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Identification and characterization of cartilage progenitor cells by single cell sorting and cloning

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IDENTIFICATION AND CHARACTERIZATION OF CARTILAGE PROGENITOR CELLS BY SINGLE CELL SORTING AND CLONING

by

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CERTIFICATE OF APPROVAL

MASTER'S THESIS

This is to certify that the Master's thesis of

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has been approved by the Examining Committee
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ABSTRACT

Cartilage lesions are a fairly common problem in orthopaedic practice. However, as an avascular and aneural tissue, articular cartilage has minimal intrinsic healing ability. Although many approaches have been proposed for cartilage repairing, the resulting fibrocartilage, with inferior mechanical and biochemical properties, will ultimately leads to osteoarthritis (OA).

For past few years, many researchers have proposed stem cell transplantation treatment for enhancing cartilage repair by using mesenchymal stem cells (MSCs) along with biocompatible scaffolds. In addition to that, chondrogenic progenitor cells (CPCs) have also been discovered in OA patients and healthy articular cartilage. However, methods for isolating CPCs are not well established and the resulting population is always heterogeneous. Also the origin and function of CPCs are yet still not clear.

Full characterization of stem/progenitor cell potential requires a purified population. Therefore, in this study, a single cell clonogenecity screening system was developed to identify and isolate putative stem/progenitor cells from intact bovine cartilage based on fluorescence-activated single cell sorting (FACS) and consequent clonogenicity screening. Isolated cells were evaluated for progenitor cell characteristics.

By using FACS sorting, we successfully isolated single cell into 96-well plate. Clonogenecity screening confirmed the existence of high efficiency colony-forming cells (HECCs) both from superficial zone and deep zone of articular cartilage, which have characteristic stem/progenitor cell gene

expression and have multi-lineage differentiation ability. In addition, HECCs also can expression inflammation-related gene (CXCL12). Differentiation study also revealed that deep zone progenitor cells have superior chondrogenic, osteogenic potential and relatively higher adipogenic ability than superficial progenitor cells.

The discovery of progenitor cell found in this study may indicate the intrinsic-healing ability of articular cartilage. Different level of differentiation potential of progenitor cells from superficial and deep zone may reveal the different function of them. The upregulation of inflammatory-related gene in progenitor cells may indicate their possible role in progressive joint injuries.

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CHAPTER 1

INTRODUCTION

Stem cells have the considerable potential to differentiate into many different cell types during early life and growth. Also, they serve as a source for internal tissue repair and replenishment. Stem cells have two distinct characteristics: self-renewal and multipotency, which means they can divide essentially without limit and become cells in different tissue with specific functions. Scientists have been working primarily with two types of stem cells: embryonic stem cells and non-embryonic "somatic" or "adult" stem cells. In the year 2006, researchers reprogrammed another new type of stem cell, called induced pluripotent stem cells (iPSCs), with embryonic stem cell characteristics[1].

As for their remarkable regenerative abilities, stem cells therapies have the potential to change how clinicians treat diseases. Many stem cell treatments are being explored. For the past 30 years, bone marrow and more recently, umbilical cord blood stem cells, have been used to treat patients with leukemia and lymphoma. Scientists anticipate that adult and embryonic stem cells will soon be able to treat cancer, Type 1 diabetes mellitus [2], Parkinson's disease [3], Huntington's disease, celiac disease, cardiac failure, muscle damage and neurological disorders [4], and many others. A lot of research is needed to understand how to use stem cells for cell-based therapies to treat diseases safely and efficiently.

Articular cartilage is a unique, hypocellular, and avascular tissue, made mostly of extracellular collagens and proteoglycans; it has a limited ability to self-heal after trauma and degenerative disease [5]. Osteoarthritis (OA) is the most common form of joint disease, affecting all population worldwide. It is the most important cause of joint pain and dysfunction, caused by progressive loss of articular cartilage on the articular surface. Although aging, genetic disorders, and long-term overuse increases the risk of OA initiation, the pathophysiology is poorly understood. Since the late 1980s, the most advanced and promising osteoarthritis treatment has been autologous chondrocyte transplantation (ACT) [6]. Although there are also other treatments like arthroscopic lavage, microfracture surgery,and etc. they only offer inferior tissue regeneration and incompetent functional restoration.

Stem/progenitor cell based therapy has recently been proposed for cartilage tissue repair. Although chondrocytes are thought to be the only cell type in articular cartilage, researchers have identified chondrogenic progenitor cell populations both in healthy and osteoarthritic cartilage [7, 8]. These cells showed high migratory ability, overexpressed stem cell surface markers (CD105, CD166, Notch-1, Etc.), and were able to undergo osteogenic and chondrogenic differentiation, but not adipogenesis [8]. Also, our previous study also found migrating CPCs on the articular surface post traumatic injuries [9].

Despite the evidence that these cells might be involved in cartilage regeneration, no study to date has provided a method for identifying stem/progenitor cells either in healthy or osteoarthritic cartilage with homogeneity in stem cell properties. However, full characterization of

stem/progenitor cells potential requires the generation of genetically identical population from a single stem/progenitor cell. Furthermore, the location where these cells reside in the cartilage matrix is still not clear. Although many studies have shown that these cells migrate from the superficial zone of articular cartilage [10, 11], others have observed massive stem cell marker expression in both the middle and deep zone [8].

In this study, we developed a single cell clonogenicity screening technique to identify and isolate putative stem/progenitor cells in healthy cartilage. We also evaluated the genetic and functional characteristics of these cells. Finally, we compared the functional differences between cells from the superficial zone and deep zone by evaluating their differentiation ability

CHAPTER 2

BACKGROUND

2.1 Basics of Stem Cell

Stem cells are found in many tissue and organs, which have the potential to give rise to many specialized cell types, both in the early period of human life and during development. Also, stem cells are considered to be the internal repair system for many tissues in human body.

2.1.1 Stem cell properties

Three important characteristics make stem cells distinguished from other type of cells: unspecialization, self-renewal capacity and pluripotency (Figure 2.1). The most fundamental property of stem cells is that they are unspecialized, without any specialized functions. Also, stem cells are capable to undergo unlimited cell division cycles without losing their undifferentiated state. In addition, stem cells they can be induced to differentiate into specialized cells types. More strictly, stem cells are either totipotent or pluripotent, while multipotent or unipotent cells are often referred to as progenitor cells. Clonogenicity is another important property of stem cells. Clonogenicity refers to the ability of a single cell to form clones, or colonies. The colony is defined to consist of at least 50 cells [12]. Stem cells are often measured in colony-forming units (CFUs). For example, in vitro expansion of pluripotent stem cells can yield multiple colonies, and each colony represents the progeny of a single pluripotent stem cell. Figure 2.10 shows a colony during embryonic stem cell culture.

Clonogenic assay or colony forming assay is used to determine each individual cell in the whole population for its “unlimited” self-renew ability. For cells from human body, either embryo or somatic tissue, as long as they can exhibit clonogenicity, they are highly likely to be stem/progenitor cells.

2.1.2 Types of stem cells

For decades, scientists have been working primarily with two types of stem cells: embryonic stem cells and “adult” stem cells. Embryonic stem (ES) cells can give rise to all the cell types of human body, while adult stem cells only have limited capacity to produce certain types of cells. Recently, stem cells from umbilical cord blood and placenta have also been discovered, which can differentiate into various blood cell types. In 2006, Shinya Yamanaka [13] successfully generated induced pluripotent stem (iPS) cells, which allow specialized cells to return to their pluripotent state by genetically defined factors. These cells are pluripotent, very similar embryonic stem (ES) cells.

2.1.2.1 Embryonic stem cell

Embryonic stem (ES) cells were first discovered in mouse embryos in 1981 [14], and in 1998 [15], human embryonic stem cells were grown in laboratory. Embryonic stem (ES) cells, as their name indicate, are derived from the epiblast of inner cell mass (ICM) of early stage embryos. Figure 2.2 showed the isolation and in vitro expansion of ESCs. Embryonic stem cells are pluripotent and essentially can replicate indefinitely over time. The pluripotency allows embryonic stem cell to differentiate into diverse cell types within virtually any tissue in human body, like cells in skin, muscle, bone, cartilage, etc.

2.1.2.2 Adult stem cell

Adult stem cells, also called somatic stem cells and germline (giving rise to gametes) stem cells, they can be found in children, as well as adults. [16] These cells reside deeply in a tissue or organ surrounded by tons of differentiated cells, and are primarily involved in replenishing and repairing the tissue or organ of their origin, especially in some organs which need constant turnover, such as skin, blood, and epithelial lining of gut. Nevertheless, adult stem cells have also been found in organ like brain which does not need a constant supply of cells.

Unlike embryonic stem cells, pluripotent adult stem cells are rare but can be found in some tissues like umbilical cord blood.[17] Most adult stem cells are somewhat specialized, and can only differentiate towards restricted lineage (multipotent), like hematopoietic stem cells and mesenchymal stem cells in bone marrow, neural stem cells in the adult brain, etc. Therefore, adult stem cells are usually classified by their tissue of origin. (Figure 2.3).

Attempts to isolate adult stem cells usually results in a heterogeneous population of cells. Firstly, cells can be labeled with a combination of molecular marker to purify and enrich the population. Many of these markers are CD (cluster of differentiation) molecules, like CD43, CD59, CD90, CD109, etc. Secondly, researchers can label cells from certain tissue with markers in vitro, and then transplant them into other animal to check their ability to regenerate their tissue of origin. Most importantly, it must be proven that a single adult stem cell can generate a line of genetically identical cells that have multi-lineage differentiation ability. For human mesenchymal stem cells, in vitro formation of

adipose tissue, cartilage, and bone is a classical standard for checking their multipotency. Other example like bone marrow stem cells can differentiate into hepatocytes, epithelial lining cells of GI tract, which are from endoderm and mesoderm.

2.1.3 Potential application of human stem cells

Human stem cells have been widely used both in research and in clinical medicine. Scientists around the world have been taking advantage of stem cell technology to understand the pathophysiology of diseases, and develop new treatments to cure them. Figure 2.4 illustrates the potential applications of embryonic and tissue-specific adult stem cells.

Human embryonic stem cells are often times be used to study complex processes during human development. Researchers have been working on identifying how undifferentiated stem cells become lineage-specific cells. They found that the "on" and "off" state of certain genes is crucial in this process, which may also play a role in some serious medical conditions, like birth defects and cancer [18].

Another significant application of human stem cells is stem cell-based therapies. Traditionally, organ donation is the primary source for replacing diseased or damaged tissue. However, the availability of organ donation is far less than the demand by patients. Human stem cells, by differentiating into specific cell types, offer an opportunity for producing transplantable tissues or organs to treat diseases like Alzheimer`s disease, stroke, skin lesions, diabetes, myocardial infarction, osteoarthritis, etc.

In recent years, the potential of stem cell for tissue engineering-base therapies have been well-established. In addition, with the development of biomaterial technology, tissue engineering has grown into a convincing field for future regenerative medicine. Stem cells are usually isolated from patients or donor, and then expanded in vitro to obtain sufficient number of cells. These cells are seeded onto scaffold, and cultured in bioreactors. The resulting tissue or organ can be transplanted into patients with tissue damage or organ dysfunction.

2.2 Articular Cartilage

Articular cartilage, also known as hyaline cartilage, is found on articulating surfaces of diarthroidal joints. Due to its relatively simple structure with no blood supply or nerves, articular cartilage has notoriously poor healing ability post injury or disease.

