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Chondrogenic progenitor cell response to cartilage injury and its application for cartilage repair

Dong Rim Seol
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CHONDROGENIC PROGENITOR CELL RESPONSE TO CARTILAGE
INJURY AND ITS APPLICATION FOR CARTILAGE REPAIR

by

Dong Rim Seol

An Abstract

Of a thesis submitted in partial fulfillment of the
requirements for the Doctor of Philosophy degree
in Biomedical Engineering
in the Graduate College of
The University of Iowa

July 2011

Thesis Supervisors: Assistant Professor James A. Martin
Professor Tae-Hong Lim

ABSTRACT

Focal damage to cartilage sustained in serious joint injuries typically goes unrepaired and may progress to post-traumatic osteoarthritis. However, in a bovine explant model we found that cartilage damage provoked the emergence of highly migratory cells that homed to the site of injury and appeared to re-populate dead zones. We hypothesized that the migrating population were chondrogenic progenitor cells engaged in cartilage repair.

The surfaces of bovine osteochondral explants injured by blunt impact were serially imaged to follow cell migration. Migrating cells harvested from cartilage surfaces were tested for clonogenic, side population, chemotactic activities and multipotency in *in vitro* assays. Gene expression in migrating cells was evaluated by microarray and their potential for spontaneous cartilage regeneration was assessed in a chondral defect model.

Migrating cells emerged from superficial zone cartilage and efficiently repopulated areas where chondrocyte death had occurred. In confocal examination with high magnification, we could clearly observe the morphology of elongated progenitor cells which were migrating toward cartilage defect area and these cells were distinguishable from round chondrocytes. The cells were also activated to migrate in cartilage defect model. Most migrated cells in fibrin were morphologically elongated and a few cells were differentiating to chondrocyte-like cells with the deposit of proteoglycans. These cells proved to be highly clonogenic and capable of chondrogenesis and osteogenesis, but not adipogenesis. They were more active in chemotaxis assays than chondrocytes, showed a significantly larger side population, and over-expressed progenitor cell markers and genes involved in migration, chemotaxis, and proliferation.

To active migration of chondrogenic progenitor cells (CPCs) short-term enzymatic method was used around edge of cartilage defect. Surprisingly, CPCs migrated into fibrin defect and were differentiating into chondrocytes with abundant deposit of proteoglycans. This result strongly supports that progenitor cells are activated in traumatic cartilage injury and have great potential for cartilage repair.

In conclusion, migrating cells on injured explant surfaces are chondrogenic progenitors from the superficial zone that were activated by cartilage damage to attempt repair. Facilitating this endogenous process could allow repair of focal defects that would otherwise progress to post-traumatic osteoarthritis.

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Graduate College
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CERTIFICATE OF APPROVAL

PH.D. THESIS

This is to certify that the Ph.D. thesis of

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The surfaces of bovine osteochondral explants injured by blunt impact were serially imaged to follow cell migration. Migrating cells harvested from cartilage surfaces were tested for clonogenic, side population, chemotactic activities and multipotency in *in vitro* assays. Gene expression in migrating cells was evaluated by microarray and their potential for spontaneous cartilage regeneration was assessed in a chondral defect model.

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CHAPTER 1

INTRODUCTION

The risk for post-traumatic osteoarthritis (PTOA) after serious joint injuries runs as high as 70% and has not improved substantially in the last 40 years despite many refinements in care [1-3]. This underscores the urgent need for new treatments to prevent articular cartilage degeneration initiated by joint damage. Cartilage is notoriously limited in its intrinsic capacity for repair; focal damage associated with joint injuries seldom heals and often worsens to engulf entire articular surfaces [1, 4]. Cell-based therapies intended to regenerate neocartilage *in situ* have shown some clinical promise. The two most common, microfracture and autologous chondrocyte implantation, promote healing of small defects in young patients [5-10]. However, results outside this limited population have been disappointing [11-13]. Findings from animal models mainly indicate that marrow stromal cells introduced by microfracture or chondrocytes introduced by implantation often form soft, fibrous or fibrocartilagenous repair tissue, [14, 15]. Although these results further dampened enthusiasm for cell-based strategies, interest has been rejuvenated by accumulating evidence of chondrogenic progenitor cells (CPCs) in adult cartilage [16-19]. These observations suggest that a rudimentary self-repair mechanism exists that might be marshaled for cartilage regeneration after traumatic injury, but complete knowledge of the post-traumatic behavior and function of CPCs is needed to evaluate this potential.

Like mesenchymal stem cells (MSCs) that originate in bone marrow, progenitor cells residing in tissues are multipotent, highly clonogenic, and chemotactic [20, 21]. These characteristics empower progenitor cells to migrate locally to sites of injury where they proliferate and differentiate as needed to replace damaged tissue [22-25]. In terms of multipotency, the progenitor cells' repertoire is typically more limited than that of MSCs. However, this is not necessarily a handicap: Unlike MSCs, which must be capable of differentiating suitably for the regeneration of multiple tissues in different organ systems, local tissue regeneration by progenitors does not require such pluripotency [24].

Chondrogenic progenitor cells (CPCs) were first identified in calf cartilage as a sub-population of superficial zone cells required for the appositional growth of articular cartilage [16, 26]. The specialized population was isolated from other cartilage cells based on enhanced binding to fibronectin. Compared with normal chondrocytes, CPCs over-expressed the stem cell-associated factor Notch-1 and the fibronectin receptor $\alpha 5\beta 1$ integrin. The cells also showed enhanced clonality in culture and multipotency when grafted to chick limb buds. Alsalameh and co-authors subsequently showed that approximately 4% of the cells in normal human cartilage expressed the mesenchymal stem cell markers CD105 and CD166 [27]. This frequency increased to near 8% in osteoarthritic cartilage. However, less than half of the CD105+/CD166+ cells were capable of adipogenic differentiation, suggesting that CD marker status overestimated the numbers of multipotent MSC-like progenitors. Using fluorescent activated cell sorting (flow cytometry) analysis, Hattori *et al.* demonstrated that 0.07% of the cells present in the superficial zone of calf stifle cartilage were capable of Hoescht 33342 dye exclusion, whereas none of the cells from the middle or deep zones excluded the dye [18]. As

expression of the multi-drug transporter responsible for dye exclusion is a typical of stem cells, the authors concluded they had found a stem-like progenitor cell population [28]. An examination of human end-stage osteoarthritic cartilage obtained at the time of total joint replacement revealed a sub-population of CD13-, CD29-, CD44-, CD73-, CD90-, and CD105-expressing cells in repair tissue around lesions. The cells appeared to migrate from subchondral bone via tidemark-spanning blood vessels and expressed both the osteoblastic transcription factor runx-2 and the chondrogenic transcription factor sox-9 [23]. Concurrently, Grogan and coworkers found high numbers of chondrocytes (>45%) that were immunohistologically positive for the MSC markers Notch-1 and Stro-1 in both normal and osteoarthritic cartilage [17]. These cells were osteogenic and chondrogenic, but not adipogenic. They included a small side population (0.14%) of Hoescht dye-excluding cells.

A number of studies have shown that blunt trauma to articular cartilage of the kind seen in many joint injuries induces acute chondrocyte necrosis and apoptosis [29-32]. These losses in cellularity have been thought to be irreversible [1]. However, in an explant trauma model we found that “dead zones” were frequently re-populated over a period of 5-12 days post-impact by cells that appeared to migrate over the cartilage surface toward the injury site. We hypothesized that these migrating cells were CPCs mounting a reparative response. To test this, we isolated surface-adherent migrating cells and determined their ability to move toward injured cartilage. The *in vitro* chemotactic activity, clonality, and multipotency of the putative CPCs (pCPCs) were compared with normal chondrocytes (NCs). Gene expression profiling was used to assess the phenotypic relatedness of migrating cells to normal chondrocytes and marrow stromal cells (MSCs).

Next, Hoescht dye exclusion assays were performed to measure the percentage of stem-like cells in each population. Lastly, we tested the ability of the migrating cells to regenerate cartilage matrix in full-thickness chondral defects made on the surfaces of explants.

CHAPTER 2

LITERATURE REVIEW

2.1. Articular Cartilage

Articulating ends of diarthrodial joints are covered by a thin layer called hyaline cartilage. Articular cartilage has no nerve supply and is therefore not sensitive to early injuries. It is alymphatic and avascular and is sheltered from the immune system. Due to relatively few cells in the tissue, the cartilage has poor repair properties.

2.1.1. Anatomy of articular cartilage

The major structures of the knee are articular cartilage, meniscus, synovial membranes, ligaments, muscles and tendons. Three bones in the knee joint are the patella, femur and tibia and each bone is covered by articular cartilage known as hyaline cartilage. Figure 2.1 explains the location of articular cartilage in the knee joint which is the end of the femur, the top of the tibia and the back of the patella.

2.1.2. Function of articular cartilage

Articular cartilage has two major functions. The tissue serves as a load-bearing elastic material that is responsible for resistance to compressive forces and distribution of mechanical load, thereby minimizing peak stress on subchondral bone. It also has an important role in decreasing the friction between the joining bones with the synovial fluid [33, 34].

2.1.3. Structure of articular cartilage

Articular cartilage can be subdivided into four horizontal layers zones depending on the alignment of collagen fibers, which give each zone particular biomechanical advantages: the superficial (tangential), middle (transitional), deep (radial) and calcified cartilage zones (Figure 2.2). The size, shape, density and metabolic activity are different in each layer [35, 36].

2.1.3.1. Superficial (tangential) zone

The superficial zone is the thinnest zone of articular cartilage (10-20% of the total cartilage volume) and contains a relatively high number of flattened chondrocytes. This zone has the highest water content, relatively low proteoglycan content, and densely packed collagen fibers [37]. This zone consists of two collagen layers. The first layer, known as the lamina splendens, is composed of collagen network within it is compromised of unique interwoven collagen bundles arranged parallel to each other to give the tissue its mechanical properties [38, 39]. The second layer of collagen fibers is aligned perpendicular to the articular surface. This specific organization of the superficial zone influences the mechanical properties of tissue and also may act as a barrier to the passage of large molecules from synovial fluid to cartilage.

2.1.3.2. Middle (transitional) zone

The middle zone (transitional zone) contains 40-60% of the total cartilage volume. This zone composed of spherical chondrocytes surrounded by extracellular matrix. The collagen fibers are thicker and randomly organized with low density of chondrocytes, and the proteoglycan content is increased. Mechanically, this zone is resistant to compression.

2.1.3.3. Deep (radial) zone

In the deep zone (radial zone, 30% of the total cartilage volume), the chondrocytes are mainly arranged in columns, and the cell volume is at its lowest. This zone provides the greatest resistance to compressive forces, given that collagen fibrils are arranged perpendicular to the surface. The proteoglycan content is the highest and water content is the lowest. This part contains the largest diameter collagen fibrils.

2.1.3.4. Calcified zone

The calcified zone contacts the underlying cortical bone, which is known as the articular end plate and acts as an anchor between articular cartilage and subchondral bone. This zone is characterized by spherical chondrocytes located in uncalcified lacunae without proteoglycans. The collagen fibers are arranged perpendicular to the articular surface and of largest diameter [38]. From the cartilage, dynamic forces are transmitted to the subchondral bone [40].

2.1.3.5. Tidemark

The tidemark which is a wavy and irregular line separates the deep zone (non-calcified zone) from the calcified zone (Figure 2.2). The tidemark and calcified cartilage play a crucial role in the transmission of mechanical forces to the subchondral bone [41]. Ultrastructurally, the tidemark has a band of fibrils which may function as a confining mechanism for the collagen fibrils of noncalcified zone to prevent them from the calcified zone. There are small gaps in the tidemark to provide channels for nutrients [42]. In osteoarthritis, the area of tidemark is significantly increased and a number of vessels penetrate through the tidemark [43].

2.1.4. Chondrocyte

Articular cartilage contains a small number of specialized and highly differentiated cells (1-5% of total tissue volume) [44], the chondrocytes, which are located in an extracellular matrix primarily composed of water, proteoglycans, collagens and noncollagenous proteins. They are located in lacunae, usually scattered individually throughout articular cartilage (Figure 2.3). During growth, chondrocytes usually have roundish shape, but their shape is variable depending on age, pathological state and the cartilage layer [36]. Chondrocytes are anaerobic, and receive their nutrition via diffusion of substances within synovial fluid.

They produce the extracellular matrix such as collagens, proteoglycans, and noncollagenous proteins [45]. Their metabolic activity differs depended on cartilage layer. For example, cells in the superficial zone synthesize different relative amounts of aggrecan and proteoglycans than chondrocytes in the deeper layer [35].

The specific structural organization of articular cartilage matrix endows this tissue with special mechanical properties such as compressive strength and elasticity, which allow them absorb and distribute loads. Chondrocytes can recognize any changes in the composition of the extracellular matrix and keep a balance between anabolism and catabolism.

Chondrocyte density is decreased with age in human articular cartilage (Figure 2.4) [46]. In particular, the number of cells in the superficial zone was significantly decreased than that of the middle and deep zones. The cell density is also distinct in different areas of cartilage (Figure 2.5) [47]. There was higher cell density in non-load-bearing area which is protected by meniscus than load-bearing area.

2.1.5. Extracellular matrix

Articular cartilage can be subdivided into the pericellular, territorial and interterritorial matrix around each chondrocyte (Figure 2.6). These different regions are classified by structural differences and a specific distribution of proteoglycans, core and link protein [48].

2.1.5.1. Pericellular matrix (microenvironment)

Chondrocytes in each layer are surrounded circumferentially by a 2-3 μm -thick pericellular matrix that is rich in hyaluronan, type II, VI, IX and XI collagen and proteoglycans such as aggrecan, decorin and biglycan [49-51]. This matrix provides hydrodynamic protection for the chondrocyte during physiological loading and plays a metabolic role in pericellular retention. The chondrocyte and its pericellular microenvironment together form a structural unit in cartilage and are called 'chondron' [52]. Ross *et al.* [53], introduced the morphologies of single and double chondron, and chondron columns, immediately after enzymatic isolation from adult canine tibial cartilage (Figure 2.7). Chondron remodeling consists of three phases (Figure 2.8). In phase I, fibrillar collagens and aggrecan are destructed by matrix metalloproteinases (MMP) and aggrecanase with hydrodynamic expansion of the chondrons. The fibrillar collagens are continuously declined and division of the progenitor chondrocyte and migration with the swollen microenvironment are shown in phase II. Clonal chondrocyte is expanded in swollen matrix and some progenitor chondrocytes migrate out in phase III. These chondron remodeling and cell migrating are visualized in Figure 2.9. In addition, the initiation and progression of chondron remodeling is associated with the early events in the osteoarthritic process [34].

2.1.5.2. Territorial matrix

The territorial region surrounds the pericellular matrix. Thin collagen fibrils near the chondrocytes appear to bind to the pericellular matrix. This region had larger diameter of collagen fibers and they are arranged in radial bundles [34]. The concentration of proteoglycans (chondroitin sulfate) is higher in this region [54].

2.1.5.3. Interterritorial matrix

The interterritorial matrix has greater diameter of collagen fibers than the territorial matrix and has a higher concentration of proteoglycans (keratan sulfate) [54].

2.1.6. Composition of extracellular matrix

There are two major phases in articular cartilage; 1) a fluid phase containing water and electrolytes, and 2) a solid phase containing chondrocyte, collagen, proteoglycan, and other glycoproteins (Figure 2.10).

2.1.6.1. Water

Water contributes up to 80% of the wet weight of articular cartilage, and 30% of this water is found in intrafibrillar space of collagen. Amount of water depends on fixed charge density, organization of collagen network and resistance to swelling. The interaction of water with the matrix macromolecules significantly influences the mechanical properties of the cartilage tissue.

2.1.6.2. Collagen

Collagen constitutes approximately 10% of the wet and 50% of the dry weight of articular cartilage. Cartilage strength depends on the extensive cross-linked collagen and its changes in fibrillar architecture. Collagen fibril has triple helix structure by three identical α -1 chains and provides the basic architecture of cartilage (Figure 2.11) [55].

The principal articular cartilage collagen, type II, accounts for 90% to 95% of the cartilage collagen, but collagens III, VI, IX, X, XI, XII and XIV also contribute to the mature matrix [56]. Microfibrillar type VI collagen is located in the pericellular microenvironment, territorial and interterritorial matrix [57]. Type X collagen is found in the calcified zone and also present around hypertrophic chondrocytes of the growth plate [58]. Cross-linked copolymer of type II, IX and XI collagens is the core fibrillar network in developing cartilage and contributes to tissue stiffness and strength [59].

In the adult cartilage, the synthetic rate of collagen type II by articular cartilage is dramatically decreased. However, the synthesis can be continued and accelerated up to 10-fold after joint injury [60]. In traumatic cartilage injury and osteoarthritis, the proteolysis and denaturation of matrix type II collagen is observed. This collagen fibril degradation is through an initial cleavage of the collagen molecule by collagenase-3 (MMP13) [61].

2.1.6.3. Proteoglycans

Proteoglycans, which have 10-20% of wet weight, are molecules consisting of a core protein, hyaluronic acid (hyaluronan), with multiple glycosaminoglycans subunits (Figure 2.12). The glycosaminoglycans include chondroitin-4-sulphate, chondroitin-6-sulphate and keratin sulphate [62]. Large aggregated proteoglycans (aggrecans) compose of 90% of the total cartilage proteoglycan mass. Their long chain polysaccharides are negatively charged and hold water within the tissue by osmotic pressure, thus responsible for compressive strength of articular cartilage. Small proteoglycans such as biglycan, decorin and fibromodulin constitute 3% of the total proteoglycan mass and help to stabilize the tissue.

In traumatic or inflammatory cartilage, the proteoglycan structure disintegrates and its water-holding capacity is lost. This leads to progressive breakdown of the collagen meshwork and finally derives the exposure of subchondral bone, causing severe pain and disability.

2.1.7. Nutrition of articular cartilage

The nutrition of articular cartilage which has no blood and nerve supply, is transported to chondrocytes predominantly by diffusion from the synovial fluid [63]. Synovial fluid is a viscous fluid found in the cavities of synovial joints and serves to reduce friction between the articular cartilage of the femur and tibia. Normal synovial fluid consists of water, glucose, electrolytes, and small molecules as well as metabolic wastes such as oxygen and carbon dioxide. Synoviocytes can synthesize hyaluronan, a polymer of disaccharides, lubricin, and mediators of inflammation [34]. Nutrients of the synovial fluid must pass through a double diffusion system, the synovial membrane and the cartilage matrix, to reach the cells [64, 65].

2.2. Articular Cartilage Injury

Widuchowski *et al.* reported that 60% of knees (total 25.124 knees) had chondral lesions [66]. The cartilage lesions were classified as 67% focal osteochondral or chondral lesions and 29% osteoarthritis. Most patients were associated with ligamentous or meniscal lesions, mostly ACL tear [67, 68].

Articular cartilage injuries can be caused by either traumatic mechanical destruction like sport-accident or progressive mechanical degeneration such as wear and tear. Immobilization can also result in cartilage damage. The damage is one of several possible lesions, softening, fissuring, fragmenting or defect. Depending on the injury

extent and location, it is sometimes possible to heal articular cartilage by resident cells. However, stable regeneration of hyaline cartilage has never been reported. Generally articular cartilage has no direct blood supply, thus it has little or no capacity to repair itself. The injuries may result in pain, swelling and subsequent loss of joint function, finally leading to osteoarthritis [69].

To determine the degree of chondral lesions and suitable treatment strategies, qualification of the chondral lesions is important. Three kinds of grading system are available and Outerbridge's grading system [70] is simple and clinically useful (Table 2.1).

2.2.1. Traumatic injury and secondary osteoarthritis (OA)

Traumatic injury activates chondrocytes and synovial cells and these cells release pro-inflammatory cytokines (tumor necrosis factor- α (TNF- α) and interleukin-1 β (IL-1 β), chemokines and catabolic mediators (matrix metalloproteinases (MMPs)) [71-73]. TNF- α and IL-1 β are distributed in the OA joint and inhibit extracellular cartilage matrix synthesis [74, 75]. In addition, the traumatic cartilage injury resulted in increased chondrocyte apoptosis (Figure 2.13) [76-78].

2.3. Articular Cartilage Repair

In mature articular cartilage, chondrocytes mainly receive their nutrition through diffusion from the synovial fluid and this limits their intrinsic capacity for cartilage healing. For this reason, cartilage defects cannot be repaired by resident chondrocytes and commonly progress to osteoarthritis if defects are untreated [79-81]

Decisions about whether and how to treat damaged cartilage remain a challenge to orthopaedic surgeon [67]. A variety of options are available nowadays such as

conservative treatment and surgical treatment. Surgical treatment consists of arthroscopic lavage and debridement, abrasion arthroplasty, subchondral drilling, microfracture, osteochondral grafting, autologous cell implantation, cartilage fragment implantation and tissue-engineered cartilage implantation.

2.3.1. Conservative treatment

Conservative treatment is considered in mild symptomatic injury where surgery will be more harmful. The purpose of conservative treatment is to reduce symptoms, not repair the damage. Several non-operative approaches can be considered according to severity of the cartilage lesion [82]. Medication is one of the conservative treatments. Non-steroidal anti-inflammatory drug (NSAID), analgesics and hormones (estrogen and growth hormone) are examples of medication. Mechanical approach is the other method and consists of bracing, canes, ice, physical therapy, resting, weight loss, and nutrition supply such as glucosamine and chondroitin phosphate [83], methylsulfonylmethane (MSM), Omega-3, calcium, and vitamins.

2.3.2. Arthroscopic lavage and debridement

Knee arthroscopy can directly visualize the cartilage joint using videofiberoptics (Figure 2.14). If lavage and debridement are located in the joint, the surgery can be accomplished with the same surgical equipment. Using saline solution, arthroscopic lavage washed out bone or cartilage fragments which are free-floating within the synovial fluid and cause synovitis and effusion. Debridement is often performed to remove loose flaps or osteophytes. It helps to reduce pain and mechanical symptoms and improve cartilage function [67]. This arthroscopic lavage and debridement technique is also used for patients with early OA and improved their daily-living activities [84, 85].

2.3.3. Abrasion arthroplasty

Abrasion arthroplasty has been performed arthroscopically for an exposed sclerotic degenerative arthritic lesion to sustain a uniformly contoured edge of cartilage surface [86, 87]. Unfortunately, the outcomes of this technique vary among studies based on doctors' experience and there are no prospective clinical studies.

2.3.4. Subchondral drilling

Arthroscopic drilling was first used by Smillie [88] in 1957 and popularized by Pridie in 1959 [89]. The surgery is performed using a high speed drill which drilled the subchondral bone. This method is one of the bone marrow stimulation techniques and used for treatment of small chondral defects due to low invasion [90, 91]. However, this technique has disadvantage such as thermal necrosis and fibrocartilage scar tissue formation [92].

2.3.5. Microfracture

Microfracture is introduced by Steadman [93] and used for repair of a focal chondral defect with fibrocartilage. This repair creates through surgical penetration of the subchondral bone and allows to attract exposes the damaged area to progenitor cells in the bone (Figure 2.15). This technique has benefits due to several reasons: (1) less destructive to the subchondral bone due to less thermal injury than drilling; (2) available in rough articular surface; (3) controlled depth penetration. Microfracture is recommended for active patients with smaller lesions ($< 2 \text{ cm}^2$) and produces good and excellent results in 60-80% of patients [94-96].

2.3.6. Osteochondral grafting

Osteochondral autograft is an obvious technique because of using same tissue and antigenicity with non-weight bearing area. This approach allows young patient with a medium-sized lesion (2.5-4 cm²). Mosaic autografts (mosaicplasty) is applied more recently using a collection of small osteochondral cylinders from usually the femoral condyle to maintain the curvature of articular surface and showed clinically promising results [97, 98]. Due to limited graft size of donor, allogeneic osteochondral graft is also used, but this approach has the risk of transmission of viral disease.

