Artificial extracellular matrix proteins for substrates in skin substitutes

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NANYANG
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**ARTIFICIAL EXTRACELLULAR MATRIX PROTEINS
FOR SUBSTRATES IN SKIN SUBSTITUTES**

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SCHOOL OF MATERIALS SCIENCE AND ENGINEERING

2015

**ARTIFICIAL EXTRACELLULAR MATRIX PROTEINS
FOR SUBSTRATES IN SKIN SUBSTITUTES**

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School of Materials Science and Engineering

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To my beloved family and friends

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ABSTRACT

For decades, there has been intense research to develop novel substrates for skin repair. Several tissue-engineered skin products are available currently, however, autograft remains the gold standard in skin repair. Recently, protein-based biomaterials have recently received great attention for use in biomedical applications as they can be genetically engineered to mimic the biological properties of the native tissue.

This research aims to develop novel protein-based biomaterials for use as tissue engineered skin. It was hypothesized that the incorporation of integrin-specific cell binding domains on aECM proteins will enhance keratinocytes adhesion and viability; promote faster re-epithelialization and in-situ human dermal fibroblast infiltration during wound healing. For this purpose, recombinant DNA technologies were used to create artificial extracellular matrix (aECM) proteins that promote integrin-specific interactions between human epidermal keratinocytes (HEKs) and the underlying substratum. Artificial ECM proteins were developed as elastin-like polypeptide fusion proteins containing different types of cell binding domains: FN910 (FN910 aECM), PPFLMLLKSTR (LN-5 aECM), and GEFYFDLRLKGDK (Col-IV aECM).

In this work, we found that keratinocyte attachment and proliferation were mediated through specific interactions between the integrins and cell-binding domain present in aECM proteins. Specifically, keratinocyte attachment on FN910 aECM protein was mediated by alpha5 beta1 integrin, while their attachment on LN-5 aECM protein was mediated by alpha3 beta1 integrin. We also showed that aECM proteins

were able to support proliferation and colony-forming abilities of keratinocytes stem cell.

The cell-aECM proteins interactions were also examined in the context of cell migration. Similar to the native FN, we found that FN910 aECM protein promoted faster wound closure compared to BSA control. In contrast, slower wound closure rate was observed on LN-5 aECM compared to native LN. This could be attributed to the lower cell speed and less directional movement observed on keratinocytes migrating on this aECM. Likewise, cell migrating individually on LN-5 aECM substrates also moved with slower speeds and little persistence. Furthermore, addition of anti-integrin antibodies led to severe inhibition of cell migration and loss of directed motility. Taken together, our results suggest that keratinocytes primarily utilized $\alpha 3 \beta 1$ integrin to migrate on LN-5 aECM, while $\alpha 5 \beta 1$ is engaged by keratinocytes to migrate on FN910 aECM.

Finally, we evaluated the feasibility of developing the artificial ECM proteins into a mechanically intact free-standing scaffold to be used as keratinocytes cell delivery system. We showed that aECM protein scaffold produced by freeze-drying method was able to support keratinocytes attachment and viability. Further, there was sufficient porosity within the aECM protein scaffolds to allow human dermal fibroblasts infiltration. Hence, we demonstrate that aECM protein scaffolds are suitable for use as a cell carrier, potentially serving as a substrate to aid wound repair in the clinic.

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Abbreviations

Ab	antibody
aECM	artificial extracellular matrix
BS ³	bis(sulfosuccinimidyl)suberate
CEA	cultured epithelial autografts
CFE	colony forming efficiency
Col-IV	type-IV collagen
DAPI	4',6-diamidino-2-phenylindole
ELPs	elastin-like polypeptides
FITC	fluorescein isothiocyanate
FN	fibronectin
HA	hyaluronic acid
HDF	human dermal fibroblasts
HEK	human epidermal keratinocytes
ITC	inverse transition temperature
LCST	lower critical solution temperature
LN	laminin
MMPs	matrix metalloproteinase
NHS-ester	N-hydrosuccinimide ester
PBS	phosphate-buffered saline
PCL	poly-epsilon-caprolactone
PDMS	polydimethylsiloxane
PGA	poly glycolic acid
PLA	poly lactic acid

PLGA	poly lactic-co-glycolic acid
SDS- PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TBS	tris-buffered saline
TCPS	tissue culture polystyrene

Symbols

E	Young's modulus
G'	elastic/storage modulus
G''	viscous/loss modulus
G*	complex modulus
p63	transcription factor p63
Tan δ	phase / loss angle
ν	Poisson's ratio

1. INTRODUCTION

1.1. Background

For centuries, people have attempted to achieve successful tissue/organ repair and regeneration. Over the years, transplantation or tissue grafting, whether it is taken from patients own body (autograft), from other individual (allografts) or from other species (xenograft), has been shown to be the most direct and very often the only way of repairing a damaged tissue/organ [1]. For skin, the history of the earliest skin transplantation dates back as early as 700 years BC where skin autograft transplant was done by the Indian surgeon Sushruta for nose reconstruction [2]. First description of successful skin grafting technique was recorded centuries later by Reverdin in 1869, which was later termed “pinch graft” after the technique was refined by John Staige Davis [2]. As the science of skin grafting continue to develop over the years, it became clear that allograft and xenograft skin was sufficient for temporary skin substitutes, but it was not a long-term skin transplant.

Currently, the gold standard for skin transplant is autologous skin grafting. However, autologous skin graft is not always available, especially in the case of patient suffering from extensive burns (e.g. 50 percent total body surface area full-thickness wounds), where donor sites are extremely limited. Temporary dressings or cadaveric skin allograft are usually used in such cases, but only serial autologous skin grafting is able to heal such injured skin fully. Impaired epidermal barrier due to awaiting epithelial regeneration in addition to reduced immunity of heavily burned patients can give rise to bacterial sepsis, which is the major complication in deep

extensive burns [3]. Further, current trend of wound care has shifted from merely achieving satisfactory survival rate to improvement in the long-term function of the healed wound and quality of life.

Thus, there is a significant need for a not only readily available and transplantable, but also functional and clinically effective skin graft to meet the ever-increasing demand. For these reasons, tremendous efforts have been devoted in the past three decades to develop bioengineered human skin substitutes, resulting in a number of clinical products and skin models for pharmaceutical and cosmetic companies. Indeed, skin grafts represent nearly 50% of tissue engineering and regenerative medicine revenues worldwide [4]. In 2009, the potential market for tissue engineered skin substitutes for approximately 5 million target patient population in United States alone summed up to \$18.9 billion. By the year 2019, the number is expected to increase to 6.4 million, resulting in a potential market of approximately \$24.3 billion. The largest potential target markets are mainly represented by products for diabetic and venous ulcers treatments, abdominal wall repair, and burn treatments. Every year approximately 190,000 people die from severe burns and thermal injuries worldwide. The American Burn Association's report in 2013 indicated approximately 450,000 burn incidences occurred in the U.S [5]. Although burns case may not be numerically significant in the western world, in developing countries this remains a major healthcare problem [6-9].

1.2. Need and criteria for tissue engineered skin replacement

Ideally, skin substitutes should promote healing and the development of a new tissue that resembles the structure and function of an uninjured tissue. Three major requirements for skin substitutes are: it must be safe for the patients (non-toxic), clinically effective, and convenient in handling and application. To date, there are no commercially available skin substitutes that possess all these characteristics nor can they fully replace the functional and anatomical properties of the native skin. There are, however, a number of bioengineered skin replacement that are currently available to clinicians and used for wound-healing purposes, some of which such as Integra[®] and Permaderm[®] have even been used fairly successfully in the clinics [6]. Nonetheless, it is also known that some of these commercial products suffer from the same problems of extremely high cost, sub-normal dermal microstructure, xenogeneic in nature and inconsistent engraftment.

Among the bioengineered skin substitutes products available (Reviewed in Chapter 2), both natural and synthetic materials are explored. The efficacy of a skin replacement depends largely on the interaction between the biomaterials used and the cells involved. For this reason, much interest have been shown in using native proteins as skin substitutes, particularly extracellular matrix (ECM) proteins, mainly because they provide intrinsic biological stimuli to support cell and tissue function, one of the desirable properties that cannot be provided by synthetic polymers alone [10]. For example, fibrin is an excellent material for promoting epithelial cells adhesion and differentiation [11-13]. Bovine collagen gels are often preferred as they have shown to support the growth of human dermal fibroblasts as well as functions as a carrier for keratinocytes. However, the use of native ECM proteins in clinical setting is often

limited by batch-to-batch variability, restricted range of materials properties (including rapid degradation of the proteins), and also concerns about disease transmission associated with the source from where the proteins were isolated.

On the other hand, synthetic polymers are attractive as they offer biocompatibility, scalability, and better control on mechanical properties for easy handling. However, synthetic polymers possess no bioactivity as they generally lack basement membrane and cannot be remodeled by the cells residing in the scaffold. Thus these types of skin substitutes often cause permanent scarring and some even require secondary surgery as they only serve as temporary epidermal barrier [14].

In this study, a novel strategy was sought to engineer an artificial ECM proteins that promotes integrin-specific interactions between human epidermal keratinocytes (HEKs) and the underlying substratum. Recently, a great interest has been given to biomimetic approach for biomaterials design in tissue engineering. Particularly, recombinant DNA technologies have enabled the possibility of creating artificial analogs of ECM proteins as integrated design having specific domains to modulate cellular behavior and at the same time possess sufficient mechanical integrity for ease-of-handling and application. For example, it has been shown that artificial proteins containing full-length FN domains engage $\alpha 5 \beta 1$ integrins with increased binding affinity, leading to rapid cell sheet migration [15, 16]. In this regard, genetically engineered artificial proteins can be designed with the relevant properties sufficient for reconstruction of specific tissues in the body. Moreover, reports have shown that recombinant proteins cause relatively little immunogenic reactions [17, 18], which could therefore possibly minimize problems in biocompatibility.

Here, three different artificial ECM proteins containing a functional cell-binding domain derived from either laminin-5, fibronectin, or type IV collagen, combined with flanking elastin-like domains are investigated. The cell-binding domains were chosen to target major integrins expressed by human epidermal keratinocytes in wound healing condition (e.g. alpha3 beta1, alpha2 beta1, and alpha5 beta1 integrins). The aECM amino acid sequence is shown in Figure 1.1 below.

FN910 aECM : MKVD {(VPGIG)₂ VPGKG(VPGIG)₂}₈ **FN910** {(VPGIG)₂ VPGKG(VPGIG)₂}₈
LN-5 aECM : MKVD {(VPGIG)₂ VPGKG(VPGIG)₂}₄ **PPFLMLLKGSTR** {(VPGIG)₂ VPGKG(VPGIG)₂}₄ HHHHHH
Col-IV aECM : MKVD {(VPGIG)₂ VPGKG(VPGIG)₂}₄ **GEFYFDLRLKGDK** {(VPGIG)₂ VPGKG(VPGIG)₂}₄ HHHHHH

FN910:
FGLDSPTGIDFSDITANSFTVHWIAPRATITGYRIRHHPEHFSGRPREDRV**PHSRN**SITLTNLTGTEYVVSIVALNGREESPLLIGQQSTVSDVPRDLEVVAATPT
LLISWDAPAVTVRRYRITYGETGGNSPVQEFTVPGSASTATISGLAPGVDYTITVYAVTGRGDS**SPASSAPIS**INYRT

Figure 1.1 Amino acid sequences of artificial ECM proteins

The PPFLMLLKGSTR sequence taken from the laminin-5 alpha3 chain globular domain 3 (LG3) has been shown to specifically bind alpha3 beta1 integrin expressed by keratinocytes [19-21]. On the other hand, full-length fragment of the 9th and 10th domains of fibronectin (FN910) has shown to increase cell-matrix interaction of keratinocytes via alpha5 beta1 integrin [22]. Finally, a short peptide sequence found in the alpha1 chain of type IV collagen, GEFYFDLRLKGDK, was chosen as it has been shown to increase HaCaT keratinocytes adhesion [23].

In order to increase the overall solubility of the aECM proteins in aqueous environment, each aECM protein was constructed such that there was at least 70-80% elastin content. Further, lysine residues were interspersed within the elastin domains to serve as crosslinking sites such that the aECM proteins can be formed into a

mechanically intact freestanding scaffold. And finally, hexahistidine tag was added to the C-terminus of the Col-IV and LN-5 aECM construct to aid protein detection.

1.3. Hypothesis and objectives

The hypothesis of this study is formulated as follow:

The incorporation of integrin-specific cell binding domains on aECM proteins will enhance keratinocytes adhesion and viability; promote re-epithelialization and in-situ human dermal fibroblast infiltration during wound healing.

The overall goal of this study is to develop novel artificial ECM protein biomaterials for use as artificial skin substitutes. The specific aims of this work are as follow:

1. Design and generate the artificial ECM proteins through recombinant genetic engineering.
2. Evaluate human epidermal keratinocytes interaction with aECM proteins *in vitro*.
3. Fabricate, characterize, and evaluate the artificial ECM protein scaffold as cell delivery system *in vitro*.

1.4. Research Novelty

To date, integrin-specific biomaterials for skin tissue engineering have not been explored. The proposed work will focus on the development of artificial ECM protein biomaterials that mimic the biological properties of native skin. Our

biomaterial design strategy is novel and has yet to be reported. In addition, attempts to synthesize elastin-based recombinant proteins containing functional laminin and collagen domains for directing keratinocytes have not been demonstrated. Further, this work aims to address missing gaps in the current understanding the role of alpha3 beta1 integrin in keratinocytes migration. These studies are expected to provide useful guidelines for future development of biomaterials in artificial skin substrates.

1.5. Thesis Organization

The thesis reports the efficacy of artificial ECM proteins in their potential use as skin substitutes by modulating specific integrin-ECM interaction, particularly in the context of wound repair condition. Briefly, Chapter 1 gives a background on the need and criteria of biomaterials for use as skin replacement and defines the hypothesis and objectives of this study.

Chapter 2 provides a detailed literature review of relevant topics on skin structure, wound healing, current strategies in healing cutaneous wound, and a brief overview of scaffold engineering. This chapter gives the basis and understanding leading to the formulation of strategies adopted in this research.

Chapter 3 describes the materials and all the techniques and assays used throughout the course of this research.

Chapter 4 documents the results and discussion obtained from the experiments performed in this study. The first section of this chapter describes the generation and purification of artificial ECM proteins. Subsequently, studies on the effect of integrin-specific artificial ECM proteins on human epidermal keratinocytes adhesion and

proliferation by performing peptide and integrin adhesion blocking assay and BrdU assay respectively, as well as their ability to form colonies through colony forming efficiency assay were examined. Section 4.2 evaluates on how FN910 and LN-5 aECM proteins affect keratinocytes migratory behavior, specifically the role of the integrins involved (alpha5 and alpha3 integrins) in cell migration. The final section of this chapter describes the fabrication and characterization of the aECM protein into intact scaffold, as well as the evaluation of this scaffold in promoting epithelial cells viability and human dermal fibroblast infiltration, to be potentially used as substrates for cell carriers.

Finally, a summary of the studies and recommended future directions are given in Chapter 5.

2. LITERATURE REVIEW

2.1. Basic Skin Structure and Function

2.1.1. The anatomy of skin

Skin or the integument is the largest organ of the human body. Skin constitutes up to 16% of the total body mass and can measure up to 2.2m^2 in an adult of 1.8m height and weighing 90kg [24]. Skin is a dynamic organ in a constant state of change. As it undergoes intricate differentiation process, the cells of the outer layers are continuously shed and replaced by inner cells moving up to the surface, creating a tough and water impermeable barrier. Although structurally consistent throughout the body, the thickness of skin varies between 1.5 - 4.0mm according to anatomical site and age of the individual [24].

Skin comprises of a very complex tissue with several distinct layers and components. There are three structural layers to the skin: the epidermis, the dermis and subcutis. Hair, nails, sebaceous, sweat and apocrine glands are regarded as derivatives of skin (Figure 2.1).

Figure 2.1 Schematic showing various layers in human skin [25]

2.1.1.1. Epidermis

Epidermis forms the outmost barrier, which comprises of densely packed squamous epithelium. The main cells of the epidermis are the keratinocytes, which typically only 70-140µm thick. Keratinocytes proliferate closely to each other; therefore they leave little space for extracellular matrix (ECM) and form a dense barrier against external environment. Epidermis undergoes constant cellular turnover by division and differentiation of keratinocytes in the basal layer, which migrate outwards to replace dead or damaged cells. Keratinocytes synthesize protein keratin that matures in differing stages over four separate layers of the epidermis: stratum basale, stratum spinosum, stratum granulosum, and stratum corneum.

