The Impact of Bioactive Collagen Peptides on Promoting Cutaneous Wound Healing

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Abstract

Chronic wounds continue to be a major clinical and financial burden to healthcare providers worldwide with increased prevalence associated with an ageing population and systemic diseases such as diabetes. An acute unmet need for innovative therapies for effective wound repair thus remains. Recent studies using bioactive collagen peptides report their ability to promote cellular differentiation, proliferation and migration in animal models of cutaneous wound healing, leading to the present study aimed at defining the potential for and the mechanistic action of porcine-derived collagen peptides (Peptan P) to increase cutaneous healing in primary human keratinocytes and dermal fibroblasts *in vitro* and in wounded full thickness *ex vivo* skin equivalents, in an age dependent context. Results demonstrated Peptan P significantly promoted wound closure of both dermal fibroblasts and keratinocytes derived from young or aged individuals by enhancing cellular proliferation, with additional studies demonstrating the ability for Peptan P to also promote keratinocyte and dermal fibroblast wound closure in a hyperglycaemic environment. Mechanistic studies revealed Peptan P induced significant increase of both integrin α2 and β1 subunit expression by both keratinocytes and dermal fibroblasts, promoting activation of an ERK-FAK signalling cascade during keratinocyte wound closure, whilst integrin ligation most likely activates other downstream signalling pathways to promote dermal fibroblast wound closure. These observations were further supported by studies showing diminished Peptan P-induced wound closure of keratinocytes and fibroblasts following siRNA-mediated knockdown of the integrin β1 subunit. Studies in optimised 3D human skin equivalent models subjected to punch biopsy-induced wounding further revealed Peptan P promoted wound closure through enhanced re-epithelialisation. Collectively, these data highlight the translational and clinical potential for Peptan P as a viable topical therapeutic to promote re-epithelialisation of superficial cutaneous wounds.

Dedication

This work is dedicated to my parents

For their continued love and support

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Declaration

This thesis is submitted for the degree of Doctor of Philosophy at Newcastle University. This research was performed in the Translational and Clinical Research Institute under the main supervision of Professor Penny Lovat. This thesis is my own work unless stated within the text. I certify that none of the material offered in this thesis has been previously submitted by me for a degree or any other qualification at this, or any other University.

List of Published Manuscripts arising from this Thesis

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'Potentiation of cutaneous wound closure using porcine-derived collagen peptides'.

British Society for Investigative Dermatology (BSID) Annual Meeting, Cardiff, UK March 2021.

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List of Abbreviations

µg	Microgram
µl	Microliter
µm	Micrometre
µM	Micromolar
AHE	Area of fully healed epidermis
ANOVA	Analysis of Variance
AP-1	Activator protein 1
APC	Allophycocyanin
α-SMA	α-Smooth muscle actin
BA	Benzoic acid
BSA	Bovine serum albumin
BV421	Brilliant Violet 421
C	Circumference
$CaCl_2$	Calcium Chloride
CCN1	Cysteine-rich angiogenic protein 61
CD	Cluster of differentiation
CD29	Cluster of differentiation 29 / Integrin β1 subunit
CD49b	Cluster of differentiation 49b / Integrin α2 subunit
CD49c	Cluster of differentiation 49c / Integrin α3 subunit
CD49e	Cluster of differentiation 49e / Integrin α5 subunit
CD49f	Cluster of differentiation 49f / Integrin α6 subunit
cDNA	Complementary DNA
CO_2	Carbon Dioxide
Ctrl	Control
Cy7	Cyanine7
DAPI	4’ 6-diamidino-2-phenylindole
DDR	Discoidin-domain receptor

DMEM	Dulbecco’s Modified Eagle’s Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGF-L	Epidermal growth-like repeats
EGFR	Epidermal growth factor receptor
ERK	Extracellular signal-regulated kinase
FACIT	Fibril-associated collagens with interrupted triple helices
FACS	Fluorescent-activated cell sorting
FAK	Focal adhesion kinase
FBS	Foetal bovine serum
FFPE	Formalin-fixed paraffin-embedded
FGF	Fibroblast growth factor
FITC	Fluorescein isothiocyanate
g	Grams
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFOGER	Glycine-Phenylalanine-Hydroxyproline-Glycine-Glutamic acid-Arginine
GLOGEN	Glycine-Leucine-Hydroxyproline-Glycine-Glutamic acid-Asparagine
Gly	Glycine
HCL	Hydrochloric Acid
HDFn	Human neonatal dermal fibroblasts
HEKn	Human neonatal keratinocytes
HKGS	Human keratinocyte growth serum
HPLC	High performance liquid chromatography
HUVEC	Human umbilical vein endothelial cell
Hyp	Hydroxyproline
IL-1	Interleukin-1
IL-6	Interleukin-6
IL-10	Interleukin-10

IL-22	Interleukin-22
ITGB1	Integrin β1 subunit
JNK	c-Jun N-terminal kinase
kDa	Kilo Daltons
kg	Kilograms
KGF	Keratinocyte growth factor
KRT	Keratin
LET	Length of newly formed epidermis
LDR	Length of newly formed dermis
LNT	Length of newly formed tissue
MAPK	Mitogen-activated protein kinase
MEK	Mitogen activated protein kinase kinase
mg	Milligrams
Mito C	Mitomycin C
ml	Millilitres
mm	Millimetres
mM	Millimolar
MMP	Matrix metalloproteinase
MTS	3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium solution
NaCl	Sodium Chloride
NaF	Sodium Fluoride
NaOH	Sodium Hydroxide
NEAA	Non-essential amino acids
NET	Neutrophil extracellular traps
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
OCT	Optimal Cutting Solution
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PCL	Poly(ε-caprolactone)
PDGF	Platelet-derived growth factor
PDGF-AA	Platelet-derived growth factor-AA
PDGF-AB	Platelet-derived growth factor-AB
PDGF-BB	Platelet-derived growth factor-BB

PDGFR	Platelet-derived growth factor receptor
PE	Phycoerythrin
PEG	Polyethylene glycol
PEPT1	Peptide transporter 1
PEPT2	Peptide transporter 2
PFA	Paraformaldehyde
PGA	Poly(glycolic acid)
PHT1	Peptide histidine transporter 1
PHT2	Peptide histidine transporter 2
PIC	Protease inhibitor cocktail
PI3K	Phosphoinositide 3-kinase
PLGA	Polylactic-co-glycolic acid
PKC	Protein kinase C
Pro	Proline
PSA	Penicillin-Streptomycin-Amphotericin
PVA	Poly(vinyl alcohol)
p75NTR	p75 neurotrophin receptor
qPCR	Real-time polymerase chain reaction
r	Radius
RGD	Arginine-Glycine-Aspartate
RNAi	RNA interference
ROS	Reactive oxygen species
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
Shc	Src homology and collagen
SiCtrl	Non-target siRNA control
siRNA	Short interfering ribonucleic acid
SPARC	Secreted protein acidic and rich in cysteine
SRP	Small proline-rich proteins
STAT3	Signal transducer and activator of transcription 3
TBS	Tris-Buffered Saline
TBS/T	Tris-Buffered Saline/ 0.1% Tween 20
TG1	Transglutaminase 1

TG2	Transglutaminase 2
TG3	Transglutaminase 3
TG5	Transglutaminase 5
TGF-β	Transforming growth factor-β
TGF-βR	Transforming growth factor-β receptor
TIMP	Tissue inhibitors of matrix metalloproteinases
TNF-α	Tissue necrosis factor-α
uPARAP/Endo180	Urokinase plasminogen activator receptor-associated protein
UPLC-MS/MS	Ultra-performance liquid chromatography tandem mass spectrometry
UV	Ultraviolet
UVR	Ultraviolet radiation
VEGF	Vascular endothelial growth factor
VHE	Volume of fully healed epidermis
VNT	Volume of newly formed tissue

Chapter 1: Introduction

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1.1 Structure and Function of Skin

The skin represents the largest organ in the human body, comprised of three main layers; the epidermis, dermis and hypodermis that principally serves as a barrier against foreign invading microorganisms, regulates body temperature and prevents dehydration (Figure 1.1) (Arda *et al.*, 2014).

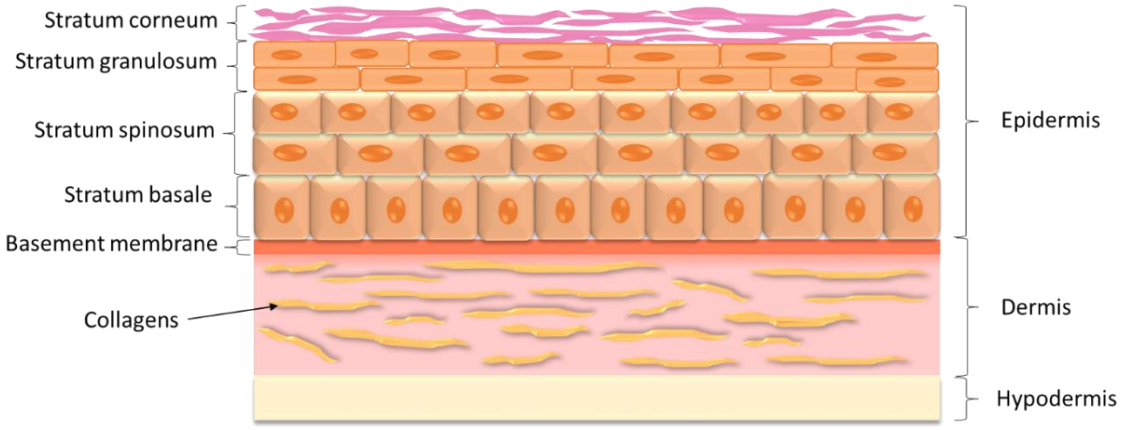

Figure 1.1 Structure of human skin. *Skin is comprised of three main layers; the epidermis, dermis and hypodermis. The epidermis can be further divided into the stratum basale, stratum spinosum, stratum granulosum and stratum corneum. The epidermis acts as a physical barrier against pathogens and helps to prevent dehydration. The dermis is mainly comprised of structural proteins such as collagens and elastins that help to maintain structural integrity whilst the hypodermis helps to prevent heat loss and is involved in energy storage and metabolism.*

1.1.1 The Epidermis

The uppermost layer of skin, termed the epidermis is a stratified epithelium composed of four sublayers over a basement membrane; the *stratum basale*, *stratum spinosum*, *stratum granulosum* and *stratum corneum*, all of which comprise of increasingly differentiated keratinocytes that help to provide a barrier to the external environment and pathogens; however, in thick skin such as the palms of the hands and soles of the feet, there is an additional layer known as the *stratum lucidum*, which is located above the *stratum granulosum* and below the *stratum corneum* (Baroni *et al.*, 2012). Melanocytes, another cell type located within the stratum basale layer of the epidermis, are melanin producing cells that form epidermal melanin units through their interactions with keratinocytes and provide photoprotection from ultraviolet radiation (UVR) (Lin and Fisher, 2007; Cichorek *et al.*, 2013). Additionally, the secretion of cytokines such as neuregulin1 by dermal

fibroblasts within the dermal layer of skin influences the growth, dendricity and pigmentation of the melanocytes found within the epidermis (Cichorek *et al.*, 2013).

Keratinocytes present within the *stratum basale* of the epidermis are characterised as proliferative and actively undergo proliferation to contribute to the renewal of the epidermis (Baroni *et al.*, 2012). In addition, the attachment of these keratinocytes to the basement membrane via hemidesmosomes helps to prevent basal keratinocyte cornification (Arda *et al.*, 2014). As keratinocytes move from the basal layers towards the upper epidermal layers, they increasingly undergo differentiation and cytomorphosis until they reach the outermost *stratum corneum* layer which is mainly comprised of terminally differentiated keratinocytes termed corneocytes, interconnected to each other by corneodesmosomes to form a barrier that protects underlying tissue from external pathogens, dehydration and mechanical stress (Madison, 2003; Baroni *et al.*, 2012). Within the intracellular space of the *stratum corneum* are a unique mixture of lipids that also function to counteract the loss of water and salts from the skin (Madison, 2003).

Epidermal differentiation is integral to the formation and maintenance of the epidermis and is regulated through changes in gene expression and keratinocyte morphology (Figure 1.2). The presence of an epidermal calcium gradient is a key aspect for epidermal differentiation, with low calcium concentrations ensuring the proliferation and replenishment of the epidermis, while higher concentrations of calcium induce keratinocyte differentiation and formation of a functioning epidermal barrier (Celli *et al.*, 2010; Elsholz *et al.*, 2014). The calcium influx also activates signalling pathways including the mitogen-activated protein kinase (MAPK) pathway as well as protein kinase C (PKC) (Pastar *et al.*, 2014). The activation of PKC activates genes associated with epidermal differentiation via transcription factors such as activator protein 1 (AP-1), while the maintenance of the epidermal calcium gradient is regulated by proteins such as skin calmodulin-related factor (Scarf) that acts as a calcium sensor in order to regulate epidermal barrier formation (Baroni *et al.*, 2012; Kypriotou *et al.*, 2012; Wikramanayake *et al.*, 2014).

Additionally, within the epidermis are many structural proteins present that are all integral in maintaining its integrity. Keratins (KRT), the main structural proteins synthesised by keratinocytes as well as filaggrin constitute around 80% of proteins

within the epidermis, with other structural proteins such as involucrin and loricrin present in the *stratum corneum* layers (Baroni *et al.*, 2012). During the final stages of normal keratinocyte differentiation, filaggrin organises keratin filaments into highly condensed tight bundles that promote the collapse of the keratinocyte into a flattened shape, characteristic of the corneocytes found with the *stratum corneum* (Baroni *et al.*, 2012). As keratinocytes become more differentiated, alterations in the expression of keratins also occurs, with keratinocytes within the basal layers expressing KRT5, KRT14 and KRT15. However, keratin expression changes to KRT1 and KRT10 as keratinocytes move upwards towards the spinous layer (Figure 1.2) (Wikramanayake *et al.*, 2014). KRT1 and KRT10 are then expressed until differentiating keratinocytes reach the granular layer where overall keratin expression diminishes (Elsholz *et al.*, 2014).

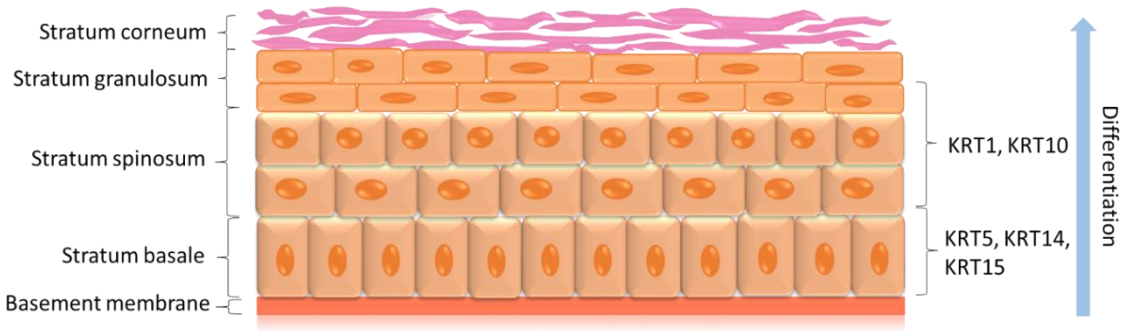

Figure 1. 2 Epidermal Differentiation. *The epidermis is comprised of four main sublayers; the stratum basale, spinosum, granulosum and corneum. As keratinocyte move from the stratum basale to the stratum corneum, they become increasingly differentiated. Keratinocytes synthesise keratins (KRT) with keratinocytes in the stratum basale expressing KRT5, KRT14 and KRT15 while more differentiated keratinocytes express KRT1 and KRT10.*

Specific enzymes within the epidermis including transglutaminases are also key to epidermal homeostasis and structure. Transglutaminases are calcium-dependent enzymes that catalyse the formation of isodipeptide cross-links between lysine and glutamine residues, contributing to the formation of insoluble macromolecular assemblies (Thibaut *et al.*, 2009). Specifically, transglutaminases 1, 3 and 5 (TG1, TG3 and TG5) are mainly expressed in the spinous and granular layers of the epidermis and are associated with the formation of the stratum corneum, with TG1 involved in cross-linking lipids and proteins within the *stratum corneum* (Stephens *et al.*, 2004; Eckert *et al.*, 2005). High concentrations of calcium activates TG1,

which catalyses the cross-linking of involucrin and loricrin to form the cell envelopes that surround the corneocytes and increases their resistance to mechanical disruptions (Wikramanayake *et al.*, 2014). Previous studies have reported that TG1-deficient animals have defective epidermal development, lacking the cornified envelope and impaired epidermal barrier function (Matsuki *et al.*, 1998). TG3 is a zymogen that requires activation through proteolytic cleavage to aid in epithelial barrier formation by catalysing the assembly of intrachain cross-links (Kypriotou *et al.*, 2012). The function of TG5 has not been fully characterised but studies have reported that TG5 has similar functions to both TG1 and TG3, being able to cross-link loricrin, involucrin and small proline-rich proteins (SRPs) as well as the assembly of corneodesmosomes (Candi *et al.*, 2002; Kypriotou *et al.*, 2012). However, studies have also reported that an upregulation of TG5 contributes to the hyperkeratotic phenotype that is observed in both lamellar and vulgaris ichthyosis (Candi *et al.*, 2002).

1.1.2 The Dermis and Hypodermis

The dermal layer of skin is divided into two main layers; the papillary dermis, which has a high fibroblast density, rich supply of blood vessels and sensory nerve endings and the reticular dermis, which is thicker and characterised by an abundance of highly organised fibrillar collagen (Lai-Cheong and McGrath, 2009; Watt, 2014). The dermis contains many structural proteins that form the extracellular matrix with around 70% comprised of collagens, predominantly type I and III collagen and 5% elastin, all of which are secreted by dermal fibroblasts, the main cell type found within the dermis (Lai-Cheong and McGrath, 2009). Collagens are key to the structural integrity and tensile strength of skin whilst elastin provide elasticity to skin (Lai-Cheong and McGrath, 2009). The hypodermis is the thickest layer of skin located below the reticular dermis and provides anchorage to underlying muscles. The hypodermis is characterised as loose connective tissue comprised of collagen and elastins and a dense network of blood vessels which means that the hypodermis is able to provide thermal insulation and fatty acid storage and help protect against mechanical shock, (Bonté *et al.*, 2019).

1.1.3 The Extracellular Matrix

The extracellular matrix (ECM) is found within the dermal layer of human skin and is a 3D extracellular macromolecule network that is assembled by dermal fibroblasts

and comprised of a large number of structural proteins such as collagens, proteoglycans, fibronectin, laminins and elastin that facilitate cell interactions critical to normal tissue homeostasis, development and regeneration (Schultz and Wysocki, 2009; Xue and Jackson, 2015; Keane *et al.*, 2018). These proteins exert a range of physical, biochemical and biomechanical properties enabling multiple functions such as anchorage to the basement membrane to maintain tissue polarity as well as modulating cell migration and proliferation (Lu *et al.*, 2012; Keane *et al.*, 2018). Specifically, collagens the main structural proteins found within the ECM, act to maintain structural integrity by providing tensile strength while fibronectin acts a biological glue, mediating interactions between cells and the ECM by providing adhesion and reinforcing the structural integrity between the dermis and epidermis (Schultz and Wysocki, 2009). Glycosaminoglycans and proteoglycans within the ECM are hydrophilic molecules that are capable of absorbing water thus providing dermal hydration in order to maintain a water balance that supports the metabolic requirements of the ECM (Schultz and Wysocki, 2009). Also within the ECM are matricellular proteins such as secreted protein acidic and rich in cysteine (SPARC), thrombospondin 1 and tenascin C which are secreted macromolecules that do not function as structural proteins but instead interact with cell surface receptors and growth factors to promote processes such as vascular growth (Sage, 2001).

The ECM can be further sub-categorised into two types; the basement membrane and the interstitial matrix (Bonnans *et al.*, 2014). The basement membrane is a specialised form of the ECM, mainly comprised of collagen IV and laminins that helps separate the epithelium from other tissue, whilst the interstitial matrix acts as a structural scaffold that controls cell organisation and differentiation through interactions with various cell surface receptors, critical to the maintenance of tissue integrity as well as acting as a selective barrier for cell transmigration (Egeblad *et al.*, 2010; Lu *et al.*, 2012; Keane *et al.*, 2018).

The ECM can also store and release growth factors and cytokines such as transforming growth factor-β (TGF-β), fibroblast growth factor (FGF) and epidermal growth factor (EGF) that have chemotactic effects on cells but also aid in the regulation and promotion of dermal fibroblast differentiation (Gelse *et al.*, 2003; Bonnans *et al.*, 2014; Keane *et al.*, 2018). The sequestration and release of growth

factors by the ECM also prolongs growth factor action and localisation of activity to the immediate environment (Schultz and Wysocki, 2009).

Additionally, within the ECM are naturally occurring ECM peptides known as matrikines that are formed through the partial proteolysis of various ECM proteins (Maquart *et al.*, 2004; Maquart *et al.*, 2005; Tran *et al.*, 2005). These matrikines are able to bind to and activate various cell surface receptors including epidermal growth factor receptor (EGFR) and integrin receptors to regulate cell activity such as cellular migration and proliferation (Maquart *et al.*, 2005). However, matrikines bind to these receptors at a lower affinity than growth factors, cytokines and chemokines so they are only able to exert their influence to a local region meaning only cells within the vicinity of the matrikine will be affected (Tran *et al.*, 2005). Epidermal growth factor-like (EGF-L) repeats present in tenascin C are an example of a matrikine that is able to bind to EGFR on dermal fibroblasts to stimulate downstream signalling pathways that are important for fibroblast migration (Tran *et al.*, 2005).

Importantly, the ECM plays a key role in cutaneous wound healing, providing a scaffold to support cellular migration and proliferation whilst also acting as a reservoir for various wound healing-associated growth factors such as TGF-β, FGF, EGF and vascular endothelial growth factor (VEGF) (Keane *et al.*, 2018).

1.2 Collagen: Structure and Function

Collagens, particularly type I, III and IV are the main proteins found within the ECM of the skin, each varying considerably in size, function and distribution (Gelse *et al.*, 2003). All collagens maintain a triple helix structure but can be classified as fibrillar, basement membrane network forming, filamentous, hexagonal network forming and fibril-associated collagens with interrupted triple helices (FACIT) (Keane *et al.*, 2018).

Collagen I, the most abundant collagen found within the body with high distribution within skin, bones, tendons and ligaments is a fibrillar collagen and is formed from three polypeptide chains; 2 pro-α1(I) and 1 pro-α2(I) chains (Gelse *et al.*, 2003). These parallel polypeptide chains are coiled into a left-handed polyproline II-type helical conformation around each other with a one residue stagger aiding the formation of a right-handed triple helix (Shoulders and Raines, 2009), comprising of

repeated glycine (Gly) residues that results in repeated sequences of X-Y-Gly, where X and Y are usually proline (Pro) and hydroxyproline (Hyp), making Pro-Hyp-Gly the most common amino acid triplet in collagen I (Shoulders and Raines, 2009; Chattopadhyay and Raines, 2014).

Collagen III is also a fibrillar collagen formed from three α1(III) chains that, like collagen I, forms a triple helix structure mainly comprised of repeated X-Y-Gly sequences; however, the diameter of collagen III fibrils are smaller than collagen I fibrils (Shoulders and Raines, 2009; Kuivaniemi and Tromp, 2019). Collagen III constitutes about 5-20% of collagen within the human body being mainly found in arteries, uterus and bowel (Kuivaniemi and Tromp, 2019). Collagen III is also found in low abundance in human skin with increased production during the early phases of cutaneous wound healing to form granulation tissue.

Dermal fibroblasts are the principal cell type responsible for collagen I and III production within the skin. Synthesised as a protocollagen precursor comprising of a central collagen domain, an N-terminal propeptide and a C-terminal propeptide, these protocollagen chains undergo post-translational modifications within the rough endoplasmic reticulum (RER) before being transferred to the golgi apparatus. During this transfer, hydroxylation of some proline and lysine residues occur through the action of prolyl hydroxylase and lysl hydroxylase to produce a triple helix procollagen (Shoulders and Raines, 2009; Parenteau-Bareil *et al.*, 2010). Once transported outside the cell, collagen peptidases cleave the N- and C-terminal propeptides, which allows for the self-assembly of multiple tropocollagen molecules to produce collagen fibrils (Figure 1.3). Multiple collagen fibrils assemble to form collagen fibres through the action of lysl oxidase and stabilisation through covalent cross-links contributing to the mechanical resilience of collagen (Shoulders and Raines, 2009; Xue and Jackson, 2015).

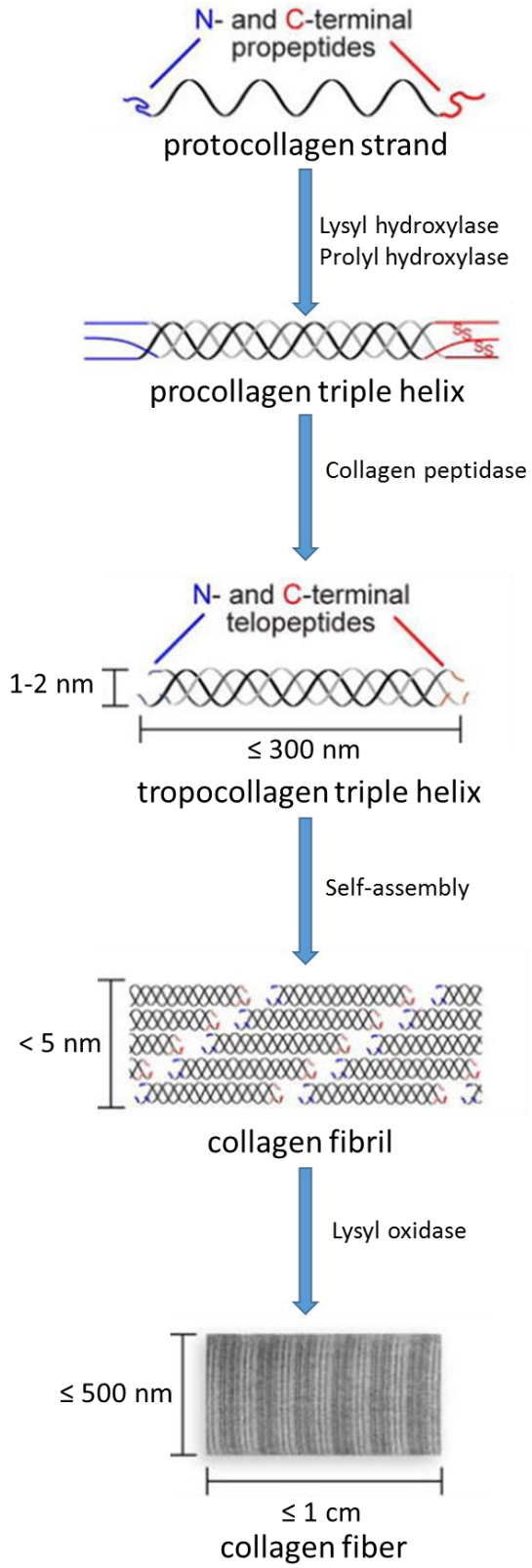

Figure 1.3 Biosynthesis of fibrillar collagens. *Collagen is the most abundant structural protein found in skin and is mainly synthesised and secreted by dermal fibroblasts. In fibrillar collagens, protocollagen strands form a procollagen triple helix. The N- and C-terminal propeptides are cleaved to form tropocollagen. Tropocollagen triple helices self-assembly to form collagen fibrils which then accumulate to form collagen fibres. Adapted from Chattopadhyay and Raines (2014).*

One of the main functions of collagen is to provide and maintain structural integrity of skin. This is achieved through the formation of cross-links with other collagen fibres and interactions with other ECM proteins that helps to support various cells by regulating their differentiation and proliferation (Xue and Jackson, 2015; Malcor *et al.*, 2016). Specifically, collagen III has been shown to influence platelet aggregation through interactions with specific glycoproteins and non-integrin receptors (Monnet and Fauvel-Lafève, 2000). Additionally, during cutaneous wound healing, increased collagen III production forms granulation tissue which acts as a temporary matrix and promotes fibroblast adhesion, migration and proliferation via interactions with integrin receptors α1β1 and α2β1 (Kim *et al.*, 2005).

Collagen I induces cell signalling through interactions with specific integrins such as α2β1, discoidin-domain receptors (DDRs), urokinase plasminogen activator receptor-associated protein (uPARAP/Endo180) and glycoprotein VI receptors (Vogel *et al.*, 1997; Koivisto *et al.*, 2014; Boraschi-Diaz *et al.*, 2017). Signalling through these specific receptors mediates cell adhesion, migration, differentiation and growth, with the binding of collagen I to DDRs specifically regulating fibroblast migration and proliferation (Gelse *et al.*, 2003; Wilgus, 2012). Additionally, the binding of collagen I to DDR2 receptors upregulates matrix metalloproteinase-1 (MMP-1) expression, which leads to degradation of fibrillar collagens such as collagen III within wounds (Vogel *et al.*, 1997). Collagen I also contributes to the storage and delivery of multiple growth factors and cytokines critical to tissue development and homeostasis. During cutaneous wound healing, collagen I acts as a scaffold to promote fibroblast migration and proliferation whilst the synthesis and remodelling of new collagen fibres increases the tensile of the mature wound (Guo and DiPietro, 2010; Young and McNaught, 2011). However, an overproduction of collagen I during cutaneous wound healing can contribute to the development of abnormal or excessive scars (Wilgus, 2012; Keane *et al.*, 2018). To prevent an overproduction of collagen, cutaneous wound healing is tightly regulated by the action of multiple biological processes.

1.3 Cutaneous Wound Healing

Cutaneous wounds arise from injury to the epidermis and/or the dermis and can be categorised as; superficial wounding, where only the epidermis is damaged or full-

thickness wounding, where there is damage to both the epidermis and the underlying dermis (Enoch and Leaper, 2008). Cutaneous wounds can then be further sub-categorised into acute and chronic wounds. Acute wounds will generally completely heal within a set period of time whilst chronic wounds have impaired healing and fail to heal in a timely manner, often resulting in further complications including infections and pro-longed hospitalisation (Young and McNaught, 2011; Gould *et al.*, 2015).

Following injury, damaged cutaneous tissue is repaired in a series of coordinated processes, defined by four main overlapping phases; haemostasis, inflammation, proliferation and remodelling (Bielefeld *et al.*, 2013).

1.3.1 Haemostasis

Haemostasis is the first phase of cutaneous wound healing and begins immediately following wounding through the constriction of vascular vessels to prevent exsanguination and formation of a fibrin clot (Figure 1.4) (Gurtner *et al.*, 2008; Bielefeld *et al.*, 2013). The constriction of vascular smooth muscle is stimulated by vasoconstrictors such as endothelin that are released from the damaged endothelium, whilst circulating hormones such as catecholamines, epinephrine and norepinephrine released from injured cells help to regulate vasoconstriction (Godo and Shimokawa, 2017). Platelets secrete platelet-derived growth factor (PDGF) which activates mesenchymal cells such as smooth muscle cells in the vessel walls to contract. However, this initial contraction of vascular smooth muscle is only temporary as the increased hypoxia and acidosis of the wound triggers the passive relaxation of the vascular muscle. Therefore, the subsequent activation of the coagulation cascade is required to further regulate vasoconstriction and resolve the bleeding in the long term (Rodrigues *et al.*, 2019).

The activation of both extrinsic and intrinsic pathways of the coagulation cascade causes platelets to aggregate and bind to the exposed subendothelial matrix via interactions with G protein-coupled receptors which stimulates integrin activation and increased attachment of platelets to the surrounding ECM (Velnar *et al.*, 2009; Pradhan *et al.*, 2017; Ellis *et al.*, 2018). The activation of factor X by either the intrinsic or extrinsic pathway causes prothrombin to be converted to thrombin which cleaves fibrinogen into fibrin. Factor XIII then covalently crosslinks fibrin to form a

fibrin mesh which binds to the aggregated platelets to form a definitive haemostasis plug known as a thrombus (Rodrigues *et al.*, 2019). Along with fibrin, the thrombus is also comprised of fibronectin, vitronectin and thrombospondins that forms a provisional wound matrix to support the migration of immune cells, keratinocytes and dermal fibroblasts to the wound site (Reinke and Sorg, 2012). Fibronectin acts as a scaffolding protein during wound healing regulating ECM organisation and stability enabling fibroblast polarisation to assist the migration of dermal fibroblasts towards the wound site, while vitronectin binds activated platelets to aid in the regulation of growth factor activity (Upton *et al.*, 2008; Keane *et al.*, 2018).

Platelets release multiple cytokines including interleukin-1 (IL-1), interleukin-6 (IL-6) and tumour necrosis factor-α (TNF-α) to stimulate the inflammatory phase and recruitment of immune cells (Reinke and Sorg, 2012). Platelets also release growth factors such as TGF-β, FGF, VEGF and PDGF that stimulates neutrophil, macrophage and fibroblast recruitment to the wound site and promotes angiogenesis (Shaw and Martin, 2009; Young and McNaught, 2011). After haemostasis is achieved, histamine is released causing capillary dilation, accelerating migration of immune cells into the wound site and initiating the inflammatory phase of wound healing (Sinno and Prakash, 2013).

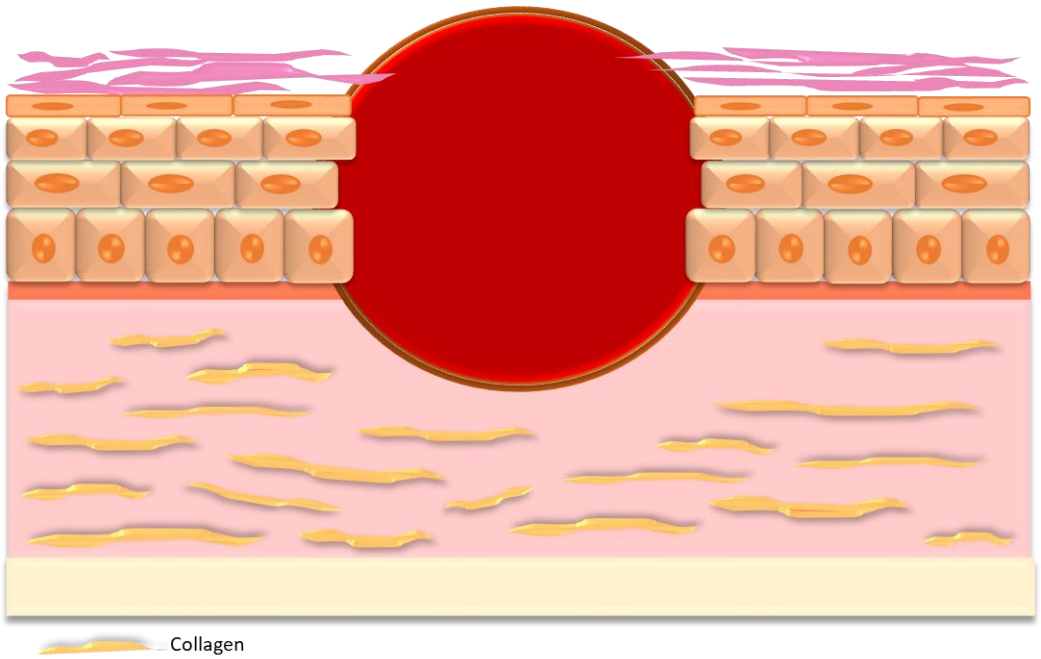

Figure 1. 4 Haemostasis phase of cutaneous wound healing. *Following injury, vascular vessels constrict to prevent blood loss and platelets aggregate to form a blood clot rich in fibrin that acts as a temporary matrix and scaffold for dermal fibroblasts. Platelets also release various cytokines and growth factors that attract other cell types such as immune cells and dermal fibroblasts to the wound site.*

1.3.2 Inflammation

The formation of the initial fibrin clot results in the activation of the inflammatory phase of cutaneous wound healing, leading to the recruitment of various immune cells to the wound site and the establishment of an immune barrier against invading pathogens (Figure 1.5). Neutrophils are the first and most abundant inflammatory cells to be recruited the wound site appearing within the first 48 hours following injury through stimulation by CXCL8 (Ridiandries *et al.*, 2018). CXCL8 along with other chemokines such as CXCL1, CXCL2, CXCL3, CXCL5, CXCL6 and CXCL7 play a role in neutrophil chemotaxis by binding to glycosaminoglycans in the ECM to create a signalling gradient that allows for directional migration of neutrophils towards the site of injury (Gillitzer and Goebeler, 2001). Neutrophils are able to phagocytose foreign pathogens with the assistance of neutrophil extracellular traps (NETs) while releasing lactoferrin and proteases that remove dead bacteria and cell debris from the wound site (Young and McNaught, 2011; Larouche *et al.*, 2018). Once the neutrophils have removed any foreign pathogens and cellular debris, they undergo apoptosis and are phagocytosed by pro-inflammatory macrophages in a

process known as efferocytosis (Ellis *et al.*, 2018). Failure to remove neutrophils via efferocytosis can lead to secondary necrosis as the lysed neutrophils release pro-inflammatory and cytotoxic molecules that increase damage to surrounding tissue and can cause the emergence of non-healing chronic wounds (Jun *et al.*, 2015). Pro-inflammatory macrophages phagocytose any remaining cellular debris before transitioning into an anti-inflammatory macrophage population that releases growth factors such as TGF-β, FGF and VEGF to stimulate angiogenesis, granulation tissue formation and promote the migration and proliferation of keratinocytes, dermal fibroblasts and endothelial cells (Velnar *et al.*, 2009; Krzyszczyk *et al.*, 2018).

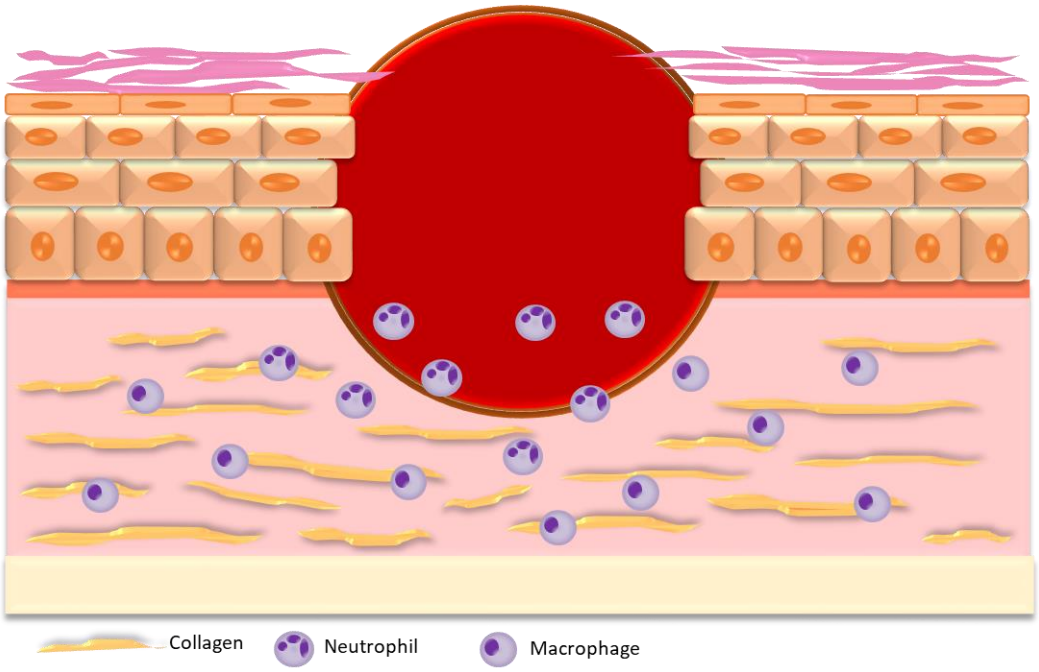

Figure 1. 5 Inflammatory phase of cutaneous wound healing. *Immune cells such as neutrophils and macrophages migrate into the wound site and phagocytose any cell debris and pathogens present within the wound before secreting growth factors such as TGF-β, FGF and VEGF that promote the migration and proliferation of keratinocytes, dermal fibroblasts and endothelial cells.*

1.3.3 Proliferation

As the inflammatory phase resolves, the proliferation phase begins. This involves re-establishing the vascular network, granulation tissue formation and re-epithelialisation (Figure 1.6). This is achieved through the proliferation and migration of keratinocytes, dermal fibroblasts and endothelial cells as well as the deposition of ECM proteins, particularly collagen I and III to synthesis a new ECM to replace

the provisional fibrin matrix initially generated following injury (Velnar *et al.*, 2009; Guo and DiPietro, 2010).

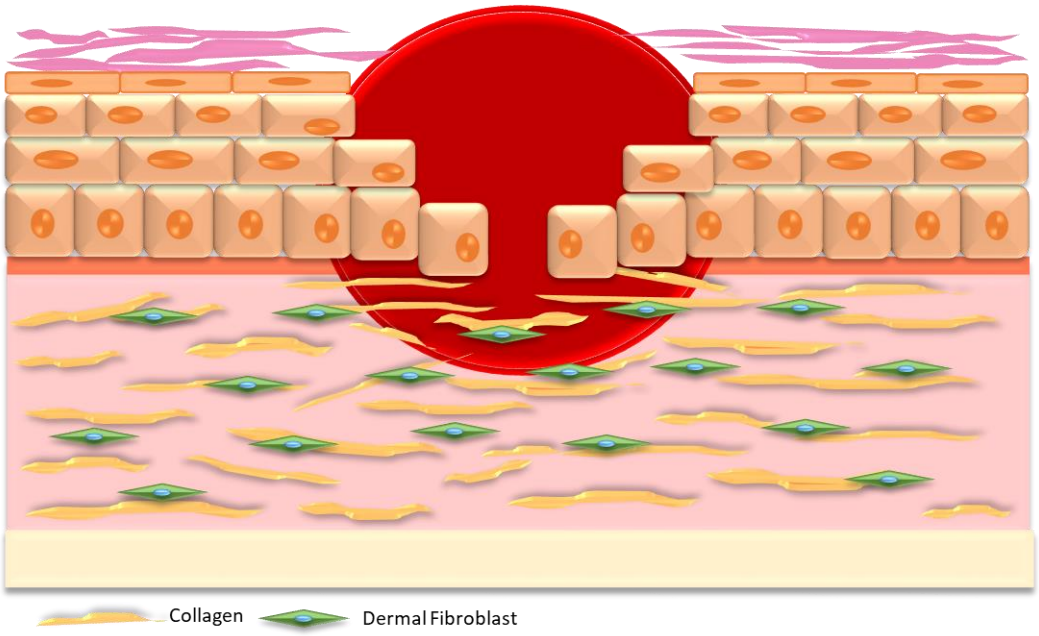

Figure 1. 6 Proliferation phase of cutaneous wound healing. *Dermal fibroblasts migrate into the wound site and proliferate synthesising collagen III which is used to form granulation tissue. New blood vessels are formed through angiogenesis which allows oxygen and nutrient supply to the wound site and keratinocytes undergo re-epithelialisation in order to re-establish the epidermis*

1.3.3.1 Angiogenesis

Angiogenesis is a crucial process during wound healing as it allows for the repair of damaged vessels along with the formation of new blood vessels through neovascularisation. Growth factors including VEGF bind to their receptors on endothelial cells within damaged vessels, activating intracellular signalling pathways and initiate new vessel formation (Reinke and Sorg, 2012). Through the action of proteolytic enzymes, endothelial cells migrate into the wound and begin proliferating. This process is known as sprouting and results in the formation of newly formed vessels from small tubular canals that then interconnect with each other before differentiating into arteries and venules. The resulting vessels are then further stabilised through the recruitment of pericytes and smooth muscle cells. The repair and formation of these blood vessels allows for the delivery of oxygen and nutrients to the wound site necessary for cell metabolism, and in particular energy

production, which helps to increase keratinocyte differentiation and migration as well as dermal fibroblast proliferation (Bao *et al.*, 2009; Guo and DiPietro, 2010).

1.3.3.2 Collagen Synthesis and Granulation Tissue Formation

Once dermal fibroblasts have migrated into the wound site, they begin to proliferate and synthesise collagen III to generate a collagen matrix termed granulation tissue enriched with proteoglycans and glycosaminoglycans that replaces the temporary fibrin matrix produced during haemostasis (Young and McNaught, 2011; Olczyk *et al.*, 2014; Smith and Melrose, 2015). This granulation tissue acts as a temporary substitute for the dermis and is characterised by a high density of capillaries, dermal fibroblasts and loosely organised collagen bundles (Reinke and Sorg, 2012).

Additionally, as the collagen content within the granulation tissue increases, sup-populations of fibroblasts are activated and differentiate into myofibroblasts in response to interactions with EDA fibronectin via α5β1 and αvβ3 integrins or stimulation by TGF-β (Serini *et al.*, 1998; Hinz, 2007). These myofibroblasts are contractile cells that express both β-cytoplasmic and γ-cytoplasmic actins as well as α-smooth muscle actin (α-SMA) in microfilament bundles which adhere to the wound edge and reduce the size of the wound through the contraction of these actin bundles (Hinz, 2007; Sarrazy *et al.*, 2011).

1.3.3.3 Re-epithelialisation

Re-epithelialisation is a key event that occurs during the proliferation phase of wound healing and is characterised by the migration and proliferation of keratinocytes over the provisional collagen matrix to re-establish the epidermis. Following skin injury, keratinocytes are activated through the action of various cytokines and growth factors causing changes in the keratinocyte cytoskeleton network and cell surface receptors that are critical for re-epithelialisation, particularly increased expression of KRT6 and KRT16 and downregulated expression of KRT1 and KRT10 (Wong and Coulombe, 2003; Patel *et al.*, 2006; Pastar *et al.*, 2014). In order to achieve wound closure, keratinocyte at the wound edge dissolve their hemidesmosomes to loosen their adhesion to each other as well as to the basement membrane in order to develop the flexibility to migrate over the provisional matrix whilst keratinocytes behind the migrating epithelial tongue proliferate to ensure adequate cell supply (Werner and Grose, 2003; Pastar *et al.*, 2014). Migrating

keratinocytes at this point display an increased expression of KRT6, KRT16 and KRT17 which contributes to increased viscoelastic properties and the assembly of actin-rich lamellar protrusions that assist in keratinocyte migration along with an upregulation of proteolytic enzymes termed matrix metalloproteinases (MMPs) (Patel *et al.*, 2006; Pastar *et al.*, 2014; Rousselle *et al.*, 2019a). Specifically, MMP-1 helps to facilitate re-epithelialisation by promoting cell elongation and activating extracellular signal-regulated kinase (ERK) signalling (Xue *et al.*, 2006; Caley *et al.*, 2015; Xue and Jackson, 2015). Interestingly, *in vivo* studies using transgenic mice overexpressing MMP-1 in the epidermis display a hyperproliferative and hyperkeratotic phenotype along with delayed wound closure suggesting that maintained regulation of MMP-1 expression is essential for effective re-epithelialisation (Di Colandrea *et al.*, 1998).

Growth factors, particularly EGF stimulate keratinocyte migration and proliferation directly whilst keratinocyte growth factor (KGF) expressed by dermal fibroblasts below the wound edge stimulate re-epithelialisation through paracrine effects, with inhibition of the KGF receptor demonstrating delayed wound re-epithelialisation due to a reduction in the proliferative rate of keratinocytes at the wound edge (Werner *et al.*, 1994b; Werner *et al.*, 2007). Once re-epithelialisation is completed and a new stratified epidermis has been formed, the migration of epidermal cells ceases and keratinocytes revert to a stationary phenotype in which they have an apical polarity and re-establish contact with the basement membrane via reconstituted hemidesmosomes (Gonzalez *et al.*, 2016; Rousselle *et al.*, 2019b).

1.3.4 Remodelling

The breakdown and remodelling of the ECM is the final phase of wound healing and is responsible for epithelium development. Matrix remodelling consists of the deposition of denser collagen I fibrils and modifications to the ECM structure through the action of MMPs, tissue inhibitors of matrix metalloproteinases (TIMPs) and tissue transglutaminase 2 (TG2) to maximise tensile strength of the mature wound (Figure 1.7) (Griffin *et al.*, 2002; Caley *et al.*, 2015). The activity of MMPs and TIMPs allows for the degradation of the damaged ECM and gradual replacement of the collagen III-rich granulation tissue with the formation and accumulation of a denser collagen I-rich matrix (Visse and Nagase, 2003; Xue and Jackson, 2015). TG2 helps to regulate ECM remodelling by cross-linking with ECM proteins such as collagen I

and fibronectin, leading to ECM stabilisation by increasing collagen I resistance to protease degradation and promoting cell-matrix interactions by enhancing dermal fibroblast adhesion (Stephens *et al.*, 2004; Chau *et al.*, 2005). As collagen III is degraded and replaced by collagen I, there is also a gradual reduction in the amount of hyaluronic acid present within the new ECM, which triggers a reduction in fibroblast migration and proliferation. Transforming growth factor-β3 (TGF-β3) promotes reorganisation and remodelling of ECM proteins, thus improving dermal architecture and reducing subsequent scar formation (Occleston *et al.*, 2008). The basement membrane also undergoes remodelling following tissue injury through the action of MMPs. Comprised primarily of collagen IV, the remodelling of the basement membrane aids in re-establishing skin integrity whilst also maintaining epithelial cell polarity (Keane *et al.*, 2018).

Decorin produced by myofibroblasts binds to and neutralises TGF-β, reducing the stimulatory effects of TGF-β on collagen production whilst secretion of PDGF helps to modulate MMP and TIMP activity (Zhang *et al.*, 2007). As the wound becomes fully healed, the expression of α-SMA decreases causing myofibroblasts to undergo apoptosis resulting in the formation of a collagen I-rich, hypocellular scar (Sarrazy *et al.*, 2011; Xue and Jackson, 2015). The remodelling of the new collagen I-rich matrix allows the ECM to become more cross-linked and orientated, thus increasing the tensile strength of the mature wound; however, the collagen I bundles found within mature wounds are smaller than those found within the ECM of unwounded skin. Furthermore, the tensile strength of the mature wound will never reach that of unwounded skin, with approximately 80% being the maximum tensile strength the mature wound can achieve.

Disruption to the remodelling of the ECM can contribute to impaired wound healing. An overexpression of MMPs and decreased expression of TIMPs can lead to excessive ECM degradation and disorganisation that is associated with the development of chronic non-healing wounds (Muller *et al.*, 2008). Dysregulation of wound matrix remodelling also results in hyperproliferation which consequently can lead to fibrosis and excessive scar formation (Hinz, 2007). Further, a decrease in MMP expression and increase in TIMP expression contributes to tissue fibrosis and hypertrophic scar formation, demonstrating the importance of MMP and TIMP

regulation during matrix remodelling to ensure effective wound healing (Xue *et al.*, 2006; Xue and Jackson, 2015).

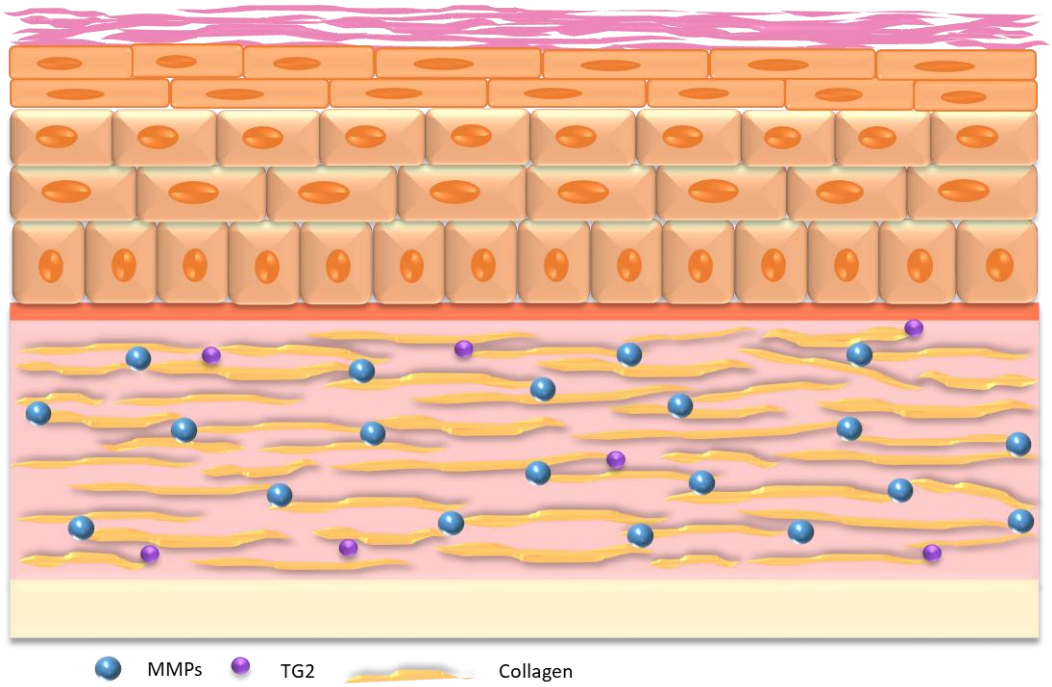

Figure 1. 7 Remodelling phase of cutaneous wound healing. *Dermal fibroblast secrete denser collagen I fibrils that replace the collagen III-rich granulation tissue. These collagen I fibrils are then remodelled through the action of MMPs and TG2 to become cross-linked and more orientated to increase the tensile strength of the mature wound.*

1.4 Impaired Cutaneous Wound Healing

Regulation of wound healing is important for effective tissue repair with dysregulation or imbalances causing impaired cutaneous wound healing and the development of chronic non-healing wounds (Guo and DiPietro, 2010). The development of chronic wounds is associated with a myriad of risk factors such as ageing and diabetes.

