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MATRIX STIFFNESS REGULATES OXIDATIVE STRESS RESPONSE OF HUMAN DERMAL FIBROBLASTS

YAO XUEFENG

SCHOOL OF MATERIALS SCIENCE AND ENGINEERING

2018
MATRIX STIFFNESS REGULATES OXIDATIVE STRESS RESPONSE OF HUMAN DERMAL FIBROBLASTS

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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

2018
Statement of Originality

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

25 JAN 2018

Date

Yao Xuefeng
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.

25 JAN 2018

Date

Assoc Prof. Tan Lay Poh
Authorship Attribution Statement

This thesis contains material from (a) paper(s) published in a/the following peer-reviewed journal(s) where I was the first and/or corresponding author.

**Chapters 4 and 7** are going to be submitted and published as XF Yao, LP Tan, *et al.*, Soft Matrix Prevents UV Induced Human Dermal Fibroblasts Senescence via Inhibition of NF-κB Activity. Manuscript is under preparation.

The contributions of the co-authors are as follows:
- A/Prof Tan Lay Poh provided the initial project direction and edited the manuscript drafts.
- I prepared the manuscript drafts.
- I co-designed the study with A/Prof Tan Lay Poh and Dr. Oliver Dressen. All the laboratory work was performed by me at the School of Materials Science and Engineering and School of Biological Science. I also analyzed the data.
- All substrates preparation, cell culture, immunostaining and qRT-PCR were conducted by me in School of Materials Science and Engineering.
- Dr Zhu Pengcheng assisted in the experiments of flow cytometry and western blotting.

**Chapters 5 and 6** are going to be submitted and published as XF Yao, LP Tan, *et al.*, Matrix Stiffness Regulates Human Dermal Fibroblasts Oxidative Stress Response via Nox4-Nrf2 redox. Manuscript is under preparation.

The contributions of the co-authors are as follows:
- A/Prof Tan Lay Poh provided the initial project direction and edited the manuscript drafts.
- I wrote the drafts of the manuscript. The manuscript was revised by A/Prof Tan Lay Poh.
- I co-designed the study with A/Prof Tan Lay Poh and A/Prof Andrew Tan. All
the laboratory work was performed by me at the School of Materials Science and Engineering and School of Biological Science. I also analyzed the data.

- All substrates preparation, cell culture, immunostaining and qRT-PCR were conducted by me in School of Materials Science and Engineering.
- Dr Zhu Pengcheng assisted in the experiments of flow cytometry and western blotting.
Abstract

Reactive oxygen species (ROS) are widely involved in many physiological and pathological processes. It has been well known that decreased antioxidant ability resulted excessive ROS accumulation leads elderly people to have more risk in ageing related diseases. However, the underlying mechanism of why the antioxidant ability decreases with age remains unknown. Recent research progress shows that aged and diseased tissues have significantly altered mechanical microenvironment. Also, the regulatory role of mechanical microenvironment in numerous cellular processes such as cell adherent, cell morphology, cell migration, cell proliferation, cell differentiation and cell apoptosis has been well established. But, little has been known about the correlation between altered microenvironment and ROS dysregulation. As above, this dissertation aims to study the effect of extracellular matrix (ECM) stiffness on ROS regulation.

In this study, polydimethylsiloxane (PDMS) substrates with different crosslinking ratio (1:10, 1:40 and 1:70, cross linker: base) were fabricated to mimic the microenvironment with different stiffness. Tissue culture plates (TCPs) and 750kPa PDMS substrates were used as stiff matrix, while 46kPa and 21kPa represented soft matrix. Human dermal fibroblasts (HDFs) were cultured on these substrates followed by additional exotic oxidative stress stimulation, and the cell behaviors were examined accordingly. Firstly, HDFs on stiff matrix were more susceptible to H$_2$O$_2$ induced ROS accumulation and apoptosis, while soft matrix significantly reduced H$_2$O$_2$ induced cellular damage. It is also suggested that the prevention is nuclear factor kappa B (NF-κB) involved and independent of the ROCK pathway. Then, it is demonstrated that the matrix stiffness might regulate cell oxidative stress tolerance via the YAP-Nox4/Nrf2 pathway. HDFs on stiff matrix exhibited high Nox4 expression, so that they were oxidative stress sensitive. Due to high expression of Nrf2, HDFs on soft matrix showed strong oxidative stress resistance. At last, matrix stiffness regulated oxidative stress was further confirmed by UV exposure. It is revealed that most HDFs accumulate excessive ROS and undergo senescence on stiff
matrix, while it is slightly affected on soft matrix, upon UV irradiation. The results proved that the mechanical microenvironment has regulatory effect on cell oxidative stress response, as well oxidative stress induced cell fate decision. It is also indicated that ECM stiffness increase is a possible cause of decreased antioxidant ability.
Lay Summary

Understanding the science behind ageing remains a promising and critical research avenue that will yield significant impact for both medical science and public health. Ageing is associated with significant morbidity and elderly individuals are known to have increased susceptibility to many diseases and cancers. In addition, ageing leads to poor disease healing ability.

Free radical, as a popular ageing mechanism, is widely involved in ageing and ageing related disease. Excessive reactive oxygen species (ROS) production as a result of decreased antioxidant ability contributes to ageing and many other age associated dysfunctions.

In past decades, numerous studies have been conducted to understand why the elderly people have decreased antioxidant capacity. However, one of the aspects that have been significantly neglected is the effect of microenvironment where cells lived in. Cells are surrounded and affected by biologically active and mechanically dynamic mechanical microenvironment. The microenvironment mechanics have close connections to ageing and ageing related diseases. Increased microenvironment stiffness is widely observed in aged tissue, fibrotic diseases and cancer. However, whether stiffness increase is a cause or a consequence of decreased cell antioxidant ability is poorly understood. It is time now to seek better knowledge about the relationship between microenvironment stiffness and cell antioxidant ability.

With the advantages of materials science and engineering related techniques, the biocompatible PDMS substrates with tunable stiffness were employed to establish an in vitro platform to mimic the mechanical microenvironment. Cells were cultured on these substrates followed by the stimulation of exotic oxidative stress such as H₂O₂ and UV. Cell behaviors and oxidative stress response were monitored and observed with related biological and materials engineering techniques accordingly. The present study revealed that the mechanics of microenvironment could modulate
cell oxidative stress responses under external stimuli. ECM stiffness could affect cell antioxidant ability and further regulates H2O2 induced apoptosis, as well as UV irradiation induced senescence. With advances of material engineering approaches, we reported physical microenvironment regulated cell oxidative stress response for the first time. From this understanding, microenvironment may be eventually used and engineered to be potent modulators of cell oxidative stress response just as in the case of stem cell differentiation in recent years.
Acknowledgements

Every small accomplishment relies on the kindness from many other people. Thanks for all the people appeared in my life.

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With sincere appreciation, I would like to thank my supervisor, Associate Professor Tan Lay Poh, for all your support in my PhD candidature. In your lab, it’s the first time I truly felt the importance of environment, the effect of environment is everywhere, even in the micro world at cellular level. From macro to micro, the world follows the same logic, the beautiful art of balance. That’s the fantasy and attractiveness of the natural science.

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# Abbreviations

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<tr>
<td>AGEs</td>
<td>Advanced Glycation End products</td>
</tr>
<tr>
<td>ARE</td>
<td>Antioxidant Response Element</td>
</tr>
<tr>
<td>AO</td>
<td>Acridine Orange</td>
</tr>
<tr>
<td>ATR-FTIR</td>
<td>Attenuated Total Reflectance Fourier Transform Infrared spectroscopy</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CCK-8</td>
<td>Cell Counting Kit-8</td>
</tr>
<tr>
<td>CuSO$_4$</td>
<td>Copper Sulfate</td>
</tr>
<tr>
<td>CDK</td>
<td>Cyclin-Dependent Kinase</td>
</tr>
<tr>
<td>DCFDA</td>
<td>Dichlorodihydrofluorescein Diacetate</td>
</tr>
<tr>
<td>ECM</td>
<td>Extra Cellular Matrix</td>
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<tr>
<td>GAPDH</td>
<td>Glyceraldehyde-3-Phosphate Dehydrogenase</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>Hydrogen Peroxide</td>
</tr>
<tr>
<td>LMP</td>
<td>Lysosome Membrane Permeabilization</td>
</tr>
<tr>
<td>MPCs</td>
<td>Myogenic Progenitor Cells</td>
</tr>
<tr>
<td>MMPs</td>
<td>Matrix Metalloproteinases</td>
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<tr>
<td>NADPH</td>
<td>Nicotinamide Adenine Dinucleotide Phosphate</td>
</tr>
<tr>
<td>Nrf2</td>
<td>Nuclear Factor Erythroid 2-Related Factor 2</td>
</tr>
<tr>
<td>Noxs</td>
<td>NAD(P)H Oxidases</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear Factor Kappa B</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene Fluoride</td>
</tr>
<tr>
<td>PDMS</td>
<td>Polydimethylsiloxane</td>
</tr>
<tr>
<td>pRB</td>
<td>p16–Retinoblastoma Protein</td>
</tr>
<tr>
<td>PDTC</td>
<td>Pyrrolidinedithiocarbamate Ammonium</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium Iodide</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative Reverse Transcription Polymerize Chain Reaction</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>sulfo-SANPAH</td>
<td>Sulfosuccinimidyl 6-(4’-azido-2’-nitrophenylamino)hexanoate</td>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>SASP</td>
<td>Senescence-Associated Secretory Phenotype</td>
</tr>
<tr>
<td>SA-β-gal</td>
<td>Senescence Associated β-Galactosidase</td>
</tr>
<tr>
<td>TIMPs</td>
<td>Tissue Inhibitors of Metalloproteinases</td>
</tr>
<tr>
<td>TCPs</td>
<td>Tissue Culture Plates</td>
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<tr>
<td>YAP</td>
<td>Yes Associated Protein</td>
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Chapter 1

Introduction

_In this chapter, a brief introduction about rationale for the research is given at the first place. The importance of physical microenvironment on cellular ROS regulation has not been explored is the current research gap. We bring out the hypothesis that the mechanics of microenvironment have great potential in regulating HDFs oxidative stress response and oxidative stress induced cell apoptosis/senescence. Then, we summarize the objectives, as well as work scopes about this study. We also describe the overview of thesis contents and the novelty of the work in this chapter. Novelty relies on that an undocumented influence of matrix stiffness on HDFs oxidative stress response has been elucidated in this dissertation._
1.1 Background

The discovery of excessive reactive oxygen species (ROS) accumulation in the elderly came with exciting hypotheses about the potential roles it may have in ageing and aging related diseases [1]. Despite intensive research on this topic in the past decades, it is only recently that solid evidence was first presented that dysregulated ROS significantly contribute to impaired wound healing and fibrotic diseases in elder individuals [2, 3]. In spite of this important progress, many challenges remain especially in understanding ageing and disease related ROS regulation in a more holistic manner. One of the aspects that has been significantly neglected is the link between extra cellular matrix (ECM) and the cellular ROS regulation.

Increasingly, there have been studies and discoveries that cellular microenvironment plays a crucial role in modulating cellular behaviors including differentiation, migration, mortality and even metastasis [4-9]. Cellular microenvironment could modulate the behaviors via both surface chemical configuration/composition as well as just physical means such as stiffness and topography [10, 11]. Even though there has been evidence showing significantly altered microenvironment in ageing and ageing related diseases, the understanding of the relationship has been vague [12-15]. Little has been known about the cause and effect of the microenvironment on oxidative stress response and ROS related diseases. Therefore, it is timely now to include the effect of microenvironment on ROS regulation into the picture of ageing and ageing related diseases.

Recent research evidences showed cellular redox state could be influenced by cytoskeleton organization. It is reported that NAD(P)H oxidase 4 (NOX4), the major enzyme responsible for ROS production, is regulated by cytoskeleton-controlled transcriptional coactivators. Down-regulation/inhibition of Yes-associated protein (YAP) abrogates NOX4 expression [16]. Meanwhile, YAP has long been identified as an important sensor of mechanical signals exerted by the cellular microenvironment. Many works reported both ECM stiffness and cell geometry have profound effects on YAP activity [17, 18] [19]. Based on these two parts, it is reasonable to speculate that ECM stiffness could affect cell oxidative...
stress response via YAP-NOX4 in an indirect manner.

1.2 Hypothesis

One of the significant physical changes that accompany aged and fibrotic tissues is increased ECM stiffness. Also, it is well known that aged and fibrotic tissues have more risk in ROS involved pathologies. Therefore, it is hypothesized that there is probably a relationship between ECM stiffness and cell oxidative stress response during stimulation. This project therefore seeks to explore the effect of matrix stiffness on cellular oxidative stress response. Here, we speculate that there is a strong correlation between the ECM stiffness and the antioxidant ability of the residential cells. Within the scope of this project, we hypothesize that the ECM stiffness could modulate cell oxidative stress responses under external stimuli. We also hypothesize that ECM stiffness might regulate oxidative stress induced apoptosis and senescence depending on the dose and source of the applied oxidative stress.

1.3 Objectives and scopes

The general objective of this study is to explore the influence of matrix stiffness on cellular oxidative stress response. A systematical study of the relationship between matrix stiffness and oxidative stress induced cell apoptosis/senescence will provide new insights in understanding cell matrix interactions in ageing and ageing related diseases.

As the largest organ and first barrier to external environment, skin has been widely employed in connective tissue ageing study [20-22]. Moreover, skin ECM is well documented with significant alterations in the process of ageing, mainly stiffness increases and topography changes [23, 24]. Therefore, under such context, human dermal fibroblasts (HDFs) were chosen as candidate cells to study ECM’s effect on cell oxidative stress response.

The specific objectives are as following:
1) To fabricate substrates with tunable stiffness for cell culture.
   - Fabrication of PDMS substrates with different stiffness.
   - Modification of PDMS surface to facilitate HDFs attachment.
   - Investigation of matrix stiffness’ effect on HDFs morphology.

2) To explore the effect of matrix stiffness on H$_2$O$_2$ induced oxidative stress response.
   - Optimization of H$_2$O$_2$ working concentration to induce HDFs apoptosis.
   - Assessment of H$_2$O$_2$ induced ROS accumulation and cell apoptosis on substrates with different stiffness.
   - Exploration of Rho\ROCK pathway’s role in stiffness mediated oxidative stress response.
   - Investigation of NF-κB activation in stiffness mediated oxidative stress response.

3) To elucidate the underlying mechanism behind matrix stiffness effect on cell oxidative stress response.
   - Evaluation of YAP, Nox4 and Nrf2 expression on substrates with different stiffness.
   - Speculation of the underlying signaling pathway.

4) To explore the effect of matrix stiffness on UV induced oxidative stress response.
   - Optimization of UV working condition to induce HDFs senescence.
   - Assessment of UV induced ROS accumulation, cell proliferation and senescence on substrates with different stiffness.
   - Investigation of NF-κB activation in stiffness mediated oxidative stress response.

1.4 Thesis outline

This dissertation is made up by eight chapters.

In Chapter 1, a brief introduction of the research background and current research gap are
given at first place, followed by elucidation of the hypothesis, then the research scopes and objectives, followed by the overview of the dissertation, finished with the novelty and significance of this dissertation.

In Chapter 2, a detailed literature review about recent research highlights related to this dissertation is given. The implications of ROS regulation and ECM stiffness alterations in ageing tissues and fibrotic diseases are elucidated. Moreover, the potential clues in connecting ROS regulation with ECM stiffness are discussed.

In Chapter 3, the main experimental techniques and methods, as well as the behind principles used in this dissertation are elucidated.

In Chapter 4, the fabrications, surface modifications and characterizations of PDMS substrates with different stiffness are introduced. The effects of matrix stiffness on HDFs morphology and focal adhesion are assessed as well.

In Chapter 5, the effects of matrix stiffness on H$_2$O$_2$ induced ROS accumulation and HDFs apoptosis are investigated. In addition, the role of Rho\ROCK pathway and NF-κB (nuclear factor kappa B) in matrix stiffness mediated H$_2$O$_2$ stress response are explored.

In Chapter 6, it is aimed to understand the mechanism about matrix stiffness regulated oxidative stress response. The effects of matrix stiffness on yes associated protein (YAP), Nox4 and Nrf2 are examined. The YAP mediated matrix stiffness regulation of Nox4/Nrf2 expression is involved in oxidative stress response.

In Chapter 7, the effects of matrix stiffness on UV induced ROS accumulation, cell proliferation and senescence are explored. Besides, the role of NF-κB activation in matrix stiffness regulated UV stress response is investigated.

In Chapter 8, the general findings of each project are discussed and concluded systematically. Also, future recommendations based on the conclusion are prospected.
1.5 Novelty and significance

To the best of our understanding, studies on the role of microenvironment in modulating cell oxidative stress response and oxidative stress related cell apoptosis/senescence have not been explored. There is evidence showing microenvironment changes in ROS dysregulated tissues and organs, but the causality of the relationship has not been established. After decades of studies on ROS regulation in the process of ageing, the physiological relevance of these studies is slowly showing. Recent data validate the early idea that ROS regulation is important for tissue ageing and ageing related diseases [16-18]. All these studies are focused around the biological aspects of senescence. However, cells are surrounded by a dynamic environment and they interact intimately with the environment. Therefore, with a strong biological understanding of ROS regulation in ageing and ageing related diseases, it is timely to include microenvironment such that the understanding would be more holistic and not isolated. Furthermore, from this understanding, microenvironment may be eventually used and engineered to be potent modulators of cell oxidative stress response just as in the case of stem cell differentiation in recent years.

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Chapter 2

Literature Review

In this literature review, detailed background information and recent research progress regarding to this dissertation, as well as current research gap, are introduced and discussed. It is mainly consisted of three parts, microenvironment stiffness alteration in ageing and diseases, ROS in ageing and diseases, as well as the possible links between extra cellular matrix (ECM) stiffness and ROS regulation. In the first part, the features of aged ECM are introduced in details. Aged and diseased tissues exhibit significantly altered microenvironment. Then, the importance and casual role of ROS in ageing and ageing related diseases are discussed. Thereafter, we review recent research highlights regarding to potential connections between ECM stiffness and ROS regulation. Therefore, more efforts should be made to understand the underlying mechanisms of age related ROS regulation. Based on this, integrated therapeutic strategies to improve human health and lifespan could be generated.
2.1 Background

The population of elderly individuals is fast increasing worldwide [1]. It is well known that elderly individuals have higher risk to diseases with poor ability to heal [2]. Understanding the science behind tissue ageing and ageing related diseases remains a promising and critical research avenue that will yield significant impact for both medical science and clinical trials.

