The Role of Flightless Protein in Hypertrophic Scarring and its Potential as a Target for a Novel Therapy.

Alexander MacGregor Cameron

University of Adelaide

Faculty of Health Sciences

School of Medicine

Department of Surgery

Supervisors:
Professor Allison Cowin
Professor Peter Anderson

Regenerative Medicine
Future Industries Institute
Mawson Lakes
September 2017

TABLE OF CONTENTS

TITLE PAGE ..... **i**

TABLE OF CONTENTS ..... **ii**

LIST OF FIGURES ..... **ix**

ABSTRACT ..... **xiii**

DECLARATION ..... **xv**

ACKNOWLEDGMENTS ..... **xvi**

PUBLICATIONS ARISING FROM THIS THESIS ..... **xix**

NATIONAL AND INTERNATIONAL MEETINGS, SCIENTIFIC ABSTRACTS

RESEARCH GRANTS AND AWARDS ARRISING FROM THIS THESIS ..... **xx**

CHAPTER ONE: LITERATURE REVIEW

1.1 Overview **2**

1.2 Skin Biology **5**

1.2.1 Epidermis **6**

1.2.2 Dermis **9**

1.3 Wound Healing **10**

1.3.1 Inflammatory Phase **10**

1.3.2 Proliferative Phase **11**

1.3.3 Remodelling Phase **12**

1.4 The Myofibroblast 13

1.4.1 Myofibroblast origin 14

1.4.2 Fibroblast-myofibroblast Differentiation and Tissue Stiffness....15

1.4.3 How do myofibroblasts sense mechanical stress? 16

1.4.4 The myofibroblast and fibroproliferative disease 17

1.5 The Cytoskeleton..... 19

1.5.1 Actin dynamics during wound healing.....20

1.5.2 Actin cytoskeleton, cell motility and cell matrix.....21

1.6 Flightless Protein 23

1.6.1 The gelsolin family of actin remodelling proteins 31

1.6.2 Flightless I (Flii) protein.....26

1.6.3 Flightless I structures, expression and molecular functions.....28

1.6.4 Flii in wound healing and tissue regeneration 33

1.7 Hypertrophic Scarring..... 37

1.7.1 Defininig hypertrophic scarring..... 37

1.7.2 Epidemiology.....39

1.7.3 Burn injury, hypertrophic scarring and current treatments.....40

1.7.3.1 Burn injury 40

1.7.3.2 History of burn injury management..... 41

1.7.3.3 Current management of hypertrophic scarring 43

1.7.4 Current understanding of the pathophysiology of hypertrophic scarring 51

1.7.4.1 Inflammation 51

1.7.4.2 Extracellular matrix 52

1.7.4.3 Keratinocyte fibroblast interaction 54

1.7.4.4 Reversibility of fibrosis 55

1.8 Pre-clinical models of hypertrophic scarring 55

1.9 Research Aims and Hypothesis 64

CHAPTER TWO: MATERIALS AND METHODS

2.1 Materials 63

2.1.1 Molecular reagents 63

2.1.2 Immunohistochemical reagents 63

2.1.3 General reagents 66

2.1.4 Osmotic pumps 66

2.1.5 Human samples 67

2.1.6 Animal tissue 68

2.1.6.1 Flii$^{+/-}$ mouse generation 71

2.1.6.2 Flii$^{Tg/Tg}$ mouse generation 72

2.1.7 Flii antibody generation 73

2.2 Methods 74

2.2.1 A novel murine model of hypertorhic scarring using subcutaneous

infusion of bleomycin **74**

2.2.2 Murine surgery **75**

2.2.3 Flii neutralizing antibody treatment **77**

2.2.4 Sample processing, histology and immunohistochemistry **77**

2.2.5 Immunohistochemistry and image analysis **79**

2.2.6 Cell lines and cell culture **80**

2.2.7 HFF isolation and culture **81**

2.2.8 Murine fibroblast isolation and culture **81**

2.2.9 Migration scratch assay **82**

2.2.10 WST-1 proliferation assay **82**

2.2.11 Immunocytochemistry, image analysis and fluorescence co-localization **83**

2.2.12 Protein extraction and quantification **84**

2.2.13 Western Blotting **85**

2.3 Statistical analysis **86**

CHAPTER THREE: EXPRESSION OF FLII IN BURNS AND HYPERTROPHIC SCARS

3.1 Introduction **88**

3.2 Results **90**

3.3 Discussion **96**

CHAPTER FOUR: A NOVEL MURINE MODEL OF HYPERTROPHIC SCARRING USING SUBCUTANEOUS INFUSION OF BLEOMYCIN

4.1 Introduction 101

4.2 Results 105

4.2.1 The bleomycin model produces tissue architecture that corresponds to hypertrophic scars 105

4.2.2 The bleomycin model displays histopathological features of hypertrophic scars 110

4.2.3 Collagen content and composition of bleomycin induced lesions is analogous to hypertrophic scars 111

4.2.4 Key determinants of fibroproliferation are increased in the bleomycin model 113

4.3 Discussion 117

CHAPTER FIVE: EFFECT OF FLIGHTLESS ON HYPERTROPHIC SCARRING *IN VIVO*

5.1 Introduction 123

5.2 Results 126

5.2.1 Decreasing Flii reduces acute and mature scar formation 126

5.2.2 Reducing Flii reduces collagen-1:3 ratio 131

5.2.3 Manipulation of Flii level affects key determinants of scarring 134

5.2.4 Flightless I neutralizing antibody treatment reduces fibrosis in day 28 scars 137

5.3 Discussion 140

CHAPTER SIX: EFFECT OF FLIGHTLESS ON FIBROBLAST PHENOTYPE IN VITRO

6.1 Introduction 144

6.2 Results 146

6.2.1 Flii is increased in human hypertrophic scar fibroblasts compared to human foreskin fibroblast 146

6.2.2 FnAb mediates fibroblast-myofibroblast phenotype change in HFFs treated with bleomycin 148

6.2.3 Treatment of primary fibroblasts from Flii$^{+/-}$, WT and Flii$^{Tg/Tg}$ mice with bleomycin and FnAb 151

6.3 Discussion 157

CHAPTER SEVEN: GENERAL DISCUSSION AND FUTURE DIRECTIONS

7.1 Discussion 162

7.2 A role for Flightless in hypertrophic scarring and the development of a novel murine model of hypertrophic scarring 162

7.3 Potential mechanisms for the role of Flii in hypertrophic scarring 166

7.3.1 TGF-β-Smad pathway 166

7.3.2 Flii and other cell signaling pathways 169

7.3.3 Flii regulation of keratinocyte function 170

7.1.2.4 Flii and cytoskeletal regulation of the fibroblast.....171

7.4 FnAb and potential as a novel therapy..... 174

7.5 Future Directions.....175

7.6 Conclusion.....178

REFERENCES.....182

APPENDIX.....198

A.1 WST-1 calculations for ideal bleomycin concentration in cell culture 198

A.2 Papers published in peer reviewed journals.....199

A.3 Consent forms and ethics approvals.....223

LIST OF FIGURES AND TABLES

CHAPTER 1

1.1 Spectrum of wound healing pathology 3

1.2 Anatomy of the skin 5

1.3 Diagrammatic representation of human skin 8

1.4 The fibroblast and the proliferative phase of wound healing 12

1.5 Fibroblast to myofibroblast differentiation as a function of tissue stiffness 15

1.6 Comparison of Flii and gelsolin proteins 25

1.7 Gastrulation and cellularisation defects in Flii homozygous embryos 27

1.8 Schematic diagram of Flii illustrating its role as a nuclear receptor co-activator and formation of the co-activator complexes at the promoter of an NR responsive gene 31

1.9. Representative partial thickness burn wounds seen in Flii mice 36

1.10 Complications of hypertrophic scarring 38

CHAPTER 2

2.1 (Table) Antibody information and concentrations used in western analyses and immunofluorescence experiments 65

2.2 Alzet micro-osmotic pump 66

2.3 Targeted disruption of Flii gene 70

2.4 Murine model of hypertrophic scarring using subcutaneous infusion of bleomycin 75

CHAPTER 3

3.1 The author collecting human tissue samples from the RAH Adult Burns'Service 90

3.2 Clinical photographs of acute burns prior to sample collection 92

3.3 Clinical photographs of hypertrophic scars prior to sample collection..... 93

3.4 Histopathological features of hypertrophic scars..... 94

3.5 Immunolocalisation of Flii in human skin, acute burns and hypertrophic scars..... 95

CHAPTER 4

4.1 The bleomycin model reproduces histologic architecture of hypertrophic scars..... 106

4.2 Quantification of dermal thickness 108

4.3 The bleomycin model reproduces histopathological features of hypertrophic scars 110

4.4 Lesions produced by the 28-day and 56 day bleomycin models have altered collagen-1:3 ratios 112

4.5 Myofibroblasts are increased in the bleomycin model scars compared to control 114-5

4.6 TGFβ1 is increased in bleomycin model scars compared with controls..116

CHAPTER 5

5.1 Decreasing Flii reduces dermal thickness in 28-day and 56-day fibrosis models 127

5.2 Decreasing Flii reduces dermal thickness in 28 day and 56 day fibrosis models 128

5.3 Decreasing Flii reduces scar cross sectional area in day 28 and day 56 fibrosis models 129

5.4 Decreasing Flii reduces scar cross sectional area in day-28 and day-56 fibrosis models 130

5.5 Decreasing Flii reduces collagen-1:3 ratio in the 28-day model..... 132

5.6 Decreasing Flii reduces collagen-1:3 ratio in the 56-day model..... 133

5.7 TGFβ-1 expression is higher as Flii levels are increased 135

5.8 Increasing Flii leads to higher number of myofibroblasts 136

5.9 FnAb treatment reduces scarring in the 28-day model of acute fibrosis. 138

5.10 FnAb treatment reduces collagen-1:3 28-day model of acute fibrosis... 139

CHAPTER 6

6.1 Immunolocalisation of Flii in human foreskin fibroblasts (HFF) and hypertrophic scar fibroblasts (HTSF) 147

6.2 Co-culture of HFF with bleomycin induces myofibroblast like phenotype changes that can be mediated by FnAb 150

6.3 Co-culture of primary fibroblasts from Flii$^{+/-}$, WT and Flii$^{Tg/Tg}$ mice with bleomycin and FnAb 152

6.4 Effects of bleomycin and FnAb on motility of cultured primary Flii$^{+/}$, WT and Flii$^{Tg/Tg}$ fibroblasts.....155

6.5 FnAb mediates the decreases in motility caused by co-culture with bleomycin 156

CHAPTER 7

7.1 Schematic illustrating potential sites of Flii intraction with TGFβ-Smad pathway..... 169

7.2 Schematic diagram summarizing potential mechanisms by which Flii may regulate the fibroproliferation underlying hypertrophic scarring.....174

ABSTRACT

Hypertrophic scarring is a poorly understood condition which affects the lives of millions of people around the world annually. Despite its common occurrence following burn injury, trauma or surgery the present treatments are of limited efficacy. Research over the past decade in the Cowin laboratory has identified Flightless (Flii), a highly conserved cytoskeletal protein, as a negative regulator of wound healing. Wounding leads to an increased expression of Flii, while Flii has been shown to inhibit cellular migration and proliferation. Reducing Flii *in vivo* leads to improved wound healing. The aim of this study was to investigate the role of Flii in the fibroproliferative process underlying hypertrophic scarring.

Chapter three shows for the first time that Flii expression in increased in human burn and hypertrophic scar tissue.

Chapter four details the development of a novel murine model of hypertrophic scarring. Previous animal models have focused on reproducing the clinical characteristics of the human hypertrophic scar, which often required significant derangement of the animal immune response. The novel model used bleomycin to stimulate the fibroproliferative process that underlies hypertrophic scarring. Results in this chapter use histology and immunohistochemistry to verify the bleomycin model as a valid model of hypertrophic scarring.

Chapter five uses the bleomycin mode to demonstrate that Flii is a key determinant of the extent of fibroproliferation that underlies hypertrophic scarring. Increasing Flii genetically in this animal model leads to increased dermal thickening and increases in key determinants of hypertrophic scarring, such as myofibroblasts, transforming growth factor β-1 (TGFβ-1) and scar collagen composition. Decreasing Flii genetically causes a reduction in hypertrophic scarring using the same measures. Decreasing Flii using a monoclonal antibody therapy in the bleomycin model also led to a reduction in hypertrophic scarring, confirming Flii as a potential target for a novel therapy for hypertrophic scarring.

Chapter six investigates potential mechanisms for the findings observed in previous chapters by using *in vivo* techniques. Focusing on the fibroblast, the key cell type in fibroproliferation, immunocytochemistry and cell migration assays, were used to show that decreasing Flii genetically or using a monoclonal antibody, reverses the fibroblast-myofibroblast phenotypic change that characterizes fibroproliferartive pathology.

Flii appears to be a key determinant of the fibroproliferative process underlying hypertrophic scarring. This study uses human tissue, a novel small animal model and *in vivo* techniques to demonstrate this and identify Flii as a potential target for a novel therapy to reduce or prevent hypertrophic scarring.

DECLARATION

"This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution to **Alexander MacGregor Cameron** and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text"

"I give consent to this copy of my thesis when deposited in the University Library, being made available for loan and photocopying, subject to the provisions of the Copyright Act 1968."

"The author acknowledges that copyright of the published work contained within this thesis (as listed in the – Publications arising from this thesis) resides with the copyright holder(s) of those works."

"I also give permission for the digital version of my thesis to be made available on the web, via the University's digital research repository, the Library catalogue, the Australian Digital Theses Program (ADTP) and also through web search engines, unless permission has been granted by the University to restrict access for a period of time."

ACKNOWLEDGEMENTS

Professor Allison Cowin

Thank you for the privilege of allowing me to work as part of your excellent team for four years. Inviting an inexperienced clinician into your laboratory presented many challenges and I owe you an enormous debt of gratitude for the patience and understanding you granted me in helping me to complete this PhD. I am in awe of the work produced by your team and look forward to seeing many more outstanding developments over the coming years.

*

Associate Professor Peter Anderson

Thank you for your excellent supervision and mentorship over the past 6 years. Without your help foresight, I would not have had the opportunity to work with Professor Cowin and her team. Your enthusiasm and passion for the role of surgical scientist has been a great inspiration. Your role in helping me to arrange time away from surgical training to complete this PhD was also much appreciated.

*

Damian Adams

Thank you for taking on the difficult task of orientating me to the world of the laboratory and the animal house. Your meticulous and professional approach to your work was a great example of the hard work required to achieve results in this kind of research. Your work in developing the bleomycin model was an integral part of this research.

*

Associate Professor Nicola Dean

Thank you for your help in preparing this manuscript over the previous year. Your understanding, interest and guidance has been unendingly helpful. I have learnt a great deal from your inspiring example, both clinically and as head of unit at Flinders' Medical Centre

*

The Cowin Laboratory

Thanks to the past and present members of the Cowin team for your help and support throughout my PhD.

*

WG Norman Trust, Sir John Lowenthal Trust &

The Royal Australasian College of Surgeons

NHMRC

Thank you for the invaluable funding that made it possible to undertake full time research

*

Dr Michelle Lodge

Thank you for your support in allowing my time away from surgical training to undertake full time research.

*

Professor John Greenwood& Mr Chris Kirby

Thank you providing me with the human tissue samples used in this research

PUBLICATIONS ARISING FROM THIS THESIS

Cameron AM, Turner CT, Adams DH, Jackson JE, Melville E, Arkell RM, Anderson PJ, Cowin AJ Flightless I is a Key Regulator of the Fibroproliferative Process in Hypertrophic Scarring and a Target For a Novel Anti-Scarring Therapy. *Br J Derm* 2016 Jan **IF 4.317 RIF 5/63**

Cameron AM, Adams DH, Greenwood JE, Anderson PJ, Cowin AJ.
A novel murine model of hypertrophic scarring using subcutaneous infusion of bleomycin. Plast Reconstr Surg. 2014 Jan;133(1):69-78. **IF 3.535 ROF 13/198**

Cameron AM, Ruzehaji N, Cowin AJ. Burns wound healing: a surgical perspective. Wound Practice & Research. Feb 2010 18(1), 34-40.

NATIONAL AND INTERNATIONAL MEETINGS, SCIENTIFIC ABSTRACTS RESEARCH GRANTS AND AWARDS ARRISING FROM THIS THESIS

Cameron AM, Anderson P, Cowin AJ "Reducing Flightless Improves Hypertrophic Scarring by Modulating Fibroblast Phenotype" Verbal Presentation, **Royal Australasian College of Surgeons Research Society Annual Meeting, Adelaide, November 2012**

Cameron AM, Adams. D, Anderson P, Cowin AJ. Reducing Flightless Improves Hypertrophic Scarring in an Animal Model" **Royal Australasian College of Surgeons RP Jepson Medal Presentations October, 2012**

Cameron AM, Adams. D, Anderson P, Cowin AJ. A novel animal model of hypertrophic scarring and a new target for a novel anti-scarring therapy **Australia and New Zealand Burns' Association Annual Meeting, Hobart, Oct 2012**

Cameron AM, Adams. D, Anderson P, Cowin AJ Reducing Flightless Improves Hypertrophic Scarring in an Animal Model **Australian Wound Tissue Repair Society, Annual Research Meeting, Sydney, July 2012**

Cameron AM, Adams. D, Anderson P, Cowin AJ. Reducing Flightless Improves Hypertrophic Scarring in an Animal Model **Royal Australian College of Surgeons A Annual Scientific Congress, Kuala Lumpur, May 2012**

Cameron AM, Adams. D, Anderson P, Cowin AJ Reducing Flightless Improves Hypertrophic Scarring in an Animal Model **Young Investigator Award, American Wound Healing Society, Atlanta, Georgia, April 2012**

Cameron AM, Anderson P, Cowin AJ Reducing Flightless Improves Scarring in an Animal Model" **Australasian Society of Plastic Surgeons, Registrar Research Meeting, November 2012**

Cameron AM, Anderson P, Cowin AJ Reducing Flightless Improves Scarring in an Animal Model **Royal Australasian College of Surgeons Research Society Annual Meeting, Adelaide, November 2011**

Cameron AM, Anderson P, Cowin AJ The Role of Flightless in Hypertrophic Scarring **Poster Presentation, Adelaide University Post-Graduate Health Faculty Conference, Adelaide Oct 2012**

Cameron AM, Cowin AJ, Anderson P "Flightless I, an actin remodelling protein, in the development of cranial scars in Apert & Crouzon syndromes" **Verbal**

Presentation, Royal Australasian College of Surgeons Annual Scientific Congress, May 2011 (ANZSCMFS Craniomaxillofacial Surgery Section)

Cameron AM, Greenwood J, Cowin AJ"The role of Flightless I, an actin-remodelling protein, in the development of hypertrophic scarring following burn injury" **Verbal Presentation, Royal Australasian College of Surgeons Annual Scientific Congress Annual Scientific Congress, May 2011 (ANZBA Burns section)**

Royal Australasian College of Surgeons WG Norman Trauma Fellowship 2011

Don Robinson Medal for Best Research Paper by a South Australian Plastic Surgery Registrar 2011

NHMRC Post-Graduate Scholarship 2012

MIGA Doctors in Training Travel Grant 2012

RP Jepson Medial for Best Research Paper by a South Australian Surgical Registrar 2012

Australia and New Zealand Burns' Association Annual Meeting Runner up Best Scientific Paper 2012

Royal Australasian College of Surgeons WG Norman Trauma Fellowship 2012

Royal Australasian College of SurgeonsSir John Lowenthal Scholarship 2013

Royal Australasian College of Surgeons Annual Scientific Meeting Best Research Presentation 2013

Adelaide University Faculty of Health Sciences 3 Minute Thesis Winner 2013

Don Robinson Medal for Best Research Paper by a South Australian Plastic Surgery Registrar 2017

Chapter 1

LITERATURE REVIEW

Parts of this chapter have been published in the following journal:

Cameron AM, Ruzehaji N & Cowin AJ (2010) Burns wound management: a surgical perspective. Wound Practice and Research **18** (1): 34-40

1.1 Overview

All multicellular organisms are equipped to repair damage to their outermost layer via the process known as wound healing. The normal wound healing process is a tightly orchestrated sequence involving numerous cell types, extracellular matrix components and signalling molecules (Singer & Clark, 1999). It balances several requirements, including prevention of infection; restoration of epithelial integrity; and approximation of normal skin structure and function (Bielefeld et al., 2013).

In humans, the inevitable result of normal wound healing is the rapid interposition of scar tissue, which is eventually remodeled so that the disturbance to normal skin structure and function is minimized. However, there are a number of circumstances in which abnormal wound healing leads to pathology. These pathologies can arbitrarily be placed on a spectrum ranging from "under-healing" (chronic wounds, diabetic ulcers, skin blistering diseases) through to those related to "over-healing" (hypertrophic scarring, keloid, malignancy).

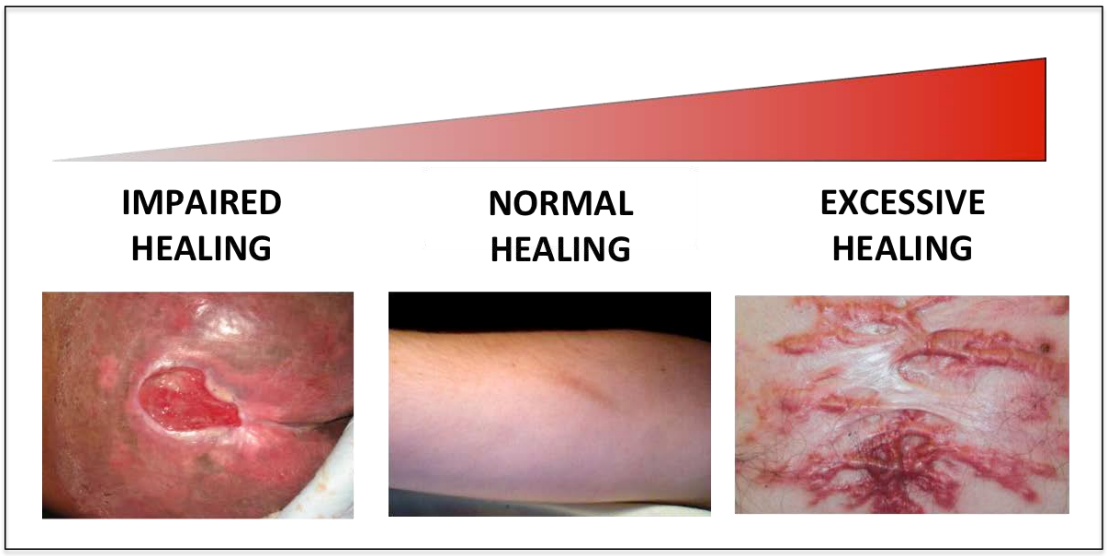


Figure 1.1 The spectrum of wound healing pathology Schematic representation of the "spectrum " of wound healing pathology, which ranges from pathology associated with impaired healing (e.g. chronic ulcers) to pathology associated with excessive healing (e.g. hypertrophic scarring).

Wound healing pathologies represent an area of unmet clinical need. Chronic wounds are estimated to affect 443 000 Australians and cost the health system $2.6 billion annually (Wound Management Innovation CRC Annual Report). Excessive scarring occurs in greater than 70% of deep burns and carries a considerable burden of disease, includes disfigurement, pain, disability and psychological co-morbidity (Brown et al., 2008). Current therapies mainly focus on managing the end-stages of abnormal wound healing rather than preventing these complications from occurring (Franz et al., 2007). The majority of these treatments relies largely on anecdotal evidence and lack a strong evidence base for their efficacy or understanding of mechanisms of action (Mustoe et al., 2002; Robson et al., 2001).

Across taxa, we see considerable diversity in how wound healing occurs. Most other mammals lack human's predilection for excessive scarring, while adult

amphibians are able to regenerate an amputated appendage through the formation of a blastema (a mass of undifferentiated cells capable of regeneration) (Bielefeld et al., 2013). Even certain human tissues demonstrate regenerative healing. The adult liver may regenerate up to 70% of its mass without scarring, while gingiva also demonstrates scar free wound healing. The skin of the human foetus is capable of scar free wound healing until late in the third trimester (Gurtner et al., 2008). As the core components of the wound healing process are highly conserved across multicellular organisms, much attention has focused on elucidating key differences in specific cell populations and molecular pathways that influence the eventual outcome. If these are unveiled, it follows that this knowledge could be used to manipulate human wound healing (Gurtner et al., 2008).

This introduction will outline the current understanding of wound healing. It will then contextualize hypertrophic scarring the outcome of the dysregulation of this process. Flightless (Flii), a novel member of the gelsolin family of proteins will be introduced as a potential regulator of the wound healing and scar formation.

1.2 Skin Biology

Mammalian skin consists of two layers: the *epidermis,* a keratinized epithelial layer comprised of keratinocytes; and the *dermis*, a spongy dermal layer consisting mainly of collagen, as well as fibroblasts and skin appendages (Martin, 1997). The skin fulfills several vital functions. It is the body's primary physical and immune barrier to trauma and infection. It is also vital for thermoregulation and gathering information about the external environment through touch, pain and temperature sensation (Alonso & Fuchs, 2006; Koster, 2009).

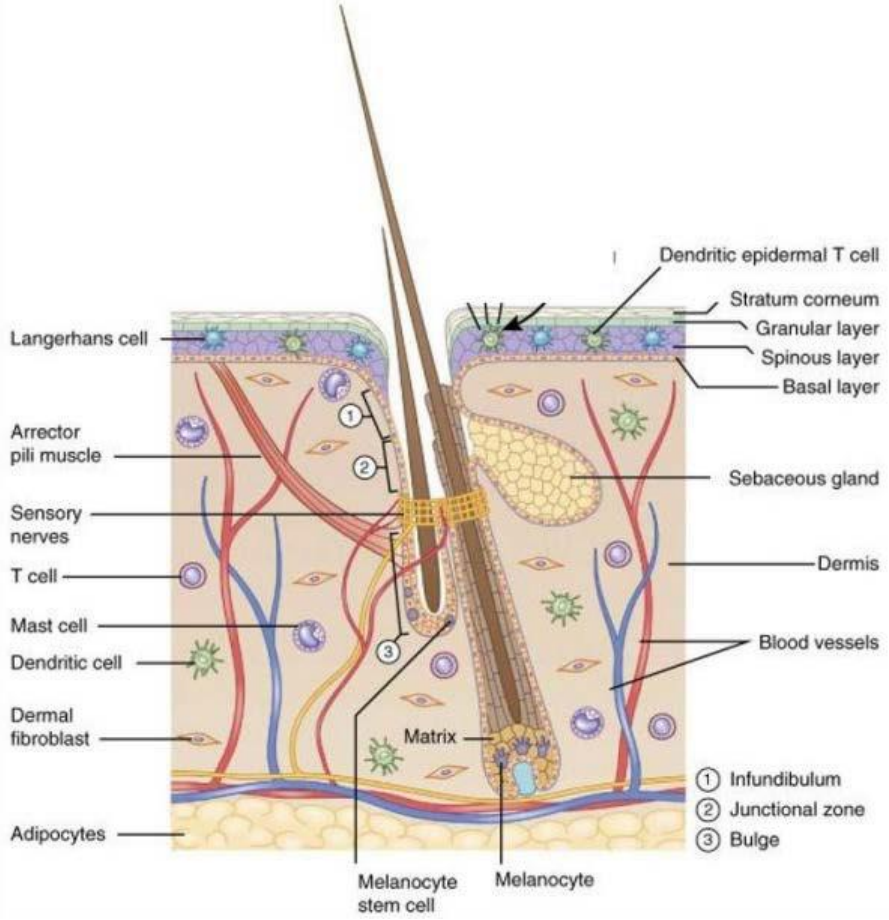


Figure 1.2 Anatomy of the skin. Illustration of skin anatomy, demonstrating epidermis, dermis and adnexal appendages (Habif, 2004)

1.2.1 Epidermis

The epidermis is comprised of keratinocytes, which continually differentiate, divide and migrate towards the skin's surface. It can be further divided into five layers depending on the stage of cell differentiation: stratum basale, stratum spinosum, stratum granulosum, stratum lucidum and stratum corneum (see Figure 1.3) (Alonso & Fuchs, 2006; Koster, 2009).

The stratum basale is comprised of undifferentiated keratinoctyes, which are able to consistently renew their population. Cells of the next layer, the stratum spinosum maintain the structural integrity of the epithelium and produce keratin-1 and keratin-10. Cells in the stratum granulosum are characterized by the production of keratohyalin granules which contain histidine rich proteins and keratin (Kanitakis, 2002). The stratum lucidum, named for its translucent microscopic appearance, is comprised of 3-5 layers of dead keratinocytes and underlies the stratum corneum. It takes 14 days for a keratinocyte to reach the stratum corneum from its origin at the stratum basale. By this time the previously living keratinocytes are transformed into non-living corneocytes, whose cell membranes are replaced by a layer of ceramides, which covalently link to the cornified envelope and are integral to barrier function (Alonso & Fuchs, 2006).

A range of keratinocyte junctions also contribute to barrier function, both structurally as well as by regulating cell proliferation and differentiation (Byrne et al., 2003). These include apical actin tight junctions in granular keratinocytes,

which augment the barrier function of the stratum corneum. Gap junctions provide intercellular communication between keratinocytes, while a network of keratin and actin filaments maintain the three dimensional architecture of the epidermis. Stratum specific distribution of cadherin subtypes help to maintain the epidermis through differential adhesion between cells at different layers and adherens junctions are important in cell proliferation (Butnaru & Kanitakis, 2002; Byrne et al., 2003).

The *basement membrane* separates the dermis from the epidermis and is comprised of a series of proteins that provide a connection between the two layers (McMillan et al., 2003). Its structural components include keratin monofilaments, outer and inner plaques of hemidesmosomes, plasma membrane, lamina lucida, lamina densa and collagen-VII anchoring fibrils (Bruckner-Tuderman, 1999).

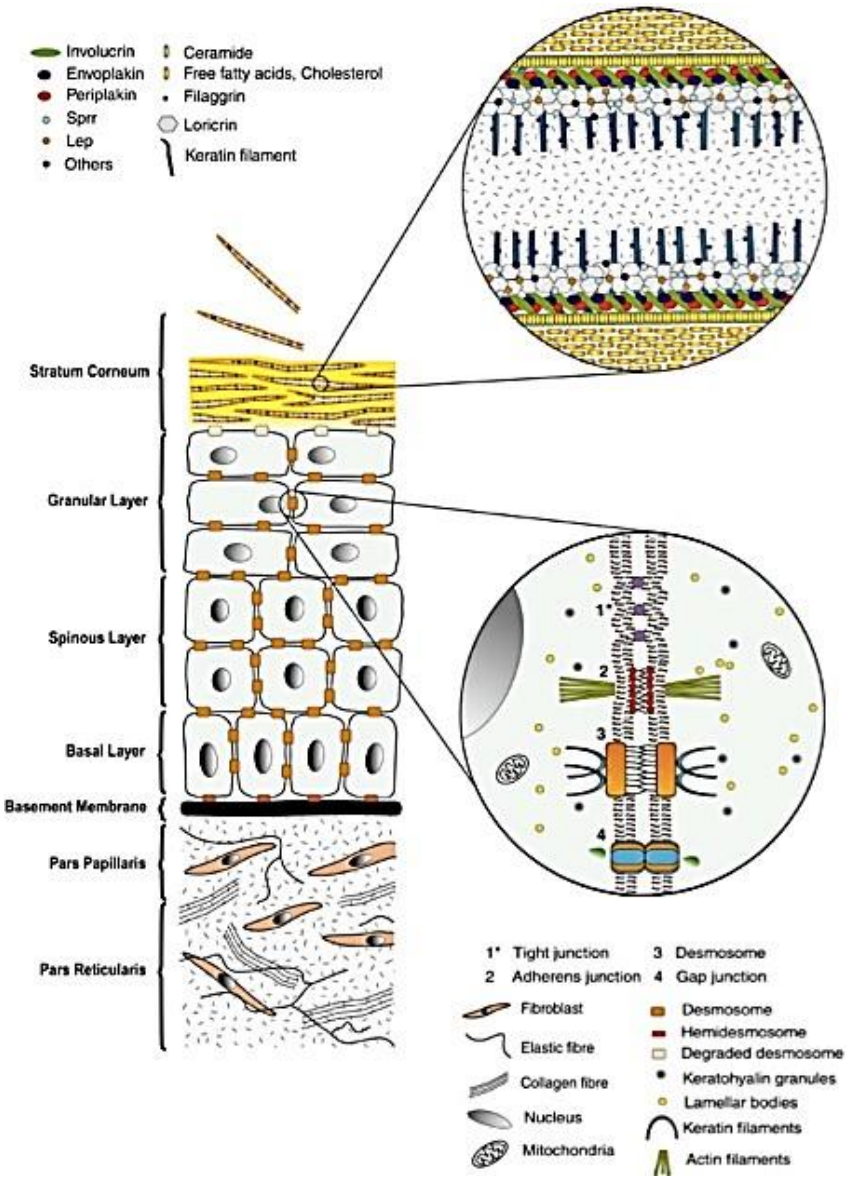


Figure 1.3 Diagrammatic representation of human skin. Diagrammatic representation of human skin illustrating epidermal layers and the basement membrane. Intercellular junctions comprised of tight junctions, adherens junctions, desmosomes and gap junctions are illustrated (Byrne et al., 2003).

1.2.2 Dermis

The dermis is the second layer of skin and varies in thickness from 0.3mm – 3mm. It is primarily comprised of elastic tissue, namely collagen, elastin and reticular fibres. The *papillary* dermis is the thin upper layer consisting of loosely arranged collagen fibres and elastin. The deeper *reticular layer*, which extends to the subcutaneous tissue, is composed of thick collagen fibres orientated parallel to the surface of the skin as well as a thicker elastic network, dermal appendages and neurovascular structures (Habif, 2004). Dermal collagen is predominately made of collagen-1 and collagen-3, which maintain structural integrity and strength of the skin. Also important are collagen-3, found in the basement membrane and blood vessels, and collagen-7 which is important in anchoring the dermis to the epidermis (Kanitakis, 2002). Mutations in collagen 7 lead to the skin blistering disease, epidermolysis bullosa (Dang & Murrell, 2008).

Fibroblasts are spindle shaped cells that produce collagen. They are thought to have a key regulatory role in the wound healing process. *Myofibroblasts* are a contractile phenotype of the fibroblast which drive tissue repair through collagen production and by contracting the ECM (Bochaton-Piallat et al., 2016).Their dysfunction is thought to be central to the fibroproliferation that leads to hypertrophic scarring (Gurtner et al., 2008). Other cells found within the dermis include macrophages that function to scavenge dead cells or debris; and *mast cells*, located primarily around blood vessels, which manufacture and release histamine in response to injury (Habif, 2004).

1.3 Wound Healing

In order to understand hypertrophic scarring as an aberration of normal wound healing, it is important to understand the normal wound healing process. The chronology and components of normal wound healing have been well described, with events being divided into three arbitrary phases: inflammation, proliferation and remodelling (Singer & Clark, 1999).

1.3.1 Haemostasis

Following damage to the skin, the coagulation cascade is initiated within the first minute after injury. Platelets are activated and clump together to achieve haemostasis. Fibrin is then incorporated and a fibrin plug is formed (Grose & Werner, 2004). Within one to two hours post-injury various cytokines, growth factors, fibronectin and pro-inflammatory mediators are released, which initiate the inflammatory phase of wound healing (Singer & Clark, 1999).

1.3.2 Inflammatory Phase

Immune cells, namely neutrophils and macrophages, enter the wound by binding to integrins and becoming internalised through endothelial gaps. These cells accumulate within the wound and eliminate foreign material, dead tissue or microbes. At the same time, hypoxia within the wound stimulates keratinocyte migration and local fibroblast proliferation (Falanga, 2005). All cells produce further cytokines, growth factors and angiogenic factors, which lead to the accumulation of fibroblasts within the wound and in time, the initiation of the proliferative phase of healing (Rappolee et al., 1988; Simpson & Ross, 1972).

1.3.2 Proliferative Phase

The proliferative phase generally lasts from 4 to 21 days. Keratinocytes, which are normally adherent to their adjacent cells via desmosomes and to the basement membrane via hemidesmosomes, take on a migratory phenotype. This involves hemidesmosome disassembly, dissolution of desmosomes and retraction of intracellular tonofilaments. At the same time lamellapodia and focal complexes develop (Santoro & Gaudino, 2005).

Re-epithelialization occurs with the migration of keratinocytes from wound margins, across the fibrin and fibronectin matrix. In addition to migration, keratinocyte proliferation is increased, stimulated by various growth factors, integrins and metalloproteinases (Falanga, 2005).

Fibroblasts and their contractile phenotype, myofibroblasts, produce collagen rich connective tissue, which replaces the provisional fibrin matrix to form granulation tissue, a new scaffold for cell migration (Figure 1.4).

Granulation tissue is predominately comprised of collagen, particularly collagen-1 and collagen-3. Collagen-3 is produced in early wound healing before being replaced by collagen-1 (W. Liu et al., 2001). Collagen-4 is important in the formation of a new basement membrane over the healing matrix.

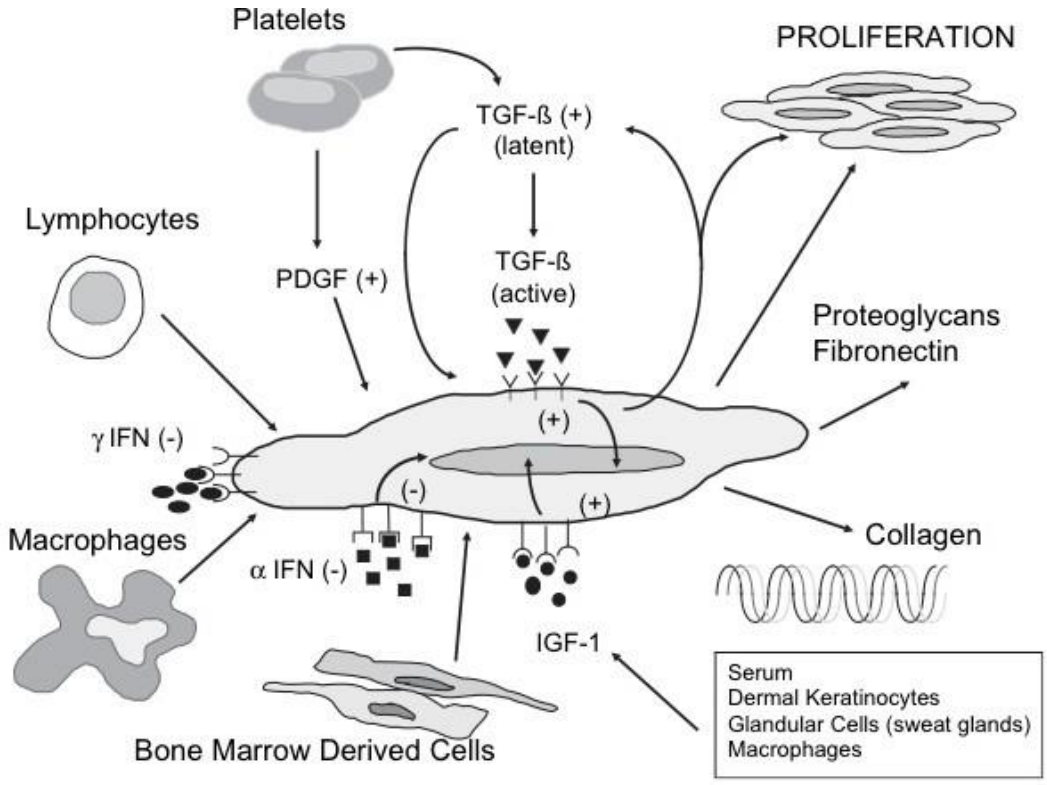


Figure 1.4 The fibroblast (centre) and the proliferative phase of wound healing. Various growth factors and cytokines released in the inflammatory phase of wound healing lead to the migration of fibroblasts to the wound, which then proliferate, undergo phenotypic change to myofibroblasts and produce collagen (Werner & Grose, 2003)(Werner & Grose, 2003)(Werner & Grose, 2003)(Werner and Grose 2003).

1.3.3 Remodelling phase

Once an abundant collagen matrix has been established, cells in the wound undergo apoptosis during the remodelling phase, which can last for up to two years and eventually results in an acellular and avascular scar. The remodelling phase involves both wound contraction and collagen remodelling. Wound contraction is brought about when fibroblasts assume a myofibroblast phenotype, developing intracellular actin microfilaments capable of force generation and

matrix contraction (Bochaton-Piallat et al., 2016; Singer & Clark, 1999). At the same time, collagen-3 is degraded by matrix metalloproteinases (MMPs) and replaced by collagen-1. This collagen remodelling, combined with increased collagen cross linking, leads to increased scar tensile strength.

This wound healing process is a tightly orchestrated sequence, which balances the synthesis and degradation of collagen in order to repair injury with as little disruption of normal skin architecture as possible. Hypertrophic scarring represents an aberration of normal wound healing where this balance is disrupted in favour of excess collagen production.

1.4 The Myofibroblast

First defined in the early 1970s, the myofibroblast was identified as the main cell type responsible for fibrotic pathology (Gabbiani et al., 1971). The classically recognised features of the myofibroblast are expression of α-smooth muscle actin (αSMA) and the fibronectin (FN) splice variant ectodomain (ED)-A FN (Hinz, 2010). As its nomenclature suggests, the myofibroblast is defined by contractile function rather than αSMA expression *per se*. It has been shown that "proto-myofibroblasts", which contain stress fibres, but not αSMA, emerge after six days of wound healing, with αSMA expressing fibroblasts appearing at around nine days signalling the beginning of wound contraction (Hinz et al., 2001).

1.4.1 Myofibroblast Origin

The exact origin of myofibroblasts in fibroproliferative disease has not been fully clarified. In the skin, it is recognised that connective tissue fibroblasts acquire contractile stress fibres and then express α-SMA (Hinz, 2009). In liver fibrosis, hepatic stellate cells and portal fibroblasts are transformed into myofibroblasts, while in the kidney epithelial cells undergo epithelial to mesenchymal transition to become myofibroblasts (Guyot et al., 2006; Zeisberg & Kalluri, 2008). Epithelial to mesenchymal transition has also been demonstrated in lung, kidney and liver fibrosis, while pericytes have been shown to transform to myofibroblasts in scleroderma scarring (Kim et al., 2006; Rajkumar et al., 2006; Zeisberg & Kalluri, 2008).

1.4.2 Fibroblast-Myofibroblast Differentiation and Tissue Stiffness

The myofibroblast receives constant feedback from the ECM, which regulates its role in the wound healing process. *In vitro,* fibroblast stress fibre formation and myofibroblast differentiation will only occur if cells are cultured on substrates providing sufficient mechanical tension (Hinz, 2009).

Attempts have been made to quantify the mechanical load required to stimulate myofibroblast differentiation and therefore to define what constitutes a "stiff" ECM. The provisional matrix of early wounds (<5 days) is estimated to be ~10-100Pa, which is comparable with newly polymerized collagen gels often used in *in vitro* studies (Carlson & Longaker, 2004). Fibroblasts cultured in this environment

show no stress fibre development or αSMA expression (Tamariz & Grinnell, 2002). Stress fibres without αSMA expression is first seen in fibroblasts grown in conditions of tissue stiffness of ~3000-6000Pa (Discher et al., 2005). 20 000Pa is required for the expression of αSMA and it has been shown that the amount of αSMA expression increases with increasing tension (Discher et al., 2005).

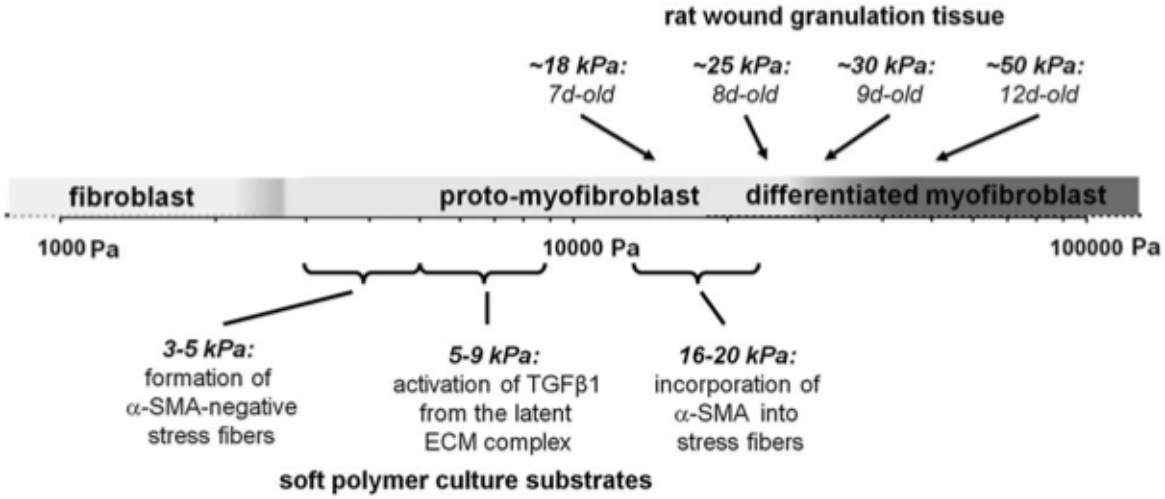


Figure 1.5 Fibroblast to myofibroblast differentiation as a function of tissue stiffness (Discher et al., 2005)

The relationship between mechanical tension and fibroblast phenotype is particularly compelling in relation to hypertrophic scarring. It has been known since the advent of modern surgery that a wound under tension is particularly prone to hypertrophic scarring (Gurtner et al., 2008). In fact, Gurtner and colleagues showed that applying mechanical force across a mouse wound (effectively counteracting the natural contraction of *panniculus carnosus*) leads to hypertrophic-like scarring (Aarabi, Bhatt, et al., 2007). Furthermore, scar development in this model was prevented by interruption of the Focal Adhesion Kinase (FAK) signalling pathway (Wong et al., 2012). This demonstrates the key importance of the myofibroblast-ECM relationship to the pathogenesis of

hypertrophic scarring and illustrates the potential for therapeutic intervention aimed at uncoupling this relationship.

1.4.3 How Do Fibroblasts Sense Mechanical Stress?

Fibroblasts attach to the ECM substrate via integrins, αβ heterodimers that form cell receptors anchoring intracellular stress fibres to the ECM (Hynes, 2002). When extracellular mechanical stress is applied to fibroblasts, integrins cluster into *nascent adhesions* and then further into *focal complexes* (~1μm^2). If further stress is applied *classical focal adhesions* develop (2-6μm^2) (Bershadsky et al., 2006).

αSMA positive myofibroblasts display significantly larger *supermature focal adhesions* (8-30μm^2) (Dugina et al., 2001). Supermature focal adhesions may be reduced to classical focal adhesions when αSMA contraction is inhibited or when extracellular substrate tension is reduced (Goffin et al., 2006).

Thus, fibroblasts gain information about mechanical tension through the interaction between their stress fibres and the ECM through their adhesion sites. This information then leads to responses in cell properties such as migration, proliferation, survival, differentiation, ECM organisation and remodelling (Vogel & Sheetz, 2006). This can occur through intracellular mechanisms, such as molecular switches or signal pathways, or extracellular protein unfolding to reveal integrin binding sites (Zhong et al., 1998). Similarly, release and activation of TGFβ1 contained in the ECM can be brought about by cellular contraction (Wipff & Hinz, 2009).

It is known that focal adhesions allow contractile activity of myofibroblasts to be transmitted to the ECM (Hinz et al., 2012), creating a more strained, stiffer environment. These mechanical conditions generated by the myofibroblast also lead to their sustained fibroproliferative activity (Hinz et al., 2001). Indeed, scar development in a murine model of hypertrophic scarring was prevented by disruption of Focal Adhesion Kinase (FAK) signalling pathway (Wong et al., 2012).

1.4.4 The Myofibroblast and Fibroproliferative Disease

The production of ECM and generation of contractile force by myofibroblasts are integral to normal wound healing. Dysregulation of these processes is the hallmark of fibroproliferative disease, whereby normal tissue is replaced with fibrotic scar tissue. In the skin this manifests as hypertrophic scars (Atiyeh, Costagliola, et al., 2005) scleroderma (Varga & Abraham, 2007) and Dupuytren's disease (Tomasek et al., 1999). Similar scar production underlies fibroproliferative disease of the organs, such as the liver, lung and kidney (Hinz, 2010).

The initiation of fibrosis marks the beginning of a vicious cycle, whereby the ECM is stiffened by myofibroblast activity, which then stimulates further myofibroblast differentiation and activity. A key research question is whether tissue stiffness or myofibroblast differentiation is the initial step in this process. Recent evidence from an animal model of liver fibrosis, points to the former, with ECM stiffness

preceding the presence of activated fibroblasts or collagen accumulation(Georges et al., 2007).

1.5 The Cytoskeleton

The cytoskeleton is vital for the maintenance of cellular architecture and is instrumental to how cells move through, sense and respond to the extracellular environment (Lambrechts et al., 2004). The cytoskeleton is comprised of microtubules, intermediate filaments, microfilaments and stress fibres (in order of decreasing size) (Oshima, 2007).

Microtubules are approximately 25nm in diameter and are concentrated in locomotive structures such as cilia and flagella. They are comprised of α and β tubulin dimers, which serve to bring about cell motility and division by GTPase dependent polymerization (Valiron et al., 2001). Microtubules are also important for vesicle transport through the cell (Holzbaur, 2004).

Intermediate filaments are approximately 10nm in diameter and consist of a central α-helical domain flanked by variable N and C terminals (Goldman et al., 2008). This structure allows interaction with a range of proteins at the cell surface such as desmosomes, hemidesmosomes, focal adhesions and the extracellular matrix (Capetanaki et al., 2007).

Considerable attention has been focused on *actin microfilaments*, the smallest of the three cytoskeletal proteins. They are thought to be important for cell motility, structure and division (Lambrechts et al., 2004). Actin microfilaments exist as

individual non-polymerized globular actin subunits (G-actin) which assemble into filamentous fibres (F-actin) (Lambrechts et al., 2004).

After injury, changes in the composition, organization and tension of the ECM , as well as the action of growth factors, stimulate fibroblast development of *stress fibres* (Werner & Grose, 2003). These stress fibres are initially composed of cytoplasmic actins (Tomasek et al., 2002). With increasing ECM tension, α-smooth muscle actin (αSMA) is expressed (see above), which marks the phenotypic transition from fibroblast to myofibroblast (Hinz & Gabbiani, 2010; Tomasek et al., 2002). Stress fibres in mammalian cells are classified according to their relationship to focal adhesions: ventral adhesions attach to focal adhesions at both ends, dorsal stress fibers attach to focal adhesions at one end and transverse arcs do not attach to focal adhesions directly (Naumanen et al., 2008). Stress fibers are thought to be important for maintaining a balance between contraction and adhesion so that actin bundles maintain a constant length under tension (Goffin et al., 2006).

1.5.1 Actin dynamics during wound healing

Actin dynamics are integral to the wound healing process. After wounding, cells respond to a variety of stimuli to remodel their actin cytoskeleton, which brings about changes in cell contraction, adhesion, motility or phenotype. In the case of keratinocytes at the wound edge, this leads to migration across the wound matrix to bring about re-epithelialization, whereas fibroblasts begin to express stress

fibres and take on a myofibroblast phenotype (Cowin et al., 2003; Jacinto & Wolpert, 2001; Lambrechts et al., 2004).

Consequently, understanding the regulation of actin dynamics and the role they play in different types of wound healing is of considerable interest. For example, previous work by Cowin has demonstrated differential expression of F-actin in scar less fetal wound healing (F-actin predominately expressed in the epidermis) versus scar forming adult wound healing (F-actin predominately expressed in the dermis (Cowin et al., 2007; Cowin et al., 2003).

1.5.2 Actin cytoskeleton, cell motility and cell matrix adhesion

The actin cytoskeleton, cell motility and cell matrix adhesion are all intimately connected. In order to migrate through the ECM cells form lamellipodia and filopodia at their leading edge, which then form adhesions with the matrix, allowing movement of the cell body (Partridge & Marcantonio, 2006).

Lamellipodia are a cell projection consisting of a network of branched actin filaments, whose assembly is regulated by the Rho GTPase family. Surface receptors are linked to the actin cytoskeleton allowing force to be produced for cell protrusion at the leading edge of the cell (Small et al., 2002).

Filopodia are long parallel actin filaments arranged into bundles that cause elongation of the cell. This elongation is regulated primarily by Cdc42GTPase and the Ena/VASP family of proteins and formins (Hotulainen & Lappalainen, 2006). *Membrane ruffles* are brought about by the dynamic movement of the cell membrane due to lamellipodia and filopodia formation. *Lamella* are formed away from the leading edge of the cell by the interaction between stress fibers and the proteins tropomyosin and myosin II (Le Clainche & Carlier, 2008).

The interaction between the actin cytoskeleton and the ECM occurs through highly dynamic, temporary adhesions known as *focal complexes*. These adhesions are formed by actin polymerization underneath lamellipodia. These have been described as a "molecular clutch" which connects the cytoskeleton to the matrix, thereby allowing actin-myosin generated tension to bring about cell traction (Le Clainche & Carlier, 2008). Focal complex function is a complex process determined by interaction between integrin-ECM, integrin-actin binding proteins and actin filament-actin binding protein interactions. In this way focal complexes link the ECM to the actin cytoskeleton, essentially functioning as mechanosensors which can then influence maturation of adhesion sites and recruitment of signaling or structural molecules (Lambrechts et al., 2004; Le Clainche & Carlier, 2008). Under certain conditions focal complexes become larger, more stable adhesions known as *focal adhesions*, which are associated with stress fiber development and fibroblast transformation to myofibroblasts (Hinz, 2010).

A number of proteins have been identified as essential mediators of the relationship between the actin cytoskeleton and cell adhesion/motility. These

proteins, including vinculin, talin, paxillin, α-actinin and Src kinase are involved in signaling pathways initiated by cell matrix adhesion. *Vinculin* has been shown to stabilize adhesions during maturation of focal complexes into focal adhesion, while *talin* helps to link integrin receptors to both the actin cytoskeleton and ECM ligands (Le Clainche & Carlier, 2008). *Paxillin* allows recruitment of a variety of different proteins to focal adhesions as well as acting as a signal transducer to produce changes in cytoskeletal structure and gene expression (Cowin et al., 2003).

Improved understanding of the specifics of the complex signaling pathways associated with adhesion sites, as well as the role of adaptor proteins like those described will help understanding of cell motility and adhesion during normal and pathological wound healing.

1.6 Flightless Protein

1.6.1 The gelsolin family of actin remodeling proteins

Actin filaments are regulated by actin remodeling proteins, which can sequester, cross link and nucleate actin filaments. More than 100 such proteins have been identified, including ADF/cofilin family, profilin family, gelsolin superfamily, thymosins, capping proteins and the Arp2/3 complex (Dos Remedios, Liew, et al., 2003)(Dos Remedios, Liew, et al., 2003). Of these, the gelsolin superfamily, which includes gelsolin, adeseverin, villin, advillin, supervillin, CapG and Flightless I

(Flii) has been shown to be integral to reorganization of the actin cytoskeleton. They function by severing pre-existing actin filaments, capping the fast-growing filament ends, while nucleating or bundling actin filaments into new cytoskeletal structures (Archer et al., 2005; Goshima et al., 1999; Y. T. Liu & Yin, 1998).

The gelsolin superfamily is characterized by 3 to 6 homologous gelsolin like structural domains (G1-G6 segmental domains), 3 actin binding regions and various calcium independent monomer and filament domains. Despite considerable homology across the gelsolin family, differences suggest that individual proteins have gained novel functions in addition to actin remodeling (Archer et al., 2005; Campbell et al., 1997). For example, villin contains an additional actin binding domain ("villin head piece"); supervillin contains an N-terminus domain capable of protein-protein interactions nuclear localization; and Flii contains an N-terminus Leucine Rich Repeat (LRR) domain capable of protein-protein interactions (Figure 1.6) (Witke et al., 2001).

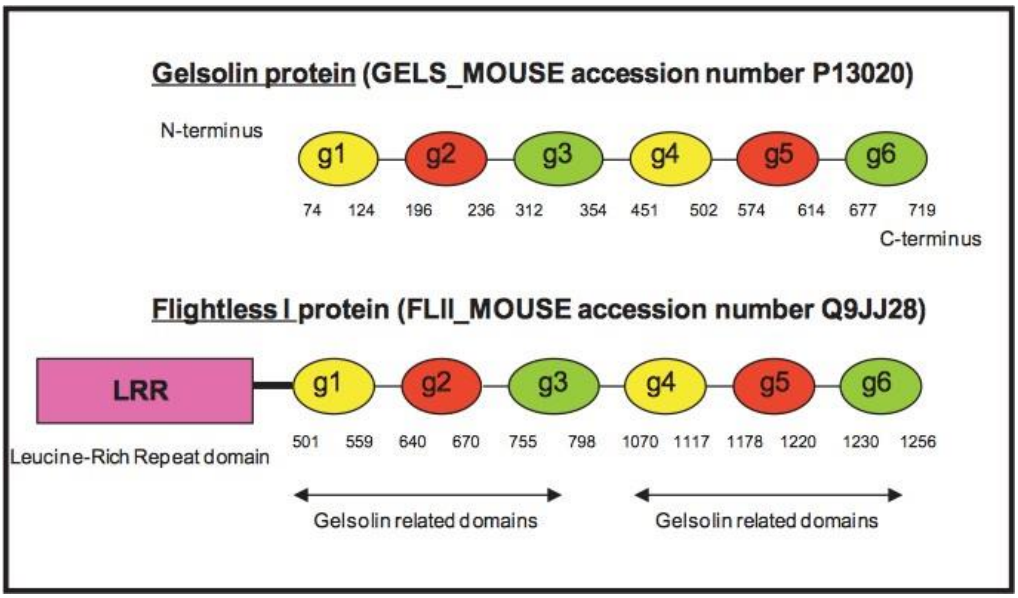


Figure 1.6 Comparison of Flii and gelsolin proteins Flii is a member of the gelsolin family and possesses two gelsolin like domains, as well as a unique Leucine Rich Repeat domain (LRR) on its N-terminus (Witke et al., 1995)

The gelsolin superfamily is essential to a variety of processes important in wound healing, such as cell motility, apoptosis and gene expression (Silacci et al., 2004). Studies indicate that members of the gelsolin family are involved in the regulation of wound repair and as such, may be targets for therapies to help improve wound healing and reduce scar formation (Cowin et al., 2007). For example, increasing cellular levels of gelsolin has been shown to enhance wound closure, while decreasing gelsolin leads to delayed wound healing (Cunningham et al., 1991; Witke et al., 1995). For these reasons, Flii was identified as a protein of interest in wound healing research upon its discovery in the 1990s (Campbell et al., 1997). Since that time it has been identified as a negative regulator of wound healing and a promising candidate as a target for novel wound healing therapies (Adams et al., 2009; Cowin et al., 2007).

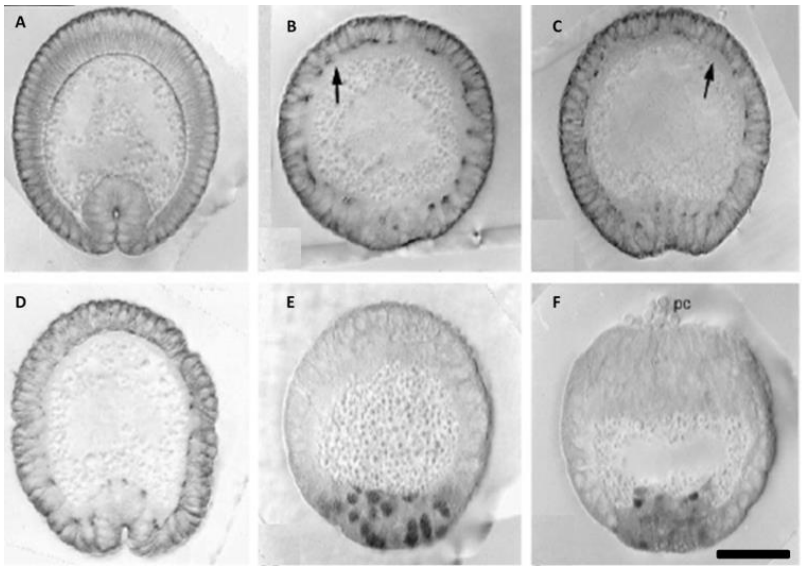
1.6.2 Flightless I (Flii) Protein

Flii was discovered as a gene mutation in *Drosophila melanogaster*, located in the subdivision 19F on the X chromosome. Flii is the most conserved protein in the gelsolin family but is unique in containing a LRR domain at its N-terminus (Claudianos & Campbell, 1995). It was named due to the irregular actin organisation observed in Flii heterozygote *Drosophila* that resulted in defective flight muscles (Campbell et al., 1993). In more severe mutations, *Drosophila* embryos suffer incomplete cellularisation with irregularities in gastrulation and mesoderm invagination during early embryogenesis leading to a disorganised cytoskeleton. Homozygous expression is embryonically lethal, failing to develop beyond the blastocyst stage. A trophoblast cell layer is formed, but the development of the egg cylinder fails, resulting in complete embryonic degeneration (Campbell et al., 2002) (Figure 1.7). The insertion of a cosmid clone containing Flii to homozygous Flii embryos restores normal development (Campbell et al., 2002).

The human homologue of the Flii gene was subsequently discovered in 1997 (Campbell et al., 1997). The human Flii locus has been mapped to human chromosome 17p11.2. The deletion of this region results in the congenital syndrome Smith-Magenis (SMS) syndrome, which comprises behavioural and developmental abnormalities (Elsea & Girirajan, 2008; Y. T. Liu & Yin, 1998). Most of the phenotypic features of Smith-Magenis have been attributed to the loss of

Retinoic Acid Induced 1 (RAI1), but Flii may contribute to this disease although this is yet to be demonstrated (Girirajan et al., 2006).

Flii's essential role in embryogenesis has also been shown in mammals as well as *Drosophila*. Normal development is restored to a homozygous Flii mouse by a human transgene containing the complete human Flii gene (Campbell et al., 2002). The human Flii transgene was used to develop a Flii transgenic mouse ($Flii^{Tg/Tg}$), while a heterozygous Flii mouse ($Flii^{+/-}$), with half the normal amount of Flii protein, was also developed. Both genotypes appear phenotypically normal and have been invaluable for *in vivo* research into the function of Flii (Campbell et al., 2002).



1.7 Gastrulation and cellularisation defects in Flii homozygous embryos.
WT embryo showing normal mesoderm invagination with cytoplasmic incorporation and gastrulation. **B-C** Unincorporated cytoplasm (arrows) seen in Flii homozygous embryos. **D** Incomplete and irregular invagination occurs in Flii homozygous embryos. **E-F** Ventral nuclei move towards the centre of the cell in slightly older Flii homozygous embryos and pole cells (pc) reformed. Scale bar = 100μM. Figure adapted with permission from (Campbell et al., 1993)

1.6.3 Flightless I structure, expression and molecular functions

Flii is a 1256 amino acid protein with a molecular weight of 143kDa (Davy et al., 2000). It possess duplicated domains containing three repeated 125-150 amino acid sequences corresponding to the six gelsolin-like (g1-6) segmental repeats which identify it as a member of the gelsolin family of proteins (Claudianos & Campbell, 1995). Flii is unique amongst the gelsolin family in containing a leucine rich repeat (LRR) domain at the N-terminal end of its mRNA sequence. Other proteins containing this domain utilise it for protein-protein or protein-lipid interactions, which may underlie the diversity of functions Flii is thought to possess (Kobe & Kajava, 2001). Flii is the only known protein in which gelsolin-like and LRR domains exist together (Kopecki & Cowin, 2008).

The unique structure of Flii has led to a number of possible mechanisms for its regulatory role in wound healing. As an actin remodeling protein, Flii is important in the regulation of the actin cytoskeleton and consequently, the motility, contraction and adhesion of cells (Davy et al., 2000). Davy demonstrated that Flii is localized to various cell migratory structures, such as filopodia, neuritis and growth cones, while co-localizing with other cytoskeletal structures related to motility (Davy et al., 2000).

A recent study has shown that Flii acts by inhibiting actin polymerization, while capping, but not severing, actin filaments (Mohammad et al., 2012). This prevents

the growth of actin and contributes to actin filament stability. When Flii is reduced filament ends are not capped leading to increased actin and focal adhesion turnover as well enhanced cell motility (Mohammad et al., 2012). There is also evidence to suggest that Flii may actually be secreted by fibroblasts and so may act in a similar manner to the secretory phenotype of gelsolin that scavenges extracellular actin following tissue injury (Cowin et al., 2012).

Flii co-localizes with several molecules involved in cytoskeletal re-organisation, such as Ras, Cdc42 and RhoA. It associates with Ras via its LRR domain. Ras proteins regulate the actin cytoskeleton by associating with Raf-1 activating mitogen-associated protein kinase (MAPK) and this represents a possible pathway by which Flii regulates cell response during wound healing (Campbell et al., 1997; Davy et al., 2000; Goshima et al., 1999).

More recent work has shown that Flii modulates focal adhesions and filamentous actin stress fibres in a Rac-1 dependent manner (Kopecki, O'Neill, et al., 2011). Flii co-precipitates with focal adhesion proteins talin, paxillin and vinculin (Kopecki et al., 2009). *In vivo*, elevated Flii leads to increased stress fiber formation by impaired focal adhesion turnover and enhanced formation of fibrillar adhesions. Conversely, Flii knockdown increases the percentage of focal complex positive cells (Kopecki, O'Neill, et al., 2011). Flii knockdown cells exhibit lower levels of activated β1-integrin and vinculin, without any change in talin levels. This suggests that Flii may promote the recruitment of focal adhesion proteins like vinculin, which through its interaction affects β1-integrin activation (Mohammad et al., 2012).