Stratum basale

The innermost layer of epidermis, stratum basale (basal cells), lies adjacent to the dermis. Basal cells remain attached to the basement membrane, a junction layer between epidermis and dermis, by hemidesmosomes. Stratum basale comprises mainly of dividing and non-dividing keratinocytes. As they divide, keratinocytes are pushed from the deeper layer to the surface and differentiated. A small proportion of basal cell layer (5 to 10%) is populated by melanocytes, which produces melanin that gives color to the skin. Melanin pigment gives protection against ultraviolet radiation. Merkel cells are also found in this basal layer, which serve as sensory receptors in association with free nerve endings in skin.

Stratum spinosum

As the basal cells reproduce and mature towards the outer layer, they form intercellular bridges, desmosomes, and connect the cells. In this layer, immunologically active Langerhans cells are mainly present. These cells are involved in the first line of defense against exogenous pathogen invasion by providing antigens [26] and thought to play a significant role in immunoregulation [27, 28]

Stratum granulosum

Continuing the migration from stratum spinosum, keratinocytes became flattened, granular, and expressed keratohyalin. At this layer pro-filaggrin, precursor of filaggrin, is expressed. Filaggrin is known to bind keratin filaments, contributing to the mechanical strength and integrity of the stratum corneum [29]. Filaggrin is also reported to regulate the structural organization of the lipid extracellular matrix of the stratum corneum, without which the skin barrier function is compromised [30, 31].

Stratum corneum

The topmost layer of the epidermis is the stratum corneum, comprises of hexagonal-shaped cornified cells known as corneocytes, at approximately 10 to 25 layers. This non-viable epidermis layer functions as protection barrier for the underlying tissue from dehydration, infection, and mechanical impact. As such, this cell layer contains a dense network of keratin protein (i.e. filaggrin, loricrin, and

involucrin) and embedded in a lipid matrix. Over time, this layer is shed off from the surface, a process termed as desquamation.

Keratinocytes

Keratinocyte is one of the few well-characterized adult stem cells. Skin keratinocytes have been shown to be able to be maintained and propagated in culture. Skin keratinocyte primary cultures are also reported to have the potential to proliferate without entering senescence [32, 33]. The ability of keratinocytes to maintain the stem cell characteristic has been shown *in vitro* by the formation of colonies from single cells grown on lethally irradiated 3T3 cells at a pre-determined density [32].

Barrandon and Green have classified keratinocytes in the epidermis into 3 populations based on their morphology in culture: Holoclones, Meraclones, and Paraclones [34] (Figure 2.2). Holoclones are colonies of progenitor cells that have a self-renewal capacity and exhibit high proliferative potential in culture [35]. Holoclones are characterized by small cells grouped together forming a smooth, typically circular, perimeters. On the other hand, paraclones are characterized with large, flattened TA (transit amplifying) cells with limited proliferative potential. Cells in paraclones are programmed for terminal differentiation. A mixture of both colony types is identified as meraclones.

Figure 2.2 Phase-contrast images on colony types of keratinocytes culture: (A) Holoclones, marked with cells with high growth potential; (B) Meraclones contain a mixture of progenitor cells and differentiated cells (*); (C) Paraclones marked by large and flattened differentiated cells. Cells were cultured on gamma-irradiated 3T3-J2 cells for 12 days, fixed with paraformaldehyde and stained with 1% Rhodamine B. Scale bar – 200μm.

Keratinocytes stem cell markers

Stem cells are defined by their functional characteristics and removing stem cells from their microenvironmental niche potentially alter their properties and irreversibly influence their fate. Some pioneering works on epidermal stem cells area have defined some cell surface markers. Apart from having a highly proliferative characteristic *in vitro*, Jones and Watt demonstrated primary keratinocytes culture had higher colony forming efficiency (CFE) when they express high level of beta1 integrin [36]. Other studies showed that keratinocyte stem cells upregulated α_6 integrin [37, 38]. Similarly, K15 and K19 were also reported to be markers for keratinocytes stem cells (particularly on hair follicle) [39, 40].

Transcription factor p63, a homologue of p53, was first described by F. McKeon and coworkers [41] and has been suggested to be an important regulator of skin keratinocytes function [42]. p63 transcription factor, particularly $\Delta Np63\alpha$, is expressed abundantly in basal cells and decreases with differentiation [42]. It has also been suggested that the overexpression of $\Delta Np63\alpha$ serves to maintain the basal cell phenotype as well as promotes cell survival [43]. Heterogeneous mutation in p63 caused developmental defects on ectodermal structures [44-47].

2.1.1.2. Basement membrane

Basement membrane (basal laminae), the boundary between the epidermis and the underlying dermis, is constructed by deposition of extracellular matrix. The formation of basement membrane is essential for normal tissue development and function [48, 49]. Many biological activities like proliferation, differentiation, growth, and cell migrations are regulated by each component of basement membrane. Basement membranes also control the cellular functions, e.g. regulate cell polarity, adhesions and spreading by modulating the cell's cytoskeleton via the engagement of surface receptors such as integrins [50, 51].

In addition, basement membrane is required to maintain stem cell characteristics in adult tissues, particularly during cell development [52]. In skin, the extracellular matrix on basement membrane provides specific ligands for anchoring and altering keratinocytes behavior via integrins. With the help of strong and stable integrins mediated anchorage (e.g. beta1 for focal adhesion and basement membrane assembly, beta4 for hemidesmosome assembly), keratinocytes stem cells at the basal

layer are inherently polarized and thus undergoes asymmetric division, producing a committed transit amplifying (TA) suprabasal cell and a proliferative progenitor/basal cell [53].

In general, basement membrane contains various isoforms of four protein families: type IV collagen, laminin, nidogen, and perlecan (reviewed in [54, 55]). In this chapter, the description will be focused on type IV collagen and laminin.

Type-IV collagen

Collagen is a major constituent of the extracellular matrix of connective tissue. It is responsible for carrying load in large range of soft tissues and therefore, very important to human physiology. Collagen molecules are linked to one another by covalent bonds, building collagen fibrils. For different functions, there will be different strength required of the tissue and thus, the diameter of collagen fibrils varies. There are as many as 12 types of collagen being reported. Most collagen molecule consists of three amino acids and they are glycine, proline, and hydroxyproline. Glycine is known to improve stability of the collagen structure. The strength of connective tissues is given by the intramolecular crosslinks of collagen and this strength varies with age and pathology. Function and mechanical integrity of organs are determined by the tension in collagen fibers [56].

The nonfibrillar type IV collagen is the major collagen found in basement membranes. Collagen IV is constructed of 6 genetically distinct alpha chain (alpha1-alpha6), but most commonly contains two alpha1 and one alpha2 chains. Each chain contains an N-terminal (7S domain), a C-terminal noncollagenous 1 (NC1) domains,

and a triple helix Gly-Xxx-Yyy repeating domain. Each individual alpha-chain associate to form a triple helix known as protomer that dimerizes via the NC1 domain to form a dimer. Four dimers come together through the interactions between the 7S domains and form into sheet-like mesh found in the BM. The structure of type IV collagen is shown in Figure 2.3 [57].

Many different cells have been shown to bind to collagen IV, including keratinocytes [58], and the interactions are mediated through either integrins or non-integrins receptors. Collagen binding integrins include alpha1beta1, alpha2beta1, alpha3beta1, alpha10beta1, and alpha11beta1. Alphavbeta3 and alphavbeta5 integrins have also been described to bind collagen IV [58]. Numerous triple helical peptide (THP) binding sites have been identified to better understand the role of the collagen binding integrins [59]. Among these sequences, the peptide sequence GEFYFDLRLKGDK found in the alpha1 chain of collagen IV was shown to increase HaCaT keratinocytes adhesion [60, 61].

Figure 2.3 Structure of type IV collagen in basement membrane. Reproduced with permission from [57].

Laminins

Laminins are heterotrimeric glycoproteins composed of three chains, alpha, beta, and gamma, with a molecular weight of 500-900 kDa. These chains can be assembled into at least 16 different isoforms [62, 63] and these isoforms are

distributed during development and in adult tissues. Similar to collagen IV, laminins are involved in important cellular signaling such as adhesion and migration. Laminin isoforms mainly found in skin basement membrane are laminin-111, laminin-332, and laminin-311. Laminin-332 (formerly known as laminin-5, kalinin, epiligrin, nicein, and BM600) plays a major role in skin function [64]. Laminin-5 regulates the stable adhesion of the epithelium to the underlying connective tissue [65] and influences the cellular behavior by interacting with $\alpha 3 \beta 1$ and $\alpha 6 \beta 4$ integrins [65, 66]. Integrin $\alpha 6 \beta 4$ forms the hemidesmosome structure of epithelial cells, mediates adhesion, migration, and wound healing [67]. The structure of Laminin-5 is described in Figure 2.4 below.

The precursor of laminin-5 is processed in keratinocytes. Keratinocytes secrete the laminin-5 precursor, followed by proteolytic conversion of the $\alpha 3$ chain and $\gamma 2$ chain. The laminin $\alpha 3$ chain contains a series of C-terminal globular (LG) domains that consists of five globular modules LG1-LG5. It is reported that the C-terminal LG3 domain is essential for the unique activity of laminin-5.

Figure 2.4 Laminin-5 Structure. Reproduced with permission from [68]

In order to investigate the function of each LG domain in laminin-5 alpha3 chain, recombinant proteins from each LG domain have been developed and tested. LG3 was shown to promote cell attachment and mobility. Within this LG3 domain, the motif PPFLMLLKWSTR was identified as an active site for integrin alpha3 beta1 binding [19]. Cell attachment and spreading were enhanced from this binding through the increased phosphorylation of focal adhesion kinase (FAK) at tyrosine residues 397 and 577 [19].

In terms of the functions in basement membrane, laminins are required for basement membrane assembly. The basement membrane is connected to the dermis underneath by loop structures of collagen VII, the anchoring fibrils, that bind to laminin-5 via the NC1 domains and interwoven with the fabric of collagen I and III fibrils. Together these adhesion complexes provide a structural and functional integrity of skin. It has been shown in *Drosophila* that the absence of laminin leads to the complete absence of basement membrane, resulting in a disorganized ECM [69]. Further, most laminin subunit knockout are lethal [49, 70], otherwise they develop severe diseases. Mutations in laminin-5 result in junctional epidermolysis bullosa, a severe skin blistering disease [71].

Nidogen

Nidogens are homologous glycoproteins widely distributed in basement membranes. Nidogen-1 and nidogen-2, the predominant nidogen isoforms, originate from fibroblasts [54]. The main role of nidogen is reportedly as a key bridge between laminins and type IV collagen, thus provide stability to the basement membranes [72, 73]. Studies have shown that the absence of either one of the two nidogen isoforms or

nidogen binding site in laminin does not affect development of basement membrane [72, 74, 75]. However, the absence of both nidogens severely impaired basement membrane deposition and structural assembly, although the amount of other basement membrane components remained unchanged [54].

Perlecan

Perlecan is a heparan sulfate proteoglycan in basement membrane that has the potential to bind laminins and type IV collagen. Different from nidogen, perlecan is made by both keratinocytes and fibroblasts. Perlecan does not appear to have a principal role in the basement membrane assembly, but rather similar to nidogen it provides stabilizing function. However, perlecan deficiency is found to be lethal for mouse embryos at the midgestational stage [76, 77].

2.1.1.3. Dermis

Dermis, the second layer of the skin, is mainly formed by dense fibroelastic connective tissue layer consists of collagen, elastin, and ground substances (GAGs, proteoglycans, and glycoproteins) [78]. Dermis constitutes the bulk of the skin and plays a major role in protecting the body from mechanical impact. It contributes to 15-20% of the total body weight. Dermis comprises of two layers: papillary layer and reticular layer.

Papillary layer is a thinner outermost part of the dermis, located right underneath the basement membrane. This layer composed of small and loosely

distributed elastin and collagen fibrils, predominantly collagen type III. At this layer lies the capillary network, which provides the epidermis with nutrients and support waste removal, and also the sensory nerve endings. On the other hand, the reticular layer makes up the largest part of the dermis. This layer has dense connective tissue comprising collagen fiber components (predominantly type I) with random bundles of elastin interspersed in between. Studies have shown that the collagen fiber bundles form an irregular network that is almost parallel to the epidermal surface [79]. The lower part of the reticular layer is in contact with the subcutis.

Dermal matrix component

Type I and Type III Collagen

The major components of dermal matrix are collagen fibers (type I and III). Collagen fibers account for 75% of the fat-free dry weight and 18-30% of the volume of the dermis [80]. The collagen fibers are poorly extensible, but are extremely tough and resistant to tension parallel to the fibers. Collagen is characterized by high tensile strength (1.5-3.5 MPa), high stiffness ($E = 0.1 \text{ GPa to } 1 \text{ GPa}$), and low extensibility ($\sim 5\text{-}6\%$). Collagen fiber molecules are produced by fibroblasts in the endoplasmic reticulum [81].

Elastin

The second main component of the dermis is elastin fibers, comprises about 4% of the fat-free dry weight of the dermis [82]. As the name implies, elastin is extremely elastic and have a reversible strains of more than 100%. Elastin is essential for various human tissues such as lung, skin, ligament, blood vessel, etc. It has a half-life about 74 years, making it the longest lasting protein in the body. The Young's modulus of elastin is reported to be in the order of 0.3-1MPa [83]. In dermis, the thickness of the elastic fibers varies: the closer it is to the papillary layer, the thinner it is and the more perpendicular it is to the skin surface [84].

Elastin is extremely insoluble due to its extensive crosslinking at Lysine residues. Elastin is synthesized as tropoelastin precursors, consisting of alternating hydrophobic and hydrophilic domains. The hydrophobic domain is rich in non-polar amino acids, specifically Gly, Val, Pro, and Ala. This domain typically occurs in repeats of three to six peptides. On the other hand, the hydrophilic domains are rich in Lys and Ala, which is typically used for crosslinking. Tropoelastin is secreted in human as approximately 72 kDa protein by various cells, including fibroblasts and endothelial cells [85-89]. Different from elastin, the human precursor tropoelastin contains an unusual highly hydrophilic domain, which is enriched in Ser residues, numerous charged residues (Glu, Asp, Arg), and the only His on tropoelastin. Human tropoelastin is soluble in cold aqueous solution and became insoluble upon raising the temperature.

Elastin-like polypeptides

Elastin like polypeptides (ELPs) are repetitive peptide sequences derived from hydrophobic domain of mammalian tropoelastin. Urry and co-workers have studied extensively that the physical properties of ELPs are highly dependent on the amino acid composition of the peptide sequence [90]. The most commonly studied motif is the pentapeptide motif (VPGXG)_m, where X is known as guest amino acid other than proline. The subscripted m describes the number of repeats which typically ranging from 20 to 330 repeats [91].

Silimar to human tropoelastin, ELPs is their ability to undergo a sharp and reversible phase transition at a specific temperature known as inverse transition temperature (T_t) or lower critical solution temperature (LCST), both can be used interchangeably [90]. ELPs undertake hydrophilic random coil conformations below their LCST, and are highly soluble in water. Above their LCST, ELPs aggregate rapidly into micron-size particles that are visible to the naked eye [92]. Such phase transitions are reversible, and can be rapidly triggered by temperature shifts. More importantly, the LCST of LEPs can be tailored to suit the application by changing the guest residue X in the (VPGXG)_m [91].

Bioactive ligands from peptide domains can also be fused with ELPs to promote cell-material or cell-cell interactions. Short sequences derived from ECM proteins, such as fibronectin, collagen, laminin, fibrinogen, vitronectin have been investigated quite extensively, although many of them are still not fully characterized. Some of these bioactive domains have been successfully synthesized as fusion proteins [10] and have the ability to support the adhesion and growth of a multitude of cell types [15, 23, 93, 94], while retaining their LCST properties.

Elastin-based fusion proteins are typically synthesized by recombinant genetic engineering approach using a Recursive Directional Ligation (RDL) method [92]. Using temperature responsive behavior, ELP and ELP fusion proteins can be purified easily by Inverse Transition Cycling (ITC). This process consists of steps where protein solutions are centrifuged at temperatures above and below their LCST, as shown in Figure 2.5. Therefore, it is possible to obtain a scalable amount of protein without having to go through tedious and costly purification methods such as chromatography.

Figure 2.5 Illustration of ITC (adopted from Hassouneh W. et al. [95])

Cellular components on dermal matrix

Fibroblasts are the main cell type in the dermis. It appears as thin spindle-shaped cells sparsely distributed in collagen matrix. Dermal fibroblasts are responsible to produce and organize extracellular matrix such as collagen, fibronectin and laminin. Other studies have also shown that fibroblasts are the main source of nidogen [81]. Dermal fibroblast plays a critical role in wound healing. Upon injury, fibroblasts undergo active division and differentiate to myofibroblasts to aid in tissue contraction.

Further, during wound healing fibroblasts release growth factors and cytokines such as keratinocytes growth factors (KGF) by regulating the activity of keratinocytes.

Other than fibroblasts, other cells such as histiocyte, mast cells, and macrophages are also present in the dermis.

