2015

Decellularized matrix and cartilage regeneration: A focus on developing a matrix with anti-inflammation and micromechanical properties

Ying Zhang

Follow this and additional works at: <https://researchrepository.wvu.edu/etd>

Recommended Citation

Zhang, Ying, "Decellularized matrix and cartilage regeneration: A focus on developing a matrix with anti-inflammation and micromechanical properties" (2015). *Graduate Theses, Dissertations, and Problem Reports*. 7035.

<https://researchrepository.wvu.edu/etd/7035>

This Dissertation is protected by copyright and/or related rights. It has been brought to you by the The Research Repository @ WVU with permission from the rights-holder(s). You are free to use this Dissertation in any way that is permitted by the copyright and related rights legislation that applies to your use. For other uses you must obtain permission from the rights-holder(s) directly, unless additional rights are indicated by a Creative Commons license in the record and/ or on the work itself. This Dissertation has been accepted for inclusion in WVU Graduate Theses, Dissertations, and Problem Reports collection by an authorized administrator of The Research Repository @ WVU. For more information, please contact researchrepository@mail.wvu.edu.

**DECELLULARIZED MATRIX AND CARTILAGE
REGENERATION: A FOCUS ON DEVELOPING A MATRIX
WITH ANTI-INFLAMMATION AND MICROMECHANICAL
PROPERTIES**

Ying Zhang

**Dissertation submitted to the Benjamin M. Statler College of
Engineering and Mineral Resources at West Virginia University
in partial fulfillment of the requirements for the degree of**

Doctor of Philosophy

Ming Pei, M.D., Ph.D., Chair

Cerasela Zoica Dinu, Ph.D.

Victor H. Mucino, Ph.D.

Konstantinos A. Sierros, Ph.D.

Nithi T. Sivaneri, Ph.D.

Nianqiang Wu, Ph.D.

in the Department of Mechanical and Aerospace Engineering

Morgantown, West Virginia

2015

Key words: Decellularized extracellular matrix, Anti-inflammation,

Chondrogenesis, Biomechanics

Copyright 2015 Ying Zhang

Abstract

DECELLULARIZED MATRIX AND CARTILAGE REGENERATION: A FOCUS ON DEVELOPING A MATRIX WITH ANTI-INFLAMMATION AND MICROMECHANICAL PROPERTIES

Ying Zhang

Osteoarthritis (OA) is predicted to be the fourth leading cause of disability worldwide by the year 2020. Stem cell-based therapy is a promising biological approach for the treatment of cartilage defects. However, adult stem cells tend to become replicatively senescent when they are expanded on conventional plastic flasks. Decellularized extracellular matrix (dECM) provides an expansion system to rejuvenate and maintain the chondrogenic phenotype of stem cells for cartilage repair. In addition, joints with cartilage defects normally have inflammation to some extent; several stimuli, such as Reactive Oxygen Species (ROS) and Interleukin-1 (IL-1), reportedly induce apoptosis of chondrocytes. The tolerance of transplanted cells to a harsh environment is a key determinant for successful cartilage repair. dECM expansion gives adult stem cells an ability to resist inflammation and oxidants. This anti-inflammatory effect was further optimized by the combined use of small molecules in inflammation-related signaling pathways. Furthermore, dECM is a three-dimensional nanofiber structure with unique micromechanical properties. The influence of dECM elasticity on expanded stem cells' chondrogenic differentiation has been studied. Our long-term goal is to develop an *in vitro* stem cell matrix with optimized micromechanical properties to enhance stem cell proliferation and boost chondrogenic differentiation in order to treat cartilage defects and OA.

Acknowledgements

The present work would not have been possible without the help of the following people.

Foremost, I would like to express my sincere gratitude to my research advisor Dr. Ming Pei for the continuous support of my Ph.D study and research, for his patience, motivation, enthusiasm, and immense knowledge. His guidance helped me during my research and writing of my dissertation. Without his support and guidance, the present dissertation would not have been possible.

Furthermore, I would like thank all my committee members (Dr. Cerasela Zoica Dinu, Dr. Victor H. Mucino, Dr. Konstantinos A. Sierros, Dr. Nithi T. Sivaneri, Dr. Nianqiang (Nick) Wu) for being my committee members, their support, encouragement and insightful comments.

I would also like to thank the Department of Orthopaedics at West Virginia University for providing me a nice lab and working atmosphere. Special thanks go to Nina Clovis and Suzanne Danley for maintenance of laboratory and preparatory work of experiments, manuscripts editing, etc. Thanks to Dr. Chenbo Dong in the Department of Chemical Engineering for his assistance in AFM experiments.

My work was financially supported by Research Grants from the AO Foundation (S-12-19P) and the National Institutes of Health (NIH) (1 R03 AR062763-01A1 and 5 R03 DE021433-02).

Table of contents

Acknowledgements.....	iii
Table of contents.....	iv
List of figures.....	vii
List of symbols/nomenclature.....	xi
Specific Aims.....	1
References.....	2
Introduction.....	3
Current progress in cartilage repair.....	3
The applications of dECM in resisting oxidative and inflammatory environment in osteoarthritis treatment.....	4
Preconditioning stem cells with small molecules targeting inflammation signals	5
Biomechanics in chondrogenic differentiation of stem cells.....	5
References.....	7
Chapter 1: Anti-inflammatory Strategies in Cartilage Repair	8
Abstract.....	9
Introduction.....	1
Pharmacologic strategy.....	2
Biomechanical Strategy	8
Mesenchymal stem cell strategy	10
DSCM-mediated stem cell preconditioning.....	11
Low oxygen and FGF-2 contribute to DSCM-mediated stem cell preconditioning	12
Small molecules targeting inflammation signals contribute to DSCM-mediated stem cell preconditioning.....	13
Extracellular matrix strategy.....	13
Summary	16
References.....	20
Chapter 2: Delineation of in vitro chondrogenesis of human synovial stem cells following preconditioning using decellularized matrix	24
Abstract.....	25
Introduction.....	26
Materials and Methods.....	27
Results.....	32
Discussion	35
Conclusion and Perspectives.....	40
References.....	41
Chapter 3: sb203580 preconditioning recharges matrix-expanded human adult stem cells for chondrogenesis in an inflammatory environment – a feasible approach for autologous stem cell based osteoarthritic cartilage repair	51
Abstract.....	52
Introduction.....	53
Materials and Methods.....	54

Results.....	58
Discussion	62
Conclusion	64
References.....	65
Chapter 4. Biomechanical signals guiding stem cell cartilage engineering: from molecular adaption to tissue functionality	74
Abstract.....	75
Introduction.....	76
Substrate elasticity influences stem cell/chondrocyte fate.....	77
Potential mechanisms of substrate elasticity underlying chondrogenic differentiation.....	82
External forces on stem cell/chondrocyte chondrogenic differentiation	87
Potential mechanisms of external forces underlying chondrogenic differentiation.....	91
Concluding remarks	95
References.....	97
Chapter 5. Optimization of decellularized extracellular matrix to reprogram infrapatellar fat pad-derived stem cells for cartilage tissue engineering	105
Abstract.....	106
Introduction.....	107
Materials and methods	109
Results.....	113
Discussion	115
References.....	120
Chapter 6. Antioxidation of decellularized stem cell matrix promotes human synovium-derived stem cell-based chondrogenesis	129
Abstract.....	130
Introduction.....	131
Materials and Methods.....	132
Results.....	137
Discussion	141
References.....	145
Chapter 7. A review of crosstalk between MAPK and Wnt signals and its impact on cartilage regeneration.....	157
Abstract.....	158
Introduction.....	159
Influence of canonical Wnt signals on the MAPK pathway.....	161
Influence of non-canonical Wnt signals on the MAPK pathway	162
Influence of MAPK signals on the Wnt pathway	164
Crosstalk of MAPK and Wnt signals in cartilage inflammation and regeneration.....	165
Conclusion and Future Directions	169
References.....	170
Chapter 8: General discussion	183
dECM expanded stem cell applications in <i>in vivo</i> animal experiments.....	184

MAPK signal pathway with dECM in chondrogenesis of stem cells in an inflammatory environment.....	185
Biomechanical stimulations influence stem cell behaviors and chondrogenic differentiation fate.....	187
References.....	191
Conclusions.....	193
References.....	196

List of figures

Chapter 1

Figure1. 1 Inflammation in cartilage repair.	17
Figure1. 2 Schematic representation of key signaling pathways in the inflammatory process and potential strategies for inflammatory inhibition in cartilage repair.	18
Figure1. 3 Therapeutic strategies for cartilage damage utilizing preconditioned stem cell and three-dimensional matrix.....	19

Chapter 2

Figure 2. 1 Morphological characterization of culture substrates and expanded cells.	43
Figure 2. 2 dECM promoted expanded human SDSCs' proliferation and changed expression of stem cell surface markers.	44
Figure 2. 3 dECM promoted expanded human SDSCs' chondrogenic differentiation.	45
Figure 2. 4 dECM promoted expanded human SDSCs' chondrogenic differentiation.	46
Figure 2. 5 hSDSCs grown in ECM ("IN") exhibited a decline in chondrogenic potential compared with those expanded on dECM ("ON").	48
Figure 2. 6 Wnt signals were actively involved in chondrogenesis of dECM expanded human SDSCs.	49
Figure 2. 7 MAPK signals were actively involved in chondrogenesis of dECM expanded human SDSCs.....	50

Chapter 3

Figure 3. 1 Preconditioning using sb203580 enhanced proliferation of dECM expanded hSDSCs.....	67
Figure 3. 2 Preconditioning using sb203580 enhanced chondrogenesis of dECM expanded hSDSCs.....	68
Figure 3. 3 Human SDSCs expanded on either dECM or Plastic for one passage were followed by a 28-day chondrogenic induction in a pellet system with or without preconditioning of sb203580.	69
Figure 3. 4 Chondrogenic induction of dECM-expanded hSDSCs in the presence of IL-1 β	70
Figure 3. 5 Chondrogenic induction of dECM-expanded hSDSCs in the presence of IL-1 β	71
Figure 3. 6 35-day-chondrogenic induction of dECM-expanded hSDSCs preconditioned with sb203580 in the presence of 1 ng/mL IL-1 β	72

Figure 3. 7 Flow cytometry analysis of CD45 and HLA-DR surface marker expression following cell expansion.	73
---	----

Chapter 4

Fig 4. 1 Potential mechanisms of substrate stiffness on stem cell chondrogenic fate.	103
Fig 4. 2 Potential mechanisms of external forces on stem cell chondrogenic fate. ...	104

Chapter 5

Fig 5. 1 Schematics of preparation of dECM with different elasticities.	121
Fig 5. 2 dECMs (different culture days) enhanced expanded hIPFP stem cells' proliferation and changed stem cell markers' expression.	122
Fig 5. 3 dECM from different culture days expanded hIPFP stem cells showed varied chondrogenic differentiation.	123
Fig 5. 4 Real-time PCR was used to evaluate chondrogenic induction of expanded hIPFP stem cells on either Plastic or dECMs prepared by different culture days.	124
Fig 5. 5 Plastic and dECMs prepared by different culture days showed varied elasticity changes, which was mirrored by elasticity changes of corresponding expanded cells.	125
Fig 5. 6 dECMs (different culture days) promoted expanded hIPFP stem cells' proliferation and changed expression of stem cell surface markers.	126
Fig 5. 7 Plastic and L-ascorbic acid phosphate-dependent dECMs expanded hIPFP stem cells showed varied chondrogenic differentiation.	127
Fig 5. 8 Plastic and dECMs prepared by different concentration of L-ascorbic acid demonstrated changed substrate and cells' elasticity.	128

Chapter 6

Figure 6. 1 Effect of DSCM pretreatment on hSDSC oxidative stress and chondrogenic potential.	147
Figure 6. 2 Effect of DSCM pretreatment on hSDSC expansion under H ₂ O ₂	148
Figure 6. 3 Effect of DSCM pretreatment with 0.05 mM H ₂ O ₂ on expanded hSDSC chondrogenic potential.	150
Figure 6. 4 Effect of DSCM pretreatment on expanded hSDSC chondrogenic differentiation with either 0.05 or 0.1 mM H ₂ O ₂	152
Figure 6. 5 Effect of H ₂ O ₂ treatment in either cell expansion or chondrogenic differentiation phase on expanded SDSC chondrogenic hypertrophy.	154
Figure 6. 6 Western blot was used to measure the levels of p38 and Erk1/2, p21 in the hSDSCs expanded on either DSCM or Plastic after the treatment with 0.05 mM H ₂ O ₂	156

Chapter 7

Figure 7. 1 The best characterized MAPK modules are the ERK pathway, the SAPK/JNK pathway, and the p38 MAPK pathway.....	175
Figure 7. 2 Three Wnt-dependent pathways have been categorized: canonical Wnt/ β -catenin and non-canonical Wnt/PCP as well as Wnt/Ca ²⁺ pathways.	176
Figure 7. 3 Influence of canonical Wnt signals on the MAPK pathway.	178
Figure 7. 4 Influence of non-canonical Wnt signals on the MAPK pathway.	180
Figure 7. 5 Influence of MAPK signals on the Wnt pathway.....	182

List of tables

Chapter 4

Table 4. 1 The influence of substrate stiffness on chondrogenic differentiation.....	101
Table 4. 2 Influences of external mechanical forces on stem cell chondrogenic differentiation.....	102

Chapter 7

Table 7. 1 Highlighted references related to the crosstalk between Wnt and MAPK pathways in varied cell types, especially its influence in the regulation of chondrogenesis.....	173
--	-----

List of symbols/nomenclature

2D	Two-dimensional
3D	Three-dimensional
ACI	Autologous chondrocyte implantation
ADAMTs	A disintegrin and metalloproteinase with thrombospondin motifs
AE	ASDSC-derived DSCM
AFM	Atomic force microscopy
AKT	Protein Kinase B
ALP	Alkaline phosphatase
ASC	Adipose stem cell
bFGF2	Basic fibroblast growth factor-2
BMP	Bone morphogenetic protein
BMSCs	Bone marrow derived mesenchymal stem cells
CaMK	Calcium/calmodulin-dependent protein kinase
CDC42	Cell division cycle 42
CHP	Cyclic hydrostatic pressure
Col I	Type I collagen
Col II	Type II collagen
Col X	Type X collagen
COMP	Cartilage oligomeric matrix protein
COX-2	Cyclooxygenase 2
DAB	3,3'-diaminobenzidine
DECM	Decellularized extracellular matrix
DMMB	Dimethylmethylene blue
DSCM	Decellularized stem cell matrix
ECM	Extracellular matrix
ESC	Embryonic stem cell
FAK	Focal adhesion kinase
G3BP2	Ras GTPase-activating protein-binding protein 2
GAGs	Glycosaminoglycans
GATA4	Transcription factor GATA-4
HGF	Hepatocyte growth factor
HIF	Hypoxia-inducible factor
IGF1	Insulin-like growth factor-1
IHP	Intermittent hydrostatic pressure
IL-1 β	Interleukin-1 β
IP3	Inositol 1,4,5-triphosphate
IPFP	Infrapatellar fat pad
JNK	c-Jun NH2-terminal kinase
LIF	Leukemia inhibitory factor
MAPK	Mitogen activated protein kinase
MEKKs	MAPK kinase kinases
MLCK	Myosin Light Chain Kinase
MMP	Matrix metalloproteinases

MSCs	Mesenchymal stem cells
NFAT	Nuclear Factor of Activated T cells
NF- κ B	Nuclear factor-kappa B
OA	Osteoarthritis
OCN	Osteocalcin
PCL	Poly(ϵ -caprolactone)
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PGA	Poly glycolic acid
PGs	Proteoglycans
PI	Proliferation index
PI3K	Phosphatidylinositol 3-Kinase
PLCL	Poly L-lactide-co-3-caprolactone
PPAR γ	Peroxisome proliferator-activated receptor gamma
RA	Rheumatoid arthritis
ROS	Reactive oxygen species
RUNX2	Runt domain transcription factor 2
SAPKs	Stress-activated protein kinases
SDSC	Synovium-derived stem cells
SEM	Scanning electron microscope
SOX9	SRY (sex determining region Y)-box 9
Src	Proto-oncogene tyrosine-protein kinase
SSEA-4	Stage specific embryonic antigen-4
STATs	Signal transducers and activators of transcription
TAZ	Transcriptional coactivator with PDZ-binding motif
TGF β	Transforming growth factor β
TIMPs	Tissue inhibitors of metalloproteinases
TNF α	Tumor necrosis factor α
TWIST1	Twist-related protein 1
VEGF	Vascular endothelial growth factor
YAP	Yes-associated protein

Specific Aims

Articular cartilage has a limited ability to self-heal because it is a unique hypocellular, and avascular tissue and often accompanied with inflammation especially after trauma and degenerative disease [1]. Mesenchymal stem cell (MSC) is a promising cell source for cartilage regeneration because they are easily obtainable in high numbers and expanded *in vitro* without losing their differentiation potential. A reconstructed *in vitro* three dimensional (3D) stem cell microenvironment was found to dramatically increase cell numbers and greatly enhanced chondrogenic capacities were observed, which inspired us to develop an efficient *in vitro* cell expansion system with proliferation and chondrogenic differentiation rejuvenating capacities [2]. The potential impact of this study is that the optimization of *ex vivo* stem cell protocols with decellularized extracellular matrix for maintaining stemness and facilitating chondrogenesis will provide high quality and a large quantity of cells for cartilage regeneration in clinic. The understanding of the rejuvenation mechanisms including its biochemical and biophysical mechanisms by matrixes will advance the knowledge of cartilage regeneration and benefit the development of cartilage defect treatment.

The **objectives** of this dissertation were to: (1) Develop a 3D stem cell-derived decellularized extracellular matrix (dECM), including surface characterization, expanded cell morphology, functional analysis on its chondrogenesis, as well as biochemical signals. (2) Define novel properties of dECM, focusing on its antioxidant and anti-inflammatory properties, which will be promising for the application of osteoarthritis (OA) treatment. (3) Explore potential mechanisms of dECM from biophysical (especially on substrate Young' s modulus) perspective.

The **central hypothesis** was that dECM deposited by human stem cells can be optimized biochemically and biophysically to serve as *ex vivo* expansion system to beter promote cartilage regeneration in the inflammatory environment.

References

1. Pei M, He F, Wei L. *J Tissue Sci Eng* 2011;**2**:104
2. He F, Chen X, Pei M. *Tissue Eng Part A* 2009;**15**:3809-21.

Introduction

Current progress in cartilage repair

Osteoarthritis (OA), also called osteoarthrosis or degenerative joint disease, is a chronic joint disease characterized by cartilage destruction, subchondral bone sclerosis, osteophyte formation, synovial inflammation, and degeneration of ligaments. OA is the most common type of arthritis, affecting nearly 27 million people in the United States alone [1]. In the late-stage of OA when the disease progresses and the symptoms increase, surgical intervention may be required. The treatment for cartilage injury remains a clinical challenge despite advances in surgical techniques. Indeed, the oxidative and inflammatory environment of the joints may induce telomere instability and chondrocyte senescence, leading to the formation of fibrocartilage, which is inferior to hyaline cartilage in respect to biochemical and biomechanical characteristics [2].

Traditional surgical methods for articular cartilage repair such as the transplantation of osteochondral grafts, microfracturing, and autologous chondrocyte implantation (ACI) can repair or replace damaged cartilage; however, these procedures do not completely restore the normal structure composition and function of cartilage [3]. ACI has undergone considerable development since its inception and it is now an established form of treatment for symptomatic osteochondral defects [4]. However, several limitations make it an inferior application when compared to other cell-based cartilage repair therapies, including cell senescence [5] and dedifferentiation [6] which may contribute to insufficient numbers of viable chondrocytes and inferior fibrocartilage at the defect site [6, 7]. Adult mesenchymal stem cells (MSCs) have the ability to differentiate into cells of chondrogenic lineage and have emerged as an ideal cell type for cell-based articular cartilage repair technology [8]. The potential for guided chondrogenesis, and the ease with which they can be isolated and expanded *in vitro* without phenotypic change before lineage-specific differentiation [9], make MSCs attractive candidates for cartilage tissue engineering.

Recently, *in vivo* investigations of stem cell niches have suggested the importance of developing an *in vitro* stem cell microenvironment for cell expansion and tissue-specific

differentiation. To successfully translate human adult stem cells into applications in cartilage repair, it is important to create a functional *in vitro* stem cell niche that consists entirely of known, xeno-free, and chemically defined factors and molecules [10]. Despite synthetic matrices providing the highly controlled composition and structure, an *in vitro* niche derived from stem cell is more physiologically and serves as a “natural” reservoir of growth factors and cytokines [11], therefore enhancing cell attachment, proliferation, and differentiation.

The applications of dECM in resisting oxidative and inflammatory environment in osteoarthritis treatment

To guarantee successful cell-based therapy or tissue engineering, measures must be taken to control the inflammatory and oxidative environment in which cartilage is regenerated. Scaffolds made by materials with natural anti-inflammatory characteristics not only provide mechanical support for stem cells to attach, migrate, and proliferate, but also suppress inflammatory reaction in cartilage repair. Since bioengineered cartilage constructs will eventually be transplanted into arthritic joints in which elevated levels of pro-inflammatory cytokines exist, it is especially important to select scaffolds that support the stability of bioengineered cartilage in an inflammatory environment. To this end, many ECM-based scaffolds are hypothesized to meet the requirements of cartilage repair.

dECM provides an *in vitro* microenvironment for synovium-derived stem cell (SDSC) rejuvenation in terms of enhancing expanded cells' proliferation and chondrogenic potential [12, 13]. Recent findings indicated that dECM expansion decreases expanded cells' reactive oxygen species (ROS) levels [14]. Furthermore, microarray data suggested that dECM expansion could upregulate SDSCs' antioxidant gene expressions, indicating that dECM expansion may benefit SDSCs by increasing their resistance to oxidative stress and promoting chondrogenic capability [15]. A study in mini-pigs successfully proved its resurfacing effect on partial-thickness cartilage defects after intra-articular injection of dECM-expanded allogeneic SDSCs [16]. Developed as an expansion system to provide a number of high-quality cell sources ready for cartilage tissue engineering, the dECM model also demonstrated

anti-inflammatory capabilities, which is of great potential for the treatment of OA patients with cartilage defects.

Preconditioning stem cells with small molecules targeting inflammation signals

The control of stem cell fate and function is an important step for clinical applications in regenerative medicine. Understanding and controlling the fate and function of adult stem cells is a challenge for developing better therapeutic approaches to regenerative medicine. Among various approaches in controlling stem cell fate, small molecules are considered a good strategy, because small molecules targeting specific signaling pathways affect various cellular events, such as DNA replication, proliferation, differentiation, and apoptosis. Pro-inflammatory factors such as IL-1 β , tumor necrosis factor α (TNF α) and oxidative stress induce phosphorylation-dependent signaling pathways, such as p38 MAPK and NF- κ B, which regulates synthesis of several inflammatory cytokines and matrix metalloproteinases (MMPs), which play major roles in pathways that are activated during the OA process. Widely used as an anti-inflammatory drug in cartilage repair, most of the p38 MAPK inhibitor was applied in the cell differentiation phase, in which stem cells experienced the committed chondrogenesis under appropriate induction conditions. However, p38 MAPK inhibitor also blocks chondrogenesis [17], adverse effects of p38 MAPK inhibitors resulting from undesired pharmacological activity are still a major concern for clinical application [18].

Biomechanics in chondrogenic differentiation of stem cells

Stem cell biologists have long appreciated the regulatory roles of soluble stem cell niche signals (e.g., growth factors and cytokines) in regulating stem cell fate. Recent evidence demonstrated that mechanical and topographical cues that exist in stem cell niche can also be sensed and transduced into intracellular biochemical and functional responses by stem cells, a

process known as mechanotransduction [19, 20]. Mechanical forces generated intrinsically within the cell in response to its extracellular environment, and extrinsic mechanical signals from extracellular environment, play a critical role in determining stem cell fate. There are two different methods of mechanotransduction: “inside-out” or “outside-in” sensing. For the active “inside-out” sensing, the generation of internal forces allows for “measurement” of the extracellular environment, for example, changes in extracellular matrix stiffness, surface topography, and ligand density will be felt and responded to by cells through generating varying traction forces. Passive “outside-in” sensing is where the cell responds to a force imparted upon itself, for example, compression, tension, or pressure [21].

Cells are regarded to differentiate optimally on the tissue level’s elasticity, which was confirmed by Engler et al. studies showing that mesenchymal stem cells differentiate into neurogenic, myogenic, and osteogenic precursor cells through cultivation on substrates with the respective elasticity [22]. However, little is known about the influence of elasticity on chondrocyte behaviors and chondrogenic differentiation fate.

dECM is an effective expansion tool for harvesting a large amount of high-quality chondrocytes for cell-based cartilage repair. The fact that stem cells maintained better chondrogenic potentials on dECM with lower elasticity suggested that during *in vitro* cell expansion, dECM could be designed with an appropriate elasticity in order to better maintain its chondrogenic potential. By controlling dECM elasticity, or changing the nanotopography of dECM, stem cells could be steered specifically toward chondrogenic differentiation. Moreover, extrinsic mechanical forces (e.g. compression, hydrostatic pressure, fluid flow) can also be applied to change stem cell properties from cellular levels, which might further influence its chondrogenic fate. However, studies of influences of mechanical properties of the *in vitro* microenvironment on chondrogenesis of stem cells are still in their infancy and the underlying mechanisms need to be explored further.

References

3. Bitton, R. *Am J Manag Care* 2009;**15**:S230-5.
4. Martin JA, Klingelhutz AJ, Moussavi-Harami F, Buckwalter JA. *J Gerontol A Biol Sci Med Sci* 2004;**59**:324-37.
5. Karnes J, Zhang Y, Pei M. 4th Edition. Taylor & Francis/CRC Press, 2014. p. 1123-1135.
6. Brittberg M, Lindahl A, Nilsson A et al. *N Engl J Med* 1994;**331**:889-95.
7. Hayflick, L. *Exp Cell Res*. 1965;**37**:614-36.
8. Benya, PD and Shaffer JD. *Cell*. 1982;**30**:215-24.
9. von der Mark K, Gauss V, von der Mark H et al. *Nature* 1977;**267**:531-2.
10. Chen FH, Rousche KT, Tuan RS. *Nat Clin Pract Rheumatol* 2006;**2**:373-82.
11. Pittenger MF, Mackay AM, Beck SC et al. *Science* 1999;**284**:143-7.
12. Martin MJ, Muotri A, Gage F et al. *Nat Med* 2005;**11**:228-32.
13. Badylak SF, Valentin JE, Ravindra AK et al. *Tissue Eng Part A* 2008;**14**:1835-42.
14. He F, Chen X, Pei M. *Tissue Eng Part A* 2009;**15**:3809-21.
15. Pei M, Li JT, Shoukry M et al. *Eur Cell Mater* 2011;**22**:333-43.
16. Pei M, He F, Kish VL et al. *Tissue Eng Part A* 2011;**17**:3067-76.
17. Pei M, Zhang Y, Li JT et al. *Stem Cells Dev* 2013;**22**:889-900.
18. Pei M, He F, Li J et al. *Tissue Eng Part A* 2013;**19**:1144-54.
19. Oh CD, Chang SH, Yoon YM et al. *J Biol Chem* 2000;**275**:5613-9.
20. Ding C. *Curr Opin Investig Drugs* 2006;**7**:1020-5.
21. Chen CS. *J Cell Sci* 2008;**121**:3285-92.
22. Geiger B, Spatz JP, Bershadsky AD. *Nat Rev Mol Cell Biol* 2009;**10**:21-33.
23. Holle AW, Engler AJ. *Curr Opin Biotechnol* 2011;**22**:648-54.
24. Engler AJ, Sen S, Sweeney HL et al. *Cell* 2006;**126**:677-89.

Chapter 1: Anti-inflammatory Strategies in Cartilage Repair

As published in Tissue Eng Part B Rev. 2014; 20:655-68. doi: 10.1089/ten.

^{1,2}Ying Zhang, MS, ^{1,3}Tyler Pizzute, BS, and ^{1,2,3}Ming Pei, MD, PhD

¹Mechanical and Aerospace Engineering, West Virginia University, Morgantown, West Virginia 26506, USA

²Stem Cell and Tissue Engineering Laboratory, Department of Orthopaedics, West Virginia University, Morgantown, West Virginia 26506, USA

³Exercise Physiology, West Virginia University, Morgantown, West Virginia 26506, USA

Running head: Anti-inflammation Promotes Cartilage Regeneration

Corresponding author: Ming Pei MD, PhD, Stem Cell and Tissue Engineering Laboratory, Department of Orthopaedics, West Virginia University, PO Box 9196, One Medical Center Drive, Morgantown, WV 26506-9196, USA, Telephone: 304-293-1072; Fax: 304-293-7070; Email: mpei@hsc.wvu.edu

Abstract

Cartilage defects are normally concomitant with posttraumatic inflammation and pose a major challenge in cartilage repair. Due to the avascular nature of cartilage and its inability to surmount an inflammatory response, the cartilage is easily attacked by pro-inflammatory factors and oxidative stress; if left untreated, osteoarthritis may develop. Suppression of inflammation has always been a crux for cartilage repair. Pharmacological drugs have been successfully applied in cartilage repair; however, they cannot optimally work alone. This review paper will summarize current pharmacological drugs and their application in cartilage repair. The development of extracellular matrix-based scaffolds and preconditioned tissue-specific stem cells will be emphasized because both of these tissue engineering components could contribute to an enhanced ability not only for cartilage regeneration but also for anti-inflammation. These strategies could be combined to boost cartilage repair under inflammatory conditions.

Introduction

Joint injuries are common in the young and active population and often result in cartilage or osteochondral lesions. If left untreated, these defects might lead to joint swelling and pain, eventually progressing toward osteoarthritis (OA). Over 27 million Americans are affected by OA, introducing huge clinical and socioeconomic burdens [1]. Traditional cartilage repair methods include the transplantation of osteochondral grafts [2], microfracturing, and autologous chondrocyte implantation (ACI) [3]; however, none of these cartilage repair strategies have generated long-lasting hyaline cartilage that meets functional demands. The causes of cartilage impairment are diverse, including inflammation, hypertrophy, and senescence (Fig. 1) [4, 5]. Excessive mechanical surface contact stress can directly damage articular cartilage and subchondral bone and adversely alter chondrocyte function [6] while the disruption of the homeostasis of chondrocytes may gradually develop into OA [7]. In the early phase of OA, disease-modifying interventions targeting inflammatory processes might be most efficacious for the prevention and treatment of OA [8]. To this end, many anti-inflammatory strategies have been discovered, such as growth factor applications, exertion of anti-cytokines or anti-inflammatory drugs, and stem cell-based therapies.

Growth factors are utilized to improve clinical cartilage repair by altering the local biological environment at the site of cartilage damage. Inflammation at the damage site may disrupt the balance between catabolic and anabolic factors; growth factors that target specific catabolic pro-inflammatory mediators such as cytokines or nitric oxide synthase (NOS), or that affect anabolism are potential candidates in slowing down the structural progression of the disease. The production and activities of many pro-inflammatory factors, such as cytokines, chemokines, growth factors, and various immune response regulators, are controlled by different signaling systems, such as nuclear factor-kappa B (NF- κ B), mitogen-activated protein kinases (MAPK), and Janus kinase/signal transducers and activators of transcription (JAK/STAT). Novel small molecule regulators targeting specific signal pathways as well as related precursor molecules have received a great deal of attention as potential candidates for treatment of inflammatory diseases (Fig. 2).

The development of stem cell technology provides the possibility of biotherapy for cartilage repair (Fig. 3). The availability of large quantities of mesenchymal stem cells (MSCs) and their multilineage differentiation potential, especially for chondrogenic differentiation, have made MSCs the ideal

progenitor source for cartilage engineering and regeneration. Cell-based therapies using undifferentiated or pre-chondrogenic stem cells in biodegradable three-dimensional (3D) scaffolds for transplantation into focal lesions could regenerate hyaline-like cartilage [9, 10]. Since bioengineered cartilage constructs will eventually be transplanted into arthritic joints in which elevated levels of pro-inflammatory cytokines exist, it is especially important to select scaffolds that support the stability of bioengineered cartilage in an inflammatory environment. To this end, many extracellular matrix (ECM)-based scaffolds are hypothesized to meet the requirements of cartilage repair (Fig. 3).

Recently, decellularized ECM deposited by stem cells (DSCM) has attracted attention due to its excellent rejuvenation of expanded stem cells' chondrogenic potential, which has been reviewed [11]. Furthermore, DSCM expanded human synovium-derived stem cells (SDSCs) demonstrated *in vitro* antioxidant and anti-inflammatory capabilities [12]. A study in mini-pigs successfully proved its resurfacing effect on partial-thickness cartilage defects after intra-articular injection of DSCM-expanded allogeneic SDSCs [13]. In this review paper, the strategies using pharmacologic drugs, biomechanical stimulation, tissue-specific stem cells, and ECM-based scaffolds are summarized. An emerging strategy using a DSCM preconditioning approach to rejuvenate tissue-specific stem cells in both cell amount and chondrogenic potential is emphasized for its vital role in cartilage repair and anti-inflammation (Fig. 3). Future goals include the utilization of 3D ECM scaffolds, in conjunction with other combined strategies, including the use of preconditioned tissue-specific stem cells in a more favorable microenvironment to reduce the inflammation in cartilage repair.

Pharmacologic strategy

Growth factors

In articular cartilage, numerous growth factors work in concert throughout life to regulate development and homeostasis of articular cartilage [14]. Disruption in the balance of regulatory factors may hinder tissue maintenance and repair, ultimately resulting in reduced synthesis of ECM, tissue degeneration, and consequently, an accelerated erosion of the articular surface [15]. Bioactive growth factors are considered promising candidates for enhanced healing of chondral injuries and modification of the arthritic disease process. Members of the transforming growth factor beta (TGF-

β)/bone morphogenetic protein (BMP) family, insulin-like growth factor-I (IGF-I), and fibroblast growth factors (FGFs) are considered to be major anabolic factors for cartilage formation. They may stimulate chondrocyte synthesis of proteoglycans, aggrecan, and type II collagen, inducing cell proliferation, driving stem cell chondrogenic differentiation, and decreasing the catabolic effects of cytokines [16, 17]. Overexpression of the growth factor progranulin (PGRN), for example, has been implicated in the stimulation of chondrocyte proliferation; PGRN also acts as a physiological antagonist of tumor necrosis factor alpha (TNF α) signaling and disturbs the binding of TNF α and TNF receptor [18], potentially inhibiting cartilage degradation [19]. Cytokines such as TNF α can stimulate vascular endothelial growth factor (VEGF) expression [20]. Therefore, some anti-inflammatory strategies inhibit inflammation-induced angiogenesis although it remains unclear to what extent angiogenesis inhibition mediates their therapeutic effects [21].

By exerting influence over inflammation, growth factors can modulate the microenvironment of chondrocytes to improve the local residence and provide a more ideal atmosphere for cartilage regeneration. For instance, growth factors such as platelet-derived growth factor (PDGF) and TGF- β obtained from platelet-rich plasma (PRP) decreased interleukin 1 beta (IL-1 β)-induced NF- κ B activation, a major pathway involved in the pathogenesis of OA (Fig. 2) [22, 23]. Furthermore, in activated PRP, Bendinelli *et al.* observed increases in hepatocyte growth factor (HGF), IL-4, and TNF α ; HGF and TNF α , by disrupting NF- κ B-transactivating activity through the enhanced cellular I κ B α expression, were important for the anti-inflammatory function of activated PRP [24]. Paradoxically, platelet lysate (PL), a PRP derivative, was thought to play a role as a proinflammatory agent, acting synergistically with the canonical proinflammatory cytokines such as IL-1 α , thus enhancing the initial inflammatory response; surprisingly, PL also contributes to the down-regulation of the NF- κ B signal pathway and cyclooxygenase 2 (COX-2) expression, thus triggering the resolution of the inflammation [25]. The anti-inflammatory role of PRP was also demonstrated in an *in vivo* study. In the antigen-induced arthritis (AIA) porcine model, the intra-articular injection of PRP attenuated the subsequent inflammatory response [26]. The use of PRP may also improve the integration of an osteochondral graft at the cartilage interface and decreased degeneration in an *in vivo* rabbit model [27].

The strategy combining growth factors and 3D scaffolds demonstrated a huge impact on tissue regeneration. Because of PRP's prominent role in anti-inflammation and fewer immunogenic as well

as more biocompatible characteristics, the cell-free polyglycolic acid (PGA)-hyaluronan scaffold combined with PRP led to cartilage repair and improved patient-reported outcomes (the Knee injury and Osteoarthritis Score, KOOS) during 12 months of follow-up [28]. Moreover, TGF- β , delivered together with calcium alginate to the sites of osteochondral defects, improved the repair of osteochondral defects in the rabbit knee [29]. However, the local production of osteophytes has been observed in clinical trials, therefore caution is advised because some growth factors favor the dedifferentiation of stem cells and promote the endochondral ossification process [30].

Anti-cytokine therapy

Like growth factors, cytokines can also be produced in joint tissues and released into the synovial fluid; they influence the surrounding cells in an “autocrine-paracrine” manner (Fig. 1). Low levels of factors are necessary for normal homeostasis; however, inflammatory or oxidative stress conditions may disrupt normal homeostasis, driving the pathogenesis of OA. Among the vast number of cytokines, IL-1 β and TNF α seem quite prominent and of major importance to cartilage destruction [31]. Treatment strategies targeting major inflammatory factors have been developed (Fig. 2). Specific inhibitors of production/activity of IL-1, such as recombinant human IL-1 receptor antagonist (IL-1ra), can block the actions of IL-1 without any detectable agonist activity [32]. The use of monoclonal antibodies against IL-1 or against type I IL-1 receptor (IL-1RI) represents another possible approach in neutralization of the cytokine [33]. Pharmacologic reagents which down-regulate the production and activity of active pro-inflammatory and pro-catabolic IL-1 β is also a feasible approach [34]. One such example is to apply the rhein, an active metabolite of the semi-synthetic anthraquinone derivative diacerein, to down-regulate the production and activity of IL-1 β in both the cartilage layer and synovial membrane [35]. Etanercept, a recombinant soluble p75 TNF receptor, has a high affinity for TNF α , preventing it from binding with its receptor [36]. A recent report indicates that etanercept enhanced preservation of osteochondral allograft viability [37]. An *in vivo* study showed that subcutaneous injection of etanercept promoted repair of osteochondral defects in the rabbit knee [38]. Application of anti-TNF α monoclonal antibody in polyarthritic transgenic mice demonstrated reversal of cartilage degradation in the young mice [39].

Besides IL-1 β and TNF α , pro-inflammatory cytokines, such as IL-6 [40], leukemia inhibitory factor (LIF) [41], and the chemokine IL-8 [42], may modulate the direct catabolic effects of some cytokines.

They usually synergize with TNF α or IL-1 β in the catabolic process of cartilage [43]. Interference with those pro-inflammatory factors is another direction for anti-inflammatory therapy in cartilage repair. A number of anti-inflammatory cytokines, such as IL-4, IL-10, IL-11, and IL-13, were found in increased levels in the synovial fluid of OA patients [44]. They decrease the production and/or activity of the pro-inflammatory cytokines *in vitro* [45], thus have been classified as anti-inflammatory cytokines. Endogenous IL-4 and IL-10 have been shown not only to reduce local IL-1 but may also have direct stimulatory or protective effects on chondrocyte metabolism [46, 47]. Since the early phase of OA is the most effective period to inhibit inflammation, administration of cytokine antagonists following cartilage repair is a safe strategy for not only promotion of cartilage integration but also the prevention of inflammation occurrence.

Chondroprotective drugs

Among pharmacological treatments, symptomatic slow-acting drugs have been largely studied over the last decade. Recently there has been an increase in the use of symptomatic slow-acting/chondroprotective drugs such as glucosamine sulfate (GS), chondroitin sulfate (CS), hyaluronic acid (HA), and diacerein. Glucosamine decreased the activation of NF- κ B in rat chondrocytes when treated with IL-1 β [48]. In normal human articular chondrocytes, GS inhibited IL-1 β and TNF α induced nitric oxide (NO) production, which is a major contributor for the inflammatory reaction in arthritis [49]. Natural sulfated glycosaminoglycan (GAG), in particular CS, seemed to have beneficial effects on the pathophysiology of OA by reducing blood markers of inflammation and the activity of destructive proteases like matrix metalloproteinases (MMPs), diminishing pain as well as improving the function of the affected joint [50, 51, 52]. In chondrocytes, CS diminished IL-1 β -induced increases in p38 MAPK and extracellular signal-regulated kinase 1/2 (ERK1/2) phosphorylation, and decreased NF- κ B nuclear translocation and as a consequence, reduced the formation of pro-inflammatory cytokines, IL-1 β and TNF α , and pro-inflammatory enzymes, such as phospholipase A2 (PLA2), COX-2, and inducible NOS (iNOS) [53]. However, other reports suggested that chondroitin-4-sulfate inhibited the enhanced expression of COX-2 and prostaglandin E (PGE) synthases 1 but had no effect on the IL-1 β -induced decrease of I- κ B α and nuclear translocation of NF- κ B [54]; the extent of sulfation influenced the responsiveness of inflammation [55].

Synthetically sulfated HA was at first characterized as an inhibitor of TNF α [56]. Hyaluronic acid is

an anionic, non-sulfated GAG which acts as a crucial structural component of ECM and as an important mediator of leukocyte adhesion and migration [57]. Hyaluronic acid is widely used in the treatment of OA and exerts significant chondroprotective effects. An *in vitro* report showed that HA altered the profile of inflammatory mediators, shifting the balance between cell matrix synthesis and degradation away from degradation [58]. An *in vivo* rabbit study showed that three weekly intra-articular injections of HA had a positive effect on the repair tissue that formed within the chondral defect at the early follow-up time point [59]. Chondroitin sulfate, diacerein, GS, and HA demonstrated pain reduction and physical function improvement with very low toxicity in OA treatment, and could be of potential interest for the symptomatic management of OA [60]. Slowing-acting drugs avoid interrupting the normal cellular function and toxicity to cells, thus could provide an anti-inflammatory strategy for cartilage repair (Fig. 2). However, high concentrations of chondroprotective drugs are not presumably attained *via* oral administration; intra-articular injections may represent a feasible and effective approach [61]. With the application of tissue engineering in cartilage repair, slow-acting drugs serve as a basic unit of a 3D scaffold and, together with stem cells, demonstrated the potential of modulating inflammatory chemokines/receptors and catabolic/inhibiting factors [62].

Small molecules targeting inflammation signals

Strategies for generating functional small molecules of synthetic and natural products targeting inflammatory signals have been considered effective in the suppression of inflammation [63]. Primary pro-inflammatory factors IL-1 β and TNF α have the capacity to activate a diverse array of intracellular signaling pathways; upon activation they can induce phosphorylation-dependent signaling pathways, such as NF- κ B, p38 MAPK, and c-Jun N-terminal kinase (JNK), which regulate the synthesis of several inflammatory cytokines and MMPs, many of which play major roles in the process of OA formation (Fig. 2) [64].

The NF- κ B signaling pathway mediates critical events in the inflammatory response by chondrocytes, leading to progressive ECM damage and cartilage destruction. There are an increasing number of reports about NF- κ B inhibitors, such as glucocorticoids, cyclosporine A, and tacrolimus (FK-506) [65]. I κ B kinase beta (IKK β) has also become a particularly appealing target because of its crucial role in the activation of the NF- κ B pathway [66]. A recent report showed that a novel butanoylated

GlcNAc derivative, 3,4,6-O-Bu3GlcNAc, an inhibitor of NF- κ B activity, has the potential to stimulate new tissue production and reduce inflammation in IL-1 β -induced chondrocytes with utility for OA and other forms of inflammatory arthritis [67]. One concern about inhibiting several of these components of the NF- κ B pathway is the specificity of such drugs. For example, the proteasome which is responsible for I κ B degradation has many other important functions. Thus, inhibition of proteasome activity could potentially cause severe side effects. Also it may not be feasible to block the NF- κ B pathway for prolonged periods, since NF- κ B plays an important role in the maintenance of host defense responses [68].

Two MAPKs, p38 MAPK and JNK, are frequently activated by a wide range of environmental stresses and cytokines to induce inflammation and joint destruction, hence the name stress-activated protein kinases (SAPKs). p38 MAPK positively regulates the expression of many genes involved in inflammation, such as those coding for TNF α , IL-1 β , IL-6, IL-8, COX-2, and collagenase-1 and -3 [69]. Many small molecule inhibitors of p38 MAPK such as Cannabidiol [70], SB203580, Doramapimod (BIRB-796) [71], VX-702 [72], RO-3201195 [73], and SB-242235 [74] have demonstrated positive anti-inflammatory effects. Among all these p38 MAPK inhibitors currently in clinical trials, SB203580 appears to be the most potent compound in terms of anti-inflammatory activity. For instance, inhibition of the p38 MAPK signaling pathway with SB203580 showed anti-inflammatory effects in both cartilage explants [75] and animal models [76]. However, there is a conflicting report showing that inhibition of the p38 MAPK pathway using SB203580 leads to OA-like changes in a rat animal model [77]. Similarly, the JNKs are activated in macrophages after stimulation with lipopolysaccharide [78]. Inhibition of the JNK signaling pathway has shown preventive effects with regard to bone and cartilage destruction in rheumatoid arthritis (RA) [79] and down-regulation of IL-1-induced MMP-13 expression in OA chondrocytes [80].

Small molecules targeting specific enzymes

Small molecules which target specific enzymes involved in OA have been developed as a possible way to alleviate inflammation in cartilage repair (Fig. 2). MMPs are synthesized and secreted by chondrocytes in response to stimulants such as IL-1 and TNF [81]. Inappropriate expression of MMP activity constitutes part of the pathogenic mechanism associated with the destruction of cartilage and bone in OA. Possible strategies include impeding the production of MMPs, blocking the active site of

MMPs, and increasing endogenous production of tissue inhibitors of metalloproteinases (TIMPs) [82]. Another representative member of cyclooxygenase, the COX-2 enzyme is primarily associated with inflammation. Cytokines and growth factors increase the expression of COX-2, mainly at inflammatory sites, producing prostaglandins that mediate inflammation, pain, and fever [83]. Targeting cyclooxygenase, non-steroidal anti-inflammatory drugs (NSAIDs) such as celecoxib play a major role in the management of inflammation and pain caused by arthritis [84, 85]. However, non-selective NSAIDs cause gastrointestinal complications in a significant number of patients, and COX-2 inhibitors have recently raised concerns regarding cardiovascular side effects/risks [86].

The possible roles of NO in OA pathophysiology have been supported by a study showing that selective inhibition of the iNOS could reduce the progression of structural changes in experimental OA in dogs, partly related to a reduction in the levels of synovial inflammation [81]. Many of these small molecule compounds have already been launched on the market as drugs acting against inflammatory conditions. For example, leflunomide was found to significantly inhibit IL-4 and IL-13-enhanced production of chemokine (C-C motif) ligand (CCL)-26 [88].

Biomechanical Strategy

Biomechanical factors play an important role in the health of diarthrodial joints. Under normal physiological loading, because articular cartilage provides a nearly frictionless surface for the transmission and distribution of joint loads, moderate mechanical loading exhibits little or no wear over decades of use. In reality, moderate loading is necessary to maintain healthy articular cartilage. Through a variety of mechanisms, including ion channels and integrin-mediated connections to the ECM that involves membrane, cytoskeletal, and intracellular deformation, chondrocytes in cartilage may perceive physical signals from the outside environment. Altered joint loading, which might be associated with obesity, malalignment, trauma, or joint instability, is a critical risk factor for joint degeneration and OA occurrence.

Biomechanical influences on cartilage are complex. Depending on the way cartilage is loaded, the effects on cartilage homeostasis can vary. An overload of mechanical stress may lead to acute joint injury such as posttraumatic OA (PTOA), which is also caused by gradual onset of structural damage

and cartilage compositional degradation due to chronic overloading of injured joints [89]. Cartilage explants subjected to static compression exhibit a significant suppression of metabolic activity that is dependent on the magnitude of applied stress [90]. High magnitude mechanical strain is pro-inflammatory and initiates cartilage destruction while inhibiting matrix synthesis, both of which are involved in the NF- κ B-related signal pathway [91, 92].

It has been reported that low magnitude mechanical strain inhibits inflammation by suppressing IL-1 β and TNF α -induced transcription of multiple pro-inflammatory mediators involved in cartilage degradation [91]. It is noteworthy, however, that the influences of mechanical stress are not independent; many mechanical and physiochemical factors that are known to affect chondrocytes are inextricably coupled to one another within the cartilage ECM. Ramachandran *et al.* reported that treatment with C-type natriuretic peptide (CNP) and dynamic compression increased anabolic activities and blocked catabolic effects induced by IL-1 β [93]. For cyclic loading, however, the cartilage reaction is different; low frequencies and amplitudes do not appear to affect biosynthesis rates. Some studies suggested that, above a certain threshold frequency, cyclic loading increases the synthesis of components of ECM, such as aggrecans, cartilage oligomeric matrix protein, and fibronectin [90].

There is increasing evidence showing that muscle function is closely related to OA. Muscle, lying anatomically adjacent to cartilage, provides the cartilage with a biomechanical stimulation that promotes nutrient distribution and maintains homeostasis [92]. Muscle loss [94] and reduced muscle strength [95] have been shown to be risk factors for knee OA. On the other hand, patients with knee OA were found to have impaired muscle function; quadriceps, hamstring, and hip muscles are significantly impaired in subjects with knee OA compared with age-matched controls [96]. Recent studies showed that a rat chondrocyte cell line co-cultured with muscle cells or cultured in muscle cell-conditioned medium in a monolayer demonstrated enhanced resistance to pro-inflammatory factors such as IL-1 β and TNF α , suggesting that non-loading biochemical effects of muscle cells have a significant influence on cartilage homeostasis and a preventative role in OA formation [97]. Mechanistically, it is known that the pro-inflammatory cytokines IL-1 β and TNF α are major inflammation initiators in cartilage by inducing the expression of MMPs, promoting chondrocyte hypertrophy, and reducing the synthesis of cartilage matrix genes. However, muscle cell-derived factors or myokines inhibited the mRNA expression of MMPs as well as hypertrophic markers in

bioengineered cartilage; it could also rescue IL-1 β -induced chondrocyte growth arrest through regulating the cell cycle. The beneficial role of muscle cells on OA indicated muscle strengthening exercises could be a potential intervention for OA [98].

Mesenchymal stem cell strategy

Inherent anti-inflammatory properties

Despite being the most advanced and promising approach for cartilage repair, chondrocyte-based cartilage repair has some disadvantages, such as morbidity caused by damage to the donor-site articular surface and cell senescence during *ex vivo* expansion [4]. Furthermore, the inflammatory synovial fluid microenvironment triggers human chondrocytes to actively take part in inflammatory processes, particularly during the initiation and progression of inflammatory joint diseases and in the disruption of cartilage repair mechanisms resulting in cartilage degradation [99]. Unlike chondrocytes, MSCs are not limited by an intrinsic tendency to lose their phenotype and dedifferentiate during expansion [100]. MSCs also play an important role in immunomodulation and tissue regeneration by secretion of soluble factors [101, 102]. In an inflammatory environment, MSCs secrete factors which cause multiple anti-inflammatory effects and influence matrix turnover in synovium and cartilage explants [103]. MSCs have also been revealed as robust sources of TIMP-mediated MMP-inhibition, capable of protecting the perivascular niche from high levels of destructive MMPs, even under pathological conditions [104].

Due to their potential to modulate the local microenvironment *via* anti-inflammatory and immunosuppressive functions, MSCs have an additional advantage for allogeneic application (Fig. 3). Moreover, by secreting various bioactive soluble factors, MSCs can protect the cartilage from further tissue destruction and facilitate regeneration of the remaining progenitor cells *in situ* [105]. The joint resurfacing function of MSCs was also demonstrated in *in vivo* studies. After intra-articular injection of autologous MSCs into a caprine OA model (complete excision of the medial meniscus and resection of the anterior cruciate ligament), there was evidence of marked regeneration of the medial meniscus and implanted cells were detected in the newly formed tissue. Degeneration of the articular cartilage, osteophytic remodeling, and subchondral sclerosis were reduced in cell-treated joints

compared with joints treated with vehicle alone without cells [106]. A clinical case report demonstrated that, 24 weeks after the injection of autologous MSCs into a knee with symptomatic and radiographic degenerative joint disease, there was significant cartilage growth, decreased pain, and increased joint mobility in the patient [107]. As a result, MSCs could be used in cartilage repair as a potential anti-inflammatory strategy especially in the context of allogeneic transplantation.

Acquired anti-inflammatory capacity

Treatment strategies focused on both reducing inflammation and increasing tissue production are necessary to effectively treat OA from a tissue-engineering perspective. Despite their multi-lineage differentiation potential and immunomodulatory properties as well as anti-inflammatory abilities, MSCs' survival after transplantation is still very low, thereby hindering their therapeutic efficacy [108].

Recent studies found that MSCs could be rejuvenated by preconditioning strategies which enhance their post-transplantation survival and functionality [109]. The concept of preconditioning was established in 1986 by Murry and colleagues, who found ischemic preconditioning of cardiomyocytes led to the activation of survival signaling [110]. In theory, any factors that might influence the proliferation and differentiation of therapeutic cells can be an advantage. For instance, hypoxic preconditioning not only has pro-survival and cytoprotective effects, it also helps the cells maintain their stemness and promote proliferation and differentiation potential post-engraftment [111]. When referring to a cartilage engineering application, preconditioning of MSCs can be derived from microenvironment factors such as low oxygen [112], growth factors [113], or potentially pharmacological substances targeting specific signal pathways involved in MSC chondrogenesis such as the MAPK [114, 115] and Wnt signal pathways [114, 116].