Flii's multifactorial potential is emphasized by its distribution throughout the cell. Although primarily nuclear Flii is also capable of nuclear to cytoplasmic translocation, a property it shares with several other members of the gelsolin superfamily (supervillin, gelsolin and CapG) (Davy et al., 2001; Lei et al., 2012). Flii is secreted from macrophages and fibroblasts in response to wounding and LPS stimulation respectively (Lei et al., 2012). Flii has been shown to localize to late endosomes/lysosomes but not to pathways associated with classical secretory mechanisms, such as the trans-Golgi network, recycling endosome-associated SNARE protein Vti1b or VAMP3 (Lei et al., 2012).

Like gelsolin, Flii also has the ability to regulate gene transcription probably through the pools of Flii found localized to cell nuclei (Archer et al., 2005). Flii binds to oestrogen and thyroid hormone receptors, as well as co-activators GRIP1 and CARM1 (Y. H. Lee et al., 2004). Flii also inhibits β-catenin and LEF1/TCF-mediated transcription (Lee & Stallcup, 2006). Flii has been identified as a substrate for cytokine-independent survival kinase (CISK) acting downstream of PI-3kinase/CISK signaling pathway. CISK phosphorylates Flii, modulating its activity as an oestrogen co-receptor and potentially impacting on actin reorganization and endosome trafficking (Xu et al., 2009).

Flii also interacts with proteins known as enhancers of nuclear receptor-directed transcription, such as GRIPI (p160), SRC-1, pCIP and TRAM1 (Claudianos & Campbell, 1995). These proteins bind to histone acetyltransferase CBP(p300) and arginine methyltransferase CARM1 and promote histone modifying activity in the promoter area (Archer et al., 2005). The gelsolin related domains of the Flii

protein also bind nuclear actin like proteins such as BAF53 and Arp4, which form integral components of the co-activator complex (Y. H. Lee et al., 2004).

As mentioned, Flii is unique among the gelsolin family of proteins in possessing a LRR domain. This allows protein-protein/protein-lipid interactions which mediate receptor-ligand affinity (Kobe & Kajava, 2001). Two proteins that bind to Flii's LRR domain have been identified and are known as Flii leucine rich repeat associated protein (FLAP)1 and FLAP2 (Y. T. Liu & Yin, 1998) (Figure 1.8). It is postulated that interaction between Flii and FLAP1 may be important in regulating the β-catenin dependent Wnt signaling pathway (Lee & Stallcup, 2006). Flii antagonizes synergy between FLAP1, p300 and β-catenin by acting synergistically in a GRIP1 co-activator complex with nuclear receptors, and therefore may regulate LEF1/TCF-mediated transcription (Lee & Stallcup, 2006).

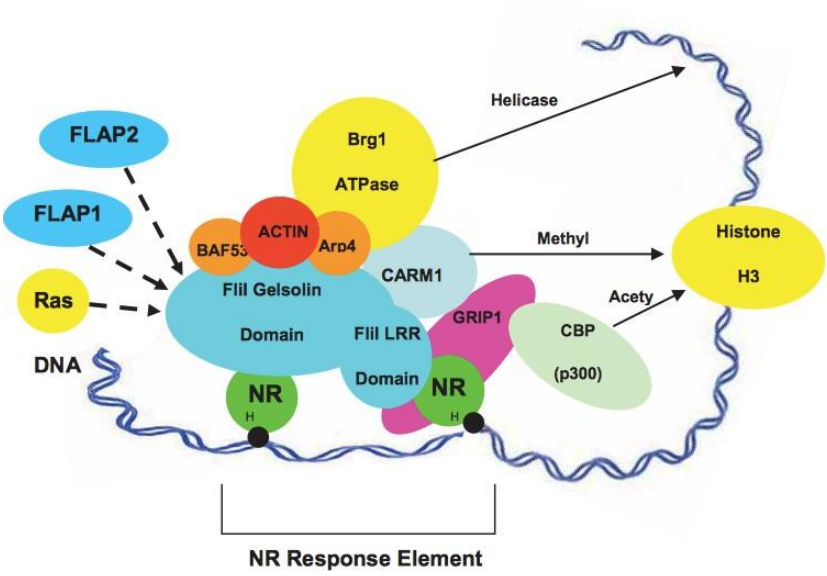


Figure 1.8 Schematic diagram of Flii illustrating its role as a nuclear receptor co-activator and formation of the co-activator complexes at the promoter of an NR responsive gene. NR denotes nuclear hormone receptor (Kopecki & Cowin, 2008)

Flii has also been shown *in vitro* to inhibit activated calcium/calmodulin dependent protein kinase type II (CaMK-II) resulting in suppression of β-catenin activity (Seward et al., 2008).

A number of recent studies have provided compelling evidence that Flii has an important role as an immune-modulator (Dai et al., 2009; Hayashi et al., 2010; Lei et al., 2012; Li et al., 2008). Flii's N-terminus LRR domains share nearly 50% homology to the LRR domains of toll-like receptor (TLR). TLRs function as detectors of injury or infection by binding to pathogen-associated molecular pattern molecules (PAMPs) (e.g. LPS from gram negative bacterial cell walls) or damage-associated molecular pattern molecules (DAMPs) (e.g. ECM cleavage products or molecules such as HMGB1 (High Mobility Group Ptorein B1) released from damaged cells) (Bianchi, 2007). After binding TLR signaling pathways are activated leading to the secretion of pro-inflammatory cytokines. *In vitro,* Flii binds to the TLR adaptor protein MyD88 through its interaction with nucleoredoxin which inhibits MyD88 binding to TLR4 and reduces inflammatory cytokine secretion (Hayashi et al., 2010; T. Wang et al., 2006). Flii has also been localized to late endosome/lysosomes in fibroblasts and macrophages where it may also dampen inflammation (Lei et al., 2012; Li et al., 2008).

The TGF-β family has been one of the most investigated cytokines in relation to wound healing, with particular focus on developing novel therapies that may modulate TGF-β1/ TGF-β3 ratio and improve wound healing (Shah et al., 1994, 1995). Flii has been shown to interact with all TGF-β1 isoforms *in vitro* and associate with activating protein-1 (AP-1), c-fos and c-jun transcription factors, as

well as nuclear Akt, suggesting Flii may regulate TGFβ activity. This is in keeping with *in vivo* data where Flii$^{+/-}$ wounds display decreased TGF-β1/ TGF-β3 ratios and Flii$^{Tg/Tg}$ wounds display increased TGF-β1/ TGF-β3 compared to controls (Campbell et al., 1993).

1.6.4 Flii in wound healing and tissue regeneration

Work over the past decade has shown that Flii is a negative regulator of wound healing (Cowin et al., 2007). Flii affects the proliferation and cell motility of keratinocytes, while also affecting dermal fibroblast motility, proliferation and collagen production (Cowin et al., 2007). *In vivo*, Flii$^{+/-}$ mice show improved wound healing, with increased epithelial migration and smaller more contracted wounds, while Flii$^{Tg/Tg}$ mice display increased wound area and dermal gap, delayed epithelial migration and reduced cell proliferation (Cowin et al., 2007; JJackson et al., 2012; Kopecki & Cowin, 2008; Kopecki et al., 2013) (Figure 1.9).

Flii has also been investigated in several other *in vivo* models of wound healing. Using a model of partial thickness burn wounds, Flii$^{+/-}$ mice displayed improved wound healing, with decreased levels of pro-scarring cytokine TGF**-**β1 and increased levels of anti-scarring TGF-β3 compared to control. On the other hand Flii$^{Tg/Tg}$ mice displayed the opposite, with impaired wound healing and increased levels of TGF**-**β1/decreased TGF-β3. Similarly reducing Flii has been shown to improve wound healing in a diabetic mouse model of wound healing. This is associated with a reduction in TLR4 expression and increased angiogenesis

(Ruzehaji, Kopecki, et al., 2013; Ruzehaji, Mills, et al., 2013). Flii has also been investigated as the target for a novel treatment of Epidermolysis Bullosa (EB). In two models of EB, $Flii^{Tg/Tg}$ displayed more severe blistering compared with control, while reducing Flii in $Flii^{+/-}$ mice less blister severity (Kopecki, Arkell, et al., 2011). A Flii neutralizing antibody has been developed and has been shown to improve healing in acute and chronic wounds in murine and porcine models (Kopecki, Ruzehaji, et al., 2013).

Recent evidence has emerged of a possible role for Flii in tissue regeneration (Strudwick et al., 2017). In a murine whisker follicle regeneration model $Flii^{+/-}$ showed repaired or delayed regenerative potential (Waters et al., 2011). In a murine distal amputation model, a regenerative phenotype was observed in $Flii^{Tg/Tg}$ mice in both distal and proximal amputations, whereas regeneration occurred only in distal models in WT mice (Strudwick et al., 2017)

Flii has also recently been investigated in relation to psoriasis (Chong et al., 2017). Psoriasis is an inflammatory autoimmune skin condition, in which a Th1 and Th17 drive abnormal keratinocyte differentiation, proliferation and expression of Toll Like Receptors 1, 2, 4, 5 and 9 (Chong et al., 2017). As mentioned, Flii has 50% homology to TLRs and is known to be involved in the TLR4 pathway (Ruzehaji et al., 2013). Elevated levels of Flii were seen in human psoriatic plaques and in a model of murine psoriasis $Flii^{+/-}$ mice demonstrated reduced inflammation, erythema and skin thickening. Analysis of plaques showed reduced inflammatory cell numbers, reduced keratinocyte proliferation and reduced TLR-4 expression (Chong et al., 2017).

Flii inhibits cell adhesion and is an important regulator of actin remodeling, cell signaling and has been shown to function as a nuclear receptor co-activator (Kopecki & Cowin, 2008; Kopecki et al., 2013). These properties are vital to the ability of tumour cells to spread and invade surrounding tissue. Flii levels have been shown to be significantly elevated in human squamous cell carcinoma (SCC) and $Flii^{Tg/Tg}$ mice demonstrated increased incidence and severity of SCC comparted to WT controls (Kopecki et al., 2015). SCC analysis in $Flii^{Tg/Tg}$ mice also revealed significantly lower levels of caspase 1 and annexin-V, suggesting decreased apoptosis of tumour cells and may contribute to tumour progression (Kopecki et al., 2015). Exogenous reduction of Flii levels also reduced tumour size and progression, which identifies Flii as a novel target for SCC therapy.

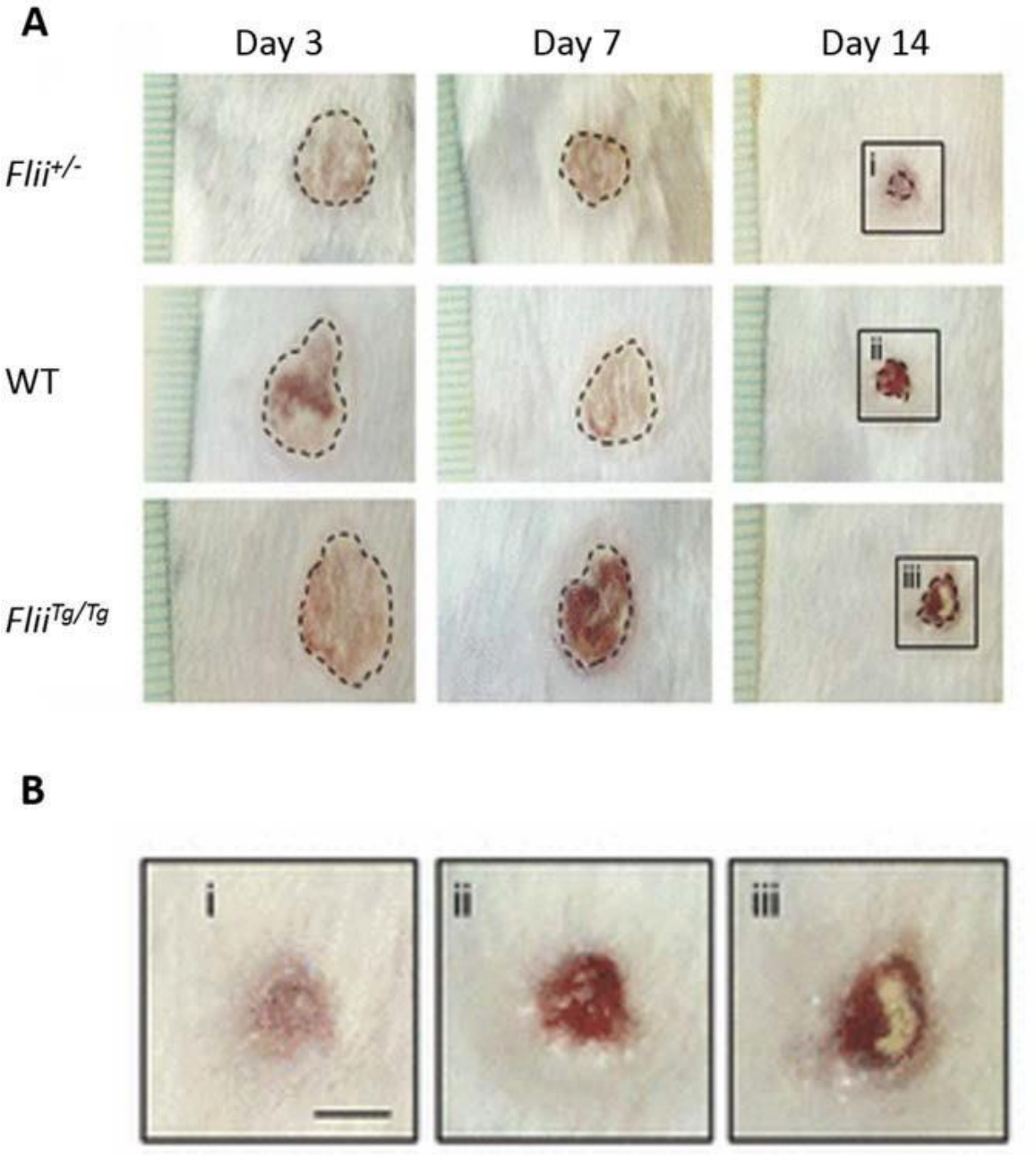


Figure 1.9. Representative partial thickness burn wounds seen in Flii mice. **A** Partial thickness burn wounds seen at 3, 7 and 14 days post injury in Flii$^{+/-}$, WT and Flii$^{Tg/Tg}$ mice. Dotted lines refer to wound margins. The ruler represents mm. **B** shows higher magnification of day 14 wounds, i, ii and iii show Flii$^{+/-}$, WT and Flii$^{Tg/Tg}$ mice respectively. Scale bar = 2mm. Adapted with permission from (Adams et al., 2009)

1.7 Hypertrophic Scarring

1.7.1 Defining hypertrophic scarring

Hypertrophic scarring is a fibroproliferative disease of the skin, in which an aberration of normal wound healing pathways result in the production of excessive collagenous extracellular matrix. Clinically this manifests as a raised, red, tender, pruritic scar, which is prone to contracture over time (Figure 1.10). These scars may cover large areas, but do not, in contrast to keloids, extend beyond the original margins of injury. In the majority of cases the scarring enlarges over a period of months, then gradually regresses over a few years (Rudolph, 1987).

Histologically, hypertrophic scars are characterized by dermal thickening due to collagen deposition, parallel collagen fibre orientation, loss of dermal appendages, absence of elastin and flattening of the epidermis (Santucci et al., 2001). A key distinguishing feature is the presence of whorls of immature collagen that consist of collagen fibrils, increased type 3 collagen, small blood vessels and acidic mucopolysaccharide (Ketchum et al., 1974).

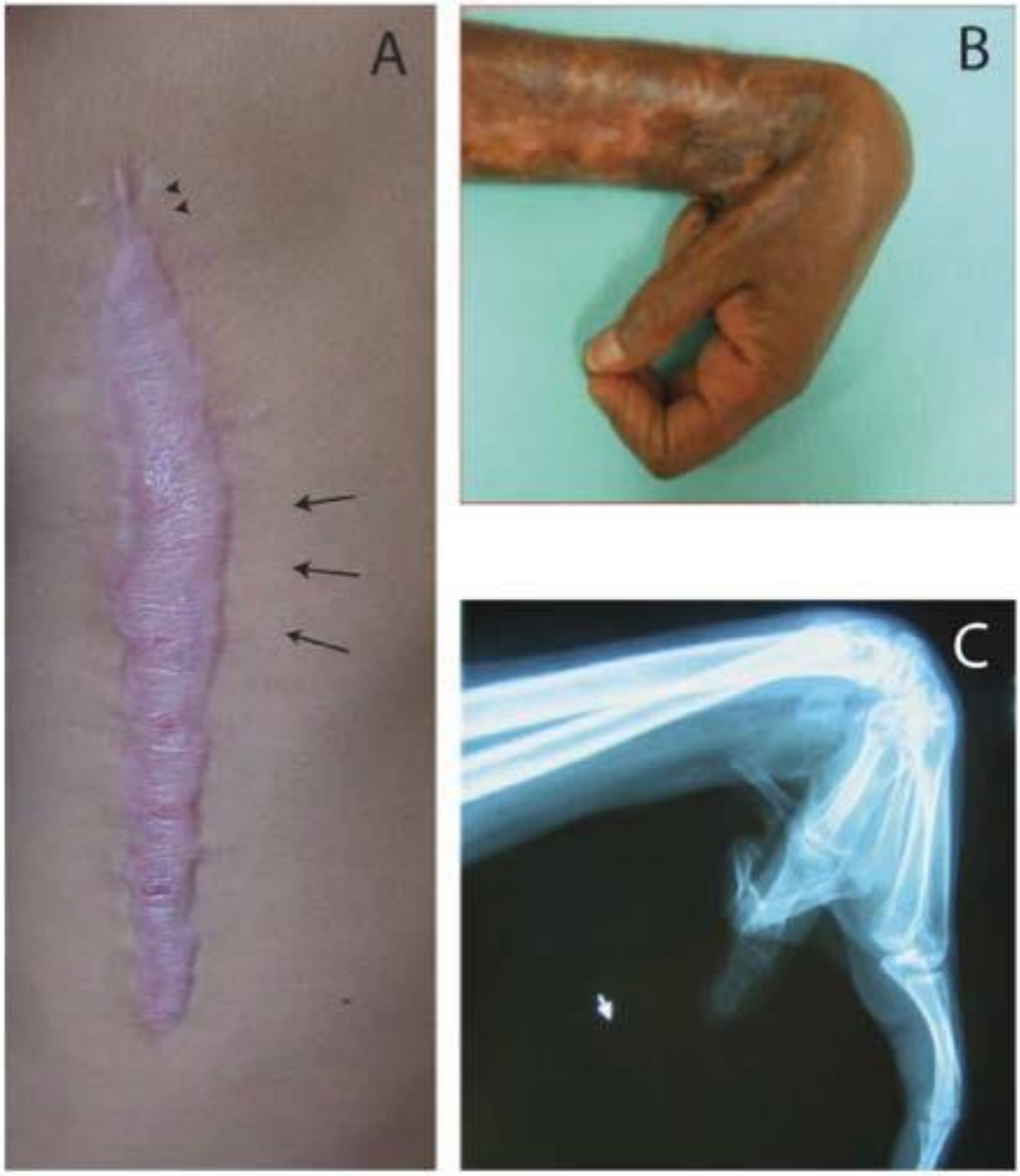


Figure 1.10 Complications of hypertrophic scarring Typical appearance of a hypertrophic scar (A) as well as an example of the deformity caused by scar contracture across the wrist joint (B and C) (Aarabi, Bhatt, et al., 2007) (Reproduced with permission)

1.7.2 Epidemiology

While most commonly associated with burn injury, hypertrophic scarring may occur after any skin injury, particularly those involving the full thickness of the dermis. Prevalence after deep dermal burn injury is estimated at 75%, and this figure is higher in children, and dark skinned individuals (Bombaro et al., 2003).

Hypertrophic scarring imparts a devastating burden of disease, including disfigurement, pain and disability. Consequently, it is associated with significant social and psychological comorbidities such as low self-esteem, social isolation, prejudicial societal reaction, depression and suicide (Engrav et al., 2007). Rehabilitation for hypertrophic scarring is laborious and costly, requiring thousands of hours of physical therapy and often, multiple surgical procedures. In the US alone, this treatment is estimated to cost in excess of $4 billion every year (Chen et al., 2017).

The antecedents to hypertrophic scarring, such as deep burn injury, wound tension, wound infection or the presence of foreign material, were identified at the beginning of modern surgery. However, how these lead to changes at the cellular and molecular level that drive fibroproliferation is poorly understood.

1.7.3 Burn injury, hypertrophic scarring and current treatments

Given its strong association with burn injury, an understanding of hypertrophic scarring is not possible without some discussion of burn injury management.

1.7.3.1 Burn Injury

The pathophysiology of the burn wound is a function of both temperature and exposure time i.e. a high temperature for a short time may cause the same tissue damage as a lower temperature for a longer time (Andrew et al 2017). Jackson described three zones of histopathological injury: coagulation, stasis and hyperaemia (Jackson, 1953). The *zone of coagulation* is comprised of eschar or necrotic tissue and is closest to the heat source. This is surrounded by the *zone of stasis*, where there is only moderate tissue damage, but slow blood flow and oedema due to capillary leakage and cell membrane disruption (Baskaran et al., 2001; Despa et al., 2005). Poor blood flow in this zone may lead to local tissue ischaemia and further necrosis (Zawacki, 1974). Surrounding the zone of stasis is the *zone of hyperaemia*, in which cell damage is minimal and blood flow gradually increases resulting in early spontaneous recovery. Burn depth may increase over 48-72 hours, as the zone of stasis becomes necrotic.

Burn depth is vital to their outcome and they are classified accordingly. *Superficial burns*, such as sunburn, affect the epidermis only and are painful. They will heal

completely within 3-5 days. *Partial thickness* burns involve the entire epidermis and portions of the dermis. They are divided into superficial partial and deep partial thickness. *Superficial partial burns* extend to the superficial dermis and are pink, moist and painful to touch. Blistering is common as a result of serum accumulating between the superficial dermis which has detached from the deep dermis (Andrew et al 2017). They will usually heal in two weeks without a scar via regenerative elements within sweat glands and hair follicles. *Deep partial thickness burns* involve the entirety of the epidermis and extend to the reticular layer of the dermis. They are a mottled white-pink colour and have variable sensation due to destruction of superficial cutaneous nerves. These wounds may heal within 3 weeks but are at increased risk of developing hypertrophic scarring. *Full thickness burns* involve the entire epidermis and dermis. They are brown-black, leathery and insensate. Healing will be extremely slow and associated with marked contraction and hypertrophic scarring.

1.7.3.2 History of burn injury management

Since 1500BC various emollients have been used to cover the burn wound, often with abysmal results. The Ebers Papyrus (1534BC) describes a five day regimen of treatments with various combinations of mud, cow dung, bees' wax, ram's horn and barley porridge (Ravage, 2004). Hippocrates proposed the application of melted pig skin mixed with bitumen (Pack, 1930) while during the Renaissance, most physicians treated acute burns with boiling hot oil (Nuland, 2008).

By the early 20th century burn treatment comprised of tannic acid spray, as well escharotic and bacteriolytic agents such as gentian violet and the flavine compounds (Heslop, 1998). The first major advance in burn wound management came during World War II, where the introduction of aerial warfare led to previously unseen numbers of large burns. The work of pioneering plastic surgeons Harold Gillies and Archibald McIndoe led the discontinuation of tannic acid sprays and the introduction of pressure absorptive dressings (Heslop, 1998). The greatest legacy of this period was the treatment of burns in specialist multidisciplinary centres, such as McIndoe's East Grinstead Hospital. McIndoe is recognized as the first surgeon to identify the importance of ongoing physical and psychological rehabilitation for burn patients, which led to the establishment of the famous "Guinea Pig Club" (McCleave, 1961).

Overwhelming infection and sepsis was still a problem that led to high mortality rates following large burns, even despite the introduction of penicillin (Heslop, 1998). During the 1950s the establishment of the Shriners' burn institutes in the US led to substantial advances in the supportive treatments for burn patients such as aggressive fluid resuscitation and the use of topical antibiotics such as silver (Heslop, 1998).

One of the most significant advancements in burn surgery came in the 1960s from the unlikely location of Slovenia, behind the "Iron Curtain". Zora Janžekovič introduced the concept of early excision of devitalized tissue to prevent it becoming a source of inflammation and a nidus of infection (Janzekovic, 1970). This led to considerable reductions in mortality and hypertrophic scarring, and,

despite initial scepticism from the West, has become standard care for burns patients (Janzekovic, 2008).

Further progress in reducing post-burn hypertrophic scarring has been slow. Promising new surgical technologies, such as cultured keratinocytes and dermal allografts have had only limited success in preventing or reducing hypertrophic scarring (Atiyeh, Hayek, et al., 2005; Janzekovic, 1970). Non-surgical treatments, such as massage, silicone dressing and pressure garments are anecdotally effective in reducing scar severity and contracture development, but they lack a strong evidence base for their efficacy or mechanisms of action. Other techniques such as steroid injection or radiotherapy are limited to small areas of scarring and carry a high risk of recurrence (Franz et al., 2000; Mustoe et al., 2002; Rahban & Garner, 2003). Thus, despite the ability to save the lives of patients with severe burn injuries, preventing and treating the sequelae of these injuries, namely hypertrophic scarring, remains limited.

1.7.3.3 Current management of hypertrophic scarring.

Minimising risk factors for hypertrophic scarring

Risk factors predisposing to hypertrophic scarring have been identified since the beginnings of modern surgery. These include factors such as deep burn injury, wound tension, wound infection or the presence of foreign material (Aarabi et al., 2007). Modifying these risk factors can prevent or reduce hypertrophic scarring. Thus, wounds should undergo appropriate debridement or excision. Closure of wounds, where possible should minimize tension and infection should be avoided.

Vigilance should be exercised in populations predisposed to hypertrophic scarring, such as children or individuals with dark skin, to ensure early detection and treatment. Burns deeper than mid or deep dermal level are at particular risk of hypertophic scarring, particularly those that take longer than three weeks to heal (Deitch et al., 1983). These patients should undergo early tangential excision and split thickness skin grafting in order to reduce the risk of hypertrophic scarring (Finnerty et al., 2016)

Management of the developing or established hypertrophic scar

1. Non-invasive management

- *Avoidance of UV exposure*

  Excessive exposure to UV light has been show to lead to hyperpigmentation of the scar, even after the resolution of hypertrophic scarring (Haedersdal et al., 1998).

- *Application of moisturizer*

  Application of moisturizer to immature scars has been associated with reduction in hypertrophic scarring. The moisture content of keratinocytes has been shown to influence keratinocyte behavior *in vitro*, while increased trans-epidermal water loss (TEWL) is associated with increased scarring (O'Shaughnessy et al., 2009).

- *Scar Massage*

  Scar massage is widely accepted to reduce development of hypertrophic scarring. The mechanism for this remains unclear, with possible theories including stimulation of ECM remodeling and induction of cell death. Scar

massage also assists with lymphatic drainage and stretching of contractures (Roques, 2002).

- *Taping*

  Taping of surgical incisions with Steri-Strips™ or Hypafix® has been shown to reduce the probability of hypertrophic scarring, especially for incisions perpendicular to relaxed skin tension lines. This is likely due to reducing ECM tnsion in the healing wound (Atkinson et al., 2005).

- *Silicone dressings*

  Silicone dressings have been widely used to reduce the incidence of hypertrophic scarring since the first evidence of their efficacy was presented in the early 1980s. Their effect is thought to be related to preventing TEWL. Dehydration of the stratum corneum is associated with an increased inflammatory response and increased scarring (Mustoe, 2008). A Cochrane review showed only weak evidence for the efficacy of silicone in preventing or treating hypertrophic scarring (O'Brien, 2013).

- *Physiotherapy*

  Physiotherapy is an integral component of burn rehabilitation particularly in preventing and managing contractures through splinting and range of motion (ROM) exercises. Splinting is particularly important for burns to body areas prone to contracture such as the neck, elbow, axilla and hands (Jordan et al., 2000).

- *Pressure garments*

  Pressure garments are used both prophylactically and to treat established hypertrophic scars. Their mechanism of action is poorly understood. Theories include: thinning of the dermis through negative balance in

collagen turnover and decreasing oedema; decreased myofibroblast activity due to reduction of oxygen tension; action via cell mechanoreceptors or stimulation of neurogenic inflammation. Pressure garments are also useful in alleviating oedema, pruritus and pain (Anzarut et al., 2009).

2. Medical therapies

- *Corticosteroids*

  Corticosteroids have been shown to inhibit extracellular matrix production and excessive collagen deposition, as well as reducing pro-inflammatory cytokine production and inhibiting angiogenesis (Cohen & Diegelmann, 1977). They are only effective as an intra-lesional treatment i.e. topical application has not been shown to be effective. Their use is limited to small areas due to the pain of multiple injections and the systemic side effects of corticosteroids, particularly immunosuppression. Hence, they are rarely used in burns' surgery.

- *Imiquimod*

  Imiquimod is an immunomodulator that acts on macrophages, monocytes and dendritic cells to reduce scarring. Evidence is limited regarding its efficacy and most evidence comes from studies involving keloids (Berman & Kaufman, 2002).

- *Bleomycin*

Bleomycin sulfate, a secondary metabolite of a strain of Streptomyces has anti-tumour, anti-viral and anti-bacterial activity. It was proposed by Bodokh and Brun in 1996 as an alternative therapy for keloids and hypertrophic scarring (Bodokh & Brun, 1996). Bleomycin has been found to cause necrosis of keratinocytes, induce inflammatory infiltration and expression of various adhesion molecules in normal skin (Rabello et al., 2014). Despite these findings the exact mechanism by which bleomycin exerts its effect on scarring is unclear (Rabello et al., 2014). Evidence for efficacy is limited to small areas of scarring due to the pain of multiple injections and systemic side-effects (Aggarwal et al., 2008).

- *5-Fluorouracil (5-FU)*

  5-FU has been used for over three decades to prevent scar formation after glaucoma surgery and is thought to act via inhibition of fibroblast proliferation. It is often combined with surgical excision and triamcinolone acetonide to avoid excessive inflammation. Evidence for its efficacy mainly comes from studies of keloids (Davison et al., 2009).

- *Verapamil*

  Verapamil is a Ca^{2+} channel antagonist known to decrease collagen synthesis and stimulate pro-collagenase. It is delivered via intra-lesional or perilesional injection. Efficacy has been shown in keloids, Dupuytren's contracture and La Peyronie's disease (Copcu et al., 2004).

- *Laser*

Laser therapy has been shown to be useful in treating various aspects of hypertrophic scarring. (Hantash et al., 2007; Hantash & Mahmood, 2007). Treatment effects are though to occur via photothermolysis of scar microvasculature, decreased cellular activity from laser induced anoxia or from collagenolysis by laser stimulation of cytokine release (Brewin, 2014). Different wavelengths and treatment regimens are tailored to the desired outcome, which include improvement in pliability, texture, erythema and pruritus (Brewin, 2014). Both pulsed dye laser, ablative and non-ablative lasers can be used, alone or in combination (Tao, 2018).

3. Surgery

Surgery is important both the prevention of hypertrophic scarring as well as managing existing hypertrophic scars. It has both functional and aesthetic considerations. As described above, surgical management of the acute burn begins with assessment of burn depth and tangential excision of any burn that extends to the deep dermis or below. Excision of the burn removes necrotic and inflamed tissue, which would otherwise act as a nidus for infection, retard wound healing and predispose to hypertrophic scarring (Ong et al., 2006). Antibacterial wound dressings, such as silver and prophylactic antibiotics are administered to minimise the risk of infection.

Surgery to counter the disability and deformity caused by scar contracture is an all too common occurrence for burn survivors, particularly those with burns over the flexor surface of joints. The “reconstructive ladder” is used to approach the problem posed by scar contracture. These techniques

range from simple excision and primary closure, to skin grafting, loco-regional flaps (including Z-plasty, W-plasty and V-Y-plasty) through to free flap transfer (Cameron et al., 2010). For large areas, tissue expansion may be used in order to increase the surface area available for local or distant

tissue transfer. This involves gradual expansion of silicone balloons under donor tissue with saline solution over 3-6 months prior to surgery.

A range of surgical techniques exist which aim to improve the appearance of hypertrophic scarring. These include:

Dermabrasion

Dermabrasion involves controlled mechanical removal of epidermis and a portion of the dermis in order to smooth scars with irregular services. Re-epithelialisation occurs from remaining dermal appendages. Dermabrasion may be used in conjunction with split thickness skin grafting or dermal substitutes such as Integra™ (Hierner et al., 2005).

Radiotherapy

Radiotherapy is described almost exclusively for treatment of keloids as an adjunct to surgical excision, with recurrence reduced to as low as 10-20% (Arnault et al., 2009).

Autologous cells

It was hoped that the use of autologous cells would mark a major breakthrough in burn surgery, which would obviate the problem of limited donor skin in large burns. However, the technique was hampered by the long delay to culture, the fragility of cultured grafts, unpredictable ingrowth and expense. The technique has been partially improved by the introduction of "spray-on" technology.

Non-cultured (ReCell®**)** cells are now also used. These cells are harvested from the dermal-epidermal junction of non-injured skin by a combined physical-enzymatic perioperative procedure. Cell population includes basal keratinocytes, papillary dermal fibroblasts, melanocytes and Langerhan's cells. Cells are then delivered by spray. Advantages of this technique are small donor sites, but a significant disadvantage is the lack of any dermal component. Autologous cell grafting can be used in conjunction with dermal substitutes and widely meshed skin grafts (Wood et al., 2007).

Lipofilling

Lipofilling (autologous fat grafting) is useful in modifying the thickness and texture of scar tissue, leading to more supple scars. Furthermore, recent findings suggest adipose derived stem cells (ADSCs) release multiple growth factors stimulating angiogenesis and extracellular matrix deposition (Coleman, 2006).

1.7.4 Current understanding of the pathophysiology of hypertrophic scarring

Hypertrophic scarring represents an aberration of the normal wound healing process (Aarabi et al. 2007). The basic chronology of hypertrophic scarring is understood as follows: a prolonged inflammatory phase results in persistence of myofibroblasts in the wound and elevated levels of fibrogenic cytokines, such as TGFβ-1 and Connective tissue growth factor (CTGF) (Wynn, 2008). Myofibroblasts continue to drive inflammation and overproduce collagen, which accumulates in

a disorganised manner (Hinz, 2010). This results in a raised, red, tender scar, which is prone to contracture over time (Baker et al. 2009).

Research has therefore focused on particular elements of the wound healing process that may play a key role in its regulation, and therefore its dysregulation (Gurtner et al. 2008). These include focusing on the inflammatory response to injury, elements of the extracellular matrix and specific cells types. The current state of research into hypertrophic scarring pathophysiology is summarized here.

1.7.4.1 Inflammation

Clinically, increased inflammation due to deep burns or infection is strongly associated with hypertrophic scarring. Despite this, anti-inflammatory therapy has not been effective in preventing or treating hypertrophic scarring (Kamp, 2003). This led to the theory that is was not the severity, but the type of inflammatory response that is important in hypertrophic scarring.

The T-helper-2 cell response (Th2) which involves interleukins-4, 5, 10 and13 is strongly linked to fibrogenesis stimulating expression of genes for procollagen-I, procollagen-III and tissue inhibitor of metalloproteinase (TIMP) (Doucet et al., 1998; Wynn, 2004). In keeping with this the T-helper-1 response (Th1) leads to expression of pro-apoptotic genes and activation of nitric oxide synthase (NOS), which promotes collagenase activity and matrix remodelling (Gordon, 2003).

The TGF-β superfamily has been extensively investigated in relation to wound healing and scar formation. TGF-β has been shown to be integral for the ontogenetic shift from scarless foetal healing to adult scar forming wound healing (Chen et al., 2005). TGF-β1 and TGF-β2 are pro-fibrotic: they are mitogenic for fibroblasts, while stimulating angiogenesis, myofibroblast differentiation and matrix deposition (Soo et al., 2000). Conversely, TGF-β3 has been shown to reduce fibroproliferative effects (Bayat et al., 2005). The role of TGF-β receptors has been investigated with an elevated TGFβ Receptor I: TGFβ Receptor II shown to increase fibroblast collagen production *in vitro* (Pannu et al., 2004). Despite this, therapies targeting TGF-β in order to reduce scarring have not been shown to be effective.

1.7.4.2 Extracellular matrix

After injury, changes in the composition, organization and tension of the ECM are integral to the regulation of wound healing. ECM tension stimulates fibroblast development of stress fibres (Hinz, 2010). These stress fibres are initially composed of cytoplasmic actins (Hinz & Gabbiani, 2010; Tomasek et al., 2002. With increasing ECM tension, α-smooth muscle actin (αSMA) is expressed, which marks the phenotypic transition from fibroblast to myofibroblast, the key cell type implicated in hypertrophic scarring {Tomasek et al., 2002)

For this reason, a considerable focus of hypertrophic scarring research has focussed on the relationship between the fibroblast and the extracellular matrix.

In particular, interest has focused on how fibroblasts detect changes in the ECM and the effects this has for fibroblast phenotype.

Integrins are the main receptor mediating fibroblast attachment to fibronectin and collagens and promote adhesive signalling through the recruitment and activation of specific cytosolic proteins, for example focal adhesion kinase (FAK) (Lagares et al., 2012). Fibroblasts in hypertrophic scars have a higher percentage of α1 integrin compared to normal skin. This difference no longer exists after serial culture, suggesting the dermal microenvironment maintains this population (Szulgit et al., 2002). β1 integrin is also overexpressed in fibrotic fibroblasts and a neutralising β1 integrin antibody reverses their excessive adhesion and contraction (Shi-Wen et al., 2007). A β1-integrin knockout mouse reduces development of fibrosis in a mouse model of skin fibrosis (Liu et al., 2009; Liu et al., 2010). It has recently been shown that specifically blocking TGF-β1 mediated differentiation of myofibroblasts by preventing αv integrin activation of latent TGF-β prevents fibroproliferation in murine models of liver and lung fibrosis (Smith et al., 2016).

As mentioned, FAK is important in mediating integrin signalling. It is also thought to participate in transduction pathways activated by growth factors via G-protein-coupled receptors and tyrosine kinases (Sieg et al., 2000). In this way, FAK is postulated to integrate signals from pro-fibrotic factors such as endothelin (ET-1), connective tissue growth factor (CTGF) or TGFβ1 (Daher et al., 2008; Liu et al., 2007; Tan et al., 2009; Teixeira et al., 1999; Thannickal et al., 2003). Pharmacologic or genetic inactivation of FAK results in attenuation of bleomycin

induced fibrosis and FAK has been shown to be required for acquisition of a contractile phenotype *in vitro* (Lagares et al., 2012). Similarly, genetic knockout or pharmacological inhibition of FAK has been shown to reduce scarring in a tension induced model of hypertrophic scarring (Wong et al., 2012).

Hic-5 is a focal adhesion protein that is induced by TGF-β and H_2O_2 which may shuttle to the nucleus to serve as a transcription factor (Shibanuma et al., 1994). It has homology to paxillin and can interact with FAK and vinculin (Thomas et al., 1999). Hic-5 has been implicated in an autocrine loop that produces and activates TGF-β1 which points to an important role in the regulation of myofibroblast proliferation

1.7.4.3 Keratinocyte fibroblast interaction

Keratinocytes have been shown to regulate fibroblasts through secretion, activation, inhibition of growth factors such as TGF β. Co-culture of hypertrophic scar keratinocytes and fibroblasts led to change in fibroblast phenotype to myofibroblast (Desmouliere et al., 2005).

1.7.4.4 Reversibility of fibrosis

It is known from the natural history of hypertrophic scarring that the characteristic changes are reversible. Findings from liver fibrosis show dynamic interaction between matric metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs), with increased levels of TIMPs associated with fibrosis and vice versa. Irreversible fibrosis occurs when MMPS are no longer

present. Therefore research into source and identity of key MMP mediators in fibroproliferation may be important to reverse fibrosis (Duffield et al., 2005; Issa et al., 2004).

1.8 Pre-clinical models of hypertrophic scarring

Development of an appropriate animal model of hypertrophic scarring has been problematic (Aarabi et al., 2007). This is due in part to the fundamental differences in wound healing between humans and other mammals, particularly the small animals commonly used for preclinical research. In contradistinction to humans, the skin of small mammals has denser hair cover with a short growth cycle; lacks dermal papillae/apocrine gland; and has a full panniculus carnosus, which rapidly apposes wound edges after wounding (Wong et al., 2011).

In vitro techniques have provided useful insights, but are limited by their inadequacy in modelling the vast complexities of the *in vitro* milieu, including the presence of various cells types, cytokines and three dimensional ECM architecture. Furthermore, phenotype changes seen in cells cultured from hypertrophic scars often do not persist after initial passages (Larsson et al., 2008; Sargent & Whitfield, 2011).

Research using *ex vivo* hypertrophic scar tissue may yield information on scar architecture and cellular constituents. However, detailed quantifiable data is very

difficult to obtain. Firstly, clinical diagnosis of hypertrophic scarring is highly arbitrary and varies from institution to institution (Lee et al., 2004). Furthermore, tissue samples are only available when it is deemed necessary for scars to be excised as part of a patient's clinical management. Developing or immature hypertrophic scars are rarely excised as this can trigger further hypertrophic scarring. Thus, samples usually represent an end stage of the scarring process and will yield no information about the process of scar development.

Thus, it is apparent that a valid animal model is needed to further research into hypertrophic scarring. The following is a summary of various approaches to this model.

Tight skin mouse 1 and 2 (Tsk1/2)

These genetic models were both originally developed to study systemic sclerosis, and have been used to test new anti-scarring therapies (Baxter et al., 2005). Tsk1 are bred with a partial in-frame duplication of the fibrillin-1 gene, located on mouse chromosome 2 (Baxter et al., 2005). Tsk2 contains a gain of function missense mutation in Col3a1 on chromosome 1 (Christner et al., 1995). Both models develop tightness of the dorsal skin, thickened dermis and with increased collagen content. However, neither of these models adequately model the excessive fibroproliferation or inflammation that characterizes hypertrophic scarring (Marttala et al., 2016).

Duroc Pig

The Duroc pig displays similar scarring properties to the human (Goodwin et al., 1976; Zhu et al., 2003). If dermal wounds are allowed to heal, they will display raised, firm, hyperpigmented scars. Histological analysis reveals increased dermal thickness, with increased myofibroblasts, mast cells as well as collagen whorls and nodules (Harunari et al., 2006). This is associated with increased TGF-β1 expression and increased collagen-1 and collagen-3. For these reasons, the Duroc pig is proposed as the most appropriate animal model to study hypertrophic scarring (Zhu et al., 2008). Unfortunately this is offset by the expense, housing and handling difficulties of such a large animal.

Rabbit ear model

The rabbit ear model involves the creation of a wound on the ventral surface of the rabbit ear, which forms a scar over 3-4 weeks post wounding (Joseph & Dyson, 1966). Histology of the resulting scar reveals some of the features that characterize human hypertrophic scars, such as parallel collagen fibres and increased inflammation. However, the extent of this increased scarring does not approach that observed in hypertrophic scarring (Wells et al., 2016).

Horse "Proud Flesh" model

Horses are well known to develop hypergranulation scar type lesions on their extremities, often following trauma (Theoret et al., 2013). Histological analysis of these lesions reveals increased haphazardly arranged collagen as well as increased myofibroblasts (Theoret et al., 2013). Limited literature on these scars points to their similarity to human hypertophic or keloid scars, but further research is limited by availability and technical difficulty of dealing with such a large animal.

Xenograft models

Xenograft models have utilized immune deficient mice(Escamez et al., 2004; Kischer et al., 1989) and rats (Polo et al., 1998), such as the athymic nude mouse in order to receive grafts of explanted human hypertrophic scars or keloids. This has the advantage of studying human tissue in an *in vivo* environment. Xenografts are viable for weeks to months after transplantation and show ongoing angiogenesis and continued collagen production (Kischer et al., 1989). Xenograft models present a preformed hypertrophic scar, and thus are unable to yield information about the fibroproliferative process in scar development. Furthermore, by manipulating the immune system, key inflammatory processes contributing to scar formation may be being altered (Seo et al., 2013).

Wound tension model

Mouse models have employed wound tension, a well-known risk factor for hypertrophic scarring, as a stimulus for scarring. The most successful of these uses

a craniofacial distraction screw to apply a constant force across an incisional wound (Aarabi et al., 2007). Resulting scars bear many of the hallmarks of hypertrophic scarring. The main drawback of this model is the technical difficult in applying and maintaing a metal screw construct on the back of the mouse.

1.9 Research Aims and Hypothesis

Hypertrophic scarring is an area of unmet clinical need that carries a significant burden of disease. Our understanding of the pathogenesis of hypertrophic scarring is limited and this has hampered the development of novel anti-scarring therapies. Flii I has been established as an important regulator of wound healing via effects on cell migration and proliferation (Adams et al., 2009; Cowin et al., 2007). Furthermore, as a member of the gelsolin family of proteins, Flii is involved in the regulation of the actin cytoskeleton, a key process in wound healing and scar formation. Thus, the aim of this thesis was to investigate the potential role of Flii in the fibroproliferative process that leads to hypertrophic scarring. The aims of this thesis were:

1. To investigate the expression of Flii in normal human tissue, acute burns, immature hypertrophic scars and mature hypertrophic scars
2. To develop and validate a murine model of hypertrophic scarring
3. To determine the effect of differential expression of Flii on fibrosis and scarring using a murine model of hypertrophic scarring
4. To investigate the effect of the differential expression of Flii on fibroblast phenotype *in vitro.*

Studies presented in this thesis have identified a role for Flii protein in regulating the fibroproliferative process that leads to hypertrophic scarring

through modulating fibroblast phenotype. This identifies Flii as a potential therapeutic target for the development of novel therapies for the prevention or reduction of hypertrophic scarring.

Chapter 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Molecular Reagents

Water Soluble Tetrazolium Salt-1 (WST-1) was purchased from Roche Diagnostics, NSW, Australia. 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI) nuclear stain used in immunocytochemistry were purchased from Sigma-Aldrich, Sydney, Australia. IgG from rabbit serum (#15006) used as a control and Ultrapure Bovine Collagen Solution (C4243) used for a floating collagen gel contraction assay were all purchased from Sigma-Aldrich, Sydney, Australia. Bleomycin sulphate (R-42569) was obtained from Hospira (Mulgrave North, VIC).

2.1.2 Immunohistochemical reagents

All the antibody information including suppliers, catalogue numbers and concentrations used for immunohistochemistry and western blotting analysis in this thesis are outlined in Table 2.1. Rabbit monoclonal anti-collagen-1 (600-401-103-0.5) and anti-collagen-3 (600-401-105-0.1 antibodies) were obtained from Rockland (Gilbertsville, PA). Anti-collagen-1 and anti-collagen-3 antibodies are raised against collagen-1 and collagen-3 from human and bovine placenta respectively. Mouse anti- Flightless I antibody is raised against amino acids 1–300 mapping at the N terminus of Flightless I of human origin (Santa-Cruz, CA).

Rabbit polyclonal anti-TGF-β1 IgG antibody (sc-146) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-TGF-β1 is mapped against the C-terminus of TGF-β1 of human origin. Mouse monoclonal anti-α-smooth muscle

actin (αSMA) IgG antibody (A2547) was obtained from Sigma (St. Louis, MO). Mouse monoclonal anti-αSMA is raised against the single isoform, α-actin. Secondary goat anti-rabbit (A-11034) and goat anti-mouse (A-11001) Alexa Fluor 488 antibodies were obtained from Life Technologies (Carlsbad, CA). Murine IgG irrelevant antibody (I8765) was obtained from Sigma (St. Louis, MO). 4', 6-diamidino-2-phenylindole (DAPI, D9564) was obtained from Sigma Aldrich (St. Louis, MO). Antibody to β-tubulin was obtained from Sigma Aldrich (St. Louis, MO).

Antibody	Manufacturer	Cat #	Raised In	Concentration for IHC	Concentration for Western Blot
β-Tubulin	Sigma Aldrich	T4026	Mouse	N/A	1µg/ml
DAPI	Sigma Aldrich	D9564	N/A	2µg/ml	N/A
Flightless	Santa Cruz	sc-30046	Rabbit	2µg/ml	1µg/ml
Flightless	Santa Cruz	sc-21716	Mouse	2µg/ml	1µg/ml
PCNA	Santa Cruz	sc-56	Mouse	2µg/ml	N/A
Collagen-1	Rockland	600-401-103-0.5	Rabbit	2µg/ml	1µg/ml
Collagen-3	Rockland	600-401-105-0.1	Rabbit	2µg/ml	1µg/ml
TGF-β1	Santa Cruz	sc-146	Rabbit	2µg/ml	1µg/ml
αSMA	Sigma Aldrich	A2547	Mouse	2µg/ml	1µg/ml
2° Anti-rabbit Alexa Fluor 488	Invitrogen	A11008	Goat	2µg/ml	N/A
2° Anti-goat Alexa Fluor 488	Invitrogen	A11055	Donkey	2µg/ml	N/A
2° Anti-goat Alexa Fluor 633	Invitrogen	A-21082	Donkey	2µg/ml	N/A
2° Anti-goat Alexa Fluor 594	Invitrogen	A-11058	Donkey	2µg/ml	N/A
2° Anti-mouse Alexa Fluor 488	Invitrogen	A-11029	Goat	2µg/ml	N/A
2° Anti-mouse Alexa Fluor 594	Invitrogen	A-11005	Goat	2µg/ml	N/A
2° Anti-mouse Alexa Fluor 633	Invitrogen	A-20980	Goat	2µg/ml	N/A
2° Anti-rabbit Alexa Fluor 633	Invitrogen	A-20981	Goat	2µg/ml	N/A

Table 2.1 Antibody information and concentrations used in Western Blot analysis and

2.1.3 General reagents

Bleomycin sulphate (R-42569, Hospira Mulgrave North, VIC) was sourced from the Women's and Children's Hospital pharmacy. It was stored and handled in keeping with protocols developed by the pharmacy and the Paediatric Oncology Unit at the Women's and Children's Hospital

2.1.4 Osmotic pumps

Alzet micro-osmotic pumps (Model 1004, Durect Corp., Cupertino CA 95014) were used. These are miniature implantable pumps for use in laboratory animals (Figure 2.1). The micro-osmotic pump has a capacity of 90µl delivers solutions continuously for 28 days at a consistent flow rate of 0.11µl/hour 0.11 without the need for external connections or frequent handling of animals.

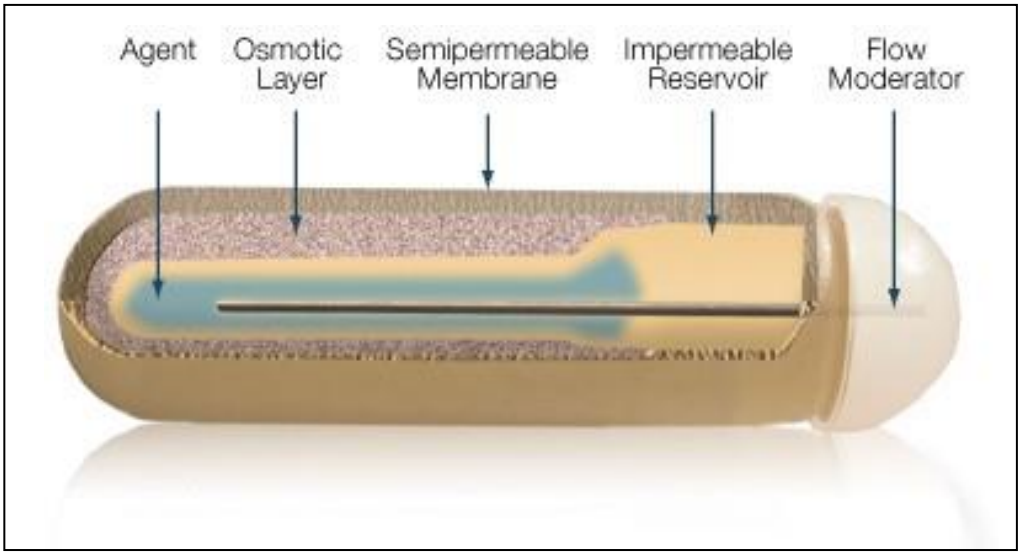


Figure 2.1 Alzet micro-osmotic pump (Model 1004, Durect Corp., Cupertino CA 95014)

2.1.5 Human Samples

Samples of normal human skin, acute burns and hypertrophic scars were obtained from the Royal Adelaide Hospital Adult Burns' Unit with ethics approval from the Royal Adelaide Hospital Human Research Ethics' Committee in accordance with the Declaration of Helsinki principles (RAH HREC Protocol 100513a). Written consent was obtained from patients undergoing surgery to obtain excised tissue that would otherwise be disposed of.

Samples of hypertrophic scars and keloid scars were obtained from Dr. Michelle Lodge, Mr. Roger Woods and Dr. Amy Jeeves from the Women's and Children's Hospital Burns' Unit with ethics approval from the Women's and Children's Health Network Ethics Committee in accordance with the Declaration of Helsinki principles (REC2399/8/14). Samples were obtained from patients undergoing emergency surgery for acute burns or elective surgery for treatment of burn related complications.

Samples of neonatal foreskins were obtained from five-day old males undergoing elective circumcision by Paediatric surgeon Mr. Chris Kirby in his private rooms at Memorial Hospital, with ethics approval from the Adelaide University Ethics Committee and Women's and Children's Health Network Ethics Committee (H-080-2011).

Normal abdominal tissue was obtained from Plastic surgeon Dr. Michelle Lodge from patients undergoing elective abdominoplasty at Calvary Hospital, with ethics

approval from the Calvary Hospital Research Ethics Committee and the Women's and Children's Health Network Ethics Committee (11-CHREC-F007).

The basic design and purpose of the research project was explained patients/guardians as well as protocols for the storage, processing and disposal of tissue. Any questions were answered and, if patients agreed to participate, written consent was obtained. Tissue collection had no bearing on surgical technique and only tissue that would ordinarily be disposed of was collected. Basic medical history was recorded e.g. age, sex, site of surgery, details of burn (if applicable), skin type and co-morbidities. Samples were de-identified.

2.1.6 Animal Tissue

All mouse strains in this study were BALB/c congenic and were maintained as homozygous colonies or by continuous backcross to BALB/c animals. Wild-type controls were obtained from BALB/c inbred litters. The murine alleles of Flii used in this study were:

1. **Flii t m*1*H d c (MGI:2 179 825),** a targeted null allele of Flii (Campbell et al. 2002). The genotype of a heterozygous carrier of this allele is written as Flii $^{+/-}$; and

2. **Tg(FLII)1Hdc (MGI:3 796 828),** a transgenic strain expressing exogenous human Flii due to the insertion of the human cosmid clone c110H8 (Campbell et al. 2002).These animals carry two copies of the

mouse Flii gene and two copies of the human Flii transgene ($Flii^{+/+}$; $Flii^{Tg/Tg}$) (Campbell et al. 2002).

$Flii^{+/-}$ and $Flii^{Tg/Tg}$ were generated by our collaborators (see 2.1.5.1), Dr. Hugh Campbell and A/Prof Ruth Arkell from Early Mammalian Development Laboratory, Research School of Biology, ANU College of Medicine, Biology & Environment, ACT, Australia (Figure 2.2). Balb/c wildtype mice were produced as litter mates to the *$Flii^{+/-}$* mice as a result of cross breeding heterozygous male mice to wild type female mice.

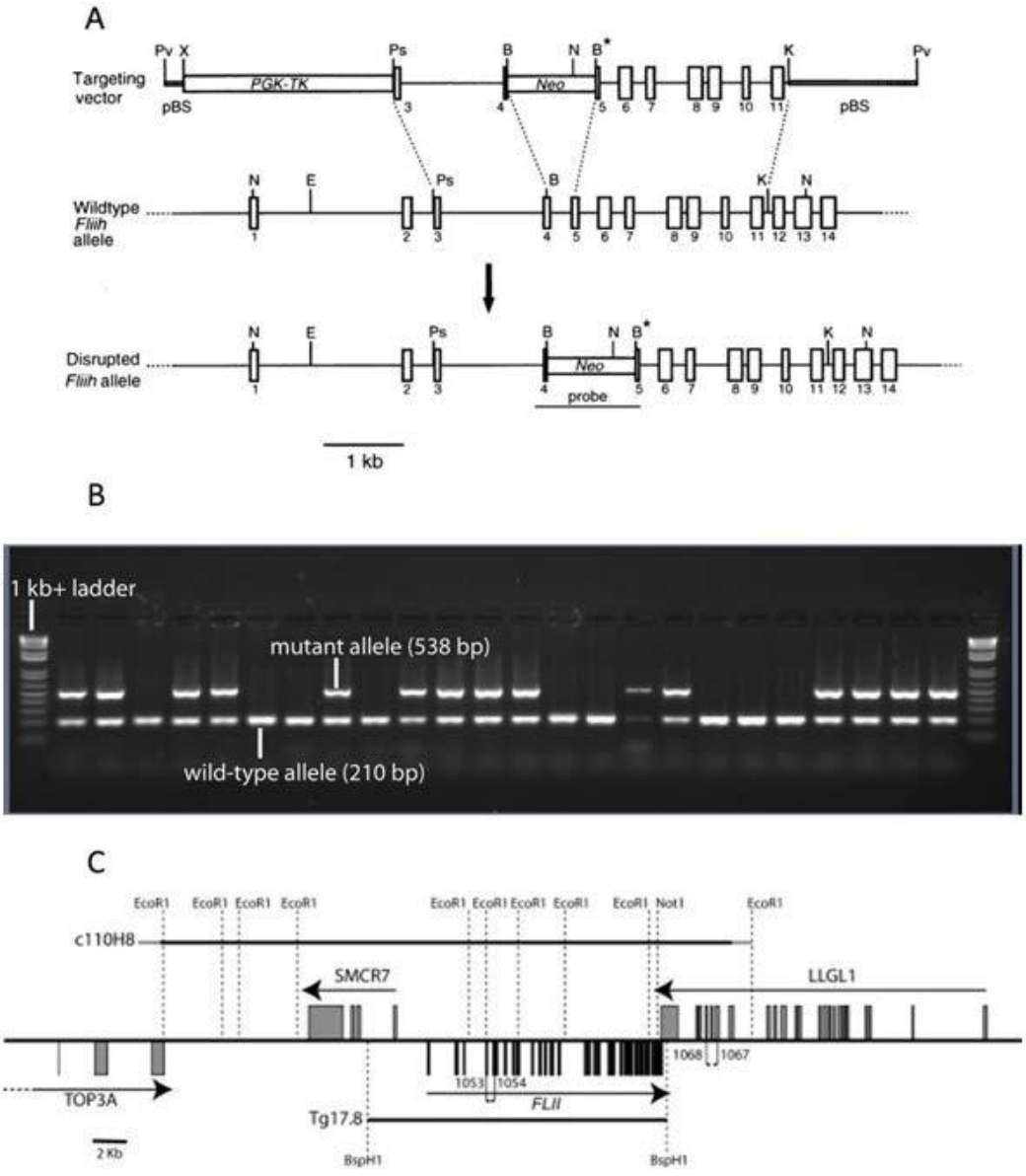


Figure 2.2: Targeted disruption of the Flii gene. A Schematic representation of the domain structure of the targeting vector, relevant portion of the Flii gene and the targeted allele after homologous recombination. . * indicates the BspEI site in exon 5 introduced by site-directed mutagenesis. *Flii* exons are represented by numbered open boxes. **B** Triple-primer PCR showing wild-type (210bp) and mutant (538bp) products. Animals with one copy of each wild-type and mutant allele express no more than 50% of regular Flii expression (Campbell et al., 2002). **C** The cosmid clone used to generate *Flii*$^{Tg/Tg}$ mice. The cosmid contains the *Flii* gene, SMCR7 gene, and partial codes for TOP3A and LLGL1 genes. Figure adapted from (Thomsen et al., 2011).

2.1.6.1 Flii$^{+/-}$ Mouse Generation

Flii$^{+/-}$ mice were generated by a positive/negative selection strategy and homologous recombination of a null allele of the mouse Flii gene into embryonic stem cells. The regions involved in this recombination only involve Flii so that changes will only affect the Flii gene. The resulting mice contain one normal copy and one mutant copy of the Flii gene, resulting in ubiquitous expression of Flii at no more than 50%. Flii deficient homozygotes do not survive embryogenesis. Homozygous Flii mutant blastocysts hatch, attach and form an outer growing trophoblast cell layer, but egg cylinder formation fails and embryos are non-viable by embryonic day 7 (Thomsen et al., 2011).

The strain was maintained by cross breeding of Flii$^{+/-}$ carriers to Balb/c mice, which produced offspring of approximately 50% Flii$^{+/-}$ and 50% Balb/C (wild type (WT)) (Campbell et al., 2002).

A three primer multiplex reaction was used to detect WT and Flii$^{+/-}$ alleles in a positive/positive PCR assay. PCR was performed on DNA extracted from 5mm tail snips of 25 day old Flii$^{+/-}$ x Balb/C offspring (see Figure 2.1B).

2.1.6.2 Flii$^{Tg/Tg}$ Mouse Generation

Mice carrying additional copies of the Flii gene were generated by introduction of a human cosmid clone (c110H8) containing the entire Flii locus into the mouse genome using transgenesis. The extent of the construct was characterised using restriction mapping (Figure 2.1). The availability of the mouse genome allowed for the estimation of the extent of the cosmid. Currently, it is known that the cosmid contains all of the neighbouring Smith-Magenis syndrome chromosome

region candidate 7 homolog (SMCR7) gene as well as patrial coding sequence for the Topoisomerase (DNA) III alpha (TOPA3A) and Lethal giant larve homolog (LLGL1) genes however we have conducted extensive studies characterising our different transgenic mice to ensure that the effects on wound healing are attributed to *Flii* gene alone (Thomsen et al., 2011). This was achieved by making a second transgenic strain using only a 17.8kb BspH1 sub-fragment of the cosmid) and assessing the strain to determine whether this minimal construct is able to provide all functions of endogenous Flii gene. We have shown that this construct contains all necessary elements to recapitulate the function of endogenous Flii and that Flii protein levels are sufficiently elevated (up to 1.52 times WT level) to cause altered Flii function in these mice (Thomsen et al., 2011).

The transgenic strain used in this project was backcrossed to BALB/c animals for 10 generations before being intercrossed and homozygous animals were classified via progeny testing following established protocols. The mouse colony was subsequently maintained by intercross of animals homozygous for the transgene. These animals carry two copies of the mouse Flii gene and two copies of the human Flii were out-crossed to BALB/c animals to generate the progeny that carry two copies of the mouse Flii gene and one copy of the human Flii transgene. A two primer reaction was been designed to detect both the mouse Flii allele and the human Flii allele in a positive/positive PCR assay. The two primers used in which the sequence of each primer is identical between mouse and human, but differences in the intervening regions generate two distinct size products. The PCR was performed on DNA extracted from 5mm tail snips from approximately 25 day old FliiTg/Tg.

2.1.6.3 FnAb Monoclonal Antibody Generation

FnAb was produced in the Cowin laboratory using standard protocols. Female mice were injected with synthetic peptides in PBS and Freund's complete adjuvant. After the initial immunisation, this injection was given weekly for 4 weeks. 35 days post immunisation, 100µL of blood was sampled and analysed for antibody levels. Mice showing a low antibody titre were given a booster immunization. All mice were euthanized at day 90 post primary immunization and spleens were harvested for fusion.

Spleens were disrupted by injection of RF10 media and connective tissue was removed. Cell suspension was agitated and cells pelleted at 400G for 5 minutes. Pellets were resuspended in 5ml red cell lysis buffer and re-pelleted under the same conditions. Cells were then resuspended in RF10 media and counted. Myeloma cells were added to the spleen cells at a ratio of 10:1 splenocytes:myelocytes. Cells were pelleted again, supernatant was removed and cells were resuspended in 1ml PEG. 3ml of RF 10 was added dropwise over 10 minutes, then a further 7ml RF10 dropwise over 10 minutes. Cells were then re-pelleted and resuspended in 50ml HAT media, sorted using FACS and plated onto a 96 well plate. The cells were left for 72 hours and culture media was then tested by ELISA for Flii reactivity and by Isostrip for IgG isoforms. Those testing positive for both Flii and IgG were expanded, purified and tested in mouse incisional wounds for an improvement in wound healing compared to an irrelevant IgG

control. The FnAb yielding the best wound healing improvement was used for this study,

2.2 Methods

The following experimental methods were used in this study. The methods were adapted or modified from different published studies as stated in the text or referenced in the text.

2.2.1 A novel murine model of hypertrophic scarring using sub-cutaneous infusion of bleomycin

We have adapted the dosage, delivery and temporal characteristics of this model in order to develop a novel model of hypertrophic scarring. Bleomycin is delivered continuously via a subcutaneous osmotic pump (Alzet Model 1004, Durect Corp., Cupertino CA 95014) in order to stimulate a fibroproliferative process in contrast to the sclerotic process described by Yamamoto et al (Yamamoto et al., 1999). Bleomycin infusion provides a continuous inflammatory stimulus, rather than the intermittent stimulus in Yamamoto model. Mice were euthanized at 28-days or 56-days in an attempt to model both immature and mature hypertrophic scars.

