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Discriminating Chondrogenic Progenitor Cells (CPCs) as a Distinct Cell Type, Apart from Normal Chondrocytes

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DISCRIMINATING CHONDROGENIC PROGENITOR CELLS (CPCS) AS A DISTINCT CELL TYPE, APART FROM NORMAL CHONDROCYTES

by

Cheng Zhou

A thesis submitted in partial fulfillment
of the requirements for the
Master of Science degree in Biomedical Engineering
in the Graduate College of
The University of Iowa

August 2013

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CERTIFICATE OF APPROVAL

MASTER'S THESIS

This is to certify that the Master's thesis of

Cheng Zhou

has been approved by the Examining Committee
for the thesis requirement for the Master of
Science degree in Biomedical Engineering at the August 2013
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ABSTRACT

Articular cartilage is an avascular, aneural, and alymphatic tissue with a structure consisting of a superficial, a middle and a deep zone, overlie a calcified zone at the cartilage border between. Each zone has biological and mechanical properties. Self-repair of damaged cartilage seldom if ever occurs, and joint injuries that harm cartilage surfaces often result in osteoarthritis. This has prompted researchers to explore diverse approaches to cartilage regeneration.

The superficial zone shows the highest cellularity and the lowest matrix density. Cartilage cells (chondrocytes) residing in the superficial zone had been thought to be a subpopulation of chondrocytes. However, our laboratory identified a second population of cells that were distinguishable from chondrocytes based on their clonogenicity, multipotency, migratory activity, higher proliferate rate and substantial morphological differences. These cells later proved to be chondrogenic progenitor cells (CPCs). Our continuing studies have shown that CPCs are less chondrogenic than normal chondrocytes and their function is to protect the cartilage surface rather than to regenerate cartilage matrix as previously supposed. In addition, we found evidence to suggest that CPCs act as pro-inflammatory cells in the context of cartilage injury. For these reasons, we undertook a more comprehensive comparison of the phenotypic differences between CPCs and normal chondrocytes and between CPCs and joint cells (tissue synoviocytes from the joint capsule and cells present in synovial fluid) which have been shown to be play roles in joint inflammation.

Gene expression microarray analysis of >25,000 genes revealed that the overall pattern of gene expression in CPCs was distinct from normal chondrocytes, but closely related to synoviocytes and synovial fluid cells. Analysis of specific genes by quantitative PCR (qPCR) showed profound differences between CPCs and normal chondrocytes in terms of cartilage matrix gene expression (Collagen Type II, Aggrecan, Link Protein and COMP) and pro-inflammatory gene expression (IL6, IL8, CCL2 and CXCL12). In contrast, the pattern of CPC gene expression closely resembled. Sulfated glycosaminoglycan assays revealed that cartilage matrix deposition by CPCs, as well as synoviocytes and synovial fluid cells, was significantly inferior to normal chondrocytes. However, chondrogenic and osteogenic differentiation assays, showed no significant differences among the four cell types.

In addition to establishing that CPCs are distinct from chondrocytes, this work suggests significant revisions to our understanding of CPC function in cartilage. The weak chondrogenic ability and higher expression of inflammatory cytokines, suggests these cells don't play a regenerative role as previously thought. On the other, we found evidence that CPCs may form a protective layer on the top of the injured cartilage surfaces, preventing further cartilage injury. *In vivo* studies are needed to fully elucidate the significance of these roles in cartilage health and disease.

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CHAPTER I

INTRODUCTION

Articular cartilage is a hypocellular, avascular, aneural and alymphatic tissue that is thought to harbor only one cell type, the chondrocyte. Osteoarthritis (OA), a disease that features cartilage degeneration, is most the widespread cause of disability worldwide [1]. Some factors that might ultimately lead to osteoarthritis are age, trauma, and long-term heavy loading.

Synovial joints such as the knee are encapsulated by a capsule lined with synovium, which produces synovial fluid. Synovial fluid contains proteoglycan 4 (PRG4) and hyaluronan (HA) which help to lubricate cartilage, protecting the whole joint from the injurious effects of excessive friction. Additionally, they help keep the cartilage nourished. The cells from synovium tissue and synovial fluid, synoviocytes and synovial fluid cells (SFCs), respectively, are actively involved in these crucial functions. It is of great significance to identify the properties and function of these two cells.

Articular cartilage is comprised mostly of extracellular collagen and proteoglycans. The arrangement and amounts of these major structural components varies through the depth of cartilage matrix, which can be divided into zones. The uppermost superficial zone is specialized to maintain low friction between joint surfaces [2]. Superficial zone cells are thought to be involved in the regulation of tissue development and growth, including morphogenesis of the diarthrodial joint [3], and the expression of many growth factors [4]. Our previous studies revealed that chondrogenic progenitor cells (CPCs) residing in

the superficial zone can be stimulated to proliferate and migrate in response cartilage trauma [5]. CPCs are morphologically distinct compared to normal chondrocytes (NCs), and show stem cell characteristics such as clonogenicity, multipotency, and high proliferate rate. Moreover, we found that CPCs over-express pro-inflammatory chemokines and cytokines relative to NCs. However, no study to date has proved that the cells from the cartilage surface are a distinct cell type, and not NCs. For these reasons, more comprehensive comparison between CPCs and NCs was carried out. We hypothesized that CPCs are closer in phenotype to synoviocytes and SFCs than to chondrocytes.

DNA Microarray is a rapidly emerging technology which can be probed with target molecules to test abundant genes simultaneously. Through this method, researchers are able to conduct large-scale quantitative experiments and compare multiple cell samples together at the genome level. Microarray technology is widely employed in illuminating mechanisms of functional pathways and predicting activities of new compounds [6].

In this study, we introduced DNA microarray technology to compare NCs, CPCs, SFCs and synoviocytes. We then found remarkable differences that successfully discriminate CPCs from NCs. We also employed quantitative real-time PCR (qPCR) to validate the microarray results in term of different categories of genes. Finally, we compared the functional differences among these four cell types by evaluating their differentiation capability and measuring glycosaminoglycan contents.

CHAPTER II

BACKGROUND

Articular Cartilage

Composition of Articular Cartilage

Articular cartilage refers to hyaline cartilage on the articular surfaces of bones. It is known as a thin connective tissue covering the surfaces in diarthrodial joints. The major components of hyaline cartilage are Type II collagen and chondroitin sulfate.

Articular cartilage consists of chondrocytes (Figure 2.1), which until recently were thought to be the only cell type existing in hyaline cartilage. Chondrocytes account for less than 5-10% of the total cartilage volume [7]. They are highly differentiated cells with remarkable properties and capabilities, setting them apart from other types of mesenchymal cells. The primary function of chondrocytes is to synthesize and maintain the matrix of the cartilage (collagens, proteoglycans, and some noncollagenous proteins). Chondrocytes are able to withstand physical deformation and also facilitate tissue function [8]. Chondrocytes reside in cavities in the matrix (cartilage lacunae), of a rounded or bluntly angular form, lying in groups of one or more in a granular or homogeneous extracellular matrix (ECM) (Figure 2.2). Chondrocytes are well differentiated to accommodate the low oxygen environment in cartilage, which can be as low as 1% compared with 21% in normal atmosphere [9]. Chondrocytes perform nutrient/waste exchange via simple diffusion from synovial fluid.

Articular cartilage is composed of a small percentage of chondrocytes and the dense ECM prevents chondrocyte mobility [10].

The distribution of chondrocytes throughout cartilage ECM varies depending upon the age of the cartilage, some pathological states and other factors [11]. Within specific articular cartilage, the amount of chondrocytes residing in different zones is substantially diverse as well.

Besides the chondrocytes, the other three key components of cartilage are fluid phase (e.g., interstitial water and electrolytes), around 65-85%, solid ECM (e.g., collagen molecules and proteoglycans), around 95% by dry weight, and noncollagenous protein, in trace amounts. Type II collagen forms a fibrillar network that entraps aggrecan, the main proteoglycan of articular cartilage [12] (Figure 2.3). Aggrecan is heavily decorated with sulfated glycosaminoglycans, which are long unbranched polysaccharides capped by a charged sulfate group that bind water. The resistance of the cartilage ECM to compression, a key physiologic function of cartilage, is due largely to this water-binding property of aggrecan. Thus, loss of aggrecan can lead to loss of function and cartilage failure [13].

Zonal Structure and Arrangement of Articular Cartilage

Articular cartilage can be divided into four vertical zones (superficial, middle (or transitional), deep (or radial), and calcified) with varying matrix composition, collagen fiber orientation, and mechanical properties (Figure 2.4). Each zone executes different activities in contributing the functions of articular cartilage. Through the whole cartilage starting from the superficial surface, water

content falls linearly from 84% wet weight (ww) in the superficial zone to 40-60% ww in the deep zone. Collagen content follows a similar gradient, decreasing from 86% to 67%. In contrast, proteoglycan content increases from around 15% dry weight (dw) to 20% dw [14].

Superficial Zone

The superficial zone is the articulating layer that provides a smooth gliding surface. It takes up 10-20% of the total articular cartilage depth. It contains the highest density of collagen fibers and cellularity among all the zones. Chondrocytes in the superficial zone are relatively flat and elongated and preferentially function to secrete proteins that lubricate and protect the articular cartilage surface [15]. Among all the proteins secreted by superficial zone, superficial zone protein (SZP), also known as lubricin, is most important in distinguishing superficial zone chondrocytes from chondrocytes in deeper layers [16].

Middle Zone

The middle zone includes 40-60% of the total cartilage thickness. This zone contains higher proteoglycan deposition and less cellularity compared to the superficial zone. Chondrocytes in this layer are generally disc-shaped and randomly distributed.

Deep Zone

The deep zone occupies 30% of the total cartilage volume. It has the highest proteoglycan concentration and lowest chondrocyte cellularity. This zone shows the largest diameter collagen fibrils, which are oriented in radial direction

and perpendicular to the surface. By being organized and integrated in this unique way, collagen fibrils strengthen the bond between cartilage and bone [17]. The chondrocytes are distributed parallel to the collagen fibrils. In addition, chondrocytes in the deep zone are ten times more synthetically active than chondrocytes in superficial zone [15].

Calcified Zone

The calcified zone is a transitional zone from cartilage to subchondral bone. This layer contains a small volume of chondrocytes, which are inert, spherical in shape, and encircled by the calcified ECM. These chondrocytes have an extremely low level of metabolic activity [17].

Tidemark

The tidemark, a visible basophilic line, separates the deep zone from calcified cartilage; it plays a pivotal role in transmitting mechanical forces through cartilage to the subchondral bone [18].

Osteoarthritis

Osteoarthritis Introduction

Osteoarthritis (OA) is the most common cause of chronic joint pain and disability [19]. OA is characterized by the structural or functional failure of the joints, which involves progressive cartilage degradation (Figure 2.5) and subchondral bone hardening [19]. This process results in the formation of osteophytes, the obliteration of the joint space, the appearance of subchondral cysts, and remodeling of the subchondral bone (Figure 2.6). The major causes of OA include endocrine imbalance, joint trauma and inflammation, metabolic,

neuropathic and other pathways. However, the pathogenesis of OA still remains poorly understood even though aging and excessive usage of joints are deemed as most common-risk factors [20]. Unlike rheumatoid arthritis, OA is not an autoimmune disease, but chondrocytes from OA patients over-produce prostaglandins, tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), IL-6, and IL-8, all of which promote joint inflammation [21].

Osteoarthritis Diagnosis and Treatment

Common methods employed to diagnose osteoarthritis, include joint fluid analysis and blood tests for markers of cartilage degeneration and by imaging using X-rays, magnetic resonance (MR), or computed tomography (CT) to detect cartilage loss and subchondral bone remodeling.

To prevent, delay, or limit osteoarthritis symptoms, it is essential to maintain general health. For example, weight loss, appropriate exercise and diet will be beneficial to prevent osteoarthritis. Orthotic devices, such as neck braces and knee braces, are used to support, correct deformities that lead to cartilage wear. Non-disease-modifying Over-the-counter (OTC) medications that control pain include acetaminophen (Tylenol) and other non-steroidal anti-inflammatory drugs (NSAIDs), and the cartilage nutrients chondroitin and glucosamine sulfate. Prescription NSAIDs such as celecoxib, an inhibitor of cyclooxygenase 2 (COX-2) may be needed if OTC medications are ineffective. A newly approved medication by the Food and Drug Administration (FDA) for chronic musculoskeletal pain is the antidepressant duloxetine which has positive effect on neurotransmitters during the pain perception in the brain.

Besides these medications, surgeries such as arthroscopy, osteotomy, chondroplasty and arthrodesis are offered to some patients to relieve pain [22]. Total joint replacement may be performed for patients who are suffering from chronic pain and disability [23].