2.1.1.4. Subcutis

The hypodermis, also known as the subcutaneous layer, is the innermost layer of the skin. It is made up of a looser connective tissue, which generally transforms into adipose tissue. The adipose cells form a layer varying in thickness, depending on its location in the body, sex, and the state of nutrition. Adipose tissue contributes to thermal insulation, storage of energy, and even acts as a shock absorber. It also permits the rapid uptake of hypodermal injection of drugs such as insulin [96].

2.1.2. Skin Functions

Fundamentally, skin has four basic functions: physical barrier to the environment, thermoregulation, sensation, and metabolic and biochemical functions while providing protection against microorganisms, ultraviolet radiation, toxic agents, and mechanical insults.

As protection, as the first line of defense, skin provides protective barrier against mechanical forces, harsh chemical, as well as thermal and physical injuries. As previously explained, basal cell keratinocytes detached from the basement membrane

and differentiated forming a dense epidermis layer, creating an impermeable, water resistant surface. The melanocytes protecting the skin from UV radiation and protects the cells' nuclei from DNA damage. Further, the Langerhans cells in the epidermis provide protection against the invasion of microorganism.

As thermoregulation, the skin plays an important role in regulating the body temperature through the changes in blood flow. Further, skin appendages like sweat glands help the body lose heat through evaporation of sweat from the surface.

As sensory organ, skin contains a variety of nerve endings to detect stimuli of touch, pressure, heat, cold and pain.

As metabolic and biochemical functions regulator, wastes and metabolites excreted through the glands and skin facilitates the formation of vitamin D and the synthesis of growth factors. Skin also functions as storage for lipids and water, while small amount of oxygen, nitrogen, and carbon dioxide can diffuse into the epidermis.

2.1.3. Mechanical behavior of human skin

Human skin displays a non-homogeneous, anisotropic, non-linear viscoelastic behavior when it is subjected to stress. Its mechanical properties vary from site to site, age dependent, and different for every person. Skin displays creep under constant and stress stress-relaxation under constant strain. Changes in skin viscoelasticity could be caused by dehydration, swelling, ageing, sun exposure, or genetic condition.

The non-linear stress-strain relationship curve of skin is shown in Figure 2.6. The curve can be divided in three stages. The stress-strain relationship in the first

region is linear, demonstrating an elastic behavior. In this stage, effect from collagen fibers can be neglected because elastin is the one responsible for skin stretching [97]. In second region, collagen fibers start to unravel and gradually align towards the applied load direction. Finally, at the third region the stress-strain relationship becomes linear again. On the second and third regions of stress-strain curve, skin exhibits visco-elastic behavior. At this region, all collagen fibers are straightened hence the mechanism of deformation is fiber stretch. Beyond the third region, collagen networks rupture. For a material such as elastin, the stress and strain at the point of rupture correspond to the strength and maximum extensibility of the material, respectively [98].

Figure 2.6 Stress-strain relationship of skin [99]

2.2. Skin injury

2.2.1. Wound Healing

Wound healing is the body first line defense response against injury or damage. Wound healing involves an intricate mechanism with several distinct phases but each phase is overlapping another throughout the process. Generally the phases

include: haemostasis, inflammation, proliferation, and remodeling [100] as shown in Figure 2.7.

Upon wounding, bleeding triggers platelets to secrete inflammatory and clotting factors. Fibrinogen is activated into fibrin and forms a clot (haemostasis) to prevent blood loss [101]. The inflammatory phase set in concurrently with haemostasis. Blood vessels become enlarged (vasodilation) due to the release of histamine and serotonin triggered by platelets [102]. This facilitates the migration of platelet into the wound site from the blood stream and adheres to exposed subendothelial and dermal fibrillar collagen [103, 104]. During inflammation, the neutrophils remove bacteria and cell debris by phagocytosis and produce cytokines to activate the proliferation and migration of keratinocytes and fibroblasts [105]. In addition, macrophages also synthesize collagenases, adhesion proteins, and secrete growth factors to promote the formation of granulation tissue [106-109]. At the end of inflammation phase, keratinocytes begin to migrate from the edge to the wound bed to cover the new tissue, a process known as re-epithelialization, followed by fibroblasts migration into the wound area to form the granulation tissue.

Re-epithelialization is a critical process as it re-establishes the important function of skin as a barrier against the external environment. Within 24 h of injury, keratinocytes at the wound edges begin to migrate with the help of the provisional matrix, which is mainly composed of fibrin and fibronectin [110]. Keratinocytes migration is induced by several chemoattractants, including fibronectin, laminin, and platelet derived growth factor (PDGF) [111, 112], while keratinocytes proliferation is stimulated by keratinocytes growth factor (KGF) secreted by fibroblasts [113].

The proliferative phase is characterized by angiogenesis and fibroplasias. Fibroblasts migration and proliferation are mainly stimulated by PDGF and basic Fibroblast Growth Factor (bFGF) released by platelets and macrophages, while PDGF and Transforming Growth Factor- β (TGF- β) induces the fibroblasts to secrete ECM proteins such as fibronectin, elastin, collagen I and III, hyaluronic acid, and sulfated proteoglycans [114]. Subsequently fibroblasts differentiate into myofibroblasts that exert contractile forces to minimize the wound size. Meanwhile, endothelial cells form new capillaries and lymphatic vessels into the wound by the stimulation of bFGF, Vascular Endothelial Growth Factor (VEGF) and Tumour Necrosis Factor- α (TNF- α) [115]. Collagen deposition continues and epithelial thickening takes place until it bridges the wound.

The final stage of wound healing process is maturation or remodeling phase. This phase involves remodeling deposited collagen by matrix metalloproteinases produced by fibroblast and microphages. The remodeling process continues progressively for months and even years, with the gradual increase in tensile strength of the new tissue formed [116].

Figure 2.7 Various phases in wound healing [117]

Fibronectin

As mentioned earlier, fibronectin plays an essential role in wound healing process, mainly in promoting cellular adhesion, migration, and mediating cell growth [101, 118-120]. Fibronectin is a large glycoprotein that is found in all kinds of tissues. Fibronectin is recognized in two forms: plasma fibronectin and tissue fibronectin. Plasma fibronectin is synthesized by hepatocytes into the blood plasma in soluble form [119, 121], while tissue fibronectin is secreted by cells like fibroblasts, keratinocytes, and endothelial cells [122]. Plasma fibronectin is required in the early phase of wound healing to bind to the platelets and fibrin. On the other hand, cellular fibronectin is important for granulation tissue formation.

FN interacts with cell through integrins, cell receptor that function as bridge between intracellular and extracellular matrix environment. Fibronectin gene is composed of three types homologous repeating units: type I, type II, and type III [123]. Only type I and II fibronectin are stabilized with intra-chain disulfide, making the type III module partially unfold under applied force.

The most widely recognized site for cell adhesion is found in type III modules of fibronectin, Arg-Gly-Asp (RGD) sequence located on 10th domain. RGD is recognized as the binding site for alpha5 beta1 integrin and known to promote cell adhesion, while Pro-His-Ser-Arg-Asn (PHSRN) sequence derived from 9th domain has been reported to enable stable binding of RGD domain to alpha5 beta1 integrin [124]. PHSRN and RGD sequences located in the 9th and 10th domains of fibronectin synergistically have shown to increase cell-matrix interactions using the alpha5beta1 integrin [15, 124-127]. During wound healing, keratinocytes are shown to upregulate

the fibronectin binding integrins [128]. Ongenae and coworkers demonstrated that the level of alpha5 beta1 integrin were upregulated, especially on acute wounds [129].

Figure 2.8 Modular structure of fibronectin and its binding domain [130]

2.2.2. Classification of wounds

Based on the depth of the injuries, wounds can be classified into three categories: epidermal, partial thickness, and full thickness wound. Depending on the each category, treatment approach differs accordingly [131]. Epidermal injuries such as sunburn and light scalds usually do not require specific surgical treatment. As only epidermis is affected, epidermal wound typically heals without scarring.

The partial thickness wound, on the other hand, affects both the epidermis and also part of the dermis. When only superficial parts of the dermis are affected (usually accompanied by epidermal blistering and severe pain), wounds heal by epithelialization from migrating basal keratinocytes [131, 132]. When the injuries

involve greater dermal damage, skin appendages are usually affected and wounds take longer to heal. In such wound, scarring is more pronounced.

Treatment for partial thickness wound is typically by using a wound dressing. Wound dressing is defined as a physical material that covers and protects a wound temporary such that physiological healing could take place undisturbed. The use of wound dressings can be traced back to 2200 BC [133] and a variety of wound dressings currently available have been reviewed extensively in the literature [134, 135]. Wound dressing usually does not participate in wound healing process and is not integrated into the new tissue.

Full thickness wound, e.g. in the case of major burn injuries, is characterized by complete destruction of the epithelial-regenerative elements. Papini reported that full thickness wound with diameter bigger than 1 cm cannot epithelialize normally and thus require skin grafting [131]. Wound size plays a major role in the outcome of the injury. Up to half a century ago, the mortality rate of patients with 60 percent burns of the total body surface area (TBSA) between the age group of 15 to 44 years was 100 percent [136], and up to 2003 the mortality rate of the same age group with 60 percent TBSA was reduced to 41.4 percent [137]. Although current advances in burns treatment technique have helped in successful treatment on burns patients, treatment of deep extensive burns remains a substantial challenge to the surgeon.

2.2.3. Strategies in skin reconstruction

When healthy skin is injured, a repair process is naturally initiated to achieve wound closure in a shortest possible time. However, in large wounds, spontaneous healing may not be possible and thus required skin grafting. The next section of this chapter will review some of current strategies to heal cutaneous wound, together with their potentials and shortcomings, which have been developed in the past few decades.

2.2.3.1. Skin grafting

Autograft

Full-thickness wound can be treated by a skin grafting. Skin grafting is divided based on its thickness. A split-thickness skin grafting is done by harvesting the epidermis with a superficial part of the dermis from an undamaged skin of the patient and applied the graft to the wound site, while a full-thickness graft includes the entire epidermis and dermis layers [138]. Ever since it was used in 1941, split-thickness autologous skin grafting has been the clinical gold standard in full-thickness wound treatment due to its immunocompatibility and superior clinical results [139]. However, in the case of more extensive injury where the donor sites are extremely limited, only delayed serial autologous split skin grafting can be used. In such case, wounds are left unhealed over the course of treatment while waiting for epithelial regeneration, within which time frame wounds are prone to severe complications and can result in death. In addition, autograft creates a new wound and thus adds pain and stress to the patient.

Allograft

Cadaveric allograft skins obtained from deceased donors. These are commonly used today as temporary dressings until permanent skin grafts are available. Cadaveric skin graft can only be used during the first few weeks post-injury, where the immune response of the patients is pathologically suppressed. Once the wounds are vascularized, the grafts are rejected triggered by the patient's immune system. Although allograft has been used for decades, its availability in skin banks, denial of application due to religious ground, and risk of disease transmission still need to be addressed [140].

Xenograft

Xenogeneic graft, a graft taken from another species, offers another possibility to overcome the limited supply of the cadaveric allograft. Porcine skin is currently used as temporary dressings, however the immunological issues and risks of pathogen transfer make this xenograft a less suitable candidate for healing full-thickness wound.

2.2.3.2. Tissue-engineered skin substitutes

Because of the acute shortage in skin grafts and demand for an alternative for skin-replacement, there is a long history of material development to create biomaterials for skin substitutes. Some of the clinically available tissue engineered skin substitutes can be classified based on the layers of the skin it regenerates, namely epidermal, dermal, and bilayered skin substitutes.

Epidermal skin substitutes

Cultivating human epidermal keratinocytes *in vitro* has been made possible through a serial culture by Rheinwald and Green [32]. This technology enables the isolation of keratinocytes from skin biopsy to form a continuous epidermal sheet that can be used for epidermal transplant or cultured epithelial autograft [141]. The clinical integration of this cultured epithelial autografts (CEA) has been shown to vary significantly [142-144]. This could be attributed to the fact that CEA contains variable number of terminally differentiated keratinocytes and thus affects the integrin expression that is used to attach to the underlying matrix [145]. Further, the disadvantages of the CEA sheets are their fragility, long culture time, and complicated handling during applications. The first commercial CEA product manufactured was Epicel® [146].

In order to improve handling difficulty of the CEA sheets, support layer from silicone (MySkin® [145], hyaluronic acid (Laserskin® [147]), and fibrin (Bioseed-S®[148], CellSpray®[149]) were formulated. Fibrin is formed when fibrinogen is crosslinked by thrombin to form a hemostatic plug or blood clot. The use of fibrin as skin graft does not improve graft ‘take’ but helps in handling, cell attachment, haemostasis and wound healing [14]. On the other hand, hyaluronic acid has been shown to promote cell proliferation, migration, and differentiation [150]. However, the efficiency of these products and their long-term outcomes are still questioned by many. Further, hyaluronic acid requires laser-microperforation for the keratinocytes to migrate down to the wound bed [147], while fibrin gel does not possess mechanical integrity

Dermal skin substitutes

The majority of dermal substitutes product are acellular. The source for decellularized matrix could be allogeneic (AlloDerm[®][151]) or xenogeneic (Matriderm[®][152], Integra[®][153]). Decellularized matrix from allogeneic source readily incorporates into the wound and does not trigger immunogenic response from the host. However, there are ethical and safety issues since these grafts are derived from human. Risks associated with transmissible human diseases such as HIV and HepB are therefore a concern. For this reason, xenogeneic source became a better option for decellularized matrix. Xenogenic sources are readily available, increasing the ease of production and lower cost. However, batch-to-batch variation remains a major drawback in using such skin grafts.

Polymer-based dermal matrix has also been explored. One of the examples includes a cryopreserved polyglactin mesh seeded with cultured neonatal fibroblasts taken from newborn foreskin (Dermagraft[®]). The matrix is recognized as Vicryl[®] mesh that is used clinically as internal haemostatic dressing and licensed and used to treat chronic diabetic foot and venous ulcers [154]. This material has also been reported to be used for burns treatment although the efficacy was not proven on clinical trials [155]. However, the disadvantages of using this graft include a necessity for repeated applications and high cost [14].

Bilayered skin substitutes

In attempt to offer an alternative to the needs of full thickness skin replacement, bilayered skin substitutes were also explored. Bilayered skin substitutes

aim to mimic the structure of normal skin with both epidermal and dermal layers present. Most of these bilayered skin substitutes products are developed by using allogeneic skin cells incorporated into a dermal matrix. Nonetheless, up to today, these bilayered construct only functions as temporary coverage [139] due to the immunogenic intolerance of the host to allogeneic cells.

Apligraf[®] is one of the bilayered allografts developed that is available commercially and been a financial success. It composes of viable allogeneic neonatal fibroblast grown in bovine type I collagen gel matrix, covered by a top layer of allogeneic neonatal keratinocytes. However, Apligraf[®] can only be used as temporary dressing due to the low viability of the allogeneic cells. Typically, allogeneic cells grown on Apligraf[®] do not survive after two months *in vivo* due to immunogenic rejection [156]. Moreover, the shelf life of Apligraf[®] is only 5 days, requires delicate handling, high cost, and there are potential risks of transmissible disease from its allogeneic source.

Other than Apligraf[®], polymer based bilayered skin substitutes have also been developed. PolyActive[®] is a polymer-based skin graft which consists of a combination of soft polyethylene oxide terephthalate and hard polybutylene terephthalate [157]. Autologous keratinocytes and fibroblasts are seeded into this matrix to prevent potential cross-contamination of infective agents or immune rejection. However, the use of autologous cells limit the product's off-the shelf availability and thus increase its costs. Further, the non-biodegradable, synthetic nature of the matrix makes it not suitable for use as a permanent skin substitutes.

There are many reports on the development of bilayered skin substitutes either from allogeneic or xenogeneic decellularized dermis, however most of these works are

only used for *in vitro* studies rather than for clinical use [158]. In addition, currently available bilayered skin substitutes are still far from ideal. One of the reasons is due to their reliance on two cell types, keratinocytes and fibroblasts, which limits the skin substitutes from performing all the skin functions.