2.2.1 Articular cartilage anatomy

Figure 2.5 shows the location of articular cartilage in the knee joint: the end of femur, the top of tibia, and the back of patella.

2.2.2 Articular cartilage function

Articular cartilage is responsible for resisting compressive stress and enables a proper distribution of mechanical loading on the subchondral bone. Another important function of articular cartilage is lubrication of the joint. Lubricants such as proteoglycan 4 (PRG4) reduces friction between contacting surfaces, thus minimizing wear and tear to the joint.

2.2.3 Structure of articular cartilage

In terms of organization of collagen fibers, articular cartilage can be subdivided into four different zones horizontally, which have their particular biomechanical properties individually: the superficial tangential zone, middle transitional zone, deep radial zone, and calcified cartilage zone (Figure 2.6). Each zone has different collagen alignment and proteoglycan density, which contribute to their difference in metabolic activity [19].

2.2.3.1 Superficial zone

The superficial zone takes up 10-20% of the total cartilage thickness, which makes it the thinnest layer of articular cartilage. It contains densely packed collagen fibers, about 85% of dry weight [20]. Superficial zone has two distinct layers of collagen fibers with different alignment. The most superficial layer known as the lamina splendens, which is made of unique interwoven collagen bundles oriented parallel with each other and to the joint surface. This gives this zone unique mechanical properties [21] [22] to resist shear stresses. The other layer has collagen fibers aligned perpendicular to the articulating surface, which makes the superficial zone be able to resist compressive stress. This unique organization of superficial zone offers the tissue better function in terms of joint protection.

2.2.3.2 Middle zone

The middle zone contains 40-60% of total cartilage thickness. It mainly contains spherical chondrocytes surrounded by extracellular matrix (ECM). This zone has thicker collagen bundles, which is randomly oriented in the ECM. Cell density is relatively low in this zone, but proteoglycan content is higher

compared with superficial zone. This special organization makes middle zone be able to resist compressive stress.

2.2.3.3 Deep zone

The deep zone takes up 30% of total cartilage thickness. The cellularity of this zone is the lowest, with chondrocytes arranged primarily in columns. This zone contains the thickest collagen fibers, which oriented perpendicular to the articulating surface. The amount of proteoglycan is highest, but water content is the lowest among all layers. This particular structure offers deep zone great resistance to compressive forces.

2.2.3.4 Calcified zone

The calcified zone is often known as the articular end plate, which lies between articular cartilage and subchondral bone. It contains spherical chondrocytes reside in uncalcified niche without proteoglycans. Calcified zone has the thickest collagen bundles oriented perpendicular to the joint surface. During joint articulating, dynamic forces can be transmitted through the calcified zone to the subchondral bone [23].

2.2.3.5 Tidemark

The tidemark is a basophilic line between calcified and uncalcified cartilage, separating hyaline cartilage from subchondral bone. It plays significant role in transmitting mechanical forces along the chondro-osseous junction to the subchondral bone [24]. In osteoarthritis, the tidemark is usually significantly damage and penetrated by pathological formed vessels [25].

2.2.4 Chondrocyte

Chondrocytes are derived from mesenchymal stem cells (mesoderm origin), and are the only cell type in articular cartilage, which are terminally differentiated cells, making up of 1-5% total cartilage volume [26]. They reside in extracellular cartilaginous matrix, consisting primarily of collagen, water, proteoglycans and some noncollagenous proteins. They usually grow in lacuna, scattered individually throughout cartilage matrix (Figure 2.7). Chondrocytes usually have rounded shape, but morphology varies in different layers, and could change during development, aging, and pathological process [27]. Chondrocytes have an anaerobic metabolism pattern, and absorb their nutrition through simple diffusion from synovial fluid. Chondrocytes can produce extracellular matrix, like collagens, proteoglycans, and some noncollagenous proteins [26]

Chondrocytes are not evenly distributed throughout cartilage matrix, the density decreases with aging and some pathological states [28]. Chondrocyte number also various in different layer of cartilage, for example, cell density in superficial zone is significantly lower than that in deep zone. In addition, cell distribution also has an area-dependent pattern: cell density is higher in non-load bearing area than in load bearing area.

2.3 Osteoarthritis

Various conditions can cause articular cartilage injuries, examples like trauma from accidents, progressive degeneration by wear and tear, and immobilization for prolonged time are common causes. Although chondrocytes are known to have the ability to repair cartilage lesions, this is highly dependent on the extent and location of the injury.

Osteoarthritis (OA), also known as degenerative joint diseases, is a group of mechanical abnormalities involving degradation of joints, which damages articular cartilage and subchondral bone [29]. It is characterized by joint pain, tenderness, stiffness; and progressively, joint contracture, muscle atrophy and limb deformity [30]. OA characterized by progressive loss of structural integrity and following by attempted repair, remodeling and sclerosis of subchondral bone, and osteophyte formation [31] (Figure 2.8). Although aging and excessive over-use are considered predisposing factors, the pathophysiology of joint degeneration leading to OA still remains poorly understood [30]. Currently, no medical treatment can offer fully restoration of disease state, or satisfactory pain relief [32]. For this reason, and its high frequency and chronic nature, OA is a substantial economic burden for patients and health care system [33].

2.3.1 Diagnosis

Most common first sign of osteoarthritis is joint pain, and decrease range of motion (ROM). Progressively, patients may notice joint enlargement, deformity, or muscle atrophy, etc. Physicians can diagnose OA based on patients` history and clinical evidences. Imaging techniques like X-rays, CT scan, MRI has been used for diagnose.

2.3.2 Classification

OA can be classified into two categories: primary osteoarthritis, and secondary osteoarthritis [33]. Primary OA, the most common type, can happen without any known causes, while secondary OA, a less frequent type, develops as

a consequence of injuries, hereditary diseases, metabolic disorders, and systemic diseases like obesity and diabetes.

2.3.3 Predisposing factors

Genetic predisposition, obesity, female gender, greater bone density, excessive mechanical loading, and repetitive joint overuse contribute to the risk for OA [34]. The most important risk factor for primary OA is age. Aging also tends tend to increase the risk for post-traumatic OA (PTOA). Studies have shown that patients older than 50 years have twofold to fourfold greater risk to develop OA than younger patients [35].

2.3.4 Post OA Function restoration

Surgeons and researchers have been seeking ways to restore joint function loss of articular cartilage in OA patients for decades. Traditional methods like penetration of subchondral bone, osteotomy, soft tissue grafts have been used in past 50 years. Recently, tissue engineering approaches have gained popularity. Transplantation of stem cells with biocompatible scaffolds, together with growth factors, has been studied and used in clinical trials. However, all of these treatments have their limitations, which make a complete regeneration of articular cartilage and cure of OA an unfulfilled goal.

2.4 Flow cytometric cell sorting

Flow cytometry have become a widespread and important method for biomedical research. The main function of flow sorter is to retrieve cells of interest from a heterogeneous population. Flow cytometry is an extremely effective tool, which offers measurement of physical and biochemical properties

of a single cell passing through a light source. If a single cell or particle can be distinguished by its physical or biochemical characteristics, it can be isolated by flow sorter.

2.4.1 Applications

Flow cytometry can be used in many situations, in which a heterogeneous population is under investigation, and a unique population is needed to be isolated for further studies. Flow cytometry is very powerful due to its multiparametric analysis ability, which allows identification of highly specific populations. More importantly, it is not only able to identify phenotypic characteristics by antibody-antigen interaction; DNA content (15), RNA content (16), or even specific function can be detected, like electron efflux ability (17), or different cell status like apoptosis and cell death (18).

A lot of research based on isolation of subpopulations by flow sorting under aseptic conditions for expansion in culture (19). Expanded cells have been used in functional assays or for cell transplantation in experimental animals (21) and in or human patients for disease treatment (22). Although the primary application of flow sorting is for mammalian cells, it has also been used for sorting yeast, bacteria, and phytoplankton (28). Moreover, for some researchers who need subcellular organelles such as chromosomes (30), flow sorter can make it possible. In addition, flow cytometirc sorting has also been used in sorting specific subpopulation for microarray analysis (34). It also plays an important role in single cell sorting into individual wells of culture plate for cell cloning (35) and subsequent gene expression studies.

2.4.2 General principles

In general, flow cytometry uses a principle involving the electrostatic deflection of charged droplets similar to that used in ink-jet printers (15). Cells are aspirated from a reservoir, forming a liquid stream, passing through one by one, and hydrodynamically focused by a beam of light (usually laser light) of a single wavelength. Various detectors are aimed at the point where the stream passes: one is parallel to light beam (Forward Scatter of FSC), some others are perpendicular to it (Side Scatter or SSC), and one or several fluorescence detectors. When the liquid steam passes through, scattered light and fluorescence signals are generated, picked up and measured by the detectors.

2.4.3 Fluorescence-activated cell sorting (FACS)

FACS is a special type of flow cytometry, which offers sorting of a heterogeneous cell population into two or more group of cells of interest, by different light scattering and fluorescent characteristics of each single cell. It is very fast, providing an objective and quantitative recording of fluorescent signals from single cell as well as effective isolating of these cells. Figure 2.9 illustrated the mechanism of FACS.

2.4.4 Single cell sorting and cloning

FACS can be used to isolate single cell, and plate individual cell into culture plate for in vitro expansion, which makes it powerful tool in cancer research, immunology, and stem cell science. FACS-base semi-automated single cell sorting has been used in human embryonic stem cell (hESC) purification and

monoclonal expansion [36]. Scientists in cancer research also take advantage of this technique to screen for cancer precursor cells [37].

2.5 Chondrogenic progenitor cells (CPCs) in OA

2.5.1 Chondrogenic progenitor cells (CPCs)

Only a few studies to date described the discovery and characterization of progenitor cells in articular cartilage. Researchers have identified mesenchymal progenitor cell markers from healthy human articular cartilage, as well as in osteoarthritis patients. Hattori et al. [10] has found progenitor/stem cell populations present in the superficial zone of bovine cartilage, by side population (SP) assay. These cells are able to differentiate towards superficial zone articular chondrocytes. Lotz et al. [38] demonstrate surprisingly high number of cells that express putative progenitor cell markers in human cartilage, which is not consistent with SP study. This result may suggest cartilage may contain a very high proportion of progenitor cells due to its avascular quality.

Researchers also discovered stem/progenitor cells in osteoarthritis patients` articular cartilage. Koelling et al. [39] described migratory chondrogenic progenitor cells from repair tissue during late stages of human osteoarthritis. These cells migrating across the tidemark into cartilage tissue may represent a mesenchymal progenitor involved in the regeneration efforts during OA [39]. Koelling also demonstrated sex differences of chondrogenic progenitor cells in late stages of OA, finding that physiologic concentrations of testosterone in men and premenopausal concentrations of estrogen in women may be beneficial on the chondrogenic potential of CPCs in vitro.

2.5.2 CPCs-base cartilage regeneration for OA

Although articular cartilage has limited capability of physiological repairing in OA, the discovery of CPCs, shade light for future therapy options. In OA defects, although superficial zone is damaged, migrating progenitor cells were discovered acrossing the tidemark into cartilage tissue [39]. This may represents a future for articular cartilage regeneration post OA.

For decades, adult stem cells-base therapies have been investigated and developed towards various diseases. This may not only avoid the ethic issue but also reduce the risk of tumor formation, which is a common side effect of embryonic stem cell treatment [40]. For OA treatment, mesenchymal stem cells (MSCs) play a significant role in regenerative strategies. Since MSCs and the skeletal-muscular system has the same origin of mesoderm. Recent studies showed that progenitor cells in OA patients may play a role for cartilage regeneration when recruited to injury site [41]. Future studies may focus on how to accelerate the migration of CPCs towards injury and how to improve the chondrogenic potential of CPCs.

