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Enhancing chondrogenesis of mesenchymal stromal cells through anti-Thy1 strategies

by

Hana Yu

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To my mother, father, and grandmother.

For I am everything that you have given me.

Abstract

The intricate arrangement and composition of articular cartilage confer the structure its unique material and mechanical properties. Degeneration of articular cartilage, especially osteoarthritis, imposes great burden not only on the patients, but also on society. While there exist various non-surgical and surgical treatment methods, continuous research is required to overcome the limitations of current therapeutic methods.

Mesenchymal stromal cell (MSC)-based therapy of cartilage defects has been developed to generate replacement articular chondrocytes for the production of extra cellular matrix. To develop a novel way of advancing MSC-based therapy, we examined the chondrogenic potential of MSCs that have undergone anti-Thy1 treatments. Although several studies have suggested a potential link between Thy-1 and chondrogenic potential of MSCs, our anti-Thy1 strategy did not enhance chondrogenic potential of MSCs. Furthermore, we report that a common lentiviral transduction strategy can compromise MSCs' chondrogenic capacity, and that Thy-1 expression during chondrogenesis is dynamic in its nature.

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List of Abbreviations

ACI = Autologous Chondrocyte Implantation

Adv = Adenoviruses

ANOVA = One-Way Analysis of Variance

BMI = Body Mass Index

CFU-Fs = Colony-Forming Unit Fibroblasts

CD = Cluster of Differentiation

DMEM = Dulbecco's modified Eagle's medium

DNA = Deoxyribonucleic Acid

dsDNAs = double-stranded DNAs

dsRNAs = double-stranded RNAs

ECM = Extracellular Matrix

GAG = Glycosaminoglycan

HEK = Human Embryonic Kidney

HIV-1 = Human Immunodeficiency Virus Type 1

hBM-MSC = human Bone Marrow Mesenchymal Stem Cells

hMSCs = Human Mesenchymal Stem Cells

HRQOL = Health-Related Quality of Life

IGF-1 = Insulin-like Growth Factor 1

ISCT = International Society for Cellular Therapy

LTRs = Long Terminal Repeats

LV = lentiviruses or lentiviral

MACI = Matrix-Assisted Chondrocyte Implantation

MFI = Geometric Mean Fluorescence Intensity

miRNA = micro RNA

mMSCs = Mature Mesenchymal Stem Cells

MNCs = Mono-Nucleated Cells

MOI = Multiplicity of Infection

MNCs = Mono-Nucleated Cells

mRNA = messenger RNA

MSCs = Mesenchymal Stem Cells

NA = Non Adherent

NSAIDs = Nonsteroidal Anti-Inflammatory Drugs

nt = nucleotide

OA = Osteoarthritis

OAT = Osteochondral Autograft Transplantation

PG = Proteoglycan

PKR = dsRNA-dependent Protein Kinase

PTGS = Post-Transcriptional Gene Silencing

qRT-PCR = Quantitative Real-Time Polymerase Chain Reaction

RA = Rapidly Adhering

RCRs = Replication-Competent Retroviruses

RISC = RNA-Induced Silencing Complex

RS-1 cells = Recycling Stem cells

RT = Reverse Transcriptase

RNA = Ribonucleic Acid

RNAi = RNA Interference

sfMPCs = Synovial Fluid Mesenchymal Progenitor Cells

sGAG = sulfated Glycosaminoglycan

shRNAs = short hairpin RNAs

SIN = Self-Inactivating

siRNAs = small interfering RNAs

ssRNAs = single-stranded RNAs

STZ = Superficial Tangential Zone

TGF- β3 = Transforming Growth Factor-β3

VSV = Vesicular Stomatitis Virus

VSV-G = Vesicular Stomatitis Virus G Protein

3D = Three-Dimensional

Part A: General Introduction

General Overview

With advancements in science and medicine, the average life expectancy of a human has increased dramatically over the past few decades. With a prolonged life span, people engage in various activities ranging from light activities, such as walking and writing, to strenuous activities, like running and cycling, until they are older. Against people's hope of maintaining active and healthy lifestyle, people's freedom of movements, and therefore their quality of life, is often restricted by degeneration of articular cartilage that comes with age or injury. While there are many types of joint injuries, osteoarthritis (OA) is especially debilitating. OA continues to progress when the material properties and structural integrity of articular cartilage are disrupted. Articular cartilage lacks the ability to heal itself owing to its avascular and aneural nature, and without effective treatment methods, the quality of life of OA patients will continue to suffer. To ameliorate the negative effects of OA on individuals and society, various treatment methods have been developed. Although many patients have benefitted from these therapeutic methods, more research must be done to overcome the obstacles that current treatment methods are facing. Our current study aims to develop a novel way of advancing mesenchymal stem cell (MSC)-based therapy via anti-Thy1 strategies. Several studies have suggested a potential link between the cell surface antigen Thy-1 and chondrogenic potential of multipotent MSCs. In order to develop a meaningful improvement in current cartilage regeneration

technique, it is essential to understand the basic anatomy and physiology of articular cartilage, health and social consequences of OA, advantages and disadvantages of current treatment methods, dynamic properties of MSCs, and current knowledge of Thy-1. Since gene silencing of Thy-1 via RNA interference (RNAi) is integral to our current research, the mechanisms and application of RNAi will also be explored. In order to devise an ideal protocol with which Thy-1 expression can be effectively reduced, several small experiments have been conducted. How the observations made in these experiments have shaped our approach to the central research question will also be demonstrated in this thesis. Finally, using the knowledge and insights gained from this review, the role of Thy-1 in chondrogenic differentiation of human bone marrow mesenchymal stem cells (hBM-MSCs) will be investigated. Healthy articular cartilage is key to maintaining productive and enjoyable life. This review will provide the basic information necessary to understand the purpose and direction of our current research.

Introduction

Degeneration of articular cartilage is a leading cause of disability resulting in a high economic and social burden on society. Owing to its avascular and aneural nature, articular cartilage cannot heal itself. Many nonsurgical and surgical techniques have been developed to help patients gain improved functionality. However, these therapeutic methods have limitations. Cell-based therapy is one of the most recent types of cartilage repair method. Current cell-based methods rely heavily on cell culture propagated autologous chondrocytes, but these cells suffer from low cell yields, undergo senescence during culture expansion and display poor cartilage forming characteristics. Because of the limitations of autologous chondrocyte implantation, mesenchymal stem cell-based cell therapy has been developed. Bone marrow mesenchymal stem cells (MSCs) are attractive as a cell source for joint articular cartilage repair and regeneration because of the cells' multi-lineage plasticity towards a variety of mesenchymal tissues including cartilage. Like any other cartilage repair techniques, MSC-based cell therapy is far from being perfect. Continuous research into finding ways of improving the chondrogenic capacity of MSCs is needed to increase the quality of cell-based therapy. Recently, Adesida *et al*. suggested a potential link between CD90 (Thy-1) expression and chondrogenic potential of bone marrow MSCs. In their experiment, CD90 protein expression was low in cells with higher chondrogenic capacity prior to chondrogenic stimulation. Although the role of CD90 has been explored for more than 40 years, hardly any studies have looked into its role in chondrogenesis of bone marrow MSCs. This review will provide an overview of

the anatomy and physiology of articular cartilage, current treatment methods for cartilage defects, properties of MSCs, and current knowledge of CD90 antigen. The background knowledge provided in this review will help one understand the importance of and the logic behind the proposed research.

Chapter 1: Anatomy and Physiology of Articular Cartilage

1.1 Articular Cartilage

Articular cartilage is a specialized hyaline cartilage found in diarthrodial joints (1). It is hypocellular, aneural, alymphatic, and avascular (2). Although it appears to be a simple inert tissue (3), the structure and composition of articular cartilage is very complex and intricate. Material properties of the hyaline articular cartilage, including thickness, cell density, matrix composition, and mechanical properties, vary between species and sites (4); however, articular cartilage in all synovial joints shares the same components, general structure, and functional purposes (3). Across all species, articular cartilage is known to have extraordinary mechanical properties. Moreover, despite the harsh and strenuous functions that the tissue serves, articular cartilage has amazing durability (1) that cannot be matched with any other synthetic materials.

1.2 Composition of Articular Cartilage

Articular cartilage is composed of two main components: cells and extracellular matrix. Cells account for only about 1% of the volume of adult human articular cartilage. Instead, water and extracellular matrix macromolecules are responsible for much of the volume and wet weight of normal articular cartilage (3, 5).

Chondrocytes

Articular cartilage is made up of only one type of cell, namely chondrocytes. Although these highly specialized cells occupy less than 5% of the total volume of articular cartilage, they are responsible for the "synthesis, remodeling and turn-over of the extracellular matrix (6)." Because each chondrocyte is surrounded by extracellular matrix, there is little cell-to-cell contact. Owing to the avascular nature of articular cartilage, chondrocytes must rely on synovial fluid for the exchange of organic and inorganic substances that are essential for maintaining the integrity of the tissue. Moreover, the fact that the synovial fluid must pass through a "double diffusion barrier" (first the synovial tissue and synovial fluid, and then the cartilage matrix) makes the cellular environment hypoxic. Consequently, chondrocytes predominantly undergo anaerobic respiration (3). The cell shape, density, metabolic activity, and organization differ in different zones of the cartilage. Although articular cartilage can be viewed as being "isolated" from the outside environment (owing to its aneural and avascular nature), chondrocytes receive, process, and respond to external stimuli that have been carried by the extracellular matrix (2). As one becomes skeletally mature,

the chondrocytes' ability to produce new tissue is largely lost; however, the chondrocytes of skeletally mature individuals will continue to regulate matrix composition (7). The metabolic activity of chondrocytes change during the course of one's development and upon varying use of articular cartilage (8). This is how articular cartilage remodels its surface and matrix in response to the changes in the external and internal environments.

Extracellular Matrix

Extracellular matrix (ECM) can be divided into two components: tissue fluid and macromolecules. The composition of extracellular matrix changes with age, the type of joint, and the site of joint (9, 10). The interaction between the two components of the ECM contributes to the tissue integrity and properties.

Tissue fluid

The major component of the ECM is water, which in the adult tissue represents about 70% of the weight (8). Water content in articular cartilage varies with the depth of the tissue. Water accounts for about 80% of the wet weight at the surface and for approximately 65% in the deep zone (11). Various organic and inorganic substances, including gases, small proteins, dissolved electrolytes, and various metabolites, are contained in the tissue fluid. The interaction between the tissue fluid and structural macromolecules helps to maintain the fluid and its solvents, within the matrix (1, 3). A small percentage of water is contained in the intracellular space (12). The remainder of the tissue fluid can be found in various parts of the matrix. About half of the water is thought to be associated with the

collagen fibers, while the other half seems to be "plasma ultrafiltrate" or free interstitial water (13). Tissue fluid plays an important role in both substance-exchange and in maintaining the biomechanical properties of articular cartilage. The flow of water through cartilage and across the articular surface aids in the transport of nutrients to the tissue and of wastes away from the tissue (1). The fluid is also critical in the fundamental mechanism of cartilage load support (14). The small pore size of the ECM causes high frictional resistance to fluid flow (1). This leads to a high interstitial fluid pressurization, which contributes to more than 90% of the load transmission function of cartilage for several hundred seconds after loading (14-18). Furthermore, high pressurization of the fluid phase establishes viscoelastic properties of cartilage (19). The resistive drag of interstitial fluid flowing through the low-permeability collagen-proteoglycan matrix, along with the intrinsic viscoelasticity of the cartilage solid matrix, is primarily responsible for the viscoelastic and dynamic response of cartilage under confined compression (14, 20). The frictional resistance to fluid flow coupled with the pressurization of the water within the ECM gives articular cartilage the compressive strength and ability to withstand high joint loads (1).

Collagens

The collagen network can be described as the "endoskeleton of cartilage" (2). The solid phase of articular cartilage is mainly composed of a three-dimensional network of collagen fibrils. About 60% of the dry weight of cartilage comes from collagens (1). Although the concentration of collagens throughout the various zones is fairly uniform, the orientation of the fibrils varies between zones (21).

There are many types of collagen, including collagen type II, III, V, VI, IX, X, and XI. Type II collagen is the predominant form of collagen (90-95%), while the contributions by types III, VI, IX, X, and XI collagens are minor (1). Regardless of the types, all collagens are composed of three identical polypeptide chains (α-1 chains) wound into a triple helix (22). Collagen II, IX, and XI together form a heterofibril (**Figure 1-1**) (23, 24). The maintenance of the structure is critical to the structural integrity of cartilage and degeneration of this heteropolymer will likely compromise the function of cartilage to a great extent (25).

Type IX collagen accounts for approximately 1% of total collagen, and its molecule are covalently bonded to the surface of type II collagen fibrils or with other collagen IX molecules (26-28). It is suspected that type IX collagen plays an important role in fibril-fibril and fibril proteoglycan interactions (29, 30). Furthermore, the covalent cross-linking of type IX collagen molecules with other collagen fibrils seems to be closely associated with the reorganization of the collagen network and the consequent network failure in osteoarthritic cartilage (28). Type XI is intimately copolymerized with type II collagen and with other collagen XI molecules (31). It is believed that the control of lateral fibril growth is an intrinsic property of appropriate collage II and collagen XI mixtures (32).

Collagen type III is found in a low abundance. Nevertheless, cross-linking studies show that it is a regular component in articular cartilage. It is known to be found pericellularly throughout the entire depth of the cartilage (33). Collagen III fibrils are linked to other collagen III molecules, as well as to the surface of type II collagen (34). From the extensive cross-linkage between collagen types II and III,

along with the fact that type III collagen is known to be prominent at sites of healing and repair in skin and other tissues (35), it is suspected that type III collagen plays a key role in matrix reinforcement and a healing response to matrix damage (34).

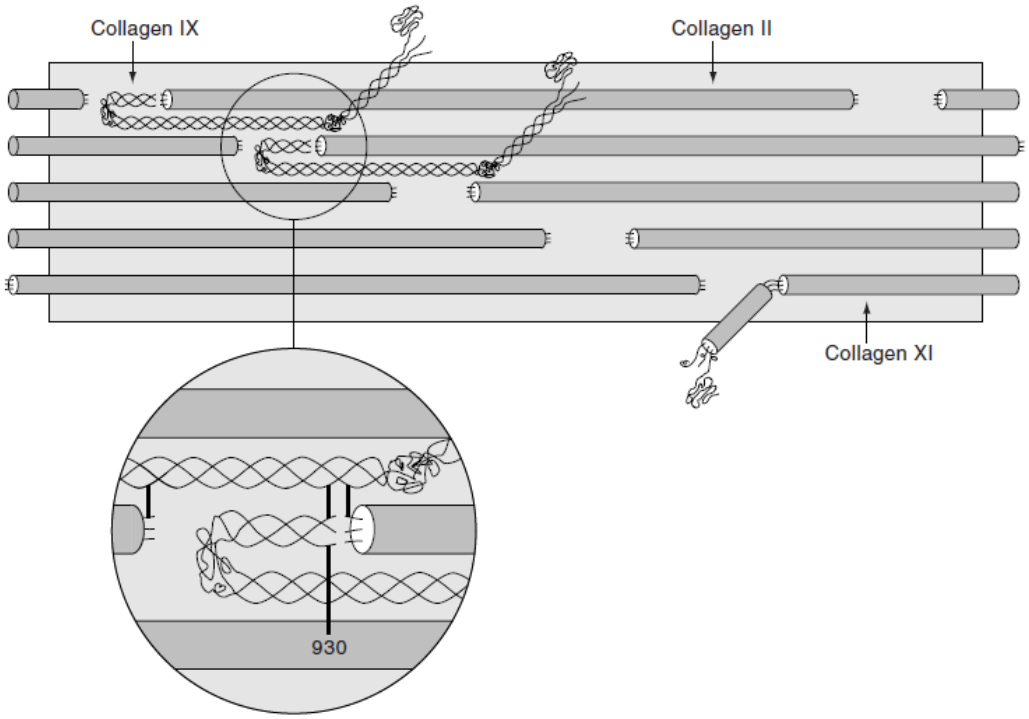

Figure 1-1 The collagen II:IX:XI heterofibril. A molecular model of the collagen type IX fold and interaction site with a collagen II microfibril that can account for all known cross-linking sites between collagen II and IX molecules (23).

Type VI collagen can be found mostly in pericellular spaces (36), although it is also interspersed loosely in spaces throughout the matrix (37). Lastly, literature has suggested that type X collagen is a skeletal-specific molecule that may be present in bone, as well as in hypertrophic cartilage (38). Overall, the intimate and intricate association between the various collagen fibrils, coupled with the

specific orientation of the fibrils in different zones, confers great tensile strength to the cartilage, thereby helping to maintain the volume and shape of the tissue.

Proteoglycan

Proteoglycan (PG) is responsible for approximately 10-15% of the wet weight of articular cartilage. PG is secreted by chondrocytes, into the ECM (1). Although its presence in our body is ubiquitous, PG is most abundantly found in the hyaline cartilages (30). A PG monomer consists of a core protein with covalently bound glycosaminoglycan (GAG) side chains (**Figure 1-2**) (1, 2, 30). The macromolecules are called the PG aggrecan molecule. Many PG aggrecan molecules interact non-covalently with hyaluronic acid to form large proteoglycan aggregates (30, 39). The hyaluronan and aggrecans are joined via link protein (**Figure 1-2**) (40). A single GAG is an unbranched chain of repeating disaccharide units. Four types of sulfated GAGs, namely chondroitin sulfate, dermatan sulfate, keratin sulfate, and heparin sulfate, and a non-sulfated GAG (hyaluronic acid) are found in articular cartilage (30, 41). Overall, these GAG side chains contain numerous negatively charged carboxyl and sulfate groups. The negative charges on the GAG side chains repel one another and make the aggregates swell. The distended volume of the PG adds to the tensile strength of the collagen network. Furthermore, the high concentration of negative charges attracts water and cations. The large number of ions creates an osmotic pressure, which enhances fluid flow into the tissue. These properties of PG give articular cartilage its elasticity and resilience (42, 43).

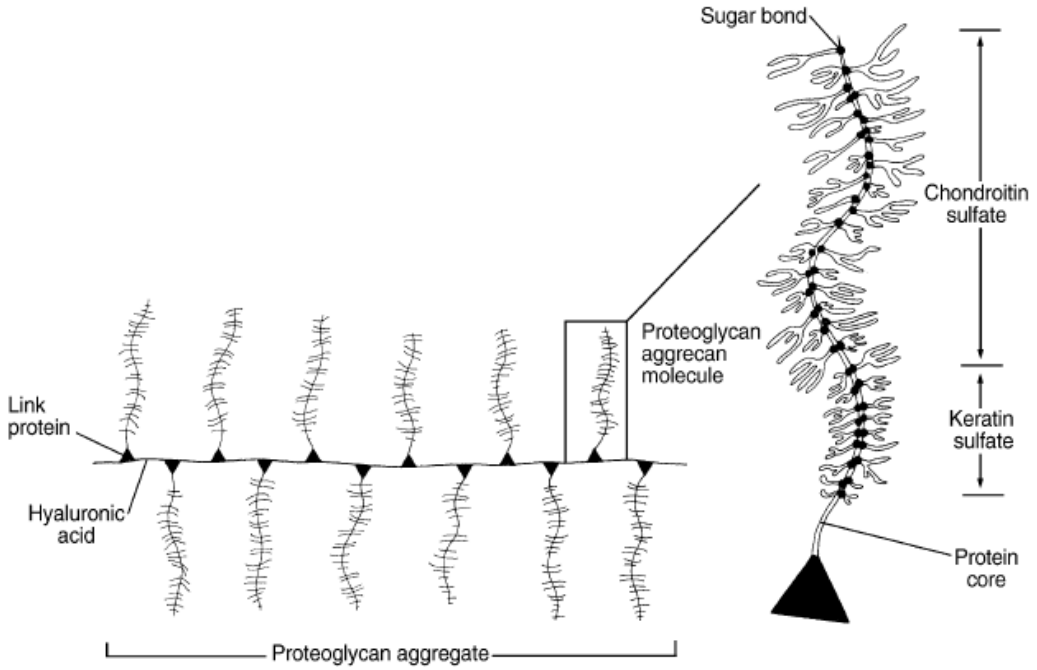

Figure 1-2 Diagram of the proteoglycan aggregate and aggrecan molecule.(44)

Non-aggregating proteoglycans, such as biglycan, decorin, and fibromodulin, are also present in articular cartilage and are thought to help stabilize the ECM (45). The structure, composition, and concentration of proteoglycans vary throughout the tissue depth and with age (46-48).

Non-collagenous proteins and glycoproteins

Articular cartilage also contains other substances, including non-collagenous proteins and various glycoproteins. Although their functions are not fully understood, it is suspected that they have roles in maintenance and organization of macromolecular structure of the matrix, cell-matrix interactions, and the responses of the tissue in inflammatory arthritis and osteoarthrosis (3).

1.3 Zones of Articular Cartilage

Articular cartilage can be roughly divided into four horizontal layers, or zones: the superficial, transitional or middle, deep or radial, and calcified cartilage zones (**Figure 1-3**).

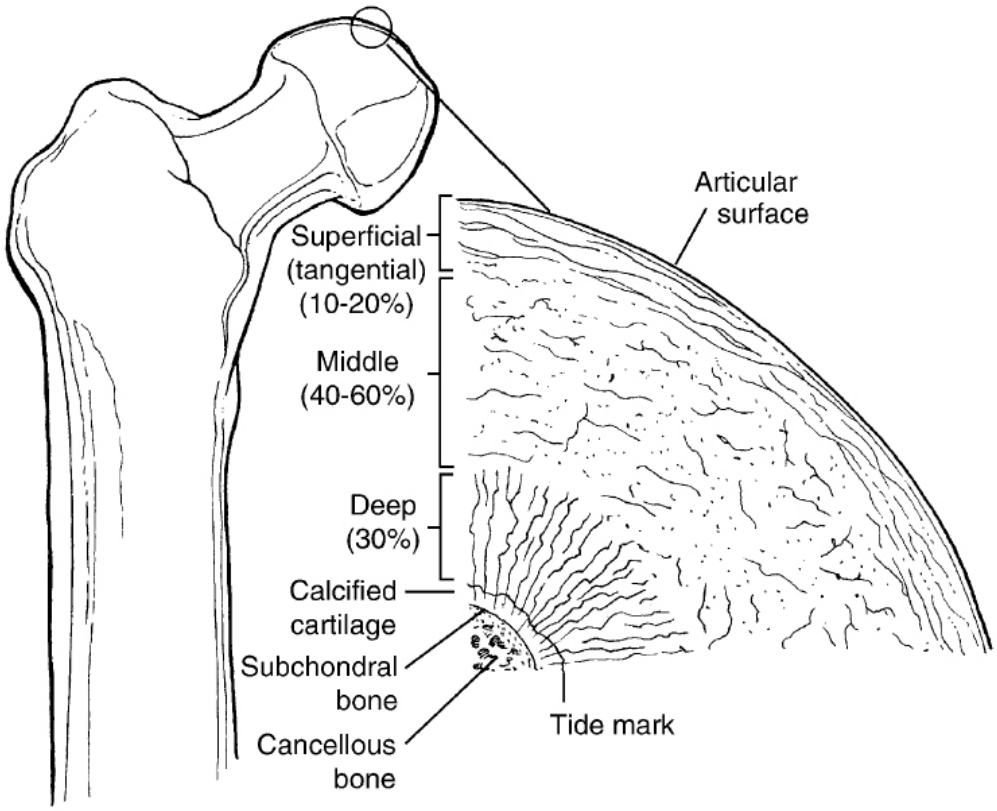

Figure 1-3 Stratified structure of cartilage demonstrating zonal arrangement. (44)

Each zone has its unique morphological, compositional, and organizational features (2, 3). In addition, articular chondrocytes from various zones are significantly different in their morphology, rate of proliferation, and biochemical activity (49, 50). These characteristics are of functional importance.

Superficial Zone

The outermost layer, or the superficial zone, is the thinnest zone of all. This layer can be divided into two sub-zones. The first sub-zone is the lamina splendens (**Figure 1-4**). The lamina splendens is acellular sheet of fine fibrils that covers the joint surface. The second-zone consists of both chondrocytes and collagen fibers. Within this sub-zone, the collagen fibrils run parallel to the surface (1, 21, 51, 52). The parallel orientation of these collagen fibrils gives this zone greater stiffness, greater strength, and less extensibility compared to the deeper zones (53). The second sub-zone contains the flattened chondrocytes that show a low PG production activity and a high PG degradation activity (49, 50, 52). Consequently, the PG content of the superficial zone is at its lowest. It should also be noted that fibronectin and water concentrations are highest in the region (1-3). These characteristics of the superficial zone contribute to the functional and biochemical properties of articular cartilage. The results from a confined compression creep experiment showed that the superficial zone plays a critical role in restricting fluid exudation and interstitial fluid movement within the tissue, thereby contributing to the compressive behavior of articular cartilage (54). In addition, biomechanical and biochemical testing on canine superficial-zone specimens demonstrated that the disruption and remodeling of the collagen network in the superficial zone may play a critical role in the development of osteoarthritis (55).

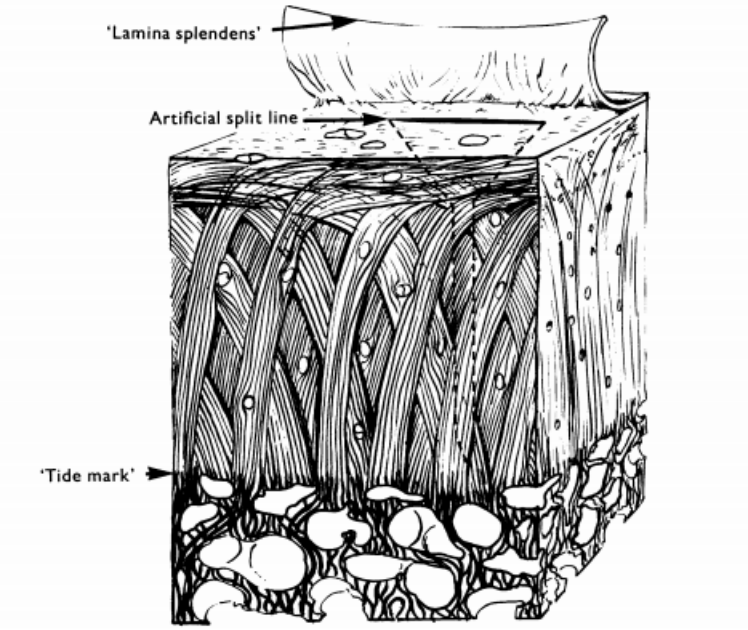

Figure 1-4 Schematic view of the collagen fibrillar organisation in human articular cartilage in a full thickness block, from the surface to the deep subchondral bone (21).

Transitional (Middle) Zone

The transitional or middle zone is sandwiched between the superficial and deep zones. The volume of this zone is typically several times greater than that of the superficial zone. The collagen fibers have larger diameters and show a random arrangement (1, 2, 52). The chondrocytes in this region are rounder. These spheroidal-shaped cells are rich in synthetic organelles, endoplasmic reticulum, and Golgi membranes. Consequently, the proteoglycan concentration is higher in this zone compared to the superficial zone.

Deep (Radial) Zone

The chondrocytes in the deep zone are round in shape, and are grouped in columns that are perpendicular to the joint surface. This zone is characterized by

collagen fibrils with largest diameters, the highest PG concentration, and the lowest water concentration (3). Collagen fibers are organized into randomly arranged large bundles (approximate diameter = 55μm) (2, 21).

Tidemark

A wavy, irregular line called the tide mark separates the deep zone from the calcified zones (**Figure 1-5a**) (2). The formation of tidemark is an active process (56). The tide mark has periodic gaps, which might provide suitable pathways for substance-exchange between the non-calcified cartilage, calcified cartilage, and subchondral bone (**Figure 1-5b**).

