

# **THE ROLE OF CYTOSKELETAL PROTEIN FLIGHTLESS I (FLII) IN DIABETIC WOUND HEALING**

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**NADIRA RUZEHAJI**

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## INTRODUCTORY STATEMENT

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, 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.

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Nadira Ruzehaji

23<sup>rd</sup> of January 2013



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## ABSTRACT

Skin lesions and ulcerations are common and severe complications of diabetes. A significant proportion of these wounds fail to respond to conventional treatment, hence amputation is a feared outcome of diabetes. Overexpression of Flightless (Flii) inhibits wound healing and ablation of Flii using specific neutralising monoclonal antibodies (FnAb) enhances cellular proliferation and migration. It was therefore hypothesized that decreasing Flii expression in diabetic wounds would create a permissive environment for cellular proliferation, enhanced neovascularization, and improved healing outcomes. The aim of this study was to determine whether genetic Flii gene knockdown or treatment with FnAb were effective in improving diabetic wound repair. A mouse model of diabetes was used in which type 1 diabetes was induced using streptozotocin. Diabetes was subsequently induced in low (Flii<sup>+/-</sup>), normal (WT) and high (Flii<sup>Tg/Tg</sup>) mice. Full-thickness dorsal wounds were created and it was found that these wounds healed more rapidly when Flii gene expression was decreased. Further studies revealed that this improved healing was accompanied by a robust pro-angiogenic response with significantly elevated von Willebrand factor and VEGF positive endothelial cell infiltration. In a separate study, wounds in WT diabetic mice were injected intradermally with FnAb and here too improved healing was observed with significantly increased rate of re-epithelialisation compared with placebo control. We investigated the angiogenic response of FnAb both in vitro and in vivo. FnAb enhanced capillary tube formation in human umbilical vein endothelial cells (HUVEC) and promoted formation of functional neovasculature in vivo. Mice with reduced Flii also showed increased numbers of mature blood vessels using an in vivo Matrigel plug assay with increased recruitment of  $\alpha$ -SMA positive cells and improved tight junction aiding cell to cell attachments. In conclusion, reducing Flii levels in wounds either genetically or using neutralising

antibodies promotes wound healing in diabetic mice by enhancing epithelialisation and improving angiogenic processes. Manipulating Flightless I may therefore be a potential approach for therapeutic intervention in the treatment of the diabetic foot.

## PUBLICATIONS ARISING FROM WORK IN THIS THESIS

**Ruzehaji, N.,** Grose, R., et al. (2012). “Cytoskeletal protein Flightless (Flii) is elevated in chronic and acute human wounds and wound fluid: neutralizing its activity in chronic but not acute wound fluid improves cellular proliferation.” *European Journal of Dermatology*; accepted for publication in July 2012.

**Ruzehaji, N.,** Mills, S., et al. (2013). “The Action of Flightless I and Toll-Like Receptors during Wound Healing in Diabetic Wounds.” *Journal of BioMed Research International*; accepted for publication in January 2013.

**Ruzehaji, N.,** Kopecki, Z., Appleby, SL., Bonder, CS., Fitridge, R., Cowin, AJ. (2013) "Attenuation of Flightless I improves healing in a murine model of type 1 diabetes through increased angiogenesis in the wounds". *Diabetes*; manuscript submitted in January 2013.

**Ruzehaji, N.,** Mills, S., et al. (2013). “The Expression of Flightless I and Toll-Like Receptors in Human Diabetic Wounds”. *International Wound Journal*; manuscript submitted in January 2013

McInnes, W., **Ruzehaji, N.,** Wright, N., Cowin, AJ., Fitridge, R. (2013) “Successful treatment of venous stasis ulcers with larvae aided wound debridement”. *Journal of Wound Care*; manuscript submitted in January 2013.

Lei, N., Franken, L., **Ruzehaji, N.,** et al. (2012). “Flightless, secreted through a late endosome/lysosome pathway, binds LPS and dampens cytokine secretion.” *Journal of Cell Science*; accepted for publication in June 2012.

Cowin, A. J., Lei, N., Franken, L., **Ruzehaji, N.**, et al. (2012) “Lysosomal secretion of Flightless upon injury has the potential to alter inflammation.” *Journal of Communicative & Integrative Biology*; accepted for publication in August 2012.

**Ruzehaji, N.**, & Cowin, AJ. (2011): Textbook: Chapter 2: “The Inhibitory Factors that Influence Wound Healing.” In: *Wound Management for the Advanced Practitioner*. Eds Asimus, Swanson, McGuinness, Publisher: IP Communications.

## NATIONAL AND INTERNATIONAL SCIENTIFIC MEETING ABSTRACTS

**Ruzehaji N**, Kopecki Z, Appleby SL, Bonder CS, Fitridge R, Cowin AJ (2012). “Ablation of Flightless protein improves healing in a murine model of type 1 diabetes through increased angiogenesis in the wounds.” Australian Society for Medical Research, South Australian Scientific Meeting, Adelaide, Australia.

**Ruzehaji N.**, Wallace, H., et al (2012). Neutralization of Flightless I (Flii) using Flii-specific monoclonal antibodies accelerates impaired healing in diabetic wounds through improved cell proliferation.” Postgraduate Conference, The University of Adelaide, Adelaide, Australia.

**Ruzehaji N**, Kopecki Z, Appleby SL, Bonder CS, Fitridge R, Cowin AJ (2012). “Ablation of Flightless protein improves healing in a murine model of type 1 diabetes through increased angiogenesis in the wounds.” The 3rd Meeting of the Australasian Wound & Tissue Repair Society, Sydney, Australia.

**Ruzehaji N**, Wallace H, Stacey M, Krumbiegel D, Zola H, Fitridge R, Cowin AJ “Neutralization of Flightless I (Flii) using Flii-specific monoclonal antibodies accelerates impaired healing in diabetic wounds through improved cell proliferation.” The 15th International Congress of Endocrinology, Florence, Italy.

**Ruzehaji N**, Wallace H, Stacey M, Krumbiegel D, Zola H, Fitridge R, Cowin AJ (2010).  
“Neutralization of Flightless I (Flii) in Chronic and Acute Wound Fluid using Flii-specific  
Monoclonal Antibodies Improves Cell Proliferation.” The 2nd Meeting of the Australasian  
Wound & Tissue Repair Society, Perth, Western Australia.

**Ruzehaji N**, Wallace H, Stacey M, Krumbiegel D, Zola H, Fitridge R, Cowin AJ (2009).  
“Extracellular Function of the Actin-Remodelling Protein Flightless I May Be important In  
Acute Wound Responses.” 5th Joint Meeting of the European Tissue Repair Society and the  
Wound Healing Society, Limoges, France.

## **AWARDS ARISING FROM WORK PRESENTED IN THIS THESIS**

- 2009                   AUGU/RC Heddle Award
- The University of Adelaide
- 
- 2009                   Australian Federation of University Women
- Brenda Nettle Award
- 
- 2009                   Postgraduate Travelling Fellowship
- The University of Adelaide
- 
- 2011                   Health Sciences Faculty Finalist
- The University of Adelaide Three Minute Thesis Competition
- 
- 2011                   Postgraduate Research Conference
- The University of Adelaide
- People’s Choice Award



2011  
Freemasons Foundation  
Trevor Prescott Memorial Award

2011  
Young Investigator Award 2011

2012  
Best Oral Award  
Australian Society for Medical Research

2012  
The Adelaide Research & Innovation Prize  
Project with most commercial potential

2012  
Best Oral Presentation  
AWTRS conference, Sydney, Australia

## LIST OF ABBREVIATION

$\alpha$ SMA	Alpha smooth muscle actin
cDNA	Complementary deoxyribonucleic acid
DNA	Deoxyribonucleic acid
EC	Endothelial cells
EM	Electron microscopy
EGF	Epidermal growth factor
ECM	Extracellular matrix
FGF	Fibroblast growth factor
Flii	Flightless I
GFR	Glomerular filtration rate
H&E	Haematoxylin and Eosin
IgG	Immunoglobulin
IL	Interleukin
MMP-9	Matrix metalloproteinase 9
mRNA	Messenger ribonucleic acid

PDGF	Platelet-derived growth factor
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
STZ	Streptozotocin
TGF	Transforming growth factor
TIMP	Tissue inhibitor of metalloproteinase
TNF- $\alpha$	Tumour necrosis factor alpha
VEGF	Vascular endothelial growth factor
vWF	von Willebrand factor

*Chapter 1*

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**LITERATURE REVIEW**

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# LITERATURE REVIEW

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## **1.1.1 Introduction**

### **1.1.1. Diabetes mellitus: a historical perspective**

The word “*diabetes*” originates from Latin *diabetes*, which literally means “a passer through”. It refers to the excessive discharge of urine – a symptom prevalent in those afflicted by the disease. The word “*mellitus*” also comes from Latin and means “sweetened with honey”. Even though the quest for causes of diabetes began in ancient times (Morton & Schwartz 2011), the term “*diabetes mellitus*” was first used by the 17<sup>th</sup> century English physician - Thomas Willis, who tasted the urine of diabetic patients and declared that “its sweet taste was imbued with honey” (Shafirir & Raz 2003). In 1922, it became clear that insulin deficiency was a cardinal feature of this otherwise fatal disease (Morton & Schwartz 2011). Banting and McLeod received the Nobel prize in 1923 for the discovery of insulin (Banting & Best 1990). From this historic breakthrough, there has evolved an era of searching for new treatment options for diabetes and its complications.

### **1.1.2. The diversity and classification of diabetes mellitus**

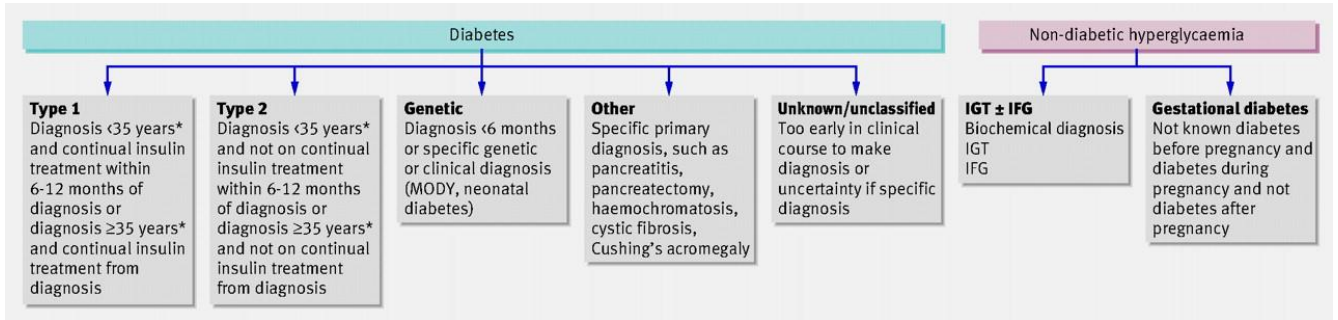
The term ‘diabetes mellitus’ covers a wide variety of conditions united only by the common principal characteristic of high blood-glucose levels (Diamond 2003). That diversity may be crudely separated into type 1 (juvenile-onset) and type 2 (adult-onset). The respective prevalence of these two types among diabetics is 10% and 90% globally (Zimmet, P, Alberti & Shaw 2001). Both diseases centre on the hormone insulin (Diamond 2003) and chronic hyperglycaemia with disturbances of carbohydrate, fat and protein metabolism resulting from defects in insulin

secretion, insulin action, or both (Alberti & Zimmet 1998). Conventional criteria for classification of diabetes include the age of onset and body mass index. However, this framework poses practical challenges for researchers and clinicians because the old stereotypes of the older obese and younger non-obese patient presenting with elevated blood glucose are no longer applicable (Dabelea et al. 2011). Due to the obesity epidemic in children and adolescents, the wider use of insulin for type 2, and a prevalence of genetic forms of diabetes, pragmatic guidelines for classifying diabetes have been set out in Figure 1.1 (Farmer & Fox 2011).

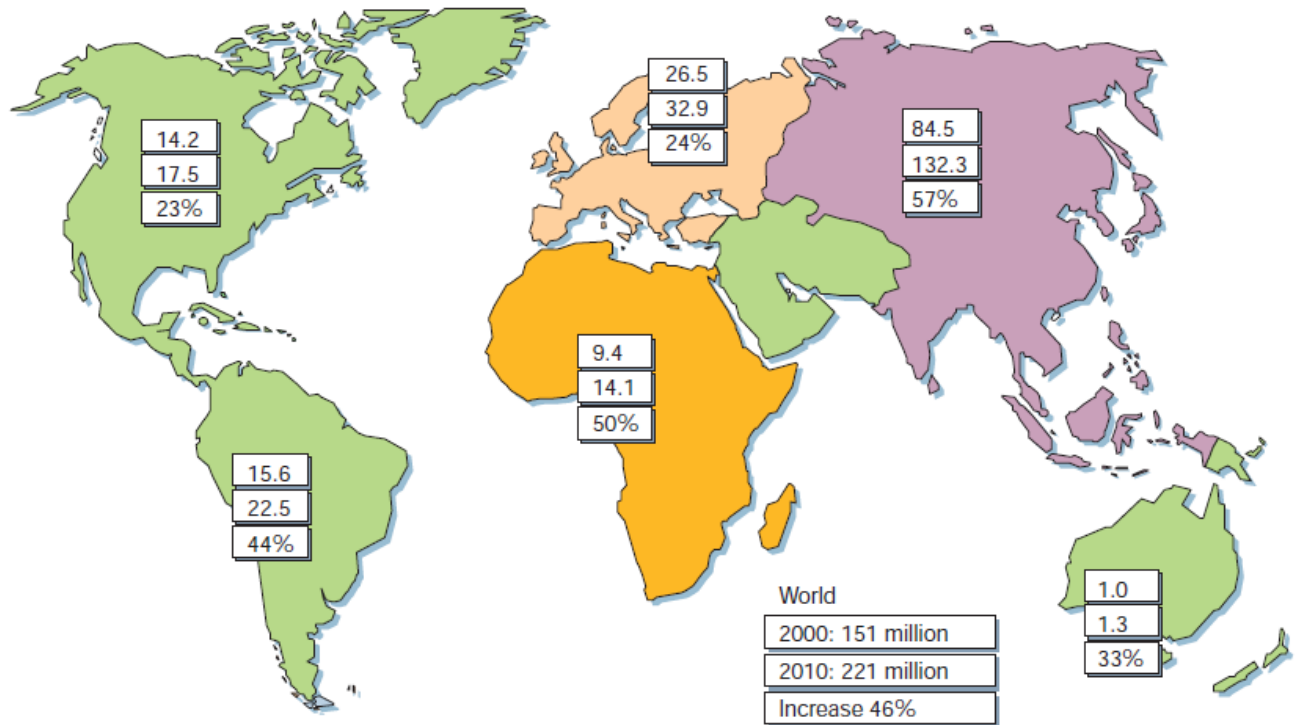
### **1.1.3 Prevalence and cost of diabetes**

Diabetes is the fastest growing chronic disease worldwide and one of the main threats to human health in the 21st century (Zimmet, P, Alberti & Shaw 2001). The global estimates for the years 2010 and 2030, using data from 216 countries of the United Nations, indicate that the world prevalence of diabetes among adults was 6.4% in 2010, and will increase to 7.7% by 2030 (Shaw, Sicree & Zimmet 2010). The number of cases worldwide, as indicated in Figure 1.2, is estimated at 221 million adults (Zimmet, P, Alberti & Shaw 2001), but this is a minimum account because, for each diagnosed case, there is thought to be one undiagnosed case in First World countries and eight in the Third World (Levitt et al. 1993). The cost of diabetes is high for both the individual and society. The total estimated cost of diabetes in 2007 is \$174 billion in the United States ('Economic costs of diabetes in the U.S. In 2007' 2008), or 15% of costs due to all diseases combined (Diamond 2003). In Australia, an estimated amount of \$636 million was spent on diabetes-attributable health-care costs in 2000 (Davis et al. 2006). The financial burden of treating diabetes and its related complications in Australia is predicted to increase by 2.5 fold in 2051 (Davis et al. 2006).





**Figure 1.1. Practical classification guidelines.** Adapted from (Farmer & Fox 2011).



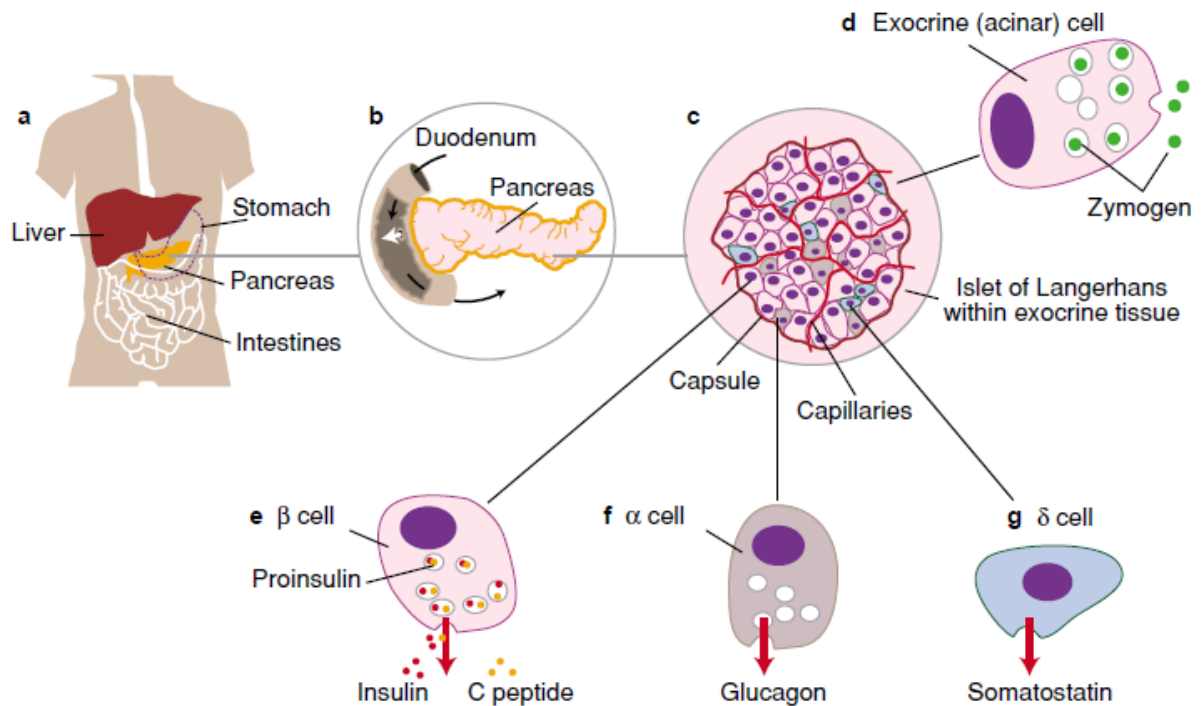
**Figure 1.2. Numbers of people with diabetes (in millions) for 2000 and 2010 (top and middle values, respectively), and the percentage increase. Adapted from (Zimmet, P, Alberti & Shaw 2001)**

## **1.2 Aetiology and pathogenesis of diabetes**

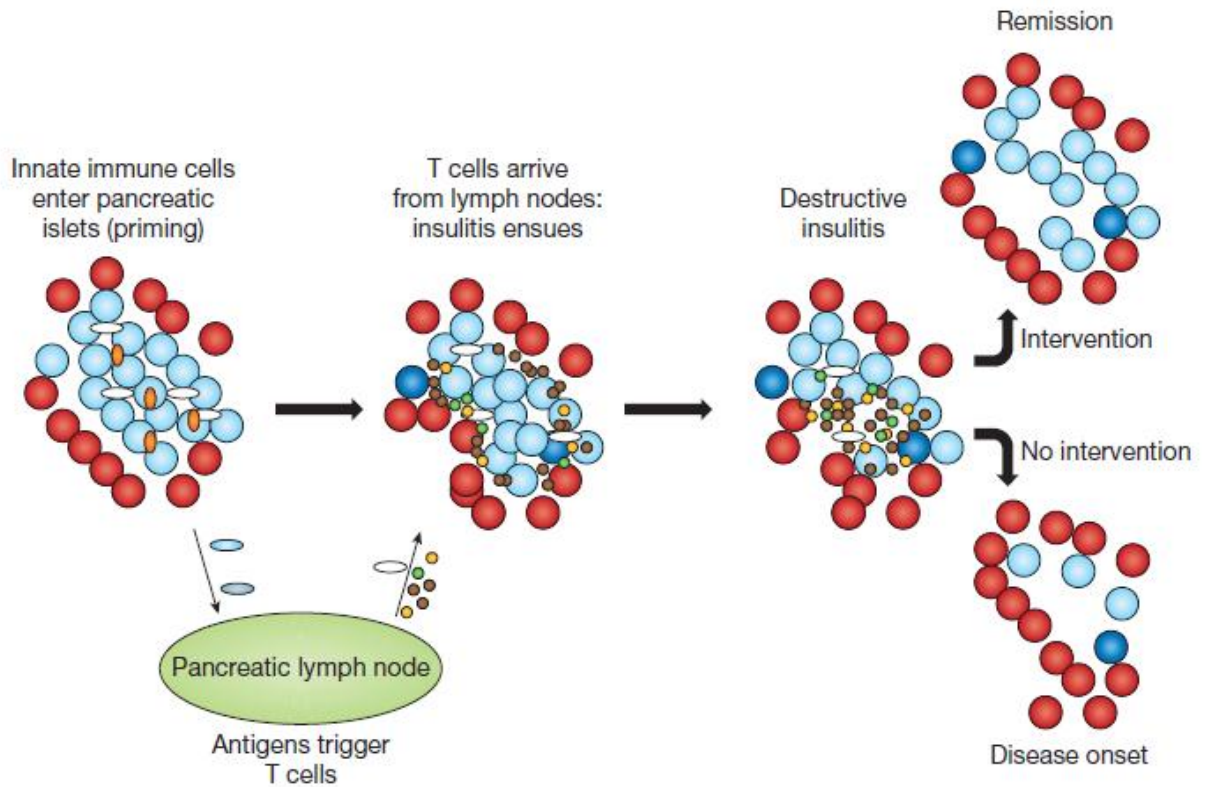
### **1.2.1. Diabetes Mellitus (Type 1)**

Type 1 diabetes is an autoimmune disease characterized by T lymphocyte-mediated destruction of insulin-producing  $\beta$ -cells of the pancreatic islets of Langerhans (Bennett & Todd 1996). The  $\beta$ -cells sense glucose and release insulin to regulate blood glucose homeostasis (Figure 1.3). As a result of destruction of the  $\beta$ -cells, patients with type 1 diabetes lose blood glucose control and rely on exogenous insulin for survival and prevention of ketoacidosis, coma and death (Alberti & Zimmet 1998). With a median age-at-onset of 12 years, the incidence of type 1 diabetes ranges from about 5 to 43 per 100,000 births (Bennett & Todd 1996).

Our current understanding of cellular and molecular mechanisms of disease aetiology and pathophysiology continue to be elucidated. Most of the knowledge concerning the immunologic picture of events have been gained from the two key animal models of type 1 diabetes: the inbred Bio Breeding (BB) rat (Mordes et al. 1996) and non-obese diabetic (NOD) mouse (Anderson & Bluestone 2005). Pathogenesis of the development of type 1 diabetes is a complex interaction between  $\beta$ -cells and cells of both the innate and adaptive immune system (Bluestone, Herold & Eisenbarth 2010). This includes natural killer cells, monocytes and other cells of the innate immune system (Figure 1.4). Analyses of the interactions between natural killer (NK) cells, NKT cells, different dendritic cell populations and T cells have highlighted how these different cell populations can influence the onset of autoimmunity (Lehuen et al. 2010).



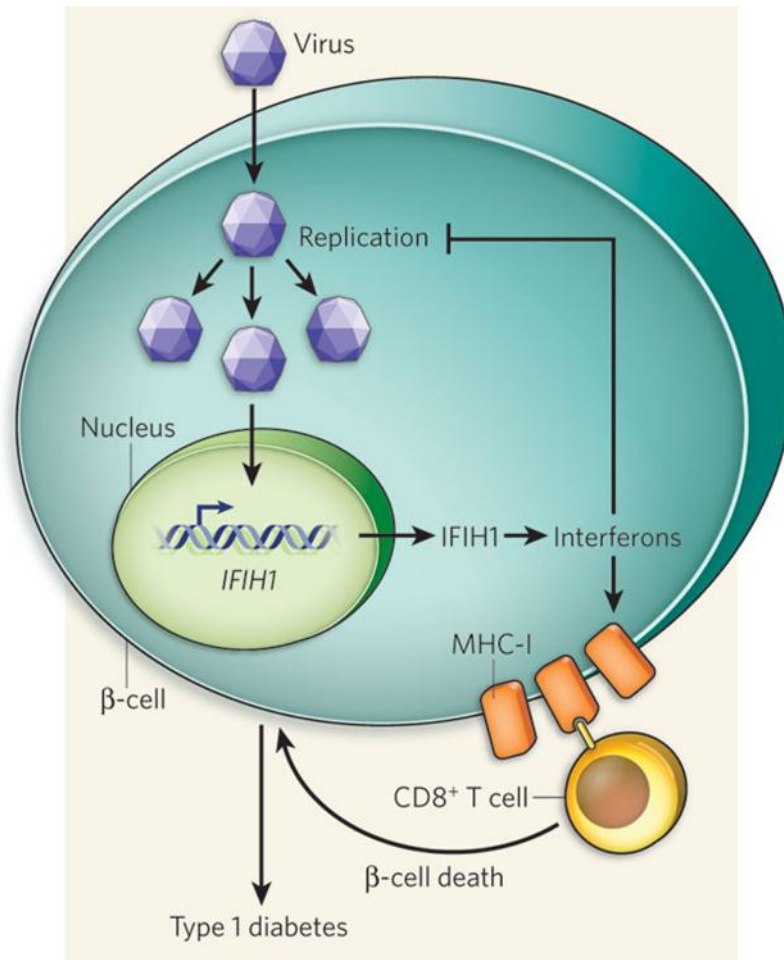
**Figure 1.3. Location of human  $\beta$ -cells in islets of the pancreas.** (a) The pancreas is located in the upper abdomen, close to the liver and behind the stomach. (b) The pancreas secretes digestive enzymes via its duct into the duodenum. (c) An islet is a collection of endocrine cells supplied by capillaries. A thin fibrous capsule separates them from the surrounding (d) exocrine cells, which produce and secrete zymogen. The endocrine cells include: (e)  $\beta$  cells, which synthesise proinsulin, which is cleaved into insulin (stored in granules) and C peptide; (f)  $\alpha$  cells, which secrete glucagon; and (g)  $\delta$  cells, which secrete somatostatin. The hormones are under homeostatic control and are secreted into the bloodstream; the islets themselves are not vascularised. Adapted from (Titus, Badet & Gray 2000).



**Figure 1.4. Immunologic history of type 1 diabetes.** Monocytes and natural killer cells (orange ovals) in the pancreatic islets. The principal site of antigen presentation is thought to be the pancreatic lymph node where islet antigens are presented by antigen-presenting cells (white ovals) to T cells (brown dots). Blue ovals are antigen-presenting cells loaded with islet antigens. B cells (green dots) and dendritic cells may be among the early antigen-presenting cells. The process specifically targets insulin producing β-cells (light blue circles), while other endocrine cells (red circles) within the islet are spared. In the lymph nodes, the cycle of antigen presentation, activation of adaptive immune cells, licensing of effector T cells and epitope spreading continues with the loss of β-cells over time. There is evidence for a regenerative attempt of β-cells in the midst of the islet inflammation (dark blue circles). Regulatory T cells (yellow dots) may arrest this process in its early and late stages but are not able to contain the amplified process in the late stages despite an increase in their numbers. With continued loss of β-cells, hyperglycaemia can be detected. Adapted from (Bluestone, Herold & Eisenbarth 2010).

The development of type 1 diabetes is under polygenic control (Table 1.1), with an additional role for environmental factors (Redondo, Fain & Eisenbarth 2001). This role for environmental factors is highlighted by the 40–60% concordance rate for diabetes onset in identical twins and also by the dramatic increase in the incidence of type 1 diabetes in recent years (Redondo et al. 2008). Improved sanitation and living conditions, together with vaccination strategies, have decreased our exposure to pathogens and development of infectious disease. Therefore, it has been proposed that a reduced exposure to pathogens might be the environmental alteration over the last 60 years that has had a role in the increased incidence of type 1 diabetes (Dunne & Cooke 2005; Lehuen et al. 2010).

Viral infection is also thought to contribute to the disease progress (von Herrath 2009). One of the environmental risk factors identified by several independent studies is represented by enteroviral infection (Dotta et al. 2007). Infected islet  $\beta$ -cells are either directly destroyed by virus-induced cytolysis or cause an inflammatory reaction as modelled in Figure 1.5. Viral infection is implicated in release of normally sequestered antigens, which then trigger pathogenic autoreactive T cell responses (von Herrath 2009).



**Figure 1.5. Viral infection, the interferon response and diabetes.** On infection with viruses such as human enteroviruses, interferon-response genes, including *IFIH1*, are activated in insulin-producing pancreatic  $\beta$ -cells, leading to increased levels of interferon. These immune mediators not only inhibit viral replication, but also enhance the expression of surface MHC-I molecules. Cytotoxic CD8<sup>+</sup> T cells recognize infected  $\beta$ -cells through the MHC-I molecules on their surface, damaging and eventually killing them. Thus, viral infection can contribute to the development of type 1 diabetes. Adapted from (von Herrath 2009).

Non-genetic	Genetic
Viral infections (for example, coxsackie, cytomegalovirus)	Human leukocyte antigen (HLA) associated
Early infant diet (early cessation of breast feeding/early introduction of cow's milk)	Non-HLA associated
Perinatal infections	
Toxins (for example, dietary nitrosamines, bafilomycin, concanamycin A)	
Vaccine administration	

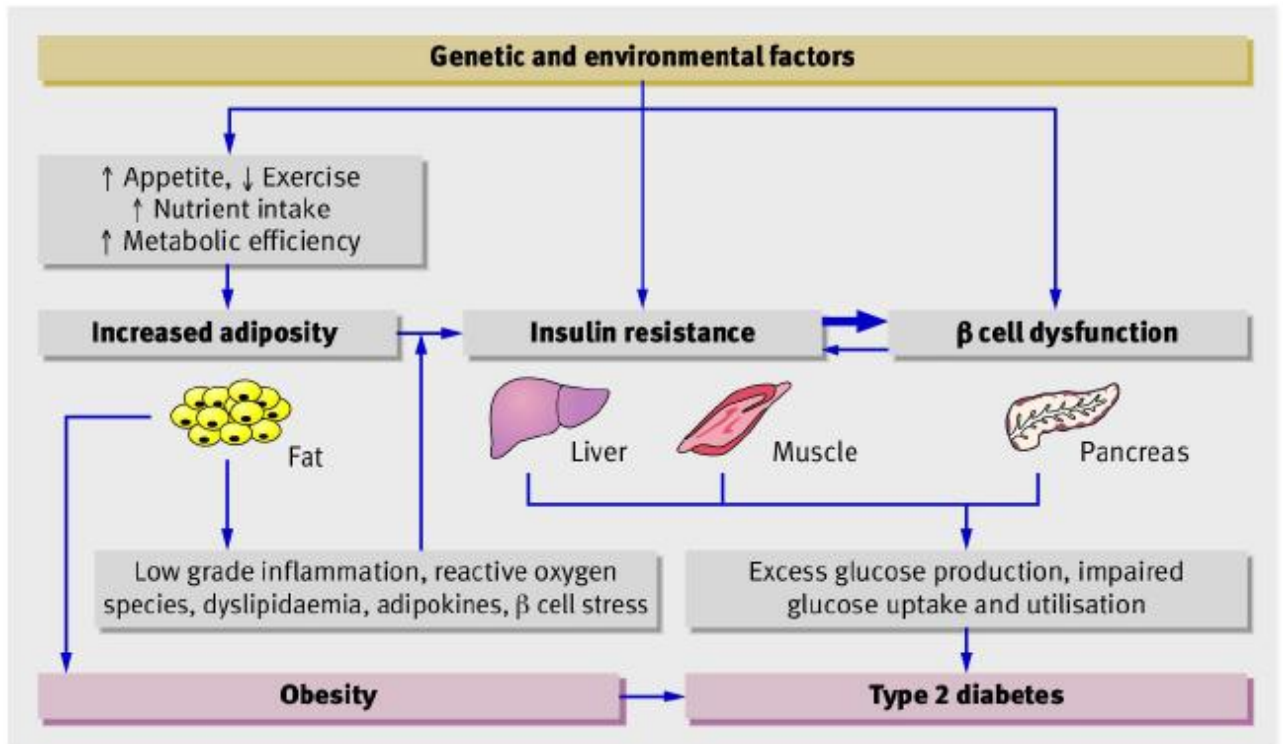
**Table 1.1. Possible aetiological factors in type 1 diabetes.** Adapted from (Zimmet, P, Alberti & Shaw 2001)



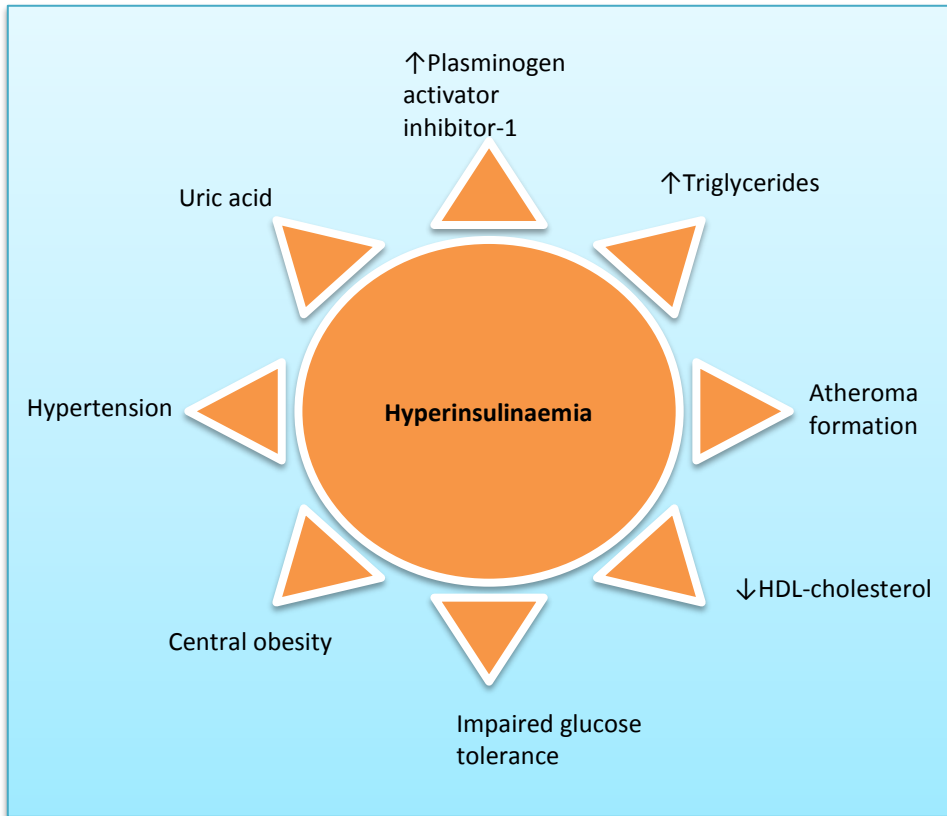
### **1.2.3 Diabetes Mellitus (Type 2)**

Diabetes of this type is prevalent in people with a resistance to the action of insulin and often do not necessarily require insulin treatment to survive (Alberti & Zimmet 1998). Type 2 diabetes is often undiagnosed for many years because the hyperglycaemia is often not severe enough to cause noticeable symptoms. Nevertheless, data suggests that such patients are at risk of developing complications many years before the onset of clinical diabetes (Zimmet, PZ & Alberti 1997). The majority of patients with this type of diabetes are obese (Alberti & Zimmet 1998). In these patients, the pancreas continues to produce insulin, yet patients remain hyperglycaemic because body tissues fail to respond efficiently to insulin (Figure 1.6). This phenomenon is known as insulin resistance (Bailey 2011). Accumulating evidence suggests that insulin resistance (Figure 1.7) may be the common aetiological factor of the metabolic syndrome (Alberti & Zimmet 1998; Haffner et al. 1992). This syndrome is associated with hypertension, obesity, dyslipidaemia and macrovascular disease (Alberti & Zimmet 1998; Bailey 2011).