Table 2.1. Differences between Adult stem cells and Embryonic stem cells

Human Adult Stem Cells	Human Embryonic Stem Cells
Stem Cells are hard to access & purify	Once isolated, the cells show high degree of proliferation
Mostly multipotent with MSCs acting as pluripotent	Pluripotent
Telomerase levels low	Telomerase levels high
Chromosomes tend to shorten with ageing	Chromosome length is maintained across serial passage
Apoptosis may be early	Apoptosis is late
No Teratoma risk	Significant Teratoma risk
No ethical issues	Serious ethical issues
Patient-specific hence less chances of immune rejection	High chance of immune rejection

(A.D. Garg: Stem Cell Therapeutics: Exploring Newer Alternatives to Human Embryonic Stem Cells. *The Internet Journal of Health*. 2008 Volume 8 Number 1)

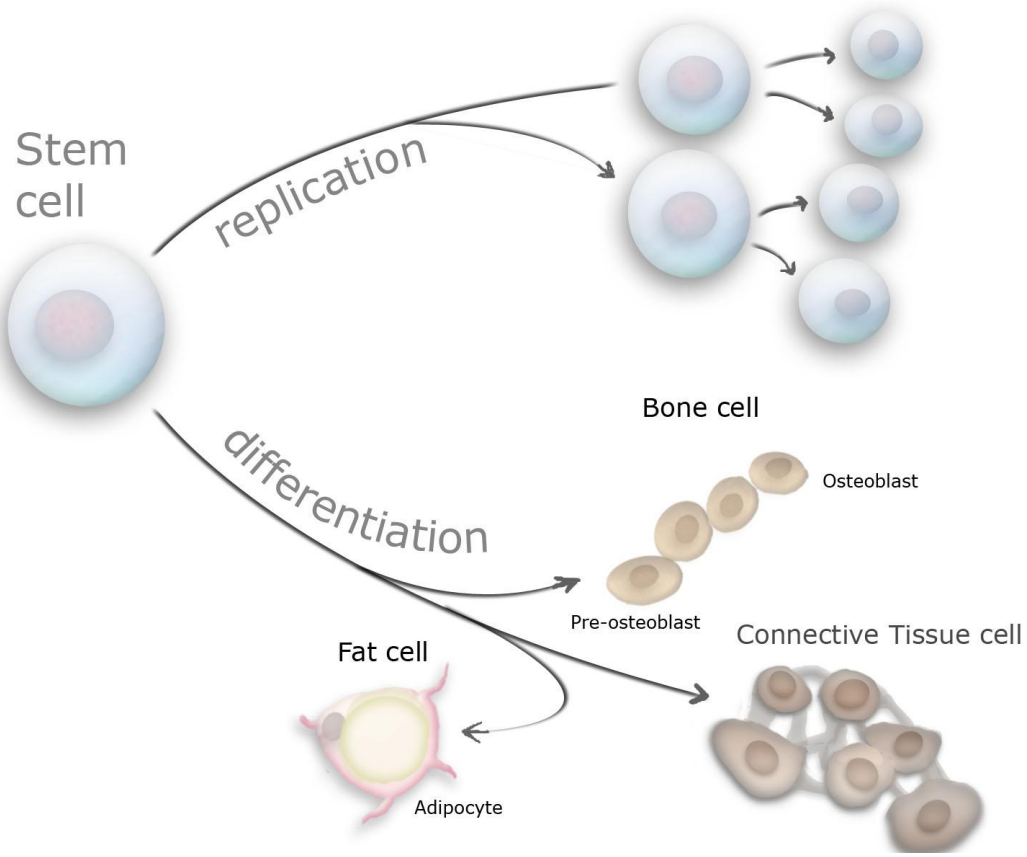


Figure 2.1. What is stem cell? Two main characteristics defines stem cell: it`s self-renewable and its potency.

Source: http://www.purstem.eu/cmsms/uploads/images/fionn/stem%20cellsdraft4.jpg

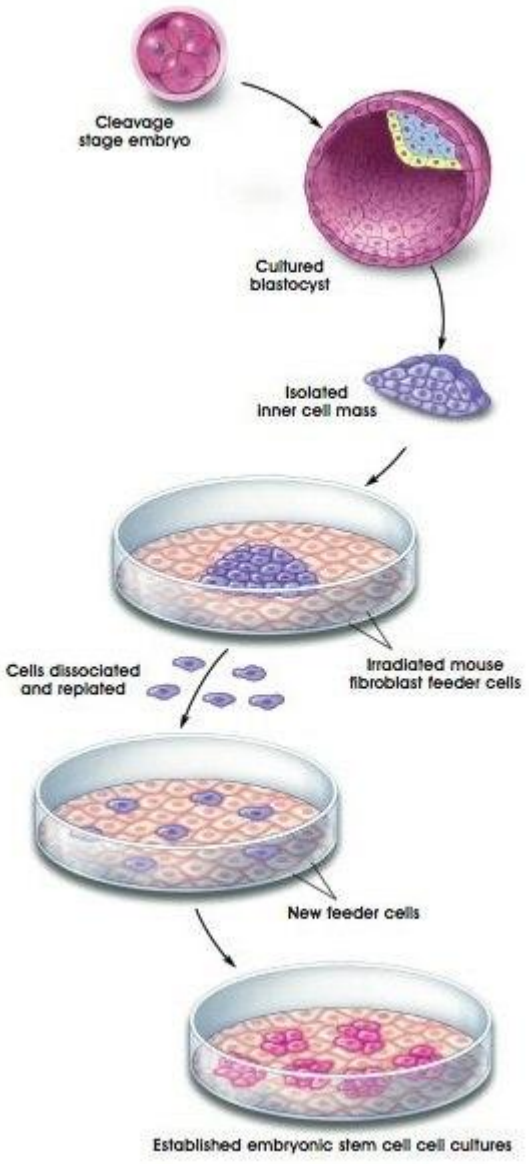


Figure 2.2. Embryonic stem cell. Techniques commonly used for generating embryonic stem cell cultures.

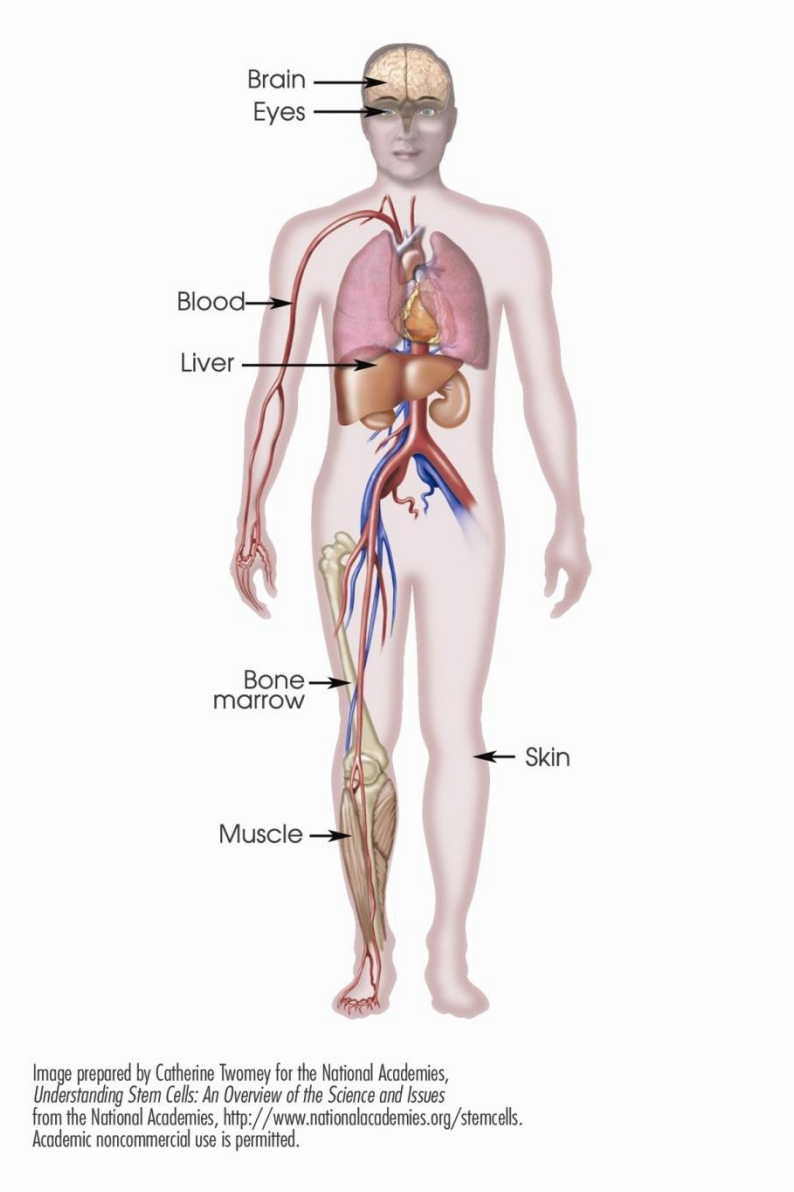


Figure 2.3. Adult stem cell. Sources for adult stem cell derivation, from many organ and tissue in human body

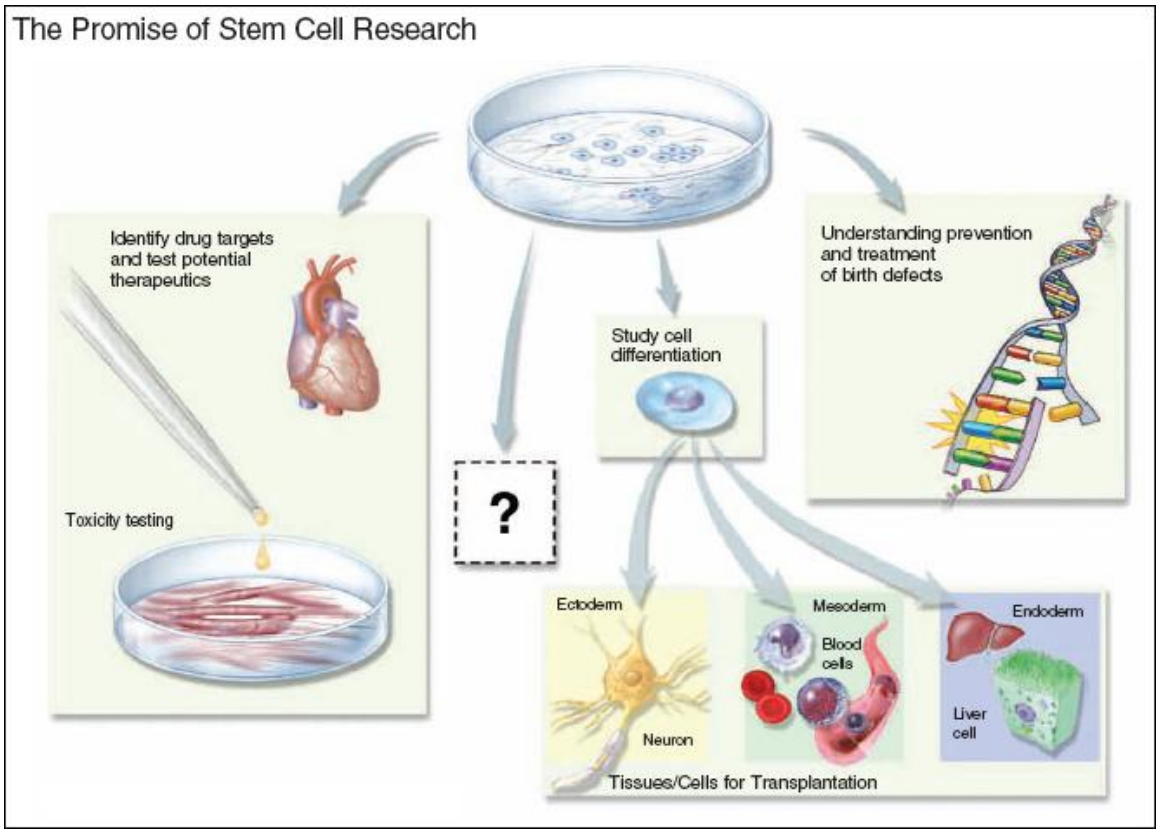


Figure 2.4. Potential applications of stem cells. This scheme illustrated potential application of embryonic and somatic stem cells in cell-base treatments and gene therapies [42]

