Construction and application of bi-functional adenoviral vectors for engineered articular chondrogenesis

Yao, Yongchang

2010

Yao, Y. C. (2010). Construction and application of bi-functional adenoviral vectors for engineered articular chondrogenesis. Doctoral thesis, Nanyang Technological University, Singapore.

<https://hdl.handle.net/10356/42512>

<https://doi.org/10.32657/10356/42512>

NANYANG
TECHNOLOGICAL
UNIVERSITY

**CONSTRUCTION AND APPLICATION OF
BI-FUNCTIONAL ADENOVIRAL VECTORS FOR
ENGINEERED ARTICULAR CHONDROGENESIS**

YAO YONGCHANG
**SCHOOL OF CHEMICAL AND BIOMEDICAL
ENGINEERING**

2010

**Construction and Application of Bi-Functional Adenoviral
Vectors for Engineered Articular Chondrogenesis**

YAO YONGCHANG

School of Chemical and Biomedical Engineering

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

2010

ACKNOWLEDGEMENTS

First and foremost, I would like to express my deepest gratitude to Associate Professor Wang Dong-An, my supervisor, for his illuminating and comprehensive instruction, as well as consistent enthusiastic support. With his constant encouragement and patient guidance, I accumulate my knowledge of gene therapy and tissue engineering from blank background and taste the joy of success from research. He also helps me like a friend when I suffer from difficulties, which would make my Ph.D. experience smooth and wonderful. I am also greatly indebted to Nanyang Technological University (NTU) for giving me a good chance to improve myself, as well as School of Chemical and Biomedical Engineering (SCBE) and all its excellent faculties, researchers and officers.

Next, I wish to express my heartfelt thanks to all the friendly lab mates and research fellows in this united and energetic team. 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 enthusiastic help; Mr Rohan Rajeev Varshney, for giving me valuable suggestions in manuscript writing; and Dr Chunming Wang, Mr Feng Zhang, Mr Kai Su and Ms Tingting Lau, for their endless support and precious friendship. 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 Ms Xiaotian Pang (2009-2010), Mr Meng Li (2008-2009) and Mr Hao Li (2007-2008), for their diligence 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 hope to express my sincere gratitude to my beloved parents and my darling Mr Ke Zeng. Without their great confidence in me and utmost support as a strong backing, I would not have confidences to conquer the difficulties; without their loving considerations and encouragement all through these years, I would not be here today.

Say big “thank you” again to all of my friends, no matter mentioned or not, and my family. Without all of them, I would not gain my harvest.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS	I
TABLE OF CONTENTS	III
LIST OF TABLES	IX
LIST OF FIGURES	X
LIST OF ABBREVIATIONS	XII
SUMMARY	XIV
 Chapter 1 Introduction	 1
1.1 Cartilage profile	1
1.1.1 Articular cartilage anatomy	1
1.1.2 Current treatments for articular cartilage repair	4
1.2 Regenerative medicine for cartilage repair	5
1.2.1 Cartilage tissue engineering	5
1.2.1.1 Cell source	6
1.2.1.1.1 MSCs.....	6
1.2.1.1.2 Chondrocytes.....	7
1.2.1.1.3 Fibroblasts.....	8
1.2.1.2 Biological molecules for cartilage tissue engineering	9
1.2.1.2.1 Growth factors.....	10
1.2.1.2.2 Other gene candidates	12
1.2.1.3 Scaffolds	12
1.2.2 Delivery Systems for regenerative medicine	14
1.2.3 Antisense makes sense in engineered regenerative medicine	16

1.3 Objective and outline of this thesis.....	20
Chapter 2 Chondrogenesis of rabbit mesenchymal stem cells via adenoviral vector-mediated TGF-β3 expression	22
2.1 Introduction	22
2.2 Materials and methods	23
2.2.1 Preparation of high-titer Ad- TGF β 3	23
2.2.1.1 Construction of Ad-TGF β 3	23
2.2.1.1.1 Obtaining target gene --- TGF- β 3	25
2.2.1.1.2 Constructing shuttle vector	25
2.2.1.1.3 Constructing recombinant adenoviral vector.....	26
2.2.1.1.4 Producing recombinant adenovirus.....	26
2.2.1.2 Amplification of Ad-TGF β 3.....	27
2.2.1.2.1 Determination titer of recombinant adenovirus.....	27
2.2.1.2.2 Preparing high-titer recombinant adenoviral stocks.....	27
2.2.1.2.3 Purification of Ad-TGF β 3.....	28
2.2.2 Isolation of rabbit SMSCs.....	28
2.2.3 3D-culture of SMSCs in alginate hydrogel	29
2.2.4 RNA isolation and qRT-PCR analysis.....	30
2.2.5 Histology and immunohistochemistry.....	30
2.2.6 Quantitative analysis of TGF- β 3 expression by specific ELISA.....	31
2.2.7 Statistical analysis	31
2.3 Results and discussion	32
2.3.1 Preparation of high-titer Ad-TGF β 3	32
2.3.1.1 Construction of Ad-TGF β 3	32
2.3.1.2 Amplification and purification of Ad-TGF β 3.....	34

2.3.2 Chondrogenesis of rabbit SMSCs via adenoviral vector-mediated TGF- β 3 expression.....	34
2.3.2.1 Cartilage-specific genes expression	34
2.3.2.2 Histology and immunohistochemistry.....	35
2.3.2.3 Kinetic expression of TGF- β 3.....	37
2.4 Summary	38

Chapter 3 Mechanism for up-regulation of type I collagen by adenoviral vector-mediated TGF- β 3.....40

3.1 Introduction	40
3.2 Materials and methods	40
3.2.1 Cell culture	40
3.2.2 Construction of adenoviral vectors	41
3.2.3 Adenoviral transduction of HDFs	41
3.2.4 3D-culture in alginate hydrogel.....	41
3.2.5 RNA isolation and qRT-PCR	41
3.2.6 Quantitative analysis of TGF- β 3 expression by specific ELISA.....	42
3.2.7 Cell proliferation.....	43
3.2.8 Statistical analysis	43
3.3 Results.....	43
3.3.1 Adenoviral vector-mediated TGF- β 3 enhanced expression of type I collagen in 3D alginate construct of HDFs.....	43
3.3.2 Adenoviral vector-mediated TGF- β 3 promoted Smad 4 expression.....	45
3.3.3 Adenoviral vector-mediated TGF- β 3 inhibited MMP-9 expression.....	46
3.3.4 Adenoviral vector-mediated TGF- β 3 promoted viability of HDFs in 3D alginate construct.....	47
3.4 Discussion.....	48

3.5 Summary.....	50
Chapter 4 Inhibition of Col I expression by RNAi.....	51
4.1 Introduction.....	51
4.2 Materials and methods	51
4.2.1 Preparation of high-titer Ad-shRNA.....	51
4.2.1.1 Construction of shuttle vector.....	53
4.2.1.2 Construction of recombinant adenoviral vector.....	54
4.2.1.3 Generation of Ad-shRNA.....	54
4.2.2 Cell culture.....	55
4.2.3 Adenoviral transduction of HDFs and hFOB5.....	55
4.2.4 RNA isolation and qRT-PCR.....	55
4.2.5 Statistical analysis	56
4.3 Results and discussion	56
4.3.1 Preparation of high-titer Ad-shRNA	56
4.3.2 Effect on inhibition of Col I by Ad-shRNA.....	57
4.4 Summary	60
Chapter 5 Effects of combinational adenoviral vector-mediated TGF-β3 transgene and anti-Col I shRNA on articular chondrogenesis of SMSCs.....	61
5.1 Introduction	61
5.2 Materials and methods	62
5.2.1 Preparation of high-titer Ad-double	62
5.2.2 Cell harvest.....	63
5.2.3 Adenoviral MOI optimization.....	63
5.2.4 3D-culture of porcine SMSCs in alginate hydrogel.....	64
5.2.5 RNA isolation and qRT-PCR analysis	65

5.2.6 Quantitative analysis of TGF- β 3 expression by specific ELISA	65
5.2.7 Flow cytometry analysis.....	66
5.2.8 Histology and immunohistochemistry.....	66
5.2.9 Statistical analysis	67
5.3 Results.....	67
5.3.1 Generation of high-titer Ad-double.....	67
5.3.2 MOI optimization of Ad-TGF β 3, Ad-shRNA and Ad-double.....	68
5.3.3 Influence of co-transducing process on transduction efficiency	75
5.3.4 Chondrogenic differentiation of SMSCs following transduction with various recombinant adenoviruses in 3D cultures.....	76
5.3.5 Histology and immunohistochemical staining.....	78
5.3.6 Corresponding transgene expression of TGF- β 3 for the 30-day culture period by ELISA analysis.....	81
5.4 Discussion.....	82
5.5 Summary.....	85

Chapter 6 Enhancing redifferentiation of dedifferentiated chondrocytes in 3D scaffold with dual transgenes of TGF- β 3 and shRNA silencing type I collagen.....87

6.1 Introduction	87
6.2 Materials and methods	88
6.2.1 Porcine chondrocytes isolation and expansion.....	88
6.2.2 Adenoviral transduction of dedifferentiated chondrocytes in monolayer.....	88
6.2.3 3D-culture of dedifferentiated chondrocytes in alginate hydrogels.....	89
6.2.4 RT-PCR and qRT-PCR.....	89
6.2.5 Quantitative analysis of TGF- β 3 expression and GAGs synthesis.....	90
6.2.6 Flow cytometry analysis.....	91

6.2.7 Histology and immunohistochemistry.....	91
6.2.8 Statistical analysis.....	91
6.3 Results.....	91
6.3.1 Chondrocyte dedifferentiation.....	91
6.3.2 MOI optimization of Ad-TGF β 3, Ad-shRNA and Ad-double.....	92
6.3.3 Influence of co-transducing process on transduction efficiency.....	98
6.3.4 Redifferentiation of dedifferentiated chondrocytes in monolayer.....	99
6.3.5 Redifferentiation of dedifferentiated chondrocytes in alginate hydrogels..	100
6.3.6 Quantitative analysis of TGF- β 3 expression.....	105
6.3.7 Analysis of GAGs deposition by histological staining and biochemical assays.....	107
6.3.8 Immunohistochemical staining for Col I and Col II.....	109
6.4 Discussion.....	111
6.5 Summary.....	114
Chapter 7 Conclusions and future work	115
7.1 Conclusions	115
7.2 Future outlook	116
REFERENCES	119
PUBLICATION LIST	144

LIST OF TABLES

Table 1.1 Composition of articular cartilage and a summary of their functions.....	2
Table 1.2 A summary of current cartilage replacement therapies and their potential drawbacks.....	4
Table 2.1 Primers used for real-time PCR in Chapter 2.....	30
Table 2.2 Expression level of recombinant protein TGF- β 3 by ELISA analysis.....	38
Table 3.1 Primers used for real-time PCR in Chapter 3.....	42
Table 5.1 Primers used in obtainment of U6 promoter and shRNA.....	63
Table 5.2 Primers used for real-time PCR in Chapter 5	65
Table 6.1 Primers used for PCR in Chapter 6.....	89
Table 6.2 Groups for 3D experiment.....	102

LIST OF FIGURES

Figure 1.1 Schematic diagram for structure of articular cartilage.....	3
Figure 1.2 Mechanism of RNAi in mammalian cells.....	18
Figure 2.1 Flow chart for construction of Ad-TGF β 3.....	24
Figure 2.2 SalI and PstI restriction analysis for shuttle vector.....	32
Figure 2.3 XhoI restriction analysis of recombinant adenoviral vector.....	33
Figure 2.4 Characterization of transfected HEK 293 cells.....	34
Figure 2.5 Gene expression of Col II and Col I.....	35
Figure 2.6 Histological and immunofluorescent staining.....	36
Figure 2.7 Expression level of adenoviral-mediated TGF- β 3 by ELISA analysis.....	37
Figure 3.1 Analysis of expression of Col I and dynamic secretion of TGF- β 3.....	44
Figure 3.2 Smad 4 gene expression.....	45
Figure 3.3 MMP-9 gene expression.....	46
Figure 3.4 Analysis of cell viability till Day 26.....	47
Figure 4.1 Flow chart for construction of Ad-shRNA.....	52
Figure 4.2 Structure of double-stranded oligonucleotide sequence encoding shRNA.....	53
Figure 4.3 Restriction analysis.....	57
Figure 4.4 Silencing effect on Col I expression of HDFs.....	58
Figure 4.5 Silencing effect on Col I expression of hFOBs.....	59
Figure 5.1 Schematic diagram of the produced Ad-double after digestion with PacI.....	63

Figure 5.2 XhoI Restriction analysis.....	68
Figure 5.3 MOI optimization of Ad-TGF β 3.....	70
Figure 5.4 MOI optimization of Ad-shRNA.....	72
Figure 5.5 MOI optimization of Ad-double.....	74
Figure 5.6 Transduction efficiency measured by flow cytometry.....	75
Figure 5.7 Analysis of expression of chondrogenic markers.....	77
Figure 5.8 Analysis of expression of Col I and Col X.....	78
Figure 5.9 Histological and immunohistochemical staining for GAGs, Col II and Col I.....	80
Figure 5.10 Quantitative analysis of hTGF- β 3 expression for the 30-day culture period by specific ELISA.....	82
Figure 6.1 Chondrocyte dedifferentiation in monolayer after serial passages.....	92
Figure 6.2 MOI optimization of Ad-TGF β 3.....	94
Figure 6.3 MOI optimization of Ad-shRNA.....	96
Figure 6.4 MOI optimization of Ad-double.....	97
Figure 6.5 Analysis of transduction efficiency by flow cytometry.....	98
Figure 6.6 Analysis of chondrocytic gene expression from genetically modified chondrocytes in monolayer cultures.....	100
Figure 6.7 Analysis of gene expression from genetically modified chondrocytes in alginate hydrogels.....	104
Figure 6.8 Dynamic changes of TGF- β 3 expression for the 30-day culture period by ELISA analysis.....	107
Figure 6.9 Analysis of GAGs production.....	109
Figure 6.10 Immunohistochemical staining for Col II and Col I.....	110

LIST OF ABBREVIATIONS

3D	three dimensional
ACT	autologous chondrocyte transplantation
Ad-null	recombinant adenovirus without any inserted gene
Ad-double	recombinant adenovirus encoding both TGF- β 3 and short hairpin RNA targeting Col I gene
Ad-shRNA	recombinant adenovirus encoding short hairpin RNA targeting Col I gene
Ad-TGF β3	recombinant adenovirus encoding TGF- β 3
BMPs	bone morphogenic proteins
BSA	bovine serum albumin
CDS	coding sequence
Col I	type I collagen
Col II	type II collagen
Col X	type X collagen
CPE	cytopathic effect
DMEM	Dulbecco's modified eagle's medium
ECM	extracellular matrix
FBS	foetal bovine serum
GAGs	glycosaminoglycans
H&E	hematoxylin and eosin
HDFs	human dermal fibroblasts
HEPES	N, 2-hydroxyethylpiperazine-N0-ethanesulfonic acid
hFOBs	human fetal osteoblasts
IGF	nsulin-like growth factor

ITR	inverted terminal repeat
MMP	matrix metalloproteinase
MOI	multiplicity of infection
MSCs	mesenchymal stem cells
NF-κB	nuclear factor κ B
OA	osteoarthritis
ODN	oligodeoxynucleotides
PBS	phosphate buffer saline
PCR	polymerase chain reaction
qRT-PCR	quantitative real-time polymerase chain reaction
RA	rheumatoid arthritis
RISC	RNA-induced silencing complex
RNAi	RNA interference
shRNA	short hairpin RNA
siRNA	small interfering RNA
SMSCs	synovium-derived mesenchymal stem cells
TGF-β	transforming growth factor beta
TNF	tumor necrosis factor
WST-1	4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio] -1,3-benzene disulfonate

SUMMARY

Articular hyaline cartilage is an avascular, aneural and alymphatic tissue that has a very limited capability to self-regenerate after damaged by injuries or degenerative diseases.

In the recent decade, novel strategies with tissue engineering and gene therapy for cartilage repair have been developed from laboratory-based investigation to clinical applications.

Nowadays, it is widely acknowledged that MSCs could differentiate into chondrocytic cells under appropriate cues. SMSCs, as a promising cell source due to their superior chondrogenic potential and proliferative ability, have been accordingly used in this study in conjunction with adenoviral vector-mediated TGF- β 3 and three dimensional culture system, which was aimed to develop engineered cartilage constructs to restore cartilage lesion. After 30-day culture period in chondrogenic medium, the constructs were analyzed by qRT-PCR and histological staining. The results proved that the transfer of TGF- β 3 by adenoviral vectors successfully induced chondrogenesis of SMSCs evidenced by the enhancement of GAGs and Col II. However, the side effect that the expression of Col I was increased by continuous supply of adenoviral-mediated TGF- β 3 showed up, thus resulting in the formation of fibrocartilage and compromising the overall quality of the engineered constructs. And it was demonstrated that the appearance of this side effect was attributed to the dual

contribution of promoting the collagen transcription via the Smad signal transduction pathway as well as restraining collagen degradation by MMP-9,

To address this issue, RNAi, making use of specific siRNA that can bind to target mRNA and subsequently turn the undesired gene off at post-transcriptional level, was introduced to inhibit the expression of Col I. A study was performed for the effects of TGF- β 3 coupled with the use of RNAi on chondrocytic differentiation of SMSCs. Episomal adenoviral vectors were used to transfer genes of interest into passage 4 SMSCs and cells were then suspended in alginate constructs for a period of 30 days. The qRT-PCR as well as histological and immunohistochemical staining results demonstrated that the application of combinational adenoviral vector-mediated transgenic TGF- β 3 and shRNA targeting Col I possessed the potential in promoting the chondrogenic differentiation of SMSCs and meanwhile suppressing fibrosis.

Apart from SMSCs, chondrocytes are an alternative cell source for cartilage tissue engineering. The ACT technique is one of the more novel and less invasive methods. One of the major setbacks faced by ACT is that cultured chondrocytes experience dedifferentiation after multiple monolayer expansion. This results in a shift in the production of Col-II to Col-I in the extracellular matrix, which impairs the overall quality of regenerated cartilage. Similar culture system to that used for SMSCs was applied for dedifferentiated chondrocytes. The expression of cartilage-specific markers such as aggrecan, Col II and Col I was monitored to determine the degree of

redifferentiation in chondrocytes. It was shown that genetically modified chondrocytes, which could express both TGF- β 3 and shRNA, were able to produce elevated amount of aggrecan and Col II, while effectively silence Col I production compared to Neg control and passage 4 monolayer cultured chondrocytes.

In summary, the combinational delivery of transgenic TGF- β 3 and shRNA specific for Col I via adenoviral vectors is effective on both inducing Col I-free chondrogenesis of SMSCs and promoting redifferentiation of dedifferentiated chondrocytes. The dual functions applied in engineered cartilage will make cell-based therapeutics more feasible and reliable for cartilage regeneration.

Chapter 1

Introduction

1.1 Cartilage profile

Cartilage is a flexible and opaque connective tissue, found in many places in the body including the joints, the rib cage, the external ear, the tip of the nose, the walls of the windpipe, the voice box (where it provides support and shape) and between intervertebral discs [1, 2]. Cartilage defects caused by traumatic damage or disease bring extensive pain and disability to patients, leading to a severe decrease in life quality. It is known that most people over the age of 65 are suffering from osteoarthritis (OA), and study reported that only 12.9% of the 55 to 65-year old were free of radiographic OA [3]. Similarly, back pain, which is closely related to degeneration of the intervertebral disc affects up to 35% people in the world [4]. With the growing population of the aged and the obese, cartilage disorders have become more and more prevalent nowadays.

1.1.1 Articular cartilage anatomy

Articular cartilage is a firm and highly elastic structure that covers the end of bones that is readily yielding to pressure, and recovering its shape when the force is removed [5]. Microscopically, hyaline articular cartilage is composed of water, collagen,

proteoglycans, other matrix proteins and lipids, together with chondrocytes making up 1-5% volume of its structure [6] (**Figure 1.1**). The composition of articular cartilage and a summary of their functions are illustrated in **Table 1.1**.

Table 1.1 Composition of articular cartilage and a summary of their functions [6-9].

Cartilage Components	Morphological Location	Functions
Chondrocytes	Make up about 1-5% of the volume of hyaline cartilage, sparsely spread within the matrix	Maintain the balance of synthesis and catabolism for extracellular matrix proteins
Collagen II	Accounts for 90-95% of total collagen in macrofibrils	Provides the articular cartilage with high tensile strength
Collagen IX	Cross-linked to surface of macrofibrils	Tensile properties and inter-fibril connections
Collagen XI	Within or on macrofibrils	Nucleates fibril formation
Proteoglycans	Interweaving macrofibrils , consist of GAGs subunits	Offers compressive strength to articular cartilage
Tissue fluid	Makes up about 65-80% of cartilage tissue by water, gases, metabolites and a large amount of cations.	Offers compressive strength and provides nutrition and lubrication for low-friction gliding surface

Figure 1.1 Schematic diagram of the structure of articular cartilage.

1.1.2 Current treatments for articular cartilage repair

Due to its aneural, avascular and alymphatic nature as well as immobilization of chondrocytes in dense ECM, cartilage tissue has a limited capacity for self-regeneration after an injury from trauma or diseases [6, 10]. Even if cartilage lesions penetrate the vasculature of the subchondral bone and cause local bleeding, the lesions fail to restore ultimately [11, 12]. The restoration of structural integrity and function of the newly repaired tissue still remain an issue.

Current approaches including microfracture, grafting and ACT, have resulted in pain relief and improvement of joint function to some extent [13]. However, these approaches still remain drawbacks such as lack of long-lasting function and graft rejection [14]. A summary of current cartilage replacement therapies and their potential drawbacks are listed in **Table 1.2**.

Table 1.2 A summary of current cartilage replacement therapies and their potential drawbacks [2, 9, 15].

Therapies	Methodology	Drawbacks
Autografting	Grafting a section of cartilage from a less load-bearing site to replace the cartilage defect	Donor site morbidity
Allografting	Cartilage is taken from another individual and then implanted in damaged site	Possibility of graft rejection
Microfracture	Penetration of subchondral bone to create an opening for migration of cartilage progenitor cells	Unpredictable results that rely on various elements: age of patient, size of the cartilage lesion etc.; High chances for relapse
Autologous Cell Transplantation (ACT)	Isolation of chondrocytes from patient, expansion and culture in the laboratory before implantation	Formation of fibrocartilage instead of hyaline cartilage

On the basis of the above, there is still no regimen that can regenerate defective cartilage with its normal function. In recent decades, more efforts have been focusing on developing regenerative medicine by combination of tissue engineering and gene therapy, by which stimulating factors such as growth factors could be delivered to therapeutic cells such as stem cells or chondrocytes via non-viral or viral vectors to engineer functional cartilage that would replace the damaged cartilage [16].

1.2 Regenerative medicine for cartilage repair

Regenerative medicine aims to repair, reconstruct or regenerate damaged tissues and organs, employing various diverse and cutting edge fields in biomedical research such as tissue engineering and gene therapy. Tissue engineering deals with the reconstruction of degenerative tissues with 3D cell-laden scaffolds, where morphogenesis is precisely induced and cell-matrix interaction is highly emphasized [17]; and gene therapy, which has been experimentally and clinically explored to treat severe diseases such as leukemia, cancer and AIDS, greatly contributes to regenerative medicine by introducing exogenous nucleotides into therapeutic cells, amplifying or silencing certain genes at molecular level to induce some desirable functionalities [18]. The review below will discuss the main elements involved in cartilage tissue engineering, including cell source, therapeutic growth factors and scaffold. Also, gene delivery systems for regenerative medicine as well as the emerging antisense technique will be introduced with respect to their applications in regenerative medicine.

1.2.1 Cartilage tissue engineering

Nowadays, cartilage tissue engineering based on therapeutic cells in conjunction with appropriate biological molecules and biodegradable materials has emerged as a

promising technique for cartilage repair [19, 20]. Here, emphasis is imposed on the following three essential factors: cell source, therapeutic biological molecules and materials for establishing 3D environment.

1.2.1.1 Cell source

The ideal therapeutic cells for cartilage tissue engineering should possess these properties: safety, easy isolation, as well as capability to proliferate and express cartilaginous molecules such as Col II and aggrecan. In recent years, several cell lineages, including MSCs, chondrocytes, fibroblasts, have been employed as therapeutic cells for cartilage regeneration and they will be discussed below.

1.2.1.1.1 MSCs

MSCs are multipotent progenitor cells which have the capability to differentiate into a wide range of cell types in various tissues, including bone, cartilage, muscle, tendon, fat and ligament [21, 22]. MSCs are considered to be a promising cell source for cartilage engineering because of a series of their characteristics. Compared to the low cell number and dedifferentiation of autologous chondrocytes [23], a large number of autologous MSCs can be easily isolated from various tissues, including bone marrow [24], synovial membrane [25], adipose tissue [26], muscle [27], skin [28], bone [29, 30] and so on. Furthermore, they can be cultured to expand their numbers in monolayer or scaffolds and their multilineage potential with passage can be maintained [21]. Lastly and most importantly, MSCs have the capacity to differentiate into chondrocytes when stimulated by appropriate chondrogenic factors [31-34].

There have been various *in vitro* systems developed for chondrogenic differentiation of MSCs. Johnstone *et. al.* [32] developed a culture system --- aggregate culture to

facilitate the chondrogenesis of rabbit bone marrow-derived mesenchymal progenitor cells. Besides, more and more 3D cultures, like agarose [35], alginate [36], silk [37], poly (ethylene glyco)-based hydrogel [38] etc. [39-42], have been explored for cartilage tissue engineering and gained some positive outcomes. In conjunction with the 3D environment, growth factors have been used and showed their potential in accelerating the production of cartilaginous ECM proteins. It has been reported that the supplement of TGF- β s [43-45], BMPs [46], IGF [19, 47], fibroblast growth factor [48] has successfully induced chondrogenesis of MSCs. The effects of growth factors will be discussed later.

A study by Mauck *et al.* demonstrated that bone marrow derived MSCs-laden hydrogels showed inferior mechanical properties to chondrocytes-laden hydrogels after a long-term culture [49]. Adipose-derived MSCs exhibited lower capability to differentiate into chondrocytic cells than other MSCs sources [26, 50-52]. Sakaguchi group [52] found that synovium-derived MSCs possessed superiority in chondrogenesis over other MSCs types. Thus more interests are attracted to the use of SMSCs for cartilage engineering.