The high prevalence of type 2 diabetes has been attributed to a mix of genetic susceptibilities, behavioural and environmental factors such as sedentary lifestyle and nutrition (Table 1.2) (Diamond 2003). The disease certainly has a genetic component as there is a concordance in diagnosis of nearly 100% for monozygotic twins, but only 20% for dizygotic twins (Hales & Barker 1992). Genetic variations in the expression of genes that affect feeding behaviour and metabolism can result in increased storage of nutrients (Bailey 2011). This in turn accentuates further genetic vulnerabilities that disturb insulin secretion and interfere with the action of insulin on tissues (McCarthy 2008). Environmental factors such as inappropriate quality and excess quantity of nutrients, insufficient physical activity, low grade inflammation, and oxidative stress combine with genetic factors to increase adiposity and insulin resistance (Bailey 2011).



**Figure 1.6. Pathogenesis of type 2 diabetes with obesity.** Genetic factors and environmental impositions confer susceptibility to weight gain, insulin resistance, and pancreatic  $\beta$  cell dysfunction. Obesity promotes insulin resistance, which is initially compensated for by increased insulin concentrations. When the insulin concentrations are unable to overcome the insulin resistance then hyperglycaemia develops. Continued deterioration of  $\beta$ -cell function causes further impairment of glucose homeostasis into type 2 diabetes. Adapted from (Bailey 2011).



**Figure 1.7. The insulin resistance syndrome.**

Genetic factors
Genetic markers, family history, 'thrifty gene(s)'
Demographic characteristics
Sex, age, ethnicity
Behavioural- and lifestyle-related risk factors
Obesity (including distribution of obesity and duration)
Physical inactivity
Diet
Stress
'Westernization, urbanization, modernization'
Metabolic determinants and intermediate risk categories of type 2 diabetes
Impaired glucose tolerance
Insulin resistance
Pregnancy-related determinants (parity, gestational diabetes, diabetes in offspring of women with diabetes during pregnancy, intra-uterine mal - or overnutrition)

**Table 1.2. Aetiological determinants and risk factors of type 2 diabetes.** Adapted from (Zimmet, P, Alberti & Shaw 2001).

One of the major debates in diabetes aetiology is the issue of the thrifty genotype (Diamond 2003). The “thrifty gene” hypothesis postulates the possible benefits of genes predisposing to type 2 diabetes (Zimmet, P, Alberti & Shaw 2001; Zimmet, PZ 1992). The ‘thrifty genotype’ hypothesis provides an explanation of the very high prevalence of obesity and type 2 diabetes in the American Pima Indians, Australian Aborigines and Pacific Islanders. Diabesity (Astrup & Finer 2000) – term to describe coexistent type 2 diabetes and obesity, could be the result of an evolutionary advantageous thrifty genotype that promoted fat deposition and storage of calories in times of plenty (Zimmet, P, Alberti & Shaw 2001). This mechanism would have been advantageous during the “famine and feast” periods. However, with Westernization, these populations now have a plentiful supply of a diet with an excess of energy. This has been accompanied by a reduction in both occupational and leisure-based physical activity. Both factors may therefore cause the previously favourable metabolic profile seen in ‘survivors’ to become a handicap, which results in obesity and type 2 diabetes (Dowse & Zimmet 1993; Zimmet, P, Alberti & Shaw 2001). All in all, the following summary can be used to describe the basis of type 2 diabetes: “it is a lifestyle disorder with the highest prevalence seen in populations that have a heightened genetic susceptibility; environmental factors associated with lifestyle unmask the disease” (Diamond 2003).

## **1.3 Diabetes and Impaired Wound Healing**

### **1.3.1 Scope of problem**

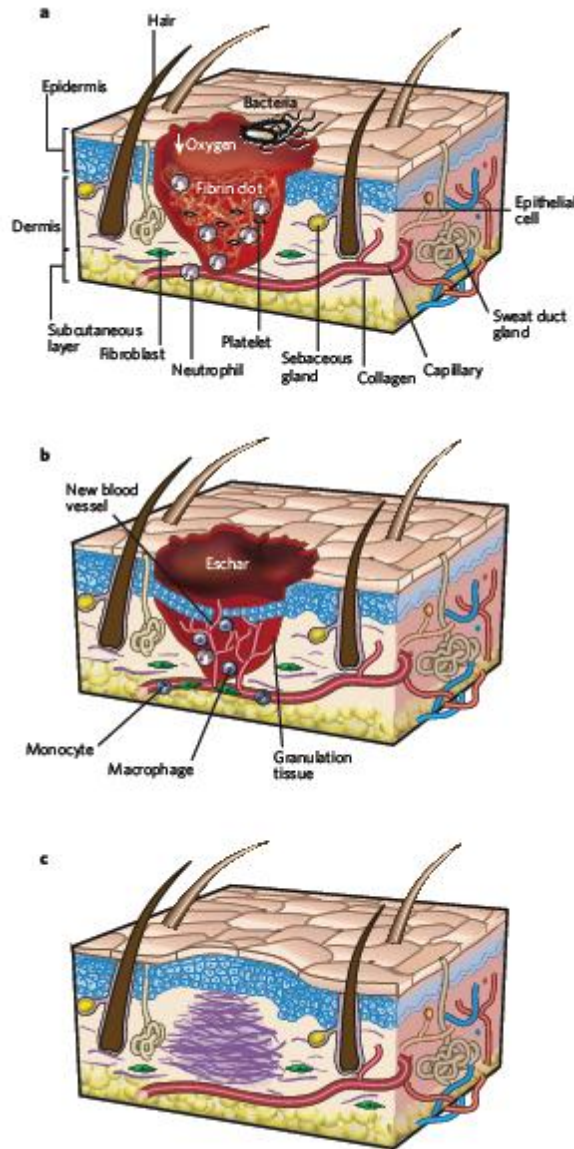
The process of wound repair is a normal reaction to injury, which sets in motion a sequence of events aimed at restoring the skin to its normal anatomical structure and physiological function. Most wounds heal without difficulty and complications. However, some wounds are subjected to inhibitory factors that impede healing. Diabetes is characterized by delayed and poor wound healing. The diabetic ulcer represents a serious wound healing complication (Goren et al. 2008) and is considered to be the most common and classic example of defective healing associated with diabetes mellitus (Lipsky et al. 2006). Diabetic foot ulcers are likely to occur in up to 25% of people with diabetes mellitus at some time in their life (Bentley & Foster 2007; Boulton et al. 2005). Furthermore, in the majority of diabetic patients, the initial condition that eventually leads to amputation is a skin ulcer (Caputo et al. 1994). Approximately 50% of all limb amputations occur in people with diabetes (Akbari & LoGerfo 1999). The average cost of an amputation in Australia is approximately \$20,416 while the cost of a single episode of a diabetic ulcer is \$15,413 (Clarke et al. 2008). The total annual care costs directly attributed to diabetes in 2000 in Australia were estimated to be \$636 million (Davis et al. 2006). With an ageing and increasingly obese population the health care expenditure associated with diabetes and indirect costs that develop as a result of lost productivity through morbidity and reduced quality of life have been predicted to escalate in the future (Cameron, AJ et al. 2003; Manuel & Schultz 2004).

### **1.3.2 Classic stages of the wound healing process**

Any consideration of diabetic wound healing must bear in mind the general principles of the wound healing process. Classic stages of wound repair are illustrated in Figure 1.8. Although the stages of wound healing are outlined below in a defined and sequential arrangement, in reality they overlap and influence each other throughout the wound healing process. It has been useful to divide the repair process into four overlapping phases of coagulation, inflammation, migration-proliferation (including matrix deposition), and remodelling (Figure 1.9).

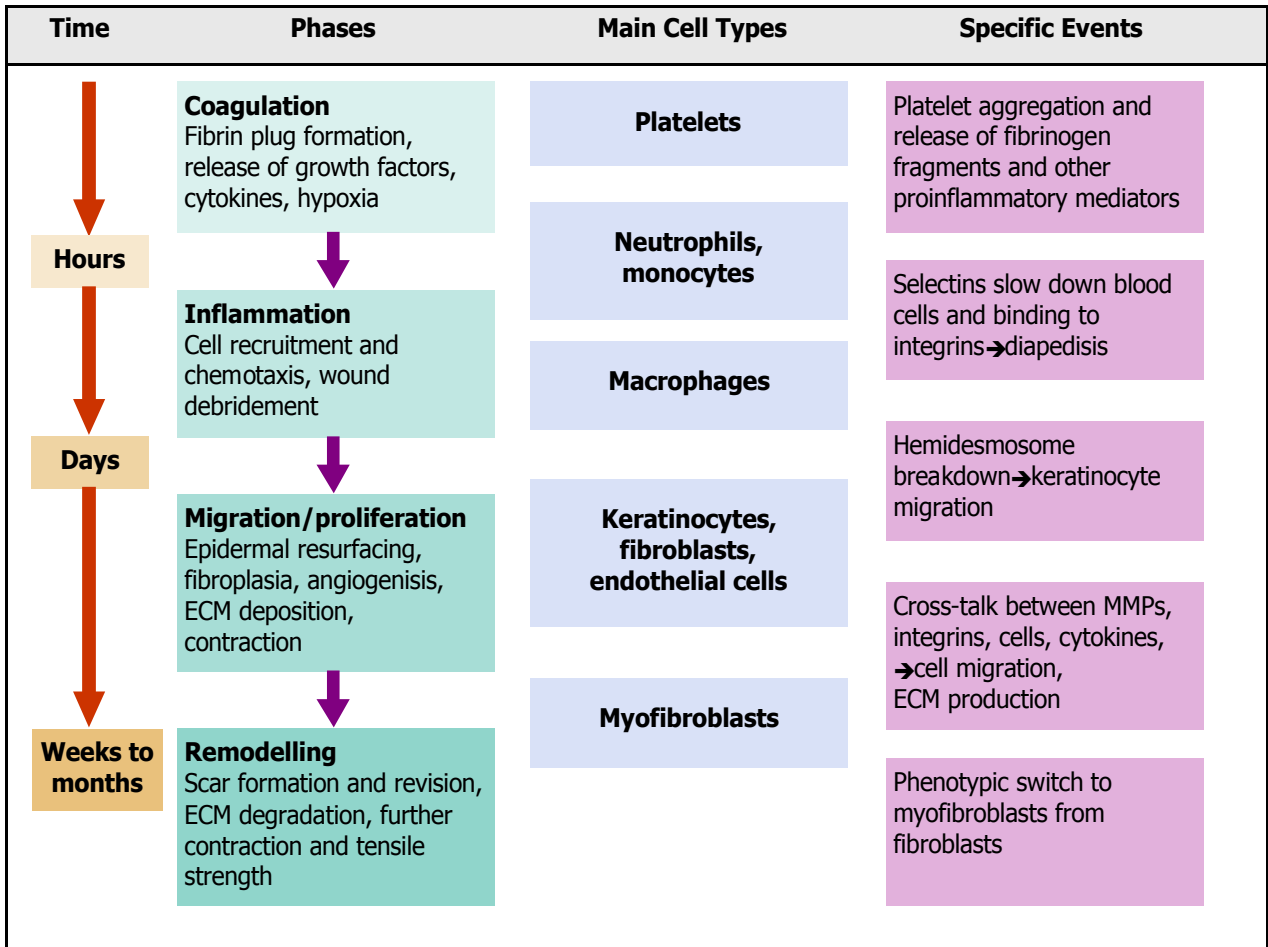
#### **Phase 1: Coagulation**

On wounding, blood vessels are disrupted such that blood begins to haemorrhage into the surrounding tissues. The first challenge for the body is to prevent further blood loss and maintain the necessary blood volume and pressure, a process termed haemostasis. Coagulation is needed for haemostasis (Falanga 2005). Secreted fibrinogen reacts with the plasma thrombin to form a fibrin clot leading to the cessation of bleeding and to the formation of a scaffold for incoming cells to migrate on (Mana M 2006).



**Figure 1.8. Classic stages of wound repair.** **a**, Inflammation. This stage lasts until about 48 h after injury. The wound is characterised by a hypoxic (ischaemic) environment in which a fibrin clot has formed. Bacteria, neutrophils and platelets are abundant in the wound. **b**, New tissue formation. This stage occurs about 2–10 days after injury. An eschar (scab) has formed on the surface of the wound. The migration of epithelial cells can be observed under the eschar. **c**, Remodelling. This stage lasts for a year or longer. Disorganised collagen has been laid down by fibroblasts that have migrated into the wound. The re-epithelialized wound is slightly higher than the surrounding surface, and the healed region does not contain normal skin appendages. Adapted from Gurtner et al (Gurtner et al. 2008).





**Figure 1.9. Phases of wound healing, major types of cells involved in each phase, and selected specific event.** Adapted from (Falanga 2005).

## **Phase 2: Inflammation**

Practically, inflammation is defined as a defence process whereby fluid and white blood cells accumulate at a site of injury. After injury or infections, the local production of cytokines and chemokines rapidly stimulates leukocyte migration into tissues (Chanson et al. 2005). First neutrophils and then macrophages and mast cells emigrate from nearby tissues and from the circulation. Neutrophils arrive on the scene very early after any tissue damage. Their prime role appears to be phagocytic clearance of microbes. This is usually achieved in phagolysosomes, but often results in neutrophils blitzing their environs with free radicals that kill many otherwise-healthy host cells as well as infectious agents (Martin and Leibovich, 2005).

Macrophages, which arrive a little later, operate as phagocytes, clearing the wound of matrix and cell debris, including fibrin and used neutrophils (Leibovich & Ross 1975). Macrophages produce numerous cytokines, growth and angiogenic factors that are believed to play an important role in the regulation of fibro-proliferation and angiogenesis (Rappolee et al. 1988). Another key leukocyte lineage, the mast cell, might be a part of the initial activation response at the wound site but is subsequently also recruited with a somewhat later time course than neutrophils and macrophages, and in this regard, has been postulated to act during the later post-inflammatory phases of repair (Martin & Leibovich 2005). Each of these inflammatory cell types release overlapping cocktails of growth factors and cytokines that are presumed to function as tissue repair signals, directing the various behaviours of the host cells as the wound is drawn closed, but also acting to amplify the inflammatory signal recruiting yet more neutrophils, macrophages and mast cells (Martin & Leibovich 2005).

### **Phase 3: Migration/Proliferation**

As the inflammatory phase of wound healing is down regulated, wound contraction begins. Formation of extracellular matrix proteins, angiogenesis, contraction, and keratinocyte migration are essential components of this phase (Falanga 2005).

#### **i) Keratinocyte migration**

Within 24 hours after wounding keratinocytes from the wound margins begin to migrate into the wound bed, proliferating to form the new epithelium (Kirfel & Herzog 2004). As the cells start to migrate across the wound bed they become flat and elongated with long cytoplasmic projections, called lamellipodia. In the unwounded epidermis the basal keratinocytes are joined to neighbouring keratinocytes by desmosomes (Green & Jones 1996) and to the basement membrane by hemidesmosomes (Borradori & Sonnenberg 1999). When cells become migratory, these structures detach and become internalised from the membrane towards a perinuclear localization (Krawczyk & Wilgram 1973).

#### **ii) Keratinocyte proliferation**

Keratinocyte proliferation is sustained by growth factors and matrix metalloproteinases (MMPs). It is well established that three growth factors, (i) EGF (epidermal growth factor), (ii) TGF- $\alpha$  (transforming growth factor- $\alpha$ ) and (iii) KGF (keratinocyte growth factor), are central players in the proliferation process (Werner & Grose 2003). Eosinophils, macrophages and epidermal keratinocytes at the wound edge have been identified as key sources of EGF and TGF- $\alpha$  (Kirfel & Herzog 2004).

#### **Phase 4: Remodelling**

Within a week of injury several events have occurred, including matrix deposition, phenotypic changes in fibroblasts to myofibroblasts (TGF- $\beta$  induced) and early remodelling (Singer & Clark 1999). It is known that myofibroblasts contract in a healing wound and that this serves to draw the edges of the wound together, reducing the size of the tissue defect (Darby & Hewitson 2007). Extracellular matrix is formed, providing initial support for cell migration, and is later degraded, as a result of serine proteases and MMPs (Singer & Clark 1999). Collagen is synthesised and secreted by fibroblasts in the granulation tissue. Sequential deposition of collagens occurs, first Type III and then Type I, and hydroxylation of these proteins peak at about 3 weeks. The wound continues to contract, with maximum tensile strength being typically 60% of the previously unwounded skin (Ramasastry 2005).

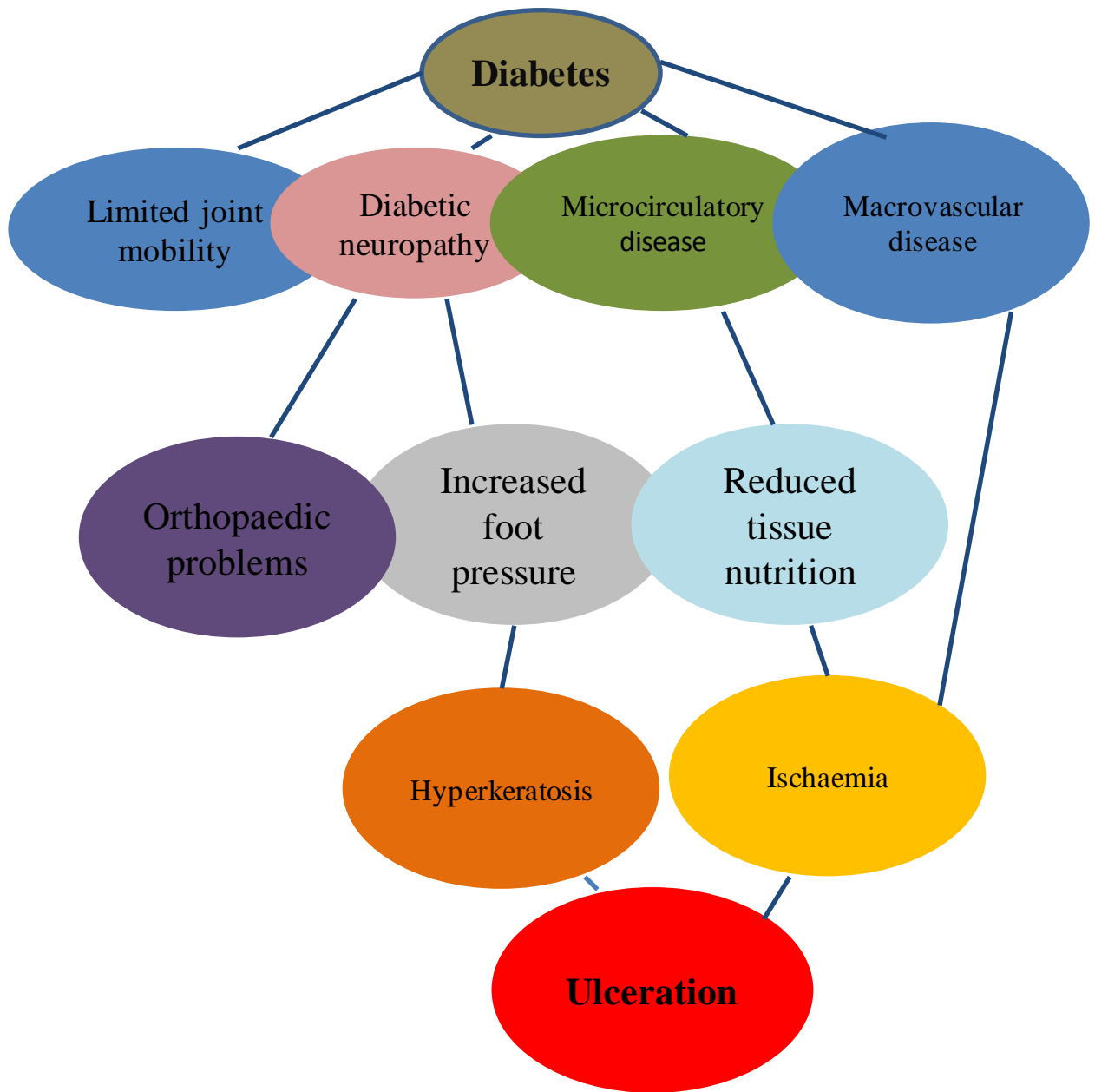
#### **1.3.3. Diabetic wound healing**

Diabetes-associated impaired wound healing results from a complex of risk factors (Figure 1.10). Neuropathy resulting in insensitivity, abnormal foot biomechanics and peripheral vascular disease are the major contributors to the development of the diabetic ulcer (Reiber et al. 1999). Diabetes is associated with both microvascular and macrovascular diseases affecting the skin. A number of biochemical and mechanical factors converge on the endothelium, resulting in endothelial dysfunction and vascular inflammation (Hartge, Unger & Kintscher 2007). Although, the pathology of atheromatous lesion in diabetes is identical to that in non-diabetic people, other factors besides hyperglycaemia may predispose to macrovascular disease. One possibility for this is hyperinsulinaemia or insulin resistance. Insulin resistance is associated with endothelial

dysfunction (Duncan et al. 2008) and is linked with increased risk of vascular disease, hyperglycaemia, dyslipidaemia, hypertension and obesity (Cade 2008).

Neuropathy plays an important role in wound healing of patients with diabetes mellitus, with disturbances of sensory, motor, and autonomic functions leading to ulceration due to trauma or excessive pressure on a deformed foot that lacks protective sensation. Once the protective layer of skin is breached, underlying tissues are exposed to bacterial colonization. Elevated glucose levels provide an ideal environment for bacterial growth. Various immunologic disturbances, such as decreased functions (chemotaxis, phagocytosis, killing) of diabetic polymorphonuclear cells and diabetic monocytes/macrophages (Geerlings & Hoepelman 1999) are likely to increase the risk and severity of wound infections (Geerlings & Hoepelman 1999; Lipsky et al. 2006).

Animal studies indicate several possible mechanisms that could potentially provide an explanation for the impaired wound-healing conditions in the diabetic patient. Of significance, insulin signalling pathway is believed to be important for differentiation and glucose uptake of skin keratinocytes and exposure to high glucose is associated with changes in their morphology, reduced proliferation and enhanced differentiation (Spravchikov et al. 2001). Furthermore, disturbances in insulin signalling coupled with the elevated levels of obesity-associated inflammatory mediator TNF- $\alpha$  are functionally connected to impaired wound healing in diabetes (Goren et al. 2006).



**Figure 1.10. Pathways to foot ulceration in the diabetic patient.**

Resident human skin cells in diabetic ulcers exhibit replicative senescence and become phenotypically altered (Falanga 2005). Evidence suggests that more chronic wound fibroblasts show decreased expression of TGF- $\beta$ 2 receptors, with impaired phosphorylation of transduction signals including Smad2, Smad3 and mitogen-activated protein kinase (Kim et al. 2003). Other cellular abnormalities include reduced keratinocyte migration and proliferation, both important for re-epithelialization (Lan et al. 2008). Elevated levels of proteases, such as matrix metalloproteinases (MMPs) have also been implicated by a number of studies as the major protease family responsible for the degradation of key factors critical to the ulcer's ability to heal (Ladwig et al. 2002; Rayment, Upton & Shooter 2008). The abnormal MMP (degradative):TIMP (protective) ratios observed in chronic wounds lead to extracellular matrix (ECM) degradation (Menke, MN et al. 2008). In summary, several pathogenic abnormalities, ranging from disease-specific intrinsic flaws in blood supply, immunological disturbances and matrix turnover to extrinsic factors due to infection and continued trauma, contribute to the delayed wound healing in individuals with diabetes.

#### **1.3.4 Pathophysiology of the diabetic wound**

The pathophysiology of a diabetic wound and associated delayed healing has been attributed to numerous factors (Liu and Velazquez 2008). These include intrinsic factors, such as the peripheral arterial disease (PAD) and neuropathy or progressive development of a sensory and autonomic neuropathy, leading to loss of protective sensation. Extrinsic factors have also been implicated in the development of diabetic foot ulcers. These include excessive pressure due to

joint and bone deformities, rocker bottom deformity with collapse of the longitudinal arch of the foot, wound infection and callus formation, all of which impair wound healing (Figure 1.11).

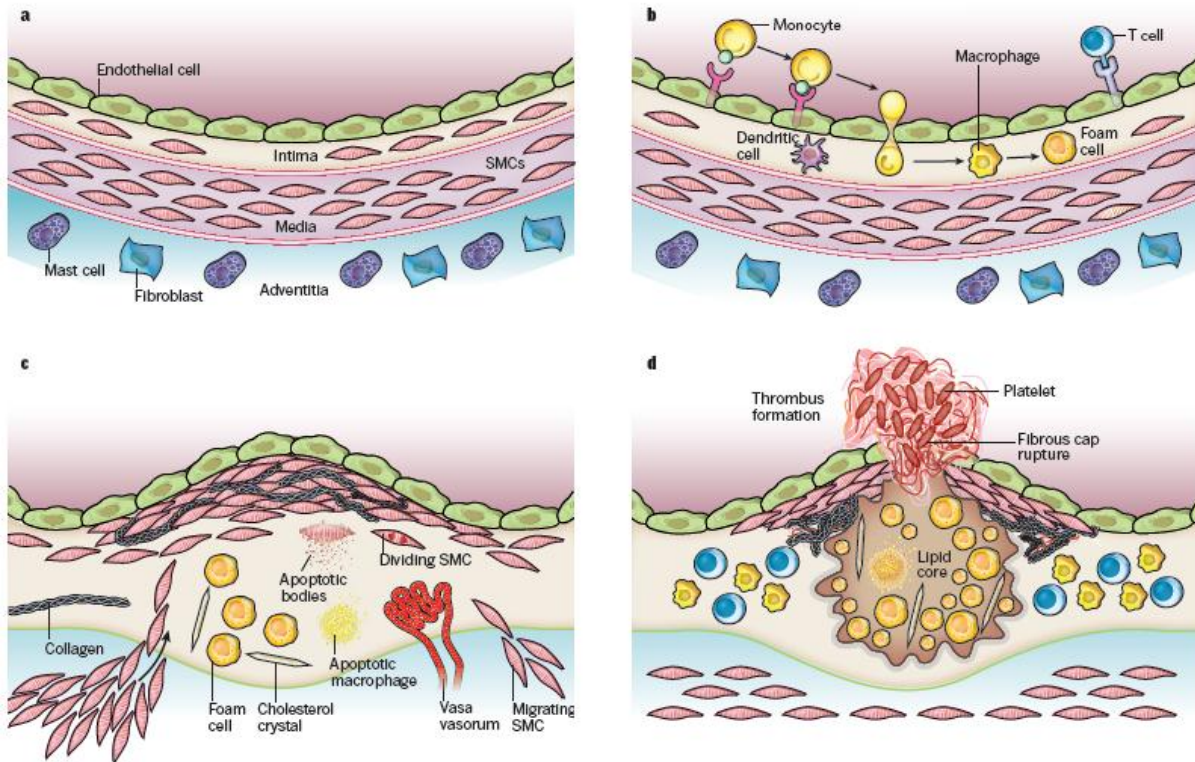
### **1.3.5 Peripheral arterial disease and diabetes mellitus**

Peripheral arterial disease (PAD) is a common vascular complication in the diabetic population. Peripheral arterial disease is characterized by a gradual reduction in blood flow to limbs (Dinh, Scovell et al. 2009). PAD most often affects the arteries that supply the legs and can result in poor perfusion of the muscles and skin of the lower extremity. As a result, PAD is a major risk factor for lower extremity amputation, especially in patients with diabetes. Diabetic wound is complicated by PVD secondary to atherosclerosis. Atherosclerosis is a chronic disease of the arterial wall characterized by the development of atheromatous plaques (Figure 1.12) in the inner lining of the arteries (Libby, Ridker et al. 2011). Plaques cause clinical manifestations by obstructing the blood flow and provoking thrombi. The initial change in the monolayer of endothelial cells that lines the inner arterial surface (Figure 1.12 a), causes the expression of adhesion molecules, aiding leukocyte adhesion (Libby, Ridker et al. 2011). Leukocyte adhesion or the capture of white blood cells followed by migration of the bound leukocytes into the innermost layer of the artery, the tunica intima (Figure 12.1 b) is caused by stimuli, such as hypertension (Libby, Ridker et al. 2011). Once resident in the artery wall, monocytes — the most numerous white blood cells in plaques — differentiate into tissue macrophages. In the nascent atheroma, these mononuclear phagocytes engulf lipoprotein particles and become foam cells — a term that reflects the microscopic appearance of these lipid-laden macrophages (Libby, Ridker et al. 2011).





**Figure 1.11. A case of diabetic foot ulcer.** A 61-year-old obese male with a 6-year history of type 2 diabetes mellitus. Images of patient's left foot and ankle demonstrate a collapse of the arch and rocker bottom midfoot deformity. The plantar blister and callus were debrided to expose an ulcer under the rocker bottom bony prominence. Adapted from (Embil and Trepman 2009).



**Figure 1.12. Stages in the development of atherosclerotic lesions.** The normal muscular artery and the cell changes that occur during disease progression to thrombosis are shown. a, The normal artery contains three layers: tunica intima, tunica media and adventitia. b, The initial steps of atherosclerosis include adhesion of blood leukocytes to the activated endothelial monolayer, directed migration of the bound leukocytes into the intima, maturation of monocytes into macrophages, and their uptake of lipid, yielding foam cells. c, Lesion progression involves the migration of SMCs from the media to the intima, the proliferation of resident intimal SMCs and media-derived SMCs, and the heightened synthesis of extracellular matrix macromolecules such as collagen, elastin and proteoglycans. d, Thrombosis often complicates a physical disruption of the atherosclerotic plaque. Shown is a fracture of the plaque's fibrous cap, which has enabled blood coagulation and thrombus formation, which in turn, impedes blood flow. Adapted from (Libby, Ridker et al. 2011).

### **1.3.6 Impaired wound arteriogenesis and diabetes**

Arteries provide bulk flow to the tissue, however, if an existing artery is occluded, the development of collateral vessels is required in an attempt to restore the blood flow. The mechanisms leading to the development of collateral vessels differ from those usually involved in angiogenesis (Carmeliet 2003; Lehoux and Levy 2006). Hence, the term “arteriogenesis” was established to encompass both angiogenesis and collateral growth, recognizing significant differences between these processes (Lehoux and Levy 2006). Arteriogenesis, hence, represents an important compensatory mechanism for atherosclerotic vessel occlusion. When an artery is occluded, its vascular territory becomes ischaemic (Carmeliet 2003). Arterial systems are often interconnected by pre-existing collateral vessels and the collaterals can enlarge and salvage the ischaemic region (Helisch and Schaper 2003).

The first clinical study focusing on the link between diabetes and collateral artery growth was published by Abaci et al. and consisted of 205 diabetic patients with significant coronary stenosis and matching controls. In diabetics, coronary collaterals were shown to be significantly reduced (Abaci, Oguzhan et al. 1999). More recently, similar results have been confirmed indicating a clear inverse relationship between the presence of diabetes and the extent of collaterals formed (Mouquet, Cuilleret et al. 2009). Endothelial function, vascular smooth muscle cell proliferation and the interaction between vessel wall and circulating cell, all which are important in collateral artery formation, have been identified as possible explanation for the attenuation of arteriogenesis in diabetes (de Groot, Pasterkamp et al. 2009). Hence, the identification of molecules regulating collateral growth offers significant potential for the treatment of ischaemic heart and limb disease (Carmeliet 2003).

Apart from collateral growth deficiency, reduced production of proangiogenic growth factors and decreased wound angiogenesis are thought to contribute to impaired wound repair in diabetic patients (Ackermann, Wolloscheck et al. 2011). Wound healing is strongly dependent on the formation of granulation tissue, which in turn is intimately correlated with the induction of new vessel formation. Wound angiogenesis relies on the formation of extracellular matrix in the wound bed and on the migration and mitogenic stimulation of endothelial cells (Singer and Clark 1999). Within this process, vascular endothelial growth factor (VEGF) enhances tissue repair by stimulating proliferation in ECs and increasing collagen deposition (Witte, Kiyama et al. 2002), whereas fibroblast growth factor (FGF) and platelet-derived growth factor (PDGF) regulate the synthesis and deposition of extracellular protein components and promote wound angiogenesis (Ackermann, Wolloscheck et al. 2011).

A number of studies have investigated whether angiogenesis stimulation can be used to promote wound healing. Of importance, adenoviral gene transfer with proangiogenic growth factors (especially VEGF) was shown to accelerate diabetic healing, whereas topical application with VEGF or PDGF showed inconsistent results (Chan, Liu et al. 2006; Ackermann, Wolloscheck et al. 2011). Murine diabetic wound healing models have shown that priming with a combination of VEGF, FGF, and PDGF led to accelerated wound closure and higher vessel densities (Ackermann, Wolloscheck et al. 2011). Several investigators have indicated that combination therapies may therefore better mimic the complex interactions of the wound microenvironment and synergistically facilitate angiogenesis in diabetic environment than monotherapy using only a single factor (Kano, Morishita et al. 2005; Ackermann, Wolloscheck et al. 2011).

### **1.3.7 Cellular and molecular basis of wound healing in diabetes**

Any diabetic individual, who suffers what may appear to be a trivial trauma such as a break in the skin of their foot, is at danger for amputation. Neuropathic and immunocompromised with an impaired ability to fight wound infections, diabetic patients become largely unable to elicit an adequate inflammatory response. Therefore, the diabetic foot ulcer becomes a portal for infection that can lead to life-threatening sepsis and require limb amputation (Brem and Tomic-Canic 2007). Currently, over 100 known physiologic factors contribute to wound healing defects in patients with diabetes (Brem and Tomic-Canic 2007). Multiple mechanisms have been proposed (Table 1.3).

<b>Documented abnormalities</b>
Growth factor deficiency (PDGF, EGF, TGF- $\beta$ 1, VEGF, CTGF, bFGF) (Thomson, McLennan & Twigg 2006)
Microvascular impairments and defects in endothelium (Pham, Economides & Veves 1998)
Pericyte degeneration and thickening of basement membrane in skeletal muscle capillaries and glomeruli due to an increased level of ECM proteins (Tilton et al. 1985)
Decreased vasodilatory response (Ajjam et al. 1985) and abnormal autoregulation (Pham, Economides & Veves 1998) of microvascular endothelium
Correlation between tissue oxygenation and ulcer healing (Schultz et al. 2011)
Altered blood flow and sensory deficits due to neuropathy (Falanga 2005)
Deficits in the bactericidal action of granulocytes (Falanga 2005)
Glycation of fibronectin that reduces collagen binding (Schultz et al. 2011)
Glycation of collagen and fibronectin that interferes with epithelial cell adherence (McDermott et al. 2003)
Glycation of albumin that leads to overproduction of certain growth factors (Cohen et al. 2002)
Effects in experimental animals: altered leukocyte infiltration and IL-6 levels in wound fluid during the late inflammatory phase (Fahey et al. 1991), differential regulation of 27 ECM genes compared with normal rats (Song & Ergul 2006)
Effects in experimental animals: altered leukocyte infiltration and IL-6 levels in wound fluid during the late inflammatory phase (Fahey et al. 1991), differential regulation of 27 ECM genes compared with normal rats (Song & Ergul 2006)

**Table 1.3: Selected disease-related abnormalities that may contribute to delayed healing of diabetic ulcers.** Adapted from (Schultz, Davidson et al. 2011).

At the cellular level, hyperglycaemia induces impaired macrophage function (Maruyama et al. 2007), monocyte response (Tchaikovski et al. 2009), keratinocyte and fibroblast migration and proliferation (Gibran et al. 2002). Biopsies taken from the epidermis of the non-healing edge of diabetic foot ulcers showed an absence of migration, proliferation and incomplete differentiation, whereas fibroblasts demonstrated a phenotypic change as well as decreased migration (Gibran et al. 2002) and apoptosis (Arya, Pokharia & Tripathi 2011). At the molecular level, chronically increased concentrations of blood glucose lead to decreased growth factor production (Falanga 2005; Galkowska, Wojewodzka & Olszewski 2006; Goren et al. 2006), collagen accumulation (Falanga 2005), disruption in the balance between the accumulation of ECM components and their remodelling by MMPs (Lobmann et al. 2002). The formation of advanced glycation end products (AGEs) has been shown as an important pathophysiological mechanism in the development of impaired wound healing in diabetes (Peppia et al. 2003). High AGE diet was associated with delayed wound healing (Peppia et al. 2003), whereas blockade of AGE restored impaired wound healing in diabetic mice (Goova et al. 2001). Molecular analyses of biopsies from the diabetic patients have identified pathogenic markers that correlate with delayed wound healing (Brem et al. 2007). It has been documented that elevated glucose levels lead to enhanced TGF- $\beta$ 1 production and signal transduction (Thomson, McLennan & Twigg 2006). Given that elevated TGF- $\beta$ 1 was associated with increased expression of cytoskeletal protein Flightless I (Flii) (Adams et al. 2009), it is reasonable to hypothesize that Flii could also be affected by hyperglycaemia. These include overexpression of c-myc and nuclear localization of  $\beta$ -catenin (Stojadinovic et al. 2005). Defective release of cytokines and growth factors such as PDGF (Kawanabe et al. 2007), CTGF (Thomson et al. 2010), EGF (Brem et al. 2007), bFGF (Servold 1991), VEGF (Glotzbach et al. 2010) and TGF- $\beta$ 1 (Mulder 2004) leads to an aberrant response

of inflammatory cells to the wound site, defective wound angiogenic processes, ECM remodelling and epithelialization.