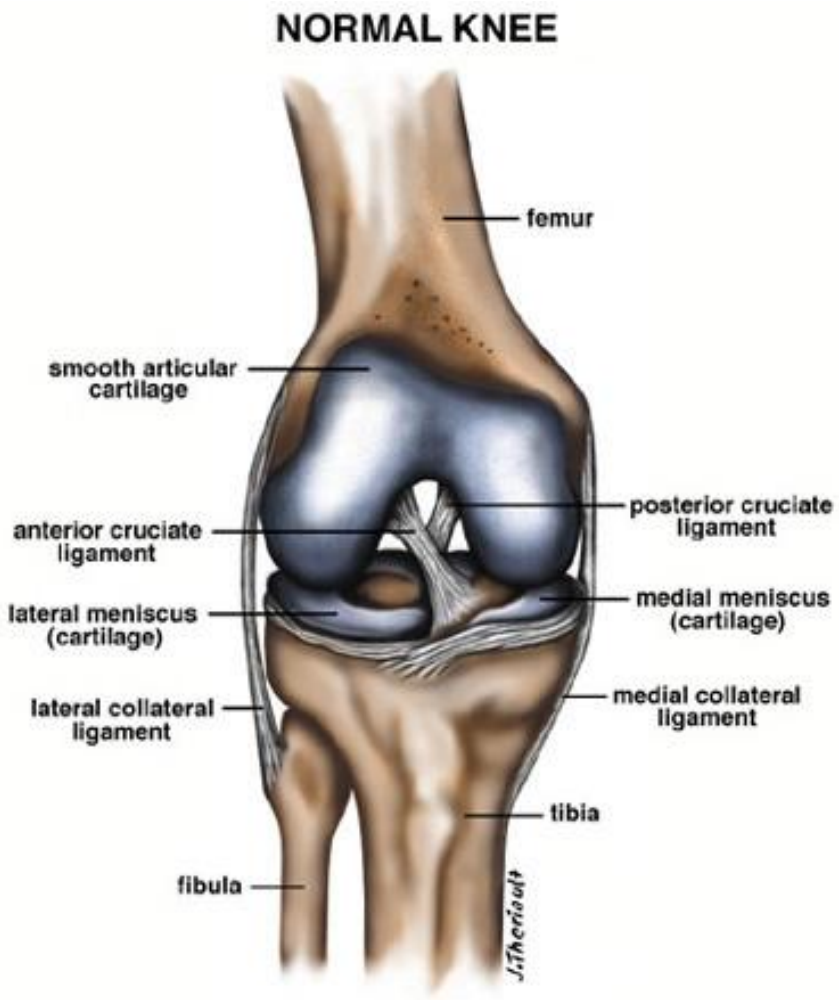


Figure 2.5. Anatomy of articular cartilage. Image shows the location of articular cartilage in the knee joint: the end of femur, the top of tibia, and the back of patella.

Source: http://www.pinnacle-ortho.com/what_hurts_specific.php?id=11

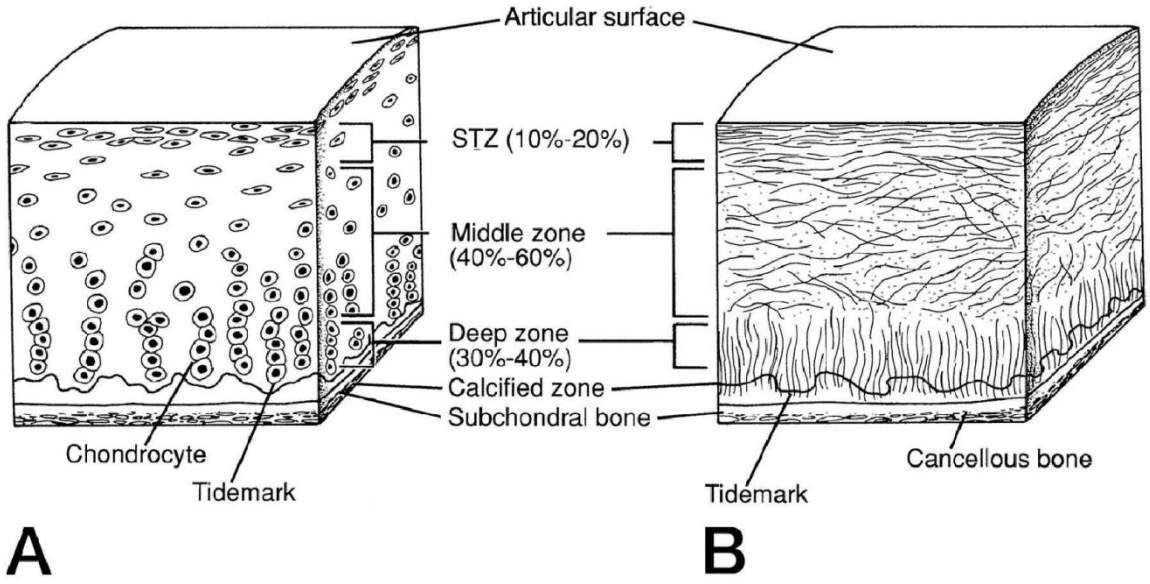


Figure 2.6. Structure of articular cartilage. Articular cartilage can be mainly divided into superficial zone, middle zone, and deep zone.

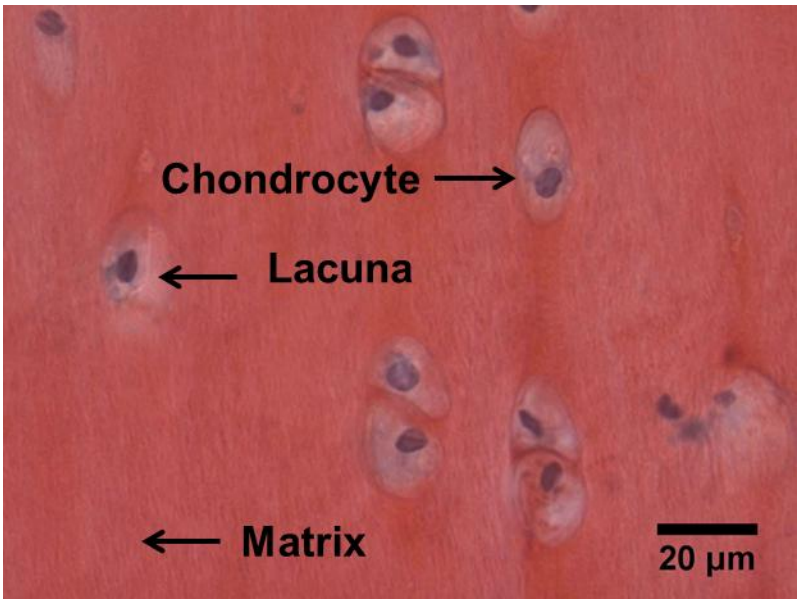


Figure 2.7. Chondrocyte. Chondrocytes reside in lacunae throughout the articular cartilage matrix.

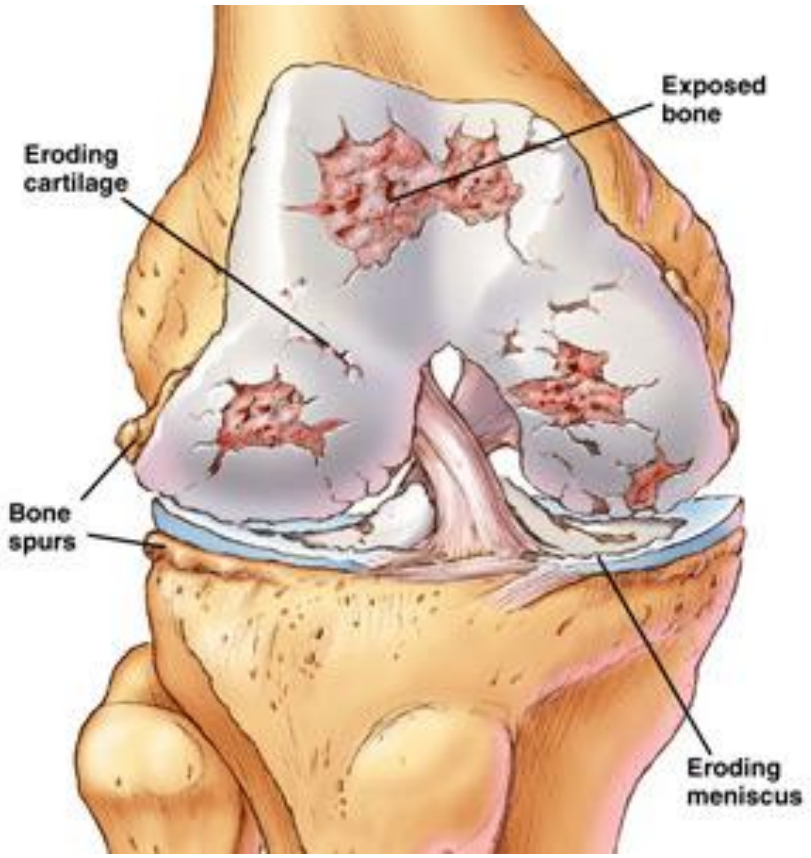


Figure 2.8. Osteoarthritis. Cartilage destruction is commonly seen in osteoarthritis. Exposed bone contact directly, causing joint dysfunction and deformity.