2.3.7. Autologous cell implantation

2.3.7.1. Autologous chondrocyte implantation (ACI)

Autologous chondrocyte implantation (ACI) is one of the promising techniques for repair of articular cartilage defects. This technique requires two surgical procedures. The articular cartilage biopsy was taken arthroscopically from non-load-bearing joint cartilage area to isolate autologous chondrocytes. After *in vitro* culturing of sufficient chondrocytes, the cells are injected into the defect with or without degradable materials which is then covered by a periosteal flap or a collagen membrane. Brittber et al. [6] and Brittber [5] have reported a good clinical outcome in their long-term study of ACI. However, ACI has hypertrophic differentiation with subsequent ossification and poor integration to host tissue [99].

2.3.7.2. Other cell sources for implantation

Besides autologous chondrocytes, bone marrow stromal cells (BMSCs) [100], periosteal cells [101], skeletal muscle [102], adipocytes [103] and synovial fibroblasts [99, 104, 105] represent possible cell sources for cell-based cartilage repair. These cells need

cell expansion to gain enough cells (at least 10 million cells) for transplantation. However, the expansion and differentiation of MSCs are required coordination and maintenance of the regular chondrogenic differentiation and are unsolved problems at present [106].

2.3.8. Enzymatic treatment techniques

Several studies have been reported enzymatic digestions in order to increase the initial adhesion of cells or cartilage tissue with host tissue. Lee et al. [107] and Hunziker et al. [108] were used chondroitinase ABC to remove proteoglycans in superficial zone of the cartilage. The chondroitinase ABC can selectively degrade the proteoglycans without affecting the collagen matrix [109]. They showed increased adhesion force and cell population after treatment. Treatment with hyaluronidase (0.1-0.3%) and collagenase (type VII, 10-30 U/ml) showed significant chondrocyte density in lesion edges and enhanced integration and interfacial strength [110-112]. However, they treated the enzymes 48 hours and showed unrecovered proteoglycan loss after 28 days. For clinical application, enzymatic treatment should be localized in cartilage defects and treated short-duration.

Collagenase is an enzyme that breaks the peptide bonds in collagen. Short-term treatment of collagenase (type I) may break just around injured site without damage of intact cartilage. Therefore, progenitor cells can easily migrate from the host tissue to injured cartilage. Moreover, reduced catabolic enzymes can help fibrin degradation, eventually enhancing tissue repair and integration.

Hyaluronidase and chondroitinase have been tested in clinical trials for pharmacologic vitreolysis which is a new treatment modality to potentially eliminate untoward effects of vitreous upon the retina [113]. However, the results are disappointing.

Hyaluronidase and chondroitinase Phase III and Phase II, respectively, of US Food and Drug Administration (FDA) trial conducted in the United States. This is due to an insufficient understanding of the molecular-effect mechanism of the agents. They could also be used for regeneration of damaged nerve tissue such as the spinal cord. These enzymes function to remove the accumulated chondroitin sulfate in glial scar which inhibits axon growth [114, 115]. Collagenase (XIAFLEX[®], Auxilium Pharmaceuticals, Inc) is the only FDA-approved nonsurgical treatment for Dupuytren's disease that is abnormal thickening of the tissue just beneath the skin known as fascia [116].

2.3.9. Cartilage fragment implantation

Main disadvantage of all cell implantation techniques require two surgical procedures; harvesting of cartilage biopsy and cell reimplantation. Moreover, this technique leads to high cost and time for *in vitro* cell expansion. Autologous cartilage fragment implantation is relatively simple and need one-step surgical procedure which implants minced cartilage fragment arthroscopically harvested from non-weight loading area in the femoral condyle. This technique showed promising results in animal studies [117, 118].

2.3.10. Tissue-engineered cartilage implantation

Tissue-engineered cartilage generally requires an artificial matrix known as a scaffold in which chondrocytes or/and progenitor cells can migrate and differentiate with new cartilage tissue formation [119]. The scaffold must be biocompatible and biodegradable and allow reasonable cell adhesion. Also, it should support sufficient mechanical property to withstand *in vivo* forces [120]. The current potential biomaterials used for tissue-engineered cartilage regeneration are shown in Table 2.2.

2.3.10.1. Fibrin hydrogel

Hydrogel is a kind of biomaterials that have great ability for many soft tissue engineered applications because of its high biocompatibility, high water content, similar mechanical properties, efficient transport of nutrients and wastes, and cell delivery (injectability) [121-123].

Fibrin is the main structural protein in the blood responsible for hemostasis. Fibrinogen is converted into fibrin monomer by thrombin (Figure 2.16). The fibrin monomers assemble into fibrils, eventually forming fibers in a 3-dimensional matrix or gel which prevents further loss of blood. Further, the blood coagulation factor XIIIa is a transglutaminase which can rapidly crosslink γ -chains in the fibrin polymer [124]. Some allogenic fibrin sealants such as Tisseel[®], Crosseal[™] and Evicel[™] are commercially available and have been approved by the Food and Drug Administration (FDA) for clinical use as hemostasis.

Fibrin hydrogel had important characteristics such as high cell chemotactic ability, uniform cell distribution, angiogenesis, and great adhesion capabilities [120, 125]. Because of these advantages, fibrin has been widely used in a variety of tissue engineering applications such as adipose [126], cardiovascular [127-129], muscle [130-132], liver [133, 134], skin [135, 136], and bone tissue [137, 138]. In particular, it has been also widely investigated for cartilage repair implanted with chondrocytes [139-154], periosteal cells [155], bone marrow stromal cells [156], adipose tissue-derived mesenchymal stem cells [157], and bone marrow-derived mononuclear cells [158].

An important disadvantage of fibrin hydrogel is an increasing instability and solubility in long-term culture due to its fibrinolysis. The fibrinolysis process can degrade

fibrin hydrogel and do not allow cell migration and proliferation [159-162]. To protect shrinkage and disintegration of fibrin, fibrinolysis inhibitors were used. Although they could help slow degradation in short-term application [159, 161, 162], it is not still inadequate for long-term shape stability. Another approach is to find optimal fibrin composition to maintain long-term shape stability and mechanical integrity for application of cartilage repair because concentration of fibrinogen and thrombin can influence gel appearance, stability and mechanical properties in previous studies [163, 164]. Eyrich *et al.*[142], found the optimal condition for cartilage tissue engineering. Fibrin hydrogel with a final fibrinogen concentration of 25 mg/ml or higher, a Ca^{2+} concentration of 20 mM and a 6.8-9 pH were stable for three weeks. This composition is available using Tisseel[®] (fibrin sealant, Baxter).

2.4. Progenitor Cells in Articular Cartilage

There are currently few studies focusing on progenitor cells in the articular cartilage. These cells exhibit stem cell characteristics such as migratory activity, high colony formation, and multipotential differentiation. During the development of articular cartilage, progenitor/stem cell population resides in the surface zone of cartilage and allows the appositional growth [16, 26]. Koelling *et al.* [23] has also found this progenitor cells from repair tissue during the late stages of human osteoarthritis. Interestingly, they observed breaks in the tidemark by progenitor cells which mean the cells oriented from subchondral bone or bone marrow. However, they could not find tidemark breaks in healthy cartilage and not observe migrated cells from healthy cartilage fragments. In contrast, Hattori *et al.* [18] identified progenitor/stem cell population in healthy bovine cartilage. 0.07% progenitor cells were resident only in superficial zone but not in middle

and deep zones of articular cartilage. Although the origin and population of progenitor cells were still controversial, discovery of these cells may be a promising advance for cartilage repair.

Table 2.1. Classifications of chondral lesions [165]

Outerbridge	
Grade 0	normal articular cartilage
Grade I	softening, blistering or swelling of the cartilage
Grade II	partial thickness fissures and clefts < 1 cm diameter
Grade III	full thickness fissures, to subchondral bone > 1 cm diameter
Grade IV	exposed subchondral bone
International Cartilage Repair Society (ICRS)	
I	superficial fissure
II	< 50% depth
III	50% to full thickness loss
IV	osteochondral lesion extends through bone
V	osteochondritis dissecans lesion (OCD)
VI	avascular necrosis (AVN)
Bauer-Jackson descriptive (I-IV traumatic/ V-VI degenerative)	
I	linear
II	stellate
III	chondral flap
IV	chondral crater
V	fibrillation
VI	exposed subchondral bone

Table 2.2. Current potential biomaterials used for tissue-engineered cartilage regeneration [166]

Biomaterial polymer	Advantages	Disadvantages
Hyaluronic acid-coated polyurethane (PU)	Cytocompatible towards fibroblasts that can be applied to articular cartilage tissue replacement [167].	No homogeneous distribution of glycosaminoglycan (GAG) within the newly engineered cartilage [167].
Hyaluronic acid / human fibrin	Paediatric articular chondrocytes can proliferate [159, 168].	Further study is needed to determine the long-term architectural and histological characteristics [159].
Oligo(poly-ethylene glycol fumarate), poly(N-isopropylacrylamide-co-acrylic acid), poly(N-isopropylacrylamide grafted gelatin), poly(ethylene oxide)	Injectable scaffolds that are easy to handle for the purpose of cartilage regeneration [169].	Do not maintain the scaffold structural integrity [170].
Platelet-rich plasma	Injectable scaffold seeded with chondrocytes that regenerates cartilage [171].	Does not maintain the scaffold structural integrity and produces inflammatory response [171].
Chitosan / hyaluronic acid	Increased amount of collagen II / cartilage formation became more marked over time [172].	No homogeneous distribution of GAG within the newly engineered cartilage [172].
Thermoreversible gelation polymer (TGP)	3-D hydrogel scaffold that delivers growth factors [173].	No homogeneous distribution of GAG within the newly engineered cartilage and produces inflammatory response [173].
Alginate	Good retention of shape and with notably less inflammatory response [174].	Further study is needed to determine the long-term architectural and histological characteristics [174].
Hyaluronic acid derivative (Hyaff 11)	Bio-resorbable scaffold that maintains the articular structure [175, 176].	Further study is needed to determine the long-term architectural and histological characteristics [176].

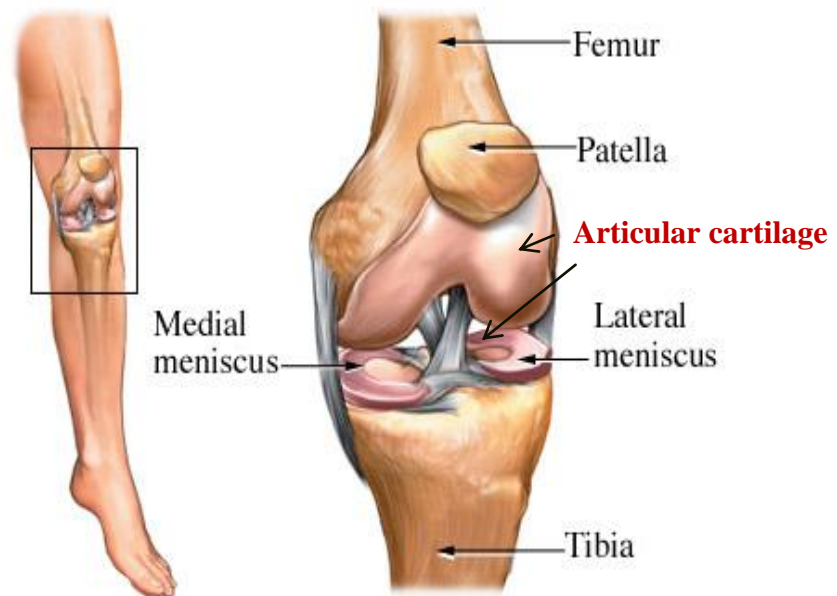


Figure 2.1. Three locations of articular cartilage in the knee joint.

Source: <http://www.orthospecmd.com/TheKnee.html>

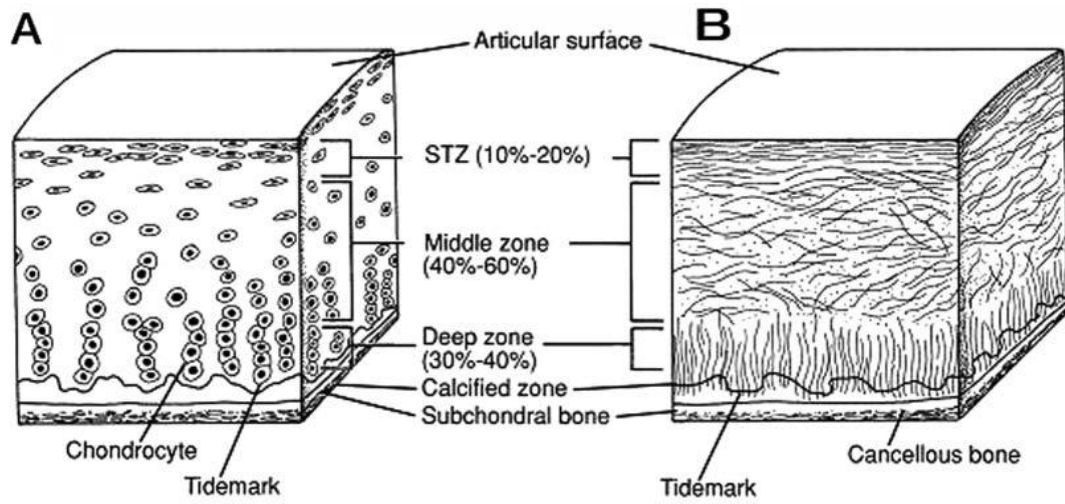


Figure 2.2. Structure of articular cartilage. Articular cartilage consists of four horizontal layers (A) and each layer has different organization of collagen fiber (B).

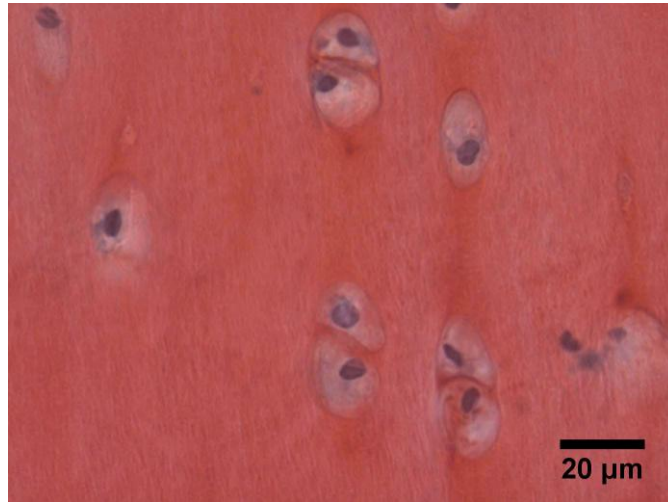


Figure 2.3. Bovine chondrocytes in middle zone. Chondrocytes have rounded shape and some cells are arranged in columns.

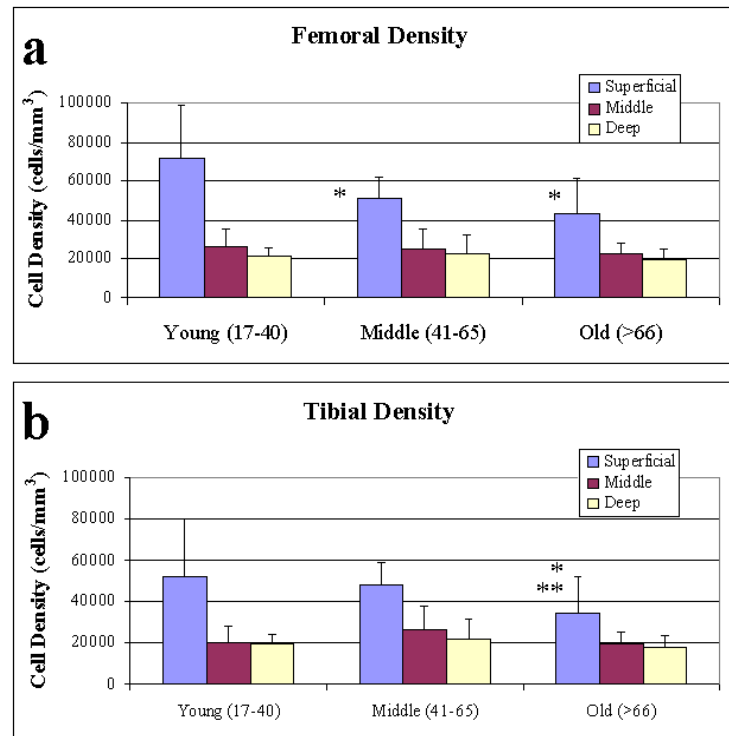


Figure 2.4. Chondrocyte density of the human femoral condyle and tibial plateau with age. Cell density in the superficial zone was significantly decreased with age [46].

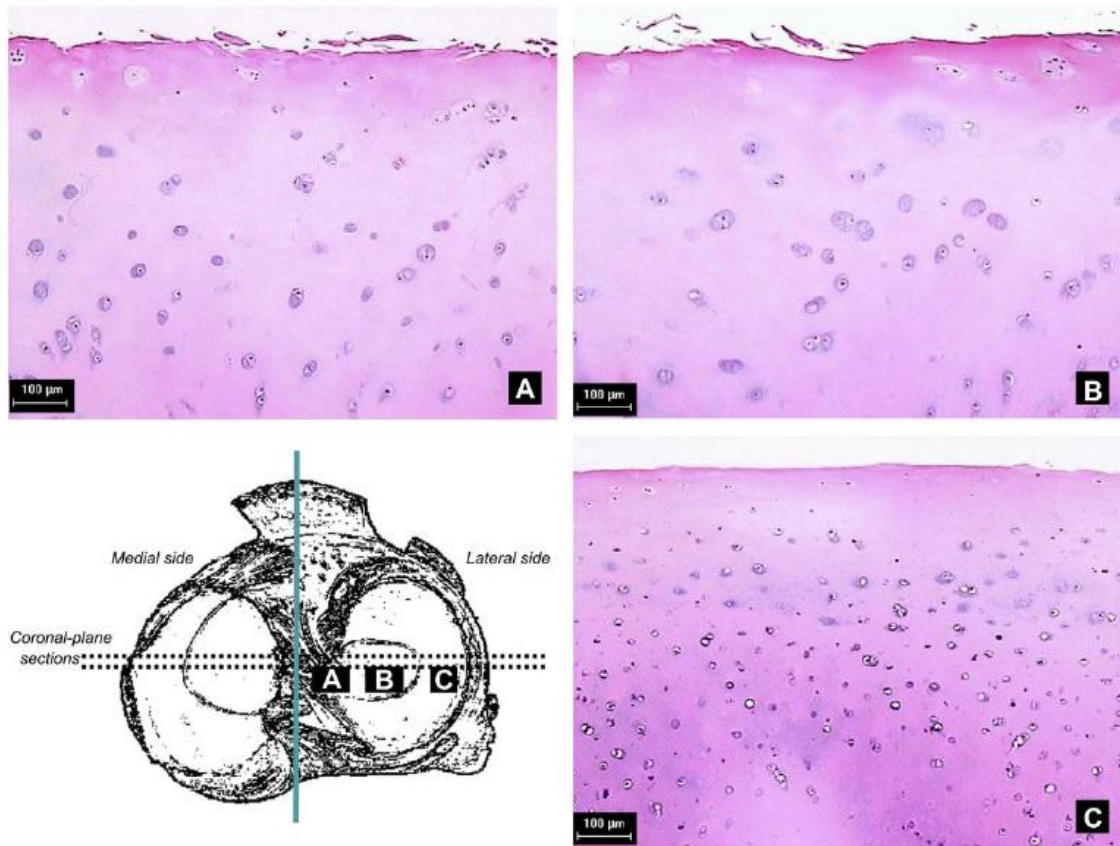


Figure 2.5. Chondrocyte density in different location of human articular cartilage.

The cell density was the lowest in load-bearing area and the highest in meniscus-covered area [47].

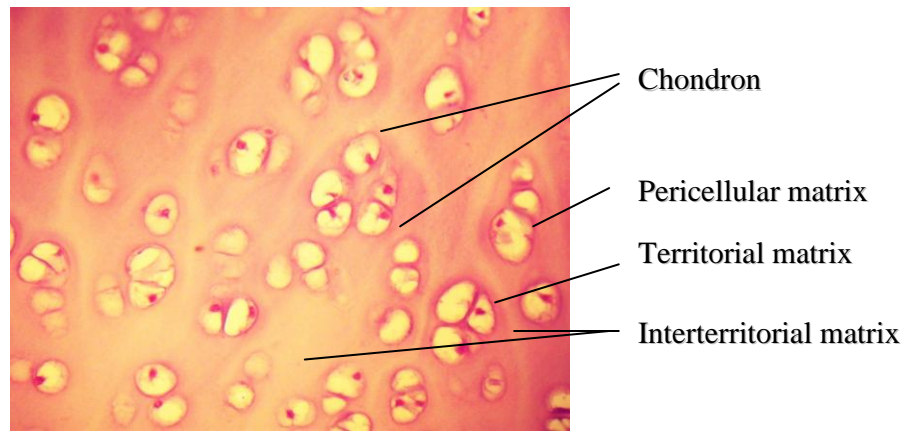


Figure 2.6. Microscopic structure of articular cartilage. The tissue can be subdivided into the pericellular, territorial and interterritorial matrix around each chondrocyte.

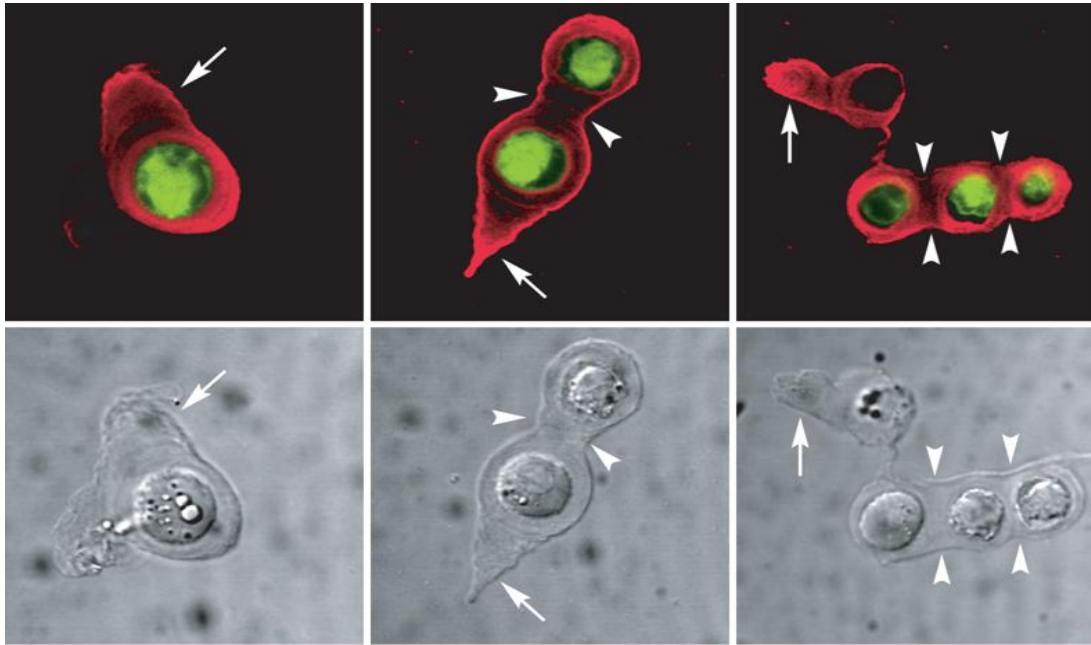


Figure 2.7. Enzymatic isolated chondron. Viable chondrocytes are shown in green with type VI collagen (red color) [53].