1.4.1 Skin Ageing

Ageing is considered a major risk factor associated with the development of chronic non-healing wounds. Skin ageing occurs through the natural passage of time (intrinsic ageing) but also as a result of exposure to external environmental factors (extrinsic ageing) (Gosain and DiPietro, 2004). Skin ageing causes a decrease in skin thickness by approximately 6.4% per decade resulting in a thinner epidermal

layer in the elderly (Farage *et al.*, 2013; Katoh *et al.*, 2018). Additionally, keratinocytes within the epidermis change shape with age becoming shorter and fatter whilst corneocytes in the stratum corneum become thicker due to decreased epidermal turnover (Farage *et al.*, 2013; Bonté *et al.*, 2019). Intrinsically aged skin can be characterised by extracellular atrophy of the dermis as well as a decrease in cell number, particularly dermal fibroblasts and mast cells present within the dermal region of skin (Baumann, 2007). Along with a decrease in cell number, intrinsic ageing also causes gradual degradation of ECM components such as collagen, elastin and glycosaminoglycans, which contribute to the appearance of aged skin; specifically wrinkles, dryness and loss of elasticity. Decreased collagen I production in aged skin affects the interactions between dermal fibroblasts and the ECM with increased collagen degradation impairing dermal fibroblast adherence and altering dermal fibroblast morphology, particularly reduced size and elongation (Varani *et al.*, 2006; Shin *et al.*, 2019). Ageing also reduces the mechanical forces exerted by dermal fibroblasts on the surrounding ECM, resulting in a downregulation of TGF-β receptor 2 (TGF-βR2) and TGF-β-induced ECM production whilst increasing secretion of cysteine-rich angiogenic protein 61 (CCN1) and MMPs (Quan *et al.*, 2011; Fisher *et al.*, 2016). The increased secretion of MMPs, particularly MMP-1, MMP-3 and MMP-9, increases degradation and fragmentation of the ECM resulting in higher fragility in elderly skin (Simonetti *et al.*, 2013).

In context of cutaneous wound healing, previous animal studies demonstrate a 20-60% delay in rate of wound healing in aged animals compared to younger animals resulting from temporal delays in cell responses to tissue injury as well as impaired vascularisation and deposition and remodelling of collagen (Swift *et al.*, 1999; Gosain and DiPietro, 2004). The decreased responses in wound healing is correlated to increased cellular senescence which alters various mechanisms such as impairing platelet function during haemostasis, diminishing expression of growth factors such as PDGF and EGF and reducing growth factor receptor phosphorylation (Jenkins, 2002; Bonté *et al.*, 2019). Studies in aged mice demonstrated a delay in EGF expression post-wounding compared to younger mice which in turn delayed re-epithelialisation (Ashcroft *et al.*, 1997a). Additional studies in aged mice demonstrated decreased phagocytic activity of wound macrophages and increased production of pro-inflammatory cytokines such as IL-1, IL-6 and TNF-

α and decreased VEGF secretion compared to younger mice (Swift *et al.*, 1999; Swift *et al.*, 2001). Previous *in vitro* studies comparing dermal fibroblasts derived from healthy young (22-30 years old) or aged (81-92 years old) individuals characterised aged dermal fibroblasts as having disorganised cytoskeletal actin which resulted in poorer migratory capabilities compared to younger dermal fibroblasts (Reed *et al.*, 2001).

1.4.2 Diabetes

Diabetes mellitus is another major risk factor with increased prevalence linked to chronic wound development and can be characterised as either type I or type II diabetes. Type I diabetes is characterised by a lack of insulin whilst type II diabetes is associated with insulin resistance (Hu and Lan, 2016). Recent reports indicate approximately 15% of all diabetic patients develop chronic wounds such as diabetic foot ulcers, with on average 2-3% of healthcare expenses spent on the treatment of chronic diabetic foot ulcers (Frykberg and Banks, 2015; Uccioli *et al.*, 2015; Li *et al.*, 2020). On average, normal blood glucose levels are 4.4-6.2mM, however the blood glucose levels of people with diabetes can reach around 20-25mM creating a hyperglycaemic environment (Güemes *et al.*, 2016). The generation of a hyperglycaemic environment has a negative impact on normal cutaneous wound healing by inhibiting both fibroblast and keratinocyte proliferation and migration whilst also increasing ROS production and cell death via apoptosis (Terashi *et al.*, 2005; Lamers *et al.*, 2011; Buranasin *et al.*, 2018; Li *et al.*, 2019). Previous *in vitro* and *in vivo* studies have demonstrated increased MMP production, particularly increased MMP-1, MMP-2 and MMP-9 secretion in diabetic patients leading to excessive ECM degradation (Derosa *et al.*, 2007; Kruse *et al.*, 2016). The hyperglycaemic environment also increases glycation of several ECM proteins such as collagen and fibronectin, impairing their ability to promote cell adhesion and subsequently causing reduced cellular proliferation and increased cellular senescence and apoptosis (McDermott *et al.*, 2003). Previous studies have demonstrated that a hyperglycaemic environment impairs re-epithelialisation during wound healing due to the impaired secretion of keratinocyte mitogens such as KGF and FGF as well as decreased expression of KRT16 and α2β1 integrins diminishing keratinocyte migration and proliferation (Werner *et al.*, 1994a; Lan *et al.*, 2009).

1.5 Cutaneous Wound Healing Strategies and Therapeutic Approaches

For the complete and effective treatment of cutaneous wounds, an appropriate wound care regime is essential. The majority of wound healing treatments are based on wound dressings, with traditional dressings being comprised of cotton gauzes and bandages (Dhivya *et al.*, 2015; Murray *et al.*, 2019). However, since these traditional dressings fail to maintain a moist environment for the wound, they have been gradually replaced by more modern dressings that are comprised of materials such as film, foam and synthetic polymers such as poly(glycolic acid) (PGA) and polyethylene glycol (PEG) that prevent dehydration but also help promote cutaneous wound healing (Lal *et al.*, 2000; Dhivya *et al.*, 2015; Zhang *et al.*, 2020). The use of hydrogels are also a common wound healing strategy as they are biocompatible and biodegradable and the incorporation of polymers such as poly(vinyl alcohol) (PVA) has demonstrated the ability to enhance cell migration and adhesion, promote granulation tissue formation and facilitate re-epithelialisation (Park *et al.*, 2018; Murray *et al.*, 2019). Additionally, many hydrogels have also been used to treat dry chronic wounds, pressure ulcers and burns as the high water content maintains a moist environment whilst also providing cooling and soothing effects to the wound site and surrounding tissue (Dhivya *et al.*, 2015).

Certain growth factors and cytokines play an important role in cutaneous wound healing such as EGF, FGF, TGF-β and PDGF, which has led to research aimed at the delivery of these growth factors to expedite the wound healing process. However, the direct application of growth factors alone has been shown to be ineffective with subsequent loss of growth factor bioactivity and poor skin penetration (Yamakawa and Hayashida, 2019; Nurkesh *et al.*, 2020). Topical treatment with EGF was considered a potential therapy after initial promising clinical results; however, the therapy failed to translate into a long-term clinical success as it could not provide prolonged stimulation of wound healing and required repetitive administration (Brown *et al.*, 1989). The three different isoforms of PDGF; platelet-derived growth factor-AA (PDGF-AA), platelet-derived growth factor-AB (PDGF-AB) and platelet-derived growth factor-BB (PDGF-BB) have also been explored as potential candidates for stimulating wound healing; however, PDGF-BB is currently the only growth factor that has been clinically approved for the treatment of chronic

wounds (Pierce *et al.*, 1991; Lepistö *et al.*, 1994; Li *et al.*, 2004; Gökşen *et al.*, 2017). The delivery of cytokines such as IL-22 and IL-10 have also been considered for resolving chronic wounds. The use of IL-22 induced keratinocyte proliferation and activation of the signal transducer and activator of transcription 3 (STAT3) in chronic diabetic wounds in mice models (Avitabile *et al.*, 2015). Also recombinant IL-10 incorporated into a dextrin nanogel matrix was considered as it enabled the slow release of biologically active IL-10 over time however, results showed that despite the slow release of IL-10, the cytokine denatured quickly and indicated that further stabilisation was required to allow for better controlled release over longer periods of time (Carvalho *et al.*, 2010; Carvalho *et al.*, 2011).

Similarly, growth factor therapy has been explored to mitigate scarring following injury. The administration of TGF-β3 demonstrated encouraging results in both phase I and II clinical trials, however the drug never made it to clinical use after failing during phase III clinical trials (Ferguson *et al.*, 2009; Evans *et al.*, 2013).

1.5.1 Collagen-Based Treatments

Due to the importance of collagen within cutaneous wound healing, various treatments have been developed that incorporate collagen. Collagen is an ideal biomaterial as it is biodegradable, has low toxicity and is readily available (Parenteau-Bareil *et al.*, 2010). Additionally, collagen-based treatments can usually be stored long-term under sterile conditions which means that they can be stockpiled in case of emergencies (Elgharably *et al.*, 2013). Natural collagen-based biomaterials can be classified into two categories; de-cellularised collagen matrices that maintain the original tissues ECM structure and properties or refined collagen-based scaffolds that are prepared via the extraction, purification and polymerisation of collagen (Chattopadhyay and Raines, 2014). Specifically, these collagen-based treatments have been shown to exert chemotactic effects on wound cells, promoting their migration and proliferation as well as encouraging the deposition of newly synthesised collagen (Fleck and Simman, 2010).

1.5.1.1 Collagen Dressings

Collagen dressings are usually developed using bovine or porcine collagen and have demonstrated the ability to create an environment that has a chemotactic effect on dermal fibroblasts whilst also encouraging the deposition of newly formed

collagen (Fleck and Simman, 2010). Wound dressings that specifically incorporate collagen I have been shown to not cause any adverse effects and can be safely used on patients with chronic wounds such as diabetic foot ulcers, with previous studies demonstrating that collagen I dressings enhanced wound healing rates and reduced the size of ulcers (Park *et al.*, 2019). The use of such collagen dressings is reported to provide anti-infective, anti-inflammatory and anti-fibrotic properties that help return skin to its normal state, offering a practical and economical advantage compared to other wound healing strategies such as growth factor therapy (Fleck and Simman, 2010; Chattopadhyay and Raines, 2014).

1.5.1.2 Collagen Sponges

Another collagen-based treatment used for treating cutaneous wounds are collagen sponges, which are insoluble forms of proteins that are usually derived from animals such as bovine and porcine. These collagen sponges are generated by lyophilising aqueous acidic or alkali swollen collagen solutions and the porosity of collagen sponges can be controlled by altering freezing rates and collagen content (Chattopadhyay and Raines, 2014). The wet-strength of collagen sponges enables them to maintain a moist environment and provide a template for new tissue growth, while also protecting against mechanical trauma or infection (Ellis and Yannas, 1996; Chattopadhyay and Raines, 2014). Previous studies have demonstrated the ability for collagen sponge implants to accelerate re-epithelialisation of the epidermis by 40% in full thickness excisional wounds (Leipziger *et al.*, 1985). Collagen sponges can be combined with natural polymers such as elastin, glycosaminoglycans and fibronectin or conjugated with synthetic polymers such as poly(hydroxylethylmethacrylate) to improve the mechanical strength of the collagen sponges (Lefebvre *et al.*, 1992; Ellis and Yannas, 1996; Chattopadhyay and Raines, 2014). Previous studies have also demonstrated that collagen sponges can be generated using synthetic collagen and the use of a sponge comprised of Pro-Hyp-Gly peptides has been shown to promote greater re-epithelialisation of full thickness wounds in animal models (Tanihara *et al.*, 2008).

Additionally, in animal models, it has been shown that the implantation of collagen sponges incorporated with growth factors such as FGF and PDGF promoted both dermal and epidermal wound healing and enhanced capillary formation (Marks *et al.*, 1991; Lepistö *et al.*, 1994). Following implantation, the collagen sponge is

gradually replaced by native collagen produced by dermal fibroblasts and the collagen sponge is degraded into peptide fragments and free amino acids by collagenases.

1.5.1.3 Collagen Gels

The use of injectable forms of collagen gels are mainly used for plastic and reconstructive surgery. The application of a modified collagen gel significantly enhanced collagen I deposition during repair of full thickness excisional wounds in animal models and in chronic ischemic wounds, the modified collagen gel promoted an up-regulation of TGF-β and VEGF whilst also enhancing the number of dermal fibroblasts and endothelial cells present at the wound site (Elgharably *et al.*, 2013; Elgharably *et al.*, 2014). Collagen gels can also be combined with synthetic polymers such as PVA and inserted with growth factors such as FGF or TGF-β which allows for the controlled release of these growth factors from the collagen gel when applied to the site of injury (Slavin *et al.*, 1992; Cascone *et al.*, 1995).

1.6 Collagen Peptides

Collagen peptides or hydrolysed collagen has for a long time been used as a food supplement with the purpose of improving health status and wellbeing. Collagen peptides are generated through the enzymatic cleavage of collagen isolated from animal skin, bones or connective tissue to produce water soluble peptides of varying molecular weight that have low immunogenicity and high bioavailability (Figure 1.8). This enzymatic hydrolysis not only affects the size of the collagen peptides produced, but also the physiochemical and biological properties of the peptides (León-López *et al.*, 2019).

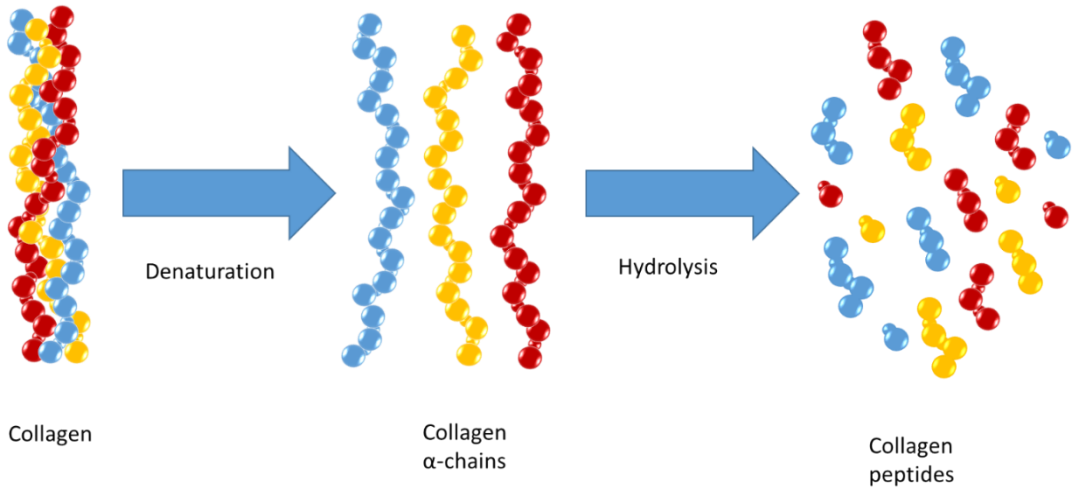

Figure 1. 8 Generation of bioactive collagen peptides. *Triple helical collagen chains derived from animal skin are denatured to form collagen α-chains which are then hydrolysed by specific enzymes to produce collagen peptides of varying molecular weight.*

Typically, collagen peptides are administered via oral ingestion, with previous studies showing that following oral ingestion these collagen peptides are absorbed and transported across the gut epithelium via peptide transporter 1 (PEPT1) into systemic circulation, where they are distributed to target tissues in the form of dipeptides, tripeptides and free amino acids (Aito-Inoue *et al.*, 2007). Previous bioavailability studies have demonstrated that the ingestion of 9.4-23g of collagen peptides results in peak concentrations of 200μM, 2 hours post-ingestion with approximately 20-60μM comprised of the dipeptide proline-hydroxyproline (Pro-Hyp), whilst other di- and tripeptides such as glycine-hydroxyproline (Gly-Hyp) and proline-hydroxyproline-glycine (Pro-Hyp-Gly) have also been detected at lower micromolar concentrations (Iwai *et al.*, 2005; Ohara *et al.*, 2007). Additional bioavailability studies carried out in Wistar rat models further revealed that radioactively-labelled collagen peptides could be detected in various tissue types such as skin, bone and muscle following oral ingestion and that these collagen peptides could still be detected within the skin of rats up to 14 days post-ingestion (Watanabe-Kamiyama *et al.*, 2010).

Currently, collagen peptides have been mainly used for their anti-ageing skin effects, with recent clinical trials demonstrating that the daily ingestion of collagen peptides over a period of 4-8 weeks improved skin hydration and elasticity whilst reducing the appearance of fine lines and wrinkles (Borumand and Sibilla, 2014;

Proksch *et al.*, 2014; Asserin *et al.*, 2015; Inoue *et al.*, 2016). Moreover, *in vivo* studies in murine animal models demonstrated the ability for collagen peptides to protect against skin ageing by maintaining collagen and elastin deposition as well as inhibiting excessive ECM degradation in chronologically aged skin (Liang *et al.*, 2010; Song *et al.*, 2017). Supporting the concept that collagen peptides inhibit excessive ECM degradation, *in vitro* studies using human dermal fibroblasts demonstrated that treatment with collagen peptides for 48 hours resulted in decreased expression of both MMP-1 and MMP-3, both of which are involved in the degradation of ECM proteins such as collagen but also are overexpressed within certain chronic wounds such as diabetic ulcers (Muller *et al.*, 2008; Edgar *et al.*, 2018).

More recently, results from *in vitro* studies have determined that the cleavage of collagen to peptide fragments exposes certain bioactive peptides that exert biological activities that are not typically observed in native collagen such as chemotactic and antioxidant effects that help to stimulate wound healing responses by promoting cellular proliferation and migration (Shigemura *et al.*, 2009; Banerjee *et al.*, 2015). Previous studies have demonstrated that treatment with animal-derived collagen peptides enhanced cellular migration of human umbilical vein endothelial cells (HUVECs) following scratch wound induction and enhanced the proliferation of dermal fibroblasts (Edgar *et al.*, 2018; Felician *et al.*, 2019). Furthermore, the use of the synthetic collagen peptide, Pro-Hyp has been shown to enhance the growth of mouse dermal fibroblasts suggesting that Pro-Hyp containing peptides may stimulate dermal fibroblast proliferation in order to promote effective wound healing (Shigemura *et al.*, 2009).

Further supporting the potential use of bioactive collagen peptides for treating cutaneous wounds, *in vivo* studies using rats demonstrated that the oral ingestion of marine collagen peptides significantly increased wound closure of both incisional and excisional wounds by increasing collagen deposition and promoting angiogenesis, thus increasing both nutrient and oxygen supply to the wound site (Zhang *et al.*, 2011; Wang *et al.*, 2015a). Additional *in vivo* studies also demonstrated that the treatment of cutaneous wounds in mice models with 0.9g/kg of marine collagen peptides significantly increased expression of FGF and TGF-β,

two growth factors that play key roles in promoting both cellular proliferation and migration during cutaneous wound healing (Felician *et al.*, 2019).

1.7 Hypothesis, Aims and Objectives

Given the financial and clinical burden that chronic non-healing wounds present to healthcare systems worldwide, there is an unmet need for more novel, reliable and cost-effective strategies in order to improve healing outcomes of chronic wounds. Therefore, the central aim of the current study was to test the hypothesis that collagen peptides promote cutaneous wound healing and to decipher the mechanisms mediating their beneficial effects. To test this hypothesis, the specific objectives of the study were to:

- Determine the effect of mixed molecular weight bovine (Peptan B), fish (Peptan F) and porcine (Peptan P)-derived collagen peptides to promote wound closure of primary keratinocytes and dermal fibroblasts derived from young or aged individuals or a hyperglycaemic environment using 2D *in vitro* scratch assays
- Determine the effect of Peptan B, Peptan F and Peptan P on proliferation of primary keratinocytes and dermal fibroblasts
- Generate, characterise and screen alternative porcine-derived collagen peptides and digests to determine their potential to promote wound closure
- Determine the mechanisms mediating the beneficial effects of collagen peptides on promoting cutaneous wound healing
- Adapt and apply 3D skin equivalent models to study full-thickness wound healing and application of collagen peptides to 3D wounded skin equivalent model to determine their wound healing potential *ex vivo*

Chapter 2: Materials and Methods

Chapter 2: Material and Methods

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2.1 Isolation, growth and maintenance of primary human keratinocytes and dermal fibroblasts

Primary human keratinocytes and dermal fibroblasts were isolated from excised human foreskin or excess normal human skin from Mohs surgery derived from differing aged individuals grouped as young (18-35 years old), middle-aged (40-55 years old) or aged (60+ years old) following informed consent (REC reference 19/NE/004_Lovat). Skin samples were placed epidermis side down into a 10cm plastic petri dish containing phosphate buffered saline (PBS) supplemented with Penicillin-Streptomycin-Amphotericin (PSA) (Lonza, Belgium). Fatty tissue, excess blood vessels and unhealthy sections of skin were removed using forceps and scissors before the remaining tissue was transferred to a new petri dish containing PBS/PSA. Tissue was then scored using a scalpel blade before being transferred to a sterile 25ml universal tube containing 8ml PBS/PSA, 1ml PSA and 1ml Dispase (Roche Diagnostics, Germany) and incubated overnight at 4°C to facilitate the separation of the epidermis and dermis. The following day, the epidermis was separated from the dermis by peeling them apart using forceps.

For keratinocyte isolation, the epidermis was placed into a sterile 25ml universal tube containing 5ml Trypsin/EDTA (Sigma-Aldrich, USA) and incubated at 37°C for 5 minutes to allow cell detachment. Five millilitres (5ml) of Dulbecco Modified Eagle Medium (DMEM) (ThermoFisher Scientific, USA) containing 10% foetal bovine serum (FBS) (Sigma-Aldrich, USA) and 1% PSA (complete DMEM) was then added to neutralise the Trypsin/EDTA before centrifugation at 300 g for 5 minutes at room temperature to obtain a cell pellet. The resulting keratinocytes were then re-suspended in 3ml EpiLife media (ThermoFisher Scientific, USA) containing 1% human keratinocyte growth factor supplement (HKGS) (ThermoFisher Scientific, USA) and 1% PSA (complete EpiLife) and transferred to a T175 tissue culture flask (Greiner, UK) containing 20ml complete EpiLife media. Primary keratinocytes were cultured at 37°C in a humidified atmosphere of 5% CO_2 with media changes every 2-3 days until keratinocytes reached 70-80% confluency. At approximately 70% confluency, keratinocytes were detached using 5ml Trypsin/EDTA and passaged at a 1:3 ratio into new T175 flasks for up to 5 passages or stored in liquid nitrogen in cryovials containing 1ml of complete EpiLife media supplemented with 10% dimethyl sulfoxide (DMSO) (ThermoFisher Scientific, USA).

To isolate primary dermal fibroblasts, the dermis was separated from the epidermis as described above and placed in a sterile petri dish containing PBS/PSA. The dermis was cut into 5-7 small pieces (approx. 2-3mm^2) using a sterile scalpel. A sterile scalpel was used to score the inside of a T75 flask (Greiner, UK). Excess liquid was removed from dermis pieces by dabbing in a fresh sterile petri dish before placing onto the scored section of the T75 flask and the addition of a drop of FBS before incubating overnight at 37°C. The following day, 10ml of complete DMEM was added to the T75 flask and continued incubation for 10-14 days to allow fibroblast migration from dermal tissue. Once dermal fibroblasts could be seen migrating out of the dermal tissue, each piece of dermis was removed from the T75 flask using sterile forceps. Adhered dermal fibroblasts were briefly washed in 3ml sterile PBS before detaching with 5ml Trypsin/EDTA at 37°C for 5 minutes and subsequent culture in complete DMEM media with media changes every 2-3 days. Dermal fibroblasts were then either used directly in experiments (up to passage 5) or stored in liquid nitrogen in cryovials containing DMEM supplemented with 10% DMSO. Demographic data for all samples used within the present study are detailed in Table 2.1

Sample ID	Gender (M= Male, F= Female)	Age	Body Site
14594	M	33	Foreskin
14595	M	55	Foreskin
14998	M	52	Foreskin
2961	F	88	Facial
2963	F	54	Facial
14610	M	18	Foreskin
14611	M	34	Foreskin
14662	M	67	Foreskin
14664	M	32	Foreskin
14668	M	73	Foreskin
14645	M	21	Foreskin
14665	M	53	Foreskin
14698	M	36	Foreskin
14701	M	32	Foreskin
14702	M	43	Foreskin
14708	M	75	Foreskin
14735	M	86	Foreskin
14735	M	86	Foreskin
14736	M	66	Foreskin
2717	M	62	Facial
2907	F	73	Facial

Table 2. 1 *Demographic data for each tissue donor used in experiments*

2.2 Growth and maintenance of human neonatal keratinocytes (HEKn) and dermal fibroblasts (HDFn)

Human neonatal keratinocytes (HEKn) and dermal fibroblasts (HDFn) were purchased from ThermoFisher Scientific, USA. HEKn cells were cultured in EpiLife media containing 1% HKGS and 1% PSA, whilst HDFn cells were cultured in DMEM media containing 10% FBS and 1% PSA. HEKn and HDFn cells were used directly in experiments for protein extraction as described in Section 2.10, for generating

full-thickness skin equivalents as described in Section 2.19 or stored in liquid nitrogen in cryovials containing either Epilife (HEKn) or DMEM (HDFn) supplemented with 10% DMSO.

2.3 Growth and maintenance of the Caco-2 cell line

Caco-2 cells, kindly provided by Dr Peter Chater (Biosciences Institute, Newcastle University, UK), were cultured in DMEM supplemented with 10% FBS, 1% L-Glutamine, 1% non-essential amino acids (NEAA) and 1% Penicillin/Streptomycin (P/S). At approximately 70% confluency, cells were detached using 5ml Trypsin/EDTA and passaged at a 1:5 ratio, with cells either used directly in experiments or frozen in cryovials containing complete growth media with 10% DMSO for long-term storage in liquid nitrogen. For experiments, 200,000 Caco-2 cells were seeded into each well of a 6 well plate (Sarstedt, Germany) with RNA extracted the following day for qPCR as described in Section 2.15.

2.4 Preparation of collagen peptides

Mixed molecular weight (average molecular weight ~ 2kDa) collagen peptides derived from bovine (Peptan B), fish (Peptan F) or porcine (Peptan P) skin were provided by Rousselot (Ghent, Belgium). For *in vitro* experiments, collagen peptides were dissolved in ultrapure water and filter sterilised to give a final concentration of 1mg/ml. For experiments, 100µl of the 1mg/ml stock solution was used to coat wells of a 96 well plate (Sarstedt, Germany) and incubated overnight at room temperature to allow adherence. The following day, excess liquid was removed and plates were air dried at room temperature. For experiments where collagen peptides were added topically to cells, collagen peptides were dissolved in PBS to a give a 15mg/ml stock solution prior to addition to culture media to give a final concentration of either 1mg/ml, 2.5mg/ml, 5mg/ml or 10mg/ml.

To quantify collagen peptide adherence, Bradford protein quantification assays (Pierce Biotech, Rockford, USA) were performed according to manufacturer's specifications, with protein absorbance measured at 595nm using a SpectraMax 250 plate reader (Molecular Devices Ltd., Wokingham, UK) (Table 2.2)

Collagen Peptide	Collagen Peptide Adherence to Plastic (µg/ml)
Peptan B	15.5 ± 8.4
Peptan F	9.3 ± 6.2
Peptan P	18.7 ± 7.6

Table 2. 2 *Concentrations of collagen peptides adhered to the surface of plastic tissue culture plates*

2.5 Generation of alternative porcine-derived collagen peptides

Alternative porcine-derived collagen peptides were generated according to standardised protocols established at Rousselot (Ghent, Belgium), with the size of these alternative collagen peptides characterised as described in Table 2.3. For *in vitro* experiments, alternative porcine-derived collagen peptides were dissolved in ultrapure water and filter sterilised to give a final concentration of 1mg/ml. For experiments, 100µl of the 1mg/ml stock solution was used to coat wells of a 96 well ImageLock plate (Essen Biosciences, UK) and incubated overnight at room temperature to allow adherence. The following day, excess liquid was removed and plates were air dried at room temperature.

Alternative porcine-derived collagen peptide ID	Peptide Size
CH1	Small
CH2	Small
CH3	Medium
CH4	Medium
CH5	Medium
CH6	Medium
CH7	Large
CH8	Large
CH9	Large
CH10	Large

*Full names of products and molecular weights are undisclosed under confidentially agreement with Rousselot

Table 2. 3 *Characterisation of alternative porcine-derived collagen peptides generated through single enzyme hydrolysis*

2.6 Generation of collagen peptide digests

Porcine-derived collagen peptide digests were provided by Rousselot (Ghent, Belgium) after digesting collagen peptide samples using an optimised version of the In Vitro Digestion protocol (Minekus *et al.*, 2014). For *in vitro* experiments, collagen

peptide digests were dissolved in PBS to a give a 10mg/ml stock solution prior to a 1:10 dilution in culture media to give a final concentration of 1mg/ml and 100µl added to cells following scratch wound induction as described in Section 2.11.

2.7 Participant recruitment and blood sampling

Full ethical permission was granted by the Newcastle University Ethics Committee (Ethics Number: 1681/11689/2019) with all participants providing informed consent. Six healthy individuals (4 females and 2 males) grouped as young (age 26 ± 6; mass 73.5 ± 9.4kg; height 1.7 ± 0.1m) or aged (age 64 ± 2; mass 55.8 ± 4.7kg; height 1.65 ± 0.01m) ingested 10g of Peptan P collagen peptides with 5ml fruit juice (Ribena, Suntory, Japan) diluted in 250ml water following an overnight fast. Blood samples were collected prior to ingesting Peptan P collagen peptides (0 hours) and 2,8 and 24 hours post-ingestion via venepuncture for subsequent analysis of hydroxyproline (Hyp), a biomarker for collagen peptide absorption, in plasma and serum (Iwai *et al.*, 2005). Participants were allowed to consume their habitual diet but were asked to keep physical activity to a minimum the day before and day of the trial. At each time-point, venous blood was collected from an antecubital vein into two separate 10ml vacutainers; one treated with EDTA to obtain plasma and the other vacutainer was untreated to obtain serum. The EDTA tube was centrifuged immediately at 3000 *g* for 10 minutes whereas the serum was centrifuged at the same speed after 30 minutes to allow time for clotting. The supernatant was aspirated from the vacutainer into aliquots and stored at -80°C until analysis

2.8 Ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS)

Total hydroxyproline levels in plasma and serum samples was quantified by ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) as previously described (Kleinnijenhuis *et al.*, 2020). Calibration samples of hydroxyproline (Sigma-Alrich, USA) were dissolved in pooled normal human serum (BioIVT, USA) to obtain concentrations ranging from 0-200µg/ml. Calibration, plasma and serum samples were spiked with an internal standard of deuterated hydroxyproline (CDN Isotopes), hydrolysed in 6M hydrochloric acid (Merck, USA), dried and re-dissolved in borate buffer before being derivatised with AccQ-Tag (Waters, USA) and diluted with ultrapure water. One microlitre of each test sample

was analysed using UPLC-MS/MS (Acquity-Waters Xevo TQ-S) and chromatographic separation was performed using an Acquity HSS T3 column (100 x 2.1mm, 1.8µm) (Waters, USA) at 60°C and a flow rate of 700µl/min. The following gradient was used: 0-0.54 min 99.9% A (0.1% formic acid (FA) in ultrapure water) and 0.1% B (0.1% FA in acetonitrile), 0.54-5.74 min 99.9-90.9% A and 0.1-9.1% B, 5.74-7.7.4 min 90.9-78.8% A and 9.1-21.2% B, 7.74-8.04 min 78.8-40.4% A and 21.2-59.6% B, 8.04-8.05 min 40.4-10.0% A and 59.6-90.0% B, 8.05-8.64 min 10% A and 90% B, 8.64-8.73 10-99.9% A and 90.0-0.1% B, 8.73-9.5 min 99.9% A and 0.1% B. Tandem mass spectrometry was carried out after positive electrospray ionisation mode, and for selected reaction monitoring, the precursor ion at m/z 302 (hydroxyproline + AccQ-Tag $[M+H]^+$) and the product ion at m/z 171 (AccQ-Tag^+) were used. The mean peak area ratio of hydroxyproline against the internal standard was determined for calibration, plasma and serum samples to control for recovery. Total hydroxyproline quantification of the plasma and serum samples was calculated using linear regression based on the calibration samples.

2.9 Cell Lysis and Protein Extraction

Protein was extracted from either 200,000 young (18-35 years old), middle-aged (40-55 years old) or aged (60+ years old) primary keratinocytes or dermal fibroblasts by cell lysis in 150µl lysis buffer (pre-prepared freshly from 850µl of lysis buffer (0.1M Tris-HCL (pH 7.4), 25mM Sodium Fluoride (NaF), 0.1M Sodium Chloride (NaCl), 2mM EDTA (pH 8), 1nM benzamidine, 0.1mM sodium orthovandate, 0.1% Triton-X100) and 150µl protease inhibitor cocktail (PIC) (Promega, Southampton, UK)).

For experiments investigating the effect of collagen peptides on collagen expression or activation of downstream signalling pathways, 100,000 young (18-35 years old) or aged (60+ years old) primary keratinocytes or dermal fibroblasts were seeded onto uncoated wells or wells coated with 1mg/ml Peptan P or 1mg/ml rat tail collagen I (Corning, USA) and incubated for 72 hours. After 72 hours cells were lysed in 150µl lysis buffer. For experiments investigating the effect of collagen peptides on activating signalling pathways post-wounding, 500,000 primary keratinocytes or dermal fibroblasts were seeded onto uncoated or 1mg/ml Peptan P coated wells to form a confluent monolayer, with a scratch wound generated using

a p200 tip. Cells were then incubated for 0, 24, 48 or 72 hours post-wounding prior to harvesting protein using 150µl lysis buffer.

Protein lysates were transferred into individual 1.5ml Eppendorf tubes and sonicated using a probe sonicator (Soniprep 150, MSE, UK) for 2 pulses of 5 seconds at an amplitude of 7 microns. Protein concentration was determined using a Bradford protein quantification assay (Pierce Biotech, USA) according to manufacturer's specifications, with protein absorbance measured at 595nm using a SpectraMax 250 plate reader (Molecular Devices Ltd., UK).

2.10 Western Blot

Ten micrograms of protein lysate was diluted 1:3 in 4x sample buffer (250mM Tris-HCL (pH 8), 8% sodium dodecyl sulphate (SDS), 40% glycerol, 10% β-mercaptoethanol and bromophenol blue) and heated to 95°C for 5 minutes using a heat block. Proteins were separated using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) through 4-20% tris-glycine gels (Bio-Rad, UK) immersed in running buffer (250mM Tris base, 1.9M glycine and 1% SDS). Proteins were subsequently transferred onto PVDF membranes using a Trans-Blot Turbo Transfer System (1.3A, 25V) (Bio-Rad, UK) and Trans-Blot Turbo Transfer Packs (Bio-Rad, UK) for 7 minutes at room temperature before blocking membranes in 5% non-fat milk diluted in 1x Tris-Buffered-Saline (200mM Tris Base, 1.37M NaCl) containing 0.1% Tween 20 (TBS/T) for 1 hour at room temperature. Membranes were then incubated with primary antibodies diluted in TBS/T containing 5% non-fat milk or 5% bovine serum albumin (BSA) (Table 2.4) overnight at 4°C. Membranes were then washed 3 times for 15 minutes in TBS/T before incubation with secondary polyclonal antibodies diluted in TBS/T containing 5% non-fat milk for 1 hour at room temperature. Following further washing (3x 15 minutes at room temperature) in TBS/T, protein expression was then detected with Clarity Western ECL Reagent (Bio-Rad, UK) for 5 minutes at room temperature according to manufacturer's specifications and visualised using a Li-COR Odyssey Fc Imager with Image Studio Software (Li-COR Biosciences, UK).

Primary Antibody	Supplier	Species (Molecular Weight)	Primary Antibody Dilution	Secondary Antibody Dilution
Collagen I	Southern Biotech (1310-01)	Goat Polyclonal (139kDa)	1:10,000 5% non-fat milk in TBS/T	Anti-goat 1:2500 5% non-fat milk in TBS/T
Collagen III	Abcam (ab184993)	Rabbit Monoclonal (139kDa)	1:1000 5% non-fat milk in TBS/T	Anti-rabbit 1:2500 5% non-fat milk in TBS/T
Transglutaminase 2	ThermoFisher Scientific	Mouse Monoclonal (77kDa)	1:500 5% non-fat milk in TBS/T	Anti-mouse 1:2500 5% non-fat milk in TBS/T
Integrin α2	Abcam (ab181548)	Rabbit Monoclonal (129kDa)	1:5000 5% non-fat milk in TBS/T	Anti-rabbit 1:2500 5% non-fat milk in TBS/T
Integrin β1	Abcam (ab179472)	Rabbit Monoclonal (130kDa)	1:1000 5% non-fat milk in TBS/T	Anti-rabbit 1:2500 5% non-fat milk in TBS/T
Phospho-FAK (Tyr397)	Cell Signalling (8556T)	Rabbit Monoclonal (125kDa)	1:100 5% BSA in TBS/T	Anti-rabbit 1:2500 5% non-fat milk in TBS/T
FAK	Cell Signalling (13009S)	Rabbit Monoclonal (125kDa)	1:1000 5% BSA in TBS/T	Anti-rabbit 1:2500 5% non-fat milk in TBS/T
Phospho-Akt (Ser473)	Cell Signalling (4060S)	Rabbit Monoclonal (60kDa)	1:1000 5% BSA in TBS/T	Anti-rabbit 1:2500 5% non-fat milk in TBS/T
Akt	Cell Signalling (2920S)	Mouse Monoclonal (60kDa)	1: 1000 5% BSA in TBS/T	Anti-mouse 1:2500 5% non-fat milk in TBS/T
Phospho-ERK 1/2 (Thr202/Tyr204)	Cell Signalling (4377S)	Rabbit Monoclonal (42kDa)	1:1000 5% BSA in TBS/T	Anti-rabbit 1:2500 5% non-fat milk in TBS/T
ERK 1/2	Cell Signalling (9102S)	Rabbit Monoclonal (42kDa)	1:1000 5% BSA in TBS/T	Anti-rabbit 1:2500 5% non-fat milk in TBS/T
GAPDH	Cell Signalling (2118S)	Rabbit Monoclonal (37kDa)	1:5000 5% BSA in TBS/T	Anti-rabbit 1:2500 5% non-fat milk in TBS/T

Table 2. 4 *Antibody dilutions for Western blot*

2.11 2D *in vitro* scratch assays

Thirty thousand primary keratinocytes or 15,000 dermal fibroblasts derived from young (18-35 years old) or aged (60+ years old) healthy donors were seeded in 100µl of complete EpiLife (keratinocytes) or DMEM (dermal fibroblasts) media into each well of a 96 well ImageLock plate (Essen Biosciences, UK) pre-coated with 1mg/ml of either Peptan B, Peptan F or Peptan P collagen peptides, 1mg/ml alternative porcine-derived collagen peptides or uncoated negative control wells. Plates were incubated overnight at 37°C to allow cellular adherence and formation of a confluent cell monolayer. Following day, cells were treated in the presence or absence of 7.5µg/ml mitomycin C (Sigma-Aldrich, USA) for 2 hours to inhibit cell proliferation prior to washing in sterile PBS and replacement with 100µl of fresh culture media. A uniform scratch was then induced in the cell monolayer using a 96-well Woundmaker (Essen Biosciences, UK), prior to washing with sterile PBS to remove cell debris and replacement with 100µl of fresh culture media.

For high glucose experiments, 5.5mM glucose was used to mimic a normal glucose environment while 25mM and 50mM glucose were used to represent a hyperglycaemic and an extreme hyperglycaemic environment (Güemes *et al.*, 2016). Thirty thousand primary keratinocytes or 15,000 dermal fibroblasts cultured in low glucose (5.5mM) EpiLife or DMEM media were seeded into each well of a 96 well ImageLock plate (Essen Biosciences, UK) pre-coated with 1mg/ml porcine collagen peptides or uncoated negative control wells and allowed to adhere overnight at 37°C to form a confluent monolayer. The following day, a uniform scratch was induced in the cell monolayer using a 96-well Woundmaker (Essen Biosciences, UK), prior to washing with sterile PBS to remove cell debris and replacement of media with 100µl of culture media containing either 5.5mM, 25mM or 50mM glucose.

All plates were then incubated at 37°C for approximately 5 minutes to prevent condensation before being placed inside an Incucyte ZOOM system (Essen Biosciences, UK) with live cell imaging and monitoring captured by time lapse microscopy at 2 hour intervals over 72 hours. Incucyte ZOOM software was used to analyse scratch assays to determine wound confluence (%) and to obtain wound widths to calculate rate of wound closure.

2.12 MTS cell viability assays

Five thousand primary keratinocytes or dermal fibroblasts were seeded in 100µl of complete EpiLife (keratinocytes) or DMEM (dermal fibroblasts) into each well of a 96 well tissue culture plate (Sarstedt, Germany) pre-coated with 1mg/ml Peptan B, Peptan F or Peptan P collagen peptides or uncoated negative control wells, before being incubated overnight at 37°C to allow cell adherence. The following day, cells were treated in the presence or absence of 7.5µg/ml mitomycin C for 2 hours, followed by washing with sterile PBS and replacement with 100µl of complete EpiLife or DMEM and continued incubation at 37°C for 72 hours.

For high glucose experiments, 5,000 primary keratinocytes or dermal fibroblasts were seeded in 100µl of complete EpiLife (keratinocytes) or DMEM (dermal fibroblasts) media containing 5.5mM glucose into each well of a 96 well tissue culture plate (Sarstedt, Germany) pre-coated with 1mg/ml Peptan B, Peptan F or Peptan P collagen peptides or uncoated negative control wells, before being incubated overnight at 37°C to allow cell adherence. Cells were then washed in sterile PBS before replacement with 100µl of culture media containing either 5.5mM, 25mM or 50mM glucose and continued incubation at 37°C for 72 hours.

Twenty microlitres of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium solution (MTS) (Cell Titre 96, Promega, Southampton, UK) was then added to each well and continued incubation at 37°C for 4 hours in the dark to allow colour development. The absorbance was then measured at a wavelength of 490nm using a SpectraMax plate reader (Molecular Devices Ltd., UK).

2.13 Immunofluorescence for Ki67 expression in primary keratinocytes and dermal fibroblasts

Fifty thousand primary dermal fibroblasts or keratinocytes were seeded onto glass coverslips pre-coated with 1mg/ml Peptan B, Peptan F or Peptan P collagen peptides or uncoated coverslips in 3ml of complete DMEM or EpiLife media per well in a 6 well tissue culture plate (Greiner, UK) for 72 hours. For high glucose experiments, 50,000 dermal fibroblasts or keratinocytes were incubated in culture media containing either 5.5mM, 25mM or 50mM glucose for 72 hours. After 72 hours, culture media was removed and cells were washed with sterile PBS and fixed

with ice cold 4% paraformaldehyde (PFA) for 15 minutes at room temperature. After a further 2 washes with PBS for 5 minutes, cells were permeablised in 0.2% Triton in PBS for 10 minutes at room temperature. After washing in PBS, cells were incubated with 2% BSA + 2% goat serum in PBS for 30 minutes at room temperature to prevent non-specific antibody binding before incubation with Ki67 rabbit polyclonal antibody (1:1000 in PBS, ab15580, Abcam, UK) for 1 hour at room temperature. Cells were then washed 3 times in PBS before incubation with secondary fluorescent antibody Alexa Fluor® 568 Goat Anti-Rabbit IgG (1:1000 in 5% BSA in PBS, Life Technologies, USA) for 1 hour in the dark at room temperature. Cells were then washed 3 times in PBS in the dark before incubation with 4',6-diamidino-2-phenylindole (DAPI, 1:10,000 in PBS) for 15 minutes in the dark. After a final two PBS washes, coverslips were mounted onto glass microscope slides (ThermoFisher Scientific, USA) using VECTASHIELD mounting medium (Vector Laboratories, USA). Immunofluorescent images were captured using a Zeiss AxioImager microscope (Carl Zeiss Ltd., UK).

2.14 RNA extraction and reverse transcription to cDNA

Ribonucleic acid (RNA) was extracted from Caco-2 cells or young (18-35 years old) and aged (60+ years old) primary keratinocytes and dermal fibroblasts using a ReliaPrep™ RNA Cell MiniPrep System (Promega, UK) as per manufacturer's instructions. The integrity of the isolated RNA was determined using a NanoDrop 2000 (ThermoFisher Scientific, USA) prior to storing RNA at -80°C. Extracted RNA was converted to single-stranded cDNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) as per manufacturer's instructions. 20µl of PCR reverse transcription reactions were prepared for each sample (Table 2.5) and subsequent PCR performed using a Gene Amp PCR system 9700 thermocycler (Applied Biosystems, USA), with cDNA stored at -20°C for long-term storage.

Reagent	Volume added for a single reaction
10x RT Buffer	2 µl
25x dNTP Mix (100mM)	0.8 µl
10x RT Random Primers	2 µl
Multiscribe Reverse Transcriptase	0.5 µl
RNase Inhibitor	0.2 µl
Nuclease-free water	4.5 µl
RNA	10 µl

Table 2. 5 *Constituents of a single reverse transcription reaction*

2.15 Real-time Polymerase Chain Reaction (qPCR)

Real-time polymerase chain reaction (qPCR) was used to determine the relative expression levels of peptide transporters PEPT1 and PEPT2, peptide histidine transporters PHT1 and PHT2, relative to the housekeeping control of GAPDH in primary keratinocytes and dermal fibroblasts. Master reaction mixes were prepared for each gene probe as described in Table 2.6, using TaqMan® Fast Advanced Master Mix (Applied Biosystems, USA) and pre-designed TaqMan® Gene Expression Assays for PEPT1 (Hs00192639_m1), PEPT2 (Hs01113665_m1), PHT1 (Hs00377326_m1), PHT2 (Hs00275455_m1) and GAPDH (Hs02786624_g1) as a housekeeping gene. Four microlitres of cDNA was combined with 16µl master mix in each well of a 96 well MicroAmp Optical Clear Reaction Plate (Applied Biosystems, USA). Triplicate samples for each gene probe were also prepared incorporating both a non-template control (H_2O only) and a positive control of Caco-2 cell cDNA for peptide transporter expression, before plates were sealed with StarSeal Advanced Polyolefin film (STARLABS, Germany), briefly centrifuged at 300 g for 1 minute and subjected to qPCR analysis using a StepOne Real-Time PCR instrument (Applied Biosystems, USA), with 40 cycles of: hold at 50^{o}C for 2 minutes, hold at 95^{o}C for 10 minutes, denaturation at 95^{o}C for 15 seconds and annealing at 60^{o}C for 1 minute. Real-time gene expression analysis was determined using StepOne software (Applied Biosystems, USA). The expression of each peptide transporter was normalised to the housekeeping gene GAPDH and the relative gene expression was calculated using the comparative ΔΔCt method (Livak and Schmittgen, 2001), with the fold change in gene expression relative to Caco-2 cells for each of the peptide transporters.

Reagents	Volume added per well
Master Mix	10 µl
Primer	1 µl
Nuclease-free water	5 µl
cDNA	4 µl

Table 2. 6 *Constituents of a single qPCR reaction*

2.16 Small interfering RNA (siRNA) knockdown of Integrin β1 Receptor

For transient knockdown of integrin β1, ON-TARGETplus SMARTpool *ITGB1* siRNA (Dharmacon, Horizon Discovery, USA) or ON-TARGETplus Non-targeting Control Pool siRNA (Dharmacon, Horizon Discovery, USA) was diluted to a final concentration of 20µM in 1x siRNA buffer (Dharmacon, Horizon Discovery, USA).

For experiments evaluating the effect of *ITGB1* knockdown on the activation of signalling pathways downstream of integrin in both unwounded and wounded cells, 100,000 or 500,000 primary keratinocytes or dermal fibroblasts were seeded onto either uncoated wells or wells pre-coated with 1mg/ml rat tail collagen I or 1mg/ml Peptan P in 3ml culture media and allowed to adhere overnight. For transient transfection in a single well, 5µl of Lipofectamine RNAi MAX (Thermofisher Scientific, USA) was added to 250µl Opti-MEM media before the addition of 5µl of either *ITGB1* or non-target control siRNA (siCtrl) in 250µl Opti-MEM media (Life Technologies, USA) and continued incubation for 15 minutes at room temperature. Cells were washed in PBS before adding 2ml of Opti-MEM and subsequent addition of 500µl of each respective siRNA and lipofectamine solution drop-wise to the wells. Cells were then incubated at 37°C for 6 hours before replacing the media with fresh DMEM or EpiLife and protein extraction at either 24, 48 or 72 hours post-transfection. For experiments looking at the effect of *ITGB1* knockdown on activation of signalling pathways post-wounding, cells were scratched using a 200µl pipette tip after incubating with *ITGB1* or siCtrl siRNA prior to washing in PBS to remove cell debris and subsequent addition of 3ml fresh culture media. After 24, 48 and 72 hours post-wounding, cells were lysed in 150µl of lysis buffer and lysates were stored at -20°C prior to analysis of protein expression by Western blotting as described in Section 2.10.

For experiments evaluating the effect of *ITGB1* knockdown on wound closure, 30,000 primary keratinocytes or 15,000 dermal fibroblasts were seeded in 100µl of complete EpiLife (keratinocytes) or DMEM (dermal fibroblasts) media into each well of a 96 well ImageLock plate (Essen Biosciences, UK) pre-coated with 1mg/ml of Peptan P. To ensure transient transfection of a single 96 well, a master mix was made consisting of 7µl of Lipofectamine RNAi MAX (Thermofisher Scientific, USA) in 133µl Opti-MEM media before combining with 7µl of either *ITGB1* or non-target control siRNA (siCtrl) in 133µl Opti-MEM media (Life Technologies, USA) and continued incubation for 15 minutes at room temperature. Cells were washed in PBS before adding 90µl of Opti-MEM and subsequent addition of 10µl of each respective siRNA and lipofectamine solution drop-wise to the wells. Cells were incubated at 37°C for 6 hours before replacing the media with 100µl fresh DMEM or EpiLife. A uniform scratch was then induced using a 96-well Woundmaker (Essen Biosciences, UK) prior to washing in 100µl PBS to remove cell debris and replacement with 100µl fresh DMEM or EpiLife. Wound closure was then monitored over 72 hours as described in Section 2.11.

2.17 Flow Cytometry

One million primary human keratinocytes or dermal fibroblasts were seeded in 10ml of either EpiLife (keratinocytes) or DMEM (dermal fibroblasts) into T75 flasks and allowed to adhere overnight at 37°C. The following day keratinocytes and dermal fibroblasts were treated with 1mg/ml Peptan P or vehicle control media before continued incubation at 37°C for 72 hours. Cells were detached using 5ml Accutase (Biolegend, UK), neutralised using 5ml DMEM and counted using a haemocytometer before $1x10^6$ cells were pipetted into 5ml fluorescent-activated cell sorting (FACS) tubes (Fisher Scientific, UK), centrifuged at 600 g for 5 minutes and re-suspended in 500µl FACS buffer (5% FBS in PBS). Cells were then centrifuged again at 600g for 5 minutes and re-suspended in 100µl FACS buffer before the addition of 5µl of Human TruStain FcX blocking buffer (Biolegend, UK) and continued incubation for 10 minutes at room temperature. Five microlitre of either APC anti-human CD49b (359309, Biolegend, UK), PE anti-human CD49c (343803, Biolegend, UK), FITC anti-human CD49e (328007, Biolegend, UK), BV421 anti-human/mouse CD49f (313623, Biolegend, UK) or APC/Cyanine7 anti-human CD29 (303014, Biolegend, UK) was added to respective FACS tubes containing either

experimental conditions, compensation controls or gating controls as described in Table 2.7 before incubation on ice for 30 minutes in the dark. Cells were then washed three times in FACS buffer with centrifugation at 300 g for 5 minutes at 4°C. Cells were then re-suspended in 300µl of FACS buffer prior to analysis using a Fortessa X20 system (BD Biosciences, USA). FCS Express 7 software (De Novo Software, USA) was used to identify CD49b+, CD49c+, CD49e+, CD49f+ and CD29+ cell populations following gating (Figure 2.1) and calculate median CD49b+, CD49c+, CD49e+, CD49f+ and CD29+ expression in control and Peptan P treated cells relative to the control.