Source: http://www.osteoarthritisblog.com

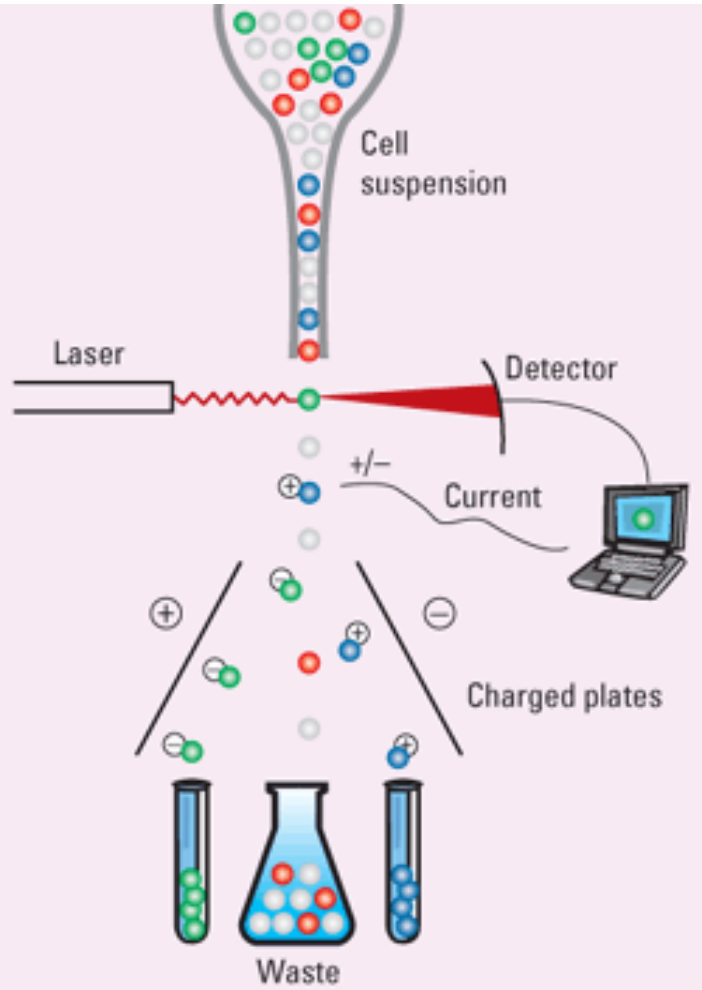


Figure 2.9. Mechanism of FACS. A scheme shows fluorescence-activated cell sorting.

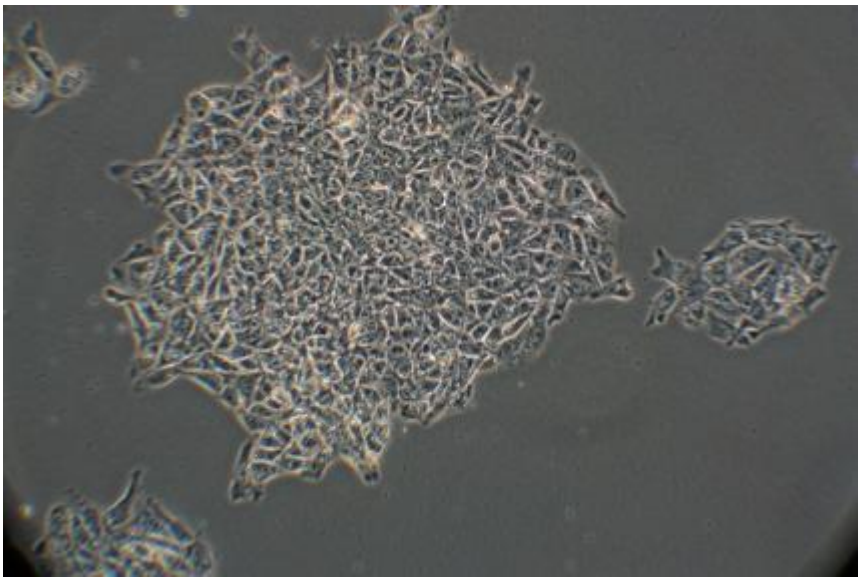


Figure 2.10. Stem cell colony. Image shows a colony formed by embryonic stem cells in gelatin-coated culture dish.

Source: http://www.primorigen.com/stemadhere

CHAPTER 3

IDENTIFICATION AND CHARACTERIZATION OF STEM/PROGENITOR CELLS BY SINGLE CELL SORTING AND CLONING

3.1 Purpose of study

Currently, only a few studies demonstrated the discovery of progenitor cells in healthy and OA cartilage. However, most of the isolated cells are actually a heterogeneous population with limited multi-lineage differentiation ability. Some researchers stated that progenitor cells only reside in the superficial zone [10], but failed to show the multipotency of these cells. Chondrogenic progenitor cells from late stage human osteoarthritis do exhibit stem cell characteristics, like clonogenicity, multipotency, and migratory activity [39]. Although various markers have been used in different studies of identify and isolate progenitor cells from cartilage, no study has shown that the resulting population is homogeneous. Nevertheless, fully characterization of stem/progenitor cells requires purified cell subpopulation. Otherwise, the phenotypic "stemness" may actually result from a mixture of different cells.

In this chapter, a simple single cell sorting technique by fluorescent-activated cell sorting (FACS) will be performed to isolate and plate single cell in 96-well culture plate. Also, clonogenicity screening technique will be established to identify putative cartilage stem/progenitor cell population in healthy bovine cartilage. A culture system is developed for maintain "stemness" of progenitor

cells in vitro. In addition, characterization (gene expression by RT-PCR and qRT-PCR) and function analysis of purified progenitor will also be covered in this chapter.

3.2 Materials and methods

3.2.1 Cartilage Tissue Harvesting

Fresh articular cartilage were harvested from the bovine femur condyle of healthy stifle joints and cultured in Dulbecco`s modified Eagle`s medium (DMEM) and Ham`s F12 (1:1 mixture) supplemented by 10% fetal bovine serum (FBS), 50 µg/µl L-ascorbate, 100 U/µl penicillin, 100 µg/ml streptomycin, and 2.5 µg/µl Fungizone after washed by Hank's Balanced Salt Solution (invitrogen, California, USA).

3.2.2 Cell Isolating and Single Cell Suspension

Cells were isolated from the cartilage tissue by 0.25 mg/ml collagenase (Sigma-Aldrich, Missouri, USA) in shaking incubator overnight. The next day, digestion solution went through different size of cell strainers (Falcon) serially (100 µm, 70 µm, and 30 µm). 5-10 x 10^6 cells were suspended in 2ml Hank`s Balanced Salt Solution (Invitrogen, California, USA) in 5ml Falcon Polystyrene Tube (BD bioscience, Maryland, USA) for cell sorting. For some experiments a customized device was used to separate the superficial zone from the middle and deep zone of the cartilage prior to collagenase/pronase digestion (Figure 3.1).

3.2.3 FACS Sorting and Single Cell Plating

Single cell suspended solution were subjected to 4 µg/ml Hoechst dye 33258 (Life technologies, NY, USA) viability labeling. Prior to single cell

sorting, 96-well culture plates were coated with 0.1% gelatin solution (Bio-Rad, CA, USA). Single cell suspension were then subjected to FACS sorting (Becton Dickinson Aria II, BD, Maryland, USA), and single cell plating to 96 well plate one cell per well in DMEM-based culture medium. Rest of the cells were either directly used for RNA isolation (Primary normal chondrocyte, PNCs) or plated for later RNA isolation (Normal chondrocyte, NCs). Figure 3.2 is a schematic representation of single cell isolation and plating.

3.2.4 Clonogenicity Screening

Post sorting and plating, cells were cultured in Dulbecco`s modified Eagle`s medium (DMEM) and Ham`s F12 (1:1 mixture) supplemented by 10% fetal bovine serum (FBS), 50 µg/µl L-ascorbate, 100 U/µl penicillin, 100 µg/ml streptomycin, and 2.5 µg/µl Fungi zone for 48 hours. From day 3, culture plates were examined every other day well by well manually to check the availability, location and size of colonies. At day 10, cells were stained with 1 µg/ml Calcein-AM (green color). Colonies were analyzed based on green fluorescent detection using Olympus IX81 Inverted Light Microscope (Olympus, PA, USA). Colony size and number were measured by ImageJ (rsb.info.nih.gov/ij).

3.2.5 Colony Isolation and In Vitro Expansion

Large size colonies were manually picked and passaged serially to 24-well, 6-well culture plate (BD Bioscience, Maryland, USA) pre-coated with 0.1% gelatin solution (Bio-Rad, CA, USA). Cells were expanded in DMEM/F12 with GlutaMax (Life technologies, NY, USA) supplemented with 2% fetal bovine

serum (FBS), 50 µg/µl L-ascorbate, 100 µg/µl penicillin, 100 µg/ml streptomycin, and 2.5 µg/µl Fungi zone.

3.2.6 Gene Expression analysis

For gene expression analysis RNA were isolated from passage three cells from big colonies, and from freshly isolated normal chondrocytes (NCs). Cells were homogenized in TRIzol® reagent (InvitrogenTM Life Technologies, Carlsbad, CA) and total RNA was extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer`s instruction. Previous study indicated that progenitor cells can have up-regulation of genes commonly expressed in MSCs (mesenchymal stromal cells). ATP-binding cassette sub-family G member 2 (ABCG2), which is a characteristic gene related to "side population" identified by flowcytometry to be stem/progenitor cells; Telomerase reverse transcriptase (TERT) gene is an indicator of length of telomere, which is a marker for both stem cells and cancer cells. Sox-9 is chondrogenic transcription factor, which is related to chondrogenic potential; RunX-2 is an osteogenic marker for progenitor cells. Many clusters of differentiation (CD) markers were also used, like CD105, CD90, and CD133, etc. all of which are stem/progenitor cell markers widely used in previous published studies.

Our previous study also showed that some pro-inflammatory factors up-regulated in CPCs (chondrogenic progenitor cells) from injured bovine cartilage, which are stromal cell-derived factor-1 (SDF-1 or CXCL-12), dictator of cytokinesis-10 (Dock-10). Real-time PCR and qRT-PCR was used to compare the

expression of these markers in normal chondrocytes (NCs) and colony-forming cells (CFCs) essentially as described [43]. Primers were purchased from Integrated DNA Technologies (Coralville, IA). Table 3.1 summarized the information of primers used in PCR analysis.

3.2.7 Multi-lineage Differentiation Assay

The multi-potency of HECCs was examined by culturing them under chondrogenic, osteogenic and adipogenic conditions [16]. For chondrogenic differentiation, 1.5 million cells were subjected to pellet culture in chondrogenic medium (DMEM containing 10 ng/ml TGF-β1, 0.1 μM dexamethasone, 25 μg/ml L-ascorbate, 100 μg/ml pyruvate, 50 mg/ml ITS+Premix and antibiotics) for 3 weeks . The pellets were then analyzed for ECM formation by Safranin-O/fast green staining of cryosections. For osteogenesis, HECCs were cultured in osteogenic medium $2x10^4$/well in 12-well plate (DMEM/F-12 containing 0.1 μM dexamethasone, 100 mM β-glycerophosphate, 50 μg/ml L-ascorbate and antibiotics) for 3 weeks and examined with Alizarin Red staining to detect calcium phosphate deposition. STEMPRO® Adipogenesis differentiation kit (GIBCO, Grand Island, NY) was used to induce adipogenesis. 3 weeks post-induction, the cells were subjected to Oil Red O staining and imaged on a Nikon XB inverted microscope.

3.3 Results

3.3.1 Discovery of High Efficiency Colony-forming Cells (HECCs)

Clonogenecity screening by both light microscopy and fluorescent microscopy revealed that some cells can form colonies in 96-well plate, but the efficiency varies.

At day 3, fluorescent detection showed that 60% of wells have live cells growing, maximum cell numbers detected was 8 cells/well. At day 5, some wells already had colony formation (>50 cells), most of wells only showed limited number of cells growing. At day 10, high efficiency colony-forming cells (HECCs) were observed, which either reached confluence (>2/3 surface area, around 5% wells), or had big-colony formation (1/3-2/3 surface area, around 10% wells). Figure 3.3 showed a formation of big colony from a single cell in a 7 days culture period. Small and medium sized colonies (<1/3 surface area) were also observed (around 40%). Table 3.1 summarized the number of different size colonies. Table 3.2 summarized the average cell number and surface area of these colonies. However, around 40% wells still had no colony formation at day 10, even after 14 days culture (data not shown). Although some cells grown in the first couple of days, cell growth were stopped later and same cell numbers were observed throughout later culture period.

