Re-purposing of clinical waste : human adipose tissue-derived extracellular matrix as wound dressing material

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**NANYANG
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UNIVERSITY**

SINGAPORE

**RE-PURPOSING OF CLINICAL WASTE: HUMAN ADIPOSE
TISSUE-DERIVED EXTRACELLULAR MATRIX AS WOUND
DRESSING MATERIAL**

CHEAM MEIN JI, NICOLE

SCHOOL OF MATERIALS SCIENCE AND ENGINEERING

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SCHOOL OF MATERIALS SCIENCE AND ENGINEERING

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

2019

Statement of Originality

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

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Abstract

A wound dressing is often needed to aid the wound healing process to enable the wound to close as quickly as possible and the skin to regain its flexibility and strength which are the two primary goals for the treatment of wound healing. Type I collagen is a common material for wound dressings as it is the most abundant protein in humans and has the ability to promote cell behaviour. However, collagen alone is not substantial for wound healing and does not prevent the formation of scars. In recent years, the extracellular matrix (ECM) has received significant attention to be used as a scaffolding material as it consists of a complex 3D architecture structure made up of largely collagen fibres and proteins (i.e. fibronectin, laminin) enabling it to have good bioinductive and biomechanical properties. In this thesis, human adipose tissue, a clinical waste was studied to determine if it can be repurposed into a potential wound dressing material. Human adipose tissue has been found to contain the matricellular protein, Angiopoietin-like 4 (ANGPTL4), which has been proven to be advantageous to wound healing as it is able to promote keratinocyte migration to the wound site as well as to enhance angiogenesis, which could further promote the wound healing process. Hence, adipose tissue derived ECM (Ad-ECM) was extracted using a physical decellularization method to obtain a delipidated and decellularized ANGPTL4-enriched ECM. The Ad-ECM was found to contain various essential proteins for wound healing such as collagen type I, III, IV, elastin, fibronectin and laminin after the extraction, without destroying the triple helical structure of collagen. In addition, ANGPTL4 was also found to be retained after the extraction process which shows that the extraction process was mild and efficient. Subsequently, it was shown that Ad-ECM has scar regulating properties as HDFs cultured onto the material were able to suppress myofibroblastic differentiation as seen from the low levels of expression of α -SMA, $\text{coll}\alpha 1$ and SPARC, and the morphology of HDFs remain unchanged after treatment with TGF- $\beta 1$, a potent inducer of fibroblast-to-myofibroblast differentiation. This phenomenon was observed to be due to the interplay of ANGPTL4. Furthermore, collective cell migration and scratch assays carried out using HaCaTs showed that Ad-ECM promotes faster cell migration than Bv Col type I which further substantiates the potential of Ad-ECM as a wound dressing material.

Lay Summary

A wound dressing is often needed to aid the wound healing process to enable the wound to close as quickly as possible and the skin to regain its flexibility and strength which are the two primary goals for the treatment of wound healing. Type I collagen is the most commonly used material for wound dressings as it is the most abundant protein in humans and has the ability to attract cells to the wound site. However, collagen alone is not substantial for effective wound healing and does not prevent the formation of scars. In recent years, the extracellular matrix (ECM), a three-dimensional network of proteins which provides structure and support for surrounding cells, has received significant attention to be used as a scaffolding material. In this thesis, human adipose tissue, a clinical waste was studied to determine if it can be repurposed into a potential wound dressing material. Human adipose tissue has been found to contain ANGPTL4, a type of protein which is loosely bounded to the ECM. This protein has been proven to be advantageous to wound healing regulating scar formation and promoting faster cell migration to close the wound. In this study, Ad-ECM was successfully extracted with the presence of several structural proteins and ANGPTL4. Subsequently, it was shown that Ad-ECM was able to suppress the differentiation of skin cells (i.e. fibroblast to myofibroblast) which often results in scarring. In addition, Ad-ECM has also been found to promote faster migration of cells on the top layer of the skin enabling faster wound closure. These findings substantiate the potential of Ad-ECM as a wound dressing material.

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Abbreviations

Ad-ECM	Adipose Tissue Derived-Extracellular Matrix
AFM	Atomic Force Microscopy
ANGPTL4	Angiopoietin-like 4
ATR-FTIR	Attenuated Total Reflectance Fourier Transform Infrared Analysis
BCA	Bicinchoninic Acid
BDE	1,4-Butanediol diglycidyl ether
Bv Col	Bovine Collagen
COL1A2	Type 1 α 2 Collagen
COL3A1	Type 3 α 1 Collagen
C _t	Threshold Cycle
DSC	Differential Scanning Calorimetry
DSRB	Domain Specific Review Board
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
FDA	Fluorescein Diacetate
FITC	Fluorescein Isothiocyanate
FSCOL	Fish Scale Collagen
Gly	Glycine
HCl	Hydrochloric Acid
HDF	Human Dermal Fibroblast
IGF-1	Insulin-like Growth Factor-1
IL	Interleukin
MMP	Matrix Metalloprotease
NaOH	Sodium Hydroxide
NHG	National Healthcare Group
PBS	Phosphate Buffer Solution
PD	Population Doubling
PDGF	Platelet-derived Growth Factor
PDMS	Polydimethylsiloxane

PFA	Paraformaldehyde
PMSF	Phenylmethane Sulfonyl Fluoride
qPCR	Quantitative Polymerase Chain Reaction
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SEM	Scanning Electron Microscopy
TBS	Tris-buffered Saline
T _d	Denaturation Temperature
TCP	Tissue Culture Plastic
TGF- β 1	Transforming Growth Factor-beta
TNF- α	Tumour Necrosis Factor-alpha
TSE	Transmittable Spongiform Encephalopathies
Yield %	Yield Percentage

Chapter 1

Introduction

This chapter entails a brief introduction to the process of wound healing and the problem of scarring. Human adipose tissue derived extracellular matrix (Ad-ECM) which is rich in extracellular matrix proteins and contains matricellular protein, ANGPTL4, with wound healing and scar regulating properties, is subsequently introduced as the research focus. Following which the hypothesis, objective and scope, and expected outcomes of the thesis will be presented.

1.1 Introduction

The primary function of the skin is to act as a barrier protecting the body from injury. When there is an injury to the skin, the protective function of the skin is lost. A series of events will occur to repair the injury. However, the wound healing process is complex and is susceptible to interruptions which may delay wound healing and lead to the formation of chronic wounds [1]. In order to facilitate the healing, a barrier is needed to maintain homeostasis and moisture of the wound. Hence, a wound dressing is applied to promote the healing process.

An ideal wound dressing is to obtain fast re-epithelization and to minimize scar formation [2, 3]. Although several dressings have been made available, most dressings only exhibit a single purpose (i.e. wound healing). A common material for wound dressings is collagen, which is the most abundant protein in humans and has the ability to promote cell behaviour [4]. However, collagen alone is not substantial for wound healing and requires other essential proteins (i.e. collagen type III, IV, laminin and fibronectin). Moreover, the use of collagen dressings during wound healing is unable to prevent the development of scars [3, 5].

In general, scarring (i.e. hypertrophic scar) is caused by the differentiation of fibroblast to myofibroblast which expresses smooth muscle actin fibres (α -SMA) that upregulates the contractile activity of fibroblast to contract the wound. In addition, myofibroblast differentiation also results in increased production of collagen. Even though myofibroblast is essential for tissue remodelling and wound closure, over contraction and also overproduction of type III collagen which is poorly reconstituted into dense parallel bundles results in scarring [3, 6]. Scars are aesthetically unpleasant and in severe cases causes tenderness, pain, severe itching, anxiety, depression, sleep disturbance and disruption of daily activities as scar tissue is stiff and results in loss in integrity and function of the skin [7, 8]. The current solution for scar management is the application of topical creams to reduce the appearance of the scar which is effective only to a certain extent [6]. Although several studies have been carried out to create an effective wound dressing,

limited studies have been done to create a multifunctional wound dressing that could promote wound healing yet regulate scar development at the point of healing. Hence, an alternative source of wound dressing material to create a multifunctional wound dressing that focuses on wound healing and minimizing scarring is highly desired.

The ECM is a complex and bioactive structure which consists of these essential proteins that can influence cell behaviour such as cell adhesion, growth and repair. Hence, several studies have been carried out to synthetically reproduce the ECM by using ECM proteins extracted from the tissue or using artificial or ECM-like proteins [9]. As seen in a study conducted by Yeo et al., elastin and elastin-like peptides were blended with other natural and synthetic moieties such as peptides, proteins, polysaccharides, and polymers to form composite elastin-based scaffolds [10]. These composites were subjected to various fabrication processes such as electrospinning, freeze drying and solvent casting to fabricate ECM-like structures to mimic the architecture and components of native tissue, with improved strength, structural integrity, elasticity, and wettability. However, due to the complexity of the ECM it is difficult to replicate natural ECM. Hence, scientists have moved towards decellularization of tissues to obtain the natural ECM [9, 11].

Adipose tissue is a rich source of extracellular matrix (ECM) that can be a potential material for wound healing [12]. Adipose tissue derived ECM (Ad-ECM) is a human clinical waste which will be more compatible with humans as compared to other animal sources decreasing risk of disease transmission. The potential risk of using human derived material is that the material may evoke an inflammatory response or immune-mediated implant rejection due to improper decellularization of the ECM and the presence of residual detergent [9]. The effective decellularization and implantation of human derived material was shown in a study conducted by Cebotari et al., where allografts of pulmonary valve were successfully decellularized and implanted into 38 children and young adults and no explantations were required following the pulmonary valve replacement for up to 5 years. This shows that with proper decellularization, human derived material is biocompatible and will not elicit in an immunogenic response [13].

In a recent study, Luo et al. demonstrated the successful decellularization and delipidation of adipose tissue with preserved ECM proteins that are crucial for the wound healing process [12]. Other than ECM proteins, adipose tissue also has high content of angiopoietin-like 4 (ANGPTL4) matricellular protein that has been shown to be able to regulate scars [14, 15]. In a particular study carried out by Teo et al. and Zhu et al., the results showed that the ANGPTL4 interacts with cadherin-11 protein in scar tissue, regulating collagen (i.e. type 1 α 2 (COL1A2) and 3 α 1 (COL3A1)) production, thus controlling scar formation [14, 15]. In addition, ANGPTL4 has also been shown to promote keratinocyte migration to the wound site as well as enhance angiogenesis, which could further promote the wound healing process [16]. Hence, ANGPTL4-rich Ad-ECM, a waste material, can be used as a potential alternative wound dressing material. Overall, this project aims to explore the use of adipose tissue derived ECM as a possible “waste-to-resource” material for functional wound healing applications.

1.2 Hypothesis

This thesis describes and validates the innovative exploitation of ECM derived from human abdominal tissue as wound dressing material with the aim to promote better wound healing and yet prevent fibroblast-to-myofibroblast differentiation. ANGPTL4 present in Ad-ECM has been shown to have anti-fibrotic properties and to promote wound healing. Hence, it is hypothesized that the extracted ANGPTL4 enriched Ad-ECM will be able to promote wound closure and at the same time suppress myofibroblast differentiation in human dermal fibroblast to prevent excessive scarring as ANGPTL4 has been shown to be a modulator of scar collagen production [14].

1.3 Objective and Scope

- To extract and characterize the biophysical and biochemical properties of Ad-ECM derived from human adipose tissue
- To investigate the adhesivity and cytocompatibility of the processed Ad-ECM
- To evaluate TGF- β 1 induced myofibroblastic differentiation of HDF cells cultured on Ad-ECM
- To examine the cell migratory behaviour on Ad-ECM using keratinocytes (HaCaT) and HDF cells.