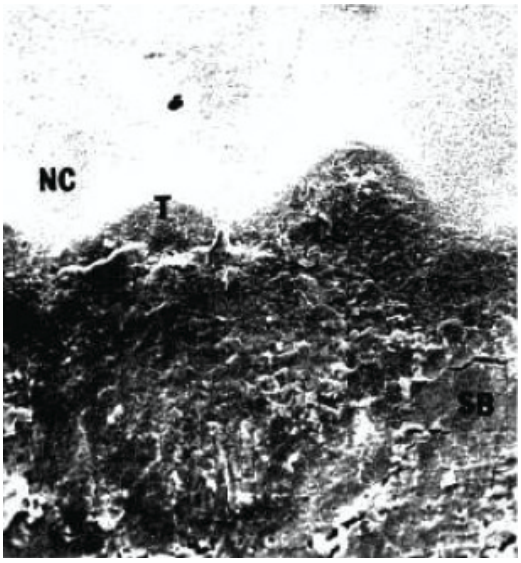

Figure 1-5a SEM micrograph of a perpendicular section of normal articular cartilage (reduced from x 300). NC: non-calcified cartilage; SB: subchondral bone; T: tidemark (57).

Figure 1-5b SEM micrograph of a perpendicular section of articular cartilage showing a gap (↓) in the Tidemark (reduced from x500). NC: non-calcified cartilage; SB: subchondral bone; T: tidemark (57)

Calcified Zone

The calcified zone is thin and is closest to the subchondral bone. This region is largely devoid of proteoglycans (2). The cells are round and contain only small amounts of endoplasmic reticulum and Golgi membranes. These chondrocytes show a low level of metabolic activity (3) and are distributed randomly in the apatitic salts-rich matrix (1). Electron microscopy scans showed that collagen fibers run perpendicular to the articular surface (51). These fibers are arranged in small bundles, which combine into larger bundles at the tidemark (**Figure 1-6**) (2, 21). This branching of fibrils diffuses and distributes the load over the entire cartilage-subchondral bone boundary (57). The collagen fibers anchor the rest of the cartilage structure to the subchondral bone underneath. Furthermore, the calcified zone likely blocks the transport of nutrients from the subchondral bone. Consequently, articular cartilage must depend on synovial fluid for nutritional support (1).

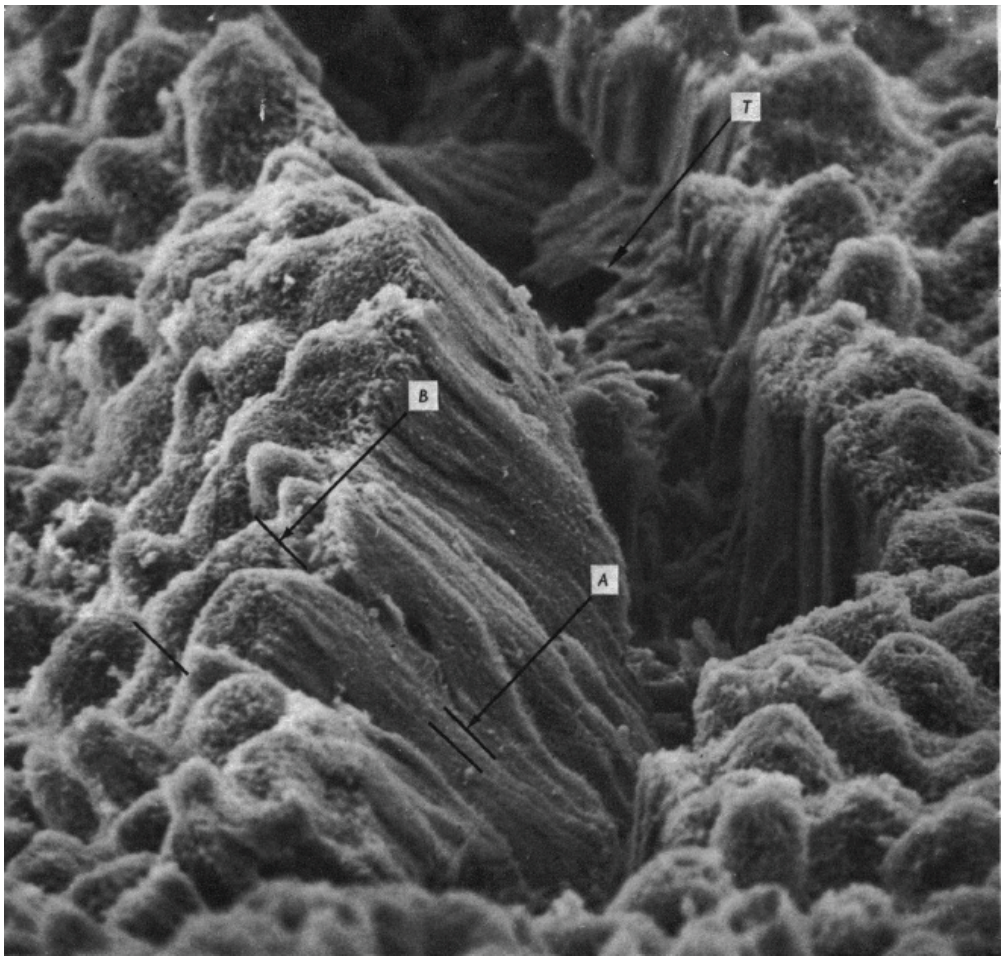

Figure 1-6 A split in the fibrillar structure of the calcified zone showing fibre bundles oriented toward the surface. Calcified fibre bundles (arrow *A*) appear to be aggregated to form larger bundles (arrow *B*) shown sectioned at the exposed surface. The underlying trabecular (*T*) are also exposed at the base of the split (reduced from x 330) (21).

1.4 Biomechanical Properties and Functions of Articular Cartilage

Articular cartilage must endure tremendous stresses and strains that develop during normal daily activities like walking, running, and jumping. The average load that a knee joint experiences during a daily life is about three times greater than the body weight (58). The ability of articular cartilage to store, transmit, and dissipate mechanical energy, coupled with its tensile and compressive properties, enables the cartilage to withstand the forces. The crosslinking of collagen fibrils is responsible for the tensile strength, while PGs and water within the collagen network confers resilience to the structure. Repulsion between the negatively charged PG side chains resists compression beyond a "point-of-no-return." The shape of the tissue is restored when water returns back to the structure as load is released (1). The fact that interstitial fluid pressure provides support to more than 95% of the applied load in normal activities partially explains why articular cartilage can withstand the high repetitive loads for many decades (1, 59) The viscoelastic nature of articular cartilage is also critical for functioning of healthy articular cartilage. The frictional drag resulting from interstitial fluid flow is the main source of the viscoelastic property. The loss of this drag as a result of increased permeability and water content leads to a loss of stress-shielding effect to protect the ECM (60). The intrinsic properties of the components of articular cartilage, their organization within the tissue, and interactions between the individual components contribute to the function of articular cartilage to provide a smooth, low-friction surface at the joint and to effectively and efficiently support and distribute load with great durability.

Chapter 2: Degeneration and Repair of Articular Cartilage

2.1 Degeneration of Articular Cartilage and its Impact on Society

When the molecular composition and organization of articular cartilage are disrupted, the material properties and structural integrity of the tissue deteriorate. Because of its aneural and avascular nature, articular cartilage has a limited ability to respond to injury (61-63). Although there are many types of joint diseases/injuries, osteoarthritis (OA) or osteoarthrosis is the leading cause of chronic disability. Although OA can be caused by multiple factors, such as trauma, joint laxity, crystal deposition, bone microfractures, and immunological factors, aging and mechanical wear resulting from excessive repetitive loading of the cartilage are the two most well-understood causes of the breakdown of articular cartilage (2). Decreases in tensile strength of weight-bearing articular cartilage resulting from changes in the organization of the collagen fiber network begin after the third decade of life (64). With increasing age, human articular cartilage experiences fundamental changes in the sizes and compositions of its proteoglycans. It has been also noted that during aging, there is "the progressive accumulation of the hyaluronic acid-binding region essentially free of the keratan sulphate-rich region and the rest of the proteoglycan monomer, and the progressive partial cleavage of the link proteins (65)." Furthermore, arthritic cartilages undergo much more collagen degradation compared to healthy cartilages (66). Loss of type II collagen content leads to softening of the tissue, and eventually to cartilage destruction (2). A basic summary of the progression of degeneration of articular cartilage in osteoarthrosis is illustrated in **Table 2-1** (61).

Table 2-1 Stages in the Development and Progression of Degeneration of Articular Cartilage in Osteoarthrosis (61).

Stage	Description
I: Disruption or alteration of cartilage matrix	Disruption or alteration of macromolecular framework of matrix associated with increase in concentration of water that may be caused by mechanical insults, degradation of matrix macromolecules, or alterations of chondrocyte metabolism. At first, concentration of type-II collagen remains unchanged, but collagen meshwork may be damaged, and concentration of aggrecans and degree of proteoglycan aggregation decrease.
II: Response of chondrocytes to disruption or alteration of matrix	When chondrocytes detect a disruption or alteration of their matrix, they can respond by increasing synthesis and degradation of the matrix and by proliferating. Their response may restore tissue, maintain tissue in an altered state, or increase volume of cartilage. They may sustain an increased level of activity for years.
III: Decline in response of chondrocytes	Failure of chondrocytic response to restore or maintain tissue leads to loss of articular cartilage accompanied or preceded by decline in chondrocytic response. The causes for this decline remain poorly understood, but they may partially result from mechanical damage to tissue with injury to chondrocytes and down-regulation of chondrocytic response to anabolic cytokines.

According to the World Health Organization's 2011 report, 9.6% of men and 18.0% of women older than 60 years of age worldwide suffer from symptomatic osteoarthritis (OA) (67). Furthermore, it is estimated that in the year 2020, about 18.2 % of the U.S. population or roughly 59.4 million people will be affected by OA (68). OA has a large impact on people's quality of life. Almost 80% of OA patients experience limitations in movement, and 25% reported that they cannot perform their daily activities (67). Moreover, health-related quality of life (HRQOL) of OA patients was measured to be lower than that of individuals

suffering other chronic diseases, including chronic obstructive pulmonary disease, low blood pressure, and irritable bowel syndrome (69). OA is not only a problem of health, but is also economically burdensome. "The economic burden of a disease comprises direct costs, such as the costs of drugs, medical care, hospitals, research, pensions and benefits, and indirect costs, such as premature mortality and chronic and short-term disability (70)." It has been reported that people with OA are less likely to be employed relative to workers without OA pain. This difference was mainly due to the OA patients' impaired productivity at work ($p < 0.0001$) (71). According to the same study, healthcare resource utilization was also significantly higher ($p < 0.0001$) among the workers with OA pain than those without OA pain (71). Another study that was conducted in 2010 also noted that as the severity of OA increased, the cost related to lost work productivity also increased (72). OA is a pervasive disease and is becoming a greater concern as the average lifespan of humans continues to increase. It is also important to note that any kind of cartilage defect can be detrimental to quality of life. Effective and efficient treatment methods are needed to help people maintain healthy life.

2.2 Nonoperative Treatments

The initial management of cartilage lesions is largely nonoperative. The nonoperative treatments include but are not limited to deep heat (diathermy) therapy (73), cryotherapy (74, 75), physical therapy, bracing (76-79), exercise (80-82), activity modification through patient education (83), topical medications (84), systemic medications (ex. analgesics, nonsteroidal anti-inflammatory drugs, glucosamine and chondroitin sulfate) (85-88), and intra-articular medications (89, 90). The primary aim of the initial treatment is to control symptoms and improve joint function. However, it is important to note that patient responses to these initial treatment methods are unpredictable. Although these treatment modalities can be effective at relieving pain and improving function in affected patients, they do not treat the underlying cause and more invasive methods of treatments are often required (1). These nonoperative treatments can also be used post-surgery to relieve pain or to enhance recovery. These treatment methods are more approachable, less invasive, and many times cheaper than operative treatments. However, some of the nonoperative treatments can cause serious complications in patients. For instance, although nonsteroidal anti-inflammatory drugs (NSAIDs) have been used many years to relieve pain, continuous use of the drugs can lead to gastrointestinal bleeding, cardiac dysfunction, and kidney dysfunction, and can even be detrimental to bone and tendon healing (91-95).

2.3 Marrow Stimulation Techniques

Both patient-specific and lesion-specific variables must be taken into account when making the decision on the surgical management of cartilage defects. Patient-specific variables include things such as age, physical fitness, body mass index (BMI), and associated injuries, while lesion-specific variables include level of acuteness, size and location, containment, and history of previous surgical interventions (96). One of the most commonly performed procedures for cartilage lesions is marrow stimulation technique. This category of surgical intervention includes strategies such as transcortical Pridie drilling, abrasion arthroplasty, microfracture, and enhanced microfracture (96, 97). In treating symptomatic cartilage damage, marrow stimulation strategies are among the most frequently used methods. Owing to the avascular nature of articular cartilage, full-thickness cartilage injuries that do not involve bone have limited intrinsic capacity to heal on their own. The principle of marrow stimulation is therefore to create blood supply and facilitate the local recruitment of multipotent bone marrow mesenchymal stem cells to the otherwise avascular joint surface. These vascular access channels also allow an inflow of fibrin, platelets, and various growth factors to the area of defect and allow the formation of what is called "super-clot (1)." This technique is effective because human mesenchymal stem cells (hMSCs) have the ability to form a type of repair cartilage. However, the mechanical properties of the newly formed tissue have been reported to be closer to those of fibro-cartilage. Fibrocartilage is predominantly made up of collagen type I, while the main constituent of hyaline cartilage is type II collagen. Consequently,

"growing fibro-cartilage into areas previously occupied by hyaline cartilage will expose the new tissue to a mechanical environment characterized by compressive forces to which it is somewhat ill-equipped (96)." However, more recent studies have reported that the newly grown tissue may be a combination of fibro-cartilage and hyaline-like cartilage (98), indicating that the newly formed tissue might be more resilient to mechanical forces than what had been expected. It is now understood that the hMSCs differentiate into fibrochondrocytes that produce varying amounts of type I and II collagens (99-101). Nevertheless, the inferior quality of the repair tissue, incomplete defect filling and new bone formation in the defect area are limitations of these methods (99). The methods and mechanisms of microfracture technique will be explored further to enhance the understanding of the marrow stimulation strategies.

Microfracture

A popular method of treating cartilage lesions is microfracture. After the destroyed and unstable cartilage is removed arthroscopically, micropenetration of the subchondral plate can be performed (**Figure 2-1**, **Figure 2-2**). Perforation of subchondral bone plate facilitates the filling of the cartilage defect with a blood clot that contains hMSCs. This technique is an appealing treatment option, because it is relatively simple to perform and carries minimal morbidity (96). Other strengths of this technique include its limited invasiveness, relatively short postoperative recovery time, and cost-effectiveness (97).

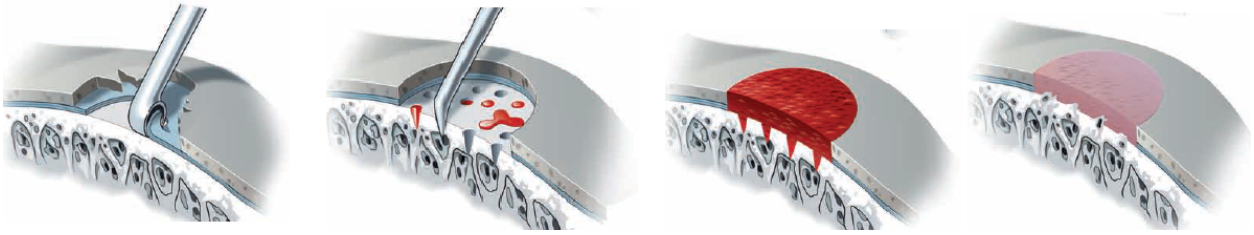

Figure 2-1 Surgical technique of microfracture: Creation of vertical margins and removal of the calcific cartilage layer with curette or Volkmann spoon. Perforation of subchondral bone plate with microfracture awl to facilitate mesenchymal stem cell clot formation. Awl tip should be driven perpendicularly into sub-chondral bone. Gradual conversion of 'super-clot' into fibro-cartilage over a period of 8 to 12 weeks (96).

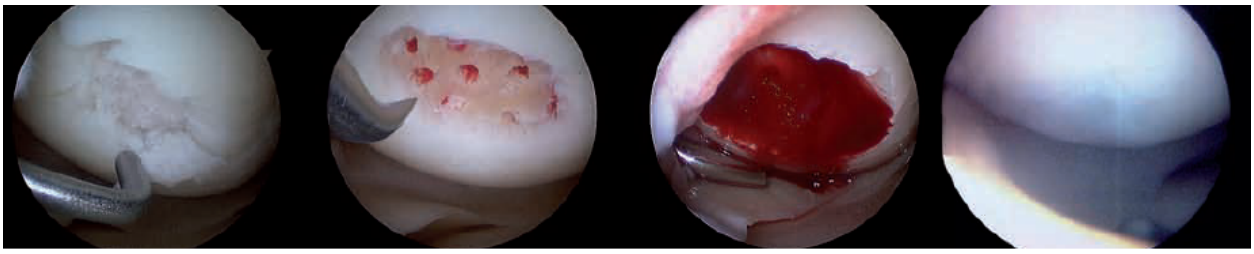

Figure 2-2 Full thickness cartilage defect on medial femoral condyle treated with microfracture in accordance to the technique shown in Figure 2.1. Second look arthroscopy at 9 months revealed complete coverage with repair cartilage (far right) (96).

In a study, 40 patients with a single symptomatic cartilage defect and no general osteoarthritis in the knee were enrolled in a randomized experiment. These patients received microfracture treatment, and a five-year follow-up evaluation showed that approximately 77% of the patients were satisfied with their results (98). Similar studies have shown that microfracture repair of articular cartilage lesions in the knee results in significant functional improvement and relief from pain (102, 103).

2.4 Replacement Techniques

Replacement techniques involve, but are not limited to osteochondral autograft transplantation, osteochondral allograft transplantation, and the use of synthetic resorbable scaffolds. The basic principle behind the techniques is to "provide instantaneous repair through structural reconstitution (96)." Two of these techniques will be reviewed further.

Osteochondral Autograft Transplantation

Osteochondral autograft transplantation (OAT), also known as mosaicplasty, is based on the transfer of one or more cylindrical osteochondral plugs into the chondral defect of the weight-bearing cartilage. Grafts that are about 2.5 to 10mm in diameter are harvested from comparatively non-weight bearing periphery of the trochlea or inter-condylar notch. These plugs are then placed into the recipient site using a press-fit technique (**Figure 2-3**) (96, 97). Hangody *et al.* reviewed 831 mosaicplasties that were done at their institution. "The results of the resurfacing procedures were evaluated at regular intervals with use of standardized clinical scores and radiography, and selected patients were also assessed with magnetic resonance imaging, second-look arthroscopy, histological analysis of biopsy materials and cartilage stiffness measurement (104)." Although complications were observed in 40 out of the 831 patients after surgery, "69 of the 83 patients who were followed arthroscopically demonstrated good gliding surfaces, histological evidence of survival of the transplanted hyaline cartilage, and fibrocartilage covering of the donor sites (104)." Likewise, Jakob

et al. examined 52 patients who had mosaicplasty of the knee. Although four patients required reoperation owing to graft failure, their two-year follow-up study showed that an increased level of knee function was found in 86% of the patients. The OAT technique has been evaluated in many other studies, and showed encouraging results (105-109). However, this technique has its limitations. These limitations include "difficulty restoring concave or convex articular surfaces, incongruity of articular surfaces that can alter joint contact pressures, short-term fixation strength and load-bearing capacity, donor site morbidity, and lack of peripheral integration with peripheral chondrocyte death associated with graft harvesting and insertion (97)." Furthermore, the technique is limited by the amount of donor tissue available, and therefore is best suited for lesions of less than $4cm^2$ (96).

Osteochondral Allograft Transplantation

The surgical procedures of osteochondral allograft transplantation are similar to those of OAT. The major difference is that this specific technique uses osteochondral grafts harvested and maintained by a tissue bank. Like OAT, many positive results have been reported on osteochondral allograft transplantation method (110-113). Although this technique avoids the donor site morbidity, the treatment method has the same kind of issues found in OAT. Additional disadvantages of osteochondral allograft transplantation include higher cost, potential for disease transmission and potential for immunologic reactions (112).

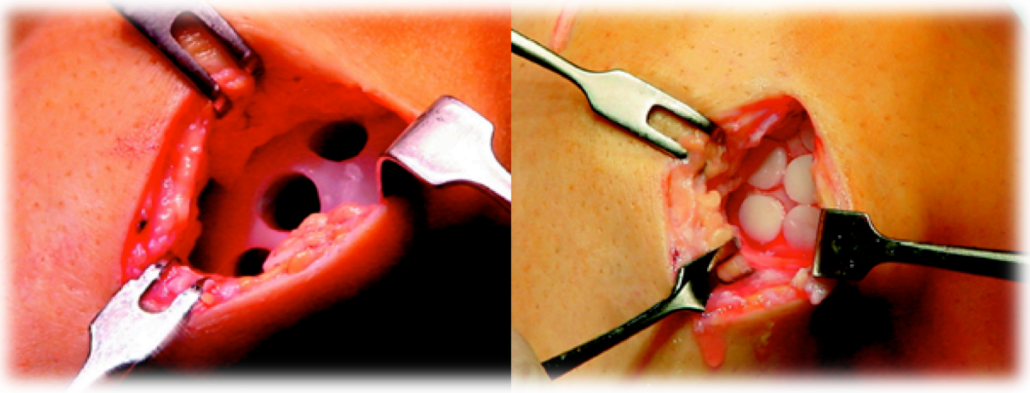

Figure 2-3 Miniarthrotomy mosaicplasty (104).

In addition, tissue availability is a major limiting factor of the treatment method, because long-term storage of the grafts is discouraged. It has been suggested that the implantation should be performed within 28 days of graft harvest, because the material properties of hypothermically stored grafts steadily decrease over time (114).

2.5 Cell Based Therapy

In the early 1970s, Bentley *et al.* demonstrated that the homografts of epiphyseal cells in suspension can survive, produce matrix and become incorporated into defects made in the tibial articular surface of adult rabbit knees. Moreover, the cells arranged themselves into layers similar to those found in surrounding cartilage and endochondral ossification occurred in the base of the graft (115). This finding sparked the development of clinical tissue engineering. One of the first forms of cell-based therapies is autologous chondrocyte implantation (ACI). However, the limited ability of chondrocytes to expand *ex vivo* has led to the investigation of MSCs as an alternative cell source. "The cellular therapies seek to generate replacement articular chondrocytes for the production of cartilage tissue thereby reducing pain, restoring joint function and delaying the onset of cartilage degradation and the need for prosthetic joint replacement (116)."

Autologous Chondrocyte Implantation

In ACI, biopsies of articular cartilage that are taken from the low weight-bearing region of the patellofemoral joint are expanded *ex vivo* for the re-implantation into debrided areas of the damaged weight-bearing surface (116-119). To reduce the risk of cell leakage from the graft site and promote more homogeneous distribution of chondrocytes throughout the graft, next generations ACI procedures, including Matrix-Assisted Chondrocyte Implantation (MACI) and autologous chondrocyte scaffolds have been developed (120-124). The results from ACI treatments have been encouraging (125-129). A review has suggested

that ACI technique might be more effective than some other techniques at repairing articular cartilage defects (130), although more studies need to be done to validate the argument. Furthermore, long-term effectiveness of the treatment method still needs to be confirmed. Disadvantages of the procedure also include technical difficulties with fixation of the membrane, problems with graft delamination and overgrowth (hypertrophy), invasiveness of the procedure, the requirement for a second surgery, dedifferentiation of mature chondrocytes into fibroblast-like cells, and high medical expenses (131-135).

Mesenchymal Cell-Based Repair

This technique shares the same principle as ACI; however, the source of cells is hMSCs rather than mature chondrocytes. In 1999, Pittenger *et al.* demonstrated multipotent nature of hMSCs by differentiating adult hMSCs into three cell lineages of adipocytes, osteoblasts and chondrocytes (136). Ever since, interest in the application of hMSCs for the development of articular cartilage cell-based therapies was reignited. Various studies have shown that the MSCs can be differentiated into chondrocytes that are capable of producing functional cartilage matrix. MSCs have been traditionally harvested from the iliac crest. The harvested MSCs can be expanded in monolayer culture until they are collected to be injected into the site of defect. Mesenchymal stem cell-based therapy has shown positive results, although long-term effectiveness of this treatment method is still unclear (137-140). One of the advantages of using MSCs for cartilage repair is their high proliferative ability. This ability allows for application of the

treatment method to very large defects. Another potential advantage is that "they may be more active than host autologous chondrocytes and produce a more regenerative matrix (141)." However, this idea is only a speculation and has not been proven. Despite its advantages, continuous research is needed to improve the technique. The biological and mechanical properties of hMSCs-derived repair cartilage are still thought to be inferior to those of normal human articular cartilage. Variability in the quality of hMSCs (142) and subsequently of differentiated chondrocytes also needs to be controlled for, if this treatment method is to become more effective. "The challenge [of the cell-therapy] is to produce a neocartilage that is sufficiently mature to withstand the biochemical environment of the joint, but immature enough to allow not only remodeling but also integration with host tissue, including the subchondral bone below the defect (141)."

Chapter 3: Mesenchymal Stem Cells and their Chondrogenic Potential

3.1 Definition of Mesenchymal Stem Cells

In general terms, MSCs are cells that have the capacity to self-renew and give rise to cells of multiple lineages. Although self-renewal and multipotency are two broad criteria that define MSCs as real stem cells (143), exact definition of MSCs is a matter of debate. Nevertheless, the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (ISCT) suggested minimal criteria to define human MSCs: (1) MSCs must be plastic-adherent when maintained in standard culture conditions and form colony-forming unit fibroblasts (CFU-Fs), (2) MSCs must express CD105, CD73, and CD90, and lack expression of CD45, CD34, CD14 or CD 11b, CD79α, or CD19, and HLA-DR surface molecules, and (3) MSCs must differentiate to osteoblasts, adipocytes, and chondroblasts *in vitro* (144). However, these characteristics might not be a correct representation of unmanipulated MSCs *in vivo*. It has been suggested that discrepancies between various reports on the characteristics of MSCs might arise due to the differences in isolation method, tissue and species of origin, and culture conditions (145). The fact that there is no efficient method of characterizing unmanipulated MSCs is of special concern, because MSCs are extremely sensitive to their physical environment.

Various studies have demonstrated that hMSCs undergo alterations in their physiology and chemistry during culture (146). For instance, a distinct expression of certain surface antigens including CD45 and CD31 was found in freshly

isolated hMSCs, while the expression was significantly lower in culture expanded hMSCs (147). MSCs were first recognized and have been primarily studied *in vitro* (145). There has been minimal information on the *in vivo* behavior and characteristics of MSCs, mainly because MSCs represent only about 0.001-0.01% of the total number of nucleated cells in the bone marrow (136). Without *in vitro* expansion, efficient and realistic examination of the MSCs is extremely difficult. Although the concept of a stem cell has been one of the organizing principles of developmental biology for more than a century, our understanding of MSC biology remains rudimentary. Like the multipotent nature of MSCs, the definition and concept of stem cells have shifted continuously and have displayed a remarkable degree of plasticity (148).

3.2 Characteristics of Mesenchymal Stem Cells

Historically, MSCs from the bone marrow stroma have been the "gold standard" of stem cell research. Furthermore, bone marrow MSCs are the most advanced MSCs with regard to clinical development (149). However, mesenchymal stem cells are known to reside in virtually all post-natal organs and tissues, including the brain, spleen, liver, kidney, bone marrow, muscle, fat, periosteum, trabecular bone, and deciduous teeth (150-155). Although various studies have reported on varying differentiation potential among the MSCs from different tissue sources (156, 157), there are certain characteristics that are common to all MSCs. Apart from what has already been discussed in **Chapter 3.1**, some important features of all types of MSCs include their immunological characteristics and response to continuous passaging *in vitro*.

