Construction and application of adenoviral and lentiviral vectors to deliver transforming growth factor β 3 and type I collagen-targeting SHRNA for engineered articular chondrogenesis

Zhang, Feng

2011

Zhang, F. (2011). Construction and application of adenoviral and lentiviral vectors to deliver transforming growth factor β 3 and type I collagen-targeting SHRNA for engineered articular chondrogenesis. Doctoral thesis, Nanyang Technological University, Singapore.

<https://hdl.handle.net/10356/44620>

<https://doi.org/10.32657/10356/44620>

NANYANG
TECHNOLOGICAL
UNIVERSITY

**CONSTRUCTION AND APPLICATION OF ADENOVIRAL
AND LENTIVIRAL VECTORS TO DELIVER
TRANSFORMING GROWTH FACTOR $\beta 3$ AND TYPE I
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ARTICULAR CHONDROGENESIS**

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**SCHOOL OF CHEMICAL AND BIOMEDICAL
ENGINEERING**

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A thesis submitted to the Nanyang Technological University
in partial fulfilment of the requirement for the degree of
Doctor of Philosophy

2011

ACKNOWLEDGEMENTS

First and foremost, I would like to express my deepest gratitude to Associate Professor Wang Dong-An, my supervisor, for his comprehensive instruction, as well as consistent enthusiastic support. Without his constant encouragement and patient guidance, I would not have acquired so much knowledge in tissue engineering and gene therapy; neither would I have experienced the joys in research and studies. Besides, Dr. Wang acts not only as a supervisor, but also like a considerate friend and consultant to provide good ideas and solutions whenever we have any problems.

I am also greatly indebted to Nanyang Technological University (NTU) as well as School of Chemical and Biomedical Engineering (SCBE) and all its excellent faculties, researchers and officers, for giving me the perfect opportunity to study here and indulge myself in the wonderful atmosphere of studies and research.

Next, I would like to express my heartfelt thanks to all the friendly lab mates and research fellows in this united and energetic team, including: Dr Jinghua Hao, for not only guiding me to learn the technical skills, but also giving me lots of advice and encouragement as a friend; Dr. Yihong Gong, Dr Ruijie Zhou, Ms. Jiangbo Shen, for providing me detailed guidance; Dr Rohan Rajeev Varshney, Dr Yan Li for giving me valuable suggestions in manuscript writing and problem-solving; and Dr Chunming Wang, Dr Yongchang Yao, Mr Kai Su and Ms Tingting Lau, for their support and precious friendship. Particularly, Dr Yongchang Yao and Dr Yingjun Wang (Key

Laboratory of Specially Functional Materials and Advanced Manufacturing Technology, South China University of Technology, Ministry of Education, Guangzhou 510641, China) contributed to the lentivirus-related work for our projects. As well, I thank Mr Xuetao Shi and Jiabing Fan (exchange students from Guangzhou, China), for showing me their professional opinions on research. In addition, I owe my thanks to all the final-year-project students including Mr. Meng Zhu, Mr. M K Mohamed Idris (2010-2011), Fudiman Citra (2009-2010), Mr Chengzhen Liu (2008-2009) and Ms Lydia Wong Kai Hui (2007-2008), for their diligent help in the lab work. All in all, the happy time I shared with all the guys in the laboratory would be treasured up as my fabulous memory.

Last but not least, I wish to send my sincere gratitude to my beloved parents and my other friends. Without their utmost support and encouragement during the four years, I would not have had the courage to conquer all the difficulties encountered and achieved all I have today.

To every friend of mine, mentioned or unmentioned above, I would say “thanks a lot” to all of you!

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LIST OF ABBREVIATIONS

RA	rheumatoid arthritis
3D	three dimensional
2D	two dimensional
ACT	autologous chondrocyte transplantation
iPS cells	induced pluripotent stem cells
Col I	type I collagen
Col II	type II collagen
Col X	type X collagen
PEG	polyethylene glycol
DEX	dexamethasone
shRNA	short hairpin RNA
siRNA	small interfering RNA
BMPs	bone morphogenic proteins
CPE	cytopathic effect
ifu	infectious unit
DMEM	Dulbecco's modified eagle's medium
CM	chondrogenic medium
ECM	extracellular matrix

FBS	foetal bovine serum
GAGs	glycosaminoglycans
H&E	hematoxylin and eosin
HEPES	N, 2-hydroxyethylpiperazine-N0-ethanesulfonic acid
IGF	insulin-like growth factor
ITR	inverted terminal repeat
MMP	matrix metalloproteinase
MOI	multiplicity of infection
MSCs	mesenchymal stem cells
BMSCs	bone marrow-derived MSCs
AMSCs	adipose-derived MSCs
SMSCs	synovium-derived MSCs
OA	osteoarthritis
PBS	phosphate buffer saline
PCR	polymerase chain reaction
qPCR	quantitative polymerase chain reaction
RISC	RNA-induced silencing complex
RNAi	RNA interference
TGF	transforming growth factor
BMP	bone morphogenetic protein

MCS	multiple cloning site
WST-1	4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio] -1,3-benzene disulfonate
Ad-N	recombinant adenoviral vector without any inserted gene
Ad-T	recombinant adenoviral vector that expresses TGF- β 3
Ad-sh	recombinant adenoviral vector that expresses Col I- targeting shRNA
Ad-D	recombinant adenoviral vector that expresses both TGF- β 3 and Col I-targeting shRNA
LV-N	recombinant lentiviral vector without any inserted gene
LV-T	recombinant lentiviral vector that expresses TGF- β 3
LV-sh	recombinant lentiviral vector that expresses Col I- targeting shRNA
LV-D	recombinant lentiviral vector that expresses both TGF- β 3 and Col I-targeting shRNA

SUMMARY

In this dissertation, we aim to induce type I collagen (Col I)-suppressed chondrogenesis in synovium-derived mesenchymal stem cells (SMSCs) or chondrocytes within 3-dimensional (3D) alginate hydrogel system with the use of adenoviral and/or lentiviral vectors to deliver both transforming growth factor β 3 (TGF- β 3) and Col I-targeting short hairpin RNA (shRNA).

Hyaline articular cartilage trauma and degeneration are major causes of suffering and pose great threat to the life quality of human beings worldwide. However, there is a limited capacity of healing for cartilage itself due to its avascular nature. In cell-based therapy, chondrocytes remain the major choice for cartilage regeneration, as they are the single type of cells within cartilage. However, chondrocytes undergo dedifferentiation during *in vitro* monolayer expansion, in which process the cells lose their phenotype by decreasing expression of chondrocytic markers- mainly type II collagen (Col II) and aggrecan, and upregulation of Col I expression. Alternatively, SMSCs are emerging as a promising source for chondrogenesis due to their relative ease in derivation and differentiation into chondrocytes. TGF- β 3 could be dosed to source cells to induce differentiation in SMSCs or redifferentiation in chondrocytes due to its ability to maintain chondrocyte morphology, promote collagen synthesis and induce chondrogenic differentiation. 3D alginate hydrogel is utilized as it is easy in manipulation and more importantly, compatible with chondrogenesis. However, a serious drawback lies in the fact that Col I is intrinsically expressed in SMSCs and upregulated in chondrocytes during monolayer culture or by the introduction of TGF- β 3 in some types of cells. Col I existence would result in the formation of fibrous cartilage, which has significant difference from hyaline articular cartilage and may not withstand the normal loading exerted on cartilage. Therefore, RNA interference (RNAi) strategy was adopted to silence or suppress the expression of Col I by degrading Col I mRNA in a posttranscriptional pathway.

To deliver both TGF- β 3 and Col I-targeting shRNA to the source cells, both adenoviral and lentiviral vectors would be applied. Among them, adenoviral vector is supposed to induce a transient expression due to its episomal performance, whereas lentiviral vector would lead to a more sustained expression since the vector can integrate its genome together with transgenes into the host genome. Therefore, in our study, a dual-functioning adenoviral vector to deliver both TGF- β 3 and Col I-targeting shRNA was firstly constructed, its transduction conditions were investigated, and its efficiency in fibroblasts, osteoblasts, chondrocytes and SMSCs in monolayer culture was proved. (Chapter 2) A dual-functioning lentiviral vector to deliver both TGF- β 3 and Col I-targeting shRNA was then constructed, and conditions for lentiviral production and titration were investigated. To improve the efficiency of lentiviral vectors in promoting articular chondrogenesis, three more dual-functioning lentiviral vectors were constructed that have various arrangements of TGF- β 3-encoding cassette and shRNA-encoding cassette. The efficiency of Col I suppression and chondrogenic induction was compared between the four dual-functioning lentiviral vectors to choose the optimal one. LV-1, with distant and reverse arrangement of the two cassettes, was proved to be the most efficient among the four. Further assessment was carried out for the delivery of both TGF- β 3 and Col I-targeting shRNA using mixed single-functioning adenoviral and lentiviral vector transduction to deliver TGF- β 3 and Col I-targeting shRNA. It was found that mixed transduction with TGF- β 3-expressing lentiviral vector and shRNA-expressing adenoviral vector resulted in the best efficiency in that timeframe. At last, TGF- β 3-expressing lentiviral vectors were also tested in chondrocyte, with results indicating that dual-functioning lentiviral vector was able to promote Col I-suppressed redifferentiation of chondrocytes in 3D culture.

In summary, a collection of single-functioning and dual-functioning adenoviral/lentiviral vectors that express TGF- β 3 and/or Col I-targeting shRNA were constructed and tested in SMSCs and chondrocytes encapsulated in 3D alginate hydrogel

for Col I-suppressed chondrogenesis. One dual-functioning lentiviral vector with particular transgene arrangement (LV-1 in Chapter 4), and the combination of TGF- β 3-expressing lentiviral vector and shRNA-encoding adenoviral vector (LV-T+Ad-sh in Chapter 5) were found to be relatively more effective than others in inducing chondrogenesis in SMSCs. Besides, the above dual-functioning lentiviral vector was also effective in inducing chondrocyte redifferentiation in 3D alginate hydrogel. These results suggest the promising potential of these viral vectors for the engineering of articular cartilage.

Chapter 1 Introduction

1.1 Cartilage

1.1.1 Cartilage-related diseases

Articular cartilage is one of the tissues that are frequently affected by various factors such as ageing and trauma. Among the many afflictions of cartilage, arthritis is a common form and comprises many different conditions caused by such factors as ageing and infection. Ageing would bring about the degeneration of articular cartilage, just as it does to many other tissues and organs. Infection may also cause arthritis, the so-called septic arthritis, in which the joint is invaded by some infectious agents such as bacteria. One major category within the arthritis family is rheumatoid arthritis (RA). The inflammation to the joint in RA may progress and migrate to many other organs such as lungs and pleura, further complicating the situation and causes a systematic and chronic disease, which is hardly curable. Cartilage degeneration caused by RA accounts for a large proportion among the people worldwide and particularly in developed countries.[1] Another more common form of arthritis is osteoarthritis (OA), which involves the gradual damage and degeneration of the articular cartilage and loss of weight-bearing functions of the joint. Risk factors that may lead to OA include trauma and sedentary lifestyle. Conditions in OA could be possibly improved with joint surgery and replacement. According to statistics, over 1,500,000 arthroscopic operations are carried out in the US, while this number reaches as high as 3,500,000 in the European Union. [2]

The major symptom in arthritis is pain, accompanied by disability and motion difficulty in many cases. All of these have threatened ordinary people's lives and therefore presented a challenge to the social and economic development of the world. Thus, the overwhelming number of cartilage degeneration and diseases calls for effective as well as cost-efficient therapies.

1.1.2 Biology of cartilage

Cartilage is a connective tissue that covers the end of bones, forming the joint. For instance, in the knee joints, articular cartilage acts as a connective tissue linking the tibia and femur of the leg. Cartilage is also present in many other tissues, such as noses, ears and intervertebral discs. It is a stiff yet flexible tissue, which allows flexible working and easy shape restoration of the tissues.

The cartilage tissue is composed of a single type of cells- chondrocytes, which are entrapped in the extracellular matrix (ECM) of cartilage produced by them. The number of chondrocytes is limited compared to the cells in many other tissues, comprising only about 1% of the tissue volume.[1] A single chondrocyte, and sometimes aggregates of several chondrocytes, are embedded in cavities called lacunae. However, chondrocytes are featured with large golgi apparatus and large areas of endoplasmic reticulum. Therefore, such limited number of chondrocytes can give rise to the enormous matrix components such as proteoglycan. Due to the avascular feature of cartilage, chondrocytes can withstand low oxygen (as low as 1% in deep cartilage layers [4]) and nutrition levels. Thus, anaerobic metabolism is preferred, in which lactate is produced from glucose by glycolysis. Due to the lack of blood vessels in cartilage, cells, nutrition and waste migrate through diffusion. Originated and differentiated from mesenchymal cells, chondrocytes diffuse into the articular cartilage, where they stop growth and cell division while start to secrete cartilage ECM.[7]

The cartilage ECM contains such components as collagen fibers, glycosaminoglycan (GAG)-rich substances and elastin fibers. These components differ in their amount in the three types of cartilage- elastic cartilage, hyaline cartilage and fibrous cartilage.

Elastic cartilage is present in ears and epiglottis. It has relatively large amount of elastic fibers in comparison to collagen fibers and proteoglycan, which endows a higher degree of flexibility to the tissue and enables the cartilage to immediately restore its original shape

after deformation imposed by compression or other external forces. A quite small cell population is also a characteristic of elastic cartilage. Comparing with other types of cartilage, elastic cartilage is relatively less prone to degeneration.

Fibrocartilage is not common over the body, and is mainly found in the intervertebral discs and meniscus. The ECM of fibrocartilage is minimal, mainly composed of some fibrous components (60-70% in total) and little proteoglycans (only around 1% of dry weight). It is a mixture of fibrous tissue and cartilaginous tissue, which endow fibrocartilage toughness and elasticity respectively. A key feature of fibrocartilage is that a significant amount of Col I is contained in the ECM. The collagen fibers in fibrocartilage are arranged in the circumferential orientation. This obviously differs from hyaline cartilage, resulting in significantly different physical properties and therefore loading capacities between the two types of cartilage.

Besides the above-mentioned two types of cartilage, the most abundant cartilage within the body is hyaline cartilage, to which category articular cartilage belongs.

1.1.2.1 Hyaline cartilage

Hyaline cartilage is present in many organs and tissues, such as nose, bronchi and joints. As a link between bones in the joint, hyaline articular cartilage covers the end of the bones. The primary function of hyaline articular cartilage is weight-loading for joint motion. The low friction between the adjacent articular cartilage tissues in the joint, due to the lubrication effect of the synovial fluid, enables convenient relative movement of the cartilage. One important feature of articular cartilage is the lack of blood vessels within the tissue. Therefore, cell proliferation is at a much lower rate than that of cells in other tissues. This has brought enormous problems when the cartilage has encountered a trauma or degeneration, under which circumstances cartilage self-repair is slow and limited, despite the fact that the underlying calcified cartilage provides as a self source for regeneration.

This is the primary reason why cartilage therapeutic intervention is necessary in case of damage.

Hyaline cartilage is highly hydrated with 60-85% of total wet weight being composed by water. Among the rest, collagen is one of the most abundant components, making up about 10-30% of total weight. Twenty-seven types of collagens have been identified, all of which share a common unit of Gly-X-Y and form triple helix structures. Of these collagen types, Col II is in the most abundance, comprising 90-95% of total collagen in cartilage, which is usually conjugated with type IX collagen and type XI collagen covalently or non-covalently at the surface or at the core. Type IX collagen and type XI collagen also provide cross-linking to aggrecan. Besides these types, types III, VI, XII and XIV collagens are found in small proportions in cartilage. The function of these collagen components is to retain the shape of cartilage and provide tensile strength. It also acts as a template for molecule binding, forming a complete network of ECM. An example of such is the binding or interaction between Col II and proteoglycan or hyaluronan acid.

Other ECM components include hyaluronan and proteoglycan. Hyaluronan is a negatively charged polysaccharide consisting of β -D-N-acetylglucosamine and β -D-glucuronic acid residues, whereas proteoglycans are composed of a protein backbone linked with many GAG branches.[39] Proteoglycans constitute around 3-10% of the wet weight in hyaline cartilage, which is much larger than the proportion of proteoglycan found in elastic cartilage and fibrocartilage. Major GAGs in cartilage ECM are chondroitin sulfate and keratan sulfate. These GAGs are covalently connected to the core protein, making up the proteoglycan, which further links to the hyaluronan backbone through link proteins. These proteoglycans and hyaluronan components are hydrated and responsible to absorb the compression imposed by external forces. The water absorption effect and the swelling of ECM are constrained by collagen to retain the shape of ECM and provide tensile strength to the cartilage. Proteoglycans also act as the bridges linking the collagen

fibrils, by binding to them every certain distance, thus shaping the overall morphology of cartilage ECM. All these ECM components serve to resist the external forces imposed on the cartilage and protect the chondrocytes embedded within the tissue. ECM also senses and transmits the physical and biochemical signals to chondrocytes, so that in abnormal situations such as trauma, chondrocytes can receive the signal and start their internal mechanisms to secrete more ECM components for regeneration.

Apart from the aforementioned components of cartilage ECM, there are also some minor constituents. Lipids, such as phosphatidylserine, are also present in the ECM of hyaline articular cartilage, which is supposed to initiate cartilage calcification.[54] Besides, lysozyme, cartilage oligomeric matrix protein (COMP) and TGFs are also included in the cartilage matrix.[53]

Within the joint there is a cavity named the synovial gap, which is filled with synovial fluid- a highly viscous fluid similar to blood in terms of its components, such as ions. It also contains some unique components, e.g., hyaluronic acid.[3] The basic function of synovial fluid is to lubricate the cartilage and the joint, so that friction between the neighboring bones would be minimal to facilitate relative movement. Synovial fluid also acts as a diagnostic agent, judging from the variations in the components. Moreover, synovial fluid functions as the source of nutrient for cartilage and the carrier of waste products, which is important as no blood vessels execute these functions within the cartilage. Synovial membrane is a soft and thin membrane acting as the barrier between the synovial fluid and the surrounding environment, so that the synovial fluid would be retained in the synovial gap without being squeezed out. In this project, synovial membrane would be the major source of cells for the engineering of articular cartilage.

1.1.3 Traditional methods for articular cartilage repair

Braces are commonly used to support those with motion disability suffering from cartilage

deficiency. Some drugs have also been extensively dosed in clinical practice, with the aim of relieving the pain of patients and slowing the degenerative process. Such drugs include diclofenac, hydrocortisone and COX-2 selective inhibitors.[4] However, these therapies only address some minor symptoms of arthritis and do not enable complete functional restoration.

The more popular therapies are surgical operations, which involve the replacement of part of or total joint. Chondrectomy and debridement aim to remove the deficient part of the cartilage in order to relieve the patient and stop inflammation, whereas abrasion and microfracture surgery induces spontaneous healing of the cartilage by causing vascular injury to the subchondral bone. Laser can also be helpful in the removal of the deficient cartilage and induction of cartilage recovery by the heat it produces. However, although widely practiced, surgical operations, especially total joint replacement, bring about huge cost to patients and society as a whole.

In other cases, grafting has become a trend for cartilage therapy. Periosteal grafts harvested from the perichondrial tissue and autologous or allogenic osteochondral plugs from the intercondylar notch in the center of the knee have been used for grafting. In the most severe cases where the above treatments do not perform satisfactorily, total joint replacement might be necessary. The surface of the defective cartilage is replaced with metal or plastic, which will substitute the defective part structurally and functionally.

Despite of the advances in cartilage repairing therapies and successful cases utilizing the above therapies, none of them can fully restore the structure and function of the native cartilage, which limits their wide application in clinical practice. In most cases, the newly-formed cartilage contains Col I, which might transform hyaline cartilage into fibrocartilage. There might be some other complications with these practices, such as immune rejection, invasive surgical procedures and infection. Therefore, it is imperative to develop novel therapies for cartilage regeneration, while regenerative medicine provides an attractive

solution.

1.2 Articular cartilage tissue engineering

Regenerative medicine is a discipline in which damaged tissues or organs are regenerated with proper treatment, aiming at restoring the structures and functions of the tissues. There are three categories of practices in regenerative medicine. Among them, regenerative drugs provide a relatively less painful treatment, with the drugs functioning biologically to rescue the diseased tissues and restore their functionality. Injection of progenitor or stem cells is a hot spot in regenerative medicine, utilizing the plasticity of progenitor or stem cells to differentiate into a certain lineage as needed *in vivo*. *In situ* environment will provide the necessary stimuli for this process and guide the differentiation of the cells. Another important area of regenerative medicine is the transplantation of engineered tissues or organs.[5-6] This involves the field of tissue engineering, which is a discipline that combines biology, material science and engineering to develop artificial substitutes to replace part of or whole tissues.[7] In tissue engineering, autologous, allogenic or even xenogenic cells are extracted from the patients themselves or donors and expanded to reach sufficiency. These cells would be loaded to a scaffold, which acts as a support or guidance for cell growth, proliferation, differentiation, and most importantly, tissue implantation to constrain cell motility and avoid cell loss. During the *in vitro* process for tissue formation, external biochemical or biophysical cues will be provided to guide cell behaviors. Particularly, in terms of the application of progenitor or stem cells, such stimuli would be imposed on the cells so that the cells would be induced to differentiate into a specific lineage for specific tissue development.