1.4 Expected Outcomes

- It is possible to use the TGF- β 1 model to understand the potential of Ad-ECM in preventing fibroblast-to-myofibroblast differentiation and to establish that Ad-ECM is a suitable material for wound healing.
- Due to time limitation of time, discovery of the very fine details of the anti-fibrotic properties of Ad-ECM may not be achieved. Instead, our study may need to make the best use of our data to propose the most rational idea to the scientific community.

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Chapter 2

Literature Review

In this chapter, a brief review will be introduced to outline the function of the skin and its components, and the wound healing process in the event of an injury to the skin. Next, the current state-of-the-art wound dressing materials, and the research gaps of wound dressings that has yet to be addressed will be discussed. Following which, the current findings of ANGPTL4-rich adipose tissue derived extracellular matrix (Ad-ECM) in preventing scarring will be delineated to understand its potential role in wound healing. Finally, the model to induce fibroblast-to-myofibroblast differentiation will be discussed.

2.1 Skin

The skin is the largest organ of the human body. It acts as a barrier between the external environment and the body and is made up of three layers: epidermis, dermis and hypodermis layer.

The epidermis is the upper most layer of the skin which is mostly made up of layers of keratinocytes. It is a constantly renewing layer due to the constant division and differentiation of keratinocytes which migrate from the basal layer to the surface of the skin to replace dead or damaged cells [1]. The interface between the epidermis and dermis is known as the basement membrane which is made up of a thin layer of extracellular matrix typically composed of Collagen type IV and laminin which provides anchorage for keratinocytes.

The middle layer of the skin is the dermis which is the bulk component of the skin conferring flexibility, elasticity and tensile strength to the skin, protecting the body from injury. This is due to the presence of collagen, elastin and proteoglycans which contributes to the structural properties of the skin. The principal component of the dermis is collagen which is a fibrous structural protein which provides the physical strength of the skin. Collagen is secreted by fibroblast which are the primary residing cells in the dermis. Dermal fibroblast plays a vital role in wound healing as they differentiate into myofibroblast during the tissue remodelling stage remodelling type I collagen to type III collagen. In addition, myofibroblast expresses alpha-smooth muscle actin (α -SMA) which enables wound contraction and thus wound closure [1].

The hypodermis layer is the innermost layer of the skin which is primarily is made up subcutaneous tissue. It is where the derivative appendages of the skin such as hair follicles, pilli, sebaceous glands and sweat glands are found [18].

2.2 Wound Healing

The skin is a major organ of the human body. When it is damaged, the body will be susceptible to pathogens which can lead to illnesses and even death. Wound healing is an important process which occurs upon injury to enable the skin to regain its protective function. Hence, it is important to first understand the normal wound healing process in response to an injury.

The wound healing process is a dynamic process which involves a cascade of events. It consists of four phases: homeostasis, inflammation, proliferation and tissue remodelling [2-5].

Homeostasis

An injury to the skin will result in damage to the blood vessels causing bleeding. The bleeding is stopped by the formation of a blood clot. This occurs immediately after the injury [2]. The blood clot is a temporary plug formed from platelets and fibrin fibres by thrombin cleavage of fibrinogen released from the platelets [2, 5]. The fibrin clot acts as a reservoir for growth factors (i.e. EGF, PDGF, TGF- β 1, and IGF-1) and cytokines to be released from degranulating platelets and mast cells. This initiates the entire wound healing process as it stimulates re-epithelization, granulation tissue formation and angiogenesis [3].

Inflammation

With an injury to the skin, the wound may be at risk of infection from invading microorganisms. Hence, an inflammatory response arises leading to extravasation and inflammation around the wound site which usually occurs within 24 to 72 hours of injury [2]. Neutrophils are first attracted to the wound site by chemotaxis signals from platelets and bacteria [4, 5]. Next, adhesion molecules from the selectin family increases and attracts leukocytes to adhere to the endothelial cells of the blood vessels [4, 5]. Following which, integrin arrest the movement of leukocytes and induce diapedesis [3]. Leukocytes will then counteract with any foreign substances in the blood vessels and surrounding tissue. The release of pro-inflammatory signals (i.e. interleukins (IL) and TNF- α) attracts macrophages

to the wound site [3, 4]. Macrophages will then engulf the dead and dying cells and bacteria, and release several types of growth factors that will attract cells such as fibroblasts and keratinocytes which are involved in the regeneration of the tissue [3].

Proliferation

The third phase of wound healing focuses on the regeneration of damaged tissue which usually occurs 3 days after the injury and can last up to 14 days [2]. Clotted blood and damaged stroma are removed from the wound space by epidermal cells of skin appendages [4]. At the same time, epithelial junctions are broken such that epidermal (i.e. keratinocytes) and dermal cells (i.e. fibroblasts) no longer adhere to one another, which allows the lateral movement of epidermal cells [3, 4]. In addition, matrix metalloproteases (MMPs) cut a pathway through the fibrin clot and extracellular matrix to enable fibroblasts and keratinocytes to migrate forward [3]. The presence of growth factors (i.e. PDGF, TGF- β) and extracellular-matrix molecules, stimulate fibroblasts of the tissue around the wound to proliferate and migrate into the wound space [2, 4]. The fibroblasts secrete ECM components, including Type III collagen to fill the wound and to replace the temporary matrix formed by the fibrin clot [4]. This leads to the formation of new granulation tissue. Migration of keratinocytes and cell proliferation behind the actively migrating cells results in wound closure. In the meantime, macrophages stimulate fibroplasia and angiogenesis by providing a continuing source of growth factors while blood vessels carry nutrients and oxygen necessary to sustain cell metabolism [4].

Tissue remodelling

The final phase of wound healing is tissue remodelling which occurs after 7 days of injury and can last up to 2 years [2]. Tissue remodelling enables the newly formed granulation tissue to be modified to regain the original mechanical strength of the skin. Some fibroblasts differentiate into myofibroblast and express smooth muscle actin which causes the wound to contract [4]. Meanwhile, collagen undergoes remodelling forming larger collagen bundles and increasing the number of intermolecular cross-links [2, 4]. Subsequently, the granulation tissue evolves into an avascular scar recovering only a maximum of 80% of the original mechanical strength of the skin is recovered [2, 4].

2.3 Wound Dressing

The wound healing process is a complex process which is susceptible to interruptions which may delay wound healing and lead to the formation of chronic wounds [6]. Often during wound healing, the skin is also unable to regain its original appearance, integrity and function. In order to facilitate the healing, a barrier is needed to maintain homeostasis and moisture of the wound.

2.3.1 Function

A wound dressing should be able to aid and promote the healing process to achieve rapid wound closure to prevent infection and water loss, and a functional and aesthetically satisfactory scar [3, 4].

2.3.1.1 Wound Closure

Rapid wound closure is essential for the restoration of the barrier between the human body and the environment to protect the body from any pathogens. This is achieved through re-epithelization. The epithelial layer can be restored by the migration of keratinocytes from the wound edge [4, 7]. However, the migration of keratinocyte cannot be achieved without a viable matrix to support the cells when a wound is deep. Thus, the wound needs to be filled with granulation tissue before re-epithelization can be achieved [4]. Therefore, in order to achieve rapid wound closure, a wound dressing is required to act as a matrix to fill the wound defect to speed up the re-epithelization process to restore the skin barrier.

2.3.1.2 Scarring

Scars are aesthetically unpleasant and in severe cases causes tenderness, pain, severe itching, anxiety, depression, sleep disturbance and disruption of daily activities [5, 8]. Scarring is not necessary for wound healing as wounds in foetus have been found to heal perfectly without the formation of scars [9]. The occurrence of hypertrophic scars is due to

the overproduction of type III collagen, which is poorly reconstituted into dense parallel bundles as seen in **Figure 2.1** [3, 10]. This is due to the differentiation of fibroblast-to-myofibroblast which is identified by smooth muscle actin (α -SMA) which is the hallmark of myofibroblast [9, 11]. The fibroblast-to-myofibroblast differentiation is important as it confers contractile forces to cells and allows the secretion of other extracellular matrices along with collagen filaments (type 1 α 2 (COL1A2) and 3 α 1 (COL3A1)) for tissue remodelling and wound closure. However, over production of collagen filaments (type 1 α 2 (COL1A2) and 3 α 1 (COL3A1)) and contraction of the wound will result in stiffening of the skin and scarring which is not desired. Although several studies have been carried out to create an effective wound dressing, limited studies have been done to create a multifunctional wound dressing that could promote wound healing yet regulate scar development at the point of healing [12-14]. Hence, a material that is able to promote wound healing and at the same time reduce fibroblast-to-myofibroblast to inhibit the over production of type III collagen and contraction of the wound is highly desired.

Figure 2.1 Collagen fibres alignment in (1) scars and (2) normal skin

2.3.2 Wound Dressing Materials

Collagen is a common scaffolding material for wound healing. In particular, Collagen type I, which is commonly found in various wound dressings is known to be the gold standard for wound healing as it makes up 70% of the skin [15, 16]. Collagen is a major extracellular matrix (ECM) protein that is abundant in the body such as the skin providing strength, structural stability and has the ability to promote cell behaviour [17]. However, the use of collagen alone is not sufficient in promoting the “perfect” wound healing as collagen alone is unable to influence cell physiology and behaviour in wound healing. Instead, ECM components such as fibronectin and elastin are required as these components plays an essential role in the attachment of keratinocytes and re-epithelization [18]. In recent years, studies have been carried out to create wound dressing materials which mimic the structure and biological function of the native extracellular matrix (ECM) which are essential in supporting and regulating cellular activity [18-20]. In addition, the use of collagen for wound healing often results in the development of scars which is undesirable [3, 16, 18].

2.3.3 Sources of ECM

In recent years, the extracellular matrix (ECM) has received significant attention to be used as a scaffolding material [21]. It consists of a complex 3D architecture structure made up of largely collagen fibres and proteins (i.e. fibronectin, laminin) enabling it to have good bioinductive and biomechanical properties [22]. Various naturally derived wound dressings are already available in the market as seen in **Table 2.1**. These dressings are derived from either porcine or bovine which are xenogeneic sources which carries risks of disease transmission. Animal sources results in trace impurities and transmissible disease contamination and mineralization which are disadvantages of animal derived collagen, which is the main component of ECM [23]. In particular, collagen derived from calf skin or bone are risk-carry materials of bovine spongiform encephalopathy due to prion-contaminated collagen [24]. Animals have been known to harbour pathogens that may cause severe infections in humans or even death. It is unknown if there may be other undiscovered animal pathogens which may be lethal to humans [25]. Hence, additional

steps are required to inactivate such viruses. In addition, the presence of gal epitopes (cell surface molecules) commonly found in non-primate mammals such as porcine poses significant immunological concerns as there is a risk of immune rejection if not removed properly during the purification process [26]. Hence, the use of alternative sources of ECM to minimize such risks is a safer alternative. As seen in **Table 2.1**, ECM derived from cadaver have also been used commercially. However, these sources are limited in supply. Recently, the emergence of human adipose tissue as a source of ECM has shown that it has the potential to be used as an alternative source of wound dressing material [27].

