was used to break the construct.
4. They were incubated at 60 ºC for 16-18 hours.
5. Digested samples were stored at -20 ºC till biochemical assays were performed.

3.5.1) DNA Assay:

The DNA content was measured using DNA Picogreen assay kit (Invitrogen) following the manufacturer's protocol:

Preparing the Assay Buffer: TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5) was used below for diluting the Quant-iT™ PicoGreen® reagent, for diluting DNA samples, and in the assay itself. The 20X TE buffer included in the Quant-iT™ PicoGreen® dsDNA Assay Kits is certified to be nucleic acid–free and DNasefree. A 1X TE working solution was prepared by diluting the concentrated buffer 20-fold with sterile, distilled, DNase-free water.

Preparing the Reagent On the day of the experiment, an aqueous working solution of the Quant-iT™ PicoGreen® reagent was prepared by making a 200-fold dilution of the concentrated DMSO solution in TE in a plastic container. The working solution was protected from light by covering it with foil or placing it in the dark, as the Quant-iT™ PicoGreen® reagent is susceptible to photo degradation.

Experimental Protocol

1) A 2 µg/mL stock solution of dsDNA in TE was prepared. The lambda DNA standard, provided at 100 µg/mL in the Quant-iT™ PicoGreen® Kits, can simply be diluted 50-fold in TE to make the 2 µg/mL working solution. A five-point standard curve was made by diluting the working solution from 25 pg/mL to 25 ng/mL, prepare a 40-fold dilution of the 2 µg/mL DNA solution to yield a 50 ng/mL DNA stock solution.

2) For the low-range standard curve, from 25 pg/mL to 25 ng/mL, the 50 ng/mL DNA stock solution was diluted into disposable tubes to make 350 µL. 100 µL of the solution was added to a 96 well plate and made upto 200 µL with the aqueous working solution of Quant-iT™ PicoGreen® reagent. It was mixed well and incubated for 2 to 5 minutes at room temperature, protected from light.

3) The experimental DNA solution (papain digested sample) was diluted in TE to a final volume of 100 µL (100X dilution) in the 96-well plate and 100 µL of the aqueous working solution of the Quant-iT™ PicoGreen® reagent was added to each well

4) After incubation, fluorescence microplate reader was used to measure the fluorescence at standard fluorescein wavelengths (excitation ~480 nm, emission ~520 nm). The fluorescence value of the reagent blank was subtracted from that of each of the samples.

5) Corrected data was used to generate a standard curve of fluorescence versus DNA concentration and find out DNA concentration for the experimental samples. All samples were measured in triplicates.

3.5.2) GAG assay:

The GAG content was quantified by using dimethylene blue spectrophotometric assays at A525 as described below.

Chondroitin Sulphate Standard: Stock CS 50mg/ml (Dissolved 0.105 g cysteine in 60 ml PBE and dissolved 50 mg CS in 1 ml PBE/cys solution), which can be stored at -20 C; Working CS 100 µg/ml in PBE/cys.

PBE buffer: Added 7.1 g Na_2HPO_4 and 1.86 g Na_2EDTA in 500ml dH_2O. Adjusted pH to 6.5 with conc. HCl, filter sterilize.

Dimethylmethylene Blue Dye (DMMB): Added 3.04 g glycine, 2.37 g NaCl and 95 ml 0.1M HCl to 905 ml H_2O. While stirring, dissolved 16mg DMMB into the solution and checked that the pH is 3and OD_{525} BETWEEN 0.31-0.34.

Spectrophotometer blank was set at 525nm against DMMB. Standard curve was made with 0-100 µl CS working solution added to 100-0 µl dH_2O and 2.5 ml DMMB dye. 100 µl of each papain digested sample was taken and mixed with 2.5 ml of DMMB to take sample reading. Standard curve was used to get CS (GAG) content of the sample.

3.5.3) Collagen assay:

Total collagen content was determined by measuring the hydroxyproline content of the constructs after acid hydrolysis and reaction with *p*-dimethylaminobenzaldehyde and chloramine-T as per the following protocol.

Reagents: pH 6 stock buffer: 25 g citric acid monohydrate, 6 ml glacial acetic acid, 60 g sodium acetate trihydrate, 17 g sodium hydroxide were mixed and made upto 500 ml

using distilled water. 150 ml isopropanol and 100 ml water were added and mixed. pH was adjusted to 6.0 with conc. HCl and finally 5 drops of toluene were added.

Hydroxyproline Standard stock: 100 µg/ml – Added 10 mg hydroxyproline to 100 ml d_2H_2O.

Chloramin-T-reagent: 0.705 g Chloramine T was dissolved in 40 ml pH 6 buffer and 5 ml isopropanol was added. Mixed by shaking well. (Note: should be made 24 hrs before the assay)

p-Dimethylaminobenzaldehyde solution: 7.5 g dimethylaminobenzaldehyde in 30 ml isopropanol and added 13 ml 60% perchloric acid (should be made 24 hrs before the assay)

1. Samples were hydrolyzed in 1 ml 6N HCl @ 115ºC for 18-24 hrs before assay (100µl papain digest + 900µl HCl).
2. Standard stock was diluted to 10 µg/ml and dilutions were made for 0, 0.25, 0.5, 1, 2, 3, 4, 5µg of hydroxyproline standard curve.
3. Hydrolyzed samples were brought to room temperature and were titrated to normalize pH (Added 2 drops of methyl red followed by 2.5M NaOH till pink color disappeared. Added 2-3 drops of 0.5M HCl to get back pink color and a few drops of 0.5M NaOH to get the final straw color).
4. dH_2O was added to bring volume to 15ml, and 1 ml of was taken in a new tube.
5. 500 µl Chloramine T was added, vortexed and incubated for 20 min @ RT.
6. 500 µl dimethylaminobenzaldehyde was added while vortexing and incubated for 30 min in a 60ºC water bath.

7. Solutions were cooled and absorbance was measured at 550nm. Standard calibration curve was used to get collagen content in experimental sample.

3.6) RT-PCR and Real-Time PCR.

Total RNA was extracted with TRIzol and reverse transcribed into cDNA by using the SuperScript First-Strand Synthesis System (Invitrogen). RT-PCR and real-time PCR were performed.

3.6.1) RNA extraction:

1) Washed each construct with 1 ml PBS and then added 1ml TRIzol.
2) Incubated the sample at room temperature for 5 minutes and disrupted construct using a homogenizer.
3) Added 0.2 ml of chloroform and shook vigorously for 1 minute, incubated at room temperature for 5-10 mins.
4) Removed cellular debris by centrifugation at 15,000 rpm for 20 min at 4°C.
5) Transferred the clear supernatant to a new tube and added 0.5 ml of isopropanol to precipitate the RNA.
6) Mixed well and incubated at room temperature for a max. of 5 minutes.
7) Centrifuged at 15,000 rpm for 20 mins at 4°C to pellet the RNA.
8) Discarded the supernatant and resuspended the pellet in 1 ml of 75% ethanol.
9) Mixed by vortexing and centrifuged at 10,000 rpm for 10 mins at 4°C and discarded supernatant. Air dried to completely get rid of the ethanol.

10) Resuspended the pellet in 40 µl diethylpyrocarbonate (DEPC) treated water. Denatured in 60°C for 10 mins and immediately cooled down on ice. Determined RNA concentration using the nano drop machine.

3.6.2) cDNA synthesis:

Initial Primer/RNA Mix: 1 µl oligo(dT) primers + 1 µl dNTP mix + 8 µl of RNA (400ng) + DEPC H20. Heated to 95°C for 5 min. Immediately chilled on ice.

Buffer/Enzyme mix (assembled in this order): 2 µl 10x cDNA synthesis buffer + 4 µl MgCl2 + 2 µl 0.1 M DTT + 1 µl RNaseOut + 1 µl Superscript III. Mixed Buffer/enzyme components and added to Primer/RNA mixed in a 0.2 ml micro-centrifuge tube. Placed the tube in the PCR machine programmed as follows: 50°C for 50 min and terminate reaction at 85°C for 5 min. Chilled on ice after reaction was complete.

Added 1 µl RNase H, mix, incubated at 37°C for 20 min and stored cDNA at -20ºC.

3.6.3) Real time PCR:

Each cDNA sample was run in triplicates for each primer with the following 25 µl reaction mixture in each well of a 96 well plate: 12.5 µl Power SYBR Green mix, 1 µl template cDNA, 0.5 µl forward primer (10 pmol/µl) , 0.5 µl reverse primer(10 pmol/µl) and 11.5 µl DEPC water. Plate was sealed with optical film and centrifuges for 2 minutes at ~2500rpm to get all liquids to the bottom of the wells The RT-PCR amplification was started (Began with a 3 min step at 95ºC. 40 cycles with a step of 10 sec at 95ºC, 45 sec at 65ºC, and 20 sec at 78ºC) in the machine. Housekeeping gene

used was β-actin and delta delta C_T method was used for analysis taking same day control as second reference.

3.6.4) Conventional PCR:

10 µl Gen Amp Master mix + 0.5 µl Forward primer + 0.5 µl reverse primer + 1 µl cDNA + 8 µl water, run for the fast PCR. The traditional PCR/RT-PCR products were then run on a 2% agarose gel (2 g agarose + 100 ml 1X TAE buffer). 5 µl of the PCR product and 5 µl TAE + 0.5 µl of DNA ladder were each loaded with 0.5 µl of loading dye and run for 50 min at 110 V of constant voltage. The bands were visualized after incubating in EtBr dye (50 µl in 500 ml TAE buffer) for 20 min. in dark under a UV lamp.

3.7) Mass spectrometry analysis:

hMSCs in MSCGM , P0 OA in CCM and P1 OA in CCM were all cultured till confluency in a 6 well plate. They were also co-cultured as described previously with hMSC in chondrogenic medium for 7 days. Following which the medium (GM/CCM/chondrogenic medium) was replaced by DMEM and collected after 48 hours for analysis of cell secreted factors in each case and their comparison. The DMEM collected was stored at -80ºC till further analysis. TCA precipitation was performed using the following protocol before mass spectrometry.

Stock Solutions: 100% (w/v) Trichloroacetic acid (TCA); recipe: dissolved 500g TCA (as shipped) into 350 ml dH2O, store at RT.

Precipitation Protocol:

1) 1 volume of TCA stock was added to 4 volumes of protein sample.

2) Samples were incubated for 10 min at 4°C.
3) Tube were spun in micro-centrifuge at 14K rpm, 5 min.
4) Supernatant was removed, leaving protein pellet intact. Pellet was formed as whitish, fluffy ppt.
5) Pellet was with 200µl cold acetone.
6) Tubes were spun in micro-centrifuge at 14K rpm, 5min.
7) Steps 4-6 were repeated for a total of 2 acetone washes.
8) Pellet was dried by placing tube in 95°C heat block for 5-10 min to drive off acetone and precipitate was dissolved in 120 µl of pure water.
9) For SDS-PAGE, 15 µl of above sample was added to 2X BioRad sample buffer and boiled for 10 min in 95°C heat block before loading sample onto a gradient polyacrylamide gel alongside a protein marker.

The sample was run in 1X running buffer (10 X buffer: Tris base 30.3g + glycine 144 g + SDS 10 g made up to 1000 ml in pure water) at 200V for 35 minutes before silver staining it using the invitrogen silver staining kit and protocol (fixation →sensitization →washing →staining →washing →developing →stopping →scanning). The protein sample was sent out to the UCSD mass spec facility for mass spectrometry.

3.8) Diffusion Modelling:

MATLAB was used as a tool to model the diffusion characteristics inside the gel during co-culture by finite difference method for a cylinder. LU factorization method in MATLAB was used for solving the matrix.

3.9) Hydrogels for in vitro expansion of chondrocytes

Acrylamide: Acryolyl-6-aminocaproic acid (A6ACA) copolymer hydrogels having a molar ration of 6:2 were tested as a possible polymer surface for chondrocyte expansion (BCC). The hydrogel was made as follows: 0.533 g Acrylamide + 0.05 g Bis Acrylamide was added to 2.75 ml dH_2O and 1.25 ml DMSO. 0.4631g of A6ACA was added to the above mixture and vortexed to dissolve completely. The mixture was kept on ice. 0.5 ml 10% v/v APS and 0.5 ml 10% w/v TEMED was added to the mixture to initiate polymerization and immediately poured into the desired cast (glass mold).

The gel was swollen in PBS overnight and then washed with 70% EtOH for 4-5 hours before washing with PBS + Pen/Strep 7 times (each wash - 2 hours). The washes were performed inside the hood and after the 3rd wash, the gel was cut using a mold to fit exactly in a 6 well plate. The gel was incubated in CCM for at least 2 hours before plating the BCC on it at ~5000cell/cm^2. The cells were allowed to attach for 2 hours before adding CCM and then it was incubated at 37ºC and 5% CO_2. Chondrocytes cultured on 6-well culture plate at same seeding density was used as control. Medium was changed every 3 days and cells were passaged after 6 days. During each passage, images were taken under the microscope every 24 hours to evaluate the cellular phenotype, samples were collected for population doubling count every 48 hours and PCR samples were collected in 1 ml TRIzol at the end of 6 days before passaging. PCR analysis was performed and compared for passages P0 to P5.

3.10) Statistical Analysis.

Data are expressed as mean+/-SD. Statistical significance was determined by ANOVA (one way/two way). PRISM software was used for graphing and performing the statistical analysis. ("*" is $p<0.05$; "**" is $p<0.01$ in terms of statistical significance).

Chemical suppliers: Appendix A

PCR primers: Appendix B

Chapter 4: RESULTS

4.1) Effect of bovine articular chondrocyte secreted factors on chondrogenesis

4.1.1) P0 BCC secreted factors promote chondrogenic differentiation of hMSCs:

We first tested our hypothesis that the chondrocyte secreted factors promote chondrogenic differentiation of MSCs into hyaline cartilage using bovine chondrocytes. Bovine chondrocytes were chosen because of their availability. Real time (qRT-PCR) analysis showed that the undifferentiated MSCs shifted their genetic expression during the culture period indicating that they are undergoing chondrogenic differentiation (Fig. 8); all PCR data was normalized against same day control). Gene expression of aggrecan (Fig. 8a) was almost entirely absent in both controls and co-cultures but increased progressively over the 21 day period. As seen from Fig 8, the co-cultured hMSCs consistently showed significantly higher levels of aggrecan and collagen type II, compared to control cultures (i.e. hMSC-laden hydrogels cultured in chondrogenic inducing conditions i.e. chondrogenic medium with TGF-β1 without chondrocyte secreted factors). Collagen type II (Fig. 8c) was not expressed in day 3 control constructs, but was present in low quantities in co-cultured constructs and increased by almost a 1000 fold by day 21.Type I collagen expression of co-cultured hMSCs were very similar to control (Fig. 8b), whereas Sox-9, a pre chondrogenic marker, was found to be peak at day 7 and decreased thereafter in co-cultured hMSCs with respect to their respective control (Fig. 8d).

In correlation with the RT-PCR, biochemical analysis of the co-cultured constructs showed a significant increase in GAG (normalized to DNA) at day 21 (Fig.

8e). GAG content increased from 0% in day 0 to 50 fold in control constructs (in presence of TGF-β1) versus a 100 fold increase in co-culture constructs on day 21.

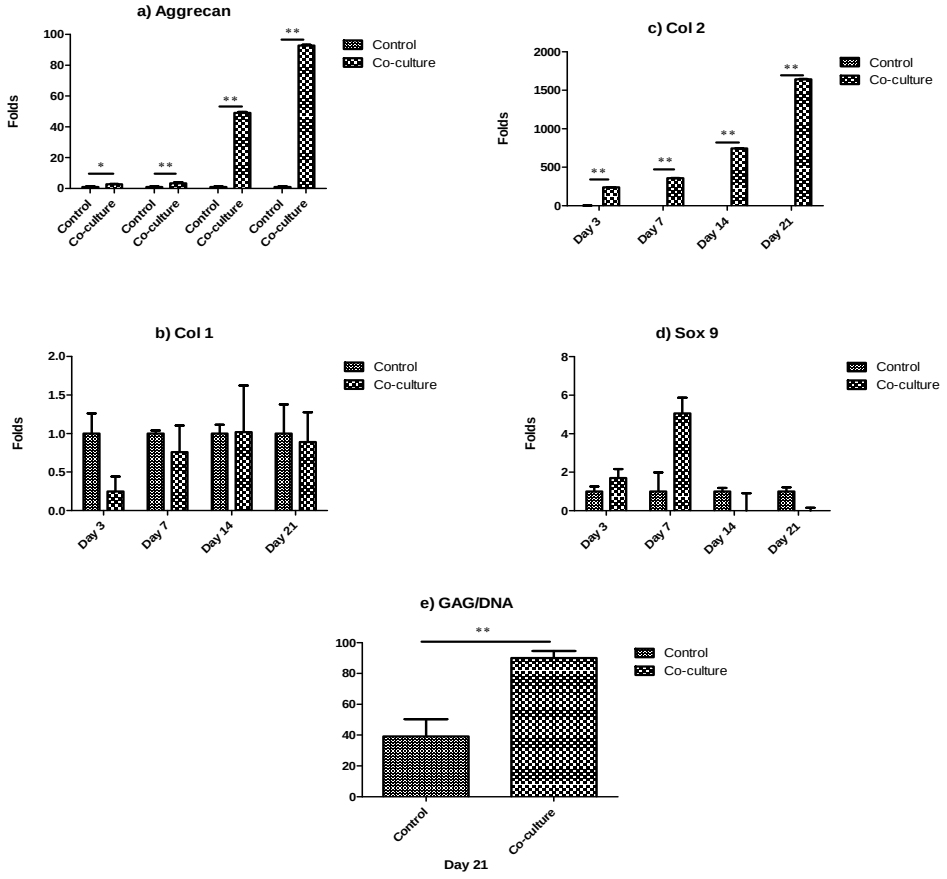


Figure 8: Co-culture with P0 BCC
A marked increase in the formation of neo-cartilage was observed in the co-culture system of hMSC-P0 BCC as compared to control (hMSCs in TGF-β1) MSCs co-cultured with primary bovine chondrocytes produced significant amounts of cartilage specific matrix components, proteoglycans, and collagen type II compared to their non-cultured counterparts. Fold change in the expression of chondrogenic markers was determined using RT-PCR: a) Aggrecan; b) Collagen type I; c) Collagen type II; d) Sox-9; e) GAG content was measured for proteoglycans and showed an increase in co-culture.

Given that the primary BCCs promote chondrogenic differentiation of hMSCs, we next investigated the effect of *in vitro* expanded chondrocytes on chondrogenic differentiation of hMSCs using the same co-culture system.

4.1.2) Passaged (P3) BCC secreted factors do not have a significant effect on chondrogenic differentiation of hMSCs:

To evaluate the effect of *in vitro* expansion on chondrocyte secreted factors, we utilized passage three chondrocytes in our co-culture experiments. In addition to the direct co-culture, we also utilized chondrocyte conditioned medium.

As seen from qRT-PCR analysis (Fig. 9), hMSCs in co-culture showed a slight upregulation of aggrecan, collagen type II, and Sox 9. Whereas the hMSCs cultured in conditioned medium showed a downregulation of these genes compared to control. In contrast to control and co-cultured hMSCs, the one cultured in conditioned medium exhibited an enhanced upregulation of collagen type 1 (Fig. 9b). Sox-9 in co-culture increased and then went down indicating pre chondrogenesis and proliferation (Fig. 9d). Overall the gene expression suggests that the conditioned medium had little or no effect on chondrogenic differentiation of hMSCs but showed an upregulation in collagen type I. However, compared to conditioned medium and control, co-culture system had a slightly favorable effect on chondrogenic differentiation of hMSCs.

Histological analysis of hMSC constructs co-cultured with P3 BCC had stronger staining for Safranin-O (which stains negatively charged glycosaminoglycans red) (Figs. 10a-b) compared to that of cultured in conditioned medium (Figs.11a-b), which showed a very week staining in the periphery of the tissue sections. Immunofluorescent staining for collagen type II (green fluorescent collagen type II with blue DAPI stained nuclei) was observed only in day 21 co-culture (Fig. 10d) constructs and was very weak for conditioned medium constructs (Fig.11c-d). However, as seen from Fig. 10e, the co-

cultured hMSCs were stained strongly for collagen type X indicating their hypertrophic differentiation.

Biochemical analysis of the DNA content (Fig. 9e) of the MSC constructs revealed a time dependant increase in DNA content irrespective of the culture conditions. These results supported the previously described increase in cell number when cultured in medium containing TGF-β1. Among the cultures, hMSCs cultured in co-cultured and conditioned medium showed higher DNA content compared to control groups. In correlation with the qRT-PCR and histologic findings, the hydrogels showed an insignificant increase in GAG (percent dry weight) from day 14 to day 21 in co-culture whereas it actually decreased slightly in control and conditioned medium constructs. Neither of the GAG results was found to be statistically significant. Overall, the results indicate that co-culture with *in vitro* expanded chondrocytes (passage 3) do not promote chondrogenic differentiation of hMSCs, while the conditioned medium was found to have some inhibiting effect i.e. promotes hypertrophy, which might be an effect of low concentration of morphogens in conditioned medium.

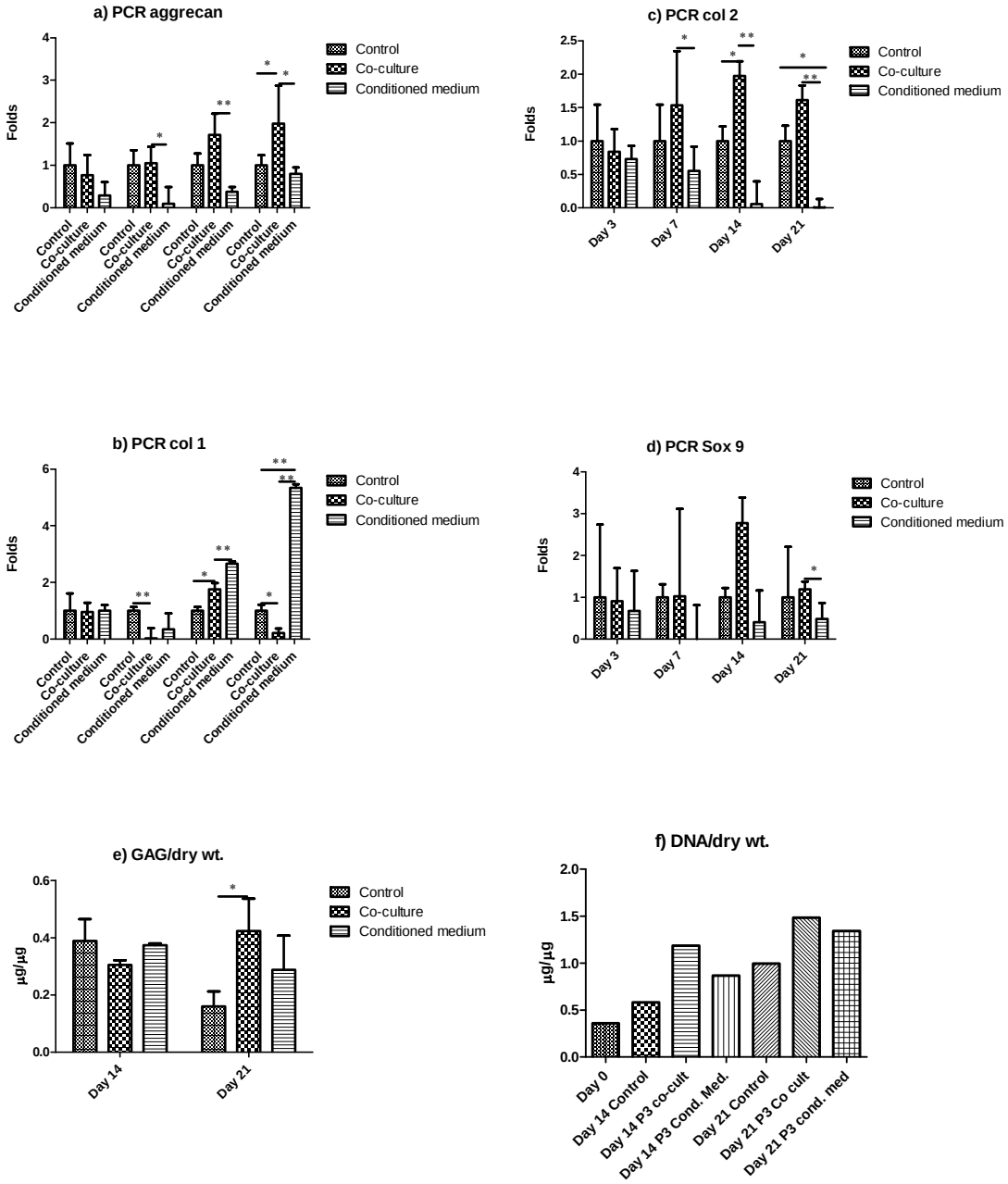


Figure 9: Gene expression and biochemical analysis results for P3 BCC.
RT-PCR analysis to determine gene expression of a) Aggrecan; b) Collagen type I; c) Collagen type II; d) Sox-9. Biochemical analysis results to estimate e) GAG content and f) DNA. Results represent the mean of triplicate culture and represented as mean+/-SD. There's not much difference between control and co-culture with dedifferentiated chondrocytes and conditioned medium does not support chondrogenesis at all.

