Tissue engineering approach using fibrous scaffold with matricellular protein for wound healing

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**NANYANG
TECHNOLOGICAL
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SINGAPORE

**TISSUE ENGINEERING APPROACH USING
FIBROUS SCAFFOLD WITH MATRICELLULAR
PROTEIN FOR WOUND HEALING**

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**INTERDISCIPLINARY GRADUATE SCHOOL
NTU INSTITUTE FOR HEALTH TECHNOLOGIES**

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PROTEIN FOR WOUND HEALING**

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

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Abstract

Electrospun fibrous scaffolds, which closely mimic native extracellular matrix (ECM) fibrous hierarchical physical structures, are promising candidates as grafts for regenerative medicine. For wound healing applications, the interactions between scaffold topographic features and cellular responses, especially the directional cell migration and phenotypic change, are critical but not well explored yet. In this regards, aiming to design superior fibrous structure for tissue-engineered skin graft and reveal the possible underlying mechanisms, interdisciplinary knowledge and techniques incorporating materials science and engineering and molecular biology techniques were employed. In this dissertation, electrospun fibrous matrices with various fiber diameters and fiber orientations were developed and applied as culture substrates to investigate the relationship between the substrate topographies and cell functional behaviors. Results showed that fibrous topography with diameter within natural ECM fibril scale could significantly advance L-929 cell migration, suggesting the essentials of both ECM biomimicking structures and appropriate fiber dimensions in promoting cell migration. Furthermore, accelerated and persistent migration of human dermal fibroblasts (HDFs) was observed on fibers with aligned orientation, evidenced by individual cell tracking using live cell imaging. This interesting phenomenon might be attributed to the relatively low expression and the linear-oriented confinement of focal adhesions. Additionally, the directional and persistent migration might be mediated by the upregulation of Cdc42 GTPase activity which serves to form filopodia protrusion along the fiber orientation. On the other hand, it was found that aligned fibers not only directed and promoted cell migration, but also induced fibroblast-to-myofibroblast differentiation of HDFs, evidenced by increased expression of alpha smooth muscle actin (α SMA) and collagen (type I and type III). The presence of myofibroblast in the wound bed contributes contractile force and ECM component deposition to advance wound repair, but

its continuous persistence might not be desired as it has been shown to be responsible for pathological scarring. However, it was remarkably noted that the introduction of matricellular protein Angiopoietin-like 4 (ANGPTL4) was able to reverse the phenotypic alteration induced by aligned fibers. Moreover, higher transforming growth factor- β 1 (TGF β 1) level in HDFs cultured on aligned fibers might result from mechanical activation, which implied the possible underlying mechanism of aligned fiber-induced myofibroblast differentiation. These discoveries indicated fibrous matrices with oriented configuration are functional in mediating directional cell migration and phenotypic change. Tissue-engineered fibrous grafts with precise fiber alignment modulation and ANGPTL4 releasing properties may thus be promising scaffolds to promote wound repair while minimizing scar formation for efficacious wound therapies in the future.

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Abbreviations

2D	Two-dimensional
3D	Three-dimensional
AF	Aligned Fibers
ANGPTL4	Angiopoietin-like 4
α SMA	Alpha Smooth Muscle Actin
COL1A1	Collagen Type I Alpha 1
COL1A2	Collagen Type I Alpha 2
COL3A1	Collagen Type III Alpha 1
DCM	Dichloromethane
DMF	Dimethylformamide
ECM	Extracellular Matrix
FFT	Fast Fourier Transform
FGF	Fibroblast Growth Factor
ELISA	Enzyme-linked Immunosorbent Assay
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
HDFs	Human Dermal Fibroblasts
HFIP	1,1,1,3,3,3,-Hexafluoro-2-Propanol
LAP	Latency-associated Peptide
MMPs	Matrix Metalloproteinases
NSD	No Significant Different
PBS	Phosphate-buffered Saline
PDGF	Platelet-derived Growth Factor
PLC	Poly (Lactic Acid-co-Caprolactone)
PLGA	Poly (Lactic Acid-co-Glycolic Acid)
RF	Random Fibers
SEM	Scanning Electron Microscopy
TFE	Tetrafluoroethylene
TGF β 1	Transforming Growth Factor β 1

Chapter 1

Introduction

This chapter begins with the background of tissue-engineered skin grafts and the hypothesis of this research (Section 1.1 and 1.2). Electrospun fibrous scaffolds with unique advantages for wound healing are subsequently introduced to the research focus, which is presented in detail in research objectives and scopes (Section 1.3). Finally, the significance and novelty of this research (Section 1.4) as well as the overview of the dissertation (Section 1.5) are also outlined and presented.

1.1 Background

Tissue-engineered skin substitutes still hold great demands in the clinical treatments for full-thickness wounds, due to the insufficient quantity of current gold-standard therapy; autograft [1, 2]. Scaffolds are one of the key factors that determine the efficacy of an artificial skin graft. In skin tissue engineering, ideal scaffolds should not only have similar mechanical properties to the skin, but also confer a compatible microenvironment for skin cell clonogenicity. In this regard, it gives rise to great attention on electrospun matrices whose fibrous configuration closely mimics native extracellular matrix (ECM) [3]. Evidently, electrospun fibrous scaffolds fabricated from a wide range of biomaterials are capable of facilitating cellular attachment, spreading and proliferation [4-7]. However, other key cell behaviors for wound healing, like cell migration and phenotypic maintenance/alteration, have not been well explored for the tissue-engineered scaffolds as skin grafts.

Directional cell migration is vital in tissue regeneration [8]. During wound healing, keratinocytes and fibroblasts migrate directionally to the wound bed and then proliferate to form granulation. However, this kind of migration is generally disabled in chronic non-healing wounds, and hence hinders the proper wound closure [8, 9]. Therefore, it should be of significant contribution to tissue regeneration if the fibrous scaffolds are functional to initiate directional migration of nearby skin cells into wound area. In addition, fibroblast-to-myofibroblast differentiation during wound healing is critical for the acquirement of contractile force to close wound and the synthesis of ECM components to restore tissue integrity [10-12]. Nevertheless, when the healing process is near complete, the persistence of myofibroblasts is responsible for excessive scar formation. Thus, the phenotypic maintenance/alteration of fibroblasts is an important factor when evaluating scaffolds.

1.2 Hypothesis

Cells are in close relationship with the surrounding microenvironment, especially the underlying substrate topography, implying the potential importance of topography on cell behaviors [13]. Thus, we hypothesize that the geometries of electrospun fibrous scaffolds can efficiently modulate motility and phenotype of skin cell. Through the investigation and understanding on cell-material interactions, it is hence promising to fabricate ideal electrospun fibrous scaffolds as functional skin grafts to achieve superior wound therapies.

1.3 Research objectives and scopes

The general objective of this thesis is to investigate the influence of fiber topography (organization and dimension) on skin cell responses in terms of the migratory and phenotypic properties, which are studied through a library of artificial fibrous matrices fabricated. Through these studies, insights regarding tunable wound closure rate could be obtained and these discoveries may significantly promote the fabrication of functional tissue replacement in regenerative medicine for efficacious therapies.

The objectives and scopes of the research are specified as:

- 1) To develop fibrous platforms with differing dimensions, fiber orientations and fiber diameters using electrospinning.
  - Investigation of the role of drum rotating speed to fiber alignment fashion.
  - Study on the role of solution properties to fiber diameter.
  - Development of 3D fibrous scaffolds as implantable ECM replacements.
- 2) To investigate the effect of fiber diameter and alignment on skin cell migration and to elucidate the possible underlying mechanisms.

- Collective and individual cell migration assessment on substrates with diverse topographic features.
  - Investigation of the relationship between cell migration and focal adhesions.
  - Inspection of the role of Rho small GTPase in directional cell migration.
- 3) To evaluate the influence of fiber alignment on fibroblast phenotypic maintenance/alteration and to explore the possible underlying mechanisms.
- Phenotypic evaluation using myofibroblastic markers.
  - Investigation of the reversibility of differentiation by introduction of ANGPTL4.
  - Evaluation of cellular TGF β 1 level.

1.4 Significance and novelty

This dissertation demonstrates that fibrous substrates with oriented fiber configuration were functional in mediating directional cell migration and fibroblast phenotypic change, which was evidenced by state-of-the-art technologies such as live cell imaging and immunochemistry techniques. It was found that human dermal fibroblasts (HDFs) migrated persistently along the fiber axis with a higher velocity on fibrous substrates of aligned configuration. Furthermore, myofibroblast differentiation of HDFs was demonstrated to be induced when the cells were cultured on aligned fibers, while this effect could be reversed through the introduction of extracellular matrix protein ANGPTL4. These discoveries pave a promising path to yield a tissue-engineered skin graft with multiple functions of accelerating cell migration, advancing wound contraction and possibility of minimizing the scar formation. Additionally, the possible molecular basis of topography-modulated cell responses has been examined to elucidate the underlying mechanisms. The novelty of this study is thus

providing insightful knowledge, via the observational research approach, in creating science-driven fibrous scaffold meant for promoting proper wound healing.

1.5 Dissertation overview

This thesis consists of the following seven chapters:

Chapter 1 briefly presents the overview background of the study, followed by an introduction of research objectives/scopes as well as significance/novelty of this project, and finally outlines the overview of the thesis.

Chapter 2 covers a detailed literature review of relevant topics on wound healing process, tissue-engineered strategies for wound therapeutics and matricellular protein ANGPTL4. The state-of-the-art studies of the cellular responses modulated by fibrous scaffold topography are highlighted to elucidate the important role of fiber organization in the design of artificial fibrous grafts.

Chapter 3 introduces the main materials and experimental methods or techniques involved in this dissertation.

Chapter 4 encompasses the development and characterization of diverse fibrous matrices using electrospinning. The roles of drum rotating speed, solution properties as well as collecting environment are experimentally assessed.

Chapter 5 demonstrates the influence of substrate topographic features on cell migration. The relationship between cell migration and focal adhesions is explored. The role of Cdc42 GTPase in directional cell migration is inspected.

Chapter 6 discusses the phenotypic alteration induced by fiber alignment. Fibroblast-to-myofibroblast differentiation is induced by aligned fibers, while the reversibility is assessed by the introduction of chemical factor ANGPTL4. The probable mechanism is discussed and speculated as the mechanical activation of latent TGF β 1.

Chapter 7 briefly summarizes and concludes the results of this study, and gives some recommendations for future perspectives.

Chapter 2

Literature Review

This chapter is split into three sections, namely; 1) skin and dermal wound healing, 2) impaired healing and solutions and 3) matricellular proteins in wound healing. The first section mainly introduces the skin structure and the process of normal wound healing. The vital role in the regulation of myofibroblast in wound healing is also summarized in this section. It also discusses how the microenvironment affects the myofibroblastic differentiation. The second section summarizes the characteristics of impaired wounds and current solutions. Moreover, the on-going research trends and applications of fibrous scaffolds for wound therapeutics have been reviewed in this section. The last section introduces the functions of matricellular proteins, especially the multifaceted modulatory role of ANGPTL4 during wound healing.

2.1 Skin and dermal wound healing

2.1.1 Skin structure

Skin as the largest organ covers the whole surface of the human body with approximately 15% of the total body weight. It exerts various critical functions, such as prevention of moisture loss, sensing on the surroundings and creation of a protective barrier against external aggressions like foreign pathogens and ultraviolet radiation [14-16]. With thickness varying between 1 and 4 mm, human skin is principally organized by three structural layers from outward to inward, namely epidermis, dermis and hypodermis [14].

The epidermis typically composes of densely packed keratinocytes, which synthesize the protein keratin, and also comprise melanocytes, Merkel cells and Langerhans cells. Epidermis keeps constant cellular turnover by division and differentiation of keratinocytes in the inner layer, which move outwards to replace dead or damaged cells in the superficial layer [17]. The boundary between epidermis and dermis is known as basement membrane, constructed by deposition of extracellular matrix (ECM) like collagen type IV and laminin, which provides the anchorage for keratinocytes. Dermis composing of abundant collagens (e.g., collagen type I and collagen type III), elastins and proteoglycans contributes the structural support and nourishment to the skin. Dermis composes the bulk of skin and is vital in protecting the body from mechanical impact. Fibroblasts are the primary residing cells in the dermis, where endothelial cells, macrophages and mast cells are also present. Dermal fibroblasts represent a vital role in wound healing by differentiating into myofibroblasts to assist in skin regeneration after injury. The hypodermis, the innermost layer of skin, is made up of fat with loose connective tissue. Derivative appendages of the skin e.g., hair follicles, arrector pilli, sebaceous glands and sweat glands are found anchored in this layer [18].

2.1.2 Cell types, events and phases in wound healing

Upon an injury, cutaneous wound healing responses with a well-orchestrated and complex series of events involving dynamic interaction and coordination between different cells, cytokines, growth factors and ECM components. As a result of microenvironmental stimulation, cellular gene expression alters to initiate cell proliferation, differentiation and migration in numerous types of cells including inflammatory cells, fibroblasts, endothelial cells and keratinocytes [19]. The healing process can be typically divided into four overlapped and considerably coordinating phases including hemostasis, inflammation, proliferation and remodeling, as shown in Figure 2.1 [20], which will be introduced in detail below.

Figure 2.1 Four phases in wound healing process. Different types of cells and specific cellular events are involved in each phase. Redrawn based on reference [20].

Hemostasis

The first reaction to skin's integrity disruption is hemostasis with the hallmark of fibrin clot formation, preventing ongoing bleeding. It also provides temporary wound coverage against bacteria and serves as provisional matrix for homing of inflammatory cells. When vascular injury and blood leakage occur after wounding, clotting cascade will be initiated. Platelets are initially triggered to activate integrin receptors on their surface to facilitate platelet adhesiveness and aggregation, accompanying with the formation of platelet plug [19]. Subsequently, fibrin, which is polymerized from fibrinogen by thrombin and along with plasma fibronectin and vitronectin, reinforces the platelet plug, leading to the construction of fibrin clot. More than assisting in the inhibition of hemorrhage, platelets presented in the clot also serve as a reservoir of multiple chemotactic signals that manage wound healing. Through degranulation, the platelets in the clot secrete mediators to attract circulating inflammatory cells to the wounded location, and meanwhile recruit growth factors (including platelet-derived growth factor (PDGF), transforming growth factor beta 1 (TGF β 1) and vascular endothelial growth factor (VEGF)) to stimulate angiogenic response and activate local fibroblast and endothelial cells [21, 22].

Inflammation

Once hemostasis is achieved, vascular permeability increases and circulating leukocytes (i.e., inflammatory cells) migrate sequentially into injurious site by a huge variety of chemo-attractants, referred as the inflammatory phase. These chemo-attractants not only originate from platelets, but also derived by bacterial degradation and other matrix components [19, 23]. Upon activation by inflammatory mediators, an increasing expression of selectins (i.e., adhesion molecules) on endothelial cells slows down the flow of leukocytes in the blood

stream, and then stronger adhesion resulting from binding with integrins will help the activated leukocytes crawl through endothelial layers into the extracellular space [20, 21]. Neutrophils are the first circulating leukocytes to be recruited and demobilized at the wound site, while the number of neutrophil keeps increasing steadily after 24~48 hrs of recruitment (before peaking) [23]. Neutrophils facilitate bacterial killing and wound decontamination, through the secretion of hydrolytic enzymes and the formation of superoxide and hydrogen peroxide (i.e., reactive oxygen species) by the respiratory burst [24]. They also release pro-inflammatory cytokines including Interleukin1 (IL1) and tumor necrosis factor alpha (TNF α) to activate macrophages, keratinocytes and fibroblasts [21]. As a result of reduction in inflammatory mediators, neutrophil infiltration ceases and neutrophil undergoes apoptosis.

As the population of neutrophils decreases and macrophages from circulating monocyte differentiation start to accumulate at the wound bed after 2~3 days, the macrophages become the predominant phagocyte in place of the neutrophil. Macrophages remove remaining pathogenic organisms, apoptotic neutrophils and matrix debris via phagocytosis, functioning as antigen-presenting cells [22]. In addition, macrophages also work as a continuing battery of multiple cytokines and growth factors that include TGF α , TGF β , VEGF, PDGF and fibroblast growth factor (FGF) [21, 22]. These growth factors recruit and trigger local fibroblasts and endothelial cells to facilitate the formation and angiogenesis granulation tissues. The inflammatory reactions appear to be essential in enabling wound healing. Nevertheless, prolonged inflammation seems to be responsible for the development of a chronically non-healing wound [20].

Proliferation

The proliferative stage overlaps with the inflammatory phase, with major events including epithelialization, ECM deposition, granulation tissue formation, wound contraction and angiogenesis.

The re-establishment of the epithelial surface (epithelialization) can occur as early as a few hours post injury, which also depends on the severity of the wound damage. In the case that the basement membrane, where the epithelial progenitor cells locate, is intact at the wound site, epithelial layer can be restored within a few days by epithelial cell (keratinocyte) division and upward migration in their normal renewing fashion. If the basement membrane has been ruined, the basal keratinocytes from the margin of wound and skin appendages (e.g., hair follicles and sweat glands) are responsible for the epithelialization [25]. However, keratinocyte migration requires viable matrix, and thus the damaged site must be firstly filled with granulation tissue if the wound is deep. During re-epithelialization, the stimuli to activate keratinocyte movement include the losing of neighboring cells at the periphery of the wound and local release of growth factors [22].

Fibroblasts become the dominant cell type in proliferative phase of healing, which fulfils various functions, such as deposition of collagen, rebuilding the skin and forming myofibroblasts to contract the wound. Growth factor cocktail (especially PDGF and TGF β 1) secreted by platelets and macrophage in wound bed stimulates fibroblast activation. The activated fibroblasts migrate from adjacent tissues and proliferate to repopulate the damage area [22]. Additionally, the fibroblasts are responsible to synthesize the matrix proteins like collagen, fibronectin, hyaluronic acid and proteoglycans. All of these structural molecules contribute to the reconstruction of ECM. Dense fibroblasts, macrophages and blood vessels form the granulation tissue after embedding the loose network of matrix molecules, which provide platform for keratinocyte migration. Over time, the latterly formed granulation tissue will replace the provisional fibrin matrix,

marking the end of the proliferative phase. Under stimulation by TGF β 1 and microenvironmental mechanics, fibroblasts differentiate into myofibroblasts, which exert contractile properties to help wound closure through expressing alpha smooth muscle actin (α SMA). The important role of myofibroblast during wound healing will be reviewed in detail in Section 2.1.3.