Table 2.1 Sources of naturally derived ECM wound dressings [31]

Naturally derived ECM Wound Dressing	Source
Matristem® (ACell Inc.)	Porcine urinary basement membrane
Oasis® (OASIS Wound Matrix, Healthpoint Ltd, Ft Worth, Tex)	Porcine small intestinal submucosa
Primatrix™ (TEI Biosciences)	Fetal bovine dermis
Alloderm® (LifeCell Corporation)	Human dermis
DermaMatrix™ (Synthes)	Human dermis
GraftJacket® (Wright Medical Technology)	Human dermis

2.4 Human Adipose Tissue Derived ECM

Adipose tissue is a loose connective tissue with a rich source of extracellular matrix (ECM) that can be a potential material for wound healing [28]. It is regarded as a form of surgical waste from liposuction and reconstructive surgeries. Hence, making it a good waste-to-resource material.

Human adipose tissue derived ECM (Ad-ECM) has been found to contain several types of collagen (i.e. Type I, III and IV) which are essential for wound healing [28-33]. Other than ECM proteins, adipose tissue has also been found to have a high content of angiopoietin-

like 4 (ANGPTL4), a matricellular protein, as compared to other tissues as the expression of ANGPTL4 is strongly up-regulated during fasting and hypoxic conditions in adipocytes (**Figure 2.2**) [34, 36].

Figure 2.2 ANGPTL4 RNA expression in TPM (transcripts per million) from different tissue sources. Image credit: Human Protein Atlas

The presence of ANGPTL4 is advantageous to wound healing as it has been shown to be able to regulate scars [11, 35]. In a particular study carried out by Teo et al. and Zhu et al., the results showed that the ANGPTL4 interacts with cadherin-11 protein in scar tissue, regulating collagen (i.e. type 1 α 2 (COL1A2) and 3 α 1 (COL3A1)) production, and thus controlling scar formation [11, 35]. In addition, ANGPTL4 has also been shown to promote keratinocyte migration to the wound site as well as enhance angiogenesis, which could further promote the wound healing process [36]. Hence, ANGPTL4-rich Ad-ECM could potentially be a good wound dressing material to promote wound healing and yet regulate scar development.

2.5 TGF- β 1 Induced Fibroblast-to-Myofibroblast Differentiation

Fibroblast and myofibroblast play an essential role in wound healing. Fibroblast proliferate and migrate during the proliferative phase of wound healing forming a provisional matrix to fill the wound after which they are activated and acquires a smooth muscle cell-like

phenotype (α -SMA) which is the hallmark of myofibroblast. The major role of myofibroblast in wound healing is to reconstruct the extracellular matrix by synthesizing ECM proteins, matrix molecules and matrix modifying proteins. In addition, the hallmark α -SMA phenotype of myofibroblast is essential to facilitate wound closure by conferring contractile forces to myofibroblast to bring the edges of a wound together. However, excessive myofibroblast formation at the wound site will result in over production of the ECM and over contraction of the wound which is undesirable as it is a major cause of tissue fibrosis and scar formation which results in a disfiguring and dysfunctional dermis where the original flexibility and strength of the skin is lost [37, 40].

TGF- β 1 is a potent inducer of fibroblast-to-myofibroblast differentiation [41, 42]. It promotes upregulation of α -SMA and ECM production which is vital in promoting wound closure and matrix deposition as it synthesizes and deposits ECM components replacing the provisional matrix. TGF- β 1 is known to increase α -SMA expression through the Smad signalling pathway [43, 44]. As seen in **Figure 2.3**, when TGF- β 1 is activated, it binds to type II receptor, resulting in the phosphorylation of type I receptor which phosphorylates effector proteins (i.e. SMAD2 and 3). The phosphorylated protein then binds to SMAD4 forming a trimer complex which translocate into the nucleus and binds to the gene promoter which induces the transcription of α -SMA and ECM production [45-47]. Therefore, the use of TGF- β 1 to induce fibroblast-to-myofibroblast is a good *in-vitro* model to study the upregulation of α -SMA.

Figure 2.3 TGF-β1 induced fibroblast-to-myofibroblast differentiation

2.6 Summary

Wound healing involves a complex process which is susceptible to interactions which prevents a wound from healing. Hence, a wound dressing is needed to act as a barrier between the wound and the environment. Collagen has been found to be a highly desired material in wound dressings. However, it often results in the undesirable formation of scars due to myofibroblastic differentiation which is often induced by TGF- β 1 through the Smad signalling pathway. The primary goals in wound healing are to achieve rapid wound closure and to induce the skin to heal perfectly without a scar. Hence, ANGPTL4 rich Ad-ECM can be a possible alternative wound dressing material as Ad-ECM contains essential proteins for wound healing other than type I collagen, and ANGPTL4 which has been shown to be able to promote wound healing, angiogenesis and yet regulate scar formation. In addition, with the use of adipose tissue derived extracellular matrix (Ad-ECM) from humans for wound healing applications, there will be decreased risk of the transmission of xenogeneic diseases. Hence, with proper delipidation and decellularization along with the retention of essential proteins, Ad-ECM can be a potential wound dressing material.

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Chapter 3

Experimental Methodology

This chapter describes the detailed description of the experimental procedures used for the extraction of the Ad-ECM. It also includes the various types of material characterization techniques such as ATR-FITR, AFM, DSC and mechanical testing were used to study the fundamental material properties of the extracted products, and the functional studies, including cellular studies of Ad-ECM.

3.1 Rationale for Selection of Materials and Methods

Freshly excised lipoaspirate and abdominal tissue samples were used to extract the extracellular matrix as it is a clinical waste which is a good waste-to-resource material rich in structural extracellular matrix proteins and matricellular protein, ANGPTL4.

Human Dermal Fibroblast (HDF, ATCC® PCS-201-012™) is a type of cell found in the dermis of the skin. During the wound healing process, fibroblast differentiates into myofibroblast which often results in scarring when myofibroblast do not undergo the normal apoptosis due to over production and over contraction of the wound [1-4]. Hence, in this study, HDF was used to study the scar regulating property of Ad-ECM.

HaCaT (Division of Genetics of Skin Carcinogenesis, Germany Cancer Research Center), an immortalized human keratinocyte cell line is found in the epidermis of the skin. The major role of keratinocytes is to promote re-epithelization of the skin [5]. Hence, HaCaT was used to study the wound closure capability of Ad-ECM.

THP-1 (American Type Culture Collection Number TIB-202™), a free floating, non-adherent human monocyte cell line is present in the human body. Monocytes are usually found in blood capillaries. When, they diffuse out into connective tissues, they differentiate into macrophages. The role of macrophages is to engulf foreign bodies invoking an immune response. Hence, in this study, THP-1 monocyte was differentiated to macrophages and used to study the biocompatibility of Ad-ECM.

Oil Red O staining and PicoGreen dsDNA assay were used to determine the presence of lipids and cellular components in the extracted Ad-ECM to ensure that the extraction process is efficient in removing these components to lower risk of immunogenic response.

Differential scanning calorimetry was used to measure the thermal stability of the extracted Ad-ECM to ensure that it is higher than that of the human body temperature.

Immunostaining and SDS-PAGE were used to detect the preservation of structural ECM proteins in the extracted Ad-ECM, while western blot and ANGPTL4 ELISA assay was used to determine the retention of matricellular protein, ANGPTL4.

ATR-FTIR and AFM was conducted to access the quality of the extracted Ad-ECM. From the ATR-FTIR analysis, the preservation of the triple helical structure present in collagen type I, the most abundant structural protein, can be determined. AFM was used to detect the visible identifiable periodic structure of 67.0 nm characteristic to collagen to determine if the protein was denatured during the extraction process.

Presto blue® was used to measure the metabolic activity of cells to assess the cytocompatibility and cell proliferation of skin cells (i.e. HDF and HaCaT) on Ad-ECM. Live/dead imaging was used to determine the cell viability and adhesion of HDF and HaCaT on Ad-ECM.

TGF- β 1, a potent inducer of myofibroblastic differentiation was used to induce fibroblast-to-myofibroblast differentiation. After which, immunohistochemistry staining was carried out to observe changes in the morphology of HDF and presence of α -SMA, the primary hall mark of myofibroblast, while real time polymerase chain reaction (qPCR) was carried out to study the expression of myofibroblastic markers (i.e. α -SMA, Coll1 α 1 and SPARC), to assess the scar regulating property of Ad-ECM.

Collective migration assay and scratch assay were conducted for the wound closure studies to determine the, cell-material interaction and cell-cell interaction of Ad-ECM respectively.

3.2 Materials

3.2.1 Source of Ad-ECM

Freshly excised lipoaspirate and abdominal tissue samples were obtained from subcutaneous depot site of patients at Tan Tock Seng Hospital, Singapore following procedures established by NHG Domain Specific Review Board (DSRB 2011/01721 and

DSRB 2012/00071, respectively).

3.2.2 Chemical Reagents

All chemicals used for the experiments in this thesis were received and purchased from Sigma–Aldrich (Singapore), unless otherwise stated.

3.2.3 Cell Culture Reagents

All cell culture reagents used for the experiments in this thesis were received and purchased from Sigma–Aldrich (Singapore), unless otherwise stated.

3.2.4 Cell Lines

Human Dermal Fibroblast (HDF, ATCC® PCS-201-012™) and HaCaT (Division of Genetics of Skin Carcinogenesis, Germany Cancer Research Center), an immortalized human keratinocyte cell line, were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin-Streptomycin (Gibco®, Life Technologies™, USA). THP-1 (American Type Culture Collection Number TIB-202™), a free floating, non-adherent human monocyte cell line was cultured in Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 10% FBS and 1% Penicillin-Streptomycin.

3.3 Physical Decellularization Extraction Method

Ad-ECM was extracted via a physical decellularization method using phosphate buffer solution (PBS) according to the previously established protocol [6]. In brief, adipose tissue was cut into small pieces of 1 × 1 cm and washed with PBS until all the blood components have been completely removed. Next, fresh PBS was added to the adipose tissue at a ratio of 3 to 1 (max 40 g). The adipose tissue mixture was blended in an ice bath at 12,500 rpm for 5 min. The tissue suspension was subsequently centrifuged at 5300 rpm for 10 min

where the lower layer of the ECM components was collected. Subsequently, the upper layer of the oil components was subjected to another 2 cycles of blending and centrifugation process to fully extract Ad-ECM.

3.4 Material Characterization

3.4.1 Extraction Yield Percentage

The extraction yield percentage (yield %) of Ad-ECM was calculated using equation (1) which is the ratio of the products obtained to the initial amount of raw material [7, 8].

$$\text{Yield \% (Ad-ECM)} = M_f / M_i \times 100 \% \dots\dots\dots (1)$$

M_f = mass of Ad-ECM

M_i = mass of the adipose tissue

3.4.2 Oil Red O Staining

The lipid content of the sample was imaged using Oil Red O staining assay (Sigma Aldrich) according to the manufacturer's protocol. Briefly, Ad-ECM was dissolved in 0.5 M acetic acid and distilled water, respectively at 1 mg/mL. Subsequently, 200 μ L of the sample was casted onto the individual well of 24-well plate and air-dried. The Oil Red O stock solution was then prepared by reconstituting 60 mg of Oil Red O powder with 20mL of 100% isopropanol. The solution was then mixed well and left undisturbed for 20 minutes. Next, the Oil Red O working solution was prepared by adding 3 parts of Oil Red O stock solution to 2 parts of water. The solution was mixed well and left undisturbed for 10 minutes. After which it was filtered with a 0.2 μ m syringe filter (Thermo Scientific) and 500 μ L of Oil Red O working solution was added to each well (24-well plate) to stain any remaining lipid for 15 minutes. At the end of the incubation, the Oil Red O solution was removed and the sample was washed with distilled water 5 times before imaging using Primo Vert light microscope (Carl Zeiss, Germany).