So far, a few artificial tissues and organs have been fabricated for repair using the principles in tissue engineering. Pancreas, livers, heart valves and cartilage are some examples. For cartilage regeneration, one of the most successful clinical trials is

autologous chondrocyte transplantation (ACT). Over 10,000 patients with cartilage deficiencies have been treated with ACT with high successful rates all over the world.[8] In ACT, biopsy is obtained from the patient or donors, chondrocytes are isolated from the biopsies and amplified *in vitro*. Afterwards, chondrocytes are transplanted into the injured site of the patient with a periosteum flap to trap the cells. This method could be regarded as a prototype of cartilage tissue engineering approach.

For the engineering of artificial cartilage, a matrix of cell types, scaffold sources and biochemical or biophysical factors has been extensively applied, providing wide options and therefore opportunities for the optimization of engineered cartilage tissues. In the next few paragraphs, these elements of cartilage tissue engineering- cells, scaffold and biological factors, are reviewed in detail.

1.2.1 Cells for cartilage tissue engineering

A few types of cells have been intensely investigated and applied in the engineering of the cartilage tissue. Some major examples include chondrocytes, fibroblasts and stem cells.

1.2.1.1 Chondrocytes

Chondrocytes are responsible for the maintenance of ECM and have been the principal type of cell used in articular cartilage tissue regeneration, as most cartilage degenerative and traumatic problems involve the loss of chondrocytes or ECM. The phenotype of chondrocytes varies at different zones of the cartilage. With the deepening of cartilage, chondrocytes tend to secrete more Col II and GAG, which endows higher mechanical strength to the deeper zone of cartilage.[9-10] The primary and most appropriate source of chondrocytes for articular cartilage repair should be articular cartilage. However, intact articular cartilage is not easily accessible and its isolation is invasive, which brings pain and complications to the patients. Therefore, chondrocytes from some other sources have

been investigated as a substitute for articular chondrocytes. These include elastic auricular cartilage and the hyaline nasal cartilage[11][12] Chondrocytes isolated from these sites have high proliferative capabilities and high secretion of Col II and GAG. Application of these chondrocytes has resulted in the construction of engineered cartilage that is similar to native articular cartilage in content and mechanical properties.[11-12]

However, chondrocytes exist in a limited number within cartilage. The isolated chondrocytes have to be passaged and expanded in order to obtain a sufficient number. A key problem involved in this process is cell dedifferentiation. Chondrocytes will gradually lose their chondrocytic phenotypes with decreased secretion of Col II and aggrecan and overexpression of Col I.[13-14] This dedifferentiation process is observed even at the first passage of expansion.[15] This would obviously lead to altered composition of ECM and the construction of cartilage tissues with different mechanical and biochemical properties from the native tissue. The newly-formed cartilage tissue thereof with altered mechanical strength may not withstand the external loading and therefore fail in the fulfillment of weight-bearing. To avoid cell dedifferentiation, some additional substrates have been applied during cell culture. Aggrecan-coating has enabled the cells grown on it to maintain their phenotype and gene expression profiles, while Col I, Col II or fibronectin do not have such capabilities.[16-17] Another method is to culture the chondrocytes in 3D scaffolds, and agarose[18], alginate[19] and fibrin[20] have all been tested as options. After expansion within poly(N-isopropylacrylamide-co-acrylic acid) (a thermoreversible hydrogel)[21] or alginate (a hydrogel that could be dissolved easily with sodium citrate), chondrocytes could maintain their phenotype and be released for loading to other materials. Alternatively, bioreactors could be utilized, not only to convey sufficient nutrition to the cells but also maintain chondrocyte phenotype.[22] The supplementation of some growth factors can also slow down or even reverse the dedifferentiation process,[16, 23] examples of which are fibroblast growth factor-2 (FGF-2) [24] and TGFs.[25] This lays the

foundation of our research in Chapter 6 to use TGF- β 3 delivered with lentiviral vectors in order to initiate redifferentiation of dedifferentiated chondrocytes.

1.2.1.2 Fibroblasts

Fibroblasts have also been used in cartilage tissue engineering in that they are in large quantity all over human body, and they are easily and noninvasively obtainable from the skin. Although belonging to a fundamentally different type of cells, fibroblasts can be guided to chondrocytic phenotypes and assume the responsibilities of chondrocytes under specific conditions. For instance, when dermal fibroblasts were treated with IGF and cultured on aggrecan, they could grow into aggregates with positive expression of GAG and Col II.[26] When fibroblasts, which endogenously expressed TGF- β 1, were injected into the injured site, neotissue formation was observed from the injected cells, indicating the transformation of fibroblasts into chondrocytic-like cells and the production of ECM components.[27] However, a major problem involved in the application of fibroblasts in cartilage tissue engineering is the production of Col I, which would lead to the formation of fibrocartilage and undermine the mechanical strength. This phenomenon has been observed when fibroblasts were implanted with polylactic acid (PLA) meshes into the injured site, where fibrocartilage was formed therein.[28] This has posed great limitations to their applications.

1.2.1.3 Mesenchymal stem cells (MSCs)

MSCs, a group of adult stem cells derived from mesenchyme, have been a major type of stem cells used in cartilage tissue engineering. As a type of stem cells, MSCs are undifferentiated cells that have potentials to differentiate into certain cell types for tissue formation during tissue development or regeneration after injuries to the tissue. They usually reside quietly in niches of tissues and when necessary, are recruited to the injured

site, where they receive signals from the *in situ* environment for reprogramming. MSCs have been a popular cell source for chondrogenesis due to their relative ease in derivation and reprogramming into chondrocytic phenotype.

Like many other stem cells, MSCs have two distinctive features, which make them superior to terminally differentiated cells. Firstly, they are able to differentiate into other cell lineages. Secondly, they are able to replicate for multiple rounds while maintaining their differentiation potential, so that a large cell number is obtainable, which overcomes the major impediment in tissue engineering.[29]

MSCs can be isolated from the adult patients themselves, which circumvent the ethical and immunogenic problems encountered by ESCs. MSCs are found in many tissues, among which the earliest was bone marrow.[30] Afterwards, MSCs from umbilical cord blood[31], peripheral blood[32] and adipose tissue[33] were also identified

Bone marrow-derived MSCs (BMSCs) exist in plenitude in the bone marrow and are easily obtainable. After the aspiration of bone marrow, BMSCs can be conveniently separated from other cells by centrifugation followed by resuspending cell pellet and plating the cells into culture vessels. The adherence of BMSCs and spindle-like morphology would distinguish BMSCs from other cell types, since BMSCs are adherent cells while other bone marrow cells remain in suspension during culture. Therefore, pure populations of BMSCs can be obtained through frequent media change and cell passaging. Alternatively, since CD105⁺, CD73⁺, CD90⁺, CD45⁻ and CD34⁻ are characteristic markers of BMSCs,[34] fluorescence-activated cell sorting (FACS) could be utilized to purify BMSCs.[35] BMSCs can be passaged while retaining their potential for differentiation, and they have been substantiated in myriads of experiments to be able to differentiate into many cell types, including osteocytes, chondrocytes, adipocytes, cardiomyocytes, etc.

Apart from BMSCs, MSCs from umbilical cord blood have also been intensively studied. Such MSCs have been found to differentiate into smooth muscle cells, neurons and endothelial cells on top of osteocytes, chondrocytes and adipocytes. Research in this area has stimulated a market where babies' umbilical cord blood is preserved in frozen form after birth to meet the possible demand in case of emergencies.

Adipose is another major source of MSCs. Adipose-derived MSCs (AMSCs) can be isolated from adipose in larger numbers with minimally invasive procedures, so that cell expansion can even be skipped. Besides, age does not pose a barrier to the application of AMSCs, since AMSCs exist in elderly patients and still possess efficient regenerative capabilities. Like BMSCs and umbilical cord blood-derived MSCs, AMSCs also possess the ability for osteogenesis, chondrogenesis, adipogenesis and myogenesis, although poorer chondrogenesis was reported with AMSCs compared with others. [36]

Besides MSCs derived from all the abovementioned sources, MSCs isolated from synovium have recently been identified.[37-38] Methods for the derivation and isolation of SMSCs have been established.[37] As illustrated previously, synovial membrane is a thin layer lining the joint surfaces, whose function is to maintain synovial fluid within the cavity around the cartilage to lubricate cartilage and facilitate motion. SMSCs can be further divided into fibrous SMSCs and adipose SMSCs, according to their origin- fibrous synovium or adipose synovium, both of which are subsynovial tissues.[39] The morphology and plasticity of SMSCs are similar to BMSCs.[40] To date, SMSCs have been induced to differentiate for osteogenesis under the stimulation of osteogenic medium, which was demonstrated to have even better osteogenic capability than AMSCs and muscle-derived MSCs [38-39]. Besides, in clinical practices, it was found that SMSCs could aid in the regeneration of tendons as well as bones.[41] SMSCs were also able to differentiate into myocyte-like cells in vitro in myogenic medium and form atypical myotubes.[37] *In vivo*, SMSCs could also acquire the phenotype of myocyte for the

regeneration of muscles after residing in the muscle tissue, where the environment would exert great influence to cell reprogramming.[42]

SMSCs possess some additional advantages in comparison to BMSCs and other stem cells. Firstly, SMSCs have a higher proliferative capacity, which is an essential characteristic particularly important in regenerative medicine. Although senescence can be a problem constraining cell application, SMSCs can be cultured for up to ten consecutive passages while retaining their proliferative capacity.[43] Another more important merit of SMSCs lies in their outstanding capacity in chondrogenesis.[44] SMSCs and chondrocytes have been found to originate from the same progenitor cells. In this sense, SMSCs and chondrocytes should share certain common features, such as the expression of such components as hyaluronan receptor (CD44), cartilage oligomeric matrix protein (COMP), SOX-9, aggrecan, collagen X and XI, although the expression level in SMSCs was lower than those in chondrocytes[45-46]. These features have conferred SMSCs as a perfect source for chondrogenesis, whereby the reprogramming efficiency could outperform many other sourced cells. Comparisons made between rat MSCs derived from various tissues, including adipose, bone marrow, periosteum, synovium and muscle, have led to the conclusion that SMSCs have higher proliferative capability and chondrogenic differentiation represented by larger pellet and more matrix deposition[43, 47]. In practice, a part of SMSC research has been donated to this area, with significant progress having been achieved in the understanding of biological fundamentals and practical applications of SMSCs in cartilage regeneration.[48-50]

In summary, a number of types of cells have been exercised for cartilage tissue engineering, including chondrocytes, fibroblasts and MSCs derived from various sources. To achieve our objective to engineer cartilage tissues that mostly resemble the native cartilage, the source cells should meet some criteria, including easy access, high proliferative capacity and efficient plasticity for the reprogramming into differentiated

chondrocytes. In light of these principles, we have selected SMSCs as the major source cell for the reprogramming into chondrocytic phenotype in this PhD research.

1.2.2 Scaffolds for cartilage engineering

Mature chondrocytes will gradually lose their chondrocytic phenotype when they proliferate on culture flasks. For example, cells would elongate, a morphology that is distinct from the normal polygonal shape of chondrocytes. The expression of cartilage ECM components would also be decreased. To maintain the cells, either chondrocytes or other types of cells such as MSCs, in the similar phenotype to native chondrocytes, 3D biomaterials are applied. Biomaterials, used as matrices to constrain cell motility, avoid cell loss and deliver the therapeutic cells to the injured defect, provide cues that can affect the behaviors of the cells. Those biomaterials that do not provide adherence sites for cells would maintain the cells in a round shape, which is a favorable phenotype of chondrocytes.

Biomaterials should be biocompatible, without adverse effect on cell viability and proliferation. They should support cell adherence and growth, or even guide the differentiation process of stem/progenitor cells. They should also have proper mechanical strength that can be comparable to the target tissue that they aim to mimic. Moreover, an ideal biomaterial should be able to integrate into the defect site, where they can gradually degrade at a certain rate to give space to the newly-formed tissue matrix.[51]

Conventional porous scaffolds and hydrogels are the two major types of 3D scaffolds used in cartilage tissue engineering.

1.2.2.1 Conventional porous scaffolds

Fabricated by freeze-drying of the substrate solution[52], solvent leaching[53], electrospinning[54], etc, conventional porous scaffolds take on a porous appearance. The substrates form the backbone, with pores of various dimensions intertwined within the

scaffold. The substrates provide the wall for cell attachment and proliferation, while the pores allow the penetration of nutrient-rich medium and exchange of cellular metabolic wastes. Due to the significant increase in surface area, porous scaffolds are particularly suitable for adherence-dependent cells.

Both synthetic and natural materials can be fabricated into porous scaffold. Some of the most studied synthetic materials for porous scaffolds are poly(L-lactic acid) (PLLA), poly(glycolic acid) (PGA) and poly(L-lactide- ϵ -caprolactone) (PCL). PCL porous scaffold has been used as a vehicle of stem cells for directed chondrogenesis, and it was found that chondrogenesis was induced within the scaffold. The result was comparable to that obtained in conventional pellet culture.[54]

To improve biocompatibility with cells, natural biomaterials are commonly applied. As a major component of hyaline cartilage ECM, collagen has been extensively used as the porous scaffold material for cartilage tissue engineering. They present the sites for chondrocyte binding mediated by chondrocyte cell surface receptors, therefore maintaining the characteristic signaling pathway of chondrocytes and their phenotype[55]. Porous scaffolds fabricated from Col I can promote chondrogenesis with seeded foetal bovine epiphyseal chondrocytes, whereas the level of GAG was lower than that in native cartilage.[56] Col II has also been fabricated into porous scaffold to support chondrogenesis.[57]

One problem involved in the application of porous scaffold in cartilage tissue engineering is that cells adhere to the internal surfaces of the pores and tend to elongate. Hydrogel system is more favorable in cartilage tissue engineering in comparison to porous scaffold.

1.2.2.2 Hydrogels

Hydrogels are fabricated by dissolving the hydrophilic substance into water, which then gels under defined conditions, e.g., a certain temperature. Hydrogels have several advantages in cartilage tissue engineering application. Firstly, hydrogels have high water content, which permits efficient exchange of nutrients and wastes with medium. This allows high cell proliferation and more ECM production. Secondly, hydrogel solution is injectable and can be easily shaped and tailored to specific needs. Cell-laden hydrogel can be injected into the injured site, where it forms the gel *in situ*, triggered either by a lower temperature or chemical/photoinitiation crosslinking. This is particularly useful because cartilage defects are usually irregular in shape. Injectability also endows it a minimally invasive practice for the patient. Besides, since hydrogels themselves do not provide sites for cell attachment, they are particularly suitable for the maintenance of chondrocyte morphology and phenotype and therefore cartilage tissue engineering.

Polyethylene glycol (PEG) is a synthetic biomaterial that can be fabricated with ease and crosslinked with rapid photoinitiation.[58] The mechanical strength can be modulated with controlled cross-linking process.[59] It has been shown that PEG hydrogel can promote chondrogenesis. A stratified MSC-laden PEG hydrogel has shown osteo/chondral differentiation after implantation into immunodeficient mice.[60-61]

Agarose, a polysaccharide derived from Asian seaweeds, is a conventional natural biomaterial that is often fabricated into hydrogels to encapsulate cells. It easily gels at room temperature, e.g., the gelation point for 1.5% agarose solution is 26-30°C. Furthermore, when brought to body temperature 37°C, the pre-formed hydrogel does not melt to the liquid state because the melting temperature is 65°C. This property has enabled agarose as a possible vehicle of cells under proper conditions.[62] Agarose hydrogel has been found to maintain chondrocyte phenotype, and the content of agarose in hydrogel was shown to affect the chondrocyte phenotype. Chondrocytes encapsulated in 2% agarose hydrogel were found to produce more Col II than those in 3% agarose, resulting in a stiffer

tissue.[63]. *In vivo* trials have also been conducted with agarose hydrogel with promising results. No immune rejections were observed and hyaline cartilage-like neotissue was formed after implantation into osteochondral defects in rabbits[64].

Alginate, a co-polymer composed of 1,4-linked β - D-mannuronic and α -L-guluronic residues, is derived from brown algae. Bivalent cations, e.g., Ca^{2+} , can bridge the residues and therefore form a network, which enable it to form a gel. Ion exchange with sodium citrate or other mild chelators can easily dissolve the gel and release the cells. This has made it an intelligent material for tissue engineering research and application. Besides, it was found that alginate hydrogel was able to maintain the chondrocytic phenotype and promote the expression of markers of hyaline articular cartilage.[65-68] The ease in manipulation and competency in chondrogenic induction of alginate hydrogel justify its application in cartilage tissue engineering. Therefore, in our research, alginate hydrogel would be selected as the biomaterial to encapsulate the cells and engineer cartilage tissues.

1.2.3 Biochemical factors in cartilage tissue engineering

In the literature currently available, most of the effort in chondrogenic induction applies a recipe of chondrogenic medium (CM) containing dexamethasone (DEX), ascorbate, ITS premix, proline, sodium pyruvate. However, the basic CM is insufficient, and other biochemical factors are usually required for chondrogenesis to guide the stem cells along the chondrocytic lineage and maintain the chondrocytic phenotype. Biochemical stimuli encompass a large family, including growth factors, hormones, cytokines, etc. Many papers have been dedicated to the application of cytokines, transcription factors or even small chemical molecules such as inhibitors for chondrogenic induction and inhibition of side effect. For instance, some of these factors are intended to foster the production of ECM components, while others may help resist inflammatory responses and cell hypertrophy. A summary of these factors can be found in the review contributed by

Steinert *et al.*[69] On top of these biochemical factors, growth factor is an important factor extensively studied for cartilage tissue engineering. Growth factors are typically signaling protein molecules responsible for a series of cellular activities, such as cell growth, proliferation, differentiation, matrix deposition, maturation and apoptosis. Fibroblast growth factors (FGFs)[70], IGF-1[71-72] and TGF- β superfamily have proved to be effective in promoting cartilage repair. Among them, TGF- β superfamily has been investigated most extensively and is the focus of our study. A brief review of TGF- β superfamily in cartilage tissue engineering is provided as below.

TGF- β superfamily is a large family which involves multiple members of growth factors, including TGF- β s, bone morphogenetic proteins (BMPs) and activin. They are categorized under the same TGF- β superfamily due to several reasons. Firstly, they are synthesized via a similar mechanism, involving the cleavage of propeptide, and the mature proteins share a similar dimeric structure. Secondly, these proteins exert their effect through similar signaling pathways, in which type I and type II serine/threonine kinase receptors are involved.

BMPs, including BMP-2, BMP-4, BMP-6, BMP-7 BMP-9 and BMP-13, have been substantiated to induce osteogenesis[73-75]. They have also been implicated in chondrogenic induction. For example, BMP-2 significantly promoted the expression of cartilage markers in SMSCs encapsulated in injectable gellan hydrogels.[76] Adenoviral vector-mediated BMP-13 expression also induced chondrogenic differentiation of murine MSCs, and results showed lower alkaline phosphatase expression and mineralization compared to those transduced with BMP-2-expressing adenoviral vector.[77]

The category of TGF- β s alone contains three subtypes: TGF- β 1, TGF- β 2 and TGF- β 3. In cartilage, TGF- β 1 expression is confined to the proliferative chondrocytes in the upper hypertrophic zones of cartilage.[78] One of its functions is to initiate cell-cell interactions for condensation. As an aftermath, cartilage ECM production can be promoted.[79-80]

Nishimura *et al* found that chondrogenesis could be enticed in pellet culture of SMSCs with high Col II expression by TGF- β 1 supplemented into CM.[48] However, some studies have reported insufficient chondrogenesis with TGF- β 1.[47, 81] A comparison between BMP-2, BMP-7 and TGF- β 1 has concluded a more potent induction of BMP-2 and BMP-7 over TGF- β 1.[82] It was also found that dosing TGF β 1 together with IGF-1 provides better chondrogenic induction against sequential dosing manner and other growth factor combinations investigated in the study by Pei *et al*[49]. TGF- β 2 is ubiquitous in all zones of cartilage, and is implicated in the induction of chondrocyte hypertrophy.[83] On top of the previous two subtypes, TGF- β 3 has been substantiated to maintain the phenotypes of chondrocytes, including their round shape and ability to produce collagen.[84-85] TGF- β 3 has also been proved to induce chondrogenesis with a variety of cell types, including MSCs[86], amniotic fluid-derived stem (AFS) cells[87], periosteum-derived progenitor cells (PDPCs)[88], pericytes and endometrial stromal cells[89-90]. A comparison between TGF- β 1, TGF- β 3 and BMP-2 using SMSCs in gellan hydrogel suggests that TGF- β 3 had a better induction of cartilage markers than others in the relatively short period of 21 days.[76] Therefore, as a potent inducer of chondrogenesis, TGF- β 3 has become the target growth factor that we aim to deliver to the target cells in our studies.

However, a problem incurred by the use of TGF- β 3 is that Col I expression is upregulated.[91-92] The introduction of Col I would alter the composition of ECM and lead to the formation of fibrous cartilage, which has varied mechanical strength different from that of the native hyaline articular cartilage. The capability of cartilage to withstand weight loading might be compromised. The problem is expected to be addressed through silencing of Col I gene, which would be reviewed in detail later.