DSCM-mediated stem cell preconditioning

The stem cell niche is a specialized microenvironment that helps sustain the stem cell pool within each tissue or organ system. It is hypothesized that stem cells can create their own microenvironment; in such a microenvironment, adult stem cells are expected to greatly expand while retaining their

stemness for a tissue-specific lineage. In 2009, He *et al.* utilized porcine SDSCs, a tissue-specific stem cell for chondrogenesis [117], as a model to reconstruct an *in vitro* 3D stem cell microenvironment, in which expanded SDSCs produced a drastic increase in cell number and chondrogenic potential [118]. Later on, this DSCM-mediated stem cell preconditioning was also demonstrated to be effective in rejuvenating human bone marrow stromal cells, in terms of enhanced proliferation and chondrogenic hypertrophy [119]. Interestingly, this DSCM-mediated expansion system also works for primary chondrocytes, such as articular chondrocytes [120, 121] and nucleus pulposus cells [122, 123].

The regulation of intracellular reactive oxygen species (ROS) is crucial for cell survival in a harsh environment and guarantees successful cell therapy [124]. DSCM preconditioning could decrease expanded stem cell ROS level [119, 125], and protect human SDSCs from oxidative stress-induced cell senescence, as it applies to cell proliferation and differentiation capacity [12]. Furthermore, our recent data showed that human SDSCs expanded on DSCM exhibited an increased chondrogenic potential as well as heightened protection against IL-1 β -induced inflammation [126]. The adaptive capacity in a harsh environment was also validated in a minipig study, in which DSCM-expanded SDSCs exhibited an enhanced *in vivo* cartilage regeneration capacity post-injection, evidenced by intensely stained type II collagen and sulfated GAGs in the partial-thickness cartilage defects with negligible staining of type I collagen [13], indicating that DSCM preconditioned SDSCs have the ability to resist inflammation. In contrast, the existence of type I collagen in the regenerated cartilage from the plastic flask-expanded SDSC groups suggested fibrocartilage formation [13], which might be explained by dedifferentiation caused by inflammatory stress [127].

Low oxygen and FGF-2 contribute to DSCM-mediated stem cell preconditioning

Low oxygen tension (hypoxia) maintains undifferentiated states of MSC phenotypes and also influences proliferation and cell-fate commitment [128]. There is increasing evidence suggesting that hypoxia (or, more appropriately, physiological hypoxia) can stimulate chondrogenesis [129, 130]. Hypoxic preconditioning can boost the expression of genes favoring MSC proliferation and growth prolongation by inducing the expression of pro-survival and pro-angiogenic markers in MSCs [131].

Recently, hypoxia-inducible factors (HIFs) have been shown to activate specific signaling pathways such as Notch and the expression of transcription factors such as octamer-binding transcription factor 4 (Oct4) which is responsible for controlling stem cell self-renewal and multipotency [132]. This finding suggests that modulation of oxygen availability and HIF expression can influence stem cell fate. Our recent report suggested that the combination of hypoxia and FGF-2 significantly enhanced DSCM-expanded SDSC proliferation and chondrogenic potential [133]. This micro-ecosystem apparently reconciled the contradiction of “Quantity” versus “Quality”, a predicament often met in stem cell-based cartilage repair. *In vivo*, numerous growth factors and morphogens are immobilized by directly binding to the ECM through specific heparin-binding domains, by direct binding to ECM molecules such as collagen, or by direct anchoring to cell membranes [134]. By immobilizing growth factors in a concentrated area, DSCM helps to amplify the effects of growth factors and reduce the inflammation reaction leading to improved proliferation and differentiation of stem cells [102].

Small molecules targeting inflammation signals contribute to DSCM-mediated stem cell preconditioning

Widely used as an anti-inflammatory drug in cartilage repair, p38 MAPK inhibitor also blocks chondrogenesis [135], indicating that p38 MAPK inhibitor has some disadvantages in the treatment of cartilage damage with concomitant inflammation. Considering its adverse effects for clinical treatment of OA, MSCs pretreated by SB203580 in the DSCM expansion phase were assumed to avoid this dilemma. Preconditioning using SB203580 significantly enhanced DSCM-expanded human SDSC chondrogenic potential; this rejuvenation of chondrogenic capacity, instilled in SDSCs by SB203580, gave the stem cell the ability to resist the influence of IL-1 β -induced inflammation [136].

Extracellular matrix strategy

Compared to natural biomaterials, synthetic polymers are more controllable and predictable in their chemical and physical properties, holding some promise of success in tissue engineering and

regenerative medicine. Of the synthetic polymers, those derived from poly(α -hydroxy esters),¹³⁶ poly(propylene fumarates) [137], polyurethanes [138], PGA [139], and poly(lactide-co-glycolide) [140] have been used for cartilage regeneration; however, inflammatory reaction due to synthetic scaffolds that impaired the quality of regenerated cartilage has also been reported in some studies [141, 142].

Natural matrix scaffolds

Naturally occurring polymers as scaffolds offer options for cartilage tissue engineering due to biocompatibility, biodegradability, low toxicity of degradation byproducts, and plasticity in processing into a variety of material formats [143]. In an attempt to repair articular cartilage, allografted articular chondrocytes, embedded in collagen gel, were transplanted into full-thickness defects in rabbit articular cartilage; 24 weeks after implantation, the defects were filled with hyaline cartilage, synthesized type II collagen, and exhibited no signs of immunologic rejection and degeneration of the reparative tissue [144, 145]. Rahfoth *et al.* found that the transplantation of allograft chondrocytes embedded in agarose gel was a suitable method to repair articular cartilage defects in rabbits because there were no signs of graft-versus-host rejection or infiltration by immune cells [146]. Other natural polymers that have been explored as bioactive scaffolds for cartilage tissue engineering include but are not limited to silk [147], hyaluronan [148], and chitosan [149]. In a recent study, the responses of articular chondrocytes under inflammatory conditions were compared after seeding within three polymeric scaffolding materials [silk, collagen, and polylactic acid (PLA)]; Kwon *et al.* found that chondrocytes grown in the silk and collagen scaffolds exhibited higher levels of cartilage matrix gene expression than those in the PLA scaffolds [150]. When using a PGA scaffold for treatment of full-thickness defects, however, there appears to be a moderate immunoreaction as evidenced by lymphocytes in transplanted joints [9, 151].

Chondrocyte sheets

A cell-sheet technique developed in 1993 [152] shows promise in cartilage repair with concomitant inflammation. Hamahashi *et al.* reported that a layered chondrocyte sheet produced the most humoral factors including PGE₂, which plays a key role in its anti-inflammatory function [153]. In chondrocyte sheets, catabolic factors such as MMP3, MMP13, and a disintegrin and metalloproteinase

with thrombospondin motifs 5 (ADAMTS5) were also observed to decrease while the expression of TIMP1 with antagonistic actions against MMP3 increased [154]. In a rabbit model with partial-thickness cartilage defects, layered chondrocyte sheets were able to maintain the cartilaginous phenotype, and could be attached to the site of cartilage damage, acting as a barrier to prevent a loss of proteoglycan from these sites and to protect them from catabolic factors in the joint [154]. This technique might have great potential for OA treatment and inflammatory prevention in cartilage repair.

Decellularized tissue matrix

In contrast to the DSCM described above, which attempts to rejuvenate expanded stem cells' chondrogenic potential as well as anti-inflammatory capacity, decellularized tissue matrix influences tissue engineering directly, providing a carrier (or scaffold) and chondrogenically induced growth factors as well as an anti-inflammatory function. The use of ECM derived from decellularized tissue is increasingly common in regenerative medicine and tissue engineering [155]. By virtue of physical, chemical, or enzymatic approaches, organ decellularization removes all cellular material without adversely affecting the composition, biologic activity, or mechanical integrity of the remaining 3D matrix [156]. The molecules that constitute ECM are largely and highly conserved across species and are well tolerated even by xenogeneic recipients. The effects of xenogeneic ECM on the innate immune response, specifically the responding macrophages, may elicit a necessary M2 phenotypic profile to support a constructive remodeling response for the scaffold [157]. In the field of cartilage regeneration, many decellularized tissue scaffolds showed positive effects and reparative capability for cartilage defects [158]. For example, the combined use of cells with decellularized aortic scaffold was able to prevent the generation of a strong inflammatory response and improve overall tracheal cartilage regeneration [159]. Compared to a PGA scaffold, an ECM scaffold derived from porcine cartilage not only strongly supported chondrogenic differentiation of rabbit MSCs, but also helped maintain its phenotype *in vivo* [160].

Summary

The treatment of cartilage injury remains a clinical challenge despite the advancements in surgical procedures and techniques. An important hurdle is the concomitant inflammation during cartilage repair. Pharmacological drugs have been successfully applied in cartilage repair; however, they cannot optimally work alone [161]. With the development of ECM-based scaffolds and preconditioned tissue-specific stem cells, both tissue engineering components could not only contribute to an enhanced ability in anti-inflammation but also to cartilage regeneration. All of these strategies could combine to boost cartilage repair under inflammatory conditions.

Figure1. 1 Inflammation in cartilage repair.

Abnormally high contact stresses such as mechanical overload transmitted to focal areas of articular cartilage result in cartilage defects and release cartilage fragments. This process stimulates the synovial membrane, leading to the activation of macrophages and inflammatory cells such as T cells, which produce interleukin-1 β (IL-1 β) and tumor necrosis factor alpha (TNF α). In an “autocrine-paracrine” manner, these activated inflammatory factors may stimulate chondrocytes to secrete degradative enzymes like proteinases, such as matrix metalloproteinases (MMPs) and a disintegrin and metalloprotease with thrombospondin motifs (ADAMTS), which are directly involved in degradation of type II collagen and aggrecans in cartilage matrix. In the meantime, chondrocytes can change phenotype and size in response to stimulation from inflammatory factors and undergo hypertrophy, which is an essential step in the endochondral ossification process.

Figure1. 2 Schematic representation of key signaling pathways in the inflammatory process and potential strategies for inflammatory inhibition in cartilage repair.

Use of anti-cytokine and chondroprotective drugs against IL-1, TNFα, or against their receptors is a direct and effective approach in the suppression of inflammation. Strategies targeting disturbance or intervention of key signal pathways in cytosol have been developed such as blocking the MAPK signal pathways or the NF-κB pathway. Using inhibitors for downstream products of degradative enzymes, such as MMPs, COX-2, iNOS, and ADAMTs, can also effectively protect cartilage from degradation and inflammation. Growth factors such as FGF-2, IGF-I, or TGF-β may interfere with inflammation by blocking the associated signal pathways. Use of growth factors is a possible anti-inflammatory strategy in cartilage repair despite the fact that growth factors can promote neo-cartilage formation.

Figure1. 3 Therapeutic strategies for cartilage damage utilizing preconditioned stem cell and three-dimensional matrix.

After *ex vivo* expansion of adult stem cells extracted from the human body, two strategies can be used for the treatment of cartilage defects: direct intra-articular injection or transplant of cells in a three-dimensional (3D) scaffold. For full-thickness cartilage defects, the second strategy is normally used. 3D scaffolds can be made of ECM materials. A structurally and mechanically stable scaffold allows for infiltration and attachment of bioactive molecules (IGF-I, FGF-2, and/or TGF- β) which can exert their anti-inflammatory effects on the surrounding environment. For partial thickness cartilage defects, direct intra-articular injection of expanded stem cells is an option. Mesenchymal stem cells can secrete growth factors, which may exert their anti-inflammatory and anti-oxidative effects in the microenvironment where MSCs reside. These growth factors also benefit tissue regeneration. A recent finding suggests that decellularized stem cell matrix (DSCM) could rejuvenate expanded MSCs in proliferation and chondrogenic potential; this process may provide a large quantity of high quality MSCs for cell-based cartilage regeneration. DSCM-expanded MSCs exhibiting an enhanced capacity against oxidation and inflammation may represent a promising anti-inflammatory strategy in the near future.

References

1. Bitton R. *Am J Manag Care* 2009;**15**:S230.
2. Hangody L and Füles P. *J Bone Joint Surg Am* 2003;**85-A suppl 2**:25.
3. Knutsen G, Engebretsen L, Ludvigsen TC et al. *J Bone Joint Surg Am* 2004;**86-A**:455.
4. Li JT, and Pei M. *Tissue Eng Part B* 2012;**18**:270.
5. Steinert AF, Ghivizzani SC, Rethwilm A et al. *Arthritis Res Ther* 2007;**9**:213.
6. Buckwalter JA, Martin JA, and Brown TD. *Biorheology* 2006;**43**:603.
7. Heijink A, Gomoll AH, Madry H et al. *Knee Surg Sports Traumatol Arthrosc* 2012;**20**:423.
8. Sokolove J, and Lepus CM. *Ther Adv Musculoskelet Dis* 2013;**5**:77.
9. Pei M, He F, Boyce BM et al. *Osteoarthritis Cartilage* 2009;**17**:714.
10. Pei M, He F, Kish V et al. *Clin Orthop Relat Res* 2008;**466**:1880.
11. Pei M, Li JT, Shoukry M. et al. *Eur Cell Mater* 2011;**22**:333.
12. Pei M, Zhang Y, Li JT et al. *Stem Cells Dev* 2013;**22**:889.
13. Pei M, He F, Li J et al. *Tissue Eng Part A* 2013;**19**:1144.
14. Goldring MB, Tsuchimochi K, and Ijiri K. *J Cell Biochem* 2006;**97**:33.
15. Alves H, Munoz-Najar U, De Wit J et al. *J Cell Mol Med* 2010;**14**:2729.
16. Goldring MB. *Curr Rheumatol Rep* 2000;**2**:459.
17. Montaseri A, Busch F, Mobasheri A et al. *PLoS One* 2011;**6**:e28663.
18. Tang W, Lu Y, Tian QY et al. *Science* 2011;**332**:478.
19. Feng JQ, Guo FJ, Jiang BC et al. *FASEB J* 2010;**24**:1879.
20. Pufe T, Harde V, Petersen W et al. *J Pathol* 2004;**202**:367.
21. Ashraf S, and Walsh DA. *Curr Opin Rheumatol* 2008;**20**:573.
22. Andia I, and Maffulli N. *Nat Rev Rheumatol* 2013;**9**:721.
23. van Buul GM, Koevoet WL, Kops N et al. *Am J Sports Med* 2011;**39**:2362.
24. Bendinelli P, Matteucci E, Dogliotti G et al. *J Cell Physiol* 2010;**225**:757.
25. Pereira RC, Scaranari M, Benelli R et al. *Tissue Eng Part A* 2013;**19**:1476.
26. Lippross S, Moeller B, Haas H et al. *Arthritis Rheum* 2011;**63**:3344.
27. Smyth NA, Haleem AM, Murawski CD et al. *J Bone Joint Surg Am* 2013;**95**:2185.
28. Siclari A, Mascaro G, Gentili C et al. *Clin Orthop Relat Res* 2012;**470**:910.
29. Mierisch CM, Cohen SB, Jordan LC et al. *Arthroscopy* 2002;**18**:892.
30. van der Kraan PM, and van den Berg WB. *Osteoarthritis Cartilage* 2007;**15**:237.
31. Malesmud CJ. *BioDrugs* 2004;**18**:23.
32. Pelletier JP, Martel-Pelletier J, and Raynauld JP. *Arthritis Res Ther* 2006;**8**:206.
33. Pelletier JP, Martel-Pelletier J, and Abramson SB. *Arthritis Rheum* 2001;**44**:1237.
34. Leeb BF. *European Musculoskeletal Review* 2010;**5**:23.
35. Martel-Pelletier J, Mineau F, Jolicoeur FC et al. *J Rheumatol* 1998;**25**:753.
36. Scott DL, and Kingsley GH. *N Engl J Med* 2006;**355**:704.
37. Linn MS, Chase DC, Healey RM et al. *Am J Sports Med* 2011;**39**:1494.
38. Kawaguchi A, Nakaya H, Okabe T et al. *Acta Orthop* 2009;**80**:606.
39. Shealy DJ, Wooley PH, Emmell E et al. *Arthritis Res* 2002;**4**:R7.
40. Guerne PA, Carson DA, and Lotz M. *J Immunol* 1990;**144**:499.
41. Villiger PM, Geng Y, and Lotz M. *J Clin Invest* 1993;**91**:1575.

42. Recklies AD, and Golds EE. *Arthritis Rheum* 1992;**35**:1510.
43. Henrotin YE, De Groote DD, Labasse AH et al. *Osteoarthritis Cartilage* 1996;**4**:163.
44. Martel-Pelletier J, Alaaeddine N, and Pelletier JP. *Frontiers in Bioscience* 1999;**4**:694.
45. Alaaeddine N, Di Battista JA, Pelletier JP et al. *Cytokine* 1999;**11**:1020.
46. Lubberts E, Joosten LA, Helsen MM et al. *Cytokine* 1998;**10**:361.
47. Lubberts E, Joosten LA, van Den Bersselaar L et al. *J Immunol* 1999;**163**:4546.
48. Gouze JN, Bianchi A, Becuwe P et al. *FEBS Lett* 2002;**510**:166.
49. Shikhman AR, Kuhn K, Alaaeddine N et al. *J Immunol* 2001;**166**:5155.
50. Hochberg M, Chevalier X, Henrotin Y et al. *Curr Med Res Opin* 2013;**29**:259.
51. Martel-Pelletier J, Kwan Tat S, and Pelletier JP. *Osteoarthritis Cartilage* 2010;**18 Suppl 1**:S7.
52. Volpi N. *Inflammopharmacology* 2011;**19**:299.
53. du Souich P, García AG, Vergés J et al. *J Cell Mol Med* 2009;**13**:1451.
54. Kunze R, Hempel U, and Dieter P. *Prostaglandins Other Lipid Mediat* 2010;**92**:8.
55. Kajahn J, Franz S, Rueckert E et al. *Biomatter* 2012;**2**:226.
56. Chang NS, Intrieri C, Mattison J. et al. *J Leukoc Biol* 1994;**55**:778.
57. Tammi M, Day AJ, and Turley EA. *J Biol Chem* 2002;**277**:4581.
58. Moreland LW. *Arthritis Res Ther* 2003;**5**:54.
59. Strauss E, Schachter A, Frenkel S et al. *Am J Sports Med* 2009;**37**:720.
60. Bruyère O, Burlet N, Delmas PD et al. *BMC Musculoskelet Disord* 2008;**9**:165.
61. Lotz MK, and Kraus VB. *Arthritis Res Ther* 2010;**12**:211.
62. Guo Y, Yuan T, Xiao Z et al. *J Mater Sci Mater Med* 2012;**23**:2267.
63. Ivanenkov YA, Balakin KV and Tkachenko SE. *Drugs R D* 2008;**9**:397.
64. Burrage PS, Mix KS, and Brinckerhoff CE. *Front Biosci* 2006;**11**:529.
65. Lee JI, and Burckart GJ. *J Clin Pharmacol* 1998;**38**:981.
66. Roman-Blas JA, and Jimenez SA. *Osteoarthritis Cartilage* 2006;**14**:839.
67. Coburn JM, Wo L, Bernstein N et al. *Tissue Eng Part A* 2013;**19**:2035.
68. Yamamoto Y, and Gaynor RB. *J Clin Invest* 2001;**107**:135.
69. Kyriakis JM, and Avruch J. *Physiol Rev* 2001;**81**:807.
70. El-Remessy AB, Al-Shabrawey M, Khalifa Y et al. *Am J Pathol* 2006;**168**:235.
71. Kuma Y, Sabio G, Bain J et al. *J Biol Chem* 2005;**280**:19472.
72. Ding C. *Curr Opin Investig Drugs* 2006;**7**:1020.
73. Goldstein DM, Alfredson T, Bertrand J. et al. *J Med Chem* 2006;**49**:1562.
74. Ward KW, Proksch JW, Salyers KL et al. *Xenobiotica* 2002;**32**:221.
75. Ridley SH, Sarsfield SJ, Lee JC et al. *J Immunol* 1997;**158**:3165.
76. Brown KK, Heitmeyer SA, Hookfin EB et al. *J Inflamm (Lond)* 2008;**5**:22.
77. Prasadam I, Mao X, Wang Y et al. *Rheumatology (Oxford)* 2012;**51**:813.
78. Chan ED, Winston BW, Jarpe MB et al. *Proc Natl Acad Sci USA* 1997;**94**:13169.
79. Bogoyevitch MA, Boehm I, Oakley A et al. *Biochim Biophys Acta* 2004;**1697**: 89.
80. Ahmed S, Rahman A, Hasnain A et al. *J Pharmacol Exp Ther* 2003;**305**:981.
81. Tanaka, S, Hamanishi C, Kikuchi H et al. *Semin Arthritis Rheum* 1998;**27**:392.
82. Jackson C, Nguyen M, Arkell J et al. *Inflamm Res* 2001;**50**:183.
83. Crofford LJ. *J Rheumatol* 1997;**24**:15.
84. Jiang D, Zou J, Huang L et al. *Int J Mol Sci* 2010;**11**:4106.
85. Noble SL, King DS, and Olutade JI. *Am Fam Physician* 2000;**61**:3669.

86. Funk CD, and FitzGerald GA. *J Cardiovasc Pharmacol* 2007;**50**:470.
87. Pelletier J-P, Jovanovic D, Fernandes JC et al. *Arthritis Rheum* 1998;**41**:1275.
88. Kagami S, Saeki H, Komine M et al. *Clin Exp Immunol* 2005;**141**:459.
89. Buckwalter JA, Anderson DD, Brown TD et al. *Cartilage* 2013.
90. Guilak F, Sah RL, and Setton LA. 2nd ed Lippincott-Raven; Philadelphia: 1997.
91. Deschner J, Hofman CR, Piesco NP et al. *Curr Opin Clin Nutr Metab Care* 2003;**6**:289.
92. Knobloch TJ, Madhavan S, Nam J et al. *Crit Rev Eukaryot Gene Expr* 2008;**18**:139.
93. Ramachandran M, Achan P, Salter DM et al. *Arthritis Res Ther* 2011;**13**:R145.
94. Scott D, Blizzard L, Fell J et al. *Arthritis Care Res* 2012;**64**:30.
95. Slemenda C, Heilman DK, Brandt KD et al. *Arthritis Rheum* 1998;**41**:1951.
96. Alnahdi AH, Zeni JA, and Snyder-Mackler L. *Sports Health* 2012;**4**:284.
97. Rainbow RS, Kwon H, Foote AT et al. *Osteoarthritis Cartilage* 2013;**21**:990.
98. Roos EM, Herzog W, Block JA et al. *Nat Rev Rheumatol* 2011;**7**:57.
99. Röhner E, Matziolis G, Perka C et al. *Immunol Res* 2012;**52**:169.
100. Chen FH, Rousche KT, and Tuan RS. *Nat Clin Pract Rheumatol* 2006;**2**:373.
101. Caplan AI, and Dennis JE. *J Cell Biochem* 2006;**98**:1076.
102. Pei M, Li JT, Zhang Y et al. *Cell Tissue Res* 2014;**356**:391-403.
103. van Buul GM, Villafuertes E, Bos PK et al. *Osteoarthritis Cartilage* 2012;**20**:1186.
104. Lozito TP, and Tuan RS. *J Cell Physiol* 2011;**226**:385.
105. Gupta PK, Das AK, Chullikana A et al. *Stem Cell Res Ther* 2012;**3**:25.
106. Murphy JM, Fink DJ, Hunziker EB et al. *Arthritis Rheum* 2003;**48**:3464.
107. Centeno CJ, Busse D, Kisiday J et al. *Pain Physician* 2008;**11**:343.
108. Toma C, Wagner WR, Bowry S et al. *Circ Res* 2009;**104**:398.
109. Yu SP, Wei Z, and Wei L. *Transl Stroke Res* 2013;**4**:76.
110. Murry CE, Jennings RB, and Reimer KA. *Circulation* 1986;**74**:1124.
111. Rosová I, Dao M, Capoccia B et al. *Stem Cells* 2008;**26**:2173.
112. Portron S, Merceron C, Gauthier O et al. *PLoS One* 2013;**8**:e62368.
113. Holland TA, Bodde EW, Cuijpers VM et al. *Osteoarthritis Cartilage* 2007;**15**:187.
114. Li J, Hansen KC, Zhang Y et al. *Biomaterials* 2014;**35**:642.
115. Stanton LA, Underhill TM., and Beier F. *Dev Biol* 2003;**263**:165.
116. Im GI, and Quan Z. *Tissue Eng Part A* 2011;**16**:2405.
117. Jones B, and Pei M. *Tissue Eng Part B Rev* 2012;**18**:301.
118. He F, Chen XD, and Pei M. *Tissue Eng Part A* 2009;**15**:3809.
119. Pei M, He F, and Kish VL. *Tissue Eng Part A* 2011;**17**:3067.
120. Pei M, He F, and Wei L. *J Tissue Sci Eng* 2011;**2**:104.
121. Pei M, and He F. *J Cell Physiol* 2012;**227**:2163.
122. He F, and Pei M. *Spine* 2012;**37**:459.
123. Pei M, Shoukry M, Li JT et al. *Spine* 2012;**37**:1538.
124. Dhalla NS, Golfman L, Takeda S et al. *Can J Cardiol* 1999;**15**:587.
125. He F, and Pei M. *J Tissue Eng Regen Med* 2013;**7**:73.
126. Zhang Y, Li JT, and Pei M. *Trans ORS* 2013;**38**:470.
127. Gelse K, Mühle C, Franke O et al. *Arthritis Rheum* 2008;**58**:475.
128. Mohyeldin A, Garzón-Muvdi T, and Quiñones-Hinojosa A. *Cell Stem Cell* 2010;**7**:150.
129. Lafont JE. *Int J Exp Pathol* 2010;**91**:99.

130. Li JT, He F, and Pei M. *Cell Tissue Res* 2011;**345**:357.
131. Chacko SM, Ahmed S, Selvendiran K et al. *Am J Physiol Cell Physiol* 2010;**299**:1562.
132. Pistollato F, Rampazzo E, Persano L et al. *Stem Cells* 2010;**28**:1918.
133. Li JT, and Pei M. *Tissue Eng Part A* 2011;**17**:703.
134. Rider CC. *Biochem Soc Trans* 2006;**34**:458.
135. Oh CD, Chang SH, Yoon YM et al. *J Biol Chem* 2000;**275**:5613.
136. Li WJ, Cooper JA Jr Mauck RL et al. *Acta Biomater* 2006;**2**:377.
137. Liao E, Yaszemski M, Krebsbach P et al. *Tissue Eng* 2007;**13**:537.
138. Werkmeister JA, Adhikari R, White JF et al. *Acta Biomater* 2010;**6**:3471.
139. Pei M, Seidel J, Vunjak-Novakovic G et al. *Biochem Biophys Res Commun* 2002;**294**:149.
140. Sittinger M, Reitzel D, Dauner M et al. *J Biomed Mater Res* 1996;**33**:57.
141. Britt JC, and Park SS. *Arch Otolaryngol Head Neck Surg* 1998;**124**:671.
142. Cao Y, Rodriguez A, Vacanti M et al. *J Biomater Sci Polym Ed* 1998;**9**:475.
143. Velema J, and Kaplan D. *Adv Biochem Eng Biotechnol* 2006;**102**:187.
144. Masuoka K, Asazuma T, Ishihara M et al. *J Biomed Mater Res B Appl Biomater* 2005;**75**:177.
145. Wakitani S, Kimura T, Hirooka A et al. *J Bone Joint Surg Br* 1989;**71**:74.
146. Rahfoth B, Weisser J, Sternkopf F et al. *Osteoarthritis Cartilage* 1998;**6**:50.
147. Altman GH, Diaz F, Jakuba C et al. *Biomaterials* 2003;**24**:401.
148. Pei M, Solchaga LA, Seidel J et al. *FASEB J* 2002;**16**:1691.
149. Suh JK, and Matthew HW. *Biomaterials* 2000;**21**:2589.
150. Kwon H, Sun L, Cairns DM et al. *Acta Biomater* 2013;**9**:6563.
151. Schreiber RE, Ilten-Kirby BM, Dunkelman NS et al. *Clin Orthop Relat Res* 1999;**S382**:367.
152. Okano T, Yamada N, Sakai H et al. *J Biomed Mater Res* 1993;**27**:1243.
153. Hamahashi K, Sato M, Yamato M et al. *J Tissue Eng Regen Med* 2015;**9**:24-30
154. Kaneshiro N, Sato M, Ishihara M et al. *Biochem Biophys Res Commun* 2006;**349**:723.
155. Badylak, S.F. *Semin Cell Dev Biol* 2002;**13**:377.
156. Gilbert TW, Sellaro TL, and Badylak SF. *Biomaterials* 2006;**27**:3675.
157. Badylak SF, Valentin JE, Ravindra AK et al. *Tissue Eng Part A* 2008;**14**:1835.
158. Benders KE, van Weeren PR, Badylak SF et al. *Trends Biotechnol* 2013;**31**:169.
159. Paz AC, Kojima K, Iwasaki K et al. *J Bioeng Biomed Sci* 2011;**S2**:001.
160. Choi KH, Choi BH, Park SR et al. *Biomaterials* 2010;**31**:5355.
161. Chevalier X, Florent Eymard F, and Richette P. *Nature Rev Rheumat* 2013;**9**:400.

Chapter 2: Delineation of in vitro chondrogenesis of human synovial stem cells following preconditioning using decellularized matrix

As published in Acta Biomater. 2015; 20:39-50. doi: 10.1016/j.actbio.2015.04.001.

Zhang Y¹, Li J², Davis ME³, Pei M^{1,2,4}.

¹Mechanical and Aerospace Engineering, West Virginia University, Morgantown, West Virginia 26506, USA

²Stem Cell and Tissue Engineering Laboratory, Department of Orthopaedics, West Virginia University, Morgantown, West Virginia 26506, USA

³Department of Physiology and Pharmacology, West Virginia University, Morgantown, WV 26506, USA.

⁴Exercise Physiology, West Virginia University, Morgantown, West Virginia 26506, USA

Corresponding author: Ming Pei MD, PhD, Stem Cell and Tissue Engineering Laboratory, Department of Orthopaedics, West Virginia University, PO Box 9196, One Medical Center Drive, Morgantown, WV 26506-9196, USA, Telephone: 304-293-1072; Fax: 304-293-7070; Email: mpei@hsc.wvu.edu

Abstract

As a tissue-specific stem cell for chondrogenesis, synovium-derived stem cells (SDSCs) are a promising cell source for cartilage repair. However, a small biopsy can only provide a limited number of cells. Cell senescence from both *in vitro* expansion and donor age presents a big challenge for stem cell based cartilage regeneration. Here we found that expansion on decellularized extracellular matrix (dECM) full of three-dimensional nanostructured fibers provided SDSCs with unique surface profiles, low elasticity but large volume as well as fibroblast-like shape. dECM expanded SDSCs yielded larger pellets with intensive staining of type II collagen and sulfated glycosaminoglycans compared to those grown on plastic flasks while SDSCs grown in ECM yielded 28-day pellets with minimal matrix as evidenced by pellet size and chondrogenic marker staining, which was confirmed by both biochemical data and real-time PCR data. Our results also found lower levels of inflammatory genes in dECM expanded SDSCs that might be responsible for enhanced chondrogenic differentiation. Despite an increase in type X collagen in chondrogenically induced cells, dECM expanded cells had significantly lower potential for endochondral bone formation. Wnt and MAPK signals were actively involved in both expansion and chondrogenic induction of dECM expanded cells. Since young and healthy people can be potential donors for this matrix expansion system and decellularization can minimize immune concerns, human SDSCs expanded on this future commercially available dECM could be a potential cell source for autologous cartilage repair.

Introduction

Articular cartilage has a limited capacity for self-repair. Once damaged, the injured cartilage will develop defects. Despite promising results from autologous chondrocyte implantation [1], donor tissue availability is a challenge. Recent advances make adult stem cells an attractive cell source for cartilage regeneration, especially tissue-specific stem cells such as synovium-derived stem cells (SDSCs) [2,3]. Since *in vitro* expansion is a necessary step before *in vivo* application, accompanying cell senescence and dedifferentiation represents a formidable challenge for stem cell-based cartilage repair [4].

We found that decellularized extracellular matrix (dECM) deposited by mesenchymal stem cells could rejuvenate stem cells [5-11] and primary cells [12-14] in both proliferation and differentiation capacity. For instance, dECM deposited by SDSCs significantly promoted expanded porcine SDSCs (pSDSCs) in both proliferation and chondrogenic potential [5]. *In vivo* transplantation of dECM expanded pSDSCs demonstrated efficacy in promoting cartilage regeneration in a partial thickness cartilage defect porcine model [15].

Our recent reports suggested that this *in vitro* cell expansion system also benefits human SDSC (hSDSC) expansion and rejuvenation of chondrogenic potential [16,17], which brings hope for the potential use of this approach in clinical treatment [18,19]. However, a concomitant up-regulation of type X collagen (*COL10A1*) was also observed in chondrogenically differentiated hSDSCs that have undergone dECM expansion, indicating a tendency toward chondrogenic hypertrophy.

In this study, we fully characterized cell morphology, volume, elasticity, and surface phenotypes in hSDSCs following dECM expansion. We not only defined proliferation and chondrogenic potential in dECM expanded hSDSCs but also examined whether increased expression of *COL10A1* could be a sign of endochondral bone formation. Since both the mitogen-activated protein kinase (MAPK) and Wnt signals are critical pathways for chondrogenesis and have crosstalk in stem cell mediated cartilage regeneration [20], these two signals were evaluated for their changes in both cell expansion and chondrogenic induction of hSDSCs after preconditioning using dECM and conventional plastic flasks, which might provide evidence for further investigation of potential mechanisms underlying the rejuvenation of hSDSCs by dECM expansion.

Materials and Methods

SDSC culture

Adult human synovial fibroblasts (4 donors, two male and two female, average 43 years old, all had no known joint disease), referred to as hSDSCs [16,17], were obtained from Asterand (North America Laboratories, Detroit, MI). Human SDSCs were plated and cultured in a growth medium [α MEM containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 μ g/mL streptomycin, and 0.25 μ g/mL fungizone (Invitrogen, Carlsbad, CA)] at 37°C in a humidified 5% CO₂ and 21% O₂ incubator. The medium was changed every three days.

dECM preparation

The preparation of dECM was described in our previous study [16,17]. Briefly, plastic flasks (Plastic) were precoated with 0.2% gelatin (Sigma-Aldrich, St. Louis, MO) at 37°C for 1 h and seeded with passage 3 (P3) hSDSCs at 6,000 cells/cm². After the cells reached 90% confluence, 50 μ M L-ascorbic acid phosphate (Wako Chemicals USA, Inc., Richmond, VA) was added for 8 days. The medium was changed every other day. The deposited matrix was incubated with 0.5% Triton X-100 containing 20 mM ammonium hydroxide at 37°C for 5 min and stored at 4°C in phosphate-buffered saline (PBS) containing 100 U/mL penicillin, 100 μ g/mL streptomycin, and 0.25 μ g/mL fungizone.

SDSC expansion

P3 hSDSCs were cultured at 3000 cells/cm² for one passage on two substrates: dECM or Plastic. The cell number was counted using a hemocytometer. Expanded cells were also evaluated for cell morphology using scanning electronic microscopy (SEM), and atomic force microscopy (AFM), cell number using a hemocytometer, and proliferation index and surface markers using flow cytometry.

Morphological observation using the SEM and AFM

Representative samples (n=2) were primarily fixed in 2.5% glutaraldehyde (Sigma-Aldrich) for 2 h, followed by secondary fixation in 2% osmium tetroxide (Sigma-Aldrich) for another 2 h. The samples

were then dehydrated in a gradient ethanol series, in hexamethyldisilazane (HMDS, Sigma-Aldrich) at a ratio of 1:1 with ethanol twice for 1 h each time, in HMDS at a ratio of 1:2 with ethanol overnight, and in HMDS three times for 4 h each time. The samples were air-dried for 24 h and gold sputter was added. The images were recorded by an SEM (Hitachi, Model S 2400).

Morphology of culture substrates (dECM and Plastic) and both morphology and elasticity of expanded hSDSCs were performed using an MFP-3D-BIO AFM (Asylum Research, TE2000-U, Santa Barbara, CA) integrated with an inverted fluorescence microscope (Nikon Eclipse, Ti-U, Nikon Instruments Inc., Melville, NY) and Olympus TR400-PB cantilevers with manufacturer spring constant of 0.09 N/m. The samples were imaged in Petri dishes after they were fixed with 1% glutaraldehyde and washed once with PBS. The location of the cantilever on the sample was confirmed using a 10× microscopy objective. For the morphology imaging, each sample was mapped in PBS buffer using contact mode. An area of 90 μm by 90 μm was imaged with the pixel resolutions of 512 and scan rate at 0.2 Hz. For the quantitative nanomechanical analysis, a Sneddon's modification of the Hertz model developed for a four-sided pyramid was employed. The fixed cell sample elasticity (Young's modulus, E) was corrected with the indentation of the tip, δ, through the

following equation: $E = \frac{\pi}{2} \frac{1-\nu^2}{\tan\alpha} \frac{F}{\delta^2}$, where E is the elastic modulus, ν is Poisson's ratio with a value

of 0.5 for ECM and cells, F is the force given by the cantilever deflection multiplied with the cantilever spring constant, α is the open angle used in this study which had a value of 36°, and lastly δ is the indentation depth [21]. The average height, projected cell area and the volume of the fixed cells were calculated using the MFP-3D Bio dedicated *Igor Pro* software (Asylum Research). The diameter of matrix fibers were determined by the measurement of full width of half maximum (FWHM) using ImageJ software.

Measurement of expanded cell proliferation index using flow cytometry

Before cell expansion, passage 3 hSDSCs were labeled with CellVue® Claret (Sigma-Aldrich) at 2×10^{-6} M for 5 min according to the manufacturer's protocol. Expanded cells were collected and measured using a BD FACS (fluorescence activated cell sorting) Calibur™ flow cytometer (dual laser) (BD Biosciences, San Jose, CA). Twenty thousand events of each sample were collected using

CellQuest Pro software (BD Biosciences) and cell proliferation index was analyzed by ModFit LT™ version 3.1 (Verity Software House, Topsham, ME).

Evaluation of cell surface phenotypes using flow cytometry

The following primary antibodies were used in flow cytometry to detect hSDSC surface phenotypes: integrin $\beta 1$ (CD29) was purchased from Abcam (Abcam, Cambridge, MA); CD105 and the stage-specific embryonic antigen-4 (SSEA-4) were from Santa Cruz Biotechnology (Santa Cruz, CA); and CD90 was from BD Pharmingen (BD Biosciences). IgG1 and IgG2a (Beckman Coulter, Fullerton, CA) were used as the isotype controls. Samples ($n=3$) of each 0.2×10^6 expanded cells were incubated on ice in cold PBS containing 0.1% ChromPure Human IgG whole molecule (Jackson ImmunoResearch Laboratories, West Grove, PA) and 1% NaN_3 (Sigma-Aldrich) for 30 min. Then, the cells were sequentially incubated in the dark in the primary antibodies for 30 min. The fluorescence was analyzed by a FACS Calibur (BD Biosciences) using FCS Express software package (De Novo Software, Glendale, CA).

Chondrogenic induction and evaluation of expanded hSDSCs

After *in vitro* expansion, 0.25×10^6 of hSDSCs from each group were centrifuged at 500 g for 5 min in a 15-mL polypropylene tube to form a pellet. After overnight incubation (day 0), the pellets were cultured for 35 days in a serum-free chondrogenic medium consisting of high-glucose Dulbecco's Modified Eagle's Medium (DMEM), 40 $\mu\text{g/mL}$ proline, 100 nM dexamethasone, 100 U/mL penicillin, 100 $\mu\text{g/mL}$ streptomycin, 0.1 mM ascorbic acid-2-phosphate, and 1 $\times$ ITS™ Premix [6.25 $\mu\text{g/mL}$ insulin, 6.25 $\mu\text{g/mL}$ transferrin, 6.25 $\mu\text{g/mL}$ selenous acid, 5.35 $\mu\text{g/mL}$ linoleic acid, and 1.25 $\mu\text{g/mL}$ bovine serum albumin (BSA), from BD Biosciences] with supplementation of 10 ng/mL transforming growth factor beta 3 (TGF- $\beta 3$, PeproTech Inc., Rocky Hill, NJ). Chondrogenic differentiation was evaluated using histology, immunostaining, biochemical analysis, microarray, and real-time polymerase chain reaction (PCR).

Representative pellets ($n=3$) were fixed in 4% paraformaldehyde at 4°C overnight, followed by dehydrating in a gradient ethanol series, clearing with xylene, and embedding in paraffin blocks. Five μm -thick sections from the center of representative pellets were histochemically stained with Alcian

blue (Sigma-Aldrich; counterstained with fast red) for sulfated glycosaminoglycans (GAGs). For immunohistochemical analysis, the consecutive sections were immunolabeled with primary antibodies against type II collagen (II-II6B3; Developmental Studies Hybridoma Bank, Iowa City, IA) and type X collagen (Sigma-Aldrich) followed by the secondary antibody of biotinylated horse anti-mouse IgG and IgM, respectively (Vector, Burlingame, CA). Immunoactivity was detected using Vectastain ABC reagent (Vector) with 3,3'-diaminobenzidine as a substrate.

Representative pellets (n=4) were digested for 4 h at 60°C with 125 µg/mL papain in PBE buffer (100 mM phosphate and 10 mM ethylenediaminetetraacetic acid, pH 6.5) containing 10 mM cysteine, by using 200 µL enzyme per sample. To quantify cell density, the amount of DNA in the papain digestion was measured using the QuantiTTM PicoGreen® dsDNA assay kit (Invitrogen) with a CytoFluor® Series 4000 (Applied Biosystems, Foster City, CA). GAG was measured using dimethylmethylene blue dye and a Spectronic BioMate 3 Spectrophotometer (Thermo Scientific, Milford, MA) with bovine chondroitin sulfate as a standard.

Global gene expression was evaluated in hSDSCs after expanded cell chondrogenic induction. The pellets from dECM expanded cells were referred to as Ep while those from Plastic expanded cells were referred to as Pp. Total RNAs were isolated from the pellets using Trizol (Invitrogen) followed by additional purification using an RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The required amount of cDNA (5.5 µg) was processed for fragmentation and biotin labeling using the GeneChip® WT Terminal Labeling Kit (Affymetrix, Santa Clara, CA). The entire reaction of fragmented and biotin-labeled cDNA (50 µL) with added hybridization controls was hybridized to the human GeneChip® 1.0 ST Exon Arrays (Affymetrix) at 45°C for 17 h in the GeneChip® Hybridization Oven 640 (Affymetrix). The raw data was uploaded into Partek (St. Louis, MO) software for initial analysis and Ingenuity Pathway Analysis (IPA, Redwood City, CA) for pathway and functional analysis. Briefly, raw intensity was background-subtracted, robust multi-array analysis (RMA) normalized, log-transformed, and fold changes determined. The batch effects (scan date) were removed before fold change calculation. Cluster analysis was used to compare the genes (the dECM group *versus* the Plastic group) annotated in IPA as affecting chondrodysplasia or bone ossification. For bone ossification, genes were filtered to 60 with the greatest differential expression. Clustering was done using heatmap.2 in R (Euclidean distance, complete linkage).

After extraction and purification of total RNA from samples (n=4), about 1 µg of mRNA was used for

reverse transcriptase with a High-Capacity cDNA Archive Kit (Applied Biosystems) at 37°C for 120 min. Chondrogenic marker genes [type II collagen (*COL2A1*; assay ID: Hs00156568_m1), aggrecan (*ACAN*; assay ID: Hs00153935_m1), and SRY (sex determining region Y)-box 9 (*SOX9*; assay ID: Hs00165814_m1)], hypertrophic marker genes [*COL10A1* (assay ID: H200166657_m1) and alkaline phosphatase (*ALPL*; assay ID: Hs01029144_m1)], Wnt signal genes [WNT3A (assay ID: Hs00263977_m1), WNT5A (assay ID: Hs00998537_m1), and WNT11 (assay ID: Hs00182986_m1)], and inflammation related genes [interleukin 1 beta (*IL1B*; assay ID: Hs01555410_m1), and a disintegrin and metalloproteinase with thrombospondin motifs 5 (*ADAMTS5*; assay ID: Hs00199841_m1)] were customized by Applied Biosystems as part of the Custom TaqMan® Gene Expression Assays. Eukaryotic 18S RNA (assay ID: Hs99999901_s1) was carried out as the endogenous control gene. Real-time PCR was performed with the iCycler iQ™ Multi-Color Real Time PCR Detection (Perkin-Elmer, Waltham, MA). Relative transcript levels were calculated as $\chi=2^{-\Delta\Delta Ct}$, in which $\Delta\Delta Ct = \Delta E - \Delta C$, $\Delta E = Ct_{exp} - Ct_{18s}$, and $\Delta C = Ct_{ctl} - Ct_{18s}$.

Characterization of both MAPK and Wnt signals using Western blot

Adult hSDSCs from cell expansion (day 1, 3, and 5) and pellets before (day 0) and after (day 1 and 7) chondrogenic induction were dissolved in the lysis buffer (Cell Signaling, Boston, MA) with protease inhibitors. Total protein was quantified using the BCA™ Protein Assay Kit (Thermo Fisher Scientific). Thirty micrograms of protein from each sample were denatured and separated using NuPAGE® Novex® Bis-Tris Mini Gels in the XCell SureLock™ Mini-Cell (Life Technologies) at 120 V at 4°C for 3 h. Bands were transferred onto a nitrocellulose membrane using an XCell II™ Blot module (Life Technologies) at 15 V at 4°C overnight. The membrane was incubated with primary monoclonal antibodies in 5% bovine serum albumin (BSA) in TBST buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween-20) for 1 h (β -actin served as an internal control), followed by the secondary antibody of horseradish peroxidase-conjugated goat anti-mouse (Thermo Fisher Scientific) for 1 h. SuperSignal West Femto Maximum Sensitivity Substrate and CL-XPosure Film (Thermo Fisher Scientific) were used for exposure. The primary antibodies used in immunoblotting included the MAPK family antibody sampler kit [extracellular signal-regulated protein kinases 1 and 2 (Erk1/2), Jun N-terminal kinase (Jnk), and p38], phosphorylated (p-) MAPK family antibody sampler kit, and

the Wnt signaling antibody sampler kit (Cell Signaling). Wnt11 polyclonal antibody was obtained from Thermo Fisher Scientific. ImageJ software was used to quantify immunoblotting bands.

Statistics

Numerical data are presented as the mean and the standard error of the mean. The Mann-Whitney U test was used for pair-wise comparison in biochemistry and real-time PCR data analysis. All statistical analyses were performed with SPSS 13.0 statistical software (SPSS Inc., Chicago, IL). A *p* value less than 0.05 was considered statistically significant. For microarray data analysis, a regulation z-score statistic was calculated to predict if the observed numbers of genes changed in the direction to produce the functional effect would occur by chance. A z-score > 2 or < -2 was considered significant.

Results

Expansion on dECM changed cell morphology and increased cell proliferation

Before plating cells, SEM data showed that, compared with a flat surface on Plastic substrate, dECM coated flasks exhibited a three-dimensional structure with interconnected nanofibers (Fig. 1A) that was corroborated by morphological data from AFM (Fig. 1B), in which the diameter of matrix fibers was 109.5 ± 39.6 nm (*n*=82). SEM data also showed that hSDSCs displayed a well-organized fibroblast-like cell shape when expanded on dECM rather than a broad and flat cell shape when grown on Plastic substrate (Fig. 1C). This finding was also in line with cell morphology shown by AFM (Fig. 1D), in which several parameters were measured for fixed cells with glutaraldehyde after expansion on Plastic and dECM substrates, including average height (1.33 ± 0.57 μm *versus* 2.34 ± 0.44 μm), projected cell area (713.7 ± 154.4 μm^2 *versus* 916.0 ± 190.9 μm^2), and volume (962.3 ± 502.7 μm^3 *versus* 2101.3 ± 354.1 μm^3). Young's moduli of the fixed cells were 91.22 ± 64.15 kPa after expansion on Plastic and 53.33 ± 53.47 kPa when expanded on dECM (Fig. 1E).

To determine whether dECM expansion also affected cell proliferation rate, 3000 cells per cm^2 were incubated on both substrates for seven days. The data from cell counting showed that the cells grown on Plastic flasks increased 6.95-fold compared with 8.57-fold on dECM (Fig. 2A), which was confirmed by proliferation index data (8.46 *versus* 22.28) measured in expanded cells (Fig. 2B). To

further characterize molecular phenotype changes following dECM expansion, flow cytometry was used to quantify both percentage and median fluorescence intensity of typical mesenchymal stem cell markers in expanded cells (Fig. 2C). Notably, despite almost 100% expression in the cells after expansion on both substrates, the median fluorescence intensity of CD29, CD90, and CD105 dramatically decreased in dECM expanded cells. In contrast, the percentage of SSEA4 doubled in dECM expanded cells concomitantly with a slight increase in median fluorescence intensity.

Expansion on dECM enhanced cell chondrogenic potential

To determine whether dECM expansion could enhance hSDSC chondrogenic potential, a pellet culture system was used for chondrogenic induction. Histology data showed that dECM expanded cells yielded 35-day-pellets with a larger size despite starting with the same initial cell density as those from Plastic expanded cells. The increased pellet size was accompanied by similarly increased staining intensity of both sulfated GAG and type II collagen, two cartilage markers, in the pellets from dECM expanded cells (Fig. 3A). The staining data were further confirmed by the quantification data at both the protein and mRNA levels. Our biochemical analysis data showed that, after a 35-day-chondrogenic induction, expansion on dECM enhanced DNA ratio (167.35 ± 4.01 %, adjusted by day 0) while expansion on Plastic decreased this ratio (82.03 ± 4.06 %); despite the lower amounts at day 0, after chondrogenic induction, both GAG amount per pellet and ratio of GAG to DNA from the dECM group dramatically increased and were higher than those from the Plastic group (Fig. 3B).

Real-time PCR data showed that all chondrogenic marker genes (*SOX9*, *COL2A1* and *ACAN*) were up-regulated during chondrogenic induction, especially in cells from the dECM group (Fig. 3C). Interestingly, despite a dramatic increase 24 h after chondrogenic induction, *SOX9* expression in dECM expanded cells reached a comparable level at day 7 and decreased afterwards compared with that from the Plastic group (Fig. 3C). Our data also showed that both *IL1B* and *ADAMTS5*, two typical inflammatory genes, were significantly down-regulated in chondrogenically induced hSDSCs from the dECM group (Fig. 3D).

Expansion on dECM promoted cell hypertrophy but not toward an osteogenic lineage

To determine whether expansion on dECM would enhance hSDSCs' hypertrophy during chondrogenic induction, we examined markers of hypertrophy. Immunostaining data showed that the

35-day-pellets from dECM expanded hSDSCs were intensely stained with type X collagen compared with those from Plastic expanded cells (Fig. 4A). These data were further confirmed by our real-time PCR data in which both *COL10A1* (Fig. 4B) and *ALPL* (Fig. 4C) were dramatically up-regulated in chondrogenically induced hSDSCs following dECM expansion.

Next, we wanted to determine whether expansion on dECM would promote hSDSCs toward bone ossification and/or chondrodysplasia. Pathway analysis of microarray data showed expression of 63 genes related to chondrodysplasia (Fig. 4D) and 377 genes related to bone ossification (the 60 genes most differentially expressed are shown in Fig. 4E). Compared with the pellets from Plastic expanded hSDSCs, chondrogenically induced hSDSCs from the dECM group exhibited an up-regulation in fibroblast growth factor receptor 2 (*FGFR2*) and noggin (*NOG*) and a down-regulation in *FGF18*, forkhead box O1 (*FOXO1*), bone morphogenetic protein 4 (*BMP4*), and caspase 3 (*CASP3*), suggesting that, after dECM expansion, ossification of bone is decreased (prediction z-score: -2.43) (Fig. 4F). An up-regulation of indian hedgehog (*IHH*), *COL2A1*, chondroitin sulfate synthase 1 (*CHSY1*), and solute carrier family 35 (UDP-GlcA/UDP-GalNAc transporter) member D1 (*SLC35D1*) indicated that, after dECM expansion, dysplasia of the skeleton (including chondrodysplasia) is decreased (prediction z-score: -2.00) (Fig. 4F).

Growth in ECM dramatically decreased expanded cell chondrogenic capacity

To determine whether expansion on dECM (“ON”, deposited by other SDSCs) was superior to SDSCs that were grown in ECM (“IN”, deposited by expanded SDSCs) in chondrogenic capacity, expanded cells were incubated in a pellet culture system supplemented with chondrogenic induction medium for 28 days. Both groups exhibited a similar pellet size at day 2 but, at day 28, SDSCs expanded on dECM yielded pellets with dramatically increased size whereas SDSCs grown in ECM yielded pellets that had no change in size visible to the naked eye, which was accompanied by less staining intensity of both sulfated GAG and type II collagen compared to those expanded on dECM (Fig. 5A). Based on the DNA ratio adjusted by day 0, cell viability at day 28 in the IN ECM group was about 90% while in the ON dECM group, it was about 168%. Despite the higher GAG amount (2.73-fold) and ratio of GAG to DNA (1.99-fold) at the initial time point (day 0), 28-day chondrogenic induction yielded pellets from the IN ECM group with a lower GAG amount (0.13-fold) and ratio of GAG to DNA (0.18-fold) compared to those from the ON dECM group (Fig. 5B). Real-time PCR data showed that,

after 28-day chondrogenic induction, all chondrogenic marker genes (*SOX9*, *COL2A1* and *ACAN*) showed a similar trend in the pellets with lower mRNA levels from the IN ECM group compared to the ON dECM group (Fig. 5C).