Free radical, as a popular ageing mechanism, is widely involved in ageing and ageing related disease. Excessive ROS production as a result of decreased antioxidant ability contributes to ageing and many other age-associated dysfunctions. In past decades, many efforts have been made to answer why the elderly people has decreased antioxidant capacity. However, one of the aspects that have been significantly neglected is the effect of microenvironment. Even though emerging evidence showing significantly altered mechanical microenvironment with ageing and diseases, the effect of ECM mechanics on antioxidant ability is poorly understood. Therefore, this study is aimed at understanding the effect of matrix stiffness on cells oxidative stress response. The detailed background information and potential links about ECM stiffness and ROS regulation in ageing and diseases will be introduced as following.

2.2 Microenvironment stiffness alteration in ageing and diseases

Extracellular matrix (ECM) is defined as the non-cellular components of tissue, it binds cells together to form connective tissues [3]. ECM provides physical support for the residing cells [4]. In past decades, ECM has been demonstrated with multiple roles in regulating cellular activities and responses [5]. In turn, residing cells control ECM deposition through synthesizing and secreting proteins, water, polysaccharides, as well as all the other essential biomolecules [6]. To date, many evidences demonstrate ECM plays essential roles in numerous cellular processes such as cell adherence, cell morphology, cell migration, cell proliferation, cell differentiation and cell apoptosis through focal adhesion [4, 7, 8]. However, the concept that ECM shows negative effects on cellular activity has
been refuted [5]. It is only recently that evidence slowly showing ECM is involved in ageing and ageing related diseases [9] As shown in Figure 2.1, multiple factors, such as protein synthesis and degradation, elastin-collagen integrity, ECM protein crosslinking and many others, could affect ECM mechanics. This altered ECM mechanics consequently regulate tissue ageing and ageing related dysfunction. In this section, the stiffness changes in connective tissue ageing, fibrotic diseases and cancer progression will be discussed.

![Figure 2.1](image)

**Figure 2.1** Age-related alterations of the extracellular matrix and it related dysfunction [10].

### 2.2.1 Increase of ECM stiffness in tissue ageing

ECM mechanics changes significantly in many connective tissues, such as skin, tendon and skeletal muscle, during ageing. The detailed biological events are introduced as following.

A consistent aspect of skin ageing is the presence of glycated dermal collagen [11] [12]. Reducing sugars (ie. glucose) abundantly circulating in the blood are known to spontaneously react with proteins in tissues over time in the course of ageing through
glycation. This nonenzymatic reaction produces advanced glycation end products (AGEs) that subsequently mediate the chemical crosslinking of collagen in the skin. The fibers of glycated collagen are also found to be more densely packed, physically stiffer and less elastic altogether, clearly representing a significant change of the physical microenvironment for the residing dermal fibroblasts. The major characteristic physical alteration of the skin ageing is the loss of elasticity, which essentially corresponds to the substantial loss of the ECM’s collagen integrity. It is recorded that the stiffness of skin increased from 80kPa to 260kPa during the process of aging, as shown in Figure 2.2[13].

Advanced glycation leads to stiffness increase in the other connective tissues ageing as well. Animal works in rat tail tendon showed young rats at 3 months old have lower collagen content [14]. However, the aged rats have significantly higher collagen content which results in tissue stiffness increase [15].

Similar character is also observed in skeletal muscle. Aged skeletal muscle is featured by excessive deposition of ECM [16]. A recent work revealed that increased muscle ECM stiffness during ageing impairs the behavior of myogenic progenitor cells (MPCs), which leads to declined muscle regenerative potential in elderly [17].
2.2.2 Increase of ECM stiffness in fibrotic diseases and cancer

Beyond connective tissue ageing, the alteration of ECM mechanics has been demonstrated with crucial roles in many diseases development and cancer progression [18, 19].

Fibrotic diseases, such as pulmonary fibrosis, cardiovascular disease, systemic sclerosis and liver fibrosis, are always associated with increased tissue stiffness resulted by elevated ECM synthesis and decreased ECM degradation [20]. In such cases, over proliferation of fibroblasts and sustain activation of myofibroblasts mediate imbalanced synthesis and secretion of ECM proteins (e.g. collagen). Meanwhile, MMPs (matrix metalloproteinases), enzymes to degrade ECM, are downregulated. On the contrary, TIMPs (tissue inhibitors of metalloproteinases), which could inhibit MMPs’ activity, are overexpressed [21]. The constitutive accumulation of ECM induces elevation of tissue stiffness and progression of organ dysfunction [22]. Alteration of ECM composition strongly affects tissue biomechanical property, as is clearly observed in fibrotic diseases [23]. For example, elastin and collagen fibers, the key ECM components of pulmonary connective tissues, exhibit substantially different elastic properties. The elastic modulus of elastin fiber is around 1 MPa, which is 1200 folds lower than collagen fibrils (around 1200 MPa) [24, 25]. In the lung, the continuous network, formed by elastin and collagen fibers, provides the mechanical forces for passive recoil during expiration. When lung is undergoing pulmonary fibrosis, the upregulated collagen synthesis and crosslinking lead to elevation of tissue stiffness and loss of elasticity, which finally result in progressive dyspnea such as shortness of breath [26, 27].

Distinctly alteration in ECM remodeling and tissue stiffness, which is closely connected to fibrotic diseases as introduced above, also plays essential role in cancer. With progressive and sustained ECM remodeling, tumor microenvironment is biologically and mechanically active and dynamic [28]. The altered microenvironment could drive malignancy by enhancing tumor cell survive. As such, increased ECM stiffness has been demonstrated as one of the hallmarks in tumor progression. In breast cancer, a tenfold stiffness increase is observed, the stiffness of cancer tissue and normal tissue is 1.5 kPa and 150 Pa,
respectively. It has also been reported that tumor stroma is significantly stiffer than normal stroma (400 Pa versus 150 Pa) [29-31]. Additionally, tissue stiffening was also occurred in pre-malignant tissue, the stiffness increases from 150 Pa to 350 Pa when compared to normal tissue, this increase has been demonstrated with effect on malignant transformation in breast [31]. Moreover, a recent study revealed that tumorigenic cells can become phenotypically normal if the microenvironment is appropriately manipulated [32]. The above information highlights and exemplifies the significance of ECM stiffness on tumor progression.

As introduced above in this section, ECM mechanics have close connections to ageing and ageing related diseases. Increased ECM stiffness is widely observed in aged tissue, fibrotic diseases and cancer. However, whether stiffness increase is a cause or a consequence of these pathological processes is poorly understood. It is time now to seek better knowledge about the relationship between ECM mechanics and ageing related disorders.

2.3 ROS in ageing and diseases

Reactive oxygen species (ROS) is considered as free radicals and molecules derived from molecular oxygen. ROS is produced by the leakage of electrons to molecular oxygen in the mitochondrial respiratory chain in the process of ATP synthesis. Oxygen is reduced to the superoxide anion (O$_2$•) after gaining one electron. Both enzymatic and non-enzymatic reactions could produce superoxide anion. The enzymes producing superoxide are cell membrane-bound and include the reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and cytochrome P450-dependent oxygenase. Without enzymatic help, superoxide production can still occur with the direct transfer of a single electron to oxygen from reduced coenzymes or prosthetic groups (e.g., flavins or iron sulfur clusters). Superoxide is the precursor of most ROS, and its subsequent reduction or dismutation leads to the production of hydrogen peroxide (H$_2$O$_2$). H$_2$O$_2$ is then partially reduced to a hydroxyl radical (OH•) or completely reduced to water. The hydroxyl radical is perhaps the strongest natural source of oxidants. In essence, O$_2$•, H$_2$O$_2$ and OH• are the relatively stable intermediates from these monovalent reduction processes and are collectively termed ROS.
ROS, with a highly reactive feature, is able to damage all forms of biomolecules, such as nucleic acids, proteins and lipids. Through lipid peroxidation reactions, ROS could damage cell membrane integrity. Moreover, ROS leads to peptide chain fragmentation, amino acid modifications and altered isoelectric points among which, will disrupt protein function and further hinder cellular function. ROS induced DNA damage has also been widely reported.

**Figure 2.3** Schematic description of ROS and oxidative stress associated process of aging.
2.3.1 ROS involved tissue ageing and ageing related diseases

Many evidences demonstrate reactive oxygen species (ROS) as a main contributor of ageing [33]. The free radical theory is popular among ageing mechanisms [34]. Chronic and irreversible accumulation of oxidative damage mediated by excessive ROS has profound impact on physiological dysfunction, elevated incidence of disease and declined lifespan [35, 36]. The general process of excessive ROS caused ageing is elucidated in Fig 2.3. An age associated intracellular ROS imbalance leads to a chronic state of inflammation. It also activates the ROS sensitive transcription factors and proinflammatory mediators, such as chemokines, cytokines and nitric oxide synthase. These ROS sensitive molecules further promote the generation of ROS. As such, a positive feedback loop of ROS production and inflammation is activated, which further exacerbates inflammation-induced cellular and tissue damage.

![Figure 2.4](image)

**Figure 2.4** ROS mediates fibrotic response through multiple feed-forward and feedback loops.

ROS has long been involved in fibrotic diseases. As shown in Fig 2.4, ROS induced by various factors such as disease, trauma, toxins and radiation contributes to fibrotic response...
in many faces through enhanced inflammation. ROS and fibrosis interacts in a positive feedback loop. Excessive ROS enhances fibrotic responses, in turn, fibrosis triggers further accumulation of ROS [37].

In specific, increased 8-isoprostane (an oxidative stress marker) and H$_2$O$_2$ were detected in the urine and exhalants of lung fibrosis patients [38, 39]. Also, elevated lipid peroxidation marker 4-hydroxy-2′-nonenal was reported in biopsy specimens of fibrotic liver [40]. Additionally, ROS showed its implication in silicosis and asbestosis, as enhanced levels of 8-hydroxy-2′-deoxyguanosine (an oxidative DNA damage indicator) and nitrotyrosine adducts were detected in these diseases [41, 42]. Moreover, ROS has strong impact on renal fibrosis, as well as myocardial infarction [43-45].

Beyond those important roles of ROS in ageing and fibrotic diseases, its impact on tumor progression has also attracted lots of attentions. Tumor cells with high ROS accumulation were observed both in vivo [46-49]. For instance, higher ROS was detected in fresh isolated tumor leukemia cells when compared with normal lymphocytes [50]. Oxidative damage products (e.g. 8OHdG) and lipid peroxidation products, are resulted from ROS accumulation, were observed in tumor biopsy, plasma and cancer cell lines [49-51]. Increased ROS accumulation in cancer cells plays important role in malignant initiation and progression [46].

### 2.3.2 ROS induced apoptosis and senescence

ROS and the resulting oxidative damage to DNA can induce senescence as well as apoptosis [52-54]. ROS might cooperate with physiological levels of p53 to convert a senescence response to apoptosis. As an important sensor of oxidative stress, tumor suppressor p53 is strongly related to ROS mediated cell fate decision. P53 activation induced by ROS is involved in either reversible or permanent growth arrest, as well as apoptosis [55-57]. As reported, at physiological p53 protein levels capable of triggering senescence, an apoptotic response was observed when the p53-induced increase in intracellular ROS levels was complemented by an exogenous ROS source, which itself was
not able to induce apoptosis in the absence of p53. These findings suggest the existence of a threshold of ROS above which the apoptotic program is initiated. This threshold may vary between cell types or as a function of other physiological factors. However, the balance between all the ROS inducers and the antioxidants present in the cell at a given moment is likely crucial in determining cell fate decisions (Fig 2.5) [58].

![Figure 2.5 ROS levels dependent cell fates decision: apoptosis or senescence.](image)

### 2.3.3 Apoptosis in ageing and diseases

Apoptosis was first defined by Kerr et al. in early 1970s [59]. It is a programmed cell suicide process initiated by either intrinsic or extrinsic signals. In past decades, apoptosis has been elucidated with multiple possible roles in ageing and ageing associated dysfunctions [60]. It, at least partially, has great significance in ageing process and age-related tumorigenesis in mammals [61]. The apoptosis rate is significantly increased in many types of ageing organs and tissues, such as skin, brain, immune system, eye, cardio-vascular system and reproductive system [62]. On one hand, accelerated apoptosis in these organs and tissues
serves as a protective mechanism against the accumulation of dysfunction cells which will maintain tissue hemostasis and avoid tumorigenesis. On the other hand, elevated apoptosis profoundly contributes to age-related decline and deterioration in function and structure integrity of aged organs and tissues, as a result of decreased cell population and related alterations in gene and protein expression [62]. As reviewed by B. Lu et al, the relationship between apoptosis and ageing can be elucidated from 3 aspects as following. Firstly, apoptosis increases with ageing. Secondly, ageing could restrain apoptosis. Thirdly, ageing is partially the result of apoptosis and ageing interaction [63]. As a sentinel homeostatic pathway, elevated apoptosis has path-physiological consequences in ageing. Increased apoptosis will lead to tissue degeneration [64], and inappropriate apoptosis may result in age-related diseases and cancer [65].

Apoptosis also has essential role in chronic wound healing [66]. Impaired wound healing is always observed in elders [67]. It is possible that delayed skin wound repair of older individuals is in part due to an apoptosis defect in older fibroblasts [68].

Recent evidence has suggested a deep involvement of ROS also in the extrinsic pathway of apoptosis. Fas and TNFR1 activation are known to generate ROS in response to stimulation, which has been hypothesized to be due to the production of superoxide (O$_2^-$) because of the formation of lipid raft-derived NADPH oxidase platforms. This lipid raft-associated ROS downstream generation may be of high importance in induction of apoptosis or necrosis [69, 70]. Moreover, ROS are required for apoptosis induction by Fas ligand (FasL) in primary lung epithelial cells. ROS mediate the downregulation of FLIP (FLICE inhibitory protein, a strong inhibitor of apoptosis) by ubiquitination and subsequent degradation by proteasome or through nitric oxide (NO) scavenging that prevents FLIP S-nitrosation and cytoprotection [71].

2.3.4 Senescence in ageing and diseases

Senescence is where cells undergo permanent growth arrest and was first defined by Leonard Hayflick in the early 1960s [72]. After which, senescent fibroblasts have long been
used as model cells to study skin and the other connective tissue ageing. As a hallmark of ageing, senescent cells are unable to divide, but they keep the metabolic ability and therefore are able to interfere with their surrounding matrix and cellular environment [73]. It is believed that the SASP (senescence-associated secretory phenotype), specific factors secreted by the senescent cells, contributes a lot to tissue ageing [74]. Moreover, the senescent cells could communicate with their neighbouring cells which will lead to both positive and negative effects. In particular, cell senescence serves as a hemostasis mechanism to limit unlimited cell division. At the same time, increase in senescent cells contributes to quite some age-related skin disorders [73].

![Figure 2.6](image_url)

**Figure 2.6** ROS induced by external factors contributes to cell senescence.

Multiple pathways about cell senescence have been reported. ROS regulation is always involved in the process of senescence and ageing. As shown in Fig 2.6, these pathways as
well as ongoing DNA damage response can promote a ROS positive feedback loop. Once
the cells are growth arrested, they start expressing senescence-associated secretory
phenotype (SASP) factors. The SASP factors can further feedback and generate more
mitochondrial ROS, and mitochondrial and nuclear DNA damage, which feeds forward into
more cycles of senescence induction. While a fraction of senescent cell population could
get cleared by immune function, a majority of cells are likely to survive and metabolically
active generating more of the SASP factors, ultimately resulting into tissue degeneration
and tissue dysfunction. ROS can also induce mitochondrial dysfunction and telomere
dysfunction, which will further feed into senescence induction pathways. Thus, ROS can
initiate and maintain senescence via multiple pathways [75].

2.3.5 ROS mediated NF-κB activation

NF-κB family is mainly consisted of RelA (p65), RelB, c-Rel, p50/p105 (NF-κB1) and
p52/p100 (NF-κB2). As an oxidative stress sensor, NF-κB could be activated by the
intracellular ROS, and in turn, NF-κB-dependent genes influence the levels of ROS [76, 77]. NF-κB usually bound with its inhibitor IκB and exists in the cytosol with an inactive
status [78-80]. Upon activation, NF-κB translocates into nucleus as a result of IκB
degradation [81]. NF-κB activation is a transient process, it only occurs under appropriate
stimulation. After simulation, it will return to the resting state. Sustained activation of NF-
κB cause disorders in cell adhesion, cell cycle and apoptosis.

Numerous works have reported that ROS could affect NF-κB activity, both positively and
negatively.

As from Fig 2.7, the alternative IκBα phosphorylation is often related to ROS induced NF-
κB activation. ROS dependent phosphorylation of RelA (p65) leads to greater NF-κB
activation. Among all the known ROS inducers related to NF-κB activation, H2O2 and some
environmental factors (e.g. UV irradiation) are the most well investigated ones. Some other
oxidative stress agents, such as IL-1, TNFa and viral proteins, also induce the ROS
accumulation and further activate NF-κB.
As a ROS sensor, NF-κB pathway plays a crucial role in ageing and ageing related disorders. Over expression of NF-κB subunits induces cultured cells senescence [82-84]. Similarly, mouse embryonic fibroblasts have been shown to escape senescence when there is loss of p65. Similarly, mouse embryonic fibroblasts have been shown to escape senescence when there is loss of p65 [85]. Moreover, sustain NF-κB activation is observed in many aged tissues, such as liver, skin kidney, cardiac muscle and cerebellum [86-88]. Furthermore, cells from elder people (aged 72–93) and HGPS progeria patients showed much higher NF-κB activation when compared to those from younger individuals (aged 22–33).

**Figure 2.7** Crosstalk of ROS with NF-κB signaling pathways. ROS interacts with NF-κB at various places within the signaling pathway [89].