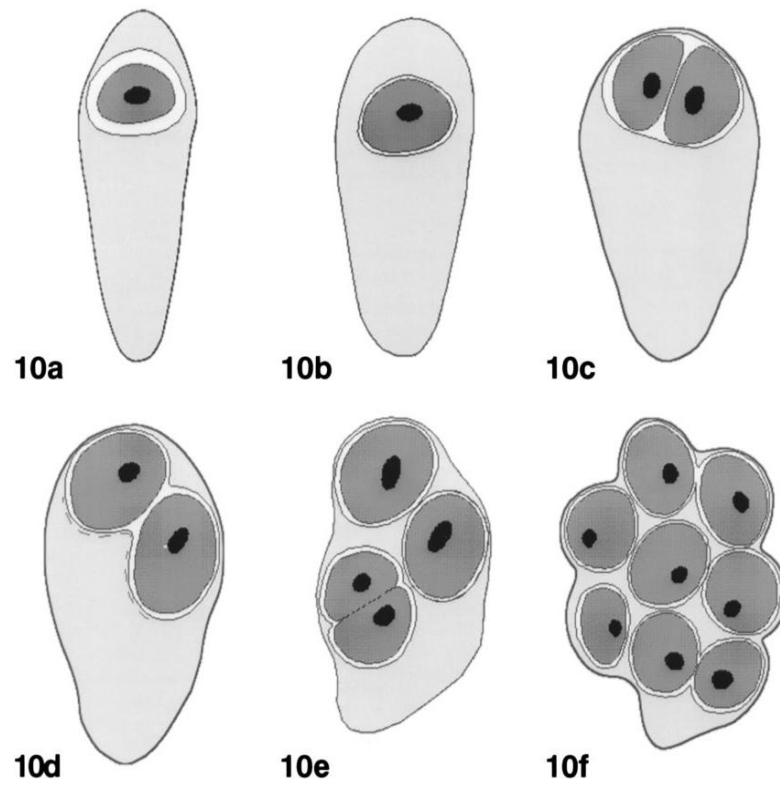


Figure 2.8. Chondron remodeling. a and b; phase I, c and d; phase II, e and f; phase III [177].

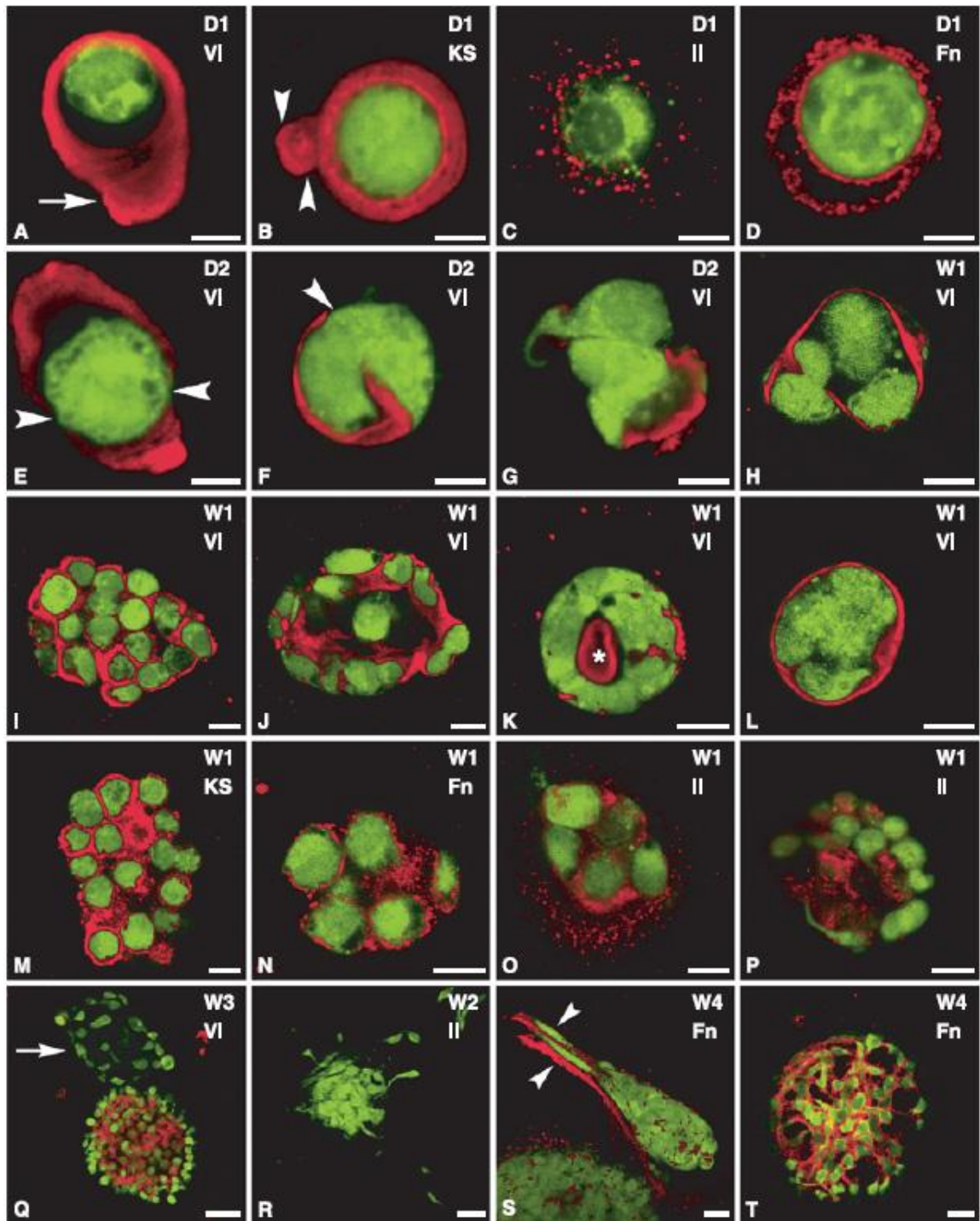


Figure 2.9. Remodeling of enzymatic isolated chondron and chondrocyte migration [53].

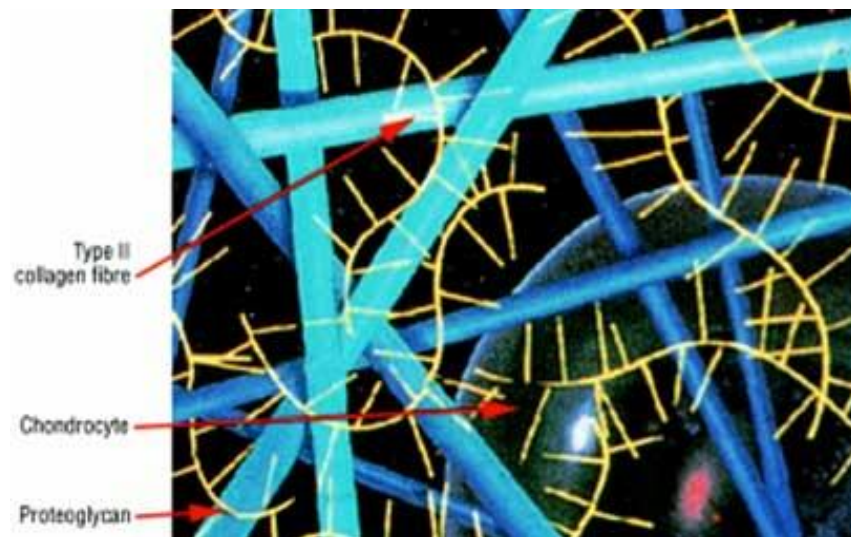


Figure 2.10. A schematic of ultrastructural articular cartilage.

Source: http://www.kneejointssurgery.com/html/articular_cartilage/anatomy.html

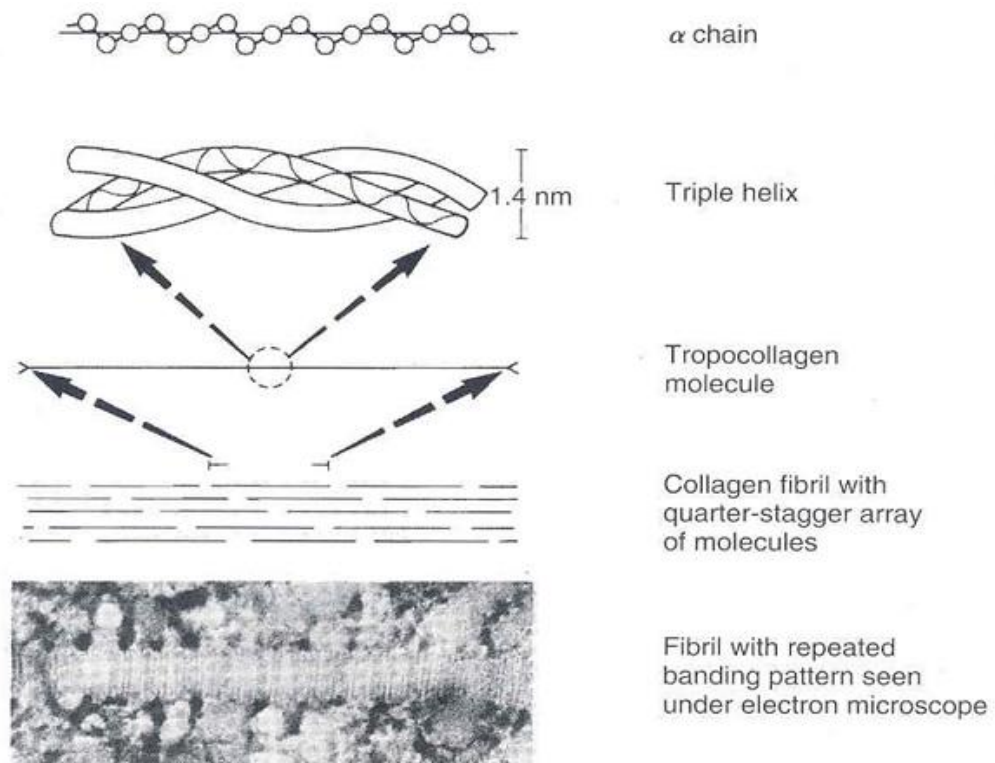


Figure 2.11. Collagen structure.

Source: <https://www.msu.edu/course/kin/831/ Cartilage.ppt#466,11,Collagen Structure>

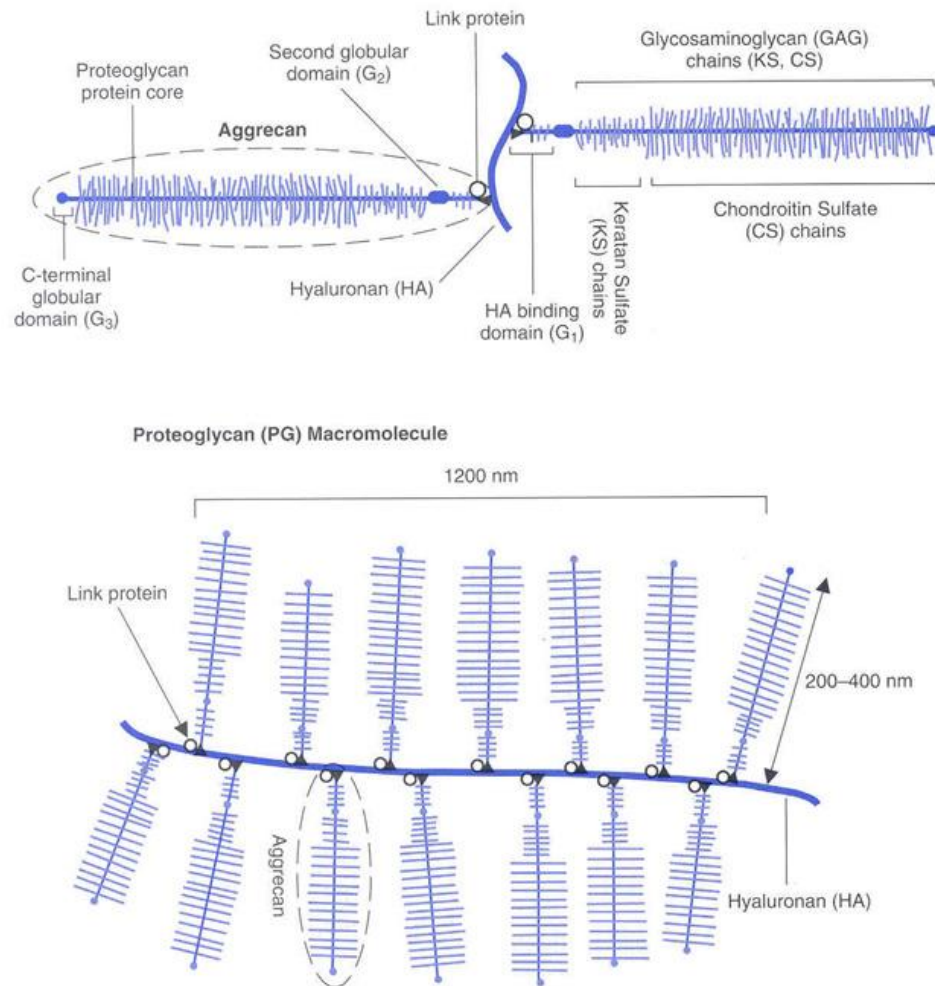


Figure 2.12. Proteoglycan structure.

Source: <https://www.msu.edu/course/kin/831/Cartilage.ppt#471,19,ProteoglycanAggregate>

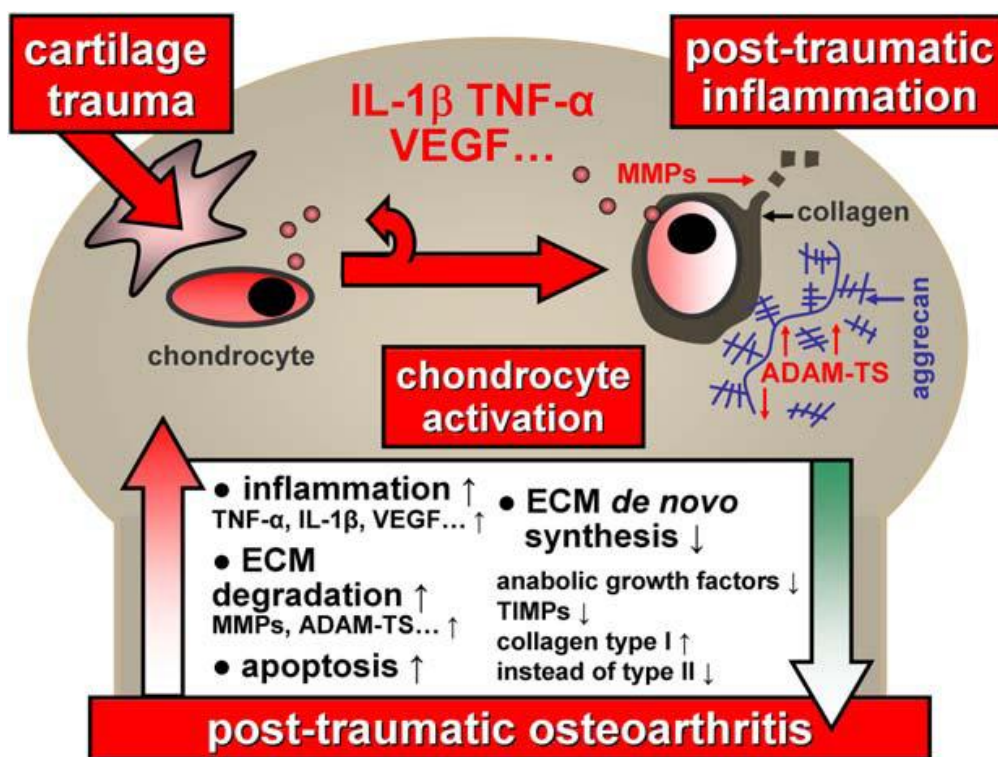


Figure 2.13. Chondrocyte activation in response to traumatic cartilage injury [178].

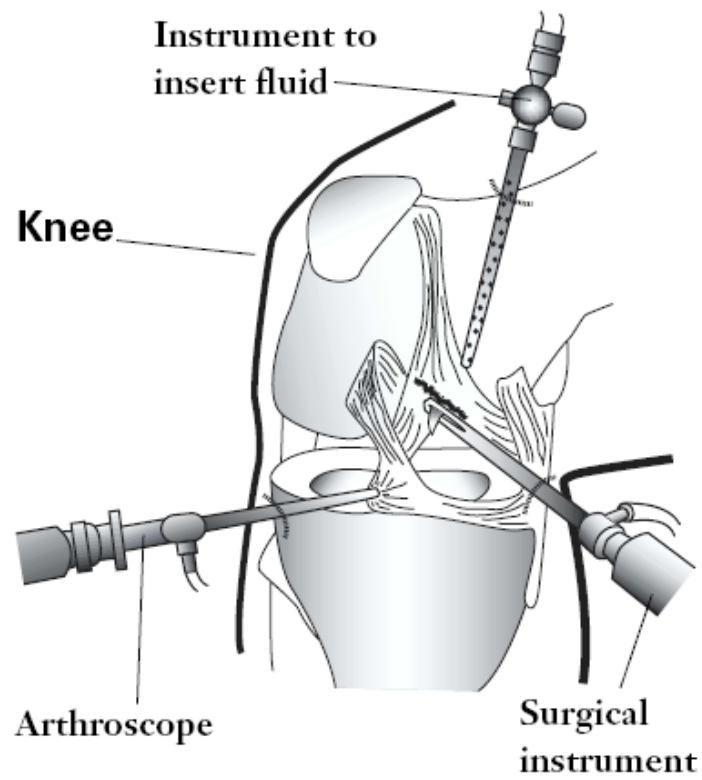


Figure 2.14. Knee arthroscopy. Knee arthroscopy gives a view of the inside of the knee joint. This procedure allows the surgeon to determine if patients have a knee injury or abnormality.



Figure 2.15. Knee microfracture [179].

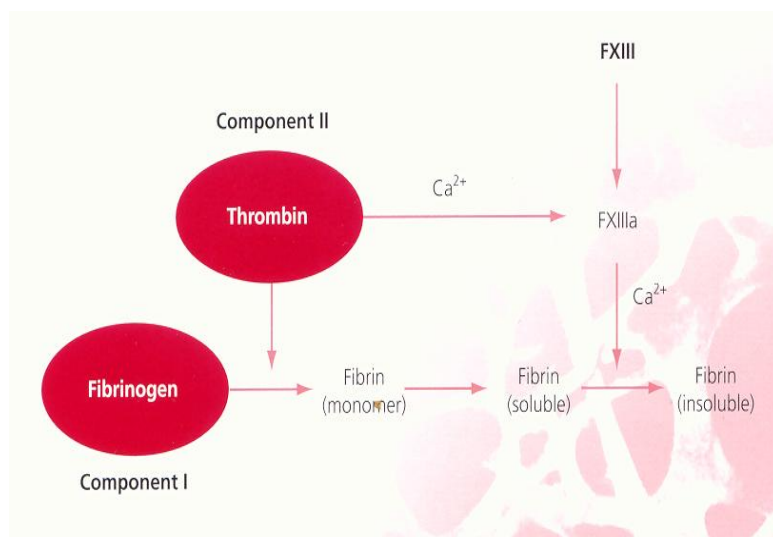


Figure 2.16. Fibrin hydrogel. Fibrinogen is converted into fibrin monomer by thrombin.

CHAPTER 3

CHONDROGENIC PROGENITOR CELL RESPONSE TO CARTILAGE INJURY

3.1. Purpose of Study

There are currently a few studies focusing on progenitor cells in cartilage. These cells exhibit stem cell characteristics such as migratory activity, high colony formation, and multipotential differentiation. During the development of articular cartilage, progenitor/stem cell population resides in the surface zone of cartilage and allows the appositional growth [16, 26]. Koelling *et al.* [23] also found progenitor cells in repaired human articular cartilage during the late stages of osteoarthritis. However, there is no report introducing cartilage progenitor cells in traumatic injured articular cartilage. In this chapter, we will find chondrogenic progenitor cells (CPCs) in injured bovine explain and identify their characteristics and cartilage repair potential. Here are specific aims.

- a. Finding of elongated fibroblast-like cells in cartilage injury
- b. Characteristics of chondrogenic progenitor cells
 - i) Colony formation
 - ii) Cell migration
 - iii) Side population
 - iii) Multipotent differentiation
- c. Identification of chondrogenic progenitor cells
 - i) Side population
 - ii) Gene expression

3.2. Materials and Methods

3.2.1. Harvesting of bovine articular cartilage explant

Mature bovine stifle joints were obtained after slaughter from a local abattoir (Bud's Custom Meats, Riverside, IA). Osteochondral explants were prepared by manually sawing an approximately 25 x 25 mm² from bovine tibial plateau, which included the central loaded area of the articular surface. The explants were rinsed in HBSS and cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA), 50 µg/ml L-ascorbate, 100 U/ml penicillin, 100 µg/ml streptomycin, and 2.5 µg/ml Fungizone. The medium was changed every 3 days.

3.2.2. Traumatic cartilage injuries

The explants were secured in custom testing fixtures for impact loading and were kept submerged in culture medium at all times. A drop tower was used to impart loads to an indenter resting on the explant surface (Figure 3.1). The indenter was a flat-faced, a 5 mm-diameter flat-ended platen. Impact energy was modulated by dropping a 2-kg mass from a height of 14 cm, resulting in an impact energy density of 14 J/cm². The mass was removed from the platen immediately after impact. Full-thickness cartilage defects were created using 4-mm biopsy punch. After 2-day pre-culture, 50 mg/ml fibrinogen and 10 U/ml thrombin (TISSEEL, Baxter Healthcare Corp., Westlake Village, CA) are equally mixed together and implanted into the defects. At one week, the explants were stained by 1 µg/ml Calcein AM (green color) and 1 µM Ethidium homodimer-2 (red color) (Invitrogen) for confocal examination. After three weeks, they were fixed in 10% neutral-buffered formalin, isolated cartilage from the bone, embedded in tissue freezing medium,

sectioned (10 μm) by a cryostat and stained with Safranin-O/fast green. Cartilage scratch injury was manually created using a 22G needle to create partial thickness tears in the matrix and localized chondrocyte death (within ~ 100 microns of the matrix tears) at the center of the explant. All explants were cultured under hypoxic culture condition (5% O_2/CO_2 at 37°C). To observe pCPC migration we embedded minced cartilage fragments (approximately 1 mm^3) from the bovine distal femur in fibrin hydrogel. The morphology and migration of cells was observed by light microscopy.

To stain collagen type VI in pericellular matrix, immunohistochemistry was performed. According to a standard protocol, sections were digested by testicular hyaluronidase (1,600 units/ml) and then incubated with primary antibody (rabbit polyclonal, diluted 1:100, Abcam Inc., Cambridge, MA) and goat anti-mouse secondary antibody (Alexa Fluor 568). Images were obtained from Zeiss LMS 710 confocal microscope (Carl Zeiss, Jena, Germany).

3.2.3. Cell isolation

After five to seven days post-injury, the explant surfaces were submerged in 0.25% trypsin-EDTA and were incubated for 20 minutes to detach migrating progenitor cells from the superficial surface of bovine explant. To recover normal chondrocytes the underlying cartilage which was not injured was then digested overnight with collagenase type 1 and pronase E (Sigma-Aldrich, St. Louis, MO) dissolved in culture medium (0.25 mg/ml each). For some experiments a custom-fabricated device was used to separate the superficial 1/3 from the deep 2/3 of the cartilage prior to collagenase/pronase digestion (Figure 3.2). This apparatus allowed attachment of a micrometer with precision of $2\ \mu\text{m}$ and a blade.

3.2.4. Green Fluorescent Protein transduction

Isolated pCPCs transduced with Lentivirus expressing Green Fluorescent Protein (GFP, 488 nm) and cultured for 5 days. The transduction efficiency reached approximately 40%. GFP-labeled cells (1×10^5) were suspended in a temperature-sensitive hydrogel, 0.6% (w/v) sodium hyaluronate (Easy Motion Horse product Inc., Niagara Falls, NY) and 18% (w/v) Pluronic F-127[®] (BASF, Gurney, IL) [180, 181]. The suspension was overlain on explant surfaces adjacent to a blunt impact site. The explants were incubated for 5 days when they were counterstained with 0.5 μ M Cell Tracker Red CMTPX (Invitrogen, 605 nm) and imaged on a Bio-Rad 1024 laser scanning confocal microscope (LSCM) (Bio-Rad Laboratories Inc., Hercules, CA) equipped with a custom built stage driver to enable repeated imaging of the same site over days in culture. The sites were scanned to an average depth of 330 μ m at 40 μ m intervals. Z-axis projections of confocal images were acquired using ImageJ (rsb.info.nih.gov/ij).