Both Wnt and MAPK pathways were actively involved in dECM mediated chondrogenesis

To determine whether Wnt signals were involved in dECM mediated cell expansion, real-time PCR was used to measure *WNT3A* (a canonical signal) and *WNT5A* and *WNT11* (noncanonical signals) in hSDSCs following cell expansion. We found that *WNT3A* was undetectable but, compared to the Plastic group, both *WNT5A* and *WNT11* were up-regulated in dECM expanded cells, particularly *WNT11* (Fig. 6A). The above data were confirmed by Western blot data in which both Wnt5a and Wnt11 exhibited a higher level of expression in dECM expanded hSDSCs (Fig. 6B). Intriguingly, we also found that wnt3a protein was detectable and exhibited a higher level in dECM expanded hSDSCs compared to those grown on Plastic (Fig. 6B).

Similar to cell expansion, *WNT3A* mRNA was undetectable while Wnt3a protein exhibited a higher level in dECM expanded hSDSCs during the early stage of chondrogenic induction. Wnt5a and Wnt11 exhibited comparable expression profiles in both mRNA (Fig. 6C) and protein (Fig. 6D). Compared to an up-regulation of Wnt5a in the beginning followed by a decrease afterwards, a continuing increase of Wnt11 at both mRNA and protein levels was found in the pellets from both expanded cells, with dECM expanded hSDSCs exhibiting a significant advantage over those expanded on Plastic.

To determine whether MAPK signals were involved in dECM mediated cell expansion, Western blot was used to measure phosphor (p)-Erk, Erk, p-p38, p38, p-Jnk, and Jnk in hSDSCs after a five-day expansion. We found that p-Erk, p-p38, p-Jnk, p38, and Jnk exhibited higher levels in dECM expanded cells than those grown on Plastic (Fig. 7A). However, during chondrogenic induction, most MAPK signals increased at day 1 but decreased afterwards (Fig. 7B).

Discussion

Our previous work found that dECM could rejuvenate pSDSCs for both proliferation and

chondrogenic potential. We recently found this *in vitro* cell expansion system also applied to hSDSCs. In order to make this approach applicable to future clinical treatment, in this study, we characterized dECM and expanded cells using both SEM and AFM and compared them to the Plastic control; we also characterized proliferation and chondrogenic capacity of adult hSDSCs after expansion on dECM and determined whether dECM expanded cells exhibited a differentiation trend toward bone formation as well as potential involvement of Wnt and MAPK signals. We found that dECM expanded hSDSCs exhibited a fibroblast shape, a low level of elasticity, enhanced proliferation, unique changes in stem cell surface markers, and enhanced chondrogenic capacity and hypertrophy, which are consistent with our previous findings [16,17]. We also found, for the first time, that dECM expansion could dramatically decrease the potential differentiation toward bone and dysplasia in the skeleton. Additionally, we discovered that both Wnt and MAPK signals are actively involved in dECM expanded hSDSC chondrogenesis.

It is known that microenvironments play important roles in stem cell lineage specifications. Cell proliferation leads to an increase of cell volume while one of the hallmarks of apoptosis is cell shrinkage [22]. In this study, cells expanded on dECM showed more than a two-fold increase in cell volume, suggesting strong mitotic activity; comparatively, the low volume value for those cells grown on Plastic substrate indicated low mitotic activity of cells that might experience senescence and apoptosis. This observation was supported by expanded cell proliferation index data (Plastic *versus* dECM: 8.46 *versus* 22.28). We also found that dECM was a nanostructured three-dimensional matrix, on which expanded cells possessed low elasticity and high chondrogenic potential compared to those grown on Plastic substrate, which might be explained by lower elasticity favoring expanded stem cells chondrogenic differentiation [16,23,24]. In this study, dECM expansion lowered cell elasticity, about 0.58-fold compared to those cells grown on Plastic substrate. This finding is consistent with our previous report, in which dECM expansion yielded cells with elasticity about 0.40-fold compared to those grown on Plastic substrate [16], despite the fact that the absolute values were much higher than previous ones due to the fixation of expanded cells using glutaraldehyde before measurement. Evidence has more recently emerged that fixation is considered the preferred method for reliable data analysis (reproducible) of both cell elasticity and cell morphology [25,26] since this method not only allows for longer scanning time but further limits changes associated with the inherent dynamics of living cells within the extended observation time of at least one hour per cell.

In this study, the increase in cell number from passage 4 to 5 in the dECM group was 1.23-fold that in the Plastic group while our previous study showed about an increase of 14.57-fold that in the Plastic group [5]. The chondrogenic index from chondrogenically induced pSDSCs in the dECM group was 1.36-fold that in the Plastic group while our previous study showed an increase of about 18.71-fold that in the Plastic group [5]. The discrepancy of nanofiber size is probably responsible for expanded SDSC proliferation rate and chondrogenic potential. Our data suggested that the diameter of dECM fibers measured by AFM was 109.5 ± 39.6 nm, which was about double the size of dECM fibers measured by SEM (45.1 ± 8.8 nm) from our previous study [5]. As described by Oh and colleagues, small (around 30-nm diameter) nanotubes promoted adhesion without noticeable differentiation, whereas larger (70- to 100-nm diameter) nanotubes elicited a dramatic stem cell elongation (10-fold increase), which induced cytoskeletal stress and differentiation [27]. Another report by Park et al. indicates that a 15-20 nm spacing was better for integrin activation, while tube diameter larger than 50 nm severely impaired cell spreading, adhesion, and spacing of 100 nm resulting in dramatically reduced cell proliferation, migration, and differentiation [28]. Further investigation needs to determine whether donor age affects the diameter of dECM fibers.

For cell proliferation, we found that hSDSCs grown on dECM became fibroblast-like in shape and exhibited enhanced proliferation; CD29, CD90, and CD105 exhibited a decreased expression while SSEA4 was up-regulated, which is consistent with our recent report using hSDSCs [16]. Interestingly, despite a similar trend in cell morphology and proliferation capacity, our early report found that dECM expanded pSDSCs displayed an enhanced expression of CD90 [5] rather than a decreased level in this study, which is probably due to the source of SDSCs from different species (porcine *versus* human) and/or donor age (3-month-old *versus* 43-year-old). This discrepancy remains to be elucidated.

As we observed in a previous report [5], dECM expanded hSDSCs had a lower level of GAG and *ACAN* before chondrogenic induction (at day 0) which may be indicative of “stemness” because these pretreated SDSCs exhibited an enhanced chondrogenesis when incubated in a chondrogenic medium, as evidenced by an improved cell survival capacity (DNA amount adjusted by day 0) and enhanced chondrogenic differentiation. In contrast, SDSCs grown in ECM yielded pellets with the lowest levels in GAG/pellet, GAG/DNA, *ACAN*, and *COL2A1*, indicating the minimum chondrogenic matrix deposition.

The aim of using dECM for cell expansion is to provide a large quantity of high-quality stem cells for cartilage regeneration. In line with a recent report [17], an up-regulation of *COL10A1* was observed in chondrogenically induced adult hSDSCs after expansion on dECM, but this outcome differed from the decreased level of hypertrophy in dECM expanded pSDSCs [5]. We expect the expanded cells to have high chondrogenic potential but less tendency to differentiate into bone. Our microarray data for pellets suggested that *FGFR2* [29] and *NOG* [30] were up-regulated and *FGF18* [31], *FOXO1* [32], *BMP4* [33], and *CASP3* [34] were down-regulated during chondrogenic induction of dECM expanded hSDSCs. BMP signaling is critical for osteogenesis; as a potent BMP antagonist, reduction of noggin enhanced BMP signaling and *in vitro* osteoblast bone formation [30]; activating mutations of FGFR2 decreased the expression of NOG [35]. In addition, mice lacking FGF18 display delayed ossification and decreased expression of osteogenic markers [31]; as an early molecular regulator in the differentiation of mesenchymal cells into osteoblasts, silencing FOXO1 in a tibia organ culture model was demonstrated to decrease the expression of RUNX2 and impair bone formation [32]. Human BMP4 protein was reported to increase (in a dose dependent manner) ossification of endochondral bone [33]. Delayed ossification and decreased bone mineral density in caspase-3-deficient (*Casp3*^{-/-} and *Casp3*^{+/-}) mice suggested that caspase-3 is crucial for the differentiation of bone marrow stromal cells by influencing the TGF-beta/Smad2 pathway and cell cycle progression [34]. Our findings suggest that dECM expansion decreased the tendency of hSDSCs toward ossification of bone. We also found that *IHH* [36], *COL2A1* [37], *CHSY1* [38], and *SLC35D1* [39] were up-regulated in dECM expanded hSDSCs, indicative of a down-regulation of dysplasia of the skeleton, including chondrodysplasia.

Wnt signaling molecules are involved in a multitude of developmental processes including chondrogenesis [40]. In this report, both canonical and noncanonical Wnt signals were activated in dECM expanded hSDSCs, indicating that Wnt signals are probably associated with enhanced cell proliferation. For example, Wnt3a promoted the proliferation of undifferentiated mesenchymal stem cells (MSCs) [41,42]. Wnt5a induced endothelial cell proliferation and enhanced cell survival under serum-deprived conditions [43]. Despite a continuing increase in both groups, especially in the dECM group, there is not sufficient evidence to support the role of Wnt11 in MSC proliferation. Intriguingly, overexpression of Wnt11 was recently demonstrated in suppressing MSC proliferation and arresting the cell cycle at the G0/G1 phase [44].

During chondrogenic induction, both WNT3A and WNT5A in dECM expanded hSDSCs exhibited a comparable trend, with an initial increase followed by a decrease; in contrast, Wnt11 expression exhibited a time-dependent increase in both groups, particularly for dECM expanded cells. This finding might indicate that the signals of both Wnt3a and Wnt5a are most related to cell expansion with a minor effect on the early stage of chondrogenic differentiation. Noncanonical Wnt signaling pathways such as Wnt5a and Wnt11 are known to promote chondrogenesis *in vitro* by increasing cartilage nodule formation [40,45]. Our data showed that Wnt5a and Wnt11 were up-regulated at both the mRNA and protein levels in dECM expanded hSDSCs except that Wnt5a exhibited a decrease at the later stage of chondrogenic induction. This outcome could possibly be explained by a previous report that Wnt5a specifically promotes entry into the prehypertrophic phase, whereas it conversely blocks chondrocyte hypertrophy, acting in a stage-specific context [46].

Wnt5a has been shown to have stage-dependent regulation of chondrocyte differentiation by promoting chondroprogenitor proliferation and inhibiting chondrocyte hypertrophy [47]; in addition, Wnt5a-infected limbs reveal a delay in the onset of type X collagen expression, a marker for hypertrophic chondrocytes [48]. It is possible that, in the early stage, the Wnt5a was highly activated in dECM expanded hSDSCs in order to boost chondrogenesis while the decreased expression of Wnt5a in the late stage contributes to enhanced hypertrophy. Interestingly, we also witnessed increased expression of Wnt11, which has been reported to greatly enhance chondrogenesis [44]. Although overexpression of Wnt11 did not appear to delay chondrocyte differentiation including type X collagen [40], more investigation is needed to confirm the correlation between Wnt11 expression and chondrogenic hypertrophy.

Similar to Wnt signals, all MAPK signals were up-regulated in dECM expanded hSDSCs, likely indicating a potential crosstalk between Wnt and MAPK signals during chondrogenesis. The MAPK pathway is one of the conserved signal transduction systems in cartilage, where it plays a crucial role in chondrogenic differentiation. Erk and p38 MAPK have central roles in mediating chondrocyte proliferation and related gene expression [49]. During the proliferation stage, dECM expanded cells showed enhanced expression of p-Erk and Erk; interestingly, Wnt3a can also promote Erk1/2 phosphorylation, and this signal was proposed to mediate proliferation of NIH3T3 cells [50]. Wnt5a also promotes Erk1/2 phosphorylation in endothelial cells and Wnt5a protects cells from apoptosis via Erk signaling [51].

During chondrogenic induction, the MAPK signals were observed to tentatively increase followed by a drop at day 7, indicating that the role of MAPK signals is mainly at the early stage of chondrogenic differentiation. This finding is in line with previous reports which maintain that the Erk pathway might be predominantly involved during early cartilage developmental stages in relaying signals down-stream of FGF actions [52,53] and Erk1/2 activities are observed to decrease as chondrogenesis proceeds and inhibition of Erk1/2 with PD98059 enhances chondrogenesis [54]. Jnk has been reported to have a minor role in chondrogenesis [55,56]. However, in this study, we did find Jnk signals to be increased during cell expansion and at the early stage of chondrogenic induction; the potential mechanisms are still under investigation.

Conclusion and Perspectives

Our study demonstrated that dECM deposited by hSDSCs formed a three-dimensional nanostructured matrix; expansion on dECM yielded a large-quantity of hSDSCs with enhanced chondrogenic potential; despite an increase of type X collagen in chondrogenically induced cells, dECM expanded cells had significantly lower potential for endochondral bone formation. Even though the composition of dECM has been characterized in a recent report [16], research on the crosstalk between expanded SDSCs and dECMs is still in its infancy. dECM expansion of hSDSCs and subsequent chondrogenic induction were accompanied by a significant change in both Wnt and MAPK signals, indicating potential mechanisms underlying dECM mediated hSDSC rejuvenation, which needs further in-depth investigation. Progress in understanding environment-oriented epigenetic regulatory mechanisms could benefit cartilage regeneration and engineering on a larger scale and provide more promising therapeutic applications [57]. For future clinical application, young and healthy donors can be selected for deposition of this ECM [16]; a decellularized process can minimize concerns about immune issues; and this dECM can be commercialized to make one-step *in vitro* expansion possible.

References

1. Karnes J, Zhang Y, Pei M. 4th Edition. Taylor & Francis/CRC Press, 2014. p. 1123-1135.
2. Jones B, Pei M. *Tissue Eng Part B Rev* 2012;**18**:301-11.
3. Pizzute T, Lynch K, Pei M. *Stem Cell Rev Rep* 2015;**11**:119-32
4. Li JT, Pei M. *Tissue Eng Part B* 2012;**18**:270-87.
5. He F, Chen XD, Pei M. *Tissue Eng Part A* 2009;**15**:3809-21.
6. He F, Pei M. *J Tissue Eng Regen Med* 2013;**7**:73-84.
7. Li JT, He F, Pei M. *Cell Tissue Res* 2011;**345**:357-65.
8. Li JT, Pei M. *Tissue Eng Part A* 2011;**17**:703-12.
9. Pei M, He F, Kish VL. *Tissue Eng Part A* 2011;**17**:3067-76.
10. Pei M, He F, Wei L. *J Tissue Sci Eng* 2011;**2**:104.
11. Pei M, Li JT, Zhang Y et al. *Cell Tissue Res* 2014;**356**:391-403.
12. He F, Pei M. *Spine* 2012;**37**:459-69.
13. Pei M, He F. *J Cell Physiol* 2012;**227**:2163-74.
14. Pei M, Shoukry M, Li JT et al. *Spine* 2012; **37**:1538-47.
15. Pei M, He F, Li JT et al. *Tissue Eng Part A* 2013;**19**:1144-54.
16. Li JT, Hansen K, Zhang Y et al. *Biomaterials* 2014;**35**:642-653.
17. Pei M, Zhang Y, Li JT et al. *Stem Cells Dev* 2013;**22**:889-900.
18. Li JT, Pei M. Springer Science + Business Media Dordrecht, 2014. p. 109-15.
19. Pei M, Li JT, Shoukry M et al. *Eur Cell Mater* 2011;**22**:333-43; discussion 343.
20. Zhang Y, Pizzute T, Pei M. *Cell Tissue Res* 2014;**358**:633-49.
21. A-Hassan E, Heinz WF, Antonik MD et al. *Biophys J* 1998;**74**:1564-78.
22. Lang F, Ritter M, Gamper N et al. *Cell Physiol Biochem* 2000;**10**:417-28.
23. Engler AJ, Sen S, Sweeney HL et al. *Cell* 2006;**126**:677-89.
24. Fernández-Muñíos T, Suárez-Muñoz M, Sanmartí-Espinal M et al. *Tissue Eng Part A* 2014;**20**:1145-55.
25. Dong C, Kashon ML, Lowry D et al. *Adv Healthc Mater* 2013;**2**:945-51.
26. Rheinlaender J, Geisse NA, Proksch R et al. *Langmuir* 2011;**27**:697-704.
27. Oh S, Brammer KS, Li YS et al. *Proc Natl Acad Sci USA* 2009;**106**:2130-5.
28. Park J, Bauer S, von der Mark K et al. *Nano Lett* 2007;**7**:1686-91.
29. Eswarakumar VP, Monsonego-Ornan E, Pines M et al. *Development* 2002;**129**:3783-93.
30. Wan DC, Pomerantz JH, Brunet LJ et al. *J Biol Chem* 2007;**282**:26450-9.
31. Liu Z, Xu J, Colvin JS et al. *Genes Dev* 2002;**16**:859-69.
32. Teixeira CC, Liu Y, Thant LM et al. *J Biol Chem* 2010;**285**:31055-65.
33. Krishnan V, Ma Y, Moseley J et al. *Endocrinology* 2001;**142**:940-7.
34. Miura M, Chen XD, Allen MR et al. *J Clin Invest* 2004;**114**:1704-13.
35. Mansukhani A, Ambrosetti D, Holmes G et al. *J Cell Biol* 2005;**168**:1065-76.
36. Chen MH, Li YJ, Kawakami T et al. *Genes Dev* 2004;**18**:641-59.
37. Gustafsson E, Aszodi A, Ortega N et al. *Ann N Y Acad Sci* 2003;**995**:140-50.
38. Wilson DG, Phamluong K, Lin WY et al. *Dev Biol* 2012;**363**:413-25.
39. Hiraoka S, Furuichi T, Nishimura G et al. *Nat Med* 2007;**13**:1363-7.
40. Church V, Nohno T, Linker C et al. *J Cell Sci* 2002;**115**:4809-18.

41. Boland GM, Perkins G, Hall DJ et al. *J Cell Biochem* 2004;**93**:1210-30.
42. Shang YC, Wang SH, Xiong F et al. *Acta Pharmacol Sin* 2007;**28**:1761-74.
43. Masckauchán TN, Agalliu D, Vorontchikhina M et al. *Mol Biol Cell* 2006;**17**:5163-72.
44. Liu S, Zhang E, Yang M et al. *Mol Cell Biochem* 2014;**390**:123-31.
45. Ryu JH, Chun JS. *J Biol Chem* 2006;**281**:22039-47.
46. Yang Y, Topol L, Lee H et al. *Development* 2003;**130**:1003-15.
47. Hosseini-Farahabadi S, Geetha-Loganathan P, Fu K et al. *Matrix Biol* 2013;**32**:252-64.
48. Hartmann C, Tabin CJ. *Development* 2000;**127**:3141-59.
49. Krens SF, He S, Spaink HP et al. *Gene Expr Patterns* 2006;**6**:1019-26.
50. Yun MS, Kim SE, Jeon SH et al. *J Cell Sci* 2005;**118**:313-22.
51. Almeida M, Han L, Bellido T et al. *J Biol Chem* 2005;**280**:41342-51.
52. Corson LB, Yamanaka Y, Lai KM et al. *Development* 2003;**130**:4527-37.
53. Lunn JS, Fishwick KJ, Halley PA et al. *Dev Biol* 2007;**302**:536-52.
54. Oh CD, Chang SH, Yoon YM et al. *J Biol Chem* 2000;**275**:5613-9.
55. Nakamura K, Shirai T, Morishita S et al. *Exp Cell Res* 1999;**250**:351-63.
56. Stanton LA, Underhill TM, Beier F. *Dev Biol* 2003;**263**:165-75.
57. Li J, Ohliger J, Pei M. *Stem Cells Dev* 2014;**23**:1178-94.

Figure 2. 1 Morphological characterization of culture substrates and expanded cells.

Surface topography of both substrates, Plastic and dECM, was characterized using SEM (A) and AFM (B). Scale bar for SEM (A): 5 μm . Expanded cells on either Plastic (three cells) or dECM (four cells) after fixation in glutaraldehyde were characterized using SEM (C) and AFM (D) for morphology and using AFM (E) for elasticity. Scale bar for SEM (C): 50 μm .

Figure 2. 2 dECM promoted expanded human SDSCs' proliferation and changed expression of stem cell surface markers.

(A) Fold change of cell number by day 0 was used to indicate cell proliferation after 7-day-expansion on 175 cm² flasks (n=6). (B) Flow cytometry was used to measure the proliferation index (PI) of expanded human SDSCs. (C) Flow cytometry was used to measure both percentage and median of stem cell surface markers (CD29, CD90, CD105, and SSEA4) of expanded ASDSCs.

Figure 2. 3 dECM promoted expanded human SDSCs' chondrogenic differentiation.

(A) Before histological staining, pellet size was measured with a scale bar as mm; Alcian blue (AB) was used to stain sulfated GAGs and immunohistochemistry staining (IHC) was used to detect type II collagen (Col II). (B) Biochemical analysis was used for DNA and GAG contents of pellets; cell viability in chondrogenic medium was evaluated using DNA ratio adjusted by day 0; a ratio of GAG to DNA indicated chondrogenic index. Real-time polymerase chain reaction (PCR) was used to evaluate (C) chondrogenic marker gene expression (*SOX9*, *ACAN*, and *COL2A1*) and (D) inflammation related genes (*IL1B* and *ADAMTS5*). Data are shown as average $\pm$ standard deviation (SD) for $n = 4$. $*p < 0.05$ indicated a statistically significant difference.

Figure 2. 4 dECM promoted expanded human SDSCs' chondrogenic differentiation.

Human SDSCs were grown on either Plastic or dECM for one passage followed by chondrogenic induction in a pellet culture system for 35 days. (A) Immunohistochemistry staining (IHC) was used to detect type X collagen. (B) Real-time PCR was used to evaluate hypertrophic marker gene expression (*COL10A1* and *ALPL*). Data are shown as average $\pm$ standard deviation (SD) for $n = 4$. $*p < 0.05$ indicated a statistically significant difference. (C) Microarray data were used to predict the effect of dECM expansion on subsequent chondrogenic induction, especially on ossification of bone and chondrodysplasia. Ossification of bone and chondrodysplasia were predicted to be decreased in the pellets from dECM expanded cells, relative to those from Plastic expanded cells. Expression results for the genes are shown as FC ($\log_2$). The orange line denotes predicted activation of the target molecule (NOG) by the observed state of the upstream molecule (FGF18). Blue lines denote predicted inhibition of the molecule or function by the observed state (up-regulation or down-regulation) of the upstream molecule. Gray lines indicate that an effect on function predicted or interaction between molecules noted to occur, but existing data do not support prediction of direction of change. Gold indicates the effect on the downstream molecule is inconsistent with that predicted by direct

interaction of the molecules.

Figure 2. 5 hSDSCs grown in ECM (“IN”) exhibited a decline in chondrogenic potential compared with those expanded on dECM (“ON”).

(A) Before histological staining, pellet size was measured with a scale bar as mm; Alcian blue (AB) was used to stain sulfated GAGs and immunohistochemistry staining (IHC) was used to detect type II collagen (Col II). (B) Biochemical analysis was used for DNA and GAG contents of pellets; cell viability in chondrogenic medium was evaluated using DNA ratio adjusted by day 0; a ratio of GAG to DNA indicated chondrogenic index. (C) Real-time PCR was used to evaluate chondrogenic marker gene expression (*SOX9*, *ACAN*, and *COL2A1*). Data are shown as average $\pm$ SD for $n=4$. * $p < 0.05$ indicated a statistically significant difference.

Figure 2. 6 Wnt signals were actively involved in chondrogenesis of dECM expanded human SDSCs.

Both real-time PCR (A, C) and Western blot (B, D) were used to evaluate canonical Wnt signal (Wnt3a) and noncanonical Wnt signals (Wnt5a and Wnt11) in human SDSCs during cell expansion (A, B) and chondrogenic induction (C, D) at both mRNA and protein levels. β -actin was used as an internal control. Data are shown as average $\pm$ standard deviation (SD) for $n = 4$. $*p < 0.05$ indicated a statistically significant difference. ImageJ software was used to quantify immunoblotting bands.

Figure 2. 7 MAPK signals were actively involved in chondrogenesis of dECM expanded human SDSCs.

Western blot were used to evaluate Erk1/2, p38, and Jnk signals in both phosphorylated and nonphosphorylated formats in human SDSCs during cell expansion (A) and chondrogenic induction (B). β -actin was used as an internal control. ImageJ software was used to quantify immunoblotting bands.

Chapter 3: sb203580 preconditioning recharges matrix-expanded human adult stem cells for chondrogenesis in an inflammatory environment – a feasible approach for autologous stem cell based osteoarthritic cartilage repair

As published in Biomaterials. 2015; 64:88-97. doi:10.1016/j.biomaterials. 2015.06.038

Zhang Y¹, Pizzute T², Li J², He F³, Pei M⁴.

¹Stem Cell and Tissue Engineering Laboratory, Department of Orthopaedics, West Virginia University, Morgantown, WV 26506, USA; Mechanical and Aerospace Engineering, West Virginia University, Morgantown, WV 26506, USA.

²Stem Cell and Tissue Engineering Laboratory, Department of Orthopaedics, West Virginia University, Morgantown, WV 26506, USA; Exercise Physiology, West Virginia University, Morgantown, WV 26506, USA.

³Stem Cell and Tissue Engineering Laboratory, Department of Orthopaedics, West Virginia University, Morgantown, WV 26506, USA; Exercise Physiology, West Virginia University, Morgantown, WV 26506, USA; Orthopaedic Institute, Soochow University, Suzhou 215007, China.

⁴Stem Cell and Tissue Engineering Laboratory, Department of Orthopaedics, West Virginia University, Morgantown, WV 26506, USA; Mechanical and Aerospace Engineering, West Virginia University, Morgantown, WV 26506, USA; Exercise Physiology, West Virginia University, Morgantown, WV 26506, USA.

Running head: sb203580 priming of matrix-mediated chondrogenesis

Abstract

Autologous stem cells are a promising cell source for cartilage regeneration; however, cell replicative senescence and joint posttraumatic inflammation provide challenges to bring this treatment modality to fruition. In this study, we hypothesized that preconditioning with p38 MAPK inhibitor (sb203580) would recharge decellularized extracellular matrix (dECM) expanded human synovium-derived stem cell (hSDSC) chondrogenesis in an inflammatory environment. We found that preconditioning with sb203580 greatly enhanced dECM expanded hSDSC proliferation and chondrogenic potential while supplementation with sb203580 in an induction medium dramatically retarded hSDSC chondrogenic differentiation, even for dECM expanded cells. We also found that sb203580 preconditioning enhanced matrix-expanded hSDSC chondrogenic capacity even in an interleukin-1 (IL-1) induced inflammatory environment. Non-detectable expression of HLA-DR in the hSDSCs grown on allogeneic dECM indicates the feasibility of commercial preparation of these dECMs from healthy, young donors for patients who need autologous transplantation. Our study indicated that p38 MAPK inhibitor has a distinctive priming effect on dECM mediated stem cell cartilage regeneration. Combined rejuvenation with sb203580 and dECM expansion can precondition hSDSCs' resurfacing capacity for osteoarthritic patients with cartilage defects.

Introduction

Cartilage defects do not heal themselves due to a shortage of blood supply. Recently, adult stem cells have become a promising cell source for autologous cartilage regeneration. Adipose-derived stem cells (ASCs) have limited chondrogenic ability and bone marrow stromal cells (BMSCs) tend to undergo endochondral ossification upon chondrogenic induction [1]. By comparison, synovium-derived stem cells (SDSCs) are a tissue-specific stem cell for cartilage regeneration [2] and have been successfully used in cartilage regeneration studies [3-5]. *In vitro* expansion is a necessary step before *in vivo* application and represents a formidable challenge since stem cells are thought to exist in “niches” where extrinsic signals modulate the intrinsic signals that drive self-renewal and cell fate determination [6]. Outside of their niche, adult stem cells lose their developmental potential quickly and tend to either randomly differentiate or undergo apoptosis over time [7].

Decellularized extracellular matrix (dECM) could provide such an *in vitro* “niche”-like nanostructured microenvironment in which porcine SDSCs (pSDSCs) were greatly expanded with enhanced chondrogenic potential [8,9]. Despite success in using adult human SDSCs (hSDSCs) in this system [10], the rejuvenation effect was not as robust as that using young pSDSCs [8]. Different species and donor age might cause this discrepancy [7]. Furthermore, posttraumatic joint inflammation, normally accompanying cartilage defects [11,12], could perhaps lead to reduced efficiency of dECM on the rejuvenation of adult hSDSCs [13].

The p38 mitogen-activated protein kinase (MAPK) signaling cascade is known to be involved in various biological responses such as cell proliferation and differentiation [14]. Recently, p38 MAPK was also found to be activated by various pro-inflammatory and stressful stimuli [15]. There is increasing evidence showing that *in vivo* application of p38 MAPK inhibitors can decrease inflammation and related damage [16]; unfortunately, these inhibitors also arrest tissue regeneration [17-19].

To maximize advantages and minimize disadvantages, in this study, a p38 MAPK inhibitor was used to precondition hSDSCs during cell expansion on either plastic flasks (Plastic) or dECM followed by chondrogenic induction in a pellet culture system. In comparison, p38 MAPK inhibitor was supplemented in induction medium instead for the assessment of the direct effect on hSDSC chondrogenesis. Expanded hSDSCs were also evaluated for chondrogenic capacity in an interleukin-1

beta (IL-1 β) induced inflammatory environment. Lastly, dECM deposited by allogeneic cells were evaluated for eliciting potential immune issues in hSDSCs after expansion. We hypothesized that preconditioning with p38 MAPK inhibitor would recharge dECM expanded hSDSC chondrogenesis in an inflammatory environment.

Materials and Methods

SDSC culture

Adult human synovial fibroblasts (4 donors, two male and two female, average 43 years old, all had no known joint disease), referred to as hSDSCs [10,20,21], were obtained from Asterand (North America Laboratories, Detroit, MI). Human SDSCs were plated and cultured in a growth medium [alpha minimum essential medium (α MEM) containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 μ g/mL streptomycin, and 0.25 μ g/mL fungizone (Invitrogen, Carlsbad, CA)] at 37°C in a humidified 5% CO₂ and 21% O₂ incubator. The medium was changed every three days.

dECM preparation

The preparation of dECM was described in our previous study [10,21,22]. Briefly, plastic flasks (Plastic) were precoated with 0.2% gelatin (Sigma-Aldrich, St. Louis, MO) at 37°C for 1 h and seeded with passage 3 (P3) SDSCs. After cells reached 90% confluence, 250 μ M L-ascorbic acid phosphate (Wako Chemicals USA, Inc., Richmond, VA) was added for 8 days. The deposited matrix was incubated with 0.5% Triton X-100 containing 20 mM ammonium hydroxide at 37°C for 5 min and stored at 4°C in phosphate-buffered saline (PBS) containing 100 U/mL penicillin, 100 μ g/mL streptomycin, and 0.25 μ g/mL fungizone.

Morphological characterization of dECM with or without hSDSCs

Representative samples (n=3) were primarily fixed in 2.5% glutaraldehyde (Sigma-Aldrich) for 2 h, followed by secondary fixation in 2% osmium tetroxide (Sigma-Aldrich) for another 2 h. The samples were then dehydrated in a gradient ethanol series, in hexamethyldisilazane (HMDS, Sigma-Aldrich) at a ratio of 1:1 with ethanol twice for 1 h each time, in HMDS at a ratio of 1:2 with ethanol overnight,

and in HMDS three times for 4 h each time. The samples were air-dried for 24 h and gold sputter was added. The images were recorded by a scanning electron microscope (SEM) (Hitachi, Model S 2400).

Preconditioning using p38 MAPK inhibitor during cell expansion

Passage 3 hSDSCs were cultured at 3000 cells/cm² for one passage (8 days) on two substrates: dECM or Plastic. Ten μ M of sb203580 (p38 MAPK inhibitor, LC Laboratories, Woburn, MA) were added 48 h after cell seeding throughout the culture. The groups without sb203580 preconditioning served as a control. There were four groups: Plastic expansion alone (Pcontrol), Plastic expansion plus sb203580 (Psb203580), dECM expansion alone (Econtrol), and dECM expansion plus sb203580 (Esb203580). The cell number in T175 flasks (n=6) from each group was counted using a hemocytometer. For proliferation index (PI), P3 hSDSCs (before expansion) were labeled with CellVue[®] Claret (Sigma-Aldrich) at 2×10^{-6} M for 5 min according to the manufacturer's protocol. After passaging, expanded cells were collected and measured using a BD FACS Calibur[™] flow cytometer (dual laser) (BD Biosciences, San Jose, CA). Twenty thousand events of each sample were collected using CellQuest Pro software (BD Biosciences) and PI was analyzed by ModFit LT[™] version 3.1 (Verity Software House, Topsham, ME).

The following primary antibodies were used to detect expanded hSDSC surface immunophenotype profiles: Alexa Fluor 488 anti-human CD29 antibody (BioLegend, San Diego, CA), FITC anti-human CD90 antibody (Biolegend), FITC anti-human CD105 antibody (BioLegend), PE anti-human SSEA4 (the stage-specific embryonic antigen 4) antibody (BioLegend), and isotype-matched IgGs (Beckman Coulter, Fullerton, CA). Samples (n=3) of each 2×10^5 expanded cells were incubated on ice in cold PBS containing 0.1% Chrom-Pure Human IgG whole molecule (Jackson ImmunoResearch Laboratories, West Grove, PA) and 1% NaN₃ (Sigma-Aldrich) for 30 min. The cells were then incubated in the dark in the primary antibody for 30 min. Fluorescence was analyzed by a FACS Calibur (BD Biosciences) using FCS Express software package (De Novo Software, Los Angeles, CA).

Chondrogenic induction of expanded hSDSCs with dECM and/or p38 MAPK inhibitor preconditioning

After *in vitro* expansion, 0.3×10^6 of hSDSCs from each group (Pcontrol, Psb203580, Econtrol, and

E203580) were centrifuged at 500 g for 5 min in a 15-mL polypropylene tube to form a pellet. After overnight incubation, the pellets were cultured for 35 days in a serum-free chondrogenic medium consisting of high-glucose Dulbecco's Modified Eagle's Medium (DMEM), 40 µg/mL proline, 100 nM dexamethasone, 100 U/mL penicillin, 100 µg/mL streptomycin, 0.1 mM ascorbic acid-2-phosphate, and 1×ITS™ Premix [6.25 µg/mL insulin, 6.25 µg/mL transferrin, 6.25 µg/mL selenous acid, 5.35 µg/mL linoleic acid, and 1.25 µg/mL bovine serum albumin (BSA), from BD Biosciences] with supplementation of 10 ng/mL transforming growth factor beta 3 (TGFβ3, PeproTech Inc., Rocky Hill, NJ). Chondrogenic differentiation was evaluated using histology, immunostaining, and biochemical analysis.

Representative pellets (n=3) were fixed in 4% paraformaldehyde at 4°C overnight, followed by dehydrating in a gradient ethanol series, clearing with xylene, and embedding in paraffin blocks. Five-µm thick sections were histochemically stained with Alcian blue (Sigma-Aldrich; counterstained with fast red) for sulfated glycosaminoglycans (GAGs). For immunohistochemical analysis, the sections were immunolabeled with primary antibody against type II collagen (Col II; II-II6B3; Developmental Studies Hybridoma Bank, Iowa City, IA) followed by the secondary antibody of biotinylated horse anti-mouse IgG (Vector, Burlingame, CA). Immunoactivity was detected using Vectastain ABC reagent (Vector) with 3,3'-diaminobenzidine as a substrate.

Representative pellets (n=4) were digested for 4 h at 60°C with 125 µg/mL papain in PBE buffer (100 mM phosphate and 10 mM ethylenediaminetetraacetic acid, pH 6.5) containing 10 mM cysteine, by using 200 µL of enzyme per sample. To quantify cell density, the amount of DNA in the papain digestion was measured using the Quant-iT™ PicoGreen® dsDNA assay kit (Invitrogen) with a CytoFluor® Series 4000 (Applied Biosystems, Foster City, CA). GAG was measured using dimethylmethylene blue dye and a Spectronic BioMate 3 Spectrophotometer (Thermo Scientific, Milford, MA) with bovine chondroitin sulfate as a standard.

Treatment using p38 MAPK inhibitor during chondrogenic induction

After *in vitro* expansion, the pellets from both the Plastic and dECM groups were cultured for 28 days in a serum-free chondrogenic medium supplemented with 10 µM of sb203580 throughout the culture. The groups without sb203580 treatment served as a control. Chondrogenic differentiation was evaluated using Alcian blue staining for sulfated GAGs, immunostaining for type II collagen, and

biochemical analysis for DNA and GAG amounts in pellets.

Preconditioning strategy for hSDSC-based chondrogenesis in an inflammatory environment

After *in vitro* expansion, the pellets from both the Plastic and dECM groups were cultured for 24 days in a serum-free chondrogenic medium. Two dosages of IL-1 β , 0.1 and 1 ng/mL, were applied either throughout the culture (day 0-24), at the early (day 0-15), or late stage (day 15-24) of chondrogenic induction. Chondrogenic differentiation was evaluated using Alcian blue staining for sulfated GAGs, immunostaining for type II collagen, biochemical analysis for DNA and GAG amounts in pellets, and TaqMan[®] real-time polymerase chain reaction (PCR) for chondrogenic markers including *SOX9* [SRY (sex determining region Y)-box 9], *ACAN* (aggrecan), and *COL2A1* (type II collagen).

After *in vitro* expansion with or without 10 μ M of sb203580, the pellets from both the Plastic and dECM groups were cultured for 35 days in a serum-free chondrogenic medium with 1 ng/mL of IL-1 β . Chondrogenic differentiation was evaluated using Alcian blue staining for sulfated GAGs, immunostaining for type II collagen, biochemical analysis for DNA and GAG amounts in pellets, and real-time PCR for chondrogenic markers in terms of *ACAN* and *COL2A1* and inflammatory factors including *PTGS2* (prostaglandin G/H synthase and cyclooxygenase), *VEGFA* (vascular endothelial growth factor A), and *MMP13* (matrix metalloproteinase 13).

Total RNA was extracted from pellets (n=4) using an RNase-free pestle in TRIzol[®] (Invitrogen). About 1 μ g of mRNA was used for reverse transcription with a High-Capacity cDNA Archive Kit (Applied Biosystems) at 37°C for 120 min. Chondrogenic marker genes [*COL2A1* (assay ID: Hs00156568_m1), *ACAN* (assay ID: Hs00153935_m1), and *SOX9* (assay ID: Hs00165814_m1)] and inflammatory genes [*PTGS2* (assay ID: Hs00153133_m1), *VEGFA* (assay ID: Hs00900055_m1), and *MMP13* (assay ID: Hs00233992_m1)] were customized by Applied Biosystems as part of the Custom TaqMan[®] Gene Expression Assays. Eukaryotic 18S RNA (assay ID: Hs99999901_s1) was carried out as the endogenous control gene. Real-time PCR was performed with the iCycler iQTM Multi-Color Real Time PCR Detection (Perkin-Elmer, Waltham, MA). Relative transcript levels were calculated as $\chi = 2^{-\Delta\Delta C_t}$, in which $\Delta\Delta C_t = \Delta E - \Delta C$, $\Delta E = C_{t_{exp}} - C_{t_{18s}}$, and $\Delta C = C_{t_{ctrl}} - C_{t_{18s}}$.

Potential immune issues in hSDSCs after expansion on dECM deposited by either allogeneic or xenogeneic cells

Human SDSCs were expanded on either Plastic or dECM deposited by either hSDSCs (HECM) or pSDSCs (PECM) followed by the evaluation of surface marker expression for CD45 using anti-CD45 APC (BD Biosciences) and HLA-DR [major histocompatibility complex (MHC), class II, DR] using anti-human HLA-DR FITC (eBioscience, San Diego, CA), and isotype-matched IgGs (Beckman Coulter). Samples (n=5) of each 3×10^5 expanded cells were incubated on ice in cold PBS containing 0.1% Chrom-Pure Human IgG whole molecule (Jackson ImmunoResearch Laboratories) and 1% NaN₃ (Sigma-Aldrich) for 30 min. The cells were then incubated in the dark in the primary antibodies (both HLA-DR and CD45 simultaneously) for 30 min. The fluorescence data were analyzed by a BD LSRFortessa™ (BD Biosciences), collected using BD FACSDiva 8.0 Software (BD Biosciences) at 10,000 events/sample. The results were analyzed using FCS Express 4 software (De Novo Software).

Statistics

Numerical data are presented as the mean and the standard error of the mean. A Mann-Whitney U test and a linear model with contrast analysis were used for pairwise comparison in biochemistry and real-time PCR data analysis. All statistical analyses were performed with SPSS 13.0 statistical software (SPSS Inc., Chicago, IL). A *p* value less than 0.05 was considered statistically significant.

Results

Preconditioning using sb203580 further promoted proliferation of dECM expanded cells

SEM data (Fig. 1A) showed that dECM deposited by hSDSCs was composed of randomly arranged fine matrix fibers and expansion on dECM yielded hSDSCs with a tiny fibroblast-like shape compared to a flattened and broad shape when grown on Plastic. Flow cytometry data (Fig. 1B) indicated that, despite a slight increase of proliferation index in hSDSCs when grown on Plastic, preconditioning using sb203580 presented a 2.62-fold increase in dECM expanded hSDSCs compared to a 1.65-fold increase in dECM expansion alone. Proliferation index data were confirmed by cell number change (Fig. 1C), in which preconditioning using sb203580 presented a 2.00-fold increase in dECM expanded hSDSCs compared to a 1.41-fold increase in dECM expansion alone. Flow cytometry data (Fig. 1D) also showed that mesenchymal stem cell (MSC) surface markers (CD29,

CD90, and CD105) were down-regulated at the median in dECM expanded hSDSCs; when combined with sb203580 preconditioning, the expression of the above markers presented a slight increase. Interestingly, sb203580 preconditioning increased SSEA4 expression in both percentage and median of Plastic expanded hSDSCs followed by dECM expansion with the co-preconditioning of sb203580 and dECM expansion having the largest.

sb203580 preconditioning enhanced dECM expanded hSDSC chondrogenic potential while use of sb203580 in the induction phase arrested hSDSC chondrogenic differentiation

To determine whether p38 MAPK inhibitor preconditioning played a role in hSDSC expansion and chondrogenic potential, 10 μ M of sb203580 was added to culture medium for hSDSC expansion on either Plastic or dECM. Both dECM expansion and sb203580 preconditioning alike led to chondrogenically differentiated 35-day pellets of a larger size with more intense staining of sulfated GAGs and type II collagen (Fig. 2A) and higher cell viability (DNA ratio adjusted by day 0) and GAG amount (chondrogenic marker) (Fig. 2B) compared to the corresponding Plastic group. Co-preconditioning with dECM and sb203580 yielded the largest hSDSC pellets (Fig. 2A) with the highest cell viability and GAG amount followed by the dECM group with the Plastic group having the lowest viability (Fig. 2B).

To determine whether p38 MAPK inhibitor played a role in hSDSC chondrogenic differentiation, 10 μ M of sb203580 was supplemented in a serum-free chondrogenic medium. Due to a dramatic decrease in pellet size from both the Plastic and dECM groups, chondrogenic induction was ended at day 28. Despite enhanced chondrogenic staining in the pellets from the dECM group, the addition of sb203580 in the induction medium significantly decreased both staining and size of pellets in both groups, even as early as day 14 (Fig. 3A). The above findings were corroborated by biochemical analysis data, in which sb203580 arrested cell viability and reduced both GAG amount per pellet and ratio of GAG to DNA in both the Plastic and dECM groups (Fig. 3B).

dECM expansion protects chondrogenically induced hSDSCs from IL-1 β induced inflammatory stress

To determine whether dECM expanded hSDSCs had the ability to resist inflammatory stress, two doses of IL-1 β , 0.1 and 1 ng/mL, were used to treat chondrogenically induced hSDSCs after

expansion on either dECM or Plastic. Continuous treatment with IL-1 β from day 0 to day 15 of chondrogenic induction yielded small pellets with less intensive staining of sulfated GAGs and type II collagen compared to the untreated control in both the dECM and Plastic groups, particularly for treatment with 1 ng/mL of IL-1 β (Fig. 4A). Without IL-1 β treatment, dECM expanded hSDSCs yielded pellets with higher GAG amount per pellet and ratio of GAG to DNA than those from Plastic expansion. With IL-1 β treatment, both GAG amount per pellet and ratio of GAG to DNA decreased in a dose-dependent fashion; however, dECM expanded hSDSCs yielded pellets with a significantly higher ratio of GAG to DNA than those from Plastic expansion (Fig. 5A).

Continuous treatment with IL-1 β from day 0 to day 24 of chondrogenic induction yielded pellets with a dramatic decrease in both size and chondrogenic staining when 1 ng/mL of IL-1 β was applied; there was no significant difference when 0.1 ng/mL of IL-1 β was used, particularly for dECM expanded cells (Fig. 4B). Interestingly, treatment with IL-1 β from day 15 to day 24 of chondrogenic induction yielded pellets with a significant improvement in both size and chondrogenic staining for both dECM and Plastic expansion even in the presence of 1 ng/mL of IL-1 β (Fig. 4C). The above histology result was supported by quantification data. Biochemical analysis data showed that continuous treatment with 1 ng/mL of IL-1 β yielded pellets with a significant decrease in both DNA ratio and ratio of GAG to DNA (Fig. 5B). However, treatment with 1 ng/mL of IL-1 β from day 15 to day 24 of chondrogenic induction yielded pellets with a significant increase in both DNA ratio and GAG amount compared to the corresponding group with continuous IL-1 β treatment (Fig. 5B). The above protein level data were also consistent with the mRNA data using real-time PCR for chondrogenic marker genes including *SOX9*, *ACAN*, and *COL2A1* (Fig. 5C).

sb203580 preconditioning promotes dECM rejuvenated hSDSCs' ability against inflammation during chondrogenic induction

The above results indicated that continuous treatment with 1 ng/mL of IL-1 β from day 0 to day 24 of chondrogenic induction yielded pellets with diminished chondrogenic differentiation in hSDSCs from dECM expansion compared with the corresponding control without IL-1 β treatment. In this study, we assumed that co-preconditioning using dECM and sb203580 would retard the diminished chondrogenic differentiation of dECM expanded hSDSCs in the presence of a high concentration of

IL-1 β . Continuous treatment for 35 days with 1 ng/mL of IL-1 β reduced the difference in chondrogenic capacity in hSDSCs from dECM and Plastic expansion. Compared to the slight increase in pellet size observed in Plastic expanded cells, preconditioning using sb203580 dramatically increased both size and chondrogenic staining in the 35-day IL-1 β treated pellets from dECM expanded hSDSCs (Fig. 6A). This histology result was also supported by both biochemical analysis and real-time PCR data. dECM expanded cells yielded pellets with a higher amount of GAG and a higher ratio of GAG to DNA compared to those from Plastic expanded cells in the presence of 1 ng/mL of IL-1 β (Fig. 6B) despite no difference in *ACAN* and *COL2A1* (Fig. 6C). Preconditioning using sb203580 dramatically increased both GAG amount and ratio of GAG to DNA (Fig. 6B) as well as *ACAN* and *COL2A1* (Fig. 6C) in hSDSC pellets from both dECM and Plastic expansion with the co-preconditioning group being the highest.

To determine whether sb203580 preconditioning had any inhibitory effect on inflammation during subsequent chondrogenic induction, real-time PCR data (Fig. 6D) showed that, without sb203580 preconditioning, dECM expanded hSDSCs yielded 35-day pellets with a significantly lower *VEGFA* level compared to those from Plastic expanded cells. With sb203580 preconditioning, dECM expanded hSDSCs yielded pellets with significantly lower levels of *PTGS2*, *MMP13*, and *VEGFA*; a notable increase in these gene expressions occurred in pellets from Plastic expanded hSDSCs.

Allogeneic dECM expanded hSDSCs exhibited a non-detectable HLA-DR expression

Flow cytometry was also used to determine whether expansion of hSDSCs on Plastic, HECM (allogeneic substrate), or PECM (xenogeneic substrate) increased HLA-DR expression. After subtracting the respective unstained negative controls (Fig. 7A/B) from the percent HLA-DR-positive values for both Plastic (Fig. 7C) and dECM substrates (Fig. 7D/E), the percent HLA-DR-positive cells in each population were 1.08% for Plastic expanded hSDSCs (Fig. 7C), 0.81% for HECM expanded hSDSCs (Fig. 7D), and 6.48% for PECM expanded hSDSCs (Fig. 7E). These cells also exhibited a similar trend when comparing the mean fluorescence intensity (MFI) values: the Plastic group was 474.19, the HECM group was 357.23, and the PECM group was 583.29. Additionally, all hSDSC samples were CD45 negative, ensuring there was no persisting contamination in the hSDSC populations with HLA-DR⁺ immune cells following their isolation from synovial tissue.

Discussion

Cartilage tissue engineering has emerged as a potential therapeutic option for cartilage repair; however, once transplanted into the joints, cells or premature cartilage often face an inflammatory environment, making cartilage resurfacing challenging [13]. Among the proinflammatory cytokines, IL-1 β acts as an important mediator of increased ECM degradation by stimulating a number of events, such as an increase of MMPs and nitric oxide production [23] and by suppressing type II collagen and proteoglycan synthesis [24]. In osteoarthritic joints, the IL-1 β concentration was reported to range from 1-10 pg/mL [25]. Xie *et al.* reported that the mean concentration of IL-1 β in synovial fluid from early osteoarthritis in dysplastic hips was 55 (SD 12), ranging from 24 to 74 pg/mL [26]. Attur *et al.* reported that 5 ng/mL of IL-1 β could significantly suppress proteoglycan synthesis from both human and bovine chondrocytes [27]. Yudoh *et al.* reported a significant reduction in production of type II collagen by rabbit chondrocytes incubated in the presence of 10 ng/mL of IL-1 β [28]. Thus, in this study, IL-1 β was added into chondrogenic medium to simulate the inflammatory environment often encountered in joint capsule; 0.1 ng/mL of IL-1 β was chosen to represent a relatively low concentration despite the fact that this dose is still higher than the reported pathological concentration of IL-1 β in osteoarthritis [25]. One ng/mL of IL-1 β was chosen to represent a relatively high concentration.

In this report, we found that dECM expansion could maintain an advantage over Plastic expansion in chondrogenic capacity of hSDSCs in the presence of a low concentration (0.1 ng/mL) of IL-1 β . However, this advantage was diminished when a high concentration (1 ng/mL) of IL-1 β was applied during chondrogenic induction. Interestingly, premature tissue pellets from dECM expanded hSDSCs could, to a certain degree, resist the decline of chondrogenic differentiation resulting from a high concentration of IL-1 β . This finding is consistent with a previous report in which more mature cartilaginous tissue was more resistant to 1 ng/mL of IL-1 β exposure [29]. Previous investigations showed that inflammatory-related genes such as *MMP13*, *PTGS2*, and *VEGF* were upregulated in the presence of IL-1 β but were blocked by application of sb203580 [30-32]. In this study, despite a higher expression in the preconditioning group by sb203580, these inflammatory genes were significantly downregulated in chondrogenically induced hSDSCs when a co-preconditioning strategy with dECM expansion was applied. This finding might be attributable to the anti-inflammatory function of

sb203580 [16,33] and antioxidant characteristics of dECM during cell expansion [10].

Consistent with previous reports [34,35], we found that the use of sb203580 in the induction medium significantly diminished TGF β mediated hSDSC chondrogenic differentiation. It is well known that TGF β induces chondrogenic differentiation classically through the SMAD pathway and also via p38 MAPK signaling [36]. For instance, TGF β induced rapid phosphorylation of SMAD2, extracellular signal-activated kinase 1/2 (ERK1/2), and p38 MAPK in ATDC5 cells; however, incubation with sb203580 repressed TGF β -induced *ACAN*, suggesting an important role for transcriptional cross-talk between the TGF β and p38 MAPK pathways in expression of early chondrocyte differentiation [37]. A recent study suggested that, following activation by TGF β , MAPKs (in particular the p38 MAPK pathway) were involved in the control of post-translational modification of SMADs by a direct phosphorylation or through their downstream effector molecules [38]. Another report indicated that SOX9 is likely a downstream target of the p38 MAPK pathway because the increased activity of the SOX9-dependent *COL2A1* enhancer in primary chondrocytes was abolished by sb203580 [39].

Our recent study demonstrated that dECM deposited by young and healthy hSDSCs could rejuvenate older hSDSCs in chondrogenic potential [22], indicating that allogeneic dECM can be a promising approach to expand patient hSDSCs for autologous transplantation. To solve the immune concerns in hSDSCs after expansion on allogeneic dECMs, expression of HLA-DR was used as an indicator since HLA-DR is known to be involved in graft rejection and other adverse immune events [40,41]. The flow cytometry data showed no increase in HLA-DR expression in the HECM (allogeneic control) expanded cells compared to the Plastic group (negative control); however, there was a greater than 6-fold increase in the percent HLA-DR-positive expression when hSDSCs were expanded on PECM (xenogeneic control) versus Plastic or HECM substrates. Not only does the PECM-expansion of hSDSCs seem to produce more HLA-DR⁺ cells than the Plastic or HECM-expansion, but the MFI values also suggest that these PECM-expanded cells seem to express more HLA-DR per cell. These increases in HLA-DR⁺ expression could be troubling for future clinical use of PECM substrates for hSDSC expansion strategies, as studies have demonstrated the ability of MHC class II-positive antigen-presenting cells, and more recently synovial fibroblasts, to cause T-cell activation [42,43]. In order to verify that all hSDSC populations were void of immune cells whose presence could create false positives for HLA-DR expression, CD45 expression was also examined. All hSDSCs, regardless

of expansion condition, were comparatively negative for CD45 expression, leading us to conclude that the hSDSCs were the cells expressing HLA-DR. Compared to potential immunogenicity from PECM-expanded hSDSCs, allogeneic HECM-expansion seems a potentially more attractive strategy for future clinical use; however, further *in vivo* studies will be necessary to investigate this issue.