NF-κB activation also contributes to age-associated diseases. For instance, upregulated NF-κB signaling is observed in osteoarthritis, osteoporosis, atherosclerosis, neurodegeneration (Parkinson’s and Alzheimer’s) and cardiovascular disease [90-94].
As discussed, oxidative stress induced ROS plays major roles in ageing and ageing related diseases. Chronic ROS accumulation and imbalanced ROS regulation contributes, as least partially, to increased diseases risk in the elderly. Among those ROS related pathological processes, at molecular and cellular level, elevated ROS accumulation leads to sustain activation of NF-κB, which will further decide cell fate in the elderly, either senescence or apoptosis. With increased rates of cell senescence and apoptosis, the old individuals are prone to diseases and these diseases are difficult to heal. As such, more efforts should be made to understand the underlying mechanisms of age related ROS regulation. Based on this, integrated therapeutic strategies to improve human health and lifespan could be established.

2.4 The potential connections between ECM stiffness and ROS regulation

So far, the involvement and importance of ECM stiffness as well as ROS regulation in ageing and diseases have been discussed and highlighted. Since it is aimed to generate better understanding between these two aspects, the possible connections between ECM stiffness and ROS regulation will be further reviewed.

2.4.1 Nox4-Nrf2 in ROS regulation

Emerging evidence revealed that NOX4-Nrf2 redox imbalance contributes to intracellular ROS accumulation in ageing and age-related fibrotic diseases [95-97] Elevated expression of the reactive oxygen species (ROS)-generating enzyme, NADPH oxidase-4 (Nox4), and an impaired capacity to induce the NFE2-related factor 2 (Nrf2) antioxidant responses altered the cellular redox homeostasis [98].

As a reactive oxygen species-generating enzyme, Nox4 has been identified as a major source of oxidative stress [99, 100]. Nox4 is an oxygen sensor to produce ROS from molecular oxygen [101]. Overexpression of Nox4 could initiate oxidative stress induced intracellular ROS accumulation and induce mitochondrial dysfunction, which will simulate NF-κB activation and initiate cytochrome C (Cyto-C) release induced cell apoptosis [100,
Nox4 knockout diabetic mice were detected with attenuated ROS production and NF-κB activation.

An increase in Nox4 activity associated with deficient Nrf2-driven cytoprotective responses characterizes this redox imbalance. Nrf2 is an oxidative stress-sensitive transcription factor that controls the expression of genes involved in antioxidant defense and detoxification [104]. Nrf2-antioxidant response element signaling pathway has been recognized as one of the powerful mechanisms in oxidative stress defense [105]. Recent research progress has elucidated the essential role of Nrf2 in activating antioxidants of cells exposure to oxidative stress via the Nrf2-Keap1 oxidative stress system [106]. For example, improving Nrf2 activity pharmacologically protects animals against oxidative stress induced damage [107]. Whereas knockout of Nrf2 consequently leads mice susceptible to various disease conditions and chemical toxicity related to oxidative stress [108-110].

2.4.2 YAP is necessary for Nox4

Matthew et al. demonstrated that YAP/TAZ plays essential role in Nox4 expression, which correlates cytoskeleton organization to cellular redox state [111]. Through silencing YAP/TAZ with YAP/TAZ siRNA, the expression of Nox4 protein, as well as Nox4 promoter, decreased significantly in LLC-PK1 cells under TGFβ induction (Fig 2.9 (A, B)). Similarly, Nox4 decreased a lot in UUO mice model by treating with TAZ/YAP inhibitor verteporfin (VP) (Fig 2.9 (C, D)). Therefore, YAP/TAZ is necessary for efficient Nox4 expression both in vitro and in vivo.
2.4.3 YAP in mechanotransduction

As a transcriptional regulator, Yes-associated protein (YAP) plays important roles in cell proliferation, suppressing apoptotic genes, control organ size and tumor growth through regulating related gene transcription [112]. With its close paralog, transcriptional co-activator with PDZ binding motif (TAZ), YAP/TAZ serves as the main downstream effector in Hippo tumor suppression pathway [113, 114]. Upon activation, YAP will be phosphorylated on a serine residue and sequestered in the cytoplasm, otherwise it will enter the nucleus and regulate gene expression [113].

Recent studies showed YAP is widely involved in mechanotransduction. It has been identified as an important sensor of mechanical signals exerted by the cellular
microenvironment. Many works reported both ECM stiffness and cell geometry have profound effects on YAP activity [115-117]. Upon their observations, *CTGF* and *ANKRD1*, 2 of the best YAP regulated genes were expressed at lower levels on soft substrate (Figure 2.8a). Additionally, YAP mainly localized in nucleus for cells cultured on stiff substrate, while it mainly expressed in the predominantly cytoplasmic area on soft substrate (Figure 2.8b). Furthermore, by controlling the MSCs spreading area with microcontact printing technique, they demonstrated that the localization of YAP shift from predominantly nuclear in spread MSCs, to predominantly cytoplasmic in cells with smaller area (Figure 2.8c). Based on this, they proved the mechano-regulation of YAP activity requires the contractile tension of cytoskeleton and Rho GTPase activity. More recently, Chakraborty *et al.* revealed that YAP dependent mechanosensing is mediated by Agrin involved integrin-focal adhesion- Lrp4/MuSK receptor pathway. Agrin signals matrix and cellular rigidity by activating FAK-ILK-PAK1 signaling that negates the Hippo tumor-suppressor pathway [118].
Figure 2.9 ECM stiffness and cell morphology regulate YAP activation and nucleus localization.

2.5 Summary

In summary, elderly people are prone to many diseases and free radical theory has been demonstrated as an important mechanism in ageing study. Many evidences revealed that the imbalance of ROS regulation is involved and plays causal roles in the process of ageing and ageing related diseases [33-36]. Furthermore, the alteration of microenvironment has been now widely reported in aged and diseased tissues, especially the increase of ECM stiffness [13-19]. Although ECM stiffness has shown its importance
in many biological processes, such as cell morphologenesis, proliferation, migration and differentiation, the interaction between ECM stiffness and ROS regulation remains unknown. Thus, it is hypothesized that the ECM stiffness could modulate cell oxidative stress response.

Moreover, both cell apoptosis and senescence contribute a lot to ageing and ageing related diseases via modulation of associated gene expression and protein secretion. Many works reported that the imbalance of ROS regulation should, as least partially, respond for cell apoptosis and senescence. Therefore, whether ECM stiffness could affect cell ROS induced cell apoptosis and senescence will also be explored.

In addition, the underlying mechanism of ECM stiffness regulated ROS balance will be investigated as well. By which, we could get more holistic and systematic understanding about cell-matrix interactions in ageing and ageing related diseases.

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Chapter 3

Experimental Methodology

This chapter aims to discuss the main methodology and techniques employed in this thesis. The underlying principle of each method is introduced as well. This study explores cellular oxidative stress responses of cells cultured on matrix with different stiffness. Therefore, related techniques at cellular level, such as cell culture, cell proliferation assay and flow cytometry are introduced. In addition, molecular biological assays, such as immunostaining, qRT-PCR and western blotting are discussed. This chapter only introduces the general methodology and techniques which are frequently used in our study. Other information regarding to materials and methods are described in following chapters accordingly.
3.1 Cell culture and cellular biology related techniques

3.1.1 Cell culture

Human Dermal Fibroblasts, neonatal (HDFs), isolated from neonatal foreskin, were purchased from Gibco (Invitrogen). During the experiments, cells were cultured in Medium 106(Gibco) with 2% Low Serum Growth Supplement (Gibco) and 1% antibiotic/antimycotic solution in a humidified atmosphere of 5% CO₂ at 37 °C. The culture medium was changed every 2-3 days. All HDFs used in the experiments were before passage 7.

3.1.2 Cell proliferation

Principle

Direct determination of total amount of DNA synthesis has been considered as a relative accurate method for the measurement of cell proliferation [1]. However, it is a challenge to measure cell proliferation from the same sample at different time point, since extraction of DNA needs to lysate the cells. Therefore, Cell counting kit-8 (CCK-8), which is mainly rely on monitoring the dehydrogenase activity of the viable cells, was used to measure the number of cells. This method does not kill the cells. With nonradioactive feature, CCK-8 is able to determine the population of viable cells in cell proliferation measurement, as well as cytotoxicity assay. The dehydrogenases produced by cells could reduce WST-8, followed by the generation of orange colored formazan (Figure 3.1). The volume of the produced forman could directly reflect the proportion of living cells.

Method

CCK-8 from Dojindo Molecular Technologies, was used to detect the cell numbers after UV treatment every 24 hours. Simply, 10 percent of CCK-8 solution was prepared with fresh medium. Then the HDFs were replaced with CCK-8 contained medium, followed by
2 hours culture in 37 °C incubator. After incubation, the absorbance value at 450 nm was measured by a plate reader. A calibration curve was performed to convert the absorbance value to cell numbers.

![Figure 3.1 Principle of the cell viability detection with Cell Counting Kit-8.](image)

### 3.1.3 Flow cytometry

**Principle**

With rapid analysis of many cellular events, such as cell size, DNA or RNA contents, cytoplasmic complexity, and many intracellular proteins, flow cytometry is a powerful tool in cellular biology [2]. It is a fluorescence based technique. Fluorescent dyes might bind to different cellular components such as DNA and RNA. Through binding to the target proteins on cell membranes as well as in cytosols, the fluorescent dyes could enter higher energy state when the fluorescent dyes labeled cells passing through the light source. The fluorochromes will emit light energy when they return to the resting state at higher wavelengths [3]. The work process of flow cytometry is elucidated in **Figure 3.2**. Briefly, fluorescence labeled cells in suspension are driven by laminar flow created by the sheath of isotonic fluid, which allows individual cell go through the interrogation point one by one. A beam of monochromatic light provided by laser could intersect the cell at the interrogation site. After which, the collection optics will collect the emitted light, and guide
the collected light go through dichroic mirrors. The reflected lights with particular wavelength bands will pass the filters. Finally, photomultiplier tubes are used to detect the light signals. The detected signals were digitized and analyzed by computer.

![Diagram of flow cytometer](image)

**Figure 3.2** Schematic illustration of flow cytometer. Single suspended cell is focused with sheath fluid. Forward light scatter detector and multiple fluorescence emission detectors (1–4) are employed to collect the signals. The detected signals were digitized and analyzed by computer after electronic amplification and conversion.

**Method**

As above, the principle of flow cytometry was briefly introduced. In this thesis, flow cytometry was employed to measure intracellular ROS accumulation and cell apoptosis. The detailed experiment procedures will be specified in the following chapters accordingly.

3.2 **Molecular biology related techniques**

3.2.1 **Immunostaining**
Principle

Fluorescence based immunostaining has developed into a powerful and popular technique to detect a variety of proteins/antigens. There are mainly two types of immunofluorescence staining, direct and indirect. Direct immunofluorescence is a one-step method, the fluorescence dyes conjugated antibodies are employed to tag the target protein directly. The alternative method is two-step procedure that the specific primary antibody conjugates to the target protein, followed by secondary antibody incubation to allow specific conjugation with the primary antibody (Figure 3.3).

![Figure 3.3 Principle of immunostaining. The picture of IgG antibody, a commonly used antibody for the purpose of immunostaining (left) and the depiction antigen-antibodies interaction that occurs during immunostaining procedure [4].](image)

Method

For fibronectin coating intensity characterization, the fibronectin coated substrates was first labeled with primary fibronectin antibodies. Then secondary antibodies, Alexa Fluor®
488 goat anti-mouse, were added. Substrates were washed three times with PBS before capture the image. Negative controls in the absence of primary antibodies were also performed.

For Vinculin, YAP, Ki-67 staining, HDFs were fixed with 4% paraformaldehyde for 15 minutes and then permeabilized with 0.1% Triton X-100 for 5 min and followed by blocking with 5% bovine serum albumin (BSA) or 5% goat serum. HDFs were then labeled with related primary antibodies and incubated at 4 °C overnight, after washed three times with PBS. Then the secondary antibodies were added and incubated for 1 hour at room temperature, after washed three times with PBS. Then, the staining solution was washed out with PBS for three times before image capture. Negative controls in the absence of primary antibodies were also performed.

For filamentous actin (F-actin) staining, HDFs were stained with Tetramethyl Rhodamine Iso- Thiocyanate (TRITC) conjugated-phalloidin (1:400, Chemicon) and cell nucleus was counter stained with DAPI together with secondary antibody.

For ROS staining, Image-iT™ LIVE Green Reactive Oxygen Species (ROS) Detection Kit from Thermofisher was applied. Simply, 25uM of carboxy-H2DCFDA solution was prepared with 5uM hochest 33342 in HBSS buffer. The HDFs were washed with PBS for three times and incubated with carboxy-H2DCFDA working solution 30 minutes at 37°C, protected from light. After incubation, the staining solution was replaced with warm medium and imaged immediately.

In all cases, the dilution of antibodies was strictly under the guidance of the manufactures, the fluorescence images were visualized with a Nikon 80i eclipse (Nikon, Japan) upright microscope and captured with the Nikon DSFi1 (Nikon, Japan), the quantification data was analyzed and generated by ImageJ software. All the primary and secondary antibodies were purchased from Abcam if not specifically indicated.
3.2.2 Quantitative reverse transcription polymerize chain reaction (qRT-PCR)

Principle

qRT-PCR has been considered as the benchmark for the detection and quantification of RNA targets [5]. Reverse transcription begins by reverse transcribing the desired mRNA transcript into cDNA with the RTase. The cDNA that is first created is single stranded and is sequentially used as the template for PCR (Figure 3.4) [6]. The RT-PCR technique is very useful because of its high sensitivity to low input RNA quantities [7].

![Diagram of qRT-PCR process](image)

**Figure 3.4** The process of quantitative reverse transcription polymerize chain reaction (qRT-PCR).

Method

RNeasy mini kit from Qiagen was used to extract the total RNA. The concentration and
quality of the RNA were then evaluated with Nano drop-N100 from Thermo Scientific. After which, cDNA was synthesized with random hesamers and superscript reverse transcriptase from Invitrogen. Then, qRT-PCR was performed with CFX96 RT-PCR system from Bio-Rad. Primers specific to the targeted genes, which listed in Table 1, were obtained from primerbank. Through comparing the target-amplified products with glyceraldehyde-3-phosphate dehydrogenase (GAPDH), we generated the quantification graph of gene expression.

<table>
<thead>
<tr>
<th>Target</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>p53</td>
<td>GAGGTTGGCTCTGACTGTACC</td>
<td>TCCGTCCCAGTAGATTACCAC</td>
</tr>
<tr>
<td>p21</td>
<td>TGTCAGTCAGAACCCATGC</td>
<td>AAAGTCGAAGTCCATCGCTC</td>
</tr>
<tr>
<td>p16</td>
<td>GATCCAGGTGGGTAAGGTC</td>
<td>CCCCTGCAAACTTCGTTCCT</td>
</tr>
<tr>
<td>Nox4</td>
<td>CAGATGTTGGGGCTAGGATTG</td>
<td>GAGTGTTCGCGACATGGGTA</td>
</tr>
<tr>
<td>GAPDH</td>
<td>TCCACTGGCGTCTTCACC</td>
<td>GCCAGAGATGATGACCCCTTT</td>
</tr>
</tbody>
</table>

3.2.3 Western blotting

**Principle**

Western blotting is a classical and powerful technique to identify and separate proteins. Since the negative charged proteins will travel to the positive electrode under applied voltage, the travel speed is dependent on their molecular weight. By gel electrophoresis, proteins with different molecular weight could separate from protein mixtures. After which, the separated proteins are transferred to a membrane, and a band is generated for each protein. The protein of interest is then labeled with specific antibody. Since antibody only binds to target protein, only one band will be observed on the membrane. The thickness of the band represents the protein volume. Therefore, the protein expression could be quantified through comparing the protein bands’ thickness [8].

**Method**
The levels of related protein expression, such as NF-κB, p-NF-κB, Nox4 and Nrf2 were determined by western blotting. Briefly, attached cells or cell pellets were lysed by M-PER protein extraction reagent (Thermo Fisher), followed by centrifugation and mixing with 1x loading buffer. Then, the protein was denatured by heating 5 minutes at 95 °C. Equal amounts of targeted protein extracts (60 μg) were resolved and separated by SDS-PAGE and then electrotransferred onto polyvinylidene fluoride (PVDF) membrane. Thereafter, the protein transferred PVDF membranes were incubated with primary and secondary antibodies according to manufactures’ guidance. Finally, the protein bands were scanned with Odyssey CLx infrared imaging system (Li-Cor, USA). Coomassie blue staining of total proteins or GADPH were used as reference.

3.3 Statistic study

Principle

Student's t-test is most frequently used to determine if two groups of data are significantly different from each other, while the mean of the sample follows a normally distribution and the population standard deviation is unknown [9].

Method

All data of experiments are expressed as mean ± standard deviations. OriginPro 2016 was used to plot graphs and carry out all statistical analysis in this thesis. To determine the statistical significance difference between two experimental groups, null hypothesis testing that “samples having the equal means in population” was performed by two-tailed student’s t-test. However, Welch correction would be done on student’s t-test when the two experimental groups have unequal variances. A value of p < 0.05 or p < 0.01 was considered as statistically significant.

References


Chapter 4

Fabrication of Fibronectin Coated PDMS Substrates with Tunable Stiffness

This chapter aims to fabricate cell attachable substrates with tunable stiffness to mimic the ECM mechanics in vitro. With a wide range of elastic moduli (E) between 1 kPa and >1 MPa, polydimethylsiloxane (PDMS) was selected. Three types of crosslinking ratio (1:10, 1:40 and 1:70, crosslinker: base) were prepared. The Young’s modulus of 1:10, 1:40, and 1:70 PDMS were 750 kPa, 46 kPa, and 21 kPa respectively. To facilitate cell attachment, PDMS surfaces and TCPs (tissue culture plates) were coated with fibronectin. With further characterizations, such as water contact angle, immunostaining and ATR-FTIR, each substrate displayed constant surface energy and surface chemistry, which ensures matrix stiffness is the only variable of our system.
4.1 Introduction

In the past decades, growing evidence demonstrated ECM mechanics has profound effect on cell behaviors, such as cell adhesion, cytoskeleton reorganization, cell shape, cell survival, cell proliferation, cell metabolism and cell differentiation [1-3]. Beyond those involvements of tissue stiffness in ageing and ageing related disease discussed in Chapter 2, the effect of ECM stiffness on cell behaviors will be introduced in this chapter. As reported, the stiffness of polyacrylamide (PA) gels regulates focal adhesion formation and migration of cultured 3T3 fibroblasts and rat kidney epithelial cells [4]. In addition, collagen coated PA gels with different stiffness was shown to be able to guide mesenchymal stem cells differentiation into different lineages [2]. Moreover, ECM remodeling induced stiffness increase in tumors leads to tumor invasion and metastasis [5, 6]. Therefore, development of in vitro platforms with precisely controlled stiffness is essential to tissue engineering scaffolds as well as regenerative medicine strategies [7].