3.2.5. Colony formation assay

For colony forming assays, 200 cells (pCPCs cells or normal chondrocytes from the superficial 1/3 or lower 2/3 of the matrix) were plated in 150-mm culture dishes and incubated in hypoxic culture condition (5% O₂/CO₂ at 37°C) for 10 days. The colonies were fixed in 10% neutral-buffered formalin and visualized by Richardson's stain. The number of colonies was counted and each colony area was measured by ImageJ. Dishes were scanned on a flat bed scanner (300 dpi) and the number of colonies and colony diameters were measured using ImageJ.

3.2.6. Cell migration assay

Cell migration assay was measured in chemotaxis assays. Two known stem cell chemokines, conditioned medium from injured explants, and chondrocyte lysates were used as chemotactic factors. The chemotactic factors used to activate cell migration in this experiment. Stromal cell-derived factor 1 beta (SDF-1 β) and Interleukin 8 (IL-8) are members of the CXC subfamily of chemokines that have been shown to attract mesenchymal stem cells and progenitor cells [182, 183]. SDF-1 β and IL-8 (Invitrogen) were dissolved in culture medium at a concentration of 500 nM. Conditioned medium was made by incubating blunt-impacted explants overnight in 10 ml serum-free medium for 24 hours after impact. The conditioned medium was concentrated 10-fold using Amicon[®] Ultra centrifugal Filters 10K (Millipore, Billerica, MA). Chondrocyte lysates were obtained by repeated freeze-thaw of 3×10^6 chondrocytes. Cell migration/chemotaxis assays were performed using CytoSelect[™] 24-Well Cell Invasion Assay kit (Cell Biolabs Inc., San Diego, CA) which contained polycarbonate membrane inserts (8 μ m pore size) with a uniform layer coated dried bovine type I collagen matrix. Briefly, cell suspensions, 3×10^5 cells in serum free media, were added to the upper insert chamber of each well with the reservoir below containing chemotactic agents in serum free media. After incubating for 24 hours, migrated cells were dissociated and stained. 200 μ l of each sample was transferred to fluorescence plate and read at 485/538 nm with a SpectraMax M5 multi-detection microplate reader (Molecular Devices Inc., Sunnyvale, CA). The data are presented as the percentage of migrating cells in wells containing a chemotactic factor (# migrated into the bottom chamber / # seeded in the top chamber) normalized to serum free medium control.

3.2.7. Flow cytometry assay for side population (SP)

Side population (SP) assays were performed essentially as described [18]. First passage pCPCs and normal chondrocytes (1×10^6) isolated from three different explants were stained with 2.5 $\mu\text{g/ml}$ Hoechst 33342 (Invitrogen) and incubated for 1.5 hours at 37°C. The cells were washed in cold HBSS once and stained 2 $\mu\text{g/ml}$ propidium iodide (PI, Sigma-Aldrich) for dead-cell discrimination. 50 mM Verapamil (Sigma-Aldrich), inhibitor of ATP-binding cassette transporter, was used for negative control. After filtering using a 70 μm nylon mesh, the stained cells analyzed SP using fluorescence-activated cell sorter (FACS, Becton Dickinson LSRII Flow Cytometer). SP cells were defined in a distinct dim tail extending first on the left side of G0/G1 cells toward the lower Hoechst Blue signal.

3.2.8. Multipotent differentiation

The multipotency of pCPCs was tested by culturing under chondrogenic, osteogenic and adipogenic conditions. For chondrogenic differentiation, 1.2 million cells were pelleted and incubated in chondrogenic medium (DMEM containing 10 ng/ml TGF- β 1, 0.1 μM dexamethasone, 25 $\mu\text{g/ml}$ L-ascorbate, 100 $\mu\text{g/ml}$ pyruvate, 50 mg/ml ITS+Premix and antibiotics) for 14 days. The pellets were analyzed for proteoglycan-rich matrix formation using Safranin-O/fast green staining of cryosections. To induce osteogenic differentiation, 3×10^4 trypsinized migrating cells were cultured in osteogenic medium (DMEM/F-12 containing 0.1 μM dexamethasone, 100 mM β -glycerophosphate, 50 $\mu\text{g/ml}$ L-ascorbate and antibiotics) for 14 days and stained with Alizarin Red to detect deposition of calcium phosphate mineralization. We used STEMPRO[®] adipogenesis differentiation kit (GIBCO, Grand Island, NY) to induce adipogenesis. After 14-day post-

induction, the cells were stained with Oil Red O and imaged on Nikon XB inverted microscope.

3.2.9. Microarray analysis

For microarray analysis we isolated RNA from primary cultures of bovine bone marrow stromal cells (BMSCs), from freshly harvested pCPCs, and directly from freshly harvested normal chondrocytes (NCs) using collagenase/pronase digestion of explant cartilage. RNA from three batches of cells or cartilage was pooled for analysis. Cell and cartilage were homogenized in TRIzol[®] reagent (Invitrogen[™] Life Technologies, Carlsbad, CA) and total RNA was extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instruction. 50 ng RNA was converted to SPIA amplified cDNA using the Ovation[™] RNA Amplification System v2 (NuGEN Technologies, Inc., San Carlos, CA) according to the manufacturer's recommended protocol. The amplified SPIA cDNA product was purified through a QIAGEN QIAquick PCR Purification column according to modifications from NuGEN. 3.75ug of this product was fragmented (average fragment size of 85 bp) and biotin was labeled using the FL-Ovation[™] cDNA Biotin Module v2 (NuGEN Technologies, Inc.). The resulting biotin-labeled cDNA was mixed with Affymetrix prokaryotic hybridization buffer, placed onto Bovine Genome Arrays (Affymetrix, Inc., San Carlos, CA), and incubated at 45° C for 18 h with 60-rpm rotation in an Affymetrix Model 640 Genechip Hybridization Oven. Following hybridization, the arrays were washed, stained with streptavidin (Thermo Fisher Scientific, Rockford, IL), signal amplified with antistreptavidin antibody (Vector Laboratories, Inc., Burlingame, CA), followed by streptavidin-phycoerythrin (Molecular Probes, Inc., Eugene, OR), using the Affymetrix Model 450Fluidics Station (Affymetrix,

Inc.). Arrays were scanned with the Affymetrix Model 3000 scanner and data were collected using the GeneChip operating software (MAS) v5.0.

3.2.10. Statistical analysis

In the colony formation and cell migration assay, statistical analysis was performed using SPSS software (Ver.10.0.7, SPSS Inc., Chicago, IL, USA) with a one-way ANOVA and post-hoc pairwise comparison. SP data were evaluated by Students t-test. All the results were expressed as mean \pm standard deviation. Statistical significance was set at $p < 0.05$ and minimum acceptable power set at 0.9.

3.3. Results

3.3.1. Finding of elongated fibroblast-like cells in cartilage injury

Confocal microscopy revealed elongated, fibroblast-like cells in various injuries of bovine cartilage. We prepared three cartilage injury models, scratch, defect and blunt impact. To simulate cartilage tear model, we applied needle scratch on the surface of cartilage. Injured scratch areas with dead cells were covered by elongated progenitor cells after 14 days (Figure 3.3A and B). We could also observe large numbers of migrated cells in a full-thickness cartilage defect embedded with fibrin hydrogel (Figure 3.3C and D). In blunt impact-injury, these cells began to accumulate 5 days after impact and gradually repopulated previously uninhabited areas where chondrocytes had been killed and the matrix damaged by impact injury (Figure 3.3E-G). To confirm that the cells were migrating to injury sites, we harvested surface adherent migrating cells, transduced them with GFP, and grafted the labeled cells 4 mm away from a freshly made impact site on another explant. The labeled cells could be observed in the impact-injured areas after 5 days (Figure 3.3I) and had filled-in depopulated areas by 12 days (Figure 3.3J).

In order to investigate the morphology and source of migrating cells, we cultured cartilage fragments (approximately 1.0 mm^3) in fibrin hydrogel. After 10 days, many elongated cells had emerged from the fragments and migrated into the surrounding fibrin (Figure 3.4A). Pockets of proliferating chondrocytes were found near the cut surface/fibrin interface. Confocal microscope images of calcein AM-stained cells clearly showed the elongated shape of the migrating cells, which was distinct from the rounded shape of normal chondrocytes. (Figure 3.4B). Migrating cells were also found in a cartilage defect model. Most cells occupying the fibrin filler were elongated, but a few cells were morphologically similar to normal chondrocytes and deposited proteoglycans in the pericellular matrix (Figure 3.4C). In the host cartilage tissue, a number of proliferating cells were distributed around defect areas and in the superficial zone of the cartilage (Figure 3.4E and F), but not in cartilage distant from the defect (Figure 3.4D). Interestingly, we could also observe empty chondrons around defect injury (Figure 3.4E and F). Occasionally, there were small, spindle-shaped cells in the middle and deep zones that resembled progenitor cells in other tissues (Figure 3.4G and H).

Cells need to break their own matrix, pericellular matrix, to migrate into injured area. To confirm any change of pericellular matrix, we stained collagen type VI which is unique collagen type in the pericellular matrix. Pericellular matrixes were well located around chondrocyte(s) in normal cartilage (Figure 3.5A). In sharp cartilage injury, the matrixes were shown dramatic morphologic changes with matrix debris after 10 days (Figure 3.5B). This phenomenon could also be observed in fibrin hydrogel embedding on cartilage surface (Figure 3.5C) and defect model (Figure 3.5D and E). Migrated cells on

the surface and inside hydrogel made their own pericellular matrix to differentiate cartilage cells.

3.3.2. Colony formation

After 5-7 days post-impact, cells migrating on the cartilage surface were detached by trypsin treatment. Most elongated cells were clearly detached from cartilage tissue after trypsin treatment (Figure 3.6A and B). In contrast, chondrocytes in adjacent impacted cartilage did not detached by trypsin (Figure 3.6C-E). The upper 1/3 of the cartilage, which included the superficial and transitional zone, was separated from the bottom 2/3, which included the transitional and deep zones. Chondrocytes were isolated separately from both layers by collagenase-pronase digestion of the matrix. Primary cultures were established and the cells harvested for colony formation assays after 5-7 days in culture. Trypsinized migrating cells were shown rapid colony growth rate at 2 days (Figure 3.7A), 3 days (Figure 3.7B), and 6 days (Figure 3.7C) than chondrocytes cultured for 13 days (Figure 3.7D). Ten days after seeding, the colonies were stained with Richardson's and the total number of colonies and colony area were measured. Trypsinized migrating cells showed the most vigorous colony formation (Figure 3.7E). Colony formation was evaluated in terms of the total number of colonies (Figure 3.7F) and average colony size (Figure 3.7G). Trypsinized migrating cells and chondrocytes from the upper 1/3 of the cartilage matrix showed significantly higher numbers of colonies (approximately 150 and 120, respectively) than chondrocytes from the bottom 2/3 of the matrix (approximately 20 colonies) ($p < 0.001$). The average colony size for trypsinized migrating cells of 20 mm^2 was significantly greater than for chondrocytes

from the upper 1/3 or lower 2/3 of the matrix, which both showed an average of less than 5 mm² ($p < 0.001$).

3.3.3. Cell migration

In *in vitro* cell migration assays (Figure 3.9), SDF-1 β induced increased trypsinized migrating cell migration compared to serum free medium as a control ($p < 0.05$). In contrast, there was no significant migratory effect of IL-8. We also prepared 10-fold concentrated medium cultured from impact-injured explants and chondrocyte lysates to simulate traumatic injury to cartilage. The number of lysed cells applied (1.5×10^6) was not cytotoxic and showed significantly increased cell proliferation ($p < 0.01$) (Figure 3.8). Chemotactic activity was more significant in 10-fold concentrated medium and chondrocyte lysates (3×10^6) compared with SDF-1 β . In particular, cell lysates induced significantly increased migration of trypsinized migrating cells than that of chondrocytes ($p < 0.001$).

3.3.4. Side population

We performed side population discrimination assay to detect stem cells/progenitor cells based on the Hoechst dye efflux properties of ATP-binding cassette transporters. The proportion of SP was highly depended on the concentration and incubating time of Hoechst dye. Based on efflux inhibitor control, verapamil, we established optimal condition, 2.5 $\mu\text{g/ml}$ for 1.5 hours. Flow cytometry analysis of Hoescht-stained revealed side populations of cells capable of dye exclusion in both pCPCs and NCs (Figure 3.10). Their numbers in pCPCs were significantly greater than in NCs (0.22 \pm 0.07% versus 0.013 \pm 0.012% respectively, $p < 0.001$) (Figure 3.10D). Verapamil treatment reduced

side populations to less than 0.005%, indicating that the efflux mechanism depended on the stem cell-associated ABCG2 transporter (Figure 3.10B).

3.3.5. Multipotent differentiation

In order to evaluate their differentiation potential, trypsinized migrating cells were cultured in chondrogenic, osteogenic, or adipogenic media for 14 days. After the induction of chondrogenic differentiation, the cultured pellets were fixed and evaluated with Safranin-O/fast green staining. The pellets developed proteoglycan-rich matrix over the whole area of the pellet culture (Figure 3.11A). Similarly, most cells in osteogenic medium deposited a calcium phosphate-rich mineralized matrix as detected by Alizarin Red staining (Figure 3.11B). However, few cells (< 1%) stained with Oil Red O after 2 weeks of culture in adipogenic medium (Figure 3.11C).

3.3.6. Microarray

Gene expression profiling revealed substantial differences in the phenotypes of pCPCs, MSCs and NCs and 5-fold up- or down- regulated genes were listed in Table 3.1 and Table 3.2. Principal component analysis of the overall relatedness of the profiles indicated that pCPCs are a distinct population that maps nearly equidistant from NCs and MSCs. NCs produced higher levels of mRNAs encoding cartilage-specific matrix proteins than either MSCs or CPCs and the Wnt pathway, which drives chondrogenic and osteogenic differentiation, appeared to be down-regulated in MSCs and CPCs relative to NCs. Expression of the Wnt pathway inhibitor dickkopf homologue 3 (DKK3) in CPCs and MSCs was 6- and 13-fold higher respectively than in NCs. Similarly, the secreted Wnt inhibitor, frizzled-related protein 4 (SFRP4) was elevated by 10-fold over NCs in pCPCs and by 25-fold in MSCs over NCs. The Wnt receptor, frizzled

homologue 9 (FZD-9), was under-expressed in CPCs and MSCs by approximately 14-fold relative to NCs. In contrast, MSCs and CPCs showed much higher levels of Wnt10b (8 and 40-fold increases respectively), an inhibitor of adipogenesis. Migration and growth-related genes were expressed at higher levels in MSCs and CPCs than NCs. Chemokines involved in stem and progenitor cell chemotaxis [184, 185] were strikingly up-regulated in CPCs versus NCs. CXCL2, CXCL5, and CXCL12 (stromal cell derived factor-1) mRNA levels were more than 10 times greater in CPCs than in NCs, while CXCL8 (interleukin 8) was increased by 41-fold. MSCs also showed increased CXCL12 expression compared with NCs (27-fold increase), but other chemokines were not elevated, CXCR7, which serves as a receptor for CXCL12 was 4-fold higher in CPCs than in MSCs and NCs. Doublecortin-like kinase 1 (DCLK), a regulator of microtubule polymerization in cell migration [186, 187] was elevated by 9-fold and 24-fold over NCs in CPCs and MSCs respectively. The migratory activity of CPCs and MSCs appeared to be further enhanced by matrix protease expression and activity: Compared with NCs, CPCs and MSCs expressed 7-fold to 12-fold higher levels of the matrix peptidase ADAMTS-1 and -4, and 135-150-fold lower levels of the serine peptidase inhibitor SERPINA1. Genes involved in cell division including Aurora kinases A and B and cyclin B were expressed at 5-12-fold higher levels in CPCs and MSCs versus NCs. The two most highly up-regulated genes in CPCs were N-cadherin (CDH2) and defensin b-1 (EBD), which were increased 68 and 74-fold respectively above NC levels. MSCs showed even higher levels of CDH2 than did NCs (165-fold increase), but EDB was only moderately elevated compared with NCs (4-fold). Interestingly, CPCs also over-expressed CD117/KIT, the hematopoietic stem cell

growth factor receptor by 17-fold compared with NCs and the dendritic cell marker CD83 [188] by 21-fold compared with NC. These genes were also modestly increased (3-fold) in MSCs versus NCs [189].

In addition to 5-fold changed genes, we analyzed other important genes. CD44 and CD73 were only available stem cell markers in our microarray and CD44 showed up-regulated expression in in both CPCs (4.3-fold) and MSCs (4.7-fold) than NCs. CPCs differently expressed cartilage ECM genes compared to NCs. Collagen type I (COL1A1 (5.0-fold) and COL1A2 (4.1-fold)) and type VI (COL6A1 (2.2-fold) and COL6A2 (3.5-fold)) were up-regulated in CPCs, but not collagen type II and X. CPCs had more migratory ability which expressed slightly elevated MMP-1, 3 and ADAMTS-1, 4 than NCs. CPCs also over-expressed cytokines such as IL-8 (41.2-fold), transforming growth factor beta 1 (TGF- β 1, 3.8-fold) which is well known to induce the formation of cartilage tissue [190-192], platelet derived growth factor C (PDGFC, 4.6-fold), and bone morphogenetic proteins (BMP2 and BMP4, 3.5- and 2.0-fold, respectively).

3.4. Discussion

The results of these experiments demonstrate that the migrating cells we observed on injured bovine osteochondral explants closely resemble chondrogenic progenitors previously identified in normal and osteoarthritic human cartilage [18, 23]. The cells' chemotactic activity, clonogenicity, limited multipotency, and side population were all notably consistent with published descriptions of progenitor cells from cartilage and other tissues. At the molecular level, pCPCs also over-expressed stem/progenitor-associated

genes compared to NCs; these included Notch-1 (3.4-fold up), $\alpha 5$ integrin (6-fold up), CD44 (4-fold up), and interleukin 6 (16-fold up).

Time-lapse confocal imaging of blunt impact sites revealed the emergence of elongated cells with multiple filipodia onto the surfaces of explants within 3-5 days of injury. Over the next several days these cells congregated in large numbers near matrix cracks, where injury-induced cell death had occurred. Such cells were also observed to migrate to cartilage that was exposed to ultraviolet radiation, (100 mW/60 seconds), which killed chondrocytes, but did not physically disrupt the tissue. Serial imaging studies also showed the migration toward impact sites of GFP-labeled pCPCs that were grafted on adjacent, uninjured cartilage. *In vitro* assays confirmed that medium conditioned by impacted cartilage or whole cell lysates were relatively strong chemoattractants for pCPCs. These data suggest that pCPC migration to impacted cartilage is driven, at least in part, by dead cell debris. Although we have no data on which components(s) are acting as chemoattractants, dead cell debris has been shown to contain a number of homing factors that draw stem and immune cells to injured tissues. Some of these, including nuclear proteins like high-mobility group B1 (HMGB1) protein, as well as mitochondrial DNA and proteins act *via* a toll-like receptors (TLRs) and the receptor for advanced glycation endproducts (RAGE), which are expressed by pCPCs. pCPCs themselves also substantially over-expressed multiple chemokines including CXCL12. Moreover, expression of CXCR7, a CXCL12 receptor, was elevated in pCPCs, suggesting autocrine/paracrine induction of chemotaxis. This would likely amplify progenitor cell recruitment to injured cartilage and continue to promote chemotaxis even after cell debris is cleared.

Histologic observations of impacted explants at 7 days post-injury showed a 3-4 cell layer-thick surface coating of pCPCs in and around damaged cartilage. The cells stained positively for lubricin by immunohistochemistry and microarray data indicated that PRG4 gene expression was elevated by ~2-fold versus NCs. In contrast, expression of PRG4 by MSCs was 15-fold lower than NCs. Thus pCPCs appear to retain some features of superficial zone chondrocytes.

Although the origin of pCPCs is still unclear, their initial appearance on cartilage surfaces in our isolated explant model and the need for cartilage injury to provoke this response strongly suggest that the cells came from the cartilage matrix itself. Cells from the top 1/3 of the cartilage matrix were significantly more clonogenic than cells from the bottom 2/3 of the matrix, suggesting that CPCs were more abundant in the superficial/transitional zones. A chondral defect model allowed us to observe cells exiting the matrix and invading a fibrin gel. Most of the cells emerged from the superficial/transitional zone, but some cells from deeper in the matrix also made the transition, indicating that CPCs may be present throughout cartilage.

pCPCs were able to migrate through collagen and fibrin matrices with relative ease, suggesting high matrix protease activity. Interestingly, gene expression profiling identified the matrix peptidases ADAMTS-1, and -4 as among the most highly up-regulated genes in pCPCs and MSCs (7-11 times higher than in NCs). Further analysis pointed to pCPC/MS-C-specific increases in the expression of cathepsin C, which was 11-fold higher in pCPCs versus NCs and 23-fold higher in MSCs versus NCs. These differences together with 150-fold decreases in serine peptidase inhibitor expression were consistent with the invasive behavior of pCPCs. Other migration-related genes were

relatively over-expressed by pCPCs compared to NCs. These included DCLK (9-fold up), Rac and Rho GTPases (6-8-fold up), and hyaluronan-mediated motility receptor (RHAMM) (8-fold up). The same genes were regulated similarly in MSCs, which are known for their migratory capability.

The rapid accumulation of hundreds of pCPCs at injury sites on explant surfaces was unlikely to be due to migration alone, as that would have noticeably depopulated the surrounding matrix. Rapid proliferation on the cartilage surface is a much more likely explanation. This was corroborated by clonogenic assay data, which showed that pCPCs formed significantly larger colonies than NCs from either the deep or superficial zones, a clear indication of a faster growth rate. Gene expression data showing large relative increases (versus NCs) in mRNAs for cyclins b1 and b2 (15-fold), aurora kinases A and B (7-fold), and dedicator of cytokinesis (32-fold) were also consistent with a highly proliferative phenotype. Expression levels for these genes in MSCs, which are also noted for rapid proliferation, were similar to pCPCs.

Gene expression findings indicated that migratory pCPCs are highly proliferative, motile and invasive. In terms of genes related to regenerative functions, pCPCs have more in common with MSCs than with NCs. However, CPCs also clearly shared many common features with NCs including their similarity in PRG4 expression, a nominal marker of superficial zone chondrocytes. The association of lubricin protein with pCPCs on cartilage surfaces suggested they may indeed specialize as superficial chondrocytes in that context. The intermediate nature of the pCPC expression profile is well illustrated by principal component plot that located pCPCs equidistant between MSCs and NCs, which occupied opposite corners of the box plot.

The multipotency of pCPCs was tested in conventional culture systems. We found that with appropriate stimulation they readily formed cartilaginous or boney matrices, but were unresponsive to adipogenic conditions: less than 1% stained with oil red O compared with more than 14% of bone marrow derived MSCs. This was consistent with the findings of Grogan *et al*, who showed that CPCs isolated from OA cartilage also failed to trans-differentiate into adipocytes [17]. These results and the low numbers of side population cells identified by flow cytometry analysis indicate that 2% or less of the pCPC population were true stem cells, on par with published data on CPCs.