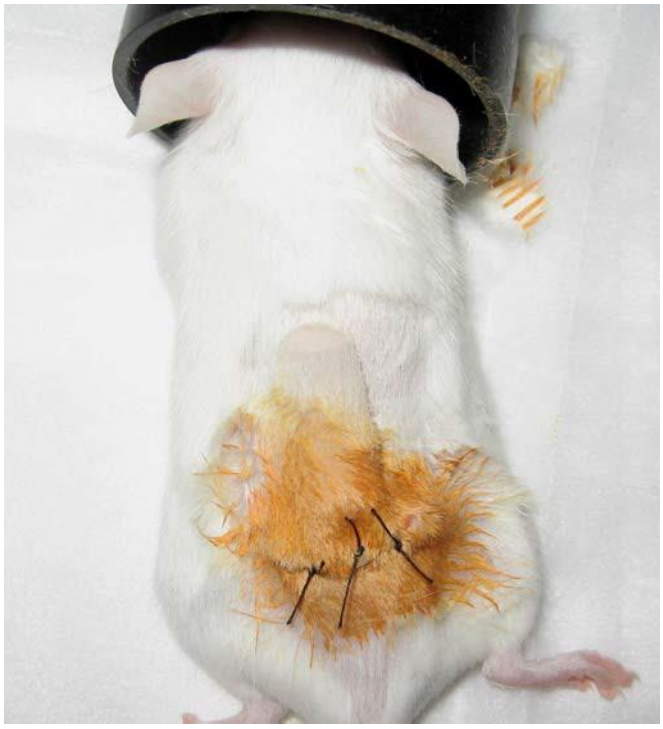


Fig. 2.3 Murine model of hypertrophic scarring using subcutaneous infusion of bleomycin Mouse post-insertion of osmotic pump

2.2.2 Murine Surgery

All experiments were approved by the Women's and Children's Health Service Animal Care and Ethics Committee and the ANU Animal Ethics Committee following the Australian Code of Practice for the Care and the Use of Animals for Scientific Purposes (WCHN AEC#892). Mice were housed in Women's and Children's Hospital animal house where cages were cleaned and food and water replaced daily.

Prior to surgery osmotic pumps (Alzet Model 1004, Durect Corp., Cupertino CA 95014) were filled to their 90µl capacity with 2.8mg/ml bleomycin sulphate (R-42569, Hospira Mulgrave North, VIC) or a sterile control solution of phosphate buffered saline (PBS). Pumps are designed to produce a consistent rate of flow of

0.11µl/hour for 28 days. Pumps were weighed before and after filling to ensure correct volume. Mice were anaesthetised using gaseous isofluorane, 5% induction, 2% maintenance. The dorsum of the mice was shaved and cleaned with 10% povidine iodine solution. A 0.5cm transverse incision was made across the midline at a point approximately 4 cm caudal to the base of the skull. Using blunt dissection, a pocket was made between the skin and muscle layers into which the osmotic pump was inserted. This pocket was carefully dissected in a suprafascial plane to ensure that pumps would not migrate from their original location. Wounds were closed with 4-0 prolene and secured with tissue adhesive (Histoacryl, TissueSeal, Ann Arbor, Michigan) (Figure 2.3). Mice were euthanized at 28 days or 56 days after pump insertion and a 4cm x 1.5cm area of skin was excised at a location corresponding to the cranial end of the osmotic pump. Unwounded skin was collected from each mouse group.

Mice were weighed upon arrival, prior to surgery and post-surgery at time points stated above to monitor their wellbeing. Mice that lost more than 10% of the body weight were excluded from the study. Mouse behaviour, coat appearance and movements were also observed regularly to ensure no adverse effects from surgery.

At the designated day of sacrifice (day 28 or day 56), the mice were weighed and euthanized using CO_2 and cervical dislocation. The mice were then re-shaved and digital photographs of the wounds were taken prior to excision. A 4cm x 1.5cm area of skin was excised around the fibrotic lesions at a location corresponding to the cranial end of the osmotic pump and unwounded skin was collected from each

mouse group/Both left and right wounds were excised and bisected, half was used for histological analyses and the other half was snap-frozen in liquid nitrogen and stored at -80°C for biochemical analyses. For each mouse group, lung, live and kidney were also collected

2.2.3 Flii neutralising antibody treatment

In a subset of WT female 12-week old mice (n=6) 200µl of 50µl/ml FnAb was injected subcutaneously at the cranial end of the osmotic pump on days 0, 7, 14 and 21. The 50µl/ml dose of FnAb had previously been used for treating acute injuries, burns and diabetic wound in murine and porcine models (J. E. Jackson et al., 2012; Kopecki, Ruzehaji, et al., 2013; Ruzehaji, Kopecki, et al., 2013). Fibrotic lesions and unwounded skin from all animals were collected at day 28 post osmotic pump insertion and bisected and fixed in 10% buffered formalin for histology and immunohistochemistry.

2.2.4 Sample processing, histology and immunohistochemistry

Human and murine samples collected from surgery were fixed in 10% formalin overnight, followed by processing in a Leica TP1020 tissue processor which dehydrated the tissues in a graded alcohol series (70% for 120 mins, 80% for 60 mins, 90% for 105 mins and 100% for 240 mins). They were then cleared in transitional solvent xylene for 180 mins followed by 240 mins of tissue infiltration with paraffin wax. Serial tissue sections (4µm) were cut from paraffin-embedded fixed tissue using a Leica RM2235 microtome and labelled. Prior to staining, skin sections were de-waxed by a series of xylene (30 mins) and graduated ethanol

washes (bringing sections to water) (100% for 1 min, 70% for 1 min & 30% for 1 min) before further processing. Skin sections were either stained with Haematoxylin and Eosin (H&E), Masson's Trichrome or subjected to antigen retrieval and immunohistochemistry using antibodies from Table 2.1. Staining the sections in H&E involved bringing sections to water as mentioned above, followed by staining in Lillie's- Mayer's Haematoxylin for 6min, "blueing" sections in bicarbonate water for 15 seconds, differentiating Haematoxylin in 0.25% Acid Alcohol for 6 sec, staining in alcohol based Eosin stain for 2 minutes dehydrating in grated alcohol series (30% for 30 sec, 70% for 30 seconds, 100% for 1 min) and clearing in transitional solvent xylene for 2 minutes before mounting in DePeX mounting medium.

H&E staining of unwounded skin was analysed microscopically using light microscopy. Dermal thickness was determined by measuring the distance between the basement membrane and the panniculus carnosus using Image Pro-Plus 5.1 program (MediaCyberneticsInc.). Epidermal thickness was measured as the distance from the stratum corneum to the basement membrane. Multiple measurements were taken across the width of the sections and the average of these was calculated. This was done in a blinded fashion. For sections showing fibrotic pathology dermal thickness measurements only included fibrotic regions. The limits of fibrotic regions were determined by the presence/absence of dermal thickening, dermal appendages, epidermal thickening and epidermal acanthosis. H&E stained wounds from Flii$^{+/-}$, WT and Flii$^{Tg/Tg}$ mice were analysed using light microscopy and fibrotic cross sectional area was measured using the Image Pro-Plus program. Serial sections

were examined until the region of maximal thickness/cross sectional area was found. These sections were used for final measurements.

2.2.5 Immunohistochemistry and image analysis

Immunohistochemistry experiments were undertaken on all human and murine samples. The following method was used as the standard antigen retrieval procedure, and Table 2.1 lists all the primary and secondary antibodies used in immunohistochemistry for different targets.

Sections were de-waxed by a series of xylene changes (30 minutes) and gradual ethanol washes (100% for 1 min, 70% for 1 minute and 30% for 1 minute), before being rinsed in 1x Phosphate Buffered Saline (PBS) and pre-treated with 250 ml Target Retrieval Solution (TRS) solution (2.8g Citric Acid, 3.76g Glycine, 0.372g EDTA, pH 5.9 in 1L 1xPBS). The sections were then microwaved for 2 minutes on "high" after which a "ballast" pot of water was added to help absorb some heat and pre-treatment continued for 2 x 5 minutes with regular "airing" to let the steam out and ensure that temperature reached 94°C but not 100°C. Sections were then allowed to cool to 50°C on the bench before they were washed in fresh 1x PBS and enzyme digested with 0.0625g of Trypsin (Sigma-Aldrich, Sydney, Australia) dissolved in 1xPBS and pre-warmed to 37°C. Following the 3 min enzyme digestion at 37°C, sections were washed in 1xPBS and then incubated for 30 minutes in NHS blocking solution (3%NHS in 1xPBS). Slides were then washed in 1xPBS for 2 min and then incubated in primary antibody in a humid air tight box overnight at 4°C. Sections were then washed 3 x 2 minutes in 1xPBS and then incubated in species specific Alexa Fluor 488 or Alex Fluor 594 at a concentration

of 2µg/ml for all primary antibodies (Table 2.1) for 1hr at room temperature in a humid box. Slides were then washed 3 x 2 minutes in 1xPBS to remove any unspecific binding of Alex Fluor and mounted in Dako Fluorescent Mounting Medium (DAKO Corporation, Botany, Australia). Slides were stored in the dark at -20°C. Integrated fluorescence intensity was determined using AnalySIS software package (Soft- Imaging System GmbH, Munster, Germany) (Cowin et al., 2007). Negative controls were included to demonstrate antibody staining specificity. Control samples undergo the exact same staining procedure outlined except omitting the primary or secondary antibody. All control sections had negligible immunofluorescence.

2.2.6 Cell lines and cell culture

Cells used in cell culture experiments were human foreskin fibroblasts (HFFs) and primary murine fibroblasts (from $Flii^{+/-}$, WT and $Flii^{Tg/Tg}$ mice). All cell cultures were incubated at 37° C and 5% CO_2. Cells were serum starved in DMEM (JRH Biosciences, USA) for at least 12 hrs or as otherwise stated prior to the start of experimenting to synchronize the cells to the same phase of cell cycle. Cells were stored in liquid nitrogen stores in 10% heat inactivated Fetal Calf Serum (FCS) (JRH Biosciences, USA) in DMEM containing following antibiotics unless otherwise stated: 100U/ml penicillin, 100µg/ml streptomycin and 100µg/ml Fungizone (Invitrogen, Mount Waverly, VIC, Australia). Cells were grown in the same 10% heat inactivated FCS solution.

2.2.7 HFF isolation and culture

Neonatal foreskins were obtained following elective circumcision performed on five day old males by paediatric surgeon Mr Chris Kirby and placed immediately into ice cold Ham's Nutrient Mixture F12 (SAFC Biosciences, Lenexa, KS), containing 100U/ml pencillin, 100µg/ml streptomycin and 100µg/ml Fungizone. A 5mm punch biopsy was used to excise explants in sterile conditions, which were placed dermal side down into 6-well plates. Explants were allowed to partially dry and fix to the bottom of the well before 2ml DMEM, 20% FCS, 100U/ml pencillin, 100µg/ml streptomycin and 100µg/ml Fungizone was added and tissue cultured at 37°C, 5% CO_2. Cell media was replaced after 24hr of initial explant culture and then every 2 days. At day 7 post explant culture once fibroblasts migrated out from the explant the skin explant was removed and cells cultured as normal in 10% FCS/DMEM, 100U/ml penicillin, 100µg/ml streptomycin and 100µg/ml Fungizone. Low passage cells (P 2–5) were used in all experiments.

2.2.8 Murine fibroblast isolation and culture

Hair was shaved from the dorsal flank of euthanized 12-week-old female BALB/c Flii$^{+/-}$, WT, and Flii$^{Tg/Tg}$ mice and skin excised and collected in ice-cold Ham's Nutrient Mixture F12 (SAFC Biosciences, Lenexa, KS), 1% Penicillin Streptomycin, 1% Fungizone (Sigma-Aldrich). A 5mm punch biopsy was used to excise explants in sterile conditions, which were placed dermal side down into 6-well plates. Explants were allowed to partially dry and fix to the bottom of the well before 2ml DMEM, 20% FCS, 100U/ml pencillin, 100µg/ml streptomycin and 100µg/ml Fungizone was added and tissue cultured at 37°C, 5% CO_2. Cell media was

replaced after 24hr of initial explant culture and then every 2 days. At day 7 post explant culture once fibroblasts migrated out from the explant the skin explant was removed and cells cultured as normal in 10% FCS/DMEM, 1% Penicillin Streptomycin. Low passage cells (P 2–5) were used in all experiments.

2.2.9 Migration scratch assay

HFFs or primary murine fibroblasts were grown in 12-well plates (Cell-Tech, Millipore, NSW, Australia) in 10% heat inactivated FCS in DMEM containing 100U/ml pencillin, 100µg/ml streptomycin and 100µg/ml fungizone at 37°C, 5% CO_2. Cells were cultured with or without the addition of bleomycin 140µg/ml (see below) or Flii neutralising antibody (FnAb) 20 µg/ml (see below). They were then scratched with a P200 pipette tip, producing equal size wounds of 2 mm x 1 cm. The cells were photographed using the Olympus DP72 camera at 0, 3, 6, 9, 12, 24, 30, 36, 48 and 96 hrs post-wounding and wound area was calculated using the cellSens digital imaging program (Olympus, Shinjuku, Japan). Reduction in scratch wound width was measured and recorded. This was replicated 3 times for each cell type and treatment

2.2.10 WST-1 Proliferation Assay

WST-1 Proliferation reagent (Roche Diagnostics, NSW, Australia) was used to assess the proliferation rate of HFFs and primary murine fibroblasts in response to different concentrations of bleomycin. The WST-1 is a tetrazolium salt based reagent that converts the soluble formazan which directly correlates with cellular metabolic activity and proliferation rate. Cells were seeded in 96-well microtitre

plates at a concentration of $1x10^5$ cells per well in 100µl of 10% or 0.5% FCS/DMEM with antibiotics. One lane of a 96-well microtitre plate was filled with media only and one lane with normal cells, which did not receive any treatment or WST-1 reagent and acted as the blank controls. Cells were incubated at 37°C 5% CO_2 overnight, cell media was aspirated off and serum-free media was added to all cells to synchronize all cells to the gap phase of the cell cycle. After 6 hrs, 100µl of 10% or 0.5% FCS/DMEM with antibiotics was added to the cells and cells were incubated overnight in 37°C 5% CO_2 incubator for 24, 48 or 72 hrs depending on the time point of assessment. Following the incubation period, 10µl of WST-1 reagent was added to the cells and mixed thoroughly on an orbital shaker. The cells were then incubated at 37°C 5% CO_2 incubator for 30 min as per manufacturer's instructions where metabolic enzymes produced by cells react with WST-1 reagent to form a formazan dye product. The amount of the formazan dye produced therefore directly correlates with metabolic activity of the cells and their proliferation rate. Formazan dye was quantified where dual absorbance of 450nm and 600nm was measured on the Tecan microplate reader. Absorbance values were blanked with absorbance values of control without cells and then compared between different cells for the assessment of proliferation. Results were normalized to the untreated control sample and expressed as a percentage.

2.2.11 Immunocytochemistry, image analysis and fluorescence co-localization

HFFs or primary murine were grown to a density of 60-70% confluence on sterile glass coverslips. Cells were serum starved for 12 hours and then media was

exchanged for 10% FCS/DMEM, 100U/ml pencillin, 100µg/ml streptomycin and 100µg/ml Fungizone + 140µcg/ml bleomycin sulfate. In a separate experiment, both control and bleomycin cells were treated with 20µg/ml FnAb or 20µg/ml irrelevant murine IgG (control). Cells were then incubated for 6 hours before being fixed and permeabilised with methanol/0.02% Tx-100/PBS using a protocol described by Smith-Clerc and Hinz (Smith-Clerc & Hinz, 2010). Cells were then blocked with bovine serum albumin for 30 minutes before incubation with primary antibodies against Flii or αSMA for 60 minutes. Cells were then washed thoroughly in 0.02% Tx-100/PBS before Flii/αSMA was detected through incubation with fluorescent Alex Fluor 488 goat anti-mouse IgG (2µg/ml, Invitrogen OR). Coverslips were mounted onto slides using Dako mounting medium (DAKO Corporation, Botany, Australia) and images were obtained using the Olympus DP72 camera and cellSens digital imaging program (Olympus, Shinjuku, Japan). Negative controls included replacing primary antibodies with species-specific IgG. For verification of staining, nonspecific binding was determined by omitting primary or secondary antibodies. All control sections had negligible immunofluorescence.

2.2.12 Protein Extraction and quantification

HFFs were grown until confluent in T75 flask. At this time they were serum starved for 24 hours, whereupon media was replaced with 10% FCS/DMEM, 5% Penicillin Streptomycin (control), 10% FCS/DMEM or 5% Penicillin Streptomycin + 140µcg/ml bleomycin sulfate. Cells were cultured for six hours and protein was then extracted by lysing cells in 50 mM Tris pH 7.5, 1 mM EDTA, 50 mM NaCl, 0.5% Triton-X-100, containing Complete Mini protease inhibitor cocktail tablet (Roche

Diagnostics, Castle Hill, NSW). Protein levels were quantified using the Thermo Scientific Pierce Bicinchoninic Acid (BCA) Protein Assay Kit (Waltham, MA). The absorbance was measured at a wavelength of 570nm in a Dynatech MR700 microplate reader using the BioLinx 2.0 computer software program.

2.2.13 Western Blotting

Protein amounts in each sample were equalized by dilution and heated at 95°C prior to electrophoresis. Protein fractions were run on a 10% SDS-PAGE (sodium dodecyl sulfate / polyacrylamide) gel consisting of a 10% separating solution (3.35ml 30% Acrylamide-Bis solution (37.5:1, 2.6% C, BioRad Laboratories, CA, USA), 1.25ml 3M Tris pH 8.9, 5.25ml distilled water, 125µl 10% SDS, 100µl 10% APS (Ammonium Persulfate)(Sigma-Aldrich Chemical Company, Sydney, Australia) and 6.25µl TEMED(N,N,N',N'-Tetramethylethylene-diamine)(Sigma-Aldrich Chemical Company, Sydney, Australia) and 4% stacking solution (0.5ml 30% Acrylamide, 0.276ml 0.5M Tris pH 6.8, 4.104ml distilled water, 50µl 10% SDS, 40µl 10% APS and 4µl TEMED). Protein fractions were run at 100V for 90 min in the electrophoresis tank. The gel was then transferred onto a 0.2µm pore nitrocellulose membrane (Advantec MFS Inc, CA, USA) by the process of Wet Transfer using the Bio-Rad Mini-ProteanII Transfer Apparatus (Bio- Rad Laboratories, NSW, Australia) and 1xWet Transfer Buffer/Tobins Buffer (3.3g Tris, 14.4g Glycine, 900ml MilliQ Water, 100ml Methanol) at 100V for 1 hr.

Membranes were then blocked in 5% skimmed milk in 0.3% Tween/PBS for 1hr and hybridized with appropriate primary antibody at 1µg/ml concentration,

diluted in blocking buffer prior to addition to the membrane overnight at 4°C. This was followed by 4 x 10 min membrane washes in 5% skim milk, 0.3% Tween/PBS blocking buffer and application of appropriate secondary antibody conjugated to horse radish peroxidise (HRP) at 1µg/ml and diluted in blocking buffer and applied to membranes in dark for 1 hr at room temperature. This was followed by further membrane washes of 4 x 10 min in 0.3% Tween/PBS and signal detection using Super Signal West Femto Maximum Sensitivity Substrate (Pierce Biotechnology, Rockford, USA) and signal capture using GeneSnap analysis software program (Syngene, Maryland, USA). Membranes were stripped by incubation in Stripping Buffer (5ml 20% SDS, 350µl 2-Mercaptoethanol (#M7522, Sigma- Aldrich, Sydney, Australia) , 6.25ml 0.5M Tris/HCl pH 6.7, 38.4ml MilliQ Water) for 30 min with gentle shaking every 10 min followed by re-probing of membranes with β-tubulin (Sigma-Aldrich, Sydney, Australia) as a loading/transfer control.

2.3 Statistical analysis

Statistical differences were determined using the InStat program v3.05 (GraphPad Software, USA) using the unpaired Student's t-test or ANOVA. For data not following a normal distribution, the Mann-Whitney U test was performed. A P value of less than 0.05 was considered significant.

Chapter 3

EXPRESSION OF FLIGHTLESS IN HUMAN BURNS AND HYPERTROPHIC SCARS

Parts of this chapter have been published in following journal:

Cameron A.M., Ruzehaji, N. Cowin A.J. (2010). Burn wound management: A surgical perspective *Wound Practice and Research* 18(1): 5

Cameron A.M., Turner C.T., Adams D.H., Jackson J.E., Melville E, Arkell R.M., Anderson P.J and Cowin A.J. (2016) Flightless I is a key regulator of the fibroproliferative process in hypertrophic scarring and a target for a novel anti-scarring therapy *Br J Dermatol* 174(4): 786-794

3.1 Introduction

Hypertrophic scarring is a fibroproliferative disease of the skin most commonly associated with burn injury, but may also occur after trauma or elective surgery(Aarabi et al., 2007; Engrav et al., 2007). It is estimated that at least 70% of deep burns in adults, and a greater percentage in children, will develop hypertrophic scarring, which can cause disfigurement (Robson et al., 2001).

Hypertrophic scarring represents an aberration of the normal wound healing process, which is a tightly orchestrated, nonlinear sequence involving numerous cell types, extracellular matrix (ECM) components and signaling molecules (Bielefeld et al., 2013). Normal wound healing balances several requirements: prevention of infection, restoration of epithelial integrity and approximation of normal skin structure and function (Wynn, 2008). Hypertrophic scarring results from a disruption of this delicate balance. A prolonged inflammatory phase results in the persistence of large numbers of myofibroblasts in the wound and elevated levels of fibrogenic cytokines, such as transforming growth factor-β1 (TGF- β1) and connective tissue growth factor (CTGF) (Hinz, 2010).Myofibroblasts drive inflammation and overproduce collagen, which accumulates in a disorganized manner (Hayakawa et al., 1982)and results in an increased collagen 1/3 ratio (Rahban & Garner, 2003. This results in a raised, red, tender scar, which is prone to contracture over time (Rahban et al., 2003 ; Zhang et al., 1995). Studies have shown that the protein Flightless I (Flii) is a regulator of the wound healing process (Cowin et al., 2007). Flii is a member of the gelsolin family of actin-remodeling proteins that regulates intracellular actin

by severing pre-existing filaments and/or capping filament ends to enable filament reassembly into new cytoskeletal structures (Kopecki et al., 2011). Flii co-localizes with molecules involved in regulating cytoskeletal reorganization, including members of the Rho family of GTPases, Ras and Cdc42 (Claudianos & Campbell, 1995). It possesses a unique leucine-rich repeat domain, which is important for protein–protein interactions (Campbell et al., 1993). Cells within the epidermis and dermis express Flii and studies have shown that Flii is able to translocate from the cytoplasm to the nucleus in response to wounding (Cowin et al., 2007). Focal adhesions and migratory structures involved in cellular motility also express Flii (Davy et al., 2000) and it is also secreted from cells. In $Flii^{+/-}$ mice, healing is enhanced, whereas in $Flii^{Tg/Tg}$ mice, healing is impaired, with delayed epithelial migration and increased scarring (Cowin et al., 2012). In vitro, $Flii^{+/-}$ fibroblasts and keratinocytes have improved cellular adhesion and spreading resulting in accelerated scratch wound closure (Cowin et al., 2007; Kopecki et al., 2009). Additionally, reducing Flii in a murine model of partial-thickness burn injury led to faster re-epithelialization and reduced scar formation (Adams et al., 2009) suggesting the reduction of this protein in wounds may be beneficial as a hypertrophic scarring therapeutic treatment. Given the relationship between impaired wound healing and the development of hypertrophic scars, we have investigated whether Flii has a regulatory role in the fibroproliferative process that leads to hypertrophic scar formation. Here we examine the expression of Flii in human hypertrophic scars.

3.2 Results

Samples of human normal skin, acute burns and hypertrophic scars at varying stages of maturity were collected from the Royal Adelaide Hospital Adult Burns' Unit, the Women's and Children's Hospital and Calvary Hospital. Normal skin was sourced mainly from patients undergoing abdominoplasty or occasionally from normal skin adjacent to scar specimens (Figure 3.1). Acute burn specimens were sourced from debridement of burn injuries (Figure 3.2). These debridements occurred at times ranging from 6 hours to 16 days post burn injury. Hypertrophic scars were obtained after surgery indicated for contracture release or scar revision (Figure 3.3). Samples were initially classified according to the patient's clinical history and the macroscopic appearance of the tissue.

Fig. 3.1 The author (left) collecting human tissue samples from the RAH Adult Burns' service.

The samples were examined as H&E sections to confirm these classifications. Mature hypertrophic scars demonstrated increased dermal thickness, with parallel sheets of collagen. Immature hypertrophic scars demonstrated collagen nodules and whorls in the dermis. Immature hypertrophic scars displayed marked epidermal hyperplasia and basal cell disarray. Mature hypertrophic scars showed a flattened epidermal layer with basal cell disarray and vacuolar change. It was also noted that there was a great deal of heterogeneity of these histological findings and that the histological classification of scars did not always correlate to their clinical classification (Figure 3.4.).

Histological examination of acute burns showed epidermal peeling, sub epidermal vesicles, collagen alteration, microthrombus and coagulative necrosis. Again, there was a great deal of heterogeneity between the histological features of these samples that did not necessarily correlate with their clinical classification.

Immunohistochemical analysis for Flii in the samples showed that Flii expression was increased in human burn wounds and hypertrophic scars compared with normal unwounded skin. This increase is Flii expression was seen throughout the epidermis and also in the dermis of acute burns and immature scars.

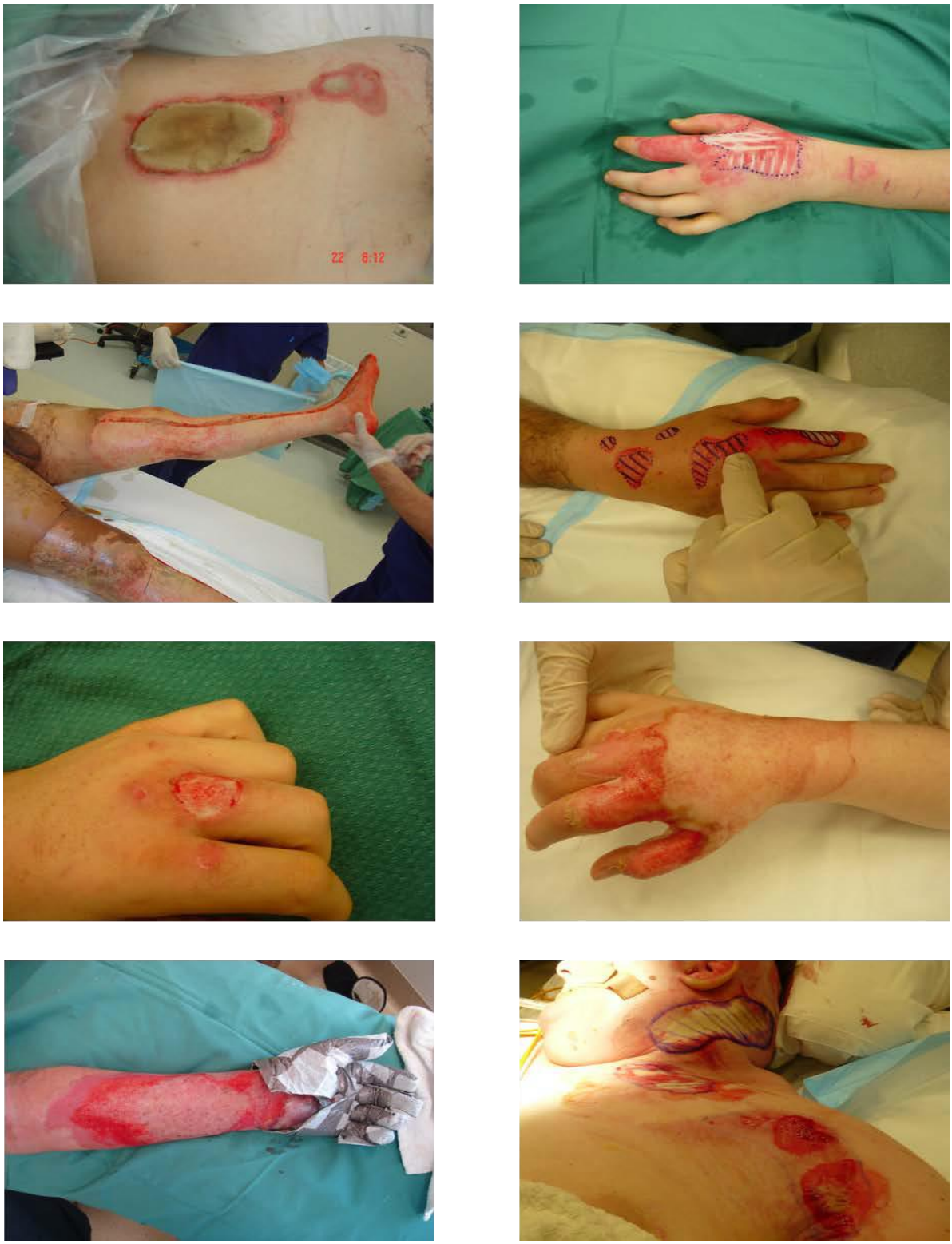


Fig. 3.2 Clinical photographs of acute burns prior to sample collection

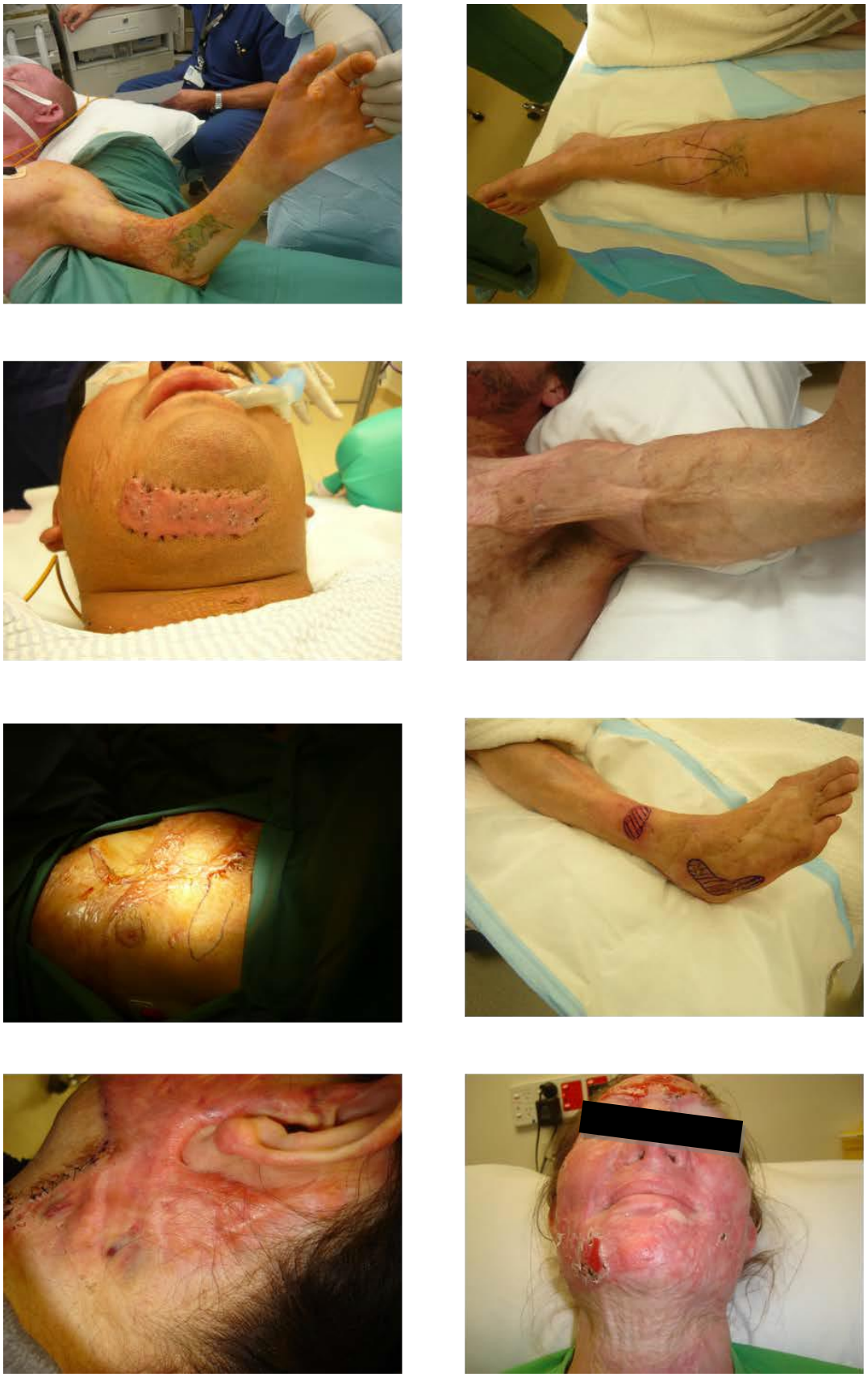


Fig. 3.3 Clinical photographs of hypertrophic scars prior to sample collection

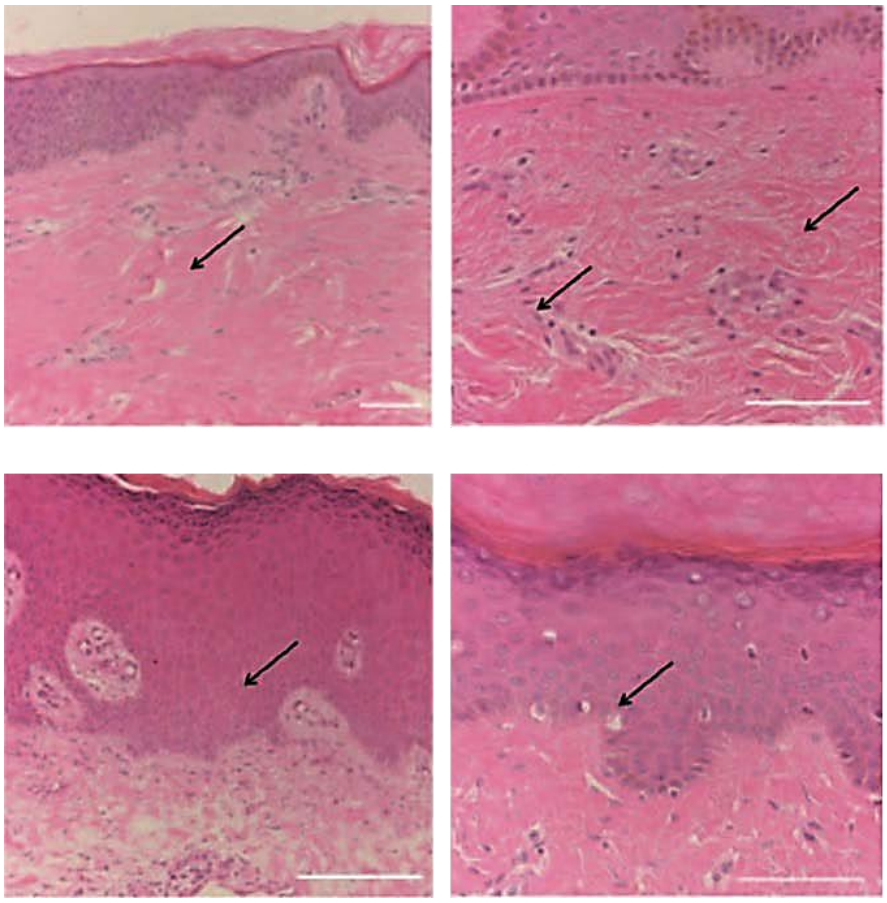


Fig. 3.4 Histopathological features of hypertrophic scars. Samples of immature hypertrophic scars and mature hypertrophic scars were stained with haemotoxylin and eosin and photographed at 20 or 40 times magnification. Mature hypertrophic scar demonstrated parallel sheets of collagen in the dermis (*top left, arrows*). Immature hypertrophic scars demonstrated collagen nodules and whorls (*top right, arrows*). Hyperplasia and basal cell disarray were seen in immature hypertrophic scars (*bottom left, arrows*). Mature hypertrophic scars displayed a flattened epidermal layer with basal cell disarray and vacuolar change (*bottom right*).

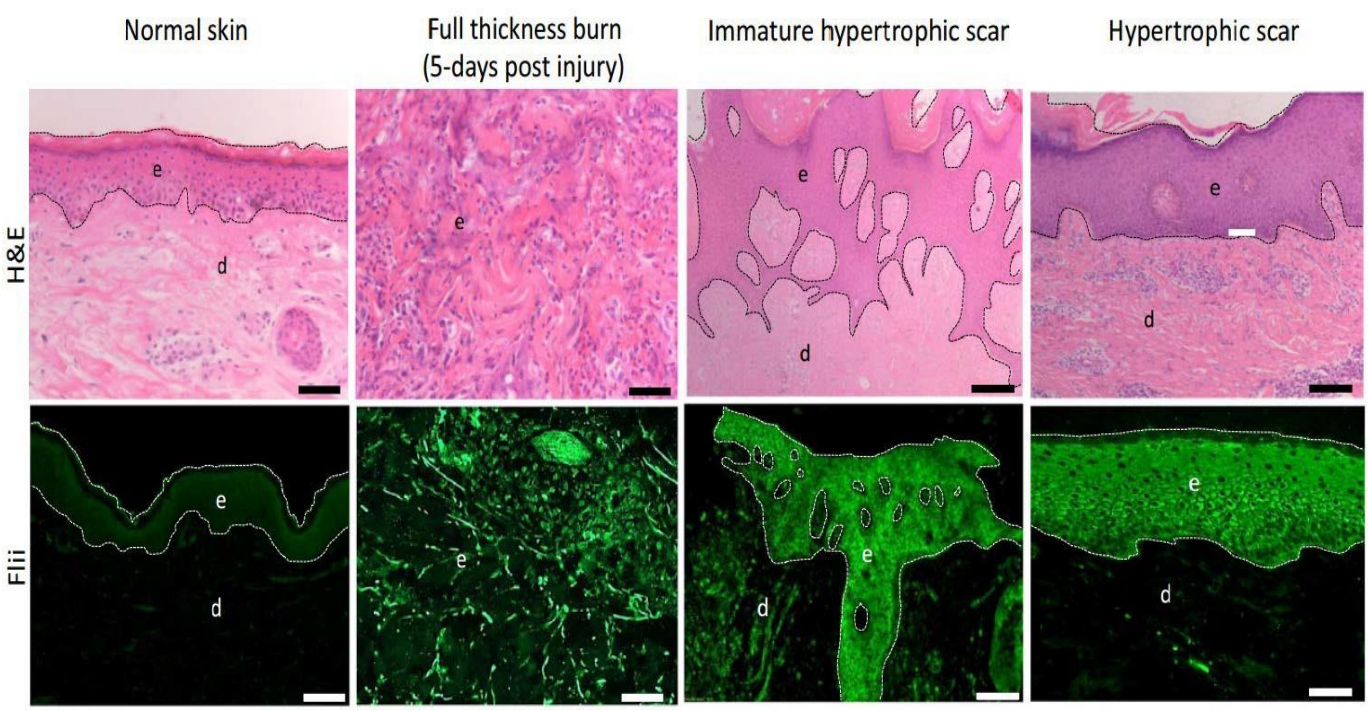


Fig. 3.5 Immunolocalisation of Flii in human skin, acute burns and hypertrophic scars. Flii expression was increased in human burn wounds and hypertrophic scars compared with normal unwounded skin. This increase in Flii expression was seen throughout the epidermis and also in the dermis of acute burns and immature scars. *Scale bars* = 100μm

3.3 Discussion

Hypertrophic scars and keloids were originally defined by their clinical characteristics, before attempts were made to define them as distinct pathologies, in terms of their histological characteristics, such as parallel collagen fibres, collagen whorls and epidermal flattening (Lee et al., 2004). However, producing evidence to support these definitions has been difficult; indeed the histological distinction between normal scars, hypertrophic scars and keloid scars is controversial (Lee et al., 2004; Muyembe-Tamfum et al., 2009). There is currently no consensus in the literature regarding the relative contribution of different collagen subtypes to hypertrophic scar tissue fibrillogenesis, nor the importance or temporal profile of key cell types, such as myofibroblasts, or well-known cytokines, such as TGF-β1 (Dale et al., 1996; Fleischmajer et al., 1990; Rawlins et al., 2006; Umezawa, 1967).

Indeed, the classification of hypertrophic versus normal scarring as distinctive entities may only be suited to clinical application. Defining a scar as "hypertrophic" ensures the patient receives treatment accordingly, such as scar massage, topical silicone or occasionally intralesional corticosteroid injection. Surgery may be indicated to release subsequent scar contracture or to improve appearance.

The lack of objective markers for hypertrophic scarring, such as a clearly defined histopathological characteristics or biochemical markers, makes research using human samples difficult. Without accurate classification of tissue samples,

quantifiable data is difficult to come by. Furthermore, the types of burn injury or scar that may be collected are limited by the clinical indications for surgery. Viz only full thickness or near full thickness burns are excised. More superficial burns are managed without surgery. Furthermore, given the natural history of hypertrophic scarring, it is unusual to operate on acute hypertrophic scars, as most will mature over 12 months.

Hence the tissue samples in this study comprised mainly full thickness burns and hypertrophic scars in the later stages of their development. In addition these tissues came from a variety of anatomical locations in a wide demographic of patients. For these reasons, in depth investigation of Flii levels or related parameters was not attempted. Although qualitative date may be taken from these samples, any quantitative analysis is problematic.

The increase in Flii expression in acute burns and hypertrophic scars suggest Flii may have a role in the pathogenesis of hypertrophic scarring. Interestingly, this increase was seen predominately in the epidermis. The epidermis has an important role in normal skin, as it contain stem cell niches and participates in complex signaling pathways to regulate mesenchymal cell function (Aarabi et al., 2007). Indeed, burn surgeons have long recognised the importance of achieving epidermal coverage of burn wounds (via autologous skin graft or otherwise) at an early stage of treatment in order to dampen inflammation and prevent hypertrophic scarring.

Keratinocytes have been shown to regulate fibroblasts through secretion, activation, inhibition of growth factors such as TGF-β. Co-culture of human keratinocytes and fibroblasts led to fibroblast- myofibroblast transition over 6 days in a process regulated by the pro-scarring cytokine TGF β (Shephard et al., 2004). Given Flii's role in regulation of keratinocyte migration after wound repair(Cowin et al., 2007), regulation of TGF β in burn wound healing (Adams et al., 2009) and key role in epithelial integrity (Kopecki et al., 2009) there is a putative role for Flii in this epidermal regulation of mesenchymal activity.

In addition a number of recent studies have provided compelling evidence that Flii has an important role as an immune-modulator (Dai et al., 2009; Hayashi et al., 2010; Lei et al., 2012; Li et al., 2008). Flii's N-terminus LRR domains share nearly 50% homology to the LRR domains of toll-like receptor-4 (TLR-4). TLRs function as detectors of injury or infection by binding to pathogen-associated molecular patterns molecules (PAMPs) (e.g. LPS from gram negative bacterial cell walls) or damage-associated molecular pattern molecules (DAMPs) (e.g. ECM cleavage products or molecules such as HMGB1 released from damaged cells). (Bianchi, 2007). After binding TLR signaling pathways are activated leading to the secretion of pro-inflammatory cytokines. *In vitro,* Flii binds to the TLR adaptor protein MyD88 through its interaction with nucleoredoxin which inhibits MyD88 binding to TLR4 and reduces inflammatory cytokine secretion (Hayashi et al., 2010; T. Wang et al., 2006). Flii has also been localized to late endosome/lysosomes in fibroblasts and macrophages where it may also dampen inflammation (Lei et al., 2012; Li et al., 2008).

Thus, the increased expression of Flii in human burn wounds and hypertrophic scars compared to normal skin is consistent with the hypothesis that Flii plays an important role in the regulation of the fibroproliferative process underlying hypertrophic scarring.

Chapter 4

A NOVEL MURINE MODEL OF HYPERTROPHIC SCARRING USING SUBCUTANEOUS INFUSION OF BLEOMYCIN

Parts of this chapter have been published in following journal:

Cameron AM, Adams DH, Greenwood JE, Anderson PJ, Cowin AJ (2014) A novel murine model of hypertrophic scarring using subcutaneous infusion of bleomycin. *Plast Reconstr Surg* 133:69-78.

4.1 Introduction

Despite the well-recognized burden of disease associated with hypertrophic scarring and considerable research in the area, we still have limited understanding of its pathogenesis. In particular, the cellular and molecular changes that drive fibroproliferation are poorly understood. As such, little progress has been made in developing new therapies to prevent or reduce hypertrophic scaring.

Laboratory research into hypertrophic scarring can be divided into three broad categories: *in vitro* cell culture, which examines particular cell types under different conditions in order to elucidate their role in scarring; analysis of *ex vivo* human hypertrophic scar tissue via various techniques; and animal models of hypertrophic scarring.

In vitro cell lines and primary cell culture have been useful in investigating the role of different cells and cytokines in hypertrophic scarring. However, this technique is limited by the considerable differences between *in vitro* conditions and the *in vivo* wound healing environment, where there are varied and complex interactions between many different cells, cytokines and extracellular matrix components. In addition, phenotype changes seen in cells cultured from hypertrophic scars have been found to no longer be present after initial passages (Larsson et al., 2008; Sargent & Whitfield, 2011).

Analysis of *ex vivo* hypertrophic scar tissue excised from human patients can provide information on scar architecture, as well as the presence of different cell types and other constituents. Unfortunately, as discussed in Chapter 3, given the nature of scar surgery, these samples usually represent an end stage of the scarring process and provide little information about mechanisms preceding this. Similarly, tissue from burn surgery is either taken from an acute burn or a mature burn scar. This means that key events in hypertrophic scar development are rarely represented in human samples. Furthermore, the clinical classification of scar type or severity is highly arbitrary and variable from institution to institution (Lee et al., 2004). This further confounds any data gained from human tissue.

Given the limitations inherent in techniques using *in vitro* and *ex vivo* human tissue, the need for an animal model of hypertrophic scarring is manifest.

The development of an appropriate animal model has been problematic (Aarabi, et al., 2007), owing much to the phylogenetic variation in wound healing (Gurtner et al., 2008). For example, differences between mouse and human skin include denser hair covering with a shorter growth cycle than human hair; absence of dermal papillae/apocrine glands; and the presence of a full panniculus carnosus, which rapidly apposes wound edges after injury (Wong et al., 2011). There have been numerous attempts to develop an animal model of human hypertrophic scarring. Several models involve xenografts of human skin or hypertrophic scar onto immunodeficient mice (Escamez et al., 2004; Kischer et al., 1989) and rats (Polo et al., 1998). Although these models produced some of the histological changes seen in hypertrophic scars, they require significant suppression of the

animals' immune system, rendering any conclusions difficult to extrapolate to the human milieu. The Duroc pig displays similar scarring properties to the human (Goodwin et al., 1976; Zhu et al., 2003), but the experimental value of such a large animal model is limited by expense, housing and handling difficulties. A model utilising applied mechanical force (a well-recognised risk factor for hypertrophic scarring) across a murine incisional wound to stimulate fibroproliferation has been developed (Aarabi et al., 2007). Some models employ silicone splints to counter the effect of panniculus carnosus (Galiano et al., 2004). Despite these attempts, the need for a practical and reproducible animal model of hypertrophic scarring remains.

The rationale behind our murine model is to recreate the pathological process underlying hypertrophic scarring by using bleomycin infusion to stimulate dermal fibroproliferation. Bleomycin is an antibiotic, originally isolated from *Streptomyces verticillus* and widely used as an anti-cancer treatment (Umezawa, 1967). Bleomycin hydrolase inactivates bleomycin by hydrolysing the amide bond in the b-aminoalanineamide moiety. However, the shortage of this enzyme in the lungs and the skin, allows bleomycin-induced fibrosis and sclerosis to occur in these organs in a dose-dependent manner (Muggia, 1983). Bleomycin was first used to create animal models of pulmonary fibrosis, before Yamamoto and colleagues established a mouse model of scleroderma using daily subcutaneous injections over a 4-week period (Moseley et al., 1986; Yamamoto et al., 1999).

We have adapted the dosage, delivery and temporal characteristics of this model to develop a novel model of hypertrophic scarring. Bleomycin is delivered

continuously via a subcutaneous osmotic pump (Alzet Model 1004, Durect Corp., Cupertino CA 95014) in order to stimulate a fibrotic process rather than the dermal sclerosis described by Yamamoto et al. Bleomycin infusion provides a continuous inflammatory stimulus, in contrast to the daily dosing used in the scleroderma model. Mice were euthanized at 28-days or 56-days in an attempt to model both immature and mature hypertrophic scars.

4.2 Results

The osmotic pump and infusion was well tolerated by the mice (Figure 4.1). There were no obvious systemic differences in the bleomycin cohort compared to the control cohort. As the model did not involve a gross epidermal breach and the pump was positioned away from the insertion site (Figure 4.1), no macroscopic scarring was observed. The appearance of the murine skin was assessed for gross changes in appearance but none were observed between the treatment groups.

4.2.1. The bleomycin model produces tissue architecture that corresponds to hypertrophic scars.

To determine whether the lesions produced in response to bleomycin infusion reproduced the features of hypertrophic scar, normal mouse skin and sections from the 28-day and 56-day models were stained with haemotoxylin and eosin. These were examined under light microscopy and compared with similarly stained normal human skin and hypertrophic scar samples from the RAH Adult Burn Centre (Figure 4.1).

No difference was observed between PBS infused control skin and untreated murine skin in terms of dermal thickening or skin architecture for the 28-day model or the 56-day model (Figure 4.1).

The 28-day model lesions displayed marked dermal thickening, compared to the control samples. This was associated with a hyperplastic epidermis, increased cellularity and a reduction in the number of dermal appendages compared to control (Figure 4.1). The 56-day model lesions had increased dermal thickness compared to the 28-day model and control samples. They also displayed a flattened epidermis and an absence of dermal appendages, both features consistent with mature hypertrophic scars in humans.

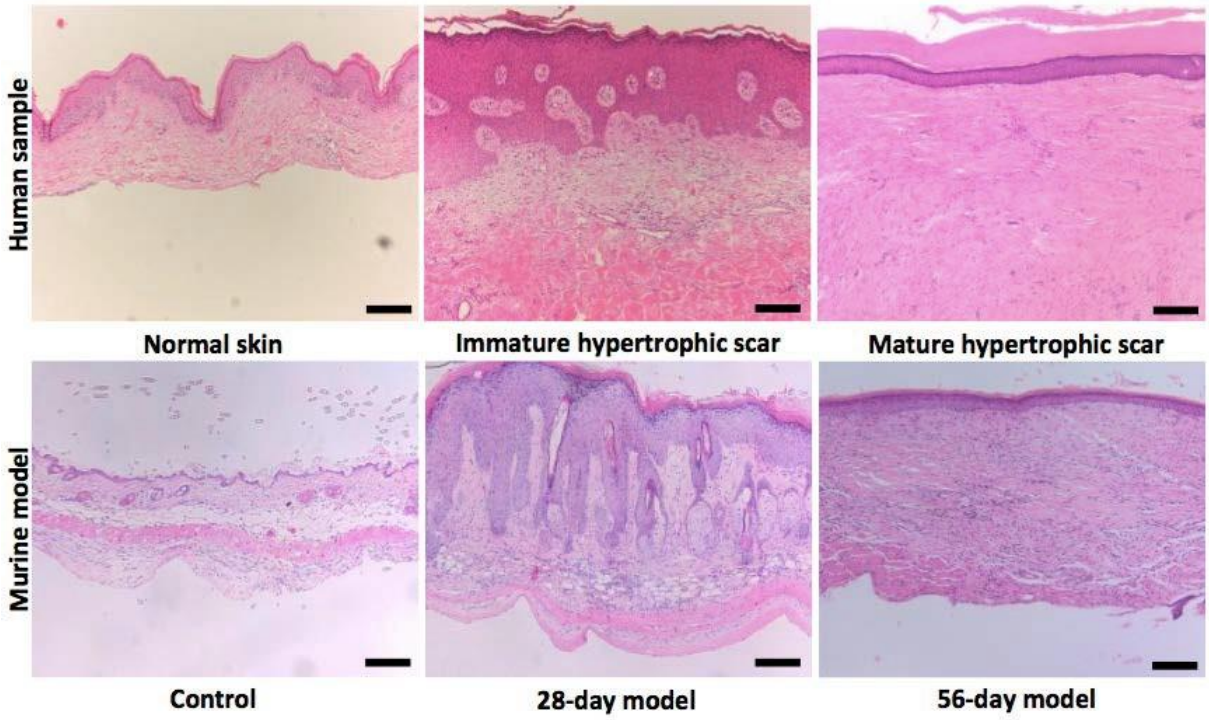


Fig. 4.1 The bleomycin model reproduces histologic architecture of hypertrophic scars. Control (PBS treated) murine skin, 28-day model lesion and 56-day model lesion compared with normal human skin, immature hypertrophic scar and mature hypertrophic scar (*above*). The 28-day model displays dermal thickening, a paucity of dermal appendages and hyperplastic epidermis. These features are consistent with those displayed in an immature hypertrophic scar. The 56-day model displays a marked dermal thickening, absence of dermal appendages and a flattened epidermis. These features are consistent with those displayed in a mature hypertrophic scar. *Scale bars* = 100μm

Quantification of dermal thickness was performed using the Image Pro-Plus 5 program. The 28-day model lesions displayed dermal thickening, which was significantly increased compared to control (202.5±30.93μm vs. 30±5μm, $p < 0.0005$).

The 56-day model lesions had significantly increased dermal thickness compared to both control samples and 28-day model samples (726.72±77.18μm vs. 30±5μm, $p < 0.0005$; 726.72±77.18μm vs. 202.5±30.93μm, $p < 0.0005$) (Figure 4.2).

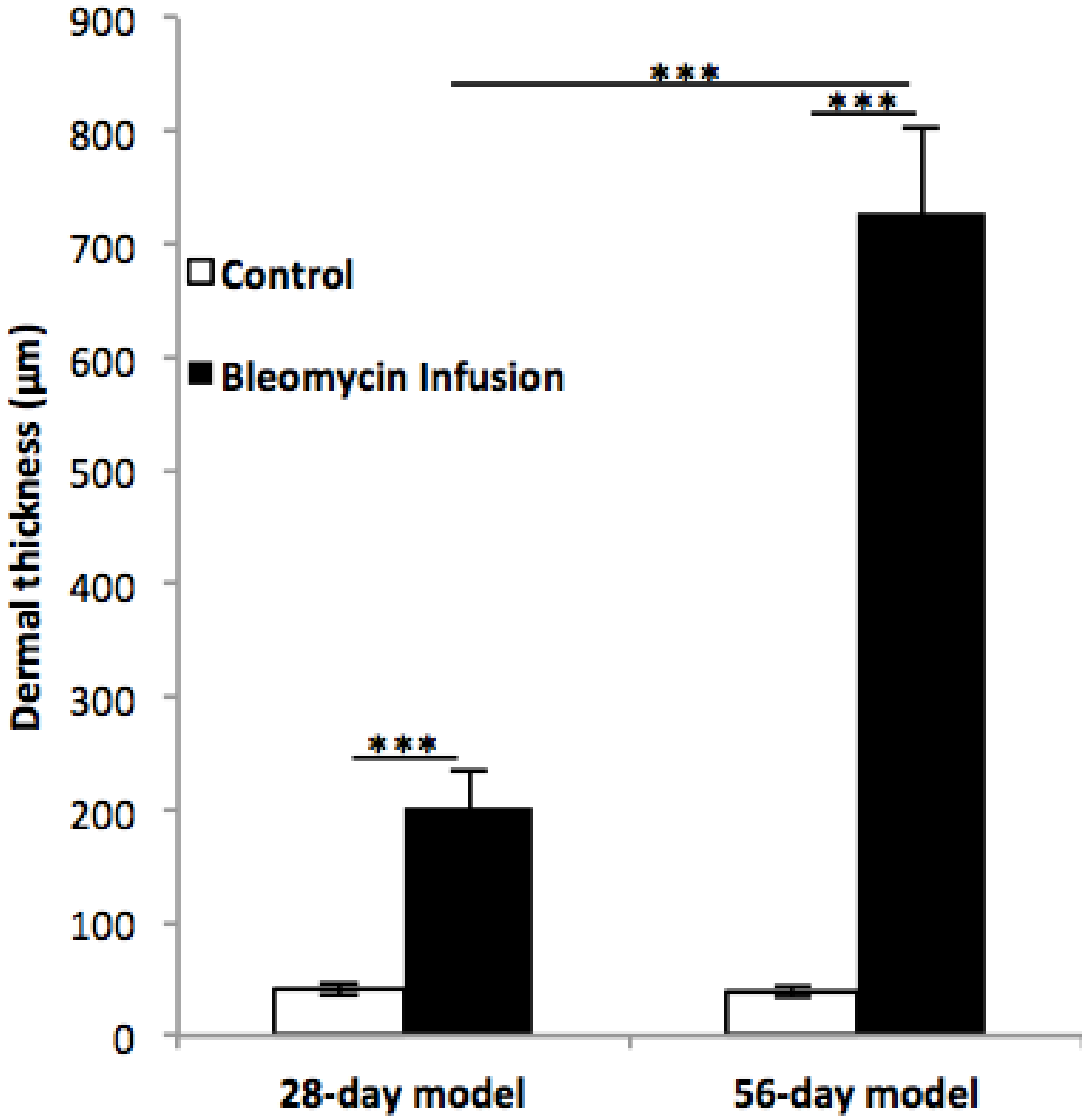


Figure 4.2 Quantification of dermal thickness: Quantification of dermal thickness was performed using the Image Pro-Plus 5.1 program. The 28-day bleomycin model produced a dermal thick more than 6 times that of the control samples. The 56-day model produced a dermal thickness approximately 30 times that of the control samples, and 3 times that of the 28-day bleomycin model. ***p<0.0005; *error bars* = SEM

4.2.2 The bleomycin model displays histopathological features of hypertrophic scars

There are a number of classic histopathological features associated with hypertrophic scars including collagen nodules and whorls, thickened hyperplastic epidermis and abnormal dermal collagen organisation (J. Y. Lee et al., 2004). Samples of immature and mature human hypertrophic scars obtained from burn patients were stained with H&E, as well as lesions from the 28-day and 56-day models. These samples examined by light microscopy in order to determine whether histopathological features of human hypertrophic scars were reproduced in bleomycin-induced lesions.

Collagen nodules and collagen whorls are features of hypertrophic scar and were present in lesions of both the 28-day model and the 56-day model, although they appeared to be most prevalent in the 28-day lesions (Figure 4.3).

In both models, abnormal dermal collagen organisation was observed. Collagen arrangement was disorganised in the 28-day model, while the 56-day model displayed collagen arranged in sheets parallel to the surface of the skin. This corresponds to the collagen arrangement seen in immature and mature hypertrophic scars respectively (Figure 4.4).

Epidermal layers of the 28-day and 56-day model lesions displayed basal disarray and vacuolar change. The epidermis of 28-day model lesions was hyperplastic and

displayed rete pegs/dermal papillae, whereas the epidermis of the 56-day model was flattened and did not display the same degree of hyperplasia. (Figure 4.3).

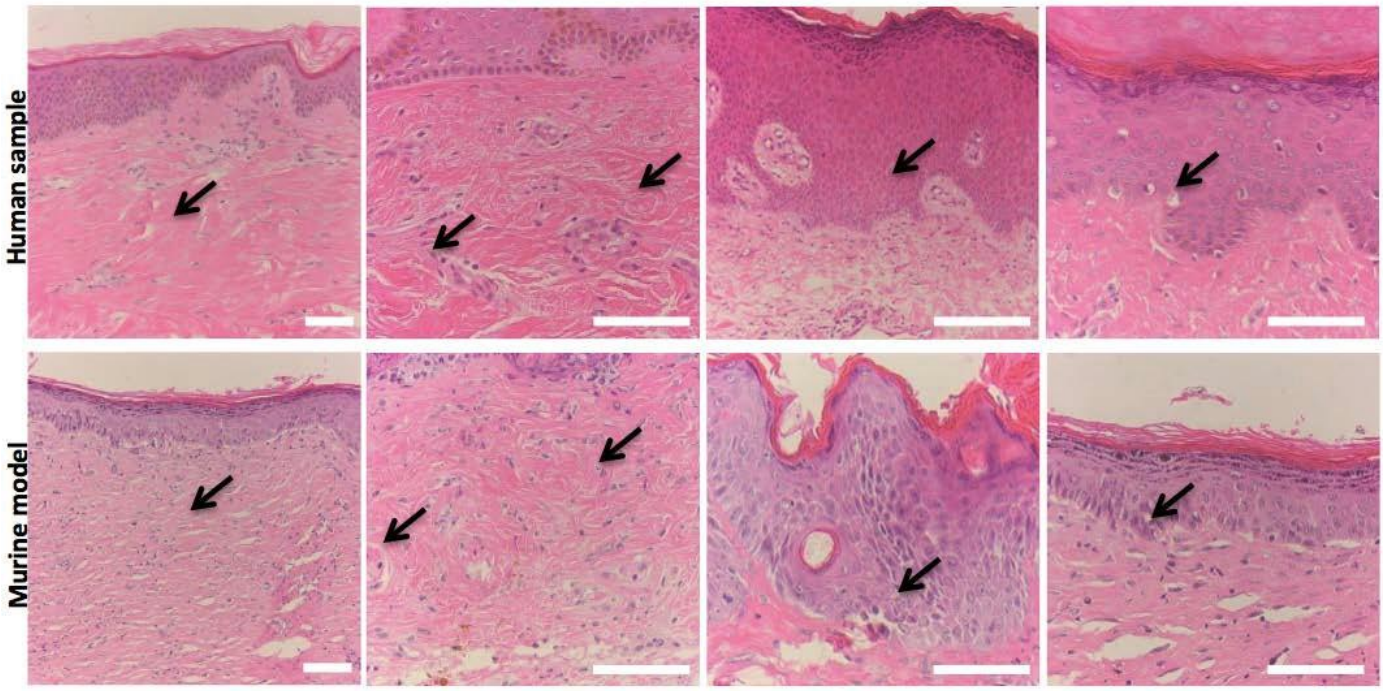
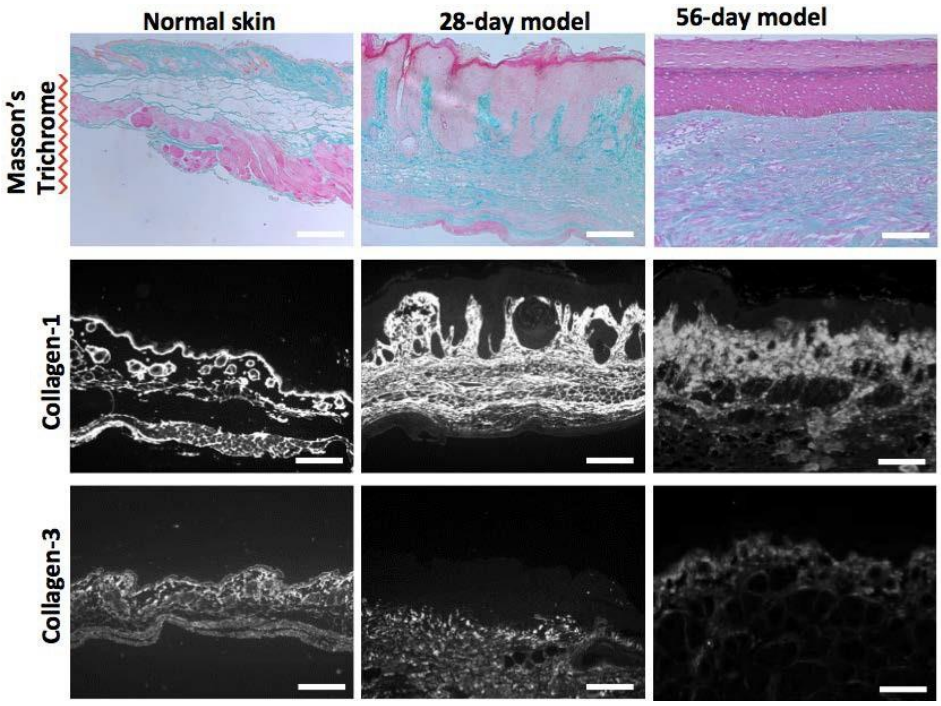


Figure 4.3 The bleomycin model reproduces histopathological features of hypertrophic scars. Samples of immature hypertrophic scars; mature hypertrophic scars; and lesions from 28-day model and 56-day model were stained with H&E and photographed at 20x or 40x magnification. Mature hypertrophic scars demonstrated parallel sheets of collagen in the dermis, and similar collagen organisation was seen in the 56-model (*arrowheads; far left*). Immature hypertrophic scars demonstrated collagen nodules and whorls; similar nodules whorls were observed in the dermis of 28-day model lesions (*arrowheads; middle left*). Hyperplasia and basal cell disarray was seen in immature hypertrophic scars; similar epidermal changes were seen in the 28-day model lesions (*arrowheads; middle right)*. Mature hypertrophic scars displayed a flattened epidermal layer with basal disarray and vacuolar change; similar epidermal changes were seen in 56 day-model lesions (*arrowheads; right)*. *Scale bars* = 100µm

4.2.3 Collagen content and composition of bleomycin induced lesions is analogous to hypertrophic scars

Alterations in the amount of collagen and the ratio of collagen-1 to collagen-3 (collagen-1:3) are associated with hypertrophic scarring (Zhang et al., 1995). Immunohistochemical localisation and quantification of collagen-1 and collagen-3 revealed a significant increase in the ratio of 28-day model lesions compared to normal murine skin (3.12±0.10 vs. 0.90±0.11, $p < 0.0005$). The collagen-1:3 ratio in the 56-day model was significantly lower than the 28-day model (0.72±0.12 vs. 3.12±0.10, $p < 0.0005$). There was no significant difference between the 56-day ratio and the ratio of normal murine skin. (Figure 4.4)



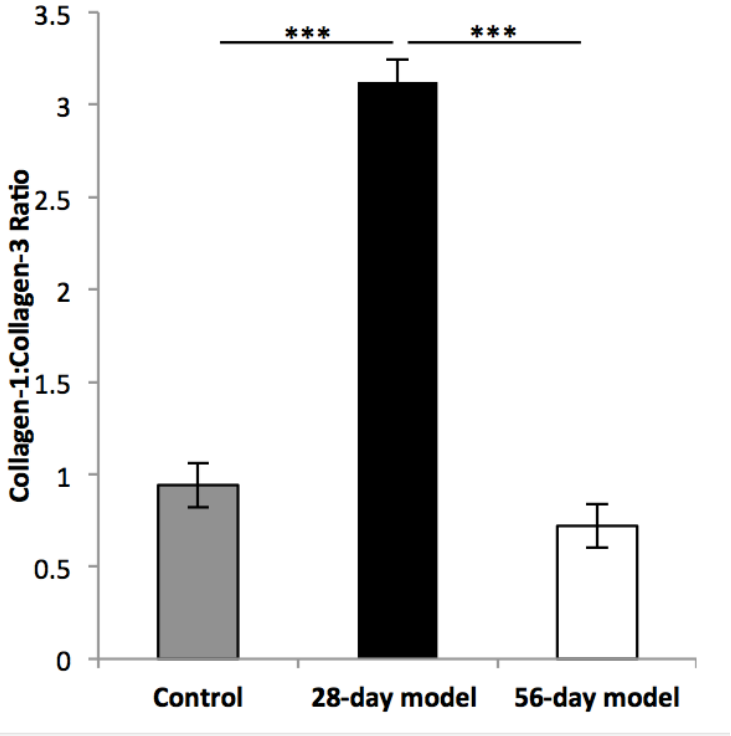


Fig. 4.4 Lesions produced by the 28-day and 56 day bleomycin models have altered collagen-1:3 ratios. Samples from control skin, 28-day model lesions and 56-day model lesions were stained using Masson's Trichrome and using immunohistochemistry for collagen-1 and collagen-3 (*above*). Collagen expression was then quantified using the AnalySIS software package and this data was used to calculate the collagen-1: collagen-3 sample for each lesion (*below*). *Scale bars* = 100μm; ***p<0.0005; *error bars* = SEM.