1.3 Gene delivery in cartilage regeneration

Given that appropriate cell source, biomaterial and biological cues have been selected for

directed chondrogenesis to construct engineered cartilage tissues, researchers have adopted various strategies to provide protein molecules, including TGF- β 1[93], TGF- β 3[94], BMP[95], IGF[96], Sox-9[97], to the cells for chondrogenesis. Conventionally, recombinant proteins such as TGF superfamily are dosed to the cells directly. However, this dosing method has its intrinsic drawbacks. Recombinant proteins usually have a short half life time, rendering them ineffective within a short time period, sometimes even only a few minutes, after being dosed to the cells. This would require repeated administration of the proteins, which is not practical particularly in the cases when the proteins are still needed for functionality after implantation of the cells into the patient. Technological advances have provided a solution to this problem with emerging gene delivery methods. Relevant genes are incorporated into gene vectors and transfected into cells, within which they express the proteins encoded by the inserted genes. This would lead to a more localized and sustained release of the proteins. Conventionally, gene delivery vehicles have been divided into two major categories: non-viral and viral delivery.

Non-viral gene delivery involves using physical forces or chemical agents to transfer the gene of interest, mainly in the form of a plasmid, into the cells. The physical methods include gene gun shooting and electroporation. Examples of chemical agents include liposome[98] and some polymers such as PEG. These chemicals are cationic, which are conducive to the condensation of anionic DNA strands and therefore facilitate gene transfer via cell membrane-associated fusion or endocytosis. As an example, IGF-1 gene has been delivered into articular chondrocytes encapsulated in alginate spheres using a lipid vector Fugene 6, which was expressed within the cells for a period as long as 32 days [99]. However, there are some serious drawbacks of these non-viral delivery methods that greatly undermine their applicability. Most of these methods function in an unnatural manner and pose destruction to the cellular structures and functions. For instance, the liposome would alter the cell membrane structure and therefore change membrane

permeability, resulting in significant cell phenotypic change or even cell death. This is also the case for the physical transfer of genes. Electroporation, for example, brings significant destruction to the cells. Another major drawback is the relatively low transfection efficiency. Only a small portion of the cell population can be successfully transfected, which entails further purification steps for analysis. Although current technologies have brought up the transfection efficiency using non-viral gene delivery methods to high levels, e.g., over 50%, the disadvantages have diverted our attention to a more efficient method-viral delivery.

In comparison with non-viral vectors, viral vectors can greatly improve the transfection efficiency, up to 90%, without obviously affecting cell phenotypes. Transduction efficiency as high as 99% was reported using adenoviral vectors.[100] So far, a few viral vectors have been developed, with increasing efficiency and safety. These vectors include adenoviral vector, retroviral vector, adeno-associated viral vector and lentiviral vector. Among them, adenoviral and lentiviral vectors have been most prevalently studied due to good understanding of the structures and functions of adenoviral and lentiviral vectors. They would be the focus of our study and are briefly reviewed below.

1.3.1 Adenoviral vector

Adenoviral vector is non-enveloped and icosahedral in morphology. Hexons and pentons are the primary units of adenoviral capsid, with a trimeric glycoprotein fiber protruding from each penton. This acts as a binding site for cell membrane receptors and facilitates adenoviral internalization and infection. Following internalization, the viral genome, which is double-stranded DNA, is released from the endocytotic vesicles into cell nucleus, where it replicates and initiates the production of capsid proteins for the assembly of progeny virions. It performs in an episomal manner, whereby it does not integrate into the host genome and functions independently. The adenoviral genome comprises early (E) and late

regions (L), flanked by two inverted terminal repeats (ITRs). Adenoviral vectors are usually engineered with deleted E1 and/or E3 regions, which are implicated in viral replication. This has conferred replication deficiency to the adenoviral vectors. The regions can be complemented by 293 cells, within which adenoviral vectors are mass produced, whilst in other cells, adenoviral vectors do not replicate with each cycle of cell replication. The above features of commercially available adenoviral vectors have determined that only a transient expression of the inserted gene is permitted.

1.3.2 Lentiviral vector

Lentiviruses belong to the retrovirus family. With a particle size ranging between 80 and 120 nm in diameter, they have an envelope structure surrounding the capsid, which is usually derived from the host cells when they are released from the cells. The genome of lentiviruses is composed of diploid RNA molecules, which are linked with viral replication enzymes. The genome consists of several encoding domains, including *gag*, *pol* and *env*, which are responsible for the production of the proteins involved in viral replication, integration and envelope. Other genes such as *tat* and *rev* exist as regulatory domains. The encoding domains are flanked by long terminal repeats (LTRs), namely, 5'-LTR and 3'-LTR, which are implicated in transcription initiation and viral integration. After infection, genomic RNA contents are transported into host cell nucleus, where they were reverse transcribed into double-stranded DNA and integrated into the host genome with the aid of integrase encoded by the lentiviral genome. New RNA copies are generated through transcription of the DNA molecules, and are encapsulated into capsid by the proteins encoded by the genome. A layer of envelope is added on as an exterior coat while lentiviral capsid particles protrude from the host cell membrane.

Similar to adenoviral vector, lentiviral vectors also share the advantage of high transduction efficiency and the ability to infect both non-dividing and dividing cells. One

distinctive feature of lentiviral vector is the persistent expression of transgene as compared to the transient expression induced by adenoviral vectors. As they are capable of integrating into the host cell genome, the lentiviral genome would replicate along with the host cell genome, resulting in a persistent existence of the viral genome and therefore a more sustained release of the gene inserted into the lentiviral vector compared with adenoviral vectors.

Table 1.1 gives some examples of the existing application of either non-viral or viral delivery of various growth factors to different types of cells. Chondrogenesis has been observed to occur in all these examples. Among these cases, liposome-mediated transfection and adenoviral vector-mediated transduction only induce a transient expression of the transgene, while transduction mediated by retroviral vector leads to a more sustained expression and release of the growth factor encoded by the transgene, due to the insertional characteristics of retroviral vectors. Since lentivirus has some additional genes that regulate synthesis and processing viral RNA and other replicative functions (e.g., *vif*, *vpr*, *vpu*, *tat*, *rev*, *nef*), lentiviral vectors are preferred for long-term expression of transgene. In our study, we will construct both recombinant adenoviral and lentiviral vectors encoding TGF- β 3 to investigate the effect of various delivery modes on chondrogenesis.

Table 1.2 Some examples of the *in vivo* or *ex vivo* applications whereby articular cartilage defects are regenerated using various gene delivery methods.

Gene	Vector	Cells
TGF- β [27]	Retrovirus	NIH3T3
IGF-1[99]	Liposome	MSC
BMP-2[101]	Adenovirus	Perichondrial cells

FGF-2[102]	Liposome	Chondrocytes
BMP-7[103]	Adenovirus	Chondrocytes

1.3.3 Advantages of articular cartilage tissue engineering over other therapies

There are several advantages of cartilage tissue engineering over other therapies such as direct dosing of therapeutic molecules and transgene vectors. These advantages are inherent in the *ex vivo* practice applied in tissue engineering. In this practice, cells are harvested either from the patient or donors, subjected to various treatments for cell fate reprogramming, and then transplanted back into the patient for regeneration. The treatments applied to the cells, which would otherwise be directly dosed to the patient, might be physical stimuli, chemical/biological molecules or gene delivery vectors- either plasmid or viral/nonviral vectors. These treatments, although effective in some aspects, may exert side effects to the cells to some extent, causing cell phenotype changes, carcinogenesis and even cell death. Viral vectors, especially retroviral and lentiviral vectors, could integrate their genome randomly into host cell genome and cause mutagenesis in the cells. However, since the *ex vivo* practice involves the application of treatments such as chemicals and viral vectors *in vitro*, it eliminates the direct contact of those treatments with the patient and effectively minimizes the systemic risks imposed by those treatments on the patient as a whole, compared to direct administration of those treatments to the patient. Besides, this practice enables the screening of the mutant cells before they are transplanted into the patient and thus minimizes the possible side effect exerted by these nocuous cells. Cells can be screened *in vitro* so that mutant cells would be selected and discarded to avoid complications. Moreover, the biomaterials in use will constrain cell movement and fix the regenerative cells at the injured sites by direct transplantation. In case of any malignancy that occurs with the transplanted cells, the anomaly can be strictly confined to the transplantation site and will not proliferate to other

tissues and exert systemic effect to the whole organism.

1.4 RNAi concept and application in cartilage regeneration

During chondrocyte amplification in monolayer culture for tissue engineering, Col I expression is upregulated.[104] For SMSCs, Col I is inherently expressed even in primary SMSCs.[105] The situation is further complicated with the introduction of TGF- β 3 into the cells.[91] Since Col I will result in fibrocartilage formation and is unwanted in cartilage ECM, we hereby apply RNAi technology to downregulate or silence its expression during chondrogenesis.

RNAi has been accepted as a potential therapeutics for the treatment of the diseases related to genetic disorders and cancers by directed cleavage and degradation of target mRNA with small interfering RNA (siRNA) molecules. The strategy was developed following the discovery in 1998 that double-stranded RNA can bind to the homologous site of mRNA and initiate mRNA degradation. The fundamental idea of RNAi is to introduce double-stranded siRNA with a length of 19-21 basepairs, into the cells. siRNA then binds to the complementary sequence in the mRNA molecule with the aid of RNA-induced silencing complex (RISC), which cleaves the corresponding sequence accordingly. RISC can be recycled for further cleavage.[106-107] The approach can effectively knock down the expression of related genes at the mRNA level, therefore providing a potent tool both in biological research and gene therapy for the gene-related diseases.

siRNA can be delivered into cells either directly or indirectly. Direct delivery methods include the use of such vectors as liposome and quantum dots [108]. However, these vectors suffer a few drawbacks. They damage the cells by permeabilizing the cell membrane, and transfection efficiency is low. Besides, they merely temporarily induce the silencing effect, without a sustained effect. Moreover, stability of siRNA also poses a challenge, due to the degradation of siRNA by endosomes. Therefore, in indirect delivery, plasmid and

viral vectors have been designed to accommodate short hairpin RNA (shRNA), which consists of the sense strand and antisense strand of siRNA to form a stem-and-loop structure. shRNA is cleaved by the enzyme Dicer into siRNA, which then implements the silencing function in a manner as mentioned above.[109] These vectors use endogenously-produced siRNA to knock down certain gene expression continuously, and induce a more sustained blocking effect. Recombinant adenoviral, retroviral, adeno-associated viral and herpes viral vectors have been used in siRNA delivery due to their high delivery efficiency.

In regenerative medicine research, RNAi strategy has been utilized for the treatment of a variety of disorders, including anti-apoptosis[110], RA[111], osteogenesis imperfect[112], cardiovascular degeneration[113] and neural degeneration such as Parkinson's disease[114]. For example, tumor necrosis factor- α (TNF- α)[115], nuclear factor- κ B (NF- κ B)[116], matrix metalloproteinase-1 (MMP-1)[117] and Cathepsin L (CL)[118] have been the targets for the treatment of RA, a cartilage-related disorder, using RNAi strategy. For the therapy of another major disease related to cartilage degeneration-OA, the fundamental task is to promote chondrocyte proliferation and cartilage ECM formation. To meet this end, multiple growth factors, e.g., TGF- β 3, have been delivered to target cells, either chondrocytes or progenitor/stem cells. However, the inherent expression of Col I in SMSCs or upregulation of Col I elicited by TGF- β 3 in the target cells is disfavored in chondrogenesis, as it would alter ECM composition and result in the formation of fibrocartilage, one type of cartilage that is distinct from hyaline articular cartilage in terms of their composition and physical properties.[119] This would cause failure of the fabricated cartilage tissue to withstand the normal load that is frequently imposed to cartilage, and thus cartilage function will not be fully implemented, leading to physical impediment and disability. For this reason, RNAi was applied in our study in conjunction with viral delivery of TGF- β 3 to downregulate the expression of Col I during chondrogenesis. shRNA sequence was designed according to specific siRNA sequence

corresponding to a segment of Col I mRNA. The DNA sequence corresponding to shRNA was recombined into viral vectors to mediate Col I knockdown in the target cells after viral transduction. From this practice, we expect to engineer a cartilage tissue that has minimal Col I within ECM.

1.5 Objective and outline of this thesis

The overall objective of this thesis is induce Col I-suppressed chondrogenesis for articular cartilage regeneration. For this purpose, biochemical factors delivered locally with sustained release are required to induce chondrogenesis; simultaneously Col I, either intrinsically produced by the cells or elevated by monolayer culture or TGF- β 3, is expected to be downregulated. The three key elements in tissue engineering, including cell source, scaffold and biochemical stimuli have been selected. SMSCs would be the major cell source in our studies due to their easy access, high proliferative capacity and relative ease in reprogramming into chondrocytic phenotype. Chondrocytes would also be tested since they are the principal type of cells in articular cartilage. Alginate would be used as 3D hydrogel scaffold for their compatibility in chondrogenesis and easy manipulation for research. TGF- β 3, a potent growth factor in chondrogenic induction, would be used as biochemical stimuli to induce chondrogenesis via genetic approach to provide a sustained and localized release. In the mean time, RNAi technology would be introduced to downregulate Col I expression that is intrinsic of the target cells or elevated by TGF- β 3. Both adenoviral and lentiviral vectors would be used to investigate their temporal effect on Col I-suppression and chondrogenesis. To meet our objective, we would incorporate TGF- β 3 and/or Col I-targeting shRNA encoding sequences into either adenoviral vector or lentiviral vector, and delivered TGF- β 3 and Col I-targeting siRNA into SMSCs or chondrocytes encapsulated in 3D alginate hydrogel to guide Col I-suppressed chondrogenesis. The two functions of TGF- β 3 expression and shRNA encoding could be

accommodated into either one dual-functioning viral vector or single-functioning viral vectors separately. Therefore, the two functions could be implemented either by one dual-functioning vector or a mixture of two single-functioning vectors (which have different functions of expressing either TGF- β 3 or Col I-targeting shRNA). Possible combinations of viral vectors to fulfill the two functions would be studied to find out their effect on Col I-suppressed chondrogenesis.

The dissertation is composed of 7 chapters, as briefly outlined below:

Chapter 1 gives a comprehensive introduction to cartilage regeneration, the elements of cartilage tissue engineering (including cell sources, biomaterials and biochemical factors), gene delivery methods and RNAi, based on which choices were made in terms of source cells, materials, delivery vehicles, etc. The objective and the outline are also provided herewith.

Chapter 2 presents the construction of a dual-functioning adenoviral vector to deliver both TGF- β 3 and Col I-targeting shRNA, whose optimal transduction conditions and efficacy in Col I inhibition and TGF- β 3 release would be tested in fibroblasts, osteoblasts, chondrocytes and SMSCs in monolayer culture.

Chapter 3 introduces steps of constructing a dual-functioning lentiviral vector. Based on this lentiviral vector, protocols for lentiviral titration, transduction and quantification of TGF- β 3 in the active form with modified ELISA would be specifically formulated and provided. Optimal medium would also be selected for the culture of transduced SMSCs.

Chapter 4 specifies the construction of a series of four dual-functioning lentiviral vectors and their efficiency in Col I downregulation and chondrogenic induction in SMSCs cultured in 3D alginate hydrogel would be compared.

Chapter 5 gives information of the efficiency of various combinations of the single-functioning adenoviral/lentiviral vectors for the delivery of both TGF- β 3 and Col I-targeting shRNA to SMSCs cultured in 3D alginate hydrogel.

Chapter 6 would extend the application of lentiviral vectors to chondrocytes, to determine whether redifferentiation could be initiated with TGF- β 3-expressing lentiviral vectors in chondrocytes after 2D monolayer expansion.

Chapter 7 would make conclusions to the above findings, and propose some future works.

Chapter 2 A dual-functioning adenoviral vector encoding both TGF- β 3 and shRNA silencing Col I: construction and controlled release for chondrogenesis

2.1 Introduction

As introduced in the previous chapter, our aim is to induce Col I-suppressed chondrogenesis for articular cartilage regeneration. To this end, TGF- β 3 would be delivered to either SMSCs or dedifferentiated chondrocytes to drive chondrogenesis in alginate hydrogel system. Simultaneously, as Col I is intrinsically expressed in SMSCs and upregulated with chondrocyte monolayer culture or by TGF- β 3, RNAi technology would be applied to suppress Col I expression. The two functions would be fulfilled efficiently with viral delivery by incorporating either one expression cassette or both cassettes (TGF- β 3 expression cassette and Col I-targeting shRNA expression cassette) into adenoviral or lentiviral vectors.

As no viral vector is commercially available that can accommodate both TGF- β 3 expression cassette for gene overexpression and Col I-targeting shRNA expression cassette for downregulation of gene expression, it poses a challenge for us to construct a recombinant dual-functioning adenoviral or lentiviral vector. In this chapter, adenoviral vector [120-121] was selected as a model recombinant dual-functioning adenoviral vector (Ad-D) for investigation in order to achieve the above two objectives simultaneously. It also serves as a model and template for the construction of dual-functioning lentiviral vectors (LV-D) in subsequent chapters.

Ad-D was constructed by inserting TGF- β 3 encoding sequence, U6 promoter sequence (a promoter that drives small RNA expression) and Col I-targeting shRNA encoding sequence into the multiple cloning site (MCS) of the plasmid. To minimize the interference between the two expression cassettes, shRNA expression cassette was inserted in the

reverse direction opposed to that of TGF- β 3 expression cassette. Quantitative real-time PCR (qPCR) and flow cytometry have revealed the effect of various MOIs on cell viability, infection efficiency, Col I inhibition and TGF- β 3 expression in three model cell types: fibroblast, osteoblast and chondrocyte, since all the types of cells express Col I. Appropriate MOIs for each type of cells were selected accordingly for subsequent investigation. qPCR, ELISA assay and immunofluorescent staining were used to evaluate the release of TGF- β 3 and downregulation of Col I expression. Besides, as SMSCs are an important source for articular chondrogenesis, we also carried out preliminary experiments to test the effect of recombinant adenoviral vectors at various MOIs in SMSCs.

2.2 Materials and methods

Construction and production of null and all other recombinant adenoviral vector

Schematic diagrams of various recombinant adenoviral vectors were given in Figure 2.1, and their construction mechanisms and procedures were shown in Figure 2.2. These adenoviral vectors include null adenoviral vector (Ad-N), recombinant adenoviral vector that specifically expresses Col I-targeting shRNA (Ad-sh), recombinant adenoviral vector that specifically expresses TGF- β 3 (Ad-T) and recombinant adenoviral vector that encodes both Col I-targeting shRNA and TGF- β 3 (Ad-D).

Ad-sh was produced using Adeno-X ViraTrak ZsGreen1 Promoterless Expression System 2 (Clontech Laboratories, Mountain View, CA, USA) by incorporating annealed shRNA-encoding double-stranded sequence with BamHI and EcoRI overhangs at the two ends (Sense:5'-GATCCGCAATCACCTGCGTACAGAATTCAAGAGATTCTGTACG-CAGGTGATTGTTTTTACGCGTG-3'; Antisense:5'-AATTCACGCGTAAAAAACA-ATCACCTGCG-TACAGAATCTCTTGAATTCTGTACGCAGGTGATTGCG-3') into pSIREN-DNR vector. Recombinant pSIREN-DNR vector was then recombined with pLP-Adeno-X-PRLS-ES-ZsGreen1 vector to produce recombinant adenoviral plasmid.

The other two recombinant adenoviral vectors (Ad-T and Ad-D) were produced using Adeno-X ViraTrak ZsGreen1 Expression System 2 (Clontech Laboratories, Mountain View, CA, USA). Ad-T was constructed by incorporating Sall/PstI-digested TGF- β 3 sequence (amplified from hTGF- β 3-pCMV6-XL5 plasmid DNA (Origene Technologies, Rockville, MD, USA)) into donor vector pDNR. [14] Ad-D was constructed by sequentially incorporating the following fragments into pDNR: Sall/PstI-digested TGF- β 3 coding sequence amplified from hTGF- β 3-pCMV6-XL5 plasmid DNA (Origene Technologies, Rockville, MD, USA) (Forward primer:ACGCGTCGACATGAAGATGCACTT; reverse:TGCACTGCAGTCAGCTACATTTTC), ApaI/XhoI-digested human U6 promoter amplified from human fibroblast genome (Primers forward:ATTTGCGGGCCCGCAGG AAGAGGGCCTAT;reverse:CCGCTCGAGTCGTCCTTTCCACAAG), and annealed shRNA-encoding double-stranded sequence with XhoI and PstI overhangs at the two ends (Sense:TCGAGCAATCACCTGCGTACAGAATTCAAGAGATTCTGTACGCAGGTGAT TGTTTTTTACGCGTCTGCA;antisense:GACGCGTAAAAACAATCACCTGCGTACA GAATCTCTTGAATTCTGTACGCAGGTGATTGC). Recombinant pDNR plasmid and adenoviral vector pLP-Adeno-X-CMV-E3-ZsGreen1 were recombined with Cre recombinase to produce recombinant adenoviral plasmids.