Table 2.1 Commercially available tissue engineered skin substitutes

Brand name	Company	Type	Cell source	Scaffold material	Ref
Epicel [®]	Genzyme Biosurgery, Cambridge, MA, USA	Epidermal	Autologous keratinocytes (<i>in vitro</i>)	-	[159]
MySkin [®]	CellTran Ltd, Sheffield, UK	Epidermal	Autologous keratinocytes (<i>in vitro</i>)	Surface modified silicone layer	[160]
Laserskin [®] / Vivoderm [®]	Fidia Advanced Biopolymers, Padua, Italy	Epidermal	Autologous keratinocytes (<i>in vitro</i>)	Recombinant HA with laser perforations	[161]
BioSeed-S [®]	BioTissue Technologies GmbH, Freiburg, Germany	Epidermal	Autologous keratinocytes (subconfluent cell suspension)	Allogeneic fibrin sealant suspension	[162]
CellSpray [®]	Clinical Cell Culture (C3), Perth, Australia	Epidermal	Autologous keratinocytes (subconfluent cell suspension)	-	[163]
Alloderm [®]	LifeCell Corporation, Branchburg, NJ,	Dermal	Acellular	Human acellular lyophilized	[164]

	USA			dermis	
Matriderm®	Dr Suwelack Skin and HealthCare AG, Billerbeck, Germany	Dermal	Acellular	Bovine non- crosslinked lyophilized dermis, coated with alpha-elastin hydrolysate	[165]
Integra®	Integra NeuroSciences, Plainsboro, NJ, USA	Dermal	Acellular	Silicone, bovine lyophilized crosslinked collagen sponge from heat- denatured collagen	[166]
Dermagraft®/ TransCyte®	Advanced BioHealing, Inc., New York, NY and La Jolla, CA, USA	Dermal	Allogeneic neonatal fibroblasts	PGA/PLA, ECM	[167]
Aligraft®	Organogenesis Inc., Canton, Massachusetts, CA, USA	Bilayered	Allogeneic keratinocytes and fibroblasts	Bovine collagen	[168]
PolyActive®	HC Implants BV, Leiden, The Netherlands	Bilayered	Autologous keratinocytes and fibroblasts	Polyethylene oxide terephthalate/ Polybutylene terephthalate	[169]

Other products for skin substitutes that are currently under investigation are reviewed in [14].

2.2.4. Natural and Synthetic materials for skin substitutes

As briefly reviewed in the previous section, both natural and synthetic materials have been researched for use as skin substitutes. Dermal and epidermal matrix developed from natural materials such as collagen (Apligraf[®], Biobrane[®], Dermagraft[®], Integra[®]), hyaluronic acid (TissueTech[®], Laserskin[®]), fibrin (BioSeed-S[®], CellSpray[®]), keratin [170], silk fibroin/alginate blend [171] and chitosan [172]; as well as synthetic materials such as PLGA [172] and PCL [173] have been explored.

Natural materials are generally attractive in the field of tissue engineering since they possess the biological cues required for modulating cellular behavior, a characteristic that is not offered by synthetic materials. However, the use of naturally derived materials has several drawbacks, such as: poor mechanical properties, difficult to process, have large batch-to-batch variations, limited source, and possibility of pathogens transfer.

Synthetic materials on the other hand, provide tunable mechanical properties to withstand cellular contraction forces and allow easy handling. Further, it minimizes risk of pathogen transfer and is easy to be processed through various processing techniques. However, as synthetic materials do not possess natural bio-inductive abilities, it usually requires surface modification to improve cell-materials interaction.

With the advancement in recombinant DNA technologies, synthetically produced ECM proteins having active domains to modulate cellular behavior and tailor-able mechanical properties. Many recombinantly produced peptide sequences encoding cell-binding domain (e.g. PPFLMLLKGSTR, GEFYFDLRLKGDK, and RGD) have been used to increase cell-matrix interaction of keratinocytes [60, 174].

One study reported the use of recombinant ELP containing RGD domain mixed with Collagen type I to recreate a full thickness scaffold for oral mucosa equivalents [23]. However, up to date, there are no reports on fully recombinant matrix for tissue engineered skin substitutes.

2.3. Scaffold fabrication

Scaffold fabrication is an important aspect for tissue engineering as it influences cellular behavior by providing the appropriate topographical cues [175]. Therefore, in attempts to engineered tissues, the scaffold properties need to be carefully controlled to match the intended application. For most tissue engineering applications, a three-dimensional porous structure is preferred as porous structure can facilitate better nutrients / waste and oxygen transport, which is critical for the survival of the tissue at the implant site.

Various techniques have been developed and used to developed porous scaffolds. Methods such as freeze-drying, solvent casting, particulate leaching, gas foaming, fiber meshes, electrospinning, phase separation, and solution casting have been developed. In this work, freeze-drying and electrospinning method were explored to create a freestanding artificial ECM scaffold.

2.3.1. Freeze-drying

Freeze-drying is a technique for phase separation to remove the solvent and leave behind a polymer foam structure. The process consists of three main stages:

freezing an aqueous solution, primary and secondary drying. When an aqueous polymer solution is frozen, ice crystals will be formed by the water molecules form and pushed the solute molecules to the boundaries of the ice crystals. Interconnected network of cells formed by the displaced polymer is left behind when the ice crystals are removed.

Drying is usually carried out under controlled temperature and vacuum pressure in a freeze-dryer. Sublimation of ice crystals at low temperatures and pressures below the triple point of water occurs during primary drying. This ensures that capillary forces from liquid water do not close up the pores after the ice is removed. Secondary drying removes water that adsorbs to the polymer walls. Primary drying usually takes up the bulk of the drying time. Complete drying can take between 24 to 72 hours. Freeze-drying has been used to image the morphology of crosslinked elastin gels. It has also been widely used to generate porous scaffolds in a wide variety of natural and synthetic materials.

2.3.2. Electrospinning

Electrospinning is a cost effective and scalable method for fabricating fibrous scaffold for biomedical applications [176]. Typically, a polymer solution is dispensed through a needle connected to a high voltage and placed a distance apart from a grounded collecting plate. The droplet dispensed from the needle end is stretched electrostatically due to high voltage applied, forming a Taylor cone. As a result, the polymer solution is drawn as a continuous fiber towards the collecting plate while the

solvent is evaporated during the process [177]. The fibers can be collected as a random, non-woven mat or an aligned scaffold depending on the type of the collector.

To increase biocompatibility of the fiber material used for biomedical purpose, bioactive molecules have been incorporated during the electrospinning process, or grafted directly onto polymer fibrous scaffold [60, 178, 179]. Natural, synthetic and recombinantly engineered proteins have also been successfully electrospun [23, 180-183].

The spinability of the polymer solution depends on the solubility, electrostatic repulsion between the molecules and also the conductivity of the solution. Organic solvents such as hexafluoroisopropanol, chloroform, ethanol, and dimethylformamide trifluoroacetic acid are commonly used to increase spinability. Electrospun scaffolds are often crosslinked with glutaraldehyde, formaldehyde, carbodiimides, genipin and methacrylate based photocrosslinker to increase the mechanical integrity of electrospun scaffold [23, 182]. Subsequently, electrospun scaffolds are subjected to extensive rinsing to remove the organic solvents. Despite so, concerns for potential cytotoxicity caused by insufficient removal of the organic solvents are still present.

3. MATERIALS AND METHODS

3.1. Development of artificial ECM proteins and protein purification

3.1.1. Materials

DNA isolation and purification was done using QIAprep Spin Miniprep Kits purchased from Qiagen (Valencia, CA). Genes encoding ELP repeats ((VPGIG)₂VPGKG(VPGIG)₂)₂ was purchased from Genscript (Piscataway, NJ) and DNA oligonucleotides and primers were obtained from 1st BASE (FCBO, Singapore). The plasmid containing the genes encoding (ELK)₆FN910(ELK)₆ amino acid sequence was a generous gift from the Tirrell laboratory at Caltech [15]. Restriction enzymes and DNA ladder were purchased from New England Biolabs (Ipswich, MA). The pET22b(+) expression vector was purchased from Novagen (Madison, WI). Enzymatic dephosphorilation of DNA fragments was done using calf intestinal alkaline phosphatase at 37°C for 5min, and DNA ligation were performed for 1.5 h at room temperature using T4 ligase, both were purchased from Roche Applied Science (Indianapolis, IN). *E. coli* XL10-Gold cloning strain was obtained from Agilent Technologies, Inc. (Stratagene, Santa Clara, CA) and *E. coli* BL21 (DE3) pLysS, BL21 (DE3), and BLR (DE3) pLysS strains were from Life Technologies (Carlsbad, CA). Ampicillin (50 µg/mL) and chloramphenicol (34 µg/mL) were purchased from Affymetrix (Santa Clara, CA). All antibodies were obtained from Merck Millipore (Rockland, MA) or Santa Cruz Biotechnology, Inc. (Dallas, TX) unless otherwise stated. Other chemicals were purchased from Bio-Rad (Hercules, CA), Merck Millipore (Rockland, MA) and Sigma Aldrich (St. Louis, MO).

3.1.2. Construction of artificial ECM proteins

DNA manipulations, bacterial growth, and electrophoresis were done using standard molecular biology techniques [184]. The schematic of DNA cloning procedure for all three aECM protein constructs by (Recursive Directional Ligation) is shown in Figure 3.1 below.

Figure 3.1 Artificial ECM protein's DNA cloning procedure by RDL. RE = Restriction Enzymes, ELP = Elastin Like Polypeptides, CBD = Cell Binding Domain.

Briefly, pET22b(+) expression vector is digested with restriction enzymes SalI (RE1) and XhoI (RE2). ELP repeats (sequence shown in APPENDIX) was digested using SalI and XhoI to obtain ELP insert shown as yellow-colored rectangle in Figure 3.1. ELP insert was subsequently ligated into pET22b(+) vector and transformed into *E. coli*. Positive clone is obtained by screening colonies formed from transformation product and verified by re-digesting with SalI and XhoI and examining the size of the

DNA insert. DNA sequencing (1st BASE, Singapore) was done to further confirm the DNA sequence of the positive clone.

The cell binding domains in LN-5 aECM and Col-IV aECM were constructed using DNA annealing. Briefly, complementary pairs of oligonucleotides were dissolved in annealing buffer containing 10 mM Tris, 100 mM NaCl and 100 nM MgCl₂. The mixture was incubated at 95°C for 5 min and cooled to room temperature gradually overnight. Annealed DNA oligonucleotides were digested with SalI and XhoI restriction enzymes, and recovered using 1.2% DNA agarose gel. Subsequently, digested DNA fragments were subcloned into the pET22b(+) vector with ELP repeats obtained previously and transformed in *E. coli* XL10-gold strain using heat shock. Correct clones were screened and verified using DNA sequencing (1st BASE, Singapore). Finally, another ELP repeats were inserted and hence complete the artificial ECM protein DNA constructs.

3.1.3. Protein expression and purification

Protein expression was performed in large-scale fermentation (Applikon 5L Microbial Bioreactor, USA) at 37°C in Terrific Broth (TB) media supplemented with ampicillin and chloramphenicol where necessary. Under the control of T7 promoter, protein expression was induced using 1 mM Isopropyl β -D-1-thiogalactopyranoside (IPTG, Gold biotechnology Inc., St Louis, MO) when the optical densities (OD₆₀₀) of the cultures reached 6 – 8. Cells were subsequently harvested via centrifugation after 4 h at 4°C, 9000 rpm (JOUAN KR25i, AKL500-11) and resuspended in TEN buffer (10

mM Tris-HCl, 1 mM EDTA, 100 mM NaCl, pH 8) at a concentration of 0.5 g/mL and stored at -80°C.

The aECM proteins were purified using thermal cycling as previously reported [185, 186]. Briefly, the frozen cells were thawed at room temperature, supplemented with 10 µg/mL Deoxyribonuclease I (DNase I), 10 µg/mL Ribonuclease A (RNase I), and 50 µg/mL phenylmethylsulfonyl fluoride (PMSF). To complete cell lysis, freeze/thaw process was repeated thrice and sonicated for 20 min on ice using a 2 mm diameter flat tip (Sonics Vibra-Cell VC130 Ultrasonic Processor, 40% amplitude with 5 s pulse). Subsequently, the pH of the cell lysate mixture was adjusted to 9 using 6N NaOH and homogenized at 4°C for 30 min and centrifuged at 9000 rpm (AKL500-11), 4°C for 1 h to pellet the cell debris. The supernatant was collected, NaCl was added to a final concentration of 1 M and warmed to 37°C for 2 h with shaking. The warmed solution was centrifuged at 9000 rpm (AKL500-11) at 37°C, 2 h and the pellet was resuspended in cold distilled H₂O overnight at 4°C (50 mg/mL). The cold and warm purification steps were repeated two to four more times with higher centrifugation speed (39750g, AKL50-22) for better separation. The final protein solution was desalted by dialysis at 4°C, lyophilized and stored at -20°C until further use.

3.1.4. SDS-PAGE and Western blot

The purity of aECM proteins was verified using SDS-PAGE and western blot. Briefly, purified aECM proteins were separated through SDS PAGE gel electrophoresis. Gel was then transferred onto nitrocellulose membranes, blocked in

5% non-fat milk in TBS, and probed with anti-His tag mouse monoclonal primary antibodies (Millipore, Rockland, MA) and subsequently secondary anti-mouse antibodies conjugated with horse-radish peroxidase. Blots were developed using ECL PlusTM chemiluminescence kit (GE Healthcare, NJ) according to manufacturer's instructions and visualized.

3.2. *In vitro* evaluation of cellular interactions with aECM proteins

3.2.1. Materials

EpiLife serum-free keratinocytes medium, EpiLife Defined Growth Supplement (EDGS), 0.05 % Trypsin/0.25 % EDTA, penicillin/streptomycin, Human plasma fibronectin, mouse LN, bovine serum albumin (BSA) and Phosphate Buffered Saline pH = 7.2 were purchased from Life Technologies (Carlsbad, CA). Cell culture flasks and multi-well culture plates were obtained from Corning (NY) and BD Falcon (BD Bioscience), respectively. Collagen type IV was obtained from Sigma Aldrich.

For integrin blocking, anti-alpha2 Ab (P1E6 and P1H5), anti-alpha3 Ab (P1B5) and anti-alpha5 (JBS5) were purchased from Millipore. For immunostaining, Rabbit monoclonal anti-paxillin Ab (clone Y113) was purchased from Millipore, while Alexa Flour 488 goat anti-mouse IgG, and Alexa Fluor 568 Phalloidin were purchased from Invitrogen. Anti-vinculin Ab (clone HVIN-1) was obtained from Sigma Aldrich.

3.2.2. Keratinocytes isolation and cell culture

Primary human epidermal keratinocytes were isolated from skin biopsies of at least two adult skin donors at the Singapore General Hospital (SGH). Cells were cultured according to the methods developed by Rheinwald and Green [32]. Briefly, HEKs were cultured on a feeder layer of lethally irradiated 5 (60 Gy) 3T3-J2 (generous gift from the Green laboratory) in culture 6 medium of Dulbecco's Modified Eagle Medium (DMEM) and Ham's 7 F12 media (3:1 mixture) containing 10% FBS, 5 mg/mL insulin, 0.18×10^{-3} M adenine, 0.4 mg/mL hydrocortisone, 0.1×10^{-9} M cholera toxin, 2×10^{-9} mM triiodothyronine, 10 ng/mL epidermal growth factor and 100 IU–100 mg/mL penicillin–streptomycin. Subsequently, primary HEKs were cultured in serum-free keratinocytes media EpiLife, supplemented with Defined Growth Supplement (EDGS) and 1% penicillin/streptomycin at cell passages 3–5 to prevent any interactions of the serum with the investigated ECMs.

3.2.3. Cell adhesion, competitive peptide, and integrin inhibition assays

The aECM proteins (1 mg/mL) and native proteins FN, LN, and Col-IV (10 μ g/mL) [187] were adsorbed on standard multi-well tissue culture plates overnight at 4°C. Subsequently, wells containing adsorbed proteins were rinsed twice with PBS, blocked with 1% heat-denatured BSA for 30 min at RT and washed twice with PBS. Primary HEKs were seeded at a density of 1×10^4 cells/cm² and incubated at 37°C, 95% air/5% CO₂. At regular time intervals, images at 5 random spots were acquired for each samples using phase contrast microscopy (Olympus IX51). The individual

cell areas were determined by tracing the contours of each cell manually using ImageJ. Percent attached cells was determined as the number of cells with cell areas greater than the average cell area on BSA control divided by the total number of cells measured. At least 50 cells were analyzed for each time point for each sample.

For competitive peptide and integrin inhibition assays, cells were pre-incubated with soluble peptides (New England Peptides, MA) or monoclonal antibodies to the integrin receptors $\alpha 5\beta 1$ (JBS5), $\alpha 3\beta 1$ (P1B5), and $\alpha 2\beta 1$ (P1E6, P1H5) for 30 min at 37°C prior to seeding. At the same time, Calcein AM (2 μ M) was also added to the media to aid cell counting. After 2 h, wells were rinsed once with pre-warmed PBS and imaged using a fluorescence microscope (Olympus IX51). Cells were counted and reported as a percentage of the total number of cells attached in the control well.

