Exploiting material softness to regulate mesenchymal stem cells secretome production for "cell-free" regenerative medicine

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**EXPLOITING MATERIAL SOFTNESS TO REGULATE
MESENCHYMAL STEM CELLS SECRETOME PRODUCTION
FOR “CELL-FREE” REGENERATIVE MEDICINE**

YANG HAIBO

SCHOOL OF MATERIALS SCIENCE AND ENGINEERING

2020

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YANG HAIBO

SCHOOL OF MATERIALS SCIENCE AND ENGINEERING

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

2020

Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research, is free of plagiarised materials, and has not been submitted for a higher degree to any other University or Institution.

May 14th 2020

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Date

Yang Haibo

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Supervisor Declaration Statement

I have reviewed the content and presentation style of this thesis and declare it is free of plagiarism and of sufficient grammatical clarity to be examined. To the best of my knowledge, the research and writing are those of the candidate except as acknowledged in the Author Attribution Statement. I confirm that the investigations were conducted in accord with the ethics policies and integrity standards of Nanyang Technological University and that the research data are presented honestly and without prejudice.

14/05/2020

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Date

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Assistant Professor Dalton Tay Chor Yong

Authorship Attribution Statement

This thesis contains material from two papers published in the following peer-reviewed journals in which I am listed as one of the co-first author.

Chapter 4 is published as H. Yang, K. T. Nguyen, D. T. Leong, N. S. Tan, and C. Y. Tay. Soft Material Approach to Induce Oxidative Stress in Mesenchymal Stem Cells for Functional Tissue Repair. *ACS Applied Materials & Interfaces* **8**, 26591-26599 (2016). DOI: 10.1021/acsami.6b09222.

The contributions of the co-authors are as follows:

- Asst. Prof Tay provided the initial project direction and edited the manuscript drafts.
- I prepared the manuscript drafts. The manuscript was revised by Dr. Nguyen, A/P Leong, A/P Tan and Asst. Prof Tay.
- I co-designed the study with Asst. Prof Tay and performed the laboratory work at the School of Materials Science and Engineering. I also analyzed the data.
- Dr. Nguyen measured storage modulus of hydrogels adopted in the study.
- Asst. Prof Tay conducted the animal study and protein array assay.

Chapters 5 and 6 are published as H. Yang, N. M. J. Cheam, H. Cao, M. K. H. Lee, S. K. Sze, N. S. Tan, and C. Y. Tay. Materials Stiffness-Dependent Redox Metabolic Reprogramming of Mesenchymal Stem Cells for Secretome-Based Therapeutic Angiogenesis. *Advanced Healthcare Materials* **8**, 1900929 (2019). DOI: 10.1002/adhm.201900929.

The contributions of the co-authors are as follows:

- Asst. Prof Tay provided the initial project direction and edited the manuscript drafts.
- I designed the research plan with the guidance of Asst. Prof Tay.
- I wrote the drafts of the manuscript. Asst. Prof Tay edited the manuscript drafts. The manuscript was revised together with Ms. Cheam, Ms. Cao, Ms. Lee, A/P Sze, A/P Tan and Asst. Prof Tay.

- I performed all the materials preparation, materials characterization, biology studies, and data analysis.
- Ms. Cao assisted in the 3D spheroid culture of mesenchymal stem cells.
- Ms. Lee conducted micro-contact printing studies and assisted in the *ex ovo* CAM assay.
- A/P Tan helped with the HUVECs migration assay.

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Abstract

Mesenchymal stem cells (MSCs) are multipotent stem cells derived from various adult tissues. They have been widely used in regenerative medicine because of their capability to differentiate into a variety of cell types. However, the paradigm of MSCs therapy shifts from differentiation to the secreted trophic factors of MSCs. MSCs secretome is a collective term for growth factors, chemokines, and cytokines and a variety of biologically active molecules secreted by MSCs. The therapeutic value of MSCs secretome has been proven in the treatment of cardiovascular diseases, liver diseases, and brain diseases, etc. To activate MSCs and engineer MSCs secretome for regenerative medicine, various preconditioning methods, including gene modification, drug treatment, hypoxia culture, etc., are adopted. Considering the sensitivity of MSCs to their microenvironment, biomaterials of different properties could be a potent regulator of MSCs secretome.

Hydrogel systems with tunable stiffness have been adopted to study the response of cells to substrates of different stiffnesses. In this thesis, polyacrylamide (PAAm) hydrogel was chosen due to its highly feasibility in tuning the stiffness mimicking to that of the human organs. Two different substrate stiffnesses, namely 0.15-1.5 kPa (brain mimicking) and 10-17.7 kPa (heart mimicking), were achieved. After conjugation of fibronectin (Fn) through sulfo-SANPAH chemistry, soft material for MSCs culture was prepared. With this PAAm hydrogel system, it was firstly found that primary MSCs are sensitive to substrate stiffness in aspects of cell spreading, cytoskeleton development, and intracellular ROS level even in the serum free condition. In addition, among the 42 cytokines screened with protein array, 15 of them were defined as “mechanosensitive” secretome since that their secretions were modulated by substrate stiffness. As a common mediator in MSCs preconditioning, reactive oxygen species (ROS) was found to regulate MSCs secretome production under the mechanical cue of substrate stiffness. Furthermore, compared with the CS of GPa stiffness, this “mechano-condition” of MSCs with soft PAAm hydrogel of kPa stiffness further improved the therapeutic efficacy of MSCs secretome in both *in vitro* scratch assay

and *in vivo* full-thickness excisional splinted wound healing mouse model with higher wound healing index. In short, soft material can be utilized for preconditioning of primary MSCs to promote “cell-free” regenerative medicine. Secondly, to address the senescence issue that bothers primary MSCs, the feasibility of “mechano-condition” on immortalized MSCs was tested. Immortalized MSCs were first confirmed to maintain differentiation potential, especially at the late passage (P10). Meanwhile, they still secreted trophic factors that promoting migration of human dermal fibroblasts and keratinocytes. These results suggest that immortalized MSCs are also an ideal cell source for tissue repair. Furthermore, the immortalization procedure was found to have no impact on the mechanosensitivity of MSCs. The 0.15 kPa PAAm hydrogel also limits spreading, hinders cytoskeleton maturation, induces intracellular ROS expression of immortalized MSCs. The expressions of VEGF and bFGF by immortalized MSCs were stimulated by the 0.15 kPa PAAm hydrogel at both transcription and protein levels, with the involvement of redox signaling. This mechano-conditioned MSCs secretome promoted the therapeutic angiogenesis because of a higher amount of proangiogenic factors. In the end, by focusing on the angiogenesis stage of tissue repair, the mechanism for MSCs redox stress induced by the 0.15 kPa PAAm hydrogel to guide proangiogenic secretion production was studied. Substrate stiffness was proven to be the main controller of the intracellular ROS level, regardless of serum availability. Nrf2 antioxidant response was triggered by excess intracellular ROS in immortalized MSCs on the 0.15 kPa PAAm hydrogel. Furthermore, the interplay between Nrf2 signaling and HIF1 signaling causes a non-canonical activation of this important regulatory factor for angiogenesis (i.e., HIF1) in immortalized MSCs under normoxic condition. ROS inhibition assay consolidates the activating role of upregulated intracellular ROS on HIF1 signaling. Overall, this thesis shows that material softness can be utilized to modulate MSCs secretome for regenerative medicine, with the redox status of MSCs as a key mediator.

Lay Summary

Tissues are highly vulnerable and susceptible to various types of injuries ranging from a simple paper cut to damage of muscles, ligaments and tendons during sports and exercise activities. As such, human body was designed to have its own ability to heal. During the healing process, mesenchymal stem cells (MSCs), which are dormant in various tissues, are being activated and produce various types of healing factors (i.e. secretome) to initiate and to facilitate the healing process. Scientists have successfully harvested secretome produced by MSCs and applied it to treat heart diseases, brain diseases, liver diseases and so on. However, to maximize the healing effect of MSCs secretome, MSCs need to be activated, just as they are stimulated by injury events *in vivo*. This process is called MSCs preconditioning. In general, MSCs can be preconditioned by changing the exposed oxygen level, by gene edition, by drug treatment, etc. In this thesis, a novel preconditioning method was explored by utilizing material softness of MSCs culture substrate.

Conventional cell culture substrates of MSCs are polystyrene petri dish and glass coverslip (CS), which have a high stiffness of GPa. In striking contrast, the stiffness of human tissues where MSCs reside is much lower. For instance, bone marrow has a stiffness ranging from 0.5-1.5 Pa. Even for bone, the stiffest tissue in human body, its stiffness is not more than 20 kPa. As such, conventional cell culture substrates are highly non-favorable for MSCs culture, which could also significantly impair their cellular function. In this thesis, soft PAAm hydrogels of 0.15-1.5 kPa and 10-17.7 kPa were fabricated for MSCs culture in order to mimic the stiffness of human brain tissue and heart tissue. With a layer of fibronectin coating, these PAAm hydrogels become a biomimicking substrate for MSCs culture and MSCs secretome production. It is found that these soft PAAm hydrogel of kPa stiffness can activate MSCs, producing more healing factors. Overall, the newly developed hydrogel system can be engineered to influence the MSCs secretome production targeted for effective skin wound healing and angiogenesis applications.

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Abbreviations

ABTS	2,2'-Azino-Bis(3-ethylbenzoThiazoline-6-Sulfonic acid)
ANOVA	ANalysis Of VAriance
BDNF	Brain-Derived Neurotrophic Factor
bFGF	basic Fibroblast Growth Factor
BMP	Bone Morphogenetic Protein
BSA	Bovine Serum Albumin
CAM	Chick Chorioallantoic Membrane
CD	Cluster of Differentiation
cDNA	complementary DeoxyriboNucleic Acid
CM	Conditioned Medium
CoM	Center of Mass
C _T	Cycle Threshold
DAB	3,3'-DiAminoBenzidine
DI water	De-Ionized water
DNA	DeoxyriboNucleic Acid
dNTP	deoxy-riboNucleoside TriPhosphate
dsDNA	double-stranded DeoxyriboNucleic Acid
ECL	Enhanced Chemiluminescence by Luminol
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
ELISA	Enzyme-Linked ImmunoSorbent Assay
ERK	Extracellular signal-Regulated Kinase
EthD-1	Ethidium homodimer-1
FAK	Focal Adhesion Kinase
Fn	Fibronectin
Fn-PAAm	Fibronectin conjugated Polyacrylamide
GDNF	Glial-Derived Neurotrophic Factor
GelMA	Gelatin MethAcrylate

HDF	Human Dermal Fibroblasts
HEPES	4-(2-HydroxyEthyl)-1-PiperazineEthaneSulfonic acid
HG-DMEM	High Glucose Dulbecco's Modified Eagles Medium
HGF	Hepatocyte Growth Factor
HIF1	Hypoxia-Inducible Factor 1
HLA-DR	Human Leukocyte Antigens-DR isotype
HMOX-1	HeMe OXYgenase-1
HRP	HorseRadish Peroxidase
hTERT	human TElomerase Reverse Transcriptase
hTERT-ADMSCs	human TElomerase Reverse Transcriptase immortalized Adipose Derived Mesenchymal Stem Cells
HUVECs	Human Umbilical Vein Endothelial Cells
ICAM-1	InterCellular Adhesion Molecule-1
IDO	Indoleamine-2,3-DiOxygenase
IGF	Insulin-like Growth Factor
IL	InterLeukin
INM	Inner Nuclear Membrane
Keap1	Kelch-like ECH-associated protein 1
LINC	LIinker of Nucleoskeleton and Cytoskeleton
LMWHA	Low Molecular Weight Hyaluronic Acid
LSD	Least Significant Difference
LVE	Linear Viscoelastic
MAPK	Mitogen-Activated Protein Kinase
MCP-1	Monocyte Chemoattractant Protein-1
miRNA	micro RNA
mRNA	messenger RNA
MS	Mass Spectrometry
MSCs	Mesenchymal Stem Cells
MT	Melting Temperature
NAC	N-Acetyl-L-Cysteine

NFκB	Nuclear Factor κ-light-chain-enhancer of activated B cells
NGF	Nerve Growth Factor
NQO1	Nicotinamide adenine dinucleotide phosphate dehydrogenase (QuinOne 1)
Nrf2	Nuclear factor erythroid 2-related factor 2
ONM	Outer Nuclear Membrane
PAAm	Polyacrylamide
PBS	Phosphate-Buffered Saline
PCR	Polymerase Chain Reaction
PDADMAC	Poly(DiAllylDiMethylAmmonium Chloride)
PDMS	PolyDiMethylSiloxane
PEG2000	Poly (Ethylene) Glycol molecular weight 2000
PEGDA	Poly (Ethylene) Glycol DiAcrylate
PGE2	ProstaGlandin E2
PKB	Protein Kinase B
PNIPAM	Poly(N-IsoPropylAcrylaMide)
PSS	Poly(4-StyreneSulfonic acid)
RANTES	Regulated upon Activation, Normal T cell Expressed and presumably Secreted
RFU	Relative Fluorescence Units
RhoA	Ras homolog family member A
RNA	RiboNucleic Acid
ROCK	Rho-associated protein kinase
ROS	Reactive Oxygen Species
RT-PCR	quantitative Reverse-Transcriptase Polymerase Chain Reaction
SAOS	Small-Amplitude Oscillatory Shear
SD	Standard Deviation
SDS-PAGE	Sodium Dodecyl Sulfate-PolyAcrylamide Gel Electrophoresis
SFM	Serum-Free Medium
SOD2	SuperOxide Dismutase 2

STC1	STannioCalcin 1
sulfo-SANPAH	N-SulfoSuccinimidyl-6-[4'-Azido-2'-NitroPhenylAmino] Hexanoate
TAN	Transmembrane Actin-associated Nuclear line
TAZ	Transcriptional coActivator with PDZ-binding motif
TGF β	Transforming Growth Factor β
TMB	3,3',5,5'-TetraMethylBenzidine
TNF α	Tumor Necrosis Factor α
TSG6	TNF α -Stimulated Gene protein 6
UGT1A1	Uridine diphosphate GlucuronosylTransferase family 1 member A1
UV	UltraViolet
VEGF	Vascular Endothelial Growth Factor
YAP	Yes-Associated Protein

Chapter 1

Introduction

The successful application of MSCs secretome has been demonstrated in regenerative medicine. Through various preconditioning methods, the performance of this “cell-free” therapy can be further improved. Even though it is established that MSCs are responsive to mechanical stimuli, the impact of cell culture substrate softness on the production of MSCs secretome is unclear. Hypothesis, objectives, and scope of the PhD project are presented here, followed by a summary of findings.

MSCs are multipotent stem cells derived from various adult tissues, whose main roles are to be activated after injury and repair local tissue damage [1]. They have the potential to differentiate into different lineages, such as adipocytes, osteoblasts, chondroblasts and so on [2]. Hence, MSCs have been widely used in cell-based therapy in regenerative medicine [3]. However, due to the complex *in vivo* environment, specific differentiation of MSCs to target cells was barely observed [4]. On the other hand, studies have shown that the paracrine action by MSCs has also been accounted for tissue regeneration [5]. As such, the growth factors, chemokines, cytokines and a variety of biologically active molecules secreted by MSCs, which is also known as MSCs secretome, have now been collected for various therapeutic studies [6]. Over the years, the therapeutic value of the MSCs secretome has been proven in the treatment of cardiovascular diseases, liver diseases, and brain diseases, etc. [7-9]. Furthermore, MSCs secretome is a cell-free therapeutic method, which devoid the issues commonly encountered by MSCs transplantation, like stemness attenuation, risk of immunological reactions as well as transmission of zoonotic disease etc. [10]. Considering the many benefits, MSCs secretome-based regenerative medicine is an emerging field of research and has gained significant traction in recent years.

Just like dormant MSCs in human body need the activation of tissue injury events to maximize their tissue repairing efficacy, *in vitro* stimulation of MSCs with stress signals also improves the therapeutic outcomes of MSCs therapy [11]. This process is nominated as preconditioning and various stimuli, including but not limited to TNF α treatment, hypoxia culture, H₂O₂ incubation, have been exploited [12-14]. Through interdisciplinary collaboration between biology and material science, the research community has made significant progress in controlling MSCs phenotype using advanced biomaterials with tunable biophysical and biochemical properties. In 2006, a landmark finding by Engler et al., demonstrated that substrate stiffness can function as a potent biophysical regulator of MSCs differentiation [15]. Specifically, MSCs cultured on PAAm hydrogels with stiffness that mimics the brain, muscle and bone tissues, were coaxed to differentiate into the respective tissue-specific cells. Since then, numerous studies have also shown that substrate stiffness can exert profound effects on MSCs spreading, proliferation, stemness

maintenance, senescence, and so on [16-18]. However, the effect of substrate stiffness on MSCs secretome production is less well understood, which raises an interesting point:

“Can substrate stiffness modulate the therapeutic potential of MSCs secretome?”

Considering the emerging role MSCs secretome plays in the contemporary regenerative medicine, and the increased research as well as clinical interest in MSCs-secretome based therapy, progress in this study will guide the design of future biomaterials to condition MSCs for “cell-free” therapy.

1.1 Hypothesis

This thesis tests the hypothesis that when the soft material is utilized as the cell culture substrate, it modulates MSCs secretome to promote tissue repair.

1.2 Objectives and Scope

The general objective of this study is to elucidate whether soft material can be used as cell culture substrate to precondition MSCs for tissue repair. This objective can be achieved by employing PAAm hydrogel, in which its stiffness can be tuned by changing its crosslinking density to mimic stiffness ($E \sim$ kPa range) of human organs.

The specific objectives are:

1. Fabricate stiffness tunable PAAm hydrogel with human plasma Fn coating for MSCs culture
2. Examine the effect of substrate stiffness on MSCs secretome production and tissue repair
3. Extend mechano-preconditioning to immortalized MSCs and explore therapeutic angiogenesis application
4. Elucidate the mechanism for substrate stiffness to modulate MSCs secretome

1.3 Dissertation Overview

The framework of the proposed research programme is based on the listed specific objectives in the previous section. The overall flow can be subdivided into three stages:

1.3.1 Stage 1: Fabricate substrate with in vivo tissue mimicking stiffness for MSCs culture and examine the effect of substrate stiffness on MSCs secretome

Recent studies confirmed the pivotal role of MSCs secretome in tissue repair, and substrate stiffness is a potent regulator of MSCs phenotype. Meanwhile, engineering MSCs secretome through various preconditioning strategies to increase its therapeutic potential (e.g. tissue repair, angiogenesis, immunomodulation, etc) have been found to be practical. Such observations regarding MSCs properties and substrate stiffness raises an interesting question:

“Can substrate stiffness be exploited to modulate MSCs secretome production for functional tissue repair?”

PAAm hydrogel, whose stiffness can cover the range of various human organs by adjusting its crosslinking density, is fabricated. To be used as cell culture substrate for MSCs, PAAm hydrogel is coated with Fn through sulfo-SANPAH chemistry. The response of MSCs to substrate stiffness, including cell spreading, cytoskeleton development, intracellular ROS level, and generation of secretome, was studied. The application of this new preconditioning method in tissue repair was tested with *in vitro* HDF and keratinocytes scratch assays and *in vivo* mouse full-thickness excisional wound healing.

1.3.2 Stage 2: Test the effect of mechano-preconditioning on immortalized MSCs and angiogenesis

Considering the limited growth potential of primary MSCs, immortalized MSCs could be a promising alternative for further large-scale manufacturing of MSCs molecular derivatives. However, even if it meets the minimal criteria of MSCs, immortalized MSCs might response to extracellular stimuli in a way different from primary MSCs. Thus, it is

critical to test:

“Whether if the concept of “mechano-preconditioning” applies to immortalized MSCs?”

hTERT-ADMSCs was used as the cell model. Its differentiation potential and secretion of trophic factors to facilitate cell migration was first tested. Subsequently, with the same PAAm hydrogel system, the response of hTERT-ADMSCs to substrates of various stiffness was studied. Finally, the tissue repair outcomes of preconditioned MSCs secretome from hTERT-ADMSCs were evaluated in *in vitro* HUVECs models and *ex ovo* CAM assay.

1.3.3 Stage 3: Elucidate how MSCs sense material stiffness and adjust secretome production

Little is known about the effect of biomaterials properties on MSCs secretome production. From the previous two stages, the role of substrate stiffness on the MSCs secretome production was assessed. Since oxidative stress has been implicated in numerous conventional preconditioning strategies, it is hypothesized that:

“Intracellular ROS level in MSCs may be modulated as a function of substrate stiffness.

The phenotypic change and the imbalance in redox metabolism may then converge towards critical associated signaling node to modulate secretome production in MSCs.”

With micro-contact printing technology, Fn was conjugated to the predetermined area of non-biofouling PAAm hydrogel. In this manner, hTERT-ADMSCs are coerced to different spreading areas while maintaining the same substrate stiffness. PAAm hydrogel bearing Fn islands becomes a useful platform to elucidate the potential of MSCs spreading in modulating intracellular ROS signaling. Furthermore, considering that HIF1 signaling is activated in various MSCs preconditioning method to overexpress proangiogenic factors, the role of HIF1 signaling in secretion of proangiogenic factors from MSCs cultured on substrates of different stiffnesses was studied.

Based on these three stages, this dissertation is made up of seven chapters.