Conclusion

This study is the first to investigate p38 MAPK inhibitor for its preconditioning in hSDSC proliferation and chondrogenic potential as well as its inhibitory effect on hSDSC chondrogenic differentiation. The joint preconditioning effect of p38 MAPK inhibitor and dECM expansion on hSDSC chondrogenesis in the presence of IL-1 β was also explored. We found that preconditioning with sb203580 favored hSDSC proliferation and chondrogenic capacity, which was enhanced in combination with dECM expansion; however, the application of sb203580 in the induction medium suppressed the growth of chondrogenic pellets in both the Plastic and dECM groups. The inhibitory effect of IL-1 β on chondrogenic differentiation of expanded hSDSCs was in a dose- and phase-dependent manner. Despite the fact that pre-differentiated hSDSCs acquired an enhanced capacity against inflammatory insult, expanded hSDSCs could only survive chondrogenic induction in the presence of a low dose of IL-1 β ; interestingly, we found that sb203580 preconditioning could enhance matrix expanded hSDSC chondrogenesis even under stimulation by a high dose of IL-1 β . This preconditioning strategy could enhance the successful rate of implantation of undifferentiated stem cells or premature tissue constructs in treating cartilage defects in which a harsh inflammatory environment exists. Non-detectable expression of HLA-DR in hSDSCs grown on allogeneic dECM indicates the feasibility of commercial preparation of these dECMs from healthy, young donors for patients who need autologous transplantation.

References

1. Pizzute T, Lynch K, Pei M. *Stem Cell Rev* 2015;**11**:119-32.
2. Jones B, Pei M. *Tissue Eng Part B Rev* 2012;**18**:301-11.
3. Lee JC, Min HJ, Park HJ et al. *Arthroscopy* 2013;**29**:1034-46.
4. Pei M, He F, Boyce BM et al. *Osteoarthritis Cartilage* 2009;**17**:714-22.
5. Pei M, He F, Li J et al. *Tissue Eng Part A* 2013;**19**:1144-54.
6. Lynch K, Pei M. *Organogenesis* 2014;**10**:289-98.
7. Li JT, Pei M. *Tissue Eng Part B* 2012;**18**:270-87.
8. He F, Chen XD, Pei M. *Tissue Eng Part A* 2009;**15**:3809-21.
9. Pei M, Li JT, Shoukry M et al. *Eur Cell Mater* 2011;**22**:333-43.
10. Pei M, Zhang Y, Li JT et al. *Stem Cells Dev* 2013;**22**:889-900.
11. Gruber J, Vincent TL, Hermansson M et al. *Arthritis Rheum* 2004;**50**:2539-46.
12. Hembry RM, Dyce J, Driesang I et al. *J Bone Joint Surg Am* 2001;**83A**:826-38.
13. Zhang Y, Pizzute T, Pei M. *Tissue Eng Part B Rev* 2014;**20**:655-68.
14. Ono K, Han J. *Cell Signal* 2000;**12**:1-13.
15. Schindler JF, Monahan JB, Smith WG. *J Dent Res* 2007;**86**:800-11.
16. Brown KK, Heitmeyer SA, Hookfin EB et al. *J Inflamm (Lond)* 2008;**5**:22.
17. Braem K, Luyten FP, Lories RJ. *Ann Rheum Dis* 2012;**71**:722-8.
18. Prasadani I, Mao X, Wang Y et al. *Rheumatology* 2012;**51**:813-23.
19. Yong HY, Koh MS, Moon A. *Expert Opin Investig Drugs* 2009;**18**:1893-905.
20. Li JT, Jones B, Zhang Y et al. *Drug Deliv Transl Res* 2012;**2**:363-74.
21. Zhang Y, Li J, Davis ME et al. *Acta Biomater* 2015;**20**:39-50.
22. Li JT, Hansen K, Zhang Y et al. *Biomaterials* 2014;**35**:642-53.
23. Van der Kraan PM, van den Berg WB. *Curr Opin Clin Nutr Metab Care* 2000;**3**:205-11.
24. Chevalier X. *Biomed Pharmacother* 1997;**51**:58-62.
25. Barakat AF, Elson CJ, Westacott CI. *Osteoarthritis Cartilage* 2002;**10**:264-9.
26. Xie J, Naito M, Maeyama A. *Acta Orthop* 2010;**81**:189-92.
27. Yudoh K, Shishido K, Murayama H et al. *Arthritis Rheum* 2007;**56**:3307-18.
28. Francioli S, Cavallo C, Grigolo B et al. *Clin Orthop Relat Res* 2011;**469**:2773-84.
29. Joos H, Albrecht W, Laufer S et al. *Br J Pharmacol* 2010;**160**:1252-62.
30. Joos H, Albrecht W, Laufer S et al. *Int J Mol Med* 2009;**23**:685-93.
31. Tanaka T, Kanai H, Sekiguchi K et al. *J Mol Cell Cardiol* 2000;**32**:1955-67.
32. Han SK, Jeon SJ, Miyazawa K et al. *Rheumatol Int* 2006;**26**:972-8.
33. Oh CD, Chang SH, Yoon YM et al. *J Biol Chem* 2000;**275**:5613-9.
34. Prasadani I, Mao X, Wang Y et al. *Rheumatology (Oxford)* 2012;**51**:813-23.
35. Horbelt D, Denkis A, Knaus P. *Int J Biochem Cell Biol* 2012;**44**:469-74.
36. Watanabe H, de Caestecker MP, Yamada Y. *J Biol Chem* 2001;**276**:14466-73.
37. Javelaud D, Mauviel A. *Oncogene* 2005;**24**:5742-50.
38. Zhang R, Murakami S, Coustry F et al. *Proc Natl Acad Sci U S A* 2006;**103**:365-70.
39. Claesson K. *Scand J Urol Nephrol Suppl* 1987;**103**:1-42.
40. Döring M, Rohrer KM, Erbacher A et al. *Ann Hematol* 2015;**94**:265-73.
41. Conte E, Gili E, Fruciano M et al. *Cell Immunol* 2013;**285**:55-61.

42. Kraft M, Filsinger S, Krämer KL et al. *Immunology* 1995;**85**:461-6.

Figure 1

Figure 3. 1 Preconditioning using sb203580 enhanced proliferation of dECM expanded hSDSCs.

(A) Scanning electron microscopy was used to characterize surface topography of both Plastic and dECM (scale bar: 20 μm) and morphology of expanded hSDSCs (scale bar: 200 μm). (B) Human SDSCs were expanded on either dECM or Plastic for one passage (8 days) with or without preconditioning of sb203580. Flow cytometry was used to measure proliferation index of expanded cells. (C) A hemocytometer was used to measure cell numbers in T175 flasks (n=6) from each group. (D) Flow cytometry was used to measure both percentage and median fluorescence intensity of MSC surface markers (CD29, CD90, and CD105) and SSEA4 of expanded cells.

Figure 2

Figure 3. 2 Preconditioning using sb203580 enhanced chondrogenesis of dECM expanded hSDSCs.

Human SDSCs expanded on either dECM or Plastic for one passage (8 days) with or without preconditioning of sb203580 were followed by a 35-day chondrogenic induction in a pellet system. (A) Pellets were photographed before histology; Alcian blue (Ab) was used to stain sulfated GAGs and immunohistochemical staining (IHC) was for Col II. (B) Biochemical analysis was used for DNA and GAG amounts in chondrogenic pellets. Cell proliferation and viability were evaluated using DNA ratio (DNA amount at days 35 and 14 adjusted by that at day 0). Chondrogenic index was evaluated using ratio of GAG to DNA. Data are shown as average $\pm$ SD for $n = 4$. * $p < 0.05$ suggests a statistically significant difference. NS indicates no significant difference.

Figure 3

Figure 3. 3 Human SDSCs expanded on either dECM or Plastic for one passage were followed by a 28-day chondrogenic induction in a pellet system with or without preconditioning of sb203580.

(A) Pellets were photographed before histology; Alcian blue (Ab) was used to stain sulfated GAGs and immunohistochemical staining (IHC) was for Col II. (B) Biochemical analysis was used for DNA and GAG amounts in chondrogenic pellets. Cell proliferation and viability was evaluated using DNA ratio (DNA amount at days 28 and 14 adjusted by that at day 0). Chondrogenic index was evaluated using ratio of GAG to DNA. Data are shown as average $\pm$ SD for $n = 4$. * $p < 0.05$ suggests a statistically significant difference.

Figure 4

Figure 3. 4 Chondrogenic induction of dECM-expanded hSDSCs in the presence of IL-1 β .

Human SDSCs were expanded on either Plastic or dECM for one passage followed by a pellet culture in a serum-free chondrogenic medium supplemented with either 0.1 or 1 ng/mL of IL-1 β treatment for 15 days (A), 24 days (B), or the later stage between the 15th and 24th days (C). Chondrogenic induction without IL-1 β treatment served as a control. Alcian blue (Ab) was used to stain sulfated GAGs. Immunohistochemistry staining (IHC) was used to detect collagen II (Col II).

Figure 5

Figure 3. 5 Chondrogenic induction of dECM-expanded hSDSCs in the presence of IL-1 β .

Human SDSCs were expanded on either Plastic or dECM for one passage followed by a pellet culture in a serum-free chondrogenic medium supplemented with either 0.1 or 1 ng/mL of IL-1 β treatment for 15 days (A), 24 days, or the later stage between the 15th and 24th days (B). Biochemical analysis was used for DNA and GAG amounts in the chondrogenically-induced pellets. Cell proliferation and viability was evaluated using DNA ratio (DNA amount adjusted by that at day 0). Chondrogenic index was evaluated using a ratio of GAG to DNA. (C) Real-time PCR was used to evaluate chondrogenic marker gene expression (SOX9, ACAN, and COL2A1) in day 24 pellets. Data are shown as average $\pm$ SD for n = 4. * p < 0.05 compared with the corresponding Plastic group. # p < 0.05 compared with the corresponding group with the same dose treatment of IL-1 β .

Figure 6

Figure 3. 6 35-day-chondrogenic induction of dECM-expanded hSDSCs preconditioned with sb203580 in the presence of 1 ng/mL IL-1 β .

Human SDSCs were expanded on either Plastic or dECM for one passage with or without preconditioning using sb203580 followed by a 35-day pellet culture in a serum-free chondrogenic medium supplemented with continuous treatment of IL-1 β at 1 ng/mL. (A) Alcian blue (Ab) was used to stain sulfated GAGs. Immunohistochemistry staining (IHC) was used to detect collagen II (Col II). (B) Biochemical analysis was used for DNA and GAG amounts in the 35-day chondrogenically-induced pellets. Chondrogenic index was evaluated using a ratio of GAG to DNA. (C) Real-time PCR was used to evaluate chondrogenic marker gene expression (ACAN and COL2A1) in day 35 pellets. (D) Real-time PCR was used to evaluate inflammatory marker gene expression (PTGS2, MMP13, and VEGFA) in day 35 pellets. Data are shown as average $\pm$ SD for $n = 4$. * $p < 0.05$ compared with the corresponding Plastic group. # $p < 0.05$ compared with the corresponding group without sb203580 treatment.

Figure 7

Figure 3. 7 Flow cytometry analysis of CD45 and HLA-DR surface marker expression following cell expansion.

Unstained Plastic-expanded (A) and dECM-expanded (B) hSDSCs were used as negative controls. CD45 and HLA-DR surface marker expression was analyzed for each sample of hSDSCs expanded on Plastic (C), HECM (D), and PECM substrates (E). Additionally, a separate macrophage/monocyte population was analyzed for CD45 expression (F) as a positive control.

Chapter 4. Biomechanical signals guiding stem cell cartilage engineering: from molecular adaption to tissue functionality

As submitted to *Eur Cell Mater*

^{1,2,#}Ying Zhang, MS, ^{3,#}Song Chen, MS, and ^{1,2,4,*}Ming Pei, MD, PhD

¹Stem Cell and Tissue Engineering Laboratory, Department of Orthopaedics, West Virginia University, Morgantown, West Virginia 26506, USA; ²Mechanical and Aerospace Engineering, West Virginia University, Morgantown, West Virginia 26506, USA; ³Department of Arthroplasty Surgery, Changzheng Hospital, Second Military Medical University, Shanghai 200003, China; ⁴Exercise Physiology, West Virginia University, Morgantown, West Virginia 26506, USA

Running head: Biomechanical Signals & Stem Cell Chondrogenic Differentiation

Key words: Biomechanics; Cartilage Regeneration; Extracellular Matrix; Stiffness; Stem Cell

***Corresponding author:** Ming Pei MD, PhD, Stem Cell and Tissue Engineering Laboratory, Department of Orthopaedics, West Virginia University, PO Box 9196, One Medical Center Drive, Morgantown, WV 26506-9196, USA, Telephone: 304-293-1072; Fax: 304-293-7070; Email: mpei@hsc.wvu.edu

Abstract

In vivo Cartilage is in a state of constant mechanical stimulation. It is therefore reasonable to deduce that mechanical forces play an important role in cartilage formation. Mechanical forces, such as compression, tension, and shear force, have been widely applied for cartilage engineering; however, relatively few review papers have summarized the influences of biomechanical signals on stem cell-based neo-cartilage formation and cartilage engineering in both molecular adaption and tissue functionality. In this review, we will discuss recent progress related to the influences of substrate elasticity on stem cell chondrogenic differentiation and elucidate the potential underlying mechanisms. Aside from active sensing and responding to the extracellular environment, stem cells also could respond to various external mechanical forces, which also influence their chondrogenic capacity; this topic will be discussed along with associated signaling pathways. We expect that these different regimens of biomechanical signals can be utilized to boost stem cell-based cartilage engineering and regeneration.

Introduction

Damage to cartilage represents one of the biggest challenges in musculoskeletal therapeutics due to its limited aptitude for healing and regenerative capabilities [1]. Lack of treatments to restore cartilage tissue function has prompted research on functional cartilage tissue substitutes in this rapidly emerging field [2]. Engineered tissue implants are attractive and offer a promising approach to cartilage restoration. Many tissue engineering techniques have been attempted such as developing alternate cell sources, optimizing scaffolds, and applying novel exogenous agents and mechanical stimulation regimens to produce cartilage constructs that could match native cartilage in biochemical and biomechanical properties [3].

Although stem cell biologists have long appreciated the regulatory roles of soluble stem cell niche signals (e.g., growth factors and cytokines) in regulating stem cell fate, recent evidence demonstrated that biophysical information such as mechanical cues that exist in the extracellular microenvironment can also be sensed by cells. This information can be transduced into intracellular biochemical and functional responses, a process known as mechanotransduction [4, 5], which plays a critical role in determining stem cell fate. There are two different regimens of mechanotransduction: “inside-out” or “outside-in” sensing. For the active “inside-out” sensing, changes in extracellular matrix (ECM) stiffness and surface topography can be felt and responded to by cells through generating traction forces. For the passive “outside-in” sensing, the cell responds to a force imparted upon itself such as shear stress, compression, or pressure [6].

The report from Evans and colleagues suggests a fundamental role for mechanosensing in mammalian development and illustrates that the mechanical environment should be taken into consideration when engineering implantable scaffolds or when producing therapeutically relevant cell populations *in vitro* [7]. While biomechanics-driven approaches have been thoroughly investigated for their potential influence on mesenchymal stem cell (MSC) differentiation [8], relatively few review papers have summarized the influences of biomechanical signals on stem cell based neo-cartilage formation and cartilage engineering in both molecular adaption and tissue functionality. In this review, we will discuss recent progress related to the influences of substrate elasticity on stem cell chondrogenic differentiation and elucidate the potential underlying mechanisms. Aside from the active sensing and responding to the extracellular environment, stem cells also could respond to various external

mechanical forces, which also influence their chondrogenic capacity; this topics will be discussed along with associated signaling pathways.

Substrate elasticity influences stem cell/chondrocyte fate

Landmark work by Engler and coworkers (2006) [9] suggests that soft (elastic modulus, E , 0.1-1 kPa), medium (E , 8-17 kPa), and hard (E , 25-40 kPa) matrices can induce MSC lineage specification toward neurons, muscles, and osteoblasts, respectively. A recent report using a three-dimensional (3D) numerical model showed that matrix stiffness plays a significant role in controlling the fate of MSCs [10]. Substrate rigidity was also found to exert long-term effects on modulation of cell proliferation [11]. Studies from different elasticity evaluation systems have provided a lot of evidence suggesting that chondrocytes and stem cells grown on soft substrates tend to chondrogenic differentiation compared to stiffer substrates. (Table 1)

Cells grown on less stiff hydrogels showed enhanced chondrogenic potential

The development of hydrogels tailored for cartilage engineering has been a research and clinical goal for over a decade. Coaxing stem cells toward a chondrogenic phenotype and promoting new matrix formation are a significant challenge that must be overcome for the successful application of hydrogels in cartilage repair. A previous report indicates that stiffer hydrogels affect ligand presentation and gel porosity, which influence cell proliferation, clustering, migration, and ECM deposition patterns [12]. In designing hydrogels for cartilage engineering, their elasticity is an important factor determining stem cell/chondrocyte chondrogenic differentiation.

Polyacrylamide (PAM) hydrogels

Xue and colleagues (2013) [13] found that, using polyacrylamide (PAM) hydrogels with different stiffnesses as culture substrates, human bone marrow MSC (BMSC) aggregation was more predominant when cultured on soft matrix (E , 1.6 ± 0.3 kPa) rather than on hard matrix (E , 40 ± 3.6 kPa); expanded on soft matrix BMSCs exhibited a dramatic upregulation of chondrogenic marker genes, *SOX9* [SRY (sex determining region Y)-box 9], *ACAN* (aggrecan), and *COL2A1* (type II

collagen). In contrast, the promotion of osteogenic marker expression by hard gel was overridden by a high seeding density. Interestingly, Allen and coworkers (2012) [14] reported that primary murine chondrocytes and ATDC5 cells grown on 0.5 MPa PAM hydrogel substrates deposited more proteoglycan and expressed more *SOX9*, *ACAN*, and *COL2A1* mRNA relative to cells exposed to substrates of other stiffnesses (E , 0.2 MPa and 1.1 MPa). This finding suggests that the chondroinductive effect of this discrete stiffness (E , 0.5 MPa), which falls within the range reported for articular cartilage [15], requires the stiffness-sensitive induction of transforming growth factor beta1 (TGF β 1).

Less stiff substrates favoring a chondrogenic lineage also apply to hybrid hydrogels. Kwon and Yasuda (2013) [16] found that mouse BMSCs cultured on the high stiffness sulfonate-coated PAM (S-PAM) hydrogels (E , ~150 kPa) spread out with strong expression of stress fibers, while BMSCs cultured on the low stiffness S-PAM hydrogels (E , ~1 kPa) had round shapes with fewer stress fibers but more cortical actins. Importantly, even in the absence of chondrogenic induction, the lower stiffness S-PAM hydrogels led to higher mRNA levels of chondrogenic markers *COL2A1*, *ACAN*, and *SOX9* and lower mRNA levels of an undifferentiation marker *SCA1* (stem cell antigen-1), indicating that the mechanical properties of S-PAM hydrogels strongly influence chondrogenesis. Similarly, Park and colleagues found that human BMSCs on stiff substrates (type I collagen-coated PAM hydrogels) had higher expression of smooth muscle cell (SMC) markers *ACTA1* (α -actin) and *CNN1* (calponin 1); in contrast, BMSCs on soft substrates had a higher expression of chondrogenic marker *COL2A1* and adipogenic marker *LPL* (lipoprotein lipase). TGF β increased SMC marker expression on stiff substrates; however, TGF β increased chondrogenic marker expression and suppressed adipogenic marker expression on soft substrates, while adipogenic medium and soft substrates induced adipogenic differentiation effectively [17]. Using a two-dimensional (2D) culturing system in which PAM gels with different concentrations of bis-acrylamide were coated with type I collagen, Schuh and coworkers found that, after porcine chondrocytes were cultivated on these hybrid hydrogels with a Young's modulus of 4, 10, 40, and 100 kPa for 7 days, cellular proliferation and actin organization were decreased on matrices of 4 kPa compared with stiffer substrates. The differentiated phenotype of the chondrocytes grown on matrices of 4 kPa was stabilized, evidenced by higher levels of *COL2A1* and *ACAN* and a lower level of *COL1A1*, indicating that chondrocytes sense the elasticity of the matrix and might be used for the design of scaffolds with mechanical properties specifically tailored

to support the chondrogenic phenotype in tissue engineering applications [18].

Polyethylene glycol (PEG) hydrogels

Results from Liu and coworkers have shown that human BMSCs expanded on PEG10-CMP (collagen mimetic peptide) hybrid hydrogel (E , 3.730 kPa) had significantly more staining intensity of aggrecan and type II collagen immunostaining than the cells grown on PEG10 hydrogels (E , 4.231 kPa), which was confirmed at mRNA levels by real-time PCR data, indicating that soft matrix induced a greater degree of chondrogenic differentiation [19]. This finding in PEG hydrogel also applies to bovine articular chondrocytes. Nicodemus and coworkers found that the deposition of aggrecan and type II collagen was the highest at both protein and mRNA levels when chondrocytes were encapsulated in hydrogels with the lowest crosslinking 60 kPa compressive modulus while the catabolic gene expression of *MMP1* (matrix metalloproteinase 1) and *MMP13* was elevated about 25-fold in hydrogels with 590 kPa compressive modulus [20]. This finding indicates that the ECM synthesis, degradation, and remodeling might be correlated with chondrocyte responses to hydrogel elasticity differences.

Defined matrix hydrogels

Wang and colleagues synthesized 3D hydrogels with varied matrix stiffnesses (3, 30, and 90 kPa) by using hydrogels with different concentrations of matrix compositions such as chondroitin sulfate (CS), hyaluronic acid (HA), and heparin sulfate (HS). Results showed that, in softer hydrogels (~ 3 kPa), increasing HA concentrations resulted in substantial upregulation of *ACAN* and *COL2A1* expression in a dose-dependent manner in human adipose-derived stem cells (ADSCs), which was reversed in HA-containing hydrogels with higher stiffness (~ 90 kPa). This finding suggested that matrix stiffness and biochemical cues interact in a nonlinear manner to regulate chondrogenesis of ADSCs in a 3D environment [21].

Cells grown on less stiff scaffolds showed enhanced chondrogenic potential

In cartilage regeneration, it is important that the implants to be transplanted to the defect sites not only maintain their mechanical integrity, but also deliver mechanical signals to adherent cells in the body.

Synthetic scaffolds

Nam and coworkers reported that pure poly(ϵ -caprolactone) (PCL) fibers with a lower modulus (7.1 MPa) provided a more appropriate microenvironment for chondrogenesis, evidenced by a marked upregulation of chondrocytic *SOX9*, *ACAN*, and *COL2A1* genes and chondrocyte-specific ECM glycosaminoglycan (GAG) production in embryonic mesenchymal progenitor cells. In contrast, the stiffer core-shell poly (ether sulfone)-poly (ϵ -caprolactone) (PES-PCL) fibers (30.6 MPa) supported enhanced osteogenesis by promoting osteogenic *RUNX2* (runt-related transcription factor 2), *ALP* (alkaline phosphatase), and *OCN* (osteocalcin) gene expression as well as ALP activity. The findings demonstrate that the microstructure stiffness/modules of a scaffold and the pliability of individual fibers may play a critical role in controlling stem cell differentiation [22]. By taking advantage of a synthetic tetrafunctional ethylene oxide/propylene oxide block copolymer (Tetronic[®] 1307), two different shear storage moduli (G') of 1 kPa or 2 kPa were fabricated and synthesized. Nucleus pulposus-derived stem cells (NP-SCs) expanded on matrices with a low shear storage modulus ($G'=1$ kPa) significantly promoted more proliferation and chondrogenic differentiation, which was evidenced by higher expression of *SOX9* and *ACAN* genes and sulfated GAG (sGAG), whereas matrices with a high modulus ($G'=2$ kPa) promoted osteogenic differentiation, which was proven by enhanced expression of osteogenesis related markers such as *OCN* and *OPN* (osteopontin) genes and higher ALP activity [23].

Natural scaffolds

Murphy and coworkers investigated the influence of ECM elasticity on stem cell differentiation. They found that, in homogeneous collagen-GAG (CG) natural scaffolds with the same composition but different stiffnesses (0.5, 1.0, and 1.5 kPa), scaffolds with the lowest stiffness facilitated a significant upregulation of *SOX9* expression and that Wistar rat BMSCs were directed toward a chondrogenic lineage in more compliant scaffolds. In contrast, the highest level of *RUNX2* expression was found in the stiffest scaffolds and BMSCs were directed toward an osteogenic lineage in stiffer scaffolds [24]. The results from the work of Fernández-Muiños and colleagues (2014) [25] suggested that mouse embryonic fibroblasts (MEFs) which were cultured in RAD16-I (PuraMatrix, self-assembling peptide nanofiber scaffolds) at a low elastic modulus value (~ 0.1 kPa) expressed the chondrogenic inductor bone morphogenetic protein 4 (BMP4) and its antagonist Noggin. On the other hand, at a higher

elastic modulus value (~ 5 kPa), the cells expressed Noggin but not BMP4, and did not engage in chondrogenesis, which suggested that the balance between BMP/Noggin could be implicated in the chondrogenic process.

Cells grown on less stiff dECMs showed enhanced chondrogenic potential

The development of decellularized ECM (dECM) maintains the stemness and specific cell morphology desired for chondrogenesis [26] and allows dECM produced by the patient's own cells to be used thereby overcoming the issues of possible exogenous pathogen transfer [27].

Cartilage-derived dECMs

During the past decade, there has been increasing interest in creating biological scaffolds composed of ECM derived from the decellularization of tissues or organs for tissue engineering applications. Schwarz and colleagues found decellularization and sterilization process (DP) treatment made the linear modulus of the scaffolds decrease significantly from 6.5 ± 2.3 MPa in native porcine nasal septal cartilage (pNSC) to 1.92 ± 0.85 MPa in processed cartilage. Processed pNSC scaffolds seeded with human primary chondrocytes were cultured for 42 days in chondrogenic induction medium. Due to the decreased linear modulus in processed pNSC, scaffolds seem to provide adequate substrate stiffness to support redifferentiation and maintenance of the chondrogenic phenotype [28]. Garrigues and coworkers (2014) [29] found that the predominant effects on human ADSCs of incorporation of cartilage-derived matrix (CDM) into electrospun PCL scaffolds were to stimulate sGAG synthesis and *COL10A1* gene expression. Compared with single-layer scaffolds, multilayer scaffolds enhanced cell infiltration and *ACAN* gene expression. Interestingly, compared with single-layer constructs, multilayer PCL constructs had a much lower elastic modulus; PCL-CDM constructs had an elastic modulus approximately 1% that of PCL constructs. These findings suggest the role of lower elastic modulus particularly from CDM in promoting chondrogenesis. Thus decellularized cartilage (tissue/organ) scaffolds with controlled elasticity hold great potential in regulating the chondrogenesis of therapeutic cells and cartilage regeneration *in vivo*.

Cell-derived dECMs

Recent reports demonstrate the influence of cell-derived matrix stiffness on chondrogenic

differentiation of adult stem cells. Zhang and coworkers (2015) [30] reported that human synovium-derived stem cells (SDSCs) expanded on stem cell-derived dECM greatly enhanced their proliferation and chondrogenic differentiation capacity. To determine the effect of mechanical properties of culture substrates on cultured cells, atomic force microscopy (AFM) was used to measure the elasticity of both substrates and expanded cells. They found that Young's moduli of the fixed cells using glutaraldehyde were 91.22 ± 64.15 kPa after expansion on plastic flasks and 53.33 ± 53.47 kPa when expanded on dECM. The finding indicates that a lower stiffness substrate might be related to enhanced chondrogenic potential. Furthermore, an AFM study showed that dECM (AECM) deposited by adult SDSCs (ASDSCs) exhibited a higher elasticity than that deposited by fetal SDSCs (FECM) (61.82 ± 35.86 versus 58.26 ± 13.37 kPa, $p = 0.000$); the elasticity of plastic flasks could be considered as infinite. Interestingly, Li and colleagues (2014) [31] found that the elasticity of expanded ASDSCs was parallel to their substrate; the comparison between plastic flasks and AECM was 9.07 ± 4.56 versus 3.60 ± 1.79 (kPa) ($p = 0.000$) while the comparison between AECM and FECM was 3.60 ± 1.79 versus 3.45 ± 2.29 (kPa) ($p = 0.024$). They also found that expanded ASDSCs on FECM yielded 21-day pellets with the highest chondrogenic markers including aggrecan and type II collagen at the both mRNA and protein levels followed by those grown on AECM; cells grown on plastic flasks had the lowest amount of chondrogenic markers. The fact that stem cells maintained better chondrogenic potentials on dECM with lower elasticity suggested that, during *in vitro* expansion, dECM could be designed with an appropriate elasticity in order to better maintain its chondrogenic potential. However, the study of influences of mechanical properties of the *in vitro* microenvironment on chondrogenesis of stem cells is still in its infancy and many potential mechanisms need to be explored.

Potential mechanisms of substrate elasticity underlying chondrogenic differentiation

The stronger mechanical feedback on stiff substrates was believed to lead to the conformational changes of tension-sensitive proteins or protein-protein topographic changes [32].

Integrin-mediated focal adhesion signaling

Integrins and other cell surface receptors provide external links to the ECM, but their functions at the cell–ECM interface require the participation of a multiprotein complex of adhesion components. Cytoskeleton includes microfilament, microtubule, and intermediate filament. The main components of actin cytoskeleton are stress fibers and focal adhesion (FA), which have been reported to influence MSC shape, mechanical properties, and differentiation down osteogenic, adipogenic, and chondrogenic pathways [36]. Focal adhesion is the attachment point of ECM and the actin cytoskeleton that could transmit the extracellular signals into cytoplasm [33]. The aggregation of FAK kinase (FAK) with integrins and other cytoskeletal proteins in focal contacts has been proposed to be responsible for FAK activation, whose phosphorylation is associated with Src activation. The activation of the FAK/Src complex serves as a potential substrate for tensin, vinculin, paxillin, p130cas [34], and talin [35]. Impaired functioning of vinculin in primary chondrocytes resulted in the reduced expression of chondrocyte-specific genes [37]. The round chondrocyte cytoskeletal structure was found to reduce vinculin expression compared with the fibroblastic chondrocyte cytoskeletal structure [38]. (Fig. 1A)

Rho, Rac, and Cdc42 GTPase were reported to regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia [39]. Small GTPases impacted chondrogenic differentiation through regulating nanomechanics of human ADSCs [40]; specifically, inhibition of Rac1 led to an increase of *COL2A1* gene expression [40] while Rac1 activation inhibited chondrogenesis [41]. In mouse limb-bud MSCs, treatment with Y27632 to inhibit Rho-associated protein kinase (ROCK) increased GAG production of the cells and caused cortical actin organization. This treatment also reduced the number of actin fibers and caused cell rounding. Conversely, RhoA overexpression inhibited GAG synthesis and *SOX9* [42]. Therefore, the RhoA/ROCK pathway plays a role in regulating the markers of chondrogenic differentiation. Cytochalasin also has been shown to increase *SOX9* [43], strongly indicating that the mechanism for this regulation is likely related to the actin cytoskeleton. Therefore, decreased actin cytoskeletal organization appears to increase chondrogenesis in MSCs. A soft substrate can only withstand low levels of force exerted by cells. When cells generate traction forces higher than what the substrate can withstand, the matrix may yield and cells could adapt by decreasing the traction force, integrin binding, and their own stiffness [44, 45], which modulate cell spreading and thus differentiation [46,

47]. Limits of cell adhesion strength on a soft substrate can provide feedback to cells to decrease stress fiber formation, which also explains why Rho activation fails to induce stress fibers on soft substrates [17] despite the fact that Rho GTPase plays an important role in actin assembly and focal adhesion formation. (Fig. 1A)

Non-muscle myosin II (NM II)-mediated contraction sensed and transmitted through substrate elasticity by cells played a critical role in chondrogenesis. For instance, the co-culture of mouse BMSCs with blebbistatin, a potent NM II inhibitor, blocked NM II-mediated mechanical sensing and suppressed chondrogenesis induced by the low stiffness of S-PAM gels, suggesting the central roles of NM II-mediated mechanical sensing in inducing chondrogenic genes [16]. Similarly, Murphy and coworker found that the treatment of Wistar rat BMSCs with blebbistatin hindered the ability of cells to sense the elasticity of the matrix and abolished the upregulation of transcription factor *SOX9* as a result of cells sensing the elasticity of the scaffold [24]. In addition, the actin structure also plays a critical role in chondrogenic differentiation; disruption of the actin cytoskeleton by cytochalasin D induced chondrogenic differentiation of limb bud mesenchymal cells [48]. Thus, the dependence of chondrogenic differentiation on substrate stiffness seems to be related to the changes of cytoskeletal structures in stem cells. (Fig. 1A)

The tension-sensitive protein pathway

The traction force generated by substrate elasticity difference might induce certain protein unfolding, which serves as ‘molecular strain gauges’ that expose binding domains of some proteins and signaling molecules. For example, stretch forces can unfold talin, which binds to integrins and actins, exposing its binding sites for vinculin, and the exposed sites on vinculin can activate mitogen-activated protein kinase (MAPK) leading to a series of cell signaling chain reactions [6]. The nuclear translocation of MAPK involves β -like importins [162]. Yang et al. [163] find that p38 MAPK directly phosphorylates Drosha, which reduces its interaction with DGCR8, promotes its nuclear export and degradation by calpain, and increases cell death under stress. On the other hand, the nuclear export of MAPK requires its dephosphorylation, and it is exported in a MAPKK-dependent manner [164, 165] After phosphorylating MAPKK, nuclear localized MAPK is dephosphorylated by MKPs. Inactivated MAPK returns to the cytosol, partly through phosphorylated MAPKK acting as a carrier, to receive the next stimulation. Tension forces may also influence the assembly, stability, and turnover of

receptor-ligand complexes and functional module components. For example, the cell membrane tension and possible membrane fluidity might affect conformational dynamics of G protein-coupled receptor (GPCR); the activated GPCR might lead to cytoskeleton re-arrangement through regulating the RhoGEF protein and its downstream RhoA/ROCK activities [49]. (Fig. 1B)

The stretch-activated channel pathway

The stronger mechanical feedback on stiff substrates may also lead to the activation of stress-sensitive ion channels [50]. Recently, it has been shown that cells on substrates of differing stiffness exhibit changes in the amplitude of calcium ion oscillations, proving that stretch activated channel permeability is related to active sensing of the substrate elasticity changes of the cell [51]. Substrate rigidity sensing was induced by intrinsic mechanical stimuli such as generation of traction forces onto the substrate *via* stress fiber/FA system. Endothelial cells cultured on stiffer substrate showed spontaneous Ca^{2+} oscillations with larger amplitude than those on soft substrate [51]. Intracellular Ca^{2+} plays a crucial role in the reorganization of the actin cytoskeleton through modulating actin-associating protein activities. Increases in cytosol Ca^{2+} induces stress fiber contractility through phosphorylation of myosin light chain kinase (MLCK) and/or activation of proteases [52]. Ca^{2+} /Calmodulin-dependent protein kinase II (CaMKII) phosphorylates Raf-1 and mediates Ras-stimulated Raf activation, which might lead to activation of downstream signals [53]. Constitutive CaMKII signaling in premature maturation of chondrocytes was a cell-autonomous effect associated with downregulation of cell-cycle regulators and upregulation of chondrocyte maturation markers [54] (Fig. 1C).

TWIST1 signaling

In the TWIST1-G3BP2 mechanotransduction pathway, increasing substrate stiffness was reported to induce integrin-dependent phosphorylation events and release of TWIST1 from its cytoplasmic anchor G3BP2 to enter the nucleus; TWIST1 is an essential mechano-mediator that promotes epithelial-mesenchymal transition (EMT) in response to increasing matrix stiffness [55]. (Fig. 1D)

YAP/TAZ signaling

Dupont and coworkers (2011) [56] reported the identification of the Yorkie-homologues YAP (Yes-

associated protein) and TAZ (transcriptional coactivator with PDZ-binding motif) as nuclear relays of mechanical signals exerted by ECM rigidity and cell shape. This regulation requires Rho GTPase activity and tension of the actomyosin cytoskeleton, but is independent of the Hippo/LATS cascade. Crucially, YAP/TAZ proteins are functionally required for differentiation of MSCs induced by ECM stiffness. This finding was mirrored by a recent report that YAP negatively regulated chondrogenic differentiation of MSCs [58]. Finally, as Moore et al. put forward, how the short-term substrate rigidity sensing is translated into the long-term sensing involved in cell differentiation remains unknown [59]; although much is shared between the two processes, they may have radically different time constants and frequencies. It is still a key challenge to link the early rigidity mechanisms to the long-term processes involved in cellular differentiation (Fig. 1E).

Nuclear deformation and gene expression

Substrate elasticity variations induce integrin-mediated cell activities leading to actomyosin contraction which is followed by transferring through nesprins to the nucleus. In addition, the chromosome is linked to the nuclear membrane through lamins, whose activities may lead to chromatin reorganization, DNA replication, and chondrogenesis-related gene changes. The plasma membrane is physically linked with the nuclear membrane [57]; it is possible that physical factors and nuclear deformation could regulate gene expressions through an as-yet-unknown mechanism. Molecular connections between integrins, cytoskeletal filaments, and nuclear scaffolds were supposed to provide a discrete path for mechanical signal transfer through cells and to produce integrated changes in cell and nuclear structure changes [60]. Many related proteins are involved in this process, such as F-actins, intermediate filaments, Nesprin1/2, Nesprin3, SUN1/2 as well as lamins. For example, nesprin-1 and nesprin-2 dimerise with nesprin-3 forms a meshwork composed of perinuclear cytoskeletal filaments around the nuclear periphery. The existence of the linker of the nucleoskeleton and cytoskeleton complex provides more evidence that the traction forces arising due to matrix rigidity can physically affect the structural organization of the nucleus. (Fig. 1F)

External forces on stem cell/chondrocyte chondrogenic differentiation

Biomechanical forces are key factors that keep mature cartilage healthy. During normal activity, *in vivo* articular cartilage undergoes cyclic compression (at a frequency of approximately 1 Hz during locomotion) and chondrocytes are exposed to a uniform compressive force within the range of 3 to 10 MPa [61]. With the emergence of tissue engineering as an independent research field and the understanding that chondrocytes are mechanically sensitive cells [62, 77], great effort has been made to determine how external mechanical stimuli, such as compression and hydrostatic pressure (HP), may improve the development of cartilage tissue *in vitro* [63]. (Table 2)

Compression

Direct compression has been used to modulate matrix composition and concomitantly influence neocartilage properties. Mauck and coworkers reported that dynamic loading yielded 28-day bovine chondrocyte-seeded agarose disks with a six-fold increase in the equilibrium aggregate modulus over free swelling controls; sGAG content and hydroxyproline content were also found to be greater in dynamically loaded disks after 21 days of loading [64]. After human BMSCs were subjected to chondrogenic differentiation in either chitosan-coated poly L-lactide-co-3-caprolactone scaffolds [66] or HA hydrogel [65], dynamic compression enhanced cartilage formation and suppressed chondrocyte hypertrophy. Using a custom built loading device, compression, shear, or a combination of both stimuli were applied onto fibrin/polyurethane composites in which human BMSCs were embedded. While no exogenous growth factors were added to the culture medium, Schätti and colleagues (2011) [67] found that either compression or shear alone was insufficient for the chondrogenic induction of human BMSCs. However, the application of shear superimposed upon dynamic compression led to significant increases in chondrogenic gene expression. Histological analysis detected sGAG and type II collagen only in the compression and shear group.

Despite a positive effect on matrix deposition, compressive loading at the early stage of chondrogenic induction is not constructive. For instance, Mouw and colleagues (2007) [68] found that the response to TGF β 1 treatment of bovine BMSCs seeded in agarose gel was determined by the timing of dynamic loading; application at the early stage (day 8 after chondrogenic induction) decreased the *ACAN* gene level while loading at the later stage (day 16 after chondrogenic induction) increased

chondrogenic gene expression. Thorpe and coworkers (2010) [69] also found that the application of dynamic compression from day 0 inhibited chondrogenesis of porcine BMSCs, indicating that MSC seeded constructs should first be allowed to undergo chondrogenesis *in vitro* prior to implantation in a load bearing environment. The influences of mechanical loading on stem cells' chondrogenic differentiation also depend on stem cell type. For example, dynamic mechanical compression significantly increased chondrogenic differentiation of goat BMSCs encapsulated in a hydrogel but decreased cartilage-specific gene expression in human embryonic stem cell-derived cells in the absence of TGF β 1 [70].

Hydrostatic pressure

As one component of the mechanical stresses present within diarthrodial joints [76], HP is the prevailing mechanical signal governing normal articular cartilage homeostasis [77], and thus has the potential to be applied in cartilage tissue engineering [78].

To date, many studies have focused on the effects of HP stimuli on chondrocyte-mediated synthesis and degradation of cartilage matrix macromolecules [79, 80, 81]. Articular chondrocytes, usually from an animal source, respond positively to pulsatile (0.0125–1 Hz) HP loadings ranging 0.3–5 MPa, by increasing GAG synthesis and deposition, as well as expression of healthy articular cartilage markers [79, 80, 82]. However, when high HP magnitudes were applied, on the order of 20 MPa, incorporation rates of $^{35}\text{SO}_4$ and [^3H] proline into adult bovine articular cartilage slices decreased but were reversible [83]; on the order of 30 MPa, stress response related genes such as heat shock protein 70 (*HSP70*), *HSP45*, growth arrest and DNA damage-inducible 45 (*GADD45*), and *GADD153* were significantly increased in immortalized human chondrocyte cell lines [84]. At 50 MPa, inflammatory factors such as interleukin 6 (*IL6*) and tumor necrosis factor alpha (*TNFA*) mRNAs were significantly increased in a human chondrosarcoma cell line [71] and apoptosis was observed in rabbit chondrocytes cultured in alginate beads [85]. The same outcome was observed when culturing human osteoarthritic chondrocytes under physiologically normal pressure magnitudes (5 MPa) [86]. Duration and magnitude of applied intermittent HP were also reported to differentially alter human chondrocyte matrix protein expression [87]. Continuous high HP could cause structural alterations in normal human chondrocytes, which obtained similar, if not identical, characteristics to those typical of osteoarthritic chondrocytes [88].

Recently, more attention has focused on the influence of HP on stem cell chondrogenic differentiation. In the absence of chondrogenic factors, such as TGF β , Miyanishi and coworkers (2006) [78] found that treatment with intermittent HP alone upregulated *SOX9*, *COL2A1*, and *ACAN* in human BMSC pellets, and co-treatment with intermittent HP and TGF β greatly enhanced chondrogenic differentiation. Puetzer and associates (2013) [89] found that cyclic HP initiated human ADSCs' chondrogenic differentiation but will not last long in the absence of chondrogenic factors. In the presence of chondrogenic factors, HP was found to increase chondrogenic differentiation in human ADSCs [90, 91] and porcine SDSCs and infrapatellar fat pad derived stem cells (FPSCs) [92]. Using porcine BMSCs and FPSCs embedded in agarose gel, Carroll and coworkers (2014) [93] found that cyclic HP not only increased the ratio of GAG to DNA and both dynamic and equilibrium moduli but also suppressed calcification of BMSC constructs.

Osmotic pressure

Cartilage matrix contains a large amount of negatively charged aggrecans entangled within the collagen matrix, which can attract a high concentration of cations relative to other physiologic fluids, leading to increased interstitial osmolarity. The interstitial osmolarity range of healthy articular cartilage lies within 350 to 450 mOsm depending on the zone [94]. The loss and gain of water exposes the chondrocytes to dynamic changes in osmotic pressure, thereby exposing chondrocytes to both hyper- and hypo-osmotic stresses [95]. The production of ECM of articular chondrocytes is also greatly influenced by the osmolarity of the culturing medium. For instance, chondrocyte alginate beads cultured in 550 mOsm medium for 48 h increased sGAG synthesis compared to culture in 380 mOsm medium, while culture in 270 mOsm medium decreased sGAG synthesis [96]. The effects of osmolarity on ECM production may be due in part to regulation of the chondrocyte transcription factor Sox9. Treatment of freshly isolated chondrocytes from osteoarthritic human articular cartilage with hyperosmotic medium led to an increase in the levels of Sox9 mRNA and protein, an effect mediated in part by an increase in the half-life of *SOX9* mRNA with hyperosmotic exposure [97]. A similar study in equine articular chondrocytes showed that hyperosmotic treatment had varying effects on *SOX9* mRNA levels depending on chondrocyte sources and whether treatment was applied in a static or cyclic manner [98].

Growth and chondrogenic differentiation of MSCs are also influenced by culture medium osmolarity.

High-osmolarity medium (485 mOsm) reduced proliferation of human ADSCs [99]. Caron and colleagues (2013) [100] found that increasing the osmolarity of chondrogenic induction medium by 100 mOsm resulted in significantly enhanced chondrogenic marker expression such as *COL2A1* and *ACAN* in two chondroprogenitors, ATDC5 and human BMSCs. Sampat and coworkers (2013) [101] found that the application of hypertonic media (400 mOsM) to constructs comprised of SDSCs or chondrocytes led to increased mechanical properties as compared to hypotonic (300 mOsM) or isotonic (330 mOsM) media. Constant exposure of SDSC-seeded constructs to 400 mOsM media from day 0 to day 49 yielded a Young's modulus of 513 ± 89 kPa and GAG content of 7.39 ± 0.52 %ww on day 49, well within the range of values of native, immature bovine cartilage. Primary chondrocyte-seeded constructs achieved a Young's modulus almost as high, reaching 487 ± 187 kPa and 6.77 ± 0.54 %ww (GAG) for the 400 mOsM condition (day 42). These findings suggest hypertonic loading as a straightforward strategy for 3D cultivation with significant benefits for cartilage engineering strategies.

Shear force

Chondrocytes in articular cartilage are subjected to synovial fluid flow-induced shear force. Shear force is known to affect chondrocyte homeostasis and induce catabolic and anabolic pathways. Fluid flow-induced shear force (1.6 Pa) caused individual chondrocytes to elongate and align tangential to the direction of cone rotation, stimulated GAG synthesis by two-fold, and increased the length of newly synthesized chains in human and bovine chondrocytes; however, it also increased prostaglandin E2 and nitric oxide synthesis and caused higher rates of apoptosis and downregulation of *COL2A1* and *ACAN* mRNA [102]. These results are consistent with reports from Mohtai et al., showing that high shear stress (1 Pa) elicits the release of pro-inflammatory cytokines such as IL-6 and mediates matrix degradation and chondrocyte cell death [103]. Goodwin and associates have shown that shear force level as low as 0.092 Pa could have an adverse effect on cells [104].

Not only for chondrocytes, caution should also be taken when applying flow-perfusion in stem cell chondrogenic induction. Alves da Silva and coworkers (2011) [105] found that 28-day flow-perfusion dramatically enhanced ECM deposition and type II collagen production in human BMSCs seeded onto fiber mesh scaffolds in chondrogenic induction medium. This finding is inconsistent with a recent report. After 18-day chondrogenic induction of human BMSCs in pellets mounted into 3D-

printed porous scaffolds followed by a continuous flow for 10 days, Kock and associates (2014) [106] found that decreased GAG release, in combination with diminished type II collagen staining, indicated reduced chondrogenesis in response to flow-perfusion. Although flow-perfusion could possibly influence the differentiation of chondrogenic differentiated MSCs, effects are inconsistent and strongly donor-dependent.

In the context of cartilage engineering, high or even moderate levels of shear are still undesirable in the formation of hyaline cartilage; the fluid flow-induced convective transport and low shear force have been exploited using a rotating-wall bioreactor. Many experiments have been proven effective at producing better cartilage constructs in rotating-wall bioreactors [107, 108,109, 110]. Although successfully applied in cartilage engineering, the effects seen in studies applying fluid shear force might be due to more than the physical stimuli of the fluid-induced shear, because fluid flow through engineered cartilage constructs also undoubtedly increases nutrient transport and oxygen diffusion.

Potential mechanisms of external forces underlying chondrogenic differentiation

Previous attempts to decipher signaling pathways that may be involved in chondrocyte mechanotransduction were primarily focused at plasma membrane proteins with putative mechanosensor functions (i.e. receptors and ion channels that are primarily exposed to mechanical stimuli), including purinergic P2 receptors [111], $\alpha 5 \beta 1$ integrins [112], transient receptor potential channel vanilloid-4 [113], N-methyl-D-aspartate type glutamate receptors (NMDAR) [114], annexin V [115], and stretch-activated Ca^{2+} channels [116, 117]. As far as signaling events downstream of plasma membrane receptors are concerned, the involvement of the Src and FAK, the extracellular signal-regulated kinase (ERK), and the phosphatidylinositol 3-kinase (PI3K)/Akt pathways have been reported in various models [118]. Of note, the majority of the above studies were performed on mature articular chondrocytes. This section will focus on potential mechanisms of external forces underlying stem cell chondrogenic differentiation.

The PKA/PP2A-CREB-SOX9 pathway

Since the Sox transcription factors, Sox9 in particular, are pivotal elements of chondrogenesis, many signaling events regulate their expression and/or function in chondrogenic cells. Among other factors, the Sox9 promoter is known to be regulated by the cyclic adenosine monophosphate (cAMP) response element binding protein (CREB) that binds to a CRE site upstream of Sox9 [119]. The cAMP-dependent Ser/Thr protein kinase A (PKA) is known to phosphorylate the Sox9 protein (at Ser 211), which induces its translocation into the nucleus and enhances its transcriptional activity [120]. Indeed, there is a complex synergism between Sox9 function and the cAMP–PKA–CREB pathway in both mature and differentiating chondrocytes [155]. Juhász and colleagues (2014) [121] applied a uniaxial intermittent cyclic load (0.05 Hz, 600 Pa; for 30 min) which provided HP and fluid shear on chondroprogenitor cells isolated from limb buds of four-day-old chicken embryos. They found that the applied mechanical load significantly augmented cartilage matrix production and elevated mRNA expression of several cartilage matrix constituents, including *COL2A1* and *ACAN*, as well as matrix-producing hyaluronan synthases through enhanced expression, phosphorylation, and nuclear signals of the main chondrogenic transcription factor Sox9. Along with increased cAMP levels, a significantly enhanced PKA activity was also detected and CREB, the archetypal downstream transcription factor of PKA signaling, exhibited elevated phosphorylation levels and stronger nuclear signals in response to mechanical stimuli. The involvement of PKA in the regulation of *in vitro* chondrogenesis was also confirmed in earlier reports [122, 123]. All of the above effects were diminished by the PKA-inhibitor H89. Meanwhile, protein phosphatase 2A (PP2A) activity was reduced following mechanical loading and treatments with the PP2A-inhibitor okadaic acid were able to mimic the effects of the intervention, which confirm an earlier report in that PP2A could counterbalance the chondrogenesis promoting effect of PKA *via* Sox9 and CREB phosphorylation [123]. These findings indicate that proper mechanical stimuli augment *in vitro* cartilage formation by promoting both differentiation and matrix production of chondrogenic cells; the opposing regulation of the PKA/CREB-Sox9 and the PP2A signaling pathways is crucial in this phenomenon. (Fig. 2A)

Calcium signaling

Mechanotransduction of HP has been proposed to differ from other mechanical loads due to the fact that HP generates a state of stress with little deformation [61], which might be partially explained by a proposed element of HP mechanotransduction with fluctuations in intracellular ion concentrations

[124, 125, 126]. Stretch activated calcium channels (SACCs) have been demonstrated to be required for mechanotransduction of a variety of loading types in chondrocytes and MSCs [116, 117, 126, 127]. Voltage gated calcium channels (VGCCs) are activated by membrane depolarization and mediate Ca^{2+} influx [128]. Ca^{2+} influx *via* SACCs and/or VGCCs increases Ca^{2+} concentrations directly and also indirectly through calcium-induced calcium release *via* calcium-sensitive receptors in the sarcoendoplasmic reticulum calcium stores (SERCS) [128]. VGCCs and SERCS were both found to be required for chondrogenesis in high-density chicken MSC culture [129] and they have both been implicated in mechanotransductive pathways [130, 131, 132]. (Fig. 2B)

In an *in vitro* study using porcine BMSCs seeded into agarose hydrogels, Steward and colleagues (2014) [133] found that chelating free Ca^{2+} , inhibiting VGCCs, and depleting intracellular calcium stores suppressed the beneficial effect of HP on chondrogenesis, indicating that Ca^{2+} mobility may play an important role in the mechanotransduction of HP. However, inhibition of SACCs in the current experiment yielded similar results to the control group, suggesting that mechanotransduction of HP is distinct from loads that generate cell deformation. Inhibition of the downstream targets calmodulin, CaMKII, and calcineurin all knocked down the effect of HP on chondrogenesis, implicating these targets in MSC response to HP. All of the pharmacological inhibitors that abolished the chondrogenic response to HP also maintained a punctate vimentin organization in the presence of HP, as opposed to the mechanoresponsive groups where the vimentin structure became more diffuse. These results suggest that Ca^{2+} signaling may transduce HP *via* vimentin adaptation to loading. Calmodulin, CaMKII, and calcineurin also have been implicated in mechanotransduction of fluid flow and compression in MSCs as well as chondrocytes [130, 132]. Moreover, transient receptor potential channels (*TRP channels*) have received the most attention because of their calcium ion permeability [134] and ability to permit transient calcium influx in stretched cells [135]. Functional gene screening has identified that TRPV4, a mechanically and osmotically sensitive ion channel [136], is a regulator of the chondrogenic differentiation of C3H10T1/2 MSCs [137].