1.2.1.1.2 Chondrocytes

Chondrocytes are the direct choice for cartilage tissue engineering due to their existence in native cartilage all over the body. Many studies have investigated various chondrocyte sources, including articular [53, 54], auricular [55, 56], nasoseptal [57, 58] and costal cartilage [59, 60], for regeneration of cartilage tissue. Chondrocytes obtained from older donors or OA patients also showed their potential in cartilage repair [61, 62].

ACT has been utilized clinically to treat cartilage defects in more than 12,000 patients

globally since 1987 [63]. Owing to the low cell number in mature cartilage tissue, one of the major challenges in ACT is to obtain sufficient cell numbers before clinical transplantation can occur. Thus, this technique involves harvest of cartilage tissue from the patient, isolation of chondrocytes from the donor tissue, cell expansion *in vitro* and followed by implantation into the cartilage lesions [53]. One of the major setbacks encountered by ACT is that cultured chondrocytes undergo a dedifferentiation process, namely a change in phenotype and losing ability to synthesize cartilaginous ECM, after a number of repeated monolayer culture [64, 65]. Dedifferentiation results in the conversion of typical spherical shape into spindle-shaped fibroblastic-like morphology and a shift in the production of ECM from cartilage-specific protein Col II and aggrecan to fibroblast-specific protein Col I [66], which may substantially impair the overall quality of the regenerated counterpart. Therefore, the maintenance of chondrocytic characteristics is of great importance for the success of ACT and cartilage tissue engineering.

Several studies reported that dedifferentiated chondrocytes could be recovered to a chondrocytic phenotype under *in vitro* 3D culture system such as agarose [67], alginate beads [68, 69], Col I gel [70, 71], Col IIgel [72], fibrin glue [73] and so on [74, 75]. Also, the delivery of growth factors has also been found to contribute to maintain the characteristics of chondrocytes [76, 77]. Although the results are encouraging, the redifferentiation effects still demand substantial improvement. The study by Darling and Athanasious demonstrated that the dedifferentiation of passaged chondrocytes was kept even after seeding these cells in alginate beads [23]. Thus, in order to use chondrocytes as a cell source for cartilage tissue engineering, a further improved culture system that prevents chondrocyte dedifferentiation should be developed.

1.2.1.1.3 Fibroblasts

Fibroblasts appear as an alternative to MSCs and chondrocytes due to the following reasons: 1) a large number of fibroblasts are easily isolated from skin in a minimally invasive way; 2) fibroblasts could be developed toward a chondrocytic phenotype under appropriate cues.

A few researchers have investigated the feasibility of fibroblasts for cartilage repair. Although transplanting fibroblasts/PLA composites directly into cartilage defects failed to form hyaline-like cartilage but fibrous tissue [78], Nicoll *et al.* found that dermal fibroblasts could be differentiated into chondrocytic cells *in vitro* under high density micromass culture with treatment of 40mM lactate [79]. Likewise, studies demonstrated that dermal fibroblasts could synthesize cartilaginous ECM proteins when cultured on aggrecan after pretreatment with IGF-1 [80] or cultured with demineralized bone powder [81], thus making fibroblasts a potential cell source for regeneration of articular cartilage. In addition, a study by Lee *et al.* suggested that 6 weeks after injection of fibroblasts expressing TGF- β 1 into the cartilage defects, there formed new hyaline cartilage by evidence of histological analyses [82]. Lately, Deng *et al.* separated dermis-isolated aggrecan-sensitive (DIAS) cells and demonstrated the cells possessed superiority in chondroinduction potential over unpurified dermis cells [83].

Although fibroblasts have been proved to be an optional cell source for cartilage tissue engineering, more work needs to be done to address some latent issues such as the possibility of fibrocartilage formation.

1.2.1.2 Biological molecules for cartilage tissue engineering

Some biofactors, no matter in the form of recombinant proteins or by a transgene

product via non-viral vectors or viral vectors, have been employed to induce chondrogenesis of mesenchymal progenitor cells [84], stimulate proliferation of chondrocytes [85, 86] and enhance synthesis of cartilaginous ECM [87]. Among the potentially useful candidates for cartilage repair, there are (1) growth factors including transforming growth factor (TGF) β superfamily, bone morphogenetic protein (BMPs), insulin-like growth factor (IGF)-1, and fibroblast growth factors (FGFs); (2) transcription factors such as Sox-9, Sox 5 and Sox-6; (3) signal transduction molecules, such as SMADS. Here, growth factors in the first category are mainly discussed because they are more widely applied for this purpose.

1.2.1.2.1 Growth factors

TGF- β is a kind of multifunctional peptide growth factors, and contains at least three known subtypes in human. TGF- β 1 plays a vital role in regulating the primary cell-cell interplay between condensing progenitor cells [43], and stimulates chondrocytes producing new ECM [88]. TGF- β 2 is known to regulate hypertrophic differentiation of chondrocytes by controlling expression of Indian hedgehog and Parathyroid Growth Hormone [89]. TGF- β 3 exhibits its greater potential in chondrocytic differentiation of MSCs [90]. They also play a role in preventing formation of blood vessels in cartilage, which prevents further mineralization and ossification [91]. Palmer et al. transfected bone marrow-derived MSCs with TGF- β 1, and the results showed the formation of cartilaginous tissue and the production of ECM consistent with that of articular cartilage [92]. An increasing number of studies also indicate that MSCs could present chondrocyte specific gene markers and form a cartilaginous matrix by addition of TGF β 3 during a 14-day to 2-month culture period [93].

BMPs are a family of growth factors with the capability to stimulate the formation of

bone and cartilage, and also belong to the TGF- β superfamily. Several studies have successfully utilized the cDNAs encoding BMPs to induce mesenchymal progenitor cells to differentiate into chondrocytes [46, 94]. Carlberg *et al.* observed that the transfer of BMP-2 cDNA by retroviral vector into C3H10T1/2 cells in micromass culture promoted cartilage formation, and its action was restricted to the infected cell population [94]. Aside from BMP-2, Sekiya *et al.* demonstrated that the supplement of BMP-6 made the pellets about 10-fold heavier and enhanced the production of proteoglycans. In addition, the expression of type II procollagen and Col X was detectable at 1 week and the levels were augmented at 3 weeks [95]. Nochi *et al.* compared the effects of expression of BMP-13 and BMP-2 in chondroprogenitor cells, finding that BMP-13 promoted chondrocytic differentiation but not terminal differentiation, whereas BMP-2 induced endochondral ossification [96]. Furthermore, the combinational transfer of TGF- β 1 and BMP-2 acted synergistically, leading to enhancement of chondrocyte redifferentiation and better ECM production [97]. Nevertheless, it should be cautious about using BMPs for cartilage repair as they can stimulate ossification potently.

IGF-1 with molecular structure similar to that of insulin, is a 7.6 kDa polypeptide growth factor which can control glycogen and protein synthesis. Particularly, IGF-1 enhances the expression of proteoglycan and Col II, two major components in cartilage, by chondrocytes. Gelse *et al.* evaluated the the repair of articular cartilage by cell transplantation in conjunction with transfer of IGF-1 cDNA, and found that cells treated with Ad-IGF-1 also led to a complete stuffing in most cartilage defects with repair tissue, and immunohistochemical staining indicated extensive Col II staining but dim Col I staining [19]. Another report by Fukumoto *et al.* also described IGF-1-mediated mesenchymal chondrogenesis in periosteal explants from rabbits [33].

Fibroblast growth factors (FGFs) are a group of growth factors playing a part in wound healing and embryonic development [98]. Studies have also shown that FGFs can induce chondrocytic differentiation, and more detailed research by Hoffmann et al. suggested the chondrogenic effects of FGF-3 in this developmental process [99]. In a study conducted by Ellsworth *et al.*, it was revealed that addition of FGF18 via adenovirus could promote the growth of articular chondrocytes and increase the expression of ECM [100].

1.2.1.2.2 Other therapeutic candidates

Sox-9, the major transcriptional factor, is capable of promoting MSCs condensation. Sox-9, together with Sox-5 and Sox-6, is identified to be essential and sufficient for cartilage formation [101, 102]. Tsuchiya et al. investigated chondrocytic differentiation of MSCs by overexpression of *sox9* gene and their histological staining results showed that both GAGs and Col II were significant but Col X was not stained [103]. In addition, signal transduction molecules SMADs could regulate the chondrogenesis intracellularly by means of gene delivery [104].

1.2.1.3 Scaffolds

Native cartilage has an extracellular protein matrix which is strengthened by a 3D network of collagen fibrils [105]. 3D construct can be a good mimic of the morphology of the *in vivo* microenvironment. In a 3D construct, most of chondrocytes showed a spherical morphology, which has been demonstrated to be associated with the production of cartilage-related ECM proteins [64]. Moreover, cells transfected by viral or nonviral vectors show a gradual decrease in transgene expression in monolayer culture, while there is a prolonged transgene expression in 3D hydrogel culture

conditions [106, 107].

To date, both natural and synthetic polymers have been studied as scaffolds for cartilage tissue engineering and both of them have their pros and cons [13]. Natural polymers including agarose, alginate, fibrin, hyaluronic acid, chitosan, collagen, silk fibroin etc. can well interact with cells so as to regulate cellular behaviors while they have inferior mechanical characteristics, vulnerability to degradation by host enzymes and potential risk in inducing immune system response. On the contrary, synthetic polymers such as poly (α -hydroxy esters), poly (ethylene glycol/oxide), poly (NipAAm) etc. have superiority in mechanical and degradation properties by modifications but are poor for cell/scaffold interactions and could produce toxic byproducts during degradation.

In addition, the structure of scaffolds is of great importance for cartilage repair. Williams *et al.* [38] encapsulated MSCs in the photopolymerizing hydrogel followed by a 6-week culture in chondrogenic medium with addition of TGF- β 1. They found that MSCs propagated in the hydrogels and aggrecan and Col II gene expression were upregulated compared with monolayer MSCs. All these results indicated that a hydrogel could provide a suitable structural support for MSCs to form cartilage-like tissue with supplement of TGF- β 1 *in vitro*. Wang and coworkers [37] compared the chondrogenesis of MSCs cultured in 3D porous aqueous-derived silk scaffolds with those cultured in aqueous-derived silk films. After 3 weeks, the expression of cartilage-specific genes at transcriptional level was not detected or very low in cells cultured in two dimensional films, whereas these genes except for Sox9 were significantly increased in cells cultured in 3D silk scaffolds.

Therefore, it can be concluded that key features for successful cartilage tissue engineering included at least three essential factors: a safe and easily acquired cell

source, stimulating factors for enhancing production of a cartilaginous matrix, and a biocompatible, degradable 3D scaffold with advantageous structural characteristics for cells to attach, proliferate and differentiate into chondrocytes.

However, every element involved in cartilage tissue engineering, such as scaffolds and bioactive molecules, is a variable that determines the mechanical and biological characteristics of the engineered cartilage. Hence, more research should be done in comprehending how each of these elements contributes to cartilage repair and optimizing the methods for cartilage regeneration.

1.2.2 Delivery Systems for regenerative medicine

Biological molecules which help to promote chondrogenesis and deposition of cartilage specific proteins, if delivered to cells in the form of recombinant proteins, are easy to lose activity. Thus, in order to achieve a sustained production of these growth factors effectively, gene delivery of their cDNAs becomes a more favorable strategy [18].

Both viral- and non-viral-based nucleic acid delivery systems serve well in gene transfer for regenerative medicine. Non-viral gene delivery utilizes chemical reagents including lipids and cationic polymers, or physical means such as electroporation and microinjection [108]. Primary advantages of non-viral methods include low immune responses and facileness in handling, while their relatively-low transfection efficiencies and cell toxicity remain major challenges. Though manufacturers have always aimed at developing non-viral delivery systems with both higher transfection efficiency and lower toxicity, and huge commercial success has been attained with products such as FuGene® and Lipofectamine™, people are still cautious in their use in clinical applications.

On the other hand, viral delivery systems, consisting of retrovirus (including lentivirus), adenovirus, adeno-associated virus and herpes virus, have attracted more attention in gene delivery on the account of their higher transfection efficiency, while concerns regarding their immunogenicity remain. Each type of viral vector has its own characteristics and thus meets the requirements in different applications. For instance, retroviruses and lentiviruses can lead to the integration of exogenous genes into the host genome with the drawback of introducing insertional mutations [109], while adenoviruses do not have this capability. Therefore, retroviruses and lentiviruses can be used to attain a stable gene expression while adenoviruses are more suitable to attain transient gene expression. Lentiviral and adenoviral vectors work in both dividing and non-dividing cells, and consequently can be used to infect terminally differentiated cells such as neurons, muscle and liver cells; in contrast, retroviruses only infect replicative cells and hence can be reliably used in cancer therapy *in vivo*. Having been used in several preclinical and clinical trials, viral vectors with specific properties provide various choices and flexibility in targeting inherited and acquired diseases with gene therapy. However, serious concerns remain in the use of viral vectors. For example retroviruses have a tendency to integrate near oncogenes in the host genome, resulting in lethal neoplastic diseases in the patients. Such issues would need to be addressed before viral vectors can be effectively used in clinical practice. Ex-vivo gene transfer into in-vitro cultured cells may result in the elimination of toxicity and immunogenicity associated with non-viral and viral vectors to a certain extent before the use of these cells in-vivo. For example, when adenoviral vectors are used for transfection, the virus undergoes disassembly inside the cells, where the capsid (an immunogen) gets degraded during the period of *in vitro* culture, while the genetic material gets transferred to the nucleus.

1.2.3 Antisense makes sense in engineered regenerative medicine

The use of antisense strategies such as ribozymes, ODNs and siRNA in gene therapy, in conjunction with the use of stem cells and tissue engineering, has opened up possibilities in curing degenerative diseases and injuries to non-regenerating organs and tissues. Belonging to the field of gene therapy, antisense technology has been widely and thoroughly explored from laboratory-based investigation to clinical trials. The rationale behind antisense technology is that specific DNA or RNA can bind to a target mRNA and subsequently turn the ‘undesired’ gene off at post-transcriptional level. These DNA or RNA molecules are designed with a sequence complimentary to that of the target mRNA; after being introduced into the cell with suitable delivery systems, this sequence guides the ‘antisense’ nucleotides to the so-called ‘sense’ segment of the target mRNA, effectively blocking the initiation of translation or resulting in the degradation of the target mRNA [110, 111]. Antisense technology has emerged to be a versatile tool that makes unique sense in engineering regenerative medicine, and its ability of suppressing certain genes has been of great assistance in promoting various kinds of tissue/organ regeneration. In this part, this review focuses on introduction of RNAi and discusses its application in the various fields of regenerative medicine.

In 1998, Andrew Fire *et al.* discovered that double stranded RNA can induce degradation of the homologous mRNA target in animals, resulting in the silencing of specific genes. This was later termed as RNAi [112]. RNAi has been widely used in gene functional analysis in mammals as well as in gene therapy for various diseases. It was found that the introduction of double stranded RNA longer than 30 base pairs into most mammalian cells elicited an antiviral immune response [113]. Therefore people started making use of small double stranded RNA fragments of 21-23 nucleotides in

length, called siRNAs for gene silencing [114, 115]. After the introduction of siRNA into the cells, the siRNA assembles with Argonaute proteins, Dicer and other cellular factors into an RISC [116], which unwinds the double stranded RNAs. One of these strands, the so-called passenger strand, gets degraded, while the other strand - antisense (guide) strand, guides the RISC to cleave the target mRNA in a sequence-specific manner, leading to the degradation or translational repression of the target gene. RISC is recycled and can process several cleavage cycles (**Figure 1.2**).

Although siRNAs can be easily introduced into the cells directly using various non-viral delivery methods, the silencing of target gene only lasts for 3-7 days, depending on the kind of target gene. Thus, plasmid vectors encoding for shRNAs have been designed to increase the duration of silencing as the shRNA can be continuously expressed within the cell for a considerable period of time [117, 118]. shRNAs consist of 19-29 base-pair stems, i.e. sense strand and antisense strand of siRNAs with a 4-9 base pair nucleotide loop at one end [119]. After the plasmid encoding for shRNA enters the cell nucleus, shRNAs are expressed and then cut to form siRNAs by Dicer, whereafter they follow the same mechanism as siRNAs to induce gene silencing (**Figure 1.2**).

siRNAs are regarded as preferred agents of gene silencing for therapeutic applications due to their low toxicity, high sequence specificity and ability to induce RNAi at low concentrations [120]. However, there are still some issues such as “off-target effects” and cellular stability that need to be addressed [121]. Chemical modification and proper sequence design may be helpful to ameliorate these issues to some extent [122].

Figure 1.2 Mechanism of RNAi in mammalian cells.

Short hairpin RNA (shRNA) is endogenously expressed via expression vectors, and then migrates into cytosol where it is cut by Dicer into smaller siRNA. Alternatively, double-strand siRNA is directly introduced into cells. siRNA produced by both means are recognized and incorporated into RISC (RNA-induced silencing complex). The antisense-combined RISC* binds to the target mRNA with a complementary sequence and cleaves it. RISC* can be recycled to carry out additional multiple reactions [123].

Antisense technology has emerged to be a versatile tool that makes unique sense in engineering regenerative medicine, and its ability of suppressing certain genes has been of great assistance in promoting various kinds of tissue/organ regeneration. Researchers in regenerative medicine are confronted by one of the greatest-ever challenges - treating bone/cartilage destruction caused by RA, which is a severe degenerative disease and a chronic inflammatory disorder. Although the detailed

pathogeny of RA remains unclear, it is widely considered that many inflammatory cytokines play a key role in RA [124]. One example is the NF- κ B, a transcription factor widely involved in immune responses and other cellular responses [125]. NF- κ B can be activated by, and then turn around to regulate the expression of, cytokines such as IL-1 β and TNF- α [126, 127]. Its potential role in joint destruction has been well documented [128]. siRNA-based strategies [129] against NF- κ B were investigated with an adenoviral delivery system and positive results were observed in the *in vivo* OA models. Chondrocyte dedifferentiation is one major intrinsic cause for cartilage degeneration in adults. Cathepsin B is a protease that indicates the extent of dedifferentiation of chondrocytes and is involved in cartilage destruction resulting from OA or RA. A trial by Zwicky *et al.* [130] demonstrated that by means of double stranded RNA and antisense DNA, silencing of Cathepsin B can be capable of preventing chondrocytes from dedifferentiation and thus protecting the cartilage from degeneration. This finding additionally provided tissue engineers and biomaterialists a unique way to prevent dedifferentiation during *in vitro* cultivation and expansion of primary chondrocytes. In another study focusing on osteopenia, a condition in which bone mineral density is abnormally lower, Gaggero *et al.* made use of siRNA [131] to down-regulate the expression of gremlin, an antagonist to BMPs, resulting in an elevated expression of osteocalcin and Runx-2 and enhanced bone formation.

Aside from its utility in osteo-/chondro- regeneration, RNAi have also been used in the treatment of myogenesis [132], cardiovascular disease [133-135], neurodegenerative disorder [136, 137]. Besides, investigators have reported the silencing of Smad2 and Smad3 of this family with specific siRNA [138, 139], resulting in a significant reduction of pro-collagen expression and ECM deposition, as well as an attenuation of fibrosis. Although more clinical trials are in demand, this technique is potentially of

great commercial value considering the huge market in scar-free wound repair and skin regeneration.

Furthermore, the application of antisense technologies could be an alternative tool to direct cell differentiation towards lineage of interest, by silencing specific genes which could block the desired route of differentiation or guide the cells to multiple lineages. Three other groups have achieved enhanced cardiac and adipocytic differentiation as well as acquired neuronal phenotypes in stem cells by the means of RNAi techniques [140-142]. These efforts in basic cell biology have substantial implications for the future use of antisense strategies in directing cell lineage for engineering purposes.

1.3 Objective and outline of this thesis

The overall object of this project is to develop engineered articular cartilage for cartilage repair. Based on the previous studies by pioneer researchers, we proposed a method by combining the approaches used in cartilage tissue engineering and gene therapy, namely the endogenous therapeutic TGF- β 3 by means of gene delivery was generated to trigger and guide the chondrogenic differentiation of SMSCs in 3D scaffold. However, we found that although continuous supply of TGF- β 3 by this means did promote the chondrogenesis of SMSCs effectively, it also stimulated the fibrosis. Hence, we introduced the RNAi technology to solve this problem. We hope that the introduction of shRNA specific for Col I coupled with the delivery of growth factor could lead to Col I-suppressed chondrogenesis of SMSCs. In the meanwhile, we expanded the study to another conventional cell source---chondrocytes, hypothesizing that this combinational delivery would also help chondrocytes maintain their phenotypes even after repeated monolayer passages.

To summarise, this project aims to evaluate the effects of TGF- β 3 and shRNA targeting

Col I on chondrocytic differentiation of SMSCs and chondrocyte redifferentiation in alginate hydrogels. This dissertation is composed of 7 chapters specified as follows:

Chapter 1 gives a review on tissue engineering involving cells source, biological molecules and scaffolds; gene delivery strategies including viral/non-viral delivery as well as antisense techniques are also discussed. The objective, together with organization of this dissertation, is indicated.

Chapter 2 would specify the preparation of of high-titer Ad-TGF β 3 and exploration of chondrogenesis of SMSCs in alginate hydrogels by TGF- β 3 transfer alone.

Chapter 3 would describe the investigation of the mechanism for up-regulation of Col I by adenoviral vector-mediated TGF- β 3.

Chapter 4 would focus on the construction of Ad-shRNA and its effect on preventing the production of Col I.

Chapter 5 would introduce the construction of recombinant adenovirus with dual functions and optimization of the MOI of the three functional recombinant adenoviruses. Afterwards, a study performed to assess the chondrogenesis of SMSCs by transduction with these adenoviruses, alone or in combination would be elaborated.

Chapter 6 would extend the application of combinational delivery of both TGF- β 3 and anti-Col I shRNA in redifferentiation of dedifferentiated chondrocytes and evaluate the effects.

Chapter 7 would conclude the above studies and propose feasible future works.

Chapter 2

Chondrogenesis of rabbit mesenchymal stem cells via adenoviral vector-mediated TGF- β 3 expression

2.1 Introduction

Tissue engineering has emerged as a promising treatment for damaged tissues and organs. Its feasibility lies in obtaining high quality and quantity of cells for transplantation, the development of suitable vehicles without toxicity and immunogenicity, as well as the use of appropriate biological molecules [143, 144]. MSCs as a cell source for cartilage tissue engineering attract more and more interests. Among various MSCs sources, SMSCs were selected in this study as they present superior chondrogenic potential and higher proliferative ability [145]. Some studies have reported that normal SMSCs could be isolated from pathological synovium and successfully proliferate [146, 147]. Since it is common for OA patients to take an arthroscopic examination, this provides convenient accessibilities for the harvest of autologous SMSCs.

MSCs could differentiate specifically into chondrocytes under suitable biological cues and microenvironment. Among the various growth factors, TGF- β superfamily including TGF- β 1, TGF- β 2 and TGF- β 3 [148, 149] has been widely used to induce the chondrogenesis of MSCs under certain culture conditions. Barry *et al.* found that TGF- β 3 was more effective than TGF- β 1 in promoting chondrogenic differentiation of MSCs [148]. In this study, adenoviral vectors were employed to transfer TGF- β 3 gene owing to its advantages of high titers, the ability to infect both nondividing and dividing cells, high transduction efficiency as well as no concern for inserted

mutagenesis. Aside from growth factors, 3D environment to support strong cell-cell interaction and mimic the in vivo microenvironment is of great importance for chondrocytic differentiation of MSCs. Among various existing biomaterials, hydrogels appear to be advantageous: they have injectable and biodegradable properties for convenient clinical use, support good mass transport to and from hydrogels, and provide an aqueous environment to maintain cells and fragile drugs [150]. It has been reported by some studies [151, 152] that alginate is a suitable carrier for MSCs to carry out chondrogenesis and was thus used in this study.

In this chapter, we aimed to induce chondrogenesis of rabbit SMSCs via adenoviral vector-mediated TGF- β 3. The preparation of high-titer recombinant adenoviruses encoding TGF- β 3 (Ad-TGF β 3) would be specified. Afterwards, rabbit SMSCs infected with Ad- TGF- β 3 were encapsulated into alginate hydrogels and cultured in chondrogenic medium for 30 days. Then a series of assays including qRT-PCR, histological and immunofluorescent staining, ELISA would be performed to determine the chondrogenesis of rabbit SMSCs.

2.2 Materials and methods

2.2.1 Preparation of high-titer Ad- TGF β 3

2.2.1.1 Construction of Ad-TGF β 3

The Adeno-X™ ViraTrak™ Expression System 2 (Clontech) was used in this study. This adenoviral genome comprises a ZsGreen1 Living Colors Fluorescent Protein expression unit, which is driven by the cytomegalovirus immediate early promoter (PCMVIE), so that cells transduced with recombinant adenovirus could be easily examined under fluorescence microscope or analyzed by flow cytometry. The general flow is illustrated in **Figure 2.1**.

Figure 2.1 Flow chart for construction of Ad-TGF β 3.

2.2.1.1.1 Obtaining target gene --- TGF- β 3

The target gene was obtained from TGF- β 3-pCMV6-XL5 plasmid DNA (Origene Technologies, Rockville, MD, USA.) by using PCR system (TITANIUM Taq PCR, Invitrogen). At the same time, Sall recognition site and PstI recognition site were added at the two ends of the hTGF- β 3 cDNA CDS by using the following primers:

Forward: 5'-ACGCGTCGACATGAAGATGCACTT-3'

Reverse: 5'-TGCACTGCAGTCAGCTACATTTAC-3'

The PCR was performed for 30 cycles by the following settings: denature for 30 seconds at 94°C, anneal for 30 seconds at 55°C, and extend for 30 seconds at 72°C, and finally extended at 72°C for 10 minutes. Then PCR products were run on 1.2% agarose gel followed by extraction/purification using QIAquick Gel Extraction Kit (Qiagen). The final products were labeled as TGF- β 3 cDNA CDS and stored at -20°C for future use.

2.2.1.1.2 Constructing shuttle vector

TGF- β 3 cDNA CDS and pDNR-CMV donor vector were digested with enzyme Sall (New England Biolabs) and enzyme PstI (New England Biolabs) followed by the ligating reaction at 16°C overnight using T4 DNA Ligase (Invitrogen). After that, the ligation products were amplified by transformation in MAX Efficiency DH5 α Competent Cells (Invitrogen) and then amplified plasmids were extracted/purified using Plasmid Midi Kit (QIAGEN). To verify the presence of ligated shuttle vector (pDNR-CMV + TGF- β 3 cDNA CDS), the purified plasmids were digested with Sall and PstI followed by gel electrophoresis. The shuttle vector, which showed positive results from gel electrophoresis was sent to 1st BASE (Singapore) for DNA sequencing.