Since ECM mechanics plays vital regulatory roles in cell behaviors and tissue development, numerous materials systems have been developed to mimic the tissue stiffness for biophysical study. With elastic moduli (E) between ~0.1 kPa to ~100 kPa, PA gels could mimic the stiffness of many soft tissues and therefore have long been used in tissue engineering [2, 4, 8]. Other materials such as hyaluron, dextran, methylcellulose, fibrin and gelatin have also been employed to fabricate mechanics tunable hydrogels [9-14]. However, many tissues such as arterial walls (E ~800 kPa), basement membranes (E ~1 MPa) and skin (E ~80 kPa to ~280 kPa) are stiffer than all these mentioned materials [15-18]. Thus, for more effective study of cell mechanobiology, substrates with a wider range of physicochemical properties in a controlled manner are necessary. Polydimethylsiloxane (PDMS) substrate has been reported to be easily manipulated to mimic soft tissues over a three order-of-magnitude range, from approximately 1 kPa to >1 MPa without significant alterations in surface chemistry and bulk properties [7]. Besides tunable elastomeric properties, PDMS substrates also exhibit many other salient features, such as high gas permeability, low cost, high optical transparency, easy moldability and low auto fluorescence, which makes them excellent candidates for mechanobiology study [19-21].
Therefore, PDMS substrates with different stiffness were employed to mimic microenvironment in this thesis.

In this chapter, PDMS substrates with different stiffness were prepared based on the crosslinking ratios. The fabricated PDMS substrates were characterized with rheometer, ATR-FTIR spectra, fluorescence staining and contact angle accordingly. Then, the well prepared and characterized PDMS substrates were employed to culture cells. Figure 4.1 shows the general process of substrate preparation, surface modification and cell seeding.

Figure 4.1 Illustration of substrate preparation, surface modification and cell seeding.

4.2 Materials and methods

4.2.1 Substrate preparation and stiffness measurement

PDMS substrates were fabricated on 35mm cell culture dishes. PDMS prepolymer (Sylgard 184, Dow Corning) with three types of crosslinking ratio (1:10, 1:40 and 1:70, cross linker: base) were thoroughly mixed for 20 minutes and vacuumed for another 20 minutes in order to obtain well mixed bubbles free solution. Small drops of prepolymer solution were then poured into 35mm dishes followed by 10 seconds spin-coating under 1000 rpm. After which the substrates were baked in oven at 80 °C overnight.

PDMS shear elastic modulus was measured using a Physica MCR 501 Rheometer (Anton Paar, Graz, Austria) with a parallel plate geometry (PP25/TG) and Rheoplus software according to the methods previously described [22]. PDMS storage shear moduli were determined by oscillatory shear experiments performed with strain amplitude of 1% and oscillation frequency of 10 rad/s. All the tests were performed with triplicates.
4.2.2 Fibronectin coating and intensity characterization

The PDMS surfaces were modified with fibronectin to facilitate cell attachment. Prior to fibronectin incubation, the surfaces were first modified with a layer of sulfo-SANPAH. Briefly, the entire PDMS surface was covered by sulfo-SANPAH solution and followed by 10 minutes UV exposure at 365nm. After which, 50 mM HEPES buffer was used to rinse the surfaces at least two to three times to eliminate excessive sulfo-SANPAH. Then, the sulfo-SANPAH coated surfaces were covered with 50μM fibronectin solution and incubated overnight at 37 °C. Prior to cell seeding, the fibronectin coated PDMS surfaces were rinsed with sterile PBS solution several times and subjected to 254nm UV for 30 minutes for sterilization.

4.2.3 Attenuated total reflectance fourier transform infrared spectroscopy (ATR-FTIR)

Attenuated total reflectance fourier transform infrared spectroscopy (ATR-FTIR) spectroscopic analysis of PDMS substrates with and without fibronectin coating was performed on PerkinElmer Spectrum GX between the ranges of 600-4000 cm\(^{-1}\). The scan resolution was 2 cm\(^{-1}\) and the scan number was 8. The correction for water and CO\(_2\) was performed during measurement.

4.2.4 Wet contact angle

To determine the wettability of PDMS substrates and TCPs with and without fibronectin coating, 260-F4 goniometer (ramé-hart Instrument Co.) was employed to measure water contact angle. With DROP image software, the contact angle was obtained under circular geometry method.

4.3 Results

4.3.1 Fabrication of PDMS substrates with different stiffness
Three batches of 1:10, 1:40 and 1:70 PDMS were prepared and the shear moduli were measured. The storage moduli $G'$ of these substrates were presented in Figure 4.2 (A). There was no statistical difference between these three batches indicates that the fabrication procedure was reproducible.

Based on literatures, the dynamic Young’s modulus of rubber elastic materials is approximately two times larger than its storage modulus, $E' \approx 3 \ G'$, so we converted the average storage modulus of PDMS into Young’ modulus, $E$ using this formula $[22]$. The calculated $E$ of 1:10, 1:40, and 1:70 PDMS was 750kPa, 46kPa, and 21kPa respectively, as shown in Figure 4.2 (B). In order to exaggerate the effect of stiffness, 750kPa substrate was used as stiff matrix, while 46kPa and 21kPa represented the soft matrix. Tissue culture plates (TCPs) with stiffness in megapascals were as also used as stiff matrix. TCPs were used as the control substrate for comparison as well.

Figure 4.2 Characterization of PDMS mechanical properties. (A) Storage modulus of PDMS substrates measured by rheometer. Three batches of samples were detected. (B) PDMS young’s modulus converted from storage modulus. Statistical significance was evaluated by Student's t-test, ** represent $p < 0.01$.

4.3.2 PDMS substrates surface modification and characterization

Despite excellent optical, chemical and mechanical properties, PDMS substrates are biologically inert and often lead to poor cell attachment. Thus, surface modification with
ECM protein is necessary to facilitate cell attachment and spreading. Fibronectin is the most widely used protein to promote cell adhesion. Fibronectin has several integrin binding peptides such as Arg-Gly-Asp (RGD), Leu-Asp-Val (LDV), Pro-His-Ser-Arg-Asn (PHSRN) and Arg-Glu-Asp-Val (REDV) [23-26]. With this unique nature of structure, fibronectin has been extensively used for cell culture related surface modification. Different methods of surface modifications have been reported, either directly incubating PDMS surface with fibronectin solution for physical crosslinking or covalently binding fibronectin residue to PDMS surface with chemical cross-linker. For a stable covalent fibronectin coating, here we used sulfo-SANPAH (sulfosuccinimidyl 6-(4′-azido-2′-nitrophenylamino) hexanoate) to modify the PDMS surface with a photo-activatable nitrophenyl azide, which provides an amine-reactive N-hydroxysuccinimide ester for fibronectin binding. After 10 minutes of sulfo-SANPAH treatment under 365nm UV, PDMS substrates were incubated with fibronectin solution for 6 hours at 37°C.

After surface modification, the samples surfaces were characterized using attenuated total reflectance fourier transform infrared spectroscopy (ATR-FTIR). As shown in the ATR-FTIR spectra in Figure 4.3, after fibronectin modification, all the PDMS substrates, as well as TCPs, exhibited specific peak at at 3384 cm⁻¹ (black curves). The broad peak at 3384 cm⁻¹ is related to N-H stretching from primary and secondary amines from fibronectin. This confirmed the successful coating of fibronectin on TCPs and PDMS substrates.
Figure 4.3 ATR-FTIR spectra of TCPs and PDMS substrates with and without fibronectin coating at the range of 600-4000 cm\(^{-1}\). Red curves represent substrates without fibronectin coating, while black curves represent fibronectin coated substrates.

To further verify the coating intensity of fibronectin, the substrates with different stiffness were characterized by fluorescent microscopy. Primary fibronectin antibodies and Alexa Fluor® 488 goat anti-mouse secondary antibodies were used to stain the coated protein according to manufacturer’s protocol. Un-coated PMDS substrates were used as the blank. Fluorescent images were taken under fluorescent microscopy (Nikon 80i) at the same exposure time. The mean fluorescent value was subtracted by the fluorescent value of blank substrate. The results showed that fibronectin is evenly coated on PDMS substrates with different compliance and there was no significant difference in protein absorption as shown in Figure 4.4.
Figure 4.4 Mean fluorescent value of fibronectin on PDMS substrates with different stiffness. Sulfo-SANPAH crosslinked PDMS substrates were incubated with 50 μM fibronectin for 6 hours at 37°C. The upper panel shows the fluorescence image of coated fibronectin, while the lower panel represents the quantified fluorescence intensity based on the upper panel.

Substrate surface energy might affect cell adhesion as well as cell behaviors [27, 28]. Thus, to further verify fibronectin coating’s effect on surface wettability, water contact angles of TCPs and PDMS substrates were measured. From Figure 4.5, the uncoated PDMS substrates exhibited significantly higher contact angles when compared to the uncoated TCPs surface. Each PDMS substrate exhibited a water contact angle ~110°, which indicated PDMS surface is hydrophobic and the value is comparable to previously documented value [29]. After fibronectin coating, both TCPs and PDMS substrates possessed highly wettable surfaces as shown in the complete spreading of water droplet.
Figure 4.5 Water contact angles of the surface of the PDMS substrate with and without fibronectin coating. The water droplets completely spread on fibronectin coated surfaces, and thus the contact angle was considered as 0, which was not displayed in the quantification.

4.4 Conclusion

Before exploring the effects of ECM stiffness on cell oxidative stress response, we assessed the ease of fabrication and the reproducibility PDMS as the substrate for tuning stiffness. PDMS was used mainly due to its high optical transparency and easily tunable and wide range of stiffness. The tunability is also highly reproducible. In addition, PDMS does not swell significantly in aqueous environments providing the material prolonged stability in dimension as well as elasticity [30]. Though the surface maybe highly hydrophobic, it is easily modified using fibronectin. This step is also crucial to ensure that the surface chemistry of all substrates tested is similar both in chemistry and concentration. Based on
ATR-FTIR spectra, fluorescence staining and contact angle characterizations, the fibronectin coated TCPs and fibronectin coated PDMS surfaces exhibit similar surface chemistry and high wettability; these features further ensured that the matrix stiffness is the only variable in our experimental system. In the following chapters, the platform will be used for cell oxidative stress response studies.

**References**


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4974-4984.


Compliant substratum guides endothelial commitment from human pluripotent stem cells. 

Chapter 5

Soft Matrix Prevents H$_2$O$_2$ Induced Human Dermal Fibroblasts Apoptosis via Inhibition of NF-κB Activation

The purpose of this study is to investigate the relationship between matrix stiffness and HDFs oxidative stress response. Molecular and cellular damages induced by sustained ROS accumulation have been considered as reasons of impaired chronic wound healing and fibrotic diseases in elderly people. Loss of tissue elasticity and increase of stiffness have long been involved in aged and fibrotic tissues. However, the interaction between matrix stiffness and ROS regulation remains unclear. It is hypothesized that matrix stiffness could affect cell oxidative stress response. To explore the hypothesis, HDFs were cultured on PDMS substrates with different stiffness, followed by H$_2$O$_2$ treatment. HDFs oxidative stress response was then measured with FACS. Based on the results, HDFs on stiff matrix were more susceptible to H$_2$O$_2$ induced ROS accumulation and apoptosis. It is further revealed that stiffness regulated oxidative stress response is independent of Rho/ROCK pathway. Moreover, soft matrix protects HDFs against H$_2$O$_2$ induced apoptosis via inhibition of NF-κB activation. The present study showed that matrix stiffness has strong implications on cell oxidative stress response.
5.1 Introduction

Reactive oxygen species (ROS), a necessary evil for cell signaling [1], plays significant biological roles in a wide spectrum that covers from physiological regulatory functions to damaging alterations involving in the pathogenesis of many diseases and ageing related issues [2]. Low and moderate levels of ROS have been demonstrated to provide beneficial effects on killing of invading pathogens, promoting cell proliferation, as well as accelerating wound closure and tissue repair processes [3]. On the contrary, it is noticeable that oxidative stress generated by excessive accumulation of ROS results in detrimental effects in the pathogenesis of many diseases. The highly reactive nature of ROS could damage many biomolecules, such as proteins, lipids and nucleic acids. Through lipid peroxidation reactions, it reacts with membrane phospholipids and further breaks the membrane integrity. Additionally, abnormal modifications of amino acid, fragmentation of peptide chain and increased susceptibility to proteolysis are associated with ROS induced protein damage [4]. As such, persistent elevation of ROS significantly contributes to chronic wound impairment, non-healing wounds and endothelial dysfunction development [5, 6]. Meanwhile, disordered ROS regulation is involved in many tissue fibrotic diseases, such as radiation exposure induced pulmonary fibrosis, therapies related peritoneal\retroperitoneal fibrosis, as well as renal fibrosis [7-9]. ROS induced fibroblast apoptosis has been proposed as a mechanism for diabetes and ageing related impaired dermal wound healing [10, 11]. Sustained accumulation of oxidative stress initiates apoptosis formation in damaged organs and tissues and elevated apoptosis signaling will further delay wound healing processes [12].

As introduced above, ROS regulation plays essential roles in ageing related impairment in wound healing and fibrotic diseases, since sustained accumulation of high levels of ROS causes serious damages at molecular and cellular level. As the antioxidant ability decreasing with age, older individuals are more prone to chronic wounds, as well as tissue fibrosis related diseases [13, 14]. Interestingly, increased tissue stiffness has long been observed in ageing and fibrotic tissue, as reviewed in Chapter 2. But, the connections between tissue stiffness alteration and ROS regulation are poorly understood.
Based on the above, we hypothesized that the matrix stiffness could affect cell oxidative stress response. The antioxidant ability of HDFs cultured on matrix with different stiffness was measured after H$_2$O$_2$ treatment. Our results revealed that HDFs on stiff matrix are prone to H$_2$O$_2$ induced ROS accumulation and apoptosis, while HDFs on soft matrix showed strong oxidative stress resistance. We further demonstrated that matrix stiffness regulated oxidative stress response is independent of Rho/ROCK pathway. Additionally, H$_2$O$_2$ induced apoptosis on stiff matrix is dependent on excessive ROS mediated NF-$\kappa$B activation. Meanwhile, soft matrix prevents H$_2$O$_2$ induced apoptosis via inhibition of NF-$\kappa$B activation. The present study showed that physical microenvironment has strong implications on cell oxidative stress response.

5.2 Materials and methods

5.2.1 H$_2$O$_2$ treatment
HDFs were seeded on surfaced treated PDMS substrates and TCPs with at a density of 30000 cells per 35mm plate, followed by 24hrs culture in the incubator to allow sufficient attachment and spreading. 30% hydrogen peroxide (Sigma-Aldrich) was mixed with medium 106 to prepare 10 $\mu$M, 100 $\mu$M and 1000 $\mu$M H$_2$O$_2$ contained medium. Then, cells were incubated with H$_2$O$_2$ mixed medium for further characterization.

5.2.2 ROS measurement
ROS accumulation was measured by previously described methods with modifications [15]. Briefly, CM-H$_2$DCFDA (Invitrogen Life Technologies, CA, USA) was mixed with medium 106 reaching a final concentration at 10 $\mu$M, HDFs were incubated with CM-H$_2$DCFDA mixture for 30 min at 37 $^\circ$C, protected from light. Then, HDFs were trypsinlized for FACS analysis with flow cytometry (BD Accuri C6, Inc., Ann Arbor, USA). Empty controls were prepared accordingly. Flowjo 7.6 software was employed to analyze data. All experiments were performed in triplicates.

5.2.3 Apoptosis analysis
After 2 hours H$_2$O$_2$ treatment, cell apoptosis was detected by Annexin V/propidium iodide (PI) staining coupled with FACS. Adhered cells were harvested by trypsinization, washed with PBS, and subjected to Annexin V/PI staining for FACS analysis. All experiments were performed in triplicates. Empty controls (without Annexin V/PI staining) were prepared accordingly. The percentage of Annexin V+/PI− and Annexin V+/PI+ cells were quantified as apoptotic cells.

5.2.4 Acridine orange (AO) staining

For AO staining, 2hrs after H$_2$O$_2$ treatment, cells were stained with 5mg/ml acridine orange in fresh M106 medium, followed by 10 minutes incubation at 37°C, then washed with PBS for 3 times and fixed with 4% paraformaldehyde for 15 minutes. Fluorescence images were visualized with confocal laser scanning microscope system (Leica TCS SP5) using 20X objective lens.

5.3 Results

5.3.1 Optimization of H$_2$O$_2$ working concentration

As one of the most predominant regulators in mitochondrial and endoplasmic reticulum (ER) stress, oxidative stress could result in many cellular damages, such as inflammation, apoptosis and other serious abnormalities [16]. Sub-lethal dose of hydrogen peroxide (H$_2$O$_2$) as an oxidative stress supplier to induce mammalian cells apoptosis has been widely used in cellular biological experiments. As reported, the optimal concentration of H$_2$O$_2$ depends on cell types and ranges from 10 $\mu$M to 1000 $\mu$M. In order to maximize the sensitivity of the experiments, we performed a calibration of H$_2$O$_2$ concentration. HDFs were seeded on tissue culture plates (TCPs) at 30000 cells per 35mm plate. After 24hrs of cell attachment and spreading, fresh mixed M106 medium with a final H$_2$O$_2$ concentration of 0 $\mu$M, 10 $\mu$M, 100 $\mu$M, 1000 $\mu$M were added to each group and cultured for another 24hrs before data was collected.
As shown in Figure 5.1, 1000 μM H₂O₂ treatment is detrimental to the cells and there were hardly any cells surviving after 24 hours. Cell viability was not significantly affected by 10 μM H₂O₂. HDFs treated with 10 μM H₂O₂ were even observed to have more proliferation, which is consistent with literature that suggests low external oxidative stress could promote cell proliferation [17]. Under 100 μM H₂O₂ treatment, cell density was distinctly less than the control group, which suggested H₂O₂ induced apoptosis. Thus, 100 μM has been established as the optimal concentration of H₂O₂ to study the effect of matrix stiffness in our further experiments.