#### **1.4 Cytoskeletal protein Flightless I (Flii)**

##### **1.4.1 Cytoskeletal and Actin-Remodelling Protein Flightless I (Flii)**

Following injury a complex series of events including cell proliferation and migration (Thomas & Harding 2002) are initiated by highly specialized cells, including immune cells, fibroblasts and keratinocytes, which interact with the extracellular matrix to synthesise new tissue to repair the damage (Goldberg & Diegelmann 2010). Changes in cell adhesion, shape and locomotion are aided by the reorganization and remodelling of the actin cytoskeleton (Cowin et al. 2003; Jacinto, Martinez-Arias & Martin 2001; Sun et al. 1999), including processes such as the lamellipodia and filopodia guided crawling of keratinocytes during wound re-epithelialisation, immune cell recruitment and fibroblast migration necessary for the deposition of the extracellular matrix and wound contraction (Cowin et al. 2007). Actin-remodelling proteins of the gelsolin family (Kwiatkowski 1999), which includes Flii (Davy et al. 2000; Davy et al. 2001), have been identified as regulators of the dynamic remodelling of the actin cytoskeleton (Goshima et al. 1999) by severing, capping, nucleating or bundling actin filaments (Campbell et al. 2002). Previous studies have provided strong evidence indicating the significance of actin remodelling proteins in tissue repair and regeneration (Cowin 2005; Cowin et al. 2003). With at least seven known members (Kopecki & Cowin 2008), a growing body of evidence indicates that one highly conserved member of the gelsolin family, Flii is involved in linking the cytoskeletal network with signal transduction pathways (Adams et al. 2009; Davy et al. 2001).



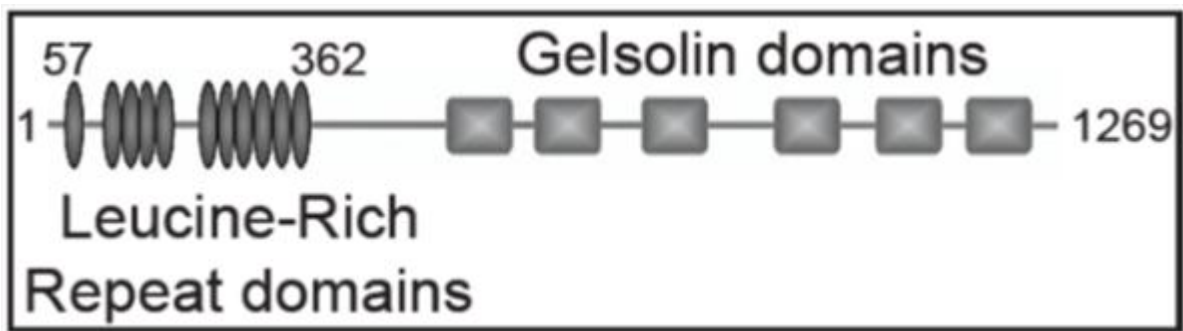
To follow is a comprehensive survey of recent advances made in experimental dermatology studies involving Flightless I (Flii), highlighting its role in wound healing which represents an important facet of complex interplay of its known functions. After the identification of the human homologue of Flii gene the main research focus evolved around its role in development and its functions as a hormone-activated nuclear receptor co-activator (Archer et al. 2004; Campbell et al. 2002; Davy et al. 2000). The role of Flii in wound healing was not identified until 2007 when it was shown to be a negative regulator of wound healing affecting both cellular migration and proliferation (Cowin, A et al. 2007). Recently, multiple papers have been published looking at the different roles of Flii in experimental dermatology including; foetal and adult wound healing, cellular adhesion, burn injury and hair follicle regeneration. These studies highlight Flii as a novel marker of impaired wound healing in different wound pathologies and describe the development of innovative Flii neutralizing antibody therapy approaches, which are currently in preclinical development.

#### **1.4.2 Flightless protein: functions and signalling pathways involved in wound healing**

Originally identified in *Drosophila melanogaster*, Campbell *et.al* reported that Flii gene mutation resulted in an irregular actin organization and degeneration in the flight muscles preventing flight and giving rise to the gene and protein name – Flightless (Campbell et al. 2002; Campbell et al. 1993). Flii is the most evolutionarily conserved member of the gelsolin family of actin-binding proteins (Claudianos & Campbell 1995). Homozygous Flii knockout in the mouse is embryonic lethal, while cultured homozygous Flii mutant blastocysts hatch, attach, and form an outgrowing trophoblast cell layer, but egg cylinder formation fails and embryos ultimately

degenerate indicating an important role for Flii in development (Campbell et al. 2002). The defining feature of Flii is its homology with two gene families, the gelsolin protein family and leucine-rich repeat (LRR)-containing proteins. This unique structure of Flii protein, which distinguishes it from the other members of the gelsolin family, allows protein-protein interaction (Kobe & Kajava 2001). The bipartite domain structure of Flii (Figure 1.13) provides the capacity to transduce cell signalling events into a remodelling of the actin cytoskeleton and is proposed to be involved in a variety of other signalling pathways (Bella et al. 2008; Davy et al. 2001; Goshima et al. 1999; Kobe & Kajava 2001). Acting as a multifunctional protein Flii has the potential to be involved in number of different signalling pathways.

Flii synergy with a GRIP1 co-activator complex negatively affects the association between Flightless associated protein 1 (FLAP1), p300 and  $\beta$ -catenin, suggesting that Flii negatively regulates the activity of  $\beta$ -catenin co-activator complexes (Lee, YH & Stallcup 2006). Further evidence of Flii involvement in regulation of  $\beta$ -catenin pathway has now emerged with studies using 3T3 fibroblasts showing direct and preferential binding of Flii to the activated form of calcium/calmodium dependant protein kinase type II (CaMK-II) affecting the progression of cell cycle (Seward et al. 2008b). Inhibition of CaMK-II and over-expression of Flii results in suppression of transcription of  $\beta$ -catenin dependent transcriptional reporters while suppression of Flii led to enhanced transcriptional activity. It was hypothesized that CaMK-II influences the  $\beta$ -catenin dependent gene expression through Flii interaction implying Flii as a major component of this signalling pathway and a possible regulator of gene transcription (Seward et al. 2008b).



**Figure 1.13. The domain structure of Flii molecule.** Schematic representation of the domain structures of Flii protein showing the two major domains: gelsolin and leucine-rich repeat domains (Lei et al. 2012).

Pathways of Flii synthesis and degradation are also still to be investigated, however studies by Davy et al., have shown that Flii is expressed in different migratory structures, including filopodia, neurites and growth cones and it specifically co-localizes with cytoskeletal structures connected with a migratory phenotype (Davy et al. 2000). Unlike gelsolin, Flii possesses F-actin binding and severing activities independent of  $\text{Ca}^{2+}$  concentration suggesting a role for Flii in dynamic assembly and disassembly of actin filaments (Goshima et al. 1999). This implies a role for Flii at different sites of dynamic actin filament reorganisation suggesting that Flii may be functionally important in cellular responses where active actin remodelling is required, including adhesion and migration.

Using a genomic cosmid clone containing the complete human Flii gene Campbell *et al.*, (2002) constructed a Flii transgenic mouse and shown that a human Flii transgene was capable of restoring normal development to homozygous Flii mutant mouse embryos. This indicates that Flii performs essential functions during early embryogenesis in both *Drosophila* and mammals (Campbell et al. 2002). Heterozygous Flii mutant mice have half the amount of Flii protein and appear phenotypically normal (Campbell et al. 2002). Although Flii was thought to be solely an intracellular protein there is new compelling evidence to suggest that it is also secreted by fibroblasts in culture and like gelsolin may also have extracellular functions following tissue injury (Lee, WM & Galbraith 1992).

Recent findings have identified Flii as a co-activator in oestrogen receptor and thyroid receptor mediated transcription (Lee, YH, Campbell & Stallcup 2004). These receptors bind directly or through other proteins to specific enhancer elements associated with the promoters of target genes, resulting in activation or repression of transcription (Lee, YH, Campbell & Stallcup

2004). Moreover, Flii is as a substrate of the cytokine-independent survival kinase (CISK) acting downstream of PI-3kinase/CISK signalling pathway. CISK association with Flii results in Flii phosphorylation at residues Ser 436 and Thr 818 and modulates Flii activity as an oestrogen receptor co-activator (Xu et al. 2009). Flii knockdown by RNA interference resulted in 293T cells being more sensitive to the interleukin-3 withdrawal-induced apoptosis suggesting that Flii may be involved in this cell cycle pathway. Protein post-translational modifications including phosphorylation by kinases are essential for development and cellular functions and Xu *et al.*, (2009) suggest that CISK phosphorylation of Flii may impact on actin reorganisation and endosome trafficking (Xu et al. 2009).

The functional activities of Flii are evident from recent reports suggesting that the Flii is involved in innate immune system responses where it down-regulates IL-1/TLR4 signalling of Toll-like receptor (TLR) pathway of immune system (Wang, T et al. 2006). Flii interacts directly with MyD88, an intracellular adaptor protein immediately downstream of most Toll-like receptors. MyD88 functions to recruit multiple proteins to precisely control signalling transduction. A novel switch-on, turn-off mechanism of TLR signalling was proposed, governed by the interactions, binding strength and competition between Flii and other MyD88 binding partners, including FLAP-1 and FLAP-2. Binding of Flii to MyD88 and the Toll/IL-1 receptor domain (TIR) of the TLR molecules regulated the signalling for activation of NF- $\kappa$ B, a protein complex, controlling the transcription of DNA and immune response to infection. The temporal and dynamic nature of specific Flii interactions with other proteins of the TLR signalling has identified Flii as a negative regulator of the TLR4 signalling pathway (Dai et al. 2009; Hayashi et al. 2010; Wang, T et al. 2006). A role for Flii in the immune system was also illustrated by its regulation of the pro-inflammatory caspases, namely caspase-1 and caspase-11. Flii modulates

the activity and intracellular localization of these caspases, distributing caspases to the actin network at the leading cell front where they can cleave and activate pro-inflammatory cytokines, including IL-1 $\beta$  and IL-18 (Li, J, Yin, HL & Yuan, J 2008).

The process of wound repair largely depends on different signalling pathways that dictate cell responses by integrating extracellular and intracellular signals. A number of studies have shown a cross-talk between pathways involved in cytoskeletal remodelling, cellular adhesion and migration during wound repair, including TGF- $\beta$  signalling and members of the MAP kinase family, Ras, RhoA, MAPK-ERK kinase and ERK1/2 (Begum, Nur & Zaman 2004; Bhowmick et al. 2001; Hall & Nobes 2000; Wang, Koka & Lan 2005). Multiple *in vivo* and *in vitro* studies have demonstrated that in mouse fibroblasts, Flii specifically colocalises with cytoskeletal structures connected with a migratory phenotype (Davy et al. 2001) and associates with both structural and signalling proteins at sites of focal adhesions, including talin, paxillin and vinculin (Kopecki et al. 2009). Flii is associated with actin arcs, membrane ruffles and is present at the leading edge of cells, where it also colocalises with the GTP-binding proteins Ras, Cdc42 and RhoA that have central roles in regulating cytoskeletal reorganization (Ben-Ze'ev 1997; Kaibuchi, Kuroda & Amano 1999). Association of Flii LRR domain with Ras and Cdc42 proteins suggests possible involvement of Flii in downstream PI3K and MAPK signalling pathways. The PI3K/Akt signalling pathways are known to regulate numerous fundamental cellular functions during wound repair, including cell growth, proliferation, motility and survival and is activated by a variety of extracellular signals (Wilkes et al. 2005). This signalling pathway is known to negatively regulate TGF- $\beta$ 1 activity due to inhibitory binding of Akt to Smad3 (Tian, B et al. 2002). Moreover, inhibitors of the PI3K prevent the translocation of Flii to actin structures, implying Flii is involved in the PI3K signalling pathway (Davy et al. 2001). In

support of Flii involvement in the PI3K signalling pathway studies have further shown the effect of Flii over-expression on signalling of different GTP-ases including cdc42, RhoA and Rac-1 and have demonstrated that the inhibitory effect of Flii on focal adhesion turnover and paxillin phosphorylation is Rac-1 dependant (Kopecki, O'Neill, et al. 2011a), implicating the involvement of these molecules in a common functional pathway.

### **1.4.3 A key role for Flightless I in skin architecture, wound repair and tissue regeneration**

Flii is an important mediator of wound healing and its attenuation leads to improved wound outcomes Flii-deficient mice have improved wound healing, whereas Flii-over-expressing mice had significantly impaired wound healing with larger wounds. *In vitro* and *in vivo* experiments suggested that Flii affects collagen I synthesis, with decreased collagen I secretion observed in Flii siRNA treated fibroblasts and Flii heterozygous mice (Cowin, AJ et al. 2007). Similarly, incisional wounds in Flii-over-expressing mice showed significantly increased levels of collagen I (Cowin, AJ et al. 2007) a major contributing factor to excessive scar formation. In agreement with these findings a recent study in NIH 3T3 cells demonstrated that decreasing Flii expression using siRNA resulted in decreased intracellular stress fibres formation (Higashi et al. 2010) suggesting that in addition to modulating collagen I secretion (Cowin, A et al. 2007) Flii may also affect cellular contraction or incorporation of  $\alpha$ -SMA into cell stress fibers. Flii is secreted in response to injury *in vitro* by skin fibroblasts (Cowin, AJ et al. 2007), suggesting that it may also have extracellular functions, which may be mediated by the LRR domain. More recently, Flii was detected as a secreted Flii protein in human bodily fluids (Lei et al. 2012) and extracellular Flii binds LPS and can affect inflammation. When considering the critical role of

inflammation in wound healing, it is conceivable that the response elicited by the immune cells must occur in a balanced manner. Insufficient inflammation in the early stages of wound healing will inhibit the capacity to clear infection, whereas excessive inflammation can be damaging and may lead to a chronic wound (Martin 1997). With this in mind Flii can alter pro-inflammatory TNF- $\alpha$  secretion by binding to LPS (Lei et al. 2012). The binding of Flii to LPS and the timing of Flii secretion suggest that Flii may act as a scavenger to help remove excess LPS and prevent hyper-activation of the immune system. The upregulation of Flii in wounds might alter inflammation and prevent excess stimulation of cytokine production.

Flii modulates integrin receptor expression through association with different structural and signalling proteins allowing Flii to regulate hemidesmosome formation, modulate focal adhesions and filamentous actin stress fiber formation (Kopecki, Arkell et al. 2009; Kopecki, O'Neill et al. 2011), all of which are needed during epithelial migration and tissue organization (Hynes 2002). Hemidesmosomes are structures that provide stable adhesion and anchoring sites, hence aiding the migration of epithelial cells (Kopecki et al. 2009). Over-expression of Flii in mice lead to impaired hemidesmosome structure and altered arrangement of ColVII anchoring fibrils suggesting that Flii is important for the maintenance of skin integrity and homeostasis at the dermal epidermal junction (Kopecki, Arkell, et al. 2011). Indeed, Flii over-expressing mice have significantly thinner and weaker skin (Kopecki et al. 2009). This suggests that Flii may be important in diseases like Epidermolysis Bullosa where skin integrity is compromised. Primary keratinocytes and fibroblasts with increased Flii expression have impaired adhesion when seeded on different extracellular matrix substrates, including laminin, collagen I and fibronectin (Kopecki et al. 2009). Flii has a negative effect on focal adhesion turnover, cell spreading and



the regulation of focal adhesions through inhibition of paxillin phosphorylation via a Rac1-dependent pathway (Kopecki, O'Neill, et al. 2011a).

A recent study has identified Flii as a regulator of Rho induced linear actin assembly mediated by Diaphanous-related formins, Daam1 and mDia1. Flii but not gelsolin associates with formins and this binding is mediated specifically by the G4-6 gelsolin motif of Flii protein which has a 41% homology and 18% identity to the corresponding G4-6 region of gelsolin (Higashi et al. 2010). These results indicate that a conserved protein like Flii which has a unique structure can act as multifunctional protein in skin with specific Flii events on different cellular responses.

The exact mechanism of Flii activity in mediating cellular adhesion is not yet clear however the inhibitory effect of Flii on paxillin phosphorylation and downstream signalling of Src, p-130Cas and different GTPases suggest that cellular adhesion might be mediated both by Flii cytoskeletal activity and its nuclear receptor co-activator function as there appears to be a regulated balance of actin dynamics and altered levels of number of adhesion signalling proteins. Adams et al., have documented that attenuation of Flii enhanced burn injury repair via modulation of TGF- $\beta$ 1 and TGF- $\beta$ 3 (Adams, Ruzehaji et al. 2009). The role of transforming growth factor TGF- $\beta$ 1 and TGF- $\beta$ 3 in wound healing has been an active area of research (Huang et al. 2002). Classic studies by Shah et al. discovered that by mimicking the embryonic profiles in adult rodent wounds through the addition of TGF- $\beta$ 3 and suppression of TGF- $\beta$ 1 via intradermal injection of TGF- $\beta$ 1 neutralising antibodies, scarring was reduced in the incisional wounds (Shah, Foreman & Ferguson 1995). Burn injury repair was improved in Flii heterozygous mice with their scald wounds healing faster than wild-type control mice and Flii over-expressing mice (Adams et al. 2009). Interestingly, pro-scarring transforming growth factor TGF- $\beta$ 1 protein and gene expression were reduced in Flii heterozygous scald wounds, whereas anti-scarring TGF- $\beta$ 3 was

significantly increased (Adams et al. 2009). Additionally, neutralising antibodies raised against the LRR domain of Flii improved burn injury repair in mice, indicating a novel approach for the treatment of thermal burn wounds (Adams et al. 2009).

Foetal wounds have the ability to regenerate an organized dermis that is identical to that of the original tissue (Larson, Longaker et al. 2010). Emerging evidence suggests that Flii has a significant role in early and late gestation foetal wound repair suggesting an important role in the healing of skin by tissue regeneration rather than fibrosis (Lin et al. 2011). Reduced Flii expression is observed in early gestation wounds that heal by regeneration suggesting that reducing Flii may be an approach for regenerative healing.

Tissue regeneration is limited in adult mammals and only several types of cells have retained this ability (Chuong 2007). A rare example of an adult mammalian organ capable of regeneration following partial amputation is the hair follicle. Using a recognized model of hair follicle regeneration recent studies by Waters et al., have shown that Flii had a positive influence on hair follicle regeneration, which is contrary to its negative influences on wound healing (Waters et al. 2011). Regenerated follicles expressing high levels of Flii, produced significantly longer terminal hair fibers while Flii deficiency resulted in an delayed or impaired regenerative potential of hair follicles (Waters et al. 2011). Flii<sup>+/-</sup> follicles that failed to regenerate the end bulb structures also displayed low expression of markers of normal hair follicle development suggesting a lack in developmental activity (Waters et al. 2011). The effect of Flii on cellular adhesion and its involvement in signalling pathways important to hair follicle development, growth and cycling might explain the delayed regeneration of hair follicles in Flii deficient mice (Waters et al. 2011). Indeed, Flii inhibits the Wnt/ $\beta$ -catenin pathway (Lee, YH & Stallcup 2006)

and plays a role as a thyroid hormone and oestrogen-activated nuclear receptor co-activator (Archer et al. 2004; Lee, YH, Campbell & Stallcup 2004) both of which would have particular significance to hair follicle development.

In conclusion, normal wound repair is coordinated by cytokines and growth factors aiding differentiation of dermal and epidermal cells, angiogenesis, migration of mature fibroblasts and keratinocytes to a temporary granulation tissue matrix. Chronic exposure to high blood glucose in diabetic patients leads to impaired wound healing, decreased wound strength, and impaired wound-related angiogenesis (Francis-Goforth, Harken & Saba 2010). Malformation of the extra cellular matrix due to abnormal fibroblasts and defective release of cytokines, along with impaired circulation and decreased oxygen to the wounded tissues, result in poor wound healing in diabetic patients. An understanding of the combined cellular and molecular factors may be a cornerstone to the development of future tissue replacement products and therapies that could be employed for prevention and/or treatment of diabetic wounds.

## **1.5 Aims and hypothesis of this thesis**

### **1.5.1 Overall hypothesis**

Diabetes is the fastest growing chronic disease and the number of cases worldwide is estimated at 221 million adults (Zimmet, P, Alberti & Shaw 2001). Diabetes interferes with a successful wound closure and impaired wound healing is a serious complication of both Type 1 and 2 diabetes (Goren et al. 2009). Diabetic foot ulcers are likely to occur in up to 25% of people with diabetes mellitus (Boulton et al. 2005). Due to poor outcomes of existing therapies complications from these wounds lead to one major amputation every 30 seconds worldwide with over 2500 limbs lost a day (Bharara et al. 2009).

Flii is a cytoskeletal protein and inhibitor of wound repair. High levels of Flii protein in wounds have been shown to impair wound healing outcomes (Cowin et.al., 2007). The overall hypothesis of this thesis is that high levels of Flii in diabetic wounds contribute to impaired healing and that altering the levels of Flii in diabetic and chronic wounds will lead to improved wound outcomes. The aim of this thesis is therefore to investigate whether Flii levels are elevated in non-healing diabetic wounds, to determine whether altering the level of Flii in diabetic wounds affects healing and to identify the mechanisms via which Flii exerts its effects on diabetic wound repair.

## 1.5 Specific aims

Specifically, the studies in this thesis aimed to:

**AIM 1:** Determine the effect of modulating Flii gene expression on diabetic wound repair using transgenic and gene knockout mice

**AIM 2:** Determine the role of extracellular Flii in healing and to characterize the possible routes taken by Flii as it moves to the plasma membrane

**AIM 3:** Determine the effect of decreasing of Flii levels in mouse diabetic wounds using intradermal injections of Flii neutralising antibodies.

**AIM 4:** Determine whether reduction of Flii levels in wounds alters the formation of new blood vessels using established *in vitro* and *in vivo* model of angiogenesis

## *Chapter 2*

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# **MATERIALS AND METHODS**

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# MATERIALS AND METHODS

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## **2.1 Human tissue collection**

### **2.1.1 Skin biopsy collection**

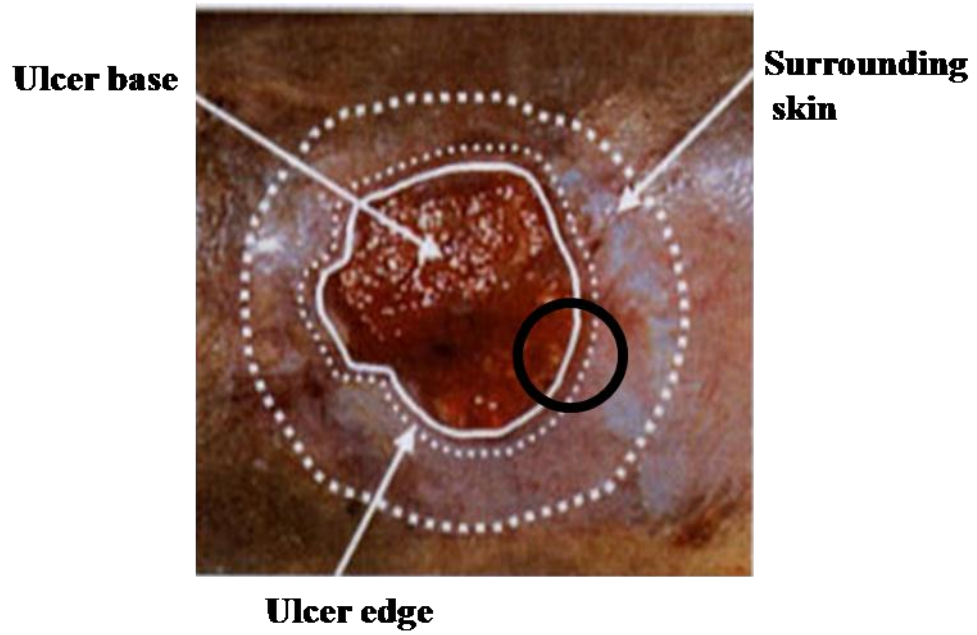
The collection of human tissue such as wound biopsies, wound fluid and blood was approved by the Health Service Human Research Ethics Committee and Central Northern Adelaide Health Service Ethics of Human Research Committee. Patients attending the outpatient clinic, multidisciplinary foot ulcer clinic, leg ulcer clinic and those admitted to the Queen Elizabeth Hospital (Adelaide, South Australia), were screened for suitability to participate in this study. No pressure was placed on any individual to participate in the study and the right of the patient to withdraw at any time was duly acknowledged. Any withdrawal by a patient from this study in no way prejudiced future treatment of their condition or any other condition at the hospital.

All potential subjects were screened for inclusion and exclusion criteria. During the screening period all relevant results from investigations such as the blood tests, quantitative microbiology, duplex and advanced wound diagnostics in the form of toe pressure, ankle brachial index and transcutaneous oxygen pressure were checked to ensure that no exclusion criteria were met. Once assigned a randomisation code, the patients were entered into one of the four study groups: diabetic ulcer, chronic venous ulcer, acute wound and normal skin (Table 2.1). As part of the inclusion criteria all members of groups 1 and 2 (chronic wound group) displayed presence of pulses and/or Ankle Brachial Index (ABI) more than 0.7, had a diabetic ulcer, skin changes, toe pressure (TP) more than 40mmHg, venous scar to confirm venous incompetence, had a chronic venous stasis ulcer with duplex proven venous insufficiency and/or well documented surgical intervention for venous disease. The diabetic or venous ulcer must have been present for at least

6 weeks. All members of group 3 (acute wound group) displayed the presence of pulses and Ankle Brachial Index (ABI) more than 0.9 and had a wound for the duration of less than 6 weeks. All members of group 4 (normal skin group) displayed presence of pulses and Ankle Brachial Index (ABI) more than 0.9 and did not have signs of venous insufficiency or diabetes. As part of the exclusion criteria all members of the three groups were checked for the absence of certain factors, which could lead to false interpretations and tampered conclusions. These included an active infection or cellulitis in the area biopsied, drugs that impair wound healing such as steroids and immunosuppressive drugs, ulcer of malignant nature and renal failure/dialysis with Glomerular Filtration Rate (GFR) less than 30 ml/min. Once the patients consented to the participation in the study specimen such as skin, wound fluid and/or blood were collected. Wound and skin biopsies were collected following the guidelines of ulcer biopsy technique mandated to exclude malignancy of some chronic ulcers and routinely practised by the surgical staff (Figure 2.1). The patients were made comfortable on an examining couch and bupivacaine 0.5% with adrenaline 1:200,000 was used for local anaesthesia. This was injected subcutaneously into the edge of the ulcer and advanced to include the ulcer base. Of twelve subjects with chronic wounds, six patients were used to collect biopsies using a 6 mm biopsy punch (SF001, Stiefel Laboratories, Castle Hill, NSW, Australia). The tissue biopsy was bisected longitudinally. Half the biopsy was snap frozen and used for biochemical/molecular analysis. The other half was further divided into two sections with one being fixed in 10% formalin for paraffin embedding and the other was embedded in Optimal Cutting Temperature (OCT) compound (#4583, Sakura Finetek, Torrance, CA, U.S.A) and stored at -80°C until further use in histological analysis. Acute wound biopsies were collected in similar fashion and as described above.

<b>Group</b>	<b>Specimen collected</b>	<b>Inclusion criteria</b>
<b>1</b> <b>(diabetic ulcer; n=6)</b>	Diabetic ulcer (n=6), plasma (n=6)	Type 1 or Type 2 diabetes, diabetic ulcer ( $\geq 6$ weeks old)
<b>2</b> <b>(chronic venous ulcer; n=12)</b>	Chronic venous ulcer biopsy (n=6) and chronic wound fluid (n=6), plasma (n=6)	Chronic venous insufficiency, venous leg ulcer ( $\geq 6$ weeks old)
<b>3</b> <b>(acute wound; n=22)</b>	Acute wound biopsy (n=6), acute wound fluid (n=22), plasma (n=22)	Acute trauma wounds such as those seen in the emergency department ( $\leq 6$ weeks old)
<b>4</b> <b>(normal skin; n=6)</b>	Normal skin (n=6), blood (n=6)	Unwounded skin from healthy volunteers

**Table 2.1 Overview of the inclusion criteria and recruitment parameters for the human biopsy collection.**



**Figure 2.1. Wound biopsy collection.** Wounds were collected from the edge of the wound using a 6 mm biopsy punch as indicated by black circle.

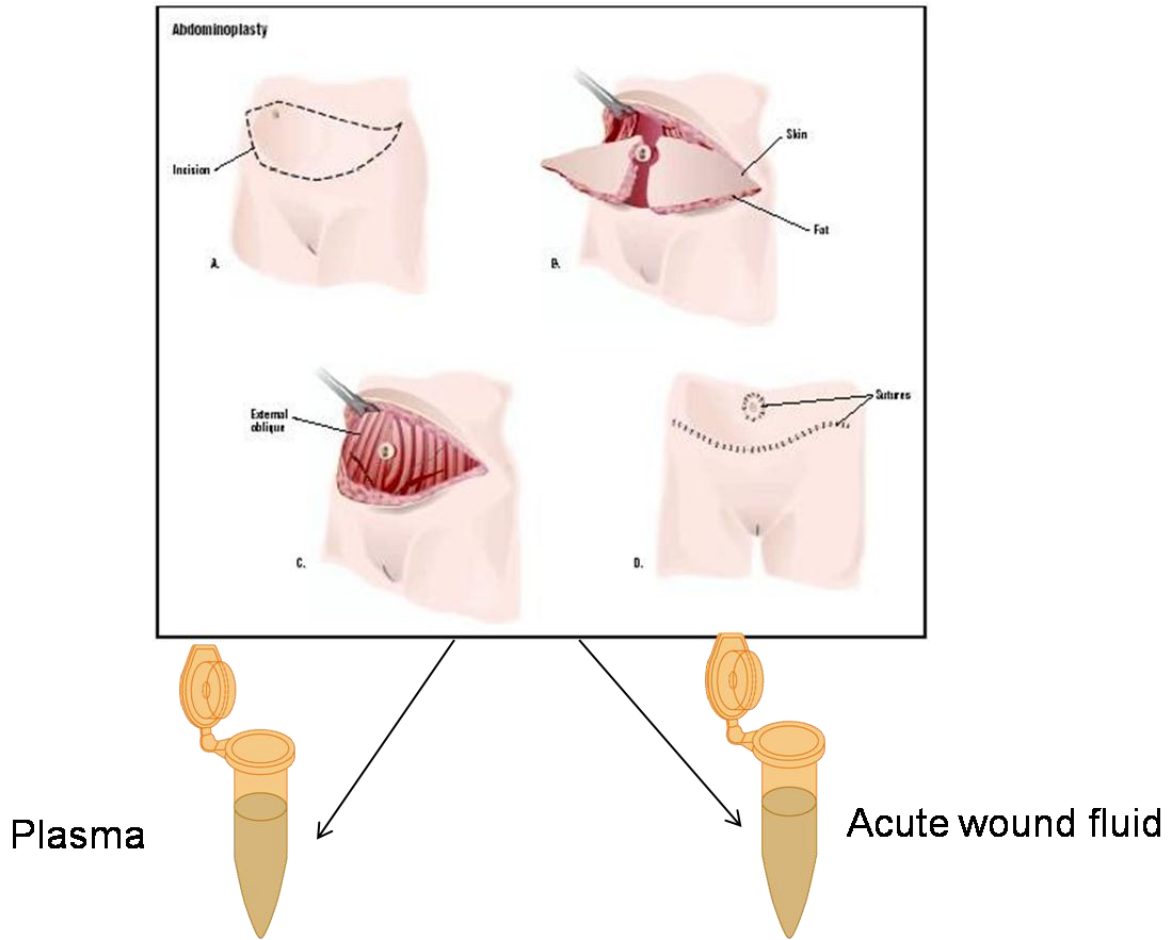
### **2.1.2 Wound fluid collection**

Of the 12 patients with chronic wounds (8 women, 4 men, and mean age of 80 years) a group of 6 patients with chronic venous ulcers were used for the compilation of chronic wound fluid. The venous ulcers were covered with polyurethane film (Op Site, Smith and Nephew, Mile End, SA, Australia) for 3 h and wound fluid was collected by aspiration using a 1 ml syringe.

Acute wound fluid was obtained from surgical drainages of twenty two patients with the mean age 52 years. These patients were undergoing abdominoplasty and breast reduction as part of the weight loss surgery (Figure 2.2). Wound fluid was placed into a pre-chilled 1.5 ml eppendorf tube and centrifuged at 400 g for 5 min at 4°C. The supernatant was collected into a clean tube and store at -20°C until further use.

### **2.1.3 Blood collection**

Human blood was collected from the brachial vein of patients by a registered nurse. Blood collected from the patients with chronic and acute wound was placed into a 9 ml EDTA treated blood collection tube (Greiner bio-one, Kremsmünster, Austria). Plasma was obtained by centrifugation at 300 x g for 5 minutes. Supernatant (plasma) was aspirated and placed into a clean pre-chilled 1.5 ml eppendorf tube and stored at -20°C until further use. Human white blood cells (WBC) were isolated from EDTA treated peripheral blood of health volunteers generously provided by the Australian Red Cross Blood Service, Adelaide, South Australia.



**Figure 2.2. Collection of body fluids from patients undergoing abdominoplasty.** Acute wound fluid was collected from surgical drainages, whereas blood was collected from the brachial vein.



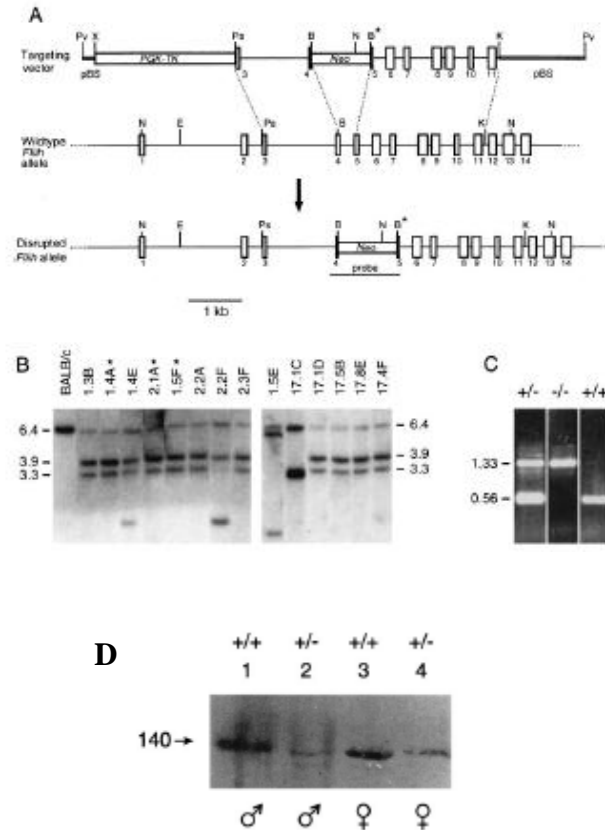
## **2. 2 Animal studies**

### **2.2.1 Mouse strain production**

Three mouse strains, i.e. Flii heterozygous (Flii<sup>+/-</sup>), wild-type (WT) and transgenic over-expressing (Flii<sup>Tg/Tg</sup>) mice were generated by our collaborator A/Prof Ruth Arkell from Molecular Genetics and Evolution group from the Early Mammalian Development Laboratory, Research School of Biological Sciences, ANU, Canberra, Australia. The methods of breeding and mouse strain generation are outlined below.

### **2.2.2 The generation of Flii heterozygous (Flii<sup>+/-</sup>) mouse strain**

Mice homozygous for Flii gene knockout are embryonic lethal (Campbell et al. 2002), hence mice heterozygous for Flii gene knockout (Flii<sup>+/-</sup>) have been generated by continuous backcross of heterozygous carriers Flii<sup>+/-</sup> to Balb/C mice. The resulting mutation has been described previously and shown in Figure 2.3. The mice were genotyped to confirm the presence of one wild-type copy of the Flii gene and one mutant copy of the Flii gene with the resultant animal expressing no more than 50% of the normal Flii gene expression (Figure 2.3 A-C). Reduced expression of Flii protein in heterozygous mutant mice was also demonstrated by Western blotting analysis (Figure 2.3 D).