The RhoA/Rac1-ROCK pathway

Rho GTPases not only function as key regulators of stress fiber assembly and FA formation [138] but also play a central role in the regulation of actin cytoskeleton [139]. Rho GTPases are involved in integrin-mediated mechanotransduction [140, 141] and regulate stress fiber formation in adult stem

cells under mechanical stimulation [142]. By the activation of Rho GTPase, the MAPK/ERK cascade is connected with the regulation of integrin β signaling [143]. Chondrocytes have been found to predominantly express $\beta 1$ subunits [144]. Along with binding a subunits, the $\beta 1$ subunits serve as receptors for collagen which further facilitate cellular cytoskeletal interaction with ECM components [145]. Formation of stress fibers, along with activation of integrin $\beta 1$, FAK, and ERK, was involved in tension stretch-mediated inhibition of chondrogenesis [146], highlighting the role of the integrin/FAK/ERK signaling axis in mediating external physical stimulation. (Fig. 2C2)

Connective tissue growth factor (CTGF), a mechanosensitive gene which provides unique binding domains for growth factors (i.e. TGF β) and integrins, is involved in cytoskeletal tension modulated by the RhoA/ROCK signaling pathway [147] (Fig. 2C1). CTGF gene expression is known to be induced through mechanical stimulation and, in particular, through mechanical stretch [148, 149]. Since CTGF does not work independently, other growth factors, integrin family proteins and possible regulatory mechanisms linking this signaling cascade remain to be investigated. Moreover, after glycocalyx is damaged, the response of cytoskeleton to shear stress is suppressed; however, the FA is almost unaffected [150]. (Fig. 2C3)

Rac1 plays an important regulatory role in cytoskeletal assembly during lamellipodia formation in cells under mechanical stimulation [151, 152, 153]. The activation of Rac1 has also been shown to promote the expression of N-cadherin and ultimately enhance the transcription of *SOX5*, *SOX6*, *SOX9*, *COL2A1*, and *ACAN* mRNAs [154]. Zhao and coworkers (2015) found that RhoA played a positive and Rac1 had a negative role in the HP-induced F-actin stress fiber assembly; RhoA and Rac1 play central roles in the pressure-inhibited ERK phosphorylation, and Rac1, but not RhoA, was involved in the pressure-promoted c-Jun N-terminal kinase (JNK) phosphorylation. Pressure promoted the expression of osteogenic marker genes in BMSCs at an early stage of osteogenic differentiation through the upregulation of RhoA activity while pressure enhanced the expression of chondrogenic marker genes in BMSCs during chondrogenic differentiation *via* the upregulation of Rac1 activity. This finding is corroborated by other studies that showed that the inhibition of RhoA-ROCK activity induced rounded cell morphologies and promoted the expression of chondrogenic marker genes in BMSCs [42, 156]. (Fig. 2C)

Other related pathways

Another possible mechanism by which mechanical stimuli may exert their positive role during *in vitro* chondrogenesis is *via* the primary cilium. Ultrastructural studies have confirmed that mature chondrocytes possess an immotile primary cilium that has a microtubule axoneme core made up of a ring of nine microtubule doublets, but devoid of the central pair of microtubules (thus designated as 9 + 0) that motile cilia always have (called a 9 + 2 axoneme) [118]. Interestingly, Wann and Knight (2012) [157] found that primary chondrocytes treated with IL-1 exhibited a 50% increase in cilia length after 3 h exposure and this elongation occurred *via* a PKA-dependent mechanism; inhibition of IL-1-induced cilia elongation by PKA inhibition also attenuated the chemokine response. Primary cilia have been well documented to serve as a nexus that integrates chemical and mechanical signals. In particular, they are considered as mechanosensors of fluid flow in many cell types including human MSCs, arthritic chondroprogenitor cells [118], and chondrocytes where they are unique sensory organelles that project into the pericellular matrix and interact with their close environment (i.e. types II and IV collagen) *via* integrins, G proteins, and various Ca^{2+} channels, many of which have been implicated as mechanoreceptors [158]. Studies show that the canonical Wnt/ β -catenin signaling pathway is closely related to the primary cilia. Glycogen synthase kinase 3 (GSK3) regulates the assembly and maintenance of flagella in *Chlamydomonas reinhardtii* [159]. Corbit and coworkers [160] demonstrated that the primary cilium restricts the activity of the canonical Wnt pathway in mouse embryos, primary fibroblasts, and embryonic stem cells. Meanwhile, the primary cilium may act as a detour to the nucleus, thereby segregating cytosolic components to keep the Wnt pathway in check [161]. (Fig. 2D)

Concluding remarks

The prevalence and severity of articular cartilage degeneration have stimulated potential methods of cartilage regeneration inspired by biomechanical approaches. Although multiple mechanical factors such as compression, hydrostatic pressure, and shear force have been introduced in cartilage engineering, the traditional method often produces fibrocartilage rather than hyaline cartilage. At the single cell or subcellular level, the way in which biomechanical factors in cell environments and external forces influence cell behaviors and fates remains elusive. While it is still important to unravel

these mechanisms, future prospects for cartilage engineering should focus more on optimization of cell niche such as changing ECM matrix stiffness. By doing so, stem cells could respond to a changing environment in a desirable mechanotransductive way, together with many other potent growth factors to regulate stem cell chondrogenic fate. A major goal of this review is to offer a perspective on this new trend in designing stem cell microenvironment and its promise for stem cell research and to enable novel, clinically relevant strategies for cartilage regeneration. Biomechanics research from tissue to molecular levels has driven recent developments in musculoskeletal medicine and will continue to be at the frontier of cartilage biology and regeneration.

References

1. Karnes J, Zhang Y, Pei M. 4th Edition. Taylor & Francis/CRC Press, 2014. pp. 1123-1135.
2. Athanasiou KA, Responde DJ, Brown WE et al. *J Biomech Eng* 2015;**137**:020901.
3. Tuan RS, Chen AF, Klatt BA. *J Am Acad Orthop Surg* 2013;**21**:303-311.
4. Chen CS. *J Cell Sci* 2008;**121**:3285-92.
5. Geiger B, Spatz JP, Bershadsky AD. *Nat Rev Mol Cell Biol* 2009;**10**:21-33.
6. Holle AW, Engler AJ. More than a feeling: discovering, understanding, and influencing mechanosensing pathways. *Curr Opin Biotechnol* 2011;**22**:648-54.
7. Evans ND, Minelli C, Gentleman E et al. *Eur Cell Mater* 2009;**18**:1-13; discussion 13-4.
8. Steward AJ, Kelly DJ. *J Anat* 2014.
9. Engler AJ, Sen S, Sweeney HL et al. *Cell* 2006;**126**:677-89.
10. Mousavi SJ, Doweidar MH. *PLoS One* 2015;**10**:e0124529.
11. Hadjipanayi E, Mudera V, Brown RA. *J Tissue Eng Regen Med* 2009;**3**:77-84.
12. Kraehenbuehl TP, Zammaretti P, Van der Vlies AJ et al. *Biomaterials* 2008;**29**:2757-66.
13. Xue R, Li JY, Yeh Y et al. *J Orthop Res* 2013;**31**:1360-5.
14. Allen JL, Cooke ME, Alliston T. *Mol Biol Cell* 2012;**23**:3731-42.
15. Kiviranta P, Lammentausta E, Töyräs J et al. *Osteoarthritis Cartilage* 2008;**16**:796-804.
16. Kwon HJ, Yasuda K. *J Mech Behav Biomed Mater* 2013;**17**:337-46.
17. Park JS, Chu JS, Tsou AD et al. *Biomaterials* 2011;**32**:3921-30.
18. Schuh E, Kramer J, Rohwedel J et al. *Tissue Eng Part A* 2010;**16**:1281-90.
19. Liu SQ, Tian Q, Hedrick JL et al. *Biomaterials* 2010;**31**:7298-307.
20. Nicodemus GD, Skaalure SC, Bryant SJ. *Acta Biomater* 2011;**7**:492-504.
21. Wang T, Lai JH, Han LH et al. *Tissue Eng Part A* 2014;**20**:2131-9.
22. Nam J, Johnson J, Lannutti JJ et al. *Acta Biomater* 2011;**7**:1516-24.
23. Navaro Y, Bleich-Kimelman N, Hazanov L et al. *Biomaterials* 2015;**49**:68-76.
24. Murphy CM, Matsiko A, Haugh MG et al. *J Mech Behav Biomed Mater* 2012;**11**:53-62.
25. Fernández-Muñoz T, Suárez-Muñoz M, Sanmartí-Espinal M et al. *Tissue Eng Part A* 2014;**20**:1145-55.
26. Pei M, Li JT, Shoukry M et al. *Eur Cell Mater* 2011;**22**:333-343.
27. Benders KE, van Weeren PR, Badylak SF et al. *Trends Biotechnol* 2013;**31**:169-76.
28. Schwarz S, Elsaesser AF, Koerber L et al. *J Tissue Eng Regen Med* 2012;
29. Garrigues NW, Little D, Sanchez-Adams J et al. *J Biomed Mater Res A* 2014;**102**:3998-4008.
30. Zhang Y, Li J, Davis ME et al. *Acta Biomater* 2015;**20**:39-50.
31. Li J, Hansen KC, Zhang Y et al. *Biomaterials* 2014;**35**:642-53.
32. Wang YL. *Sci STKE* 2007; **2007**:pe10.
33. Geiger B, Yamada KM. *Cold Spring Harb Perspect Biol* 2011;**3**.Pii: a005033.
34. Guan JL. *Int J Biochem Cell Biol* 1997;**29**:1085-96.
35. del Rio A, Perez-Jimenez R, Liu R et al. *Science* 2009;**323**:638-41.
36. Mathieu PS, Lobo EG. *Tissue Eng Part B Rev* 2012;**18**:436-444.
37. Koshimizu T, Kawai M, Kondou H et al. *J Biol Chem* 2012;**287**:15760-75.
38. Mahmood TA, de Jong R, Riesle J et al. *Exp Cell Res* 2004;**301**:179-88.
39. Nobes CD, Hall A. *Cell* 1995;**81**:53-62.

40. Jungmann PM, Mehlhorn AT, Schmal H et al. *Tissue Eng Part A* 2012;**18**:1035-44.
41. Gao L, McBeath R, Chen CS. *Stem Cells* 2010;**28**:564-72.
42. Woods A, Wang G, Beier F. *J Biol Chem* 2005;**280**:11626-34.
43. Arnsdorf EJ, Tummala P, Kwon RY et al. *J Cell Sci* 2009b;**122**:546-53.
44. Huebsch N, Arany PR, Mao AS et al. *Nat Mater* 2010;**9**:518-26.
45. Solon J, Levental I, Sengupta K et al. *Biophys J* 2007;**93**:4453-61.
46. Chowdhury F, Na S, Li D et al. *Nat Mater* 2010;**9**:82-8.
47. McBeath R, Pirone DM, Nelson CM et al. *Dev Cell* 2004;**6**:483-95.
48. Zanetti NC, Solursh M. *J Cell Biol* 1984;**99**:115-23.
49. Siehler S. *Br J Pharmacol* 2009;**158**:41-49.
50. Lee J, Ishihara A, Oxford G et al. *Nature* 1999;**400**:382-6.
51. Kobayashi T, Sokabe M. *Curr Opin Cell Biol* 2010;**22**:669-76.
52. Ridley AJ, Schwartz MA, Burridge K et al. *Science* 2003;**302**:1704-9.
53. Salzano M, Rusciano MR, Russo E et al. *Cell Cycle* 2012;**11**:2100-6.
54. Taschner MJ, Rafigh M, Lampert F et al. *Dev Biol* 2008;**317**:132-46.
55. Wei SC, Fattet L, Tsai JH et al. *Nat Cell Biol* 2015;**17**:678-88.
56. Dupont S, Morsut L, Aragona M et al. *Nature* 2011;**474**:179-83.
57. Ingber DE. *J Cell Sci* 2003;**116**:1397-408.
58. Karystinou A, Roelofs AJ, Neve A et al. *Arthritis Res Ther* 2015;**17**:147.
59. Moore SW, Roca-Cusachs P, Sheetz MP. *Dev Cell* 2010;**19**:194-206.
60. Maniotis AJ, Chen CS, Ingber DE. *Proc Natl Acad Sci U S A* 1997;**94**:849-54.
61. Elder BD, Athanasiou KA. *Tissue Eng Part B Rev* 2009;**15**:43-53.
62. Mow VC, Guo XE. *Annu Rev Biomed Eng* 2002;**4**:175-209.
63. Grad S, Eglin D, Alini M et al. *Clin Orthop Relat Res* 2011;**469**:2764-72.
64. Mauck RL, Soltz MA, Wang CC et al. *J Biomech Eng* 2000;**122**:252-60.
65. Bian L, Zhai DY, Zhang EC et al. *Tissue Eng Part A* 2012;**18**:715-24.
66. Zhang T, Wen F, Wu Y et al. *Biomaterials* 2015;**38**:72-85.
67. Schätti O, Grad S, Goldhahn J et al. *Eur Cell Mater* 2011;**22**:214-25.
68. Mouw JK, Connelly JT, Wilson CG et al. *Stem Cells* 2007;**25**:655-63.
69. Thorpe SD, Buckley CT, Vinardell T et al. *Ann Biomed Eng* 2010;**38**:2896-909.
70. Terraciano V, Hwang N, Moroni L et al. *Stem Cells* 2007;**25**:2730-8.
71. Takahashi I, Nuckolls GH, Takahashi K et al. *J Cell Sci* 1998a;**111**:2067-76.
72. Huang CY, Hagar KL, Frost LE et al. *Stem Cells* 2004;**22**:313-23.
73. Haugh MG, Meyer EG, Thorpe SD et al. *Tissue Eng Part A* 2011;**17**:3085-93.
74. Campbell JJ, Lee DA, Bader DL. *Biorheology* 2006;**43**:455-70.
75. Li Z, Yao SJ, Alini M et al. *Tissue Eng Part A* 2010;**16**:575-84.
76. Hall AC, Horwitz ER, Wilkins RJ. *Exp Physiol* 1996;**81**:535-45.
77. Grodzinsky AJ, Levenston ME, Jin M et al. *Annu Rev Biomed Eng* 2000;**2**:691-713.
78. Miyanishi K, Trindade MC, Lindsey DP et al. *Tissue Eng* 2006;**12**:1419-28.
79. Toyoda T, Seedhom BB, Yao JQ et al. *Arthritis Rheum* 2003;**48**:2865-72.
80. Jortikka MO, Parkkinen JJ, Inkinen RI et al. *Arch Biochem Biophys* 2000;**374**:172-80.
81. Sakao K, Takahashi KA, Arai Y et al. *Osteoarthr Cartilage* 2008;**16**:805-14.
82. Mizuno S, Tateishi T, Ushida T et al. *J Cell Physiol* 2002;**193**:319-27.
83. Hall AC, Urban JP, Gohl KA. *J Orthop Res* 1991;**9**:1-10.

84. Sironen RK, Karjalainen HM, Törrönen K et al. *Biorheology* 2002;**39**:111-7.
85. Nakamura S, Arai Y, Takahashi KA et al. *J Orthop Res* 2006;**24**:733-9.
86. Wenger R, Hans MG, Welter JF et al. *Front Biosci* 2006;**11**:1690-5.
87. Ikenoue T, Trindade MC, Lee MS et al. *J Orthop Res* 2003;**21**:110-6.
88. Fioravanti A, Benetti D, Coppola G et al. *Clin Exp Rheumatol* 2005;**23**:847-53.
89. Puetzer J, Williams J, Gillies A et al. *Tissue Eng Part A* 2013;**19**:299-306.
90. Correia C, Pereira AL, Duarte AR et al. *Tissue Eng Part A* 2012;**18**:1979-91.
91. Ogawa R, Orgill DP, Murphy GF et al. *Tissue Eng Part A* 2015;**21**:257-66.
92. Vinardell T, Rolfe RA, Buckley CT et al. *Eur Cell Mater* 2012;**23**:121-32; discussion 133-4.
93. Carroll SF, Buckley CT, Kelly DJ. *J Biomech* 2014;**47**:2115-21.
94. Urban JP, Hall AC, Gohl KA. *J Cell Physiol* 1993;**154**:262-70.
95. Mow VC, Ratcliffe A, Poole AR. *Biomaterials* 1992;**13**:67-97.
96. Hopewell B, Urban JP. *Biorheology* 2003;**40**:73-7.
97. Tew SR, Peffers MJ, McKay TR et al. *Am J Physiol Cell Physiol* 2009;**297**:C898-906.
98. Peffers MJ, Milner PI, Tew SR et al. *Osteoarthritis Cartilage* 2010;**18**:1502-8.
99. Liang C, Li H, Tao Y et al. *J Transl Med* 2012;**10**:49.
100. Caron MM, van der Windt AE, Emans PJ et al. *Bone* 2013;**53**:94-102.
101. Sampat SR, Dermksian MV, Oungouljian SR et al. *J Biomech* 2013;**46**:2674-81.
102. Smith RL, Donlon BS, Gupta MK et al. *J Orthop Res* 1995;**13**:824-31.
103. Mohtai M, Gupta MK, Donlon B et al. *J Orthop Res* 1996;**14**:67-73.
104. Goodwin TJ, Prewett TL, Wolf DA et al. *J Cell Biochem* 1993;**51**:301-11.
105. Alves da Silva ML, Martins A, Costa-Pinto AR et al. *J Tissue Eng Regen Med* 2011;**5**:722-32.
106. Kock LM, Malda J, Dhert WJ et al. *J Biomech* 2014;**47**:2122-9.
107. Freed LE, Vunjak-Novakovic G. *Biotechnol Bioeng* 1995;**46**:306-13.
108. Vunjak-Novakovic G, Martin I, Obradovic B et al. *J Orthop Res* 1999;**17**:130-8.
109. Pei M, Seidel J, Vunjak-Novakovic G et al. *Biochem Biophys Res Commun* 2002;**294**:149-54.
110. Pei M, Solchaga LA, Seidel J et al. *FASEB J* 2002;**16**:1691-4.
111. Garcia M, Knight MM. *J Orthop Res* 2010;**28**:510-5.
112. Orazizadeh M, Cartlidge C, Wright MO et al. *Biorheology* 2006;**43**:249-58.
113. Guilak F, Leddy HA, Liedtke W. *Ann N Y Acad Sci* 2010;**1192**:404-9.
114. Salter DM, Wright MO, Millward-Sadler SJ. *Biorheology* 2004;**41**:273-81.
115. Lucic D, Mollenhauer J, Kilpatrick KE et al. *Connect Tissue Res* 2003;**44**:225-39.
116. Mobasheri A, Carter SD, Martín-Vasallo P et al. *Cell Biol Int* 2002;**26**:1-18.
117. Wright M, Jobanputra P, Bavington C et al. *Clin Sci (Lond)* 1996;**90**:61-71.
118. Muhammad H, Rais Y, Miosge N et al. *Cell Mol Life Sci* 2012;**69**:2101-7.
119. Piera-Velazquez S, Hawkins DF, Whitecavage MK et al. *Exp Cell Res* 2007;**313**:1069-79.
120. Huang W, Zhou X, Lefebvre V et al. *Mol Cell Biol* 2000;**20**:4149-58.
121. Juhász T, Matta C, Somogyi C et al. *Cell Signal* 2014;**26**:468-82.
122. Lee YS, Chuong CM. *J Cell Physiol* 1997;**170**:153-65.
123. Zákány R, Szucs K, Bakó E et al. *Exp Cell Res* 2002;**275**:1-8.
124. Browning JA, Saunders K, Urban JPG et al. *Biorheology* 2004;**41**:299-308.
125. Hall AC. *J Cell Physiol* 1999;**178**:197-204.

126. Mizuno S. *Am J Physiol Cell Physiol* 2005;**288**:C329-37.
127. McMahon LA, Campbell VA, Prendergast PJ. *J Biomech* 2008;**41**:2055-9.
128. Catterall WA. *Cold Spring Harb Perspect Biol* 2011;**3**:a003947.
129. Fodor J, Matta C, Oláh T et al. *Cell Calcium* 2013;**54**:1-16.
130. Riddle RC, Taylor AF, Genetos DC et al. *Am J Physiol Cell Physiol* 2006;**290**:C776-C784.
131. Shakibaei M, Mobasheri A. *Histol Histopathol* 2003;**18**:343-351.
132. Valhmu WB, Raia FJ. *Biochem J* 2002;**361**:689-696.
133. Steward AJ, Kelly DJ, Wagner DR. *Eur Cell Mater* 2014;**28**:358-71.
134. Clapham DE. *Nature* 2003;**426**:517-24.
135. Follonier L, Schaub S, Meister JJ et al. *J Cell Sci* 2008;**121**:3305-16.
136. Gao X, Wu L, O'Neil RG. *J Biol Chem* 2003;**278**:27129-37.
137. Muramatsu S, Wakabayashi M, Ohno T et al. *J Biol Chem* 2007;**282**:32158-67.
138. Darling EM, Guilak F. *Tissue Eng. Part A* 2008;**14**:1507-15.
139. Shifrin Y, Arora PD, Ohta Y et al. *Mol Biol Cell* 2009;**20**:1269-79.
140. BurrIDGE K, Wennerberg K. *Cell* 2004;**116**:167-79.
141. Matthews BD, Overby DR, Mannix R et al. *J Cell Sci* 2006;**119**:508-18.
142. Discher DE, Mooney DJ, Zandstra PW. *Science* 2009;**324**:1673-7.
143. Saleem S, Li J, Yee SP et al. *J Pathol* 2009;**219**:182-92.
144. Aszodi A, Hunziker EB, Brakebusch C et al. *Genes Dev* 2003;**17**:2465-79.
145. Millward-Sadler SJ, Salter DM. *Ann Biomed Eng* 2004;**32**:435-46.
146. Takahashi I, Onodera K, Sasano Y et al. *Eur J Cell Biol* 2003;**82**:182-92.
147. Xu Y, Wagner DR, Bekerman E et al. *PloS one* 2010;**5**:e11279.
148. Kadota H, Nakanishi T, Asaumi K et al. *J Bone Miner Metab* 2004;**22**:293-302.
149. Wong M, Siegrist M, Goodwin K. *Bone* 2003;**33**:685-93.
150. Thi MM, Tarbell JM, Weinbaum S et al. *Proc Natl Acad Sci USA* 2004;**101**:16483-8.
151. Hu YL, Li S, Miao H et al. *J Vasc Res* 2002;**39**:465-76.
152. Masuda M, Fujiwara K. *Biol Cell* 1993;**77**:237-45.
153. Masuda M, Fujiwara K. *Front Med Biol Eng* 1993;**5**:79-87.
154. Woods A, Wang G, Dupuis H et al. *J Biol Chem* 2007;**282**:23500-8.
155. Zhao YH, Lv X, Liu YL et al. *Stem Cell Res* 2015;**14**:283-96.
156. Woods A, Beier F. *J Biol Chem* 2006;**281**:13134-40.
157. Wann AK, Knight MM. *Cell Mol Life Sci* 2012;**69**:2967-77.
158. Wann AK, Zuo N, Haycraft CJ et al. *FASEB J* 2012;**26**:1663-71.
159. Wilson NF, Lefebvre PA. *Eukaryotic cell* 2004;**3**:1307-19.
160. Corbit KC, Shyer AE, Dowdle WE et al. *Nat Cell Biol* 2008;**10**:70-6.
161. Lancaster MA, Schroth J, Gleeson JG. *Nat Cell Biol* 2011;**13**:700-7.
162. Zehorai E, and Seger R. *Molecular and cellular biology* 2014;**34**:259-270.
163. Yang Q, Li W, She H et al. *Molecular cell* 2015;**57**:721-734.
164. Adachi, M., M. Fukuda, and E. Nishida. *The Journal of cell biology* 2000;**148**:849-856.
165. Gong X, Ming X, Deng P et al. *Journal of cellular biochemistry* 2010;**110**:1420-1429.

Table 4. 1 The influence of substrate stiffness on chondrogenic differentiation

Substrate	Cell	Substrate stiffness	Chondrogenic effects	References
Hydrogels	Human BMSCs	PEG10 hydrogel (E , 4.231 kPa) vs PEG-CMP hybrid hydrogel (E , 3.730 kPa)	More staining intensity of aggrecan and type II collagen as well as higher levels of <i>ACAN</i> and <i>COL2A1</i> when grown on softer hybrid hydrogel	Liu et al., 2010
	Human BMSCs	Type I collagen-coated PAM hydrogels with varied stiffnesses, “1 kPa” vs “3 kPa” vs “15 kPa”	Upregulation of <i>COL2A1</i> when expanded on soft hydrogels	Park et al., 2011
	Human BMSCs	PAM hydrogels with varied Young’s modulus, “ 1.6 ± 0.3 kPa” vs “ 40 ± 3.6 kPa”	Upregulation of <i>SOX9</i> , <i>ACAN</i> , and <i>COL2A1</i> when expanded on soft PAM hydrogel	Xue et al., 2013
	Mouse BMSCs	S-PAM hydrogels with varied elastic moduli, “1 kPa” vs “15 kPa” vs “150 kPa”	The highest mRNA levels of <i>SOX9</i> , <i>ACAN</i> , and <i>COL2A1</i> and the lowest mRNA level of <i>SCA1</i> when grown on the lowest stiffness hydrogels	Kwon and Yasuda, 2013
	Porcine chondrocytes	Type I collagen-coated PAM hydrogel with varied Young’s moduli, “4 kPa” vs “10 kPa” vs “40 kPa” vs “100 kPa”	The highest mRNA levels of <i>COL2A1</i> and <i>ACAN</i> and lowest levels of <i>COL1A1</i> when grown on the softest hydrogel	Schuh et al., 2010
	Primary murine chondrocytes and ATDC5 cells	PAM hydrogels with varied stiffnesses, “0.2 MPa” vs “0.5 MPa” vs “1.1 MPa”	The most proteoglycan deposition and mRNA levels of <i>SOX9</i> , <i>ACAN</i> , and <i>COL2A1</i> when expanded on 0.5 MPa PAM hydrogel	Allen et al., 2012
	Bovine articular chondrocytes	PEG hydrogels with different compressive moduli, “60 kPa” vs “320 kPa” vs “590 kPa”	The highest level of aggrecan and type II collagen when expanded on 60 kPa PEG hydrogel	Nicodemus et al., 2011
	Human ADSCs	Defined matrix hydrogels (CS/HA/HS) with varied stiffnesses, “3 kPa” vs “30 kPa” vs “90 kPa”	Increasing HA resulting in upregulation of <i>ACAN</i> and <i>COL2A1</i> in softer hydrogels, which was reversed in HA-containing hydrogels with higher stiffness	Wang et al., 2014
Synthesized scaffold	C3H10T1/2 murine embryonic MPCs	PCL fibers (7.1 MPa) vs core-shell PES-PCL fibers (30.6 MPa)	Upregulation of chondrocytic <i>SOX9</i> , <i>ACAN</i> , and <i>COL2A1</i> genes and GAG production when grown on PCL fiber scaffold	Nam et al., 2011
	Porcine NP-SCs	Tetronic®1307 with two different shear storage moduli (G') of “1 kPa” vs “2 kPa”	Promoted cell proliferation and increased expression of <i>SOX9</i> and <i>ACAN</i> genes and sulfated GAGs when grown on scaffolds with a low shear storage modulus	Navaro et al., 2015
	Wistar rat BMSCs	CG scaffolds with varied stiffnesses, “0.5 kPa” vs “1 kPa” vs “1.5 kPa”	Upregulation of <i>SOX9</i> genes when grown on soft scaffold	Murphy et al., 2012
	MEFs	RAD16-I with varied elastic moduli, low (~0.1 kPa) vs high (~5 kPa)	A spontaneous process of cartilage-like formation when grown on scaffolds with a low elastic modulus	Fernández-Muñoz et al., 2014
dECM	Human primary chondrocytes	DP treatment decreasing the linear modulus of pNSC (6.5 ± 2.3 MPa) to processed cartilage (1.92 ± 0.85 MPa)	Increased aggrecan and type II collagen at both mRNA and protein levels in pNSC constructs during chondrogenic induction	Schwarz et al., 2012
	Human ADSCs	Elastic modulus of PCL-CDM scaffolds approximately 1% that of PCL scaffolds	Enhanced expression of sulfated GAG synthesis and <i>COL10A1</i> gene when grown on PCL-CDM compared to PCL	Garrigues et al., 2014
	Human SDSCs	FECM (58.26 ± 13.37 kPa) vs AECM (64.82 ± 35.86 kPa) vs Plastic flasks (infinite)	Expanded ASDSCs on FECM yielded 21-day pellets with the highest chondrogenic markers followed by on AECM with grown on plastic flasks being the least	Li et al., 2014
	Human SDSCs	dECM vs Plastic flasks	Expanded ASDSCs on dECM yielded 35-day pellets with the higher chondrogenic markers compared to plastic flasks	Zhang et al., 2015

Abbreviation: ADSCs: adipose-derived stem cells; AECM: decellularized ECM deposited by adult SDSCs; ASDSCs: adult SDSCs; BMSCs: bone marrow stromal cells; CG: collagen-glycosaminoglycan; CDM: cartilage-derived matrix; CMP: collagen mimetic peptide; CS: chondroitin sulfate; dECM: decellularized ECM; DP: decellularization and sterilization process; ECM: extracellular matrix; FECM: decellularized ECM deposited by fetal SDSCs; HS: heparin sulfate; HA: hyaluronic acid; MPCs: mesenchymal progenitor cells; MEFs: mouse embryonic fibroblasts; NP-SCs: nucleus pulposus-derived stem cells; pNSC: porcine nasal septal cartilage; PAM: polyacrylamide; PES: poly(ether sulfone); PEG: poly(ethylene glycol); PCL: poly(ϵ -caprolactone); RAD16-I: PuraMatrix, self-assembling peptide nanofiber scaffolds; S-PAM: sulfonate-coated PAM; SCA1: stem cell antigen-1; SDSCs: synovium-derived stem cells; Tetronic®1307: synthetic tetrafunctional ethylene oxide/propylene oxide block copolymer

Table 4. 2 Influences of external mechanical forces on stem cell chondrogenic differentiation

Forces	Cell type and culture conditions	Force conditions	Effects	References
Compression	Mouse limb bud mesenchymal cells in 3D type I collagen gel	20%-30% strain	Increased type II collagen and aggrecan expression, enhanced <i>SOX9</i> mRNA, down-regulation of IL-1 β	Takahashi et al., 1998a
	Rabbit BMSCs in 2% agarose gel (10 ⁷ cells/ml)	Subjected to a cyclic, unconfined compression test	Increased aggrecan and type II collagen over non-loaded controls	Huang et al., 2004
	Porcine BMSCs embedded in 2% agarose gel and grown in CIM	1 h/day, 1 Hz, 10% strain	Increased <i>ACAN</i> , <i>COL1A1</i> , and <i>COL2A1</i> mRNA in a temporal and spatial-dependent manner	Haugh et al., 2011
	Porcine BMSCs seeded in 2% agarose gel	1 Hz, 10% strain, 1 h/day	The application of dynamic compression from day 0 inhibited chondrogenesis of MSCs	Thorpe et al., 2010
	Human BMSCs encapsulated in HA hydrogels in CIM	10% strain, 1 Hz, 4 h/day, 5 days/wk	Increased mechanical properties as well as the GAG and collagen contents but reduced hypertrophic markers	Bian et al., 2012
	Human BMSCs in 3% alginate	1.5 h on/4.5 h off, 8 day, 1 Hz, 15% strain	Increased <i>SOX9</i> , <i>COL2A1</i> , <i>COL10A1</i> , and <i>ACAN</i> mRNA	Campbell et al., 2006
	Human BMSCs embedded in fibrin/polyurethane gels and grown in CIM with 1ng/mL TGF β 1	1 h/day, 7 days, 0.1 or 1 Hz, 10-15, 20 or 30% strain	Higher load frequency and higher amplitude induced higher GAG synthesis, higher chondrocytic gene expressions	Li et al., 2010
	Human BMSCs embedded in fibrin/polyurethane composites in defined medium w/o GFs	1 Hz 0.4 mm strain and/or shear force (ball oscillation of $\pm 25^{\circ}\text{C}$ at 1 Hz)	Combined forces led to significant increases in chondrogenic gene expression and sGAG and type II collagen staining	Schätti et al., 2011
	Human BMSCs seeded onto the PLCL/chitosan scaffolds in CIM	5% of strain, 1 Hz, 2 h/day, 7 days/week for 3 weeks	Enhanced cartilage formation and suppressed hypertrophy	Zhang et al., 2015
Hydrostatic pressure	Porcine BMSCs and FPSCs embedded in agarose gel and cultured in CIM with 10 ng/mL TGF β 3	CHP at 10 MPa, 1 Hz, 4 h/day, 5 days/week, for 5 weeks	Increased ratio of GAG to DNA and both dynamic and equilibrium moduli but suppressed calcification of BMSC constructs	Caroll et al., 2014
	Porcine SDSCs and FPSCs formed in pellets and cultured in CIM with 1/10 ng/mL TGF β 3	CHP at 10 MPa, 1 Hz, 4 h/day, for 14 days	Increased and maintained chondrogenic differentiation only in the presence of a low dose of TGF β 3 (1 ng/mL) rather than none (0 ng/mL) or a high dose (10 ng/mL)	Vinardell et al., 2012
	Human ADSCs encapsulated in GG hydrogel and cultured in CIM with 10 ng/mL TGF β 3	HP at 0.4 and 5 MPa, 0.5 Hz, 4 h/day, 5 days/week for 4 weeks	Physiologic loading (5 MPa) increased chondrogenic differentiation and matrix deposition	Correia et al., 2012
	Human ADSCs embedded in collagen gel and cultured in CIM with 10 ng/mL TGF β 1	CHP at 0-0.5 MPa (4.93 atm), 0.5 Hz for one week	Increased matrix deposition and <i>COL2A1</i> , <i>ACAN</i> , and <i>SOX9</i> mRNA levels but decreased <i>COL1A1</i> level	Ogawa et al., 2015
	Human BMSCs formed in pellets and cultured in CIM containing 10 ng/mL TGF β 3	IHP at 10 MPa, 1 Hz, 4 h/day for 14 days	The treatment with IHP upregulated <i>SOX9</i> , <i>COL2A1</i> , and <i>ACAN</i> ; the combined group yielded the most chondrogenic differentiation at both mRNA and protein levels	Miyaniishi et al., 2006
	Human SDSCs embedded in agarose gel and cultured in a growth medium with 10% FBS	CHP at 7.5 MPa, 1 Hz, 4 h/day, for 21 days	CHP initiated human ADSC chondrogenic differentiation but will not last long in the absence of chondrogenic factors	Puetzer et al., 2013
Osmotic pressure	Bovine SDSCs and chondrocytes encapsulated in 2% w/v agarose in 10% FBS + GF cocktail	300 mOsm vs 330 mOsm vs 400 mOsm for 7 weeks	The application of hypertonic media to constructs led to increased mechanical properties and GAG contents.	Sampat et al., 2013
	ATDC5 and human BMSCs in a monolayer culture in CIM	ATDC5: 310 $\pm$ 5 mOsm BMSCs: 285 $\pm$ 5 mOsm	An increase of 100 mOsm in medium enhanced levels of <i>COL2A1</i> , <i>ACAN</i> , <i>SOX9</i> , <i>RUNX2</i> , and <i>COL10A1</i>	Caron et al., 2013
Shear forces	Human BMSCs seeded onto CPBTA and grown in CIM (1 ng/mL TGF β 3)	A continuous flow of 100 $\mu\text{L}/\text{min}/\text{fiber}$ mesh for 28 days	An enhanced ECM deposition and type II collagen production	Alves da Silva et al., 2011
	Human BMSCs in a pellet culture system with CIM	A continuous flow of 1.22 mL/min for 10 days	Decrease production of chondrogenic and hypertrophic matrix components	Kock et al., 2014

Abbreviations: ADSCs: adipose derived stem cells; BMSCs: bone marrow stromal cells; CHP: cyclic hydrostatic pressure; CIM: chondrogenic induction medium; CPBTA: fiber mesh scaffolds, consisting of a blend of 50:50 chitosan:poly(butylene terephthalate adipate); ECM: extracellular matrix; FBS: fetal bovine serum; FPSCs: infrapatellar fat pad derived stem cells; GF: growth factor; GG: gellan gum; HA: hyaluronic acid; IHP: intermittent hydrostatic pressure; IL-1 β : interleukin 1beta; PLCL: poly L-lactide-co-3-caprolactone; SDSCs: synovium-derived stem cells; TGF β : transforming growth factor beta

Fig 4. 1 Potential mechanisms of substrate stiffness on stem cell chondrogenic fate.

A) Integrin-mediated focal adhesion signaling; B) The tension-sensitive protein pathway; C) The stretch-activated channel pathway; D) TWIST1 signaling; E) YAP/TAZ signaling; and F) Nuclear deformation and gene expression. [Abbreviation: CaMK: Calcium/calmodulin-dependent protein kinase; CDC42: Cell division cycle 42; FA: Focal adhesion; FAK: Focal adhesion kinase; G3BP2: Ras GTPase-activating protein-binding protein 2; MEK: MAPK kinase; MLCK: Myosin light chain kinase; SAM: stretch activated channels; Src: Proto-oncogene tyrosine-protein kinase; TAZ: transcriptional coactivator with PDZ-binding motif; TF: Transcriptional factor; TWIST1: Twist-related protein 1; YAP: Yes-associated protein.]

Fig 4. 2 Potential mechanisms of external forces on stem cell chondrogenic fate.

A) The PKA/PP2A-CREB-SOX9 pathway; B) Calcium signaling; C) The RhoA/Rac1-ROCK pathway; and D) Other related pathways. [Abbreviation: AKT: Protein Kinase B (PKB); CaMK: Calcium/calmodulin-dependent protein kinase; FAK: Focal Adhesion Kinase; GATA4: Transcription factor GATA-4; GR: Growth Factors; IP3: Inositol 1,4,5-triphosphate; Mechano-sensitive genes: Egr1, lex1, Fos, Jun, Cox2; MLCK: Myosin Light Chain Kinase; NFAT: Nuclear Factor of Activated T cells; PI3K: Phosphatidylinositol 3-Kinase; PKC: Protein Kinase C (PKC); PLC: Phospholipase C; STATs: Signal transducers and activators of transcription.]

Chapter 5. Optimization of decellularized extracellular matrix to reprogram infrapatellar fat pad-derived stem cells for cartilage tissue engineering

Abstract

Adult stem cells gradually lose their stemness and become senescent when plated in monolayer culture *in vitro*. This obstacle could be overcome by developing a decellularized extracellular matrix (dECM) for stem cell expansion. Stem cells expanded on *in vitro* dECM produce a large amount of high quality therapeutic cells and have proved to be successful in cartilage tissue engineering. To obtain a maximal stem cell numbers with optimal chondrogenic potential, optimization of the *in vitro* dECM niche is urgently needed. Driven by biomechanics research on stem cells, in this study, we hypothesized that the elasticity of dECMs could be designed by controlling the culture days or changing L-ascorbic acid concentration in the normal growth medium. Stem cells expanded on dECMs of varied elasticity were compared for their responses to expanded substrates, cell surface phenotypes, proliferation and chondrogenic differentiation. Stem cells expanded on the substrates in the 1-10 kPa substrate elasticity range have demonstrated enhanced chondrogenesis compared to their counterparts on other substrate elasticities. Furthermore, stem cells on passage senescent stem cell-derived dECMs had decreased chondrogenesis but did not show significant differences with respect to their elasticity compared to early passage stem cell dECM. Future work about proteomics is desirable to further determine the potential biochemical and molecular mechanisms contributing to chondrogenic differentiation differences.

Introduction

Two challenges need to be overcome for the advancement of regenerative cartilage medicine. One challenge is to generate articular cartilage-like chondrocytes that do not undergo hypertrophy as a terminal differentiation stage. Infrapatellar fat pad (IPFP) cells have advantages over synovium-derived stem cells (SDSCs) and bone marrow-derived stem cells (BMSCs) because they are less likely to become hypertrophic when incubated in a chondrogenic induction medium. Even under chondrogenic induction, BMSCs have showed a 5- to 10-fold increase in osteocalcin and Alkaline phosphatase (ALP) compared to SDSCs [1]. Another challenge is to prevent stem cell dedifferentiation and enhance proliferation and chondrogenic capacity during *in vitro* cell expansion. Previously, series of studies were conducted in our lab, indicating that decellularized extracellular matrix (dECM) deposited by stem cells is a promising and novel cell expansion system for cartilage tissue engineering, not only enhancing cell proliferation but also promoting expanded cell chondrogenic potential instead of (or even against) hypertrophy. Our lab has pioneered the investigations of cartilage engineering and regeneration based on the use of SDSCs and dECM rejuvenation.

To optimize the application of adult stem cells, an efficient expansion system that can rejuvenate or at least maintain the self-renewal and differentiation potentials of adult stem cells is urgently needed. Work has been done in our laboratory to maximize its rejuvenation effect by combining dECM with fibroblast growth factor (FGF-2) and low oxygen [2]. However, few studies have been reported to focus on improving the dECM system itself.

dECM is a complex mixture of molecules comprised of collagen, elastic fibers, heparin and proteoglycans, as well as associated non-matrix molecules such as growth factors [3, 4]. It not only provides a natural reservoir of growth factors and other signaling molecules, it also serves as a physical support for cells [5]. While collagen fibers, the most abundant ECM molecules, provide tissues with mechanical strength, elastic fibers provide extensibility, elastic recoil and resiliency.

In recent years, the importance of substrate mechanics has been increasingly appreciated. The mechanical properties of substrates have been shown to significantly influence cell functions such as morphogenesis, proliferation and differentiation. Many synthetic three-dimensional (3D) gels of fibrin, collagen I and Matrigel have been studied with a variety of compressive and tensile techniques

and the findings vary widely, ranging from 175 to 112,000 Pa [6]. It is believed that in this range, substrates can clearly affect cell growth and differentiation [7].

Ascorbic acid (AA) was proven to promote mesenchymal stem cell (MSC) proliferation with extended culture times [8]; different AA concentrations have been used to stimulate the secretion of decellularized ECM in our previous studies [9, 10]. AA has been widely applied in cell sheet technology, the use of cell sheets is advantageous because an entirely natural neo-tissue assembled by the cells, with mature extracellular matrix (ECM), can be engineered [11, 12].

By application of AA, dECMs with varied elasticity were produced and manipulated by controlling culture days or different AA concentrations. The elasticity of cell-derived decellularized ECMs has demonstrated influence on the chondrogenic differentiation of stem cells. Previous work in our lab showed that Adult SDSC-derived ECM (AE) exhibited a higher elasticity than fetal SDSC-derived ECM (FE) [61.82 ± 35.86 versus 58.26 ± 13.37 (kPa)]. Interestingly, the elasticity of expanded ASDSCs (Adult SDSCs) was mirrored by their substrates; living cells expanded on plastic and AE were 9.07 ± 4.56 versus 3.60 ± 1.79 (kPa) while the comparison between AE4 and FE4 was 3.60 ± 1.79 versus 3.45 ± 2.29 (kPa) [9]. The lower elasticity in FE4 is likely to be associated with enhancement of ASDSC chondrogenic potentials. A recent study by Zhang and coworkers confirmed that human SDSCs expanded on dECM exhibited a fibroblast shape, a low level of elasticity, enhanced proliferation, unique changes in stem cell surface markers and enhanced chondrogenic capacity and hypertrophy [10].

Many papers have been published on the effects of ascorbic acid on ECM production, cell proliferation and differentiation [13]. However, few studies reported on the effects of various concentrations of ascorbic acid on the *in vitro* behaviors of stem cells including proliferation and differentiation [8]. Studies from Choi and coworkers showed that cell growth was significantly enhanced in proportion to the increasing AA concentration; MSCs cultured in 250 μ M AA showed the highest cell proliferation activity. Cell proliferation was demonstrated to be the highest in 250 μ M AA containing culture medium.

Some studies have shown that ascorbic acid induces various cellular responses in a variety of cells and that the dose- and environmental parameter-dependent ascorbic acid effects also modulate various cellular responses such as ECM synthesis, proliferation and differentiation. Generally, a low concentration of ascorbic acid increases cell proliferation while a higher concentration of ascorbic

acid will slow down cell growth or even be cytotoxic to cultured cells. AA concentration during culture can modulate MSC proliferation and differentiation and specific levels of ascorbic acid serve as potent positive modulators of MSC proliferation without causing a loss in the differentiation capacity of the cells [8]. While the biochemical and molecular mechanisms of ascorbic acid on cellular responses and which concentration of ascorbic acid induces a selective response are not clear, studies from a biophysical perspective might provide a new mechanism of dECMs on cell response. Previously in our lab, we conducted a series of studies by changing culturing days or using different concentrations of AA; however, the optimum culture days and ascorbic acid concentration have never been determined. We hypothesized that dECMs of different culture days and different concentrations of AA during cell culture will also produce dECM with varied elasticity which can influence expanded stem cells' chondrogenic fates. Our ultimate goal is to optimize dECM synthesis by controlling the ascorbic acid concentration and choosing appropriate culture days. By optimizing the dECM (for example, a well-fitted elasticity range) which will influence the expanded stem cells, chondrogenic differentiation could be maximized to better facilitate cartilage tissue engineering.

Materials and methods

dECM preparation

The preparation method for human IPFP stem cell-derived dECM was described in our previous study [10]. Briefly, plastic flasks (P-Flask) were precoated with 0.2% gelatin (Sigma-Aldrich, St. Louis, MO) at 37°C for 1 h and seeded with passage 2 IPFPs at 6000 cells/cm². In experiment 1, dECMs were prepared by controlling the culture days with a fixed concentration of L-ascorbic acid (AA) in 10% fetal bovine serum (FBS)-containing complete medium. D0 (Cells reaching confluence without addition of AA), D10 (AA stimulation for 10 days) and D20 (AA stimulation for 20 days) dECMs were decellularized and stored in phosphate buffered saline (Invitrogen, Thermo Fisher Scientific, Inc., Grand Island, NY). In experiment 2, dECMs were prepared by changing the concentration of L-ascorbic acid and culture for 12 days. Different working concentrations of AA (50 uM, 250 uM, and 500 uM) were used to make decellularized ECM which was stored in PBS solution (Fig. 1). After all dECMs were prepared, Passage 2 human IPFPs were seeded at 3000 cells/cm² for growth with plastic

flasks as a control.

Cell counting and proliferation index determination

Passage 2 human infrapatellar fat pad (hIPFP) stem cells were expanded on T175 plastic flasks with growth medium containing alpha-minimum essential medium (α MEM), 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μ g/mL streptomycin and 0.25 μ g/mL fungizone for 12 days. After detaching human IPFP cells, Passage 3 hIPFP cells were seeded into Plastic and dECM-coated flasks. For the proliferation index (PI) test, before cell expansion, passage 3 hIPFPs were labeled with CellVue[®] Claret (Sigma-Aldrich) at 2×10^{-6} M for 5 min according to the manufacturer's protocol (containing negative control and pre-stained group) at a seeding density is 3000 cells/cm². After five days, expanded cells were collected and measured using a BD FACS Calibur[™] flow cytometer (dual laser) (BD Biosciences, San Jose, CA). Twenty thousand events of each sample were collected using CellQuest Pro software (BD Biosciences) and cell proliferation index was analyzed by ModFit LT[™] version 3.1 (Verity Software House, Topsham, ME).

Measurement of expanded cell proliferation index using flow cytometry

On day 6 after Passage 3 hIPFP expansion, cells from each group were detached. The following primary antibodies were used in flow cytometry to detect hIPFP surface phenotypes: integrin b1 (CD29) (Abcam, Cambridge, MA); CD105 and the stage-specific embryonic antigen-4 (SSEA-4) (Santa Cruz Biotechnology; Santa Cruz, CA) and CD90 (BD Pharmingen; BD Biosciences). Samples ($n = 3$) of each 0.2×10^6 expanded cells were incubated in ice cold PBS containing 0.1% ChromPure Human IgG whole molecule (Jackson ImmunoResearch Laboratories, West Grove, PA) and 1% NaN₃ (Sigma–Aldrich) for 30 min. The cells were incubated in the dark in the primary antibodies for 30 min. Fluorescence was analyzed by a FACS Calibur (BD Biosciences) using FCS Express software package (De Novo Software, Glendale, CA).

Chondrogenic induction and evaluation of expanded stem cells

HIPFP stem cells from each group (0.25×10^6) were centrifuged at 500 g for 5 min in a 15-mL polypropylene tube to form a pellet. After overnight incubation (day 0), the pellets were cultured for 35 days in a serum-free chondrogenic medium consisting of high-glucose Dulbecco's Modified

Eagle's Medium (DMEM), 40 µg/mL proline, 100 nM dexamethasone, 100 U/mL penicillin, 100 µg/mL streptomycin, 0.1 mM ascorbic acid-2-phosphate and 1 × ITSTM Premix [6.25 µg/mL insulin, 6.25 µg/mL transferrin, 6.25 µg/mL selenous acid, 5.35 µg/mL linoleic acid and 1.25 µg/mL bovine serum albumin (BSA), from BD Biosciences] with supplementation of 10 ng/mL transforming growth factor beta 3 (TGF-β3, PeproTech Inc., Rocky Hill, NJ). In order to evaluate the passage-senescent hIPFPs' chondrogenic changes, P5 hIPFP cells were seeded at 1038 cells/cm²; after 12 days of expansion, 4.5×10⁶ were used to make pellets. Chondrogenic differentiation was evaluated using histology, immunostaining, biochemical analysis and real-time polymerase chain reaction (RT-PCR). Representative pellets (n=3) were fixed in 4% paraformaldehyde at 4°C overnight, followed by dehydrating in a gradient ethanol series, clearing with xylene and embedding in paraffin blocks. Five-µm thick sections were histochemically stained with Alcian blue (Sigma-Aldrich; counterstained with fast red) for sulfated glycosaminoglycans (GAGs). For immunohistochemical analysis, the sections were immunolabeled with a primary antibody against type II collagen (Col II; II-II6B3; Developmental Studies Hybridoma Bank, Iowa City, IA) followed by the secondary antibody of biotinylated horse anti-mouse IgG (Vector, Burlingame, CA). Immunoactivity was detected using Vectastain ABC reagent (Vector) with 3,3'-diaminobenzidine as a substrate.

Representative pellets (n = 4) were digested for 4 h at 60 °C with 125 µg/mL papain in PBE buffer (100 mM phosphate and 10 mM ethylenediaminetetraacetic acid, pH 6.5) containing 10 mM cysteine, by using 150 µL enzyme per sample. To quantify cell density, the amount of DNA in the papain digestion was measured using the QuantiTTM PicoGreen_{dsDNA} assay kit (Invitrogen) with a CytoFluor_{Series 4000} (Applied Biosystems, Foster City, CA). GAG was measured using dimethylmethylene blue dye and a Spectronic BioMate 3 Spectrophotometer (Thermo Scientific, Milford, MA) with bovine chondroitin sulfate as a standard.

TaqMan[®] real-time polymerase chain reaction (PCR)

After extraction and purification of total RNA from samples (n = 4), about 1 µg of mRNA was used for reverse transcriptase with a High-Capacity cDNA Archive Kit (Applied Biosystems) at 37°C for 120 min. Chondrogenic marker genes [type II collagen (COL2A1; assay ID: Hs00156568_m1), aggrecan (ACAN; assay ID: Hs00153935_m1), [type I collagen (COL1A1; assay ID: Hs00164004_m1)] and SRY (sex determining region Y)-box 9 (SOX9; assay ID: Hs00165814_m1)]

and hypertrophic marker genes [type X (COL10A1 (assay ID: H200166657_m1)), were customized by Applied Biosystems as part of the Custom TaqMan_Gene Expression Assays. Eukaryotic 18S RNA (assay ID: Hs99999901_s1) was carried out as the endogenous control gene. Real-time PCR was performed with the iCycler iQ™ Multi-Color Real Time PCR Detection (Perkin-Elmer, Waltham, MA). Relative transcript levels were calculated as $v = 2^{-\Delta\Delta Ct}$, in which $\Delta\Delta Ct = \Delta E - \Delta C$, $\Delta E = Ct_{exp} - Ct_{18s}$, and $\Delta C = Ct_{ctl} - Ct_{18s}$.