All the above recombinant adenoviral plasmids were amplified in DH10 β , electrocompetent *Escherichia coli* cells (New England Biolabs, Beverly, MA, USA) and purified with QIAfilter plasmid extraction kit (Qiagen, Hilden, Germany) The purified recombinant adenoviral plasmids were linearized by PacI digestion and transfected into HEK 293 cells (ATCC, Manassas, VA) using Lipofectamine reagents (Invitrogen, Carlsbad, CA, USA) for viral packaging. The packaged adenoviral vector was then purified using Adeno-XTM Virus Purification Kit (Clontech Laboratories, Mountain View, CA, USA) and titrated using Adeno-XTM Rapid Titer Kit (Clontech Laboratories, Mountain View, CA, USA) for subsequent experiments.

Figure 2.1 Schematic diagrams of the produced adenoviral vectors. These vectors include Ad-N, Ad-sh, Ad-T and Ad-D.

Figure 2.2 Constructing recombinant adenovirus with Creator technology. DNA sequences are rapidly transferred from pDNR-CMV to pLP-Adeno-X-CMV-E3-ZsGreen-Express using Cre-loxP recombination. Donor Vectors contain two loxP sites and Acceptor Vectors contain one loxP site. Once the Cre recombinase binds, it cleaves the DNA in the loxP spacer region, and then reattaches the 5' end to the 3' end of another loxP site having the same orientation. Multiple reactions between the two sites in the Donor Vector and the

single loxP site in the Acceptor Vector occur simultaneously, resulting in the transfer of gene of interest (GOI) and the chloramphenicol resistance gene into the Acceptor Vector. Products of recombination between or within individual Donor Vectors are eliminated in the next step, when transformants are plated on LB/Cm/sucrose. Donor Vectors and their derivatives all encode SacB, whose product is lethal to *E. coli* grown on sucrose-containing media. (Reproduced from Clontech)

Cell isolation and culture

Human fibroblast and human fetal osteoblast were purchased from American Type Culture Collections (ATCC, Manassas, VA, USA). Porcine chondrocyte and SMSCs were isolated according to previously published papers. [122-123] Briefly, porcine cartilage tissue was obtained from the joint and cut into small pieces. The cartilage pieces were incubated in DMEM culture medium supplemented with 10% (v/v) fetal bovine serum (FBS) and 1 mg/mL collagenase type II at 37 °C for 12 hours. Chondrocytes were centrifuged and resuspended in high glucose DMEM supplemented with 20% (v/v) FBS, 0.1 mM nonessential amino acids (NEAA), 0.01 M 4-(2-hydroxyethyl)-piperazine-1-ethanesulfonic acid (HEPES), 0.05 mg/mL vitamin C, 0.4 mM proline, 100 mg/mL streptomycin and 100 units/mL penicillin. Cells were plated in culture flasks at a density of around 2×10^4 cells/cm² and incubated in humidified air with 5% CO₂ at 37 °C.

For SMSCs, synovial membrane tissues were harvested aseptically from knee joint of mature swine, washed with phosphate buffered saline (PBS) supplemented with penicillin/streptomycin three times and minced. After digestion with 0.1% collagenase II, cells were plated in high-glucose DMEM supplemented with 10% FBS, 100 µg/mL streptomycin and 100 units/mL penicillin. Medium was replaced every 3 days. Cells were subcultured at 1:3 dilution ratio when cultures reached 90% confluence.

The above cell culture-related reagents were purchased from Gibco (Invitrogen,

Singapore).

Cell transduction

The cells were cultured and seeded in 24-well or 6-well plates at a density of 5×10^4 cells/cm². The next day recombinant adenoviral vectors were diluted in DMEM or DMEM/F12 medium (for osteoblast culture, Invitrogen, Carlsbad, CA, USA) without FBS and added to the cells at certain MOIs. Two hours later, the medium was removed and replaced with fresh medium. Cells without transduction were used as negative controls (Neg).

Flow cytometry analysis

72 hours after infection, cells were trypsinized, washed with PBS and stained with propidium iodide (50 µg/mL, BD, Heidelberg, Germany). The cells were then subjected to FACSCalibur flow cytometer (BD, Heidelberg, Germany) to determine the percentages of dead cells (stained in red), infected cells (with green fluorescence emission) and viable infected cells. 5 000 cells were counted in each measurement.

Quantitative real-time PCR (qPCR)

72 hours after infection with various recombinant adenoviral vectors, cells were lysed and total RNA was isolated using TRIzol kit (Invitrogen, Carlsbad, CA, USA). For cDNA synthesis, 1 µg of total RNA was reverse transcribed using Superscript® First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA). qPCR for Col I and TGF-β3 was performed using IQ™ SYBR Green Supermix system (Bio-Rad, Hercules, CA, USA) according to the manual. The reaction conditions were as follows: initial denaturation at 95°C for 3 min, followed by 40 cycles of denaturation at 95°C for 30 sec, annealing at 53°C for 30 sec, and extension at 72°C for 30 sec. Human β-actin and porcine RPL4 were

used for normalization, respectively. The relative gene expression values were calculated with the comparative $\Delta\Delta C_T$ (threshold cycle) method. Sequences of all the primers (including those to be used in the following chapters) are listed in Table 2.1.

Table 2.1 Primers for qPCR. AT: annealing temperature (°C).

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	AT
Col I	CCTGCGTGTACCCCACTCA	ACCAGACATGCCTCTTGTCTT	58
TGF- β 3	5'-GCGGAGCACAACGAACTG-3'	CTGCTCATTCCGCTTAGAG	58
Col II	GCTATGGAGATGACAACCTGGCTC	ACAACGATGGCTGTCCCTCA	58
Col X	CAGGTACCAGAGGTCCCATC	CATTGAGGCCCTTAGTTGCT	58
Comp	GGCACATTCCACGTGAACA	GGTTTGCCTGCCAGTATGTC	58
Aggrecan	CGAGGAGCAGGAGTTTGTCAAC	ATCATCACCACGCAGTCCTCTC	58
RPL4	CAAGAGTAACTACAACCTTC	GAACTCTACGATGAATCTTC	58
Human	CCTGGCACCCAGCACAAAT	GGGCCGGACTCGTCATACT	58
β -actin			

Immunofluorescent staining of Col I

72 hours after transduction, immunofluorescent staining was carried out to quantitate Col I protein. Briefly, the monolayer cells were fixed with 2.5% glutaraldehyde at room temperature for 30min, followed by three PBS washes. The cells were then blocked by 10% goat serum, followed by incubation with mouse anti-pig Col I primary antibody (1:200, Santa Cruz Biotechnology, Santa Cruz, CA, USA) at room temperature for 1hr. Afterwards AlexaFluor 546 rabbit anti-mouse IgG (1:500, Invitrogen, Carlsbad, CA, USA) was added to the cells and the cells were incubated at 37°C for 1hr. Finally, DAPI (1ng/ml, dilute 1000 times) was added to the cells at 37°C for 5min. After three washes with PBS, the cells were observed under fluorescent microscope. Infinite 200 fluorescent microplate

reader (TECAN Systems, San Jose, CA, USA) was used to determine the fluorescent density in each well, with the excitation wave length of 560nm and emission wave length of 590nm. Immunostaining without cells was used as blank. Data were expressed as mean $\pm$ SEM (standard error of mean).

Quantitative analysis of TGF- β 3 expression by specific ELISA

Conditioned medium for the cells transduced with Ad-D at both MOI 200 and 500 was collected at Day 2, 4 and 6 and stored at -80°C for further analysis by specific ELISA (R&D Systems, Minneapolis, MN). Triplicates were performed for each time point. Briefly, a 96-well microplate was coated with capture antibody overnight. Samples were activated with hydrochloric acid and, together with two-fold serially diluted standards, added into the 96-well plate, followed by incubation for 2 hrs at room temperature. After 3 washes with PBS+0.05% Tween, the microplate was incubated with detection antibody, streptavidin-HRP, and substrate solution sequentially. The absorbance in each well was determined using a microplate reader at a wavelength of 450 nm, and the concentration of TGF- β 3 was calculated automatically against the standard curve.

2.3 Results

Optimal MOI of Ad-D in human fibroblast, human foetal osteoblast and porcine chondrocyte

Flow cytometry was carried out to determine the appropriate MOI for Ad-D in each of the three types of cells: fibroblast, osteoblast and chondrocyte (Figure 2.3.A-C). According to the charts, there was no significant difference between the groups of all three cell types in terms of cell viability from MOI 0 to 500, since the percentages of dead cells remained at a relatively stable level, with or without transduction. The percentages of infected cells and viable infected cells, for fibroblast and osteoblast, increased significantly from MOI 0 to

50, and then remained relatively stable for the following MOIs of 100, 200, with transduction efficiency falling between 80% to 90% and the percentage of viable infected cells falling between 70% to 80%. For porcine chondrocyte, these two values increased significantly from MOI 0 to MOI 500, reaching as high as 90% for transduction efficiency and 80% for viable infected cells.

qPCR was also performed to determine the suitable MOI in terms of Col I inhibition efficiency and TGF- β 3 expression efficiency at mRNA levels, as an indicator of the amount of released TGF- β 3 and shRNA. From the results as shown in Figure 2.3 D-F, for the three types of cells, Ad-D inhibited the expression of Col I in an MOI-dependent manner. As MOI increased till 100, Col I mRNA level tended to decline remarkably, while from MOI 100 till 500, the inhibition efficiency did not exhibit significant disparities.

On the other hand, with the increase in MOI from 0 to 100, TGF- β 3 mRNA level increased significantly (Figure 2.3 G-I). Particularly, TGF- β 3 was much more highly transcribed at MOI 500 than that at MOI 200, with a fold increase of up to 4.7 for fibroblast, 3 for osteoblast, 2 for porcine chondrocyte.

Taking all of the above results into account, MOI 200 and 500 were selected for comparison in the following experiments to characterize the functions of recombinant adenoviral vectors in fibroblast, osteoblast and chondrocyte. Release profiles of both TGF- β 3 and shRNA can be manipulated by the amount of adenoviral vectors to be transduced.

Figure 2.3 The effect of various Ad-D MOIs on cell viability, infection efficiency, number of viable infected cells as well as Col I and TGF-β3 mRNA levels. Panels A-C represent the cell viability, infection efficiency, number of viable infected cells at different MOIs for A) Human fibroblast, B) human foetal osteoblast and C) porcine chondrocyte. Panels D-F and G-I give Col I (D,E,F) and TGF-β3 (G,H,I) mRNA levels at various MOIs in fibroblast (D,G), osteoblast (E,H) and porcine chondrocyte (F,I) obtained from qPCR.

Col I inhibition at mRNA and protein levels with various adenoviral vectors in 2D culture

Each type of cells was transduced with either of the four adenoviral vectors: Ad-N, Ad-sh, Ad-T and Ad-D, and qPCR was performed to estimate Col I mRNA levels in various groups. (Figure 2.4 A-C) From the results, it was found that the expression of Col I was increased upon infection with Ad-N, which was more apparent at MOI 500 in fibroblast

and osteoblast. Col I mRNA level was effectively reduced by the infection with Ad-sh, particularly in fibroblast and osteoblast. In comparison, Ad-T had a diversified effect on the expression of Col I. In fibroblast, Col I was remarkably increased by the infection with Ad-T, while in osteoblast, Ad-T tended to decrease its production. In porcine chondrocyte under 2D culture, Col I mRNA level was not significantly affected by the infection with Ad-T. For Ad-D, Col I expression at mRNA level was knocked down compared to either the Neg group or the group transduced with Ad-N.

Cells transduced with various adenoviral vectors at MOI 500 were stained with fluoro-conjugated antibodies against Col I and the fluorescence density was quantified using fluorescent microplate reader. (Figure 2.4. D-F). In all the three types of cells, Ad-D decreased the production of Col I protein to a certain level, which was comparable to the effect of Ad-sh. The images of the stained cells in the Neg group and Ad-D group are shown in Figure 2.4. G-I. Red fluorescence intensity in Ad-D group as shown in the right picture of each panel was found to be greatly reduced compared to that in the Neg group as shown in the left picture.

Figure 2.4 Quantification of Col I and TGF-β3 in three cell types under 2D culture.

Col I mRNA levels relative to respective housekeeping genes by qPCR (A,B,C) and protein (D,E,F) levels with pictures (G,H,I. Left panel: Neg; Right panel: Ad-D) by immunofluorescent staining were obtained 3 days after transduction with various recombinant adenoviral vectors. Panels J-L show the concentration of released TGF-β3 in culture media from different cells transduced with Ad-D at MOI 200 and 500. A,D,G,J human fibroblast; B,E,H,K human fetal osteoblast; C,F,I,L porcine chondrocyte.

TGF- β 3 quantification

By ELISA, TGF- β 3 release curves from the various types of cells under 2D culture at both MOI 200 and 500 were obtained (Figure 2.4. J-L). In the short term from transduction to day 6, TGF- β 3 protein levels tended to increase in fibroblast and porcine chondrocyte, except that there was a slight decline for fibroblast at MOI 500. In fibroblast, TGF- β 3 protein level reached as high as 15 ng/mL in medium at day 4 at MOI 500, while in porcine chondrocyte, the value is much lower at approximately 2ng/mL in medium at day 6 at MOI 500. In osteoblast, TGF- β 3 protein level increased till day 4, and then started to decline. The peak value reached about 4.5ng/mL in medium. It was also found that TGF- β 3 protein level at MOI 500 was much higher than that at MOI 200 in both fibroblast and porcine chondrocyte. However, TGF- β 3 protein level in osteoblast remained almost unchanged at both MOI 200 and 500.

Functions of Ad-D in SMSCs at various MOIs

Since SMSCs are a major target of Ad-D for therapeutic application, we also carried out preliminary studies on the functions of Ad-D in SMSCs and investigated the effect of various MOIs on Col I inhibition and TGF- β 3 expression. Since the studies were carried out in two separate sets of experiments, the MOIs used in various assays may differ. However, they still present a general trend and show the effect of Ad-D in SMSCs.

According to Figure 2.5.A, the percentage of dead cells remained relatively stable from MOI 10 to 500, whereas transduction at MOI 1000 resulted in almost double cell death compared to other groups. Infection efficiency and the percentage of viable infected cells were not significantly changed from MOI 100 to 1000. Over 95% of SMSCs were infected at MOI 200 and 500, and the percentage of viable infected cells reached around 90%.

From qPCR result shown in Figure 2.5.B, Ad-D can also reduce the transcription of Col

I in SMSCs in an MOI-dependent manner. The amount of Col I mRNA was significantly decreased at MOI 100 or higher MOIs. No remarkable difference existed among MOI 100, 200 and 500. However, Col I mRNA amount was further reduced at MOI 1000 compared to that at MOI 100, 200 and 500. The fluorescence intensity of immunofluorescently-stained Col I in SMSCs at various MOIs also followed such a trend, with decreasing intensity at higher MOIs. (Figure 2.5.C) In Figure 2.5.D, as MOI increased, fewer and fewer red dots were visible, indicating the decreased Col I secretion at higher MOIs.

Contrary to the trend in Col I production, TGF- β 3 was greatly increased with the increase of MOI, both at mRNA and protein level, as assessed by qPCR (Figure 2.5.E) and ELISA (Figure 2.5.F). The amount of TGF- β 3 at MOI 500 was 3.5 times of the amount at MOI 200. And the amount at MOI 1000 was even higher, reaching over 5ng/mL in medium.

Figure 2.5 The effect of various MOIs on viability, infection efficiency, number of viable infected cells as well as Col I and TGF- β 3 mRNA and protein levels in SMSCs. Graph A represents the cell viability, infection efficiency, number of viable infected cells at different MOIs. Graph B shows Col I mRNA levels at various MOIs obtained from qPCR. Fluorescence intensity and pictures at various MOIs after staining with Col I antibodies and DAPI are given in Graphs C and D. Graphs E and F demonstrate TGF- β 3 mRNA levels relative to housekeeping genes and protein levels 3 days after infection at various MOIs obtained from qPCR and ELISA. Values at X-axis denote MOI.

2.4 Discussion

TGF- β 3 has been substantiated to promote chondrocytic differentiation of stem or

progenitor cells, and we thus aim to deliver TGF- β 3 gene to cells to induce chondrogenesis. On the other hand, Col I is disfavored by hyaline articular cartilage, whereas the isolated chondrocytes or adult stem cells such as SMSCs express this protein, and the level could even be elevated when treated by TGF- β 3. Hence, inhibition of Col I is also our concern. In this study, we aim to integrate the two functions into one adenoviral vector by accommodating the two expression cassettes, TGF- β 3 encoding cassette and shRNA-encoding cassette, in the same vector. By inserting the three components - TGF- β 3 encoding sequence, U6 promoter and Col I-targeting shRNA-encoding sequence, the recombinant dual-functioning adenoviral vector were successfully constructed, as assessed by enzymatic digestion and DNA sequencing. The recombinant adenoviral vectors were subjected to characterization methods such as qPCR, ELISA and immunofluorescent staining to assess their functions in the cells. Human fibroblast, human fetal osteoblast and porcine chondrocyte all express Col I and were therefore used as model cells to test the efficiency of recombinant lentiviral vectors. SMSCs, as a promising source of cells for chondrogenesis, were also used in the preliminary study to investigate the functions of Ad-D.

For viral application, optimal MOI is an essential parameter and needs to be determined for individual type of cells. Flow cytometry was performed to assess the effect of various MOIs on cell viability and transduction efficiency. At appropriate MOI, a large majority, if not all, of the cells should be infected with the viral vector. At various MOIs the percentages of dead cells did not change significantly, indicating that the recombinant dual-functioning adenoviral vector did not affect cell viability significantly. Transduction efficiency reached up to 80-90%, while the viable infected cells accounted for 70-80% of total at both MOI 200 and 500. The percentages are high enough for application or cell sorting. qPCR for TGF- β 3 and Col I mRNA levels was also performed. No significant difference was found between MOI 200 and 500 in terms of Col I mRNA level, whereas

TGF- β 3 mRNA level was much higher at MOI 500. Therefore, MOI 500 seems to be optimal for all the types of cells. However, for discretion, we tried both MOI 200 and 500 in the following experiments. If the adenoviral vector performs well at MOI 200 which is comparable MOI 500, we may as well reduce its amount of usage to MOI 200 in order to minimize its usage and possible side effect, rather than simply applying MOI 500

For Col I inhibition efficiency, we used the single-functioning adenoviral vectors (Ad-sh and Ad-T) as well as Ad-N to compare their effect on Col I expression. It was found that even Ad-N can raise the expression of Col I mRNA to some extent, which was consistent with a study reporting that malignant rabbit fibroma virus (MV) was able to elicit the increase of Col I mRNA level *in vitro*. [124] It is to our expectation that Ad-sh showed strong inhibitive effect on Col I expression. However, Ad-T seemed to have diversified effects depending on cell types. It raised Col I mRNA level in fibroblast while reducing mRNA level in osteoblast. In porcine chondrocyte, Ad-T did not exert remarkable effect. This discovery differs from previous studies, which showed that TGF- β 3 could decrease Col I amount and improves scar-free wound healing. [125] Comparatively, Ad-D consistently reduced Col I mRNA level significantly compared to either the Neg control or Ad-N group especially in fibroblast and osteoblast at both mRNA and protein levels.

It should be noted that expression of Col I is only partially blocked. Since adenoviral vector works in an episomal fashion, the expression of delivered genes will be attenuated after each cycle of cell replication. Hence, the function of Col I inhibition might be undermined after a certain period of time. Improvement can be made by applying the strategies in this model dual-functioning adenoviral vector to a dual-functioning retroviral vector, which is integrative and thus potentially improves the Col I blocking efficiency..

It is also notable that for porcine chondrocyte, Col I knock-down efficiency was not as high as that in fibroblast and osteoblast. The underlying reason might be that there are two base-pair mismatches between the designed siRNA sequence specific to human Col I α 1

chain and porcine Col I gene sequence, which can reduce the blocking efficiency significantly. [126]

Through qPCR and ELISA, it is clearly evidenced that TGF- β 3 is expressed in the three types of cells both at mRNA and protein levels. At protein level, TGF- β 3 expression at MOI 500 was much higher than that at MOI 200 in fibroblast and chondrocyte. However, for osteoblast TGF- β 3 protein released at MOI 500 did not differ significantly from that at MOI 200. This might be due to the short half-life of TGF- β 3 mRNA in osteoblast before its translation into proteins. It is also noted that TGF- β 3 protein level increased until six days after transduction in fibroblast and porcine chondrocytes, while the amount started to decrease at day 4 for osteoblast. This indicates that TGF- β 3 is more transiently expressed in osteoblast than the other two types of cells. In porcine chondrocytes, the amount of released TGF- β 3 was relatively lower. In order to reach the amount that has therapeutic effect, cell population can be increased for therapeutic applications.

Since SMSCs are a possible source for chondrogenesis, we carried out preliminary studies on the efficacy of Ad-D in this cell type. Results have shown that after infection with Ad-D, Col I production was inhibited while TGF- β 3 was expressed, indicating the successful functioning of Ad-D in SMSCs. Therefore, the construction of Ad-D has provided a potential tool to promote Col I suppressed chondrogenesis using SMSCs.

In conclusion, we have developed a model dual-functioning adenoviral vector that can simultaneously deliver TGF- β 3 and Col I-targeting shRNA to cells toward Col I-suppressed chondrogenesis. The recombinant adenoviral vector was characterized by qPCR, ELISA and immunofluorescent staining to assess its functions in the cells. Human fibroblast, human foetal osteoblast, porcine chondrocyte and SMSCs were used. Results have demonstrated that Ad-D could perform the two functions as expected. It could efficiently downregulate the expression of Col I and produce TGF- β 3 in the mean time. Particularly, hereby we have also provided preliminary results showing that Ad-D can

function well in SMCs, the major cell source in this PhD study. Therefore, this adenoviral vector, when applied to therapeutic cells, has great potential to promote Col I suppressed chondrogenesis and cartilage repair.