Cartilage Repair and Regeneration Post Osteoarthritis

Treatments to promote cartilage regeneration have been under development for decades [24]. Tissue engineering and stem cells therapy have been studied and attracted much attention, becoming more and more promising for potential treatment of cartilage repair.

Synovium and Synoviocytes

The synovium, also known as synovial membrane, is the soft tissue between the articular capsule and the joint cavity (Figure 2.7). Unlike cartilage, the synovium is porous and vascularized. This thin lining is responsible for maintaining normal function and homeostasis of joints through synthesizing hyaluronan and lubricin [25]. In addition, the synovium functions to mediate nutrient exchange between circulating blood and joint fluid.

The lining layer is comprised of a few macrophage-like type A and many fibroblast-like type B synovial cells. Type A cells, which are important in phagocytosis of antigens, are derived from bone-marrow myeloid precursors. Type B cells, which are of mesenchymal lineage, produce synovial fluid. Some researchers term the cells with features of both type A and type B cells as distinct type C cells. These cells are nourished mainly through the blood vessel network in the sub-lining layer [26]. Synoviocytes are fibroblast-like cells that

synthesize hyaluronan, which makes synovial fluid viscous and contributes to its lubricating effects [27]. Synoviocytes show prominent expression of the ECM protein type I collagen, the ECM adhesion molecules CD44 and VCAM-1 [28] and CD44 is a receptor for hyaluronan [29] and has relatively high expression in synoviocytes compared to other connective tissue cells.

Some studies have revealed inflammatory changes in the synovium of OA patients, including synovium hypertrophy, elevated vascularity and infiltration of the underlying tissue [30].

Synovial Fluid and Synovial Fluid Cells (SFCs)

Synovial fluid is a viscous and non-Newtonian fluid found in the cavities of synovial joints (e.g., knee, elbow). Synovial fluid is produced by Type A cells in synovium tissue and is secreted and regulated by synovium into the joint cavity. A healthy human knee normally contains around 2mL of synovial fluid, which is a complex mixture of hyaluronan, lubricin, proteinase, collagenases and prostaglandins [31]. The major functions of synovial fluid are lubricating articular cartilage, absorbing shock and providing nourishment of cartilage through diffusion.

Boundary lubrication of cartilage surfaces is a significant factor in preventing cartilage damage and maintaining normal function [32]. Proteoglycan 4 (PRG4) and hyaluronan (HA), both of which adhere to the surface of the cartilage, are the two most pivotal lubricating molecules in synovial fluid [33]. PRG4 is one of the mucinous glycoproteins and is synthesized by synoviocytes and chondrocytes residing in the superficial zone of the cartilage to enhance the

boundary lubrication and protect the joint from tissue wear damage. Furthermore, HA also contributes hydrodynamic and boundary lubrication in the knee joint.

In addition to proteins from plasma, synovial fluid contains molecules secreted from the cells of adjacent joint tissue. Moreover, synovial fluid contains most candidate biomarkers for pathogenesis of relevant disease [34]. This unique property offers the reason why synovial fluid is significantly associated with specific diagnoses, such as osteoarthritis, rheumatoid arthritis, pyogenic bacterial infection and tumors.

Chondrogenic Progenitor Cells in Osteoarthritis

With respect to their potential to form multiple tissues, progenitor cells are midway between stem cells, which are multipotent (able to form a wide range of tissue) and fully differentiated cells, which are described as unipotent or oligopotent (able to form only one or two related tissue types). Progenitor cells do have certain capacity to differentiate to multiple cell types, which makes them similar to stem cells. However, unlike stem cells that can replicate indefinitely, progenitor cells have a limited replicative lifespan. Most progenitor cells act to repair and maintain tissues. While under normal conditions, progenitor cells appear quiescent in the tissue where they reside, they migrate, proliferate and differentiate locally to injury sites where they repair damaged tissues [35, 36].

Chondrogenic progenitor cells (CPCs) were first found in bovine cartilage as a subpopulation of superficial zone cells [37, 38] (Figure 2.8). Side population assays, revealed that relatively few CPCs reside in the middle and deep zones [39]. Additional studies showed that CPCs are particularly abundant in cartilage

during the later stages of osteoarthritis [40]. In addition, results from our laboratory revealed that CPCs actively migrate to injury sites [5] (Figure 2.9). All these views combined together suggest that cartilage might have a unique proportion of progenitor cells, especially in the superficial zone. These special cells over-express the stem cell marker Notch 1 when compared to normal chondrocytes and are more fibronectin adhesive. In addition, CPCs exhibit many stem cell features, such as multipotency, clonogenicity and migratory activity [40]. Around 4% of normal human chondrocytes express CD105 and CD166, which are important markers for Mesenchymal stem cells (MSC) [41].

Some transcriptional genes are commonly used to test the properties of chondrogenic progenitor cells, such as Runx2, an osteogenic transcription factor. RUNX2 is widely used as a marker gene of osteogenic differentiation potential. Also SOX9, an important transcription factor in chondrocyte regulation and is frequently used to identify chondrogenic differentiation [42]. Studies have been performed to show that downregulation of the RUNX2 could promote SOX9 expression in CPCs [40]. Our gene expression analyses recently showed that compared to mesenchymal stem cells, CPCs over-expressed proteoglycan 4 (PRG4), the gene encoding lubricin. Immunohistochemistry revealed that multilayer CPCs expressed lubricin on damaged cartilage surfaces after migrating to injury sites (Figure 2.10). This behavior strongly suggests that CPCs repair the vital lubricant coating that is lost from cartilage surfaces as a result of mechanical damage.

The discovery of chondrogenic progenitor cells illuminates the path to restore damaged cartilage despite the limited self-repairing capacity of cartilage. During the past decades, people have investigated and developed various stem cell based therapies to treat many diseases. A few studies suggest that progenitor cells in OA patients play a regenerative role, leading to proposals to stimulate and accelerate progenitor migration and proliferation in degenerating cartilage. However, recent findings from our laboratory suggest CPCs also participate in inflammatory reactions to cartilage injury, suggesting that excessive stimulation could do harm.

DNA Microarray

Microarray Introduction

The DNA microarray is widely used to measure the expression of an abundance of genes simultaneously. Microarray technology evolved from southern blotting, in which fragmented DNA is adsorbed to a solid substrate and probed with a specific DNA sequences [43]. Microarray analysis has been widely adopted to examine cells from different sites including normal or pathologic tissue. This mRNA representative method is extremely sensitive and has a high output [44]. Microarray technology represents a rapid-emerging and powerful new set of tools that enables researchers to connect hypothesis testing with data. It allows for rapid measurement and visualization of differential expression between genes at the scale of the whole genome. Since microarray has been compiled to simultaneously detect large numbers of analytes in a sample, this

strategy is faster and more convenient than serial tests for each analyte [45] (Figure 2.11).

Principles

Individual nucleic acid sequences are synthesized and printed on a glass surface by a sophisticated robot. The essence of microarray is the hybridization of two DNA strands. Complementary nucleic acid will pair with each other through hydrogen bonds, and increasing numbers of these bonds generates tighter non-covalent bonding between two strands. The tightly bound strands will remain hybridized after washing off the non-specific bonding sequences. The target sequences, which are fluorescently labeled, will create a signal that depends on the hybridization conditions. The amount of target sample bound to the probe determines the total signal strength from a spot. This is then measured across the array with a scanning confocal microscope. Afterwards, image acquisition and following data processing are also needed to achieve the satisfactory results through different software (Figure 2.12).

Applications

Microarray technology is especially useful for comparing mRNA from two or more cell or tissue types or from the same cell or tissue treated in different ways [46]. This then can be used to monitor gene expression patterns in great depth. A growing number of fields are applying the microarray technology as it has been rapidly developing. These fields include pharmacogenomics, epidemiology, cardiovascular, oncology and disease classification. The future direction of microarray may drift from single-layer platform to microspheres,

which are solution-phase kinetics instead of solid-phase kinetics. In this way, the surface can be more easily bound by the substrate [47].

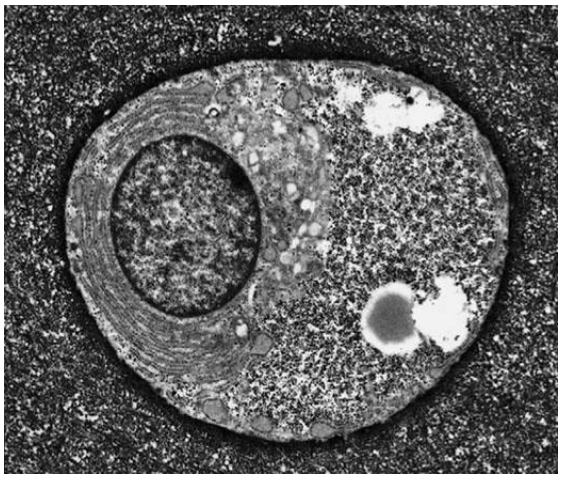

Figure 2.1. Chondrocyte.

Electron micrograph of a typical articular chondrocyte [8]

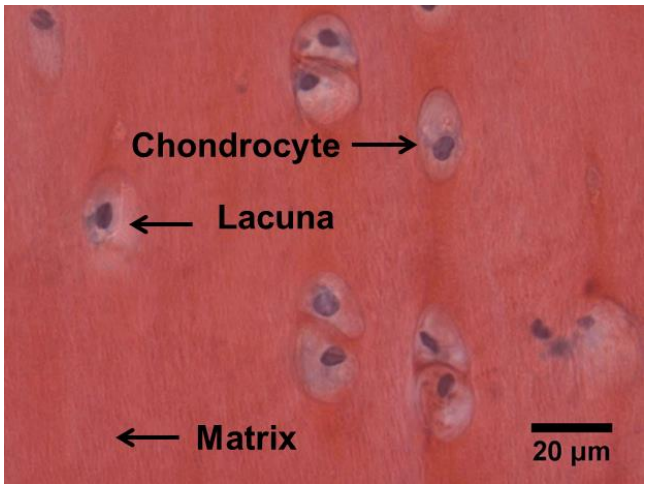

Figure 2.2. Location of chondrocytes.

Chondrocytes reside in lacunae throughout the articular cartilage matrix.

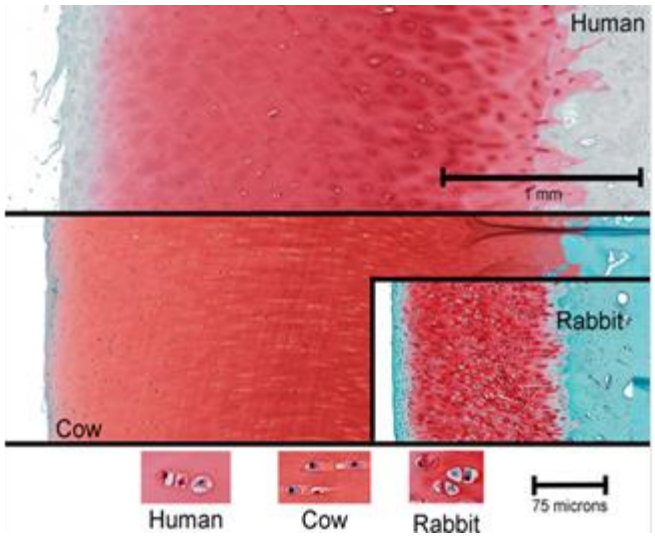

Figure 2.3. Proteoglycan deposition in cartilage of different species.

Red: Positive safranin O staining of proteoglycan deposition in cartilage

Blue: Counterstain (Bone, proteoglycan-depleted cartilage)

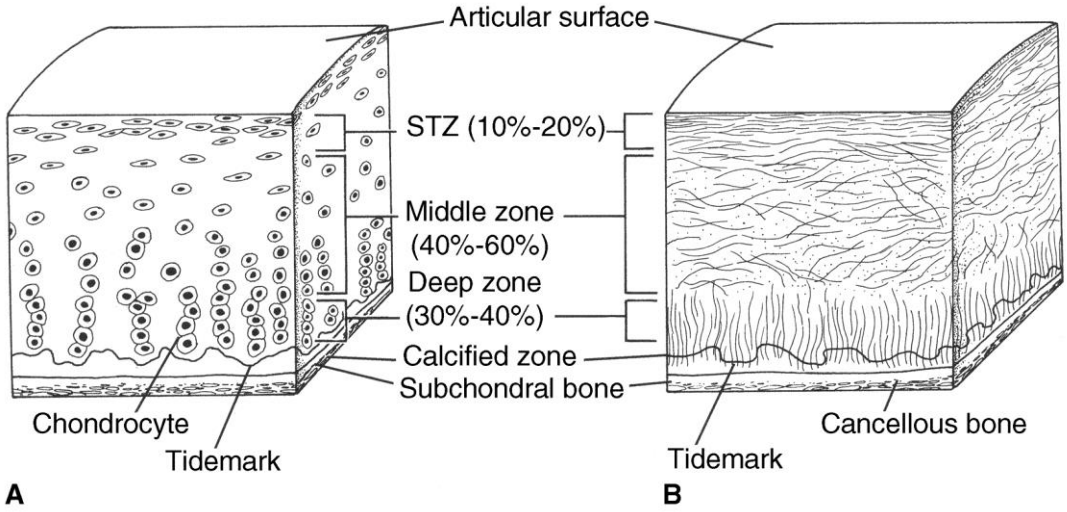

Figure 2.4. Zonal arrangement of articular cartilage.