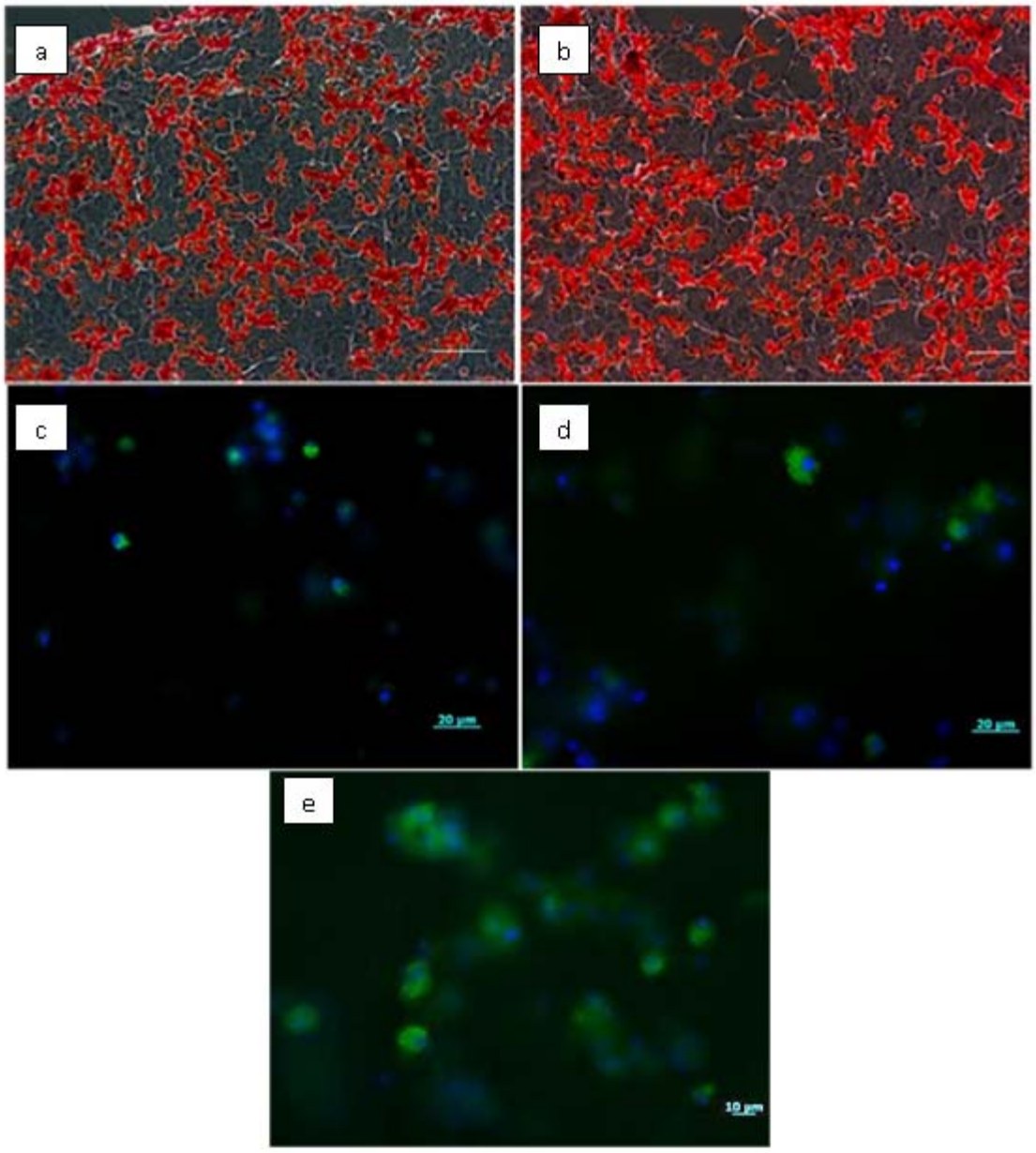


Figure 10: hMSC construct histology samples co-cultured with P3 BCC.
a) d14 saf-O staining ; b) d21 saf-O staining: original magnification was 10X and scale bars show 100 µm; c) d14 collagen type II staining; d) d21 collagen type II staining; e) d21 collagen type X staining : original magnification was 40X. There is a very strong type X staining compared to type II on d21 indicating that the stem cells might be undergoing hypertrophy. This was not seen in conditioned medium constructs.

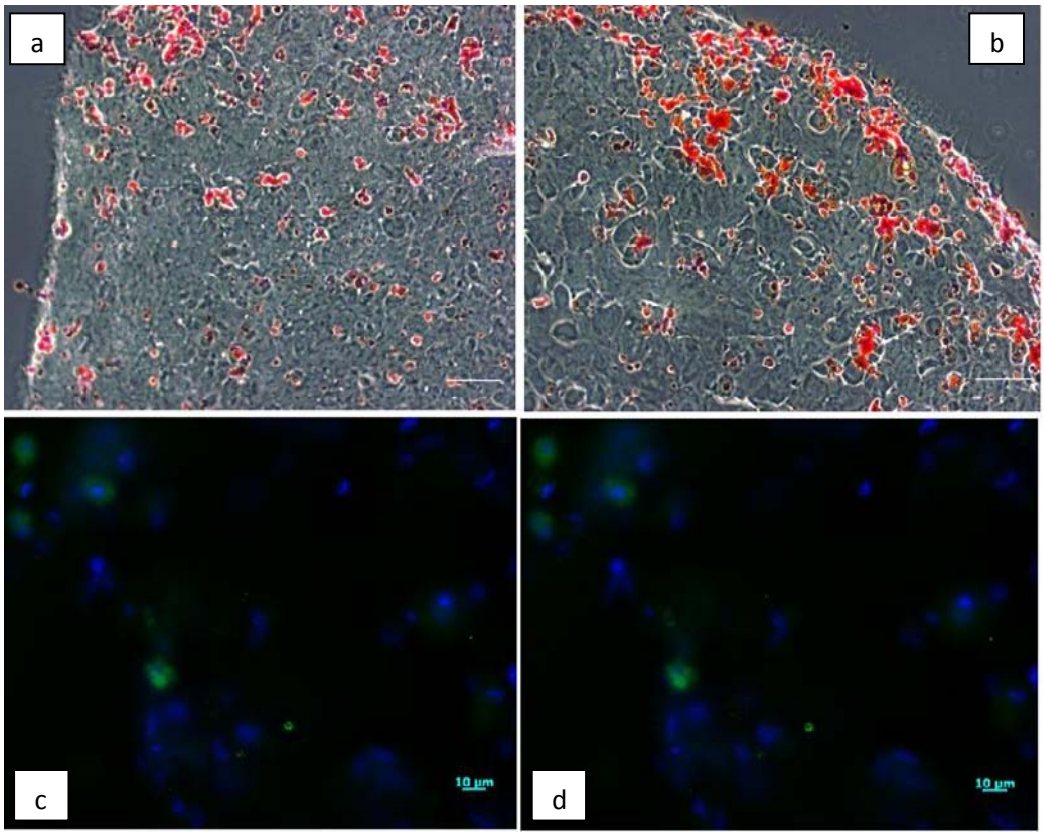


Figure 11: hMSC construct histology samples cultured in BCC Conditioned medium.
a) d14 saf-O staining ; b) d21 saf-O staining: original magnification was 10X and scale bars show 100 µm; c) d14 collagen type II staining; d) d21 collagen type II staining: original magnification was 40X.

4.2) OA chondrocyte secreted factors promote chondrogenic differentiation of hMSCSs

In the previous section, we discussed the positive effect of primary chondrocyte secreted factors on chondrogenic differentiation of hMSCs. Here we discuss the effect of OA chondrocyte secreted factors on chondrogenic differentiation of hMSCs. There are no reports on the effect of OA chondrocytes on hMSCs. It is important to understand the effect of OA environment on hMSCs if we intend to use hMSCs to treat

OA pathology, as the potential of using MSCs to repair osteoarthritic cartilage tissue is highly dependent upon how these cells respond to the cell secreted morphogens from the diseased chondrocytes.

Here, we used both human P0 OA (osteoarthritic chondrocytes) and passaged OA chondrocytes (P1) to evaluate the effect of OA-secreted morphogens on hMSCs utilizing the same co-culture setup described previously. But given the lack of prior knowledge about the effect of OA cells on hMSCs, the limited literature on *in vitro* expanded OA cells and the huge variability in properties of human tissue between donors, we first characterized the OA chondrocytes.

4.2.1) Characterization of OA cells

The OA cells were characterized for their adhesion, population doubling, gene profile, and ability to expand *in vitro* in a 2D environment. Continuous observation of primary OA cells plated on a standard culture plate showed that they took a week's time to attach before starting to proliferate. Hence, we decided to change medium on OA cell culture for the first time after 7 days and every 3 days thereafter. Passaged OA chondrocytes (i.e., after passage one) however, attached within 24 hours and started proliferating. Microscopic images were taken every day to keep a track of morphological changes of the plated OA cells. Cells were passaged till P3 and analyzed by PCR and staining.

4.2.1.1) P0 and P1 OA cells differ significantly in their morphology:

Figure 12 shows the drastic difference in cell attachment between P0 and P1 OA chondrocytes where P0 cells still retained their round, clustered morphology even on day 6 (Fig. 12a) as compared to P1 OA which spread out on day 1 itself (Fig. 12b).

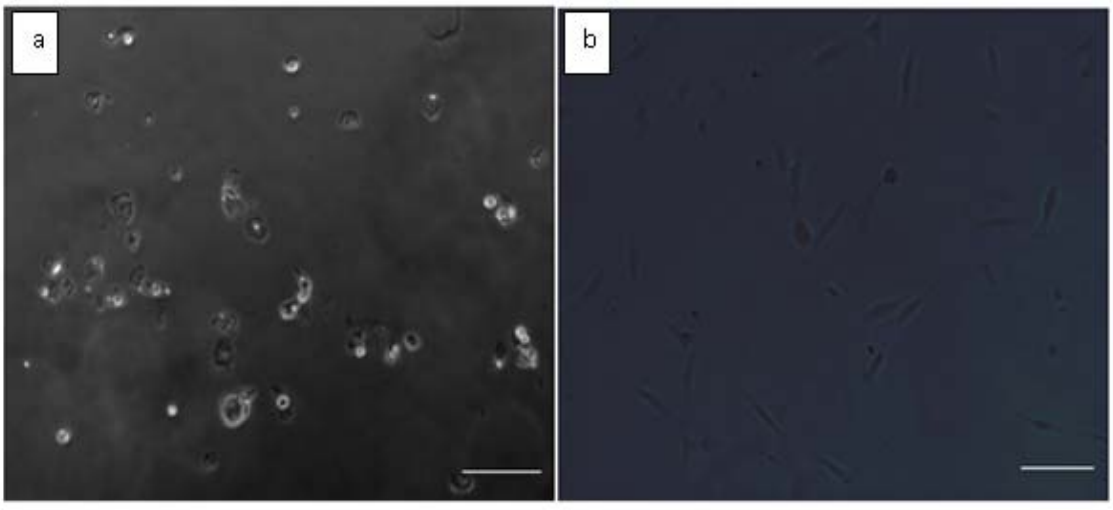


Figure 12: Cell morphology of OA cells.
A) P0 OA cells on day 6 after plating. B) P1 OA cells 24 hours after plating. Both cultures were started at the same initial cell density. All pictures were taken at an original magnification of 10X and the scale bar shows 100 μm.

4.2.1.2) Histochemical Staining of OA cells:

Clearly, OA chondrocytes de-differentiate and become fibroblastic in appearance after the first passage itself. The morphological appearances were further supported by alcian blue staining (for extra cellular matrix) and saf-O staining (for sulphated glycosamines) where the chondrocytes were cultured as micromass. P0 OA has a high amount of saf-o staining (Fig. 13a) compared to their P1 OA counterparts (Fig. 13b). A similar behavior was also observed with alcian blue staining (Fig. 13d).

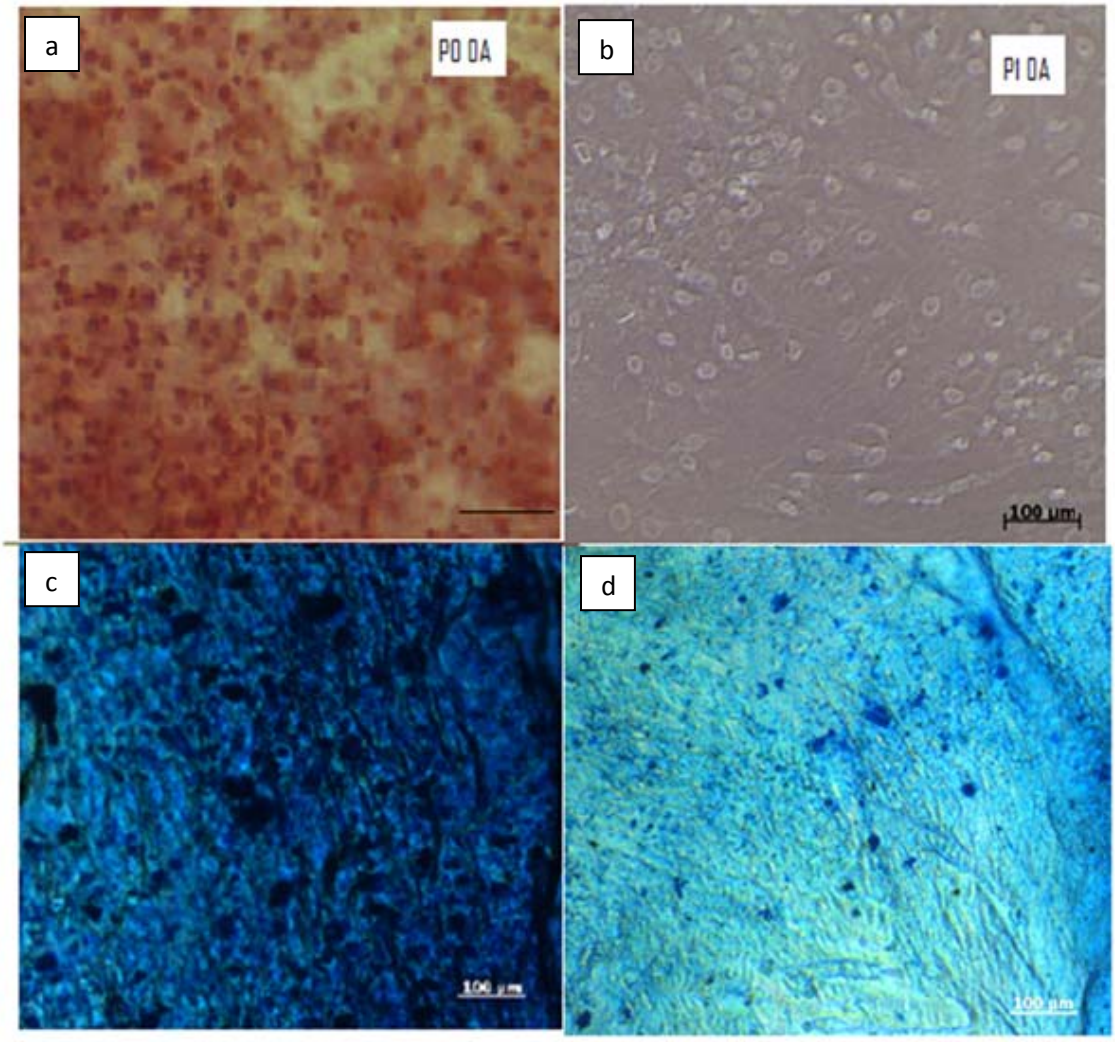


Figure 13: Histochemical staining of OA chondrocytes.
A) Saf-O stained P0 OA. B) Saf-O stained P1 OA. C) Alcian blue staining of P0 OA micromass culture. D) Alcian blue staining of P1 OA micromass culture. All pictures were taken at an original magnification of 10X and scale bars show 100 µm.

4.2.1.3) P1 OA cells were highly proliferative than P0 OA cells.

Population doubling calculations were done to know the proliferation time for OA cells and the difference between subsequent passages. P0 OA had the highest

doubling time because of the initial lag in attachment. The results were given in Table 2, while Fig. 14 showed the growth curve.

Table 2 : Population doubling time and equation.

PASSAGE	DOUBLING TIME	EQUATION
P0 OA	474.87=ln(2)/0.0015	Amount= 16572.4002*e0.0015*time
P1 OA	51.51=ln(2)/0.0135	Amount= 10815.592*e0.0135*time
P2 OA	37.9=ln(2)/0.0183	Amount= 10754.0022*e0.0183*time
P3 OA	51.88=ln(2)/0.0134	Amount= 9957.8308*e0.0134*time

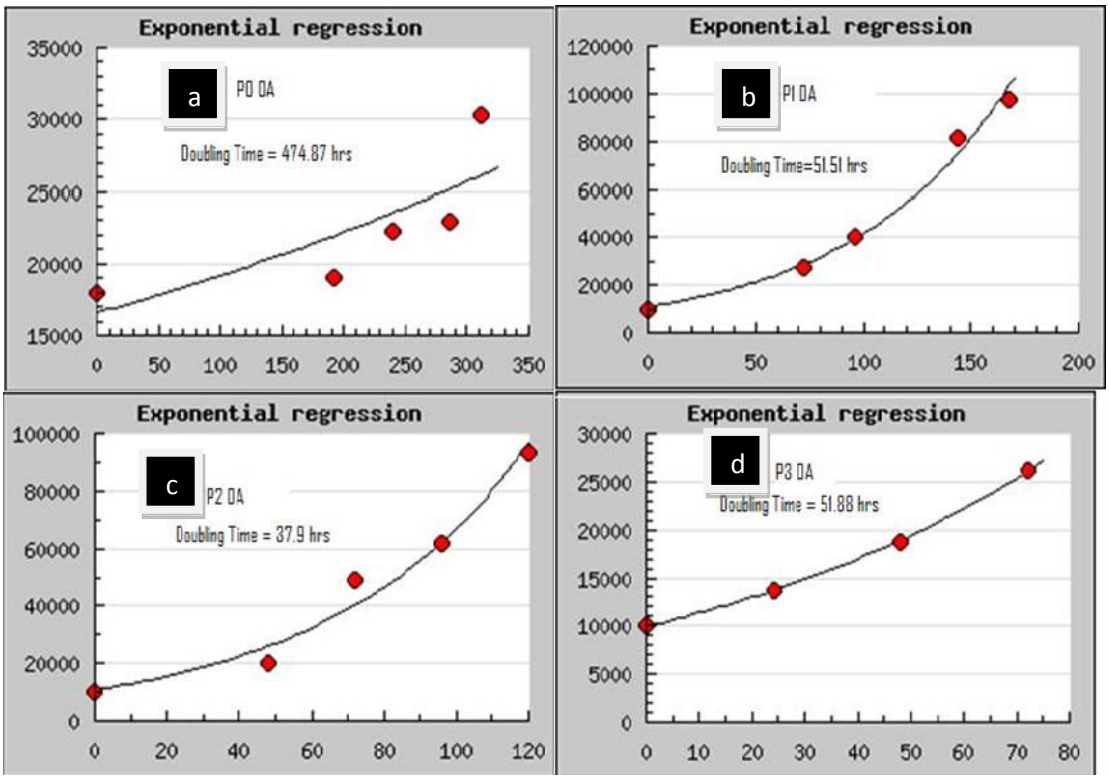


Figure 14: Population Doubling curves. a) P0 OA; b) P1; c) P2 ; d) P3

4.1.2.4) OA chondrocytes expressed genes related to hypertrophic state

The gene expression profile of OA chondrocytes was analyzed using RT-PCR and then the bands were visualized using agarose gels as well. Quantitative analysis of the gene expressions was carried out using qRT-PCR. P0 OA showed expression of collagen type X and ALP, in addition to characteristic chondrocyte markers such as collagen type II and aggrecan. All the chondrogenic markers however were lost or reduced during *in vitro* expansion as shown in (Fig. 15a and 15b).

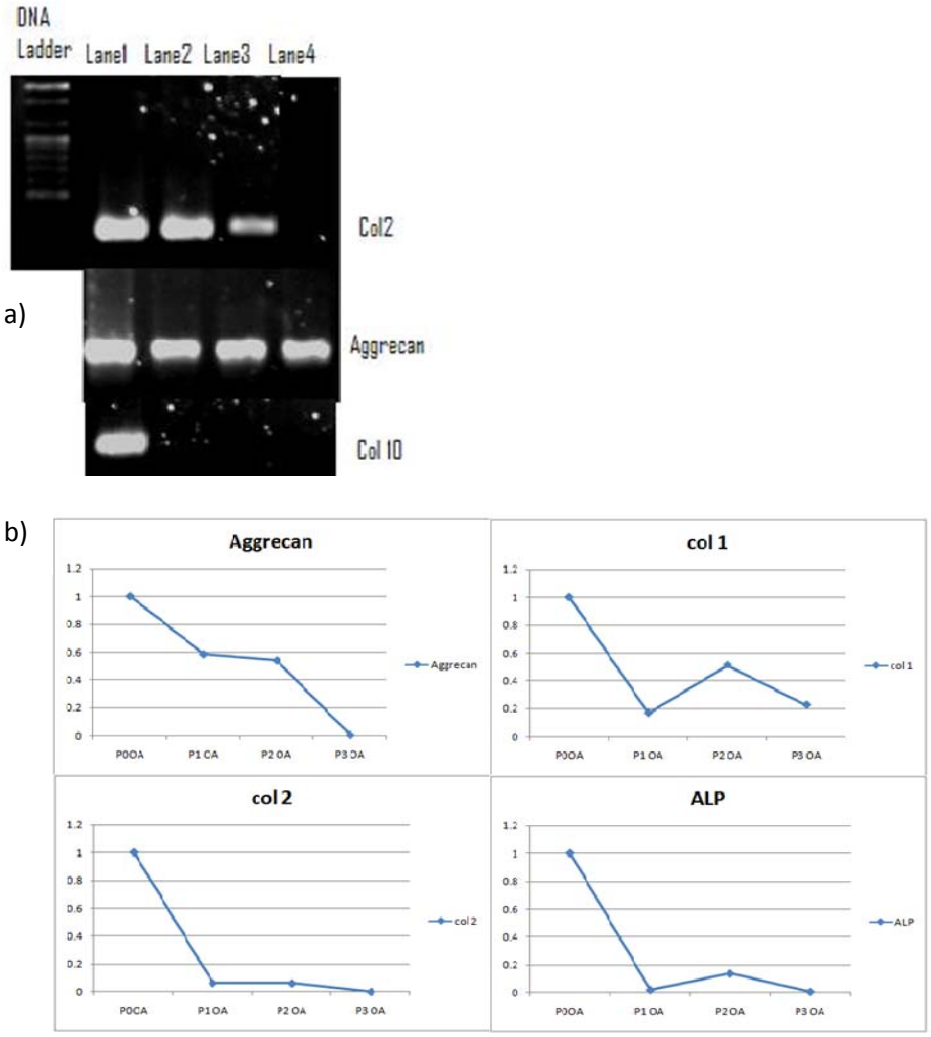


Figure 15: Gene analysis of OA chondrocytes using PCR:

a) Conventional PCR bands for chondrogenic markers; Lane 1 is P0 OA, Lane 2 P1 OA, Lane 3 P2 OA and Lane 4 P3 OA.
b) RT-PCR curves for OA from P0 to P3 with the reference being P0.

4.2.2) Primary OA chondrocytes promote chondrogenic differentiation of MSCs.

Comparison of hMSC constructs grown in co-culture with primary OA chondrocytes with those grown alone (control) or in conditioned medium showed significant differences including the consistent expression of features of the

chondrocyte phenotype. Initial viability of hMSCs after encapsulation was tested using live dead assay (Figure 16) where green fluorescent signal represents the live cell cytoplasm and the red dots are dead nuclei, which shows high cell viability (> 90%).