Chapter 3 Construction of a dual-functioning lentiviral vector and optimization of culture condition, titration and ELISA protocols

3.1 Introduction

Ad-D, which incorporates TGF- β 3 expression cassette and Col I-targeting shRNA expression cassette into one adenoviral vector, has been constructed and described in the previous chapter. It was found to be effective in suppressing Col I expression. [92] However, adenoviral vector only induces a short-term expression of inserted genes due to its episomal feature. In our endeavor to promote Col I-suppressed chondrogenesis, we need to know whether a more sustained release of TGF- β 3 is beneficial to support chondrogenesis. A more important factor is that Col I is expected to be permanently suppressed, even after successful chondrogenesis, so that Col I protein would not be adulterated into cartilage ECM. These considerations drive us to apply a new vector-lentiviral vector, which integrates its genome together with transgene into host genome and therefore induces a more sustained release profile.

In this chapter, we demonstrated the construction of one dual-functioning lentiviral vector (LV-D) that expresses both TGF- β 3 and Col I-targeting shRNA. Since the insert in LV-D is directly “copied” from Ad-D, LV-D shares the same arrangement of transgene with Ad-D, in which TGF- β 3 expression cassette and U6-shRNA expression cassette are in reverse directions. Using this LV-D as a model, we established a set of protocols, including lentiviral production, titration and ELISA for TGF- β 3 in the active form. Optimal culture medium was also determined with SMSCs transduced with LV-D.

3.2 Materials and methods

Construction of dual-functioning recombinant lentiviral vectors (LV-D)

To construct lentiviral plasmid (pLVX-D) that has the same arrangement of TGF- β 3 and shRNA-expressing cassettes in Ad-D as demonstrated in Chapter 2, the recombined adenoviral shuttle vector pDNR was digested with the restriction endonuclease PspOM I. PCR purification kit was utilized to purify the linearized plasmid, which was further digested with the restriction endonuclease Sal I and subjected to gel electrophoresis for extraction of the fragment containing TGF- β 3 cDNA sequence, U6 promoter and Col I-targeted shRNA, which was about 1600bp long. Meanwhile, the pLVX-IRES-ZsGreen vector was double digested with restriction endonucleases Xho I and Not I, and the digestion product was extracted from agarose gel after gel electrophoresis. The fragment from the recombined adenoviral shuttle vector pDNR and the linearized pLVX-IRES-ZsGreen vector were mixed and ligated using T4 DNA Ligase (Invitrogen) at 16 °C overnight. The recombinant plasmid pLVX-D was amplified and extracted from DH5 α using PureLink™ HiPure Plasmid Filter Purification Kits (Invitrogen, USA) and verified through enzymatic digestion and sequencing.

For verification via enzymatic digestion, the restriction endonucleases Sal I and MluI were used separately. Briefly, 1 μ g of the recombinant plasmid was digested with 1 μ L of the endonucleases respectively at 37°C for 2hrs. The digested products were subjected to agarose gel electrophoresis to visualize the DNA bands. The recombinant pLVX-D was also sequenced to verify that the sequence of the insert was the same as expected and there was no point mutation.

Figure 3.1 Lentivirus production with the Lenti-X Packaging System and 293T cells. Initially, cotransfection of a Lenti-X Vector and the Lenti-X HT Packaging Mix (Step 1) results in the production of the corresponding recombinant lentiviral genomic RNA and the required viral packaging proteins (Step 2). A vector in the Packaging Mix expresses the Tet-Off transactivator (tTA) to produce extra-high expression of specific viral components. Recognition of the packaging sequence (Ψ) on the viral RNA by the packaging proteins (Step 3) results in the assembly of viral cores, which are transported to the cell membrane (Step 4). Cores are then enveloped by cellular membrane containing aggregated VSV-G envelope protein. Mature, infectious virions then bud from the cell (Step 5) and are collected in the medium (Step 6). While infectious, the virions lack several critical genes required for their subsequent replication and production in target cells. (Reproduced from Clontech)

Production of recombinant lentiviral vectors

pLVX-D was then used to generate lentiviral vectors LV-D according to established methods. (Clontech, USA) Briefly, 5×10^6 cells/100 mm plate were plated in 10 ml of complete growth medium containing Tc-free FBS. pLVX-D was mixed with packaging mix and transfected into 293T cells using Lentiphos HT transfection system. Ten ml of fresh complete growth medium (with Tc-free FBS) was replaced 8hrs post-transfection. Forty-eight hours after transfection, the supernatant was collected and filtered through a 0.45µm filter. The virus stock was then aliquoted into single-use cryotubes and stored at -80°C refrigerator for subsequent use.

Titration of the lentiviral vector produced from human embryonic kidney (HEK) 293T cells

To titrate the produced lentiviral vector, flow cytometry was utilized. Briefly, serial ten-fold dilutions of the vector in medium were made and add to 293T cells in the presence of polybrene at a final concentration of 2µg/mL. After culturing for additional 8hrs at 37°C , the medium was changed and the cells were incubated for up to 72 hrs post-infection and analyzed by flow cytometry to determine the percentage of cells with green fluorescence. Viral dilution that resulted in 10- 20% infected cells was selected for calculation to ensure one infectious unit (ifu) per cell. Virus titer was calculated using the formula below:

$$(\text{number of cells/well at the time of harvest}) \times (\% \text{ positive cells by FACS}) / \text{volume of virus in ml} = \text{IFU/ml}$$

An alternative method was used to measure the number of RNA genomic copies of the viral stock. Briefly, lentiviral genomic RNA was purified using NucleoSpin® RNA Virus Kit. Residual plasmid DNA was removed by treatment with DNase I. The purified RNA was subjected to qPCR and the threshold cycle (C_t) was obtained for the sample using Lenti-X qRT-PCR Titration Kit. Meanwhile, standard samples with known number of genomic RNA copies were also tested and a standard curve of C_t versus lg (genomic copies)

was derived. The genomic RNA copy number for the sample lentiviral vector was obtained from the standard curve and compared with the infectious units obtained through flow cytometry. A ratio of genomic RNA copy number to IFU was established so that in subsequent titration experiments, Lenti-X qRT-PCR Titration Kit can be indirectly used to determine infectious units for ease.

Transduction of SMSCs with recombinant lentiviral vector and subsequent cell sorting

SMSCs were plated in complete growth medium 12–18 hr before transduction. Lentiviral stocks were thawed and polybrene was supplemented to the viral stock to obtain the desired final concentration of 2µg/mL during the transduction step. The viral supernatant was added to the cells and allowed for transduction. Eight hours post-infection, the viral vector-containing transduction medium was discarded and replaced with fresh growth medium. Cells were incubated for another 3 to 4 days to allow the expressed protein to accumulate in the target cells.

After the appearance of green fluorescence observed under fluorescent microscope, cells were trypsinized and washed with PBS. The cells were then subjected to fluorescence-activated cell sorting (FACS) to select the ZsGreen positive cells, which had been successfully transduced with recombinant lentiviral vector. Cells without transduction were used as a negative control. The excitation wavelength was 470nm, and the emission wavelength was 520nm. The sorted cells were then plated in dishes for amplification, marked as P0. Subsequent passages would be labeled as P1, P2, etc.

Optimization of culture medium conditions for the amplification of SMSCs

After cell sorting, both ZsGreen-positive and –negative cells were cultured in either of the following four media: DMEM supplemented with 10% FBS (Hyclone), MSCGM (Lonza), MesenPRO (Invitrogen) and CM which contained high-glucose DMEM (Gibco), 100 nM

DEX (Sigma), ascorbic acid 2-phosphate (50 mg/mL; Sigma), sodium pyruvate (100 mg/mL; Gibco), proline (40 mg/mL; Sigma), penicillin (100 U/mL), streptomycin (100 mg/mL) (Gibco), and 5 mL of ITS premix (insulin [6.25 mg/mL], transferrin [6.25 mg/mL], selenous acid [6.25 mg/mL], linoleic acid [5.35 mg/mL], and bovine serum albumin [1.25 mg/mL] (BD Biosciences, Bedford, MA) in 500 mL of medium. The cells were passaged over and at Passage 4, RNA was isolated to determine the gene expression profiles in the cells cultured in different media.

RNA isolation and qPCR analysis

The detailed procedures have been described in section 2.2, page 39.

3-D culture of SMSCs

ZsGreen-positive cells were amplified in 2D culture flasks according to the standard culture protocols. When the number of ZsGreen-positive cells had reached a certain number, usually at Passage 4, cells were trypsinized and rinsed with washing buffer [0.15M NaCl (Sigma-Aldrich) + 25mM N, 2-hydroxyethylpiperazine-N0-ethanesulfonic acid (HEPES) (Hyclone) in distilled water]. After that, the cells were resuspended in sterile 1.2% alginate (Sigma) at 7×10^6 cells/mL. Then 40 μ L of cell suspension was slowly dropped into a beaker containing 102 mM CaCl_2 (Sigma-Aldrich) solution for polymerization for 10 min. Finally four beads were placed into each well of the 24-well plate with 1 mL of CM. Medium was replaced and collected for ELISA assay every 3 days.

Quantitative analysis of TGF β 3 expression by specific ELISA

The detailed procedures can be found in section 2.2, page 53. The amount of total TGF- β 3 was measured by activating the protein with hydrochloride acid, while the amount of TGF- β 3 in the active form was obtained without the activation step.

Statistical analysis

The results are indicated as mean $\pm$ SD. Where appropriate, student t-test was used to analyze results and difference was considered to be statistically significant at $P < 0.05$.

3.3 Results

pLVX-D construction and LV-D production

After recombination of pLVX plasmid vector, digestion with SalI and MluI was performed for verification. Results are displayed in Figure 3.2. a. Digestion of pLVX with SalI gave rise to bands with approximate lengths of 1998bp, 2186bp and 4023bp (Lane 2), while the bands were 1998bp, 2186bp and 5593bp for pLVX-D (Lane 3). Alternatively, a linearized DNA strand at 8204bp appeared after digestion of pLVX with MluI (Lane 4), whereas bands at 8144bp and 1656bp were generated with pLVX-D (Lane 5). The comparison between pLVX and pLVX-D after digestion with SalI and MluI clearly demonstrated the incorporation of the insert into pLVX vector.

After LV-D packaging, the aliquot of LV-D stock was used to transduce 293T cells. Three days post transduction, green fluorescence was detected in 293T cells, as shown in Figure 3.2.b. This suggested that LV-D had been successfully packaged and live lentiviral stock was produced.

a

b

Figure 3.2 Recombinant lentiviral vector construction. a. Verification of recombinant lentiviral plasmid with SalI and MluI. Markers in the 1st lane designate 10kb, 8kb, 6kb, 5kb, 4kb, 3kb, 2kb, 1.5kb, 1kb and 0.5kb, respectively. b. 293T cells transfected with recombinant lentiviral plasmids to produce viable lentiviral vectors.

LV-D titration

Flow cytometry was used to determine the percentage of transduced 293T cells, as assessed by the emission of green fluorescence due to ZsGreen protein. With 100 μ L of the lentiviral stock, the proportion of ZsGreen-positive cells reached up to around 13% of the total 1.2×10^6 cells. From the value it could be calculated that the titer of the LV-D stock was around 1.6×10^6 ifu/mL.

Lenti-X qRT-PCR Titration Kit was also applied for the purpose of obtaining the genomic RNA copy number in the viral stock. A standard curve of threshold cycles versus lg (genomic RNA copy numbers) was generated and linearly fitted as shown in Figure 3.3. From the C_t of LV-D genomic RNA samples, a concentration of 7.95×10^8 copies/mL was obtained. The number of genomic RNA copies was correspondent to the above 1.6×10^6 ifu/mL obtained from flow cytometry, with a ratio of around 500:1. This ratio could be used in subsequent lentiviral titer determination using qRT-PCR, while flow cytometry would no longer be used for its tedious procedures as well as safety considerations.

Figure 3.3 Standard curve obtained for the determination of RNA copy number of lentiviral vectors.

Optimization of culture medium conditions for the amplification of SMSCs

From the photos taken at Passage 4 in Figure 3.4.a, hardly any cell survived in CM, and the few observed cells showed a round-shaped morphology. For the cells cultured in MesenPRO, there were a much higher number of survived cells. However, most of these cells were hypertrophic, a significantly different morphology from the primary cells. In comparison, cells cultured in MSCGM and DMEM supplemented with 10% FBS (Hyclone) had higher proliferative capacity as assessed by the higher cell number after passages. Moreover, they maintained the good spindle-shaped morphology of SMSCs. Since both DMEM and MSCGM were found to maintain SMSC phenotype, these two types of medium were further tested to see their effect on Col I expression levels in ZsGreen+ SMSCs.

qPCR was carried out for the determination of Col I gene expression in ZsGreen+ cells cultured in MSCGM and DMEM supplemented with 10% FBS (Hyclone), and the results are shown in Figure 3.4.b. It was found that SMSCs cultured in DMEM had lower levels

of Col I mRNA level than those cultured in MSCGM. Since Col I expression is expected to be minimal at all times, DMEM supplemented with 10% FBS was selected for SMSC monolayer expansion in all subsequent experiments.

Figure 3.4 Medium selection for 2-D SMSC expansion. (a) Morphology of ZsGreen+ cells cultured in various media at Passage 4. (b) Col I gene expression relative to the housekeeping gene RPL4 in ZsGreen+ cells cultured in MSCGM and DMEM at Passage 4. * $p < 0.05$.

ELISA for TGF- β 3 expression SMSCs in alginate hydrogel beads

ELISA was adopted to quantify the protein level of both inactive (or total) and active TGF- β 3 in SMSCs encapsulated in alginate beads (Figure 3.5). Initially, there was a high expression of TGF- β 3, while there was a drastic decline between day 6 and day 12. After that, the expression of both total and active TGF- β 3 remained at a relatively stable level. Comparing the amount of total and active TGF- β 3, the active TGF- β 3 accounted for approximately one third of the total initially, while most of the total TGF- β 3 was in the active form from day 12.

Figure 3.5 Quantification of total and active TGF- β 3 in 3-D culture of LV-D-transduced SMSCs for 42 days by specific ELISA.

3.4 Discussion

In this chapter, a dual-functioning lentiviral vector LV-D was constructed and used as a model to establish a set of methods suitable for the application of lentiviral vectors in

SMSCs. Except for the routine methods such as for the titration, which are essential in its application, the selection of appropriate medium is the focus in this chapter. Here four types of media were assayed - CM, MesenPro, MSCGM and DMEM. The proper medium should in the first place support adequate cell growth and proliferation, and maintain cell viability during cell amplification. Undoubtedly, CM did not support cell proliferation as we had expected, due to the lack of serum as nutritional source. MesenPro, which is suitable for the culture of BMSCs, resulted in cellular hypertrophy in our study and therefore may not be suitable for the culture of our lentiviral vector-transduced SMSCs. Under such criteria, MSCGM and DMEM were shortlisted. Besides, SMSCs cultured in DMEM were found to have lower Col I expression levels than those cultured in MSCGM. Since our final objective is to construct a Col I-suppressed cartilage tissue, Col I expression is disfavored in the cells; therefore, DMEM was determined to be optimal for SMSC monolayer culture.

Besides the selection of proper medium for 2-D cell amplification, we also determine the relationship between total and active TGF- β 3 released during 3-D culture by specific ELISA. Although quite a large quantity of total TGF- β 3 was expressed initially, only about one third of it was truncated into the active form, which accounts for chondrogenic induction. Several theories may contribute to this observed outcome. TGF- β 3 is originally synthesized within the cells in the form of propeptide, which is an inactive form. The propeptide has to undergo enzymatic cleavage to obtain full activity. Besides, the propeptide may still be bound to other substances, such as ECM components, even after cleavage, rendering it latent[127]. Since the active form of TGF- β 3 contributes to chondrogenic induction, in the following experiments, we will only measure the quantity of TGF- β 3 in the active form and assess its ability to induce chondrogenesis in the following chapters.

After the establishment of a set of methods for the application of lentiviral vectors, we then set out to compare the effect of various delivery vehicles to deliver both TGF- β 3 and Col I-targeting shRNA as demonstrated in subsequent chapters.

Chapter 4. Optimal Construction and Delivery of Dual-Functioning Lentiviral Vectors for Col I-Suppressed Chondrogenesis in SMSCs

4.1 Introduction

Previously, a dual-functioning lentiviral vector encoding both TGF- β 3 and Col I-targeting shRNA (LV-D) has been successfully constructed and general protocols have been established for the utilization of lentiviral vectors, including viral production, titration, ELISA protocols for active TGF- β 3 quantification and SMSC culture medium. However, since the two expression cassettes in LV-D are functioning in the same vector, transcriptional interference might exist to promote or compromise the transcription of each other. The orientation and location of the two cassettes would affect the expression levels. RNA polymerase binds to the promoter of an expression cassette and initiates the elongation of the transcriptional process. In the theory of position transcriptional effect, an enhancer of a promoter may function to affect the expression of an adjacent gene, leading to the variation of the expression levels.[128] On the other hand, negative transcriptional interference occurs due to either of the following mechanisms: promoter competition[129], sitting duck mechanism [130], occlusion[131], collisions[132] and roadblock[133]. When two expression cassettes are located in proximity, whether they are in convergence or tandem, transcriptional interference might occur. Even if the two cassettes are a distance away from each other, there might also be transcriptional interference, whereby one transcriptional activity would impact a second transcriptional activity.

Besides transcriptional interference, the expression of a green fluorescent protein, ZsGreen, under a component of internal ribosome entry site (IRES) further complicates the construction of the recombinant vector. IRES is located between MCS and ZsGreen encoding sequence, so that both the transgene and ZsGreen encoding sequence would be

transcribed into a single mRNA strand. It allows the ribosome to bind at this internal site of mRNA strand and initiate translation from it, rather than from the start of the mRNA sequence. Therefore, unexpected translational termination of the upstream transgenes in MCS may interfere with the expression of ZsGreen and even the transcription of whole lentiviral genome. This would lead to failure in lentiviral production. In consideration of all the above factors, cautions should be exercised to accommodate the transgenes into MCS for rational design of the recombinant lentiviral vectors. In this chapter, four LV-Ds were constructed by altering the direction and position of the shRNA-expressing cassette, and the details are described in this chapter. SMSCs were transduced with these LV-Ds and encapsulated in 3D alginate hydrogel beads. Expression of Col I and chondrogenic markers was tested. The overall effect of the viral genomic configuration on Col I-suppressed chondrogenesis was assessed to obtain the optimal vector for Col I-suppressed chondrogenesis.

4.2 Materials and methods

Construction of single-functioning and dual-functioning recombinant lentiviral vectors (LV-D)

The schematic structures of the recombinant lentiviral vectors that can express TGF- β 3 and/or suppress Col I expression and their roles in Col I-suppressed chondrogenesis were shown in Figure 4.1. a and Figure 4.1.b.

Figure 4.1. Schematic roles and structures of recombinant lentiviral vectors. (a) The relationship of the molecules in our research. (b) The structures of the recombinant lentiviral vectors.

Among the four LV-Ds, LV-3 is the one that we constructed as described in Chapter 3. LV-4 differs from the existing LV-3 in that the shRNA expressing cassette is in the same direction with TGF- β 3 expressing cassette in LV-4, whereas in LV-3 the directions for the two cassettes are reverse. In LV-1 and LV-2, the shRNA expressing cassette was moved to

elsewhere between MfeI and FseI restriction sites, out of the MCS, to minimize the potential interference between the two expressing cassettes and attenuate the impact on ZsGreen expression and transcription of whole lentiviral genome. In LV-1, the two cassettes are in reverse directions, while in LV-2 they are in the same direction.

For detailed procedures, lentiviral plasmid vector carrying only TGF- β 3 expressing cassette (pLVX-T) was firstly constructed as the backbone for the others and also as a control in subsequent experiments. TGF- β 3 sequence was amplified through PCR using primers as shown in Table I. Both the TGF- β 3 sequence and pLVX vector were digested with XhoI and NotI, and the two linear segments were ligated.

shRNA expressing cassette (U6 and shRNA encoding sequence) was PCR amplified from recombinant dual-functioning adenoviral plasmid described in Chapter 2 as template, with primers carrying various restriction sites at the ends. The segment was digested with corresponding restriction endonucleases and ligated with pLVX-T which had been digested with the same restriction endonucleases. The primers for the amplification of TGF- β 3 and shRNA expressing cassettes for pLVX-1, pLVX-2 and pLVX-4, respectively, were designed and listed in Table 1.

Recombinant lentiviral plasmids (pLVX-T, pLVX-sh, pLVX-1, pLVX-2, pLVX-3, pLVX-4) were verified with restriction endonuclease digestion followed by gel electrophoresis and gene sequencing. Subsequently, these plasmids were used to generate lentiviral vectors (LV-T, LV-sh, LV-1, LV-2, LV-3, LV-4) according to the manual of Lenti-XTM Lentiviral Expression Systems (Clontech, Mountain View, CA), as described in Chapter 3, page 69. Among them, LV-T referred to the recombinant lentiviral vector that expresses TGF- β 3 only, while LV-sh was the one that expresses shRNA against type 1 collagen. LV-1, LV-2, LV-3 and LV-4 all belonged to the LV-D family, which express both TGF- β 3 and shRNA.