3.4.3 PicoGreen dsDNA Assay

Quant-iTTM PicoGreen[®] dsDNA Reagent Kit (Invitrogen) is commonly used to quantify the amount of double stranded deoxyribonucleic acid (DNA) present within cells. The DNA content in the extracted Ad-ECM was analysed according to the manufacturer's instructions. Briefly, Ad-ECM was dissolved in 0.5M acetic acid and distilled water respectively at 10 mg/mL. Subsequently, 150 μ L of Quant-iTTM PicoGreen[®] reagent working solution, which was prepared by diluting Quant-iTTM PicoGreen[®] reagent with 1 $\times$ TE buffer (10mM Tris-HCl, 1mM EDTA, pH 7.5) working solution at 200-folds, was added to 150 μ L of sample. The sample was then incubated for 5 minutes at room temperature in dark condition. At the end of the incubation, the fluorescence intensity of the sample was measured using a SpectraMax M2 microplate reader at an excitation wavelength of 480nm and an emission wavelength of: 520nm. The DNA content was then calculated using a standard curve correlating the fluorescence intensity to the known concentration of DNA.

3.4.4 DSC Analysis

The thermal stability of Ad-ECM was assessed by DSC analysis. The sample was heated from 15 – 85 °C at a rate of 2 °C /min using DSC-Q10 Analyzer (TA Instruments, USA) against an empty reference pan under nitrogen atmosphere (50 mL/min). The denaturation temperature (T_d) was measured as the peak temperature of the endothermic peak.

3.5 Protein Analysis

3.5.1 Immunostaining

The presence of various essential proteins in Ad-ECM was determined by immunostaining. Briefly, Ad-ECM was dissolved in 0.5M acetic acid at 10 mg/mL, where 200 μ L of the sample was coated onto individual wells of a 24-well plate and air-dried for 18 h. Subsequently, the sample was incubated with rabbit anti-human primary antibodies against Type I (ab34710, Abcam, UK), Type III (ab7778, Abcam, UK), and Type IV (ab6586,

Abcam, UK) collagen, elastin (ab23747, Abcam, UK), fibronectin (ab23750, Abcam, UK) and laminin (ab11575, Abcam, UK) at a ratio of 1:500 in 1× PBS overnight at 4 °C. Subsequently, the sample was washed thrice with 1× PBS followed by staining with goat anti-rabbit secondary antibody (ab96885, Abcam, UK) for 60 min at 37 °C in dark condition. The sample was then washed thrice with 1× PBS and imaged using Zeiss Axio Observer Z1 inverted fluorescence microscope (Carl Zeiss, Germany) fitted with a camera at excitation wavelength of 561 nm and emission wavelength of 594 nm.

3.5.2 SDS-PAGE Analysis

The extracted Ad-ECM was subjected to Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) to separate and analyse the protein present according to a protocol established by Laemmli [9]. Briefly, 10 µg of the lyophilized Ad-ECM was added to 1× SDS Loading Dye and heated at 95 °C for 15 min. Subsequently, the sample was loaded to 10 % polyacrylamide gel where the migration was done with a current of 0.03A for 2h. After the electrophoresis process, the polyacrylamide gel was stained with Bio-Safe™ Coomassie Brilliant Blue R-250 (Bio-Rad, USA) and destained with 10 and 40% of methanol and acetic acid respectively until protein bands appear.

3.5.3 Western Blot Analysis

The presence of the ANGPTL4 was identified by Western blotting. Briefly, Ad-ECM was dissolved in 1 mL of PBS containing 10 µL phenylmethane sulfonyl fluoride (PMSF) and 0.1 µL SDS lysis buffer at 10 mg/mL. Subsequently, 50 µL of the sample was mixed with 10 µL 6× SDS loading dye and heated at 65 °C for 15 min. Protein samples were then resolved using 10% SDS-PAGE at 0.03 A for 90 min and electrotransferred onto Immobilon-FL polyvinylidene fluoride membrane (IPFL00010, Merck Millipore). The membranes were then blocked with 1× Odyssey Blocking buffer (#927–40000, LI-COR Biotechnology, USA) for 1 hour at room temperature before incubating with ANGPTL4 primary antibodies (Prof. Tan Nguan Soon) in 0.5× Odyssey Blocking buffer diluted with TBS (50 mmol/l Tris HCl, pH 7.6 and 150 mmol/l NaCl) overnight at 4 °C. At the end of

the incubation, membranes were washed thrice with TBS, and incubated with appropriate IRDye680 goat anti-rabbit IgG secondary antibodies (1:10,000, LI-COR Biotechnology) for 1 hour at room temperature. The membranes were then washed thrice with TBS and dried at 37 °C before imaging using the Odyssey CLx Infrared Imaging System.

3.5.4 ANGPTL4 Enzyme-linked Immunosorbent Assay (ELISA)

The ELISA method was used for quantification of ANGPTL4 present in Ad-ECM. The Human ANGPTL4 ELISA kit (Thermo Fisher Scientific) was performed according to the manufacturer's instructions. Briefly, all assay reagents from the kit, samples and standards were prepared as instructed. The following steps were carried out with 4x washes with wash buffer between each step. 100 μ L of standard or sample was added to each well of 96-well plate and incubated for 2.5 h at room temperature with gentle shaking. 100 μ L of biotinylated antibody prepared at 4°C was then added and incubated for 1 h at room temperature with gentle shaking. Next, 100 μ L of streptavidin working solution was added and incubated for 45 min at room temperature with gentle shaking. Subsequently, 100 μ L of TMB substrate reagent was added and incubated for 30 min at room temperature with gentle shaking in dark condition. Finally, 50 μ L of stop solution was added to each well to stop the reaction and the plate was read at 450 and 550 nm immediately using a SpectraMax M2 microplate reader. The standard curve was plotted and used to calculate the concentration of ANGPTL4 present in the samples.

3.5.5 ATR-FTIR Analysis

The conformational structure of the protein present in Ad-ECM was assessed by ATR-FTIR using Spectrum GX (Perkin Elmer Inc., USA). The ATR-FTIR spectrum of the sample was recorded from 4000 – 650 cm^{-1} at a resolution of 4 cm^{-1} . The peak intensity ratio was calculated using equation (2). The closer the ratio is to 1, the higher the quality of the extracted product [10].

$$\text{Peak intensity ratio} = A_{\text{Amide III}} / A_{1450} \dots\dots\dots (2)$$

$A_{\text{Amide III}}$ = absorbance peak intensity ratio at Amide III

A_{1450} = absorbance peak intensity ratio at 1450 cm^{-1}

3.5.6 Atomic Force Microscopy

AFM was used to analyse the topography of collagen fibres present in Ad-ECM using Cypher S atomic force microscope (Oxford Instruments, UK) in ambient air environment. The analysis was carried out using the tapping mode and 500mV set point so as to avoid any changes and damages to the sample surface. The scan rate of 1.0 Hz was used for a scan size of a square of area $(1.30 \times 1.30) \mu\text{m}^2$.

3.6 Cellular and *In-vitro* Wound Healing Studies

3.6.1 THP-1 Monocyte to Macrophage Differentiation

THP-1 monocytes (free floating cells) were treated for 48 h with 100 ng/mL of phorbol 12-myristate 13-acetate (PMA; Sigma) in 6-well cell culture plates to obtain THP-1 macrophages (adherent cells) according to previously established protocols [11, 12]. Briefly, cells were seeded at a density of 1×10^6 cells/well. After treatment, the differentiated, adherent cells were washed twice with fresh RPMI culture medium and incubated for another 24 h in the culture medium to obtain the resting state of macrophages (M0). M0 macrophages were seeded in 12-well plates (uncoated and coated with 10mg/mL of Ad-ECM) at a density of 5×10^5 cells/well.

3.6.2 THP-1 Macrophages Polarization

In order to polarize resting macrophages (M0) to M1 phenotype which induces cells to secrete pro-inflammatory cytokines (positive control), cells were treated using the following previously established protocols [11, 12]. Briefly, the resting macrophages (M0) were primed with fresh medium supplemented with $1 \mu\text{g/mL}$ of Lipopolysaccharides (LPS) and incubated for 6h to differentiate M0 macrophages into the M1 phenotype.

3.6.3 Cell Proliferation

In order to determine the cell cytocompatibility of Ad-ECM, HaCaT and HDF cells were seeded at a density of 10,000 cells/cm² onto well plates coated with 200µL of Ad-ECM. In this study, TCP was used as the control. Cell proliferation was measured using Presto Blue® cell viability reagent (Molecular Probes®, USA) following the manufacturer's protocol on days 1, 3, 5 and 7. The principle of Presto Blue® is through measuring the reduction of resazurin present in Presto Blue® reagent, to quantitatively measure cell proliferation. Resazurin is non-fluorescent and appears blue. When added to cells, the reducing environment of the viable cells reduces resazurin to resorufin which is highly fluorescent and appears red. Briefly, the cells were incubated with 10% Presto Blue® working solution for 30 min and the fluorescence intensity was measured using a SpectraMax M2 microplate reader at an excitation wavelength of 560 nm and an emission wavelength of 590 nm. The number of cells was then calculated using a standard curve correlating the fluorescence intensity to known number of cells.

The population doubling (PD) of the cells was also calculated using equation (3). The sum of the PDs was then plotted against time of culture to obtain the cumulative population doubling profile of the cells.

$$PD = [\ln(N_f) - \ln(N_i)] / [\ln(2)] \dots\dots\dots (3)$$

N_f = number of cells at respective time

N_i = number of cells on the previous time point

3.6.4 Cell Viability Imaging

Cell viability was imaged using LIVE/DEAD® staining. Briefly, the cells were stained with 200 µL of the working solution (i.e. 8 µL fluorescein diacetate and 50 µL propidium iodide in 5mL of serum-free cell culture medium) for 5 min and viewed under Zeiss Axio

Observer Z1 inverted fluorescence microscope (Carl Zeiss, Germany) fitted with a camera at an excitation wavelength of 488 nm and an emission wavelength of 517 nm.

3.6.5 TGF- β Treatment to Induce Myofibroblast Differentiation

In this study, TGF- β 1 was used as positive control to induce the differentiation of fibroblast to myofibroblast. Briefly, HDFs were seeded on Ad-ECM at a density of 10,000 cells/ cm² and allowed to proliferate for 3 days after which they were starved in serum free medium for 48 hours followed by TGF- β 1(10ng/mL) treatment for 72 hours.

3.6.6 Antibody Treatment

Anti-ANGPTL4 antibody (Prof. Tan Nguan Soon) was added at 0.1 μ g/mL prior to cell seeding.

3.6.7 Real time quantitative polymerase chain reaction (qPCR)

In order to determine the immunogenic response and scar regulating properties of Ad-ECM, mRNA was extracted from THP-1 macrophages and HDF respectively and was evaluated by real-time polymerase chain reaction (qPCR) according to the previously established protocol [13]. Real time quantitative polymerase chain reaction (qPCR) was used to determine expression of specific genes from mRNA obtained from cells cultured under different conditions by amplifying that specific gene sequence and determining the expression. Primer sequences as seen in **Table 3.1**, were checked for specificity using BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>), and melt curve analysis.