3.2.4. BrdU labeling

The number of proliferated cells was determined using 5-bromo-2'-deoxyuridine staining (BrdU Cell Proliferation Assay Kit, Roche). The assay was performed using the manufacturer's instructions. Briefly, $1 \times 10^4/\text{cm}^2$ cells were seeded on chamber slides (ibidi®, Germany) pre-coated with various aECM and native proteins. Cells were left to attach for 24 h and subsequently incubated with BrdU labeling solution (10 μ M final concentration) for 24 h at 37°C. Cells were washed three times, fixed with ethanol and probed with BrdU antibody conjugated to FITC. Cell nuclei were co-stained with DAPI (1:5000, Molecular Probes, Invitrogen). The

percent of BrdU-positive cells was determined by counting the number of BrdU-labeled cells divided by the total number of DAPI-labeled cells.

3.2.5. Immunofluorescence staining

For p63 transcription factor staining, primary HEKs were grown to 70% confluency on chamber slides pre-adsorbed with the aECM and native proteins. Cells were washed with once with prewarmed PBS, fixed in 4% paraformaldehyde for 15 min at RT and subsequently permeabilized with 0.1% Triton X-100. Samples were blocked with 10% normal goat serum in PBS for 30 min RT. Primary antibodies targeting the p63 transcription factor (4A4, Santa Cruz, 1:100) were added to each well. Samples were washed three times with PBS and probed with FITC-conjugated secondary antibodies (1:100) and DAPI (1:5000, Molecular Probes, Invitrogen). Chamber slides were mounted on glass coverslips and visualized with Nikon Eclipse TE2000 laser scanning confocal microscope. Images at 4 random positions were acquired for each sample using the same parameters (gain/exposure time adjustment).

To determine the relative expression levels of p63 of each sample, images were converted to binary images by thresholding to 50% of maximum intensity and the mean gray value of each cell was determined using ImageJ. The number of cells with mean gray values larger than the average mean gray value of cells on glass control was counted, and reported as a percentage of the total number of cells in each sample.

Immunofluorescence staining for paxillin was performed with rabbit monoclonal antibody against paxillin and Alexa Fluor 488 goat anti-mouse IgG. Staining for F-actin was performed with Alexa Fluor 568 Phalloidin.

3.2.6. Reverse Transcript PCR (RT-PCR)

Pre-confluent (70%) HEKs were trypsinized from culture dishes pre-coated with aECM and native proteins and the total RNA was extracted with RNA extraction kit (RNeasy Mini Kit, Qiagen, Valencia, CA). RNA collected was quantified by spectrophotometer and cDNA was generated from 2µg total RNA using QuantiTect Reverse Transcription Kit (Qiagen). The samples were subjected to PCR for 30 cycles at annealing temperature of 55°C, elongation temperature of 72°C, and DNA strand dissociation temperature of 94°C. The PCR primers used are found in Table 3.1. The PCR products were ran on 1.2% agarose gel, and the molecular weights of the products were compared to that of their respective cDNA bands.

Table 3.1 List of PCR primers used for RT-PCR assay

Primers	Sequence
ΔNp63alpha	Forward: 5'-GGAAAACAATGCCCAGACTC-3' Reverse: 5'-ATGATGAACAGCCCAACCTC-3'
alpha2	Forward: 5'-TGGTCTCATCAATCTCATCT-3' Reverse: 5'-TGACATCAGTTGTAATGCAG-3'
alpha3	Forward: 5'-TCCATGAACTACTCTTTACCTTTGCGGATGC-3' Reverse: 5'-TGCCAGACTCACCCATCACTGTC-3'
alpha5	Forward: 5'-CATTTCCGAGTCTGGGCCAA-3' Reverse: 5'-TGGAGGCTTGAGCTGAGCTT-3'
GAPDH	Forward: 5'-GAAGGTGAAGGTCGGAGTC-3' Reverse: 5'-GAAGATGGTGATGGGATTTC-3'

3.2.7. Colony forming efficiency (CFE) assay

Pre-confluent (70%) cultures of HEKs were trypsinised from culture dishes pre-coated with aECM and native proteins and subsequently counted with haemocytometer. 200 cells were inoculated into 100 mm petri dishes pre-seeded with gamma-irradiated 3T3-J2 cells. Cells were cultured for 12 days and subsequently fixed with formalin and stained with 1% rhodamine B. The percentage of colonies formed was counted as the total number of colonies grown divided by 200 while the percentage of non-aborted (growing) colonies was calculated by dividing the number of non-aborted colonies by the total number of colonies formed.

3.2.8. ‘Wounding’ and single-cell migration assays

For ‘wounding’ assay, thin blocks of PDMS (sterilized) were placed onto multi-well culture plates that have been pre-coated with proteins and air-dried. Primary HEKs were subsequently seeded into each well in EpiLife supplemented with EDGS. Prior imaging, the PDMS blocks were removed and cells were washed with EpiLife media to remove cell debris. Subsequently, the ‘wound’ was re-coated with respective proteins that were to be tested (or heat-inactivated BSA as control) for 30 min at 37°C. Several images on the wound edge area were acquired every 30 min for 48 h and analyzed using ImageJ. The wound area was traced manually at various time points. Wound closure is represented as the ratio of the wound area after migration at time = t , over the wound area at $t = 0$. Values shown represent the means $\pm$ s.e.m. of five independent experiments. For individual cell tracking, cells at the edge of the

wound sheet were tracked for 10 h from the start of wounding. An average of 50 cells was tracked for each protein surface for each independent experiment.

To analyze single-cell migration, 5×10^3 cells/cm² primary HEKs were seeded on tissue culture plates coated with aECM or native proteins in EpiLife with supplement. Phase contrast images were captured every 15 min intervals at 37°C, 95% air/5%CO₂ using a Nikon TE2000 inverted microscope mounted in a thermally controlled chamber over a period of 15 h. Cells were tracked manually using imageJ plugin, MTrackJ (<http://www.bigr.nl/>). For integrin-function blocking experiment, anti-integrin antibodies (anti alpha3, P1B5, 2 µg/mL, or anti-alpha5, JBS5, 2 µg/mL) were added prior imaging. Cell migration speeds and cell trajectories of single-cell migration were computed using the tracking data from at least 50 cells per experiment and the graphs represent the averages $\pm$ s.e.m. from five independent experiments. Cell speed is determined by the total distance travelled by the cells divided by time. Cells were considered as polarized when they extend a single stable lamellipodia, forming a fan-shaped morphology, for at least 2 h after attachment.

3.3. Artificial ECM protein as biomaterials for skin substitutes

3.3.1. Artificial ECM protein scaffold fabrication

3.3.1.1. Freeze-dried scaffold

Protein solutions were prepared by dissolving 10 wt% of artificial ECM proteins in PBS at 4°C. Protein solutions were then mixed with crosslinker bis(sulfosuccinimidyl) suberate (BS3) at a 1:3 stoichiometric ratio of activated lysines

to ester. The mixture was pipetted between two flat glass plates with a thickness of 1 mm and allowed to crosslink for 2h at RT. Subsequently, crosslinked scaffolds were freeze dried to create porous structure and stored at -80°C until further use. Prior mechanical testing and cell culture, freeze dried scaffolds were washed in ddH₂O at 4°C overnight to remove the excess crosslinker.

3.3.1.2. Electrospun scaffolds

Protein solutions were prepared as described previously except lyophilized aECM proteins were dissolved in 1,1,1,2,2,2-hexafluoro-2-propanol (HFIP) at 4°C. The solution was dispensed through a 21½G blunt-ended needle at a rate of 2.5 mL/h. The solution was charged by applying a voltage of 20 kV. Fibers were collected on a flat grounded aluminum foil placed 12cm apart from the needle end. Collected fibers were chemically crosslinked to stabilize their structures with glutaraldehyde (GA) vapor. Briefly, scaffolds were placed together with a separate 25% (v/v) aqueous GA solution in an open stage desiccator for 24 h at ambient temperature. Unreacted GA in the scaffolds was neutralized by immersing them into 0.2 M glycine solution overnight. Electrospun scaffolds were then rinsed extensively in ddH₂O before further use.

3.3.2. Artificial ECM scaffold characterization

3.3.2.1. Scaffold thickness measurement

Thickness of both freeze-dried and electrospun aECM scaffolds were measured using a standard micrometer with a sensitivity of 0.1 μm .

3.3.2.2. Morphology and microstructure visualization

The scanning electron microscope (JEOL) was used to visualize the scaffold morphology and microstructure. Samples were mounted on SEM stubs using carbon tape and gold sputtered for 60s at 18 mA before viewing under SEM at 10 kV.

3.3.2.3. Porosity and pore diameter quantification

Scaffold porosity was quantified from the scaffold's cross-section images. Briefly, scaffolds were cut to size and infiltrated in tissue embedding medium (Tissue-Tek® O.C.T™ Compound, Sakura, Netherlands), overnight at 4°C. Subsequently, samples were snap-frozen in tissue embedding medium using liquid nitrogen and sectioned on a cryo-microtome (Leica CM1950 Cryostat, Leica Microsystems). 7 μm thickness sections were collected on glass slides, air dried, and stored at -20°C prior to use.

Cryo-sections obtained from 3 different samples were imaged using an inverted microscope (Olympus IX51) with a total of 5 images per sample. Section

images were converted to binary format with imageJ. Percent porosity is defined by the percentage of pore area relative to the total scaffold section surface area.

Pore diameter is quantified by measuring the longest axes of each pore in the section image. A total of 50 pores within an image and a total of 3 images per sample (n=3) were quantified using imageJ.

3.3.2.4. Mechanical properties of aECM scaffolds

3.3.2.4.1. Tensile testing

Films were cut to 5 mm x 24 mm size with gauge length of 18mm. Tensile testing was performed with Instron Micro Tester 5848 with a 50N load cell at a constant strain rate of 10% of gauge length per minute (0.00167%/s) until failure (n=5). Prior testing, scaffold was swollen in water to equilibrium and subsequently the excess water was patted dry after mounting prior to the tensile test. Experiment was performed at room temperature to prevent sample's rapid dehydration. Young's modulus was obtained from the slope of the stress-strain curve generated over the linear portion of the strain range, while tensile strength was determined as the ultimate stress at break.

The recovery of scaffold was measured by stretching the sample to 100%. Subsequently the sample was immediately removed from the microtester and soaked in water for 10minutes to remove the residual stress. The length of the sample before

stretching was marked and after recovery the final length was measure and recorded.

Sample recovery was calculated by the formula below.

$$\% \text{ Elastic Recovery} = \frac{(L_M - L_R)}{(L_M - L_O)} \times 100\%$$

Where L_M is the maximum sample length at elongation, L_R is sample length after recovery, and L_O is the initial length.

3.3.2.4.2. Rheology testing

The rheological data of the aECM scaffold were acquired using Anton Paar, Physica MCR 501 Rheometer with a 8mm diameter parallel plate. Briefly, protein scaffold was prepared as described in section 2.1.1. Dynamic amplitude sweep test was first conducted to obtain the linear viscoelastic region (LVE). The test was performed at angular frequency of 10 rad/s (0.1% to 10% strain). Upon determining the LVE, dynamic frequency sweep test was carried out with the range of strain amplitudes obtained from the first test. Dynamic frequency sweep test was carried out within angular frequency range of 0.05-50 rad/s and torsional shear was at maximum shear strain, γ_0 of 0.05. Linear viscoelastic theory was used to calculate the magnitude of complex shear modulus, $|G^*|$, and loss angle, $\tan \delta$. Elastic modulus is estimated by converting G^* using the formula $E = 2G^*(\nu+1)$, where $\nu = 0.5$.

3.3.3. *In vitro* studies

3.3.3.1. HEKs cell culture on aECM scaffold

Prior cell seeding, aECM protein scaffolds were rinsed thoroughly with PBS. Primary HEKs or HDFs ($1 \times 10^4/\text{cm}^2$) were seeded onto the scaffolds and grown for 7 days in EpiLife supplemented with EDGS or complete DMEM supplemented with 10% FBS, respectively. Cell culture medium was changed every 2 days. MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) metabolic assay was used to quantify cell proliferation on the aECM scaffold. Briefly, MTS reagent (Promega) was added to the seeded wells according to manufacturer's instructions at the respective time points and incubated for 4 h at 37 °C, 95% air/ 5% CO₂ in the dark. The absorbance of formazan product from the reaction was measured by a microplate spectrophotometer (Tecan, Infinite M200) at 490 nm (reference wavelength = 650 nm). To test the cellular viability on aECM scaffold, Live/dead assay was performed using Calcein AM and ethidium homodimer-1 (Molecular Probes) as live/dead fluorescence indicator.

3.3.3.2. Human dermal fibroblasts cell culture on aECM scaffold

Cell adhesion assay was conducted as described previously [22]. Briefly, FN910 aECM protein (1mg/mL) and native FN (10µg/mL) were adsorbed on standard multi-well tissue culture plates overnight and non-specific interactions were blocked with 1% heat-denatured BSA. 1×10^4 primary HDF cells were seeded into each wells and incubated at 37°C, 95%air/5%CO₂. Images at 5 random spots were acquired for each samples at regular time intervals using phase contrast microscopy (Olympus

IX51). Individual cell areas from the images were determined using ImageJ. Percent attached cells was defined as the number of cells with cell areas greater than the average cell area on BSA control divided by the total number of cells counted. Minimum of 100 cells were analyzed for each time point for each sample.

For cellular infiltration, all substrates were rinsed thoroughly with PBS prior to cell seeding. Human dermal fibroblasts ($3 \times 10^5/\text{cm}^2$) were seeded onto three dimensional aECM scaffold and maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS and 100 IU-100mg/mL penicillin-streptomycin. Cell culture media was changed every 2 days and cells were grown for 18 days before they were washed, fixed in 4% buffered paraformaldehyde and sectioned.

3.4. Statistical analysis

The results obtained in all assays were reported as a mean $\pm$ standard deviation. The experiments were performed in triplicates. Statistical significance was determined by One-Way ANOVA and Student's *t*-test. Differences were considered significant at $p < 0.05$.

4. RESULTS AND DISCUSSIONS

4.1. Human epidermal keratinocyte cell response on integrin-specific artificial extracellular matrix proteins

4.1.1. Synthesis and analysis of aECM proteins

4.1.1.1. Cloning

DNA plasmids for each of the artificial ECM protein constructs were digested with XhoI and SalI enzymes for 2 h at 37°C and verified by agarose gel electrophoresis. Digested products corresponding to the correct molecular weight was observed for each aECM construct (FN910 aECM, 1.8 kbp; Col-IV aECM, 696 bp; LN-5 aECM, 699bp; Figure 4.1). Each of these plasmids was further verified using DNA sequencing (APPENDIX).

Figure 4.1 Verification of final aECM protein DNA constructs through restriction enzymes digestion ran on 1.2% DNA agarose gel.

4.1.1.2. Protein expression and purification

Table 4.1 Typical yields for aECM proteins from bacterial fermentation

aECM protein	Protein yield
FN910	120mg/L
LN-5	60mg/L
Col-IV	60mg/L

Artificial ECM proteins were obtained from protein expression. Bacterial expression using 5L fermentation typically yielded purified aECM proteins ranging from 50 mg to 120 mg/L (Table 4.1). The purity and molecular weight of each protein were verified by polyacrylamide gel electrophoresis (SDS-PAGE) and western blot (Figure 4.2).

Figure 4.2 (A) Coomassie SDS-PAGE gel analysis of aECM proteins; lane 1: protein ladder, lane 2: FN910 aECM, lane 3: Col-IV aECM, lane 4: LN-5 aECM. (B). Western blot analysis of Col-IV aECM (left) and LN-5 aECM proteins (right).

4.1.2. Keratinocytes adhesion on aECM proteins

Primary human epidermal keratinocytes were cultured on surfaces coated with various aECM proteins and their respective native proteins that serve as controls. Figure 4.3A shows the number of attached cells on various substrates over a certain period of time. From this graph, all three aECM proteins were shown to be able to support keratinocyte adhesion in a similar fashion compared to their respective native controls. The most rapid cell attachment and spreading were on both FN910 aECM and FN surfaces; where almost 100% of the cells were attached within 4 h. This observation was in agreement with other reports where fibronectin was shown to promote rapid keratinocytes attachment and spreading [188]. On the other hand, cell attachment on native LN and LN-5 aECM substrates were also rapid, compared to native Col-IV and Col-IV aECM. Moreover, the number of attached cells found on Col-IV aECM and native Col-IV were not quite different than that on TCPS control after 4 h.

Figure 4.3B shows representative phase contrast images obtained from each substrate after 4 h. From this figure, there were no significant differences observed in cell morphologies between the aECM proteins and their respective native controls.

Figure 4.3 (A) Percent attached cells for various adsorbed protein surfaces over time. Data represent means $\pm$ standard error of the mean (SEM) from three independent experiments. * denotes significant difference from TCPS control ($p < 0.05$). (B) Phase contrast images of HEK cultured on various protein substrates acquired after 4 h. Scale bar: 100 μ m.