The creation of new blood vessels is essential for the delivery of oxygen and nutrients to the wound. The active angiogenic process is stimulated by the hypoxic state of wound environment and facilitated by migration and proliferation of endothelial cells from the pre-existing and intact capillaries at the wound bed [22]. Multiple cytokines and growth factors, including FGF, VEGF, TGF β , TNF α and thrombospondin, are concerted in the neovascularization. In clinical complications from diseases such as diabetes, poor capillary formation leads to an insufficient nutrient supply to sustain the tissue deposition in the granulation phase, and thus results in the development of a chronically unhealed wound [26].

Remodeling

The remodeling of wound tissue will last a prolonged time up to 1~2 year or even longer, which can be alternatively called the maturation. The remodeling phase involves ECM reorganization coupled with diminishing cellularity and ceasing cellular activities [19]. The reduction of cellularity is indicated by the decline of cellular activity, the decrease and regression of blood vessels and the apoptosis of inflammatory cells and myofibroblasts. During remodeling, the ECM components undergo active metabolism and turnover. Collagen type III, the prevalent matrix component deposited within the proliferative phase, is replaced gradually by the collagen type I which is the major matrix protein of the dermis [27]. This matrix remodeling is profoundly carried out by proteolytic enzymes, such as matrix metalloproteinases (MMPs) and their inhibitors-tissue

inhibitors of metalloproteinases (TIMPSs) [27]. The dysfunction of collagen remodeling from type III to type I is responsible for the formation of excessive scar [27]. During wound maturation, wounded skin gradually becomes stronger over time with increasing tensile strength, owing to the appearance of elastin [27]. Nevertheless, the tissue never recovers the complete properties of uninjured skin. The maximum strength that can be restored is roughly 80% of the strength of healthy unwounded skin [28].

2.1.3 Myofibroblast involvement in wound repair

It is well accepted that smooth muscle cell-like phenotype of fibroblast serves a critical function during the wound healing process [12], known as myofibroblast. Myofibroblasts appear normally five days after wounding in human body [29], which are differentiated from fibroblasts during the proliferative phase. Myofibroblasts are capable of speeding up wound closure and promoting matrix reconstruction [27]. The incorporation of neo-expressed α SMA into microfilament bundles and stress fibers contributes to the high contractile forces in myofibroblasts, which assists to contract the edges of the wound. Moreover, myofibroblasts are active in synthesis and deposition of ECM components (especially collagen) for replacing the provisional matrix and providing platform for epithelialization. Myofibroblasts typically synthesize collagen 1~2 times more than fibroblasts [30]. Experimentally, the increasing expression of α SMA and the excessive production of collagens (type I and type III) are generally used as molecular indicators for myofibroblast formation. It is well documented that fibroblast-to-myofibroblast differentiation is precisely regulated by the combination of growth factors (e.g., TGF β 1), specialized ECM (e.g., ED-A fibronectin) and the mechanical properties of microenvironment both *in vivo* and *in vitro* [12, 31, 32].

TGF β 1 is widely considered as the pivotal growth factor in myofibroblastic transformation with upregulation of α SMA expression and ECM production. The induction of myofibroblastic differentiation by TGF β 1 is dependent on ED-A fibronectin and matrix stiffness, suggesting the key functions of chemical and physical properties of ECM in cell activity mediators [27, 31, 33]. It is canonically accepted that TGF β 1 increases α SMA expression through Smad signaling pathway [34, 35]. Active TGF β 1 binds to TGF β type II receptor, rendering the phosphorylation and recruitment of TGF β type I receptor which phosphorylates Smad2 and Smad3. Phosphorylated Smad2 and Smad3 separately bind to Smad4 to form a complex and translocate into the nucleus to regulate the transcription of targeting genes coupled with other DNA transcription factors [36-38]. Independently from TGF β 1, only a few molecular factors were reported as agonists to induce α SMA expression, e.g., IL6, nerve growth factors, Fizz1 and angiotensin-II, while the molecular mechanism remains unclear [12].

Besides biomolecules, the mechanical properties of cellular microenvironment also play a key role in regulating myofibroblast development, in particular, the regulation by matrix stiffness. Higher level of α SMA expression has been observed in fibroblasts cultured on gel substrates with increasing rigidity [33, 39, 40]. In addition, the TGF β 1-induced myofibroblast differentiation is dependent on the matrix stiffness. Suppressed myofibroblastic differentiation is observed on compliant substrate (< 1 kPa) under TGF β 1 stimulation, while the provisional matrix formed after tissue injury is considered as very soft (10~1000 Pa). This may partly explain why myofibroblast is absent in early wounds despite the high levels of active TGF β 1 [32]. It seems that a matrix stiffness of 20 kPa or higher is required to permit myofibroblast differentiation. Recently, Hinz B and co-workers revealed that the mechanoregulation of α SMA expression and myofibroblastic differentiation is correlated with the mechanical activation of latent TGF β 1, as illustrated in Figure 2.2 [32, 41, 42].

TGF β 1 is synthesized by cells and stored as an element in a latent complex within ECM. The latency-associated peptide (LAP), another component of the latent complex, connects the TGF β 1 and cell via integrins [43, 44]. The TGF β 1 is activated and released mechanically through deformation of the latent complex, which is triggered by mechanical stress from matrix and cell traction force [41, 45, 46].

Figure 2.2 Illustration of mechanical activation of latent TGF β 1. TGF β 1 is synthesized by cells and stored in a latent complex with LAP. Under mechanical stress from matrix and cell traction force, TGF β 1 is released mechanically through deformation of the latent complex. Redrawn based on reference [41].

The presence of myofibroblasts is beneficial and required for wound contraction and ECM deposition during wound healing. When the healing finishes, myofibroblasts normally disappear through apoptosis. However, the pathological persistence of myofibroblasts will produce expansive ECM, leading to keloids or hypertrophic scars with contraction [27].

2.2 Impaired healing and solutions

Skin is serving as the protective barrier primarily against the surrounding environment. The loss of integrity of large portions of skin, causing from burn, venous stasis or diabetes mellitus, may result in major disability or even death. The failure in yielding a durable, structural and cosmetic closure via a spatially and temporally continuum of events will lead to chronic wound formation [47]. These non-healing wounds, including diabetic foot ulcers, pressure ulcers and venous ulcers, represent one of the most significant medical burdens in the world today [48].

Though it is challenging to measure the costs correlated to chronic wounds, the worldwide prevalence of diabetes is apparent. Meanwhile, diabetic foot ulceration is the most common reason for hospital admission and deserves the responsibilities for most lower-limb amputations [49]. Furthermore, a diabetic wound that heals poorly is patulous to infections, usually leading to chronic inflammation, sepsis, dehiscence and even subsequent death. In spite of the various impacts of these chronic wounds, effective therapies are still lacking. To effectively address these problems, it is critical to understand the healing process and to create a salubrious environment physically and biologically to promote healing.

2.2.1 Characteristics and therapies of non-healing wounds

Normal wound healing undergoes a series of events that includes inflammatory response, proliferative activity and remodeling (see Section 2.1.2 for detailed description) [21, 50]. These events carry out a complex interplay between connective tissue generation, cellular behaviors and biomolecule activities. However, all these physiologic processes are altered in the state of diabetes, where the chronic wound may be stuck in diverse events, losing the ideal congruousness in the pathway to wound closure [20]. This can be characterized by the formation of devitalized tissue, increased and prolonged inflammation, poor angiogenesis and deficiencies in ECM proteins [51]. Chronic wounds exhibit increased presence of matrix metalloproteinases and enhanced proteolytic degradation of ECM components, resulting in a corrupt microenvironment unable to support healing [20, 52].

Primary clinical protocols for treating chronic wounds include debridement, prevention of infection, maintaining moist wound environment and off-loading [53-56]. Additional therapies may be applied if wounds fail to heal after the treatments above for three weeks. The ideal goal of wound care would be to regenerate tissue with restoring structural and functional properties as before injury. Current therapies for chronic wounds employ growth factor administration for improved tissue restoration [57], but limited amount of available growth factors may be insufficient to restore tissue homeostasis. Although tissue-engineered skin substitutes have already been used for chronic wounds, there are several intrinsic shortcomings such as fragile epidermal grafts, creation of new wounds in autografts, and possible transmission of infectious diseases and immune rejection in the case of allografts or xenografts [58, 59].

2.2.2 Fibrous scaffolds for wound therapeutics

The deficiencies in ECM and the accumulation of devitalized tissues are significant characteristics of non-healing wounds [51]. From this view,

introduction of a biofunctional construct to replace the missing or dysfunctional ECM may be beneficial. Ideally, such an artificial replacement should closely mimic the fibrous construction of natural ECM [60] and provide both physical and chemical cues for recruiting nearby skin cells into the wound bed. To date, various studies have been conducted to fabricate fibrous matrices that not only resemble the morphological network of ECM but also possess tunable physical properties and good biocompatibility [61]. It was reported that enhanced cell responses were revealed on artificial fibrous matrices, including improved cell attachment, progressive proliferation, directed migration and modulated gene expression signature [62, 63]. The improved cell performance by fibrous scaffolds may render accelerated tissue repair and thus the scaffolds may work as attractive matrices for skin tissue engineering [64].

Electrospinning is recognized to be a simple, rapid and cost-effective approach for the fabrication of fibrous matrices. In electrospinning processing, polymer solution is loaded in a capillary tube and confined by its surface tension. The ejection of polymer solution is accomplished when it is exposed to an electric field with the electrostatic repulsion overcoming the surface tension. Meanwhile, the solvent evaporates when the solution-jet travels in air, leaving behind polymeric fibers which will deposit on the collector [65-67]. Finally, continuous fibers are collected to generate a non-woven fabric that mimics the fibrous network of native ECM, with the fiber sizes closely similar to ECM fibrils [68, 69]. By varying the processing parameters (e.g., applied voltage, solution flow rate and spinneret-collector distance) and solution properties (e.g., surface tension, viscosity and conductivity), the fiber diameter, fiber orientation (aligned vs. random) and pore size of the electrospun fibrous scaffolds are able to be tailored and optimized for specific applications [66]. For the applications of skin tissue engineering, various designs of electrospun fibrous scaffolds have been achieved, which is summarized as below in terms of 1) selection of materials; 2) involvement of bioactive molecules; 3) topographic cues.

Selection of materials

As a material for the fabrication of electrospun fibers for wound healing, the material must generally show good biocompatibility. Meeting this basic requirement, a variety of polymers have been employed for such fiber construction, including synthetic and natural materials, as well as composites, which can confer both mechanical support and biomimetic stimulation.

In addition to biocompatibility, tunable mechanical and biodegradable properties enable synthetic polymers to attract widespread attention for the fabrication of electrospun scaffolds for biomedical applications. Currently, synthetic polymers available in electrospinning include poly (vinyl alcohol) (PVA), poly (hydroxybutyrate-co-hydroxyvalerate) (PHBV), poly (ϵ -caprolactone) (PCL), poly (lactic acid) (PLA), poly (lactic acid-co-glycolic acid) (PLGA) and poly (lactic acid-co-caprolactone) (PLC), and so on [63, 70-73]. Owing to its absorbable and semipermeable abilities, PVA is frequently used as a basic material in electrospun fibers to prevent the accumulation of wound fluid [74]. While PLGA degrades relatively fast, PCL exhibits relatively small degradation kinetics, being advantageous to facilitate structural stability and prolonged usage [75, 76]. PLC is more elastic and less rigid as compared to most other aliphatic polyesters, and the tensile properties of PLC electrospun fibers falls within the range of native human skin [77-79]. Besides these materials that are widely-used, some newly synthetic polymers have been developed to construct optimized electrospun scaffolds. For instance, thermoresponsive polymers poly (di(ethylene glycol) methyl ether methacrylate) (PDEGMA) and poly (N-isopropylacrylamide) (PNIPAAm) exhibit varying hydrophilicity (from hydrophilic to hydrophobic) under temperature stimulation. As wound dressing materials, their thermoresponsive properties enable

manipulation over cell adhesion and detachment, reducing secondary injuries when replacing wound dressing [80, 81].

In order to mimic the natural ECM in a more accurate manner, natural materials have also been investigated as electrospinning polymers, including collagen, chitosan, gelatin, hyaluronic acid and small intestine submucosa (SIS) [6, 82-84]. However, these commonly used natural materials often lack the appropriate physical properties to facilitate electrospinning. The addition of synthetic polymers is able to improve the electrospinnability of natural materials, resulting in hybrid materials consisting of synthetic and natural materials. For instance, Chitosan, an amino polysaccharide obtained from chitin, possesses antimicrobial properties that are beneficial for infection-related wound healing, but it is hardly used alone as an ideal material for electrospinning [85]. To facilitate its processability, a common approach is to blend it with synthetic materials such as PVA, PLA and poly (ethylene oxide) (PEO) [86]. Collagen is one of the main structural proteins in skin ECM, representing an attractive material to develop skin substitute. Blending collagen with zein (a maize protein), which has better biocompatibility over synthetic polymers, makes it easier to form fibers by electrospinning [87]. Although hybrid materials may have the advantage in both mechanical and biological properties, the blended processing causes challenges to produce all scaffolds with identical surface chemistry throughout all batches.

Involvement of bioactive molecules

To better manage the process of wound healing, the involvement of bioactive molecules in/on the electrospun fibers appears to be essential. Anti-inflammatory and anti-bacterial ingredients were often incorporated in fibers by directly blending in electrospinning solutions to yield multi-functional wound healing materials [7, 74, 82]. It is widely noted that growth factors play vital

roles in wound healing. Due to difficulty in getting a uniform solution and harmful effects from organic solvents, the administration of growth factors through direct dispersion into polymer solution would not be effective. Through linker heparin, growth factor bFGF have been successfully grafted onto the surface to achieve bioactive fibers for accelerated wound healing [88]. Alternatively, introduction of core-sheath structure into fibers has also been investigated for sustained delivery of bioactive proteins, which could be achieved by coaxial electrospinning or emulsion electrospinning. By incorporating cyclodextrin as formulation excipient, core-sheath fibers loaded with bFGF was successfully fabricated through emulsion electrospinning, which showed to accelerate skin regeneration in diabetic rats [89].

Topographic cues

Electrospun fabrics hold great promise in biomedical applications due to their ECM-mimicking fibrous structure, providing topographic cues to guide and modulate crucial cell behaviors involved in the regenerative processes. Studies showed that the fibrous nature of electrospun scaffolds was capable of promoting cell attachment, advancing cell growth as well as modulating cytoskeletal organization [4, 90]. Besides the fibrous nature, other topographic characteristics (like pore size, fiber diameter and fiber alignment) of electrospun scaffolds appeared to play a key role in regulating cellular behaviors.

The pore size of fibrous scaffolds could be manipulated by blending water-soluble PEO into the polymer solution, in which the PEO would be removed post electrospinning [5]. It has been reported that architectures with pore size ranging from 6 to 12 μm further benefited the progressive proliferation of human dermal fibroblasts, while fibers with pore size larger than 12 μm would restrain cellular spreading morphologies. Recently, a single-step process using customized collectors was assessed to fabricate fibrous scaffolds with diverse

porosities (0~70%) and pore shapes (circle, rhomboid and square) [91]. However, the cell responses over this kind of platforms have not been well understood.

Various studies have been conducted to investigate the cellular response as a function of fiber diameter. Fibers with differing diameters can be easily produced through adjusting the electrospinning parameters, like polymer concentration, solvent conductivity, jet-to-collector distance and applied voltage. Results demonstrated that fibers with diameter smaller than 1 μm were able to stimulate cell growth and collagen deposition as part of the repair process [92-95].

Fiber alignment has been evidenced to be critical to guide cellular behaviors and tissue assembly [88, 96]. The fabrication of parallel fiber alignment can be achieved by rotating drum or a pair of electrodes, while a composited collector with a central point electrode as well as a peripheral ring electrode facilitates the formation of radial alignment [97]. Despite the alignment pattern, it was well reported that aligned fibers stimulated elongated morphologies and enhanced directional cell migration [88, 96, 97]. Cells would be guided to migrate along the long axis of fiber. Indeed, the modulation of cell migration is vital in many physiological and pathological processes, including embryonic development, tissue regeneration, tumor metastasis, etc [9]. Particularly, during wound healing process, keratinocytes and fibroblasts migrate directionally to the wound bed and then proliferate to advance wound repair. Besides the directional cell migration, it has been showed that aligned fibers were able to induce human mesenchymal stem cell differentiation towards myogenic lineage [98]. These findings delineate the important role of fiber alignment as design parameters in the fabrication of biomimetic scaffolds. Nevertheless, the behind signaling pathways of aligned fiber-induced cellular responses are still unclear to date. To better construct artificial and functional tissue architecture to

improve wound healing, it is highly desired to understand how fiber alignment regulates skin cell migration as well as phenotypic alteration, and the underlying intracellular signaling pathways.

2.3 Matricellular proteins in wound healing

Ideally, ECM replacement should be multifaceted and interactive in nature, and closely approximate the components of the normal ECM. The major compositions of ECM include collagens and fibronectins, which serve as primarily a constructional function in the matrix to maintain the structural integrity [99]. Apart from that, matricellular proteins are a group of proteins that present in ECM but do not serve as stable structural elements in extracellular environment. These proteins are turned over rapidly and dynamically, playing crucial role in the modulation of cell-matrix interactions [100]. The major structure characteristic of matricellular proteins is possessing adhesive sites for ECM structural proteins, cell surface receptors as well as biomolecules (e.g., cytokines, growth factors, proteases and proteases inhibitors). These binding sites facilitate the association with diverse proteins in ECM reservoir and bridge the intersection of cell-matrix communications and cell-cell communications [100-102]. As a result, they act temporally and spatially to provide signals that influence cell activities such as migration, adhesion, inflammation and proliferation [100, 102]. Members of matricellular proteins include the CCN family [100], thrombospondins [103], osteonectin (also known as SPARC) [104], osteopontin [105], tenascin C [106] and the recently discovered angiopoietin-like protein 4 (ANGPTL4) [107, 108].