In order to determine the effect of chondrocyte secreted factors on gene expression profile of differentiating hMSCs, we performed qRT-PCR analysis of RNA isolated from hMSC co-cultured with OA chondrocytes; hMSC constructs cultured in OA chondrocyte conditioned medium, and hMSC constructs grown in chondrogenic medium without chondrocyte secreted factors (control constructs). The qRT-PCR results were interesting in the sense they mirrored the results obtained from co-culture with P0 BCC and clearly indicate that even diseased chondrocytes like OA chondrocytes can induce chondrogenic differentiation of hMSCs (Fig. 17). Gene expression for aggrecan was almost entirely absent in conditioned medium constructs with respect to control (Fig. 17a) but was strongly present in the co-culture constructs reaching up to 16 folds compared to control by day 21. As shown in Fig. 17c and 17d, co-cultured hMSC constructs showed an upregulation in collagen type II and Sox 9 expression compared to control. Collagen type II was again not expressed in conditioned medium but went up to 10 fold in co-cultured hMSCs with respect to the control (Fig. 17c). Sox-9 peaked around day 14 before reducing again marking the proliferative pre-chondrogenic stage (Fig. 17d).

The biochemical analysis showed that the DNA content of constructs at different times during co-culture was consistently higher than that of control, indicating proliferation of hMSCs in presence of chondrocyte secreted factors (Fig. 17f). No significant proliferation of hMSCs was observed in conditioned medium. Significantly

higher levels of GAG were found in hMSC co-cultured with chondrocytes than in hMSC grown alone (Fig. 17e) or in conditioned medium.

Histological analysis substantiated these findings; hMSC cultured in co-culture displayed dense extracellular matrix, which is stained dark red for Safranin-O (Fig. 18a and 18b). Immunofluorescent staining with antibodies for collagen type II showed a strong presence of collagen type II matrix (Fig. 18c and 18d). In comparison, hMSC constructs grown in conditioned medium showed weak staining for Saf O and collagen type II, indicating lack of matrix productions (Fig. 19c and 19d). Most importantly collagen type X did not show up in any of the PCR gene analysis compared to the control during co-culture implying that terminal differentiation was being arrested. Immunofluorescent staining for collagen type X further indicates the absence of hypertrophic differentiation (Fig. 25 b and 25e).

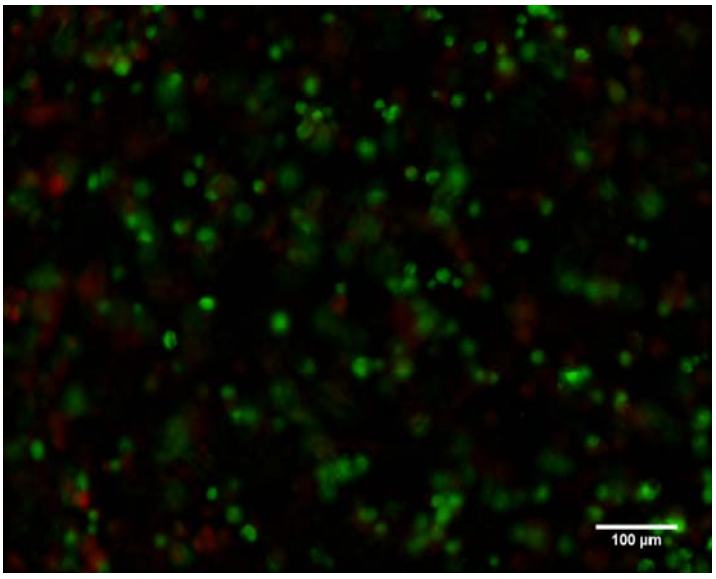


Figure 16: Live Dead assay
Live-dead staining of hydrogel constructs 24 hours after encapsulation. Image was taken at an original magnification of 10X.

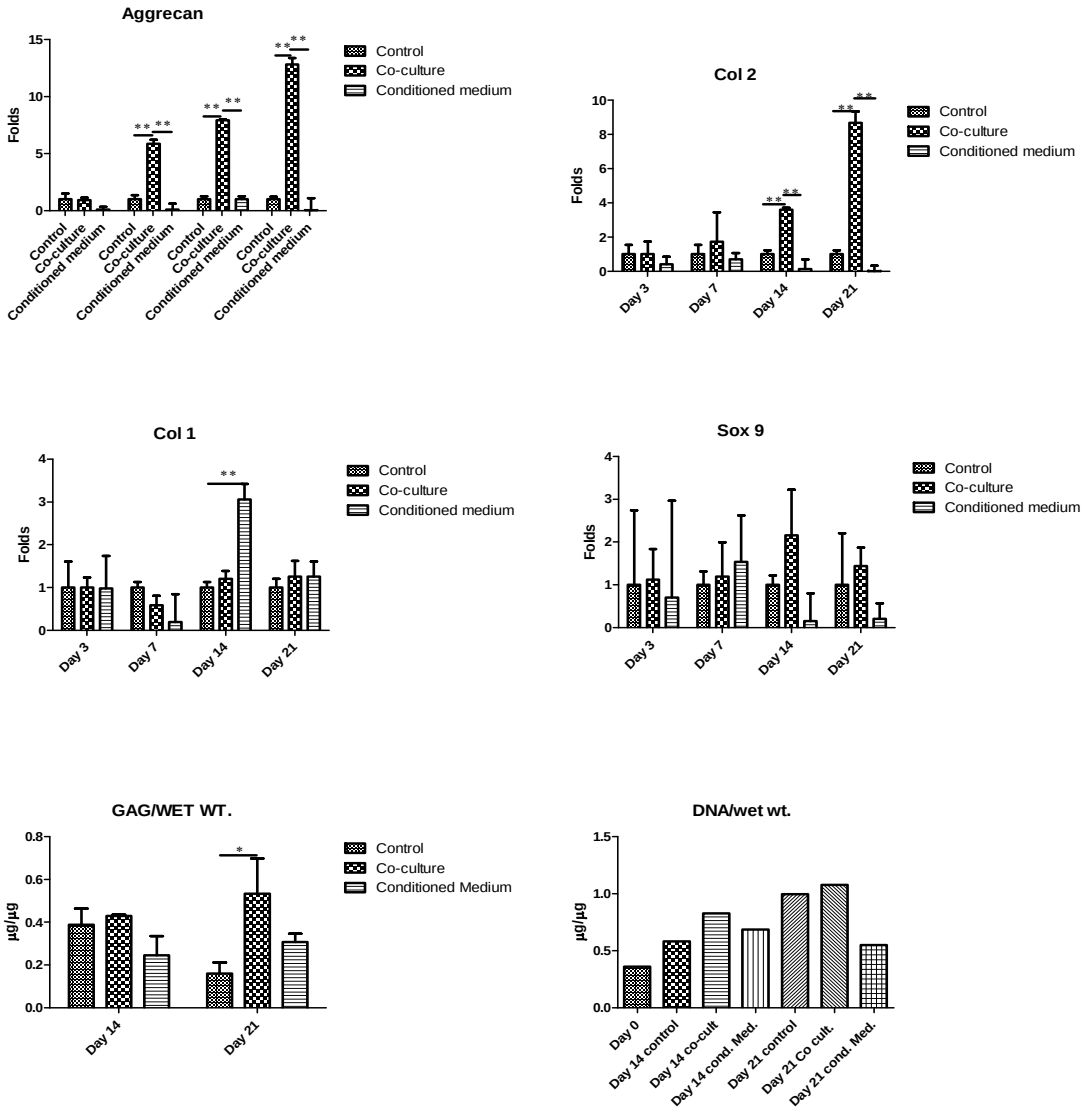


Figure 17: Gene expression and biochemical analysis results of hMSC constructs for co-culture with P0 OA.

RT-PCR analysis to determine gene expression of a) Aggrecan; b) Collagen type I; c) Collagen type II; d) Sox-9. Biochemical analysis results to estimate e) GAG content (percent wet weight) and f) DNA content (percent wet weight). Results represent the mean of triplicate culture and represented as mean+/-SD. There's not much difference between control and conditioned medium constructs but co-culture with P0 OA clearly supports chondrogenesis.

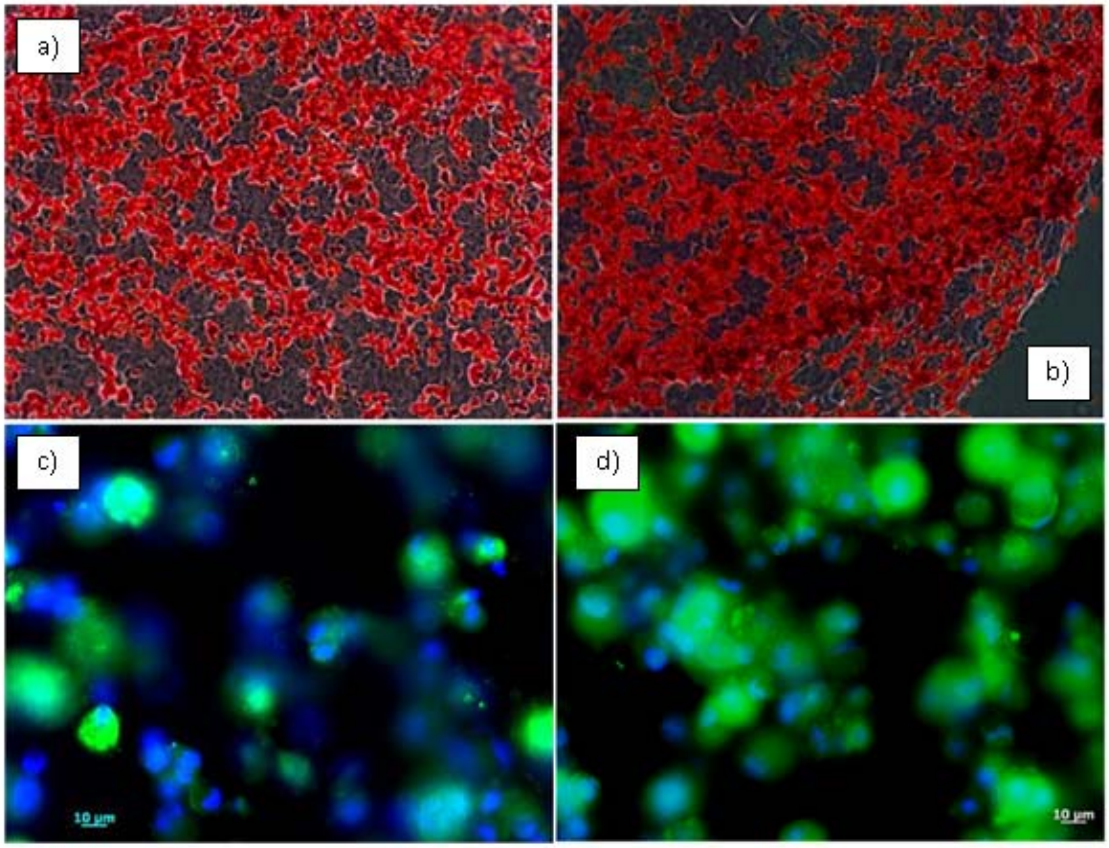


Figure 18: hMSC construct histology samples co-cultured with P0 OA.
a) d14 saf-o staining ; b) d21 saf-0 staining: original magnification was 10X and scale bars show 100 µm; c) d14 collagen type II staining; d) d21 collagen type II staining;: original magnification was 40X. There's a considerable collagen type II staining at day 14 which increases significantly by day 21.

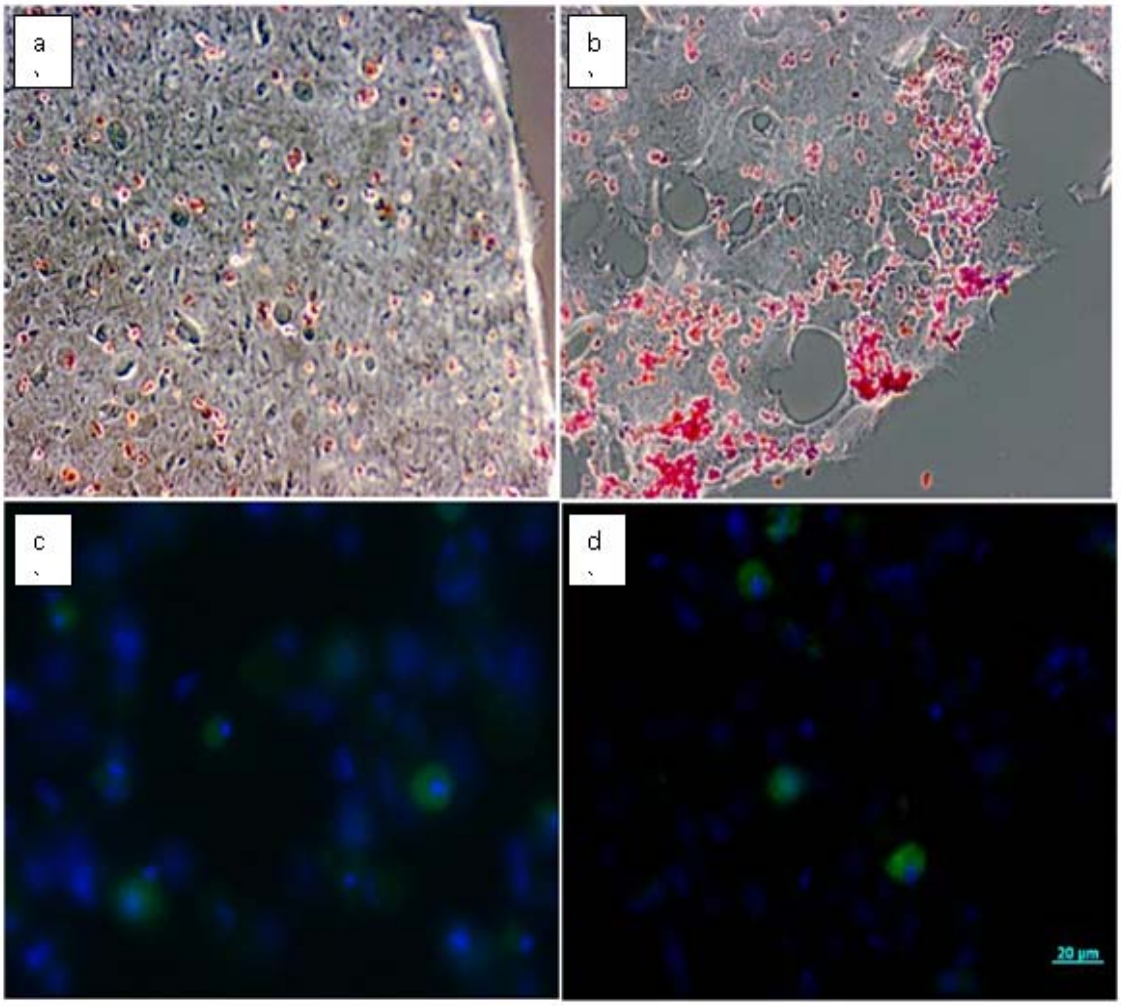


Figure 19: hMSC construct histology samples cultured in conditioned medium of P0 OA.
a) d14 saf-o staining ; b) d21 saf-0 staining: original magnification was 10X and scale bars show 100 µm; c) d14 collagen type II staining; d) d21 collagen type II staining;: original magnification was 40X. There's a considerable collagen type II staining at day 14 which increases significantly by day 21.

Passaged OA cells (P1) on the other hand did not support chondrogenesis any more than the control constructs with TGF-β1. The qRT-PCR analysis showed an upregulation of collagen type 1 expression in hMSC constructs, which were cocultured with P1 OA chondrcoytes. The expression levels of Aggrecan (Fig. 20a) and collagen

type II (Fig. 20c) levels were similar to that of control indicating that the P1 OA chondrocyte secreted factors does not have any significant effect on chondrogenic differentiation of hMSCs.

Biochemical analysis corroborated the findings from the RT-PCR analysis. The GAG content (normalized to the DNA) of the co-culture constructs was found to be slightly lower than the control (Fig. 20e).

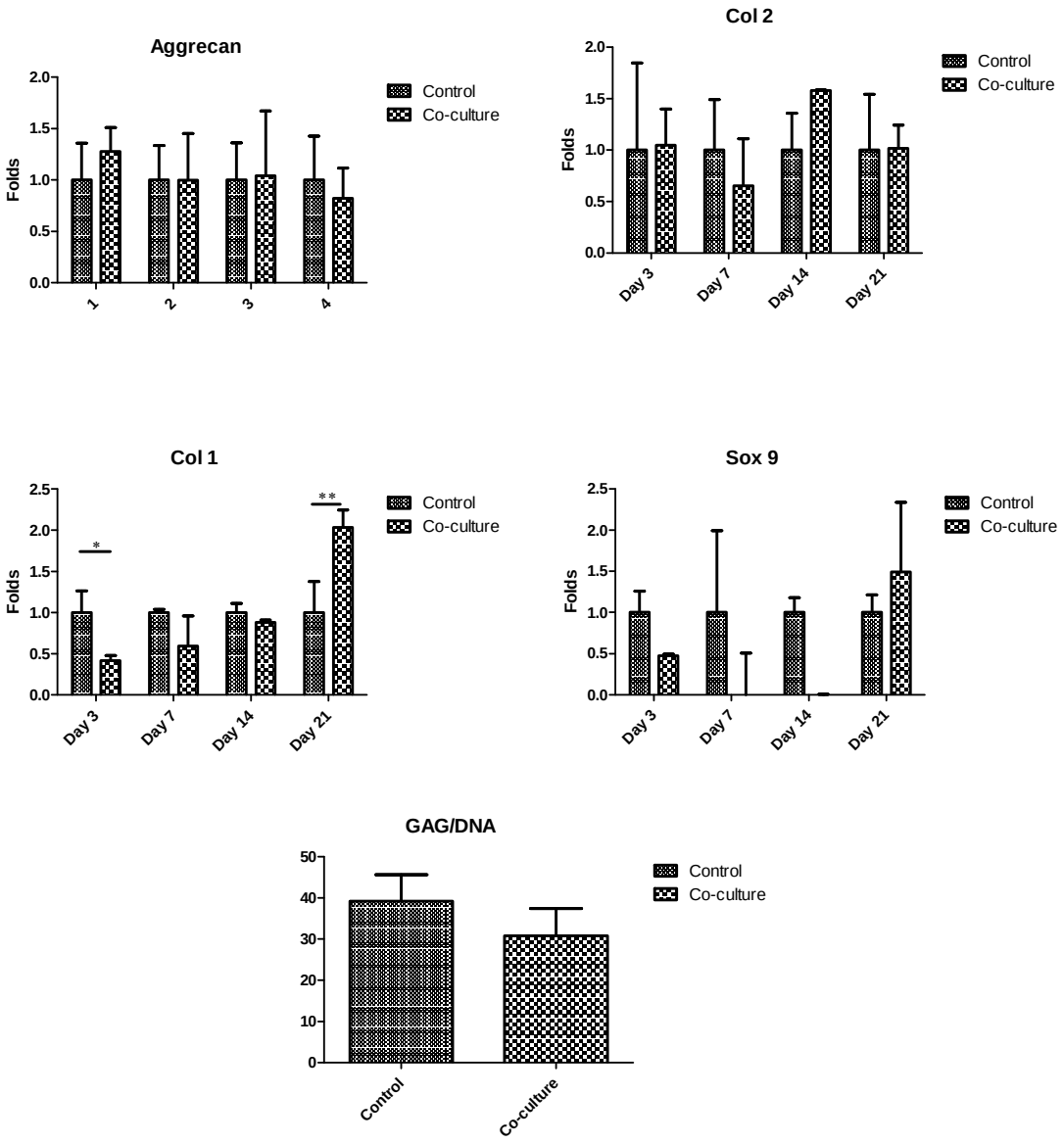


Figure 20: Gene expression and biochemical results for co-culture with P1 OA.
RT-PCR analysis to determine gene expression of a) Aggrecan; b) Collagen type I; c) Collagen type II; d) Sox-9. Biochemical analysis results to estimate e) GAG content (w.r.t. DNA content). Results represent the mean of triplicate culture and represented as mean+/-SD. P1 OA does not support chondrogenesis.

4.2.3) Exogenous supplementation of PTHrP and TGF-β1 promote chondrogenic differentiation of MSCs while repressing their hypertrophic differentiation

Once the effect of chondrocyte secreted factors on chondrogenic differentiation of hMSCs was established, we wanted to look into the pathways that might be involved in promoting chondrogenic differentiation of hMSCs and/or repressing the terminal differentiation of such MSC-derived chondrocytes into hypertrophic chondrocytes. To that effect, co-culture studies were carried out in the presence as well as absence of TGF-β1 to explore its role in chondrogenesis. hMSC constructs for both co-culture and control were grown in chondrogenic medium containing TGF-β1and compared against constructs grown in chondrogenic medium without TGF-β1. We also examined the role of PTHrP on chondrogenic differentiation of hMSCs, which is known to prevent hypertrophy in chondrocytes. In order to evaluate the role of PTHrP, studies were carried out in the presence of PTHrP (1-34 a.a. chain) and its chemical blocker cyclopamine. The effect on hypertrophy was examined mainly by collagen type X staining.

4.2.3.1) Chondrocyte secreted factors promote chondrogenic differentiation of hMSCs even in the absence of TGF-β1:

The real-time PCR results (Fig. 21) showed co-culture alone significantly increases gene expression of chondrogenic markers such as aggrecan (10 folds), and collagen type II (4 folds) of hMSC constructs. Whereas, a combination of co-culture with TGF-β1 had strongly increased gene expression of aggrecan (60 folds), and collagen type II (30 folds) compared to the same day control sample. However, control

without TGF-β1 showed no gene expressions of chondrogenic markers. Collagen type I and Sox-9 during co-culture in the presence of TGF-β1 follow the same trend as the previous experiment however they're minimally expressed with respect to control in presence of TGF-β1 in co-culture constructs without TGF-β1. Histological examination of hMSC constructs co-cultured with chondrocytes in the presence of TGF-β1 showed an extensive chondrogenic differentiation of hMSCs. Immunofluorescent staining for collagen type II showed strong presence of collagen type II in co-cultured hMSC constructs (Fig. 24c and 24d). Control constructs in the presence of TGF-β1 on the other hand showed only slightly positive immunoflourescent staining for collagen type II (Fig. 24a and 24c). Collagen type II was entirely absent in control constructs cultured in the absence of TGF-β1 (Fig. 24e). But what was interesting was the presence of collagen type II staining in co-cultured constructs even in the absence of TGF-β1 (although less than that of co-cultures in TGF-β1 presence- Fig. 24f) showing that co-culture conditions by themselves are enough to induce chondrogenesis in hMSCs. All co-culture constructs of hMSC displayed significant sulfated proteoglycans, which stained positive for Safrain-O (again absence of TGF-β1 displayed reduced staining compared to co-culture in presence of TGF-β1 but nevertheless was present) (Fig. 23). Control cultures in presence of TGF-β1 had safranin-O staining that increased from day14 to day 21 but without TGF-β1 exhibited a very weak staining for SafO, (Fig 23g).

Biochemical analysis of the hMSC constructs support the histology staining with collagen content (normalized to DNA content) being highest in constructs grown in co-culture with TGF-β1 which significantly increased from day 14 to day 21 (Fig. 22a).

There was also significantly more total collagen in the co-cultured constructs than the controls. Controls with no TGF-β1 showed no detectable collagen content but co-culture without TGF-β1 had more collagen than the control with TGF-β1 on day 21 (Fig. 22a). Interestingly there was less DNA content in all constructs grown without TGF-β1 as compared to their TGF-β1counterparts indicating that TGF-β1might play a role in cell proliferation (Fig. 22e). There was significantly more GAG in the co-cultured constructs than the controls at all time points and increased from day 14 to day 21 considerably (Fig. 22 c and d). The controls without TGF-β1 showed minimal amount of GAG corroborating with the Saf-O staining. Co-cultures without TGF-β1 on the other hand showed significant ($p<0.05$) GAG on day 21 compared to same day control with TGF-β1 (Fig. 22c).