Tube	**Condition**	**Fluorochrome**				
Tube 1	Unstained control	-	-	-	-	-
Tube 2	Experimental control	CD49b APC	CD49c PE	CD49e FITC	CD49f BV421	CD29 APC/Cy7
Tube 3	Treated with Peptan P	CD49b APC	CD49c PE	CD49e FITC	CD49f BV421	CD29 APC/Cy7
Tube 4	Compensation Controls (Single stains)	APC	-	-	-	-
Tube 5		-	PE	-	-	-
Tube 6		-	-	FITC	-	-
Tube 7		-	-	-	BV421	-
Tube 8		-	-	-	-	APC/Cy7
Tube 9	Gating Controls (Fluorescence minus one (FMO))	-	PE	FITC	BV421	APC/Cy7
Tube 10		APC	-	FITC	BV421	APC/Cy7
Tube 11		APC	PE	-	BV421	APC/Cy7
Tube 12		APC	PE	FITC	-	APC/Cy7
Tube 13		APC	PE	FITC	BV421	-

Table 2. 7 *Experimental conditions for flow cytometry*

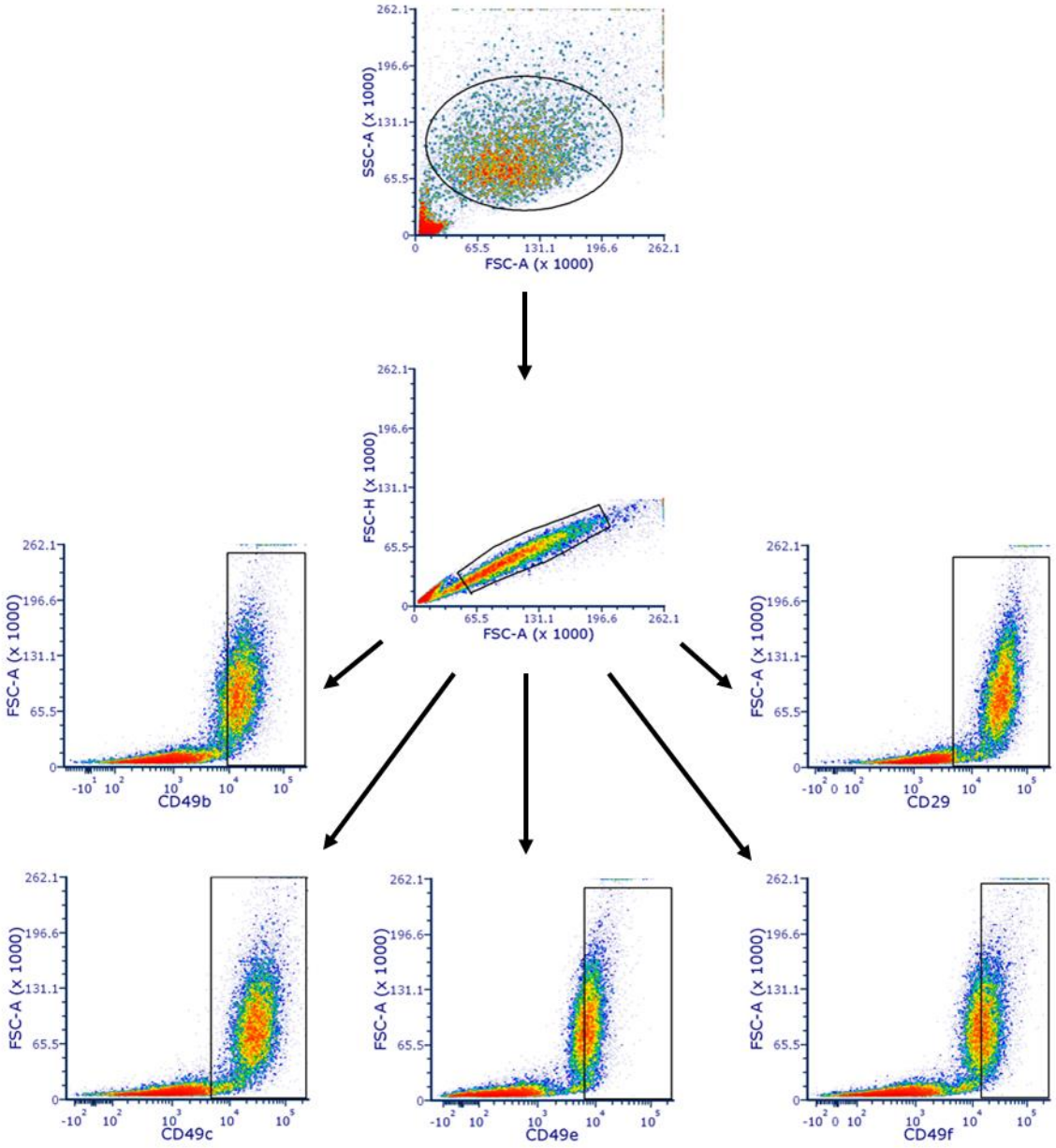

Figure 2. 1 FACS gating strategy to identify CD49b+, CD49c+, CD49e+, CD49f+ or CD29+ cells. *Single cells were defined and gates were set based on relevant fluorescent minus one (FMO) used for CD49b (APC), CD49c (PE), CD49e (FITC), CD49f (BV421) or CD29 (APC/Cy7).*

2.18 Generation of 3D full thickness skin equivalents

The generation of full thickness skin equivalents was based on the method published by Hill *et al.* (Hill *et al.*, 2015). Young (18-35 years old) or aged (60+ years old) primary patient-matched keratinocytes and dermal fibroblasts were isolated from excised human skin as described in Section 2.1. Alvetex® scaffold inserts (ReproCell Ltd., UK) were rehydrated in 70% ethanol for 10 seconds before being

washed twice with sterile PBS and incubated in 9ml DMEM media at 37°C in a 6 well plate (TPP, Switzerland) until use. Five-hundred thousand dermal fibroblasts were seeded in 200µl DMEM per Alvetex® scaffold and incubated for 3 hours at 37°C to allow cell adherence to scaffold. After 3 hours, 9ml DMEM supplemented with 100µg/ml Vitamin C (Sigma-Aldrich, USA) was added to each scaffold and media was changed every other day. After 7 days, another $5x10^5$ dermal fibroblasts were seeded onto the dermal equivalent in 200µl DMEM per Alvetex® scaffold and incubated for 3 hours at 37°C prior to addition of 9ml DMEM supplemented with 100µg/ml Vitamin C. This dermal layer was maintained for a further 21 days with media changes every other day prior to the seeding of $1.5x10^6$ primary keratinocytes in 200µl Epilife media per Alvetex® scaffold and incubation for 3 hours at 37°C to allow cell adherence. After 3 hours, the skin equivalents were supplemented with 9ml EpiLife media supplemented with 100µg/ml Vitamin C and 10ng/ml Keratinocyte Growth Factor (KGF) (ThermoFisher Scientific, USA) before incubating at 37°C. After 2 days, the full-thickness skin equivalents were raised to the air-liquid interface using a deep well petri dish (ReproCell Ltd., UK) to allow epidermal differentiation. Skin equivalents were maintained at the air-liquid interface for 14 days using EpiLife media containing high calcium (1.5mM), 10ng/µl KGF and 100µg/ml Vitamin C, with media changes every other day. After 14 days, full-thickness skin equivalents were removed from inserts, washed in sterile PBS and placed in histology cassettes, fixed in 10% formalin and sent to Cellular Pathology (NHS Foundation Trust, Royal Victoria Infirmary, Newcastle upon Tyne, UK) for embedding in paraffin.

2.19 Generation of 3D wounded skin equivalents

For the generation of wounded skin equivalents, full thickness skin equivalents were generated using young (18-35 years old) primary patient-matched keratinocytes and dermal fibroblasts as described above in Section 2.19. To support the wounded skin equivalents, an extra dermal layer for each full-thickness skin equivalent was also generated as described above in Section 2.19 and based on the method previously described in the PhD thesis of Dr Moyassar Al-Shaibani (Deciphering the Role of Mesenchymal Stem Cells (MSCs) in Cutaneous Wound Healing, 2018, Newcastle University). Full-thickness skin equivalents were harvested from inserts, washed in PBS before using a 1mm punch biopsy to generate a full-thickness wound. Wounded skin equivalents were placed on top of the extra dermal layer to

provide support for the wounded full-thickness skin equivalent prior to incubation at 37°C and supplementation with 5ml 1:1 EpiLife/DMEM containing 100µg/ml Vitamin C. Wounded skin equivalents were then treated in the presence or absence of 300µl of 1mg/ml Peptan P applied topically. Full-thickness wounded skin equivalents were harvested on weeks 1, 2, 3 and 4 post-wounding, washed in sterile PBS, fixed in 10% formalin and sent to Cellular Pathology (NHS Foundation Trust, Royal Victoria Infirmary, Newcastle upon Tyne, UK) for embedding in paraffin.

2.20 Haematoxylin & Eosin (H&E) staining of formalin-fixed paraffin-embedded (FFPE) human skin or skin equivalent sections

Four micrometre sections of formalin-fixed paraffin-embedded (FFPE) blocks of human skin or skin equivalents were cut using a microtome, placed on glass microscope slides (ThermoFisher Scientific, USA) and baked overnight at 60°C. Slides were immersed in Histoclear (Scientific Laboratory Supplies Limited, UK) for 15 minutes before washing with 100% ethanol followed by 90% ethanol for 10 seconds. Slides were then placed in Mayer's Haematoxylin (Sigma-Aldrich, USA) for 10 minutes at room temperature before rinsing in tap water for 2 minutes to remove excess solution. Slides were immersed in Eosin for 30 seconds (human skin section) or 3 minutes (skin equivalents) before again rinsing in tap water for 2 minutes to remove excess solution. Slides were then washed once in 90% ethanol, 100% ethanol and Histoclear before dabbing to remove any excess liquid. Coverslips were then applied to slides using DPX mounting medium (Thermofisher Scientific, USA) and allowed to dry at room temperature. Images of H&E stained sections of human skin or skin equivalents were acquired by scanning slides at 40x magnification using an Aperio AT2 Slide Scanner (Leica Biosystems, Germany).

2.21 Analysis of wound closure in 3D wounded skin equivalents

Aperio ImageScope software (Leica Biosytems, Germany) was used to measure both epidermal and dermal width and thickness of the full-thickness wounded 3D skin equivalents at 1, 2, 3 and 4 weeks post-wounding. Since the use of a punch biopsy generates a circular wound further mathematical equations were used in conjunction in order to calculate 3D wound healing as previously described in the PhD thesis of Dr Moyassar Al-Shaibani (Deciphering the Role of Mesenchymal Stem Cells (MSCs) in Cutaneous Wound Healing, 2018, Newcastle University). The

following formula (Equation 2.1) was applied to measure the area of the circular wound at week 0 for further comparisons of recovery of wound area over time.

Equation 2. 1 $A = \pi r^2$

Where:

A= Area of circular punch wound

π= 3.14

r= radius of the punch wound

The formula for the area of the circular punch wound (Equation 2.1) was further adapted to evaluate the area of epidermal recovery (re-epithelialisation) over time. The re-epithelialisation of the epidermis could be calculated by measuring the length of the newly formed epidermal tongue (LET) instead of the radius used in Equation 2.1. Therefore, the newly derived formula for re-epithelialisation is presented as Equation 2.2

Equation 2. 2 $Area\ of\ Re-epithelialisation = \pi(LET)^2$

Where:

π= 3.14

LET= length of epidermal tongue

To evaluate the area of dermal recovery over time, Equation 2.2 was adapted to measure the length of dermal recovery and was presented as Equation 2.3

Equation 2. 3 $Area\ of\ Dermal\ recovery = \pi(LDR)^2$

Where:

π= 3.14

LDR= length of dermal recovery

To account for irregularities in the shape of the newly formed dermis or epidermis e.g. triangular shape of newly formed dermis in weeks 1 and 2 post-wounding (Figure 2.2), Equation 2.4 was used to calculate area when the shape of newly regenerated tissue was irregular.

Equation 2. 4 $Area = \frac{1}{2} \times b \times h$

Where:

b=base

h=height

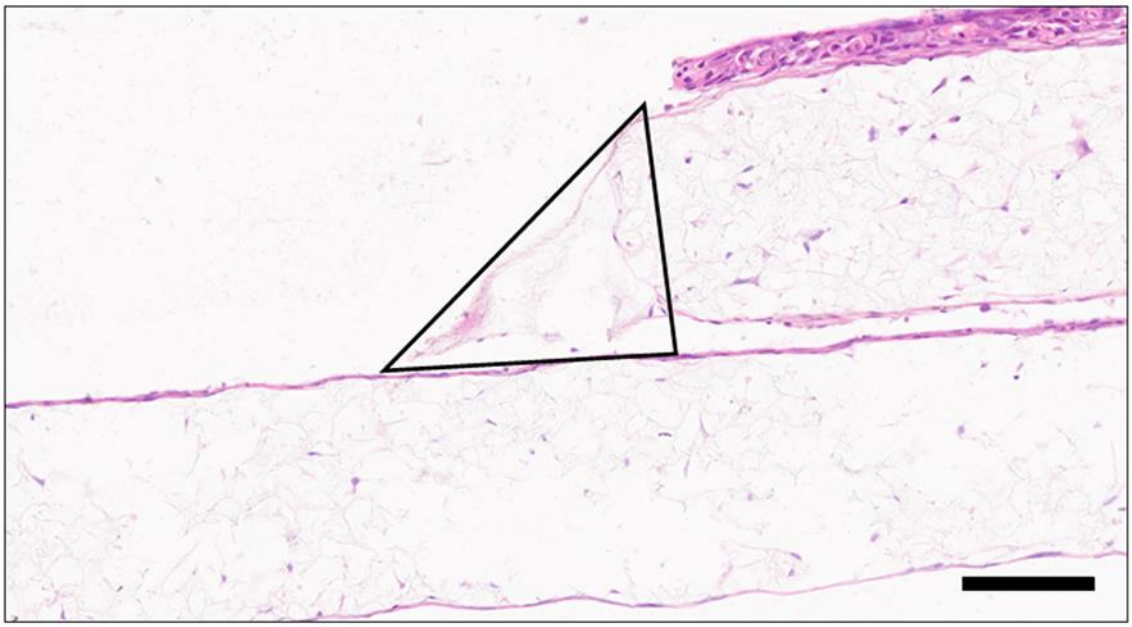

Figure 2. 2 Deposition of newly formed dermis has an irregular shape. *A representative H&E image of a wounded 3D skin equivalent showing that the initial deposition of newly formed dermis has a triangular shape. The black triangle represents the area of newly formed dermis that equation 2.6 was applied to. Magnification = 20x. Scale bar = 100µm.*

Given that the punch biopsy generates a cylindrical void, the general equation for cylindrical volume (Equation 2.5) was used to measure the void volume of the punch and allow for further analysis of reduction in the volume of the wound area over time.

Equation 2. 5 $Cylindrical\ volume = \pi r^2 h$

Where:

π= 3.14

r= radius of the punch wound

h= height of the punch area

To calculate the volume of newly formed epidermis or dermis that has a triangular shape, Equation 2.4 was further adapted. Since the newly dermis and epidermis will generate in the round area of the punch wound, the formula was adjusted to include the circumference (C) and Equation 2.6 was used to calculate the volume of newly formed dermis or epidermis that is triangular in shape.

Equation 2. 6 $VNT = \frac{1}{2} \times T \times LNT \times C$

Where:

VNT= volume of newly formed tissue

T=thickness of newly formed tissue

LNT=length of newly formed tissue

C= circumference

To calculate the volume of the fully healed epidermis observed at later timepoints post-wounding that has a semi-circular shape, Equation 2.7 was used to calculate the area of the newly healed epidermis, whilst Equation 2.8 was used to calculate the volume of the newly healed epidermis generated in the round area of the punch wound.

Equation 2. 7 *AHE* = $\frac{1}{2}(\pi r^2)$

Where:

AHE = area of fully healed epidermis

π = 3.14

r = radius of fully healed epidermis

Equation 2. 8 $VHE = \frac{2}{3}\pi r^3$

Where:

VHE = volume of fully healed epidermis

π = 3.14

r = radius of fully healed epidermis

2.22 Immunofluorescence of FFPE human skin or skin equivalents sections

Four micrometre sections derived from FFPE blocks of either normal human skin or skin equivalents were cut using a microtome, placed on glass microscope slides (ThermoFisher Scientific, USA) and baked overnight at 60°C. Slides were placed in Histoclear (Scientific Laboratory Supplies Limited, UK) for 15 minutes and dehydrated in 100% ethanol, 75% ethanol, 50% ethanol and distilled water, each for 10 seconds. Slides were then placed in 10mM Sodium Citrate (pH 6) buffer and heated twice for 6 minutes in a microwave on 80% power before being left to cool in buffer solution at room temperature for 15-20 minutes. Next, sections were rehydrated by incubating in PBS + 0.05% Tween 20 (PBS/T) for 3 minutes prior to permeablising sections with PBS/T + 0.2% Triton X100 for 10 minutes at room temperature. Slides were then washed in PBS/T before incubating with PBS containing 2% goat serum and 2% BSA for 20 minutes at room temperature. Primary antibodies were diluted in PBS containing 2% BSA and slides were incubated with primary antibodies overnight at 4°C (Table 2.8). Slides were then washed 3 times in PBS/T before incubating with secondary fluorescent antibodies Alexa Fluor® 488 Goat Anti-Rabbit IgG (1:300 in 2% BSA in PBS), Alexa Fluor® 488 Goat Anti-Mouse IgG (1:300 in 2% BSA in PBS) or Alexa Fluor® 568 Goat Anti-Rabbit IgG (1:300 in 2% BSA in PBS) for 1 hour in the dark at room temperature. Slides were then washed 3 times in PBS/T before incubation with DAPI (1:10,000 in PBS) for 15 minutes at room temperature in the dark. Finally, slides were washed a further 3 times in PBS/T before 1 wash in distilled water and 1 wash in 100% ethanol and mounting with a glass coverslip using VECTASHIELD mounting medium (Vector Laboratories, USA). Immunofluorescent images were captured using a Leica SP8 confocal microscope (Leica Biosystems, Germany).

Primary Antibody (Supplier)	Species	Primary Antibody Dilution
Collagen I (ab34710, Abcam)	Rabbit	1:100 in PBS + 2% BSA
Collagen III (ab7778, Abcam)	Rabbit	1:100 in PBS + 2% BSA
Keratin 1 (ab93652, Abcam)	Rabbit	1:100 in PBS + 2% BSA
Keratin 14 (ab7800, Abcam)	Mouse	1:100 in PBS + 2% BSA
Loricrin (905014, Biolegend)	Rabbit	1:100 in PBS + 2% BSA

Table 2. 8 *Primary antibody dilutions for immunofluorescent staining of FFPE sections*

2.23 Statistics

Data were analysed using statistical software Prism 9 (GraphPad, San Diego, USA) and presented as mean ± SD. A One-way ANOVA with Tukey's multiple comparisons test was used to analyse wound closure rates, relative cell viability, collagen I and III expression. A One-way ANOVA with Dunnett's post hoc correction was used to compare Ki67 expression in the presence or absence of collagen peptides. Pearson's correlation co-efficient was used to determine the correlation between basal collagen expression and rate of wound closure. Unpaired t-tests were used to determine relative CD49b, CD49c, CD49e, CD49f and CD29 expression. Paired t-tests were used to compare total Hyp concentrations at baseline and at different timepoints post-ingestion. A two-way ANOVA with Tukey's multiple comparisons test was used to compare the effect of collagen peptides on integrin α2 integrin β1, p-FAK/FAK, p-Akt/Akt and p-ERK/ERK expression in unwounded and wounded keratinocytes and fibroblasts treated in the presence or absence of *ITGB1* siRNA and also compare wound healing of 3D wounded skin equivalents treated in the presence or absence of 1mg/ml Peptan P.

Chapter 3: Clinically achievable concentrations of Peptan P promote cutaneous wound healing *in vitro*

Chapter 3: Clinically achievable concentrations of Peptan P promote cutaneous wound healing *in vitro*

Table of Contents

3.1 Introduction

Chronic non-healing wounds are an increasing clinical and financial burden to healthcare systems worldwide, costing the USA $20 billion per annum and consuming on average 5.5% of all NHS expenditure in the UK (Sen *et al.*, 2009; Järbrink *et al.*, 2017). Prevalence of chronic wound development is further linked to an ageing population and systemic diseases such as diabetes (Gould *et al.*, 2015; Järbrink *et al.*, 2017). Hence, there is an acute unmet need for the development of novel therapeutic strategies for the acute and long-term management of cutaneous wounds.

Ageing negatively impacts normal wound healing responses, with age-related changes affecting structure and function of the ECM as well as changes to growth factor secretion. Skin ageing causes a decline in the synthesis of key ECM proteins such as collagen I and increased MMP secretion, leading to increased collagen degradation and disorganisation of collagen fibres which results in reduced skin tensile strength (Shuster *et al.*, 1975; Ashcroft *et al.*, 1997b; Gosain and DiPietro, 2004). Additionally, skin ageing results in delayed cellular responses to tissue injury demonstrated by delayed re-epithelialisation of wounds in aged mice (Swift *et al.*, 1999). Consequently, whilst younger skin is able to mount an effective and timely wound healing response, aged skin exhibits prolonged inflammatory and remodelling phases that delay tissue repair, reduce skin tensile strength and increase the incidence of medical complications associated with chronic wound development in the elderly (Quirinia and Viidik, 1991). Along with increasing age, systemic diseases such as diabetes have increased prevalence linked to chronic wound development. In diabetic patients, the presence of a hyperglycaemic environment generated through high blood glucose levels has been shown to negatively impact normal cutaneous wound healing responses by inhibiting dermal fibroblast and keratinocyte migration and proliferation, further contributed to by increased ROS production and cell death via apoptosis (Terashi *et al.*, 2005; Lamers *et al.*, 2011; Buranasin *et al.*, 2018; Li *et al.*, 2019). The hyperglycaemic environment increases secretion of MMPs and inflammatory cytokines such as interleukin-1 (IL-1) and tissue necrosis factor-α (TNF-α) by macrophages and dermal fibroblasts which results in an extended inflammatory responses and increased ECM degradation (Liu *et al.*, 2009b; Uccioli *et al.*, 2015). Therefore, more

novel, reliable and cost-effective strategies are required to help improve healing outcomes of chronic wounds in both the elderly and diabetic patients.

The structural and mechanical properties of collagen within the ECM are key to tissue integrity and wound healing. Therefore, many therapeutic strategies have explored the incorporation of collagen to create various biomaterials such as collagen gels and dressings. While many of these collagen-based treatments have shown some efficacy in increasing wound moisture, reducing pain and decreasing risk of infection, few have established significant effects on stimulating consistent and effective wound repair in different types of wounds (Brett, 2008; Fleck and Simman, 2010; Dhivya *et al.*, 2015). Consequently, recent focus has been directed to the potential use of collagen peptides for treating cutaneous wounds. Collagen peptides are produced through the enzymatic hydrolysis of collagen derived from animal skin to produce a mixture of low molecular weight, water soluble peptide of differing lengths (Asserin *et al.*, 2015). Previous bioavailability studies have indicated that hydroxyproline-containing collagen peptides can be detected in the bloodstream at approximately 20μM concentrations 1 hour after oral administration of 10g of collagen peptides, with distribution to the skin retained for up to 14 days (Ohara *et al.*, 2007; Watanabe-Kamiyama *et al.*, 2010; Shigemura *et al.*, 2014). Currently, collagen peptides are mainly used to help reduce signs of skin ageing, with recent human clinical trials demonstrating that daily ingestion of collagen peptides helps to improve skin elasticity and hydration (Asserin *et al.*, 2015). Additionally, *in vivo* murine models have demonstrated that the administration of collagen peptides protect against extrinsic skin ageing by maintaining deposition of collagen and elastin but also by inhibiting the degradation of collagen in chronologically aged skin (Zhuang *et al.*, 2009; Liang *et al.*, 2010).

Currently, there are limited studies that support the use of collagen peptides for the treatment of cutaneous wounds; however, recent *in vitro* studies have illustrated that collagen peptides possess chemotactic properties that are not typically observed in native collagen, stimulating both keratinocyte and fibroblast migration and proliferation (Postlethwaite *et al.*, 1978; Shigemura *et al.*, 2009; Ohara *et al.*, 2010; Edgar *et al.*, 2018). Further supporting the use of collagen peptides for the treatment of cutaneous wounds, *in vivo* studies using rat models demonstrated that oral administration of marine collagen peptides significantly enhanced collagen

deposition, increased angiogenesis and increased wound closure rates (Zhang *et al.*, 2011; Wang *et al.*, 2015a). In the context of diabetes, few studies have investigated the effect of collagen peptides on alleviating a hyperglycaemic environment and promoting wound healing in diabetic patients. Interestingly, previous studies have demonstrated that treatment of human umbilical vein endothelial cells (HUVECs) cultured in a hyperglycaemic environment with marine collagen peptides resulted in reduced expression of apoptosis marker caspase-3 and also increased cell proliferation (Zhu *et al.*, 2017). Furthermore, streptozotocin-induced diabetic mice treated with marine collagen peptides in combination with whey proteins improved wound healing and also increased expression of wound healing associated growth factors such as EGF, FGF and TGF-β (Xiong *et al.*, 2020).

While collagen peptides have been shown to promote cutaneous wound healing in animal models, there is limited data surrounding their potential to promote cutaneous wound healing in humans but also in the context of chromic wounds that have increased prevalence linked to ageing or systemic diseases such as diabetes. Therefore the specific aims of this chapter were to determine the potential for bovine (Peptan B), fish (Peptan F) or porcine (Peptan P)-derived collagen peptides to promote wound closure *in vitro* in context of age using both young and aged dermal fibroblasts and keratinocytes, determine clinically achievable concentrations of collagen peptides achievable *in vivo* following ingestion and determine the potential for collagen peptides to promote cutaneous wound healing in a hyperglycaemic environment *in vitro*.

3.2 Results

3.2.1 Ageing reduces basal collagen I expression but not basal collagen III expression by dermal fibroblasts

Collagen I and III play important roles within the proliferation and remodelling phases of cutaneous wound healing by providing a scaffold for cells to migrate along whilst also increasing skin tensile strength (Gelse *et al.*, 2003). To determine the impact of ageing on collagen expression, Western blotting was performed to evaluate the basal expression of collagen I and collagen III in dermal fibroblasts derived from young (18-35 years old), middle-aged (40-55 years old) or aged (60+ years old) individuals. Results revealed a significant decrease in the basal expression of collagen I in aged dermal fibroblasts compared to young dermal fibroblasts (Figure 3.1A and B, One-way ANOVA with Tukey's multiple comparisons test, *P<0.05). However, the basal expression of collagen III in dermal fibroblasts did not significantly differ between young, middle-aged or aged individuals (Figure 3.1C and D, One-way ANOVA with Tukey's multiple comparisons test, ns). Collectively these data demonstrate an age related decline in collagen I expression by dermal fibroblasts.

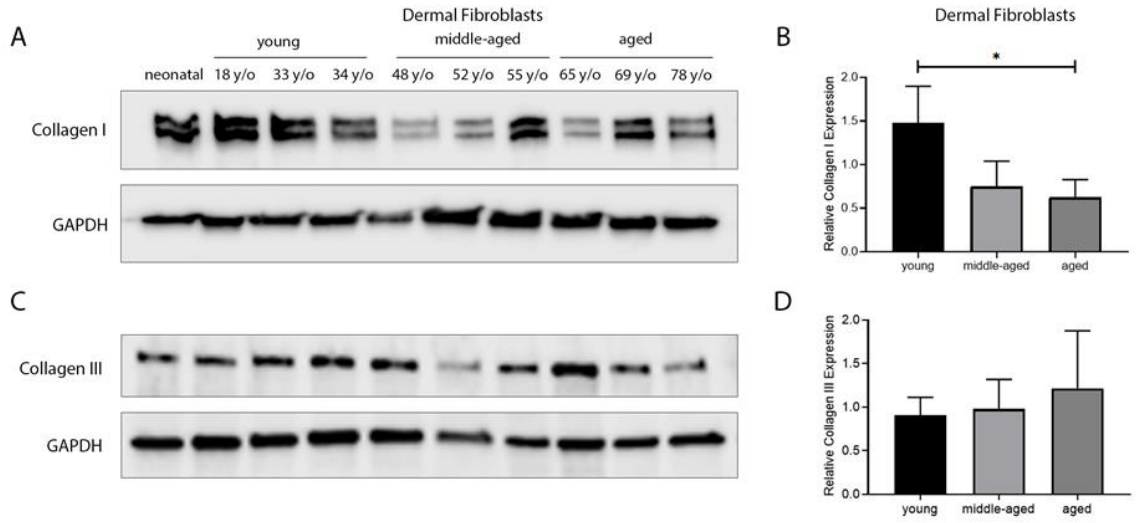

Figure 3. 1 Basal expression of collagen I but not collagen III declines with age. *Representative Western blot for (A) collagen I (139kDa), (C) collagen III (139kDa) and GAPDH (37kDa) expression in foreskin-isolated dermal fibroblasts derived from young (18-35 years old), middle-aged (40-55 years old) or aged (60+ years old) individuals. Densitometric expression of (B) collagen I or (D) collagen III relative to GAPDH expression in young, middle-aged or aged dermal fibroblasts (mean ± SD, N=3, *P<0.05) (Mistry et al., 2021).*

3.2.2 Basal collagen I or collagen III expression does not affect dermal fibroblast wound closure *in vitro*

Collagen is crucial for effective cutaneous wound healing as it acts as a scaffold for cells to migrate along as well as stimulating cellular proliferation (Gelse *et al.*, 2003). To determine whether the basal level of collagen expressed by dermal fibroblasts is a factor that can impair cutaneous wound closure within the elderly, 2D scratch assays using dermal fibroblasts derived from 18-78 year old individuals were used to determine any correlation between basal collagen I or collagen III expression and wound closure rate. Results revealed no significant correlation between wound closure rates of dermal fibroblasts and basal collagen I or collagen III expression (Figure 3.2A and B, Pearson's correlation, R^2= 0.0001 and 0.008), suggesting that basal collagen expression does not affect dermal fibroblast wound closure *in vitro* and that other age-related changes are associated with impaired wound closure in the elderly.

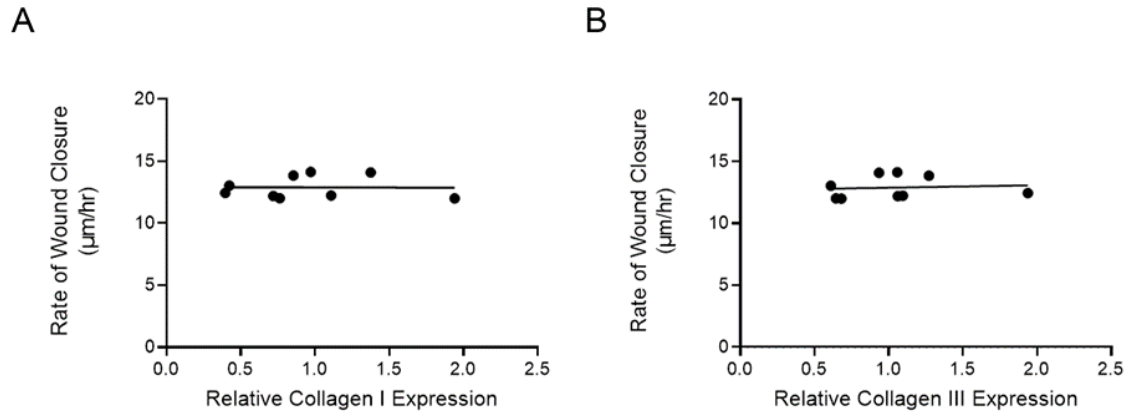

Figure 3. 2 Basal expression of collagen I or collagen III does not affect the basal rate of wound closure of dermal fibroblasts. *Correlation of relative (A) collagen I or (B) collagen III expression and rate of dermal fibroblast wound closure derived from 18-78 year old individuals. (A) R^2= 0.0001, (B) R^2= 0.008. (Mistry et al., 2021)*

3.2.3 Basal transglutaminase 2 expression by primary dermal fibroblasts or keratinocytes is not altered by age or wound induction

Transglutaminase 2 (TG2) is an enzyme involved in ECM remodelling with increased expression reportedly linked to wounding (Haroon *et al.*, 1999). Western blotting was performed using both dermal fibroblasts and keratinocytes derived from young (18-35 years old), middle-aged (40-55 years old) or aged (60+ years old)

individuals to determine any changes in basal TG2 expression with age or following scratch induction in 2D culture. Results demonstrated no significant difference in basal expression of TG2 in differing aged dermal fibroblasts or keratinocytes (Figure 3.3A, B, E and F, One-way ANOVA with Tukey's multiple comparisons test, ns). Results also revealed no significant difference in TG2 expression in unwounded and wounded dermal fibroblasts or keratinocytes (Figure 3.3C, D, G and H, Unpaired T-test, ns). Collectively, these data suggest that age does not affect basal expression of TG2 in dermal fibroblasts or keratinocytes nor expression following wounding in 2D.

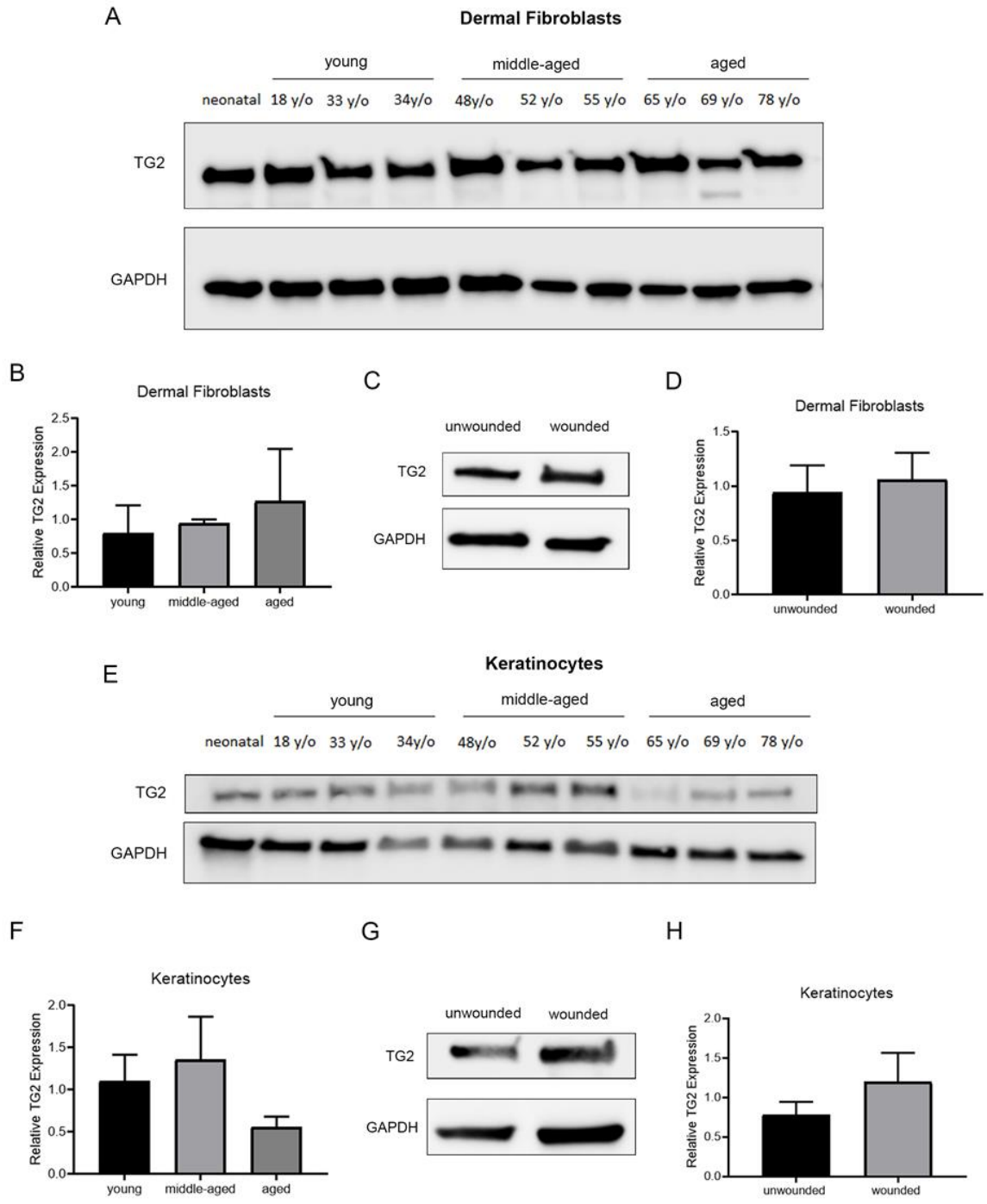

Figure 3. 3 Transglutaminase 2 expression by dermal fibroblasts or keratinocytes is not altered by age or following scratch wound induction. *Representative Western blot for transglutaminase 2 (TG2) (77kDa) and GAPDH (37kDa) expression in foreskin-isolated (A) dermal fibroblasts or (E) keratinocytes derived from young, middle-aged or aged individuals. Densitometric expression of TG2 relative to GAPDH expression in young, middle-aged or aged (B) dermal fibroblasts or (F) keratinocytes (mean ± SD, N=3). Representative Western blot for TG2 (77kDa) and GAPDH (37kDa) expression in unwounded and wounded (C) dermal fibroblasts or (G) keratinocytes. Densitometric expression of TG2 relative to GAPDH expression in unwounded and wounded (D) dermal fibroblasts or (H) keratinocytes (mean ± SD, N=3).*

3.2.4 Peptan B and Peptan P collagen peptides significantly enhance wound closure of both young and aged dermal fibroblasts

Ageing has been associated with delayed cellular responses to injury resulting in reduced fibroblast migration and proliferation, thereby impairing normal wound healing responses in the elderly (Swift *et al.*, 1999; Reed *et al.*, 2001). To determine the potential for collagen peptides to promote wound closure of dermal fibroblasts in the context of age, dermal fibroblasts derived from young (18-35 years old) or aged (60+ years old) individuals were seeded in the presence or absence of 1mg/ml bovine (Peptan B), fish (Peptan F) or porcine-derived (Peptan P) collagen peptides prior to wound induction and monitoring over 72 hours. Results demonstrated that collagen peptides were able to promote dermal fibroblast wound closure with both Peptan B and Peptan P significantly enhancing wound closure of both young and aged dermal fibroblasts (Figure 3.4 and Figure 3.5A, B, E and F, One-way ANOVA with Tukey's multiple comparisons test, *P<0.05, **P<0.01, ***P<0.001. However, Peptan F was unable to significantly enhance wound closure of either young or aged dermal fibroblasts (Figure 3.5E and F, One-way ANOVA with Tukey's multiple comparisons test, ns). Collectively, these data demonstrate the potential for Peptan B and Peptan P collagen peptides to promote dermal fibroblast wound closure in both young and aged individuals.

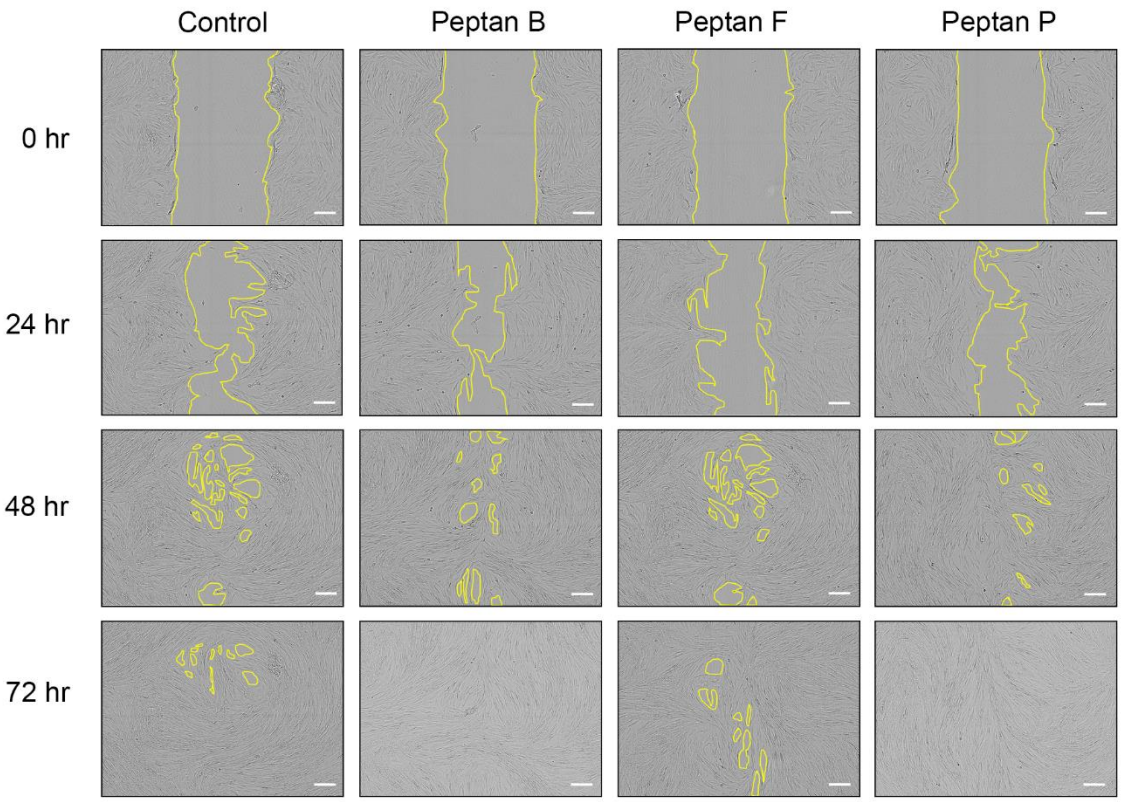

Figure 3. 4 Peptan B and Peptan P collagen peptides promote wound healing of primary dermal fibroblasts. *Representative photomicrographs of wound closure of dermal fibroblasts derived from young individuals seeded onto uncoated control or 1mg/ml of bovine, fish or porcine collagen peptide coated wells at 0, 24, 48 and 72 hours post-scratch wound induction. Magnification = 10x. Scale bar = 100µm. (Mistry et al., 2021)*

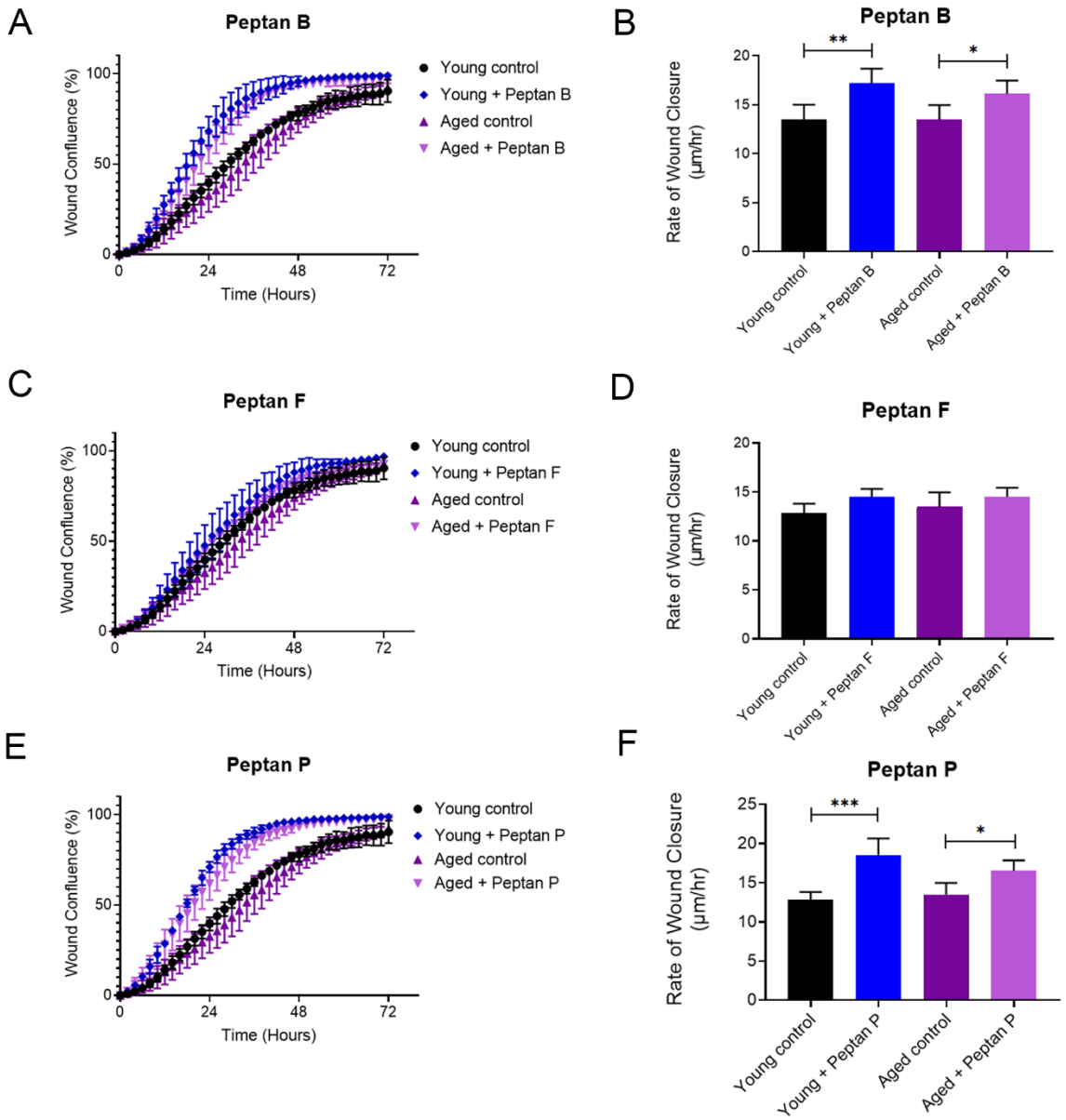

Figure 3. 5 Peptan B and Peptan P collagen peptides significantly enhance wound closure of both young and aged dermal fibroblasts. Wound confluence (%) of young (18-35 years old) or aged (60+ years old) fibroblasts seeded onto 1mg/ml (A) bovine, (C) fish or (E) porcine coated wells or uncoated control wells measured every 2 hours over 72 hours (mean ± SD, n=3). Rate of wound closure (µm/hr) of young and aged fibroblasts seeded onto 1mg/ml (B) bovine, (D) fish or (F) porcine collagen peptide coated wells or uncoated control wells (mean ± SD, n=9, N=3, *P<0.05, **P<0.01, ***P<0.001). (Mistry et al., 2021)

3.2.5 Peptan B and Peptan P collagen peptides promote dermal fibroblast proliferation

To determine whether collagen peptides mediate dermal fibroblast wound closure primarily through enhanced migration or proliferation, dermal fibroblasts (<45 years old) were treated in the presence or absence of 7.5µg/ml mitomycin C for 2 hours to inhibit cell proliferation prior to scratch wound induction and monitoring of wound closure over 72 hours. Results demonstrated Peptan B and Peptan P induced wound closure was significantly inhibited in the presence of mitomycin C (Figure 3.6A, C and D, One-way ANOVA with Tukey's multiple comparisons test, **P<0.01), while again, there was no significant potentiation of wound closure by Peptan F collagen peptides or indeed any observed inhibition by mitomycin C (Figure 3.6B and D, One-way ANOVA with Tukey's multiple comparisons test, ns). Collectively, these data suggest that Peptan B and Peptan P collagen peptides may promote dermal fibroblast wound closure through enhanced cellular proliferation.

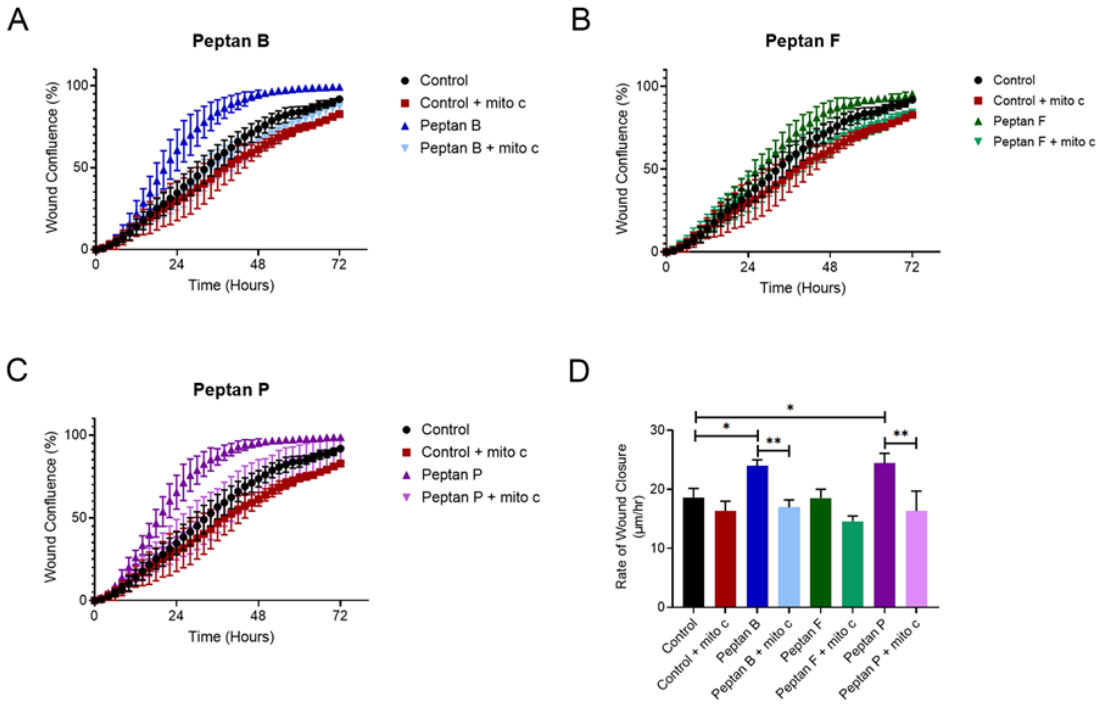

Figure 3. 6 Mitomycin C significantly inhibits peptide-induced wound closure of dermal fibroblasts. *Wound confluence (%) of dermal fibroblasts (<45 years old) seeded onto 1mg/ml (A) bovine, (B) fish or (C) porcine collagen peptide coated wells or uncoated control wells in the presence or absence of 7.5µg/ml mitomycin C (mito c) measured every 2 hours for 72 hours (mean ± SD, N=3). (D) Rate of wound closure of dermal fibroblasts seeded onto 1mg/ml bovine, fish or porcine collagen peptide coated wells or uncoated control wells in the presence or absence of 7.5µg/ml mitomycin C (mito c) (mean ± SD, n=9, N=3, *P<0.05, **P<0.01). (Mistry et al., 2021)*

To confirm the potential for collagen peptides to promote dermal fibroblast proliferation, MTS cell viability assays and the immunofluorescent expression of Ki67 were assessed using dermal fibroblasts derived from individuals less than 45 years old. Results demonstrated that both Peptan B and Peptan P collagen peptides significantly enhanced cell viability and increased Ki67 expression (Figure 3.7A-C, One-way ANOVA with Tukey's multiple comparisons test, One-way ANOVA with Dunnett's post-hoc test, *P<0.05, **P<0.01). Supporting their effect on cellular proliferation results also confirmed a significant inhibition of Peptan B and Peptan P induced fibroblast cell viability by mitomycin C (Figure 3.7A, One-way ANOVA with Tukey's multiple comparisons test, ***P<0.001). Conversely, while mitomycin C significantly inhibited dermal fibroblast cell viability cultured on Peptan F collagen peptides (Figure 3.7A, One-way ANOVA with Tukey's multiple comparisons test, ***P<0.001), there was no significant effect of Peptan F collagen peptides on increasing cell viability compared to control (Figure 3.7A, One-way ANOVA with Tukey's multiple comparisons test, ns). Taken together, these data suggest the beneficial effects of Peptan B and Peptan P collagen peptides on cutaneous wound healing are predominantly mediated by their ability to promote dermal fibroblast proliferation.

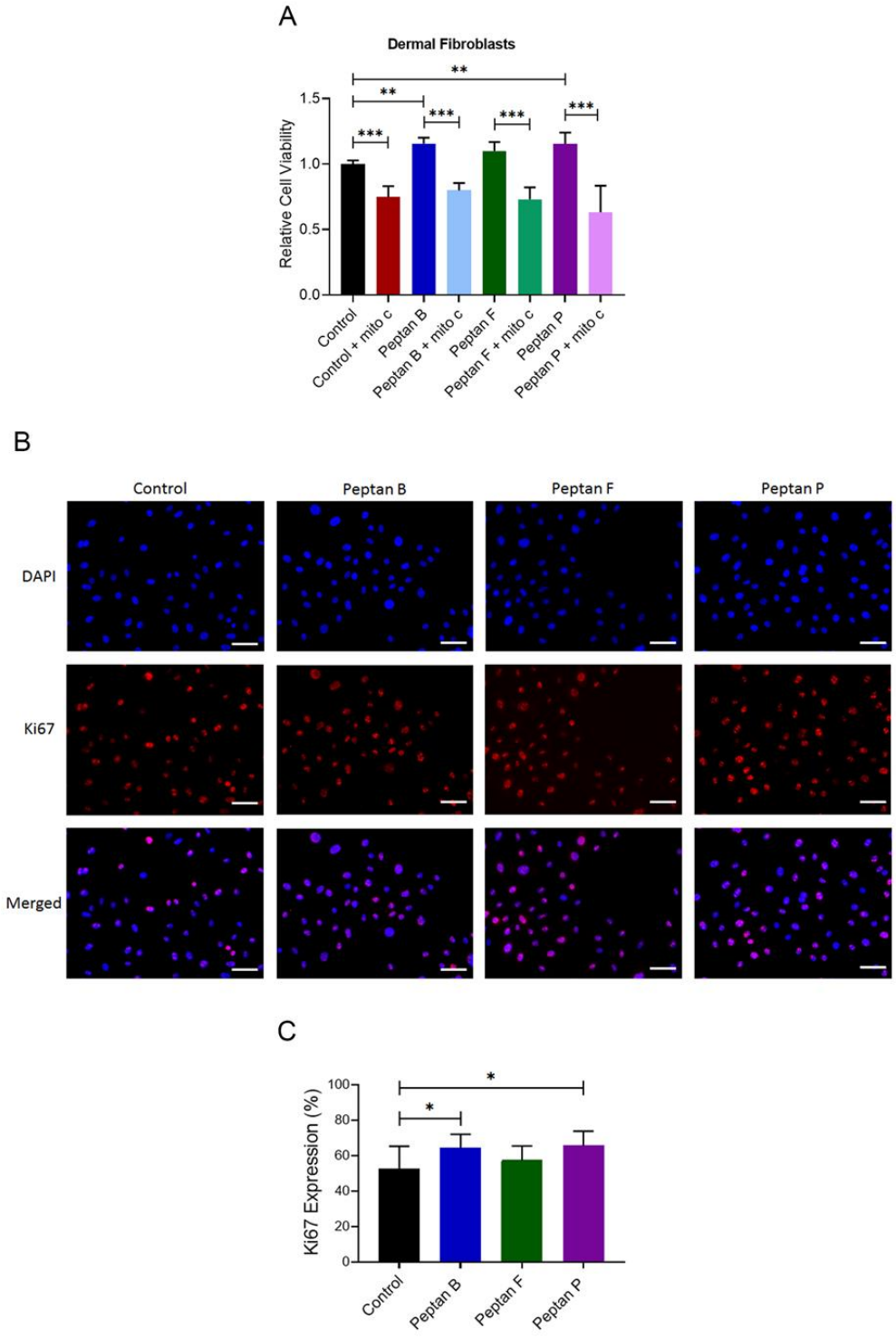

Figure 3. 7 Peptan B and Peptan P collagen peptides promote proliferation of dermal fibroblasts. *(A) Relative cell viability of primary dermal fibroblasts (<45 years old) seeded onto uncoated control wells or 1mg/ml bovine, fish or porcine collagen peptide coated wells in the presence or absence of 7.5µg/ml mitomycin C (mito c) for 72 hours (mean ± SD, N=3, *P<0.05, **P<0.01, ****P<0.0001). (B) Representative photomicrographs of Ki67 (red) expression by primary fibroblasts grown in the presence or absence of 1mg/ml bovine, fish or porcine collagen peptides for 72 hours. Nuclei are stained with DAPI (blue). Magnification = 20x. Scale bar = 50µm. (D) Mean percentage of Ki67 expressing fibroblasts grown in the presence or absence of 1mg/ml bovine, fish or porcine collagen peptides relative to DAPI expression (mean ± SD, N=3, *P<0.05). (Mistry et al., 2021)*

3.2.6 Peptan P collagen peptides significantly promote wound closure of young but not aged keratinocytes

Since ageing has also been associated with reduced keratinocyte migration and proliferation, thereby delaying re-epithelialisation following injury, the potential for collagen peptides to promote keratinocyte wound closure in the context of age was also explored (Swift *et al.*, 1999). Keratinocytes derived from young (18-35 years old) or aged (60+ years old) individuals were seeded in the presence or absence of 1mg/ml Peptan B, Peptan F or Peptan P collagen peptides prior to scratch wound induction and monitoring of wound closure over 72 hours. However, unlike their promotion of dermal fibroblast wound closure, results revealed that Peptan B collagen peptides were unable to promote wound closure of young or aged keratinocytes (Figure 3.8 and Figure 3.9A and B, One-way ANOVA with Tukey's multiple comparisons test, ns). Furthermore. Peptan P collagen peptides were only able to significantly enhance wound closure of young keratinocytes and not aged keratinocytes (Figure 3.8 and Figure 3.9E and F, One-way ANOVA with Tukey's multiple comparisons test, *P<0.05), while no effect was observed with Peptan F (Figure 3.8 and Figure 3.9C and D, One-way ANOVA with Tukey's multiple comparisons test, ns). Collectively, these data suggest that collagen peptides have limited effect on keratinocyte wound closure *in vitro* with Peptan P only enhancing wound closure of keratinocytes derived from young individuals.