Articular cartilage can be mainly separated into the superficial, middle, deep and calcified zone

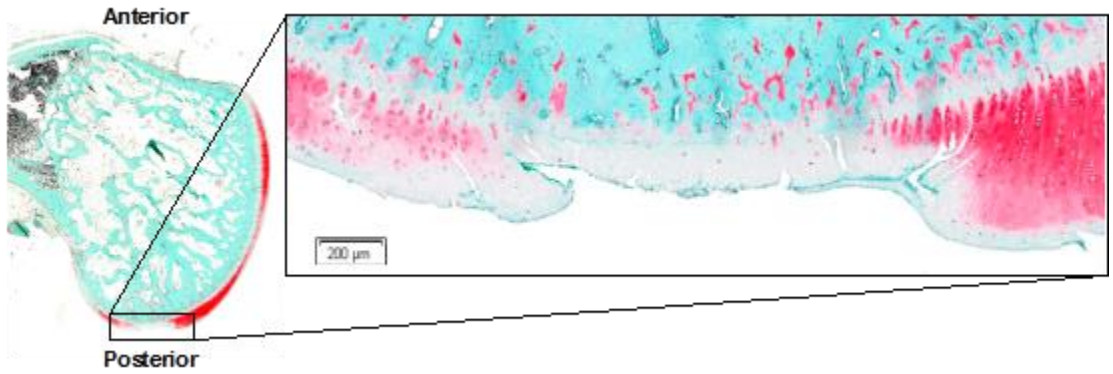

Figure 2.5. Proteoglycan & cartilage loss in osteoarthritis.

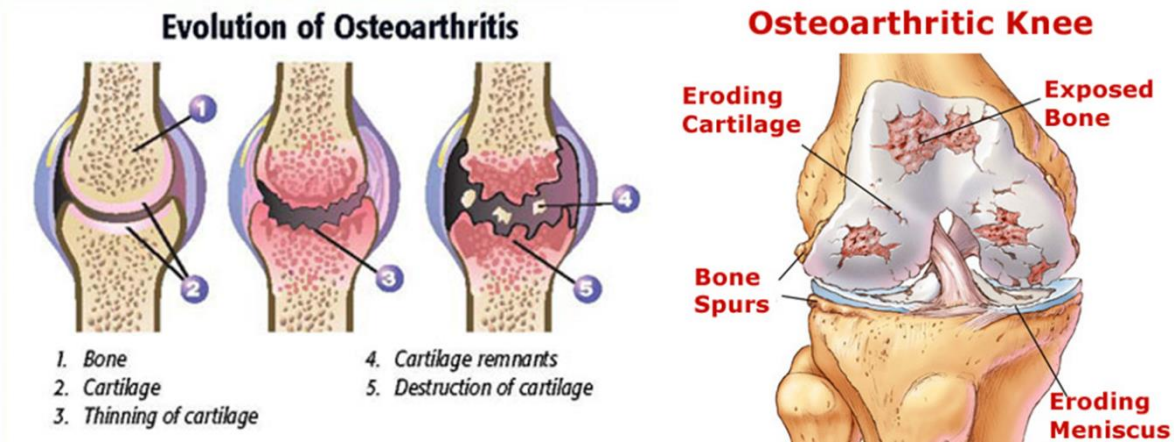

Figure 2.6. Evolution of osteoarthritis (left) & illustration of osteoarthritic knee joint (right).

(Left: http://www.bestphysicaltherapistnyc.com/dealing-with-the-pain-of-osteoarthritis-in-the-knee/ Right: http://www.physioflex.com.au/the-secret-to-improving-knee-osteoarthritis/)

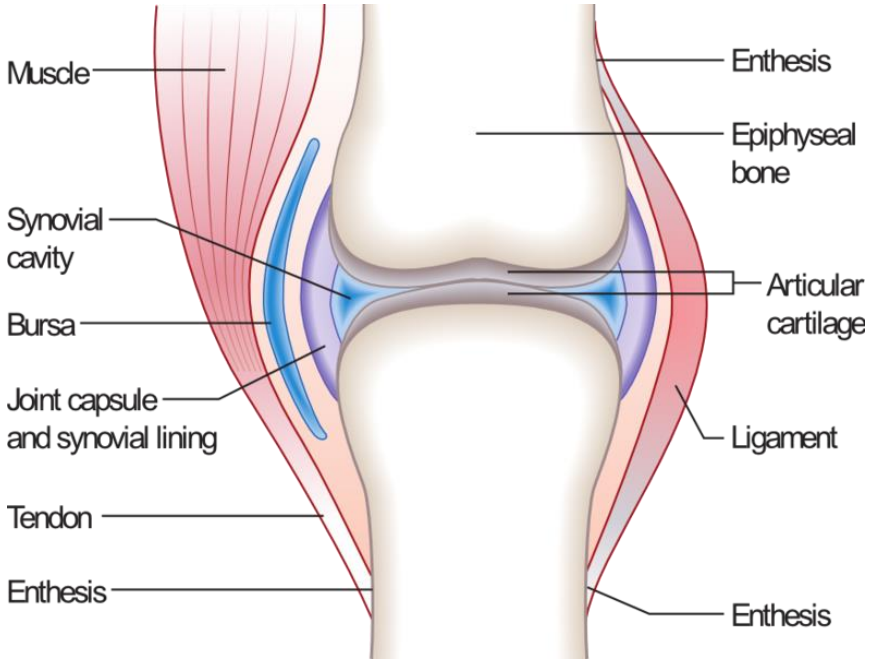

Figure 2.7. Illustration of synovial joint.

(http://en.wikipedia.org/wiki/Synovial_membrane)

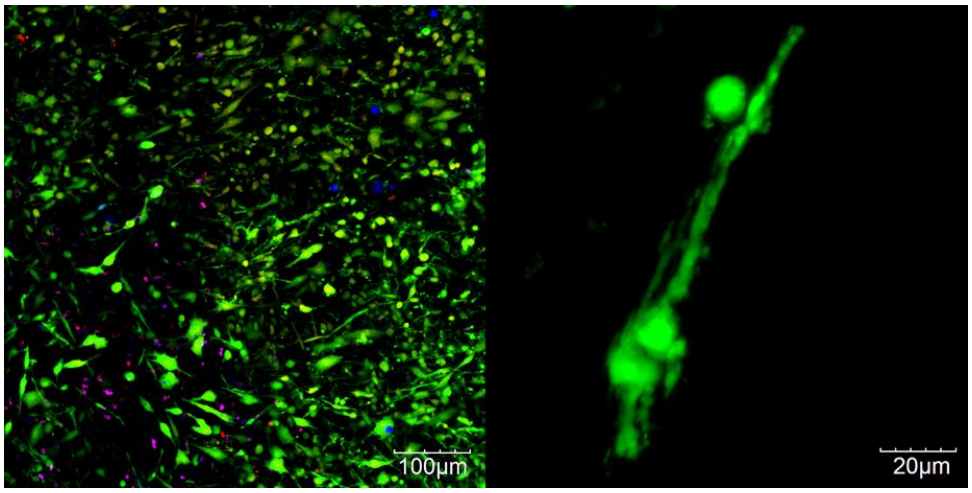

Figure 2.8. Morphology of chondrogenic progenitor cells (CPCs)

Confocal images of CPCs (Calcein AM staining)

Left: CPCs (green) are gathered together after certain injury on cartilage

Right: Higher magnification figure showing the morphology of a specific CPC

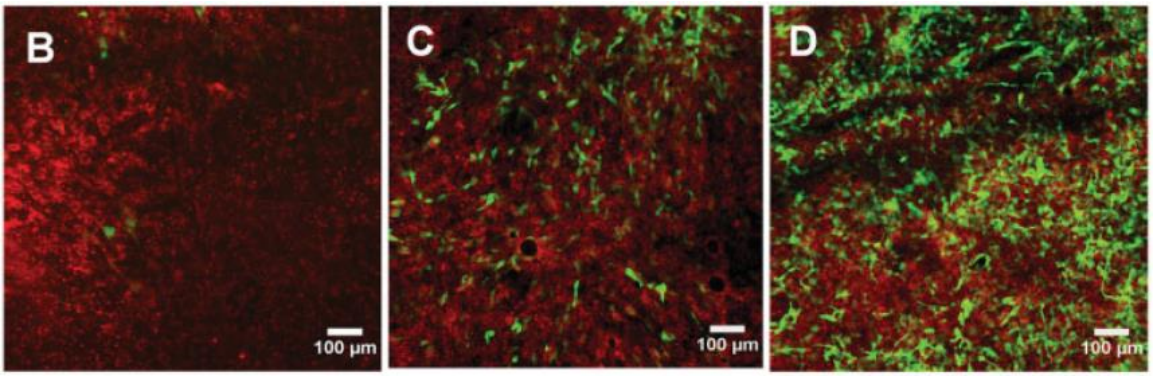

Figure 2.9. Migration of grafted putative chondrogenic progenitor cells (pCPCs) [5]

Exactly the same impact site was imaged by confocal microscopy at various times after grafting (B: 2 days, C: 5 days, D: 12 days). Grafted GFP-labeled cells (green) can be seen against the background of host cells labeled with a red tracking stain.

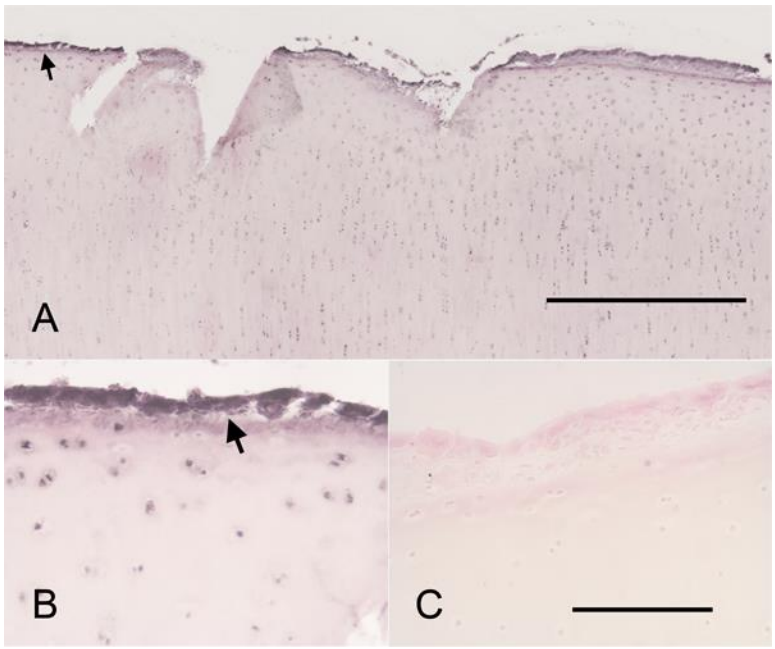

Figure 2.10. Immunostaining of injured cartilage for lubricin.