Atomic force microscopy (AFM)

Elasticity of culture substrates (dECM and Plastic) and expanded hIPFP stem cells was determined using an MFP-3D-BIO AFM (Asylum Research, TE2000-U, Santa Barbara, CA) integrating with an inverted fluorescence microscope (Nikon Eclipse, Ti-U, Nikon Instruments Inc., Melville, NY) and Olympus TR400-PB cantilevers with manufacturer spring constant of 0.09 N/m. For the human IPFP stem cell elasticity measurement, Passage 2 hIPFP cells were seeded on Petri dishes with and without dECM coated substrates at 20,000 cells/cm² for overnight, then the cells were washed with phosphate buffered saline (Invitrogen) two times for 5 min each and then fixed with 4% glutaraldehyde solution (Sigma=Aldrich) for 30 min, washed again and further analyzed in PBS. The location of the cantilever above the sample was confirmed using a 10× microscopy objective. For the quantitative nanomechanical analysis, a Sneddon's modification of the Hertz model developed for a four-sided pyramid was employed. The fixed cell samples elasticity (Young's modulus, E) were corrected with the indentation of the tip, δ , through the following equation: $E = \frac{\pi}{2} \frac{1-\nu^2}{\tan \alpha} \frac{F}{\delta^2}$, where E is the elastic modulus, ν is Poisson's ratio with a value of 0.5 for dECM and cells, F is the force given by the cantilever deflection multiplied with the cantilever spring constant, α is the open angle used in this study which has a value of 36° and lastly, δ is the indentation depth (A-Hassan et al., 1998).

Statistics

Numerical data are presented as the mean and the standard error of the mean. A Mann-Whitney U test and a linear model with contrast analysis were used for pairwise comparison in biochemistry and real-time PCR data analysis. AFM data analysis was performed by Kruskal Wallis nonparametric tests; different letters represent the statistical significance ($p < 0.05$) between groups. All statistical analyses

were performed with SPSS 13.0 statistical software (SPSS Inc., Chicago, IL). A *p* value less than 0.05 was considered statistically significant.

Results

Expansion on dECM increased cell proliferation and changed cell surface phenotypes

Flow cytometry data indicated that, despite a comparable proliferation index of hIPFP stem cells between the D20 and Plastic groups, D0, D10, P5 dECM expanded cells presented increases in proliferation index, D0 dECM expanded cells demonstrated the highest proliferation index, while the D20 dECM group was minimal in proliferation index value (Fig. 2 A&B). Similarly, hIPFP cells expanded on 50 μ M, 250 μ M and 500 μ M AA prepared dECM demonstrated enhanced proliferation index compared to Plastic, 50 μ M and 250 μ M dECM-expanded hIPFP cells showed much higher proliferation index than their counterparts in 500 μ M (4.78 vs 3.96) (Fig. 6A&B).

Flow cytometry data also showed that mesenchymal stem cell (MSC) surface markers (CD29, CD90 and CD105) were down-regulated at the median in dECM expanded hIPFP stem cells (Fig. 2 C&D; Fig. 6 C&D). Not surprisingly, the SSEA-4 expression in both percentage and median of dECMs expanded stem cells were enhanced compared to those on Plastic (Fig. 2 C&D; Fig. 6 C&D).

dECMs of varied elasticity due to different culture days distinctly enhanced expanded stem cells' chondrogenic potential

To determine whether dECM (from different culture days with a fixed L-ascorbic acid concentration) expanded hIPFP stem cells played distinct roles in chondrogenic potential, cells expanded on either Plastic or dECM (D0, D10, D20, P5ECM) for one passage were induced for chondrogenic differentiation. dECM expansion led to chondrogenically differentiated 35-day pellets of a larger size with more intense staining of sulfated GAGs and type II collagen (Fig. 3A), higher cell viability (DNA ratio adjusted by day 0) and GAG amount (chondrogenic marker) as well increased GAG/DNA (chondrogenic index) (Fig. 3C) compared to the corresponding Plastic group. Stem cells expanded on D20 dECM had the highest GAG amount and GAG/DNA ratio (chondrogenic index), followed by the D10 dECM and D0 dECM groups. Although expanded cells from P5 dECM had a higher GAG

amount and chondrogenic index than their counterparts on plastic, they had a decreased GAG amount and chondrogenic index compared to P2 dECM with the same number of culture days (Fig. 3C). The above protein level data were also consistent with mRNA data using real-time PCR for chondrogenic marker genes including *SOX9*, *ACAN*, *COL2A1*, *COL1A1* and *COL10A1* (Fig. 4).

dECMs from different culture days showed distinct elasticity which was mirrored by expanded cells

To determine the effects of elasticity of culture substrates on grown cells, AFM was used to measure the Young's modulus of both substrate and expanded cells. The elasticity (Young's modulus) of PL could be considered as infinite (Bhana et al., 2010). D10 dECM exhibited a higher Young's modulus median value when compared with D0 dECM [6.12 (kPa) versus 3.33 (kPa)] ($p < 0.05$), although there is no statistical significance with P5 dECM (Fig. 5A). Interestingly, the elasticity of expanded stem cells was parallel to their substrate; the median value of Young's modulus between PL and D0 ($p < 0.05$) was [193.1 (kPa) versus 36.78 (kPa)] while the median value between D0 and D10 ($p < 0.05$) was [36.78 (kPa) versus 64.05 (kPa)]. However, cells on D10 and P5 ECM showed no statistical significance (Fig. 5B).

dECMs prepared by different L-ascorbic acid concentrations had different influences on expanded hIPFP stem cells' chondrogenesis

To determine whether dECMs (due to different concentrations of L-ascorbic acid stimulation) had different influences on preconditioned hIPFP stem cells in chondrogenic potential, cells expanded on either Plastic or dECM (50 μ M, 250 μ M and 500 μ M dECM) for one passage were induced for chondrogenic differentiation. Similarly, cells expanded on dECMs had enhanced chondrogenesis capabilities compared to Plastic; cells on 250 μ M dECM produced chondrogenically differentiated 35-day pellets with the biggest size. Alcian blue and immunohistochemistry staining results showed more intense staining of sulfated GAGs and type II collagen than 50 μ M and 500 μ M groups (Fig. 7A). The quantitative data further confirmed that 250 μ M group had higher cell viability (DNA ratio adjusted by day 0) and GAG amount (chondrogenic marker) as well as increased GAG/DNA (chondrogenic index) (Fig. 7B) compared to the other dECM groups and the plastic group.

dECMs prepared by different concentrations of L-ascorbic acid showed distinct elasticity which reflected by expanded cells

To determine the effect of elasticity of culture substrates on grown cells, AFM was used to measure the Young's modulus of both substrate and expanded cells. The elasticity (Young's modulus) of PL could be considered as infinite (Bhana et al., 2010); 250 μ M dECM exhibited a higher median Young's modulus value than 50 μ M dECM [0.64 (kPa) versus 0.31 (kPa)] ($p < 0.05$) (Fig. 8A). Similarly, the Young's modulus of expanded cells was parallel to their substrate. The median value of Young's modulus between PL and 250 μ M dECM was [193.14 (kPa) versus 9.97 (kPa)] ($p < 0.05$); the comparison of hIPFP stem cells between 250 μ M dECM and 50 μ M dECM was [9.97 (kPa) versus 8.85 (kPa)] ($p < 0.05$) (Fig. 8B).

Discussion

Ascorbic acid is necessary to biosynthesize ECM as well as to mimic the *in vivo* physiological environment of mesenchymal stem cells (MSCs) and regulate their proliferation and differentiation. Ascorbic acid alone was predicted to induce cell sheet formation to benefit cell-based tissue engineering. Furthermore, ascorbic acid improved the DNA synthesis and proliferation ability of cells without causing premature differentiation of the cells, which indicates that ascorbic acid delays premature cell aging and inactivity. In addition, more collagen and other ECM constituents were produced and preserved during AA-induced cell sheet construction, including Col I, integrin β 1 and fibronectin, which were reported by previous studies [14, 15].

Cells sense their surroundings not through sight or sound but through the “touch” or “feel” of insoluble mechanical cues and “smell” or “taste” of various soluble factors [16]. ECM is comprised of a scaffold of collagens and other structural proteins that are interlaced with proteoglycans which, together, control the local mechanical environment. The interlaced proteoglycans, glycoproteins and adhesion molecules of dECM also contribute to the “stem cell niche” microenvironment through their own signaling moieties and their ability to bind growth factors, cytokines, enzymes and other diffusible molecules [17]. While various biochemical factors attracted stem cell biologists to elucidate the potential mechanisms of dECM's promoting effects on stem cells, few studies focus on the

mechanical cues of dECM on stem cell differentiation. Understanding the mechanical properties of cell-derived matrices is critical to design tissue engineering scaffolds in order to mimic the *in vivo* environment and to optimize stem cell growth and differentiation. Conventionally used polystyrene plastic culture system has a Young's modulus in the GPa range [18], whereas a Young's modulus that is five orders of magnitude smaller has been demonstrated to have profound effects upon cell growth and differentiation [7]. Reconstituted basement membrane has been reported to have a Young's modulus of 175 Pa while 2 mg/ml collagen gel has a Young's modulus of 328 Pa [19]. Cells are thought to differentiate optimally on tissue level elasticity; landmark work by Engler and coworkers (2006) suggests that soft (elastic modulus, E , 0.1-1 kPa), medium (E , 8-17 kPa) and hard (E , 25-40 kPa) matrices can induce MSC lineage specification toward neurons, muscles and osteoblasts, respectively. However, little is known about the effect of elasticity on chondrocyte behaviors and chondrogenic differentiation fate. Compared to a substrate with higher elasticity, stem cells expanded on a substrate with an appropriate elasticity range are generally regarded to prefer chondrogenesis.

In this study, elasticity differences of various expanded substrates were compared. Not surprisingly, hIPFP stem cells expanded on Plastic, which is assumed to have GPa elasticity range [6] had the highest cell elasticity, which might significantly influence cell morphology, cell proliferation and chondrogenic differentiation, as evidenced by its minimal cell proliferation index and chondrogenic differentiation compared to cells expanded on other dECM groups. dECMs from D0 showed the lowest elasticity (~ 3kPa), lower than that from D10 and Passage 5 derived dECM (~10 kPa); P2 and P5 dECMs showed no statistical significance of their elasticity. Cell types grown on soft substrates usually display *in vivo*-like morphological and functional properties of compliance similar to that of particular tissue from which they are derived. It has been reported that chondrocytes exhibit increased growth and proliferation markers on gels with compliance similar to hard cartilage (~10 kPa) [20]. D10 dECM had ~10 kPa substrate elasticity value, indicating their tendency toward chondrogenesis.

Engler et al. reported that, in the 0.1~1 kPa substrate elasticity range, stem cells tend toward neurogenic differentiation. In preparing dECM, an elasticity value that is matched with *in vivo* chondrocyte tissue is critical to guide stem cell chondrogenesis. Therefore, an elasticity range value should be established. Intermediate elasticity value (1-10 kPa) was regarded as benefiting chondrogenesis. We assume that 3 kPa D0 dECM, while having the capability to promote chondrogenic differentiation of stem cells, might also promote stem cells toward myogenic

differentiation. Similarly, expanded on substrate with an elasticity lower than 1 kPa, stem cells might tend more towards to neurogenic differentiation; therefore, a higher elasticity value of dECM (250 uM dECM) and expanded stem cells in chondrogenic induction medium had better performance than its counterpart (50 uM) with a lower Young's modulus value, as evidenced by bigger pellet size, more intense staining of chondrogenic marker and higher chondrogenic index (Fig. 7).

We noticed the significant value changes between substrate elasticity and cell elasticity values. In measuring the elasticity of expanded cells, 4% glutaraldehyde solution was used to fix expanded cells for 30 min, which led to increased cell elasticity values. The glutaraldehyde fixation method has been used in our previous study [10], and it has been proven to be the preferred method for reliable data analysis (reproducible) of both cell elasticity and cell morphology [21, 22]. There is still an ongoing discussion about whether cell elasticity measurements performed on fixed cells could be used for determination of the relative elasticity changes of unfixed cells. A recent publication made a case, for the legitimacy of cell fixation for cell elasticity measurements; the trend of elasticity alternations of endothelial cells exposed to TNF- α has been preserved by glutaraldehyde fixation [23].

Other possible influential factors, such as biochemical and molecular factors, might also exert influences on chondrogenic differences. For example, compared to D0 dECM, D10 and D20 dECM contain more ECM components such as collagen, fibronectin and laminin, which might serve as a better reservoir for various growth factors, cytokines and other stimuli for stem cell chondrogenic differentiation. However, one limitation of this study is that the dECM elasticity is not a fully independent variable, as differences in crosslinking density can also alter ECM protein anchoring and substrate porosity [24]. Undoubtedly the elasticity of dECM is a significant contributing factor influencing chondrogenic differentiation.

Some investigators have used cell-derived extracellular matrix without ascorbic acid stimulation for the application of tissue engineering (chondrogenesis or osteogenesis) and these experiments have demonstrated good effects [25, 26, 27]. Unlike our conventional dECM preparation method, they removed the cellular components either physically or chemically when cells reached to confluence instead of allowing confluent cells grow under stimulation of ascorbic acid. In this experiment, we set up such a parallel group, named the D0 group, in order to evaluate whether it is a better idea to use pure decellularized extracellular matrix without ascorbic acid stimulation. The AFM data showed that expanding substrate of the D0 group did have lower elasticity than the D10 group and expanded cells

also mirrored their expanding substrate: on D0 substrate, hIPFP cells demonstrated lower elasticity than their counterparts on D10. However, in regard to their chondrogenic differentiation capabilities, hIPFP cells expanded on the D0 substrate had decreased chondrogenesis compared to those on D10 and D20 dECM. Biophysically, D0 dECM have an advantage over D10 and D20 groups with respect to their lower elasticity, which theoretically will be beneficial for expanded cells' chondrogenesis; however, our observed results suggested that D20 and D10 dECM might have an edge over D0 dECM with respect to their biochemical and molecular mechanisms, which is not yet elucidated.

Aging is another important factor that might affect the cell cytoskeletal organization and focal adhesion. The loss of tissue elasticity has a close relationship with aging, as observed in human epithelial tissue. It has been suggested that the loss of tissue elasticity results from increased crosslinking of extracellular matrix proteins; however, Berdyyeva and coworkers demonstrated that the increased rigidity of aging cells could also be attributed to a higher density of cytoskeletal fibers [28]. Our previous study indicated that decellularized ECM, especially from young donors, rejuvenated ASDSCs in expansion, as evidenced by increased cell proliferation and resistance to apoptosis; chondrogenic differentiation also improved. In the characterization of cell and matrix interaction, some unique proteins such as collagen and elastin in fetal ECM favored ASDSC rejuvenation [9]. In this study, passage senescent stem cell derived dECM was used to compare its rejuvenating effects on chondrogenesis of expanded stem cells. P5 dECM decreased the expanding stem cells' proliferation and chondrogenic differentiation compared to stem cells expanded on P2 dECM. The elasticity difference between P2 and P5 dECM was compared for both expanded substrates and expanded stem cells. Our results suggested that there is no statistical significance between P2 and P5 dECM substrates, which led us to conclude that other biochemical and molecular factors contribute to this process. A wide variety of soluble growth factors, such as basic fibroblast growth factor (FGF-2), TGF- β , vascular endothelial growth factor and hepatocyte growth factor binds to components of dECM (such as heparin sulfate), which greatly slows their diffusion and serves to fine-tune their local concentrations and gradients [17].

Since its inception, dECM has demonstrated great potential for application in cartilage tissue engineering [29, 30]. Acquisition of a large amount of high-quality cells from dECM has been a great advantage for cell-based cartilage regeneration. In this paper, we explored the possible optimized methods for dECM by changing its elasticity, which proved to be useful for stem cell-based cartilage

repair. However, the underlying mechanisms of promoting effects of dECMs on chondrogenesis are still in their infancy, much work needs to be done in order to optimize dECM for this application. Furthermore, future work will focus on elucidating other possible factors that might influence chondrogenesis from a biochemical perspective; for example, by applying proteomics methods, specific protein components will be screened and their roles in promoting chondrogenesis will be determined. Eventually they can be integrated to optimize dECM system.

References

1. Djouad F, Bony C, Häupl T et al. *Arthritis Res Ther* 2005;**7**:R1304-15.
2. Li J, Pei M. *Tissue Eng Part A* 2011;**17**:703-12.
3. Brown B, Lindberg K, Reing J et al. *Tissue Eng* 2006;**12**:519-26.
4. Badylak, SF. *Biomaterials* 2007;**28**:3587–3593.
5. Vorotnikova E, McIntosh D, Dewilde A et al. *Matrix Biol* 2010;**29**:690-700.
6. Soucy PA, Werbin J, Heinz W et al. *Acta Biomater* 2011;**7**:96-105.
7. Engler AJ, Sen S, Sweeney HL et al. *Cell* 2006;**126**:677-89.
8. Choi KM, Seo YK, Yoon HH et al. *J Biosci Bioeng* 2008;**105**:586-94.
9. Li J, Hansen KC, Zhang Y et al. *Biomaterials* 2014;**35**:642-53.
10. Zhang Y, Li J, Davis ME et al. *Acta Biomater* 2015;**20**:39-50.
11. Ng KW, Hutmacher DW. *Biomaterials* 2006;**27**:4591–8.
12. Laplante AF, Germain L, Auger FA et al. *FASEB J* 2001;**15**:2377–89.
13. Sommer F, Kobuch K, Brandl F et al. *Tissue Eng* 2007;**13**:1281-9.
14. Murad S, Grove D, Lindberg KA et al. *Proc Natl Acad Sci U S A* 1981;**78**:2879-82.
15. Prockop DJ, Kivirikko KI. *N Engl J Med* 1984;**311**:376–386.
16. Wells RG, Discher DE. *Sci Signal* 2008;**1**:pe13.
17. Pei M, Li JT, Shoukry M et al. *Eur Cell Mater* 2011;**22**:333-43.
18. Callister WD. 5th ed. Somerset, NJ: John Wiley & Sons; 2000.
19. Paszek MJ, Zahir N, Johnson KR et al. *Cancer Cell* 2005;**8**:241-54.
20. Ilya Levental, Penelope C. Georges, Paul A. Janmey. *Soft Matter* 2007;**3**:299–306.
21. Dong C, Kashon ML, Lowry D et al. *Adv Healthcare Mater* 2013;**2**:945–51.
22. Rheinlaender J, Geisse NA, Proksch R et al. *Langmuir* 2011;**27**:697–704.
23. Targosz-Korecka M, Brzezinka GD, Danilkiewicz J et al. *Cytoskeleton (Hoboken)* 2015;**72**:124-30.
24. Trappmann B, Gautrot JE, Connelly JT et al. *Nat Mater* 2012;**11**:642-9.
25. Park YB, Seo S, Kim JA et al. *Biomed Mater* 2015;**10**:035014.
26. Evans ND, Gentleman E, Chen X et al. *Biomaterials* 2010;**31**:3244-52.
27. Rao Patabhi S, Martinez JS, Keller TC. *Differentiation* 2014;**88**:131-43.
28. Berdyyeva TK, Woodworth CD, Sokolov I. *Phys Med Biol* 2005;**50**:81-92.
29. He F, Chen X, Pei M. *Tissue Eng Part A* 2009;**15**:3809-21.
30. Pei M, Li JT, Shoukry M, Zhang Y. *Eur Cell Mater* 2011;**22**:333-43;discussion 343.

Figure 1

Fig 5. 1 Schematics of preparation of dECM with different elasticities.

In experiment 1, dECMs were prepared by controlling the culture days with fixed 250 μM L-ascorbic acid (AA) in 10% FBS-containing complete medium. D0 (HIPFP stem cells reach to confluence without addition of AA), D10 (AA stimulation for 10 days), and D20 (AA stimulation for 20 days) were harvested and decellularized. In experiment 2, dECMs were prepared by changing the concentration of L-ascorbic acid but culture for fixed days. 50 μM, 250 μM, and 500 μM AA working concentration were chosen to make decellularized ECM. After all dECMs were ready, Passage 2 HIPFPs were seeded at 3000 cells/cm² for growth with Plastic as control.

Figure 2

Fig 5. 2 dECMs (different culture days) enhanced expanded hIPFP stem cells' proliferation and changed stem cell markers' expression.

(A&B) Human IPFPs were expanded on either dECM or Plastic for one passage (5 days). Flow cytometry was used to measure proliferation index of expanded cells. (C) Flow cytometry was used to measure both percentage and median fluorescence intensity of MSC surface markers (CD29, CD90, and CD105) and SSEA4 of expanded cells.

Figure 3

Fig 5. 3 dECM from different culture days expanded hIPFP stem cells showed varied chondrogenic differentiation.

Human IPFP stem cells expanded on either dECM of different culture days (D0, D10, D20 and P6) or Plastic for one passage (9 days) were followed by a 35-day chondrogenic induction in a pellet system. (A) Pellets were photographed before histology; Alcian blue (Ab) was used to stain sulfated GAGs and immunohistochemical staining (IHC) was for Col II. (B) Biochemical analysis was used for DNA and GAG amounts in chondrogenic pellets. Cell proliferation and viability were evaluated using DNA ratio (DNA amount at days 35 and 14 adjusted by that at day 0). Chondrogenic index was evaluated using ratio of GAG to DNA. Data are shown as average $\pm$ SD for $n = 4$. $*p < 0.05$ suggests a statistically significant difference.

Figure 4

Fig 5. 4 Real-time PCR was used to evaluate chondrogenic induction of expanded hIPFP stem cells on either Plastic or dECMs prepared by different culture days.

Human IPFP stem cells were expanded on either Plastic or dECM for one passage followed by a pellet culture in a serum-free chondrogenic medium for 35 days. Real-time PCR was used to evaluate chondrogenic marker gene expression (SOX9, COL2A1, ACAN, COL1A1 and COL10A1) in day 35 pellets. Data are shown as average $\pm$ SD for n = 4. * p < 0.05 compared with the corresponding Plastic group.

Figure 5

Fig 5. 5 Plastic and dECMs prepared by different culture days showed varied elasticity changes, which was mirrored by elasticity changes of corresponding expanded cells.

AFM was used to measure Young's modulus of both expanded substrate (A) and expanded hIPFP cells (B). Different letters represent statistical significance ($p < 0.05$) between different groups by applying Kruskal Wallis nonparametric tests.

Figure 6

Fig 5. 6 dECMs (different culture days) promoted expanded hIPFP stem cells' proliferation and changed expression of stem cell surface markers.

(A&B) Human IPFP stem cells were expanded on either dECMs or Plastic for one passage (5 days). Flow cytometry was used to measure proliferation index of expanded cells. (C&D) Flow cytometry was used to measure both percentage and median fluorescence intensity of MSC surface markers (CD29, CD90, and CD105) and SSEA4 of expanded cells.

Figure 7

Fig 5. 7 Plastic and L-ascorbic acid phosphate-dependent dECMs expanded hIPFP stem cells showed varied chondrogenic differentiation.

Human IPFP stem cells expanded on either dECM prepared by different concentration of L-ascorbic acid phosphate (50 μ M, 250 μ M, 500 μ M) or Plastic for one passage (9 days) were followed by a 35-day chondrogenic induction in a pellet system. (A) Pellets were photographed before histology; Alcian blue (Ab) was used to stain sulfated GAGs and immunohistochemical staining (IHC) was for Col II. (B) Biochemical analysis was used for DNA and GAG amounts in chondrogenic pellets. Cell proliferation and viability were evaluated using DNA ratio (DNA amount at days 35 and 14 adjusted by that at day 0). Chondrogenic index was evaluated using ratio of GAG to DNA. Data are shown as average $\pm$ SD for $n = 4$. * $p < 0.05$ suggests a statistically significant difference.

Figure 8

Fig 5. 8 Plastic and dECMs prepared by different concentration of L-ascorbic acid demonstrated changed substrate and cells' elasticity.

AFM was used to measure Young's modulus of both expanded substrate (A) and expanded hIPFP cells (B). Different letters represent statistical significance ($p < 0.05$) between different groups by applying Kruskal Wallis nonparametric tests.

Chapter 6. Antioxidation of decellularized stem cell matrix promotes human synovium-derived stem cell-based chondrogenesis

As published in Stem Cells Dev. 2013; 22(6):889-900. doi:10.1089/scd.2012.0495.

^{1,2,3}Ming Pei, MD, PhD, ^{1,2}Ying Zhang, MS, ^{1,3}Jingting Li, MS, and ⁴Dongquan Chen, PhD

¹Stem Cell and Tissue Engineering Laboratory, Department of Orthopaedics, West Virginia University, Morgantown, West Virginia 26506, USA

²Mechanical and Aerospace Engineering, West Virginia University, Morgantown, West Virginia 26506, USA

³Exercise Physiology, West Virginia University, Morgantown, West Virginia 26506, USA

⁴Division of Preventive Medicine, University of Alabama at Birmingham, Birmingham, Alabama 35205, USA

Running head: DSCM rescues human SDSCs from oxidative stress

Key words: Decellularized stem cell matrix, Monolayer expansion, Oxidative stress, Cell senescence, Chondrogenesis, Synovium-derived stem cell

Corresponding author: Ming Pei MD, PhD, Stem Cell and Tissue Engineering Laboratory, Department of Orthopaedics, West Virginia University, PO Box 9196, One Medical Center Drive, Morgantown, WV 26506-9196, USA, Telephone: 304-293-1072; Fax: 304-293-7070; Email:mpei@hsc.wvu.edu

Abstract

Clinical treatment of cartilage defects is challenging due to concomitant posttraumatic joint inflammation. This study was to demonstrate that the antioxidant ability of human adult synovium-derived stem cells (SDSCs) could be enhanced by *ex vivo* expansion on decellularized stem cell matrix (DSCM). Microarray was used to evaluate oxidative, antioxidative, and chondrogenic status in SDSCs after expansion on DSCM and induction in chondrogenic medium. Hydrogen peroxide (H_2O_2) was added to create oxidative stress in either expanded SDSCs or chondrogenically induced premature pellets. The effect of H_2O_2 on SDSC proliferation was evaluated using flow cytometry. Chondrogenic differentiation of expanded SDSCs was evaluated using histology, immunostaining, biochemical analysis, and real-time PCR. MAPK signaling pathways and p21 were compared in DSCM and plastic flask expanded SDSCs with or without H_2O_2 treatment. We found that expansion on DSCM upregulated antioxidative gene levels and chondrogenic potential in human SDSCs, retarded the decrease in cell number and the increase in apoptosis, and rendered SDSCs resistant to cell-cycle G1 arrest resulting from H_2O_2 treatment. Treatment with 0.05 mM H_2O_2 during cell expansion yielded pellets with increased chondrogenic differentiation; treatment in premature SDSC pellets showed that DSCM expanded cells have a robust resistance to H_2O_2 -induced oxidative stress. Erk1/2 and p38 were positively involved in antioxidative and chondrogenic potential in SDSCs expanded on DSCM in which p21 was downregulated. DSCM could be a promising cell expansion system to provide a large number of high-quality human SDSCs for cartilage regeneration in a harsh joint environment.

Introduction

Cartilage defects, especially from trauma-induced cartilage injuries, do not heal or self regenerate well due to the absence of blood supply. Current treatment options include microfracture, osteochondral transplantation, and autologous chondrocyte implantation (ACI) [1]. Compared to other treatments, ACI has been shown to work in older, active populations with larger defects. Some limitations of ACI, however, prevent its ultimate success. For example, trauma-induced cartilage injuries may lead to early posttraumatic osteoarthritis [2]. A major source of the destructive power of inflammation is the direct and indirect generation of reactive oxygen species (ROS) and free radicals after the inflammatory cytokine response [3]. Despite studies investigating the inflammatory environment in cartilage repair [4,5], there are few reports focusing on the effect of oxidative stress on stem cell-based chondrogenesis. Oxidative stress induces chondrocyte senescence [6]; oxidative DNA damage has been demonstrated in osteoarthritic articular cartilage in both porcine [7] and human samples [8], indicating that oxidative stress is one of the most important hurdles to overcome in order to increase the efficacy of ACI.

Adult stem cells could be an excellent cell candidate not only because of their self-renewal and multilineage differentiation capacity but also due to their antioxidant capacity [9]. A recent report demonstrated that there were distinct DNA responses to damage and repair mechanisms in stem cells that render them tolerant to stressors [10], making them superior to their more differentiated counterparts [11,12]. Synovium-derived stem cells (SDSCs), a tissue-specific stem cell for chondrogenesis, are an appropriate stem cell candidate for cartilage engineering and regeneration [13,14]. The regenerative capacity of synovium has been demonstrated after surgical and chemical synovectomy [15]. Synovium can be obtained in a minimally invasive fashion with few complications during arthroscopy. Although stem cells exhibit some intrinsic degree of antioxidant capacity [16,17], this intracellular defense system may be rapidly overwhelmed in an inflammatory environment, resulting in poor cell survival and engraftment [18-20].

For successful cell therapy or tissue engineering, measures must be taken to control the inflammatory and oxidative environment in which cartilage is regenerated. The regulation of intracellular ROS is crucial for cell survival in the harsh environment and guarantees successful cell

therapy. Our previous work suggested that decellularized stem cell matrix (DSCM) provides an *in vitro* microenvironment for SDSC rejuvenation in terms of enhancing expanded cell proliferation and chondrogenic potential [21-23]. It is possible that a small punch biopsy plus our DSCM approach would be sufficient for growth of a clinically useful quantity of cells. Our recent findings indicated that DSCM expansion decreases expanded cell ROS level [24,25]. In addition, our microarray data suggested that DSCM expansion could upregulate SDSCs' antioxidant gene levels, indicating that DSCM expansion may benefit SDSCs by increasing the resistance to oxidative stress and promoting cell chondrogenic capacity. In this study, we hypothesized that human SDSCs expanded on DSCM acquired an ability to resist oxidative stress induced by hydrogen peroxide (H₂O₂) and enjoyed expanded cell chondrogenic potential.

Materials and Methods

SDSC culture

Adult human synovial fibroblasts [4 donors, two male (39 and 42 years old) and two female (43 and 47 years old), average 43 years old with no known joint disease], referred to as hSDSCs [26], were obtained from Asterand (North America Laboratories, Detroit, MI). Human SDSCs were plated and cultured in a growth medium [Alpha Minimum Essential Medium containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 µg/mL streptomycin, and 0.25 µg/mL Fungizone (Invitrogen, Carlsbad, CA)] at 37°C in a humidified 5% CO₂ and 21% O₂ incubator. The medium was changed every three days.

DSCM preparation

The preparation of DSCM was described in our previous study [21]. Briefly, plastic flasks (Plastic) were precoated with 0.2% gelatin (Sigma, St. Louis, MO) at 37°C for 1 h and seeded with passage 3 (P3) hSDSCs. After cells reached 90% confluence, 50 µM L-ascorbic acid phosphate (Wako Chemicals USA, Inc., Richmond, VA) was added for 8 days. The deposited matrix was incubated with 0.5% Triton X-100 containing 20 mM ammonium hydroxide at 37°C for 5 min and stored at 4°C in phosphate-buffered saline (PBS) containing 100 U/mL penicillin, 100 µg/mL streptomycin, and

0.25 µg/mL Fungizone.

Global gene expression by microarrays and data analyses

A preliminary study was conducted to evaluate global gene expression changes in hSDSCs during DSCM expansion and chondrogenic induction. P3 hSDSCs were expanded on either DSCM (Ecell) or Plastic (Pcell) for one passage. Expanded cells were chondrogenically induced in a pellet culture system (Ep and Pp, respectively) for 21 days. Total RNAs were isolated from expanded cells and 21-day pellets using Trizol (Invitrogen) followed by additional purification using the RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The required amount of cDNA (5.5 µg) was processed for fragmentation and biotin labeling using the GeneChip® WT Terminal Labeling Kit (Affymetrix, Santa Clara, CA). The entire reaction of fragmented and biotin-labeled cDNA (50 µL) with added hybridization controls was hybridized to the human GeneChip® 1.0 ST Exon Arrays (Affymetrix) at 45°C for 17 h in the GeneChip® Hybridization Oven 640 (Affymetrix). Calculation of the scaling factor, background, noise, and percent present, *etc.* was performed according to manufacturer's procedures (Affymetrix). The raw data was uploaded into GeneSpring (Agilent, Santa Clara, CA) and Partek (St. Louis, MO) software for initial analysis and Ingenuity Pathway Analysis (IPA, Redwood City, CA) for pathway and functional analysis. Briefly, raw intensity was background-subtracted, robust multi-array average (RMA) normalized, log-transformed, and fold changes determined. The batch effects (scan date) were removed before fold change calculation.

SDSC expansion and H₂O₂ treatment

P3 hSDSCs were cultured at 3000 cells/cm² for one passage on two substrates: DSCM or Plastic. To determine the effects of H₂O₂ on expanded cells, oxidative stress was induced 48 h after cell seeding by exposing cells to 0.05, 0.5, and 5 mM H₂O₂ throughout the culture. Since higher concentrations (0.5 and 5 mM) of H₂O₂ caused obvious cell death, 0.05 mM H₂O₂ was chosen for our cell expansion study. Non-treated groups served as a control. The cell number was counted using a hemocytometer. Expanded cells were also evaluated for proliferation index, apoptosis, and cell cycle using flow cytometry.

Cell proliferation, apoptosis, and cell cycle analysis

P3 hSDSCs were pre-labeled with CellVue® Claret (Sigma) at 2×10^{-6} M for 5 min according to the manufacturer's protocol. After a 7-day expansion, cells treated with or without 0.05 mM H₂O₂ in DSCM and Plastic groups were collected and analyzed using BD FACSCalibur (BD Biosciences, San Jose, CA). For each sample, 20,000 events were collected using CellQuest™ Pro Software (BD Biosciences) and cell proliferation index was analyzed by ModFit LT 3.1 (Verity Software House, Topsham, ME).

After cell expansion, an Annexin V-FITC Apoptosis Detection Kit (Biovision, Mountain View, CA) was used to detect apoptosis. Briefly, 2×10^5 cells treated with or without 0.05 mM H₂O₂ in DSCM and Plastic groups (n=3 each) were labeled with FITC annexin V and propidium iodide for 15 min. For each sample, 10,000 events were collected; samples were analyzed using BD FACSCalibur. FCS Express V3 (De Novo Software, Los Angeles, CA) was used to generate the histograms.

After hSDSCs were treated with 0.05 mM H₂O₂ for 2 h, the medium was switched to fresh medium for cell recovery. Detached cells were washed with PBS containing 1% FBS, and then fixed with 80% ethanol at 4°C for 15 min. Fixed cells were stained with 20 µg/mL propidium iodide in PBS buffer containing 1% FBS, 0.05% Triton X-100, and 50 µg/mL RNase. After a 30 min incubation at room temperature, a flow cytometer was used to determine the percentage of cells in different phases of the cell cycle (G1, S, and G2/M phase) with CFlow software (Accuri Cytometers, Inc., Ann Arbor, MI).

Chondrogenic differentiation

The effect of H₂O₂ treatment on SDSC-based chondrogenesis was evaluated in two phases. During cell expansion on either DSCM or Plastic, 0.05 mM H₂O₂ was added to the medium throughout the culture period. A 21-day chondrogenic induction followed in a pellet culture system. We also evaluated the effect of H₂O₂ on premature SDSC pellets, in which both DSCM and Plastic expanded SDSCs were incubated in a chondrogenic medium for 21 days, followed by the medium being supplemented with 0.05 or 0.1 mM H₂O₂ for 7 days. The chondrogenic induction protocol is briefly described below: After *in vitro* expansion, 0.3×10^6 of SDSCs from each group were centrifuged at 500 g for 5 min in a 15-mL polypropylene tube to form a pellet. After overnight incubation, the pellets were cultured in a serum-free chondrogenic medium consisting of high-glucose Dulbecco's Modified Eagle's Medium, 40 µg/mL proline, 100 nM dexamethasone, 100 U/mL penicillin, 100

μg/mL streptomycin, 0.1 mM ascorbic acid-2-phosphate, and 1×ITS™ Premix [6.25 μg/mL insulin, 6.25 μg/mL transferrin, 6.25 μg/mL selenous acid, 5.35 μg/mL linoleic acid, and 1.25 μg/mL bovine serum albumin (BSA), from BD Biosciences] with supplementation of 10 ng/mL transforming growth factor beta 3 (TGF-β3, PeproTech Inc., Rocky Hill, NJ). Chondrogenic differentiation was evaluated using histology, immunostaining, biochemical analysis, and real-time PCR.

Histology and immunostaining

The pellets (n=3) were fixed in 4% paraformaldehyde at 4°C overnight, followed by dehydrating in a gradient ethanol series, clearing with xylene, and embedding in paraffin blocks. Sections that were 5 μm thick were histochemically stained with Alcian blue (Sigma; counterstained with fast red) for sulfated glycosaminoglycans (GAGs). For immunohistochemical analysis, the sections were immunolabeled with primary antibodies against collagen II (II-II6B3; Developmental Studies Hybridoma Bank, Iowa City, IA) and collagen X (Sigma), followed by the secondary antibody of biotinylated horse anti-mouse IgG (Vector, Burlingame, CA). Immunoactivity was detected using Vectastain ABC reagent (Vector) with 3,3'-diaminobenzidine as a substrate.

Biochemical analysis

The pellets (n=4) were digested for 4 h at 60°C with 125 μg/mL papain in PBE buffer (100 mM phosphate and 10 mM EDTA, pH 6.5) containing 10 mM cysteine, by using 200 μL enzyme per sample. To quantify cell density, the amount of DNA in the papain digestion was measured using the QuantiT™ PicoGreen® dsDNA assay kit (Invitrogen) with a CytoFluor® Series 4000 (Applied Biosystems, Foster City, CA). GAG was measured using dimethylmethylene blue dye and a Spectronic BioMate 3 Spectrophotometer (Thermo Scientific, Milford, MA) with bovine chondroitin sulfate as a standard.

Real-time PCR

Total RNA was extracted from samples (n=4) using an RNase-free pestle in TRIzol® (Invitrogen). About 1 μg of mRNA was used for reverse transcriptase with a High-Capacity cDNA Archive Kit (Applied Biosystems) at 37°C for 120 min. Chondrogenic marker genes [collagen II (*COL2A1*) aggrecan (*ACAN*) and SRY (sex determining region Y)-box 9 (*SOX9*)] and hypertrophic marker gene

[collagen X (*COL10A1*)] were customized by Applied Biosystems as part of the Custom TaqMan[®] Gene Expression Assays (Li *et al.*, 2011). Eukaryotic 18S RNA (Assay ID HS99999901_s1) was carried out as the endogenous control gene. Real-time PCR was performed with the iCycler iQ[™] Multi-Color Real Time PCR Detection (Perkin-Elmer, Waltham, MA). Relative transcript levels were calculated as $\chi=2^{-\Delta\Delta C_t}$, in which $\Delta\Delta C_t=\Delta E-\Delta C$, $\Delta E=C_{t_{exp}}-C_{t_{18s}}$, and $\Delta C=C_{t_{ctl}}-C_{t_{18s}}$.

Western blot

To further determine potential mechanisms underlying DSCM-mediated cell proliferation and differentiation under oxidative stress, the expanded cells from each group were homogenized and dissolved in the lysis buffer (Cell Signaling, Danvers, MA) with protease inhibitors. Total proteins were quantified using BCA[™] Protein Assay Kit (Thermo Fisher Scientific, Rockford, IL). Thirty micrograms of protein from each sample were denatured and separated using NuPAGE[®] Novex[®] Bis-Tris Mini Gels (Invitrogen) in the XCell SureLock[™] Mini-Cell (Invitrogen) at 120 V at 4°C for 3 h. Bands were transferred onto a nitrocellulose membrane (Invitrogen) using an XCell II[™] Blot module (Invitrogen) at 15 V at 4°C overnight. The membrane was incubated with primary monoclonal antibodies in 5% BSA, 1×TBS (10 mM Tris-HCl, 150 mM NaCl, pH 7.5), and 0.05% Tween-20 at room temperature for 1 h (β -actin served as an internal control), followed by the secondary antibody of horseradish peroxidase-conjugated goat anti-mouse (Thermo Fisher Scientific) at room temperature for 1 h. SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific) and CL-XPosure Film (Thermo Fisher Scientific) were used for exposure. The primary antibodies used in immunoblotting included the mitogen-activated protein kinase (MAPK) family antibody sampler kit (catalog number: 9926) and phosphor (p)-MAPK family antibody sampler kit (catalog number: 9910) as well as p21 Waf1/Cip1 (12D1) rabbit monoclonal antibody (catalog number: 2947) were ordered from Cell Signaling.

Statistics

Numerical data are presented as the mean and the standard error of the mean. The Mann-Whitney U test was used for pair-wise comparison in biochemistry and real-time PCR data analysis. All statistical analyses were performed with SPSS 13.0 statistical software (SPSS Inc., Chicago, IL). *P* values less

than 0.05 were considered statistically significant.

Results

DSCM expansion promoted hSDSC antioxidation and chondrogenic potential

To explore the possible mechanisms of DSCM interaction with expanded cells, a microarray was conducted for global analysis of gene expression in expanded hSDSCs and subsequent chondrogenic differentiation in a pellet system. The top 100 genes (50 up- and 50 down-regulated) were analyzed for gene-gene interaction networks and signaling pathways (Fig. 1A). The top associated networks included: a) antigen presentation, cell-to-cell signaling and interaction, and inflammatory response; b) cellular development; and c) cell death, cellular growth, and proliferation, *etc.* Top molecular and cellular functions with *p* values ranging from 1.59E-08 to 5.07E-03 included: a) cellular movement and development; b) cellular growth and proliferation; and c) cell-to-cell signaling and interaction, *etc.* The top pathways included: a) endothelin-1 signaling; b) acute phase response signaling; and c) G-protein coupled receptor signaling, *etc.*

During the cell expansion phase, we found DSCM expansion upregulated the genes for antioxidation including prostaglandin F_{2α} receptor (*PTGFR*), heme oxygenase (decycling) 1 (*HMOX1*), superoxide dismutase 2 (*SOD2*), selenoprotein P, plasma, 1 (*SEPP1*), and interleukin 6 (*IL-6*). In the meantime, DSCM expansion also downregulated the genes for oxidative stress including nephroblastoma overexpressed (*NOV*), insulin-like growth factor binding protein 5 (*IGFBP5*), connective tissue growth factor (*CTGF*), A disintegrin and metalloproteinase with thrombospondin motifs 5 (*ADAMTS5*), and thioredoxin reductase 1 (*TXNRD1*) (Fig. 1B). In the subsequent chondrogenic differentiation, the genes of antioxidation as above were downregulated in DSCM pretreated hSDSCs; chondrogenic marker genes were also upregulated including collagen 9 alpha 1 (*COL9A1*), collagen 2 alpha 1 (*COL2A1*), aggrecan (*ACAN*), *SOX9*, and collagen 11 alpha 1 (*COL11A1*) (Fig. 1C).

DSCM expansion alleviated oxidative stress-mediated proliferation reduction through lowering the apoptosis rate and elevating the G1 transition

To determine an appropriate concentration of H₂O₂ for this study, a series of concentrations (0, 0.05, 0.5, and 5 mM) were chosen to evaluate toxicity in hSDSCs (Fig. 2A). After hSDSCs were plated on either DSCM or Plastic for 48 h, the medium was added with varying concentrations of H₂O₂. When treated with high concentrations of H₂O₂ (0.5 and 5 mM), a number of floating cells and attached round cells were in both groups, indicative of cell death. At a low concentration of H₂O₂ treatment (0.05 mM), both DSCM and Plastic groups showed that a decrease in cell number compared with the non-treated group despite a similarity in cell morphology. Human SDSCs grown on Plastic displayed random arrangements and flattened shapes while DSCM expanded hSDSCs exhibited an ordered arrangement and glistening “fiber-like” shapes. Thus, 0.05 mM of H₂O₂ was chosen for cell expansion to evaluate its effect on cell proliferation and subsequent multi-lineage differentiation.

To determine the effect of DSCM on cell proliferation under oxidative stress, expanded hSDSCs were evaluated using both proliferation index (Fig. 2B) and cell number counting (Fig. 2C) approaches. Under treatment with H₂O₂, proliferation index in the DSCM group was 9.20 compared to 4.36 in the Plastic group; without H₂O₂ treatment, the proliferation index in the DSCM group was 18.75 compared to 10.62 in the Plastic group. Despite the fact that H₂O₂ treatment affected the proliferation index in both groups, DSCM expanded cells retained more than twice the proliferation compared to Plastic expanded cells, which is also consistent with cell number counts. Despite the same initial seeding density (0.525×10^6 in a 175 cm² flask), after a seven-day incubation under H₂O₂ treatment, DSCM expansion yielded 1.99×10^6 cells compared to 1.21×10^6 cells in the Plastic group; without H₂O₂ treatment, the cell number in the DSCM group was 4.69×10^6 compared to 3.24×10^6 in the Plastic group. The proliferation difference in both DSCM and Plastic groups may be related to a lower apoptosis rate in the DSCM group (Fig. 2D). There was a slightly lower apoptosis rate in the DSCM group (3.30%) compared to the Plastic group (4.40%); under treatment with H₂O₂, the apoptosis rate in the DSCM group decreased to 2.87% compared to an increase in the Plastic group (8.77%).

The discrepancy in cell proliferation may also be explained by cell cycle data (Fig. 2E). Flow cytometric analysis revealed concomitant cell cycle arrest at the G1 checkpoint when expanded on Plastic versus DSCM and incubated in the medium supplemented with versus without 0.05 mM H₂O₂. After hSDSCs were treated with 0.05 mM H₂O₂ for 20 hours, we found that Plastic-expanded cells were 85.00% (versus 75.20% without treatment) in the G1 phase, 8.63% (versus 18.18% without treatment) in the S phase, and 6.36% (versus 6.62% without treatment) in the G2/M phase, suggesting

that H₂O₂ treatment resulted in an arrest at the G1 phase of the cell cycle. In contrast, we found that DSCM-expanded cells were 73.62% (versus 72.70% without treatment) in the G1 phase, 16.81% (versus 16.76% without treatment) in the S phase, and 9.57% (versus 10.54% without treatment) in the G2/M phase, suggesting that DSCM-expanded cells were able to resist the G1 arrest from H₂O₂. We also found that, after 20 hours of H₂O₂ treatment, DSCM expansion could greatly enhance cell proliferation ability, as evidenced by the cells either without H₂O₂ treatment (10.54% versus 6.62% in the G2/M phase) or with H₂O₂ treatment (9.57% versus 6.36% in the G2/M phase), which was in accord with the initial cells before H₂O₂ treatment (5.11% on DSCM versus 3.73% on Plastic in the G2/M phase) (data not shown).

DSCM expanded hSDSCs acquired enhanced chondrogenic potential after treatment with H₂O₂ in both proliferation and chondrogenic phases

The effect of oxidative stress on expanded hSDSC chondrogenic differentiation was evaluated in two distinct phases: the proliferation phase (H₂O₂ was added during cell expansion followed by chondrogenic induction) and the chondrogenic phase (H₂O₂ was added 21 days after expanded cell chondrogenic induction). For the proliferation phase study, the expanded hSDSCs with or without H₂O₂ treatment were incubated in a pellet system with a chondrogenic medium for 21 days. DSCM expanded hSDSCs yielded pellets with larger size and intense staining of cartilage markers, in terms of sulfated GAGs and collagen II, compared with Plastic expanded hSDSCs (Fig. 3A). Interestingly, H₂O₂ treatment during cell expansion enhanced the resulting pellet in both size and chondrogenic differentiation, especially for the DSCM group, which is supported by biochemical analysis data. DSCM expanded hSDSCs yielded pellets with higher GAG amount and ratio of GAG to DNA (chondrogenic index) when treated with H₂O₂ compared with the non-treated group. In contrast, Plastic expanded hSDSCs yielded pellets with a relatively low GAG amount and chondrogenic index when treated with H₂O₂, but did not show a significant difference when compared with the non-treated group (Fig. 3B). Despite enhanced chondrogenic differentiation in DSCM expanded hSDSCs as evidenced by histology and biochemical analysis data, H₂O₂ treatment did downregulate chondrogenic marker gene expression (*SOX9*, *ACAN*, and *COL2A1*) in the pellets after a 21-day chondrogenic induction. On the other hand, for Plastic expanded hSDSCs, H₂O₂ treatment upregulated chondrogenic marker gene expression, despite the fact that all chondrogenic marker genes from

Plastic expanded hSDSCs were significantly lower than those from DSCM expanded hSDSCs under treatment either with or without H₂O₂ (Fig. 3C).

For the differentiation phase study, expanded hSDSCs incubated in a pellet system with a chondrogenic medium for 21 days were subsequently supplemented with H₂O₂ for an additional 7 days. DSCM expanded hSDSCs yielded pellets with larger size and intense staining of cartilage markers, with or without H₂O₂ treatment, compared with Plastic expanded hSDSCs (Fig. 4A). There was no difference in the size and chondrogenic staining of pellets from either DSCM or Plastic expanded cells between H₂O₂ treated and non-treated groups. The histology data were supported by biochemical data, in which DSCM expanded hSDSCs yielded pellets with a higher DNA ratio, GAG amount, and chondrogenic index than those from Plastic expanded cells, despite no significant difference from H₂O₂ treatment in either DSCM or Plastic expanded cells being found (Fig. 4B). Real-time PCR data showed that, compared to pellets from Plastic expanded cells, DSCM expanded cells yielded pellets with a higher chondrogenic marker gene expression with or without H₂O₂ treatment. DSCM expanded hSDSCs exhibited an upregulation of all three chondrogenic marker genes (*SOX9*, *ACAN*, and *COL2A1*) when treated with a low concentration of H₂O₂ (0.05 mM) compared to an upregulation of two chondrogenic marker genes (*SOX9* and *COL2A1*) when treated with a higher concentration of H₂O₂ (0.1 mM). In contrast, Plastic expanded hSDSCs displayed a dramatic decrease of all three chondrogenic marker genes under treatment with H₂O₂ at both 0.05 mM and 0.1 mM (Fig. 4C).

Similar to the *SOX9* mRNA level, for the proliferation phase study, DSCM expanded hSDSCs exhibited a downregulation of the *COL10A1* level compared to an upregulation in Plastic expanded cells after H₂O₂ treatment followed by a 21-day pellet culture (Fig. 5A/C). For the differentiation phase study, DSCM expanded hSDSCs displayed an upregulation of *COL10A1* level compared to a downregulation in Plastic expanded cells after a 21-day pellet culture followed by a seven-day H₂O₂ treatment (Fig. 5B/D).

Under oxidative stress, DSCM expanded hSDSCs exhibited an upregulation of p38 and Erk1/2 and a downregulation of p21

To further determine potential mechanisms underlying DSCM mediated cell proliferation and differentiation under oxidative stress, western blot was used to investigate the expression of the

MAPK signaling pathway [extracellular signal-regulated kinases 1 and 2 (Erk1/2), p38] and a senescence-associated marker (p21) in hSDSCs after exposure to 0.05 mM H₂O₂. Image J analysis showed that, with H₂O₂ treatment, DSCM expanded cells exhibited higher levels of p-p38/p38 (Fig. 6A) and p-Erk/Erk (Fig. 6B) compared to those from Plastic expanded cells; in contrast, DSCM expanded cells exhibited a lower level of p21 (Fig. 6C), a senescence-associated marker, compared to those from Plastic expanded cells.

Discussion

Chondrocytes produce superoxide, hydrogen peroxide, and hydroxyl radicals under resting conditions [27,28] and nitric oxide during chondrogenesis [29]. ROS are produced at a low level in articular chondrocytes and play an important as integral actors of intracellular signaling mechanisms; they modulate gene expression and are likely to contribute to the maintenance of cartilage homeostasis [30]. Increased production of ROS contributes to the pathology observed in inflammatory joint diseases [30], which is believed to be responsible for the fact that cartilage defects are difficult to repair except in the presence of inflammatory factors [interleukin 1 beta (IL-1 β) and tumor necrosis factor alpha (TNF- α)] [5]. Our recent studies demonstrated that DSCM expansion enhanced SDSC proliferation and chondrogenic potential [21,22]. It would be interesting to know if DSCM expansion could resist H₂O₂-induced oxidative stress while enhancing cell expansion and chondrogenic capacity. Our microarray data suggested that DSCM expansion not only dramatically enhanced hSDSC chondrogenic potential but also promoted expanded cell antioxidation ability, indicating that DSCM expansion could rejuvenate hSDSCs by decreasing the senescence-associated secretory phenotypes [31].

Our study found that, during the cell proliferation phase, DSCM expansion decreased the decline in cell proliferation and the increase in cell apoptosis in hSDSCs under H₂O₂-induced oxidative stress; this finding may be because DSCM expanded cells were able to resist the G1 arrest from H₂O₂. In contrast to the cell proliferation phase, expanded SDSCs were differently affected in chondrogenic potential by oxidative stress in terms of an upregulation in Plastic expanded SDSCs compared to a downregulation in DSCM expanded SDSCs. Our result differed from a previous report [32], in which

H₂O₂ inhibited cartilage formation in chicken micromass cultures. This difference could be because they used 1 mM H₂O₂ treatment for 30 min and we used 0.05 mM H₂O₂ as a continuous treatment. Moreover, different types of cells have different responses and tolerances to H₂O₂-induced oxidative stress [33]. In fibroblasts, low concentrations of H₂O₂ (<0.01 mM) stimulate cell proliferation; intermediate concentrations (~0.15 mM) cause growth arrest or senescence; and high levels of H₂O₂ (>0.4 mM) induce rapid apoptosis [34]. Our dose of H₂O₂ causing growth arrest is also in accordance with a previous study [35], in which inflammatory conditions (rheumatoid arthritis and osteoarthritis) were imitated; synoviocytes were inhibited in both hyaluronan and DNA synthesis at concentrations of H₂O₂ (10 – 100 µM), less than those which caused loss of cell integrity (>200 µM). The preconditioned effect from 0.05 mM H₂O₂ might be responsible for the upregulation of chondrogenic marker genes [36].