Our work in an *in vitro* bovine injury model leaves many unknowns regarding the clinical significance of CPCs. However, the finding that such a vigorously chondrogenic cell population is activated by acute mechanical injury and homes to damaged cartilage certainly endorses further exploration of their therapeutic potential. Cartilage degeneration in PTOA is thought to start with focal matrix cracking together with the local loss of superficial zone cells and the critical lubricants they provide. In our model, which was isolated from the bleeding, inflammation, and mechanical stresses that would be present *in vivo*, it appeared that both kinds of cartilage damage were repairable by resident CPCs. That this does not seem to occur spontaneously in people or in animals with clinically significant joint injuries, even when a defect-filling scaffold is provided, suggests that conditions *in vivo* in the early post-trauma phase are detrimental to CPCs: Cell mortality, oxidative damage, and physical dislodgement from joint surfaces could all plausibly retard CPC-mediated healing. Thus, we may need to develop interventions to mitigate the effects of secondary pathogenic factors on these cells to exploit their full potential.

Table 3.1. List of 5-fold (CPC vs. NC) up-regulated genes

Gene	Description	I.D.	Fold change		
			CPC	BMSC	BMSC
			vs. NC	vs. NC	vs. CPC
VCL	vinculin	XM_001790292	5.0	7.3	1.4
DKK3	dickkopf homolog 3 (Xenopus laevis)	NM_001100306	5.8	13.2	2.3
ITGA5	integrin, alpha 5	NM_001166500	5.6	3.9	-1.5
ITGA3	integrin, alpha 3	NM_001101900	5.8	12.7	2.2
RND3	Rho family GTPase 3	NM_001099104	6.0	11.6	1.9
TNFSF13B	tumor necrosis factor (ligand) 13b	NM_001114506	6.6	-1.4	-8.9
ADAMTS1	ADAM metallopeptidase with thrombospondin 1	NM_001101080	6.8	11.0	1.6
CXCL2	chemokine (C-X-C motif) ligand 2	NM_174299	12.8	-1.9	-29.1
CCL5	chemokine (C-C motif) ligand 5	NM_175827	7.7	-2.1	-16.0
AURKB	aurora kinase B	NM_183084	7.8	4.9	-1.6
RACGAP1	Rac GTPase activating protein 1	XM_592496	8.0	4.8	-1.7
HMMR	hyaluronan-mediated motility receptor	XM_590028	8.3	3.3	-2.5
WNT10b	wingless member 10b	XM_586498	8.3	41.1	4.9
ADAMTS4	ADAM metallopeptidase with thrombospondin 4	NM_181667	8.5	2.3	-3.8

Table 3.1. Continued

DCLK1	doublecortin-like kinase 1	NM_001109962	8.6	23.8	2.8
SFRP4	secreted frizzled-related protein 4	NM_001075764	9.5	24.6	2.6
AURKA	aurora kinase A	NM_001038028	9.8	4.5	-2.3
CD83	CD83 molecule	NM_001046590	11.3	5.2	-2.2
CXCL5	chemokine (C-X-C motif) ligand 5	NM_174300	14.2	11.4	-1.2
CCNB1	cyclin B1	NM_001045872	14.8	9.1	-1.6
CCNB2	cyclin B2	NM_174264	15.6	5.1	-3.1
KIT	v-kit feline sarcoma viral oncogene homolog	NM_001166484	17.2	4.0	-4.4
CXCL12	chemokine (C-X-C motif) ligand 12	NM_001113174	28.1	27.4	-0.1
CD83	CD83 molecule	NM_001046590	29.9	5.0	-6.0
DOCK10	dedicator of cytokinesis 10	XM_001787477	32.4	67.9	2.1
IL8	interleukin 8	NM_173925	41.2	-1.7	-71.4
CDH2	cadherin 2, type 1, N-cadherin (neuronal)	NM_001166492	67.8	165.3	2.4
EBD	defensin, beta 1	NM_175703	74.5	4.5	-16.7

Table 3.2. List of 5-fold (CPC vs. NC) down-regulated genes

Gene	Description	I.D.	Fold change		
			CPC	BMSC	BMSC
			vs. NC	vs. NC	vs. CPC
SOD2	superoxide dismutase 2, mitochondrial	NM_201527	-5.1	-7.7	-1.5
SIRT3	sirtuin 3	XM_873980	-5.1	-5.1	1.0
COL9A1	collagen type IX, alpha1	XM_601325	-5.5	-5.4	0.0
FGFRL1	fibroblast growth factor receptor-like 1	XM_610839	-5.6	-10.8	-2.0
INSR	insulin receptor	XM_590552	-5.6	-6.0	-1.1
TIMP4	TIMP metalloproteinase inhibitor 4	NM_001045871	-6.1	-5.7	1.1
HSPA1A	heat shock 70kDa protein 1A	NM_174550	-6.1	-3.8	1.6
NKIRAS2	NFKB inhibitor interacting Ras-like 2	NM_001075387	-6.7	-5.5	1.2
ARHGEF6	Rac/Cdc42 guanine nucleotide exchange factor 6	XM_865157	-7.2	-4.2	1.7
FOXA3	forkhead box A3	NM_001033119	-8.6	-10.2	-1.2
ACAN	aggrecan	NM_173981	-10.1	-23.6	-18.7
FZD9	frizzled homolog 9 (Drosophila)	XM_599625	-12.3	-14.1	-1.1
WNK4	WNK lysine deficient protein kinase 4	XM_001790559	-12.8	-10.9	1.2

Table 3.2. Continued

GPX3	glutathione peroxidase 3 (plasma)	NM_174077	-13.1	-16.8	-1.3
COL9A2	collagen type IX, alpha2	XM_582312	-41.0	-73.7	-1.8
COL10A1	collagen, type X, alpha 1	NM_174634	-42.6	-42.6	1.0
FRZB	frizzled-related protein	NM_174059	-112.3	-96.1	1.2
COMP	cartilage oligomeric matrix protein	NM_001166517	-125.6	-116.8	1.1
CHAD	chondroadherin	NM_174019	-130.3	-109.9	1.2
SERPINA1	serpin peptidase inhibitor, clade A	NM_173882	-150.3	-135.0	0.1

Table 3.3. List of cartilage-related genes

Gene	Description	I.D.	Fold change		
			CPC	BMSC	BMSC
			vs. NC	vs. NC	vs. CPC
Stem cell markers					
CD44	CD44 molecule	NM_174013	4.3	4.7	1.1
NT5E	5'-nucleotidase, ecto (CD73)	NM_174129	-1.5	-1.1	1.3
Cartilage-related matrix protein genes					
COL1A1	collagen, type I, alpha 1	NM_001034039	5.0	6.9	1.3
COL1A2	collagen, type I, alpha 2	NM_174520	4.1	5.2	1.3
COL2A1	collagen, type II, alpha 1	NM_001001135 /NM_001113224	-11.2	-19.1	-2.0
COL6A1	collagen, type VI, alpha 1	NM_001143865 /XM_588755	2.2	-1.1	-2.6
COL6A2	collagen, type VI, alpha 2	NM_001075126	3.5	2.1	-1.6
COL10A1	collagen, type X, alpha 1	NM_174634	-42.6	-42.6	1.0
PRG4	proteoglycan 4	XM_606494	2.9	-13.6	-31.4
ACAN	aggrecan	NM_173981	-10.1	-187.6	-18.7
Matrix-degrading enzymes					
MMP1	matrix metalloproteinase 1 (interstitial collagenase)	NM_174112	3.0	-9.4	-27.9

Table 3.3. Continued

MMP2	matrix metallopeptidase 2 (gelatinase A, 72kDa gelatinase, 72kDa type IV collage)	NM_174745	2.8	7.5	2.7
MMP3	matrix metallopeptidase 3 (stromelysin 1, progelatinase)	XM_586521	-1.8	-32.8	-18.5
ADAMTS1	ADAM metallopeptidase with thrombospondin type 1 motif, 1	NM_00110108 0	6.8	11.0	1.6
ADAMTS2	ADAM metallopeptidase with thrombospondin type 1 motif, 2	NM_174631	1.2	2.5	2.0
ADAMTS4	ADAM metallopeptidase with thrombospondin type 1 motif, 4	NM_181667	8.5	2.3	-3.8
FN1	fibronectin 1	NM_00116377 8 / XM_864390 / XM_873485 / XM_873966 / XM_874054 / XM_8741	1.4	-1.4	-2.1
Cytokines					
IL8	interleukin 8	NM_173925	41.2	-1.7	-71.4
TNF	tumor necrosis factor (TNF superfamily, member 2)	NM_173966	-1.1	1.0	1.1
TGF- β 1	transforming growth factor, beta 1	NM_00116606 8 /XM_592497	3.8	4.8	1.2

Table 3.3. Continued

SDF2	stromal cell-derived factor 2	NM_00103432 1	-1.2	-1.2	-1.1
SDF4	stromal cell derived factor 4	NM_00103537 5	-1.0	-1.1	-1.1
PDGFC	platelet derived growth factor C	XM_864899	4.6	8.1	1.8
BMP2	bone morphogenetic protein 2	NM_00109914	3.5	8.0	2.3
BMP4	bone morphogenetic protein 4	NM_00104587 7	2.0	4.7	2.3

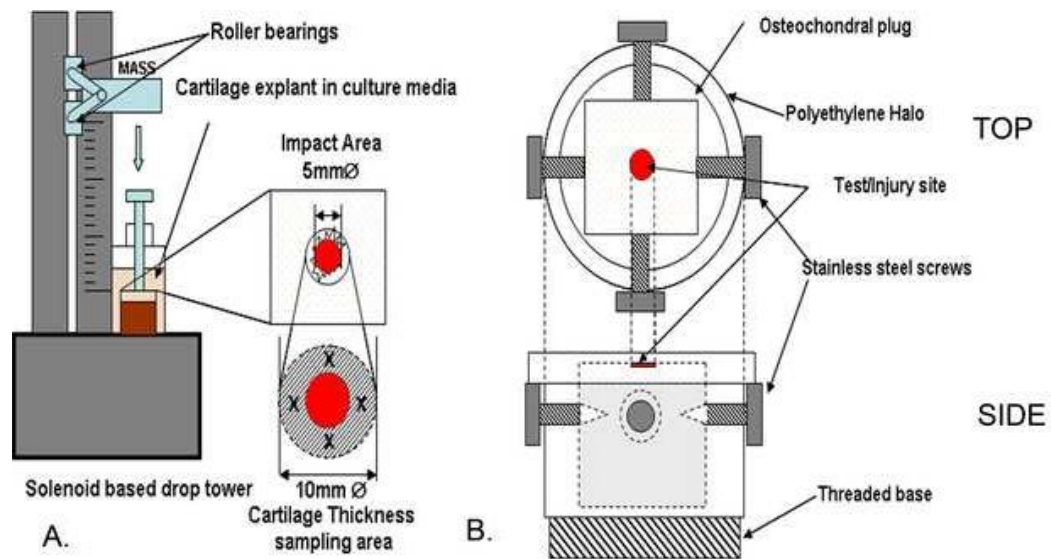


Figure 3.1. Drop tower device. The drop tower assembly (A) was used to induce the impact injury on the osteochondral explant. The explant was secured in the impactor using steel pins interfaced with the subchondral bone to ensure rigidity. The polyethylene halo (B) facilitated convenient handling of the tissue during mechanical testing. Prior to mechanical testing, cartilage thickness at the testing site was estimated manually (by the operator) using a K-wire/polymer stopper instrument and a digital caliper [193].

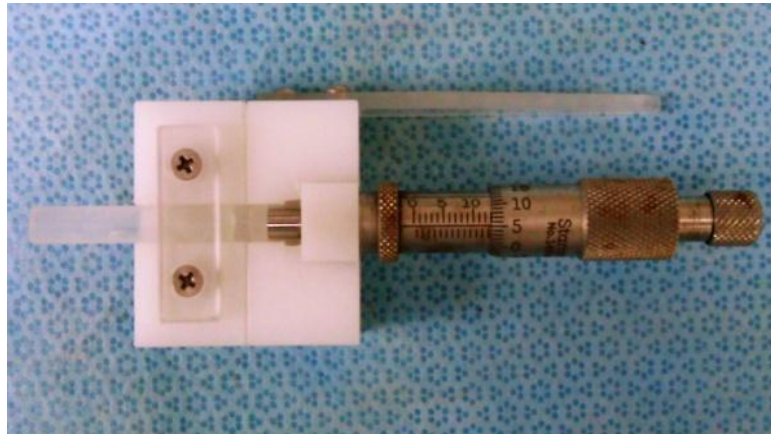


Figure 3.2. Custom-made measurement device. The device was used to separate the superficial 1/3 from the deep 2/3 of the cartilage and allowed attachment of a micrometer with precision of 2 μm and a blade.

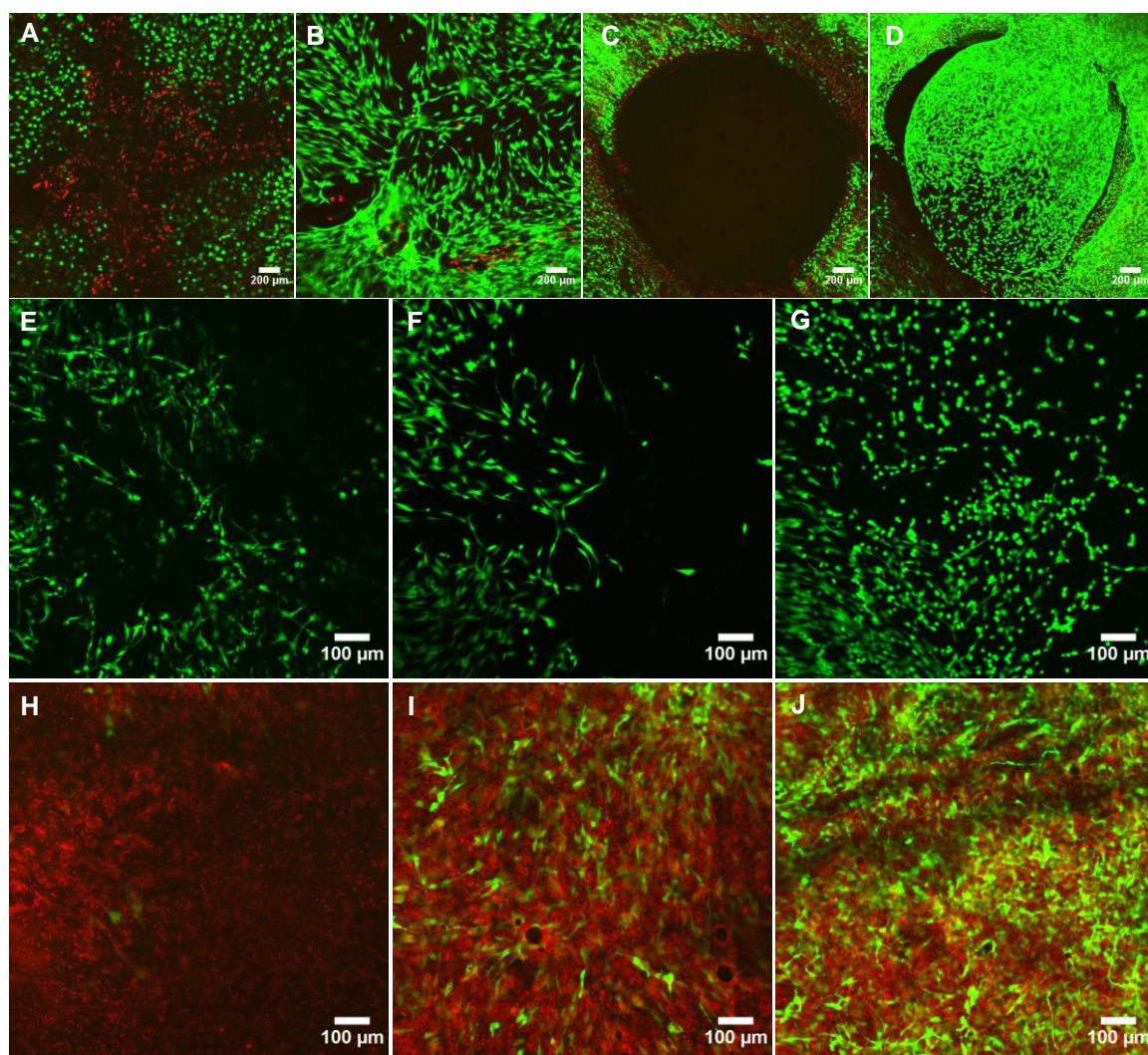


Figure 3.3. Repopulation of progenitor cells in injured cartilage. (A and B) Confocal images of a scratch injured explant. Needle scratch created sharp injury (approximately 300 μm) with cell death (Ethidium homodimer-2, red) (A). After 14 days, the scratch defects were covered by elongated progenitor cells (Calcein AM, green) (B). (C and D) Confocal images of a full-thickness defect injured explant. The defect induced cell death around the edge (C). After 7 days, a number of progenitor cells migrated into fibrin hydrogel implanted in defect (D). (E-G) Confocal images of an impact-injured explant. These elongated progenitor cells were migrated into the injury site at 7 days (E), 11 days

(F), and 15 days (G) post-impact. (H-J) Confocal images of migrated GFP-labeled progenitor cells in an impact-injured explant. GFP-labeled cells (green) implanted adjacent an impact area were migrated into the injured site at 2 days (H), 5 days (I), and 12 days (J) post-impact. Red: endogenous chondrocytes.

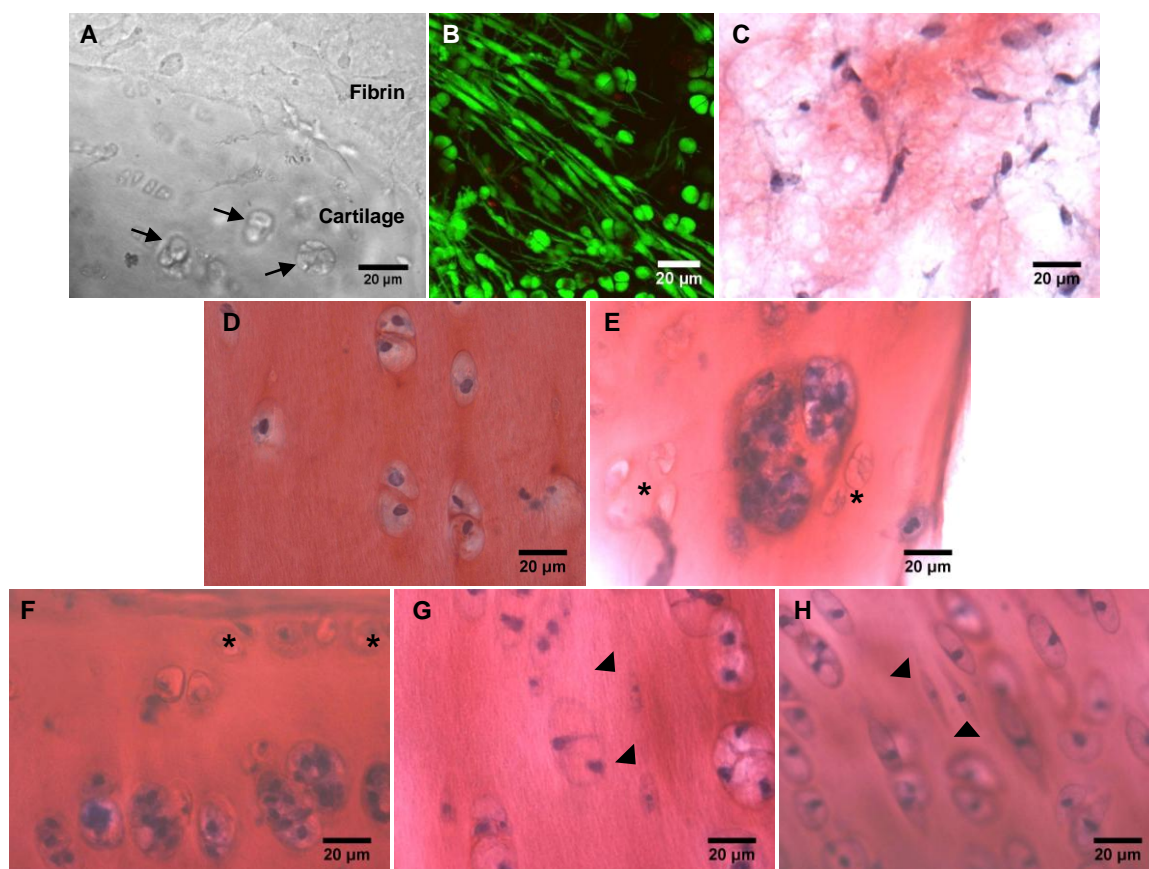


Figure 3.4. Morphologic examination of trypsinized migrating cells. (A) Light microscopic image of cells migrating out of a bovine cartilage fragment into fibrin hydrogel at 10 days. The arrow indicates proliferating cells in the cartilage tissue. (B) Confocal image in defect-injured cartilage. Elongated cells were migrating toward a cartilage defect at 3 days. (C-I) Safranin-O/fast green stain of a defect-injured cartilage. The defect was filled with cell-free fibrin. After 18 days of culture the fibrin was filled with cells (C). In the host cartilage tissue, many proliferating progenitor cells were found in the edge of defect injury (E) and the superficial zone (F), but not in the cultured normal cartilage (D). Interestingly, we could also observe empty chondrons around defect injury (asterisk) Atypically small, spindle-shaped cells were observed in the middle (G) and deep (H) zones (arrowheads).

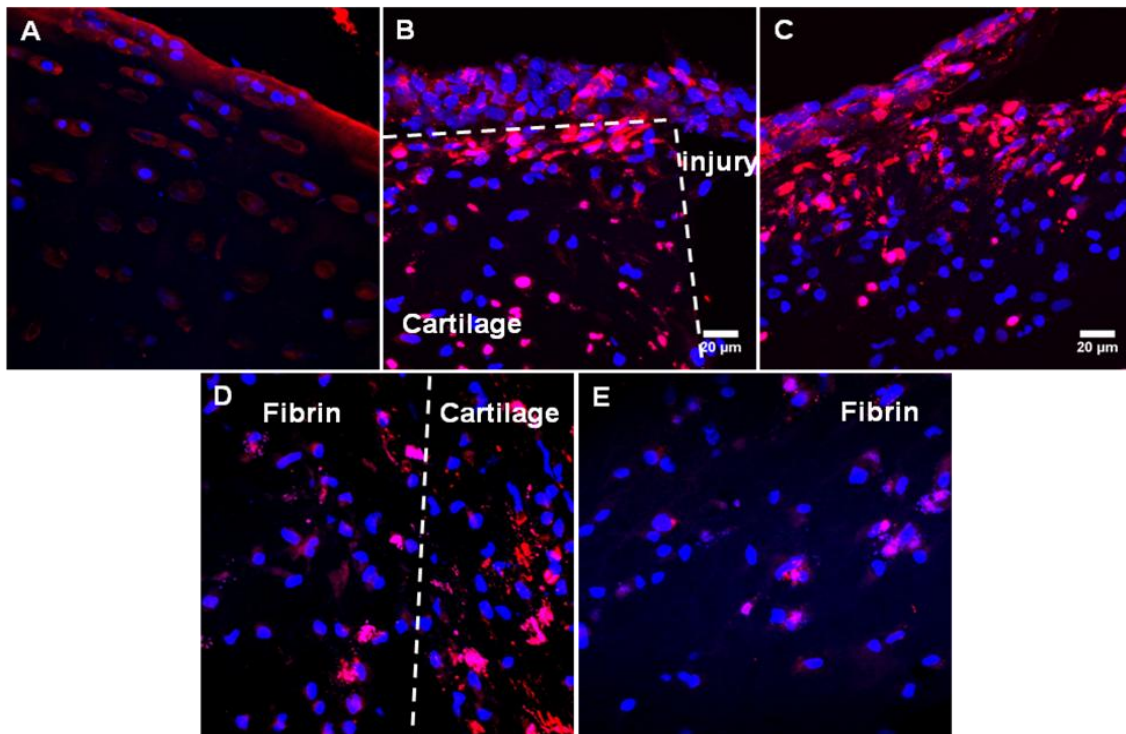


Figure 3.5. Pericellular matrix of migrating cells. Cells stained with DAPI (blue) and collagen type VI (red) and visualized by confocal microscope. Pericellular matrixes were well located around chondrocyte(s) in normal cartilage (A). In sharp cartilage injury, the matrixes were shown dramatic morphologic changes with matrix debris after 10 days (B). This is a strong evidence that the migrating cells broke their own pericellular matrix to migrate. This phenomenon could also be observed in fibrin hydrogel embedding on cartilage surface (C) and defect model (D and E). Migrated cells on the surface and inside hydrogel made their own pericellular matrix to differentiate cartilage cells.