![Figure 5.1](image)

**Figure 5.1** Optimization of H₂O₂ working concentration. 24 hours after cell seeding, fresh mixed M106 medium with a final H₂O₂ concentration of 0 μM, 10 μM, 100 μM, 1000 μM was applied to induce HDFs apoptosis. Images were collected by optical microscope after 24hr of H₂O₂ induction. The scale bar is 200μm.

### 5.3.2 Soft matrix inhibits H₂O₂ induced HDFs apoptosis

Fibronectin coated PDMS substrates with different crosslinking ratios were employed to mimic the microenvironment with different stiffness. TCPs (with stiffness in the range of megapascals) and 750kPa substrate were used to represent the stiff ECM, while 46kPa and 21kPa PDMS substrates represented the soft ECM. Then, HDFs were seeded on these substrates and cultured for 24hrs, followed by 100 μM H₂O₂ treatment.

To explore the effect of matrix stiffness on oxidative stress induced apoptosis, ROS accumulation and cell apoptotic levels induced by H₂O₂ treatment were monitored by flow cytometry. CM-H₂DCFDA is widely used for intracellular ROS detection. It is cell permeable and non-fluorescent in the absence of ROS. Once oxidized by excessive ROS production, it exhibits green fluorescence [18]. According to Figure 5.2 (A-D), HDFs on
each stiffness exhibit a narrow range of DCF fluorescence intensity distribution without H$_2$O$_2$ treatment (dashed grey lines), while the range distribution became much wider after H$_2$O$_2$ treatment (solid red lines). This result indicated that H$_2$O$_2$ affects ROS production of HDFs. But the effect, using this treatment time and concentration, may not be uniform across all cells as shown by the diverse distribution of ROS intensity after H$_2$O$_2$ treatment. Additionally, after H$_2$O$_2$ treatment, significant right shift of the peaks was observed on stiff matrix (Figure 5.2 (A-B)), while no significant difference was observed on soft matrix (Figure 5.2 (C-D)). This indicates H$_2$O$_2$ treatment causes robust effect on ROS accumulation on stiff matrix, but minimum effect on soft matrix. Based on the quantification in Figure 5.2 (E), the mean DCF fluorescence intensity indicated that intracellular ROS accumulation increases more than 3 times on stiff matrix, while it was only slightly increased on soft matrix. Thus, soft matrix reduces H$_2$O$_2$ induced intracellular ROS accumulation.

![Figure 5.2](image)

**Figure 5.2** Soft matrix reduces H$_2$O$_2$ induced intracellular ROS accumulation. After 24 hours attachment on matrix with different stiffness, HDFs were treated with 100μM H$_2$O$_2$. After 1 hour H$_2$O$_2$ treatment, the intracellular ROS production was detected by H$_2$DCFDA flow cytometry. (A-D) Representative DCF fluorescence intensity histograms on matrix with different stiffness and (E) quantification of mean DCF fluorescence intensity from three independent experiments. (A-D)
Solid grey areas represent experiments without H$_2$O$_2$ treatment and H$_2$DCFDA staining, dashed grey lines represent ROS fluorescence intensity without H$_2$O$_2$ treatment, red lines represent ROS fluorescence intensity after H$_2$O$_2$ treatment. (E) Light grey and dark grey represent the mean ROS fluorescence intensity without and with H$_2$O$_2$ treatment respectively. Three independent experiments were performed. Statistical significance was evaluated by Student’s t-test, ** represent p < 0.01.

Interestingly, significant morphological alteration was also observed under H$_2$O$_2$ treatment. 2hrs after H$_2$O$_2$ treatment, HDFs morphology was observed with optical microscope. As shown in Figure 5.3 (A-B), on stiff matrix, many cells detached and floated, other attached cells showed distinctly round cataplastic morphology with membrane blebbing, which indicated that cells are undergoing apoptosis. However, cell morphology on soft matrix was not affected much after H$_2$O$_2$ treatment (Figure 5.3 (C-D))

**Figure 5.3** H$_2$O$_2$ induces distinct morphological alteration on stiff matrix. 2 hours after 100 μM H$_2$O$_2$ treatment, HDFs morphology on different matrix stiffness was compared. The upper and lower panels represent HDFs before and after H$_2$O$_2$ treatment respectively. (A-D) Representative images of HDFs on matrix from stiff to soft under H$_2$O$_2$ treatment. Images were collected by optical microscope. The scale bar is 200μm.

As above, more apoptotic cells were observed on stiff matrix after H$_2$O$_2$ treatment. To further quantify the proportion of apoptotic cells on each matrix, annexin V/ PI staining was performed and analyzed with flow cytometry. During early apoptosis,
phosphatidylserine (PS) will transfer to the outer surface of the plasma membrane, which can be detected by FITC labeled annexin V [19]. During late apoptosis, the plasma and nuclear membranes will break, which allows PI to go into the nucleus and generate red fluorescence [20].

Thus, the Annexin V/PI method is a classic approach to evaluate cell apoptosis [21]. From Figure 5.4 (B), on matrix with different stiffness, HDFs mainly distributed in the lower left section, which indicated cells exhibit neither positive annexin V nor positive PI staining, and thus they maintained viable (Figure 5.4 (B), upper panel). When subject to H$_2$O$_2$ treatment, HDFs on stiff matrix mainly displayed in the upper right area, which indicated cells exhibit both positive annexin V and positive PI staining and represented late apoptosis. However, HDFs on soft matrix still mainly distributed in the lower left section and are still viable (Figure 5.4 (B), lower panel). Based on the quantification data in Figure 5.4 (C), only low levels of apoptotic cells were detected on each matrix (light grey bars) when they were not subjected to H$_2$O$_2$ treatment, though the percentage of apoptotic cells was slightly higher on soft matrix (up to 7%) when compared to stiff matrix (up to 3%). After H$_2$O$_2$ treatment, up to 65% HDFs were detected with apoptosis on stiff matrix, while it was only 9% on soft matrix (dark grey bars). These findings indicated that H$_2$O$_2$ induce universal cell apoptosis on stiff matrix, while cells on soft matrix showed greater resistance against apoptosis probably due to lower oxidative stress.
Figure 5.4 Soft matrix protects HDFs against H$_2$O$_2$ induced apoptosis. 2 hours after H$_2$O$_2$ treatment, HDFs were labeled with Annexin V/propidium iodide (PI) staining, and the levels of cell apoptosis were determined by flow cytometry. (A) Flow cytometry results of cells without staining, with only PI staining and only annexin V staining. (B) Flow cytometry results of cells on different stiffness with annexin V/PI counterstaining. The upper and lower panel represent before and after H$_2$O$_2$ treatment respectively. The gate selection in (B) is compensated to (A). (C) Quantification of the percentage of apoptotic cells extracted from (B), HDFs displayed in right side of each grid was counted as apoptotic cells. Light grey and dark grey represent cell apoptosis percentage without and with H$_2$O$_2$ treatment respectively. Three independent experiments were performed. Statistical significance was evaluated by Student’s t-test, ** represent p < 0.01 and *** represent p < 0.001.
respectively.

In oxidative stress induced apoptosis, cell membrane rupture is caused by the abnormal release of membrane protein hydrolase, which is mediated by lysosome membrane permeabilization (LMP) and the consequent leakage of lysosomal content [22]. It has also been demonstrated that susceptibility of lysosomes to rupture is a determinant factor for plasma membrane disruption in cell death [23].

![Figure 5.5](image.png)

Figure 5.5 Soft matrix inhibits H$_2$O$_2$ induced lysosome membrane permeabilization (LMP). 2 hours after H$_2$O$_2$ treatment, HDFs were labeled with acridine orange and visualized by confocal laser microscope. The upper panel represents AO emits green fluorescence when it dispersedly distributes in cytosol and nucleus. The intermediate panel represents AO emits red fluorescence concentrated exists in lysosomes. The lower panel is the merged images of the green and red channel. The scale bar is 100μm.

As a lysosomotropic metachromatic fluorochrome, acridine orange (AO) is often used to determine LMP [24], we performed AO staining to monitor LMP of HDFs under H$_2$O$_2$ treatment on each substrate. The fluorescence emission of AO is concentration dependent,
from red at high concentrations (in lysosomes) to green at low concentrations (in the cytosol and nucleus) [25]. As such, once the lysosome membrane breaks down, the red fluorescence will decrease in AO-loaded cells. As shown in Figure 5.5, on stiff matrix, weak red color was displayed, which revealed the lysosome membrane has been broken down. On the contrary, bright red color was detected in cells cultured on soft matrix, which indicated the integrity of lysosome membrane has not been disrupted by H\textsubscript{2}O\textsubscript{2} treatment. Obviously, LMP only occurred in cells cultured on stiff substrates under H\textsubscript{2}O\textsubscript{2} treatment, which is in accordance with the observation that soft matrix inhibits H\textsubscript{2}O\textsubscript{2} induced HDFs apoptosis.

5.3.3 Matrix stiffness mediated HDFs apoptosis is ROCK independent

As above, matrix stiffness plays essential roles in oxidative stress mediated ROS accumulation, LMP and apoptosis. Soft matrix significantly protected HDFs against H\textsubscript{2}O\textsubscript{2} induced damage, but the mechanism behind is not known yet. ROCK pathway has been shown to be widely involved in both stiffness mediated mechanotransduction and oxidative stress induced apoptosis [26-31]. On one hand, tensile force exerted on stiff matrix results in compensatory Rho/ROCK activation, which could increase actomyosin-mediated cellular tension and reestablish force equilibrium. On another hand, pharmacological inhibition of Rho- kinase (ROCK) signaling improves oxidative stress resistance and inhibits oxidative stress induced apoptosis [32-34].
Therefore, it is speculated that Rho/ROCK pathway may be involved in the observations of our results thus far. To elucidate the role of Rho/ROCK pathway in matrix stiffness mediated oxidative stress response, HDFs on both stiff and soft matrix were pretreated with Y27632 (a widely used ROCK inhibitor) followed by H$_2$O$_2$ treatment. The intracellular
ROS accumulation and cell apoptotic levels were examined with flow cytometry. As presented in Figure 5.6 (A-D), the black lines (pretreated with Y27632 followed by H$_2$O$_2$) were almost coincided with the red lines (only treated with H$_2$O$_2$) and no significant peak shift was observed on each matrix with different stiffness (black lines versus red lines). Meanwhile, the quantification data in Figure 5.6 (E) indicated the mean ROS fluorescence intensity induced by H$_2$O$_2$ was similar with or without Y27632 treatment (dark grey bars versus grey bars). Therefore, pretreatment with Y27632 did not affect H$_2$O$_2$ induced ROS accumulation indicating that soft matrix reduces H$_2$O$_2$ induced ROS accumulation is ROCK independent.

The effect of Y27632 on H$_2$O$_2$ induced apoptosis was further examined by annexin V/ PI flow cytometry. As from the lower two panels of Figure 5.7 (A), Y27632 did not affect the distribution trends of HDFs on each matrix with different stiffness. No matter with or without Y27632 treatment, HDFs on stiff matrix mainly distributed in the right upper area, while HDFs on soft matrix mainly stayed in the left lower area, after H$_2$O$_2$ treatment. Based on the quantification in Figure 5.7 (B), Y27632 had no effect on the percentage of H$_2$O$_2$ induced cell apoptosis on each matrix with different stiffness (light blue bars versus dark grey bars).

Taken together, both ROS production and the proportion of apoptotic cells induced by H$_2$O$_2$ were not affected by Y27632 treatment (Figure 5.6 and Figure 5.7). Therefore, matrix stiffness mediated oxidative stress response is Rho/ROCK independent, although initially it was speculated to be the pathway involved.
Figure 5.7 Inhibition of Rho/ROCK has no effect on H$_2$O$_2$ induced apoptosis. HDFs were pretreated with 10μM Y27632 for 1 hour, followed by 1 hour H$_2$O$_2$ treatment. Then, cells were labeled with Annexin V/ PI staining, and the levels of cell apoptosis were determined by flow cytometry. (A) Flow cytometry results of cells on different stiffness with annexin V/ PI counterstaining. The upper and intermediate panels represent before and after H$_2$O$_2$ treatment respectively, and the lower panel represents cells pretreated with Y27632 followed by H$_2$O$_2$. (B) Quantification of the percentage of apoptotic cells extracted from (A), HDFs displayed in right side of each grid was counted as apoptotic cells. Light grey and dark grey represent cell apoptosis percentage without and with H$_2$O$_2$ treatment, and the light blue represent cells pretreated with Y27632 followed by H$_2$O$_2$. Three independent experiments were performed. Statistical significance
was evaluated by Student's t-test. ** represent p < 0.01 and *** represent p < 0.001, respectively.

**5.3.4 NF-κB signaling pathway is involved in matrix stiffness regulated oxidative stress response**

The other pathway that was studied is NF-κB signaling pathway. NF-κB (nuclear factor kappa B) proteins, a family of transcription factors, have profound importance in inflammation and immunity. Many NF-κB-regulated genes play important roles in regulating ROS accumulation. In turn, ROS has stimulatory roles in NF-κB signaling [35]. Numerous works demonstrated that oxidative stress induced apoptosis is mediated by NF-κB activation [36-39]. Under normal state, NF-κB resides in the cytoplasm in an inactive complex with the inhibitor kappa B (IκB). Under oxidative stress, IκB will release from the complex and phosphorylated NF-κB will translocate into the nucleus, bind to DNA control elements and influence the transcription of specific genes that determine cellular fate and also illicit an immune response to stressed tissue [40].

Therefore, we next investigated whether NF-κB signaling pathway is involved in matrix stiffness regulated oxidative stress response. The subunit of NF-κB, p65, was examined by western blotting. As shown in Figure 5.8 (A), strong phosphorylated p65 blotting bands were only observed on stiff matrix (TCPs and 750kPa PDMS) under H$_2$O$_2$ treatment. Among experiments without H$_2$O$_2$ treatment, as well as soft matrix (46kPa and 21kPa) exposed to H$_2$O$_2$, cells only exhibited weak phosphorylated p65 bands. We then quantified the intensity of each bands and normalized to total p65 expression. Based on the quantification in Figure 5.8 (B), the expression of phosphorylated p65 increased up to 7 folds on stiff matrix under H$_2$O$_2$ treatment, while it was only slightly increased on soft matrix. Interestingly, in control groups without H$_2$O$_2$ treatment, HDFs on soft matrix were detected with slightly higher p65 phosphorylation. Based on these findings, we speculated that stiff matrix stimulated HDFs apoptosis under H$_2$O$_2$ treatment is mediated by NF-κB activation, while soft matrix mediated protection may be NF-κB signaling independent.
### 5.3.5 Soft matrix prevents H$_2$O$_2$ induced apoptosis via inhibition of NF-$\kappa$B activation

To further examine our speculation, Pyrrolidinedithiocarbamate ammonium (PDTC), an NF-$\kappa$B inhibitor, was applied to inhibit NF-$\kappa$B expression. With PDTC treatment, distinctly morphological alteration was observed in HDFs on stiff matrix, while cell morphology was not affected on soft matrix, as shown in the lower panel of **Figure 5.9**. On stiff matrix, most cells showed a rounder shape after treatment. Meanwhile, the cell density decreased as a result of PDTC cytotoxicity [41]. However, no significant effect was observed on cells cultured on soft matrix both in morphology and cell density. These
evidences further supported our prediction that stiff matrix mediated cell apoptosis is NF-κB activation dependent while it may not be involved in the mechanism of survival of cells on the soft matrix.

![Image of cell morphology](image)

**Figure 5.9** Inhibition of NF-κB changes HDFs morphology on stiff matrix. 24 hours after cell seeding, cells were treated with 100μM PDTC to inhibit NF-κB activity. 1 hour after PDTC treatment, images were collected by optical microscope. The upper panel represents control groups without PDTC treatment. The lower panel represents HDFs treated with PDTC for 1 hour. The scale bar is 200μm.

Next, we examined PDTC’s effect on H₂O₂ stimulated oxidative stress response via flow cytometry. As shown in **Figure 5.10 (A-D)**, on each stiffness, PDTC pretreatment led to distinct left shift of the ROS fluorescence intensity peak (green lines versus grey lines). Moreover, H₂O₂ did not stimulate ROS accumulation in PDTC pretreated HDFs on all stiffness, since significant left shift of the peak was observed when comparing PDTC treated cells to non-PDTC treated cells under H₂O₂ treatment (blue lines versus red lines). In addition, PDTC pretreatment caused HDFs to maintain low and almost fixed levels of ROS, no matter under normal condition or oxidative stress, since similar ROS intensity distribution curves were detected in PDTC pretreated cells (blue lines versus green lines).

According to the quantified mean ROS intensity in **Figure 5.10 (E)**, PDTC treatment resulted in around 3 folds decrease of ROS production without H₂O₂ treatment. H₂O₂ did not induce ROS accumulation in PDTC treated cells on all matrix with different stiffness.

Taken together, PDTC locked down ROS accumulation in HDFs on both stiff and soft
matrix, even under H$_2$O$_2$ treatment. Therefore, H$_2$O$_2$ induced ROS accumulation on stiff matrix is NF-κB activation dependent since inhibiting NF-κB activation could block H$_2$O$_2$ induced ROS accumulation. Meanwhile, since no significant NF-κB activation was observed on soft matrix, it is deduced that soft matrix reduces H$_2$O$_2$ induced ROS accumulation via inhibition of NF-κB activity.