4.1.3. Effect of competitive peptides on HEK cell attachment

We next wondered if human epidermal keratinocytes attachment on the aECM proteins was due to specific interactions with the cell-binding domains present in each aECM protein construct. In order to examine this, we tested HEK attachment to various aECM proteins in the presence of soluble competitive peptides. Figure 4.4 below shows the number of attached cells for each surface in the presence of GEFYFDLRLKDGK, PPFLMLLLKGSTR, GPHSRN and GRGDSP soluble peptides.

The cell attachment on native FN and FN910 aECM surfaces in the presence of various competitive peptides is shown in Fig 4.4A. Here, the presence of 300 μ M GPHSRN did not have any effect on the cell attachment on native FN, but at the same concentration, GPHSRN peptides inhibited cell attachment on FN910 aECM to 60%. In the presence of 300 μ M GRGDSP peptides, cell attachment was diminished to 60% and 30% on both native FN and FN910 aECM surfaces respectively. Cell attachment on native FN was further reduced to 30% when both GPHSRN and GRGDSP peptides were present (Figure 4.4A, black bars), similar to the FN910 aECM. The presence of GEFYFDLRLKGDGK and PPFLMLLLKGSTR peptides had no effect on cell

attachment, suggesting that HEK attachment on FN910 aECM was mediated by both PHSRN and RGD sequences present on the cell-binding domain.

Figure 4.4 Effect of competitive peptides on cell attachment to (A) FN native and FN910 aECM, (B) Col-IV native and Col-IV aECM and (C) LN native and LN-5 aECM. Data are means $\pm$ SEM from three independent experiments. *, significant difference from no peptide control.

Subsequently, the addition of GEFYFDLRLKGDGK peptides (200 μ M) also significantly reduced cell attachment (by almost 80%) on both Col-IV aECM and native Col-IV substrates (Figure 4.4B). Other peptides (GPHSRN, GRGDSP) had no effect on cell attachment to either native Col-IV or Col-IV aECM substrates. Surprisingly, PPFLMLLKGSTR peptides reduced cell attachment on native Col-IV

but had no effect on Col-IV aECM. This could be due to the $\alpha3\beta1$ integrins expressed by keratinocytes that are known to bind weakly to native Col-IV [189-191]. It is likely that addition of PPFLMLLKGSTR competes with the $\alpha3\beta1$ integrins for binding to native Col-IV substrates. Taken together, our data suggests that HEK attachment to Col-IV aECM was due to the specific interactions with the GEFYFDLRLKGDK sequence present within the aECM construct.

Lastly, HEK adhesion on LN-5 aECM substrate was also specific to PPFLMLLKGSTR (Figure 4.4C). In the presence of PPFLMLLKGSTR peptides (200 μ M), nearly 80% of cells were inhibited on LN-5 aECM compared to only about 50% on native LN.

4.1.4. Integrin blocking

In the previous section, by using competitive peptides, we have shown that HEK cell attachment on various aECM substrates was specific to the cell-binding domain present in each aECM protein construct. Here, we used integrin-blocking antibodies to determine which integrins were utilized for cell attachment to the various aECM proteins. Figure 4.5A shows the number of attached cells on various surfaces in the presence of integrin-blocking antibodies.

Figure 4.5 (A) Effect of integrin blocking to cell attachment. Cells were incubated with various integrin-blocking antibodies and allowed to attach on various substrates for 2 h. * denotes significant difference from no antibody control. (B, C) RT-PCR of keratinocytes integrin expression cultured on Col-IV and Col-IV aECM. #, significant difference from each other ($p < 0.05$). Data are means $\pm$ SEM from three independent experiments.

Consistent with our previous data on competitive peptide experiments shown in Figure 4.4, cell attachment on native FN and FN910 aECM surfaces were significantly reduced by the presence of anti-alpha5 antibodies. We also observed that anti-alpha3 antibodies also slightly inhibited cell attachment on FN910 aECM. It has been shown that the alpha3beta1 integrins are able to bind a wide variety of ligands, including fibronectin [192, 193], laminin [194, 195], collagen, and epiligrin [196].

Elices et al. showed that $\alpha 3$ integrins also recognize the RGD site in fibronectin [195]. Taken together, we conclude that keratinocytes primarily use $\alpha 5\beta 1$ integrin to adhere to FN910 aECM, either through recognition of the RGD domain, or synergistically with the PHSRN sequence [15, 197, 198].

Previously, we showed that the Col-IV aECM protein was also able to support attachment of keratinocytes via specific recognition of the GEFYFDLRLKGDK sequence present within the aECM construct. This observation was consistent with the observations of Grafahrend and coworkers [60], where they showed that electrospun PLGA decorated with GEFYFDLRLKGDK peptides were able to increase HaCaT adhesion. However, there is little known about how keratinocytes interact with the GEFYFDLRLKGDK domain in the literature. $\alpha 2\beta 1$ integrin is widely known as the major receptor for binding to native Col-IV, and thus is most likely to interact with GEFYFDLRLKGDK for cell attachment. However, in our hands, the addition of anti- $\alpha 2$ integrin antibodies (both P1E6 and P1H5 clones) had no inhibitory effect on cell attachment to Col-IV aECM (as well as to native Col-IV), even up to the highest concentration (20 $\mu\text{g/mL}$, $\text{IC}_{50} = 2 \mu\text{g/mL}$) tested. This result was unexpected as the same anti- $\alpha 2$ antibody (P1E6 and P1H5) had been previously shown to inhibit keratinocyte cell adhesion to native Col-IV [199, 200].

Similarly, the addition of anti- $\alpha 3$ antibodies, which is known to bind weakly to native Col-IV [189-191], also had no inhibitory effect on cell attachment. While addition of anti- $\alpha 2$ antibodies did not inhibit cell attachment to Col-IV aECM, there was however, a slight reduction in cell attachment on Col-IV aECM in the presence of anti- $\alpha 5$ antibodies.

In order to investigate if alpha2 integrin was expressed by keratinocytes cultured on Col-IV and Col-IV aECM, we tested the expression levels of various integrins using RT-PCR. Figure 4.5B shows the relative intensities of alpha2, alpha3 and alpha5 integrins expressed by keratinocytes cultured on Col-IV and Col-IV aECM surfaces. Alpha2 integrins were expressed by keratinocytes on both native and aECM surfaces in an expression level comparable to each other. Alpha3 and alpha5 integrins were also expressed, with a lower level of alpha5 integrin expression observed on Col-IV aECM. At this point, we were unable to conclude if alpha2 integrins were primarily utilized for cell attachment to Col-IV aECM since alpha2 integrins were expressed by cells. Yet, addition of anti-alpha2 antibodies did not inhibit cell attachment on native Col-IV and Col-IV aECM. Hence, it is likely that other Col-IV integrins such as alpha1beta1 were utilized for cell attachment on both Col-IV and Col-IV aECM[201].

Finally, on native LN, the addition of the anti-alpha3 antibodies inhibited cell adhesion to almost 40%, while with both anti-alpha2 and anti-alpha5 antibodies cell adhesion was only slightly reduced (to 80%). On the other hand, anti-alpha3 antibodies dramatically reduced cell attachment on LN-5 aECM to nearly 20%, suggesting that HEK cell attachment on LN-5 aECM was likely to be primarily mediated by alpha3beta1 interactions with the PPFLMLLKGSTR domain. This result is in agreement with findings by Kim et al [19]. Our data collectively show that keratinocytes interact specifically with PPFLMLLKGSTR cell-binding domain in LN-5 aECM proteins for cell attachment via alpha3 (and likely alpha3beta1) integrins.

4.1.5. Keratinocytes proliferation and colony forming efficiency

BrdU labeling was used to determine if the various aECM protein substrates were able to support keratinocyte proliferation. Figure 4.6A shows the relative amount of BrdU-positive cells on various substrates normalized to the control with no coating (TCPS). Here, Almost 2-fold increase in cell proliferation on FN910 and LN-5 aECM protein substrates was observed, while cell proliferation was only slightly higher (almost 1.3-fold) on Col-IV aECM protein compared to uncoated control.

Figure 4.6 (A) Percent BrdU-labeled cells on various substrates. (B) p63 expression level in keratinocytes cultured on various protein substrates. Data are means $\pm$ SEM from three independent experiments. *, #, significant difference from glass ($p < 0.05$ and $p < 0.01$ respectively).

Subsequently, we tested if various aECM surfaces also able to promote colony forming of keratinocytes stem cells. The p63 transcription factor is a widely recognized marker of epidermal stem cells [42, 202, 203]. The p63 gene is known to be involved in maintaining progenitor-cell populations which are necessary for

epithelial development and morphogenesis [204]. This gene transcribes both transactivating (TA-p63) and non-transactivating (Δ N-p63) isoforms.

The p63 mAb antibody (clone 4A4), which has been reported to recognize all p63 isoforms, is widely used as a reporter for keratinocyte skin stem cells [204]. Fixed cultured keratinocytes on various protein surfaces were probed with p63 mAb antibodies (1:100). We found that significantly higher numbers of p63-positive cells were present on all of the aECM proteins compared to control without coating. This observation was consistent with our BrdU data, where the number of p63-positive cells on FN910 aECM and LN-5 aECM were 2-fold higher than that of control with no coating.

Finally, we examined the abilities of keratinocytes cultured on aECM proteins to form colonies, as indirect measurement of the cells' clonogenic ability and growth capacity. Figure 4.7A are representative images showing the colony forming abilities of keratinocytes subcultured from various aECM and native protein-coated culture dishes.

There were no significant differences between the colony forming efficiencies and percentage of non-aborted (growing) colonies between all native and aECM protein substrates tested (Figure 4.7B and 4.7C), validating the p63 immunostaining results. These findings collectively show that aECM proteins were able to support proliferation and colony forming abilities of keratinocyte stem cells, comparable to their native controls.

Figure 4.7 (A) Colony forming efficiency of HEKs (passage 4) grown on native (left) and aECM (right) proteins. (B) Comparison of HEK's CFE values between native and aECM proteins.

4.2. Cell motility on integrin specific artificial ECM substrates

4.2.1. Collective migration on artificial ECM proteins

Re-epithelialization is one of the most important phases in wound healing as it restores the skin function as protective barrier. In wound healing, regeneration of the basement membrane is essential to restore tissue compartmentalization and provide structural support to the new epidermis. In normal wound healing, newly deposited basement membrane proteins as well as ECM proteins that appear in the wound bed, provide signals to the regenerating epidermis. *In vitro*, many studies have shown that ECM is indeed required to support cell migration and ensure rapid coverage of the wound area [15, 205, 206]. Integrins are cell surface receptors responsible for adhesion to the ECM, and they mediate both outside-in and inside-out signal transduction pathway. Hence, there is an increasing interest to understand how integrins regulate cell-ECM interactions related to cell motility.

In our previous work, we have designed and developed aECM proteins that target key integrins expressed by keratinocytes. We saw significant differences in keratinocyte attachment and proliferation behavior, due to differences in integrin-ECM interactions. Specifically, we showed that keratinocytes utilize the $\alpha3\beta1$ integrins for attachment to LN-5 aECM while the $\alpha5\beta1$ integrins utilize for attachment to the FN910 aECM (Figure 4.5A). However, apart from promoting cellular attachment and proliferation, aECM protein should also promote keratinocytes migration to be functional as substrates in skin substitutes.

In this work, we wondered if these integrin-specific aECM proteins influenced the migratory behavior of keratinocytes in the context of wound healing. Hence, first

we performed a “wounding” experiment to study the migration of keratinocyte cell sheets on FN910 and LN-5 aECM substrates.

Figure 4.8B and 4.8C show the percent wound closure over time on various adsorbed protein surfaces. We found that the FN910 aECM substrates promoted rapid wound closure, comparable to the native fibronectin control. In contrast, wound closure on LN-5 aECM substrates was significantly slower than native LN substrates. Nonetheless, both FN910 and LN-5 aECM were able to promote significantly faster wound closure compared to the BSA control.

Figure 4.8 (A) Schematic diagram of ‘wounding’ assay. Tissue culture dish were coated with various protein substrates and sterile PDMS blocks were then secured on the protein surface before cells were seeded. Upon confluency, PDMS blocks were removed and ‘wounded’ area was re-coated before wound closure was observed using time lapse microscopy. (B) Wound healing behavior observed on various native and aECM surfaces. (C) Wound closures rate on various native and aECM surfaces. Wound closure rates are slopes from linear fit of wound closure area over time (Appendix).

To quantify these differences, cells at the wound edge were tracked manually for 8 h and their cell speeds were calculated. First, we quantified the trajectories of the keratinocytes migration on these substrates. The trajectories of individual migrating cells can be described using a persistent random walk model characterized by two phenomenological parameters: cell speed and persistence [207-209]. These two parameters allow a quantitative analysis on the qualitative differences in migration behavior observed in the wind-rose diagram. Persistent migration is characterized by stable cell polarization that was tightly coupled to persistent, linear migration in the

absence of a chemotactic agent. The quantification of persistent migration is defined as persistence index (PI), which is calculated by the following equation:

$$PI = \frac{l}{d}$$

where l is linear distance between the beginning and final position of the cells, and d defines the total distance travelled by the cells [210].

Figure 4.9 (A) Wind-rose migration plots of wound edge keratinocytes on various substrates. All scales are measured in μm . (B) Average cell speed and (C) its persistence for individual cells migrating on the wound area within the first 8 h. Error bars represent SEM from five independent experiments. * denotes significant difference from LN native control.

Here we found that the cells trajectories were similar on both native FN and FN910 aECM. On the other hand, shorter trajectories and lower the persistence index were observed for cells migrating on LN-5 aECM compared to the native LN substrates (Figure 4.9A,C). Further, Figure 4.9B shows the average cell speeds on various substrates. Indeed, the average cell speeds on LN-5 aECM substrates were 2-folds lower than that on native LN substrates, while the average cell speeds on FN910 aECM were comparable to the native FN.

Hence, we questioned if such difference in wound closure rate is due to the different binding domains that the cells utilized and therefore the migratory behavior of the cells were different. In order to find out, we first observed more carefully on the cell migratory behavior, particularly on LN-5 aECM compared to its native substrates.

Figure 4.10 (A) Keratinocytes migratory behavior on LN-5 aECM (left) and LN native (right). Scale bar = 100 μ m. (B) Keratinocytes on LN-5 aECM exert multiple lamellipodia (i, white arrowheads), while on LN native keratinocytes migrate with persistent migration (ii). White arrows denote the direction of cell migration, leading edge is marked by yellow arrowheads. Scale bar = 50 μ m.

Figure 4.10 showed that indeed, keratinocytes migratory behaviors on both substrates were distinct. On LN-5 aECM, cells appeared to prefer cell-cell interactions and often exert multiple lamellipodia. On the other hand, keratinocytes on LN native established polarized shapes and appeared committed to move in single direction defined by the leading edge [211]. Therefore, we hypothesize that such differences is due to the engagement of $\alpha 3 \beta 1$ integrin used by the cells to migrate on LN-5 aECM.

4.2.2. Single cell migration on aECM proteins

We saw slower migration of keratinocyte cell sheets on LN-5 aECM compared to all the other substrates in the “wounding” experiment. This suggests that integrin-ECM interactions might be responsible for these differences in migratory behavior on LN-5 and FN910 aECM substrates. To probe this hypothesis, we next examined the migratory behavior of individual HEK cells on surfaces coated with various aECM and native proteins.

Figure 4.11 Keratinocytes single cell migratory behavior on LN-5 aECM versus LN native. Note that high cell-cell interactions was prominent on LN-5 aECM sample and less directed motility. Scale bar = 100 μ m.

Figure 4.11 shows keratinocytes migrating on LN-5 aECM and native LN over 6 h period. Consistent with what was observed in the wound-healing experiments, cell migration was impaired on LN-5 aECM, with cells moving randomly, in a circular trajectory. We also noted that cells often came into physical contact with other cells

(white arrow). Carter et al. reported that the $\alpha 3\beta 1$ integrin mediates cell-substrate adhesion as well as cell-cell adhesion [193]. Hence, it is likely that such behavior is due to the engagement of $\alpha 3\beta 1$ integrin that is used by the keratinocytes to interact with LN-5 aECM.

4.2.3. Effect of anti-integrin antibodies on cell migration

We showed that cells utilize the $\alpha 3\beta 1$ integrin for attachment on LN-5 aECM and $\alpha 5\beta 1$ for attachment to FN910 aECM. Hence, it is likely that the same integrins are also utilized for migration on the aECM substrates. To test this hypothesis, we next observed the migratory behavior of keratinocytes on various protein substrates in the presence of anti-integrin antibodies. Cells were followed for 8 h, and their migration paths were manually tracked and represented using wind-rose diagrams.

Figure 4.12 (A) Migration plots of HEK cells on native FN and FN910 aECM in the presence or absence of antibodies. All scales are in μm . (B) Cell speed and Persistence Index comparison between native FN and FN910 aECM. * represents significant

difference ($p < 0.05$). (C) Effect of anti- $\alpha 3$ antibodies on cell spreading. Scale bar = 100 μm .