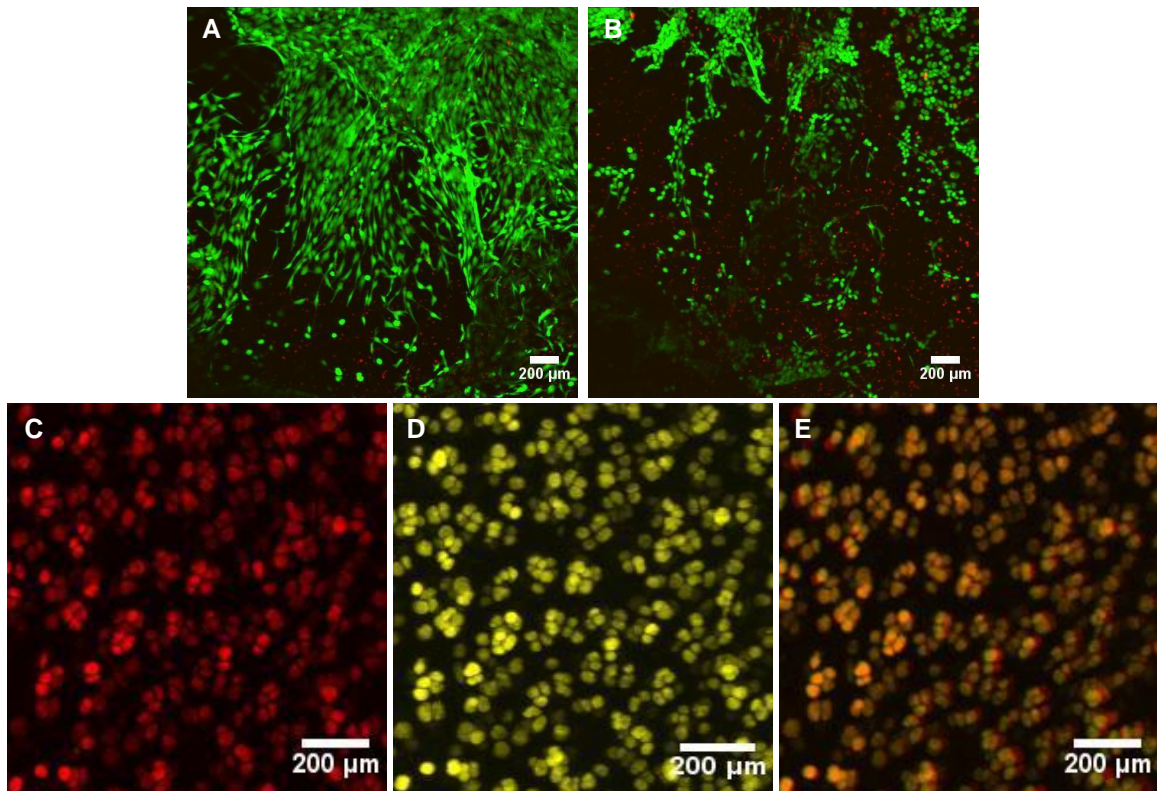


Figure 3.6. Isolation of trypsinized migrating cells. After 5-7 days post-impact, trypsinized migrating cells were isolated by trypsin. Most elongated cells which were abundant in left below were clearly detached from cartilage tissue after trypsin treatment (A; before trypsinization, B; after trypsinization). In contrast, chondrocytes in adjacent impacted cartilage did not detached by trypsin (C; before trypsinization, D; after trypsinization, E; merged image C and D).

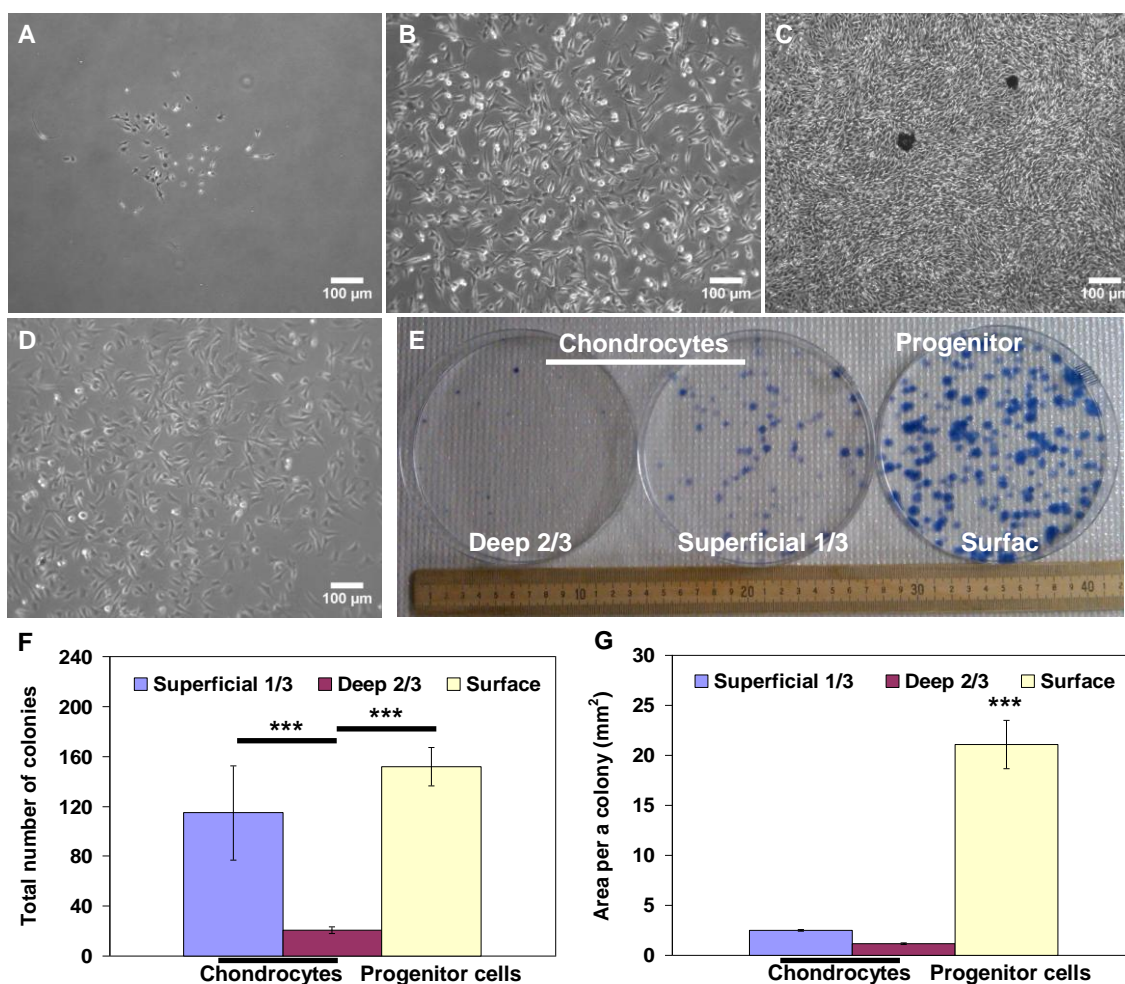


Figure 3.7. Colony formation of trypsinized migrating cells and chondrocytes. (A-D) Light microscopic image of single colony. Trypsinized migrating cells were shown rapid colony growth rate at 2 days (A), 3 days (B), and 6 days (C) than chondrocytes cultured for 13 days (D). (E) Macroscopic image of the ability of 200 cells from different areas of cartilage to form colonies 10 days post-seeding. The colonies were calculated by total number (F) and area (G) using ImageJ. Both chondrocytes and trypsinized migrating cells from 1/3 superficial zone showed higher total colony number than chondrocytes from 2/3 deep zone. However, trypsinized migrating cells had significantly higher colony area. Error bars are Ave. \pm SD. (n=4-5, ***p<0.001)

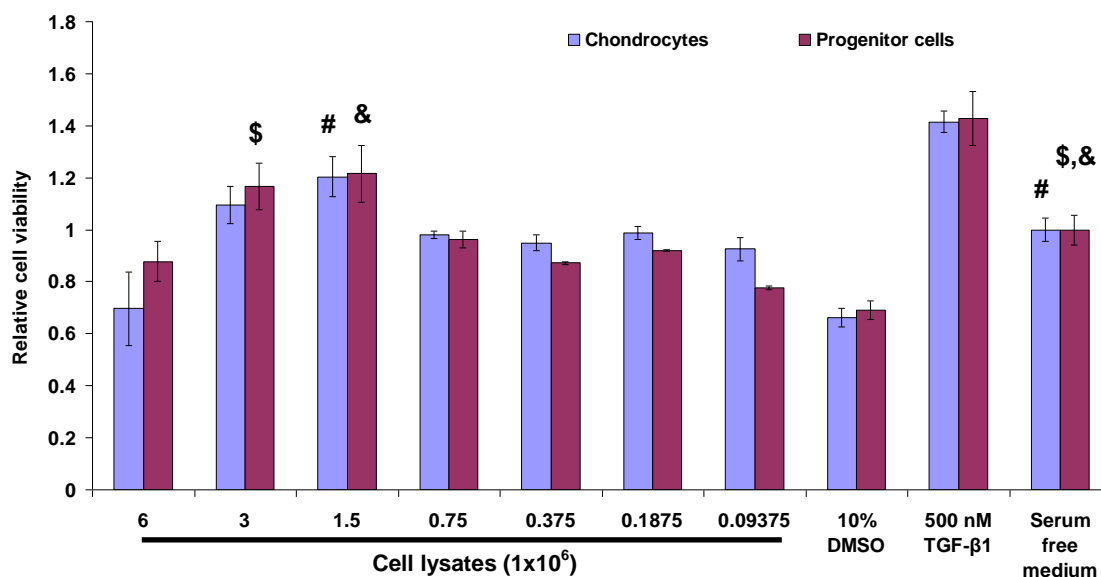


Figure 3.8. Viability of trypsinized migrating cells and chondrocytes. In order to determine the optimal number of cell lysates, serially diluted cell lysates were performed for cell viability test. 10% Dimethyl sulfoxide (DMSO) and 500 nM Transforming growth factor beta 1 (TGF-β1) were used for negative and positive control, respectively. Chondrocytes and trypsinized migrating cells were cultured with or without cell lysates and controls for 24 hours and the viability was quantified by colorimetric method. All data were normalized by viability in serum free medium. In all cell lysate groups, cells maintained almost 100% viability except 6×10^6 . Particularly, cell lysates with 1.5 and 3×10^6 induced cell proliferation. These two groups used for cell migration assay. Error bars are Ave. \pm SD. \$; $p < 0.01$, # and &; $p < 0.001$.

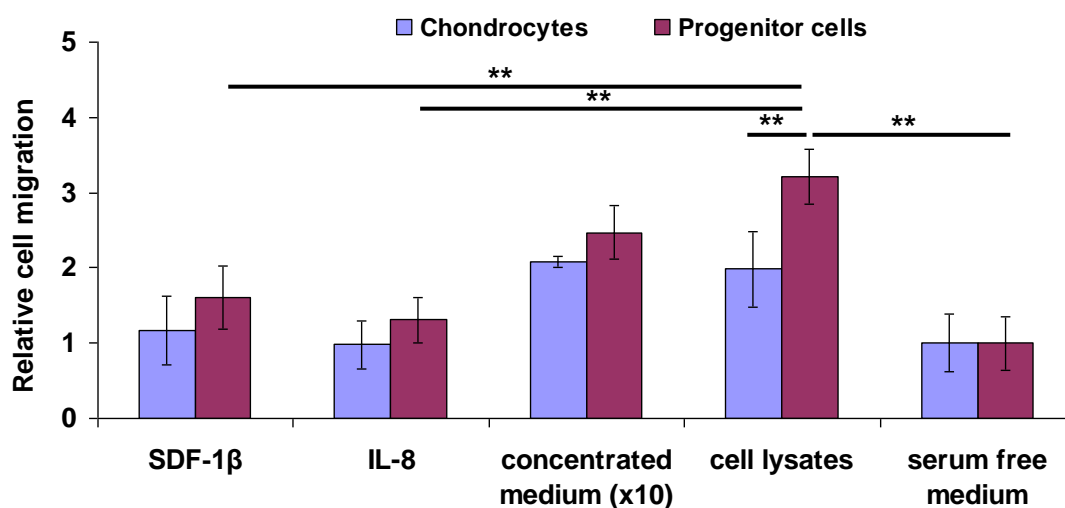


Figure 3.9. Chemotactic activity of trypsinized migrating cells and chondrocytes in collagen membrane. Various chemotatic factors were used to activate cell migration in this experiment. Stromal cell-derive factor 1β (SDF-1β) induced increased trypsinized migrating cell migration compared to serum free medium as a control ($p < 0.05$). This cell migration activity was more significant in 10-fold concentrated medium from cultured impact-injured cartilage explant and chondrocyte lysates (3×10^6). In particular, cell lysates induced significantly increased migration of trypsinized migrating cells than that of chondrocytes ($p < 0.001$). Error bars are Ave. \pm SD. ($n=3-9$, $***p < 0.001$)

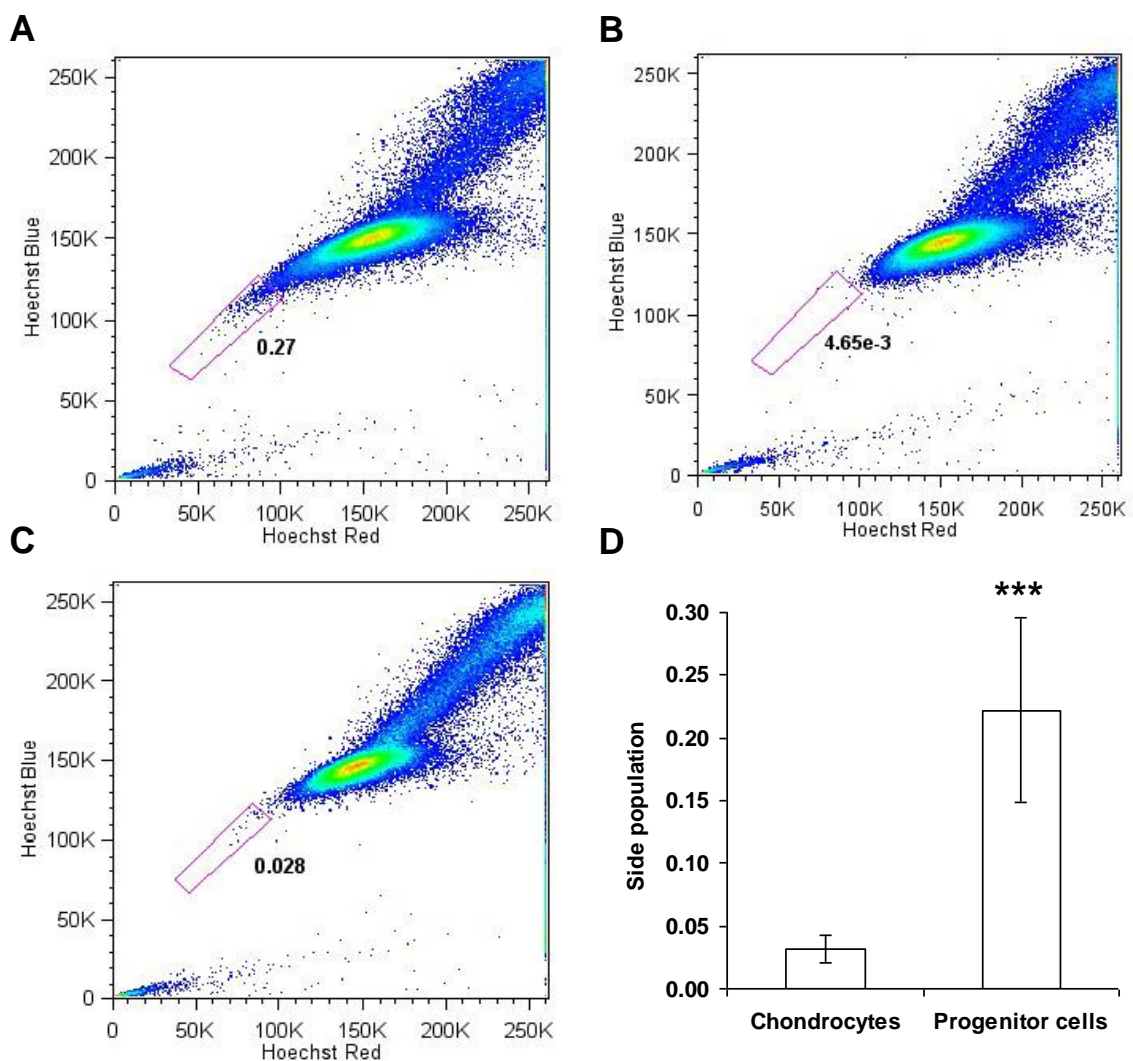


Figure 3.10. Side population (SP) analysis. (A-C) Cell distribution by fluorescence-activated cell sorting (FACS). The proportion of SP cells were calculated in a dim tail extending first on the left side of G0/G1 cells. Progenitor cells (A) were defined higher proportion of side population cells than chondrocytes (C). Verapamil inhibited the fractionation of SP progenitor cells (B). Both progenitor cell and chondrocytes were repeated using cells harvested from different explants and compared each other (D). The proportion of SP was significantly higher in progenitor cells than chondrocyte. Error bars are Ave. \pm SD. (n=5, ***p<0.001)

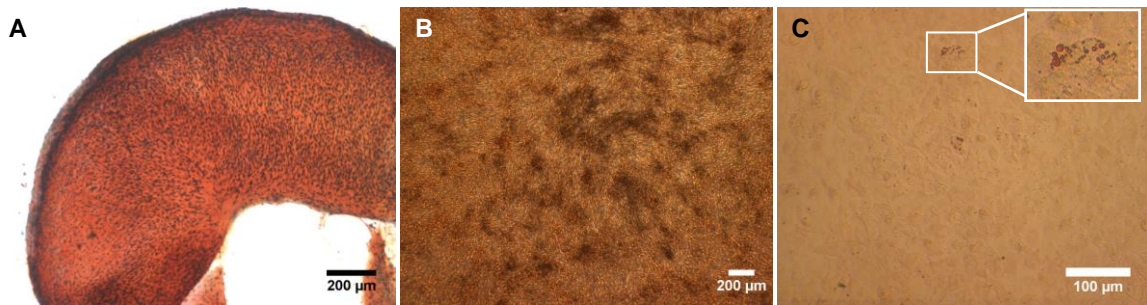


Figure 3.11. Differentiation of trypsinized migrating cells. (A) Chondrogenic differentiation in pellet culture. The pellet showed intense red Safranin-O/fast green staining indicating the presence of cartilage proteoglycans. (B) Osteogenic differentiation in monolayer culture. Deposit of calcium phosphate was detected by staining with Alizarin Red (dark red spots). (C) Adipogenic differentiation in monolayer culture. Only few cells produced positive fat vacuoles in Oil Red O staining.

CHAPTER 4

APPLICATION OF PROGENITOR CELLS ON CARTILAGE REPAIR

4.1. Purpose of Study

In the chapter 3, we identified apparent migration, colony formation, side population (SP), and differentiation characteristics of chondrogenic progenitor cells (CPCs) in traumatic cartilage injury. This finding of cells suggests the existence of a mechanism for repairing matrix damage. In this chapter, we apply the progenitor cells to repair cartilage defects. Our strategy is not implantation of isolated cells, but induction of active cell migration into the defect injury. For this purpose, we use short-term enzymatic method using collagenase to accelerate cell migration. Moreover, we examine the effect of cell lysate on migration of CPCs using defect explants. Here are specific aims.

- a. Location-dependant cellularity in the bovine cartilage
- b. Effect of short-term collagenase treatment using cartilage fragments
- c. Effect of short-term collagenase treatment in cartilage defect on cartilage repair
- d. Effect of cell lysate on migration in explants

4.2. Materials and Methods

4.2.1. Harvesting of bovine articular cartilage explant

Mature bovine stifle joints were obtained after slaughter from a local abattoir (Bud's Custom Meats, Riverside, IA). Osteochondral explants were harvested from two regions of bovine tibial plateau, meniscus-covered area and load-bearing area, and prepared by manually sawing an approximately 25 x 25 mm². The explants were rinsed in HBSS and cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with

10% fetal bovine serum (Invitrogen, Carlsbad, CA), 50 µg/ml L-ascorbate, 100 U/ml penicillin, 100 µg/ml streptomycin, and 2.5 µg/ml Fungizone. All explants were cultured under hypoxic culture condition (5% O₂/CO₂ at 37°C).

4.2.2. Full-thickness cartilage defect

The ability of putative progenitor cells to repopulate full-thickness cartilage defects was tested *in vitro* using bovine osteochondral explants. The defects were created four places in the explants using 4-mm biopsy punches. This procedure also resulted in the death of chondrocytes within ~100 microns of the cut edges. The cartilage around the full-thickness defects was treated with type I collagenase (Sigma) to facilitate cell migration. 0.25 mg/ml or 0.5 mg/ml collagenase dissolved in culture medium defects (20 µl) and incubated for 10 or 30 minutes. After 2-day pre-culture, the defects were filled with fibrin hydrogel (final concentration; 25 mg/ml fibrinogen and 5 U/ml thrombin). To assess the cellularity of the fibrin filler the explants were stained with 1 µg/ml calcein AM and 1 µM ethidium homodimer (Invitrogen) and imaged by confocal microscopy. Cell migration into the filler was evaluated by imaging the same sites after one week of incubation. Explants with defects were cultured an additional 3 weeks to evaluate the accumulation of hyaline cartilage matrix in the fibrin filler. The explants were fixed in 10% neutral-buffered formalin and cryo-processed. 10 µm-thick cryosections were stained with Safranin-O/fast green and imaged in transmitted light mode on an Olympus BX-60 microscope.

4.2.3. Cartilage cell density analysis

We examined cell density of the bovine cartilage in different area. The full-thickness cartilages were harvested in various location, femoral condyle or tibial plateau,

load-bearing area or meniscus-covered area (Figure 4.1). The samples were fixed in 10% neutral-buffered formalin and cryo-processed. 10 μm -thick cryosections were stained with Safranin-O/fast green and imaged in transmitted light mode on an Olympus BX-60 microscope. Each sample was taken pictures in three zone, top, center and bottom and counted the number of cells.

4.2.4. Cartilage fragment culture

Cartilage tissues were isolated from the bovine femur and minced into approximately 1- mm^3 fragments. The minced fragments was treated with type I collagenase and washed several times by culture medium. The fragments treated collagenase or not were embedded into fibrin hydrogel and cultured for 10 days. We examined the effect of collagenase on pCPC migration.

4.2.5. Preparation of chondrocyte lysate

Cartilage was isolated from the femoral condyle and digested with 0.25 mg/ml collagenase type 1 and pronase E (Sigma-Aldrich) to isolate chondrocytes. Cell lysates were obtained by repeated freeze-thaw ($-196\text{ }^\circ\text{C}$ - $37\text{ }^\circ\text{C}$) of $2.5 \times 10^4/\text{ml}$, $2.5 \times 10^5/\text{ml}$ and $2.5 \times 10^6/\text{ml}$ chondrocytes. The lysates were implanted with fibrin hydrogel in full-thickness cartilage defect pre-treated by collagenase (0.25 mg/ml for 10 min) as a chemotaxis.