Chapter 1 provides a rationale for the research and outlines the goals and scope.

Chapter 2 reviews the literature concerning the application and modulation of MSCs secretome for tissue repair, followed by an introduction to the phenomenon of mechanotransduction where cell function can be controlled. The development of PAAm hydrogel for mechanobiology studies is introduced. In the end, current trials of preconditioning MSCs with substrates of different stiffnesses are shared, pointing to the fact that barely anything is known about the mechanism for preconditioning MSCs with different stiffness to control secretion production.

Chapter 3 introduces the principles behind the main experimental techniques used in this dissertation and describes the detailed methods.

Chapter 4 first characterizes the PAAm hydrogel with Fn coating adopted in this dissertation for MSCs culture. Subsequently, the bio-characterization of primary MSCs culture on this PAAm hydrogel were compared with those on the conventional CS. In the end, the application of MSCs secretome in skin wound healing was presented.

Chapter 5 begins with the function tests of hTERT-ADMSCs, finding that hTERT-ADMSCs have some advantages over primary MSCs for regenerative medicine applications. Considering the potential difference between primary and immortalized MSCs, the feasibility of preconditioning hTERT-ADMSCs with the soft substrate was evaluated. Finally, the therapeutic efficacy of preconditioned MSCs secretome on angiogenesis was assessed.

Chapter 6 focuses on the explanation of this soft substrate-based preconditioning method. As overexpression of intracellular ROS was found to be a key mediator for the soft substrate to modulate MSCs secretome, various parameters were scrutinized first. Then, the Nrf2 and HIF1 signaling related to how redox metabolism regulates the secretion of proangiogenic factors was studied.

Chapter 7 discusses the main findings and proposes future studies.

1.4 Findings and Outcomes/Originality

This research led to several novel outcomes by:

1. Established a stiffness tunable PAAm hydrogel system for MSCs preconditioning
2. Examined the effect of substrate stiffness on primary and immortalized MSCs
3. Demonstrated MSCs secretome harvested from cells cultured on mechanically compliant PAAm hydrogel can outperform the therapeutic potential of MSCs secretome derived from cells grown on conventional hard substrates in terms of skin wound healing and therapeutic angiogenesis applications
4. Implicated changes to intracellular ROS level as an important upstream mechano-transducible molecular event
5. Examined the effects of culture conditions and morphological changes on the redox metabolism of MSCs
6. Elucidated how the shift in MSCs redox status instructs MSCs secretome production by activating the HIF1 signaling under normoxia condition

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Chapter 2

Literature Review

This chapter introduces MSCs secretome and current trials of using mechanical stimuli to precondition MSCs. The role of MSCs in tissue repair is first illustrated, with the importance of MSCs secretome emphasized. At the end of the first part, different established methods to modulate MSCs secretome are shared. In the second part, mechanotransduction, an important biophysical phenomenon, is introduced. The development of PAAm hydrogel system for mechanobiology studies are then presented. Eventually, a comprehensive review examining the engineering of MSCs secretome using current mechanobiology tools is given.

2.1 Overview

MSCs are multipotent cells that can differentiate into cells belong predominantly but not limited to the mesodermal lineages [1]. Various adult tissues, including bone marrow, adipose tissue, lung, umbilical cord, etc., could be the source of MSCs [2]. MSCs are promising candidates for regenerative medicine, where aged, damaged, and dead cells and tissues are rejuvenated or replaced [3]. The topic of MSCs therapy is popular worldwide, with more than 1000 clinic studies at different stages recorded by December of 2019 (<https://clinicaltrials.gov/>). Encouraging results of MSCs therapy have been reported for spinal cord injury, heart disease, osteoarthritis, neurological disorders, etc. [4-7].

For a long time, it was assumed that the therapeutic outcome of MSCs is caused by their differentiation into functional cells to replenish the damaged cells and tissues [8]. Because of the researches finding that the engraftment efficacy of MSCs is strikingly low, the secretory roles that MSCs play in the repair of MSCs are being appreciated and emphasized now [9-11]. Consequently, the trophic and immunomodulatory properties of MSCs have gained a lot of attention and is also taken as the primary pro-reparative mechanism of MSCs therapy [12-14]. These secretory products released by MSCs into extracellular space, including soluble proteins, free nucleic acids, lipids, and extracellular vesicles, are collectively nominated as MSCs secretome [15]. Accordingly, the advanced technologies to characterize and engineer MSCs secretome are being developed [16].

Mechanotransduction is the process where mechanical stimuli are converted into cellular signaling events [17]. It is a universal phenomenon but only applicable for all eukaryotic cells, but also observed in even bacterial [18]. Mechanotransduction event is a potent regulator of cell functions [19]. For the mechanobiology study of MSCs, a hallmark study is that matrix elasticity directs their lineage specification [20]. Besides differentiation, mechanotransduction also influences other biological process of MSCs, such as integrin binding, cytoskeleton reorganization, migration, immunomodulation, etc. [21-23]. Based on this knowledge, biomaterials with different features have been designed to control the phenotype and function of MSCs [24-26]. However, studies regarding whether

mechanotransduction events modulate MSCs secretome are limited and the underlying mechanism is unknown.

2.1.1 MSCs Secretome in Regenerative Medicine

2.1.1.1 MSCs and Their Mechanisms in Regenerative Medicine

According to the minimal criteria of International Society for Cellular Therapy, MSCs are plastic-adherent when maintained in standard culture conditions, express CD105, CD73 and CD90 and lack expression of CD45, CD34, CD14 or CD11b, CD79 α or CD19 and HLA-DR, and have the potential to differentiate into osteoblasts, adipocytes and chondroblasts *in vitro* [27]. They have been a subject that attracts intense scientific investigation since their discovery [28]. In the early studies, MSCs were found to have the capacity to differentiate into several mesenchymal tissue lineages. Researchers have attempted to use MSCs for the treatment of several diseases such as heart diseases, osteoarthritis, neurological disorders, etc [5-7]. The original hypothesis was that administrated MSCs will migrate to injured sites, engraft, and differentiation into functional cells [29] (**Figure 2.1.1.1a**). Besides the direct differentiation of MSCs, nuclear reprogramming of damaged somatic cells after the fusion between MSCs and somatic cells was also observed [30] (**Figure 2.1.1.1b**). However, occurrence and direct observations of both events is rare. Engraftment and differentiation were found to be infrequent and transient. Cellular fusion happens only at low level. Therefore, the long-term benefits of MSCs transplantation cannot be explained solely by these mechanisms [31].

Recently, the trophic effects of MSCs has gained intense experimental and biomedical interest. The paracrine activity of MSCs was first noted in the 1970s when the long-term culture of hematopoietic stem cells without growth factor supplementation was proven to be possible with the co-culture of MSCs and hematopoietic stem cells [32]. Numerous studies have documented the secretion of the impressive repertoire of tissue repair enhancing secretion of growth factors, cytokines, exosomes, mRNA and hormones by MSCs from different tissue sources. (**Figure 2.1.1.1c**). Additionally, novel mechanism of

MSCs mediated tissue regeneration such as cell-cell interactions between MSCs and somatic cells through tunneling nanotubes (**Figure 2.1.1d**), and the release of extracellular vesicles that encapsulating reparative peptides and proteins, mRNA and miRNA (**Figure 2.1.1e**) have emerged in recent years. Noteworthy, the International Society for Cell & Gene Therapy Mesenchymal Stromal Cell committee has also suggested the production of paracrine factors and extracellular vesicles as the primary therapeutic mode of MSCs [33].

Figure 2.1.1.1 The Mechanisms of MSCs in regenerative medicine. (a) Differentiation

to replace damaged cells. (b) Cell fusion to rescue damaged or dying cells. (c) Secretion of paracrine factors. (d) Organelles and molecules transfer through tunneling nanotubes. (e) Realizing of extracellular vesicles to transfer molecules. Adapted with permission [29]. Copyright 2016 Jeffery L. Spees et. al.

2.1.1.2 MSCs Secretome and Its Application in Regenerative Medicine

MSCs secretome refers to the whole secretory products, including soluble fraction and extracellular vesicles fraction, of MSCs, containing growth factors, cytokines, metabolites, mRNA, miRNA, etc. (**Figure 2.1.1.2**) [34]. With a huge number of successful applications of MSCs secretome in regenerative medicine, MSCs are even suggested to be renamed as “medicinal signaling cells” to better reflect the secretion of immunomodulatory and trophic bioactive factors as the primary modes of mechanism-of-actions [11].

Figure 2.1.1.2 Overview of MSCs Secretome. The MSCs secretome comprises of soluble proteins and extracellular vesicles. The soluble proteins include cytokines, chemokines and growth factors. The extracellular vesicles include exosomes and microvesicles. Adapted with permission [34]. Copyright 2019 Julia Driscoll et. al.

2.1.1.2.1 MSCs Secretome and Its Effects on Heart Disease

Heart failure is a growing public health problem worldwide [35]. The effects of MSCs secretome on cardiac progenitor cells growth and health have been tested *in vitro* [36]. When incubated with MSCs secretome, the viability of cardiac progenitor cells was improved significantly with reduced apoptosis and caspase 3 activity. MSCs secretome also attracted more cardiac progenitor cells to migrate through chemotaxicell filters (124.8 ± 14.8 cells/well versus 4.7 ± 1.9 cells/well). After induction of MSCs secretome for two to four weeks, the transcription of both atrial natriuretic peptide and β -myosin heavy chain was upregulated in cardiac progenitor cells. However, spontaneous cell beating was not observed, indicating that pristine MSCs secretome may not be potent enough to initiate functional beating of the heart [36].

As one of the most classic application areas of MSCs therapy, conditions related to myocardial infarction was also found to be alleviated from MSCs secretome administration [37]. The effects of MSCs secretome on blood vessel formation was first investigated with *in vitro* angiogenesis assays. MSCs secretome significantly induced more capillary sprouting from HUVEC spheroids and increased the total length of sprouts. In another HUVECs/Matrigel angiogenesis assay, longer average tube length was observed after MSCs secretome treatment. Consistent with the *in vitro* results, significantly more capillaries were formed in the border areas of a myocardial infarction pig model. Three weeks after induction of myocardial infarction, less ventricle remodeling was observed for pigs treated with MSCs secretome. At the same time, a higher fractional area shortening reflects increased systolic cardiac performance after MSCs secretome treatment, together with improved ejection fraction, stroke volume, and stroke work. Hemodynamic parameters and diastolic function were improved, and myocardial infarct size was also much smaller ($11.6 \pm 2.0\%$ versus $16.6 \pm 1.2\%$) [37].

2.1.1.2.2 MSCs Secretome and Its Effects on Liver Disease

Application of MSCs secretome have also been reported to ameliorate ischemic injury of

the liver [38]. 6 h and 12 h after reperfusion of MSCs secretome, the level of serum proinflammatory IL-6 was reduced significantly. 24 h after reperfusion, histologic hallmarks of hepatic ischemia/reperfusion injury showed a considerable restoration of the liver architecture. Meanwhile, mice receiving secretome-free medium demonstrated more hepatocellular necrosis, congestion, and cytoplasmic vacuolization. The expression of CD31 and ICAM-1, two major adhesion molecules promoting inflammatory cell infiltration and causing liver damage, was suppressed at 24 h after MSCs secretome treatment. Thus, through histologic examinations and myeloperoxidase assay, the inflammatory cell infiltration was confirmed to be reduced in MSCs secretome group. All the reduction of inflammatory proteins secretion and inflammatory cell infiltration into the liver contribute to lessen liver damage.

2.1.1.2.3 MSCs Secretome and Its Effects on Brain Disease

Both trophic and immunomodulatory properties of MSCs secretome were found to be useful in curing brain diseases. Multiple sclerosis is a common autoimmune disease that will eventually cause progressive neuron functional deficits. MSCs secretome was observed to reduce the functional deficits of neurons and promote oligodendrocytes and neuron development [39]. *In vitro* treatment of neurosphere with MSCs secretome increased A2B5⁺ oligodendrocyte precursor cells, oligodendrocytes and neurons, while reducing the number of astrocytes. For *in vivo* study, injection of MSCs secretome at the peak of disease alleviated functional deficits in all treated mice, with a small number of the specimens exhibiting a 100% recovery. The demyelination region in the spinal cord was also observed to reduce after MSCs secretome treatment.

Besides the therapeutic effect on autoimmune brain disease, MSCs secretome also protected against brain damage after hypoxic-ischemic injury [40]. 7 days after injury, the rats treated with the control medium showed severe brain-tissue atrophy, losing 32.8% tissue in the cortical region and 34.6% tissue in the hippocampal region. However, injection of 250-fold concentrated MSCs secretome significantly reduced brain tissue loss. Pretreatment with MSCs secretome 1 h before the injury limited hippocampus tissue loss

to only 2.7% and cortex tissue loss to 11%. If MSCs secretome administration was carried out 24 h after the injury, 12.2% of the hippocampus and 17.3% of the cortical were lost. A long-term checking of brain tissue revealed chronic changes. At 2 months after hypoxic-ischemic brain injury, rats treated with MSCs secretome showed attenuated spatial and working memory deficits. At 91 days post-injury, neuroprotective effects of MSCs secretome were noticed in thalamus and striatum. Mechanism study revealed the critical role of MAPK signaling, which mediates growth factor-dependent cell survival, in this neuroprotective event [40].

2.1.1.2.4 MSCs Secretome and Its Effects on Kidney Disease

An acute kidney injury rat model was established by continuous injection of gentamicin for 15 days. A single injection of MSCs secretome on the 10th day significantly reduced serum creatinine, serum urea, and fractional excretion of sodium [41]. However, if MSCs secretome was pretreated with either ribonuclease or trypsin for 12 h, the protective effect of MSCs secretome was partially blunted, indicating that both RNA and protein in MSCs secretome are essential for the best therapeutic outcome. Histological scoring also confirmed that MSCs secretome treatment mitigated kidney damage, with fewer lesions and apoptotic cell death and more cell proliferation. Similarly, the protective role of MSCs secretome against cisplatin-induced kidney injury was also studied with *in vitro* and *in vivo* assays [42]. MSCs secretome reduced detachment and death of mouse proximal tubule cells exposed to cisplatin. For *in vivo* study, by day 6, 6 of 15 mice in the cisplatin control group had died, while no mice died among those receiving MSCs secretome administration twice daily. At the same time, blood urea nitrogen value and other key parameters of renal function were improved significantly, and renal cell apoptosis was reduced by MSCs secretome treatment [42].

Besides all the listed therapeutic benefits, MSCs secretome has also been successfully applied for the treatment of skin wound healing, bladder dysfunction, osteoarthritis, and so on [43-45].

2.1.1.3 MSCs Preconditioning Strategies

While extensive studies have demonstrated the potential clinical utility of MSCs secretome, there is a need to future improve the therapeutic potency of MSCs. For example, even though MSCs secretome incubation promoted the expression of key cardiac markers in cardiac progenitor cells, none spontaneously cell beating was observed [36]. Meanwhile, it should be noticed that for most of animal studies utilizing MSCs secretome, the original MSCs secretome has been concentrated for more than 50-folds before injected into diseased animals [40]. Finally, the therapeutic potential of MSCs secretome is now known to be severely influenced by many extrinsic factors, such as tissue source of MSCs, age and health status of MSCs donors, MSCs passage number, to name a few (**Figure 2.1.1.3a**) [46-48]. Therefore, to maximize the therapeutic potential of MSCs secretome, MSCs can be subjected to an *in vitro* pre-conditioning process whereby the cells are exposed to a variety of stimuli to trigger the increased production of several pro-healing factors (**Figure 2.1.1.3b**) [49].

Figure 2.1.1.3 Flexibility of MSCs secretome. (a) The secretion profile of MSCs could be altered by many extrinsic factors. (b) Various preconditioning methods have been adopted to modulate MSCs secretome. Adapted with permission [50]. Copyright 2018 Jolene Phelps et. al.

2.1.1.3.1 Biochemical Cues

As a classical example of biochemical stimuli, lipopolysaccharide has been used to treat MSCs to mimic the inflammatory microenvironment after tissue damage [51-53]. By injecting MSCs exosome derived from MSCs preconditioned with lipopolysaccharide, the expression of anti-inflammatory cytokines and activation of M2 macrophage was promoted in the healing of diabetic wounds [51]. With the same lipopolysaccharide preconditioning method, the administration of MSCs secretome lowered the secretion of proinflammatory IL-6 and TNF α , enhancing liver regeneration [54]. Besides lipopolysaccharide, other inflammatory stimuli like interferon γ and TNF α have also been used [55-57]. Overall, these inflammatory stimuli enhance the immunoregulatory and trophic role of MSCs secretome. To increase the secretion of VEGF to restore heart function, MSCs were incubated with angiotensin II [58]. Since activation of ERK signaling through angiotensin II receptor type 1, a 3-fold increase in VEGF secretion was achieved. Biochemical stimuli are not limited to peptides and proteins. By activating the melatonin receptors, pineal hormone melatonin induced expression of antioxidant enzyme catalase and Cu/Zn superoxide dismutase and secretion of proangiogenic and mitogenic bFGF and HGF by MSCs [59].

2.1.1.3.2 3D Culture

In contrast to 2D cultures, where MSCs are typically grown in a monolayer fashion, MSCs grown in 3D self-aggregates to form compact 3D spheroids. Spheroids can be fabricated by hanging drop method, low attachment plates, and suspension bioreactor with high cell inoculating density [60-62]. Compared with conventional 2D monolayer culture, MSCs grown as 3D spheroids exhibited increased anti-inflammatory, proangiogenic, and tissue regenerative potential [63]. Thus, MSCs secretome derived from 3D spheroids showed stronger capacity to suppress the inflammatory response of stimulated macrophages [64]. This enhanced immunomodulatory function of MSCs secretome has been attributed to overexpression of anti-inflammatory factors TSG6, STC1, and PGE2, etc. Besides immunomodulators, the secretion of angiogenesis promoter VEGF and bFGF,

antioncogenic proteins TNF α and CD82 are also stimulated in 3D culture [65-67]. 3D culture of MSCs can also be achieved by encapsulation of MSCs into 3D hydrogel network. Chierchia et. al. used semi-interpenetrated hydrogel of PEG2000/collagen or collagen/LMWHA to encapsulate MSCs [68]. The secretome released by encapsulated MSCs was found to ameliorate the toxicity of dopaminergic selective toxin 6-hydroxydopamine to human neuroblastoma and upregulate the expression of antioxidant enzyme sirtuin 3. Silva and coworkers compared how gellan gum of its commercially available form and a fibronectin-modified form will influence the behavior and secretome of MSCs [69]. Secretome derived from MSCs cultured in modified gellan gum induced higher metabolic viabilities and cell densities of neuronal cells *in vitro*. Cai et. al. fabricated dual-network hydrogel composed of PNIPAM and PEG with the weight percentage of PNIPAM varied from 0% to 4% [70]. A high PNIPAM weight percentage of 4% was found to impair the survival and proliferation of encapsulated MSCs. However, a middle PNIPAM weight percentage of 1.25% and 2.5% significantly promoted the transcription of proangiogenic factors by MSCs. Thus, MSCs secretome derived from 1.25% and 2.5% PNIPAM-PEG hydrogel enhanced *in vitro* tube formation of human microvascular endothelial cells. Murphy et. al. studied the effect of gel stiffness and gel contraction on the secretion of VEGF and PGE2 by entrapping MSCs spheroids with a fibrin gel [71]. MSCs spheroids in stiffer gels secreted the most VEGF and the least PGE2. To simultaneously activate VEGF and PGE2 secretion, the gel of intermediate mechanical properties was chosen to produce MSCs secretome. This collected MSCs secretome stimulated endothelial cell proliferation, enhanced macrophage polarization and promoted angiogenesis when it was applied to a wounded 3D human skin equivalent. Although a comprehensive understanding of the effect of 3D culture on MSCs secretion has not been achieved, the increase in cell-cell interaction, altered cell morphology and the hypoxic core of the spheroid are believed to have a large effect on the composition of MSCs secretome.

2.1.1.3.3 Hypoxia Culture

The hypoxia environment is well-known to modulate MSCs secretome. Under the standard *in vitro* cell culture condition, the cells are exposed to an oxygen level of 21%. However,

MSCs are rarely exposed to such high level of oxygen in their *in vivo* niche. For example, the local oxygen concentration in bone marrow where MSCs resides, was measured to be approximately 2% [72]. Thus, 21% of oxygen can be deemed a hyperoxic environment for MSCs, a condition that might induce oxidative stress, DNA damage, and reduced lifespan [73]. Under careful control of exposure time, hypoxia simulates ischemic injury *in vitro* and stimulates the release of chemotactic and proangiogenic factors by MSCs [74]. Usually, the oxygen level of hypoxia culture condition is defined as 1-5%, with a lower oxygen level taken as anoxia. Considering the survival of MSCs, hypoxia is more commonly used than anoxia as the condition is not so extreme [75]. A critical pathway that activates during hypoxia culture is HIF1 signaling [76]. Because of this, both the secretion profile and quantity of released exosomes are enhanced [77-79]. miRNA11, miRNA22, miRNA223, etc. were observed to be overexpressed under hypoxia condition to direct inflammation, proliferation, and differentiation [80]. At the same time, the soluble factors that enhance survival, proliferation, immunomodulation, angiogenesis, neuroregulation, and differentiation, including VEGF, IGF, IL-6, FGF, etc., are also upregulated [77]. It was found that the differing oxygen level, exposure time and cell type led to different upregulation or downregulation of factors. For instance, TGF β 1 were upregulated at 1% oxygen level but downregulated at 5% oxygen level [81]. Exposure of MSCs to the hypoxic environment for 30, 60, or 90 min also resulted in different secretome profiles [82].