4.2.4 Key determinants of fibroproliferation are increased in the bleomycin model.

Normal mouse skin, 28-day lesions and 56-day lesions were co-stained with αSMA (α-smooth muscle actin) and DAPI (4',6-diamidino-2-phenylindole**)** to identify myofibroblasts. Myofibroblasts are the key cell type found in hypertrophic scars and express αSMA, which distinguishes them from fibroblasts and gives them their contractile properties. (Hinz, 2010). DAPI is a fluorescent stain that binds strongly to A-T rich regions in DNA and therefore is used in immunofluorescent staining to identify cell nuclei.

Myofibroblasts were identified as αSMA-positive cells within the dermis of the samples and expressed as a percentage of total cells/high powered field (cells/HPF). There was a significant increase in the percentage of αSMA-positive cells/HPF in both the 28-day (87.67±2.30% vs. 18.19±3.05%, $p<0.0005$) and 56-day models (76.84±4.14% vs. 18.19±3.05%, $p<0.0005$) compared to normal skin. The reduction in the percentage of αSMA-positive cell/HPF in the 56-day model lesions compared to the 28-day model lesions was statistically significant (87.67±2.30% vs. 76.84±4.14%, $p<0.05$) (Fig. 4.5)

TGF-β1 is the most investigated pro-inflammatory and pro-scarring cytokine in wound healing (Bayat et al., 2003). Normal mouse skin, 28-day lesions and 56-day lesions were stained using immunohistochemistry for TGF-β1. TGF-β1 was expressed in the epidermal layer of 28-day lesions and 56-day lesions as well as

in cells throughout the dermis. These images then quantified using the AnalySIS software package. There was a significant increase in TGF-β1 expression in the epidermal layer of both 28-day model lesions (14.00±1.95 vs 3.00±0.91, p<0.0005) and the 56-day model lesions (18.00±2.34 vs. 3.00±0.91, p<0.0005). There was also a significant increase in dermal TGFβ1 in both the 28-day model lesions (9.11±1.74 vs. 1.72±0.18, p<0.0005) and the 56-day model lesions (3.62±0.50 vs 1.72±0.18, p<0.005). There was a statistically significant reduction in dermal TGF-β1 in the 56-day model lesions compared to the 28-day model lesions (3.62±0.50 vs. 9.11±1.74 p<0.005) (Figure 4.6).

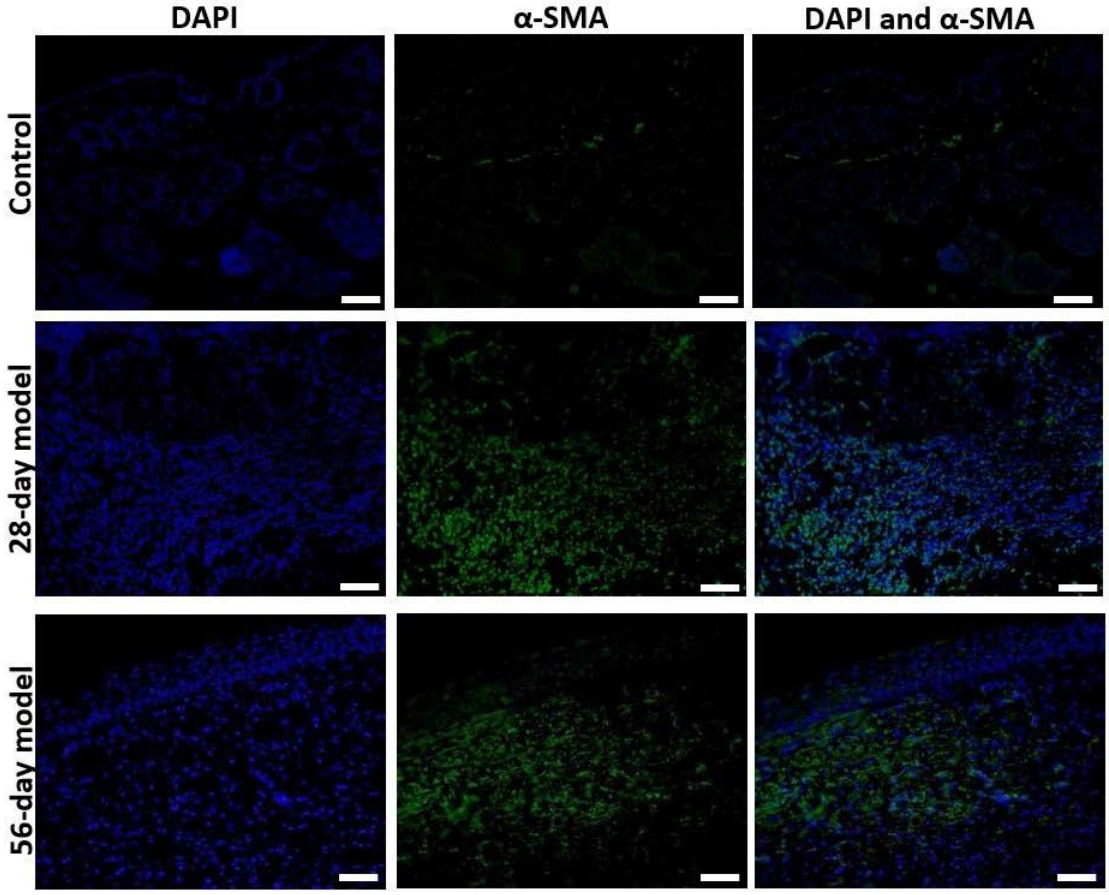


Figure 4.5 Myofibroblasts are increased in the bleomycin model scars compared to control Normal mouse skin, 28-day lesions and 56-day lesions were co-stained using immunohistochemistry for αSMA and DAPI

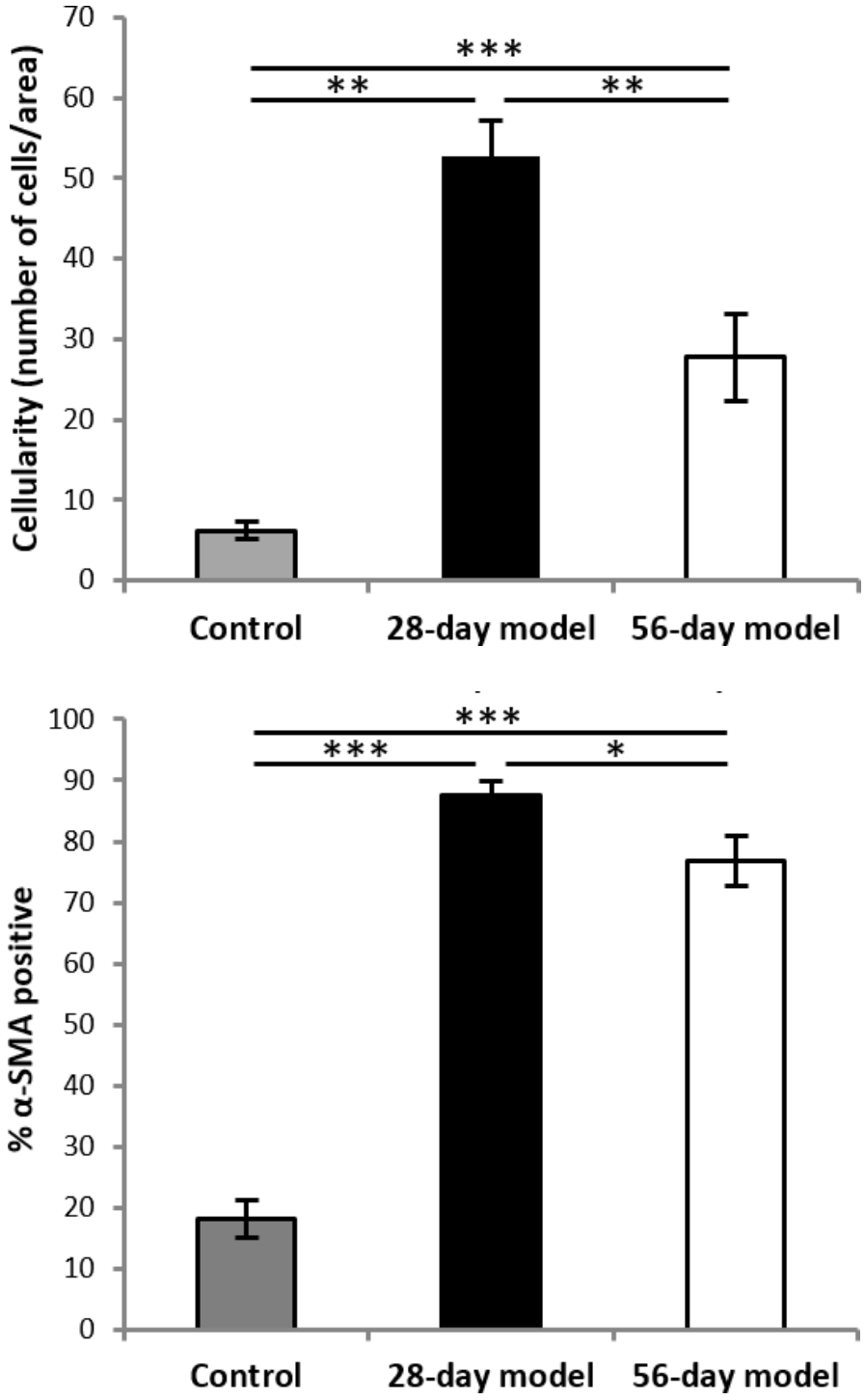


Figure 4.5 (cont.) Myofibroblasts are increased in the bleomycin model scars compared to control images were then quantified using the AnalySIS software package in terms of cellularity per high power field (HPF) and the percentage of these cells positive for αSMA (*right*). *Scale bars* = 100μm; ***p<0.0005; **p<0.0005; *p<0.0005; *error bars* = SEM, n=6.

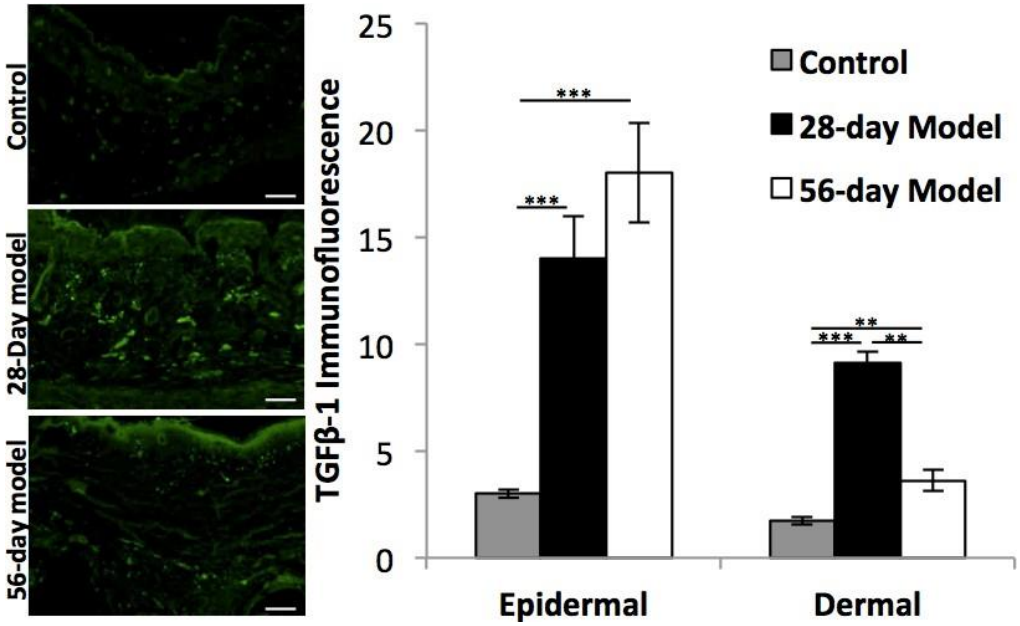


Figure 4.6 TGFβ1 is increased in bleomycin model scars compared with controls. Normal mouse skin, 28-day lesions and 56-day lesions were stained using immunohistochemistry for TGFβ1 (*left*). TGFβ1 was expressed in the epidermal layer of 28-day lesions and 56-day lesions and also in cells throughout the dermis. These images then quantified using the AnalySIS software package (*right*). *Scale bars* = 50μm; ***p<0.0005; **p<0.0005; *p<0.0005; *error bars* = SEM, n=6.

4.3 Discussion

Most existing models of hypertrophic scaring have focused on reproducing the histopathological characteristics of the hypertrophic scar, rather than modeling the process that leads to its development (Ramos et al., 2008). Hypertrophic scars and keloids were originally defined by their clinical characteristics, before attempts were made to define them as distinct pathologies with distinct histological characteristics, such as parallel collagen fibres, collagen whorls and epidermal flattening (Lee et al., 2004). However, producing evidence to support these definitions has been difficult; indeed the histological distinction between normal scars, hypertrophic scars and keloid scars is controversial (Lee et al., 2004; Muyembe-Tamfum et al., 2009). There is currently no consensus in the literature regarding the relative contribution of different collagen subtypes to hypertrophic scar tissue fibrillogenesis, nor the importance or temporal profile of key cell types, such as myofibroblasts, or well-known cytokines, such as TGF-β1 (Dale et al., 1996; Fleischmajer et al., 1990; Rawlins et al., 2006; Umezawa, 1967).

It may be important to re-contextualise hypertrophic scarring as a fibroproliferative disease, sharing a common mechanism of fibrosis with systemic scleroderma, lung fibrosis or glomerulosclerosis, albeit it with a unique organ (the skin) and aetiology (Wynn, 2008). It follows that, instead of settling on the hypertrophic scar phenotype as an endpoint, an ideal animal model would be one that focuses on the fibroproliferation that characterises hypertrophic scarring and other fibrotic diseases.

Previous studies have used a daily injection of bleomycin into the skin of mice to induce scleroderma (Yamamoto et al., 1999). In these trials, bleomycin-treated dermis showed sclerosis with thickened, homogenous collagen bundles, perivascular infiltrates and thickening of the vascular wall. We adapted this method by delivering bleomycin via a subcutaneous osmotic pump, which delivered bleomycin at a constant rate of 0.11μL/hour for 28 days. This technique not only limited animal pain and distress but also provided a continuous inflammatory stimulus, in contrast to once daily doses (Yamamoto et al., 1999). Bleomycin has a relatively short half-life and has been demonstrated to exert a greater dose response when delivered as an infusion rather than individual doses (Sikic et al., 1978; Sikic et al., 1978)

This model generated all the histopathological features that characterise human hypertrophic scarring, including dermal thickening due to collagen deposition, parallel collagen fibre orientation, collagen whorls, loss of dermal appendages and flattening of the epidermis.

Immature and mature hypertrophic scars were obtained depending on the length of time that samples were taken post treatment with bleomycin. After 28 days of bleomycin infusion, murine skin exhibited a thickened epidermis and inflammatory cell infiltrate consistent with a developing or immature hypertrophic scar. However, at day 56, dermal thickness had continued to increase, and an absence of dermal appendages and a flattened epidermis were observed. This ongoing fibroproliferation occurred even though the bleomycin

infusion had ceased at day 28; this is consistent with Yamamoto's results showing sustained phenotypic changes in fibroblasts after exposure to bleomycin (Takagawa et al., 2003).

Changes in extracellular matrix composition and collagen fibrillogenesis are key features of fibrotic disease (Karsdal et al., 2013). It is known that the ratio of collagen-1:collagen-3 is altered compared in fibrotic diseases, including hypertrophic scarring, although, there is conflicting evidence as to the exact nature of this alteration due to most studies using heterogeneous groups of human samples and varying considerably in their methodology {Rawlins, 2006 #648}. The 28-day mouse model showed a significant increase in the ratio of collagen-1: collagen-3 compared to normal mouse skin. The 56-day model had a collagen-1: collagen-3 ratio that was not significantly different from normal skin. These changes may reflect the reorganization and remodeling of collagen subtypes similar to that occurring in normal wound healing (collagen-3 produced early in the proliferative phase of wound healing is replaced by collagen-1). Further investigation of collagen production and fibrillogenesis by more informative methods, such as electron microscopy, is required

Bleomycin is known to induce production of reactive oxygen species and cause damage to endothelial and other cells types (Yamamoto & Nishioka, 2005a). In addition, bleomycin directly stimulates the pro-fibrotic TGF-β pathway, as well as a range of other pro-inflammatory and pro-fibrotic mediators such as chemo-attractant protein 1, PDGF, Il-4, Il-6 and Il-13 (Yamamoto & Nishioka, 2005a). In this way, bleomycin causes scarring by direct activation of inflammatory and

fibrotic mediators that have been shown to be important to the development of human hypertrophic scarring. This process does not involve an epidermal injury, which is the first event in any human wound leading to hypertrophic scarring. The benefit of avoiding epidermal wounding in the mouse model is that it circumvents the need to deal with the confounding differences between murine and human wound healing. In mice, the panniculus carnosus causes early wound contraction, limiting the requirement for healing by secondary intention (Cameron et al., 2013). By using bleomycin, we directly stimulate the fibroproliferative pathway common to every hypertrophic scar.

It follows that one criticism of the bleomycin model is that it does not allow investigation of the role of restoring epidermal integrity in reducing hypertrophic scarring, which is known to be important in the reduction of hypertrophic scarring in burn injury (Cameron et al., 2013). This might be better investigated using a model designed specifically to measure re-epithelialisation (such as an excisional or burn wound).

Any study using animals in order to model human disease should always acknowledge the significant differences between that animal and the human phenotype. Indeed a recent publication showed that genomic inflammatory responses in mouse models of human disease differed considerably from the genomic response seen in the corresponding human disease. In fact, the genes activated in the mouse model are close to random in matching the human disease genes. For this reason, murine models of human disease should always be viewed

with some skepticism and only be considered in conjunction with *in vivo* and human data (Seok et al., 2013).

A novel model of immature and mature hypertrophic scarring has been created, using the infusion of bleomycin to stimulate dermal fibroproliferation. This model reproduces morphological, histological and biochemical characteristics of human hypertrophic scars and presents two distinct windows in which to investigate scar formation and test potential anti-scarring therapies. For investigations directed at inflammatory response which drives fibroproliferation, the 28-day model would be most appropriate. For therapies designed to increase scar remodeling, the 56-day model would potentially be useful.

The bleomycin model has considerable potential to be used to understand the complex processes involved in the formation of hypertrophic scars and allows for preclinical evaluation of anti-scarring therapies, whether they are directed at either the aberrant inflammatory response that leads to hypertrophic scarring or aimed at altering the composition of a mature scar.

Chapter 5

EFFECT OF FLIGHTLESS ON HYPERTROPHIC SCARRING *IN VIVO*

Parts of this chapter have been published in following journal:

Cameron A.M., Turner C.T., Adams D.H., Jackson J.E., Melville E, Arkell R.M., Anderson P.J and Cowin A.J. (2016) Flightless I is a key regulator of the fibroproliferative process in hypertrophic scarring and a target for a novel anti-scarring therapy *Br J Dermatol* 174(4): 786-794

5.1 Introduction

Studies have shown that the protein Flightless I (Flii) is a regulator of the wound healing process (Adams et al., 2009; Cowin et al., 2007; Jackson et al., 2012). Flii is a member of the gelsolin family of actin-remodeling proteins that regulate actin by severing pre-existing filaments and/or capping filament ends to enable filament reassembly into new cytoskeletal structures (Kopecki et al., 2011). Flii co-localizes with molecules involved in regulating cytoskeletal reorganization, including members of the Rho family of GTPases, Ras and Cdc42 (Campbell et al., 1993). It possesses a unique leucine-rich repeat domain, which is important for protein–protein interactions (Claudianos & Campbell, 1995). Cells within the epidermis and dermis express Flii and Cowin et al. have shown that Flii is able to translocate from the cytoplasm to the nucleus in response to wounding (Cowin et al., 2007). Focal adhesions and migratory structures involved in cellular motility also express Flii (Davy et al., 2000) and it is also secreted from cells.

Flii has been investigated in a number of wound healing models of the past decade. In normal wound healing, In $Flii^{+/-}$ mice, wound healing under normal conditions is enhanced, whereas in $Flii^{Tg/Tg}$ mice, healing is impaired, with delayed epithelial migration and increased scarring (Cowin et al., 2007). In vitro, $Flii^{+/-}$ fibroblasts and keratinocytes have improved cellular adhesion and spreading resulting in accelerated scratch wound closure (Cowin et al., 2007; Kopecki et al., 2009). Additionally, reducing Flii in a murine model of partial-thickness burn injury led to faster re-epithelialization and reduced scar formation (Adams et al., 2009)

compared to wild type, suggesting the reduction of this protein in wounds may be beneficial as a treatment for hypertrophic scarring. Flii has also been shown to improve wound healing in models of impaired wound healing, namely murine models of diabetic wounds (Ruzehaji et al., 2013) and skin blistering/EB (Kopecki et al., 2013).

These studies have identified Flii as a potential target for a novel therapy to improve wound healing. However, in many of these initial findings, Flii was reduced by virtue of a heterozygous $Flii^{+/-}$. For this reason, a monoclonal antibody was developed as a topical or injectable therapy which inactivates Flii in the wound healing environment. This has been shown to improve wound healing in a similar manner to what has been seen in the heterozygous animals across the different models used (Jackson et al., 2012; Kopecki et al., 2013; Ruzehaji et al., 2013).

Interestingly, Flii, like other gelsolin proteins is secreted in response to tissue injury and is likely to be a mediator of inflammation (Lin et al., 2011). Secretion of gelsolin family proteins into the circulation has been linked to functions in scavenging actin (Bucki et al., 2008). The FnAb has been shown to deplete Flii in conditioned media by over 90% (Lei et al., 2012). Thus, FnAb appears to "mop-up' this extracellular to bring about improved wound healing. Flii secretion has been characterized (Lei et al., 2012) but Flii cell surface receptors have not yet been identified. However, FnAb has been shown to be taken up into cells (Kopecki et al., 2013) so it may have wound healing effects in both the extracellular and intracellular milieus.

Given the relationship between impaired wound healing and the development of hypertrophic scars, we have investigated whether Flii has a regulatory role in the fibroproliferative process that leads to hypertrophic scar formation. Here we examine the expression of Flii in human hypertrophic scars and investigate the effect of differential expression of Flii in a murine model of hypertrophic scarring as well as determining whether injection of a neutralizing antibody to Flii (FnAb) (Jackson et al., 2012) can reduce hypertrophic scar formation. Several determinants were used to measure scarring severity. Scar architecture was measured via dermal thickness and scar cross-sectional area. Collagen composition has also been shown to be linked to hypertrophic scarring severity, with an increased ration of collagen-1: collagen-3 indicating hypertrophic scarring (Hayakawa et al., 1982; Zhang et al., 1995). Myofibroblasts are differentiated fibroblasts involved in wound contraction and contribute to the development of hypertrophic scars. αSMA is used as a marker of myofibroblasts and distinguishes them from undifferentiated dermal fibroblasts (Hinz et al., 2007). Collagen content and αSMA positive cells were also measured to determine scarring severity.

5.2 Results

5.2.1 Decreasing Flii reduces acute and mature scar formation

After 4 weeks of bleomycin treatment, $Flii^{+/-}$ mice displayed significantly thinner dermis compared with WT (wild type) (145.4μm ± 5.7μm vs. 203.0μm ± 14.8μm, $p < 0.05$) and $Flii^{Tg/Tg}$ (145.4μm ± 5.7μm vs. 344.7μm ± 16.9μm, $p < 0.0005$) in the developing/immature hypertrophic scars (Fig. 5.1, 5.2). Similarly, but to a much greater extent, $Flii^{Tg/Tg}$ displayed significantly thicker dermis mice compared with WT (1120.9μm ± 140.8μm vs. 627.8μm ± 73.7μm, $p < 0.05$) and $Flii^{+/-}$ (1120.9μm ± 140 8μm vs. 726.7μm ± 128.9 μm, $p < 0.05$) at day 56 (Fig. 5.1). The developing/immature hypertrophic scar at day 28 showed that $Flii^{Tg/Tg}$ mice had a significantly higher cross-sectional area compared with both $Flii^{+/-}$ (76 361.9μm^2 ± 2895.6μm^2 vs. 42 608 4μm^2 ± 6784.3μm^2, $p < 0.0005$) and WT (76 361.9 μm^2 ± 2895.6μm^2 vs. 48 144.9μm^2 ± 7135.2μm^2, $p < 0.005$) (Fig. 5.3, 5.4). There was also a significantly higher cross-sectional area in the mature scars of the $Flii^{Tg/Tg}$ mice compared with both WT and $Flii^{+/-}$ (106 454.8μm ± 21 838.1μm^2 vs. 51 566.2 μm^2 ± 3134.3 μm^2: $p < 0.05$; vs. 36 597.8μm^2 ± 7564.3μm^2, $p < 0.0005$) at day 56. A significant reduction in scar area in $Flii^{+/-}$ mice compared with WT was also observed (36 597.8μm^2 ± 7564.3μm^2 vs. 51 566.2μm^2 ± 3134.3μm^2, P < 0.05) at this later time.

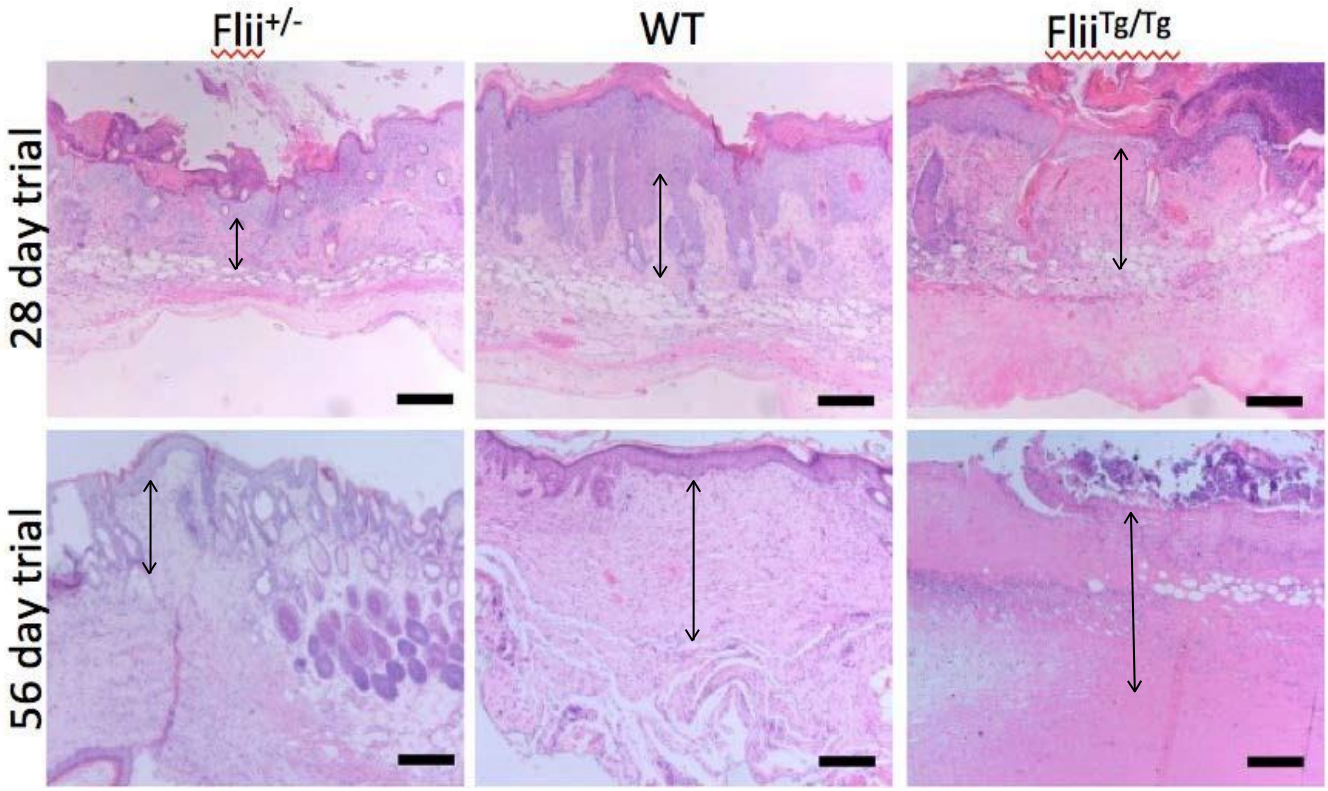


Fig. 5.1 Decreasing Flii reduces dermal thickness in 28-day and 56-day fibrosis models. Representative haematoxylin and eosin images of dermal thickness of scars after 28 days and 56 days. Microscopic analysis showed a significant decrease in dermal thickness in Flii$^{+/-}$ scars at 28 days compared with WT and Flii$^{Tg/Tg}$. Original magnification x6. Scale bar = 50 μm^2.

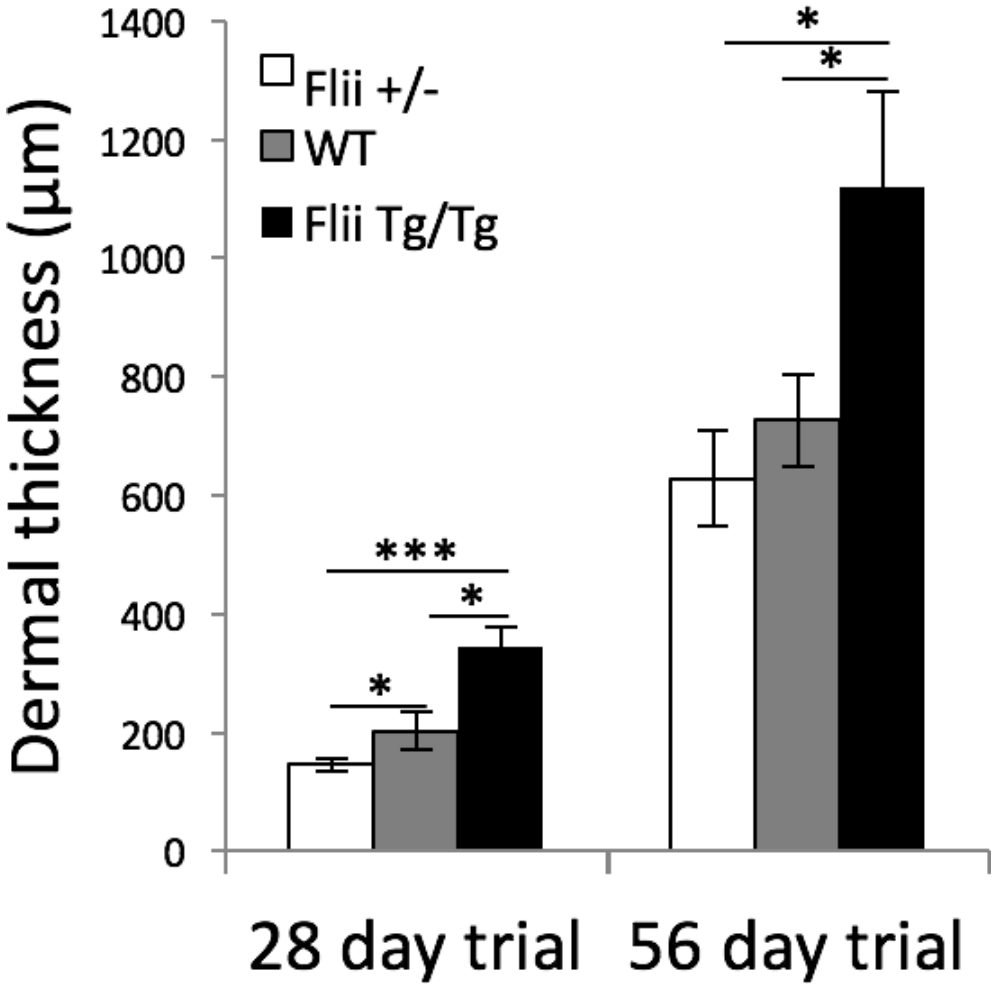


Figure 5.2 Decreasing Flii reduces dermal thickness in 28-day and 56-day fibrosis models. Graph showing changes in dermal thickness after 28 and 56 days. There was a significant increase in dermal thickness in the $Flii^{Tg/Tg}$ compared with WT and $Flii^{+/-}$ at 56 days. n = 6. Mean ±SEM. *$p < 0.05$, **$p < 0.005$, *** $p < 0.0005$.

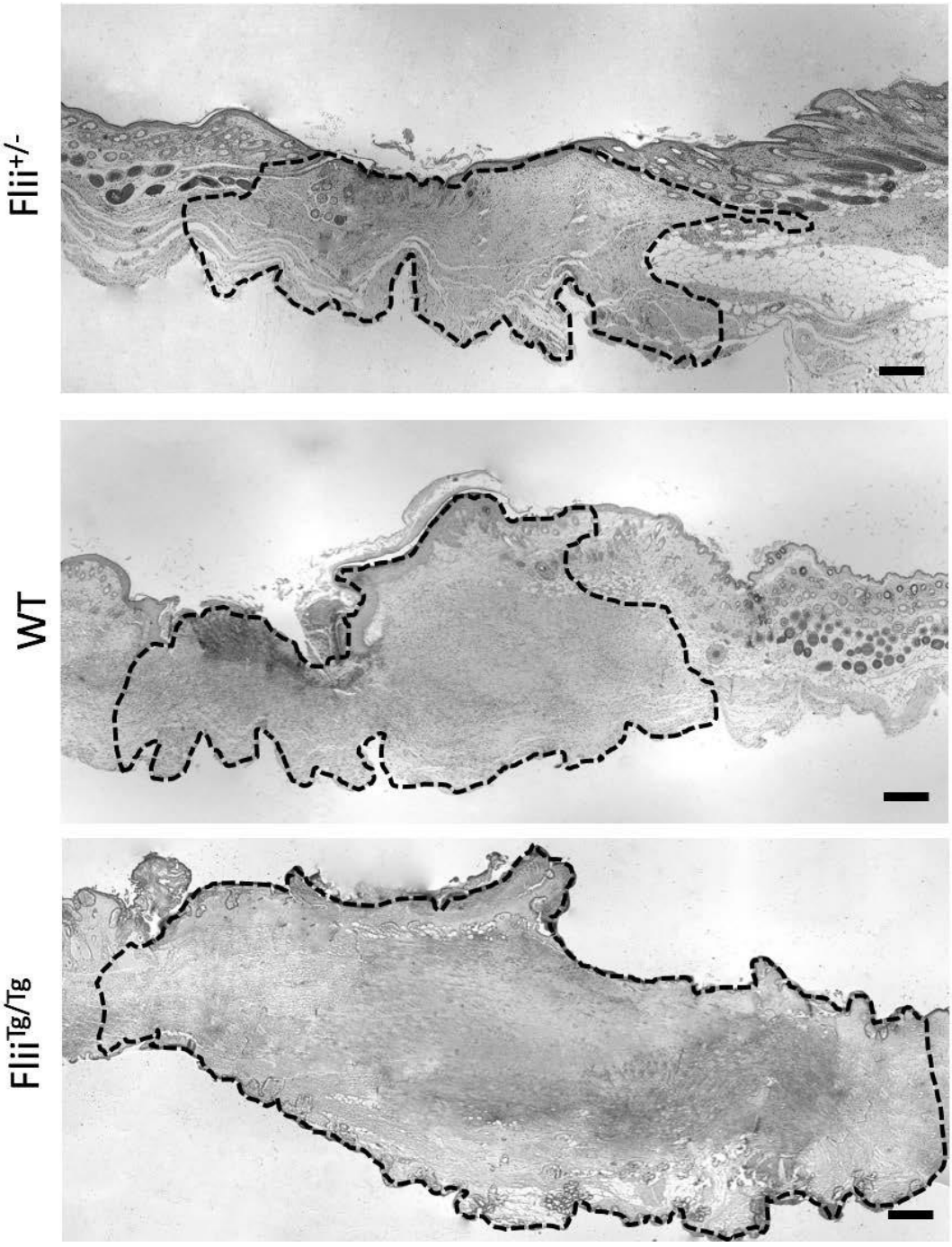


Figure 5.3 Decreasing Flii reduces scar cross sectional area in day-28 and day-56 fibrosis models. Representative stitched haemotoxylin and eosin images of total scar cross-sectional area at 28-days. Scale bar = 50 μm^2

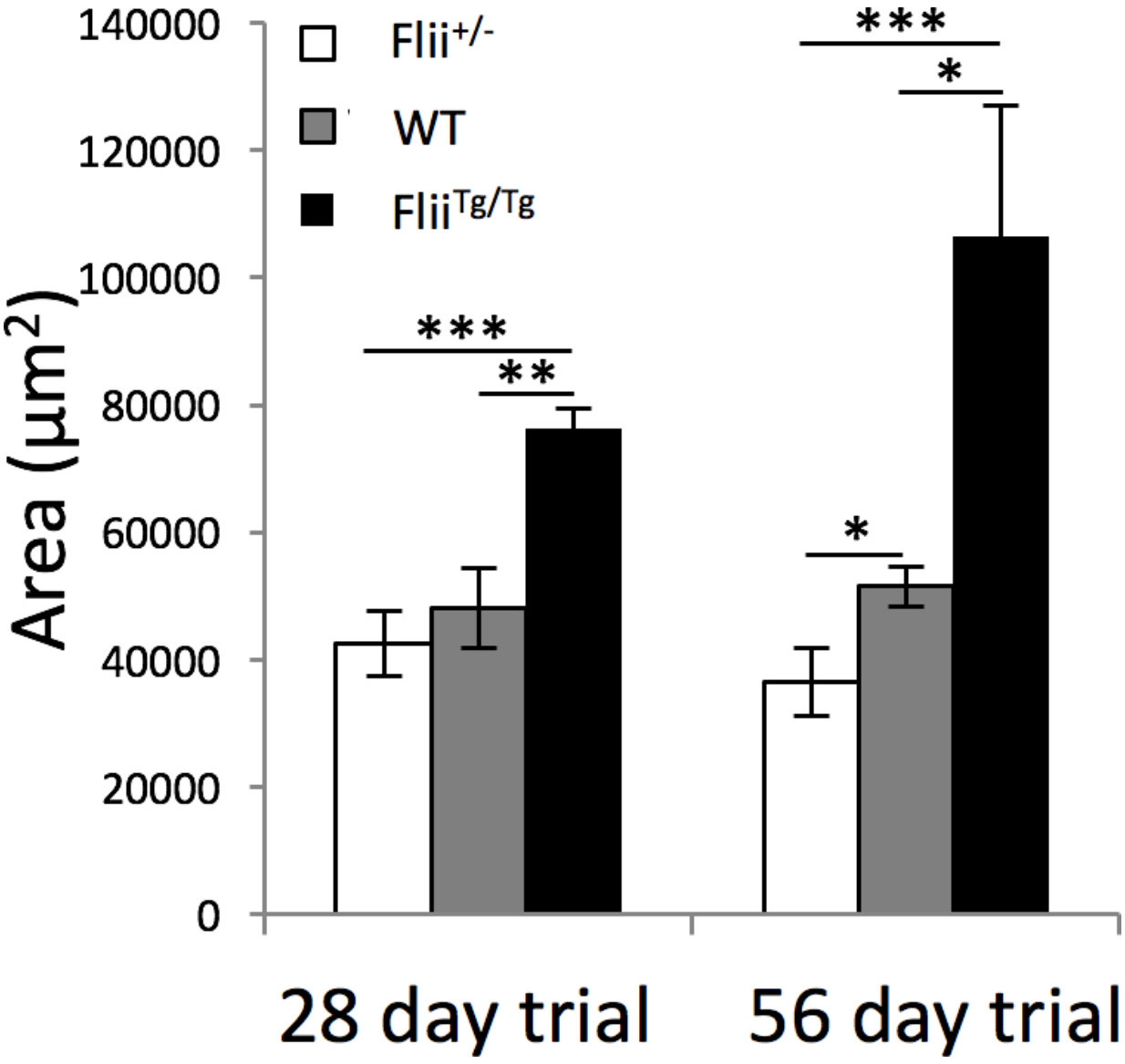


Figure 5.4 Decreasing Flii reduces scar cross sectional area in day-28 and day-56 fibrosis models. Microscopic analysis of images represented in Fig. 5.3 showed that Flii$^{+/-}$ scars had significantly less cross sectional area at 56 days compared with WT and Flii$^{Tg/Tg}$, n=6. Mean ±SEM. *p < 0.05, **p < 0.005, *** p < 0.0005.

5.2.2 Reducing Flii reduces collagen-1:3 ratio

More dermal collagen was observed in both 28-day and 56-day scars using Masson's trichrome staining compared with controls. Collagen-1 expression was higher at day 28 and day 56 in $Flii^{Tg/Tg}$ mice. Conversely collagen-3 was highest in the $Flii^{+/-}$ mice scars and lowest in the $Flii^{Tg/Tg}$ mice (Fig. 5.5, 5.6). When formulated as a ratio, collagen-1:3 was significantly higher in $Flii^{Tg/Tg}$ developing/immature scars compared with both WT (1.44±0.19 vs. 0.720±.15, $p<0.005$) and $Flii^{+/-}$ at 28 days (1.44±0.19 vs. 0.57±0.13, $p<0.0005$). In 56-day mature scars, the collagen-1:3 ratio in $Flii^{Tg/Tg}$ scars was also significantly higher compared with WT (3.13±0.13 vs. 0.92±0.012, $p < 0.0005$) and $Flii^{+/-}$ (2.60±0.38 vs. 0.92±0.012, $p<0.0005$). A lower collagen-1: collagen-3 ratio was observed in these mature scars at day 56 in $Flii^{Tg/Tg}$ compared to WT but this was not statistically significant (Fig 5.5-5.6).

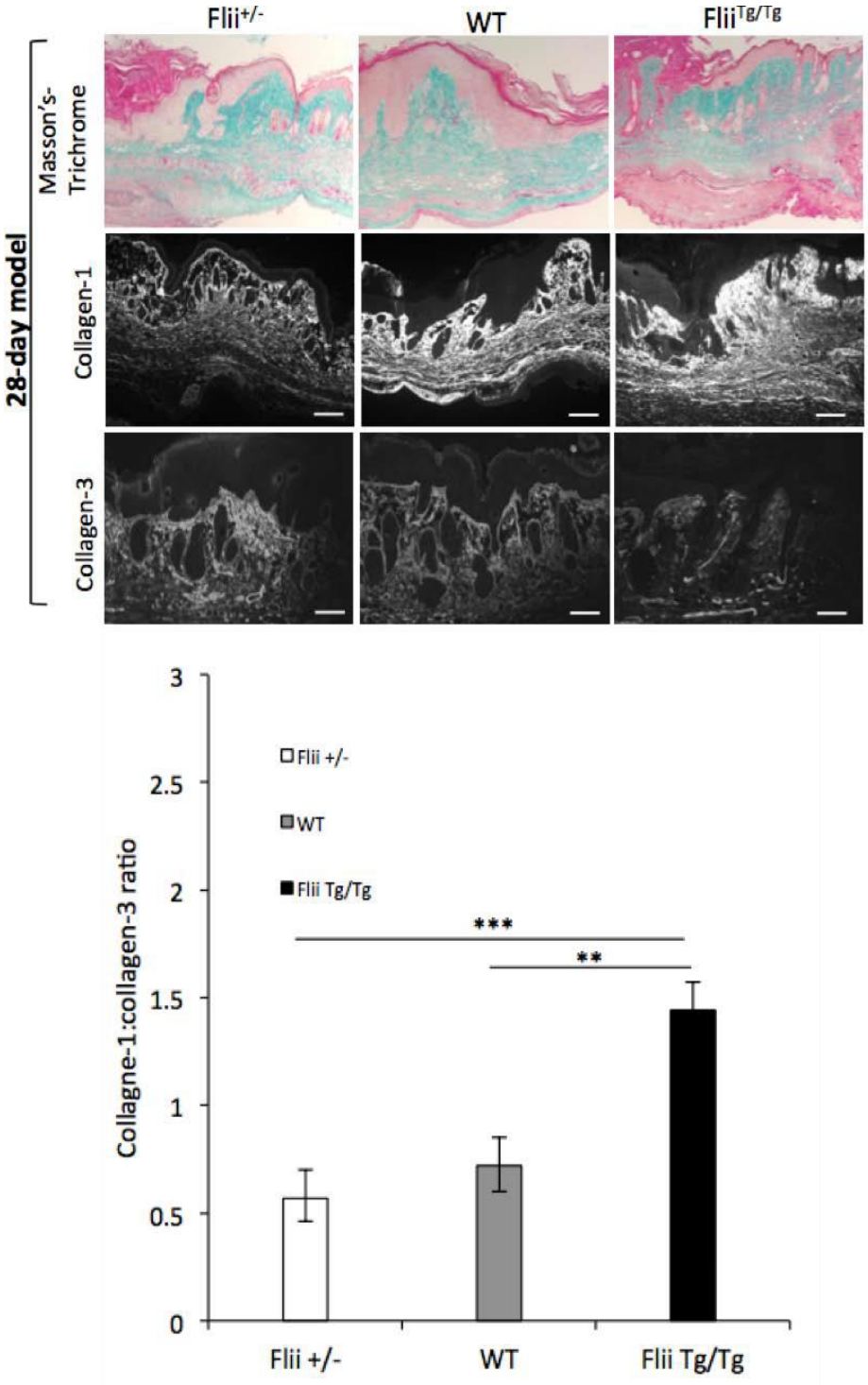


Fig. 5.5 Increasing Flii increases collagen-1:3 ratio in the 28-day model. (above) Representative Masson's trichrome images and immunofluorescent images showing total collagen and collagen 1 and 3 distribution in day-28 scars. (below) Optical density analysis of immunofluorescent images shows a significant increase in the collagen-1:3 ratio in Flii$^{Tg/Tg}$ compared with WT and Flii$^{+/-}$. n = 6. Mean SEM. *P < 0 05, **P < 0 005, ***P < 0 .0005.

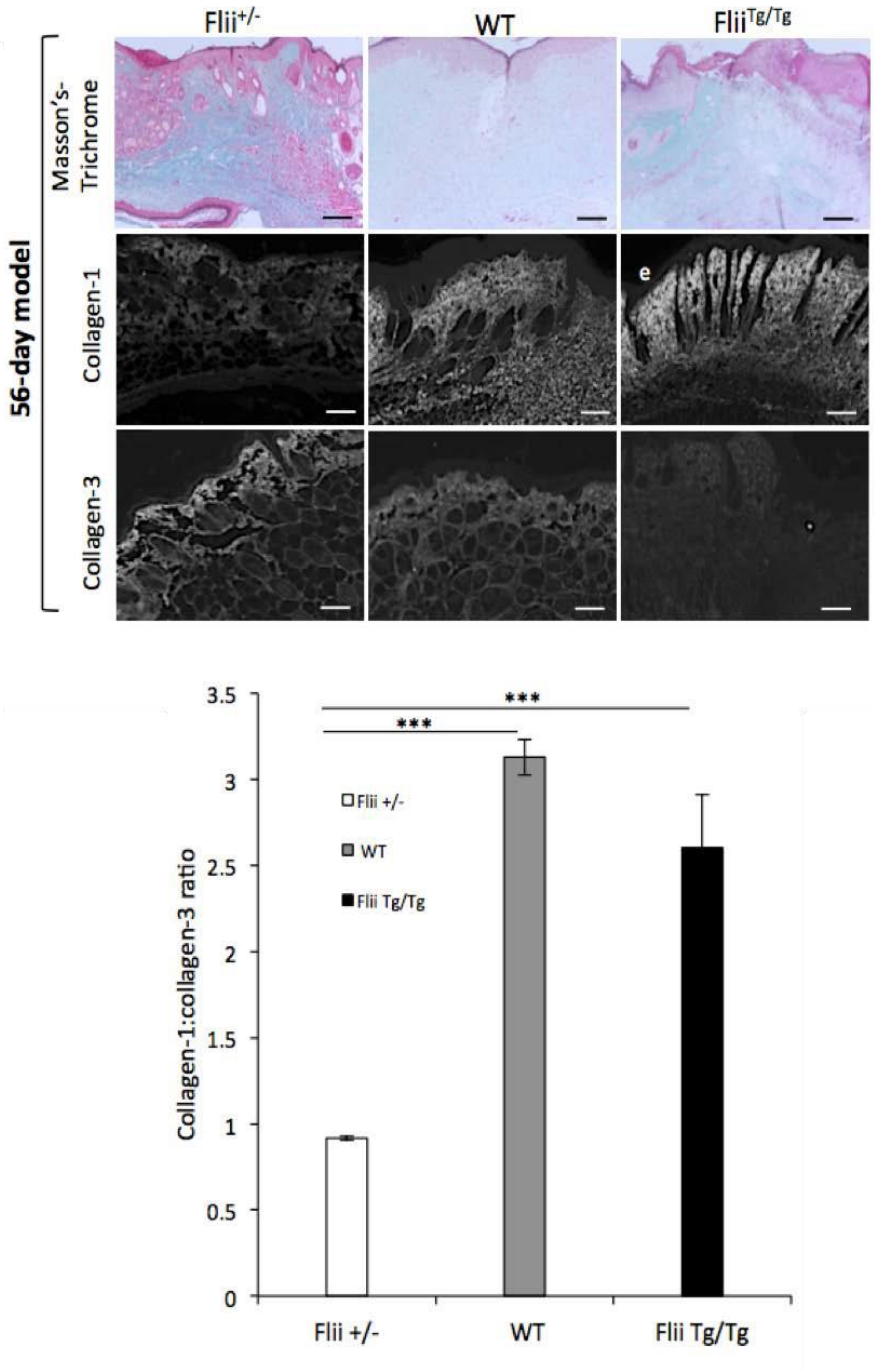


Fig 5.6 Decreasing Flii reduces collagen-1:3 ratio in the 56-day model
(above) Representative Masson's trichrome images and immunofluorescent images showing total collagen and collagen-1 and 3 distribution in day-56 scars. (below) Optical density analysis of immunofluorescent images shows a significant decrease in the collagen-1:3 ratio in Flii$^{+/-}$ scars compared with WT and Flii$^{Tg/Tg}$ scars. n = 6. Mean = SEM. *P < 0 05, **P < 0 005, ***P < 0 .0005.

5.2.3 Manipulation of Flii level affects key determinants of scarring.

The 28-day developing/immature scars in the Flii$^{+/-}$ mice had significantly less epidermal and dermal TGF-β1 expression compared with WT (*epidermal* - 42±8.7 vs. 145±4.3, p<0.05; *dermal* – 40.7±1.3 vs. 113±4.8, p<0.005) and Flii$^{Tg/Tg}$ (*epidermal* - 42±8.7 vs. 264±10.1, p<0.005; *dermal* - 40.7±1.3 vs. 130±12.1) (Fig. 5.7 a,c). Similarly, in the 56-day mature scars, Flii$^{+/-}$ mice displayed significantly less TGF-β1 in both skin layers compared with WT (*epidermal* – 103.3±1.3 vs. 246.3±4.8, p<0.005; *dermal* – 23.8±0.8 vs. 40.5±0.12, p<0.05) and Flii$^{Tg/Tg}$ (*epidermal* - 103.3±1.3 vs. 348.8±17.6, p<0.0005; *dermal* - 23.8±0.8 vs. 127.5±9.8, p<0.0005). (Fig. 5.7).

α- SMA-positive cells within the dermis were expressed as a percentage of total cells per field of view. Significantly fewer myofibroblasts were observed in 28-day developing/immature scars in the Flii$^{+/-}$ mice compared with WT (61±4.3 cells vs. 174.5±14.8 cells, p <0.005) and Flii$^{Tg/Tg}$ (61±4.3 cells vs. 195.4±13.7cells, p < 0.0005). Similarly, there were significantly fewer myofibroblasts in the mature scars of the Flii$^{+/-}$ mice compared with WT at 56 days (26.8±2.2 cells vs. 96±6.73 cells, p<0.0005) and Flii$^{Tg/Tg}$ (26.8±2.2 cells vs. 139.8±5.5 cells, p<0.0005). (Fig. 5.8). There were also significantly fewer myofibroblasts in WT mature scars compared with FliiTg/Tg (96±6.73 cells vs. 139.8±5.5 cells, p <0.005). (Fig. 5.8).

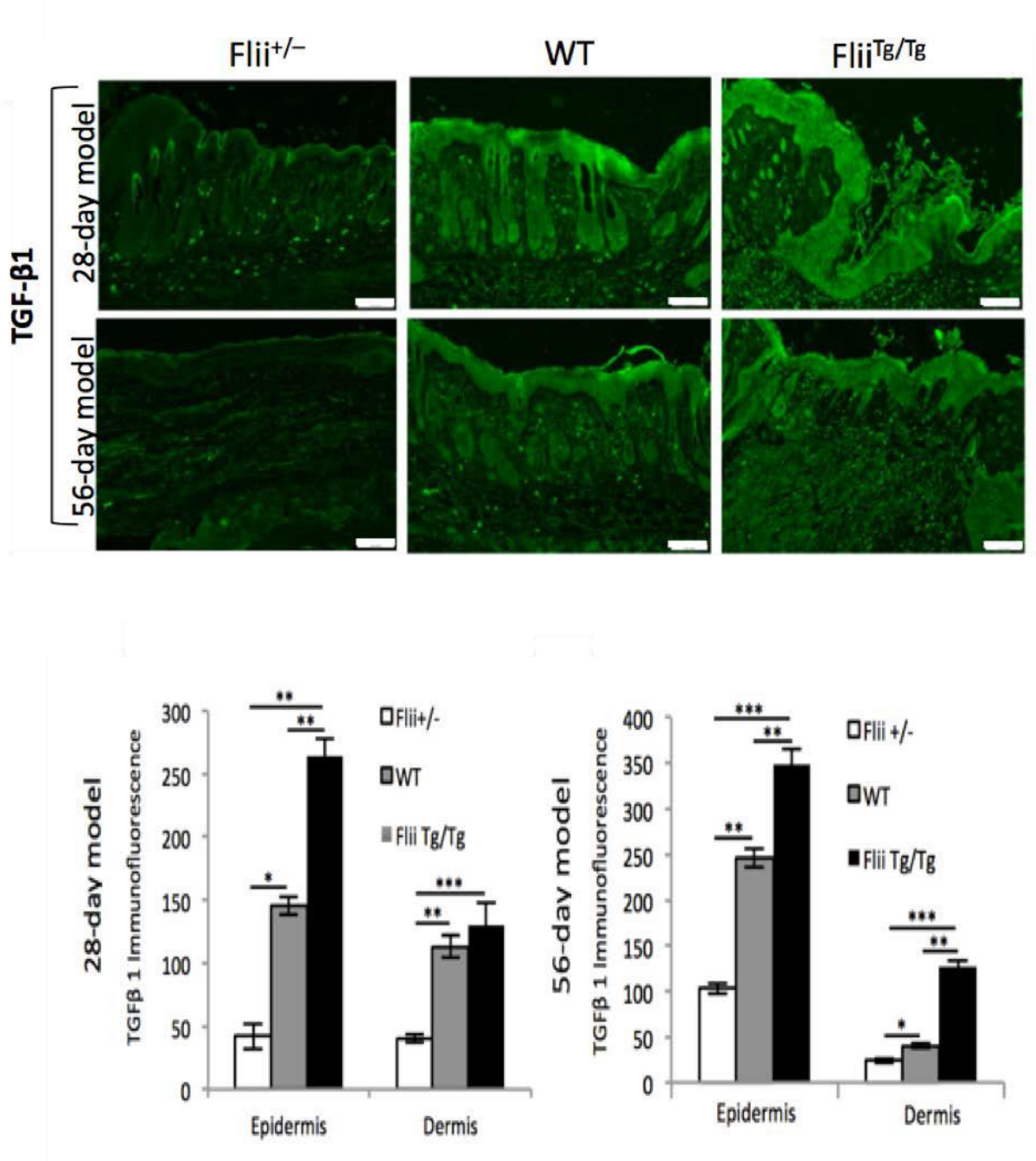


Figure 5.7. TGFβ-1 expression is higher as Flii levels are increased (above) Representative immunofluorescent images showing TGFβ-1 expression throughout the epidermis and dermis of Flii$^{+/-}$, WT and Flii$^{Tg/Tg}$ scars. Optical density analysis shows a significant increase in TGF-β1 as Flii levels increase across the genotypes at 28 days (below left) and 56 days (below right). Scale bar = 50 lm. n = 6. Mean SEM. *P < 0 05, **P < 0 005, ***P < 0 0005.

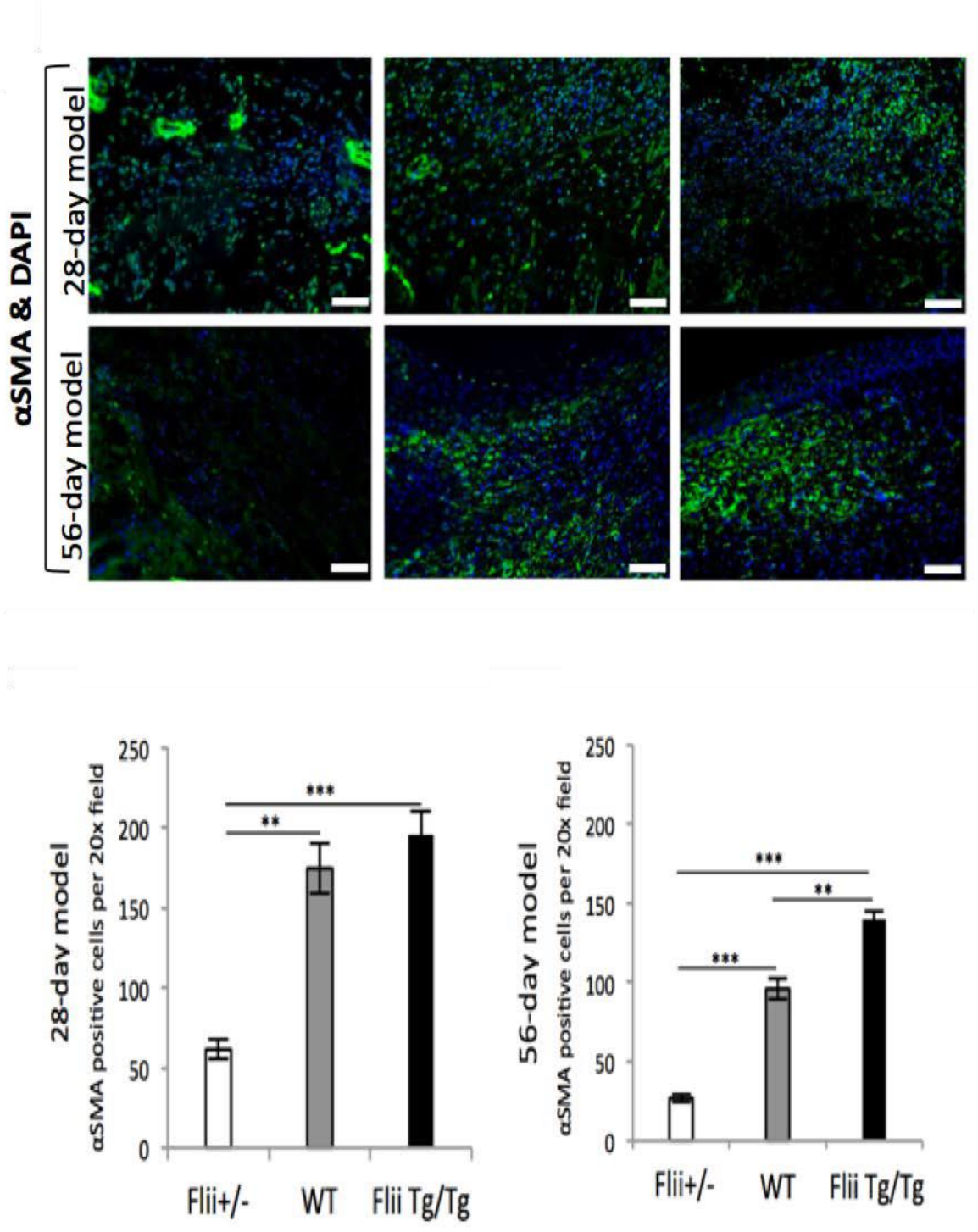


Figure 5.8 Decreasing Flii leads to a decrease in myofibroblasts Representative immunofluorescent images showing a-SMA and DAPI positive cells (above). Microscopic analysis showed significantly fewer myofibroblasts in $Flii^{+/=}$ scars compared with WT and $Flii^{Tg/Tg}$ at 28 days (below left) and 56 days (below right). In the 56 day model increasing Flii in the $Flii^{Tg/Tg}$ model led to a significantly increased number of fibroblast. Scale bar = 50 lm. n = 6. Mean SEM. *P < 0 05, **P < 0 005, ***P < 0.0005

0005.

5.2.4 Flightless I neutralizing antibody treatment reduces fibrosis in day 28 scars

FnAbs have previously been shown to decrease the level of Flii in wounds and improve healing. To determine if FnAb could reduce the development of hypertrophic scars, bleomycin-induced fibrotic lesions were injected with FnAb or IgG (control) at days 0, 7, 14 and 21. Histological assessment of day 28 scars showed that FnAb-treated mice had significantly smaller scar areas compared with IgG-treated controls (39324.6 μm^2 ± 5126.4 μm^2 vs. 71115.9μm^2 ± 4897.3, $p < 0.05$) (Fig. 5a, b) and dermal thickness 154μm ± 5.3μm vs. 209.7μm ± 31.7μm, $p < 0.05$). Furthermore, FnAb-treated scars also had significantly reduced collagen-1:3 ratios compared with IgG-treated controls (0.824 ± 0.31 vs. 2.92 ± 0.98, $p<0.05$). (Fig. 5.9 c, d).

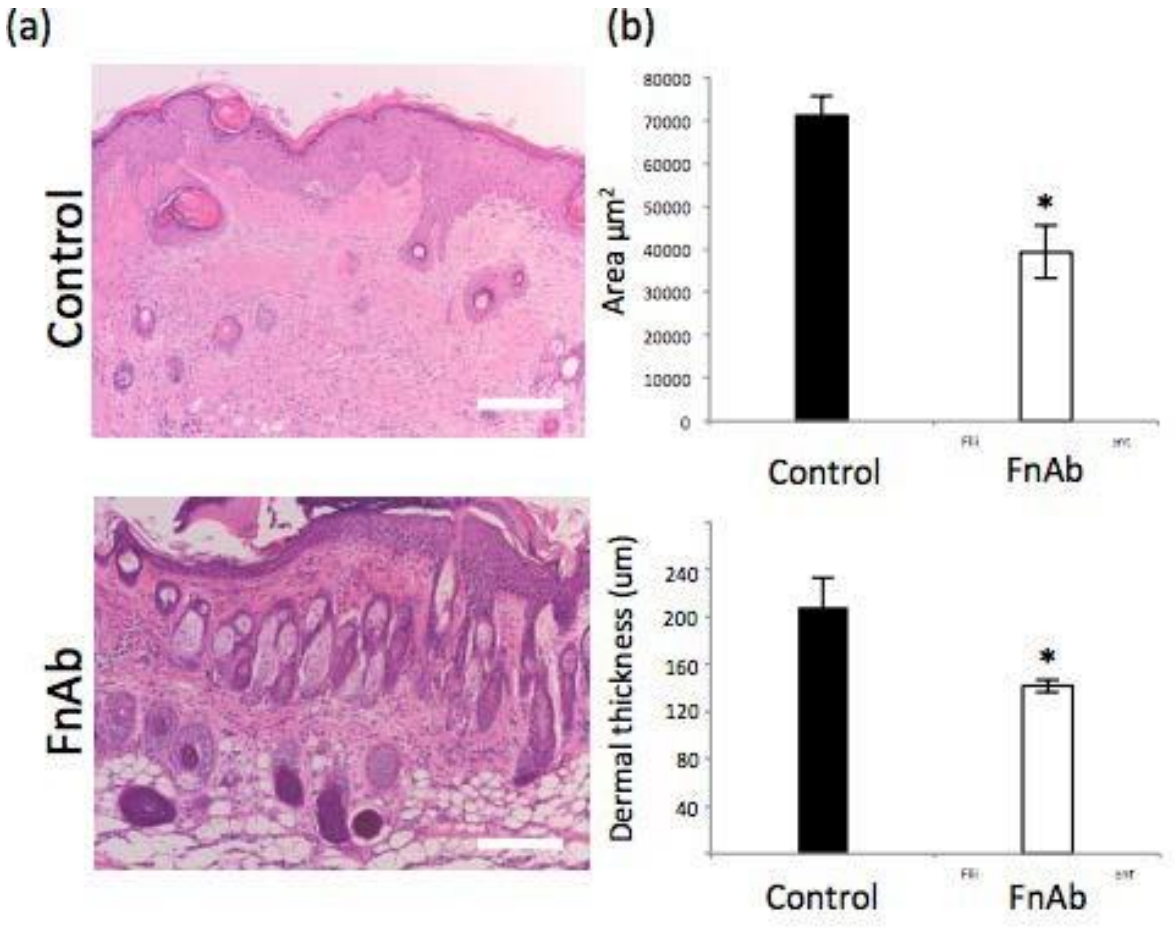


Fig 5.9 FnAb treatment reduces scarring in the 28-day model of acute fibrosis. (a) Treatment with 20 μg/m. FnAb caused a significant reduction in both dermal thickness and scar cross-sectional area compared with IgG-treated controls. Scale bar = 50 lm. n = 3. (b) Graph showing changes in significant reduction in dermal thickness and cross-sectional area in the 28-day model of acute fibrosis compared with IgG-treated controls. Mean SEM. *P < 0 05.