2.2.1.1.3 Constructing recombinant adenoviral vector

TGF- β 3 cDNA CDS from Shuttle Vector was transferred into Acceptor Vector (pLP-Adeno-X-ViraTrak), which depends on the catalytic activity of Cre recombinase. Recombinants were harvested and amplified by transforming ElectroMAX™ DH10BTM Cells (Invitrogen), and then screened against Shuttle Vector and the non-recombinant Acceptor Vector by plating cells onto LB-agar/chloramphenicol (30 μ g/mL)/sucrose (7% w/v) plates. At last, the recombinant adenoviral vectors were extracted/purified by QIAfilter Plasmid Midi Kit (QIAGEN) and confirmed by XhoI restriction digestion. Also, the recombinant adenoviral vectors, which showed positive results from XhoI restriction analysis, was sent to 1st BASE (Singapore) for DNA sequencing.

2.2.1.1.4 Producing recombinant adenovirus

The adenoviral vector plasmids were linearized by digestion with PacI to expose the ITRs for supporting the formation of the replication complex. Lower passage of HEK 293 cells (ATCC) were transfected with PacI-digested Adenoviral Vector complex using Lipofectamine™ 2000 (Invitrogen). Two days later, expression of the ZsGreen1 Living Colors Fluorescent Protein was examined under fluorescence microscope. When CPE was observed, recombinant adenoviruses were collected by lysing cells with three consecutive freeze-thaw cycles. Then the collected adenoviruses were used to transfect HEK 293 cells with 50-70% confluence again. After appearance of CPE, viral stock was collected by lysing cells with three consecutive freeze-thaw cycles and labeled as “primary amplification”.

2.2.1.2 Amplification of Ad- TGF β 3

2.2.1.2.1 Determination titer of recombinant adenovirus

Titer of “primary amplification” was determined using Adeno-X™ Rapid Titer Kit (Clontech) as the following steps. First of all, HEK 293 cells were plated at 5×10^5 cells/well in a 12-well plate. 100 μ L of viral dilutions which were prepared 10-fold serially from 10^{-5} to 10^{-8} mL was added dropwise to each well. Cells were then maintained in a 37°C /5% CO₂ incubator for 48 hr. Afterwards, cells in each well were fixed with 1 mL of ice-cold 100% methanol (Sigma) and then incubated at -20°C for 10 min. After three washes with 1 mL of PBS (1st Base) + 1% BSA (Sigma), 0.5 mL of Anti-Hexon Antibody dilution (1:1000) (Clontech) was added to each well and the plate was kept at 37°C on an orbital shaker for 1 hr. And then the wells were rinsed three times and 0.5 mL of Rat Anti-Mouse Antibody (HRP conjugate) (Clontech) dilution (1:500) was added to each well. After 1 hr incubation at 37°C, color was developed using DAB working solution (Clontech). At last, a minimum of three fields which should contain 5 to 50 brown/black cells were counted under a microscope with a 20X objective and the average of positive cells in each well was calculated. Infectious units (ifu/mL) were obtained using the following formula:

$$\frac{(\text{infected cells/field}) \times (\text{fields/well})}{\text{volume virus (mL)} \times (\text{dilution factor})}$$

2.2.1.2.2 Preparing high-titer recombinant adenoviral stocks

HEK 293 cells were seeded in multi- T150 flasks and incubated at 37°C in humidified air with 5% CO₂. When cell monolayer reached 50-70% confluence, medium in each flask was replaced with 10 mL of fresh medium with supplement of recombinant adenovirus from “Primary Amplification” Stock at a multiplicity of infection (MOI)

≥ 5 . Then the flasks were returned to the incubator for 90 min. Thereafter the medium containing recombinant adenovirus in each flask was replaced with 20 mL of fresh growth medium. The cells were continued culturing in the incubator. Cell fluorescence and CPE were checked each day until most of cells have detached owing to CPE. Then lysates containing recombinant adenovirus were harvested with three consecutive freeze-thaw cycles.

2.2.1.2.3 Purification of Ad-TGF β 3

Recombinant adenoviruses were purified using Adeno-X™ Virus Purification Kit (Clontech) according to the manual. Briefly, after amplification of recombinant adenovirus, the lysates were filtrated using Bottle-Top Filter Unit. Benzonase® Nuclease was added to the filtrate to decrease the viscosity of the solution and degrade cellular DNA. Then the solution was incubated at 37°C for 30 min. The Benzonase Nuclease-treated filtrate was thereafter mixed with an equal volume of 1X Dilution Buffer. The dilution was loaded onto the purification filter. After that, the filter was washed and eluted.

Finally, recombinant adenovirus titer was determined by the procedure described in **Section 2.2.1.2.1**.

2.2.2 Isolation of rabbit SMSCs

Rabbit SMSCs were isolated from rabbit synovium as described by De Bari *et al* [153]. Briefly, after being harvested aseptically from knee joint of mature New Zealand rabbits in the age of 3-5 months, the synovial tissues were washed triply with PBS solution with penicillin/ streptomycin and minced finely. Following digestion with 0.1% collagenase II, cells were released and then propagated in complete growth

medium containing high-glucose DMEM supplemented with 10% FBS, 100 units/mL penicillin, and 100 µg/mL streptomycin. The medium was changed every 3-4 days. Subculture with 1:3 dilution ratio was carried out when cultures reached 90% confluence. Cells at passage 4 (P4) were used in this study.

The above mentioned cell culture-related reagents were purchased from Gibco (Invitrogen, Singapore).

2.2.3 3D-culture of SMSCs in alginate hydrogel

P4 rabbit SMSCs on individual 150-cm² flasks were infected with MOI 300 of (defined as ifu per cell) Ad-null, Ad- TGF β3. After 2h infection, the medium was aspirated and replaced with 20 mL of fresh complete growth medium. After 48h incubation, the cells were collected and rinsed with washing buffer [0.15M NaCl (Sigma-Aldrich) + 25mM HEPES (Hyclone) in distilled water]. After that, the cells were resuspended in sterile 1.2% alginate (Sigma) at 6x10⁶ cells/mL. Then 40 µL of cell suspension was slowly dropped into a beaker containing 102 mM CaCl₂ (Sigma-Aldrich) solution for polymerization for 10 min. Finally alginate beads were cultured in chondrogenic medium which contained high-glucose DMEM (Gibco), 100 nM dexamethasone (Sigma), ascorbic acid 2-phosphate (50 µg/mL; Sigma), sodium pyruvate (100 µg/mL; Gibco), proline (40 µg/mL; Sigma), penicillin (100 U/mL), streptomycin (100 µg/mL) (Gibco), and 5 mL of ITS premix in 500 mL of medium (insulin [6.25 µg/mL], transferrin [6.25 µg/mL], selenous acid [6.25 µg/mL], linoleic acid [5.35 µg/mL], and BSA [1.25 µg/mL]; (BD Biosciences, Bedford, MA). Medium was replaced and collected for ELISA assay every 3 days. In parallel, SMSCs without transduction were embedded into alginate hydrogel as Neg control. One day after gelation was defined as Day 0.

2.2.4 RNA isolation and qRT-PCR analysis

For RNA extraction from alginate hydrogel constructs cultured for 30 days, the cells were firstly released by dissolving the alginate beads in 55mM sodium citrate (Sigma, Singapore) for 5 min and then RNA was obtained with TRIZOL[®] Reagent. Then 500 ng of RNA from each group was converted to cDNA for the subsequent real-time PCR experiments using M-MLV reverse transcriptase (Promega, USA). The qRT-PCR was performed using iQTM qPCR system (Bio-Rad, USA) under the following conditions: 40 cycles in total, each cycle including 30 s for denaturation at 95°C, 30 s for annealing at 58°C and 30s for extension at 72°C. The threshold cycle values of each sample were normalized against corresponding housekeeping gene GAPDH. Sequences of all the primers for real-time PCR in this work are shown in **Table 2.1**.

Table 2.1 Primers used in real-time PCR.

Genes	Primer sequence (both 5'-3')	Annealing temperature (°C)
GAPDH	F: TCGTCCTCCTCTGGTGCTCT R: CCACTTTGTGAAGCTCATTTTCCT	58
Type II collagen	F: TATCCAGTAGTCACCGCTCTTCC R: ACGCTCAAGTCCCTCAACAAC	58
Type I collagen	F: ATCAAGGAAGGGCAAACGAG R: GGCAACAGCAGGTTCACTTACA	58

2.2.5 Histology and immunohistochemistry

Samples from each group (n = 3) at Day 30 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 hematoxylin and eosin (H&E) and Safranin-O, respectively. 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 antibody for Col II (Chemicon) was applied overnight (4 °C). Following three PBS washes, the sections for Col II were incubated with AlexaFluoro 546-labeled secondary antibodies (Invitrogen) at room temperature for 1 h. Finally, all samples staining were washed again, mounted with DAPI (Invitrogen) to locate the cell nucleus and analyzed using fluorescence microscopy.

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

Conditioned medium was collected at various time points as indicated in the text and figures and stored at -20 °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 in advance. Then, the standards with two-fold serial dilutions and the pretreated samples were added into each well of the pre-coated 96-well plate. The plate was then incubated for 2 h at room temperature. After 3-time washes with washing buffer [PBS+0.05% Tween 20(bio-rad)], the plate was incubated with detection antibody, streptavidin-HRP, and substrate solution in sequence. Finally, the absorbance at 450nm was measured using a microplate reader. The concentration of TGF- β 3 from each group was calculated using SkanIt Software 2.2.

2.2.7 Statistical analysis

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

2.3 Results and discussion

2.3.1 Preparation of high-titer Ad-TGF β 3

2.3.1.1 Construction of Ad-TGF β 3

To verify the successful construction of shuttle vector (pDNR-CMV + TGF- β 3 cDNA CDS), the purified plasmids were digested with Sall and PstI and used for gel electrophoresis. The plasmids of successfully constructed shuttle vector should be digested into two segments --- about 5.6 kb (size of pDNR-CMV) and 1.25 kb (size of TGF- β 3 cDNA CDS). As shown in **Figure 2.2**, two bands appeared between 1000 bp & 1500 bp and 5000 bp & 6000 bp indicating presence of both TGF- β 3 cDNA CDS and pDNR-CMV. These plasmids of shuttle vector were stored at -20°C as stock for future use.

Figure 2.2 Sall and PstI restriction analysis for shuttle vector.

Plasmids of shuttle vector extracted from colonies were digested with enzyme Sall and PstI and run on agarose gel.

For further verification, the plasmids of shuttle vector were sent to 1st BASE (Singapore) for DNA sequencing. The feedback from 1st BASE indicated that only one nucleotide was mismatched. However, this mutation would not influence the

expression of TGF- β 3 for the codon, which was composed of the mismatched nucleotide also coded for the same amino acid.

To determine whether the recombinant adenoviral vectors were constructed successfully, the plasmids of recombinant adenoviral vector were digested with XhoI enzyme. Expected bands were: 14.6 kb, 9.7 kb, 5.6 kb, 4.2 kb, 2.5 kb, 1.4 kb and 0.6 kb. As shown in **Figure 2.3**, all of the above bands appeared in the samples indicating successful construction of recombinant adenoviral vector. Additionally, the sequencing result from 1st BASE confirmed the success of construction of recombinant adenoviral vectors.

Figure 2.3 XhoI restriction analysis of recombinant adenoviral vector.

Plasmids of recombinant adenoviral vector extracted from colonies were digested with enzyme XhoI and run on agarose gel.

Two days after infection of HEK 293 cells by Ad-TGF β 3, expression of the ZsGreen1 Living Colors Fluorescent Protein was evident by inspecting the cells under

fluorescence microscope (**Figure 2.4 A**). In a week, CPE showed up (**Figure 2.4 B**). Then the collected viruses were used to transduce HEK 293 cells again and harvested as “primary amplification” when CPE appeared.

Figure 2.4 Characterization of transfected HEK 293 cells.

(A) Green fluorescence was observed under fluorescence microscope two days after transfection. Magnification is 40; (B) CPE was observed under light microscope 7 days after transfection. Magnification is 100.

2.3.1.2 Amplification and purification of Ad-TGF β 3

According to the methods as described in **Section 2.2.1.2.1 & 2.2.1.2.2**, the adenoviral vectors were amplified and purified. Titer of these viruses (Ad-TGF β 3) was obtained at $\sim 10^9$ pfu/mL and could be used in the subsequent experiments. Moreover, Primary Stocks of these viruses have been stored in aliquots at -80°C for future expansion, which helps to reduce the risk of emergence of RCA.

2.3.2 Chondrogenesis of rabbit SMSCs via adenoviral vector-mediated TGF- β 3 expression

2.3.2.1 Cartilage-specific genes expression

After a 15- or 30-day culture period in chondrogenic medium, total RNA was extracted from alginate beads of three groups followed by a qRT-PCR analysis. As shown in

Figure 2.5A, the expression of Col II was not detected in three groups at Day 15 while that in Ad- TGF- β 3 group but not in Neg or Ad-null groups showed a significant increase at Day 30. Interestingly, the expression level of Col I in Ad- TGF- β 3 group was the highest among the three groups at both Day 15 and Day 30 (**Figure 2.5B**). Expression of Col I in cartilage would result in the formation of fibrocartilage, which cannot withstand the stress as the native hyaline cartilage and leads to considerable impairment in the overall quality of the regenerated counterpart.

Figure 2.5 Gene expression of Col II and Col I.

Expression of Col II (A) and Col I (B) from rabbit SMSCs in the three groups: Neg, Ad-null, Ad-TGF β 3 were quantified by qRT-PCR analysis.

2.3.2.2 Histology and immunohistochemistry

The rabbit SMSCs-laden alginate beads from Neg and Ad-TGF β 3 groups were cultured in chondrogenic medium for 30 days and sectioned followed by the H&E and Safranin-O staining (for GAGs) as well as immunofluorescent staining for Col II. It was showed in **Figure 2.6** that no GAGs and Col II staining were seen in Neg group, indicating that rabbit SMSCs even in 3D environmental culture failed to produce

cartilaginous ECM. On the contrary, cells with treatment of Ad-TGF β 3 showed its ability to enhance the deposition of GAGs and Col II, as evidenced by the considerably intensive staining. Moreover, H&E staining suggested that a large number of chondrocytic lacunae, which is a marker of interstitial growth of cartilage, were observed in Ad-TGF β 3 group while no lacuna in Neg group, indicating that rabbit SMSCs could proliferate and differentiate into chondrocytic cells under the stimulus of TGF- β 3.

Figure 2.6 Histological and immunofluorescent staining.

The constructs were sectioned after a 30-day culture period and subject to H&E, safranin-o staining and Immunofluorescent staining specific for Col II.

2.3.2.3 Kinetic expression of TGF- β 3

To determine the release level of TGF- β 3, conditioned medium was collected every three days and analyzed by ELISA assay. Medium collected from Neg group was also examined and there is no expression of TGF- β 3 in these samples (data not shown).

As shown in **Figure 2.7**, TGF- β 3 was expressed at a concentration around 22 ng/mL at 3 days and then a prominent decrease in TGF- β 3 transgene expression appeared between 6 days and 9 days. Afterwards, the release of TGF- β 3 decreased slowly till Day 30 (around 3 ng/mL).

Figure 2.7 Expression level of adenoviral-mediated TGF- β 3 by ELISA analysis

Meanwhile, ELISA assay was also conducted for recombinant protein TGF- β 3 concentration at various time points as indicated in **Table 2.2**. Uninfected rabbit SMSCs-laden alginate beads were cultured in the medium with manual addition of

recombinant protein TGF- β 3 at the concentration of 10ng/mL at the beginning. Our data showed that concentration of exogenous TGF- β 3 fell rapidly from its original concentration of 10 ng/mL to approximately 2 ng/mL in only 1 hr (**Table 2.2**) [93], indicating that recombinant protein has a very short pharmacological half-life.

Time	Concentration (ng/mL)
0 min	10
15 min	5.352 $\pm$ 1.025
30 min	3.142 $\pm$ 0.836
45 min	3.345 $\pm$ 1.739
1 hr	2.140 $\pm$ 0.571
2 hr	2.154 $\pm$ 0.276
4 hr	1.838 $\pm$ 0.385
8 hr	1.963 $\pm$ 0.453
24 hr	1.962 $\pm$ 0.182
48 hr	1.375 $\pm$ 0.227
72 hr	0.814 $\pm$ 0.133

Table 2.2 Expression level of recombinant protein TGF- β 3 by ELISA analysis.

As showed in the Table, recombinant protein has a very short pharmacological half-life, about 15 min as indicated in bold [93].

2.4 Summary

Our general aim was to induce chondrocytic differentiation of rabbit SMSCs by means of gene transfer of TGF- β 3 in conjunction with 3D culture environment. Both the qRT-PCR and histological staining results demonstrated that Ad-TGF β 3 played a vital

role in promoting the chondrocytic differentiation of rabbit SMSCs. Moreover, delivery of TGF- β 3 via adenoviral vectors could supply sustained expression of TGF- β 3 and is cost-effective compared to approach of supplement with recombinant protein TGF- β 3. Hence, this culture system was effective in stimulating rabbit SMSCs to differentiate into chondrocyte phenotype. However, it was noticed that Ad-TGF β 3 would bring out a side effect---up-regulation of Col I expression, which would make the engineered cartilage fibrous and result in the degeneration in the end. Thus this issue should be addressed so that this culture system could be more feasible for cartilage repair.

Chapter 3

Mechanism for up-regulation of type I collagen by adenoviral vector-mediated TGF- β 3

3.1 Introduction

TGF- β s play crucial roles in tissue regeneration [154], cell differentiation [38], embryonic development [155], and regulation of the immune system [156]. Several studies [157-159] suggest that unlike TGF- β 1 and TGF- β 2, TGF- β 3 has distinct roles in wound healing, resulting in the inhibition of fibrosis and scar formation. However, as mentioned in Chapter 2, our findings, by continuous supply of TGF- β 3 via adenoviral vector, suggest an upregulating profile of Col I production. This could arise from different culture or delivery conditions; the mechanism was investigated and presented in this chapter.

In this study, HDFs were used as model cells to explore the mechanism by which adenoviral vector-mediated TGF- β 3 contributed to promote the expression of Col I. Cells were transduced with Ad- TGF- β 3, which lead to the secretion of TGF- β 3 from the cells *per se* continuously for at least one month, and then encapsulated into alginate hydrogels and cultured for 30 days.

3.2 Materials and methods

3.2.1 Cell culture

HDFs (passage 8-15) were purchased from Cambrex (North Brunswick, NJ) and cultured in high-glucose DMEM containing 1.5g/L sodium bicarbonate, 10% FBS, 100 units/mL penicillin and 100 μ g/mL streptomycin.

The above mentioned cell culture-related reagents were purchased from Gibco (Invitrogen, Singapore).

3.2.2 Construction of adenoviral vectors

Adenoviral vector encoding TGF- β 3 was constructed as described in Chapter 2. Also an adenoviral vector without any inserted gene was constructed as a control and designated as Ad-null.

3.2.3 Adenoviral transduction of HDFs

HDFs were seeded into 10-cm culture dishes at a density of 1.5×10^6 cells per dish 12–24 h before infection. Then, the cells in individual dishes were incubated in 4 mL of serum-free DMEM with MOI 300 of Ad-TGF β 3 and Ad-null in a cell culture incubator. After incubation for 2 h, the culture fluids were replaced with 10 mL of fresh complete growth medium. Following an additional 48 h incubation, the cells were trypsinized.

3.2.4 3D-culture in alginate hydrogel

The alginate beads were made as described in previous chapters. One bead (around 8×10^4 cells) was placed into each well of the 24-well plate with 1 mL of complete growth medium. Medium was replaced every 3 days. Also HDFs without transduction were seeded into alginate hydrogel as Neg control. In total, there were three groups: Neg (Neg control), Ad-null, Ad-TGF β . One day after gelation was defined as Day 0.

3.2.5 RNA isolation and qRT-PCR

Total RNA was extracted from alginate hydrogel constructs of HDFs cultured for Day

5, 12, 19 and 26 using the combination of TRIZOL[®] Reagent (Invitrogen) and RNeasy[®] Mini Plant Kit (Qiagen, Düsseldorf, Germany) [160]. Then cDNA for the subsequent real-time PCR experiments was synthesized from 500 ng of total RNA from each group using M-MLV reverse transcriptase (Promega, Madison, MI, USA). In real-time PCR, the relative expression values of genes were acquired from iQ[™] qPCR system (Bio-Rad, Hercules, CA, USA) and calculated with the comparative CT method (DDCT). All the primers for real-time PCR in this work are shown in **Table 3.1**.

Table 3.1 Primers used in real-time PCR [161].

Genes	Primer sequence (both 5'-3')	Annealing temperature (°C)
Type I collagen	F: CCTGCGTG TACCCCACTCA R: ACCAGACATGCCTCTTGTCCTT	60
Smad4	F: TGGAGGTGGCCTGATCTTCA R: ACAACGATGGCTGTCCCTCA	60
Mmp-9	F: CCCAGACAGCGCCAGTC R: GCATCTCTGCCACCCGAGTG	60
RPL13A	F: CCTGGAGGAGAAGAGGAAAGAGA R: TTGAGGACCTCTGTGTATTTGTCAA	60

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

Conditioned medium collected from monolayer or SMSCs alginate constructs from Ad-TGF β 3 group, Ad-double group and Ad-combination group was stored at -20 °C for further analysis by specific ELISA kit (R&D Systems, Minneapolis, MN) following the instruction described in Chapter 2. Triplicates were performed for each time point. The concentration of TGF- β 3 from each group was calculated using SkanIt Software 2.2.

3.2.7 Cell proliferation

The viability of HDFs in alginate constructs of the three groups was determined at Day 0, 5, 12, 19 and 26, respectively, using the WST-1 assay (4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate, Roche Diagnostics, Germany). Briefly, the samples were collected and subsequently incubated in 500 μ L of growth medium containing 50 μ L of WST-1 reagent for 2 h. Afterwards, the absorbance of each sample was determined using a microplate reader set to 450 nm with reference to 620 nm.

3.2.8 Statistical analysis

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

3.3 Results

3.3.1 Adenoviral vector-mediated TGF- β 3 enhanced expression of type I collagen in 3D alginate construct of HDFs

Real-time PCR results at various time points, Day 5, 12, 19 and 26, all showed that TGF- β 3 significantly elevated the expression of type I collagen in 3D alginate construct of HDFs (**Figure 3.1 A-D**).

Figure3. 1 Analysis of expression of Col I and dynamic secretion of TGF-β3.

Real-time PCR was performed for gene expression of type I collagen from 3D alginate construct of HDFs at Day 5 (A), Day 12 (B), Day 19 (C) and Day 26 (D). Dynamic changes of TGF-β3 secreted by infected fibroblasts using ELISA assay (E) [161].

The relative expression of type I collagen in Ad-TGF β3 group reached between 9.55 and 30 folds of that in Neg group. The expression of collagen I in Ad-null group was slightly higher when compared with that in the Neg group.

As shown in **Figure 3.1E**, TGF-β3 was expressed at a concentration of around 68 ng/mL at Day 3 and then a marked decrease in TGF-β3 transgene expression appeared

between Day 6 and Day 12. Finally, the concentration of TGF- β 3 secreted by infected HDFs was maintained within the range of 7 to 11 ng/mL for about 2 weeks.

3.3.2 Adenoviral vector-mediated TGF- β 3 promoted Smad 4 expression

Since transcription of Col IA2 gene is regulated via the Smad signal transduction pathway and study demonstrated that Col IA2 promoter activity was promoted by transcriptional coactivators p300, which involved interaction with Smad 4 [162], we then investigated how Smad 4 was influenced by TGF- β 3.

Figure 3.2 Smad 4 gene expression.

Expression of Smad 4 from 3D alginate constructs of HDFs at Day 5 (A), Day 12 (B), Day 19 (C) and Day 26 (D) using real-time PCR [161].

The results shown in **Figure 3.2 A-D** exhibited that TGF- β 3 strongly stimulated the expression of Smad 4. Thus, it was rational that after upregulation of Smad 4 by

TGF- β 3, HDFs could respond to p300, which in turn enhanced the Col IA2 promoter activity and accordingly increased Col I gene transcription.

3.3. 3 Adenoviral vector-mediated TGF- β 3 inhibited MMP-9 expression

ECM remodeling is partly modulated by proteins of the MMP family [163]. As a component of ECM, Col I can be degraded by MMP-9 [164].

Figure 3.3 MMP-9 gene expression.

Expression of MMP-9 from 3D alginate constructs of HDFs at Day 5 (A), Day 12 (B), Day 19 (C) and Day 26 (D) using real-time PCR [161].

Using real-time PCR, we detected that the expression of MMP-9 in Ad- TGF- β 3 group was attenuated greatly compared with that in Neg group at different time point as indicated in **Figure 3.3 A-D**. The expression of MMP-9 in Ad-null group was higher than that in Ad- TGF- β 3 group at most time points. Therefore, it was reasonable to

conclude that TGF- β 3 had an effect on decreasing the expression of MMP-9.

3.3.4 Adenoviral vector-mediated TGF- β 3 promoted viability of HDFs in 3D alginate construct

HDFs proliferation in 3D constructs of alginate hydrogel in the three groups of Neg, Ad-null, Ad- TGF- β 3 was examined with WST-1 colorimetric assay in parallel (**Figure 3.4**). HDFs infected with adenovirus encoding TGF- β 3 grew up to about 120% of the starting cell population at Day 5 and then had a slight decline in proliferative tendency and were about 75% of the original population till Day 26. On the contrary, cells in the other two groups demonstrated a rapid drop in proliferation at Day 5 and kept decreasing till Day 26. After 26 days of culture, the cell amount in Neg and Ad-null group was 29% and 41% of the starting cell population, respectively. The above quantitative results showed that cell viability was enhanced in Ad- TGF- β 3 group but inhibited in the other three groups.

Figure 3.4 Analysis of cell viability till Day 26.

The cell proliferation in the three groups was assessed with WST-1 colorimetric assay [161].

3.4 Discussion

The three isoforms of TGF- β s (TGF- β 1, TGF- β 2 and TGF- β 3) have 70-80% sequence homology [90] and share most cell-surface receptors. Also, they have been shown to involve common features in their downstream Smad signal transduction pathway [165]. Smads are a class of intracellular proteins that play a critical role in propagating the TGF- β signals from cell-surface receptors to the cell nucleus. In response to the interaction of TGF- β with type II receptors, type I receptors are recruited to form heterotetramers with type II receptors. Type I receptors are transphosphorylated by type II receptors, and activated type I receptors in turn activate Smad 2 and Smad 3. Then these Smads with the common mediator Co-Smad4 form heteromeric complexes, which move into the nucleus to regulate the transcription of specific genes [166].