(A) Low magnification image shows positively stained CPCs on the damaged cartilage surface (bar = 250 microns). (B) The area over the arrow in A is shown at higher magnification. (C) Negative control at the same magnification as in B (bar = 100 microns)

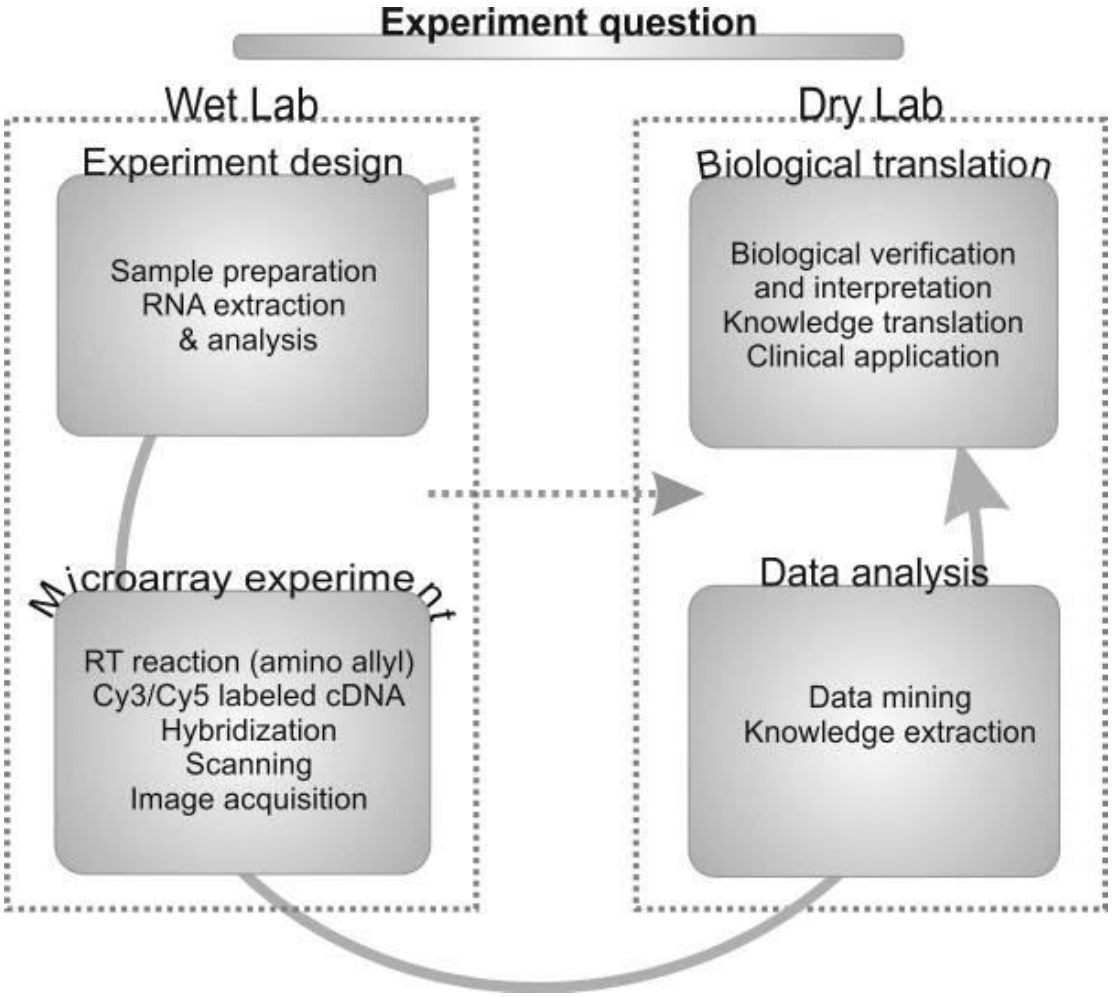

Figure 2.11. Schematic steps of DNA microarray technology [46]

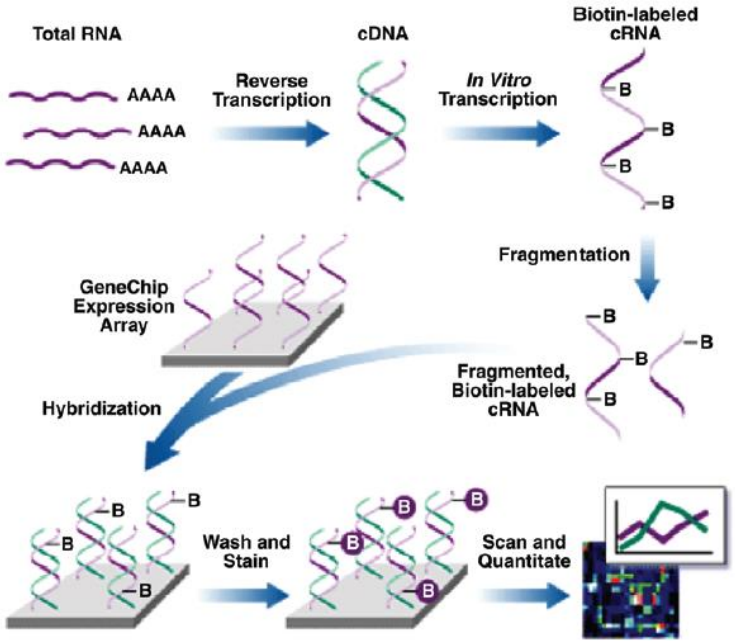

Figure 2.12. Schematic illustration of Affymetrix GeneChip Oligo microarrays.

RNA is extracted from different cell/tissue groups and reverse transcribed to cDNA, which is then transcribed to cRNA. After a period of incubation on the microarray slide, the unbound probe is washed off and stained with biotin-conjugated antibodies, which can emit fluorescence. The microarray slide is then scanned at certain laser intensity and the emitted light will be quantified and analyzed. (Adapted from www.affymetrix.com)

CHAPTER III

DISCRIMINATING CHONDROGENIC PROGENITOR CELLS (CPCS) AS A DISTINCT CELL TYPE, APART FROM NORMAL CHONDROCYTES

Purpose of Study

Articular cartilage is an avascular, aneural and alymphatic tissue, which can be functionally and structurally divided into four different zones: superficial zone, middle zone, deep zone and calcified zone [13]. Synovium and synovial fluid are crucial components preventing cartilage degradation in the knee joint. For decades, people have held the universal notion that cells from superficial zone are subpopulation of chondrocytes, but very few studies were performed to compare these cells to normal chondrocytes. Chondrogenic progenitor cells from post-traumatic cartilage superficial zone did exhibit the potential capacity to regenerate and recover to the native cartilage [5], indicating the heterogeneous properties between the cells from the cartilage surface and normal chondrocytes.

From various perspectives, we hypothesized the chondrogenic progenitor cells (CPCs) are essentially distinct from normal chondrocytes. However based on many evidences, these cells appear more closely related to synoviocytes and synovial fluid cells (SFCs), as they serve to form a protective layer on the uppermost the OA cartilage.

In this chapter, DNA microarray, which is a precise measurement of the expression levels of large number of genes simultaneously, will be performed to

examine the identity of CPCs, compared to the normal chondrocytes (NCs), synoviocytes and synovial fluid cells (SFCs). In addition, gene expression will be analyzed by quantitative real-time PCR (qPCR), as well as several function analyses will be used to confirm the results.

Materials and Methods

Cartilage Harvesting and Culture

Fresh articular cartilage was harvested from bovine femur condyles or tibia plateaus of healthy stifle joints (Bud's, IA, USA). The cartilage was then washed using Hank's Balanced Salt Solution (Invitrogen, California, USA) containing antibiotic and Fungizone and cultured in Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 with a 1:1 mixture. This mixture was supplemented with 10% fetal bovine serum (FBS), 50 µg/µl L-ascorbate, 100 U/µl penicillin, 100 µg/ml streptomycin, and 2.5 µg/µl Fungizone.

Synovial Fluid Collecting and Culture

Synovial fluid was obtained from the same bovine knee joint with the cartilage by sterile syringe. Synovial fluid was then 1:1 mixed with culture media (same ingredients with the media for cartilage culture) and cultured in a low oxygen incubator being sure to change the culture media the every other day.

Synovium Tissue Harvesting and Culture

The synovium tissue was washed with Hank's Solution then cut into many smaller pieces (0.5cm × 0.5cm) to attach the bottom of the tissue to the culture dishes (Falcon, NJ, USA). After around two hours of dry attachment, the culture media (same ingredients with the media for cartilage culture) was added onto the

synovium tissue drop by drop using a 1ml pipette. The following days, the same culture media adding method was used to keep the tissue in a nutritious environment until the synovium tissue was no longer attached. The synovium tissue was then removed and the synoviocytes remained in low oxygen incubator to culture while changing the culture media every other day. Figure 3.1 illustrates the attachment and culture system for synovium tissue.

Chondrogenic Progenitor Cells (CPCs) Obtainment and Isolation

After 2-day equilibrium of culture media, a sterile needle was dragged on the cartilage surface of each explant to create multiple X-shape matrix tears. The explants were then washed by Hank's Solution once prior to being returned into a sterile specimen container (Kendall, MA, USA) filled with 60ml culture media. Then the explants were cultured for 7-10 days with changing the culture media the every other day until the CPCs were ready to be isolated. To isolate the CPCs, each explant was first washed with 60ml Hank's solution to eliminate residual serum and then flipped each explant upside down. Next, 10ml 0.25% Trypsin-EDTA (GIBCO, NY, USA) was added to each explant and returned to the low oxygen incubator. After 10mins, 20ml of culture media was added to end the trypsinization. Then the containers were shaked to ensure the complete detachment of CPCs. After, the tissue debris was removed with cell strainers (Falcon, NJ, USA) and the cell suspensions were centrifuged at 2000rpm for 10mins at room temperature. Then, the cells were resuspended in culture media and seeded in different size of flasks (Corning, NY, USA) according to the cell

number. Lastly, the CPCs were cultured in a low oxygen incubator while making sure to change the culture media every other day.

Cartilage Digestion and Normal Chondrocytes (NCs) Isolation

After CPCs were isolated, the whole thickness of cartilage tissue was shaved off and minced into smaller pieces. Then the cartilage tissue was digested in 0.4% protease (Sigma, USA) dissolved in serum free media (DMEM and F12 with 1:1 mixture supplemented with 50 µg/µl L-ascorbate, 100 U/µl penicillin, 100 µg/ml streptomycin, and 2.5 µg/µl Fungizone) in a spinner flask and incubated in a high oxygen incubator for one and a half hours. Then, the product was washed the cartilage tissue with serum free media three times. This was followed with incubating the cartilage tissue in 0.02% collagenase (Sigma, USA) in a high oxygen incubator for 16 hrs. The next day, centrifuged the digestion solution was centrifuged at 2000rpm for 10mins and resuspended with culture media. Lastly, the cells were seeded in flasks with appropriate sizes according to the cell number.

Cell Culture and Passaging

Every other day, the culture media must be changed to keep fresh nutrients for the cells. When the cell confluence reached 70-80%, the cells need to be passaged and washed with Hank's solution once. Then the proper volume of Trypsin-EDTA (5–10ml depends upon the size of the flask or dish) was added into the flask. Then, this was mixed well and returned into the low oxygen incubator. Two to three minutes later, the cell condition was checked until cells were in rounded shape and were about to detach. Then added two volumes of

culture media were added to end the trypsinization. Lastly, the pellet cells were centrifuged at 2000rpm for 10mins and resuspended the cells with culture media and seeded them into new flasks.

RNA Extraction

The RNA for gene expression analysis was isolated from passage two of four different kinds of cells (CPCs, NCs, synoviocytes and SFCs) which were previously seeded into 6-well plates. The cells were homogenized in Trizol reagent (InvitriogenTM Life Technologies, CA, USA) and the total RNA was extracted by the RNeasy Mini Kit (Qiagen, CA, USA) according to the manufacturer's protocol.

After total mRNA was successfully extracted, the RNA concentration was measured according to the manufacturer's instruction through the use of a Nano-Drop spectrophotometer located in the DNA Facility (Iowa City, IA). The total RNA was further processed using microarray analysis, qPCR or stored at -80°C freezer until ready for use.

DNA Microarray Analysis

RNA was harvested from three independent batches of synoviocytes, SFCs, NCs, two independent batches of CPCs. The RNA extraction procedure for each kind of cells is essentially identical as previously described. The RNA (50ng) was then reverse transcribed to single primer isothermal amplification-amplified complementary DNA (cDNA) using an Ovation RNA Amplification System version 2 (NuGEN). Biotinylated cDNA was hybridized to Bovine Genome Arrays (Affymetrix). Arrays were scanned with an Affymetrix Model 3000, and

the data was collected using GeneChip operating software (MAS 5.0). Statistical fold change expression was then applied via 1-way analysis of variance (ANOVA) model by using Method of Moments [48]. Fisher's Least Significant Difference (LSD) was employed as the contrast method. Heat maps and annotated gene groups (Metalloendopeptidase related, Extracellular matrix related, Collagen related, Inflammatory related, Cytokine related) were retrieved from the internal microarray database which discriminates a 5-fold change (either +5 or -5-fold change) between normal chondrocytes and CPCs. Then 3D Principle Component Analysis (PCA) plot and dendrogram were generated using Partek Genomics Suite software.