**Figure 2.3. Targeted disruption of *Fliih*.** (A) The structures of the targeting vector, the relevant portion of the *Fliih* gene, and the targeted allele after homologous recombination are depicted. Restriction enzyme sites are indicated (B, *BspEI*; E, *EcoRV*; K, *KpnI*; N, *NcoI*; Ps, *PshAI*; Pv, *PvuI*; and X, *XhoI*). The asterisk denotes the *BspEI* site in exon 5 introduced by site-directed mutagenesis. *Fliih* exons are depicted by the numbered open boxes. The *tk-neo* and *pgk-thymidine kinase* cassettes and the pBluescript vector are indicated. The dotted lines indicate the regions of identity between the targeting vector and the wild-type *Fliih* allele. (B) Southern analysis of targeted ES cell lines. Genomic DNA was digested with *NcoI* and *EcoRV*, electrophoresed, blotted to a nitrocellulose membrane, and hybridized to the [<sup>32</sup>P]-labelled probe fragment indicated in panel A. Untargeted ES cell DNA was run as a control (BALB/c). The three lines injected into blastocysts to generate chimeric mice are indicated by asterisks. Sizes of bands (in kilobases) are indicated. (C) PCR results on individual blastocysts. Sizes of bands (in kilobases) are indicated. (D) Reduced expression of *Fliih* protein in heterozygous mutant mice. Protein extracts from livers of heterozygous (+/-) *Fliih* mutant mice (line 2.1A) and wild-type (+/+) littermate controls were subjected to SDS-gel electrophoresis and Western analysis using an anti-*Fliih* antipeptide antibody (Flig). Samples from male and female mice are as indicated. Sizes of markers and *Fliih* are indicated in kilodaltons (Campbell et al. 2002).

### **2.2.3. The generation of Flii transgenic (Flii<sup>Tg/Tg</sup>) mouse strain**

To generate a transgenic mouse strain (Flii<sup>Tg/Tg</sup>) a human cosmid clone (c110H8) that contained the entire FLII locus was used (Thomsen, Chappell et al. 2011) (Figure 2.4). Heterozygous carriers were intercrossed and carrier progeny identified by PCR assay to distinguish animals that carry one copy of the transgene from those that carry two (Thomsen et al. 2011). Carrier animals were therefore test mated with wild-type Balb/C mice. Animals that, when crossed with a wild-type animal, produced at least 12 offspring all of which carried the transgene, were presumed to be themselves homozygous for the transgene. To produce strains of mice that were homozygous for a targeted mutation a three primer multiplex PCR assay was established that could distinguish between heterozygous and homozygous mutant animals. Heterozygous animals were then intercrossed and homozygous carrier progeny identified by PCR assay (Thomsen et al. 2011).

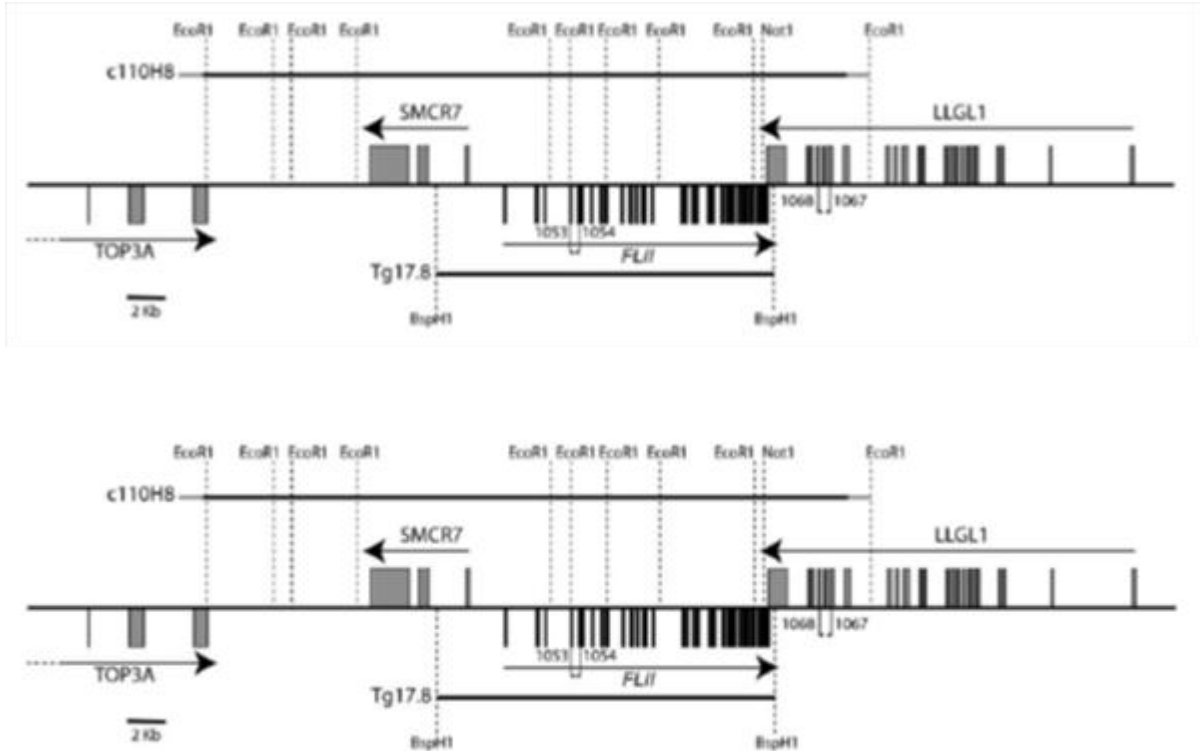
## **2.3. Mouse model of Type 1 diabetes**

### **2.3.1 Multiple low-dose streptozotocin (MLDS) mouse model of diabetes**

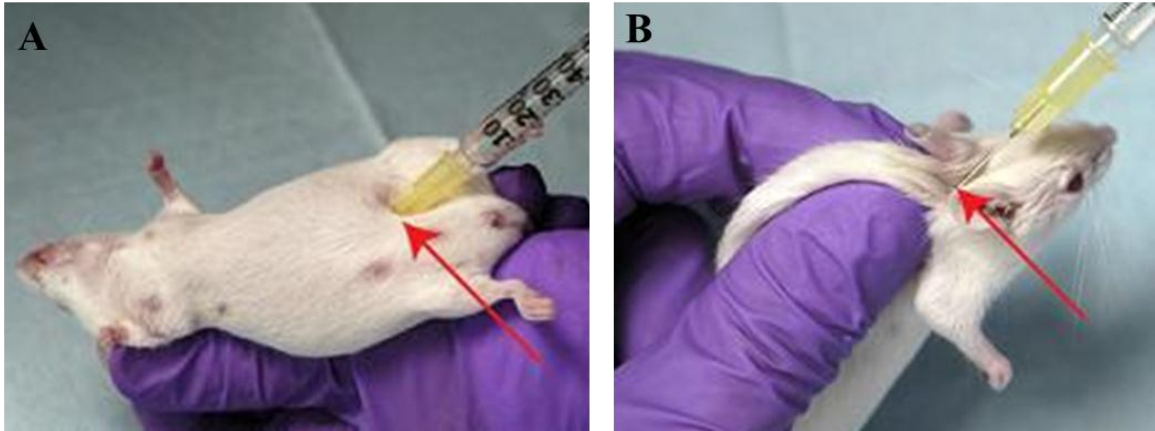
All animal experiments were approved by the Adelaide Women's and Children's Hospital Animal Care and Ethics Committee following the Australian Code of Practice for the Care and the Use of Animals for Scientific Purposes. All studies were performed in mice with the Balb/C background. All the animals were maintained on tap water and standard food pellets ad libitum, in a 12 h light/darkness cycle at 22 °C. Female Flii<sup>+/-</sup>, WT and Flii<sup>Tg/Tg</sup> mice of 12-16 weeks of

age and weighing 20-35g, were used. Streptozotocin (STZ) was used to induce type 1 diabetes (S0130, Sigma-Aldrich, St. Louis, MO, USA). STZ is toxic to the pancreatic beta-islet cell, rendering the mouse unable to produce adequate amount of insulin. After one week of acclimatization the mice were given one intraperitoneal (IP) injection of streptozotocin for 5 consecutive days (STZ:50mg/kg, Sigma-Aldrich, St. Louis, MO, USA) in citrate buffer pH 6.5 (Figure 2.5). This dose was chosen based on previously reported studies (Johnson, Ryals & Wright 2008). Mice were fasted for 4 hours before STZ injection as recommended by the Animal Models of Diabetic Complications Consortium, <http://www.amdcc.org/> (Johnson, Ryals & Wright 2008). Age matched non-diabetic control animals were treated with an equivalent dose of vehicle (citrate buffer alone).

Diabetic symptoms were observed closely, with the weight being monitored daily. Non fasting blood glucose levels (BGL) were tested weekly by tail vein sampling. To maintain body weight and prevent ketoacidosis, animals with confirmed diabetes were maintained with subcutaneous injection of insulin as shown in Figure 6. Insulin (1 IU, Mixtard insulin 30/70, Novo-Nordisk, Baulkham Hills, NSW, Australia) was administered every second day. If animals failed to maintain weight within 10% of the start weight and/or have non fasting BGL levels greater than 25mmol/l the insulin dose was increased to 2 IU every second day. In addition, mice had access to extra water and the cages were changed every second day due to excessive urination. Mice were tested for sufficient levels of hyperglycaemia at 6 weeks after the last STZ injection and only those with blood glucose level greater than 15 mmol/L were wounded and utilized for this study. Animals that were not hyperglycaemic at this time were excluded from this study.



**Figure 2.4. The generation of a transgenic mouse strain (*Flii*<sup>Tg/Tg</sup>).** The cosmid clone (c110H8) used to generate Tg1 is shown (top) aligned with the human genome. The exact extent of the cosmid is not known and the limits of certainty are represented by the black bar with the possible extent represented by a grey bar. The relative position of genes in the region is shown with the shaded boxes representing exons and the direction of transcription indicated by the arrow with each gene symbol. The *BspHI* sites used to isolate the 17.8 kb fragment for the Tg2 strain are shown (bottom) and indicate that this transgene contains only the first exon of the flanking *SMCR7* gene. The position of PCR primers used for genotyping the strains (Ark1053, Ark 1054, Ark 1067, and Ark 1068) are shown (Thomsen et al. 2011).



**Figure 2.5. Intra-peritoneal and subcutaneous injection.** (A) The mouse was restrained by the scruff method. The ventral side of the animal was exposed, tilting the head down at a slight angle. The sterile needle was placed bevel up, in the lower right or left quadrant of the animal's abdomen. The needle was inserted at a 30° angle. (B) Subcutaneous injection of insulin. The mouse was restrained by the scruff method. The thumb and forefinger were used to make a tent of skin over the scruff. The needle was inserted bevel up, at the base of the tent. The needle was inserted parallel to the skin and directed toward the posterior of the animal.

### 2.3.2 Murine model of excisional wound healing

6 animals per group (diabetic vs. non diabetic controls) were used (2 wounds per animal or 12 wounds per group) and 3 time-points (7, 14 and 21 days post-wounding). Wild type (total of 36 mice), Flii<sup>+/-</sup> (total of 36 mice) and Flii<sup>Tg/Tg</sup> (total of 36 mice) animals were wounded using a 6 mm biopsy punch (SF001, Stiefel Laboratories, Castle Hill, NSW, Australia). At the time of the procedure, anaesthesia was induced by inhalation of isoflurane (5% induction at 2 L/min and 2% maintenance at 500 ml/min). To expose the skin, hair was clipped from the back of the mouse using hand held clippers (Figure 2.6 A) and treated with hair removal cream (Veet®, Reckitt Benckiser, West Ryde, NSW, Australia) for 2-3 minutes (Figure 2.6 B). Two 6 mm wounds, one on each side of the midline were created on the dorsum of the mouse (Figure 2.6 D) by excising the skin to the subcutaneous fascia below the panniculus carnosus on the prepped dorsal surface of the mouse. Digital photographs were taken of the wounds at 0, 7, 14 and 21 days post-wounding. A ruler was aligned next to the wound to allow direct wound area and wound gape (diameter of the wound) measurements to be made (Figure 2.6 D). Temgesic (buprenorphine 0.05 mg/kg) was administered post-operatively to provide analgesia for up to 8 hours. After creation of the wound the animals were allowed to recover in an individual cage. In diabetic mice, diabetes was maintained throughout the wound study and confirmed by BGL testing once a week. To prevent ketoacidosis diabetic animals were given subcutaneous insulin (1 IU, Mixtard insulin 30/70, Novo-Nordisk, Baulkham Hills, NSW, Australia) every second day. The animals remained individually caged until the time of euthanasia. Animals were checked twice daily for the first two days post-procedure and then daily for the first week thereafter, for normal weight gain, normal behaviour, signs of infection, haemorrhage and distress. Animals that exhibited behaviour indicative of profound or prolonged pain or distress and those with an

excessive weight-loss ( $\geq 10\%$  of pre-surgery weight) were excluded from the study and euthanized. Animals were killed by CO<sub>2</sub> asphyxiation and cervical dislocation and biopsies of the wounds were taken for histological, biochemical and molecular analysis. Wounds were harvested at 7, 14 and 21 days and bisected. One half was fixed in 10% buffered formalin and processed so that the midpoint of the wound was sectioned and compared between groups. The other half was micro dissected to remove any contaminating normal, unwounded skin and snap frozen in liquid nitrogen for RNA extraction.

#### **2.3.4 Treatment of excisional wounds with Flii neutralising antibody (FnAb)**

Two excisional wounds were created using a 6 mm biopsy punch on the back of mice, which had been made diabetic as previously described (n=10; 20 wounds in total). The wounds were injected intradermally with 200 $\mu$ l of 50 $\mu$ g/ml of FnAb at day 1, 2 and 3. Control diabetic mice were treated with an equivalent dosage of IgG antibodies (n=10, 20 wounds in total). As demonstrated in Figure 2.7, FnAb or IgG antibodies were injected around the wound using insulin syringe with ultra-fine short needle (#329411, BD Bioscience, NJ, U. S. A). The allocation of animals into various arms of the experiment and the total number of animal usage are indicated in Figure 2.8. Digital images of the wounds were taken daily for analysis of change in wound size. All wounds were harvested at 7 days post-wounding for subsequent assessment. Histological wound measurements of wound size, dermal gape and reepithelialisation were measured to determine the effect of antibody treatment on diabetic wound repair. Animals were killed by CO<sub>2</sub> asphyxiation and cervical dislocation and biopsies of the wounds were taken for histological, biochemical and molecular analysis. Wounds were harvested and bisected with one



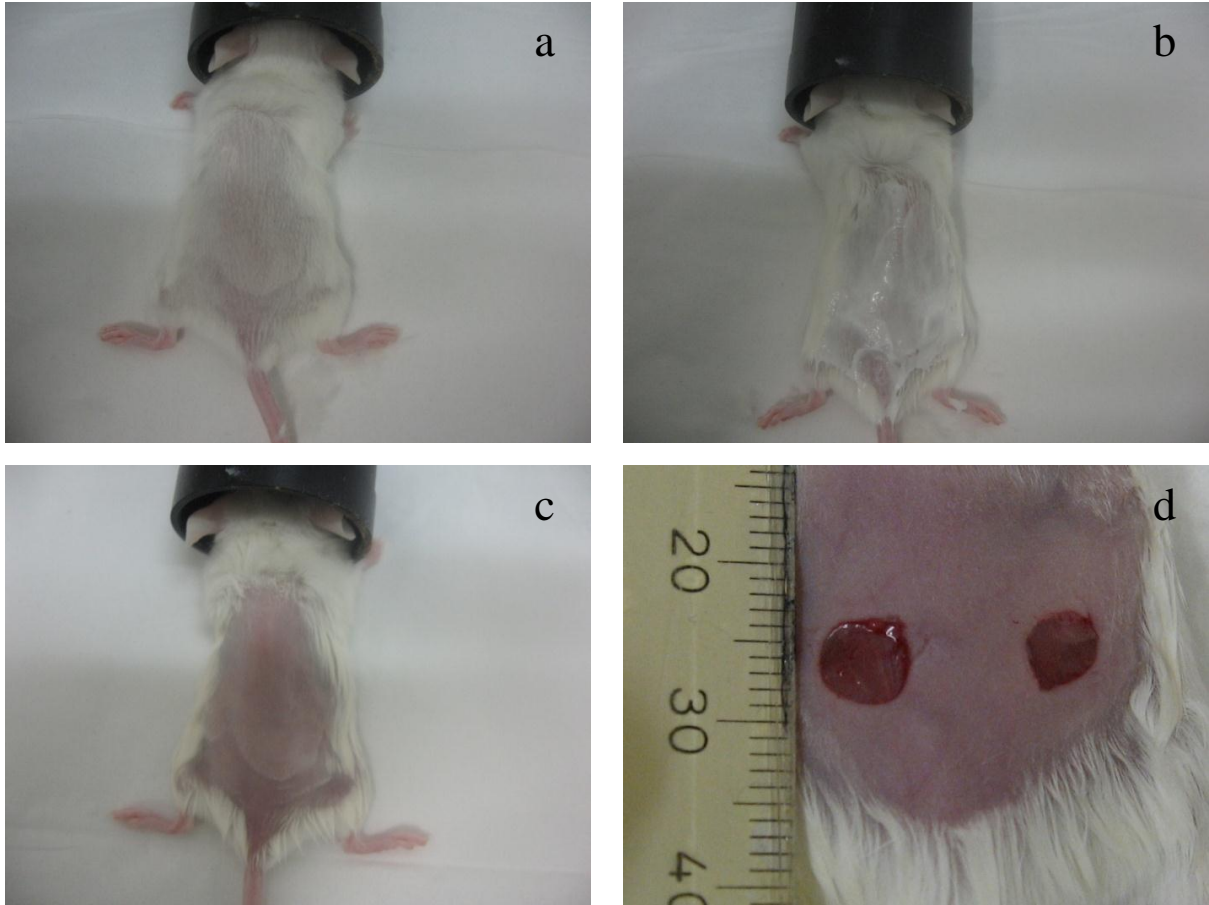
half fixed in 10% buffered formalin and processed so that the midpoint of the wound was sectioned and compared between groups. The other half was micro dissected to remove any contaminating normal, unwounded skin and snap frozen in liquid nitrogen for RNA extraction.

## **2.4 Angiogenesis studies**

### **2.4.1 *In vivo* therapeutic angiogenesis model (Matrigel plug assay)**

Wild type Balb/C, 6-8 weeks of age female mice (n=6) had one week of acclimatization, after which they were given one subcutaneous injection (500  $\mu$ l) of Matrigel (#354248, BD Biosciences, Bedford, MA, U. S. A.) on either side of the abdominal region as described previously (Kano et al. 2005). Two plugs with different ligands (FnAb or IgG antibodies were mixed by pipetting; 500  $\mu$ l at 50  $\mu$ g/ml) were injected into each mouse to avoid differences between the individual mice (Kano et al. 2005). The plug on the left side of the abdomen contained Matrigel compound mixed with 50  $\mu$ g/ml of FnAb and the plug on the right (control) contained an equivalent dose of rabbit IgG. The injections are non-toxic as the major component of this injectable Matrigel substance is laminin and collagen.

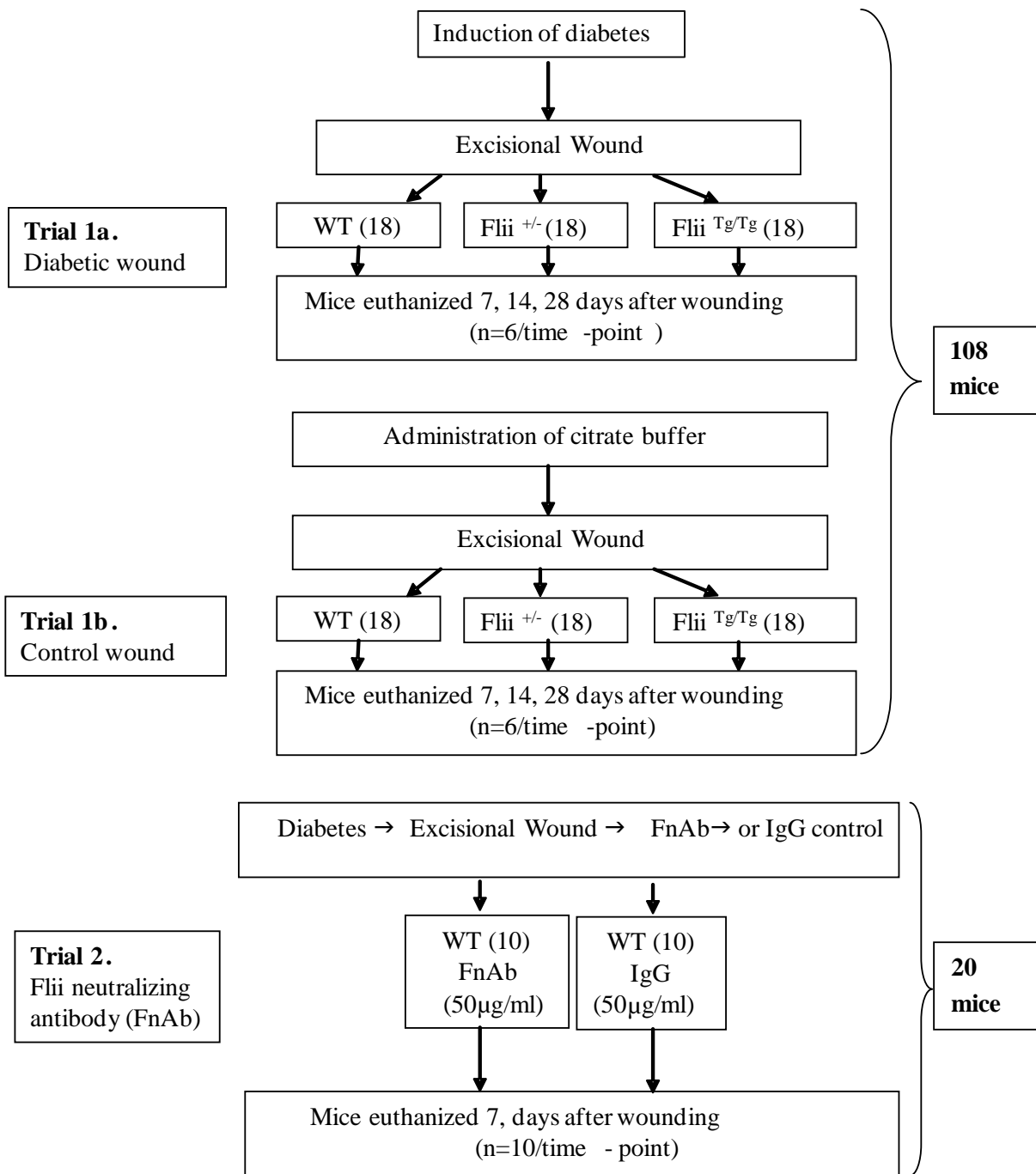
Animals were checked daily for 7 days post-procedure for normal weight gain, normal behaviour, signs of infection, haemorrhage and distress. Animals remained individually caged until the time of euthanasia. After an incubation period of 7 days mice were euthanized by carbon dioxide asphyxiation followed by cervical dislocation. The Matrigel plugs were removed. Half the plug was snap frozen in liquid nitrogen and used for blood vessel visualisation by immunofluorescence and the other half was fixed in 10% formalin and processed for histology.



**Figure 2.6. Murine model of excisional wound healing.** Mouse skin was exposed by clipping the hair (a) and then treated with hair removal cream for 2-3 minutes (b and c). Two 6 mm wounds, one on each side of the midline were created on the dorsum of the mouse (d) by excising the skin to the subcutaneous fascia on the dorsal surface of the mouse. Digital photographs were taken of the wounds at 0, 7, 14 and 21 days post-wounding. A ruler was aligned next to the wound to allow direct wound area and wound gape (diameter of the wound) measurements to be made (d).



**Figure 2.7. Intradermal injection with FnAb or IgG antibodies.** Two excisional wounds were created using a 6 mm biopsy punch on the back of diabetic mice. FnAb or IgG antibodies were injected intradermally using insulin syringe with ultra-fine short needle. Digital images of the wounds were photographed daily and harvested at 7 days post-wounding for subsequent assessment.



**Figure 2.8. Summary of experimental design.** A diagram demonstrating the number of animals and how these animals were allocated to the various arms of the project.

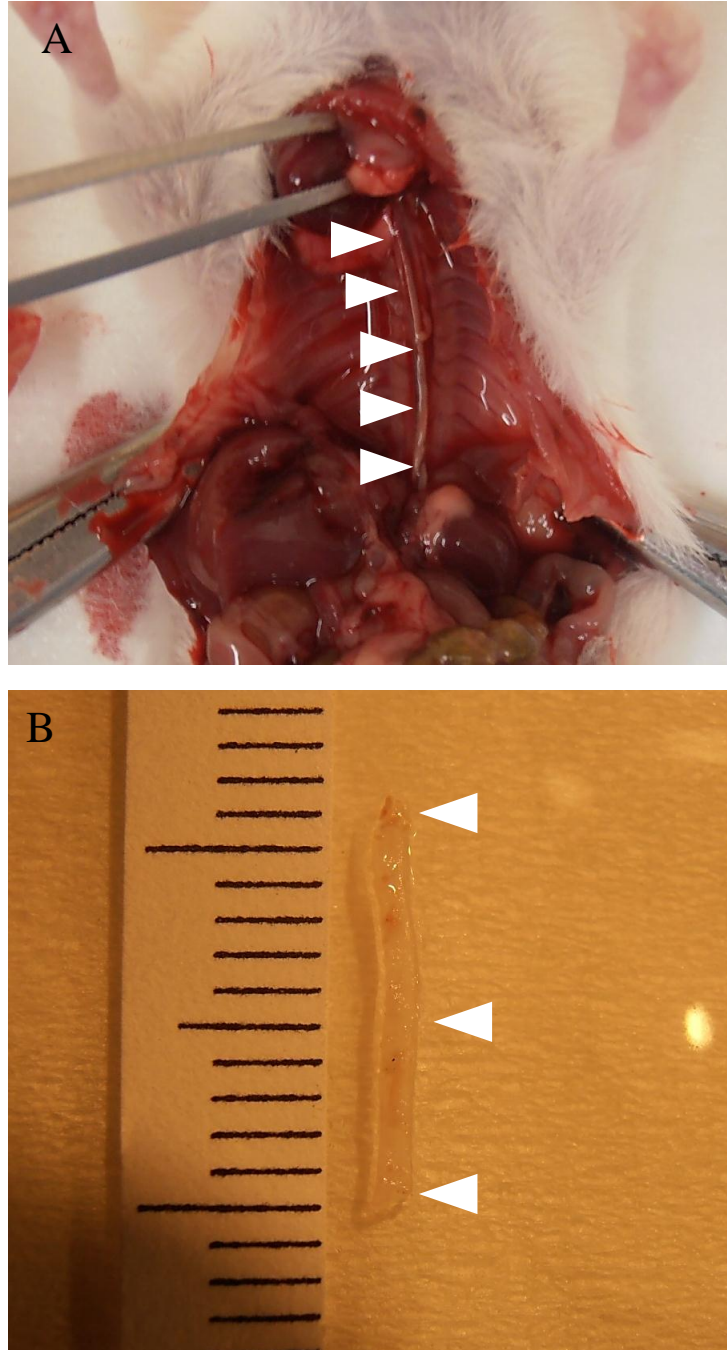
#### 2.4.2 Matrigel tube formation assay

Human umbilical-vein endothelial cells (HUVEC) were obtained from Dr Claudine Bonder (IMVS, Hanson Cancer Research Centre, Adelaide, South Australia) and isolated from the umbilical cord/vein of neonates born at the Adelaide's Women's and Children's Hospital as described previously (Ferrante et al. 2006). *In vitro* angiogenesis and tube formation assays were performed in Vascular Biology and Cell Trafficking Laboratory (IMVS, Hanson Cancer Research Centre, Adelaide, South Australia) headed by Dr Claudine Bonder. Passage 1-2 HUVEC were cultured on gelatine plated T25 flasks and maintained in culture media containing 10% FCS in EGM®-2 BulletKit (#CC-3162, Lonza, Basel, Switzerland). The cells were allowed to reach 50-60% confluence and harvested using trypsin. Cell counts was performed and HUVEC were seeded on angiogenesis plate (#01367, 1 µ-Slide Angiogenesis ibiTreat, Ibidi, Munich, Germany) coated with 12 µl of Matrigel (#354234, BD Biosciences, Bedford, MA, U.S.A.). The Matrigel was thawed and added slowly to the side of the well to avoid air bubbles. Matrigel-coated plates were placed into an incubator for 30 min to set. Harvested cells were diluted to a cell density of  $3.0 \times 10^5$  cells/ml. 50 µl of suspended HUVEC (i.e.  $1.5 \times 10^4$  cells/well) were added into each well and the plate was placed in the incubator. The number of cells in each well was assessed at 30 minutes post-plating to ensure that the cells were plated at similar densities and only those with equal densities were used in experiments. Tube formation was assessed under the light microscope equipped with  $\times 4$  and  $\times 10$  objectives with the images taken at 6 hours post-seeding. Captured images were used to produce a panoramic view of the entire well using a stitching software program (PTGui 9.0.4. New House Internet Services B.V., Rotterdam, The Netherlands). The number of tubes was counted by manually drawing lines

along the formed vessels using Adobe Photoshop (Adobe systems, Inc). The total number of lines was calculated and the data was derived from average of 4 donors.

### **2.4.3 Mouse aortic ring assay**

Aortae were obtained from Flii<sup>+/-</sup>, WT and Flii<sup>Tg/Tg</sup> mice (n = 6 for each mouse genotype) and cut into rings (Figure 2.9). The protocol involved dissection of the aortae, serum starvation, embedding and feeding of the aortic rings, imaging and quantifying microvessel sprouts as described previously (Baker et al. 2012). The aortae from Flii<sup>+/-</sup>, WT and Flii<sup>Tg/Tg</sup> mice were flushed out to remove blood from the lumen and cut into rings (0.5 mm in width) with a scalpel. A total of 15 rings per mouse were obtained. To create a uniform baseline state and equilibrate growth factor responses, the aortic rings were serum-starved overnight in Opti-MEM®I + GlutaMAX™-I Reduced Serum Medium (Gibco® Life Technologies, Grand Island, NY, USA, Cat. No. 51985-034) at 37°C and 5% CO<sub>2</sub>. The next day the rings were embedded into Collagen I, Rat Tail a total concentration of 1 mg/ml (Gibco® Life Technologies, Grand Island, NY, USA, Cat. No. A10483-01) in a 96-well plate. The embedded aortic rings were fed with 150 µl of Opti-MEM supplemented with 2.5% foetal calf serum and VEGF at a final concentration of 30 ng/ml (PeproTech Rocky Hill, NJ, USA, Cat No. 450-32). The growth medium was changed first on day 3 and then every other day until the experiment ended. The microvessel growth from the aortae rings was quantified on days 5, 6, 7 and 8-post embedding by live phase-contrast microscopy. Starting from a specific point of the ring, emerging microvessels were counted and the data was plotted as mean microvessel numbers per ring.



**Figure 2.9. Dissection of mouse aorta from the thoracic cavity.** (A) The mouse aorta was dissected by opening of the chest cavity and cutting around the rib cage and through the diaphragm. The aorta is visible along the spine (white arrowheads) from the anterior end. The aorta was removed by cutting once near the abdominal branch and once at the anterior end. (B) Dissected mouse aorta (white arrowheads point to the anterior, middle and posterior ends). Under a dissection microscope, all extraneous fat was removed with forceps and blood was flushed by inserting a 27-G needle fixed to a 1-ml syringe.

## **2.5 Histology, immunohistochemistry and image analysis**

### **2.5.1 Mouse Skin Tissue Processing**

Mouse skin and wound samples were placed in cassettes and fixed in 10% formalin prior to being processed using Leica TP 1020 Tissue Processor (Leica Microsystems, North Ryde, NSW, Australia), which dehydrated the tissues in graded alcohol series, cleared in transitional solvent xylene and infiltrated the tissues with paraffin wax (Table 2.2).

Histological sections (4µm) were cut from paraffin-embedded formalin fixed tissue. Leica RM 2235 microtome (Leica Microsystems, North Ryde, NSW, Australia) was used to cut the sections. Two sections of tissue were placed on each slide and put in the oven overnight at 50°C. Sections were stained with Haematoxylin and Eosin or subjected to immunohistochemistry as described below.



Step	Reagent	Time	Temperature (°C)
1	70% ethanol	1 h	Room Temperature
2	70% ethanol	1 h	Room Temperature
3	80% ethanol	1 h	Room Temperature
4	90% ethanol	1 h 45 min	Room Temperature
5	100% ethanol	2 h	Room Temperature
6	100% ethanol	2 h	Room Temperature
7	xylene	1 h 30 min	Room Temperature
8	xylene	1 h 30 min	Room Temperature
9	Paraffin wax	2 h	Room Temperature
10	Paraffin wax	2 h	Room Temperature

**Table 2.2. The protocol for mouse tissue processing and embedding of sections in wax.** The conditions used were recommended by Leica Microsystems, North Ryde, NSW, Australia.

### **2.5.2 Haematoxylin and Eosin staining**

Sections were deparaffinised in 2 changes of xylene, 10 minutes each, then re-hydrated in 2 changes of absolute alcohol, 1 minute each, after which they were transferred into 70% and 30% alcohol for 1 minute each. Sections were washed briefly in distilled water and placed for 8 minutes in Mayer's Haematoxylin (Surgipath Medical Industries, Peterborough, UK). 50 ml of 10% alcoholic haematoxylin solution was added and heated to boil for 1 minute. The solution was removed from heat and 2.5 g of mercuric oxide was added slowly. The solution was cooled in cold water bath and 20 ml of glacial acetic acid was added. Meyer's Haematoxylin solution was filtered before use). The sections were washed in running tap water for 5 minutes and blued-up in lithium carbonate solution (1.54 g lithium carbonate and 100 ml distilled water were mixed together) for 15 seconds, after which they were differentiated in 1% acid alcohol (1 ml of hydrochloric acid and 100 ml of 70% ethanol were mixed well) for 30 sec. Excess water was blotted from the slides before going into eosin. The sections were counterstained in 0.25% eosin solution (Surgipath Medical Industries, Peterborough, UK. Eosin Y stock solution (1%): 10 g of eosin Y, 200 ml of distilled water, 800 ml of 95% ethanol. Eosin Y Working Solution (0.25%): 250 ml of eosin Y stock solution, 750 ml of 80% ethanol, and 5 ml of glacial acetic acid). All sections were dehydrated in 100%, 70% and 30 % alcohol for 1 min each and transferred in 2 changes of xylene for 5 minutes each. Coverslips were applied using DePex mounting medium taking care to leave no bubbles. Light microscopy was performed using a Nikon Eclipse 50i Microscope (Nikon Corporation, Melville, NY, U.S.A) equipped with a 10X objective and Digital Microscope Camera ProgRes® C5 (Jenoptik, Jana, Germany).

### 2.5.3 Masson's Trichrome Staining

Sections were deparaffinised in 2 changes of xylene, 10 minutes each, brought to water through graduated ethanol baths and mordant in Bouin's fixative (375 ml picric acid: Sigma-Aldrich Chemicals, Germany; 125 ml 37% w/w formaldehyde: AnalaR, Victoria, Australia; 25 ml glacial acid: APS Ajax Finechem, NSW, Australia) for 30 minutes at 60°C. The sections were washed in water, mordant in 0.5% celestine blue (Sigma-Aldrich Chemicals, Steinheim, Germany) in 5% iron alum for 3 minutes and rinsed in water again. The slides were placed in Mayer's haematoxylin (Sigma Diagnostics, St. Louis, USA) for 3 minutes, washed in water and blued in bicarbonate solution. The sections were then stained in fuchsin ponceau for 5 minutes, rinsed in distilled water, treated with 5% aqueous phosphotungstic acid (Sigma-Aldrich Chemicals, Steinheim, Germany) for 10 minutes, drained and stained in 2% light green (Sigma-Aldrich Chemicals, Steinheim, Germany) in 1% acetic acid. The sections were dipped in 1% acetic acid, and then transferred in distilled water, after which they were dehydrated and cleared. Coverslips were applied using DePex mounting medium (BDH Lab Supplies, Poole, England) taking care to leave no bubbles. Light microscopy was performed using a Nikon Eclipse 50i Microscope (Nikon Corporation, Melville, NY, U.S.A) equipped with a  $\times 10$  objective and Digital Microscope Camera ProgRes® C5 (Jenoptik, Jana, Germany).