Many of matricellular proteins have been shown to take part in various processes related to wound healing and tissue repair. *In vivo* studies using knockout mice have shown that the deficiency in one or more of these matricellular proteins would impair the wound healing [108, 109]. Furthermore,

upregulation of matricellular proteins have been observed during wound healing, where they modulate communications between cells and the ECM to exert control over events that are essential for efficient tissue repair [110, 111]. Presumably, the managed pathways consist of complex networks with many opportunities for compensatory adjustments required for wound repair. Therefore, targeting or replacing the necessary matricellular proteins may be more efficient than individual cytokine-mediated candidates for wound therapeutics.

2.3.1 Angiopoietin-like protein 4 (ANGPTL4) as wound-healing agent

The matricellular protein ANGPTL4 of interest has been recently reported to serve a vital function in wound healing [112]. It is a secreted glycosylated protein, releasing a coiled-coil N-terminal domain (nANGPTL4, fragments 17~207) and a fibrinogen-like C-terminal domain (cANGPTL4, fragments 207~460) under proteolytic cleavage. While nANGPTL4 plays a well-established role on regulation of circulating triglyceride metabolism by inhibiting the lipolysis of triglyceride-rich lipoproteins [113, 114], cANGPTL4 modulates multifaceted cellular functions to improve wound repair process [107, 108, 115, 116]. Herein, the regulatory functions of cANGPTL4 will be discussed in detail.

During the normal process of wound healing, the expression of cANGPTL4 increased progressively in wound epithelia and returned back to basal level after complete wound closure [108]. cANGPTL4 correlates with vitronectin and fibronectin in the wound bed to decrease their proteolytic degradation of these local ECM proteins, which assists in providing a provisional matrix for epidermal cell migration [108]. In keratinocytes, cANGPTL4 interacts with integrin $\beta 1$ and $\beta 5$, activating the integrin-mediated intracellular signaling to enhance keratinocyte migration during wound re-epithelialization [107].

Moreover, the cANGPTL4/integrin signaling axis in keratinocytes mediates upregulation of inducible nitric oxide synthase (iNOS) expression to increase nitric oxide (NO) production within the wound bed, and thus advances wound angiogenesis to accelerate wound repair [116]. The signaling pathways modulated by cANGPTL4 during wound healing are illustrated in detail as shown in Figure 2.3. In chronic diabetic wounds, the expression of cANGPTL4 remained low throughout the healing process. Consistently, the deficiency in ANGPTL4 of mice resulted in delayed wound re-epithelialization, decreased matrix proteins expression, elevated inflammation and an impaired wound-related angiogenesis, which are common characteristics of diabetic wound [107, 108, 117].

Figure 2.3 Schematic illustration of the multifaceted modulatory roles of cANGPTL4 during wound healing. Adapted from Chong H.C.'s PhD thesis.

It is noteworthy that the role of cANGPTL4 in fibroblasts and its corresponding influence on wound scar formation has been revealed recently [118]. Under

treatment with cANGPTL4, enhancement in fibroblast migration and proliferation is observed, while the production of scar-associated collagen type 1a2 and 3a1 is attenuated. The reduction of collagen deposition is mediated through interaction of cANGPTL4 with fibroblast cadherin-11. The interaction trigger β -catenin translocation into the nucleus and lead to upregulated expression of inhibitor of DNA-binding/differentiation protein 3 (ID3) in fibroblasts. Finally, ID3 binds to scleraxis, a basic helix-loop-helix transcription factor, reducing the transcriptional expression of scar-associated collagen type 1a2 and 3a1. The signaling mechanism is illustrated in Figure 2.4. Taking the above information into account, cANGPTL4 is suggested to be a potential multifunctional agent for advancing scar-free wound repair and regeneration.

Figure 2.4 Illustration depicting the signaling mechanism of cANGPTL4 on COL1A2 and COL3A1 expression in fibroblasts [118].

2.4 Summary

From the literature, most of the previous studies focus on the influence of the fiber alignment on cell migration and concluded that alignment generally improved migration, just as important as migration, phenotypic alteration and maintenance investigation is essential especially in the case of wound healing where fibroblast to myofibroblast differentiation dynamics is paramount to the success of a fast healing and minimal scarring therapy. Moreover, the effect of fiber diameter has not been explored. Therefore, it is in the interest of this thesis to shed light on the phenotypic alternation by topographic cues and possible ways to arrest this alteration at the appropriate timing to balance rapid wound closure with possible reduced scarring. The scope will cover *in vitro* studies for fundamental understanding and possible molecular basis of topography modulated cell behaviors will be examined to elucidate the mechanism. Through these studies, insights regarding cell-materials interactions could be obtained, and these discoveries may significantly promote the creation of science-driven fibrous scaffolds for efficacious therapies.

Chapter 3

Experimental Methodology

This chapter summarizes the main experimental methods and techniques used in this thesis and it is split into four sections. The first section starts by revealing the techniques used in scaffold fabrication, followed by the second section describing scaffold characterization methods. Subsequently, general cellular studies are presented in the third section. Finally, in order to demonstrate whether there were significant differences among and between groups of specimens, statistical analysis was conducted whenever necessary in this work, the methods of which are described in the fourth section.

3.1 Scaffolds fabrication and experimental setup

3.1.1 Development of electrospun scaffolds

Polymer solution at desired concentration was prepared by dissolving polymer granules in organic solvent. The solution was stirred for 12 hours before electrospinning to obtain a clear, homogenous, and viscous solution. Fabrication of electrospun scaffold was achieved with the electrospinning apparatus NANON-01A (MECC, Japan). For electrospinning processing, appropriate amount of polymer solution was loaded into a 5 mL (diameter = 13.4 mm) plastic syringe, with a tube connecting a syringe to a metal blunt needle equipped spinneret. In this thesis, collectors were selected depending on different purposes [65]. For the fabrication of two-dimensional (2D) fibrous mats, conductive metal collectors (rotating drum or flat plate) were used. The collectors were covered with aluminium foil for the deposition of electrospun fibers, with a fixed working distance of 15 cm for product collection. For three-dimensional (3D) fibrous sponge development, fibers were collected inside liquid phase with working distance of 4 cm [73]. The liquid consists of 7:3 (v/v) isopropyl alcohol and distilled deionized water with 0.05% (w/v) Koppiphor P188. The eventual dimension of scaffolds can be controlled by customized molds. Collected electrospun scaffold was dried in vacuum oven at 37 °C for 7 days to remove any residual organic solvent.

3.1.2 Film formation

Film construction started from dissolving polymer granules in organic solvent at desired concentration. The polymer solution was then cast onto glass slides using Sheen automatic film applicator (Sheen Instruments, England) at a speed of 50 mm/s with a wet thickness of 500 µm. The cast PLC membranes were left

in ambient conditions for 24 hours and subsequently placed in a vacuum oven at 37 °C for 7 days to remove the remaining solvent.

3.1.3 Microfiber melt drawing

A cylindrical cup with an orifice at the bottom was used to hold polymer melt. In addition, a rotating mandrel was introduced to collect microfibers after it was drawn manually from the melt. To initiate the drawing process, a needle was inserted into the melt through the orifice to break the melt surface and pull the melt down, creating a single strand of microfibers. To facilitate the continuous collection, the drawn microfiber was wound onto the rotating mandrel [119].

3.2 Characterization of scaffolds

3.2.1 Morphological assessment of electrospun scaffolds

The morphology of the scaffolds was examined under a JSM-6360F (JEOL, Japan) scanning electron microscopy (SEM). The SEM helps to directly visualize the sample topography by bombarding the surface of the sample with its electrons, and detecting the resulting secondary electrons generated by the sample surface due to the bombardment. It is noted that resolution of SEM imaging is affected by charging of samples, especially biological specimens. Hence, dried samples were sputtered with platinum at 20 mA for 60 sec and analyzed at a working voltage of 5 kV so as to avoid charging issues which might lead to poor quality images. SEM images were taken at different magnifications. At least 5 random SEM images (2000×) were taken for fiber diameter and pore size measurement by ImageJ, and a total of 150 data points were used to determine the average diameter. OriginPro 2016 was used to plot histogram of fiber diameter distribution enabling further analysis.

3.2.2 Fiber alignment quantification by FFT analysis

2D fast Fourier transform (FFT) analysis was applied to characterize the degree of fiber alignment as a function of rotating speed during electrospinning, which transformed the spatial information of the original image to output frequency distribution [120]. The process of FFT analysis was demonstrated using aligned fibers and random fibers, as shown in Figure 3.1. Firstly, digitized SEM images in 8-bit grayscale TIF files (1280×960 pixels) were cropped into 762×762 pixels (Figure 3.1 A and C) and converted to FFT output image in 1024×1024 pixels (Figure 3.1 B and D). Then the FFT images were 90° rotated, and the grayscale pixel distribution of FFT output images were circularly quantified for each degree from 0° to 360° to reflect the fiber alignment (Figure 3.1 E and G), using a plugin Oval Prolife (authored by Bill O'Connell) in ImageJ. The gray intensities were normalized to a baseline of 0 and plotted within first 180° (Figure 3.1 F and H). The degree of alignment was finally determined by the peak value and the shape of peak presented in the plot.

Figure 3.1 Demonstration of FFT analysis on fiber alignment. A, C: Cropping squares on SEM images for aligned fibers (AF) and random fibers (RF), respectively. B, D: FFT output image post 90 ° rotation from the cropped SEM images, and their selected oval for quantification (yellow circled area). E, G: the plot of FFT frequency gray value between 0 ° and 360 ° of aligned and random fibers. F, H: the plot of normalized FFT gray intensity between 0 ° and 180 ° of aligned and random fibers.

3.2.3 Analysis of mechanical properties

The mechanical properties of fibers were measured by electromechanical tester 5567 (Instron, USA). Investigations on the mechanical properties of the fiber samples were conducted with static film tensile mode by pulling the sample with two clamps until it breaks [121]. It is noted that results with false accuracy may be generated if the sample slips during the pulling process. Thus, samples must be firmly held by two clamps during the measurements. Prior to mechanical testing, the measured thickness values of the fibrous samples were keyed into the software for calculation. The thickness of the fiber samples was found to be 30~50 μm according to the measurement by micrometer screw gauge. Samples were stretched until they broke at a ramping rate of 5 mm/min. The ultimate tensile strength and strain at break point were determined from the stress-strain curves generated from 3 individual experiments for each sample type. The Young's modulus was determined by evaluating the initial linear slope of the stress-strain curve using the following expression.

$$\text{Young's Modulus } (E) = \frac{\text{stress}}{\text{strain}} = \frac{F/A}{\Delta l/l_o}$$

Where F is the force required for sample failure; A is the cross-sectional area of test sample; Δl is sample extension length; l_o is the sample original length.

3.2.4 Analysis of chemical properties

The chemical properties of fibrous scaffolds were analyzed by attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR). FTIR is an absorption technique whereby IR radiation, which passes through the sample, will get absorbed by the functional group existed in the sample. The technique

determines the chemical composition of sample in terms of functional groups via analysis of the acquired spectral adsorption bands. For FTIR analysis, it is noted that moisture can interfere with absorption bands and then result in broadening of the bands. Thus, strict drying process of samples was conducted to eliminate the moisture impact before FTIR characterization. Absorbance spectra of the samples were recorded with a resolution of 2 cm^{-1} at wavenumbers from 600 to 4000 cm^{-1} using a FTIR spectrometer (Perkin Elmer, Singapore).

3.3 Cell culture and cellular studies

3.3.1 Materials and reagents

L-929, a cell line of mouse fibroblast, was obtained from ATCC, USA. Human dermal fibroblast (HDFs), harvested from human neonatal foreskin, and Medium 106 were purchased from Invitrogen, Singapore. High glucose Dulbecco's Modified Eagle Medium (DMEM) and PBS were purchased from Lonza, Switzerland. DiI, Fetal bovine serum (FBS), trypsin-ethylenediaminetetraacetic acid (EDTA) and antibiotic-antimycotic were purchased from Gibco (Thermo Fisher Scientific, USA). Hoechst 33342, 4',6-diamidino-2-phenylindole (DAPI) and rhodamine phalloidin were purchased from Life Technologies (Thermo Fisher Scientific, USA). Rabbit monoclonal to alpha smooth muscle actin (Alexa Fluor 488) and fluoroshield mounting medium with DAPI were purchased from Abcam, Hong Kong. Anti-vinculin antibody was obtained from Merck, Germany. All primers used in this thesis were obtained from IDT, Singapore. Matricellular protein angiopoietin-like 4 (ANGPTL4) was provided by Prof Andrew Tan's lab [118].

For cellular assays, scaffolds were sterilized by UV irradiation (wavelength = 254 nm) and washed by DI water and PBS three times prior to cell seeding.

3.3.2 Cell culture

The L-929 cells were cultured in high glucose DMEM containing L-glutamine supplemented with 1% penicillin-streptomycin and 10% FBS. The HDFs cells were routinely expanded and maintained in Medium 106 prior to experiment. Experiments were carried out using HDFs at Passage 5, which were cultured in high glucose DMEM containing L-glutamine supplemented with 1% penicillin-streptomycin and 10% FBS. Culture medium was refreshed every 2~3 days. The cells were maintained at 37 °C in 5% CO₂ humidified atmosphere. PBS and 0.05% trypsin-EDTA were used for washing and cell detachment purposes respectively.

3.3.3 Immunocytochemistry

At predetermined time points, cells on different groups of substrates were respectively fixed in 4% PFA for 15 min after rinse with PBS. Subsequently, the cells were permeabilized in 0.1% Triton-X for 10 min at room temperature, followed by 3-times washing. Non-specific binding sites were blocked with 10% goat serum for 60 min at room temperature. The samples were then incubated with respective primary antibodies at 4 °C overnight, with PBS washing for 3 times post incubation. Corresponding secondary antibodies were subsequently added and allowed to stand at room temperature for 1 hr in dark, and the samples were then washed with PBS for 3 times. The treated cells were counterstained with DAPI for nucleus and rhodamine phalloidin for intracellular actin filaments, which were finally mounted on a glass slide with DAKO mounting agent and imaged with an upright fluorescence microscope (Nikon 80i eclipse, Japan).

3.3.4 Quantitative polymerase chain reaction (qPCR)

Prior to RNA extraction, the cells were rinsed with PBS. EZ-10 Spin Column Total RNA Mini-Preps Super Kit (Bio Basic, Canada) was employed to isolate total RNA according to the manufacturer's protocol. The isolated RNA was quantified and qualified spectrophotometrically using Nano drop-N100 (Thermo Fisher Scientific, USA). 500 ng of RNA were used to generate first-strand complementary DNA (cDNA) by iscript^{TM} cDNA Synthesis Kit (Bio-Rad Laboratories, USA). The cDNA synthesis was performed on Applied Biosystems 2720 Thermal Cycle (Thermo Fisher Scientific, USA) using the following thermal programme: 25 °C for 5 min, 46 °C for 20 min and 95 °C for 1 min. The synthesized cDNA was 10-times diluted and stored in -20 °C for further usage. qPCR was performed with KAPA SYBR FAST Master Mix (2×) Universal (KAPA Biosystems, USA) using CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, USA) according to protocol provided by manufacturer. The thermal cycling profile was 95 °C for 3 min, followed by 45 cycles of 95 °C for 3 sec and finally 60 °C for 20 sec. Melt curve analysis was included post PCR to confirm that only one amplified product was formed. A 0.5 °C temperature increment from 65 °C to 95 °C and a hold time of 5 sec for each temperature was applied in the melt curve protocol.

Referring references or primerbank (<http://pga.mgh.harvard.edu/primerbank/>), PCR primers for each targeted gene were designed to form a PCR amplification product of 80 to 220 bp. The primer pairs giving unique amplification products were subsequently used for qPCR. Gene expression are calculated with $2^{-\Delta C_q}$ method and presented in fold change related to housekeeping gene GAPDH (glyceraldehyde-3-phosphate dehydrogenase). The sequences of qPCR primers are listed in Table 3.1.

Table 3.1 List of compiled gene targets and primer sequences.

Target Gene	Sequence (5'-3')	Amplicon Length (bp)
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	CATGAGAAGTATGACAACAGCCT AGTCCTTCCACGATACCAAAGT	113
Alpha smooth muscle actin (α SMA)	CCGACCGAATGCAGAAGGA ACAGAGTATTTGCGCTCCGAA	88
Collagen type I alpha 1 chain (COL1A1)	ATCAACCGGAGGAATTTCCGT CACCAGGACGACCAGGTTTTC	218
Collagen type I alpha 2 chain (COL1A2)	GGCCCTCAAGGTTTCCAAGG CACCTGTGGTCCAACAACCTC	166
Collagen type III alpha 2 chain (COL3A1)	TTGAAGGAGGATGTTCCCATCT ACAGACACATATTTGGCATGGTT	83
Transforming growth factor beta 1 (TGF β 1)	CTAATGGTGGAAACCCACAACG TATCGCCAGGAATTGTTGCTG	209

3.4 Statistical analysis

All data of experiments are expressed as mean $\pm$ 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.

Chapter 4

Development of Diverse Fibrous Scaffolds by Electrospinning

This chapter describes the development and characterization of electrospun fibrous platforms differing in dimensions, fiber orientations and fiber diameters for various applications (e.g., fundamental cellular studies and implantable ECM replacement). After a brief introduction and materials and methods, studies on two-dimensional (2D) and three-dimensional (3D) fibrous scaffold fabrication will be presented in Section 4.3 (Results and discussions). The Section 4.3.1 presents the fabrication of 2D fibrous mats for fundamental cellular studies. The tunability of fiber morphology (e.g., fiber orientation and fiber diameter) through controlling processing parameters and solution properties is discussed in this section. The Section 4.3.2 introduces an approach to produce 3D fibrous sponge matrices via liquid-collecting electrospinning. Additionally, in vitro cellular studies showed that it has great potential as an implantable ECM replacement.