Gene Expression Analysis Using Quantitative

Real-time Polymerase Chain Reaction (qPCR)

50ng of RNA was reverse transcribed to complimentary DNA according to the manufacturer's instructions using TaqMan reverse transcription reagents (Applied Biosystem, CA, USA) containing 10X TaqMan RT Buffer, 25mM $MgCl_2$, dNTPs Mixture (2.5mM each dNTP), 50μM Random Hexamers, 20U/μL RNase Inhibitor, and 50U/μL MultiScribe^{TM} Reverse Transcriptase. qPCR reactions were performed with SYBR Green reagents (Applied Biosystem) and custom specific primers which were purchased from Integrated DNA Technologies (Coralville, IA, USA). Table 3.1 summarizes the information of the primers. All the qPCR experiments were performed in triplicate using PCR System to maintain the stability of the technique. Each gene expression level was normalized to β-actin levels, where β-actin was regarded as reference gene, and

the raw data was processed by SDS Software for the 7900HT Fast Real-Time PCR System (Applied Biosystems). Then the fold change was calculated by the $2^{-\Delta\Delta Ct}$ method (The C_t value will be disregard if it exceeds 35).

Multi-lineage Differentiation Potential Assay

Chondrogenic and osteogenic differentiation assays were performed aiming to check the functionalities of these four different cell types. The major principle when examining differentiation potential is to keep all the cells in the exact same passage.

Chondrogenic Differentiation Assay

The multi-potency of passage two for CPCs, NCs, synoviocytes and SFCs was examined by culturing them under chondrogenic and osteogenic conditions. For the chondrogenesis differentiation assay, 200µl of chondrogenic differentiation media (DMEM supplemented with 10ng/ml TGF-β1, 0.1µM dexamethasone, 25µg/ml L-ascorbate, 100µg/ml pyruvate, 50mg/ml ITS + Premix and antibiotics) containing 0.25×10^6 cells of all four kinds cells were seeded into each well of a 96-well, V-bottom, non-treated, sterile, polystyrene microplate (Costar, NY, USA). The microplates were then centrifuged for 5mins at 500×g and placed in a low oxygen incubator. The next day, the cells aggregated as cell pellets. Then the cell pellets were cultured for two weeks with changing chondrogenic differentiation media every other day [49]. After a 14-day culture, the cell pellets were embedded in Tissue Freezer Media and then sectioned in cryostat with thickness of each sample equaling 10µm. The slides

were then subjected to Safranin O staining and images were taken under an Olympus VS110 microscope (Olympus America Inc, PA, USA).

Osteogenic Differentiation Assay

For the osteogenesis differentiation assay, StemPro® Osteogenesis Differentiation Kit (GIBCO, NY, USA) was used for inducing osteogenesis. Four different kinds of cells of passage two were cultured in osteogenic differentiation media with cell density 2×10^4 cells/well in 12-well plate for 21 days being sure to change osteogenic differentiation media every three days. Three weeks post osteogenesis induction, all the cells were subjected to Alizarin Red S staining and images were taken by camera.

Sulfated Glycosaminoglycan (sGAG) Assay

Utilizing Dimethylmethylene Blue

The pellets of four different kinds of cells, triplet of each sample were prepared (12 pellets in total). To consolidate the results, they were cultured under the same conditions with the chondrogenic differentiation assay. After five days of chondrogenic differentiation culture, the cell pellets were transferred to a -80°C freezer for future use.

When ready to use, the cell pellets were digested using 40µl papain digest buffer (0.01 mM/mL L-Cysteine HCl, 0.2 mM/mL Na_2HPO_4, 0.01 mM/mL Papain type III) for four hours. The pH was pre-adjusted to 7.5, and all samples were vortexed all samples every 30mins over the four hours digestion period to ensure complete digestion of the samples. Then the samples were centrifuged at

12000g for 10mins to pellet any insoluble material once the digestion was completed.

sGAG content of cell pellets was quantified using the dimethylmethylene blue assay. Absorbance was spectrophotometrically measured on a kinetic microplate reader (VMaz, Molecular Devices), which adjusted the wavelength to 530nm, and then compared the absorbance to a standard curve generated from known concentrations of the sulfated GAGs. sGAG content was then normalized to the DNA content of each cell pellets, where the measurement of the DNA content was performed by using Quant-iT ^{TM} PicoGreen® dsDNA Reagent and Kits (Invitrogen, California, USA) according to the manufacturer's manual. sGAG contents were reported as µg sGAG/µg DNA.

Statistical Analysis

Statistical assay was performed using a one-way analysis of variance (ANOVA) through SPSS software version 21 (SPSS, Sigma Stat). A P value less than 0.05 via Tukey's post hoc test was considered to indicate statistical differences among groups (*: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$).

Results

Microarray analysis

Gene Expression Profiling of NCs, CPCs, SFCs and Synoviocytes

24128 genes were found and processed in the chip designed for the bovine species. Table 3.2 summarizes the selected gene expression fold change from microarray data. These results showed that NCs possess significant increases in expression of cartilage forming markers such as Collagen II (COL2A1),

Aggrecan (ACAN), Cartilage Oligomeric Matrix Protein (COMP) and Hyaluronan and Proteoglycan Link Protein (HAPLN) than CPCs, (20.5-fold, 5.9-fold, 76.9-fold and 1.8-fold, respectively). The same gene expression pattern appeared in NCs versus SFCs (15.4-fold, 29.1-fold, 180-fold and 221-fold, respectively) and NCs versus synoviocytes (16.1-fold, 39.1-fold, 145-fold and 433-fold, respectively).

As for the genes which are related to inflammation, Interleukin 6 (IL6), IL 8, Chemokine (C-C motif) ligand 2 (CCL2), Chemokine (C-X-C motif) Ligand 12 (CXCL12), CPCs are all up-regulated to NCs (225-fold, 9.7-fold, 32.3-fold and 12.1 fold, respectively). A similar scenario also happens for both SFCs (84.6-fold, 17.2 fold, 50-fold and 4.8-fold, respectively, over NCs) and synoviocytes (33-fold, 15-fold, 51.6-fold and 10.8-fold, respectively, over NCs).

In terms of the fibroblastic marker gene, Collagen I, all CPCs, SFCs and synoviocytes showed up-regulated expression over NCs (44.5-fold, 46.1-fold and 47.1-fold, respectively). However, no information about the transcriptional genes (SOX9 and RUNX2) can be taken from the microarray data.

Heat Maps and Hierarchical Cluster Analysis

Utilizing the genes whose fold change are five or larger (either +5-fold or -5-fold) between NCs and CPCs, a whole gene heat map and several annotated heat maps were created for all four cell types aiming to distinguish cell types.

Considering the gene expression pattern of NCs, CPCs, SFCs and synoviocytes, the whole 5-fold based heat map clearly showed that the CPCs are

remarkably different when compared to NCs. Additionally, most gene expression patterns of CPCs are tremendously similar to SFCs and synoviocytes (Figure 3.2).

Five annotated heat maps (collagen related, cytokine related, extracellular related, inflammatory related and metalloendopeptidase related) were generated based on the genes that are functionally filtered by the annotations of the gene database (Figure 3.3). Almost all the five heat maps exhibited data that suggests that the CPCs as a distinct cell type, apart from NCs, despite the fact in some heat maps some noisy genes did exist. This data thus influences the clarity of the distinction among the four cell types to some extent.

Strikingly, the hierarchical clustering analysis straightforwardly resulted in two main groups, classified as NCs and the other combined cell types. In addition, the length of the line path on the top is indicative of how related or unrelated the cell type is to any other cell type. In general, all of these elements indicate that NCs possess disparate properties from CPCs, SFCs and synoviocytes.

3D Principal Component Analysis (PCA) Plot

A 3D PCA plot was generated, based on all the genes detected by the bovine genome chip, to demonstrate the relatedness level of different cell types in a more visual way (Figure 3.4). Synoviocytes and SFCs are tightly distributed to each other showing highest relatedness among all the cell types. CPCs are fairly much closer to synoviocytes and SFCs rather than NCs, suggesting CPCs are more like synoviocytes and SFCs genetically. Due to the existence of inevitable noises, the three trials of NC are not as close as expected to each other.

Quantitative Real-time PCR Validation of Microarray Results

qPCR was performed and analyzed in order to validate the expression profiles determined by microarray analysis.

Matrix Forming Gene Expression

Gene expression analysis exhibited dramatically lower expression of matrix forming marker genes in CPCs, synoviocytes, and SFCs compared to NCs, while CPCs had relatively up-regulated expression over synoviocytes and SFCs in term of matrix forming marker genes. qPCR results showed that Collagen II expression in NCs was 26-fold over synoviocytes, 34-fold over SFCs and 20-fold over CPCs. Aggrecan expression in NCs was 368-fold over synoviocytes, 129-fold over SFCs and 3-fold over CPCs. Link Protein expression in NCs was more than 10000-fold change over synoviocytes, 215-fold over SFCs and 14-fold over CPCs. COMP expression in NC was 12-fold over synoviocytes, 25-fold over SFCs and 138-fold over CPCs. It should be noted that Collagen II in CPCs was 1.3-fold over synoviocytes and 1.7-fold over SFCs. Aggrecan expression in CPCs was 119-fold over synoviocytes and 42-fold over SFCs. Link Protein expression in CPCs was approximately 1000-fold over synoviocytes and 15-fold over SFCs (Figure 3.5). As for the fibroblastic marker gene Collagen I, NCs were down-regulated compared to synoviocytes, SFCs, and CPCs (0.19-fold, 0.32-fold and 0.41-fold, respectively). In CPCs, howeer, Collagen I expression was 0.45-fold over synoviocytes and 0.8-fold over SFCs. For the transcriptional marker genes, SOX9 expression showed similar gene expression trend when compared to the matrix forming marker genes. NCs were 7-fold over synoviocytes, 2.6-fold over

SFCs, and there was no significant difference between NCs and CPCs. Similarly, SOX9 expression in CPCs was 6.2-fold over synoviocytes and 2.3-fold over SFCs. RUNX2's expression trend was comparable with Collagen I (Figure 3.7).

Inflammatory Related Gene Expression

qPCR results showed the inflammatory gene expression in NCs are down-regulated compared to the CPCs, SFCs and synoviocytes. These results are fairly consistent with microarray data, except for the expression of IL6. IL6 expression in NCs was 6.36-fold over synoviocytes, 2.05-fold over SFCs and 2.86-fold over CPCs. IL8 expression in NCs was dramatically down-regulated, 0.0085-fold of synoviocytes, 0.0005-fold of SFCs and 0.122-fold of CPCs. CCL2 expression in NCs was 0.012-fold of synoviocytes, 0.003-fold of SFCs and 0.402-fold of CPCs. CXCL12 expression in NCs was 0.21-fold of synoviocytes, 0.835-fold of SFCs and 0.69-fold of CPCs. Meanwhile, IL6 expression in CPCs was 2.23-fold over synoviocytes and 0.72-fold of SFCs. IL8 expression in CPCs was 0.07-fold of synoviocytes, 0.005-fold of SFCs. CCL2 expression in CPCs was 0.03-fold of synoviocytes and 0.008-fold of SFCs. CXCL12 expression in CPCs was 0.3-fold of synoviocytes and 1.21-fold over SFCs (Figure 3.6).

Sulfated Glycosaminoglycan (GAG) Assay

The GAG assay was examining the quantified glycosaminoglycan deposition in each cell type. The DNA contents were employed to be normalized to eliminate the influence of cell pellets difference (size, cell number, etc.).

The sGAG/DNA (μg sGAG/μg DNA) level in NCs is around 0.34 units, and 0.21 units in CPCs. A significant decrease (37.4%) did exist in the

comparison between CPCs and NCs ($p<0.05$). As expected, the sGAG/DNA level in both SFCs and synoviocytes, 0.13 units and 0.16 units, respectively, were dramatically lower (63.2% and 53.0%, respectively) than NCs ($p<0.001$ in SFCs and $p<0.01$ in synoviocytes). There is no significant difference both in the comparison of the sGAG/DNA between CPCs and SFCs and the comparison between CPCs and synoviocytes. This reveals that the NCs contain the highest glycosaminoglycan contents, while CPCs stay in the same level with SFCs and synoviocytes (Figure 3.8).

Multipotent Differentiation Capacity

CPCs, NCs, synoviocytes and SFCs were cultured in chondrogenic and osteogenic media for 14 days and 21 days, respectively, to evaluate the difference of their multilineage differentiation potential.

After the pellet culture under the chondrogenic condition, the samples were fixed and stained with Safranin-O/fast green. After this staining, the pellet of NCs was completely pinkish, indicating the NCs have the strongest proteoglycan deposition throughout the whole pellet. The pellet of CPCs did show strong pinkish signal as well, except some specific regions, however no significant differences of proteoglycan deposition among CPCs, NCs and SFCs observed. The pellets of synoviocytes showed limited pinkish signal, revealing that the proteoglycan deposition in synoviocytes is an extremely small amount (Figure 3.9).