Table 4.1 Primers designed for vector construction. (TA: Annealing temperature)

Amplicon	Restriction sites	Primer sequence 5'-3'	Length (bp)	TA (°C)
TGF- β 3	XhoI/NotI	F:CCGCTCGAGATGAAGATGCACTT R:ATAAGAATGCGGCCGCTCAGCTACATTAC	1259	50
shRNA cassette-1	FseI/MfeI	F:AGACTACAATTGTGCAGGAAGAGGGCCTAT R:CATCAAGGCCGCGCCACGCGTAAAAACAATCACCTG	~350	55
shRNA cassette-2	MfeI/FseI	F:AGACTAGGCCGCGCCGACCATGTTCACCTAC R:CATCAACAATTGACGCGTAAAAACAATCACCTG	~400	58
shRNA cassette-4	NotI/BamHI	F:AAGGAAAAAAGCGGCCGCATATAGCAGGAAGAGGG CCTAT R:CGGGATCCACGCGTAAAAACAATCACCTG	~350	55

Porcine SMSC isolation and culture

Detailed procedures can be found in section 2.2, page 38.

Lentiviral transduction and selection of ZsGreen-positive cells

Procedures can be found in section 3.2, page 54. The sorted cells were then plated in dishes for amplification, marked as P0. Subsequent passages were labeled as P1, P2, etc.

Amplification and 3D culture of pSMSC

For detailed procedures, please refer to section 3.2, page 55. Medium was replaced and collected for ELISA assay every 3 days. In parallel, SMSCs without transduction were embedded into alginate hydrogel as negative control. pSMSCs without transduction are also cultured with manually added TGF- β 3 protein every three days at a concentration of 10ng/mL as a positive control. In total, there were 7 groups: Neg (negative control),

Neg/TGF β 3 (positive control), LV-T, LV-1, LV-2, LV-3 and LV-4. One day after gelation was defined as Day 0. At day 21 and day 42, the beads were collected for RNA analysis, CGC quantification as well as staining.

RNA isolation and qPCR analysis

Detailed procedures can be found in section 2.2, page 39.

Quantitative analysis of active TGF β 3 expression by specific ELISA

Detailed procedures can be found in section 3.2, page 58.

Cell viability test

Cell viability was tested using WST-1 assay {4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate assay, Roche Diagnostics, Germany}. Briefly, at day 42 of the culture period, 10 μ L of WST solution was added into each well containing one sample bead in 100 μ L of medium. After 2 hrs incubation the absorbance at 450 nm of the medium was determined by a microplate reader (Multiskan_ spectrum, Thermo). Each group was conducted in triplicate.

Quantitative immunofluorescent staining of Col II protein

At day 42, SMSCs-alginate constructs from each group were dissolved in sodium citrate. The suspension was homogeneously aliquoted into the wells of 96-well plate and frozen. Then the solid phase of the mixture was freeze-dried overnight. The product was fixed with glutaraldehyde for 1hr and blocked with 10% (v/v) goat serum for 30min. After washing with PBS, the residual proteins on the surface of the wells were incubated with mouse anti-pig Col II primary antibody (2 ng/mL in PBS, Chemicon)) at room temperature for 2 hrs. After three washes with PBS, the samples were further incubated with

AlexaFluor 546 rabbit anti-mouse IgG (1:200, Invitrogen, Carlsbad, CA, USA) for 1.5 hrs. After three washes with PBS, Infinite 200 fluorescent microplate reader (TECAN Systems, San Jose, CA, USA) was used to determine the fluorescent density in each well, with the excitation wave length of 560nm and emission wave length of 590nm. Each sample was performed in triplicate.

Biochemical analysis

After 42 days of culture, constructs were taken out, washed 3 times with deionized water to remove the salt in the medium followed by freeze drying (36 hrs), and digested with 1 mL of papain per sample for biochemical analysis.[134-135] Dimethylmethylene blue dye was added into the digested solution, and absorption at 525 nm was measured using a UV-VIS spectrophotometer (Multiskan spectrum, Thermo) to quantify sulfated GAG content.[136-137]

Histology and immunohistochemistry

For 3D constructs, samples from each group at Day 42 were fixed in 4% (w/v) neutral buffered paraformaldehyde, embedded in paraffin and sectioned (5µm thick). Then sections from all groups were deparaffined and stained with Safranin-O for GAGs and Masson's Trichrome for total collagen (Invitrogen, Carlsbad, CA). For immunohistochemistry, the specimens were fixed in glutaraldehyde (2.5%, 30min) and blocked with 1% goat serum (w/v, in PBS) for 1 h. Afterwards, primary antibodies for Col I (2 ng/mL in PBS, Santa Cruz Biotechnology) and Col II (2 ng/mL in PBS, Chemicon) were applied for 2hrs (4 °C), respectively. Following three PBS washes, the sections for Col II or Col I were respectively incubated with HRP-conjugated secondary antibodies (1 µg/mL in PBS, Invitrogen, Carlsbad, CA) at room temperature for 1 h. The presence of Col II was observed using the DAB Substrate kit (Clontech, Mountain View, CA).

Statistical analysis

The results are indicated as mean $\pm$ SD. Where appropriate, student t-test was used to analyze results and difference was considered to be statistically significant at $P < 0.05$.

4.3 Results

Construction of single-functioning and dual-functioning recombinant lentiviral vectors

The configurations of the recombinant vectors are given in Figure. 4.1b. Among them, LV-1 is characterized with two distant and reversely oriented cassettes, while LV-2 has two distant and same oriented cassettes. On the other hand, LV-3 has two proximal and reversely oriented cassettes, while LV-4 has two proximal and same oriented cassettes. MluI and EcoRI digestion proved successful recombination of the plasmids.

3-D gene expression of Col I, Col II and aggrecan

21 and 42 days after 3-D culture of non-transduced and transduced pSMSCs in CM, gene expression of Col I, Col II and aggrecan was assessed with qPCR, as shown in Figure. 4.2. It was observed that Col I was elevated either in Neg/TGF- β 3 or LV-T group at both day 21 and day 42. Col I expression was lower in LV-1 and LV-3 than that in Neg/TGF- β 3 at day 21 and day 42. On the contrary, LV-2 transduction resulted in a comparable Col I level at day 21 with Neg/TGF- β 3, while it decreased Col I expression significantly at day 42. In comparison, LV-4 elicited the expression of Col I to a higher extent at day 42 compared to Neg/TGF- β 3.

Quantitative analysis of Col II and aggrecan mRNA levels was also performed, since the two are the primary components of cartilage ECM and therefore can be trusted as markers for chondrogenesis. Groups of Neg/TGF- β 3, LV-T, LV-1 and LV-4 presented much higher expression of Col II relative to that in Neg at both day 21 and 42. Results obtained for LV-

2 and LV-3 showed some differences between day 21 and day 42. Col II expression was much higher in LV-2 at day 21 compared to Neg, while the level decreased significantly at day 42. Contrarily, LV-3 remarkably promoted Col II expression at day 42. The trends for aggrecan expression were similar to those for Col II, except that the level in LV-1 was very low at day 21.

Figure 4.2. Relative gene expression determined by qPCR at day 21 (a,b,c) and day 42 (d,e,f). RNA was isolated and reverse transcribed. 1 μ L of cDNA was used for real-

time PCR. (a,d) Col I; (b,e)Col II; (c,f) aggrecan.

Quantitative analysis of TGF- β 3 expression by specific ELISA

The curves of TGF- β 3 expression at the protein level in LV-T, LV-1, LV-2, LV-3 and LV-4 groups were obtained throughout the culture period of 42 days, as shown in Figure. 4.3. In all the five groups, there was a high expression level initially, reaching up to 120 ng/mL for LV-4 and 8 ng/mL for LV-T, followed by a dramatic decline within the first 18 days. The expression levels then remained at a relatively stable platform afterwards, although there were minor fluctuations or rebounding particularly for LV-4. By comparing the expression levels among these five groups, LV-4 always had the highest expression of TGF- β 3, whereas LV-T seemed to have the lowest expression level of all. The platform expression levels of LV-1, LV-2 and LV-3 in the latter phase were close to one another, around 2 ng/mL.

Figure 4.3. Quantity of TGF- β 3 determined by specific ELISA. Medium was collected every 3 days and subjected to ELISA with standard. Concentration was calculated against the standard curve obtained from the standard.

Cell proliferation test at day 42 by WST assay

Comparison of absorbance levels obtained from WST assay is demonstrated in Figure. 4.4a. All of the six groups (Neg/TGF- β 3, LV-T, LV-1, LV-2, LV-3 and LV-4) displayed more or less higher absorbance values in relation with the value in Neg group, indicating higher cell proliferative capability in these groups. Among them, LV-4 group had the highest cell proliferation.

Quantitative analysis of Col II protein expression

The intensity following fluorescent staining of Col II was illustrated in Figure. 4.4b. In the vertical axis for percentage of induction, the level of blank was set as 0, while the fluorescent level in Neg was labeled as 100%, with all other groups being demonstrated as the ratio relative to that in Neg group. All of the six groups with either manually-supplemented TGF- β 3 or TGF- β 3 expressed endogenously via the lentiviral vectors (Neg/TGF- β 3, LV-T, LV-1, LV-2, LV-3 and LV-4) showed higher amount than the Neg group. Among them, LV-2 and LV-4 showed the highest Col II protein levels.

Quantitative analysis of GAG

From Figure. 4.4c, it is seen that there was a basal expression of GAG even in Neg group. In comparison, all other groups showed higher production of GAG. Among them, cells transduced with LV-3 had the highest GAG levels at day 42.

Figure 4.4. Cell proliferation and quantity of ECM components at day 42. (a) Cell proliferative capability was determined by WST assay. Col II (b) was determined by immunofluorescent staining. (c) The amount of GAG was measured by biochemical assay. n=3; error bars represent SEM. *p<0.05.

Immunohistochemical analysis

Safranin O staining was performed in order to visualize GAG in the cross sections of the

beads cultured for 42 days. GAG was stained in red surrounding the cells, as shown in Figure. 4.5a. In the Neg group, there was a minimal amount of GAG observed in between the cells, if not zero amount. With Neg group being the ground mark, the rest of the groups were above the ground in terms of GAG assessment, with LV-1 group showing the most obvious GAG staining. LV-3 also demonstrated a large production and homogeneous distribution of GAG within the cross section.

Other means of staining techniques was applied as well. Masson's Trichrome staining was performed to visualize total collagen, including Col I, Col II and other types. In Figure. 4.5b, the LV-D groups (LV-1, LV-2, LV-3 and LV-4) all showed aggregation of collagens around the cells. Amongst them, LV-2 and LV-4 had the most obvious visualization of collagens.

In immunohistochemistry staining for type I and Col II, Col I and Col II were stained as brown against the background. In Figure. 4.5c, Col II was visually apparent in LV-1, LV-2 and LV-4, while LV-3, LV-T and Neg/TGF- β 3 also showed visualization of Col II to some extent. All these groups demonstrated higher expression of Col II in comparison with Neg.

In Figure. 4.5d, more Col I was observable in Neg and Neg/TGF- β 3. Comparatively, Col I was rarely detected in LV-D groups.

Figure 4.5. Chemical and immunohistochemical staining on the cross sections of the constructs cultured for 42 days. (a) Safranin O staining for GAG; (b) Masson's Trichrome staining for total collagen; (c) Immunohistochemical staining for Col II; (d) Immunohistochemical staining for Col I.

4.4 Discussion

Four lentiviral vectors were designed and constructed by altering the location and orientation of the shRNA-expressing cassette. Col I-suppressed chondrogenesis was examined to assess the overall effect of these various lentiviral vectors. Among these four LV-Ds, TGF- β 3 cassette and shRNA cassette are closely proximal in LV-3 and LV-4, while the two cassettes are far apart in LV-1 and LV-2. Therefore, transcriptional interference is expected to be lower in LV-1 and LV-2. Similarly, as the shRNA cassette is in the reverse orientation of TGF- β 3 cassette in LV-1 and LV-3, the interference would be lower than that in LV-2 and LV-4, where the two cassettes are in the same direction and therefore transcription activities on the two cassettes proceed on the same strand of the double-stranded DNA. By altering the relative positions and orientations of TGF- β 3 and Col I-

targeting shRNA cassettes, we could expect various degrees of transcriptional interference between the two cassettes, which would result in different efficiencies in Col I-suppressed chondrogenesis. We aim at finding out the optimal construct that performs best in both Col I suppression and chondrogenic induction in engineered cartilage tissues.

TGF- β 3 secretion was shown to be the lowest in LV-T according to ELISA results, while all LV-Ds expressed higher amount of TGF- β 3. This phenomenon suggests that the expression of TGF- β 3 could be elevated by the introduction of shRNA cassette. This elevation was more obvious when the two cassettes are located close to each other and when they are in the same direction, as the expression level in LV-4 was much higher than that in LV-3, and the expression in LV-3 was higher than that in LV-2 and LV-1. The results have indicated a positive effect of proximity and same direction on the transcription of TGF- β 3 cassette. A possible hypothesis might be the attraction or recruit of type II RNA polymerase, which binds to CMV promoter, by type III RNA polymerase, which binds to U6 promoter. An alternative hypothesis to explain this phenomenon might be the higher local concentration of nucleotides and other regulation factors at the site of the two cassettes recruited by type III RNA polymerase. A higher concentration of type II RNA polymerase, nucleotides and regulation factors by this effect would therefore result in higher expression of TGF- β 3.

For the mRNA level of Col I, it was found that TGF- β 3, whether manually supplemented or endogenously expressed through the recombinant lentiviral vectors, was able to elicit the transcription of Col I. This has complicated the situation where Col I, an undesired protein of cartilage ECM, is inherently produced in SMSCs, which adds on more justification to the necessity for Col I suppression. Some LV-Ds were found to be effective in reducing the Col I mRNA level. The expression levels of Col I in these groups may not be significantly lower than that in the Neg group. However, by comparing the expression levels of Col I in these groups with Neg/TGF- β 3 or LV-T group, they did serve to down-

regulate the expression of Col I and compromise the elevating effect imposed by TGF- β 3. Of the four LV-Ds, LV-4 had the least effect, or the expression level of Col I in LV-4 was even elevated at day 42 in 3-D culture compared to either Neg/TGF- β 3 or LV-T group. LV-1 was more efficient in Col I suppression in the shorter term (day 21), whereas LV-2 had a high Col I inhibition efficiency in the longer term (day 42). LV-3 was supposed to be the most effective among all the vector configurations, by down-regulating Col I expression compared to either Neg/TGF- β 3 or LV-T at both time points. Comparatively, LV-1 and LV-2 are suboptimal.

Higher cell viability was observed in all the groups than that in Neg group. As TGF- β 3 was either manually supplemented to the cells or endogenously expressed with the transduction of the lentiviral vectors in the cells in these groups, the higher cell viability could be attributed to the presence of TGF- β 3, which is a growth factor that controls such cellular activities as cell proliferation, differentiation and apoptosis. The highest cell proliferation was found in the cells transduced with LV-4, which is corresponding to the ELISA result where LV-4 group had the highest expression of TGF- β 3 protein. This leads to the assumption that TGF- β 3 promotes cell proliferation on a concentration-dependent basis. Higher amount of TGF- β 3 within a certain limit may better promote cell viability and proliferation.

With endogenously expressed TGF- β 3, chondrogenesis induced by these lentiviral vectors were explored. Based on qPCR results, LV-1 and LV-4 had high Col II expression levels at both day 21 and 42. In comparison, LV-2 promoted Col II expression in the shorter term, as opposed to LV-3, which resulted in a high Col II expression in the long run. Aggrecan expression had similar patterns, except that LV-1 only promoted aggrecan expression in the longer term.

Since qPCR only represents the real time status of transient expression of the markers at the time of testing, we further assessed the overall Col II accumulation during the 42 days

of culture by quantitative immunofluorescent staining, which takes account of the overall 42-day time period for accumulative protein levels. Therefore, despite some differences between the day 21 and day 42 qPCR results, e.g., Col II mRNA level in LV-2 was quite low at day 42 while it was much higher at day 21, all the groups had higher levels of Col II protein compared to Neg group in quantitative immunofluorescent staining. Immunohistochemical staining further substantiates the above observations, with LV-1 and LV-4 showed highest demonstration of Col II. Since Col II is a major component of the ECM of cartilage, the expression of Col II at both mRNA and protein levels corroborates the assumption that the cells are differentiating towards the chondrocyte phenotype.

GAG, as the brick for the organization of cartilage ECM, was also quantified with biochemical assays. The amount of GAG with lentiviral transduction all showed higher levels than that in the Neg group. This was also observed in Safranin O staining. These results provide the evidence that the lentiviral vectors were capable of inducing the cells into the chondrocyte lineage, by producing more constituents to construct cartilage ECM. By comparing the four LV-Ds, LV-1 and LV-3 seemed to have a better expression profile of GAG, and also a better distribution as observed by Safranin O staining on the cross sections. It is noteworthy that in our hydrogel system, both the biochemical assay and immunofluorescent staining for Col II are adopted as qualitative and semi-quantitative references with lower resolution compared to qPCR assay. Therefore, hereby we are making comparisons relative to the Neg group, with the result being that all groups showed higher Col II levels than Neg. The quantitative relations between the experimental groups and Neg, as well as among all the experimental groups, are analyzed based on qPCR results for examination at transcriptional level.

The result obtained from Masson's Trichrome staining was, to some degree, consistent with the result from immunohistochemical staining of Col II. This is primarily because Col II constitutes the principal collagen matrix in cartilage, about 90-95% of the macrofibrillar

framework, while Col I, in spite of its existence, makes up only a small portion of total collagen, as observed in the Neg group in Col I immunohistochemical staining.[119] Other collagen subtypes such as type IX and type X collagen (Col X) are also in small amount. [119] Although we are expecting an increase in Col II and decrease in Col I, the two effects would not be offset due to the imbalance between the quantities of the various collagen subtypes.

Comprehensively in consideration of all the above factors, LV-4 was able to express the highest level of TGF- β 3, which led to the best induction of chondrogenesis and Col I expression simultaneously. However, as the increase of Col I level was unable to be compensated by the insertion of shRNA cassette, LV-4 goes out of our option to meet our objective of Col I-suppressed chondrogenesis. LV-3 had the best Col I suppression efficiency, while it tended to induce chondrogenesis only in the longer term. LV-1 and LV-2 had suboptimal Col I down-regulation, whereas LV-1 had a better profile for chondrogenic induction. Therefore, LV-1 was determined as optimal among the four LV-Ds investigated. However, more work has to be done in order to improve the inhibitive efficiency and coordinate between Col I suppression and chondrogenic induction.

Chapter 5. Articular chondrogenesis of transgenic SMSCs in 3D scaffolds: a co-delivery of growth factor and shRNA genes via adeno-/lentiviral vectors

5.1 Introduction

Dual-functioning lentiviral vectors with various configurations to deliver the genes of both TGF- β 3 and Col I-targeting shRNA have been constructed and demonstrated in the previous chapter. Among them, LV-1 was found to be effective in Col I-suppressed chondrogenesis. However, even the optimal one, LV-1, had some fluctuation in the short term and long term expression of Col I and aggrecan. More importantly, lentiviral vectors can integrate its genome together with transgene into host genome and induce a sustained expression of the transgene, whereas whether a transient or sustained transgene expression is favorable for Col I-suppressed chondrogenesis remain unknown. It is possible that adenoviral vector-mediated transient expression of TGF- β 3 or Col I-targeting shRNA is sufficient for our purpose of articular chondrogenesis, while sustained expression does no good or even do harm to the engineered cartilage tissue. Therefore, in this chapter, a collection of single-functioning adenoviral and lentiviral vectors encoding either TGF- β 3 or Col I-targeting shRNA, constructed either previously or as described in this chapter, was used to modulate the expression duration of transgenes and direct Col I-suppressed chondrogenesis. To implement the two functions in SMSC - induction of chondrogenesis and suppression of Col I, co-delivery with these single-functioning viral vectors was adopted. For instance, adenoviral vector expressing TGF- β 3 and lentiviral vectors expression Col I-targeting shRNA can be used to transduce the same batch of SMSCs, or vice versa. Alternatively, the co-delivery system could be composed of both adenoviral vectors that express TGF- β 3 and shRNA, respectively. These combinations of the single-functioning vectors were introduced into SMSCs, which were then incorporated in alginate

hydrogel to achieve Col I-suppressed chondrogenesis. The various combinations of delivery systems would lead to differential TGF- β 3 and shRNA expression profiles over the culture period and eventually resulted in different degrees in Col I suppression and chondrogenesis. Based on the results, optimal strategies for co-delivery of TGF- β 3 and shRNA could be determined. Schematic illustration of the co-delivery design is demonstrated in Figure 5.1.

Figure 5.1 Schematic illustration of co-delivery strategies for TGF- β 3 and Col I-targeting shRNA using adeno-/lentiviral vectors. RISC: RNA-induced silencing complex.