4.2.6. Biochemical assay

The fibrin-embedded explants were cultivated for 3 week and isolated from the defect. The fibrin was digested in papain buffer (500 $\mu\text{g}/\text{ml}$ papain, 5 mM L-cysteine HCl, 100 mM Na_2HPO_4 , and 5 mM $\text{Na}_2\text{-EDTA}$) at $65\text{ }^\circ\text{C}$ for 2~4 hours until complete dissolution.

4.2.6.1. DMMB assay

Glycosaminoglycan (GAG) content was determined using the dimethylmethylen blue (DMMB) dye-binding assay (Farndale et al., 1986). Briefly, serially diluted sample were prepared and DMMB solution was added. Absorbance was measured at 530 nm using VMax Kinetic ELISA microplate reader (Molecular devices, Inc., Sunnyvale, CA). GAG standard was established using chondroitin-6-sulfate (Sigma-Aldrich).

4.2.6.2. DNA quantification assay

Amount of migrated cells was determined by the fluorometric DNA quantitation method using Quant-iT™ PicoGreen® dsDNA Assay Kit (Molecular Probes Inc.). DNA standard was also loaded in order to allow the conversion of selective fluorescent units to a cell contents. Digest fibrin hydrogel without implantation prepared as a blank. Fluorescence was measured on fluorescence microplate reader using wavelengths of 480-nm excitation and 520-nm emission.

4.2.7. Statistical analysis

For cartilage cell density and biochemical assay, statistical analysis was performed using SPSS software (Ver.10.0.7, SPSS Inc., Chicago, IL, USA) with a one-way ANOVA and post-hoc pairwise comparison. All the results were expressed as mean \pm standard deviation. Statistical significance was set at $p < 0.05$ and minimum acceptable power set at 0.9.

4.3. Results

4.3.1. Location-dependant cellularity

The cellularity in the host cartilage is one of the important factors for cartilage repair. Therefore, we first examined cellularity of the bovine cartilage in different area

before collagenase treatment. The cartilage was harvested in various location, femoral condyle or tibial plateau, load-bearing area or meniscus-covered area. Each cartilage was taken pictures in thress zone, top, center and bottom. In safranin-O staining, the number of cells of meniscus-covered area in the tibial plateau was apparently higher than those of load-bearing area (Figure 4.2A). The number of cells was quantified by cell counting (Figure 4.2B). In the tibial plateau, meniscus-covered area had significantly higher number of cells than load-bearing area in all zones ($p < 0.01$). It means that the relative CPC density is low in load-bearing area and there will be limitation to repair that area of tissues even if we treat collagenase.

4.3.2. Effect of short-term collagenase treatment using cartilage fragments

We examined the effect of short-term collagenase treatment on cell migration using cartilage fragments. After collagenase treatment, we could observe proteoglycan loss from the edge of tissues (Figure 4.3B and C) compared to that of no treated control (Figure 4.3A). The proteoglycan loss was more apparent in 30 min treated group by 0.25 mg/ml (Figure 4.3C). This physically loosed cartilage allowed cell migration ease and fast. These treated or non-treated cartilage fragments were cultured in fibrin hydrogel and observed cell migration. At day 5, some PCPs were migrated into fibrin in collagenase-treated groups (Figure 4.3E and F). In contrast, only a few of cells started to migrate in control (Figure 4.3D). The number of migrated cells was more distinct between control and treated groups at day 10. Microscopically, a number of cells were migrated from fragments and proliferated in hydrogel (Figure 4.3H and I). In particular, the number of migrated cells was highest in 30-min treatment group (Figure 4.3I).

4.3.3. Effect of short-term collagenase treatment in cartilage defect on cartilage repair

Bovine cartilage explants were separately harvested from load-bearing area or meniscus-covered area and created 4-mm diameter defect injury. During 2-day pre-culture before collagenase treatment, the tissue morphology around defect injury was macroscopically different between two areas (Figure 4.4). Meniscus-covered area maintained the original size of defect. However, cartilage in load-bearing area was swelled into defect and covered approximately 50% of original defect. This phenomenon could be confirmed in histologic examination. The host cartilage tissue around defect was structurally loosed and swelled in load-bearing area (Figure 4.5). Sometimes, the fibrin hydrogel was shrinkage or lifted fibrin up by pushing of swelled tissue. Collagenase treatment also accelerated the swelling due to breaking down the edge of cartilage tissue.

This structurally unstable swelling induced poor tissue integration between host cartilage and fibrin hydrogel. In all groups, migrated CPCs were locally distributed in hydrogel. Sometimes, there were no migrated cells in untreated control (Figure 4.5A). In collagenase-treated groups, a number of activated CPCs were observed in the host tissue (Figure 4.5E, G and H). The tissue showed dense cell population and the cells included morphologically elongated CPCs. Although CPCs were not uniformly distributed in fibrin, collagenase treatment induced more cell migration compare to control (Figure 4.5F and I).

The effect of short-term collagenase treatment was also examined in the meniscus-covered cartilage. After one-week culture, the explants were stained and taken confocal examination to observe cell migration in the defect. Compared to control (Figure 4.6A) which had no migrated cells, a number of cells were migrated into fibrin

hydrogel (Figure 4.6B and C). In particular, migrated cells were covered all defect area in 30-min treatment group. After three weeks in culture sections from the explants were fixed and stained with Safranin-O/fast green (Figure 4.7). In untreated control, no cell migrated into the fibrin (Figure 4.7A). On the other hand, the effect of collagenase was dramatic. CPCs were perpendicularly migrated into the hydrogel in 0.25 mg/ml collagenase treated for 10 min (Figure 7.4B). When collagenase was treated for 30 min, elongated cells were found embedded throughout the fibrin hydrogel and evenly distributed (Figure 4.7C). A pericellular halo of heavy proteoglycan deposition was observed around some cells (Figure 4.7F, arrowhead). The highest density of cells was across the surface of the fibrin and at the cartilage/fibrin interface. (Figure 4.7E and G). We also observed high density of cell sheet on the surface of fibrin in treated groups. Higher concentration of collagenase (10 min) also allowed active cell migration with heavy proteoglycan deposition (Figure 4.7D).

The result of histologic examination was summarized in Table 4.1. Load-bearing cartilage and meniscus-covered cartilage which had at least three samples were graded in terms of fibrin stability, cell migration and proteoglycan deposit. Fibrin stability was estimated by any observation of fibrin degradation, shrinkage and movement. CPC population and distribution in fibrin were indicators for cell migration. These migrated cells were observed morphologic change like chondrocyte and proteoglycan deposit around the cells. Although collagenase treatment showed slightly better grading than untreated group in load-bearing cartilage, all groups were graded 'bad' or 'poor'. Short-term enzymatic treatment enhanced cell migration and proteoglycan deposit in full-thickness defect of meniscus-covered cartilage. In particular, 0.25 mg/ml for 30 min and

0.5 mg/ml for 10 min showed the highest grading, ‘good’ or ‘excellent’ in terms of fibrin stability, cell migration and proteoglycan deposit.

For quantifying of proteoglycan and migrated cells, we performed DMMB and DNA quantification assays. Unlike histologic examination, most samples showed low proteoglycan content (Figure 4.8A). In contrast, a large number of CPCs were migrated into fibrin. Collagenase treatment, especially 0.25 mg/ml for 30 min, induced slightly better cell migration than the untreated group (Figure 4.8B). However, there was no significant difference among the groups.

4.3.4. Effect of cell lysate on migration in explants

We showed the positive effect of chondrocyte lysates on migration of CPCs in migration assay (Figure 3.8). The cell lysate can also be applicable for CPC attraction in defect injury of bovine cartilage. The optimal concentration of cell lysate ranged from 1.5 millions/ml to 3 millions/ml were prepared based on previous chemotactic assay. Serially diluted cell lysates were also prepared since cartilage injury produced necrotic cells around the defect. 2.5×10^4 /ml of cell lysate showed increased DNA content compare to 2.5×10^6 /ml ($p < 0.05$) (Figure 4.9). However, there was no significant difference between 2.5×10^4 /ml of cell lysate and control.

4.4. Discussion

Autologous chondrocyte implantation (ACI) is one of the promising techniques for repair of articular cartilage defects. For ACI, articular cartilage biopsy was taken arthroscopically from non-load-bearing joint cartilage area to isolate autologous chondrocytes. After *in vitro* expansion, the cells are injected into the defect which is then covered by a periosteal flap or a collagen membrane. Brittber et al. [5, 6] have reported a

good clinical outcome in their long-term study of ACI. However, ACI also showed hypertrophic differentiation with subsequent ossification and poor integration to host tissue [194, 195].

Besides autologous chondrocytes, bone marrow stromal cells (BMSCs) [100], periosteal cells [101], skeletal muscle [102], adipocytes [103] and synovial fibroblasts [99, 104, 105] represent possible sources for cell-based cartilage repair. Based on our results, CPCs may also be harvested to serve as a source for cell implantation. However, the main appeal of a CPC-based strategy lies in their ability to heal by self-congregating at injury sites, thereby eliminating the costs and risks associated with *ex vivo* cell expansion and the additional surgery needed for grafting.

Since cartilage cells, chondrocytes and progenitor cells, are surrounded by the proteoglycan-rich pericellular matrix and by the capsular basket-like matrix characterized by the presence of a network of fibrillar collagen, their migration is an extremely challenging. During cartilage injury, the collagen network is disrupted, providing a permissive environment for cell migration [196]. However, the process still takes long time to recover the cartilage defect by migrated progenitor cells.

Several studies have been reported several enzymatic digestions in order to increase the initial adhesion of cells or cartilage tissue with host tissue. Lee *et al.* [107] and Hunziker *et al.* [108] were used chondroitinase ABC to remove proteoglycans in superficial zone of the cartilage. They showed increased adhesion force and cell population after treatment. Treatment with hyaluronidase (0.1-0.3%) and collagenase (type VII, 10-30 U/ml) showed significant chondrocyte density in lesion edges and enhanced integration and interfacial strength [110-112]. However, they treated the

enzymes 48 hours and showed unrecovered proteoglycan loss after 28 days. For clinical application, enzymatic treatment should be localized in cartilage defects and treated short-duration. In this study, we used short-time collagenase enzymatic digestion method in the defect to accelerate cell migration. The method can locally damage the edge of cartilage defect with collagen and proteoglycan loss. We confirmed the effect of enzymatic treatment on progenitor cell migration using *in vitro* cartilage fragment culture with fibrin hydrogel. A number of progenitor cells were migrated from enzymatic treated fragments at day 5, while only few cells started to migrate in untreated control. This method was also effective in cartilage defect healing. Migrated progenitor cells were covered all defect area and produced abundant proteoglycan deposit with chondrocyte-like differentiation. In contrast, there were no migrated cells at 3 weeks in non-treated group.

In order to attract progenitor cells, we also used fibrin hydrogel which has excellent characteristics as a scaffold such as high cell chemotactic ability, uniform cell distribution, angiogenesis, and great adhesion capabilities [120, 125]. Because of these advantages, fibrin has been widely used in a variety of tissue engineering applications such as articular cartilage [142, 150], adipose [126], bone tissue [137, 138], cardiovascular [127, 129], liver [133, 134], muscle [131, 132] and skin [135, 136]. However, the fibrin hydrogel has some major disadvantages; shrinkage, low mechanical strength and rapid degradation. In particular, catabolic enzymes such as matrix metalloproteinase (MMP), ADAMTS, fibronectin and tumor necrosis factor alpha (TNF- α) secreted from damaged cartilage and during cell migration can dramatically accelerate fibrin degradation. In addition to accelerated cell migration, enzymatic treatment could

protect fibrin degradation since the cells easily migrated through loosed cartilage matrix without secreting catabolic enzymes.

Bovine cartilage explants were separately prepared from load-bearing area or meniscus-covered area and created 4-mm diameter defect injury. The result of load-bearing cartilage regeneration was disappointing even in collagenase treated groups. Only a few of cells were able to migrate into fibrin hydrogel. In contrast, a great number of CPCs migrated into hydrogel with abundant proteoglycan deposit. One possible reason is different cell density between two areas. In human cartilage, the number of chondrocytes in non-weight bearing area is significantly higher than that in load-bearing area [47]. This trend was similar in bovine cartilage. The number of cells of meniscus-covered area was 2 and 2.5 times in lateral and medial tibial plateau, respectively, than load-bearing area. Although load-bearing area had relatively low number of cells, the number was still enough to heal the cartilage. We could observe a lot of actively migrating cells in the edge of cartilage defect. They were already ready to migrate, but could not move fibrin hydrogel like unconnected bridge.

Another important factor is stability of implanted material for tissue regeneration. Macroscopically, we could observe physically unstable matrix in load-bearing area. Regardless of collagenase treatment, the tissue morphology around defect injury was different between two areas. Cartilage in load-bearing area was swelled into defect and covered approximately 50% of original defect. Sometimes, the fibrin hydrogel was shrinkage or lifted fibrin up by pushing of swelled tissue. This phenomenon was occurred due to low mechanical property in load-bearing area. Compared to load-bearing cartilage, the articular cartilage beneath the meniscus showed a significantly larger modulus by as

much as 70% [47]. This structurally unstable swelling induced poor tissue integration between host cartilage and fibrin hydrogel.

We have several strategies to overcome problematic load-bearing cartilage. One approach is reinforcement of implanted material with higher mechanical strength to protect host tissue swelling. Structural modulus of fibrin hydrogel is dependant on the concentration of fibrinogen and thrombin [197]. In this study, we used 25 mg/ml fibrinogen and 5 U/ml thrombin in which modulus is approximately 1.2 KPa. In higher concentration of two components, 50 mg/ml fibrinogen and 50 U/ml thrombin, the modulus is increased 5 times. However, we have to consider the negative effect of cell migration in higher concentration which has smaller pore size. Fibrin/hyaluronic acid composit hydrogel can be also another good candidate to reinforce hydrogel. In presence of high molecular weight hyaluronic acid, the fibrin chondrograft showed improved biomechanical properties and cartilage healing ability *in vivo* animal studies [198-200]. As well as material reinforcement, two more approaches will be useful for improving cartilage tissue regeneration (Figure 4.10). First one is cartilage fragment implantation. Isolated autologous cartilage fragments are treated collagenase and implanted with fibrin hydrogel. Progenitor cells can migrate from the fragment to fibrin hydrogel as well as from the host tissue. Second approach is autologous CPC implantation like ACI. We expect that the isolated CPCs have much better healing ability based on our results than chondrocytes.

Safranin-O histology of meniscus-covered cartilage showed cells that invaded the fibrin filler deposited a proteoglycan-rich pericellular matrix. This signature chondrogenic activity involves coordinated expression of numerous structural proteins

(e.g. aggrecan, hyaluronan, collagens, fibronectin) and processing enzymes (e.g. MMPs, lysyl oxidase, prolyl hydroxylase). Staining immediately around the cells (50-100 microns) was often as intense as in normal cartilage matrix, but in only 1 of 4 cases did the entire fibrin structure contain normal proteoglycan levels. On the other hand, the DNA content of the fibrin gels was modestly greater than normal cartilage, an indication of near normal cellularity. It remains to be seen if all of these cells will eventually engage in high levels of matrix production spontaneously, or will require intervention with chondrogenic growth factors, which we saw drive 90% of pCPCs in pellet cultures to vigorously synthesize proteoglycans. Control cartilage in our explant model begins to deteriorate after ~3 weeks, due possibly to lack of mechanical stimulation in culture. This may not have been long enough to fully evaluate the potential for matrix regeneration in defects. Multiple *in vivo* joint injury models offer solutions to this problem.

Chondrocyte lysate showed the most effective chemotaxis in our cell invasion assay. This lysate can also be applicable for CPC attraction in defect-injured cartilage. Unfortunately, there was no significantly higher effect of cell lysate ranged from 2.5×10^4 /ml to 2.5×10^6 /ml even if 2.5×10^4 /ml of cell lysate showed slightly increased DNA content compared to control. Moreover, DNA content was strongly associated with number of cell lysate. Major difference between *in vitro* cell invasion assay and defect explant model is the number of resident necrotic chondrocyte in explants. Defect injury has already created cell lysate around injury area and the number may be sufficient as a cell attractor. In clinic, the degree of traumatic cartilage injury and the number of necrotic cells are unpredictable. Therefore, direct application of cell lysate into the traumatic

cartilage has limitation. Instead, it is more useful to find proteins related to cell migration like high mobility group box-1 (HMGB1) [201-203].

In the chapter, we applied the progenitor cells to repair cartilage defects. Our strategy was not isolating the cells, but inducing the cell migration into the defect. For this purpose, we used short-term enzymatic method using collagenase to accelerate cell migration. Surprisingly, numerous CPCs migrated into fibrin defect and differentiated into chondrocyte-like cells with abundant deposit of proteoglycans. This result strongly supports that progenitor cells are activated in traumatic cartilage injury and have great potential for cartilage repair.

Table 4.1. Summary of collagenase treatment study

		No treatment	0.25 mg/ml for 10 min	0.25 mg/ml for 30 min	0.5 mg/ml for 10 min
Load-bearing cartilage	Fibrin stability	+ or ++	+ or ++	+ or ++	+ or ++
	Cell migration	+	++	++	++
	Proteoglycan deposit	+	+	+	++
Meniscus-covered cartilage	Fibrin stability	+++ or +++++	+++ or +++++	+++ or +++++	+++ or +++++
	Cell migration	+	+++	++++	++++
	Proteoglycan deposit	+	+++	++++	+++ or +++++

+ bad, ++ poor, +++ good, +++++ excellent

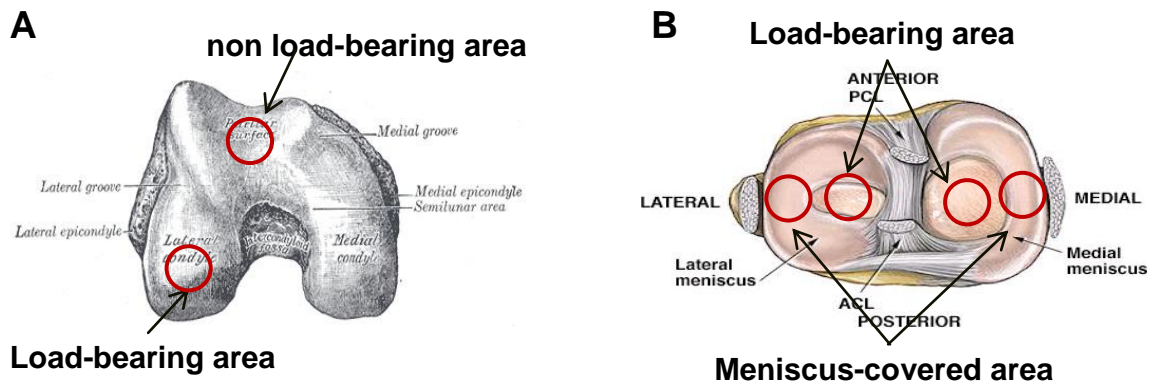


Figure 4.1. Harvested sample location to examine cell density. Two samples were harvested from femoral condyle (A) and four samples were harvested from from tibial plateau (B). The samples were separated by load-bearing area and meniscus-covered area (non load-bearing area).

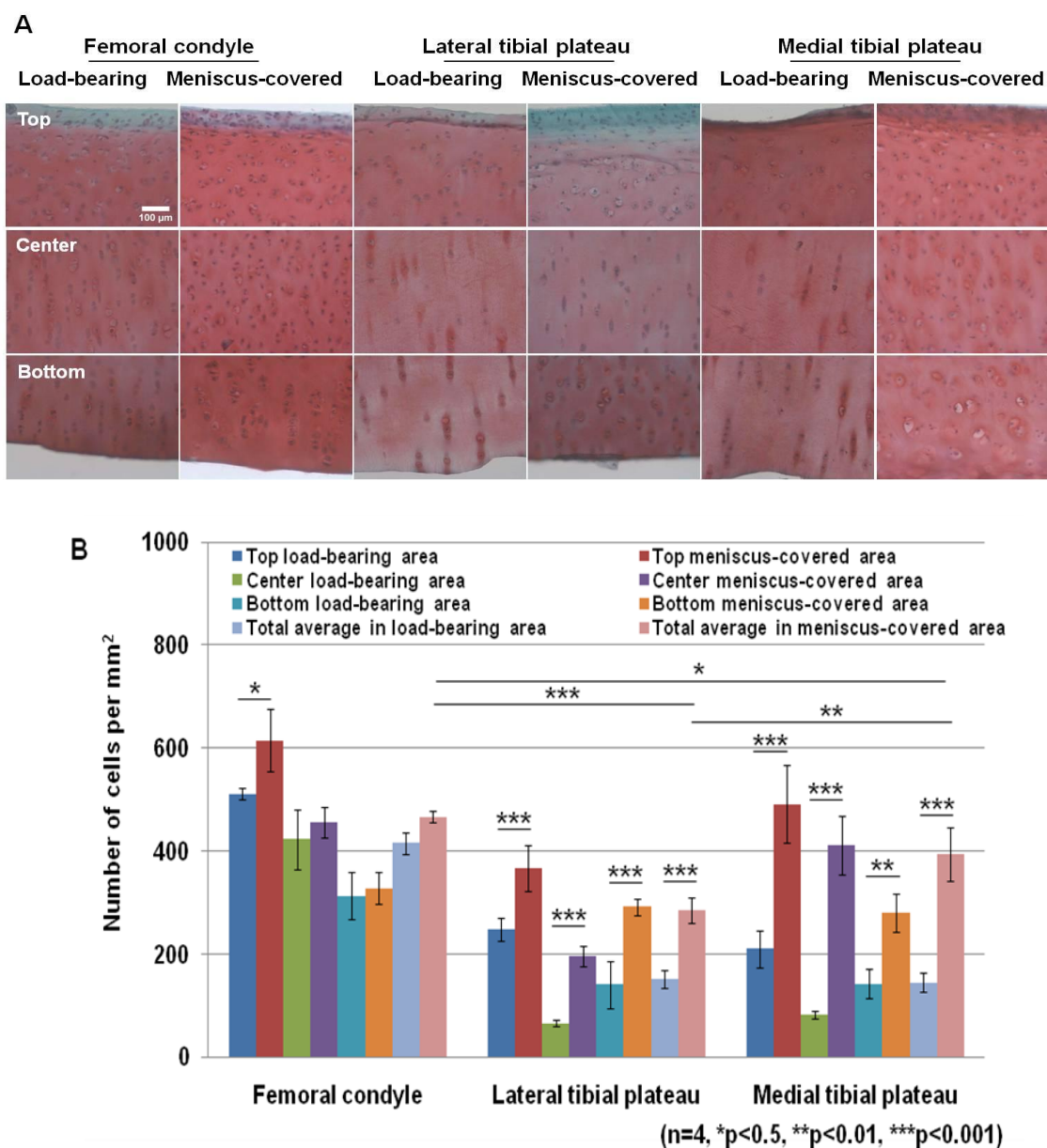


Figure 4.2. Location-dependant cell density in the bovine cartilage. (A) Safranin-O staining in different locations. Each cartilage was taken pictures in three zones, top, center and bottom. (B) Quantitative result of cellularity. In the tibial plateau, the number of cells in medial plateau was higher than those in lateral plateau.