4.2.3.2) Presence of PTHrP in culture promotes chondrogenic differentiation of hMSCs while actively repressing their terminal differentiation into hypertrophic chondrocytes

qRT-PCR results demonstrated that in the presence of PTHrP protein there was an elevated expression of chondrogenic markers compared to cultures done in its absence (Fig. 21). Whereas, in the presence of blocker cyclopamine there was a significant expression of collagen type I and type X, which was not seen in other groups (Fig. 21 c and d). Gene expression of aggrecan was almost entirely absent in the presence of blocker (both control and co-culture), but was strongly present in the d21 co-culture with PTHrP higher than in co-culture without PTHrP. Type I collagen was not expressed in d21 co-culture with PTHrP, was almost absent in the d21 normal co-

culture, but was weakly present in the control constructs with blocker and was 4-folds in co-culture with blocker. Type II collagen was not expressed in the presence of blocker, was strongly present in the d21 co-culture, but was present in even higher quantities in the co-culture constructs grown in PTHrP. Interestingly control constructs grown in the presence of PTHrP but without TGF-β1 show very little expression of Aggrecan and Collagen type II but have an increased expression of collagen type I and Sox-9 indicating a possibly delayed chondrogenesis in the absence of TGF-β1.

Biochemical analysis showed that the DNA content (percent dry weight) (Fig. 22 e and f) of the MSC constructs revealed a statistically significant increase in constructs cultured in the presence of PTHrP (both control and co-culture). These results supported the previously described increase in cell number by proliferation in its presence. In correlation with the RT-PCR and histological analysis, the hydrogels showed a significant increase in GAG and total collagen content (normalized to DNA) at day 21 when the constructs were cultured with PTHrP in controls (Fig. 22). Interestingly however, co-cultured hMSC constructs with and without PTHrP showed almost equal amounts of GAG and collagen content whereas co-cultured hMSC constructs with blocker showed significantly lesser GAG (Fig. 22 c and d) production but similar amount of collagen content as compared to co-culture + PTHrP (Fig. 22 a and b). The similar amount of collagen observed in the co-cultured systems with and without the PTHrP blocker is attributed to the measurement of total collagen content, which does not distinguish the type of collagens. GAG synthesis increased significantly from day 0 to day 14 and finally to day 21 in all co-culture groups, except in the presence of blocker.

Histological analysis of the hMSC constructs showed that the experimental groups cultured with PTHrP were strongly positive for GAG compared with the other groups for both control and co-culture (Fig. 23). However, in the presence of blocker cyclopamine, there was negligible saf-O staining observed (Fig. 23 i and j). Figure 23 depicts histologic sections of the ten groups with safranin-O, which stains negatively charged glycosaminoglycans red. Sections from the d21 co-culture construct (Fig. 23 b and d) in TGF-β1 revealed intense positive staining for GAG, particularly around the pericellular regions. The positive staining was enhanced in the presence of PTHrP in co-cultured group (Fig. 23f). It was perceptibly more in the control group grown in PTHrP too (Fig. 23e). Conversely, in the sections taken from control constructs grown in both PTHrP and blocker, there was only a small amount of GAG and was entirely absent in the co-culture sections grown in the presence of blocker only (Fig. 23 i and j). Immunoflourescent staining for collagen type II (Fig. 24) show similar trends with hMSC constructs grown in the presence of PTHrP showing the most intense green labeling for collagen type II (blue shows DAPI stained nuclei) followed by co-culture in presence of TGF-β1 at day 21. There were negative or sporadic, weak positively stained cells for collagen type II present in culture with cyclopamine, the blocker. However, staining for type X collagen was strongly positive in the presence of blocker (Fig. 25 e and f) on both the control and the co-culture sections, more so in co-culture and was almost entirely absent in co-culture with PTHrP. The co-cultured hMSC constructs supplemented with PTHrP show weaker type X collagen than that co-cultured hMSCs without PTHrP on both day 14 and day 21. In fact, collagen type X in hMSCs co-cultured without PTHrP supplementation was weaker than that of the same day control.

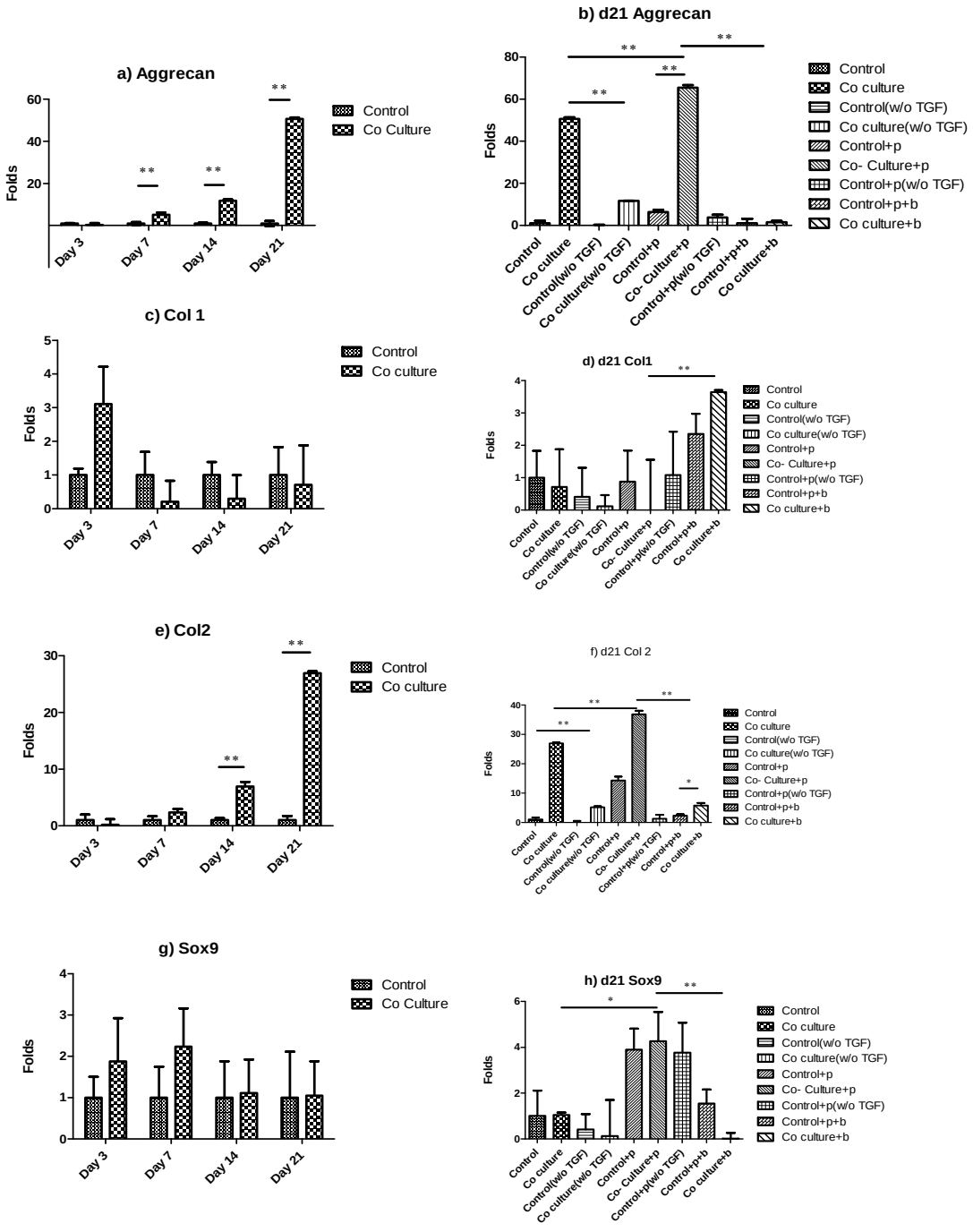


Figure 21: qRT-PCR analysis results for study of TGF-β1 and PTHrP pathways during co-culture with P0 OA.

a) Timed study for Aggrecan; b) d21 Aggrecan; c) Timed study for Collagen type I; d) d21 Collagen I; e) Timed study for Collagen type II; f) d21 Collagen II; g) Timed study for Sox-9; h) d21 Sox-9

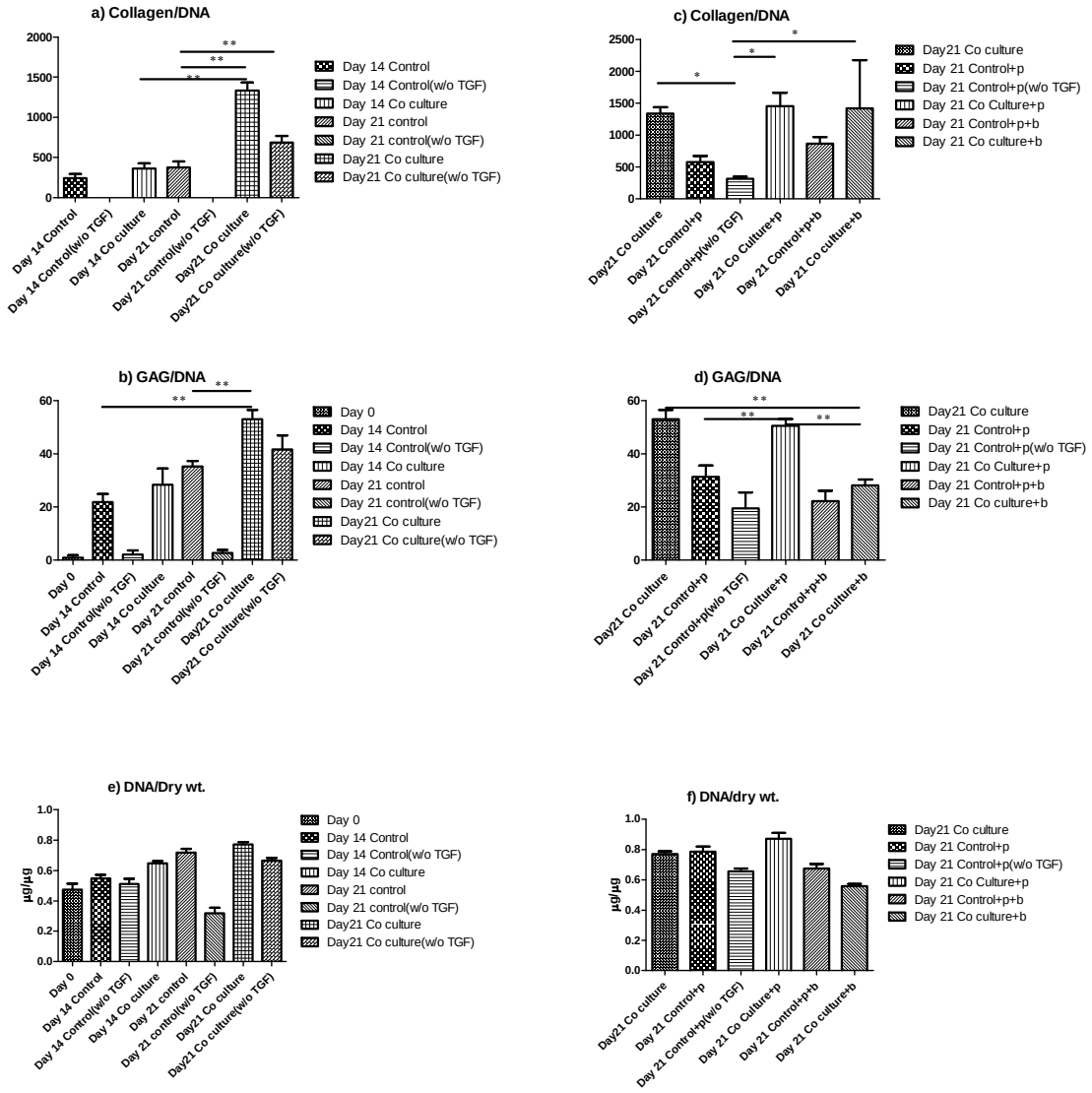


Figure 22: Biochemimcal analysis results for pathway studies.

a) Collagen/DNA timed study; b) Collagen /DNA day 21; c) GAG/DNA timed study; d) GAG/DNA day 21; e) DNA timed study; f) DNA assay for day 21. Results represent the mean of triplicate culture and represented as mean+/-SD.

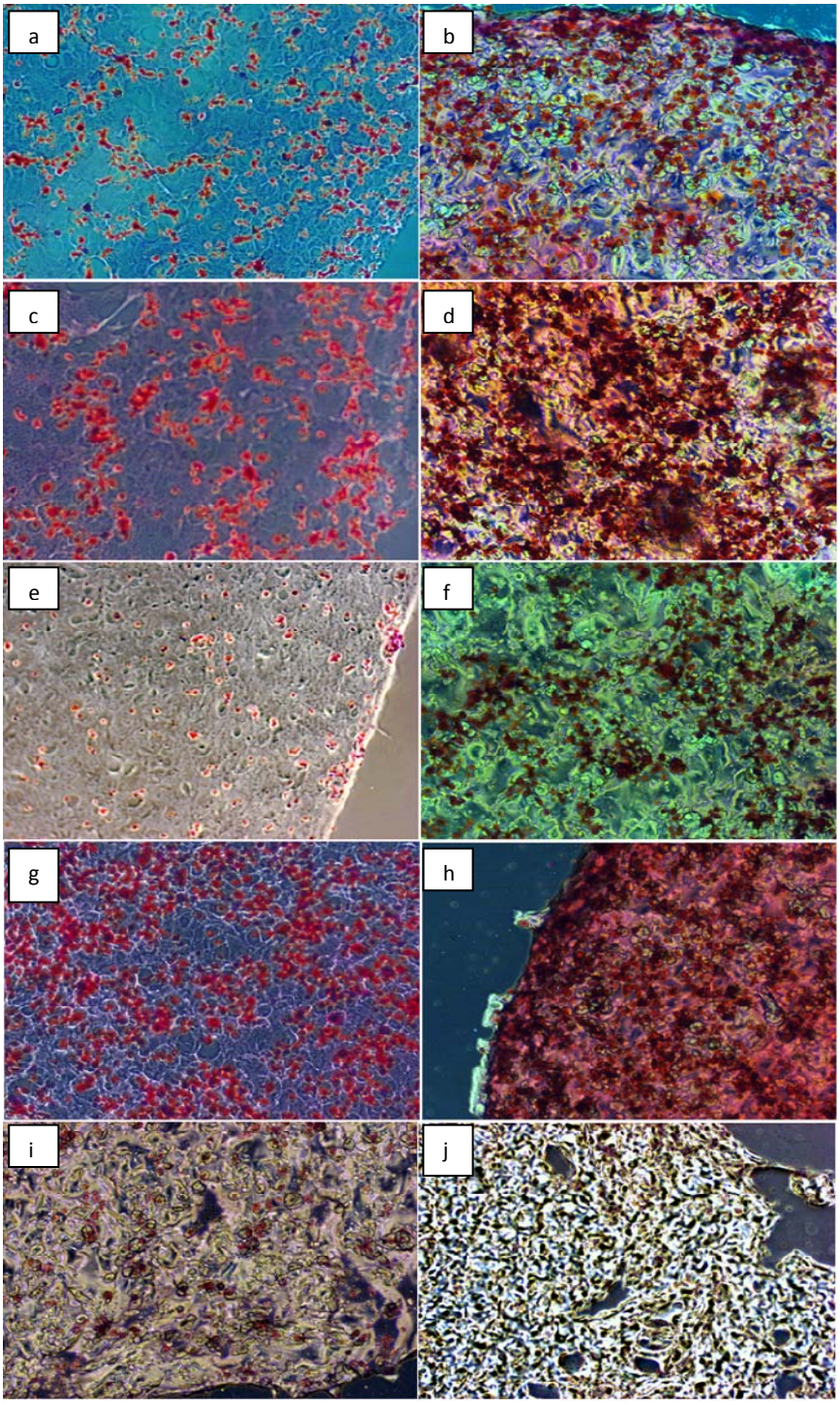


Figure 23: Safranin-O staining images for pathways studies.
a) d14 control with TGF-β1; b) d14 co-culture with TGF-β1; c) d21 control with TGF-β1; d) d21 co-culture with TGF-β1; e) d21 control without TGF-β1; f) d21 co-culture without TGF-β1; g) d21 control with PTHrP; h) d21 co-culture with PThrP; i) d21 control + blocker + PTHrP; j) d21 co-culture + blocker. ***Blocker = cyclopamine. All images were taken at an original magnification of 10X.

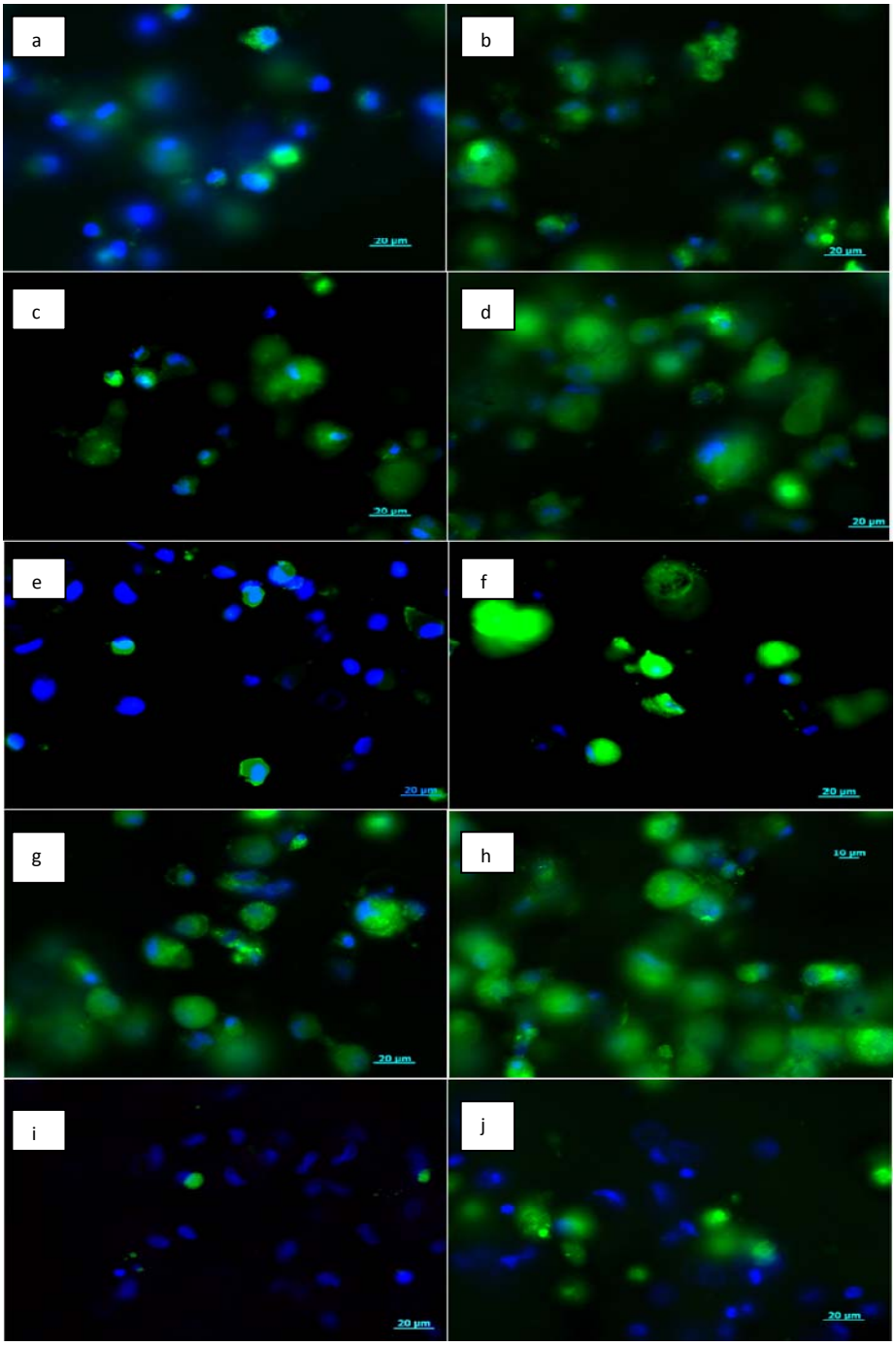


Figure 24: Collagen type II immunostaining images for pathway studies.
a) d14 control with TGF-β1; b) d14 co-culture with TGF-β1; c) d21 control with TGF-β1; d) d21 co-culture with TGF-β1; e) d21 control without TGF-β1; f) d21 co-culture without TGF-β1; g) d21 control with PTHrP; h) d21 co-culture with PThrP; i) d21 control + blocker + PTHrP; j) d21 co-culture + blocker. ***Blocker = cyclopamine. All images were taken at an original magnification of 40 X. Green is collagen type 2 with DAP I stained blue nuclei.

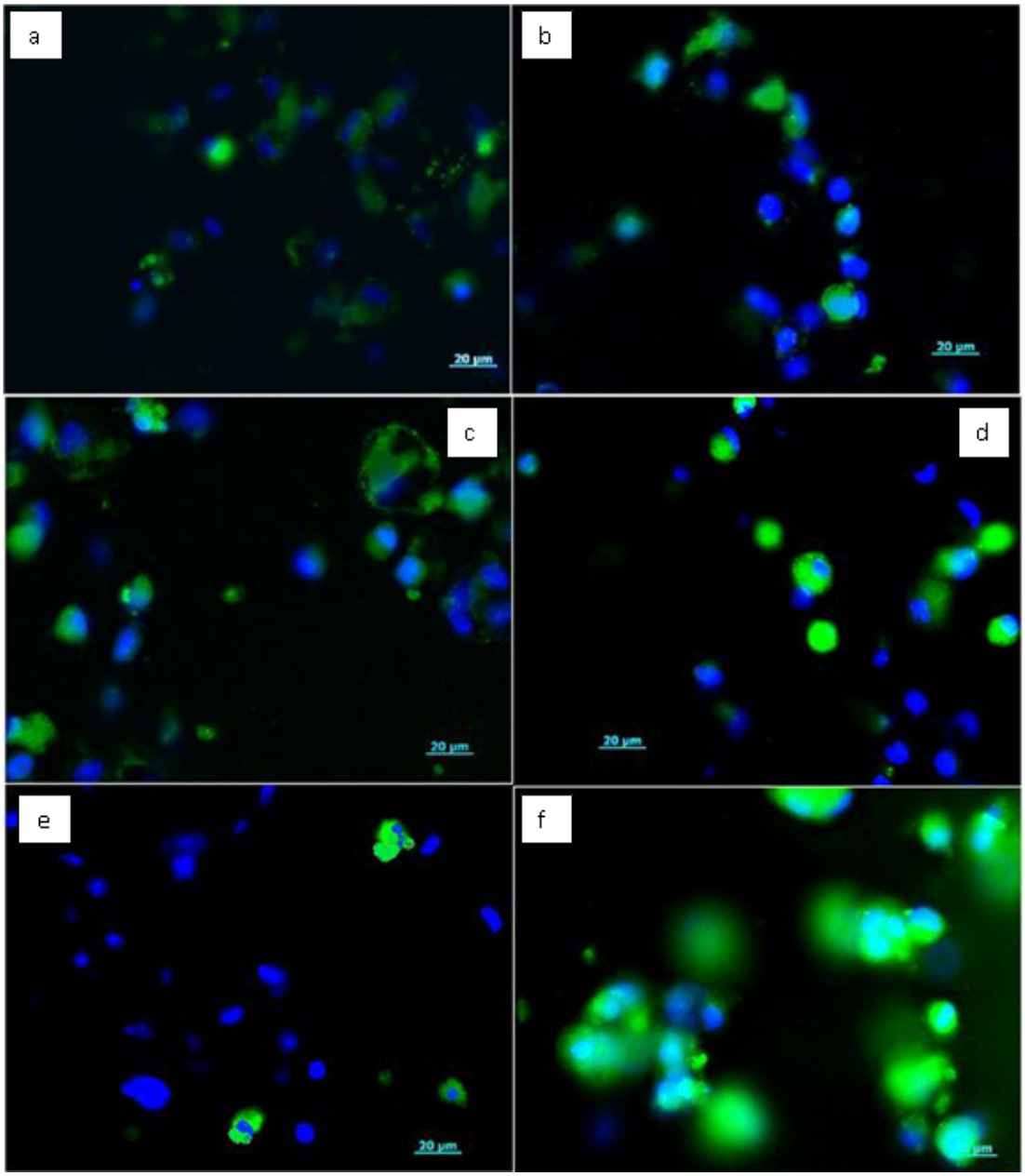


Figure 25: Collagen type X immunostaining images.
a) d14 control with TGF-β1; b) d14 co-culture with TGF-β1; c) d21 control with TGF-β1; d) d21 co-culture with TGF-β1; e) d21 co-culture with PTHrP; f) d21 co-culture in the presence of blocker cyclopamine.All images were taken at an original magnification of 40 X. Green is collagen type 10 with DAPI stained blue nuclei.