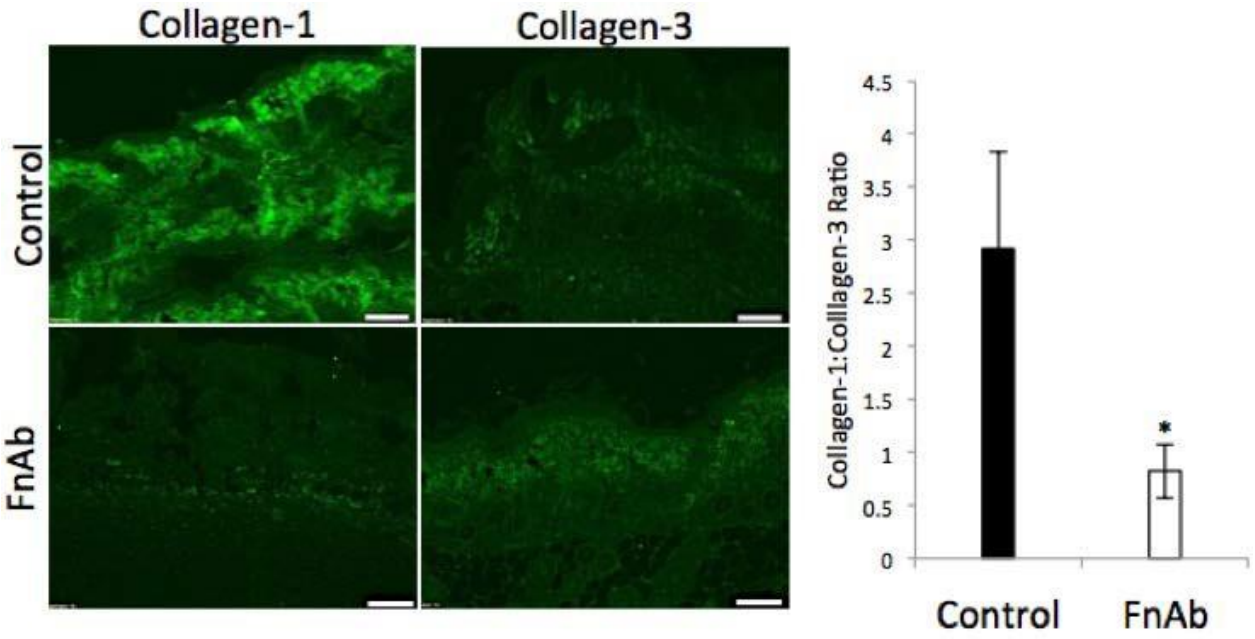


Fig 5.10 FnAb treatment reduces collagen-1:3 28-day model of acute fibrosis. FnAb-treated scars also show a significant reduction in the collagen-1:3 ratio compared with IgG-treated controls. Scale bar = 50 lm. (b) Graph showing significant reduction in the collagen-1:3 ratio of FnAb-treated scars compared with IgG-treated controls. *P < 0 05.

5.3 Discussion

As shown in Chapter 3, Flii is upregulated in human burn wounds and hypertrophic scars compared with normal unwounded skin, suggesting a potential role for Flii in the regulation of the scarring process. However, given the heterogeneity of human samples, an animal model of hypertrophic scarring was required to investigate the role of Flii in more detail. Chapter 4 showed the development of an effective mouse model using bleomycin-induced fibrosis. This has been developed to induce developing and mature hypertrophic scarring, with histopathology that correlates well with human hypertrophic scars (Cameron et al., 2013). Bleomycin acts on fibroblasts, keratinocytes, endothelial and immune cells via TGF-β1 and CTGF to drive fibroproliferative events (Yamamoto et al., 2000).

This chapter demonstrates that increasing the level of Flii ($Flii^{Tg/Tg}$ mice) resulted in a significant increase in the severity of scar formation. Conversely, lowering Flii using a heterozygous knockout mouse (homozygous knockout being embryonic lethal) led to a reduction in scarring in terms of both dermal thickness and cross-sectional area. Additionally, when Flii levels were reduced using a neutralizing antibody (FnAb) administered in conjunction with the bleomycin treatment, a significant reduction in scar severity was observed, suggesting that decreasing the level of Flii could reduce the fibrosis observed with scar formation.

TGF-β1 is an important fibrogenic factor (Wynn, 2008). Reducing Flii expression has previously been reported to decrease TGF-β1/ SMAD signalling, possibly through Flii forming a transcription complex with AP-1 proteins (Kopecki, Arkell, et al., 2011). In this study, scars of $Flii^{+/-}$ mice had decreased levels of fibrogenic TGF-β1 compared with scars of both WT and $Flii^{Tg/Tg}$ mice lesions, suggesting that Flii may potentially assist in reducing scarring severity via an effect on TGF-β1. Myofibroblasts have also been implicated in the process of hypertrophic scarring and the detection of myofibroblasts and TGF-β1 is highly significant for determining the degree of fibrosis (Hinz, 2010). In addition to producing the collagenous ECM comprising the hypertrophic scar, myofibroblasts can also promote fibrosis by secreting angiogenic, pro-inflammatory and fibrogenic factors (Darby et al., 2007). Scars in $Flii^{+/-}$ mice had significantly fewer myofibroblasts in the healing environment, suggesting that the anti-scarring effects of Flii may be mediated by an effect on myofibroblast differentiation, migration or survival.

The collagen-1:3 ratio has been identified as an important factor in determining severity of scar formation (Dale et al., 1996). Collagen-3 is a member of the fibrillar collagen family and is colocalised with collagen-1 in the skin. Collagen-3 is essential for normal collagen-1 fibrillogenesis by regulating the diameter of the collagen-1 fibril (Liu et al., 1997). An increased collagen-1:3 ratio is associated with hypertrophic scarring (Hayakawa et al., 1982). In keeping with the improvement in scar thickness and area as seen in $Flii^{+/-}$ mice, $Flii^{+/-}$ scars had significantly reduced collagen-1:3 ratios compared with WT and $Flii^{Tg/Tg}$ scars. Similarly, day 28 model mice treated with FnAb had significantly lower collagen-1:3 ratios compared with IgG-treated controls.

This chapter demonstrates that Flii is a key regulator of the fibroproliferative process underpinning hypertrophic scarring. Reducing Flii in a bleomycin model of hypertrophic scarring, either genetically via gene knockdown, or via treatment with FnAbs, leads to significant improvements in scarring. Reducing Flii also led to a reduction in key determinants of scarring including TGF-β1 and myofibroblast numbers. This study therefore identifies Flii as a potential therapeutic target for a novel therapy that may prevent or reduce hypertrophic scarring.

Chapter 6

EFFECT OF FLIGHTLESS ON FIBROBLAST PHENOTYPE *IN VITRO*

6.1 Introduction

To examine possible cellular mechanisms for the reduction in scarring seen in the bleomycin model, we investigated a putative role for Flii in the regulation of myofibroblast function and the fibroblast-myofibroblast transition.

In skin, myofibroblasts arise from various precursor cells that differ according to the characteristics of injury(Hinz et al., 2012) and cytokines and express α-SMA and the fibronectin (FN) splice variant ectodomain (ED)-A FNfibre (Hinz, 2010). The myofibroblast receives constant feedback from the extracellular matrix (ECM), which regulates its role in the wound healing process. This information then leads to responses in cell properties such as migration, proliferation, survival, differentiation, ECM organization and remodeling (Vogel & Sheetz, 2006). The dysregulation of this process is the hallmark of fibroproliferative disease, which in the skin is characterized as hypertrophic scarring.

Flii has a number of potential roles in the regulation of fibroblast-myofibroblast function. As an actin remodeling protein, Flii is important in the regulation of the actin cytoskeleton and consequently the motility, contraction and adhesion of cells (Davy et al., 2000). Flii has also been shown to act by inhibiting actin polymerization by capping, but not severing actin filaments (Mohammad et al., 2012). When Flii is reduced, increased actin and focal adhesion turnover results and leads to increased cell motility. Flii modulates focal adhesions and filamentous actin stress fibres in a Rac-1 dependent manner (Kopecki, O'Neill, et al., 2011). Flii co-precipitates with focal adhesion proteins talin, paxillin and vinculin (Kopecki

et al., 2009). *In vivo*, elevated Flii leads to increased stress fibre formation by impaired focal adhesion turnover and enhanced formation of fibrillar adhesions. Conversely, Flii knockdown increases the percentage of focal complex positive cells (Kopecki, O'Neill, et al., 2011). Flii knockdown cells exhibit lower levels of activated β1-integrin and vinculin, without any change in talin levels. This suggests that Flii may promote the recruitment of focal adhesion proteins like vinculin, which through its interaction affects β1-integrin activation (Mohammad et al., 2012). It is known that focal adhesions allow contractile activity of myofibroblasts to be transmitted to the ECM (Hinz et al., 2012), creating a more strained, stiffer environment. These mechanical conditions generated by the myofibroblast also lead to their sustained fibroproliferative activity (Hinz et al., 2001). Indeed, scar development in a murine model of hypertrophic was prevented by disruption of Focal Adhesion Kinase (FAK) signalling pathway (Wong et al., 2012). Reducing Flii may potentially alter cell focal adhesions and prevent this sustained myofibroblast activation.

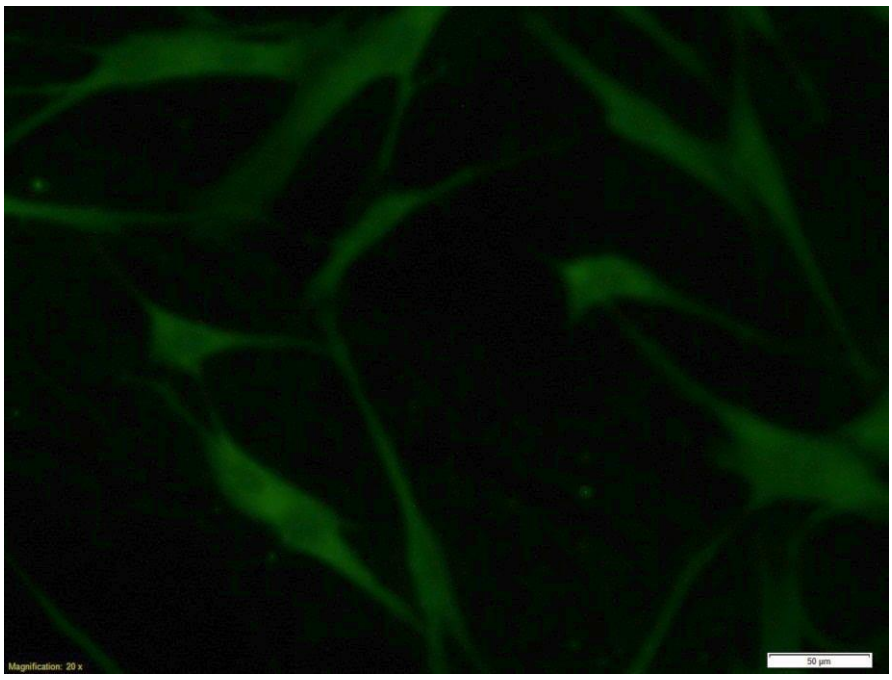
It has recently been shown that specifically blocking TGF-β1 mediated differentiation of myofibroblasts by preventing αv integrin activation of latent TGF-β prevents fibroproliferation in murine models of liver and lung fibrosis (Smith & Henderson, 2016). Flii may act in a similar manner to regulate myofibroblast function in fibroproliferation.

Thus, this chapter investigates the effective of Flii on fibroblast function, specifically in regulating the fibroblast-myofibroblast transition associated with hypertrophic scarring.

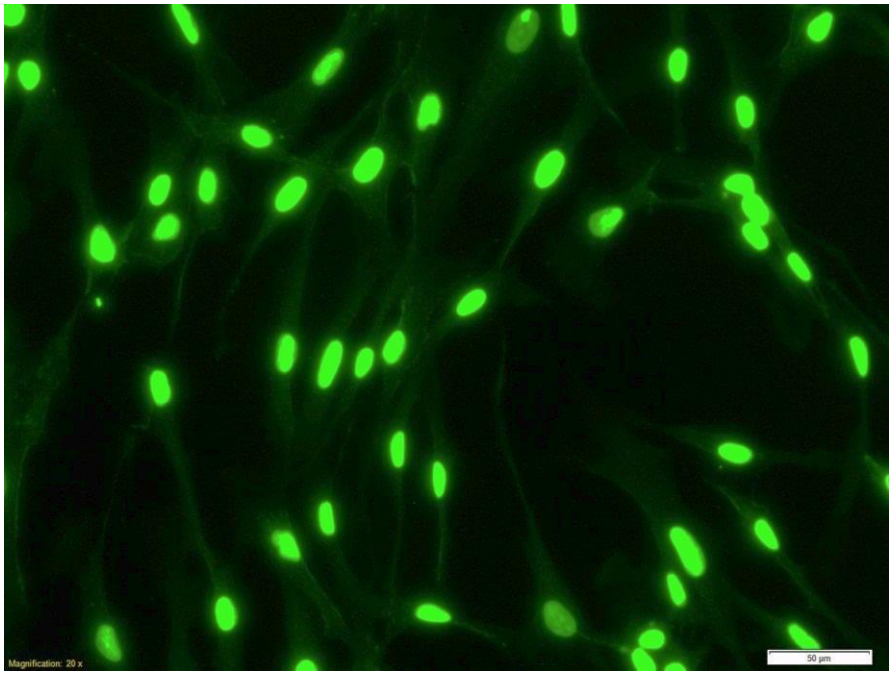
6.2 Results

6.2.1 Flii is increased in human hypertrophic scar fibroblasts compared to human foreskin fibroblast

Hypertrophic scar fibroblasts (HTSF) and human foreskin fibroblasts (HFFs) were cultured and subjected to immunocytochemistry for Flii as detailed in Chapter 2. There was a marked increase of Flii in HTSFs compared to HFFs. This was localized to the nucleus of the cells compared to the cytosolic distribution seen in HFFs. Morphological differences were also observed - with HTSFs appearing more spindle shaped compared with HFFs (Figure 6.1).



HFF



HTSF

Fig 6.1 Immunolocalisation of Flii in human foreskin fibroblasts (HFF) and hypertrophic scar fibroblasts (HTSF). Flii upregulated in HTSF and was localized to the nucleus of the cell. Scale bar = 50µm.

6.2.2 FnAb reduces fibroblast-myofibroblast phenotype change in HFFs co-cultured with bleomycin

It has previously been shown that culturing fibroblasts in the presence of bleomycin induces a fibrotic fibroblast phenotype (Yamamoto, Eckes, et al., 2000).

A bleomycin concentration of 140μg/ml was used based on concentrations used in Yamamoto's work. A WST-1 assay was used to confirm that this dose did not cause significant cell toxicity (Appendix 1). Cellular lysates were collected and Western Blot Protein analysis was performed to determine relative levels of Flii and αSMA, with β-tubulin used to normalise protein levels. Levels of Flii protein and αSMA were increased in HFFs cultured with bleomycin compared to control and the addition of FnAb led to a reduction in Flii and αSMA levels, although not to control levels (Fig 6.2 (c)).

Immunocytochemistry was then performed to correlate these findings. Bleomycin treated cells showed increased Flii expression, in a nuclear/peri-nuclear distribution, as well as increased expression of αSMA. Bleomcyin treated cells also appeared to have a more spindle like phenotype compared to control. Treatment with FnAb led to a reduction in Flii and αSMA expression and less pronounced spindle phenotype (6.2 (a)).

It is known that myofibroblasts are less motile than their fibroblast counterparts (Ronnov-Jessen & Petersen, 1996). Scratch migration assays were therefore performed to determine whether FnAb treatment has an effect on cell motility.

Confluent monolayers of HFF grown in 24 well plates were scratch wounded and cultured in the presence or absence of 140μg/ml bleomycin with 20μg/ml FnAb or 20μg/ml irrelevant murine IgG (control). Residual wound area was examined over 96 hours. HFFs cultured with bleomycin had significantly reduced motility compared with control HFFs. However, when treated with FnAb this motility was significantly increased. Addition of FnAb to control HFFs also resulted in a small but significant increase in motility (Fig. 6.2 (b)).

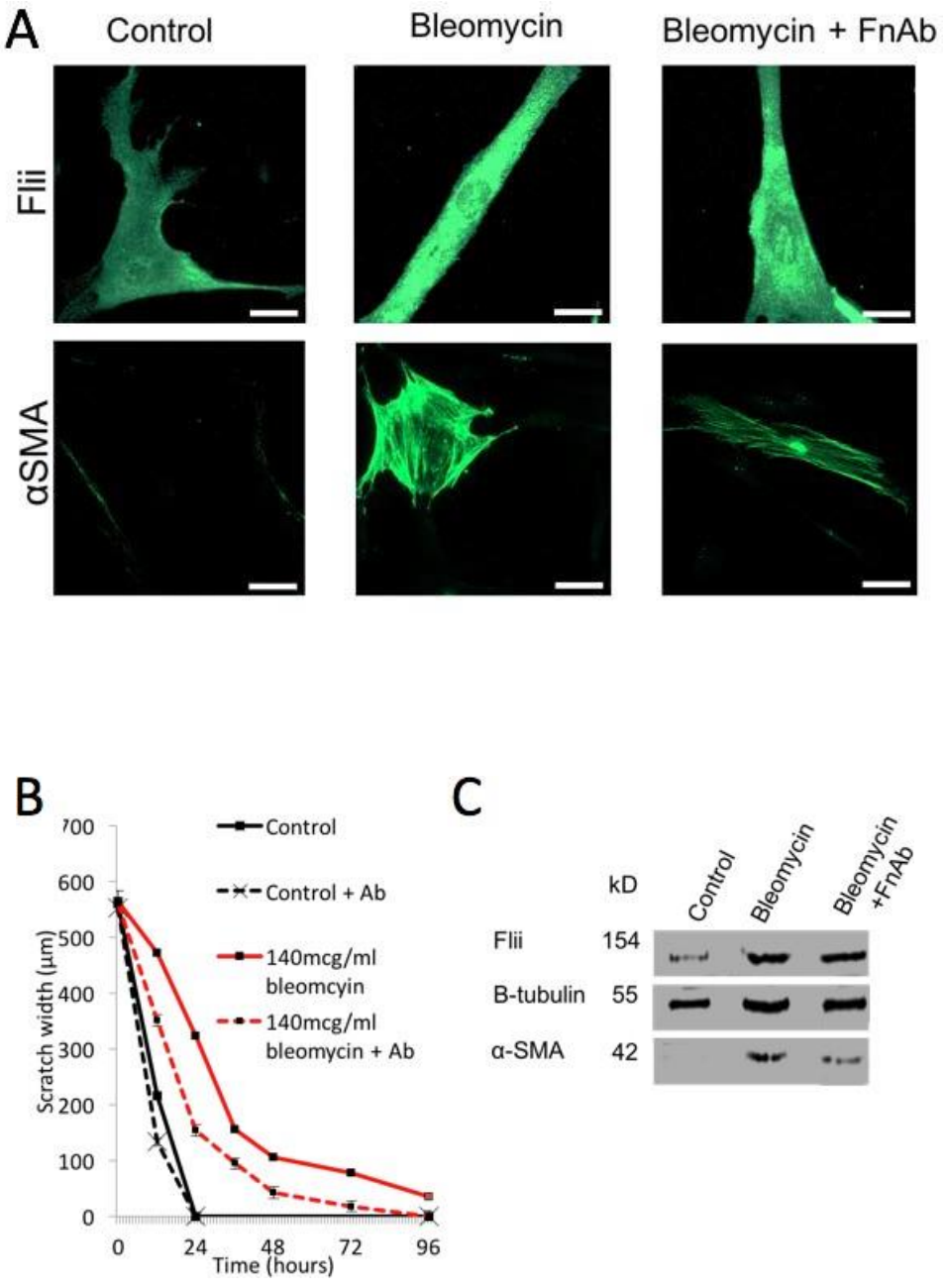


Fig 6.2 Treatment of HFFs with bleomycin induces myofibroblast like phenotype changes that can be reduced by FnAb. (a) Immunocytochemistry for Flii in HFFs, HFFs treated with bleomycin and HFFs treated with bleomycin and FnAb. Treatment with bleomycin led to an increase in Flii and αSMA. Addition of FnAb caused a reduction of Flii and αSMA, although not to control levels. (b) Western blot of cellular lysates of HFF, HFF treated with bleomycin and HFF treated with bleomycin and FnAb. Treatment with bleomycin led to an increase in Flii and αSMA, which was reduced after addition of FnAb. (c) Scratch assay showing decrease in motility of bleomycin treated HFFs compared with control HFFs. This decrease in motility is lessened by addition of FnAb.

6.2.3 Treatment of primary fibroblasts from Flii$^{+/-}$, WT and Flii$^{Tg/Tg}$ mice with bleomycin and FnAb

Immunocytochemistry for Flii and αSMA was performed on primary fibroblasts cultured from Flii$^{+/-}$, WT and Flii$^{Tg/Tg}$ mice. As expected, there was increased Flii expression in WT fibroblasts compared to Flii$^{+/-}$ and in Flii$^{Tg/Tg}$ compared to WT and Flii$^{+/-}$(Figure 6.3). Flii was upregulated in all three genotypes when cells were cultured with 140μg/ml bleomycin (Figure 6.3). Treatment with FnAb had little effect on Flii expression as detected by immunocytochemistry (Figure 6.3). αSMA was upregulated in WT fibroblasts compared to Flii$^{+/-}$ and in Flii$^{Tg/Tg}$ compared to WT and Flii$^{+/-}$ (Figure 6.4). This trend was enhanced by culture with bleomycin. Treatment with FnAb reduced αSMA expression in both control and bleomycin groups across all genotypes (Figure 6.4).

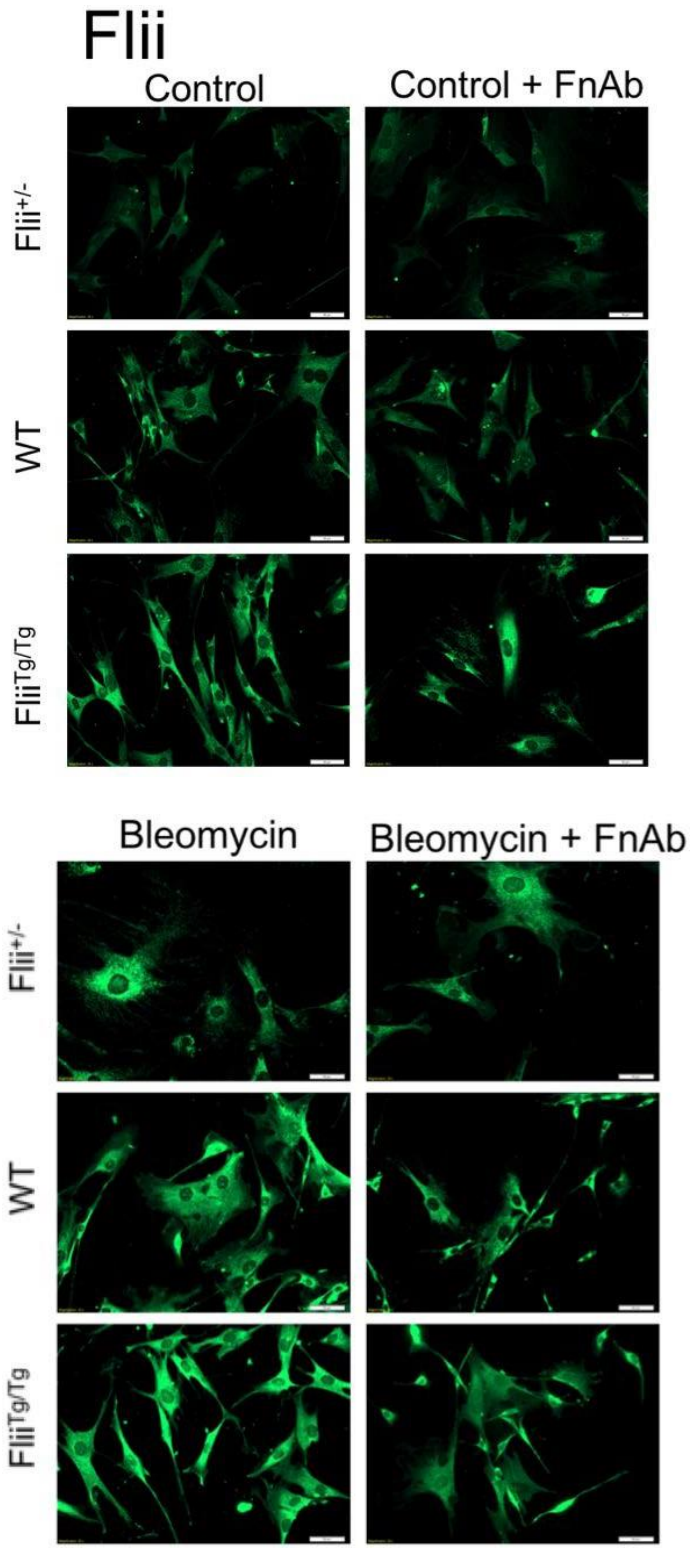


Figure 6.3 Treatment of primary fibroblasts from Flii$^{+/-}$, WT and Flii$^{Tg/Tg}$ mice with bleomycin and FnAb. Flii expression in three genotypes is increased after co-culture with bleomycin. There was no significant decrease of Flii expression in any genotype following addition of FnAb.

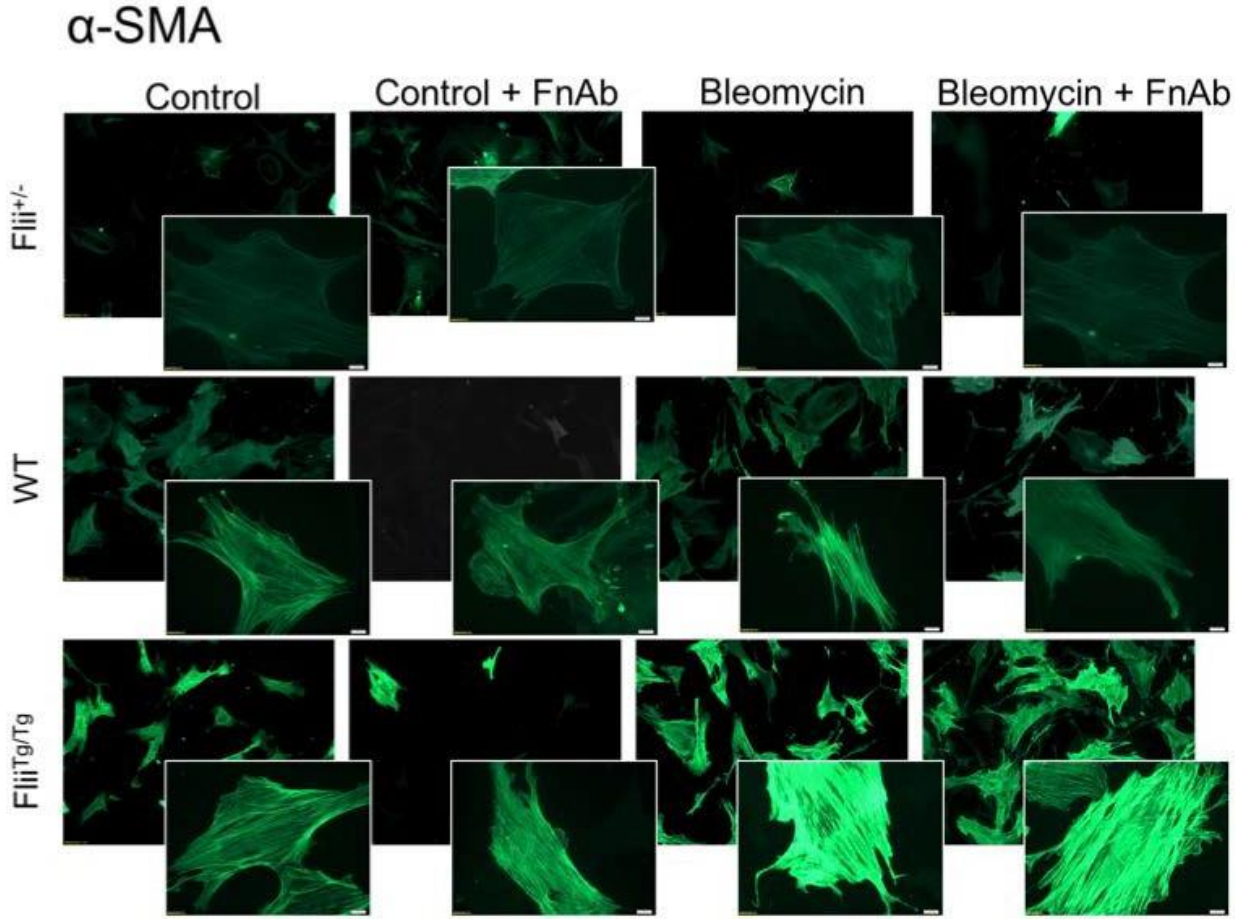


Figure 6.4 Treatment of primary fibroblasts from Flii$^{+/-}$, WT and Flii$^{Tg/Tg}$ mice with bleomycin and FnAb. αSMA expression in three genotypes is increased after addition of bleomycin. This increase in expression is reduced by co-culture with FnAb. Main images x20 magnification, inset images x 50 magnification of a single cell from main image.

Scratch migration assay was used to assess the motility of the primary fibroblasts. As shown in our previous work (Cowin et al., 2007), $Flii^{+/-}$ displayed increased motility compared to WT, while motility of $Flii^{Tg/Tg}$ compared to control was reduced. Culture with 140μg/ml bleomycin led to a significant decrease in motility of all genotypes compared to control. (Fig 6.5 (a)). Culture with 20μg/ml FnAb led to a significant increase in motility in $Flii^{Tg/Tg}$ fibroblasts across all time points and a significant increase in motility of WT fibroblasts from 0-12 hours. There was no significant increase in motility of $Flii^{+/-}$ fibroblasts with addition of FnAb (Fig. 6.5 (b)). No differences were observed when cells were cultured with murine irrelevant IgG (control).

$Flii^{+/-}$, WT and $Flii^{Tg/Tg}$ fibroblasts were then cultured with 140μg/ml bleomycin and treated with either 20μg/ml FnAb or control (20μg/ml murine irrelevant IgG). In all genotypes, the addition of FnAb increased the motility of bleomycin treated fibroblasts significantly. This treatment effect was most pronounced in $Flii^{Tg/Tg}$ and WT, with a smaller but still significant effect observed in WT fibroblasts (Fig 6.6 (a-c)).

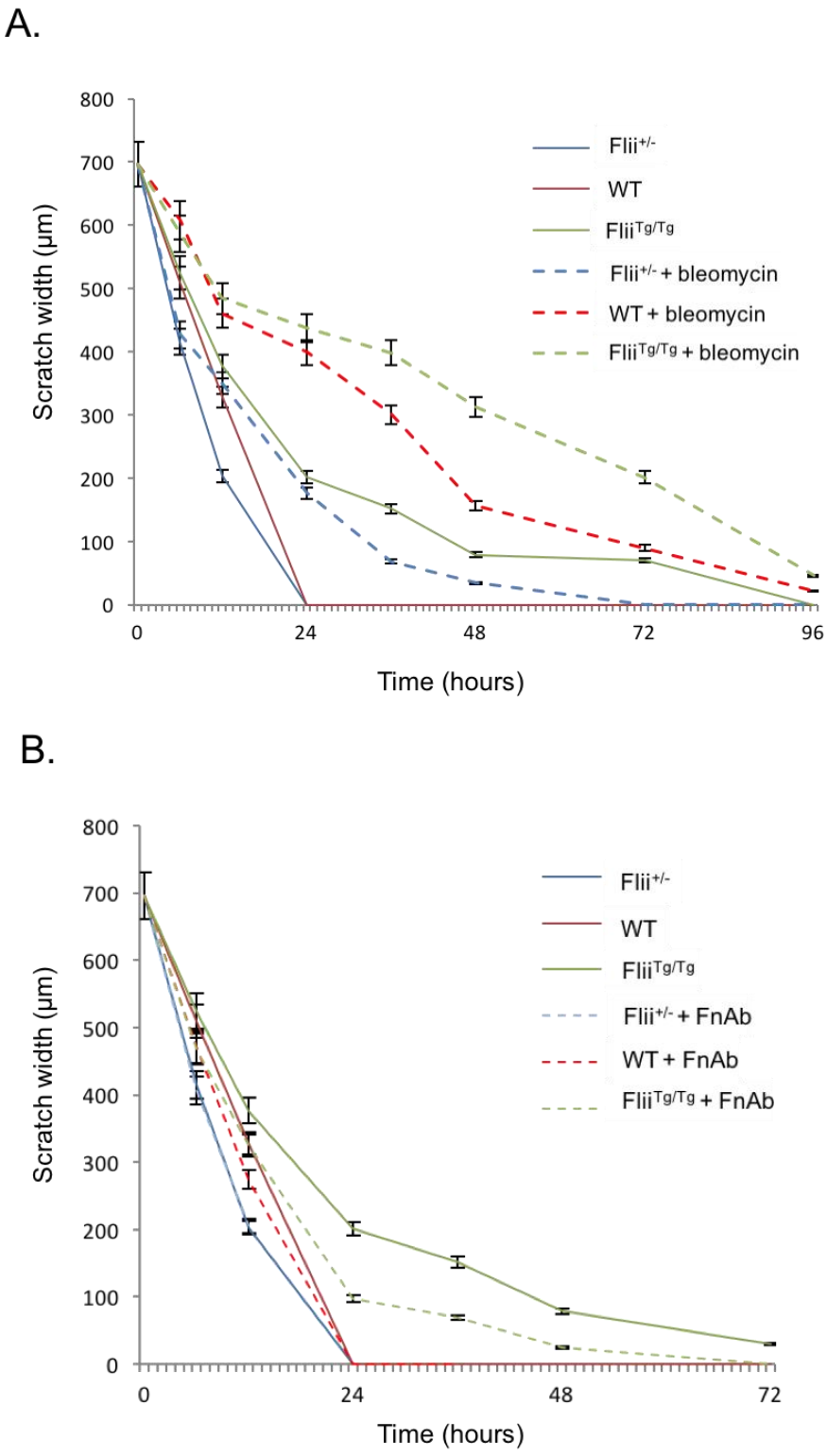


Figure 6.5 Effects of bleomycin and FnAb on motility of cultured primary Flii$^{+/-}$, WT and Flii$^{Tg/Tg}$ fibroblasts. (a) Treatment with bleomycin decreases the motility of Flii$^{+/-}$, WT and Flii$^{Tg/Tg}$ fibroblasts compared to control. (b) Addition of FnAb to primary fibroblast culture led to a significant increase in the motility of WT and Flii$^{Tg/Tg/-}$ but not Flii$^{+/-}$.

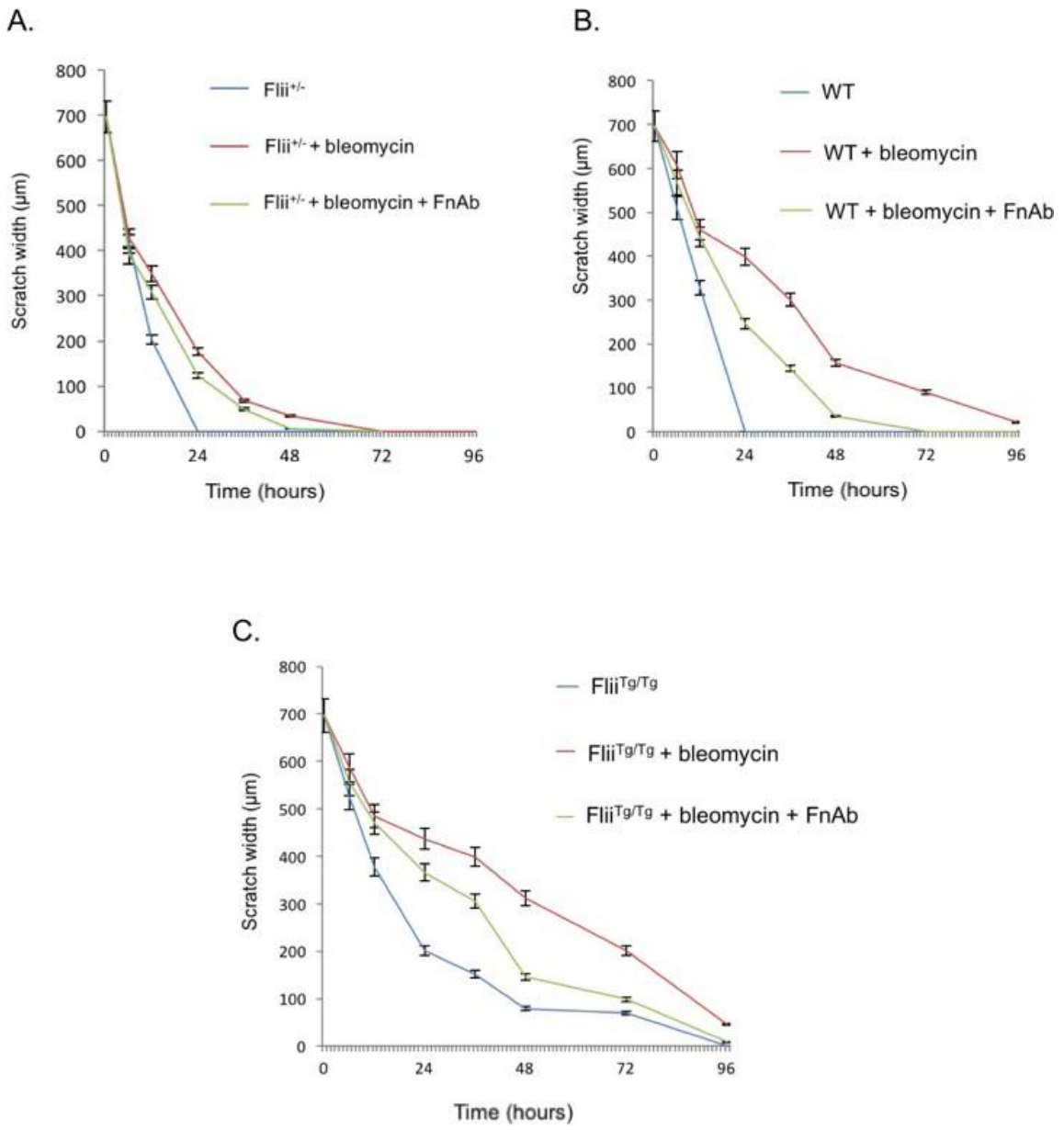


Figure 6.6 FnAb mediates the decreases in motility caused by treatment with bleomycin. In all genotypes, the addition of FnAb increased the motility of bleomycin treated fibroblasts significantly. This treatment effect was most pronounced in Flii$^{Tg/Tg}$ and WT, with a smaller but still significant effect observed in WT fibroblasts. (a) Flii$^{+/-}$ (b) WT (c) Flii $^{Tg/Tg}$

6.3 Discussion

Myofibroblasts are key cells involved in hypertrophic scarring (Junker et al., 2008). In addition to producing the collagenous extracellular matrix comprising the hypertrophic scar, myofibroblasts can also promote fibrosis by secreting angiogenic, pro-inflammatory and fibrogenic factors (Badid et al., 2000; Darby, 2007). Chapter 5 showed that scars in *Flii*$^{+/-}$ mice had significantly fewer myofibroblasts in the healing environment, suggesting that the anti-scarring effects of *Flii* may be mediated by an effect on myofibroblast differentiation, migration or survival. Given the importance of the fibroblast/myofibroblast to the scarring process, it was pertinent to focus our *in vitro* studies on the effect of Flii on these cells.

In keeping with this, the first section of this chapter shows that Flii expression is increased in fibroblasts cultured from human hypertrophic scars compared to human foreskin fibroblasts (Fig. 6.1).

In keeping with the animal model used in this thesis, bleomycin was then used to induce a myofibroblast phenotype, as previously described (Yamamoto et al., 2000). Fibroblasts cultured with bleomycin showed increased expression of αSMA, as well as decreased motility, characteristics of myofibroblasts. These cells also showed an increased expression of Flii and these changes could be partly prevented by treatment with FnAb (Fig. 6.2)

These trends were also evident when fibroblasts from Flii$^{+/-}$, WT and Flii$^{Tg/Tg}$ were cultured with bleomycin. Bleomycin treated cells showed increased Flii expression, increased αSMA and decreased motility. Treatment FnAb led to reduced αSMA and partially restored the migratory capacity observed in control groups. In future studies, image analysis and Western Blot protein analysis could be used to quantify immunohistochemical findings shown here, although time constraints did not permit this for this study.

There are a number of putative mechanisms via which Flii may modulate fibroblast-myofibroblast behavior. Actin dynamics are integral to the wound healing process. After wounding cells respond to a variety of stimuli to remodel their actin cytoskeleton, which brings about changes in cell contraction, adhesion, motility or phenotype. Ras proteins are involved in the regulation of the actin cytoskeleton where their interaction with Raf-1 connects it to the mitogen-activated protein kinase (MAPK) pathway (Goshima et al., 1999).

Flii can be induced by serum to translocate from the nucleus to leading edge of the cell, membrane ruffles and actin arcs where Flii colocalizes with Ras and GTPases (Davy et al., 2001).The co-localization of Flii with Ras is consistent with findings using kinetic analysis based on competitive inhibition of Ras-dependent adenylyl cyclase activity in which the results showed the association of the LRR domain of Flii with Ras (Davy et al., 2000; Goshima et al., 1999). Therefore, the interaction of Flii with Ras proteins provided a link to

the MAPK pathway. The connection of Flii to a signaling pathway is also supported by the interaction of Flii with the phosphoinositol-signaling pathway in the regulatory events during ovulation (Campbell, 1993)

There is also evidence to suggest that Flii may actually be secreted by fibroblasts and so may act in a similar manner to the secretory phenotype of gelsolin that scavenges extracellular actin following tissue injury.

The interaction between the actin cytoskeleton and the ECM occurs through highly dynamic, temporary adhesions known as *focal complexes*. These adhesions are formed by actin polymerization underneath lamellipodia. These have been described as a "molecular clutch" which connects the cytoskeleton to the matrix, thereby allowing actin-myosin generated tension to bring about cell traction (Le Clainche & Carlier, 2008). Focal complex function is a complex process determined by interaction between integrin-ECM, integrin-actin binding proteins and actin filament-actin binding protein interactions. In this way, focal complexes link the ECM to the actin cytoskeleton, essentially functioning as mechanosensors which can then influence maturation of adhesion sites and the recruitment of signaling or structural molecules (Lambrechts et al., 2004; Le Clainche & Carlier, 2008). Under certain conditions focal complexes become larger, more stable adhesions known as *focal adhesions*, which are associated with stress fibre development and fibroblast transformation to myofibroblasts (Hinz, 2010).

More recent work has shown that Flii modulates these focal adhesions and filamentous actin stress fibres in a Rac-1 dependent manner (Kopecki et al., 2011).

Flii co-precipitates with focal adhesion proteins talin, paxillin and vinculin (Kopecki et al., 2009). *In vivo*, elevated Flii leads to increased stress fibre formation by impaired focal adhesion turnover and enhanced formation of fibrillar adhesions. Conversely, Flii knockdown increases the percentage of focal complex positive cells (Kopecki et al., 2011). Flii knockdown cells exhibit lower levels of activated β1-integrin and vinculin, without any change in talin levels. This suggests that Flii may promote the recruitment of focal adhesion proteins like vinculin, which through its interaction affects β1-integrin activation (Mohammad et al., 2012). It is known that focal adhesions allow contractile activity of myofibroblasts to be transmitted to the ECM (Hinz et al., 2012), creating a more strained, and stiffer environment. These mechanical conditions generated by the myofibroblast also lead to their sustained fibroproliferative activity (Hinz et al., 2001). Reducing Flii may potentially alter cell focal adhesions and prevent this sustained myofibroblast activation.

Chapter 7

GENERAL DISCUSSION AND FUTURE DIRECTIONS

7.1 Discussion

Hypertrophic scarring represents an area of unmet clinical need. Occurring in more than 70% of deep burns, as well as after surgery or trauma it carries a considerable burden of disease including disfigurement, pain, disability and psychological co-morbidity (Brown et al., 2008).

Current therapies focus mainly on managing hypertrophic scars once they have developed. These interventions are limited in their efficacy and the majority lack strong evidence for their efficacy (Mustoe et al., 2002).

Improving our knowledge of the pathophysiology of hypertrophic scarring will contribute to a better understanding of the complex regulation of wound healing. This will potentially inform novel therapies to prevent or mitigate hypertrophic scarring. The work in this thesis focused on understanding the role of Flightless (Flii) in the fibroproliferative process that underlies hypertrophic scarring.

7.2 A role for Flightless in hypertrophic scarring and the development of a novel murine model of hypertrophic scarring

The human homologue of the Flii gene was identified by Campbell in 1997 (Campbell et al., 1997) and was initially investigated in relation to embryogenesis and its role as a hormone-activated nuclear receptor co-activator (Campbell et al.,

2002; Davy et al., 2000). In 2007, our laboratory demonstrated that Flii deficient mice had improved wound healing, with increased epithelial migration and improved cellular proliferation, whereas Flii over expressing mice had impaired wound healing (Cowin et al., 2007). Since that time, we have shown that Flii is a key regulator of the wound healing process, in vitro and in vivo studies of normal wound healing, burn injury, diabetic wound healing and skin blistering disease (Kopecki et al., 2009; Kopecki et al., 2011; Kopecki et al., 2013; Kopecki et al., 2013; Ruzehaji et al., 2013). This study marks the first time that Flii has been investigated in the fibroproliferative process that underlies hypertrophic scarring.

Chapter 3 showed, for the first time, that Flii is overexpressed in acute human burns and hypertrophic scars, suggesting a role for Flii in the pathogenesis of hypertrophic scarring (Cameron et al., 2016). Analysis of Flii expression in human samples comes with the caveat that these samples provide limited windows into the process of hypertrophic scar formation. In general, clinical practice dictates that only acute burns wounds (<14 days old) or mature hypertrophic scar are excised. Thus, little human tissue is available from the proliferative phase of wound healing, which is when the fibroproliferative process underlying hypertrophic scarring will be at its peak. Indeed, mature hypertrophic scars will have few cells at all in their dermis, tissue from acute burns wounds is usually so damaged that little architecture is preserved.

Furthermore, what actually qualifies as a hypertrophic scar is unclear and problematic for research using human samples. Hypertrophic scars were originally described by clinical characteristics (red, raised, painful scars prone to

contracture) (Herndon, 2012). Attempts were subsequently made to delineate a histological classification, via features such as parallel collagen fibres, collagen whorls and epidermal flattening (Lee et al., 2004). However, defining reliable histological characteristics to differentiate between normal scars, hypertrophic scars and keloid scars has proved difficult (Lee et al., 2004; Verhaegen et al., 2009). There is no consensus in the literature regarding the relative contribution of different collagen subtypes to hypertrophic scar fibrillogenesis, nor the importance or temporal profile of key cell types, such as myofibroblasts, or cytokines, such as TFG-β1 (Rawlins et al., 2006).

This confusion has hampered the development of an adequate animal model of hypertrophic scarring, with many models overly focused on reproducing the pathological endpoint of the process rather than modelling the process leading to this. By shifting focus from the pathological endpoint to the process by which this arises, hypertrophic scarring may be re-contextualised as a fibroproliferative disease, sharing a common mechanism with systemic scleroderma, lung fibrosis or glomerulosclerosis. This tenet provided the basis for the development of a novel model of hypertrophic scarring described in Chapter 4. Bleomycin had previously been used to model the fibroproliferation seen in lung fibrosis and scleroderma (Moseley et al., 1986; Yamamoto et al., 1999). By adapting the dosage, delivery and temporal characteristics of these models a novel model of the process of hypertrophic scarring was developed. This was based in the process of dermal fibroproliferation that underlies hypertrophic scarring, while also reproducing the morphological, histological and biochemical characteristics of human hypertrophic scars. Two time points (28 and 56 days) were chosen at which to

analyse the scars, in order to ensure the inflammatory and maturation phases of the wound healing process were captured.

The bleomycin model does not involve a breach of the epidermis. Rather an osmotic pump is placed in a subdermal pocket and bleomycin is leeched out to exert a direct effect on the murine dermis. A model of wound healing lacking an epidermal breach may appear controversial. Upon closer examination there are a number of reasons why this may be considered a strength. Foremost, there are already major differences between the anatomy and physiology of murine compared to human skin. Murine skin has denser hair with a shorter growth cycle than human hair while lacking dermal papillae and apocrine glands. Importantly the presence of the panniculus carnosus muscle layer, which is vestigial absent in humans, rapidly apposes murine wound edges after injury (Wong et al., 2011). The bleomycin model effectively excludes these potentially confounding mechanical and architectural differences and specifically targets dermal fibroproliferation. Indeed, during hypertrophic scarring dermal fibroproliferation is occurring long after the epidermis has healed(Cameron et al., 2010). The role of restoring epithelial integrity is undoubtedly of much interest in understanding the development of hypertrophic scarring; however, this would be best investigated using an alternative model.

Some may also question the validity of using bleomycin, a toxic substance, in our model. Bleomycin directly stimulates the pro-fibrotic TGF-β pathway, as well as other pro-inflammatory and pro-fibrotic mediators such as chemo-attractant protein 1, PDGF, Il-4, Il-6 and Il-13 (Yamamoto & Nishioka, 2005b). Bleomycin

thus causes scarring by direct activation of inflammatory and fibrotic mediators that have been shown to be important to the development of human hypertrophic scarring.

Any study employing animal models of human disease should always acknowledge the significant differences between that animal and the human phenotype. Indeed, recent evidence shows that genomic inflammatory responses in mouse models differ considerably from the genomic response seen in the corresponding human disease (Seok et al. 2013). In fact, the genes activated in the mouse model are close to random in matching the human disease genes. For this reason, murine models of human disease should always be viewed with some skepticism and only be considered in conjunction with in vivo and human data

7.3 Potential mechanisms for the role of Flii in hypertrophic scarring

7.3.1 TGF-β-Smad pathway

Chapter 5 demonstrated that an increased level of Flii in $Flii^{Tg/Tg}$ led to a significant increase in scarring in the murine model, while a reduced level of Flii in $Flii^{+/-}$ led to a significant decrease. This findings were also associated with increased and decreased levels of TGF-β1 respectively, suggesting that this effect may Flii may exert an effect on scarring via TGF-β1.

The TGF-β superfamily has been extensively investigated in relation to wound healing and scar formation (Wynn, 2008). The TGF-β-Smad signalling pathway is a central mediator in fibrogenic events such as chemotaxis, extracellular matrix synthesis and autocrine activation of growth factors (Pannu et al., 2007; Wilkes et al., 2005). TGF-β is integral to the ontogenetic shift from scarless foetal healing to adult scar forming wound healing in the third trimester (Chen et al., 2005). TGF-β signalling in fibroblasts has been shown to be important for production of extracellular matrix components, as well as regulating keratinocyte and the fibroblast to myofibroblast transition (Ong et al., 2009).

Flii has been shown to be a modulator of TGF/Smad signalling, while studies by the Cowin team have shown that Flii affects wound healing through modulation of TGF-β1 and TGF-β3 expression *in vivo* (Adams et al., 2009; Adams et al., 2008) (Figure 7.1). Reducing Flii gene expression via siRNA in *in vivo* wounds or *in vitro* fibroblasts reduced TGF-β1 levels while Flii overexpression led to upregulation of TGF-β1. Lim demonstrated that Flii functions as a coactivator in the expression of the type I collagen gene COL1A2 induced by TGF-β in cultured epithelial cells (Lim & Jeong, 2014). This suggested that Flii is required for TGF-β induced chromatin remodelling carried out by the SWI/SNF complex. TGF-β may also be involved in Flii interaction with extracellular matrix proteins, with Kopecki demonstrating that addition of TGF-β reduces the Flii association with Collagen VII (Kopecki et al., 2011). Thus, there are several possible mechanisms by which Flii could regulate wound healing through the TGF-β-Smad pathway. In this study, TGF-β1

was seen to be decreased concomitant with improved scarring across both the 28-day and 56-day model.

It has recently been shown that specifically blocking TGF-β1 mediated differentiation of myofibroblasts by preventing αv integrin activation of latent TGF-β prevents fibroproliferation in murine models of liver and lung fibrosis (Smith & Henderson, 2016). Blocking Flii may act in a similar manner via the TGF-β pathway to regulate myofibroblast function and reduce scarring.

Interestingly, Flii, like other gelsolin proteins is secreted in response to tissue injury and is likely to be a mediator of inflammation (Lin et al., 2011). Secretion of gelsolin family proteins into the circulation has been linked to functions in scavenging actin (Bucki et al., 2008) The FnAb has been shown to deplete Flii in conditioned media by over 90% (Lei et al., 2012). Thus, FnAb appears to "mop-up' this extracellular to bring about improved wound healing. Flii secretion has been characterized (Lei et al., 2012) but Flii cell surface receptors have not yet been identified. However, FnAb has been shown to be taken up into cells (Kopecki et al., 2013) so it may have wound healing effects in both the extracellular and intracellular milieus.

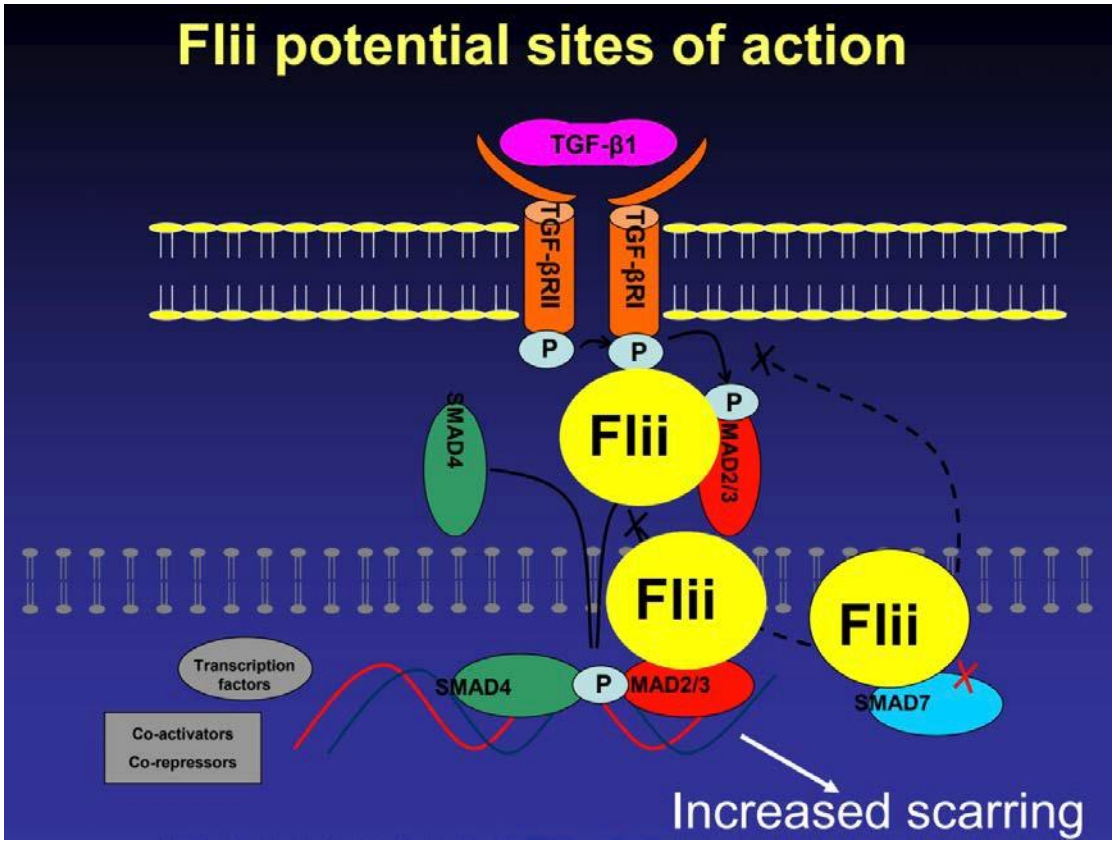


Figure 7.1. Schematic illustrating potential sites of Flii interaction with TGFβ-Smad pathway

7.3.2 Flii and other cell signaling pathways

Numerous pathways involved in wound healing evens such as cytoskeletal remodeling, cellular adhesion and migration have been identified including TGF-β. MAP kinase family, Ras, RhoA, MAPK-ERK kinase and ERK1/2 (Begum et al., 2004; Bhowmick et al., 2001; Hall & Nobes, 2000; Wang et al., 2005). Flii has both cytoskeletal and nuclear regulatory functions, and has been shown to co-localise with different molecules involved in the aforementioned pathways, such as Ras and Cdc42 (Campbell et al., 2002; Lee & Stallcup, 2006). The ability of Flii to bind both Ras and Cdc42 suggests it may be involved in both PI3K and MAPK signaling pathways. Kopecki demonstrated an effect of Flii on modulation of small GTPases, including Rac1, RhoA and Cdc42 suggesting that Flii could be involved in

mediating various signaling pathways through such interactions (Kopecki, O'Neill, et al., 2011). The effect of Flii on these pathways presents further possible mechanisms by which Flii may cause the effects on fibroproliferation demonstrated in this thesis.

7.3.3 Flii regulation of keratinocyte function

Immunohistochemistry of human burn samples in chapter 3 showed that Flii was seen predominately in the epidermis. This was also evident in murine model scars in Chapters 4 and 5, although significant dermal expression was shown presumably due to these scars being more representative of the inflammatory stage of wound healing. Clinicians have long understood the epidermis to be a key regulator of the wound healing process, with wounds that are slow to achieve epithelial coverage being more likely to undergo hypertrophic scarring (Herndon, 2012). In keeping with this, keratinocytes have been shown to regulate fibroblasts, the key cell in hypertrophic scarring, through secretion, activation and inhibition of growth factors such as TFG-β (Santoro & Gaudino, 2005). As our previous work has shown Flii's role in keratinocyte migration after wound repair (Cowin et al., 2007), regulation of TGF-β in burn wound healing (Adams et al., 2009) and key role in epithelial integrity (Kopecki et al., 2009) there is a potential role for Flii in this epidermal regulation of mesenchymal activity.

7.3.4 Flii and cytoskeletal regulation of the fibroblast

Migrating cells undergo a series of structural changes involving protrusions of the leading edge of the cell, adhesion of this leading edge to substrate then freeing of adhesions at the trailing edge before forward movement of the body of the cell (Lauffenburger & Horwitz, 1996). When cell adhesion forces are low, cells cannot generate forces necessary for forward movement. Similarly, when adhesion forces are high, cell movement may be slowed or impaired (Palecek et al., 1997).

Migration and adhesion represents the interaction between the actin cytoskeleton and the ECM. This occurs through focal complexes and adhesions, the "molecular clutch" of the cell. These are specialized cellular domains that connect the cell to its extracellular milieu, providing continuity between the extracellular matrix and actin filaments (Izzard & Lochner, 1976). Actin filaments undergo changes that enable formation of lamellipodia and formation of focal adhesions (Pollard & Borisy, 2003; Webb et al., 2004).

More than 100 actin binding proteins have been described, which bind to actin filaments and variably sever, bundle, cross-link in order to regulate actin structure, function and remodeling (dos Remedios et al., 2003). Flii is a member of a subset of these actin remodeling proteins, the gelsolin family, which includes adseverin, Flii, villin, advillin, supervillain and CapG (Sun et al., 1995).

Mohammad et al showed that Flii is a focal adhesion associated actin-capping protein that regulates cell migration (Mohammad et al., 2012). Flii associates with

actin filaments and actin monomers, inhibiting actin polymerization and capping but not severing actin filaments. Cells with less expression of Flii are less adherent than wild type, with reduced numbers of focal adhesions containing activated β1 integrins and vinculin and increased incorporation of actin monomers into new filaments at focal adhesions (Mohammad et al., 2012). In keeping with this, Flii knockdown enhanced cell migration, while overexpression reduced cell migration with increased numbers of focal adhesions (Kopecki et al., 2011). Kopecki showed that Flii inhibition of paxillin phosphorylation at Y118 site inhibits paxillin mediated focal adhesion disassembly. This also affects the Rac1-rhoA pathway, affecting adhesion and leading edge protrusion (Zvibel et al., 2008).

Focal adhesions in fibroblasts are associated with stress fibre development and the transition from fibroblast to myofibroblast (Hinz, 2010). As Flii has is associated with stress fibre formation and increased focal adhesion complexes, this presents a possible mechanism via which Flii regulates the formation and function of myofibroblasts, the key cell in hypertrophic scarring. It is known that focal adhesions allow contractile activity of myofibroblasts to be transmitted to the ECM (Hinz et al., 2012), creating a more strained, stiffer environment. These mechanical conditions generated by the myofibroblast also lead to their sustained fibroproliferative activity (Hinz et al., 2001). Indeed, scar development in a murine model of hypertrophic was prevented by disruption of Focal Adhesion Kinase (FAK) signalling pathway (Wong et al., 2012).

Chapter 6 focussed on this potential regulatory mechanism. Fibroblasts were cultured with bleomycin to induce a myofibroblast phenotype in a technique

previously described by Yamamato (Yamamoto et al., 2000). By using bleomycin this provided a single cell insight into our murine model of hypertrophic scarring.

As expected, fibroblasts cultured with bleomycin showed increased levels of αSMA and decreased motility, indicative of the myofibroblast phenotype. This was associated with an increase in Flii. Flii$^{+/-}$ mice showed increased motility and less αSMA expression, suggesting a partial restoration of normal fibroblast phenotype. Similar results were found when bleomycin treated cells were treated with FnAb. In future studies, image analysis and Western Blot protein analysis could be used to quantify immunocytochemical findings shown here, although time constraints did not permit this for this study.

A role for Flii in the regulation of how fibroblasts sense and respond to mechanical ECM forces is compelling evidence that Flii may be an important regulator of hypertrophic scarring. Clinicians have long been aware that wound tension is one of the most important risk factors for hypertrophic scarring (Gurtner et al., 2008). Wong et al. recently demonstrated that focal adhesion kinase (FAK) is activated by cutaneous injury and that this process is potentiated by mechanical loading. FAK acts through extra-cellular related kinase (ERK) to mechanically trigger monocyte chemoattractant protein-1 (MCP-1) which is a potent chemoattractant and pro-inflammatory mediator. Blocking FAK was shown to block this affect and reduce scarring in a mouse model by uncoupling physical force from the molecular pathway of scar formation(Wong et al., 2011). The evidence prevented in this thesis suggests that Flii could function in a similar manner and treatment to

reduce Flii activity could therefore interrupt the pathway between the physical stimuli for hypertrophic scarring and fibroproliferation (Figure 7.2).