2.1.1.3.4 Gene Modification

A standard biological method for improving the effectiveness of MSCs secretome is gene modification. Since soluble proteins are critical for tissue repair caused by MSCs secretome, overexpression of growth factors with gene editing is the easiest and most direct approach. Both original MSCs and HGF transfected MSCs were cultured in the hypoxic condition to harvest MSCs secretome [83]. The secretome of HGF transfected was found to be more effective in rescuing apoptosis of neonatal cardiomyocytes induced by the hypoxic challenge. Even though only HGF was targeted at the beginning, VEGF, EGF, and bFGF were also overexpressed by MSCs with HFG transfection. This increase in the secretion of other factors might be caused by positive feedback after uptake of secreted HGF by MSCs

themselves. Different from targeting a single trophic factor, multiple signaling cascades could be activated by the transfection of transcription factors into MSCs. As a key transcription factor for cardiac development, overexpression of GATA4 led to increased proangiogenic factors secretion, including IGF and VEGF [84]. Thus, MSCs secretome from GATA4 transfected cells promoted migration and tube formation of HUVECs.

2.1.1.3.5 Role of Oxidative Stress in MSCs Preconditioning

ROS are a group of highly reactive ions and molecules derived from oxygen, including free radicals such as hydroxyl radical, superoxide anion, as well as non-radical molecules like hydrogen peroxide [85]. All ROS are extremely harmful to organisms at high concentration [86]. Meanwhile, a moderate level of ROS is also essential for regulation of physiological processes through its interaction with PKB, NF κ B, HIF1, integrins, etc [87]. Even though the MSCs preconditioning methods can be classified into different stimuli, ROS is found to be a common mediator in several preconditioning strategies (**Figure 2.1.1.3.5**). Under hypoxia conditions, intracellular ROS generation is induced by switching the function of complex II in mitochondria from succinate dehydrogenase to fumarate reductase [88]. Hydrogen peroxide treatment of MSCs raises ROS levels in MSCs mitochondria, activating ERK signaling and leading to the overexpression of pro-survival genes [89]. Incubation of MSCs with proinflammatory TNF α also stimulates mitochondrial ROS production through ROS modulator 1 and B-cell lymphoma-extra large [90]. Preconditioning of MSCs by serum deprivation also activates ROS modulator 1 [91]. Several important signaling pathways involved in MSCs preconditioning, like NF κ B, Nrf2, PKB, etc. are known to interact with ROS [92]. Furthermore, in cell-based MSCs therapy, oxidative stress preconditioning has already been adopted to promote the survival of MSCs in harsh environment. It was reported that preconditioning MSCs with 50 μ M hydrogen peroxide for 8 h improved the viability of MSCs under the treatment of 1000 μ M hydrogen peroxide. Activation of Nrf2 signaling and overexpression of antioxidant enzymes after preconditioning MSCs with low oxidative stress ameliorated the damage of DNA and mitochondria under high oxidative stress [93]. Similarly, Nouri and coworkers observed upregulated HIF1 and PKB signaling in MSCs pretreated with 20 μ M hydrogen peroxide

for 12 h. This decreased MSCs apoptosis in following 100 μ M hydrogen peroxide challenging [94]. Thus, changes to intracellular levels of ROS can be considered as an important signaling event in MSCs preconditioning strategies.

Figure 2.1.1.3.5 Involvement of intracellular ROS in various MSCs preconditioning methods. Adapted with permission [95]. Copyright 2018 Luisa Silva et. al.

2.1.2 Mechanotransduction and MSCs Function

2.1.2.1 Mechanotransduction and Current Understanding

Mechanotransduction is the process that cells sense mechanical stimuli and respond accordingly by converting into biochemical signals [96]. Mechanotransduction events are known as regulators of cell functions [20]. On the cell membrane, integrins, growth factor receptors, stretch-activated ion channels, and G protein coupled-receptors are in charge of initiating different mechanotransduction pathways [97-99]. A number of intracellular molecules have also been found to undergo post-translational modifications, intracellular shuttling, protein unfolding, or creation of novel interactions after imposing of mechanical stimulation [100-102]. These proteins are defined as mechanotransducers [103]. In this section, the up-to-date knowledge of mechanotransduction is introduced.

As the interface between cells and ECM, the cell membrane is the starting point of mechanotransduction. Focal adhesion is an integrin containing multi-protein structure that links intracellular actin bundles with ECM [104]. Its mechanosensing activity is rendered by its two-layer structure, including the transmembrane layer and the intracellular layer. The intracellular layer contains scaffolding, docking and signaling proteins (vinculin, talin, FAK, etc.) that work together to link integrins in the transmembrane layer with the actin cytoskeleton (**Figure 2.1.2.1.1**). Integrins are heterodimers composed of α and β subunits. The integrin affinity to its ECM ligand is regulated in both “inside-out” and “outside-in” manner [105]. In an “outside-in” manner, integrins are activated through confirmation change, clustering and reinforcing molecular links at the cell-matrix interface [106]. The establishment of interaction between ECM and cytoskeleton actin through focal adhesion allows the cells to respond in “inside-out” manner [107].

FAK is one of the very first proteins recruited to developing focal adhesion. After autophosphorylation, FAK serves as a trigger of intracellular mechanotransduction, activating downstream mechanotransducers that will further reinforce FAK signaling [100, 108]. Talin is a 270 kDa protein with one binding site for integrin, two binding sites for actin and several binding sites for vinculin [109]. After unfolding, talin will expose its hydrophobic binding sites for vinculin head, releasing vinculin from autoinhibited state [101]. This process is nominated as talin-vinculin mechanosensitivity, stabilizing talin-F-actin interaction and transferring mechanical signal “outside-in” [102]. Because of the talin-vinculin mechanosensitivity, the extent of vinculin presence at focal adhesion correlates to the force applied on the same focal adhesion [110]. After binding to talin, vinculin switches between a low affinity state and an inactive state [101]. Paxillin is another phosphotyrosine-containing docking protein. After being phosphorylated by FAK or Proto-oncogene tyrosine-protein kinase, paxillin stabilizes the interaction between focal adhesion and cytoskeleton by binding to activated vinculin and paxillin LD motif-binding protein. Phosphorylated paxillin also recruits Crk, which activates the MAPK signaling [111]. After the assembly of focal adhesion structure, the regulation of cytoskeleton tension achieves the propagation of extracellular and cell-generated forces [112]. Cytoskeleton not only provides mechanical support to the cells but also influences cell motility, morphology, and

gene expression [113-115]. Therefore, changes to the dynamics, geometry and polarity of to any of the cytoskeleton components (i.e. F-actin, microtubules, and intermediate filaments) can profoundly influence cell mechanics [116].

Figure 2.1.2.1.1 Structure of focal adhesion after cell adhesion on ECM. Adapted with permission [117]. Copyright 2015 Liviu Feller et. al.

The mechanical information received by focal adhesion and propagated by the cytoskeleton modifies the structure and the location of some proteins. Through their nuclear translocation, the mechanical information perceived at the cell membrane can be delivered into the cell nucleus. Tight junction protein 1, c-Abl tyrosine kinase, and β -catenin are the first proteins identified to shuttle across the nuclear envelope after mechanical stimulation [118-120]. Interestingly, nuclear translocation of two focal adhesion proteins under mechanical stress has also been observed. In vascular smooth muscle cells exposed to cyclic stretch, zyxin dissociates from focal adhesion and accumulates in the nucleus, modulating the expression of endothelin B receptor and tenascin-C [121]. Fibroblasts seeded on micro-contact printed circular fibronectin islands showed faster focal adhesion turnover and increased intranuclear translocation of paxillin compared with those on triangular islands. On the other hand, FAK hampers nuclear transport of paxillin [122]. Some other mechanotransducers move forth and back from the nucleus without any

physical association to focal adhesion, with YAP/TAZ as one typical example. A typical activator of YAP/TAZ is the stiff substrate, translocating YAP/TAZ from the cytoplasm to the nucleus [123]. YAP/TAZ in the nucleus could initiate the transcription of several factors, like c-Jun, activating protein 2, CCAAT enhancer-binding protein α , etc., to control cell functions [124].

Besides the transfer of mechanotransducers into cell nucleus, the nucleus also possess its own mechanosensitive apparatus [125]. The existence of nuclear-cytoskeletal coupling was observed in studies showing that there is nuclear reorganization and deformation shortly after the application of mechanical force on integrins [126]. Indeed, there is LINC complex on the INM and ONM of the nuclear envelope to link nucleoskeleton, nuclear envelope, and cytoskeleton together (**Figure 2.1.2.1.2**).

Similar to the formation of focal adhesion, TANs are formed by gathering of LINC components at the apical region of the nucleus [127]. This gathering happens under mechanical stimulation at the positions where LINC proteins come into contact with the nucleoskeleton and cytoskeleton [128]. On the internal side of the nucleus, lamin A links nuclear lamina with nesprins bind SUN proteins [129]. This structure allows the transduction of mechanical force from cytoskeleton to nucleoskeleton. As the connector between the cytoskeleton and nucleoskeleton, expression of lamin A was found to be modulated by substrate stiffness: soft substrates induce its phosphorylation and dissociation from nucleoskeleton [130]. LINC components and emerin, a regulatory protein in the nucleus, have been reported as mechanosensors. For example, nesprin-1 knockdown impaired nuclear translocation of YAP after mechanical stimulation [131]. In isolated nuclei, mechanical stimulation leads to phosphorylation of emerin by Src kinase, causing lamin A accumulation and nuclear stiffening [132]. Emerin also helps lamin A-chromatin interaction promote nuclear F-actin polymerization [133]. INM proteins can also modulate gene expression more directly, reducing the accessibility of chromatin to transcription regulators [134-136]. Some other signaling, like Wnt, Mega-karyoblastic leukemia 1, NF κ B, etc., also interacts with LINC components [137-139].

Figure 2.1.2.1.2 LINC components link nucleoskeleton and cytoskeleton. Adapted with permission [140]. Copyright 2018 Fabiana Martino et. al.

2.1.2.2 MSCs Behaviors Under Mechanotransduction Control

In nature, MSCs niche is a hydrated protein and proteoglycan-based gel network with diverse elements, with mechanical stimuli affect the behaviors of MSCs [141]. The most important mechanical cues are flow shear stress, tensile forces, substrate stiffness, and substrate geometry and topography [142]. Mechanical stimuli from the extracellular microenvironment are transmitted into the cell nucleus, becoming biochemical signaling that controls cell function. Besides basic cell processes like adhesion, proliferation, and migration, the important feature of MSCs, differentiation, has been well studied under various mechanical stimulation [143-145].

2.1.2.2.1 Substrate Stiffness

The stiffness of a substrate determines its resistance to deform under applied forces and usually represented by Young's modulus E [146]. The most well-documented substrate system with tunable stiffness is hydrogel. The hydrogels are crosslinked swollen

macromolecules forming a 3D structure. The design of ideal hydrogels for cell culture requires reproducing both the biochemical and mechanical properties of an *in vivo* microenvironment. Water-swollen polymers like PAAm, polyethylene glycol, polyvinyl alcohol, etc. have been utilized in previous studies to fabricate hydrogels to reproduce some mechanical features of human tissue [147]. Stiffness of hydrogels can be tuned by changing the crosslinking density, crosslinker length and molecular weight of the precursors to mimic the stiffness of human tissues and organs [148].

MSCs are contractile cells and their mechanotransduction can be explained by the tensegrity theory [149]. MSCs sense the stiffness of ECM by exerting contractile force through adhesion complexes that connect intracellular structures with ECM [150]. The regulation of several biological processes by substrate stiffness has been proven to be driven by integrin-mediated focal adhesion signaling under the control of both extracellular forces and contractility of the cytoskeleton [151]. In the seminal study done by Engler and coworkers, they showed that manipulating substrate stiffness can trigger the differentiation of MSCs towards the main cell type of the tissue with similar stiffness [20]. Stiffer hydrogel composed of gelatin-hydroxyphenyl propionic acid enhanced both proliferation and neuronal differentiation of MSCs [152]. On stiffer gels, osteogenic differentiation was favored over adipogenic differentiation [153]. In the coculture of MSCs and endothelial cells, decreasing substrate stiffness enhanced vessel network formation [154]. In another work of Engler and coworkers, they found that MSCs seeded on stiffness gradients first migrate to higher stiffness and then differentiate into a more contractile myogenic phenotype, with some cells retain a neural marker $\beta 3$ tubulin [155]. Because ECM interacts with growth factors *in vivo*, combining varying stiffness with growth factors is meaningful [156]. By controlling both substrate stiffness and TGF β exposure, more smooth muscle cells-specific markers were observed in stiff substrate with TGF β , while increased chondrogenic marker expression was observed on soft substrate with TGF β [157]. In another study interpreting the interaction between substrate stiffness and BMP2 mimetic peptide, it was first confirmed that 13-17 kPa substrate promotes myogenic differentiation while 45-49 kPa substrate enhances osteogenic differentiation. However, after chemical grafting of BMP2 mimetic peptide onto both substrates, only osteogenic differentiation

was induced. Meanwhile, if even softer substrate with 0.5-3.5 kPa stiffness is utilized, the BMP2 mimetic peptide has no effect on MSCs differentiation [158]. Similarly, soft substrate and addition of VEGF synergistically guided MSCs differentiation into endothelial cells [159].

2.1.2.2.2 Tensile Force

By sample stretching, tensile forces can be created in parallel to the stretching direction [160]. Cyclic and static stretching can be performed in either multiple or uniaxial direction [161]. Tensile force can be applied to MSCs in different ways, including stretching cell-embedded substrates, via magnetic beads, stretching the Flexcell device with 3D scaffolds or coated plates in 2D [162-164]. The concept that MSCs respond to tensile force originates from the observing about embryonic development. For example, during gastrulation, cells are exposed to several types of forces when the embryo transforms from a group of cells into three germ layers [165]. As the nature residences of MSCs, bone, cartilage, muscle, and other tissues are exposed to tensile force in nature.

Osteogenic differentiation of MSCs under tensile force is well studied. By exposing MSCs embedded in type 1 collagen to tensile strain of 3-5% amplitude, the expression of osteogenic markers were increased significantly, with a downregulation of chondrogenic, adipogenic, and neurogenic markers [166]. Using MSCs gene engineered with BMP2 seeded in a polyethylene glycol-fibrinogen hydrogel, compression was found to increase cell metabolism, alkaline phosphatase activity, BMP2 secretion, and formation of mineralized tissue [167]. Tensile forces boosted both angiogenic factors secretion and osteogenic differentiation of MSCs collected from osteoporotic donors [168]. By applying 0.4% mechanical strain for 2 h and then resting for 22 h, 63 totally distinct genes were upregulated, with most of them involving in cell surface receptor signal transduction and metabolic and cell division regulation, together with the osteogenic transcription factor RUNX2. A persistent mechanical loading of 24 h also induced osterix expression. Meanwhile, mechanical loading led to upregulation of ERK1/2 phosphorylation, with a number of genes identified as possible regulators of this process [169]. Similarly, the

impact of integrin-mediated mechanical forces resulted in the upregulation of collagen 1, VEGF, and SRY-box transcription factor 9 expression in MSCs, respectively indicating osteogenesis, angiogenesis, and chondrogenesis [170]. Without addition of growth factors, MSCs differentiated to smooth muscle cells under application of uniaxial tensile stretching, with higher proliferation rate and reorientation in the stretching direction [171]. Uniaxial stretching was reported to upregulate smooth muscle markers, while equiaxial cyclic loading led to their downregulation [161]. MSCs differentiation towards fibroblasts was also observed under external cyclic forces [172]. Uniaxial stretching of poly(3-caprolactone) films seeded with MSCs induced a contractile smooth muscle cells like phenotype and the formation of vascular engineered tissue with organized and oriented vascular cells [173]. Static stretching given to cell-embedded 3D collagen matrices reduced glycosaminoglycan synthesis, while cyclic stretching increased its expression, indicating chondrogenic differentiation [174].

2.1.2.2.3 Shear Stress

Shear stress is the force applied parallel to the plane of activation [175]. It plays a critical role in the function of blood vessels as the inner part of the blood vessel senses the shear stress caused by blood flow at the points of contact [176]. Therefore, a lot of efforts have been put on understanding the effect of shear stress on the behavior, shape, organization, and functionality of blood vessel cells, especially endothelial cells [177]. In recent years, it is also found that shear stress regulates differentiation and other behaviors of MSCs.

Using parallel plate system to generate shear stress, the expression of mature endothelial cell-specific markers, such as CD31, von Willebrand factor, and vascular endothelial-cadherin, was induced at both transcription and protein level [178]. In addition, shear stress promoted uptake of acetylated low-density lipoproteins, a phenotype of mature endothelial cells, and formation of capillary like structures on Matrigel. Meanwhile, upregulation of angiogenic growth factors were observed, together with downregulation of growth factors associated with smooth muscle cells differentiation. Upregulation of endothelial markers upon growth of MSCs under fluid flow was also reported by other study [179]. The

oscillatory shear stress forces the directional reorganization of F-actin [180]. Besides to its role in vascular differentiation, very low shear stress was also reported to induce osteogenic differentiation [181-183]. Chondrogenic differentiation of MSCs can also be achieved by combining shear stress with induction media supplemented with chondrogenic factors [184].

2.1.2.2.4 Substrate Geometry and Topography

To apply mechanical constraints on cells, diverse substrate patterns can be fabricated with innovative processes, with micro-contact printing as one widely adopted method [185]. Within patterned islands, MSCs are subjected to different constraints, which could control MSCs differentiation. With multi cells seeded on one island, the core of the island promoted adipogenic differentiation, while the perimeter enhanced osteogenic differentiation [186]. It has been proven that higher tension forces generated by non-muscle myosin II exerted at island edges induced osteogenesis [187]. Under the treatment of mixed adipogenic and osteogenic differentiation medium at 1:1 ratio, most of single MSCs seeded on 10000 μm^2 fibronectin island underwent osteogenesis while MSCs on 1024 μm^2 island became adipocytes [188]. The curvature of the islands also direct fate decision of MSCs. For example, star shapes promote osteogenesis and flower shapes enhance adipogenesis [189]. Micropatterned surfaces can also be prepared through photolithography. Using cell nonadhesive polyvinyl alcohol, cell adhesive polystyrene geometries of triangle, square, pentagon, hexagon, and circle with the same surface area of around 1140 μm^2 were fabricated by Song et. al. [190]. Under the induction of adipogenic differentiation medium, all these geometries did not induce difference in the fate decision of MSCs.

Photolithography and electron beam lithography are the two most common techniques to fabricate substrates with well-controlled topographies [191]. Some widely studied topographies include grooves, pits and pillars. With electron beam lithography, Dalby et. al. fabricated 100 nm deep nanopits in hexagonal, square, displaced square and random placements [192]. The displaced square distributed nanopits alone was found to have similar osteoinduction efficacy as osteogenic medium. The same features have also been

fabricated with polycaprolactone, finding that square placements of the nanopits maintain the stemness and multipotential differentiation of MSCs [193]. When the cells are cultured on grooves, cells typically elongate and align along the grooves, which is referred to as “contact guidance” [194]. By coercing MSCs to elongate on the grooves, neurogenesis can be induced at the similar efficacy as induction medium [195].

Besides well-control topography fabricated through lithography, nanomaterials have also been deposited to tune surface topography. Deposition of single wall carbon nanotubes increased the surface roughness and facilitated protein adsorption, which further promotes MSCs adhesion and spreading on the substrate [196]. At the same time, a transient upregulation of neuronal markers after one-week culture was reported. Thin film of oxygen plasma treated carbon nanotubes was also observed to enhance osteogenic differentiation of MSCs without any biochemical treatment [197]. Carbon nanotubes can also be micropatterned to manipulate the morphology and organization of MSCs [198]. Substrate with vertically standing TiO₂ nanotubes has been fabricated through anodization of titanium in suitable electrolyte. A optimal diameter of 15 nm was shown to promote MSCs adhesion, proliferation, migration, and differentiation [199].

2.1.2.3 MSCs Secretome Under Mechanotransduction Control

Even though a big number of studies regarding mechanotransduction events influence MSCs differentiation have been reported, how mechanical stimulation controls MSCs secretome production is largely unknown because the importance of MSCs secretome on tissue repair has not been recognized until recent years.

Shear stress is one of the widely studied mechanical stimulation, as its regulatory role on endothelial cells has been known for decades [200]. In one secretome study, by applying shear stress of 10^{-4} newtons/cm² to MSCs for up to 96 h, MSCs overexpressed VEGF in a nitric oxide dependent way without inducing endothelial cells differentiation [201]. Similarly, multiaxial mechanical load was also found to increase nitric oxide release of MSCs, together with overexpression of angiogenin, activated leukocyte cell adhesion

molecule, latency-associated peptide, VEGF, etc. [202]. With a mechanical stretch of 10% extension at a frequency of 30 cycles/min, nuclear localization of NF κ B p65 was promoted, inducing expression of VEGF. This preconditioning rendered a stronger pro-angiogenic potential to MSCs secretome [203]. Culturing MSCs on microcarriers with bioreactor system was initially utilized for the expansion of MSCs to large numbers. The stirring of microcarriers also causes a shear force to MSCs on the microcarriers. As a result, the secretions of cystatin C, glia-derived nexin, galectin-1, and pigment epithelium-derived factor were stimulated [204]. These overexpressed neuroregulatory molecules promoted the neuronal differentiation of both human neural progenitor cells and hippocampal dentate gyrus *in vitro*.