Noticeable morphological differences were observed between HECCs and Non-colony forming cells: a large portion of HECCs displayed fibroblast-like morphology, while non-colony forming cells showed characteristic cobblestone shape of chondrocyte. (Figure 3.4) However, no significant differences were observed after prolonged culture, both group displayed fibroblast-like morphology.

Both superficial zone and deep zone have cells form colonies, with superficial zone having more HECCs (around 5%) than deep zone (around 15%) at day 10 (Table 3.2).

3.3.2 Stem/Progenitor cell marker expression

Gene expression analysis revealed substantially higher expression of stem cell marker genes in HECCs versus NCs. qRT-PCR showed that ABCG2 was increased by over 8-fold, TERT by 3.2-fold, Sox-9 by 3-fold, Runx-2 by 2.5-fold and CD105, CD90, CD71, CD29, were increase around 2-fold in HECCs than in NCs (Figure 3.5). RT-PCR assay showed positive gene expression of ABCG2, and pro-inflammatory CXCL-12 and Dock-10. Stronger bands were observed in HECCs compared with NCs (Figure 3.5).

RT-PCR analysis of superficial and deep HECCs for ABCG2, Dock 10, and CXCL 12 showed similar expression pattern, which are up-regulated in HECCs. However, although primary normal chondrocytes (PNCs) from both superficial and deep zone showed similar expression level, which are lower than HECCs, passage one normal chondrocytes (NCs) from deep zone showed increased expression of all three genes, with NCs from superficial zone had decreased level expression.

3.3.3 Multipotent differentiation ability

HECCs were cultured in chondrogenic, osteogenic, or adipogenic media for 21 days to evaluate their multi-lineage differentiation potential. After chondrogenic induction with pellet culture, samples were fixed and stained with Safranin-O/fast green, revealing a strong proteoglycan deposition throughout the

pellets. Similarly, HECCs cultured in osteogenic medium had some calcium phosphate deposition in extracellular matrix as detected by Alizarin Red staining. However, only a few cells (< 1%) were positive for Oil Red O staining after 3 weeks culture in adipogenic medium (Figure 3.6).

3.3.4 Zonal differences of HECCs multipotency

HECCs from both superficial zone and deep zone were cultured in chondrogenic, osteogenic or adipogenic media for 21 days to compare their function. Safranin-O/fast green staining revealed higher proteoglycan deposition in pellet of deep zone HECCs compared with superficial zone HECCs both at day 7 time point and after 3 week chondrogenic induction. Superficial HECCs showed negative staining on the out surface of pellet while deep HECCs had strong proteoglycan deposition throughout the pellet (Figure 3.7). Similarly, more calcium phosphate deposition was observed in deep zone HECCs with around 2-fold up-regulation of osteoblast marker collagen type I (COL-1) and Runt-related transcription factor 2 (Runx-2). Although both deep and superficial HECCs showed limited adipogenic ability, deep HECCs showed much higher level positive rate for Oil Red O staining (Figure 3.8).

Table 3.1. Primer information for PCR

	Forward	Reverse
ABCG2	CCTTGGTTGTCATGGCTTCA	AGTCCTGGGCAGAAGTTTTGTC
CD 105	CCACTGCCCCAGAGACTGCGC	GCCCCCACAGTGAGTGCTTAGGT
CD 90	CGGTGGTGTTTGGCCATGTAATGA	GAGAGAGGGGAGTCCTATCCTGGT
CD 73	AGCTTTCCCAGCCTTCCATGCG	GGGTGTCCTCTTGAGTCCTGCA
CD 29	GCGGCCTCCGGGTGGATTCC	GCCGGGAAGGTCCAGGGGC
Runx-2	GCATGAAGCCCTATCCAGAGTCT	GCTGATGGAGCTGTTGGTGTAG
Sox-9	CGGTGGTGTTTGGCCATGTAATGA	GAGAGAGGGGAGTCCTATCCTGGT
B-actin	TCGACACCGCAACCAGTTCGC	CATGCCGGAGCCGTTGTCGA
CXCL-12	AGATGCCCTTGCCGATTC	TCTTCAGCCTTGCCACGA
Dock-10	ATCCCAGTAGCAACGAGC	ATCATGTGGTCAGCGAAG

Table 3.2. Summary of clonogenicity screening study

(A) Full thickness articular cartilage

		Plate 1 (# of wells)	Plate 2
Confluent (**C**)	(>2/3 SA)	**3**	**4**
Big (**B**)	(1/3-2/3 SA)	**12**	**8**
Small	(<1/3 SA)	**41**	**43**
Non-colony forming	(<50 cells)	**40**	**41**

*** Time point : Day 10**

(B) Superficial and deep zone articular cartilage

		Deep zone (# of wells)		Superficial zone	
		Plate A	Plate B	Plate A	Plate B
Big colony & Confluent	(>1/3 SA)	**3**	**7**	**11**	**15**
Small	(<1/3 SA)	**35**	**38**	**34**	**41**
Non-Colony	(<50 cells)	**58**	**51**	**51**	**50**

*** Time point : Day 10**

Table 3.3. Sizes and cell numbers of colonies

	Cell number	Surface area
Small	**475**	**17.2%**
Big	**8664**	**53.6%**
Confluent	**10974**	**78.8%**

*data were averaged by colony numbers

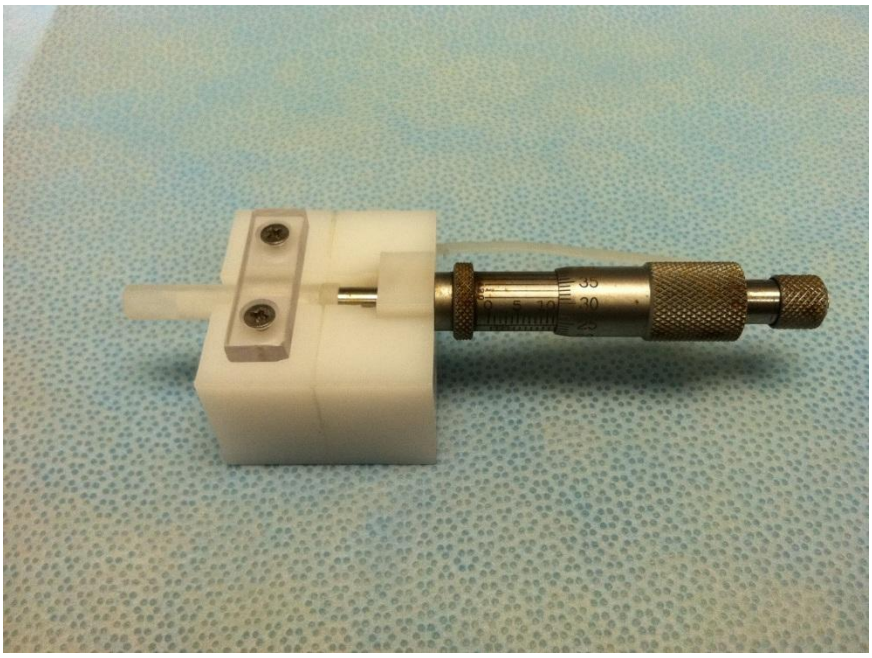


Figure 3.1. Customized cutting device. Device used for separating the Superficial 1/3 and the deep 2/3 of the articular cartilage with accuracy up to 2 µm.

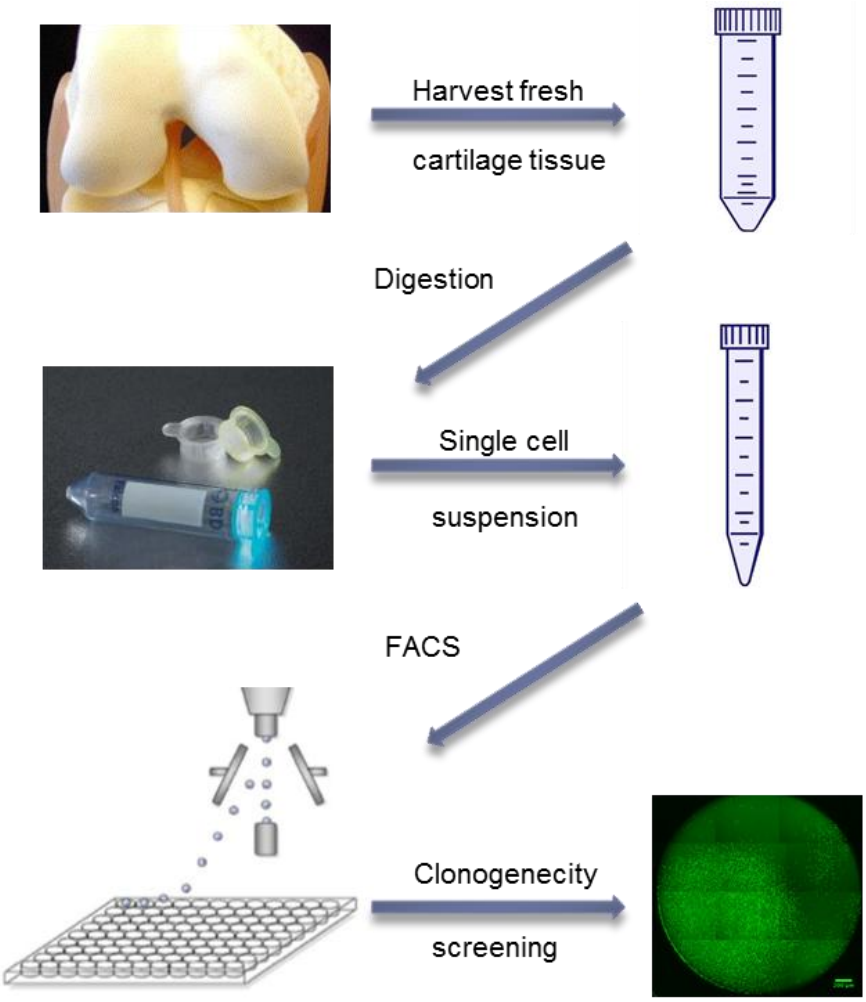
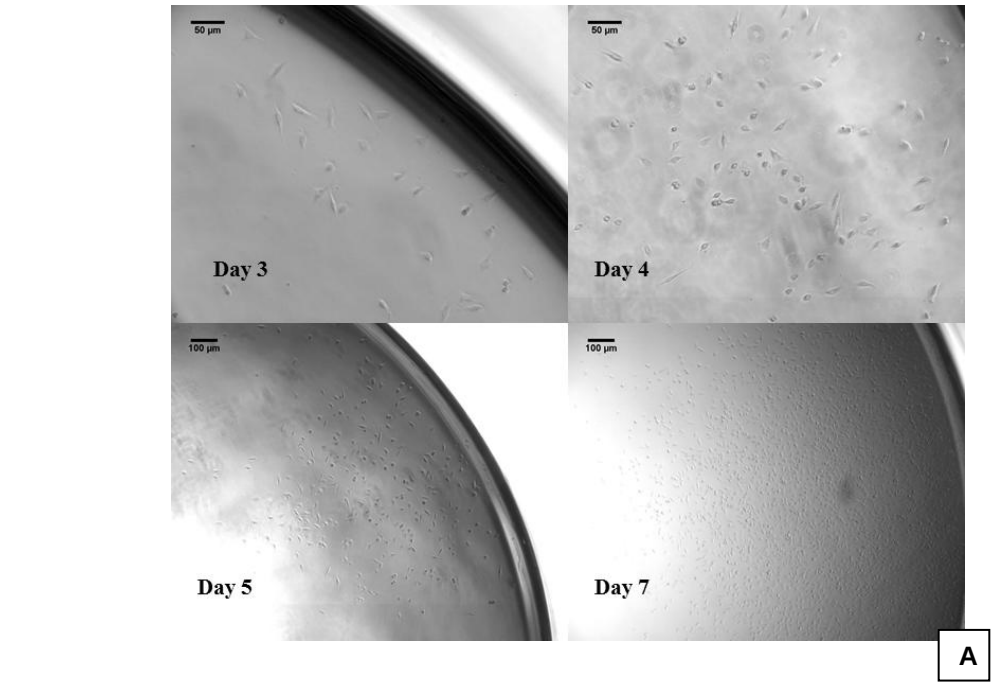


Figure 3.2. Single cell isolation and clonogenicity screening. Schematic representation for single cell isolation, sorting and clonogenicity assay.