**Figure 5.10** Inhibition of NF-κB blocks H$_2$O$_2$ induced ROS accumulation. HDFs were pretreated with 100μM PDTC for 1 hour, followed by 1 hour H$_2$O$_2$ treatment. Then, cells were labeled with CM-H$_2$DCFDA, and the levels of intracellular ROS were determined by flow cytometry. **(A-D)** Representative DCF fluorescence intensity histograms on matrix with different stiffness and **(E)** quantification of mean DCF fluorescence intensity from three independent experiments. **(A-D)** Solid grey areas represent experiments without H$_2$O$_2$ treatment and H$_2$DCFDA staining. Dashed grey lines represent experiments with only H$_2$DCFDA staining and no H$_2$O$_2$ treatment. Red lines represent ROS fluorescence intensity after H$_2$O$_2$ treatment. Green lines represent experiments with only PDTC treatment and no H$_2$O$_2$ treatment. Blue lines represent ROS fluorescence intensity of cells pretreated with PDTC followed by H$_2$O$_2$ treatment. **(E)** Light grey and grey represent the mean ROS fluorescence intensity of cells without and with H$_2$O$_2$ treatment respectively. Light blue and blue represent the mean ROS fluorescence intensity of cells only treated with PDTC and pretreated with PDTC followed by H$_2$O$_2$ treatment respectively. Statistical significance was evaluated by
Student's t-test, * represents p < 0.05 and ** represent p < 0.01.

PDTC’s effect on H₂O₂ stimulated apoptosis was next examined by annexin V/ PI flow cytometry. As shown in Figure 5.11 (A), PDTC itself caused slight cell apoptosis oneach matrix, as shown by some cells distributed in the lower right area of each grid, which represents early apoptosis (from top to bottom, the first panel versus the third panel). PDTC pretreatment significantly attenuated H₂O₂ induced apoptosis on stiff matrix, as shown by the shift from right upper area to lower left area (from top to bottom, the second panel versus the last panel). Based on the quantification in Figure 5.11 (B), PDTC treatment itself slightly increased cell apoptosis on stiff matrix, from around 3% to 9%, which is consistent with what we observed from optical microscope (Figure 5.9). Meanwhile, PDTC pretreatment sharply down-regulated H₂O₂ stimulated apoptosis from 64% to 20% and 59% to 11%, on stiff TCPs and 750kPa PDMS, respectively. No significant difference in cell apoptosis was detected on soft matrix with or without PDTC treatment.

Taken together, NF-κB signaling pathway is involved in matrix stiffness regulated oxidative stress response and H₂O₂ induced apoptosis on stiff matrix is NF-κB activation dependent. Once NF-κB activation is blocked with PDTC, the apoptosis will be significantly ameliorated. Meanwhile, soft matrix inhibits NF-κB activation indicates soft matrix prevents H₂O₂ induced apoptosis via inhibition of NF-κB activation.
Figure 5.11 Inhibition of NF-κB attenuates H₂O₂ induced apoptosis. HDFs were pretreated with 100μM PDTC for 1 hour, followed by 1 hour H₂O₂ treatment. Then, cells were labeled with Annexin V/PI staining, and the levels of cell apoptosis were determined by flow cytometry. (A) Flow cytometry results of cells on different stiffness with annexin V/PI counterstaining. From top to bottom, each panel represents experiments without PDTC and H₂O₂ treatment, with only H₂O₂ treatment, with only PDTC treatment, and with PDTC treatment followed by H₂O₂ treatment accordingly. (B) Quantification of the percentage of apoptotic cells extracted from (A), HDFs...
displayed in right side of each grid were counted as apoptotic cells. Light grey and dark grey represent cell apoptosis percentage without and with H$_2$O$_2$ treatment, light blue and blue represent cell apoptosis percentage of HDFs only treated with PDTC and pretreated with PDTC followed by H$_2$O$_2$ treatment respectively. Three independent experiments were performed. Statistical significance was evaluated by Student's t-test, * represents p < 0.05, ** represent p < 0.01 and *** represent p < 0.001, respectively.

5.4 Conclusion

In summary, the results presented in this chapter show matrix stiffness plays regulatory roles in oxidative stress induced ROS production and excessive ROS mediated cell apoptosis. Under H$_2$O$_2$ induction, most of the HDFs were detected with elevated ROS accumulation and could not survive on stiff matrix, while HDFs were not affected on soft matrix. It reveals that HDFs cultured on stiff matrix are more susceptible to oxidative stress, while HDFs on soft matrix exhibit oxidative stress resistance. Inhibiting Rho/ROCK pathway with Y27632 did not attenuate H$_2$O$_2$ induced ROS and apoptosis on stiff matrix, which indicates matrix stiffness regulated oxidative stress response is Rho/ROCK independent. Elevated NF-κB activation triggered by H$_2$O$_2$ was observed on stiff matrix, but not on soft matrix. Blocking NF-κB activation with PDTC significantly attenuated H$_2$O$_2$ induced ROS and apoptosis on stiff matrix. As significant NF-κB activation was not observed in cells on soft matrix with H$_2$O$_2$ exposure, it is reasonable to deduce that soft matrix prevents H$_2$O$_2$ induced apoptosis via inhibition of NF-κB activation.

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Chapter 6

Matrix Stiffness Regulates Human Dermal Fibroblasts Oxidative Stress Response via Nox4/Nrf2 Regulation

This chapter aims to understand why HDFs on matrix with different stiffness exhibit different oxidative stress tolerance. Since Nox4/Nrf2 balance is responsible for oxidative stress response, the levels of Nox4 and Nrf2 expression of HDFs on different stiffness were detected. Then, the connections between matrix stiffness and Nox4/Nrf2 were proposed and examined. Based on the results, the schematic illustration of matrix stiffness regulation of human dermal fibroblasts oxidative stress response via Nox4/Nrf2 regulation was mapped. In specific, soft matrix inhibits YAP nucleus localization, which further blocks Nox4 expression and elevates Nrf2 expression. For cells cultured on soft matrix, decreased Nox4 leads to poor oxidative stress sensitivity, while increased Nrf2 results in effective oxidative stress defense. To sum up, matrix stiffness dependent oxidative stress response is regulated by YAP-Nox4/Nrf2 signaling pathway.
6.1 Introduction

In Chapter 5, it has been demonstrated that matrix stiffness has significant effect on oxidative stress induced ROS accumulation. HDFs on stiff matrix were sensitive to oxidative stress, while HDFs on soft matrix showed strong antioxidant ability. However, how matrix stiffness regulates cells’ oxidative stress response remains unanswered. Therefore, this chapter aims to elucidate the possible mechanisms behind such observations. As reviewed in Chapter 2, Nox4-Nrf2 redox imbalance contributes to intracellular ROS accumulation in ageing and age-related fibrotic diseases [1-3]. Elevated expression of the ROS-generating enzyme, Nox4, and an impaired capacity to induce the Nrf2 antioxidant response alters the cellular redox homeostasis [4]. Since Nox4/Nrf2 regulation is responsible for oxidative stress response, this chapter mainly explored the relationship between matrix stiffness and Nox4/Nrf2 expression.

Separately, recent studies showed Yes-associated protein (YAP) is involved in both ECM stiffness mediated mechanotransduction and Nox4 expression. On one hand, YAP has been identified as an important sensor of mechanical signals exerted by the cellular microenvironment where many studies have reported both ECM stiffness and cell geometry have profound effects on YAP activity [5-7]. On another hand, YAP is necessary for efficient Nox4 expression both in vitro and in vivo, inhibition of YAP leads to decreased Nox4 expression [8].

Based on the above information, it was speculated that matrix stiffness could regulate Nox4/Nrf2 related oxidative stress response via modulating YAP expression. To verify this hypothesis, Nox4 and Nrf2 expression were monitored for cells cultured on different substrate stiffness using western blotting and qRT-PCR. The results indicated that matrix stiffness has profound effects on Nox4/Nrf2 balance. Stiff matrix triggered Nox4 expression and blocked Nrf2 expression, while an opposite manner was observed on soft matrix. With further analysis, YAP was confirmed to be involved in matrix stiffness mediated redox regulation. In summary, matrix stiffness dependent oxidative stress response is probably regulated by YAP-Nox4/Nrf2 signaling pathway.
6.2 Materials and methods

Western blotting, qRT-PCR and immunostaining were employed in this chapter. The detailed methods were introduced in Chapter 3.

6.3 Results

6.3.1 Soft matrix inhibits Nox4 expression

NAD(P)H oxidases (Noxs) are major enzymes responsible for superoxide (O$_2^-$) production and they could transfer electrons across the membrane from NAD(P)H to molecular oxygen [9, 10]. Thus, Noxs play essential roles in ROS regulation. Nox4, one of the Noxs, has been identified as a major source of oxidative stress [11]. Nox4 dependent ROS production is involved in many pathological processes. Since matrix stiffness affected ROS accumulation induced by H$_2$O$_2$, we are interested to know whether matrix stiffness regulated oxidative stress response is mediated by Nox4.

![Image](A) Western blotting bands of Nox4 expression and coomassie staining of total protein. Protein loading was normalized to coomassie staining. (B) Nox4 protein

Figure 6.1 Soft matrix inhibits Nox4 expression. The effect of matrix stiffness on Nox4 expression was determined by western blotting and qRT-PCR. 24 hours after cell seeding, the proteins and mRNA were extracted respectively. (A) Western blotting bands of Nox4 expression and coomassie staining of total protein. Protein loading was normalized to coomassie staining. (B) Nox4 protein
expression normalized to total protein. The intensity of the protein bands was quantified by ImageJ. (C) mRNA fold change of Nox4 on different stiffness, the relative gene expression was normalized to GAPDH. Three independent experiments were performed. Statistical significance was evaluated by Student's t-test, * represents p < 0.05, ** represent p < 0.01 and *** represent p < 0.001, respectively.

The levels of Nox4 expression was examined by qRT-PCR and western blotting. As shown in Figure 6.1 (A), strong Nox4 protein bands were only observed on stiff matrix. The quantification in Figure 6.1 (B) indicated cells on stiff matrix expressed significant more Nox4 protein when compared to cells on soft matrix. The expression of Nox4 mRNA presented in Figure 6.1 (C) showed same trends as protein expression. Taken together, HDFs on stiff matrix expressed more Nox4 both at mRNA and protein level. It could be concluded that high level of Nox4 expression makes HDFs on stiff matrix more sensitive to H$_2$O$_2$ induced ROS accumulation.

### 6.3.2 Soft matrix promotes Nrf2 expression

Failure to prevent intracellular ROS accumulation accounts for oxidative stress induced cellular damage [12]. Nuclear factor erythroid 2-related factor 2 (Nrf2) is responsible for the antioxidant response element (ARE)-dependent gene regulation induced by oxidative stress [13]. Over the past decade, Nrf2 has been identified to play a major role in resistance to oxidant stress. For instance, improving Nrf2 activity pharmacologically protects animals against oxidative stress induced damage [14]. Whereas knockout of Nrf2 consequently leads the mice to be susceptible to various disease conditions and chemical toxicity related to oxidative stress [15-18]. To verify HDFs antioxidant ability on substrates with different stiffness, the levels of Nrf2 expression was monitored by western blotting. As presented in Figure 6.2 (A), strong Nrf2 protein bands were only observed on soft matrix. The quantification in Figure 6.2 (B) indicated cells on stiff matrix expressed significant lower Nrf2 protein when compare to cells on soft matrix. To sum up, HDFs on soft matrix have significantly elevated Nrf2 expression, HDFs on stiff matrix showed very low Nrf2 expression. Therefore, improvement of antioxidant ability resulted by upregulation of Nrf2 expression makes HDFs on soft matrix escape from H$_2$O$_2$ induced ROS accumulation and
cellular damages.

![Cellular damages](image.png)

**Figure 6.2** Soft matrix elevates Nrf2 expression. The effect of matrix stiffness on Nrf2 expression was determined by western blotting. 24 hours after cell seeding, the proteins were extracted. (A) Western blotting bands of Nrf2 expression and coomassie staining of total protein. Protein loading was normalized to coomassie staining. (B) Nrf2 protein expression normalized to total protein. The intensity of the protein bands was quantified by ImageJ. Three independent experiments were performed. Statistical significance was evaluated by Student's t-test, * represents $p < 0.05$ and ** represent $p < 0.01$, respectively.

As above, HDFs on stiff matrix exhibited high Nox4 and low Nrf2 expression. Hence, they showed strong oxidative stress sensitivity and weak antioxidant ability. An opposite manner was observed on soft matrix.

### 6.3.3 Soft matrix inhibits YAP nucleus translocation

So far, it has been shown that matrix stiffness regulated HDFs oxidative stress response and it is probably mediated by Nox4/Nrf2 balance. This was supported by the high levels of Nox4 expressed in HDFs cultured on stiff matrix where they were more prone to H$_2$O$_2$ induced ROS production and apoptosis. Meanwhile, the increased Nrf2 expression on soft matrix leaded to prevention of cellular damage induced by H$_2$O$_2$. However, there is still a missing link between Nox4/Nrf2 redox regulation and ECM stiffness. As reviewed in Chapter 2, YAP shows great potential to connect matrix stiffness and Nox4/Nrf2. Recent studies showed YAP is widely involved in mechanotransduction. It has been identified as an important sensor of mechanical signals exerted by the cellular microenvironment. Many
works reported both ECM stiffness and cell geometry have profound effect on YAP activity [5-7]. Meanwhile, it has been demonstrated that YAP plays essential role in Nox4 expression. Silencing of YAP gene could inhibit Nox4 expression at cellular level. Pharmaceutical inhibition of YAP activity could decrease Nox4 expression in mice [8]. Encouraged by these two demonstrations, it is assumed that YAP activity could correlate matrix stiffness to cellular redox state. Therefore, immunostaining was performed to check YAP activity in HDFs cultured on different stiffness. As shown in Figure 6.3, on stiff matrix, strong bright green fluorescence was observed in the nucleus area rather than in the cytosol (red arrows). On soft matrix, fluorescence emitted in nucleus was much weaker than in cytosol (white arrows). As such, predominant nuclear staining on stiff matrix and diffuse staining on soft matrix indicate YAP activity is mechanically regulated in HDFs. The observation is consistent with previous report that ECM stiffness regulates YAP activity and its subcellular localization [6].

![Figure 6.3](image)

**Figure 6.3** Soft matrix inhibits YAP activity and nucleus localization. 24 hours after cell seeding, the effect of matrix stiffness on YAP activity was visualized by immunostaining. The arrows indicate the nucleus area of representative cells. The scale bar is 50 μm.

**6.3.4 Matrix stiffness regulates oxidative stress response via YAP-Nox4/Nrf2 pathway**

This chapter aims to understand why HDFs on matrix with different stiffness exhibit different oxidative stress tolerance. Based on the results presented in this chapter, the relationship between matrix stiffness and Nox4/Nrf2 balance via YAP activity has been proposed in Figure 6.4. It is well recognized that ECM stiffness and cell geometry are essential for YAP activity and subcellular localization in many cell types [5-7]. The result presented in Figure 6.3 indicates stiff matrix promotes nucleus localization of YAP in
HDFs as well. Increased YAP nucleus localization further triggers the upregulation of Nox4 and downregulation of Nrf2. Since YAP nucleus localization is necessary for Nox4 expression [8], the Nox4 expression is decreased accordingly while Nrf2 expression is increased in HDFs cultured on soft matrix as a result of decreased YAP nucleus localization (Figure 6.1 and 6.2). In this manner, HDFs on stiff matrix exhibit high oxidative stress sensitivity and weak antioxidant ability, while HDFs on soft matrix are non-responsive to oxidative stress. This could be the mechanism by which HDFs on stiff matrix are more susceptible to oxidative stress. As such, when H$_2$O$_2$ is applied, HDFs on stiff matrix generate excessive intracellular ROS. The toxic ROS and ROS mediated NF-κB activation further induce cell apoptosis. On the contrary, soft matrix blocks intracellular ROS accumulation induced by oxidative stress, so that HDFs on soft matrix exhibit robust antioxidant ability and cellular damage resistance when exposed to oxidative stress.

Figure 6.4 Schematic illustration of matrix stiffness regulated oxidative stress response and oxidative stress induced apoptosis.
6.4 Conclusion

In current study, the mechanism on matrix stiffness regulated oxidative stress tolerance has been proposed. Briefly, HDFs cultured on soft matrix exhibit weak nucleus localization of YAP, which further blocks Nox4 expression and elevates Nrf2 expression. For cells cultured on soft matrix, decreased Nox4 leads to poor oxidative stress sensitivity, while increased Nrf2 results in effective oxidative stress defense. To sum up, matrix stiffness dependent oxidative stress response is probably regulated by YAP-Nox4/Nrf2 signaling pathway.

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Chapter 7

Soft Matrix Prevents UV Induced Human Dermal Fibroblasts Senescence via Inhibiting NF-κB Activation

In Chapters 5 and 6, we have studied the effects of H$_2$O$_2$ on cells cultured on different substrates in terms of oxidative stress response. Here, we are interested to study the effect of UV on cellular behavior, particularly cell senescence, when HDFs were cultured on different stiffness. It has been widely reported that skin ageing results in significantly increased skin stiffness. Thus, in this work, the main aim is to explore how the matrix stiffness will affect HDFs senescence. Experimentally, we cultured HDFs on PDMS substrates with different stiffness followed by UV exposure. The results revealed that most HDFs undergo senescence on stiff matrix, while it is slightly affected on soft matrix. With further characterization, we observed cells on stiff substrates have enhanced activation of nucleus factor kappa-B (NF-κB) under UV exposure, while cells on soft matrix only exhibited low NF-κB activation. Elevated activation of NF-κB is a prominent feature and cause of accelerated senescence. In summary, this work has demonstrated soft matrix protects HDFs against UV induced senescence via inhibition of NF-κB activation.
7.1 Introduction

In Chapters 5 and 6, it is revealed that matrix stiffness could regulate H$_2$O$_2$ induced apoptosis via modulation of cells antioxidant ability. As reviewed in Chapter 2, ROS induced cell senescence also plays significant causal roles in ageing and its related diseases. Therefore, the research interest of this chapter is to explore the effect of matrix stiffness on HDFs senescence.

Cellular senescence is where cells developed into an irreversible growth arrest status after a certain amount of cell division, which was first defined by Leonard Hayflick in the early 1960s [1]. From then on, cellular senescence has been demonstrated as a great potential in regulating tumor suppression, tumor promotion, connective tissue repair and tissue ageing [2-4]. The accumulation of senescent HDFs has been reported as the most predominant feature of skin ageing [5]. The population of HDFs decreases dramatically as a result of decreasing cell proliferation ability [6]. It is widely accepted that this progressive stoppage of HDFs function during skin ageing consequently erodes the dermal collagen matrix which eventually decreases the skin’s structural integrity, leading to problems of skin barrier dysfunction, impaired wound healing, skin fragility and cosmetic problems [7-10].