Briefly, mRNA was extracted from the harvested cells using RNeasy® Mini Kit (Qiagen, Germany) according to the manufacturer's protocol. Subsequently, 100 ng of the RNA was used for the synthesis of cDNA using Superscript® III First-Strand Synthesis Supermix (Bio-Rad, USA). The qPCR analysis was then carried out using Ssoadvanced universal SYBR green mastermix (Bio-Rad, USA) performed under a Bio-Rad C1000™ thermal cycle (Bio-Rad, USA) according to the manufacturer's protocol. All the gene and primers

used are listed in **Table 3.1**. The threshold cycle (C_t) values obtained were then used to calculate the gene fold expression using $\Delta\Delta C_t$ method as shown in equation (4) where 18S was used as the housekeeping gene.

$$\text{Gene fold expression} = 2^{-(\Delta C_t (\text{Control}) - \Delta C_t (\text{Experimental}))} \dots\dots\dots (4)$$

$\Delta C_t (\text{Control}) = \text{mean } C_q \text{ value (gene of interest)} - \text{mean } C_q \text{ value (housekeeping gene)}$

$\Delta C_t (\text{Experimental}) = \text{mean } C_q \text{ value (gene of interest)} - \text{mean } C_q \text{ value (housekeeping gene)}$

Table 3.1 Sequences of primers used for gene expression studies by HDF

Gene	Primer Sequence (5' to 3')
IL-1 β F	AAATACCTGTGGCCTTGGGC
IL-1 β R	TTTGGGATCTACACTCTCCAGCT
IL 6 F	GTAGCCGCCCCACACAGA
IL 6 R	CATGTCTCCTTTCTCAGGGCT G
IL 23 F	TGCAAAGGATCCACCAGGGTCTGA
IL 23 R	TAGGTGCCATCCTTGAGCTGCTGC
TNF- α F	ATGAGCACTGAAAGCATGATC
TNF- α R	TCACAGGGCAATGATCCCAAAGTAGACCTGCCC
ACTA2 F	CCGACCGAATGCAGAAGGA
ACTA2 R	ACAGAGTATTTGCGCTCCGAA
Coll α 1 F	GGGCAAGACAGTGATTGAATA
Coll α 1 R	ACGTCGAAGCCGAATTCCT
SPARC F	TGATGAGACAGAGGTGGTGG
SPARC R	AAGTGGCAGGAAGAGTCGAAG
18S F	GCGGCGGAAAATAGCCTTTG
18S R	ATCACACGTTCCACCTCATCC
GAPDH F	GAAGGTGAAGGTTCGGAGT
GAPDH R	GAAGATGGTGTGATGGGATTTC
RPL27 F	CGCAAAGCTGTCATCGTG

RPL27 R	GTCACCTTTGCGGGGGTAG
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3.6.8 Immunofluorescence Staining

Immunohistochemistry staining was carried out to visualize the cell morphology and to indicate fibroblast-to-myofibroblast differentiation tendency. After TGF- β Treatment, cells (i.e. HDF) were fixed with 4% paraformaldehyde (PFA) for 15 min at room temperature. The cells were permeabilized with 0.2% Triton-X 100 and subsequently blocked with 2% bovine serum albumin (BSA) for 60 min at room temperature. The samples were then incubated with 1:250 Alexa Fluor 488 α -SMA (Thermo Fisher Scientific) and 1:500 TRITC-conjugated phalloidin for 60 min at room temperature in dark condition. After incubation, samples were washed thrice with 1 $\times$ PBS and subsequently mounted using Fluoroshield™ with DAPI. Fluorescence images were acquired using Zeiss Axio Observer Z1 inverted fluorescence microscope (Carl Zeiss, Germany) fitted with a camera.

3.6.9 Collective Cell Migration

For collective cell migration studies, PDMS blocks were used to block half of the wells in 24 well plates while the other half was coated with Ad-ECM and Bv Col (control) and allowed to dry. HaCaT was seeded at 40 000 cells per well in (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin-Streptomycin. Upon confluence, cells were incubated in serum free medium for another 24hrs. Thereafter, PDMS blocks was gently removed and the cell movement onto the coated surface was monitored over 12 and 24 hrs using a Nikon eclipse ts100 inverted microscope. The cell migration was quantified by comparing the uncovered surface area at each time point to the original gap area at 0 h.

3.6.10 Wound Scratch Assay

In order to mimic re-epithelization due to keratinocyte migration during wound healing, an *in vitro* scratch wound assay was carried out using silicone culture inserts (Ibidi, Germany)

following previously established protocols [14]. Bovine type I collagen (Bv Col) was used as the control. Briefly, Ad-ECM and Bv Col was coated onto 24 well plates at a concentration of 1mg/mL and left to dry. Individual inserts with a cell-free gap ($500\ \mu\text{m} \pm 50\ \mu\text{m}$), was placed and attached to each well. HaCaT were seeded at a density of 35,000 cells per well of each insert and cultured in serum free DMEM with 1% penicillin-streptomycin in order to limit cell proliferation for 16hrs. Thereafter, the inserts were gently removed and the progression of the cell migration was monitored over 4, 8, 12 and 24 hrs using a Nikon eclipse ts100 inverted microscope. The cell migration was quantified by comparing the uncovered surface area at each time point to the original gap area at 0 h.

3.7 Statistical Analysis

All data were expressed as mean and standard deviation of the mean with 3 replicates ($n = 3$), unless otherwise specified. The significant level was analysed using Kruskal-Wallis non-parametric one-way analysis of variance and Mann-Whitney U test with $p < 0.05$ was considered statistically significant.

3.8 Overview of Methodologies

Summary of the overall strategy and the various characterization techniques used for this thesis are presented in the *Figure 3.1*.

Study A: Extraction and characterization of Ad-ECM	Study B: Influence of Ad-ECM on wound healing
<p>1) Fundamental characterization</p> <ul style="list-style-type: none"> • Yield % • Thermal stability analysis (DSC) • Oil Red O staining • Picogreen dsDNA Assay • Biocompatibility Analysis (qPCR) <p>2) Protein Analysis</p> <ul style="list-style-type: none"> • Immunostaining • Western blot (ANGPTL4) • ANGPTL4 (ELISA Assay) • ATR-FTIR (Collagen type I) • AFM 	<p>1) Cytocompatibility (HaCaT and HDF)</p> <ul style="list-style-type: none"> • Cell viability • Cell doubling • LIVE/DEAD imaging <p>2) Scar regulating property of Ad-ECM</p> <ul style="list-style-type: none"> • Immunohistochemistry staining • Qualitative polymerase chain reaction (qPCR) <p>3) Wound closure</p> <ul style="list-style-type: none"> • Collective cell migration • Scratch assay

Figure 3.1 Outline of experimental methodologies used in this thesis

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Chapter 4

Extraction and Characterization of Ad-ECM

This chapter first gives a short introduction on the purpose of this study and details the materials and methods used in this study. Next, it illustrates the fundamental characterization of the extracted Ad-ECM such as the extraction yield percentage and thermal stability, biocompatibility and protein analysis of Ad-ECM. Lastly, a brief conclusion will be given.

4.1 Introduction

Adipose tissue is found abundantly in the human body and is considered a clinical waste from procedures such as liposuction and reconstructive surgeries. Therefore, it can be a potential waste-to-resource material. It is composed of 60 to 85% of adipocytes, 5 to 30% of water and 5 to 10% of ECM [1]. Recent studies have shown that adipose tissue derived ECM (Ad-ECM) is able to promote wound healing [2, 3]. However, these studies are only a proof of concept where pro-healing mechanisms and in depth understanding of Ad-ECM in wound healing were not study. Hence, in this study, Ad-ECM was extracted and characterized to determine the yield percentage, thermal stability, biocompatibility and protein types present in Ad-ECM, which are important in understanding biophysical and biochemical properties of the extracted Ad-ECM.

4.2 Experimental Methods

For the experimental methods used in chapter 4, please refer to *Table 4.1*.

Table 4.1 Experimental Methods used in Chapter 4

Description		Section
Extraction	Physical Decellularization	3.3
Characterization	Yield %	3.4.1
	Thermal stability analysis	3.4.4
	Oil Red O staining	3.4.3
	PicoGreen dsDNA assay	3.4.4
Biocompatibility analysis	THP-1 monocyte differentiation	3.6.1
	Polarization of THP-1 macrophage	3.6.2

	qPCR	3.6.7
Protein analysis	SDS-PAGE analysis	3.5.2
	ATR-FTIR	3.5.5
	AFM	3.5.6
	Immunostaining	3.5.1
	Western blot analysis	3.5.3
	ANGPTL4 ELISA	3.5.4

4.3 Results and Discussion

4.3.1 Physical Characterization of Ad-ECM

Ad-ECM was extracted from adipose tissue and the potential of the extracted product as a scaffolding material for biomedical applications was assessed in terms of its physical properties. The extracted Ad-ECM is as shown in **Figure 4.1** where it has a white appearance which is an indication that the cleaning and physical extraction process were effective in removing the blood and lipid contents from the adipose tissue. The yield % of the extracted product was calculated based on the weight ratio of the final product (i.e. decellularized Ad-ECM) to the raw material (i.e. adipose tissue). The yield % of Ad-ECM is $14.37 \pm 2.20\%$ which is relatively low. This could be due to adipose tissue having a high lipid and low protein content.

In order for a material to be suitable for use in biomedical applications, a high T_d is essential as it prevents rapid degradation when in contact with the human body (37°C). This prolongs the mechanical strength and increases the lifespan of the material. The thermal stability of the extracted Ad-ECM was measured using DSC analysis. From the DSC analysis, the denaturation temperature (T_d) of the extracted Ad-ECM was found to be $62.48 \pm 1.11^\circ\text{C}$ which shows that it has good thermal stability for tissue engineering.

Figure 4.1 Successful extraction of ECM from human adipose tissue waste. Schematic representation of the extraction of Ad-ECM using a chemical-free decellularization method. Scale bar, 1cm.

4.3.2 Confirmation of Delipidation and Decellularization of Ad-ECM

The trace amount of remaining lipid and cellular components in the extracted Ad-ECM was characterized by Oil Red O staining and PicoGreen dsDNA assay respectively. As seen from **Figure 4.2a**, lipid droplets were visible after staining with Oil Red O on the adipose tissue. However, after the physical delipidation process, no visible lipid droplets were being observed in the extracted Ad-ECM. This is an indication that the lipid components were successfully removed from the raw material. The lipid can be easily removed through separation with simple centrifugation as lipid is much lighter than ECM. Hence, after centrifugation, the lipid can be discarded as the supernatant.

Subsequently, PicoGreen dsDNA assay was carried out to determine the residual DNA left in the extracted Ad-ECM (**Figure 4.2b**). As seen from the figure, the amount of dsDNA present in Ad-ECM dissolved in acetic acid was found to be 0.194 ± 0.038 ng/mg, which is within the acceptable range of 50 ng/mg for clinical applications [4]. The same assay was carried out by suspending the extracted Ad-ECM in distilled water, and the amount of

dsDNA was found to be 0.261 ± 0.088 ng/mg which is within the threshold value stipulated for clinical applications. Hence, the results show the potential of the extracted Ad-ECM as a material for biomedical applications.

Figure 4.2 Confirmation of delipidation and decellularization of Ad-ECM. a) Oil Red O staining shows Lipid (red stains) successfully removed. Scale bar, 100 μ m. b) PicoGreen assay shows dsDNA present is much lower than acceptable range of 50ng/mg.