Besides a direct application of force, numerous mechanobiology studies have been conducted on substrate with different features [20, 205]. Through electrospinning, 2D fibrous scaffolds with various kinds of fiber alignment, like random, aligned and mesh pattern, can be fabricated [206]. Compared with traditional flat 2D polystyrene plate, all these three topographies induced the secretion of a list of key proteins by MSCs, like PGE₂, TGF β , VEGF, HGF, etc. In *in vitro* cell models, secretome collected from MSCs cultured on mesh pattern was found to possess stronger anti-inflammatory and proangiogenic efficacy. Thus, in *in vivo* rat shin wound healing, administration of this engineered secretome promoted wound closure, together with enhanced M2 macrophage infiltration to the wound area. By injecting polystyrene onto metal cylinders of different roughness, the roughness of polystyrene cell culture surface can be tuned [207]. It was found that if the roughness of substrate surfaces fits in size range of a single cell, FAK/ERK and Rho/ROCK pathways are activated by the increased high affinity β 1 integrin level, inducing secretion of VEGF. This preconditioning method successfully enhanced the proangiogenic potential of MSCs secretome as proven in *in vitro* HUVECs migration and tube formation, and in *ex ovo* CAM assay. Lithography is another conventional method to manipulate the topography of substrates. Different from the periodical arranged nanopits with a fixed center-to-center spacing of 300 nm, the substrate with a random arrangement of the same nanopits was found to promote the immunomodulatory effect of MSCs, inhibiting the proliferation of T cells with IDO as a key mediator [25]. Mechanism studies

revealed the contribution of decreased intracellular tension and changes in cellular respiration in this phenomenon.

Fabricating hydrogel of different stiffness by tuning crosslink density is almost the most direct and simple way to prepare substrate for mechanobiology studies [208]. Even though limited, studies are exploring the relationship between substrate stiffness and MSCs secretome production. For example, it was recently reported that compared with a soft substrate of 3 kPa, stiff substrate of 30 kPa activates NF κ B p65 signaling of MSCs, promoting secretion of immunosuppressive IDO, cytochrome oxidase, osteopontin and stromal cell-derived factor [26]. However, a single study cannot draw the conclusion. Abdeen et al. fabricated PAAm hydrogel of different Young's modulus (0.5 kPa, 10 kPa, or 40 kPa) with a coating of various ECM proteins (Fn, collagen type I, or laminin) by hydrazine activation of the surface [209]. At the same time, the glass surface was used as a control for MSCs culture. Not much difference between the ECM coating was observed regarding the cytoskeleton development and cell spreading of MSCs. However, when Fn was used as the coating ECM, PAAm hydrogel of all the three stiffness promoted the secretion of a list of proteins, including VEGF, angiogenin, EGF, HGF, etc. Especially for VEGF, among the three different PAAm stiffness, there is a positive correlation between the stiffness and VEGF secretion. Thus, when MSCs secretome collected from MSCs cultured on the three PAAm hydrogels was applied in *in vitro* human microvascular endothelial cells tube formation, the 40 kPa group stimulated the biggest tube area.

However, there is also a study reporting a reverse relationship between substrate stiffness and secretion of proangiogenic factors. Inspired by the fact that MSCs with a smaller diameter showed stronger therapeutic efficacy in improving hematopoietic recovery than those MSCs with a bigger diameter, Liu et al. cultured MSCs on PDMS of 1 kPa, 10 kPa, and 100 kPa, as cells cultured on a soft substrate will reduce their diameter [210]. Without ECM protein conjugation, plasma treatment allows the attachment of MSCs onto the PDMS. As expected, when MSCs were cultured on soft PDMS, more proangiogenic proteins were secreted and promoted hematopoietic recovery. As compared to the previous two studies where the most abundant proangiogenic factors secretion was observed on the

stiffest or softest substrate, Nasser et al. got the strongest HUVECs tube formation and VEGF secretion from MSCs cultured on PEGDA/GelMA hydrogel of 5 kPa rather than 1.3, 13 and 23 kPa [211].

It should be noticed that even though all these three reports intend to elucidate the relationship between substrate stiffness and proangiogenic factors secretion, there are a lot of discrepancies in their experimental details, with hydrogel type (PAAm versus PDMS versus PEGDA/GelMA) and MSCs adhesion ligand (fibronectin versus non-specific absorbed protein versus GelMA) as the two most important difference. Since there was no mechanism study conducted in all these three reports, the divergences between them could be caused by the activation of various signaling under different experimental environments, or the collection of unreliable data. Overall, how substrate stiffness controls MSCs secretome production and its underlying mechanism is largely unknown.

2.2 Summary

Stem cell therapy has been proven to be a useful treatment solution for several tissue injuries. In contrast to the original attribution of healed damaged tissue to the differentiation of injected MSCs into functional cells, the secretome released by MSCs was later recognized as a main contributor to the healing outcomes. At the same time, the paracrine effect of MSCs is influenced by the status of MSCs. Many preconditioning methods are explored to modulate the composition of MSCs secretome, which could further improve the therapeutic efficacy of MSCs secretome. The phenotype of cells can also be controlled using mechanical stimuli, as extracellular mechanical force can be transmitted into the cell nucleus and converted into a biochemical signal through a process called mechanotransduction. The trials of using mechanical stimuli to modulate MSCs secretome is limited, with barely any understanding regarding its mechanism. In this situation, whether and how substrate stiffness, a kind of mechanical stimulus, modulates MSCs secretome is a topic worth studying to realize a “cell-free” stem cell therapy.

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Chapter 3

Experimental Methodology

Chapter 3 summarizes the experimental protocol development during the progress of the PhD thesis. The selection criteria for hydrogel system and surface modification method are first explained. Rationales for tests employed, method developments and validation to evaluate the properties of hydrogel system and phenotypes of MSCs are detailed.

3.1 PAAm Hydrogel as Cell Culture Substrate

Principle

Adhered cells sense and respond to ECM. The ECM guides cellular behavior by presenting both biochemical signals and biophysical cues [1]. Among these biophysical stimuli, stiffness, or elastic modulus E in material science, is a key variable of cell-ECM interaction [2]. Most of soft tissues in human have the E ranging from 0.01 kPa to 100 kPa. However, polystyrene petri dish and glass CS, two of the most conventional substrates for *in vitro* cell growth and biological studies, have an E in the GPa range (**Figure 3.1.1**). To examine the response of cells in a biomimicking environment, several hydrogel systems, including PAAm, collagen, PDMS, and many more have been employed [3-5]. Even though E can be controlled by tuning the crosslinking density, the range of E that can be achieved varies for different hydrogel systems (**Figure 3.1.1**). PAAm was chosen as the model hydrogel system in this thesis.

Figure 3.1.1 The stiffness of various human tissues is compared with the stiffness of various hydrogels and polystyrene employed in cell culture. Adopted with permission [6]. Copyright 2012 Kevin S. Kolahi et. al.

As mentioned, PAAm is a popular substrate that have been used in numerous mechanobiological studies [3, 7-8]. The *E* of PAAm hydrogel can be precisely controlled by altering the cross-linking density of the polyacrylamide network. This can be easily achieved by changing the concentrations of the monomer acrylamide and the crosslinker bis-acrylamide. Various methods have been established to conjugate ECM proteins to the PAAm hydrogel covalently [9-11]. For this thesis, sulfo-SANPAH was used because it is commercially available and widely used [12-14]. The sulfo-SANPAH crosslinker contains a nitrophenyl azide group that can react with amino groups of PAAm hydrogel and an NHS ester that conjugates with primary amines of ECM proteins [11]. In this way, cyto-adhesive PAAm hydrogel of various stiffness can be employed as cell culture substrates. For ease of handleability, the PAAm hydrogel can be polymerized onto a cover slip in situ with the aid of silane coupling agents [15]. To bind PAAm hydrogel with CS, 3-(trimethoxysilyl)propyl methacrylate was used to treat CS first through the hydrolytic deposition. After that, the vinyl group of 3-(Trimethoxysilyl)propyl methacrylate reacts with the radical initiated copolymerization of acrylamide and bis-acrylamide to form a covalently bound PAAm hydrogel. In an earlier study, it was shown that MSCs can sense stiffness of materials to up to 3.4 μm deep [16]. Therefore, to ensure that the cells do not sense the stiffness of CS, the PAAm hydrogel thickness was designed to be around 70 μm .

Method

22*22 mm square No. 1 glass CS (Marienfeld #0101050) was cleaned by one round of acetone sonication and two rounds of ethanol sonication for 5 minutes each round. Silane working solution was prepared by diluting 10 μL of 3-(Trimethoxysilyl)propyl methacrylate (Sigma #440159) with 200 μL of acetic acid (Sigma A6283), 1.8 mL of deionized water and 8 mL of ethanol (Merck #100983). Air-dried glass CS were immersed in silane working solution for 15 mins, protected from light, to prepare surface for covalent binding of PAAm hydrogel. Surface treated glass CS were then air-dried. To prepare PAAm hydrogel of two different stiffness, the amounts of acrylamide and bis-acrylamide were tuned. Two combinations of acrylamide/bis-acrylamide weight percentages were used, namely 4%/0.15% and 10%/0.1%, with deionized water as the solvent.

Polymerization of acrylamide monomers was initiated by adding 0.4% ammonium persulfate (Bio-Rad #1610700) and 0.04% tetramethylethylenediamine (Bio-Rad #1610800). For fabricating PAAm hydrogel with glass coverslip as the base, the prepared solution of acrylamide monomers and crosslinking initiators was sandwiched between the surface-treated glass CS and a hydrophobic plastic sheet. After 30 min of polymerization, the PAAm hydrogel coated glass CS were washed thoroughly thrice with pH 8.5 50 mM HEPES buffer (HyClone SH30237.01). After that, 350 μ L of sulfo-SANPAH (ProteoChem c1111) was dropped onto every piece of PAAm hydrogel, followed by irradiation for 15 min with 365 nm UV to modify the surface of PAAm hydrogel. Residual sulfo-SANPAH was washed off with another three rounds of HEPES buffer washing. Human Fn (Merck #341635) was conjugated to PAAm hydrogel by incubating the hydrogel and Fn working solution (50 μ g/ml in pH 8.5 50 mM HEPES buffer) overnight at 4 °C. On the following day, Fn-PAAm hydrogels were washed once more with 1X PBS (HyClone SH30378.03) before cell seeding. **Figure 3.1.2** depicts the procedure of fabricating Fn-PAAm hydrogels.

Figure 3.1.2 Fabrication of Fn-PAAm hydrogels.

3.2 Rheological Characterization of Hydrogels

Principle

Rheology studies the deformation and flow of materials [17]. The main rheological technique for the characterization of hydrogels is SAOS [18]. The oscillation test can be explained using the two-plate-model (**Figure 3.2.1**). The upper plate moves forth and back under the shear force $\pm F$, while the lower plate stays fixed. The oscillation movement

shears the sample that is placed between the two plates. The sample should adhere to both plates without sliding or slipping, and the sample fills the whole shear gap h . s is the deflection distance, and φ is the deflection angle during the oscillatory test.

Figure 3.2.1 A schematic figure of oscillatory test on the sample in the shear gap h under shear force $\pm F$, with s as deflection distance and φ as deflection angle.

The strain sweep (**Figure 3.2.2a**), which varies strain and fix frequency, and the frequency sweep (**Figure 3.2.2b**), which varies frequency and fix strain, are two modes used to measure the mechanical properties of hydrogels. The strain sweep should be first done, typically at a frequency of 1 Hz, to determine the LVE range and the moduli of hydrogels. For example, in **Figure 3.2.2a**, the given hydrogel shows a constant storage modulus G' up to 15% strain, beyond which the hydrogel structure breaks down. Meanwhile, loss modulus G'' is too small to be measured, as is to be expected for fully crosslinked hydrogels. According to the LVE range obtained from the strain sweep, a strain of 10% can be chosen for the following frequency sweep to examine the behavior of G' and G'' in the LVE range using constant deformation (**Figure 3.2.2b**).

Figure 3.2.2 Characterize hydrogels with SAOS. (a) The strain sweep and (b) The frequency sweep. Adopted with permission [18]. Copyright 2014 Wiley.

G' is the elastic modulus of a sample and measures the deformation energy stored in the sample during the shear process. On the other hand, G'' is the viscous modulus of a sample and measures the deformation energy used up in the sample during the shear process [19]. Meanwhile, the complex shear modulus G^* represents the complete viscoelastic behavior of a sample, consisting of both the elastic and the viscous components. Therefore, G^* is the vector sum of G' and G'' , as shown in **Figure 3.2.3** [20].

Figure 3.2.3 G^* as the vector sum of G' and G'' .

According to **Figure 3.2.3** and the Pythagorean theorem, G^* can be calculated with the following equation:

$$|G^*| = \sqrt{(G')^2 + (G'')^2}$$

For fully crosslinked hydrogels, $G' \gg G''$. In this situation, $|G^*| \approx |G'|$. To compare this thesis with related studies where Young's modulus E is adopted, E of hydrogels can be calculated using the following equation [7, 21-23]:

$$E = 2G^*(1 + \nu)$$

In this equation, ν is Poisson ratio. For PAAm hydrogel, $\nu = 0.45$ [24].

Method

PAAm hydrogels were characterized using an MCR501 rheometer from Anton Paar with the parallel plate measuring system. A strain sweep from 0.01 to 100% strain was first conducted at a fixed frequency of 1 Hz to confirm the LVE range of PAAm hydrogels. An appropriate strain value, which is within the LVE range, was chosen for following frequency sweep. A frequency sweep from 0.1 to 3.98 Hz was then done at the chosen strain value. The storage modulus of PAAm hydrogel can be obtained from the linear equilibrium modulus plateau of the frequency sweep. Finally, Young's modulus of PAAm hydrogels was converted from the complex shear modulus, which is close to the storage modulus of fully crosslinked PAAm hydrogels.

3.3 Micro-Contact Printing on PAAm Hydrogel Substrate

Method

Micro-contact printing is a well-established soft-lithography technique for printing biomolecules (**Figure 3.3**) [25]. In this thesis, Fn micropatterns were printed on the PAAm hydrogel substrate as previously described [26]. PDMS stamps bearing circular micropatterns with a diameter of 60 and 100 μm were sterilized by acetone and ethanol washing and dried with nitrogen flow. 50 $\mu\text{g/ml}$ Fn was spread on the patterned side of PDMS stamps. After being left at room temperature for 1 h, Fn was absorbed onto the stamps and excess solution was removed. The stamps were then dried with nitrogen flow. The Fn-coated stamps were then placed in conformal contact with sulfo-SANPAH activated PAAm hydrogel. After that, PAAm hydrogels bearing the Fn patterns were removed from the stamps, washed with PBS and then blocked with 1%

BSA in PBS. The success patterning of PAAm hydrogel with Fn was confirmed through immunostaining.

Figure 3.3 Graphical illustration of protein patterning through micro-contact printing. Adopted with permission [25]. Copyright 2016 Luisa Filipponi et. al.

3.4 Cell Culture and MSCs CM Collection

Method

Primary human MSCs derived from the bone marrow were purchased from Lonza (Lonza PT-2501) and cultured with MSCGM™ Mesenchymal Stem Cell Growth Medium BulletKit™ (Lonza PT-3001). Cells between passage 4 to 6 were used. hTERT-ADMSCs from ATCC (ATCC SCRC-4000) were cultured in Mesenchymal Stem Cell Basal Medium (ATCC PCS-500-030) supplement with Mesenchymal Stem Cell Growth Kit (ATCC PCS-500-040). Cells between passage 6 to 10 were used. HDF and HaCaT keratinocytes were cultured in high glucose Dulbecco's Modified Eagles Medium (HG-DMEM, HyClone SH30243.01) with 10% fetal bovine serum (HyClone SV30160.03HI). HUVECs (Merck SCCE001) were cultured with EndoGRO-LS Complete Culture Media Kit (Merck SCME001). Cells between passage 10 to 12 were used.

CM is spent medium of MSCs culture, containing secretome produced by MSCs. Thus, MSCs CM is collected for characterization and therapeutic evaluation of MSCs secretome [27-29]. For MSCs CM collection, MSCs were seeded on Fn-PAAm hydrogel or Fn-coated coverslip at a density of 5000 cells/cm² in complete growth medium overnight. On the following day, the cells were washed with PBS and then cultured in SFM (HyClone

SH30243.01) for 48 h. Thereafter, the CM containing the MSCs secretome was retrieved. The CM was then centrifuged at 2000 g for 5 min to remove the cell debris, the supernatant was collected and filtered through a 0.22 μm syringe filter (Sartorius 16532) to remove any residual debris. For fair comparison, the harvested CM of various groups was normalized against the total dsDNA amount of every group measured with the AccuClear Ultra High Sensitivity dsDNA Quantification Kit (Biotium 31029). The regenerative potential of the CM was then evaluated in terms of dermal tissue repair and angiogenesis (**Figure 3.4**).

Figure 3.4 Collection and evaluation of MSCs CM.

3.5 Immunofluorescence Staining

Method

The samples were first washed with PBS (HyClone SH30378.03). During the sample fixation, to achieve optimal immunofluorescence staining outcome, 4% paraformaldehyde (Sigma P6148) fixation for 15 min at room temperature was conducted for vinculin, F-actin and Nrf2 staining, while prechilled methanol (Sigma 322415) fixation for 20 min at -20 °C was done for HIF1 α staining. After that, the cells were permeated with 0.2% Triton X-100 (Bio-Rad #1610407) for 10 min at room temperature. The samples were then blocked with 2% BSA (Sigma 05479) in PBS for 1 h at room temperature. Primary antibody incubation was carried out overnight at 4 °C with 100 times dilution of individual antibodies in the blocking buffer. Primary antibodies used in this thesis: monoclonal mouse anti-human vinculin antibody (Merck Millipore 90227); monoclonal rabbit anti-human Nrf2 antibody (Cell Signaling Technology 12721); monoclonal rabbit anti-human HIF1 α

antibody (Cell Signaling Technology 36169). On the following day, the cells were washed thrice with PBS to remove unbound primary antibodies and incubated with 100 times diluted respective secondary antibodies for 1 h in the dark: chicken anti-mouse Alexa Fluor 488 antibody (Thermo A-21200); goat anti-rabbit Alexa Fluor 488 antibody (Thermo A-11008); goat anti-rabbit Alexa Fluor 594 antibody (Thermo A-11012). 10^4 times diluted Hoechst 33342 (Thermo H3570) and 100 times diluted TRITC-conjugated phalloidin (Merck Millipore 90228) were mixed with corresponding secondary antibody. At the end of the incubation, the samples were washed thrice with PBS and mounted with Fluoroshield Mounting Medium (Abcam ab104139). The samples were imaged with Zeiss Observer Z1 inverted fluorescence microscope. All image processing and analysis were done using ImageJ.

3.6 Intracellular ROS Measurement and Inhibition

Principle

ROS are short-lived and highly reactive small molecules, including free radicals, hydroxyl radical and non-radical molecules [30]. Intracellular ROS level is controlled by an equilibrium between ROS generation and a wide variety of antioxidant defenses [31]. Low to modest doses of ROS is essential for the regulation of normal physiological functions, while excess intracellular ROS is deleterious or even lethal [31-33]. In this thesis, CellROX Orange was utilized for the detection of intracellular ROS. Compared with H_2DCFDA , CellROX Orange exhibits higher specificity and greater resistance to light-induced oxidation [34]. The cell permeable CellROX Orange is nonfluorescent or very weakly fluorescent in its reduced state. Upon oxidation, CellROX Orange becomes brightly fluorescent, with excitation/emission maxima at 545/565nm, and is localized in the cytoplasm.

NAC is an aminothiols and precursor of intracellular cysteine and glutathione [35]. NAC works as an antioxidant by not only increasing intracellular glutathione levels, but also through its thiol-disulfide exchange activity [35]. Special attention should be given to NAC,

since that it will oxidize and degrade when in solution and exposed to air. Therefore, to attain long duration of antioxidant effects, NAC needs to be replenished [36].

Method

The intracellular ROS of MSCs was detected with CellROX Orange (Thermo C10443). In brief, MSCs were incubated with 5 μ M CellROX Orange and 1 μ g/ml Hoechst 33342 (Thermo H3570) in SFM for 30 min at 37 °C. After that, the cells were washed thrice with PBS and replenished with fresh medium. The CellROX Orange stained live MSCs were imaged with Zeiss Observer Z1. For the ROS inhibition assay, NAC treatment protocol was optimized at a dosage of 700 μ M, with NAC solution refreshed every 12 h over the 48 h of MSCs culture.