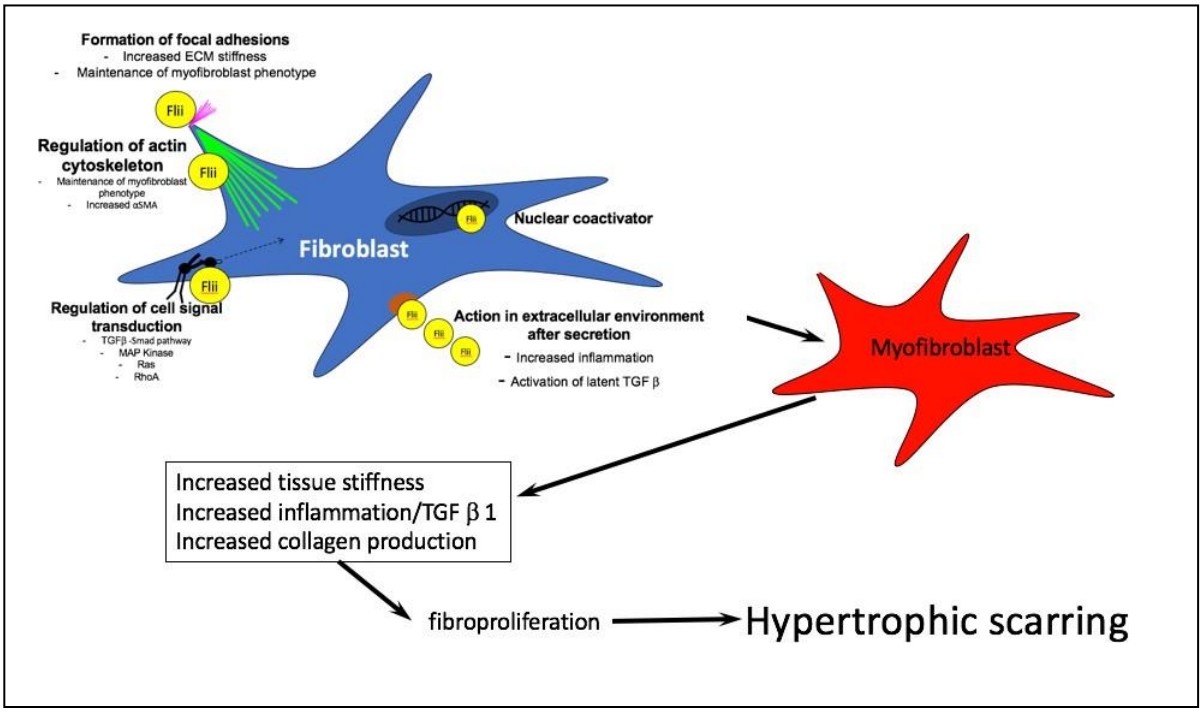


Figure 7.2 Schematic diagram summarizing potential mechanisms by which Flii may regulate the fibroproliferation underlying hypertrophic scarring

7.4 FnAb and potential as a novel therapy

Chapters 5 and 6 showed the efficacy of the FnAb monoclonal antibody therapy to reduce scarring and alter myofibroblast function in *in vivo* and *in vitro* respectively. Interestingly, Flii, like other gelsolin proteins is secreted in response to tissue injury and is likely to be a mediator of inflammation (Lin et al., 2011). Secretion of gelsolin family proteins into the circulation has been linked to functions in scavenging actin (Bucki et al., 2008) The FnAb has been shown to deplete Flii in conditioned media by over 90% (Lei et al., 2012). Thus, FnAb

appears to "mop-up' this extracellular to bring about improved wound healing. Flii secretion has been characterized (Lei et al., 2012) but Flii cell surface receptors have not yet been identified. However, FnAb has been shown to be taken up into cells (Kopecki et al., 2013) so its effects in this study may be explained by both intracellular and extracellular activity. Regardless, the FnAb has promise as a potential therapy in the clinical setting.

7.5 Future directions

This study is the first to identify a role for Flii in regulation of the fibroproliferation underlying hypertrophic scarring. Reducing Flii led to reduced scarring in an animal model of hypertrophic scarring, associated with significant changes in collagen-1:3 ratios, TGFβ expression and myofibroblast numbers. Flii exerts an effect on the fibroblast-myofibroblast transition, which may occur through its regulation of focal complex and focal adhesion dynamics. Further investigation of the details of this proposed mechanism would be of important regulator if hypertrophic scarring but it may also reveal other potential targets for novel therapies.

Fibroproliferation underlies many other human pathologies. The role of Flii in other skin and soft tissue fibrotic conditions such as Dupuytren's contracture or scleroderma is an area for further research. Fibroproliferative disease affecting other organs, such as cirrhosis, lung fibrosis and glomerulosclerosis would also be of interest. The success of many surgical procedures rely on wounds healing

efficiently with a minimum of scarring. When this does not occur, complications can occur, such as bowel adhesions, tendon adhesions or stiff joints. Further studies into Flii as a target for therapies to improving would healing to suit a given clinical scenario could be of great benefit for many types of surgery.

Investigation into hypertrophic scarring is hampered by the lack of suitable animal models. The bleomycin model validated by this study is useful for investigating the fibroproliferative process underlying hypertrophic scarring. However, further studies using larger animal models are necessary to confirm that these findings are applicable to the human milieu.

The Flii neutralizing antibody presents a potential therapy to reduced or prevent the development of hypertrophic scarring. Further work is required to investigate the safety and efficacy of this antibody for use in human subjects. In addition, a suitable delivery system will need to be designed.

7.6 Limitations of this study

This thesis was successful in establishing and validating a novel model of hypertrophic scarring by using bleomycin to stimulate fibroproliferation in a murine milieu. The strength of this model was its focus on the fibroproliferative pathway that underlies hypertrophic scarring in the human setting. However, it has limitations. Firstly, it has been shown that the murine response to inflammation, including that at a genomic level, is significantly different to that seen in humans (Seok J, 2013). As inflammation is a key event in hypertrophic scarring this presents a caveat for any conclusions drawn from a murine model. Secondly the model does not involve wounding of the epidermis/dermis as an initiation of the scarring process as would occur in the clinical setting. As mentioned, conclusions regarding the role of Flii in this study are limited to its role in the fibroproliferative process, rather than the entirety of hypertrophic scarring *per se*.

This animal model enabled use of knockout Flii $^{+/-}$ and transgenic Flii$^{Tg/Tg}$ murine models to investigate the effects of altering Flii levels on fibroproliferation. Histology and immunohistochemistry performed on tissue from these models provided the majority of data for this thesis. While these techniques were suitable in providing information on the structural and cellular make up of the tissue, the results would have been strengthened by the inclusion of different quantitative methods such as Western Blot protein analysis or PCR. Time limitations prevented this.

Chapter 6 comprised of preliminary *in vitro* investigation of the effect of Flii on fibroblast phenotype. Most of the data in this chapter was limited to qualitative immunohistochemistry and cell motility data. As above, these results would have been strengthened by the addition

of Western Blot protein analysis and PCR. Furthermore more work is required to further elucidate the mechanism by which Flii exerts it effect on fibroproliferation and whether the myofibroblast is indeed the key cell in this regard.

This thesis contained only a small amount of data derived from human samples. As detailed in this thesis, human skin represents an extremely unique wound healing environment. Indeed hypertrophic scarring is essentially a condition unique to humans. It is difficult to obtain human sample. Samples which are obtained are extremely heterogenous making significant findings difficult to obtain. Biopsy of human skin at various stages of scar formation or systemic measurements of Flii would provide more information about the role of Flii in hypertrophic scarring. Ethical approval was not possible for these measurements in this study. Ultimately studies in the human milieu will be necessary if treatments targeting Flii are developed.

7.7 Conclusions

The aim of this study was to investigate the potential role of Flii in the formation of hypertrophic scars. Flii had been previously shown to be involved in the regulation of normal wound healing, with reductions in Flii leading to wounds that healed more quickly with smaller scars. For the first time this study showed that reducing Flii improves hypertrophic scarring, in terms of scar architecture, TGFβ expression, collagen-1:3 ratio and myofibroblast numbers. This involved the development of a novel murine model of hypertrophic scarring, which allowed targeted investigation of the fibroproliferative stage of scar formation. *In vitro*

work showed that increasing Flii led to fibroblast expression of a myofibroblast

phenotype, with increased αSMA and decreased motility. This effect may be due to Flii role in actin cytoskeletal regulation and its effect on how fibroblasts sense and respond to physical stimuli. Reducing Flii in both *in vitro* and *in vivo* conditions reduced scarring and myofibroblast expression respectively. This confirms Flii as a potential target for a novel therapy to reduce or prevent hypertrophic scarring.

REFERENCES

Aarabi, S., Bhatt, K. A., Shi, Y., Paterno, J., Chang, E. I., Loh, S. A., . . . Gurtner, G. C. (2007). Mechanical load initiates hypertrophic scar formation through decreased cellular apoptosis. *FASEB J, 21*(12), 3250-3261. doi:10.1096/fj.07-8218com

Aarabi, S., Longaker, M. T., & Gurtner, G. C. (2007). Hypertrophic scar formation following burns and trauma: new approaches to treatment. *PLoS Med, 4*(9), e234. doi:10.1371/journal.pmed.0040234

Adams, D. H., Ruzehaji, N., Strudwick, X. L., Greenwood, J. E., Campbell, H. D., Arkell, R., & Cowin, A. J. (2009). Attenuation of Flightless I, an actin-remodelling protein, improves burn injury repair via modulation of transforming growth factor (TGF)-beta1 and TGF-beta3. *Br J Dermatol, 161*(2), 326-336. doi:10.1111/j.1365-2133.2009.09296.x

Adams, D. H., Strudwick, X. L., Kopecki, Z., Hooper-Jones, J. A., Matthaei, K. I., Campbell, H. D., . . . Cowin, A. J. (2008). Gender specific effects on the actin-remodelling protein Flightless I and TGF-beta1 contribute to impaired wound healing in aged skin. *Int J Biochem Cell Biol, 40*(8), 1555-1569. doi:10.1016/j.biocel.2007.11.024

Aggarwal, H., Saxena, A., Lubana, P. S., Mathur, R. K., & Jain, D. K. (2008). Treatment of keloids and hypertrophic scars using bleom. *J Cosmet Dermatol, 7*(1), 43-49. doi:10.1111/j.1473-2165.2008.00360.x

Alonso, L., & Fuchs, E. (2006). The hair cycle. *J Cell Sci, 119*(Pt 3), 391-393. doi:10.1242/jcs02793

Anzarut, A., Olson, J., Singh, P., Rowe, B. H., & Tredget, E. E. (2009). The effectiveness of pressure garment therapy for the prevention of abnormal scarring after burn injury: a meta-analysis. *J Plast Reconstr Aesthet Surg, 62*(1), 77-84. doi:10.1016/j.bjps.2007.10.052

Archer, S. K., Claudianos, C., & Campbell, H. D. (2005). Evolution of the gelsolin family of actin-binding proteins as novel transcriptional coactivators. *Bioessays, 27*(4), 388-396. doi:10.1002/bies.20200

Arnault, J. P., Peiffert, D., Latarche, C., Chassagne, J. F., Barbaud, A., & Schmutz, J. L. (2009). Keloids treated with postoperative Iridium 192* brachytherapy: a retrospective study. *J Eur Acad Dermatol Venereol, 23*(7), 807-813. doi:10.1111/j.1468-3083.2009.03190.x

Atiyeh, B. S., Costagliola, M., & Hayek, S. N. (2005). Keloid or hypertrophic scar: the controversy: review of the literature. *Ann Plast Surg, 54*(6), 676-680.

Atiyeh, B. S., Hayek, S. N., & Gunn, S. W. (2005). New technologies for burn wound closure and healing--review of the literature. *Burns, 31*(8), 944-956. doi:10.1016/j.burns.2005.08.023

Atkinson, J. A., McKenna, K. T., Barnett, A. G., McGrath, D. J., & Rudd, M. (2005). A randomized, controlled trial to determine the efficacy of paper tape in preventing hypertrophic scar formation in surgical incisions that traverse Langer's skin tension lines. *Plast Reconstr Surg, 116*(6), 1648-1656; discussion 1657-1648.

Badid, C., Mounier, N., Costa, A. M., & Desmouliere, A. (2000). Role of myofibroblasts during normal tissue repair and excessive scarring: interest of their assessment in nephropathies. *Histol Histopathol, 15*(1), 269-280.

Baskaran, H., Toner, M., Yarmush, M. L., & Berthiaume, F. (2001). Poloxamer-188 improves capillary blood flow and tissue viability in a cutaneous burn wound. *J Surg Res, 101*(1), 56-61. doi:10.1006/jsre.2001.6262

Baxter, R. M., Crowell, T. P., McCrann, M. E., Frew, E. M., & Gardner, H. (2005). Analysis of the tight skin (Tsk1/+) mouse as a model for testing antifibrotic agents. *Lab Invest, 85*(10), 1199-1209. doi:10.1038/labinvest.3700331

Bayat, A., McGrouther, D. A., & Ferguson, M. W. (2003). Skin scarring. *BMJ, 326*(7380), 88-92.

Bayat, A., Walter, J. M., Bock, O., Mrowietz, U., Ollier, W. E., & Ferguson, M. W. (2005). Genetic susceptibility to keloid disease: mutation screening of the TGFbeta3 gene. *Br J Plast Surg, 58*(7), 914-921. doi:10.1016/j.bjps.2005.04.009

Begum, R., Nur, E. K. M. S., & Zaman, M. A. (2004). The role of Rho GTPases in the regulation of the rearrangement of actin cytoskeleton and cell movement. *Exp Mol Med, 36*(4), 358-366. doi:10.1038/emm.2004.47

Berman, B., & Kaufman, J. (2002). Pilot study of the effect of postoperative imiquimod 5% cream on the recurrence rate of excised keloids. *J Am Acad Dermatol, 47*(4 Suppl), S209-211.

Bershadsky, A. D., Ballestrem, C., Carramusa, L., Zilberman, Y., Gilquin, B., Khochbin, S., . . . Kozlov, M. M. (2006). Assembly and mechanosensory function of focal adhesions: experiments and models. *Eur J Cell Biol, 85*(3-4), 165-173. doi:10.1016/j.ejcb.2005.11.001

Bhowmick, N. A., Zent, R., Ghiassi, M., McDonnell, M., & Moses, H. L. (2001). Integrin beta 1 signaling is necessary for transforming growth factor-beta activation of p38MAPK and epithelial plasticity. *J Biol Chem, 276*(50), 46707-46713. doi:10.1074/jbc.M106176200

Bianchi, M. E. (2007). DAMPs, PAMPs and alarmins: all we need to know about danger. *J Leukoc Biol, 81*(1), 1-5. doi:10.1189/jlb.0306164

Bielefeld, K. A., Amini-Nik, S., & Alman, B. A. (2013). Cutaneous wound healing: recruiting developmental pathways for regeneration. *Cell Mol Life Sci, 70*(12), 2059-2081. doi:10.1007/s00018-012-1152-9

Bochaton-Piallat, M. L., Gabbiani, G., & Hinz, B. (2016). The myofibroblast in wound healing and fibrosis: answered and unanswered questions. *F1000Res, 5*. doi:10.12688/f1000research.8190.1

Bodokh, I., & Brun, P. (1996). [Treatment of keloid with intralesional bleomycin]. *Ann Dermatol Venereol, 123*(12), 791-794.

Bombaro, K. M., Engrav, L. H., Carrougher, G. J., Wiechman, S. A., Faucher, L., Costa, B. A., . . . Honari, S. (2003). What is the prevalence of hypertrophic scarring following burns? *Burns, 29*(4), 299-302.

Brewin, M.P., Lister T.S., Prevention or treatment of hypertrophic burn scarring: A review of when and how to treat with the Pulsed Dye Laser. *Burns,* 40 (5), 797-804.

Brown, B. C., McKenna, S. P., Siddhi, K., McGrouther, D. A., & Bayat, A. (2008). The hidden cost of skin scars: quality of life after skin scarring. *J Plast Reconstr Aesthet Surg, 61*(9), 1049-1058. doi:10.1016/j.bjps.2008.03.020

Bruckner-Tuderman, L. (1999). Biology and pathology of the skin basement membrane zone. *Matrix Biol, 18*(1), 3-4.

Bucki, R., Byfield, F. J., Kulakowska, A., McCormick, M. E., Drozdowski, W., Namiot, Z., . . . Janmey, P. A. (2008). Extracellular gelsolin binds lipoteichoic acid and modulates cellular response to proinflammatory bacterial wall

components. *J Immunol, 181*(7), 4936-4944.

Butnaru, C. A., & Kanitakis, J. (2002). Structure of normal human skin. *Eur J Dermatol, 12*(6), II-IV.

Byrne, C., Hardman, M., & Nield, K. (2003). Covering the limb--formation of the integument. *J Anat, 202*(1), 113-123.

Cameron, A. A., DH; Greenwood, JE; Anderson PJ; Cowin AJ. (2013). A novel murine model of hypertrophic scarring using subcutaneous infusion of bleomycin. *Plastic and Reconstructive Surgery (in press)*((in press)), (in press).

Cameron, A. M., Turner, C. T., Adams, D. H., Jackson, J. E., Melville, E., Arkell, R. M., . . . Cowin, A. J. (2016). Flightless I is a key regulator of the fibroproliferative process in hypertrophic scarring and a target for a novel antiscarring therapy. *Br J Dermatol, 174*(4), 786-794. doi:10.1111/bjd.14263

Cameron, A. M. R., N.; Cowin, A.J. (2010). Burn wound management: A surgical perspective. *Wound Practice and Research, 18*(1), 5.

Campbell, H. D., Fountain, S., McLennan, I. S., Berven, L. A., Crouch, M. F., Davy, D. A., . . . Matthaei, K. I. (2002). Fliih, a gelsolin-related cytoskeletal regulator essential for early mammalian embryonic development. *Mol Cell Biol, 22*(10), 3518-3526.

Campbell, H. D., Fountain, S., Young, I. G., Claudianos, C., Hoheisel, J. D., Chen, K. S., & Lupski, J. R. (1997). Genomic structure, evolution, and expression of human FLII, a gelsolin and leucine-rich-repeat family member: overlap with LLGL. *Genomics, 42*(1), 46-54. doi:10.1006/geno.1997.4709

Campbell, H. D., Schimansky, T., Claudianos, C., Ozsarac, N., Kasprzak, A. B., Cotsell, J. N., . . . Miklos, G. L. (1993). The Drosophila melanogaster flightless-I gene involved in gastrulation and muscle degeneration encodes gelsolin-like and leucine-rich repeat domains and is conserved in Caenorhabditis elegans and humans. *Proc Natl Acad Sci U S A, 90*(23), 11386-11390.

Capetanaki, Y., Bloch, R. J., Kouloumenta, A., Mavroidis, M., & Psarras, S. (2007). Muscle intermediate filaments and their links to membranes and membranous organelles. *Exp Cell Res, 313*(10), 2063-2076. doi:10.1016/j.yexcr.2007.03.033

Carlson, M. A., & Longaker, M. T. (2004). The fibroblast-populated collagen matrix as a model of wound healing: a review of the evidence. *Wound Repair Regen, 12*(2), 134-147. doi:10.1111/j.1067-1927.2004.012208.x

Chen, L., Li, J., Li, Q., Yan, H., Zhou, B., Gao, Y., & Li, J. (2017). Non-Coding RNAs: The New Insight on Hypertrophic Scar. *J Cell Biochem, 118*(8), 1965-1968. doi:10.1002/jcb.25873

Chen, W., Fu, X., Ge, S., Sun, T., Zhou, G., Jiang, D., & Sheng, Z. (2005). Ontogeny of expression of transforming growth factor-beta and its receptors and their possible relationship with scarless healing in human fetal skin. *Wound Repair Regen, 13*(1), 68-75. doi:10.1111/j.1067-1927.2005.130109.x

Chong, H. T., Yang, G. N., Sidhu, S., Ibbetson, J., Kopecki, Z., & Cowin, A. J. (2017). Reducing Flightless I expression decreases severity of psoriasis in an imiquimod-induced murine model of psoriasiform dermatitis. *Br J Dermatol, 176*(3), 705-712. doi:10.1111/bjd.14842

Christner, P. J., Peters, J., Hawkins, D., Siracusa, L. D., & Jimenez, S. A. (1995). The tight skin 2 mouse. An animal model of scleroderma displaying cutaneous

fibrosis and mononuclear cell infiltration. *Arthritis Rheum, 38*(12), 1791-1798.

Claudianos, C., & Campbell, H. D. (1995). The novel flightless-I gene brings together two gene families, actin-binding proteins related to gelsolin and leucine-rich-repeat proteins involved in Ras signal transduction. *Mol Biol Evol, 12*(3), 405-414.

Cohen, I. K., & Diegelmann, R. F. (1977). The biology of keloid and hypertrophic scar and the influence of corticosteroids. *Clin Plast Surg, 4*(2), 297-299.

Coleman, S. R. (2006). Structural fat grafting: more than a permanent filler. *Plast Reconstr Surg, 118*(3 Suppl), 108S-120S. doi:10.1097/01.prs.0000234610.81672.e7

Copcu, E., Sivrioglu, N., & Oztan, Y. (2004). Combination of surgery and intralesional verapamil injection in the treatment of the keloid. *J Burn Care Rehabil, 25*(1), 1-7. doi:10.1097/01.BCR.0000105097.36706.5D

Cowin, A. J., Adams, D. H., Strudwick, X. L., Chan, H., Hooper, J. A., Sander, G. R., . . . Campbell, H. D. (2007). Flightless I deficiency enhances wound repair by increasing cell migration and proliferation. *J Pathol, 211*(5), 572-581. doi:10.1002/path.2143

Cowin, A. J., Hatzirodos, N., Teusner, J. T., & Belford, D. A. (2003). Differential effect of wounding on actin and its associated proteins, paxillin and gelsolin, in fetal skin explants. *J Invest Dermatol, 120*(6), 1118-1129. doi:10.1046/j.1523-1747.2003.12231.x

Cowin, A. J., Lei, N., Franken, L., Ruzehaji, N., Offenhauser, C., Kopecki, Z., & Murray, R. Z. (2012). Lysosomal secretion of Flightless I upon injury has the potential to alter inflammation. *Commun Integr Biol, 5*(6), 546-549. doi:10.4161/cib.21928

Cunningham, C. C., Stossel, T. P., & Kwiatkowski, D. J. (1991). Enhanced motility in NIH 3T3 fibroblasts that overexpress gelsolin. *Science, 251*(4998), 1233-1236.

Daher, Z., Noel, J., & Claing, A. (2008). Endothelin-1 promotes migration of endothelial cells through the activation of ARF6 and the regulation of FAK activity. *Cell Signal, 20*(12), 2256-2265. doi:10.1016/j.cellsig.2008.08.021

Dai, P., Jeong, S. Y., Yu, Y., Leng, T., Wu, W., Xie, L., & Chen, X. (2009). Modulation of TLR signaling by multiple MyD88-interacting partners including leucine-rich repeat Fli-I-interacting proteins. *J Immunol, 182*(6), 3450-3460. doi:10.4049/jimmunol.0802260

Dale, P. D., Sherratt, J. A., & Maini, P. K. (1996). A mathematical model for collagen fibre formation during foetal and adult dermal wound healing. *Proc Biol Sci, 263*(1370), 653-660. doi:10.1098/rspb.1996.0098

Dang, N., & Murrell, D. F. (2008). Mutation analysis and characterization of COL7A1 mutations in dystrophic epidermolysis bullosa. *Exp Dermatol, 17*(7), 553-568. doi:10.1111/j.1600-0625.2008.00723.x

Darby, I. A., & Hewitson, T. D. (2007). Fibroblast differentiation in wound healing and fibrosis. *Int Rev Cytol, 257*, 143-179. doi:10.1016/S0074-7696(07)57004-X

Darby, M. (2007). Debate: a teaching-learning strategy for developing competence in communication and critical thinking. *J Dent Hyg, 81*(4), 78.

Davison, S. P., Dayan, J. H., Clemens, M. W., Sonni, S., Wang, A., & Crane, A. (2009). Efficacy of intralesional 5-fluorouracil and triamcinolone in the treatment of keloids. *Aesthet Surg J, 29*(1), 40-46. doi:10.1016/j.asj.2008.11.006

Davy, D. A., Ball, E. E., Matthaei, K. I., Campbell, H. D., & Crouch, M. F. (2000). The flightless I protein localizes to actin-based structures during embryonic development. *Immunol Cell Biol, 78*(4), 423-429. doi:10.1046/j.1440-1711.2000.00926.x

Davy, D. A., Campbell, H. D., Fountain, S., de Jong, D., & Crouch, M. F. (2001). The flightless I protein colocalizes with actin- and microtubule-based structures in motile Swiss 3T3 fibroblasts: evidence for the involvement of PI 3-kinase and Ras-related small GTPases. *J Cell Sci, 114*(Pt 3), 549-562.

Deitch E.A., Wheelahan T.M., Rose M.P., Clothier., Cotter., (1983) Hypertrophic burn scars: analysis of variables. *J Trauma* (23) 895-8.

Desmouliere, A., Chaponnier, C., & Gabbiani, G. (2005). Tissue repair, contraction, and the myofibroblast. *Wound Repair Regen, 13*(1), 7-12. doi:10.1111/j.1067-1927.2005.130102.x

Despa, F., Orgill, D. P., Neuwalder, J., & Lee, R. C. (2005). The relative thermal stability of tissue macromolecules and cellular structure in burn injury. *Burns, 31*(5), 568-577. doi:10.1016/j.burns.2005.01.015

Discher, D. E., Janmey, P., & Wang, Y. L. (2005). Tissue cells feel and respond to the stiffness of their substrate. *Science, 310*(5751), 1139-1143. doi:10.1126/science.1116995

dos Remedios, C. G., Chhabra, D., Kekic, M., Dedova, I. V., Tsubakihara, M., Berry, D. A., & Nosworthy, N. J. (2003). Actin binding proteins: regulation of cytoskeletal microfilaments. *Physiol Rev, 83*(2), 433-473. doi:10.1152/physrev.00026.2002

Dos Remedios, C. G., Liew, C. C., Allen, P. D., Winslow, R. L., Van Eyk, J. E., & Dunn, M. J. (2003). Genomics, proteomics and bioinformatics of human heart failure. *J Muscle Res Cell Motil, 24*(4-6), 251-260.

Doucet, C., Brouty-Boye, D., Pottin-Clemenceau, C., Canonica, G. W., Jasmin, C., & Azzarone, B. (1998). Interleukin (IL) 4 and IL-13 act on human lung fibroblasts. Implication in asthma. *J Clin Invest, 101*(10), 2129-2139. doi:10.1172/JCI741

Duffield, J. S., Forbes, S. J., Constandinou, C. M., Clay, S., Partolina, M., Vuthoori, S., . . . Iredale, J. P. (2005). Selective depletion of macrophages reveals distinct, opposing roles during liver injury and repair. *J Clin Invest, 115*(1), 56-65. doi:10.1172/JCI22675

Dugina, V., Fontao, L., Chaponnier, C., Vasiliev, J., & Gabbiani, G. (2001). Focal adhesion features during myofibroblastic differentiation are controlled by intracellular and extracellular factors. *J Cell Sci, 114*(Pt 18), 3285-3296.

Elsea, S. H., & Girirajan, S. (2008). Smith-Magenis syndrome. *Eur J Hum Genet, 16*(4), 412-421. doi:10.1038/sj.ejhg.5202009

Engrav, L. H., Garner, W. L., & Tredget, E. E. (2007). Hypertrophic scar, wound contraction and hyper-hypopigmentation. *J Burn Care Res, 28*(4), 593-597.

Escamez, M. J., Garcia, M., Larcher, F., Meana, A., Munoz, E., Jorcano, J. L., & Del Rio, M. (2004). An in vivo model of wound healing in genetically modified skin-humanized mice. *J Invest Dermatol, 123*(6), 1182-1191. doi:10.1111/j.0022-202X.2004.23473.x

Falanga, V. (2005). Wound healing and its impairment in the diabetic foot. *Lancet, 366*(9498), 1736-1743. doi:10.1016/S0140-6736(05)67700-8

Fleischmajer, R., Perlish, J. S., Burgeson, R. E., Shaikh-Bahai, F., & Timpl, R. (1990). Type I and type III collagen interactions during fibrillogenesis. *Ann N Y Acad Sci, 580*, 161-175.

Finnerty C.F., Jeschke M.G., Branksi L.K., Barret J.P., Dziewulski P., Herndon D.N., (2016) Hypertrophic scarring: the greatest unmet challenge following burn injury. Lancet, Oct 388 (10052)

Franz, M. G., Kuhn, M. A., Wright, T. E., Wachtel, T. L., & Robson, M. C. (2000). Use of the wound healing trajectory as an outcome determinant for acute wound healing. *Wound Repair Regen, 8*(6), 511-516.

Franz, M. G., Steed, D. L., & Robson, M. C. (2007). Optimizing healing of the acute wound by minimizing complications. *Curr Probl Surg, 44*(11), 691-763. doi:10.1067/j.cpsurg.2007.07.001

Gabbiani, G., Ryan, G. B., & Majne, G. (1971). Presence of modified fibroblasts in granulation tissue and their possible role in wound contraction. *Experientia, 27*(5), 549-550.

Galiano, R. D., Michaels, J. t., Dobryansky, M., Levine, J. P., & Gurtner, G. C. (2004). Quantitative and reproducible murine model of excisional wound healing. *Wound Repair Regen, 12*(4), 485-492. doi:10.1111/j.1067-1927.2004.12404.x

Georges, P. C., Hui, J. J., Gombos, Z., McCormick, M. E., Wang, A. Y., Uemura, M., . . . Wells, R. G. (2007). Increased stiffness of the rat liver precedes matrix deposition: implications for fibrosis. *Am J Physiol Gastrointest Liver Physiol, 293*(6), G1147-1154. doi:10.1152/ajpgi.00032.2007

Girirajan, S., Vlangos, C. N., Szomju, B. B., Edelman, E., Trevors, C. D., Dupuis, L., . . . Elsea, S. H. (2006). Genotype-phenotype correlation in Smith-Magenis syndrome: evidence that multiple genes in 17p11.2 contribute to the clinical spectrum. *Genet Med, 8*(7), 417-427. doi:10.109701.gim.0000228215.32110.89

Goffin, J. M., Pittet, P., Csucs, G., Lussi, J. W., Meister, J. J., & Hinz, B. (2006). Focal adhesion size controls tension-dependent recruitment of alpha-smooth muscle actin to stress fibers. *J Cell Biol, 172*(2), 259-268. doi:10.1083/jcb.200506179

Goldman, R. D., Grin, B., Mendez, M. G., & Kuczmarski, E. R. (2008). Intermediate filaments: versatile building blocks of cell structure. *Curr Opin Cell Biol, 20*(1), 28-34. doi:10.1016/j.ceb.2007.11.003

Goodwin, M. N., Silverstein, P., Raulston, G. L., & Eatherly, J. (1976). Hypertrophic Dermal Scar - Study of Porcine Model. *Laboratory Investigation, 34*(3), 339-339.

Gordon, S. (2003). Alternative activation of macrophages. *Nat Rev Immunol, 3*(1), 23-35. doi:10.1038/nri978

Goshima, M., Kariya, K., Yamawaki-Kataoka, Y., Okada, T., Shibatohge, M., Shima, F., . . . Kataoka, T. (1999). Characterization of a novel Ras-binding protein Ce-FLI-1 comprising leucine-rich repeats and gelsolin-like domains. *Biochem Biophys Res Commun, 257*(1), 111-116.

Grose, R., & Werner, S. (2004). Wound-healing studies in transgenic and knockout mice. *Mol Biotechnol, 28*(2), 147-166. doi:10.1385/MB:28:2:147

Gurtner, G. C., Werner, S., Barrandon, Y., & Longaker, M. T. (2008). Wound repair and regeneration. *Nature, 453*(7193), 314-321. doi:10.1038/nature07039

Guyot, C., Lepreux, S., Combe, C., Doudnikoff, E., Bioulac-Sage, P., Balabaud, C., & Desmouliere, A. (2006). Hepatic fibrosis and cirrhosis: the

(myo)fibroblastic cell subpopulations involved. *Int J Biochem Cell Biol, 38*(2), 135-151. doi:10.1016/j.biocel.2005.08.021

Habif, T. P. (2004). *Clinical dermatology : a color guide to diagnosis and therapy* (4th ed.). Edinburgh ; New York: Mosby.

Haedersdal, M., Bech-Thomsen, N., Poulsen, T., & Wulf, H. C. (1998). Ultraviolet exposure influences laser-induced wounds, scars, and hyperpigmentation: a murine study. *Plast Reconstr Surg, 101*(5), 1315-1322.

Hall, A., & Nobes, C. D. (2000). Rho GTPases: molecular switches that control the organization and dynamics of the actin cytoskeleton. *Philos Trans R Soc Lond B Biol Sci, 355*(1399), 965-970. doi:10.1098/rstb.2000.0632

Hantash, B. M., Bedi, V. P., Chan, K. F., & Zachary, C. B. (2007). Ex vivo histological characterization of a novel ablative fractional resurfacing device. *Lasers Surg Med, 39*(2), 87-95. doi:10.1002/lsm.20405

Hantash, B. M., Bedi, V. P., Kapadia, B., Rahman, Z., Jiang, K., Tanner, H., . . . Zachary, C. B. (2007). In vivo histological evaluation of a novel ablative fractional resurfacing device. *Lasers Surg Med, 39*(2), 96-107. doi:10.1002/lsm.20468

Hantash, B. M., & Mahmood, M. B. (2007). Fractional photothermolysis: a novel aesthetic laser surgery modality. *Dermatol Surg, 33*(5), 525-534. doi:10.1111/j.1524-4725.2007.33110.x

Harunari, N., Zhu, K. Q., Armendariz, R. T., Deubner, H., Muangman, P., Carrougher, G. J., . . . Engrav, L. H. (2006). Histology of the thick scar on the female, red Duroc pig: final similarities to human hypertrophic scar. *Burns, 32*(6), 669-677. doi:10.1016/j.burns.2006.03.015

Hayakawa, T., Hashimoto, Y., Myokei, Y., Aoyama, H., & Izawa, Y. (1982). The effect of skin grafts on the ratio of collagen types in human post-burn wound tissues. *Connect Tissue Res, 9*(4), 249-252.

Hayashi, T., Funato, Y., Terabayashi, T., Morinaka, A., Sakamoto, R., Ichise, H., . . . Miki, H. (2010). Nucleoredoxin negatively regulates Toll-like receptor 4 signaling via recruitment of flightless-I to myeloid differentiation primary response gene (88). *J Biol Chem, 285*(24), 18586-18593. doi:10.1074/jbc.M110.106468

Herndon, D. N. (2012). *Total burn care* (4th ed.). Edinburgh ; New York: Saunders Elsevier.

Heslop, J. (1998). The Murray Clarke Oration: a brief history of burn treatment and the contribution of four New Zealand pioneers of plastic surgery. *Aust N Z J Surg, 68*(10), 746-751.

Hierner, R., Degreef, H., Vranckx, J. J., Garmyn, M., Massage, P., & van Brussel, M. (2005). Skin grafting and wound healing-the "dermato-plastic team approach". *Clin Dermatol, 23*(4), 343-352. doi:10.1016/j.clindermatol.2004.07.028

Hinz, B. (2009). Tissue stiffness, latent TGF-beta1 activation, and mechanical signal transduction: implications for the pathogenesis and treatment of fibrosis. *Curr Rheumatol Rep, 11*(2), 120-126.

Hinz, B. (2010). The myofibroblast: paradigm for a mechanically active cell. *J Biomech, 43*(1), 146-155. doi:10.1016/j.jbiomech.2009.09.020

Hinz, B., & Gabbiani, G. (2010). Fibrosis: recent advances in myofibroblast biology and new therapeutic perspectives. *F1000 Biol Rep, 2*, 78. doi:10.3410/B2-78

Hinz, B., Mastrangelo, D., Iselin, C. E., Chaponnier, C., & Gabbiani, G. (2001). Mechanical tension controls granulation tissue contractile activity and myofibroblast differentiation. *Am J Pathol, 159*(3), 1009-1020. doi:10.1016/S0002-9440(10)61776-2

Hinz, B., Phan, S. H., Thannickal, V. J., Galli, A., Bochaton-Piallat, M. L., & Gabbiani, G. (2007). The myofibroblast: one function, multiple origins. *Am J Pathol, 170*(6), 1807-1816. doi:10.2353/ajpath.2007.070112

Hinz, B., Phan, S. H., Thannickal, V. J., Prunotto, M., Desmouliere, A., Varga, J., . . . Gabbiani, G. (2012). Recent developments in myofibroblast biology: paradigms for connective tissue remodeling. *Am J Pathol, 180*(4), 1340-1355. doi:10.1016/j.ajpath.2012.02.004

Holzbaur, E. L. (2004). Motor neurons rely on motor proteins. *Trends Cell Biol, 14*(5), 233-240. doi:10.1016/j.tcb.2004.03.009

Hotulainen, P., & Lappalainen, P. (2006). Stress fibers are generated by two distinct actin assembly mechanisms in motile cells. *J Cell Biol, 173*(3), 383-394. doi:10.1083/jcb.200511093

Hynes, R. O. (2002). Integrins: bidirectional, allosteric signaling machines. *Cell, 110*(6), 673-687.

Issa, R., Zhou, X., Constandinou, C. M., Fallowfield, J., Millward-Sadler, H., Gaca, M. D., . . . Iredale, J. P. (2004). Spontaneous recovery from micronodular cirrhosis: evidence for incomplete resolution associated with matrix cross-linking. *Gastroenterology, 126*(7), 1795-1808.

Izzard, C. S., & Lochner, L. R. (1976). Cell-to-substrate contacts in living fibroblasts: an interference reflexion study with an evaluation of the technique. *J Cell Sci, 21*(1), 129-159.

Jacinto, A., & Wolpert, L. (2001). Filopodia. *Curr Biol, 11*(16), R634.

Jackson, D. M. (1953). [The diagnosis of the depth of burning]. *Br J Surg, 40*(164), 588-596.

Jackson, J. E., Kopecki, Z., Adams, D. H., & Cowin, A. J. (2012). Flii neutralizing antibodies improve wound healing in porcine preclinical studies. *Wound Repair Regen, 20*(4), 523-536. doi:10.1111/j.1524-475X.2012.00802.x

Janzekovic, Z. (1970). A new concept in the early excision and immediate grafting of burns. *J Trauma, 10*(12), 1103-1108.

Janzekovic, Z. (2008). Once upon a time ... how west discovered east. *J Plast Reconstr Aesthet Surg, 61*(3), 240-244. doi:10.1016/j.bjps.2008.01.001

Jordan, R. B., Daher, J., & Wasil, K. (2000). Splints and scar management for acute and reconstructive burn care. *Clin Plast Surg, 27*(1), 71-85.

Joseph, J., & Dyson, M. (1966). Effect of anabolic androgens on tissue replacement in the ear of the rabbit. *Nature, 211*(5045), 193-194.

Junker, J. P., Kratz, C., Tollback, A., & Kratz, G. (2008). Mechanical tension stimulates the transdifferentiation of fibroblasts into myofibroblasts in human burn scars. *Burns, 34*(7), 942-946. doi:10.1016/j.burns.2008.01.010

Kamp, D. W. (2003). Idiopathic pulmonary fibrosis: the inflammation hypothesis revisited. *Chest, 124*(4), 1187-1190.

Kanitakis, J. (2002). Anatomy, histology and immunohistochemistry of normal human skin. *Eur J Dermatol, 12*(4), 390-399; quiz 400-391.

Karsdal, M. A., Nielsen, M. J., Sand, J. M., Henriksen, K., Genovese, F., Bay-Jensen, A. C., . . . Leeming, D. J. (2013). Extracellular matrix remodeling: the common

denominator in connective tissue diseases. Possibilities for evaluation and current understanding of the matrix as more than a passive architecture, but a key player in tissue failure. *Assay Drug Dev Technol, 11*(2), 70-92. doi:10.1089/adt.2012.474

Ketchum, L. D., Cohen, I. K., & Masters, F. W. (1974). Hypertrophic scars and keloids. A collective review. *Plast Reconstr Surg, 53*(2), 140-154.

Kim, K. K., Kugler, M. C., Wolters, P. J., Robillard, L., Galvez, M. G., Brumwell, A. N., . . . Chapman, H. A. (2006). Alveolar epithelial cell mesenchymal transition develops in vivo during pulmonary fibrosis and is regulated by the extracellular matrix. *Proc Natl Acad Sci U S A, 103*(35), 13180-13185. doi:10.1073/pnas.0605669103

Kischer, C. W., Pindur, J., Shetlar, M. R., & Shetlar, C. L. (1989). Implants of hypertrophic scars and keloids into the nude (athymic) mouse: viability and morphology. *J Trauma, 29*(5), 672-677.

Kobe, B., & Kajava, A. V. (2001). The leucine-rich repeat as a protein recognition motif. *Curr Opin Struct Biol, 11*(6), 725-732.

Kopecki, Z., Arkell, R., Powell, B. C., & Cowin, A. J. (2009). Flightless I regulates hemidesmosome formation and integrin-mediated cellular adhesion and migration during wound repair. *J Invest Dermatol, 129*(8), 2031-2045. doi:10.1038/jid.2008.461

Kopecki, Z., Arkell, R. M., Strudwick, X. L., Hirose, M., Ludwig, R. J., Kern, J. S., . . . Cowin, A. J. (2011). Overexpression of the Flii gene increases dermal-epidermal blistering in an autoimmune ColVII mouse model of epidermolysis bullosa acquisita. *J Pathol, 225*(3), 401-413. doi:10.1002/path.2973

Kopecki, Z., & Cowin, A. J. (2008). Flightless I: an actin-remodelling protein and an important negative regulator of wound repair. *Int J Biochem Cell Biol, 40*(8), 1415-1419. doi:10.1016/j.biocel.2007.04.011

Kopecki, Z., O'Neill, G. M., Arkell, R. M., & Cowin, A. J. (2011). Regulation of focal adhesions by flightless i involves inhibition of paxillin phosphorylation via a Rac1-dependent pathway. *J Invest Dermatol, 131*(7), 1450-1459. doi:10.1038/jid.2011.69

Kopecki, Z., Ruzehaji, N., Turner, C., Iwata, H., Ludwig, R. J., Zillikens, D., . . . Cowin, A. J. (2013). Topically applied flightless I neutralizing antibodies improve healing of blistered skin in a murine model of epidermolysis bullosa acquisita. *J Invest Dermatol, 133*(4), 1008-1016. doi:10.1038/jid.2012.457

Kopecki, Z., Yang, G. N., Arkell, R. M., Jackson, J. E., Melville, E., Iwata, H., . . . Cowin, A. J. (2013). Flightless I overexpression impairs skin barrier development, function and recovery post skin blistering. *J Pathol*. doi:10.1002/path.4323

Kopecki, Z., Yang, G. N., Jackson, J. E., Melville, E. L., Calley, M. P., Murrell, D. F., . . . Cowin, A. J. (2015). Cytoskeletal protein Flightless I inhibits apoptosis, enhances tumor cell invasion and promotes cutaneous squamous cell carcinoma progression. *Oncotarget, 6*(34), 36426-36440. doi:10.18632/oncotarget.5536

Koster, M. I. (2009). Making an epidermis. *Ann N Y Acad Sci, 1170*, 7-10. doi:10.1111/j.1749-6632.2009.04363.x

Lagares, D., Busnadiego, O., Garcia-Fernandez, R. A., Kapoor, M., Liu, S., Carter, D. E., . . . Rodriguez-Pascual, F. (2012). Inhibition of focal adhesion kinase

prevents experimental lung fibrosis and myofibroblast formation. *Arthritis Rheum, 64*(5), 1653-1664. doi:10.1002/art.33482

Lambrechts, A., Van Troys, M., & Ampe, C. (2004). The actin cytoskeleton in normal and pathological cell motility. *Int J Biochem Cell Biol, 36*(10), 1890-1909. doi:10.1016/j.biocel.2004.01.024

Larsson, O., Diebold, D., Fan, D., Peterson, M., Nho, R. S., Bitterman, P. B., & Henke, C. A. (2008). Fibrotic myofibroblasts manifest genome-wide derangements of translational control. *PLoS One, 3*(9), e3220. doi:10.1371/journal.pone.0003220

Lauffenburger, D. A., & Horwitz, A. F. (1996). Cell migration: a physically integrated molecular process. *Cell, 84*(3), 359-369.

Le Clainche, C., & Carlier, M. F. (2008). Regulation of actin assembly associated with protrusion and adhesion in cell migration. *Physiol Rev, 88*(2), 489-513. doi:10.1152/physrev.00021.2007

Lee, J. Y., Yang, C. C., Chao, S. C., & Wong, T. W. (2004). Histopathological differential diagnosis of keloid and hypertrophic scar. *Am J Dermatopathol, 26*(5), 379-384.

Lee, Y. H., Campbell, H. D., & Stallcup, M. R. (2004). Developmentally essential protein flightless I is a nuclear receptor coactivator with actin binding activity. *Mol Cell Biol, 24*(5), 2103-2117.

Lee, Y. H., & Stallcup, M. R. (2006). Interplay of Fli-I and FLAP1 for regulation of beta-catenin dependent transcription. *Nucleic Acids Res, 34*(18), 5052-5059. doi:10.1093/nar/gkl652

Lei, N., Franken, L., Ruzehaji, N., Offenhauser, C., Cowin, A. J., & Murray, R. Z. (2012). Flightless, secreted through a late endosome/lysosome pathway, binds LPS and dampens cytokine secretion. *J Cell Sci, 125*(Pt 18), 4288-4296. doi:10.1242/jcs.099507

Li, J., Yin, H. L., & Yuan, J. (2008). Flightless-I regulates proinflammatory caspases by selectively modulating intracellular localization and caspase activity. *J Cell Biol, 181*(2), 321-333. doi:10.1083/jcb.200711082

Lim, M. S., & Jeong, K. W. (2014). Role of Flightless-I (Drosophila) homolog in the transcription activation of type I collagen gene mediated by transforming growth factor beta. *Biochem Biophys Res Commun, 454*(3), 393-398. doi:10.1016/j.bbrc.2014.10.100

Lin, C. H., Waters, J. M., Powell, B. C., Arkell, R. M., & Cowin, A. J. (2011). Decreased expression of Flightless I, a gelsolin family member and developmental regulator, in early-gestation fetal wounds improves healing. *Mamm Genome, 22*(5-6), 341-352. doi:10.1007/s00335-011-9320-z

Liu, S., Kapoor, M., Denton, C. P., Abraham, D. J., & Leask, A. (2009). Loss of beta1 integrin in mouse fibroblasts results in resistance to skin scleroderma in a mouse model. *Arthritis Rheum, 60*(9), 2817-2821. doi:10.1002/art.24801

Liu, S., Xu, S. W., Blumbach, K., Eastwood, M., Denton, C. P., Eckes, B., . . . Leask, A. (2010). Expression of integrin beta1 by fibroblasts is required for tissue repair in vivo. *J Cell Sci, 123*(Pt 21), 3674-3682. doi:10.1242/jcs.070672

Liu, S., Xu, S. W., Kennedy, L., Pala, D., Chen, Y., Eastwood, M., . . . Leask, A. (2007). FAK is required for TGFbeta-induced JNK phosphorylation in fibroblasts: implications for acquisition of a matrix-remodeling phenotype. *Mol Biol Cell, 18*(6), 2169-2178. doi:10.1091/mbc.E06-12-1121

Liu, W., Cao, Y., & Longaker, M. T. (2001). Gene therapy of scarring: a lesson learned from fetal scarless wound healing. *Yonsei Med J, 42*(6), 634-645.

Liu, X., Wu, H., Byrne, M., Krane, S., & Jaenisch, R. (1997). Type III collagen is crucial for collagen I fibrillogenesis and for normal cardiovascular development. *Proc Natl Acad Sci U S A, 94*(5), 1852-1856.

Liu, Y. T., & Yin, H. L. (1998). Identification of the binding partners for flightless I, A novel protein bridging the leucine-rich repeat and the gelsolin superfamilies. *J Biol Chem, 273*(14), 7920-7927.

Martin, P. (1997). Wound healing--aiming for perfect skin regeneration. *Science, 276*(5309), 75-81.

Marttala, J., Andrews, J. P., Rosenbloom, J., & Uitto, J. (2016). Keloids: Animal models and pathologic equivalents to study tissue fibrosis. *Matrix Biol, 51*, 47-54. doi:10.1016/j.matbio.2016.01.014

McCleave, H. (1961). *McIndoe: Plastic Surgeon*. London: Frederick Muller Limited.

McMillan, J. R., Haftek, M., Akiyama, M., South, A. P., Perrot, H., McGrath, J. A., . . . Shimizu, H. (2003). Alterations in desmosome size and number coincide with the loss of keratinocyte cohesion in skin with homozygous and heterozygous defects in the desmosomal protein plakophilin 1. *J Invest Dermatol, 121*(1), 96-103. doi:10.1046/j.1523-1747.2003.12324.x

Mohammad, I., Arora, P. D., Naghibzadeh, Y., Wang, Y., Li, J., Mascarenhas, W., . . . McCulloch, C. A. (2012). Flightless I is a focal adhesion-associated actin-capping protein that regulates cell migration. *FASEB J, 26*(8), 3260-3272. doi:10.1096/fj.11-202051

Moritz, A. R., & Henriques, F. C., Jr. (1947). The reciprocal relationship of surface temperature and time in the production of hyperthermic cutaneous injury. *Am J Pathol, 23*(5), 897.

Moseley, P. L., Hemken, C., & Hunninghake, G. W. (1986). Augmentation of fibroblast proliferation by bleomycin. *J Clin Invest, 78*(5), 1150-1154. doi:10.1172/JCI112695

Mustoe, T. A. (2008). Evolution of silicone therapy and mechanism of action in scar management. *Aesthetic Plast Surg, 32*(1), 82-92. doi:10.1007/s00266-007-9030-9

Mustoe, T. A., Cooter, R. D., Gold, M. H., Hobbs, F. D., Ramelet, A. A., Shakespeare, P. G., . . . International Advisory Panel on Scar, M. (2002). International clinical recommendations on scar management. *Plast Reconstr Surg, 110*(2), 560-571.

Muyembe-Tamfum, J. J., Veyi, J., Kaswa, M., Lunguya, O., Verhaegen, J., & Boelaert, M. (2009). An outbreak of peritonitis caused by multidrug-resistant Salmonella Typhi in Kinshasa, Democratic Republic of Congo. *Travel Med Infect Dis, 7*(1), 40-43. doi:10.1016/j.tmaid.2008.12.006

Naumanen, P., Lappalainen, P., & Hotulainen, P. (2008). Mechanisms of actin stress fibre assembly. *J Microsc, 231*(3), 446-454. doi:10.1111/j.1365-2818.2008.02057.x

Nuland, S. B. D. t. i. h. o. m. p. B. D. a. L. P., Inc.: New York, NY. (2008). *Doctors: The illustrated history of medical pioneers*. New York, NY: Black Dog and Leventhal Publishers, Inc.

O'Brien L., (2013) Silicone gel sheeting for preventing and treating hypertrophic scars *Cochrane Database Syst Rev* Sept 12 (9)

O'Shaughnessy, K. D., De La Garza, M., Roy, N. K., & Mustoe, T. A. (2009). Homeostasis of the epidermal barrier layer: a theory of how occlusion

reduces hypertrophic scarring. *Wound Repair Regen, 17*(5), 700-708. doi:10.1111/j.1524-475X.2009.00534.x

Ong, V. H., Carulli, M. T., Xu, S., Khan, K., Lindahl, G., Abraham, D. J., & Denton, C. P. (2009). Cross-talk between MCP-3 and TGFbeta promotes fibroblast collagen biosynthesis. *Exp Cell Res, 315*(2), 151-161. doi:10.1016/j.yexcr.2008.11.001

Ong, Y. S., Samuel, M., & Song, C. (2006). Meta-analysis of early excision of burns. *Burns, 32*(2), 145-150. doi:10.1016/j.burns.2005.09.005

Oshima, R. G. (2007). Intermediate filaments: a historical perspective. *Exp Cell Res, 313*(10), 1981-1994. doi:10.1016/j.yexcr.2007.04.007

Pack, G. T. (1930). *Burns: Types, pathology and management*. Philadelphia, PA: J.B. Lippincott Company.

Palecek, S. P., Loftus, J. C., Ginsberg, M. H., Lauffenburger, D. A., & Horwitz, A. F. (1997). Integrin-ligand binding properties govern cell migration speed through cell-substratum adhesiveness. *Nature, 385*(6616), 537-540. doi:10.1038/385537a0

Pannu, J., Gore-Hyer, E., Yamanaka, M., Smith, E. A., Rubinchik, S., Dong, J. Y., . . . Trojanowska, M. (2004). An increased transforming growth factor beta receptor type I:type II ratio contributes to elevated collagen protein synthesis that is resistant to inhibition via a kinase-deficient transforming growth factor beta receptor type II in scleroderma. *Arthritis Rheum, 50*(5), 1566-1577. doi:10.1002/art.20225

Pannu, J., Nakerakanti, S., Smith, E., ten Dijke, P., & Trojanowska, M. (2007). Transforming growth factor-beta receptor type I-dependent fibrogenic gene program is mediated via activation of Smad1 and ERK1/2 pathways. *J Biol Chem, 282*(14), 10405-10413. doi:10.1074/jbc.M611742200

Partridge, M. A., & Marcantonio, E. E. (2006). Initiation of attachment and generation of mature focal adhesions by integrin-containing filopodia in cell spreading. *Mol Biol Cell, 17*(10), 4237-4248. doi:10.1091/mbc.E06-06-0496

Pollard, T. D., & Borisy, G. G. (2003). Cellular motility driven by assembly and disassembly of actin filaments. *Cell, 112*(4), 453-465.

Polo, M., Kim, Y. J., Kucukcelebi, A., Hayward, P. G., Ko, F., & Robson, M. C. (1998). An in vivo model of human proliferative scar. *J Surg Res, 74*(2), 187-195. doi:10.1006/jsre.1997.5251

Rabello, F. B., Souza, C. D., & Farina, J. A. (2014). Update on hypertrophic scar treatment. *Clinics, 69*(8), 565-573. doi:10.6061/clinics/2014(08)11

Rahban, S. R., & Garner, W. L. (2003). Fibroproliferative scars. *Clin Plast Surg, 30*(1), 77-89.

Rajkumar, V. S., Shiwen, X., Bostrom, M., Leoni, P., Muddle, J., Ivarsson, M., . . . Abraham, D. J. (2006). Platelet-derived growth factor-beta receptor activation is essential for fibroblast and pericyte recruitment during cutaneous wound healing. *Am J Pathol, 169*(6), 2254-2265.

Ramos, M. L., Gragnani, A., & Ferreira, L. M. (2008). Is there an ideal animal model to study hypertrophic scarring? *J Burn Care Res, 29*(2), 363-368. doi:10.1097/BCR.0b013e3181667557

Rappolee, D. A., Mark, D., Banda, M. J., & Werb, Z. (1988). Wound macrophages express TGF-alpha and other growth factors in vivo: analysis by mRNA phenotyping. *Science, 241*(4866), 708-712.

Ravage, B. B. u. s. l. a. t. f. D. C. P. C., MA. (2004). *Burn Unit: Saving lives after the flames*. Cambridge, MA: Da Capo Press.

Rawlins, J. M., Lam, W. L., Karoo, R. O., Naylor, I. L., & Sharpe, D. T. (2006). Pentoxifylline inhibits mature burn scar fibroblasts in culture. *Burns, 32*(1), 42-45. doi:10.1016/j.burns.2005.08.004

Robson, M. C., Steed, D. L., & Franz, M. G. (2001). Wound healing: biologic features and approaches to maximize healing trajectories. *Curr Probl Surg, 38*(2), 72-140. doi:10.1067/msg.2001.111167

Ronnov-Jessen, L., & Petersen, O. W. (1996). A function for filamentous alpha-smooth muscle actin: retardation of motility in fibroblasts. *J Cell Biol, 134*(1), 67-80.

Roques, C. (2002). Massage applied to scars. *Wound Repair Regen, 10*(2), 126-128.

Rudolph, R. (1987). Wide spread scars, hypertrophic scars, and keloids. *Clin Plast Surg, 14*(2), 253-260.

Ruzehaji, N., Kopecki, Z., Melville, E., Appleby, S. L., Bonder, C. S., Arkell, R. M., . . . Cowin, A. J. (2013). Attenuation of flightless I improves wound healing and enhances angiogenesis in a murine model of type 1 diabetes. *Diabetologia*. doi:10.1007/s00125-013-3107-6

Ruzehaji, N., Mills, S. J., Melville, E., Arkell, R., Fitridge, R., & Cowin, A. J. (2013). The influence of Flightless I on Toll-like-receptor-mediated inflammation in a murine model of diabetic wound healing. *Biomed Res Int, 2013*, 389792. doi:10.1155/2013/389792

Santoro, M. M., & Gaudino, G. (2005). Cellular and molecular facets of keratinocyte reepithelization during wound healing. *Exp Cell Res, 304*(1), 274-286. doi:10.1016/j.yexcr.2004.10.033

Santucci, M., Borgognoni, L., Reali, U. M., & Gabbiani, G. (2001). Keloids and hypertrophic scars of Caucasians show distinctive morphologic and immunophenotypic profiles. *Virchows Arch, 438*(5), 457-463.

Sargent, J. L., & Whitfield, M. L. (2011). Capturing the heterogeneity in systemic sclerosis with genome-wide expression profiling. *Expert Rev Clin Immunol, 7*(4), 463-473. doi:10.1586/eci.11.41

Seo, B. F., Lee, J. Y., & Jung, S. N. (2013). Models of abnormal scarring. *Biomed Res Int, 2013*, 423147. doi:10.1155/2013/423147

Seok, J., Warren, H. S., Cuenca, A. G., Mindrinos, M. N., Baker, H. V., Xu, W., . . . Host Response to Injury, L. S. C. R. P. (2013). Genomic responses in mouse models poorly mimic human inflammatory diseases. *Proc Natl Acad Sci U S A, 110*(9), 3507-3512. doi:10.1073/pnas.1222878110

Seward, M. E., Easley, C. A. t., McLeod, J. J., Myers, A. L., & Tombes, R. M. (2008). Flightless-I, a gelsolin family member and transcriptional regulator, preferentially binds directly to activated cytosolic CaMK-II. *FEBS Lett, 582*(17), 2489-2495. doi:10.1016/j.febslet.2008.06.037

Shah, M., Foreman, D. M., & Ferguson, M. W. (1994). Neutralising antibody to TGF-beta 1,2 reduces cutaneous scarring in adult rodents. *J Cell Sci, 107 (Pt 5)*, 1137-1157.

Shah, M., Foreman, D. M., & Ferguson, M. W. (1995). Neutralisation of TGF-beta 1 and TGF-beta 2 or exogenous addition of TGF-beta 3 to cutaneous rat wounds reduces scarring. *J Cell Sci, 108 (Pt 3)*, 985-1002.

Shephard, P., Martin, G., Smola-Hess, S., Brunner, G., Krieg, T., & Smola, H. (2004). Myofibroblast differentiation is induced in keratinocyte-fibroblast co-cultures and is antagonistically regulated by endogenous transforming growth factor-beta and interleukin-1. *Am J Pathol, 164*(6), 2055-2066.

Shi-Wen, X., Renzoni, E. A., Kennedy, L., Howat, S., Chen, Y., Pearson, J. D., . . . Leask, A. (2007). Endogenous endothelin-1 signaling contributes to type I collagen and CCN2 overexpression in fibrotic fibroblasts. *Matrix Biol, 26*(8), 625-632. doi:10.1016/j.matbio.2007.06.003

Shibanuma, M., Mashimo, J., Kuroki, T., & Nose, K. (1994). Characterization of the TGF beta 1-inducible hic-5 gene that encodes a putative novel zinc finger protein and its possible involvement in cellular senescence. *J Biol Chem, 269*(43), 26767-26774.

Sieg, D. J., Hauck, C. R., Ilic, D., Klingbeil, C. K., Schaefer, E., Damsky, C. H., & Schlaepfer, D. D. (2000). FAK integrates growth-factor and integrin signals to promote cell migration. *Nat Cell Biol, 2*(5), 249-256. doi:10.1038/35010517

Sikic, B. I., Collins, J. M., Mimnaugh, E. G., & Gram, T. E. (1978). Improved therapeutic index of bleomycin when administered by continuous infusion in mice. *Cancer Treat Rep, 62*(12), 2011-2017.

Sikic, B. I., Young, D. M., Mimnaugh, E. G., & Gram, T. E. (1978). Quantification of bleomycin pulmonary toxicity in mice by changes in lung hydroxyproline content and morphometric histopathology. *Cancer Res, 38*(3), 787-792.

Silacci, P., Mazzolai, L., Gauci, C., Stergiopulos, N., Yin, H. L., & Hayoz, D. (2004). Gelsolin superfamily proteins: key regulators of cellular functions. *Cell Mol Life Sci, 61*(19-20), 2614-2623. doi:10.1007/s00018-004-4225-6

Simpson, D. M., & Ross, R. (1972). The neutrophilic leukocyte in wound repair a study with antineutrophil serum. *J Clin Invest, 51*(8), 2009-2023. doi:10.1172/JCI107007

Singer, A. J., & Clark, R. A. (1999). Cutaneous wound healing. *N Engl J Med, 341*(10), 738-746. doi:10.1056/NEJM199909023411006

Small, J. V., Geiger, B., Kaverina, I., & Bershadsky, A. (2002). How do microtubules guide migrating cells? *Nat Rev Mol Cell Biol, 3*(12), 957-964. doi:10.1038/nrm971

Smith-Clerc, J., & Hinz, B. (2010). Immunofluorescence detection of the cytoskeleton and extracellular matrix in tissue and cultured cells. *Methods Mol Biol, 611*, 43-57. doi:10.1007/978-1-60327-345-9_4

Smith, J. R., & Henderson, N. C. (2016). Recent progress on targeting the alphavbeta1 integrin for the treatment of tissue fibrosis. *Expert Opin Drug Discov, 11*(8), 749-751. doi:10.1080/17460441.2016.1193486

Soo, C., Hu, F. Y., Zhang, X., Wang, Y., Beanes, S. R., Lorenz, H. P., . . . Ting, K. (2000). Differential expression of fibromodulin, a transforming growth factor-beta modulator, in fetal skin development and scarless repair. *Am J Pathol, 157*(2), 423-433.

Strudwick, X. L., Waters, J. M., & Cowin, A. J. (2017). Flightless I Expression Enhances Murine Claw Regeneration Following Digit Amputation. *J Invest Dermatol, 137*(1), 228-236. doi:10.1016/j.jid.2016.08.019

Sun, H. Q., Kwiatkowska, K., & Yin, H. L. (1995). Actin monomer binding proteins. *Curr Opin Cell Biol, 7*(1), 102-110.

Szulgit, G., Rudolph, R., Wandel, A., Tenenhaus, M., Panos, R., & Gardner, H. (2002). Alterations in fibroblast alpha1beta1 integrin collagen receptor expression in keloids and hypertrophic scars. *J Invest Dermatol, 118*(3), 409-415. doi:10.1046/j.0022-202x.2001.01680.x

Takagawa, S., Lakos, G., Mori, Y., Yamamoto, T., Nishioka, K., & Varga, J. (2003). Sustained activation of fibroblast transforming growth factor-beta/Smad signaling in a murine model of scleroderma. *J Invest Dermatol, 121*(1), 41-50. doi:10.1046/j.1523-1747.2003.12308.x

Tamariz, E., & Grinnell, F. (2002). Modulation of fibroblast morphology and adhesion during collagen matrix remodeling. *Mol Biol Cell, 13*(11), 3915-3929. doi:10.1091/mbc.E02-05-0291

Tao J., (2018). Treatment of burn scars in Fitzpatrick phototype III patients with a combination of pulsed dye laser and non-ablative fractional resurfacing 1550nm erbium:glass/1927 thulium laser devices., Scars Burn Heal doi: 10.1177/2059513118758510

Tan, T. W., Lai, C. H., Huang, C. Y., Yang, W. H., Chen, H. T., Hsu, H. C., . . . Tang, C. H. (2009). CTGF enhances migration and MMP-13 up-regulation via alphavbeta3 integrin, FAK, ERK, and NF-kappaB-dependent pathway in human chondrosarcoma cells. *J Cell Biochem, 107*(2), 345-356. doi:10.1002/jcb.22132

Teixeira, A., Chaverot, N., Schroder, C., Strosberg, A. D., Couraud, P. O., & Cazaubon, S. (1999). Requirement of caveolae microdomains in extracellular signal-regulated kinase and focal adhesion kinase activation induced by endothelin-1 in primary astrocytes. *J Neurochem, 72*(1), 120-128.

Thannickal, V. J., Lee, D. Y., White, E. S., Cui, Z., Larios, J. M., Chacon, R., . . . Thomas, P. E. (2003). Myofibroblast differentiation by transforming growth factor-beta1 is dependent on cell adhesion and integrin signaling via focal adhesion kinase. *J Biol Chem, 278*(14), 12384-12389. doi:10.1074/jbc.M208544200

Theoret, C. L., Olutoye, O. O., Parnell, L. K., & Hicks, J. (2013). Equine exuberant granulation tissue and human keloids: a comparative histopathologic study. *Vet Surg, 42*(7), 783-789. doi:10.1111/j.1532-950X.2013.12055.x

Thomas, S. M., Hagel, M., & Turner, C. E. (1999). Characterization of a focal adhesion protein, Hic-5, that shares extensive homology with paxillin. *J Cell Sci, 112 (Pt 2)*, 181-190.

Thomsen, N., Chappell, A., Ali, R. G., Jones, T., Adams, D. H., Matthaei, K. I., . . . Arkell, R. M. (2011). Mouse strains for the ubiquitous or conditional overexpression of the Flii gene. *Genesis, 49*(8), 681-688. doi:10.1002/dvg.20735

Tomasek, J. J., Gabbiani, G., Hinz, B., Chaponnier, C., & Brown, R. A. (2002). Myofibroblasts and mechano-regulation of connective tissue remodelling. *Nat Rev Mol Cell Biol, 3*(5), 349-363. doi:10.1038/nrm809

Tomasek, J. J., Vaughan, M. B., & Haaksma, C. J. (1999). Cellular structure and biology of Dupuytren's disease. *Hand Clin, 15*(1), 21-34.

Umezawa, H. (1967). [Bleomycin]. *Gan No Rinsho, 13*(10), 735.

Valiron, O., Caudron, N., & Job, D. (2001). Microtubule dynamics. *Cell Mol Life Sci, 58*(14), 2069-2084.