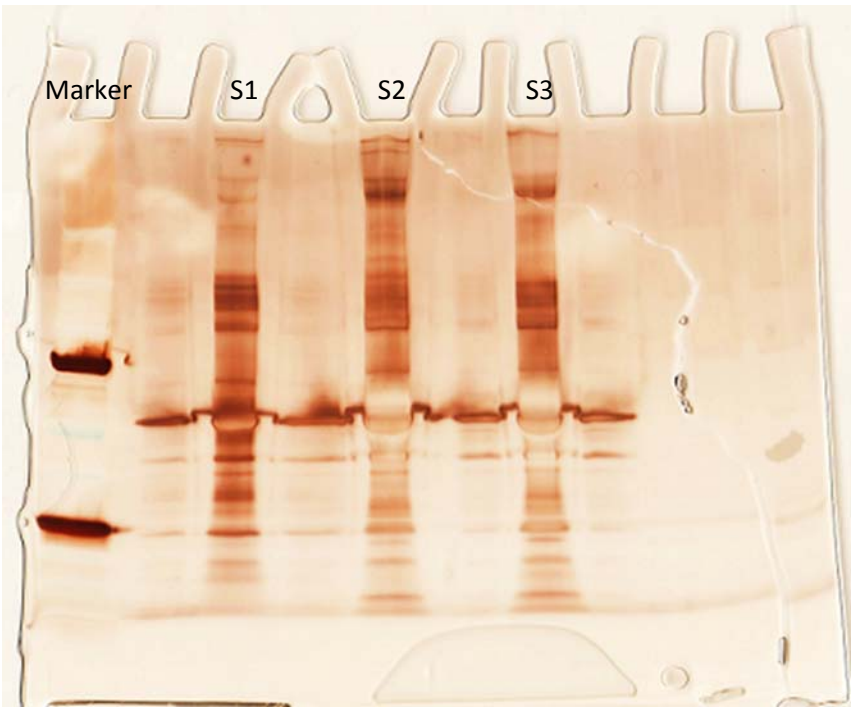
4.3) Identification of Chondrocyte secreted factors

Mass spectroscopic analysis was performed to identify soluble factors being secreted by chondrocytes in co-culture and which were different from the one that is secreted by hMSC or chondrocytes when they were cultured alone. To that effect, conditioned DMEM without FBS from each of the following conditions was collected after 48 hours:

1) P4 hMSC cultured in growth medium (GM).
2) P4 hMSC in chondrogenic medium (CM).
3) P0 OA cells in chondrocyte culture medium (CCM).
4) P1 OA cells in CCM.
5) P0 OA-P4 hMSC co-culture in CM.
6) P1 OA-P4 hMSC co-culture in CM

After protein precipitation with TCA, the proteins were run on an SDS-PAGE gel. The following (Fig. 26) images were obtained and sent to UCSD Mass Spectrometry facility to identify the proteins.

There were 22 proteins identified in sample 1, 30 in sample 2, 35 each in samples 3 and 4, 106 in sample 5 and 61 in sample 6. The list of the identified proteins are tabulated in tables 3-8 To identify the unique proteins of co-cultures systems we created Venn diagrams constituting the above proteins (Figs. 27-29).



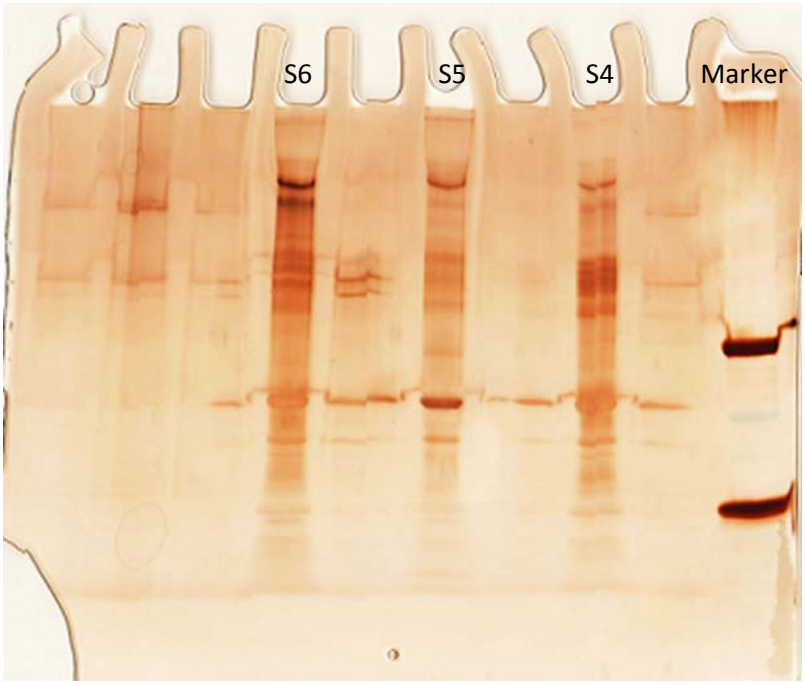


Figure 26: SDS-PAGE gel images after silver staining.

The list of proteins was:

Table 3: Mass spec identified proteins from sample 1

	Name	Abbreviation
1	alpha 1 type I collagen preproprotein [Homo sapiens]	Col1(a1)
2	alpha 2 type I collagen [Homo sapiens]	Col2(a2)
3	keratin 1 [Homo sapiens]	Ker1
4	albumin precursor [Homo sapiens]	Alb-pre
5	fibronectin 1 isoform 1 preproprotein [Homo sapiens]	F1I1P
6	fibronectin 1 isoform 2 preproprotein [Homo sapiens]	F1I2P
7	fibronectin 1 isoform 4 preproprotein [Homo sapiens]	F1I4P
8	fibronectin 1 isoform 5 preproprotein [Homo sapiens]	F1I5P
9	fibronectin 1 isoform 6 preproprotein [Homo sapiens]	F1I6P
10	fibronectin 1 isoform 3 preproprotein [Homo sapiens]	F1I3P
11	transferrin [Homo sapiens]	Transfe
12	insulin-like growth factor binding protein 7 [Homo sapiens]	IGFBP7
13	vimentin [Homo sapiens]	Viment
14	PREDICTED: hypothetical protein [Homo sapiens]	HP
15	keratin 6B [Homo sapiens]	Ker6B
16	keratin 6A [Homo sapiens]	Ker6A
17	keratin 6C [Homo sapiens]	Ker6C
18	keratin 2 [Homo sapiens]	Ker2
19	keratin 6L [Homo sapiens]	Ker6L
20	keratin 5 [Homo sapiens]	Ker5
21	keratin 75 [Homo sapiens]	Ker75
22	tissue inhibitor of metalloproteinase 1 precursor [Homo sapiens]	TIMP1pre

Table 4: Mass spec identified proteins from sample 2

	Name	Abbreviation
1	fibronectin 1 isoform 4 preproprotein [Homo sapiens]	F1I4P
2	fibronectin 1 isoform 3 preproprotein [Homo sapiens]	F1I3P
3	fibronectin 1 isoform 5 preproprotein [Homo sapiens]	F1I5P
4	fibronectin 1 isoform 6 preproprotein [Homo sapiens]	F1I6P
5	fibronectin 1 isoform 1 preproprotein [Homo sapiens]	F1I1P
6	fibronectin 1 isoform 2 preproprotein [Homo sapiens]	F1I2P
7	alpha 2 type I collagen [Homo sapiens]	Col1(a2)
8	alpha 1 type I collagen preproprotein [Homo sapiens]	Col1(a1)
9	albumin precursor [Homo sapiens]	Alb-pre

Table 4 continued…

10	insulin-like growth factor binding protein 7 [Homo sapiens]	IGFBP7
11	keratin 1 [Homo sapiens]	Ker1
12	plasminogen activator inhibitor type 1, member 2 [Homo sapiens]	PAI1mem2
13	alpha-2-macroglobulin precursor [Homo sapiens]	Mcpra2
14	pregnancy-zone protein [Homo sapiens]	PZP
15	thrombospondin 1 precursor [Homo sapiens]	Thr1pre
16	matrix metalloproteinase 2 preproprotein [Homo sapiens]	MMP2P
17	actin, gamma 1 propeptide [Homo sapiens]	Acting1p
18	beta actin [Homo sapiens]	b-actin
19	protein expressed in prostate, ovary, testis, and placenta 2 [Homo sapiens]	ppotp2
20	secreted protein, acidic, cysteine-rich [Homo sapiens]	SPAC
21	fibrillin 1 precursor [Homo sapiens]	FIB1PRE
22	periostin, osteoblast specific factor [Homo sapiens]	PEROSTIN
23	vitamin D-binding protein precursor [Homo sapiens]	Vit-DBPP
24	plasminogen activator inhibitor-1 [Homo sapiens]	PAI1
25	EGF-containing fibulin-like extracellular matrix protein 1 precursor [Homo sapiens]	EGFECM11
26	EGF-containing fibulin-like extracellular matrix protein 1 precursor [Homo sapiens]	EGFECM12
27	EGF-containing fibulin-like extracellular matrix protein 1 precursor [Homo sapiens]	EGFECM13
28	collagen, type VI, alpha 1 precursor [Homo sapiens]	Col6(a1)
29	carboxypeptidase D precursor [Homo sapiens]	carDpre
30	transforming growth factor, beta-induced, 68kDa [Homo sapiens]	TGF-B-I

Table 5: Mass spec identified proteins from sample 3

	Name	**Abbreviation**
1	alpha 2 type I collagen [Homo sapiens]	Col1(a2)
2	fibronectin 1 isoform 4 preproprotein [Homo sapiens]	F1I4P
3	fibronectin 1 isoform 5 preproprotein [Homo sapiens]	F1I3P
4	fibronectin 1 isoform 6 preproprotein [Homo sapiens]	F1I5P
5	fibronectin 1 isoform 3 preproprotein [Homo sapiens]	F1I6P
6	fibronectin 1 isoform 1 preproprotein [Homo sapiens]	F1I1P
7	fibronectin 1 isoform 2 preproprotein [Homo sapiens]	F1I2P
8	albumin precursor [Homo sapiens]	Alb-pre
9	alpha 1 type I collagen preproprotein [Homo sapiens]	Col1(a1)
10	keratin 1 [Homo sapiens]	Ker1

Table 5 continued…

11	PREDICTED: hypothetical protein [Homo sapiens]	PreHP
12	insulin-like growth factor binding protein 7 [Homo sapiens]	IGFBP7
13	fibrillin 1 precursor [Homo sapiens]	FIB1PRE
14	chitinase 3-like 1 [Homo sapiens]	Chit3
15	EGF-containing fibulin-like extracellular matrix protein 1 precursor [Homo sapiens]	EGFECM11
16	EGF-containing fibulin-like extracellular matrix protein 1 precursor [Homo sapiens]	EGFECM12
17	EGF-containing fibulin-like extracellular matrix protein 1 precursor [Homo sapiens]	EGFECM13
18	insulin-like growth factor binding protein 3 isoform a precursor [Homo sapiens]	IGFBP3a
19	insulin-like growth factor binding protein 3 isoform b precursor [Homo sapiens]	IGFBP3b
20	alpha-fetoprotein precursor [Homo sapiens]	afetpre
21	transforming growth factor, beta-induced, 68kDa [Homo sapiens]	TGF-b-I
22	collagen, type VI, alpha 1 precursor [Homo sapiens]	Col6(a1)
23	keratin 2 [Homo sapiens]	Ker2
24	keratin 6B [Homo sapiens]	Ker6B
25	keratin 6L [Homo sapiens]	Ker6L
26	keratin 6C [Homo sapiens]	Ker6C
27	keratin 6A [Homo sapiens]	Ker6A
28	keratin 5 [Homo sapiens]	Ker5
29	keratin 75 [Homo sapiens]	Ker75
30	vanin 1 precursor [Homo sapiens]	vanin1pre
31	G-gamma globin [Homo sapiens]	gamma(g)
32	A-gamma globin [Homo sapiens]	gamma(a)
33	delta globin [Homo sapiens]	globin(d)
34	beta globin [Homo sapiens]	globin(b)
35	epsilon globin [Homo sapiens]	globin(e)

Table 6: Mass spec identified proteins from sample 3

	Name	**Abbreviation**
1	alpha 1 type I collagen preproprotein [Homo sapiens]	Col1(a1)
2	alpha 2 type I collagen [Homo sapiens]	Col1(a2)
3	fibronectin 1 isoform 1 preproprotein [Homo sapiens]	F1I1P
4	fibronectin 1 isoform 3 preproprotein [Homo sapiens]	F1I3P
5	fibronectin 1 isoform 5 preproprotein [Homo sapiens]	F1I5P

Table 6 continued…

6	fibronectin 1 isoform 4 preproprotein [Homo sapiens]	F1I4P
7	fibronectin 1 isoform 2 preproprotein [Homo sapiens]	F1I2P
8	fibronectin 1 isoform 6 preproprotein [Homo sapiens]	F1I6P
9	albumin precursor [Homo sapiens]	Alb-pre
10	fibrillin 1 precursor [Homo sapiens]	FIB1PRE
11	transferrin [Homo sapiens]	transfe
12	keratin 1 [Homo sapiens]	Ker1
13	follistatin-like 1 precursor [Homo sapiens]	fol1pre
14	keratin 9 [Homo sapiens]	Ker9
15	insulin-like growth factor binding protein 7 [Homo sapiens]	IGFBP7
16	keratin 2 [Homo sapiens]	Ker2
17	keratin 6B [Homo sapiens]	Ker6B
18	keratin 6A [Homo sapiens]	Ker6A
19	keratin 6C [Homo sapiens]	Ker6C
20	keratin 5 [Homo sapiens]	Ker5
21	keratin 75 [Homo sapiens]	Ker75
22	keratin 6L [Homo sapiens]	Ker6L
23	latent transforming growth factor beta binding protein 2 [Homo sapie]	L-TGF-b
24	periostin, osteoblast specific factor [Homo sapiens]	PEROSTIN
25	PREDICTED: hypothetical protein [Homo sapiens]	HP1pre
26	plasminogen activator inhibitor-1 [Homo sapiens]	PAI(1)
27	secreted protein, acidic, cysteine-rich [Homo sapiens]	SPAC
28	alpha 3 type VI collagen isoform 1 precursor [Homo sapiens]	Col6(a3)1
29	alpha 3 type VI collagen isoform 2 precursor [Homo sapiens]	Col6(a3)2
30	alpha 3 type VI collagen isoform 4 precursor [Homo sapiens]	Col6(a3)4
31	alpha 3 type VI collagen isoform 5 precursor [Homo sapiens]	Col6(a3)5
32	alpha 3 type VI collagen isoform 3 precursor [Homo sapiens]	Col6(a3)3
33	proinsulin precursor [Homo sapiens]	Proinsulin
34	connective tissue growth factor [Homo sapiens]	CTGF
35	alpha 2 type V collagen preproprotein [Homo sapiens]	Col5(2)

Table 7: Mass spec identified proteins from sample 5

	Name	**Abbreviation**
1	alpha 2 type I collagen [Homo sapiens]	Col1(a2)
2	alpha 1 type I collagen preproprotein [Homo sapiens]	Col1(a1)
3	fibronectin 1 isoform 4 preproprotein [Homo sapiens]	F1I4P
4	fibronectin 1 isoform 2 preproprotein [Homo sapiens]	F1I2P

Table 7 continued…

5	fibronectin 1 isoform 1 preproprotein [Homo sapiens]	F1I1P
6	fibronectin 1 isoform 5 preproprotein [Homo sapiens]	F1I5P
7	fibronectin 1 isoform 3 preproprotein [Homo sapiens]	F1I3P
8	fibronectin 1 isoform 6 preproprotein [Homo sapiens]	F1I6P
9	keratin 1 [Homo sapiens]	Ker1
10	insulin-like growth factor binding protein 3 isoform b precursor [Homo sapiens]	IGFBP3b
11	insulin-like growth factor binding protein 3 isoform a precursor [Homo sapiens]	IGFBP3a
12	albumin precursor [Homo sapiens]	Alb-pre
13	lumican precursor [Homo sapiens]	lumican
14	tissue inhibitor of metalloproteinase 1 precursor [Homo sapiens]	TIMP1pre
15	insulin-like growth factor binding protein 7 [Homo sapiens]	IGFBP7
16	keratin 10 [Homo sapiens]	Ker10
17	fibrillin 1 precursor [Homo sapiens]	FIB1PRE
18	vitamin D-binding protein precursor [Homo sapiens]	Vit-DBPP
19	keratin 9 [Homo sapiens]	Ker9
20	PREDICTED: hypothetical protein [Homo sapiens]	HP3
21	collagen, type III, alpha 1 preproprotein [Homo sapiens]	Col3(a1)
22	serine (or cysteine) proteinase inhibitor, clade C (antithrombin), member 1 [Homo sapiens]	serinePI
23	vimentin [Homo sapiens]	Vim
24	alpha-fetoprotein precursor [Homo sapiens]	afetopre
25	secreted protein, acidic, cysteine-rich [Homo sapiens]	SPAC
26	TIMP metallopeptidase inhibitor 2 precursor [Homo sapiens]	TIMPinh2
27	complement component 3 precursor [Homo sapiens]	CC3P
28	PREDICTED: hypothetical protein, partial [Homo sapiens]	HPP
29	chitinase 3-like 1 [Homo sapiens]	Chit3
30	keratin 6B [Homo sapiens]	Ker6B
31	keratin 6C [Homo sapiens]	Ker6C
32	keratin 6A [Homo sapiens]	Ker6A
33	keratin 2 [Homo sapiens]	Ker2
34	keratin 75 [Homo sapiens]	Ker75
35	keratin 5 [Homo sapiens]	Ker5
36	keratin 6L [Homo sapiens]	Ker6L
37	transferrin [Homo sapiens]	Transfe
38	clusterin isoform 1 [Homo sapiens]	CI1
39	clusterin isoform 2 [Homo sapiens]	CI2
40	EGF-containing fibulin-like extracellular matrix protein 1 precursor	EGFECM11

Table 7 continued…

41	EGF-containing fibulin-like extracellular matrix protein 1 precursor [Homo sapiens]	EGFECM12
42	EGF-containing fibulin-like extracellular matrix protein 1 precursor [Homo sapiens]	EGFECM13
43	insulin-like growth factor binding protein 4 precursor [Homo sapiens]	IGFBP4
44	beta-2-microglobulin precursor [Homo sapiens]	b2micglo
45	actin, gamma 1 propeptide [Homo sapiens]	actin(g1)
46	hypothetical protein LOC345651 [Homo sapiens]	HPLOC
47	beta actin [Homo sapiens]	b-actin
48	protein expressed in prostate, ovary, testis, and placenta 2 [Homo sapiens]	ppotp2
49	prostate, ovary, testis expressed protein on chromosome 2 [Homo sapiens]	ppotchro
50	alpha 1 actin precursor [Homo sapiens]	actin(a1)
51	cardiac muscle alpha actin 1 proprotein [Homo sapiens]	CMAA1
52	actin, gamma 2 propeptide [Homo sapiens]	Actin(g2)
53	alpha 2 actin [Homo sapiens]	actin(a2)
54	PREDICTED: hypothetical protein isoform 5 [Homo sapiens]	HPI5
55	PREDICTED: hypothetical protein isoform 4 [Homo sapiens]	HPI4
56	triosephosphate isomerase 1 [Homo sapiens]	TI1
57	PREDICTED: similar to rcTPI1 isoform 1 [Homo sapiens]	rcTPI11
58	PREDICTED: similar to rcTPI1 isoform 2 [Homo sapiens]	rcTPI12
59	PREDICTED: similar to rcTPI1 isoform 6 [Homo sapiens]	rcTPI16
60	PREDICTED: similar to rcTPI1 isoform 4 [Homo sapiens]	rcTPI14
61	follistatin-like 1 precursor [Homo sapiens]	fol1pre
62	transforming growth factor, beta-induced, 68kDa [Homo sapiens]	TGF-b-I

******Sample 5 also had a bunch of histones (44 in number which have not been shown here):*** Different isoforms of H2a and H2b

Table 8: Mass spec identified proteins from sample 6

	Name	**Abbreviation**
1	alpha 2 type I collagen [Homo sapiens]	Col1(a2)
2	alpha 1 type I collagen preproprotein [Homo sapiens]	Col1(a1)
3	fibronectin 1 isoform 3 preproprotein [Homo sapiens]	F1I3P
4	fibronectin 1 isoform 4 preproprotein [Homo sapiens]	F1I4P
5	fibronectin 1 isoform 5 preproprotein [Homo sapiens]	F1I5P

Table 8 continued…

6	fibronectin 1 isoform 6 preproprotein [Homo sapiens]	F1I6P
7	fibrillin 1 precursor [Homo sapiens]	FIB1PRE
8	periostin, osteoblast specific factor [Homo sapiens]	PEROSTIN
9	elastin isoform d [Homo sapiens]	elastind
10	elastin isoform c [Homo sapiens]	elastinc
11	elastin isoform a [Homo sapiens]	elastina
12	elastin isoform e [Homo sapiens]	elastine
13	elastin isoform b [Homo sapiens]	elastinb
14	secreted protein, acidic, cysteine-rich [Homo sapiens]	SPAC
15	keratin 1 [Homo sapiens]	Ker1
16	latent transforming growth factor beta binding protein 2 [Homo sapiens]	L-TGF-b
17	insulin-like growth factor binding protein 7 [Homo sapiens]	IGFBP7
18	tissue inhibitor of metalloproteinase 1 precursor [Homo sapiens]	TIMP1pre
19	albumin precursor [Homo sapiens]	Alb-pre
20	connective tissue growth factor [Homo sapiens]	CTGF
21	thrombospondin 1 precursor [Homo sapiens]	Thr.1pre
22	alpha 2 type V collagen preproprotein [Homo sapiens]	Col5(2)
23	transferrin [Homo sapiens]	Transfe
24	follistatin-like 1 precursor [Homo sapiens]	fol1pre
25	insulin-like growth factor binding protein 3 isoform b precursor [Homo sapiens]	IGFBP3b
26	insulin-like growth factor binding protein 3 isoform a precursor [Homo sapiens]	IGFBP3a
27	transforming growth factor, beta-induced, 68kDa [Homo sapiens]	TGF-b-I
28	keratin 9 [Homo sapiens]	Ker9
29	alpha 1 type V collagen preproprotein [Homo sapiens]	Col5(1)
30	collagen, type III, alpha 1 preproprotein [Homo sapiens]	Col3(a1)
31	PREDICTED: hypothetical protein [Homo sapiens]	HP2
32	lumican precursor [Homo sapiens]	lumican
33	cartilage oligomeric matrix protein precursor [Homo sapiens]	COMP
34	keratin 2 [Homo sapiens]	Ker2
35	keratin 6B [Homo sapiens]	Ker6B
36	keratin 6A [Homo sapiens]	Ker6A
37	keratin 75 [Homo sapiens]	Ker75
38	keratin 6L [Homo sapiens]	Ker6L
39	keratin 6C [Homo sapiens]	Ker6C
40	keratin 5 [Homo sapiens]	Ker5
41	TIMP metallopeptidase inhibitor 2 precursor [Homo sapiens]	TIMPinh2

Table 8 continued…

42	hyaluronan and proteoglycan link protein 1 [Homo sapiens]	GAG
43	plasminogen activator inhibitor-1 [Homo sapiens]	PAI(1)
44	alpha 2 type IV collagen preproprotein [Homo sapiens]	Col4(a2)
45	vimentin [Homo sapiens]	Vim
46	alpha 3 type VI collagen isoform 4 precursor [Homo sapiens]	Col6(a3)1
47	alpha 3 type VI collagen isoform 1 precursor [Homo sapiens]	Col6(a3)2
48	alpha 3 type VI collagen isoform 2 precursor [Homo sapiens]	Col6(a3)4
49	alpha 3 type VI collagen isoform 5 precursor [Homo sapiens]	Col6(a3)5
50	alpha 3 type VI collagen isoform 3 precursor [Homo sapiens]	Col6(a3)3
51	biglycan preproprotein [Homo sapiens]	biglypp
52	procollagen C-endopeptidase enhancer [Homo sapiens]	procolc
53	keratin 10 [Homo sapiens]	Ker10
54	beta-2-microglobulin precursor [Homo sapiens]	b2micglo
55	lysyl oxidase preproprotein [Homo sapiens]	lysyloxi
56	fibulin 5 precursor [Homo sapiens]	fibu(5)
57	scrapie responsive protein 1 [Homo sapiens]	SRP1
58	cystatin C precursor [Homo sapiens]	cstatin
59	actin, gamma 1 propeptide [Homo sapiens]	actin(g1)
60	beta actin [Homo sapiens]	b-actin
61	hypothetical protein LOC345651 [Homo sapiens]	HPLOC

Venn Diagrams:

4.3.1) P0 OA

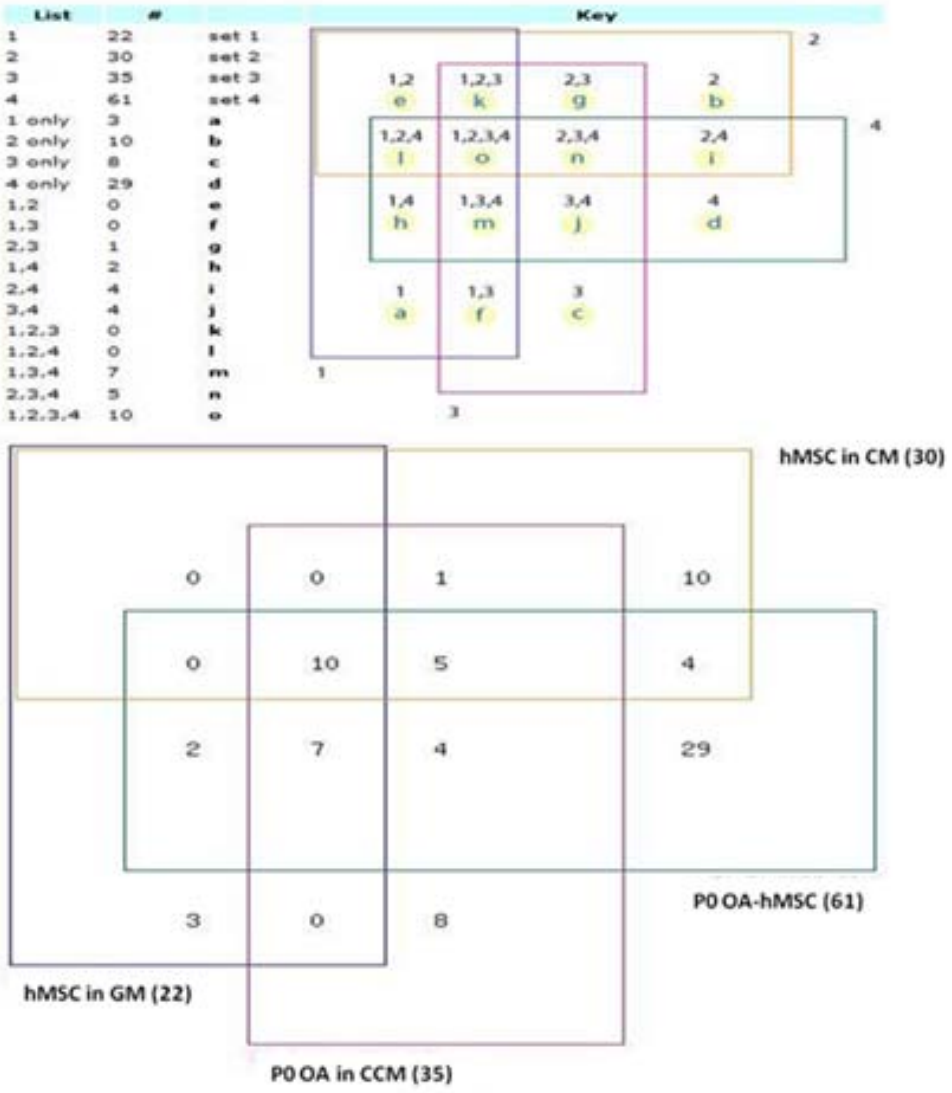


Figure 27: Venn diagram for P0 OA co-culture.
CM = Chondrogenic medium; CCM = Chondrocyte Culture Medium; GM = Growth Medium.