We also evaluated the effect of H₂O₂ on 21-day SDSC pellets in chondrogenic medium and found that predifferentiated SDSCs in a pellet could better resist H₂O₂-induced oxidative stress, as evidenced by comparable staining of sulfated GAGs and collagen II as well as stable cell viability and chondrogenic index. Our data were in accord with a study investigating the effects of peroxynitrite (0.1-0.6 mM) and H₂O₂ (0.1-4 mM) on poly (adp-ribose) polymerase (PARP) activation and extracellular matrix production of high-density micromass cultures prepared from chick limb bud mesenchymal cells [37]. They found that both oxidative species strongly inhibited matrix formation of high-density micromass cultures treated on day 2 but not on day 5. In micromass cultures, the major steps of cartilage differentiation occur on days 2 and 3 when young cartilage cells start to secrete a specific extracellular matrix (ECM) rich in collagen II and aggrecan [38], suggesting that ECM could protect chondrogenically differentiated mesenchymal cells from oxidative stress. Despite resistance at the protein level, DSCM expanded SDSCs yielded pellets with enhanced levels of chondrogenic marker genes upon stimulation by H₂O₂; in contrast, Plastic expanded SDSCs yielded pellets with downregulation of chondrogenic marker genes, indicating DSCM expansion renders SDSCs with enhanced chondrogenic potential.

Despite an enhanced antioxidation level in DSCM expanded cells, chondrogenic induction made these cells lose their antioxidation capacity when compared with Plastic expanded cells. Since enhanced endogenous antioxidant levels can protect cells from oxidative stress and inhibit chondrocyte hypertrophy [39], a decline in the antioxidative level may explain why DSCM expanded hSDSCs

exhibited a higher level of collagen X after chondrogenic induction compared to those grown on Plastic. When H_2O_2 was used to treat chondrogenically differentiated SDSCs, it was also understandable that DSCM expanded SDSCs exhibited an enhanced collagen X expression compared with Plastic expanded SDSCs. Following H_2O_2 treatment during monolayer culture, Plastic expanded SDSCs exhibited an increased ROS level, which is responsible for a higher level of collagen X expression in these chondrogenically differentiated cells. Interestingly, we found DSCM expanded SDSCs displayed a different response when incubated in a chondrogenic induction medium, while H_2O_2 treated SDSCs had a lower level of collagen X than non-treated cells. The mechanism is still under investigation.

The Erk cascade is a prominent component of the MAPK family that in particular plays an integral role in both growth factor and stress signaling, although it preferentially regulates cell growth and differentiation [40]. p38MAPKs are described as stress activated protein kinases because they are frequently activated by a wide range of environmental stresses such as oxidative stress and cytokines to induce inflammation, a key process in the host defense system [41]. The activation of Erk1/2 and p38MAPK after treatment with H_2O_2 was demonstrated in different types of cells, such as PC12 cells [42], skeletal myoblasts [43], alveolar epithelial cells [44], and ATDC5 cells [39], indicating that Erk1/2 and p38MAPK have universal protection against oxidative stress in different cell types. Our western blot data showed that the MAPK pathway was involved in the H_2O_2 -mediated and DSCM-mediated cell activities in terms of proliferation and differentiation; both Erk1/2 and p38MAPK were upregulated in DSCM expanded SDSCs, indicating that DSCM expansion might activate the MAPK signal pathway, helping combat H_2O_2 -induced oxidative stress. A higher level of p38MAPK in DSCM expanded SDSCs after H_2O_2 treatment might benefit subsequent differentiation due to its positive role in mesenchymal stem cell chondrogenesis [45]. p21, a potent cyclin-dependent kinase (CDK) inhibitor (CKI), functions as a regulator of cell cycle progression at G1 through binding and inhibiting the activity of cyclin-CDK2 or CDK4 complexes. Compared to SDSCs grown on Plastic, DSCM expanded SDSCs exhibiting a lower level of p21 might result from the observation that DSCM expanded cells were able to resist G1 arrest from H_2O_2 , demonstrating that DSCM expansion could rejuvenate SDSCs from oxidative stress-induced cell senescence [46].

In the present study, for the first time, we demonstrated that DSCM could enhance human SDSC *in vitro* expansion and chondrogenic potential; more importantly, we found that DSCM expansion could

prevent human SDSCs from oxidative stress-induced cell senescence, in terms of cell proliferation and differentiation. Since most patients with cartilage defects have diseased joints with oxidative stress, this finding provides important information for the potential application of using DSCM in cartilage engineering and regeneration. DSCM could be a promising cell expansion system to provide a large number of high-quality hSDSCs for cartilage regeneration in a harsh joint environment. Our DSCM model, as a new strategy and intervention aimed at reducing oxidative damage in implanted cells, might be a promising alternative in the treatment of osteoarthritis patients with cartilage defects.

References

1. Fitzpatrick K and JM Tokish. *J Knee Surg* 2011;**24**:159-166.
2. John T, PF Stahel, SJ Morgan et al. *Histol Histopathol* 2007;**22**:781-790.
3. Dhalla NS, L Golfman, S Takeda et al. *Can J Cardiol* 1999;**15**:587-593.
4. Francioli S, C Cavallo, B Grigolo et al. *Clin Orthop Relat Res* 2011;**469**:2773-2784.
5. Wehling N, GD Palmer, C Pilapil et al. *Arthritis Rheum* 2009;**60**:801-812.
6. Brandl A, A Hartmann, V Bechmann et al. *J Orthop Res* 2011;**29**:1114-1120.
7. Chen AF, CM Davies, M De Lin et al. *J Cell Physiol* 2008;**217**:828-833.
8. Grishko VI, R Ho, GL Wilson and AW 4th Pearsall. *Osteoarthritis Cartilage* 2009;**17**:107-113.
9. Urish KL, JB Vella, M Okada et al. *Mol Biol Cell* 2009;**20**:509-520.
10. Blanpain C, M Mohrin, PA Sotiropoulou et al. *Cell Stem Cell* 2011;**8**:16-29.
11. Dernbach E, C Urbich, RP Brandes et al. *Blood* 2004;**104**:3591-3597.
12. He T, TE Peterson, EL Holmuhamedov et al. *Arterioscler Thromb Vasc Biol* 2004;**24**:2021-2027.
13. Jones B and M Pei. *Tissue Eng Part B Rev* 2012;**18**:301-311.
14. Pei M, F He and G Vunjak-Novakovic. *Differentiation* 2008;**76**:1044-1056.
15. Bentley G, A Kreutner and AB Ferguson. *J Bone Joint Surg Br* 1975;**57**:454-462.
16. Kim WS, BS Park, HK Kim et al. *J Dermatol Sci* 2008;**49**:133-142.
17. Valle-Prieto A and PA Conget. *Stem Cells Dev* 2010;**19**:1885-1893.
18. Angelos MG, VK Kutala, CA Torres et al. *Am J Physiol Heart Circ Physiol* 2006;**290**:H341-347.
19. Bredesen DE. *Ann Neurol* 1995;**38**:839-851.
20. Yao EH, Y Yu and N Fukuda. *Curr Pharm Biotechnol* 2006;**7**:101-108.
21. He F, X Chen and M Pei. *Tissue Eng Part A* 2009;**15**:3809-3821.
22. Li JT and M Pei. *Tissue Eng Part A* 2011;**17**:703-712.
23. Pei M, JT Li, M Shoukry et al. *Eur Cell Mater* 2011;**22**:333-343.
24. He F and Pei M. *J Tissue Eng Regen Med* 2013;**7**:73-84.
25. Pei M, F He and VL Kish. *Tissue Eng Part A* 2011;**17**:3067-3076.
26. Li JT, B Jones, Y Zhang et al. *Drug Deliv Transl Res* 2012;**2**:363-374.
27. Henrotin Y, G Deby-Dupont, C Deby et al. *Br J Rheumatol* 1993;**32**:562-567.
28. Hiran TS, PJ Moulton and JT Hancock. *Free Radic Biol Med* 1997;**23**:736-743.
29. Johnson K, A Jung, A Murphy et al. *Arthritis Rheum* 2000;**43**:1560-1570.
30. Henrotin YE, P Bruckner and JP Pujol. *Osteoarthritis Cartilage* 2003;**11**:747-755.
31. Freund A, AV Orjalo, PY Desprez et al. *Trends Mol Med* 2010;**16**:238-246.
32. Zákány R, Z Sziógyártó, C Matta et al. *Exp Cell Res* 2005;**305**:190-199.
33. Yoon SO, CH Yun and AS Chung. *Mech Ageing Dev* 2002;**123**:1597-1604.
34. Kim BY, MJ Han and AS Chung. *Free Radic Biol Med* 2001;**30**:686-698.
35. Hutadilok N, MM Smith and P Ghosh P. *Ann Rheum Dis* 1991;**50**:219-226.
36. Ramakrishnan P, BA Hecht, DR Pedersen et al. *J Orthop Res* 2010;**28**:914-920.
37. Zákány R, E Bakondi, T Juhász et al. *Int J Mol Med* 2007;**19**:597-605.
38. Sandell LJ and P Adler. *Front Biosci* 1999;**4**:D731-742.
39. Morita K, T Miyamoto, N Fujita et al. *J Exp Med* 2007;**204**:1613-1623.

40. Nishida E and Y Gotoh. *Trends Biochem Sci* 1993;**18**:128-131.
41. Coulthard LR, DE White, DL Jones et al. *Trends Mol Med* 2009;**15**:369-379.
42. Su QJ, XW Chen, ZB Chen et al. *Neurosci Bull* 2008;**24**:244-250.
43. Kefaloyianni E, C Gaitanaki and I Beis. *Cell Signal* 2006;**18**:2238-2251.
44. Carvalho H, P Evelson, S Sigaud et al. *J Cell Biochem* 2004;**92**:502-513.
45. Li J, Z Zhao, J Liu et al. *Cell Prolif* 2010;**43**:333-343.
46. Ju Z, AR Choudhury and KL Rudolph. *Ann N Y Acad Sci* 2007;**1100**:333-344.

Figure 1

Figure 6. 1 Effect of DSCM pretreatment on hSDSC oxidative stress and chondrogenic potential.

In the heatmap (A), the expression intensity was log2-transformed and visualized by color ranging from low expression (blue) to high expression (red). The comparisons of Ecell versus Pcell in the genes (>2 fold change) related to oxidative stress and antioxidant (B) and Ep versus Pp in the genes (>2 fold change) related to antioxidant and cartilage markers (C), were clustered by hierarchical clustering methods.

Figure 2

Figure 6. 2 Effect of DSCM pretreatment on hSDSC expansion under H₂O₂.

(A) Cell morphology of hSDSCs expanded on either DSCM or Plastic was shown under phase contrast microscope after treatment with varying concentration (0, 0.05, 0.5, and 5 mM) of H₂O₂ for four days. Scale bar is 200 μ m. (B) The effect of 0.05 mM H₂O₂ on the proliferation of hSDSCs expanded on either DSCM or Plastic was evaluated using proliferation index by flow cytometry. (C) The effect of 0.05 mM H₂O₂ on the proliferation of hSDSCs expanded on either DSCM or Plastic was

evaluated using cell count (n=8 flasks each group) by hemocytometer. **(D)** The effect of 0.05 mM H_2O_2 on apoptosis of hSDSCs expanded on either DSCM or Plastic was evaluated using flow cytometry. **(E)** The effect of 0.05 mM H_2O_2 on the cell cycle of hSDSCs expanded on either DSCM or Plastic was evaluated using flow cytometry.

Figure 3

Figure 6. 3 Effect of DSCM pretreatment with 0.05 mM H₂O₂ on expanded hSDSC chondrogenic potential.

hSDSCs expanded on either DSCM or Plastic with H₂O₂ treatment were chondrogenically induced in a pellet culture system for 21 days. Alcian blue (AB) was used to stain sulfated GAGs. Immunohistochemistry staining (IHC) was used to detect collagen II (Col II). **(B)** Biochemical analysis was used for DNA and GAG contents in the 21-day chondrogenically-induced pellets. Cell

proliferation and viability was evaluated using DNA ratio (DNA content at day 21 adjusted by that at day 0). Chondrogenic index at day 21 was evaluated using a ratio of GAG to DNA. (C) Real-time PCR was used to evaluate chondrogenic marker gene expression (*SOX9*, *ACAN*, and *COL2A1*) in day 21 pellets. Data are shown as average $\pm$ SD for $n = 4$. * $p < 0.05$ compared with the corresponding DSCM group. # $p < 0.05$ compared with the corresponding group without H_2O_2 treatment.

Figure 4

Figure 6. 4 Effect of DSCM pretreatment on expanded hSDSC chondrogenic differentiation with either 0.05 or 0.1 mM H_2O_2 .

(A) hSDSCs expanded on either DSCM or Plastic with H_2O_2 treatment were chondrogenically induced in a pellet culture system for 21 days and treated with H_2O_2 for the following 7 days. Alcian blue (AB) was used to stain sulfated GAGs. Immunohistochemistry staining (IHC) was used to detect collagen II (Col II). Scale bar is 1 mm. (B) Biochemical analysis was used for DNA and GAG contents in the 28-day chondrogenically-induced pellets. Cell proliferation and viability was evaluated

using DNA ratio (DNA content at day 28 adjusted by that at day 0). Chondrogenic index at day 28 was evaluated using ratio of GAG to DNA. (C) Real-time PCR was used to evaluate chondrogenic marker gene expression (*SOX9*, *ACAN*, and *COL2A1*) in day 28 pellets. Data are shown as average $\pm$ SD for n = 4. * $p < 0.05$ compared with corresponding DSCM group. # $p < 0.05$ compared with the corresponding group without H₂O₂ treatment. \$ $p < 0.05$ compared with the corresponding group treated with 0.05 mM H₂O₂.

Figure 5

Figure 6. 5 Effect of H_2O_2 treatment in either cell expansion or chondrogenic differentiation phase on expanded SDSC chondrogenic hypertrophy.

(A and C) hSDSCs expanded on either DSCM or Plastic with 0.05 mM H_2O_2 treatment were chondrogenically induced in a pellet culture system for 21 days. (B and D) hSDSCs expanded on either DSCM or Plastic were chondrogenically induced in a pellet culture system for 21 days and treated with H_2O_2 for the following 7 days. Immunohistochemistry staining (IHC) was used to detect collagen X (A and B). Scale bar is 1 mm. Real-time PCR was used to evaluate hypertrophy marker

gene expression (*COL10A1*) (**C** and **D**). Data are shown as average $\pm$ SD for $n = 4$. * $p < 0.05$ compared with corresponding DSCM group. # $p < 0.05$ compared with the corresponding group without H_2O_2 treatment. \$ $p < 0.05$ compared with the corresponding group treated with 0.05 mM H_2O_2 .

Figure 6

Figure 6. 6 Western blot was used to measure the levels of p38 and Erk1/2, p21 in the hSDSCs expanded on either DSCM or Plastic after the treatment with 0.05 mM H₂O₂.

β -actin served as an internal control. Image J software was used to quantify immunoblotting bands.

Chapter 7. A review of crosstalk between MAPK and Wnt signals and its impact on cartilage regeneration

As published in Cell Tissue Res. 2014; 358(3):633-49. doi: 10.1007/s00441-014-2010-x

^{1,2}Ying Zhang, MS, ^{1,3}Tyler Pizzute, BS, and ^{1,2,3}Ming Pei, MD, PhD

¹Mechanical and Aerospace Engineering, West Virginia University, Morgantown, West Virginia 26506, USA

²Stem Cell and Tissue Engineering Laboratory, Department of Orthopaedics, West Virginia University, Morgantown, West Virginia 26506, USA

³Exercise Physiology, West Virginia University, Morgantown, West Virginia 26506, USA

Running head: Crosstalk Signaling Affects Cartilage Regeneration

Corresponding author: Ming Pei MD, PhD, Stem Cell and Tissue Engineering Laboratory, Department of Orthopaedics, West Virginia University, PO Box 9196, One Medical Center Drive, Morgantown, WV 26506-9196, USA. Telephone: 304-293-1072. Fax: 304-293-7070. Email: mpei@hsc.wvu.edu

Abstract

Chondrogenesis is a developmental process that is controlled and coordinated by many growth and differentiation factors as well as environmental factors that initiate or suppress cellular signaling pathways and transcription of specific genes in a temporal-spatial manner. As key signaling molecules in regulating cell proliferation, homeostasis, and development, both mitogen-activated protein kinases (MAPK) and the Wnt family participate in morphogenesis and tissue patterning and play important roles in skeletal development, especially chondrogenesis. Recent findings suggest that both signals are also actively involved in arthritis and related diseases. Despite the fact that the crosstalk between MAPK and Wnt signaling has been implicated to play a significant role in cancer, few studies have summarized this interaction and crosstalk in regulating chondrogenesis. In this review, we focus on MAPK and Wnt signaling in reference to their relationships in different types of cells and particularly how this crosstalk might influence chondrogenesis and cartilage development. We also discuss how the interactions between MAPK and Wnt signaling might relate to cartilage related diseases such as osteoarthritis and explore the potential therapeutic targets for disease treatments.

Key words

Chondrogenesis; MAPK signal; Wnt signal; Stem cell; Osteoarthritis

Introduction

Chondrogenesis is a morphogenetic event that includes proliferation, condensation, and differentiation of mesenchymal cells into chondrocytes with the production of a cartilage-specific extracellular matrix (ECM) rich in type II collagen and sulfated proteoglycans [12]. Mitogen-activated protein kinase (MAPK) is one of the conserved signal transduction systems in cartilage, which plays a crucial role in chondrogenic differentiation. The MAPK cascades constituting three sequentially activated kinase complexes, which include p38 MAPK, c-Jun N-terminal kinase (JNK), and extracellular regulated kinase (ERK), are substrates for phosphorylation by MAPK kinases (MKKs) (Fig. 1). The MKKs are in turn phosphorylated by MAPK kinase kinases (MEKKs). Regarding chondrogenesis and chondrocyte differentiation, ERK and p38 MAPK have central roles in mediating chondrocyte proliferation and related gene expression [55], whereas JNK has a minor role in chondrogenesis as JNK phosphorylation is not affected during the process [66, 87]. p38 MAPK is usually phosphorylated during chondrogenesis and is generally accepted as a positive regulator in chondrogenesis and chondrocyte differentiation [73, 87, 101]; however, the role of the ERK MAPK pathway (also known as the MEK-ERK kinase cascade) is still controversial. Murakami et al. reported that ERK is a positive regulator in chondrogenesis, as the increase in SRY (sex determining region Y)-box 9 (*SOX9*) levels induced by basic fibroblast growth factor (FGF2) was inhibited by a specific ERK kinase (MEK) inhibitor (U0126) in primary chondrocytes. Co-expression of a constitutively active mutant of MEK1 increased the activity of the Sox9-dependent enhancer in primary chondrocytes and C3H10T1/2 cells [68]. However, some studies interpreted MEK-ERK as a negative factor for chondrogenesis. For example, ERK1/2 activities were observed to decrease as chondrogenesis proceeded and inhibition of ERK1/2 with PD98059 enhanced chondrogenesis [73]; other studies also showed similar results [7, 15].

The Wnt family of secreted glycoproteins are signaling molecules that play important roles in controlling a wide range of developmental processes, including tissue patterning, cell proliferation, and cell fate, through two distinct signaling pathways, in terms of the canonical and non-canonical Wnt pathways (Fig. 2). In the canonical Wnt signaling pathway, binding of secreted Wnts to the Frizzled family of cell surface receptors inactivates glycogen synthase kinase 3beta (GSK3 β),

resulting in stabilization and nuclear translocation of β -catenin and activation of Wnt target genes. The non-canonical pathways also signal through the Frizzled receptors; the planar cell polarity (PCP) pathway activates the rho family of GTPases and JNK and modifies cytoskeletal organization and epithelial cell polarization. The Wnt/ Ca^{2+} pathway stimulates the intracellular increase of Ca^{2+} through activation of protein kinase C (PKC) and calmodulin-dependent kinase II (CaMKII) [1]. During embryonic skeletogenesis, Wnt components act as both positive and negative regulators of key events, including chondroblast differentiation, chondrocyte maturation, and joint formation [16].

In embryos, low levels of Wnt/ β -catenin signaling stimulate chondrogenic differentiation of stem cells whereas high levels of Wnt/ β -catenin signaling inhibit that process [42, 49, 102]. Wnts have also been shown to both inhibit and stimulate chondrogenic differentiation of adult progenitor cells [19, 43, 44]. Removal of β -catenin early in mesenchymal progenitor cells promoted chondrocyte differentiation while ectopic expression of an activated form of β -catenin in early differentiating chondrocytes induced ectopic joint formation both morphologically and molecularly [38]. In adult progenitor cells, osteoblast precursors lacking β -catenin are blocked in differentiation and develop into chondrocytes instead. Detailed *in vivo* and *in vitro* loss- and gain-of-function analyses reveal that β -catenin activity is necessary and sufficient to repress the differentiation of mesenchymal cells into runt-related transcription factor 2 (*RUNX2*) and *SOX9*-positive skeletal precursors [43], suggesting that Wnt/ β -catenin signaling controls osteoblast and chondrocyte formation when they differentiate from mesenchymal progenitors.

It has been reported that the MAPK pathway also regulates Wnt/ β -catenin signaling. Wnt/ β -catenin signaling was activated by *LIT1* and *MOM4*, which separately encode a homologue of the MAPK-related Nemo-like Kinase (NLK) and a homologue of transforming growth factor beta ($\text{TGF}\beta$)-activated kinase (TAK-1) [66]. TAK-1, a MEKK activated by $\text{TGF}\beta$, bone morphogenetic protein (BMP), and other MAPK signaling components, plays a critical role in chondrogenesis. Deletion of *TAK1* in chondrocytes resulted in novel embryonic developmental cartilage defects including decreased chondrocyte proliferation, reduced proliferating chondrocyte survival, delayed onset of hypertrophy, and reduced matrix metalloproteinase-13 (*MMP13*) expression [36]. Since both MAPK and Wnt signaling pathways play critical regulatory roles in the development of cartilage and bone formation, studying their interactions and crosstalk is of extreme importance to elucidate the complex signaling networks in chondrogenesis and to explore potential therapeutic targets for related diseases

such as osteoarthritis (OA).

Influence of canonical Wnt signals on the MAPK pathway

In totipotent mouse F9 teratocarcinoma cells, the canonical Wnt- β -catenin-JNK signaling pathway was found to be activated by G-proteins, which can propagate the signals downstream through Dishevelled isoforms. Suppression of Dishevelled-1 or Dishevelled-3 abolished Wnt3a activation of JNK [5]. Wnt3a treatment enhanced the mRNA and protein expression of c-Jun and stimulated the phosphorylation of c-Jun and JNK. Furthermore, Wnt3a activation of activator protein-1 (AP-1) was blocked by the inhibition of JNK with SP600125 and by the inhibition of AP-1 with N-acetyl-L-cysteine and nordihydroguaiaretic acid [45]. AP-1 was also activated by ERK1/2 in C3H10T1/2 cells [83]. In NIH3T3 fibroblast cells, ERK pathway activation by Wnt signaling could occur at multiple levels, including β -catenin-independent direct signaling resulting from a Wnt3a (Wnt3a-Raf-1-MEK-ERK) and a β -catenin-/Tcf-4-dependent post gene transcription event [108]. In addition to JNK and ERK, p38 MAPK was strongly activated by Wnt3a in mouse F9 teratocarcinoma cells and the activated p38 MAPK regulated canonical Wnt- β -catenin signaling through regulation of GSK3 β . Chemical inhibitors of p38 MAPK (SB203580) and expression of a dominant negative-version of p38 MAPK attenuated Wnt3a-induced accumulation of β -catenin, Lef/Tcf-sensitive gene activation, and primitive endoderm formation [6]. The above evidence indicates the influence of canonical Wnt signals on the MAPK pathway (Fig. 3).

It is known that reduced expression of adhesion molecules is associated with formation and differentiation of cartilage nodules, which is supported by the fact that N-cadherin is expressed in prechondrogenic mesenchymes during cell condensation but not in differentiated chondrocytes [72, 91]. One study indicated that inhibition of p38 MAPK results in sustained expression of N-cadherin and eventually inhibits the chondrogenic differentiation in chick limb mesenchymal micromass cultures [73]. Wnt regulation of limb mesenchymal chondrogenesis is also involved in the modulation of N-cadherin. Wnt7a signaling has been shown to inhibit chondrogenic differentiation of limb mesenchymal cells *in vitro* by modulating the expression of N-cadherin and the turnover of N-cadherin-dependent cell-cell adhesion complexes [98]. The combination of Wnt7a misexpression and

ERK inhibition partially recovers Wnt7a inhibition of chondrogenic differentiation, whereas the combination of Wnt7a misexpression and p38 inhibition acts in a synergistic chondro-inhibitory fashion [97].

Wnt3a can also induce a rapid and transient activation of p38 MAPK, which in turn regulates alkaline phosphatase activity and mineralization of nodules, directing the differentiation of mesenchymal cells into osteoprogenitors. Dickkopf1, a selective antagonist of Wnt proteins, did not influence the activation of p38 MAPK and ERK induced by Wnt3a [13], implying that non-canonical Wnt pathways might participate in the regulatory process of mesenchymal cell differentiation into osteogenic cells.

Influence of non-canonical Wnt signals on the MAPK pathway

As a non-canonical Wnt signal, Wnt5a specifically promotes entry into the prehypertrophic phase, whereas it conversely blocks chondrocyte hypertrophy, acting in a stage-specific context [51, 106]. This finding was confirmed by a study showing that Wnt5a misexpression delays the maturation of chondrocytes and the onset of bone collar formation [41]. Wnt5a increased chondrocyte differentiation at an early stage through CaMK/calcineurin (CaN)/nuclear factor of activated T cells (NFAT)-dependent induction of Sox9 while repressing chondrocyte hypertrophy *via* I κ B kinase (IKK)/nuclear factor- κ B (NF- κ B)-dependent inhibition of Runx2 expression [9]. In mouse F9 embryonal teratocarcinoma cells, a strong activation of p38 MAPK was observed in response to Wnt5a; treatment with SB203580 effectively abolished the stimulatory effects of Wnt5a [61]. Both exogenous TGF β 3 and overexpression of Wnt5a stimulated PKC α and p38 MAPK activation early in the culture, resulting in cellular condensation and chondrogenesis. Comparatively, inhibiting PKC α or p38 MAPK activity abolished the promotion of chondrogenic differentiation by overexpressing Wnt5a or exogenous TGF β 3. On the other hand, partial reduction of endogenous *WNT5A* by small interfering RNA diminished TGF β 3-stimulated chondrogenesis through inhibition of PKC α and p38 MAPK activity [48]. Wnt5a was also found to promote ERK1/2 phosphorylation in endothelial cells [62]; the expression of Wnt5a blocked canonical Wnt signaling in endothelial cells and other cell types [95]. (Fig. 4)

However, non-canonical Wnt signaling more commonly functions through the Wnt-JNK pathways [59]. Activation of Wnt5a signaling by interleukin 1beta (IL-1 β) induced the expression of MMPs *via* the JNK pathways in rabbit temporomandibular joint (TMJ) condylar chondrocytes, whereas blockage of JNK signaling impaired the Wnt5a-induced up-regulation of MMPs [31]. The highly homologous non-canonical Wnt signals, Wnt5a and Wnt5b, have differential effects on cartilage development in regard to cell proliferation and expression of type II collagen. Unlike Wnt5a, Wnt5b repressed chondrocyte differentiation in both the initial stages of cartilage condensation and the late hypertrophic stage [106]. Wnt5b activated JNK, a component of the PCP pathway, contributing to an increase in cellular migration and Wnt5b-mediated decreases in cell-cell adhesion through an activation of Src and subsequent cadherin receptor turnover [10]. (Fig. 4)

Wnt5a also plays an important role in osteoblast differentiation. The MAPK pathway was altered in Wnt5a-deficient mouse calvarial cells, suggesting that Wnt5a signaling influenced the MAPK/JNK pathway [37]. Other studies provide evidence for crosstalk between Wnt5a and MAPK. Ishitani et al. found that overexpression of Wnt5a in HEK293 cells activated NLK MAPK through TAK-1; furthermore, overexpression of Wnt5a antagonized the canonical Wnt/ β -catenin pathway [46]. Through CaMKII-TAK1-TAB2-NLK, non-canonical Wnt signaling transcriptionally repressed peroxisome proliferator-activated receptor gamma (*PPARG*) transactivation and induced *RUNX2* expression, promoting osteoblastogenesis in preference to adipogenesis in bone marrow mesenchymal progenitors [88]. Wnt4, conventionally regarded as a non-canonical Wnt class [103], was found to potently enhance osteogenic differentiation of mesenchymal stem cells (MSCs) isolated from human adult craniofacial tissue *in vitro* and bone formation *in vivo*, through activating p38 MAPK, which is known to positively regulate osteogenic differentiation induced by BMPs and other growth factors [30, 35]. The inhibition of p38 MAPK abolished osteogenic differentiation of MSCs promoted by Wnt4.

Wnt11 belongs to the Wnt5a subclass which exerts diverse effects through activation of the non-canonical Wnt signaling pathway [25]. Recently, Rye and Chun demonstrated that Wnt11 stimulates the accumulation of type II collagen in articular chondrocytes [78]. In three-dimensional alginate gels, *WNT11* expression peaked at the late stage of chondrogenic differentiation of human MSCs [105]. In *Xenopus laevis* and mouse P19 cells, signaling cascades activated by Wnt11 were crucial for initiating cardiogenesis; furthermore, Wnt11 not only inhibited β -catenin signaling, but also activated JNK,

suggesting crosstalk between Wnt11 and MAPK signals [74]. In human hepatocellular carcinoma (HCC) cell lines, overexpression of Wnt11 activated PKC signaling, which antagonized canonical Wnt signaling through phosphorylation of β -catenin and reduction of T-cell factor (TCF)-mediated transcriptional activity [96]. However, few studies have been reported about the interplay between Wnt11 and MAPK signaling in the regulation of chondrogenesis.

Influence of MAPK signals on the Wnt pathway

Many studies showed that MAPKs participated in the regulation of Wnt pathway activities (Fig. 5). Expression of constitutively active MKK6, an upstream activator of p38 MAPK, in 293T cells was sufficient to increase the expression of β -catenin proteins, through direct phosphorylation of GSK3 β protein both *in vitro* and *in vivo*; this phosphorylation was blocked by SB203580 or the knock-out of MKK3 and MKK6 [25, 93]. Since MAPK signals were required for the phosphorylation of PPPS/TP motifs of endogenous LDL-related protein 6 (LRP6), Wnt3a-induced phosphorylation of endogenous LRP6 was significantly attenuated by knock-down of JNK1 and p38 β . These results were further confirmed by pharmacological inhibition of p38 MAPK by SB203580 and that of JNK by SP600125 [14]. Rac1 can activate JNK2 to phosphorylate β -catenin, which is responsible for controlling limb outgrowth in mouse embryos [104]. In *Xenopus* embryos, activation of JNK antagonized the canonical Wnt pathway through activating the nuclear export of β -catenin rather than its cytoplasmic stability [58]. Many receptor tyrosine kinase (RTK) systems facilitated Wnt/ β -catenin signaling by the phosphatidylinositol 3-kinase (PI3K)/AKT (or alternatively Protein Kinase B, PKB) pathway through inhibiting GSK3 activity [18]. Interestingly, RTKs were also found to utilize ERK/LRP6 pathways for a direct phosphorylation of β -catenin to activate WNT/ β -catenin signaling [50].

By changing key signals of the Wnt pathways, some critical transcriptional factor activities such as Sox9 or Runx2 are regulated, and eventually determine the differentiation fate of cells. BMP2, for instance, promotes chondrogenesis by activating p38 MAPK, which in turn down-regulates Wnt7a/ β -catenin signaling. Inhibition of p38 MAPK using a dominant negative mutant led to sustained Wnt7a increase and decreased Sox9 expression, with consequent inhibition of pre-cartilage condensation and chondrogenic differentiation [47]. Similarly, TGF β -1 mediated MAPK activation, which controls

WNT7A gene expression and Wnt-mediated signaling through the intracellular β -catenin-TCF pathway, likely regulates N-cadherin expression and subsequent N-cadherin-mediated cell-adhesion complexes during the early steps of mesenchymal progenitor cell chondrogenesis [99].

Environmental factors such as mechanical stress and cytokines may also activate the MAPK pathway. Static compressive loading of cartilage activates the MAPK pathway, which is also known as the stress-activated protein kinase (SAPK) pathway [28, 94]. Wnt/ β -catenin signaling was not only involved in the bone response to mechanical loading [76, 81] but also associated with the response to mechanical damage to cartilage, which results in an increase in Wnt16 expression [20]. In MC3T3-E1 osteoblastic cells, activation of the pathway by treatment with a GSK3 β inhibitor resulted in an anabolic bone formation response whereas the application of inhibitor combined with mechanical loading produced a synergistic effect on the expression of Wnt/ β -catenin pathway target genes [76]. These results indicated that mechanical loading activated the Wnt/ β -catenin signaling pathway, at least in part, through the MAPK signaling pathway [93].

Crosstalk of MAPK and Wnt signals in cartilage inflammation and regeneration

Osteoarthritis is a common disease, clinically manifested by joint pain, swelling, and impairment of joint function, which leads to disability and the need for joint replacement. Levels of β -catenin and cyclooxygenase 2 (COX2) were increased in osteoarthritic and rheumatoid arthritic (RA) cartilage, suggesting that accumulation of β -catenin may contribute to the inflammatory responses of cartilage by inducing *COX2* expression in chondrocytes of arthritis-affected cartilage (Kim et al. 2002). Activation of β -catenin in mature chondrocytes stimulated hypertrophy and matrix mineralization, evidenced by the expression of *MMP13* and vascular endothelial growth factor (*VEGF*) [19, 90]. Overexpression of β -catenin in chondrocytes markedly increased expression of matrix degradation enzymes such as MMP-2, MMP-3, MMP-7, MMP-9, membrane-type 3 MMP (MT3-MMP), and A Disintegrin And Metalloproteinase with Thrombospondin Motifs 5 (ADAMTS5) [90]. Evidence from animal and *in vitro* models showed that knock-out of *FRZB*, which encodes a secreted Frizzled-related protein that can bind Wnt proteins, is more prone to lose proteoglycans from the articular cartilage in

the knee [60]. Through β -catenin stabilization and its nuclear translocation, Wnt signaling is associated with negative regulation of early chondrogenesis and stimulation of chondrocyte hypertrophy during development. Overexpression of *Frzb1* lowered the expression of β -catenin [26]. The *FRZB*^{-/-} mice may induce OA formation through up-regulation and stabilization of β -catenin in the canonical Wnt pathway. Interestingly, *Frzb* deficiency also resulted in thicker cortical bone, with increased stiffness and higher cortical appositional bone formation after loading, seemingly supporting the hypothesized inverse relationship between OA and osteoporosis [21]. In addition, canonical Wnt signaling is influenced by local factors, including alterations in glycosaminoglycan sulfation, cartilage matrix content, TGF β , and vitamin D. It is interesting to note that the MMPs and ADAMTSs boosted by experimental activation of the Wnt/ β -catenin pathway are quite similar to those triggered by treatment with IL-1 β or tumor necrosis factor alpha (TNF α) in chondrocytes [11]. However, the role of β -catenin in the homeostasis of cartilage is still controversial, as suggested by Zhu et al. that inhibition of β -catenin signaling in articular chondrocytes caused increased cell apoptosis and articular cartilage destruction in COL2A1-ICAT-transgenic mice [110]. It is assumed that the Wnt/ β -catenin pathway may be part of integrated signal transduction mechanisms through which chondrocytes respond to deranging and catabolic cues, activate the expression of *MMP* and *ADAMTS* genes and corresponding proteolytic activity, and undermine their phenotypic status and ultimate tissue function [107].

Accumulating evidence supports a central regulatory role of MAPK in mediating inflammatory and matrix degrading processes that contribute to joint tissue destruction in OA. Both OA and normal chondrocytes expressed p38 MAPK; however, OA chondrocytes showed a much higher phosphorylated p38 MAPK level compared to normal chondrocytes [27, 89]. Activated JNK was detected in the cytoplasm of OA chondrocytes, but not in healthy controls [17]. In a dog model of surgically induced OA, p38 MAPK, JNK, and ERK1/2 were all activated to a greater degree compared to those in normal tissue [8]. Among the possible MAPK therapeutic targets for OA or RA, p38 MAPK is generally considered to be the most promising, as p38 MAPK isoforms have been implicated in the regulation of processes (such as migration and accumulation of leucocytes and production of cytokines and pro-inflammatory mediators and angiogenesis) which promote disease pathogenesis [54, 82]. p38 MAPK inhibitors have been proven effective in reducing clinical severity, paw swelling, inflammation, cartilage breakdown, and bone erosion in a rat streptococcal cell wall

arthritis model [63, 64], a collagen-induced-arthritis (CIA) model in mice [65], and adjuvant and CIA models in rats [2, 70]. JNK appears to be a critical MAPK pathway for IL-1-induced collagenase gene expression in synoviocytes and joint arthritis (Han et al. 1999; Han et al. 2001). The JNK inhibitor SP600125 completely blocked not only IL-1-induced accumulation of phosphorylated Jun and induction of c-Jun transcription in synoviocytes but also the AP-1 binding and collagenase mRNA accumulation [40]. ERK is known to be involved in the regulation of IL-6, IL-12, IL-23, and TNF- α synthesis, suggesting a possible involvement of ERK in joint damage associated with pro-inflammatory cytokine production by macrophages [29, 34]. ERK inhibitors have been found to be successful in reducing inflammation in an experimental OA model in rabbits [75]; therapeutic intervention with the goal of MEK1/2 inhibition may have interesting potential for the development of agents for the treatment of OA. Due to important roles in transducing inflammation and joint destruction, MAPK signals are key molecular targets for therapeutic intervention in inflammatory diseases such as OA and RA. However, inhibitors targeting ablation or reduction of MAPK activity are likely to have serious side effects [92].

Recently, several non-canonical Wnt isoforms such as Wnt5a and Wnt11 were reported to be involved in IL-1 β -induced dedifferentiation of articular chondrocytes [78]. Wnt5a was detectably expressed in OA and RA and was involved in IL-1 β -induced up-regulation of MMP-1, MMP-3, MMP-9, and MMP-13 in primary TMJ condylar chondrocyte *via* the JNK pathway, suggesting the role of Wnt5a in arthritic pathology and regulation of cartilage destruction. Furthermore, blockage of JNK signaling impaired the Wnt5a-induced up-regulation of MMPs [31]. This finding indicates that crosstalk between Wnt5a and JNK contributes to the pathogenesis of OA; meanwhile, disturbance of or intervention in the interaction between these signals might provide new targets for OA treatment. Several studies have indicated that signaling pathways involving MAPKs mediate the catabolic response of chondrocytes to those inflammatory cytokines; specific inhibitors to those pathways can counteract cytokine effects on matrix protease gene expression [33, 45, 56, 57, 79].

Besides activation of inflammatory cytokines such as IL-1, the occurrence of OA may also take the route in which articular chondrocytes lose their differentiated phenotype and obtain a behavior with similarities to terminal differentiating chondrocytes (hypertrophy-like), as can be found in the growth plate of growing individuals [24, 100]. Chondrocytes in OA cartilage show an aberrant phenotype and actively produce cartilage-degrading enzymes, such as MMP-13 and aggrecanases [67, 85, 86]. The

higher expression of hypertrophic chondrocyte markers, type X collagen and MMP-13 [53, 71] in OA, suggests a correlation between hypertrophy and OA. Some studies demonstrated that Wnt signaling promoted chick chondrocyte hypertrophy through induction of the bone and cartilage-related transcription factor Runx2. Dong et al. reported that β -catenin is able to induce *RUNX2* and *COL10A1* transcription as the molecular mechanism through which Wnt signaling regulates chondrocyte hypertrophy [23]. Protein levels of β -catenin, which accumulates in OA chondrocytes, are very low in differentiated articular chondrocytes; however, low levels of β -catenin were up-regulated during phenotypic loss after a serial monolayer culture. Ectopic expression or inhibition of β -catenin degradation caused cessation of cartilage-specific ECM molecule synthesis *via* activation of β -catenin-Tcf/Lef transcriptional activity [79]. Activation of Wnt/ β -catenin signaling is usually accompanied by a shift in chondrocyte cytoarchitecture. This event may result from a reduction of proteoglycan pericellular matrix, or interactions between chondrocyte surface and substrate or fibrillar components such as collagen or fibronectin, which could change intracellular signaling and up-regulate cell adhesion pathways such as that of MAPK [32]. Because the loss of a differentiated phenotype of chondrocytes is associated with cartilage destruction during arthritis [80], the canonical Wnt pathway-mediated cell phenotype change *via* crosstalk with MAPK signals is another pathway through which OA forms. For the non-canonical Wnt signal pathway, Wnt5a has been reported as a key parameter influencing the phenotypic stability of chondrocytes [4, 107]. Wnt5a inhibits type II collagen expression in rabbit TMJ condylar chondrocytes [31], suggesting that Wnt5a signaling may regulate pathologic cartilage degeneration by inducing chondrocyte dedifferentiation [78]. Increased p38 activity is accompanied by type X collagen staining in osteochondrocytes and marginal synovial cells in a mouse OA model [84]. During monolayer culture, p38 MAPK was responsible for the loss of chondrocyte phenotypes including type II collagen and Sox9 while the blockade of p38 MAPK enhanced chondrocyte phenotypes, which suggests a blockade of dedifferentiation (Rosenzweig et al. 2013). Inhibition of p38 signaling in chondrocytes resulted in decreased expression of the *COL10A1* gene [3, 87, 109].

Conclusion and Future Directions

Although there is an increasing awareness of the importance of MAPK and Wnt signaling pathways in regulating cell activities and relevant diseases such as cancer, their interaction networks and potential roles in disease are still not fully appreciated, especially in the cartilage regeneration area (Table 1). Cartilage differentiation and maintenance of homeostasis are finely tuned by a complex network of signaling molecules; interplay of these signaling pathways leads to changes in cell activities and eventually influences their differentiation fates. Over the past two decades, extensive studies on Wnt and MAPK regulation of chondrogenesis and cartilage development have shown that Wnt and MAPK signals have both positive and negative regulatory effects on cartilage development. Increasing evidence indicates the involvement of Wnt and MAPK signals in the regulation of differentiated chondrocyte functions and cartilage disease.

Recent evidence from both animal experiments and clinical samples demonstrated the role of both Wnt and MAPK signaling in OA pathology, making these pathways attractive targets for therapy. Some chemicals and drugs targeting MAPK or Wnt have been designed and applied clinically; while some are effective in treatment of OA, their side effects have drawn concern. Direct targeting to Wnt or MAPK has been reported to be too risky, because of the critical role of these signals in the maintenance of articular chondrocyte stability. A promising method would be to identify the mis-regulated genes in the Wnt or MAPK pathways, and try to determine balanced therapeutic targets. The ideal therapeutic goal would be treatment with few or even no side effects to patients. In order to achieve the goal of clinical application, comprehensive appreciation and meticulous evaluation of interactions of these signaling pathways are not only necessary but also required. Future research may focus on elucidation of the network between MAPK and Wnt signaling interplays and exploration of clinical application in cartilage regeneration by intervening in specific signaling pathways.

References

1. Akiyama H, Lyons JP, Mori-Akiyama Y et al. *Genes Dev* 2004;**18**:1072-1087.
2. Badger AM, Griswold DE, Kapadia R et al. *Arthritis Rheum* 2000;**43**:175-183.
3. Beier F, LuValle P. *Biochem Biophys Res Commun* 1999;**262**:50-54.
4. Benya PD, Shaffer JD. *Cell* 1982;**30**:215-224.
5. Bikkavilli RK, Feigin ME, Malbon CC. *J Cell Sci* 2008a;**121**:234-245.
6. Bikkavilli RK, Feigin ME, Malbon CC. *J Cell Sci* 2008b;**121**:3598-3607.
7. Bobick BE, Kulyk WM. *Exp Cell Res* 2006;**312**:1079-192.
8. Boileau C, Martel-Pelletier J, Brunet J et al. *Ann Rheum Dis* 2006;**65**:573-580.
9. Bradley EW, Drissi MH. *Mol Endocrinol* 2010;**24**:1581-1593.
10. Bradley EW, Drissi MH. *J Cell Physiol* 2011;**226**:1683-1693.
11. Burrage PS, Mix KS, Brinckerhoff CE. *Front Biosci* 2006;**11**:529-43.
12. Cancedda R, Castagnola P, Cancedda FD et al. *Int J Dev Biol* 2000;**44**:707-714.
13. Caverzasio J, Manen D. *Endocrinology* 2007;**148**:5323-5330.
14. Červenka I, Wolf J, Mašek J et al. *Mol Cell Biol* 2011;**31**:179-189.
15. Chang SH, Oh CD, Yang MS et al. *J Biol Chem* 1998;**273**:19213-19219.
16. Church VL, Francis-West P. *Int J Dev Biol* 2002;**46**:927-936.
17. Clancy R, Rediske J, Koehne C et al. *Osteoarthritis Cartilage* 2001;**9**:294-299.
18. Dailey L, Ambrosetti D, Mansukhani A et al. *Cytokine Growth Factor Rev* 2005;**16**:233-247.
19. Day TF, Guo X, Garrett-Beal L et al. *Dev Cell* 2005;**8**:739-750.
20. Dell'accio F, De Bari C, Eltawil NM et al. *Arthritis Rheum* 2008;**58**:1410-1421.
21. Dequeker J, Aerssens J, Luyten FP. *Aging Clin Exp Res* 2003;**15**:426-439.
22. Ding Q, Xia W, Liu JC et al. *Mol Cell* 2005;**19**:159-170.
23. Dong YF, Soung do Y, Schwarz EM et al. *J Cell Physiol* 2006;**208**:77-86.
24. Dreier R. *Arthritis Res Ther* 2010;**12**:216.
25. Du SJ, Purcell SM, Christian JL et al. *Mol Cell Biol* 1995;**15**:2625-2634.
26. Enomoto-Iwamoto M, Kitagaki J, Koyama E et al. *Dev Biol* 2002;**251**:142-156.
27. Fan Z, Söder S, Oehler S et al. *Am J Pathol* 2007;**171**:938-946.
28. Fanning PJ, Emkey G, Smith RJ et al. *J Biol Chem* 2003;**278**:50940-50948.
29. Feng GJ, Goodridge HS, Harnett MM et al. *J Immunol* 1999;**163**:6403-6412.
30. Gallea S, Lallemand F, Atfi A et al. *Bone* 2001;**28**:491-498.
31. Ge X, Ma X, Meng J et al. *Arthritis Rheum* 2009;**60**:2714-2722.
32. Gemba T, Valbracht J, Alsalameh S et al. *J Biol Chem* 2002;**277**:907-911.
33. Geng Y, Valbracht J, Lotz M. *J Clin Invest* 1996;**98**:2425-2430.
34. Goodridge HS, Harnett W, Liew FY et al. *Immunology* 2003;**109**:415-425.
35. Guicheux J, Lemonnier J, Ghayor C et al. *J Bone Miner Res* 2003;**18**:2060-2068.
36. Gunnell LM, Jonason JH, Loiselle AE et al. *J Bone Miner Res* 2010;**25**:1784-1797.
37. Guo J, Jin J, Cooper LF. *Bone* 2008;**43**:961-971.
38. Guo X, Day TF, Jiang X et al. *Genes Dev* 2004;**18**:2404-2417.
39. Han Z, Boyle DL, Aupperle KR et al. *J Pharmacol Exp Ther* 1999;**291**:124-130.
40. Han Z, Boyle DL, Chang L et al. *J Clin Invest* 2001;**108**:73-81.
41. Hartmann C, Tabin CJ. *Development* 2000;**127**:3141-3159.

42. Hartmann C. *Trends Cell Biol* 2006;**16**:151-158.
43. Hill TP, Später D, Taketo MM et al. *Dev Cell* 2005;**8**:727-738.
44. Hu H, Hilton MJ, Tu X et al. *Development* 2005;**132**:49-60.
45. Hwang SG, Yu SS, Lee SW et al. *FEBS Lett* 2005;**579**:4837-4842.
46. Ishitani T, Kishida S, Hyodo-Miura J et al. *Mol Cell Biol* 2003;**23**:131-139.
47. Jin EJ, Lee SY, Choi YA et al. *Mol Cells* 2006b;**22**:353-359.
48. Jin EJ, Park JH, Lee SY et al. *Int J Biochem Cell Biol* 2006a;**38**:183-195.
49. Johnson ML, Rajamannan N. *Rev Endocr Metab Disord* 2006;**7**:41-49.
50. Krejci P, Aklia A, Kaucka M et al. *PLoS One* 2012;**7**:e35826.
51. Kawakami Y, Wada N, Nishimatsu SI et al. *Dev Growth Differ* 1999;**41**:29-40.
52. Kim SJ, Im DS, Kim SH et al. *Biochem Biophys Res Commun* 2002;**296**:221-226.
53. Kirsch T, von der MK. *Bone Miner* 1992;**18**:107-117.
54. Korb A, Tohidast-Akrad M, Cetin E et al. *Arthritis Rheum* 2006;**54**:2745-2756.
55. Krens SF, Spaink HP, Snaar-Jagalska BE. *FEBS Lett* 2006;**580**:4984-4990.
56. Kumar S, Votta BJ, Rieman DJ et al. *J Cell Physiol* 2001;**87**:294-303.
57. Liacini A, Sylvester J, Li WQ et al. *Matrix Biol* 2002;**21**:251-262.
58. Liao G, Tao Q, Kofron M et al. *Proc Natl Acad Sci U S A* 2006;**103**:16313-16318.
59. Logan CY, Nusse R. *Annu Rev Cell Dev Biol* 2004;**20**:781-810.
60. Lories RJ, Peeters J, Bakker A et al. *Arthritis Rheum* 2007;**56**:4095-4103.
61. Ma L, Wang HY. *J Biol Chem* 2007;**282**:28980-28990.
62. Masckauchán TN, Agalliu D, Vorontchikhina M et al. *Mol Biol Cell* 2006;**17**:5163-5172.
63. Mbalaviele G, Anderson G, Jones A et al. *J Pharmacol Exp Ther* 2006;**317**:1044-1053.
64. Mclay LM, Halley F, Souness JE et al. *Bioorg Med Chem* 2001;**9**:537-554.
65. Medicherla S, Ma JY, Mangadu R et al. *J Pharmacol Exp Ther* 2006;**318**:132-141.
66. Meneghini MD, Ishitani T, Carter JC et al. *Nature* 1999;**399**:793-797.
67. Moldovan F, Pelletier JP, Hambor J et al. *Arthritis Rheum* 1997;**40**:1653-1661.
68. Murakami S, Kan M, McKeehan WL et al. *Proc Natl Acad Sci U S A* 2000;**97**:1113-1118.
69. Nakamura K, Shirai T, Morishita S et al. *Exp Cell Res* 1999;**250**:351-363.
70. Nishikawa M, Myoui A, Tomita T et al. *Arthritis Rheum* 2003;**48**:2670-2681.
71. Nurminskaya M, Linsenmayer TF. *Dev Dyn* 1996;**206**:260-271.
72. Oberlender SA, Tuan RS. *Development* 1994;**120**:177-187.
73. Oh CD, Chang SH, Yoon YM et al. *J Biol Chem* 2000;**275**:5613-5619.
74. Pandur P, Läsche M, Eisenberg LM et al. *Nature* 2002;**418**:636-641.
75. Pelletier JP, Fernandes JC, Brunet J et al. *Arthritis Rheum* 2003;**48**:1582-1593.
76. Robinson JA, Chatterjee-Kishore M, Yaworsky PJ et al. *J Biol Chem* 2006;**281**:31720-31728.
77. Rosenzweig DH, Ou SJ, Quinn TM. *J Cell Mol Med* 2013;**17**:508-517.
78. Ryu JH, Chun JS. *J Biol Chem* 2006; **281**:22039-22047.
79. Ryu JH, Kim SJ, Kim SH et al. *Development* 2002;**129**:5541-5550.
80. Sandell LJ, Aigner T. *Arthritis Res* 2001;**3**:107-113.
81. Sawakami K, Robling AG, Ai M et al. *J Biol Chem* 2006;**281**:23698-23711.
82. Schett G, Tohidast-Akrad M, Smolen JS et al. *Arthritis Rheum* 2000;**43**:2501-2512.
83. Seghatoleslami MR, Roman-Blas JA, Rainville AM et al. *J Cell Biochem* 2003;**88**:1129-1144.
84. Seto H, Kamekura S, Miura T et al. *J Clin Invest* 2004;**113**:718-726.