3.7 Cell Viability Staining

Method

Calcein AM (Thermo C3099) and EthD-1 (Thermo E1169) were diluted 1000 times with SFM as the working solution. Tested MSCs were incubated with the working solution for 30 min in cell culture incubator. Image acquisition of cell viability staining was done with Zeiss Observer Z1. The number of calcein AM stained (green) cells and EthD-1 stained (red) cells were counted. Cell viability was then calculated with the following equation:

$$Cell\ Viability = \frac{N_g}{N_g + N_r} \times 100\%$$

Where N_g denotes the number of calcein AM-stained live cells, and N_r indicates the number of EthD-1-stained dead cells. Cell viability of 100% means all cells are alive. This experiment was replicated 3 times for each group.

3.8 RT-PCR

Method

Total RNA was extracted with the PureLink RNA Mini Kit (Thermo 12183025). Reverse transcription of extracted RNA was done using the iScript cDNA Synthesis Kit (Bio-Rad #1708891) by following the thermal cycle on T100 Thermal Cycler: Priming at 25 °C for 5 min, reverse transcription at 46 °C for 20 min, and reverse transcriptase inactivation at 95 °C for 1 min. KAPA SYBR FAST (Sigma KK4618) and CFX Connect Real-Time PCR Detection System were used for relative PCR. In detail, polymerase activation and DNA denaturation were first done at 95 °C for 30 s. The template DNA was then amplified for 40 cycles. In each cycle, denaturation was carried out at 95 °C for 15 s, then DNA extends at 60 °C for 30 s, and finished with signaling reading at 60 °C. After the whole amplification stage, melting curve analysis was conducted by continuous monitoring of fluorescence signaling while increasing the temperature from 65 to 95 °C with a 0.5 °C increment every 5 s. The sequence of the respective primers used in this thesis was obtained from primer bank (<https://pga.mgh.harvard.edu/primerbank/>), as listed in **Table 3.1** and synthesized by Sigma. The expression level of targeted genes was normalized to housekeeping gene β -actin following $\delta\delta C_T$ method.

Table 3.1 Primer sequence of genes tested in relative PCR.

Gene	Primer sequence [5'-3']	Amplicon size (bp)
IL-6	Forward: ACTCACCTCTTCAGAACGAATTG Reverse: CCATCTTTGGAAGGTTTCAGGTTG	149
MCP-1	Forward: CAGCCAGATGCAATCAATGCC Reverse: TGGAATCCTGAACCCACTTCT	190
RANTES	Forward: CCAGCAGTCGTCTTTGTCAC Reverse: CTCTGGGTTGGCACACACTT	54
IL-8	Forward: ACTGAGAGTGATTGAGAGTGAGAC Reverse: AACCCTCTGCACCCAGTTTTC	112
HMOX-1	Forward: AAGACTGCGTTCCTGCTCAAC Reverse: AAAGCCCTACAGCAACTGTCTG	247
UGT1A1	Forward: CATGCTGGGAAGATACTGTTGAT Reverse: GCCCGAGACTAACAAAAGACTCT	214

SOD2	Forward: GCTCCGGTTTTGGGGTATCTG Reverse: GCGTTGATGTGAGGTTCCAG	92
NQO1	Forward: GAAGAGCACTGATCGTACTGGC Reverse: GGATACTGAAAGTTCGCAGGG	196
VEGF	Forward: AGGGCAGAATCATCACGAAGT Reverse: AGGGTCTCGATTGGATGGCA	75
bFGF	Forward: AGAAGAGCGACCCCTCACATCA Reverse: CGGTTAGCACACACTCCTTTG	82
β -actin	Forward: CATGTACGTTGCTATCCAGGC Reverse: CTCCTTAATGTACGCACGAT	250

3.9 Characterization of MSCs CM

Principle

A large portion of active biomolecules in MSCs CM are proteins, such as growth factor and chemokines [37-39]. Coupling of 2D SDS-PAGE with MS, and MS testing of protein samples with proper labeling method are conventional approach to analysis multiple protein expression levels [40-42]. However, these methods are expensive, labor-intensive, and require special equipment. Furthermore, they provide a comprehensive understanding of the whole secretome, but the sensitivity is compromised. In this thesis, only targeted proteins and cytokines that contribute to tissue repair will be probed. Specifically, ELISA and protein array are selected to compare the secretome profile of MSCs under different culture conditions.

Similar to immunofluorescence staining, both ELISA and protein array are based on immune interaction between antigen and antibody (**Figure 3.9**). Antibody targeting protein of interest is immobilized onto the 96-well plate for ELISA and nitrocellulose membrane for protein array. For ELISA, there is only one kind of antibody in every well; while for protein array, different antibodies are localized at different positions of the membrane. During the sample incubation, for ELISA, only targeted protein will be trapped in the well, with all nontargeted protein removed during washing; for protein array, targeted proteins will be captured with corresponding antibodies at different positions of the membrane.

Figure 3.9 Mechanism of targeted protein detection in ELISA and protein array.

HRP is an enzyme that oxidizes various organic substrates with hydrogen peroxide as the oxidizing agent [43]. When chromogenic substrates (e.g., TMB, DAB, ABTS) are oxidized, HRP will produce colored products; when chemiluminescent substrates (e.g., ECL) are oxidized, light will be emitted. Thus, HRP is used extensively in biochemistry applications [44-46]. For the ELISA kits used in this thesis, colorimetric analysis was used, while chemiluminescence signal was measured for protein array experiments.

Method

Characterization of MSCs secretome was done with VEGF and bFGF ELISA kits (Shanghai Xitang Biotech F03060, F00760), and human cytokine array C3 (RayBiotech AAH-CYT-3). For ELISA, the plates were first incubated with MSCs conditioned medium for 1 h at room temperature, then 30 min of biotinylated antibody incubation for 30 min at room temperature, followed by streptavidin conjugated HRP incubation for another 30 min at room temperature. The plates were washed thoroughly with washing buffer between every incubation. Following which, TMB was added into the wells as the substrate of HRP. The oxidation reaction was terminated 10 min later by adding 1 M sulfuric acid. The intensity of the colorimetric products was measured at 450 nm with SpectraMax M2 from Molecular Devices. For protein array assay, the membranes were first blocked with a buffer for 30 min at room temperature. CM samples were then incubated with the membrane at 4 °C overnight. Similarly, biotinylated antibody and streptavidin conjugated HRP

incubation was also carried out overnight at 4 °C. The membranes were washed thoroughly with washing buffer between every incubation. After applying detection buffer onto the membranes for 2 min at room temperature, chemiluminescent signals were detected with the Syngene gel doc system and analyzed using the protein array analyzer for ImageJ. To compare the secreted levels of proteins between groups, chemiluminescence signals were normalized to the positive and negative controls.

3.10 Statistical Analysis

Method

Data were either presented as mean $\pm$ SD in a scatterplot with trendline fitting, as mean + SD in bar plots, or as box and whisker plots to illustrate the variance better. Statistical significance was determined by one-way ANOVA using SPSS. F-test was first done to check the homogeneity of variance, with equal variance as the null hypothesis. After this, the LSD method was used as the post hoc test for equal variance, while the Games-Howell method was used as the post hoc test for unequal variance, with no significant difference between groups as the null hypothesis. The differences are considered as statistically significant with $p < 0.05$.

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Chapter 4*

Effect of Substrate Stiffness on MSCs Secretome Production and Tissue Repair

Chapter 4 examines the effect of substrate stiffness on MSCs secretome. PAAm hydrogel with tissue mimicking Young's modulus (i.e. 1.5 and 17.7 kPa) was used as the MSCs culture substrate. The successful conjugation of Fn to PAAm hydrogel was confirmed by immunostaining, providing attachment sites for MSCs. Cytoskeleton development and secretome production of MSCs were found to be directed by substrate stiffness in serum-free condition. Furthermore, compared to the control Fn coated CS substrate, increased expression of intracellular ROS level was observed in MSCs cultured on the PAAm hydrogels. At the same time, protein arrays analysis revealed a group of "mechano-sensitive" cytokines, whose expression varies significantly as function of substrate stiffness. Importantly, CM harvested from MSCs cultured on the PAAm hydrogel was also observed to significantly enhance wound healing both in vitro as well as in an in vivo full-thickness excisional skin wound healing.

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4.1 Introduction

MSCs are multipotent cells that can differentiate into various cell types such as adipocytes, osteoblasts, and chondrocytes *in vitro* [1]. Besides their well-established differentiation potential, MSCs also possess other properties that foster tissue repair, like immune-modulatory effects, self-renewability and homing to diseased sites [2]. MSCs secretome, which contains all growth factors, cytokines, metabolites, etc. secreted by MSCs, also regulated fundamental biological processes during tissue repair [3]. Therefore, MSCs is a promising cell source for regenerative medicine, especially in the treatment of diabetes, cardiovascular diseases, neurodegeneration and so on [4]. MSCs are guided by soluble molecules and solid components of their microenvironment to carry out a plethora of fundamental cell behaviors, like proliferation, migration and differentiation [5-7]. Thus, the interaction between MSCs and matrix materials attracts the attention of biomaterials and regenerative medicine researchers. It has been found that biophysical and biochemical cues of matrix substrate, such as stiffness, nanotopography, surface functionalization, etc., impact fate decision of MSCs via mechanotransduction [8-10]. Furthermore, some proteins, like FAK, Rho, YAP, etc., have been identified as mechanosensitive signaling proteins [11-13]. Despite the critical contribution of MSCs secretome in tissue repair, effects of substrate stiffness on MSCs secretome generation remains elusive.

4.2 Experimental Methods

4.2.1 Fabrication of PAAm Hydrogel as Cell Culture Substrate and Characterization

As described in section 3.1, PAAm hydrogels of two different stiffness were prepared using two combinations of acrylamide/bis-acrylamide weight percentages. To allowing MSCs adhesion onto anti-biofouling PAAm hydrogel, Fn was conjugated into PAAm hydrogel with sulfo-SANPAH treatment. To measure the stiffness of prepared PAAm hydrogel, samples of column shape with a diameter of 10 mm and a height of 3 mm were tested on the MCR501 rheometer with a parallel plate PP10 geometry, following principle and method in section 3.2. Successful conjugation of Fn onto all cell culture substrate was first

confirmed with immunostaining. Following the principle in section 3.5 of using immunofluorescence staining for protein detection, the substrates were first blocked and then incubated with 100 times diluted anti-Fibronectin antibody (Sigma F3648) overnight at 4 °C. On the following day, the substrates were washed with PBS and incubated with 100 times diluted goat anti-rabbit Alexa Fluor 488 antibody for 1 h at room temperature. Substrates that skip the anti-Fibronectin incubation serve as the negative control. The fluorescence images were captured with Zeiss Observer Z1 to confirm the conjugation of Fn onto cell culture substrates.

4.2.2 Cell Culture and Collection of MSCs CM

In this chapter, primary MSCs derived from bone marrow, HDF and HaCaT keratinocytes were used. The detailed method was described in section 3.4.

4.2.3 Bio-characterization of MSCs on Various Cell Culture Substrates

To observe the MSCs focal adhesion and cytoskeleton development, vinculin antibody, TRITC-conjugated phalloidin was used. Cell nucleus was counter-stained with Hoechst 33342. This is illustrated in section 3.5. Intracellular ROS was detected with CellROX Orange and intracellular ROS inhibition was achieved by NAC treatment, with detailed principle and method listed in section 3.6. The viability of MSCs was tested using Calcein AM and EthD-1, following steps in section 3.7. To quantify the spreading area and intracellular ROS level of MSCs, raw fluorescence images captured with Zeiss Observer Z1 were processed with ImageJ.

4.2.4 MSCs CM Characterization

The composition of MSCs CM was analyzed with the human cytokine array C3 from RayBiotech according to the manufacturer's protocol. To examine the effect of intracellular ROS on secretome production, the expression of IL-6, MCP-1, RANTES and IL-8 by MSCs with or without NAC inhibition were measured at transcription level with the

method described in section 3.8.

4.2.5 *In Vitro* Scratch Assay

HDF and HaCaT keratinocytes were seeded into the 24-well plates at a density of 10^5 cells/cm² and grown to confluence. After that, the cells were serum starved for 24 h in SFM and a scratch was made in each well to the monolayer of cells using a p200 pipet tip. Three rounds of PBS washing were done to remove floating cells. MSCs CM was applied and the phase contrast images of the samples were obtained with the Olympus DP21 microscope at predetermined time points (6 h, 12 h, 24 h). The area of the scratch was quantified with ImageJ. Wound closure index was calculated as follows:

$$\text{Wound Closure Index} = \frac{A_0 - A_t}{A_0} \times 100\%$$

Where A_0 denotes the original scratch area at time point 0 right after scratch making and A_t represents the scratch area at predetermined time point t . A wound closure index of 100% indicates full closure of the scratch area. This experiment was done in triplicate.

4.2.6 *In Vivo* Wound Healing Study

Eight-week-old male C57BL/6J wild-type mice with a body weight between 18-22 grams were used in *in vivo* wound healing study. Two circular 5 mm full-thickness excisional wounds were first created on the dorsomedial back of each mice. Silicon donut-shaped splints with a diameter of 10 mm were then centered over the wounds and adhered to the skin with cyanoacrylate glue. The surgery day was designed as day 0. Various samples (MSCs CM or PBS) were mixed separately with 4% carboxymethyl cellulose and topically applied onto respectively wound on day 0. Treated wounds were protected with an occlusive dressing over the whole study. At predetermined time points (day 1, day 3, day 5), the mice were euthanized to collect the wound samples, which was measured and fixed with 4% PFA at 4 °C overnight. For paraffin embedding, the samples were dehydrated with

a series of increasing graded ethanol followed by xylene. Dehydrated samples were soaked in molten paraffin wax and tissue embedding was done with Leica EG1160. Paraffin sections of 5 μm thickness were used in Haematoxylin and Eosin staining. Images of the stained samples were captured with Zeiss Axio Scan.Z1. The animal studies were approved by the Institutional Animal Care and Use Committee of Nanyang Technological University (ARF-SBS/NIE-A0174AZ). The wound closure index was calculated with this equation:

$$\text{Wound Closure Index} = \frac{A_1 - A_t}{A_1} \times 100\%$$

Where A_1 denotes the original wound area on day 1 and A_t represents the wound area at predetermined day t . A wound closure index of 100% indicates full closure of mouse skin wounds.

4.3 Principle Outcomes

4.3.1 PAAm Hydrogel as Cell Culture Substrate

4.3.1.1 Fabrication of Cell Culture Substrates with Various Stiffness

Cytocompatible PAAm hydrogels are ideal substrates to study the mechanobiology of adherent cells [14-16]. In this thesis, the acrylamide/bis-acrylamide weight percentages of 4%/0.15% and 10%/0.1% were used to prepare PAAm hydrogel of two different stiffness [17]. To measure the storage modulus G' , a strain sweep was first conducted with a fixed frequency of 1 Hz (**Figure 4.3.1.1a**). After the trendline fitting of measured points, 4%/0.15% PAAm hydrogel displayed linear behavior over the whole tested strain range. However, decrease in storage modulus G' was observed for the 10%/0.1% PAAm hydrogel when the given strain is larger than 10%. Thus, a 1% strain was chosen for the following frequency sweep to examine the behavior of G' in the LVE range (**Figure 4.3.1.1b**). The Young's modulus, E , can then be calculated with the measured G' using the following equation: $E = 2G'(1 + \nu)$, where ν equals 0.45 as the Poisson ratio of PAAm [18]. In this thesis, fresh

PAAm hydrogels were prepared for every study. Because the radical initiator ammonium persulfate is instable, 4%/0.15% and 10%/0.1% PAAm hydrogel used in chapter 4 has an E of 1.5 kPa and 17.7 kPa respectively, while 4%/0.15% and 10%/0.1% PAAm hydrogel adopted in chapter 5 and 6 has an E of 0.15 kPa and 10 kPa respectively. Even though there is a difference in E for PAAm hydrogel of the same formulation, 4%/0.15% PAAm hydrogel always mimics the stiffness of brain tissue and 10%/0.1% PAAm hydrogel consistently mimics the stiffness of heart tissue [19]. Meanwhile, the E of CS that serves as a baseline comparison fits in GPa range. Sulfo-SANPAH chemistry was employed in this thesis to covalently conjugate 50 $\mu\text{g/ml}$ Fn onto both 0.15-1.5 kPa and 10-17.7 kPa PAAm hydrogel, with 50 $\mu\text{g/ml}$ Fn coated Cs serves as a control substrate. Successful coating of Fn onto the substrates was validated using immunostaining (**Figure 4.3.1.1c**).

Figure 4.3.1.1 Fabrication of cell culture substrates with various stiffness. (a) Strain sweep and (b) frequency sweep of PAAm hydrogel substrates prepared with acrylamide/bis-acrylamide weight percentages of 4%/0.15% and 10%/0.1%. Data presented as mean $\pm$ SD with linear trendline fitting ($n = 5$ per group). (c) Immunostaining detecting Fn confirms the conjugation of Fn onto various substrates (Scale bar = 100 μm). Samples without the addition of primary antibodies serves as negative controls.

4.3.1.2 Influence of Substrate Stiffness on Cell Spreading and Cytoskeleton Development of MSCs in Serum Deplete Condition

Multiple studies have established the effects of substrate stiffness on cell morphology [20]. However, most of these studies were conducted in a serum-containing medium. In order to examine the effect of substrate stiffness on MSCs secretome production, the cells would need to be cultured under serum free condition, otherwise growth factors present in the FBS may interfere with secretome analysis. Therefore, the first critical question asked is: can MSCs mechano-sense the substrate stiffness in a serum-deplete culture condition? After the cells were grown on the various substrates in SFM for 48 h, the samples were fixed and immunostained to evaluate the effect of substrate stiffness on cellular morphology as well as focal adhesion and cytoskeleton development (**Figure 4.3.1.2a**). MSCs on the GPa substrate were observed to be well spread ($\sim 6500 \mu\text{m}^2$) and developed matured focal adhesion plaques as well as thick F-actin stress fibers (**Figure 4.3.1.2**). Conversely, MSCs on the 1.5 kPa substrate displayed a predominantly rounded morphology ($\sim 2000 \mu\text{m}^2$), diffused vinculin staining in the cytoplasm and presence of stress fibers was absence. Meanwhile, MSCs on the 17.7 kPa substrate exhibited an intermediate cellular spreading area ($3500 \mu\text{m}^2$). The substrate stiffness-dependent changes to the cell spreading and cytoskeleton development corroborate the trend that are reported in earlier studies [21-23]. Therefore, it can be concluded that MSCs are still responsive to the effect of substrate stiffness even in serum-free condition.

Figure 4.3.1.2 Substrate stiffness exerts a dramatic effect on MSCs cytoskeleton development and spreading even in serum free condition. (a) Immunostaining of MSCs cultured on various substrates, with nucleus in blue, vinculin in green and F-actin in red (Scale bar = 50 μm). (b) The spreading area of MSCs is dependent on substrate stiffness, presented as mean + SD, $n > 30$. * denotes statistical difference at $p < 0.05$.

4.3.1.3 Influence of Substrate Stiffness on the Production of MSCs CM

Next, the effect of substrate stiffness on the CM production of MSCs was evaluated using protein array [14, 24-26]. Using human cytokine array C3 from RayBiotech, the secretion of 42 proteins was semi-quantified, and 15 of them were shown to be sensitive to substrate stiffness (expression level > 2 -fold change between the three groups) (**Figure 4.3.1.3a,c**).

Refer to the positive control and negative control, the secretion of these 15 proteins were compared across the three experimental groups (**Figure 4.3.1.3b**). 9 of the 15 (IL-1 α , IL-3, IL-10, M-CSF, MIP-1 δ , RANTES, SDF-1, TNF α , OSM) displayed a positive correlation with substrate stiffness, and 5 of the 15 (IL-8, MCP-1, TARC, TNF β , VEGF) exhibited a bi-modal-stiffness expression profile, with IL-6 as the only cytokine whose secretion had an inverse relationship with substrate stiffness.

Figure 4.3.1.3 Substrate stiffness guides the production of MSCs CM. (a) Protein array

detects 15 “mechanosensitive” proteins secreted by MSCs. (b) Secretion levels of these 15 “mechanosensitive” proteins by MSCs under the guidance of substrate stiffness, presented as heatmap. (c) Array map identifies all the 42 proteins targeted and all the 15 “mechanosensitive” proteins detected.

4.3.2 Role of Intracellular ROS in Mechano-conditioning of MSCs

4.3.2.1 Influence of Substrate Stiffness on Intracellular ROS Level in MSCs

The intracellular ROS level of MSCs cultured on various substrates was assessed by staining the cells with CellROX Orange dye (**Figure 4.3.2.1a-c**). Bright orange fluorescence was emitted after the oxidation of CellROX Orange by intracellular ROS. Interestingly, the intracellular ROS level of MSCs increased with decreasing substrate stiffness (**Figure 4.3.2.1d**). Compared with MSCs in the GPa group, around a 3-fold intracellular ROS level was expressed by MSCs in the 1.5 kPa group. Besides the involvement of ROS in the regulation of mechanotransduction proteins, a potential outcome of ROS overexpression is cell death [30-33]. However, even for MSCs on the 1.5 kPa substrate whose intracellular ROS level is the highest, the viability was still maintained at a high level (> 90%) (**Figure 4.3.2.1e**). Overall, substrate stiffness has a direct effect on the intracellular ROS level of primary MSCs without compromising cell viability.