4.3.3 Biocompatibility Analysis

From the results obtained from the Oil Red O staining and PicoGreen dsDNA assay, it was shown that the extracted Ad-ECM was successfully delipidated and decellularized. This suggests that there is a lower risk of immunogenic response. It is essential for a biomaterial to elicit minimum foreign body response and toxic effect. Macrophages play a key role in the phagocytosis of cellular debris and pathogens, as well as in the foreign body responses resulting from events such as biomaterial implantation. Upon contact with a foreign body object, macrophages will actively adhere to the material and try to phagocytise the foreign body, and in the process result in the secretion of pro-inflammatory cytokines such as TNF-

α , IL-1 β and IL-6 [5, 6]. Hence, in this study THP-1 monocytes were differentiated to macrophages (M0) using PMA and cultured onto Ad-ECM.

In order to study whether Ad-ECM would elicit an immune response, RT-qPCR was carried out to determine the production of pro-inflammatory cytokine by the macrophage. As a positive control, when treated with lipopolysaccharide (LPS), the macrophage can be induced to adopt a polarized M1 phenotype, which is characterized by the overproduction of pro-inflammatory cytokines such as TNF- α , IL-1 β , IL-6 and IL-23 [7, 8].

In this study, the morphology of cells cultured on Ad-ECM was similar to that of the control (TCP) whereas, cells on the positive control (TCP LPS) had increased spreading (**Figure 4.3a**). From the qPCR results, it was observed that Ad-ECM had similar gene expression of pro-inflammatory cytokines: IL-1 β (**Figure 4.3b**), IL 6 (**Figure 4.3c**), IL 23 (**Figure 4.3d**) and TNF- α (**Figure 4.3e**) to the control which is much lower than that of the positive control. Hence, it can be concluded that Ad-ECM is biocompatible as it does not elicit immune response.

Figure 4.3 Ad-ECM does not elicit immunogenic response. (a) Macroscopic images illustrating THP-1 macrophage after differentiation from THP-1 monocytes. Scale bar, 50 μ m Relative gene expression of pro-inflammatory cytokines (b) IL-1 β (c) IL 6, (d) IL 23 and (e) TNF- α .

4.3.4 Protein Analysis

Adipose tissue is composed of ground substance and fibers. However, to be used for wound healing, presence of Type I collagen is essential [9, 10]. The presence of type I collagen in Ad-ECM was characterized by SDS-PAGE analysis. As seen from **Figure 4.4**, three distinct bands were observed, including a β -band near 200 kDa and 2 α -bands (i.e. α_1 - and α_2 -chains) near 130 kDa, similar to that of commercially available type I bovine collagen (Bv Col). In addition, the width ratio of the α_1 - and α_2 -chains was found to be approximately 2:1, which confirms that Ad-ECM contains type I collagen [11, 12].

Figure 4.4 SDS-PAGE analysis shows that extracted Ad-ECM contains type I collagen [A: Ladder, B: Ad-ECM, C: Bv Col]

The quality of the extracted Ad-ECM was assessed by ATR-FTIR analysis. In general, the structural stability of the protein is highly dependent on the formation of the triple helix. Hence, the quality of the extracted product can be deduced by measuring the amount of triple helical structure retained after the extraction process. As seen in **Figure 4.5a**, the various characteristic amide peaks of collagen were detected for Ad-ECM. In general, the peaks of Amide A, Amide B, Amide I, Amide II and Amide III were near 3300, 2920, 1630, 1550 and 1200 cm^{-1} respectively [13, 14]. Subsequently, the retention of the triple helical structure was assessed by calculating the absorbance peak intensity ratio of Amide III to 1450 cm^{-1} . The absorbance peak intensity at 1450 cm^{-1} was used for the calculation

as it has been found to be a useful internal standard as it remains unchanged even after the denaturation of collagen [14, 15]. The peak intensity ratio of Amide III to 1450 cm^{-1} for Ad-ECM was calculated to be 1.03 ± 0.03 (**Figure 4.5b**). The peak intensity ratio is approximately 1, thus indicating that the triple helical structure is being preserved without denaturation during the extraction process [15]. Subsequently, this was confirmed by conducting AFM which shows the retention of the triple helical structure of collagen with identifiable periodic structure of 67.0 ± 2.6 nm (**Figure 4.5c**). This shows that the physical extraction method of Ad-ECM is highly effective as it does not involve harsh chemicals and the process was carried out on ice, allowing the maximum preservation of the quality of the extracted Ad-ECM.

Figure 4.5 Chemical analysis showing a) ATR-FTIR spectra of Ad-ECM b) Retention of triple helical structure of collagen in Ad-ECM c) AFM confirms the preservation of the triple helical structure of collagen.

The extracellular matrix is known to contain various types of protein such as collagen I, III, IV, elastin, fibronectin and laminin which are essential in promoting wound healing (**Table 4.2**) [16, 17]. Hence, immunostaining was carried out to assess the presence of these essential proteins in the extracted Ad-ECM. As seen in **Figure 4.6**, all the key proteins were well preserved in the extracted Ad-ECM [18]. Hence, this shows that Ad-ECM has huge potential as a wound healing material.

Table 4.2 Essential proteins found in Ad-ECM for wound healing

Components	Physiological function	Ad-ECM
Collagens		
Type I, III, IV	Provides structure and support and promotes cell adhesion and migration	✓
Elastin	Confers tissue elasticity	✓
Glycoproteins		
Fibronectin	Directs organization of interstitial ECM and, mediates mediating cell attachment, proliferation and migration	✓
Laminin	Influences cell adhesion, proliferation and migration	✓

Figure 4.6 Results from immunostaining of Ad-ECM shows the retention of Col I, III, IV, elastin, fibronectin and laminin. Scale bar, 500 μ m.

In addition, western blot analysis was also carried out to investigate the presence of a matricellular protein, ANGPTL4, which is known to be abundantly present in adipose tissue. In general, ANGPTL4 exhibits a molecular weight in the range between 45 to 65 kDa [19]. As seen in **Figure 4.7**, a distinct band was being detected near 65kDa, thus confirming the presence of ANGPTL4. Subsequently, the amount of ANGPTL4 present in Ad-ECM was characterized by conducting ANGPTL4 ELISA assay. The concentration of ANGPTL4 present in Ad-ECM was found to be 14.07 ± 2.28 pg/mg. ANGPTL4 has been shown to be able to regulate collagen production and the orientation of the collagen formation, thus regulating fibrosis (i.e. scarring) [19, 20]. Furthermore, ANGPTL4 has also been found to be able to promote wound healing by helping keratinocyte migration to the wound site and enhancing angiogenesis [21, 22].

Conc. = 14.07 ± 2.28 pg/mg

Figure 4.7 Western blot confirms the presence of matricellular protein (ANGPTL4) in Ad-ECM [A: Ladder, B: ANGPTL4]

4.4 Summary

Overall, we showed that human lipoaspirate “waste” is an excellent source of numerous valuable ECM components [23-26]. It has good thermal stability and biocompatibility which is essential for a biomaterial. Specifically, the extraction method used for Ad-ECM was effective in retaining essential proteins such as collagen type I, III, IV, elastin,

fibronectin, laminin and ANGPTL4. Particularly, the retention of ANGPTL4 in Ad-ECM from the extraction process is highly beneficial for use in clinical applications as it is able to promote cell proliferation, angiogenesis and has anti-fibrotic properties. In addition, the extracted Ad-ECM has suitable thermal stability and mechanical properties for use as a wound healing material. Hence, Ad-ECM has the potential to be a superior wound dressing material.

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Chapter 5

Influence of Ad-ECM on Wound Healing

This chapter first gives a short introduction on the purpose of this study and delineates the materials and methods used in this study. Next, it describes the wound healing potential of Ad-ECM where the cytocompatibility of Ad-ECM on skin cells is first discussed followed by the scar regulating properties and re-epithelization capabilities of Ad-ECM. Lastly, a brief conclusion will be given.

5.1 Introduction

The goals of an ideal wound dressing is to obtain fast re-epithelization and to minimize scar formation [1, 2]. Ad-ECM has been found to contain various essential structural proteins for wound healing such as Collagen type I, III and IV, elastin, fibronectin and laminin. In addition, it is a rich source of matricellular protein, ANGPTL4, which has been shown to be able to regulate scar formation and to promote keratinocyte migration and proliferation accelerating the re-epithelization process [3-6]. Hence, in this study skin cells (i.e. HDF and HaCaT) were cultured on Ad-ECM to investigate the adhesivity and cytocompatibility of the processed Ad-ECM. Next, the scar regulating property of Ad-ECM was studied using HDF to study the effect of Ad-ECM on suppressing myofibroblastic differentiation. Following which, the wound closure capability of Ad-ECM was studied using HaCaT cells to determine the influence of Ad-ECM on re-epithelization.

5.2 Experimental Methods

For the experimental methods used in chapter 5, please refer to *Table 5.1*.

Table 5.1 Experimental Methods used in Chapter 5

Description		Section
Cytocompatibility analysis	Cell proliferation	3.6.3
	Cell viability Imaging	3.6.4
Scar regulating property of Ad-ECM	TGF- β 1 induced myofibroblastic differentiation	3.6.5
	Antibody treatment	3.6.6
	Immunofluorescence staining	3.6.8
	qPCR	3.6.7

Wound closure capability of Ad-ECM	Collective cell migration	3.6.9
	Wound scratch assay	3.6.10

5.3 Results and Discussion

5.3.1 Cytocompatibility Analysis

Cytocompatibility study was carried out to examine the ability of Ad-ECM to support cell adhesion and proliferation which are important parameters for wound dressing materials [7]. In this study, skin cells such as HDFs (*Figure 5.1a-c*) and HaCaTs (*Figure 5.1d-f*) were used to assess the cell-material interactions of the extracted Ad-ECM by evaluating the attachment and the proliferation of the cells over 7 days of culture. Subsequently, the viability and the distribution of HDFs on Ad-ECM were imaged on day 7. TCP was used as the control. As seen from *Figure 5.1a and d*, a positive cell proliferation was observed for Ad-ECM, similar to the TCP control. When the cell proliferation rate was being evaluated, it was observed that Ad-ECM supported better proliferation of HaCaTs and HDFs as compared to TCP with higher cell doubling rate (*Figure 5.1b and e*). This suggests that Ad-ECM has good cell-material interaction for long term applications. The distribution of HaCaTs and HDFs was being imaged on day 7 (*Figure 5.1c and f*). As seen from the figures, cells were able to adhere well to Ad-ECM where a confluent number of viable cells were being observed. This suggest that the extraction process of Ad-ECM is mild and sterilizable enabling it to support cell adhesion and proliferation, thus suggesting the potential applicability of the extracted products.

Figure 5.1 Ad-ECM has excellent cytocompatibility supporting the attachment and proliferation of Human Dermal Fibroblast (HDF) and keratinocyte (HaCaT) cells. (a,d) Prestoblu cell proliferation assay over 7 days. (b,e) Cell doubling over 7 days. (c,f) LIVE/DEAD staining after 7 days of in vitro culture. : (a-c) HDF (d-f) HaCaT with TCP as the control. Scale bar, 200 μ m.

5.3.2 Ad-ECM Partially Suppresses TGF- β 1 Induced Myofibroblast Differentiation

A major goal of wound healing is to induce the skin to heal with minimum scarring [2]. In this study, the potential of Ad-ECM as a multifunctional wound healing material to induce wound closure and to minimize scar formation is being studied. A scar is formed during the tissue remodeling phase of wound healing whereby fibroblast differentiates into myofibroblast. The hallmark of fibroblast-to-myofibroblast differentiation is the production of α -SMA which causes the differentiated fibroblast to exhibit contractile forces enabling it to contract to close the wound. This process is necessary for wound closure. However, over production of α -SMA results in the formation of scars which is aesthetically unpleasant and at the same time prevents the skin from regaining its original function.