85. Shlopov BV, Gumanovskaya ML, Hasty KA. *Arthritis Rheum* 2000;**43**:195-205.
86. Song RH, Tortorella MD, Malfait AM et al. *Arthritis Rheum* 2007;**56**:575-585.
87. Stanton LA, Underhill TM, Beier F. *Dev Biol* 2003;**263**:165-175.
88. Takada I, Mihara M, Suzawa M et al. *Nat Cell Biol* 2007;**9**:1273-1285.
89. Takebe K, Nishiyama T, Hayashi S et al. *Int J Mol Med* 2011;**27**:329-335.
90. Tamamura Y, Otani T, Kanatani N et al. *J Biol Chem* 2005;**280**:19185-19195.
91. Tavella S, Raffo P, Tacchetti C et al. *Exp Cell Res* 1994;**215**:354-362.
92. Thalhamer T, McGrath MA, Harnett MM. *Rheumatology* 2008;**47**:409-414.
93. Thornton TM, Pedraza-Alva G, Deng B et al. *Science* 2008;**320**:667-670.
94. Tibbles LA, Woodgett JR. *Cell Mol Life Sci* 1999;**55**:1230-1254.
95. Topol L, Jiang X, Choi H et al. *J Cell Biol* 2003;**162**:899-908.
96. Toyama T, Lee HC, Koga H et al. *Mol Cancer Res* 2010;**8**:254-265.
97. Tufan AC, Daumer KM, DeLise AM et al. *Exp Cell Res* 2002;**273**:197-203.
98. Tufan AC, Tuan RS. *FASEB J* 2001;**15**:1436-1438.
99. Tuli R, Tuli S, Nandi S et al. *J Biol Chem* 2003;**278**:41227-41236.
100. von der Mark K, Kirsch T, Nerlich A et al. *Arthritis Rheum* 1992;**35**:806-811.
101. Watanabe H, de Caestecker MP, Yamada Y. *J Biol Chem* 2001;**276**:14466-14473.
102. Westendorf JJ, Kahler RA, Schroeder TM. *Gene* 2004;**341**:19-39.
103. Wong GT, Gavin BJ, McMahon AP. *Mol Cell Biol* 1994;**14**:6278-6286.
104. Wu X, Tu X, Joeng KS et al. *Cell* 2008;**133**:340-353.
105. Xu J, Wang W, Ludeman M et al. *Tissue Eng Part A* 2008;**14**:667-680.
106. Yang Y, Topol L, Lee H et al. *Development* 2003;**130**:1003-1015.
107. Yuasa T, Otani T, Koike T et al. *Lab Invest* 2008;**88**:264-274.
108. Yun MS, Kim SE, Jeon SH et al. *J Cell Sci* 2005;**118**:313-322.
109. Zhen X, Wei L, Wu Q et al. *J Biol Chem* 2001;**276**:4879-4885.
110. Zhu M, Chen M, Zuscik M et al. *Arthritis Rheum* 2008;**58**:2053-2064.

Table 7. 1 Highlighted references related to the crosstalk between Wnt and MAPK pathways in varied cell types, especially its influence in the regulation of chondrogenesis

Cell type	Approach	Signal	Result	Reference
Mouse F9 teratocarcinoma cells	Gene knockout (siRNAs targeting p38 α MAPK) and chemical inhibitor (SB203580 targeting p38 MAPK)	P38 MAPK and Wnt5a	Wnt5a activated p38 MAPK pathway, which feeds into the Wnt5a/cyclic GMP/Ca ²⁺ /NF-AT pathway	Ma and Wang 2007
	Gene knockout (siRNA targeting Dvl-1, Dvl-2, and Dvl-3) and chemical inhibitors (SB203580 targeting p38 MAPK, SP600125 targeting JNK)	Wnt3a and JNK	Suppression of either Dvl-1 or Dvl-3 but not Dvl-2 abolished Wnt3a activation of JNK; SP600125 but not SB203580 blocked Wnt3a activation of JNK	Bikkavilli et al. 2008a
	Gene knockout (siRNA targeting G α_o , G α_s , G α_q , G α_{11} , JNK1) and chemical inhibitor (SB203580 targeting p38 MAPK)	Wnt3a and JNK	Wnt3a activated p38 MAPK while G α_s and G α_q knockout attenuated this effect; SB203580 attenuated Wnt3a induced accumulation of β -catenin	Bikkavilli et al. 2008b
Mouse C3H10T1/2 mesenchymal cells	Gene transfection (dominant-negative p38, MEK3, and MEK6) and chemical inhibitors (SB203580 targeting p38 MAPK, MEK inhibitor U0126)	Wnt3a, ERK and P38 MAPK	Wnt3a induced a rapid and transient activation of p38 MAPK and ERK; SB203580 and dominant-negative p38, MEK3 and MEK6 led to inhibition of β -catenin expression	Caverzasio and Manen 2007
Mouse NIH3T3 cells	Gene transfection (recombinant Wnt3a) and gene knockout (siRNA targeting β -catenin, ERK1, ERK2)	ERK and Wnt/ β -catenin	Wnt3a stimulated the proliferation of fibroblast cells, at least in part, via activation of the ERK and Wnt/ β -catenin pathways	Yun et al. 2005
Mouse Stromal cell clone (ST2)	Gene transfection (retroviruses expressing GqI, Dkk1, Dvl-2 derivatives, N17Rac1, N17Cdc42, and V12Rac1) and gene knockout (siRNA targeting β -catenin)	Wnt/ β -catenin and JNK2	JNK2 kinase activated Rac1 resulting in β -catenin phosphorylation and canonical Wnt signaling activation	Wu et al. 2008
Chicken mesenchymal cells from embryo wing buds	Gene transfection (dominant-negative Tcf-4)	Wnt3a and JNK	Wnt3a inhibited chondrogenesis by stabilizing cell-cell adhesion; Wnt3a caused dedifferentiation of chondrocytes by activation of the β -catenin-Tcf/Lef transcriptional complex and the c-Jun/AP-1 pathway	Hwang et al. 2005
	Gene transfection (retrovirus transfection targeting Wnt5a, Wnt7a)	N-cadherin (MAPKs) and Wnt7a, Wnt5a	Retrovirally mediated misexpression of Wnt7a inhibited in vitro chondrogenesis whereas Wnt5a did not; Wnt signaling in chondrogenesis was likely to involve modulation of N-cadherin expression	Tufan and Tuan 2001
	Gene transfection (RCAS constructs of Wnt5a or RCAS vector); gene knockout (siRNA targeting Wnt5a); and chemical inhibitors (PD169316 targeting p38 MAPK, GF109203X targeting PKC- α)	Wnt5a and p38 MAPK	Overexpression of Wnt5a or treatment with TGF β 3 stimulated the activation of PKC- α and p38 MAPK, both positively regulated chondrogenic differentiation; inactivation of PKC- α and p38 MAPK by specific inhibitors abrogated chondrogenesis stimulated by both TGF β 3 and Wnt5a	Jin et al. 2006a
	Gene transfection (dominant-negative p38 MAPK)	Wnt7a/ β -catenin and p38 MAPK	BMP2 promoted chondrogenesis by activating p38 MAPK, which in turn down-regulated Wnt7a/ β -catenin signaling responsible for proteasomal degradation of Sox9	Jin et al. 2006b
	Chemical inhibitors (SP600125 targeting JNK, Src kinase inhibitor I targeting Src)	Wnt5b and JNK	Wnt5b not only inhibited chondrocyte hypertrophy but also promoted cellular migration through the JNK-dependent activation of Src and subsequent cadherin receptor turnover	Bradley and Drissi 2011
Human embryonic kidney 293 (HEK 293) cell line	Gene transfection (GSK3 β mutant constructs, dominant-negative ERK1/2, constitutively active GSK3 β); gene knockout (siRNA targeting β -catenin, siRNA targeting ERK1/2); and chemical inhibitors (PD98059 targeting MEK1, LY294002 targeting PI3K, calphostin C targeting PKC)	P38 MAPK; ERK1/2; and Wnt/ β -catenin	ERK activation led to phosphorylation and inactivation of GSK3 β , resulting in release of β -catenin, which was translocated into nucleus and facilitated cell proliferation	Ding et al. 2005

	Gene transfection (plasmids for wild type p38 MAPK, constitutively active MEK6, mutant GSK3 β) and chemical inhibitors (SB203580 targeting p38 MAPK, Wortmanin targeting PI3K)	Tak1 MAPK and Wnt/ β -catenin	p38 MAPK inactivated GSK3 β by direct phosphorylation at its C terminus; this inactivation led to an accumulation of β -catenin in nucleus	Thornton et al. 2008
	Gene transfection (FGFR3 vectors, LRP6 variant vectors, plasmids expressing V5-tagged FGFR2, EGFR, and TRKA) and chemical inhibitor (U0126 targeting ERK1/2)	Wnt/ β -catenin and ERK1/2	Wnt/ β -catenin was activated by FGFR2/3, EGFR, and TRKA kinases, which depend on ERK-mediated phosphorylation of Wnt co-receptor LRP6	Krejci et al. 2012
	Gene knockout (siRNAs targeting MAPK1, MAPK3, MAPK8, MAPK11) and chemical inhibitors (BIRB796 and SB203580 targeting p38 MAPK, SP600125 targeting JNK, U0126 targeting ERK1/2)	MAPKs and Wnt/ β -catenin	Several MAPKs, such as p38, ERK1/2, and JNK1, were sufficient and required for the phosphorylation of PPPS/TP motifs of LRP6, which is a co-receptor of Wnts and a key regulator of Wnt/ β -catenin pathway	Cervenka et al. 2011
Human trabecular bone-derived mesenchymal progenitor cells (MPCs)	Gene transfection (plasmid containing human type II collagen α 1, plasmid of pAGC1 containing human aggrecan promoter) and chemical inhibitors (SB203580 targeting p38 MAPK, PD98059 targeting ERK-1 specific MEK1, SP600125 targeting JNK)	MAPKs and Wnt7a	TGF β 1 treatment initiated and maintained chondrogenesis of MPCs through the differential chondro-stimulatory activities of p38, ERK1, and to a lesser extent, JNK; TGF β 1-mediated MAPK activation controlled Wnt7a gene expression and Wnt-mediated signaling through the intracellular β -catenin-TCF pathway, which likely regulated N-cadherin expression and subsequent cell-adhesion complexes during the early steps of MPC chondrogenesis	Tuli et al. 2003
Rabbit temporomandibular joint (TMJ) condylar chondrocytes	Gene transfection (β -catenin-Tcf/Lef expression plasmid, Wnt5a expression vector)	Wnt5a and JNK	Activation of Wnt5a signaling by IL-1 β induced the expression of MMPs via the JNK pathway	Ge et al. 2009
Xenopus embryo	Gene transfection [constitutive-active JNK plasmid (Flag-MKK7-hJNK1), antisense oligo against Dsh] and chemical inhibitor (SP600125 targeting JNK)	Wnt/ β -catenin and JNK	JNK antagonized the canonical Wnt pathway by regulating the nucleocytoplasmic transport of β -catenin	Liao et al. 2006

Figure 7. 1 The best characterized MAPK modules are the ERK pathway, the SAPK/JNK pathway, and the p38 MAPK pathway.

The MAPK cascades consist of an MEKK, an MEK, and an MAPK. MEKKs are activated through a large variety of extracellular signals such as growth factors, cytokine factors, and stress. The activated MEKKs can phosphorylate and activate one or several MEKs, which, in turn, phosphorylate and activate a specific MAPK. Activated MAPK phosphorylates and activates various substrates in the cytoplasm and the nucleus of the cell, including transcription factors. These downstream targets control cellular responses (e.g., apoptosis, proliferation, and differentiation).

Figure 7. 2 Three Wnt-dependent pathways have been categorized: canonical Wnt/β-catenin and non-canonical Wnt/PCP as well as Wnt/Ca²⁺ pathways.

Canonical Wnt/β-catenin pathway: In cells, with an inactive state of canonical Wnt signaling, cytosolic β-catenin is targeted to proteolytic degradation through phosphorylation by the APC–Axin–GSK3β complex and further ubiquitination through action of βTrCP-dependent E3 ubiquitin ligase complex. On stimulation by Wnt ligands through binding to Fzd receptors and its co-receptor Lrp, Fzd recruits and phosphorylates Dsh, and inhibits APC–Axin–GSK3β complex formation by the recruitment and inhibition of GSK3β. Consequently, β-catenin can accumulate in the cytoplasm and enter the nucleus, activating transcription of target genes through association with the Lef1/Tcf transcription factor family. Non-canonical Wnt/Ca²⁺ pathway: Interaction of Wnt ligands with Fzd receptors can lead to an increase in intracellular calcium level, through possibly the activation of phospholipase C

(PLC). Intracellular calcium will subsequently activate Ca^{2+} /calmodulin-dependent protein kinase II (CAMKII) and protein kinase C (PKC) in cells, as well as the transcription factor NFAT. This pathway is particularly important for convergent-extension movements during gastrulation. Additionally, Fzd receptors can also activate JNK, promoting expression of specific genes through activation of AP-1. Non-canonical Wnt/PCP pathway: This pathway is characterized by an asymmetric distribution of Fzd and related receptors, resulting in the polarization of the cell. Also, Wnt-signaling activates Cdc42, RhoA, and Rac1 leading to cytoskeleton rearrangement. Rac1 can also activate JNK, activating specific gene transcription through modulation of the AP-1 protein complex.

Figure 7. 3 Influence of canonical Wnt signals on the MAPK pathway.

Wnt3a treatment activated the Raf-1-MEK-ERK cascade and the JNK pathways. Furthermore, Wnt3a activation of activator protein-1 (AP-1) was blocked by the inhibition of JNK with SP600125 and by the inhibition of AP-1 with N-acetyl-L-cysteine and nordihydroguaiaretic acid. In C3H10T1/2 cells, AP-1 was activated by ERK1/2. In totipotent mouse F9 teratocarcinoma cells, canonical Wnt-β-catenin-JNK signaling was found to be activated by G-proteins, which propagated the signals downstream through Dishevelled (Dsh) isoforms; suppression of Dsh-1 or Dsh-3 abolished Wnt3a activation of JNK. In addition to JNK and ERK, p38 MAPK was strongly activated by Wnt3a and the

activated p38 MAPK regulated canonical Wnt- β -catenin signaling through regulation of GSK3 β . Chemical inhibitors of p38 MAPK (SB203580) and expression of a dominant negative (DN)-version of p38 MAPK attenuated Wnt3a-induced accumulation of β -catenin, Lef/Tcf-sensitive gene activation, and primitive endoderm formation.

Figure 7. 4 Influence of non-canonical Wnt signals on the MAPK pathway.

In mouse F9 teratocarcinoma embryonal cells, a strong activation of p38 MAPK was observed in response to Wnt5a and treatment with SB203580 effectively abolished the ability of Wnt5a's stimulatory effects. Wnt5a was also found to promote ERK1/2 phosphorylation, enhancing endothelial cell survival and proliferation, and the expression of Wnt5a blocked canonical Wnt signaling in endothelial cells and other cell types. However, non-canonical Wnt signaling more commonly functions through the Wnt-JNK pathway. Activation of Wnt5a signaling by IL-1 β induced the expression of MMPs via the JNK pathway in rabbit temporomandibular joint (TMJ) condylar chondrocytes, whereas blockage of JNK signaling impaired the Wnt5a-induced up-regulation of MMPs. Wnt5a increased chondrocyte differentiation at an early stage through CaMK/NFAT-dependent induction of Sox9 while repressing chondrocyte hypertrophy via NF- κ B-dependent inhibition of Runx2 expression. Wnt5b activated JNK, a component of the planar cell polarity pathway, contributed to an increase in cellular migration while Wnt5b also decreased cell-cell

adhesion through an activation of Src and subsequent cadherin receptor turnover.

Figure 7. 5 Influence of MAPK signals on the Wnt pathway.

Expression of constitutively active MKK6, an upstream activator of p38 MAPK, in 293T cells increased the expression of β -catenin proteins through direct phosphorylation of GSK3 β protein both in vitro and in vivo; this phosphorylation was blocked by SB203580 or the knock-out (KO) of MKK3 and MKK6. Members of MAPKs such as ERK1/2, p38 MAPK, and JNK contributed to the phosphorylation of PPPS/TP clusters of endogenous LDL-related protein 6 (LRP6) phosphorylation, stimulating Wnt/ β -catenin expression. Rac1, a small signaling G protein, could activate JNK2 to phosphorylate β -catenin. In *Xenopus* embryos, activation of JNK antagonized the canonical Wnt pathway through activating the nuclear export of β -catenin rather than maintaining its cytoplasmic stability. Receptor tyrosine kinase (RTK) systems facilitated Wnt/ β -catenin signaling by the PI3K/AKT pathway through inhibiting GSK3 activities; RTKs could also phosphorylate β -catenin through involving ERK/LRP6 pathways to activate Wnt/ β -catenin signaling.

Chapter 8: General discussion

The objectives of this dissertation were to develop a novel *in vitro* expansion system (decellularized extracellular matrix) for harvesting a large amount of high-quality stem cells for cartilage regeneration. Surface characterization and mechanical properties of dECM have been determined; the *in vitro* chondrogenesis of human SDSCs following preconditioning using decellularized matrix was delineated. Oxidative stress and inflammatory environment are commonly seen in the joints of patients with OA. To overcome these obstacles, we further explored dECM's anti-oxidant and anti-inflammatory properties, and proved its effectiveness in a mini-pig model. MAP kinase signaling pathways play important roles in dECM-mediated chondrogenesis; p38 MAP kinase inhibitor (sb203580) also has anti-inflammatory properties and have been applied in an *in vivo* animal model. dECM not only provides biochemical and molecular factors to influence cell differentiation, it also provide physical support as a 3D nano-scale structure with micromechanical properties. Thus, we conducted some preliminary studies about the potential biophysical mechanisms of dECM on stem cells' chondrogenesis by testing its mechanical properties such as elasticity. In the synthesis of dECM, ascorbic acid is an effective agent to control the micromechanical properties of the dECM microenvironment, providing the capability to manipulate micromechanical properties such as elasticity of dECM to influence expanded stem cells' chondrogenic fate. Our **long-term goal** is to develop an efficient *ex vivo* matrix niche with anti-inflammatory and appropriate micromechanical properties to provide a large quantity of qualified stem cells for chondrogenesis even under the oxidative and inflammatory environment in OA patients. The **central hypothesis** is that dECM could rejuvenate expanding stem cells for both proliferation and chondrogenic potential in a harsh (oxidative, inflammatory) environment. Due to the need for a large quantity of high quality chondrocytes for cartilage repair, there is increasing need for developing *ex vivo* matrix for stem cell expansion. Although previous studies of dECM demonstrated promising results in promoting chondrogenic differentiation of stem cells, its functionalities are not fully explored. By taking advantage of its anti-oxidant and anti-inflammatory properties, dECM has demonstrated great potentials for enhanced cell proliferation and chondrogenesis under harsh environment. In addition, strategies for generating functional small molecules of synthetic and natural products targeting

inflammatory signals have been considered effective in the suppression of inflammation. For instance, inhibition of the p38 MAPK signaling pathway with SB203580 showed anti-inflammatory effects in both cartilage explants and animal models. Preconditioning using SB203580 was assumed to significantly enhance dECM-expanded human SDSC chondrogenic potential and acquired anti-inflammatory characteristics. While biomechanics-driven approaches have greatly motivated the development of functional cartilage tissue engineering at the tissue level, few studies reported the influences of biomechanics on chondrogenesis from the cellular level, its molecular adaption, and its signals on neo-cartilage formation. Although stem cell biologists have long appreciated the regulatory roles of soluble stem cell niche signals (e.g., growth factors and cytokines) in regulating stem cell fate, recent evidence demonstrated that mechanical and topographical cues existing in the stem cell niche can also be sensed and transduced into intracellular biochemical and functional responses by stem cells, a process known as mechanotransduction [1, 2]. Mechanical forces generated intrinsically within the cell in response to its extracellular environment, and extrinsic mechanical signals from extracellular environment, play a critical role in determining stem cell fate. By controlling dECM elasticity or changing the nanotopography of dECM, stem cells could be steered specifically toward chondrogenic differentiation.

dECM expanded stem cell applications in *in vivo* animal experiments

dECM-expanded SDSCs not only exhibited enhanced *in vitro* chondrogenesis, they also demonstrated improved cartilage regeneration by repairing partial-thickness cartilage defects through intra-articular injection in a minipig model. Intra-articular injection of dECM-expanded SDSCs yielded the most improved cartilage resurfacing, evidenced by intensified staining of hyaline cartilage markers and comparable depth of regenerated cartilage with surrounding host cartilage tissue. The existence of collagen I in the regenerated cartilage suggested fibrocartilage, which was nondetectable in the resurfacing cartilage in the group implanted with dECM-expanded SDSCs. These results might suggest the existence of a harsh environment in minipig knees where the partial-thickness cartilage defects were created. The harsh environment, including reactive oxygen species, inflammatory cytokines, metalloproteinases, nitric oxide, or mechanical forces, might cause implanted cell necrosis

or apoptosis [3]. Inflammatory stress reportedly causes dedifferentiation of chondrogenic cells to fibroblast-like cells, resulting in the defects being filled with a mix of fibrous and cartilaginous tissues [4].

In tissue engineering, mesenchymal stem cells or tissue constructs to be transplanted into the human body for therapeutics often face a hostile environment. In order to prepare MSCs to better adapt to the undesirable environment, many preconditioning strategies have been studied. Reconstruction of an *in vitro* microenvironment is one preconditioning strategy. Other strategies such as controlling oxygen concentration, mechanical stimuli, growth factors, or chemicals can be used for priming the MSCs. Through application of basic fibroblast growth factor-2 (bFGF-2) and controlling oxygen concentration, in addition to the 3D dECM, porcine SDSCs demonstrated dramatic enhancement of both cell proliferation and chondrogenic potential [5]. Those environmental factors demonstrated better effects than using dECM alone which proved to be a potential application strategy for cartilage repair.

MAPK signal pathway with dECM in chondrogenesis of stem cells in an inflammatory environment

Accumulating evidence supports a central regulatory role of the MAP kinases in mediating inflammatory and matrix degrading processes that contribute to joint tissue destruction in osteoarthritis (OA). Once activated, the MAP kinases in turn activate other protein kinases and several transcriptional regulatory proteins [6]. Some characterized transcriptional regulatory proteins include Elk-1 activated by ERK, c-jun activated by JNK, and ATF-2 and MEF2 activated by p38 MAPK. These transcriptional factors regulate the expression of OA-related genes involved in the inflammatory response, regulation of cell proliferation, and production of matrix degrading enzymes such as MMPs.

In previous studies, we found that p38 MAPK inhibitor SB203580 was able to precondition hSDSCs, dramatically enhancing chondrogenic differentiation. The pellets derived from DSCM and p38 MAPK inhibitor preconditioning exhibited enhancements in resistance to IL-1 β . This strategy successfully solved the toxicity concerns of p38 MAPK inhibitors. Since JNK inhibitors are similar to p38 MAPK

inhibitors in respect to safety profile, we wondered whether JNK inhibitor SP600125 would be able to precondition hSDSCs with DSCM and to enhance chondrogenesis. Inhibition of JNK was once reported to suppress chondrogenesis in ATDC5 cells [7, 8]; in chondrocytes, however, inhibition of JNK resulted in slightly enhanced Safranin O staining and deposition of collagen type II as compared with DMSO controls. At the molecular level, there was significantly higher mRNA expression of the chondrogenic markers collagen type II (Col2 α 1), aggrecan, cartilage oligomeric matrix protein (COMP), and Sox9 in chondrocytes treated with JNK inhibitors [9]. However, there are no studies concerning the effects of JNK inhibitors on chondrogenesis in human mesenchymal stem cells; additionally, we are also curious about the chondrogenic effects of JNK inhibition in a 3D microenvironment derived from stem cells. We expect that preconditioning stem cells with JNK inhibitor SP600125 and dECM would significantly enhance chondrogenesis and also be resistant to an inflammatory environment.

As possible OA treatment strategies targeting c-jun and AP-1 component, specific JNK inhibitors such as SP600125 have been developed and found to suppress IL-1-induced and JNK MAPK-mediated c-jun phosphorylation and AP-1 up-regulation, resulting in consequent down-regulation of MMP-1 expression. An *in vivo* experiment demonstrated that SP600125 had a beneficial effect in rat adjuvant arthritis and it modestly decreased paw swelling in rat model. Moreover, radiographic evaluation of rats treated with JNK inhibitor showed significantly less joint damage and remodeling than control groups [10].

Considering the effects of JNK inhibitor on chondrogenesis and its anti-inflammatory effects on OA, we hypothesized that preconditioning stem cells with JNK inhibitor as well as 3D dECM would boost the chondrogenesis in an inflammatory environment. The overexpression of mitogen-activated kinase-extracellular signal-regulated kinase (MAPK-ERK) has been reported in previous studies and these findings have generated interest in MAPK-ERK as a potential target for OA therapies. Additionally, the phosphorylated mitogen-activated protein kinase kinase-extracellular signal-regulated kinase (MEK-ERK) was developmentally overexpressed in articular cartilage and subchondral bone in rat OA models. Several *in vitro* studies also demonstrated that modulating the MEK-ERK1/2 signaling pathway using pharmacological inhibitors proved to have beneficial effects on OA [11]. Inhibition of ERK can enhance the cartilage phenotype by negatively regulating the hypertrophic [12] and degradative changes [13, 14, 15].

MEK inhibitor U0126 is a chemically synthesized organic compound that inhibits the activation of MAPK (ERK1/2) by inhibiting the kinase activity of MAP kinase kinase (MAPKK or MEK1/2) and has been widely used in *in vivo* and *in vitro* studies to explore the function of the ERK pathway. U0126 has been demonstrated to attenuate the synthesis of inflammatory cytokines and MMPs in cartilage cells in previous studies [16, 17]. Preclinical *in vivo* studies have shown that U0126 successfully reduced inflammatory rheumatoid arthritis [18].

Previous studies confirmed that ERK is a strong candidate for the discriminating switch point between lineages and the integration of 3D information; the addition of ERK inhibitor induced a significant amplification of osteogenic gene expression and matrix mineralization in 3D collagen I cultures [19]. ERK can be activated indirectly by matrix adhesion signaling, which can be a potential link between matrix interaction and cellular behavior. It is therefore supposed that 3D cell matrix-induced chondrogenesis requires the participation of ERK.

However, the study of the ERK1/2 kinase cascade has not progressed as rapidly as the development of p38 inhibitors which have already been applied in clinical trials. In some anti-cancer therapies, ERK inhibitors have revealed certain toxicities, with side effects being perhaps the most frequent amongst them [20]. On the other hand, there are no studies about the influences of ERK inhibition in 3D environment for the chondrogenesis of hMSCs. Considering the antioxidant and anti-inflammatory effects of the dECM model, we hypothesized that the interference with ERK signals in the 3D dECM microenvironment would significantly enhance chondrogenesis of stem cells; also, we expect such chondrogenesis would perform better in an inflammatory environment.

Biomechanical stimulations influence stem cell behaviors and chondrogenic differentiation fate

The control of stem cell fate via cell-exerted forces (e.g. by modulating substrate stiffness) and exogenous mechanical stimulation is increasingly being explored as a possible differentiation strategy. By changing the substrate stiffness, the resultant cell-exerted forces can be altered to control stem cell lineage commitment. At the single cell level, differentiating stem cells exhibit altered mechanical properties. For example, mouse stem cells demonstrate increased stiffness after 6 days of chemical

differentiation, with up to 3-fold higher stiffness values compared to undifferentiated cells [21]. The results from the González-Cruz et al. study indicate that cellular mechanical properties are predictive of adipose stem cell (ASC) differentiation and synthetic potential. Significant correlations existed between mechanical properties and lineage-specific metabolite production by ASC clones [22].

Mechanical forces play important roles in stem cell adhesion, spreading, proliferation, migration, and differentiation [23]. It is difficult to precisely determine the biomechanical mechanisms *in vivo* related to the cytoskeletal conformational changes. Therefore, biomimetic mechanical stimulation, including compression, stretching, and fluid-induced shear stress, have been used to investigate the mechanism *in vitro* [24]. Cyclic compression is able to alter MSC phenotype. MSCs subjected to dynamic compression or hydrostatic pressure induced an increase of chondrocyte lineage markers and enhanced ECM deposition [25, 26]. It was hypothesized that biomechanical stimuli can be transduced into molecular signals during early chondrogenesis by a combination of positive and negative effectors of cartilage specific extracellular matrix macromolecules. Although the underlying molecular mechanisms remain elusive, it is believed that biomechanical signals converting into biochemical signals might involve integrin or non-integrin ECM receptors, cell-cell adhesion molecules, or alteration of cytoskeletal architecture mediated by MAP kinase [27], rho kinase [28], or focal adhesion associated with tyrosine kinase signal transduction pathways [29, 30].

Mammalian cells not only sense applied mechanical forces, but also sense the mechanical properties of their environment, such as the elasticity of the substrate on which they grow. Substrate stiffness influences cell adherence and cell migration and spreading [31, 32]. Peyton et al. reported that cells proliferate more quickly on stiff than soft substrates [33]; in addition, DNA synthesis proceeds more rapidly in flattened, well-spread cells [34]. Furthermore, substrate stiffness also has fundamental effects on cellular differentiation. Embryonic stem cell (ESC) differentiation has been shown to be regulated by substrate stiffness. Engler et al. showed that mesenchymal stem cells (MSCs) grown on soft substrates differentiated preferentially into neurons, while those on substrates with stiffness similar to muscle and bone tissue differentiated into myocytes and osteoblasts, respectively [35]. In regarding to chondrogenic differentiation, chondrocytes on PA gels with Young's moduli of 4 to 100 kPa showed that chondrocytes on very soft substrates (4 kPa) promoted the maintenance of the chondrogenic phenotype as indicated by a diffuse orientation of the actin in the cytoskeleton, low proliferation, high collagen type II and aggrecan, and slightly lower collagen type I expression [36].

Comparatively, chondrocytes cultured on stiff substrates showed an accelerated loss of differentiated phenotype, which was also supported by the significant decrease of collagen type II and aggrecan production on stiffer matrices compared to cells on soft substrates. These findings indicate that chondrocytes sense the elasticity of the matrix and might be used for the design of scaffolds with mechanical properties specifically tailored to support the chondrogenic phenotype in tissue engineering applications. It was proposed that a cell does not have a predefined stiffness; rather, cells increase their internal stiffness until they match that of the substrate. This way, cells mechanically probe their microenvironment and change their corresponding behaviors, such as adhesion, migration, and proliferation [37].

Ni and coworkers explained this phenomenon through proposing a mathematical model in which the morphology of a cell adhering to a substrate is characterized by competition between strain energies (cell and substrate) and interfacial energy (work of adhesion at the cell periphery) and that the final configuration of cell morphology is determined by the minimum of total free energy of the cell-substrate system. Thus, the cell changes into its energetically favorable shape by assembling/disassembling of focal adhesions distributed around the cell periphery [38]. Daniel et al. reported that substrate elasticity modulated the TGF- β 1 stimulated re-differentiation of expanded/differentiated human articular chondrocytes (HAC). Specifically, human articular chondrocytes on soft substrate were restricted cortically while, on the stiffer substrates, F-actin assembled into stress fibers, while type II collagen mRNA expression on the soft substrate (Young's modulus of 0.26 ± 0.08 kPa) was dramatically enhanced [39].

Cells attached to a substrate have been shown to exert contractile forces, resulting in tensile stresses in the cytoskeleton. The relationship between these forces and the mechanical stiffness, or elasticity, of the ECM can have a major influence on cell behaviors such as migration, apoptosis [24], and proliferation [40]. Through changing a series of cell activities, ECM stiffness can influence cell differentiation fate. MSCs grown on variably compliant polyacrylamide gel were found to alter their properties in relation to the stiffness of the substrate (i.e., stiffer substrates induced stiffer cells). It is well known that chondrocytes expanded on plastic usually experienced “dedifferentiation”, which might hinder its subsequent differentiation capabilities. Cells can regain their differentiation phenotype; however, in order to produce functional cartilage tissue, after transferring and expansion into a 3D environment [41], the redifferentiation process needs to induce the production of a

substantial amount of GAGs to enable load bearing of the developed tissue, and the stimulation of collagen type II over type I secretion in order to avoid the formation of fibrocartilage [42]. While a 3D environment plays a crucial role in regaining chondrogenic phenotype, few systematic studies investigate the influence of isolated matrix properties on this process [43].

Aging is another important factor that might influence the cell cytoskeletal organization and focal adhesion. The loss of tissue elasticity has a close relationship with aging, as observed in human epithelial tissue. It is believed that the loss of tissue elasticity results from rigidification of the extracellular matrix due mostly to an increase of polymerization of collagen and elastin [44]. Berdyyeva et al. reported that tissues become more rigid through intracellular changes that enhance cell rigidity of individual cells [45], which might have a positive correlation with the surface density of cytoskeletal fibers. In the past decade, increasing research has been reported about the benefits of a young microenvironment to cell differentiation compared to an old microenvironment in different types of cells [46, 47, 48]. However, most researchers tried to explain the potential mechanisms from the biochemical and molecular perspective. How aging-related elasticity changes influence the cell behaviors and differentiation fate has rarely been investigated.

The mechanical properties of tissues are not only important for maintaining macroscale mechanical integrity but also essential regulators of cellular function. Cells sense stiffness using structures such as integrins to attach to the ECM and often remodel their environment by generating traction forces via actomyosin contractility. When alterations are made to the extracellular mechanical environment, cells can react to these mechanical stimuli by influencing tissue development, cellular differentiation, or disease progression. An understanding of how the mechanical properties of the dECM contribute to cell responses and tissue formation will ultimately further the understanding of disease states associated with aberrant mechanosensing and guide the design parameters of successful biomaterials and tissue-engineered constructs. Future cartilage tissue engineering strategies should work to produce biomaterials and implants that are not only chemically favorable, but also integrate mechanical cues that dictate cellular behavior to aid in cellular differentiation and cartilage regeneration.

References

1. Chen CS. *J Cell Sci* 2008;**121**:3285-92.
2. Geiger B, Spatz JP, Bershadsky AD. *Nat Rev Mol Cell Biol* 2009;**10**:21-33.
3. Vinatier, C., Mrugala, D., Jorgensen, C. et al. *Trends Biotechnol* 2009;**27**:307.
4. Gelse, K., Muühle, C., Franke, O. et al. *Arthritis Rheum* 2008;**58**:475.
5. Li, J. and M. Pei. *Tissue Eng Part A* 2011;**17**:703-12.
6. Avruch J. *Biochim Biophys Acta* 2007;**1773**:1150-60.
7. Mitsugi S, Ariyoshi W, Okinaga T et al. *Biochem Biophys Res Commun* 2012;**420**:380-4.
8. Nakajima M, Negishi Y, Tanaka H et al. *Biochem Biophys Res Commun* 2004;**320**:1069-75.
9. Rosenzweig DH, Ou SJ, Quinn TM. *J Cell Mol Med* 2013;**17**:508-17.
10. Han Z, Boyle DL, Chang L et al. *J Clin Invest* 2001;**108**:73–81.
11. Pelletier JP, Fernandes JC, Brunet J et al. *Arthritis Rheum* 2003;**48**:1582–1593.
12. Papachristou DJ, Pirttiniemi P, Kantomaa T et al. *Histochem Cell Biol* 2005;**124**:215–223.
13. Fan Z, Yang H, Bau B et al. *Rheumatol Int* 2006;**26**:900–3.
14. Sondergaard BC, Schultz N, Madsen SH et al. *Osteoarthritis Cartilage* 2010;**18**:279-88.
15. Thalhamer T, McGrath MA, Harnett MM. *Rheumatology (Oxford)* 2008;**47**:409–14.
16. Appleton CT, Usmani SE, Mort JS et al. *Lab Invest* 2010;**90**:20–30.
17. Shakibaei M, Schulze-Tanzil G, de Souza P et al. *J Biol Chem* 2010;**276**:13289–13294.
18. Thiel MJ, Schaefer CJ, Lesch ME et al. *Arthritis Rheum* 2007;**56**:3347–3357.
19. Lund AW, Stegemann JP, Plopper GE. *Stem Cells Dev* 2009;**18**:331-41.
20. Manousaridis I, Mavridou S, Goerdts S et al. *J Eur Acad Dermatol Venereol* 2013;**27**:11-8.
21. Pillarisetti A, Desai JP, Ladjal H et al. *Cell Reprogram* 2011;**13**:371-80.
22. González-Cruz RD, Fonseca VC, Darling EM. *Proc Natl Acad Sci U S A* 2012;**109**:E1523-9.
23. Guilak F, Cohen DM, Estes BT et al. *Cell Stem Cell* 2009;**5**:17-26.
24. Wang JH, Thampatty BP. *Int Rev Cell Mol Biol* 2008;**271**:301-46.
25. Saha S, Ji L, de Pablo JJ, Palecek SP. *J Cell Physiol* 2006;**206**:126-37.
26. Mauck RL, Byers BA, Yuan X et al. *Biomech Model Mechanobiol* 2007;**6**:113-25.
27. Kimura K, Ito M, Amano M et al. *Science* 1996;**273**:245-8.
28. Chrzanowska-Wodnicka M, Burridge K. *J Cell Biol* 1996;**133**:1403-15.
29. Yamada KM, Miyamoto S. *Curr Opin Cell Biol* 1995;**7**:681-9.
30. Yamada KM, Geiger B. *Curr Opin Cell Biol* 1997;**9**:76-85.
31. Yeung T, Georges PC, Flanagan LA et al. *Cell Motil Cytoskeleton* 2005;**60**:24-34.
32. Discher DE, Janmey P, Wang YL. *Science* 2005;**310**:1139-43.
33. Peyton SR, Raub CB, Keschrumrus VP et al. *Biomaterials* 2006;**27**:4881-93.
34. Folkman J, Moscona A. *Nature* 1978;**273**:345-9.
35. Engler AJ, Sen S, Sweeney HL et al. *Cell* 2006;**126**:677-89.
36. Schuh E, Kramer J, Rohwedel J et al. *Tissue Eng Part A* 2010;**16**:1281-90.
37. Solon J, Levental I, Sengupta K et al. *Biophys J* 2007;**93**:4453-61.
38. Ni Y, Chiang M. *Soft Matter* 2007;**3**:1285-1292.
39. Daniel Vonwil, Andreas Trüssel, Olivia Haupt et al. *Drug Delivery and Translational Research* 2012;**2**:351-362.
40. Hadjipanayi E, Mudera V, Brown RA. *J Tissue Eng Regen Med* 2009;**3**:77-84.

41. Buschmann MD, Gluzband YA, Grodzinsky AJ et al. *J Orthop Res* 1992;**10**:745-58.
42. Peterson L, Minas T, Brittberg M et al. *Clin Orthop Relat Res* 2000;(374):212-34.
43. Miot S, Woodfield T, Daniels AU et al. *Biomaterials* 2005;**26**:2479-89.
44. Dimri GP, Lee X, Basile G et al. *Proc Natl Acad Sci U S A* 1995;**92**:9363-7.
45. Berdyyeva TK, Woodworth CD, Sokolov I. *Phys Med Biol* 2005;**50**:81-92.
46. Yin T, Li L. *Cell Res* 2010;**20**:504-5.
47. Vas V, Wandhoff C, Dörr K et al. *PLoS One* 2012;**7**:e31523.
48. Conboy IM, Conboy MJ, Wagers AJ et al. *Nature* 2005;**433**:760-4.

Conclusions

Cartilage defects, especially from trauma-induced cartilage injuries, do not heal or self-regenerate well due to the absence of blood supply [1]. For the most commonly used autologous chondrocyte implantation (ACI) technique, chondrocyte senescence and cell dedifferentiation are still bothering clinicians in orthopaedics. The development of mesenchymal stem cell (MSC) may help to improve the current situation. However, how to harvest a large amount of high-quality of stem cells for cartilage repair remains a challenge for both stem cell biologists and orthopaedics surgeons. Decellularized extracellular matrix provides an *in vitro* cell expansion system that applied to human synovium-derived stem cells. In order to apply dECM cell expansion system for future clinical treatment, the characterization of dECM and expanded cells using both SEM and AFM was compared to the plastic control; Moreover, the proliferation and chondrogenic capacity of adult human SDSCs after expansion were compared. dECM expanded hSDSCs exhibited a fibroblast shape, a low level of elasticity, enhanced proliferation, unique changes in stem cell surface markers and enhanced chondrogenic capacity and hypertrophy, which are consistent with our previous findings [3]. These results suggested that dECM holds the potential for stem cell expansion and for future cartilage regeneration.

Furthermore, cartilage joint is a harsh environment where a lot of reactive oxygen species (ROS) and inflammatory factors might exist. Clinical treatment of cartilage defects is challenging due to concomitant post-traumatic joint inflammation [2]. A major source of destructive power of inflammation is the direct and indirect generation of reactive oxygen species (ROS) and free radicals after the inflammatory cytokine response [3]. For successful cell therapy or tissue engineering, measures must be taken to control the inflammatory and oxidative environment in which cartilage is regenerated [2]. The regulation of intracellular ROS and inflammatory factors is crucial for cell survival in the harsh environment and guarantee successful cell therapy. Novel properties of dECM in respect to its antioxidant and anti-inflammation characteristics have been defined. Our experiments suggested that dECM-expanded hSDSCs acquired enhanced chondrogenic potential after treatment with H_2O_2 in both proliferation and chondrogenic phase [2]. Without the IL-1 β induced inflammatory environment, dECM expanded hSDSCs yielded pellets with higher GAG amount per pellet and ratio of GAG to DNA than those from plastic expansion. In the IL-1 β induced inflammatory environment,

both GAG amount per pellet and ratio of GAG to DNA decreased in a dose-dependent fashion; however, dECM expanded hSDSCs yielded pellets with a significantly higher ratio of GAG to DNA than those from plastic expansion [4]. After defining the anti-oxidant and anti-inflammatory roles of dECM, some critical inflammation-related signals such as p38 MAPK has been proposed to co-preconditioning with stem cells to positively influence its chondrogenesis. This biochemically-modified dECM has proved its effectiveness in promoting chondrogenesis under the inflammatory environment [4].

Thirdly, to obtain a maximal stem cell numbers with optimal chondrogenic potential, optimization of the *in vitro* dECM niche is urgently needed. Driven by biomechanics research on stem cells, we hypothesized that the Young's modulus of dECMs could be designed by controlling the culture days or changing ascorbic acid concentration in the normal growth medium. Stem cells expanded on dECMs of varied elasticity were compared for their responses to expanded substrates, cell surface phenotypes, proliferation and chondrogenic differentiation. Stem cells expanded on the D10 dECM with higher Young's modulus demonstrated enhanced chondrogenesis compared to D0 dECM. Compared to 50 μ M ascorbic acid prepared dECM, 250 μ M ascorbic acid produced dECM showed higher Young's modulus value and consistently enhanced chondrogenesis. Furthermore, stem cells on passage senescent stem cell-derived dECMs had decreased chondrogenesis but did not show statistical significance in respect to their Young's modulus values compared to stem cells on early passage stem cell-derived dECM. Future work about proteomics analysis is desirable to further determine the potential biochemical and molecular mechanisms that might contribute to chondrogenic differentiation differences.

In conclusion, to overcome the dedifferentiation and aging problems during cell expansion and successfully translated human adult stem cell into applications in regenerating cartilage, it is important to recreate a functional stem cell niche *in vitro*. Cell-derived decellularized extracellular matrix (dECM) scaffolds harvested from cultured cells have attracted attentions in respect to their rejuvenation and regeneration effects on stem cell. Decellularized extracellular matrix deposited by stem cells also demonstrated promising applications in cartilage tissue engineering, not only enhancing cell proliferation but also promoting expanded stem cells' chondrogenic potential instead of (or even against) hypertrophy, which is undesirable for neo-cartilage formation. The dECM expanded stem cells also exhibited enhanced anti-oxidant and anti-inflammatory properties, suggestive of its potential applications for the repair of

cartilage defects in osteoarthritic patients. Therefore, the dECM approach combined with stem cells may provide a novel and promising approach to overcome replicative senescence and chondrogenic hypertrophy, which are two significant hurdles for cell-based cartilage engineering and regeneration. The development of biomechanics has advanced the development of optimization of dECM in respect of its elasticity, dECMs with appropriate Young's modulus not only promote stem cell proliferation and chondrogenic differentiation, but may also optimize stem cells to adapt to undesirable environment such as oxidative or inflammatory environment. Our ultimate goal is to optimize the dECM from biochemical and biophysical perspective to facilitate the cartilage engineering and tissue regeneration in an inflammatory environment.

References

1. Pei M, He F, Wei L. *J Tissue Sci Eng* 2011;**2**:104
2. Pei M, Zhang Y, Li J, Chen D. *Stem Cells Dev* 2013;**22**:889-900.
3. Li J, Hansen KC, Zhang Y, Dong C, Dinu CZ, Dzieciatkowska M, Pei M. *Biomaterials* 2014;**35**:642-53.
4. Zhang Y, Pizzute T, Li J, He F, Pei M. *Biomaterials* 2015;**64**:88-97.

CURRICULUM VITAE

Ying Zhang

EDUCATION

2011-2015 Ph.D. in Department of Mechanical and Aerospace Engineering, Benjamin M. Statler College of Engineering and Mineral Resources at West Virginia University
2007-2010 Master in College of Life Science, Sichuan University, Chengdu, China
2003-2007 Bachelor in College of Life Science, Linyi University, Linyi, China

TRAINING

2011-2015 Graduate Research Assistant in Exercise Physiology, Stem Cell and Tissue Engineering, Department of Orthopaedics, West Virginia University

FELLOWSHIPS AND AWARDS

2011-2015 Graduate Research Assistant Fellowship, West Virginia University

WORK EXPERIENCES

2010-2011 Technician in Lunan Pharmaceutical Group Corporation, Linyi, China

PEER-REVIEWED PUBLICATIONS:

1. **Zhang Y**, Chen S, **Pei M***. Biomechanical signals guiding stem cell cartilage engineering: from molecular adaption to tissue functionality. *Eur Cell Mater* 2015 (Submitted). (IF: 4.886)
2. **Zhang Y**, Pizzute T, Li JT, He F, **Pei M***. sb203580 preconditioning recharges matrix-expanded human adult stem cells for chondrogenesis in an inflammatory environment – a feasible approach for autologous stem cell based osteoarthritic cartilage repair. *Biomaterials* 2015;64:88-97. PMID: 26122165. doi: 10.1016/j.biomaterials.2015.06.038. (IF: 8.557) (**highlighted in F1000Prime**)
3. **Zhang Y**, Li JT, Davis ME, **Pei M***. Delineation of in vitro chondrogenesis of human synovial stem cells following preconditioning using decellularized matrix. *Acta Biomaterialia* 2015;20:39-50. PMID: 25861949. pii: S1742-7061(15)00162-2. doi: 10.1016/j.actbio.2015.04.001 (IF: 6.025) (**feature article in Mesenchymal Cell News 6.14 April 16, 2015**)
4. **Zhang Y**, **Pei M***. A review of crosstalk between MAPK and Wnt signals and its impact on cartilage regeneration. *Cell Tissue Res* 2014;358:633-49. PMID: 25312291. PMCID: PMC4234693 (IF: 3.677)
5. **Zhang Y**, Pizzute T, **Pei M***. Anti-inflammatory strategies in cartilage repair. *Tissue Eng Part B* 2014;20:655-68. PMID: 24846478 (IF: 4.64) (**feature article in Mesenchymal Cell News 5.20 May 29, 2014**)

6. Li JT, Hansen K, **Zhang Y**, Dong CB, Dinu C, Dzieciatkowska M, **Pei M***. Rejuvenation of chondrogenic potential by young stem cell microenvironment. *Biomaterials* 2014;35:642-653. PMID: 24148243 (IF: 8.557)
7. **Pei M***, Li JT, **Zhang Y**, Liu GH, Wei L, Zhang YY. Expansion on matrix deposited by nonchondrogenic urine stem cells strengthens repeated passage bone marrow stromal cells' chondrogenic capacity. *Cell Tissue Res* 2014;356:391-403. PMID: 24705582. PMCID: PMC4128485 (IF: 3.677) (*feature article in Extracellular Matrix News 5.13 April 10, 2014*)
8. **Pei M***, **Zhang Y**, Li JT, Chen DQ. Antioxidation of decellularized stem cell matrix promotes human synovium-derived stem cell-based chondrogenesis. *Stem Cells Dev* 2013;22(6):889-900. PMID: 23092115. PMCID: PMC3585742 (IF: 3.727)
9. Li JT, Jones B, **Zhang Y**, Vinardell T, **Pei M***. Low density expansion rescues human synovium-derived stem cells from replicative senescence. *Drug Deliv Transl Res* 2012;2(5):363-374. PMID: 25787175. (IF: n/a)
10. **Pei M***, Li JT, Shoukry M, **Zhang Y**. A Review of Decellularized Stem Cell Matrix: a Novel Cell Expansion System for Cartilage Tissue Engineering. *Eur Cell Mater* 2011;22:333-343. PMID: 22116651. (IF: 4.886)

Books and Patents:

Karnes J, **Zhang Y**, **Pei M***. Cell Therapy for the Creation of Cartilage and Related Clinical Trials. N. S. Templeton. Gene and Cell Therapy: Therapeutic Mechanisms and Strategies. 4th Edition. Taylor & Francis/CRC Press, 2014. pp. 1123-1135. (K16570_C048) (*invited book chapter*)

CONFERENCE PRESENTATIONS:

1. **Zhang Y**, Li JT, Davis M, Pei M. Extracellular matrix mediated human synovial stem cell chondrogenesis. *61th Annual Meeting of the Orthopaedic Research Society*. MGM Grand Hotel, Las Vegas, NV (2015) March 28-April 1. Trans ORS 40:xxx.
2. **Zhang Y**, Li JT, Pei M. Adult stem cells are chondrogenically rejuvenated after preconditioning "on" rather than "in" decellularized matrix. *61th Annual Meeting of the Orthopaedic Research Society*. MGM Grand Hotel, Las Vegas, NV (2015) March 28- April 1. Trans ORS 40:xxx.
3. Pei M, **Zhang Y**. p38 MAPK inhibitor priming of matrix expanded human synovium-derived stem cells for chondrogenesis. *International Cartilage Research Society (ICRS) 2015*. Chicago USA, May 8-11, 2015.
4. Donnelly B, **Zhang Y**, Branyan K, Suggs P, Pei M. Heparin sulfate proteoglycans enhance cell expansion and growth for cartilage engineering. *SURE (Summer Undergraduate Research Experience) Research Symposium*, July 24, 2014.
5. Li JT, Hansen KC, **Zhang Y**, Pei M. Define the rejuvenating effect of young matrix microenvironment in stem cell chondrogenic potential. *60th Annual Meeting of the Orthopaedic Research Society*. Hyatt Regency New Orleans, New Orleans, Louisiana (2014) March 15-18. Trans ORS 39:1146.
6. Li JT, **Zhang Y**, Pizzute T, Pei M. Contribution of FGF2 versus FGF10 to

- synovium-derived stem cell expansion and tri-lineage differentiation and potential mechanisms. 60th Annual Meeting of the Orthopaedic Research Society. Hyatt Regency New Orleans, New Orleans, Louisiana (2014) March 15-18. Trans ORS 39:384.
7. **Zhang Y**, Li JT, Pei M. SB203580 facilitates decellularized stem cell matrix-expanded human synovium-derived stem cells in chondrogenic potential in IL-1 β induced inflammatory conditions. 60th Annual Meeting of the Orthopaedic Research Society. Hyatt Regency New Orleans, New Orleans, Louisiana (2014) March 15-18. Trans ORS 39:382.
 8. **Zhang Y**, Li JT, Pei M. Preconditioning with p38 MAPK inhibitor boosts matrix expanded human synovium-derived stem cells in chondrogenic potential. 60th Annual Meeting of the Orthopaedic Research Society. Hyatt Regency New Orleans, New Orleans, Louisiana (2014) March 15-18. Trans ORS 39:1206.
 9. **Zhang Y**, Pei M. Matrix-mediated stem cell chondrogenesis and associated MAPK and Wnt signals. 2014 E. J. Van Lier Convocation and Research Day, WVU, Feb 28.
 10. Li JT, **Zhang Y**, Dong CB, Dinu C, Pei M. Rejuvenation of chondrogenic potential by young stem cell microenvironment. 2013 E. J. Van Lier Convocation and Research Day, WVU, Feb 28 (Poster presentation). **Zhang Y**, Li JT, Pei M. P38 inhibitor facilitates stem cell matrix in enhancing chondrogenesis and anti-inflammation. 2013 E. J. Van Lier Convocation and Research Day, WVU, Feb 28 (Poster presentation).
 11. **Zhang Y**, Li JT, Pei M. Decellularized Matrix Benefits Expanded Human Stem Cell Chondrogenesis in Resistance to an Inflammatory Environment. 59th Annual Meeting of the Orthopaedic Research Society. Henry B Gonzalez Convention Center, San Antonio, Texas (2013). Jan 26-29. Trans ORS 38:470.
 12. Li JT, **Zhang Y**, Kish VL, Pei M. Decellularized Stem Cell Matrix: A “Fountain of Youth” for Human Adult Stem Cell-Based Cartilage Regeneration. 2012 E. J. Van Lier Convocation and Research Day, WVU, March 9.
 13. **Zhang Y**, Li JT, Kish VL, Pei M. Decellularized Stem Cell Matrix Protects Adult Stem Cell-Based Chondrogenesis from Oxidative Stress. 2012 E. J. Van Lier Convocation and Research Day, WVU, March 9. (Poster presentation)
 14. Jones B, Li JT, **Zhang Y**, Pei M. Low density monolayer expansion rescues human synovial stem cells from replicative senescence. 2012 E. J. Van Lier Convocation and Research Day, WVU, March 9.
 15. Li JT, **Zhang Y**, Kish VL, Pei M. Young microenvironment rejuvenates aged human synovium-derived stem cells toward chondrogenesis. 58th Annual Meeting of the Orthopaedic Research Society. Moscone West Convention Center, San Francisco, CA (2012). Feb 4-7. Trans ORS 37:1748.
 16. **Zhang Y**, Li JT, Kish VL, Pei M. Extracellular matrix rescues human synovium-derived stem cells from oxidative stress and enhances expanded stem cell chondrogenesis. 58th Annual Meeting of the Orthopaedic Research Society. Moscone West Convention Center, San Francisco, CA (2012). Feb 4-7. Trans ORS 37:1747.

17. Li JT, Jones B, **Zhang Y**, Pei M. Low seeding density favors human synovium derived stem cell proliferation and multi-lineage differentiation capacity. 58th Annual Meeting of the Orthopaedic Research Society. Moscone West Convention Center, San Francisco, CA (2012). Feb 4-7. Trans ORS 37:1750.