#### **2.5.4 Immunohistochemistry of paraffin-embedded samples**

Sections were deparaffinised in 2 changes of xylene, 15 minutes each. Sections were re-hydrated in 2 changes of absolute alcohol, 1 minute each, after which they were transferred into 70% and 30% alcohol for 1 minute each. Sections were brought to water, rinsed in 1 x PBS and placed into 250 ml TRS (2.8g citric acid, 3.76 glycine, 0.372g ethylenediaminetetraacetic acid, pH=5.9 in 1 L PBS). The slides were heated in microwave for 2 minutes on high power, after which ballast pot of water was added to help absorb some of the heat. The slides were heated further in microwave using medium power (2 x 5 minutes). Occasionally, the microwave was stopped and opened to allow the hot air to escape. It was important to ensure that the TRS solution did not boil over. After the pre-treatment, the pot was removed from the microwave, placed on the bench at room temperature and allowed to cool to 50°C. The sections were washed in 1 x PBS and enzyme digested (pre-warmed 250 ml of 1 x PBS to 37° was mixed with 0.0625g of Trypsin (Sigma-Aldrich, Sydney, Australia) and incubated for 3 minutes at 37°C. Following blocking in 3% serum, primary antibodies were applied. Species-specific, biotinylated secondary antibodies were used (see Appendix 1). Images were captured with Leica Leitz DMRB Fluorescence Microscope (Leica Microsystems, Wetzlar, Germany) and Olympus DP72 Microscope Camera (Olympus, Centre Valley, PA, U.S.A.). Integrated fluorescence intensity was determined using AnalySIS software package (Soft Imaging System GmbH, Munster Germany). Negative controls included replacing primary antibodies with normal rabbit IgG, normal mouse or normal goat IgG. Non-specific binding was determined by omitting primary or secondary antibodies.

### **2.5.5 Immunohistochemistry of cryosections**

Cryosections (5 µm) were cut from Optimal Cutting Temperature (OCT) compound embedded frozen tissue samples. These sections were stained with haematoxylin and eosin or subjected to fluorescence immunohistochemistry. Acetone fixed cryosections of human skin biopsies were blocked in 1% BSA for 1 hour at room temperature; primary antibodies against Flii (1 µg/ml) (Santa Cruz, CA, U.S.A.) were applied and incubated overnight at 4°C. Biotinylated mouse IgG (7.5 µg/ml) was used and detection was by CY3-conjugated streptavidin (1:200) (Sigma-Aldrich, St Louis, MO, U.S.A.). AnalySIS software package (Soft Imaging System GmbH, Munster, Germany) was used to measure fluorescence intensity per unit area as described previously (Adams, Strudwick et al. 2008). Control immunostaining was carried out by omitting primary or secondary antibodies for verification of staining and non-specific binding.

### **2.5.6 Dual immunofluorescence staining of human wounds with Flii and CD16**

5 µm methanol fixed cryosections of chronic venous ulcers were co-stained with Flii (Santa Cruz, CA, U.S.A.) and CD16 (550383BD, San Jose, CA, U.S.A.). Immunofluorescence staining was performed by blocking the sections with 1% BSA and incubating with primary antibodies against Flii and CD16 at 4°C overnight. The secondary antibodies were applied for 1 hour with Alexa Fluor 488 goat anti-mouse (Invitrogen, Oregon, U.S.A.) being used to detect CD16 and Cy3 goat anti-rabbit (Invitrogen, Oregon, U.S.A.) antibodies used to detect Flii. Coverslips were mounted onto slides using Dako mounting medium. Confocal microscopy was performed using a

Leica SP5 spectral scanning confocal microscope. A confocal Microscope equipped with a  $\times 100$  oil objective was used to capture confocal images using Leica Confocal Software.

### **2.5.7 Immunofluorescence of cultured cells**

Dual immunofluorescence experiments were performed in Dr Rachael Murray's Laboratory (Kids Research Institute, Children's Hospital at Westmead, Sydney, N.S.W., Australia) and are as described previously (Murray, Kay, et al. 2005). Cells were cultured in a T75 flask (#156499, Nunc™, Roskilde, Denmark), maintained in 10% FCS (#SH30084.03, HyClone® Laboratories, Mordialloc, Victoria, Australia), 1% Penicillin-Streptomycin solution (#P4458, Sigma Aldrich, St. Louis, MO, U.S.A.), DMEM (SH30243.01, HyClone® Laboratories, South Logan, Utah, U. S. A.) and allowed to reach confluence. Upon confluence, media was decanted and cells washed with PBS (SH30028.02, HyClone® Laboratories, South Logan, Utah, U. S. A.). 2 ml of 1x Trysin-EDTA solution (#T4174, Sigma Aldrich, Steinheim, Germany) was added to cells and incubated for 5 min. Cells were collected and centrifuged at 1400rpm for 5min. The pellet was re-suspended and cell count performed. Cells were seeded on glass coverslips ( $3 \times 10^5$  per coverslip) and placed inside 24-well plates (#150628, Nunc™, Roskilde, Denmark). The wells were covered with 2 ml of 10% FCS/1% PenStrep/DMEM to allow the cells to adhere overnight. The next day, media was removed and cells were washed in PBS. Cells were fixed in pre-cooled 100% methanol at -20 C for 4 min. Following methanol fixation the coverslips were removed and placed in a foil covered dish to keep the fluorescent tag from bleaching. Parafilm was placed on top of wet paper to prevent drying out of coverslips. Coverslips were placed with cell side up on the parafilm with sufficient space between the coverslips. Cells were blocked in 1% BSA

(bovine serum albumin, 1 g of BSA in 100 ml of 1 x PBS) for 30min. 100 µl of 1% BSA was used to completely cover the surface area of each coverslip. Cells were rinsed with 1 x PBS (3 times 2 min each). Primary antibodies were made up in blocking buffer and gently pipetted onto the coverslips and incubated for 60 minutes. Coverslips were washed 5 X with washing buffer (0.5% BSA in 1 x PBS) using the Pasteur pipet and vacuum pump. Secondary antibodies were made up in washing buffer and incubated on the coverslips for 60min. Coverslips were washed 5 X with blocking buffer using the Pasteur pipet and vacuum pump. Coverslips were adhered on glass slide using Dako fluorescent mounting media (#S3023, Dako, Glostrup, Denmark) and stored in -4 °C or for longer storage at -20 °C. Confocal microscopy was performed using a Leica TCS SP2 Confocal Microscope equipped with a ×100 oil objective. Confocal images were pseudocoloured where appropriate and overlaid using Adobe Photoshop CS3.

## **2.6 Mouse wound analysis**

### **2.6.1 Macroscopic wound analysis**

A ruler was aligned next to the wound to allow direct macroscopic measurement of the excisional wound area. Digital images of the wounds were taken at days 0, 7, 14 and 21 post-wounding using Canon PowerShot A530 digital camera. Image analysis was performed using the Image ProPlus program (Media Cybernetics Inc., Bethesda, MD, U.S.A.). Macroscopic wound size was determined by manually drawing around the wound edge. Total wound area was calculated and expressed as the “average wound area” in mm<sup>2</sup>.

## **2.6.2 Histological Image Analysis**

Image analysis was performed using the ImageProPlus program (MediaCybernetics Ins., Maryland, U.S.A.). Wound size was determined by manually drawing below the zone of coagulation or between the wound margins. Dermal gape was determined by measuring between the dermal wound margins. Total length of the wound and length of the dermal gape were measured as demonstrated in Figure 2.10.

## **2.7 Tissue culture**

### **2.7.1. Cell culture and maintenance**

Cultured cells were kept in Sanyo CO<sub>2</sub> incubator (#MCO-18 AIC, Sanyo Electric Co., Tokyo, Japan) and maintained at 37°C and 5% CO<sub>2</sub>. Human Skin Fibroblasts (HFF), Human Monocyte Cell-Line (THP-1), Human Embryonic Kidney Cell-Line (HEK-293), Mouse Fibroblast Cell-Line (NH3T3) were obtained from ATCC and stored in liquid nitrogen at Women's and Children's Health Research Institute. The base medium for all above mentioned cell lines was 10% DMEM with added 1% antibiotic agent (Sigma Aldrich, Castle Hill, NSW, Australia).

### **2.7.2 Primary fibroblast isolation**

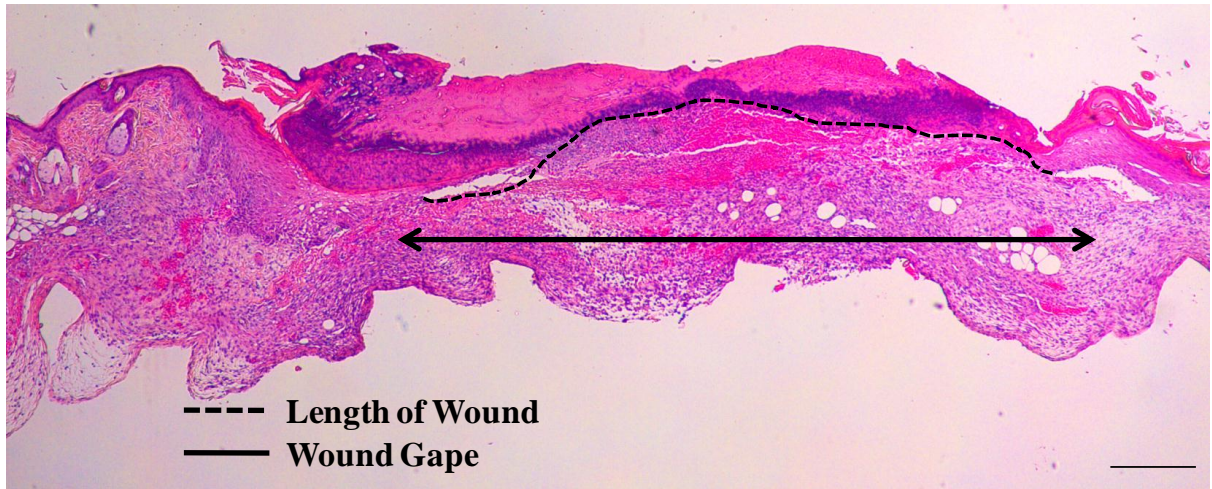
Flii<sup>+/-</sup>, wild-type and Flii<sup>Tg/Tg</sup> mouse skin was used to isolate primary skin fibroblasts. Hair was removed from the back of the euthanized mice using hand held hair clippers. Skin was excised



using scissors and collected in ice-cold Nutrient Mixture F12 (SAFC Biosciences, Lenexa, KS), containing 1% penicillin (Sigma Aldrich, Castle Hill, NSW, Australia). Skin was dissected into 3 x 3 mm portions and placed into a 6-well plate with 4 pieces of skin per well. Skin was placed with the dermal side down and allowed to adhere to plastic for 45 minutes at room temperature. 2 ml of 20% FCS/1% Penicillin was added and incubated overnight at 37°C, 5% CO<sub>2</sub>. Cell media was replaced after 24 hours and every second day thereafter. At day 7, skin explants were removed and healthy fibroblasts were collected using trypsin. Low passage (1-3) primary skin fibroblasts were used in all experiments.

### **2.7.3 Skin explant outgrowth assay**

Diabetic and non-diabetic mice were used to collect skin explants to perform outgrowth assays. 2 mm biopsies were taken from the excised skin of mouse back using a biopsy punch (Acu-Punch<sup>®</sup>, Acuderm, Fort Lauderdale, FL, U.S.A.). Biopsies were collected in ice-cold Nutrient Mixture F12 (SAFC Biosciences, Lenexa, KS), 1% penicillin (Sigma Aldrich, Castle Hill, NSW, Australia). Four biopsies were added to each well in a 6 well plate. Individual biopsies were placed with the dermal side down and allowed to adhere for 45 minutes in room temperature. Skin explants were cultured in 2 ml 20% FCS/DMEM, 1% penicillin (Sigma Aldrich, Castle Hill, NSW, Australia) at 37°C, and 5% CO<sub>2</sub>. Media was replaced after 24 hours and then every second day thereafter. Images were taken to capture and measure cellular outgrowth with 3 images per explant and 8 explants per group. The proximal distance of cellular migration outgrowth from each skin explant was measured at days 0 to 7 post-plating and measurements recorded using AnalySIS software (Soft-Imaging System GmbH, Munster, Germany).



**Figure 2.10. Microscopic assessment of excisional wound.** Illustration of different measurement parameters used as part of microscopic assessment excisional wounds. Scale bar = 200  $\mu\text{m}$ . Magnification  $\times 4$ .

## 2.8 Transfection studies

### 2.8.1 Plasmid DNA Transfection

Human Embryonic Kidney cells (HEK-293) were cultured in T75 flask and allowed to reach 90% confluence. On the day of transfection, the cells were incubated with 10 ml of growth medium without antibiotics and serum. Plasmid DNA transfection was performed using Lipofectamine™ 2000 (#11668-027, Invitrogen, Mulgrave, Victoria, Australia) in accordance with manufacturer's recommendation. To elucidate the possible importance of gelsolin or leucine-rich repeat (LRR) domain in secretion of Flii protein, three different DNA plasmids were used for cell transfection and Western blotting analysis of collected media.

The cDNA sequences of Flightless I and homologues have been determined (Campbell et al. 1993) (*D. melanogaster* GenBank accession no. U01182, *C. elegans* GenBank U01183 and *Homo sapiens* GenBank U01184; U80184). Variant 1 plasmid DNA was provided by Dr Hugh Campbell (Australian National University) and contained a plasmid with the complete human *FLII* coding region (Davy et al. 2000). Variant 2 plasmid DNA contained gelsolin-like domain only and consisted of (amino acids from 424-1269) with C terminal EGFP fusion (Figure 2.11). This Flii plasmid DNA variant was generated by Dr James Waters (Wound Healing Laboratory, WCHRI, South Australia). It was created by NheI digest of PEGFP-N1-Flii to remove LRR, heat inactivation and re-ligation of vector. Variant 3 contained LRR only (amino acids from 1-478) with C terminal EGFP fusion. This variant was also created by Dr James Waters (Wound Healing Laboratory, Women's and Children's Health Research Institute, Adelaide) by AgeI

digestion of pEGFP-N1-Flii followed by column clean-up, then PflMI digestion, column clean-up, followed by single stranded nuclease treatment, column clean-up, then re-ligation of vector. In preparation for plasmid DNA transfection, 1.5 ml of DMEM and 60  $\mu$ l of lipofectamine were mixed in one 10 ml tube. 1.5 ml of DMEM and 24  $\mu$ g of plasmid DNA were added in another 10 ml tube. Both tubes were incubated for 5 minutes at room temperature and mixed together. Mixed solution was incubated for further 30 min at room temperature in the dark. Incubated solutions were mixed together and added directly to cells and incubated for 7 hours. 7 hours post-transfection media was replaced with fresh DMEM/1% Penicillin (Sigma Aldrich) and incubated overnight. The cells were analysed for positive transfection using Nikon Eclipse inverted Microscope. At 48-hours post-transfection media was collected for detection of secreted Flightless with either gelsolin domain or LRR domain only. Media was analysed by Western blotting using mouse anti-human monoclonal antibodies to Flii (Santa Cruz, San Jose, CA, U.S.A.).

## **2.9 Flow cytometry**

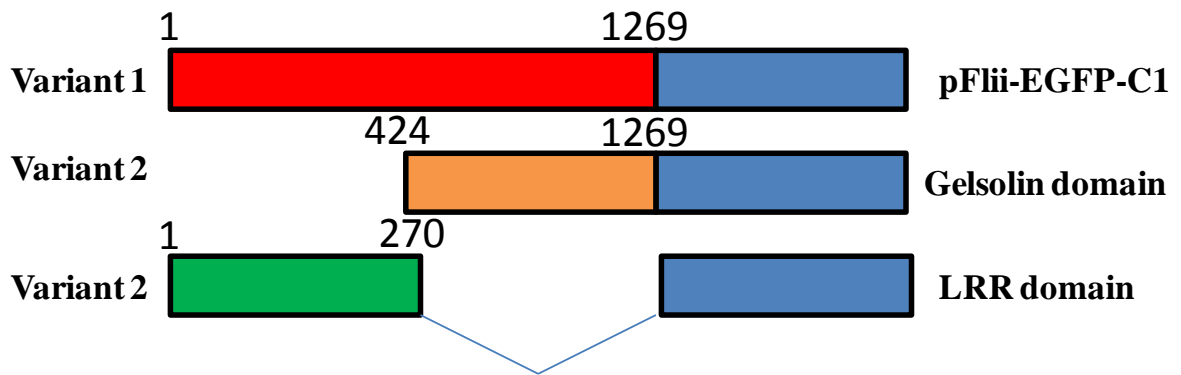
### **2.9.1 Fluorescence-activated cell sorting (FACS)**

Whole blood was collected from healthy adult volunteers by brachial venepuncture in EDTA-treated collection tubes. 200  $\mu$ l of EDTA-treated blood was pipetted into a clean tube and blocked for 30 minutes with human serum. Without washing 5  $\mu$ l of undiluted monoclonal antibodies were added and incubated for 30 minutes (Figure 2.12). 3 ml of 0.05% Azide in 1 x PBS was added to the tube and centrifuged at 1300 rpm for 5 minutes. This washing step was repeated three times. Red blood cell lysis was performed by adding 2 ml of 1 x BD FACSTM

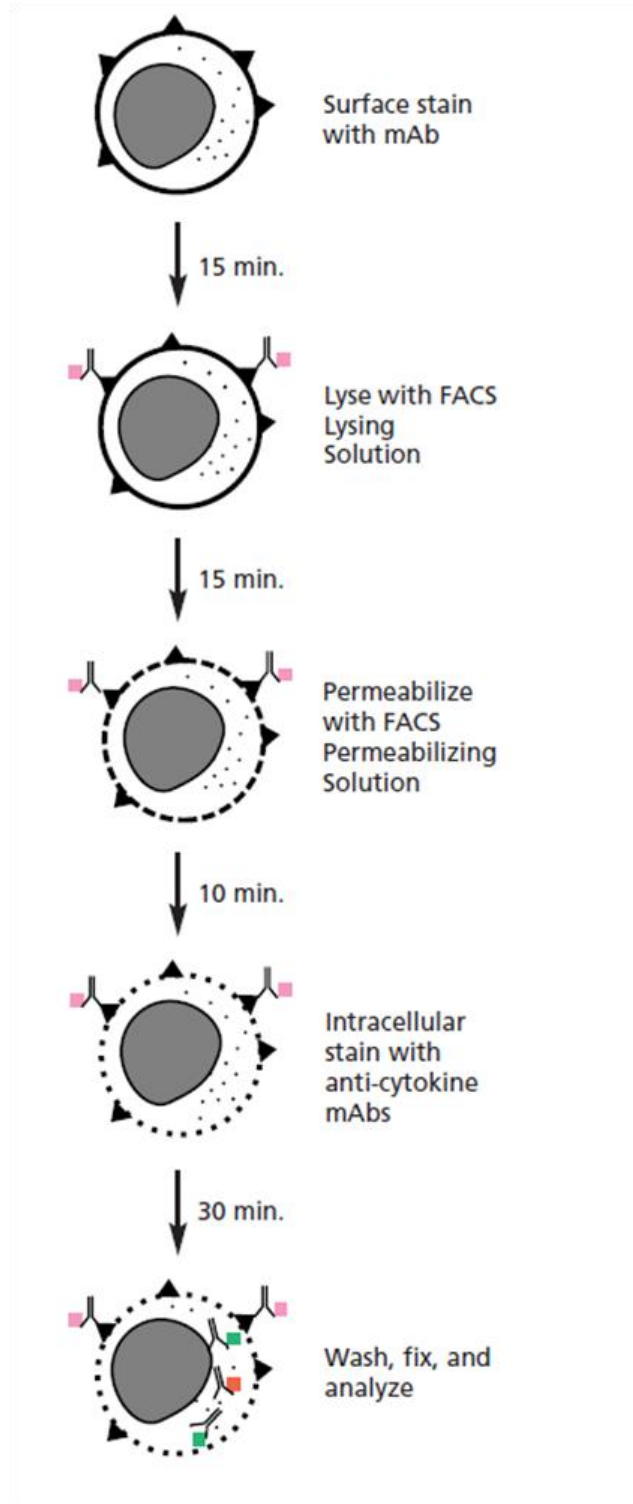
Lysing Solution (#349202, BD Biosciences, San Jose, CA, U.S.A.) and incubating the sample for 10 minutes. The cells were permeabilized prior to intracellular antibody staining by adding 250 µl of BD Cytotfix/Cytoperm (554722, San Jose, CA, U.S.A.) for 20 minutes at 4°C followed by the washing step with 3 ml of BD Perm/Wash Buffer, (# 554723, BD Biosciences, San Jose, CA, U.S.A.). Cells were washed two times in a Perm/Wash™ buffer (BD Perm/Wash™ buffer, # 554723, BD Biosciences, San Jose, CA, U.S.A.), which was also used as an antibody dilutant. The samples were then incubated with 5 µl of undiluted intracellular antibody (Flightless, Cytochrome C or IgG control) for 30 minutes at 4°C. This was followed by another washing step (x 3). All tubes were analysed using FACS AriaII (BD Biosciences, San Jose, CA, U.S.A.).

### **2.9.2 Red Blood Cell Lysis**

To isolate white blood cells for cell sorting the whole blood (white blood cell enriched), also referred to as the “buffy coat” was obtained from the Australian Red Cross Blood Service (Pirie Street Donor Centre, Adelaide, South Australia). 5 ml of the collected buffy coat was pipetted into a 50 ml conical flask and mixed with 40 ml of ACK Lysing Buffer (#A1049201, Invitrogen, Carlsbad, U.S.A.). The mixture was incubated for minutes at room temperature at which time the solution turned a clear deep red colour. The white blood cells were collected by centrifugation at 300 x g for 5 minutes at room temperature. The supernatant was aspirated and the pellet was resuspended in 50 µl of 1 x PBS for cell sorting.



**Figure 2.11. Plasmid DNA generation.** Three different DNA plasmids were used for HEK293T cell transfection. Variant 1 plasmid DNA was provided by Dr Hugh Campbell (Australian National University) and contained a plasmid with the complete (amino acids 1-1269) human Flii coding region (Davy et al. 2000). Variant 2 plasmid DNA contained gelsolin-like domain only and consisted of (amino acids from 424-1269) with C terminal EGFP fusion. This Flii plasmid DNA variant was generated by Dr James Waters (Wound Healing Laboratory, WCHRI, South Australia). Variant 3 contained LRR only (amino acids from 1-478) with C terminal EGFP fusion.



**Figure 2.12.** Cell surface and intracellular stain with monoclonal antibodies for multi-coloured flow cytometry analysis.

## **2.10 Regulated trafficking and exocytosis studies**

### **2.10.1 Isolation of exosomes from cultured cells**

Eight flasks (T75) of confluent Human Foreskin Fibroblasts (HFF) were trypsinized and cell counts performed.  $15\text{-}20 \times 10^6$  cells were seeded into three separate T75 flasks and incubated for 7 hours in FCS free media and in the presence of monensin (#M5273, Sigma-Aldrich, Saint Louis, Missouri). The three different concentrations of monensin were trialled  $0 \mu\text{M}$ ,  $1 \mu\text{M}$ ,  $5 \mu\text{M}$  (Savina et al. 2003). Following the 7 hour incubation at  $37^\circ\text{C}$ ,  $5\%$   $\text{CO}_2$ , culture media was collected on ice and centrifuged at  $800 \times g$  for 10 minutes to sediment the cells, and then centrifuged at  $12000 \times g$  for 30 minutes to remove the cellular debris. Exosomes were separated from the collected supernatant by centrifugation at  $100,000 \times g$  for 3 hours using Beckman Coulter Class S centrifuge, Optima L Series Optima L, Rotor SW32T, rotor radius 129.33 mm (Beckman Coulter, Brea, CA, U.S.A.). Exosome pellet was collected by firstly washing the pellet in 1 x PBS and then re-suspending it in  $100 \mu\text{l}$  of PBS. This was followed by protein quantification and Western blotting analysis to determine the levels of Flii and Hsp70/Hsc70 in the exosome fraction.

### **2.10.2 Preparation of Nuclear and Cytoplasmic Extract**

The Nuclear Extract Kit (#40010, Active Motif, Carlsbad, CA, U.S.A.) was used for the preparation of nuclear and cytoplasmic extracts from HFF cells. To obtain proteins contained in cytoplasmic and nuclear compartments of the cell,  $8.8 \times 10^6$  cells were collected by scraping the flask with a cell scraper. The cells were transferred to a pre-chilled 15 ml conical tube. The cell suspensions were centrifuged for 5 minutes at 500 rpm in a centrifuge pre-cooled at  $4^\circ\text{C}$ . The



supernatant was discarded and the cell pellet was kept on ice. For the cytoplasmic fraction collection the cells were resuspended 500 µl 1X Hypotonic Buffer provided in the kit (Nuclear Extract Kit, #40010, Active Motif, Carlsbad, CA, U.S.A.) by pipetting up and down several times. The suspension was transferred to a pre-chilled microcentrifuge tube and incubated for 15 minutes on ice. 25 µl of detergent provided in the kit was added and vortexed for 10 seconds at highest setting. The suspension was centrifuged for 30 seconds at 14,000 x g in a microcentrifuge pre-cooled at 4°C. The supernatant was transferred (cytoplasmic fraction) into a pre-chilled microcentrifuge tube and stored at –80°C until ready to use. The pellet was used for nuclear fraction collection. To collect the nuclear fraction of the cell compartment the nuclear pellet was resuspended in 50 µl Complete Lysis Buffer (provided in kit) by pipetting up and down. The suspension was vortexed for 10 seconds at highest setting. The suspension was incubated for 30 minutes on ice on a rocking platform set at 150 rpm. The sample was vortexed for 30 seconds at highest setting and centrifuged for 10 minutes at 14,000 x g in a microcentrifuge pre-cooled at 4°C. The supernatant (nuclear fraction) was transferred into a pre-chilled microcentrifuge tube and stored at –80°C. Freeze/thaw cycles were avoided.

## **2.11 Cellular Proliferation Studies**

### **2.11.1 Cell Proliferation Assay**

Human foreskin fibroblasts (HFF) were used in the *in vitro* assays. 96-well plate (n=6 for each group) was used to seed HFF at  $2 \times 10^3$  cells/well. Cell proliferation using the WST-1 reagent was performed according to the manufacturer's direction (#05015944001, Roche Applied

Science, Munich, Germany). On day 1 of the experiment, the cells were seeded and incubated overnight in 10% FCS/DMEM, 1% penicillin (Sigma Aldrich, Castle Hill, NSW, Australia) at 37°C, and 5 % CO<sub>2</sub>. On day 2, the media was replaced with serum-free DMEM and incubated for 6 hours to synchronise the cell cycle. Media was aspirated and replaced with serum free DMEM, 2% penicillin, 2% Fungizone (Sigma Aldrich, Castle Hill, NSW, Australia) media mixed with acute and chronic wound fluid at a ratio of 1:1. Flightless neutralizing antibody (FnAb) has been described elsewhere (Adams et al. 2008; Campbell et al. 2002) and was added to wound fluid with the final antibody concentration of 20 µg/ml. Wound fluid was incubated with FnAb at 37°C, 5 % CO<sub>2</sub> for 30 min to counteract the negative effects of Flii. 100 µl of the mixture containing wound fluid, DMEM and FnAb was added into each well with HFF and incubated for 24 hours at 37°C, 5% CO<sub>2</sub>. On day 3, the cell proliferation reagent WST-1 was added to determine spectrophotometric value of cell growth and viability at 24 hours post-addition of FnAb. 10 µl of WST-1 reagent was added to the cells and left at 37°C for 30 minutes. The presence of the formazan product was quantified at a dual absorbance of 450nm and 600nm using ELISA plate reader. HFF cells grown in the same media and cells treated with an irrelevant rabbit IgG were used as a control population.

## **2.12 Protein Expression and Western Blotting**

### **2.12.1 Protein Isolation from Human Wound and Skin Biopsies**

Human skin specimens were defrosted and dissected into 0.5 mm x 0.5 mm pieces. Following manual microdissection specimens were placed into a 1.5 ml eppendorf tube and mixed with 0.5 ml of RIPA buffer (1% TritonX-100: Ajax Chemicals, Auburn, Australia; 1% Na deoxycholate: Sigma Aldrich, Castle Hill, NSW, Australia, 50mM Tris pH = 7.0; 0.1% SDS: Medicago AB, Uppsala, Sweden; 150mM NaCl: Unilab, Seven Hills, NSW, Australia, 2mM EDTA pH 8.0: Unilab, Seven Hills, NSW, Australia; adjusted to pH = 7.2 with 193.25 ml Milli Q water to make stock solution up to 275ml). Before use 5 ml of RIPA buffer was combined with 1mM Na<sub>3</sub>VO<sub>4</sub> pH 10.0, 1mM NaF, 2mM Perfabloc and 1 x complete protease inhibitors (#1836153 Boehringer Mannheim, Ingelheim Germany). Samples were homogenised on ice by using a DiAx 600 homogenizer (Heidolph, Kelheim, Germany) set at 22000 rpm for 1 min. Homogenised samples were incubated for 30 minutes at 4°C and centrifuged at 12000rpm for 15 min. Supernatant containing protein was transferred to a clean pre-cooled 1.5 eppendorf tube and stored at -20°C until further use.

### **2.12.2 Protein isolation from wound fluid and plasma**

Samples of plasma, acute and chronic wound fluid were also collected and centrifuged to precipitate cellular debris. The supernatant was used to isolate proteins by precipitation with trichloroacetic acid (TCA) (Gruhler et al. 2005). Flii protein levels were quantified by Western Blotting as previously described below. Western blots were probed with Flii, albumin and β-

tubulin antibodies. Protein bands were visualized and captured using Syngene G:BOX Chemi HR16 and GeneSnap image capture software (Syngene, Cambridge, UK). Quantification of protein bands was carried out using the Image Quant program by Molecular Dynamics; Amersham Pharmacia Biotech. (GE Healthcare, NSW, Australia).

### **2.12.3 Protein isolation from cultured cells and determining the protein yield**

The protein was extracted using RIPA buffer as described above. Before use 5 ml of RIPA buffer was combined with 1mM Na<sub>3</sub>VO<sub>4</sub> pH 10.0, 1mM NaF, 2mM Perfabloc and 1 x complete protease inhibitors (#1836153 Boehringer Mannheim, Ingelheim Germany). Samples were mixed with 200 µl of RIPA buffer and incubated for 30 minutes at 4°C. Following centrifugation at 12, 000 rpm for 15 minutes, supernatant containing protein was transferred to a clean pre-cooled 1.5 eppendorf tube and stored at -20°C until further use.

### **2.12.4 Protein quantification**

Protein was quantified using Pierce BCA (Bicinchoninic Acid) Quantification kit (#23227, Thermo Fisher Scientific, Rockford, IL USA). The albumin standards were made up to the following concentrations (µg/ml): 0, 25, 125, 250, 500, 750, 1000, 1500 and 2000. The working reagent was made from 50 parts of BCA reagent A and 2 parts for BCA reagent B. 10 µl of the samples were pipetted, in duplicate, into a 96 well plate (#167008, Nunc™, Roskilde, Denmark). 200 µl of the working solution was then pipetted into each well and the contents were mixed using a plate mixer for 30 seconds. The plates were then incubated at 37°C for 30 minutes. The

absorbance was then measured at a wavelength of 562 nm using a plate reader (Sunrise Basic, Tecan Group Ltd., Mannedorf, Switzerland). The data was transferred to a Magellan software programme (Magellan V 6.3, Tecan, Mannedorf, Switzerland). Using the reading values a standard curve from the controls and also the sample concentrations was constructed.

#### **2.12.5 Western Blotting**

Western blotting was performed on the total wound protein and whole cell lysate to determine individual protein levels using a Mini Trans-Blot® Cell and Mini Protean® Tetra Cell (BioRad Laboratories, Hercules, CA, U.S.A.) electrophoresis apparatus. A separating gel was made up and covered with propan-2-ol to ensure a smooth level surface. The separation gel was made up as indicated in Table 2.3. The gel was allowed to set for 30 minutes. The propan-2-ol was then discarded and washed with distilled water. A stacking polyacrylamide gel was then prepared and layered on top of the separating gel. The stacking gel was made up as shown in Table 2.3.

	10% Separating	4% Stacking
30% Acrylamide	3.35 ml	0.5 ml
3M Tris pH8.9	1.25 ml	
0.5 Tris pH 6.8		0.276 ml
Distilled water	5.25 ml	4.104 ml
10% SDS	125 $\mu$ l	50 $\mu$ l
10% APS	100 $\mu$ l	40 $\mu$ l
TEMED	6.25 $\mu$ l	4 $\mu$ l

**Table 2.3. The recipe used to prepare the separating and staking gels (SDS PAGE gel).**

Once the gel was poured a comb was placed into the stacking gel and then allowed to set. 20 µg of individual protein samples were prepared with the final solutions being made up to 35 µl. 7 µl of 5X SDS PAGE loading buffer (25mM Tris pH 6.8, 8% glycerol, 1% SDS, 0.02% bromophenol blue) containing 5% of 2-mercaptoethanol was added to each sample. Samples were then incubated at 95°C for 5 minutes to denature and placed on ice. Protein samples were loaded on to a 10% SDS-PAGE gel and run at 100V for 1.5 hours and then transferred to nitrocellulose membrane (#66485, BioTrace™ NT, Pall Corporation, Pensacola, FL, U.S.A.) by wet transfer. Membranes were blocked in 5% skimmed milk powder for one hour and primary antibody added in PBS/3% SMP/0.3% Tween20 overnight at 4°C. The following day 4 washes (10 minutes each) in 5% milk in PBS/3% Tween20 were performed. Horse Radish Peroxidase (HRP) linked secondary antibodies and a peroxide substrate was then used to amplify the signal and provide a means for detection. The secondary antibody was added for an hour. Stringent washes were performed before detection of HRP by ECL (#32132, Pierce® ECL Plus Western Blotting Substrate, Thermo Scientific, Rockford, IL, U.S.A.). Detection was measured via dark bands developed on gel imaging system (G:BOX Chemi, Syngene, Cambridge, U.K.). Once the membrane was developed a JPEG image was saved and opened in image processing program (Image J, public domain, <http://rsb.info.nih.gov/ij/>). The intensity of each of the protein bands on the membrane were digitally analysed and measured with Image J calibrated software. Once the intensity values were obtained each sample was normalised to a loading control protein intensity of that particular sample.

### **2.13 Statistical analysis**

Statistical differences were determined using the Student's T-test. A  $p$  value of less than 0.05 was considered significant.



## *Chapter 3*

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# FLIGHTLESS I IN A MURINE MODEL OF DIABETIC WOUND HEALING

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Results in this chapter have been submitted to the *Journal of Biomedicine and Biotechnology*

**Title:** The Action of Flightless I and Toll-Like Receptors during Wound Healing in Diabetic Wounds

**Authors:** Nadira Ruzehaji, Stuart J. Mills, Elizabeth Melville, Ruth Arkell, Robert Fitridge, Allison J Cowin

Manuscript of the article is in Appendix 3

# FLIGHTLESS I IN A MURINE MODEL OF DIABETIC WOUND HEALING

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### 3.1 Introduction

Diabetic foot ulceration is a leading cause of lower limb amputation (Reiber et al. 1999). Peripheral neuropathy, peripheral vascular disease and reduced joint mobility, all of which are associated with diabetes, increase the risk of ulceration induced by a trivial injury from shoes and other minor physical trauma (Oyibo et al. 2001). Up to 25% of people with diabetes can expect to develop a non-healing ulcer (Bentley & Foster 2007) and 3% will have a lower-limb amputation (Oyibo et al. 2001). The financial and personal burden placed on the individual and the society is significant. Despite numerous approaches, the therapeutic results are still very limited. Therefore, studies aimed at developing effective treatments for diabetic ulcers are of paramount importance.

The actin-remodelling protein Flii has been identified as an important regulator of wound repair, affecting cell proliferation, motility and matrix deposition (Cowin, AJ et al. 2007). Previously, we have studied incisional wounds recovered from mice heterozygous for Flii gene knockout and found that healing is significantly improved, whereas Flii overexpressing mice have significantly impaired healing with reduced dermal cell proliferation and delayed re-epithelialization (Cowin, AJ et al. 2007). Our previous work demonstrated that neutralising antibodies to Flii (FnAbs) have the capacity to increase cellular proliferation and adhesion *in vitro* and promote the rate of healing in cutaneous wounds *in vivo* (Adams et al. 2009). To date, FnAbs have been tested in several murine (including incisional and burn models of wound healing) (Adams et al. 2009; Cowin, AJ et al. 2007) and porcine models (excisional and incisional) (Jackson et al. 2012). These studies have recorded a measurable efficacy of FnAb in wound healing with the treated wounds healing faster and producing better scars than placebos.