4.1 Introduction

Fibrous scaffolds, which closely mimic natural extracellular matrix (ECM) fibrous structure, have attracted much attention as replacement of the missing or dysfunctional ECM in tissue regeneration [69]. Electrospinning is an easy and versatile approach for the development of diverse fibrous platforms made from a wide range of polymers. Figure 4.1 illustrates the electrospinning setup, which mainly include a syringe pump, a high-voltage power supply, a conductive spinneret and a grounded collector. Polymer solution loaded in the syringe can flow through a needle at a constant and controllable rate, and restricted by its surface tension at the nozzle. When it is exposed to an electric field, the conductive liquid drop of polymer solution will deform into a Taylor cone because of the Coulombic force exerted by the external electric field. The ejection of polymer solution is accomplished when the electrostatic force overcomes the surface tension. The solvent evaporates when the solution-jet travels in air undergoing a whipping and bending process associated with the electrostatic repulsion between the surface charges, leaving behind continuous polymeric fibers on the grounded collector to form a non-woven fabric [65-67]. The key principle of fiber construction described above guides our electrospinning development. Fibers will be naturally deposited in random orientation on static plate collector as a result of jet instabilities and bending spinning (Figure 4.1A), while applying a rotating drum as collector can align fibers orientation (Figure 4.1B) on collector surface.

Figure 4.1 Schematic illustration of electrospinning setup with a plate collect (A) or a rotating collector (B), and the corresponding SEM image of fibers deposited on the collectors.

Fabrication of aligned fibers was initially attempted in this work by investigating the rotating speed of drum. Fast Fourier Transform (FFT) analysis [120] was employed to assess the degree of alignment of the resulting fibers, which helped to get fibers with the best alignment. Additionally, other processing parameters (e.g., applied voltage, feed rate, working distance and needle gauge) and solution parameters (e.g., solvents conductivity and polymer concentration) were investigated to control the fiber diameter. It was found in our studies that solvent conductivity and polymer solution concentration played major role on the diameter, which will be discussed in detail in this chapter.

Though 3D fibrous highly porous matrices are ideal for implantable scaffolds, it is a challenge to produce fibrous mats greater than 200 μm in thickness through conventional electrospinning methods. A few methods have been explored to enhance the thickness and porosity of electrospun fibrous matrices. One common strategy involves easily-eliminated materials (such as salt and water-soluble polymer) during electrospinning, which will be selectively removed after electrospinning [122-125]. However, the scaffold's stability is often altered after sudden elimination. Another strategy is the application of

ultrasonication post electrospinning where physical manipulation can enlarge the pore size of electrospun scaffolds [126], but this strategy does not produce a true 3D scaffolds, especially in terms of the thickness. In place of traditional collectors, cotton-ball-like scaffolds could be electrospun in a spherical collector with an array of needles [127]. However, this is not easy to replicate due to precise control over parameters such as the dimensions of the spherical dish, needle length, needle distance, *etc.*

Herein, a simple and versatile approach to produce 3D fibrous porous scaffolds via liquid-collecting electrospinning will be introduced in our work. *In vitro* cellular studies showed that the 3D fibrous platforms could support cellular proliferation and penetration. Such an ECM mimicking matrix providing structural support for cell growth may be potential as a bio-artificial implant.

4.2 Materials and methods

4.2.1 Preparation of 2D PLC fibrous scaffolds

Poly (L-lactide-co- ϵ -caprolactone) (PLC) with the molar ratio of 70:30 and an inherent viscosity of 1.5 dl/g was obtained from Purac, the Netherlands. PLC granules were dissolved in various organic solvents at various concentrations to examine the influence of solution properties. Rotating drum at desired rotating speed was used as collector with working distance of 15 cm. Applied voltage was fixed at 20 kV while flow rate was fixed at 1 mL/h.

4.2.2 Preparation of 3D PLGA fibrous scaffolds

Copolymer poly (lactic acid-co-glycolic acid) (PLGA) with the molar ratio of 80:20 and an inherent viscosity of 1.05 dl/g was purchased from Purac, the Netherlands. PLGA granules were dissolved at 15% in a solvent mixture of 7:3

(v:v) TFE and chloroform to fabricate 3D fibrous scaffolds. A bath containing 7:3 (v:v) isopropyl alcohol and distilled deionized (DI) water with 0.05 % (w/v) Koppiphor P188 was used as collector. The bath was placed 4 cm vertically from the needle tip. Applied voltage was fixed at 18 kV while flow rate was fixed at 0.5 mL/h.

4.2.3 Surface modification of scaffolds with biomolecules

Optimized method for scaffold surface modification was carried out based on the previous report [128]. Briefly, scaffolds were firstly immersed in 0.05% NaOH solution at room temperature for 30 min. Upon completion of hydrolysis by NaOH, the samples were rinsed with DI water thoroughly. For activation, the hydrolyzed scaffolds were incubated with a mixture of 40 mM EDC and 80 mM NHS in 50 mM MES buffer (pH = 6) for 4 hrs at 4 °C. Activated scaffolds were washed with DI water thoroughly and soaked in 50, 100, and 500 µg/mL of collagen type I solution overnight at 4 °C. Finally, the samples were washed with phosphate-buffered saline (PBS).

4.2.4 Cellular proliferation assay

Cell counting kit-8 (CCK-8) (Dojindo Molecular Technologies, USA) was employed to investigate cellular proliferation. At testing time point, scaffolds loaded with cells were incubated in a mixture of 1:10 (v:v) CCK-8 reagent and cell culture medium at 37 °C for 2 hrs prior to absorbance measurements ($\lambda = 450$ nm). The cell number was calculated by comparing with absorbance standards of known cell numbers.

4.2.5 Cellular infiltration analysis

Cell suspension of 1×10^5 cells was initially loaded on scaffold top surface with area of 400 mm^2 . Cellular response was examined after one, three, and seven days of cell loading. For each time point at least three specimens per experimental group were examined. At the specified time points, scaffolds were collected and fixed with 4% paraformaldehyde (PFA) for cellular infiltration analysis. After washing with PBS, samples were soaked into 15% sucrose and then transferred into 30% sucrose. The scaffolds were then embedded in OCT freezing medium overnight for efficient penetration, followed by being frozen at -20°C to become rock-hard. Cryosections of $30 \text{ }\mu\text{m}$ thickness were picked up on glass slides. Subsequently, immunofluorescence staining was carried out by mounting with DAPI. Images were visualized by a fluorescence microscope and captured with a $4\times$ objective lens.

4.3 Results and discussion

4.3.1 Exploration of processing parameters in 2D fibrous scaffolds development

Generally, a variety of tissues have ECMs composed of aligned protein fibrils and bundles [129]. Additionally, it is known that contact guidance from artificial ECM-mimicking fibrous substrates may control cell migration, which is advantageous for biomedical applications in wound healing. Furthermore, studies on the effect of fiber diameter on cell migration may help provide further information beneficial for wound healing. Therefore, in this study, the author developed (through the versatile electrospinning technique) and investigated 2D fibrous scaffolds, which provided topographical alignment cues in diverse fiber diameters.

4.3.1.1 Tuning fiber alignment by controlling rotating speed of drum collector

A pair of split electrodes and rotating drum has been used to produce aligned fibers [130-134]. In this study, drum collector was used to tailor fiber orientation via applying various drum rotation speed (50, 500, 1000, 1500, and 2000 rpm). Random-oriented fibrous mat collected by flat plate was used as a control, expressed as 0 rpm. The feed rate of the Poly (L-lactide-co- ϵ -caprolactone) (PLC) solution was fixed at 1 mL/h. For universal comparisons, the drum rotation speed was converted to their corresponding surface velocity by taking the size of the rotating drum into account, as shown in Table 4.1. The surface velocity was determined by multiplying the speed of the rotating drum in rpm with drum perimeter ($2\pi r$) in which r is the radius of the rotating drum (i.e., 100 mm).

Table 4.1 Drum surface velocity converted from corresponding rotation speed.

	50 rpm	500 rpm	1000 rpm	1500 rpm	2000 rpm
Surface					
Velocity	31.40	314.00	628.00	942.00	1256.00
(m/min)					

Morphology of fibers produced by various rotation speeds was observed by scanning electron microscopy (SEM), and the degree of alignment was evaluated by FFT analysis, which is displayed in Figure 4.2. FFT peak value is known to be directly proportional to the degree of alignment [120]. As seen in Figure 4.2A-C, when the rotation speed was 50 rpm, the electrospun fibers distributed randomly with no obvious FFT peak which was similar to the random control (0 rpm). This might be due to the lower surface velocity of the drum collector than that of the polymer jet coming out from the needle tip [135], resulting in a faster rate of electrospun fiber deposition that prevented their

sufficient alignment with the rotation of drum collector. Despite the results from 50 rpm, results from other rotation speeds (0~2000 rpm) clearly shows a velocity dependent degree of alignment/FFT values as a function of rotation speed. Figure 4.2D presents a quantitative analysis of the images. While speeding up the drum rotation from 50 to 1500 rpm, the fiber alignment was improved with FFT peak value increasing from 0.050 ± 0.006 to 0.345 ± 0.015 with significant difference. Directional fiber deposition was observed from SEM image in 1500 rpm sample (Figure 4.2A), which indicated surface velocity of the drum (942 m/min at 1500 rpm) was larger or at least close to the polymer jet velocity at this point. When the rotation speed increased to 2000 rpm, the fibers collected remained aligned and the FFT value has no significant difference comparing with that of 1500 rpm (0.345 ± 0.015 vs. 0.352 ± 0.04) indicating that at 1500 rpm the threshold surface velocity has been met. As higher rpm may also increase the occurrence of fiber breakage, 1500 rpm was chosen as our optimized rotation speed for fiber alignment in future studies.

Figure 4.2 The role of drum rotating speed on the degree of fiber alignment. (A)

Representative SEM images; (B) the corresponding FFT output images; (C) plot of FFT quantification; and (D) the average FFT peak value of produced fibers using various rotating speed (0, 50, 500, 1000, 1500, and 2000 rpm) during electrospinning. N = 3.

Apart from degree of fiber alignment, the effect of drum rotation speed on fiber diameter was also studied in order to investigate their correlation, which may be further beneficial for the development of fibers. However, no significant difference was observed between the diameters of fibers produced with various drum rotating speeds (Figure 4.3). Other factors on fiber diameters like polymer solution properties during electrospinning were considered and the results on their correlation will be presented the following section.

Figure 4.3 Average diameters of PLC fibers produced by drum collector at different rotation speed. No significant difference of fiber diameter was observed between all the groups.

4.3.1.2 Tuning fiber diameter by varying polymer concentration and solvent conductivity

In the process of electrospinning, polymeric fibers with submicron-to-micro diameters are fabricated when a droplet of viscoelastic polymer solution is subjected to a high electrical field. It has been established that the processing parameters (e.g., applied voltage, solution feed-rate and working distance) [136-139] and solution properties (e.g., viscosity, surface tension and conductivity) [140-142] affect the morphology and diameter of electrospun fibers. In our work, thorough studies on the electrospinning parameters were performed to fabricate fibers with diameters ranging from submicron to micrometer. We found that solvent conductivity and polymer solution concentration were major influential factors determining the fiber diameters, which will be shown and discussed below. The discovery helped tune the fiber diameter for purposed studies and applications. In this investigation, flow rate was fixed at 1 mL/h while applied voltage was fixed at 20 kV.

Dichloromethane (DCM) is an ideal solvent for PLC in terms of dissolution. However, in our preliminary tests, due to the high volatility and low polarity, DCM used as sole solvent did not support continuous spinning since the fluid jet solidifies prematurely and clogged at the needle tip. Addition of dimethylformamide (DMF), which has a higher polarity, was able to increase the spinnability to produce defect-free fibers. Thus, the combination of DCM and DMF was used as solvent for PLC electrospinning. To study the effect of electric conductivity of solution (resulted from the solvent polarity) on fiber diameter, DCM:DMF at volume ratio of 7:1, 7:2 and 7:3 was employed as solvent for PLC at fixed concentration of 10 w/v %, and the results are shown in Figure 4.4 A and C. It can be visually observed in the SEM images (Figure 4.4A) that higher conductivity (more DMF) led to slightly smaller fiber diameter ($0.91 \pm 0.23 \mu\text{m}$ for DCM:DMF of 7:1 vs. $0.69 \pm 0.19 \mu\text{m}$ for DCM:DMF of 7:3, Figure 4.4C). It is due to the fact that fiber jets of higher conductivity are subjected to a greater tensile force in the presence of an electric field.

Figure 4.4 Influence of solvent conductivity and polymer concentration on fiber diameter. (A) SEM images and (C) average fiber diameters of electrospun scaffolds produced with solvents DCM:DMF 7:1, 7:2 and 7:3; (B) SEM images and (D) average fiber diameters (D) of electrospun scaffolds produced with polymer concentration of 10, 15 and 20 w/v %.

Solution viscosity is known to be an important factor affecting fiber diameter in electrospinning, which mostly and empirically relies on the polymer inherent viscosity and polymer concentration. In our investigation, PLC with a polymer inherent viscosity of 1.5 dl/g and mixed solvent DCM:DMF of 7:1 were employed to prepare polymer solutions of concentration 10, 15 and 20 w/v %, which were electrospun to examine the effect of concentration on the morphology and diameter of the spun fibers. Increasing polymer concentration

resulted in larger diameter (Figure 4.4B), varying from $0.91 \pm 0.23 \mu\text{m}$ (10 w/v %) to $2.08 \pm 0.62 \mu\text{m}$ (20 w/v %), as shown in Figure 4.4D.

To produce fibrous scaffolds with smallest possible fiber diameter, lower concentration (i.e., 8 w/v %) in DCM:DMF 7:3 was employed according to the previous results. The resultant fibers were as thin as $0.32 \pm 0.07 \mu\text{m}$. However, beads-containing fibers were formed (indicated by arrows in Figure 4.5A), probably because insufficient viscosity caused the jet of fluid to congregate under the action of strong surface tension [143]. To solve this issue, a fluorinated alcohol, heptafluoro-2-propanol (HFIP) with correspondingly higher polarity was introduced under the same concentration. This was able to facilitate the formation of thinner and defect-free fibers with an average diameter of $0.30 \pm 0.02 \mu\text{m}$ Figure 4.5B.

Figure 4.5 SEM characterizations of electrospun fibers from (A) 8 w/v % PLC in DCM:DMF 7:3; (B) 8 w/v % PLC in HFIP.

To summarize, the values of fiber diameters resulting from various solution and processing parameters for fabrication of aligned electrospun PLC fibers are included in Table 4.2.

Table 4.2 Electrospinning parameters and resultant diameters of aligned PLC fibers.

Conc. (w/v %)	Solvent	Applied Voltage (kV)	Feed Rate (mL/h)	Drum Speed (rpm)	Fiber Diameter (μm)
8	HFIP	20	1	1500	0.30 ± 0.02
10	DCM:DMF (7:3)	20	1	1500	0.69 ± 0.19
10	DCM:DMF (7:2)	20	1	1500	0.79 ± 0.16
10	DCM:DMF (7:1)	20	1	1500	0.91 ± 0.23
15	DCM:DMF (7:1)	20	1	1500	1.54 ± 0.42
20	DCM:DMF (7:1)	20	1	1500	2.08 ± 0.63

4.3.2 Development of 3D fibrous scaffolds as implantable ECM replacement

The ECM-mimicking microenvironment of electrospun scaffolds could enhance cellular behaviors in various aspects, including cell attachment, proliferation, migration, and gene expression signature [4, 62, 63, 88, 90]. However, cellular growth and infiltration are usually limited to the superficial layer of the flat, sheet-like fiber mat prepared by conventional electrospinning, presenting a significant obstacle in developing tissue replacement [127]. From that point of view, an easy and versatile approach were developed to fabricate 3D network scaffolds incorporating fibrous morphologies and interconnected pore structure, which will be introduced in detail below. In this section focusing on the fabrication of 3D network scaffolds, biodegradable and biocompatible polyester poly (lactic acid-co-glycolic acid) (PLGA) with an inherent viscosity of 1.05 dl/g was used as the polymer material.

4.3.2.1 3D fibrous scaffolds development via liquid-collecting electrospinning

In traditional electrospinning, a flat and grounded platform is normally used as a collector, and fibers are deposited on the platform layer by layer to form a densely-packed 2D structure. Instead of collecting in air, fiber collecting in liquid can decrease the bulk density of the fiber matrix [144]. The setup of liquid-collecting electrospinning is illustrated in Figure 4.6A. In order to achieve fiber penetration and sinking into the liquid rather than floating on the liquid surface, the surface tension and density should be lower than that of polymer solution. Thus, isopropyl alcohol (IPA) and surfactant Kollphor® P188 were added into water to reduce the surface tension and density. The shape and size of scaffolds produced from liquid-collecting electrospinning are shown in Figure 4.6B. The scaffolds were 3D spongiform and shaped into cylinders with a diameter of 20 mm and thickness of 5 mm. From SEM characterization images, the 3D fibrous scaffolds (Figure 4.6C) collected by the liquid bath exhibited large pores in both horizontal and vertical directions, while interweaving fibers were closely packed vertically in the 2D non-woven mat (Figure 4.6D) fabricated by the conventional system. The scaffold parameters were further examined quantitatively from SEM characterization images and the results are graphically shown in Figure 4.6 E and F. The fiber diameter of the 3D fibrous scaffolds ranged from 1.0 to 1.8 μm (Figure 4.6E) with an average diameter of $1.34 \pm 0.12 \mu\text{m}$. The pore sizes mainly ranged from 5 to 40 μm , and most of them were between 5 and 20 μm with an average pore size of $14.54 \pm 6.47 \mu\text{m}$ (Figure 4. 6F).

Figure 4.6 (A) Illustration of liquid-collecting electrospinning setups and (B-F) characterization of the fabricated scaffolds. (B) Relative shape and size of spongy fibrous scaffolds; (C) SEM characterization of electrospun scaffolds collected from liquid bath and (D) conventional flat platform; (E) Distribution of fiber diameter and (F) pore size within 3D electrospun fibrous scaffolds. One hundred measurements of fiber diameter and pore size were randomly made from each scaffolds; $N = 3$. Reprinted with permission from reference [73].