Table 9: List of proteins corresponding to Figure 27

A(3)	B(10)	C(8)	D(29)	E(0)	F(0)	G(1)	H(2)	I(4)	J(4)	K(0)	L(0)	M(7)	N(5)
Col2(a2)	Acting1p	PreHP	Actin(g2)			Col6(a1)	TIMP1pre	SPAC	Chit3			Ker2	Col1(a2)
HP	MMP2P	afetpre	CC3P				Transfe	Vit-DBPP	IGFBP3a			Ker5	EGFECM11
Viment	Mcpra2	gamma(a)	CI1					b-actin	IGFBP3b			Ker6A	EGFECM12
	PAI1	gamma(g)	CI2					ppotp2	TGF-b-I			Ker6B	EGFECM13
	PAI1mem	globin(b)	CMAA1									Ker6C	FIB1PRE
	PEROSTIN	globin(d)	Col3(a1)									Ker6L	
	PZP	globin(e)	HP3									Ker75	
	TGF-B-I	vanin1pre	HPI4										
	Thr1pre		HPI5										
	carDpre		HPLOC										
			HPP										
			IGFBP4										
			Ker10										
			Ker9										
			TI1										
			TIMPinh2										
			Vim										
			actin(a1)										
			actin(a2)										
			actin(g1)										
			afetopre										
			b2micglo										
			fol1pre										
			lumican										
			ppotchro										
			rcTPI11										
			rcTPI12										
			rcTPI16										
			serinePI										

4.3.2) P1 OA:

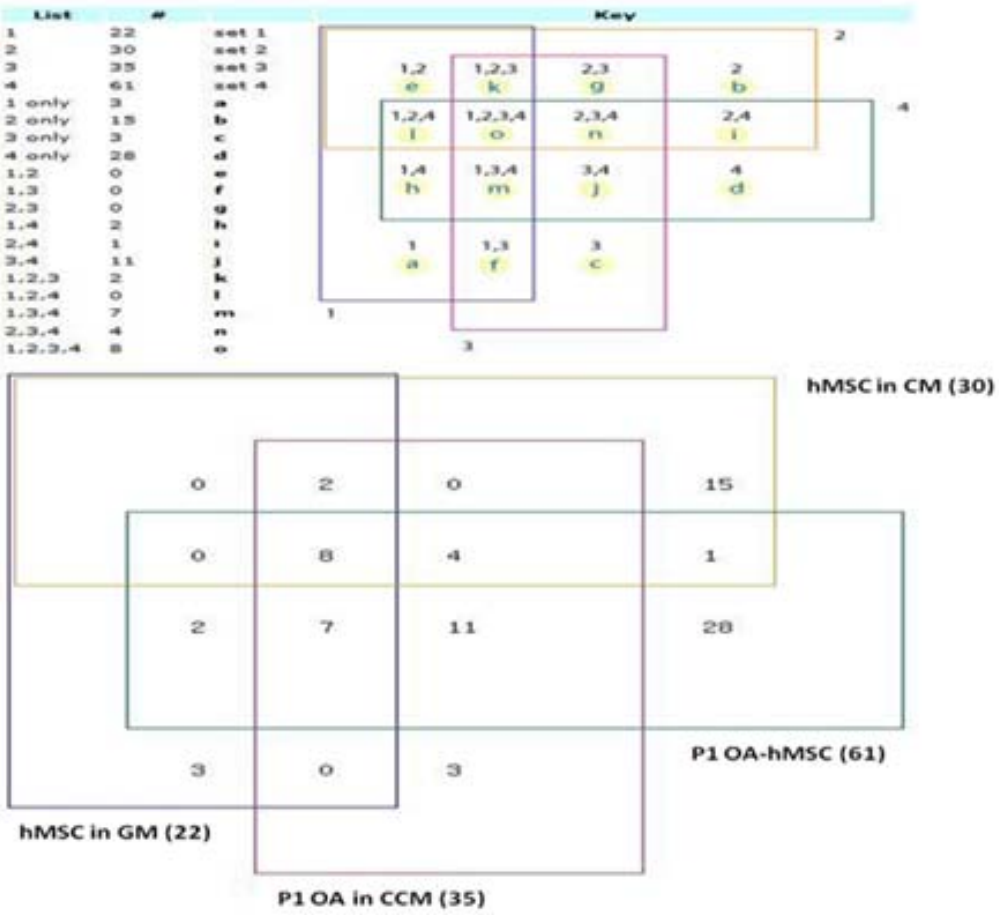


Figure 28: Venn diagram for P0 OA co-culture.
CM = Chondrogenic medium; CCM = Chondrocyte Culture Medium; GM = Growth Medium.

Table 10: List of proteins corresponding to Figure 28

A(3)	B(15)	C(3)	D(28)	E(0)	F(0)	G(0)	H(2)	I(2)	J(11)	K(2)	L(0)	M(7)	N(4)
Col2(a2)	Acting1p	HP1pre	COMP				TIMP1pre	b-actin	CTGF	F1I1P		Ker2	Col1(a2)
HP	Col6(a1)	proinsulin	Col3(a1)				Transfe		Col5(2)	F1I2P		Ker5	FIB1PRE
Viment	EGFECM11	trans	Col4(a2)						Col6(a3)1			Ker6A	PEROSTIN
	EGFECM12		Col5(1)						Col6(a3)2			Ker6B	SPAC
	EGFECM13		GAG						Col6(a3)3			Ker6C	
	MMP2P		HP2						Col6(a3)4			Ker6L	
	Mcpra2		HPLOC						Col6(a3)5			Ker75	
	PAI1		IGFBP3a						Ker9				
	PAI1mem2		IGFBP3b						L-TGF-b				
	PZP		Ker10						PAI(1)				
	TGF-B-I		SRP1						fol1pre				
	Thr1pre		TGF-b-I										
	Vit-DBPP		TIMPinh2										
	carDpre		Thr.1pre										
	ppotp2		Vim										
			actin(g1)										
			b2micglo										
			biglypp										
			cstatin										
			elastina										
			elastinb										
			elastinc										
			elastind										
			elastine										
			fibu(5)										
			lumican										
			lysyloxi										
			procolc										

4.3.3) Comparison of P0 OA and P1 OA:

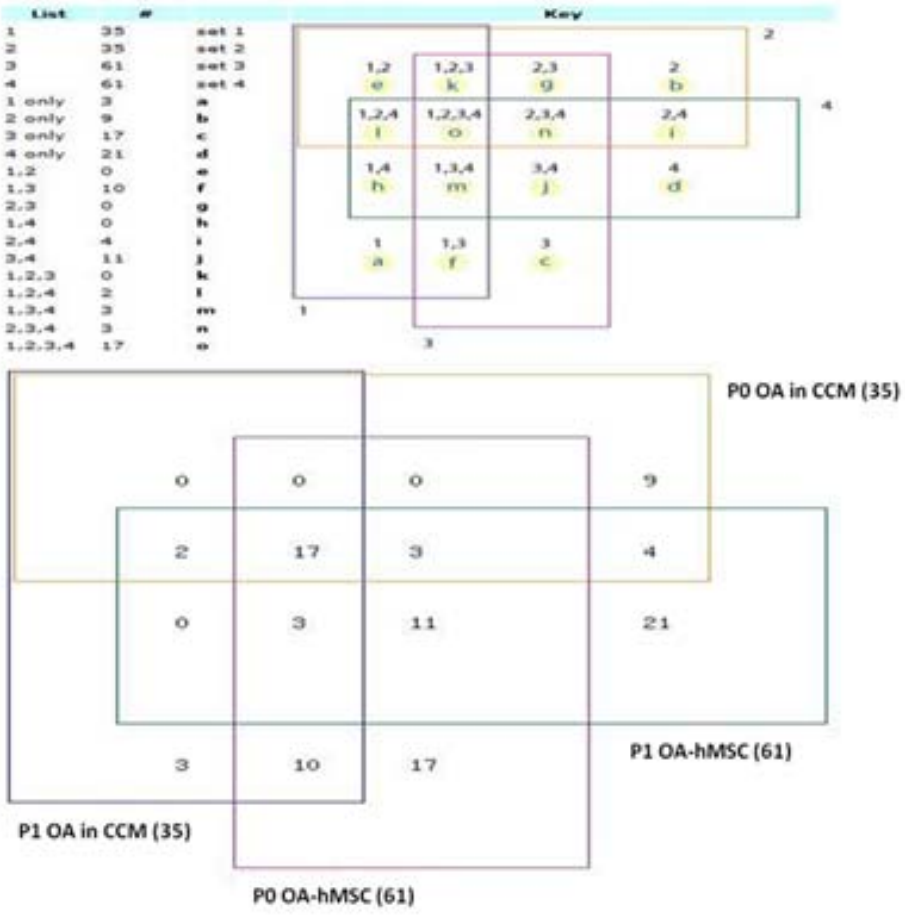


Figure 29: Venn diagram for P0 OA and P1 OA co-culture comparison.
CM = Chondrogenic medium; CCM = Chondrocyte Culture Medium; GM = Growth Medium.

Table 11: List of proteins corresponding to Figure 29.

A(3)	B(9)	C(17)	D(21)	E(0)	F(10)	G(0)	H(0)	I(4)	J(11)	K(0)	L(2)	M(3)	N(3)	O(17)
HP1pre	Col6(a1)	COMP	Actin(g2)		CTGF			Chit3	Col3(a1)		F111P	Ker9	IGFBP3a	Alb-pre
proinsulin	PreHP	Col4(a2)	CC3P		Col5(7)			EGFECM11	HPLOC		F112P	SPAC	IGFBP3b	Col1(a1)
trans	afetpre	Col5(1)	C1		Col6(a3)1			EGFECM12	Ker10			fol1pre	TGF-b-I	Col1(a2)
	gamma(a)	GAG	C2		Col6(a3)2			EGFECM13	TIMP1pre					F113P
	gamma(g)	HP2	CMAA1		Col6(a3)3				TIMPinh2					F114P
	globin(b)	SRP1	HP3		Col6(a3)4				Transfe					F115P
	globin(d)	Thr.1pre	HP14		Col6(a3)5				Vim					F116P
	globin(e)	biglypp	HP15		L-TGF-b				actin(g1)					F1B1PRE
	vanin1pre	cstatin	HPP		PAI(1)				b-actin					IGFBP7
		elastina	IGFBP4		PERIOSTIN				b2micglo					Ker1
		elastinb	TI1						lumican					Ker2
		elastinc	Vit-DBPP											Ker5
		elastind	actin(a1)											KerGA
		elastine	actin(a2)											KerGB
		fibu(5)	afetopre											KerGC
		lysyloxi	ppotchro											KerGL
		procolc	ppotp2											Ker75
			nCTP1L1											
			nCTP1L2											
			nCTP1L6											
			serineP1											

It is evident from figure 27 that there are 29 proteins that are unique to the co-culture system with primary OA chondrocytes. This however, does not include the 44 histones that are only present in the conditioned medium of P0 OA-hMSC co-culture system. Some of these 29 proteins are matrix producing, some are growth factors and some are collagen specific as well. Interestingly, there are reports indicating that the histones produced as extracellular components induce growth hormone production, which in turn induces chondrogenesis [62]. Figure 28 on the other hand shows the interaction between P1 OA and hMSC and the unique proteins present in P1 OA are

mostly cartilage specific matrix proteins leading us to anticipate the possibility that P1 OA are undergoing re-differentiation in presence of hMSCs.

4.4) Diffusion modeling:

Diffusion plays an important role in the movement of morphogens from the surrounding medium into the hydrogel containing hMSCs. The concentration profile of morphogens across the cylindrical construct was analyzed using finite difference scheme. This was attempted in an effort to understand the few interesting observations that we made during the course of our histological analysis.

Firstly, Safranin-O staining was highest in terms of intensity in the peripheral regions of the constructs and progressively decreased from the outside to the inside. As seen from Figs. 17b, 23a and 23h, the Saf-O staining of the sections (circular sections because of the 2D nature of the thin sections) of co-cultured hMSCs showed higher intensity at the boundaries which steadily decreases inwards. The same phenomenon was observed in the case of collagen type II staining. On the other hand, collagen type X staining was mostly clustered in the center of the constructs where concentrations of the morphogens are the lowest. This is also evident in Figs. 9b and 18b for conditioned medium studies (sporadic chondrogenesis only in the periphery) implying that due to the low concentrations of morphogens in conditioned medium, they never diffused to the inside cells.

We hence modeled the diffusion gradient across the cylindrical hMSCs constructs based on these observations to draw a concentration profile of the morphogens. This analysis along with the histological analysis clearly suggests a strong

dependency between morphogenic concentration and pattern of chondrogenesis of hMSCs. Based on our findings, we hypothesize that below a critical concentration of the morphogens, the chondrocyte secreted factors promote either hypertrophic or osteogenic differentiation of hMSCs. This is further supported by the chondrocyte conditioned medium results wherein hMSC constructs cultured in chondrocyte conditioned medium (which has lower concentration of morphogens) showed higher levels of collagen type 1. However, these analyses do not provide the minimum amount of morphogens required to maintain the phenotypic stability of MSC-derived chondrocytes.

Diffusion model problem:

1) Using cylindrical hMSC-laden PEG hydrogels having a pore size of 10 nm.
2) Number of cells initially per construct ~ 1 million (Assuming constant and homogeneous cell proliferation over 21 days).
3) Pore size of co culture well = 0.4 um

   The phenomenon occurring is shown in the figure below:

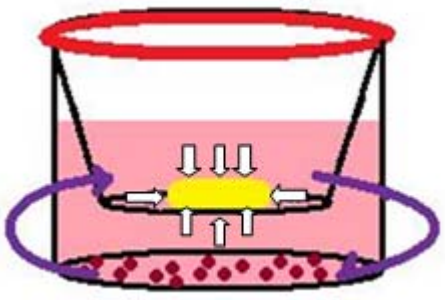


Figure 30: Schematic of the diffusion process in co-culture system

4) Equation to be used; $\frac{\partial c}{\partial t} = Dab * \nabla^2 Ca + Ra + \nabla \text{Ca} * \text{V}$;

Where,

$\nabla \text{Ca} * \text{V} = 0$ (convective transfer)

D_a = Diffusion constant

C_a = Concentration at any given point

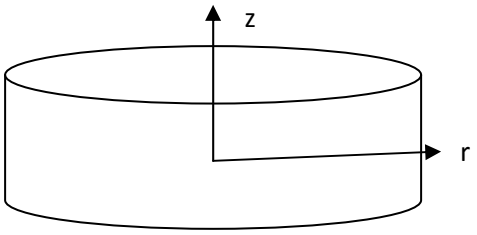
R_a= -K*C_a is the reaction factor term for the medium components being consumed by the peripheral cells which is a first order reaction. (K=reactivity constant)

LHS=0; Therefore,

$Dab * \nabla^2 Ca - k\text{Ca} = 0$;

Governing equation→ $Dab * \nabla^2 Ca = k\text{Ca}$

Need to solve this using finite difference method.



For this profile; the governing equation transforms to:

$$\left(\frac{k}{Dab}\right) * \text{Ca} = \frac{1}{r}\frac{\partial}{\partial r}\left(r\frac{\partial Ca}{\partial r}\right) + \frac{\partial Ca^2}{\partial z^2}$$

Which can also be written as:

$$r * Ca\left(\frac{k}{Dab}\right) = \frac{\partial Ca}{\partial r} + r\frac{\partial^2 Ca}{\partial r^2} + \frac{\partial Ca^2}{\partial z^2} \qquad \rightarrow \quad 1$$

Difference calculus using finite difference method was used to solve for this;

Centered difference – $\frac{\Delta Ca}{\Delta x}$: is calculated numerically at 2 different matrix points each time i-1 and i+1 each separated by a infinitesimal distance "2h" with their middle point being i. Hence,

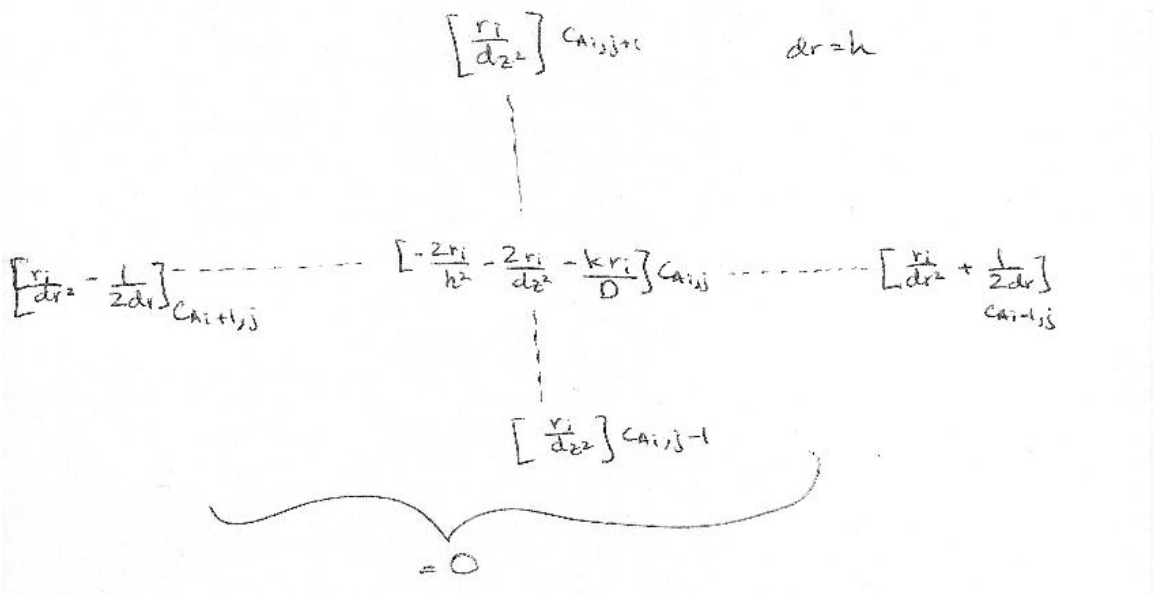
$$\frac{dCai}{dx} = (Ca_{i+1} + Ca_{i-1})/2h$$

$$\frac{d\left(\frac{dCai}{dx}\right)}{dx} = (Ca_{i+1} + Ca_{i-1} - 2\,Ca_i)/h^2$$

Discretizing equation 1 we get,

$$r_i\left(\frac{K}{D}\right) Ca_{i,j} = (Ca_{i+1,j} + Cai\text{-}1,j)/2h + r_i\,(Ca_{i+1,j} + Ca_{i-1,j} - 2\,Ca_{i,j})/h^2 + (Ca_{i,j+1} + Ca_{i,j-1} - 2\,Ca_{i,j})/dz^2$$

The nodes were set up as shown in:



We assumed that in both r and z directions there are 25 nodes where the concentration is unknown. Hence, there are 25*25 unknown concentrations represented as $C_1, C_2, C_3 \ldots C_{625}$.

There will be a total of 4 boundary conditions:

1) At i, j=1: $Ca_{i,1} = Ca_{i,2} = 0$ ➔ $Ca_{i,1} - Ca_{i,2} = 0$ because @ r=0; $\frac{\partial Ca}{\partial r} = 0$
2) Similarly, at i=1, j: $Ca_{1,j} = Ca_{2,j} = 0$ ➔ $Ca_{1,j} - Ca_{2,j} = 0$
3) $r = R_0$; $c = C_0$
4) $z = Z$; $c = C_0$

Where, R_0 and Z are the radius and height of the cylinder and C_0 is the concentration outside the gel construct.