Adipose tissue has been found to contain a matricellular protein, ANGPTL4. Recent studies have shown that ANGPTL4 is able to regulate scars [3, 8]. Hence, it was hypothesized that the extracted Ad-ECM may be able to suppress the upregulation of myofibroblast-associated marker, α -SMA.

5.3.2.1 TGF- β 1 Induced Fibroblast-to-Myofibroblast Differentiation

Transforming Growth Factor-beta (TGF- β 1) is a growth factor that stimulates the differentiation of fibroblasts to myofibroblast [9, 10]. Hence, to verify this model, HDF cultured on TCP were treated with varying concentrations of TGF- β 1 (0, 1, 2.5, 5, 10 ng/mL) to induce fibroblast-to-myofibroblast differentiation. qPCR was carried out to determine the gene expression of α -SMA (ACTA2), a primary myofibroblast-associated marker, which was normalized to HDFs cultured on TCP without TGF- β 1 treatment. As seen in **Figure 5.2a**, HDFs were being successfully differentiated into myofibroblast as there was an increasing upregulation of α -SMA (1-, 8.4-, 16.5-, 18.0- and 70.9 fold) with increasing concentrations of TGF- β 1, with 10ng/mL of TGF- β 1 showing the highest upregulation of α -SMA. Changes in the morphology of HDFs after TGF- β 1 treatment were also observed, where cells had increased the spreading and thickening of F-actin fibers (**Figure 5.2b-d**). Hence, the differentiation model was proven to be successful and was used for the rest of the study.

Figure 5.2 TGF- β 1 model induces myofibroblast differentiation. (a) Relative gene expression of myofibroblast-associated marker α -SMA normalized to TCP shows that varying concentrations of TGF- β 1 (0, 1, 2.5, 5, 10 ng/mL) results in an increase in upregulation of α -SMA (b) Representative immunofluorescence images of HDFs cultured on TCP (ii) with and (i) without TGF- β 1 treatment costained for F-actin (red) and cell nucleus (blue). Scale bar, 300 μm . Change in cell morphology from (i) spindle-like elongated cells to (ii) dense cell clusters with increased (c) spreading area and (d) F-actin stress fibers in HDFs cultured on TCP after TGF- β 1 treatment are hallmarks of myofibroblast differentiation.

5.3.2.2 Cell Morphology and Expression of α -SMA

The changes in morphology of the cells were observed using fluorescence imaging for F-actin fibers. As seen in **Figure 5.3a** only slight changes in morphology of cells cultured on Ad-ECM after TGF- β 1 treatment were observed, with non-significant (p-value < 0.005) increase of cell spreading area of $3337.55 \pm 935.97 \mu\text{m}^2$ to $3586.79 \pm 914.62 \mu\text{m}^2$ (**Figure 5.3b**). This is unlike that of HDFs cultured on TCP where undifferentiated fibroblasts exhibited dendritic spindle-like morphology with a spreading area of $3929.72 \pm 945.77 \mu\text{m}^2$ whereas myofibroblast-like cells formed dense clusters with increased spreading of $5226.52 \pm 921.74 \mu\text{m}^2$ after TGF- β 1 treatment (**Figure 5.3b**). In addition, the intensity of the staining with TRITC-conjugated phalloidin (**Figure 5.3c**) which shows the organization of intracellular bundles of F-actin stress fibers were observed to be more prominent in the TGF- β 1 treated fibroblast (16.06 ± 3.48 a.u) as compared to that of untreated fibroblast (9.43 ± 2.46 a.u.) which is an indication of myofibroblastic differentiation [11-13].

In addition to F-actin staining, staining for α -SMA was also carried out as it is the primary hallmark of myofibroblast. As seen in **Figure 5.3a**, presence of α -SMA was only observed in TGF- β 1 treated cells cultured on conventional TCP but not on the Ad-ECM nanofibrous mat. Hence, suggesting that Ad-ECM is able to suppress myofibroblast differentiation

Figure 5.3 Ad-ECM prevents morphological changes in HDF and presence of α -SMA (a) Representative immunofluorescence images of HDFs cultured on Ad-ECM and TCP, with and without TGF- β 1 treatment triple stained for F-actin (red), α -SMA (green) and cell nucleus (blue). Scale bar, 50 μ m. Changes in (b) Cell Spreading area and (c) F-actin intensity of HDF cultured on Ad-ECM before and after TGF- β 1 has no significant changes.

5.3.2.3 Expression level of Myofibroblastic mRNA transcripts

Besides observing the morphological changes of the cells, qPCR was also carried out using various myofibroblastic markers, α -SMA, $\text{Col1}\alpha 1$ and SPARC, to further study the scar regulating property of Ad-ECM. As seen in **Figure 5.4a**, HDFs cultured on Ad-ECM (2.3 folds) shows a higher gene expression of α -SMA as compared to that of TCP (1 fold). However, with the addition of TGF- $\beta 1$, Ad-ECM (2.2 folds) is able to prevent further upregulation of α -SMA as compared to that of TCP (70.9 folds). A similar trend was observed for other myofibroblastic markers as seen in **Figure 5.4b**, where the expression of $\text{Col1}\alpha 1$ in HDFs cultured on Ad-ECM before and after TGF- $\beta 1$ treatment was 1.3 and 3.4 folds respectively whereas that of TCP before and after TGF- $\beta 1$ treatment was 1 and 19.2 folds respectively. Also, as seen in **Figure 5.4c**, the upregulation of the expression of SPARC, another myofibroblastic marker, was significant in the positive control but not in HDFs cultured on Ad-ECM. The gene expression of SPARC in HDFs cultured on Ad-ECM before and after TGF- $\beta 1$ treatment was found to be 0.4 and 0.9 folds respectively while that of HDFs cultured on TCP before and after TGF- $\beta 1$ treatment was found to be 1 and 4.6 folds respectively.

This shows that Ad-ECM has the potential to prevent the formation of scars. Studies have shown that the expression of $\text{Col1}\alpha 1$ and SPARC is upregulated in myofibroblast [11-13]. In this study, it was shown that Ad-ECM is able to suppress the expression of these myofibroblastic markers in addition to α -SMA, the primary hallmark of myofibroblast. α -SMA in the tissue remodeling phase of wound healing is still needed for wound closure. However, overproduction is not desired as α -SMA confers contractile forces to the cells which will result in excessive contraction of the wound which is undesirable as it is a major cause of tissue fibrosis and scar formation which will result in a disfiguring and dysfunctional dermis where the original flexibility and strength of the skin is lost [14-17]. Hence, with only a slight upregulation of α -SMA, the wound will still be able to close without excessive contraction of the wound preventing the formation of scar.

Figure 5.4 Ad-ECM prevents excessive upregulation of myofibroblastic markers. Relative gene expression of myofibroblast-associated marker (a) α -SMA (b) Coll α 1 (c) SPARC normalized to TCP shows that Ad-ECM reduces the upregulation of myofibroblastic markers when HDFs were treated with TGF- β 1 (10ng/mL) to induce myofibroblast differentiation.

5.3.3 Role of ANGPTL4

5.3.3.1 Cell Morphology and Expression of α -SMA

From the results above, it was shown that Ad-ECM was able to prevent the upregulation of α -SMA in HDFs treated with TGF- β 1. In previous studies, ANGPTL4 has been shown to be able to decrease gene expression of myofibroblast markers [18]. Therefore, it was hypothesized that the anti-myofibroblastic differentiation properties of Ad-ECM could be attributed to the presence of ANGPTL4 in the extracted Ad-ECM. Hence, in order to determine if the presence of ANGPTL4 prevents fibroblast-to-myofibroblast differentiation and thus expression of α -SMA, Ad-ECM was coated onto TCP plates and ANGPTL4 was blocked using ANGPTL4 antibody. HDFs were then cultured and treated with TGF- β 1. As seen in **Figure 5.5a** changes in morphology of cells cultured on Ad-ECM after blocking of ANGPTL4 with TGF- β 1 treatment were observed, with a significant increase in cell spreading area of $3586.79 \pm 914.62 \mu\text{m}^2$ to $4403.27 \pm 1065.00 \mu\text{m}^2$ (**Figure 5.5b**), and f-actin intensity of 8.38 ± 1.37 a.u. to 10.39 ± 2.64 a.u. (**Figure 5.5c**). However, from the α -SMA staining, there was no presence of α -SMA. Hence, in order to confirm the role of ANGPTL4 in suppressing myofibroblastic differentiation, qPCR was carried out.

Figure 5.5 ANGPTL4 plays role in suppressing myofibroblastic differentiation. (a) Representative immunofluorescence images of HDFs cultured on Ad-ECM and TCP, with and without TGF- β 1 treatment triple stained for F-actin (red), α -SMA (green) and cell nucleus (blue). Changes in (b) Cell Spreading area and (c) F-actin intensity of HDF, $n = 15$, Mann–Whitney U test.

5.3.3.2 Expression levels of Myofibroblastic mRNA transcripts

As seen in **Figure 5.6a**, a higher upregulation of α -SMA was found for HDFs cultured on Ad-ECM coated plates treated with ANGPTL4 antibody after TGF- β 1 treatment of 10.6 folds as compared to that of HDFs cultured onto Ad-ECM plates without ANGPTL4 antibody treatment of 2.2 folds. This trend was also observed in other myofibroblastic markers (i.e. Coll α 1 and SPARC). As seen in **Figure 5.6b and c**, where there was an increase in upregulation of Coll α 1 from 3.3 to 6.0 folds and SPARC from 0.9 to 1.7 folds after the blocking of ANGPTL4. It was also observed that the upregulation of α -SMA, Coll α 1 and SPARC was much higher in samples where HDFs were cultured on TCP with TGF- β 1 treatment. This shows that Ad-ECM itself is able to prevent extreme upregulation of α -SMA. This could be due to (in part) the presence of the ECM proteins (i.e. Col I, III, IV, elastin, fibronectin and laminin) and growth factors released from the extracted Ad-ECM which may provide signals to mediate ECM synthesis and thus resulting in less fibroblast-to-myofibroblast differentiation [19]. Hence, with the presence of ECM proteins and ANGPTL4, Ad-ECM is able to prevent fibroblast-to-myofibroblast differentiation.

Figure 5.6 ANGPTL4 present in Ad-ECM prevents excessive upregulation of myofibroblastic markers. Relative gene expression of myofibroblast-associated marker (a) α -SMA, (b) Coll α 1 and (c) SPARC normalized to TCP shows that blocking ANGPTL4 results in an upregulation myofibroblastic markers when HDFs were treated with TGF- β 1 (10ng/mL) to induce myofibroblast differentiation.

Overall, our findings suggest that Ad-ECM may have an intrinsic anti-scarring modulatory property, which could in part be attributed to the presence of ANGPTL4.

5.3.4 Cell Migration Study

Another major goal of wound healing is to achieve rapid wound closure [1, 2]. In order to study the effect of cell-material interaction on influencing cell migration, well plates were half coated with Ad-ECM and the other half was blocked with PDMS to ensure that the TCP surface remains uncoated as seen in **Figure 5.7a**. Here, Bv Col (type I) was used as the control as it is being widely used in wound healing substrates and type I collagen is known to be the gold standard for wound healing [20, 21]. The collective cell migration was assessed by culturing HaCaT cells onto the pristine TCP surface as seen in **Figure 5.7a** to ensure that the only difference is the interaction between the cell and material. The movement of cells onto the coated surfaces were observed over 24h. As seen from **Figure 5.7b and c**, similar trends of collective cell migration was being observed on both Ad-ECM and Bv Col which shows that Ad-ECM has comparable influence on attracting cells towards itself resulting in similar cell migration rate as BV Col. This suggests that Ad-ECM can be a good alternative to Bv Col.