As mentioned before, fiber formation was influenced by the solution and processing parameters during electrospinning, among which viscosity and polarity of the polymer solution were vital to spinnability and fiber

morphology. Solution viscosity depends on the inherent viscosity and concentration of the polymer. In this study, using PLGA with the inherent viscosity of 1.05 dl/g, the polymeric solution at the concentration of 15 w/v % produced continuous and smooth fibers. PLGA which were dissolved in the sole solvent of tetrafluoroethylene (TFE) resulted in coiled fibers. The addition of chloroform with a lower dielectric constant produced coil-free fibers, and hence we hypothesized that a stronger electric field leads to coiled fibers. The fact that the application of higher voltage also produced coiled fibers has supported the hypothesis. Beyond the intrinsic properties of the solution, the processing parameters including voltage (i.e., 20 kV), flow rate (i.e., 0.5 mL/h), and tip-to-target distance (i.e., 4 cm) were optimized to obtain defect-free fibers. Taken together, 3D fibrous scaffolds with interconnected 5~20 μm pores were successfully developed and fabricated by liquid-collecting electrospinning.

4.3.2.2 Evaluation of cellular proliferation and penetration

PLGA as a biodegradable, biocompatible and FDA-approved polyester has been considered an attractive candidate for scaffolding material in regenerative medicine [95, 145]. However, its bio-inert surface is incapable of supporting cellular adhesion. Cells will attach on the PLGA surface only after protein deposition on the PLGA surface, from the culture medium or as secreted by cells [146]. Collagen type I is the major component of skin-native ECM with abundant arginine-glycine-aspartate (RGD) peptide to stimulate cell attachment [147]. With the purpose of fabricating biologically active matrices, we engineered the PLGA fiber surface with collagen type I through chemical modification.

The immobilization of proteins on fiber surface was accomplished as section 4.3.2. Briefly, carboxylic groups were generated through the hydrolysis of PLGA by immersion in NaOH solution, and chemical bonds were formed

between the amine groups from the proteins and the carboxylic groups on the PLGA fiber's surface. The scaffolds immobilized with collagen type I at concentrations of 50, 100 and 500 $\mu\text{g/mL}$ were designated as P-C50, P-C100 and P-C500 respectively, and the control scaffold without surface modification was designated as P-BLK. The immobilized protein was quantified by microBCA assay, and the results are shown in Figure 4.7A. The protein amount of P-C100 was determined to be 10.27 ± 4.06 μg per scaffold, which was significantly larger than that of P-C50 (4.77 ± 0.70 μg per scaffold), while no significant difference was detected between P-C100 and P-C500 (12.19 ± 7.07 μg per scaffold). It indicated that saturation of grafting was reached at 100 $\mu\text{g/mL}$ collagen.

Figure 4.7 Characterizations of protein-modified scaffolds. (A) Encapsulated collagen type I within scaffolds immobilized at concentrations of 50 (P-C50), 100 (P-C100), and 500 (P-C500) $\mu\text{g/mL}$. *Both protein amount of P-C100 and P-C500 were more than that of P-C50 ($P < 0.05$). NSD = no significant difference. $N = 3$. (B) ATR-FTIR analysis of P-BLK (light gray), P-C50 (gray), and P-C100 (black). Reprinted with permission from reference [73].

To characterize the surface chemistry of various scaffolds, attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR) was employed and the spectra are shown in Figure 4.6B. The intense peaks at 1750 cm^{-1} found

in common belong to the C=O stretching of PLGA backbone; however, three specific peaks were detected at 3384, 1647, and 1568 cm^{-1} in the P-C50 and P-C100 spectra. The extensive peak detected at 3384 cm^{-1} is relevant to N-H stretching from primary and secondary amines, while peaks at 1647 and 1568 cm^{-1} correspond to N-H bending from primary amines. These results indicated amine-containing substances were present in P-C50 and P-C100. The successful grafting of collagen type I was indicated by the distinct presence of amine signals (3384, 1647, and 1568 cm^{-1}) on the spectra of P-C50 and P-C100.

To investigate cellular infiltration and support of these scaffolds, *in vitro* studies were carried out using human dermal fibroblasts (HDFs). The cellular proliferation on various scaffolds was quantitatively evaluated and is presented in Figure 4.8. The cell loading density was 100 k per scaffold. After one day of culturing, the cell counts on P-C50 and P-C100 were 133.91 ± 11.61 k and 136.32 ± 27.13 k, respectively, while a significantly lower cell number (117.34 ± 11.04 k) was detected for P-BLK. This indicated that the collagen-immobilized fibrous scaffolds improved cellular attachment. On day 3, the cell number for all scaffolds increased to over 200 k. They were 238.38 ± 16.65 , 242.82 ± 27.47 , and 272.50 ± 45.66 k for P-BLK, P-C50, and P-C100, respectively, and a significant difference was only exhibited between P-BLK and P-C100. The most noticeable change was observed between day 3 and day 7. Over this time, the cell number on P-BLK increased to 302 ± 33.39 k, while the cell number on P-C50 and P-C100 markedly increased to 529.72 ± 31.16 k and 661.05 ± 152.73 , respectively, which was significantly greater than that on P-BLK. No significant difference was shown between P-C50 and P-C100 over the culturing time. The results demonstrated clearly that blank 3D polymeric fibrous scaffolds could support cellular proliferation within 7 days, however, cell proliferation were further promoted with scaffolds surface-modified with collagen type I. No obvious improvement was detected between P-C50 and P-

C100, which suggested that improved cellular proliferation was fulfilled by $4.77 \pm 0.70 \mu\text{g}$ collagen per scaffold.

Figure 4.8 HDFs proliferated on P-BLK (white), P-C50 (light gray), and P-C100 (black) within seven days. * The cell number was significantly greater as compared with P-BLK ($p < 0.05$). NSD = no significant difference. Error bar represents standard deviation of means; $n = 3$. Reprinted with permission from reference [73].

Cellular infiltration into the scaffolds was examined by studying cross-sections of the scaffolds using cryosectioning coupled with 4',6-diamidino-2-phenylindole (DAPI) staining. Figure 4.9 shows cross-sectional images of various scaffolds seeded with cells on the top surface after one, three, and seven days. Over the culturing time, cells gradually proliferated and invaded into the scaffolds. On day 1 the cells had adhered on the scaffolds and their infiltration was limited to the upper layer ($\sim 200 \mu\text{m}$), while the cells penetrated deep into $\sim 700 \mu\text{m}$ on day 3. By day 7, cells completely migrated into the inner part of the scaffolds at a depth of $\sim 1400 \mu\text{m}$. The cellular infiltration patterns were

comparable on all 3D scaffolds in both the presence and absence of collagen type I.

Figure 4.9 Cellular infiltration assay. Fluorescence microscope images of 4',6-diamidino-2-phenylindole (DAPI) stained cross-sections of P-BLK, P-C50, and P-C100 scaffolds seeded with human dermal fibroblasts (HDFs) on day 1, 3, and 7. The yellow dotted lines indicate the top margin of scaffolds. Section thickness = 30 μm . Scale bar = 500 μm . Reprinted with permission from reference [73].

Engineered scaffolds with a 3D ECM-mimicking architecture and interconnected pore structures allow cells and nutrients to penetrate into the internal environment. It was reported that HDFs have a propensity for pore sizes of 6 to 20 μm , where larger pores (>20 μm) lead to cells growing along fibers instead of branching out in a 3D configuration [5]. As shown in our *in vitro* cell infiltration study, HDFs initially seeded on the surface were shown to

migrate into the inner part of all scaffolds progressively over time, reaching as deep as $\sim 1400\ \mu\text{m}$ after seven days of culturing. The cellular infiltration patterns were similar on all 3D scaffolds with or without immobilized collagen type I, indicating that the highly porous structure appears to have a greater impact on cell infiltration than the surface properties for this scaffold-material combination while the latter has significant effect on cell proliferation.

4.4 Summary

The versatility of electrospinning is capable of the fabrication of fibrous scaffolds mimicking ECM structure as well as conferring diverse topographic cues. Fibers can be aligned by using a rotating drum as collector. Through tuning the rotating speed of drum, the degrees of fiber alignment could be manipulated. The resulting fiber diameter could be easily controlled by solution conductivity and polymer solution concentration. In the later chapter, we attempt to investigate how the fiber diameter and fiber alignment affect skin cell behaviors.

To fabricate a 3D fibrous scaffold out of electrospinning, collecting the fibers in alcohol-based liquid seemed to be able to facilitate the construction of 3D fibrous substrates. The 3D highly porous substrate enabled excellent cell infiltration and proliferation within the time frame studied. This result demonstrates the possibility of such artificial 3D fibrous scaffolds as potential ECM replacement in reconstructing tissues.

Chapter 5

Influence of Topographic Cues in Directing Cell Migration

This chapter investigates the influence of substrate topographic cues on cell migration. Substrates with diverse topographical features were fabricated, and skin cells which are mechanosensitive were employed to understand the effect of varying features on cell migration. It was found that substrates which possess extracellular matrix (ECM)-mimicking fibrous nature have significantly advanced cell migration, as evidenced by fluorescent images taken across a time frame of 48 hours. Notably, real-time imaging results demonstrated that fiber alignment could indeed persistently induce directional cell migration. The underlying mechanisms of these interesting phenomena were postulated to be due to the focal adhesion assembly as well as Rho small GTPase activity, where the detailed discussion will be given in the later sections. Through these understanding, insights regarding promoting faster wound closure could be obtained and these knowledge could be employed in the fabrication of functional tissue replacements for regenerative medicine with better therapeutic efficacy.

5.1 Introduction

Directional cell migration plays a vital role in many physiological and pathological processes, including embryonic development, tissue regeneration, and tumor metastasis and so on [8, 9]. For instance, in the proliferation phase during wound healing, keratinocytes and fibroblasts migrate directionally to the wound bed and then proliferate to form granulation. However, this kind of migration is generally disabled in chronic wounds, and hence affecting the proper wound closure. Thus, there is a considerable interest to study directional cell migration which may probably open a new avenue to novel therapies, especially in the fabrication of functional tissue replacements for regenerative medicine.

It is generally perceived that cells migrate randomly in an isotropic system (known as Brownian movement), while they move in response to an asymmetric cue [148]. Directional cell migrations occur when the cells are being subjected to certain stimulation cues presented in the surrounding microenvironment, such as chemo-attractant gradient [149, 150], substrate stiffness gradient [151], and substrate topographical cues [152]. Notably, the use of topographical cues in guiding cell movement has gained enormous attention due to the ease of operation as well as the effect was long lasting. It was demonstrated that aligned electrospun nanofibers significantly directed and enhanced cell migration [88, 97]. These evidences strongly drive the motivation on the development of advanced substrates with topographical cues for directional cell migration. Fabricated by electrospinning, artificial fibrous scaffolds confer ECM-resembling architecture and provide aligned topographical cues at the same time [129]. Furthermore, the versatility of electrospinning enables the development of fibrous scaffolds with varying topographies, like the size of fiber diameter and the alignment of fiber orientation. Previous studies mainly investigated the effect of fiber alignment

on cell migration, but the role of such topographic cues with varying fiber diameter (submicrometer vs. micrometer) on migratory properties has never been studied in detail. In this study, diverse substrates were designed and fabricated, including films with flat topography, random-oriented fibers and aligned fibers with different diameters. These substrates were evaluated in terms of their abilities to promote cell migration via the usage of the cellular migration assay.

Traditionally, scratch assay is usually applied for the study of cellular migration *in vitro*, in which a “wound” is created by scratching into a monolayer of cells using a pipette tip [153]. However, the process of scratching will cause physical damage to the underlying substrates, which makes it not ideal for our experiments. Alternatively, introduction of an insert as cell stopper is able to create a cell-free-zone into which cell can migrate upon removal of the insert [96]. Therefore, we used a stopper-based assay for the present study. As shown in Figure 5.1, a wound gap of 500 μm can be induced on diverse substrates for cell migration assessment.

Figure 5.1 Illustration of cell seeding with an insert to create a cell-free “wound gap” of 500 μm for cell migration assay.

The speed of cell migration is closely related to cell adhesion. Cells weakly attached on the substrates fail to obtain sufficient traction force to move forward, while cells forcefully adhered cannot overcome the adhesion to move

[154]. Furthermore, the directed migration appears to rely on the confinement of adhesion sites along the movement direction [155]. With regards to molecular basis, Rho family of small GTPase Cdc42 is deemed to be the master regulator for forming the leading edge to direct the cell movement [8, 156]. Thus, after the evaluation of fibroblast mobility on substrate with diverse topographies, focal adhesion expression and Cdc42 activity were investigated to reveal the underlying mechanism which might affect the cell movement.

5.2 Materials and methods

5.2.1 Cell migration assay

The adherent cells were trypsinized and their cell membranes were stained with cell tracker DiI. Silicon culture-inserts (ibidi, Germany) were applied to proceed cell seeding. There are two separated wells ($0.22 \text{ cm}^2 \times 0.5 \text{ cm}$) for cell suspension, forming a $500 \pm 50 \text{ }\mu\text{m}$ cell-denuded gap between the two designed areas. Cells were seeded at a density of 2.5×10^4 cells per well and cultured for 16 hrs. Thereafter, the culture-inserts were removed, and the cells were cultured in DMEM with 2% FBS and 1% penicillin-streptomycin solution instead of complete medium (DMEM with 10% FBS and 1% penicillin-streptomycin solution), in order to limit cell proliferation in cell migration assay. The progression of cell migration was monitored through fluorescence imaging at the wound edge every 24 hrs using a Nikon 80i eclipse upright microscope. After 48 hrs, cells were fixed by 4% PFA for 15 min and permeablized with 0.1% Triton-X 100 for 10 min. At last, the cells were stained for nuclei using DAPI to quantify the migrated cell number.

5.2.2 Single cell tracking

For individual cell tracking, cells were staining for nuclei using Hoechst 33342 prior to cell migration assay. Cells at the edge of the wound sheet were tracked for 6 hrs since the start of wounding. Images were acquired every 30 min by live cell imaging system (JuLI Stage, Korea). Individual cells were tracked manually using ImageJ with Manual Tracking plugins. Migration velocity is determined by the total distance travelled by the cells divided by time. Euclidean distance is the straight-line distance between the first point and the last point during migration tracking.

5.2.3 G-LISA activation assay

Activation of Cdc42 and Rac1 GTPase of HDFs was analyzed using G-LISA activation assay (Cytoskeleton, USA). The Cdc42 and Rac1 G-LISA activation assays are ELISA-based assays measure the level of GTP-loaded Cdc42 and Rac1 in cell lysates, respectively. The level of activation is measured with absorbance at 490 nm. Each assay was performed strictly according to manufacturer's instructions.

5.3 Results and discussion

5.3.1 The effect of topographic features on L-929 migration

To study the influence of substrate topography on skin cell migration, substrates with diverse topographies were firstly fabricated. The topography of each substrate was assessed by SEM and the images are displayed in Figure 5.2A. According to the results from SEM imaging, the topographic properties of the substrates including fiber alignment mode and fiber diameter are qualitatively and quantitatively listed in Table 5.1, respectively. Based on these results, the samples were named as casting film (Film), aligned fibers with diameter of 0.3 (A-0.3), 0.8 (A-0.8), 2 (A-2) and 13 (A-13) μm , and random fibers with

diameter of 0.8 (R-0.8) μm . The films fabricated by casting were employed for comparison with the fibrous substrates. For fabrication of aligned fiber using electrospinning with rotating drum as collector, solution parameters were varied to tune the fiber diameter from 0.3 to 2 μm . Due to the limitation of electrospinning, 2 μm represented the greatest possible fiber diameter obtainable. Thus, melt drawing was used to construct a fiber diameter up to 13 μm . Finally, a substrate was fabricated with random fibers with 0.8 μm fiber diameter.

Table 5.1 Topographic properties of various substrates used for cell migration assay.

Sample	Fabrication	Fiber alignment	Fiber diameter (μm)
Film	Casting	Not applicable	Not applicable
A-0.3	Electrospinning (rotating drum)	Aligned	0.30 ± 0.02
A-0.8	Electrospinning (rotating drum)	Aligned	0.79 ± 0.16
A-2	Electrospinning (rotating drum)	Aligned	2.08 ± 0.63
A-13	Melt drawing	Aligned	13.2 ± 0.34
R-0.8	Electrospinning (rotating drum)	Random	0.80 ± 0.09

To study skin cell migration over time, L-929 murine fibroblasts were seeded as monolayers on different substrates. A cell-denuded gap of around 500 μm was created as “wound gap” perpendicularly to fiber orientation. To determine the initial wound edges and visualize the progression of cell migration, the L-929 cells were stained with red-fluorescent cell tracker DiI prior to seeding on scaffolds. To ensure that cell migration is the main event, the cells were cultured in low-serum medium to refrain cell proliferation. As illustrated in Figure 5.2A, the cultured cells were allowed to migrate into the wound gap upon the removal of insert. The progression of wound closure was captured under fluorescent microscopy every 24 hrs, and the images are shown in Figure

5.2 B-D. Images captured at 0 h (Figure 5.2B) verified the successful creation of wound gap, with the yellow dotted lines indicating initial wound edges. As expected, cells migrated into wound gap over time in all kinds of substrates. After 24 hrs (Figure 5.2C), cells almost covered the gap on samples A-0.3, A-0.8, A-2 and R-0.8, while relatively small amount of cells were presented in the wound gap on Film and A-13. Similar trend was observed after 48 hrs of cell seeding (Figure 5.2D), where more cells appeared to be present in the gap on sample A-0.3, A-0.8, A-2 and R-0.8 compared to Film and A-13.

Figure 5.2 L-929 migration assay on diverse substrates. (A) Topographies of diverse substrates characterized by SEM, including casting film (Film), aligned fibers with diameter of 0.3 (A-0.3), 0.8 (A-0.8), 2 (A-2) and 13 (A-13) μm and random fibers with diameter of 0.8 (R-0.8) μm . (B-D) L-929 cells cultured on different substrates over time with a gap of around 500 μm . To monitor cell migrating progression, the cells were stained with cell tracker DiI before cell seeding. The yellow dotted lines indicated the initial wound edges. Fluorescent images were captured at 0 (B), 24 (C) and 48 (D) h, respectively. Scale bar = 100 μm .