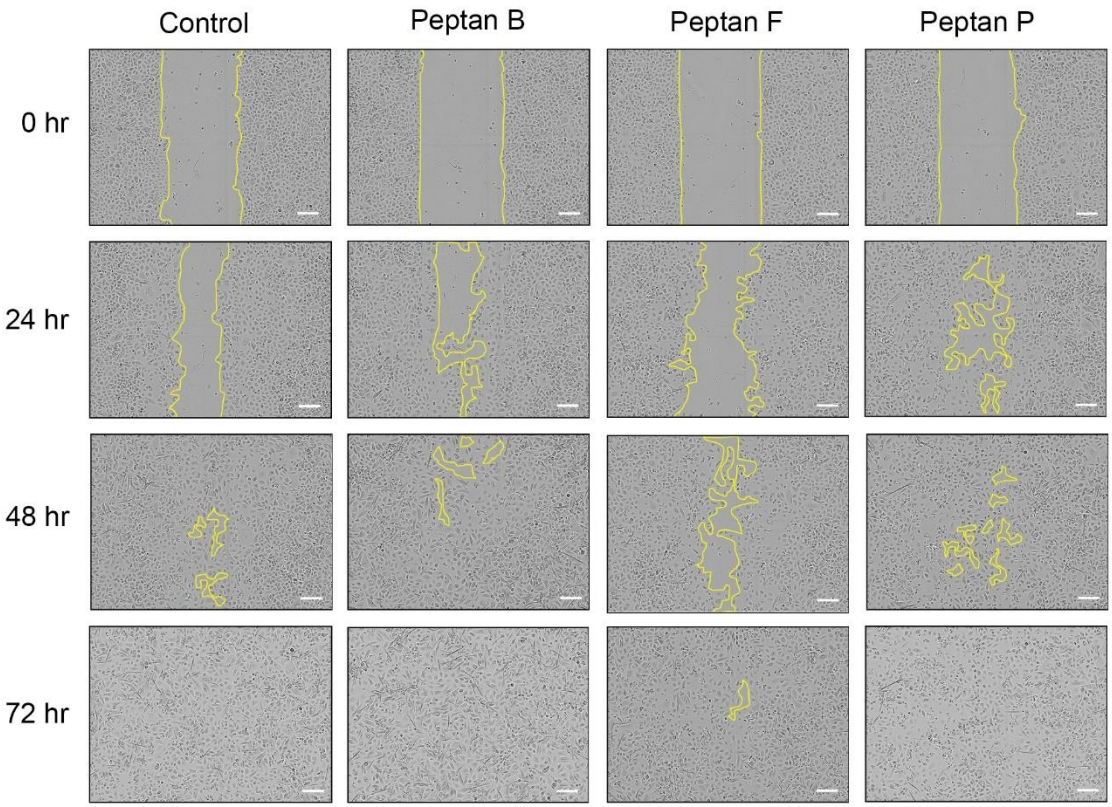

Figure 3. 8 Peptan P collagen peptides promote wound closure of young keratinocytes. *Representative photomicrographs of wound closure of keratinocytes derived from young individuals seeded onto uncoated control or 1mg/ml of bovine, fish or porcine collagen peptide coated wells at 0, 24, 48 and 72 hours post-scratch. Magnification = 10x.Scale bar = 100µm. (Mistry et al., 2021)*

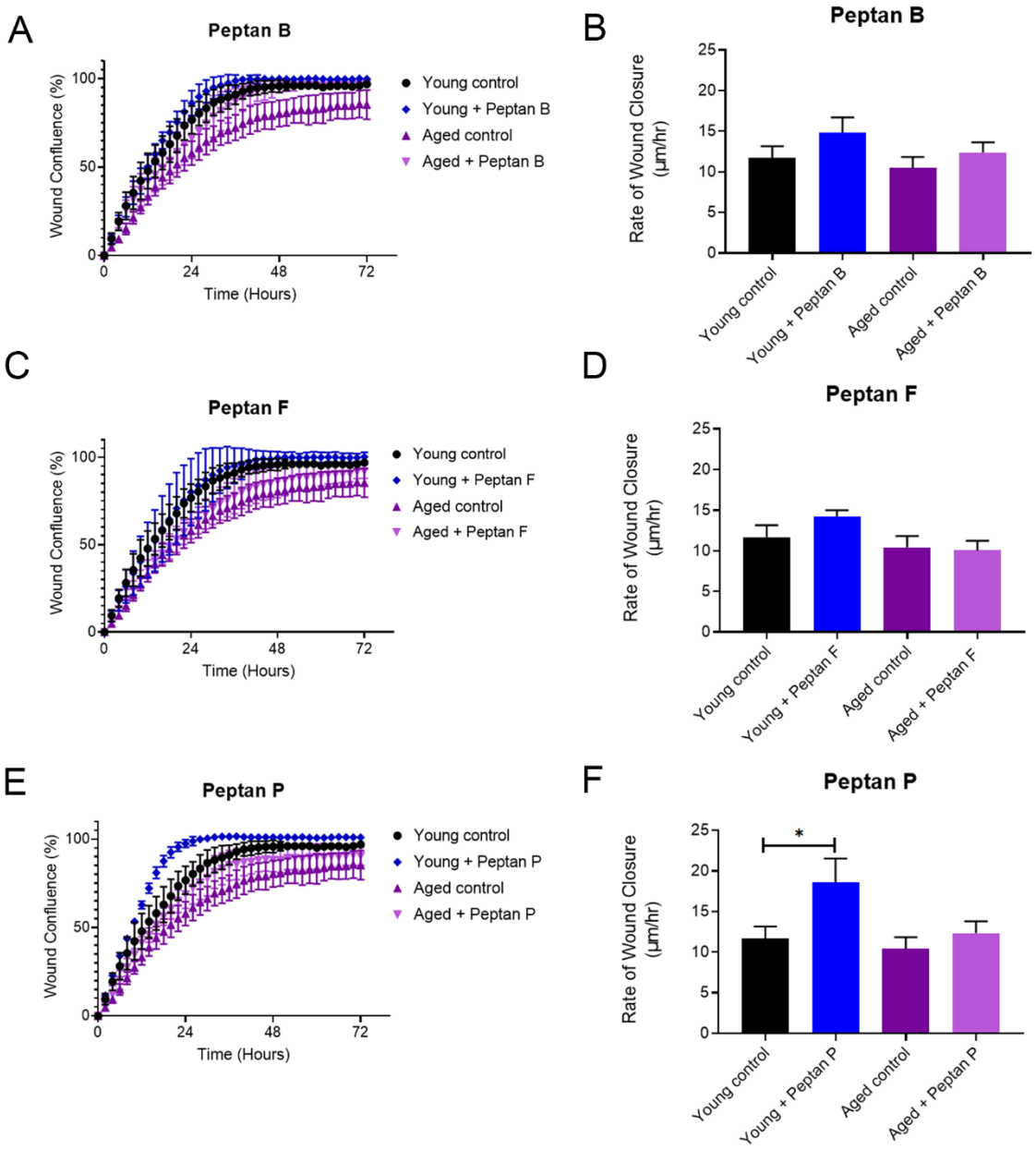

Figure 3. 9 Peptan P collagen peptides significantly enhanced wound closure of young but not aged keratinocytes. Wound confluence (%) of young (18-35 years old) or aged (60+ years old) keratinocytes seeded onto 1mg/ml (A) bovine, (C) fish or (E) porcine collagen peptide coated wells or uncoated control wells measured every 2 hours over 72 hours (mean ± SD, N=3). Rate of wound closure (µm/hr) of young and aged keratinocytes seeded onto 1mg/ml (B) bovine, (D) fish or (F) porcine collagen peptide coated wells or uncoated control wells (mean ± SD, n=9, N=3, *P<0.05). (Mistry et al., 2021)

3.2.7 Collagen peptides do not promote proliferation of keratinocytes

To further probe whether the effect of Peptan P collagen peptides on enhanced wound closure of young keratinocytes resulted from their induction of cellular proliferation, as well as to question if other species-specific collagen peptides may exert a proliferative effect on keratinocytes, wound closure experiments with keratinocytes derived from individuals less than 45 years old were repeated in the presence or absence of 2 hour treatment with 7.5µg/ml mitomycin C to inhibit cellular proliferation prior to scratch wound induction and monitoring of wound closure over 72 hours. Results revealed that while Peptan P was able to significantly enhance keratinocyte wound closure, treatment with mitomycin C significantly decreased Peptan P-induced wound closure (Figure 3.10C and D, One-way ANOVA with Tukey's multiple comparisons test, *P<0.05). Interestingly, although mitomycin C significantly inhibited Peptan B-induced wound closure, Peptan B collagen peptides were unable to significantly enhance keratinocyte wound closure compared to control (Figure 3.10A and D, One-way ANOVA with Tukey's multiple comparisons test **P<0.01). Furthermore, there was no effect of Peptan F on keratinocyte wound closure in the presence or absence of mitomycin C (Figure 3.10B and D, One-way ANOVA with Tukey's multiple comparisons test, ns). Taken together, these data suggest that Peptan P promotes keratinocyte wound closure by enhancing cellular proliferation.

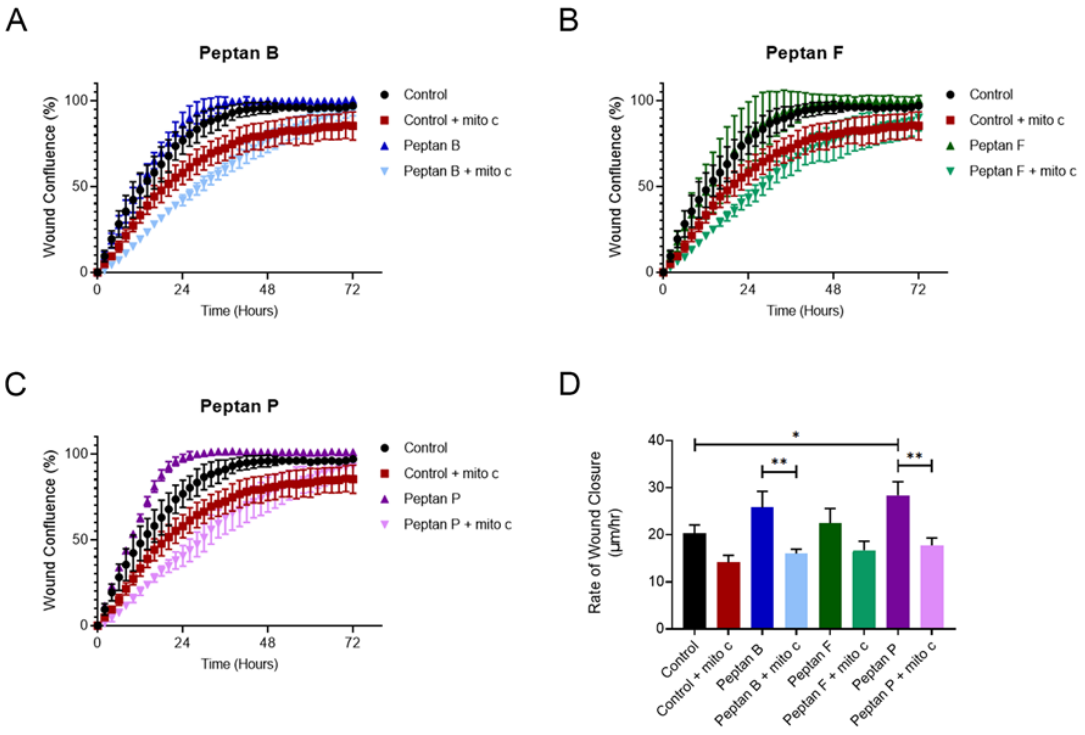

Figure 3. 10 Mitomycin C inhibits peptide-induced wound closure of keratinocytes. *Wound confluence (%) of keratinocytes (<45 years old) seeded onto uncoated control wells or wells pre-coated with 1mg/ml (A) Peptan B, (B) Peptan F or (C) Peptan P in the presence or absence of 7.5µg/ml mitomycin c (mito c) measured every 2 hours for 72 hours. (D) Rate of wound closure of keratinocytes seeded onto uncoated wells or wells pre-coated with 1mg/ml Peptan B, Peptan F or Peptan P in the presence or absence of 7.5µg/ml mitomycin c (mito c) (mean ± SD, n=9, N=3, *P<0.05, **P<0.01). (Mistry et al., 2021)*

To confirm any effect of collagen peptides on keratinocyte proliferation, MTS cell viability assays and immunofluorescent expression of Ki67 using keratinocytes derived from individuals less than 45 years old were performed in the presence or absence of mitomycin C. Consistent with 2D wound healing assay results, there was no enhanced effect on cell viability in the presence of Peptan B, Peptan F or Peptan P collagen peptides compared to control, although in all cases, mitomycin C significantly inhibited the effects of collagen peptides on cell viability (Figure 3.11A, One-way ANOVA with Tukey's multiple comparisons test, *P<0.05, **P<0.01, ****P<0.0001). Additionally, results from immunofluorescent analysis of Ki67 expression revealed that none of the collagen peptides significantly increased expression of Ki67 in keratinocytes (Figure 3.11B and C, One-way ANOVA with Dunnett's post hoc correction, ns). Collectively, these data suggest that Peptan P-induced wound closure likely arises predominantly from their ability to enhance cellular migration.

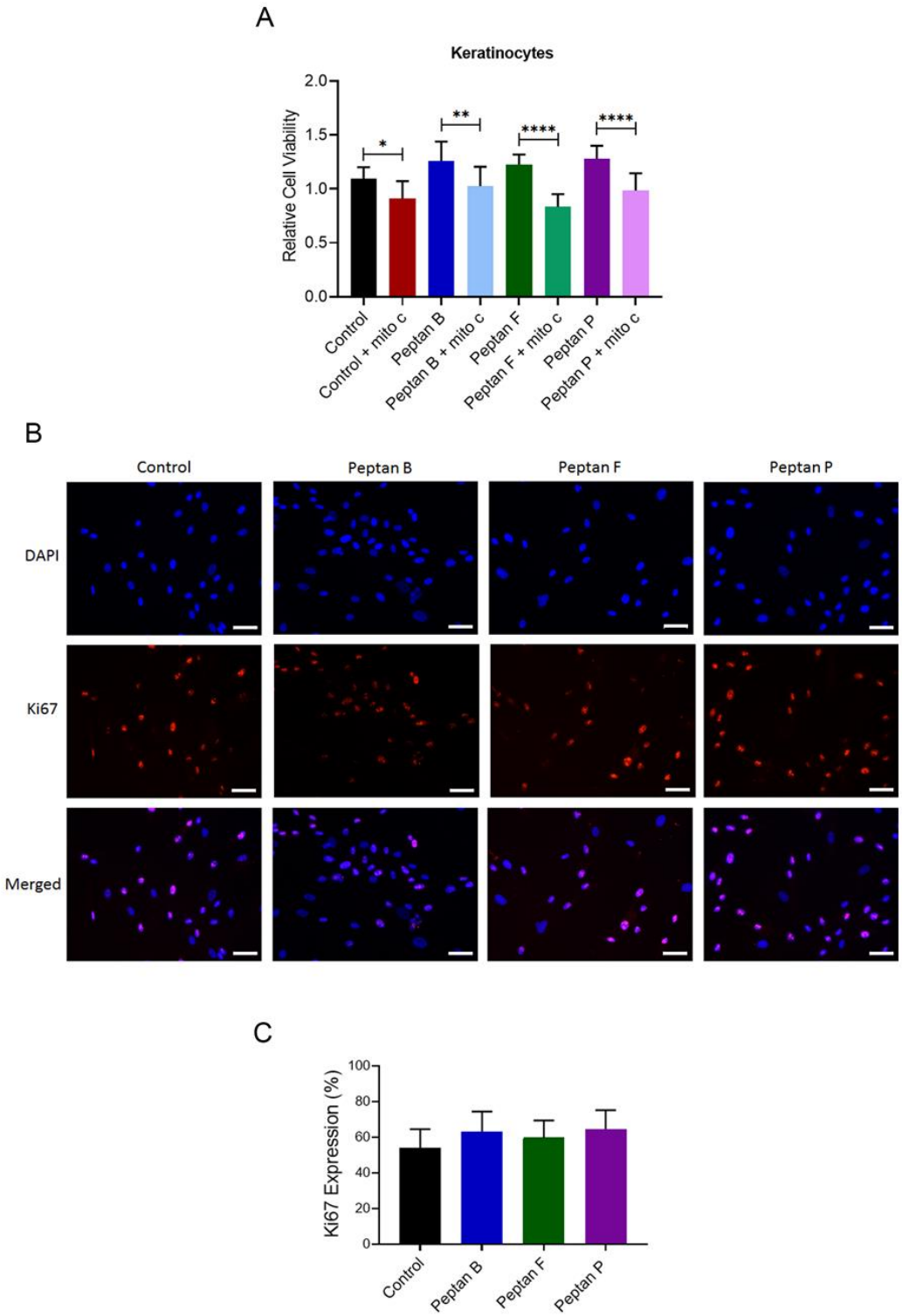

Figure 3. 11 Collagen peptides do not significantly promote proliferation of keratinocytes. *(A) Relative cell viability of keratinocytes (<45 years old) seeded onto uncoated control wells or 1mg/ml Peptan B, Peptan F or Peptan P coated wells in the presence or absence of treatment with 7.5µg/ml mitomycin C (mito c) for 72 hours (mean ± SD, N=3, *P<0.05, **P<0.01, ****P<0.0001). (B) Representative photomicrographs of Ki67 (red) expression by primary keratinocytes grown in the presence or absence of 1mg/ml Peptan B, Peptan F or Peptan P collagen peptides for 72 hours. Nuclei are stained with DAPI (blue). Magnification = 20x. Scale bar = 50µm. (D) Mean percentage of Ki67 expressing keratinocytes grown in the presence or absence of 1mg/ml Peptan B, Peptan F or Peptan P relative to DAPI expression (mean ± SD, N=3). (Mistry et al., 2021)*

3.2.8 Age does not affect porcine-derived collagen peptide absorption

Collagen peptides are typically administered orally. Given the potential for Peptan P collagen peptides to exert a biological effect on cutaneous wound healing *in vitro*, as evidenced in particular by peptide-induced proliferation and/or migration of dermal fibroblasts and keratinocytes, pharmacokinetic studies were carried out to determine the bioavailability of Peptan P collagen peptides following ingestion *in vivo* in both young and aged individuals as previously described (Kleinnijenhuis *et al.*, 2020). Total hydroxyproline (Hyp) concentration, a biomarker for collagen peptide absorption in the blood, was assessed in serum and plasma samples taken from healthy volunteers before (0 hour (baseline)) and 2, 8 and 24 hours after ingestion of 10g of Peptan P collagen peptides (Table 3.1) (Iwai *et al.*, 2005; Ohara *et al.*, 2007). Results demonstrated a significant increase in total Hyp concentration in both serum and plasma derived from all individuals following collagen peptide ingestion between baseline (0 hours) and 2 hours (Table 3.1, paired t-test **P<0.01), with maximum concentrations reached at 2 hours post-ingestion (serum = 20 ± 4 µg/ml, plasma = 23 ± 3 µg/ml) which steadily decreased to baseline concentrations after 24 hours (Figure 3.12, Table 3.1). Furthermore, results revealed no significant difference in total Hyp concentrations in serum and plasma samples between young and aged individuals (Figure 3.12A and B). Collectively, these data demonstrate clinical concentrations of collagen peptides are readily achievable in the blood of both young and aged individuals 2 hours post-ingestion of 10g of Peptan P collagen peptides and to equivocal concentrations that exert a positive effect on wound healing *in vitro*.

Volunteer ID	Age	Time (hr) Post Porcine Collagen Peptide Ingestion	Total Hyp Concentration (µg/ml) in serum	Total Hyp Concentration (µg/ml) in plasma
1	<35	Baseline	11.20	12.80
		2	19.00	19.20
		8	22.40	23.80
		24	12.20	15.30
2	<35	Baseline	8.13	13.80
		2	15.10	24.20
		8	16.80	18.00
		24	12.60	13.60
3	>60	Baseline	7.29	9.45
		2	19.70	21.50
		8	19.00	20.20
		24	10.50	15.40
4	>60	Baseline	9.47	11.20
		2	20.30	25.80
		8	15.50	20.00
		24	11.50	14.00
5	<35	Baseline	9.32	12.00
		2	25.00	26.10
		8	15.50	20.90
		24	11.60	11.90
6	<35	Baseline	10.20	10.50
		2[1]	-	-
		8	17.00	20.70
		24	8.83	15.70

[1]Unable to collect a sample

Table 3. 1 *Total hydroxyproline (Hyp) concentration levels in the serum and plasma of healthy volunteers following ingestion of 10g Peptan P collagen peptides*

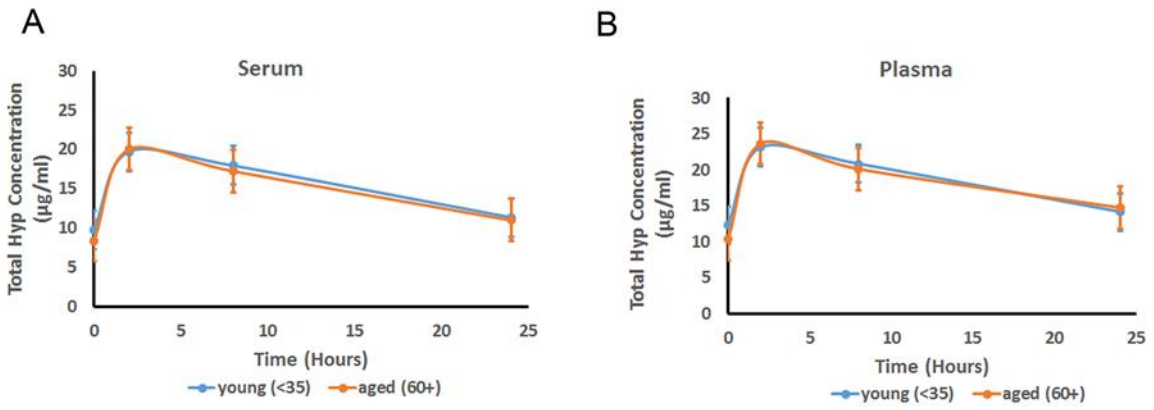

Figure 3. 12 Age does not affect the absorption of Peptan P collagen peptides. *Total hydroxyproline (Hyp) concentrations (µg/ml) in (A) serum and (B) plasma taken at 0 (baseline), 2, 8 and 24 hours post ingestion of 10g Peptan P collagen peptides in young (<35 years old) and aged (60+ years old) individuals. N=6 (4 young (mean age = 26), 2 aged (mean age = 64)). (Mistry et al., 2021)*

3.2.9 Peptan P promotes dermal fibroblast and keratinocyte wound closure in a normoglycaemic and hyperglycaemic environment

Along with ageing, diabetes is considered a key risk factor associated with the development of chronic wounds. Hyperglycaemia is one of the main symptoms of diabetes with previous studies reporting that a hyperglycaemic environment affects the migratory and proliferative capacity of dermal fibroblasts and keratinocytes (Xuan *et al.*, 2014; Hu and Lan, 2016; Buranasin *et al.*, 2018; Li *et al.*, 2019).

Given the ability for Peptan P to promote cutaneous wound healing in the context of age, further studies were carried out to determine their potential to promote dermal fibroblast or keratinocyte wound closure in a hyperglycaemic environment. 2D scratch assays were performed using dermal fibroblasts (<35 years old) cultured in either a normoglycaemic environment (5.5mM D-Glucose), a hyperglycaemic (25mM D-Glucose) or an extreme hyperglycaemic environment (50mM D-Glucose) in the presence or absence of 1mg/ml Peptan P, with monitoring of wound closure over 72 hours. Results revealed a glucose-dependent decrease in dermal fibroblast wound closure, with a significant decrease in wound closure rates of fibroblasts cultured in an extreme hyperglycaemic environment compared to a normoglycaemic or moderate hyperglycaemic environment (Figure 3.13D, One-way ANOVA with

Tukey's multiple comparisons test, *P<0.05, **P<0.01). Furthermore, Peptan P was able to significantly enhance dermal fibroblast wound closure rates in a normoglycaemic and hyperglycaemic environment (Figure 3.13A, B and D, One-way ANOVA with Tukey's multiple comparisons test, *P<0.05, ***P<0.001), but were unable to promote dermal fibroblast wound closure in an extreme hyperglycaemic environment (Figure 3.13C and D, One-way ANOVA with Tukey's multiple comparisons test, ns).

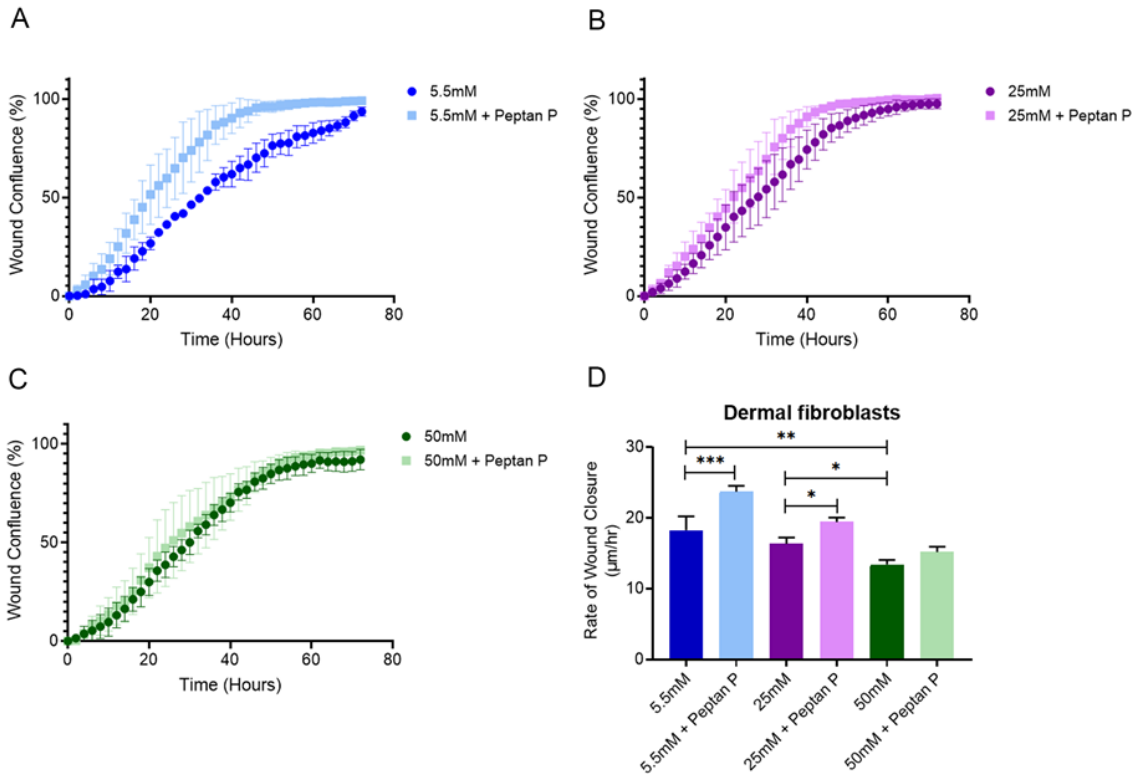

Figure 3. 13 Peptan P promotes dermal fibroblast wound closure in a normoglycaemic and hyperglycaemic environment. *Wound confluence (%) of dermal fibroblasts (<35 years old) seeded onto uncoated wells or wells coated with 1mg/ml Peptan P prior to scratch wound induction and culturing in (A) 5.5mM, (B) 25mM, (C) 50mM glucose for 72 hours. (D) Rate of wound closure of dermal fibroblasts seeded on uncoated wells or wells coated with 1mg/ml Peptan P cultured in 5.5mM, 25mM or 50mM glucose (mean ± SD, n=9, N=3, *P,0.05, ***P<0.001).*

To determine the potential for Peptan P to promote keratinocyte wound closure in a hyperglycaemic environment, 2D scratch assays were also performed using primary keratinocytes (<35 years old) seeded onto 1mg/ml Peptan P coated plates and cultured in either a normoglycaemic (5.5mM), hyperglycaemic (25mM) or extreme hyperglycaemic (50mM) environment over 72 hours. Results revealed a glucose concentration-dependent decrease in keratinocyte wound closure rates with a

significant decrease in wound closure rate of keratinocytes cultured in an extreme hyperglycaemic environment compared to a normoglycaemic environment (Figure 3.14D, One-way ANOVA with Tukey's multiple comparisons test, *P<0.05). Peptan P significantly enhanced keratinocytes wound closure rates in a normoglycaemic and hyperglycaemic environment (Figure 3.14A, B and D, One-way ANOVA with Tukey's multiple comparisons test, *P<0.05, **P<0.01), but again was unable to promote keratinocyte wound closure in an extreme hyperglycaemic environment (Figure 3.14C and D, One-way ANOVA with Tukey's multiple comparisons test, ns). Collectively, these data suggest that Peptan P to promote cutaneous wound healing in a moderate hyperglycaemic environment.

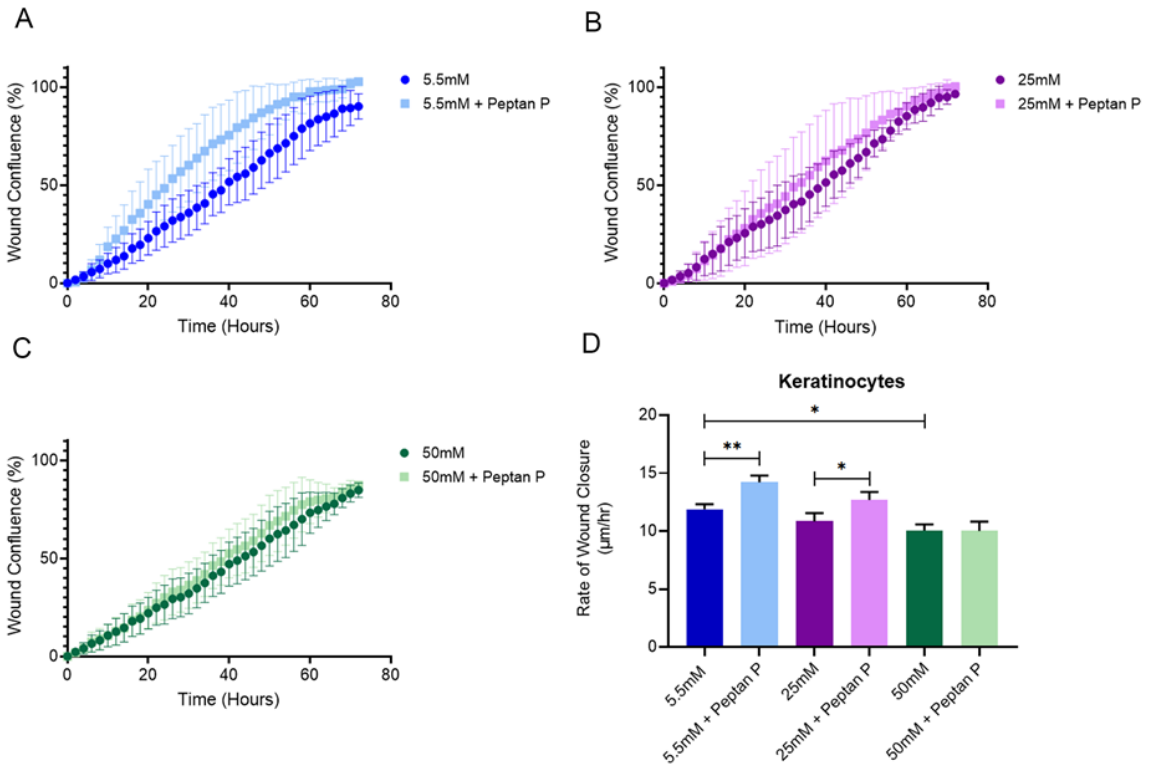

Figure 3. 14 Peptan P enhances keratinocyte wound closure in a normoglycaemic and hyperglycaemic environment. *Wound confluence (%) of keratinocytes (<35 years old) seeded onto 1mg/ml Peptan P coated wells or uncoated control wells cultured in either (A) 5.5mM, (B) 25mM or (C) 50mM glucose for 72 hours. (D) Rate of wound closure of keratinocytes seeded onto 1mg/ml Peptan P coated wells or uncoated control wells cultured in either 5.5mM, 25mM or 50mM glucose (mean ± SD, n=9, N=3, *P<0.05, **P<0.01).*

3.2.10 Peptan P does not promote dermal fibroblast or keratinocyte proliferation in a hyperglycaemic environment

Previous data suggests that Peptan P promotes cutaneous wound healing by promoting cellular proliferation. To determine whether Peptan P promotes cutaneous wound healing in a hyperglycaemic environment through enhanced cellular proliferation, MTS cell viability assays and immunofluorescent analysis of Ki67 expression in the presence or absence of 1mg/ml Peptan P was performed in primary dermal fibroblasts and keratinocytes cultured in a normoglycaemic (5.5mM), hyperglycaemic (25mM) or an extreme hyperglycaemic (50mM) environment for 72 hours. Results demonstrated the culture of either keratinocytes or dermal fibroblasts in an extreme hyperglycaemic environment resulted in the significant inhibition of cell viability compared to a normoglycaemic or hyperglycaemic environment (Figure 3.15A and B, One-way ANOVA with Tukey's multiple comparisons test, **P<0.01, ***P<0.001, ****P<0.0001). Although, Peptan P demonstrates a trend to enhance dermal fibroblast cell viability in a hyperglycaemic and extreme hyperglycaemic environments, this effect was only significant for dermal fibroblasts cultured in a normoglycaemic environment (Figure 3.15B, One-way ANOVA with Tukey's multiple comparisons test, ***P<0.001). Conversely, Peptan P had no significant effect on enhancing keratinocyte cell viability in a normoglycaemic, hyperglycaemic or extreme hyperglycaemic environment (Figure 3.15A, One-way ANOVA with Tukey's multiple comparisons test, ns).

Studies of Ki67 expression in keratinocytes and dermal fibroblasts cultured in a normoglycaemic, hyperglycaemic or extreme hyperglycaemic environment revealed a significant reduction in Ki67 expression of both keratinocytes and dermal fibroblasts cultured in a hyperglycaemic and extreme hyperglycaemic environment compared to a normoglycaemic environment (Figure 3.15C-E, One-way ANOVA with Tukey's multiple comparisons test, *P<0.05, ***P<0.001). Furthermore, results revealed that Peptan P only significantly increased Ki67 expression in dermal fibroblasts cultured in a normoglycaemic environment (Figure 3.15E, One-way ANOVA with Tukey's multiple comparisons test, **P<0.01), collectively suggesting that Peptan P may exert its beneficial effects on keratinocytes and dermal fibroblast

wound closure in a hyperglycaemic environment through other mechanisms rather than through enhancing cellular proliferation.

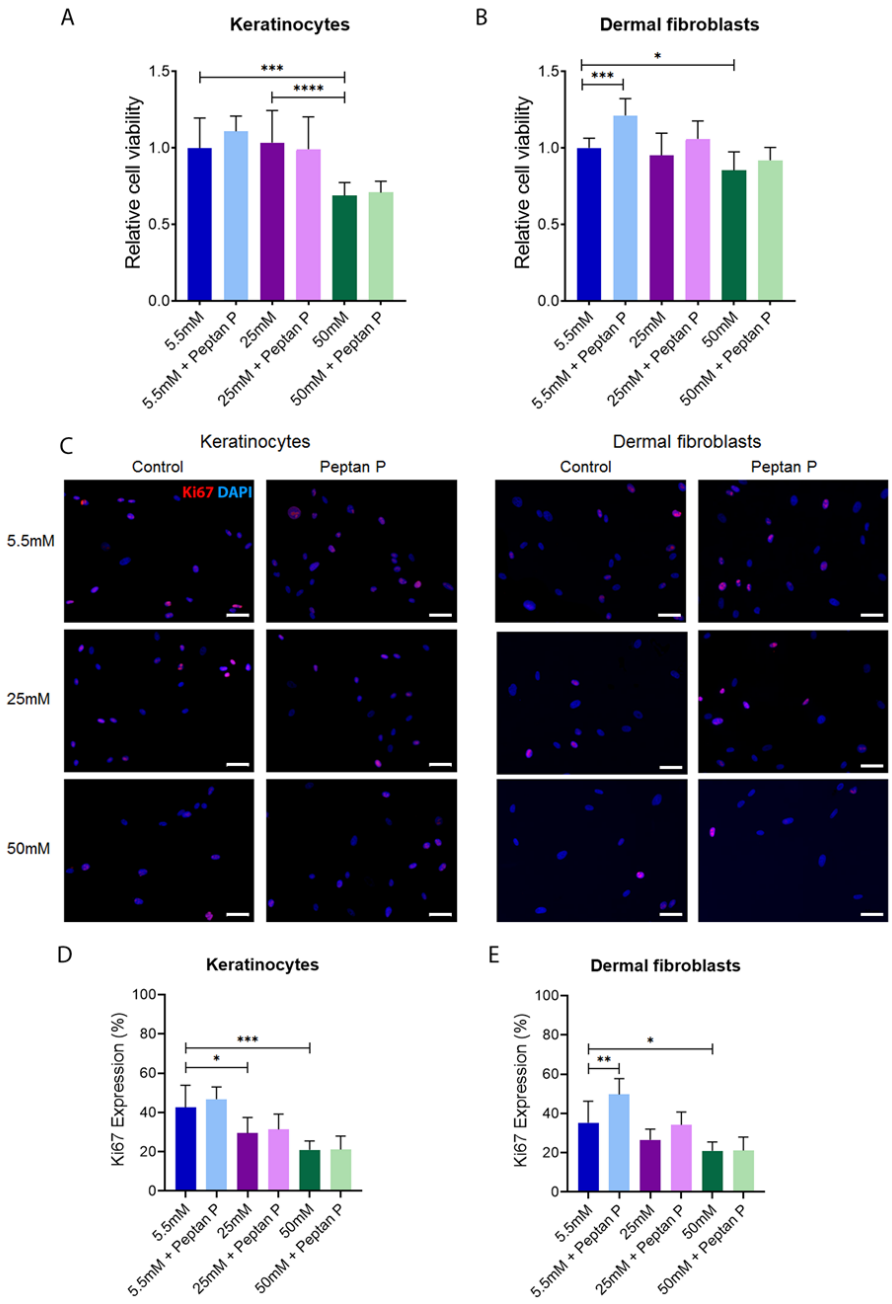

Figure 3. 15 Peptan P does not promote keratinocyte or dermal fibroblast proliferation in a hyperglycaemic environment. *Relative cell viability of (A) keratinocytes or (B) dermal fibroblasts (mean age = 45 years old) seeded onto 1mg/ml Peptan P coated wells or uncoated wells in either 5.5mM, 25mM or 50mM glucose containing culture media for 72 hours (mean ± SD, N=3, *P<0.05, ***P<0.001, ****P<0.0001). (C) Representative microphotographs for Ki67 expression (red) by keratinocytes or dermal fibroblasts cultured in either 5.5mM, 25mM or 50mM glucose containing culture media over 72 hours in the presence or absence of 1mg/ml Peptan P. Nuclei stained with DAPI (blue) Magnification = 20x. Scale bar = 50µm. Mean percentage of Ki67 expression in (D) keratinocytes or (E) dermal fibroblasts cultured in either 5.5mM, 25mM or 50mM glucose containing culture media over 72 hours in the presence or absence of 1mg/ml Peptan P relative to DAPI expression (mean ± SD, N=3, *P<0.05, **P<0.01, ***P<0.001).*

3.3 Discussion

Chronic non-healing wounds continue to be a major burden to worldwide healthcare systems with increased prevalence linked to an ageing population and systemic diseases such as diabetes (Sen *et al.*, 2009; Järbrink *et al.*, 2017). Ageing impairs cellular responses to injury, confounding the ability of aged skin to heal at comparable rates to young skin, whilst the hyperglycaemic environment generated by diabetic patients impairs the physiological function of cutaneous cells, affecting both cell proliferation and migration (Swift *et al.*, 1999; Lan *et al.*, 2008; Guo and DiPietro, 2010).

A hallmark of aged skin is reduced collagen synthesis, with results confirming a significant reduction in basal collagen I expression by aged dermal fibroblasts (60+ years old) compared to young dermal fibroblasts (18-35 years old) (Figure 3.1A and B), consistent with the reported diminished ability of dermal fibroblasts to synthesis collagen I by 1% for each life year (Shuster *et al.*, 1975). Although similar age-related changes in collagen III expression have been reported, results revealed no significant difference in basal collagen III expression by differing aged dermal fibroblasts (Figure 3.1C and D), which is likely explained by the naturally low abundance of collagen III in unwounded skin (<20%), as collagen III is largely synthesised during early phases of cutaneous wound healing as granulation tissue (Shuster *et al.*, 1975; Merkel *et al.*, 1988). Additionally, data revealed no correlation between basal collagen I or collagen III expression and the rate of wound closure of differing aged dermal fibroblasts (Figure 3.2) suggesting that basal collagen expression does not affect dermal fibroblast wound closure *in vitro* and therefore highlighting the importance of other ECM-associated changes such as cell-ECM interactions, ECM contractile forces and cell responses to growth factors that may affect normal wound healing responses (Stanulis-Praeger and Gilchrest, 1986; Ashcroft *et al.*, 1997b; Swift *et al.*, 1999).

The crosslinking of proteins within the ECM and at the dermal-epidermal junction enhances tissue stabilisation and has been previously reported to play a significant role in wound healing and tissue repair. TG2 is a key enzyme, mainly expressed in the dermis, that catalyses the formation of Nε-(γ-glutamyl) lysine bonds that covalently crosslink various ECM proteins such as collagen and fibronectin making them more stable to proteolytic and mechanical degradation (Akimov *et al.*, 2000;

Telci and Griffin, 2006). In addition to crosslinking and stabilisation of the ECM, TG2 has recently been linked to fibroblast adhesion and motility, with previous studies demonstrating that adhesion and spreading of 3T3 mouse fibroblasts is mediated through cell surface expression of TG2 and inhibition of TG2 impairs fibroblast migration (Balklava *et al.*, 2002). Given the proposed role of TG2 in fibroblast migration, coupled with studies highlighting increased TG2 expression with age following exogenous stimulation by ROS or UVA radiation, basal expression of TG2 was evaluated in young and aged primary dermal fibroblasts and keratinocytes (Gross *et al.*, 2003; Lee *et al.*, 2003). However, results revealed no significant difference in basal TG2 expression between differing aged primary dermal fibroblasts or keratinocytes isolated from human foreskin, suggesting that basal TG2 expression is unaffected by chronological ageing (Figure 3.3A, B, E and F). Impaired wound healing responses by TG2-deficient mice and the observed increase in TG2 expression and activity post-injury, suggests a role for TG2 in promoting effective wound closure (Haroon *et al.*, 1999; Balklava *et al.*, 2002). However again, results revealed no significant change in total TG2 expression following scratch wound induction of primary dermal fibroblasts or keratinocytes *in vitro* (Figure 3.3C, D, G and H). The fact that no significant change in TG2 expression was observed post-wounding is perhaps not surprising given that the upregulation of TG2 in response to wounding is due to the activation of TG2 already present within the microenvironment and additional TG2 synthesis at later time points post-injury, factors which likely would not have been captured within the utilised 2D assay (Upchurch *et al.*, 1991; Haroon *et al.*, 1999). Furthermore, TG2 expression by keratinocytes is mainly localised to the basal layer of keratinocytes at the dermal-epidermal junction of skin, and therefore it is possible that the confluent monolayer used in 2D scratch assays is not a representative of TG2 expression by unwounded and wounded basal keratinocytes in human skin (Haroon *et al.*, 1999; Eckert *et al.*, 2005).

Increasing evidence supports the potential for collagen peptides to promote cutaneous wound healing whilst also protecting against age-associated reductions in ECM deposition in animal models (Liang *et al.*, 2010; Zhang *et al.*, 2011; Wang *et al.*, 2015a); however, their potential to promote cutaneous wound healing in humans in the context of age remains undefined. Results demonstrated that both

Peptan B and Peptan P were able to significantly enhance wound closure of both young and aged dermal fibroblasts whilst Peptan F was unable to enhance wound closure of young and aged dermal fibroblasts (Figure 3.4 and Figure 3.5). The observed enhancement of collagen peptide-induced wound closure was diminished following treatment with mitomycin C (Figure 3.6) suggesting that these collagen peptides potentially promote dermal fibroblast wound closure by enhancing cellular proliferation. Supporting this potential, both Peptan B and Peptan P significantly increased dermal fibroblast cell viability and expression of the proliferation marker, Ki67 (Figure 3.7). These results corroborate with previous studies that have demonstrated that both mouse and human fibroblast proliferation was enhanced by the addition of exogenous collagen peptides at similar concentrations used in the present study (Shigemura *et al.*, 2009; Ohara *et al.*, 2010; Edgar *et al.*, 2018). Furthermore, previous studies identified that prolyl-hydroxyproline containing peptides were responsible for the enhancement of mouse fibroblast proliferation, therefore it is possible that the increase in dermal fibroblast proliferation induced by both Peptan B and Peptan P may result from a high content of prolyl-hydroxyproline present within these peptides, whilst the inability for Peptan F to enhance dermal fibroblast proliferation may reflect a low content or complete absence of prolyl-hydroxyproline containing peptides (Shigemura *et al.*, 2009). Despite results from the present and previous studies, both demonstrating that collagen peptides promote dermal fibroblast proliferation, the mechanism by which these collagen peptides promote cellular proliferation remains undefined. Nevertheless, previous studies have suggested that collagen peptides may have specific amino acid sequences exposed that are not accessible within native collagen, allowing them to directly or indirectly activate cell surface receptors and activate downstream signalling pathways to induce cellular proliferation (Ohara *et al.*, 2010; Yang *et al.*, 2019). It has also been suggested that collagen peptides may increase the expression and secretion of wound healing associated growth factors, with previous studies demonstrating that treatment with collagen peptides increased expression of FGF, EGF, VEGF and TGF-β by 3T3 mouse fibroblasts (Yang *et al.*, 2019). Alternatively, collagen peptides may be transported into the cells themselves via the expression of proton coupled oligopeptide transporters such as peptide transporter 1 (PEPT1) and 2 (PEPT2), and peptide histidine transporters 1 and 2 (PHT1 and PHT2) to induce cellular proliferation (Kudo *et al.*, 2016).

Interestingly, only Peptan P was able to significantly enhance wound closure of young keratinocytes but not aged keratinocytes, with no significant effect observed in response to Peptan B and Peptan F in either young or aged keratinocytes (Figure 3.8 and Figure 3.9). Collagen peptides derived from different animal species are known to produce different peptides with unique amino acid compositions which have different physiological properties (Wang *et al.*, 2008). The fact that both Peptan B and Peptan F were unable to significantly enhance keratinocyte wound closure compared to Peptan P may therefore reflect variances in the composition of individual peptides present and their ability to bind to or activate keratinocyte receptors to promote wound closure. Additionally, treatment with mitomycin C significantly diminished Peptan P-induced wound closure of keratinocytes, suggesting that like dermal fibroblasts, Peptan P promotes keratinocyte wound closure through enhanced cellular proliferation (Figure 3.10). Interestingly though, results from MTS cell viability assays and immunofluorescent analysis of Ki67 revealed that none of the collagen peptides significantly enhanced the proliferation of keratinocytes (Figure 3.11). However, these proliferation assays were performed on unwounded keratinocytes and therefore may reflect the previously described incapability of inactivated keratinocytes to proliferate in response to collagen and the need for the release of growth factors and cytokines from wounded keratinocytes to enable cell activation and subsequent induction of proliferation by collagen peptides (Dawson *et al.*, 1996; Komine *et al.*, 2000).

The ability for orally administered collagen peptides to exert a physiological effect on cutaneous wound healing is predominantly determined by their absorption and distribution to the skin. Previous studies have demonstrated that orally administered collagen peptides are absorbed into the blood stream and are able to penetrate the skin, where they are retained for up to 14 days post-ingestion (Ohara *et al.*, 2007; Ichikawa *et al.*, 2010; Watanabe-Kamiyama *et al.*, 2010). Analysis of hydroxyproline concentration, a biomarker of collagen peptide absorption, in the blood of healthy individuals following ingestion of 10g of Peptan P revealed peak concentrations of total hydroxyproline were achieved 2 hours post-ingestion with approximately 20µg/ml (150nmol/ml) of total hydroxyproline detectable in serum and approximately 23µg/ml (177nmol/ml) detectable in plasma, with subsequent reductions in total hydroxyproline concentration to baseline level at 24 hours post-

ingestion (Table 3.1, Figure 3.12). These findings correlate with previous studies that demonstrated that approximately 60-120nmol/ml of total hydroxyproline was detectable in the blood 1-2 hours post-ingestion of either porcine or fish-derived collagen peptides, with subsequent decreases in hydroxyproline concentration after 2 hours and no detection of hydroxyproline-containing peptides after 7 hours post-ingestion (Iwai *et al.*, 2005; Ohara *et al.*, 2007). Furthermore, results revealed that concentrations of total hydroxyproline detectable in the blood are similar to concentrations of Peptan P that enhance cutaneous wound healing *in vitro*, further highlighting the potential for collagen peptides to promote cutaneous wound healing at small micromolar concentrations. Previous studies have identified that peptide transporters such as PEPT1 and PEPT2 are involved in transporting di- and tri-peptides across the gut epithelium into the blood and therefore may also be involved in transporting collagen peptides across the bloodstream and into cutaneous cells where they exert their beneficial effect on cutaneous wound healing (Aito-Inoue *et al.*, 2007; Sontakke *et al.*, 2016). Given that no significant difference in total hydroxyproline concentrations between young and aged volunteers was observed (Figure 3.12), it can be theorised that age does not affect the expression or activity of peptide transporters and thereby does not affect collagen peptide absorption and bioavailability.

Along with ageing, diabetes is also a key risk factor associated with the development of chronic non-healing wounds such as diabetic ulcers. Given the ability for clinically achievable concentrations of Peptan P to promote cutaneous wound healing *in vitro* in the context of age, further studies were carried out to determine the potential for Peptan P to promote cutaneous wound healing in a hyperglycaemic environment. Similar to results from previous studies, 2D scratch assays using dermal fibroblasts and keratinocytes cultured in media representative of a normoglycaemic (5.5mM), hyperglycaemic (25mM) and an extreme hyperglycaemic (50mM) environment revealed a glucose-dependent inhibition of wound closure of both keratinocytes and dermal fibroblasts, with Peptan P significantly enhancing wound closure of both cell types in an normoglycaemic and hyperglycaemic environment (Figure 3.13 and 3.14) (Lamers *et al.*, 2011; Buranasin *et al.*, 2018; Li *et al.*, 2019). However, Peptan P was unable to enhance keratinocyte or dermal fibroblast wound closure in an extreme hyperglycaemic environment, suggesting that high levels of glucose

impacts collagen peptides function and thereby limits their potential to promote cutaneous wound healing. Previous studies have shown that a hyperglycaemic environment reduces expression of αv and α5 integrin subunits on the surface of rat dermal fibroblasts, thereby inhibiting their migratory capacity and suggesting that the extreme hyperglycaemic environment used in the present study may have inhibited the expression of these integrin subunits contributing to the significant decrease in wound closure rates and the ability of Peptan P to enhance cellular migration through interactions with these integrin subunits (Almeida *et al.*, 2016). Furthermore, previous studies in keratinocytes have demonstrated that a hyperglycaemic environment impairs keratinocyte migration through downregulation of the p38/MAPK signalling pathway, evidenced by decreased expression of phosphorylated p38, thereby suggesting the requirement for p38/MAPK activation for effective wound healing and that Peptan P may induce the activation of p38/MAPK to promote keratinocyte wound closure (Li *et al.*, 2019). Poor wound healing in diabetic patients has also been linked to increased MMP secretion, suggesting that the ability for Peptan P to promote wound closure in a hyperglycaemic environment may be due to their ability to inhibit MMP secretion, correlating with previous studies that have shown treatment of dermal fibroblasts with collagen peptides inhibited MMP-1 and MMP-3 secretion (Liu *et al.*, 2009b; Edgar *et al.*, 2018). However, the inability for Peptan P to promote wound closure in an extreme hyperglycaemic environment could suggest that high levels of MMPs may lead to the degradation of the collagen peptides, since they are originally derived from the ECM of porcine skin and therefore impair their ability to activate dermal fibroblasts or keratinocytes to promote wound closure or inhibit MMP secretion.