We solved for concentration [C] in the form of matrices; [A][C]=[r] and [A][C]=[z] in two different directions using MATLAB and drew the concentration profiles. The MATLAB code written is given below:

```
clear all
r = 3; %in mm
dr = 0.12; %in mm
z = 2.3; % in mm
dz = 0.092; %in mm
k = 0.1; %0.0001 is magic number
D = 0.5; %in cm2/s %0.5 is magic number
Co = 1; %magic number is 10
A126to150row=zeros(5,9);

for x = 1:25;
   if x == 1,
      for i=1:25;
         if i == 1,
            A126to150row(i,i) = -1;
            A126to150row(i,i+25) = .5;
```

```
            A126to150row(i,i+1) = .5;
         %elseif i == 2,
            %   A1to25row(i,i-1) = -1;
            %  A1to25row(i,i) = 1;
         else
            %A1to25row(i,i-2) = -1;  %i,i
            A126to150row(i,i)= -1; %i,i+1
            %A1to25row(i,i)= [(i-2)/(dr^2) - 1/(2*dz)]; %i,i+2
            A126to150row(i,i+25)= 1; %i,i+26
         end
      end
   elseif x<25,
      for i=1:25;
         if i == 1,
            A126to150row(i+25*(x-1),i+(25*(x-1)))= -1;
            A126to150row(i+25*(x-1),i+(26+(25*(x-2))))= 1;
         elseif (i<25),
            A126to150row(i+25*(x-1),i+(24+(25*(x-2)))) = 0.12*(i-1)/(dr^2) - 1/(2*dr);
            A126to150row(i+25*(x-1),i+(25+(25*(x-2)))) = [-2*0.12*(i-1)/(dr^2) -
2*(0.12*(i-1))/(dz^2) - k*0.12*(i-1)/D];
            A126to150row(i+25*(x-1),i+(26+(25*(x-2)))) = 0.12*(i-1)/(dr^2) + 1/(2*dr);
            A126to150row(i+25*(x-1),i+(50+(25*(x-2)))) = 0.12*(i-1)/(dz^2);
            A126to150row(i+25*(x-1),i+25*(x-2)) = 0.12*(i-1)/(dz^2);
         else
            A126to150row(i+25*(x-1),i+(24+(25*(x-2)))) = 0.12*(i-1)/(dr^2) - 1/(2*dr);
            A126to150row(i+25*(x-1),i+(25+(25*(x-2)))) = [-2*0.12*(i-1)/(dr^2) -
2*(0.12*(i-1))/(dz^2) - k*0.12*(i-1)/D];
            %A126to150row(i+25*(x-1),i+126)= [0.12*(i-1)/(dr^2) - 1/(2*dz)];
            A126to150row(i+25*(x-1),i+(50+(25*(x-2))))= 0.12*(i-1)/(dz^2);
            A126to150row(i+25*(x-1),i+25*(x-2)) = 0.12*(i-1)/(dz^2);
         end
      end
   else
      for i=1:25;
         if i == 1,
            A126to150row(i+25*(x-1),i+(25*(x-1)))= -1;
            A126to150row(i+25*(x-1),i+(26+(25*(x-2))))= 1;
         elseif (i<25),
            A126to150row(i+25*(x-1),i+(24+(25*(x-2)))) = 0.12*(i-1)/(dr^2) - 1/(2*dr);
            A126to150row(i+25*(x-1),i+(25+(25*(x-2)))) = [-2*0.12*(i-1)/(dr^2) -
2*(0.12*(i-1))/(dz^2) - k*0.12*(i-1)/D];
            A126to150row(i+25*(x-1),i+(26+(25*(x-2)))) = 0.12*(i-1)/(dr^2) + 1/(2*dr);
            %A126to150row(i+25*(x-1),i+(50+(25*(x-2)))) = 0.12*(i-1)/(dz^2);
            A126to150row(i+25*(x-1),i+25*(x-2)) = 0.12*(i-1)/(dz^2);
         else
```

```
            A126to150row(i+25*(x-1),i+(24+(25*(x-2)))) = 0.12*(i-1)/(dr^2) - 1/(2*dr);
            A126to150row(i+25*(x-1),i+(25+(25*(x-2)))) = [-2*0.12*(i-1)/(dr^2) -
2*(0.12*(i-1))/(dz^2) - k*0.12*(i-1)/D];
            %A126to150row(i+25*(x-1),i+126)= [0.12*(i-1)/(dr^2) - 1/(2*dr)];
            %A126to150row(i+25*(x-1),i+(50+(25*(x-2))))= 0.12*(i-1)/(dz^2);
            A126to150row(i+25*(x-1),i+25*(x-2)) = 0.12*(i-1)/(dz^2);
         end
      end

   end
end




A = inv(A126to150row);


for x = 1:25;
   if x == 1,
      for i=1:25;
         R(i,1) = 0;
      end
   elseif x<25,
      for i=1:25;
         if i<25,
            R(i+25*(x-1),1)= 0;
         else
            R(i+25*(x-1),1) = - [0.12*(i-1)/(dr^2) + 1/(2*dr)]*Co;
         end
      end
   else
      for i=1:25;
         if i == 1,
            R(i+25*(x-1),1)= 0;
         elseif (i<25),
            R(i+25*(x-1),1) = - [0.12*(i-1)/(dz^2)]*Co;
         else
            R(i+25*(x-1),1) = - [0.12*(i-1)/(dr^2) + 1/(2*dr)]*Co - [0.12*(i-
1)/(dz^2)]*Co;
            %R(i+25*(x-1),1) = - [0.12*(i-1)/(dz^2)]*Co;

         end
      end
```

```
   end
end

X = A*R;

for y = 1:26;
   if y == 1;
      for i = 1:26;
         J(y,i) = Co;
      end
   else
      for i = 1:26;
         if i<26,
            J(28-y,i) = X(i+25*(y-2),1);
         else
            J(28-y,i) = Co;
         end
      end
   end
end
```

The 3D plot obtained
was:

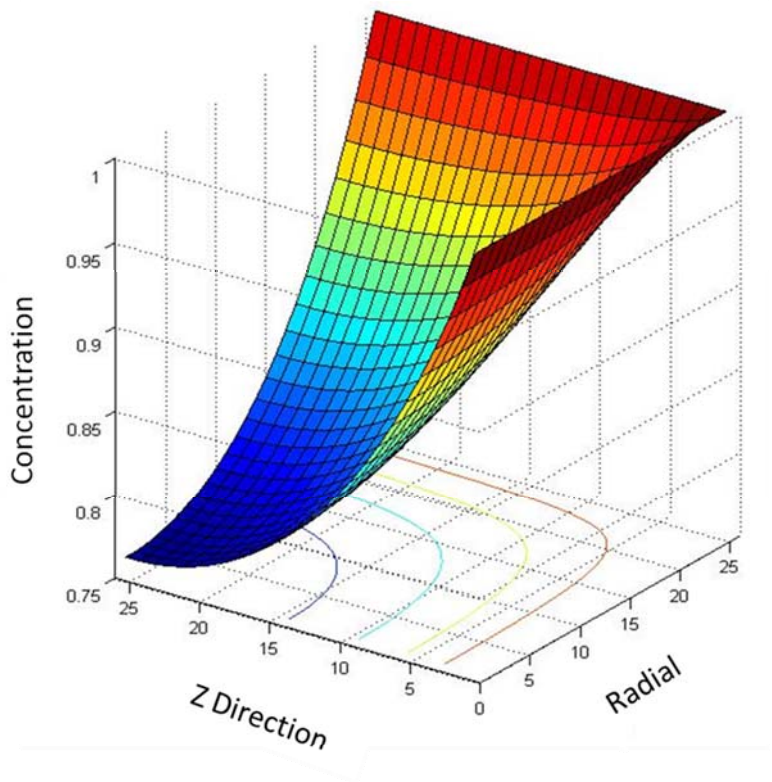


Figure 31: Diffusion gradient model
3D-concentration profile inside the gel construct as a result of the diffusion gradient in z and r directions. Co, Z and Ro were assumed as any random number

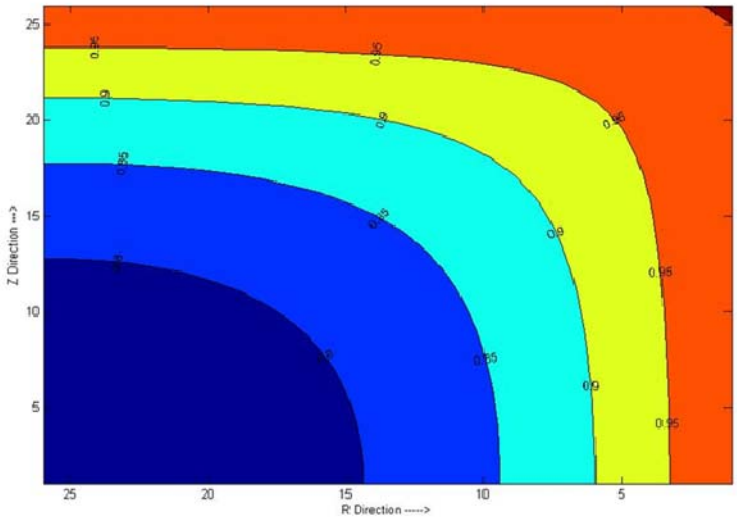


Figure 32: 2-D contour for the concentration profile

4.5) Hydrogels containing a balance of hydrophilic-hydrophobic balance support chondrocyte *in vitro* expansion:

Hydrogels comprising of acrylamide and acryloyl 6-amino caproic acid (A6ACA) moieties were prepared as described in materials and methods. Primary BCCs were seeded onto the gel surface at a concentration of ~5000cells/cm^2 and passaged on day 7. The phenotype was compared against that of cells cultured on standard tissue culture plate. The figures 33, 34 and 35 give an idea of the effectiveness of hydrogel. Figure 34e and 35f show chondrocytes with round morphology on day 5 even after few passages, P1 and P2 respectively. The control cells on the other hand are spread out from day 1 itself for both P1 and P2. (Fig. 34b and 35b). The main difference thus observed between the cells cultures on the hydrogel versus the plastic control was the cell morphology, shape and proliferation capacity.

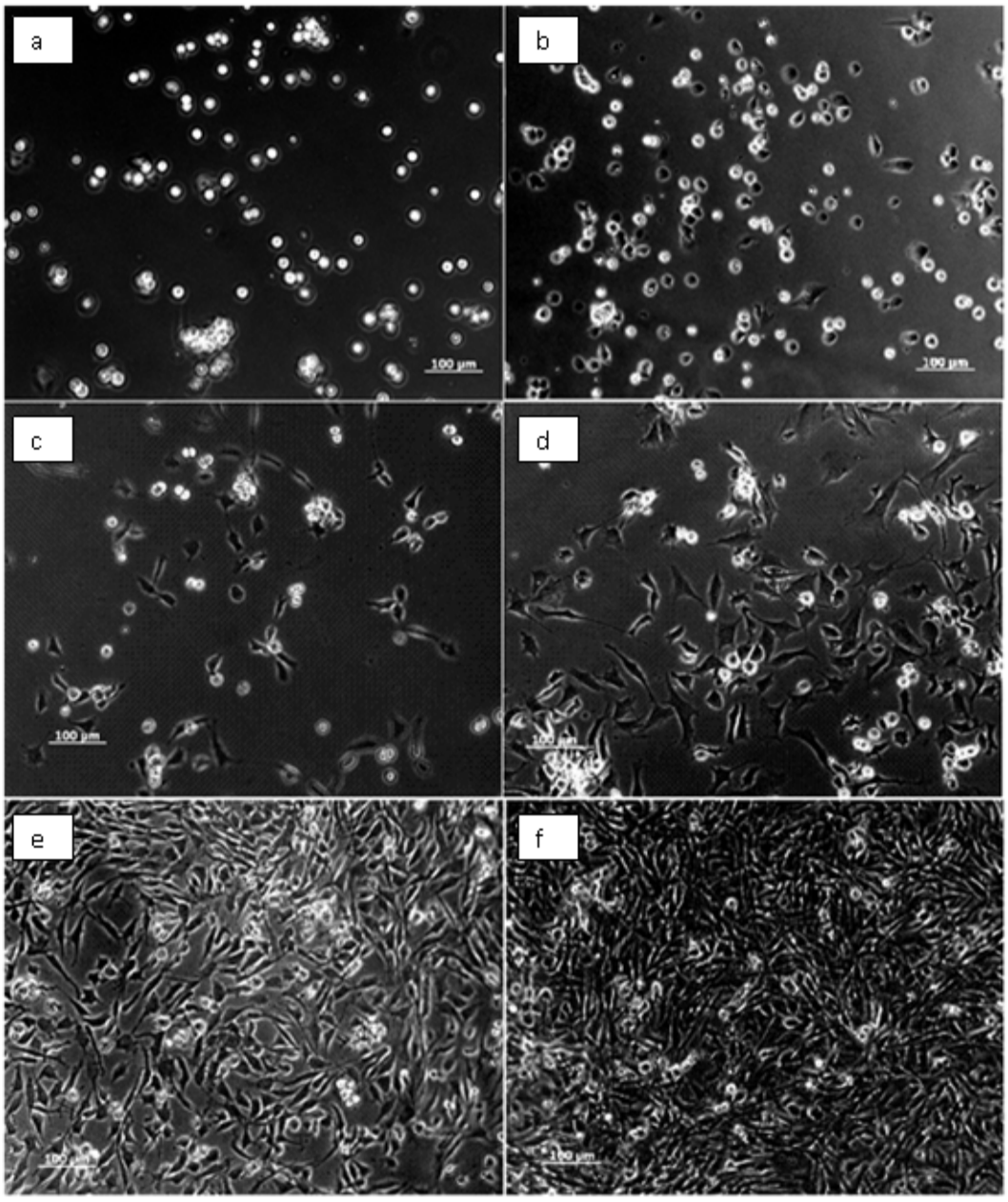


Figure 33: P0 cells morphology comparison on A6ACA gel versus plastic culture plate.

a) Day 1 P0 BCC on gel; b) Day 1 P0 BCC on control; c) Day 3 P0 BCC on gel; d) Day 3 P0 BCC on control; e) Day 5 P0 BCC on gel; f) Day 5 P0 BCC on control. All pictures were taken at an original magnification of 10X and the scale bar shows 100µm.

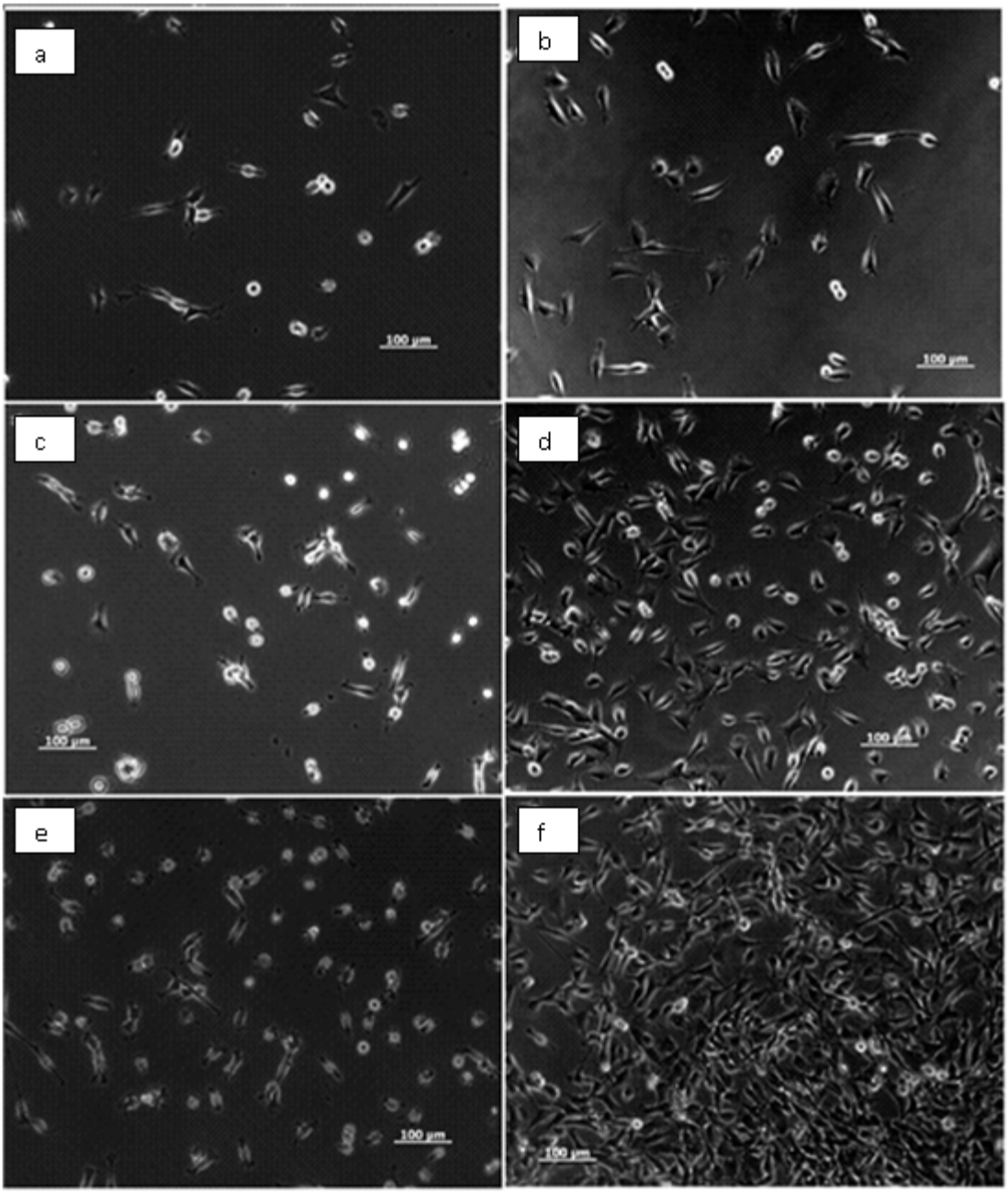


Figure 34: P1 cells morphology comparison on A6ACA gel versus plastic culture plate.

a) Day 1 P1 BCC on gel; b) Day 1 P1 BCC on control; c) Day 3 P1 BCC on gel; d) Day 3 P1 BCC on control; e) Day 5 P1 BCC on gel; f) Day 5 P1 BCC on control. All pictures were taken at an original magnification of 10X and the scale bar shows 100µm.

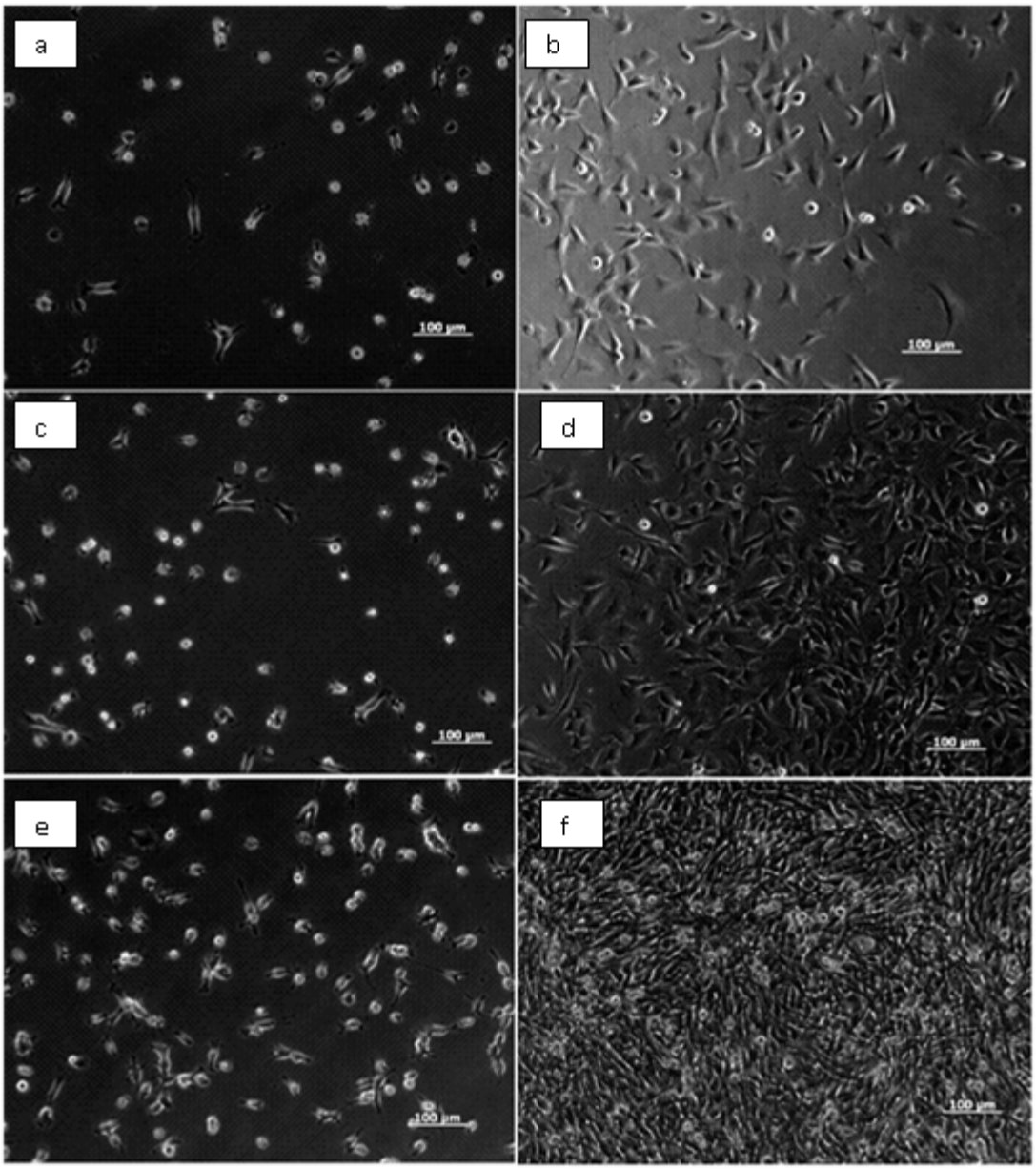


Figure 35: P2 cells morphology comparison on A6ACA gel versus plastic culture plate.

a) Day 1 P2 BCC on gel; b) Day 1 P2 BCC on control; c) Day 3 P2 BCC on gel; d) Day 3 P2 BCC on control; e) Day 5 P2 BCC on gel; f) Day 5 P2 BCC on control. All pictures were taken at an original magnification of 10X and the scale bar shows 100µm.

Chapter 5: DISCUSSION

Bone marrow-derived mesenchymal stem cells have been reported to differentiate into multiple cell types of mesenchymal origin and to contribute to tissues such as cartilage, bone, fat, and tendon. The differentiation of MSCs into different tissues is regulated by the cues arising from the tissue microenvironment, which involves both insoluble and soluble factors. While insoluble factors such as matrix components and soluble factors such as growth factors have been studied in great depth, little is known about the role of cell secreted morphogens on stem cell differentiation, especially in the case of diseased tissue.

In striving towards this goal, I have more accurately defined a co-culture system for chondrogenesis to study the effect of cell secreted factors on chondrogenic differentiation of hMSCs and identified a few key signaling pathways involved in this process. Chondrogenesis is a process that results in the formation of the cartilage tissue, either permanent cartilage (which covers the articular surface) or transient cartilage which provides foundation for long bone formation during vertebrate skeletal development. This process requires precise control of cellular interactions with the surrounding matrix and differentiation factors, in a temporal-spatial manner. Developmental complexity presents a great challenge for in vitro replication of chondrogenesis when devising a stem cell based therapeutic platform for clinical purposes. For instance, direct implantation of MSCs often results in heterotrophic osseous tissue formation followed by mineralization of the transplant; use of preconditioned MSCs and knowledge of their precise chondrogenic differentiation status are therefore crucial to the efficacy of MSC-based cartilage regeneration. We thus

established a co-culture system in which MSCs were directed toward chondrogenesis under the influence of mature chondrocytes, thereby creating an *in vitro* model system to understand the role of cell-cell interactions in the form of morphogens on regulating stem cell commitment using chondrocytes and hMSCs.

5.1) Co-culture with BCC:

First, with the establishment of a co-culture system, we found that the cell secreted factors produced through crosstalk between chondrocytes and MSCs promote chondrogenic differentiation of undifferentiated hMSCs. In our co-culture system, the hMSCs showed an up-regulation of genes associated with chondrogenic differentiation including increased levels of SOX9, Collagen type II, and aggrecan, as detected by qRT-PCR. Among these genes, SOX9 expression in the co-cultured hMSCs was higher than the control on day 7 marking the pre-chondrogenic stage and proliferation of differentiating hMSCs. In addition biochemical assays demonstrated an upregulation in GAG content. These findings confirm that the hMSCs are capable of differentiating into chondrocytes under a co-culture system. Our results were supported by the observations from studies by previous investigators, in which it was shown that the differentiation lineages of MSCs depend on their co-cultured cell types and, possibly, their activated adhesion molecules and respective signaling pathways.

Having established the feasibility of chondrogenesis of hMSCs in co-culture with primary chondrocytes, I investigated the effect of *in vitro* expanded (passage 3) bovine chondrocytes on the chondrogenic differentiation of hMSCs. It is very well accepted that when chondrocytes are cultured in monolayer they progressively lose their

chondrocyte phenotype with each passage to become fibroblastic in nature. However, it is essential to examine the effect of expanded chondrocytes on hMSCs because of the limited number of chondrocytes that can be extracted from a donor necessitating the need for in vitro expansion. Hence, we evaluated the effect of de-differentiated chondrocytes on chondrogenic differentiation of hMSCs. In addition to co-culture we also used chondrocyte conditioned medium from P3 chondrocytes as in the latter case the cell secreted factors are not influenced by the cross talk between chondrocytes and MSCs. As seen from our results, cell secreted factors from de-differentiated chondrocytes did not have any significant effect on inducing chondrogenic differentiation of hMSCs. There was about a 2-fold increase in the expression of Aggrecan and Collagen type II in the co-culture system but was insignificant compared to the results obtained from co-culture with primary chondrocytes. Findings from the gene profile were further supported by the biochemical analysis (GAG content). Histological analysis showed a weak staining for safranin-O, which increased from day 14 to day 21. The dense collagen type X staining of the sections indicates the terminal differentiation of MSC-derived chondrocytes into hypertrophic chondrocytes. The differential effect of primary and de-differentiated chondrocytes on chondrogenic differentiation of hMSCs could be attributed to their phenotypes, which may have a significant effect on the concentration and/or type of cell secreted morphogens.