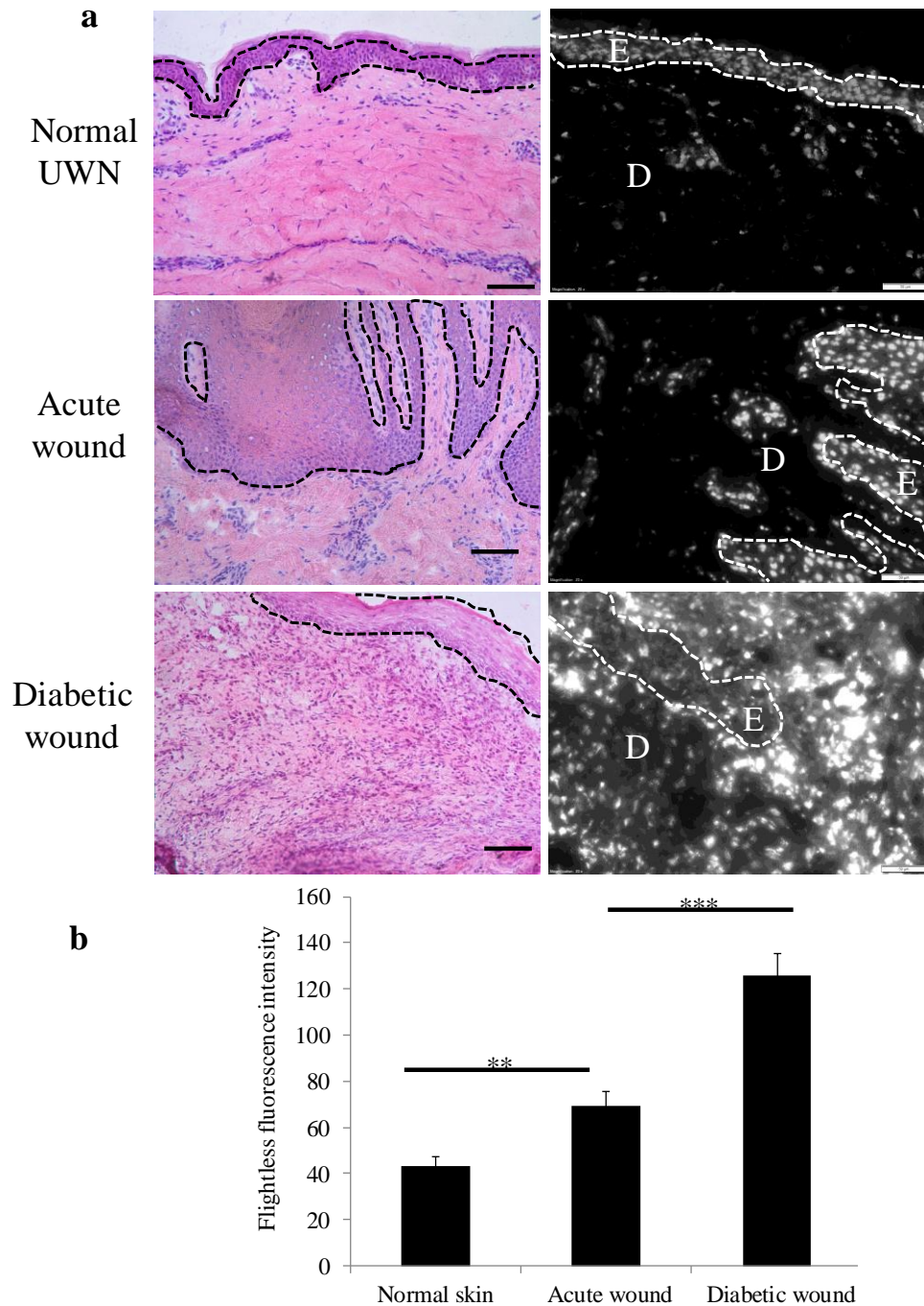
Given that Flii is an important negative regulator of wound repair and its deficiency leads to significantly faster and enhanced re-epithelialization, the role of Flii on diabetic wound healing may be of potential significance. The aim of this study was to determine the differential effect of Flii gene expression on epithelial migration and wound closure using an *in vivo* model of diabetes. We used Flii heterozygous mice (Flii<sup>+/-</sup>, homozygous knockout mice are embryonic-lethal), wild-type (WT) and Flii transgenic mice (Flii<sup>Tg/Tg</sup>) to investigate the role of Flii gene expression in murine model of diabetic wound healing. To test the efficacy of exogenous addition of FnAbs to diabetic wounds we treated WT diabetic wounds with either FnAbs or isotype and dose-matched control antibodies. We assessed the macroscopic and histological wound sizes of FnAb-treated diabetic wounds and compared these to IgG-treated controls.

## 3.2 Results

### 3.2.1 Flii is elevated in human diabetic wounds

To study the effect of diabetes on Flii protein expression in humans, six diabetic wound biopsies were collected from patients diagnosed with diabetic foot ulcers with duration  $\geq 6$  weeks (4 men and 2 women with a mean age of 64). All wounds studied showed features characteristic of diabetic ulcers with loss of epithelium that extended into the dermis and deeper layers of skin. The epithelial margin was characterized by marked hyperkeratosis. Acute wounds ( $\leq 4$  weeks old) were collected from 6 patients with acute trauma wounds such as those seen in the emergency room (4 women and 2 men with a mean age of 40 years). Unwounded skin specimens were collected from 6 healthy donors (4 women and 2 men with a mean age of 42) and used as controls. Representative H&E stained views of a diabetic ulcer, acute wound and normal skin are shown in Figure 3.1a.

Flii is a negative regulator of wound healing and Flii overexpressing mice have impaired healing (Adams et al. 2009). To assess the expression of Flii protein in diabetic wounds we proceeded to stain these wounds with anti-Flii antibody. Figure 3.1 shows that Flii is expressed in unwounded skin in both the epidermis and the dermis (a). This expression is significantly increased ( $p = 0.005$ ) upon wounding in acute wounds (b). There is, however, a much greater and highly significant increase of Flii in the diabetic wounds (b) when compared to the acute wounds ( $p = 0.0008$ ).



**Figure 3.1. Flii protein expression is increased in diabetic wounds compared to acute and normal unwounded skin.** (a) Illustrates representative immunohistochemistry images of Flii and haematoxylin and eosin-stained sections of normal unwounded skin, acute wounds and diabetic wounds. E denotes the epidermis, D the dermis and W denotes the wound. Scale bars = 50µm. (b) Graphical representation of the intensity of Flii staining in the three groups. \*\*  $p \leq 0.01$  and \*\*\* $p \leq 0.001$ ; n = 6.

### **3.2.2 Diabetes was successfully induced by STZ in mice expressing low, normal, and high levels of Flii**

Groups of 12-16 weeks of age mice expressing low (Flii<sup>+/-</sup>), normal (Flii<sup>+/+</sup>), and high (Flii<sup>Tg/Tg</sup>) levels of Flii have been treated with STZ and the effect on blood glucose levels determined. STZ-treated mice had consistently higher fasting blood glucose levels ( $\geq 14.9$  mmol/L) over entire experimental period. STZ-treated mice with whole blood glucose higher than 14.9 mmol/L were considered as diabetic. Mice serving as vehicle controls were given the same volume of sodium citrate and showed consistently lower fasting blood glucose levels ( $\leq 7.5$  mmol/L) than mice treated with STZ. Changes in Flii gene manipulation did not affect blood glucose levels in diabetes (Figure 3.2).

### **3.2.3 Inhibitory effect of Flii on cellular growth and migration**

The skin explants (2 mm) were collected from diabetic and non-diabetic mouse skin using a biopsy punch. The explants were incubated in normal glucose (5.5 mM D-glucose) or high glucose (25 mM D-glucose) supplemented media for 7 days. The micrographs were taken daily for 7 days. On day 2 post-culture a halo appeared around the explants. The cellular growth and migration potential was determined by measuring the distance between the explant and the end of the growing halo. This *in vitro* assay assessed the effect of Flii gene expression on cell migration in normal and diabetic cells (Figure 3.3a). Diabetic explants displayed a significant reduction in their capacity to grow and migrate compared to non-diabetic skin tissue ( $p = 0.02$ ; day 6; WT diabetic vs. WT non diabetic). A dose-dependent effect of Flii gene expression on



cellular outgrowth was observed with the  $Flii^{+/-}$  cells migrating faster than WT, which in turn grew faster than  $Flii^{Tg/Tg}$ . Diabetic  $Flii^{Tg/Tg}$  explants displayed the worst phenotype, with the cellular outgrowth being slower (Figure 3.3b and c), suggesting that over expression of Flii gene has a negative effect on cellular migration.

### **3.2.4 Excisional wounds heal faster in mice with low levels of Flii gene and protein expression**

To determine the effect of Flii expression on excisional wound healing, three lines of mice were used expressing low ( $Flii^{+/-}$ ), normal ( $Flii^{+/+}$ ), and high ( $Flii^{Tg/Tg}$ ) levels of Flii. The rates of healing and representative images of wounds at 0, 7, 14 and 21 days are shown in Figure 3.4a. As a result of wounding some bleeding was inevitable followed by coagulation and development of non-viable eschar surrounding the circular wounds. Wound healing resulted in decreased wound areas, when assessed macroscopically (Figure 3.4b). Wound area was decreased by almost half in  $Flii^{+/-}$  ( $p = 0.01$ ) and wild-type ( $p = 0.012$ ) mice relative to  $Flii^{Tg/Tg}$  mice at day 7 post-wounding (Figure 3.4b). Wound areas in all three mice lines were similar on day 14 and 21 post-wounding (Figure 3.4b).

Histological assessment of excisional wounds showed that at day 7 the dermal wound gape (Figures 3.5b;  $p = 0.05$ ) and the percentage of re-epithelialization (Figures 3.8a, b;  $p = 0.01$ ) were significantly smaller in non-diabetic Flii deficient mice ( $Flii^{+/-}$ ) compared with non-diabetic Flii overexpressing ( $Flii^{Tg/Tg}$ ) littermates. Representative microscopic images of days 0, 7, 14 and 21 wounds are shown in Figure 3.5a. Healing in non-diabetic Flii overexpressing ( $Flii^{Tg/Tg}$ ) mice was delayed with histological wound length, wound gape and the percentage of re-

epithelialization being significantly larger than those in non-diabetic wild-type at 7 days after wounding (Figure 3.5b and Figure 3.8d). There was no significant difference in the percentage of re-epithelialization of the non-diabetic wild-type compared with diabetic  $Flii^{+/-}$  wounds at day 7 post-wounding (Figure 3.8d).

### 3.2.5 Flii deficiency attenuates diabetes-induced impaired wound healing

The influence of Flii gene expression on diabetic wound healing was determined by creating two 6 mm full thickness excisional wounds on the dorsum of mice with low ( $Flii^{+/-}$ ), normal ( $Flii^{+/+}$ ), and high ( $Flii^{Tg/Tg}$ ) level of Flii expression. Decreased Flii gene expression resulted in a significant increase in wound size as determined by the planimetric analysis of surface wound area ( $p = 0.049$   $Flii^{+/-}$  vs.  $Flii^{Tg/Tg}$ ; Figure 3.6b) and dermal gape (Figure 3.7b;  $p = 0.05$  WT vs.  $Flii^{Tg/Tg}$ ;  $p = 0.049$   $Flii^{+/-}$  vs.  $Flii^{Tg/Tg}$ ). Representative digital images of 0, 7, 14 and 21 day diabetic wounds are shown in Figure 3.6a.

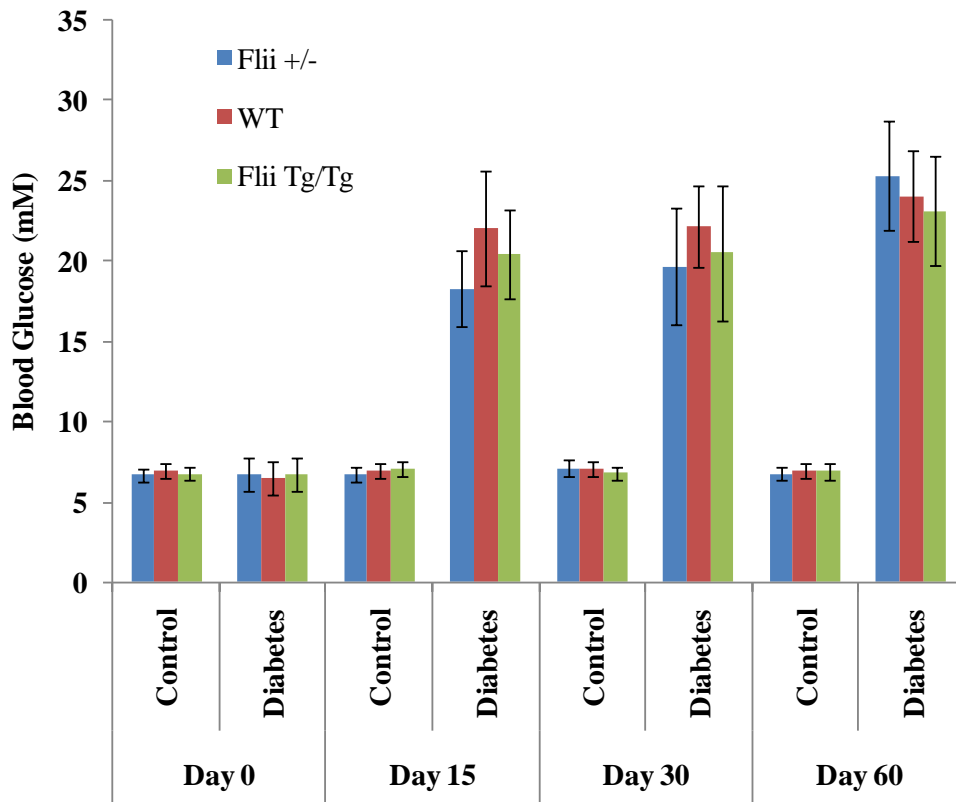
Histological analysis of the wounds revealed that the distance between the dermal wound margins was longer in wounds with high levels of Flii ( $Flii^{Tg/Tg}$ ) at day 7 compared to WT and Flii-deficient wounds ( $Flii^{+/-}$ ) (Figure 3.7b;  $p = 0.05$  WT vs.  $Flii^{Tg/Tg}$ ; (\*b)  $p = 0.007$   $Flii^{+/-}$  vs.  $Flii^{Tg/Tg}$ ). Representative haematoxylin and eosin stained wound sections for  $Flii^{+/-}$ , WT and  $Flii^{Tg/Tg}$  mice are shown in Figure 3.7a. The percentage of wound re-epithelialization was evaluated by measuring the length of the neopeidermis at day 7 post-wounding (Figure 3.8d). This histologic measurement confirmed that healing was significantly impaired in Flii transgenic mice ( $Flii^{Tg/Tg}$ ) with the wound area being larger and the rate of re-epithelialization being

severely delayed in  $Flii^{Tg/Tg}$  wound compared with WT and  $Flii^{Tg/Tg}$  age-matched cohorts (Figure 3.8d;  $p = 0.02$   $Flii^{+/-}$  vs.  $Flii^{Tg/Tg}$ ). Healing was significantly impaired in all three diabetic mice lines ( $p \leq 0.05$ ) compared to non-diabetic controls (Figure 3.8d) confirming that this model of STZ-induced diabetes has successfully induced impaired wound healing seen in human diabetes (Type 1 and Type 2).

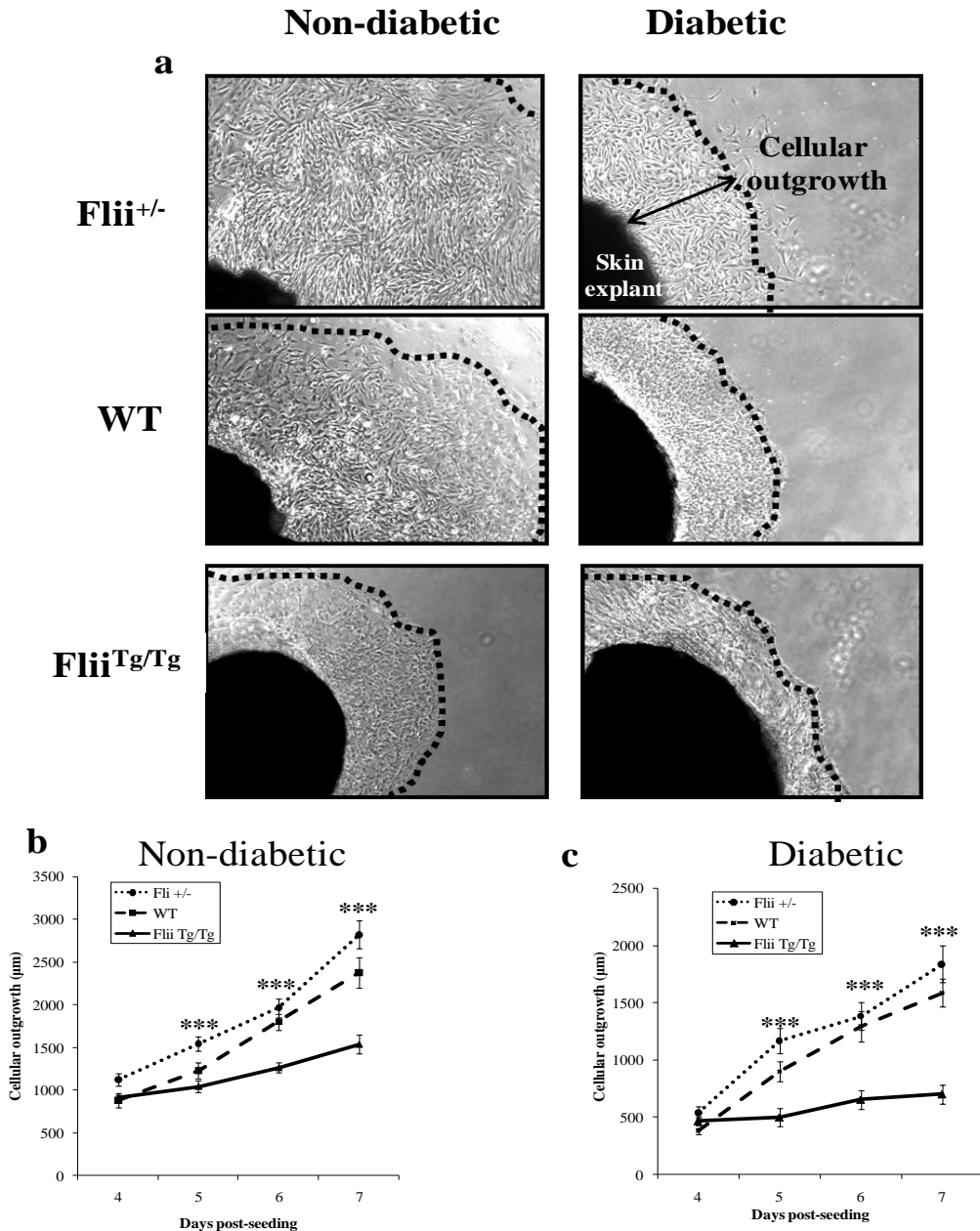
### 3.2.6 Flii is upregulated in response to excisional wounding

The effect of wounding on protein levels of Flii was determined in non-diabetic, normal wounds in mice with low ( $Flii^{+/-}$ ), normal ( $Flii^{+/+}$ ), and high ( $Flii^{Tg/Tg}$ ) expression of Flii. Wounds collected on 0, 7 and 14 days post-wounding were immunostained for Flii protein expression (Figure 3.9a). Reduced Flii immunofluorescence was observed in  $Flii^{+/-}$  skin ( $p \leq 0.001$ ; unwounded  $Flii^{+/-}$  vs. unwounded  $Flii^{Tg/Tg}$ ) was observed compared to WT and  $Flii^{Tg/Tg}$  mouse skin (Table 3.1).

Under non-diabetic control conditions, wounding significantly increased Flii protein level in  $Flii^{+/-}$ , WT and  $Flii^{Tg/Tg}$  skin (Figure 3.9a, b). An 87% increase was observed in response to wounding in non-diabetic Flii overexpressing ( $Flii^{Tg/Tg}$ ) mice in the dermis, which peaked at 7 days post-wounding ( $p \leq 0.001$ ; unwounded  $Flii^{Tg/Tg}$  vs. day 7  $Flii^{Tg/Tg}$ ; Figure 3.9b) and decreased by half (50%) on day 14 post-wounding in all mice lines (Figure 3.9b).

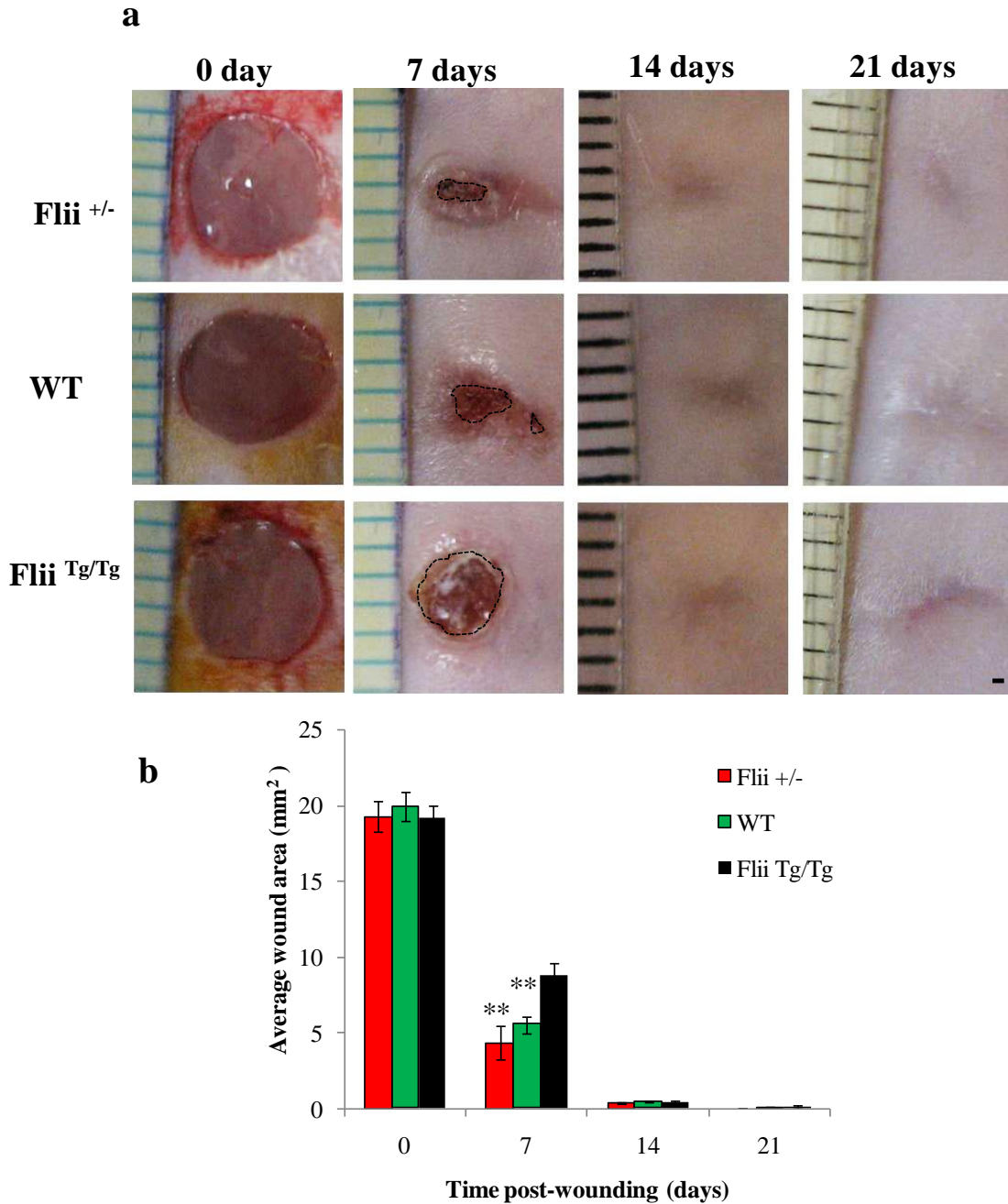


**Figure 3.2. Induction of diabetes and effect of STZ on blood glucose levels.** Mice expressing low (Flii<sup>+/-</sup>), normal (Flii<sup>+/+</sup>), and high (Flii<sup>Tg/Tg</sup>) levels of Flii were treated with five consecutive daily injections of 50mg/kg STZ or sodium citrate buffer. Fasting blood glucose levels as determined by the tail vein sampling were measured weekly and data obtained on 0, 15, 30 and 60 days post-STZ injection are shown above. Mice treated with STZ showed higher fasting blood glucose ( $\geq 14.9$  mmol/L) than those serving as vehicle controls ( $\leq 7.5$  mmol/L).

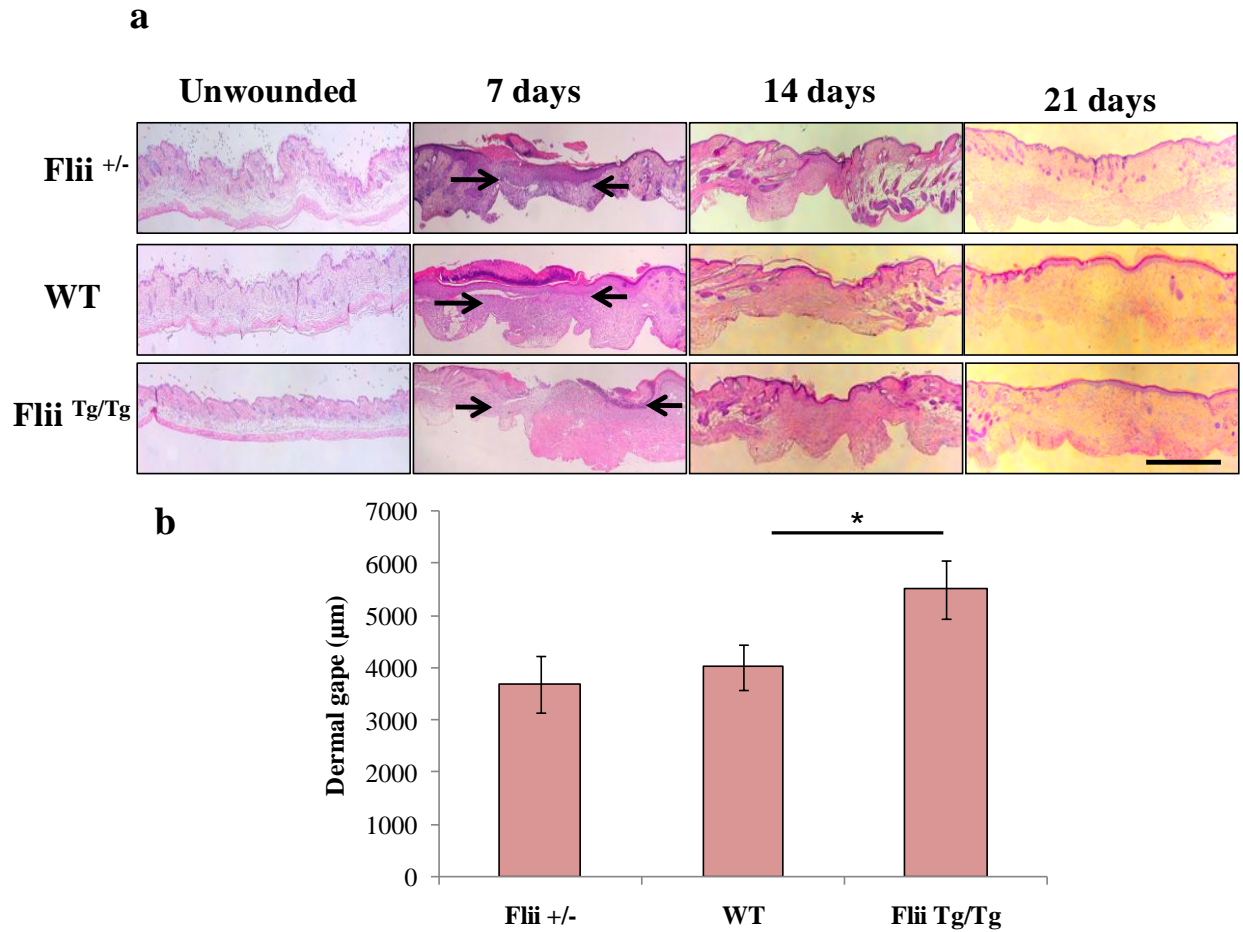


**Figure 3.3. Differential effect of Flii gene on primary mouse cellular growth and migration.**

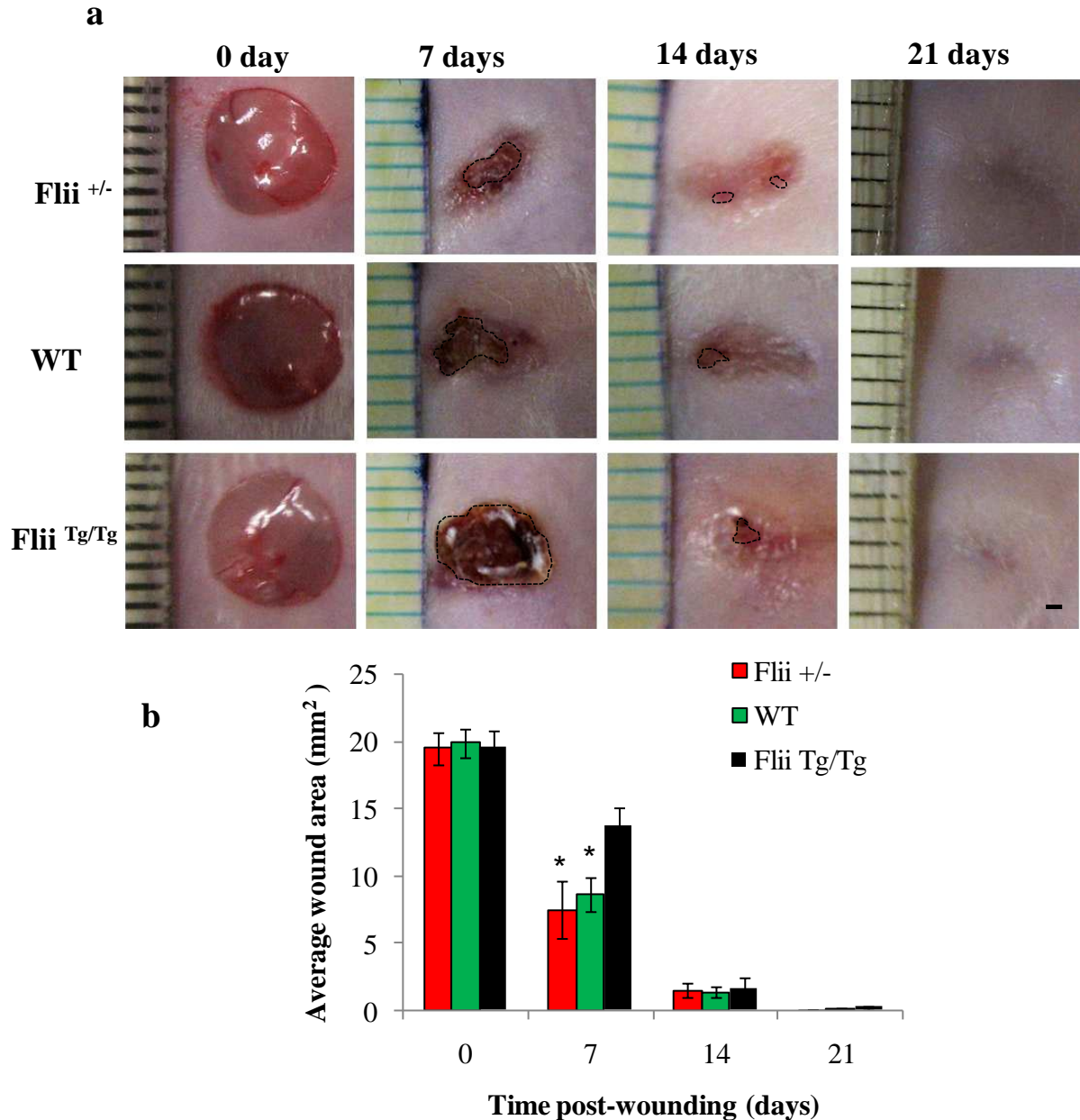
(a) Representative (day 7) micrographs showing cellular migration upon *in vitro* incubation of skin explants from diabetic (n=4) and non-diabetic mice (n=4). Cellular outgrowth was higher under (b) non-diabetic conditions than (c) diabetic ( $p = 0.02$ ; day 6; WT diabetic vs. WT non-diabetic). Cellular outgrowth was significantly slower in (a) non-diabetic Flii<sup>Tg/Tg</sup> cells compared to non-diabetic Flii<sup>+/-</sup> and WT fibroblasts on days 5, 6, 7 (three asterisks,  $p \leq 0.001$ ; Flii<sup>Tg/Tg</sup> vs. Flii<sup>+/-</sup>). The worst phenotype was recorded in (b) diabetic Flii<sup>Tg/Tg</sup> cells, with the cellular outgrowth being the least efficient. (c) Flii<sup>Tg/Tg</sup> diabetic cells migrated significantly slower than their Flii<sup>+/-</sup> counterparts on days 5, 6 and 7 post-culture, suggesting that Flii gene has a negative effect on cellular growth and migration (three asterisks,  $p \leq 0.001$ ; Flii<sup>Tg/Tg</sup> vs. Flii<sup>+/-</sup>). Scale bar = 100  $\mu\text{m}$ . All values represent means and s.e.m. \*\*\* $p \leq 0.001$



**Figure 3.4. Flii overexpression impairs healing in mouse excisional wounds.** Two full-thickness 6mm circular excisions were made on the dorsal skin in mice expressing low (Flii<sup>+/-</sup>), normal (Flii<sup>+/+</sup>), and high (Flii<sup>Tg/Tg</sup>) levels of Flii. Representative images taken on days 0, 7, 14 and 21 post-wounding are shown in (a). (b) Surface wound area at 0, 7, 14 and 21 days post-wounding in Flii<sup>+/-</sup>, WT and Flii<sup>Tg/Tg</sup> mice. Scale bar 500  $\mu$ m. Results represent mean  $\pm$  S.E.M. (6 mice, 12 wounds; n = 12 for each group). (\*\*b)  $p = 0.012$  WT vs. Flii<sup>Tg/Tg</sup>; (\*\*b)  $p = 0.01$  Flii<sup>+/-</sup> vs. Flii<sup>Tg/Tg</sup>.

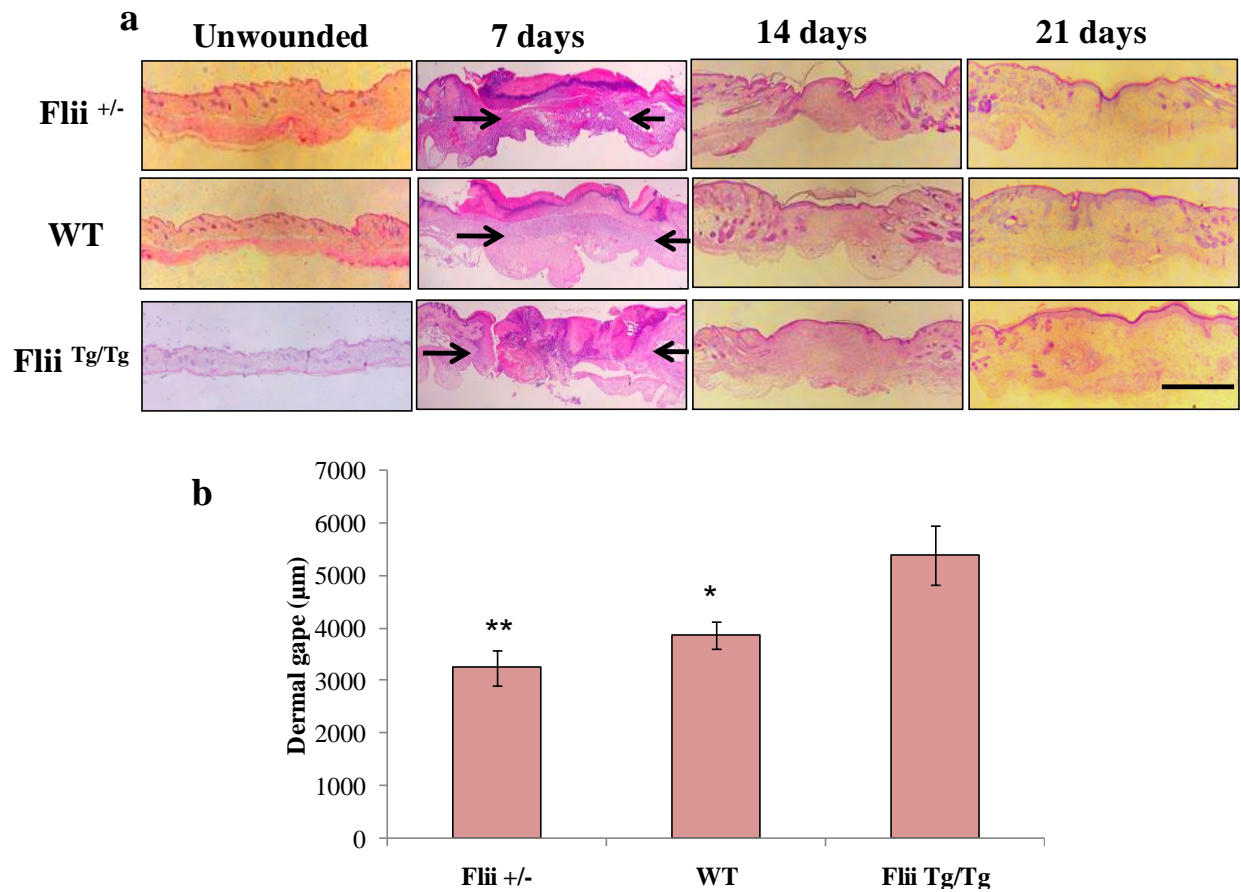


**Figure 3.5 Microscopic analysis of excisional wounds in mice expressing low (Flii<sup>+/-</sup>), normal (Flii<sup>+/+</sup>), and high (Flii<sup>Tg/Tg</sup>) levels of Flii.** (a) Representative haematoxylin and eosin-stained wound sections at 0, 7, 14 and 21 post-wounding. Arrows indicate wound margins. (b) Histological dermal wound gape was determined by measuring the distance between the dermal wound margins on day 7 post-wounding. Scale bar in (a) = 100 µm. Results represent mean ± S.E.M. (6 mice, 12 wounds; n = 12 for each group). (\*b)  $p = 0.052$  WT vs. Flii<sup>Tg/Tg</sup>; (\*b)  $p = 0.05$  Flii<sup>+/-</sup> vs. Flii<sup>Tg/Tg</sup>.