Figure 4.3.2.1 PAAm hydrogel with kPa stiffness induces oxidative stress of MSCs without compromising cell viability. CellROX Orange staining of MSCs cultured on (a) 1.5 kPa, (b) 17.7 kPa, and (c) GPa substrate. Scale bar = 100 μ m. (d) Quantification of intracellular ROS level in MSCs cultured on various substrates, presented as mean + SD, $n > 30$. * denotes statistical difference at $p < 0.05$. (e) Quantification of MSCs viability after being cultured on various substrates in serum free condition for 48 h, presented as mean + SD, $n > 300$. No statistical difference was observed between all groups.

4.3.2.2 Effect of Manipulating Intracellular ROS on MSCs Secretome

Due to ROS signaling plays complex physiological roles in the determination of MSCs phenotype, the elevated intracellular ROS level of MSCs on the 1.5 kPa substrate might affect the production of MSCs secretome [31-33]. To examine this possibility, attention was focused on 4 of the 15 proteins whose expression levels were shown to change the most significant as a function of substrate stiffness, namely IL-6, MCP-1, RANTES and IL-8. In detail, IL-6 secretion correlated inversely with substrate stiffness, peaking at the 1.5 kPa substrate group. On the other hand, MCP-1 and RANTES overexpressed with increased substrate stiffness and their expression levels are the highest for MSCs of the GPa group. Meanwhile, IL-8 production exhibited a non-linear dependency on substrate

stiffness (**Figure 4.3.2.1**). If intracellular ROS level is an upstream factor of these “mechanosensitive” proteins, then treating MSCs on the 1.5 kPa substrate with NAC, a potent ROS scavenger, should force the MSCs to produce these proteins like MSCs on the GPa substrate. This assumption is based on the observation that compared with MSCs on the GPa substrate, MSCs on the 1.5 kPa substrate and 17.7 kPa substrate expressed elevated intracellular ROS (**Figure 4.3.1.3b**). Continuous exposure of MSCs on the 1.5 kPa substrate to 700 μ M NAC suppressed the transcription level of IL-6 by 11.6-fold compared with the untreated group. At the same time, expression level of MCP-1 and RANTES transcripts were upregulated by 13-fold and 28-fold respectively after NAC treatment. Because of the complex regulation between intracellular ROS level and IL-8 secretion, NAC treatment did not result in a significant difference to the transcription of IL-8 (**Figure 4.3.2.2a**). Taken together, our results showed that substrate stiffness can function as potent regulator of MSCs secretome production in part via a ROS-dependent manner (**Figure 4.3.2.2b**).

Figure 4.3.2.2 Substrate stiffness directs secretome production of MSCs through ROS signaling. (a) Effects of NAC treatment expression levels of IL-6, MCP-1, RANTES and IL-8 mRNA transcripts in MSCs, presented as mean + SD, $n = 4$. * denotes statistical difference at $p < 0.05$. (b) A schematic diagram depicting that substrate stiffness modulate MSCs secretome in a ROS dependent manner.

4.3.3 Skin Wound Healing Potential of CM Harvested from MSCs Cultured on Substrates of Different Stiffness

4.3.3.1 Effect of MSCs CM on Migration of HDF and Keratinocytes *in vitro*

MSCs secretome contains numerous growth factors, chemokines, and cytokines, including but not limited to VEGF, bFGF, SDF-1, and IL6, which are important for tissue repair [3, 34]. Therefore, the therapeutic implications of the mechano-conditioned MSCs CM were tested for skin tissue repair, with the scratch assay as an *in vitro* model [35]. This assay was conducted with HDF and HaCaT keratinocytes, two representative cell types of skin (**Figure 4.3.3.1**) [36]. Interestingly, experiment groups treated with CM from MSCs cultured on the 1.5 kPa and 17.7 kPa substrate showed significant promotion of wound closure for both cell types. On the other hand, MSCs on the GPa substrate did not produce secretory proteins that help cell migration. After 24 h of CM incubation, the HDF wound closure index of the CM_{1.5 kPa} and CM_{17.7 kPa} groups improved significantly by around 2.6-fold and 2.1-fold over the CM_{GPa} group and the SFM control group (**Figure 4.3.3.1a**). For HaCaT keratinocytes, a marked improvement in wound repair was also observed for the CM_{1.5 kPa} and CM_{17.7 kPa} groups compared with the CM_{GPa} group by approximately 1.4-fold and 1.3-fold respectively (**Figure 4.3.3.1b**). These results indicate that mechano-conditioning MSCs with the Fn-PAAm hydrogel of kPa stiffness can confer a therapeutic advantage for skin wound healing.

Figure 4.3.3.1 Mechano-conditioned MSCs CM promotes migration of HDF and keratinocytes. Quantification of wound closure after treating (a) HDF and (b)

keratinocytes with MSCs CM, presented as mean + SD, $n = 4$. * denotes statistical difference at $p < 0.05$.

4.3.3.2 Effect of MSCs CM on Mouse Skin Wound Healing

Encouraged by the promising *in vitro* result (**Figure 4.3.3.1**), the therapeutic efficacy of the mechano-conditioned MSCs CM was further tested using a full-thickness excisional splinted wound healing mouse model [37]. The MSCs CM and PBS was separately mixed with 4% carboxyl methylcellulose in PBS and applied topically to the wound region (**Figure 4.3.3.2a**). Wound biopsy was collected at day 1, 3, and 5 postinjury, with the wound area captured and quantified (**Figure 4.3.3.2b**). Compared with mice in the PBS group, *in vivo* wound healing of mice in the CM_{GPa} group was promoted significantly (71% versus 60% wound closure at day 5). Strikingly, mechano-conditioning of MSCs further improved the pro-wound healing effect of MSCs CM. The wound closure index of mice in the CM_{1.5 kPa} and CM_{17.7 kPa} group was 76% and 79% respectively at day 5, significantly higher than that of mice in the CM_{GPa} and PBS group. Histological examination of the wound section with hematoxylin and eosin staining also revealed that the re-epithelialization process was accelerated for mice of the CM_{1.5 kPa} and CM_{17.7 kPa} group compared with those of the CM_{GPa} and PBS group (**Figure 4.3.3.2c**). Collectively, all these results suggest that preconditioning MSCs with the Fn-PAAm hydrogel with E in the range of kPa can be exploited to enhance the therapeutic effect of MSCs secretome for skin wound healing.

Figure 4.3.3.2 Enhancement of *in vivo* mouse skin wound healing by CM derived from MSCs cultured on PAAm hydrogel of kPa stiffness. (a) Two full-thickness wounds were made on each side of the mouse back to evaluate the therapeutic effect of MSCs CM. (b) Quantification of mouse *in vivo* skin wound healing after MSCs CM treatment by normalizing to wound area on day 1. Data is presented as mean + SD, n = 7. * denotes statistical difference at p < 0.05. (c) Hematoxylin and eosin staining of the skin wound samples, with red arrows indicating the position of epithelial tongues to show re-epithelialization. Scale bar = 1 mm.

4.4 Conclusions

Using Fn coated PAAm hydrogel with biomimicking stiffness, it is showed that MSCs can be “mechano-conditioned” to alter its secretome production. Among the 42 cytokines screened, 15 (~35% hit rate) of them were shown to be sensitive to culture substrate mechanics. MSCs cultured on the PAAm hydrogel mechano-sense the substrate stiffness by exhibiting a marked reduction in cell spreading and the cytoskeleton system is less

developed compared to cells cultured on the CS substrate. Interestingly, these morphological changes are accompanied with an increased expression of intracellular ROS with decreasing substrate stiffness. Despite the enhancement in endogenous ROS production, the cells remained viable (>90%). Furthermore, the preliminary mechanistic studies revealed that the mRNA transcripts corresponding to these mechano-sensitive cytokines could be modulated using the well-established antioxidant, NAC, suggesting that changes to ROS level is an upstream event, regulating MSCs secretome production. Most importantly, application of CM (containing the secretome) harvested from MSCs grown on the PAAm hydrogel of kPa stiffness significantly accelerated wound closure both *in vitro* and *in vivo*. The term “mechano-conditioning” was coined to refer to the use of substrate stiffness to precondition MSCs secretome production. As far as anyone can tell, this study is the first to demonstrate a link between mechanobiology and modulation of oxidative stress level in MSCs as well as to demonstrate that the mechano-conditioned MSCs secretome can be exploited to foster functional tissue repair.

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Chapter 5*

Extending Mechano-preconditioning to Immortalized MSCs and Its Effect on Angiogenesis

Chapter 5 intends to exploit hTERT-ADMSCs as a factory to produce CM for proangiogenic applications. hTERT-ADMSCs were found to retain multipotency even at later passages (P10), allowing a long-term CM harvesting. Meanwhile, the secretions of two established proangiogenic factors, VEGF and bFGF, by hTERT-ADMSCs were induced by the 0.15 kPa PAAm hydrogel with intracellular ROS as a mediator. Interestingly, the overexpression of VEGF and bFGF triggered by 0.15 kPa PAAm hydrogel is even higher than traditional 3D spheroid culture preconditioning. By collecting CM of hTERT-ADMSCs, it was found that after mechano-conditioning of 0.15 kPa PAAm hydrogel, CM enhanced a list of key events in angiogenesis, including endothelial cells proliferation, migration and tube formation. In the end, mechano-conditioned CM also promoted angiogenesis of chick embryo in ex ovo CAM assay.

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5.1 Introduction

Preconditioning MSCs enhances their anti-apoptotic, pro-angiogenesis, and regenerative trophic capabilities [1]. However, the clinical application of primary MSCs faces a number of problems, including but not limited to donor variance, epigenetic reprogramming and senescence, which represents an intrinsic limitation on production capacity [2]. Cell immortalization is one approach to solve this issue [3]. Ashiba et. al. immortalized MSCs by introducing human papillomavirus E6 and E7 genes and hTERT gene [4]. Using gene arrays, they concluded that immortalization procedure did not disturb the molecular characteristics of primary MSCs, thus immortalized MSCs were expected to generate a large quantity of MSCs CM while maintaining the MSCs features. Park and coworkers established immortalized MSCs by transfection of the SV40 large T antigen [5]. After applying CM of this immortalized MSCs to various types of skin cells, it was found that PKB or ERK signaling cascades in fibroblasts, keratinocytes, and vascular epithelial cells was activated, ultimately accelerating wound contraction in mouse full-thickness excisional model. Overall, immortalized MSCs could be a factory for large scale production of MSCs CM, which can be used in regenerative medicine.

Angiogenesis is the process where new blood vessels are formed from a preexisting vascular bed through the tightly regulated process of endothelial cells growth, migration, and tubulogenesis [6]. Angiogenesis plays a critical role for tissue growth and repair, as new vessels deliver oxygen, nutrients, and functional cells to site of injury [7]. Endothelial cells in the vasculature also release survival factors to promote cell proliferation and impede cell death in regenerating tissues [8]. Furthermore, angiogenesis is a powerful solution for several fatal tissue damages, like ischemic brain injury, heart infarction and acute renal failure [9-11]. Thus, MSCs CM has been utilized to enhance angiogenesis in tissue repair. Boomsma et. al. detected abundant VEGF, MCP-1, MIP-1 α , all of which are proangiogenic factors, in MSCs CM and application of this MSCs CM to canine vascular endothelial cells induced development of complex capillary networks [12]. The secretion of proangiogenic microRNAs from MSCs into CM promoted formation of tube-like structure by HUVECs *in vitro*, increased mobilization of endothelial cells and blood flow

into Matrigel plug subcutaneously transplanted into mice [13]. Proper preconditioning strategies will further improve the proangiogenic efficacy of MSCs CM. Thus, this chapter seeks to extend the concept of mechano-conditioning to hTERT-ADMSCs, with therapeutic angiogenesis as targeted application. Meanwhile, this is the first time to conduct mechano-conditioning study with immortalized MSCs.

5.2 Experimental Methods

5.2.1 *In Vitro* Adipogenesis and Osteogenesis of MSCs

DMEM (Gibco, 11995-065) with 10% fetal bovine serum (GE Hyclone, SV30160.03HI) and 1% Penicillin-Streptomycin (GE Hyclone, SV30010) was used as the basal culture medium in the preparation of the adipo and osteo differentiation medium. For adipogenic differentiation medium, the following chemicals were used: 0.5 mM 3-Isobutyl-1-methylxanthine (Sigma, I5879), 10 μ M insulin (Sigma, I3536), 1 μ M dexamethasone (Sigma, D4902) and 200 μ M indomethacin (Sigma, I7378). For osteogenic differentiation medium the following chemicals were used: 200 μ M L-ascorbic acid (Sigma, A4403), 10 mM β -glycerophosphate disodium salt hydrate (Sigma, G9422) and 100 nM dexamethasone (Sigma, D4902). Primary MSCs (P6) and hTERT-ADMSCs (P6, P10) were seeded into 12-well plates with a density of 5k/cm². Cells were cultured in growth medium until 70%-80% confluence. Thereafter, the medium was switched to the respective differentiation medium and changed twice every week. Duration of the adipogenic differentiation lasted for two weeks and osteogenic differentiation lasted for three weeks. At pre-determined post differentiation time points, cells were washed with PBS thrice and fixed with 4% paraformaldehyde for 1 h at room temperature. After three rounds of washing with DI water, 0.3% oil red (Sigma, O0625) in mixed solution of isopropyl alcohol and DI water (3:2) was used to stain for lipid droplets, while 0.1% alizarin red (Sigma, A5533) in 0.1 M Tris-HCl buffer (pH4.2, Sigma, RES3098T) was used to stain for calcium. The staining lasted for 1 h at room temperature, followed by another three rounds of DI water washing to remove excess dye.

5.2.2 Cell Culture Substrate Fabrication, Cell Culture and CM Collection

Cell culture substrate fabrication was mentioned in section 3.1. In this chapter, hTERT-ADMSCs, HDF, HaCaT keratinocytes, and HUVECs were used. Their culture method and MSCs CM collection procedure were described in section 3.4.

5.2.3 Formation of MSCs Spheroids for 3D Culture

The hTERT-ADMSCs spheroids were formed with the help of MicroTissues 3D Petri Dish micro-mold (Sigma, Z764116) by following the manufacturer's recommended protocol. Preheated 1% agarose solution (Sigma, A2790) in DI water was first poured into the micro-mold. After cooling, the complementary agarose mold bearing the micro-wells was formed. hTERT-ADMSCs were then seeded at a density of 1.5×10^6 per agarose mold. Since agarose is anti-biofouling, the cells were coerced to aggregate and form 3D spheroids [14].

5.2.4 *In Vitro* Scratch Assay

In vitro scratch assay procedure and quantification were described in section 4.2.5.

5.2.5 Bio-characterization of hTERT-ADMSCs on Various Cell Culture Substrates

The hTERT-ADMSCs on various cell culture substrates were characterized in aspects of cytoskeleton development, intracellular ROS expression and inhibition assay, and cell viability as mentioned in section 4.2.3.

5.2.6 Quantification of Proangiogenic Factors Secretion

To study the secretion of proangiogenic factors by hTERT-ADMSCs, both transcription and protein level experiments were conducted as described in section 3.8 and section 3.9. The expressions of two established proangiogenic factors, VEGF and bFGF, were used as indicators for proangiogenic potential of MSCs CM.

5.2.7 *In Vitro* HUVECs Proliferation

CM collected from hTERT-ADMSCs cultured on various substrates was mixed separately with EndoGRO-LS Complete Culture Medium at an optimized mixing ratio of 30%:70% to ensure viability of the endothelial cell since HUVECs cannot be maintained in SFM. HUVECs suspended in EndoGRO-LS Complete Culture Medium was first seeded into 96-well plate at a density of 5000 cells/cm². The next day, the HUVEC complete growth medium was replaced with the mixed CM samples. HUVECs proliferation was measured 48 h post culture using PrestoBlue (Thermo A13262) in accordance with the manufacture's protocol. Briefly, HUVECs were incubated with PrestoBlue for 30 min at 37°C and the supernatant was transferred into a black polystyrene 96-well plate (Corning CLS3925). Fluorescence intensity of the reduced product by the metabolically active cells was measured with 560 nm excitation and 590 nm emission. Experiments were repeated 4 times.

5.2.8 *In Vitro* HUVECs Migration

The effect of immortalized MSCs secretome on the migration of HUVECs was tested using the μ -Slide Chemotaxis (ibidi 80306) in accordance with the protocol provided by the manufacturer. In this study, a seeding density of 1.8×10^4 HUVECs per chip was used. To create a chemotactic gradient, 18 μ L of the CM was separately applied into the upper chamber, with the low chamber filled with SFM. Following which, HUVEC migration was monitored for 6 h with the JuLI Stage Real-Time Cell History Recorder (NanoEntek), with 30 mins as recording interval. The migration path of 15 HUVECs from each group was tracked manually with the Manual Tracking plugin of ImageJ. In detail, 13 images taken over the 6 h monitoring for each group were first stacked together according to the sequence. After this, the migration of single cell were traced by manually clicking its position at different time points, forming its migration path. Centre of mass (CoM) represents the spatial averaged position of all 15 HUVECs endpoints for each group. It was calculated with the online Chemotaxis and Migration Tool from ibidi using the following equation:

$$\text{CoM} = \frac{1}{n} \sum_{i=1}^n (X_{i,end}, Y_{i,end})$$

Based on CoM, the chemotactic index was further derived from the Y-axis value of CoM and used to indicate the tendency for HUVECs to migrate towards the samples.

5.2.9 *In Vitro* HUVECs Tube Formation

In vitro HUVECs tube formation assay was performed using the μ -Plate Angiogenesis 96 Well (ibidi 89646). The microwells were first coated with 10 μ L of Matrigel (Corning 356237) and allowed to gel for 30 min in the cell culture incubator. Thereafter, 2×10^4 HUVECs suspended in a mixture of 15 μ L CM and 35 μ L EndoGRO-LS Complete Culture Medium were seeded into each well. HUVECs were incubation with the samples for 3 h to allow the formation of the capillary-like tube. The tube structure was imaged with a Zeiss Primo Vert Inverted Microscope and analyzed with the Angiogenesis Analyzer plugin of ImageJ. The extent of tube formation was then quantified with the number of nodes and the total tube length.

5.2.10 *Ex Ovo* CAM Assay

Fresh embryonic day 0 specific-pathogen-free chicken eggs were collected from the Agri-Food & Veterinary Authority of Singapore and kept at 37 °C under 60% humidity to allow embryonic development. 48 h later, the eggs of day 2 were cracked and every chicken embryo was transferred to an artificial culture vessel composed of a plastic cup lined with a support plastic wrap. 100 μ L of Penicillin-Streptomycin (Gibco 15140-122) was applied to each embryo every 3 days since E2 to prevent bacteria contamination. From day 5 onwards, 100 μ L of 20 mg/ml CaCl_2 (Sigma C5670) in DI water was fed to each embryo every 3 days to help the embryo development. By day 11, clear blood vessels network has been formed for every embryo and the embryos were transferred to plastic petri dishes. 10 μ L of the CM sample was locally applied to the developing embryo with the help of pre-

cut CS. With the pre-cut CS in the center, a 10 mm * 15 mm area was set as area of interest. After 3 days of treatment at 37 °C, blood vessels in this area of interest were imaged with a Plugable Digital Viewer. To maximize the contrast between the blood vessels and the background, the blood vessels were traced manually with painting tool of Photoshop. The exported blood vessels tracing images were analyzed with the Angiogenesis Analyzer plugin of ImageJ, which typically used to detect and analyze the pseudo vascular organization of endothelial cells cultured in gel medium. Total tube length represents the total length of traced blood vessels in the images, and number of nodes represents the number of jointing points formed by two traced blood vessels in the images.

5.3 Principle Outcomes

5.3.1 Evaluation of hTERT-ADMSCs as Potential Cell Source for “Cell-Free” Therapy

5.3.1.1 Comparative Study of Differentiation Potential of hTERT-ADMSCs and primary MSCs

The differentiation of MSCs is a two stages process, namely (i) lineage commitment and (ii) maturation when progenitor cells become specific cell types [15]. During adipocyte maturation, the expression of intracellular lipid binding proteins that promote fatty acid uptake and lipid-droplet accumulation is activated [16]. For differentiated osteoblasts, vast extracellular calcium will be deposited because of osteocalcin upregulation [17]. To evaluate the adipogenic and osteogenic differentiation events, oil red O that stains lipid and alizarin red S that form complex with calcium were employed [18]. As shown in **Figure 5.3.1.1a**, both primary MSCs and hTERT-ADMSCs at P6 can differentiate into adipocytes and osteoblasts as indicated from the positive staining of oil droplets or calcium deposits respectively. Conversely, lipid and calcium staining were evidently absent for cells grown in basal culture medium. On the other hand, the detrimental effect of senescence stopped the proliferation of primary MSCs after P8. Thus, confluent monolayer of primary MSCs was not formed at P10 to conduct *in vitro* adipogenesis and osteogenesis assays. Oil red O

staining and alizarin red S staining was not applicable to primary MSCs at P10 (**Figure 5.3.1.1b**). Differentiation cannot be conducted with primary MSCs because of limited proliferation caused by senescence. Nonetheless, the differentiation potential and fibroblast-like morphology were preserved for hTERT-ADMSCs. Thus, only hTERT-ADMSCs retain multipotency after long term culture.

Figure 5.3.1.1 hTERT-ADMSCs maintained multipotency over long term culture. Differentiation of MSCs at (a) P6 and (b) P10. N.A.: not available. Scale bar = 100 μ m.