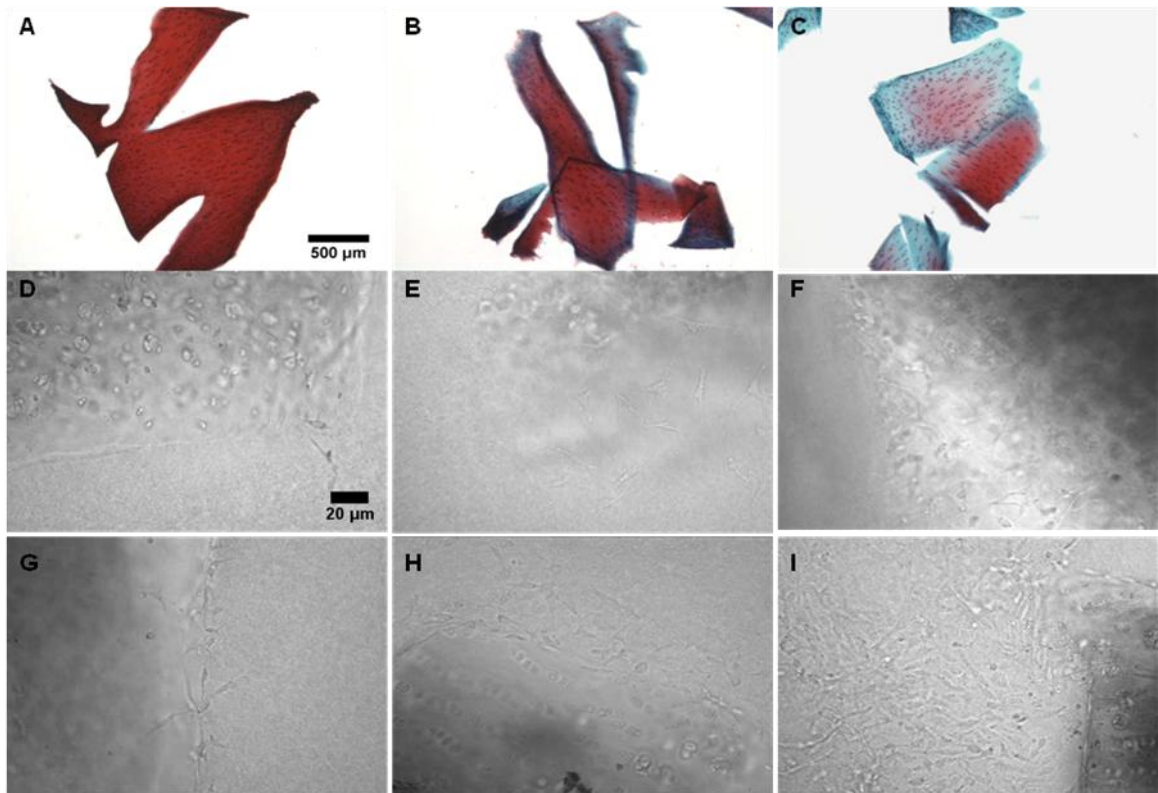


Figure 4.3. Cell migration from collagenase-treated cartilage fragments. (A-C) Safranin-O staining after 0.25 mg/ml collagenase treatment. Compared to control (no treatment, A), cartilage lost proteoglycans after 10 min (B) and 30 min (C) collagenase treatment. (D-I) Light microscopic examination; 5-day culture (D-F) and 10-day culture (G-I). Collagenase treatment induced active cell migration from the fragments. In particular, the number of migrated cells was highest in 30-min treatment group (F and I).

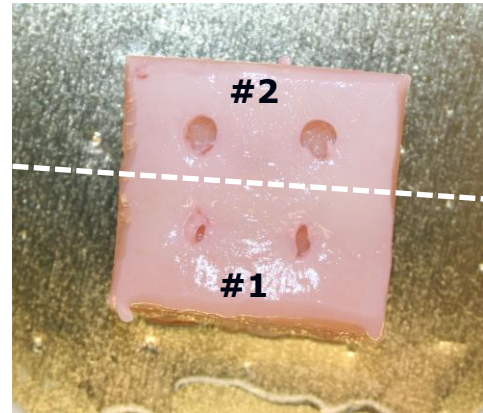
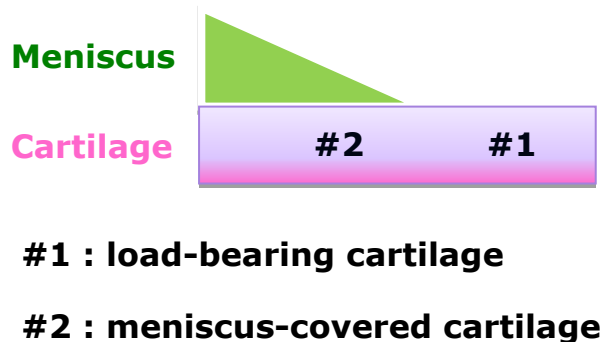


Figure 4.4. Morphologic change of defect injury in load-bearing and meniscus-covered cartilage. During 2-day pre-culture before collagenase treatment, the cartilage around defect injury was swelled into defect in load-bearing cartilage. In contrast, meniscus-covered cartilage was maintained their structure.

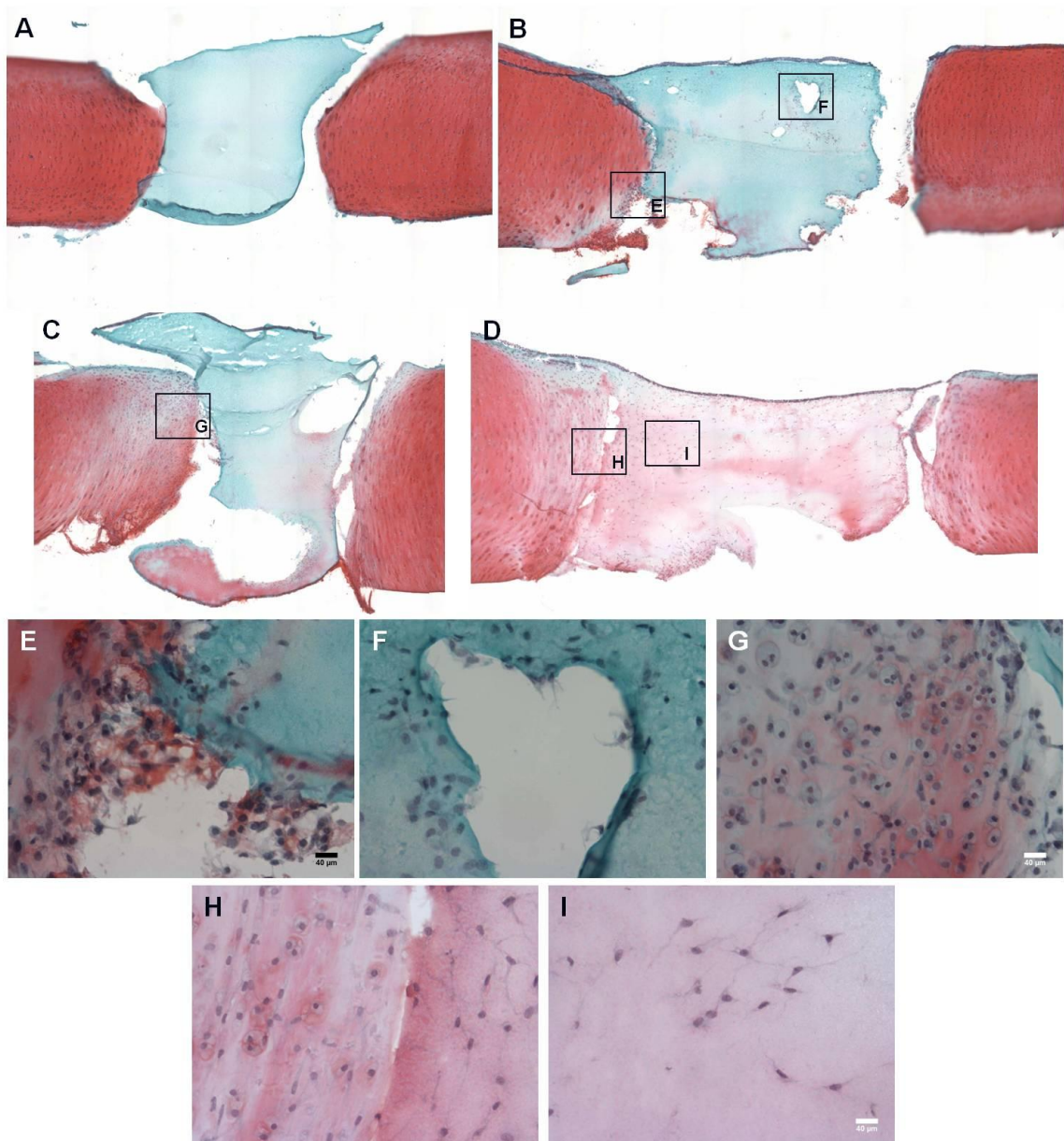


Figure 4.5. Repair of load-bearing cartilage using collagenase treatment. Defect-injured explants were cultured for 3 weeks and stained Safranin-O; (A) no treatment of collagenase, (B) 0.25 mg/ml collagenase for 10 min, (C) 0.25 mg/ml collagenase for 30 min, and (D) 0.5 mg/ml collagenase for 10 min. All groups showed poor cell migration inside fibrin hydrogel. Progenitor cells were actively migrated in structural loosed host

tissue treated by collagenase (E, G and H). However, just a few of cells were migrated in collagenase treatment groups (F and I).

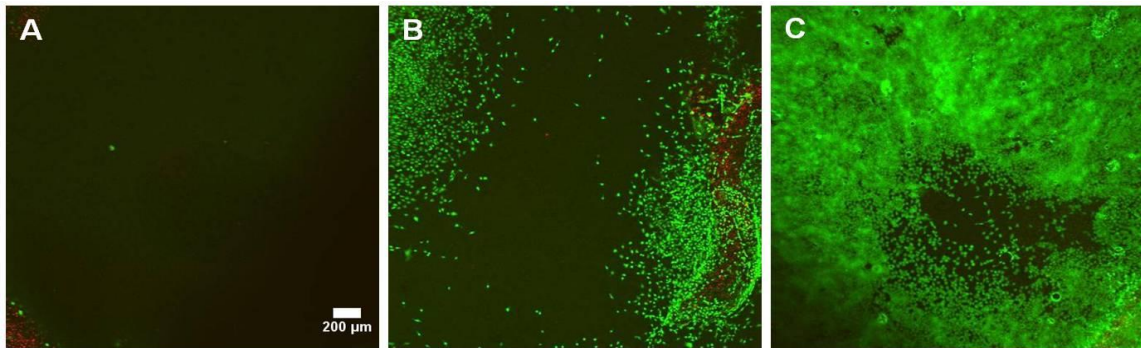


Figure 4.6. Repair of meniscus-covered cartilage using collagenase treatment (confocal examination). Defect-injured explants were cultured for 1 week and performed confocal examination; (A) no treatment of collagenase, (B) 0.25 mg/ml collagenase for 10 min, and (C) 0.25 mg/ml collagenase for 30 min. Compared to control (A), a number of cells were migrated into fibrin hydrogel (B and C). In particular, migrated cells were covered all defect area in 30-min treatment group (C).

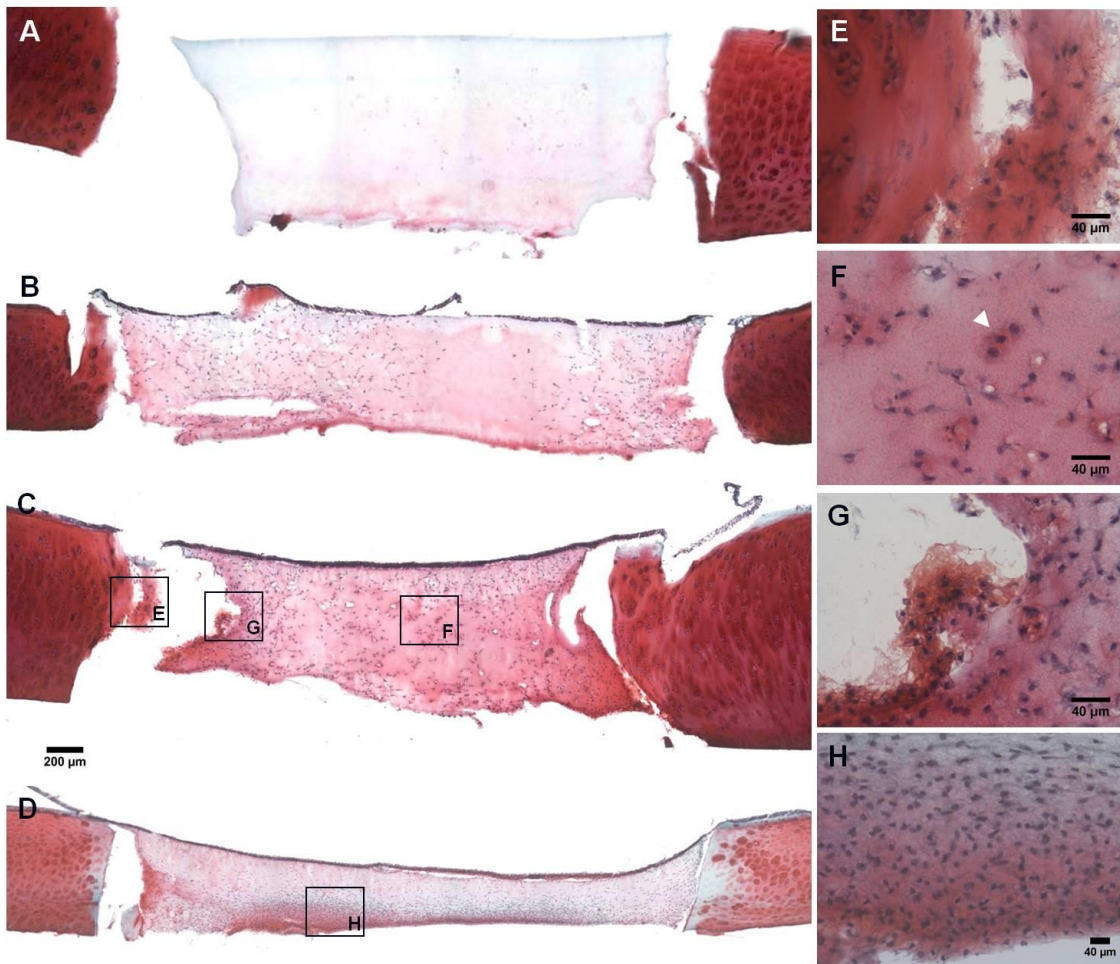


Figure 4.7. Repair of meniscus-covered cartilage using collagenase treatment (histologic examination). Defect-injured explants were cultured for 3 weeks and stained Safranin-O; (A) no treatment of collagenase, (B) 0.25 mg/ml collagenase for 10 min, (C) 0.25 mg/ml collagenase for 30 min, and (D) 0.5 mg/ml collagenase for 10 min. There was no cell migration in non-collagenase treatment group (A). On the other hand, abundant cells were migrated into fibrin in collagenase-treated groups (B-D). Progenitor cells were highly dense in the edge of host tissue (E and G) and evenly distributed in hydrogel (F). Some migrated cells were differentiated to chondrocyte-like cells with abundant proteoglycan deposit around cells (F and H, arrowhead).

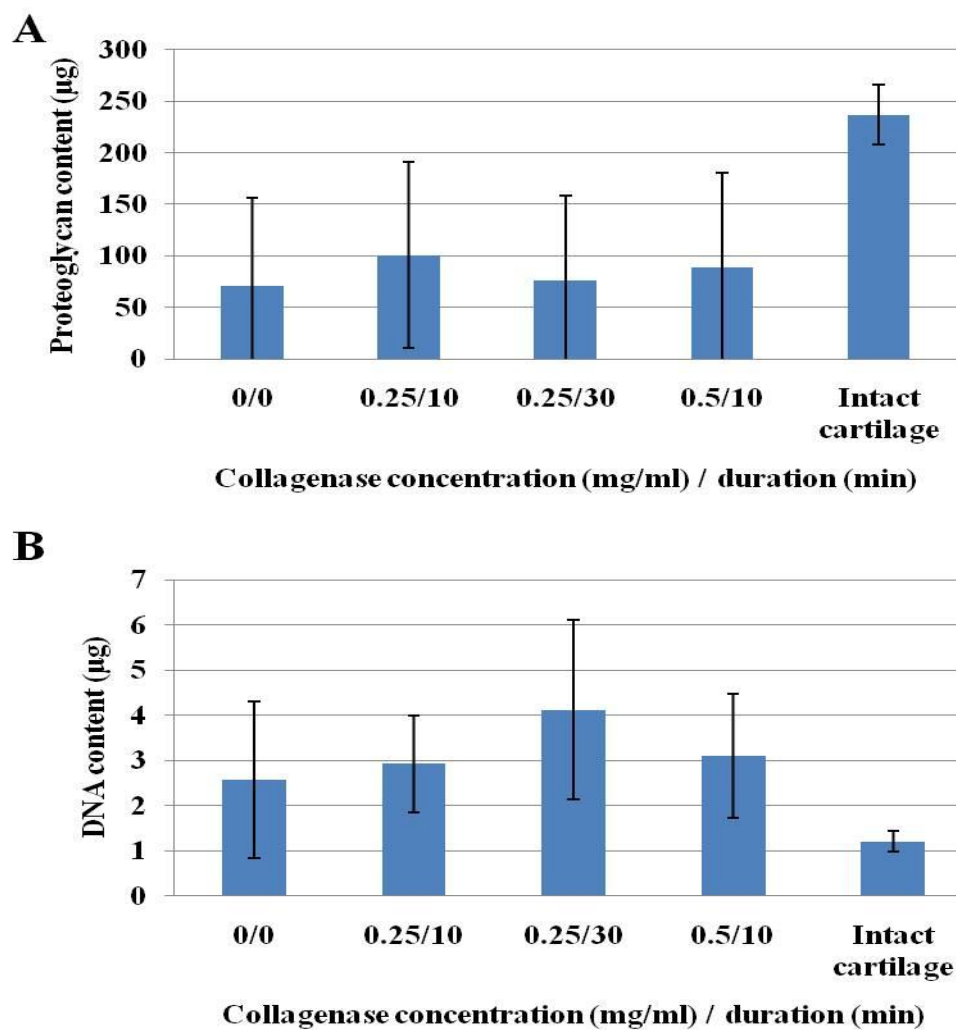


Figure 4.8. Proteoglycan and DNA content in collagenase treated defect cartilage.

Defect-injured explants harvested from meniscus-covered area were treated by collagenase using various concentrations and durations and cultured for 3 weeks; (A) proteoglycan content and (B) DNA content. Proteoglycan content in all defect-injured cartilage was lower than that in intact cartilage and was no difference among the groups (A). In contrast, collagenase treated groups showed higher DNA content compared to non-treated control (B). However, there was no significant difference. Error bars are Ave. \pm SD. (n=3-4)

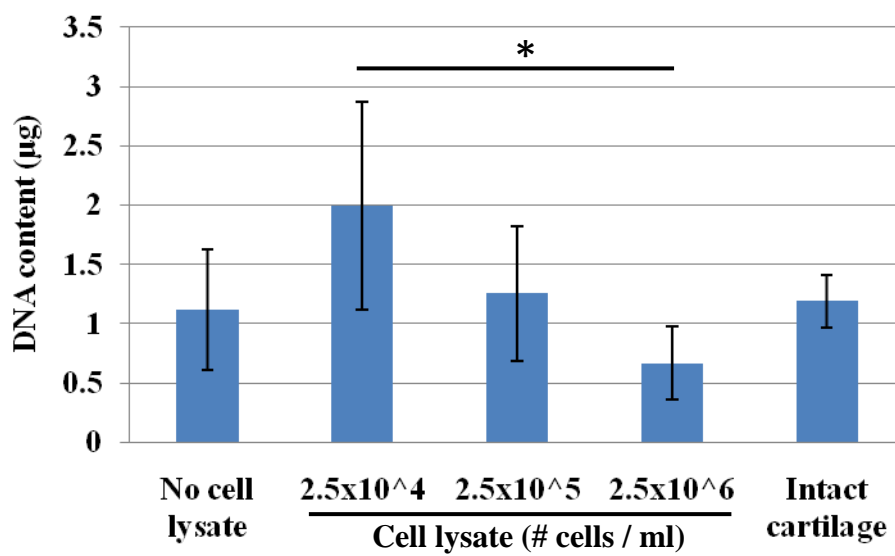


Figure 4.9. DNA content in defect cartilage embedded chondrocyte lysates. Defect-injured explants harvested from meniscus-covered area were treated by 0.25 mg/ml collagenase for 30 min. Cell lysates were mixed with fibrin and implanted into the defect. High number of cell lysate (2.5×10^6) induced decreased cell migration compared to no cell lysate group. On the other hand, higher number of progenitor cells was migrated into fibrin hydrogel with 2.5×10^4 lysate than control, but no significant difference. Error bars are Ave. \pm SD. (n=3-4, *p<0.05)

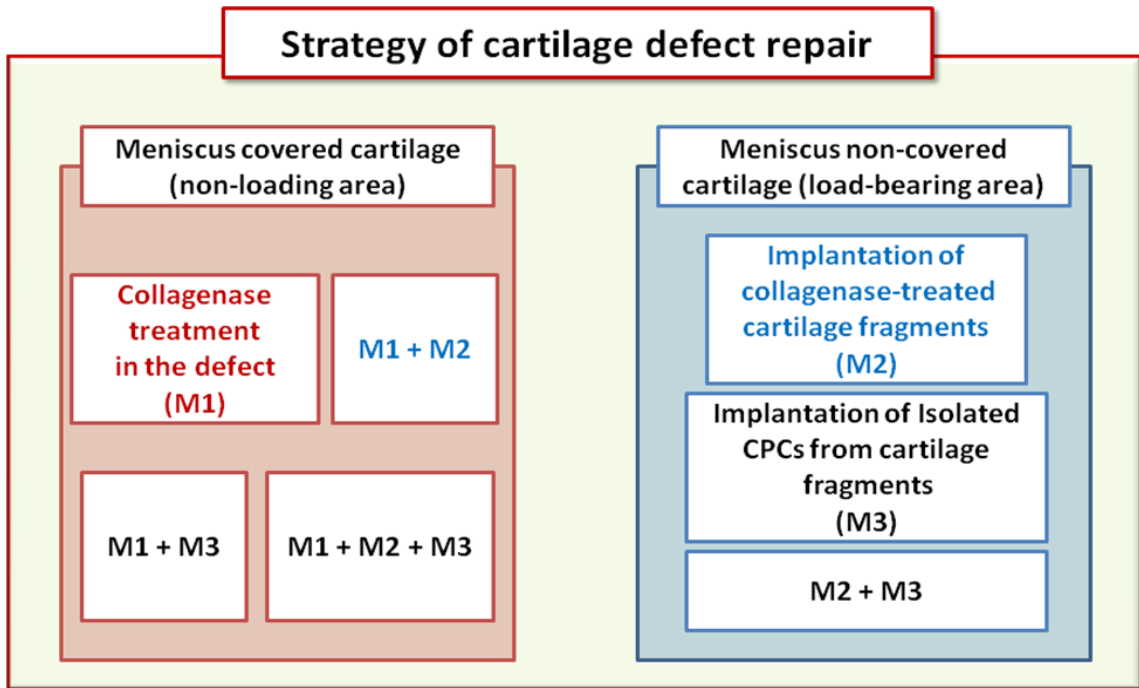


Figure 4.10. Repair strategy of cartilage defect injury.

CHAPTER 5

CONCLUSION

Our work in an *in vitro* bovine injury model leaves many unknowns regarding the clinical significance of CPCs. However, the finding that such a vigorously chondrogenic cell population is activated by acute mechanical injury and homes to damaged cartilage certainly endorses further exploration of their therapeutic potential. Cartilage degeneration in PTOA is thought to start with focal matrix cracking together with the local loss of superficial zone cells and the critical lubricants they provide. In our model, which was isolated from the bleeding, inflammation, and mechanical stresses that would be present *in vivo*, it appeared that both kinds of cartilage damage were repairable by resident CPCs. That this does not seem to occur spontaneously in people or in animals with clinically significant joint injuries, even when a defect-filling scaffold is provided, suggests that conditions *in vivo* in the early post-trauma phase are detrimental to CPCs. Cell mortality, oxidative damage, and physical dislodgement from joint surfaces could all plausibly retard CPC-mediated healing. Thus, we may need to develop interventions to mitigate the effects of secondary pathogenic factors on these cells to exploit their full potential.

In summary, migrating CPCs on injured explant surfaces are chondrogenic progenitors from the superficial zone that were activated by cartilage damage to attempt repair. Facilitating this endogenous process could allow repair of focal defects that would otherwise progress to post-traumatic osteoarthritis.

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