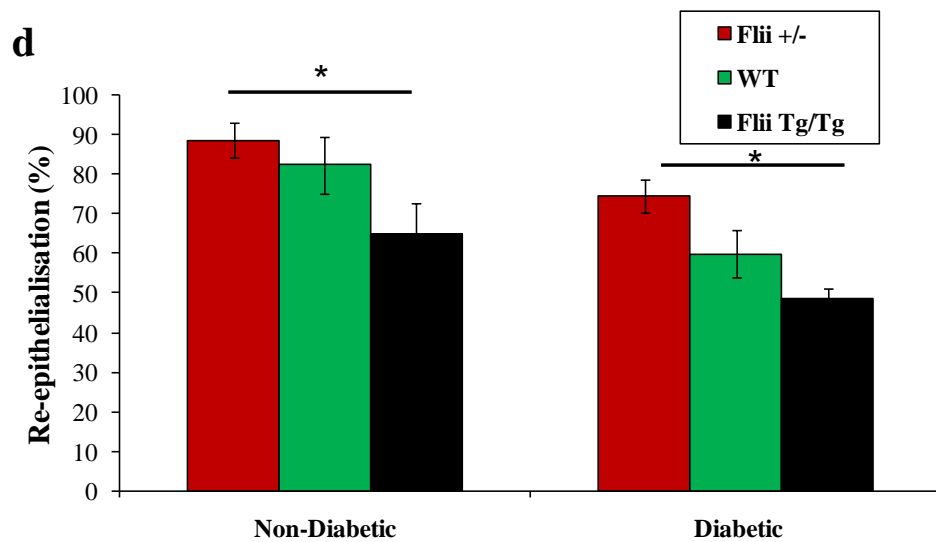
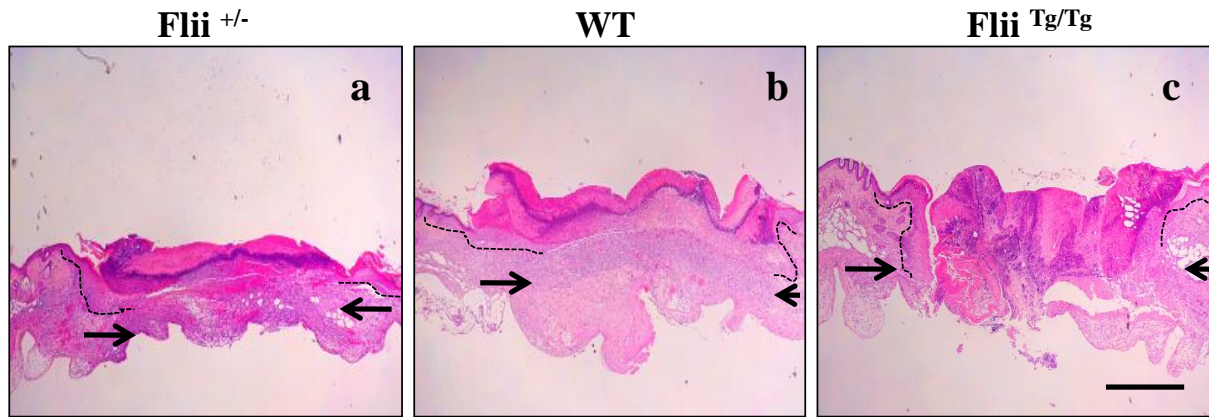


**Figure 3.6. Flii overexpression decreased the rate of wound closure in diabetic mice.** STZ-induced diabetic mice with low (Flii<sup>+/-</sup>), normal (Flii<sup>+/+</sup>), and high (Flii<sup>Tg/Tg</sup>) levels of Flii were wounded and the effect of differential Flii gene expression on average wound area was determined. Representative macroscopic images of diabetic wounds at 0, 7, 14 and 21 days after wounding are shown in (a). The change in resulting wound size was measured using planimetry measurements and showed that Flii-deficient (Flii<sup>+/-</sup>) wounds were significantly smaller at day 7 compared with Flii-overexpressing (Flii<sup>Tg/Tg</sup>) wounds (\**p* = 0.049). Similarly, day 7 Flii<sup>Tg/Tg</sup> wounds were significantly larger than day 7 Flii<sup>+/-</sup> wounds (\**p* = 0.05). Scale bar 500 μm. Results represent mean ± S.E.M. (6 mice, 12 wounds; n = 12 for each group). (\*b) *p* = 0.05 WT vs. Flii<sup>Tg/Tg</sup>, (\*b) *p* = 0.049 Flii<sup>+/-</sup> vs. Flii<sup>Tg/Tg</sup>.

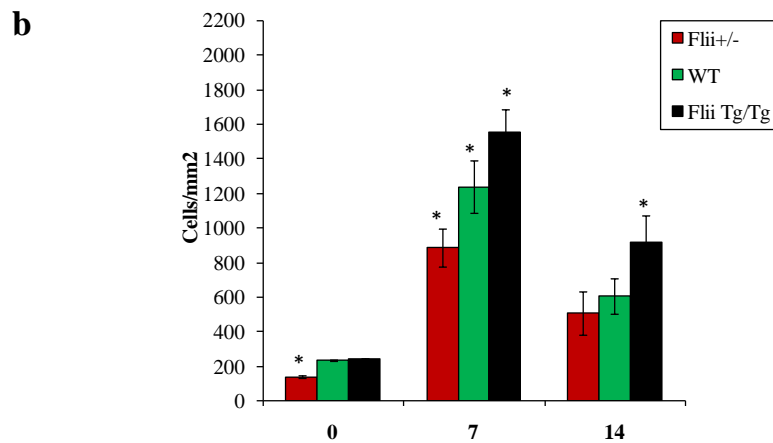
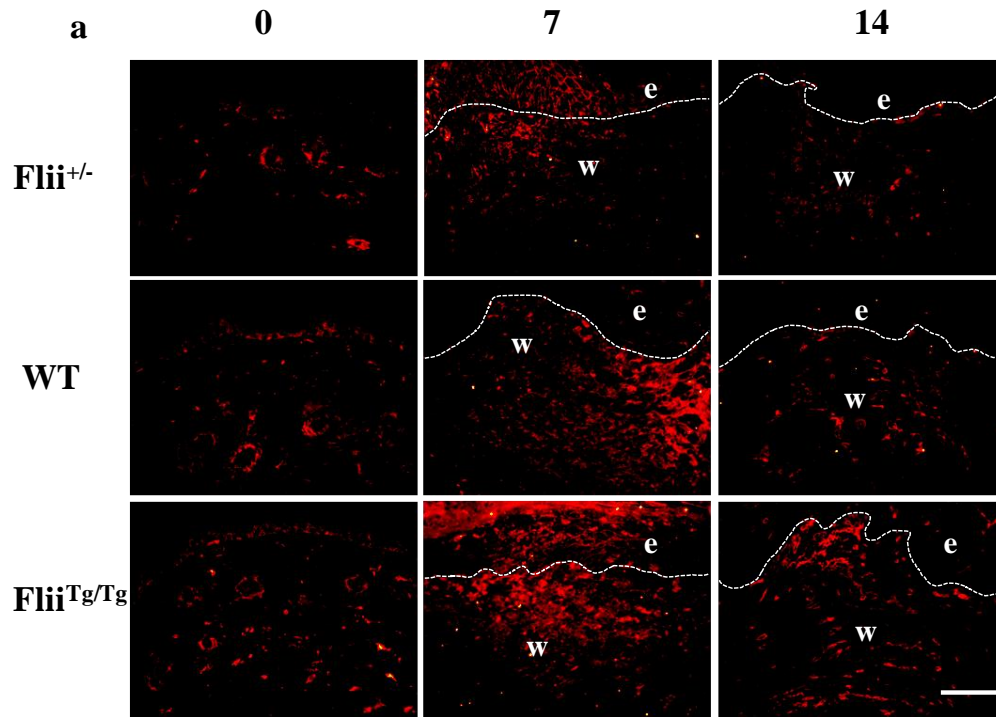




**Figure 3.7. The rate of diabetic wound closure in mice with varying expression levels of Flii.** Representative H&E stained wound sections collected at days 0, 7, 14 and 21 post-wounding from diabetic mice with low (Flii<sup>+/-</sup>), normal (Flii<sup>+/+</sup>), and high (Flii<sup>Tg/Tg</sup>) levels of Flii are shown in (a). Histological analysis of samples at day 7 post-wounding showed significant difference in the total length of wound and dermal gape (b) with Flii-deficient and WT wounds being significantly smaller compared with Flii-overexpressing wounds. Arrows indicate wound margins. (b) Histological dermal wound gape was determined by measuring the distance between the dermal wound margins. Scale bar in (a) = 100 µm. Results represent mean ± S.E.M. (6 mice, 12 wounds; n = 12 for each group). Statistical differences were determined using the Student's t-test. A p value of less than 0.05 was considered significant (\*\*b)  $p = 0.05$  WT vs. Flii<sup>Tg/Tg</sup>; (\*b)  $p = 0.007$  Flii<sup>+/-</sup> vs. Flii<sup>Tg/Tg</sup>.



**Figure 3.8. Flightless deficiency increased rate of wound re-epithelialization in both non-diabetic and diabetic wounds.** Wound re-epithelialization was evaluated by measuring the length of the neopidermis at day 7. Representative haematoxylin and eosin stained 7 day wound sections for (a)  $Flii^{+/-}$ , (b) WT and (c)  $Flii^{Tg/Tg}$  mice are demonstrated. Wound re-epithelialisation was determined by measuring the percentage of the wound that had epidermal covering at day 7. Scale bar in (a-c) = 100  $\mu$ m. Results represent mean  $\pm$  S.E.M. (6 mice, 12 wounds; n = 12 for each group) (\*d)  $p = 0.02$  non-diabetic  $Flii^{+/-}$  vs. non-diabetic  $Flii^{Tg/Tg}$ ; (\*d)  $p = 0.01$  diabetic  $Flii^{+/-}$  vs. diabetic  $Flii^{Tg/Tg}$ ;  $p = 0.03$  non diabetic WT vs. diabetic WT.

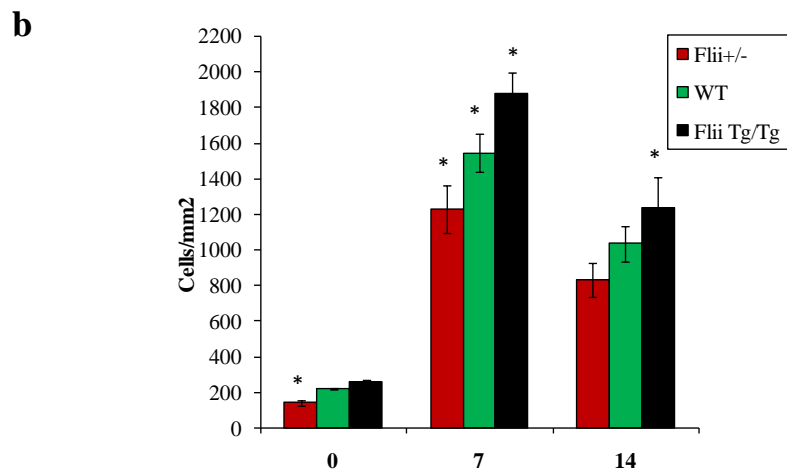
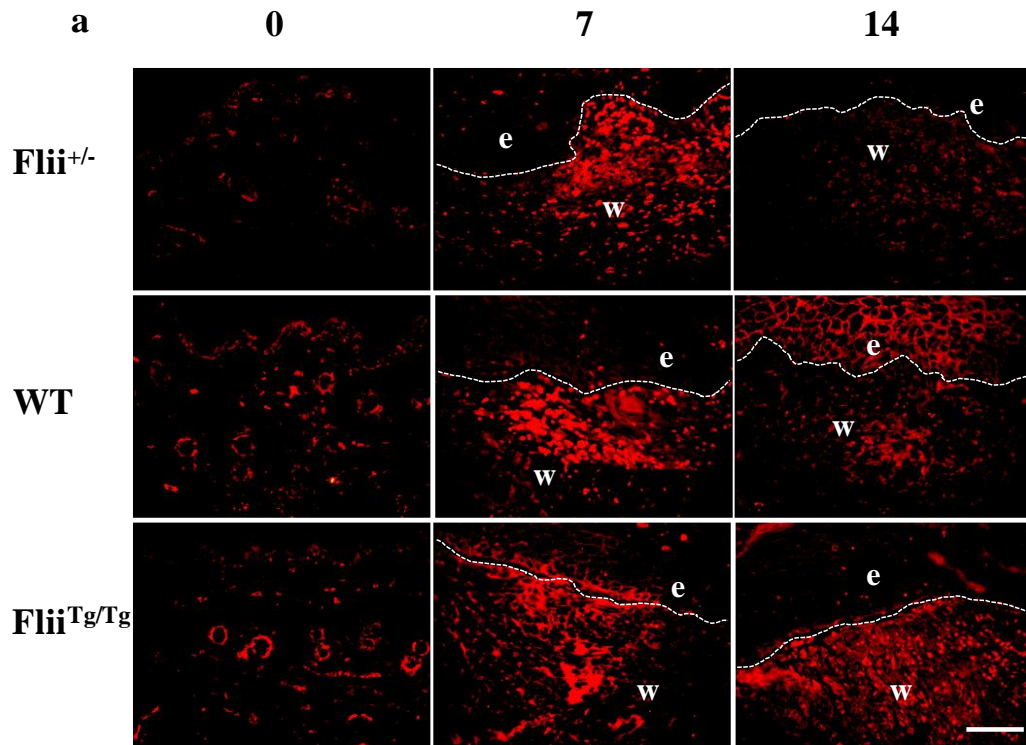


**Figure 3.9. Flii is upregulated in response to wounding.** (a) Immunofluorescence analysis of Flii protein was performed on excisional wounds in Flii<sup>+/-</sup>, WT and Flii<sup>Tg/Tg</sup> mice. Representative images are shown for 0, 7, and 14 day wounds. In all images, e denotes epidermis and w indicates position of wound. Scale bar refers to all and = 50  $\mu$ m. (b) Flii fluorescence intensity was quantified in the dermis with a temporal effect of wounding on Flii expression being observed in all three mouse lines at days 7 and 14 post-wounding ( $p \leq 0.05$  between Flii<sup>+/-</sup> vs. Flii<sup>Tg/Tg</sup> wounds). Results represent mean  $\pm$  S.E.M. (n=6 mice, 12 wounds each group/time-point).

### 3.2.7 Diabetes further increases Flii expression in response to wounding

The effect of wounding on Flii protein expression was determined in diabetic Flii<sup>+/-</sup>, WT and Flii<sup>Tg/Tg</sup> mice, at 7 and 14 days post-wounding (Figure 3.10 and Table 3.1). In unwounded skin, diabetes did not change Flii expression when compared to non-diabetic conditions. Although insignificant, once wounded, diabetes increased Flii protein level in diabetic wounds compared to non-diabetic control wounds at day 7 post-wounding (Figure 3.9 and 3.10). With diabetic Flii<sup>+/-</sup> mice having reduced Flii gene expression (Campbell et al. 2002) and with diabetic Flii<sup>Tg/Tg</sup> having increased gene expression (Thomsen et al. 2011), significant difference in Flii protein expression was observed in unwounded skin of diabetic Flii<sup>+/-</sup> and diabetic Flii<sup>Tg/Tg</sup> mice ( $p \leq 0.001$ ; Day 0 Flii<sup>+/-</sup> wounds vs. day 0 Flii<sup>Tg/Tg</sup> wounds; Figure 3.10b). Immunofluorescence staining of Flii was detected predominantly in cytoplasm of keratinocytes, fibroblasts and the outer root sheath and dermal sheath of hair follicles (Figure 3.10a).

In diabetic mice, wounding upregulated Flii protein expression by 83% in Flii<sup>+/-</sup> mice, by 86% in WT and by 89% in Flii<sup>Tg/Tg</sup> mice ( $p \leq 0.001$ ; day 0 diabetic Flii<sup>+/-</sup> wounds vs. day 7 diabetic Flii<sup>+/-</sup> wounds; Figure 3.10b). Wounding increased Flii expression in all three mice lines peaking at day 7 post-wounding and with the expression reducing by 37.5% in diabetic Flii<sup>Tg/Tg</sup> wounds on day 14 post-wounding. Significantly less Flii staining was observed in diabetic Flii<sup>+/-</sup> wounds at days 7 and 14 post-wounding compared with diabetic Flii<sup>Tg/Tg</sup> wounds ( $p = 0.04$ ; Flii<sup>+/-</sup> vs. Flii<sup>Tg/Tg</sup> at day 7; Figure 3.10b).



**Figure 3.10. Wounding induces Flii up-regulation in diabetic excisional wounds.** (a) Immunohistochemistry was performed on diabetic mouse wounds. Representative images are shown for 0, 7 and 14 day wounds. In all images, e denotes epidermis, w indicates position of wound and white dotted line represents the dermal-epidermal junction. Scale bar refers to all and = 50  $\mu$ m. (a) In unwounded (day 0) skin strong expression of Flii was detected in keratinocytes of the epidermal layer, fibroblasts and cells lining the hair follicle in the dermal layer. (b) Fluorescence intensity quantification in Flii<sup>+/-</sup>, WT and Flii<sup>Tg/Tg</sup> diabetic mouse excisional wounds (\*denotes significance  $p \leq 0.05$  Flii<sup>+/-</sup> vs. Flii<sup>Tg/Tg</sup>). Results represent mean  $\pm$  S.E.M. (n=6 mice, 12 wounds each group/time-point).

	<b>Controls</b>		
<b>Day</b>	<b>0</b>	<b>7</b>	<b>14</b>
Flii <sup>+/-</sup>	134.4±8.7‡	884.6±109.4	507±124.2
WT	231.8±4.5	1234±152.4	602.3±102
Flii <sup>Tg/Tg</sup>	236.9±8.4	1553.6±134.2	913±159.7§

	<b>Diabetic</b>		
<b>Day</b>	<b>0</b>	<b>7</b>	<b>14</b>
Flii <sup>+/-</sup>	137.5±11.7§	1224±134.2	828.4±95.9
WT	218.74±5.8	1543±109	1032.2±97
Flii <sup>Tg/Tg</sup>	253.2±13.8	1967±121.2	1234±170

Values are mean ±S.E.M.; NS = not significant

\* $p \leq 0.05$  different to WT in the same group (T-test)

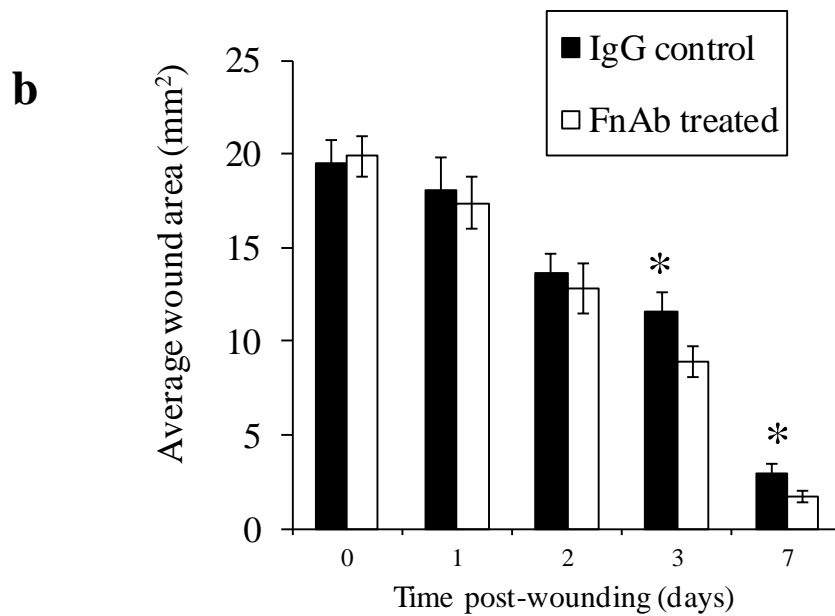
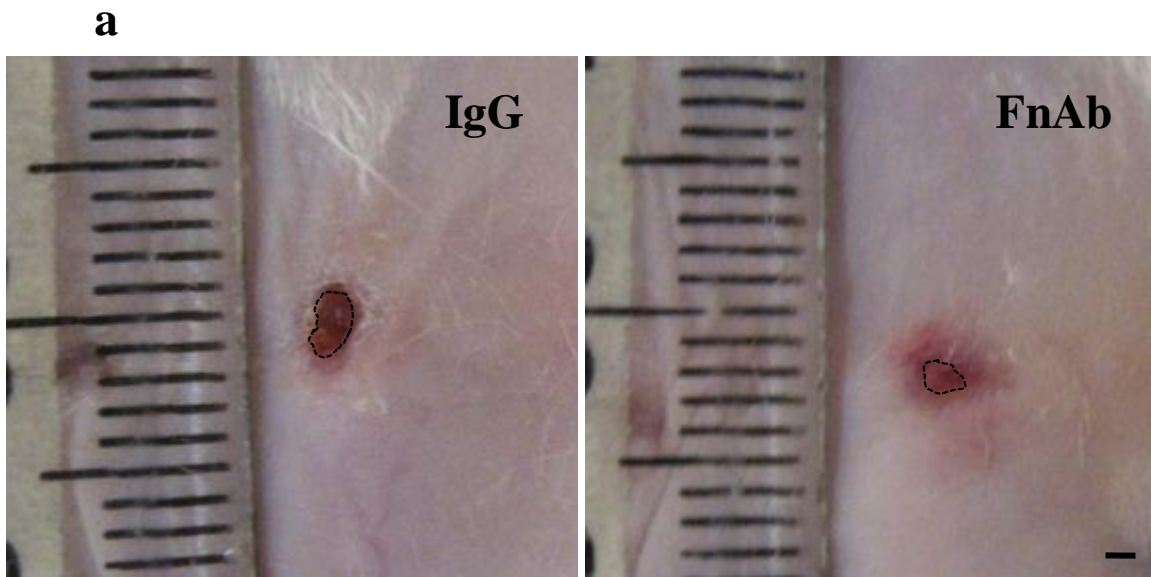
§  $p \leq 0.01$  different to WT in the same group (T-test)

‡  $p \leq 0.001$  different to WT in the same group (T-test)

**Table 3.1. Comparison of Flii protein expression in mouse non-diabetic control and diabetic wounds at 0, 7 and 14 days post-wounding.**

### **3.2.8 Accelerated diabetic wound closure is induced by FnAb *in vivo***

Flii is accumulated in high levels as a result of wounding. Flii is upregulated in response to wounding in non-diabetic (Figures 3.9) and diabetic wounds (Figure 3.10) suggesting Flii may contribute to the impaired healing observed in chronic diabetic wounds by adversely affecting cellular migration, proliferation and cytokine synthesis. We addressed the negative contribution of extracellular Flii to the process of diabetic wound healing by reducing exogenous expression of Flii protein using neutralising antibodies. FnAbs were injected intradermally and marked differences were recorded in the macroscopic appearance of healing wounds at 3 and 7 days post-wounding (Figure 3.11a, b). By the end of the first week post-wounding the control wounds were still covered with a non-viable necrotic material which included the clot (Figure 3.11a). In contrast, the appearance of wounds treated with FnAb was markedly improved than that seen in control wounds, i.e. faster clearance of non-viable tissue and rapid appearance of granulation tissue, which was promptly replaced by shiny epidermal layer (Figure 3.11a). Intradermal administration of FnAb (200  $\mu$ l of 50 $\mu$ g/ml injected at day 1, 2 and 3 post-wounding), but not control antibodies (irrelevant affinity purified IgG; 200  $\mu$ l of 50 $\mu$ g/ml injected at day 1, 2 and 3 post-wounding), resulted in a 1.9-fold decrease in wound healing (Figure 3.11b). Treated mice had significantly smaller wound areas on days 3 and 7 post-wounding compared to IgG-treated controls ( $p \leq 0.05$ ; IgG vs. FnAb).



**Figure 3.11. Positive effect of FnAb on diabetic wound healing.** (a) Images of punch wounds (6mm) treated IgG or FnAb on day 7 (n=6; two wounds per mouse; 12 wounds). Dotted line delineates the wound edge. Scale bar = 1 mm. (b) Quantification of wound surface area. Treatment with FnAb had a positive effect on diabetic wound closure *in vivo*. In wound assays, injection of FnAb accelerated wound closure and reduced the size of diabetic wound compared to IgG control on days 3 and 7 post-wounding. One asterisk,  $p \leq 0.05$ . All values represent means and s.e.m.



### **3.2.9 Microscopic appearance of healing wounds**

Wounds from diabetic WT mice treated with FnAb and IgG were harvested on day 7 post-wounding and stained with H&E and microscopic measurements of the average wound length and gape were made as described in Materials and Methods. Representative images are shown in Figure 3.12a. Microscopic analysis of wound biopsies showed that intradermal injection of FnAb accelerated wound closure compared to their IgG-treated counterparts. FnAb-treated wounds had significantly smaller wound length and gape than IgG-treated control wounds on day 7 after wounding (Figure 3.12b and c;  $p \leq 0.05$ , IgG vs. FnAb; day 7 post-wounding). At day 7 IgG control wounds remained open and were characterized by an exaggerated inflammatory response with a higher infiltration of inflammatory cells in the subepidermal region.

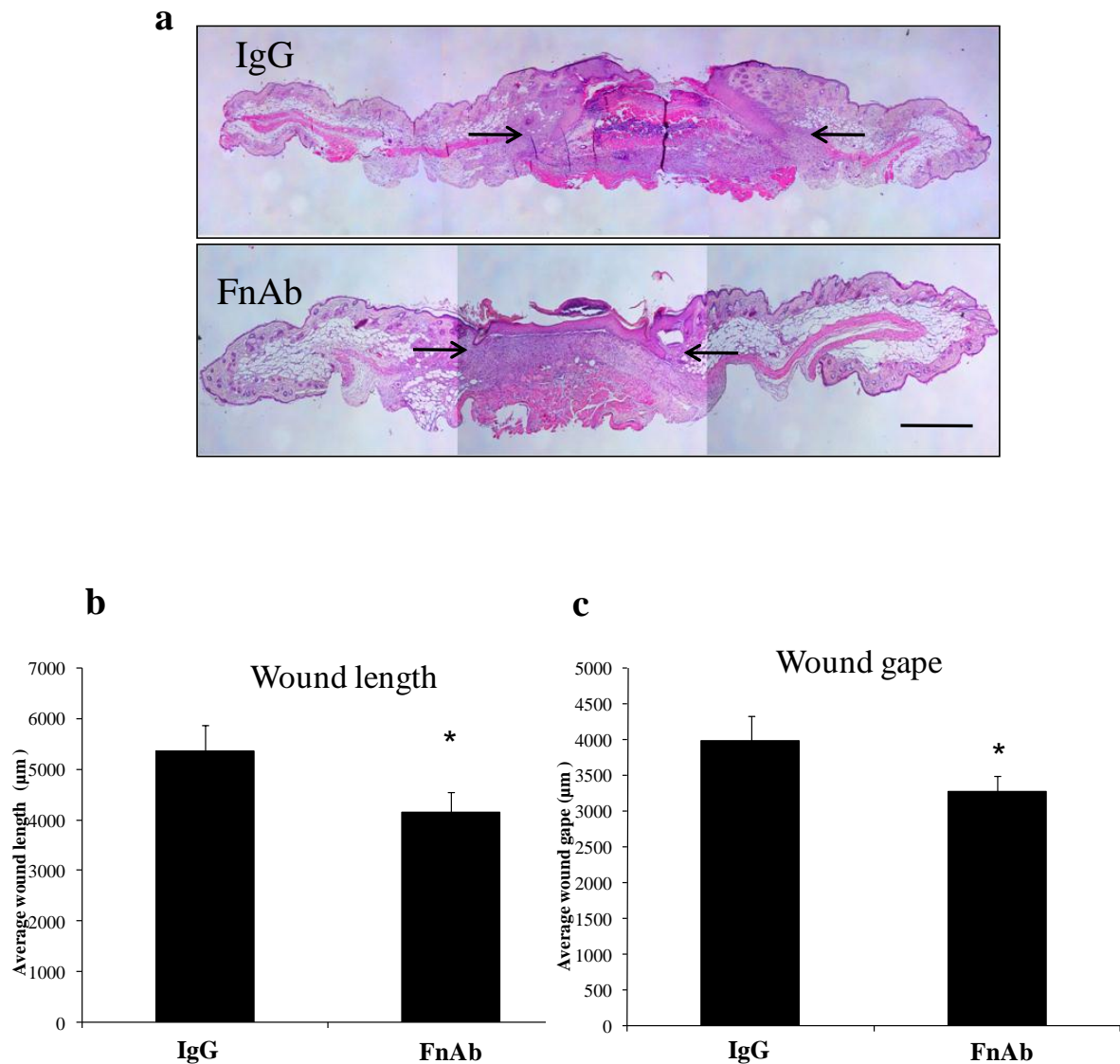
### **3.2.10 Altered macrophage recruitment contributes to pathogenesis of diabetic wounds**

Having established the role of Flii as a negative regulator of wound healing, we speculated that Flii over expression might promote infiltration of macrophages and increase the inflammatory response in diabetic and non-diabetic wounds. Abundant inflammatory infiltrate was previously reported in days 3 and 7 Flii<sup>Tg/Tg</sup> mouse incisional wounds compared to WT (Adams et al. 2008). The inflammatory cell invasion was investigated using F4/80 staining to mark the macrophages in day 7 diabetic (Flii<sup>+/-</sup>, WT, Flii<sup>Tg/Tg</sup>) and day 7 non-diabetic (Flii<sup>+/-</sup>, WT, Flii<sup>Tg/Tg</sup>) wounds. The results of these immunohistochemistry experiments did not show significant difference between mice with low and high expression of Flii (Figure 13a, b). However, a substantial effect of

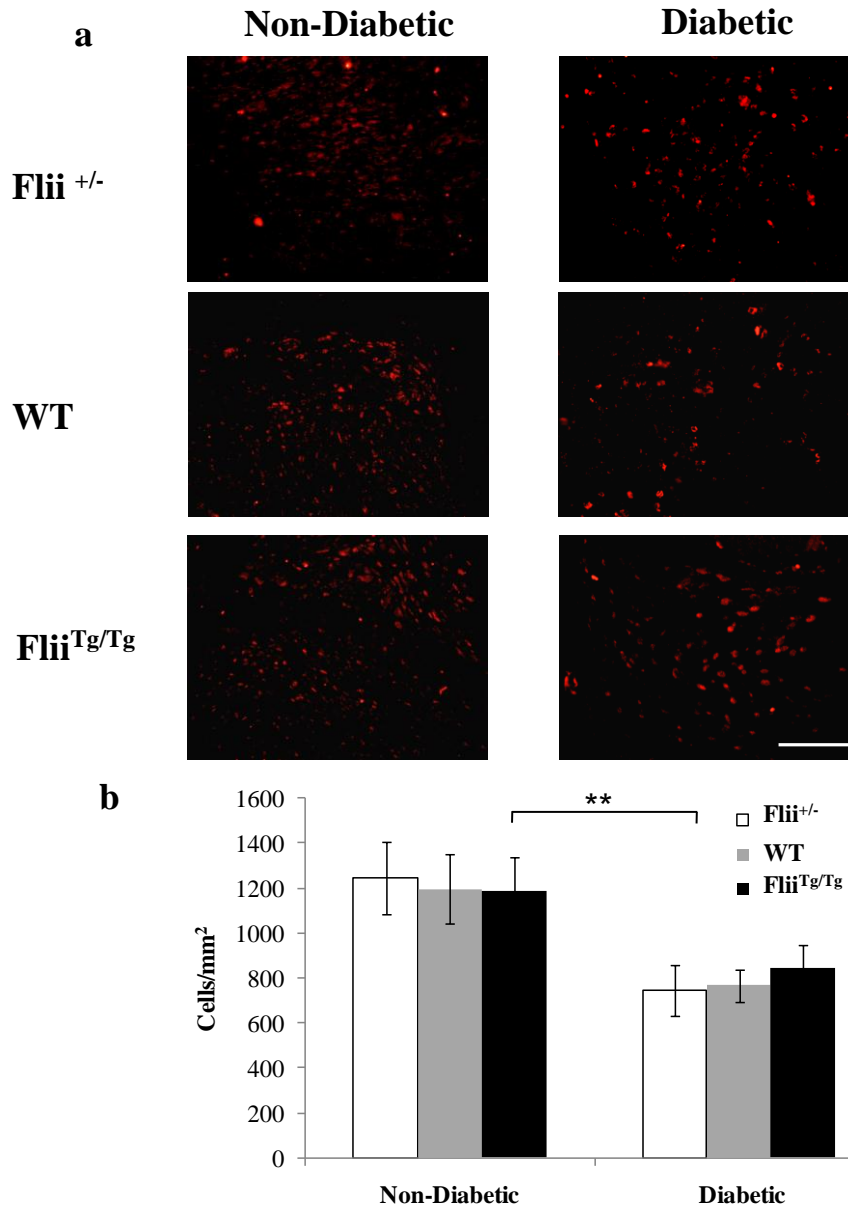
diabetes on macrophage infiltration was seen in diabetic compared to non-diabetic wounds. In punch wounds diabetes resulted in up to 40% decrease of the macrophage infiltration.