5.2 Materials and methods

Construction of recombinant adenoviral and lentiviral vectors

The schematic configurations of null and recombinant adenoviral/lentiviral vectors are

given in Figure 5.2. Adenoviral vector expressing TGF- β 3 (Ad-T), adenoviral vector expressing Col I-targeting shRNA (Ad-sh) and null adenoviral vector (Ad-N) were constructed as described in our previous study. [92]

Lentiviral plasmid vector expressing only Col I-targeting shRNA (pLVX-sh) was constructed by PCR amplifying the U6-shRNA cassette from Ad-D and incorporating this DNA fragment into NotI/BamHI gap of pLVX-IRES-ZsGreen1 (Clontech Laboratories Inc., Mountain View, CA). Primers for PCR are as follows: Forward: AAGGAAAAAAGCGGCCGCATATAGCAGGAAGAGGGCCTAT; Reverse: CGGGATC CACGCGTAAAAACAATCACCTG.

pLVX-sh and null lentiviral plasmid (pLVX-N) were then used to generate single-functioning lentiviral vector that only expresses Col I-targeting shRNA (LV-sh) and null lentiviral vector (LV-N), as previously described.

Figure 5.2. Schematic configurations of null and recombinant adenoviral/lentiviral vectors.

Porcine SMSC isolation, culture and transduction

Detailed procedures can be found in section 2.2, page 38.

3-D culture of pSMSCs transduced with various combinations of adenoviral and lentiviral vectors

Detailed protocol for the viral transduction in each group is given as below:

i) Ad-N, Ad-sh, Ad-T

SMSCs at passage 4 were infected with either of the above four vectors at MOI 200 and encapsulated 3 days later in alginate hydrogels. The beads were cultured in CM for 30 days.

ii) LV-N, LV-sh, LV-T

Low-passage SMSCs were infected with either of the above four vectors. Successfully infected cells carrying green fluorescence were sorted out via FACS and allowed to proliferate in a well-defined cell culture medium for expansion. At passage 4, cells were detached from flasks and encapsulated in alginate hydrogels for 30-day culture.

iii) Combination of Ad-T and LV-sh (LV-sh+Ad-T)

iv) Combination of Ad-sh and LV-T (LV-T+Ad-sh)

For groups ii and iii, freshly-isolated SMSCs were infected with LV-T or LV-sh and subjected to FACS, and the successfully infected cells carrying green fluorescence were sorted out. These positive cells were cultivated in a well-defined cell culture medium optimized previously for expansion. At passage 4, the cells were infected again with Ad-sh or Ad-T at MOI 200. After infection, the cells were collected and encapsulated 3 days later in alginate hydrogels for culture in CM for 30 days.

v) Combination of Ad-T and Ad-sh (Ad-T+Ad-sh)

SMSCs at passage 4 were infected with Ad-T and Ad-sh at MOI 200 simultaneously and encapsulated 3 days later in alginate hydrogels. The beads were cultured in CM for 30 days.

There is one more group using cells without viral transduction but with manually

supplemented TGF- β 3 at 10ng/mL with medium change every three days (Neg/TGF- β 3). Therefore, in total we have 11 groups: Neg, Neg/TGF- β 3, Ad-N, LV-N, Ad-sh, LV-sh, Ad-T, LV-T, LV-T+Ad-sh, LV-sh+Ad-T and Ad-T+Ad-sh. Among them, LV-T+Ad-sh, LV-sh+Ad-T and Ad-T+Ad-sh are the ones we are particularly interested in, while the rest were used as controls.

For 3D culture, cells were used. For detailed procedures for 3D culture, please refer to section 3.2, page 58, except that cell density here was 2×10^6 cells/mL in alginate..

RNA isolation and qPCR analysis

Detailed procedures can be found in section 2.2, page 39.

Quantitative analysis of active TGF- β 3 expression by specific ELISA

Detailed procedures can be found in section 3.2, page 58.

Quantitative analysis of Col I protein levels with immunofluorescent staining

At day 30, SMSCs-alginate constructs from each group were dissolved in sodium citrate. The suspension was homogeneously aliquoted into the wells of 96-well plate and frozen. Then the solid phase of the mixture was freeze-dried overnight. The product was fixed with glutaraldehyde for 1hr and blocked with 10% (v/v) goat serum for 30min. The following procedures can be found in section 2.2, page 40.

Biochemical analysis

After 30 days of culture, constructs were taken out, washed 3 times with deionized water to remove the salt in the medium followed by freeze drying (36 hrs), and digested with 0.5 mL of papain per sample for biochemical analysis. [134] DNA content was assessed by applying the Hoechst 33258 dye assay (7.7pg DNA/cell). [22, 138] Dimethylmethylene

blue dye was added into the digested solution, and absorption at 525 nm was measured using a UV-VIS spectrophotometer (Multiskan spectrum, Thermo) to quantify sulfated GAG content. [136-137] Total collagen was quantified from the hydroxyproline content after hydrolysis (6N HCl at 115 °C for 18 hrs) and reaction with p-dimethylaminobenzaldehyde and chloramine-T. Absorption at 550 nm was measured using the spectrophotometer. [139] Results were normalized with cell number.

Histological analysis

Safranin-O and Masson's Trichrome staining were performed to detect GAG and collagen. For detailed procedures, please refer to Section 4.2, page 72.

5.3 Results

Gene expression profiles with various delivery vehicles

Three genes including Col II, aggrecan and Cartilage oligomeric matrix protein (Comp) were used as markers of chondrogenic differentiation. The mRNA levels of Col II in all the groups at day 15 and 30 were presented in Figure 5.3.a,b. All the control groups without TGF- β 3-encoding cassette (Neg, Ad-N, LV-N, Ad-sh, LV-sh) did not increase Col II levels as expected, except that Ad-sh enhanced the level by 2 fold at day 15. Ad-T elevated the expression of Col II significantly, especially in the short term by 3 fold at day 15, whereas LV-T had a better effect at day 30 by increasing the level by 2 fold. Among the three groups with mixed adenoviral or lentiviral transduction, LV-sh+Ad-T showed the highest level of Col II at both time points, with a fold increase of 9, while Ad-T+Ad-sh showed the least inductive effect.

Figure 5.3. Gene expression of Col II at day 15 (a) and 30 (b). RNA was isolated and reverse transcribed. 1 μ L of cDNA was used for real-time PCR. RPL4 was used as house-keeping gene for normalization.

As another important component of cartilage ECM apart from Col II, the levels of aggrecan mRNA levels were also investigated. According to Figure 5.4.a,b, similar to the expression profiles of Col II, the control groups without supplemented or endogenously

produced TGF- β 3 (Neg, Ad-N, LV-N, Ad-sh, LV-sh) showed little effect in aggrecan expression, whereas all other groups with supplemented TGF- β 3 (Neg/TGF- β 3) or endogenously expressed TGF- β 3 (Ad-T, LV-T, LV-T+Ad-sh, LV-sh+Ad-T, Ad-T+Ad-sh) elevated aggrecan levels remarkably. Among them, LV-T+Ad-sh demonstrated the highest aggrecan level of all at both times points.

Figure 5.4. Gene expression of aggrecan at day 15 (a) and 30 (b). RNA was isolated and reverse transcribed. 1 μ L of cDNA was used for real-time PCR. RPL4 was used as house-keeping gene for normalization.

Comp is a noncollagenous ECM protein and is another marker of cartilage turnover. According to Figure 5.5.a,b, it is observed that the control groups (Neg, Ad-N, LV-N, Ad-sh, LV-sh) did not increase the levels of Comp, or even decreased its expression, except that LV-N increased the level at day 15. All other groups with TGF- β 3 supplementation or expression had some effect on the induction of Comp expression. Among them, LV-T+Ad-sh demonstrated the highest induction. However, the other four groups were not stable in Comp induction, where elevation compared to Neg was seen at only one time point, either day 15 or day 30.

Figure 5.5. Gene expression of Comp at day 15 (a) and 30 (b). RNA was isolated and

reverse transcribed. 1 μ L of cDNA was used for real-time PCR. RPL4 was used as house-keeping gene for normalization.

Apart from the above three genes used as chondrogenic markers, the levels of Col I mRNA were measured with some of the groups to confirm Col I knock-down in those groups with viral vectors having Col I-targeting shRNA encoding cassette, as shown in Figure 5.6.a,b. Distinct expression profiles were observed between the two time points. At day 15, there was no significant difference among the control groups without Col I-targeting shRNA encoding cassette, except that LV-T elevated Col I level remarkably. However, at day 30, all these groups decreased Col I expression. Particularly, Col I mRNA level in LV-N was much lower than that in Neg. Among those groups with shRNA encoding cassette, Ad-sh, LV-T+Ad-sh and Ad-T+Ad-sh always reduced Col I expression compared with either Neg or Ad-N. In contrast, although LV-sh and LV-sh+Ad-T demonstrated lower levels of Col I compared to Neg, the levels were not significantly lower than LV-N, indicating a low efficiency of shRNA-encoding lentiviral vectors.

Figure 5.6. Gene expression of Col I at day 15 (a) and 30 (b). RNA was isolated and reverse transcribed. 1μL of cDNA was used for real-time PCR. RPL4 was used as house-keeping gene for normalization.

Besides, we also examined the expression of Col X, which is a marker of chondrocyte hypertrophy. It could be seen from Figure 5.7.a,b that either adenoviral or lentiviral transduction was able to decrease Col X expression significantly compared with Neg, regardless of whether TGF-β3 was expressed. Exogenous supplementation of TGF-β3 also served to decrease Col X expression, since Neg/TGF-β3 also showed a lower level of Col

X compared to Neg. This has implied a positive effect on inhibition of cell hypertrophy with either viral transduction or TGF- β 3 dosing.

Figure 5.7. Gene expression of Col X at day 15 (a) and 30 (b). RNA was isolated and reverse transcribed. 1 μ L of cDNA was used for real-time PCR. RPL4 was used as house-keeping gene for normalization.

Active TGF- β 3 expression with various delivery vehicles

The amount of TGF- β 3 in the active form was measured in all the five groups that were transduced with viral vectors carrying TGF- β 3 encoding cassette. TGF- β 3 amount in Neg

was under detectable limit of current ELISA method. From Figure 5.8, it was observed that in the groups of LV-sh+Ad-T, Ad-T+Ad-sh and Ad-T, there was a much higher expression at the beginning of the culture and declined drastically in the short term till day 15, while the levels sustained during the latter 15 days. Among them, LV-sh+Ad-T and Ad-T+Ad-sh had the highest expression levels of TGF- β 3, reaching up to around 14ng/mL initially. Ad-T had a significantly lower initial expression levels, around 2ng/mL. The other two groups (LV-T+Ad-sh and LV-T) had even lower levels of TGF- β 3 expression. Despite the marked differential in the initial expression levels among these groups, they converged to a platform that ranged from 100 to 500pg/mL.

Figure 5.8. The amount of TGF- β 3 release during the culture period of 30 days. Medium was collected every 3 days and subjected to ELISA together with standard. Concentration was calculated against the standard curve.

GAG and total collagen levels with various delivery vehicles

With biochemical assays, the relative amount of GAG and total collagen normalized by cell number was obtained. The trends of GAG produced by each cell as shown in Figure

5.9.a were approximately consistent with the qPCR results of aggrecan. Control groups without TGF- β 3 supplementation or expression had comparable levels compared to Neg, while all the groups with TGF- β 3 demonstrated significantly higher levels of GAG produced by each cell than Neg. Among them, LV-T+Ad-sh had the highest level, reaching above $19 \times 10^{-4} \mu\text{g}/\text{cell}$.

The relative amount of total collagen expression in each cell was given in Figure 5.9.b. Similarly to Figure 5.9.a, the expression in the groups with either exogenously supplemented or endogenously produced TGF- β 3 showed higher levels of total collagen. Although Col I is expected to be suppressed in some of the groups, Col II remains the major ECM component and overwhelms Col I in quantity. Therefore, the quantity of total collagen gives a rough indicator of Col II, and it is no wonder that the amount was higher under the influence of TGF- β 3.

Figure 5.9. Biochemical assay for GAG (a) and total collagen quantification (b). After 30 days of culture, the beads were washed three times with deionized water, freeze-dried for 36hrs and digested with 1 mL of papain per sample for biochemical analysis. Dimethylmethylene blue dye was added into the digested solution, and absorption at 525 nm was measured using a UV-VIS spectrophotometer (Multiskan spectrum, Thermo) to quantify sulfated GAG content. The relative amount of total collagen was measured according to the hydroxyproline content after hydrolysis in 6N HCl at 115 °C for 18 hrs

and reaction with p-dimethylaminobenzaldehyde and chloramine-T. The absorption of the reaction product at 550 nm was determined using the spectrophotometer.

Quantity of Col I by quantitative immunofluorescent staining

With quantitative immunofluorescent staining, the relative amount of Col I could be approximately compared. From Figure 5.10, a decrease in the amount of Col I could be observed in groups of Ad-sh, LV-sh, LV-T+Ad-sh, LV-sh+Ad-T and Ad-T+LV-sh, all of which carried Col I-targeting shRNA cassette.

Figure 5.10. Quantitative analysis of Col I with immunofluorescent staining. At day 30, one bead from each group was dissolved in 55mM sodium citrate, and the suspension was homogeneously aliquoted into the wells of 96-well plate and frozen. Samples were freeze-dried for 12 hrs, then fixed with glutaraldehyde for 1hr and blocked with 10% (v/v) goat serum for 30min. After PBS wash, the specimen on the surface of the wells were incubated with mouse anti-pig Col I primary antibody (1:200, Santa Cruz Biotechnology, Santa Cruz, CA, USA) at room temperature for 2 hrs and AlexaFluor 546 rabbit anti-mouse IgG (1:200, Invitrogen, Carlsbad, CA, USA) for 1.5 hrs sequentially. After PBS washes, fluorescent

intensity in each well was measured at the excitation wavelength of 560nm and emission wavelength of 590nm. Each group was measured in triplicate.

Chemical staining

Safranin O and Masson's trichrome staining were performed in some of the groups to visualize GAG and total collagen, respectively. According to Figure 5.11.a,b, the groups of LV-T+Ad-sh, LV-sh+Ad-T and Ad-T+Ad-sh all showed the appearance of GAG and total collagen, as stained in red and blue, respectively. Among them, LV-T+Ad-sh had the most obvious visualization of GAG. (Figure 5.11.a) This is in correspondence to the qPCR result of aggrecan. In Figure 5.11.b, it could be observed that LV-sh+Ad-T had the most apparent staining of collagen, which is consistent with the qPCR result for Col II.

Figure 5.11. Safranin O (a) and Masson's trichrome staining (b) for GAG and collagen, respectively. Scale bar: 100μm.

5.4 Discussion

As introduced previously, in the application of SMSCs to engineer articular cartilage tissue, TGF-β3 has been substantiated as a potent inducer for

chondrogenesis. On the other hand, RNAi is essential to reduce the expression of Col I so that the engineered articular cartilage tissue is Col I-free. Since adenoviral and lentiviral vectors are both mediators for gene delivery with different performance manners, we constructed both adenoviral and lentiviral vectors, and co-transduced SMSCs with adenoviral and/or lentiviral vectors in order to deliver both TGF- β 3 and Col I-targeting shRNA to the cells in 3D culture, aiming at finding out if they are capable of engineering competent cartilage construct. As chondrogenesis was obvious at day 42 as seen in the previous chapter, in this chapter and the following, we cultured the cells in 3-D hydrogel beads for 30 days to test if chondrogenesis could occur within a shorter period of time. This is of practical significance, since a minimum in vitro culture period is conducive for the treatment of the patient. Besides, the group of LV-T+LV-sh was absent in our study due to technical difficulties. Since only a proportion of cells were successfully transduced with lentiviral vectors as observed with green fluorescence, if we want to transduce both LV-T and LV-sh into the same cells, it's impractical to do the transduction of both two viral vectors simultaneously. Rather, the cells should be transduced sequentially. That means the cells would have to go through two rounds of lentiviral transduction and FACS, which will bring much more damage to cell viability. Besides, the duration for the preparation of this group would be enormous, since the cells would be transduced, sorted and amplified before the next round of transduction, cell sorting and amplification. This is also impractical in terms of minimizing preparation period for patient treatment.

According to ELISA results, TGF- β 3 secretion in LV-sh+Ad-T, Ad-T+Ad-sh and Ad-T was much higher initially, while the amount declined dramatically and remained stable

afterwards. All these groups share a common feature, that is, they all contain Ad-T. Since adenoviral vectors perform in an episomal manner, they gradually degenerate with each cycle of cell replication and cell apoptosis, which contributed to the decline of TGF- β 3 release. Besides, these groups with Ad-T transduction had much higher expression levels initially. This is attributed to the high transduction MOI (200) of adenoviral vectors, whereas MOI for lentiviral vectors was below 10. Even at the end of the 30-day culture period, TGF- β 3 release amount was still a bit higher than LV-T transduced groups. The various TGF- β 3 release profiles in these groups, including release timescale and amount, have contributed to the distinct induction efficiencies.

For Col I suppression, no significant difference was observed at both time points between the three groups of mixed adenoviral and lentiviral transduction (LV-T+Ad-sh, LV-sh+Ad-T and Ad-T+Ad-sh). In terms of the chondrogenic induction efficiency of SMSCs, the levels of Col II, aggrecan and Comp were assessed, all of which are essential ECM constituents of cartilage. Comprehensively in consideration of the above three factors, among the three groups with mixed adenoviral and lentiviral transduction, LV-sh+Ad-T had higher efficiency in Col II induction but lower expression of aggrecan and Comp than the other two groups, although the expression levels were still higher than Neg. LV-T+Ad-sh had good effect in the induction of aggrecan and Comp, while Col II induction efficiency is lower than LV-sh+Ad-T. Ad-T+Ad-sh gives intermediate induction levels of all the three markers.

In conclusion, we have used various combinations of adenoviral and lentiviral vectors to deliver both TGF- β 3 and Col I-targeting shRNA to SMSCs and examined their effect on Col I-suppressed chondrogenesis in 3-D culture. The various vectors conferred different expression levels of TGF- β 3 and Col I-targeting shRNA, which account for the difference in the expression profiles of all the markers during the chondrogenic process. Among the three modes of co-transduction with adenoviral and/or lentiviral vectors, LV-T+Ad-sh was

able to elicit the best chondrogenic effect in comprehensive consideration, although Col II induction was not significant. However, longer effect of these modes remains to be explored in the future.

Chapter 6 *In Vitro* Study of Chondrocyte Redifferentiation with Lentiviral Vector-Mediated Transgenic TGF- β 3 and shRNA Suppressing Col I in 3D Culture

6.1 Introduction

In our previous chapters, we have been utilizing SMSCs as the source of therapeutic cells for Col I-suppressed chondrogenesis. In this chapter, we are trying to use another conventional cell source- chondrocytes, for the construction of the neotissue. Chondrocytes are isolated, amplified *in vitro*, encapsulated in alginate hydrogel for *in vitro* chondrogenesis and then transplanted into the injured site of the patient. However, a serious drawback inherent to this strategy is the incidence of chondrocytic dedifferentiation during *in vitro* amplification in monolayer culture. [140-141] In the course of dedifferentiation, chondrocytes gradually lose their normal morphology and undergo phenotypic changes with down-regulation of hyaline cartilage-specific markers such as Col II and aggrecan, accompanied with up-regulation of Col I that is a marker of fibrous cartilage. [142-143] All these events in dedifferentiation would result in formation of tissues that differ significantly from native cartilage and thus cannot withstand the demanded mechanical load. Strategies have been adopted to suppress the dedifferentiation and enable redifferentiation in dedifferentiated chondrocytes. It has been proved that TGF- β 3 is able to maintain and regain the chondrocytic phenotype from dedifferentiation. [84-85] It is also reported that 3D cultural conditions *per se* could lead to the redifferentiation of chondrocytes, whereby cells retain their phenotype in such scaffolds as Col II sponge [144], agarose [62], alginate [145-146], fibrin glue [147] and PLGA-collagen hybrid mesh[148]. In light of these facts, our dual-functioning viral vectors described in previous chapters provide a pathway to deliver both TGF- β 3 and Col I-targeting shRNA for Col I-

suppressed redifferentiation of chondrocytes in 3D cultural condition.

We have previously constructed a single-functioning lentiviral vector that expresses TGF- β 3 (LV-T) and optimized the configuration of dual-functioning lentiviral vectors that express both TGF- β 3 and Col I-targeting shRNA (LV-D) in terms of GOI inserting sites and orientations in vector genome. [149] In this chapter, we applied LV-T and the optimized LV-D (LV-1 in Chapter 4) to deliver genes of TGF- β 3 and Col I-targeting shRNA in pursuit of chondrocyte redifferentiation. Non-transduced and transduced chondrocytes were cultivated in monolayer over five passages to produce dedifferentiated chondrocytes with 2D cell sub-cultures. We then assessed if the lentiviral vectors were able to initiate and facilitate chondrocyte redifferentiation in 3D culture. Moreover, Col I silencing was also conducted and assessed in 3D culture aiming at constructing a Col I-suppressed cartilaginous tissue for cartilage repair.

6.2 Materials and methods

Materials

Single-functioning lentiviral vector that expresses TGF- β 3 (LV-T) and dual-functioning lentiviral vector that expresses both TGF- β 3 and Col I-targeting shRNA (LV-D; LV-1 as in Chapter 4) have been constructed and described in Chapter 4. [149]

Porcine chondrocyte isolation and culture

Detailed procedures can found in section 2.2, page 38.