Our previous study demonstrated that the expression of Col I was elevated in HDFs growing in culture plates when cells were treated with adenoviral vector encoding TGF- β 3 [167]. In this study, our observations showed that TGF- β 3 increased Col I expression in HDFs cultured in 3D constructs of alginate hydrogel. Interestingly, some other studies exhibited different experimental outcomes that indicated discrepancies of Col I production when exposing to TGF- β 3 [157, 158]. Such discrepancy may result from different dosage, source, or target of TGF- β 3 addition. For example, they mostly apply high magnitude of growth factor powders (in protein) to in-vivo tissues instead of the engineered cellular models in this study. Our previous findings also showed that exogenous TGF- β 3 had a short pharmacological half-life as its concentration fell rapidly from its original concentration of 10 ng/mL to approximately 2 ng/mL in only 1 hr [93]. Unlike manual exogenous addition leading to a pulse supply, in the current study the delivery of TGF- β 3 gene via adenoviral vector allows a continuous and relatively stable supply of growth factor to the cells over a long period, which was later

shown by ELISA analysis.

We observed that Adenoviral vector-mediated TGF- β 3 would promote the expression of Col I in 3D constructs of HDFs for up to at least 26 days. Col I undergoes turnover by both synthesis and degradation. Considering the previous studies that there was a functional interaction between p300/CBP and endogenous Smad involved in the control of TGF- β -stimulated Col1A2 transcription [162, 168, 169], we believed that in response to stimulation by TGF- β 3, the Smad signal transduction pathway would be activated, which in turn induced Col I synthesis. On the other hand, our findings suggested that TGF- β 3 restricted MMP-9 expression, which thereby inhibited the degradation of Col I. Other studies showed that level of MMP-9 was low or undetectable in areas expressing excessive collagen [170, 171]. Thus, taking into account all of the above considerations, it suggests that the intracellular homeostasis of Col I synthesis and degradation gets interfered by TGF- β 3, leading to the increase in expression of Col I. Furthermore, we found that the survival rate of HDFs infected with adenovirus encoding TGF- β 3 was greater than that of cells in the other groups. Taken together with other studies that the interactions between integrins, cell surface adhesion receptors, and ECM regulated the survival of fibroblasts in Col I matrices [172-174], these findings demonstrate that integrins acting as mechanoreceptors, can perceive ECM-derived mechanical stimuli and transform them to chemical signals to direct the pathways of cell viability. Sebra *et al.* [175] illustrated the role of Col I grafts in promoting cell adhesion through cell-adhesive peptide moieties situated within the structure of Col I. Therefore, we believe that an increase in the expression of Col I provided more cell-adhesive peptide moieties for HDFs to anchor, leading to better cell interactions with positive signals being transmitted through integrins to avoid undergoing apoptosis.

3.5 Summary

In this chapter, it has been demonstrated that TGF- β 3 in a continuous supply mode could significantly enhance the expression of type I collagen. The findings have shown that TGF- β 3 functions in stimulating the Smad signal transduction pathway so as to promote the synthesis of type I collagen. Moreover, TGF- β 3 prohibited the degradation of type I collagen by inhibition of MMP-9. This duality results in the ascending expression of type I collagen and thereby benefits HDFs proliferation in alginate hydrogels, which will be helpful for the application of hydrogels for anchorage-dependent cells.

Chapter 4

Inhibition of Col I expression by RNAi

4.1 Introduction

As demonstrated in Chapter 2, Ad-TGF β 3 could enhance the expression of Col I, which will need to be removed so as to improve the overall quality of engineered cartilage. To this end, RNAi, well acknowledged for its capability in inhibiting the expression of pathogenic genes, would provide an effective tool to ameliorate this issue [123].

This chapter will focus on the construction of Ad-shRNA and its effect on preventing the production of Col I.

4.2 Materials and methods

4.2.1 Preparation of high-titer Ad-shRNA

Ad-shRNA was produced using Adeno-X™ ViraTrak ZsGreen1 Promoterless Expression System 2 (Clontech Laboratories, Mountain View, CA, USA). A U6 promoter together with shRNA sequence can be transferred from the pSIREN-DNR vector into the adenoviral genome (pLP-Adeno-X-PRLS-E3-ZsGreen1 Acceptor Vector) using Cre-loxP cloning. Also the adenoviral genome has been engineered with a ZsGreen1 expression cassette for easy identification of cells infected with recombinant adenovirus. The general flow is illustrated in **Figure 4.1**.

Figure 4.1 Flow chart for construction of Ad-shRNA.

4.2.1.1 Construction of shuttle vector

The target sense and antisense sequences were obtained from Hs_COL1A1_6_HP

Validated siRNA (Qiagen) as follows:

Sense: CAAUCACCUGCGUACAGAA

Antisense: UUCUGUACGCAGGUGAUUG

After that, two complementary oligonucleotides for the above sequences were annealed to form a double-stranded oligonucleotide with respectively engineered 5'-BamH I restriction site and 5'-EcoR I restriction site overhang on the top and bottom strand. The structure of a double-stranded oligonucleotide encoding shRNA specific for Col I was illustrated in Figure 4.2.

Figure 4.2 Structure of double-stranded oligonucleotide sequence encoding shRNA.

Then the pSIREN-DNR Vector was digested with enzyme BamH I (New England Biolabs) and enzyme EcoR I (New England Biolabs). Afterwards, the obtained linearized pSIREN-DNR Vector and the double-stranded oligonucleotide were ligated using T4 DNA ligase (Invitrogen) at 16°C overnight to generate shuttle vectors. The constructed shuttle vectors were extracted/purified using Plasmid Midi Kit (QIAGEN) after expansion by transformation in MAX Efficiency DH5α Competent Cells (Invitrogen). To confirm the success of the shuttle vector construction, the purified plasmids were identified by digestion with enzyme MluI (New England

Biolabs), which restriction site has been engineered into the double-stranded oligonucleotide as well as the pSIREN-DNR Vector, followed by gel electrophoresis. The shuttle vector, which showed positive results from gel electrophoresis, was sent to 1st BASE (Singapore) for DNA sequencing.

4.2.1.2 Construction of recombinant adenoviral vector

The sequence encoding shRNA specific for Col I was transferred from between the two *loxP* sites in the shuttle vector into the single *loxP* site in the Acceptor Vector using the bacteriophage P1 enzyme, Cre recombinase. In addition, a chloramphenicol resistance gene (Cm^r) is accompanied with the shRNA sequence to move into the adenoviral Acceptor Vector during recombination, which helps to screen against Shuttle Vector and the non-recombinant Acceptor Vector. After the amplification in ElectroMAXTM DH10BTM Cells (Invitrogen), the recombinant adenoviral vectors were extracted/purified by QIAfilter Plasmid Midi Kit (QIAGEN) and confirmed by Xho I restriction analysis.

4.2.1.3 Generation of Ad-shRNA

Lower passage of HEK 293 cells (ATCC) were transfected with linerized PacI-digested Adenoviral Vector complex using LipofectamineTM 2000 (Invitrogen). After appearance of CPE, cells were lysed with three consecutive freeze-thaw cycles to collect recombinant adenoviruses. This transduction process was repeated to obtain “primary amplification”. Afterwards, high-titer Ad-shRNA were produced after amplification, purification and titration following the steps described in **Section 2.2.1.2.1, Section 2.2.1.2.2 & Section 2.2.1.2.3 in Chapter 2.**

4.2.2 Cell culture

HDFs (passage 8-15; Cambrex, North Brunswick, NJ) were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM) (Gibco) containing 1.5g/L sodium bicarbonate, 10% fetal bovine serum (FBS) (Gibco), 100 units/mL penicillin and 100 µg/mL streptomycin.

Human fetal osteoblasts (hFOBs 1.19,) were obtained from ATCC (Manassas, VA, USA) and cultured in DMEM/Ham's F12 (1:1) (Gibco) with supplement of 2.5 mM L-glutamine (ATCC), 0.3 mg/mL G418 (PAA Laboratories) and 10% (v/v) FBS at 33.5 °C.

4.2.3 Adenoviral transduction of HDFs and hFOBs

HDFs or hFOBs were seeded into 12-well culture plates at a density of 2×10^5 cells per well 12–24 h before infection. Then, the cells in individual wells were kept in 0.5 mL of serum-free DMEM with various MOI of Ad-shRNA as follows: MOI 0, 10, 50, 100, 200 and 500. 2 h post infection, the culture fluids were replaced with 1 mL of fresh complete growth and cells were incubated at 37°C in 5% CO₂.

4.2.4 RNA isolation and qRT-PCR

After 7 days incubation, total RNA was extracted from each well using the combination of TRIZOL[®] Reagent (Invitrogen) and converted into for the subsequent real-time PCR experiments using M-MLV reverse transcriptase (Promega, Madison, MI, USA). In real-time PCR, the relative expression values of genes were acquired from iQ[™] qPCR system (Bio-Rad, Hercules, CA, USA) and calculated with the comparative CT method (DDCT). All the primers for real-time PCR in this work are shown in **Table 4.1**.

Table 4.1 Primers used in real-time PCR.

Genes	Primer sequence (both 5'-3')	Annealing temperature (°C)
Type I collagen	F: CCTGCGTGTACCCCACTCA R: ACCAGACATGCCTCTTGTCTT	60
RPL13A	F: CCTGGAGGAGAAGAGGAAAGAGA R: TTGAGGACCTCTGTGTATTTGTCAA	60

4.2.5 Statistical analysis

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

4.3 Results and discussion

4.3.1 Preparation of high-titer Ad-shRNA

The constructed shuttle vector with inserted sequence encoding shRNA was identified using *Mlu* I restriction analysis. Since both shRNA oligonucleotide and the pSIREN-DNR Vector have been engineered with one *Mlu*I restriction site, desired shuttle vector with inserted shRNA sequence was expected to produce two segments whose lengths were 500 and 4700 bp accordingly. After digestion by enzyme *Mlu*I, bands shown in **Figure 4.3A** matched the expected lengths, Additionally, the sequencing result got from 1st BASE showed 100% match of the inserted sequence, thus indicating that the recombinant shuttle vectors have been successfully constructed.

Figure 4.3 Restriction analysis.

Plasmids of shuttle vector (A) and adenoviral vectors (B) extracted from colonies were digested with enzyme MluI or XhoI, respectively, and run on agarose gel.

To confirm the presence of shRNA sequence in the constructed adenoviral vectors, the restriction analysis using XhoI restriction site was conducted. According to the plasmid map of adenoviral vector, there were six Xho I restriction sites in the adenoviral vector and the expected band sizes were: 14.5, 9.7, 7.6, 2.5, 1.4 & 0.6 kbp. It was indicated in **Figure 4.3B** that all of the above bands appeared in the samples, demonstrating successful construction of recombinant adenoviral vector encoding anti-Col I shRNA. Afterwards, these positive recombinants were transferred into HEK 293 cells to package as recombinant adenoviruses. The packaged adenoviruses were then amplified, purified, titrated and aliquoted for the subsequent experiment.

4.3.2 Effect on inhibition of Col I by Ad-shRNA

To examine the silencing effect on Col I expression by Ad-shRNA, fibroblasts and

osteoblasts were used as target cells for their characteristics of abundant Col I expression. Fibroblasts and osteoblasts were infected respectively with various MOI of Ad-shRNA as indicated in **Figure 4.4** & **Figure 4.5**.

Figure 4.4 Silencing effect on Col I expression of HDFs.

Fibroblasts were transfected with various MOI of Ad-shRNA and observed under fluorescent microscope 7 days postinfection (A). The Col I gene expression was determined by qRT-PCR (B).

For fibroblasts, the number of fluorescent green cells increased with higher MOI, indicating that transduction efficiency exhibited in a viral dose-dependent manner (**Figure 4.4A**). The data from qRT-PCR showed that there was a stronger silencing effect on Col expression when increasing MOIs of Ad-shRNA were applied. As shown in **Figure 4.4B**, MOI 10 had faint influence on inhibiting Col I expression while MOI

50, 100, 200 and 500 showed a significant decrease in Col I production, which was 54%, 67%, 84% and 87%, respectively, less than that of Neg control.

Figure 4.5 Silencing effect on Col I expression of hFOBs.

Osteoblasts were transfected with various MOI of Ad-shRNA and observed under fluorescent microscope 7 days postinfection (A). The Col I gene expression was determined by qRT-PCR (B).

Similarly, for osteoblasts, the higher transduction efficiency of Ad-shRNA was accompanied with the increasing MOI (**Figure 4.5A**). Diverse MOIs of Ad-shRNA displayed their silencing function in suppression of Col I gene expression by a viral dose-dependent decrement within the range of 33%-83% (**Figure 4.5B**).

4.4 Summary

It is widely acknowledged that shRNA, which is cut to form siRNA in cytosol, knocks down the specific gene by cleaving the target mRNA with a complementary sequence. From the results shown in the previous sections, it can be concluded that recombinant adenoviruses encoding shRNA targeting Collagen I gene have been successfully constructed. And it has been proved by employment of fibroblasts and osteoblasts that Ad-shRNA could be an effective tool to restrain the production of Col I which need be eliminated for cartilage tissue engineering.

Chapter 5

Effects of combinational adenoviral vector-mediated TGF- β 3 transgene and anti-Col I shRNA on articular chondrogenesis of SMSCs

5.1 Introduction

As mentioned in previous chapters, cartilage tissue engineering based on MSCs in combination with appropriate biological molecules and biodegradable materials has attracted more and more attention to regenerate damaged cartilage tissue. And SMSCs are considered as a promising cell source thanks to their superior chondrogenic potential and higher proliferative ability. We demonstrated that Ad-TGF β 3 was a necessary and effective stimulator for induction of chondrogenesis of SMSCs in our previous study. However, we also found that Ad-TGF β 3 would lead to the rise in Col I expression, which will potentially cause impairments to the mechanical functions of the constructs. Moreover, even freshly-isolated SMSCs display fibroblastic characteristics more or less which would undermine the effect of chondrogenesis [176, 177]. RNAi, well acknowledged for its capability in inhibiting the expression of pathogenic genes, would provide an effective tool to ameliorate this issue. In chapter 4, Ad-shRNA has been successfully generated and we attempted to make use of shRNA to suppress the expression of Col I in SMSCs.

Hence, we hypothesized that the combination of TGF- β 3 and shRNA specific for Col I could promote the chondrogenic differentiation of SMSCs as well as inhibit the formation of fibrocartilage. Toward this end, we designed two ways to combine TGF- β 3 and shRNA: 1) to construct a recombinant adenovirus with dual functions (Ad-double), i.e. insert TGF- β 3 and shRNA to the same vector; and 2) to produce two

kinds of recombinant adenoviruses expressing either TGF- β 3 (Ad- TGF β 3) or shRNA (Ad-shRNA) and co-transduce them into SMSCs simultaneously. As controls, SMSCs were also infected respectively with Ad- TGF β 3 or Ad-shRNA alone. The level of cartilage-specific gene expression and cartilage matrix accumulation were investigated by qRT-PCR and histological staining after 30 days' cultivation.

5.2 Materials and methods

5.2.1 Preparation of high-titer Ad-double

The shuttle vector of Ad-TGF β 3 was used as a template for that of Ad-double. Then human U6 promoter gene obtained from human fibroblast genome with engineered Apa I and Xho I restriction sites was inserted into the shuttle vector followed by the incorporation of the double-stranded oligonucleotide encoding shRNA with 5'-Xho I and 5'-Pst I restriction sites overhang at the two ends. Then the two cassettes each encoding TGF- β 3 and shRNA were transferred from the constructed shuttle vector into Acceptor Vector (pLP-Adeno-X-ViraTrak) using Cre-loxP cloning. After expansion in ElectroMAXTM DH10BTM Cells (Invitrogen), the recombinant adenoviral vectors were extracted/purified by QIAfilter Plasmid Midi Kit (Qiagen) and confirmed by Xho I restriction analysis. Afterwards, the constructed recombinant adenoviral vector was delivered into HEK 293 cells (ATCC) using Lipofectamine reagents (Invitrogen) for viral packaging after linearization by Pac I digestion. Then the recombinant adenoviruses encoding both TGF- β 3 and anti-Col I shRNA were amplified, purified and titrated following the procedures described in previous chapters. The schematic diagram of the produced Ad-double was illustrated in **Figure 5.1**.

Figure 5.1 Schematic diagram of the produced Ad-double after digestion with *PacI* [167].

Table 5.1 Primers used in obtainment of U6 promoter and shRNA [167].

Genes	Primer sequence (both 5'-3')
human U6 promoter	F: ATTTGCGGGCCCGCAGGAAGAGGGCCTAT R: CC-GCTCGAGTCGTCCTTTCCACAAG
shRNA	F: TCGAGCAATCACCT-GCGTACAGAATTCAAG AGATTCTGTACGCAGGTGATTGTTTTTACGCGTC TGCA R: GACGCGTAAAAACAATCACCTGCGTACAGAAT CTCTTGAATTCTGTACGCAGGTGATTGC

5.2.2 Cell harvest

Porcine SMSCs were isolated from porcine synovial tissue as described in chapter 2. The cells were cultured in complete growth medium containing high-glucose DMEM supplemented with 10% FBS, 100 units/mL penicillin, and 100 µg/mL streptomycin after harvest. The medium was changed every 3-4 days. Subculture with 1:3 dilution ratio was carried out when cultures reached 90% confluence. Cells at passage 4 (P4) were used in this study.

5.2.3 Adenoviral MOI optimization

P4 porcine SMSCs were seeded into 12-well plates at a density of 1×10^5 cells/well. In the following day, the cells in individual wells were infected with Ad- TGF β3, Ad-shRNA and Ad-double respectively at various MOIs as indicated in the figures and incubated in 400 µL of serum-free DMEM for 2h at 37°C in 5% CO₂. Following 2h

incubation, the culture fluids were replaced with 1 mL of fresh complete growth medium. Then after 24h incubation, the medium was changed. To evaluate the expression of TGF- β 3, the culture medium was collected after an additional 24h incubation to measure the concentration of TGF- β 3 by ELISA analysis. RNA was extracted from the cells and the expression of TGF- β 3 at transcriptional level was determined by qRT-PCR. To estimate the effect of shRNA, qRT-PCR and immunofluorescent staining for Col I at 72h post-transduction were carried out respectively to check the expression of Col I at transcriptional level and protein level. To examine the transduction efficiency and cell viability, the percentage of infected cells and dead cells were counted by flow cytometry.

5.2.4 3D-culture of porcine SMSCs in alginate hydrogel

P4 porcine SMSCs on individual 150-cm² flasks were infected with Ad-null, Ad- TGF β 3, Ad-shRNA, Ad-double and co-transfected with Ad- TGF β 3 and Ad-shRNA simultaneously as well. After 2h infection, the medium was aspirated and replaced with 20 mL of fresh complete growth medium. After 48h incubation, the cells were encapsulated into alginate hydrogels following the steps described in previous chapters. Finally one alginate bead was placed into each well of the 24-well plate with 1 mL of chondrogenic. Medium was replaced and collected for ELISA assay every 3 days. In parallel, SMSCs without transduction were embedded into alginate hydrogel as Neg control. In total, there were 6 groups: Neg (negative control), Ad-null, Ad-TGF β 3, Ad-shRNA, Ad-double and Ad-combination. One day after gelation was defined as Day 0.

5.2.5 RNA isolation and qRT-PCR analysis

RNA from monolayer was extracted using TRIZOL[®] Reagent (Invitrogen, Singapore) according to the procedure described in the manual. For alginate hydrogel constructs cultured for 30 days, RNA extraction and real-time PCR were described in previous chapters. The threshold cycle values of each sample were normalized against corresponding housekeeping gene RPL4. Then quantitative data were normalized against that of the Neg control sample to yield the relative fold change. Triplicates were conducted for each sample. Sequences of all the primers for real-time PCR in this work are shown in **Table 5.2**.

Table 5.2 Primers used in real-time PCR [178].

Genes	Primer sequence (both 5'-3')	Annealing temperature (°C)
Type I collagen	F: CCTGCGTGTACCCCACTCA R: ACCAGACATGCCTCTTGTCCTT	58
Type II collagen	F: GCTATGGAGATGACAACCTGGCTC R: ACAACGATGGCTGTCCCTCA	58
Type X collagen	F: CAGGTACCAGAGGTCCCATC R: CATTGAGGCCCTTAGTTGCT	58
COMP	F: GGCACATTCCACGTGAACA R: GGTTTGCCCTGCCAGTATGTC	58
Aggrecan	F: CGAGGAGCAGGAGTTTGTCAAC R: ATCATCACCACGCAGTCCTCTC	58
RPL4	F: CAAGAGTAACTACAACCTTC R: GAACTCTACGATGAATCTTC	58

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

Conditioned medium collected from monolayer or SMSCs alginate constructs from Ad-TGF β 3 group, Ad-double group and Ad-combination group was stored at -20 °C for further analysis by specific ELISA kit (R&D Systems, Minneapolis, MN) following

the manufacture's instruction. Triplicates were performed for each time point. The concentration of TGF- β 3 from each group was calculated using SkanIt Software 2.2.

5.2.7 Flow cytometry analysis

The percentage of GFP-positive cells for transduction efficiency and the ratio of propidium iodide (50 μ g/mL, BD) staining cells for cell viability were distinguished at 48 h post transduction by using a FACSCalibur flow cytometer and CellQuest software (BD, Heidelberg, Germany). In each measurement, 10,000 cells were counted.

5.2.8 Histology and immunohistochemistry

For 3D constructs, samples from each group (n = 3) at Day 30 were fixed in 4% (w/v) neutral buffered paraformaldehyde, embedded in paraffin and sectioned (5 μ m thick). Then sections from all groups were deparaffined and stained with Safranin-O for glycosaminoglycans (GAGs). 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 (Santa Cruz Biotechnology) and Col II (Chemicon) were applied overnight (4 °C), respectively. Following three PBS washes, the sections for Col II or Col I were respectively incubated with HRP-conjugated (Clontech) at room temperature for 1 h. Finally, the presence of Col II or Col I was observed using the DAB Substrate kit (Clontech).

For monolayer cultures, cells were gently washed with PBS and then fixed with 2.5% glutaraldehyde at room temperature for 30min. And then cells were blocked with 1% goat serum, incubated with primary antibodies for Col I overnight and AlexaFluoro 546-labeled secondary antibodies (Invitrogen) for 1 h in sequence. After that, all samples were washed again, mounted with DAPI (Invitrogen) to locate the cell nucleus

and analyzed using fluorescence microscopy. Fluorescent density in each well was determined using Infinite 200 fluorescent microplate reader (TECAN Systems, San Jose, CA, USA) with the excitation wave length of 560nm and emission wave length of 590nm.

5.2.9 Statistical analysis

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

5.3 Results

5.3.1 Generation of high-titer Ad-double

The constructed recombinant adenoviral vector with two cassettes each encoding shRNA and TGF- β 3 was identified using XhoI restriction analysis. According to the plasmid map of adenoviral vector, there were six XhoI restriction sites in the adenoviral vector and one was engineered into shRNA-encoding cassette. Therefore, there should be seven expected bands with the following sizes: 14.5, 9.7, 6.3, 4.5, 2.5, 1.4 & 0.6 kbp. It was indicated in **Figure 5.2** that all of the above bands appeared in lane 3, demonstrating that the plasmids of recombinant adenoviral vectors extracted from this clone could be successfully constructed with two inserted functional cassettes; while only six bands shown in lane1, indicating that those extracted from this clone were not positive recombinants. Afterwards, these positive recombinants were transferred into HEK 293 cells to package as Ad-double. The packaged adenoviruses were then amplified, purified, titrated and aliquoted for the subsequent experiment.

Figure 5.2 XhoI Restriction analysis

Plasmids of constructed adenoviral vectors extracted from colonies were digested with enzyme XhoI and run on agarose gel. Lane 1: negative recombinants; Lane 2: DNA ladder; Lane 3: positive recombinants [167].

5.3.2 MOI optimization of Ad-TGF β 3, Ad-shRNA and Ad-double

In our present study, there are three kinds of functional recombinant adenoviruses, including Ad- TGF β 3, Ad-shRNA and Ad-double. In order to determine the optimal MOI of these adenoviral vectors for SMSCs infection, we infected P4 monolayer cultures of SMSCs with increasing MOI of Ad- TGF β 3, Ad-shRNA and Ad-double respectively.

As shown in **Figure 5.3A**, 48h after infection, microscopic inspection revealed that the percentage of cells expressing ZsGreen1 Living Colors Fluorescent Protein was augmented with increasing viral dose. However, it indicated no significant difference in cell viability with increasing MOI. The statistic data from flow cytometer analysis

(**Figure 5.3B**) indicated that the percentage of fluorescent green cells exhibited a viral dose-dependent increase in the range of ~77%-97% and the PI staining cells (dead cells) showed no obvious difference from MOI 100 to MOI 1000. The percentage of SMSCs, which expressed ZsGreen1 Living Colors Fluorescent Protein but were PI-negative, were 74.24%, 89.89%, 92.56%, 92.63% at MOI 100, 300, 600 and 1000, respectively. To measure the relative level of TGF- β 3 synthesis, qRT-PCR analysis for TGF- β 3 was performed and conditioned medium collected at 48h post-transduction was analyzed for TGF- β 3 content using ELISA. **Figure 5.3C & D** exhibited that the trend of TGF- β 3 transgene expression showed an ascent at transcriptional level and at protein level with increasing MOI. According to the rule that sufficiently high transduction efficiency and expression of TGF- β 3 by applying relatively low MOI could be achieved, MOI 300 for Ad- TGF β 3 was regarded as optimal MOI and used in the subsequent experiments.

Fig 5.3 MOI optimization of Ad-TGF β3.

Monolayer cultures of SMSCs were transduced with increasing MOI of Ad-TGF β3. At 48 h post-transduction, representative fluorescence of SMSCs infected with various MOI was observed under optical fluorescent microscope (A). The percentage of infected cells and dead cells were counted by flow cytometry to check transduction efficiency and cell viability (B). The expression of TGF-β3 was analyzed at transcriptional level by qRT-PCR (C) and protein level using ELISA assay (D). Magnification: 40X [178].