To quantify the number of migrated cells, the cells were fixed and immunostained for nuclei imaging (blue) after 48 hrs, and the staining images are shown in Figure 5.3A. It is clear that lesser cells were presented in the gap on Film and A-13. The number of migrated cells was calculated with ImageJ Cell Counter plugin and normalized by the gap area, as shown in Figure 5.3B. The cell number of A-0.3, A-0.8, A-2 and R-0.8 was significantly higher than that of Film, while no significant difference was showed between Film and A-13. No improved cell-migration on A-13 indicated that the topographic cues induced by larger fiber diameter of 13 μm were not able to significantly confer contact-guidance to the cell movement. There was no difference on migration within aligned fiber in the smaller diameter ranges (A-0.3, A-0.8 and A-2). There is also no difference observed on cell migration between fibers with different alignments (A-0.8 and R-0.8). The results showed that L-929 migration was enhanced by fibrous substrates with diameter of 0.3~2 μm , which is within the native ECM fibrils scale range (0.01~3 μm). It was well accepted that aligned fiber with diameter in submicrometer ($< 1 \mu\text{m}$) significantly enhanced cells migration [88, 97], but the effect of fibers dimension beyond native ECM fibrils scale (e.g. more than 10 μm) have not been reported yet. Thus, the results here emphasized the importance of fiber dimensional similarity to native ECM.

Figure 5.3 Quantitative number of L-929 cells migrated into the gap area. (A) Cells were stained for nuclei by DAPI after 48 hrs in cell migration assay. The dotted yellow lines indicated the initial edges of gaps. Scale bar = 100 μm . (B) The number of cells migrated into the gap area was quantified per unit area. Data were collected from 5 independent captures per experimental group. Single asterisk (*) and double asterisks (**) indicate statistical significant difference of $p < 0.05$ and $p < 0.01$ when comparing with Film.

5.3.2 The influence of fiber alignment on directing HDFs migration

In the previous work (Section 5.3.1), L-929 (murine fibroblast) migration in similar manner was observed generally on both aligned fibers and random fiber with fiber diameter in submicrometer. We wondered if the migratory behavior of human skin cells would be affected by fiber alignment. Hence, human dermal fibroblasts (HDFs) were employed in the following works to investigate the migrating direction and velocity in individual cells. As fibrous substrates, aligned fibers (AF) with a diameter of 0.79 ± 0.16 and random fibers (RF) with a diameter of 0.80 ± 0.09 were used in this work.

5.3.2.1 Assessment of collective and individual cell migration

Using the same cellular migration assay, HDFs were seeded on AF and RF and their movement was investigated. Figure 5.4 A and B show the cell migrating progression by cell membrane staining at 0 and 24 h, respectively. From Figure 5.4B, it is found that the wound gap in AF was almost covered by migrated cells after 24 hrs. Furthermore, HDFs displayed typical elongated morphology along the fiber orientation. In contrast, lesser cells were observed on RF within the wound gap, and the cells oriented randomly. Subsequently, the quantitative migrated cell number was performed by nucleus immunostaining after fixation and plotted, as shown in Figure 5.4 C and D. The number of migrated HDFs on AF was significantly higher than that on RF, which indicated AF advanced HDFs migration when comparing with RF.

Figure 5.4 HDFs migration assay on aligned fibers (AF) and random fibers (RF). HDFs were cultured on different substrates with a gap of 500 μm . To monitor cell migrating progression, the cells were stained with cell tracker DiI before cell seeding. The dotted yellow lined indicated the initial edges of gaps. Fluorescent images were captured at 0 (A) and 24 (B) h. Cells were stained for nuclei by DAPI after 24 hrs (C), and cells migrated into gap area was quantified per unit area (D). Data were collected from 7 independent captures per

experimental group. Double asterisk (**) indicates statistical significant difference of $p < 0.01$ between the two experimental groups. Scale bar = 100 μm .

In order to minimize the potential interference which arise from cell proliferation during end-point cell migration assay, it is of interest to investigate the real-time individual cell motility, thus single cell tracking would be carried out as reported in following section. To study the migratory behavior in single cell, HDFs were stained for nuclei with Hoechst 33342 prior to cell migration assay. Cells at the wound edge were tracked manually every 30 min through live imaging. Figure 5.5 A and B show the trajectories of individual migrating HDFs on AF and RF for 6 hrs, respectively. The migrating paths of 60 single cells from each experimental group are plotted in wind-rose diagram. It clearly shows that HDFs on AF migrated linearly and persistently along the fiber axis even in the absence of a chemotactic agent, while a few of cells migrated in opposite direction to the wound gap. In contrast, cells traveled in non-persistent (disperse) model on the substrates with random morphology. In addition, the migrating trajectories were characterized in quantitative analysis by three phenomenological parameters: velocity, Euclidean distance, persistence index [157], as shown in Figure 5.5 E-G. Herein, velocity refers to the average migration speed, while Euclidean distance describes the linear distance between the initial and final position of the cell. Persistence index was used to characterize quantitatively the persistent migration, which is calculated by the following equation:

$$\text{Persistence Index (PI)} = \frac{E}{A}$$

Where E is Euclidean distance (linear distance between the initial and final position of the cell), and A defines as the accumulated distance travelled by the

cell. PI closer to 0 indicates a more disperse migration; PI closer to 1 indicated a more linear and persistent migration.

Figure 5.5 The migration of HDFs on aligned fibers (AF) and random fibers (RF) was tracked and analyzed individually. (A-B) Migration path of individual cells at the wound edge were tracked manually and (C-D) plotted in wind-rose graft. (E) Average migration velocity, (F) Euclidean distance and (G) persistence index were quantified from 60 cells per experimental group. Single asterisk (*) and double asterisks (**) indicate statistical significant difference of $p < 0.01$ and $p < 0.05$ between the two experimental groups, respectively. Scale bar = 100 μm .

Here it was found that HDFs migrated on AF at an average velocity of $0.30 \pm 0.11 \mu\text{m/min}$, which is significantly higher than that of RF ($0.26 \pm 0.09 \mu\text{m/min}$) (as shown in Figure 5.5E). Further, Figure 5.5 F and G show the average

Euclidean distance and persistent index of HDFs migrated on fibers with different alignment. The average Euclidean distance on AF ($92.2 \pm 37.6 \mu\text{m}$) was 1.53 folds higher than that on RF ($60.5 \pm 27.7 \mu\text{m}$). Indeed, the persistence index on AF (0.92 ± 0.08) was closer to 1 than that on RF (0.68 ± 0.18), confirming the directional migration guided by AF.

As in the following, the underlying molecular mechanisms of AF-induced directional and persistent migration were investigated by studying the focal adhesion expression and Cdc42 activity.

5.3.2.2 Expression and assembly of focal adhesions

Cell migration is a cyclical and orchestrated process through dynamic spatially and temporally arrangement of adhesion sites and cytoskeleton. It is initiated by extension of protrusion forms and adheres along the migrating direction as a leading edge. Following, contraction force of cell cytoskeleton moves the cell body forward. Lastly, trailing edge detaches and retracts to complete the cyclical process [8, 158]. The frequency of the cycle determines the migration speed. In addition, studies showed that the speed and direction of migration closely relied on the adhesion strength and the spatial positioning of focal adhesion [155, 159, 160]. Thus, the expression and assembly of vinculin, a key membrane-cytoskeletal component in focal adhesion, was studied in this work.

HDFs cultured on AF and RF were immunostained for vinculin (green), F-actin (red) and nucleus (blue) for visual observation and showed as merged fluorescence images in Figure 5.6 A. It was showed that HDFs elongated strictly along the fiber alignment on AF with greatest staining intensity of vinculin being observed at two edges of cells. Additionally, the shape of vinculin on AF were confined into linear form and arranged preferentially along the axis of cell, as shown in the magnified insert (Figure 5.6 A). On RF, cell

morphologies mostly exhibited as spindle but irregular shapes. Vinculins in linear pattern were concentrated around the cell periphery in a seemingly random-oriented fashion, as shown in the magnified insert (Figure 5.6 A). Xia *et al* demonstrated that directional cell motility could be controlled by focal adhesion positioning spatially through contact patterning. Thus, the confined assembly of focal adhesions aligned along the fiber axis might be associated with the resultant persistent migration induced by AF.

Figure 5.6 Vinculin immunostaining (A) and quantification (B-D) of HDFs cultured on aligned fibers (AF) and random fibers (RF). (A) Cells with typical morphologies were immunostained with vinculin (green), F-actin (red) and nucleus (blue). Magnifications of vinculin are inserted in dotted white rectangle. Scale bar (white) = 50 μm . Insert scale bar (yellow) = 20 μm . (B) The size of vinculin was measured by area and sorted into classes: 5~10, 10~15 and >15 μm^2 . The number of vinculins in each class is expressed as a percentage of the total number of vinculin. (C) The total number of vinculins in each cell was calculated and plotted. (D) Vinculin density was measured the

total area of vinculins in each cell normalized with cell area. Data were collected from 50 cells ($n = 50$) per experimental group. Single asterisk (*) indicates statistical significant difference of $p < 0.05$ between the two experimental groups, respectively.

To characterize the expression of focal adhesion, the vinculin was analyzed quantitatively by projected area in each cell with the fluorescent images, as shown in Figure 5.6 B-D. Based on the area, vinculins were sorted into four classes: < 5 , $5\sim 10$, $10\sim 15$ and $> 15 \mu\text{m}^2$. Focal adhesions with an area of smaller than $5 \mu\text{m}^2$ are considered to be immature and hence they were not included in the analysis [161]. Figure 5.6B presents the number of vinculins with the area greater than $5 \mu\text{m}^2$ in each class as a percentage of the total number of vinculin on different substrates. It showed that larger vinculin (with the area greater than $15 \mu\text{m}^2$) distributed more frequently on RF when comparing with that on AF. Total number of vinculins and vinculin density were plotted in Figure 5.6 C and D. Vinculin density referred to the total area of vinculins in each cell and normalized with cell spread area. Both vinculin number and vinculin density were significantly greater on RF than that on AF. These results indicated RF governed a larger degree of cell-material contact. In other words, HDFs had stronger focal adhesion on RF.

Adhesion is the first machinery of local cell-substrate interaction [162]. From the results above, topographic alignment was capable of mediating the expression and assembly of focal adhesions. The confinement of adhesion sites along the axis of the fiber on AF might be associated with the directional migration. Additionally, it has been well accepted that adhesion strength influences the speed of cell motility [163]. Horwitz et al demonstrated that maximum migration speed decreases reciprocally as cell-substrate adhesiveness increases [154]. Herein, the lower expression of focal adhesion on AF might confer faster migration.

5.3.2.3 Evaluation of Rho small GTPase activity

Cdc42 and Rac1, members of Rho family of small GTPase, are responsible for cell motility. The activation of Cdc42 and/or Rac1 is a master regulator to trigger actin polymerization at the leading edge, resulting in the extension of protrusion towards the migrating direction [8, 156]. Additionally, it was recently found that Cdc42 stimulating spike-like filopodia formation contributes to persistent migration, while Rac1 induce broad lamellipodia formation is responsible to random migration [164]. Hence, it is of interest to study the cellular activity of Cdc42 and Rac1 to reveal the potential signaling molecules, which might contribute to the phenomena as shown in Section 5.3.2, where AF promoted persistent HDF migration.

Using GLISA assay, the activity of Cdc42 and Rac1 GTPase of HDFs cultured on AF/RF was investigated. The results (as shown in Figure 5.7) displayed that Cdc42 GTPase activity was significantly higher by 1.58 fold in HDFs on AF, while Rac1 GTPase was lower at 0.75 fold comparing to that of RF. The results suggested that the activation of Cdc42 was stimulated by fiber alignment, which is known to regulate actin cytoskeleton reorganization to form filopodia protrusion in persistent cell migration [165, 166]. It implied that directional migration triggered by aligned fibers might be mediated by Cdc42 forming finger-like filopodia along the fiber orientation.

Figure 5.7 Cdc42 and Rac1 GTPase activation in HDFs culture on aligned fibers (AF) and random fibers (RF) as measured by GLISA. Fold activation of Cdc42 and Rac1 is normalized by RF samples. Each reaction was performed in triplicate. Single asterisk (*) and double asterisk (**) indicate statistical significant difference of $p < 0.05$ and $p < 0.01$ between the two experimental groups, respectively.

5.4 Summary

L-929 migration was promoted on fibrous substrates with diameter range within the native ECM fibril scale. It suggested that it is essential to mimic ECM properties not only from the aspect of fiber topography but also the fiber dimension. In addition, accelerated and persistent migration in HDFs was demonstrated on aligned fibers, where the higher migratory velocity might be attributed to the lower expression of focal adhesions in the cells. Furthermore, focal adhesions on aligned fibers were confined into linear and oriented along fiber direction, which may be associated to the directional motility. Besides the

confinement of focal adhesions, aligned fibers also stimulated the activation of Cdc42 GTPase. The results suggested that directional migration triggered by aligned fibers may be mediated by Cdc42 forming finger-like filopodia along the fiber orientation. The results of this study emphasized the important role of fibrous topography in directing persistent cell migration.

Chapter 6

Influence of Scaffold Topographies on Cellular Phenotypes

This chapter investigates the influence of substrate topographic properties on cellular phenotypes. Electrospun fibers with different alignments (aligned fibers vs. random fibers) were used as substrates, and human dermal fibroblasts were employed to study the effect of varying topographies on fibroblast phenotypic maintenance/alteration. It was found that fibroblasts on aligned fibers exhibited elongated morphologies and transited into more myofibroblast-like phenotype over a time frame of 14 days. Notably, the addition of extracellular matrix protein ANGPTL4 was able to reverse this fiber alignment-induced differentiation. The underlying mechanisms of the differentiation induced by fiber alignment were speculated to be the mechanical activation of latent TGF β 1 by aligned fibers. These discoveries pave a promising path to yield an artificial scaffold with dual functions of helping wound contraction and minimizing the scar formation.

6.1 Introduction

Upon injury, fibroblasts migrate to the wound site and transform into “muscle-like” contractile cells, myofibroblasts, to advance wound closure. Myofibroblasts are responsible for wound contraction and synthesis of new extracellular matrix (ECM) proteins, which are essential for reducing wound size and restoring tissue integrity [10-12]. Thus, the fibroblast-to-myofibroblast differentiation acts as a critical and necessary event in normal tissue repair and wound healing. This phenotypic alteration can be characterized by upregulated expression of alpha smooth muscle actin (α SMA) and increased deposition of the ECM components, including collagen (type I and III) and fibronectin [167]. It is well accepted that active transforming growth factor β 1 (TGF β 1) is the major inducer for myofibroblast differentiation [168, 169]. However, recent investigations suggested that mechanical properties, especially the matrix stiffness, of microenvironment appeared to serve as an essential and modulatory function in myofibroblast differentiation. Evidently, stiff matrix could promote myofibroblast differentiation [39-41], by activating the latent transforming growth factor- β 1 (TGF β 1) through mechanotransduction [41, 170]. This demonstrated a close correlation between fibroblast-to-myofibroblast differentiation and their microenvironment.

The presence of myofibroblasts is beneficial to advance wound closure through conferring contraction force. Upon completion of contraction phases, the number of myofibroblasts is thought to reduce through apoptosis [171]. Otherwise, the accumulated myofibroblasts are responsible for the development of pathological scarring, which is marked by excessive ECM deposition and unregulated contraction [27]. Due to the critical roles of myofibroblasts in both wound healing and pathological scarring formation, it is important to know if myofibroblast is a terminally differentiated phenotype or it could be reversibly modulated to minimize scar formation. Recently, some studies demonstrated

the possibility to reversibly modulate myofibroblast differentiation. Specific mitogenic growth factors (like basic fibroblast growth factor (bFGF) and platelet-derived growth factor (PDGF)) could markedly down-regulate α SMA expression through the activation of mitogen-activated protein kinase (MAPK) signaling pathway, and thus inhibited the myofibroblast differentiation [39, 172-174]. These evidences suggest the reversibility possibility of myofibroblast differentiation, and appropriate balance of fibroblast-to-myofibroblast may be critical for advanced wound healing and reduced fibrotic response to wound repair.

Employment of electrospun fibrous matrices for skin wound healing holds great promise owing to their biomimetic morphology. Thus, it is worthwhile to investigate the influence of tissue-engineered fibrous scaffolds on cellular phenotype to determine the functionality of artificial skin grafts. In our previous studies (Chapter 5), HDFs showed elongated morphologies on electrospun aligned fibers (AF), and the AF enhanced and directed cell migration, which greatly benefits wound closure. Thus, it would be of interest to investigate if such scaffold topography will affect phenotypic maintenance/alteration of HDFs over the time frame of wound healing (e.g., 14 days). Moreover, it is interesting to study the possibility of reversibility of phenotypic change by chemical factors if necessary. These investigations may help present a deeper understanding of how the fibroblastic/myofibroblast phenotype can be modulated by microenvironment and allow for the better application of fibrous scaffolds as tissue-engineered matrices.

6.2 Materials and methods

6.2.1 Flow cytometry

For determination of α SMA protein expression, intracellular staining for α SMA was carried out on fixed and permeabilized cells. Prior to staining, cells were blocked with 10% goat serum in PBS for 30 min at room temperature. The blocked cells were incubated with staining buffer (10% goat serum in PBS) containing Alexa Fluor 488 conjugated rabbit anti- α SMA for overnight at 4 °C. Cells were then washed by PBS before flow cytometric analysis. Flow cytometry was carried out using BD LSRII flow cytometer (BD Biosciences, Singapore) and data analysis was carried out using Flowjo software version 7.6.1.

6.2.2 Enzyme-linked immunosorbent assay (ELISA)

The cellular concentrations of active TGF β 1 were measured with ELISA using a human TGF β 1 ELISA kit (Sigma Aldrich, Singapore). Briefly, the cells on various substrates were washed thoroughly, harvested by treatment with trypsin, then cell number calculated by hemocytometer and collected by centrifugation. The cell pellet was lysed in M-PER Mammalian Protein Extraction Reagent (Thermo Fisher Scientific, USA) and the debris was removed by centrifugation at 14,000 $\times$ g for 15 min. The concentration of TGF β 1 in the supernatant was assessed by ELISA according to instructions provided by manufacturer.