Lentiviral transduction and selection of ZsGreen+ cells

Detailed procedures can be found in section 3.2, page 57.

2D monolayer culture of chondrocytes

Chondrocytes were cultured in flasks for 5 passages. Cell passaging was performed when cells reach 80~90% confluence. Briefly, cells were detached by incubation with trypsin for 3 minutes and neutralized with fresh medium conditioned with FBS. After being centrifuged, the cells were resuspended in fresh medium and split into flasks at a ratio of 1:3.

At each passage, 1×10^3 cells were transferred into one well of 96-well plate for quantitative Col II immunofluorescent staining or MTT assay 3 days later. 1×10^4 cells were transferred into one well of 24-well plate. Medium was collected 3 days later for the quantification of TGF- β 3 with ELISA. RNA of P2 and P5 cells was isolated from the cells for PCR.

3D redifferentiation of chondrocytes

At Passage 5, cells were encapsulated in alginate beads as stated in Section 3.2. Medium was replaced and collected for ELISA assay every 3 days. Chondrocytes without transduction were used as negative control (Neg). In total, there were three groups: Neg, LV-T and LV-D.

RNA isolation and qPCR analysis

Detailed procedures can be found in section 2.2, page 39, except that 300 ng of RNA was used for reverse transcription.

Quantitative analysis of active TGF β 3 expression by specific ELISA

Detailed procedures can be found in section 3.2, page 58.

MTT assay

Cells in monolayer culture of 96-well plates were incubated with 100 μ l of MTT solution

for 4 hrs. Then MTT solution was replaced with 100 μ l of DMSO for 20 min. The absorbance of the solution was detected at 490nm.

Quantitative immunofluorescent staining of Col I and Col II protein

Detailed procedures can be found in section 2.2, page 40 and section 4.2, page 71.

Biochemical analysis

Detailed procedures can be found in section 5.2, page 88.

Statistical analysis

The results are indicated as mean $\pm$ SD. Where appropriate, student t-test was used to analyze results and difference was considered to be statistically significant at $P < 0.05$.

6.3 Results

Chondrocyte dedifferentiation during monolayer culture

The expression of Col II and aggrecan, the two markers of chondrocytes, was explored with P1, P2 and P5 cells during monolayer culture. From Figure 1a and Figure 1b, it was observed that in each group (Neg, LV-T and LV-D), there was a declining trend for the expression of both Col II and aggrecan. P2 cells demonstrated remarkably lower expression of both genes. The levels were even lower in P5 cells, indicating the incidence of dedifferentiation of chondrocytes during monolayer culture for five passages. Col II and aggrecan expression in LV-T and LV-D groups were comparable to or lower than that of Neg group, indicating that delivery of TGF- β 3 or TGF- β 3 in conjunction with shRNA targeting Col I does not induce redifferentiation in 2D monolayer culture. The dedifferentiated cells produced by passaging can be used for redifferentiation purpose in 3D culture.

Figure 6.1. Gene expression levels in monolayer culture for 5 passages. a) Col II expression; b) Aggrecan expression. RNA of chondrocytes with various treatments in 2D culture was isolated and reverse transcribed. qPCR was used to quantify relative mRNA levels with the comparative $\Delta\Delta C_T$ (threshold cycle) method, with which gene expression levels were normalized by the housekeeping gene PRL4.

TGF- β 3 expression during monolayer culture

As insufficient P1 cells were obtained from FACS, we performed the ELISA and MTT

assays in the absence of P1 cells. From ELISA result shown in Figure 2a, an increased secretion of TGF- β 3 was observed with higher passages in both LV-T and LV-D. The expression level was only between 400 and 500 ng/mL in P2 cells, while the amount increased with each passage and reached 4000~5000 ng/mL in P5 cells. Comparing LV-T and LV-D, there was no significant difference at P2 and P5, whereas the expression in LV-D was slightly higher than that in LV-T in P3 and P4 cells.

Cell viability during monolayer culture

From MTT result shown in Figure 2b, an increasing trend was observed with higher passages for each group. P2 cells showed much lower proliferative activity than P3, P4 and P5 cells, while the activity generally stabilized afterwards, although there were some fluctuations between P3, P4 and P5 cells. Difference was insignificant between LV-T and LV-D.

Figure 6.2. TGF-β3 quantification and cell proliferation in monolayer culture over 5 passages. a) The amount of TGF-β3 measured by ELISA. Conditioned medium collected from 2D monolayer culture in the two groups (LV-T, LV-D) was tested using the specific TGF-β3 ELISA kit. Triplicates were performed for each sample. b) Cell proliferation measured with MTT assay. Cells in monolayer culture in 96-well plates were tested with MTT reagents to determine the relative cell proliferative activity. * $p < 0.05$ as compared to Neg.

3.4 Morphological changes during monolayer culture

The morphology of cells at each passage during 2D culture is presented in Figure 6.3. With the increase of cell passages, the cells in all the three groups tended to elongate, turning from the common cobblestone shape to spindle-like morphology, one of the typical characteristics of chondrocyte dedifferentiation. Among the three groups, LV-T and LV-D resulted in a more apparent morphological change. P3 cells in LV-T and LV-D groups demonstrated even higher degree of elongation than those in the Neg group.

Figure 6.3. Chondrocyte morphology in monolayer culture over 4 passages. At each passage during monolayer culture, photos were taken for the cells to visualize their morphology.

3.5 Gene expression profiles in 3D culture

The expression of Col II and aggrecan was assessed with qPCR at day 0, 15 and 30 to see if redifferentiation occurred in 3D culture. It could be seen from Figure 4a and Figure 4b that Neg group exhibited negligible amount of Col II and aggrecan expression whereas both LV-T and LV-D groups had significantly higher expression levels of both genes at day 15 and 30 than Neg group, except that there was no significant difference in aggrecan expression at day 30 between LV-D and Neg groups.

The expression of Col I was also assessed with qPCR to find out if Col I-targeting shRNA encoding cassette functioned to suppress Col I expression (Figure 4c). At all the three time points, LV-T transduction resulted in a significantly higher level of Col I. However, in LV-D, with the introduction of Col I-targeting shRNA cassette, the expression of Col I was significantly down-regulated to a level similar to that in Neg group and much lower than that in LV-T group.

Figure 6.4. Gene expression of chondrocytes at day 0 (P5 cells), 15 and 30 in 3D hydrogel culture. a) Col II; b) aggrecan; c) Col I. Alginate beads containing chondrocytes in each group were dissolved with sodium citrate, and RNA was isolated from the released chondrocytes. qPCR was performed to determine the relative mRNA levels of various genes. * $p < 0.05$ as compared to Neg; ** $p < 0.05$ as compared to LV-T for Col I levels.

3.6 TGF- β 3 release profiles

TGF- β 3 expression (Figure 5) reached 15~20 ng/mL during the first few days, while the amount declined significantly to around 5ng/mL in the latter days. Generally, LV-T group showed a higher expression of TGF- β 3 than LV-D group.

Figure 6.5. Quantification of TGF- β 3 expressed by chondrocytes in 3D culture for 30 days. Conditioned medium collected from 3D culture in the two groups (LV-T, LV-D) was tested using the specific TGF- β 3 ELISA kit. Triplicates were performed for each sample.

Cell number, protein and GAG quantification in 3D culture

After 30 days of culture in 3D alginate beads, there were around $2.3\text{--}2.5 \times 10^5$ cells in each bead of Neg or LV-T group, and about 1.4×10^5 cells in each bead of LV-D group, as determined by biochemical assays shown in Figure 6a. The amount of GAG and total

collagen in each bead was also determined using biochemical assays and represented in Figure 6b1 and Figure 6c1. The results indicate that, in both LV-T and LV-D groups, gene transduction promoted the production of GAG and total collagen, among both of which, if counting per construct, production in LV-T was elevated to higher levels than that in LV-D; if counting per cell, the production profile turned reversely - LV-D exceeding LV-T in yields of extracellular matrices.

In quantitative immunofluorescent staining, Col I and Col II protein levels were determined (Figure 6d and Figure 6e). In accordance with qPCR results, LV-T elevated Col I levels, whereas LV-D offset the elevation by significantly decreasing Col I expression. On the other hand, both LV-T and LV-D showed higher levels of Col II after 30 days of 3D culture.

Figure 6.6. Quantification of cell number, Col I and Col II proteins, GAG and total collagen in each bead 30 days after 3D culture. a) Cell number in each alginate bead at day 30; b1) The amount of GAG in each bead as determined with biochemical assay; b2) The amount of GAG produced in each cell as determined with biochemical assay; c1) The amount of total collagen in each bead as determined with biochemical assay; c2) The

amount of total collagen produced in each cell as determined with biochemical assay; d) Relative amount of Col I protein as measured by quantitative immunofluorescent staining; e) Relative amount of Col II protein as measured by quantitative immunofluorescent staining. * $p < 0.05$ as compared to Neg; ** $p < 0.05$ as compared to LV-T for Col I levels.

6.4 Discussion

In the practice for curing cartilage diseases and trauma, a noteworthy problem involves the incidence of chondrocyte dedifferentiation during monolayer culture. In this process, chondrocytes lose their phenotype and decrease the expression of chondrocyte-specific markers such as Col II and aggrecan. The alterations in phenotype and gene expression profiles result in dedifferentiated chondrocytes that are not suitable for transplantation. 3D culture of chondrocytes was substantiated to be able to reverse this process. Biochemical stimuli such as TGF- β 3 have also been explored in terms of their capability in initiating chondrocyte redifferentiation. Gene delivery methods have been investigated to deliver recombinant proteins to the cells. Among the methods, viral delivery received special attention due to its high efficiency.

In our research, we constructed lentiviral vectors carrying either TGF- β 3-encoding cassette or a combination of TGF- β 3-encoding cassette and Col I-targeting shRNA encoding cassette. These viral vectors were transduced into early passage chondrocytes before cell amplification in monolayer. According to our results obtained in 2D experiments, transduction of the lentiviral vectors accelerated chondrocyte dedifferentiation during monolayer culture. A phenomenon observed was the increase of cell proliferation after viral transduction, as assessed with MTT assay. The proliferative activity also increased along the passages, which might be a reason contributing to the increase of TGF- β 3 expression at later passages. Despite the increase in cell viability and TGF- β 3 expression, dedifferentiation still occurred in chondrocytes. Col II and aggrecan

expression decreased from P2 to P5 in LV-T and LV-D, as occurred in Neg. Moreover, the expression of these two chondrocytic markers in LV-T and LV-D was even lower than that in Neg. Drastic decrease was observed as early as P1. Therefore, transduction of LV-T or LV-D exacerbated chondrocyte dedifferentiation in monolayer culture. At the end of monolayer culture, we have obtained dedifferentiated chondrocytes, which were used in subsequent experiments for redifferentiation in 3D culture.

On the other hand, despite the dedifferentiation in monolayer culture, LV-T and LV-D were effective in promoting chondrocyte redifferentiation in 3D culture within alginate hydrogel. 3D culture has been substantiated to be helpful to restore the expression of chondrocyte markers. [62, 144, 148] In this study, it was further proved that lentiviral vector-mediated transgenic TGF- β 3 was efficient in inducing chondrocyte redifferentiation in 3D culture. After 15 or 30 days of culture in hydrogel, the expression of Col II and aggrecan significantly increased in LV-T and LV-D groups compared to Neg group, indicating re-expression of these two ECM components and cell redifferentiation towards chondrocytic phenotype. However, it was noted that aggrecan mRNA level in LV-D was much lower than that in LV-T at day 30. We hypothesized that with the accumulation of aggrecan along the culture period, higher amount of aggrecan (measured as GAG per cell) poses negative feedback to aggrecan gene expression, which results in low aggrecan expression at mRNA level at day 30, as indicated in Figure 6b2. Further tests are to be carried out to substantiate the hypothesis. TGF- β 3 expression, in general, declined over the 30 days of culture and stabilized at a platform of around 5ng/mL. The secreted TGF- β 3 was supposed to be responsible for the redifferentiation process in 3D culture. Besides, Col I-targeting shRNA encoding cassette was incorporated in LV-D group, and it was observed to be effective in suppressing Col I expression. Although Col I expression was not completely inhibited, Col I expression was greatly reduced in LV-D group compared to that of LV-T. TGF- β 3 has been proved to elicit the expression of Col I according to

references. [92] Incorporation of Col I-targeting shRNA in LV-D group was able to offset the increase elicited by TGF- β 3. Therefore, incorporation of Col I-targeting shRNA encoding cassette is beneficial to Col I-suppressed chondrocyte redifferentiation.

In conclusion, lentiviral vector-mediated transgenic TGF- β 3 expression has been successfully manipulated to facilitate chondrocyte redifferentiation in 3D culture, based on which the incorporation and expression of transgenic Col I-silencing shRNA further manages to offset the undesired upregulation of Col I production elicited by the transgenic TGF- β 3 in a post-transcriptional manner. Establishment and investigations of this transgene-enhanced chondrocytic redifferentiation model would benefit the efforts in reconstruction of non-fibrous cartilaginous tissue for regenerative applications.

Chapter 7 Conclusions and future work

7.1 Conclusions

In our study, a collection of recombinant adenoviral and lentiviral vectors were constructed that could express TGF- β 3 or Col I-targeting shRNA (single-functioning), or both of them (dual-functioning), aiming at directing chondrogenesis while suppressing Col I expression with SMSCs or chondrocytes in 3D alginate hydrogel system. TGF- β 3 expression was expected to initiate chondrocyte differentiation, while shRNA was targeted toward downregulating Col I expression, which was either intrinsically expressed in SMSC and dedifferentiated chondrocytes, or upregulated by the introduction of TGF- β 3.

In Chapter 2, the dual-functioning adenoviral vector (Ad-D) was constructed, and the optimal transduction condition- MOI of 200 or 500 was determined for fibroblasts, osteoblasts and chondrocytes. Ad-D was found to be effective in the delivery of TGF- β 3 and Col I suppression in these types of cells and SMSCs in 2D culture.

In Chapter 3, in consideration that a sustained expression of transgene (particularly for Col I-targeting shRNA) might be favored for Col I-suppressed articular chondrogenesis, lentiviral vector might be more beneficial due to its integrative manner. Therefore, a dual-functioning lentiviral vector (LV-D) that has the same arrangement of transgenes with Ad-D was constructed. Its titration and transduction methods were established. A modified ELISA protocol without hydrochloric acid activation step was also established with LV-D to measure the amount of TGF- β 3 in the active form. Besides, DMEM was selected as the optimal medium for 2D monolayer culture of SMSCs due to better phenotype maintenance and Col I suppression.

As the arrangement of two expression cassettes may affect their transcription and even lentiviral production, it will lead to various expression profiles of TGF- β 3 and Col I-targeting shRNA, and finally different results in Col I-suppressed chondrogenesis.

Therefore, in Chapter 4, three more LV-Ds were constructed in addition to the one described in Chapter 3. These vectors differ in the arrangement of TGF- β 3 and shRNA expression cassettes, which have different relative positions and orientations with each other. Among them, LV-1, in which the two expression cassettes were arranged far apart in reverse directions, was found to be optimal in inducing Col I-suppressed chondrogenesis in SMSCs within 3D alginate hydrogel culture.

The above dual-functioning viral vectors induce either a transient (adenoviral) or sustained (lentiviral) expression of both transgenes simultaneously. However, the delivery of TGF- β 3 and Col I-targeting shRNA might possibly require different delivery vectors to modulate the expression duration and profiles. Therefore, in Chapter 5, instead of using dual-functioning viral vectors, various combinations of single-functioning adenoviral and lentiviral vectors were devised for the co-delivery of TGF- β 3 and Col I-targeting shRNA. It was concluded that the combination of lentiviral-mediated TGF- β 3 expression and adenoviral-mediated shRNA expression was optimal in Col I-suppressed chondrogenesis in 3D alginate hydrogel.

In Chapter 6, a preliminary study was conducted by introducing lentiviral vectors into another important cell source for articular cartilage engineering – chondrocytes, to investigate their effect on chondrocyte dedifferentiation and redifferentiation. LV-D (LV-1 as in Chapter 4) was proved to be effective in inducing redifferentiation and suppressing Col I expression in dedifferentiated chondrocytes within 3D alginate hydrogel system.

In conclusion, in this PhD study, various strategies were adopted to deliver both TGF- β 3 and Col I-targeting shRNA to SMSCs or chondrocytes, including the delivery of dual-functioning viral vectors and co-delivery of two single-functioning viral vectors. One LV-D with particular transgene arrangement (LV-1), and the combination of TGF- β 3-expressing lentiviral vector and shRNA-encoding adenoviral vector were found to be relatively more effective than others in inducing chondrogenesis in SMSCs in 3D alginate

hydrogel system. Besides, LV-D was also found to be effective in initiating chondrocyte redifferentiation in 3D alginate hydrogel. Our results suggest that our pool of recombinant adenoviral and lentiviral vectors could be promising candidates for the engineering of articular cartilage.

7.2 Future works

In this PhD study, the potential of dual-functioning and single-functioning adenoviral/lentiviral vectors to deliver both TGF- β 3 and Col I-targeting shRNA was investigated to see their effect on Col I-suppressed chondrogenesis with SMSCs or chondrocytes in 3D alginate hydrogel culture. Some of these vectors or their combinations, e.g., LV-1, have proved to be promising in initiating chondrogenesis while suppressing Col I expression. However, there are still some problems that need to be addressed and studied in the future.

Firstly, Col I-targeting shRNA was not able to downregulate Col I expression to sufficiently low levels. In most of the cases, the level of Col I in groups of dual-functioning viral vectors, although lower than that in TGF- β 3 dosed groups, was comparable to, or even higher than that in negative controls. shRNA encoding cassette only partially offset the elevated Col I level, but not completely. Therefore, more specific and efficient siRNA sequences should be designed to select those having better function of minimizing Col I expression. This would be of great significance to our current study.

Secondly, in Chapter 5, it was found that the combined delivery of TGF- β 3 and Col I-targeting shRNA with LV-T and Ad-sh (LV-T+Ad-sh) was better in the induction efficiency than other combined delivery methods. However, as adenoviral vector performs in the episomal manner while lentiviral vector is integrative, a sustained or even permanent release of TGF- β might adversely affect cell functions. Once chondrogenesis has occurred within a certain period of time, the release of TGF- β 3 might have to be terminated.

Therefore, more work should be done to study whether there is any adverse effect of sustained conditioning of TGF- β 3 to the cells, either SMSCs or chondrocytes. If this is the case, we can work out with adenoviral vectors to optimize the transduction conditions such as MOI, in order to control the release of TGF- β 3 in timing and amount. Besides, it is worthwhile to study whether chondrocytes, either differentiated from SMSCs or redifferentiated from dedifferentiated chondrocytes after a period of 3D culture, still express Col I. If this is the case, then lentiviral vector is required to permanently suppress its expression. Therefore, a longer study period, e.g., six months, is needed to determine the optimal gene delivery strategy.

Thirdly, some other cell sources could be investigated for chondrogenesis with the delivery strategies of the viral vectors described in previous chapters. Although chondrocytes and SMSCs are relatively more accessible than some other cell source such as BMSCs, their derivation still brings pain to the patients. Nowadays, induced pluripotent stem cells (iPS cells) are emerging as a promising cell source for reprogramming due to their pluripotency and non-invasive derivation. Therefore, we could determine the efficiency of our viral delivery systems on iPS cells for chondrogenesis.

Moreover, in our study we mainly focused on the biological behaviors of the source cells under the treatment of recombinant viral vectors, e.g., gene expression, production of individual ECM components (GAG, Col II). However, the final objective is to engineer an articular cartilage construct that could possibly be used for transplantation to withstand the mechanical loading and facilitate joint motion. In other words, mechanical properties of the engineered tissue are crucial in determining the potential of the construct. Therefore, in our future work, mechanical characteristics, e.g., Young's modulus, of the engineered tissue would be tested and compared to that of the native cartilage. In cases that the properties are distinctively different between the engineered and native tissues, strategies in our articular cartilage engineering should be adjusted such as in biomaterial selection.

We have had some preliminary data indicating the efficiency of dual-functioning lentiviral vector in inducing chondrocyte redifferentiation. As an extension of the previous work, we can apply other vectors, e.g., dual-functioning adenoviral vector and combinations of single-functioning adenoviral/lentiviral vectors, to chondrocytes in order to find out the optimal delivery mode.

Last but not least, after all the above *in vitro* work, *in vivo* experiment should be initiated to assess the efficacy of our engineered articular cartilage tissues. At first, nude mice can be utilized to observe cell behaviors *in vivo*. A further step will be the use of pathological animal models which have cartilage degeneration, e.g., rabbits with cartilage deficiency, for the appraisal of the engineered articular cartilage constructs, in the final objective to investigate if they could restore the structure and functions of native articular cartilage.

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PUBLICATION LIST

Zhang, F., Yao, Y. C., Zhou, R. J., Su, K., Citra, F., Wang, D. A.*, “Optimal Construction and Delivery of Dual-Functioning Lentiviral Vectors for Type I Collagen-Suppressed Chondrogenesis in Synovium-Derived Mesenchymal Stem Cells”, *Pharmaceutical Research*, in press. DOI: 10.1007/s11095-010-0305-5

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