As for Ad-shRNA, it was shown in **Figure 5.4 A & B** that there was a slight increase in transduction efficiency with rising viral dose. The percentages of viable fluorescent green SMSCs at various MOI were listed as follows: MOI 100, 89.46%; MOI 300, 92.42%; MOI 600, 94.69%; MOI 1000, 93.62%. To detect the silence effect of Col I, qRT-PCR and immunofluorescent staining for Col I were performed. As shown in **Figure 5.4C**, the expression of Col I with Ad-shRNA treatment was effectively inhibited at transcriptional level. However, the silencing effect at MOI 300 was more significant than that at MOI 100, while there is no obvious difference between those at MOI 300, MOI 600 and MOI 1000. The result from immunofluorescent staining (**Figure 5.4D & E**) was also consistent with qRT-PCR data. Based on these results, MOI 300 for Ad-shRNA was selected for the following experiments.

Figure 5.4 MOI optimization of Ad-shRNA.

Monolayer cultures of SMSCs were transduced with increasing MOI of Ad-sh RNA. At 48 h post-transduction, representative fluorescence of SMSCs infected with various MOI was observed under optical fluorescent microscope (A). The percentage of infected cells and dead cells were counted by flow cytometry to check transduction efficiency and cell viability (B). The expression of Col I was analyzed at transcriptional level by qRT-PCR (C) and protein level by Immunofluorescent staining (D, E). Magnification: 40X (A, D) [178].

As similar as the methods used for Ad-TGF β 3 and Ad-shRNA, the optimal MOI of Ad-double was determined from the following three facets: 1) transduction efficiency; 2) expression level of TGF- β 3; and 3) silencing effect of Col I. From the pictures by fluorescent microscope (**Figure 5.5A**), the transduction efficiency in MOI 1000 was the highest among all the groups evidenced by both the amount and intensity of cells showing fluorescent green. The statistic data from flow cytometry (**Figure 5.5B**) were in accord with the observation from fluorescent microscope. The release of TGF- β 3 transgene was increased with rising MOI, at both transcriptional (**Figure 5.5C**) and protein level (**Figure 5.5D**). Nevertheless, the level of TGF- β 3 assessed by ELISA showed a lesser extend than that by Ad-TGF β 3, reaching around 6 ng/mL at MOI 1000. As far as effect on inhibiting Col I expression, it was shown in **Figure 5.5G** that Ad-double did have capability to suppress Col I expression compared with Neg group, exhibiting in MOI-dependent manner. Also, the immunofluorescent staining for Col I results indicated the consistent trend, with declining intensity at higher MOI. Amongst all the groups, MOI 1000 showed the best efficacy. Based on the above data, MOI 1000 for Ad-double was used in the subsequent study.

Figure 5.5 MOI optimization of Ad-double.

Monolayer cultures of SMSCs were transduced with increasing MOI of Ad-double. At 48 h post-transduction, representative fluorescence of SMSCs infected with various MOI was observed under optical fluorescent microscope (A). The percentage of infected cells and dead cells were counted by flow cytometry to check transduction efficiency and cell viability (B). The expression of TGF-β3 was analyzed at transcriptional level by qRT-PCR (C) and protein level using ELISA assay (D). The expression of Col I was analyzed at transcriptional level by qRT-PCR (G) and protein level by immunofluorescent staining (E, F). Magnification: 40X (A, E) [167].

5.3.3 Influence of co-transducing process on transduction efficiency

To study whether Ad- TGF β 3 and Ad-shRNA could influence each other when co-transducing the same target cells, we transduced SMSCs in monolayer with the following groups of adenoviruses: 1) MOI300 of Ad- TGF β 3; 2) MOI 300 of Ad- TGF β 3 and MOI300 of Ad-null; 3) MOI 300 of Ad-shRNA; 4) MOI 300 of Ad-shRNA and MOI 300 of Ad-null; 5) MOI 300 of Ad- TGF β 3 and MOI 300 of Ad-shRNA. 2 days after transduction, transduction efficiencies were determined by flow cytometry. Here, Ad-null shared the same adenoviral vector as we used for constructing functional adenoviruses except insertion of ZsGreen1 Living Colors Fluorescent Protein, i.e. the cells infected with Ad-null could not display fluorescent green.

As shown in **Figure 5.6**, the transduction efficiency for Ad- TGF β 3 would not be interfered under the presence of Ad-null. Similarly, the presence of Ad-null showed no significant influence on the infection effect of Ad-shRNA. Therefore, it could draw a conclusion that there would be no mutual interference in each other's transduction efficiency when SMSCs were treated with Ad-shRNA and Ad- TGF β 3 at the same time.

Figure 5.6 Transduction efficiency measured by flow cytometry.

SMSCs were infected with various adenoviruses as identified in the text. Two days after transduction, cells were collected and the number of fluorescent green cells in each group was counted by flow cytometry.

5.3.4 Chondrogenic differentiation of SMSCs following transduction with various recombinant adenoviruses in 3D cultures

In this study, we employed an alginate hydrogel system to evaluate the chondroinductive activity of SMSCs with the treatment of various functional recombinant adenoviruses. We infected P4 monolayer cultures of SMSCs with the following doses of recombinant adenoviruses, respectively: Ad- TGF β 3, MOI 300; Ad-shRNA, MOI 300; Ad-double, MOI 1000 and one combination (Ad- combination: infecting the cells with MOI 300 of Ad- TGF β 3 and MOI 300 of Ad-shRNA). 48h after transduction, we encapsulated the monolayer SMSCs into alginate hydrogel and cultured them for 30 days in a defined chondrogenesis medium. In parallel, SMSCs transfected with MOI 300 of Ad-null or without infection (designated as Neg) were also seeded into alginate hydrogel as control groups.

30 days after encapsulation, we determined the expression of genes specific for chondrogenesis from genetically modified SMSCs-laden alginate constructs in the six groups using qRT-PCR. The data from each group were normalized against those of Neg control. As shown in **Figure 5.7A**, we observed there was a significant increase in expression of mRNA for Col II in Ad-TGF β 3, Ad-shRNA, Ad-double and Ad-combination group, but not Ad-null group in comparison with Neg group. Thereinto, elevation of Col II expression in Ad- TGF β 3 group (171-fold) was greatly superior to the other three groups. **Figure 5.7B** depicted that expression of aggrecan in Ad- TGF β 3, Ad-double and Ad-combination group dramatically increased compared with Neg group, ranging from 5.7-fold for Ad-combination to 25.8-fold for Ad- TGF β 3 group; while a slight decrease in Ad-shRNA group. **Figure 5.7C** exhibited that transduction groups except Ad-null group resulted in remarkably elevated expression of cartilage oligomeric matrix protein (COMP). Ad- TGF β 3 group (832.9-fold) and

Ad-double group (511.8-fold) yielded much more significance in COMP expression than Ad-shRNA group (38.3-fold) and Ad-combination group (55.9-fold).

Figure 5.7 Analysis of expression of chondrogenic markers from transgenic SMSC-laden gels in the six groups of Neg, Ad-null, Ad-TGF β 3, Ad-shRNA, Ad-double and Ad-combination.

After 30 days, total RNA was extracted from alginate constructs (3/group) and the expression of Col II (A), aggrecan (B), COMP (C) was determined using quantitative qRT-PCR as described in Materials and Methods section. * $p < 0.05$, significant effects *versus* Neg [178].

Meanwhile, we detected the expression of Col I, which leads to fibrosis and damages

the mechanical load of cartilage, among these groups. **Figure 5.8A** revealed that Ad-TGF β 3 group brought the highest Col I expression level (4.3-fold), exceeding Ad-shRNA group (0.48-fold), Ad-double (1.7-fold) and Ad-combination (0.47-fold). Additionally, Col X is a marker for hypertrophic chondrocytes. SMSCs infected with Ad-shRNA (25.8-fold) expressed the highest yield of Col X, significantly exceeding those in the other groups, as shown in **Figure 5.8B**.

Figure 5.8 Analysis of expression of Col I and Col X from transgenic SMSC-laden gels in the six groups of Neg, Ad-null, Ad-TGF β 3, Ad-shRNA, Ad-double and Ad-combination.

After 30 days, total RNA was extracted from alginate constructs (3/group) and the expression of Col I (A), Col X (B) was determined using quantitative qRT-PCR as described in Materials and Methods section. * $p < 0.05$, significant effects *versus* Neg; # $p < 0.05$, significant effects *versus* Ad-TGF β 3 [178].

5.3.5 Histology and immunohistochemical staining

After 30-day culture, the cell/alginate constructs in the six groups were harvested and sectioned. Then the sections were subjected to safranin-O staining for GAGs and

immunohistochemical staining specific for Col II or Col I.

As shown in **Figure 5.9**, there showed faint or unperceivable GAGs and Col II staining in Neg, Ad-null and Ad-shRNA groups while more apparent GAGs and Col II staining in Ad-TGF β 3, Ad-double and Ad-combination groups, indicating that TGF- β 3 played an important role in chondrocytic differentiation of SMSCs and was beneficial to the accumulation of cartilage-specific matrix molecules in the cell/alginate constructs. However, more intense Col I staining was observed in Ad- TGF β 3 group. The dim Col I staining in Ad-shRNA, Ad-double and Ad-combination groups confirmed the silencing effect of Col I by shRNA specific for Col I.

Figure 5.9 Histological and immunohistochemical staining for GAGs, Col II and Col I.

The constructs in Neg, Ad-null, Ad-TGF β3, Ad-shRNA, Ad-double and Ad-combination groups were harvested at Day 30, sectioned and then stained with Safranin-O for GAGs, or immunohistochemical stained with antibodies specific for Col II and Col I. Magnification, 100x [178].

5.3.6 Corresponding transgene expression of TGF- β 3 for the 30-day culture period by ELISA analysis

Conditioned medium was collected from Ad-TGF β 3 group, Ad-double group and Ad-combination group every 3 days during the 30-day culture period. Afterwards, the medium was analyzed using ELISA assay to measure the dynamic change of TGF- β 3 concentration.

As shown in **Fig5.10A**, the concentration of TGF- β 3 transgene in Ad- TGF β 3 group was around 17 ng/mL at Day 3 and then a marked decline appeared between Day 6 and Day 12. Finally, the concentration of TGF- β 3 secreted by infected SMSCs was maintained at about 2 ng/mL for the final twenty days. Since the same MOI of Ad-TGF β 3 was used in Ad-combination group, the trend of TGF- β 3 secretion was very similar (**Figure 5.10C**).

The expression profile of TGF- β 3 in Ad-double group shown in **Figure 5.10B** was similar to those of the other two groups. However, the peak concentration of TGF- β 3 was around 7.5 ng/mL at Day 3 and then was kept at about 2 ng/mL till the end of the culture period.

Figure 5.10 Quantitative analysis of hTGF-β3 expression for the 30-day culture period by specific ELISA.

Values represent TGF-β3 levels in conditioned medium collected from Ad-TGF β3 group (A), Ad-double group (B) and Ad-combination group (C) every 3 days [178].

5.4 Discussion

In recent decades, MSC chondrogenesis catalyzed by adenoviral vector-mediated transgenic growth factor such as IGF1, TGF β1 or BMP2 [19, 92] has been widely studied, from which favorable outcomes have been achieved. Further studies have reported simultaneous/sequential delivery of growth factors or combination of

mechanical and chemical stimulation for cartilage engineering [179, 180]. In contrast, few studies have been performed on whether combinational application of transgenic growth factors and RNAi could offer synergistic effect in chondrogenic differentiation of SMSCs. In this study, we figured out two strategies to deliver genes encoding both TGF- β 3 and anti-Col I shRNA into SMSCs, in the hope that this cocktail could bring beneficial effects towards Col I-suppressed chondrogenesis by SMSCs. We demonstrated that at an optimal MOI 300 for Ad- TGF β 3, there was around 18 ng/mL of TGF- β 3 expressed in the medium that could meet the general requirement for chondrocytic differentiation [37]. We also observed that at MOI 300 for Ad-shRNA, there was a two-fold reduction in expression of Col I at protein level and 90% reduction at transcriptional level. These data suggested that adenovirus-mediated transgenic TGF- β 3 or shRNA with optimal MOI could successfully function in expected role.

Nowadays, SMSCs appear as an attractive cell source for cartilage regeneration due to the ease of yielding and efficiency in undergoing chondrogenesis. Moreover, as an autologous source tissue, synovium could readily self-regenerate after surgery [181]; while the quality and multipotency of the yielded SMSCs could be maintained for a long time regardless of the donor age, cell passage or cryopreservation conditions [153]. SMSCs at passage 4 were usually populated and selected as target cells, in which the cellular behavior and phenotype tended stable. However, one significant issue to be addressed is the expression of Col I in SMSCs during the monolayer culture *in vitro* [182], which would attenuate the efficacy of chondrogenesis. Besides, our previous [161] and current data further showed that adenoviral vector-mediated TGF- β 3 up-regulated the expression of Col I in alginate hydrogel constructs of SMSCs. Hence, despite the beneficial effect on chondrogenic differentiation of SMSCs,

TGF- β 3 treatment alone might bring severe side-effect of fibrogenesis and therefore impair the overall quality of the engineered cartilage.

siRNA is a short and double-stranded RNA molecule that can cleave the mRNA of target gene with complementary sequence [123]. This process termed as RNAi, emerged as an effective tool with the function to silence the undesired genes. At present, RNAi has been employed widely in gene functional analysis in mammalian cells and gene therapy for various diseases, including cartilage regeneration [129]. However, since both the efficacy and efficiency of direct delivery of siRNA remains unsatisfactory, shRNA, which encodes the gene of siRNA and enables endogenous supply of it in host cells, would be delivered to SMSCs via adenoviral vectors in this study. qRT-PCR analysis confirmed the Col I- silencing effect of Ad-shRNA in alginate constructs in 30-day culture. Nevertheless, Ad-shRNA treatment alone also posed its own side-effect: it led to the highest expression of Col X, the marker for hypertrophic chondrocytes, among the six groups.

The trial with simultaneous cotransduction of both Ad- TGF β 3 and Ad-shRNA, namely the Ad-combination group, resulted in effective chondrogenic differentiation of SMSCs. In comparison with Neg group, Ad-combination group enhanced the expression of chondrocytic markers: Col II (7.7-fold), aggrecan (5.7-fold) and COMP (55.9-fold). Moreover, Ad-combination group attenuated the expression of Col I (0.47-fold) and Col X (0.9-fold), proving that Ad-combination could repress cell fibrosis and hypertrophy. Ad-combination showed similar capability to Ad-shRNA in inhibiting the Col I expression.

The trial with Ad-double group indicated that it favored the expression of cartilage-specific genes as well; besides, in comparison with Ad- TGF β 3 group, Ad-double group also mitigated the expression of Col I, though generally this silencing

effect was observably lower than that in Ad-shRNA and Ad-combination groups. Furthermore, Ad-double group led to the lowest expression of Col X among all six groups.

These analyses were found consistent with histological and immunohistochemical staining results. Additionally, the data from ELISA assay indicated that profiles of transgenic TGF- β 3 expression posed large similarities among Ad- TGF β 3, Ad-double and Ad-combination groups, in all of which the release of growth factor reached the peak value at Day 3 and kept declining with time. Compared with our previous findings [93] that suggested a short pharmacological half-life of the directly dosed growth factor, the results from this study indicated that in the functional time of adenovirus, adenovirus-mediated transgenic TGF- β 3 could provide localized, continuous, and longer-term supply of growth factors to the target cells.

Although adenovirus-mediated coexpression of TGF- β 3 and shRNA has showed capability in guiding the chondrogenesis of SMSCs as well as effective inhibition of fibrosis and cell hypertrophy, the extent of chondrogenesis in Ad-double and Ad-combination groups is inferior to that in Ad- TGF β 3 group. To fulfill the dual function more effectively, it is necessary to figure out a more sophisticated strategy to construct Ad-double with different arrangement of the expression cassettes that might have higher efficiencies. Some other parameters such as transduction timing, the proportion of Ad- TGF β 3 and Ad-shRNA should be optimized. Besides, the sequential transduction scheme might be tried. Thus, it is possible to produce the regenerated cartilage more similar to the native one.

5.5 Summary

In this study, we demonstrated that although individual treatment of Ad- TGF β 3 or

Ad-shRNA could exert its efficacy on promoting chondrogenesis of SMSCs or inhibiting the expression of Col I, they also have the drawbacks of their own. Contrastively, evidence was provided that combination of a growth factor and shRNA possessed the potential in enhancing the *in vitro* chondrogenesis of SMSCs as well as suppressing the production of Col I. The dual functions applied in engineered cartilage will make cell-based therapeutics more feasible and reliable for cartilage regeneration..

Chapter 6

Enhancing redifferentiation of dedifferentiated chondrocytes in 3D scaffold with dual transgenes of TGF- β 3 and shRNA silencing type I collagen

6.1 Introduction

Growth factors including transforming growth factor (TGF- β), bone morphogenetic proteins (BMPs), etc. show their capability of improving the proliferation of chondrocytes and stimulating the synthesis of cartilage ECM [183-185]. TGF- β 3 has also been identified as a potent biological factor for the promotion of chondrogenesis and expression of cartilaginous genes [186, 187]. Yun *et al.* reported that composite hydrogel co-encapsulated with TGF- β 3 helped to maintain chondrocytes in a spherical morphology and helped in the synthesis of the total collagen content [188]. Hence, it should be helpful to preserve the chondrocyte quality by genetically engineering passaged chondrocytes with Ad-TGF β 3. On the other side, with the complete development of antisense technology, which serves to turn the undesired gene off at post-transcriptional level by specific small interfering RNA (siRNA) [123], we can take advantage of this promising tool to eliminate the expression of Col I in dedifferentiated chondrocytes.

We designed this study to observe whether the introduction of shRNA specific for Col I coupled with the delivery of TGF- β 3 could make dedifferentiated chondrocytes re-express Col II and aggrecan but halt the expression of Col I. Two ways were used to deliver both anti-Col I shRNA and TGF- β 3 to passaged chondrocytes: first method was to transduce cells with Ad-double while the second method involved the simultaneous co-transduction of cells with Ad-shRNA and Ad- TGF β 3. Redifferentiation effects of

dedifferentiated chondrocytes treated with the above-mentioned ways were evaluated by culturing the cells in monolayer or alginate beads.

6.2 Materials and methods

6.2.1 Porcine chondrocytes isolation and expansion

Cartilage tissues were harvested aseptically from the knee joint of a 5-month-old swine and washed twice with PBS solution containing penicillin. The tissue was then minced finely and digested with 0.1% collagenase II at 37 °C for 12 hours under gentle stirring, which will result in cells being released from the collagen matrix. The released cells (designated P0) were collected by centrifuge and then propagated in complete growth medium comprising of high-glucose DMEM supplemented with 20% (v/v) FBS, 0.01 M 4-(2-hydroxyethyl)-piperazine-1-ethanesulfonic acid (HEPES), 0.1 mM nonessential amino acids (NEAA), 0.4 mM proline, 0.05 mg/mL vitamin C, 100 units/mL penicillin and 100 µg/mL streptomycin. The cells were passaged successively upon confluence until Passage 3 (P3) cells were obtained. The above-mentioned reagents for cell culture were purchased from Gibco (Invitrogen, Singapore).

6.2.2 Adenoviral transduction of dedifferentiated chondrocytes in monolayer

P3 chondrocytes (1.5×10^5 cells) were seeded into each well of the 12-well plate in advance. After 24h incubation, the cells in each well were infected with various MOIs of recombinant adenoviruses as stated specifically in the text or figure legends and incubated in 400 µL of serum free DMEM for 2h at 37°C in 5% CO₂. After 2 h infection, the medium was replenished with complete growth medium and the cells were left in the incubator for the serial assays.

6.2.3 3D-culture of dedifferentiated chondrocytes in alginate hydrogels

Individual 150-cm² flasks of P3 chondrocytes were infected with specific MOI of Ad-null, Ad-TGF β 3, Ad-shRNA, Ad-double as well as co-transfected simultaneously with both Ad-TGF β 3 and Ad-shRNA as indicated in the text. And then the cells were performed the same procedure as that described in previous chapter to make alginate beads. Lastly, each well of a 24-well plate was filled with 2 beads and 1 mL of chondrogenic medium. The medium was replaced and harvested for ELISA analysis every 3 days. The experiment was conducted for a period of 30 days. One day after gelation would be defined at Day 0.

6.2.4 RT-PCR and qRT-PCR

RNA from monolayer culture was isolated using TRIZOL[®] Reagent (Invitrogen) following the manufacture's instruction. For alginate beads, the ways for RNA extraction and qRT-PCR were referred to the steps mentioned in previous chapters.

The conventional PCR reactions were performed using GoTaq[®] Flexi DNA Polymerase (Promega) and the amplification products were visualized on 1.2% agarose gels with ethidium bromide (EB) staining. All the primer sequences for RT-PCR and qRT-PCR are shown in **Table 6.1**.

Table 6.1 Primers for PCR.

Genes	Primer sequence (both 5'-3')	Size (bP)	Annealing temperature (°C)
RT-PCR:			
Type II collagen	F: TGCCTACCTGGACGAAGC R: CCCAGTTCAGGCTCTTAG	449	55
Aggrecan	F: CGAGGAGCAGGAGTTTGTCAAC R: ATCATCACCACGCAGTCCTCTC	177	58

β -actin	F: CGAGGAGCAGGAGTTTGTCAAC R: ATCATCACCACGCAGTCCTCTC	498	55
qRT-PCR:			
Type I collagen	F: CCTGCGTGTACCCCACTCA R: ACCAGACATGCCTCTTGTCTCTT	84	58
Type II collagen	F: GCTATGGAGATGACAACCTGGCTC R: ACAACGATGGCTGTCCCTCA	256	58
Type X collagen	F: CAGGTACCAGAGGTCCCATC R: CATTGAGGCCCTTAGTTGCT	117	58
COMP	F: GGCACATTCCACGTGAACA R: GGTTCGCCTGCCAGTATGTC	127	58
Aggrecan	F: CGAGGAGCAGGAGTTTGTCAAC R: ATCATCACCACGCAGTCCTCTC	177	58
RPL4	F: CAAGAGTAACTACAACCTTC R: GAACTCTACGATGAATCTTC	140	58

6.2.5 Quantitative analysis of TGF- β 3 expression and GAGs synthesis

To determine TGF- β 3 concentration, conditioned medium in Ad-TGF β 3 group, Ad-double group and Ad-combination group were collected from monolayer or 3D culture system at various time points and stored at -20 °C for further assay by specific ELISA kit (R&D Systems, Minneapolis, MN). Triplicates were performed for each time point. All the data were analyzed using SkanIt Software 2.2.

To determine quantity of DNA and GAGs, at 30 days of 3D culture, construct samples were taken out and rinsed with PBS to remove excess medium. After frozen and lyophilized, the constructs were digested with papain (Sigma). Then the amount of DNA was assayed fluoremetrically by mixing the papain-digested samples with Hoechst 33258 (Sigma) [189]. The sulfated GAGs content in each specimen was quantified by mixing an aliquot of the digested samples with 1, 9-dimethylmethylene blue and reading the absorbance at 525 nm using a UV-VIS spectrophotometer (Multiskan_ spectrum, Thermo) [190].

6.2.6 Flow cytometry analysis

At 48 h post transduction, cells were trypsinized and washed with PBS. To check cell viability, cells were treated with propidium iodide (PI, 50 $\mu\text{g/mL}$, BD) for dead cells. Afterward, the number of fluorescent green cells and the amount of PI-staining cells was counted by using a FACSCalibur flow cytometer and CellQuest software (BD, Heidelberg, Germany). In each measurement, the total number of cells was 10,000.

6.2.7 Histology and Immunohistochemistry

Histological and immunohistochemical staining for 3D constructs and monolayer cultures were described in chapter 5.

6.2.8 Statistical analysis

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

6.3 Results

6.3.1 Chondrocyte dedifferentiation

Microscopic inspection of the cells shown in **Figure 6.1A** illustrated that the spherical chondrocytes found in the first passage transformed over the repeated subcultures into P4 cells with an increasingly fibroblastic morphology.

On top of that, the data of PCR (**Figure 6.1B**) for the expression of aggrecan and Col II also indicated that dedifferentiation had occurred during the subculture process. We noticed that the expression of both aggrecan and Col-II dwindled until expression was lost by the fourth passage, while the expression of β -actin remained fairly constant. Both of these results suggested that dedifferentiation had indeed occurred by the time

chondrocytes were cultured to P4. Therefore, we chose P4 dedifferentiated chondrocytes as the target cells in the subsequent experiments.

Figure 6.1 Chondrocyte dedifferentiation in monolayer after serial passages. Chondrocytes at P1 (A) and P4 (B) were inspected under microscope. RT-PCR followed by gel electrophoresis (C) was conducted to indicate the expression of cartilage-specific genes, namely aggrecan and Col II in various passages of chondrocytes. Housekeeping gene β -actin acted as internal control. Magnification: 100X.

6.3.2 MOI Optimization for Ad-TGF β 3, Ad-shRNA and Ad-double

The purpose of this experiment is to identify the optimum MOI of the recombinant

adenoviruses used to infect target chondrocytes. As documented, three types of adenoviruses, namely Ad-TGF β 3, Ad-shRNA and Ad-double were constructed for this research. P3 chondrocytes in monolayer were infected with Ad- TGF β 3, Ad- shRNA and Ad-double respectively at various MOIs: MOI 100, MOI 300, MOI 600 and MOI 1000. At 24 h post infection, the medium was replaced. Subsequent after another 24 h, RNA extraction was conducted and the expression of TGF- β 3 and Col I was measured at transcriptional level by qRT-PCR; the medium was collected for ELISA assay to evaluate the expression of TGF- β 3; immunofluorescent staining for Col I was performed to observe the expression of Col I at protein level. Also, transduction efficiency and cell viability were determined using flow cytometry. Thus, optimal MOI was selected based on the principle that the lowest MOI could fulfill sufficiently high transduction efficiency as well as expression of TGF- β 3 or/and effective Col I silencing.

As for Ad- TGF β 3, fluorescent microscopic inspection indicated that the ratio of fluorescent green (infected) cells increased generally when adenoviruses of increasing MOIs were used (**Figure 6.2A**). The statistical data obtained from the flow cytometer analysis (**Figure 6.2B**) indicated that there was no considerable change in the viability of the cells despite an increased viral dosage. Compared with MOI 100, the group infected with Ad-TGF β 3 at MOI 300 showed a significant increase in transduction efficiency, subsequent increments in viral dosage did not affect the transduction efficiency. At transcriptional level, qRT-PCR data (**Figure 6.2C**) revealed that the expression of TGF- β 3 was augmented in a viral dose-dependent manner. Furthermore, results of ELISA analysis (**Figure 6.2D**) showed that the expression of TGF- β 3 at protein level exhibited the ascending profile with increasing viral dosages. Based on the above analysis and the selection principle, MOI 300 for Ad-TGF β 3 was regarded

as optimal MOI and used in the subsequent experiments.