Figure 5.7 Ad-ECM has similar cell-material influence on cell migration as Bv Col. (a) Schematic illustration of experimental design. (b,c) Cell migration of HaCaT from TCP onto Ad-ECM and Bv Col were monitored over 24 hours (white line: initial cell position; red line: migrated cell position) with Bv Col type I as the control. Scale bar, 250 μm .

Subsequently, the cell-cell interaction on Ad-ECM was studied by conducting a scratch assay where wound closure rate of HaCaT on Ad-ECM was compared to that of Bv Col to study the rate of re-epithelization. A scratch assay is an *in vitro* test usually carried out by creating a scratch on a monolayer of cells using a pipette tip to study the migration of cells to close a “wound” [22]. However, for this study, the creation of a scratch will cause damage to the coated surface. Hence, a PDMS insert was used to create a cell-free gap in between a monolayer of cells (**Figure 5.8a**). Upon removal of the PDMS insert, cells will be able to migrate over the coated surface to close the gap [23]. As seen in **Figure 5.8b**, an

initial cell-free gap of $500 \pm 50 \mu\text{m}$ was obtained on Ad-ECM and Bv Col and the cells were allowed to migrate over 24 hours (**Figure 5.8b and c**). The wound closure rate of HaCaT over Ad-ECM was faster than Bv Col over the first 20 hours (**Figure 5.8d**). Such cell-type dependence observation may be attributed to the presence of the extracellular matrix protein, ANGPTL4, which has been found to be able to promote wound healing by helping keratinocyte migration to the wound site [4, 5]. This phenomenon was observed only in the scratch assay and not the collective cell migration. This could be attributed to paracrine effect due to the design of the experiment whereby in the scratch assay, cells are seeded directly onto the material with a gap in between. In addition, enzymes secreted by the cells could have induced degradation of the coated material [24]. In the case of Ad-ECM, the degradation of Ad-ECM may have resulted in the release of ANGPTL4, thus influencing the migration of keratinocytes enabling faster re-epithelization. This is unlike the collective migration assay where cells are seeded onto pristine TCP instead of the coated material. Hence, there is no release of ANGPTL4 until cells migrate onto the coated surface which explains the slight increase in migration rate of keratinocytes only after 12 hours for Ad-ECM as compared to Bv col as seen in **Figure 5.7c**. Overall, the wound closure rate of HaCaT on Ad-ECM is faster than that of commercially available Bv Col which shows that Ad-ECM is able to obtain faster re-epithelization making it an ideal material for wound healing.

Figure 5.8 Ad-ECM promotes cell migration. (a) Schematic illustration of experimental design HaCaT cultured on Ad-ECM and Bv Col with (c) an initial gap of around 500 μm to monitor (b,d) cell migration over 24 hours (white line: initial gap length; red line: migrated cell position) with Bv Col type I as the control. Scale bar, 250 μm .

5.4 Summary

Overall, Ad-ECM is cytocompatible to skin cells (i.e. HDF and HaCaT) as cells were able to proliferate over 7 days and the cell proliferation was even better than that of TCP. Besides being able to enhance cell proliferation, Ad-ECM is able to suppress myofibroblastic differentiation where there were no changes in cell morphology and

upregulation of myofibroblastic markers such as α -SMA, Col 1 α 1 and SPARC. It was also observed that HaCaT cells migrated faster in the presence of Ad-ECM as compared to Bv Col which suggests that faster re-epithelization can be achieved. This could be attributed (in part) to the presence of proteins such as collagen type I, III, IV, elastin, fibronectin, laminin which are essential for wound healing (**Table 4.2**) and also presence of ANGPTL4 which has been found to be able to regulate scar formation and promote keratinocyte migration.

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Chapter 6

Conclusion and Future Work

This chapter concludes the findings from the studies carried out in this thesis and discusses other reconnaissance work not included in the main chapters which were helpful in further understanding the results obtained in the studies conducted in the main chapters. Lastly, outstanding questions will be discussed for the potential future works.

6.1 Conclusion

This dissertation aims to address the issues of re-epithelization and scar formation for an ideal wound dressing. Wound dressings typically made up of type I collagen are insufficient in supporting effective wound healing as it is unable to create a microenvironment similar to that of the skin to promote efficient wound healing with minimal scar formation and fast re-epithelization. Hence, in recent years there has been the emergence of the use of naturally derived extracellular matrix as a material for wound dressings. Recent studies have shown the potential of Ad-ECM in supporting wound closure. However, these studies were only a proof of concept and the pro-healing mechanisms of Ad-ECM were not being explored. Therefore, in this thesis, the scar regulating properties and wound closure capabilities of the extracted ANGPTL4 enriched Ad-ECM were studied.

From the results and discussion, it can be concluded that the extraction method of Ad-ECM was efficient. The extracted Ad-ECM was successfully delipidated and decellularized enabling it to be suitable for use for clinical applications with a lower risk of immunogenic response, which was substantiated by the results obtained from the immunogenicity study where THP-1 macrophages did not elicit an immunogenic response when in contact with Ad-ECM. After the extraction process, essential proteins for wound healing such as collagen type I, III, IV, elastin, fibronectin and laminin, and matricellular protein, ANGPTL4 were found to be retained. The cellular studies conducted showed that Ad-ECM is cytocompatible to skin cells, HDF and HaCaT which is essential for a wound dressing. Functional studies carried out on Ad-ECM showed that Ad-ECM was able to suppress myofibroblastic differentiation and promote faster re-epithelization through the interplay of ANGPTL4 and presence of other structural ECM proteins. Hence, Ad-ECM is a potential ideal wound dressing material.

6.2 Reconnaissance Work Not Included in Main Chapters

6.2.1 Loss of ANGPTL4 During Extraction Process

ANGPTL4 has been found to be advantageous to wound healing. Hence, a material that consists of ANGPTL4 will be able to further aid the wound healing process. Previous studies carried out to study the role of ANGPTL4 on wound healing used concentrations ranging from 6 to 12 $\mu\text{g/mL}$ [1, 2]. However, the amount of ANGPTL4 obtained after the extraction process in this thesis was found to be 140.7 pg/mL which is much lower than that of previous studies even though adipose tissue is known to be a rich source of ANGPTL4. Therefore, western blot was carried out on samples obtained from different steps of the extraction process (i.e. Ad-ECM before washing and the solution used to wash the extracted Ad-ECM) to determine if there was a loss in ANGPTL4 during the extraction process. As seen in **Figure 6.1**, it was observed that both samples contained ANGPTL4 which shows that some ANGPTL4 was lost during the extraction process. Nonetheless, as the extracted Ad-ECM is still enriched with ANGPTL4, and the purpose of this thesis is to study the scar regulating property and wound closure capabilities of the extracted ANGPTL4 enriched Ad-ECM, all the studies in this thesis were still carried out using the extracted Ad-ECM. However, future studies should focus on the optimization of the extraction process to minimize the loss of ANGPTL4 such as using other solutions other than PBS for the extraction process to change the charge distribution in the mixture and centrifugation speed used to separate the contents of the mixture [3, 4].

Figure 6.1 Leaching of ANGPTL4 during the extraction process

6.2.2 Ad-ECM Influence on HDF Cell Migration

Fast re-epithelization is required for efficient wound healing to ensure quick reformation of the skin barrier [5, 6]. This can be achieved by keratinocytes found in the epidermis of the skin. Likewise, the reformation of the structural framework of the skin is also important and this is achieved by fibroblasts found in the dermis of the skin. Hence, collective cell migration assay and wound scratch assays were also carried out using HDF to study if Ad-ECM is able to attract fibroblasts towards it and if it can influence cell migration to fill a wound gap.

As seen from **Figure 6.2a and b**, similar trends of collective cell migration were being observed on both Ad-ECM and Bv Col which shows that Ad-ECM has a comparable influence on the fibroblast migration rate. Ad-ECM was also found to be able to support cell migration to fill a wound gap as seen in **Figure 6.2c-e**, where fibroblasts were observed to have migrated from the scratch edge of $500 \pm 50 \mu\text{m}$ to infiltrate the gap. This suggests that Ad-ECM can be a good alternative to Bv Col as it is able to influence the migration of fibroblast which is essential for the formation of a provisional matrix to fill the wound gap. With the presence of various ECM structural proteins in Ad-ECM, a 3D scaffold will be able to provide a temporary matrix similar to the skin to influence fibroblast to infiltrate the scaffold and replace it with a provisional matrix. Hence, in addition to the 2D *in vitro* studies carried out in this study, there is a need to further investigate the migration of HDF into a 3D Ad-ECM scaffold.

Figure 6.2 Cell-material and cell-cell influence of HDF on Ad-ECM. (a,b) Collective cell migration of HDFs onto Ad-ECM and Bv Col were monitored over 24 hours (white line: initial cell position; red line: migrated cell position) with Bv Col type I as the control. Scale bar, 250 μm . (c,d) HDF migration from the edges of the scratch onto Ad-ECM and Bv was monitored over 24 hours (white line: initial gap length; red line: migrated cell position) with Bv Col type I as the control. Scale bar, 250 μm . With (d) an initial gap of around 500 μm .

6.3 Outstanding Questions

In summary, it was shown that ANGPTL4 enriched Ad-ECM has the potential to be a material for a wound dressing as it has scar regulating property and promotes fast re-epithelization. However, some questions have not been addressed in this study and require further understanding. First, the role of ANGPTL4 present in Ad-ECM in promoting faster re-epithelization as compared to Bv Col (type I) is unclear. Second, other than the interplay of ANGPTL4 in suppressing myofibroblastic differentiation, it is unknown what other factors resulted in this phenomenon and whether the structural proteins present in Ad-ECM played a role in this mechanism. Third, it is still unclear how Ad-ECM will influence wound healing in a 3D model. In vitro organotypic skin culture model can be carried out to study the infiltration and proliferation of fibroblast into Ad-ECM. In order to confirm the potential of Ad-ECM for wound healing, it is vital to move away from *in vitro* testing to *in vivo* tests such as the *in vivo* full thickness skin defect model as carried out by Yang et al.. Briefly, full thickness excisional wounds can be created on the dorso-medial back of each mouse by creating a biopsy punch and placing the Ad-ECM on the wound [7]. The evaluation of the effectiveness or healing potential of Ad-ECM can be studied by examining the time taken for wound closure and the area of wound contraction. In addition, the tensile strength of the skin can be measured by conducting tensile test to determine if the skin is able to regain its original strength [8]. Finally, the collagen formation after wound closure can be analysed through histology staining [9]. In addition, further confirmation of the biocompatibility of Ad-ECM can be done by analysing the complement activation system and expression of major histocompatibility complex (MHC) class II molecules in the presence of Ad-ECM. Collectively, the critical findings on the role of ANGPTL4 present in Ad-ECM have unravelled the novel understanding on the pro-healing mechanisms of Ad-ECM derived from human clinical waste which has the potential to be translated from bench-to-bedside in the future.

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Appendix

Figure A.1 Ad-ECM has excellent cytocompatibility supporting the attachment and proliferation of Human Dermal Fibroblast (HDF) and keratinocyte (HaCaT) cells. (a,d) Prestoblu cell proliferation assay over 7 days. (b,e) Cell doubling over 7 days. (c,f) LIVE/DEAD staining after 7 days of in vitro culture. : (a-c) HDF (d-f) HaCaT with Col as the control. Scale bar, 200 μ m.