The presence of a hyperglycaemic environment has been shown to inhibit cellular proliferation and thereby impair proliferation of cutaneous cells required to promote effective wound healing (Hehenberger *et al.*, 1998; Terashi *et al.*, 2005). Given that Peptan P has been shown to enhance cellular proliferation, dermal fibroblasts and keratinocytes were cultured in a normoglycaemic, hyperglycaemic or an extreme hyperglycaemic environment in the presence or absence of 1mg/ml Peptan P, prior to evaluation of cell viability and Ki67 expression. Results revealed a glucose concentration-dependent decrease in cell viability and Ki67 expression in both

dermal fibroblasts and keratinocytes (Figure 3.15). These data are in line with previous studies that showed that high glucose-induced inhibition of cell viability and proliferation is mediated by increased ROS production and apoptosis through increased DNA damage and caspase expression, suggesting the decrease in dermal fibroblast and keratinocyte viability and proliferation in a hyperglycaemic environment may result from increased cell death (Lan *et al.*, 2013; Buranasin *et al.*, 2018; Rizwan *et al.*, 2020). Results also revealed that Peptan P was only able to increase cell viability of dermal fibroblasts cultured in a normoglycaemic environment but not a hyperglycaemic environment (Figure 3.13B), suggesting that high glucose concentrations impair Peptan P-induced proliferation, which is further supported by data showing their inability to increase Ki67 expression in a hyperglycaemic or extreme hyperglycaemic environment (Figure 3.15 C and E). Additionally, Peptan P was also unable to increase cell viability or Ki67 expression of keratinocytes cultured in a hyperglycaemic or extreme hyperglycaemic environment (Figure 3.15A, C and D). In this context, given that a hyperglycaemic environment may result in stimulation of other detrimental signalling mechanisms such as excessive ROS production, it is possible that such mechanisms also impair any beneficial effect of Peptan P in stimulating cellular proliferation during wound healing (Buranasin *et al.*, 2018; Volpe *et al.*, 2018). It has also been previously suggested that collagen peptides may be transported into cells via the expression of peptide transporters on the cell surface to stimulate downstream signalling pathways and therefore a hyperglycaemic environment may additionally affect expression and function of these peptide transporters (Kudo *et al.*, 2016). Nevertheless, the fact that Peptan P still enhanced both keratinocyte and dermal fibroblast wound closure in a hyperglycaemic environment (Figure 3.13 and 3.14) suggests that Peptan P may promote wound closure through other unknown mechanisms. Additionally, given the observed trend for increased Peptan P-induced dermal fibroblast viability in both a hyperglycaemic and extreme hyperglycaemic environment (Figure 3.15B) suggests that these collagen peptides may potentially inhibit ROS production and apoptosis to prevent cell death, with previous studies demonstrating that some collagen peptides exert anti-oxidant effects (Chi *et al.*, 2014; Onuh *et al.*, 2014; Sabeena Farvin *et al.*, 2016; Pozzolini *et al.*, 2018).

Even though 50mM concentrations of glucose represents an extreme hyperglycaemic environment, the blood glucose levels of most diabetic patients throughout the day are between 5.5-25mM, with highs of 50mM only occurring in diabetic patients who do not monitor and manage their blood glucose levels (Güemes *et al.*, 2016). Given that Peptan P exerted significant effects on both dermal fibroblast and keratinocyte wound closure in a 2D hyperglycaemic wounded environment, suggests that collagen peptides may be a potential therapeutic strategy to promote wound healing in diabetic patients, however further studies are warranted in more representative *in vitro* and *ex vivo* models using keratinocytes and dermal fibroblasts derived from patients with diabetic ulcers.

Collectively, these data suggest the potential for clinically achievable concentrations of Peptan P collagen peptides as a potential viable strategy for the treatment of cutaneous wounds in both young and aged individuals as well as a potential wound healing strategy for patients with diabetes. To further explore this potential, subsequent studies were focussed on comparing the effects of Peptan P collagen peptides with newly synthesised alternative porcine-derived collagen peptides or digested porcine-derived collagen peptides on dermal fibroblast and keratinocyte wound healing as well as investigating the mechanisms by which these porcine-derived collagen peptides exert their beneficial effect on cutaneous wound healing.

3.4 Summary

- Collagen I expression declines with age
- Peptan P significantly promotes wound closure of both young and aged keratinocytes and dermal fibroblasts *in vitro*
- Peptan P significantly enhances proliferation of dermal fibroblasts
- Inhibition of proliferation impairs Peptan P-induced wounded closure, suggesting their ability to promote wound closure likely arises from promoting cellular proliferation
- Age does not affect the absorption of orally ingested collagen peptides
- Concentrations of Peptan P detected in the blood following ingestion are equivocal to concentrations that promote wound closure *in vitro*
- Peptan P significantly enhanced wound closure of keratinocytes and dermal fibroblasts in a hyperglycaemic environment
- A hyperglycaemic environment impairs Peptan P-induced proliferation of keratinocytes and dermal fibroblasts

Chapter 4: Peptan P-induced wound closure is mediated through activation of integrin signalling

Chapter 4: Peptan P-induced wound closure is mediated through activation of integrin signalling

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4.1 Introduction

Bioactive peptides generated from the hydrolysis of ECM proteins such as collagen found in animal bones, skin and connective tissue have shown potential for the management of varying health conditions, with previous studies demonstrating their anti-ageing effects in both skin and joints as well as their ability to promote cutaneous wound healing both *in vitro* and *in vivo* (Zhang et al., 2011; Liu et al., 2014; Wang et al., 2015a; Zhu et al., 2020). However, whilst these bioactive collagen peptides have shown some potential for promoting cutaneous wound healing both *in vitro* and *in vivo*, the mechanisms by which they promote wound healing still remains undefined.

Previous studies exploring the anti-ageing effects of collagen peptides demonstrated that fish-derived collagen peptides stimulated TGF-β signalling-induced collagen synthesis and synthesis of other ECM proteins such as elastin and hyaluronic acid in human dermal fibroblasts *in vitro* and in mouse models following exposure to UV radiation (Edgar *et al.*, 2018; Liu *et al.*, 2019). Additionally fish-derived collagen peptides with a molecular weight between 0.3-8kDa have been shown to inhibit expression and secretion of MMPs such as MMP-1 and MMP-3 thereby preventing excessive collagen degradation within the skin (Edgar *et al.*, 2018). This is also important in the context of cutaneous wound healing as an overexpression or over-secretion of MMPs delays wound healing through increased degradation of the provisional collagen matrix, impairing the ability for keratinocytes to migrate over the provisional matrix (Reiss *et al.*, 2010; Simonetti *et al.*, 2013).

Studies focussing on orally ingested collagen peptides have shown that these peptides are transported into the bloodstream from the gut via proton coupled oligopeptide transporters such as peptide transporter 1 and 2 (PEPT1 and PEPT2) present on enterocytes within the intestinal lining (Aito-Inoue *et al.*, 2007; Wang *et al.*, 2017; Jochems *et al.*, 2018). Therefore it is possible that collagen peptides may interact with peptide transporters present on the cell surface of cutaneous cells to activate downstream signalling pathways associated with cutaneous wound healing and supported by previous studies demonstrating that PEPT2 is expressed by basal keratinocytes whilst peptide histidine transporter 2 (PHT2) is expressed by dermal fibroblasts (Kudo *et al.*, 2016). Additionally, studies using mouse fibroblasts have

demonstrated that the collagen dipeptide, Pro-Hyp, is detectable in the cytoplasm of p75 neurotrophin receptor (p75NTR) expressing fibroblasts resulting in enhanced cell growth and suggesting that the expression of p75NTR may also play an important role in the transport of collagen peptides into the cell in order to activate signalling pathways that promote cellular proliferation during cutaneous wound healing (Asai *et al.*, 2020).

Previous studies using porcine bone-derived collagen peptides at concentrations between 0.05-5mg/ml have demonstrated that these low molecular weight peptides are able to promote osteoblast proliferation and differentiation through the activation of the PI3K/Akt pathway whilst inhibition with a PI3K inhibitor, abolished collagen peptide-induced activation of Akt thereby impairing osteoblast proliferation, collectively suggesting that collagen peptides may activate PI3K/Akt signalling in cutaneous cells as well and that the activation of the PI3K/Akt pathway is linked to the enhancement of cellular proliferation during cutaneous wound healing (Zhu *et al.*, 2020). In the context of cutaneous cells, studies using NIH-3T3 mouse fibroblasts have also shown that treatment with 12.5-50µg/ml marine collagen peptides also results in enhanced cell proliferation during wound healing through the activation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signalling pathway (Yang *et al.*, 2019). Additionally, *in vitro* studies have demonstrated marine collagen peptides are also able to increase the expression of wound healing-associated growth factors such as EGF, FGF, VEGF and TGF-β in mouse fibroblasts, suggesting collagen peptides may in fact promote wound healing through multiple mechanisms rather than the activation of just one signalling pathway (Yang *et al.*, 2019).

Another signalling pathway that plays an important role in wound healing is the integrin signalling pathway. In particular, integrin α2β1 is a collagen receptor, expressed by both keratinocytes and dermal fibroblasts (Koivisto *et al.*, 2014). Through the recognition of specific motifs present within native collagen such as GFOGER and GLOGEN, leads to autophosphorylation of focal adhesion kinase (FAK) and downstream activation of Akt and ERK to promote cellular proliferation (Figure 4.1) (Moreno-Layseca and Streuli, 2014). This suggests that collagen peptides may also contain motifs such as GFOGER that allows for the interaction

and binding of integrins to activate downstream signalling pathways such as ERK and Akt in order to promote cutaneous wound healing.

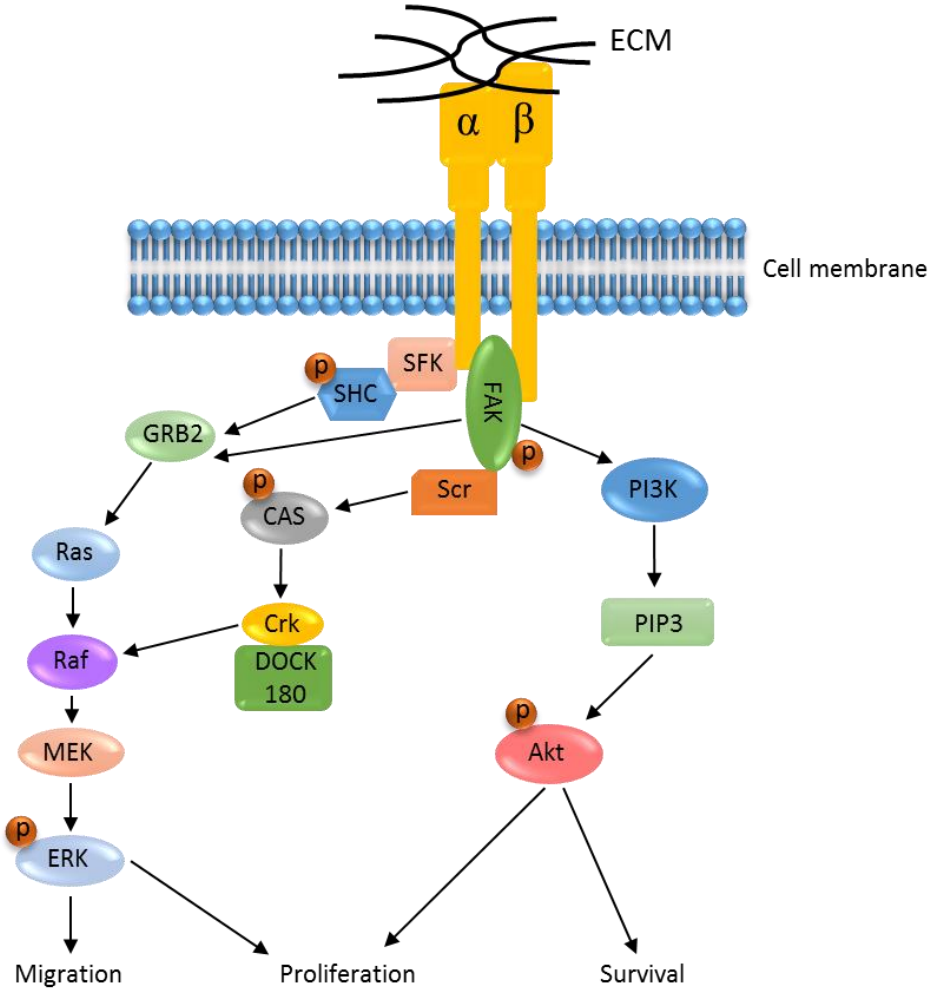

Figure 4. 1 Signalling pathways downstream of integrins that promote cellular proliferation. *Following ECM binding with cell surface integrin receptors leads to the downstream activation of both the PI3K/Akt and ERK signalling pathways to promote cellular proliferation.*

Nevertheless, despite many attempts to elucidate the mechanistic action of collagen peptides in various cell types and animal models, the mechanism by which collagen peptide promote cutaneous wound healing still remains undefined. In addition to evaluating the potential for alternative undigested and digested porcine-derived collagen peptides to promote cutaneous wound healing *in vitro*, the aim of the present chapter was to therefore determine the mechanisms mediating Peptan P-induced wound closure of human keratinocytes and dermal fibroblasts *in vitro*.

4.2 Results

4.2.1 Alternative CH2, CH7 and CH10 porcine-derived collagen peptides promote dermal fibroblast wound closure

Since initial studies carried out using 2D scratch assays demonstrated the potential for Peptan P collagen peptides to promote wound closure of both young and aged keratinocytes and dermal fibroblasts, additional alternative porcine-derived collagen peptides; CH1, CH2, CH3, CH4, CH5, CH6, CH7, CH8, CH9 or CH10 were screened to evaluate their potential to promote dermal fibroblast wound closure compared to the original Peptan P collagen peptides. For scratch assays, 1mg/ml of all peptides were used to coat wells prior to addition of dermal fibroblasts (<35 years old), scratch wound induction and monitoring of wound closure over 72 hours. Results demonstrated that CH2, CH7 and CH10 significantly enhanced dermal fibroblast wound closure compared to uncoated control wells and with equal significance to wound closure rates induced by Peptan P (Figure 4.2A, One-way ANOVA with Tukey's multiple comparisons test, *P<0.05). However, CH1, CH3. CH4, CH5, CH6, CH8 and CH9 had no significant effect on dermal fibroblast wound closure.

To determine the potential for alternative CH2, CH7 and CH10 porcine-derived collagen peptides to potentiate dermal fibroblast wound closure in the context of age, 2D scratch assays were repeated using young (18-35 years old) and aged (60+ years old) dermal fibroblasts, with Peptan P used as a positive control (Figure 4.2B). Results demonstrated that both CH7 and CH10 were able to significantly enhance wound closure of both young and aged dermal fibroblasts (Figure 4.2D and E, One-way ANOVA with Tukey's multiple comparisons test, *P<0.05, **P<0.01), while CH2 were only able to significantly enhance wound closure of young dermal fibroblasts (Figure 4.2C, One-way ANOVA with Tukey's multiple comparisons test, **P<0.01). Collectively, these data suggest that while CH7 and CH10 may offer additional bioactive collagen peptides with wound healing properties, neither peptide enhance young or aged dermal fibroblast wound closure more significantly than the original Peptan P collagen peptides.

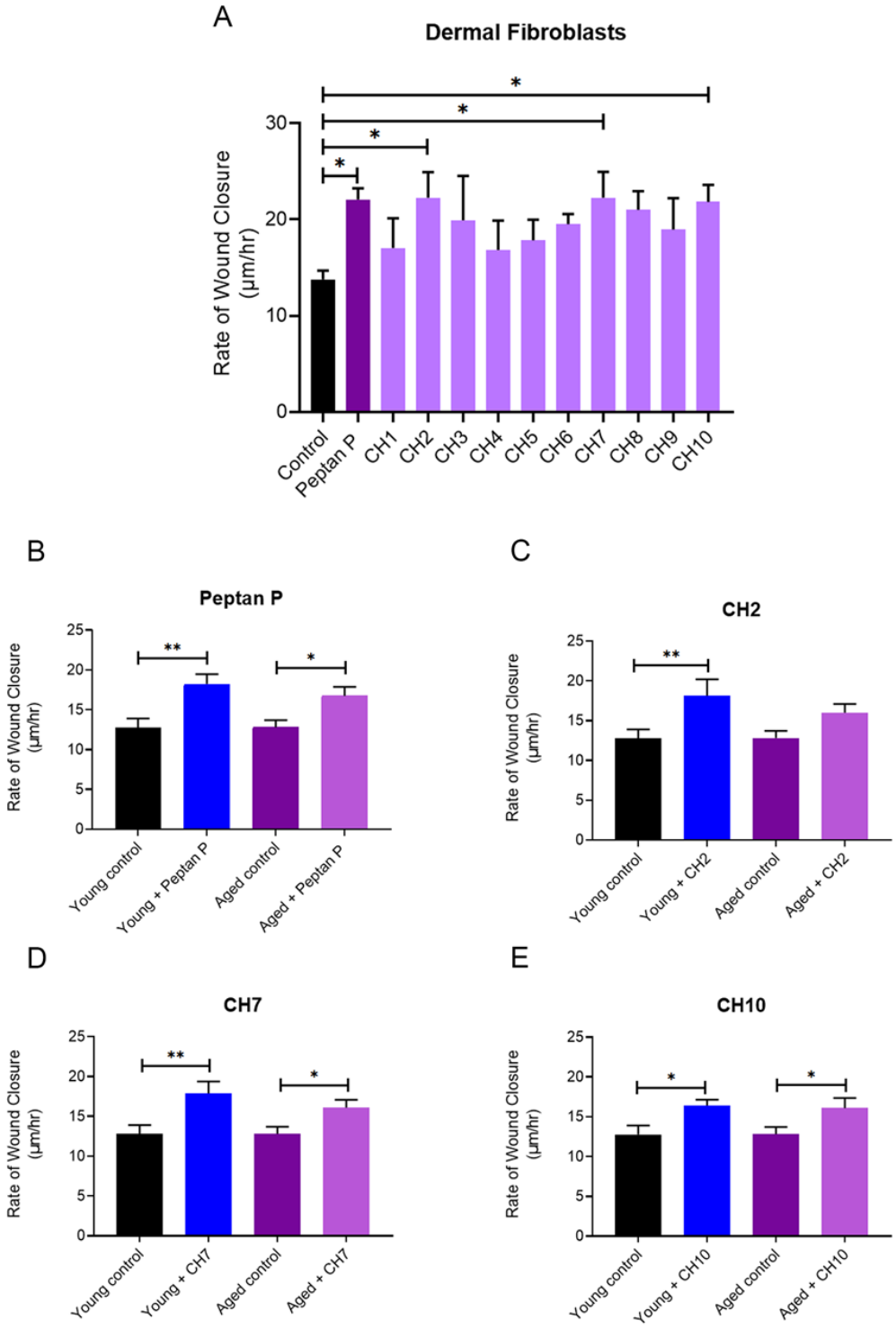

Figure 4. 2 Alternative CH2, CH7 and CH10 porcine-derived collagen peptides promote dermal fibroblast wound closure. *(A) Rate of wound closure of dermal fibroblasts (<35 years old) seeded onto uncoated control wells or wells pre-coated with 1mg/ml of either Peptan P or alternative collagen peptides; CH1, CH2, CH3, CH4, CH5, CH6, CH7, CH8, CH9 and CH10 coated wells (mean ± SD, n=9, N=3, *P<0.05). Rate of wound closure of young (18-35 years old) or aged (60+ years old) dermal fibroblasts seeded onto uncoated control wells or wells pre-coated with either 1mg/ml (B) Peptan P, (C) CH2, (D) CH7 or (E) CH10 collagen peptides. (mean ± SD, n=9, N=3, *P<0.05, **P<0.01).*

4.2.2 Alternative porcine-derived collagen peptides do not enhance keratinocyte wound closure

To determine the potential for alternative porcine-derived collagen peptides to promote keratinocyte wound closure, primary keratinocytes (<35 years old) were seeded onto uncoated wells or wells pre-coated with 1mg/ml Peptan P (positive control) or 1mg/ml CH1, CH2, CH3, CH4, CH5, CH6, CH7, CH8, CH9 or CH10 alternative collagen peptides, prior to scratch wound induction and monitoring of wound closure over 72 hours. In comparison to the significant increase in wound closure rates induced by Peptan P (Figure 4.3, One-way ANOVA with Tukey's multiple comparisons test, *P<0.05), results showed no significant effect on keratinocyte wound closure induced by any of the alternative collagen peptides (Figure 4.3, One-way ANOVA with Tukey's multiple comparisons test, ns). These data therefore suggest that the alternative porcine-derived collagen peptides do not contain any bioactive peptides that are able to exert a beneficial effect on keratinocyte wound closure.

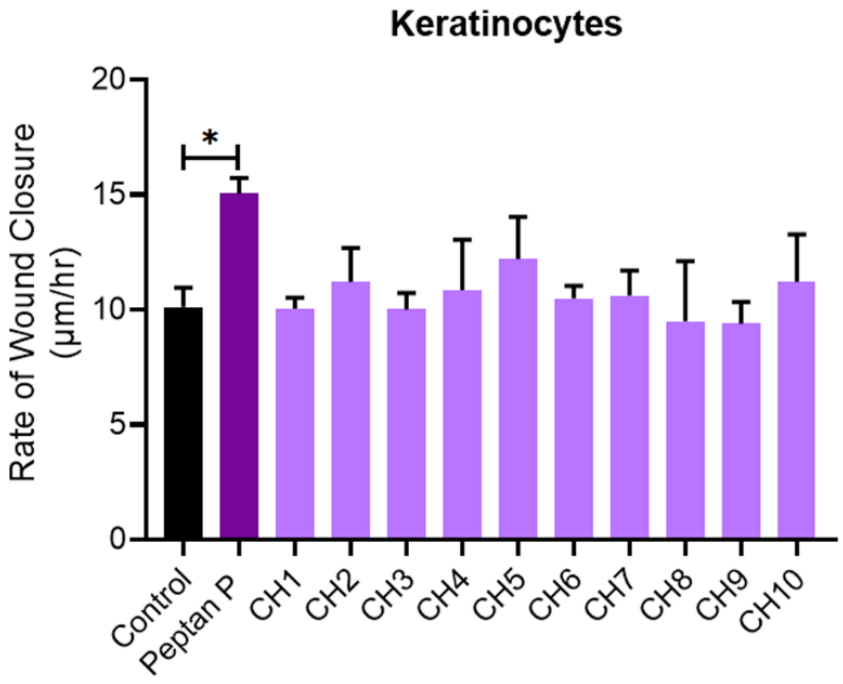

Figure 4. 3 Alternative porcine-derived collagen peptides do not significantly enhance wound closure of keratinocytes. *Rate of wound closure of keratinocytes (<35 years old) seeded onto uncoated control wells or wells pre-coated with 1mg/ml of either Peptan P or alternative collagen peptides; CH1, CH2, CH3, CH4, CH5, CH6, CH7, CH8, CH9 and CH10 coated wells (mean ± SD, n=9, N=3, *P<0.05).*

4.2.3 Digested collagen peptides do not significantly promote dermal fibroblast wound closure

To assess the potential of orally administered collagen peptides to promote dermal fibroblast wound closure, porcine-derived collagen peptides were subjected to an *in vitro* digestion to mimic human digestion in order to create collagen peptide digests. Primary dermal fibroblasts (<35 years old) were seeded onto wells pre-coated with 1mg/ml Peptan P (positive control) or uncoated wells. Following scratch induction, uncoated wells were treated with culture media only (negative control) or treated with 1mg/ml empty digest (internal control) or 1mg/ml of collagen peptides digests; H22D2, H34D1, H144D1, H212D1, H165D1, H221D1, H68D2, H58D2, H123D2, H93D1, H80D1, H47D1, H155D1 in culture media, with wound closure monitored over 72 hours. Results demonstrated that whilst Peptan P induced a significant effect on the wound closure of dermal fibroblasts (Figure 4.4, One-way ANOVA with Tukey's multiple comparisons test, **P<0.01), none of the collagen peptide digests were able to significantly enhance dermal fibroblast wound closure.

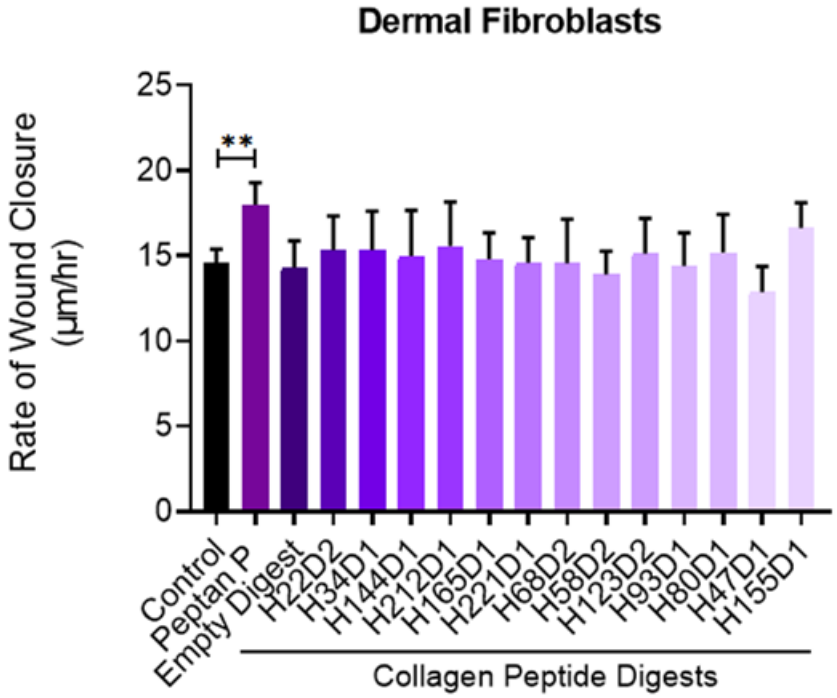

Figure 4. 4 Digested collagen peptides do not significantly promote wound closure of dermal fibroblasts. *Rate of wound closure of dermal fibroblasts (<35 years old) seeded onto wells pre-coated with 1mg/ml of either Peptan P or uncoated wells that were left untreated or treated with 1mg/ml of either empty digest or 1mg/ml of digested collagen peptides; H22D2, H34D1, H144D1, H212D1, H165D1, H221D1, H68D2, H58D2, H123D2, H93D1, H80D1, H47D1, H155D1 (mean ± SD, n=9, N=3, *P<0.05).*

4.2.4 Digested collagen peptides do not significantly promote keratinocyte wound closure

To assess the potential for digested collagen peptides to promote keratinocyte wound closure, primary keratinocytes (<35 years old) were seeded onto uncoated or wells pre-coated with 1mg/ml Peptan P (positive control). Following scratch induction, uncoated wells were either treated with culture media only (control) or treated with 1mg/ml empty digest (internal control) or 1mg/ml of collagen peptide digests; H22D2, H34D1, H144D1, H212D1, H165D1, H221D1, H68D2, H58D2, H123D2, H93D1, H80D1, H47D1, H155D1 in culture media, with wound closure monitored over 72 hours. Results demonstrated that again, whilst Peptan P induced a significant effect on the wound closure of keratinocytes (Figure 4.5, One-way ANOVA with Tukey's multiple comparisons test, *P<0.05), none of the collagen peptide digests were able to significantly enhance keratinocyte wound closure. Collectively, these results demonstrate that oral ingestion impairs the bioactivity of collagen peptides to promote dermal fibroblast and keratinocyte wound closure.

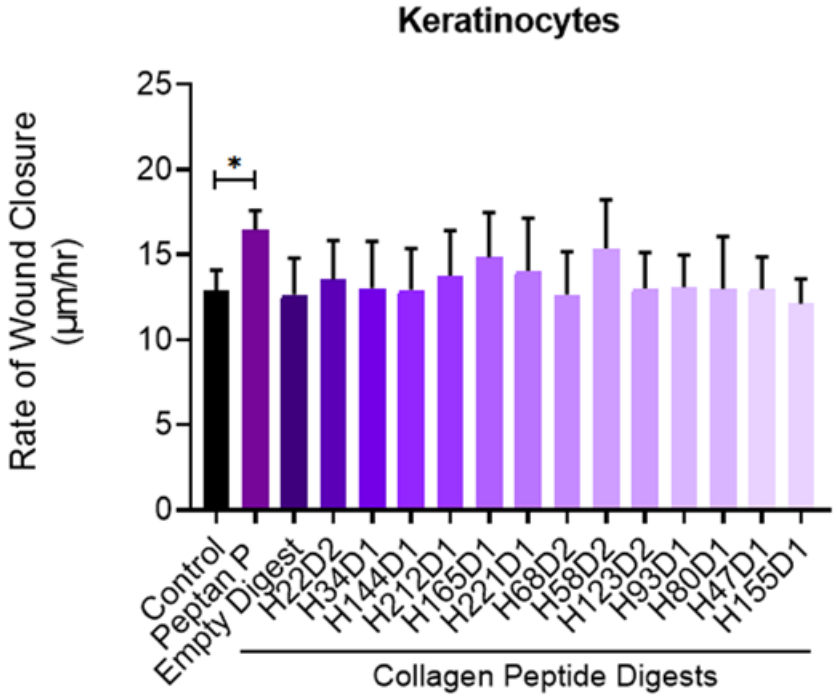

Figure 4. 5 Digested collagen peptides do not significantly promote wound closure of keratinocytes. *Rate of wound closure of keratinocytes (<35 years old) seeded onto wells pre-coated with 1mg/ml of either Peptan P or uncoated wells that were left untreated or treated with 1mg/ml of either empty digest or 1mg/ml of digested collagen peptides; H22D2, H34D1, H144D1, H212D1, H165D1, H221D1, H68D2, H58D2, H123D2, H93D1, H80D1, H47D1, H155D1 (mean ± SD, n=9, N=3, *P<0.05).*

4.2.5 Keratinocytes and dermal fibroblasts do not significantly express peptide transporters, PEPT1, PEPT2, PHT1 and PHT2

Previous studies have shown that collagen peptides are transported across the gut epithelium into the bloodstream via the expression of peptide transporters that are present on the cell surface of enterocytes (Aito-Inoue *et al.*, 2007). Specifically, studies have identified the expression of peptides transporters 1 and 2 (PEPT1 and PEPT2) and peptide histidine transporters 1 and 2 (PHT1 and PHT2) within the skin (Kudo *et al.*, 2016; Kudo *et al.*, 2020). However, results revealed that while age does not affect the expression of PEPT1, PEPT2, PHT1 or PHT2 in young or aged keratinocytes and dermal fibroblasts, these peptide transporters were expressed at low to undetectable levels when compared to the expression by the positive control of Caco-2 cells (Figure 4.6A-D, One-way ANOVA with Tukey's multiple comparisons test, ns). Collectively, these results suggest that although keratinocytes and dermal fibroblasts express low levels of peptide transporters, it is unlikely to be the mechanism by which collagen peptides promote cutaneous wound closure.

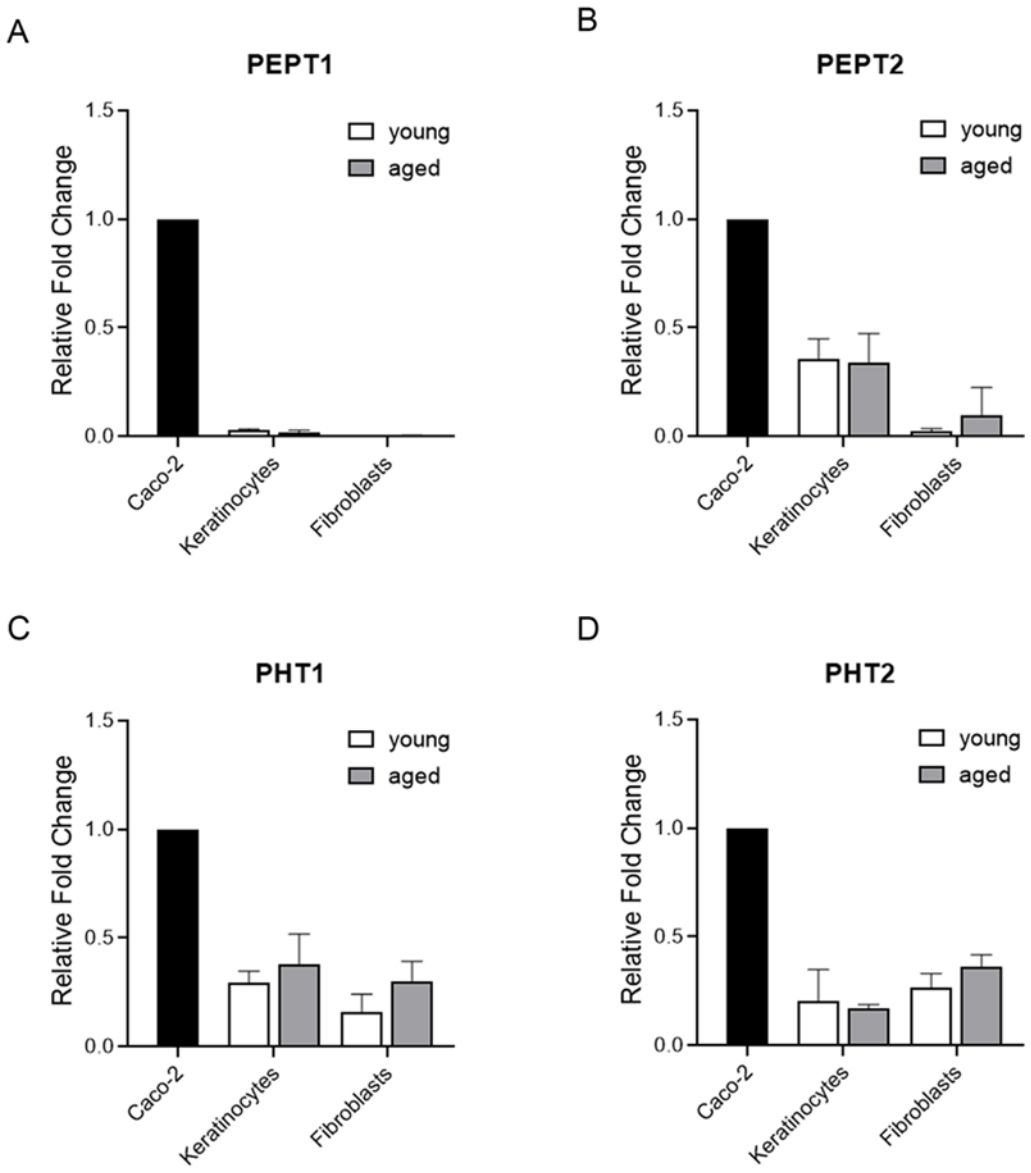

Figure 4. 6 Keratinocytes and dermal fibroblasts do not significantly express peptide transporter; PEPT1, PEPT2, PHT1 or PHT2. *Relative mRNA levels of (A) PEPT1, (B) PEPT2, (C) PHT1 and (D) PHT2 in Caco-2 cells or young and aged keratinocytes and dermal fibroblasts (mean ± SD, N=3).*

4.2.6 Age does not affect expression of the integrin α2 or β1 subunit by keratinocytes

Integrin receptors such as integrin α2β1 are collagen receptors that are able to recognise specific motifs within native collagen leading to activation of the integrin signalling pathway and activation of downstream signalling pathways that are involved in promoting cellular proliferation, migration and differentiation. Previous studies have also implicated ageing with decreased integrin expression in

keratinocytes (Bosset *et al.*, 2003). To determine whether age has any impact on the expression of the integrin α2β1 receptor in keratinocytes, Western blotting was used to evaluate the expression of the integrin α2 and integrin β1 subunits in primary human keratinocytes derived from young (18-35 years old), middle-aged (40-55 years old) or aged (60+ years old) individuals. Results however, demonstrated that age had no significant effect on the expression of either the integrin α2 or integrin β1 subunit in differing aged keratinocytes (Figure 4.7, One-way ANOVA with Tukey's multiple comparisons test, ns).

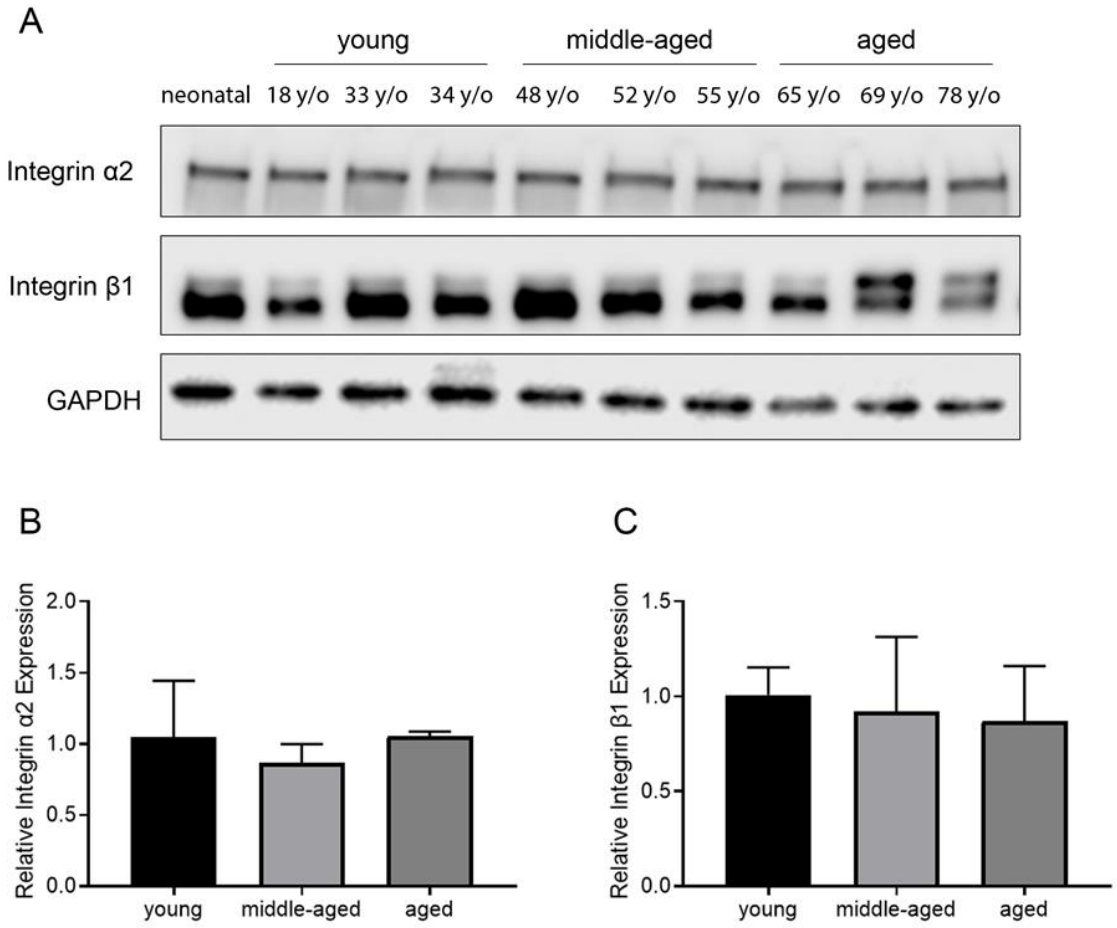

Figure 4. 7 Age does not significantly affect expression of the integrin α2 or integrin β1 subunits in keratinocytes. *(A) Representative Western blot for integrin α2 (130kDa), integrin β1 (130kDa) and GAPDH (37kDa) expression in keratinocytes derived from young (18-35 years old), middle-aged (40-55 years old) and aged (60+ years old) individuals. Densitometric expression of (B) integrin α2 or (C) integrin β1 relative to GAPDH expression in young, middle-aged and aged keratinocytes (mean ± SD, N=3).*

4.2.7 Age does not affect expression of the integrin α2 or β1 subunit by dermal fibroblasts

To determine whether age has any impact on the expression of the integrin α2β1 receptor in dermal fibroblasts, Western blotting was again used to evaluate the expression of the integrin α2 and integrin β1 subunits in primary human dermal fibroblasts derived from young (18-35 years old), middle-aged (40-55 years old) or aged (60+ years old) individuals. As in keratinocytes, results again demonstrated age did not have a significant effect on the expression of either the integrin α2 or integrin β1 subunit in differing aged dermal fibroblasts (Figure 4.8, One-way ANOVA with Tukey's multiple comparisons test, ns). Collectively, these data suggest that age does not affect integrin α2β1 expression in both keratinocytes and dermal fibroblasts.

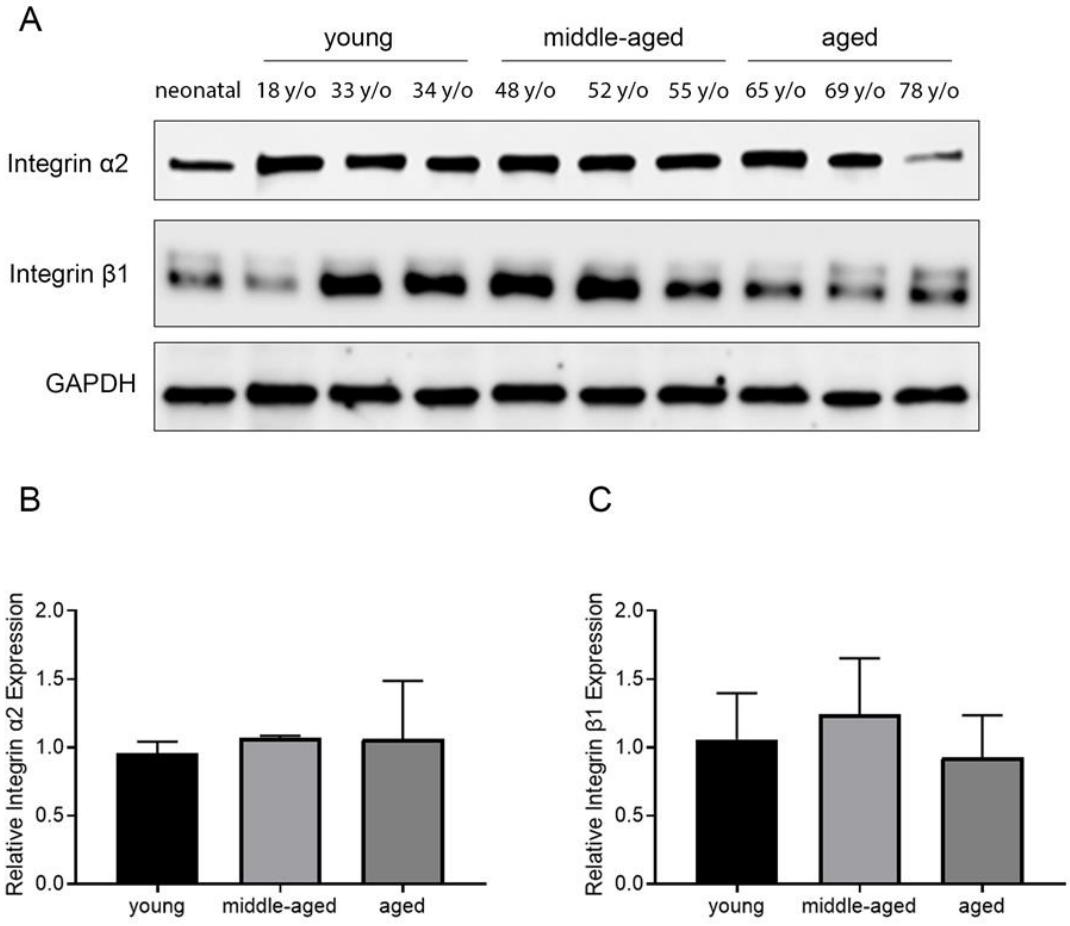

Figure 4. 8 Age does not significantly affect expression of the integrin α2 or integrin β1 subunits in dermal fibroblasts. *(A) Representative Western blot for integrin α2 (130kDa), integrin β1 (130kDa) and GAPDH (37kDa) expression in dermal fibroblasts derived from young (18-35 years old), middle-aged (40-55 years old) and aged (60+ years old) individuals. Densitometric expression of (B) integrin α2 or (C) integrin β1 relative to GAPDH expression in young, middle-aged and aged dermal fibroblasts (mean ± SD, N=3).*

4.2.8 Peptan P significantly activates Akt signalling in unwounded keratinocytes

Motifs present within native collagen such as GFOGER and GLOGEN are known to interact with integrin receptors such as integrin α2β1 causing autophosphorylation of FAK at tyr397 and downstream activation of Akt and ERK signalling pathways to promote cellular proliferation (Moreno-Layseca and Streuli, 2014). To determine whether Peptan P activated the integrin signalling pathway to promote cellular proliferation, primary keratinocytes were seeded onto either uncoated wells or wells pre-coated with 1mg/ml rat tail collagen I (positive control) or Peptan P and incubated for 72 hours. Results revealed that neither rat tail collagen I nor Peptan P significantly increased expression of either the integrin α2 or integrin β1 subunit compared to the uncoated control (Figure 4.9A-C, One-way ANOVA with Tukey's multiple comparisons test, ns). Results also demonstrated that rat tail collagen I significantly promoted activation of FAK, Akt and ERK in unwounded keratinocytes compared to the uncoated control (Figure 4.9A, D-F, One-way ANOVA with Tukey's multiple comparisons test, *P<0.05, **P<0.01, ***P<0.001). Furthermore, Peptan P was able to significantly promote the activation of Akt compared to the uncoated control (Figure 4.9A and E, One-way ANOVA with Tukey's multiple comparisons test, **P<0.01), but did not however, significantly enhance activation of FAK or ERK in unwounded keratinocytes (Figure 4.9A, D and E). Taken together, these results suggest that Peptan P exerts its beneficial effects on keratinocytes by activating the Akt signalling pathway.

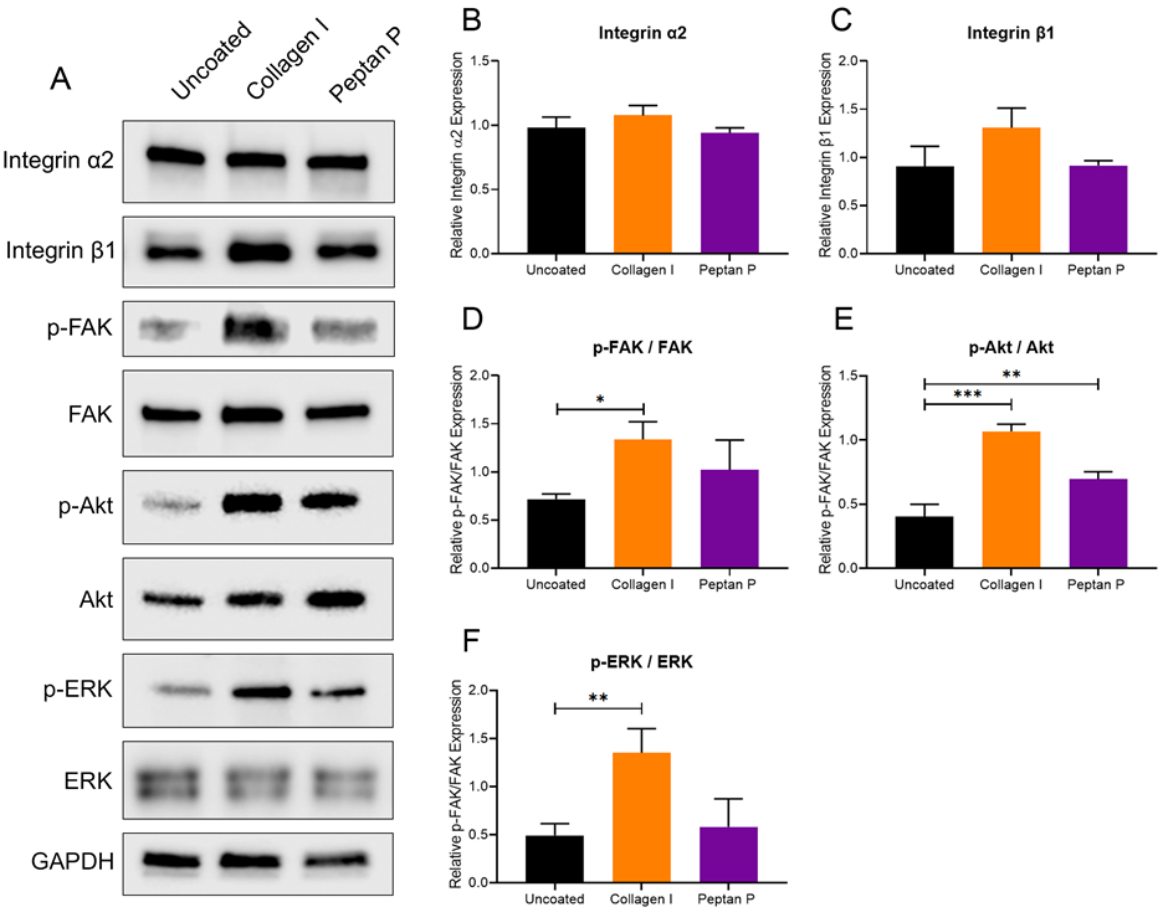

Figure 4. 9 Peptan P significantly promotes the activation of Akt in unwounded keratinocytes. *(A) Representative Western blot for integrin α2 (130kDa), integrin β1 (130kDa), p-FAK (110kDa), FAK (110kDa), p-Akt (60kDa), Akt (60kDa), p-ERK (42-44kDa), ERK (42-44kDa) and GAPDH (37kDa) expression in keratinocytes seeded onto uncoated wells or wells pre-coated with 1mg/ml rat tail collagen I or Peptan P. Densitometric expression of (B) integrin α2, (C) integrin β1, (D) p-FAK/FAK, (E) p-Akt/ Akt or (F) p-ERK/ERK expression relative to GAPDH expression in keratinocytes seeded onto uncoated wells or wells pre-coated with 1mg/ml rat tail collagen I or Peptan P (mean ± SD, N=3, *P<0.05, **P<0.01, ***P<0.001).*

4.2.9 Peptan P does not significantly activate FAK, Akt or ERK signalling in unwounded dermal fibroblasts

To determine whether Peptan P activated the integrin signalling pathway to promote cellular proliferation in unwounded dermal fibroblasts, primary dermal fibroblasts were seeded onto either uncoated wells or wells pre-coated with 1mg/ml rat tail collagen I (positive control) or Peptan P and incubated for 72 hours. Results demonstrated that collagen I was able to significantly increase expression of integrin α2 and promoted activation of FAK and Akt in unwounded dermal fibroblasts (Figure 4.10A, B, D and E, One-way ANOVA with Tukey's multiple comparisons test,

*P<0.05, **P<0.01). However, Peptan P was unable to significantly increase expression of the integrin α2 or integrin β1 subunit compared to the uncoated control (Figure 4.10A-C, One-way ANOVA with Tukey's multiple comparisons test, ns). Additionally, Peptan P had no significant effect on the activation of FAK, Akt or ERK in unwounded dermal fibroblasts (Figure 4.10A, D-F, One-way ANOVA with Tukey's multiple comparisons test, ns). Taken together, these data suggest that Peptan P may not activate the integrin signalling pathway in order to promote dermal fibroblast proliferation.

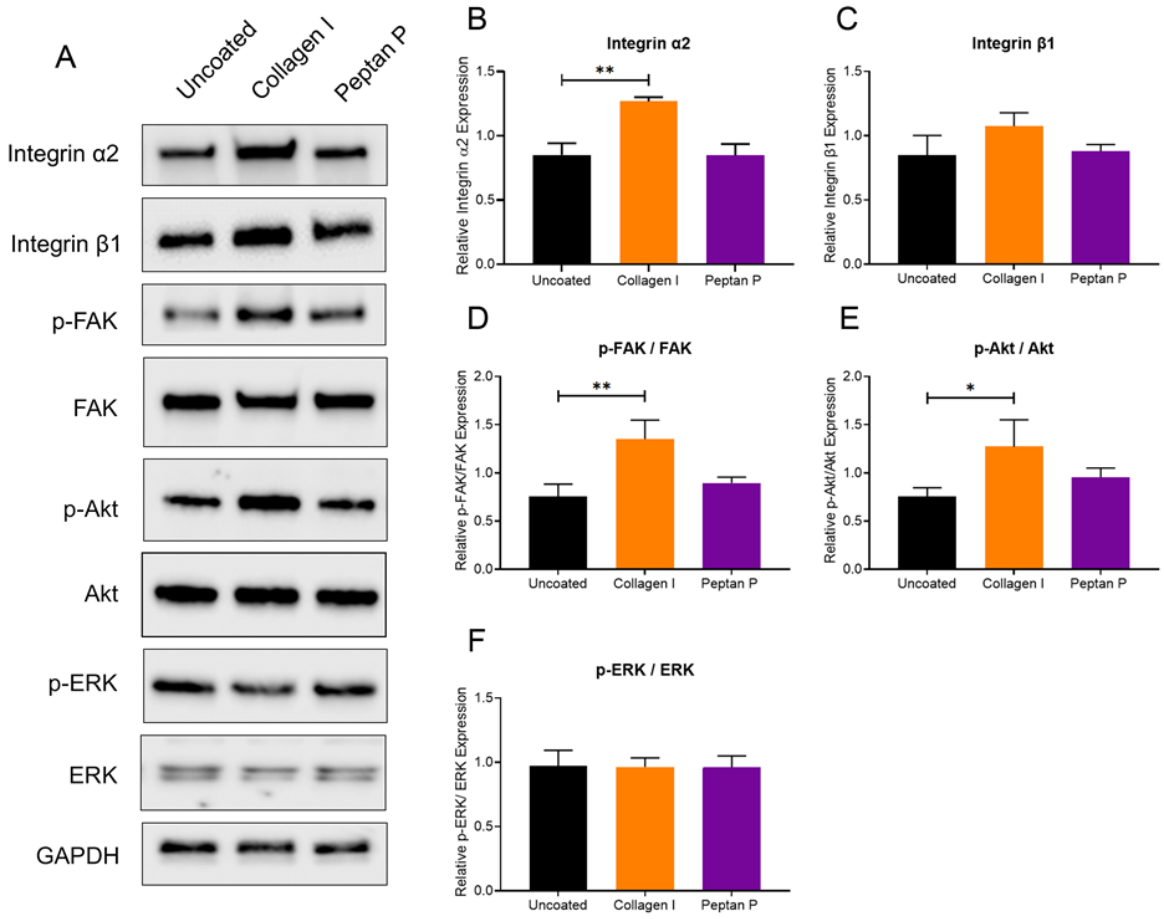

Figure 4. 10 P does not significantly promote the activation of FAK, Akt or ERK in unwounded dermal fibroblasts. *(A) Representative Western blot for integrin α2 (130kDa), integrin β1 (130kDa), p-FAK (110kDa), FAK (110kDa), p-Akt (60kDa), Akt (60kDa), p-ERK (42-44kDa), ERK (42-44kDa) and GAPDH (37kDa) expression in dermal fibroblasts seeded onto uncoated wells or wells pre-coated with 1mg/ml rat tail collagen I or Peptan P. Densitometric expression of (B) integrin α2, (C) integrin β1, (D) p-FAK/FAK, (E) p-Akt/Akt or (F) p-ERK/ERK expression relative to GAPDH expression in dermal fibroblasts seeded onto uncoated wells or wells pre-coated with 1mg/ml rat tail collagen I or Peptan P (mean ± SD, N=3, *P<0.05, **P<0.01).*

4.2.10 Peptan P does not significantly activate FAK, Akt or ERK signalling in wounded keratinocytes

Given that Peptan P was able to activate Akt in unwounded keratinocytes, further experiments were conducted to determine whether Peptan P activated Akt via the integrin signalling pathway in wounded keratinocytes. Primary keratinocytes were seeded onto uncoated wells or wells pre-coated with 1mg/ml Peptan P to form a confluent monolayer before scratch wound induction and harvesting of cell protein at 0, 24, 48 and 72 hours post-wounding. However, Peptan P did not significantly increase expression of integrin α2 or integrin β1 at 0, 24, 48 and 72 hours post-wounding (Figure 4.11A-C, One-way ANOVA with Tukey's multiple comparisons test, ns). Results also revealed that Peptan P did not significantly activate FAK, Akt or ERK in wounded keratinocytes at either 0, 24, 48 or 72 hours post-wounding; however, there was a trend for increased activation of both FAK and ERK by Peptan P compared to the control from 24 hours post-wounding onwards (Figure 4.11A, D and F, One-way ANOVA with Tukey's multiple comparisons test, ns). Taken together, these data suggests that Peptan P unlikely interacts with integrin receptors to promote keratinocyte wound closure.