Figure 6.2 MOI optimization of Ad-TGF β3.

P3 chondrocytes in monolayer were infected with Ad- TGF β3 at various MOIs: MOI 100, MOI 300, MOI 600 and MOI 1000. 2 day after transduction, representative fluorescence of chondrocytes was observed under fluorescent microscope (A). Transduction efficiency and cell viability were determined using flow cytometry (B). The expression of TGF-β3 was measured at transcriptional level using qRT-PCR (C) and at protein level using ELISA assay (D). Magnification: 40X.

As for Ad-shRNA, the transduction efficiency at MOI 300 presented a significant rise compared with that at MOI100, while there was no significance compared with that at MOI 600 or MOI 1000 (**Figure 6.3B**). These data were also consistent with the inspection by fluorescent microscope as shown in **Figure 6.3A**. To observe the

capability of inhibiting Col I by Ad-shRNA, the level of Col I expression was measured at transcriptional level using qRT-PCR and at protein level by immunofluorescent staining. Both qRT-PCR data (**Figure 6.3C**) and immunofluorescent staining results (**Figure 6.3D&E**) indicated that chondrocytes treated with Ad-shRNA showed signs of Col I gene silencing, as evident in the decreased expression of Col I. qRT-PCR data showed that MOI 300 led to a drop in expression level of Col I that was half of the Neg control. Subsequent increase in viral doses of Ad-shRNA did not result in significant reduction in Col I expression. Immunofluorescent staining results revealed that expression level of Col I declined with increasing MOI of Ad-shRNA. Conclusively, MOI 300 was chosen for the subsequent study.

Like those for Ad-TGF β 3 and Ad-shRNA, the analyses for Ad-double were conducted in three facets: 1) transduction efficiency; 2) release of TGF- β 3; and 3) silencing effect of Col I. It was shown in **Figure 6.4A&B** that viral doses of MOI 600 and MOI 1000 showed notably higher transduction efficiency compared with those in MOI 100 and MOI 300. The expression of TGF- β 3 transgene was ascended with increasing viral dosage at both transcriptional level and protein level (**Figure 6.4 C&D**). However, it was observed from ELISA results that the maximum release of TGF- β 3 was around 7 ng/mL at MOI 1000. In the case of gene knockout of Col I, qRT-PCR data seen in **Figure 6.4F** coupled with immunofluorescent staining results illustrated in **Figure 6.4E&G**, showed that Ad-double did have effect on inhibition of Col I compared with Neg control and MOI 1000 induced the most significant inhibition of Col I production. Concluding from these results, MOI 1000 for Ad-double was selected since this viral dose could produce sufficient TGF- β 3 and simultaneously offer efficient silencing of Col I production.

Figure 6.3 MOI optimization of Ad-shRNA.

P3 chondrocytes in monolayer were infected with Ad-shRNA at various MOIs: MOI 100, MOI 300, MOI 600 and MOI 1000. 2 day after transduction, representative fluorescence of chondrocytes was observed under fluorescent microscope (A). Transduction efficiency and cell viability were determined using flow cytometry (B). The expression of Col I was measured at transcriptional level using qRT-PCR (C) and at protein level by immunofluorescent staining (D, E). Magnification: 40X (A, D).

Figure 6.4 MOI optimization of Ad-double.

P3 chondrocytes in monolayer were infected with Ad- double at various MOIs: MOI 100, MOI 300, MOI 600 and MOI 1000. 2 day after transduction, representative fluorescence of chondrocytes was observed under fluorescent microscope (A). Transduction efficiency and cell viability were determined using flow cytometry (B). The expression of TGF- β 3 was measured at transcriptional level using qRT-PCR (C) and at protein level using ELISA assay (D). The expression of Col I was determined at transcriptional level using qRT-PCR (F) and at protein level by immunofluorescent staining (E, G). Magnification: 40X (A, E).

6.3.3 Influence of co-transducing process on transduction efficiency

Since the target cells have been changed from SMSCs to chondrocytes, we performed this experiment again following the procedure described in chapter 5.

As shown in **Figure 6.5**, there was no significance in transduction efficiencies between group 1 and group 2 as well as between group 3 and group 4. Namely, when we infected cells with Ad-null and Ad- TGF β 3 simultaneously, the presence of Ad-null could not interfere severely with the transduction efficiency of Ad- TGF β 3. Similarly, the presence of Ad-null showed no significant influence on the infection effect of Ad-shRNA. Therefore, we could draw a conclusion that there would be no mutual interference in each other's transduction efficiency when cells were treated with Ad-shRNA and Ad- TGF β 3 at the same time.

Figure 6.5 Analysis of transduction efficiency by flow cytometry.

Chondrocytes were infected with various adenoviruses as identified in the text. Two days after transduction, cells were collected and the number of fluorescent green cells in each group was counted by flow cytometry.

6.3.4 Redifferentiation of dedifferentiated chondrocytes in monolayer

Before encapsulating the cells in alginate hydrogels, we performed a trial by using monolayer culture system to observe chondrocytic gene expression of genetically modified dedifferentiated chondrocytes. Passaged chondrocytes were infected with optimal doses of recombinant adenoviruses as follows: Ad- TGF β 3, MOI 300; Ad-shRNA, MOI 300; Ad-double, MOI 1000 as well as co-transduced with Ad- TGF β 3 (MOI 300) and Ad-shRNA (MOI 300) (this group was designated as Ad-combination). Additionally, cells without transduction (Neg control) and cells infected with adenovirus without inserted gene (Ad-null) were used as controls. Cells were then kept in chondrogenic medium for 7 days. Subsequently, RNA from each group was extracted followed by qRT-PCR analysis.

As shown in **Figure 6.6A**, after 7 days monolayer culture, Ad-TGF β 3, Ad-double and Ad-combination groups indicated increased expression of aggrecan, compared to Neg control group. These three groups also showed high expression of Col II (**Figure 6.6B**) while Ad-shRNA group showed the lowest expression levels in aggrecan and Col II. Looking at **Figure 6.6C**, we observed that shRNA against Col I was very successful in the inhibition of Col I production, as evident in Ad-shRNA and Ad-combination groups. Although the highest expression of chondrocytic markers was seen in Ad-TGF β 3 group, Ad- TGF β 3 also appeared to result in the highest production of Col I among all the groups. Compared to Ad-TGF β 3 group, Ad-double group seemed still effective on suppressing the Col I expression.

Figure 6.6 Analysis of chondrocytic gene expression from genetically modified chondrocytes in monolayer cultures.

7 days post infection, total RNA was extracted from cells in the six groups of Neg, Ad-null, Ad-TGF β 3, Ad-shRNA, Ad-double and Ad-combination followed by qRT-PCR for the expression of aggrecan (A), type II collagen (B), type I collagen (C).

* $P < 0.05$, significant effects versus Neg.

6.3.5 Redifferentiation of dedifferentiated chondrocytes in alginate hydrogels

For better evaluation of redifferentiation of genetically modified chondrocytes, we seeded cells in alginate hydrogels and cultured the alginate beads in chondrogenic medium for 30 days. Here, we conducted two sets of experiments: For set A, the

optimum MOI of each functional recombinant adenovirus was selected in this experiment. i.e. MOI 300 was used for Ad-TGF β 3 and Ad-shRNA while MOI 1000 was used for Ad-double. In Ad-combination group, chondrocytes were infected with both MOI 300 of Ad-TGF β 3 and MOI 300 of Ad-shRNA simultaneously. For set B, after observing the results in monolayer culture, we noticed that the results offered by Ad-combination group (Ad-TGF β 3-MOI 300/ Ad-shRNA-MOI 300) showed effective Col I inhibition but the expression levels of chondrogenesis markers, aggrecan and Col II were lower than those in both Ad-TGF β 3 and Ad-double groups. Hence, we up-regulated the MOI of Ad-TGF β 3 in Ad-combination group from MOI 300 to MOI 700. Also, the sum of viral dosage used in Ad-combination group was equivalent to the optimum MOI of Ad-double, which made the results comparable. Furthermore, we added two more groups which were cultured in the chondrogenic medium with supplement of recombinant TGF- β 3 protein at a concentration of 10 ng/mL. All the groups' information for these two sets of 3D experiments was listed in **Table 6.2**.

In the meanwhile, the data for two groups, namely P4 monolayer chondrocytes and native cartilage were included to facilitate comparison. The values obtained in native cartilage group indicated the direction we intended to bio-mimic, while P4 monolayer chondrocytes was used as a control group that represented prolonged monolayer cultured cells.

Table 6.2 Groups for 3D experiment.

Set A			Set B		
No.	Groups	Remarks	No.	Groups	Remarks
1	Neg	Without virus treatment	1	Neg	Without virus treatment
2	Ad-null	MOI 300	2	Ad-null	MOI 300
3	Ad-TGF β 3	MOI 300	3	Ad-TGF β 3	MOI 300
4	Ad-shRNA	MOI 300	4	Ad-shRNA	MOI 300
5	Ad-double	MOI 1000	5	Ad-double	MOI 1000
6	Ad-combination	Ad- TGF β 3 (MOI 300) / Ad-shRNA (MOI 300)	6	Ad-combination	Ad- TGF β 3 (MOI 700) / Ad-shRNA (MOI 300)
			7	r TGF- β 3	In the presence of recombinant TGF- β 3 at 10 ng/ml
			8	rTGF- β 3/Ad-shRNA	Alginate beads with MOI 300 Ad-shRNA infected chondrocytes & in the presence of recombinant TGF- β 3 at 10 ng/ml

From the results of Set A, it was evident in **Figure 6.7A** that there was a significant increase in the production of aggrecan in groups of Ad-TGF β 3, Ad-double and Ad-combination as compared with the Neg control group. The expression level in the Ad-double group was most similar to that of native cartilage tissue, while expression in Ad-TGF β 3 was 2.4 times more than native tissue. In the case of Col II, significant

expression could be seen in Ad-TGF β 3, Ad double and Ad-combination. Although the production of Col II was not as high as that of native cartilage tissue, the genetically modified chondrocytes showed higher Col II expression compared with P4, Neg control and Ad-null chondrocytes, among which Ad-TGF β 3 group displayed the greatest expression of Col II. As far as the expression of cartilage oligomeric matrix protein (COMP), we observed elevated production of COMP in Ad-TGF β 3 (44.2-fold), Ad-double (12.7-fold) and Ad-combination (4.2-fold) groups compared to the Neg control group. However, these values were still uncomparable to the native tissue's production which stood at 95 times more than the Neg control.

As shown in **Figure 6.7B**, native cartilage tissue produced 4 times less Col I expression compared to the Neg control while Ad-TGF β 3 group appeared to promote the expression of Col I. Both Ad-shRNA and Ad-combination produced closely similar amount of Col I compared to native chondrocytes. In the case of Ad-double, we observed a significant inhibition of Col I expression compared with Ad-TGF β 3 groups. Considering the expression of Col X, a marker for hypertrophic chondrocytes, native cartilage expressed the highest Col X among all the groups, which could be attributed to the fact that the deep layer of cartilage tissue was mainly composed of hypertrophic chondrocytes.

Figure 6.7 Analysis of gene expression from genetically modified chondrocytes in alginate hydrogels.

Total RNA was extracted from alginate beads followed by qRT-PCR for the expression of cartilage-specific genes including aggrecan, type II collagen, COMP (A) as well as type I collagen and type X collagen (B). The upper row was for set A while the lower row was for set B. * $P < 0.05$, significant effects versus Neg. # $P < 0.05$, significant effects versus Ad-TGF $\beta 3$.

For set B experiment, in general, there was a close resemblance in the expression profiles of Ad-TGF β 3 and Ad-double groups (**Figure 6.7A**) while there was a significant elevation in the expression of aggrecan in the Ad-combination group that was not seen in set A experiment. Furthermore, manual addition of recombinant TGF- β 3 protein also resulted in an increased production of aggrecan compared with Neg control and monolayer culture of P4 chondrocytes. We noticed that the expression trends of Col II was comparable to the results gathered from set A experiment. Exogenous TGF- β 3 protein did not increase Col II expression compared with Neg group, despite the fact that the expression did exceed P4 monolayer-cultured cells. For COMP, Ad-TGF β 3, Ad-double, Ad-combination and both groups with manual addition of TGF- β 3 exhibited improved COMP expression compared to the Neg control and P4 monolayer culture of cells.

As for Col I expression (**Figure 7.7B**), it could be seen in both Ad-shRNA groups, with and without the presence of recombinant TGF- β 3 protein, the expression level of Col I closely mimicked that of native cartilage tissue. In addition, Ad-double and Ad-combination groups showed their effect on suppressing Col I expression compared with Ad-TGF β 3 group, which expressed the highest Col I expression level among all the genetically modified groups. And the results obtained in Col-X expression appeared to be comparable with the results in set A experiment.

6.3.6 Quantitative analysis of TGF- β 3 expression

Conditioned medium was collected from Ad-TGF β 3, Ad-double and Ad-combination groups every 3 days during the 30-day culture period followed by analysis with ELISA to measure protein concentration changes of TGF- β 3.

The general trend in all graphs shown in **Figure 6.8** indicated a general decrement in

the TGF- β 3 concentration towards the end of the 30-day culture period. In the case of Ad-TGF β 3 shown in **Figure 6.8A**, the initial concentration of TGF- β 3 at Day 3 was about 15ng/mL. The concentration faced a sharp decline between Day 3 and Day 6, followed by a slower descent. The eventual concentration at the end of the 30 days registered at around 2ng/mL.

As shown in **Figure 6.8B**, we found that the initial concentration in Ad-double group appeared to be the lowest compared to the other two groups, with a magnitude of 8ng/mL. However, the decline in concentration appeared to be the most gradual compared to Ad-TGF β 3 and Ad-combination groups. The concentration of TGF- β 3 maintained above 2ng/mL until Day 21 before dropping to about 1.2ng/mL by Day 30. In the case of Ad-combination group illustrated in **Figure 6.8C&D**, the initial concentration in set B experiment was found to be 18.4ng/mL while that in set A experiment was 10.5 ng/mL. On top of that, the concentration in these two sets of experiments exhibited a similar profile: gradually declined between Day 3 and Day 15 as well as maintained at around 0.5ng/mL for the rest of the culture period.

Figure 6.8 Dynamic changes of TGF-β3 expression for the 30-day culture period by ELISA analysis.

Values represented TGF-β3 concentrations in conditioned medium collected every 3 days from Ad-TGF β3 group (A), Ad-double group (B), Ad-combination group (set A) (C) and Ad-combination group (set B) (D).

6.3.7 Analysis of GAGs deposition by histological staining and biochemical assays

To observe GAGs accumulation in alginate constructs, the alginate beads in the eight groups were collected and sectioned at Day 30, followed by safranin-O staining for detection of GAGs. Also, biochemical assays were performed to quantify GAGs.

As shown in **Figure 6.9A**, the groups which were cultured in the presence of TGF-β3, namely Ad-TGF β3, Ad-double, Ad-combination, rTGF β3 and Ad-shRNA/rTGF β3

groups, showed abundant staining for GAGs, while Neg, Ad-null and Ad-shRNA groups showed faint staining. In accordance with the qRT-PCR data and histological findings, the genetically modified groups (Ad-TGF β 3, Ad-double, Ad-combination) synthesized significantly more GAGs than Neg control group (**Figure 6.9B**) by biochemical assays. Also, rTGF β 3 and Ad-shRNA/rTGF β 3 groups had an evident increase in GAG accumulation when compared with Neg control group.

It should be highlighted that the staining for GAGs in genetically modified groups were stronger than that in rTGF β 3 and Ad-shRNA/ rTGF β 3 groups. And the consistent results were obtained by biochemical assays. All these results demonstrated that the extent of redifferentiation was greater when TGF- β 3 was provided by means of a transgene than a recombinant protein.

Figure 6.9 Analysis of GAGs production.

The constructs in all of the eight groups were harvested at Day 30, sectioned and subjected to Safranin-O for GAGs (A). Also, GAGs of all the samples were quantified by biochemical assays (B). Magnification, 200x.

6.3.8 Immunohistochemical staining for Col I and Col II

To observe the deposition of cartilaginous ECM, all the samples were collected and sectioned at Day 30, followed by immunohistochemical staining specific for Col II or Col I.

As shown in **Figure 6.10**, Ad-TGF β 3, Ad-double, Ad-combination groups

considerably yielded more Col II production, as evidenced by apparent staining for Col II in these groups while invisible or dim staining in Neg, Ad-null, Ad-shRNA, rTGF β 3 and Ad-shRNA/rTGF β 3 groups. In addition, we observed that Col I staining in Ad-TGF β 3 group was more intensive. The negligible or undetectable production in Ad-shRNA, Ad-double, Ad-combination and Ad-shRNA/rTGF β 3 groups substantiated that shRNA targeting Col I did effectively inhibit the expression of Col I.

Figure 6.10 Immunohistochemical staining for Col II and Col I.

The constructs in all of the eight groups were harvested at Day 30, sectioned and immunohistochemical stained with antibodies specific for Col II and Col I. Magnification, 200x.

6.4 Discussion

In native conditions, healthy chondrocytes maintain a spherical morphology and have their ability to produce cartilage-specific ECM molecules such as Col II and aggrecan. In order to use chondrocytes for future implantation, these characteristics must be maintained. Based on our results in qRT-PCR, we identified a direct contrast between the native cartilage tissue we hoped to bio-mimick and the P4 chondrocytes that could potentially be used in ACT. Dedifferentiation was likely to have significantly affected in P4 chondrocytes onwards, evidently from the high Col I expression but low Col II and aggrecan production compared with the native cartilage. This could be expected that the use of these dedifferentiated chondrocytes as therapeutic cells for the construction of neocartilage would very likely cause suboptimal tissue quality with reduced biochemical content and compromised mechanical properties.

As such, a large number of studies have been performed on this issue, aiming to revert the dedifferentiated chondrocytes back to their chondrocytic phenotype. Besides the employment of 3D culture system, interests gradually focus on the introduction of growth factors by means of gene delivery. Lin and coworkers [76] found that adenoviral vector-mediated BMP-4 had capability to recover chondrocytic phenotype of dedifferentiated chondrocytes *in vitro* and *in vivo*. A series of studies by Sung *et al.* [77, 97] demonstrated that transfer of BMP-2 or TGF- β 1 via baculovirus could induce redifferentiation of dedifferentiated chondrocytes. Especially, the co-transduction of baculovirus-BMP-2 and Baculovirus- TGF- β 1 elevated the production of cartilaginous ECM molecules and maintained the chondrocytic phenotype.

Hence, to make use of chondrocytes as therapeutic cells for cartilage regeneration, we should figure out a way to preserve chondrocytic phenotype while inhibiting the formation of fibrocartilage. Our previous study showed that combinational delivery of

TGF- β 3 and shRNA specific for Col I could stimulate the chondrogenesis of SMSC but suppress the expression of Col I, thus bringing synergistic effects on inducing Col I-free chondrogenesis of SMSCs. Upon that, we attempted to make use of the coexpression of TGF- β 3 and anti-Col I shRNA to fulfill the mission. We constructed three kinds of functional recombinant adenovirus: Ad- TGF- β 3, Ad-shRNA and Ad-double, followed by performing experiments to determine their respective optimal MOI. Dedifferentiated chondrocytes were treated with these recombinant adenoviruses alone or in combination followed by encapsulation into alginate hydrogels and cultured in chondrogenic medium for 30 days.

In this study, we found that by using the proposed viral delivery system, TGF- β 3 could be released continuously for at least 30 days, thus providing a sustained and easily accessible supply for cells in proximity. This would also potentially resolve the challenge of feeding growth factors to the engineered implants *in vivo* encountered in future clinical practices. Although there was a declining tendency in the production of TGF- β 3 with time due to episomal characteristics of adenovirus, the qRT-PCR data and the histological staining results indicated that this release profile of TGF- β 3 was effective in helping chondrocytes maintain the chondrogenic phenotype. In Ad-combination group, we used two different titers for Ad- TGF- β 3: MOI 300 and MOI 700 respectively. We observed that this discrepancy only influenced the original concentration of TGF- β 3, where higher MOI resulted in higher initial concentration. With time, the releasing level of TGF- β 3, no matter at MOI 300 or MOI 700, was similar and kept in a stably low level.

From our findings, it is evident that that TGF- β 3 played a vital role in stimulating the expression of cartilage specific markers. Ad-shRNA alone had no effect on promoting the redifferentiation of chondrocytes, the condition of which was comparably similar to

that of P4 cells at transcriptional level. In Ad- TGF β 3, Ad-double and Ad-combination groups, the expression levels of chondrocytic genes, including aggrecan, Col II and COMP were remarkably augmented when compared to Neg or P4 control group and comparable with that in native cartilage. Moreover, the deposition of cartilaginous ECM molecules was enhanced as evidenced by the visible staining for GAGs and Col II, as well as the positive data obtained from biochemical assays. Recombinant TGF- β 3 protein added in a manual manner did improve the expression of aggrecan, Col II and COMP modestly but offered results that were inferior to the genetically modified groups.

Our previous studies demonstrated that sole delivery of transgene TGF- β 3 to cells via adenoviral vectors in their effective period could remarkably increase the expression of Col I [161, 167, 178, 191]. Since the rising amount of Col I expression is one of the signs for dedifferentiation of chondrocytes, it was hypothesized that introducing shRNA specific for Col I would help to attenuate this situation. The results from qRT-PCR showed evidence that Col I-specific shRNA was effective in the inhibition of Col I expression, no matter how shRNA was delivered --- alone (Ad-shRNA, with or without presence of recombinant TGF- β 3 protein), in combination (Ad-combination) or in conjugation (Ad-double). Moreover, the histological staining further proved the silencing effect by shRNA. The results of this study also indicated that long-term 3D cultural conditions alone could also influence Col I expression to a certain degree. Chondrocytes in Neg group (P4 cells encapsulated into alginate hydrogels and cultured for 30 days) expressed less Col I than P4 cells in monolayer culture, suggesting 3D microenvironment helped to retain low Col I expression in the cells. In spite of that, it is preferable to suppress Col I expression at the start of the culture. With RNA interference, the expression of Col I gene could be knocked down specifically, thus the

matrix of the cartilage repair tissue would not be populated with Col I.

In addition, the expression of Col X did not vary significantly amongst all the genetically modified groups. This result showed that the transduction of recombinant adenoviruses did not promote Col X production and chondrocytes were able to sustain their phenotype without entering the endochondral pathway.

6.5 Summary

The idea of transferring bi-genes to fulfill two functions did take synergic effect. Both Ad-combination and Ad-double have capability to make dedifferentiated chondrocytes recover chondrocytic properties as well as suppress Col I expression. For Ad-combination, the extent of redifferentiation was similar no matter whether MOI 700 of Ad-TGF β 3 or MOI 300 was used, which only influenced the original concentration of TGF- β 3. This also substantiated the MOI 300 was optimum MOI for Ad- TGF β 3 in Ad-combination group.

Chapter 7

Conclusions and future work

7.1 Conclusions

In these studies, two approaches for combinational transfer of transgene TGF- β 3 and anti-Col I shRNA via adenoviral vector were proposed and their effects for engineered cartilage chondrogenesis were evaluated. The investigation of sole delivery of transgene TGF- β 3 to SMSCs indicated that Ad-TGF β 3 could significantly promote the expression of Col I in its effective period, which was also found in other studies by our lab [167, 191]. Interestingly, exogenously supplemented TGF- β 3 may lead to various results under different experimental conditions (cell lineage, material, dosage, cell cure or tissue cure and so on). Whatever the experimental conditions are, its effect on the change of Col I expression does not seem as significant as that by Ad- TGF β 3. Also, through the exploration of mechanism, we found that adenoviral vector-mediated TGF- β 3 could incite the Smad signal transduction pathway to increase the Col I gene expression and inhibit MMP-9 synthesis to restrain Col I degradation, thus making synergic effect on up-regulation of Col I expression.

Owing to the declined mechanical property under the presence of Col I, the issue brought about by Ad-TGF β 3 should be addressed to improve the overall quality of the engineered cartilage. To this end, RNAi as an effective tool to silence the undesired gene was introduced, aiming to effectively eliminate the production of Col I. SMSCs were infected with Ad- TGF β 3 and/or Ad-shRNA separately, simultaneously (Ad-combination) or conjugately (Ad-double, mediated by one vector encoding both). After the 30-day culture period in chondrogenic medium, we compared the data from various groups and demonstrated that the application of combinational adenoviral

vector-mediated transgenic TGF- β 3 and shRNA targeting Col I possesses the potential in promoting the chondrogenic differentiation of SMSCs as well as inhibiting the formation of fibrocartilage.

On the other hand, one obstacle that is faced by the use of chondrocytes as cell source for cartilage tissue engineering is that monolayer expansion of chondrocytes causes dedifferentiation. This results in a change of phenotype as well as increased production of Col I, which may decrease the repaired tissue quality of the constructs. Similarly, we employed the dual functional delivery system to passaged chondrocytes. Our findings indicated that genetically modified chondrocytes that could express TGF- β 3 and shRNA were able to express elevated amount of cartilaginous ECM genes as well as effectively silence Col I production.

Although Ad- TGF β 3 caused the up-regulation of Col I which should avoid presenting in cartilage, it was an effective stimulator for both chondrogenesis of SMSCs and maintenance of chondrocytic phenotype. And the introduction of RNAi would become a helpful assistant to eliminate the side effect arose from Ad- TGF β 3. Besides, with the adenoviral vectors administered *ex vivo* to the therapeutic constructs, chances of inducing inflammatory and immune responses by virions will be greatly lowered. Therefore, such a system with dual functions would help produce a safe and efficient cartilage constructs without Col I interference, thus making the repaired cartilage more similar to a native articular counterpart.

7.2 Future works

One of the characteristics of adenovirus is its transient expression of transgene so that there appears a decrease in expression level over time. Although the previous studies demonstrated that the dual delivery system via adenoviruses did have effect on

engineering Col I –free cartilage construct during the 30-day culture period which was still the effective time of adenovirus, it is worthy of investigation whether a more sustained expression/release of shRNA and/or TGF- β 3 would perform better. It is therefore of great significance to study the effect of the sustained release/expression of TGF- β 3 and shRNA mediated by integrative lentiviral vectors.