6.2.3 Western-blot analysis

The cellular expression level of phosphorylated TGF β -receptor II was determined using western blotting [175]. Briefly, cells were pelleted and lysed in M-PER Mammalian Protein Extraction Reagent (Thermo Fisher Scientific, USA) with protease inhibitor cocktail. After lysis, the protein extracts were collected as supernatant post centrifugation. Equal amount of total protein extracts (100 μ g) were resolved and separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 4% stacking gel and 12%

resolving gel. PageRuler Plus Prestained Protein Ladder (1 μ L) (Thermo Fisher Scientific, USA) was employed under the same condition to indicate the molecular weights of the samples' bands. The resolved and separated proteins on SDS-PAGE gels were then electrotransferred onto polyvinylidene fluoride (PVDF) membranes. Membranes were processed with primary and secondary antibody incubation following the instruction from manufacturers, and proteins of interest were detected by chemiluminescence. Briefly, PVDF membranes were incubated in Odyssey blocking buffer for 1 hr at room temperature on the rocker, followed by incubation with phosphorylated TGF β -receptor II antibody at 4 °C overnight and 3-times washing with TBS. Subsequently, the membranes were incubated with IRDye 680RD secondary antibody for 1 hr at room temperature and washed with TBS three times. Finally, infrared imaging system Odyssey CLx (Li-Cor Biotechnology, USA) in 700 nm channel was applied to detect the samples' bands. Coomassie blue-stained membrane or GAPDH was used to check for equal loading and transfer.

6.3 Results and discussion

6.3.1 Effect of topographical alignment on HDFs morphology and cytoskeletal configuration

To determine the cell morphology on fibers with different alignments, HDFs were cultured on aligned fibers (AF) and random fibers (RF) respectively and visualized by immunostaining. Figure 6.1 displays representative fluorescence images of individual cells after 1 day of culture. The F-actin cytoskeleton and nucleus of cells were stained by rhodamine phalloidin and 4',6-diamidino-2'-phenylindole dihydrochloride (DAPI) respectively to examine how the cellular shape and subcellular components were influenced by the topography of substrate. On AF, cells attained an elongated and bipolarized morphology conformably along the fiber orientation (Figure 6.1A). The F-actin organized

into stress fibers and oriented parallel to the same direction as aligned fibers (Arrow a). Additionally, DAPI staining showed that the cell nuclei were highly elliptical and oriented to fiber alignment (Arrow b). In contrast, cells on RF exhibited polygonal morphologies with well spreading, as shown in Figure 6.1B. Cells were arranged into multipolarized (Arrow d) or bipolarized (Arrow e) shapes. The organization of actin filaments exhibited certain orientation but remained as crossed bundles. Furthermore, the cell nuclei were more circular in shape (Arrow c).

Figure 6.1 Nuclear shape and cytoskeletal development of HDFs cultured on fibrous substrates. HDFs seeded on AF (A) and RF (B) were immuno-labeled

for cell nucleus (blue) and F-actin (red). Cells were cultured for 1 day prior to fixation and staining. Scale bar = 50 μm .

Morphological differences between the cells cultured on the AF and RF were analyzed from individual cells. Measurable cellular parameters including cell spreading area, bipolarity index (BI), projected cell nucleus area and nucleus shape index (NSI) were quantified and analyzed. The BI and NSI were examined using the following equations (6.1 and 6.2).

$$BI = \frac{\text{Cell Length}}{\text{Cell Width}} \quad (6.1)$$

Where Cell Length and Cell Width refer to the extents of the major and minor nucleus-crossing axes of the cell, respectively. A higher BI value indicates a more elongated cell.

$$NSI = \sqrt{1 - \left(\frac{AB}{CD}\right)^2} \quad (0 \leq NSI \leq 1) \quad (6.2)$$

Where AB and CD refer to the extents of the minor and major axes of the cell nucleus, respectively. NSI closer to 0 presents a rounder nucleus; NSI closer to 1 presents a more elliptical nucleus.

The measured morphological differences between the HDFs on AF and RF were subjected to statistical analysis. Altogether, 50 cells from each experimental group were analyzed to evaluate the respective morphological indexes. The quantitative results are presented in Figure 6.2. HDFs grew on RF displayed a mean spreading area of $2864.2 \pm 1357.5 \mu\text{m}^2$ (Figure 6.2A), a BI value of ~ 6.5 (Figure 6.2B), a nucleus area of $206.7 \pm 61.3 \mu\text{m}^2$ (Figure 6.2C) and a NSI value of approximately 0.82 (Figure 6.2D). On the contrary, the morphological indices for the cells on AF differed significantly to that of cells

on RF. Compared to the case of RF, the cells on AF exhibited a much smaller spreading area of $1410.7 \pm 769.3 \mu\text{m}^2$ (Figure 6.2A), which corresponded to 50 % reduction in cell area relative to cells on RF. This suggested that HDFs spreading was confined on AF. A BI value of ~ 16.4 indicated the cells adopted a highly elongated form on AF (vs. 6.5 for RF). A projected cell nucleus area of $151.3 \pm 58.7 \mu\text{m}^2$ and NSI of ~ 0.90 signified that cell nuclei were well elliptical while cultured on AF. These results demonstrated that HDFs on AF displayed reduced cell spreading area with consistently elongated morphologies compared to those on RF, indicating fiber topography was able to guide and confine cellular morphology. From the understanding of the correlation between cellular morphology and phenotype, we therefore hypothesized that phenotype of HDFs could also be regulated by the scaffold topography, namely fiber orientation.

Figure 6.2 Measured morphological parameters including (A) cell spreading area, (B) bipolarity index (BI), (C) cell nucleus area and (D) nucleus shape

index (NSI). The vaules were examined from immune-stained images of HDFs cultured on AF and RF. Data were collected from 50 cells per experimental group. Double asterisks (**) indicates statistically significant difference at level of $p < 0.01$ between the two experimental groups.

6.3.2 Influence of topographical alignment on fibroblast phenotypic alteration

Differentiation of fibroblast-to-myofibroblast and the enhancing contractile force are key effectors in advancing wound closure [167]. To reveal whether the fiber organization is capable of regulating the myofibroblast differentiation, HDFs cultured on AF and RF were examined for α SMA expression with samples retrieved at 1, 3, 7, and 14 days post cell seeding. Besides AF and RF, casted film (Film) and tissue culture polystyrene (TCPS) were employed as control culture substrates.

The synthesis of α SMA is considered a hallmark for myofibroblastic phenotype, which is responsible for “muscle-like” contraction [167, 172]. In our study, the gene expression of α SMA (encoded by gene ACTA2) was determined by performing qPCR, and all qPCR data were normalized to the values of cells before seeding (considered as the basic level). As shown in Figure 6.3, HDFs retrieved from RF, film and TCPS retained the similar α SMA gene expression to basic level ranging from 1.0 to 1.7 times within 14 days of culture. In contrast, HDFs on AF exhibited an increasing tendency in α SMA transcription level. At day 1, the α SMA expression increased approximately 1.8 folds to the basic level, which was significantly higher than that of RF (1.0 fold) and TCPS (1.1 fold) groups. Additionally, the elongated HDFs cultured on AF displayed a progressive increment of α SMA gene expression from day 3 to day 14. By 14 days of culture, expression of α SMA transcript was approximately 3.8 times higher than the basic level. It suggested that AF enhances α SMA gene

expression as a function of time within the time frame of wound healing (e.g., 14 days).

Figure 6.3 Relative gene expressions of myofibroblast-associated marker α SMA. HDFs cultured on different substrates (AF: aligned fiber; RF: random fiber; Film: casted film; TCPS: tissue culture polystyrene) were retrieved after 1, 3, 7 and 14 days of cell seeding. Relative expression levels were normalized to the values of the cells before seeding. The symbols α , β , γ and δ indicate statistically significant difference ($p < 0.05$) between the experimental group and AF on day 1, 3, 7 and 14 respectively. The asterisk (*) indicates statistically significant difference ($p < 0.05$) between the time points in AF group.

Myofibroblasts produce incremental amounts of ECM components to reconstruct tissue, especially for collagen types I and III, with typically 1~2 times more than fibroblasts. Thus, increased levels of collagen expression are

also considered as myofibroblast signature [30, 176, 177]. Collagen type I consists of two chains of alpha-1(I) (encoded by COL1A1) and one chain of alpha-2(I) (encoded by COL1A2). On the other hand, collagen alpha-1(III) (encoded by COL3A1) forms the collagen type III, the abundant collagen in granulation tissue. Herein, the transcriptional level of COL1A1, COL1A2 and COL3A1 of HDFs cultured on different substrates were examined.

As presented in Figure 6.4, RF, Film and TCPS upregulated expression of COL1A1, COL1A2 and COL3A1 to 1.9~2.3, 1.6~2.1 and 1.1~1.7 folds of basic level respectively within 14 days. The upregulation of collagen transcriptional activity may also be contributed by the activation during subculturing and transferring of cells to a new microenvironment. Furthermore, HDFs cultured on AF possessed a significantly higher level of COL1A1 (2.7~3.3 folds of basic level) and COL3A1 (1.6~3.0 folds of basic level) compared with the other substrates, while there was no significant difference observed in COL1A2 expression. Additionally, it appeared that AF increased COL3A1 level as a function of time, which was in a similar manner to that of induced α SMA expression. To sum up, the upregulation of α SMA and collagen (type I and type III) gene expression preliminarily indicated that AF could promote a phenotypic transforming of HDFs into a more myofibroblast-like phenotype.

Figure 6.4 Relative gene expressions of myofibroblast-associated markers (A) COL1A1, (B) COL1A2 and (C) COL3A1. HDFs cultured on different substrates (AF: aligned fiber; RF: random fiber; Film: casted film; TCPS: tissue culture polystyrene) were retrieved after 1, 3, 7 and 14 days of cell seeding. Relative expression levels were normalized to the values of the cells before seeding. The symbols α , β , γ and δ indicate statistically significant difference ($p < 0.05$) between the experimental group and AF on day 1, 3, 7 and 14 respectively. The asterisk (*) indicate statistically significant difference ($p < 0.05$) between the time points in AF group.

To further confirm the influence of fiber alignment on phenotypic changes, intracellular staining and flow cytometry were employed to examine α SMA protein expression in HDFs. The HDFs were cultured on different substrates for 1, 3, 7 and 14 days, and the representative fluorescent plots are displayed in Figure 6.5A-D. The mean fluorescence intensities were compared and

normalized to that of TCPS, which stood for the basic level as 1, and the corresponding quantitation is shown in Figure 6.5E. At day 1, no change of α SMA protein expression was detected among all the experimental groups (Figure 6.5A), with all the values retained at 0.9~1.1 folds of the basic level. Although AF slightly induced α SMA gene expression at day 1, higher protein level was observed only after 3 days (Figure 6.5B). After 3-day-culture on AF, HDFs possessed a higher α SMA protein level (around 1.9 folds to basic level), while the values in both RF group and Film group were retained at 1.1 folds. The protein quantitation at day 7 and day 14 exhibited the similar manner as that of day 3. Higher fluorescence intensities of immunostaining α SMA on AF further evidenced that aligned fibers indeed stimulated the myofibroblast differentiation of HDFs.

Figure 6.5 (A-D) Representative fluorescent intensity histograms and (E) corresponding quantitation of α SMA protein expression in flow cytometry. HDFs were cultured on different substrates and retrieved on day (A) 1, (B) 3,

(C) 7 and (D) 14, respectively. AF: aligned fiber; RF: random fiber; Film: casting film; TCPS: tissue culture polystyrene. (E) The fluorescent intensity of α SMA expression was normalized to that of TCPS (as baseline) and displayed in fold change. The symbols α , β and γ indicate statistically significant difference ($p < 0.05$) of the experimental group against AF group on day 3, 7 and 14 respectively. Values were collected from three independent experiments.

6.3.3 Investigation on the reversibility of myofibroblast differentiation induced by aligned fibers

6.3.3.1 The introduction of ANGPTL4

The upregulation of α SMA and collagen (type I and type III) expression demonstrated that AF could induce fibroblast-to-myofibroblast differentiation. The consequent contractile enhanced force may help to advance wound closure [167]. However, during the ultimate stage of wound healing, the excessive ECM deposition from accumulated myofibroblast would contribute to pathological scar formation. Therefore, it is ideal to abrogate the myofibroblastic activity in later event to yield an enhancing and beneficial wound healing as well as minimum scar formation. In this regard, a matricellular protein as a potential and multifunctional wound-healing agent, angiopoietin-like protein 4 (ANGPTL4), attracted our interests. It was recently reported that ANGPTL4 not only enhanced fibroblast migration and proliferation but also reduce the production of scar-associated collagen [107, 108, 115, 116]. Thus, we specifically examined if the presence of ANGPTL4 can reverse increased expression of α SMA and collagen induced by AF substrate.

With the addition of ANGPTL4 (6 μ g/mL) into culture medium from day 0 (i.e., when cell seeding), HDFs cultured on AF were retrieved and analyzed by the

qPCR within 14 days as previously described, nominated as AA group. The qPCR results were statistically analyzed and plotted in Figure 6.6 A-D. Evidently, comparing with the case in AF sample, the presence of ANGPTL4 significantly decreased the gene expression of myofibroblastic markers. AF was known to up-regulate α SMA expression to 3.8 folds after 14 days, while ANGPTL4 retained the expression at 1.2~1.8 folds throughout the time frame. Furthermore, collagen expressions were also attenuated by introduction of ANGPTL4. Within 14 days, the expression of COL1A1 of AF was reduced from 2.7~3.3 to 1.9~2.3 folds. Similarly, COL1A2 and COL3A1 expression was reduced from 2.3~2.4 to 1.2~1.8 folds and from 1.6~3.0 to 1.5~1.9 folds, respectively. These results suggested that addition of ANGPTL4 could inhibit AF-induced myofibroblastic differentiation. This might be due to the interaction between ANGPTL4 and cadherin-11 [118], as described in Section 2.3.1.

Figure 6.6 Relative gene expressions of myofibroblast-associated markers (A) α SMA, (B) COL1A1, (C) COL1A2 and (D) COL3A1. HDFs were cultured on

aligned fiber in the medium without (AF) or with (AA) ANGPTL4, which were retrieved after 1, 3, 7 and 14 days of cell seeding. Relative expression levels were normalized to the values of the cells before seeding. The symbols α , β , γ and δ indicate statistically significant difference ($p < 0.05$) between AA and AF on day 1, 3, 7 and 14 respectively. The asterisk (*) indicates statistically significant difference ($p < 0.05$) between the time points in AF group.

6.3.3.2 Effect of ANGPTL4 after the initiation of myofibroblast differentiation

The above results showed that presence of ANGPTL4 from the beginning was able to inhibit AF-induced fibroblast-to-myofibroblast differentiation. However, myofibroblast is essential in the initial stage of wound healing. Here, we would like to investigate if this differentiation could be reversed by ANGPTL4 only after wound closure has taken place. Thus, HDFs were firstly cultured on AF, and ANGPTL4 were added only after 7, 10 and 13 days of culture (denominated as AA7, AA10 and AA13 respectively). Subsequently, the α SMA gene expression levels were measured at day 14. The results are displayed in Figure 6.7. AA7 and AA10 possessed a significantly reduced expression of 1.2 and 1.7 folds of basic level respectively than that of AF (3.8 folds). However, there is no difference between the fold changes of AA13 (3.6 folds) and AF. This indicated that ANGPTL4 could reverse AF-stimulated myofibroblast differentiation, but probably in a time-dependent manner whereby if ANGPTL4 was added by day 13, there is terminal differentiation of myofibroblast which is irreversible. In summary, the AF-stimulated fibroblast-to-myofibroblast differentiation may be beneficial for wound contraction during the initial and middle stages in wound healing. The addition of ANGPTL4 in later stage (e.g., day 10) to reverse the differentiation after wound contraction may be a plausible strategy to reduce scarring without compromising healing time. In the future, the optimal combination of AF and ANGPTL4 could prove to be a

promising strategy for tunable and controllable myofibroblast differentiation for on-demand tissue engineering and wound healing.

Figure 6.7 Fold change of α SMA gene expression of 14-days cultured HDFs on different substrates or with different ANGPTL4 addition time. AF: aligned fiber; RF: random fiber; Film: casting film; TCPS: tissue culture polystyrene; AA7, AA10 and AA13: aligned fibers with ANGPTL4 addition in the medium after 7, 10 and 13 days of culture respectively. The asterisks (*) indicates statistically significant difference ($p < 0.05$) between two experimental groups.

6.3.4 Evaluation of cellular TGF β 1 level and its mechanoregulation

It is well accepted that TGF β 1 is the main stimulator of myofibroblast differentiation [168, 178]. Thus, to gain possible insight into the mechanism by which AF regulates myofibroblast differentiation, we examined the TGF β 1 level of HDFs. According to the previous results, HDFs cultured on AF showed increased α SMA protein expression after 3 days. Thus, HDFs cultured on different substrates were retrieved at day 3 for subsequent analysis. Concentration of TGF β 1 was assessed using ELISA, and the gene expression was determined by qPCR. The results are presented in Figure 6.8. Significantly

higher concentration of active TGF β 1 in HDFs was detected on AF, in compared with that of RF, Film and TCPS. However, no change between AF group and AA group was observed. As expected, consistent results were observed from the analysis of TGF β 1 gene expression. These results implied that AF might induce fibroblast-to-myofibroblast differentiation by activating TGF β 1 expression. Additionally, as the AA sample (with interference from ANGPTL 4) also showed increase in TGF β 1, it shows that ANGPTL reversed the differentiation through TGF β 1-independent pathway.

Figure 6.8 (A) ELISA and (B) qPCR quantitation of TGF β 1 level. HDFs cultured on different substrates (AF: aligned fiber; RF: random fiber; Film: casting film; TCPS: tissue culture polystyrene; AA: aligned fibers and presence of ANGPTL4 in the medium) were retrieved at day 3. Relative gene levels were normalized to the cells before seeding. The asterisk (*) indicates statistically significant difference ($p < 0.05$) between two experimental groups.

Phosphorylation of TGF β receptor II is one of the key downstream events during TGF β 1 stimulating pathway [179] and thus investigation on TGF β receptor II phosphorylation may help verify the possibility stated above. Therefore, the expression of phosphorylated TGF β receptor II (pTGF β -RII) was determined by western-blot analysis. The quantitative results (Figure 6.9) showed that AF possessed a slightly higher protein expression of pTGF β -RII, further supporting the assumption on AF-induced TGF β 1 activation.