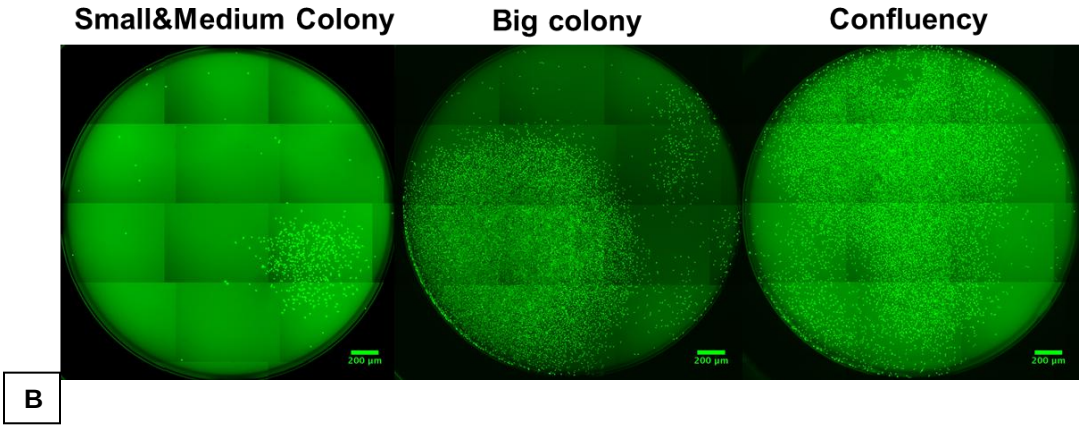


Figure 3.3. Colony forming cells . Time course of cell growth and formation of a big colony from a single cell (**A**). Various sizes of colonies formed from a single cell, small size colony has average cell number around 475, covering 17.2% surface area, big colony has average cell number around 8664, covering 53.6% surface area, and confluent colony has >10974 cells, with over 78.8% surface area coverage (**B**).

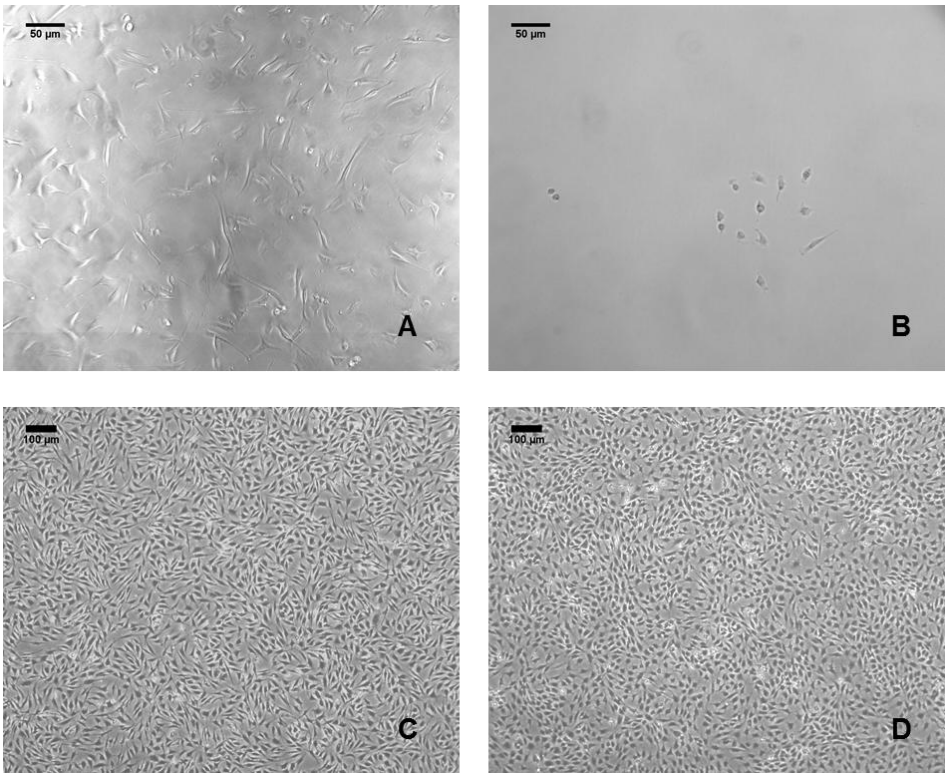
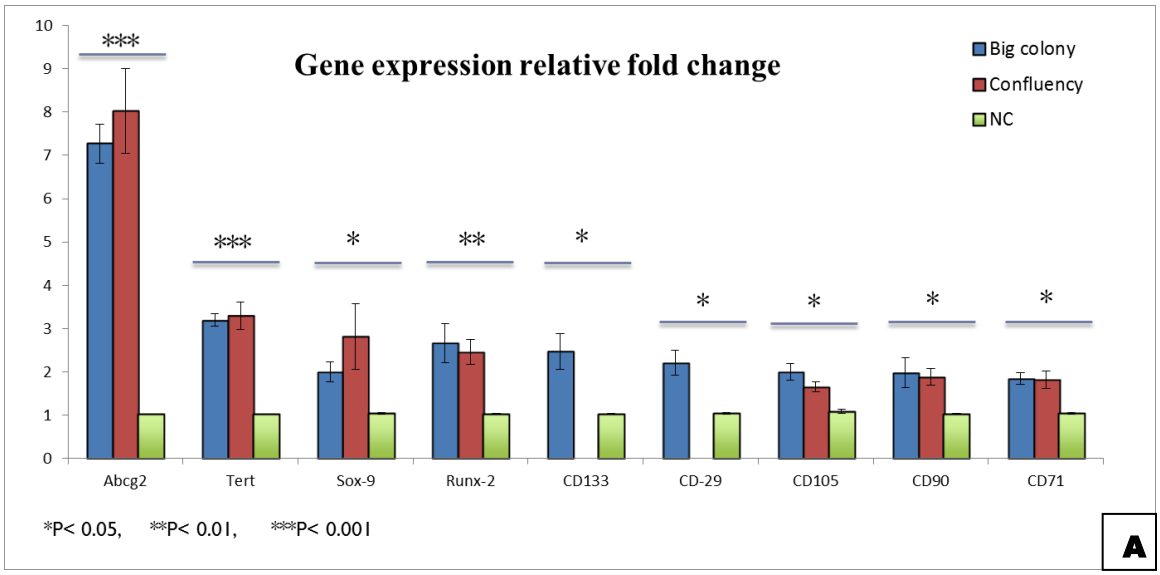


Figure 3.4. Morphological differences between HECCs and non-HECCs. HECCs displayed fibroblast-like morphology (A), while non-HECCs were characteristic chondrocyte shape (B); Morphological difference became unnoticeable after prolonged monolayer in vitro expansion: passage 3 HECCs (C), passage 5 non-HECCs (D).



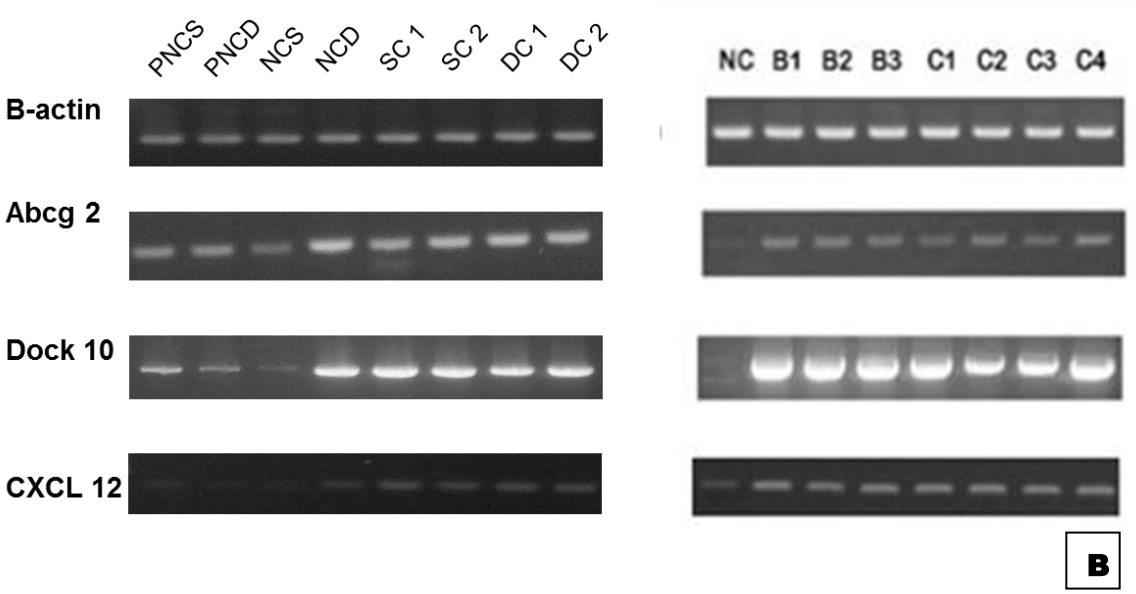


Figure 3.5. Gene expression analysis. Quantative RT-PCR showed relatively higher stem/progenitor markers expression of HECCs (**A**); PCR electrophoresis displayed stronger signal for Abcg2 stem cell marker and Dock10 and CXCL12 compared with normal chondrocyte.

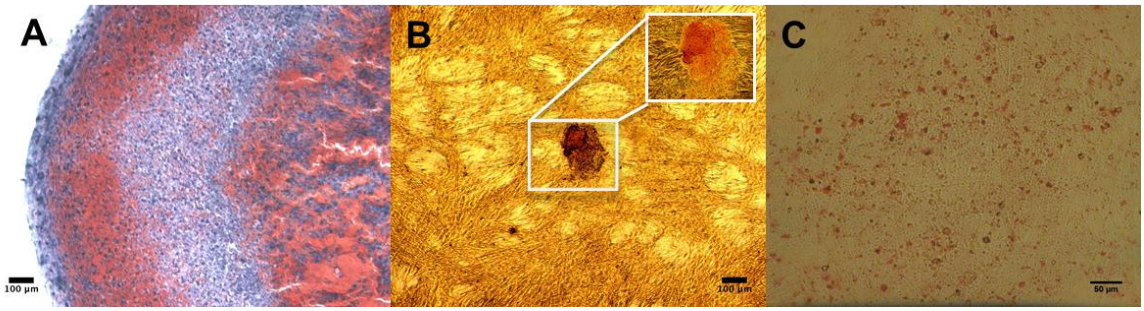


Figure 3.6. Multi-lineage differentiation ability. (A) Chondrogenic differentiation in pellet culture. Safranin-O/fast green staining displayed strong proteoglycan deposition. (B) Osteogenic differentiation in monolayer culture. Alizarin Red (dark red) staining showed massive calcium phosphate deposition. (C) Adipogenic differentiation in monolayer culture. Only few cells were positive for Oil Red O staining.