Once these lentiviruses have been demonstrated to function properly by releasing TGF- β 3 and/or suppressing Col I, we can use them for further trials to test if they can work effectively to promote chondrogenesis of SMSCs and finally produce a well-defined Col I-free cartilage plug for transplantation. However, it is still unknown whether a sustained release/expression of TGF- β 3 or shRNA would better promote Col I-suppressed chondrogenesis. Hereby we can make use of the episomal adenovirus, and apply various combinations of recombinant adenovirus and/or lentivirus to execute the two functions in order to control the release/expression profiles of TGF- β 3 and/or shRNA, in the hope of finding out the optimal protocol that has the best performance in chondrogenesis. The various combinations of either recombinant adenovirus or lentivirus are supposed to implement a controlled release/expression of either TGF- β 3 or anti-Col I shRNA in a transient or permanent manner. These viral vector transducing systems will be applied coupling with a hydrogel scaffold based 3D culture system. By comparison between the various groups in terms of chondrogenic induction and Col I suppression, the optimal scheme will be selected and established as a sound system for the engineering of a successful cartilage construct.

Through the experiments illustrated above, with a comprehensive consideration of all the results, we can select a protocol with the best performance. The criteria in the selection process include the following:

- i) Col I inhibition efficiency should be as high as possible, to leave as little Col I protein as possible remaining in the construct.
- ii) TGF- β 3 expression levels should fall within a reasonable range. Low concentration would render no or little inductive effect, while high expression of TGF- β 3 might do harm to the cells. Since TGF- β 3 concentration of 10ng/mL has been intensively applied for induction of chondrocytic differentiation, we can then optimize our 3-D culture system by adjusting the cell number to be encapsulated in one construct, so that TGF- β 3 concentration will be reasonable for optimal inductive effect.
- iii) The best protocol should result in the best, or at least moderate induction of chondrocytic phenotypes, including the expression of the specific genes at both mRNA and protein levels.

REFERENCES

1. Mankin HJ: **The response of articular cartilage to mechanical injury.** *J Bone Joint Surg Am* 1982, **64**(3):460-466.
2. O'Driscoll SW: **The healing and regeneration of articular cartilage.** *J Bone Joint Surg Am* 1998, **80**(12):1795-1812.
3. Meulenbelt I, Bijkerk C, de Wildt SC, Miedema HS, Valkenburg HA, Breedveld FC, Pols HA, Te Koppele JM, Sloos VF, Hofman A *et al*: **Investigation of the association of the CRTM and CRTL1 genes with radiographically evident osteoarthritis in subjects from the Rotterdam study.** *Arthritis Rheum* 1997, **40**(10):1760-1765.
4. Urban JP, Roberts S: **Degeneration of the intervertebral disc.** *Arthritis Res Ther* 2003, **5**(3):120-130.
5. Henry Gray aHVC: **Articulations.** In: *Anatomy, Descriptive and Surgical.* Blanchard and Lea; 1859: 133-140
6. Buckwalter JA, Mankin HJ: **Articular cartilage: tissue design and chondrocyte-matrix interactions.** *Instr Course Lect* 1998, **47**:477-486.
7. Bhosale AM, Richardson JB: **Articular cartilage: structure, injuries and review of management.** *Br Med Bull* 2008, **87**:77-95.
8. Stockwell RA: **Biology of cartilage cells:** Cambridge University Press; 1979.
9. Temenoff JS, Mikos AG: **Review: tissue engineering for regeneration of articular cartilage.** *Biomaterials* 2000, **21**(5):431-440.
10. Hunziker EB: **Biologic repair of articular cartilage. Defect models in experimental animals and matrix requirements.** *Clin Orthop Relat Res* 1999(367 Suppl):S135-146.
11. Coletti JM, Jr., Akeson WH, Woo SL: **A comparison of the physical behavior**

- of normal articular cartilage and the arthroplasty surface.** *J Bone Joint Surg Am* 1972, **54**(1):147-160.
12. Jackson DW, Lalor PA, Aberman HM, Simon TM: **Spontaneous repair of full-thickness defects of articular cartilage in a goat model. A preliminary study.** *J Bone Joint Surg Am* 2001, **83-A**(1):53-64.
 13. Chung C, Burdick JA: **Engineering cartilage tissue.** *Adv Drug Deliv Rev* 2008, **60**(2):243-262.
 14. Weng HH, Fitzgerald J: **Current issues in joint replacement surgery.** *Curr Opin Rheumatol* 2006, **18**(2):163-169.
 15. Hunziker EB: **Articular cartilage repair: are the intrinsic biological constraints undermining this process insuperable?** *Osteoarthritis Cartilage* 1999, **7**(1):15-28.
 16. Schek RM, Wilke EN, Hollister SJ, Krebsbach PH: **Combined use of designed scaffolds and adenoviral gene therapy for skeletal tissue engineering.** *Biomaterials* 2006, **27**(7):1160-1166.
 17. Shieh SJ, Vacanti JP: **State-of-the-art tissue engineering: from tissue engineering to organ building.** *Surgery* 2005, **137**(1):1-7.
 18. Trent RJ, Alexander IE: **Gene therapy: applications and progress towards the clinic.** *Intern Med J* 2004, **34**(11):621-625.
 19. Gelse K, von der Mark K, Aigner T, Park J, Schneider H: **Articular cartilage repair by gene therapy using growth factor-producing mesenchymal cells.** *Arthritis Rheum* 2003, **48**(2):430-441.
 20. Hao J, Varshney RR, Wang DA: **Engineering osteogenesis and chondrogenesis with gene-enhanced therapeutic cells.** *Curr Opin Mol Ther* 2009, **11**(4):404-410.

21. Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR: **Multilineage potential of adult human mesenchymal stem cells**. *Science* 1999, **284**(5411):143-147.
22. Song L, Baksh D, Tuan RS: **Mesenchymal stem cell-based cartilage tissue engineering: cells, scaffold and biology**. *Cytotherapy* 2004, **6**(6):596-601.
23. Darling EM, Athanasiou KA: **Rapid phenotypic changes in passaged articular chondrocyte subpopulations**. *J Orthop Res* 2005, **23**(2):425-432.
24. Yoo JU, Barthel TS, Nishimura K, Solchaga L, Caplan AI, Goldberg VM, Johnstone B: **The chondrogenic potential of human bone-marrow-derived mesenchymal progenitor cells**. *J Bone Joint Surg Am* 1998, **80**(12):1745-1757.
25. Nishimura K, Solchaga LA, Caplan AI, Yoo JU, Goldberg VM, Johnstone B: **Chondroprogenitor cells of synovial tissue**. *Arthritis Rheum* 1999, **42**(12):2631-2637.
26. Huang JI, Kazmi N, Durbhakula MM, Hering TM, Yoo JU, Johnstone B: **Chondrogenic potential of progenitor cells derived from human bone marrow and adipose tissue: a patient-matched comparison**. *J Orthop Res* 2005, **23**(6):1383-1389.
27. Peng H, Huard J: **Muscle-derived stem cells for musculoskeletal tissue regeneration and repair**. *Transpl Immunol* 2004, **12**(3-4):311-319.
28. Young HE, Steele TA, Bray RA, Hudson J, Floyd JA, Hawkins K, Thomas K, Austin T, Edwards C, Cuzzourt J *et al*: **Human reserve pluripotent mesenchymal stem cells are present in the connective tissues of skeletal muscle and dermis derived from fetal, adult, and geriatric donors**. *Anat Rec* 2001, **264**(1):51-62.

29. Noth U, Osyczka AM, Tuli R, Hickok NJ, Danielson KG, Tuan RS: **Multilineage mesenchymal differentiation potential of human trabecular bone-derived cells.** *J Orthop Res* 2002, **20**(5):1060-1069.
30. Tuli R, Seghatoleslami MR, Tuli S, Wang ML, Hozack WJ, Manner PA, Danielson KG, Tuan RS: **A simple, high-yield method for obtaining multipotential mesenchymal progenitor cells from trabecular bone.** *Mol Biotechnol* 2003, **23**(1):37-49.
31. Adachi N, Sato K, Usas A, Fu FH, Ochi M, Han CW, Niyibizi C, Huard J: **Muscle derived, cell based ex vivo gene therapy for treatment of full thickness articular cartilage defects.** *J Rheumatol* 2002, **29**(9):1920-1930.
32. Johnstone B, Hering TM, Caplan AI, Goldberg VM, Yoo JU: **In vitro chondrogenesis of bone marrow-derived mesenchymal progenitor cells.** *Exp Cell Res* 1998, **238**(1):265-272.
33. Fukumoto T, Sperling JW, Sanyal A, Fitzsimmons JS, Reinholz GG, Conover CA, O'Driscoll SW: **Combined effects of insulin-like growth factor-1 and transforming growth factor-beta1 on periosteal mesenchymal cells during chondrogenesis in vitro.** *Osteoarthritis Cartilage* 2003, **11**(1):55-64.
34. Park Y, Sugimoto M, Watrin A, Chiquet M, Hunziker EB: **BMP-2 induces the expression of chondrocyte-specific genes in bovine synovium-derived progenitor cells cultured in three-dimensional alginate hydrogel.** *Osteoarthritis Cartilage* 2005, **13**(6):527-536.
35. Huang CY, Reuben PM, D'Ippolito G, Schiller PC, Cheung HS: **Chondrogenesis of human bone marrow-derived mesenchymal stem cells in agarose culture.** *Anat Rec A Discov Mol Cell Evol Biol* 2004, **278**(1):428-436.

36. Coleman RM, Case ND, Guldberg RE: **Hydrogel effects on bone marrow stromal cell response to chondrogenic growth factors.** *Biomaterials* 2007, **28**(12):2077-2086.
37. Wang Y, Kim UJ, Blasioli DJ, Kim HJ, Kaplan DL: **In vitro cartilage tissue engineering with 3D porous aqueous-derived silk scaffolds and mesenchymal stem cells.** *Biomaterials* 2005, **26**(34):7082-7094.
38. Williams CG, Kim TK, Taboas A, Malik A, Manson P, Elisseeff J: **In vitro chondrogenesis of bone marrow-derived mesenchymal stem cells in a photopolymerizing hydrogel.** *Tissue Eng* 2003, **9**(4):679-688.
39. Chen G, Liu D, Tadokoro M, Hirochika R, Ohgushi H, Tanaka J, Tateishi T: **Chondrogenic differentiation of human mesenchymal stem cells cultured in a cobweb-like biodegradable scaffold.** *Biochem Biophys Res Commun* 2004, **322**(1):50-55.
40. Li WJ, Tuli R, Okafor C, Derfoul A, Danielson KG, Hall DJ, Tuan RS: **A three-dimensional nanofibrous scaffold for cartilage tissue engineering using human mesenchymal stem cells.** *Biomaterials* 2005, **26**(6):599-609.
41. Pang Y, Cui P, Chen W, Gao P, Zhang H: **Quantitative study of tissue-engineered cartilage with human bone marrow mesenchymal stem cells.** *Arch Facial Plast Surg* 2005, **7**(1):7-11.
42. Fan H, Hu Y, Zhang C, Li X, Lv R, Qin L, Zhu R: **Cartilage regeneration using mesenchymal stem cells and a PLGA-gelatin/chondroitin/hyaluronate hybrid scaffold.** *Biomaterials* 2006, **27**(26):4573-4580.
43. Tuli R, Tuli S, Nandi S, Huang X, Manner PA, Hozack WJ, Danielson KG, Hall DJ, Tuan RS: **Transforming growth factor-beta-mediated**

- chondrogenesis of human mesenchymal progenitor cells involves N-cadherin and mitogen-activated protein kinase and Wnt signaling cross-talk.** *J Biol Chem* 2003, **278**(42):41227-41236.
44. Lisignoli G, Cristino S, Piacentini A, Toneguzzi S, Grassi F, Cavallo C, Zini N, Solimando L, Mario Maraldi N, Facchini A: **Cellular and molecular events during chondrogenesis of human mesenchymal stromal cells grown in a three-dimensional hyaluronan based scaffold.** *Biomaterials* 2005, **26**(28):5677-5686.
 45. Huang AH, Yeger-McKeever M, Stein A, Mauck RL: **Tensile properties of engineered cartilage formed from chondrocyte- and MSC-laden hydrogels.** *Osteoarthritis Cartilage* 2008, **16**(9):1074-1082.
 46. Grande DA, Mason J, Light E, Dines D: **Stem cells as platforms for delivery of genes to enhance cartilage repair.** *J Bone Joint Surg Am* 2003, **85-A Suppl 2**:111-116.
 47. Indrawattana N, Chen G, Tadokoro M, Shann LH, Ohgushi H, Tateishi T, Tanaka J, Bunyaratvej A: **Growth factor combination for chondrogenic induction from human mesenchymal stem cell.** *Biochem Biophys Res Commun* 2004, **320**(3):914-919.
 48. Akaogi H, Akimoto T, Miyaki S, Ushida T, Ochiai N, Tateishi T, Tanaka J: **Basic fibroblast growth factor supports in vitro chondrogenesis of bone marrow-derived mesenchymal stem cells from patients with osteoarthritis.** *Mater Sci Eng C-Biomimetic Supramol Syst* 2004, **24**(3):403-406.
 49. Mauck RL, Yuan X, Tuan RS: **Chondrogenic differentiation and functional maturation of bovine mesenchymal stem cells in long-term agarose culture.** *Osteoarthritis Cartilage* 2006, **14**(2):179-189.

50. Winter A, Breit S, Parsch D, Benz K, Steck E, Hauner H, Weber RM, Ewerbeck V, Richter W: **Cartilage-like gene expression in differentiated human stem cell spheroids: a comparison of bone marrow-derived and adipose tissue-derived stromal cells.** *Arthritis Rheum* 2003, **48**(2):418-429.
51. Im GI, Shin YW, Lee KB: **Do adipose tissue-derived mesenchymal stem cells have the same osteogenic and chondrogenic potential as bone marrow-derived cells?** *Osteoarthritis Cartilage* 2005, **13**(10):845-853.
52. Sakaguchi Y, Sekiya I, Yagishita K, Muneta T: **Comparison of human stem cells derived from various mesenchymal tissues: superiority of synovium as a cell source.** *Arthritis Rheum* 2005, **52**(8):2521-2529.
53. Brittberg M, Lindahl A, Nilsson A, Ohlsson C, Isaksson O, Peterson L: **Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation.** *N Engl J Med* 1994, **331**(14):889-895.
54. Klein TJ, Schumacher BL, Schmidt TA, Li KW, Voegtline MS, Masuda K, Thonar EJ, Sah RL: **Tissue engineering of stratified articular cartilage from chondrocyte subpopulations.** *Osteoarthritis Cartilage* 2003, **11**(8):595-602.
55. Chung C, Mesa J, Miller GJ, Randolph MA, Gill TJ, Burdick JA: **Effects of auricular chondrocyte expansion on neocartilage formation in photocrosslinked hyaluronic acid networks.** *Tissue Eng* 2006, **12**(9):2665-2673.
56. Van Osch GJ, Mandl EW, Jahr H, Koevoet W, Nolst-Trenite G, Verhaar JA: **Considerations on the use of ear chondrocytes as donor chondrocytes for cartilage tissue engineering.** *Biorheology* 2004, **41**(3-4):411-421.
57. Miot S, Woodfield T, Daniels AU, Suetterlin R, Peterschmitt I, Heberer M, van Blitterswijk CA, Riesle J, Martin I: **Effects of scaffold composition and**

- architecture on human nasal chondrocyte redifferentiation and cartilaginous matrix deposition.** *Biomaterials* 2005, **26**(15):2479-2489.
58. Vinatier C, Magne D, Moreau A, Gauthier O, Malard O, Vignes-Colombeix C, Daculsi G, Weiss P, Guicheux J: **Engineering cartilage with human nasal chondrocytes and a silanized hydroxypropyl methylcellulose hydrogel.** *J Biomed Mater Res A* 2007, **80**(1):66-74.
 59. Kato Y, Gospodarowicz D: **Sulfated proteoglycan synthesis by confluent cultures of rabbit costal chondrocytes grown in the presence of fibroblast growth factor.** *J Cell Biol* 1985, **100**(2):477-485.
 60. Tay AG, Farhadi J, Suetterlin R, Pierer G, Heberer M, Martin I: **Cell yield, proliferation, and postexpansion differentiation capacity of human ear, nasal, and rib chondrocytes.** *Tissue Eng* 2004, **10**(5-6):762-770.
 61. Li Y, Tew SR, Russell AM, Gonzalez KR, Hardingham TE, Hawkins RE: **Transduction of passaged human articular chondrocytes with adenoviral, retroviral, and lentiviral vectors and the effects of enhanced expression of SOX9.** *Tissue Eng* 2004, **10**(3-4):575-584.
 62. Mesa JM, Zaporozhan V, Weinand C, Johnson TS, Bonassar L, Randolph MA, Yaremchuk MJ, Butler PE: **Tissue engineering cartilage with aged articular chondrocytes in vivo.** *Plast Reconstr Surg* 2006, **118**(1):41-49; discussion 50-43.
 63. Marlovits S, Zeller P, Singer P, Resinger C, Vecsei V: **Cartilage repair: generations of autologous chondrocyte transplantation.** *Eur J Radiol* 2006, **57**(1):24-31.
 64. von der Mark K, Gauss V, von der Mark H, Muller P: **Relationship between cell shape and type of collagen synthesised as chondrocytes lose their**

- cartilage phenotype in culture.** *Nature* 1977, **267**(5611):531-532.
65. Benya PD, Padilla SR, Nimni ME: **Independent regulation of collagen types by chondrocytes during the loss of differentiated function in culture.** *Cell* 1978, **15**(4):1313-1321.
 66. Huch K, Stove J, Puhl W, Gunther KP: **[Review and comparison of culture-techniques for articular chondrocytes].** *Z Orthop Ihre Grenzgeb* 2002, **140**(2):145-152.
 67. Benya PD, Shaffer JD: **Dedifferentiated chondrocytes reexpress the differentiated collagen phenotype when cultured in agarose gels.** *Cell* 1982, **30**(1):215-224.
 68. Bonaventure J, Kadhon N, Cohen-Solal L, Ng KH, Bourguignon J, Lasselin C, Freisinger P: **Reexpression of cartilage-specific genes by dedifferentiated human articular chondrocytes cultured in alginate beads.** *Exp Cell Res* 1994, **212**(1):97-104.
 69. Stevens MM, Qanadilo HF, Langer R, Prasad Shastri V: **A rapid-curing alginate gel system: utility in periosteum-derived cartilage tissue engineering.** *Biomaterials* 2004, **25**(5):887-894.
 70. Chaipinyo K, Oakes BW, Van Damme MP: **The use of debrided human articular cartilage for autologous chondrocyte implantation: maintenance of chondrocyte differentiation and proliferation in type I collagen gels.** *J Orthop Res* 2004, **22**(2):446-455.
 71. Takahashi T, Ogasawara T, Asawa Y, Mori Y, Uchinuma E, Takato T, Hoshi K: **Three-dimensional microenvironments retain chondrocyte phenotypes during proliferation culture.** *Tissue Eng* 2007, **13**(7):1583-1592.
 72. Malesmud CJ, Stevenson S, Mehraban F, Papay RS, Purchio AF, Goldberg VM:

- The proteoglycan synthesis repertoire of rabbit chondrocytes maintained in type II collagen gels.** *Osteoarthritis Cartilage* 1994, **2**(1):29-41.
73. Ting V, Sims CD, Brecht LE, McCarthy JG, Kasabian AK, Connelly PR, Elisseeff J, Gittes GK, Longaker MT: **In vitro prefabrication of human cartilage shapes using fibrin glue and human chondrocytes.** *Ann Plast Surg* 1998, **40**(4):413-420; discussion 420-411.
 74. Reginato AM, Iozzo RV, Jimenez SA: **Formation of nodular structures resembling mature articular cartilage in long-term primary cultures of human fetal epiphyseal chondrocytes on a hydrogel substrate.** *Arthritis Rheum* 1994, **37**(9):1338-1349.
 75. Yamaoka H, Asato H, Ogasawara T, Nishizawa S, Takahashi T, Nakatsuka T, Koshima I, Nakamura K, Kawaguchi H, Chung UI *et al*: **Cartilage tissue engineering using human auricular chondrocytes embedded in different hydrogel materials.** *J Biomed Mater Res A* 2006, **78**(1):1-11.
 76. Lin L, Zhou C, Wei X, Hou Y, Zhao L, Fu X, Zhang J, Yu C: **Articular cartilage repair using dedifferentiated articular chondrocytes and bone morphogenetic protein 4 in a rabbit model of articular cartilage defects.** *Arthritis Rheum* 2008, **58**(4):1067-1075.
 77. Sung LY, Lo WH, Chiu HY, Chen HC, Chung CK, Lee HP, Hu YC: **Modulation of chondrocyte phenotype via baculovirus-mediated growth factor expression.** *Biomaterials* 2007, **28**(23):3437-3447.
 78. Yan H, Yu C: **Repair of full-thickness cartilage defects with cells of different origin in a rabbit model.** *Arthroscopy* 2007, **23**(2):178-187.
 79. Nicoll SB, Wedrychowska A, Smith NR, Bhatnagar RS: **Modulation of proteoglycan and collagen profiles in human dermal fibroblasts by high**

- density micromass culture and treatment with lactic acid suggests change to a chondrogenic phenotype.** *Connect Tissue Res* 2001, **42**(1):59-69.
80. French MM, Rose S, Canseco J, Athanasiou KA: **Chondrogenic differentiation of adult dermal fibroblasts.** *Ann Biomed Eng* 2004, **32**(1):50-56.
 81. Yates KE, Forbes RL, Glowacki J: **New chondrocyte genes discovered by representational difference analysis of chondroinduced human fibroblasts.** *Cells Tissues Organs* 2004, **176**(1-3):41-53.
 82. Lee KH, Song SU, Hwang TS, Yi Y, Oh IS, Lee JY, Choi KB, Choi MS, Kim SJ: **Regeneration of hyaline cartilage by cell-mediated gene therapy using transforming growth factor beta 1-producing fibroblasts.** *Hum Gene Ther* 2001, **12**(14):1805-1813.
 83. Deng Y, Hu JC, Athanasiou KA: **Isolation and chondroinduction of a dermis-isolated, aggrecan-sensitive subpopulation with high chondrogenic potential.** *Arthritis Rheum* 2007, **56**(1):168-176.
 84. Iwasaki M, Nakata K, Nakahara H, Nakase T, Kimura T, Kimata K, Caplan AI, Ono K: **Transforming growth factor-beta 1 stimulates chondrogenesis and inhibits osteogenesis in high density culture of periosteum-derived cells.** *Endocrinology* 1993, **132**(4):1603-1608.
 85. Rosier RN, O'Keefe RJ, Crabb ID, Puzas JE: **Transforming growth factor beta: an autocrine regulator of chondrocytes.** *Connect Tissue Res* 1989, **20**(1-4):295-301.
 86. Vivien D, Galera P, Lebrun E, Loyau G, Pujol JP: **Differential effects of transforming growth factor-beta and epidermal growth factor on the cell cycle of cultured rabbit articular chondrocytes.** *J Cell Physiol* 1990,

- 143(3):534-545.
87. Mankin HJ, Jennings LC, Treadwell BV, Trippel SB: **Growth factors and articular cartilage.** *J Rheumatol Suppl* 1991, **27**:66-67.
 88. Shuler FD, Georgescu HI, Niyibizi C, Studer RK, Mi Z, Johnstone B, Robbins RD, Evans CH: **Increased matrix synthesis following adenoviral transfer of a transforming growth factor beta1 gene into articular chondrocytes.** *J Orthop Res* 2000, **18**(4):585-592.
 89. Alvarez J, Sohn P, Zeng X, Doetschman T, Robbins DJ, Serra R: **TGFbeta2 mediates the effects of hedgehog on hypertrophic differentiation and PTHrP expression.** *Development* 2002, **129**(8):1913-1924.
 90. Hao J, Varshney RR, Wang DA: **TGF-beta3: A promising growth factor in engineered organogenesis.** *Expert Opin Biol Ther* 2008, **8**(10):1485-1493.
 91. Joyce ME, Roberts AB, Sporn MB, Bolander ME: **Transforming growth factor-beta and the initiation of chondrogenesis and osteogenesis in the rat femur.** *J Cell Biol* 1990, **110**(6):2195-2207.
 92. Palmer GD, Steinert A, Pascher A, Gouze E, Gouze JN, Betz O, Johnstone B, Evans CH, Ghivizzani SC: **Gene-induced chondrogenesis of primary mesenchymal stem cells in vitro.** *Mol Ther* 2005, **12**(2):219-228.
 93. Hao J, Yao Y, Varshney RR, Wang L, Prakash C, Li H, Wang DA: **Gene transfer and living release of transforming growth factor-beta3 for cartilage tissue engineering applications.** *Tissue Eng Part C Methods* 2008, **14**(4):273-280.
 94. Sato K, Urist MR: **Bone morphogenetic protein-induced cartilage development in tissue culture.** *Clin Orthop Relat Res* 1984(183):180-187.
 95. Sekiya I, Colter DC, Prockop DJ: **BMP-6 enhances chondrogenesis in a**

- subpopulation of human marrow stromal cells.** *Biochem Biophys Res Commun* 2001, **284**(2):411-418.
96. Nochi H, Sung JH, Lou J, Adkisson HD, Maloney WJ, Hruska KA: **Adenovirus mediated BMP-13 gene transfer induces chondrogenic differentiation of murine mesenchymal progenitor cells.** *J Bone Miner Res* 2004, **19**(1):111-122.
 97. Sung LY, Chiu HY, Chen HC, Chen YL, Chuang CK, Hu YC: **Baculovirus-mediated growth factor expression in dedifferentiated chondrocytes accelerates redifferentiation: effects of combinational transduction.** *Tissue Eng Part A* 2009, **15**(6):1353-1362.
 98. Osborn KD, Trippel SB, Mankin HJ: **Growth factor stimulation of adult articular cartilage.** *J Orthop Res* 1989, **7**(1):35-42.
 99. Hoffmann A, Czichos S, Kaps C, Bachner D, Mayer H, Kurkalli BG, Zilberman Y, Turgeman G, Pelled G, Gross G *et al*: **The T-box transcription factor Brachyury mediates cartilage development in mesenchymal stem cell line C3H10T1/2.** *J Cell Sci* 2002, **115**(Pt 4):769-781.
 100. Ellsworth JL, Berry J, Bukowski T, Claus J, Feldhaus A, Holderman S, Holdren MS, Lum KD, Moore EE, Raymond F *et al*: **Fibroblast growth factor-18 is a trophic factor for mature chondrocytes and their progenitors.** *Osteoarthritis Cartilage* 2002, **10**(4):308-320.
 101. Bi W, Deng JM, Zhang Z, Behringer RR, de Crombrughe B: **Sox9 is required for cartilage formation.** *Nat Genet* 1999, **22**(1):85-89.
 102. Magne D, Vinatier C, Julien M, Weiss P, Guicheux J: **Mesenchymal stem cell therapy to rebuild cartilage.** *Trends Mol Med* 2005, **11**(11):519-526.
 103. Tsuchiya H, Kitoh H, Sugiura F, Ishiguro N: **Chondrogenesis enhanced by**

- overexpression of sox9 gene in mouse bone marrow-derived mesenchymal stem cells.** *Biochem Biophys Res Commun* 2003, **301**(2):338-343.
104. Trippel SB, Ghivizzani SC, Nixon AJ: **Gene-based approaches for the repair of articular cartilage.** *Gene Ther* 2004, **11**(4):351-359.
 105. Aigner T, Stove J: **Collagens--major component of the physiological cartilage matrix, major target of cartilage degeneration, major tool in cartilage repair.** *Adv Drug Deliv Rev* 2003, **55**(12):1569-1593.
 106. Stove J, Fiedler J, Huch K, Gunther KP, Puhl W, Brenner R: **Lipofection of rabbit chondrocytes and long lasting expression of a lacZ reporter system in alginate beads.** *Osteoarthritis Cartilage* 2002, **10**(3):212-217.
 107. Madry H, Cucchiaroni M, Stein U, Remberger K, Menger MD, Kohn D, Trippel SB: **Sustained transgene expression in cartilage defects in vivo after transplantation of articular chondrocytes modified by lipid-mediated gene transfer in a gel suspension delivery system.** *J Gene Med* 2003, **5**(6):502-509.
 108. Partridge KA, Oreffo RO: **Gene delivery in bone tissue engineering: progress and prospects using viral and nonviral strategies.** *Tissue Eng* 2004, **10**(1-2):295-307.
 109. Kurian KM, Watson CJ, Wyllie AH: **Retroviral vectors.** *Mol Pathol* 2000, **53**(4):173-176.
 110. Sazani P, Vacek MM, Kole R: **Short-term and long-term modulation of gene expression by antisense therapeutics.** *Curr Opin Biotechnol* 2002, **13**(5):468-472.
 111. Khoury M, Jorgensen C, Apparailly F: **RNAi in arthritis: prospects of a future antisense therapy in inflammation.** *Curr Opin Mol Ther* 2007,

- 9(5):483-489.
112. Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC: **Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans***. *Nature* 1998, **391**(6669):806-811.
 113. Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T: **Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells**. *Nature* 2001, **411**(6836):494-498.
 114. Tuschl T, Zamore PD, Lehmann R, Bartel DP, Sharp PA: **Targeted mRNA degradation by double-stranded RNA in vitro**. *Genes Dev* 1999, **13**(24):3191-3197.
 115. Zamore PD, Tuschl T, Sharp PA, Bartel DP: **RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals**. *Cell* 2000, **101**(1):25-33.
 116. Hammond SM, Bernstein E, Beach D, Hannon GJ: **An RNA-directed nuclease mediates post-transcriptional gene silencing in *Drosophila* cells**. *Nature* 2000, **404**(6775):293-296.
 117. Brummelkamp TR, Bernards R, Agami R: **A system for stable expression of short interfering RNAs in mammalian cells**. *Science* 2002, **296**(5567):550-553.
 118. Sui G, Soohoo C, Affar el B, Gay F, Shi Y, Forrester WC: **A DNA vector-based RNAi technology to suppress gene expression in mammalian cells**. *Proc Natl Acad Sci U S A* 2002, **99**(8):5515-5520.
 119. Paddison PJ, Caudy AA, Bernstein E, Hannon GJ, Conklin DS: **Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells**. *Genes Dev* 2002, **16**(8):948-958.