Osteogenic potential was assessed by Alizarin Red S staining. Each kind of cells did show a strong positive staining when compared to the negative

control in which cells were culture with normal culture media. Staining showed that all the cells cultured in osteogenic differentiation media had high calcium phosphate deposition in their extracellular matrix. Interestingly, there was no significant difference among the experimental groups for these four kinds of cell types (Figure 3.10).

Table 3.1. Primer information for quantitative real-time PCR

Gene	Forward primer 5'-3'	Reverse primer 5'-3'
β-actin	TCGACACCGCAACCAGTTCGC	CATGCCGGAGCCGTTGTCGA
Collagen I	CCCACCCCAGCCGCAAAGAG	GAGCAGGAGCCGGAGGTCCA
Collagen II	AAGACGCAGAGCGCTGCTGG	GGGTCTCTACCGCGCCCTCA
Aggrecan	CAGCCAGGCCACCCTAGAG	GGGTGTAGCGCGTGGAGAT
Link Protein	ACTTCTTCTGGTGCTGATT	CTGTAGGGTCTCGGTAAA
COMP	TTCGGAACGCACTGTGG	TGCAGGAACCAGCGGTA
SOX9	ACGCCGAGCTCAGCAAGA	CACGAACGGCCGCCTCT
RUNX2	CGCACCGACAGCCCCAACTT	CTTGAAGGCCACGGGCAGGG
IL6	GGCTGCTCCTGGTGATGACT	CTCCTTGCTGCTTTCACACTCA
IL8	CCACACCTTTCCACCCCAAA	CCTTGGGGTTTAGGCAGACC
CCL2	TCGCTGCAACATGAAGGTCT	TATAGCAGCAGGCGACTTGG
CXCL12	GCTCCACGTAGAACTGCCAT	AGGGAGAGGCTGGGAATGAT

Table 3.2. Summary of selected gene expression fold change from microarray data

Gene Symbol	Gene Title	p-value (CPC vs. NC)	Fold-Change (CPC vs. NC)	p-value (CPC vs. SFC)	Fold-Change (CPC vs. SFC)	p-value (CPC vs. Synoviocyte)	Fold-Change (CPC vs. Synoviocyte)	p-value (NC vs. SFC)	Fold-Change (NC vs. SFC)	p-value (NC vs. Synoviocyte)	Fold-Change (NC vs. Synoviocyte)
COL2A1	collagen, type II, alpha 1	1.62E-05	-20.536	0.356252	-1.33137	0.432558	-1.27289	1.50E-05	15.4247	1.34E-05	16.1333
ACAN	aggrecan	0.0384056	-5.93773	0.0575955	4.89456	0.0310472	6.57791	0.00102891	29.0626	0.000628423	39.0578
COMP	cartilage oligomeric matrix protein	3.33E-07	-76.9226	0.00835722	2.34509	0.0304477	1.88578	4.47E-08	180.391	6.01E-08	145.059
HAPLN1	hyaluronan and proteoglycan link protein 1	0.366302	-1.83963	0.000127642	120.114	5.50E-05	235.431	2.87E-05	220.965	1.32E-05	433.105
IL6	interleukin 6 (interferon, beta 2)	0.00287415	225.322	0.444957	2.66233	0.156617	6.81856	0.00456514	-84.6332	0.0144052	-33.0454
IL8	interleukin 8	0.106465	9.74559	0.657751	-1.76583	0.736805	-1.53727	0.0360697	-17.209	0.0433658	-14.9816
CCL2	chemokine (C-C motif) ligand 2	0.00277838	32.261	0.622576	-1.48713	0.560955	-1.60079	0.000804649	-47.9762	0.000720209	-51.6431
CXCL12	chemokine (C-X-C motif) ligand 12	0.0133413	12.1079	0.26324	2.51598	0.881543	1.12437	0.0537136	-4.81238	0.00995026	-10.7686
COL1A1	collagen, type I, alpha 1	0.00159777	44.4758	0.962923	-1.03738	0.942041	-1.05908	0.000796027	-46.1383	0.00077123	-47.1035

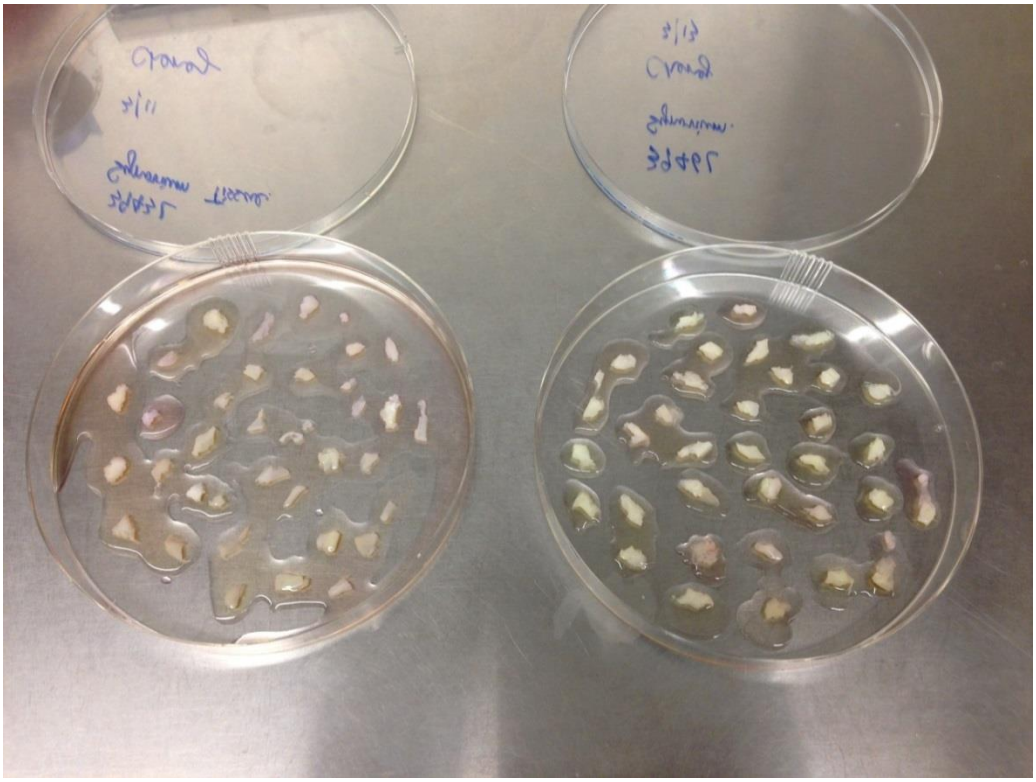

Figure 3.1. Synovium tissue attachment and culture technique.

Dissect and stretch the synovium tissue to its maximal extension. After couple hours of dry attachment, the tissue was replenished with small amount of culture media for the following days to keep the synovium nutritious.

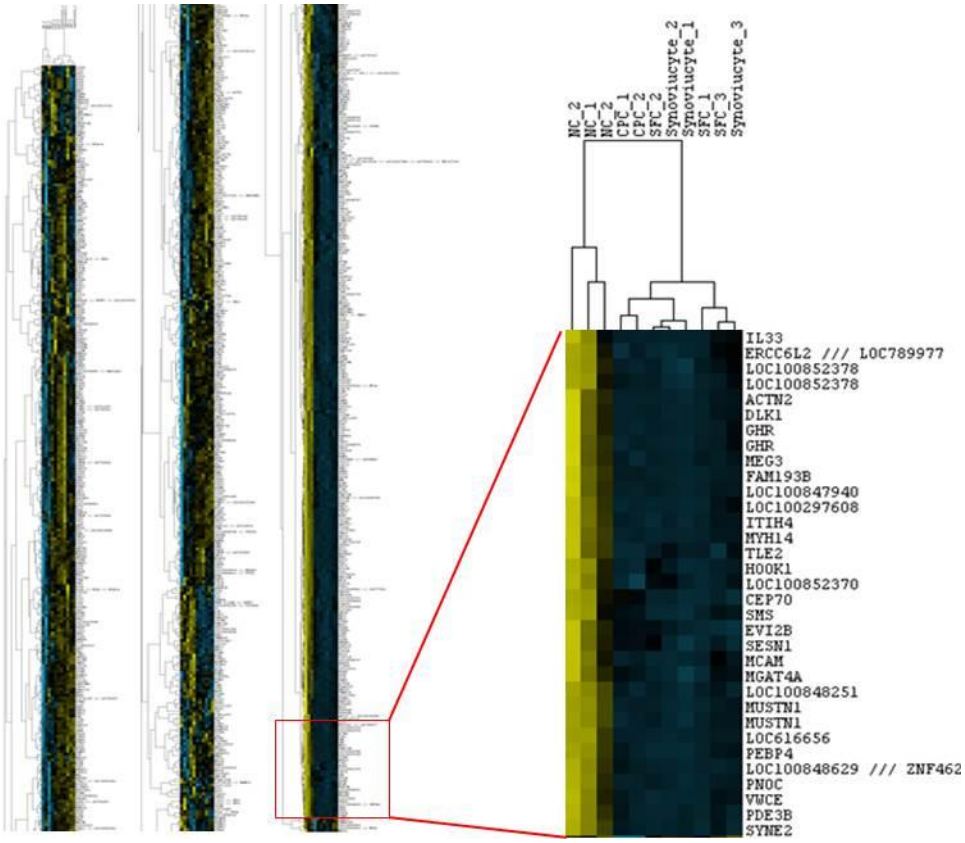

Figure 3.2. Whole heat map showing differences among NCs, CPCs, synoviocytes and SFCs.

The 5-fold (>+5-fold or <-5-fold) change between NCs and CPCs based heat map was generated to reveal the enormous difference between NCs and CPCs as well as to show the similarities among CPCs, synoviocytes and SFCs. The hierarchical cluster analysis showed the similarities among four cell types and directly divided all four cell types into two major categories (NCs and the other combined cell types).

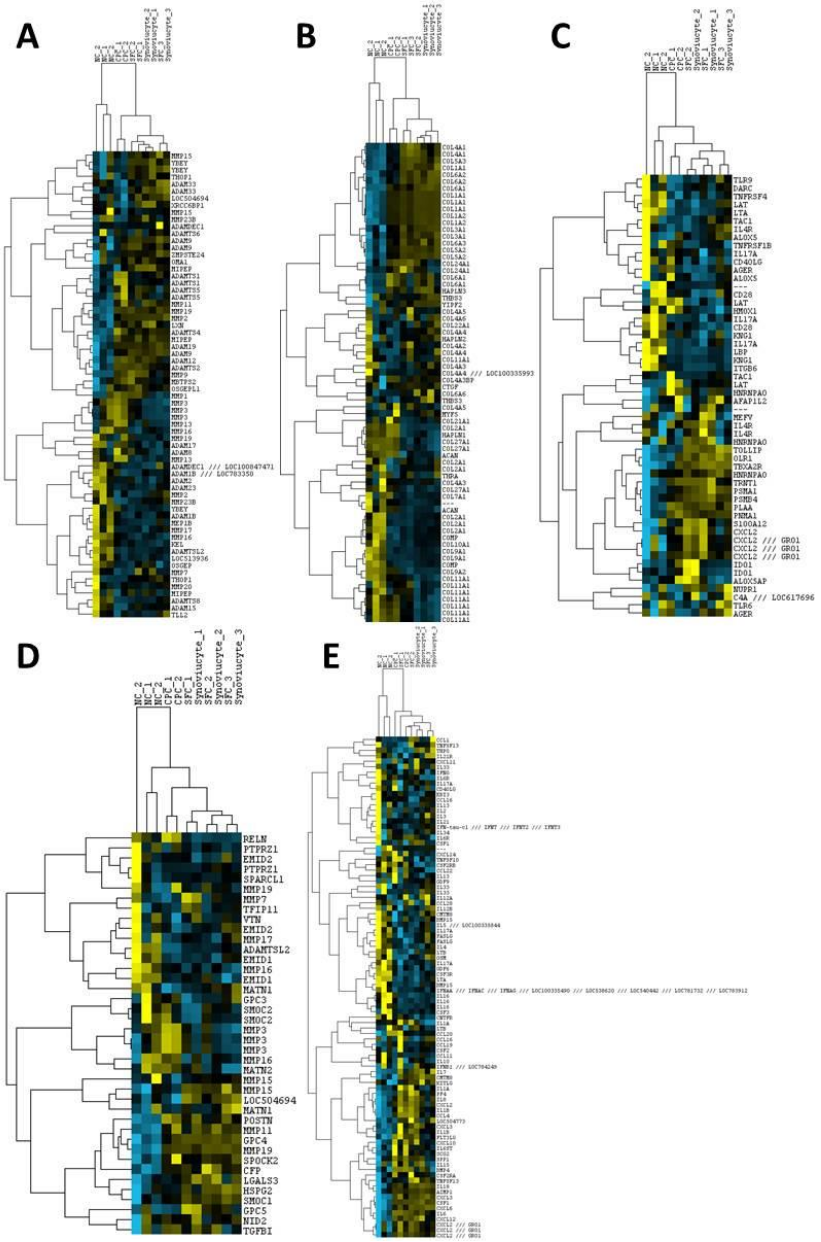

Figure 3.3. Annotated heat maps based on various gene functions.