Immunological Characteristics

Several studies reported that MSCs escape immune recognition and inhibit immune responses (158). In conjunction with this observation, it was also reported that MSCs and their differentiated derivatives do not express HLA-Class II antigens *in vitro* and possess only low level expression of co-stimulatory molecules (159). More specifically, the immune phenotype of cultured MSCs is widely described as MHC Class I^+, MHC Class II^-, $CD40^-$, $CD80^-$, and $CD86^-$ (160). These phenotypes suggest the nonimmunogenic nature of MSCs and imply that MSCs might be effective in inducing tolerance. These immunological characteristics make MSCs suitable for clinical use in an allogeneic manner in

diverse regenerative medicine approaches (161). Moreover, MSCs have the capacity to engraft into various tissues and organs when infused systemically, and this engraftment has been shown to be stable in the long term (162, 163). Although the exact mechanism of immunomodulatory effects of MSCs is not clear, their low expression of class II Major Histocompatibility Complex and costimulatory molecules in their cell surface augments their potential utility in tissue engineering, and cellular and gene therapy (164).

Effect of Continuous Passaging

Despite its great potential as a clinical tool, MSCs have their limitations. MSCs have a limited lifespan *in vitro* as any normal, somatic cell. After a certain number of cell divisions, MSCs enter senescence, which is morphologically characterized by enlarged and irregular cell shapes and is ultimately a stop of proliferation (165). It has been shown that MSCs demonstrate loss of multipotentiality (166), morphological abnormalities, enlargement, attenuated expression of specific surface markers, and stop in proliferation within 43 to 77 days of cultivation (7 to 12 passages). These changes are thought to be a continuous process starting from the first passage onwards (167). Because MSCs must be expanded *in vitro* in order to generate enough cells to be used during cell therapy, the 'mortal' property of MSCs can be a limiting factor for the clinical application of MSCs.

3.3 Heterogeneous Population of Mesenchymal Stem Cells

Human MSCs are known to be heterogeneous in terms of their morphology and their differentiation potential (163). Moreover, a change in the composition of hMSC population during the course of culture expansion has been reported (168). Heterogeneity of MSC can refer to various aspects (168): (A) **Proliferation rate**: MSCs can be divided into slow-growing and fast-growing categories; (B) **Morphology**: spindle-shaped, large flat and small round MSCs have been observed (169); (C) **Growth pattern**: tight or disperse (170); (D) **Immunophenotype**: different subsets of the MSC population expressed distinct cell surface antigens (171, 172); (E) **Multilineage differentiation potential**: MSCs are known to be multipotent. However, clonal analysis of single-cell derived colonies has demonstrated that not every cell possesses differentiation potential toward osteogenic, adipogenic, and chondrogenic lineages and that MSCs tend to lose adipogenic and chondrogenic potential at increasing cell doubling (173-176); (F) **Heterogeneity in the level of differentiation toward a specific lineage**: it has been suspected that different subsets of MSCs are committed to different differentiation pathways. The gradation of osteogenic, adipogenic, or chondrogenic differentiation varies between different clones but this has hardly been addressed so far (175); and finally (G) **Cellular aging**: MSCs are diverse in their states of cellular aging (177).

A subject of particular interest is the existence of uncommitted mesenchymal progenitor cells in MSC cultures (178). According to Colter *et al.* (179), the

developmental and growth stages of cultured MSCs can be divided into three phases: a lag phase, a log phase or rapid growth, and a stationary phase. In stationary cultures of bone marrow, a majority of MSCs were large and moderately granular (also called mature MSCs or mMSCs), while a minority of MSCs was small and agranular. The small and agranular cells are termed recycling stem cells (RS-1 cells). It has been shown that these RS-1 cells give rise to a new population of small and densely granular cells (RS-2 cells) during the lag phase. During the late log phase, the RS-2 cells decrease in number and regenerate the pool of RS-1 cells found in stationary culture. The authors suggest that the earliest progenitors in the cultures are RS-1 and RS-2 cells. Therefore the number of RS-1 and RS-2 cells in any sample of MSCs should reflect the number of cells that generate single-cell derived colonies in cfu-assays. All of the cells in the cultures of MSCs were consistently negative for markers for hematopoietic cells (CD34, CD11B, CD43, and CD45). More interestingly, the mMSCs were positive for CD90 (Thy-1), while RS-1 cells were dimly positive and the RS-2 cells were negative for this specific cell marker.

The authors suggest a mechanism (**Figures 3-1** and **3-2**) by which interactions between the RS-1 and RS-2 cells give rise to mMSCs. mMSCs are relatively mature and slow dividing and become the predominant cell as the cultures approach senescence (166). The findings by Colter *et al.* have been consistent throughout various studies, including the work by Mets and Verdonk (180). Mets and Verdonk reported that there are two cell types in human bone marrow derived stromal cells: type I cells, which look like typical fibroblast-like cells, and type II cells that are large and resemble epithelial-like cells in morphology.

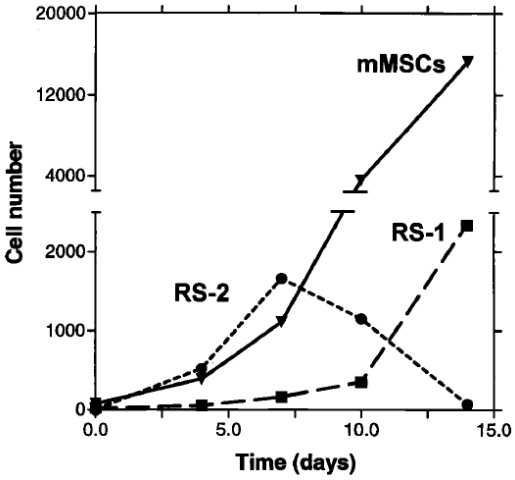

Figure 3-1 Time course of number of cells observed after initial plating of cells. Values are adjusted for the total number of cells in the cultures (179).

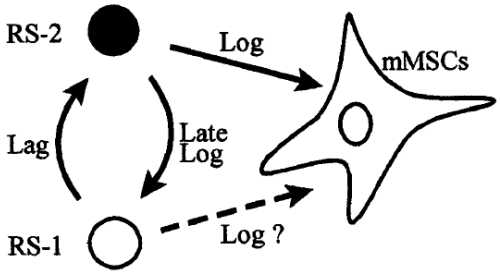

Figure 3-2 Scheme for the precursor-product relationships of cells in cultures of MSCs. The large mMSCs replicate poorly. Therefore, the RS-2 cells that appear during the lag phase must arise from RS-1 cells. During the early log phase of growth, the RS-2 cell decline in number as the mMSCs appear in large numbers. Therefore, the RS-2 cells are probably precursors of the mMSCs. However, the data do not completely exclude the possibility that RS-2 cells rapidly generate RS-1 cells, and the RS-1 cells then give rise to mMSCs (dashed arrow). Also, the earliest mMSCs probably continue to replicate. During the late log phase, the RS-2 cells decline in number, and the subpopulation of RS-1 expands. Therefore, the RS-2 cells probably recycle into RS-1 cells (179).

The authors observed that type II cells were largely non-dividing, while type I cells were rapidly dividing. Phase I cultures consisted mainly of type I cells, but this cell population decreased over subsequent passages. Because the type II cells formed an increasing fraction of the population as the culture aged, the authors suspect that type I cells act as progenitor cells, giving rise to non-dividing type II cells. More interestingly, another study by the same authors found that the donor age is inversely related to type I cell population and directly related to type II cell population (181). It would also be interesting to test if the decrease in the number of type 1 cells (or RS-1 or RS-2 cells) is related to the loss of multipotentiality following serial passage in culture (166). These results from various authors highlight the fact that identification of different subgroups within the MSC culture will be very important when using MSCs for cell and gene therapy (179).

Another topic of special interest is the notion of specific lineage-priming. Lineage-priming is a molecular model of stem cell differentiation in which proliferating stem cells express a subset of gene associated to the differentiation pathways to which they can commit (174). Two models of stem cell differentiation have been proposed (**Figures 3-3**). The second model corresponds to lineage-priming hypothesis. Delorme *et al.* suggested that the later model of stem cell differentiation is a more accurate representation of MSC differentiation (174).

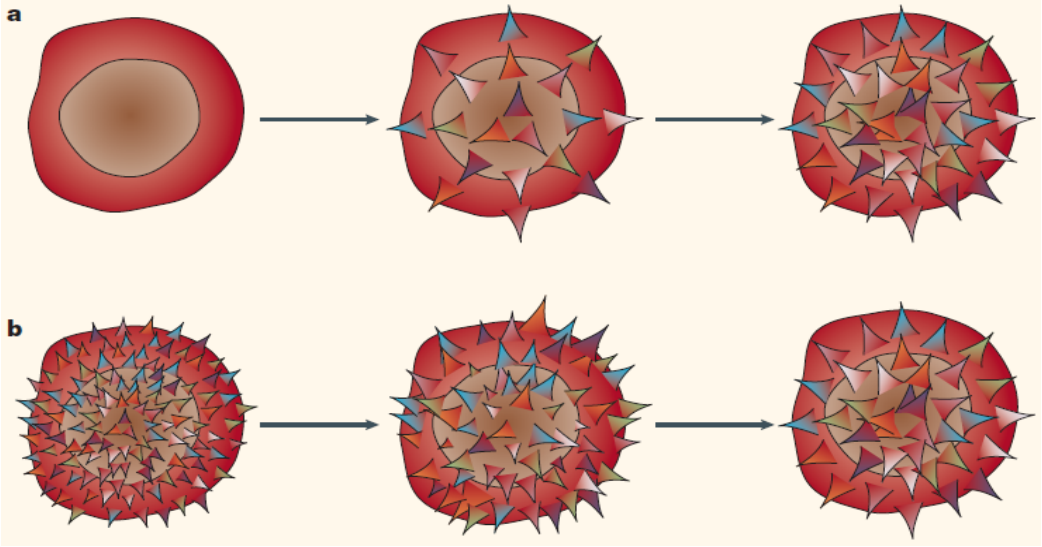

Figure 3-3. Gene-expression patterns as a function of transition from a stem-cell to a maturing-cell state. (a) One possibility is that the stem cell is a 'blank slate' and that differentiation entails acquisition of different gene-expression capacities (indicated by protrusions of different colours). (b) An opposing possibility is that stem cells express many genes at a low level (indicated by small protrusions), and that the expression of many of these is reduced during differentiation, with the expression of a small collection of the rest increased to a higher level (indicated by larger protrusions)(182). (Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Genetics, Zipori D. *The nature of stem cells: state rather than entity*. 2004, 5(11): p.873-8. Copyright 2004. http://www.nature.com/nrg/index.html)

They showed that proliferating primary layers and clones of bone marrow MSCs have precise priming to the osteoblastic, chondrocytic, and adipocytic lineages and further demonstrated that MSCs cultured in the presence of inducers differentiate into the lineages for which they are primed. In support of this view, various other studies have shown that not every cell is tripotent and that MSCs are heterogeneous in terms of lineage commitment (173, 175, 176). For example, Muraglia *et al.* analyzed the ability of 185 non-immortalized human bone marrow stromal cell clones to differentiate into osteogenic, chondrogenic, and adipogenic lineages (173). They found that "all clones but one differentiated into the

osteogenic lineage. About one third of the clones differentiated into all three lineages analyzed, [but] most clones (60-80%) displayed an osteo-chondrogenic potential." Similar to **Figure 3-3b**, the authors suggest "a possible model of predetermined bone marrow stromal cells differentiation where the tripotent cells can be considered as early mesenchymal progenitors that display a sequential loss of lineage potentials, generating osteochondrogenic progenitors, which in turn, give rise to osteogenic precursors."

From many studies, it is evident that MSCs are a heterogeneous ensemble of progenitors and lineage-committed cells, with a broad range of regenerative properties (176). For an effective clinical use of MSCs, recognizing and isolating the different subgroups would be crucial. More advanced understanding about the phenotypic characteristics of the different subgroups might lead to a development of "cell-engineering." Through cell-engineering, one might be able to program individual cells to behave like highly proliferative and multipotent MSCs or even to select a specific differentiation pathway the MSCs must follow.

3.4 Microenvironments and Chondrogenesis of Mesenchymal Stem Cells

Although why, when, and how the heterogeneous nature of MSCs arises are unclear, some studies have suggested that environmental influences might induce heterogeneity in MSC culture (163). "Physical factors in the local cellular microenvironment, including cell shape and geometry, matrix mechanics, external mechanical forces, and nanotopographical features of the extracellular matrix, can all have strong influences on regulating stem cell fate (183)." The *in vivo* stem cell niche presents a wide range of molecular and cellular scale physical, biological, and chemical signals. These environmental cues act to regulate tissue regeneration based on physiological demands (184). For instance, it has been reported that a mechanical stimulation can be an efficient method to induce chondrogenic differentiation of MSCs *in vitro* for cartilage tissue engineering in a three-dimensional environment (185). The natural niche of MSCs is neither stationary nor uninterrupted. *In vivo*, MSCs are exposed to both structural changes in the surrounding environment and many mechanical stresses that are caused by addition or removal of surrounding cells, muscle contraction and relaxation, as well as bone compression and decompression (183). Therefore, understanding and reproducing the unique aspects of the dynamic cellular microenvironments in which the MSCs are naturally found are critical when unlocking the full potential of stem cells. Among various environmental factors, the effect of growth factors, oxygen tension, and biomaterials in chondrogenic differentiation of MSCs will be explored further.

Growth Factors

Chondrogenic differentiation of MSCs can be induced by various intrinsic and extrinsic factors, but growth factors are some of the most important modulators in this process (186). Growth factors are a group of biologically active polypeptides produced by the body that can stimulate various biological activities including cellular division, growth, and differentiation (187). *In vivo,* interactions between various growth factors regulate development and maintain homeostasis of articular cartilage (188). Common growth factors that are used for chondrogenic differentiation of MSCs include transforming growth factor-β3 (TGF-β3) and insulin-like growth factor 1 (IGF-1). TGF-β3 is known to increase cartilaginous ECM production (189, 190), while IGF-1 increases proliferation and cartilaginous ECM production (191-193). Specific growth factors have been shown to promote cell proliferation and differentiation; however, some animal studies have raised biosafety concerns around application of growth factors *in vitro* and on animal models (194, 195). Further investigations are necessary to find the safest way of their utilization in the regenerative medicine.

Normoxia vs. Hypoxia

Another important regulatory factor which influences the chondrogenic potential of MSCs is oxygen tension. It has been consistently shown that low oxygen tension promotes chondrogenesis of MSCs in many aspects, including increased GAG content, higher and more homogeneous distribution of PG and collagen type II, and increased Sox 9 expression (196-199). For instance, Sheehy *et al.* showed

that expansion of MSCs at 5% pO_2 was faster than expansion at 20% pO_2 (200). The authors demonstrated that both pellets and hydrogels cultured under the low oxygen tension stained more intensely for type II collagen when undergoing chondrogenesis. The low oxygen tension also appeared to inhibit hypertrophy in both pellets and hydrogels. It has even been shown that low oxygen tension is a more potent promoter of chondrogenesis than 1 hour/day of dynamic compression (201). Current research and regenerative medicine would benefit from closer observation of phenotypic changes that occur in MSCs undergoing differentiation in hypoxic and normoxic conditions.

Biomaterials

In vivo, the ECM provides natural scaffolding for cells. Hence, when culturing cells for tissue engineering purposes *in vitro*, it is important to mimic the structural integrity of native ECM. Compared to the traditional monolayer culture, 3D culture environment has been reported to yield significantly higher level of collagen and protein production by fibroblasts (202). Scaffolds can be built using various types of biomaterials. Regardless of its type, a biomaterial should be able to "replace part of a living system or to function in intimate contact with living tissues" (203). Furthermore, a scaffold is expected to support chondrogenic cell growth and the formation of new tissue without eliciting harmful responses from the surrounding tissues *in vivo* (204). To fulfill this purpose, a scaffold should ideally have suitable surface chemistry to support cellular behaviors, have mechanical properties similar to the tissue at the site of implantation, and be

highly porous with an interconnected pore network for cell growth and dynamic exchange of nutrients and metabolic waste (205). Today, a wide range of biomaterials are available for use in tissue engineering. The biomaterials include, but are not limited to, biodegradable synthetic polymers such as poly(lactic acid) and polyglycolide, and poly(lactic-coglycolide) copolymers (205-209) , naturally-derived or synthetic-based hydrogels (210, 211), and natural polymers including collagen (202, 212, 213). Synthetic polymers are versatile tools for tissue engineering, because they are easy to process, control and modify relative to natural materials (214, 215). However, degradation of these synthetic polymers can release acidic degradation products, which can create an unfavorable environment for surrounding tissue (203). These byproducts can cause local disturbances such as inflammatory reactions, especially if the tissue has poor vascularization or low metabolic rate (205, 216). Furthermore, synthetic polymer scaffolds suffer from relatively low-cell seeding efficacy and poor retention of the ECM formed by the seeded cells (210). On the other hand, hydrogels can achieve homogeneous distribution of cells and can better maintain the neotissues formed within the scaffold (210). Hydrogels are composed of synthetic or natural hydrophilic polymer chains, and have properties similar to the natural ECM (211). Their highly hydrated 3D structure is especially similar to the macromolecular structure of cartilage, making hydrogels a suitable shelter for chondrocytes and MSCs. However, due to their nanoporous structure, hydrogels cannot support the proliferation and migration of seeded cells like scaffold sponges can support (202, 217). Scaffold sponges built from natural polymers have been widely used in

tissue engineering. Of various natural polymers, collagen is particularly suitable for biomedical application, because it is the most abundant protein in mammalian tissues (213, 218). Collagen type I is the most commonly used natural polymer, because of its abundance, ubiquity, and biocompatibility (219). While some additional strengths of collagen as a biomaterial include its innate low antigenicity, biodegradability, bioreabsorbability, high porosity, and its easily modifiable nature, the disadvantages include high cost of purifying type I collagen, variability of isolated collagen, lack of inherent rigidity, and potential for antigenicity through telopeptides (213, 219-222). Despite its drawbacks, collagen type I sponges have been used successfully with various cell types, including hBM-MSCs (212, 223). Furthermore, chondrocytes cultured in 3D porous collagen sponges have been reported to maintain the production of cartilage-specific ECM and expression of chondrocyte genes in contrast to the decreases seen in monolayer 2D cultures (219, 224, 225). These properties of collagen type I sponges make them an attractive tool for ACI and MSC-based cell therapy. By restoring a 3D structure which mimics the *in vivo* environment, natural interactions between cells and the ECM are facilitated, and chondrogenic differentiation of MSCs can therefore be effectively induced.

The influence of environmental factors on the behavior of MSCs has been well recognized. However, it is also important to note the trophic capacities of MSCs. In 1996, Haynesworth *et al.* documented that newly committed progenitor cells synthesize a wide range of growth factors and cytokines that have effects on cells

in their vicinity (226). "All cells secrete various bioactive agents that reflect both their functional status and the influence of their local microenvironments. … The pattern and quantity of such secreted factors is well known to feed back on the cell itself and govern both its functional status and physiology."(227) The bioactive molecules secreted by MSCs can have either direct or indirect effect on local cellular dynamics. Here, the indirect activity is referred to as "trophic." For many years, it has been thought that the regeneration of cells or tissues is influenced by MSCs' multilineage differentiation potential. However, various studies have shown that bioactive factors released by MSCs play a critical role in tissue repair. For instance, Park *et al.* (228) demonstrated that pancreatic islets co-cultured with MSCs showed lower ADP/ATP ratios, higher glucose stimulated insulin secretion indexes and viability, and higher levels of anti-apoptotic signal molecules. Furthermore, diabetic mice that received islet transplants cultured in MSC-conditioned medium for 48 hours demonstrated significantly lower blood glucose levels and enhanced blood vessel formation. These results suggest that the trophic factors secreted by hMSCs enhance islet survival and function after transplantation. Trophic effects of MSCs have also been observed with various other tissues including myocytes, neurons, and chondrocytes (227, 229, 230). Wu *et al.* (231) cocultured MSCs with primary chondrocytes and found that "increased cartilage deposition in cocultures is mainly due to a trophic role of the MSCs in stimulating chondrocyte proliferation and matrix deposition rather than MSCs actively undergoing chondrogenic differentiation."(231) These findings suggest an even greater role of MSCs as a therapeutic tool. In addition to

providing replacement cells for damaged or "expired" cells, MSCs can secrete bioactive factors that function to trophically assist the repair and regeneration process (227).

In order for cell-based therapies to be both efficient and effective, the ability to manipulate the local microenvironments of MSCs *in vitro* is crucial. Furthermore, understanding the potential influence MSCs can have on their environment is important when designing tissue repair strategies. Outlining the interaction between MSCs and their local environments, as well as identifying factors that increase the proliferative and chondrogenic potential of MSCs will significantly advance mesenchymal stem cell-based therapy.

Chapter 4: CD90 (Thy-1) and Mesenchymal Stem Cells

4.1 Current Knowledge of CD90 (Thy-1)

"Thy-1 (CD90) is a developmentally regulated, evolutionarily conserved cell surface glycoprotein, the biological role of which remains somewhat enigmatic despite hundreds of intriguing publications" over many years (232). CD90 is "expressed in a heterogeneous range of cell types, including thymocytes, lymphocytes, fibroblasts, neuronal cells, hematopoietic and mesenchymal stem cells, ovarian follicular cells, and some cancer cells."(232) The structural gene for human CD90 is localized in the long arm of chromosome 11, and although the structure of the Thy-1 gene is highly conserved across different species, the regulatory mechanism of the Thy-1 gene is fundamentally different (233). Previous studies have explored both immunologic and nonimmunologic roles of CD90 (234) . Immunologically, CD90 is thought to be involved in T cell activation (235) and to regulate neutrophil functions (236). In addition, some of its nonimmunological functions involve its role in inhibition of neurite outgrowth (237), induction of apoptosis in thymocytes and mesangial cells (238-240), leukocyte and melanoma cell adhesion and migration (241-243), tumor suppression (244, 245), and fibroblast proliferation and migration (246). Although the functions and behaviors of CD90 are still mysterious, CD90 is thought to be an important regulator of cell-cell and cell-matrix interactions. To the minds of many, Thy-1 has been relegated to the role of a mere marker (232). This is especially true for MSCs. Besides from the fact that CD90 antigen is one of the cell surface antigens that are used to identify mesenchymal stem cells, there

has been hardly any research on the relationship between MSCs' level of CD90 expression and their chondrogenic capacity.

4.2 CD 90 (Thy-1) Expression on Mesenchymal Stem Cells

In effort to better characterize MSCs, several antibodies have been raised. Along with many other cell marker antibodies, CD90 antibody was reported to react with undifferentiated MSCs (247). Furthermore, the ISCT has proposed that MSC populations must be positive at least for several antigens, including CD73, CD90, and CD105 (144, 247). Therefore, it has been generally accepted that CD90 is found in all MSCs. However, it must be noted that the phenotypic properties of MSCs is usually based on analyses of *in vitro* expanded cells. Very little is known about their *in vivo* phenotype. In 2005, Boiret *et al*. reported something striking (248). The authors characterized various antigenic expressions on fresh, unmanipulated MSCs and on MSCs after short-time adherence. Interestingly, they found that 95.5±2.1% of initial, unmanipulated bone marrow cells were CD90- (n = 5). After 1 to 3 days of culturing, the proportion of CD90- cells decreased to 82.6±5.5% (n = 10). Although defining CD90- cells as MSCs violates the ISCT guideline, none of the MSCs that were used in their study expressed hematopoietic markers like CD34, CD45, and glycophorin A. Furthermore, by eliminating cells with CD14 expression, it was ensured that the adherent cells were not monocytes (249). These results suggest that an alteration of antigen expression occurs when MSCs are removed from their natural niche (or *in vivo* environment). It is highly likely that unusual environmental conditions, such as adherence to a two dimensional surface, induce the conversion of CD90- cells into CD90+ cells. Furthermore, the authors sorted the MSCs into CD90+ and CD90- fractions, and the cells were cultured in basic mesenchymal medium

for four weeks. After four weeks, only 22.7±11.3% of the "CD90- population" remained CD90- (n = 3). This change in CD90 expression within the negative subset cannot be explained by an initial contamination because the purity of sorting was over 98.5%. The result suggests that the culture condition continues to induce the conversion from CD90- to CD90+ in MSCs. This study is important because it highlights the fact that *in vitro* characterization of MSCs can be different from *in vivo* characterization of the cells.

4.3 Potential Significance of CD90 (Thy-1)

The results acquired by Veyrat-Masson *et al*. become more interesting when they are compared to the observations made by Colter *et al.*. Colter *et al.*(179) identified RS-1 cells which are weakly CD90+, RS-2 cells which are CD90-, and mMSCs cells which are CD90+. The authors also remarked that the earliest progenitors in the cultures were RS-1 and RS-2 cells, and that the population of mMSCs rapidly expanded during the log phase growth, while the RS-2 cells declined in number (**Figure 3-1**). The observation made by Colter *et al.* on the RS-2 and possibly RS-1 cells corresponds with the observation made by Veyrat-Masson *et al.* on CD90- cells. As discussed in **Chapter 3.3**, RS-1 and RS-2 cells are likely to be the same group of cells as type I cells described by Mets *et al*. (180). Mets *et al*. noted that the population of type I cells is inversely proportional to a donor's age (181). This result indicates that the conversion of CD90- MSCs into CD90+ MSCs can be induced not just by the unfavorable conditions that are normally introduced during *in vitro* cultivation, but also by unfavorable physiological changes that arise from aging. In addition, the data from the four studies suggest that CD90- cells that were observed in abundance in unmanipulated MSCs are likely to be the earliest progenitor cells, which give rise to mMSCs. In this sense, CD90- can be thought to be more "flexible" or "malleable" than mMSCs (CD90+ cells).

In support of this view, Adesida *et al.* reported that cell populations which contain a higher percentage of CD90- cells were the ones to produce better cartilage (250).

In their study, bone marrow MSCs were cultured either in a hypoxic condition (3% O_2) or in a normoxic condition (21% O_2). After 14 days of culturing using αMEM supplemented with 10% heat inactivated fetal bovine serum, penicillin-streptomycin, HEPES, sodium pyruvate, and 5ng/ml FGF-2, the number of MSC colonies developed under hypoxia was generally higher by 5% to 38% than those developed under normoxia. Furthermore, the authors found that regardless of the oxygen tension during pellet culture, hypoxia-expanded MSC pellets underwent a more robust chondrogenesis than normoxia-expanded MSC pellets after three weeks of culture using chondrogenic media. The enhancement of chondrogenic capacity of bone marrow MSCs was marked by increased GAG synthesis and Safranin O staining, along with an increase in mRNA expression of aggrecan, collagen type II, and Sox 9. In addition to these analyses, the effect of oxygen tension on the expression of cell surface markers was investigated. The results were surprising: the CD90 expression was consistently lower in MSCs expanded under a hypoxic condition. This observation is important, because it might imply that there is a potential link between CD90 expression and chondrogenic potential of bone marrow MSCs. It has been reported that MSCs tend to lose adipogenic and chondrogenic potential at increasing cell doubling (173-176). It has also been reported that with subsequent cell doublings, the proportion of CD90- cells decreases while the percentage of CD90+ cells increases (248). Based on these observations, it can be hypothesized that a loss of multipotentiality in MSCs is a result of a loss of the CD90- MSC population over time or that a decrease in the CD90- expression is a phenotypic representation of MSCs' loss of

multipotentiality. If this assumption is true, the definition of MSCs that has been used widely by many scientists must be redefined, and cell-based therapy for cartilage repair will benefit from selecting for MSCs lacking CD90 expression.

In contrast to the results found by Adesida *et al*., Krawetz *et al*. reported that CD90+ synovial fluid mesenchymal progenitor cells (sfMPCs) had increased chondrogenic potential compared to the CD90- population (251). In their study, sfMPCs were derived from synovial fluid from normal and osteoarthritic knee joints. Fresh sfMPC were plated in untreated culture dishes and after 1 -2 hours at 37°C/5%CO_2, culture media (DMEM with 10% fetal bovine serum, 1% penicillin-streptomycin, 1% non-essential amino acids, and 0.2% beta-mercaptoethanol) was added. When the cells reached about 60-70% confluency, the cells were harvested and sorted under sterile conditions. CD105+, CD73+, CD44+, CD45-, CD11b- cell populations were selected for further culturing for 1 passage. Then, these cells were sorted using magnetic enrichment process. Both the CD90+ and CD90- fractions were cultured for one additional passage and then induced to differentiate in chondrogenic media for 14 days with or without micro-mass aggregation. In their analyses, the authors found that CD90- fraction displayed reduced levels of Sox9, collagen type II and Aggrecan mRNA, as well as less intense alcian blue and collagen type II staining at day 14 of chondrogenesis compared to the CD90+ population. Although more insight into the role of CD90 antigen in chondrogenesis of MSCs has been gained from this study, it is unclear whether the CD90- fraction was truly CD90-. It has been

reported that CD90- cells quickly convert to CD90+ cells upon the initial plating of extracted MPCs on a two-dimensional plastic surface (248). Although passaging the sorted cells for an additional passage would have given the cells time to recover from the stressful sorting procedure and to proliferate further, the "once-pure" CD90- sfMPCs population could have developed into a mixture of CD90+ and CD90- sfMPCs population. It should also be noted that although sfMPCs and bone marrow MSCs are both MSCs, it is uncertain how the source of MSCs might affect their behavior *in vitro*. Nevertheless, further investigation is needed to accurately portray the relationship between CD90 and chondrogenic potential of bone marrow MSCs.