In order to simplify the complex senescence process, plenty of candidates have been explored as agents to accelerate HDFs senescence in vitro. Hydrogen peroxide (H$_2$O$_2$), copper sulfate (CuSO$_4$), Cyr61/CCN1 and UV irradiation have shown their success in accelerating HDFs senescence [11-14]. Among which, UV irradiation is not only a lab on a bench method, but also a main cause of photoageing in everyone’s daily life [15]. Recent evidence also showed that UV induced senescence shows similar molecular features with replicative cell senescence, both of them have altered signal transduction pathways that promote MMP expression and decrease precollagen synthesis [16].

The distinct alteration of microenvironment is another significant feature of aged skin. The major characteristic of skin ageing is the loss of elasticity, which essentially corresponds to the substantial loss of the skin’s collagen integrity [17]. A consistent aspect of skin ageing is the presence of glycated dermal collagen. Reducing sugars (ie. glucose)
abundantly circulating in the blood are known to spontaneously react with proteins in tissues over time in the course of ageing through glycation [18]. This nonenzymatic reaction produces advanced glycation end products (AGEs) that subsequently mediate the chemical cross linking of collagen in the skin. The fibers of glycated collagen are observed to be more densely packed, physically stiffer and less elastic altogether. This clearly represents a significant change of the physical microenvironment for the residing dermal fibroblasts [19]. It has been documented that human skin’s stiffness increases from 80kpa to 260kpa from 5 years old to 90 years old [20].

Increasingly, there have been studies and discoveries showing that the stiffness of microenvironment plays a crucial role in modulating cellular behaviors including differentiation, migration, mortality and even metastasis [21-23]. Although the previous introduction shows solid evidence that senescent HDFs are surrounded with significantly altered microenvironment, the link between cellular microenvironment and the cellular senescence process is still significantly neglected.

To the best of our understanding, studies on the role of microenvironment in modulating senescence have not been explored. There is evidence showing microenvironment changes in senescence but the causality of the relationship has not been established. After decades of studies on senescence, the physiological relevance of these studies is slowly showing. For example, recent data validate the early idea that cellular senescence is important for tumor suppression and also strongly suggest that cellular senescence contributes to aging, and that senescence-associated phenotypes can contribute to both tumor progression and normal tissue repair. All these studies are focused around the biological aspects of senescence. However, cells are surrounded by a dynamic environment and they interact intimately with the environment. Therefore, with a strong biological understanding of senescence it is timely to include microenvironment such that the understanding would be more holistic and not isolated. Furthermore, from this understanding, microenvironment may be eventually used and engineered to be potent modulators of cellular senescence just as in the case of stem cell differentiation in recent years.
7.2 Materials and methods

7.2.1 UV exposure

OSRAM ultra-vitalux 300 Watt 230V E27/ES UVB lamp was chosen to provide UVB rays. To ensure consistent results between experiments, UV lamp was fixed in iron support at fixed height. The lamp was placed 35cm above the ground of the cell culture hood. The lamp was warmed up for 5 minutes before exposure to ensure the stable irradiation. The medium was then removed from the 35mm tissue culture plate, 1 ml of 1xPBS was added to each plate to prevent cells from drying up. Following, cells were exposed to UV lamp for 4 minutes. Then, PBS solution was replaced with 3 ml fresh fibroblast culture medium. After which, the plate was put into incubator for further culture.

7.2.2 Cell proliferation assay

Cell counting kit-8 (CCK-8) from Dojindo Molecular Technologies, was used to detect the cell numbers after UV treatment every 24 hours. Simply, 10 percent of CCK-8 solution was prepared with fresh medium. Then the HDFs were immersed with CCK-8 contained medium, followed by 2 hours culture in 37 °C incubator. After incubation, the absorbance value at 450 nm was measured by a microplate reader. A calibration curve was performed to convert the absorbance value to cell numbers.

7.2.3 Senescence associated β-Galactosidase staining

HDFs senescence was evaluated with Senescence β-Galactosidase Staining Kit from Cell Signaling. Simply, HDFs were fixed with 4% paraformaldehyde for 15 minutes and then incubated with the β-gal staining solution at 37°C overnight in a CO2 free incubator. Then the HDFs were visualized with an optical microscope.

7.2.4 UV lamp setup and exposure time optimization
UV lamp was set up in the hood as shown in Figure 7.1 (A), the distance between UV lamp and cell culture plate is fixed at 35cm for all the experiments. To ensure full attachment and spreading, the HDFs were cultured 24 hours before UV exposure. UV induced HDFs senescence is a dose dependent process. Strong UV irradiation would lead to cell death rather than senescence, while weak UV would not affect cell growth. The effect of UV irradiation time on proliferation of HDFs cultured on TCPs is monitored by cck-8 kit. As shown in Figure 7.1 (B), during the 8 days culture, the absorbance value at 490nm decreased under UV irradiation when compared to the control groups without UV irradiation, indicating lower cell proliferation for the group irradiated with UV. Meanwhile, the value decreased in an exposure time dependent manner. UV exposure of 2 and 3 minutes slightly decreased the absorbance value, while 4 minutes UV irradiation triggered sharp decrease of the absorbance. Since the absorbance of cck-8 test could reflect cell proliferation, the absorbance curve showed that the HDFs proliferation significantly slowed down with 4 minutes of UV exposure.

**Figure 7.1** Optimization of UV exposure time. (A) UV lamp setup. (B) HDFs proliferation curve under different UV exposure time. During the 8 days cell culture, cell proliferation was monitored by cell counting kit-8 (cck-8) every 24 hours. The proliferation curve was plotted accordingly. Grey, black, orange and red curve represent experiments without UV exposure, 2 minutes, 3 minutes and 4 minutes UV exposure, respectively. Three independent experiments were performed.

UV induced HDFs senescence under different exposure time was further examined by SA-
β-gal histochemical staining. As shown in Figure 7.2, with 2 and 3 minutes of UV exposure, a small proportion of HDFs were observed with positive SA-β-gal staining. Under 4 minutes UV irradiation, most of cells exhibited positive SA-β-gal staining and enlarged cell area. With 5 minutes exposure, HDFs were observed with abnormal shrinkage and the cells could not survive. Taken together, 4 minutes of UV irradiation was sufficient to arrest HDFs proliferation and trigger HDFs senescence. Therefore, 4 minutes of UV exposure was chosen for further experiments.

![Image of Figure 7.2](image)

**Figure 7.2** Determination of cell senescence under different UV exposure time. After 24 hours attachment on TCPs, HDFs were subjected to 0-5 minutes UV exposure. 7 days after UV exposure, SA-β-gal staining (blue stain indicates positive staining) was performed to detect HDFs senescence. The scale bar is 50μm.

### 7.3 Results

#### 7.3.1 Soft matrix inhibits UV induced ROS accumulation

Reactive oxidative species (ROS) production of the cells, the earliest response of UV irradiation, was monitored 30mins after UV treatment using the 2’,7’-dichlorodihydrofluorescein diacetate (DCFDA)–ROS detection assay. The relative change
between HDFs cultured on each substrate with or without UV was examined by fluorescence microscopy and flow cytometry. **Figure 7.3 (A)** presented the fluorescent images of ROS staining. In control groups without UV irradiation (upper panel), no significant fluorescence was detected on each substrate. In UV irradiated group (lower panel), strong fluorescence was observed on stiff substrates while weak fluorescence was detected on soft substrates. In **Figure 7.3 (B)**, the flow cytometry histograms indicated UV irradiation triggers distinct right shift of ROS intensity peaks on stiff matrix, while no peak shift was observed on soft matrix (red curves versus dashed grey curves).

Meanwhile, under UV exposure, two prominent peaks displayed on stiff matrix (red curves), where the left peak remained closer to the control group (grey curves) and represented no excessive ROS production is triggered among this population; distinctive right shift peak denotes the population of cells with excessive ROS production since the curve falls outside of the control group. According to the quantification in **Figure 7.3 (C)**, the ROS fluorescence intensity increased up to 2 folds on stiff matrix, while it only slightly increased on soft matrix. Taken together, the results mentioned above indicate that soft matrix reduces UV induced intracellular ROS accumulation.
Figure 7.3 Soft matrix inhibits UV irradiation induced intracellular ROS accumulation. After 24 hours attachment on matrix with different stiffness, HDFs were exposed to UV for 4 minutes. 1 hour after UV exposure, cells were labeled with H$_2$DCFDA. Then, the levels of intracellular ROS were visualized by fluorescence microscope (A) and quantified by flow cytometry (B, C). (A) The upper and lower panel represents experiments without and with UV irradiation respectively. (B) Representative DCF fluorescence intensity histograms on matrix with different stiffness and (C) quantification of mean DCF fluorescence intensity based on (B). (B) Solid grey areas represent experiments without UV irradiation and H$_2$DCFDA staining, dashed grey lines represent ROS fluorescence intensity without UV irradiation, red lines represent ROS fluorescence intensity after UV irradiation. (C) Light grey and dark grey represent the mean ROS fluorescence intensity without and with UV irradiation respectively. Three independent experiments were performed.
Statistical significance was evaluated by Student's t-test, ** represent p < 0.01. The scale bar is 100μm.

### 7.3.2 Soft matrix reduces UV induced HDFs proliferation arrest

Cell senescence is the permanent cell growth arrest, which means cell will stop proliferation when it is ageing. Thus, lack of proliferation is an indication of the senescent phenotype development. Here, a series of experiments were performed to check the proliferation status of HDFs.

**Figure 7.4 (A)** presented the optical images of HDFs density 7 days after UV irradiation. Obviously, HDFs without UV treatment reached confluence regardless of the substrate stiffness. Under UV treatment, only HDFs on soft substrates reached confluence. HDFs on stiff substrates showed significantly enlarged, spread, stellate morphology rather than the normal elongated, spindle morphology, which clearly indicated they were senescent.

Meanwhile, we monitored the cell numbers with cell counting kit-8 (cck-8) every 24 hours after cell seeding. **Figure 7.4 (B)** showed HDFs cell numbers during the 8 days culture, where HDFs without UV treatment were proliferating well on all substrates. Under UV irradiation, HDFs proliferation significantly slowed down on stiff substrates, while the proliferation slightly decreased on soft substrates. Thus, we demonstrated that soft substrate significantly prevented UV induced HDFs proliferation arrest.
Figure 7.4 Soft matrix protects HDFs from UV induced growth arrest. (A) Visualization of HDFs morphology and density at day 7 after UV treatment. (B) The proliferation curve of HDFs with/without UV exposure on different stiffness. After cell seeding, cell number was monitored by cell counting kit-8 (cck-8) every 24 hours. The proliferation curve on each matrix was plotted accordingly. Three independent experiments were performed. The scale bar is 100μm.

To reinforce the above conclusion, we next analyzed the biomarker of cell proliferation via ki-67 immunostaining and quantified the percentage of proliferating cells (Figure 7.5 (A)). The upper row represents HDFs without UV treatment, which shows HDFs on all four different stiffness had extensive positive ki-67 staining. The bottom row represents HDFs with UV irradiation, where positive stained HDFs were the minority on stiff substrates while positive stained HDFs were still the majority on soft substrates. When the staining
was quantified (Figure 7.5 (B)), HDFs showed similarly high percentage of proliferation when not exposed to UV, regardless of substrate stiffness. After UV irradiation, the percentage of proliferative HDFs decreased from 61% to 28% and 69% to 36% on TCPs and 750kPa PDMS substrate, respectively while those on soft matrices (46kPa and 21kPa) remained high at about 70%. Collectively, matrix stiffness does not have significant effect on HDFs proliferation under normal culture condition. However, the effect was profound under UV stress. Since more ki-67 positive HDFs were observed on soft matrix under UV treatment, we concluded that soft substrate reduces UV induced proliferation arrest.

Figure 7.5 Soft matrix prevents UV induced senescence. HDFs proliferation was quantified 3 days after 4 minutes UV exposure. The proliferation ability of HDFs on different stiffness was determined by Ki-67 staining. (A) Fluorescent images of Ki-67 (green) staining, DAPI (blue) was counterstained. The upper panel represents control groups without UV irradiation. The lower panel represents experiments with UV irradiation. (B) The percentage of positive Ki-67 staining HDFs based on (A). Three independent experiments were performed. Statistical significance was evaluated by Student’s t-test, *** represent p < 0.001. The scale bar is 100μm.
7.3.3 Soft matrix inhibits UV induced HDFs senescence

The activity of the cellular senescence biomarker, SA-β-gal, was examined by SA-β-gal histochemical staining assay. According to Figure 7.6 (A), on different matrix stiffness, no significant positive SA-β-gal staining was observed without UV irradiation. However, upon UV irradiation, the percentage of SA-β-gal positive cells was increased in a stiffness dependent manner. On stiff matrix, SA-β-gal activity markedly increased, up to 70% cells were observed with positive staining. On soft matrix, only less than 20% cells had positive staining (Figure 7.6 (B)). Therefore, soft matrix significantly reduced UV induced HDFs senescence.

Figure 7.6 Soft matrix protects UV induced HDFs senescence. 7 days after 4 minutes UV exposure, SA-β-gal staining was performed to stain HDFs on different stiffness. (A) The levels of cell senescence were detected by SA-β-gal staining. The upper panel represents control groups without UV irradiation. The lower panel represents experiments with UV irradiation. (B) The percentage of senescent cells was quantified based on (A). Three independent experiments were performed.
Statistical significance was evaluated by Student's t-test, ** represent p < 0.01 and *** represent p < 0.001, respectively. The scale bar is 50μm.

In order to elucidate the molecular mechanism behind, qRT-PCR experiment was performed to check the senescence related gene expression. It is widely accepted that either p53 or p16 tumour suppressor pathways should be responsible for induced premature senescence [24]. Under senescence stimuli, such as oxidative stress, DNA damage, p53 gene will be activated [25, 26].

**Figure 7.7** Soft matrix inhibits UV induced senescence associated gene expression. The mRNA expression levels of p53, p21 and p16 were determined by qRT-PCR. 7 days after 4 minutes UV exposure, the total mRNA was extracted and examined. (A) The fold change of p53 gene expression. (B) The fold change of p21 gene expression. (C) The fold change of p16 gene expression. The fold change was normalized to GAPDH. Black bars represent control groups without UV irradiation. Grey bars represent experiments with UV irradiation. Three independent experiments were performed. Statistical significance was evaluated by Student's t-test, ** represent p < 0.01 and *** represent p < 0.001, respectively.
The expression of p21, a cyclin-dependent kinase (CDK) inhibitor will be activated by the active p53 [27]. It will further lead to cell growth arrest and senescence. The expression of p16, another CDK inhibitor that prevents p16–retinoblastoma protein (pRB) phosphorylation and inactivation, can also be activated by senescence stimuli. Thus, the related gene expressions, such as p53, p21 and p16 gene, were analyzed. As shown in Figure 7.7, on different stiffness, the fold change of these genes were similar without UV irradiation. However, the fold change of p53 and p21 increased significantly on stiff matrix when compared to soft matrix under UV irradiation (Figure 7.7 (A, B)), the fold change of p16 was not much affected by UV treatment (Figure 7.7 (C)). Therefore, the effect of substrate itself on senescence related gene expression is not significant when there is no UV irradiation, but it would trigger great elevation of these genes expression when UV irradiation is applied. Since p53 and p21 were remarkably increased, rather than p16, in HDFs cultured on stiff substrate, p53-p21 pathway probably plays the major role in UV induced HDFs senescence.

### 7.3.4 NF-κB signaling pathway is involved in matrix stiffness regulated oxidative stress response

So far, it is demonstrated that matrix stiffness could regulate UV induced HDFs senescence, where HDFs on soft matrix displayed strong senescence resistance against UV exposure. Emerging evidence reported by other researchers, as well as our demonstrations in Chapters 5 and 6, suggested that NF-κB activity might be involved in matrix stiffness mediated HDFs senescence fate. NF-κB activation has long been linked with tumour suppression, cell apoptosis and cell senescence [28]. Many studies showed that NF-κB activity increases with chronologic ageing and NF-κB activation controls the generation of senescence associated secretory phenotype [29]. It is also reported that intact NF-κB function is required for therapy induced cell senescence [30]. Furthermore, inhibition of NF-κB activation can downregulate ROS production as well as oxidative stress induced cellular damage (refer to Chapter 5). Inhibition of NF-κB also could promote lymphomas chemoresistance via a failure to engage the senescence response that contributes to cell cycle arrest [29]. From recent studies, microenvironment also showed its great potential in
regulating NF-κB expression and translocation [31]. In order to investigate whether substrate stiffness has effect on NF-κB signaling pathway under UV exposure, NF-κB activation was determined by western blotting. As shown in Figure 7.8 (A), on stiff matrix (TCPs and 750kPa PDMS), phosphorylated p65 blotting bands were much stronger after UV irradiation. Intensity of each band was quantified and normalized to total p65 expression. Based on the quantification shown in Figure 7.8 (B), the expression of phosphorylated p65 increased up to 2.5 folds on stiff matrix under UV irradiation, while it was only slightly increased on soft matrix under same condition. Therefore, it is possible that soft matrix prevents UV induced HDFs senescence via inhibition of NF-κB activation, since phosphorylated NF-κB increased significantly on stiff matrix, while it was only slightly upregulated on soft matrix, upon UV treatment.

![Figure 7.8](image)

**Figure 7.8** Soft matrix inhibits UV induced NF-κB activation. Total p65 and phosphorylated p65 protein expression were determined with western blotting. The proteins were extracted 1 hour after UV exposure. (A) Total p65 and phosphorylated p65 protein expression. Protein loading was normalized to GAPDH. (B) The intensity of the protein bands was quantified by ImageJ. Three independent experiments were performed. Statistical significance was evaluated by Student's t-test,
** represent \( p < 0.01 \).

### 7.3.5 UV induced HDFs senescence on stiff matrix is NF-\( \kappa \)B activation dependent

Since soft matrix reduces UV induced ROS accumulation and cell senescence when comparing to stiff matrix, and UV induced NF-\( \kappa \)B activation was only observed on stiff matrix, it is speculated that UV induced HDFs senescence on stiff matrix is NF-\( \kappa \)B activation dependent; while soft matrix may have prevented UV induced senescence via inhibition of NF-\( \kappa \)B activity. To confirm this hypothesis, pyrrolidinedithiocarbamate ammonium (PDTC), a potent inhibitor of NF-\( \kappa \)B activation [30], was applied to inhibit NF-\( \kappa \)B expression.