Conditioned medium studies on the other hand, did not have any effect on chondrogenesis of hMSCs. The gene expression of chondrogenic markers was less than that of control on day 21. However, they expressed a higher Collagen type 1 level on

day 21 compared to the control and co-cultured hMSCs. This could be either due to the low concentration and/or type of cell secreted factors in the absence of a co-culture.

5.2) Co-culture with P0 OA:

Thus far, we have shown that our co-culture system utilizing primary chondrocytes can sustain and promote chondrogenesis of hMSCs. However, for a potential application of MSCs to treat osteoarthritis we need to evaluate the effect of such an environment on MSCs. Therefore, we further used our co-culture system to study the effect of OA chondrocytes secreted morphogens on chondrogenic differentiation of hMSCs.

The adult chondrocytes plays a critical role in the pathogenesis of OA in responding to adverse environmental stimuli by promoting matrix degradation and downregulating processes like recruiting healing molecules essential for cartilage repair. In the absence of disease, the chondrocytes maintain a low turnover rate of replacement of cartilage matrix proteins with a half-life for collagen of greater than 100 years [63]. In contrast, the glycosaminoglycan constituents on the aggrecan core protein are more readily replaced and the half-life of aggrecan sub-fractions has been estimated in the range of 3–24 years [64]. In early OA, there is evidence of increased synthetic activity, which is viewed as an attempt to regenerate the matrix with cartilage-specific components, including types II, IX, and XI collagens, aggrecan, and pericellular type IV collagen [65]. Evidence of phenotypic changes is reflected in the presence of collagens not normally found in adult articular cartilage which include the hypertrophic

chondrocyte marker, type X collagen, as well as other chondrocyte differentiation genes, suggesting a recapitulation of endochondral developmental program [66, 67].

Given the scarcity of data available on the phenotype, gene expression and monolayer expansion of OA chondrocytes *in vitro*, we first carried out characterization of OA chondrocytes. The objective of the study was to characterize the human OA chondrocytes, and to investigate the effect of *in vitro* expansion on their phenotype. Primary OA chondrocytes expressed all the chondrogenic markers along with some hypertrophy markers like Collagen type X and ALP. We however did not have healthy human chondrocytes to compare the results against. It took a long time to culture the OA cells in monolayer and reach confluency which might be species dependent or an artifact decreased matrix secretion by OA chondrocytes because of which they take much longer to attach and proliferate. A concrete conclusion cannot be reached till normal human chondrocytes are compared to human OA chondrocytes. While P0 OA cells expressed the chondrocyte specific markers there was a drastic change in the chondrocytic phenotype from the first passage itself which progressively decreased with continuing passage. The staining for matrix proteins i.e. Saf-O and alcian blue gave negative results for P1 OA chondrocytes compared to primary and the cellular morphology was also completely different.

Based on our data, we conclude that the gene expression profile of the OA chondrocytes is largely dependent on its cellular passages during cultivation. Gene expression of OA chondrocytes changes during monolayer passaging, suggesting that a variance in differentiated phenotypes of hMSCs may occur in co-culture studies as well. The further understanding of chondrocyte dedifferentiation in passaged monolayer

culture may help to generate new approaches for cultivating more phenotypically stable chondrocytes and/or redifferentiating chondrocytes for increased therapeutic gain.

Results from the P0 OA co-culture studies showed that the cell secreted factors promote chondrogenic differentiations of hMSCs and similar to the findings from P0 BCC co-cultures. As seen from qRT-PCR, the hMSC constructs co-cultured with P0 OA chondrocytes expressed significantly more type II collagen and aggrecan than the control constructs. This was further supported by biochemical analysis, which showed higher amounts of proteoglycans and collagen content in co-cultured hMSC constructs. The histological analysis further supports the findings from qRT-PCR and biochemical analysis. Although, the biomaterial supported cartilage tissue engineering approaches were successful in engineering cartilage tissues with GAG content very similar to native cartilage, the amount of collagen content in most of these tissue engineered constructs has been very minimal. This limitation is more apparent when stem cells were used as a cell source. In this study however, we showed that the hMSC constructs cultured with chondrocytes produced much higher amounts of collagen. Moreover, immunofluorescent staining along with gene analysis suggests that a majority of the collagens presented is collagen type 2. The continuous enhancement of DNA content observed in the co-cultured hMSCs suggests that the cell secreted factors promote proliferation of hMSCs and hMSC-derived chondrocytes. Compared to control, the co-cultured hMSCs showed very weak type X expression indicating the positive effect of cell secreted factors on repressing the terminal differentiation of MSC-derived chondrocytes.

We also evaluated the effect of P0 OA conditioned medium on chondrogenic differentiation of hMSCs. The two main differences between conditioned medium and co-culture are the absence of cross talk between MSCs and chondrocytes and the concentration of cell secreted factors.

Conditioned medium results of P0 OA chondrocytes mirrored the results obtained with the bovine chondrocytes. There was no significant effect on chondrogenesis as indicated by the qRT-PCR gene expression analysis, biochemical results and histology staining. However the hMSC constructs cultured in conditioned medium showed significant upregulation in collagen type I expression compared to control and it could be attributed to an effect of concentration and or type of cell secreted factors in conditioned medium compared to that in co-culture. These constructs also exhibited higher levels of type X collagen. We hypothesize that the upregulation of collagen type 1 and X while maintaining an inhibitory effect on other cartilage specific markers such as collagen type II and aggrecan is due to the types and/or lower concentration of cell secreted factors. This is highly conceivable given that the only difference between the conditioned medium and the chondrogenic medium used to culture constructs was the cell secreted factors.

Co-culture with passaged OA cells did not induce chondrogenesis in hMSCs any better than control suggesting that the observed chondrogenic differentiation is solely from TGF-β1 and other chondrogenesis inducing factors in the medium

We inferred that chondrocytes in co-culture therefore provide signals to the hMSCs, possibly by the production of growth factors that stimulate differentiation. It was also demonstrated that the co-culture environment supports cell growth and

expression of mature phenotypes. These co-culture systems indicated the existence of coordinated signaling between the cells, thereby stimulating their normal growth and differentiation as would be observed *in vivo*. An improved understanding of pathways reacting to factors released from the chondrocytes as well as the regulatory mechanisms controlling chondrogenic differentiation, we realized would provide further insights into developing new methods of generating cells for cartilage repair. Therefore, we evaluated the effect of PTHrP and TGF-β1 on chondrogenic differentiation of hMSCs. These candidates were chosen based on their reported role in regulating chondrogenic differentiation pattern of hMSCs.

5.3) Pathway studies:

To evaluate the effect of PTHrP and TGF-β1, we used the same co-culture model. Studies were designed with and without TGF-β1 and with PTHrP protein and its blocker cyclopamine.

Interestingly, using the hMSC-chondrocyte co-culture system, chondrogenesis was effectively induced even in the absence of TGF-β1 as demonstrated by the time-dependent up-regulated expression of cartilage-specific gene markers, Col2, Sox9 and Aggrecan, and the deposition of chondrogenic ECM protein collagen II. This effect was completely absent in the control groups cultured without TGF-β1. It was not surprising that the basic chondrogenic media itself without any growth factors could increase the expression of these chondrogenic markers as the co-culture system produces signaling molecules which promote chondrogenic differentiation of hMSCs. Addition of TGF-β1 further increased the chondrogenic differentiation as apparent from the qRT-PCR, biochemical and histology results. The most interesting aspect of this study was the

reduced DNA content in all constructs (control and co-culture) grown without TGF-β1. We thus surmised that, in a co-culture system, chondrogenesis can be induced even in the absence of TGF-β1 but it is essential for the proliferation of pre chondrogenic hMSCs and hMSC-derived chondrocytes which has been corroborated by previous studies as well. Additionally, exogenous TGF-β1 increased cartilage specific extracellular matrix production in co-cultured hMSCs.

PTHrP on the other hand is a signaling molecule that controls endochondral ossification which is expressed at low levels throughout the growth plate and at high levels in proliferating chondrocytes as shown in Fig. 4. It has been shown that activation of PTHrP signaling leads to delay of hypertrophic differentiation by Ihh-PTHrP negative feedback loop. To block PTHrP signaling, we used the alkaloid cyclopamine, which has previously been demonstrated to inhibit Hedgehog signal transduction in target cells [68, 69] and hedgehog signaling is necessary for high-levels of PTHrP expression. Treatment of chicken tibia explants with cyclopamine led to a decrease in PTHrP expression due to its ability to block endogenous Ihh activity [70]. PTHrP has also been proposed to treat cartilage defects (Goomer et al.).

As seen from the described results, PTHrP was found to promote chondrogenesis and to suppress hypertrophy, as evidenced by gene expression studies, and histological findings. The signaling mechanism by which PTHrP induces cellular phenotypes has been widely investigated. PTHrP signals through a G protein-coupled receptor that activates proteins A and C. Moreover, it has been shown that the phenotypic effects of PTHrP in chondrocytes are primarily facilitated downstream of protein kinase A. PTHrP treatment increases cellular proliferation by suppressing the

expression of the p57 gene, and this causes cell growth arrest in chondrocytes . At the dose used in the present study, PTHrP also increased the cell proliferation as indicated by the DNA assay of hMSCs. In the present study, levels of Collagen type II, aggrecan and SOX-9 mRNA were increased by PTHrP. Exogenous PTHrP, however, did not have a profound effect on the accumulation of extracellular matrix components as seen from biochemical analysis. Interestingly, the presence of PTHrP along with TGF-β1 supressed collagen type I and type X expression while consistently upregulated of cartilage specific markers. The observed down regulation of collagen type 1 and X are mainly attributed to the PTHrP, as previous studies have shown that type I and X collagen mRNA is persistent or up regulated after prolonged treatment with TGF-β1. On the other hand in the presence of PTHrP blocker cyclopamine, there was a significant upregulation in the expression of collagen type I and collagen type X while none of the chondrogenic markers were present. Our results suggest that this pathway is operational during the chondrogenesis of MSCs. However, the total collagen content in the presence of blocker was comparable to that found in co-culture with or without PTHrP and is mainly due to tha fact that when PTHrP pathway is blocked hMSCs secretes collagen type 1 and X instead of II. In conclusion, our study provides robust *in vitro* data which supports the benefit of using PTHrP and TGF-β1 along with chondrocyte secreted factors for MSC-based cartilage tissue engineering.

Our results show the usefulness of human OA chondrocytes in directing the differentiation of hMSC and creating tissue that macroscopically and phenotypically resembles cartilage. Further steps, including purification, chemical definition of the medium, three-dimensional growth, and testing in a suitable *in vivo* model are necessary

to advance these findings into a clinical setup, which will have significant clinical implications. We were thus able to show, for the first time, differentiation of hMSC to chondrocytes in co-culture with a diseased chondrocytes.

5.4) Identification of cell secreted factors using mass spectrometry

Our *in vitro* results raised the as yet unresolved questions of the identity of key morphogenetic factors influencing osteo-chondrogenic differentiation of MSCs. Previous studies have indicated possible roles of the secreted exogenous chondroitin sulfate capable of inducing chondrogenic differentiation [71]. Other studies have described the range of molecular weight of morphogenetic factors that have specific inducing abilities [72]. Initial SDS-gel analysis and Coomasie blue staining of soluble factors secreted by chondrocytes show two distinct molecular weight groups [27]. The low molecular weight proteins ranging from 37 to 50 kDa and higher molecular weight proteins ranging from 75 to 100 kDa were detected. These two groups were in the approximate molecular weight ranges for secreted growth factors and extracellular macromolecules, respectively [27]. Identification of specific morphogenetic factors promoting MSC differentiation and induction may elucidate specific chondrocyte-MSC interactions leading to skeletal development.

To this effect we carried out the next step for our studies where in conditioned medium from MSCs, OA chondrocytes and co-culture system for both primary and passaged OA was collected for analysis. The results obtained from mass spectrometry analysis were in line with our hypothesis. There were a significantly higher number of proteins produced in co-culture compared to their non co-cultured counter parts as shown in the Venn diagram which include matrix and growth proteins. Among the co-

cultured samples, P0 OA had more number of cell secreted factors compared to P1 OA. The most interesting aspect was the sheer number of histones present in the P0 OA co-culture secreted as extracellular components. In contrast, the morphogens unique to the co-culture with P1 OA mainly comprised of chondrocyte matrix producing factors which may indicate the effect of hMSC secreted factors during co-culture on P1 OA. Various previous studies have suggested that in addition to their structural role in chromatin, histones may have hormone-like activities when present in extracellular fluids and influence the secretory effects of somatotrophic cells. Ref [62], Brown et al reported that histones H2A and H2B possess growth hormone (GH)-releasing activity *in vitro* and described the specificity and signal transduction pathways involved in these effects. Furthermore, numerous studies Ref. [73] have shown that GH promotes chondrogenesis. These investigators observed that GH-treated explants were about 4 times larger in cell mass than controls after 6 days of culture, providing strong evidence for an in vitro effect on cartilage growth. GH selectively promoted the formation of large chondrocyte clusters, and interacts with pre-chondrocytes or young differentiating cells that have a high inherent capacity to divide. Given the large number of histones in the P0 OA co-culture conditioned medium and its role in enhancing the secretion of GH, we surmised that histones might play a very important role in the cross talk during co-culture to promote chondrogenesis along with other factors identified to be unique to the co-culture system.

We have thus established a co-culture system in which the chondrogenic differentiation stages of MSCs could be specifically manipulated. Our findings not only provide insights into the stage-specific molecular pathways and cell secreted soluble

factors involved in *in vitro* chondrogenesis of MSCs but also contribute to the advancement of cell-based transplantation techniques for future clinical use.

Having obtained a list of the cell secreted morphogens and possible candidates that play a role in chondrogenesis, it is essential in the future to characterize them according to their functions, pinpoint the key morphogens, their concentration, and identify the pathways involved to better understand the chondrogenic differentiation of hMSCs. It will enable us to do further in vivo studies and obtain a neo cartilage similar in properties and phenotype to that of native cartilage. This will also provide an insight into the progression of osteoarthritis and pathways involved in chondrocyte dedifferentiation.

5.5) Diffusion modeling:

Analysis of histological sections showed a change in intensity of cartilage matrix proteins from periphery to centre. There was a significantly higher cartilage specific matrix staining in the outer areas of the cylindrical constructs compared to the center of the construct. On the other hand, collagen type X expression, a hypertrophy marker was mostly clustered at the center. This clearly suggests the effect of concentration of morphogens on chondrogenic differentiation of hMSCs, which was diffusion mediated in our co-culture system. The concept of morphogen gradients has always played an important role in understanding how the “naïve” progenitor cells determine their position and differentiation commitment during development. A recent study by Lander et al have demonstrated that the morphogen mediated cell responses are largely governed by diffusive mechanisms of morphogen transport [74]. The concept of morphogen gradience across the cylindrical construct was verified and

explained using a finite difference modeling shown in Fig. 31. This figure along with our histological findings suggest that the diffusion mediated concentration profile inside the gel construct (where diffusion gradient is a function of radius and height of the cylindrical construct) controls the fate of stem cell differentiation at each point inside the gel.

Based on these analysis, we hypothesized that lower than a critical concentration of morphogens leads to the differentiation of stem cells into osteoprogenitors by undergoing hypertrophy and a higher concentration is required for sustainable chondrogenesis.

5.6) Engineering matrix interfacial properties to regulate chondrocytes phenotype during ex vivo expansion

For therapies based on injection/transplant of autologous chondrocytes in the defect site, a large number of healthy chondrocytes is needed which poses a problem. Chondrocytes are known to de-differentiate under normal 2-D monolayer culture conditions [16, 20, 21]. Although 3-D culture conditions have been devised to maintain the phenotype of chondrocytes during expansion like the alginate bead method [21, 75] and as spheroids in agarose [18]; there is still a need for the identification and development of polymer surfaces that can support the monolayer expansion of chondrocyte. Use of hydrogels might be the answer to the riddle here [19] where hydrogels are three-dimensional networks of hydrophilic polymers which have the ability to imbibe a large quantity of water and biological fluids. We thus looked into Am-co-A6ACA gel surface as an alternate and found a difference in phenotype as

observed under the microscope, when compared to the control plastic surface on passaging. More detailed studies involving gene expression profiles, histology etc. are needed to come to any definite conclusions. The choice of this hydrogel was based on findings from Dr. Ayala from our group who has shows that hydrogels with a balance of hydrophilic and hydrophobic balance can promote cell-matrix interactions and thereby cell fate.

Chapter 6: CONCLUSION, IMPLICATIONS AND FUTURE WORK

In this study, we demonstrated the effect of chondrocyte secreted morphogens on chondrogenic differentiation commitment of hMSCs using a co-culture system. Unlike healthy chondrocytes, OA chondrocytes express hypertrophic markers such as collagen type X and ALP in addition to the cartilage specific markers. Despite their hypertrophic stage, primary OA chondrocytes induced chondrogenic differentiation of hMSCs, and their effect was very similar to healthy bovine chondrocytes. In fact, the co-cultured hMSCs underwent chondrogenic differentiation even in the absence of TGF-β1, indicating the potential of cell secreted factors as chondrogenic inducers. However, de-differentiated chondrocytes have minimal or no effect on chondrogenic differentiation of hMSCs. But hMSC constructs co-cultured with de-differentiated chondrocytes showed upregulated collagen type I expression. A similar observation was made in the case of hMSCs constructs cultured in conditioned medium. Additionally, these constructs showed higher levels of collagen type X. In contrast, the co-cultured hMSCs showed weak collagen type X staining, which could be further inhibited using exogenous PTHrP, which is known to repress hypertrophic differentiation and mineralization of chondrocytes. Co-cultured hMSC constructs along with exogenous PTHrP and TGF-β1 exhibited higher levels of matrix production, collagen type II and glycosaminoglycans. PTHrP in the absence of TGF-β1 did not contribute significantly towards the matrix production, but prevented hypertrophic differentiation of hMSC-derived chondrocytes. PTHrP alone in the culture medium was sufficient to totally prevent the hypertrophy of hMSCs derived chondrocytes in co-culture conditions.

Blocking PTHrP pathways using cyclopamine reversed the effect. Histological analysis along with theoretical analysis showed that the differentiation pattern of hMSCs is strongly dependent upon the concentration of morphogens. High concentrations of morphogens promoted chondrogenic differentiation of hMSCs, the low concentration of morphogens supported endochondral ossification of differentiating hMSCs. Based on the findings from this study we anticipate that the differentiation of hMSCs could be directed employing gradience of morphogens.

Implications: The afore-summarized results of this thesis have significant clinical implications as hMSCs have been touted as a clinically viable cell source for cartilage tissue regeneration. However, regulating the lineage specific differentiation of MSCs into structurally and functionally stable hyaline cartilage tissue is a daunting task because of its multipotent differentiation potential. In this thesis, we systematically investigated the effect of cell-cell communications in the form of soluble factors on chondrogenic differentiation of hMSCs. One of the most important findings of this study with practical implications is the chondrogenic inducing effect of OA chondrocyte secreted factors without imparting any detrimental effects to the differentiating MSCs. This suggests the potential applications of hMSCs for treating cartilage lesions associated with OA pathology. The morphogen gradient mediated differentiation pattern of hMSCs also necessitates the need of biomimetic tissue engineering approaches for creating functional tissues. It also provides an explanation for the previously reported conflicting results demonstrating the effect of chondrocyte secreted factors on both chondrogenic and osteogenic differentiation of hMSCs.

Additionally, the adopted co-culture system also provides new insights into how a microenvironment comprising of extracellular and cellular components may be harnessed to generate hierarchically complex tissues from multipotent "naïve" progenitor cells.

The future studies emerging from this thesis can be:

- Evaluation of the phenotypic stability of co-cultured hMSC constructs *in vivo.*
- Comparison of the difference between co-culture of hMSCs with human OA chondrocytes and human healthy chondrocytes. In this study we used bovine chondrocytes because of their availability and pending IRB approval. A species matching comparison is essential to pin-point various effects identified in this thesis.
- Study the effect of hMSC-secreted factors on P1 OA chondrocytes based on the mass spec analysis results that indicate a potential phenotype change in co-cultured P1 OA cells.
- Evaluation of the effect of ratio of chondrocytes to MSCs on chondrogenic differentiation of hMSCs.
- Evaluation of the *in vivo* chondrogenic differentiation of hMSCs using 3D constructs containing a random mixture of chondrocytes and MSCs (to eliminate diffusion gradience).
- Investigate the effect of pre-conditioning (using co-culture systems) before transplantation on *in vivo* chondrogenic differentiation of hMSCs

- Detailed evaluation of the hydrophobic A6ACA-based matrix on *in vitro* expansion of chondrocytes.
- Investigate the effect of A6ACA-based matrix on re-differentiation of de-differentiated chondrocytes
- Although we identified several proteins unique to a co-culture system using mass spectrometry, it still remains to be answered which of these specific components or combination of the chondrocyte-secreted factors stimulated chondrogenic differentiation of stem cells. Evaluate of a critical concentration of each of these key elements to promote and maintain chondrogenesis and prevent endochondral ossification.

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Appendix A: List of chemicals

Chemical Name	Supplier	Catalog Number
Trypsin-EDTA, 0.25%	GIBCO	25200
HEPES buffer	GIBCO	15630
Non essential amino acids	GIBCO	11140
L-Glutamine	GIBCO	25030
Sod. Bicarbonate (7.5%)	GIBCO	25080
Sod. Pyruvate (100X)	GIBCO	11360
2-Mercaptothenol	GIBCO	21985-023
Collagenase Type IV	GIBCO	17104-019
DMEM (high glucose)	GIBCO	11965
Trizol-R reagent	Invitrogen	15596-018
DNA picogreen assay kit	Invitrogen	D7589
SS III First strand synthesis	Invitrogen	548818
Alcian Blue (8X)	National Diagnostics	75881-23-1
Chondroitin Sulphate	Sigma	C 9819
Albumin (bovine serum)	Sigma	A7906-100 G
TEMED	Sigma	T-9281
PTHrP	Sigma	12955-31-4

DMSO	Sigma	D2650L
L-Cysteine	Sigma	C7352
APS	Sigma	A-3678
Collagenase type II	Worthington	4177
Papain	Worthington	3126
Acrylamide	Aldrich	148660
N,N-methylene bisarylamide	Aldrich	146072
Trans-4-hydroxyproline	Aldrich	H54409
Power SYBR green PCR master mix	Applied Biosystems	4367659
FBS Atlanta advantage	Applied Biosystems	S11050
FBS Premium Select	Applied Biosystems	S11550
TGF-β1	Fitzgerald	30R-AT027
Glycine	Fisher	G46-500
SDS	Fisher	B0166-500
Triton-X	Fisher	G337-100
OCT Compound	Tissue-Tek	4583
Safranin-O	Scholar Chemistry	9466802
Ready SDS gels (4-15%, Tris-HCl)	BioRad	161-1104
Silver Staining Kit	Invitrogen	LC6070
Cyclopamine	LC Laboratories	C8700

Appendix B: Primers used

Gene	Forward	Reverse
β-actin	5'-CTG-CGG-CAT-TCA-CGA-AAC-TA	5'-ACC-GTG-TTG-GCG-TAG-AGG-TC
Collagen 1(a2)	5' -ATC-CAG-CTG-ACC-TTC-CTG-CG	5'-TCG-AAG-CCG-AAT-TCC-TGG-TCT
Aggrecan	5'-AGC-TGG-GTT-CGG-GGC-ATC-T	5'-TGG-TAG-TCT-TGG-GCA-TTG-TTG-TTG-A
Collagen 2(a1)	5'-CTG-GTC-CTT-CTG-GTC-CTG-TTG	5'-GTG-CGA-GCT-GGG-TTC-TTT-CTA
ALP	5'-TGG-AGC-TTC-AGA-AGC-TCA-ACA-CCA	5'-ATC-TCG-TTG-TCT-GAG-TAC-CAG-TCC
Collagen 10	5'-TGG-ATC-CAA-AGG-CGA-TGT-G	5'-GCC-CAG-TAG-GTC-CAT-TAA-GGC
PTHrP	5'-ACC-TCG-GAG-GTG-TCC-CCT-AA	5'-GCC-CTC-ATC-ATC-AGA-CCC-AA
Sox-9	5'-CTA-CGA-CTG-GAC-GCT-GGT-GC	5'-CGA-TGT-CCA-CGT-CGC-GGA-AG