Conclusion

Across several different studies, CD90- subset of MSC population has been noted as "interesting" and "mysterious." However, the exact role of CD90 in chondrogenic differentiation of bone marrow MSCs is still unknown. In-depth investigation into the properties and function of CD90 can improve the current understanding of MSCs. To determine if bone marrow MSCs lacking CD90 surface expression have enhanced chondrogenic propensity, a gene knock-down experiment can be performed. By doing so, it is possible to investigate whether anti-Thy1 treatment of bone marrow MSCs can enhance bone marrow-MSCs' *in* vitro chondrogenic capacity. In this review, the anatomy and physiology of articular cartilage has been explored. Poor intrinsic healing capacity of articular cartilage that arises from its unique structure has also been described. Furthermore, the serious negative impacts that cartilage defects can have on society and individuals, as well as the limitations of current cartilage repair strategies have been carefully reviewed. From this review, it is evident that a new method of increasing the chondrogenic capacity of bone marrow MSCs is critical. Advancement in mesenchymal stem cell research is crucial if MSC-based therapy of cartilage defects is to become more efficient and effective.

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Part B: Introduction to RNA interference and Development of a Working Gene Knockdown Protocol

Introduction

In the previous section of the thesis, an overview of the basic anatomy and physiology of articular cartilage, treatment methods for cartilage defects, properties of MSCs, and current understanding of CD90 was provided in order to help the reader gain the background knowledge necessary to understand the purpose of our proposed research. The objective of this section is to provide basic information on RNAi technology in order to show how we have optimized our CD90 knockdown protocol. In **Chapter 5**, RNAi will be described to help the reader understand the mechanisms behind gene silencing technology. In **Chapter 6**, various vector systems will be explored and in **Chapter 7**, lentivirus (LV) vector system will be investigated in greater details. In **Chapters 8** and **9**, we demonstrate how we have developed our final CD90 knockdown protocol through experimental means. Finally in **Chapter 10**, we investigate how the protein expression of CD90 is affected by transduction. RNAi is a powerful tool which can help scientists study gene functions. However, this tool cannot be used safely and effectively without understanding the nature of this technology. This review will provide the basis on which our experiments can build.

Chapter 5: RNA Interference

5.1 Basic Aspects of RNA Interference

RNA interference (RNAi), also known as post-transcriptional gene silencing (PTGS) in plants, is "the inhibition of expression of specific genes by double-stranded RNAs (dsRNAs)" (1). During this innate, evolutionarily conserved cellular process (2), "dsRNA molecule of greater than 19 duplex nucleotides" is known to cause the degradation of both the invading dsRNA molecule and single-stranded RNAs (ssRNAs) of identical sequences, including endogenous mRNAs (3). It has been speculated that RNA-mediated gene silencing has a biologically significant role in stabilizing the genome by sequestering repetitive sequences, in development of multicellular organisms, and in defense against viral infections (4-7). RNAi is a multistep process whose basic mechanism is conserved among virtually all eukaryotes, including mammals (8). RNAi can be roughly divided into two phases: (1) Initiation and (2) Silencing or effector. In human cells, RNAi pathway takes places in the cytoplasm (5, 9, 10). During the initiation phase, dsRNAs are cleaved by a dsRNA-specific riboendonuclease called Dicer into 21-25 nt duplex RNAs called small interfering RNAs (siRNAs) (10, 11). During the silencing or effector phase, these small dsRNAs are unwound by RNA helicase activity and "the siRNA strand antisense to the target RNA (known as guide strand) is incorporated into the RNA-induced silencing complex (RISC), while the complementary passenger strand is destroyed" (10). It is this guide strand, which is complementary to the target mRNA, that allows for a highly sequence-specific gene silencing. The binding between the guide strand and the RISC is

followed by recognition of the complementary mRNA, which is cleaved by a component of RISC (12, 13). **Figure 5-1** schematically summarizes the RNAi machinery.

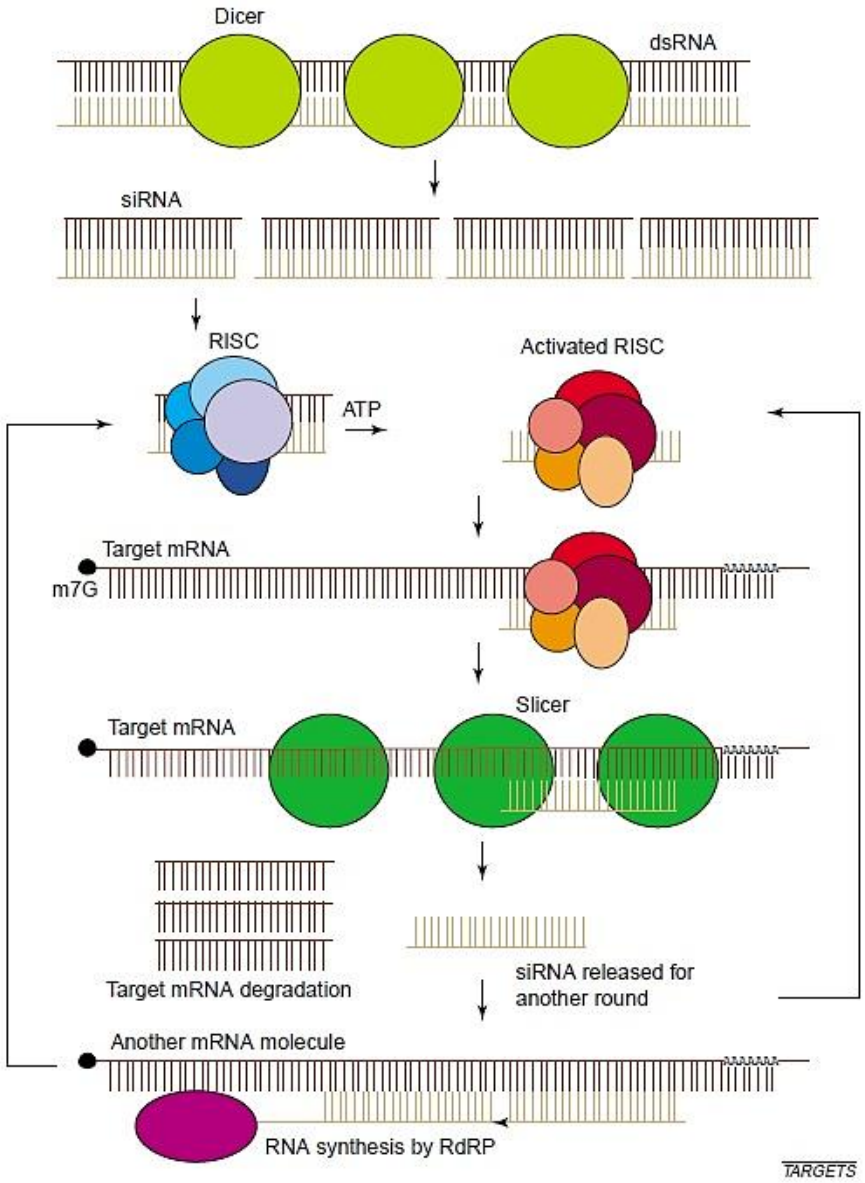

Figure 5-1 Mechanism of RNA interference. Double-stranded RNA is cleaved inside the cell by Dicer enzyme into 21-23 nucleotide fragments called short interfering RNA (siRNA). The siRNA is assembled in a hetero-multimedia protein complex called RNA induced silencing complex (RISC). The RISC complex is activated by ATP, and the unwound siRNA in the complex guides the target mRNA degradation by a ribonuclease, tentatively called Slicer (11).

Due to the destruction of mRNA templates, protein synthesis of the specific gene is inhibited. Previous studies have suggested that RNAi can "inhibit expression of gene of interest in almost every type of cells," although siRNA-mediated gene inhibition can never completely eliminate the gene product (4). Nevertheless, RNAi has proven to be an efficient and effective method for investigating gene functions in mammalian cells.

5.2 Triggers of RNA Interference in Mammals

As the definition of RNAi suggests, dsRNA is an important precursor for RNAi. However, complications can arise when dsRNA longer than 30 nt length is directly introduced to mammalian cells. The long dsRNA can activate a dsRNA-dependent protein kinase (PKR), leading to the global gene silencing and cell toxicity (phenomena collectively known as nonspecific off-target effects) (3, 14, 15). This undesirable consequence can be circumvented by using smaller dsRNAs (less than 30 nt length), namely siRNAs and short hairpin RNAs (shRNAs). shRNA is a fold-back stem loop structure of siRNA whose sense and antisense strands are linked together by a small linker sequence (16). Dicer degrades the linker sequence as shRNA is exported from nucleus to cytoplasm, and the resulting siRNAs enter the usual RNAi mechanism (17).

Although chemically synthesized siRNAs and vector based shRNAs can both achieve target-specific silencing, they are intrinsically different molecules, and therefore can have different molecular mechanisms of action and off-target effects (18). While both types of molecules are valuable tools for RNAi technology, they have their advantages and disadvantages. siRNAs are simple to manufacture and easier to chemically modify. This property of siRNAs is especially useful when trying to reduce unintended effects on gene expression mediated by RNAi or "off-target effects." Specific off-target effects are caused when partial sequence complementarity of the RNAi construct (either the guide or the passenger strand) to non-target mRNAs leads to off-target knockdown (18, 19). Unlike shRNA

approach which does not permit specific chemical modification of the silencing construct, siRNA approach allows for chemical modifications which can reduce direct off-target effects (20-22). Furthermore, chemical modification of siRNAs can make them less immunostimulatory, thereby reducing nonspecific off-target effects. However, chemical modifications are costly and must be applied carefully, because reductions in off-target effects can decrease the overall potency of suppression (18).

Unlike siRNA, shRNA is endogenously expressed. While siRNAs can be delivered directly to the cytosol where they can enter the RNAi pathway, shRNA constructs must be delivered to the nucleus of cells. Moreover, the resulting shRNAs must overcome an additional challenge of having to be transported from the nucleus to the cytoplasm to enter the effector phase of the RNAi pathway. Despite this disadvantage, several studies have shown that shRNAs act as "a better substrate for dicer and displays improved RISC loading" (23-25) compared to siRNAs. In addition, shRNA allows for a longer period of expression as compared with the siRNA, because it can be amplified by transcription. Although the transient nature of siRNA might be preferential in certain occasions, this property of shRNA can be especially useful when applying RNAi technology to mammalian cells, because they lack amplification steps which are available in other systems like *C. elegans*, plants, and fungi (26). More importantly, some studies have suggested that shRNA is more potent than siRNA at mediating gene silencing (23, 27). For instance, McCaffrey *et al.* (27) achieved a significant

(>80%) knockdown of luciferase expression when hydrodynamic dosing 40 μg of siRNA with 2 μg of a plasmid encoding luciferase (pGL3), while only 10 μg of shRNA hydrodynamically coadministed with 2 μg of pGL3 was required to achieve >95% luciferase knockdown under the same conditions. Another benefit of using shRNA comes from the fact that shRNAs are processed via endogenous mechanisms, making shRNA-induced inflammatory responses less likely. In support, Rao *et al.* (19) argued that using siRNA is more likely to result in off-target effects, because siRNA "requires higher concentrations and more frequent dosing to achieve similar levels of knockdown" as shRNA. The same research group added that because unprotected siRNA may be more susceptible to intracellular metabolism, its risk for nonspecific target recognition increases. While shRNA is a potent, stable, and effective tool for RNAi technology, it has its disadvantages. In contrast to siRNA, with which precise control of its intracellular level is possible, shRNA lacks the means to adjust levels of suppression. Because there exists no exact and reliable way of preventing the overexpression of shRNA, shRNA can saturate the endogenous miRNA pathway resulting in significant lethality (19, 28, 29). However, using an RNA polymerase II promoter-driven shRNA expression cassette rather than highly active RNA polymerase III type promoters allows for greater regulation, thereby alleviating toxic effects (30).

5.3 Using RNA Interference Technology

RNAi technology is a potent and efficient method of gene suppression which utilizes cells' innate, evolutionarily conserved system of self-defense and regulation. Some of the important factors that must be considered in order to achieve efficient and effective knockdown include the cell type, type of RNAi triggers used, specific designs of siRNA or shRNA, and methods of siRNA or shRNA delivery (31). Research can take full advantage of RNAi technology, only when these key factors are carefully determined. After close review, we have decided to use shRNA for our current study, because we must achieve an effective and sustained knockdown of the gene CD90 in human primary cells. Understanding the mechanisms and components of RNAi will contribute to the development of an optimal knockdown protocol for our future experiments.

Chapter 6: Methods of Delivering RNAi Triggers

6.1 Transfection of Mammalian Cells

The cytosolic delivery of "naked" siRNA oligonucleotides is the simplest method for RNAi (32). However, it is unlikely to achieve a successful knockdown using this simple method in mammalian cells, because of the following reasons: (1) "mammalian cells appear to lack the effective dsRNA-uptake machinery that is found in other species such as *C. elegans*; (2) siRNA is highly charged and cannot pass freely through the cytoplasmic membrane; and (3) uptake of siRNA by fluid-phase endocytosis does not result in the release of siRNA into the cytoplasm. If siRNA is not delivered effectively to the cytoplasmic compartment, it will not interact with other RISC components and, thus, will not induce RNAi. (31)" To improve transfection efficiency in cells, including those that are otherwise incapable of transfection, a plethora of chemical delivery agents have been developed. The basic principles underlying the design of the chemical delivery agents are similar for all nucleic acids, including plasmid DNA and siRNA. Nucleic acid transfection reagents must be capable of interacting with the nucleic acid cargo and of fusing and/or disrupting biological membranes to deliver the cargo to the cell cytoplasm. Transfection reagents often interact with nucleic acids via electrostatic forces. This interaction enables the formation of transfection reagents-nucleic acid complexes, and the two components can be presented simultaneously to the biological membranes. The three different types of complexes are lipoplexes, polyplexes, and lipopolyplexes (31). Lipoplexes, polyplexes, and lipopolyplexes refer to cationic lipid-nucleic acid complexes,

cationic polymer-nucleic acid complexes, and complexes with both polycationic polymers and cationic lipids, respectively (33). It has been proposed that the positively charged nano-sized lipoplex or polyplex particles bind to the negatively charged cell membrane by non-specific, electrostatic interactions, and enter the cell via endocytosis (34-36). Upon a subsequent endosomal/lysosomal disruption, the nucleic acid is released into the cytoplasm. The method of release is fundamentally different for lipoplexes and polyplexes. The interaction between the cationic lipids of the lipoplex and the anionic lipids of the endosomal membrane weakens the electrostatic interaction between the nucleic acid and the lipoplex, releasing the content into the cytoplasm. On the other hand, the polyplex approach involves endosome disruption through increased osmotic pressure. Many cationic polymers have pH-dependent functional groups with pK_a values of 5-7. When these buffering groups act as an extensive "proton sponge" (37), the number of protons required for acidification of endosomes during their maturation to lysosomes is increased. Consequently, the concentration of their counter-ions inside the lumen also increases, resulting in osmotic swelling due to water entry (31, 38). Finally, "lipopolyplex reagents combine the action of cationic lipids and polymers to deliver nucleic acids" (31). Once the nucleic acid is released into the cytoplasm, it may be transported into the nucleus. Although the exact mechanism of transport is still unclear, it has been hypothesized that nucleic acid passively enters the nucleus during mitosis when the nuclear membrane disintegrates temporarily (39) and/or that it is actively transported through nuclear pores into the nucleus (34, 40). Pollard *et al.* (41) has suggested

that unlike cationic lipids, cationic polymers promote gene delivery from the cytoplasm to the nucleus potentially by enhancing intracytoplasmic mobility of nucleic acid and by protecting nucleic acid from endogenous nuclease degradation. The same research group (41) also argued that transgene expression in the nucleus is prevented by complexation with cationic lipids. In support, Zabner *et al.* (34) found that cationic lipid-mediated transfection is an inefficient process, because only a small percentage of nucleic acid delivered to the cytoplasm is released from the endosomes, and only a small percentage of the free nucleic acid actually enters the nucleus. Despite many years of efforts to optimize transfection efficiency of non-viral, chemical delivery reagents, transfection continues to be an inefficient delivery method for mammalian primary cells. In other words, siRNA or plasmids encoding shRNA cannot enter the cells' cytoplasm and/or nucleus using simple chemical delivery reagents. The fact that siRNAs cannot be efficiently delivered to the cytoplasm of mammalian primary cells supports our previous decision to take shRNA-approach to our current study. Furthermore, the limitations of transfection method suggests that a more potent and efficient method of delivery is required to achieve a sufficient level of RNAi in target cells.

6.2 Transduction of Mammalian Cells

"For most untransfectable cells, adenoviral, retroviral, or lentiviral-based shRNA technology remains the only viable technology for successful delivery of RNAi" (32). While there exist health and safety concerns over viral methods for delivering RNAi triggers, transduction of mammalian cells with viral vectors has been widely used as a powerful tool to achieve a stable knockdown in various biological systems. Although there are many different kinds of viral vectors, only the most commonly used ones, namely standard retroviruses, adenoviruses (Adv), and lentiviruses (LV), will be discussed in this chapter (42).

Standard Retroviruses

One of the most distinguishing properties of retroviruses is their ability to reverse the transcription of their ssRNA genome into dsDNA, which is subsequently integrated into the host cell genome (42). Retroviruses were "among the first vectors used as transfer vehicles for hairpin-RNA expressing plasmids," and have been used effectively for many years to transduce most cell lines and many primary cell types (43, 44). However, one drawback of retroviral vectors is that they require cell division for expression (31, 45). **Figure 6-1** represents a schematic overview of the mechanism of RNA silencing after retroviral delivery of shRNA.

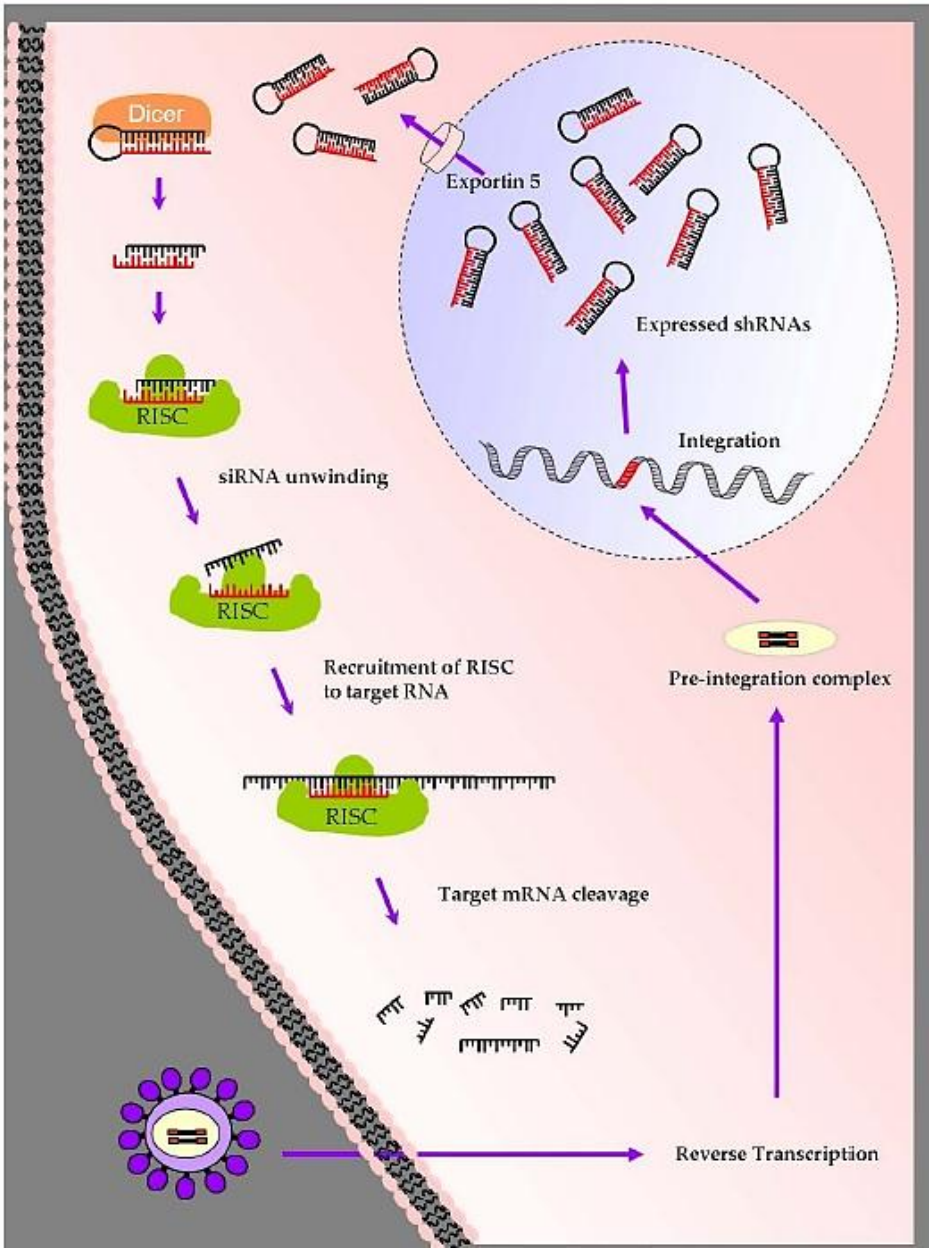

Figure 6-1 Schematic overview of the mechanism of RNA silencing in the host cell that leads to transcriptional silencing after retroviral delivery of shRNA. Retroviruses (or vectors) deliver therapeutic shRNA-expressing transgenes that integrate into the genome of the host cell and lead to stable shRNA expression. Expressed shRNAs require the activity of endogenous Exportin 5 for nuclear transport. Several proteins are recruited and form a dimer with Dicer which receives and subsequently cleaves the dsRNA generating duplex siRNAs with 2 nt 3' overhangs. These siRNAs activate the RNA-induced silencing complex (RISC) which unwinds the RNA and recruits only the guiding strand to target mRNA which is subsequently cleaved and degraded. The figure is schematic, and the Dicer and RISC complexes can vary dependent on cellular process (44).

Adenoviruses

Adenoviruses are "medium-sized, non-enveloped viruses with a nucleocapsid and a linear dsDNA genome" (44). Unlike standard retroviruses, Adv can infect both dividing and non-dividing cells (31). Despite this advantage, adenoviral vector approach might not be as effective in stem cells because it appears that the primary receptors for Adv are poorly expressed in stem cells, resulting in low transduction rates (46).

Lentiviruses

LV "constitute a subclass of retroviruses which also carry two copies of ssRNA genome in an enveloped capsid" (44). The most widely-known example of LV, the human immunodeficiency virus type-1 (HIV-1), provided the basis for the development of first LV vectors (47, 48). Like Adv, LV can effectively transduce both dividing and non-dividing cells (32, 48, 49). Because of this property, the LV-mediated gene-delivery system can lead to an efficient and stable expression of RNAi in human cells, including primary non-dividing cells (50). Other advantages of LV vectors include their ability to accommodate large (up to 7.5 kb) amounts of DNA (51), and their lower immunogenicity and toxicity compared to Adv (32, 44).

6.3 The Choice of a Suitable Gene Delivery System

While there are many different ways of delivering RNAi triggers, the properties of each method must be carefully reviewed in order to determine the most suitable vector for a specific biological system. Since achieving a long-term knockdown of gene CD90 in primary, human mesenchymal stem cells (hMSCs), is an important requirement for our current study, LV-based vectors were chosen over other delivery systems. Lentiviral expression system will be explored further in the following chapter.

Chapter 7: Lentiviral Expression System

7.1 Structure of Lentivirus

Understanding the structure of LV is useful when using lentiviral expression system, because LV vectors' biological behaviors are closely related to the biology of parental LV. LV, which belong to a subclass of complex retroviruses, are characterized by their ability to reverse transcribe their ssRNA genome into dsDNA (52). Similar to other retroviruses, LV contain three basic genes, namely *gag*, *pol*, and *env*. The *gag* gene codes for the precursor structural proteins of the LV particle, including the matrix, capsid, and nucleocapsid, while the *pol* gene codes for reverse transcriptase (RT) and integrase. Finally, the *env* gene codes for surface and transmembrane components of the viral envelope proteins (42, 53). In addition to these genes, the lentiviral genome also comprises six additional genes (i.e. *rev*, *tat*, *nef*, *vpr*, *vpu*, and *vif*) which code for proteins critical for viral replication, binding, infection, and release (54). Another important genomic feature of LV is the homologous regions of 600 to 900 nt, also called long terminal repeats (LTRs). LTRs flank both ends of the lentiviral proviruses (i.e. the viral cDNA integrated in the host genome), and each LTR is segmented into three regions called U3, R, and U5. LTRs play a critical role in virus replication, integration, and expression (53).

7.2 Life Cycle of Lentivirus

The life cycle of LV is similar to that of other retroviruses in that the interaction between the glycoproteins of the viral envelope and specific cell receptors leads to the fusion of the envelope with the host cell's membrane, which in turn triggers the release of the lentiviral core into the host cell's cytoplasm (42). Once inside the cytoplasm, the viral capsid disintegrates, and the complex process of reverse transcription begins. To quickly summarize, the enzyme RT converts the viral RNA genome into a dsDNA. Subsequently, its DNA genome is integrated into the host cell chromosome. Unlike other retroviruses, disassembly of the nuclear membrane is not a prerequisite for the integration of the lentiviral cDNA into the host cell's genome. Upon integration, the virus uses the host cell's replication machinery to express viral genes (53, 55). Once the virus reassembles in the cytoplasm, it exits the host cell by budding from the cellular membrane, where the LV acquires the capsid (56). After budding, it matures into an infectious particle that is capable of starting a fresh cycle of replication (57).