Varga, J., & Abraham, D. (2007). Systemic sclerosis: a prototypic multisystem fibrotic disorder. *J Clin Invest, 117*(3), 557-567. doi:10.1172/JCI31139

Verhaegen, P. D., van Zuijlen, P. P., Pennings, N. M., van Marle, J., Niessen, F. B.,

van der Horst, C. M., & Middelkoop, E. (2009). Differences in collagen architecture between keloid, hypertrophic scar, normotrophic scar, and

normal skin: An objective histopathological analysis. *Wound Repair Regen, 17*(5), 649-656. doi:10.1111/j.1524-475X.2009.00533.x

Vogel, V., & Sheetz, M. (2006). Local force and geometry sensing regulate cell functions. *Nat Rev Mol Cell Biol, 7*(4), 265-275. doi:10.1038/nrm1890

Wang, Q., Yerukhimovich, M., Gaarde, W. A., Popoff, I. J., & Doerschuk, C. M. (2005). MKK3 and -6-dependent activation of p38alpha MAP kinase is required for cytoskeletal changes in pulmonary microvascular endothelial cells induced by ICAM-1 ligation. *Am J Physiol Lung Cell Mol Physiol, 288*(2), L359-369. doi:10.1152/ajplung.00292.2004

Wang, T., Chuang, T. H., Ronni, T., Gu, S., Du, Y. C., Cai, H., . . . Chen, X. (2006). Flightless I homolog negatively modulates the TLR pathway. *J Immunol, 176*(3), 1355-1362.

Waters, J. M., Lindo, J. E., Arkell, R. M., & Cowin, A. J. (2011). Regeneration of hair follicles is modulated by flightless I (Flii) in a rodent vibrissa model. *J Invest Dermatol, 131*(4), 838-847. doi:10.1038/jid.2010.393

Webb, D. J., Donais, K., Whitmore, L. A., Thomas, S. M., Turner, C. E., Parsons, J. T., & Horwitz, A. F. (2004). FAK-Src signalling through paxillin, ERK and MLCK regulates adhesion disassembly. *Nat Cell Biol, 6*(2), 154-161. doi:10.1038/ncb1094

Wells, A., Nuschke, A., & Yates, C. C. (2016). Skin tissue repair: Matrix microenvironmental influences. *Matrix Biol, 49*, 25-36. doi:10.1016/j.matbio.2015.08.001

Werner, S., & Grose, R. (2003). Regulation of wound healing by growth factors and cytokines. *Physiol Rev, 83*(3), 835-870. doi:10.1152/physrev.00031.2002

Wilkes, M. C., Mitchell, H., Penheiter, S. G., Dore, J. J., Suzuki, K., Edens, M., . . . Leof, E. B. (2005). Transforming growth factor-beta activation of phosphatidylinositol 3-kinase is independent of Smad2 and Smad3 and regulates fibroblast responses via p21-activated kinase-2. *Cancer Res, 65*(22), 10431-10440. doi:10.1158/0008-5472.CAN-05-1522

Wipff, P. J., & Hinz, B. (2009). Myofibroblasts work best under stress. *J Bodyw Mov Ther, 13*(2), 121-127. doi:10.1016/j.jbmt.2008.04.031

Witke, W., Sharpe, A. H., Hartwig, J. H., Azuma, T., Stossel, T. P., & Kwiatkowski, D. J. (1995). Hemostatic, inflammatory, and fibroblast responses are blunted in mice lacking gelsolin. *Cell, 81*(1), 41-51.

Witke, W., Sutherland, J. D., Sharpe, A., Arai, M., & Kwiatkowski, D. J. (2001). Profilin I is essential for cell survival and cell division in early mouse development. *Proc Natl Acad Sci U S A, 98*(7), 3832-3836. doi:10.1073/pnas.051515498

Wong, V. W., Rustad, K. C., Akaishi, S., Sorkin, M., Glotzbach, J. P., Januszyk, M., . . . Gurtner, G. C. (2012). Focal adhesion kinase links mechanical force to skin fibrosis via inflammatory signaling. *Nat Med, 18*(1), 148-152. doi:10.1038/nm.2574

Wong, V. W., Sorkin, M., Glotzbach, J. P., Longaker, M. T., & Gurtner, G. C. (2011). Surgical approaches to create murine models of human wound healing. *J Biomed Biotechnol, 2011*, 969618. doi:10.1155/2011/969618

Wood, F. M., Stoner, M. L., Fowler, B. V., & Fear, M. W. (2007). The use of a non-cultured autologous cell suspension and Integra dermal regeneration

template to repair full-thickness skin wounds in a porcine model: a one-step process. *Burns, 33*(6), 693-700. doi:10.1016/j.burns.2006.10.388

Wynn, T. A. (2004). Fibrotic disease and the T(H)1/T(H)2 paradigm. *Nat Rev Immunol, 4*(8), 583-594. doi:10.1038/nri1412

Wynn, T. A. (2008). Cellular and molecular mechanisms of fibrosis. *J Pathol, 214*(2), 199-210. doi:10.1002/path.2277

Xu, J., Liao, L., Qin, J., Xu, J., Liu, D., & Songyang, Z. (2009). Identification of Flightless-I as a substrate of the cytokine-independent survival kinase CISK. *J Biol Chem, 284*(21), 14377-14385. doi:10.1074/jbc.M807770200

Yamamoto, T., Eckes, B., & Krieg, T. (2000). Bleomycin increases steady-state levels of type I collagen, fibronectin and decorin mRNAs in human skin fibroblasts. *Arch Dermatol Res, 292*(11), 556-561.

Yamamoto, T., & Nishioka, K. (2005a). Cellular and molecular mechanisms of bleomycin-induced murine scleroderma: current update and future perspective. *Exp Dermatol, 14*(2), 81-95. doi:10.1111/j.0906-6705.2005.00280.x

Yamamoto, T., & Nishioka, K. (2005b). Increased expression of p53 and p21 (Waf1/Cip1) in the lesional skin of bleomycin-induced scleroderma. *Arch Dermatol Res, 296*(11), 509-513. doi:10.1007/s00403-005-0550-3

Yamamoto, T., Takagawa, S., Katayama, I., Yamazaki, K., Hamazaki, Y., Shinkai, H., & Nishioka, K. (1999). Animal model of sclerotic skin. I: Local injections of bleomycin induce sclerotic skin mimicking scleroderma. *J Invest Dermatol, 112*(4), 456-462. doi:10.1046/j.1523-1747.1999.00528.x

Yamamoto, T., Takagawa, S., Kuroda, M., & Nishioka, K. (2000). Effect of interferon-gamma on experimental scleroderma induced by bleomycin. *Arch Dermatol Res, 292*(7), 362-365.

Zawacki, B. E. (1974). The natural history of reversible burn injury. *Surg Gynecol Obstet, 139*(6), 867-872.

Zeisberg, M., & Kalluri, R. (2008). Fibroblasts emerge via epithelial-mesenchymal transition in chronic kidney fibrosis. *Front Biosci, 13*, 6991-6998.

Zhang, K., Garner, W., Cohen, L., Rodriguez, J., & Phan, S. (1995). Increased types I and III collagen and transforming growth factor-beta 1 mRNA and protein in hypertrophic burn scar. *J Invest Dermatol, 104*(5), 750-754.

Zhong, C., Chrzanowska-Wodnicka, M., Brown, J., Shaub, A., Belkin, A. M., & Burridge, K. (1998). Rho-mediated contractility exposes a cryptic site in fibronectin and induces fibronectin matrix assembly. *J Cell Biol, 141*(2), 539-551.

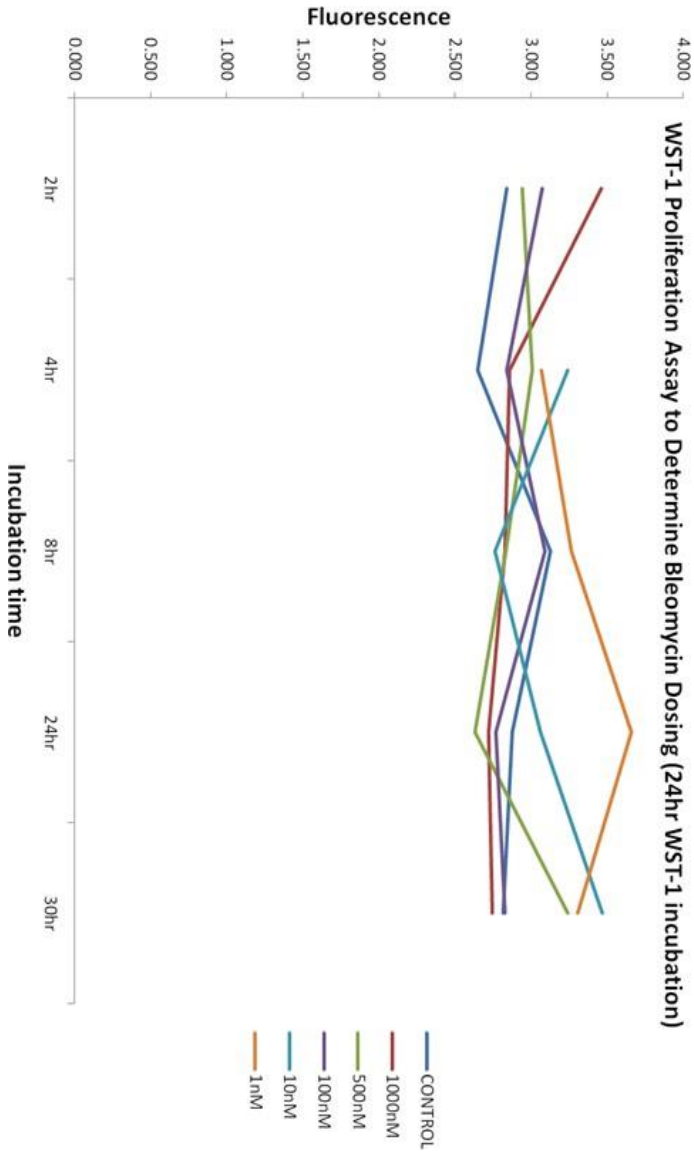
Zhu, K. Q., Carrougher, G. J., Couture, O. P., Tuggle, C. K., Gibran, N. S., & Engrav, L. H. (2008). Expression of collagen genes in the cones of skin in the Duroc/Yorkshire porcine model of fibroproliferative scarring. *J Burn Care Res, 29*(5), 815-827. doi:10.1097/BCR.0b013e3181848141

Zhu, K. Q., Engrav, L. H., Gibran, N. S., Cole, J. K., Matsumura, H., Piepkorn, M., . . . Yang, T. M. (2003). The female, red Duroc pig as an animal model of hypertrophic scarring and the potential role of the cones of skin. *Burns, 29*(7), 649-664.

Zvibel, I., Bar-Zohar, D., Kloog, Y., Oren, R., & Reif, S. (2008). The effect of Ras inhibition on the proliferation, apoptosis and matrix metalloproteases activity in rat hepatic stellate cells. *Dig Dis Sci, 53*(4), 1048-1053. doi:10.1007/s10620-007-9984-0

APPENDIX

A.1 WST-1 calculations for ideal bleomycin concentration in cell culture



A.2 Papers published in peer reviewed journals

TRANSLATIONAL RESEARCH

BJD
British Journal of Dermatology

Flightless I is a key regulator of the fibroproliferative process in hypertrophic scarring and a target for a novel antiscarring therapy*

A.M. Cameron,[1,2] C.T. Turner,[1] D.H. Adams,[1] J.E. Jackson,[1] E. Melville,[1] R.M. Arkell,[3] P.J. Anderson[4] and A.J. Cowin[1]

[1]Regenerative Medicine, Future Industries Institute, University of South Australia, Mawson Lakes, SA 5095, Australia
Disciplines of [2]Surgery and [4]Paediatrics, School of Medicine, Faculty of Health Sciences, The University of Adelaide, Adelaide, SA, Australia
[3]Research School of Biology, College of Medicine, Biology and Environment, Australian National University, Acton, ACT 2601, Australia

Correspondence
Allison Cowin.
E-mail: allison.cowin@unisa.edu.au

Accepted for publication
26 October 2015

Funding sources
A.J.C. is the recipient of scholarships/awards from the NHMRC (GNT#1038104), Royal Australasian College of Surgeons and the Australian Society of Plastic Surgeons. A.J.C. is supported by a Senior Research Fellowship (GNT#1002009) from the National Health and Medical Research Council of Australia.

Conflicts of interest
The intellectual property relating to this study is owned by AbRegen Pty Ltd of which A.J.C. is a shareholder.

*Plain language summary available online

DOI 10.1111/bjd.14263

Summary

Background Hypertrophic scarring carries a large burden of disease, including disfigurement, pain and disability. There is currently no effective medical treatment to reduce or prevent hypertrophic scarring. Flightless I (Flii), a member of the gelsolin family of actin remodelling proteins, is an important negative regulator of wound repair.

Objectives The objective of this study was to investigate the role of Flii as a potential regulator of hypertrophic scarring.

Methods Using human skin samples and an animal model of bleomycin-induced hypertrophic scarring in mice that overexpress or have reduced expression of Flii, we investigated its effect on dermal fibrosis and hypertrophic scarring.

Results Flii expression was increased in human burns and hypertrophic scars. A similar increase in Flii was observed in hypertrophic scars formed in mice post-treatment with bleomycin. However, Flii-deficient ($Flii^{+/-}$) mice had reduced scarring in response to bleomycin evidenced by decreased dermal thickness, smaller cross-sectional scar areas, fewer myofibroblasts and a decreased collagen I/III ratio. In contrast, bleomycin-treated Flii-overexpressing mice ($Flii^{Tg/Tg}$) showed increased scar dermal thickness, larger cross-sectional scar areas, more myofibroblasts and an increased collagen I/III ratio. Injecting developing scars with a Flii neutralizing antibody led to a significant reduction in the size of the scars and a reduction in the collagen I/III ratio.

Conclusions This study identifies Flii as a profibrotic agent that contributes to excessive scar formation. Reducing its activity using neutralizing antibodies is a promising approach for reducing hypertrophic scarring.

What's already known about this topic?

- Flightless I (Flii) adversely affects wound healing by inhibiting migration, proliferation and adhesion.
- Increased Flii in transgenic mice models impairs re-epithelialization and in a burn model leads to increased deposition of collagen and profibrotic growth factors.

What does this study add?

- Flii is elevated in human hypertrophic scars and reducing its level in a mouse model of hypertrophic scarring either genetically or by using an Flii neutralizing antibody decreases the severity of fibrosis and scarring.

Hypertrophic scarring is a fibroproliferative disease of the skin most commonly associated with burn injury, but may also occur after trauma or elective surgery.[1] It is estimated that at least 70% of deep burns in adults, and greater in children, will develop hypertrophic scarring,[1] which can cause disfigurement. Hypertrophic scarring represents an aberration of the normal wound healing process, which is a tightly orchestrated, nonlinear sequence involving numerous cell types, extracellular matrix (ECM) components and signalling molecules.[2] It balances several requirements: prevention of infection, restoration of epithelial integrity and approximation of normal skin structure and function.[3] Hypertrophic scarring results from a disruption of this delicate balance. A prolonged inflammatory phase results in the persistence of myofibroblasts in the wound and elevated levels of fibrogenic cytokines, such as transforming growth factor (TGF) β1 and connective tissue growth factor (CTGF).[4] Myofibroblasts drive inflammation and overproduce collagen, which accumulates in a disorganized manner[5] and results in an increased collagen I/III ratio.[6,7] This results in a raised, red, tender scar, which is prone to contracture over time.[8]

Studies have shown that the protein Flightless I (Flii) is a regulator of the wound healing process.[9] Flii is a member of the gelsolin family of actin-remodelling proteins that regulate actin by severing pre-existing filaments and/or capping filament ends to enable filament reassembly into new cytoskeletal structures.[10] Flii co-localizes with molecules involved in regulating cytoskeletal reorganization, including members of the Rho family of GTPases, Ras and Cdc42.[11] It possesses a unique leucine-rich repeat domain, which is important for protein–protein interactions.[12] Cells within the epidermis and dermis express Flii and studies have shown that Flii is able to translocate from the cytoplasm to the nucleus in response to wounding.[9] Focal adhesions and migratory structures involved in cellular motility also express Flii[13] and it is also secreted from cells.[14] In $Flii^{+/-}$ mice, healing is enhanced, whereas in $Flii^{Tg/Tg}$ mice, healing is impaired, with delayed epithelial migration and increased scarring.[9] *In vitro*, $Flii^{+/-}$ fibroblasts and keratinocytes have improved cellular adhesion and spreading resulting in accelerated scratch wound closure.[9,15] Additionally, reducing Flii in a murine model of partial-thickness burn injury led to faster re-epithelialization and reduced scar formation,[16] suggesting the reduction of this protein in wounds may be beneficial as a hypertrophic scarring therapeutic treatment.

Given the relationship between impaired wound healing and the development of hypertrophic scars, we have investigated whether Flii has a regulatory role in the fibroproliferative process that leads to hypertrophic scar formation. Here we examine the expression of Flii in human hypertrophic scars and investigate the effect of differential expression of Flii in a murine model of hypertrophic scarring as well as determining whether injection of a neutralizing antibody to Flii (FnAb)[17] can reduce hypertrophic scar formation.

Materials and methods

Reagents and antibodies

Rabbit anti-collagen I and III antibodies: Rockland Immunochemicals (Gilbertsville, PA, U.S.A.). Mouse anti-Flii (H-300), and rabbit anti-TGF-β1 (V) antibody: Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Mouse anti-α-smooth muscle actin (α-SMA), mouse immunoglobulin (Ig) G, and 4′,6-diamidino-2-phenylindole (DAPI): Sigma Aldrich (Castle Hill, NSW, Australia). Secondary antibodies, Alexa Fluor 488 goat anti-rabbit and Alexa Fluor 488 goat anti-mouse were from Life Technologies (Carlsbad, CA, U.S.A.). Affinity purified mouse monoclonal anti-Flightless antibody (FnAb) raised against the leucine-rich repeat domain of the Flii protein was made in house. Murine nonspecific IgG (I8765) was from Sigma (St Louis, MO, U.S.A.).

Human samples

Samples of normal human skin, acute burns and hypertrophic scars ($n = 5$ per group) were obtained from the Royal Adelaide Hospital Adult Burns Unit with ethics approval from the Royal Adelaide Hospital Human Research Ethics Committee (11-CHREC-F007) in accordance with the Declaration of Helsinki principles. Written consent was obtained from patients undergoing surgery to obtain excised tissue. Skin and scar samples were bisected and fixed in 10% (v/v) neutral buffered formalin for histology and/or immunofluorescence or frozen in liquid nitrogen for biochemical analysis.

Animal studies

Mice were maintained according to the Australian code for the care and use of animals for scientific purposes under protocols approved by the Australian National University Animal Ethics Committee and the Child, Youth and Women's Health Service Animal Ethics Committee. Additionally, all protocols were in accordance with the ARRIVE Guidelines (Animal Research: Reporting *In Vivo* Experiments). All strains were BALB/c congenic and were maintained as homozygous colonies or by continuous backcross to BALB/c animals. Wild-type (WT) controls were obtained from BALB/c inbred litters. The murine alleles of Flii used in this study were (i) Flii tm1Hdc (MGI:2 179 825), a targeted null allele of Flii; and (ii) Tg(FLII)1Hdc (MGI:3 796 828), a transgenic strain expressing exogenous human Flii due to the insertion of the human cosmid clone c110H8.[18,19] The genotype of the heterozygous carrier of this allele is written as $Flii^{+/-}$ and $Flii^{Tg/Tg}$, respectively.

Murine surgical technique and Flightless I neutralizing antibody treatment

Prior to surgery, osmotic pumps (Alzet Model 1004; Durect Corp., Cupertino, CA, U.S.A.) were filled to capacity with

2·8 mg mL^{-1} bleomycin sulphate (R-42569; Hospira, Mulgrave North, VIC, Australia) or phosphate-buffered saline, pH 7·2. Pumps are designed to produce a consistent rate of flow of 0·11 µL h^{-1} for 28 days. Pumps were weighed before and after filling to ensure the correct volume. $Flii^{+/-}$, WT and $Flii^{Tg/Tg}$ female 12-week-old mice were anaesthetized using gaseous isoflurane, 5% induction, 2% maintenance. The dorsum of the mice was shaved and cleaned with 10% (v/v) povidone iodine solution. A 0·5-cm transverse incision was made across the midline at a point approximately 4 cm caudal to the base of the skull. Using blunt dissection, a pocket was made between the skin and muscle layers into which the osmotic pump was inserted.[20] Wounds were closed with 4-0 prolene and secured with tissue adhesive (Histoacryl; TissueSeal, Ann Arbor, MI, U.S.A.). Mice were euthanized at 28 days, when the pumps were removed, or 56 days after pump insertion to represent a developing/immature hypertrophic scar (28 days) and a mature hypertrophic scar (56 days). A 4 cm × 1·5 cm area of skin was excised around the fibrotic lesions at a location corresponding to the cranial end of the osmotic pump and unwounded skin was collected from each mouse group.

In a subset of WT female 12-week old mice ($n = 6$), 200 µL of 50 µg mL^{-1} FnAb was injected subcutaneously at the cranial end of the osmotic pump on days 0, 7, 14 and 21. The 50 µg mL^{-1} dose of FnAb had previously been used for treating acute injuries, burns and diabetic wounds in murine and porcine models.[9,16,17] The weekly treatment regime was chosen to provide prolonged neutralization of Flii activity over the 4-week induction period. A control group ($n = 6$) was injected with mouse IgG (200 µL, 50 µg mL^{-1}) at the same time points. Fibrotic lesions and unwounded skin from all animals were collected at days 28 and 56 post osmotic pump insertion and bisected and fixed in 10% (v/v) buffered formalin for histology and/or immunohistochemistry.

Histology, immunohistochemistry and image analysis

Tissue samples were fixed in 10% buffered formalin and embedded in paraffin. Serial histological sections (4 µm) were prepared and sections stained with haematoxylin and eosin, Masson's trichrome or subjected to immunohistochemistry for DAPI, Flii, collagen I, collagen III, TGF-β1 or α-SMA. Immunohistochemistry was performed following antigen retrieval. After blocking in 3% normal goat serum, primary antibodies were applied at a concentration of 2 µg mL^{-1}. Slides were incubated overnight at 4 °C in a humidified chamber. Species-specific Alexa Fluor 488 was used for detection at a concentration of 2 µg mL^{-1} for all primary antibodies. Fluorescence intensity per unit area was determined using AnalySIS software package as previously described.[9] Negative controls included replacing primary antibodies with species-specific IgG. All control sections had negligible immunofluorescence.

Analysis of scarring was performed using Image Pro-Plus 5.1 program (MediaCybernetics Inc., Rockville, MD, U.S.A.). Dermal thickness was measured as the average distance from the basement membrane to the panniculus carnosus. Scar cross-sectional area was measured as the total area of dermis that showed evidence of fibrotic change. This area was identified by the presence of increased dermal thickness and paucity of dermal appendages compared with normal unwounded skin.

Statistical analysis

Statistical differences were determined using Student's t-test or an ANOVA. For data not following a normal distribution, the Mann–Whitney *U*-test was performed. A *P*-value of less than 0·05 was considered significant.

Results

Flightless I is upregulated in human acute burns and hypertrophic scars

Samples of human normal skin, acute burns and hypertrophic scars at varying stages of maturity were analysed using immunohistochemistry for Flii (Fig. 1). Increased Flii expression was observed in human burn wounds and hypertrophic scars compared with normal unwounded human skin similar to that observed in murine models of burn injury (Fig. 1a).[16] This increase in Flii expression was seen throughout the epidermis and also in the dermis in acute burns and immature scars. A mouse model of hypertrophic scarring was used to further assess the effect of scarring on the expression of Flii. In this model bleomycin was delivered subcutaneously over a 28-day period via an osmotic pump with the resulting fibrotic lesions being similar histologically to those observed in human immature and mature hypertrophic scars (Fig. 1b). Using this mouse model of hypertrophic scarring, increased Flii expression was observed in both the day 28 (developing/immature hypertrophic scar) (Fig. 1c) and day 56 (mature hypertrophic scar) fibrotic lesions (data not shown) similar to human skin (Fig. 1a). Flii was expressed throughout the epidermis and dermis similar to that observed in human scars (Fig. 1c).

Decreasing Flightless I reduces acute and mature scar formation

To determine the effect of changing the level of Flii on dermal fibrosis, bleomycin was delivered subcutaneously for 4 weeks via osmotic pumps to $Flii^{+/-}$, WT and $Flii^{Tg/Tg}$ mice and the resulting scars were assessed at days 28 and 56. A significant decrease in dermal thickness was observed at day 28 in $Flii^{+/-}$ mice compared with WT (145·4 µm ± 5·7 µm vs. 203·0 µm ± 14·8 µm, $P < 0·05$) and $Flii^{Tg/Tg}$ (145·4 µm ± 5·7 µm vs. 344·7 µm ± 16·9 µm, $P < 0·0005$) in the developing/immature hypertrophic scars (Fig. 2a,c). Similarly, but to a much greater extent, a significant increase in scar dermal thickness was observed in the mature scars of the $Flii^{Tg/Tg}$ mice compared with WT (1120·9 µm ± 140·8 µm vs.

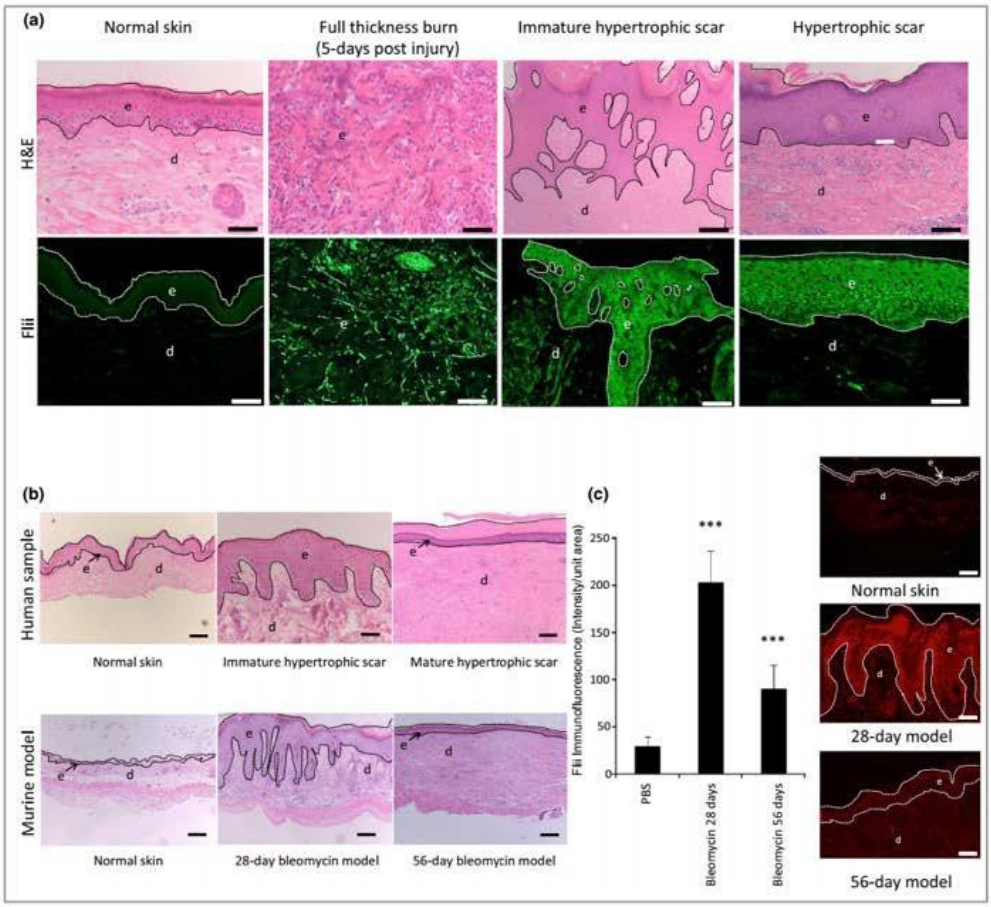


Fig 1. Flii is increased in acute burns and hypertrophic scars. (a) Haematoxylin and eosin staining and Flii immunohistochemistry (green) was performed on samples of human normal skin, acute burn, immature and mature hypertrophic scars. Flii is upregulated in acute burns and hypertrophic scars in the epidermis and throughout the dermis. n = 5 per group. (b) The fibrosis model reproduces histopathological changes seen in human hypertrophic scarring, with the 28-day model corresponding to an immature hypertrophic scar and the 56-day model corresponding to a mature hypertrophic scar. Original magnification ×10. n = 6. (c) Flii is significantly increased in the day-28 and day-56 bleomycin model compared with control. n = 6. Mean ± SEM. *P < 0·05, **P < 0·005, ***P < 0·0005. Scale bar = 50 μm. d, dermis; e, epidermis; Flii, Flightless I; H&E, haematoxylin and eosin; PBS, phosphate-buffered saline.

627·8 μm ± 73·7 μm, P < 0·05) and $Flii^{+/-}$ (1120·9 μm ± 140·8 μm vs. 726·7 μm ± 128·9 μm, P < 0·05) at day 56 (Fig. 2a,c). The developing/immature hypertrophic scar at day 28 showed that $Flii^{Tg/Tg}$ mice had a significantly larger cross-sectional area compared with both $Flii^{+/-}$ (76 361·9 μm^2 ± 2895·6 μm^2 vs. 42 608·4 μm^2 ± 6784·3 μm^2, P < 0·0005) and WT (76 361·9 μm^2 ± 2895·6 μm^2 vs. 48 144·9 μm^2 ± 7135·2 μm^2, P < 0·005) (Fig. 2b,d). There was also a significant increase in scar cross-sectional area in the mature scars of the $Flii^{Tg/Tg}$ mice compared with both WT and $Flii^{+/-}$ (106 454·8 ± 21 838·1 μm^2 vs. 51 566·2 μm^2 ± 3134·3 μm^2: P < 0·05; vs. 36 597·8 μm^2 ± 7564·3 μm^2, P < 0·0005) at day 56. A significant reduction in scar area in $Flii^{+/-}$ mice compared with WT was also observed (36 597·8 μm^2 ± 7564·3 μm^2 vs. 51 566·2 μm^2 ± 3134·3 μm^2, P < 0·05) at this later time point.

Decreasing Flightless I improves collagen deposition

An increase in the ratio of collagen I/III is associated with hypertrophic scar formation so any agent that can decrease this ratio would be considered beneficial for the reduction

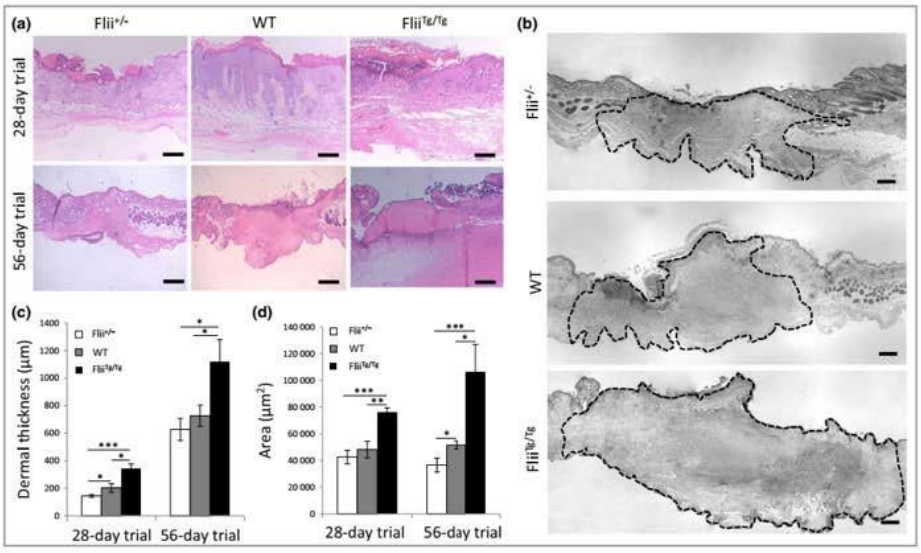


Fig 2. Decreasing Flii reduces scar severity in day-28 and day-56 fibrosis models. (a) Representative haematoxylin and eosin images of dermal thickness of scars after 28 and 56 days. Microscopic analysis showed a significant decrease in dermal thickness in $Flii^{+/-}$ scars at 28 days compared with WT and $Flii^{Tg/Tg}$. Original magnification ×10. $n = 6$. Mean ± SEM. (b) Representative stitched haematoxylin and eosin images of total scar cross-sectional area at 28 days. Microscopic analysis showed that $Flii^{+/-}$ scars had significantly less cross-sectional area at 56 days compared with WT and $Flii^{Tg/Tg}$. $n = 6$. Mean ± SEM. (c) Graph showing changes in dermal thickness after 28 and 56 days. There was a significant increase in dermal thickness in the $Flii^{Tg/Tg}$ scars compared with WT and $Flii^{+/-}$ at 56 days. (d) Graph showing changes in scar cross-sectional area at 28 and 56 days. $Flii^{Tg/Tg}$ scars had significantly greater cross-sectional area at 28 and 56 days than WT and $Flii^{+/-}$. *$P < 0.05$, **$P < 0.005$, ***$P < 0.0005$. Flii, Flightless I; WT, wild type. Scale bar = 50 µm (a,b).

or prevention of scar formation. The effect of Flii on collagen I and III deposition was therefore assessed at day 28 and 56. Increased amounts of dermal collagen were observed in both 28-day and 56-day scars using Masson's trichrome staining compared with controls (Fig. 3a–d). Collagen I expression was increased at both day 28 and day 56 in $Flii^{Tg/Tg}$ mice while conversely collagen III was most highly expressed in the $Flii^{+/-}$ mice scars and lowest in the $Flii^{Tg/Tg}$ mice (Fig. 3a–d). When expressed as a ratio, a significant decrease in the collagen I/III ratio was observed in $Flii^{+/-}$ developing/immature scars compared with both WT (0·57 ± 0·13 vs. 0·720 ± 0·15, $P < 0.005$) and $Flii^{Tg/Tg}$ (0·57 ± 0·13 vs. 1·44 ± 0·19, $P < 0.0005$) at 28 days. In the 56-day mature scars, the collagen I/III ratio in $Flii^{+/-}$ scars was also significantly reduced compared with WT (0·92 ± 0·012 vs. 3·13 ± 0·13, $P < 0.0005$) and $Flii^{Tg/Tg}$ (0·92 ± 0·012 vs. 2·60 ± 0·38, $P < 0.0005$), which is indicative of reduced scar formation. A slight decrease in the collagen I/III ratio was observed in these mature scars at day 56 between WT and $Flii^{Tg/Tg}$ but this was not statistically significant.

Manipulation of Flightless I level affects key determinants of scarring including transforming growth factor-β1 and α-smooth muscle actin

$Flii^{+/-}$, WT and $Flii^{Tg/Tg}$ scars at day 28 and day 56 were investigated for the presence of profibrotic agent TGF-β1 using immunohistochemistry (Fig. 4a). The 28-day developing/immature scars in the $Flii^{+/-}$ mice had significantly less epidermal and dermal TGF-β1 expression compared with WT (epidermal: 42 ± 8·7 vs. 145 ± 4·3, $P < 0.05$; dermal: 40·7 ± 1·3 vs. 113 ± 4·8, $P < 0.005$) and $Flii^{Tg/Tg}$ (epidermal: 42 ± 8·7 vs. 264 ± 10·1, $P < 0.005$; dermal: 40·7 ± 1·3 vs. 130 ± 12·1, $P < 0.0005$) (Fig. 4a,c). Similarly, in the 56-day mature scars, $Flii^{+/-}$ mice displayed significantly less TGF-β1 in both skin layers compared with WT (epidermal: 103·3 ± 1·3 vs. 246·3 ± 4·8, $P < 0.005$; dermal: 23·8 ± 0·8 vs. 40·5 ± 0·12, $P < 0.05$) and $Flii^{Tg/Tg}$ (epidermal: 103·3 ± 1·3 vs. 348·8 ± 17·6, $P < 0.0005$; dermal: 23·8 ± 0·8 vs. 127·5 ± 9·8, $P < 0.0005$) (Fig. 4a,d).

Myofibroblasts are differentiated fibroblasts involved in wound contraction and contribute to the development of

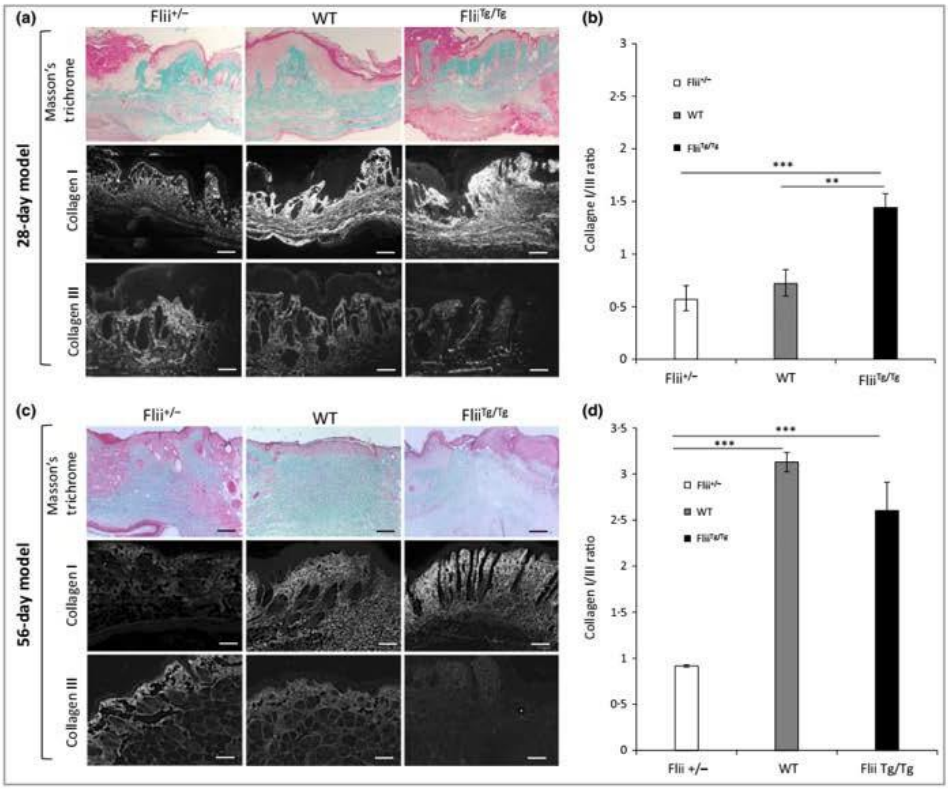


Fig 3. Decreasing Flii reduces collagen I/III ratio in the day-28 and day-56 fibrosis models. (a) Representative Masson's trichrome images and immunofluorescent images showing total collagen and collagen I and III distribution in day-28 scars. (b) Optical density analysis of immunofluorescent images shows a significant decrease in the collagen I/III ratio in $Flii^{+/-}$ compared with WT and $Flii^{Tg/Tg}$. (c) Representative Masson's trichrome images and immunofluorescent images showing total collagen and collagen I and III distribution in day-56 scars. (d) Optical density analysis of immunofluorescent images shows a significant decrease in the collagen I/III ratio in $Flii^{+/-}$ scars compared with WT and $Flii^{Tg/Tg}$ scars. Scale bar = 50 μm. n = 6. Mean ± SEM. *P < 0·05, **P < 0·005, ***P < 0·0005. Flii, Flightless I; WT, wild type.

hypertrophic scars. α-SMA is used as a marker of myofibroblasts and distinguishes them from undifferentiated dermal fibroblasts.[5] Day 28 and day 56 scars were stained for α-SMA and co-stained with the nuclear marker DAPI to identify myofibroblasts present within the fibrotic lesions (Fig. 4b). α-SMA-positive cells within the dermis were expressed as a percentage of total cells per field of view. Significantly fewer myofibroblasts were observed in 28-day developing/immature scars in the $Flii^{+/-}$ mice compared with WT (61 ± 4·3 cells vs. 174·5 ± 14·8 cells, P < 0·005) and $Flii^{Tg/Tg}$ (61 ± 4·3 cells vs. 195·4 ± 13·7cells, P < 0·0005) (Fig. 4e). Similarly, there were significantly fewer myofibroblasts in the mature scars of the $Flii^{+/-}$ mice compared with WT at 56 days (26·8 ± 2·2 cells vs. 96 ± 6·73 cells, P < 0·0005) and $Flii^{Tg/Tg}$ (26·8 ± 2·2 cells vs. 139·8 ± 5·5 cells, P < 0·0005) (Fig. 4f). There were also significantly fewer myofibroblasts in WT mature scars compared with $Flii^{Tg/Tg}$ (96 ± 6·73 cells vs. 139·8 ± 5·5 cells, P < 0·005) (Fig. 4f).

Flightless I neutralizing antibody treatment reduces fibrosis in day 28 scars

FnAbs have previously been shown to decrease the level of Flii in wounds and improve healing.[9,17] To determine if FnAb could reduce the development of hypertrophic scars, bleomycin-induced fibrotic lesions were injected with FnAb or IgG (control) at days 0, 7, 14 and 21. Histological assessment of day 28 scars showed that FnAb-treated mice had significantly smaller scar areas compared with IgG-treated controls (39 324·6 μm^2 ± 5126·4 μm^2 vs. 71 115·9 μm^2 ± 4897·3,

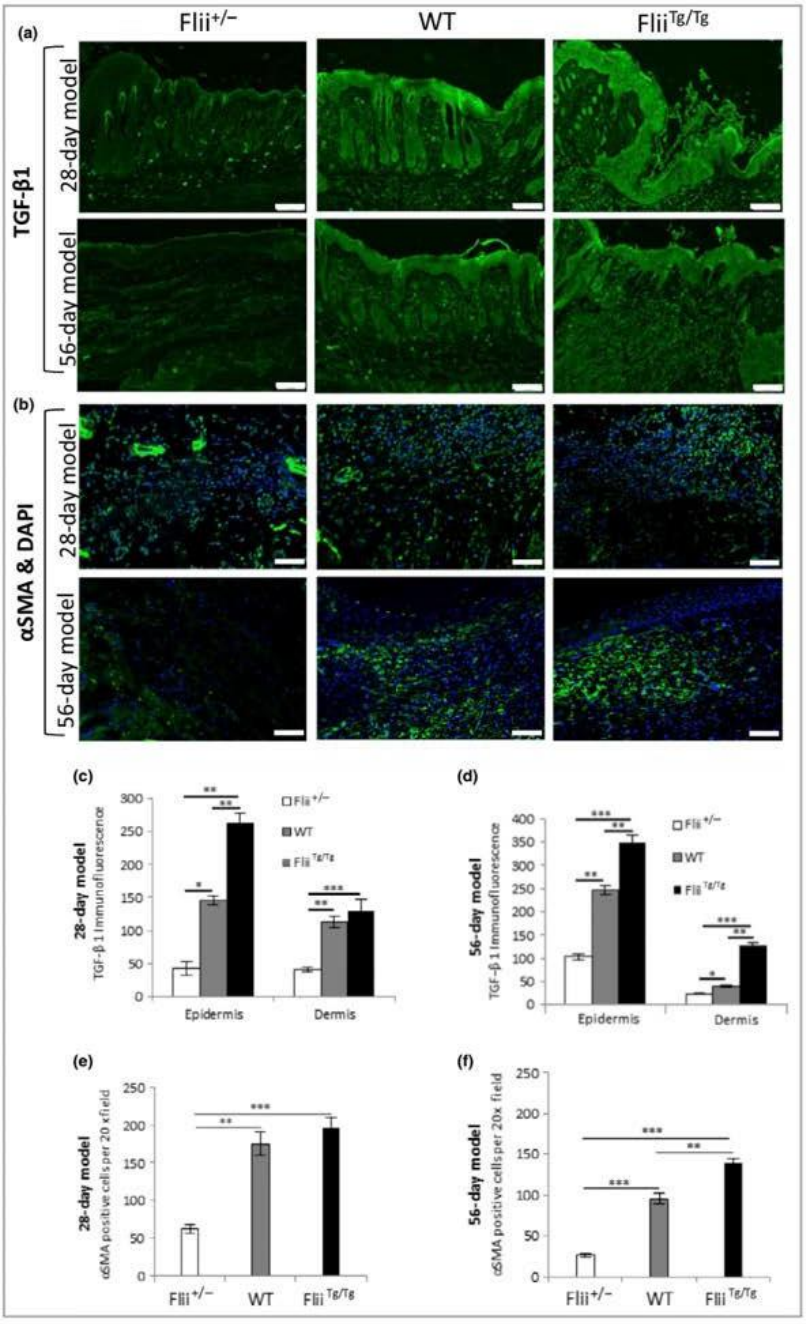


Fig 4. Manipulation of Flii affects key determinants of scarring. (a) Representative immunofluorescent images showing TGF-β1 expression throughout the epidermis and dermis of $Flii^{+/-}$, WT and $Flii^{Tg/Tg}$ scars. (b) Representative immunofluorescent images showing α-SMA and DAPI positive cells. Optical density analysis shows a significant increase in TGF-β1 as Flii levels increase across the genotypes at (c) 28 days and (d) 56 days. Microscopic analysis showed significantly fewer myofibroblasts in $Flii^{+/-}$ scars compared with WT and $Flii^{Tg/Tg}$ (e) 28 days and (f) 56 days. Scale bar = 50 μm. $n = 6$. Mean ± SEM. $^{*}P < 0{\cdot}05$, $^{**}P < 0{\cdot}005$, $^{***}P < 0{\cdot}0005$. α-SMA, α-smooth muscle actin; DAPI, 4′,6-diamidino-2-phenylindole; Flii, Flightless I, TGF, transforming growth factor; WT, wild type.

$P < 0{\cdot}05$) (Fig. 5a,b) and dermal thickness (154 μm ± 5·3 μm vs. 209·7 μm ± 31·7 μm, $P < 0{\cdot}05$). Furthermore, FnAb-treated scars also had significantly reduced collagen I/III ratios compared with IgG-treated controls (Fig. 5c,d) (0·824 ± 0·31 vs. 2·92 ± 0·98, $P < 0{\cdot}05$).

Discussion

This study has shown that Flii is upregulated in human burn wounds and hypertrophic scars compared with normal unwounded skin, suggesting a potential role for Flii in the regulation of the scarring process. However, given the heterogeneity of human samples, an animal model of hypertrophic scarring was required to investigate the role of Flii in more detail. An effective mouse model is that of bleomycin-induced fibrosis and this has been developed to induce developing and mature hypertrophic scarring, with histopathology that correlates well with human hypertrophic scars.[20,21] Bleomycin acts on fibroblasts, keratinocytes, endothelial and immune cells via TGF-β1 and CTGF to drive fibroproliferative events.[22] Additionally, the confounding influence of the panniculus carnosus present in rodent models, which causes earlier apposition of wound edges, is not a factor in this model.[21]

The study showed that increasing the level of Flii ($Flii^{Tg/Tg}$ mice) resulted in a significant increase in the severity of scar formation. Conversely, lowering Flii using a heterozygous knockout mouse (homozygous knockout being embryonic lethal) led to a reduction in scarring in terms of both dermal thickness and cross-sectional area. Additionally, when Flii levels were reduced using a neutralizing antibody (FnAb) administered in conjunction with the bleomycin treatment, a significant reduction in scar severity was observed, suggesting that decreasing the level of Flii could reduce the fibrosis observed with scar formation.

TGF-β1 is an important fibrogenic factor.[4] Reducing Flii expression has previously been reported to decrease TGF-β1/SMAD signalling, possibly through Flii forming a transcription complex with AP-1 proteins.[23] In this study, scars of $Flii^{+/-}$ mice had decreased levels of fibrogenic TGF-β1 compared with scars of both WT and $Flii^{Tg/Tg}$ mice lesions, suggesting that Flii may potentially assist in reducing scarring severity via an effect on TGF-β1. Myofibroblasts have also been implicated in the process of hypertrophic scarring[24] and the detection of myofibroblasts and TGF-β1 is highly significant for determining the degree of fibrosis.[5] In addition to producing the collagenous ECM comprising the hypertrophic scar, myofibroblasts can also promote fibrosis by secreting angiogenic, pro-inflammatory and fibrogenic factors.[25] Scars in $Flii^{+/-}$ mice had significantly fewer myofibroblasts in the healing environment, suggesting that the antiscarring effects of Flii may be mediated by an effect on myofibroblast differentiation, migration or survival.

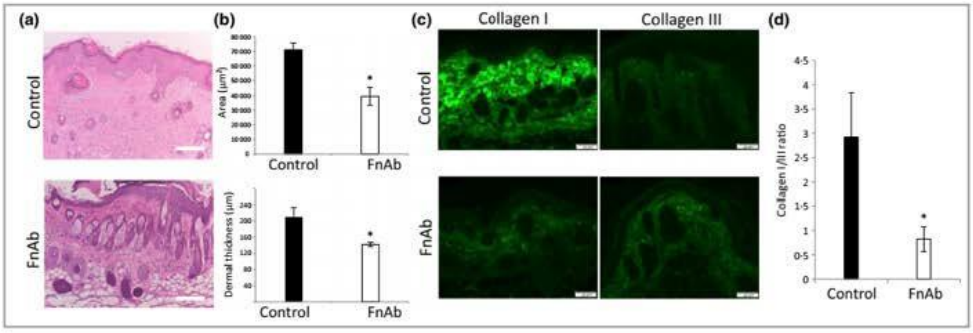


Fig 5. FnAb treatment reduces scarring in the 28-day model of acute fibrosis. (a) Treatment with 20 μg mL^{-1} FnAb caused a significant reduction in both dermal thickness and scar cross-sectional area compared with IgG-treated controls. Scale bar = 50 μm. $n = 3$. (b) Graph showing changes in significant reduction in dermal thickness and cross-sectional area in the 28-day model of acute fibrosis compared with IgG-treated controls. Mean ± SEM. $^{*}P < 0{\cdot}05$. (c) FnAb-treated scars also show a significant reduction in the collagen I/III ratio compared with IgG-treated controls. Scale bar = 50 μm. (d) Graph showing significant reduction in the collagen I/III ratio of FnAb-treated scars compared with IgG-treated controls. $n = 3$. Mean ± SEM. $^{*}P < 0{\cdot}05$. FnAb, Flightless I neutralizing antibody; IgG, immunoglobulin G; WT, wild type.

The collagen I/III ratio has been identified as an important factor in determining severity of scar formation.[26] Collagen III is a member of the fibrillar collagen family and is colocalized with collagen I in the skin. Collagen III is essential for normal collagen I fibrillogenesis by regulating the diameter of the collagen I fibril.[27] An increased collagen I/III ratio is associated with hypertrophic scarring.[6] In keeping with the improvement in scar thickness and area as seen in $Flii^{+/-}$ mice, $Flii^{+/-}$ scars had significantly reduced collagen I/III ratios compared with WT and $Flii^{Tg/Tg}$ scars. Similarly, day 28 model mice treated with FnAb had significantly lower collagen I/III ratios compared with IgG-treated controls.

This study has demonstrated that Flii is a key regulator of the fibroproliferative process underpinning hypertrophic scarring. Reducing Flii in a bleomycin model of hypertrophic scarring, either genetically via gene knockdown, or via treatment with FnAbs, leads to significant improvements in scarring. Reducing Flii also led to a reduction in key determinants of scarring including TGF-β1and myofibroblast numbers. This study therefore identifies Flii as a potential therapeutic target for a novel therapy that may prevent or reduce hypertrophic scarring.

References

1 Engrav LH, Garner WL, Tredget EE. Hypertrophic scar, wound contraction and hyper-hypopigmentation. *J Burn Care Res* 2007; **28**:593–7.

2 Robson MC, Steed DL, Franz MG. Wound healing: biologic features and approaches to maximize healing trajectories. *Curr Probl Surg* 2001; **38**:72–140.

3 Bielefeld KA, Amini-Nik S, Alman BA. Cutaneous wound healing: recruiting developmental pathways for regeneration. *Cell Mol Life Sci* 2012; **70**:2059–81.

4 Wynn TA. Cellular and molecular mechanisms of fibrosis. *J Pathol* 2008; **214**:199–210.

5 Hinz B. The myofibroblast: paradigm for a mechanically active cell. *J Biomech* 2010; **43**:146–55.

6 Hayakawa T, Hashimoto Y, Myokei Y *et al.* The effect of skin grafts on the ratio of collagen types in human post-burn wound tissues. *Connect Tissue Res* 1982; **9**:249–52.

7 Zhang K, Garner W, Cohen L *et al.* Increased types I and III collagen and transforming growth factor-beta 1 mRNA and protein in hypertrophic burn scar. *J Invest Dermatol* 1995; **104**:750–4.

8 Rahban SR, Garner WL. Fibroproliferative scars. *Clin Plast Surg* 2003; **30**:77–89.

9 Cowin AJ, Adams DH, Strudwick XL *et al.* Flightless I deficiency enhances wound repair by increasing cell migration and proliferation. *J Pathol* 2007; **211**:572–81.

10 Kopecki Z, O'Neill GM, Arkell RM, Cowin AJ. Regulation of focal adhesions by flightless I involves inhibition of paxillin phosphorylation via a Rac1-dependent pathway. *J Invest Dermatol* 2011; **131**:1450–9.

11 Claudianos C, Campbell HD. The novel flightless-I gene brings together two gene families, actin-binding proteins related to gelsolin and leucine-rich-repeat proteins involved in Ras signal transduction. *Mol Biol Evol* 1995; **12**:405–14.

12 Campbell HD, Schimansky T, Claudianos C *et al.* The *Drosophila melanogaster* flightless-I gene involved in gastrulation and muscle degeneration encodes gelsolin-like and leucine-rich repeat domains and is conserved in *Caenorhabditis elegans* and humans. *Proc Natl Acad Sci USA* 1993; **90**:11386–90.

13 Davy DA, Ball EE, Matthaei KI *et al.* The flightless I protein localizes to actin-based structures during embryonic development. *Immunol Cell Biol* 2000; **78**:423–9.

14 Lei N, Franken L, Ruzehaji N *et al.* Flightless, secreted through a late endosome/lysosome pathway, binds LPS and dampens cytokine secretion. *J Cell Sci* 2012; **125**(Pt 18):4288–96.

15 Kopecki Z, Arkell R, Powell BC, Cowin AJ. Flightless I regulates hemidesmosome formation and integrin-mediated cellular adhesion and migration during wound repair. *J Invest Dermatol* 2009; **129**:2031–45.

16 Adams DH, Ruzehaji N, Strudwick XL *et al.* Attenuation of Flightless I, an actin-remodelling protein, improves burn injury repair via modulation of transforming growth factor (TGF)-beta1 and TGF-beta3. *Br J Dermatol* 2009; **161**:326–36.

17 Jackson JE, Kopecki Z, Ali RG *et al.* Flii neutralizing antibodies improve wound healing in porcine preclinical studies. *Wound Repair Regen* 2012; **20**:523–36.

18 Thomsen N, Chappell A, Ali RG *et al.* Mouse strains for the ubiquitous or conditional overexpression of the Flii gene. *Genesis* 2011; **49**:681–8.

19 Campbell HD, Fountain S, McLennan IS *et al.* Fliih, a gelsolin-related cytoskeletal regulator essential for early mammalian embryonic development. *Mol Cell Biol* 2002; **22**:3518–26.

20 Cameron AM, Adams DH, Greenwood JE *et al.* A novel murine model of hypertrophic scarring using subcutaneous infusion of bleomycin. *Plast Reconstr Surg* 2014; **133**:69–78.

21 Wong VW, Sorkin M, Glotzbach JP *et al.* Surgical approaches to create murine models of human wound healing. *J Biomed Biotechnol* 2011; **2011**:969618.

22 Yamamoto T. Bleomycin and the skin. *Br J Dermatol* 2006; **155**:869–75.

23 Kopecki Z, Arkell RM, Strudwick XL *et al.* Overexpression of the Flii gene increases dermal–epidermal blistering in an autoimmune ColVII mouse model of epidermolysis bullosa acquisita. *J Pathol* 2011; **225**:401–13.

24 Junker JP, Kratz C, Tollback A, Kratz G. Mechanical tension stimulates the transdifferentiation of fibroblasts into myofibroblasts in human burn scars. *Burns* 2008; **34**:942–6.

25 Darby IA, Hewitson TD. Fibroblast differentiation in wound healing and fibrosis. *Int Rev Cytol* 2007; **257**:143–79.

26 Dale PD, Sherratt JA, Maini PK. A mathematical model for collagen fibre formation during foetal and adult dermal wound healing. *Proc Biol Sci* 1996; **263**:653–60.

27 Liu X, Wu H, Byrne M *et al.* Type III collagen is crucial for collagen I fibrillogenesis and for normal cardiovascular development. *Proc Natl Acad Sci USA* 1997; **94**:1852–6.

EXPERIMENTAL

A Novel Murine Model of Hypertrophic Scarring Using Subcutaneous Infusion of Bleomycin

Alexander M. Cameron, M.B.B.S., B.Med.Sci.
Damian H. Adams, B.Biotech.(Hons.)
John E. Greenwood, M.B.Ch.B., M.D.
Peter J. Anderson, M.D., Ph.D.
Allison J. Cowin, Ph.D.

Mawson Lakes, Adelaide, and North Adelaide, South Australia, Australia

Background: The development of new therapies for hypertrophic scarring has been hampered by the lack of an appropriate animal model. The authors' objective was to establish a reproducible murine model of hypertrophic scarring by infusing bleomycin over a prolonged period to stimulate dermal fibroproliferation.
Methods: Osmotic pumps filled with 90 μl of 2.8 mg/ml bleomycin or a control solution (phosphate-buffered saline) were inserted subcutaneously under the dorsal skin of BALB/c mice. The pumps delivered their content at a constant rate of 0.11 μl/hour for 28 days before mice were euthanized or kept alive for a further 28 days and euthanized at day 56. The resulting lesions were analyzed using histological and immunohistochemical techniques.
Results: The lesions displayed histopathological features of hypertrophic scar similar to those observed in humans and had increased cellularity, abnormal collagen I–collagen III ratios, elevated levels of the proscarring cytokine transforming growth factor β1, and increased numbers of myofibroblasts. The 28-day model displayed features analogous to those of a developing human hypertrophic scar, while the 56-day model was analogous to a mature hypertrophic scar.
Conclusions: The bleomycin infusion model stimulates dermal fibroproliferation, creating reproducible murine scars that are comparable to human hypertrophic scars in terms of histological features, collagen content and organization, cellularity, the presence of myofibroblasts, and expression of transforming growth factor β1. The bleomycin model represents a promising technique for studying scar formation and testing new antiscarring therapies. (*Plast. Reconstr. Surg.* 133: 69, 2014.)

Hypertrophic scarring is commonly associated with burn injury, but it may also occur after trauma or elective surgery.[1,2] It carries a considerable burden of disease, which includes disfigurement, pain, disability, and psychological comorbidity.[3] Treatment of hypertrophic scarring involves hundreds of hours of dressing changes, physical therapy, and, often, numerous surgical procedures. In the United States alone, this treatment is estimated to cost in excess of $4 billion every year.[4] The antecedents to hypertrophic scarring, such as deep burn injury, wound tension, wound infection, or the presence of foreign material, were identified at the beginning of modern surgery.[5–9] However, how these lead to changes at the cellular and molecular levels that drive fibroproliferation is poorly understood, and little

From the Center for Regenerative Medicine, Mawson Institute, University of South Australia; the Disciplines of Surgery and Pediatrics, School of Medicine, Faculty of Health Sciences, The University of Adelaide; the Adult Burns Service, Royal Adelaide Hospital; and the Australasian Craniofacial Unit, Women's and Children's Hospital.
Received for publication May 7, 2013; accepted June 26, 2013.

DOI: 10.1097/01.prs.0000436821.26709.a7

Presented in part at the Wound Healing Society 22nd Annual Meeting, in Atlanta, Georgia, April 19 through 22, 2012 (Young Investigator Award finalist and Travel Scholarship recipient); Royal Australasian College of Surgeons 81st Annual Scientific Congress, in Kuala Lumpur, Malaysia, May 6 through 10, 2012; the 36th Annual Scientific Meeting of the Australian and New Zealand Burn Association, in Hobart, Australia, October 9 through 12, 2012 (Best Scientific Presentation runner-up); Conjoint Third Australian Wound and Tissue Repair Society and Ninth Australian Society for Dermatological Research Conference, in Sydney, Australia, May 22 through 24, 2012 (Travel Scholarship recipient).

Disclosure: *The authors have no financial interest to declare in relation to the content of this article.*

progress has been made in developing new therapies to prevent or reduce hypertrophic scaring.

In vitro cell lines and primary cell culture have been used to investigate the role of the different cell types and cytokines in hypertrophic scarring. However, the usefulness of this technique is limited by the considerable differences that exist between controlled culture conditions and the wound-healing environment, where there are varied and complex interactions among many different cell types, cytokines, and extracellular matrix components. Other studies have attempted analysis of ex vivo hypertrophic scar tissue excised from human patients. However, these samples usually represent an end stage of the scarring process and provide little information about mechanisms preceding this. Given these limitations, the need for an animal model of hypertrophic scarring is manifest.

The development of an appropriate animal model has been problematic,[4] owing much to the phylogenetic variation in wound healing.[10] For example, differences between mouse and human skin include denser hair covering (with a shorter growth cycle than human hair); absence of dermal papillae/apocrine glands; and the presence of a full panniculus carnosus, which rapidly apposes wound edges after injury.[11] There have been numerous attempts to develop an animal model of human hypertrophic scarring. Several models involve xenografts of human skin or hypertrophic scar onto immunodeficient mice[12,13] and rats.[14] Although these models produced some of the histological changes seen in hypertrophic scars, they required significant suppression of the animals' immune system, rendering any conclusions difficult to extrapolate to the human milieu. The Duroc pig displays scarring properties similar to those of humans,[15,16] but the experimental value of such a large animal model is limited by expense, housing, and handling difficulties. Recently, a new model utilizing applied mechanical force (a well-recognized risk factor for hypertrophic scarring) across a murine incisional wound to stimulate fibroproliferation has been developed.[4] Some models employ silicone splints to counter the effect of panniculus carnosus.[17] Despite these attempts, the need for a practical and reproducible animal model of hypertrophic scarring remains.

The rationale behind our murine model is to recreate the pathological process underlying hypertrophic scarring by using bleomycin infusion to stimulate dermal fibroproliferation. Bleomycin is an antibiotic, originally isolated from *Streptomyces verticillus* and widely used as an anticancer treatment.[18] Bleomycin hydrolase inactivates bleomycin by hydrolyzing the amide bond in the b-aminoalanineamide moiety. However, the lack (or shortage) of this enzyme in the lungs and the skin allows bleomycin-induced fibrosis and sclerosis to occur in these organs in a dose-dependent manner.[2] Bleomycin was first used to create animal models of pulmonary fibrosis, before Yamamoto and colleagues established a mouse model of scleroderma using daily subcutaneous injections over a 4-week period.[19,20]

We have adapted the dosage, delivery, and temporal characteristics of this model to develop a novel model of hypertrophic scarring. Bleomycin is delivered continuously via a subcutaneous osmotic pump (Alzet Model 1004; Durect Corp., Cupertino, Calif.) in order to stimulate a fibrotic process rather than the dermal sclerosis described by Yamamoto et al.[19] Bleomycin infusion provides a continuous inflammatory stimulus, in contrast to the daily dosing used in the scleroderma model. Mice were euthanized at 28 days or 56 days in an attempt to model both immature and mature hypertrophic scars.

MATERIALS AND METHODS

Reagents and Antibodies

Rabbit monoclonal anti-collagen I (600-401-103-0.5) and anti-collagen III (600-401-105-0.1 antibodies) were obtained from Rockland (Gilbertsville, Pa.). Anti-collagen I and anti-collagen III antibodies are raised against collagen I and collagen III from human and bovine placenta, respectively. Rabbit polyclonal anti–transforming growth factor (TGF)-β1 immunoglobulin G antibody (sc-146) was obtained from Santa Cruz Biotechnology (Santa Cruz, Calif.). Anti-TGF-β1 is mapped against the C-terminus of TGF-β1 of human origin. Mouse monoclonal anti–α-smooth muscle actin (αSMA) immunoglobulin G antibody (A2547) was obtained from Sigma Aldrich (St. Louis, Mo.). Mouse monoclonal anti-αSMA is raised against the single isoform, α-actin. Secondary goat anti-rabbit (A-11034) and goat anti-mouse (A-11001) Alexa Fluor 488 were obtained from Life Technologies (Carlsbad, Calif.). Murine immunoglobulin G irrelevant antibody (I8765) was obtained from Sigma Aldrich. 4',6-Diamidino-2-phenylindole (D9564) was obtained from Sigma Aldrich.

Human Samples

Samples of normal human skin and hypertrophic scars were obtained from the Royal Adelaide Hospital Adult Burn Unit, with ethics approval

from the Royal Adelaide Hospital Ethics Committee. Consent was obtained from patients undergoing surgery to obtain excised tissue that would otherwise be discarded.

Animal Studies

All experiments were approved by the Adelaide Women's and Children's Hospital Animal Care and Ethics Committee and followed the Australian Code of Practice for the Care and the Use of Animals for Scientific Purposes. Studies were performed using mice with the BALB/c background.

Murine Surgical Technique

Before surgery, osmotic pumps (Alzet Model 1004) were filled to their 90-μl capacity with 2.8 mg/ml bleomycin sulphate (R-42569; Hospira, Mulgrave North, Victoria, Australia) or a sterile control solution of phosphate-buffered saline (Fig. 1). Pumps are designed to produce a consistent rate of flow of 0.11 μl/hour for 28 days. Pumps were weighed before and after filling to ensure correct volume. Mice were anaesthetized using gaseous isofluorane, 5% induction, 2% maintenance. The dorsum of the mice was shaved and cleaned with 10% povidine iodine solution. A 0.5-cm transverse incision was made across the midline at a point approximately 4 cm caudal to the base of the skull. Using blunt dissection, a pocket was made between the skin and muscle layers into which the osmotic pump was inserted. Wounds were closed with 4-0 Prolene and secured with tissue adhesive (Histoacryl; TissueSeal, Ann Arbor, Mich.) (Fig. 2). Mice were euthanized at 28 days or 56 days after pump insertion, and a 4 × 1.5-cm area of skin was excised at a location corresponding to the cranial end of the osmotic pump. Unwounded skin was collected from each mouse group.