Five annotated heat maps (**A.** metalloendopeptidase related, **B.** collagen related, **C.** inflammatory related, **D.** extracellular related, **E.** cytokine related related) were generated based on specific gene function, exhibiting the differences among four cell types. These five annotated heat maps are essentially in accord with the entire heat map.

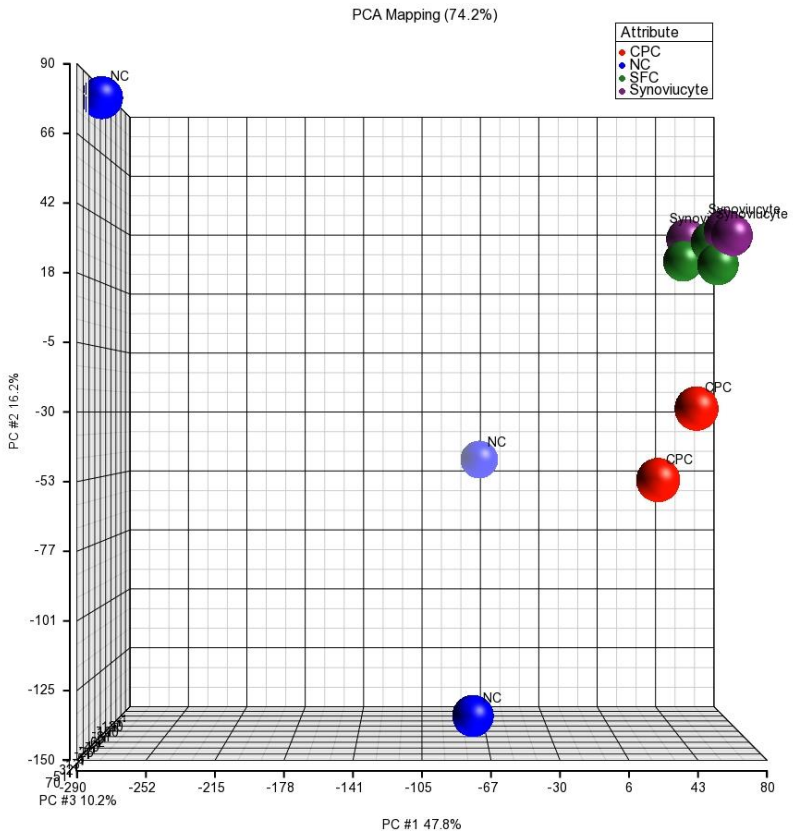

Figure 3.4. 3D PCA plot demonstration.

The 3D PCA plot visually showed the closeness of each cell type. Synoviocytes and SFCs are extremely close to each other, while CPC are much closer to these two cell types than to NCs. Noisy effect exists among the three independent NCs due to the sample variation.

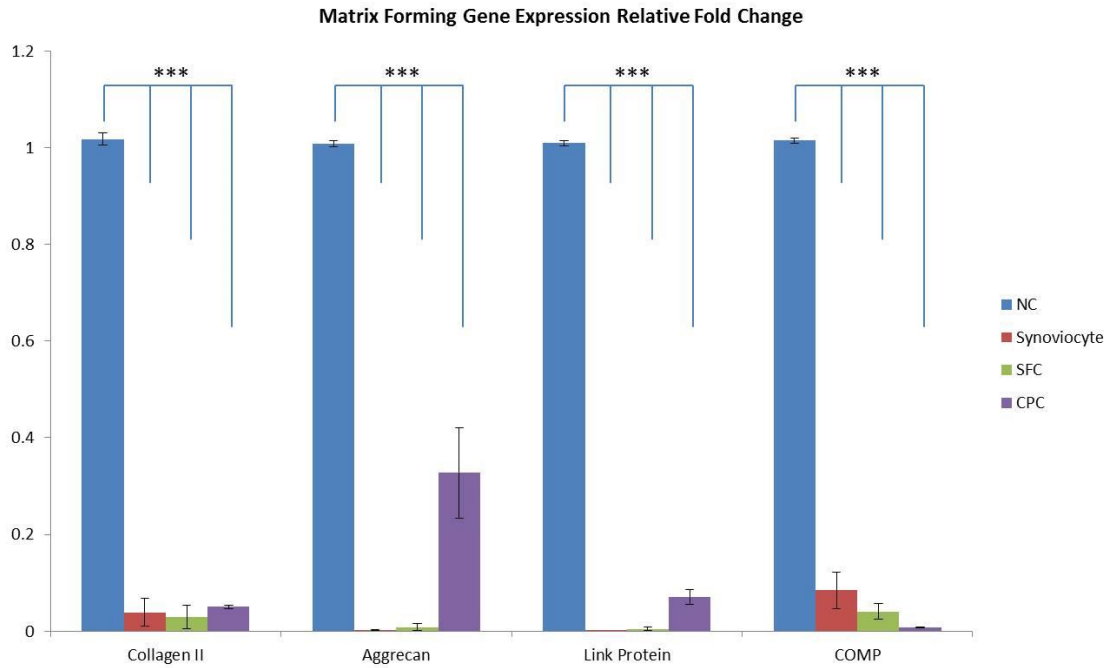

Figure 3.5. Matrix forming gene expression.

qPCR showed dramatically higher expression of all matrix forming genes (Collagen II, Aggrecan, Link protein and COMP) in NCs than in the other three cell types. (*** indicates $p<0.001$)

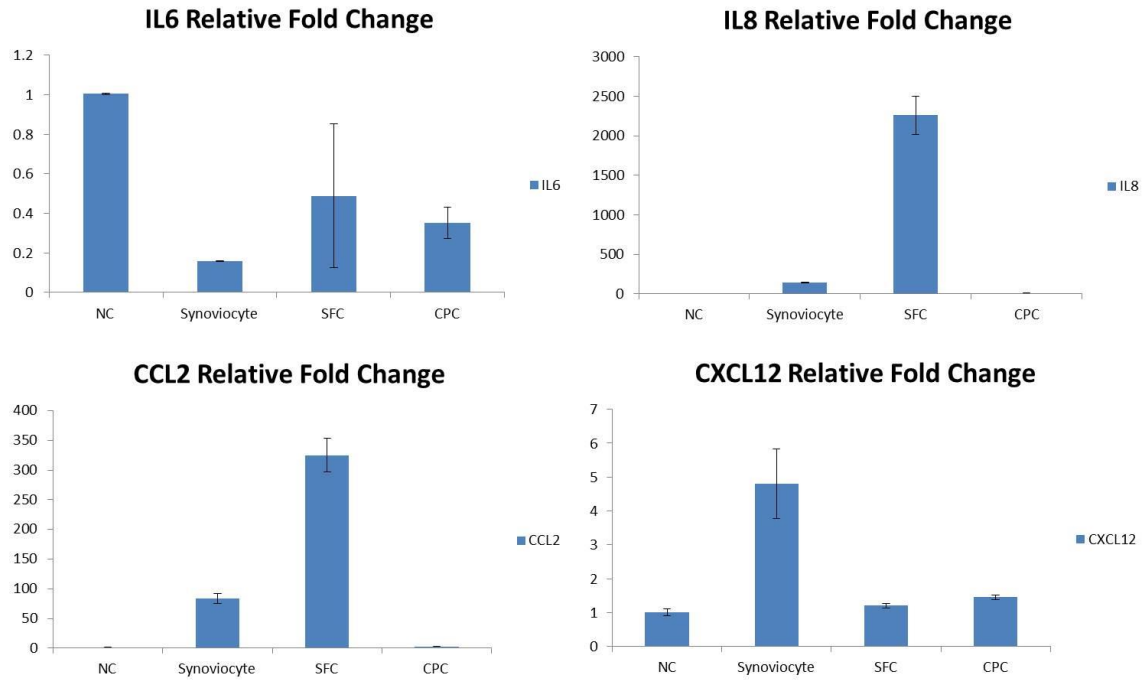

Figure 3.6. Inflammatory related gene expression.

qPCR showed dramatically lower expression of most inflammatory related genes (IL8, CCL2 and CXCL12) in NCs than in the other three cell types. However, it should be noted that IL6 expression in NCs is higher than the other three cell types.

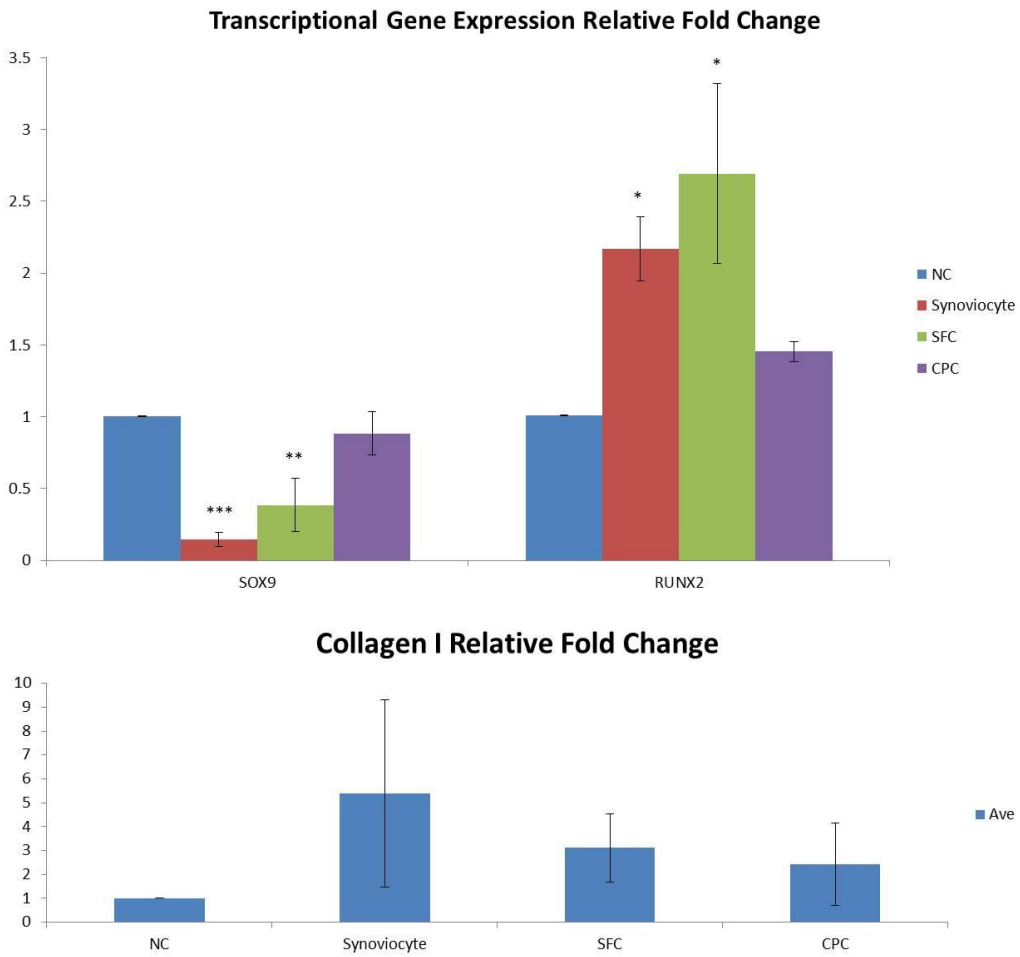

Figure 3.7. Other related gene expression.