Figure 4.12A shows the wind-rose diagrams for keratinocytes cell trajectories native FN and FN910 aECM. The average cell speeds and persistence indices for these substrates are also shown in Figure 4.12B. Here, we found that in the absence of inhibitory integrin antibodies, the cell speed and persistence of keratinocytes migration on FN910 aECM were similar to native FN. On both native FN and FN910 aECM, keratinocytes migrated with average cell speeds of $49.2 \pm 4.0 \mu\text{m/h}$ and $43.7 \pm 4.4 \mu\text{m/h}$ respectively. Our observed cell speeds were consistent with the previous report by Hamill and colleagues [212]. Keratinocytes also moved with persistence on both native FN and FN910 aECM substrates ($\text{PI} = 0.75 \pm 0.1$ and 0.58 ± 0.1 respectively).

When we subjected cells to the anti- $\alpha 5$ antibodies, we found that keratinocytes cell migration behaviors were significantly inhibited on native FN and FN910 aECM surfaces. Figure 4.12A (iii) shows that the trajectories of cells treated with anti- $\alpha 5$ antibodies on native FN (top) and FN910 aECM (bottom) were significantly different than untreated cells (Figure 4.12A (i)). Furthermore, the average speeds of antibody-treated cells were reduced by nearly 1.5-fold on both native FN and FN910 aECM (Figure 4.12B). Addition of anti- $\alpha 5$ antibodies also reduced the persistent index (PI) on both substrates, although the reduction of PI was more severe on FN910 aECM substrates (Figure 4.12B). Taken together, our result suggests that the $\alpha 5$ (likely $\alpha 5\beta 1$) is primarily utilized for cell migration on native FN and FN910 aECM and is necessary for cells to move in a directed fashion on these substrates.

Interestingly, anti- α 3 antibodies affected cell migration on both fibronectin substrates as well. From Figures 4.12A (ii), we showed that the addition of anti- α 3 antibody affected both keratinocytes migration speed and persistence on native FN and FN910 aECM. Further, anti- α 3 antibody affected keratinocytes spreading (Figure 4.12C). Consistently, we have shown earlier that anti- α 3 antibodies slightly inhibited keratinocytes attachment to FN910 aECM (Figure 4.5A). Hence, it suggests that the α 3 β 1 integrin plays an important role in keratinocytes migration on both native FN and FN910 aECM by affecting cell spreading, even though the α 5 β 1 is preferred for cell adhesion to FN910 aECM [22].

Figure 4.13 (A) Migration plots of HEK cells on native LN and LN-5 aECM in the presence or absence of antibodies. All scales are in μm . (B) Cell speed and Persistence Index comparison between native LN and LN-5 aECM. * represents significant difference ($p < 0.05$).

Figure 4.13A shows the wind-rose diagrams for keratinocytes cell trajectories native LN and LN-5 aECM, while the average cell speeds and persistence indices for these substrates are shown in Figure 4.13B. Consistent with Figure 4.11 earlier, the cell trajectories in Figure 4.13A show that the keratinocytes on LN-5 aECM moved with little persistence, as indicated by the lower PI values (0.22 ± 0.1) compared to the native LN-5 (0.65 ± 0.16). In addition, the average cell speed on LN-5 aECM substrates ($32.1 \pm 3.0 \mu\text{m/h}$) was also lower than that on native LN-5 ($53.1 \pm 2.6 \mu\text{m/h}$). Thus, our results suggest that LN-5 aECM were unable to promote cell migration in the fashion comparable to native LN.

In the presence of anti-alpha3 antibody, cell speed and persistence were reduced on both LN native and LN-5 aECM substrates, with a more severe effect on LN-5 aECM. Here, the addition of anti-alpha3 antibodies to cells on LN-5 aECM almost completely inhibited keratinocytes migration (Figure 4.13A(ii)). Further, similar to effect on both fibronectin samples, anti- $\alpha 3$ integrin antibodies affected cell spreading on both laminin samples (data not shown).

Apart from alpha3beta1 integrin, alpha6beta4 integrin has been widely known to be involved in keratinocytes migration on laminin 5 [65, 67, 213, 214]. Therefore, we tested the effect of anti-alpha6 antibodies to keratinocytes migration on LN native as well as LN-5 aECM. As expected, since LN-5 aECM does not possess the binding

domain for alpha6beta4 integrin, the presence of anti-alpha6 antibody did not affect cell speed or persistence on LN-5 aECM. In contrast, anti-alpha6 antibody affected both speed and persistence of cell migrating on LN native substrate. Hence, it suggests that cells also preferentially use alpha3beta1 for migration on LN-5 aECM substrates.

Numerous reports in the literature have showed that laminin promotes keratinocytes migration [174, 213-217], and alpha3beta1 integrin is the key integrin required for keratinocytes to migrate in a directed manner [211]. However, our results suggested that the engagement of alpha3beta1 integrin alone did not seem to be adequate to support cell migration with directed motility on LN-5 aECM. Since the LN-5 aECM contains only the PPFLMLLKSTR domain, which we have shown to primarily bind to alpha3beta1, it is likely that ligands for alpha6beta4 integrins were absent for LN-5 aECM. Consistent with our results, Russell and Seghal separately showed that the ligation alpha6beta4 integrin with LN-5 is required to establish a directed motility. In the absence of either alpha6beta4 integrin or LN-5, cells migrate through an alternative mechanism that was likely by engaging alpha3beta1 integrin, and the cell movement is aberrant with no persistent trajectories [216, 218].

Interestingly, the addition of anti-alpha5 antibodies significantly altered the trajectories of the cells migrating on native LN and LN-5 aECM substrates. Cells moving on LN native and LN-5 aECM exhibited significantly lower persistence, although their average cell speeds were not affected (Figure 4.13A(iv) and B). Consistently, in our integrin-inhibition assay, we found that the anti-alpha5 antibody also slightly blocked cell attachment to both native LN and LN-5 aECM substrates (Figure 4.5A). Hence, it is likely that the alpha5beta1 integrin is also utilized by cells for migration on both native LN and LN-5 aECM substrates.

In vitro studies have shown that both fibronectin and laminin (individually) supported epithelial cell migration [16, 213]. However, in the *in vivo* setting, fibronectin and laminin are both present on the provisional matrix, over which the keratinocytes migrate during re-epithelialization. Therefore, it is likely that the complex mix of the matrix proteins differentially regulate cell migration. Here in our study, we noted cross inhibition effects of integrin antibodies on cellular migration on aECM substrates even though these substrates did not contain ligands for those integrins (e.g. anti-alpha3 antibodies affected cell speed and persistence on FN910 aECM, while anti-alpha5 antibodies affected cell persistence on LN-5 aECM). Hence this study suggests that there is interplay between both FN and LN matrix in regulating keratinocytes migratory behavior. Further, as we showed that keratinocytes mainly utilized different integrins to migrate on LN-5 aECM (alpha3beta1 integrin) and FN910 aECM (alpha5beta1 integrin), it is likely that keratinocytes migrated on these substrates by using different mechanism in order to coordinately regulate its speed and persistence. In agreement to this, Kligys et al. recently reported that in the absence of alpha6 integrin, deposition of FN rescues bronchial epithelial cells persistent migration [219].

Taken together, this study showed that the role of alpha3beta1 integrin in keratinocytes motility is likely to promote attachment and spreading (by initiating lamellipodia protrusions). However, the engagement of alpha6beta4 integrin to LN-5 (and/or the engagement of alpha5beta1 integrin to FN) is required to stabilize the exerted lamellipodia and thus enable the cells to migrate in a directed fashion.

4.2.4. Paxillin staining

To further understand the differences in cell motility as a result of different integrin-substratum interactions, we examined the distribution of paxillin present in cells migrating on various substrates. Paxillin has been identified as a key coordinator of the Rho GTPase family and the signaling processes in the context of cell spreading and migration [220]. It is also believed to be a very early component of the focal contact assembly [221]. Studies have also shown that cells that form paxillin-rich focal adhesion plaques have increased levels of Rho signaling, increasing directed motility [222, 223].

In this work, we performed paxillin immunostaining on keratinocytes seeded on both FN910 aECM and LN-5 aECM samples. Here at 4h, we found that the paxillin was expressed in both fibronectin and laminin samples (Figure 4.14A). Both our FN910 and LN-5 aECM samples showed similar paxillin distribution pattern compared to their respective native counterparts.

Figure 4.14 (A) Paxillin expression of HEKs interacting with various surfaces by immunofluorescence. Paxillin (green) was conjugated with FITC; Cell morphology was stained with phalloidin conjugated actin and cell nuclei was counterstained with DAPI. (B) Quantification of paxillin expression as a ratio of cell area. * represents significant difference from control ($p < 0.05$).

We observed differences in the paxillin distribution between fibronectin (native FN and FN910 aECM) and laminin (native LN and LN-5 aECM) samples. Even though both cells on fibronectin and laminin displayed well-organized actin stress fibers, clear and mature focal contacts were detected in cells spread on fibronectin samples. In contrast, such paxillin-rich focal adhesions were absent in cells cultured on both native LN and LN-5 aECM substrates. This striking difference could be due to the different integrin-substratum interactions involved for fibronectin (i.e., $\alpha 5\beta 1$) and laminin (i.e., $\alpha 3\beta 1$).

Upon treatment with antibodies, as expected, F-actin staining showed that cell spreading on all samples was affected, which is consistent with the cell adhesion as well as the single cell migration assays we have done previously. Specifically on both native FN and FN910 aECM samples, focal contacts were diminished in the presence of anti-integrin antibodies. Compared to the control sample without antibody, we can see that the filopodia were present but actin filaments were not able to form a stable membrane ruffles and lamellipodia. On both native FN and FN910 aECM, $\alpha 3$ antibody blocking partly disrupted cell spreading. On the other hand, $\alpha 5$ antibody severely disturbed the F-actin organization, with cells mostly rounded and the paxillin expression was diminished completely on FN910 aECM sample (Figure 4.14B, left).

Similarly on both native LN and LN-5 aECM, both alpha3 and alpha5 antibodies impaired actin organization and cell spreading. Paxillin expression was also suppressed with antibodies treatment, with a significantly more prominent effect on the addition of alpha3 (Figure 4.14B, right), suggesting that alpha3 is necessary for keratinocytes attachment and spreading to native LN and LN-5 aECM.

In order to understand the implications of the differences in the paxillin-focal adhesion arrangement on the downstream signalling pathway (and thus its implications in cellular migratory behavior), further investigations must be performed. In wounded epithelial tissues, EGF-induced paxillin phosphorylation by c-Jun amino-terminal kinase (JNK) in human corneal epithelial cells is required for association of paxillin with focal adhesion kinase (FAK), which is necessary to support cell migration[224]. However, due to the time constraint, this downstream analysis is not included in this thesis.

4.3. Evaluation of artificial ECM scaffold for skin tissue engineering

4.3.1. Characterization of aECM protein scaffold

Upon skin injuries, damage could be introduced to only the epidermal or both epidermal and dermal layers. Under the circumstances where the latter wound is introduced, skin basement membrane is breached and blood vessels are damaged. Fibronectin was then released to the wound bed along with fibrin that is deposited by the platelets [225] and subsequently these two molecules crosslink and form a provisional matrix plug for re-epithelialization and tissue remodeling. As keratinocytes migrate onto the provisional matrix, they degrade and remodel the matrix while depositing new matrix proteins, especially LN-5, while fibroblasts deposit collagen and contracting the wound size.

Previously we have evaluated the effect of integrin-specific aECM proteins in directing keratinocytes behavior and found that FN910 aECM construct promotes keratinocytes attachment, proliferation, and migration. Hence, in this section, we are interested to examine if FN910 aECM proteins can serve as cell carrier for skin substitutes. In order to facilitate nutrients transport as well as cellular infiltration (e.g. fibroblasts infiltration in simulating the case where both epidermis and dermis are injured), we attempted to generate an aECM scaffold with porous structure.

Here, electrospinning and freeze-drying methods were employed to develop a porous scaffold construct that could potentially simulate full thickness skin equivalents. Both electrospun and freeze-dried scaffolds have successfully been generated (Figure 4.15, top panel). Electrospun aECM protein scaffold displayed flat, ribbon-like morphology with a mean thickness of $190.88 \pm 10.24\mu\text{m}$. The fiber

diameter of this scaffold is ranging from 80-500nm with a mean fiber diameter of $283 \pm 47\text{nm}$, falling closely into the range of native protein fibers in the extracellular matrix that typically have diameter between 50-500nm [226]. Fiber diameter distribution of aECM protein scaffold is shown in Figure 4.16A.

Figure 4.15 Top panel: Representative image of aECM protein scaffolds generated by freeze-drying (left) and electrospinning (right) methods. Bottom panel: SEM micrographs of freeze-dried (left) and electrospun (right) aECM protein scaffold, scale bar = $10\mu\text{m}$.

On the other hand, freeze-drying method enabled us to produce a relatively thicker scaffold (mean thickness = $1122.96 \pm 56.38\ \mu\text{m}$) compared to electrospinning,

generating a nearly 6-fold difference in scaffold thickness. In terms of pore diameter and porosity, freeze-drying method is also shown to be a more effective method to produce scaffolds with a significantly higher porosity and pore diameter. In the electrospun aECM scaffold, pore sizes ranged from 1 – 10 μ m. Instead, a larger and broader pore size distribution ranging from 10 – 200 μ m was obtained in freeze-dried aECM scaffold, leading to a higher porosity of up to 66% compared to only 44% on electrospun aECM scaffold.

Scaffold with nano-scale fibrous structure has been preferred in tissue engineering as the structure mimics the native ECM [226] and its topographical cues have been shown to affect cellular behavior such as cell adhesion and migration [227-230]. However, the interplay between fiber diameter and pore size determines whether or not cells are able to infiltrate and proliferate in the scaffold. It has been suggested that in order for cells to infiltrate into the interstitial space of a matrix, the majority of the pores must be significantly larger than the dimension of a cell, such that both the cell and its cellular processes can easily enter the interstitial space. In skin, the major cell type of the dermis is human dermal fibroblast, which size varies between 10 to 100 μ m [231]. It was reported that an optimal pore size between 20 – 120 μ m was proposed for effective cell infiltration [232], and that fibroblast migration decreases as scaffold pore size increases above 90 μ m [233].

Figure 4.16 (A) Fiber diameter distribution of electrospun aECM protein scaffold. (B) Pore diameter distribution of electrospun (left) and freeze-dried (right) aECM protein scaffold. (C) Thickness of electrospun and freeze-dried aECM protein scaffold

Based on the characterization of both scaffolds' morphology, we found that the electrospun scaffold is not suitable for cellular infiltration since the pores were

relatively small compared to the freeze-dried scaffold (mainly in a range of 1 – 3 μ m). Studies have shown that electrospun scaffold with pore size less than 5 μ m tend to completely prevent cell infiltration (forming cell barrier) [234]. Furthermore, organic solvents such as HFIP and glutaraldehyde were required to fabricate electrospun aECM scaffold. Although many have reported that these solvents did not cause significant cytotoxicity to cells [235], trace amount of such solvents might still be present and there are possible environmental concerns of using fluorinated alcohols such as HFIP.

The disadvantages of using freeze-drying method to create porous scaffold, however, is that there's little control over the pore size as well as the interconnectivity of the generated pores. However in the *in vivo* wound healing setting, interconnecting pores may not be a critical issue for a protein-based template such as collagen or elastin, because proteases (e.g. collagenases, MMPs, etc.) and elastases are synthesized by most inflammatory cells during wound healing and remodeling processes. Therefore, freeze-drying method is preferred and used for our subsequent experiments.

4.3.2. Mechanical properties of freeze-dried aECM protein scaffold

4.3.2.1. Tensile properties

The major component of the aECM protein scaffold is elastin-like component (70% Elastin-like-polypeptide (ELP)). The basic structure of ELPs is a repeating sequence identified in the hydrophobic domain found in the mammalian elastic protein elastin. As elastin is one of the major components in skin, therefore it is predictable

that the mechanical behavior of this aECM protein should follow the viscoelastic behavior of human skin. In general the stress-strain relationship curve of skin can be divided into three stages. In the first stage (phase I) at low strain, stress-strain has a linear relationship, defining the Low Modulus (E_{Low}), or generally known as Young's modulus. In the second stage (phase II), a gradual straightening of the fibers causes an increase in stiffness. Finally in the third stage (phase III), all fibers are straight and at high level of strain. The stress-strain relationship becomes linear again, showing a steep angle and defined as the high Modulus (stiffness) until it reached the ultimate tensile strength (UTS) before fracture (Figure 4.17A).