120. Zamore PD, Aronin N: **siRNAs knock down hepatitis**. *Nat Med* 2003, **9**(3):266-267.
121. Scacheri PC, Rozenblatt-Rosen O, Caplen NJ, Wolfsberg TG, Umayam L, Lee JC, Hughes CM, Shanmugam KS, Bhattacharjee A, Meyerson M *et al*: **Short interfering RNAs can induce unexpected and divergent changes in the levels of untargeted proteins in mammalian cells**. *Proc Natl Acad Sci U S A* 2004, **101**(7):1892-1897.
122. Jackson AL, Burchard J, Leake D, Reynolds A, Schelter J, Guo J, Johnson JM, Lim L, Karpilow J, Nichols K *et al*: **Position-specific chemical modification of siRNAs reduces "off-target" transcript silencing**. *RNA* 2006, **12**(7):1197-1205.
123. Yao Y, Wang C, Varshney RR, Wang DA: **Antisense makes sense in engineered regenerative medicine**. *Pharm Res* 2009, **26**(2):263-275.
124. Feldmann M, Brennan FM, Maini RN: **Role of cytokines in rheumatoid arthritis**. *Annu Rev Immunol* 1996, **14**:397-440.
125. Karin M, Delhase M: **The I kappa B kinase (IKK) and NF-kappa B: key elements of proinflammatory signalling**. *Semin Immunol* 2000, **12**(1):85-98.
126. Collart MA, Baeuerle P, Vassalli P: **Regulation of tumor necrosis factor alpha transcription in macrophages: involvement of four kappa B-like motifs and of constitutive and inducible forms of NF-kappa B**. *Mol Cell Biol* 1990, **10**(4):1498-1506.
127. Libermann TA, Baltimore D: **Activation of interleukin-6 gene expression through the NF-kappa B transcription factor**. *Mol Cell Biol* 1990, **10**(5):2327-2334.
128. Marok R, Winyard PG, Coumbe A, Kus ML, Gaffney K, Blades S, Mapp PI,

- Morris CJ, Blake DR, Kaltschmidt C *et al*: **Activation of the transcription factor nuclear factor-kappaB in human inflamed synovial tissue.** *Arthritis Rheum* 1996, **39**(4):583-591.
129. Chen LX, Lin L, Wang HJ, Wei XL, Fu X, Zhang JY, Yu CL: **Suppression of early experimental osteoarthritis by in vivo delivery of the adenoviral vector-mediated NF-kappaBp65-specific siRNA.** *Osteoarthritis Cartilage* 2008, **16**(2):174-184.
 130. Zwicky R, Muntener K, Goldring MB, Baici A: **Cathepsin B expression and down-regulation by gene silencing and antisense DNA in human chondrocytes.** *Biochem J* 2002, **367**(Pt 1):209-217.
 131. Gaggero E, Smerdel-Ramoya A, Zanotti S, Stadmeier L, Durant D, Economides AN, Canalis E: **Conditional deletion of gremlin causes a transient increase in bone formation and bone mass.** *J Biol Chem* 2007, **282**(43):31549-31557.
 132. Morosetti R, Mirabella M, Gliubizzi C, Broccolini A, De Angelis L, Tagliafico E, Sampaolesi M, Gidaro T, Papacci M, Roncaglia E *et al*: **MyoD expression restores defective myogenic differentiation of human mesoangioblasts from inclusion-body myositis muscle.** *Proc Natl Acad Sci U S A* 2006, **103**(45):16995-17000.
 133. Watanabe A, Arai M, Yamazaki M, Koitabashi N, Wuytack F, Kurabayashi M: **Phospholamban ablation by RNA interference increases Ca²⁺ uptake into rat cardiac myocyte sarcoplasmic reticulum.** *J Mol Cell Cardiol* 2004, **37**(3):691-698.
 134. Natarajan R, Salloum FN, Fisher BJ, Kukreja RC, Fowler AA, 3rd: **Hypoxia inducible factor-1 activation by prolyl 4-hydroxylase-2 gene silencing**

- attenuates myocardial ischemia reperfusion injury.** *Circ Res* 2006, **98**(1):133-140.
135. Fechner H, Suckau L, Kurreck J, Sipo I, Wang X, Pinkert S, Loschen S, Rekittke J, Weger S, Dekkers D *et al*: **Highly efficient and specific modulation of cardiac calcium homeostasis by adenovector-derived short hairpin RNA targeting phospholamban.** *Gene Ther* 2007, **14**(3):211-218.
 136. Ahmed Z, Dent RG, Suggate EL, Barrett LB, Seabright RJ, Berry M, Logan A: **Disinhibition of neurotrophin-induced dorsal root ganglion cell neurite outgrowth on CNS myelin by siRNA-mediated knockdown of NgR, p75NTR and Rho-A.** *Mol Cell Neurosci* 2005, **28**(3):509-523.
 137. Pfeifer A, Eigenbrod S, Al-Khadra S, Hofmann A, Mitteregger G, Moser M, Bertsch U, Kretzschmar H: **Lentivector-mediated RNAi efficiently suppresses prion protein and prolongs survival of scrapie-infected mice.** *J Clin Invest* 2006, **116**(12):3204-3210.
 138. Gao Z, Wang Z, Shi Y, Lin Z, Jiang H, Hou T, Wang Q, Yuan X, Zhao Y, Wu H *et al*: **Modulation of collagen synthesis in keloid fibroblasts by silencing Smad2 with siRNA.** *Plast Reconstr Surg* 2006, **118**(6):1328-1337.
 139. Wang Z, Gao Z, Shi Y, Sun Y, Lin Z, Jiang H, Hou T, Wang Q, Yuan X, Zhu X *et al*: **Inhibition of Smad3 expression decreases collagen synthesis in keloid disease fibroblasts.** *J Plast Reconstr Aesthet Surg* 2007, **60**(11):1193-1199.
 140. Wegmuller D, Raineri I, Gross B, Oakeley EJ, Moroni C: **A cassette system to study embryonic stem cell differentiation by inducible RNA interference.** *Stem Cells* 2007, **25**(5):1178-1185.
 141. Xu Y, Mirmalek-Sani SH, Yang X, Zhang J, Oreffo RO: **The use of small interfering RNAs to inhibit adipocyte differentiation in human**

- preadipocytes and fetal-femur-derived mesenchymal cells.** *Exp Cell Res* 2006, **312**(10):1856-1864.
142. Zhang P, Pazin MJ, Schwartz CM, Becker KG, Wersto RP, Dilley CM, Mattson MP: **Nontelomeric TRF2-REST interaction modulates neuronal gene silencing and fate of tumor and stem cells.** *Curr Biol* 2008, **18**(19):1489-1494.
 143. Green DW, Leveque I, Walsh D, Howard D, Yang XB, Partridge K, Mann S, Oreffo ROC: **Biomaterialized polysaccharide capsules for encapsulation, organization, and delivery of human cell types and growth factors.** *Adv Funct Mater* 2005, **15**(6):917-923.
 144. Griffith LG, Naughton G: **Tissue engineering--current challenges and expanding opportunities.** *Science* 2002, **295**(5557):1009-1014.
 145. Fan J, Varshney RR, Ren L, Cai D, Wang DA: **Synovium-derived mesenchymal stem cells: a new cell source for musculoskeletal regeneration.** *Tissue Eng Part B Rev* 2009, **15**(1):75-86.
 146. Nagase T, Muneta T, Ju YJ, Hara K, Morito T, Koga H, Nimura A, Mochizuki T, Sekiya I: **Analysis of the chondrogenic potential of human synovial stem cells according to harvest site and culture parameters in knees with medial compartment osteoarthritis.** *Arthritis Rheum* 2008, **58**(5):1389-1398.
 147. Zimmermann T, Kunisch E, Pfeiffer R, Hirth A, Stahl HD, Sack U, Laube A, Liesaus E, Roth A, Palombo-Kinne E *et al*: **Isolation and characterization of rheumatoid arthritis synovial fibroblasts from primary culture--primary culture cells markedly differ from fourth-passage cells.** *Arthritis Res* 2001, **3**(1):72-76.
 148. Barry F, Boynton RE, Liu B, Murphy JM: **Chondrogenic differentiation of**

- mesenchymal stem cells from bone marrow: differentiation-dependent gene expression of matrix components.** *Exp Cell Res* 2001, **268**(2):189-200.
149. Mehlhorn AT, Schmal H, Kaiser S, Lepski G, Finkenzeller G, Stark GB, Sudkamp NP: **Mesenchymal stem cells maintain TGF-beta-mediated chondrogenic phenotype in alginate bead culture.** *Tissue Eng* 2006, **12**(6):1393-1403.
 150. Drury JL, Mooney DJ: **Hydrogels for tissue engineering: scaffold design variables and applications.** *Biomaterials* 2003, **24**(24):4337-4351.
 151. Diduch DR, Jordan LC, Mierisch CM, Balian G: **Marrow stromal cells embedded in alginate for repair of osteochondral defects.** *Arthroscopy* 2000, **16**(6):571-577.
 152. Ma HL, Hung SC, Lin SY, Chen YL, Lo WH: **Chondrogenesis of human mesenchymal stem cells encapsulated in alginate beads.** *J Biomed Mater Res A* 2003, **64**(2):273-281.
 153. De Bari C, Dell'Accio F, Tylzanowski P, Luyten FP: **Multipotent mesenchymal stem cells from adult human synovial membrane.** *Arthritis Rheum* 2001, **44**(8):1928-1942.
 154. O'Kane S, Ferguson MW: **Transforming growth factor beta s and wound healing.** *Int J Biochem Cell Biol* 1997, **29**(1):63-78.
 155. Roberts AB, Sporn MB: **Differential expression of the TGF-beta isoforms in embryogenesis suggests specific roles in developing and adult tissues.** *Mol Reprod Dev* 1992, **32**(2):91-98.
 156. Letterio JJ, Roberts AB: **Regulation of immune responses by TGF-beta.** *Annu Rev Immunol* 1998, **16**:137-161.
 157. Hosokawa R, Nonaka K, Morifuji M, Shum L, Ohishi M: **TGF-beta 3**

- decreases type I collagen and scarring after labioplasty. *J Dent Res* 2003, **82**(7):558-564.
158. Shah M, Foreman DM, Ferguson MW: **Neutralisation of TGF-beta 1 and TGF-beta 2 or exogenous addition of TGF-beta 3 to cutaneous rat wounds reduces scarring.** *J Cell Sci* 1995, **108** (Pt 3):985-1002.
 159. Li WY, Huang EY, Dudas M, Kaartinen V, Warburton D, Tuan TL: **Transforming growth factor-beta3 affects plasminogen activator inhibitor-1 expression in fetal mice and modulates fibroblast-mediated collagen gel contraction.** *Wound Repair Regen* 2006, **14**(5):516-525.
 160. Wang C, Hao J, Zhang F, Su K, Wang DA: **RNA extraction from polysaccharide-based cell-laden hydrogel scaffolds.** *Anal Biochem* 2008, **380**(2):333-334.
 161. Yao YC, Zhang, F., Zhou, R. J., Li, M., Wang, D. A.: **Continuous supply of TGF- β 3 via adenoviral vector promotes type I collagen and viability of fibroblasts in alginate hydrogel.** *Journal of Tissue Engineering and Regenerative Medicine* 2010(DOI: 10.1002/term.263).
 162. Ghosh AK, Yuan W, Mori Y, Varga J: **Smad-dependent stimulation of type I collagen gene expression in human skin fibroblasts by TGF-beta involves functional cooperation with p300/CBP transcriptional coactivators.** *Oncogene* 2000, **19**(31):3546-3555.
 163. Mauch C, Krieg T, Bauer EA: **Role of the extracellular matrix in the degradation of connective tissue.** *Arch Dermatol Res* 1994, **287**(1):107-114.
 164. Okada Y, Naka K, Kawamura K, Matsumoto T, Nakanishi I, Fujimoto N, Sato H, Seiki M: **Localization of matrix metalloproteinase 9 (92-kilodalton gelatinase/type IV collagenase = gelatinase B) in osteoclasts: implications**

- for bone resorption.** *Lab Invest* 1995, **72**(3):311-322.
165. Heldin CH, Miyazono K, ten Dijke P: **TGF-beta signalling from cell membrane to nucleus through SMAD proteins.** *Nature* 1997, **390**(6659):465-471.
 166. Cutroneo KR: **TGF-beta-induced fibrosis and SMAD signaling: oligo decoys as natural therapeutics for inhibition of tissue fibrosis and scarring.** *Wound Repair Regen* 2007, **15** Suppl 1:S54-60.
 167. Zhang F, Yao Y, Hao J, Zhou R, Liu C, Gong Y, Wang DA: **A dual-functioning adenoviral vector encoding both transforming growth factor-beta3 and shRNA silencing type I collagen: construction and controlled release for chondrogenesis.** *J Control Release*, **142**(1):70-77.
 168. Pouponnot C, Jayaraman L, Massague J: **Physical and functional interaction of SMADs and p300/CBP.** *J Biol Chem* 1998, **273**(36):22865-22868.
 169. Topper JN, DiChiara MR, Brown JD, Williams AJ, Falb D, Collins T, Gimbrone MA, Jr.: **CREB binding protein is a required coactivator for Smad-dependent, transforming growth factor beta transcriptional responses in endothelial cells.** *Proc Natl Acad Sci U S A* 1998, **95**(16):9506-9511.
 170. Neely AN, Clendening CE, Gardner J, Greenhalgh DG, Warden GD: **Gelatinase activity in keloids and hypertrophic scars.** *Wound Repair Regen* 1999, **7**(3):166-171.
 171. Tanriverdi-Akhisaroglu S, Menderes A, Oktay G: **Matrix metalloproteinase-2 and -9 activities in human keloids, hypertrophic and atrophic scars: a pilot study.** *Cell Biochem Funct* 2009, **27**(2):81-87.
 172. Fluck J, Querfeld C, Cremer A, Niland S, Krieg T, Sollberg S: **Normal human**

- primary fibroblasts undergo apoptosis in three-dimensional contractile collagen gels.** *J Invest Dermatol* 1998, **110**(2):153-157.
173. Grinnell F, Zhu M, Carlson MA, Abrams JM: **Release of mechanical tension triggers apoptosis of human fibroblasts in a model of regressing granulation tissue.** *Exp Cell Res* 1999, **248**(2):608-619.
 174. Tian B, Lessan K, Kahm J, Kleidon J, Henke C: **beta 1 integrin regulates fibroblast viability during collagen matrix contraction through a phosphatidylinositol 3-kinase/Akt/protein kinase B signaling pathway.** *J Biol Chem* 2002, **277**(27):24667-24675.
 175. Sebra RP, Reddy SK, Masters KS, Bowman CN, Anseth KS: **Controlled polymerization chemistry to graft architectures that influence cell-material interactions.** *Acta Biomater* 2007, **3**(2):151-161.
 176. Kurth T, Hedbom E, Shintani N, Sugimoto M, Chen FH, Haspl M, Martinovic S, Hunziker EB: **Chondrogenic potential of human synovial mesenchymal stem cells in alginate.** *Osteoarthritis Cartilage* 2007, **15**(10):1178-1189.
 177. Yokoyama A, Sekiya I, Miyazaki K, Ichinose S, Hata Y, Muneta T: **In vitro cartilage formation of composites of synovium-derived mesenchymal stem cells with collagen gel.** *Cell Tissue Res* 2005, **322**(2):289-298.
 178. Yao YC, Zhang, F., Zhou, R. J., Su, K., Fan, J. B., Wang, D. A.: **Effects of combinational adenoviral vector-mediated TGF β 3 transgene and shRNA silencing type I collagen on articular chondrogenesis of synovium-derived mesenchymal stem cells.** *Biotechnology and Bioengineering* 2010, DOI: **10.1002/bit.22733**
 179. Salzmann GM, Nuernberger B, Schmitz P, Anton M, Stoddart MJ, Grad S, Milz S, Tischer T, Vogt S, Gansbacher B *et al*: **Physicobiochemical**

- Synergism Through Gene Therapy and Functional Tissue Engineering for In Vitro Chondrogenesis.** *Tissue Eng Part A* 2009, **15**(9):2513-2524.
180. Shen BJ, Wei AQ, Tao HL, Diwan AD, Ma DDF: **BMP-2 Enhances TGF-beta 3-Mediated Chondrogenic Differentiation of Human Bone Marrow Multipotent Mesenchymal Stromal Cells in Alginate Bead Culture.** *Tissue Eng Part A* 2009, **15**(6):1311-1320.
 181. Ostergaard M, Ejbjerg B, Stoltenberg M, Gideon P, Volck B, Skov K, Jensen CH, Lorenzen I: **Quantitative magnetic resonance imaging as marker of synovial membrane regeneration and recurrence of synovitis after arthroscopic knee joint synovectomy: a one year follow up study.** *Ann Rheum Dis* 2001, **60**(3):233-236.
 182. Vandenabeele F, De Bari C, Moreels M, Lambrichts I, Dell'Accio F, Lippens PL, Luyten FP: **Morphological and immunocytochemical characterization of cultured fibroblast-like cells derived from adult human synovial membrane.** *Arch Histol Cytol* 2003, **66**(2):145-153.
 183. Morales TI, Roberts AB: **Transforming growth factor beta regulates the metabolism of proteoglycans in bovine cartilage organ cultures.** *J Biol Chem* 1988, **263**(26):12828-12831.
 184. Redini F, Daireaux M, Mauviel A, Galera P, Loyau G, Pujol JP: **Characterization of proteoglycans synthesized by rabbit articular chondrocytes in response to transforming growth factor-beta (TGF-beta).** *Biochim Biophys Acta* 1991, **1093**(2-3):196-206.
 185. Sellers RS, Peluso D, Morris EA: **The effect of recombinant human bone morphogenetic protein-2 (rhBMP-2) on the healing of full-thickness defects of articular cartilage.** *J Bone Joint Surg Am* 1997, **79**(10):1452-1463.

186. Moioli EK, Mao JJ: **Chondrogenesis of mesenchymal stem cells by controlled delivery of transforming growth factor-beta3.** *Conf Proc IEEE Eng Med Biol Soc* 2006, **1**:2647-2650.
187. Miyanishi K, Trindade MC, Lindsey DP, Beaupre GS, Carter DR, Goodman SB, Schurman DJ, Smith RL: **Dose- and time-dependent effects of cyclic hydrostatic pressure on transforming growth factor-beta3-induced chondrogenesis by adult human mesenchymal stem cells in vitro.** *Tissue Eng* 2006, **12**(8):2253-2262.
188. Yun K, Moon HT: **Inducing chondrogenic differentiation in injectable hydrogels embedded with rabbit chondrocytes and growth factor for neocartilage formation.** *J Biosci Bioeng* 2008, **105**(2):122-126.
189. Kim YJ, Sah RL, Doong JY, Grodzinsky AJ: **Fluorometric assay of DNA in cartilage explants using Hoechst 33258.** *Anal Biochem* 1988, **174**(1):168-176.
190. Enobakhare BO, Bader DL, Lee DA: **Quantification of sulfated glycosaminoglycans in chondrocyte/alginate cultures, by use of 1,9-dimethylmethylene blue.** *Anal Biochem* 1996, **243**(1):189-191.
191. Varshney RR, Zhou, R. J., Hao, J. H., Yeo, S. S., Chooi, W. H., Fan, J. B., Wang, D. A.: **Chondrogenesis of Synovium-Derived Mesenchymal Stem Cells in Gene-Transferred Co-Culture System.** *Biomaterials* 2010, DOI: **10.1016/j.biomaterials.2010.05.038**

PUBLICATION LIST

1. Review papers:

YC Yao, CM Wang, RR Varshney, DA Wang. ‘Antisense makes sense in engineered regenerative medicine’. *Pharmaceutical Research*, 2009, 26:263-275;

2. Research articles:

Yao, Y. C., Zhang, F., Pang, X. T., Su, K., Zhou, R. J., Wang, D. A., “Enhancing redifferentiation of dedifferentiated chondrocytes in 3D scaffold with dual transgenes of TGF- β 3 and shRNA silencing type I collagen”, submitted

Yao, Y. C., Zhang, F., Zhou, R. J., Su, K., Fan, J. B., Wang, D. A., “Effects of combinational adenoviral vector-mediated TGF β 3 transgene and shRNA silencing type I collagen on articular chondrogenesis of synovium-derived mesenchymal stem cells”, *Biotechnology and Bioengineering*, 106 (2010), 5: 818-828.

Yao, Y. C., Zhang, F., Zhou, R. J., Li, M., Wang, D. A., “Continuous supply of TGF- β 3 via adenoviral vector promotes type I collagen and viability of fibroblasts in alginate hydrogel”, *Journal of Tissue Engineering and Regenerative Medicine*, 4 (2010), 7: 497-504.

Zhang, F.*, **Yao, Y. C.*** (*equal contribution), Hao, J. H., Zhou, R. J., Liu, C. Z., Gong, Y. H., Wang, D. A., “A dual-functioning adenoviral vector encoding both transforming growth factor- β 3 and shRNA silencing type I collagen: construction and controlled release for chondrogenesis”, *Journal of Controlled Release*, 142 (2010), 1: 70-77.

Hao, J. H., **Yao, Y. C.**, Varshney, R. R., Wang, L. C., Prakash, C., Li, H., Wang, D. A., "Gene Transfer and Living Release of Transforming Growth Factor-Beta 3 for Cartilage Tissue Engineering Applications", *Tissue Engineering Part C Methods*, 14 (2008), 4: 273-280.

Wang, Y. J., Shi, X. T., Ren, L., **Yao, Y. C.**, Zhang, F., Wang, D. A., "Poly(lactide-co-glycolide)/ titania composite microspheres sintered scaffolds for bone tissue engineering applications", *Journal of Biomedical Materials Research Part B - Applied Biomaterials*, 93B (2010), 1: 84-92.

Wang, Y. J., Shi, X. T., Ren, L., **Yao, Y. C.**, Wang, D. A., "In Vitro Osteogenesis of Synovium Mesenchymal Cells Induced by Controlled Release of Alendronate and Dexamethasone from Sintered Microspherical Scaffold", *Journal of Biomaterials Science Polymer Edition*, 21 (2010), 9: 1227-1238.

Wang, C. M., Li, X., **Yao, Y. C.**, Wang, D. A., "Concurrent extraction of proteins and RNA from cell-laden hydrogel scaffold free of polysaccharide interference", *Journal of Chromatography B*, 877 (2009), 29: 3762-3766.

Wang, C. M., Gong, Y. H., Zhong, Y., **Yao, Y.C.**, Su, K., Wang, D. A., "The control of anchorage-dependent cell behavior within a hydrogel / microcarrier system in an osteogenic model", *Biomaterials*, 30 (2009), 13: 2259-2269.