Figure 6.9 Expression of pTGFβ-RII evaluated by western-blot analysis, with GAPDH served as housekeeping control. HDFs were cultured on diverse substrates (AF: aligned fiber; RF: random fiber; Film: casting film) and retrieved at day 3. Band intensities were quantified and normalized to that of Film group. Single asterisk (*) and double asterisks (**) indicate statistically significant difference of $p < 0.01$ and $p < 0.05$ between two experimental groups, respectively.

It has been reported that latent TGFβ1 secreted by cells can be activated through mechanoregulation [32, 41, 42], as reviewed in Section 2.1.3. Therefore, the mechanical properties of fibers with different alignments (AF and RF) were evaluated to reveal the possible underlying mechanisms of AF-induced TGFβ1 activation. The stress-strain curves of AF and RF obtained from tensile tester, Instron are presented in Figure 6.10. As the results show, it appeared that AF was stiffer but had lower strain at break than RF.

Figure 6.10 Stress-strain curve of aligned fibers (AF) and random fibers (RF). Results distinctively showed that AF was stiffer while RF had higher strain at break.

Apparent Young's modulus, ultimate tensile strength and strain at break were obtained from three independent stress-strain curves and included in Table 6.1. As seen in Table 6.1, AF possessed an apparent Young's modulus of 14.13 ± 1.92 MPa, which is significantly stiffer than that of RF (5.92 ± 0.88 MPa). Besides, AF had a much higher ultimate tensile strength of 30.82 ± 4.48 MPa as compared to RF with ultimate tensile strength of 8.51 ± 0.39 MPa. By contrast, RF tended to have a higher strain at break of 275.39 ± 9.53 % (vs. 133.97 ± 17.48 % for AF). Thus, the higher level of TGF β 1 observed on AF might be associated with the stiffer mechanical properties of AF. In others words, the stiffer AF might activate latent TGF β 1 through mechanoregulation.

Table 6.1 Mechanical properties of PLC electrospun fibers determined from the stress-strain curves.

Sample	Fiber diameter (μm)	Apparent Young's Modulus (MPa)	Ultimate Tensile Strength (MPa)	Strain at Break (%)
Aligned fibers (AF)	0.83 ± 0.19	14.13 ± 1.92	30.82 ± 4.48	133.97 ± 17.48
Random fibers (RF)	0.85 ± 0.10	5.92 ± 0.88	8.51 ± 0.39	275.39 ± 9.53

6.4 Summary

HDFs exhibited different morphologies on fibers with aligned or random alignments. HDFs on AF exhibited elongated morphologies while HDFs on RF possessed polygonal shapes. In addition, increased expressions of αSMA and collagen (type I and type III) of HDFs were demonstrated on AF, suggesting that fiber alignment might promote fibroblast-to-myofibroblast differentiation. Furthermore, it is noteworthy that the presence of matricellular protein ANGPTL4 could reverse the phenotypic alteration induced by AF. Higher TGF β 1 level detected on AF might result from mechanical regulation, which suggested the possible underlying mechanism of AF-induced myofibroblast differentiation. These findings suggested the important role of mechanical properties in regulating the fiber alignment-induce fibroblast-to-myofibroblast differentiation, and it was implicated that fiber alignment should be a critical design factor for artificial fibrous scaffolds. With this knowledge obtained, several recommendations regarding the incorporation of ANGPTL4 in the fibrous scaffolds in order to advance wound repair with enhancing contraction but with reducing scar formation have been suggested and discussed in the next chapter.

Chapter 7

Summary and Future Recommendations

In this chapter, Section 7.1 summarizes the work conducted for the three objectives with the defined research scopes, and leads to a discussion on how objectives were thus achieved. These results give important hints on the future development of superior fibrous matrices for optimal cell migration and myofibroblast differentiation to advance wound healing. Subsequently, by incorporating the results obtained and literature review, Section 7.2 proposes recommendations on the possible future work as a continuation to the various findings of this study.

7.1 Summary

The aim of this dissertation was to investigate the relationship between fiber topographies and skin cell responses in terms of cell migration and phenotypic maintenance/alteration which are critical for wound healing but have not been fully understood. To achieve this aim, the dissertation is subdivided into three specific objectives: 1) To fabricate fibrous platforms with differing dimensions, fiber orientations and fiber diameters through manipulation of electrospinning processing parameters and solution parameters; 2) To investigate the effect of fiber diameter and alignment on skin cell migration and to elucidate the possible underlying mechanisms; 3) To evaluate the influence of fiber alignment on fibroblast phenotypic maintenance/alteration and to explore the possible underlying mechanisms. By adopting an interdisciplinary approach involving the technical knowledge of materials science and engineering, as well as the molecular biology techniques, this project revealed that fibrous substrates with oriented fiber configuration were functional in steering directional migration and myofibroblast differentiation. Additionally, the underlying mechanisms regarding the biophysical cues in cell-material interaction were explored and discussed, which is essentially important since the insights obtained will contribute significantly to the rational design of future biomedical scaffolds.

As the first objective, which is to fabricate fibrous substrates with desired features, different parameters in electrospinning have been explored and optimized. Electrospinning technique was chosen due to its versatility and the resultant structure that highly mimics native extracellular matrix (ECM) [180-182]. In addition, a relatively new material poly (lactic acid-co-caprolactone) (PLC), which is biocompatible, biodegradable and elastic, has been chosen to evaluate the feasibility in creating skin grafts. PLC has been commonly used for vascularization application due to its unique elastic nature [79, 183, 184].

However, its usage in skin graft application is still not explored yet. With the use of traditional electrospinning, we aimed to generate 2D fibrous scaffolds conferring diverse topographic features (e.g., fiber alignment and diameter) through manipulation of solution and processing parameters. In order to get align-oriented fibers, rotating-collection strategy was employed. Although it has been well established that a pair of split electrodes can perfectly align fibers, both the size and thickness of forming fibrous membrane are limited [130, 131]. Actually fibers can also be aligned well by rotation where the rotating speed plays a pivotal role [132, 134]. By tuning the rotating speed of fiber collecting drum, the degrees of fiber orientation could be manipulated. When the surface velocity of rotating drum is as high as that of electrospinning jet, it is sufficient to orient the fibers along the axis of rotation. In our study, it was found that the best fiber alignment could be attained at threshold surface velocity of 15.70 m/s, indicating the velocity of electrospinning jet might be lower or close to this point, which fell within the reported range of average velocity of electrpinning jet (2 to 186 m/s) [185-187]. As for fabrication of fibrous substrates with different diameter, it has been reported to be achieved by tailoring the processing parameters (e.g., solution feed-rate, applied voltage and working distance) and solution properties (e.g., viscosity, surface tension and conductivity) [138, 139, 141, 142, 188]. Increasing feed-rate leads to an increment in fiber diameter, while the influence of applied voltage seems to be less consistent and not as dominant. Several studies have demonstrated that the diameter decreased with increasing applied voltage, but others showed converse trends [136, 137, 189]. In our studies on the electrospinning parameters, we found that the dominant factors were solution conductivity as well as polymer concentration. Higher conductivity yielded thinner fibers, while increasing polymer concentration resulted in thicker fibers. Park *et al* has reported similar phenomenon with other polymers by changing these two parameters [141]. Notably, the successful generation of PLC fibers ranging from 300 nm to 2 μm was achieved, and using the combination of DCM and DMF as solvent was able

to eliminate bead formation which generally occurs in the use of solo solvent [76]. It is worth noting that thinner fibers (300~1000 nm) have been known to promote cell adhesion and growth, while the effects of thicker fibers (>1000 nm) were seldom explored [64, 93, 190]. These 2D fibrous matrices with differing fiber diameter and fiber alignment were employed in the later sections on investigating the influence of substrate topography on cellular responses.

In this dissertation, the feasibility in creating 3D PLC electrospun scaffolds was also a significant study. One common strategy in obtaining 3D scaffolds involves easily-eliminated materials (such as salt and water-soluble polymer) during electrospinning, which will be selectively removed after electrospinning [122-125]. However, the scaffold's stability is often compromised due to the sudden elimination of sacrifice materials. Another strategy is the application of ultrasonication post-electrospinning where physical manipulation can enlarge the pore size of electrospun scaffolds, but this strategy does not produce a true 3D structure, especially in terms of the thickness [126]. In place of traditional collectors, cotton-ball-like scaffolds could be electrospun in a spherical collector with an array of needles [127]. However, this is not easy to replicate due to the requirements on precise control over parameters such as the dimensions of the spherical dish, needle length, needle distance, *etc.* Herein, a simple and versatile approach to produce 3D fibrous porous scaffolds via liquid-collection has been developed and optimized in our work. This design is easy to achieve via replacing the traditional conductive collector to alcohol-water bath during electrospinning. However, this method seemed to rely on the properties of the polymer used. When PLC was used, the fibers collected in liquid were found to aggregate easily, leading to small pores (<10 μm) within the scaffold. This might be due to the unique elastic property of PLC (with T_g of $\sim 11^\circ\text{C}$). PLC fibers in the liquid tend to recover from their stretching, which is generated by repulsive forces in response to applied electrical field [191], resulting in fiber aggregation. However, when another biocompatible and

biodegradable polymer poly (lactic acid-co-glycolic acid) PLGA (with T_g of 40~48 °C [183]) was used, 3D scaffolds with relatively large pores (average pore size of $14.54 \pm 6.47 \mu\text{m}$) were formed through liquid-collecting electrospinning. It should be noted that *in vitro* cellular studies using human dermal fibroblasts (HDFs) demonstrated advanced cell proliferation and infiltration within a time frame of 7 days, suggesting the great potential of our artificial 3D fibrous scaffolds as ECM replacement in reconstructing tissues.

As the second objective, the influence of substrate topographic features on cell migration was investigated. Collective and individual cell migration assay of skin cells were employed to understand the effect of varying substrate features, which were obtained from previously optimized electrospinning. Fibers in various diameters (0.3, 0.8, 2 and 13 μm) were assayed with L929 cell migration. As presented by fluorescent images taken across a time frame of 48 hours, it was found that substrates possessing ECM-mimicking fibrous nature significantly advanced L-929 cell migration, when compared with flat topography or fibrous substrates with diameter beyond native ECM fibril scale (e.g., 13 μm). This highlights that it is essential to mimic ECM features not only in terms of fiber topography but also fiber dimensions. Additionally, scaffolds possessing aligned configuration were demonstrated to promote HDFs migration, which are consistent with published reports [88]. Notably, the migration assays employed in the dissertation has been optimized in order to minimize the potential interference which arise from cell proliferation [88, 96], through utilizing low serum content media as well as real-time cell tracking technology. It was demonstrated that fiber alignment could indeed persistently induce directional cell migration in HDFs with higher migratory velocity. The underlying mechanisms of these interesting phenomena were speculated to be due to the focal adhesion assembly as well as Rho small GTPase activity. As similarly reported in other literature [192], lower expression of focal adhesions was observed on cells cultured on aligned fibers. Additionally, it has been well

accepted that adhesion strength influences the speed of cell motility [163]. Horwitz *et al* demonstrated that maximum migration speed decreases reciprocally as cell-substrate adhesiveness increases [154]. Herein, the lower expression of focal adhesion on aligned fibers might confer the faster migration. Furthermore, the oriented configuration confined the focal adhesions to be linear and aligned along fiber direction, which might contribute to the directional motility. Besides the confinement of focal adhesions, aligned fiber also stimulated the activation of Cdc42 GTPase. Cdc42 has been commonly accepted as a master regulator trigger actin polymerization at the leading edge, stimulating filopodia extension towards the migrating direction [156, 164, 166]. The results here indicated that, for the first time, directional migration triggered by aligned fibers might be mediated through Cdc42 forming finger-like filopodia along the fiber orientation. Thus, artificial fibrous matrices with oriented fiber configuration might promote directional migration of adjacent skin cell into wound bed and hence advance wound repair.

Finally, the effect of fiber alignments on cellular morphology and phenotypic alteration was examined. Results showed that HDFs on aligned fibers exhibited elongated morphologies while cells on random fibers possessed polygonal shapes. Similar observations were showed in some other cell lines (e.g., Schwann cell, embryonic stem cell, breast cancer cell, *etc*) [193-195], supporting the indication that cell morphology is corresponding consistently to substrate topography. In addition, when cultured on aligned fibers, HDFs transited into a more myofibroblast-like phenotype over a time frame of 14 days, evidenced by myofibroblastic marker alpha smooth muscle actin (α SMA) in both gene expression and protein expression. The consequent contractile force generated from this kind of myofibroblast-like phenotype may help to advance wound closure [167]. However, during the ultimate stage of wound healing, the excessive ECM deposition from accumulated myofibroblast is undesired as it will give rise to pathological scarring [27]. Several efforts have been made to

address this kind of undesirable scarring through applying mitogenic growth factors such as bFGF and PDGF, which are capable of dedifferentiation of myofibroblasts [39, 172-174]. Notably, our work which employed matricellular protein Angiopoietin-like 4 (ANGPTL4) [118] to reverse this fiber-alignment-induced differentiation at the later stage of healing was shown to be a promising alternative solution. Besides, ANGPTL4 was found to modulate multifaceted cellular functions to benefit wound repair process, such as decreasing ECM degradation, enhancing keratinocyte migration and advancing wound angiogenesis [107, 108, 116]. Additionally, significantly higher cellular level of transforming growth factor- β 1 (TGF β 1), a major inducer of myofibroblast differentiation, was detected in HDFs cultured on aligned fiber. It is reported that latent TGF β 1 can be activated by substrate stiffness [41, 42]. Herein, this activated TGF β 1 might be correlated with mechanoregulation through the stiffer mechanical properties of aligned fibers. These findings suggested the important role of mechanical properties in regulating the fiber alignment-induced fibroblast-to-myofibroblast differentiation, and it was implied that fiber alignment should be a critical design factor for artificial fibrous scaffolds. Therefore, in the future study, introduction of fiber alignment with controlled release of ANGPTL4 at the appropriate time may advance wound repair with enhancing contractile force while reducing scar formation.

The results in this study have yielded valuable insights into biophysical parameters that regulate skin cells interactions with engineered fibrous scaffolds. These understanding obtained may lead to the superior development of multifunctional tissue-engineered skin grafts capable of accelerating wound closure and reducing scarring formation for efficacious therapies.

7.2 Future recommendations

7.2.1 Fabrication of 3D aligned fibrous scaffolds

The results from this study have demonstrated that the fibrous scaffolds with oriented fiber configuration are multifunctional and beneficial for wound healing. Thus, it is significant to develop a 3D aligned fibrous scaffold which is capable of not only promoting wound healing but also fulfilling implantable purposes, since most electrospun scaffolds reported in the literature inevitably suffer from cell infiltration issues which limited their wide applications in actual medical practices [64]. As shown in our previous studies (Chapter 4), collection of fibers in liquid facilitates the production of 3D fibrous matrices [73]. To achieve the fabrication of 3D aligned fibrous scaffolds, we performed a preliminary trial to rotate the collecting bath while collecting fibers, as seen in Figure 7.1A. As a result, a tubular 3D sponge was formed, as visualized in Figure 7.1B. From the SEM characterization (Figure 7.1C), fibers with aligned orientation were observed, with fiber density lower than that of 2D aligned fiber mats. It seemed that 3D aligned fibrous matrices could be achieved by this approach. However, it should be noted that high rotating speed might be restricted while using this approach, since high speed rotating might lead to liquid splashing. Alternatively, the introduction of a rotating jig into the liquid bath may help to enhance the controllability and facilitate the fabrication [196].

Figure 7.1 Illustration of the fabrication of 3D aligned fibrous scaffolds via liquid-collecting electrospinning. (A) Fibers are collected in liquid bath with rotating. (B) The visual images and (C) SEM characterization of the formed fibrous matrices.

7.2.2 Incorporation of ANGPTL4 into electrospun fibers

According to our previous results in Chapter 6, ANGPTL4 has shown promising ability of reversing myofibroblast differentiation. It is therefore an ideal complementary strategy for our aligned fibrous scaffold, if used appropriately. Ideally, at the initial stage of wound healing (e.g., 1~7 days) wound contraction is required and could be modulated by aligned fibers-induced myofibroblast differentiation, while dedifferentiation by ANGPTL4 could be promoted to reduce scarring at the later stage (e.g., 7~14 days). Thus, it would be desirable to have a delayed release of ANGPTL4 from electrospun scaffolds, preferably after one week of wound healing. Herein, we propose to incorporate ANGPTL4 into aligned fibers via emulsion electrospinning [197]. Via this approach, ANGPTL4 protein can be incorporated into polymer solvent while the presence of emulsifier could stabilize the emulsions as well as protect the proteins therein [198]. Figure 7.2 is presents the illustrated process of emulsion electrospinning. Briefly, ANGPTL4 protein is dissolved in water phase containing surfactant, and subsequently the aqueous solution is dropped into oil solution containing polymer and organic solvent to form stable W/O emulsion by stirring and ultra-sonication. Finally, the emulsion will be electrospun into continuous fibers loaded with ANGPTL4. As for the surfactant candidates, Span 80 [199], cyclodextrin [89] and sodium lauryl sulfate (SLS) [200] will be preferentially considered. Moreover, the amounts of protein will be examined for *in vitro* controlled release assay. After the mature development of formulation and fabrication, the resultant fibrous scaffolds incorporating ANGPTL4 can be evaluated for their wound healing efficacy *in vitro* before proceeding to *in vivo* studies.

Figure 7.2 Illustration of the formation of biphasic suspension and the process of emulsion electrospinning [201].

7.2.3 Animal trial

The tissue-engineered skin grafts of electrospun 3D aligned fibrous matrices with controlled ANGPTL4 release can then be employed for *in vivo* animal test to examine their effect on wound healing and scar formation. Briefly, circular full-thickness excisional splint wounds of 10 mm will be created on high fed diet and high glucose (HF-HS) induced mice [202]. After surgery, various matrices will be applied locally to the wounds and covered by an occlusive dressing. Digital images will be captured to assess the rate of wound closure. Additionally, at different time points (day 3, 7, 10 and 14 post-wounding),

wound biopsies will be collected for tensile test and histological analysis, such as routine hematoxylin and eosin staining, immunofluorescence staining and Masson's Trichrome staining. Through these analyses, the presence of myofibroblast and the amount of collagen deposition within the biopsies will be investigated to examine and evaluate the healing process.

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