7.3 Designing and Producing Lentivirus Vectors

LV vectors are attractive gene transfer tools, because (1) they can integrate efficiently and stably into the chromosomes of their targets; (2) they do not transfer viral genes, thus reducing the risk of vector-mediated toxicity and destruction of transduced cells by virus-specific cytotoxic T cells; and (3) they can efficiently transduce a wide variety of cells including slowly- and non-dividing cells (45, 58). LV vectors are "replication-defective, hybrid viral particles made by the core proteins and enzymes of a LV, and the envelope of a different virus, most often the vesicular stomatitis virus (VSV)" (59), whose purpose is to safely obtain a single transduction event into the target host cell, without disturbing normal function of the host cell genome (47). LV vectors are traditionally produced through transient cotransfection of 3-4 viral elements into the highly transfectable, human embryonic kidney (HEK) 293T cells (60, 61). The viral elements that are required to produce LV vectors are "the LV packaging helper proteins consisting of at least the *gag-pol* genes, the LV transfer vector RNA containing the transgene expression cassette, and an heterologous glycoprotein" (62). While the packaging cassette is critical for the "packaging" functions of LV vectors and encodes all lentiviral vector *trans*-elements, the transfer or vector cassette contains all the *cis*-acting elements required for efficient packaging, reverse transcription, nuclear import, and integration in the target cells (47). Furthermore, the genetic template for the transgene of interest is also incorporated in the transfer vector. Finally, the envelope cassette often expresses the vesicular stomatitis virus G protein (VSV-G), rather than the

parental HIV-1 envelope. VSV-G pseudotyped LV vectors can transduce a broad range of tissues and cell types, and are less dependent on viral accessory proteins for full infectivity, because they can enter target cells through an endocytic pathway instead of direct fusion with the cell membrane (47, 63). While the efficiency and potency of LV vectors are important, safety concerns surrounding the use of the vectors must not be overlooked. Over the past several decades, various biosafety features of LV vectors have been developed. These features include modifications in all three viral elements: the packaging, transfer, and envelope plasmids. To prevent the formation of replication-competent retroviruses (RCRs), split genome design, in which the *cis*-acting elements of the LV genome are segregated from the *trans*-acting elements, has been developed (64, 65). Separation of an intact LV genome into three or four components reduces the risk of generating RCRs. Furthermore, most of sequences from the LV genome were eliminated or replaced by heterologous sequences. The first generation of LV vectors lacked most of the *env* gene sequence and certain *cis*-elements, but maintain the nonessential or accessory genes *vpu, vpr, vif, nef, rev,* and *tat (48, 66)*. In the second generation of LV vectors, all the accessory genes, excluding *tat* and *rev*, were deleted to advance the biosafety of LV vectors (67, 68). Finally, even the *tat* gene was deleted in the third generation of LV vectors, and the *gag/pol* and *rev* genes, which had been placed together on one packaging vector in previous generations of LV vectors, were split into two packaging cassettes (69). Several studies have confirmed that the elimination of the accessory genes from a packaging construct does not compromise the vector yield

or the transduction efficiency of LV vector system (67, 68). Owing to these safety modifications, neither second nor third generation LV vectors have been found to form RCRs (48, 70, 71). In addition to these biosafety features, further modifications have been made to the LTRs of the transfer vector to create what is called a self-inactivating (SIN) vector (72-74). The deletion of the enhancer/promoter sequences in the U3 region of the 3' LTR abolishes the transcriptional activity of the SIN provirus. The inactivity of the LTR subsequently reduces the risk of promoter interference and susceptible oncogenic derivations (58, 62). For many years, scientists have sought ways to improve the efficiency and biosafety of LV vectors, and as a consequence, LV expression system has become a more reliable and attractive tool for gene delivery.

Chapter 8: Selecting Suitable Expression Construct for Knockdown of CD90

Proper application of RNAi technology depends on selection of a suitable target sequence for a given gene of interest. Although many different constructs are commercially available for research purposes, "there is no guarantee of effective gene silencing for a given shRNA until experimentally proven" (32). Therefore, experimentally testing several shRNA constructs that have been designed to silence CD90 is crucial for our current study (from the RNAi Consortium). Five different shRNA expression constructs (all from Thermo Scientific, Ottawa, Ontario, Canada) were tested (n=3) for their ability to trigger effective gene silencing in osteosarcoma cells U_2OS. U_2OS cells were chosen as the target cells, because they are easily transfectable and are known to express antigen CD90 (75-77).

The target sequence information for each shRNA construct is given in **Table 8-1**. The gene silencing effects of each construct can be studied by comparing the CD90 mRNA expression level of U_2OS cells transfected with each of the five constructs, to that of untreated U_2OS cells (n=2, negative control) and U_2OS cells transfected with a control vector pLKO.1 GFP (n=2, non-targeting shRNA control; Addgene 30323).

Table 8-1 Basic Construct Information. All five constructs have the pLKO.1 backbone. For convenience, each construct was given simple codes.

Code	Construct	Vector	Target Sequence
E8	TRCN0000057023	pLKO.1	GCCATGAGAATACCAGCAGTT
E9	TRCN0000057024		CGAACCAACTTCACCAGCAAA
E10	TRCN0000057025		GCTCAGAGACAAACTGGTCAA
E11	TRCN0000057026		GTCACAGTGCTCAGAGACAAA
E12	TRCN0000057027		CACCAGCAAATACAACATGAA

In this short experiment, U_2OS cells in early passages were cultured and maintained under normal oxygen tension (21% O_2) at 37°C in a standard culture medium: high glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 4.5 mg/mL D-Glucose, 0.1mM non-essential amino acids, 1 mM sodium pyruvate, 100 mM HEPES buffer, 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.29 mg/mL L-glutamine, and 10% heat inactivated fetal bovine serum (all from Invitrogen, Mississauga, Ontario, Canada). Adherent U_2OS cells were detached with 0.05% trypsin-EDTA (Invitrogen) approximately 18-24 hours before transient transfection and were plated in 2.5ml standard culture medium per well in a 6-well plate at a density of 200,000 cells/well. The cells reached 60-70% confluency approximately one day after plating. At this stage, *Trans*IT-LT1 Reagent:DNA complex was distributed to U_2OS cells in the complete culture medium. The *Trans*IT-LT1 Reagent:DNA complex was prepared by mixing and incubating 2 μg of each construct with 500 μl of serum-free standard culture medium and 6 μl *Trans*IT-LT1 Reagent (Mirus, Madison, Wisconsin, USA). After approximately 72 hours of incubation, the cells were harvested for gene expression analysis. The negative control group was cultured and harvested under the same conditions, but without undergoing transfection procedures.

After 72 hours of incubation, the culture medium from the culture vessel was aspirated, and Trizol (Invitrogen) was directly and immediately added to U_2OS cells, and total RNA of U_2OS cells was subsequently extracted according to the

manufacturer's instruction. Contaminating genomic DNA was removed by DNase treatment. Total RNA (50 ng) in a 40-µl reaction was reverse-transcribed to cDNA by using GoScript RT primed with oligo (dT)15 primer (Fisher Scientific, Whitby, ON, Canada). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed in DNA Engine Opticon II Continuous Fluorescence Detection System (Bio-Rad) by using hot start Taq and SYBR Green detection (Eurogentec North America Inc., San Diego, CA, USA). Primer sequences for CD 90 and β-actin were obtained from Invitrogen. The mRNA expression level for CD 90 was normalized to the expression level of β-actin by the $2^{-\Delta c(t)}$ method (78, 79).

Data are presented as mean ± standard error of mean of measurements. Statistical analyses were performed using SPSS version 21 (IBM Corp. Released 2012. IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp). Experimental and control groups were compared with one-way analysis of variance with Tukey's multiple comparison post hoc tests. All statistical differences were considered to be significant with a p-value of less than 0.05.

The relative mean expression of CD90 was evaluated at the mRNA level in U_2OS cells which had undergone different transfection (or no transfection) treatments. There was no statistical significance between any groups (**Figure 8-1**). The results suggested that all five constructs equally failed at generating statistically significant gene silencing in U_2OS cells.

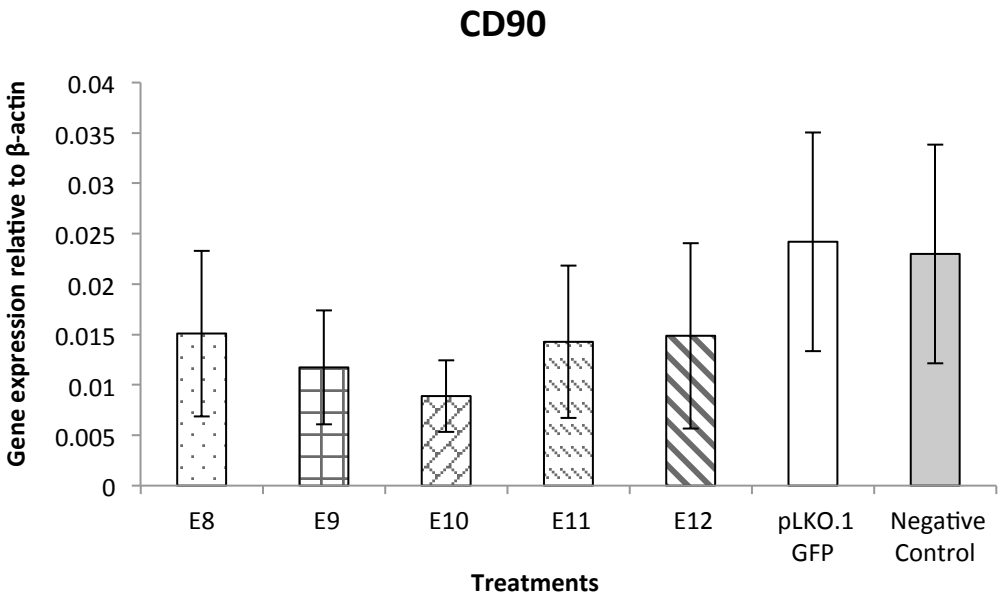

Figure 8-1 Gene expression analysis of U_2OS cells after 72h. incubation with (1) a construct of interest (E8~E12); (2) a non-targeting shRNA construct; or (3) no vector. CD90 expression was evaluated by quantitative mRNA gene expression analysis via SYBR Green detection.

Because no statistical difference was found between and within the experimental and control groups, selection of suitable expression construct became more difficult. Despite a lack of clear indication, both the average percentage knockdown and consistency of knockdown were carefully reviewed to choose the most appropriate shRNA expression construct. As a result, construct E9, which

generated 34.9%, 37.6%, and 18.8% knockdown of CD90 mRNA expression in each of the three trials when compared to the negative control group, was chosen over other constructs. Although making such a decision without sufficient statistical evidence is suboptimal, selection of suitable expression construct had to be made, and construct E9 seemed to demonstrate a reasonably consistent knockdown pattern. Furthermore, the specific construct yielded the highest viral titer (data not shown) when cotransfected with packaging and envelope vectors into HEK 293T cells, making it more appealing for use in our future transduction experiments.

As discussed in **Chapter 5.2**, transduction approach is much more potent than transfection approach. Although the gene silencing effect demonstrated by construct E9 in this chapter is not as significant as we had hoped it to be, the shRNA construct's knockdown effects become much more clear in the following chapters where hBM-MSCs are transduced with LV vectors containing the construct of our choice. Hereafter, shRNA construct coded as E9 will be called CD90 shRNA.

Chapter 9: Transduction of Human Mesenchymal Stem Cells

A variety of studies have reported successful transduction of MSCs using LV vectors (80-82). An important factor that must be determined before transducing MSCs is multiplicity of infection (MOI). MOI refers to the ratio between the number of infectious units and the number of target cells. Determining MOI is an important step towards the development of an optimal knockdown protocol for our proposed research. In this chapter, we aimed to (1) check the gene silencing effect of CD90 shRNA using LV vectors and to (2) determine a reasonable MOI that can be used for our future experiments by measuring the CD90 mRNA expression levels in hBM-MSCs that have been transduced with CD90 shRNA at four different MOIs.

In order to isolate hBM-MSCs, bone marrow aspirates were obtained from surgically discarded material after approval and a waiver of informed consent of the local ethical committee of the University of Alberta (Edmonton, Canada) during orthopedic procedures from the iliac crest of a 43-year old female patient. After counting the number of nucleated cells in the aspirates by using crystal violet nuclei staining and a hemacytometer, 15 million mono-nucleated cells (MNCs) were seeded per 150 cm^2 tissue culture flask. The cells were cultured in α-MEM supplemented with 10% heat inactivated fetal bovine serum, 1 mM sodium pyruvate, 100 mM HEPES buffer, 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.29 mg/mL L-glutamine (all from Invitrogen, Mississauga, Ontario, Canada) and 5 ng/ml basic fibroblast growth factor (from Humanzyme,

Medicorp Inc., Montreal, Quebec, Canada). Nucleated cells were allowed to adhere and grow for seven days before the first media change under normal oxygen tension (21% O_2) at 37°C in a humidified incubator with 5% CO_2. Thereafter, the culture medium was changed twice per week until 70% to 80% cell confluency was attained. The adherent cells were detached using 0.05% trypsin-EDTA (Invitrogen) and expanded until passage 1. The isolated cells were resuspended in freezing medium (high glucose Dulbecco's modified Eagle's medium supplemented with 4.5 mg/mL D-Glucose, 20% fetal bovine serum, 10% dimethyl sulfoxide, 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.29 mg/mL L-glutamine; all from Invitrogen) and were kept in liquid N_2 until experimental use. Prior to experiments, the cells were thawed and were cultured until passage 3 following the same protocol as previously described.

To transduce hBM-MSCs, second generation SIN HIV-1 based, VSV-G pseudotyped LV vectors were used. LV vectors were produced by standard transient cotransfection of a three-plasmid system into HEK 293T cells. CD90 transfer plasmid (Thermoscientific), packaging plasmid ps-PAX2 (Addgene plasmid 12260) and envelope plasmid pMD2.G (Addgene plasmid 12259) were transfected into HEK 293T cells using *Trans*IT-LT1 Reagent (Mirus, Madison, Wisconsin, USA). Standard culture medium was replaced with 30%-fetal bovine serum culture medium 18 hours post-transfection. LV vector-containing supernatants were collected 48 h and 72 h post-transfection. The titer of the pooled LV vectors was determined by using qPCR Lentivirus Titration (Titer) Kit

(Applied Biological Materials, Richmond, British Columbia, Canada) and FAST qPCR Kit Master Mix (2X) Universal (KAPA Biosystems, Woburn, Massachusetts, USA), according to the manufacturers' instructions.

Approximately 18-24 hours before lentiviral transduction, adherent hBM-MSCs were detached using 0.05% trypsin-EDTA (Invitrogen) and were plated in 4 ml standard culture medium per well in a 6-well plate at a density of 60,000 cells/well. Just prior to transduction, adherent hBM-MSCs in one well were detached using 0.05% trypsin-EDTA, and viable cells were counted using trypan blue exclusion test and a hemacytometer. Using the cell number, the number of infectious units (and therefore the volume of viral supernatant) that must be added to each well was determined. Thereafter, the culture medium in each well was replaced with 4 ml of transduction cocktail containing standard culture medium, LV vector supernatant (MOI = 0, 2, 6, and 18) and final concentration of 8 μg/ml Polybrene (Sigma-Aldrich).

After approximately 72 hours of incubation, the transduction cocktail was aspirated from the culture vessel, and Trizol (Invitrogen) was directly and immediately added to the adherent hBM-MSCs, and total RNA of hBM-MSCs was subsequently extracted according to the manufacturer's instruction. Contaminating genomic DNA was removed by DNase treatment. Total RNA (50 ng) in a 40-μl reaction was reverse-transcribed to cDNA by using GoScript RT primed with oligo (dT)15 primer (Fisher Scientific, Whitby, ON, Canada). qRT-

PCR was performed in DNA Engine Opticon II Continuous Fluorescence Detection System (Bio-Rad) by using hot start Taq and SYBR Green detection (Eurogentec North America Inc., San Diego, CA, USA). Primer sequences were taken from previously published work or were custom designed by Dr. Adetola Adesida using the Primer Express software (Applied Biosystems, Foster City, California, USA). All primers were obtained from Invitrogen. The mRNA expression level for CD90 was normalized to the expression level of β-actin by the $2^{-\Delta ct}$ method (78, 79).

The relative expression of CD90 was evaluated at the mRNA level in hBM-MSCs. **Figure 9-1** represents a decrease in CD90 mRNA expression with increasing MOI. **Figure 9-2** shows the percentage knockdown of CD90 mRNA expression when normalized to the negative control group (MOI=0).

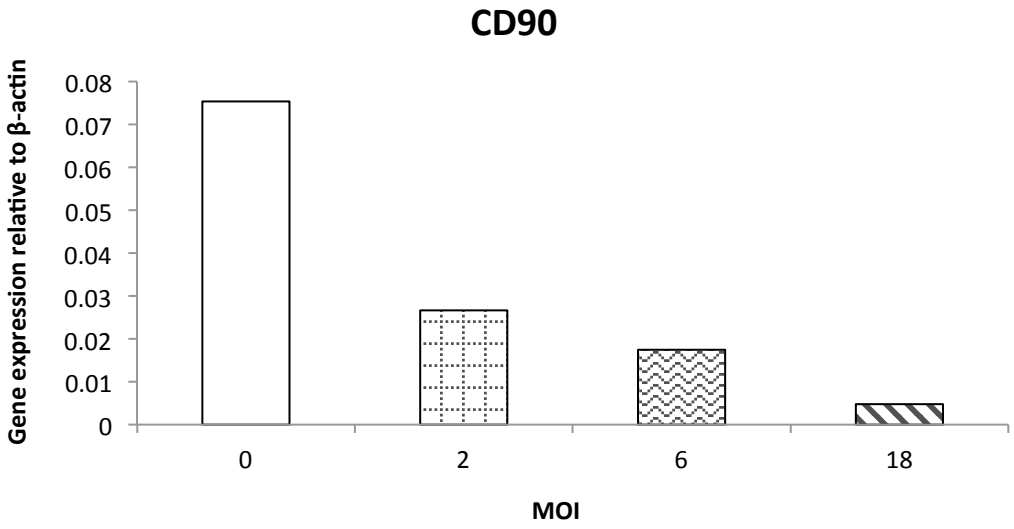

Figure 9-1 Gene expression analysis of hBM-MSCs after 72h. incubation with varying MOI of lentiviral vectors. CD90 expression was evaluated by quantitative mRNA gene expression analysis via SYBR Green detection.

In this chapter, we intended to determine the MOI with which a sufficient knock down of CD90 expression can be achieved. The results showed a dose-dependent reduction of CD90 mRNA expression level. About 65% knockdown of CD90 expression was achieved with MOI of 2, and thereafter, the percentage knockdown continued to increase slowly with increasing MOI. As discussed in **Chapter 5.2**, high concentrations of shRNAs may cause cell toxicity. While using high MOIs can help achieve near 100% knockdown of the gene of interest, oversaturating target cells with shRNAs may harm the cells. Considering these facts, MOI of 10 was determined to be appropriate. While determining MOI using one donor might not account for donor variability and potential errors, this test was meant to serve as a simple check on a reasonable MOI that can be used for our proposed research.

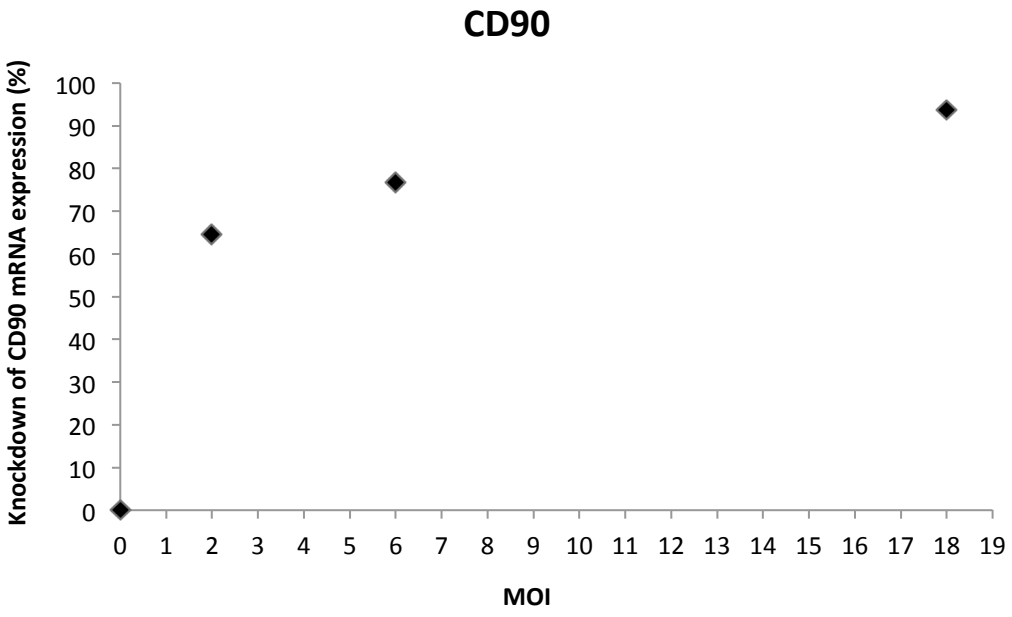

Figure 9-2 Gene expression analysis of hBM-MSCs after 72h. incubation with varying MOI of lentiviral vectors. Percentage knockdown of CD90 expression at MOI= 2, 6, and 18 when normalized to the CD90 expression at MOI=0.

Furthermore, the results support the gene silencing effect of the CD90 shRNA that was selected in **Chapter 8**. Using these results, hBM-MSCs can be successfully silenced to illuminate the functions of CD90 during chondrogenic differentiation.

Chapter 10: Flow Cytometry Analysis of Transduced hBM-MSCs

In order to verify the gene silencing effect of CD90 using our CD90 shRNA construct, the level of protein knockdown was also observed via flow cytometry analysis. In the previous chapters, qRT-PCR results confirmed a decrease in CD90 mRNA expression level. As the number of mRNA transcripts available for translation into the cell surface antigen CD90 decreases, the number of end-products (the cell markers themselves) which are presented on cellular membrane should also decrease. However, it is important to acknowledge that the degree of protein knockdown depends on the half-life of the protein. In other words, "proteins with longer half-lives will take longer to become depleted than those with shorter half-lives" (31). In this chapter, flow cytometry analysis was done on hBM-MSCs transduced with CD90 shRNA at two different time points. We hypothesize that over time, the degree of CD90 expression at protein level should decrease.

hBM-MSCs were isolated and maintained as described in **Chapter 9**. hBM-MSCs used in this experiment came from three female donors (age=23, 38, and 44). Lentiviral vectors containing either CD90 shRNA or pLKO.1 GFP (non-targeting negative control) were also produced as described in **Chapter 9**, and the viral supernatant was concentrated using Lenti-X^{TM} Concentrator (Clontech Laboratories, CA, USA) according to the user manual. hBM-MSCs were incubated with transduction cocktail for 48 hours. After 48 hours, transduction cocktail was replaced with fresh standard culture medium supplemented with

5ng/ml basic fibroblast growth factor. Thereafter, hBM-MSCs were detached using 0.05% trypsin-EDTA (Invitrogen) on Day 3 and Day 7 of experiment (a day and 5 days after transduction cocktail was replaced, respectively). The isolated cells were washed once with FACS buffer (1X PBS supplemented with 5% fetal bovine serum and 0.1% sodium azide), and were resuspended in FACS buffer at 250,000 cells/100µl buffer. Thereafter, the experimental group was incubated with PE-CD90 antibodies (BD Pharmingen), while the isotype control group was incubated with mIgG1 sc-2866/PE (Santa Cruz Biotechnology). Incubation was implemented on ice, in the dark. After 30 minutes of incubation, the cells were washed twice with FACS buffer and were resuspended in 500µl of FACS buffer. All washing steps were performed by a combination of centrifugation (1500 rpm, 5 minutes, at 4°C) and aspiration of supernatant. The cells were analyzed using FACS Calibur (BD Bioscience) and Cell Quest Pro software (BD Bioscience). The level of expression of CD90 was calculated as the ratio between geometric median fluorescence intensity (MFI) of the experimental group and that of the isotype control.

Figure 10-1 shows corrected MFI of each treatment group measured 3 and 7 days after the first introduction of transduction cocktail to the target cells. Data are presented as median ± standard error of mean of measurements. Three treatment groups within each time group were compared with one-way analysis of variance with Tukey's multiple comparison post hoc tests using SPSS version 21 (IBM Corp. Released 2012. IBM SPSS Statistics for Windows, Version 21.0. Armonk,

NY: IBM Corp). Within each time group, no significant statistical difference was found between different treatment groups at p=0.05.

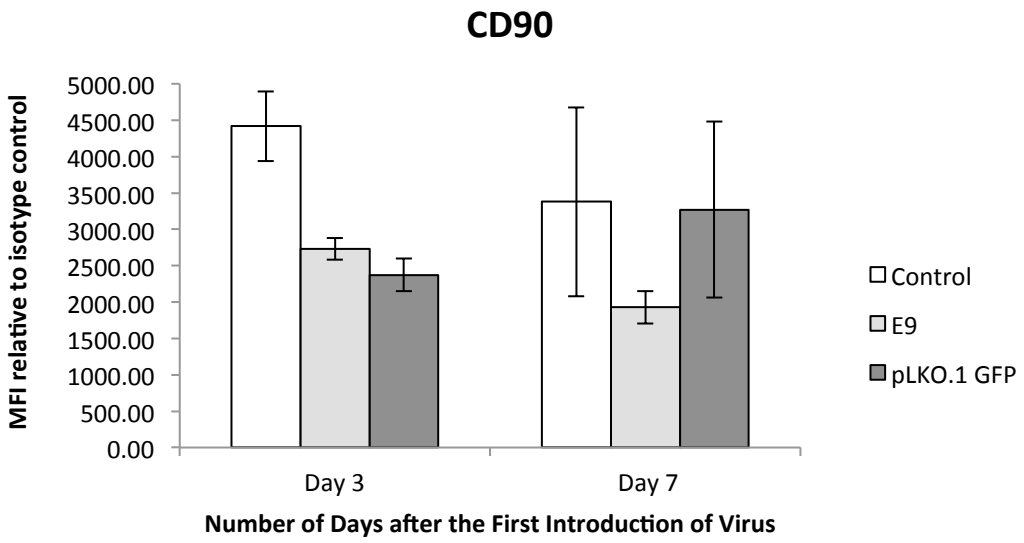

Figure 10-1 Comparisons of MFI on day 3 and day 7. MFI of three groups were measured 3 and 7 days after the first introduction of transduction cocktail.

To generate **Figure 10-2**, MFI of CD90 shRNA-treated group was normalized to MFI of pLKO.1 GFP-treated group using simple division. Then, the calculated values from Day 3 and Day 7 were compared. Data are presented as median ± standard error of mean of measurements. When paired t-test was performed on the normalized values using SPSS version 21, no statistical difference was found at p=0.05.

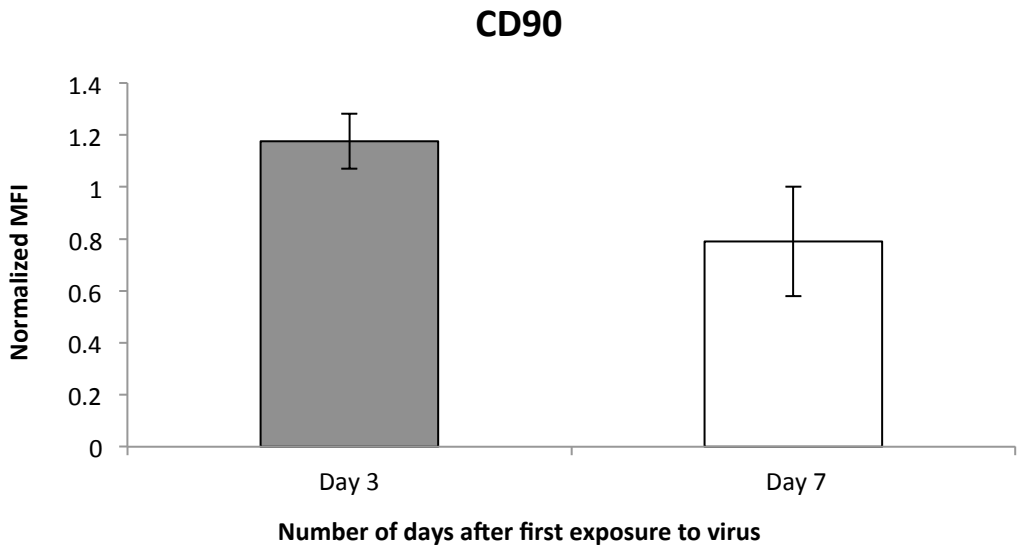

Figure 10-2 MFI of CD90 shRNA group normalized to MFI of pLKO.1 GFP group on day 3 and day 7 of transduction. MFI of three groups were measured 3 and 7 days after the first introduction of transduction cocktail. MFI of CD90 shRNA group was then normalized to that of pLKO.1 GFP group.

The flow cytometry analysis results suggest two possibilities. It is possible that although CD90 was silenced successfully at the mRNA transcript-level, the change was not significant at the protein level because the half-life of CD90 is "long." While a decrease in mRNA expression indicates that there are fewer CD90 templates that can be translated, transduction itself cannot eliminate the already-existing antigens. Alternatively, it is also possible that 48-hour incubation using MOI=10 was not sufficient to generate a significant reduction CD90 antigen-expression. Despite a lack of statistical significance, **Figures 10-1 and 10-2** show a trend of decreasing CD90 antigen over time. The fact that large donor variability existed suggests that the difference in CD90 antigen expression between CD90 shRNA-treated group and the control groups may become clearer with a larger sample size.