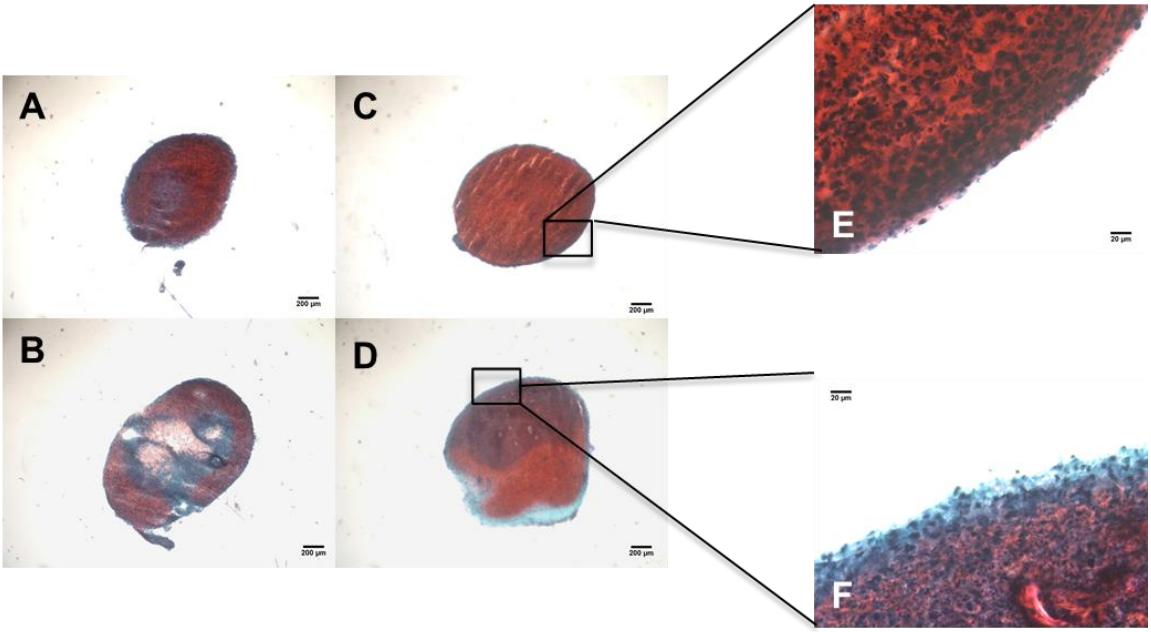


Figure 3.7. Difference of chondrogenic ability of superficial and deep HECCs. Deep HECCs (**A**) showed stronger proteoglycan deposition than superficial HECCs (**B**) after 1 week pellet culture. Even after 3 weeks culture, superficial HECCs (**D**) still showed inferior proteoglycan deposition than deep HECCs (**C**); The surface of pellet showed no proteoglycan in superficial HECCs (F), while strong positive staining in deep HECCs (E).

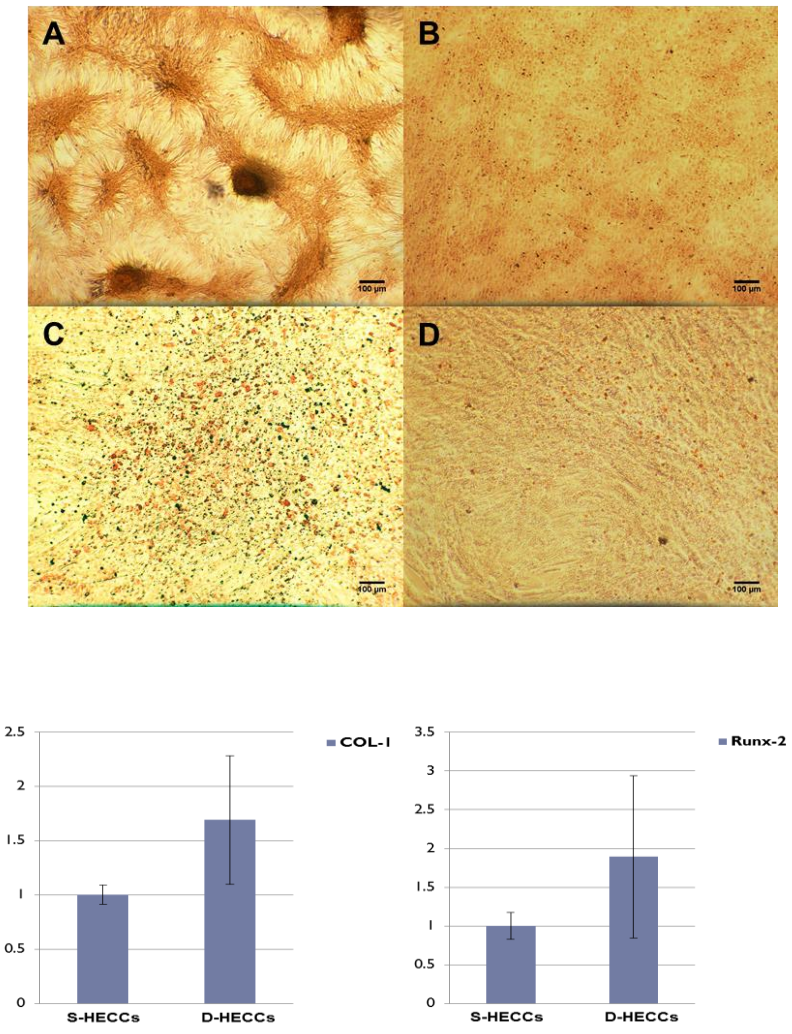


Figure 3.8. Difference of osteogenic and adipogenic ability of superficial and deep HECCs. Deep HECCs (A&C) showed superior osteogenic and adipogenic potential than superficial HECCs (B&D); Real-time PCR revealed relative higher osteogenic marker expression in deep HECCs after induction.

CHAPTER 4

DISCUSSION AND CONCLUSION

The results of these experiments demonstrate that fluorescence-activated cell sorting (FACS) is effective in single cell isolation. HECCs cloned from each single cell closely resembled chondrogenic progenitor cell (CPCs) identified in our previous study and by other groups [8]. HECCs` identifiable stem cell markers expression, clonogenicity, multipotency are consistent with published descriptions about progenitor cells from cartilage and other tissues [44].

Our enzymatic treatment together with serial cell strainer processing is an effective and safe method for getting single cell suspension with initial cell viability over 80% (confirmed by Hoechst day 33258 staining). Fluorescence-activated cell sorting (FACS) is a simple and powerful method for sterile single cell isolation and plating in our system, with cell viability over 60% on gelatin coated 96-well culture plate. It allows us to perform cloning from a single cell, which gives us genetically identical population. Since complete and accurate characterization of stem/progenitor cells requires clonal expanded cells rather cells of a heterogeneous nature, FACS is a crucial starting point for the success of our study.

Clonogenicity screening confirmed the presence of colony-forming units (CFUs) in articular cartilage. Nearly half of cells can form colonies, but colony size varies in certain time point (Day 7), which indicating CUFs have different proliferating rate. A small portion of cells (around 15%) can form big colonies at

day 7, which were named high efficiency colony-forming cells (HECCs). Characterization of high efficiency colony-forming cells (HECCs) revealed their similarities to progenitor cells.

Compared with NCs, HECCs over-expressed a few stem/progenitor cell-related molecular markers. ABCG2 (side population marker) expression were significantly up by 8-fold in HECCs. Tert, Sox-9, Runx-2 overexpressed by 3-fold, and the expression of some CD markers (CD 133, CD29, CD105 CD90 CD71) were increased by around 2-fold. These results were mainly consistent with previous work [39, 45, 46]. The up-regulation of both Sox-9 and Runx-2 demonstrated both chondrogenic and ostoelgenic potential of HECCs and distinguished them from terminal differentiated cells from either lineage, strongly supporting their progenitor cell nature.

In addition, chemokines involved in progenitor cell and leukocyte recruitment [47] were also significantly up-regulated in HECCs compared to NCs. CXCL-12 showed much stronger band in RT-PCR analysis, which may indicate the ability of HECCs to attract more progenitor cells or inflammatory cells when activated (isolation, injury, etc.). Dock-10, a activator of small G proteins, also showed stronger expression level in HECCs, meaning these cells have high proliferative phenotype compared with NCs. Lastly, relative over-expression of CSF (colony stimulating factor) in HECCs were consistent with their intrinsic clonogenic ability.

Interestingly, HECCs were observed from single cells both from superficial zone and deep zone, with superficial zone having more big colonies at day 10.

This result differs from previous publications, which stated that progenitor cells only reside in superficial zone cartilage [10, 39, 48]. However, Grogan [38] identified mesenchymal progenitor cell markers in both superficial and deep zone articular cartilage in healthy individuals and OA patients, with substantial positive rate. In terms of ABCG2 expression, PNCs (primary normal chondrocyte) from superficial and deep zone showed same level. NCs (passage 1 normal chondrocyte) from deep zone showed up-regulation after monolayer culture, while superficial NCs had decrease expression, which indicate prolonged culture may lose ABCG2 phenotype (Confirmed by PCR, data not shown). Nevertheless, deep zone may have more HECCs, whose ABCG2 expression were shut down by collagenase digestion, and regained in culturing, overwhelming the phenotypic loss by monolayer culture. Same phenomenon was observed for Dock 10, CXCL 12. Superficial HECCs and PNCs showed relatively higher level of Dock 10 expression than that from deep zone which may indicate they are in a more proliferative state.

The multi-lineage differentiation potential is the milestone for defining stem/progenitor cells. Therefore, our study evaluated HECCs mutipotency in classic differentiation culture system. We found that HECCs can be readily induced towards chondrogenic and osteogenic differentiation. However, they only have very limited adipogenic ability, with positive rate less than 1%. This result is consistent with published data for cartilage progenitor cell [8]. Therefore, HECCs are more likely to be chondrogenic progenitor cells reside in

articular cartilage matrix, which can be activated under certain conditions (traumatic injuries, OA, etc.), for restoring damaged cartilage tissue.

Multiple differentiation study was also applied to superficial and deep HECCs respectively. Progenitor cells from deep zone showed superior chondrogenic, osteogenic and relatively higher adipogenic potential than superficial zone progenitor cells, while superficial zone progenitor cells displayed a more proliferative phenotype. This observation may indicate that deep zone HECCs are more multipotent, which are more likely to function as progenitor cells in articular cartilage matrix.

In conclusion, although articular cartilage is notorious for poor healing ability post injuries, the discovery of progenitor cells in our study demonstrated its intrinsic self-repairing potential. These cells may be activated by traumatic injuries and become chondrogenic progenitor cells, migrating towards injury sites (confirmed by our previous study). However, the relatively high level of chemokine gene expression by these cells could cause pro-inflammatory reactions followed by progressing joint degeneration. Weather these activated progenitor cells are beneficial or detrimental to injured joint is still unknown.

Future studies are needed to confirm the existence and location of cartilage progenitor cells by immunostaining for stem/progenitor cell markers on tissue sections. Further, the activation pathway of these cells should be identified to establish one or more activators. Lastly, an in vivo model is needed to evaluate the function of these progenitor cells in a physiological environment.

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