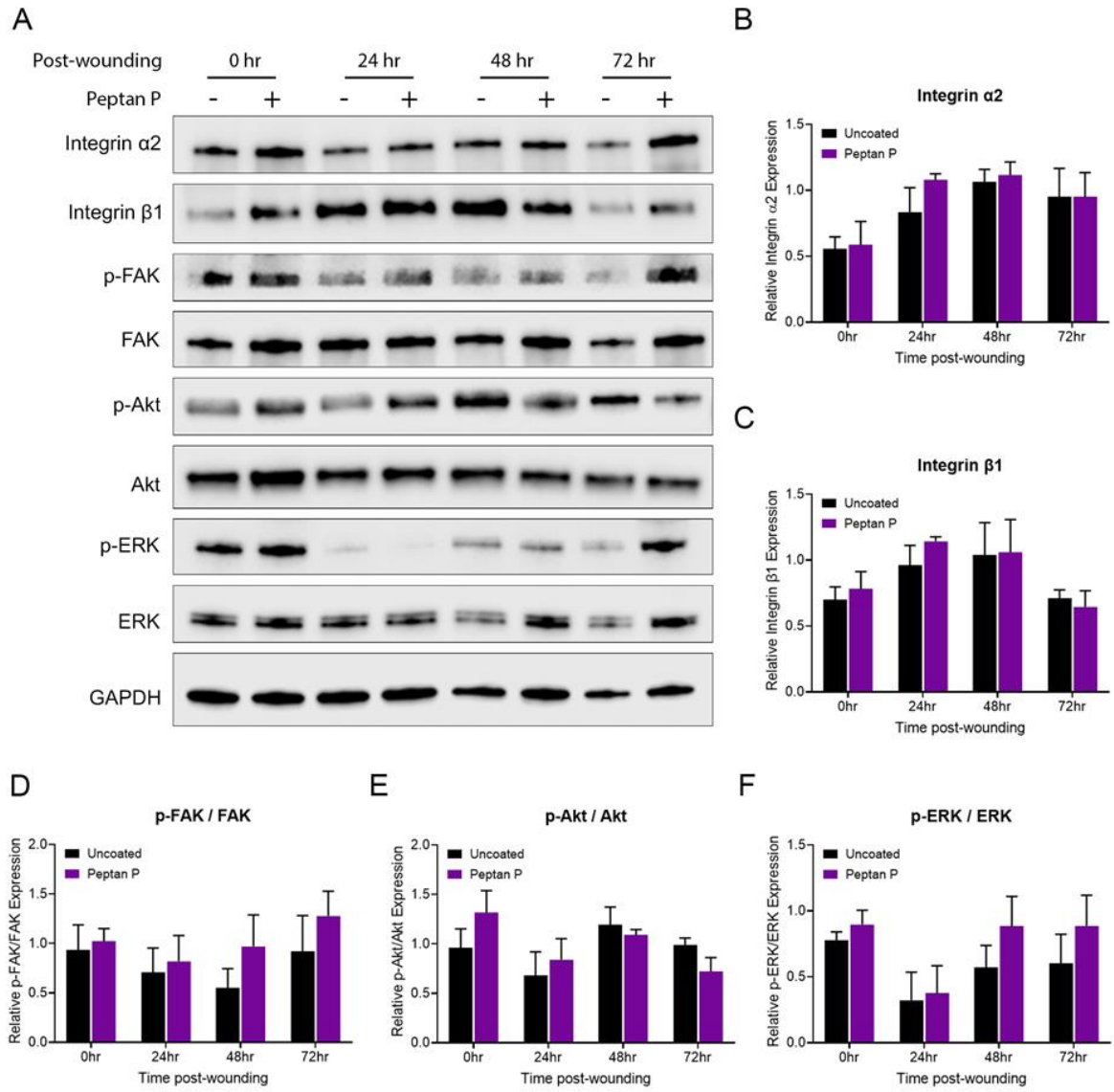

Figure 4. 11 Peptan P does not significantly activate FAK, Akt or ERK in wounded keratinocytes. *(A) Representative Western blot for integrin α2 (130kDa), integrin β1 (130kDa), p-FAK (110kDa), FAK (110kDa), p-Akt (60kDa), Akt (60kDa), p-ERK (42-44kDa), ERK (42-44kDa) and GAPDH (37kDa) expression in wounded keratinocytes taken at 0, 24, 48 and 72 hours post-wounding in the presence or absence of 1mg/ml Peptan P. Densitometric expression of (B) integrin α2, (C) integrin β1, (D) p-FAK/FAK, (E) p-Akt/Akt or (F) p-ERK/ERK expression relative to GAPDH expression in wounded keratinocytes taken at 0, 24, 48 and 72 hours post-wounding the presence or absence of 1mg/ml Peptan P (mean ± SD, N=3).*

4.2.11 Peptan P significantly activates FAK and ERK signalling in wounded dermal fibroblasts

To determine whether Peptan P promoted wound closure of dermal fibroblasts through the activation of the integrin signalling pathway, dermal fibroblasts were seeded on uncoated wells or wells pre-coated with 1mg/ml Peptan P to form a confluent monolayer, prior to scratch wound induction and harvesting of cell protein at 0, 24, 48 and 72 hours post-wounding. Results demonstrated that treatment with Peptan P significantly enhanced activation of FAK at 0 and 72 hours post-wounding compared to uncoated dermal fibroblasts (Figure 4.12A and D, One-way ANOVA with Tukey's multiple comparisons test, *P<0.05), whilst also showing a trend wise increase in ERK activation at 0 and 24 hours post-wounding. However, Peptan P-induced phosphorylation of ERK was only significant at 48 hours post-wounding compared to uncoated dermal fibroblasts (Figure 4.12A and F, One-way ANOVA with Tukey's multiple comparisons test, *P<0.05). In addition, Peptan P did not significantly increase expression of integrin α2 or integrin β1 or enhance activation of Akt at any of the timepoints post-wounding (Figure 4.12A-C and E, One-way ANOVA with Tukey's multiple comparisons test, ns). Taken together, these data suggest that Peptan P may promote dermal fibroblast wound closure through the activation of a FAK-ERK signalling cascade.

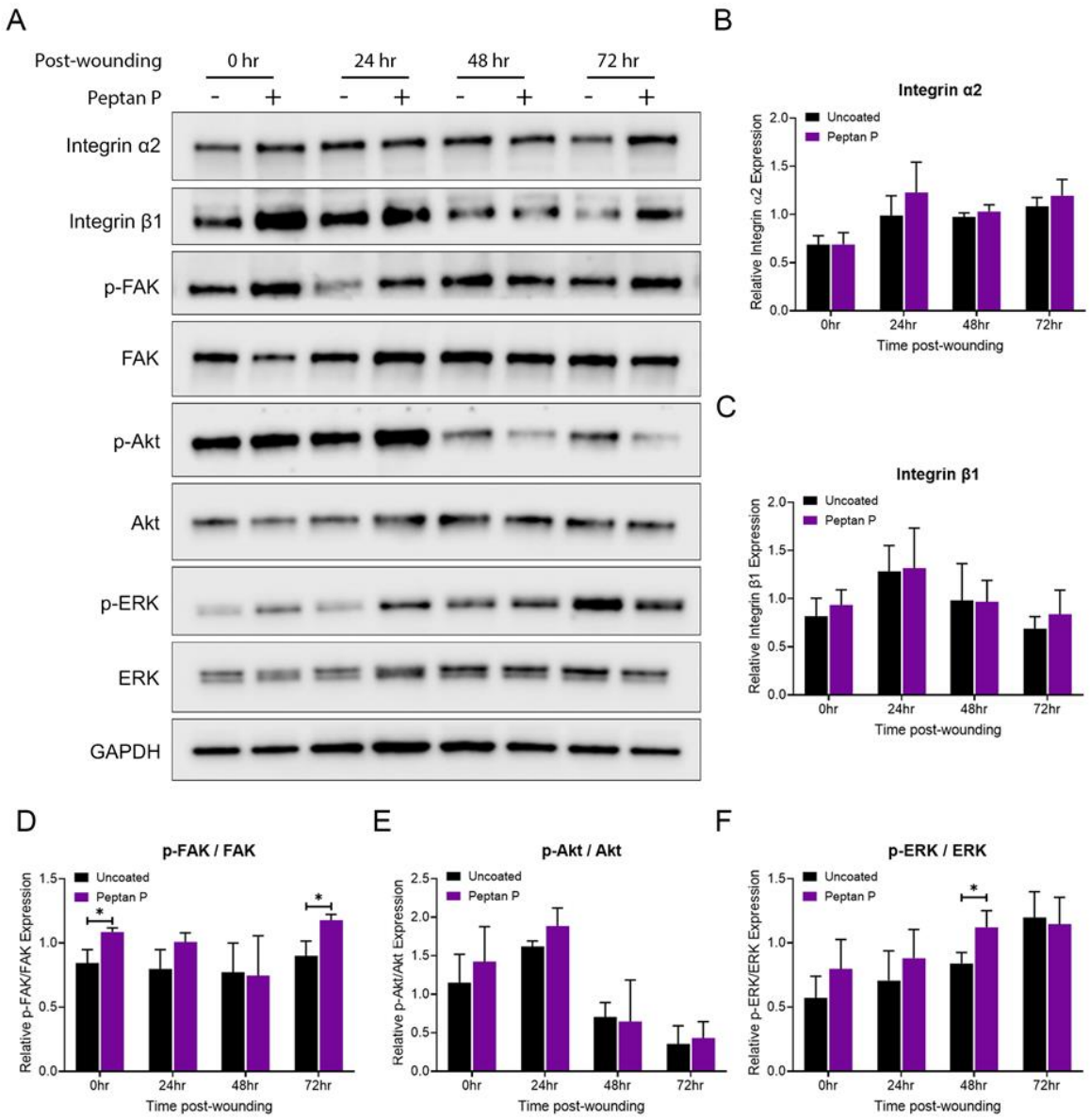

Figure 4. 12 Peptan P significantly activates FAK and ERK in wounded dermal fibroblasts. *(A) Representative Western blot for integrin α2 (130kDa), integrin β1 (130kDa), p-FAK (110kDa), FAK (110kDa), p-Akt (60kDa), Akt (60kDa), p-ERK (42-44kDa), ERK (42-44kDa) and GAPDH (37kDa) expression in wounded dermal fibroblasts taken at 0, 24, 48 and 72 hours post-wounding in the presence or absence of 1mg/ml Peptan P. Densitometric expression of (B) integrin α2, (C) integrin β1, (D) p-FAK/FAK, (E) p-Akt/Akt or (F) p-ERK/ERK expression relative to GAPDH expression in wounded dermal fibroblasts taken at 0, 24, 48 and 72 hours post-wounding the presence or absence of 1mg/ml Peptan P (mean ± SD, N=3, *P<0.05).*

4.2.12 Knockdown of integrin β1 significantly decreases integrin β1 expression in both keratinocytes and dermal fibroblasts

In order to confirm that the transient knockdown of the integrin β1 subunit using *ITGB1* siRNA is stable over 72 hours, both primary keratinocytes and dermal fibroblasts were seeded into uncoated wells prior to treatment with either ITGB1 siRNA or siCtrl and harvesting of cells at either 24, 48 or 72 hours post-treatment. Results confirmed that treatment with *ITGB1* siRNA significantly decreased integrin β1 expression in both primary keratinocytes and dermal fibroblasts at 24, 48 and 72 hours post-treatment (Figure 4.13, One-way ANOVA with Tukey's multiple comparisons test, *P<0.05, **P<0.01, ****P<0.0001). Taken together, these results demonstrate a stable transient knockdown of the integrin β1 subunit in both keratinocytes and dermal fibroblasts over a 72 hour period.

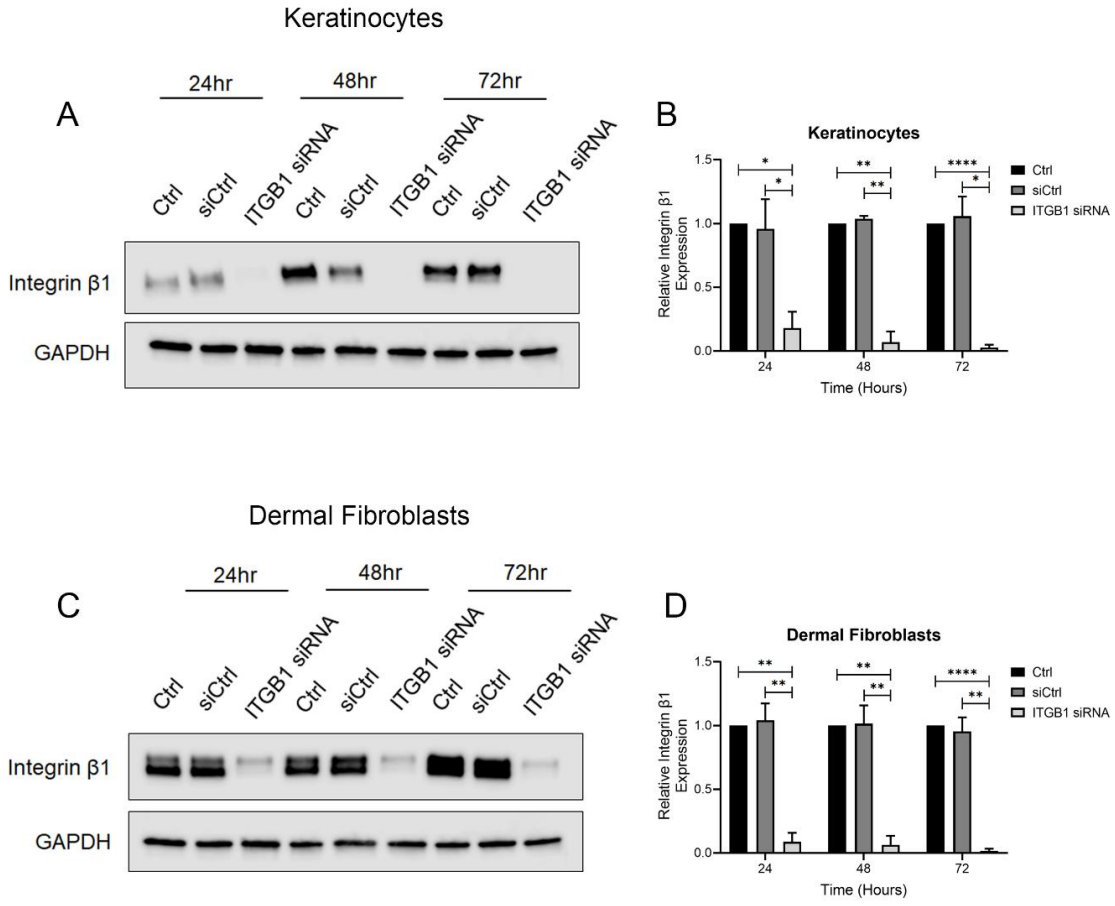

Figure 4. 13 Knockdown of integrin β1 significantly decreases integrin β1 expression in both keratinocytes and dermal fibroblasts. *(A) Representative Western blot for integrin β1 (130kDa) and GAPDH (37kDa) expression in primary keratinocytes taken at 24, 48 and 72 hours post-treatment with ITGB1 siRNA. (B) Densitometric expression of integrin β1 expression relative to GAPDH expression in primary keratinocytes taken at 24, 48 and 72 hours post-treatment with ITGB1 siRNA (mean ± SD, N=3, *P<0.05, **P<0.01, ****P<0.0001). (C) Representative Western blot for integrin β1 (130kDa) and GAPDH (37kDa) expression in primary dermal fibroblasts taken at 24, 48 and 72 hours post-treatment with ITGB1 siRNA. (D) Densitometric expression of integrin β1 expression relative to GAPDH expression in primary dermal fibroblasts taken at 24, 48 and 72 hours post-treatment with ITGB1 siRNA (mean ± SD, N=3, **P<0.01, ****P<0.0001).*

4.2.13 Knockdown of integrin β1 significantly decreases Peptan P-induced wound closure of keratinocytes

To determine whether Peptan P interacts with integrin receptors in order to promote keratinocyte wound closure, primary keratinocytes were seeded onto uncoated wells or wells pre-coated with 1mg/ml Peptan P prior to using *ITGB1* siRNA to knockdown expression of the integrin β1 subunit, scratch wound induction and monitoring of wound closure over 72 hours. Results demonstrated that Peptan P significantly enhanced the wound closure rate of keratinocytes compared to the rate observed in keratinocytes seeded and wounded in the uncoated Ctrl wells (Figure 4.14, One-way ANOVA with Tukey's multiple comparisons test, *P<0.05). However, whilst there was an observable trend for increased rate of wound closure by keratinocytes treated with both siCtrl and Peptan P, there was no significant difference in the rate of wound closure between keratinocytes subjected to both siCtrl and Peptan P and keratinocytes treated with siCtrl alone (Figure 4.14 One-way ANOVA with Tukey's multiple comparisons test, ns). Conversely, results demonstrated knockdown of the integrin β1 subunit significantly impaired the wound closure of keratinocytes in the presence or absence of treatment with Peptan P (Figure 4.14, One-way ANOVA with Tukey's multiple comparisons test, **P<0.01, ***P<0.001, ****P<0.0001). Taken together, these data suggest that the knockdown of the integrin β1 subunit impairs the ability of Peptan P to interact with integrin receptors in order to enhance keratinocyte wound closure.

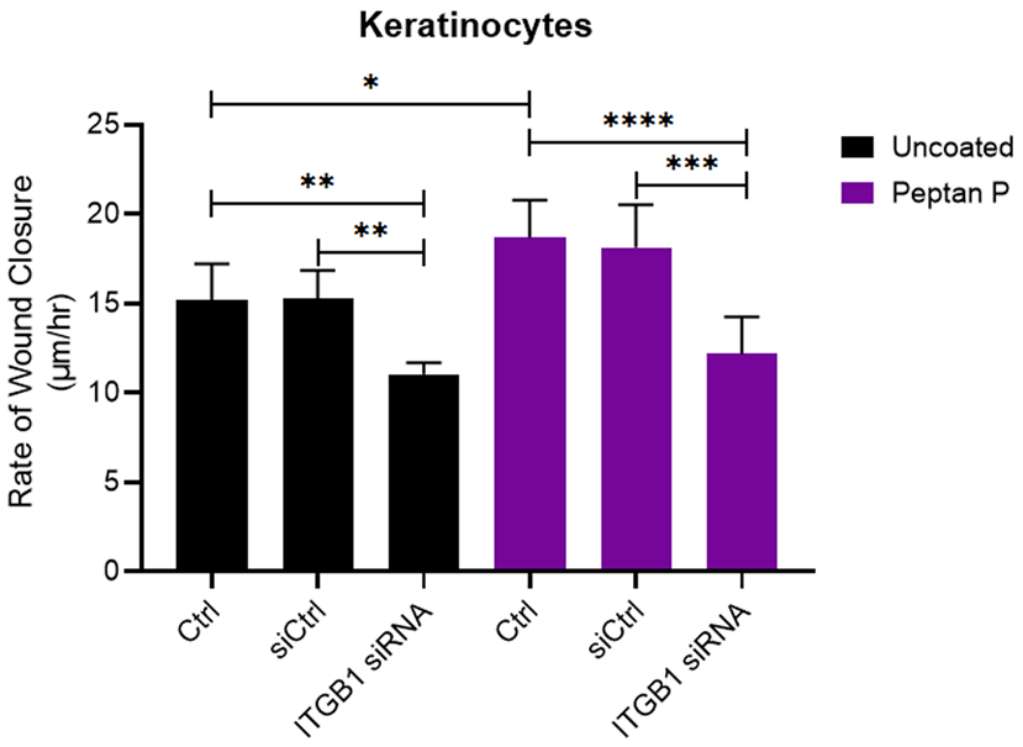

Figure 4. 14 Knockdown of integrin β1 subunit significantly decreases Peptan P-induced wound closure of primary keratinocytes. *Primary keratinocytes (<35 years old) were seeded onto either uncoated wells or wells pre-coated with 1mg/ml Peptan P before being treated with ITGB1 siRNA or a non-target siRNA (siCtrl), scratch wound induction and monitoring of wound closure over 72 hours (mean ± SD, n=9, N=3, *P<,0.05, **P<0.01, ***P<0.001, ****P<0.0001).*

4.2.14 Knockdown of integrin β1 significantly decreases Peptan P-induced wound closure of dermal fibroblasts

To determine whether Peptan P interacts with integrin receptors in order to promote dermal fibroblast wound closure, primary dermal fibroblasts were seeded onto uncoated wells or wells pre-coated with 1mg/ml Peptan P prior to using *ITGB1* siRNA to knockdown expression of the integrin β1 subunit, scratch wound induction and monitoring of wound closure over 72 hours. Results again, demonstrated that Peptan P significantly enhanced wound closure of dermal fibroblasts of both Ctrl and siCtrl conditions (Figure 4.15, One-way ANOVA with Tukey's multiple comparisons test, ***P<0.001, ****P<0.0001). As with keratinocytes, the knockdown of the integrin β1 subunit significantly impaired wound closure of dermal fibroblasts in the presence or absence of treatment with Peptan P (Figure 4.15, One-way ANOVA with Tukey's multiple comparisons test, ***P<0.001. ****P<0.0001) Collectively, these results suggest that again knockdown of integrin β1 impairs the

ability for Peptan P to interact with integrin receptors to enhance dermal fibroblast wound closure.

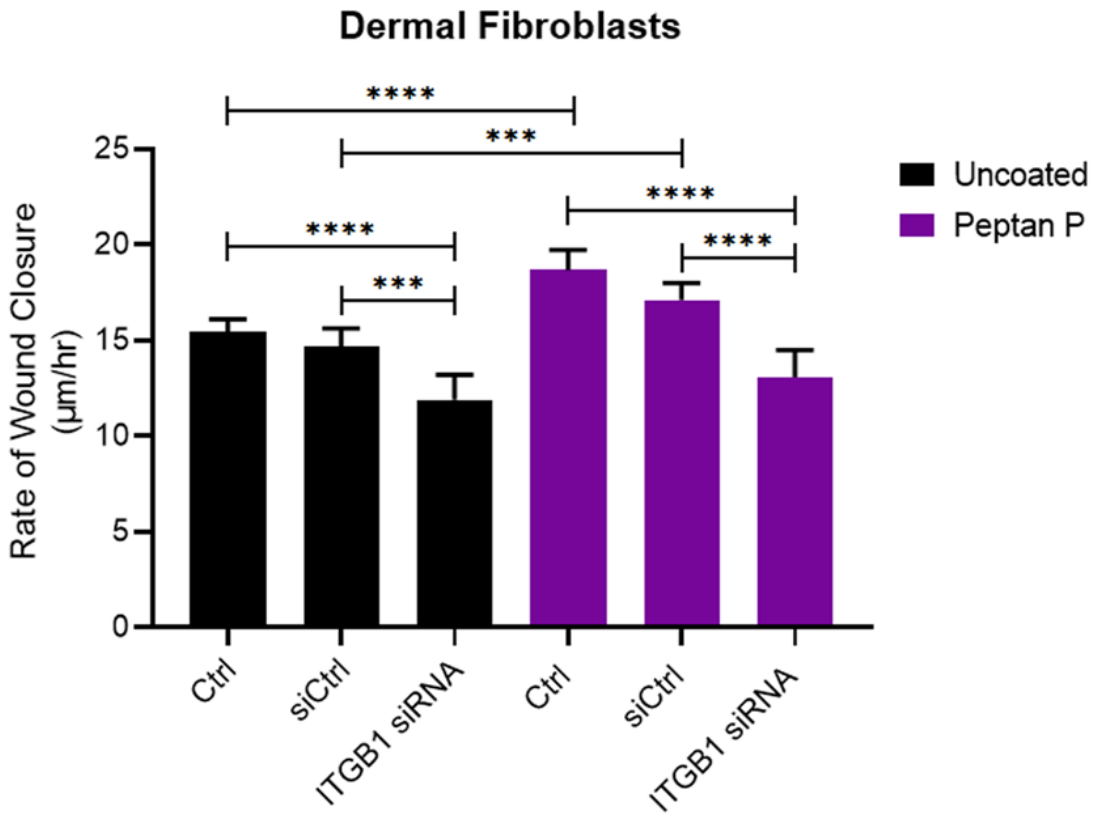

Figure 4. 15 Knockdown of integrin β1 subunit significantly decreases Peptan P-induced wound closure of primary dermal fibroblasts. *Primary dermal fibroblasts (<35 years old) were seeded onto either uncoated wells or wells pre-coated with 1mg/ml Peptan P before being treated with ITGB1 siRNA or a non-target siRNA (siCtrl), scratch wound induction and monitoring of wound closure over 72 hours (mean ± SD, n=9, N=3, ***P<0.001, ****P<0.0001).*

4.2.15 Knockdown of integrin β1 significantly inhibits Peptan P-induced expression of integrin α2 and activation of FAK in unwounded keratinocytes

To confirm whether Peptan P interacts with integrin receptors in order to activate downstream signalling pathways associated with cellular proliferation in keratinocytes, primary keratinocytes were seeded onto either uncoated wells or wells pre-coated with 1mg/ml rat tail collagen I or Peptan P prior to using *ITGB1* siRNA to knockdown expression of the integrin β1 subunit and continued incubation for 72 hours. Results demonstrated that both collagen I and Peptan P significantly increased expression of the integrin β1 subunit compared to the uncoated control

(Figure 4.16C, Two-way ANOVA with Tukey's multiple comparisons test, *P<0.05). Furthermore, knockdown of the integrin β1 subunit resulted in a significant decrease expression in integrin β1 expression in uncoated keratinocytes as well as keratinocytes exposed to collagen I or Peptan P (Figure 4.16A and C, Two-way ANOVA with Tukey's comparisons test, **P<0.01, ***P<0.001, ****P<0.0001). Additionally, knockdown of the integrin β1 subunit significantly decreased expression of integrin α2 in both collagen I and Peptan P-treated keratinocytes (Figure 4.16A and B. Two-way ANOVA with Tukey's multiple comparisons test, *P<0.05, **P<0.01). The knockdown of the integrin β1 subunit also significantly decreased the activation of FAK in both collagen I and Peptan P-treated keratinocytes (Figure 4.16A and D, Two-way ANOVA with Tukey's multiple comparisons test, *P<0.05, **P<0.01, ***P<0.001). However, knockdown of the integrin β1 subunit did not result in the significant decrease of Akt or ERK activation in uncoated, collagen I or Peptan P-treated keratinocytes (Figure 4.16A, E and F, Two-way ANOVA with Tukey's multiple comparisons test, ns). Collectively, these data suggests that Peptan P interacts with the integrin α2β1 receptor to activate FAK in unwounded keratinocytes.

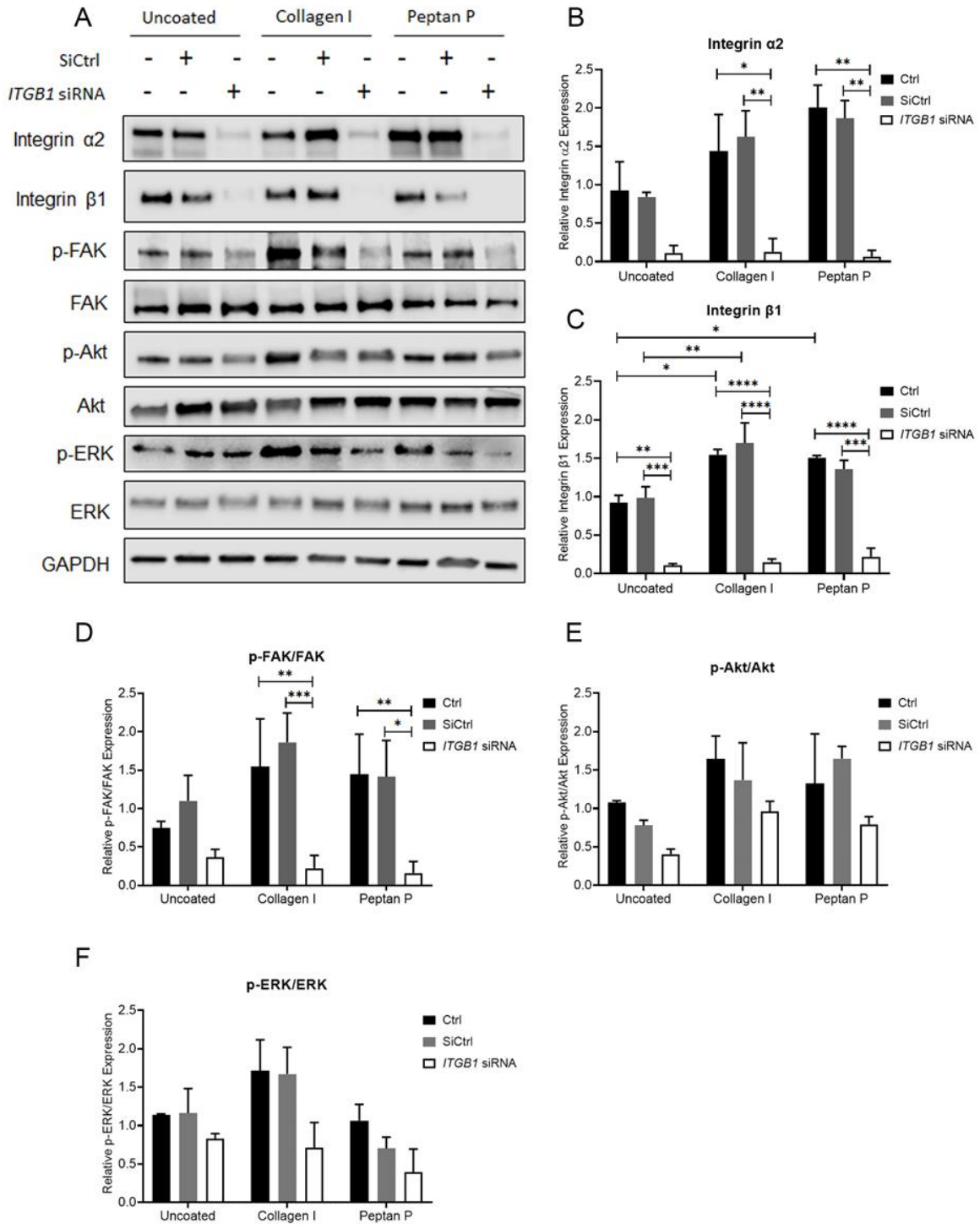

Figure 4. 16 Knockdown of integrin β1 significantly inhibits expression of integrin α2 and activation of FAK in unwounded keratinocytes. *(A) Representative Western blot for integrin α2 (130kDa), integrin β1 (130kDa), p-FAK (110kDa), FAK (110kDa), p-Akt (60kDa), Akt (60kDa), p-ERK (42-44kDa), ERK (42-44kDa) and GAPDH (37kDa) expression for keratinocytes seeded on uncoated wells or wells pre-coated with 1mg/ml collagen I or Peptan P following treatment with either SiCtrl or ITGB1 siRNA. Densitometric expression of (B) integrin α2, (C) integrin β1, (D) p-FAK/FAK, (E) p-Akt/Akt or (F) p-ERK/ERK expression relative to GAPDH expression in keratinocytes seeded on uncoated wells or wells pre-coated with 1mg/ml collagen I or Peptan P following treatment with either SiCtrl or ITGB1 siRNA (mean ± SD, N=3, *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001).*

4.2.16 Knockdown of integrin β1 significantly inhibits Peptan P-induced expression of integrin α2 and activation of FAK and ERK in unwounded dermal fibroblasts

To confirm whether Peptan P also interacts with integrin receptors in order to activate downstream signalling pathways associated with proliferation in dermal fibroblasts, primary dermal fibroblasts were seeded onto either uncoated wells or wells pre-coated with 1mg/ml rat tail collagen I or Peptan P prior to using *ITGB1* siRNA to knockdown expression of the integrin β1 subunit and continued incubation for 72 hours. Results confirmed that knockdown of the integrin β1 subunit significantly decreased integrin β1 expression in uncoated, collagen I and Peptan P-treated dermal fibroblasts (Figure 4.17A and C, Two-way ANOVA with Tukey's multiple comparisons test, ***P<0.001, ****P<0.0001). Results also revealed that collagen I and Peptan P significantly increased integrin α2 expression in unwounded dermal fibroblasts compared to the uncoated dermal fibroblasts (Figure 4.17A and B, Two-way ANOVA with Tukey's multiple comparisons test, *P<0.05, **P<0.01). Furthermore, as seen in studies using unwounded keratinocytes, knockdown of the integrin β1 subunit significantly diminished integrin α2 expression in uncoated, collagen I or Peptan P-treated dermal fibroblasts (Figure 4.17A and B, Two-way ANOVA with Tukey's multiple comparisons test, *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001). Additionally, knockdown of integrin β1 resulted in the significant inhibited FAK activation by both collagen I and Peptan P as well as inhibiting the activation of Akt in collagen I-treated cells (Figure 4.17A, D and E, Two-way ANOVA with Tukey's multiple comparisons test, *P<0.05). Finally, knockdown of the integrin β1 subunit significantly inhibited ERK activation in uncoated, collagen I and Peptan P-treated dermal fibroblasts (Figure 4.17A and F, Two-way ANOVA with Tukey's multiple comparisons test, *P<0.05, **P<0.01, ***P<0.001). Taken together, these data suggest that Peptan P interacts with the integrin α2β1 receptor to activate both FAK and ERK in unwounded dermal fibroblasts.

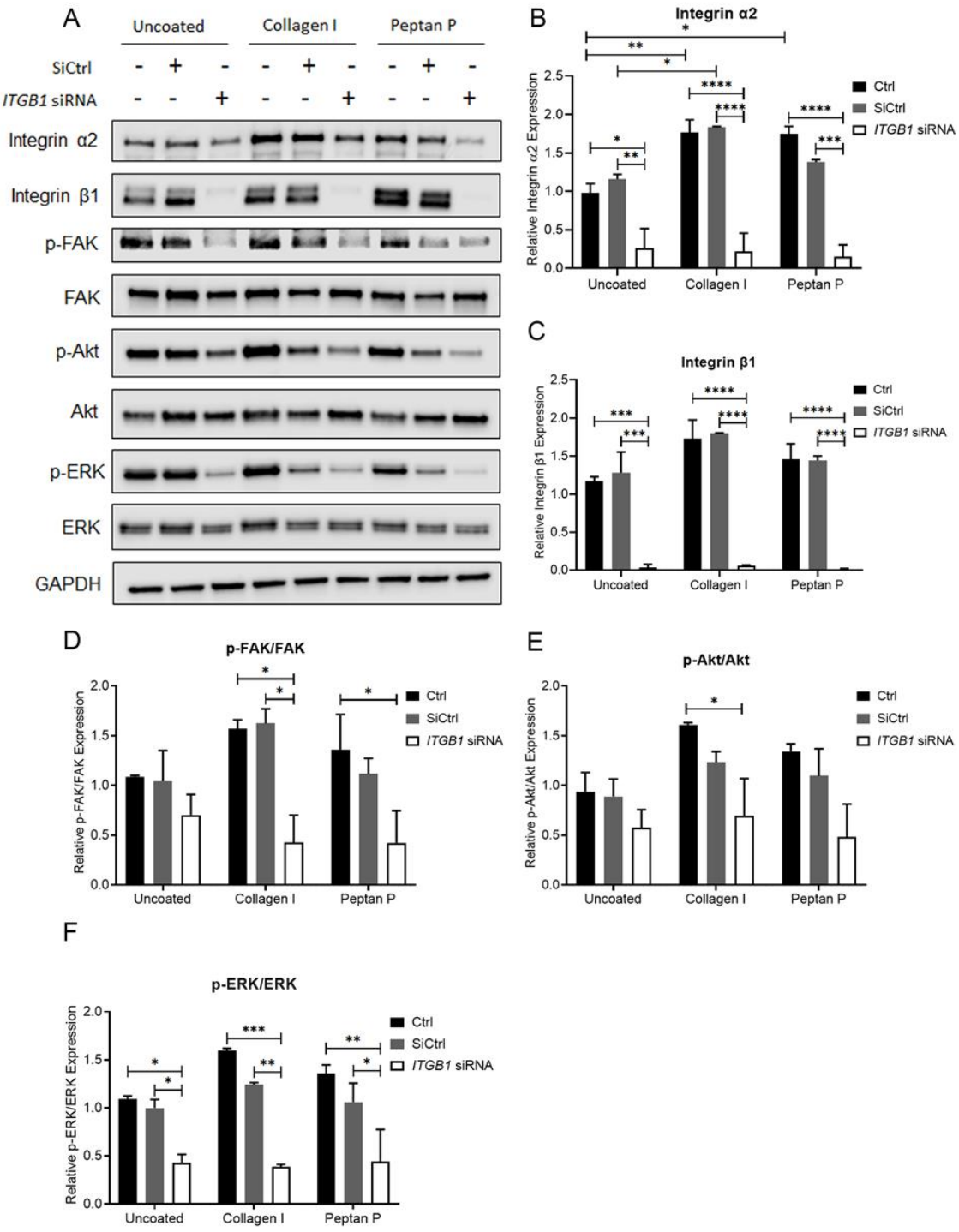

Figure 4. 17 Knockdown of integrin β1 significantly inhibits expression of integrin α2 and activation of FAK and ERK in unwounded dermal fibroblasts. *(A) Representative Western blot for integrin α2 (130kDa), integrin β1 (130kDa), p-FAK (110kDa), FAK (110kDa), p-Akt (60kDa), Akt (60kDa), p-ERK (42-44kDa), ERK (42-44kDa) and GAPDH (37kDa) expression for dermal fibroblasts seeded on uncoated wells or wells pre-coated with 1mg/ml collagen I or Peptan P following treatment with either SiCtrl or ITGB1 siRNA. Densitometric expression of (B) integrin α2, (C) integrin β1, (D) p-FAK/FAK, (E) p-Akt/Akt or (F) p-ERK/ERK expression relative to GAPDH expression in dermal fibroblasts seeded on uncoated wells or wells pre-coated with 1mg/ml collagen I or Peptan P following treatment with either SiCtrl or ITGB1 siRNA (mean ± SD, N=3, *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001).*

4.2.17 Knockdown of integrin β1 significantly decreases expression of integrin α2 and activation of FAK and ERK in wounded keratinocytes

To confirm the interaction of Peptan P with the integrin α2β1 receptor to activate downstream signalling pathways associated with cellular proliferation in wounded keratinocytes, studies were also performed in primary keratinocytes subjected to transient knockdown of *ITGB1* prior to scratch-wound induction and harvesting of protein at 24, 48 and 72 hours post-wounding. Results confirmed that knockdown of the integrin β1 subunit using *ITGB1* siRNA significantly decreased both uncoated and Peptan P-induced expression of integrin β1 by keratinocytes at 24, 48 and 72 hours post-wounding (Figure 4.18 and Figure 4.19B, Two-way ANOVA with Tukey's multiple comparisons test, *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001). Additionally, knockdown of the integrin β1 subunit also significantly decreased integrin α2 expression in both uncoated and Peptan P-treated cells at 24, 48 and 72 hours post-wounding (Figure 4.18 and Figure 4.19A, Two-way ANOVA with Tukey's multiple comparisons test, *P<0.05, **P<0.01, ***P<0.001). Furthermore, the knockdown of the integrin β1 subunit significantly inhibited Peptan P-induced activation of FAK in wounded keratinocytes at both 48 and 72 hours post-wounding (Figure 4.18 and Figure 4.19C, Two-way ANOVA with Tukey's multiple comparisons test, *P<0.05, **P<0.01, ***P<0.001). However, knockdown of the integrin β1 subunit only significantly decreased Peptan P-induced activation of ERK at 24 hours post-wounding (Figure 4.18 and Figure 4.19E, Two-way ANOVA with Tukey's multiple comparisons test, *P<0.05, ***P<0.001) with no significant effect on Peptan P-induced activation of Akt at either 24, 48 or 72 hours post-wounding (Figure 4.18 and Figure 4.19D, Two-way ANOVA with Tukey's multiple comparisons test, ns). Collectively, these results confirm that Peptan P interacts with the integrin α2β1 receptor to activate FAK during keratinocyte wound closure (Figure 4.19F).

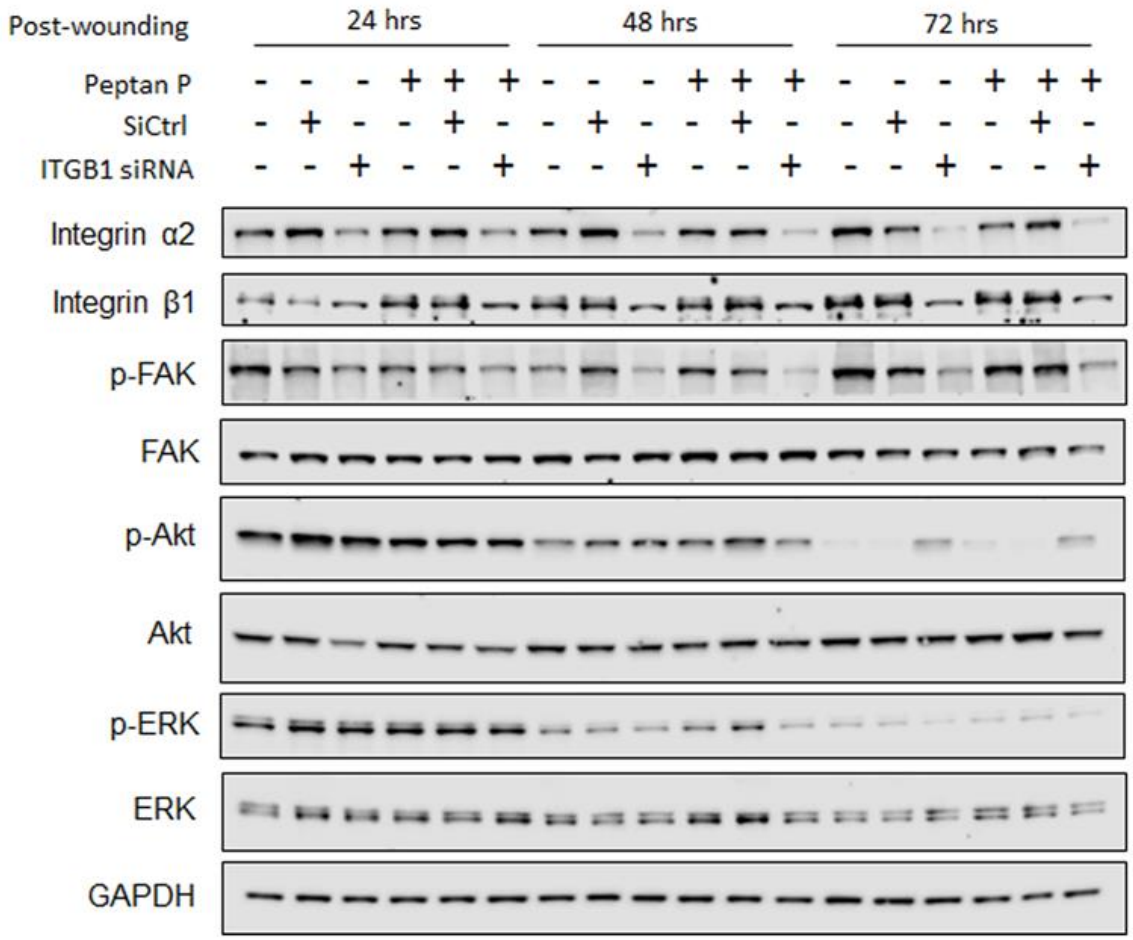

Figure 4. 18 Knockdown of integrin β1 significantly decreases expression of integrin α2 and activation of FAK and ERK in wounded keratinocytes. *(A) Representative Western blot for integrin α2 (130kDa), integrin β1 (130kDa), p-FAK (110kDa), FAK (110kDa), p-Akt (60kDa), Akt (60kDa), p-ERK (42-44kDa), ERK (42-44kDa) and GAPDH (37kDa) expression in wounded keratinocytes harvested at 24, 48 and 72 hours post-wounding in the presence or absence of 1mg/ml Peptan P following treatment with either SiCtrl or ITGB1 siRNA.*

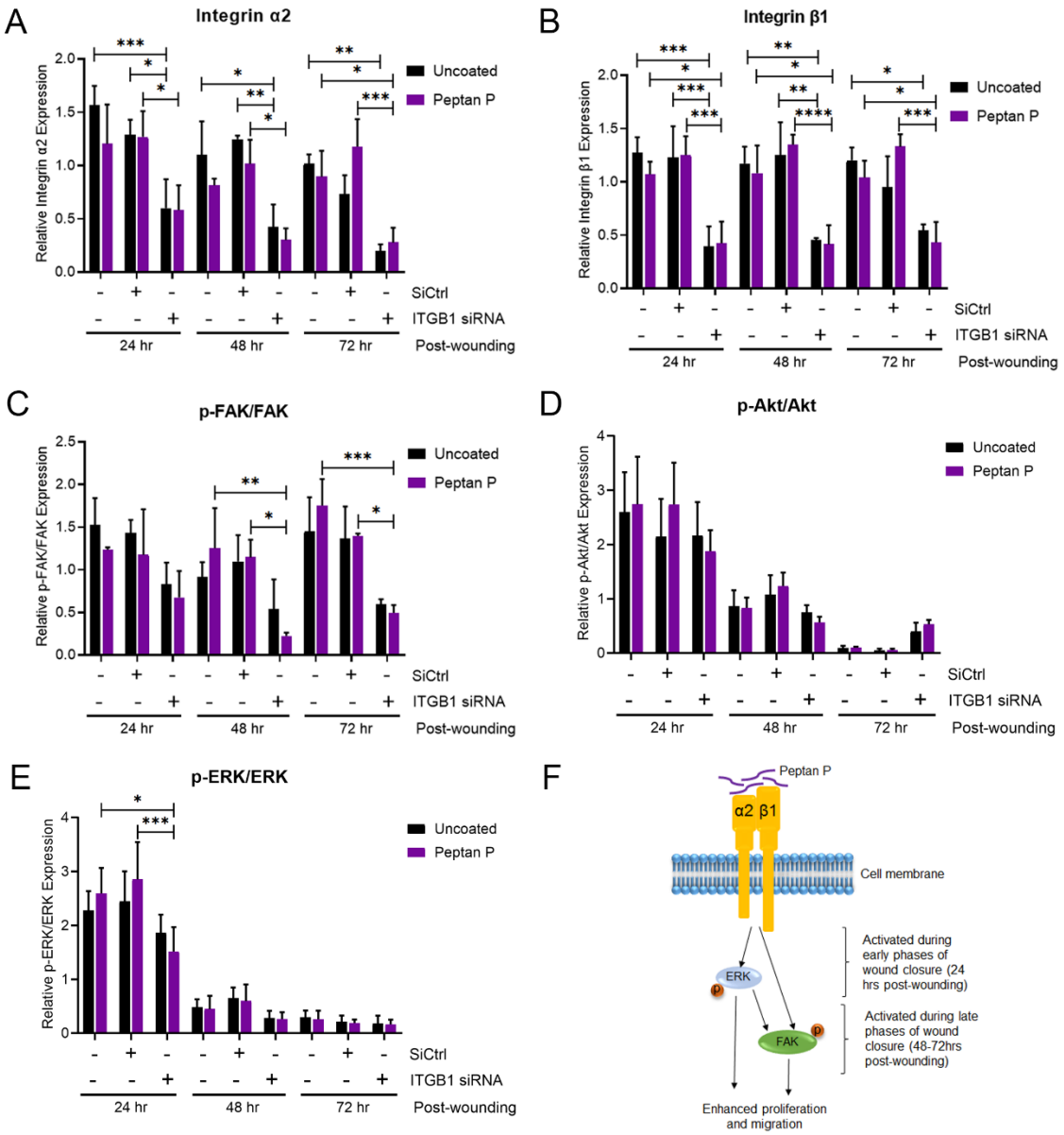

Figure 4. 19 Knockdown of integrin β1 significantly decreases expression of integrin α2 and activation of FAK and ERK in wounded keratinocytes. *Densitometric expression of (A) integrin α2, (B) integrin β1, (C) p-FAK/FAK, (D) p-Akt/Akt or (E) p-ERK/ERK expression relative to GAPDH expression in wounded keratinocytes harvested at 24, 48 and 72 hours post-wounding in the presence or absence of 1mg/ml Peptan P following treatment with either SiCtrl or ITGB1 siRNA (mean ± SD, N=3, *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001). (F) Schematic diagram showing potential downstream pathway activation following Peptan P binding to the integrin α2β1 receptor in order to promote keratinocyte wound closure*

4.2.18 Knockdown of integrin β1 significantly decreases expression of integrin α2 in wounded dermal fibroblasts

To confirm Peptan P interaction with the integrin α2β1 receptor to activate downstream signalling pathways associated with cellular proliferation in wounded dermal fibroblasts, primary dermal fibroblasts were subjected to transient knockdown of *ITGB1* prior to scratch-wound induction and harvesting of protein at 24, 48 and 72 hours post-wounding. Again, results confirmed that knockdown of the integrin β1 subunit using *ITGB1* siRNA significantly decreased both uncoated and Peptan P-induced expression of integrin β1 at 24, 48 and 72 hours post-wounding (Figure 4.20 and Figure 4.21B, Two-way ANOVA with Tukey's multiple comparisons test, *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001). Results also demonstrated that knockdown of the integrin β1 subunit significantly decreased Peptan P-induced expression of integrin α2 expression in dermal fibroblasts at 48 and 72 hours post-wounding (Figure 4.20 and Figure 4.21A, Two-way ANOVA with Tukey's multiple comparisons test, *P<0.05). However, whilst results demonstrated a trend wise decrease in Peptan P-induced activation of FAK following treatment with *ITGB1* siRNA at 24, 48 and 72 hours post-wounding, this effect was not significant (Figure 4.20 and Figure 4.21C, Two-way ANOVA with Tukey's multiple comparisons test, ns). Additionally, knockdown of the integrin β1 subunit did not significantly affect Peptan P-induced activation of either Akt or ERK in dermal fibroblasts at 24, 48 or 72 hours post-wounding (Figure 4.20 and Figure 4.21D and E, Two-way ANOVA with Tukey's multiple comparisons test, ns). Taken together, these data confirm that while Peptan P interacts with the integrin α2β1 receptor, other downstream signalling pathways are likely activated to promote dermal fibroblast wound closure (Figure 4.21F).