5.3.1.2 CM Derived from hTERT-ADMSCs Promoted Wound Closure *in vitro*

As a simple and quick way to evaluate the potential therapeutic value of hTERT-ADMSCs CM, *in vitro* wound scratch assay was employed again as in the previous chapter. Briefly, CM was collected from 90% confluency hTERT-ADMSCs after 2 days of culture. Thereafter, the CM was added to the scratched HDF and HaCaT keratinocytes samples. Compared to the SFM control, samples treated with hTERT-ADMSCs CM displayed faster migration. Specifically, hTERT-ADMSCs CM was found to increase wound closure of

HDF by 35% (64% versus 29%) (**Figure 5.3.1.2b**). Similarly, under the stimulation of hTERT-ADMSCs CM, 42% of wound closure was observed, while SFM control only induced a 16% of wound closure (**Figure 5.3.1.2d**). Taken together, these results showed that hTERT-ADMSCs CM is bioactive.

Figure 5.3.1.2 hTERT-ADMSCs secrete trophic factors that benefit tissue repair. Time dependent migration of (a) HDF and (c) keratinocytes. Scale bar = 100 μ m. Quantification of wound closure after treating (b) HDF and (d) keratinocytes with CM of hTERT-ADMSCs, presented as box and whisker plots, n = 4. * denotes statistical difference at p < 0.05.

5.3.2 Mechanosensitivity of hTERT-ADMSCs

5.3.2.1 Influence of Substrate Stiffness on Cell Spreading and Cytoskeleton Development of hTERT-ADMSCs in Serum Deplete Culture Condition

Whether hTERT-ADMSCs can respond to the cell culture substrate mechanics was tested. hTERT-ADMSCs cultured on the extremely stiff CS was observed to develop prominent thick F-actin fibers and elongated vinculin-containing mature focal adhesion (**Figure 5.3.2.1a**). In contrast, only shorter F-actin fiber can be seen for hTERT-ADMSCs on PAAM hydrogel of 0.15 kPa and 10 kPa, indicating limited polymerization of G-actin. At the same time, elongated focal adhesion micro-plaques were absent. Furthermore, numerous thin filopodia-like structures were formed for hTERT-ADMSCs on the 0.15 kPa substrate, suggesting that the cells were actively probing the substrate and searching for an anchorage point. Because of the difference in cell anchoring, the spreading area of hTERT-ADMSCs on the 10 kPa and 0.15 kPa substrate was significantly reduced when compared with those on GPa substrate (**Figure 5.3.2.1b**). The mean spreading area of hTERT-ADMSCs was $425 \mu\text{m}^2$ and $2393 \mu\text{m}^2$ for the 0.15 kPa and 10 kPa group respectively. However, the spreading area of the cells of the GPa group was $3719 \mu\text{m}^2$, which is significantly bigger than the 0.15 kPa group by approximately eight-fold. These results suggest that hTERT-ADMSCs is mechanosensitive and hTERT-immortalization did not hinder the ADMSCs to mechanosensing their microenvironment.

Figure 5.3.2.1 Cytoskeleton development and spreading of hTERT-ADMSCs on the various substrates. (a) Representative immunofluorescence staining images of hTERT-ADMSCs cultured on the various substrates, with blue staining nucleus, green staining vinculin and red staining F-actin. The white arrows point to filopodia. Scale bar = 50 μm . (b) Box and whisker plots of cell spreading area on the various substrates, $n > 30$. * denotes statistical difference at $p < 0.05$.

5.3.2.2 Influence of Substrate Stiffness on Intracellular ROS Level in hTERT-ADMSCs

In the previous chapter, it was found changes to intracellular ROS level is an important molecular event, that is intimately tight and upstream of the modulation of primary MSCs secretome production. To examine the intracellular ROS level of hTERT-ADMSCs as a

function of substrate stiffness, CellRox staining was employed (**Figure 5.3.2.2a**). **Figure 5.3.2.2b** shows that the measured intracellular ROS levels of hTERT-ADMSCs cultured on the PAAm hydrogel of 0.15 kPa and 10 kPa were around 3.5 and 1.2 times higher compared with that of the cells on the CS of GPa stiffness. Additionally, increased expression of intracellular ROS also did not impair hTERT-ADMSCs viability (**Figure 5.3.2.2c**). These results showed that both primary MSCs and hTERT-ADMSCs displayed similar substrate stiffness-dependent behaviors on the PAAm hydrogel.

Figure 5.3.2.2 PAAm hydrogel of kPa stiffness stimulates intracellular ROS generation of hTERT-ADMSCs without causing cell death. (a) CellROX Orange staining and (b) Box and whisker plots of intracellular ROS level in hTERT-ADMSCs cultured on various substrates. Scale bar = 100 μ m. $n > 30$. * denotes statistical difference at $p < 0.05$. (c) Box and whisker plots of hTERT-ADMSCs after being cultured on various substrates in serum free condition for 48 h. $n > 300$. No statistical difference was observed between all groups.

5.3.3 Mechano-Redox Regulation of Angiogenic Factors Production in hTERT-ADMSCs

5.3.3.1 Influence of Substrate Stiffness on VEGF and bFGF Secretion in hTERT-ADMSCs

The effect of substrate stiffness on the expression of VEGF and bFGF mRNA transcripts, two of the well-established proangiogenic factors was first investigated [19]. Compared to the GPa group, a significant upregulation of both VEGF (> fourfold) and bFGF (> sixfold) mRNA in the 0.15 kPa group was observed (**Figure 5.3.3.1a**). In contrast, only a moderate change in VEGF and bFGF transcripts was observed for the 10 kPa group. Interestingly, when compared with the 3D spheroids culture, a known preconditioning technique to promote angiogenic growth factors production of MSCs, expression level of the VEGF and bFGF mRNA transcripts is significantly higher in the 0.15 kPa group, suggesting that this novel mechano-conditioned approach may be superior to 3D spheroid culture. Consistent with the RT-PCR results, among the different CM examined, secretion of VEGF and bFGF were observed to be the highest in the CM harvested from hTERT-ADMSCs grown on the 0.15 kPa PAAM substrate, which is approximately 3 times more than that of the CS group (**Figure 5.3.3.1b**). To gain further mechanistic insights, hTERT-ADMSCs were treated with NAC (700 μ M) to examine the link between stiffness-regulated level of intracellular ROS and VEGF and bFGF production. Specifically, NAC treatment was observed to suppress both VEGF and bFGF secretion in hTERT-ADMSCs grown on the 0.15 kPa PAAM substrate (**Figure 5.3.3.1c**). Consistent with the observations made in primary MSCs (Chapter 4.3.2.2), these results revealed that substrate stiffness can regulate endogenous production of ROS in hTERT-ADMSCs, which in turn stimulates VEGF and bFGF secretion.

Figure 5.3.3.1 0.15 kPa PAAm hydrogel promotes secretion of proangiogenic factors by hTERT-ADMSCs through intracellular ROS. (a) Bar plots of relative VEGF and bFGF transcription (n = 4) and (b) Bar plots of VEGF and bFGF secretion by hTERT-ADMSCs (n = 2), presented as mean + SD. * denotes statistical difference at p < 0.05. (c) Bar plots of VEGF and bFGF secretion by hTERT-ADMSCs in the 0.15 kPa group with or without 700 μ M NAC treatment, presented as mean + SD, n = 2.

5.3.3.2 Angiogenic Potential of CM Harvested from hTERT-ADMSCs Grown on Substrate with Different Stiffness

During sprouting angiogenesis, blood vessels are grown into the portions of tissues previously lacking blood vessels through regulated endothelial cells growth, migration, and tube formation [20]. Therefore, the angiogenic potential of hTERT-ADMSCs CM was evaluated via three biological endpoints, namely, (i) cell proliferation, (ii) direct cell migration and (iii) formation of blood vessels.

In terms of cell proliferation, it was noted that the proliferative capacity of HUVECs cultured with CM_{0.15 kPa} was significantly increased by around 20% compared to the SFM control (**Figure 5.3.3.2a**). In contrast, no obvious difference was observed in HUVECs proliferation for the rest of the experimental groups. The angiogenic potential of CM_{0.15 kPa} was further scrutinized for its chemoattractant activity using a commercialized chemotaxis chip. The single-cell migratory trajectories of the HUVECs were monitored over the course of 6 h using time-lapse microscopy. Whereas directed migration of HUVECs was absent under the attraction of the CM_{GPa} and SFM, chemotaxis of HUVECs in response to the CM_{0.15kPa} was evident (**Figure 5.3.3.2b**). In support of this observation, the computed

chemotactic index of the CM_{0.15 kPa} group is significantly higher than that of the SFM control by 2.4-fold (66 versus 27.9), while this difference between the CM_{GPa} group and SFM control is negligible (31.2 versus 27.9).

Next, the effect of CM on endothelial tube formation was examined. In the CM_{0.15 kPa} group, HUVECs formed a well-developed tubular network with an average of 140 nodes (**Figure 5.3.3.2c**). However, CM_{GPa} and SFM control were clearly less effective in inducing HUVECs tube formation, with an average of 100 and 80 nodes recorded respectively. Accordingly, the average tube length of HUVECs treated with CM_{0.15 kPa} was also significantly greater compared to the CM_{GPa} and SFM groups. Finally, the angiogenic potency of the various hTERT-ADMSCs CM was evaluated by applying them to 11 days old chick embryos topically. The local blood vessel developments were scored after 3 days of treatment (**Figure 5.3.3.2d**). Consistent with the *in vitro* angiogenesis assay, CM_{0.15kPa} treatment lead to the extensive formation of blood vessel network. Compare with the SFM group, the CM_{0.15 kPa} group enhanced angiogenesis with a 1.2-fold increase in blood vessel length and a 1.6-fold increase of node number. Taken together, in both *in vitro* angiogenesis and *ex ovo* CAM assay, CM_{0.15 kPa} exhibited enhanced angiogenic potential.

Figure 5.3.3.2 Mechano-conditioning of hTERT-ADMSCs with 0.15 kPa PAAM hydrogel facilitates the application of MSCs CM in therapeutic angiogenesis. (a) Box and whisker plots of HUVECs proliferation under the treatment of MSCs CM and SFM control. (b) Single cell tracking of HUVECs migration under the gradient of MSCs CM and SFM control over 6 h. (c) *In vitro* HUVECs tube formation (scale bar = 100 μm) and (d) *Ex ovo* CAM assay (scale bar = 1 mm) under MSCs CM and SFM control treatment, with box and whisker plots of quantified total tube length and number of nodes. * denotes statistical difference at $p < 0.05$.

5.4 Conclusions

Through *in vitro* MSCs differentiation study and wound closure assay, hTERT-ADMSCs were found to retain their multipotency and secret healing factors that promote migration of HDF and HaCaT keratinocytes. This concludes that hTERT-ADMSCs is an ideal cell source for “cell-free” regenerative medicine as MSCs secretome can be derived on a large scale. hTERT-ADMSCs are also mechanosensitive, as their spreading and cytoskeleton maturation were limited by PAAM hydrogel with biomimicking stiffness, indicating that

they can sense the softness of substrate material. Same as primary MSCs, overexpressed intracellular ROS was also induced by 0.15 kPa PAAm hydrogel in hTERT-ADMSCs. Through mechano-conditioning of hTERT-ADMSCs, more VEGF and bFGF were expressed even compared with 3D spheroid culture. In ROS inhibition assay, ROS was proven to mediate the overexpression of both VEGF and bFGF triggered by mechano-conditioning of hTERT-ADMSCs with 0.15 kPa PAAm hydrogel. This increased secretion of proangiogenic factors rendered hTERT-ADMSCs CM a stronger angiogenic potency. CM_{0.15} kPa enhanced HUVECs proliferation, attracted HUVECs migration, promoted HUVECs tube formation and stimulated blood vessel growth of chick embryo. This is the first study reporting preconditioning immortalized MSCs by controlling substrate stiffness.

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Chapter 6*

Mechanism of Substrate Stiffness-Dependent Redox Metabolism Modulation of MSCs

Chapter 6 aims to understand how PAAm hydrogel of 0.15 kPa promotes the secretion of proangiogenic factors by hTERT-ADMSCs. Reduced cell spreading of hTERT-ADMSCs on PAAm hydrogel contributed to inducing intracellular oxidative stress, while the serum-free condition was dispensable. This intracellular oxidative stress triggered the antioxidant response of hTERT-ADMSCs. Because of the interplay between Nrf2 antioxidant response element and HIF1 α signaling, HIF1 α protein in hTERT-ADMSCs cultured on the 0.15 kPa substrate was stabilized, initiating the transcription of pro-angiogenic factors. This HIF1 α activation of hTERT-ADMSCs on the 0.15 kPa substrate was proven to be induced by intracellular ROS overexpression, while serum level is dispensable.

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6.1 Introduction

In Chapter 4 and 5, ROS were identified as an important mediator for mechano-conditioning of both primary MSCs and hTERT-ADMSCs. Mechano-conditioning of hTERT-ADMSCs with 0.15 kPa PAAm hydrogel induced intracellular ROS, accompanied with limited cell spreading and disrupted cytoskeleton. It was reported that by limiting cell spreading through latrunculin-A treatment, intracellular oxidative stress was induced in HDF [1]. This leads to the assumption that reduced hTERT-ADMSCs spreading on 0.15 kPa PAAm hydrogel could be the reason for overexpression of intracellular ROS. To test this assumption, the spreading area of hTERT-ADMSCs should be manipulated with the same matrix substrate. Micro-contact printing technology, which can form patterns of ECM proteins with different shapes and areas, is a good tool [2]. Circular micropatterns with a diameter of 60 and 100 μm can well control the position of Fn on 10 kPa PAAm hydrogel which varies the adhesion of hTERT-ADMSCs on the substrates with the same stiffness. This brings new knowledge regarding how substrate stiffness modulates intracellular oxidative stress.

By learning from current MSCs preconditioning studies, it can be known that ROS interacts with several signaling pathways, like PKB, NF κ B, HIF1, etc [3]. Additional attention should be devoted to HIF1 signaling, as it is a main regulator of angiogenesis [4]. Considering that hTERT-ADMSCs mechano-conditioned with 0.15 kPa PAAm hydrogel secreted abundant proangiogenic VEGF and bFGF, activation of HIF1 signaling could be the mechanism for 0.15 kPa PAAm hydrogel to stimulate the secretion of proangiogenic factors by hTERT-ADMSCs. Meanwhile, Nrf2 signaling, a redox defense pathway, could also be activated in hTERT-ADMSCs culture on 0.15 kPa PAAm hydrogel to achieve a redox balance. It has been documented that through its downstream product thioredoxin, Nrf2 is able to activate HIF1 signaling. In a feedforward fashion, HIF1 signaling suppress thioredoxin reductase level to strengthen Nrf2 [5]. Overall, this interplay between Nrf2 and HIF1 signaling initiated by elevated intracellular oxidative stress might explain how 0.15 kPa PAAm hydrogel triggered the secretion of proangiogenic factors by hTERT-ADMSCs.

6.2 Experimental Methods

6.2.1 Cell Culture Substrate Fabrication and Cell Culture

Cell culture substrate fabrication was mentioned in section 3.1. In this chapter, hTERT-ADMSCs was used. Its culture method was described in section 3.4.

6.2.2 Micro-Contact Printing of Fn on PAAm Hydrogel Substrate

Procedure of micro-contact printing was described in section 3.3.

6.2.3 Bio-characterization of hTERT-ADMSCs on Fn Patterned PAAm Hydrogel Substrate

The hTERT-ADMSCs on Fn patterned PAAm hydrogel substrate were characterized in aspects of cytoskeleton development and intracellular ROS expression as mentioned in section 4.2.3. ROS inhibition assay of hTERT-ADMSCs culture on 0.15 kPa PAAm hydrogel was conducted by following procedures in section 3.6.

6.2.4 Quantification of Antioxidant Proteins Transcription by hTERT-ADMSCs

Transcription of four antioxidant proteins, namely HMOX-1, UGT1A1, SOD2 and NQO1, was measured with RT-PCR as described in section 3.8.

6.2.5 Redox Metabolism of hTERT-ADMSCs

Nrf2 and HIF1 α expressions were studied through immunofluorescence staining by the following method in section 3.5.

6.2.6 Total Antioxidant Capacity Measurement of hTERT-ADMSCs

The total antioxidant capacity of hTERT-ADMSCs was assessed through the Trolox Equivalent Antioxidant Capacity assay (Beyotime Institute of Biotechnology S0119). 100 μ L of cell lysates derived from hTERT-ADMSCs cultured on various substrates were collected by sonication. The reaction substrate was first oxidized by adding an oxidizing agent provided by the manufacturer. The lysate samples of hTERT-ADMSCs were mixed separated with oxidized reaction substrate, with Trolox as the control to prepare a standard curve. The colorimetric changes at 734 nm absorption were measured using a microplate reader (SpectraMax M2). At the same time, the total protein concentration of the cell lysates was measured using the Micro BCA Protein Assay Kit (Thermo 23235). In the end, total antioxidant capacity measurements were presented as “mmol/g” by normalizing equivalent Trolox amount (mM) to the total cellular protein amount (mg/ml).

6.2.7 HIF1 α Inhibition and ELISA Assay

HIF1 α inhibition was achieved by treating hTERT-ADMSCs with 100 μ M of chrysin (Abcam ab141230). The secretions of VEGF and bFGF by hTERT-ADMSCs were measured with ELISA kits from Shanghai Xitang Biotech by following method written in section 3.9.

6.3 Principle Outcomes

6.3.1 Relationship Between Cell Spreading Area, Serum Content and Intracellular ROS Level

6.3.1.1 Influence of Cell Spreading on Intercellular ROS Level

It has been reported that treating HDF with latrunculin-A to disrupt the actin cytoskeleton led to reduced cell spreading and elevated levels of endogenous ROS [1]. In the previous chapters, hTERT-ADMSCs cultured on the PAAm hydrogel of 0.15 kPa have been

observed to develop immature cytoskeleton structure, spread smaller and express abundant ROS, suggesting that there could be a link between cell spreading and intracellular ROS level. To delineate both determinants, micro-contact printing was employed. Effect of substrate stiffness was normalized by transferring arrays of Fn islands with a diameter of 60 or 100 μm onto the 10 kPa PAAm substrate (**Figure 6.3.1.1a**). 24 h post cell seeding, hTERT-ADMSCs were observed to assume a rounded morphology that follows the pattern of the transfer printed Fn micropatterns. (**Figure 6.3.1.1b**). The micropatterned cells were then counterstained with CellROX Orange to probe the expression level of intracellular ROS (**Figure 6.3.1.1c**). As shown in **Figure 6.3.1.1d**, cells coerced to adhere within the Fn islands with 60 μm diameter displayed higher ROS expression by ~ 1.6 -fold relative to the cells with 100 μm diameter. This result shows that intracellular ROS level of hTERT-ADMSCs can indeed be modulated by controlling cell spreading area. Along the same vein, this may imply that the heightened ROS expression in hTERT-ADMSCs grown on the 0.15 kPa PAAm hydrogel may be due to a lack of cell traction generation and reduced cell spreading.

Figure 6.3.1.1 Cell spreading area modulates redox metabolism of hTERT-ADMSCs.

(a) Fn islands patterned on PAAm hydrogel of 10 kPa visualized by immunofluorescence staining. Scale bar = 100 μ m. (b) Representative immunofluorescence staining images of hTERT-ADMSCs cultured on Fn islands, with blue staining nucleus, green staining vinculin and red staining F-actin. Scale bar = 50 μ m. (c) CellROX Orange staining and (d) Box and whisker plots of hTERT-ADMSCs cultured on Fn islands. Scale bar = 50 μ m. n = 30. * denotes statistical difference at $p < 0.05$.

6.3.1.2 Influence of Serum Content on Substrate Stiffness-Dependent Regulation of Intracellular ROS Level

Unique to this thesis, MSCs were cultured under serum-deplete condition to prevent interference of FBS on secretome analysis. However, serum deprivation was also shown to enhance ROS production via activating reactive oxygen species modulator 1 [6]. Therefore, it would be of interest to examine whether if the mechano-regulation of ROS level is conditional on the serum content in the culture medium (**Figure 6.3.1.2a**). As shown in **Figure 6.3.1.2b**, the ROS level is consistently higher for hTERT-ADMSCs in the 0.15 kPa group compared to those cultured on the CS of GPa stiffness regardless of the serum level. This suggests that in PAAm hydrogel system, the effect of cell substrate mechanics dominates the effect of serum availability in terms of the modulating intracellular ROS level.

Figure 6.3.1.2 Substrate stiffness, rather than serum availability, dominates the modulation of intracellular ROS by PAAm hydrogel system. (a) CellROX Orange staining and (d) Box and whisker plots of intracellular ROS level in hTERT-ADMSCs cultured with different substrates and serum levels. Scale bar = 50 μ m. n > 30. * denotes

statistical difference at $p < 0.05$. No significant difference was observed between serum-free and serum-containing condition for the same substrate.