SOX9, chondrogenic transcriptional gene, and RUNX2, osteogenic transcriptional gene were tested for in each cell type. NCs showed significantly up-regulated expression over synoviocytes and SFCs and significantly down-regulated expression of synoviocytes and SFCs. No significant differences were found between NCs and CPCs in both two transcriptional genes. The fibroblastic marker Collagen I, showed NCs have the lowest expression when compared to the other three cell types, but no significant differences exist. (*: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$)

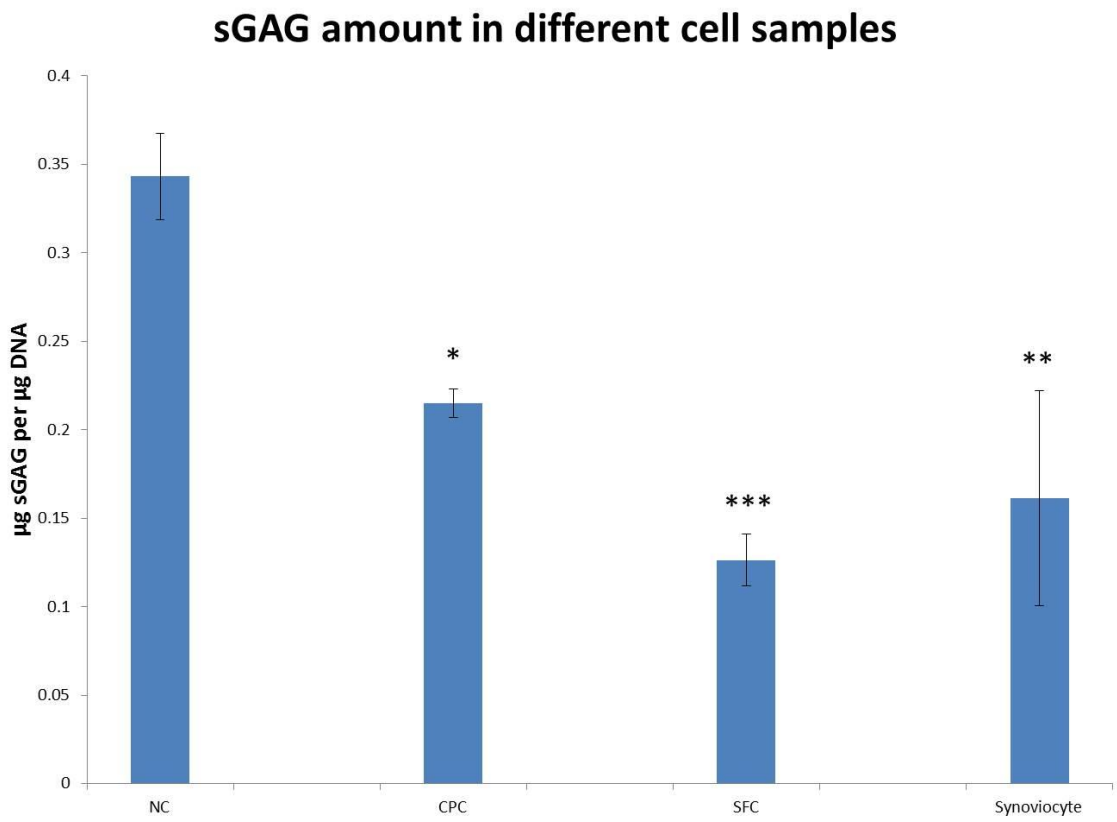

Figure 3.8. sGAG assay of NCs, CPCs, SFCs and synoviocytes.

sGAG assay legibly demonstrated the glycosaminoglycan contents in each cell type, revealing that NCs contain the highest amount, and that significant differences exist when comparing NCs to CPCs, SFCs and synoviocytes, respectively. (*: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$)

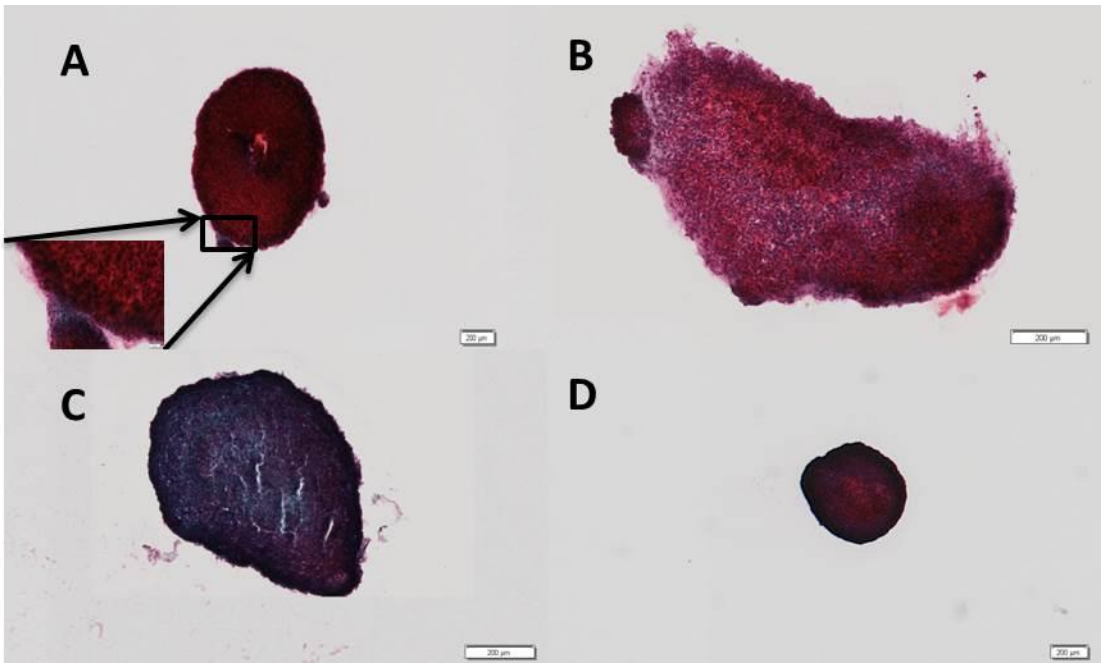

Figure 3.9. Chondrogenic differentiation ability comparison among CPCs, NCs, synoviocytes and SFCs.

No significant differences of proteoglycan deposition among CPCs (A), NCs (B) & SFCs (D) observed. However, CPCs do have relatively less proteoglycan contents in some specific regions. Synoviocytes (C) contain the weakest proteoglycan deposition.

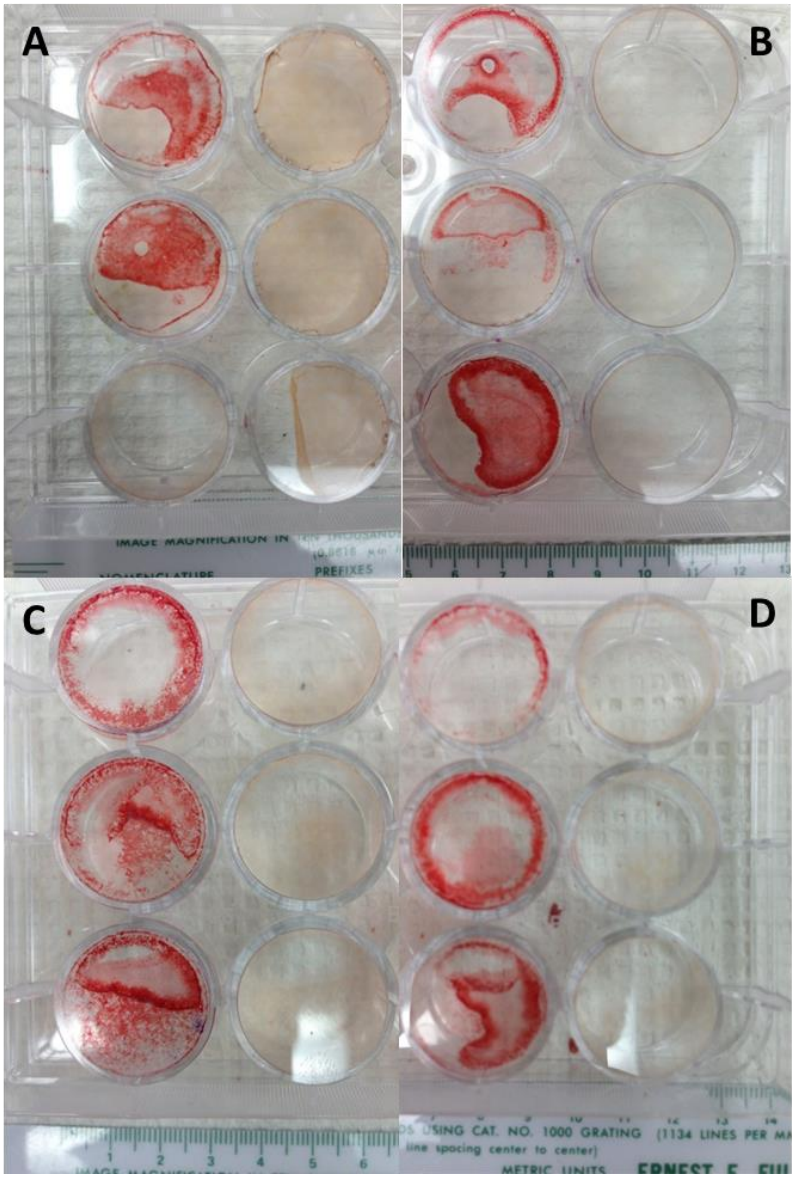

Figure 3.10. Osteogenic differentiation ability comparison among CPCs, NCs, synoviocytes and SFCs.

No significant difference observed among CPCs (**A**), NCs (**B**), synoviocytes (**C**) and SFCs (**D**) despite each cell type having been cultured under osteogenic condition. The osteogenic condition (left column in each sub-figure) did show positive staining when compared with the negative controls which were cultured using normal culture media (right column in each sub-figure).

CHAPTER IV

DISCUSSION AND CONCLUSION

Microarray technology confirmed that CPCs are a distinct cell type, contradicting the universal opinion that all cells residing in cartilage superficial zone are chondrocytes. Heat maps and 3D PCA plot clearly and visually offered us the concept that CPCs are far away from NCs in term of large scale of genes and share substantially large amount of similarities with SFCs and synoviocytes. The hierarchical clustering analysis straightforwardly divided all the cell types into chondrocyte and non-chondrocyte phenotypes. Annotated heat maps conveyed essentially the same idea as the whole heat map but in terms of selected functional classes of genes that are relevant to OA.

The results of qPCR validated the microarray data on ECM-related genes and pro-inflammatory genes. NCs showed significantly higher expression of cartilage-specific ECM genes than the other three cell types. In contrast, they showed lower expression of pro-inflammatory genes than the other three cell types. These two categories of genes are of vital importance in determining the unique cell properties, which turns out to be an efficient and novel approach to classify different cell types. The grouping indicated by overall differences in gene expression and in the expression of specific ECM and inflammatory genes paralleled differences in the expression of SOX9, an important chondrogenic transcriptional factor. Due to the sample variation, the inflammatory related genes in different batches of cow knees were fairly different, and a possible reason for this might have been that osteoarthritis already occurred in the cow

knee joints before harvesting cells, thus resulting in varying gene expression results. Interestingly, IL6 expression in all the batches is higher expression in NCs than CPCs, SFCs and synoviocytes, opposing the result from the microarray data. The reason for this is mainly because the RNA for NCs was extracted directly from cartilage tissue, rather than the monolayer cultured cells, which may have substantial influences on some specific genes. The high expression of these inflammatory related genes results also suggest that CPCs might be closely related with osteoarthritis pathogenesis.

The sGAG assay was applied to confirm the differences among these four cell types from ECM compositional view. The results were fairly consistent with microarray data and qPCR results, revealing NCs contain highest glycosaminoglycan deposition, while CPCs were in the middle between NCs and SFCs, synoviocytes. To test the function of these different cells, we checked the multi-lineage differentiation potential, which is deemed as the milestone for defining stem/progenitor cells. Chondrogenesis assays showed that NCs were superior to CPCs, SFCs and synoviocytes, which might provide some clues in selecting a cell source for restoring injured cartilage tissue. No significant differences existed among these four different cell types in term of osteogenesis.

In conclusion, cartilage is thought to possess poor healing capacity post injury due to its native properties (avascular, aneural, alymphatic). However the chondrogenic progenitor cells (CPCs), which can be activated and migrate onto the cartilage surface by the injury, may provide us a new insight to self-repair the cartilage. Through these series experiments, we validated that these CPCs

with less cartilage matrix gene expression, as well as higher inflammatory gene expression are essentially distinct from normal chondrocytes. Since many intrinsic properties of CPCs are shared with SFCs and synoviocytes, we could thus build the concept that the function of these cells is to prevent the cartilage from further injury through the forming of a protective layer on the very top of the cartilage. According to the function of these cells, a more appropriate name, surface chondro-protective cells (SCCs), might be given to these cells.

Further studies are urgently needed to detail the function of CPCs. Furthermore, since the inflammatory gene expression in CPCs is relatively much higher than NCs, we need to identify whether these properties are beneficial or detrimental for the damaged cartilage and to figure out the activation pathway of the CPCs. Last but not least, the initial location and the origin of CPCs should be identified to better understand the role CPCs play in cartilage maintenance and repair, an *in vivo* model would be the best option for these purposes.

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