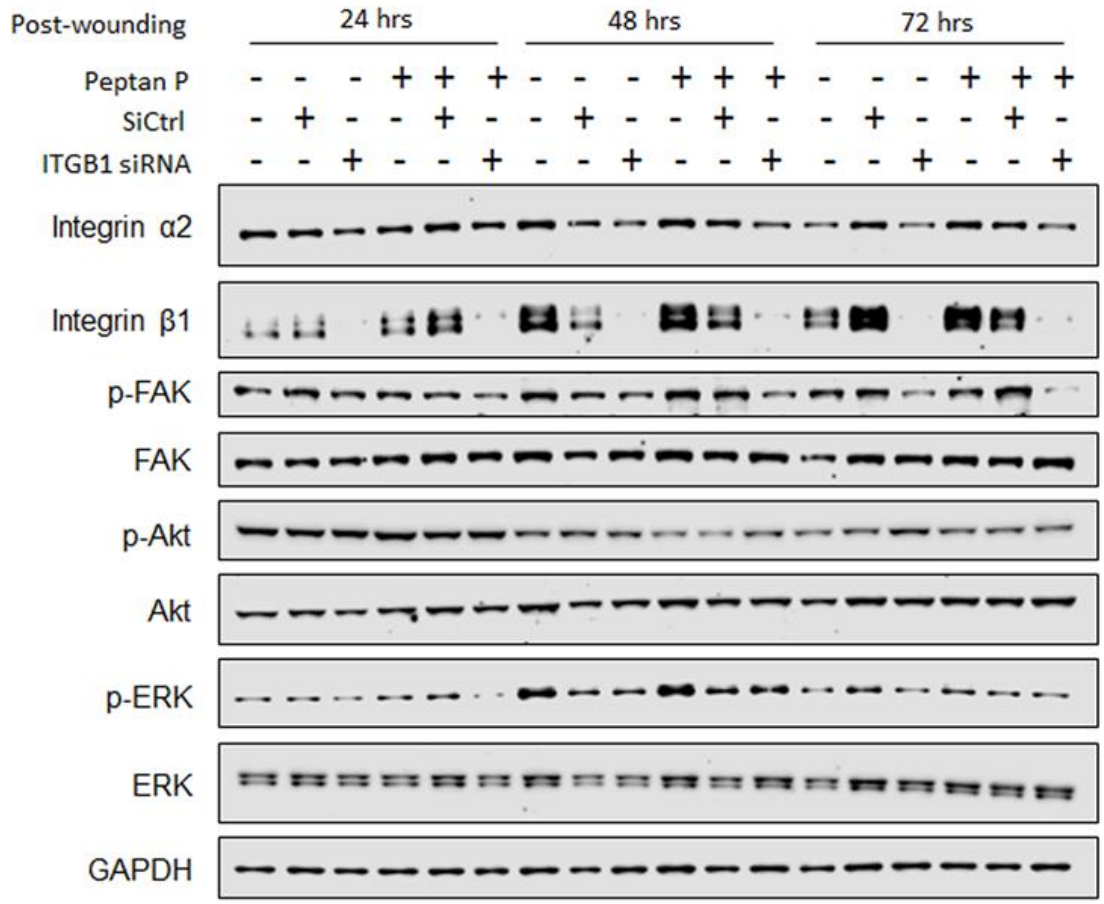

Figure 4. 20 Knockdown of integrin β1 significantly decreases expression of integrin α2 in wounded dermal fibroblasts. *(A) Representative Western blot for integrin α2 (130kDa), integrin β1 (130kDa), p-FAK (110kDa), FAK (110kDa), p-Akt (60kDa), Akt (60kDa), p-ERK (42-44kDa), ERK (42-44kDa) and GAPDH (37kDa) expression in wounded dermal fibroblasts harvested at 24, 48 and 72 hours post-wounding in the presence or absence of 1mg/ml Peptan P following treatment with either SiCtrl or ITGB1 siRNA.*

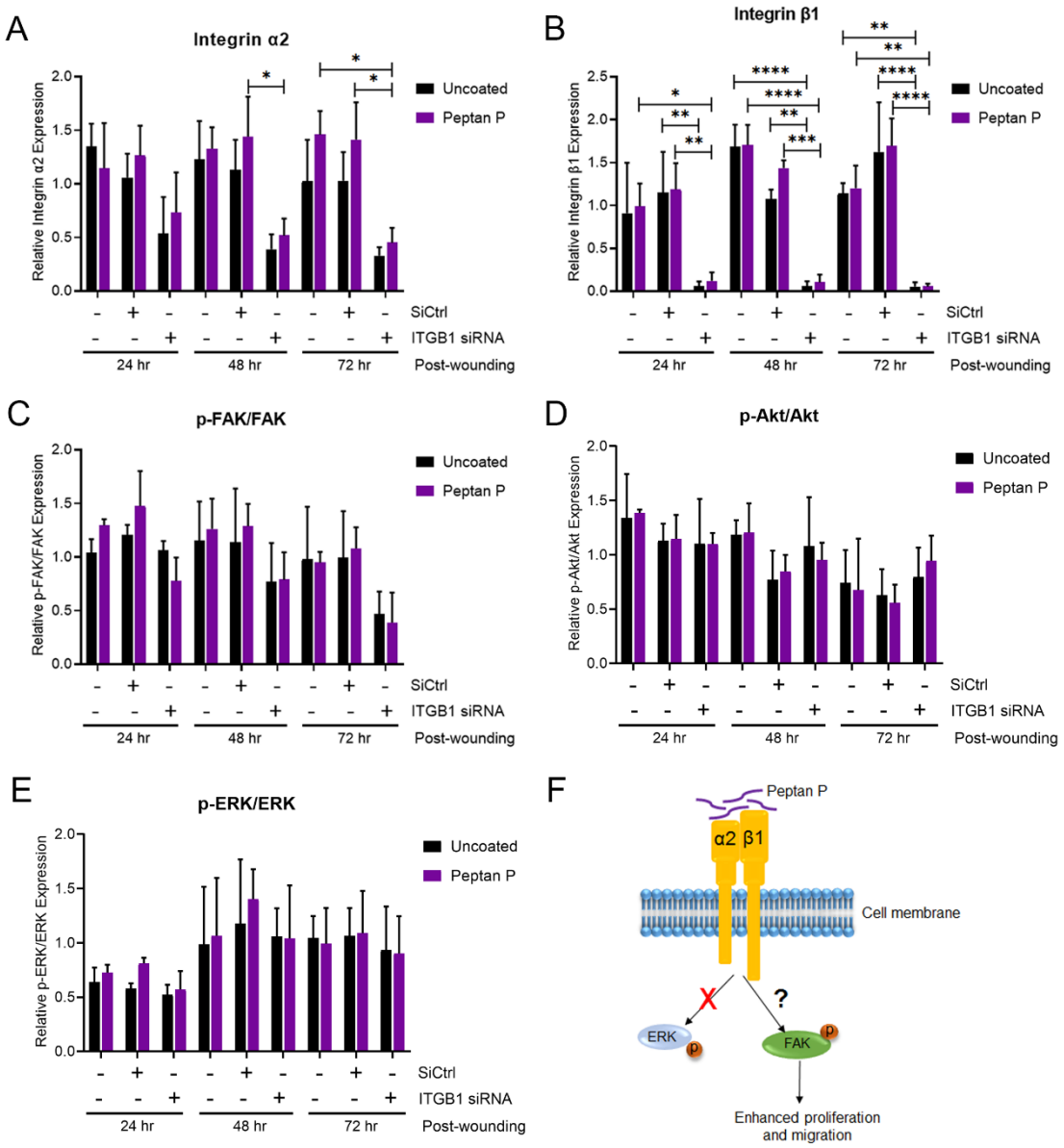

Figure 4. 21 Knockdown of integrin β1 significantly decreases expression of integrin α2 in wounded dermal fibroblasts. *Densitometric expression of (A) integrin α2, (B) integrin β1, (C) p-FAK/FAK, (D) p-Akt/Akt or (E) p-ERK/ERK expression relative to GAPDH expression in wounded dermal fibroblasts harvested at 24, 48 and 72 hours post-wounding in the presence or absence of 1mg/ml Peptan P following treatment with either SiCtrl or ITGB1 siRNA (mean ± SD, N=3, *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001). (F) Schematic diagram showing potential downstream pathway activation following Peptan P binding to the integrin α2β1 receptor in order to promote dermal fibroblast wound closure.*

4.2.19 Peptan P increases expression of CD49b, CD49e and CD29 in keratinocytes

Previous studies have suggested that the hydrolysis of native collagen to collagen peptides exposes motifs and binding sites that would typically be inaccessible to cells. Furthermore, previous studies have suggested that some of the exposed motifs in collagen peptides are capable of binding to other ECM receptors on cells and not just collagen receptors such as integrin α2β1 (Agrez *et al.*, 1991; Taubenberger *et al.*, 2010). To explore this potential, primary keratinocytes were treated in the presence or absence of 1mg/ml Peptan P for 72 hours before using flow cytometry to measure cell surface expression of different integrin subunits using their corresponding cluster of differentiation (CD) marker; CD49b (integrin α2), CD49c (integrin α3), CD49e (integrin α5), CD49f (integrin α6) and CD29 (integrin β1). Results demonstrated that Peptan P significantly increased cell surface expression of CD49b, CD49e and CD29 on keratinocytes (Figure 4.22, unpaired t-test, *P<0.05, **P<0.01). However, Peptan P did not significantly increase cell surface expression of CD49c and CD49f (Figure 4.22, unpaired t-test, ns). Taken together, these data demonstrate the ability for Peptan P to interact with integrin receptors, specifically integrin α2β1 and integrin α5β1 receptors on the cell surface of primary keratinocytes.

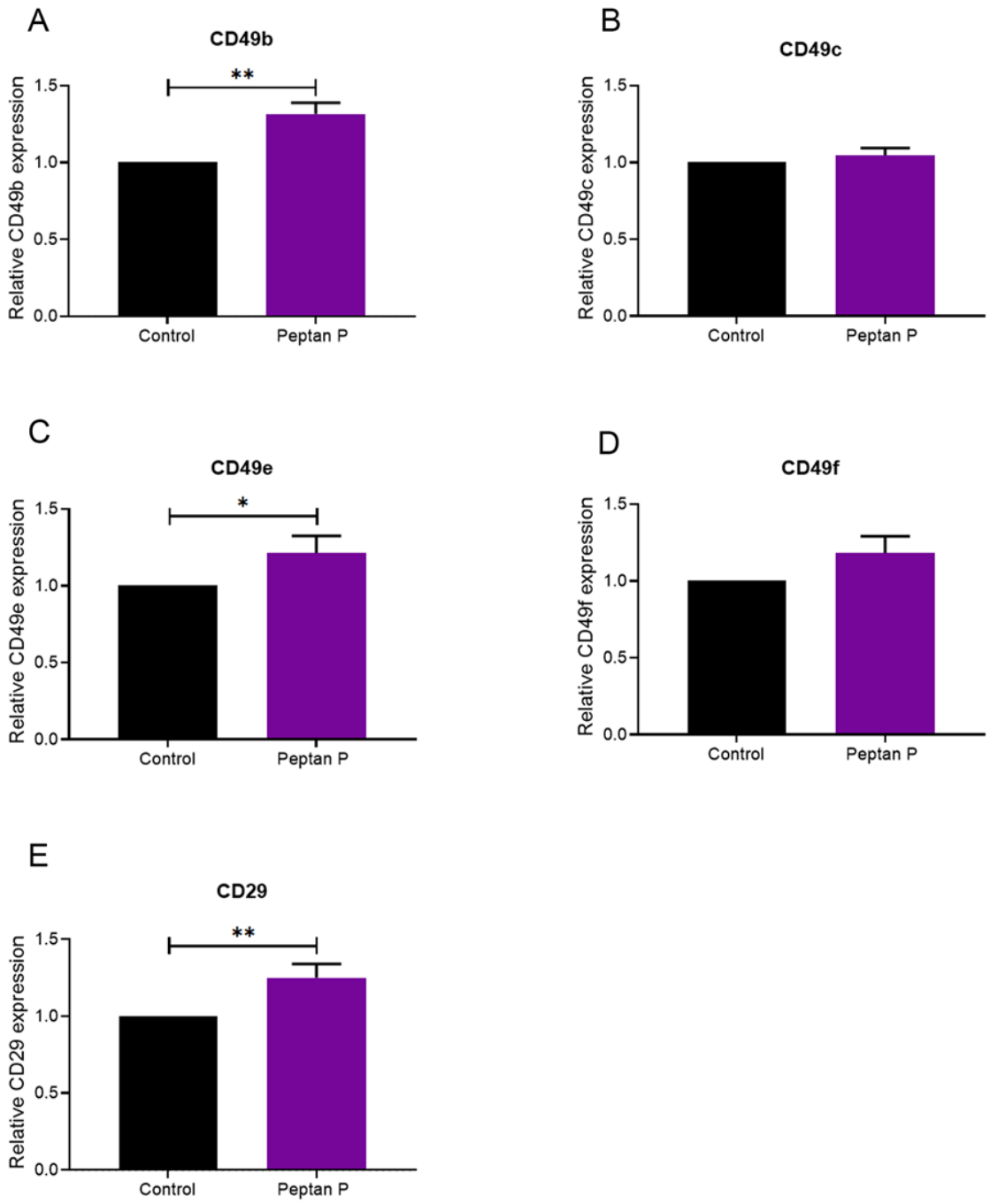

Figure 4. 22 Peptan P increases expression of CD49b, CD49e and CD29 in keratinocytes. *Relative cell surface expression of (A) CD49b, (B) CD49c, (C) CD49e, (D) CD49e and (E) CD29 compared to the control in primary keratinocytes treated in the presence or absence of 1mg/ml Peptan P for 72 hours before analysis using flow cytometry (mean ± SD, N=3, *P<0.05, **P<0.01).*

4.2.20 Peptan P increases expression of CD49b and CD49e in dermal fibroblasts

To determine the potential for Peptan P to interact with other ECM receptors on the cell surface of dermal fibroblasts, primary dermal fibroblasts were treated in the presence or absence of 1mg/ml Peptan P for 72 hours before using flow cytometry to measure the cell surface expression of CD markers; CD49b, CD49c, CD49e, CD49f and CD29. Results demonstrated that Peptan P significantly increased cell surface expression of CD49b and CD49e on dermal fibroblasts (Figure 4.23, unpaired t-test, *P<0.05, **P<0.01). However, there was no effect of Peptan P on cell surface expression of CD49c, CD49f and CD29 compared to the control (Figure 4.23, unpaired t-test, ns). Collectively, these results demonstrate the ability for Peptan P to interact with integrin receptors, specifically integrin α2β1 and integrin α5β1 receptors on the cell surface of dermal fibroblasts.

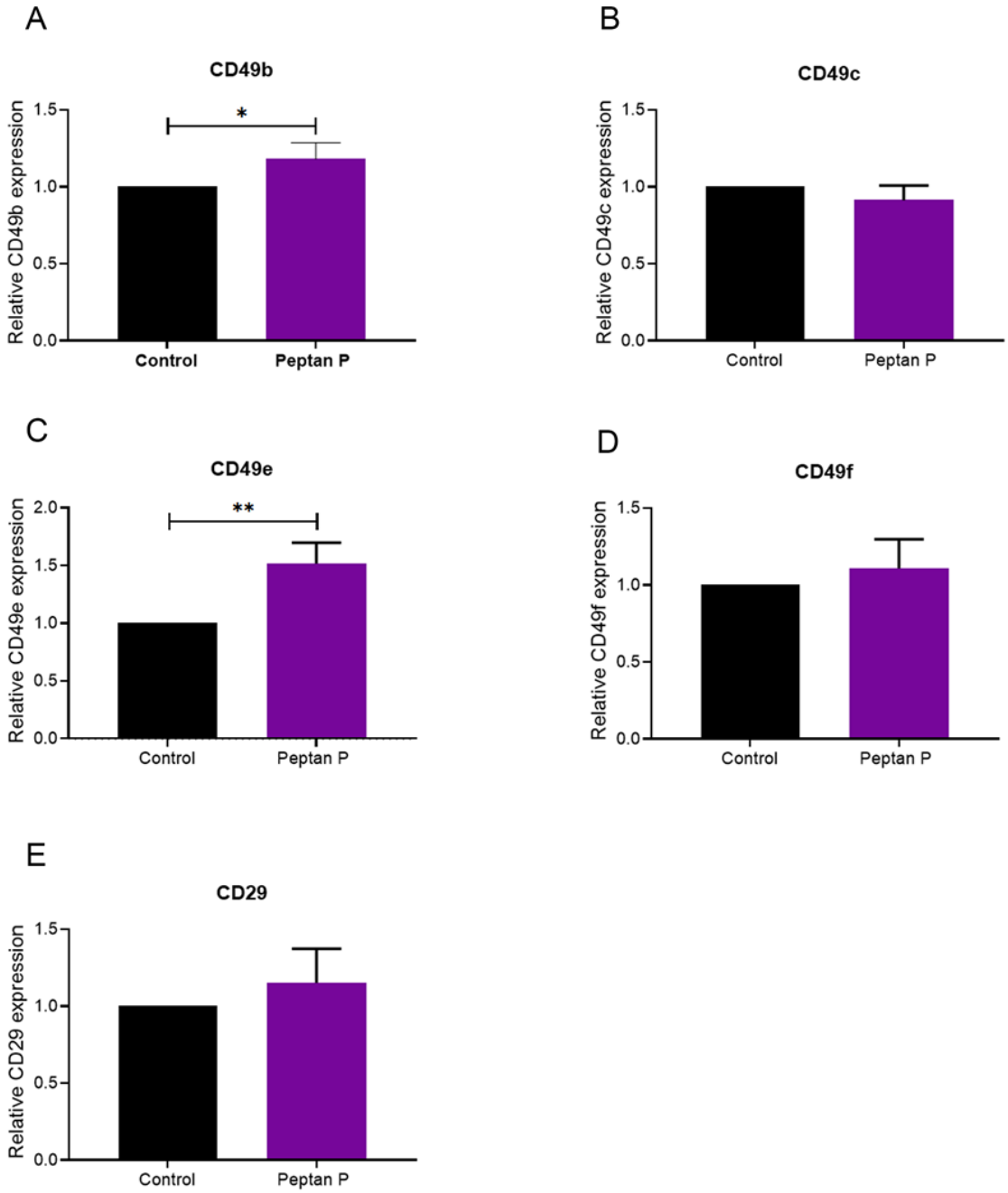

Figure 4. 23 Peptan P increases expression of CD49b and CD49e in dermal fibroblasts. *Relative cell surface expression of (A) CD49b, (B) CD49c, (C) CD49e, (D) CD49e and (E) CD29 compared to the control in primary keratinocytes treated in the presence or absence of 1mg/ml Peptan P for 72 hours before analysis using flow cytometry (mean ± SD, N=3, *P<0.05, **P<0.01).*

4.3 Discussion

Biomaterials developed from native ECM proteins such as collagen have been used to create various types of therapeutics for the treatment of acute and chronic wounds due to their biodegradability and low toxicity; however, they are not always effective in treating every type of wound. Focus over the years has therefore shifted towards the potential use of bioactive collagen peptides as a therapeutic strategy for the treatment of cutaneous wounds. The hydrolysis of native collagen to peptide fragments generates various peptides that differ in both molecular weight and bioactivity, with previous studies highlighting that collagen peptides exert biological effects that are not typically observed within native collagen such as antioxidant activity and enhancement of cellular proliferation (Ohara *et al.*, 2010; Banerjee *et al.*, 2014).

It has been suggested that the size of the collagen peptide determines its bioactivity, with previous studies suggesting that smaller peptides such as di- and tri-peptides exert more bioactivity that larger peptides, evidenced by the ability for low molecular collagen peptides to enhance cellular proliferation of both dermal fibroblasts and osteoblasts (Shigemura *et al.*, 2009; Ohara *et al.*, 2010; Asai *et al.*, 2019; Zhu *et al.*, 2020). However, results from the present study demonstrated that the size of the collagen peptide did not necessarily determine its bioactivity as alternative porcine-derived collagen peptides whose size was classified as being small (CH2) or large (CH7 and CH10) both enhanced the wound closure of dermal fibroblasts in 2D scratch assays (Figure 4.2A). Whilst these alternative collagen peptides were also able to promote dermal fibroblast wound closure in the context of age, they did not significantly enhance wound closure greater than Peptan P (Figure 4.2B-E), which suggests that the difference in bioactivity between these alternative collagen peptides is more likely to be due to the abundance of motifs present within the peptides that dermal fibroblasts are able to interact with in order to promote wound closure. Moreover, unlike Peptan P, the alternative porcine-derived collagen peptides were unable to significantly enhance keratinocyte wound closure (Figure 4.3), further demonstrating that the size of the peptide does not correlate with its ability to promote wound closure but more likely depends on the presence of specific motifs that the cells are able to interact with in order to stimulate wound healing.

Previous studies have demonstrated that hydroxyproline-containing peptides can be detected within the blood and that the concentrations detectable are similar to concentrations that are capable of significantly promoting wound healing *in vitro* (Ohara *et al.*, 2007; Mistry *et al.*, 2021). However, the collagen peptides used in *in vitro* scratch assays are not the same as the peptides detectable in blood as they have not undergone human digestion. To overcome this, porcine-derived collagen peptides were subjected to an *in vitro* digestion in order to determine whether collagen peptides that have undergone digestion still exert the same bioactivity for wound healing as undigested collagen peptides, with results revealing that the digested collagen peptides were unable to significantly promote keratinocyte or dermal fibroblast wound closure, unlike Peptan P, an undigested collagen peptide product (Figure 4.4 and 4.5). Additionally, the inclusion of an internal control, referred to as the empty digest, demonstrated that none of the traces of enzymes or reagents that may have been carried over from the *in vitro* digestion process significantly affected both keratinocyte and dermal fibroblast wound closure (Figure 4.4 and 4.5). This suggests that during the digestion process, the interactions of the collagen peptides with various enzymes and other microenvironment factors within the gut such as pH induce changes to the protein structure of the collagen peptides, thereby altering the structural properties of the motifs and binding sites present on the collagen peptides that are responsible for interacting with cell surface receptors in order to promote wound healing. Previous bioavailability studies have shown that collagen peptides have a high bioavailability, with up to 90% of ingested collagen peptides being absorbed from the intestine into systemic circulation (Oesser *et al.*, 1999; Ichikawa *et al.*, 2010; Wang *et al.*, 2015b). However, a limitation of the mass spectrometry techniques used in these previous studies is that they don't allow for the detection of both small and large peptides at the same time, meaning that these previous studies never fully captured the full scope of collagen peptides detectable post-ingestion, thereby warranting the design of experiments using labelled peptides to accurately identify and quantify which peptides are absorbed into systemic circulation. Given results from an *in vitro* digestion suggest that digestion may affect the bioactivity of collagen peptides for wound healing, additional experiments using peptides detected post-ingestion *in vivo* should be conducted in order to conclusively determine whether digestion affects collagen peptide bioactivity for wound healing. However, given the current uncertainty surrounding

whether digestion affects the bioactivity of collagen peptides for promoting wound closure, other routes of administration such as the topical application of collagen peptides should also be considered for the treatment of cutaneous wounds.

Orally ingested collagen peptides are transported into systemic circulation via the action of peptide transporters such as PEPT-1 (Aito-Inoue *et al.*, 2007). Therefore, it is possible that the expression of peptide transporters such as PEPT-1 on cutaneous cells allows for the transportation of bioactive collagen peptides into the cells to activate intracellular downstream signalling pathways that promote cutaneous wound healing. Previous studies using both keratinocyte and fibroblast cell lines revealed that these cells express various peptide transporters such as PEPT-1, PEPT-2, PHT1 and PHT2 with PEPT-2 expressed more by keratinocytes and PHT2 expressed more by dermal fibroblasts (Kudo *et al.*, 2016). This may indicate that the expression of PEPT-2 on keratinocytes mediates collagen peptide-induced wound closure of keratinocytes whilst the expression of PHT2 may mediate collagen peptide-induced wound closure of dermal fibroblasts. However, gene expression studies carried out in both young and aged primary human keratinocytes and dermal fibroblasts revealed that both of these cell types express PEPT-1, PEPT-2, PHT1 and PHT2 in low to undetectable levels when compared to Caco-2 cells, a cell known to express high levels of these peptide transporters, indicating that while peptide transporters play a role in the transport of collagen peptides from the gut into systemic circulation, they are unlikely to play a significant role in the promotion of cutaneous wound healing. (Figure 4.6). Furthermore, studies have only identified the transportation of small di- and tri-peptides via peptide transporters, which may imply that potentially larger bioactive collagen peptides are unable to interact with these peptide transporters and therefore must interact with cells through another unknown mechanism (Aito-Inoue *et al.*, 2007; Ichikawa *et al.*, 2010; Sontakke *et al.*, 2016).

The ECM plays a critical role in cellular proliferation, migration and differentiation through various cell-ECM interactions. Loss of structural proteins such as collagen through intrinsic ageing can impair the biological function of the ECM, thereby affecting cell-ECM interactions that promote cellular adhesion and proliferation (Le Varlet *et al.*, 1998). Cell-ECM interactions are mediated through interactions between specific motifs present within ECM proteins and specific cell surface

receptors present on the cells such as integrin receptors. Integrins are transmembrane receptors that facilitate cell-cell and cell-ECM interactions, allowing them to activate various intracellular signalling pathways following ECM binding that promote cellular adhesion, proliferation and survival (Hynes, 2002). Specifically, integrin β1 mediates adhesion of basal keratinocytes in the epidermis to the ECM helping to maintain their proliferative potential and regulate their differentiation (Levy *et al.*, 2000). However, previous studies have shown that skin ageing decreases integrin β1 expression by keratinocytes within the basal layer of the epidermis (Bosset *et al.*, 2003). Results from the present study revealed that age does not affect expression of either the integrin α2 or β1 subunit in differing aged keratinocytes or dermal fibroblasts (Figure 4.7 and 4.8), suggesting that the observed decline in integrin β1 expression in the epidermis may be due to the fact that the skin samples analysed were from sun-exposed areas and therefore extrinsic ageing such as overexposure to UV radiation negatively affects integrin β1 expression whilst intrinsic ageing has no significant effect on integrin β1 expression (Bosset *et al.*, 2003).

Motifs present within native collagen such as GFOGER and GLOGEN interact with collagen receptors on the surface of cells such as integrin α2β1 leading to autophosphorylation of FAK and downstream activation of signalling pathways such as MAPK and PI3K/Akt, which promote cellular proliferation (Figure 4.1) (Moreno-Layseca and Streuli, 2014). Results from the present study demonstrated that native rat tail collagen I significantly activated FAK, ERK and Akt in unwounded keratinocytes demonstrating that the presence of these motifs within collagen mediates keratinocyte proliferation via integrin ligation (Figure 4.9A, D-F). However, while Peptan P significantly activated Akt, there was no significant effect on FAK activation observed, suggesting that Peptan P may activate Akt independently of integrins, perhaps via growth factor receptor ligation instead (Figure 4.9A, D and E). This would correlate with previous studies that have shown that some ECM peptides contain binding sites that allow them to bind to other receptors such as growth factor or G-coupled receptors in order to activate downstream signalling pathways (Tran *et al.*, 2004; Schultz and Wysocki, 2009). Additionally, previous studies have demonstrated that marine collagen peptides increase expression of wound healing-associated growth factors such as EGF and TGF- β (Yang *et al.*, 2019). This may

also suggest that Peptan P could increase expression and secretion of other wound healing-associated growth factors such as PDGF and IGF-1, which are known to activate the PI3K/Akt signalling pathway in order to stimulate keratinocyte proliferation (Haase *et al.*, 2003). The use of *ITGB1* siRNA to knockdown expression of the integrin β1 subunit impaired Peptan P-induced expression of both the integrin α2 and β1 subunit as well as decreased activation of FAK in unwounded keratinocytes, confirming that Peptan P interacts with the integrin α2β1 receptor in order to induce autophosphorylation of FAK (Figure 4.16A-D). However, Peptan P did not significantly enhance activation of Akt and the loss of integrin α2β1 receptor expression did not impair Peptan P-induced activation of Akt, implying that Akt activation is not reliant on Peptan P-induced activation of the integrin signalling pathway and therefore must also require activation from other sources such as growth factors in order to enhance keratinocyte proliferation (Figure 4.16E). Growth factors such as PDGF can also activate FAK, with previous studies demonstrating that treatment of hepatic stellate cells with PDGF leads to phosphorylation of FAK via Ras signalling and downstream activation of Akt to promote cellular proliferation, suggesting that Peptan P may need to co-operate with growth factors and their receptors such as PDGF and PDGFR in order to induce keratinocyte proliferation (Reif *et al.*, 2003; Yang *et al.*, 2019). This would also corroborate with previous work that has suggested that integrins cannot promote proliferation independently of growth factor receptors and vice versa, implying that keratinocyte proliferation cannot occur without activation of both integrin and growth factor receptor signalling (Nikolopoulos *et al.*, 2005; Moreno-Layseca and Streuli, 2014).

Previous studies have demonstrated Peptan P enhanced proliferation of dermal fibroblasts (Mistry *et al.*, 2021). However, results from the present study using unwounded dermal fibroblasts demonstrated no significant activation of ERK or Akt by Peptan P suggesting that Peptan P may not promote dermal fibroblast proliferation via these signalling pathways (Figure 4.10A, E and F). Additionally, Peptan P was unable to significantly activate FAK, suggesting that the interactions between Peptan P and dermal fibroblasts may not occur directly via integrins (Figure 4.10A and D). Previous studies have demonstrated that the enhanced proliferation of mouse 3T3 fibroblasts by collagen peptides is mediated by the activation of the NF-κB pathway, implying that Peptan P could potentially promote dermal fibroblast

proliferation via the activation of the NF-κB pathway (Yang *et al.*, 2019). Despite that fact that present results revealed no activation of FAK, ERK or Akt by Peptan P in unwounded dermal fibroblasts, the use of *ITGB1* siRNA to knockdown expression of the integrin β1 subunit resulted in decreased expression of the integrin α2β1 receptor, which in turn decreased Peptan P-induced activation of FAK and ERK demonstrating that Peptan P-induced interaction with this integrin receptor may activate a FAK-ERK signalling cascade in order to promote dermal fibroblast proliferation (Figure 4.17A, B, D and F) . Again, Peptan P had no significant effect on Akt activation and knockdown of the integrin β1 subunit did not significantly affect Akt activation in control or Peptan P-treated dermal fibroblasts, suggesting that again, Akt signalling is not dependent on activation via integrin binding alone and may also rely on activation by other growth factors and cytokines in order to promote dermal fibroblast proliferation (Streuli and Akhtar, 2009).

During wound healing, cells interact with the wound ECM via integrin receptors, upregulating their expression and activating downstream signalling pathways associated with cellular adhesion, proliferation and migration. Given that Peptan P has been shown to increase cellular proliferation to enhance wound closure and this effect may be warranted through interactions with integrin receptors, further analysis was carried out to determine whether Peptan P activated signalling pathways associated with proliferation such as ERK and Akt via integrin ligation post-wounding. Unlike in unwounded keratinocytes, Peptan P was unable to significantly enhance activation of Akt in wounded keratinocytes, with a trend wise decrease observed from 48 hours post-wounding onwards, suggesting that Akt signalling may not play a prominent role in promoting the wound closure of keratinocytes (Figure 4.11A and E). Additionally, Peptan P also was unable to significantly increase expression of integrin α2 and integrin β1 or increase activation of FAK and ERK (Figure 4.11A-D and F). Although, Peptan P did not significantly increase activation of FAK and ERK in wounded keratinocytes, there was still a trend wise increase in activation by Peptan P when compared to the uncoated control from 24 hours post-wounding onwards, suggesting that Peptan P may activate a FAK-ERK signalling cascade to promote keratinocyte wound closure (Figure 4.11D and F) (Teranishi *et al.*, 2009). Furthermore, in wounded dermal fibroblasts, Peptan P significantly enhanced activation of both FAK and ERK (Figure 4.12A, D and F), supporting the

potential for Peptan P to activate an FAK-ERK signalling cascade in order to promote dermal fibroblast wound healing as well. However, Peptan P did not increase expression of either integrin α2 or integrin β1 in wounded dermal fibroblasts, which may imply that the activation of a FAK-ERK signalling cascade during dermal fibroblast wound healing may occur independently of integrin binding (Figure 4.12A-C).

The binding of integrins to the ECM to promote cell adhesion is crucial in wound healing. Too strong of an adhesion between the ECM and cells impairs cell motility causing cells to become stationary whilst too weak of an adhesion prevents the cells from building up sufficient traction in order to enhance their motility (Koivisto *et al.*, 2014). Therefore, it could be hypothesised that Peptan P binds to cells via integrin receptors to provide cellular adhesion but also act as anchorage points for cells to interact with during wound healing in order to build up traction and enhance the speed at which the wound closes (Figure 4.24). The knockdown of integrin β1 expression impaired both keratinocyte and dermal fibroblast wound closure, significantly impairing the speed of both Peptan P-induced keratinocyte and dermal fibroblast wound closure (Figure 4.14 and 4.15). This reduction in cell motility following knockdown of the integrin β1 subunit confirms that Peptan P must form an adhesion complex with integrin receptors on the cell surface of both keratinocytes and dermal fibroblasts in order to provide anchorage points that allows for the build-up of traction in order to enhance cell motility and promote wound closure.

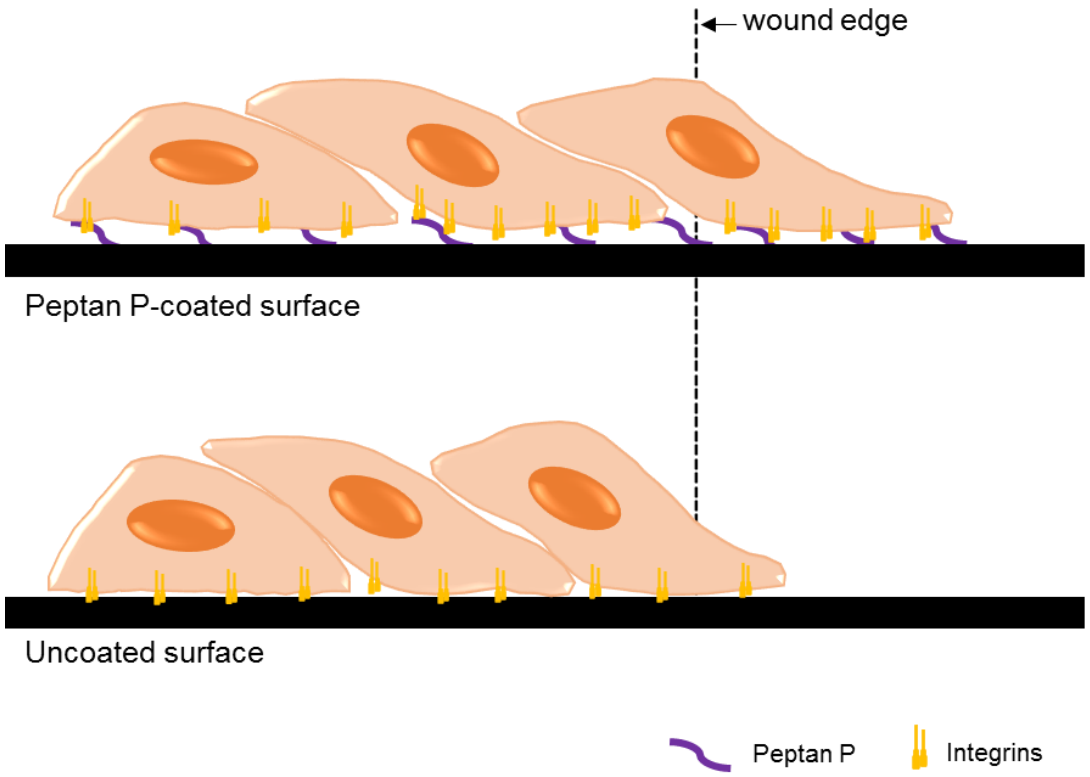

Figure 4. 24 Schematic diagram of Peptan P-induced wound closure. *Peptan P provides anchorage points that act to promote cell adhesion and help generate traction in order to enhance the speed at which the wound heals.*

Whilst the knockdown of integrin β1 impaired both keratinocyte and dermal fibroblast wound closure, it also significantly reduced integrin α2 expression in uncoated and Peptan P-treated keratinocytes post-wounding, with a significant decrease in Peptan P-induced activation of ERK observed at 24 hours post-wounding and FAK at 48 and 72 hours post-wounding (Figure 4.18 and 4.19A, C and E). This suggests that ERK activation during early phases of Peptan P-induced keratinocyte wound closure is independent of FAK activation and therefore may be activated by other proteins such a members of the Src homology and collagen (Shc) family. (Figure 4.1 and 4.19F) (Vindis *et al.*, 2003; Ahmed and Prigent, 2017). Additionally, while previous studies have linked FAK activation to cellular proliferation through modulation of cell cycle progression, FAK does not appear to always be required as FAK -/- cells and cells treated with FAK RNAi both demonstrated the ability to proliferate without FAK activation (Ilić *et al.*, 1995; Duxbury *et al.*, 2003; Pirone *et al.*, 2006). This may imply that Peptan P-induced

activation of FAK at later stages of keratinocyte wound closure is also linked to the activation of other signalling pathways such as Rho GTPases, which are associated with actin cytoskeleton organisation and cellular migration to ensure efficient wound closure (Desai *et al.*, 2004). Further investigations would therefore be warranted to further elucidate any other potential downstream signalling pathways that may be activated following Peptan P binding to integrin. Previous studies have implicated the role of an ERK-FAK-Paxillin signalling cascade in corneal epithelial cell wound healing, demonstrating that inhibition of ERK using a MEK1 inhibitor impairs wound closure but also inhibited phosphorylation of FAK and the formation of focal adhesions (Teranishi *et al.*, 2009). This signalling cascade may also play a role in Peptan P-induced wound closure of keratinocytes, with early activation of ERK being required in order to promote Peptan P-induced activation of FAK during later stages of keratinocyte wound closure (Figure 4.19C, E and F). Additionally, phosphorylated ERK has been shown to be localised to cells at the wound margin, regulating the formation of lamellipodia and focal adhesions through interactions with FAK and Paxillin, further supporting the potential for Peptan P-induced activation of ERK to promote FAK activation and stimulate additional signalling pathways to promote keratinocyte wound closure by potentially enhancing both cellular migration and proliferation (Teranishi *et al.*, 2009).

However, whilst knockdown of the integrin β1 subunit also diminished Peptan P-induced expression of integrin α2 in wounded fibroblasts, the knockdown of the integrin β1 subunit did not significantly impair Peptan P-induced activation of FAK, Akt or ERK (Figure 4.20 and 4.21A-E). Previous studies have shown that the loss of integrin β1 in mammary epithelial cells reduced proliferation but did not impair their migration and while ERK has been linked to cellular proliferation, other studies have identified that during wound healing, ERK activity is required more for cellular migration rather than cellular proliferation (Matsubayashi *et al.*, 2004; Teranishi *et al.*, 2009; Jeanes *et al.*, 2012). Therefore, it could also be theorised that the continued activation of ERK in wounded dermal fibroblasts despite knockdown of integrin β1 could be a result of its activation for promoting dermal fibroblast migration rather than dermal fibroblast proliferation. However, this continued increase in ERK activation in wounded dermal fibroblasts occurs regardless of Peptan P treatment or *ITGB1* siRNA suggesting that Peptan P does not play a role

in the activation of ERK to promote dermal fibroblast migration, indicating the possibility that other signalling pathways such as growth factor signalling have a more prominent role in activating ERK during dermal fibroblast migration. Despite the fact that FAK activation was not significantly impaired by knockdown of the integrin β1 subunit, there was still a trend wise decline in Peptan P-induced activation of FAK following knockdown of integrin β1, indicating that FAK may still play a role in Peptan P-induced wound closure of dermal fibroblasts and may activate other downstream signalling pathways that stimulate dermal fibroblast migration and proliferation (Figure 4.20 and 4.21C and F). Therefore, further studies are warranted to explore additional signalling pathways that may be activated following Peptan P-induced activation of the integrin signalling pathway in dermal fibroblasts and whether FAK is associated with Peptan P-induced wound closure of dermal fibroblasts.

Most understanding of cell-ECM interactions and mechanotransduction is derived from population-based or whole-cell assays such as Western blots and immunostaining, where the cells are viewed in a uniform state. While these analyses provide averaged metrics, they do not reveal important relationships at the cell-ECM interface (Zhou *et al.*, 2021). In particular, the use of these assays within the present study does not allow the for observation of interactions between leading wound edge and trailing cells within the wound at various timepoints so it makes it difficult to reach definitive conclusions on how Peptan P is activating various signalling pathways to promote both keratinocyte and dermal fibroblast wound closure. The heterogeneity and plasticity of cells during wound healing means that cells at the wound edge and the trailing cells will not be expressing the same proteins or genes at the same time, as indicated by previous studies showing that ERK activation occurs in waves during collective cell migration, with ERK activation occurring first at the leading wound edge before being activated by the trailing cells (Hino *et al.*, 2020). Despite the fact that the use of Western blotting within the present study did not clearly define how Peptan P may activate FAK, ERK and Akt signalling pathways and which cells within wounds (leading edge vs trailing cells) are being stimulated to activate these different pathways, the results still clearly demonstrate that Peptan P is mediating its effects via interactions with the integrin α2β1 receptor and loss of

expression of this receptor impairs Peptan P-induced wound closure of both keratinocytes and dermal fibroblasts (Figure 4.14, 4.15, 4.18-4.21).

The hydrolysis of collagen to peptide fragments exposes cryptic motifs that would typically be inaccessible to cells, enabling collagen peptides to interact with other ECM receptors, unlike native collagen which can only interact with collagen receptors in order to promote cutaneous wound healing. Previous studies have revealed that some of these cryptic motifs exposed within collagen peptides are capable of interacting with other ECM receptors such as integrin α5β1, a known fibronectin receptor found on the cell surface of various cell types such as dermal fibroblasts (Agrez *et al.*, 1991; Taubenberger *et al.*, 2010; Koivisto *et al.*, 2014). Supporting this, Peptan P demonstrated the ability to increase the cell surface expression of CD49b (integrin α2), CD49e (integrin α5) and CD29 (integrin β1) in both keratinocytes and dermal fibroblasts, implying that while Peptan P can interact with the integrin α2β1 receptor, it also has the capability to interact with fibronectin receptors like integrin α5β1 (Figure 4.22A, C and E and 4.23A and C). While the present study has predominantly focussed on the effect of collagen peptides on the proliferation phase of cutaneous wound healing by exploring the mechanisms of collagen peptides on promoting both keratinocyte and dermal fibroblast wound closure, this data suggests that Peptan P could also have an effect on earlier phases of cutaneous wound healing such as haemostasis. Fibronectin is produced in high abundance during early phases of wound healing to generate the provisional matrix, which in turn allows wound keratinocytes to migrate along the provisional matrix through interactions with integrin α5β1 in order to re-establish the damaged epidermis (Koivisto *et al.*, 2011; Bornes *et al.*, 2020; Di Russo *et al.*, 2021). Therefore, it could be theorised that Peptan P may provide additional anchorage points within the provisional matrix, allowing wound keratinocytes to interact with Peptan P via integrin α5β1 and enhance the re-epithelialisation of damaged skin.

Peptan P was unable to increase cell surface expression of CD49c (integrin α3) and CD49f (integrin α6) of both keratinocytes and dermal fibroblasts, indicating that Peptan P is not able to interact with integrin α3β1 and α6β1 receptors (Figure 4.22B and D and 4.23B and D). Integrin α3β1 and α6β1 are both receptors for laminin, allowing them to promote cell adhesion to the basement membrane. In particular, integrin α3β1 is expressed by endothelial cells and allows for cross-talk with

epidermal keratinocytes to promote angiogenesis, with deletion of the integrin α3 subunit impairing wound angiogenesis, thus implying integrin α3β1 plays a more prominent role in neovascularisation rather than the promotion of epidermal and dermal wound closure (Mitchell *et al.*, 2009).

Overall, the data from the present study confirms that Peptan P exerts its beneficial effects on keratinocytes and dermal fibroblast wound repair by binding to integrin α2β1 and α5β1 receptors, thus activating the integrin signalling pathway and promotion of cutaneous wound healing.

4.4 Summary

- The size of collagen peptides does not correlate with their bioactivity for wound healing
- *In vitro* digestion affects the ability of collagen peptides to promote both keratinocyte and dermal fibroblast wound closure
- Age does not affect integrin α2β1 receptor expression in keratinocytes or dermal fibroblasts
- Knockdown of integrin β1 impairs Peptan P-induced wound closure of both keratinocytes and dermal fibroblasts
- Peptan P promotes keratinocyte and dermal fibroblast wound closure via the integrin α2β1 receptor and activation of the integrin signalling pathway
- Peptan P promotes keratinocyte wound closure by enhancing ERK activation at early phases and FAK activation during late phases of wound healing
- Peptan P does not promote dermal fibroblast wound closure by increasing ERK or Akt activation
- Peptan P increases expression of integrin α5β1 on the cell surface of both primary human keratinocytes and dermal fibroblasts

Chapter 5: Peptan P promotes re-epithelialisation in *ex vivo* full thickness wounded 3D human skin equivalents

Chapter 5: Peptan P promotes re-epithelialisation in *ex vivo* full thickness wounded 3D human skin equivalents

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5.1 Introduction

The majority of drug testing assays for wound healing rely mainly on 2D culture-based techniques involving the seeding of cells onto uncoated or coated surfaces of tissue culture plates. Although, the use of 2D assays for wound healing studies are advantageous for high throughput screening of potential wound healing therapeutics presenting a quick, simple and inexpensive screening model, such assays do not fully capture a cutaneous wound healing environment *in vivo* (Grada *et al.*, 2017; Chen *et al.*, 2019). Both dermal fibroblasts and keratinocytes cultured in 2D are physiologically and morphologically different to cells cultured in 3D, with 2D culture systems lacking environmental factors such as the ECM and spatial orientation that are found within an *in vivo* environment (Ralston *et al.*, 1997; Sun *et al.*, 2006; Randall *et al.*, 2018).

Various attempts have therefore been made to develop *ex vivo* skin equivalent models that mimic the *in* vivo environment and can be used for modelling various health conditions and testing new therapeutics (Carlson *et al.*, 2008; Safferling *et al.*, 2013; Antoni *et al.*, 2015). However, many of the current available human skin equivalent models are limited in terms of viability due to factors such as the time-dependent contraction of an incorporated collagen gel-base or the limited use of skin tissue components (Vidal *et al.*, 2019). The generation of full thickness skin models using fibroblasts integrated into natural polymers such as collagen hydrogels provides good support for keratinocyte differentiation; however, the longevity of such models is limited with batch-to-batch variability of animal-derived collagen leading to inconsistent and unreproducible results (Gangatirkar *et al.*, 2007; Carlson *et al.*, 2008). In an attempt to overcome the batch-to-batch variability associated with 3D skin equivalents incorporating natural polymers, synthetic polymers have been developed including polyesters such as poly(ε-caprolactone) (PCL), polylactic-co-glycolic acid (PLGA) and polyethers such as polyethylene glycol (PEG) and PEG co-polymers for the production of tissue scaffolds applicable for 3D culture (Antoine *et al.*, 2014; Randall *et al.*, 2018). However, while the use of these synthetic polymers allows for the fabrication of 3D skin models with a matrix that bears a strong resemblance to the native ECM, synthetic polymers generally display poor cell adhesion properties and therefore still need to be used in combination with natural polymers such as collagen or fibronectin (Wang *et al.*,

2013; Randall *et al.*, 2018). Other studies have tried to generate human skin equivalents by layering sheets of fibroblasts to mimic the dermal compartment of skin before overlaying with keratinocytes (Lee *et al.*, 2009). Whilst this approach resulted in the formation of a fully stratified epidermis, the generated skin equivalent nevertheless lacked a true 3D structure through incomplete basement membrane formation (Lee *et al.*, 2009).

To date, many researchers have relied on commercially available 3D skin models such as EpiDerm™, StrataTest® and Phenion® for a wide range of assays including skin hydration and drug delivery, however, coupled with the limitations in methodology, the limited viability and robustness of such models reduces their applicability for specific downstream applications such as wound healing (Ackermann *et al.*, 2010; Rasmussen *et al.*, 2010). Further limitations of current 3D skin models made using collagen gels include their inability to correctly properly mimic skin ageing and the associated features of skin ageing such as loss of ECM proteins in the dermal compartment and also any effects of an aged dermis on epidermal function and formation, thus meaning that these models cannot fully represent the alterations in cell-cell and cell-ECM interactions that occur during skin ageing (Adamus *et al.*, 2014).

To counteract this, recent approaches have focussed on creating an environment that enables dermal fibroblasts to generate their own ECM matrix (El Ghalbzouri *et al.*, 2009; Hill *et al.*, 2015; Roger *et al.*, 2019). Alvetex® (ReproCell Europe Ltd.) is a highly porous cross-linked polystyrene membrane with approximately 36-40 micron sized pores that allows cells and specifically dermal fibroblasts to grow in a microenvironment that is similar to a cutaneous *in vivo* environment whilst retaining their morphological and physiological characteristics (Bokhari *et al.*, 2007). In this context, the use of the Alvetex® scaffold enables the production of reliable, robust and reproducible 3D human skin equivalent models that supports the generation of its own ECM by dermal fibroblasts resulting in a viable skin equivalent for up to 20 weeks, with expression of collagen I and III, keratin 1 and 14 and loricrin mirroring the expression of these dermal and epidermal differentiation markers in normal skin (Hill *et al.*, 2015; Roger *et al.*, 2019). Preliminary wound healing studies have additionally shown that the inclusion of an extra dermal fibroblast layer to support this model improves its robustness and thereby their potential for monitoring the

repair of a full thickness wound inflicted to the 3D skin equivalent model (Moyassar Al-Shaibani, PhD Thesis; Deciphering the Role of Mesenchymal Stem Cells (MSCs) in Cutaneous Wound Healing, 2018, Newcastle University).

The specific aims of the present chapter were to adapt and optimise this Alvetex® based 3D human skin equivalent model to enable the mimicking of full thickness wound healing and determine the potential for the topical application of Peptan P to promote cutaneous wound healing *ex vivo*.

5.2 Results

5.2.1 Topical application of Peptan P significantly enhances wound closure of keratinocytes

Previous data from Chapter 4 suggests the potential for the topical application of collagen peptides to promote wound repair, therefore 2D scratch assays were first performed using primary keratinocytes to determine the potential for Peptan P applied topically to enhance keratinocyte wound closure. Primary keratinocytes were seeded onto uncoated wells or, as a positive control, onto wells pre-coated with 1mg/ml Peptan P. Following cell adherence, a scratch wound was induced prior to the topical application of either 1mg/ml, 2.5mg/ml, 5mg/ml or 10mg/ml of Peptan P and continued monitoring of wound closure for 72 hours. Results confirmed that pre-coating wells with 1mg/ml Peptan P resulted in the significant enhancement of keratinocytes wound closure compared to the control (Figure 5.1, One-way ANOVA with Tukey's multiple comparisons test, **P<0.01). Results also demonstrated a concentration dependent decline in the ability for the topical application of Peptan P to promote keratinocyte wound closure, with only 1mg/ml Peptan P added topically resulting in a significant effect (Figure 5.1, One-way ANOVA with Tukey's multiple comparisons test, *P<0.05).

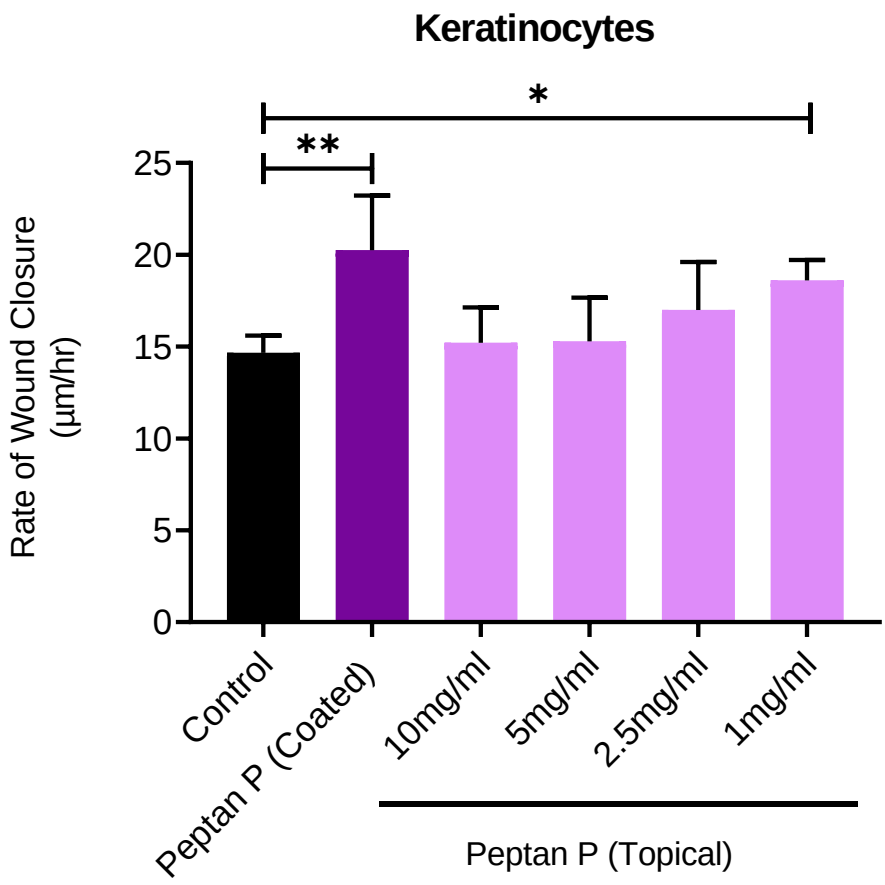

Figure 5. 1 Topical application of Peptan P significantly enhances wound closure of primary keratinocytes. *Primary keratinocytes were seeded onto uncoated control wells or wells pre-coated with 1mg/ml Peptan P. Following cell adherence, cells were scratched with either 1mg/ml, 2.5mg/ml, 5mg/ml or 10mg/ml Peptan P dissolved in culture media added to uncoated wells or addition of culture media alone (control) before monitoring of wound closure over 72 hours (mean ± SD, n=9 N=3, *P<0.05, **P<0.01).*

5.2.2 Topical application of Peptan P significantly enhances wound closure of dermal fibroblasts

Similarly, to experiments carried out in primary keratinocytes, dose response experiments of Peptan P applied topically were carried out in primary dermal fibroblasts subjected to a scratch wound induction and monitoring of wound closure over 72 hours. Results again confirmed the significant enhancement of dermal fibroblast wound closure on wells pre-coated with 1mg/ml Peptan P (Figure 5.2, One-way ANOVA with Tukey's multiple comparisons test, ***P<0.001). Additionally, results again revealed a concentration dependent decline in the ability for Peptan P applied topically to promote dermal fibroblast wound closure, with only 1mg/ml

Peptan P significantly enhancing dermal fibroblast wound closure (Figure 5.2, One-way ANOVA with Tukey's multiple comparisons test, **P<0.01).

Taken together, these data suggest that the topical application of 1mg/ml Peptan P is able to promote both keratinocyte and dermal fibroblast wound closure in 2D culture.