6.3.2 Influence of Substrate Stiffness on the Nrf2-Antioxidant Response Signaling Pathway

In general, increased intracellular ROS level can lead to a state of oxidative stress which may be detrimental to cell viability, should redox balance be not restored [7]. The Nrf2-Keap1 signaling is the master regulator of redox homeostasis and protects the cells by coordinating their adaptive response to oxidative stress [8]. While it has been shown that intracellular ROS level is significantly increased in hTERT-ADMSCs cultured on 0.15 kPa PAAm hydrogel, it is unclear whether if the excess free oxygen radicals are at a level that is sufficiently high to stress the cells. Therefore, the expression level of the redox-responsive transcription factor Nrf2 in hTERT-ADMSCs was examined. Under basal conditions, Keap1 binds to Nrf2, resulting in the rapid degradation of Nrf2 through the proteasome pathway [9]. However, excess intracellular ROS modifies the cysteine of Keap1 to prevent the formation and ubiquitination of Nrf2-Keap1 complex, thereby escaping proteasomal degradation [10]. In turn, the complex will dissociate and Nrf2 will translocate into the cell nucleus to induce antioxidant gene expression. For hTERT-ADMSCs cultured on the 10 kPa substrate, although the intracellular ROS level is higher than cells cultured on the CS, no strong staining for Nrf2 was observed (**Figure 6.3.2.1a**). Conversely, enhanced expression of Nrf2 was observed in both cytosolic and nucleus of hTERT-ADMSCs cultured on the 0.15 kPa substrate. Specifically, the measured intranuclear Nrf2 level of MSCs in the 0.15 kPa group was around 3 times that of hTERT-ADMSCs cultured on the CS with GPa stiffness (**Figure 6.3.2.1b**). This implies that the Nrf2 antioxidant machinery is activated in hTERT-ADMSCs grown on the 0.15 kPa PAAm substrate, presumably because the cells are under oxidative stress.

Figure 6.3.2.1 0.15 kPa PAAm hydrogel promotes the nuclear translocation of Nrf2.

(a) Representative immunofluorescence staining images and (b) Box and whisker plots of intranuclear Nrf2 expression of hTERT-ADMSCs preconditioned on various substrates, with blue staining nucleus and green staining Nrf2. Scale bar = 10 μm. $n > 30$. * denotes statistical difference at $p < 0.05$.

Furthermore, nuclear translocation of Nrf2 in the hTERT-ADMSCs grown on the 0.15kPa PAAm substrate resulted in the concomitant upregulation of several Nrf2 downstream antioxidant transcripts, such as HMOX-1, UGT1A1, SOD2 and NQO1 (**Figure 6.3.2.2a**). The substrate stiffness-regulated antioxidant capacity was further examined using the total antioxidant capacity assay. As shown in **Figure 6.3.2.2b**, while the mean total antioxidant capacity of the hTERT-ADMSCs in the CS group was around 0.37 mmol/g, total antioxidant capacity values of hTERT-ADMSCs cultured on the PAAm hydrogel of 10 kPa and 0.15 kPa were significantly higher at 1.11 and 1.83 mmol/g respectively. Taken together, these findings strongly suggest that substrate stiffness is a potent biophysical regulator of redox metabolism in hTERT-ADMSCs. Additionally, a threshold stiffness of ~0.15 kPa was required to activate the Nrf2-antioxidant stress signaling response pathway.

Figure 6.3.2.2 0.15 kPa PAAm hydrogel enhances the expression of antioxidant components by hTERT-ADMSCs. (a) Bar plots of relative transcription of various Nrf2 downstream target antioxidant genes by hTERT-ADMSCs as a function of substrate stiffness, presented as mean + SD, $n = 4$. * denotes statistical difference at $p < 0.05$. (b) Box and whisker plots of total antioxidant capacity of hTERT-ADMSCs cultured on various substrates. $n = 4$. * denotes statistical difference at $p < 0.05$.

6.3.3 Regulation of Angiogenic Potential of hTERT-ADMSCs by Substrate Stiffness-Dependent Activation of HIF1 Signaling

HIF1 α is traditionally known as a key transcriptional regulator of cellular and developmental response to hypoxia by promoting angiogenesis to facilitate oxygen and nutrient delivery [11]. In a normoxic environment, HIF1 α is hydroxylated by prolyl hydroxylase domain-containing proteins and further degraded by the proteasome [12]. Under hypoxia condition, the activity of prolyl hydroxylase decrease, allowing the stabilization and accumulation of HIF1 α [13]. Accumulated HIF1 α forms heterodimer with HIF1 β and the heterodimer acts as a pro-angiogenic transcription factor after translocating into the nucleus [14]. Since PAAm hydrogel of 0.15 kPa stiffness was observed to stimulate the secretion of proangiogenic factors (i.e. VEGF and bFGF) in hTERT-ADMSCs (**Figure 5.3.3.1**), it is hypothesized that HIF1 signaling may be implicated. This postulation was further supported by evidence documenting that Nrf2 stress response and HIF1 signaling can work in concert to reinforce each other [5].

Figure 6.3.3.1a shows the immunofluorescence staining of HIF1 α in hTERT-ADMSCs grown on the various substrates under the normoxic condition. As expected, HIF1 α protein was not expressed by hTERT-ADMSCs that were cultured on the CS substrate with GPa stiffness. However, hTERT-ADMSCs grown on the 10 kPa PAAm hydrogel displayed an increase in cytoplasm-residing HIF1 α staining. Significantly, cytosolic and nuclear staining of HIF1 α was apparent for the cells that were cultured on the 0.15 kPa substrate. Compared to cells on the CS of GPa stiffness, cells on the 0.15 kPa PAAm substrate displayed a 2-fold increment in nuclear-bound HIF1 α expression (**Figure 6.3.3.1b**). To gain additional molecular insights into the role of HIF1 α activation and the increased secretion of VEGF and bFGF from hTERT-ADMSCs cultured on the 0.15 kPa PAAm substrate, the cells were treated with 100 μ M chrysin, a naturally occurring antioxidant flavonoid that can function as a potent inhibitor of HIF1 signaling. Chrysin treatment led to a marked suppression of VEGF and bFGF secretion by the cells (**Figure 6.3.3.1c**). This suggests that the enhanced proangiogenic potential of hTERT-ADMSCs on the 0.15 kPa PAAm hydrogel was mediated in part via the HIF1 signaling pathway.

Figure 6.3.3.1 0.15 kPa PAAm hydrogel stimulates the secretion of proangiogenic factors from hTERT-ADMSCs by activating HIF1 signaling. (a) Representative immunofluorescence images and (b) Box and whisker plots of intranuclear HIF1 α expression of hTERT-ADMSCs cultured on various substrates. Scale bar = 10 μ m. $n > 30$. * denotes statistical difference at $p < 0.05$. (c) Bar plots of VEGF and bFGF secretion from hTERT-ADMSCs on the 0.15 kPa substrate with or without 100 μ M chrysin treatment, presented as mean + SD, $n = 2$. * denotes statistical difference at $p < 0.05$.

To further examine the mechanistic links between substrate stiffness dependent regulation of intracellular ROS level and HIF1 α signaling, hTERT-ADMSCs on the 0.15 kPa PAAM substrate was treated with NAC (700 μ M) (**Figure 6.3.3.2a**). In agreement with the earlier observations, the increased in the HIF1 α protein expression was substantial, such that positive nuclear staining was readily detected in hTERT-ADMSCs on the 0.15kPa PAAM hydrogel. However, the use of NAC led to the total abolishment HIF1 α expression (**Figure 6.3.3.2a**). It was postulated that the interplay between Nrf2 and HIF1 signaling activates HIF1 signaling of hTERT-ADMSCs on the 0.15 kPa substrate under the normoxic condition. This hypothesis is supported by NAC inhibition result. For hTERT-ADMSCs on the 0.15 kPa substrate with NAC treatment, Nrf2 was not required anymore to restore redox hemostasis. Lacking the help of Nrf2, HIF1 α also cannot escape from degradation under the normoxic condition. Overall, the increased in the intracellular endogenous ROS level lies upstream to the activation of the HIF1 α signaling pathway under normoxia condition.

After this, the role of serum level on HIF1 activation was also studied (**Figure 6.3.3.2b**). Same as the effect of serum availability on intracellular ROS level of MSCs (**Figure 6.3.1.2**), tuning the serum level barely has any impact on the status of HIF1 α protein: On the 0.15 kPa substrate, HIF1 α was always stabilized; while on the GPa substrate, HIF1 α was consistently degraded. In **Figure 6.3.3.2a**, it has been concluded that the intracellular ROS level determines HIF1 α protein condition. Meanwhile, serum availability has a marginal effect on the intracellular ROS level (**Figure 6.3.1.2b**). Thus, serum accessibility cannot influence HIF1 α stabilization as the redox metabolism of MSCs is not manipulated by serum availability.

Figure 6.3.3.2 Intracellular ROS level, but not the serum availability, determines HIF1 signaling activation under normoxic condition. (a) Representative immunofluorescence images of hTERT-ADMSCs cultured on the 0.15 kPa substrate with or without NAC treatment, with blue staining nucleus and red staining HIF1 α . Scale bar = 20 μ m. (b) Representative immunofluorescence images of hTERT-ADMSCs cultured on the 0.15 kPa or GPa substrate under different serum availability, with blue staining nucleus and red staining HIF1 α . Scale bar = 20 μ m.

6.4 Conclusions

In summary, substrate stiffness was showed to be a potent biophysical cue to reprogram the redox metabolism and conditional activation of the HIF signaling of hTERT-ADMSCs under normoxic conditions (**Figure 6.4**). It was first found that on the same 10 kPa substrate, reducing the spreading of hTERT-ADSCs by 64% is potent enough to induce a ~1.6-fold increase in intracellular ROS. Thus, compared with GPa substrate, the limited cell spreading of hTERT-ADMSCs cultured on 0.15 kPa substrate caused by immature cytoskeleton development could be an important contributor of their elevated intracellular oxidative stress. This ROS overexpression was triggered by the 0.15 kPa PAAm hydrogel,

with negligible influence of serum availability. The ROS imbalance in hTERT-ADMSCs cultured on the 0.15 kPa PAAm hydrogel activates the Nrf2-ARE signaling pathway to induce the antioxidant defenses, as indicated by transcription of antioxidants and increase in total antioxidant capacity of hTERT-ADMSCs [8]. Because of the interplay between Nrf2 and HIF signaling, HIF1 α in hTERT-ADMSCs cultured on 0.15 kPa PAAm hydrogel is stabilized under normoxic condition, initiating the secretion of proangiogenic VEGF and bFGF [5]. NAC inhibition assay double confirmed the intracellular ROS overexpression is an upstream event to the activation of the HIF1 α signaling pathway in hTERT-ADMSCs on 0.15 kPa PAAm hydrogel under normoxia condition, while serum deprivation is dispensable.

Figure 6.4 Proposed mechanism of substrate stiffness functioning as a biophysical regulator of HIF signaling in hTERT-ADMSCs under normoxic conditions.

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Chapter 7

Conclusions and Future Work

This chapter first summarizes the mechanism for substrate stiffness to modulate MSCs secretome. The importance of this engineered MSCs secretome in tissue repair is also illustrated. According to the current understanding of this novel preconditioning method based on mechanotransduction, reconnaissance work regarding the possible exploitation of zwitterionic polymer coating as an engineered surface to precondition MSCs is presented. Possible elucidation studies to decipher the involvement of specific subset of integrins within the framework of mechano-redox regulation of MSCs secretome production is proposed.

7.1 Conclusions

In Chapter 4, a facile but effective materials-driven approach to modulate the therapeutic efficacy of MSCs was presented. Using Fn conjugated PAAm hydrogels with tissue mimicking stiffness ($E \sim \text{kPa}$ range) as a model culture system, the effects of substrate stiffness on adhesion, spreading and secretome production of primary human bone marrow derived MSCs under serum deplete culture condition were examined. Fn coated CS with GPa level stiffness was employed as a control culture substrate. The non-biased cytokines array-based screening exercise revealed that the secretion level of a subset ($\sim 35\%$) of cytokines (i.e. IL-1 α , IL-3, IL-10, M-CSF, MIP-1 δ , RANTES, SDF-1, TNF α , OSM, IL-8, MCP-1, TARC, TNF β , VEGF, and IL-6) could be modulated as a function of substrate stiffness. At the mechanistic level, this phenomenon is mediated, in part, by a change in intracellular ROS level. Importantly, the conditioned medium (containing the secretome) harvested from MSCs grown on the PAAm hydrogel substrate could significantly increase *in vitro* wound closure in human HaCaTs and HDF models. Furthermore, using an *in vivo* C57BL/6J wild-type mice based full thickness skin wound healing, application of conditioned medium from MSCs on the PAAm hydrogel substrate significantly accelerated wound healing by 5-8% compared to wounds that were treated with conditioned medium from MSCs cultured on the CS substrate. Within the framework of MSCs secretome-based therapy, these findings clearly reveal that conventional “hard” culture materials such as polycarbonate, glass, is far from being the optimal. This study provides the first-in-class preclinical demonstration in the enhancement of the therapeutic value of MSCs secretome for functional tissue repair. The use of substrate mechanics to precondition MSCs is nominated as *mechano-conditioning*.

Large-scale production of MSCs secretome requires a stable cell source. In Chapter 5, to circumvent the many limitations (e.g. donor variance, epigenetic reprogramming and senescence) that are inherent to the use of primary MSCs, research focus was shifted to hTERT-ADMSCs. Superiority studies showed that the mesodermal lineage differentiation potential of hTERT-ADMSCs can be maintained up to 10 passages. Conversely, primary MSCs failed to differentiate at equivalent passage number. Furthermore, conditioned

medium derived from hTERT-ADMSCs was observed to accelerate *in vitro* wound healing, suggesting that hTERT-ADMSCs secretome is therapeutically active. The concept of mechano-conditioning was then extended to hTERT-ADMSCs and showed that secreted level of angiogenic factors such as VEGF and bFGF were higher in cells grown on 0.15 kPa PAAm hydrogel, compared to cells on CS in a ROS dependent manner. The angiogenic potential of the conditioned medium obtained from hTERT-ADMSCs under different growth conditions were further evaluated using a series of *in vitro* HUVECs models and *ex ovo* CAM assays. Compared with using CS as substrate, mechano-conditioning of hTERT-ADMSCs with 0.15 kPa PAAm hydrogel produced conditioned medium that further promotes HUVECs proliferation by 20% and attracts HUVECs migration by 2-fold. Meanwhile, this engineered conditioned medium also enhanced tube formation of HUVECs and blood vessel generation of chick embryo. Collectively, these results showed that hTERT-ADMSCs can be mechano-conditioned to augment its angiogenic potential, which may be useful for the treatment of chronic wounds and other vascular diseases.

In Chapter 6, the mechanism by which PAAm hydrogel induces intracellular ROS expression in hTERT-ADMSCs was further scrutinized. Micro-contact printing technique was first employed to evaluate the effect of morphological constraints on intracellular ROS level [1]. It was found that limiting the spreading of MSCs on the PAAm hydrogel of 10 kPa by 64% is sufficient to induce a 1.6-fold higher intracellular ROS level. This is consistent with a similar study conducted with HDF, where limiting the spreading of HDF through drug treatment also increased the intracellular ROS level [2]. Therefore, the stiffness-dependent mediated ROS level could be attributed to a reduction in cell spreading on the PAAm hydrogel substrate. Additionally, it is noted that the effect of substrate stiffness on the regulation of intracellular ROS generation is independent on serum content. Further mechanism studies revealed that when hTERT-ADMSCs were cultured on the CS substrate, intracellular ROS level is maintained at a relatively low level, so is the expression level of Nrf2 and HIF1 α [3-5]. However, when MSCs were cultured on the 0.15 kPa substrate, nuclear translocation of Nrf2 was observed, indicating that the cells are under oxidative stress. Consistently, the transcription of antioxidant enzymes, downstream effectors of Nrf2 signaling was activated, leading to the increase in total antioxidant

capacity of hTERT-ADMSCs [6]. This is also accompanied with the noncanonical activation of the HIF signaling, which subsequently upregulate VEGF and bFGF transcripts expression as well as secretion of VEGF and bFGF upregulated the secretion of proangiogenic factors [7-9]. These findings are consistent with the improved angiogenic potential of the 0.15 kPa PAAm hydrogel substrate cultured hTERT-ADMSCs. Moreover, 0.15kPa was identified as the critical threshold stiffness to activate the HIF1 α signaling under normoxia culture condition.

7.2 Reconnaissance and Future Work

7.2.1 Zwitterionic Polymer Coating as MSCs Preconditioning Substrate

Using the micro-contact printing technique, it was showed that the reduction in MSCs spreading is sufficient to increase production of intracellular ROS level (**Figure 6.3.1.1**). With NAC inhibition assay, this overexpression of intracellular ROS was found to be critical in modulating MSCs secretome production, as it activates HIF1 signaling through Nrf2 antioxidant response and promotes secretion of pro-angiogenic factors. Based on this, it is reasonable to hypothesize that other substrates that can limit the spreading of MSCs can also be used for MSCs preconditioning.

Formation of hydration layer is a common method to reduce nonspecific protein binding to surface, thus inhibits cell adhesion [10]. Hydrophilic materials, like polyethylene glycol, tetraglyme, and dextran, etc., achieve hydration via hydrogen bonding. However, zwitterionic-based materials can bind water molecules more strongly via electrostatically induced hydration and greatly reduce nonspecific protein absorption [11-13]. A simple method to fabricate zwitterionic polymer coating is coating cationic and anionic polymers alternatively onto a substrate. PDADMAC, a cationic polymer, and PSS, an anionic polymer, are two useful candidates for this purpose because of their commercial availability [14].

PDADMAC and PSS can be individually diluted in DI water to a final concentration of 1 mM (based on the monomer repeat unit), together with 1 M of NaCl. After adjusting the pH of polymer solution to 7, PDADMAC and PSS solution is ready to prepare zwitterionic polymer coating on polystyrene cell culture plates. Starting with PDADMAC solution, the plate surface is treated alternatively by PDADMAC and PSS for 5 mins at room temperature, forming layer by layer coating of zwitterionic polymer. According to the preliminary trial, on zwitterionic polymer coating composing two bilayers of PDADMAC and PSS, and one more additional layer of PDADMAC (nominated as [PDADMAC/PSS]₂[PDADMAC]), the spreading of MSCs were limited successfully (**Figure 7.2.1.a**). Same as the PAAm hydrogel of 0.15 kPa, this zwitterionic polymer coating also induced overexpression of intracellular ROS and activation of HIF1 signaling (**Figure 7.2.1.b,c**). Further investigation is required to clarify the impact of this zwitterionic polymer coating on MSCs secretome, its application in regenerative medicine and the mechanism under the triggering of intracellular oxidative stress in MSCs by both PAAm hydrogel and zwitterionic polymer coating.

Figure 7.2.1 Zwitterionic polymer coating is a potential MSCs preconditioning substrate. (a) Cell spreading (scale bar = 200 μ m), (b) Box and whisker plots of quantified intracellular ROS level (* denotes statistical difference at $p < 0.05$), and (c) HIF1 α staining (scale bar = 50 μ m) of MSCs cultured on polystyrene and zwitterionic polymer coating.

7.2.2 Understand the Role of Integrins in Mechano-conditioning of MSCs

The process of mechanotransduction begins from the binding of integrin to ECM ligands. At the same time, different integrins bind to different ECM proteins [15]. For example,

integrin $\alpha 3 \beta 1$ binds to laminins, $\alpha 2 \beta 1$ and $\alpha 1 \beta 1$ bind to collagens, while $\alpha 5 \beta 1$ specifically binds to Fn (**Figure 7.2.2.1**) [16].

Figure 7.2.2.1 Specificity of various ligands on the binding of different integrins. Adapted with permission [16]. Copyright 2006 The Company of Biologists.

At the same time, accordingly to the studies about how substrate stiffness guides the differentiation of MSCs, it is now proposed that on substrate of different stiffnesses, various integrins are involved (**Figure 7.2.2.2**) [17]. When MSCs are cultured on soft substrate of 0.1-1 kPa, $\beta 1$ integrin is uptake through caveolae/raft-dependent endocytosis, blocking BMP/Smad signaling and inducing neurogenic differentiation. As a contrast, stiff substrate of 40 kPa activates $\alpha 2$ integrin-ROCK-FAK-ERK signaling, thus increasing RUNX2 activity and promoting osteoblast differentiation.

Figure 7.2.2.2 Integrin engagement, cytoskeleton organization and signal activation of MSCs induced by various substrate stiffness. (A) Integrin binding and cytoskeleton organization of MSCs cultured on substrates of various stiffness. (B) Activation of differentiation related signal in MSCs by substrate stiffness. Adopted with permission [17]. Copyright 2015 Springer Nature.

To clarify whether specific integrin is critical for this soft substrate-based preconditioning, blocking study can be carried out by targeting integrins that are responsible for attachment of MSCs onto Fn, like $\alpha 5\beta 1$, $\alpha V\beta 1$, etc. If certain integrin is indispensable, after blocking, some or all the downstream biological events, including reprogramming of redox metabolism and HIF1 signaling activation, will not happen. Results obtained from the blocking studies can be further validated using recombinant proteins. For example, recombinant proteins with domains designed to bind $\alpha 5\beta 1$ and $\alpha 3\beta 1$ have been constructed before [18]. Using the same principle, recombinant proteins targeting different integrins could be synthesized and be decorated onto the PAAm hydrogel with different stiffness to

systematically decouple the relative contributions of substrate stiffness and specific integrin recruitment in this mechano-conditioning process.

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