Conclusion

In Part B of the thesis, the basic knowledge about RNAi and various RNAi-triggering systems has been provided. RNAi is an endogenous cellular mechanism which can be used to silence specific genes through post-transcriptional means. Our current study relies on this cellular mechanism to knockdown the expression CD90. Hence, the important factors governing the success of gene silencing, including the choice of RNAi triggering molecules, vector delivery system, and MOI, must be determined carefully. From the background knowledge gained from literature review and observations made in our experiments, we decided that shRNA-approach would be most suitable for our proposed research, because a stable and potent knockdown of CD90 was desirable. Furthermore, lentiviral expression system was determined to be most effective at transducing primary hBM-MSCs in comparison to other vector systems. The next step was to select a specific shRNA sequence that can successfully knockdown the expression of CD90 in hBM-MSCs. In addition, the shRNA sequence of choice was used to effectively knockdown hBM-MSCs from a female donor, and MOI of 10 was experimentally determined to be sufficient for eliciting a reasonable reduction of CD90 expression in hBM-MSCs. Finally, flow cytometry analysis of transduced MSCs suggests that gene silencing at the mRNA level is not readily represented at the protein level. The knowledge gained from this section of the thesis will help our pursuit for a novel method of increasing chondrogenic capacity of BM-MSCs via anti-CD90 strategies.

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Figure/Table References

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Part C: Main Experiments

Chapter 11: Changes in mRNA expression of CD90 in shRNA-transduced human mesenchymal stem cells with varying incubation time with viral supernatant

Chapter 11.1: Introduction

RNA interference (RNAi) is an innate and evolutionarily conserved cellular process that can be used to suppress expression of specific genes [1, 2]. Investigation into functions of various genes has benefitted greatly from the use of lentiviral (LV) vectors [3-5]. LV vectors have been used for many years to achieve stable gene silencing in human primary cells, including human bone marrow-mesenchymal stem cells (hBM-MSCs) [4-7]. Modification of hBM-MSCs via RNAi is of special interest because of the cells' ability to differentiate into various mesenchymal lineages, including osteocytes, adipocytes, and chondrocytes [8]. Investigation into the functions of certain genes that may play a critical role in chondrogenic differentiation of hBM-MSCs is especially valuable, because the knowledge gained from such research can help improve the quality of mesenchymal cell-based therapy for articular cartilage damage.

In order to achieve a successful transduction of hBM-MSCs, various factors such as incubation time with a transduction cocktail must be determined cautiously. Although there is evidence to suggest that increased exposure to transduction cocktail, which contains lentiviral vectors and the most common

transduction-enhancing additive called polybrene (hexadimethrine bromide) [9], can increase transduction efficiency, it has also been suggested that a greater exposure to RNAi reagents can disrupt target cells' cellular metabolism [4, 10, 11]. For instance, Lin *et al.* has reported that the commonly used concentration of polybrene (final concentration of 8μg/ml) can inhibit proliferation of MSCs and that this inhibitory effect of polybrene persisted even 3 weeks after exposure. Other researchers have also noted that lentiviral vectors themselves or the expression of the transgene of interest can be toxic to target cells [6, 12]. Similarly, our lab has observed mass cell death as a result of transduction (data not shown). In that experiment, MSCs were incubated with transduction cocktail containing 8μg/ml of polybrene and pLKO.1-based vector at multiplicity of infection (MOI) of 10 for 72 hours, at which time transduction medium was replaced with standard culture medium supplemented with 5 ng/ml basic fibroblast growth factor. Approximately 3 days after the removal of transduction cocktail, only 70% of the cells remained attached to plastic culture vessel, and after approximately 7 days, almost all the cells were detached from the flask. Trypan blue exclusion assay of the detached cells confirmed that the targets cells were dead. Grinev *et al.* suggests disruption in target cells' gene integrity by multiple integrations of virus genome copies may be responsible for the mass cell death [11, 13].

While various studies have looked into the effect of varying MOI on transduction efficiency and cellular metabolism, the relationship between transduction time and transduction efficiency has not been studied explicitly. The

fact that transduction efficiency may vary between different lentiviral vectors and target cell types further necessitates the need to find an optimal incubation time for transducing hBM-MSCs with shRNA vectors of interest to our research group. The purpose of this experiment was to investigate how different incubation times with transduction cocktail affect the degree of knockdown of our gene of interest, CD90, in hBM-MSCs. As we propose that CD90 may have an important role in guiding chondrogenic differentiation of MSCs [14], it is critical to find the minimum incubation time with which a reasonable degree of gene silencing can be achieved. It was hypothesized that the amount of gene silencing achieved will increase with transduction time until maximum copies of virus genome has integrated into the target cells genome at a given MOI.

Chapter 11.2: Materials and Methods

Lentiviral vector production, concentration and titration

HEK 293T packaging cells were co-transfected with packaging plasmid ps-PAX2 (Addgene plasmid 12260), envelope plasmid pMD2.G (Addgene plasmid 12259), and hairpin pLKO.1-RNAi vectors using *Trans*IT-LT1 Reagent (Mirus, Madison, Wisconsin, USA). The two hairpin pLKO.1-RNAi vectors used in the experiment are TRCN0000057024 (CD90 shRNA; Thermoscientific) and pLKO.1 GFP (Addgene 30323; non-targeting shRNA control). Lentiviral vector-containing supernatants were collected 48 h and 72 h post-transfection. The LV vector supernatant was concentrated using Lenti-X^{TM} Concentrator (Clontech Laboratories, CA, USA) following the user manual. The titer of the pooled LV vectors was then determined using qPCR Lentivirus Titration (Titer) Kit (Applied Biological Materials, Richmond, British Columbia, Canada) and FAST qPCR Kit Master Mix (2X) Universal (KAPA Biosystems, Woburn, Massachusetts, USA), according to the manufacturers' instructions.

Isolation and transduction of human bone marrow mesenchymal stem cells

Bone marrow aspirates were obtained from surgically discarded material after approval and a waiver of informed consent of the local ethical committee at the University of Alberta (Edmonton, Canada) during orthopedic procedures from the iliac crest of five donors (**Table 11-1**; three females, 23 to 44 years old, and two males, 24 and 51 years old). After counting the number of nucleated cells in the aspirates by using crystal violet nuclei staining and a hemacytometer, 15 million

mono-nucleated cells (MNCs) were seeded per 150 cm^2 tissue culture flask. The cells were cultured in α-MEM supplemented with 10% heat inactivated fetal bovine serum, 1 mM sodium pyruvate, 100 mM HEPES buffer, 100 U/mL penicillin, 100 µg/mL streptomycin, and 0.29 mg/mL L-glutamine (all from Invitrogen, Mississauga, Ontario, Canada) and 5 ng/ml basic fibroblast growth factor (from Humanzyme, Medicorp Inc., Montreal, Quebec, Canada). Nucleated cells were allowed to adhere and grow for seven days before the first media change under normal oxygen tension (21% O_2) at 37°C in a humidified incubator with 5% CO_2. Thereafter, the culture medium was changed twice per week until 70% to 80% cell confluence was attained. The adherent cells were detached using 0.05% trypsin-EDTA (Invitrogen) and expanded until passage 1. The isolated cells were resuspended in freezing medium (high glucose Dulbecco's modified Eagle's medium supplemented with 4.5 mg/mL D-Glucose, 20% fetal bovine serum, 10% dimethyl sulfoxide, 100 U/mL penicillin, 100 µg/mL streptomycin, and 0.29 mg/mL L-glutamine; all from Invitrogen) and were kept in liquid N_2 until experimental use.

Table 11-1 Donor information of bone marrow aspirates.

Donor	Age	Gender
BM135	23	Female
BM 140	51	Male
BM 143	44	Female
BM 147	38	Female
BM 149	24	Male

Prior to experiments, the cells were thawed and cultured until passage 3 following the same protocol as before. When 70-80% confluence was reached, the adherent cells were detached using 0.05% trypsin-EDTA and plated in 1.28ml standard culture medium per well in a 6-well plate at a density of 60,000 cells/well. Approximately one day later, when the cells reached 60-70% confluence, the culture medium was replaced with transduction cocktail, which consisted of fresh standard culture medium supplemented with 5 ng/ml basic fibroblast growth factor, lentiviral vector supernatant (multiplicity of infection of 10) and 8 μg/ml polybrene (Sigma-Aldrich). The culture medium of the no-treatment group and the polybrene-control group was replaced with fresh standard culture medium containing basic fibroblast growth factor, and fresh standard culture medium containing basic fibroblast growth factor and 8 μg/ml polybrene, respectively. After 24, 48, and 72 hours of incubation, the cells were harvested for gene expression analysis.

Gene expression analysis

Trizol (Invitrogen) was applied quickly and directly to the adherent cells at 24, 48, and 72 hour time points. Total RNA of hBM-MSCs was subsequently extracted by using RNeasy mini kit (Qiagen, Mississauga, Ontario, Canada), and contaminating genomic DNA was removed by DNase treatment. Total RNA (50 ng) in a 40-μl reaction was reverse-transcribed to cDNA by using GoScript RT primed with oligo (dT)15 primer (Fisher Scientific, Whitby, ON, Canada). qRT-PCR was performed in DNA Engine Opticon II Continuous Fluorescence

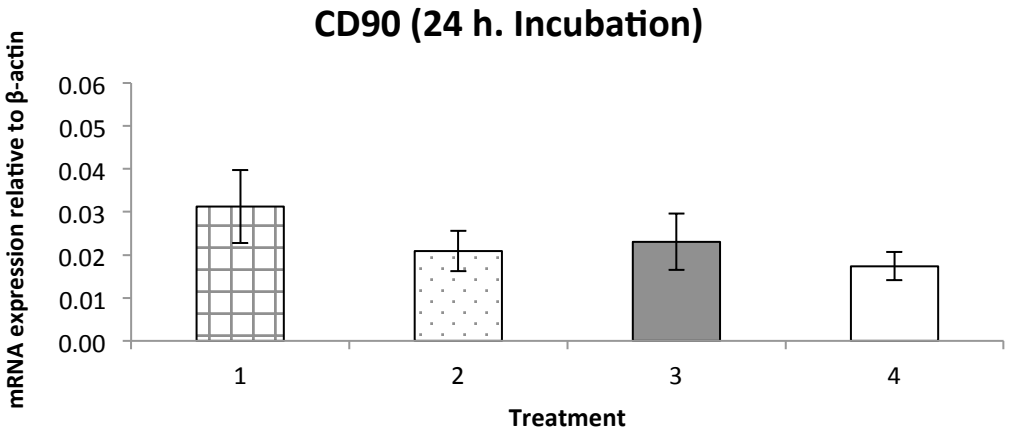
Detection System (Bio-Rad) by using hot start Taq and SYBR Green detection (Eurogentec North America Inc., San Diego, CA, USA). Primer sequences for CD90 and β-actin were obtained from Invitrogen. The mRNA expression level for CD90 was normalized to the expression level of β-actin by the $2^{-\Delta ct}$ method [15, 16].

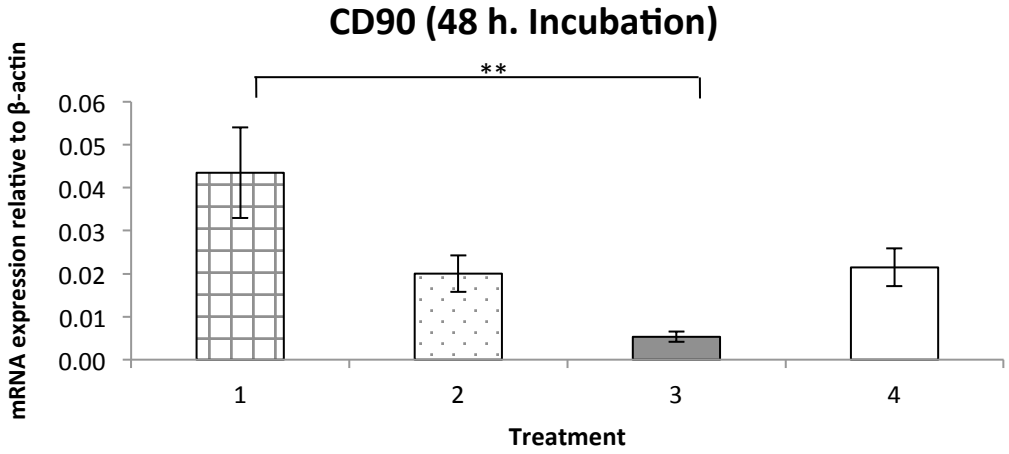
Statistical analysis

Data are presented as mean ± standard error of mean of measurements. Statistical analyses were performed using SPSS version 21 (IBM Corp. Released 2012. IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp). Different treatment groups within each incubation time group were compared with one-way analysis of variance with Tukey's multiple comparison post hoc tests. All statistical differences were considered to be significant with a p-value of less than 0.05.

Chapter 11.3: Results

For each time group, MSCs were further divided into 4 different groups: hBM-MSCs cultured in (1) normal culture medium; (2) normal culture medium containing 8 μg/ml polybrene but no LV vectors; (3) CD90 shRNA transduction cocktail; and (4) pLKO.1 GFP transduction cocktail. No significant difference was found between the groups after 24 hours (**Figure 11-1**). There was a statistically significant decrease in expression of CD90 mRNA by the CD90 shRNA-transduced MSCs (group 3) compared to the hBM-MSCs cultured in normal culture medium (group 1) after 48h and 72h incubations (**Figure 11-1**). However, no statistical difference was found between treatment groups 1, 2, and 4 regardless of the lengths of incubation.





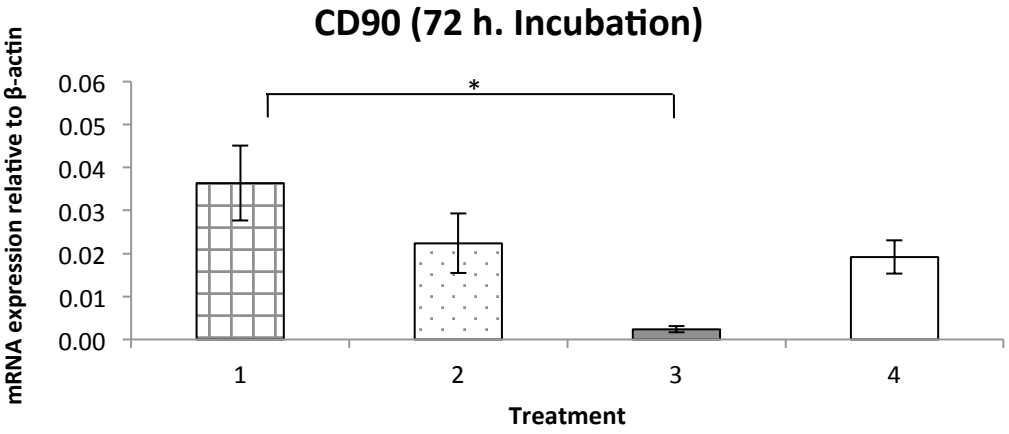


Figure 11-1 mRNA expression of CD90 relative to β-actin. Data represent mean ± standard error (n=5); one-way ANOVA with Tukey's post hoc test was used: * = $p < 0.05$ and ** = $p < 0.01$.

Chapter 11.4: Discussion

hBM-MSCs at passage 3 were cultured in four different conditions for three different incubation time to test transduction efficiency that can be achieved with each transduction time. CD90 mRNA expression of the resulting hBM-MSCs were analyzed using qRT-PCR. The results showed that neither polybrene nor pLKO.1-GFP changed CD90 expression in target cells while the CD90 shRNA group showed a significant decrease in CD90 gene expression compared to that of the hBM-MSCs cultured in normal culture medium. These results indicate that the changes that were observed in CD90 shRNA treated group are due to CD90 shRNA alone. Furthermore, it demonstrates that pLKO.1-GFP is a suitable non-targeting shRNA that can serve as a reliable control for CD90 shRNA. As it can be seen from **Figure 11-1**, incubating hBM-MSCs with CD90 shRNA for 24h. was not sufficiently long enough to generate a significant silencing of CD90. Instead, a length of incubation equal to or greater than 48h. was required to significantly knock down CD90 expression in target cells. It can also be concluded that exposing target cells to LV vectors for a longer period of time does not necessarily result in more effective gene silencing. In fact, the comparisons showed that the degree of CD90 knockdown achieved at 48h. and 72h. was very similar. As hypothesized earlier, saturation of target cell genome with viral genome appeared to have occurred, and we suggest here that with MOI=10 this plateau was reached with 48h of incubation with LV supernatant. It is also interesting to observe that no significant difference was found between non-targeting control group (group 4) and CD90 shRNA group (group 3) even though

the graph suggests a difference exists. In the future, this concern can be addressed by using a higher MOI. With a greater dose of LV vectors, a statistical difference between the two treatment groups (groups 3 and 4) may become apparent. Despite this concern, our results showed CD90 shRNA-treated MSCs consistently had lower CD90 mRNA expression relative to pLKO.1-GFP-treated MSCs. The lack of statistical difference may be attributed to donor variability, and this problem can be improved by increasing the sample size. It should also be noted that RNAi can never completely eliminate the gene product [17]. However, a complete knockout of the gene of interest is not always required to produce a biological phenomenon [18].

Chapter 11.5: Conclusion

Genetic modification of hBM-MSCs allows scientists to alter the behaviors of multipotent cells and to study functions of specific genes. There is evidence to suggest that CD90 is closely related to *in vitro* chondrogenic differentiation of hBM-MSCs, and to investigate the relationship between the level of CD90 expression and chondrogenic potential of hBM-MSCs, determining a suitable transduction time is crucial. In order to avoid cell toxicity and changes in metabolism of hBM-MSCs from the transduction procedure itself, we aimed to investigate the minimum length of incubation time necessary to generate a significant gene silencing of CD90. Here, we showed that exposing hBM-MSCs to CD90 shRNA for 48h. was sufficient to achieve a meaningful knockdown of CD90 when compared to hBM-MSCs that are cultured in standard culture medium. Interestingly, no significant difference in CD90 mRNA expression was found between CD90 shRNA-treated group and non-targeting shRNA-treated group. In conclusion, we report that LV vectors are an effective gene delivery tool and that incubating hBM-MSCs with CD90 shRNA for 48h. or more will produce a significant silencing of the gene.

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Chapter 12: Enhancing chondrogenesis of mesenchymal stromal cells through anti-Thy1 strategies

Chapter 12.1: Introduction

Degeneration of articular cartilage, especially osteoarthritis (OA), is a leading cause of disability resulting in a high economic and social burden on society. Not only does the disruption to the intricate structure and composition of articular cartilage jeopardize patients' health and quality of life, but it also imposes great economic burden to patients and society due to direct and indirect costs of the disease (1-4). Because of its low cellularity, and avascular and aneural nature (5-8), articular cartilage cannot heal itself. Therefore, effective and efficient treatment methods are highly desirable. While there exist many different kinds of nonoperative (9-13) and surgical (14-17) treatment methods, these therapeutic methods have limiting factors, and continuous effort to advance the quality of current cartilage repair strategies is critical for ameliorating OA-associated problems.

Cell-based therapy using mesenchymal stem cells (MSCs) has developed to "generate replacement articular chondrocytes for the production of cartilage tissue thereby reducing pain, restoring joint function and delaying the onset of cartilage degradation and the need for prosthetic joint replacement" (18). MSCs are an attractive cell source for cell-based therapy, because of their ability to differentiate into various cell lineages including chondrocytes (19). Furthermore,

MSCs have high proliferative ability, thereby enabling application of the treatment method to very large defects. Despite these advantages, there are concerns that the quality of human mesenchymal stem cells (hMSCs)-derived repair cartilage is inferior to that of normal human articular cartilage. In addition, this treatment method cannot be used effectively unless a wide variability in the quality of hMSCs, the "ingredients" for cell-therapy, can be controlled for (20).

Recently, Adesida *et al*. suggested a potential link between CD90 (Thy-1) expression and the chondrogenic potential of bone marrow MSCs (21). In their experiment, hBM-MSCs with higher chondrogenic potential consistently and significantly expressed less CD90 protein prior to chondrogenic stimulation. Although CD90 has been explored for nearly half a century, its role in chondrogenesis of MSCs is still unclear. In order to elucidate the relationship between CD90 and chondrogenic capacity of hBM-MSCs, we silence CD90 expression via RNA interference (RNAi). RNAi is a powerful method of gene suppression, and short hairpin RNA (shRNA)-mediated transduction can achieve an efficient and stable knockdown of a specific gene (22-27). In our current research, second generation lentiviral (LV) vectors will be used to deliver CD90-targeting shRNA into hBM-MSCs (28-33).

In this study, chondrogenic capacity of hBM-MSCs whose CD90 expression has been knocked down will be compared to that of hBM-MSCs that have been (1) cultured normally in standard culture medium; and (2) transduced with LV

vectors containing non-targeting shRNA pLKO.1 GFP. Chondrogenic potential of hBM-MSCs expressing different levels of CD90 will be explored by culturing hBM-MSCs in collagen sponge scaffolds, which mimic natural extracellular matrix (ECM) by providing highly organized, three-dimensional (3-D) cell culturing environment in which MSCs can proliferate, migrate, and differentiate (34-37).

Chapter 12.2: Materials and Methods

Lentiviral vector production, concentration, and titration

HEK 293T packaging cells were co-transfected with packaging plasmid ps-PAX2 (Addgene plasmid 12260), envelope plasmid pMD2.G (Addgene plasmid 12259), and hairpin pLKO.1-RNAi vectors using *Trans*IT-LT1 Reagent (Mirus, Madison, Wisconsin, USA). The two hairpin pLKO.1-RNAi vectors used in the experiment were TRCN0000057024 (CD90 shRNA; Thermoscientific) and pLKO.1 GFP (Addgene 30323; non-targeting shRNA control). Lentiviral vector-containing supernatants were collected 48 h and 72 h post-transfection. The LV vector supernatant was concentrated using Lenti-X^{TM} Concentrator (Clontech Laboratories, CA, USA) following the user manual. The titer of the pooled LV vectors was then determined using qPCR Lentivirus Titration (Titer) Kit (Applied Biological Materials, Richmond, British Columbia, Canada) and FAST qPCR Kit Master Mix (2X) Universal (KAPA Biosystems, Woburn, Massachusetts, USA), according to the manufacturers' instructions.

Isolation and transduction of human bone marrow mesenchymal stem cells

Bone marrow aspirates were obtained from surgically discarded material after approval and a waiver of informed consent of the local ethical committee of the University of Alberta (Edmonton, Canada) during orthopedic procedures from the iliac crest of four donors (**Table 12-1**; four males, 40 to 56 years old). After counting the number of nucleated cells in the aspirates using crystal violet nuclei staining and a hemacytometer, 15 million mono-nucleated cells (MNCs) were

seeded per 150 cm^2 tissue culture flask. The cells were cultured in α-MEM supplemented with 10% heat inactivated fetal bovine serum, 1 mM sodium pyruvate, 100 mM HEPES buffer, 100 U/mL penicillin, 100 µg/mL streptomycin, and 0.29 mg/mL L-glutamine (all from Invitrogen, Mississauga, Ontario, Canada) and 5 ng/ml basic fibroblast growth factor (from Humanzyme, Medicorp Inc., Montreal, Quebec, Canada). Nucleated cells were allowed to adhere and grow for seven days before the first media change under normal oxygen tension (21% O_2) at 37°C in a humidified incubator with 5% CO_2. Thereafter, the culture medium was changed twice per week until 70% to 80% cell confluency was attained. The adherent cells were detached using 0.05% trypsin-EDTA (Invitrogen) and expanded until passage 1. The isolated cells were resuspended in freezing medium (high glucose Dulbecco's modified Eagle's medium supplemented with 4.5 mg/mL D-Glucose, 20% fetal bovine serum, 10% dimethyl sulfoxide, 100 U/mL penicillin, 100 µg/mL streptomycin, and 0.29 mg/mL L-glutamine; all from Invitrogen) and were stored in liquid N_2 until experimental use.

Table 12-1 Donor information of bone marrow aspirates.

Donor	Age	Gender
BM133	40	Male
BM 134	56	
BM 140	51	
BM 142	44	

Prior to experiments, the cells were thawed and were cultured until passage 3 following the same protocol as before. When 70-80% confluency was reached, the cells were divided into three groups: (1) No treatment group; (2) Group E9;

and (3) Group pLKO.1 GFP. While the no treatment group was cultured in normal culture medium until its subsequent scaffold culture, the culture medium for Group E9 and pLKO.1 GFP was replaced with 20ml of transduction cocktail containing fresh standard culture medium supplemented with 5 ng/ml basic fibroblast growth factor, lentiviral vector supernatant (multiplicity of infection (MOI) of 10) and 8 μg/ml polybrene (Sigma-Aldrich). After 48 hours of incubation, the medium for all three groups was changed to standard culture medium supplemented with 5 ng/ml basic fibroblast growth factor. The cells were left to recover for 3 additional days in the normal culture medium, after which they were detached to be cultured in chondrogenic medium or to be analyzed by flow cytometry.

Flow cytometry analysis

The isolated cells were washed once with FACS buffer (1X PBS supplemented with 5% fetal bovine serum and 0.1% sodium azide), and were resuspended in FACS buffer at 250,000 cells/100μl buffer. Then, the experimental group was incubated with PE-CD90 antibodies (BD Pharmingen), while the isotype control group was incubated with mIgG1 sc-2866/PE (Santa Cruz Biotechnology). Incubation was implemented on ice, in the dark. After 30 minutes of incubation, the cells were washed twice with FACS buffer and were resuspended in 500μl of FACS buffer. All washing steps were performed by a combination of centrifugation (1500 rpm, 5 minutes, at 4°C) and aspiration of supernatant. The cells were analyzed using FACS Calibur (BD Bioscience) and Cell Quest Pro

software (BD Bioscience). The level of expression of CD90 was calculated as the ratio between geometric median fluorescence intensity (MFI) of the experimental group and that of the isotype control. Unless otherwise stated, the three treatment groups were compared with one-way analysis of variance with Tukey's multiple comparison post-tests using SPSS version 21 (IBM Corp. Released 2012. IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp).

In vitro chondrogenic differentiation

hBM-MSCs at passage 3 were seeded into three-dimensional (3-D) collagen scaffolds. DuraGen® collagen matrix (Integra Lifesciences, PlainsBoro, NJ, USA; 10cmx12.5cm; ~3.5mm total thickness collagen sponge with pore size of 115±20µm) was cut into 6mm diameter disks using a sterile biopsy punch. The scaffolds were placed in a 24-well plate, and each scaffold was seeded with 500,000 MSCs resuspended in 20µl of serum-free chondrogenic medium consisting of high glucose Dulbecco's modified Eagle's medium (DMEM) containing 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 100 mM HEPES buffer, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 µg/ml streptomycin, 0.29 mg/ml L-glutamine (all from Invitrogen), and supplemented with 0.1 mM ascorbic acid 2-phosphate, 40 µg/ml L-proline, 10^{-5} M dexamethasone, 1× ITS+1 premix (Sigma-Aldrich, Oakville, Canada), and 10 ng/ml TGF-β3 (Humanzyme-Medicorp Inc.). To allow initial cell attachment, the scaffolds seeded with MSCs were incubated in a humidified incubator maintained at 37°C with 21% O_2 and 5% CO2 for 15 minutes. Thereafter, 100µl of

chondrogenic medium was gently added to the base of each well, and the scaffold discs were incubated in the humidified incubator for an additional 30 minutes. Finally, 650µl of chondrogenic medium was added along the side of each well, and the scaffolds were cultured for 3 weeks under normoxic condition (21% O_2) at 37°C in a humidified incubator with 5% CO_2. The chondrogenic medium was changed twice a week until the scaffolds were collected for biochemical, histological, and gene expression analysis.

Biochemical analysis

Scaffolds were rinsed with 1X PBS (Invitrogen) and were digested in proteinase K (1mg/ml in 40mM Tris with 1mM EDTA, 1mM iodoacetamide and 10mg/ml pepstatin A; all from Sigma-Aldrich) for 16 hours at 56°C. The sulfated glycosaminoglycan (sGAG) content was measured by 1,9-dimethymethylene blue binding (Sigma-Aldrich) using chondroitin sulfate (Sigma-Aldrich) as standard. The DNA content was determined using the CyQuant cell proliferation assay kit (Invitrogen) with supplied bacteriophage l DNA as standard. Statistical differences between test groups were evaluated by one-way analysis of variance (ANOVA) with Tukey's multiple comparison post-tests. Statistical analyses were performed using SPSS version 21 (IBM Corp. Released 2012. IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp). All statistical differences with p-value of less than 0.05 were considered to be significant.