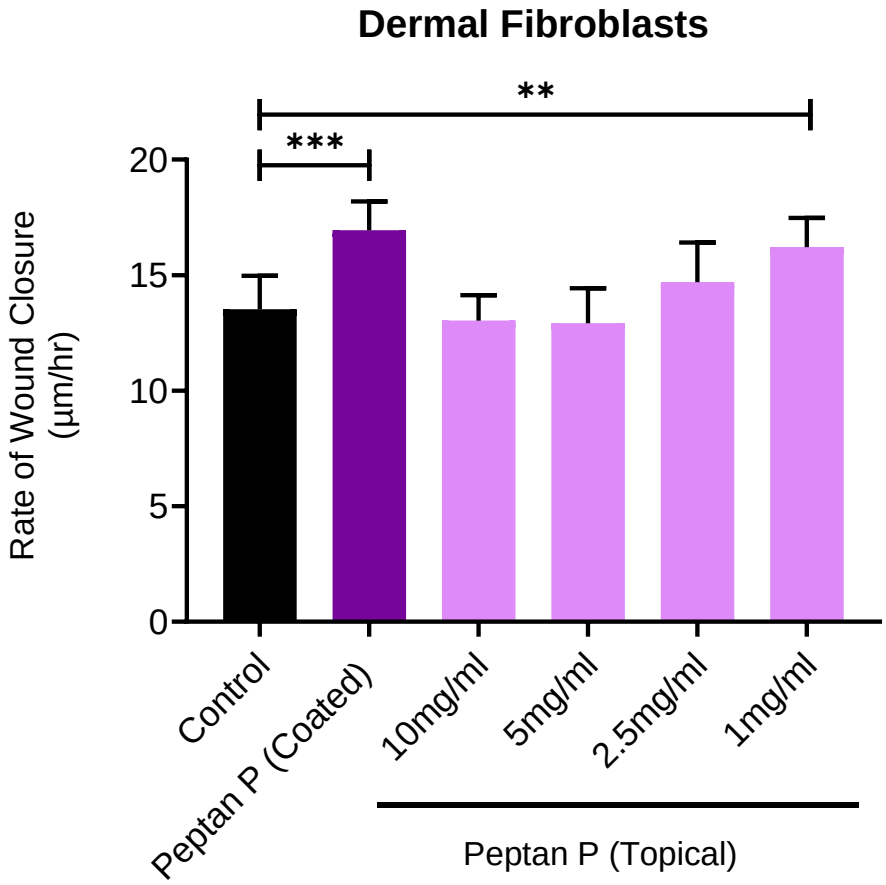

Figure 5. 2 Topical application of Peptan P significantly enhances wound closure of dermal fibroblasts. *Primary dermal fibroblasts were seeded onto uncoated control wells or wells pre-coated with 1mg/ml Peptan P. Following cell adherence, cells were scratched with either 1mg/ml, 2.5mg/ml, 5mg/ml or 10mg/ml Peptan P dissolved in culture media added to uncoated wells or addition of culture media alone (control) before monitoring of wound closure over 72 hours (mean ± SD, n=9, N=3, ***P<0.001, ****P<0.0001).*

5.2.3 Development and validation of full thickness 3D skin equivalents

Three-dimensional human skin equivalent models were generated through organotypic culture of patient-matched dermal fibroblasts overlaid with matched keratinocytes, initially derived from young individuals (18-35 years old) as previously described (Hill *et al.*, 2015). Correct formation of 3D skin equivalents was verified by comparing structural features and expression of key epidermal and dermal markers to normal human skin derived from a similar aged individual (Figure 5.3). Compared to normal human skin, H&E staining of the 3D human skin equivalents revealed two identically distinct layers of dermis and epidermis (Figure 5.3B). Confirmation of the generation of the ECM within the dermal layer of the 3D human skin equivalent was distinguished through immunofluorescent expression of both collagen I and collagen III, revealing similar content and localisation as normal human skin (Figure 5.4). Additionally, the epidermal later of the 3D skin equivalent showed apparent stratification, indicating differentiation of keratinocytes occurred to the different epidermal sublayers observed in normal human skin (Figure 5.3A and B). Confirmation of epidermal stratification was confirmed by immunofluorescent expression of loricrin in terminally differentiated keratinocytes, keratin 1 in stratified epidermal layers and keratin 14 in the basal epidermal layer of both normal human skin and the 3D human skin equivalent (Figure 5.5). Taken together, these data confirm the successful formation of a 3D human skin equivalent that mimics an *in vivo* cutaneous microenvironment.

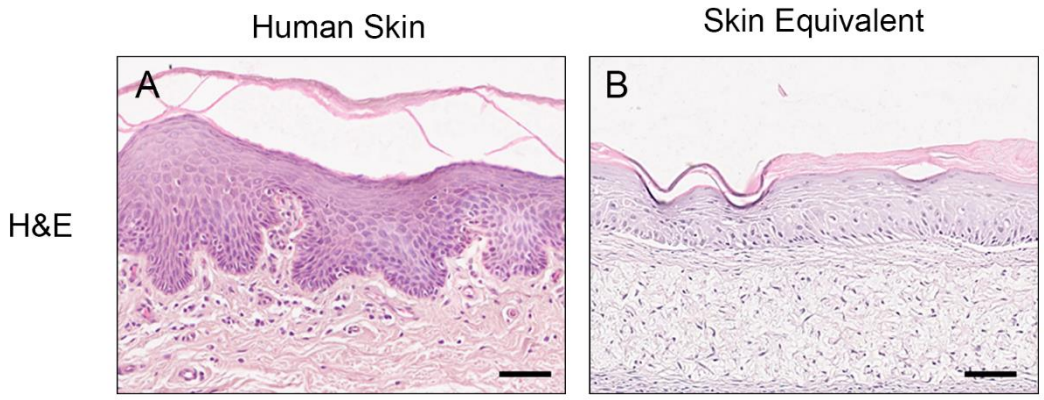

Figure 5. 3 Development of patient-matched 3D human skin equivalents. *Representative photomicrographs of H&E stained (A) normal skin (28 years old) and (B) a 3D human skin equivalent generated using cells derived from a 33 year old individual. Magnification = 10x. Scale bar = 200µm.*

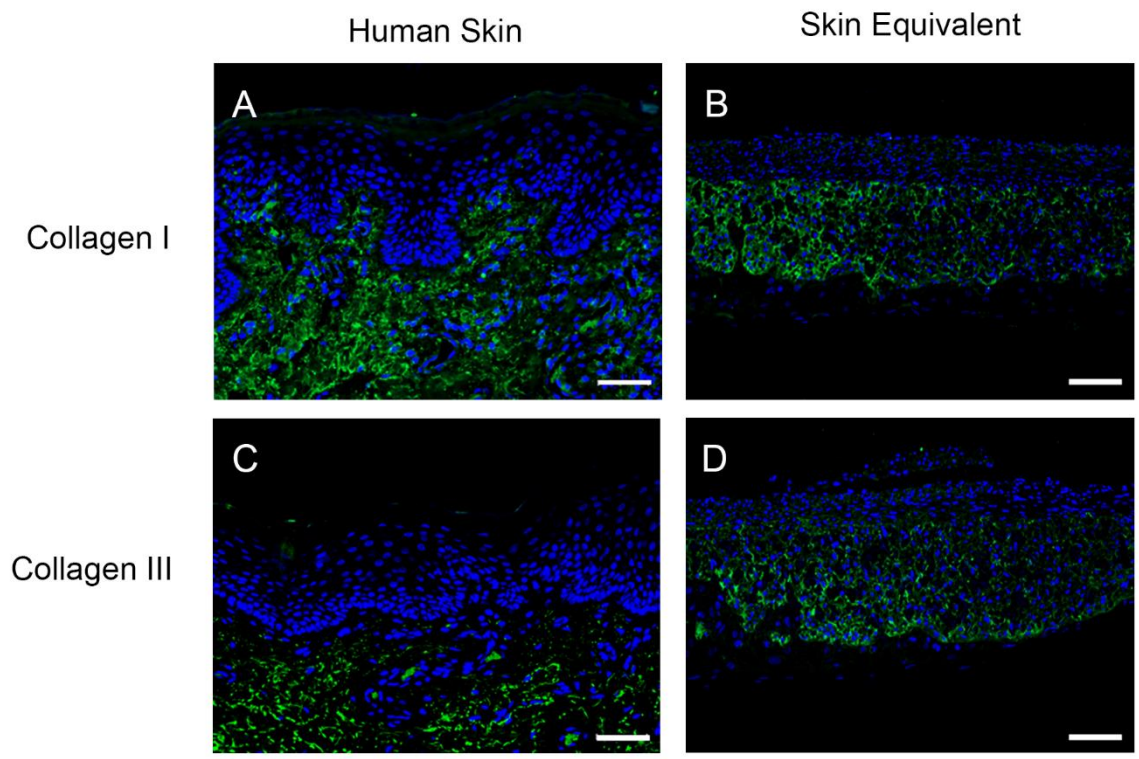

Figure 5. 4 Characterisation and validation of the dermal compartment in 3D human skin equivalents. *Representative photomicrographs of immunofluorescent staining for collagen I and collagen III expression (green) in normal human skin (A and C) and 3D human skin equivalents (B and D). Nuclei are stained with DAPI (blue). Magnification = 10x. Scale bar = 200µm.*

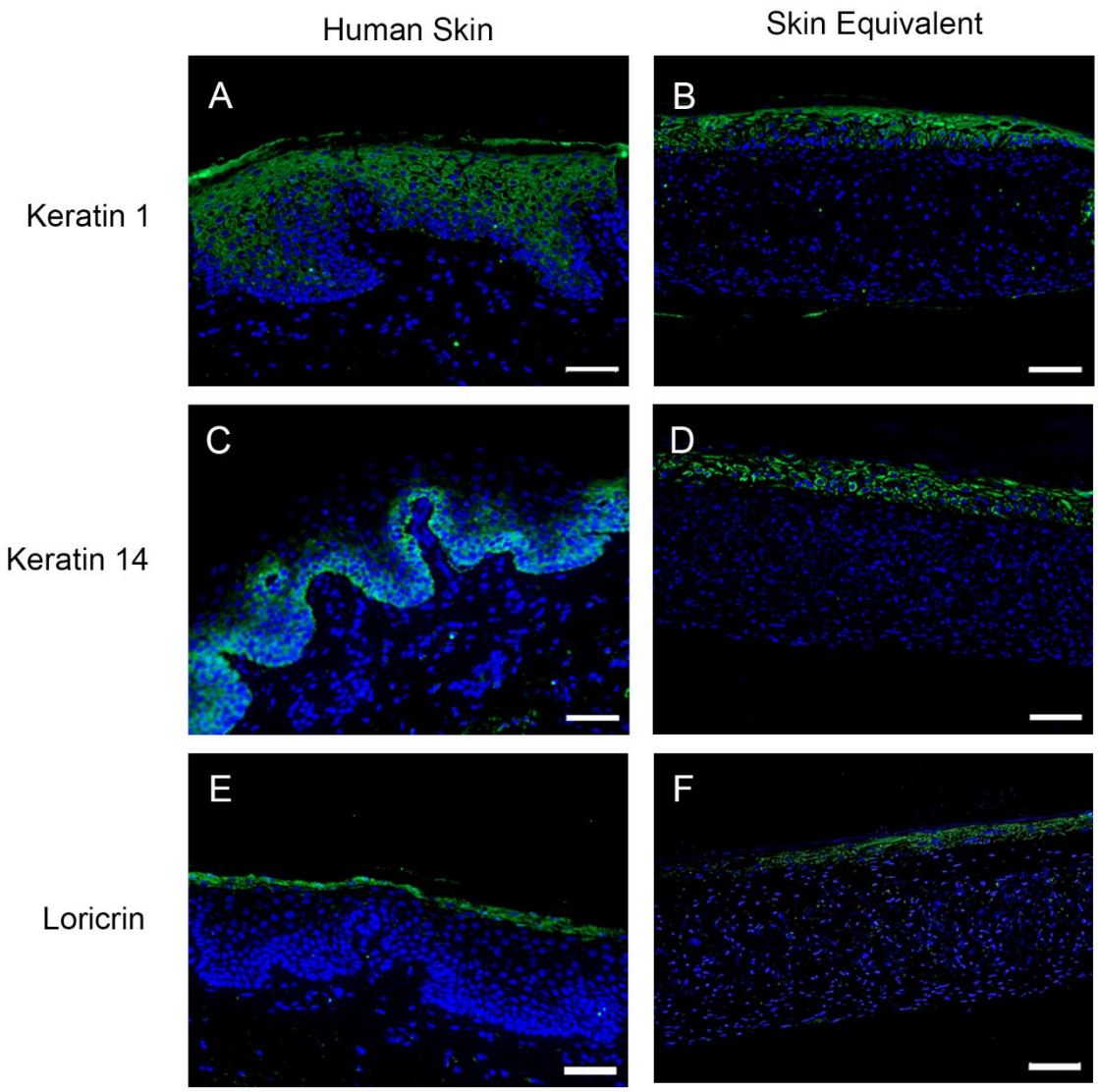

Figure 5. 5 Characterisation and validation of epidermal differentiation in human 3D skin equivalents. *Representative photomicrographs of immunofluorescent staining for keratin 1, keratin 14 and loricrin expression (green) in normal human skin (A, C and E) and 3D human skin equivalents (B, D and F). Nuclei are stained with DAPI (blue). Magnification = 10x. Scale bar = 200µm.*

5.2.4 Ageing results in the formation of a thinner epidermal layer in 3D human skin equivalents

To study the effects of ageing on 3D skin equivalent formation, patient-matched keratinocytes and dermal fibroblasts derived from young (18-35 years old) or aged (60+ years old) were used to generate young and aged 3D human skin equivalents. Results demonstrated that both young and aged patient-matched 3D human skin equivalents formed distinct dermal and epidermal layers (Figure 5.6A). However, the epidermal layer of the aged 3D human skin equivalents was significantly thinner compared to the epidermal layer of young 3D human skin equivalents (Figure 5.6B, Unpaired T-test, **P<0.01). Collectively, these data suggest that patient-matched cells derived from both young and aged individuals can be used to encapsulate ageing skin in 3D human skin equivalents *ex vivo*.

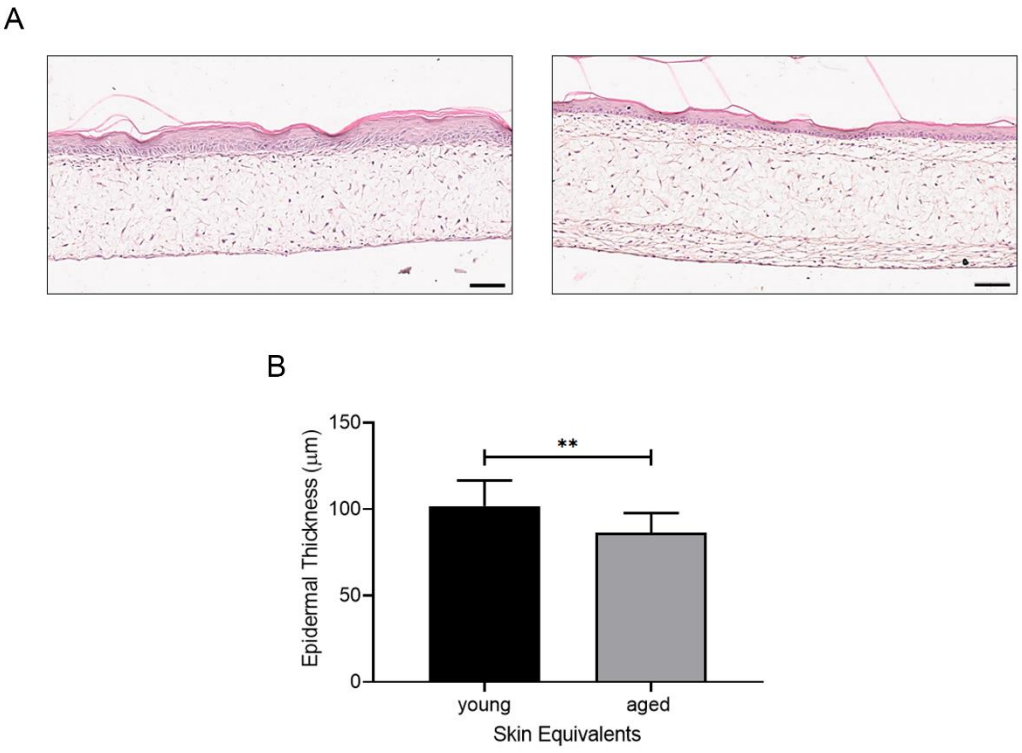

Figure 5. 6 Ageing results in the formation of a thinner epidermal layer in 3D human skin equivalents. *(A) Representative photomicrographs of H&E stained patient-matched 3D human skin equivalents derived from either young (18-35-years old) or aged (60+ years old) individuals. Magnification = 5x. Scale bar = 400µm. (B) Epidermal thickness (µm) of young and aged 3D human skin equivalents (mean ± SD, N=6 (3 young and 3 aged), **P<0.01).*

5.2.5 Peptan P significantly enhances re-epithelialisation of wounded 3D human skin equivalents

Previous data from Chapter 3 has shown that Peptan P promotes keratinocyte and dermal fibroblast wound closure in a 2D environment (Mistry *et al.*, 2021). To evaluate the potential for Peptan P to promote cutaneous wound healing in a 3D environment, 3D human skin equivalents derived from young individuals (18-35 years old) were wounded with a 1mm punch biopsy before placing onto an extra dermal layer to provide support and analysis of wound closure at 1, 2, 3 and 4 weeks post-wounding (Figure 5.5). Results from 3 independent experiments demonstrated that Peptan P significantly enhanced the wound closure of wounded 3D human skin equivalents by 3 weeks post-wounding compared to the untreated control (Figure 5.7 and 5.8A, Two-way ANOVA with Tukey's multiple comparisons test, **P<0.01). Further analysis revealed that Peptan P enhanced wound closure of 3D wounded human skin equivalents by promoting re-epithelialisation, with a significant difference compared to the control observed at 3 weeks post-wounding (Figure 5.7 and 5.8B, Two-way ANOVA with Tukey's multiple comparisons test, *P<0.05). However, despite the ability for Peptan P to promote dermal fibroblast wound closure in 2D scratch assays, Peptan P did not have a significant effect on dermal regeneration in wounded 3D human skin equivalents (Figure 5.7 and 5.8C, Two-way ANOVA with Tukey's multiple comparisons test, ns).

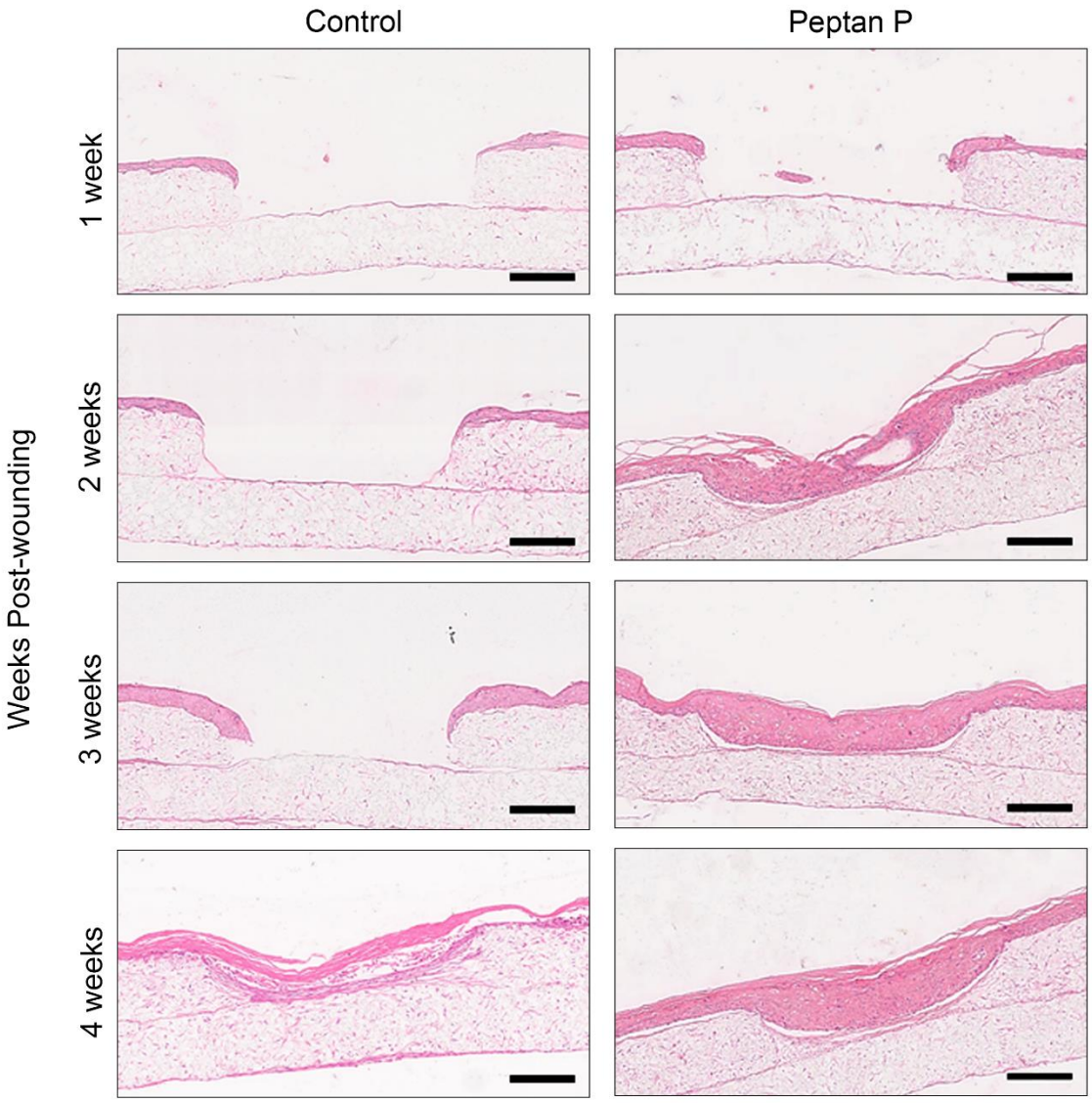

Figure 5. 7 Peptan P promotes re-epithelialisation of 3D full thickness wounded human skin equivalents. *Representative photomicrographs of H&E stained patient-matched 3D full thickness wounded human skin equivalents derived from young (18-35 years old) individuals that were treated in the presence of absence of 1mg/ml Peptan P added topically at 1, 2, 3 and 4 weeks post-wounding. Magnification = 10x. Scale bar = 200µm.*

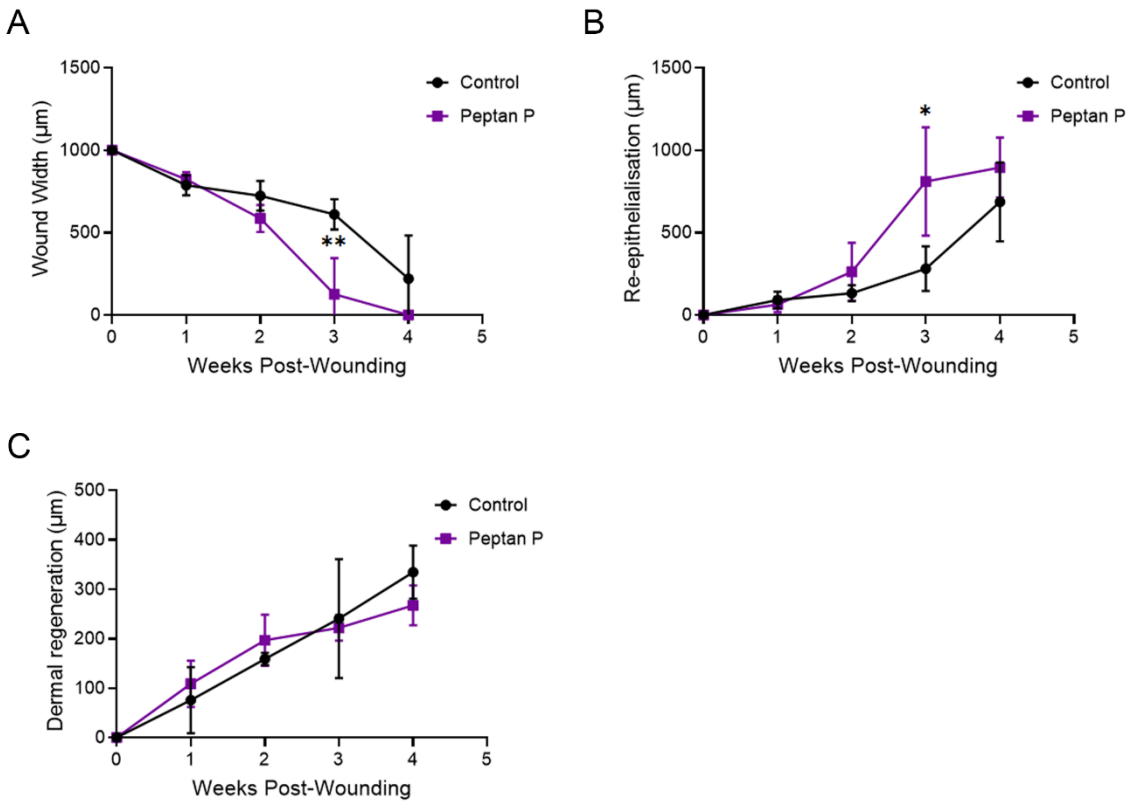

Figure 5. 8 Peptan P significantly enhances wound closure of 3D wounded human skin equivalents by promoting re-epithelialisation. *(A) Wound width (µm), (B) re-epithelialisation (µm) and (C) dermal recovery (µm) of 3D wounded human skin equivalents treated in the presence or absence of 1mg/ml Peptan P added topically and harvested at 1, 2, 3 and 4 weeks post-wounding. (Mean ± SD, N=3, *P<0.05, **P<0.01).*

Theoretically, the induction of a 1mm punch wound to the 3D skin equivalents creates a cylindrical void within the 3D human skin equivalents. Assuming that equal healing occurs across the entire cylindrical void of the punch wound, the equations described in Section 2.21 were used to calculate the volume of epidermal and dermal regeneration in both control and Peptan P-treated wounded 3D skin equivalents. Results again, confirmed that Peptan P-induced re-epithelialisation of the 3D wounded skin equivalents promoted the increased the volume of newly regenerated epidermis at 3 and 4 weeks post-wounding compared to the untreated control (Figure 5.9A, Two-way ANOVA with Tukey's multiple comparisons test, **P<0.01, ***P<0.001). However again, results revealed no significant effect of Peptan P on the regeneration of the dermal layer compared to the untreated control (Figure 5.9B, Two-way ANOVA with Tukey's multiple comparisons test, ns). Collectively, these data suggest Peptan P promotes cutaneous wound healing

through enhanced re-epithelialisation thereby supporting its potential as a therapeutic strategy for the treatment of superficial cutaneous wounds.

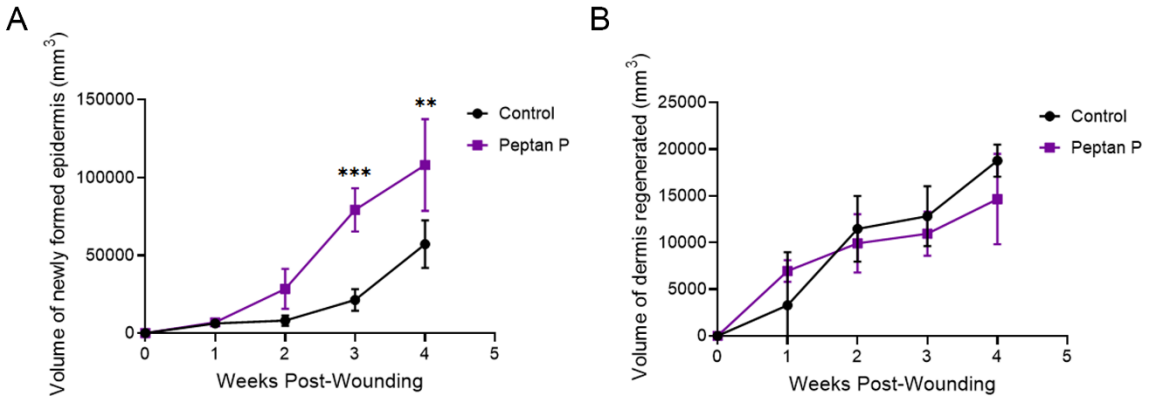

Figure 5. 9 Peptan P significantly enhances the volume of newly regenerated epidermis in 3D wounded human skin equivalents. *Volumetric analysis of newly regenerated (A) epidermis and (B) dermis in 3D wounded skin equivalents treated in the presence or absence of 1mg/ml Peptan P added topically at 1, 2, 3 and 4 weeks post-wounding. (Mean ± SD, N=3, **P<0.01, ***P<0.001).*

5.3 Discussion

While 2D scratch assays are quick, cost effective and reliable for the screening of new drugs and compounds for wound healing applications, they do not fully represent the 3D cutaneous environment and events that occur during *in vivo* wound healing such as cell-cell and cell-ECM interactions. Therefore, the use of 3D human skin equivalents are necessary in order to accurately mimic a 3D cutaneous environment during wound healing and truly evaluate the potential for new drugs and compounds such as bioactive collagen peptides for promoting cutaneous wound healing.

Collagen peptides are typically administered orally, however current *in vitro* scratch assays used in the previous chapters do not full represent this type of administration and therefore further *in vitro* scratch assays would need to be conducted in the presence of serum derived from patients who had ingested Peptan P in order to better determine the effects of orally ingested collagen peptides on promoting wound healing *in vitro*. Given the difficulty to mimic oral administration in the current assays used, other routes of administration such as a topical approach were considered for wound healing applications. Experiments mimicking the topical application of Peptan P to wounded keratinocytes and dermal fibroblasts in 2D scratch assays revealed that 1mg/ml Peptan P applied topically induced a similar significant effect on the promotion of wound closure as seen when the same concentration of this collagen peptide is used to coat tissue culture wells prior to wounding (Figure 5.1 and 5.2). The fact that high concentrations such as 5mg/ml and 10mg/ml of Peptan P added topically had no effect on both keratinocyte and dermal fibroblast wound closure in 2D scratch assays suggests such doses are too concentrated for a beneficial effect to be exerted. Data in Chapter 4 suggests Peptan P promotes wound closure through its interaction with integrin receptors suggesting that higher concentrations of Peptan P may generate strong adhesion complexes with integrin receptors that prevent cells from generating sufficient traction in order to enhance cell motility (Koivisto *et al.*, 2014). Whilst the topical application of 1mg/ml Peptan P is optimal for promoting keratinocyte and dermal fibroblast wound closure in a 2D environment, the use of optimised full thickness wounded 3D skin equivalents was used to confirm the beneficial effects of Peptan P on wound healing in a more representative cutaneous environment.

The use of 3D skin equivalents helps to mimic the cutaneous environment *ex vivo*, allowing for better analyses of cell-cell and cell-ECM interactions that cannot be observed in 2D culture. Many researchers have developed skin equivalent models that rely on the encapsulation of dermal fibroblasts into an exogenous ECM source such as collagen or fibronectin in order to generate the dermal layer before overlaying with keratinocytes and allowing for epidermal differentiation (Gangatirkar *et al.*, 2007; Carlson *et al.*, 2008). However, most previously described human skin equivalent models have a short longevity limiting their viability and use for downstream applications such as wound healing (Ackermann *et al.*, 2010; Vidal *et al.*, 2019). To counteract these limitations, recent studies have demonstrated that the use of Alvetex® scaffolds creates an environment that supports dermal fibroblasts to produce their own ECM, allowing for the generation of a more robust and reproducible 3D human skin equivalent models that is viable for up to 20 weeks, making them more suitable for downstream applications such as the modelling of melanoma invasion and wound healing (El Ghalbzouri *et al.*, 2009; Hill *et al.*, 2015). Similar to previous 3D human skin equivalents generated using Alvetex® scaffolds, the patient-matched 3D human skin equivalent models were generated and verified by their distinct epidermal and dermal layers similar to normal human skin, with immunofluorescent expression of collagen I and collagen III demonstrating the production of a cutaneous ECM within the dermal layer as well as by the immunofluorescent expression of keratin 1, keratin 14 and loricrin in the 3D human skin equivalents mirroring epidermal differentiation observed in normal human skin (Figure 5.3 – Figure 5.5) (Hill *et al.*, 2015; Roger *et al.*, 2019).

Whilst various attempts have tried to mimic an ageing cutaneous microenvironment within 3D skin equivalent models, with previous studies incorporating aged keratinocytes (53-66 years old) onto collagen gels exhibiting a thinner epidermis as well as increased $p16^{INK4a}$ expression compared to younger keratinocytes (30-40 years old). However, these 3D human skin equivalent models do not fully capture an ageing cutaneous microenvironment as the use of a fibroblast-collagen gel matrix does not allow for the study of intrinsic ageing on the ECM and dermal layer (Adamus *et al.*, 2014). Additional studies using mitomycin C-treated dermal fibroblasts to generate 3D skin equivalents have demonstrated features of ageing such as decreased dermal fibroblast populations and fragmented dermal

architecture. However, these models still provide limitations as it is unclear whether these observations are due to an ageing phenotype or because treatment with mitomycin C is impairing normal dermal fibroblast function (Diekmann *et al.*, 2016). To evaluate the potential for 3D human skin equivalents generated using Alvetex® scaffolds to mimic an ageing cutaneous microenvironment, additional 3D skin equivalents were generated using patient-matched keratinocytes and dermal fibroblasts derived from young (18-35 years old) or aged (60+ years old) individuals, with results demonstrating that the use of patient-matched keratinocytes and dermal fibroblasts derived from aged individuals also resulted in the formation of a thinner epidermis layer compared to 3D skin equivalents generated using young keratinocytes and dermal fibroblasts (Figure 5.6). Whilst these models demonstrate the ability to mimic an ageing cutaneous microenvironment, particularly with the epidermal layer, further studies are warranted to explore cell-cell and cell-ECM interactions between the epidermis and dermis and how ageing influences these interactions.

Cutaneous wound healing is a multicellular process that involves various cell-cell and cell-ECM interactions that cannot be analysed in 2D culture alone. To mimic a wounded cutaneous microenvironment *ex vivo*, 3D human skin equivalents were wounded using a 1mm punch biopsy before placing onto an extra dermal layer to enhance robustness and provide support for the repair of the wounded 3D skin equivalent as previously described (Dr Moyassar Al-Shaibani, Deciphering the Role of Mesenchymal Stem Cells (MSCs) in Cutaneous Wound Healing, 2018, Newcastle University). The use of this adapted wounded 3D human skin equivalent model mimicked cutaneous wound healing *in vivo* by mirroring the re-epithelialisation of the damaged epidermis as well as the regeneration of the dermal layer, as evidenced by keratinocytes within the epidermal layer migrating collectively as a sheet, moving from the intact wound edges towards the centre of the wound (Figure 5.7) (Chavez *et al.*, 2012; Evans *et al.*, 2013). Dermal regeneration on the other hand occurred more slowly with larger areas of regenerated dermis appearing closer to the wound edges and with an observed gradual decrease in thickness of the newly regenerated dermis towards the centre of the wound (Figure 5.7). The fact that the dermal region heals at a slower rate compared to the epidermis coincides with previous studies demonstrating that

during cutaneous wound healing dermal fibroblasts usually exhibit a relative slow migration into provisional matrix whilst keratinocytes migrate more rapidly in order to re-establish the epidermal barrier to prevent infections (Friedl and Wolf, 2010). Additionally, the remodelling of the ECM can take up to a year post-wounding to restore tensile strength which may further explain the slow regeneration of the dermis observed in the present wounded 3D skin equivalents, where the secretion of various matrix proteins are required for remodelling the newly synthesised ECM in order to increase tensile strength and support the newly regenerated epidermis (Xue and Jackson, 2015).

To determine the potential for Peptan P to promote cutaneous wound healing in an environment more representative of *in vivo* wound healing, Peptan P was added topically to wounded 3D skin equivalent models before evaluating its effects on both epidermal and dermal regeneration. Peptan P significantly promoted wound closure of the 3D human skin equivalents by enhancing re-epithelialisation, with the complete restoration of the epidermis observed 3 weeks post-wounding (Figure 5.7 and 5.8A and B). The prompt re-epithelialisation of the wounded 3D skin equivalent suggests the potential for Peptan P as a treatment for superficial cutaneous wounds as the restoration of an effective epidermal barrier is crucial to prevent further complications such as wound infections and dehydration (Singh *et al.*, 2009; Wikramanayake *et al.*, 2014). Previous data described in Chapter 4 demonstrated that Peptan P promotes keratinocyte wound closure through its interactions with integrin receptors, suggesting that this interaction with Peptan P may be responsible for the enhanced re-epithelialisation and formation of the newly generated epidermis across the wound bed of the 3D skin equivalents. This potential is supported by previous studies that demonstrate the interactions with integrin receptors are crucial to effective re-epithelialisation. Furthermore the loss of integrin expression impairs the migration of the epithelium across the wound, thus further supporting the mechanistic action of Peptan P to provide anchorage points for migrating keratinocytes in order to enhance wound closure (Singh *et al.*, 2009).

The use of the current wounded 3D skin equivalent model also allowed for the investigation for the potential for Peptan P to promote dermal regeneration, providing an advantage compared to other skin equivalent models generated using an collagen gel matrix, as the collagen gel in this context usually contracts into the

wound bed over time, making it difficult to distinguish whether new dermal tissue has been formed or whether the decrease in wound width is due to the natural contraction of the gel (Safferling *et al.*, 2013). Whilst the present optimised wounded 3D skin equivalents were suitable for modelling dermal regeneration, results however revealed no significant effect of Peptan P on promoting dermal regeneration, contradicting previous results in 2D scratch assays that suggested Peptan P promoted dermal fibroblast wound closure through enhanced cellular proliferation (Figure 5.7 and 5.8C) (Mistry *et al.*, 2021). These results likely reflect the lack of environmental factors in the 2D environment such as the ECM that affects the physiology, morphology and spatial orientation of dermal fibroblasts and thereby the potential response of dermal fibroblasts to Peptan P treatment (Ralston *et al.*, 1997; Sun *et al.*, 2006; Randall *et al.*, 2018).

The cross talk between keratinocytes and dermal fibroblasts is crucial for wound healing as dermal fibroblasts can secrete growth factors such as KGF, which is known to promote both keratinocyte migration and proliferation (Werner *et al.*, 1994b; Pastar *et al.*, 2014). Previous studies have revealed that collagen peptides can increase the expression and secretion of wound healing-associated growth factors, suggesting that while Peptan P did not affect dermal regeneration, it could perhaps promote the secretion of growth factors such as KGF by dermal fibroblasts, in turn inducing paracrine effects on nearby wounded keratinocytes to promote the re-epithelialisation of the damaged epidermis (El Ghalbzouri *et al.*, 2003; Safferling *et al.*, 2013; Yang *et al.*, 2019). Additionally, cytokines such as MMP-9 play a crucial role during wound repair as its expression at the wound edges is thought to be responsible for the detachment of basal keratinocytes from the basement membrane in order to promote their migration, with previous studies demonstrating expression of MMP-9 at the leading edge of epidermal cells in both human and mouse models (Hattori *et al.*, 2009; Rakita *et al.*, 2020). Additional studies are thus warranted to explore the role of Peptan P on growth factor and cytokine activity as well as keratinocyte and dermal fibroblast cross talk in order to determine the potential impact of Peptan P on such signalling mechanisms and their contribution to the observed enhancement in re-epithelialisation.

Given that the use of a punch biopsy generates a cylindrical void, further analyses were conducted to determine the effect of Peptan P on the volume of newly

regenerated epidermis and dermis in the wounded 3D skin equivalents, with results again demonstrating that treatment with Peptan P significantly enhanced the volume of newly regenerated epidermis but had no effect on dermal regeneration, thus further supporting the potential use of Peptan P for the treatment of superficial cutaneous wounds (Figure 5.9). Whilst mathematical equations were used to calculate volumetric healing, this analysis was carried out under the assumption that there is equal healing across the entire cylindrical void of the punch wound, however wound healing does not always occur uniformly. Sometimes, the epidermis will heal unevenly, with faster re-epithelialisation being observed from one area of the wound compared to the other making the equations used in the present study void. Previous studies have used non-invasive fluorescent cell tracer dyes to accurately monitor re-epithelialisation in real time in *ex vivo* human skin models with complete restoration of the damaged epithelium observed by 10 days post-wounding. Therefore, similar studies should be conducted using the wounded 3D skin equivalents in order to further assess the effects of Peptan P on wound healing and observe any cell-cell and cell-ECM interactions in real time that will help to elucidate any further additional mechanisms activated by Peptan P (Safferling *et al.*, 2013; Nasir *et al.*, 2019).

While the use of the current wounded 3D skin equivalent models can accurately mimic the proliferation and remodelling phases of cutaneous wound healing, providing insights into keratinocyte and dermal fibroblast behaviour in responses to new therapeutics such as collagen peptides, they are unable to mirror inflammatory responses or angiogenesis due to the lack of immune and endothelial cells present within the current 3D human skin equivalent model. The further modification of the 3D human skin equivalent model to incorporate immune and endothelial cells is currently being undertaken in the Lovat lab at Newcastle University for studies of melanoma invasion and as such these models may also be useful for *ex vivo* wound healing studies. For example, angiogenesis is an important process during cutaneous wound healing, allowing for the delivery of nutrients and oxygen that are required by cells in order to promote cellular migration and proliferation (Reinke and Sorg, 2012). Therefore, the incorporation of both immune and endothelial cells into the wounded 3D skin equivalent model would not only allow for the development of a more complex *ex vivo* model that can mimic additional events that occur in an *in*

vivo wounded cutaneous environment but also allow the possibility to further evaluate the potential of any future wound healing therapeutics in a more representative cutaneous environment.

Overall, these data demonstrate that Peptan P promotes wound closure by enhancing re-epithelialisation, thus suggesting that it would be a viable strategy for the treatment of superficial cutaneous wounds.

5.4 Summary

- Topical application of 1mg/ml Peptan P significantly enhances wound closure of both primary keratinocytes and dermal fibroblasts in 2D culture
- Patient-matched aged 3D human skin equivalents display features on aged human skin *ex vivo*
- Peptan P promotes wound closure of full thickness wounded 3D human skin equivalents by promoting re-epithelialisation
- Peptan P has no significant effect on dermal regeneration in full thickness wounded 3D skin equivalents
- Peptan P may be a viable strategy for the treatment of superficial cutaneous wounds

Chapter 6: Final Discussion and Concluding Remarks

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Cutaneous wound healing is a complex biological process involving multiple overlapping phases and events that are required to repair damaged tissue. Any imbalance to this process contributes to the development of chronic non-healing wounds that present further complications such as infections and pro-longed hospitalisation. The emergence of chronic non-healing wounds thereby inflicts a heavy burden onto worldwide healthcare providers, emphasising the need for the development of novel and reliable treatment options that can be used to treat cutaneous wounds and ease both the clinical and financial burden associated with chronic non-healing wounds (Sen *et al.*, 2009; Järbrink *et al.*, 2017).

Bioactive collagen peptides that are generated through the hydrolysis of animal bones, skin and connective tissue are currently used to manage various health conditions ranging from skin ageing to osteoarthritis (Inoue *et al.*, 2016; Boonmaleerat *et al.*, 2018; Czajka *et al.*, 2018; Bolke *et al.*, 2019). More recently, a growing interest is emerging in the potential therapeutic application for bioactive collagen peptides as a treatment of cutaneous wounds, with preliminary studies demonstrating chemotactic properties that stimulate cellular migration *in vitro* and enhance wound closure in animal models (Postlethwaite *et al.*, 1978; Zhang *et al.*, 2011; Wang *et al.*, 2015a). The present study was aimed at evaluating the potential for collagen peptides to promote cutaneous wound healing both *in vitro*, in the context of age and in *ex vivo* models of wounded human skin as well as elucidating the mechanistic action mediating their effect.

Similar to previous findings, results from the present study demonstrated the ability for collagen peptides to promote wound closure *in vitro*, with collagen peptides derived from porcine skin (Peptan P) exerting a greater effect on the promotion of both keratinocyte and dermal fibroblast wound closure in 2D scratch assays compared to collagen peptides derived from either bovine (Peptan B) or fish (Peptan F) skin, implying the presence of more peptides within Peptan P that appear to be more beneficial for wound healing applications compared to Peptan B or Peptan F (Mistry *et al.*, 2021). Further supporting the potential of Peptan P to promote cutaneous wound healing, 2D scratch assays conducted in the context of age demonstrated that Peptan P enhanced wound closure of both young and aged

keratinocytes and dermal fibroblasts. Additional studies also demonstrated that Peptan P most likely promotes wound healing through its promotion of cellular proliferation (Mistry *et al.*, 2021). These results corroborate with previous studies that demonstrated an enhancement of dermal fibroblast proliferation following treatment with the collagen dipeptide Pro-Hyp, suggesting that a high abundance of this dipeptide within Peptan P may be responsible for the observed enhancement of cellular proliferation (Shigemura *et al.*, 2009; Ohara *et al.*, 2010). Two-dimensional *in vitro* studies of wound healing conducted in a hyperglycaemic environment to mimic that of diabetic ulcers also showed the ability for Peptan P to promote both keratinocyte and dermal fibroblast wound closure in concentrations of glucose up to 25mM. However, there was no notable effect of Peptan P on the enhanced proliferation of either primary keratinocytes or dermal fibroblasts cultured in a hyperglycaemic environment. Whilst a hyperglycaemic environment observed in diabetic ulcers is defined as glucose concentrations higher than 25mM (Güemes *et al.*, 2016), further studies using more representative models of diabetic ulcers such as using keratinocytes or dermal fibroblasts that have been derived from patients with diabetic ulcers in *in vitro* scratch assays or in wounded 3D skin equivalents *ex vivo* are required in order to determine whether the use of Peptan P could represent a novel therapeutic strategy for diabetic ulcers.

In an attempt to define the mechanistic action of collagen peptides, multiple studies explored their potential interactions with various cell types and subsequent ability to activate various signalling pathways associated with cellular proliferation, migration and differentiation (Banerjee *et al.*, 2014; Liu *et al.*, 2014). Previous studies indicated that the promotion of osteoblast proliferation and differentiation by porcine-derived collagen peptide was due to the activation of the PI3K/Akt signalling pathway (Zhu *et al.*, 2020), whilst studies of collagen peptide-induced proliferation of mouse dermal fibroblasts suggest activation of the NF-κB signalling pathway is key (Yang *et al.*, 2019). Integrin signalling is a key signalling pathway associated with wound healing whereby integrin receptors recognise specific motifs present within ECM proteins such as collagen, leading to the activation of downstream signalling pathways that invoke cellular proliferation such as FAK, ERK and Akt (Pirone *et al.*, 2006; Moreno-Layseca and Streuli, 2014; Bezerra *et al.*, 2018). These observations prompted an investigation into the potential contribution of integrin

signalling to Peptan P-induced cutaneous wound healing *in vitro*. Results indicated that Peptan P promotes cutaneous wound healing through its interaction with integrin receptors present on the cell surface of both keratinocytes and dermal fibroblasts, evidenced by increased expression of the integrin α2β1 receptor. Further supporting the interaction of Peptan P with integrin receptors, RNAi-mediated knockdown of the integrin β1 subunit significantly impaired Peptan P-induced wound closure of both cell types. Collectively these data suggest, at least *in vitro*, that this Peptan P-integrin interaction mediates cutaneous wound healing by providing anchorage points that help to increase cell motility.

Further studies investigating the activation of downstream signalling pathways linked to cellular proliferation revealed Peptan P had no significant effect on the activation of Akt signalling in either unwounded or wounded keratinocytes or dermal fibroblasts. However, consistent with studies showing the activation of ERK and FAK is linked to epithelial wound closure (Teranishi *et al.*, 2009), Peptan P significantly enhanced ERK activation during early phases of keratinocyte wound closure, with subsequent activation of FAK, implying that Peptan P-induced activation of ERK occurs independently of FAK activation but may also play a role in the activation of FAK during later phases of keratinocyte wound closure. Collectively, these data suggest that in keratinocytes, Peptan P promotes the activation of an ERK-FAK signalling cascade that contributes to enhanced cellular migration and proliferation during wound healing *in vitro*. Surprisingly however, studies in wounded dermal fibroblasts revealed activation of ERK regardless of collagen peptide treatment, suggesting the activation of alternative downstream signalling pathways that promote dermal fibroblast wound closure following integrin ligation and warranting further studies to explore the potential contribution of other downstream signalling mechanisms such as the Rho GTPases; Rac1 and cdc42 in promoting Peptan P-induced wound closure of dermal fibroblasts (Desai *et al.*, 2004; Liu *et al.*, 2009a; Pothula *et al.*, 2013). Taken together, these data support the hypothesis that Peptan P provides anchorage points within the cutaneous wound through integrin ligation, thereby facilitating subsequent activation of integrin signalling and the promotion of wound closure. Future experiments should explore the use of reporter genes and live-cell imaging techniques to better understand Peptan P-induced activation of both ERK and FAK in order to determine whether

these increase in activation is observed by all cells within the wound or whether its only activated with a subpopulation such as the cells at the leading edge. This will provide a better understanding of the mechanistic action of Peptan P to promote cutaneous wound healing.

Notably, Peptan P not only enhanced cell surface expression of integrin α2β1 by both dermal fibroblasts and keratinocytes but also increased expression of integrin α5β1 by both cell types, demonstrating the ability for Peptan P to interact with other ECM receptors apart from collagen receptors. Integrin α5β1 is a receptor for fibronectin, which is predominately produced during earlier phases of wound healing to generate the provisional matrix that wound keratinocytes and dermal fibroblasts migrate along (Enoch and Leaper, 2008; Di Russo *et al.*, 2021). The fact that Peptan P enhanced the expression of integrin α5β1 by both keratinocytes and dermal fibroblasts suggests this collagen peptide may provide additional anchorage points within the provisional matrix of the cutaneous wound, thus further enhancing the promotion of re-epithelialisation and also the subsequent promotion of later phases of cutaneous wound healing such as proliferation and remodelling. Given that Peptan P has been shown to contain motifs that allow it interact with other receptors rather than just collagen receptors, further studies should be conducted to sequence all the collagen peptides present within Peptan P in order to determine whether there may be other cryptic motifs that are capable of interacting with other receptors such as growth factor receptors and thereby eliciting other mechanisms during wound healing that are not typically associated with native collagen. Further studies can then be conducted to determine affinity at which these peptides can bind to other receptors and there ability to activate their downstream signalling pathways.

Typically, collagen peptides are administered orally with previous studies in both animals and humans demonstrating the ability for collagen peptides to be retained for up to 14 days in various tissues such as skin and bone (Watanabe-Kamiyama *et al.*, 2010; Taga *et al.*, 2019). Consistent with previous studies that explored the bioavailability of collagen peptides post-ingestion (Iwai *et al.*, 2005; Ohara *et al.*, 2007), results from the present study demonstrated that oral ingestion of 10g Peptan P resulted in the detection of similar micromolar concentrations in the bloodstream for up to 24 hours post-ingestion and moreover, at similar concentrations that have been shown to promote wound closure *in vitro* (Mistry *et*

al., 2021). Taken together, these data suggested that the oral administration of collagen peptides could be a quick and easy strategy for the treatment of cutaneous wounds. However, the analysis of collagen peptides that had been subjected to an *in vitro* digestion revealed that this simulated digestion process impaired their ability to promote both keratinocyte and dermal fibroblast wound closure *in vitro*. Given the limitations of the current *in vitro* assays used in the present study to reflect the oral administration of collagen peptides, further studies are warranted using labelled peptides *in vivo* in order to accurately identify which peptides are present in systemic circulation post-ingestion as well as additional *in vitro* screening assays using serum derived from patients who have ingested Peptan P in order to better define whether the oral administration of collagen peptides is a suitable approach for wound healing applications.

To investigate the potential for the topical application of Peptan P as a therapeutic approach for cutaneous wound healing, Peptan P was added to both keratinocytes and dermal fibroblasts post-wounding in 2D scratch assays as well as to 3D human skin equivalents *ex vivo*. Initial dose response studies conducted in 2D scratch assays demonstrated that the topical application of 1mg/ml Peptan P was the optimal concentration to promote both keratinocyte and dermal fibroblast wound *in vitro*. Subsequent studies in wounded 3D human skin equivalents further confirmed the ability of Peptan P to promote wound closure by enhancing re-epithelialisation, thus suggesting Peptan P as a more suitable treatment for superficial cutaneous wounds rather than full-thickness wounds. Further experiments are warranted to explore in more detail the effect of Peptan P on re-epithelialisation such as changes in expression of keratins as well as whether Peptan P has any effect on the secretion or expression of growth factors or cytokines associated with re-epithelialisation. Additional experiments should also look to generate more complex wounded skin equivalents such as the inclusion of reticular and papillary dermal fibroblasts, immune cells and endothelial cells to better characterise wound healing *ex vivo* and determine any beneficial effect of Peptan P on other cutaneous cells in a more representative 3D environment.

Noteworthy, were studies showing the suitability of optimised *ex vivo* skin equivalents to mimic an ageing cutaneous environment, particularly a thinning epidermis. Further studies are required to validate *ex vivo* skin equivalents can truly

mimic an ageing cutaneous environment. These should include histological staining's such as Picrosirius red and Weigert's haemotoxylin to stain collagen and elastic fibres within the skin equivalents. Additional studies should look at whether the aged skin equivalents exhibit features of ageing skin *in vivo* such as decreased proliferation and increased senescence. Given that ageing increases the prevalence of chronic wound development and results showing Peptan P promotes cutaneous wound healing in the context of age *in vitro*, further studies are warranted to look at the potential for Peptan P to promote wound healing in aged wounded 3D skin equivalents. Such studies would allow better characterisation of how ageing impacts cutaneous wound healing responses *ex vivo* and also determine the potential for Peptan P to enhance re-epithelialisation in an ageing context. Additionally, further experiments looking at expression of precursor (markers specific for the N- and C-terminals) and mature collagen should be explored in order to better define whether Peptan P has any effect on dermal regeneration by stimulating the synthesis of new collagen within the cutaneous wound.

In summary, the results from this thesis highlight the translational and clinical potential for Peptan P as a viable topical therapeutic to promote re-epithelialisation of superficial cutaneous wounds.

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