Histologic, Immunohistochemical, and Image Analysis

Each tissue sample was fixed in 10% buffered formalin and embedded in paraffin. Serial histological sections (4 μm) of the 4 × 1.5-cm sample were prepared to ensure that all areas of the lesions were sectioned. Sections were stained with hematoxylin and eosin or Masson's trichrome stain or subjected to immunohistochemical analysis for 4',6-diamidino-2-phenylindole, collagen I, collagen III, TGF-β1, and αSMA.

Analysis of the area of scarring was performed using the Image Pro-Plus 5.1 program (Media Cybernetics, Inc., Rockville, Md.). Serial sections were stained and examined until the point of maximal dermal thickness or scar cross-sectional area was found. Dermal thickness was measured as the distance from the basement membrane to the panniculus carnosus.

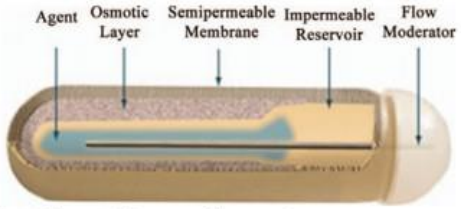


Fig. 1. Schematic diagram of the osmotic pump.

Immunochemical analysis was performed after antigen retrieval. After they were blocked in 3% normal goat serum, primary antibodies were applied at a concentration of 2 μg/ml. Slides were incubated overnight at 4°C in a humidified chamber. Species-specific Alexa Fluor 488 was used for detection at a concentration of 2 μg/ml for all primary antibodies, except antibodies for collagen 1 and collagen 3, for which species-specific biotinylated secondary antibodies and signal enhancement with streptavidin was used.

Image analysis was performed using the AnalySIS software package (Soft Imaging System GmbH, Munster, Germany). Negative controls included replacing primary antibodies with normal rabbit immunoglobulin G or normal mouse immunoglobulin G. For verification of staining, nonspecific binding was determined by omitting primary or secondary antibodies. All control sections had negligible immunofluorescence.

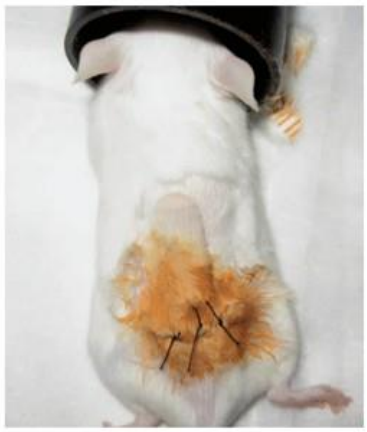


Fig. 2. The appearance of the osmotic pump after insertion.

Statistical Analysis

Statistical differences were determined using *t* test or analysis of variance. For data not following a normal distribution, the Mann-Whitney *U* test was performed. A *p* value of less than 0.05 was considered significant.

RESULTS

The osmotic pump and infusion were well tolerated by the mice (Fig. 2). There were no obvious systemic differences in the bleomycin cohort compared with the control cohort. As the model did not involve a gross epidermal breach and the pump was positioned away from the insertion site (fig. 2), no macroscopic scarring was observed. The appearance of the murine skin was assessed for gross changes in appearance, but none was observed between the treatment groups.

Bleomycin Model Produces Tissue Architecture That Corresponds to Hypertrophic Scars

To determine whether the lesions produced in response to bleomycin infusion reproduced the features of hypertrophic scar, normal mouse skin and lesions from the 28-day and 56-day models were stained with hematoxylin and eosin. They were examined under light microscopy and compared with similarly stained normal human skin and hypertrophic scar samples from the RAH Adult Burn Center (Fig. 3).

No difference was observed between phosphate-buffered saline–infused control skin and untreated murine skin in terms of dermal thickening or skin architecture for the 28-day model or the 56-day model (data not shown). The 28-day model lesions displayed marked dermal thickening, which was significantly increased compared with control (202.5 ± 30.93 μm versus 30 ± 5 μm, $p < 0.0005$). This was associated with a hyperplastic epidermis, increased cellularity, and a reduction in the number of dermal appendages compared with control (Fig. 3). The 56-day model lesions had significantly increased dermal thickness compared with both control samples and 28-day model samples (726.72 ± 77.18 μm versus 30 ± 5 μm, $p < 0.0005$; 726.72 ± 77.18 μm versus 202.5 ± 30.93 μm, $p < 0.0005$) (Fig. 3).

Bleomycin Model Displays Histopathologic Features of Hypertrophic Scars

There are a number of classic histopathologic features associated with hypertrophic scars, including collagen nodules and whorls, thickened hyperplastic epidermis, and abnormal dermal collagen organization.[21] Samples of immature and mature human hypertrophic scars obtained from burn patients, as well as lesions from the 28-day and 56-day models, were stained with hematoxylin and eosin. These samples were examined by light microscopy to determine whether histopathologic features of human hypertrophic scars were reproduced in bleomycin-induced lesions.

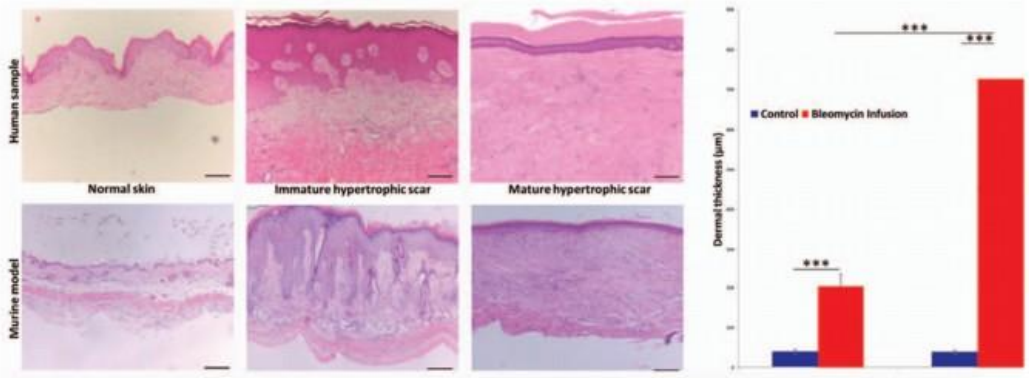


Fig. 3. The bleomycin model reproduces histologic architecture of hypertrophic scars. (*Left*) Control (phosphate-buffered saline–treated) murine skin, 28-day model lesion, and 56-day model lesion compared with normal human skin, immature hypertrophic scar, and mature hypertrophic scar. The 28-day model displays dermal thickening, a paucity of dermal appendages, and hyperplastic epidermis. These features are consistent with those displayed in an immature hypertrophic scar. The 56-day model displays a marked dermal thickening, absence of dermal appendages, and a flattened epidermis. These features are consistent with those displayed in a mature hypertrophic scar. (*Right*) Quantification of dermal thickness was performed using the Image Pro-Plus 5.1 program. *Scale bars* = 100 μm; ***$p < 0.0005$; *error bars* = SEM.

Collagen nodules and collagen whorls are features of hypertrophic scar and were present in lesions of both the 28-day model and the 56-day model, although they appeared to be most prevalent in the 28-day lesions (Fig. 4).

In both models, abnormal dermal collagen organization was observed. Collagen arrangement was disorganized in the 28-day model, while the 56-day model displayed collagen arranged in sheets parallel to the surface of the skin. This corresponds to the collagen arrangement seen in immature and mature hypertrophic scars, respectively (Fig. 4).

Epidermal layers of the 28-day and 56-day model lesions displayed basal disarray and vacuolar change. The epidermis of 28-day model lesions was hyperplastic and displayed rete pegs and dermal papillae, whereas the epidermis of the 56-day model was flattened and did not display the same degree of hyperplasia (Fig. 4).

Collagen Content and Composition of Bleomycin-Induced Lesions Are Analogous to Those of Hypertrophic Scars

Alterations in the amount of collagen and the ratio of collagen I to collagen III are associated with hypertrophic scarring.[22,23] Immunohistochemical localization and quantification of collagen I and collagen III revealed a significant increase in the ratio of 28-day model lesions compared with normal murine skin (3.12 ± 0.10 versus 0.90 ± 0.11, $p < 0.0005$). The collagen I–collagen III ratio in the 56-day model was significantly lower than that in the 28-day model (0.72 ± 0.12 versus 3.12 ± 0.10, $p < 0.0005$). There was no significant difference between the 56-day ratio and the ratio of normal murine skin (Fig. 5).

Key Determinants of Fibroproliferation Are Increased in the Bleomycin Model

Normal mouse skin, 28-day lesions, and 56-day lesions were costained with αSMA and 4',6-diamidino-2-phenylindole to identify myofibroblasts. Myofibroblasts are the key cell type found in hypertrophic scars and express αSMA, which distinguishes them from fibroblasts and gives them their contractile properties.[24] 4',6-Diamidino-2-phenylindole is a fluorescent stain that binds strongly to A-T–rich regions in DNA and therefore is used in immunofluorescent staining to identify cell nuclei.

Myofibroblasts were identified as αSMA-positive cells within the dermis of the samples and expressed as a percentage of total cells per

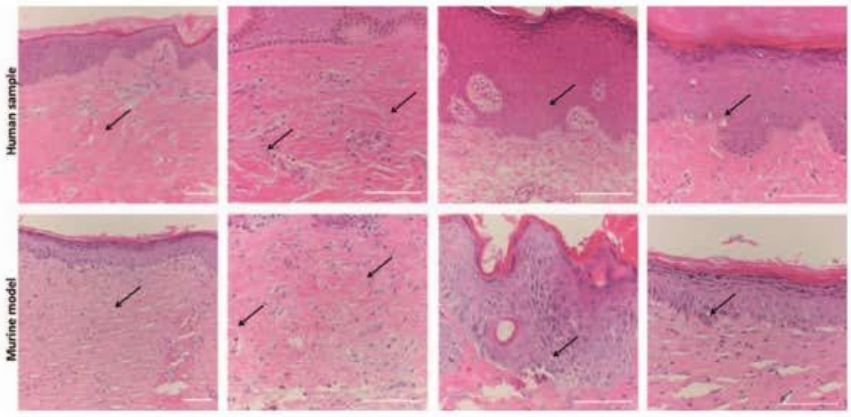


Fig. 4. The bleomycin model reproduces histopathologic features of hypertrophic scars. Samples of immature hypertrophic scars, mature hypertrophic scars, and lesions from 28-day model and 56-day model were stained with hematoxylin and eosin and photographed at 20 times or 40 times magnification. Mature hypertrophic scars demonstrated parallel sheets of collagen in the dermis, and similar collagen organization was seen in the 56-day model (*left; arrows*). Immature hypertrophic scars demonstrated collagen nodules and whorls; similar nodules whorls were observed in the dermis of 28-day model lesions (*second from left; arrows*). Hyperplasia and basal cell disarray were seen in immature hypertrophic scars; similar epidermal changes were seen in the 28-day model lesions (*second from right; arrows*). Mature hypertrophic scars displayed a flattened epidermal layer with basal disarray and vacuolar change; similar epidermal changes were seen in 56-day model lesions (*right; arrows*). *Scale bars* = 100 μm.

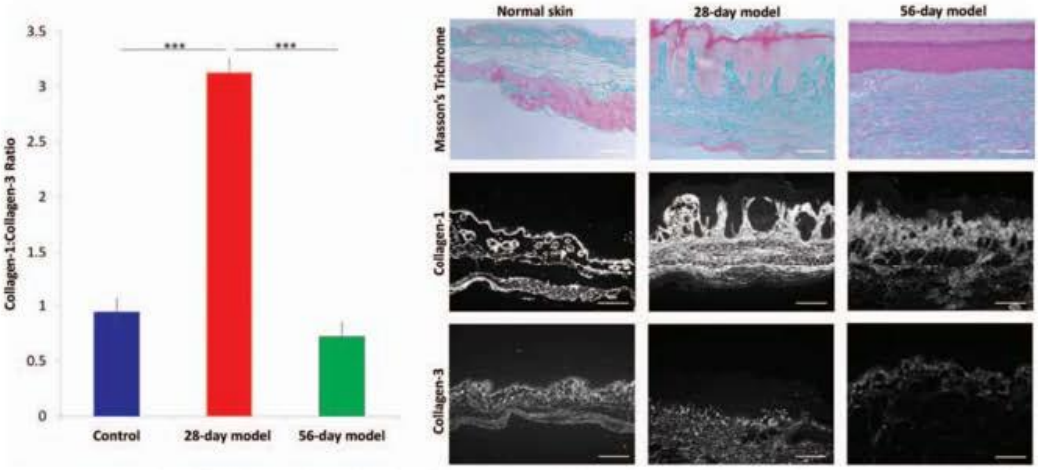


Fig. 5. Lesions produced by the 28-day and 56-day bleomycin models have altered collagen I–collagen III ratios. Samples from control skin, 28-day model lesions, and 56-day model lesions were stained using Masson's trichrome and using immunohistochemistry for collagen I and collagen III (*right*). Collagen expression was then quantified using the AnalySIS software package, and these data were used to calculate the collagen I:collagen III sample for each lesion (*left*). *Scale bars* = 100 μm; ***$p < 0.0005$; *error bars* = SEM.

high-powered field. There was a significant increase in the percentage of αSMA-positive cells per high-powered field in both the 28-day (87.67 ± 2.30 percent versus 18.19 ± 3.05 percent, $p < 0.0005$) and 56-day models (76.84 ± 4.14 percent versus 18.19 ± 3.05 percent, $p < 0.0005$) compared with normal skin. The reduction in the percentage of αSMA-positive cells per high-powered field in the 56-day model lesions compared with the 28-day model lesions was statistically significant (87.67 ± 2.30 percent versus 76.84 ± 4.14 percent, $p < 0.05$) (Fig. 6).

TGF-β1 is the most investigated proinflammatory and proscarring cytokine.[22] Normal mouse skin, 28-day lesions, and 56-day lesions were stained using immunohistochemistry for TGF-β1. TGF-β1 was expressed in the epidermal layer of 28-day lesions and 56-day lesions and also in cells throughout the dermis. These images then quantified using the AnalySIS software package. There was a significant increase in TGF-β1 expression in the epidermal layer of both the 28-day model lesions (14.00 ± 1.95 versus 3.00 ± 0.91, $p < 0.0005$) and the 56-day model lesions (18.00 ± 2.34 versus 3.00 ± 0.91, $p < 0.0005$). There was also a significant increase in dermal TGF-β1 in both the 28-day model lesions (9.11 ± 1.74 versus 1.72 ± 0.18, $p < 0.0005$) and the 56-day model lesions (3.62 ± 0.50 versus 1.72 ± 0.18, $p < 0.005$). There was a statistically significant reduction in dermal TGF-β1 in the 56-day model lesions compared with the 28-day model lesions (3.62 ± 0.50 versus 9.11 ± 1.74 $p < 0.005$) (Fig. 7).

DISCUSSION

Most existing models of hypertrophic scarring have focused on reproducing the pathological characteristics of the hypertrophic scar rather than modeling the process that leads to its development.[25] Hypertrophic scars and keloids were originally defined by their clinical characteristics, before attempts were made to define them as distinct pathologies, in terms of their histologic characteristics, such as parallel collagen fibers, collagen whorls, and epidermal flattening.[21] However, producing evidence to support these definitions has been difficult; indeed, the histologic distinction between normal scars, hypertrophic scars, and keloid scars is controversial.[21,26] There is currently no consensus in the literature regarding the relative contribution of different collagen subtypes to hypertrophic scar tissue fibrillogenesis nor the importance or temporal profile of key cell types, such as myofibroblasts, or well-known cytokines, such as TGF-β1.[18,27–29]

It may be important to recontextualize hypertrophic scarring as a fibroproliferative disease, one sharing a common mechanism of fibrosis with systemic scleroderma, lung fibrosis, or

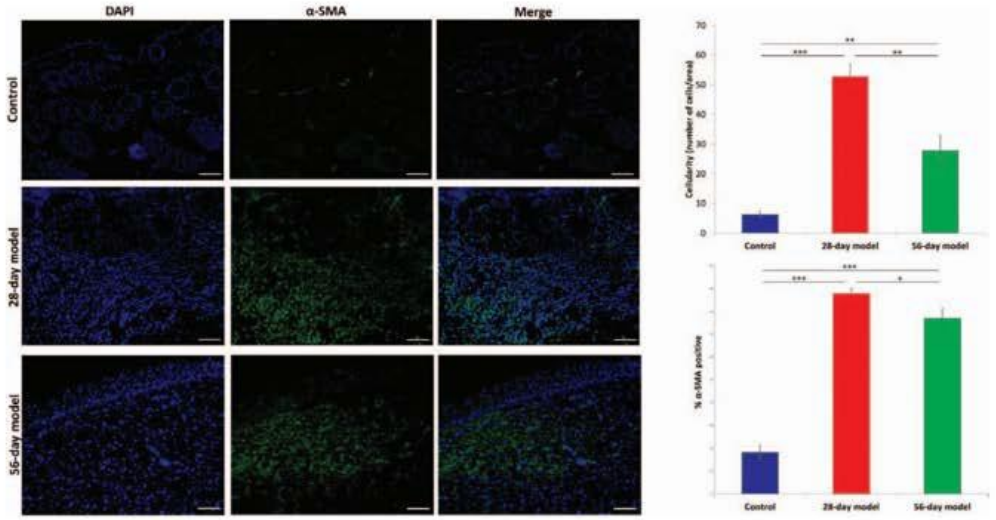


Fig. 6. Normal mouse skin, 28-day lesions, and 56-day lesions were costained using immunohistochemistry for αSMA and 4′,6-diamidino-2-phenylindole (*DAPI*) (*left*). These images were then quantified using the AnalySIS software package in terms of cellularity per high-powered field and the percentage of these cells positive for αSMA (*right*). *Scale bars* = 100 μm; ***$p < 0.0005$; **$p < 0.005$; *$p < 0.05$; *error bars* = SEM.

glomerulosclerosis, albeit it with a unique organ (the skin) and etiology.[30] It follows that, instead of settling on the hypertrophic scar phenotype as an endpoint, an ideal animal model would be one that focuses on the fibroproliferative process that characterizes hypertrophic scarring and other fibrotic diseases.

Previous studies have used a daily injection of bleomycin into the skin of mice to induce scleroderma.[19] In these trials, bleomycin-treated dermis showed sclerosis with thickened, homogenous collagen bundles, perivascular infiltrates, and thickening of the vascular wall. We adapted this method by delivering bleomycin via a subcutaneous osmotic pump, which delivered bleomycin at a constant rate of 0.11 μl/hour for 28 days. This technique not only limited animal pain and distress but also provided a continuous inflammatory stimulus, in contrast to once-daily doses.[14] Bleomycin has a relatively short half-life and has been demonstrated to exert a greater dose response when delivered as an infusion rather than in individual doses.[31,32]

This model generated all the histopathologic features that characterize human hypertrophic scarring, including dermal thickening due to collagen deposition, parallel collagen fiber orientation, collagen whorls, loss of dermal appendages, and flattening of the epidermis.

Immature and mature hypertrophic scars were obtained depending on the length of time that samples were taken after treatment with bleomycin. After 28 days of bleomycin infusion, murine skin exhibited a thickened epidermis and inflammatory cell infiltrate consistent with a developing or immature hypertrophic scar. However, at day 56, dermal thickness had continued to increase, and an absence of dermal appendages and a flattened epidermis were observed. This ongoing fibroproliferation occurred even though the bleomycin infusion had ceased at day 28; this is consistent with Takagawa et al.'s results showing sustained phenotypic changes in fibroblasts after exposure to bleomycin.[33]

Changes in extracellular matrix composition and collagen fibrillogenesis are key features of fibrotic disease.[34] It is known that the ratio of collagen I to collagen III is altered in fibrotic diseases, including hypertrophic scarring, although there is conflicting evidence as to the exact nature of this alteration due to most studies using heterogeneous groups of human samples and varying considerably in their methodology.[29,35] The 28-day mouse model showed a significant increase in the ratio of collagen I to collagen III compared with normal mouse skin. The 56-day model had a collagen I–collagen III ratio that was not significantly different from that

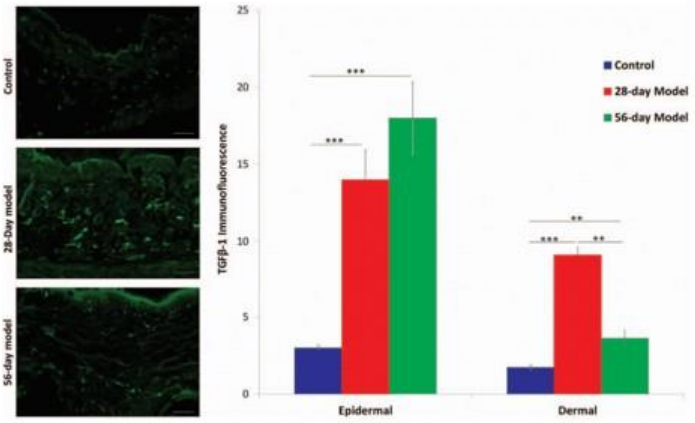


Fig. 7. Normal mouse skin, 28-day lesions, and 56-day lesions were stained using immunohistochemistry for TGF-β1 (*left*). TGF-β1 was expressed in the epidermal layer of 28-day lesions and 56-day lesions and also in cells throughout the dermis. These were images then quantified using the AnalySIS software package (*right*). *Scale bars* = 50 μm; $^{**}p < 0.0005$; *error bars* = SEM.

of normal skin. These changes may reflect the reorganization and remodeling of collagen subtypes similar to that occurring in normal wound healing, where under normal conditions collagen III produced early in the proliferative phase of wound healing is replaced by collagen I. Further investigation of collagen production and fibrillogenesis by more informative methods, such as electron microscopy, is required

Bleomycin is known to induce production of reactive oxygen species and cause damage to endothelial and other cells types.[36] In addition, bleomycin directly stimulates the profibrotic TGF-β pathway, as well as a range of other proinflammatory and profibrotic mediators, such as chemoattractant protein 1, platelet-derived growth factor, and interleukins 4, 6, and 13.[36] In this way, bleomycin causes scarring by direct activation of inflammatory and fibrotic mediators that have been shown to be important to the development of human hypertrophic scarring. This process does not involve an epidermal injury, which is the first event in any human wound leading to hypertrophic scarring. The benefit of avoiding epidermal wounding in the mouse model is that it circumvents the need to deal with the confounding differences between murine and human wound healing. In mice, the panniculus carnosus causes early wound contraction, limiting the requirement for healing by secondary intention.[37] By using bleomycin, we directly stimulate the fibroproliferative pathway common to every hypertrophic scar.

It follows that one criticism of the bleomycin model is that it does not allow investigation of the role of restoring epidermal integrity in reducing hypertrophic scarring, which is known to be important in the reduction of hypertrophic scarring in burn injury.[38] This might be better investigated using a model designed specifically to measure re-epithelialization (such as an excisional wound).

A novel model of immature and mature hypertrophic scarring has been created, using the infusion of bleomycin to stimulate dermal fibroproliferation. This model reproduces morphologic, histologic, and biochemical characteristics of human hypertrophic scars and presents two distinct windows in which to investigate scar formation and test potential antiscarring therapies. For investigations directed at the inflammatory response that drives fibroproliferation, the 28-day model would be most appropriate. For therapies designed to increase scar remodeling, the 56-day model would be potentially useful.

The bleomycin model has considerable potential to be used to understand the complex processes involved in the formation of hypertrophic scars and allows for preclinical evaluation of antiscarring therapies, whether they are directed at the aberrant inflammatory response that leads to hypertrophic scarring or aimed at altering the composition of a mature scar.

Alexander M. Cameron, M.B.B.S., B.Med.Sci.
Center for Regenerative Medicine
Mawson Institute, Building V
University of South Australia
Mawson Lakes Campus
Mawson Lakes Boulevard,
Mawson Lakes, South Australia, Australia 5095
alexander.cameron@adelaide.edu.au

ACKNOWLEDGMENTS

Dr. Cowin was supported by the National Health and Medical Research Council's Senior Research Fellowship (1002009). Dr. Cameron was supported by the National Health and Medical Research Council's Postgraduate Scholarship, the Royal Australasian College of Surgeons' WG Norman Trauma Fellowship, the Royal Australasian College of Surgeons' Sir John Lowenthal Scholarship, and the Australian Society for Plastic Surgery Foundation for Plastic Surgery Award.

REFERENCES

1. Engrav LH, Garner WL, Tredget EE. Hypertrophic scar, wound contraction and hyper-hypopigmentation. *J Burn Care Res.* 2007;28:593–597.
2. Muggia FM, Louie AC, Sikic BI. Pulmonary toxicity of antitumor agents. *Cancer Treat Rev.* 1983;10:221–243.
3. Brown BC, McKenna SP, Siddhi K, McGrouther DA, Bayat A. The hidden cost of skin scars: Quality of life after skin scarring. *J Plast Reconstr Aesthet Surg.* 2008;61:1049–1058.
4. Aarabi S, Bhatt KA, Shi Y, et al. Mechanical load initiates hypertrophic scar formation through decreased cellular apoptosis. *FASEB J.* 2007;21:3250–3261.
5. On the anatomy and physiology of the skin: Conclusions by Professor K. Langer. *Br J Plast Surg.* 1978;31:277–278.
6. On the anatomy and physiology of the skin: IV. The swelling capabilities of skin by Professor K. Langer. Presented at the meeting of 27th November 1861. *Br J Plast Surg.* 1978;31:273–276.
7. On the anatomy and physiology of the skin: III. The elasticity of the cutis by Professor K. Langer. Presented at the meeting of 27th November 1861. *Br J Plast Surg.* 1978;31:185–199.
8. On the anatomy and physiology of the skin: II. Skin tension by Professor K. Langer. Presented at the meeting of 27th November 1861. *Br J Plast Surg.* 1978;31:93–106.
9. On the anatomy and physiology of the skin. I. The cleavability of the cutis. [Translated from Langer, K. (1861). Zur Anatomie und Physiologie der Haut. I. Uber die Spaltbarkeit der Cutis. Sitzungsbericht der Mathematisch-naturwissenschaftlichen Classe der Kaiserlichen Academie der Wissenschaften, 44, 19]. *Br J Plast Surg.* 1978;31:3–8.
10. Gurtner GC, Werner S, Barrandon Y, Longaker MT. Wound repair and regeneration. *Nature* 2008;453:314–321.
11. Wong VW, Sorkin M, Glotzbach JP, Longaker MT, Gurtner GC. Surgical approaches to create murine models of human wound healing. *J Biomed Biotechnol.* 2011;2011:969618.
12. Kischer CW, Pindur J, Shetlar MR, Shetlar CL. Implants of hypertrophic scars and keloids into the nude (athymic) mouse: Viability and morphology. *J Trauma* 1989;29:672–677.
13. Escámez MJ, García M, Larcher F, et al. An in vivo model of wound healing in genetically modified skin-humanized mice. *J Invest Dermatol.* 2004;123:1182–1191.
14. Polo M, Kim YJ, Kucukcelebi A, Hayward PG, Ko F, Robson MC. An in vivo model of human proliferative scar. *J Surg Res.* 1998;74:187–195.
15. Goodwin MN, Silverstein P, Raulston GL, et al. Hypertrophic dermal scar: Study of porcine model. *Lab Invest.* 1976;34:339–339.
16. Zhu KQ, Engrav LH, Gibran NS, et al. The female, red Duroc pig as an animal model of hypertrophic scarring and the potential role of the cones of skin. *Burns* 2003;29:649–664.
17. Galiano RD, Michaels J 5th, Dobryansky M, Levine JP, Gurtner GC. Quantitative and reproducible murine model of excisional wound healing. *Wound Repair Regen.* 2004;12:485–492.
18. Umezawa H. [Bleomycin]. *Gan No Rinsho* 1967;13:733.
19. Yamamoto T, Takagawa S, Katayama I, et al. Animal model of sclerotic skin. I: Local injections of bleomycin induce sclerotic skin mimicking scleroderma. *J Invest Dermatol.* 1999;112:456–462.
20. Moseley PL, Hemken C, Hunninghake GW. Augmentation of fibroblast proliferation by bleomycin. *J Clin Invest.* 1986;78:1150–1154.
21. Lee JY, Yang CC, Chao SC, Wong TW. Histopathological differential diagnosis of keloid and hypertrophic scar. *Am J Dermatopathol.* 2004;26:379–384.
22. Zhang LQ, Laato M, Muona P, Penttinen R, Oikarinen A, Peltonen J. A fibroblast cell line cultured from a hypertrophic scar displays selective downregulation of collagen gene expression: Barely detectable messenger RNA levels of the pro alpha 1(III) chain of type III collagen. *Arch Dermatol Res.* 1995;287:534–538.
23. Uitto J, Perejda AJ, Abergel RP, Chu ML, Ramirez F. Altered steady-state ratio of type I/III procollagen mRNAs correlates with selectively increased type I procollagen biosynthesis in cultured keloid fibroblasts. *Proc Natl Acad Sci USA.* 1985;82:5935–5939.
24. Hinz B. The myofibroblast: Paradigm for a mechanically active cell. *J Biomech.* 2010;43:146–155.
25. Ramos ML, Gragnani A, Ferreira LM. Is there an ideal animal model to study hypertrophic scarring? *J Burn Care Res.* 2008;29:363–368.
26. Verhaegen PD, van Zuijlen PP, Pennings NM, et al. Differences in collagen architecture between keloid, hypertrophic scar, normotrophic scar, and normal skin: An objective histopathological analysis. *Wound Repair Regen.* 2009;17:649–656.
27. Dale PD, Sherratt JA, Maini PK. A mathematical model for collagen fibre formation during foetal and adult dermal wound healing. *Proc Biol Sci.* 1996;263:653–660.
28. Fleischmajer R, Perlish JS, Burgeson RE, Shaikh-Bahai F, Timpl R. Type I and type III collagen interactions during fibrillogenesis. *Ann N Y Acad Sci.* 1990;580:161–175.
29. Rawlins JM, Lam WL, Karoo RO, Naylor IL, Sharpe DT. Quantifying collagen type in mature burn scars: A novel approach using histology and digital image analysis. *J Burn Care Res.* 2006;27:60–65.
30. Wynn TA. Cellular and molecular mechanisms of fibrosis. *J Pathol.* 2008;214:199–210.
31. Sikic BI, Collins JM, Mimnaugh EG, et al. Improved therapeutic index of bleomycin when administered by continuous infusion in mice. *Cancer Treat Rep.* 1978;62:2011–2017.
32. Harrison JH Jr, Lazo JS. Plasma and pulmonary pharmacokinetics of bleomycin in murine strains that are sensitive and resistant to bleomycin-induced pulmonary fibrosis. *J Pharmacol Exp Ther.* 1988;247:1052–1058.
33. Takagawa S, Lakos G, Mori Y, Yamamoto T, Nishioka K, Varga J. Sustained activation of fibroblast transforming growth factor-beta/Smad signaling in a murine model of scleroderma. *J Invest Dermatol.* 2003;121:41–50.

34. Karsdal MA, Nielsen MJ, Sand JM, et al. Extracellular matrix remodeling: The common denominator in connective tissue diseases—Possibilities for evaluation and current understanding of the matrix as more than a passive architecture, but a key player in tissue failure. *Assay Drug Dev Technol.* 2013;11:70–92.
35. Hayakawa T, Hashimoto Y, Myokei Y, Aoyama H, Izawa Y. Changes in type of collagen during the development of human post-burn hypertrophic scars. *Clin Chim Acta* 1979;93:119–125.
36. Yamamoto T, Nishioka K. Cellular and molecular mechanisms of bleomycin-induced murine scleroderma: Current update and future perspective. *Exp Dermatol.* 2005;14:81–95.
37. Davidson, J. M. Animal models for wound repair. *Arch Dermatol Res.* 1998;290(Suppl):S1–S11.
38. Atiyeh BS, Hayek SN, Gunn SW. New technologies for burn wound closure and healing: Review of the literature. *Burns* 2005;31:944–956.

Evidence-Based Medicine: Questions and Answers

Q: Level of Evidence grading is new to me as an author (or reviewer). Are there resources to help me determine the LOE for my paper?

A: There are numerous resources in a variety of formats available to you as an author or reviewer. Those resources include:

- *PRS* Information for Authors (http://journals.lww.com/plasreconsurg/Pages/InformationforAuthors.aspx)
- ASPS LOE Grading Recommendations Table (see Table 1)
- PRS Evidence-Based Medicine Article Collections
  - "Evidence-Based Medicine: How-To Articles" collection (http://journals.lww.com/plasreconsurg/pages/collectiondetails.aspx?TopicalCollectionId=24
  - "Evidence-Based Medicine: Outcomes" collection (http://journals.lww.com/plasreconsurg/pages/collectiondetails.aspx?TopicalCollectionId=19)
- Author Tutorial: ASPS "Evidence-Based Medicine and the Critical Appraisal Process" (give PRS, ASPS, and Enkwell web addresses)
- Reviewer Tutorial: ASPS "Reviewing Concepts in Study Design and Critical Appraisal" (give PRS, ASPS, and Enkwell web addresses)
- See the companion article "The Levels of Evidence and their role in evidence-based medicine" article in this issue.

Additional Level of Evidence and Evidence-Based Medicine training is available at the ASPS and ASAPS annual meetings. We encourage you to register and sign up for these training courses.

Burn wound management: a surgical perspective

Cameron AM, Ruzehaji N & Cowin AJ

Abstract

Any patient who survives a large burn injury will be left with some degree of scarring. As well as affecting the form and function of the skin, scarring can have severe psychological consequences such as post-traumatic stress disorder and depression [1]. This is particularly the case for hypertrophic or keloid scars, which are common after serious burns. Despite this, the process underlying their formation is incompletely understood and limited effective options are available for their treatment. This paper reviews current understanding of the pathophysiology of the wound healing process in relation to burns and reviews the current management for burn wounds.

Introduction

Major burn injury is probably the most painful and devastating trauma a person can sustain and survive. Burns patients often require multiple surgical episodes and dressing changes followed by prolonged rehabilitation and victims can be left with lifelong dysaesthetic scarring and potential dysfunction. The scale of this clinical problem is demonstrated by the statistics from the AIHW National Hospital Morbidity Database, Australia's Health 2004. During the period of 2001-02, throughout Australia, burns and scalds were responsible for 6,248 hospitalisations in public hospitals with the average length of stay being 7.1 days entailing an estimated cost of $132 million. Despite this, scarring is an area of largely unmet medical need.

Alex Cameron MBBS, B. Med Sci
Women's & Childrens' Health Research Institute, 72 King William Road North Adelaide, South Australia. 5006

Nadira Ruzehaji B App Sc (Podiatry), BSc Hons
Women's & Childrens' Health Research Institute, South Australia

Allison Cowin PhD
Women's & Childrens' Health Research Institute, South Australia

The Wound Healing Process

Any consideration of burn wound healing must bear in mind the general principles of the wound healing process. The fundamental biological and molecular events after cutaneous injury cannot be separated and categorised in a clear-cut way. However, it has been useful to divide the repair process into four overlapping phases of coagulation, inflammation, migration-proliferation (including matrix deposition), and remodelling. These phases are shown in **Figure 1**, which also highlights the main events during each phase and the key types of cells implicated.

Pathophysiology of the burn wound

The pathophysiology of the burn wound is related to the initial distribution of heat onto the skin, which is a function of both the temperature and the exposure time i.e. a high temperature for a short time may cause the same tissue damage as a lower temperature for a longer time [2]. Jackson [3] described three zones of histopathological injury: coagulation, stasis and hyperaemia. The zone of coagulation is comprised of eschar or necrotic tissue and is closest to the heat source. This is surrounded by the zone of stasis, where there is only moderate tissue damage, but slow blood flow and oedema due to capillary leakage and cell membrane disruption [4,5]. Poor blood flow in this zone may lead to local tissue ischaemia and further necrosis [6]. Surrounding the zone of stasis is the zone of hyperaemia, in which cell damage is minimal and blood flow gradually increases resulting in early

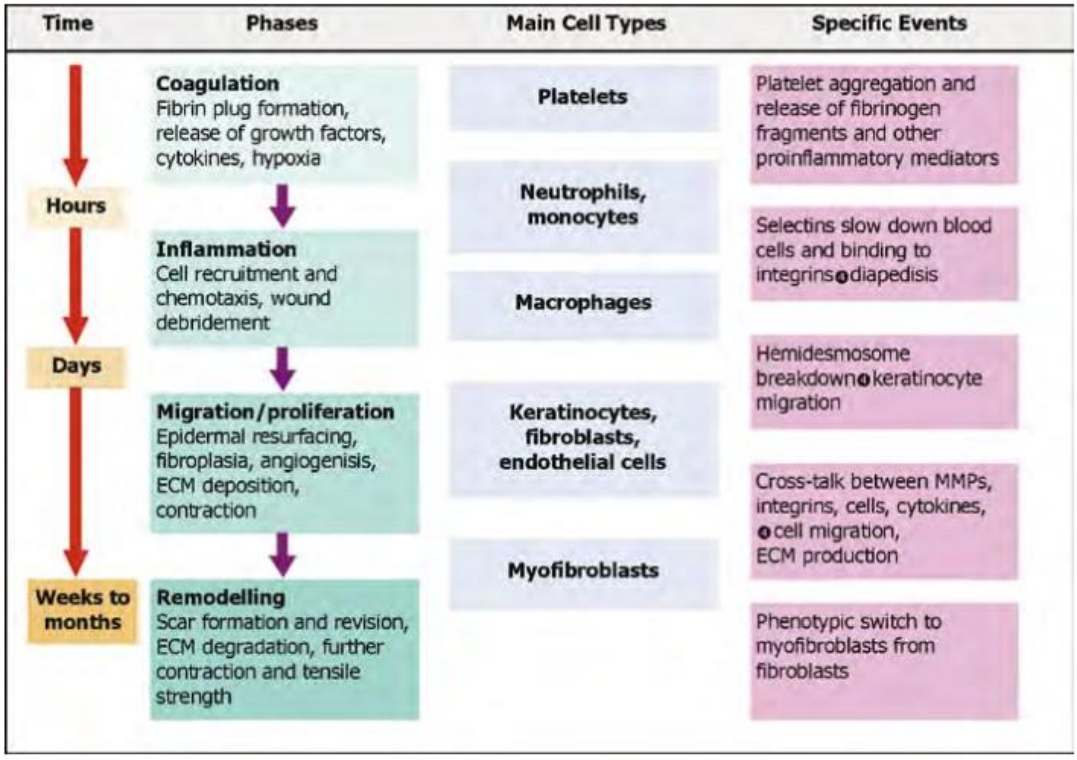


Figure 1. Phases of wound healing, major types of cells involved in each phase, and selected specific event. Adapted from Falanga, (2005).

spontaneous recovery. Burn depth may increase over 48-72 hours, as the zone of stasis becomes necrotic[7].

Superficial burns, such as sunburn, affect the epidermis only and are painful. They will heal completely within 3-5 days.

Partial thickness burns involve the entire epidermis and portions of the dermis. They are divided into superficial partial and deep partial thickness.

Superficial partial burns extend to the superficial dermis and are pink, moist and painful to touch. Blistering is common as a result of serum accumulating between the superficial dermis which has detached from the deep dermis. They will usually heal in two weeks without a scar by regeneration of epidermis from keratinocytes within sweat glands and hair follicles[8]. Burns from water scald are a common example of superficial partial burns.

Deep partial thickness burns involve the entirety of the epidermis and extend to the reticular layer of the dermis. They are a mottled white-pink colour and have variable sensation due to destruction of superficial cutaneous nerves. These wounds may heal within 3 weeks but are at risk of fibroproliferative disorders.

Full thickness wounds involve the entire epidermis and dermis. They are brown-black, leathery and insensate. Healing will be extremely slow and associated with marked contraction.

Management of the burn wound

This article will focus on the management of the burn wound itself; however, it should be noted that a multi-disciplinary approach to burn management is essential for optimal functional and cosmetic outcome[9]. This should include the following team members: anaesthetist, surgeon, nurses, physiotherapist, occupational therapist, psychologist, dietitian, social worker and rehabilitation specialist.

Assessment of burn depth

The depth of the burn wound is the most important determinant of its healing potential and appropriate management [10]. Thus, accurate assessment of this is vital. Clinical evaluation by the clinician using the characteristics described above for different burn thicknesses is the most widely used and least expensive method of assessing burn depth. However, it is accurate in only 2/3 of cases and has poor inter-observer reliability [11]. Other methods such as biopsy, thermography, videography with indocyanine green with laser fluorescence and laser Doppler perfusion imaging are also available [12]. Of these, only laser Doppler has been approved for clinical use. It has been shown to predict with 95% accuracy burns that will heal before 14 days, burns that will heal between 14 and 21 days and burns that will not heal within 21 days [13].

Superficial burns

Superficial burns do not require any specific therapy to aid healing but topical non-steroidal anti-inflammatory drugs or aloe vera may be used to reduce discomfort.

Superficial partial thickness burns

Superficial second-degree burns should be treated with a topical antimicrobial agent or an absorptive occlusive dressing.

The general principles of wound management with dressings are as follows [14]:

- Treat and prevent infection (reduce colonization by micro-organisms);
- Cleanse the wound to remove debris and facilitate the body's repair process;
- Debride the wound to remove necrotic or dead tissue and foreign matter;
- Provide an optimal healing environment by ensuring a degree of moisture at the wound surface; and
- Relieve pain and discomfort

Burn Wound Dressings

The range of dressing options is innumerable and differences between trade names in different regions can cause confusion. The Australia-New Zealand Burns Association (ANZBA) provides the following overview:

1. **Retention Dressings**: Low profile dressings used to assist adherence of other dressings or as primary dressing for superficial (minimally exudating) wounds e.g. *Fixomull®*
2. **Hydrocolloids**: Low profile, waterproof, highly conformable, wound interactive dressing e.g. *Duoderm®*
3. **Alginates**: Haemostatic dressing for moderately exudating or bleeding / oozing wounds e.g. *Sorbsan®, Algisite®.*
4. **Topical Anti-microbial Dressings**: Very important for burn wounds – used for control of or elimination of infectious organisms in the wound. May be used in various forms such as creams e.g. SSD, dressings / sheets e.g. *Acticoat®, Aquacel Ag®*, formulations e.g. Betadine® soaked gauze, AgNO3 soaks, sulfamylon soaks.
5. **Foams**: Used to control moderate to highly exudating wounds or protect fragile healed or almost healed areas e.g. *Mepilex®, Allevyn®*. Provided in thick and thin varieties.
6. **Hydrogels**: Used to maintain or introduce moisture into a wound. May be used to protect and hydrate exposed tendons or bone. Provided in liquid e.g. *Intrasite®* or sheet e.g. *Clearsite®* forms.
7. **Combination Dressings** e.g. *Combiderm®, Acticoat Absorbent®*
8. **Absorbent Dressings**: Used to mop up heavily exudating wounds e.g. combine, *Zetuvit®*, gauze, *Exu-Dry®.*
9. **Non-stick Dressings**: Used as anti-shear layers in dressing systems e.g. *Jelonet®, Melolin®.*
10. **Wound Growth Factor Impregnated Dressings**: Low profile, interactive, (relatively expensive) dressings / films which are often used acutely to reduce / prevent wound progression e.g. *Transcyte®, Biobrane®.*
11. **Biological Dressings**: Preparations used to provide another (potentially less expensive) option for introducing growth factors onto the wound e.g. xenograft (commonly pigskin), allograft (cadaver skin).
12. **Films**: Low profile, waterproof, highly conformable, adhesive dressing. Often used to secure IV cannulae.

Deep dermal burns/Full thickness burns

For any burns that extend to the deep dermis or beyond, excision and split skin grafting is required. Excision of the eschar removes necrotic and inflamed tissue which would otherwise act as a nidus for infection and retard wound healing [15]. Grafting minimises fluid loss and protects the wound against infection. New substances are now available to augment the traditional surgical approach. These include cultured epithelial autograft (CEA) and synthetic dermal templates such as *Integra®*.

Management of hypertrophic and keloid scars

It is difficult to differentiate between hypertrophic and keloid scars in their early phases. Phenotypic differences between the two will not become evident until the keloid has invaded adjacent normal tissue [16]. In terms of natural history, hypertrophic scars will tend to regress over time, albeit leaving a widened gap of thinned dermis between wound edges. Keloid scars will not regress, but rather grow to a certain size at which they will remain indefinitely [17].

Management of these scars is a major challenge in burn care. Treatments include:

Local administration of corticosteroid or non-steroidal anti-inflammatory drugs (NSAIDS) which interfere with the inflammatory process.

Occlusive dressings, such as elastic pressure wrap or silicone gel sheeting, whose exact mode of action is unknown, but is thought to involve accelerating scar degradation rate.

Calcium antagonists (e.g. verapamil) increase collagenase activity and scar tissue degradation.

Surgery: only indicated to under certain conditions e.g. very large scars unlikely to respond to medical therapy or scars impairing musculoskeletal function. It should be noted that the rate of recurrence of both hypertrophic and keloid scars is high following surgery.

Summary

In summary, burn wound management remains an area of great clinical challenge. Understanding the processes involved in burn wound healing and applying the appropriate wound management response is imperative to aid healing of the burn and reduce the risk of scarring. The remodelling stage of wound healing is associated with the formation of fibrous tissue or the scar and is considered to be the longest and least understood stage of healing. Pathologic fibrosis and scarring often lead to poor functional and aesthetic results, hence understanding the underlying cellular and molecular mechanisms involved in scar formation allows the surgeon and other health care practitioners to better address these issues, and suggests new avenues to explore therapeutic modalities designed to target the various biologic causes of dysregulated wound healing and abnormal scarring. As Australian health care becomes increasingly focused on providing multidisciplinary burn wound care, which in turn has been associated with dramatically improved outcomes for burn victims, we can look forward to more optimal burn care and improved patient outcomes.

References

1. Van Loey NE, Faber AW, Taal LA. Do burn patients need burn specific multidisciplinary outpatient aftercare: research results. Burns 2001;27:103-10.
2. Moritz AR, Henriques FC. The relative importance of time and surface temperature in the causation of cutaneous burns. Am J Pathol 1947;23:695-720.
3. Jackson DM. The diagnosis of the depth of burning. Br J Surg 1953;40:588-96.
4. Despa F, Orgill DP, Neuwalder J, Lee RC. The relative thermal stability of tissue macromolecules and cellular structure in burn injury. Burns 2005;31:568-77.
5. Baskaran H, Toner M, Yarmush ML, Berthiaume F. Poloxamer-188 improves capillary blood flow and tissue viability ina cutaneous burn wound. J Surg Res 2001;101:56-61.
6. Zawacki BE. The natural history of reversible burn injury. Surg Gynecol Obstet 1974; 139: 867-72
7. Deutsch A, Braun S and Granger C (1996): The FIM and the WeeFIM: 10 years ofdevelopment Critical Reviews in Physical and Medicine Rehabilitation 8: 267 – 281
8. Papini R. Management of burn injuries of various depths. BMJ 2004 329: 158-60
9. Serghiou M, Evans EB, Ott S, Calhoun JH, Morgan D, Hannon L (2002) Comprehensive rehabilitation of the burned patient. Chapter 45. In Hemdon DN (Ed) Total Burn Care (1st ed). London: Saunders.
10. Monstrey S, Hoeksema H, Verbelen J, Pirayesh A, Blondeel P. Assessment of burn depth and burn wound healing potential. Burns. 2008. 761-769
11. Monstrey S, Hoeksema H, Verbelen J, Pirayesh A, Blondeel P. Assessment of burn depth and burn wound healing potential. Burns. 2008. 761-769
12. Devgan L, Bhat S, Aylward S, Spence RJ. Modalities for the assessment of burn wound depth. J Burns Wounds 2006;5:e2.
13. Monstrey S, Hoeksema H, Verbelen J, Pirayesh A, Blondeel P. Assessment of burn depth and burn wound healing potential. Burns. 2008. 761-769
14. David, J.A. (1986). Wound management: A comprehensive guide to dressing and healing. Practical Nursing Handbook. London, Martin Dunitz.
15. Ong YS, Samuel M, Song C. Metaanalysis of early excision of burns. Burns 2006;32:145-50.
16. Santucci M, Borgognoni L, Reali U. Keloids and hypertrophic scars of Caucasians show distinctive morphologic and immunophenotypic profiles. Virchows Arch. 2001 ;438:457-463.
17 Roseborough IE, Grevious MA, Lee RE. Prevention and treatment of excessive dermal scarring J Natl Med Assoc. 2004;96:108-116
18. Ketchum LD, Cohen IK, Masters FW Hypertrophic scars and keloids. A collective review. Plast Reconstr Surg. 1974;53: 140-154.

DISCLOSURE

The authors have no conflict of interest to declare and received no funding for this article.

REFERENCES

1. Cameron AM, Adams DH, Greenwood JE, Anderson PJ, Cowin AJ. A novel murine model of hypertrophic scarring using subcutaneous infusion of bleomycin. *Plast Reconstr Surg.* 2014;133:69–78.
2. Hecht SM. Bleomycin: New perspectives on the mechanism of action. *J Nat Prod.* 2000;63:158–168.
3. Hillmer MP, MacLeod SM. Experimental keloid scar models: A review of methodological issues. *J Cutan Med Surg.* 2002;6:354–359.
4. Morris DE, Wu L, Zhao LL, et al. Acute and chronic animal models for excessive dermal scarring: Quantitative studies. *Plast Reconstr Surg.* 1997;100:674–681.

Reply: A Novel Murine Model of Hypertrophic Scarring Using Subcutaneous Infusion of Bleomycin

Sir:

I thank Sacak et al. for their considered response to the article entitled "A Novel Murine Model of Hypertrophic Scarring Using Subcutaneous Infusion of Bleomycin." The rationale behind our murine model is to recreate the pathologic process underlying hypertrophic scarring by using bleomycin infusion to stimulate dermal fibroproliferation. Bleomycin is an antibiotic, originally isolated from *Streptomyces verticillus* and widely used as an anticancer treatment.[1] Bleomycin hydrolase inactivates bleomycin by hydrolyzing the amide bond in the b-amino alanine amide moiety. However, the lack (or shortage) of this enzyme in the lungs and the skin allows bleomycin-induced fibrosis and sclerosis to occur in these organs in a dose-dependent manner.[2] Bleomycin was first used to create animal models of pulmonary fibrosis, before Yamamoto and colleagues established a mouse model of scleroderma using daily subcutaneous injections over a 4-week period.[3,4]

Bleomycin is known to induce production of reactive oxygen species and cause damage to endothelial and other cells types.[5] In addition, bleomycin directly stimulates the profibrotic transforming growth factor-β pathway, and a range of other proinflammatory and profibrotic mediators such as chemoattractant protein 1, platelet-derived growth factor, interleukin-4, interleukin-6, and interleukin-13.[5] In this way, bleomycin causes scarring by direct activation of inflammatory and fibrotic mediators that have been shown to be important to the development of human hypertrophic scarring.

Regarding the reference by Sacak et al. to the fate of the fibroproliferative changes of the skin, my colleagues and I reported that the histopathologic changes consistent with hypertrophic present after 28 days of bleomycin treatment were still evident at day 56, 28 days after the cessation of bleomycin treatment. However, inflammatory changes, such as epidermal acanthosis, transforming growth factor-β1 expression, and myofibroblast numbers were significantly decreased in the 56-day model compared with the 28-day model. Therefore, we concluded that for testing therapies directed at dampening the inflammatory response that drives fibroproliferation, the 28-day model would be most appropriate. For therapies intended to increase scar remodeling, the 56-day model would potentially be useful. The exact dose scheduling would be determined by the therapy in question.

I agree that there are other valid animal models, such as the rabbit ear model.[6,7] Obviously, trials using other animal species will restrict access to transgenic animals, which are of great utility in wound healing research. Furthermore, I believe that the utility of the bleomycin model is that it restricts the research focus to a pathologic process (fibroproliferation) rather than trying to reproduce a pathologic condition unique to humans. I contend that this approach will yield more relevant data about the key mechanism underlying hypertrophic scarring than existing animal models that attempt to reproduce the end-stage disease seen in humans at all costs.

DOI: 10.1097/PRS.0000000000000257

Alexander M. Cameron, M.B.B.S., B.Med.Sci.
Centre for Regenerative Medicine
Mawson Institute, Building V
University of South Australia Mawson Lakes Campus
Mawson Lakes Boulevard
Mawson Lakes, South Australia 5095, Australia
amacgcameron@gmail.com

DISCLOSURE

The author has no financial interest to declare in relation to the content of this communication.

REFERENCES

1. Umezawa H. Bleomycin (in Japanese). *Gan No Rinsho* 1967;13:735.
2. Muggia FM, Louie AC, Sikic BI. Pulmonary toxicity of antitumor agents. *Cancer Treat Rev.* 1983;10:221–243.
3. Yamamoto T, Takagawa S, Katayama I, et al. Animal model of sclerotic skin. I: Local injections of bleomycin induce sclerotic skin mimicking scleroderma. *J Invest Dermatol.* 1999;112:456–462.
4. Moseley PL, Hemken C, Hunninghake GW. Augmentation of fibroblast proliferation by bleomycin. *J Clin Invest.* 1986;78:1150–1154.
5. Yamamoto T, Nishioka K. Cellular and molecular mechanisms of bleomycin-induced murine scleroderma: Current update and future perspective. *Exp Dermatol.* 2005;14:81–95.
6. Davidson JM. Animal models for wound repair. *Arch Dermatol Res.* 1998;290(Suppl):S1–11.
7. Atiyeh BS, Hayek SN, Gunn SW. New technologies for burn wound closure and healing: Review of the literature. *Burns* 2005;31:944–956.

A.3 Consent forms & ethics approvals

INFORMATION SHEET

1. **TITLE OF PROJECT**

   THE ROLE OF FLIGHTLESS IN HYPERTROPHIC SCARRING

2. **PRINCIPAL INVESTIGATOR**

Professor Allison Cowin BSc (Hons) PhD
NHMRC RD Wright Research Fellow
Head, Wound Healing Team
Women & Children's Health Research Institute

t. 08 8161 7077
e. Allison.cowin@adelaide .edu.au

3. **CO-INVESTIGATOR**

Dr. Alexander Cameron B. Med Sci MBBS
Royal Australasian College of Surgeons WG Norman Fellow
Plastic & Reconstructive Surgery Trainee
PhD Student, Wound Healing Team
Women's & Children's Hospital Research Institute
Department of Surgery, University of Adelaide

t. 08 8161 7061
e. amacgcameron@gmail.com

4. **EXPLANATION OF THE RESEARCH**

The process of wound healing and scar formation will affect almost everyone at some point during their lifetime. Scars range from a fine line (e.g. a surgical scar) to extensive scarring and contracture associated with burn injuries or trauma. Severe scars lead to loss of movement, restricted growth, deformity and disfigurement for which patients often require multiple surgical procedures and long rehabilitation stays. Such processes are associated with significant psychological stress and an impaired quality of life.

Knowledge of the mechanisms involved in wound healing and scar formation will ultimately lead to the development of new therapies to improve the rate and quality of wound repair and reduce scarring. This offers a tremendous opportunity to enhance the quality of life for any person suffering from traumatic or burn injuries.

This project is investigating a protein named "Flightless". Through the work of Professor Allison Cowin and her team at the Women's and Children's Health Research Institute this protein has been shown to be important in the wound healing process. This project will mark the first time that Flightless has been identified in human tissue. Ultimately, it is hoped that this research will help in developing novel therapeutic agents (e.g. treatments) to improve scarring after burn injuries, trauma or surgery.

The foreskin from your son's circumcision, which would otherwise be discarded is a very useful source of keratinocytes (skin cells). We will use the foreskin as a source of the cells in order to investigate various aspects of the Flightless protein and related proteins. The tissue will not be used for any other research. Some of the tissue may be stored for use later in the project (projected end date: late 2013).

5. CONFIDENTIALITY

We will not know your name or that of your son; only your son's age will be recorded. Confidentiality will be strictly adhered to , including any results of the research that may be published.

6. WITHDRAWAL OF CONSENT/COMPLAINTS

Should you wish to withdraw your consent at any time for the use of the skin tissue please notify Dr. Kirby or if you wish to speak to an independent body contact the Human Research Ethics Committee's Secretary on phone (08) 8303 6028. (See attached sheet "Contacts for information and independent complaints procedure".)

The Role of Flightless in Hypertrophic Scarring

Dear Sir/Madam:

Thank you for considering taking part in this study, entitled "The Role of the Flightless 1 Protein in the Hypertrophic Scarring".

Background to the project

The process of wound healing and scar formation will affect almost everyone at some point during their lifetime. Scars range from a fine line (e.g. a surgical scar) to extensive scarring and contracture associated with burn injuries or trauma. Severe scars lead to loss of movement, restricted growth, deformity and disfigurement for which patients often require multiple surgical procedures and long rehabilitation stays. Such processes are associated with significant psychological stress and an impaired quality of life.

Knowledge of the mechanisms involved in wound healing and scar formation will ultimately lead to the development of new therapies to improve the rate and quality of wound repair and reduce scarring. This offers a tremendous opportunity to enhance the quality of life for any person suffering from traumatic or burn injuries.

This project is investigating a protein named "Flightless". Through the work of Professor Allison Cowin and her team at the Women's and Children's Health Research Institute this protein has been shown to be important in the wound healing process in a mouse model. This project will mark the first time that Flightless has been identified in human tissue. Ultimately, it is hoped that this research will help in developing novel therapeutic agents (e.g. treatments) to improve scarring after burn injuries, trauma or surgery.

Your involvement

As part of your surgery, you are having some scar tissue/burn tissue excised. If you agree to participate this tissue will be transported to the Wound Healing Laboratory at the Women's and Children's Hospital. **No more tissue than what is required for your surgery will be excised. Thus, the use of this tissue will not affect the outcome of your surgery in any way.**

Who will use my tissue?

Your tissue will be processed and stored by members of the Wound Healing team at the Women's and Children's Health Research Institute.

At the laboratory the tissue will be stored in a de-identified manner (i.e. the samples will be identified with a code and your name will not be recorded). .

What will happen to the tissue at the laboratory?

The tissue will be processed in various ways to allow the identification and quantification of the Flightless protein and other important proteins in the wound healing process.. Remaining tissue will be kept in storage for seven years and may be used for future Wound Healing research.

Cells obtained from your tissue may also be used to establish cell lines. A cell line consists of cells cultured to grow for a very long time in the laboratory. Cell lines, therefore, allow the creation of a large supply of material for research. Only researchers who have special approval can establish cell lines.

Your tissue samples will not be used by any other organisations or research group.

Will I receive results from the research?

You will receive all relevant information about your treatment and surgery from the RAH Adult Burns' Centre doctors. However, you will not receive specific research results . This is because research by its very nature, is experimental, can take many years, and uses samples and data from a large number of people, so what a researcher discovers in this context may be of little value to you, your family or your future health.

What benefits can I expect from my involvement?

It is unlikely that there will be a direct benefit to you. However, you involvement may benefit future patients by helping to discover treatments that improve scarring after burns or other trauma.

Further details of study

This research is done under the supervision of:

- Professor Allison Cowin (Head, Wound Healing Laboratory, Women's and Children's Hospital)
- Associate Professor Peter Anderson (Senior Consultant Surgeon, Australian Craniofacial Unit)

and with the collaboration of:

- Associate Professor John Greenwood (Head of Burns' Unit, Royal Adelaide Hospital)

This project will form part of my Masters of Surgery degree through Adelaide University.

This study has been approved by the Royal Adelaide Hospital Research Ethics Committee. If you have any questions in regard to this the committee can be contacted as follows:

t. 8222 4139
e. rah.ethics@health.sa.gov.au

Should you have any questions please contact me at

amacgcameron@gmail.com.

Dr. A. Cameron MBBS BMed Sci

The Role of Flightless in Hypertrophic Scarring

Consent form

I,

_____

(insert full name of participant)

of_____

_____

(address of participant)

agree to be a participant in the study entitled "The Role of Flightless in the Pathology of Fibrosis and Scarring". I have been supplied with an information sheet and what my role in the project has been explained. In understand that tissue from my surgery that would otherwise be discarded will be processed and stored in a de-identified in the Wound Healing Laboratory at the Women's and Children's Hospital Research Institute. I understand that my involvement will have no bearing on the outcome of my surgery of future treatment.

_____

(Signature of Participant)

_____

(Signature of Doctor)

Government of South Australia
SA Health

Women's & Children's Hospital

Research Secretariat
72 King William Road
North Adelaide SA 5006
Tel 08 8161 6521
Tel 08 8161 6390
Fax 08 8161 8177
www.cywhs.sa.gov.au

30th August 2011

Prof A Cowin
WCHRI
WCHN

Dear Prof Cowin

Re: The role of flightless in hypertrophic scarring and potential as a target for a novel therapy. REC2399/8/14

Thank you for submitting the above protocol to the Women's & Children's Health Network Human Research Ethics Committee. It was approved at the Committee's meeting on 24th August 2011 subject to:

1. In keeping with 1.4(b) of the *National Statement on Ethical Conduct In Human Research,* that 'the process of recruiting participants is fair' clarification on whether Aboriginal and/or Torres Strait Islander people are excluded from the study and, if they are, the reason for the exclusion. The HREC noted that there is conflicting advice in 6.2.4 of your NEAF regarding this.

2. Data being retained in accordance with Function 6 of the Records Disposal Schedule for SA Public Hospitals, available on: http://www.wch.sa.gov.au/research/committees/humanethics/documents/Retention_of_data_schedule.pdf. The HREC noted, 8.3.6 of the NEAF, that you were planning to retain data for only seven years. For studies involving adults or a non-legal disability, data should generally be retained for a period of fifteen years after research project completion. For studies involving minors or a legal disability, data should generally be retained for a period of 30 years after research project completion. However, it is recommended that you refer to Function 6 for specific requirements and advise the Committee in relation to Function 6 as to the data retention period for your study.

3. In keeping with 2.2.16 of the *National Statement*, clarification on the future use of the collected tissue in research. The HREC noted, 9.5.4 of the NEAF that the tissue may be used and questioned whether participants can change their mind regarding future use. Information on this should be included in the Information Sheet and Consent Form.

4. A lay title being included in the Information Sheet and Consent Form. Additionally, the complete scientific title should be used in both documents.

5. In the Information Sheet – in keeping with 2.2.1 of the *National Statement on Ethical Conduct in Human Research* to provide sufficient information to potential participants in order to facilitate informed consent, the following points should be addressed:

   i) Inclusion of sub-headings above the relevant text for readability; some suggestions are *What the study involves for participants; Who is doing the study; Confidentiality; Researchers' contact details; Human Research Ethics Committee contact details, etc.*
   ii) The last sentence in the first paragraph being amended to "This may lead to treatment to prevent or reduce excessive scarring".
   iii) Under *Background to the project:*
      a) Provide lay explanations for the terms 'hypertrophic', 'contracture', 'novel therapeutic agents' and 'excised'.
      b) Commencement of the second paragraph with "It is hoped that knowledge …" and deletion of the second sentence. The Committee considered the current wording of this paragraph could be potentially misleading to participants.
      c) Inclusion of advice that both scar and normal tissue is required.
   iv) Advise what will be done with the tissue in lay terms.

iii) Advise on the accessing of medical records and the information that will be reviewed.
iv) Advise that participation is voluntary and that participants may withdraw from the study without prejudice to their future treatment or relationship with the WCHN.
v) Advise there is no payment for participation.
vi) Inclusion of the statement - *Your information will remain confidential except in the case of a legal requirement to pass on personal information to authorised third parties. This requirement is standard and applies to information collected both in research and non-research situations. Such requests to access information are rare; however we have an obligation to inform you of this possibility.*
vii) Advice being included that the study has the approval of the Women's & Children's Health Network Human Research Ethics Committee. The name and contact details of the Executive Officer of the Committee (Ms Brenda Penny, 08 8161 6521) should be included in case participants/potential participants wish to discuss the approval process, or have any concern or complaint. The contact details for the Ethics Committee should also be under a separate heading to that for the researchers.

6. The Consent Form being based on the HREC's proforma (available on http://www.wch.sa.gov.au/research/committees/humanethics/index.html .
It should also include a clause agreeing/not agreeing to tissue being stored for future research projects.

7. Approval of indemnification from the Department of Health Insurance Services Unit. Please email the Information Sheets and Consent Forms to Mr John Markic, (john.markic@health.sa.gov.au) for assessment and approval.

Institutional requirements - Please also advise on the status of the following:

a. **National Police Certificates for students (excluding students from University of South Australia) and non-WCHN staff involved in the project on WCHN sites.** The Certificates are to be provided to the WCHN Human Resources Department for verification (telephone 81617249 for further information) and copies forwarded to the Ethics Committee.

b. **Confidentiality Agreements**. If the project involves patients/clients/staff of WCHN or their personal information, signed Confidentiality Agreements are to be provided for all students and non-WCHN staff to the Committee. Please refer to http://www.wch.sa.gov.au/research/committees/humanethics/ConfidentialityAgreement.html

The above requirements relate to current and future students and non-WCHN staff on the project. If the students and non-WCHN staff on this project are subsequently involved on other projects approved by the Committee, a copy of the National Police Certificate will need to be re-sent to the Committee and a Confidentiality Agreement signed for each specific project.

I look forward to your response and remind you that the study may not proceed until you receive a letter of final approval. In order to facilitate the review process and a timely reply, your response should clearly address each of the above points. **Additionally, modifications to the Information Sheet and Consent Form should either be tracked or highlighted so that the amended wording (where relevant) is obvious.**

Yours sincerely

TAMARA ZUTLEVICS (DR)
CHAIR
WCHN HUMAN RESEARCH ETHICS COMMITTEE