Histological analysis

Tissues generated from the scaffold cultures were fixed in 4% phosphate buffered formalin, processed into paraffin wax, sectioned at 5 μm and stained with 0.1% safranin-O and counterstained with 1% fast green to reveal sulfated GAG (sGAG) matrix depositions. Other sections were stained with 1% alcian blue and counterstained with 1% neutral red stain to reveal GAG matrix depositions. It should be noted that alcian blue may or may not stain only for sGAG depending on the pH achieved during staining. Images were captured using an Omano OM159T biological trinocular microscope (Microscope Store, Virginia, USA) fitted with an Optixcam summit series 5MP digital camera and Optixcam software and assembled in Adobe Photoshop (Adobe Systems Inc. San Jose, USA).

Gene expression analysis

Trizol (Invitrogen) was applied quickly and directly to scaffolds on Day 0 (before chondrogenic culture) or Day 21 (after 3 weeks of chondrogenic culture on scaffolds), and total RNA of hBM-MSCs was subsequently extracted according to the manufacturer's instruction. Contaminating genomic DNA was removed by DNase treatment. Total RNA (50 ng) in a 40-μl reaction was reverse-transcribed to cDNA by using GoScript RT primed with oligo (dT)15 primer (Fisher Scientific, Whitby, ON, Canada). qRT-PCR was performed in DNA Engine Opticon II Continuous Fluorescence Detection System (Bio-Rad) using hot start Taq and SYBR Green detection (Eurogentec North America Inc., San Diego, CA,

USA). The mRNA expression levels for aggrecan (AGG), Collagen1a2, Collagen2a1, Collagen10a1, cartilage oligomeric matrix protein (COMP), and CD90 were normalized to the expression level of β-actin by the $2^{-\Delta ct}$ method (38, 39). Primer sequences (**Table 12-2**) were based on previous published work (40, 41) or were custom designed by Dr. Adetola Adesida using the Primer Express software (Applied Biosystems, Foster City, California, USA). All primers were obtained from Invitrogen (Mississauga, Ontario, Canada). Unless otherwise stated, statistical analyses were evaluated by ANOVA with Tukey's multiple comparison post-tests using SPSS version 21 (IBM Corp. Released 2012. IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp). Statistical differences were considered to be significant with a p-value of less than 0.05.

Table 12-2 Primer sequences used in quantitative real-time PCR.

Gene	Primer Sequence	Direction	Reference
β-Actin	5'-AAGCCACCCCACTTCTCTCTAA-3' 5'-AATGCTATCACCTCCCCTGTGT-3'	(Forward) (Reverse)	[41]
COMP	5'-CCGACAGCAACGTGGTCTT-3' 5'-CAGGTTGGCCCAGATGATG-3'	(Forward) (Reverse)	[40]
Aggrecan	5'-AGGGCGAGTGGAATGATGTT-3' 5'-GGTGGCTGTGCCCTTTTTAC-3'	(Forward) (Reverse)	[41]
Collagen1A2	5'-TTGCCCAAAGTTGTCCTCTTCT-3' 5'-AGCTTCTGTGGAACCATGGAA-3'	(Forward) (Reverse)	[41]
Collagen2A1	5'-CTGCAAAATAAAATCTCGGTGTTCT-3' 5'-GGGCATTTGACTCACACCAGT-3'	(Forward) (Reverse)	[41]
Collagen10A1	5'-CTGCAAAATAAAATCTCGGTGTTCT-3' 5'-GGGCATTTGACTCACACCAGT-3'	(Forward) (Reverse)	[40]
Thy1 (CD90)	5'-ACCATGAACCTGGCCATCAG-3' 5'TCGGGAGACCTGCAAGACT-3'	(Forward) (Reverse)	

Chapter 12.3: Results

Effect of anti-Thy1 treatment on the expression of cell surface marker Thy1

To investigate the effect of anti-Thy1 treatment on the expression of cell surface protein Thy-1, we analyzed the cell surface molecule expression from four different donors: BM 133, 134, 140, and 142. Data are presented as median ± standard error of mean of measurements. **Figure 12-1** shows normalized CD90 cell surface expression as determined by MFI of each treatment group measured 5 days after the first introduction of transduction cocktail to the target cells. No significant statistical difference was found between different treatment groups at p = 0.05.

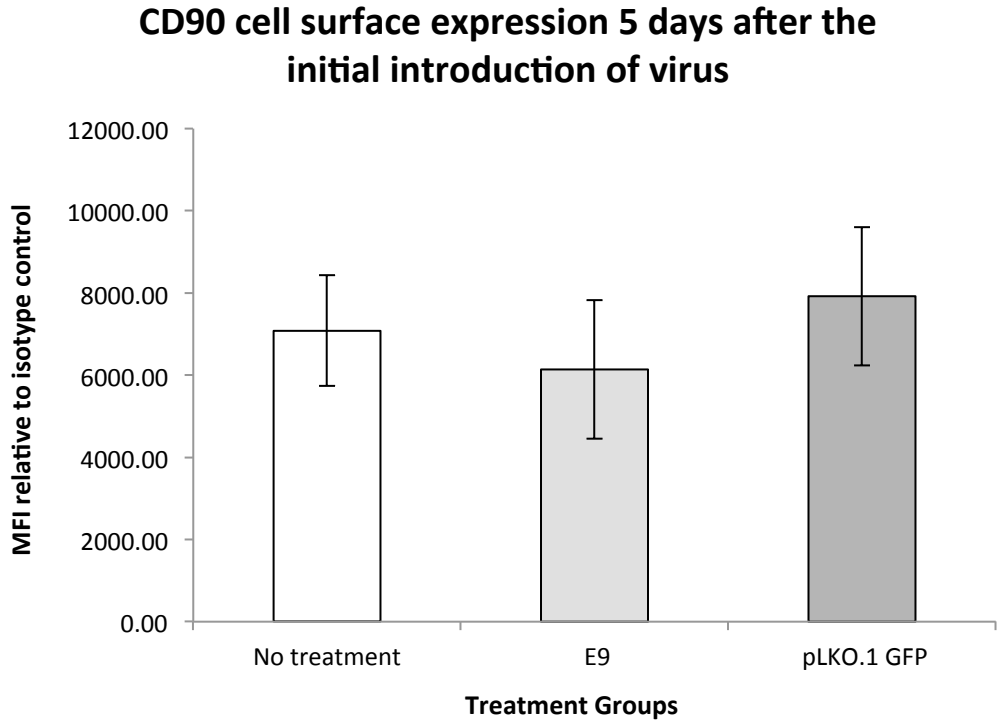

Figure 12-1 Comparisons of MFI between different groups.
MFI of three different groups were measured 5 days after the first introduction of transduction cocktail.

Downstream effect of anti-Thy1 treatment on extracellular matrix formation

The deposition and distribution of GAG in the extracellular matrix of Day 21 scaffolds was visualized by alcian blue and safranin-O staining of 5μm thickness paraffin embedded sections (**Figures 12-2**). Positive alcian blue staining was observed only in the no treatment group as indicated by greenish-blue color.

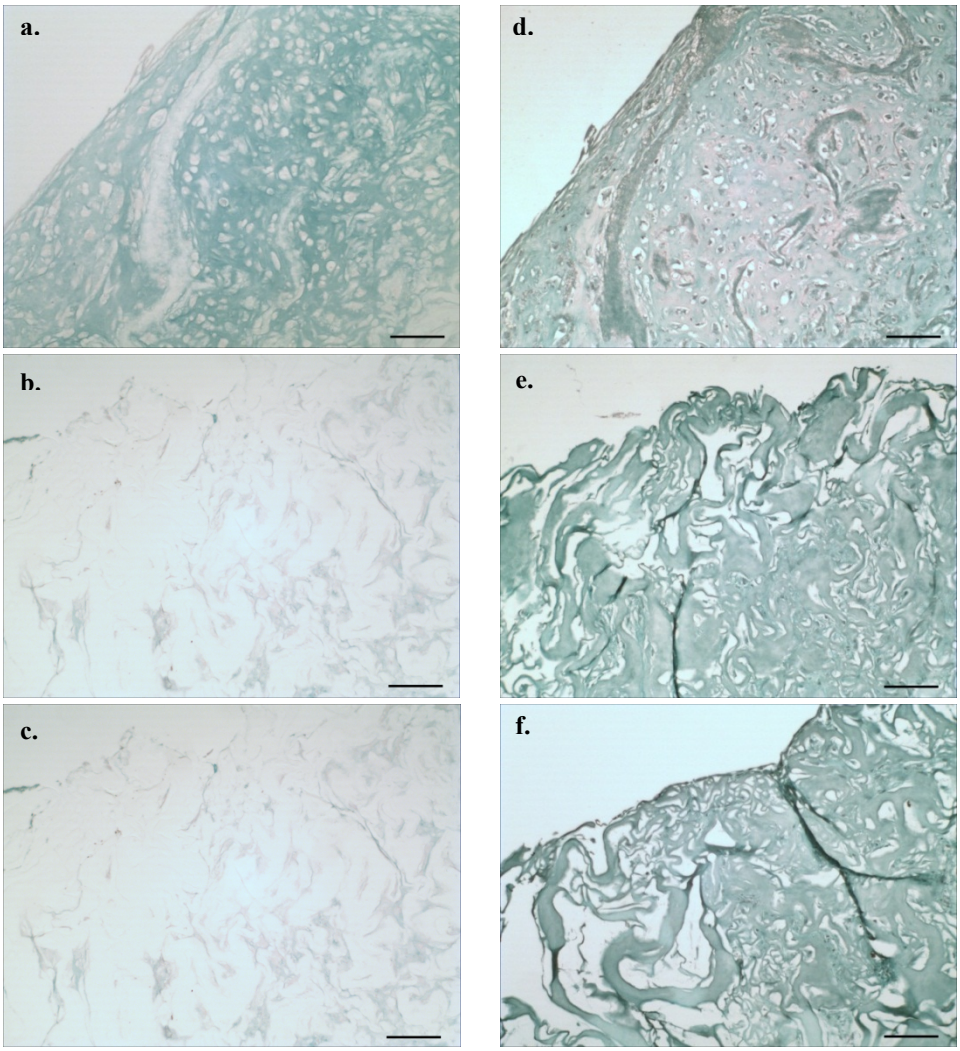

Figure 12-2 Histological analysis of scaffold constructs after three weeks of chondrogenic culture. All photomicrographs represent medium (10x) images with a 100um scale bar. (a)~(c) alcian blue/neutral red staining of no treatment group, Group E9, and Group pLKO.1 GFP, respectively. (d)~(f) safranin-O/fast green staining of no treatment group, Group E9, and Group pLKO.1 GFP, respectively.

Similarly, safranin-O staining showed that the scaffolds belonging to the no treatment group were the only constructs which stained positive for safranin-O as indicated by pink color (**Figure 12-2b**). In contrast, those constructs of the E9 and pLKO.1 GFP groups did not demonstrate any positive staining for sulfated proteoglycan (**Figure 12-2c** and **d**, respectively). Most of the cells that are embedded in the ECM, when visible, showed a rounded chondrocyte-like morphology.

Quantitative GAG matrix normalized to DNA content of the scaffolds was determined (**Figure 12-3**). In the pooled data set, no statistically significant difference was found between the three groups with regards to their GAG per DNA content.

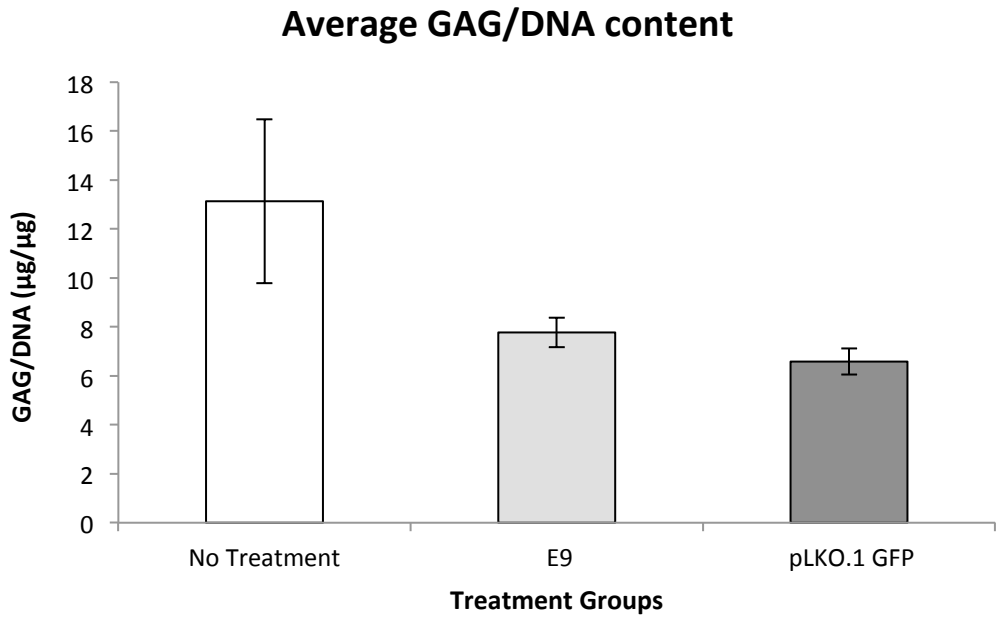

Figure 12-3 Comparisons of GAG/DNA between three groups.
Mean GAG/DNA (µg/µg) levels of three different groups was measured after three weeks of scaffold culture in chondrogenic media. A total of four independent scaffold culture experiments were performed in duplicate.

Effect of anti-Thy1 treatment on Thy1 (CD90) gene

Quantitative RT-PCR was performed for CD90 gene expression analysis for the three groups before and after three weeks of chondrogenic culture. The mean mRNA expression level of CD90 before (Day 0) and after (Day 21) three weeks of chondrogenic culture are presented in **Figure 12-4**. Before three weeks of chondrogenic culture, the mRNA expression level of CD90 was significantly lower in the E9 group compared to both the no treatment (8.4-fold at $p < 0.001$) and pLKO.1 GFP (5.4-fold at $p = 0.014$) control groups. Before chondrogenic culture, no statistically significant difference in the mean mRNA expression level of CD90 was found between the no treatment and pLKO.1 GFP groups. After three weeks of chondrogenic stimulation on scaffolds, the difference in CD90 mRNA expression level in the E9 group compared to the no treatment group (1.7-fold at $p = 0.051$) approached significance, while the difference between the E9 group and the pLKO.1 GFP group became statistically insignificant. No statistically significant difference was found between the no treatment and pLKO.1 GFP groups after three weeks of scaffold culture.

Further statistical analyses using paired samples T test revealed that the CD90 mRNA expression level of hBM-MSCs increased over the three weeks of chondrogenic culture for both the E9 (10.4-fold at $p = 0.005$) and pLKO.1 GFP groups (2.4-fold at $p = 0.035$), while approaching statistical significance for the no treatment group (2.2-fold at $p = 0.059$). **Figure 12-4** illustrates this change in CD90 mRNA expression.

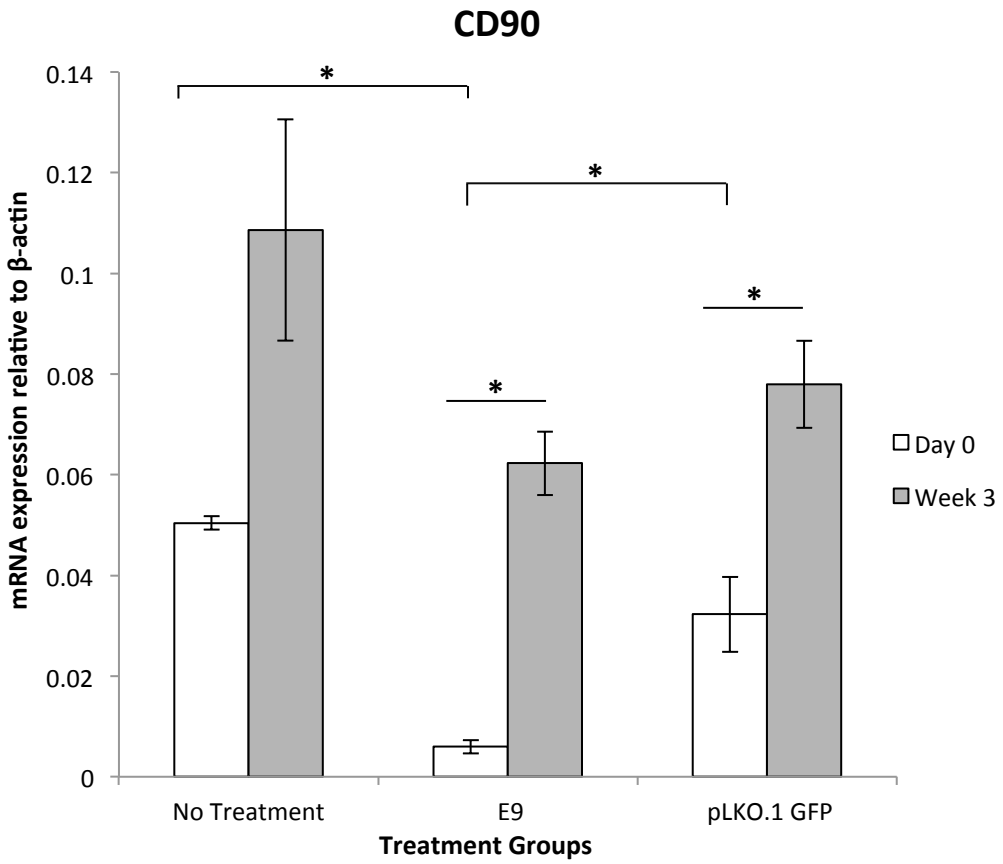

Figure 12-4 CD90 gene expression analysis of hBM-MSCs before and after three weeks of chondrogenic culture across three different groups. CD90 expression was evaluated by quantitative mRNA gene expression analysis via SYBR Green detection. * = $P < 0.05$ by (1) one-way ANOVA with Tukey's multiple comparison post-tests for in-between groups and (2) paired samples T test for within groups.

Downstream effect of anti-Thy1 treatment on chondrogenic gene

The mean mRNA expression level of cartilage oligomeric matrix protein (COMP), aggrecan (AGG), collagen I (Col1a2), collagen II (Col2a1*)*, and collagen X (Col10a1) were compared across the three groups. Before chondrogenic culture, no statistical difference was found between the three groups for all of the chondrogenic genes stated above. However, after three weeks of chondrogenic culture on scaffolds, the no treatment group showed higher mean mRNA

expression level of COMP (1.8-fold at $p < 0.001$), AGG (32.5-fold at $p = 0.024$), and Col2a1 (127.0-fold at $p = 0.045$) compared to the E9 group. Similarly, the no treatment group showed higher mean mRNA expression level of COMP (1.9-fold at $p < 0.001$), AGG (45.2-fold at $p = 0.022$), and Col2a1 (126.2-fold at $p = 0.045$) in comparison to the pLKO.1 GFP group. In addition, the expression level of Col1a2 (2.8-fold at $p = 0.03$) and Col10a1 (14.6-fold at $p = 0.039$) was significantly lower in the E9 group compared to the no treatment group, while the significance of the difference in the level of Col1a2 (2.3-fold at $p = 0.062$) and Col10a1 (9.0-fold at $p = 0.051$) expression between the no treatment and pLKO.1 GFP groups approached significance. No statistically significant difference was found between the E9 and pLKO.1 GFP groups with regards to the mRNA expression level of all five chondrogenic genes. qRT-PCR results obtained from the hBM-MSCs of the three groups that received three weeks of chondrogenic stimulation on scaffolds are presented in the next five figures (**Figure 12-5 - 12-9)**.

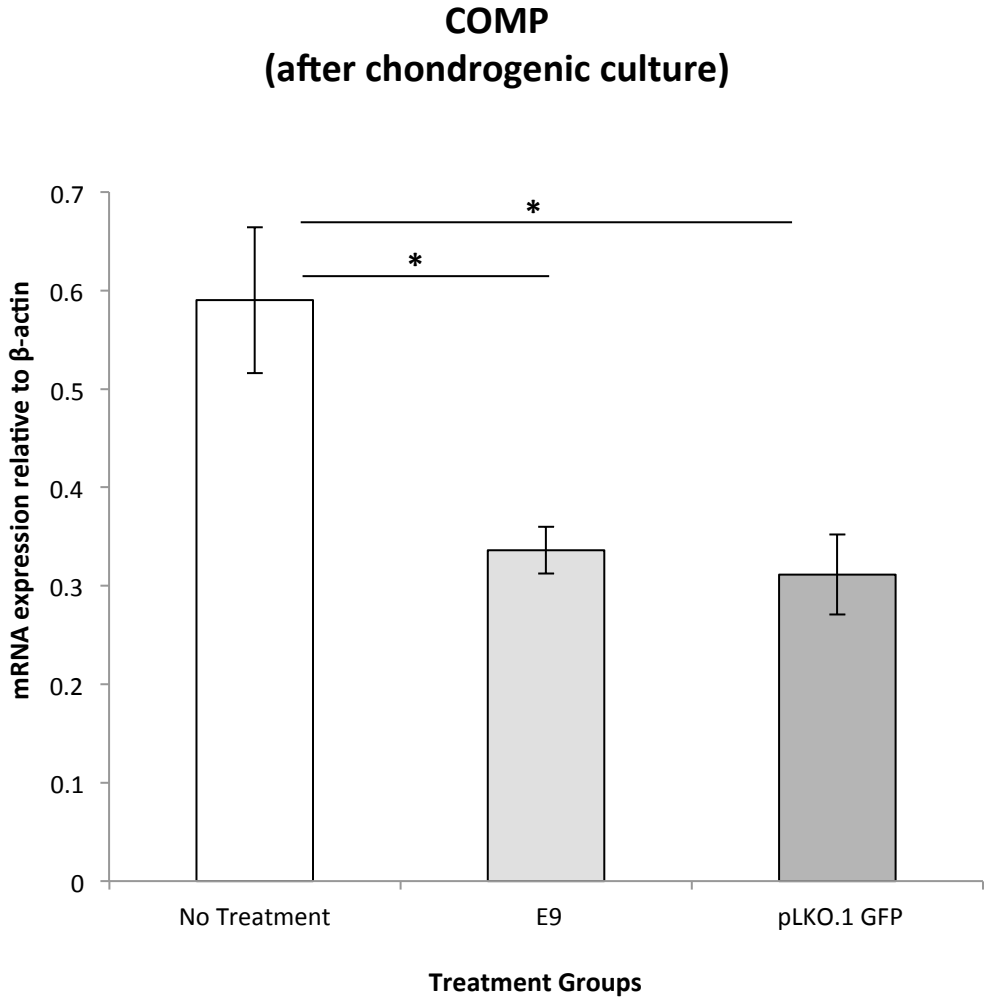

Figure 12-5 COMP gene expression analysis of hBM-MSCs after three weeks of chondrogenic culture across three different groups. COMP expression was evaluated by quantitative mRNA gene expression analysis via SYBR Green detection. * = $P < 0.05$ by one-way ANOVA with Tukey's multiple comparison post-tests.

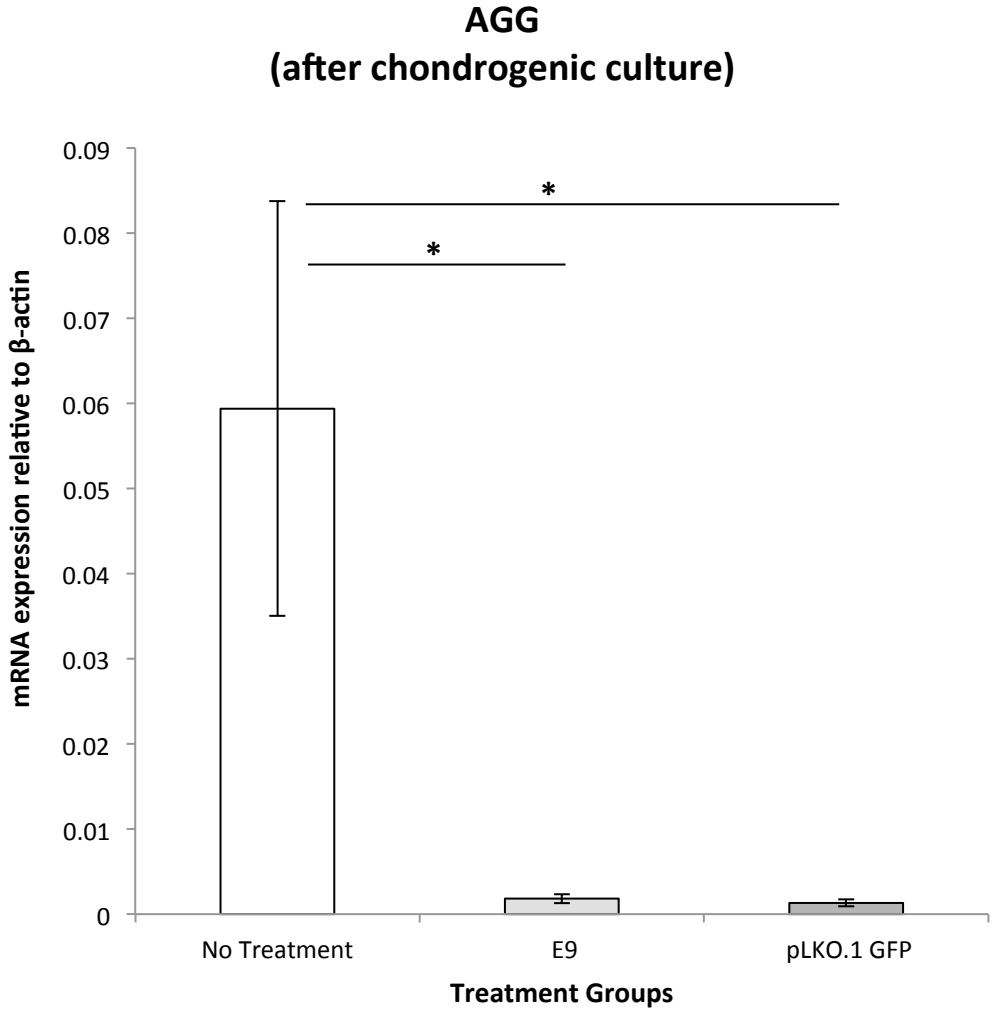

Figure 12-6 AGG gene expression analysis of hBM-MSCs after three weeks of chondrogenic culture across three different groups. AGG expression was evaluated by quantitative mRNA gene expression analysis via SYBR Green detection. * = $P < 0.05$ by one-way ANOVA with Tukey's multiple comparison post-tests.