Figure 4.17 (A) Stress-strain relationship of skin (B) Experimental result on stress-strain relationship of aECM protein scaffold

Table 4.2 below summarized the tensile properties of aECM protein scaffold produced by freeze-drying method. The Young's Modulus of the fabricated materials was found to be within the range of 14.22-22.13 kPa, comparable to those reported in the literature. Srokowski et al. reported that in the low strain range of 20-80%, the modulus of a porous foam-like ELP was between 4.48 to 146.50 kPa [236]. The degree of crosslinking of this aECM protein scaffold is determined by the number of

primary amines from the lysines presented periodically in the FN910 aECM construct. We found that increasing the amount of NHS-ester crosslinker did not significantly affect the tensile properties of the aECM protein scaffold, suggesting that the scaffold has reached the maximum degree of crosslinking.

Table 4.2 Tensile properties of freeze-dried aECM protein scaffold

Sample	Young's Modulus	Stiffness	Strength	Extensibility (%)	Recovery (%)
	E_{low} (kPa)	E_{high} (MPa)	(Stress at break) (MPa)		
FN910 aECM	14.22 - 22.13	1.47±0.06	0.32 ±0.04	286.31 ±19.66	80.36 ±1.91

Elastin is known to be responsible for elasticity and resilience in skin tissue, and is well known for its extreme durability [237]. Consistent with the literatures, aECM protein scaffold also exhibited a very high extensibility (~290%) and high resilience (about 80% of recovery after 100% deformation) [98, 238]. Therefore, aECM protein is extensible to almost three times its original length, which is well beyond the extensible range of tissues.

4.3.2.2. Rheological properties

Viscoelastic properties of the aECM protein scaffold were measured by using rheometer with 8mm diameter parallel plates. The storage modulus G' , loss modulus G'' , and complex shear modulus, $|G^*|$, was measured in wet condition at room temperature. Firstly, amplitude sweep was carried out to determine the linear viscoelastic region, LVR (Figure 4.18A). We found that within this strain range, the complex modulus is constant, suggesting that at these range the material's strength

response occurs at a constant rate. From here, strain amplitude of 1% was chosen for the dynamic frequency sweep test.

Figure 4.18 Rheological behavior of aECM protein scaffold. (A) Amplitude sweep test to determine LVR. (B) Dynamic frequency sweep test. Storage modulus (G'), loss modulus (G''), and complex viscosity (η^*) plotted as a function of frequency. (C) Loss angle ($\tan \delta$) as a function of frequency.

From Figure 4.18B, we can observe that aECM protein scaffold shows a frequency independent modulus, with a complex modulus of 7.75 ± 0.46 kPa. The storage modulus, G' , is dominant over the entire frequency range tested, indicating that the material behaves like a visco-elastic solid. This was again confirmed by the loss angles values. From Table 4.3, the loss angles values ($\tan \delta$) obtained which indicates the dissipation inherent in the material ($\delta = 0^\circ$ for an elastic solid; $\delta = 90^\circ$ for Newtonian viscous liquid) was nearly close to 0 ($0.12 \pm 0.01^\circ$).

Table 4.3 Rheological properties of freeze-dried aECM protein scaffold

Samples	Storage Modulus G' (kPa)	Loss Modulus G'' (kPa)	Complex Shear Modulus $ G^* $ (kPa)	Young's Modulus (kPa) [$E = 3G^*$]	Loss Angle ($\tan \delta$)
FN910		$1.15 \pm$			$0.12 \pm$
aECM	7.66 ± 0.45	0.18	7.75 ± 0.46	23.25 ± 1.38	0.01

* Storage, loss, and complex modulus were measured at 1% strain, $\omega = 11.4$ rad/s.

We computed the Young's Modulus (assuming ν equals to 0.5 for elastic solid) and found that the modulus was quite comparable with our tensile measurement in the previous section. The mechanical properties obtained were also comparable (or better than) other studies on ELP hydrogels, considering that the length of ELP backbone was significantly shorter than those reported in the literature [239-241].

4.3.3. *In vitro* studies

4.3.3.1. Primary HEKs interactions with aECM protein scaffold

In this section, we aim to test the efficacy of our aECM protein to support attachment of human epidermal keratinocytes (HEKs). We have previously shown that keratinocytes attachment and proliferation were mediated through specific interactions between integrins and cell-binding domains present in the aECM proteins. Specifically for the FN910 construct, it has been shown that keratinocytes attachment was enhanced by the aECM protein and was likely to be mediated by integrin $\alpha 5 \beta 1$ [22].

Here, primary HEKs were cultured on freeze-dried FN910 aECM protein scaffolds. The less porous surface of the scaffold was used to prevent keratinocytes from infiltrating through the scaffold. H&E staining (Figure 4.19) shows that keratinocytes were able to grow to confluency on the matrix within 4 days (based on a cell density of 1×10^5 cells/cm²), indicating the biocompatibility of the FN910 aECM protein scaffold and its ability to support keratinocytes attachment and proliferation, consistent with our previous data on 2D adsorbed protein surface.

Figure 4.19 (A) Schematic diagram of freeze dried scaffold's surface used for keratinocytes and fibroblast seeding. (B) H&E stained section (10x) of keratinocytes layers grown on FN910 aECM protein scaffold after 4 days (black arrows). Insert = zoomed in section of keratinocytes layer grown on aECM protein scaffold.

4.3.3.2. Human dermal fibroblast interactions with aECM protein scaffold

Next, we determined if the FN910 aECM proteins were able to support the attachment and growth of human dermal fibroblasts. We seeded HDF cells onto tissue culture plate coated with artificial ECM protein (FN910) and monitored the cell

spreading over a period of time against native fibronectin as positive control and BSA as negative control.

Figure 4.20 Evaluation of keratinocytes viability on aECM protein scaffold. (A) Percent spread cells on adsorbed aECM protein surface over time. * represents significant difference from BSA control. (B) Live-dead fluorescence image of keratinocytes grown on freeze-dried aECM protein scaffold after 7 days. (B) MTS cell proliferation assay of HDF and HEK cells on aECM protein scaffold.

Figure 4.20A showed that the attachment and spreading of HDF cells on the aECM protein began as early as 30 minutes post-seeding, about four times faster

compared to uncoated TCPS. This result is consistent with other study done by Rnjak-Kovacina and coworkers [243], which also reported the attachment and spreading of HDF cells on electrospun synthetic human elastin scaffold within 30 minutes after seeding.

Next, we tested the ability of the aECM protein scaffold to maintain the viability of HDF for up to 7 days. Cellular viability was tested by live-dead assay (Figure 4.20B) and fluorescence imaging revealed a notably high number of viable cells, marked in green Calcein AM staining. Furthermore, MTS proliferation assay showed a nearly six-fold increase for HDF cells over a 7-days period of time (Figure 4.20C). While five-fold increase in proliferation was observed for HEK cells grown on FN910 aECM protein scaffold, confirming the H&E result in the previous section.

4.3.3.3. Human dermal fibroblast infiltration into 3D aECM protein scaffold

In previous section we have seen that both human epidermal keratinocytes as well as human dermal fibroblasts attached and proliferated well on the aECM protein scaffold. Here, we further evaluated the potential of this aECM protein scaffold to be used as cell carriers for skin repair.

In this work, we examined if the aECM protein scaffolds are indeed able to promote the infiltration of human dermal fibroblasts. Briefly, human dermal fibroblasts were first seeded onto one side of the aECM protein scaffold and grown to up to 18 days. Subsequently, the samples are fixed, sectioned, and stained to observe the extent of cell infiltration.

Fig 4.21A showed immunostaining section of the aECM protein scaffold infiltrated by human dermal fibroblasts. From this section, HDF cells were able to infiltrate almost one-third of the scaffold thickness within 7 days. At the end of 18 days, a significant number of HDF cells have migrated through the scaffold (Fig 4.21B).

Figure 4.21 Immunofluorescence staining of HDF infiltration and growth on aECM protein scaffold *in vitro* on day 7 (A) and 18 (B). Cell nuclei are labeled in blue (DAPI), protein scaffold is labeled in red. Scale bar = 200μm.

This preliminary *in vitro* evaluation showed that not only the aECM protein scaffold is able to support the human dermal fibroblasts attachment and proliferation, but also promote cellular infiltration. Although further investigations are still much required to evaluate to which extent the aECM protein can be used as skin replacement, this work nevertheless has provided an insight on the potential of aECM proteins to serve as scaffolds for cell carriers in skin substitutes.

5. CONCLUSIONS AND RECOMMENDATIONS

5.1. Conclusions

Within the past three decades, significant progress has been made in skin tissue engineering. With the development of modern science, it seems that growing functional tissues and eventually organs on the lab bench could realistically be achieved. However, despite many success stories that have been reported, tissue engineered skin has not gained widespread use. Until today, the autograft remains the gold standard in skin grafting.

Recently, protein-based biomaterials have received great interest as potential candidates in biomedical applications as they provide intrinsic biological cues to support cell and tissue function. With the help of the maturation in genetic engineering, developing an artificial protein that mimics a specific site of the natural protein has been made possible.

In this work, artificial ECM (aECM) proteins were engineered to contain specific cell binding domains that engage major keratinocyte integrins in the context of wound healing. We have demonstrated that keratinocytes attachment and proliferation were enhanced through specific interactions between the integrins and cell-binding domain present in aECM proteins. Keratinocytes attachment to the aECM proteins (particularly FN910 and LN-5 aECM proteins) are likely to be mediated by $\alpha 5\beta 1$ and $\alpha 3\beta 1$ integrin, both of which are major integrins used by keratinocytes to adhere to the basement membrane. Further, we also showed that the aECM proteins were also able to maintain epidermal stem cells in culture, where high

levels of p63 expression could be observed on all aECM protein surfaces similar to native ECM proteins. These findings demonstrate that the ability of aECM proteins to maintain the growth of keratinocytes stem cells, while at the same time improve keratinocytes viability by enhancing cell attachment and proliferation, ultimately could help in faster tissue repair.

Following this work, we also evaluated the aECM proteins containing FN910 and PPFLMLLLKGSTR domains in influencing cellular migration. We found that both aECM proteins promote faster wound closure compared to the uncoated control. However the wound closure on LN-5 aECM was found to be slower than its native counterpart, likely due to the slower cell speed and less directional cell movement. We further analyze the single cell migratory behavior on both aECM proteins and found that the keratinocytes engaged alpha3 integrin for migration on LN-5 aECM. In addition, we further showed that in order for the keratinocytes to migrate with directed motility, it is likely that cells require both alpha3beta1 and alpha6beta4 integrins.

The integrin-specific aECM proteins also provide a platform for us to study the isolated effects of integrin-matrix interactions on cell motility. Conventionally, such studies were performed through ablation of specific integrins in the cell. However, complete ablation of the integrin could lead to unwanted changes in the cellular pathways (e.g. by overexpressing other types of integrins [244, 245]), or impair the cells ability in secreting and depositing its autocrine ECM [216], eventually resulting in a different phenotype and behavior. Our approach allows us to observe the effects of specific integrin engagement on cellular behavior without compromising the phenotype of the cells.

Finally, this study also demonstrated the feasibility of developing aECM protein scaffold that can be used as material to deliver cells, particularly keratinocytes, from the lab bench to the bedside. Currently in the clinic, cell delivery/carriers are often necessary to facilitate transplantation of fragile cultured epithelial autografts (CEA) sheets. An ideal delivery system should not only provide sufficient mechanical support for easy handling during surgery but also provide the appropriate biological signals to promote keratinocyte attachment and growth as well as enhance host-wound healing response [151].

As we have shown previously that FN910 aECM protein promotes HEKs, here we found that FN910 aECM also promotes HDFs attachment. By using the classical freeze-drying method, we were able to fabricate three-dimensional, elastic and highly porous aECM protein scaffolds. *In vitro* cell culture studies further showed that the aECM protein scaffolds supported the growth of keratinocytes. Subsequently, the aECM scaffold also contains sufficient porosity to facilitate infiltration and viability of human dermal fibroblasts for up to 18 days. In clinical setting, this would be an additional advantage as the in-situ fibroblast infiltration aids the integration of the scaffold with the wound bed. Taken together, this preliminary *in vitro* evaluation on the aECM protein scaffold shows promise for use as cell delivery system (and eventually skin substitutes).

5.2. Future Recommendations

The outcomes of this PhD research have led to the development of genetically engineered artificial ECM proteins which targeting specific integrin expressed by keratinocytes. However, we observed that although LN-5 aECM promoted keratinocytes adhesion, keratinocytes migration on this substrate were impaired, most likely due to the absence of pairing integrin for the $\alpha3\beta1$ integrin that is required to establish a directed motility. However, the underlying mechanism behind this integrin pairing in coordinating cell migration is unclear. In order to investigate this, a few recommendations are put forth here for future research:

1. Apart from laminin-5, provisional matrix deposited during wound healing includes fibronectin as well. However, as most studies on keratinocytes migration focused on laminin-5, future studies should look into the role of fibronectin in regulating keratinocytes migratory behavior.
2. Studies have shown that keratinocytes migratory behavior is closely related to the deposition of laminin-5 [214], and the engagement of different integrin affects the pattern of deposited laminin-5 [216]. Future experiment should aim to observe if the absence of $\alpha6\beta4$ integrin ligation indeed affects the deposition pattern of laminin-5. A comparison study using an $\alpha3$ -null as well as $\beta4$ deficient keratinocytes could also be performed.
3. Together with the laminin-5 deposition pattern, the downstream signaling related to $\alpha3\beta1$ integrin activation should be investigated. $\alpha3\beta1$ is known to activate Rac1 signaling and the balance between both Rac1 and RhoA signaling is required to prevent impaired cell migratory behavior. Thus,

a relevant study could focus on the crosstalk between these two signaling pathways.

The subsequent part of this research has also shown that the aECM proteins can be processed into a mechanically intact freestanding scaffold that supported the growth and viability of keratinocytes and infiltration of human dermal fibroblasts. However, further assessments are required to evaluate the feasibility of aECM protein scaffold before they can be used as clinically viable skin substitutes. Therefore, some of the future directions that we suggest are as follow:

1. To be a functional keratinocytes delivery system, it is important that the aECM protein scaffold is able to maintain the viability of keratinocytes stem cell populations *in vitro*, before it is transferred to the wound bed. In this regard, keratinocytes stem cell populations on the aECM scaffold should be identified.
2. As fibroblasts migrate from the surrounding tissues into the wound site, it deposits ECM proteins that aid in wound contraction and re-epithelialization. Therefore, ECM proteins such as collagen I and fibronectin deposited by fibroblasts infiltrated through the aECM matrix should be evaluated.
3. Finally, studies to evaluate the aECM protein scaffold immunocompatibility and degradation rates should be tested *in vivo* (e.g. via subcutaneous implantation).

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APPENDIX

A. Plasmid map for ELK repeat synthesized from Genscript

[illegible]

B. Plasmid map for FN910 aECM protein

[illegible]

[illegible]

His tag

C. Plasmid map for LN-5 aECM protein

[illegible]

D. Plasmid map for Col-IV aECM protein

[illegible]

D. Wound closure rate curve fit (Fig 4.14(A))

List of Publications and Conferences

Original Research Articles

- 1) **Tjin MS***, Chua AWC, Ma DR, Lee ST, Fong E. Human Epidermal Keratinocyte Cell Response on Integrin-Specific Artificial Extracellular Matrix Proteins. *Macromolecular Bioscience*. 2014;14:1125.
- 2) **Tjin MS***, Low P, Fong E. Recombinant elastomeric protein biopolymers: progress and prospects. *Polym J*. 2014;46:444.

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Conferences – Oral Presentation

- 1) **Tjin MS***, Fong E. Development of Multi-Signaling Artificial Extracellular Matrix (ECM) Biomaterials For Use As Skin Substitutes. World Biomaterials Congress 2012. 1-5 June, 2012. Chengdu, China
- 2) **Tjin MS***, Fong E. Artificial Extracellular Matrix for Keratinocytes Regeneration. UK-Singapore Translational Skin Biology Symposium 2012. 3-5 Dec 2012. Singapore

Conferences – Poster Presentation

- 1) **Tjin MS***, Fong E. Recombinant Fusion Proteins as Instructive Artificial Extracellular Matrix for Skin Tissue Engineering. 5th MRS-S Conference on Advanced Materials. 20-22 March 2012. Singapore
- 2) **Tjin MS***, Chua AWC, Ma DR, Lee ST, Fong E. An *In Vitro* Examination of Engineered Extracellular Matrix Proteins For Use As Skin Substitutes. International Conference of Young Researchers on Advanced Materials (IUMRS – ICYRAM) 2012. 1-6 July, 2012. Singapore.
- 3) **Tjin MS***, Fong E. Artificial Extracellular Matrix for Keratinocytes Regeneration. Materials for Smart Wound Dressing. 15-16 January, 2013. Singapore.
- 4) **Tjin MS***, Chua AWC, Lee ST, Fong E. Integrin Specific Elastin-based Artificial Extracellular Matrix Proteins for Directing Human Epidermal Keratinocytes. World Conference on Regenerative Medicine. 23-25 October, 2013. Leipzig, Germany.