We first examined PDTC’s effect on UV stimulated ROS accumulation via flow cytometry. Similar to that observed in Chapter 5, PDTC locked down ROS accumulation in HDFs on both stiff and soft matrix. As presented in Figure 7.9 (A-D), UV irradiation failed to stimulate ROS accumulation in PDTC pretreated HDFs on all substrates as shown by the significant left shift of the peak when comparing PDTC treated cells to non-PDTC treated cells under UV exposure (green curves versus red curves). According to the quantified mean ROS intensity in Figure 7.9 (E), PDTC pretreatment resulted in sharply reduced ROS production in cells stimulated by UV exposure, where the related ROS production was even much lower than the control groups without UV exposure. Therefore, UV induced ROS accumulation on stiff matrix is NF-\( \kappa \)B activation dependent and inhibition of NF-\( \kappa \)B activity could attenuate UV induced ROS. Hence, soft matrix reducing UV induced ROS accumulation is probably through inhibition of NF-\( \kappa \)B activity.
Figure 7.9 Inhibition of NF-κB blocks UV induced intracellular ROS. Prior to 4 minutes UV exposure, HDFs were treated with 100 μm PDTC for 1 hour. 1 hour after UV exposure, cells were labeled with CM-H$_2$DCFDA, and the levels of intracellular ROS were determined by flow cytometry. (A-D) Representative DCF fluorescence intensity histograms on matrix with different stiffness and (E) quantification of mean DCF fluorescence intensity from three independent experiments. (A-D) Solid grey areas represent experiments without UV irradiation and H$_2$DCFDA staining, dashed grey lines represent experiments with only H$_2$DCFDA staining and no UV irradiation, red lines represent ROS fluorescence intensity after UV irradiation, green lines represent ROS fluorescence intensity of cells pretreated with PDTC followed by UV irradiation. (E) Light grey and grey represent the mean ROS fluorescence intensity of cells without and with UV irradiation respectively. Black represents the mean ROS fluorescence intensity of cells pretreated with PDTC followed by UV irradiation. Statistical significance was evaluated by Student's t-test, ** represents p < 0.01 and *** represent p < 0.001, respectively.

Next, the effect of PDTC pretreatment on UV induced HDFs senescence was examined by SA-β-gal staining. As seen in Figure 7.10 (A), PDTC pretreatment resulted in significant decrease of positive SA-β-gal stained cells in the group with UV exposure. (right image versus medium image). Based on the quantification in Figure 7.10 (B), PDTC significantly down regulated SA-β-gal positive cells from ~80% to ~40%. Therefore, inhibition of NF-
κB activity through pretreatment with PDTC significantly attenuated UV induced HDFs senescence on stiff matrix.

Figure 7.10 Inhibition of NF-κB prevents UV induced senescence. HDFs on TCPs were pretreated with 100μM PDTC for 1 hour, followed by 4 minutes UV exposure. After 7 days, culture, (A) the levels of cell senescence were detected by SA-β-gal staining. From left to right, representative images of experiments without PDTC treatment and UV irradiation, with only UV irradiation, and with both PDTC treatment and UV irradiation, respectively. (B) The percentage of senescent cells was quantified based on (A). Three independent experiments were performed. Statistical significance was evaluated by Student’s t-test. ** represent p < 0.01. The scale bar is 100μm.

7.4 Conclusion

Although mechanical property of microenvironment has proven its profound implication on regulating stem cell differentiation [32], epithelial–mesenchymal transition (EMT) in tumour progression [33], and many other physiological processes, the results presented here report a significant and previously undocumented influence of microenvironment stiffness on HDFs senescence. Although stiffness increase and HDFs senescence have been
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well documented as two predominant features of skin ageing, prior studies do not demonstrate a relationship between ECM stiffness and HDFs senescence.

This study demonstrated that HDFs cultured on soft substrate have resistance against UV initiated ROS upregulation. Although the ROS production still slightly increased in response to UV exposure, it is not profound enough to activate extensive cellular damage in HDFs cultured on soft substrate. In contrast, ROS production and cell proliferation arrest remarkably increased in HDFs cultured on stiff substrate. Excessive ROS accumulation stimulated by UV irradiation triggered HDFs to lose their proliferation capacity on stiff substrate, while slightly increased ROS production did not affect HDFs’ proliferation on soft substrate significantly. Due to loss of proliferation capacity, HDFs on stiff substrate finally developed into permanent growth arrest and are represented in senescent phenotype. In particular, the percentage of SA-β-gal positive HDFs and the senescence-related gene expression dramatically increased. In conclusion, soft matrix inhibits UV induced ROS production, which ensures HDFs proliferation and avoided UV induced senescence. On further analysis, under UV treatment, extensively elevated NF-κB activation was detected on stiff matrix. Inhibition of NF-κB activity with PDTC significantly attenuated UV induced ROS accumulation and HDFs senescence on stiff matrix. These results indirectly suggested that soft matrix probably prevented HDFs against UV induced senescence via inhibition of NF-κB activation.

Although the precise mechanism underlying ECM stiffness mediated NF-κB expression and consequently HDFs senescence remains to be determined, the study provides new insights into understanding cell-matrix interactions in HDFs senescence.

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Chapter 8

Conclusion and Future Work

In this last chapter, a brief overview of study background was given. Then, the general conclusions were summarized, followed by the explanation of future work strategies. This chapter highlighted the importance of ECM stiffness in regulating HDFs oxidative stress response. In specific, matrix stiffness regulates H2O2 induced apoptosis and UV induced senescence. In future work, we first intended to study the effect of microenvironment’s topography on HDFs oxidative stress response. By which, we could gain more knowledge about cell-matrix interaction in ROS regulation. In addition, we are keen to explore physical microenvironment’s effect on HDFs oxidative stress in 3 dimensional. Part of preliminary outcomes was presented as well
8.1 General discussion and conclusion

Emerging evidence shows ageing is partially caused by cumulative and deleterious ROS [1-4]. Uncontrolled accumulation of free radicals and declined antioxidant ability make the elderly population prone to oxidative stress and its related diseases [5, 6]. In past decades, much effort has been made to understand why the elderly people have decreased antioxidant capacity. However, one of the aspects that have been significantly neglected is the effect of microenvironment. Even though emerging evidences are showing significantly altered mechanical microenvironment with ageing and diseases (as reviewed in Chapter 2), the effect of ECM mechanics on antioxidant ability is poorly understood. Therefore, this study is aimed at understanding the effect of matrix stiffness on cells oxidative stress response and the underlying molecular mechanism. To achieve this goal, the interdisciplinary approach involving techniques of materials science and engineering, as well as cellular and molecular biology techniques were employed in this study. Due to high optical transparency and easily tunable and wide range of stiffness [7-9], polydimethyilsloxane (PDMS) substrates were employed to mimic the mechanical environment with different stiffness. As the most widely distributed cells in the connective tissues of the body [10], human dermal fibroblasts (HDFs) were chosen as the model cells in this dissertation. Then, the platform of HDFs cultured on PDMS substrate was set up as an *in vitro* model to mimic cells under different mechanical environment. The exotic source of oxidative stress, such as H$_2$O$_2$ and UV, was used to trigger cell oxidative stress response.

Firstly, we examined the effect of matrix stiffness on H$_2$O$_2$ induced ROS accumulation and apoptosis. HDFs were cultured on both stiff matrix (TCPs and 750kPa PDMS) and soft matrix (46kPa PDMS and 21kPa PDMS), followed by 100 $\mu$M H$_2$O$_2$ treatment. According to the flow cytometry results, most of the HDFs on stiff matrix were detected with elevated ROS accumulation followed by apoptosis, while HDFs were not affected when cultured on soft matrix. It was revealed from our studies that HDFs cultured on stiff matrix were more susceptible to oxidative stress, while HDFs on soft matrix exhibited oxidative stress resistance. By further investigation of H$_2$O$_2$ treatment involved pathways, we found inhibition of Rho/ROCK pathway with Y27632 did not attenuate H$_2$O$_2$ induced ROS and
apoptosis on stiff matrix, and therefore matrix stiffness regulated oxidative stress response is Rho/ROCK independent. Elevated NF-κB activation triggered by H\textsubscript{2}O\textsubscript{2} was only observed on stiff matrix, but not on soft matrix. Blocking NF-κB activation with PDTC significantly reduced H\textsubscript{2}O\textsubscript{2} induced ROS and apoptosis on stiff matrix. As significant NF-κB activation was not observed in cells on soft matrix with H\textsubscript{2}O\textsubscript{2} exposure, it could be deduced that soft matrix prevented H\textsubscript{2}O\textsubscript{2} induced apoptosis via inhibition of NF-κB activation. These results demonstrated that matrix stiffness has profound implications on cell oxidative stress response.

As above, HDFs on stiff matrix were sensitive to oxidative stress, while HDFs on soft matrix showed strong antioxidant ability. We next explored the possible underlying mechanism in regards to matrix stiffness regulated HDFs oxidative stress tolerance. The effect of matrix stiffness itself (without exotic oxidative stress) on cell antioxidant ability at molecular level was examined. Since Nox4-Nrf2 redox imbalance is commonly related to intracellular ROS accumulation [11-13], the expression of Nox4 and Nrf2 in HDFs on different stiffness was monitored. The expression of Nox4, a major source of oxidative stress [14, 15], elevated at both transcripts and translational levels in HDFs cultured on stiff matrix. The expression of Nrf2, a transcription factor responsible for antioxidant defense and detoxification [16], increased only on soft matrix. According to literature and our result in Chapter 6, YAP activity is involved in matrix stiffness regulated Nox4-Nrf2 balance [17, 18]. In specific, stiff matrix promotes YAP nucleus localization, which triggers the expression of Nox4 and the suppression of Nrf2. While soft matrix inhibits YAP nucleus localization, which further blocks Nox4 expression and elevates Nrf2 expression. For cells cultured on soft matrix, decreased Nox4 leads to poor oxidative stress sensitivity, while increased Nrf2 results in effective oxidative stress defense. Collectively, matrix stiffness dependent oxidative stress response is probably regulated by YAP-Nox4/Nrf2 signaling pathway.

So far, it is confirmed that matrix stiffness could regulate HDFs antioxidant ability, and therefore regulates H\textsubscript{2}O\textsubscript{2} induced cell apoptosis decision. As reviewed in Chapter 2, ROS induced cell senescence also plays significant causal roles in ageing and its related diseases.
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UV irradiation is a main cause of photoageing in everyone’s daily life [19]. Therefore, we were next interested to study the effect of UV on cellular behavior, particularly cell senescence, when HDFs were cultured on PDMS substrates with different stiffness. We observed that HDFs cultured on soft substrate have resistance against UV initiated ROS upregulation, as well as UV induced cellular damage. On the contrary, UV irradiation resulted in elevated ROS accumulation, followed by distinctive cell proliferation arrest on stiff matrix. Due to loss of proliferation capacity, HDFs on stiff substrate finally developed into permanent growth arrest and were represented in senescent phenotype. In particular, the percentage of SA-β-gal positive HDFs and the senescence-related gene expression dramatically increased. In contrast, soft matrix inhibited UV induced ROS production, which ensured HDFs proliferation and avoided UV induced senescence. On further analysis, UV irradiation only elevated NF-κB activation on stiff matrix, but not on soft matrix. Inhibition of NF-κB activity with PDTC significantly attenuated UV induced ROS accumulation and HDFs senescence on stiff matrix. These results indirectly suggested that soft matrix probably reduced UV induced HDFs senescence through inhibition of NF-κB activation.

The findings presented above fully support our hypothesis that the ECM stiffness could modulate cell oxidative stress responses under external stimuli. ECM stiffness could regulate H$_2$O$_2$ induced apoptosis, as well as UV irradiation induced senescence, via YAP-Nox4/Nrf2 signaling pathway. Although stiffness increase and ROS dysregulation have been well documented as two predominant features of many aged tissues and age-related diseases, prior studies do not demonstrate a relationship between ECM stiffness and cell ROS regulation. Unlike the conventional regulation of ROS from biological aspects, we reported physical microenvironment regulated cell oxidative stress response for the first time. From this understanding, microenvironment may be eventually used and engineered to be potent modulators of cell oxidative stress response just as in the case of stem cell differentiation in recent years.

8.2 Future work
8.2.1 Study the effect of microenvironment’s topography on HDFs oxidative stress response

Apart from stiffness alteration, the ECM topography also changes a lot in ageing and diseases. For instance, in young (20–30 years) skin, intact collagen fibrils are abundant, tightly packed, and well-organized. In contrast, in aged skin (>80 years), collagen fibrils are fragmented and disorganized (Figure 8.1) [20]. Therefore, it may be interesting to understand the effect of microenvironment’s topography on HDFs oxidative stress response in our further study.

Figure 8.1 Nano-topography of dermal collagen significantly altered with aging. Representative AFM images of dermal collagen fibrils in young (25 years) and aged (84 years) sun-protected underarm skin. Scale bar=100 nm.

Microcontact printing technique is the most commonly used ECM patterning technique to precisely tune matrix topography and study cell-matrix interactions [21]. Briefly, the ECM protein will be inked on stamps with desired micro or nano features and then the inked protein will be transfer onto cell culture substrates to form cell attachable regions.

Microcontact printing is a top-down surface patterning technique and it is an immensely popular method due to its simplicity and wide range of applications in the field of cell
matrix interactions [22].

![Image](image.png)

**Figure 8.2** Fibronectin lane patterns were transferred onto TCPs via soft lithography technique. (A) The narrow PDMS stamp consisted of 20 μm wide features and 50 μm gaps. (B) The wide PDMS stamp had 30 μm wide features and 150 μm gaps. Fluorescent staining of coated fibronectin showed the success that fibronectin protein was transferred on TCPs precisely (C, D). (E, F) revealed the distribution of printed fibronectin was uniform and regular. Scale bar= 100 μm.

In one of our ongoing project, microcontact patterning technique was used to modulate HDFs elongation, and study cell morphology’s effect on oxidative stress response. PDMS
stamps were prepared with the method described in Chapter 3. Briefly, the silicon wafer was designed with micro features on the surface, then the PDMS mixed solution was poured on the side with micro features. After full gelation, the micro features were transferred on PDMS stamp. In order to clearly visualize the micro features of PDMS stamps, the stamps were characterized with field emission scanning electron microscopy (FESEM). The results presented in Figure 8.2 (A, B) showed the micro lanes were successfully and precisely transferred onto PDMS surface, all the lanes had smooth and straight boundary. The stamp in Figure 8.2 (A) showed it has 30 μm width lanes and 150 μm gaps between every 2 lanes. Figure 8.2 (B) represented the stamp with narrow lanes, the width of the lane was 20 μm and the gap had a distance at 50 μm.

Then, the stamp was used to print the fibronectin on TCPs. In order to determine the fidelity of the printing, the fibronectin coated TCPs were characterized with fibronectin immunostaining. The images presented in Figure 8.2 (C, D) showed fibronectin uniformly distributed on the TCPs with regular spaces. Thereafter, we further characterized the fluorescence intensity of fibronectin with imageJ. Figure 8.2 (E, F) revealed that the fluorescence intensity is around 20 A.U. to 23 A.U. on each lane of both stamps. So far, the fibronectin coated TCPs with different lanes to guide the HDFs morphogenesis have been successfully prepared. The precise fibronectin printing could further ensure the success in HDFs morphology modulation.

To further determine HDFs morphology on fibronectin patterned TCPs, the immunostaining of f-actin and nuclei were performed. Generally, extensive thick bundles of stress fibers were observed in unpatterned HDFs (Figure 8.3 (B)), while relative linear stress fibers aligned parallel to fibronectin lanes were observed in patterned HDFs (Figure 8.3 (D, F)). Highly elliptical nucleus was observed in HDFs cultured on 20um width fibronectin pattern (Figure 8.3 (E)). These findings indicated that HDFs morphology and nucleus shape were successfully modulated by microcontact printing. This micro printed platform will be further employed to study the effect of matrix topography on cell oxidative response.
8.2.2 Study physical microenvironment’s effect on HDFs oxidative stress in 3-Dimension

The study conducted in this thesis revealed the effect of ECM stiffness on HDFs oxidative stress response and oxidative stress induced apoptosis/senescence when cells are cultured on a 2-Dimensional plate. Though the results generated fundamental knowledge and provided good insights into this unexplored area, 3 dimensional interaction is a better reflection of the in vivo conditions \[23\]. 3-D culture models have proven to be more realistic for translating the study findings for in vivo applications \[24\]. Therefore, study of physical microenvironment effect on HDFs oxidative stress response in 3-D cell culture will be one of our future directions. According to our group’s recent progress in 3-D electrospinning and 3-D printing, several 3-D scaffolds with tunable mechanics and
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topography features have been proposed, as shown in Figure 8.4 [25]. With the advantages of these 3-D scaffolds, we could understand more about the interaction between biophysical microenvironment and cellular oxidative stress response in 3D system.

![Figure 8.4](image)

**Figure 8.4** (a) The device setup electrospinning; (b) Shape and size of 3-D electrospun fibers; (c) SEM images of 3-D fibers collected from liquid bath and (d) conventional flat platform (Courtesy of group member Chen Huizhi).

The current work presented in the thesis demonstrated ECM stiffness has significant effect on cells oxidative stress response and senescence. Apart from stiffness, ECM topography is another important parameter of the microenvironment. Therefore, the topography’s effect on cell oxidative stress response was proposed as the immediate future work. Combination of the findings of these two parts will yield a more detailed understanding of the relationship between physical microenvironment and cell oxidative stress response. The shortcoming of this current system is it only focused on the 2-

Dimensional study. With the latest research advances in electrospinning and 3D printing, it is achievable to investigate microenvironments’ effect on cell oxidative stress response
in 3-Dimension. The work presented in this thesis could serve as the foundation for better understanding and further adaptation into 3-D systems. With this, new knowledge linking oxidative stress to microenvironment could be mapped. Eventually with better understanding, the knowledge could be translated into applications such as in the cosmetic surgery industry.

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