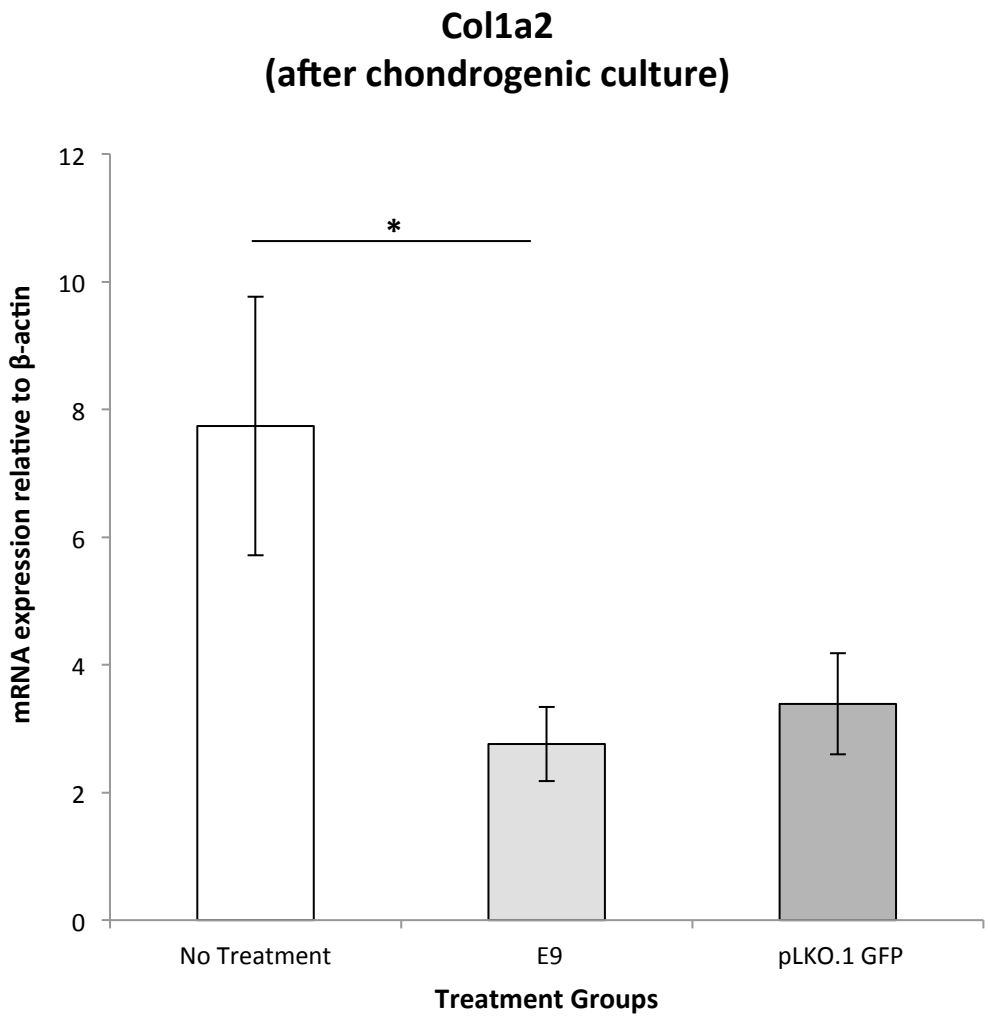

Figure 12-7 Col1a2 gene expression analysis of hBM-MSCs after three weeks of chondrogenic culture across three different groups. Col1a2 expression was evaluated by quantitative mRNA gene expression analysis via SYBR Green detection. * = $P < 0.05$ by one-way ANOVA with Tukey's multiple comparison post-tests.

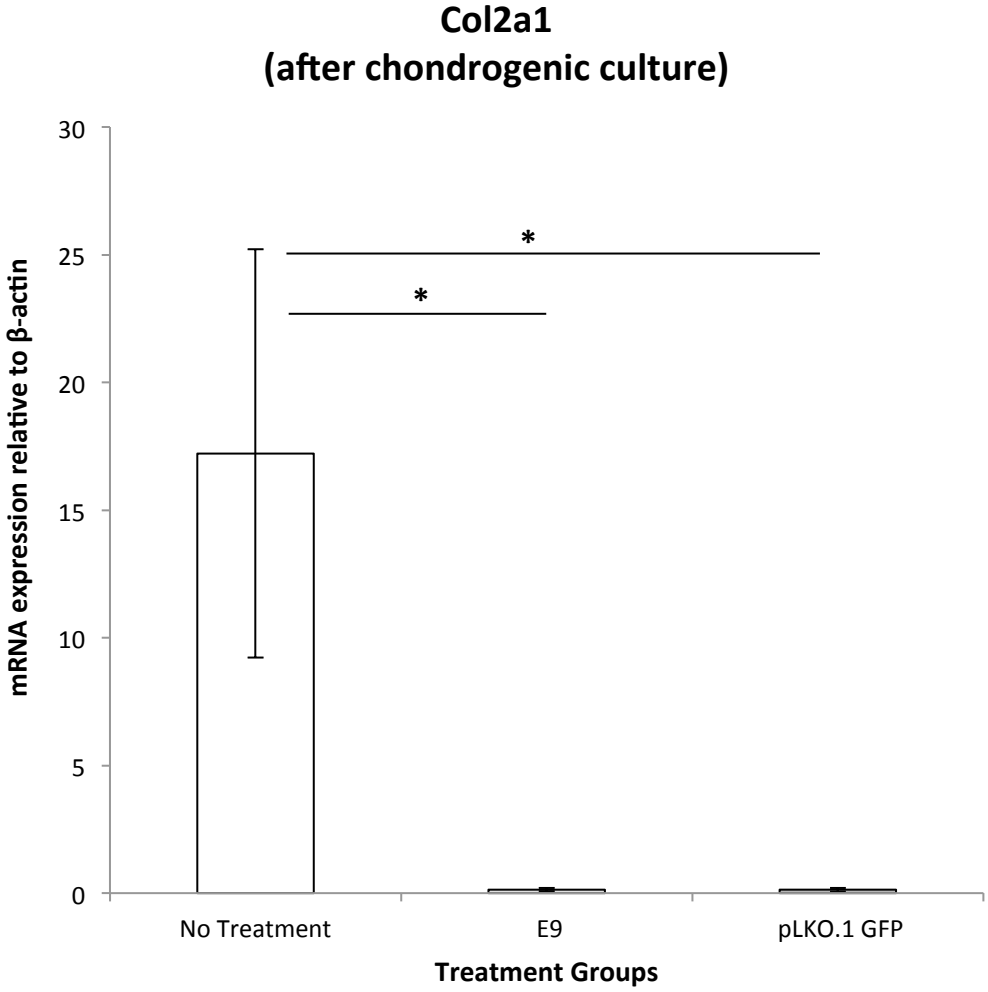

Figure 12-8 Col2a1 gene expression analysis of hBM-MSCs after three weeks of chondrogenic culture across three different groups. Col2a1 expression was evaluated by quantitative mRNA gene expression analysis via SYBR Green detection. * = $P < 0.05$ by one-way ANOVA with Tukey's multiple comparison post-tests.

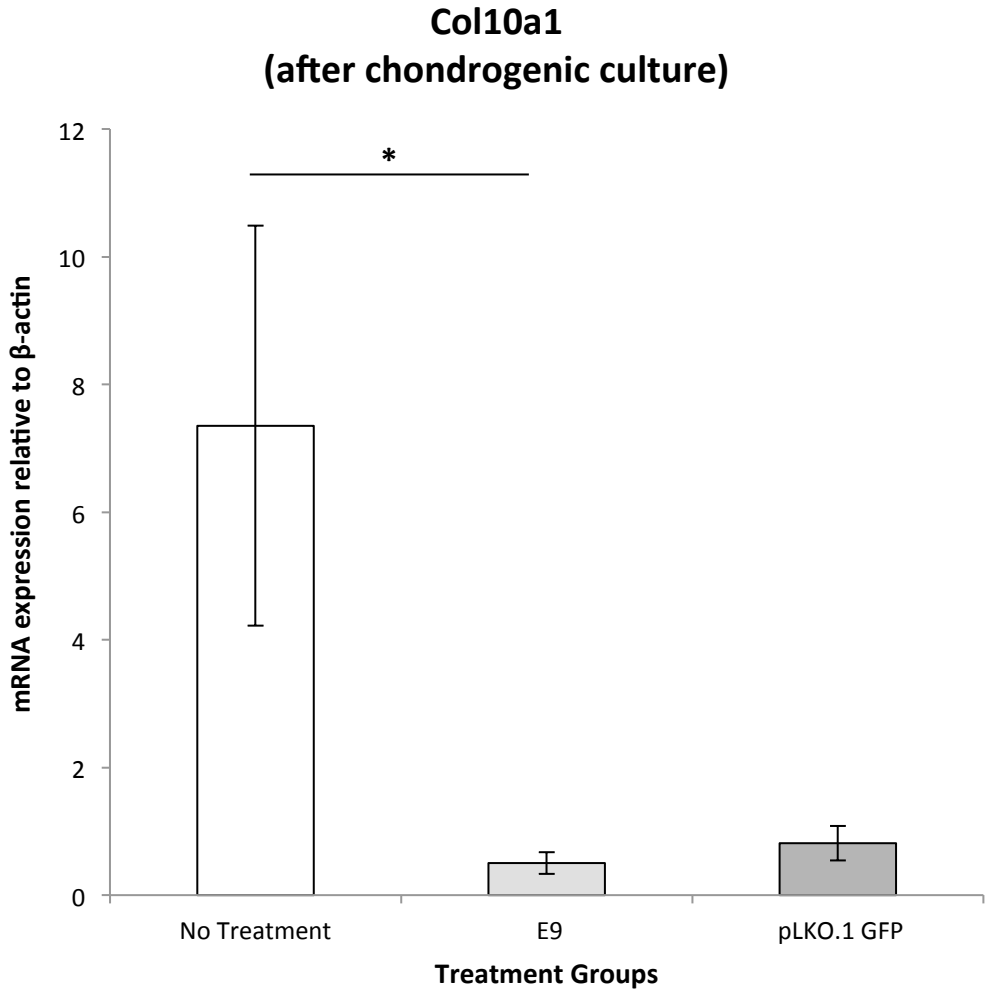

Figure 12-9 Col10a1 gene expression analysis of hBM-MSCs after three weeks of chondrogenic culture across three different groups. Col10a1 expression was evaluated by quantitative mRNA gene expression analysis via SYBR Green detection. * = $P < 0.05$ by one-way ANOVA with Tukey's multiple comparison post-tests.

Chapter 12.4: Discussion

In this study, we have compared the chondrogenic potential of hBM-MSCs that were mono-layer cultured (P3) in three different culture media: (1) standard α-MEM culture media; (2) α-MEM culture media containing CD90 shRNA transduction cocktail; and (3) α-MEM culture media containing pLKO.1 GFP non-targeting shRNA transduction cocktail. The BM-MSCs were selected via plastic adherence and cell culture mediated propagation. Their chondrogenic capacity was determined by further culturing these cells in 3-D scaffold constructs for three weeks.

Flow cytometry analysis and quantitative RT-PCR were performed to monitor the effectiveness of our gene knockdown treatment in silencing CD90. This is important since a meaningful investigation of the effect of anti-Thy1 treatment on chondrogenic potential of hBM-MSCs can only be achieved through a successful knockdown of the gene CD90 in the E9 group. The result from flow cytometry analysis showed no difference in protein expression of cell surface marker CD90 between the three groups. However, qRT-PCR suggested that initially (before scaffolding), the mRNA expression of CD90 in the E9 group was significantly lower compared to the control groups (i.e. no treatment and pLKO.1 GFP groups). Two major factors can be responsible for this discrepancy. But firstly, it is important to understand the mechanism of our anti-Thy1 strategy. The treatment achieves gene silencing by destroying CD90 mRNA templates rather than the actual cell surface proteins. Therefore, while transduction of hBM-MSCs using

CD90 targeting shRNA will reduce the expression of CD90 at the genetic level, it cannot directly eliminate the proteins that are already expressed on the cell surface. Then, the regulation of the cell surface protein expression depends on the half-life of the protein. Naturally, it will take longer for proteins with longer half-lives to be depleted compared to those with shorter half-lives (42). The lack of statistical significance in the results obtained from flow cytometry analysis could indicate that (1) no significant gene silencing occurred; or that (2) the half-life of CD90 is "long." Because our genetic analysis confirmed that CD90 was silenced successfully at the mRNA transcript-level, we suspect that the turnover rate of Thy1 surface markers is low, and that there is a delay in observation of decreased CD90 expression at the protein level. Interestingly, the level of CD90 mRNA expression increased after three weeks of chondrogenic culture, even in the E9 group. This result was surprising because we predicted that the suppression of CD90 would be maintained throughout the chondrogenic culture.

There are several possible explanations for this change. Firstly, it is possible that the gene-silencing effect was wearing off in Group E9. Although lentiviral-based shRNA technology is considered to be one of the most stable means of gene knockdown, it has its limitations (27, 43). With subsequent passages, expression of various transgenes can decrease (44). However, considering reports which indicate that successfully transduced human mesenchymal stem cells continued to express the desirable effect for at least four months in culture (44), it is not likely that the gene silencing effect of the CD90-targeting lentiviral-based shRNA

diminished over the course of three weeks. Moreover, considering that this trend of increasing CD90 mRNA expression was also seen in the pLKO.1 GFP group and, to a certain degree, with the no treatment group, instability of CD90-targeting lentiviral-based shRNA is not likely to be responsible for the recovery of CD90 mRNA transcript level in hBM-MSCs.

A second possible explanation is that the transduced cells whose CD90 expression was suppressed overcame the effect of gene knockdown by producing more mRNA transcripts than can be destroyed. No previous research has outlined the change in CD90 mRNA expression in hBM-MSCs before and after chondrogenic culture. While this mechanism is currently not fully understood, there are two plausible mechanisms by which the level of CD90 can be recovered. It could be that chondrogenic culture itself induced CD90 expression. During chondrogenic culture on scaffolds, the cells are exposed to various growth factors and other chemicals, including ascorbic acid, dexamethasone, L-proline, human insulin and TFG-β3, which were absent in the media that was used for monolayer culture on plastic. This sudden change in culture environment could have induced the upregulation of the gene CD90. Alternatively, it can also be hypothesized that an increase in CD90 mRNA expression is a natural phenomenon during chondrogenic stimulation of hBM-MSCs. From gene analysis of CD90, it is evident that the CD90 mRNA expression level of the Group E9 and pLKO.1 GFP increased significantly during the three weeks of chondrogenic culture until the expression of CD90 mRNA transcripts of all three groups became similar (or not

statistically significantly different). Therefore, it can be hypothesized that a certain level of CD90 expression is actually a necessity for chondrogenesis of hBM-MSCs. To test this hypothesis, it would be useful to completely knockout the gene CD90 instead of using the knockdown approach. Using this extreme measure, the function of CD90 during chondrogenesis can be explored further.

Comparison of chondrogenic capacity of the three groups via histological, biochemical, and genetic means led to very intriguing results. While GAG per DNA content analysis showed no significant difference across the three groups, the no treatment group performed significantly better compared to the E9 and pLKO.1 GFP groups in safranin-O/fast green staining, alcian blue/neutral red staining, and in gene analysis of chondrogenic genes. In these three types of analyses, the no treatment group was the only group which demonstrated a reasonable degree of chondrogenesis. The E9 and pLKO.1 GFP groups performed equally poorly in producing cartilage.

Several factors can be attributed to the low chondrogenic capacity of the E9 and pLKO.1 GFP groups. According to various literatures, polybrene, the most common transduction-enhancing additive, can have harmful effects to cells. For instance, Lin *et al.* reported that exposure to the commonly used concentration of polybrene can inhibit proliferation and differentiation capabilities of human mesenchymal stem cells (45, 46). In addition to the potential negative effect of polybrene, other RNAi reagents can disrupt target cells' cellular metabolism (47,

48). Considering these reports and the fact that the only difference distinguishing the no treatment group from the E9 and pLKO.1 GFP groups is the treatment with lentiviral transduction cocktail, it can be hypothesized that the chondrogenic potential of the transduced hBM-MSCs was compromised due to the treatment itself. However, there is also evidence suggesting that transduced hMSCs retained their differentiation potentials even after lentiviral transduction using polybrene (49, 50). As there are many variables that can influence the outcome of a gene knockdown experiment, such as MOI, incubation time with transduction cocktail, and the sequence of shRNA delivered to target cells, this discrepancy in results is, to a degree, expected. Nonetheless, it should be acknowledged that no "one-for-all" gene knockdown protocol, that is both perfectly safe and effective, exists. In the future, having another control group, in which hBM-MSCs are treated with the standard culture medium that contains an equal final concentration of polybrene as the E9 or pLKO.1 GFP groups, would be beneficial. With this additional control group, we will be able to better understand the impact of polybrene and other transduction reagents on cellular metabolism. Furthermore, protamine sulfate has been suggested to be a safer alternative to polybrene that can preserve proliferation and differentiation capabilities of transduced hBM-MSCs (46). Advantages and disadvantages of each transduction-enhancing additive can be explored further for future experiments.

Because the differentiation potential of the E9 and pLKO.1 GFP groups was considerably compromised, it is increasingly more difficult to investigate the relationship between CD90 and chondrogenic capacity of hBM-MSCs. From the biochemical, histological, and gene analysis results, there was no significant difference between the E9 and pLKO.1 GFP with regards to the cells' chondrogenic potential. This result can be interpreted in two ways.
Firstly, it is possible that any difference in chondrogenic potential that existed between the two treatment groups due specifically to CD90 was "masked" by the powerful negative influence the knockdown procedure had on the two groups.

Alternatively, it is possible that the anti-Thy1 strategy made no difference to the cells' ability to undergo chondrogenesis. In this experiment, however, it is important not to prematurely conclude that CD90 has no role in chondrogenesis of hBM-MSCs. In our experiment, CD90 mRNA expression of the E9 group was knocked down by a factor of 5.4 ($p=0.014$) compared to that of the pLKO.1 GFP group, while no difference was seen in their phenotypic expression of CD90. Because no previous work has explicitly investigated the role of CD90 in chondrogenesis, it is difficult to determine if there is a threshold CD90 expression level that must be achieved in order to have a significant impact on the cells' differentiation potential. Considering that mRNA silencing was not maintained throughout chondrogenesis, it is important to ask if the results would have changed if a more complete (both in degree and duration) knockdown of CD90 was achieved or if the protein expression of CD90 was significantly different

between the E9 and pLKO.1 GFP groups. To address this issue, a higher MOI or prolonged incubation time can be used to induce a more powerful knockdown of CD90. However, these adjustments should be made cautiously, because the changes can be toxic to cells.

While it is possible that no apparent difference between the E9 and pLKO.1 GFP groups with regards to their chondrogenic potential was observed due to experimental limitations, it is also possible that CD90 does not have a direct influence over hBM-MSCs' chondrogenic potential. In the work of Adesida *et al.* (21), the potential link between a lower expression of CD90 and increased chondrogenic potential is illustrated. Two different interpretations can be drawn from this observation: (1) CD90 has a direct role in chondrogenesis, and hence, reducing its expression will enhance chondrogenesis of MSCs directly; or (2) the cell surface maker CD90 is the "marker" (rather than a "cause") for a subset of heterogeneous hBM-MSCs that has high chondrogenic potential. In this scenario, unlike our initial hypothesis, CD90 can be indirectly associated with chondrogenesis. In other words, while it is not a cause of enhanced chondrogenesis, it may still be associated with increased chondrogenic potential. In this case, anti-Thy1 strategy will have no effect on the chondrogenic capacity of hBM-MSCs, because their intrinsic potential to undergo chondrogenesis is not altered by the treatment.

In 2005, Boiret *et al.* reported that 95.5±2.1% of initial, unmanipulated bone marrow cells were CD90- (n=5), but after 1 to 3 days of monolayer culturing, the proportions of CD90- cells decreased significantly until most of the hBM-MSCs became CD90+ (51). Similarly, several other scientists have identified subpopulations of hBM-MSCs as CD90- or CD90+, and noted that they ultimately become CD90+ with subsequent passaging *in vitro* or aging *in vivo* (52, 53). To study if a subset of MSCs that can maintain their natural CD90- phenotype (observed as having a lower level of CD90 expression) can better maintain their chondrogenic potential *in vitro*, a natural separation of the two cell populations (i.e. CD90- and CD90+), rather than an artificial alteration of the cell phenotype, is desirable.

In fact, Ahearne *et al.* recently reported separating two subpopulations of porcine adipose MSCs by exploiting the adhesive behavior of MSCs (54). Interestingly, the most noticeable difference between the subpopulation of cells which adhered to cell culture plastic within 30 minutes of collagenase digestion (termed rapidly adhering (RA) cells) and the other subpopulation of cells which did not adhere to cell culture plastic within that time frame (termed non adherent (NA) cells), was the presence of more CD90+ cells in the RA group. There were significantly ($p = 0.021$) fewer CD90+ cells in the NA subpopulation (57.8%) compared to the RA subpopulation (79.5%). When chondrogenic potential of these two groups was examined via 21 days of pellet culture in chemically defined chondrogenic media, the RA group was found to show a diminished chondrogenic capacity

compared to the NA group (in terms of total sGAG produced). Furthermore, the RA group displayed enhanced osteogenic capacity relative to the NA group, suggesting that MSC-based therapy can benefit from isolating and analyzing the behavior of different subpopulations of MSCs based on specific cell markers, such as CD90.

This is the first study investigating the effect of anti-Thy1 strategy on chondrogenic potential of hBM-MSCs. Taken together, our findings indicate that further adjustments to the experiment to overcome the limitations of current study are necessary to investigate the role of CD90 in chondrogenesis more accurately. The results suggest a common lentiviral transduction protocol can negatively impact the differential potential of hBM-MSCs. Furthermore, our study highlights different ways in which CD90 can be associated with chondrogenesis (direct or indirect).

Chapter 12.5: Conclusion

While the existence of CD90 has been acknowledged for nearly half a century, its exact role in chondrogenesis of hBM-MSCs is still unknown. Recently, it has been suggested that there is a potential link between a reduced expression of CD90 and increased chondrogenic potential of MSCs. In this study, we intended to investigate the properties and function of CD90, in order to better understand the surface marker's role in chondrogenic differentiation of MSCs. To examine the role of CD90 in chondrogenesis, chondrogenic potential of three groups—(1) MSCs grown under standard culture condition; (2) MSCs that underwent anti-Thy1 treatment; and (3) MSCs treated with non-targeting shRNA—were compared via histological, biochemical, and genetic means. The results showed that anti-Thy1 strategy had no effect on chondrogenic potential of hBM-MSCs. However, there were various limitations of this study which made our investigation difficult. It is hoped that the observations made in this research will benefit future research in its investigation into the role of CD90 in chondrogenesis. For the first time, we report changes in CD90 mRNA expression during chondrogenic stimulation. While the mystery of CD90 is still unsolved, our current study documents observations that bring us one step closer to understanding the dynamics of CD90 expression during chondrogenesis of hBM-MSCs.

Chapter 12.6: Acknowledgements

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Chapter 13: Concluding Remarks and Future Directions

Among many types of joint disorders, osteoarthritis (OA) is the leading cause of chronic disability. The World Health Organization (WHO) reported that 9.6% of men and 18.0% of women older than 60 years of age worldwide suffer from symptomatic OA [1]. Disruption to and degeneration of the intricate structure of articular cartilage imposes great burden on individuals and society. OA patients experience a decrease in their quality of life and work productivity as their articular cartilage loses its unique material and biomechanical properties [1, 2]. Furthermore, indirect and direct costs of OA are social and economic problems that our society cannot overlook [3].

To reduce the burden of OA, various non-surgical and surgical treatments have been developed. Nonoperative treatments can be effective at alleviating pain and improving joint function, but individual responses to the treatments can vary widely. Furthermore, non-surgical treatment options do not solve the underlying cause of OA, and consequently, more invasive methods are often required [4]. On the other hand, surgical interventions provide a more direct solution to the problems of OA. However, these methods are invasive, costly, and have significant limitations such as the inferior quality of the repair tissue, incomplete defect filling, new bone formation, and limited availability of donor tissue.

Despite the limitations of operative treatments, OA patients must rely on surgical interventions for repair of cartilage defects, because articular cartilage lacks the

ability to respond to injury [5-7]. Continuous effort by scientists and clinicians to improve treatment methods led to the development of mesenchymal stromal cell (MSC)-based therapy [8]. This technique benefits from the ability of human mesenchymal stromal cells (hMSCs) to differentiate into articular chondrocytes, which in turn, produce functional cartilage matrix [9]. MSC-based therapy has great therapeutic potential, because of MSCs' ability to proliferate quickly and produce regenerative matrix actively [10]. However, better ways of controlling for the variability in the quality of hMSCs is necessary [11].

Interestingly, recent work by Adesida *et al*. suggested a potential link between CD90 (Thy-1) and the chondrogenic potential of bone marrow MSCs [12]. Similarly, various scientists have alluded to the significance of CD90 by illustrating that (1) contrary to the definition of MSCs provided by the International Society for Cellular Therapy (ISCT), MSCs *in vivo* are mostly CD90- [13]; (2) MSCs gain CD90 surface antigens when they are removed from their natural niche and are subsequently cultured *in vitro* [13]; and (3) within the heterogeneous population of MSCs, there exists a subset of MSCs, with no or low level of CD90 expression, which seems to act as early progenitor cells [14, 15]. Despite the potential benefits it could bring to the advancement of MSC-based therapy, no previous studies have explicitly explored the role of CD90 in chondrogenesis. Current knowledge of the cell marker is very limited, and our study aimed to deepen the understanding of CD90.

To investigate the role of CD90 in chondrogenesis, our specific gene of interest (CD90) was silenced via RNA interference (RNAi). There are many important factors that govern the safety and effectiveness of gene silencing, and we developed our current knockdown protocol through various troubleshooting experiments. To achieve a balance between minimizing harm and maximizing efficiency of transduction, we carefully reviewed and selected the appropriate method of delivering RNAi triggers, CD90-targeting shRNA sequences, multiplicity of infection, and incubation time.

With this protocol, we successfully knocked down the mRNA expression of CD90 in hMSCs. However, our results suggested that the chondrogenic potential of hMSCs was compromised in the transduced MSCs. While the results showed that our anti-Thy1 strategy had no effect on chondrogenic capacity of hMSCs, the limitations of this study made the interpretation of our results more challenging.

Concerns over the safety of RNAi technology remain [16-19], and more research is required to develop a standard gene knockdown protocol, with which scientists can effectively and efficiently achieve specific gene silencing while minimizing disruption to cellular metabolism. With such advancements in RNAi technology, the role of CD90 can be examined more accurately.

Because our knowledge of CD90 is limited, certain aspects of our results could not be fully explained. It was observed that the protein expression of CD90 did

not correlate with the decrease in CD90 mRNA expression. It is possible that our anti-Thy1 treatment was not successful at enhancing chondrogenesis of MSCs, because we failed to inactivate or abolish the cell surface proteins. Therefore, future experiments will benefit from developing ways to disassemble or to blocking the surface antigen to effectively limit its functions. To our surprise, we also observed that the expression level of CD90 increased during the three weeks of chondrogenic culture regardless of the treatments the MSCs received during their monolayer culture. From this observation, it can be speculated that achieving a certain level of CD90 expression is necessary during chondrogenesis, but its mechanism is not fully understood. By knocking out the gene CD90 completely, the role of CD90 during chondrogenesis can be explored further.

Review of previously published work and our results combined together, we suspect that CD90 may be associated with chondrogenesis of MSCs by (1) having a direct role in chondrogenesis; and/or by (2) serving as a marker for a subpopulation of MSCs with high chondrogenic potential. Our work is one of the initial investigations of CD90, and much work needs to be done to identify the role of CD90 in chondrogenesis. As was done by Adesida *et al.* [12], the correlation between the protein expression of CD90 and the quality of MSCs (with regards to their differential potential) can be investigated further by profiling the expression of CD90 on MSCs that have been "prompted" to have high chondrogenic capacity. For instance, it has been reported that mechanical stimulation can be an efficient method of inducing chondrogenic differentiation of

MSCs *in vitro* [20]. It would be interesting to investigate whether the cell surface expression of CD90 differ between the MSCs that have received mechanical stimuli versus those that did not receive additional stimuli.

In addition to these investigations, our understanding of CD90 will increase immensely from observing the characteristics (such as size and shape) and behaviors (such as colony-forming ability, proliferation rate, and differentiation potential) of naturally occurring CD90- and CD90+ MSCs. Upon isolation of the two subsets of MSCs, further observation can be made on the changes of CD90 expression during monolayer expansion, and during and after chondrogenic culture in a three-dimensional environment (as in pellet or scaffold culture). There is evidence to suggest that the population of CD90+ cells increases with age [21] (similar to how the CD90+ population increases with subsequent passages *in vitro* until they reach senescence), and therefore, further exploration of the dynamic expression of CD90 can potentially increase our understanding of how the human body's ability to "heal" changes with age.

Our current study provides the basis on which future experiments can expand. It is hoped that the knowledge and insights gained from the current and future research will advance MSC-based therapy. Engineering individual MSCs to enhance their chondrogenic potential, or identifying a subpopulation of MSCs that is more ideal for use in tissue engineering can significantly improve the effectiveness of cell-based therapy. A method of isolating, expanding, and applying high-quality MSCs will greatly aid the management of OA by allowing

for the generation of high-quality cartilage and by achieving uniformity in the outcomes of cell-based therapy.

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