### **3.2.11 Reduced Flii induces neutrophil infiltration**

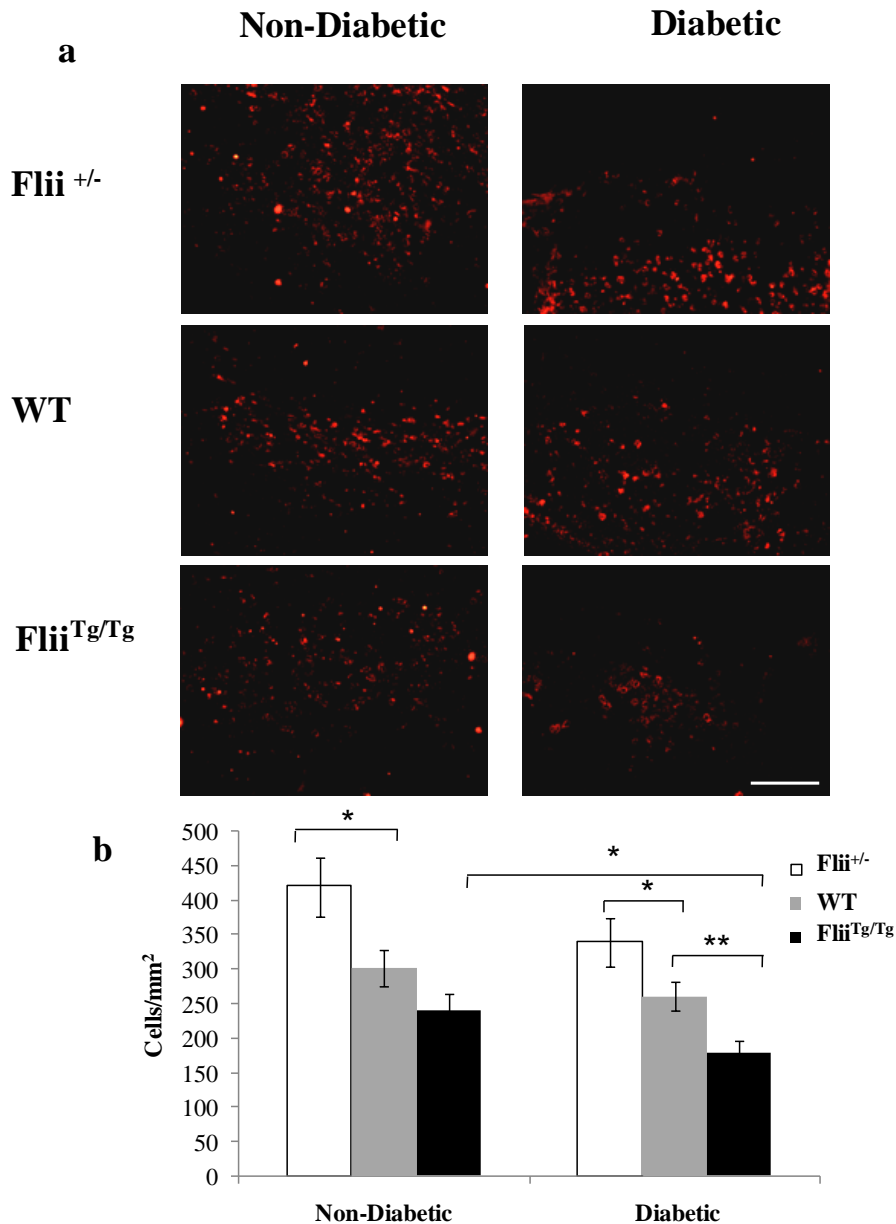
To determine whether Flii expression level has an effect on neutrophil profile, we assessed the presence of NIMP-R14-positive neutrophils in punch wounds (Figure 3.14). Neutrophils represent the very first immune cells, infiltrating within minutes into the area of injury (Martin 1997). With respect to our previously published data, which indicated that Flii over expressing wounds showed higher inflammatory cell score (Adams et al. 2008), we hypothesized that increased levels of Flii protein might be associated with higher neutrophil wound infiltrate. Paradoxically, higher Flii expression in wounds significantly reduced the magnitude of neutrophil infiltration into both diabetic and non-diabetic healing wounds (Figure 3.14a and b). Overall, the number of NIMP-R14-positive cells was significantly higher in Flii<sup>+/-</sup> than in WT and Flii<sup>Tg/Tg</sup> wounds (Figure 3.14b; non-diabetic Flii<sup>+/-</sup> vs. non-diabetic WT; day 7;  $p \leq 0.05$ ). Examination of non-diabetic Flii<sup>+/-</sup>, WT and Flii<sup>Tg/Tg</sup> control wounds revealed low Flii expression was associated with higher neutrophil infiltration (Figure 3.14a). Although lower than non-diabetic, Flii<sup>+/-</sup> diabetic wounds also showed a significantly higher neutrophil profile than their WT and Flii<sup>Tg/Tg</sup> counterparts (Figure 3.14b; diabetic WT vs. diabetic Flii<sup>Tg/Tg</sup>;  $p \leq 0.01$ ). The fact that day 7 diabetic wounds expressed lower levels of NIMP-R14 than non-diabetic controls suggests that leukocyte infiltration is impaired in these mice (non-diabetic Flii<sup>Tg/Tg</sup> vs. diabetic Flii<sup>Tg/Tg</sup>;  $p \leq 0.05$ ).



**Figure 3.12. Microscopic analysis of diabetic wounds treated with IgG and FnAb.** (a) Representative paraffin sections of 7 day wounds stained with H&E (n=6; 12 wounds per group). At least 2 histological sections from identical sites of day 7 wounds were studied to determine two important parameters and indicators of rate of wound repair: (b) wound length and (c) wound gape. Wound length was determined by manually drawing below the epidermis or clot between the wound margins. Dermal gape was recorded as the distance or a straight line between the dermal wound margins. Both parameters i.e. average wound length and gape were significantly smaller in FnAb-treated diabetic wounds than IgG-treated controls ( $p \leq 0.05$ , IgG vs. FnAb; day 7 post-wounding). Scale bar in (a) = 100 µm. One asterisk,  $p \leq 0.05$ . All values represent means and s.e.m.



**Figure 3.13. F4/80, a marker of macrophages, is reduced in day 7 diabetic wounds.** (a) Sections of wounds (n=6) harvested 7 days post-wounding were immunostained for F4/80 and the number of positively stained cells was recorded. (b) We observed no effect of Flii expression levels on macrophage profile in both diabetic and non-diabetic wounds. By contrast, diabetic (Flii<sup>+/-</sup>, WT, Flii<sup>Tg/Tg</sup>) wounds showed a marked reduction in immunostaining for F4/80-positive macrophages than the non-diabetic control wounds (diabetic Flii<sup>+/-</sup>, WT, Flii<sup>Tg/Tg</sup> vs. non-diabetic Flii<sup>+/-</sup>, WT, Flii<sup>Tg/Tg</sup>; two asterisks,  $p \leq 0.01$ ). Scale bar in (a) = 100  $\mu$ m. All values represent means and s.e.m.



**Figure 3.14. Infiltration of neutrophils (NIMP-R14 immunostaining) in diabetic and non-diabetic day 7 wounds.** (a) Representative NIMP-R14 staining in granulation tissue of Flii<sup>+/-</sup>, WT and Flii<sup>Tg/Tg</sup> diabetic and non-diabetic punch wounds (n=6, 12 wounds per group). Diabetes suppresses neutrophil infiltration (non-diabetic Flii<sup>Tg/Tg</sup> vs. diabetic Flii<sup>Tg/Tg</sup>;  $p \leq 0.05$ ). (b) Both diabetic and non-diabetic mouse wounds with low expression of Flii (Flii<sup>+/-</sup>) showed significantly higher neutrophil profile, than those with higher expression of Flii i.e. WT and Flii<sup>Tg/Tg</sup> (non-diabetic Flii<sup>+/-</sup> vs. non-diabetic WT;  $p \leq 0.05$  and diabetic Flii<sup>+/-</sup> vs. diabetic WT;  $p \leq 0.05$ ). Scale bar in (a) = 100  $\mu$ m. All values represent means and s.e.m.

### 3.3 Discussion

In humans diabetes causes impaired wound repair leading to human suffering and financial burden to the healthcare system. Epidemiological studies indicate that the prevalence of diabetes is predicted to escalate in the future (Scully 2012) meaning the problem of impaired healing will only worsen unless improved therapies for diabetic ulcers are found. A growing body of evidence suggests that Flightless I protein is of significant importance in the cutaneous wound healing process and decreasing Flii activity in incisional and burn wounds improves healing, whereas increasing Flii impairs wound repair (Adams et al. 2009).

No studies to date have examined the effect of differential levels of Flightless in diabetes. Given the role of Flii as a negative regulator of wound healing, we embarked on our studies seeking to elucidate whether augmented Flii gene expression has a similar effect on diabetic wound healing as it does on incisional and partial-thickness burn injury repair. Diabetes was induced by STZ, confirmed by measuring blood glucose levels and subsequently, excisional wounds were created on mice with low Flii <sup>+/-</sup>, normal (WT) and high (Flii <sup>Tg/Tg</sup>) expression of Flii gene. As predicted, diabetic wounds appeared larger in size and healed slower than non-diabetic control wounds. This suggests that STZ-induced murine model of diabetes is an efficient method of studying impaired wound healing and 6 weeks duration of diabetes is sufficient to develop complications such as deficient wound repair. It has been well-known for many years that the duration of diabetes and level of fasting diabetic hyperglycaemia are reliable predictors of the likelihood of the development of direct complications of diabetes. Over the five year study period the Diabetes Control and Complications Trial demonstrated that in subjects with lower and tighter fasting

glucose levels showed a 60% reduction in the risk of development of complications over the five year study period. Whilst the onset of diabetes related complications may vary from a patient to patient and the requirements for individual treatment are often “tailor made” (Alberti & Zimmet 1998), several studies have identified that people with fasting plasma glucose concentrations of  $7.0 \text{ mmol l}^{-1}$  (McCance et al. 1994) and over are at risk for development of microvascular and macrovascular complications (Charles et al. 1996). Since individuals with Type 2 diabetes are frequently undiagnosed for many years (Mooy et al. 1995), because of the mild symptoms (Alberti & Zimmet 1998), many of them show signs of macrovascular and microvascular complications at the time of medical diagnosis. However, our results indicate that a subset of diabetic mice left for 6 weeks (i.e. time between the induction of diabetes and creation of wounds) have successfully developed a delayed wound healing phenotype.

Our studies revealed that the inhibitory effect of Flii on wound repair, at least in part, is mediated by its anti-proliferative property down-regulating cellular migration. In a cellular migration assay, increased Flii gene expression reduced proliferation and migration under both diabetic and non-diabetic conditions, whereas cells originating from mice heterozygous for the Flii gene knockout (cultured in high glucose media) displayed more than twofold increase in cellular growth and migration. The negative effect of Flii on cellular migration could be mediated by its negative effect on focal adhesion turnover and cell spreading (Kopecki, O'Neill, et al. 2011b). Focal adhesions function as cellular adhesion sites and touch receptors, the latter two being responsible for initiating cellular migration and locomotion (Bershadsky et al. 2006).

Reduced Flii significantly improved diabetic wound healing and accelerated non-diabetic and diabetic wound closure as determined by planimetric analysis of surface wound area. Improved wound outcomes in wounds with reduced Flii expression were confirmed at a microscopic level

where rate of re-epithelialisation was higher in the mice with reduced Flii gene expression (Flii<sup>+/-</sup>). Whilst partial deletion of Flii resulted in a significant decrease in dermal wound gape and rate of re-epithelialisation compared to Flii overexpressing mice, the present study demonstrated that there was a small, although not statistically significant histological difference in wound closure between WT and Flii<sup>+/-</sup> mice. This observation was made in both the STZ-diabetic and non-diabetic control mice. With global depletion of Flii resulting in embryonic lethality, Flii heterozygous mice (Flii<sup>+/-</sup>) still carry one copy of Flii gene. Partial preservation of Flii gene activity may provide an explanation for the lack of significant difference of wound size between Flii<sup>+/-</sup> and their WT counterparts. Given that rapid epithelialisation and closure of disrupted dermal matrix are key determinants of successful wound healing our results suggest that modulation of Flii may prove useful in the treatment of diabetic wounds and reduction of local levels of Flii may exert a positive effect on re-epithelialisation.

Another finding of this study is that genetic overexpression of Flii reduces the rate of re-epithelialisation under both diabetic and non-diabetic conditions. Flii overexpressing mice (Flii<sup>Tg/Tg</sup>) showed significant delays in wound healing in response to hyperglycaemia and had the most severely dysfunctional wound healing phenotype. Additionally, our studies revealed that Flii levels are further upregulated in diabetic mice, with the level of this protein being consistently higher on 7 and 14 days post wounding under hyperglycaemic conditions when compared to normal non-diabetic environment. Healing of Flii overexpressing (Flii<sup>Tg/Tg</sup>) diabetic wounds was significantly impaired with wounds gaping wider, healing slower and having significantly delayed re-epithelialisation, indicating that not only is Flii a negative regulator of wound repair but also that delayed healing in the diabetic wound may be partly exacerbated by the increased expression on this cytoskeletal protein. In support of this finding, our previous



studies have revealed overexpression of Flii has inhibiting effects on migration, cellular proliferation and fibroblast adhesion (Adams et al. 2008; Kopecki, O'Neill, et al. 2011b). Additionally, increased TGF- $\beta$ 1 gene expression was observed in Flii-overexpressing acute incisional wounds (Cowin, AJ et al. 2007), which may contribute to chronicity and prolonged inflammatory response in chronic wounds (Ashcroft et al. 2000).

In the present study, using a diabetic model of wound healing we found that Flii was upregulated in response to wounding in both diabetic and non-diabetic animals. Flii was upregulated in the initial phase of healing of diabetic wounds and may have contributed to the impairment in the wound repair process. Initially high levels of Flii were seen at day 7 post-wounding followed by significantly diminished levels detected at later time points. This result is similar to what was observed in our previously published *in vitro* and *in vivo* studies, where significant changes in the levels of Flii were induced by wounding (Adams et al. 2009; Cowin, AJ et al. 2007). Our *in vivo* data demonstrated that the expression of Flii in response to wounding follows a “bell curve” shape where diabetic wounds expressed initially high levels of Flii peaking at day 7 and later returned to normal levels found in unwounded skin.

We previously showed that Flii is secreted *in vitro* (Cowin, AJ et al. 2007). We now show that human plasma, acute and chronic wound fluids contain secreted Flii protein (Chapter 4), which exits the cell via late endosome/lysosome trafficking pathway (Chapter 5). Having established three important facts (i) reduced Flii gene expression enhances diabetic wound closure, (ii) Flii is up regulated in response to wounding, (iii) Flii is secreted, our next step was to progress to the next stage – neutralization of extracellular Flii activity.

Exogenous application of neutralising antibodies is an effective method of reducing local protein expression (Shah, Foreman & Ferguson 1995). In this proof-of-concept study we show that diabetic wounds positively respond to treatment with Flii specific monoclonal antibodies (FnAb). Diabetic wounds treated with FnAb show accelerated wound closure, which may be promoted by the positive effect of FnAb on proliferation. Dose dependent effects of neutralising antibodies to Flii (FnAb) were demonstrated previously (Cowin, AJ et al. 2007), where addition of FnAb *in vitro* increased fibroblast and keratinocyte proliferation. We examined the extent to which FnAbs will stimulate wound healing in diabetic wounds. It was revealed that FnAb-treated diabetic wounds developed an overtly superior wound healing phenotype compared to IgG-treated controls. The difference was associated with a 1.9-fold decrease in average wound size in day 3 and day 7 WT-FnAb treated diabetic wounds. Similar effects were reported in previous studies where FnAbs increased the rate of incisional (Cowin, AJ et al. 2007), excisional (Jackson et al. 2012) and burn wound repair (Adams et al. 2009).

Inflammation is an important component of the normal wound healing process and occurs even in the absence of infection (Ashcroft et al. 2012). The cascade of events that occurs immediately after injury begins with clotting and the recruitment of inflammatory cells including neutrophils and macrophages (Koh & DiPietro 2011). Within the first few days after injury (1-2 days), circulating monocytes are recruited into the wound site where they differentiate into macrophages (Koh & DiPietro 2011). Macrophages influence wound healing through the generation of growth factors that promote cell proliferation (Maruyama et al. 2007) and positively influence angiogenesis by the production of pro-angiogenic VEGF (Koh & DiPietro 2011). In this regard, our study showed that diabetic wounds recruited significantly lower number of macrophages, than non-diabetic wounds, which is consistent with previous reports

(Tian, H et al. 2011). Diabetic hyperglycaemic suppresses macrophage infiltration and denies diabetic wounds of the pro-healing functions of macrophages, including promotion of endothelial cell migration and vasculature formation (Tian, H et al. 2011).

Our studies have demonstrated that macrophage infiltration in diabetic wounds was significantly halted. Compared with non-diabetic wounds, diabetic wounds expressed reduced levels of F4/80-positive macrophages (Figure 3.12). Given that macrophage transplantation into wounds promotes healing (Maruyama et al. 2007), whereas macrophage depletion results in delayed healing (Lucas et al. 2010), it is reasonable to hypothesize that diabetic impairment of macrophage infiltration, at least in part, contributes to delayed wound closure seen in diabetic mice. Of note, genetic modulation of *Flii* doesn't alter the number of infiltrating macrophages at day 7 post-wounding, suggesting that *Flii* may not be involved in altering macrophage profile in diabetic wound healing.

One important function of wound macrophages is the capacity to facilitate the phagocytic removal of neutrophils (Koh & DiPietro 2011). Neutrophils arrive at the wound site within minutes of injury and are involved in decontamination and clearing the microbes, but emerging evidence suggests that neutrophils also secrete pro-inflammatory cytokines that activate local fibroblasts and keratinocytes (Martin 1997).

Our studies showed that neutrophil profile was increased in *Flii*-deficient wounds compared to WT and *Flii*-over expressing wounds (Figure 3.13). This data suggest a reparative role of *Flii*-deficiency via action on neutrophils - a source of pro-inflammatory cytokines driving re-epithelialisation and extracellular matrix production. Non-diabetic wounds have slightly higher neutrophil profile than their non-diabetic counterparts. WT non-diabetic wounds have similar

neutrophil profiles as the Flii-deficient diabetic wounds. It is possible that in the early stages of wound healing Flii-deficiency has the capacity to redeem negative effects of diabetes on neutrophil profile and counteract such impairment by increasing the number of neutrophils recruited to the wound site.

An intact immune system and an appropriate inflammatory response during wound healing is important for generation of soluble mediators that modulate and activate proliferation and extracellular matrix production of skin fibroblasts (Ashcroft et al. 2012). Our transgenic mouse studies suggest that Flii may have a potential to alter inflammation during wound healing, since Flii deficient wounds showed significantly higher NIMP-R14 -positive neutrophil profile. The initial burst of inflammation is necessary for normal wound healing and reduced levels of Flii in wounds at day 7 have proven to be beneficial, because Flii-deficiency could be promoting inflammation and recruitment of neutrophils to diabetic wounds. It must be noted that, although initial outburst of inflammation is necessary for effective wound closure, a prolonged inflammatory response, on the other hand, is a significant contributing factor to impaired wound healing. The responsiveness of neutrophils to Flii is poorly understood and should be investigated in future studies.

In summary, the current study provided evidence that reduced activity of the actin-remodelling protein Flii is beneficial in diabetic wound healing. Healing is severely impaired in diabetic Flii overexpressing wounds suggesting that by reducing the inhibitory effect of Flii on cell proliferation and migration, wounds heal faster. Therefore we hypothesised that localised treatments aimed at reducing Flii expression in diabetic wounds will improve healing. Indeed, we showed the beneficial effect of exogenous addition of FnAb on diabetic wound repair. Armed

with this knowledge we are now in a strong position to test this new treatment in large animal models of diabetes.

## *Chapter 4*

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# NEUTRALIZING FLIGHTLESS I IN CHRONIC WOUND FLUID IMPROVES CELLULAR PROLIFERATION

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Results included in this chapter were published in the *European Journal of Dermatology*.

**Title:** Cytoskeletal protein Flightless (Flii) is elevated in chronic and acute human wounds and wound fluid: neutralizing its activity in chronic but not acute wound fluid improves cellular proliferation.

**Authors:** Nadira Ruzehaji, Randall Grose, Doreen Krumbiegel, Heddy Zola, Pallave Dasari, Hilary Wallace, Michael Stacey, Robert Fitridge, Allison J. Cowin

Manuscript of the article is in Appendix 3

# NEUTRALIZING FLIGHTLESS I ACTIVITY IN CHRONIC WOUND FLUID IMPROVES CELLULAR PROLIFERATION

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## 4.1 Introduction

Chronic wounds are an area of growing concern within the health care community (Boulton et al. 2005). The sharp rise of the aged population within the community is leading to an increase in the numbers of chronic, non-healing wounds (Cameron, AJ et al. 2003). Understanding the processes involved in impaired healing will help the development of new approaches to address these hard to heal wounds. Acute wounds heal in an efficient manner involving dynamic interaction between various cells such as fibroblasts, keratinocytes and immune cells. Apart from numerous cellular interactions, acute wound healing is characterized by dynamic reciprocity of extracellular matrix (ECM), cytokines, chemokines, growth factors, inhibitors and their receptors (Macri & Clark 2009; Schultz & Wysocki 2009). Chronic wounds, on the other hand, fail to proceed through the normal stages of healing and become arrested in a pathological inflammatory state subsequently resulting in delayed healing with poor anatomical and functional outcomes (Menke, NB et al. 2007). High levels of protease activity in chronic wounds lead to degradation and imbalance of proteins including growth factors, cytokines and extracellular matrix proteins, (Liu et al. 2009) causing the establishment and maintenance of wound chronicity (Schultz & Wysocki 2009). Biochemical analysis of acute and chronic wound fluids provide important clues about molecular profiles of healing and non-healing wounds (Tarnuzzer & Schultz 1996). Wound fluid from acute wounds has a positive effect on wound healing, whereas chronic wound fluid has an adverse effect on healing inhibiting cellular proliferation (Schultz et al. 2003).

The actin cytoskeleton is an essential network of filaments found in all cells; it is dynamic and remodelled in response to a variety of stimuli to generate the mechanical forces necessary for



changes in cell contraction and motility that underpin tissue repair (Kopecki & Cowin 2008). Actin-remodelling proteins of the gelsolin family (Lee, WM & Galbraith 1992; Sun et al. 1999), are instrumental in reorganizing the actin cytoskeleton, which is the first step in enabling cells to reorientate their cytoskeleton to drive changes in motility, adhesion and contraction (Kopecki et al. 2009). Cytoskeletal protein Flightless I (Flii) is the most evolutionarily conserved member of the gelsolin family of actin-binding proteins (Claudianos & Campbell 1995). Apart from its intracellular actin-remodelling role (Kopecki & Cowin 2008), emerging evidence suggests that Flii has potentially important extracellular functions (Lei et al. 2012). Gelsolin – a member of actin-binding family of proteins was shown to have an important extracellular function (Lee, WM & Galbraith 1992). Gelsolin has the ability to scavenge extracellular actin that is exposed to extracellular spaces or released into the circulation after tissue injury (Lee, WM & Galbraith 1992). The persistence of actin within the circulation can contribute to the pathogenesis of organ injury (Dahl et al. 1999).

In Chapter 3 we established that Flii expression is elevated in human non-healing diabetic wounds. In this chapter we aimed to determine if Flii is involved in the impaired healing associated with chronic venous ulcers. Given that both chronic wound fluid (Schultz et al. 2003) and Flii (Adams et al. 2008) have previously been shown to down-regulate fibroblast proliferation, in this chapter, we aimed to determine whether Flii may contribute to the inhibitory effect of chronic wound fluid. Additionally, we tested the hypothesis that chronic wound fluids depleted of Flii would have the capacity to improve cellular proliferation.

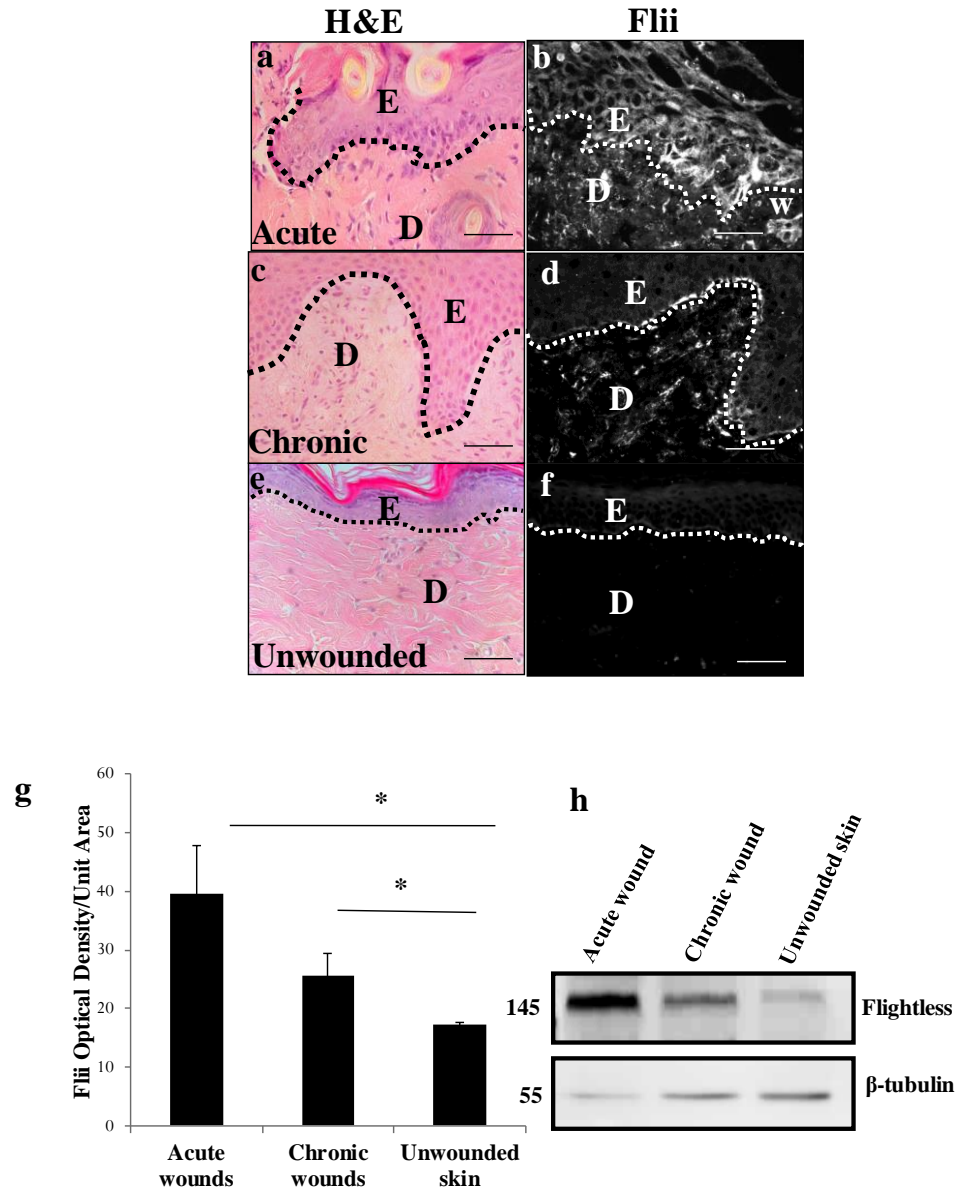
## 4.2 Results

### 4.2.1 Flii expression is up-regulated in chronic and acute human wounds

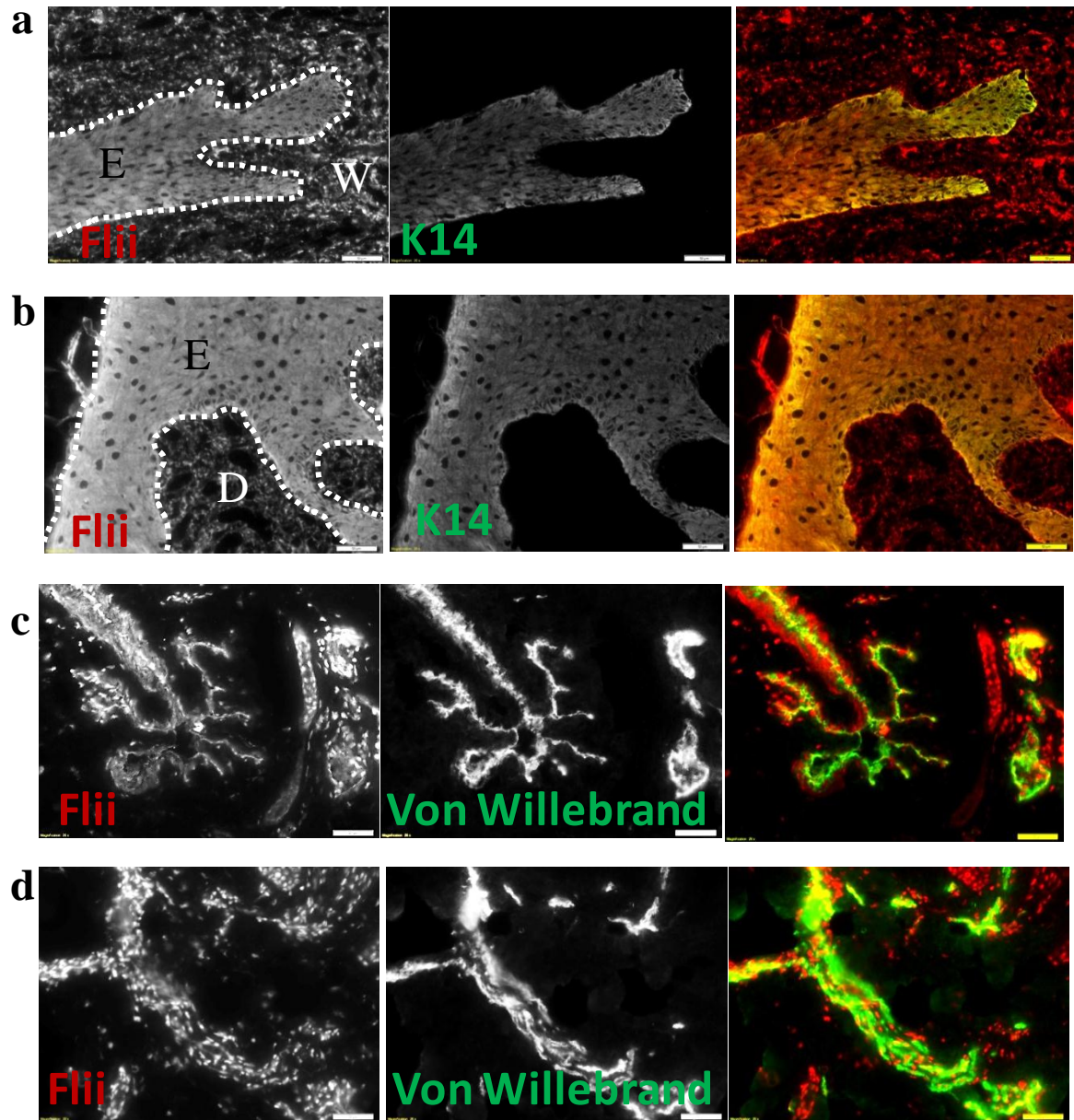
Chronic wound biopsies were collected from 6 patients diagnosed with venous insufficiency and with venous ulcers  $\geq 6$  weeks old (4 women, 2 men, mean age of 80 years). All wounds studied showed features characteristic of chronic venous ulcers. The ulcer wound base was characterized by areas of vascular granulation tissue overlying disorganized collagen bundles. A prominent inflammatory infiltrate was present in most wounds, often extending into the surrounding dermis. Characteristic hemosiderin deposits and capillary cuffing were noted. The epithelial margin was characterized by a marked hyperplastic acanthosis. Acute wounds ( $\leq 6$  weeks old) were collected from 6 patients with acute trauma wounds such as those seen in the emergency room (4 women, 2 men, and mean age of 40 years). Unwounded skin specimens were collected from 6 healthy donors (4 women, 2 men, mean age 42 years) and used as controls. Representative H&E stained views of a chronic ulcer, acute wound and normal skin are shown in Figure 4.1a, c, e, respectively and represent the views shown in the subsequent immunofluorescence studies (Figure 4.1b, d, f).

Immunofluorescence staining revealed that Flii was increased in response to wounding with both acute and chronic wounds (Figure 4.1b, d) having higher Flii expression than unwounded skin (Figure 4.1 f). Flii expression was particularly strong at the leading edge of acute wounds (Figure 4.1b). Although statistically insignificant, chronic wounds showed reduced Flii staining compared to acute wounds, particularly in the dermis (Figure 4.1b, d). Quantitation of Flii immunofluorescence showed significantly elevated expression in both wound types compared to

control unwounded skin (Figure 4.1g). Western analysis showed increased levels of Flii in both chronic and acute wounds compared to unwounded skin with acute wounds having higher Flii levels than chronic wounds (Figure 4.1h). To determine which specific cell types Flii was expressed in, dual immunofluorescence was performed using Keratin 14 as a marker of keratinocytes and Von Willebrand factor as a marker of endothelial cells. Flii was observed colocalised with Keratin 14 positive cells in both acute (Figure 4.2a) and chronic (Figure 4.2b) wounds particularly in the cytoplasm of these keratinocytes. However, Flii was not observed to colocalise with endothelial cells associated with blood vessels in these types of wounds. Instead, Flii positive cells (red) could clearly be seen distinct from the green endothelial cells around blood vessels in both acute (Fig 4.2c) and chronic (Figure 4.2d) wounds. These Flii positive cells could be pericytes, which are cells that surround endothelial cells and provide contractile properties to vessels.



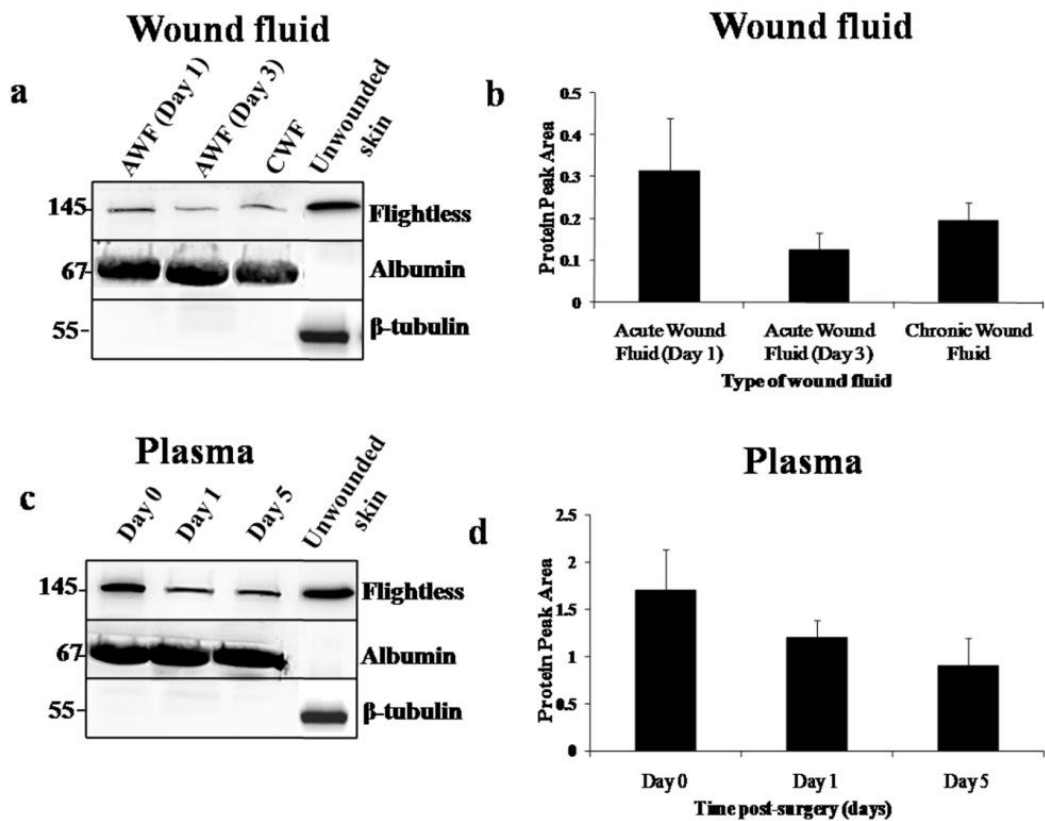
**Figure 4.1. Flii expression is up-regulated in the dermis of acute wounds.** Cryosections of skin biopsies (5 $\mu$ m thick) were used for histology and to perform immunohistochemistry for Flii protein on human acute wounds ( $\leq 6$  weeks old) (b), human chronic wounds ( $\geq 6$  weeks old; skin ulcers secondary to venous stasis) (d) and human unwounded skin (f). In all images, E denotes the position of the epidermis, D denotes the position of the dermis. Representative H&E images (40 $\times$ ) (a, c, e). Graph showing fluorescence optical density of Flii immunofluorescence is shown in (g). Western blotting analysis of skin samples from acute, chronic and normal unwounded skin is shown in (h) (n = 6 for each group). \*Denotes significance in (g)  $p = 0.02$  acute wounds vs. unwounded skin;  $p = 0.04$  chronic wounds vs. unwounded skin. Data are expressed as mean  $\pm$  s.e.m. (n=6). Scale bar is 50  $\mu$ m.



**Figure 4.2. Flii is expressed by wound keratinocytes.** (a) Acute and (b) chronic wounds were dual immunostained for Flii and K14 (keratinocyte marker). Flii localizes mainly to the cytosol in keratinocytes in both wound types. (c) Acute and (d) chronic wounds were dual immunostained for Flii with the endothelial cell marker von Willebrand factor. Flii localizes to the nucleus and cytosol of some, but not all endothelial cells and possibly pericytes. Scale bar is 50 μm.

#### **4.2.2 Flii is present in chronic, acute wound fluid and human plasma**

Our previous studies have identified that Flii is secreted by fibroblasts in response to wounding in vitro (Cowin, AJ et al. 2007). To determine if Flii was present in the wound fluid, we collected acute wound exudate from patients undergoing abdominoplasty (collected on day 1 and day 3 post-surgery) and those with chronic venous ulcers. These samples were analysed using Western blotting and representative blots are shown in Figure 4.3. Flii was present in both acute and chronic wound fluids (Figure 4.3a), but no statistical difference was observed between the levels of Flii in these different wound fluids (Figure 4.3b). To determine if Flii was also present in peripheral blood, plasma samples were collected from patients undergoing abdominoplasty at day 0 (prior to surgery), 1 and 5, and assessed using Western Blotting. Flii was present in all samples of plasma (Figure 4.3c). Flii levels were decreased in plasma at day 1 and 3 post surgery compared to those collected prior to surgery (day 0), but again this was not statistically significant. All blots were stripped and re-probed for albumin (loading control) and  $\beta$ -tubulin (negative control), which is an intracellular protein and therefore a marker of cell lysis. The absence of  $\beta$ -tubulin indicates that the presence of Flii in the wound fluid and plasma is through an active secretory process and not just through cellular debris.

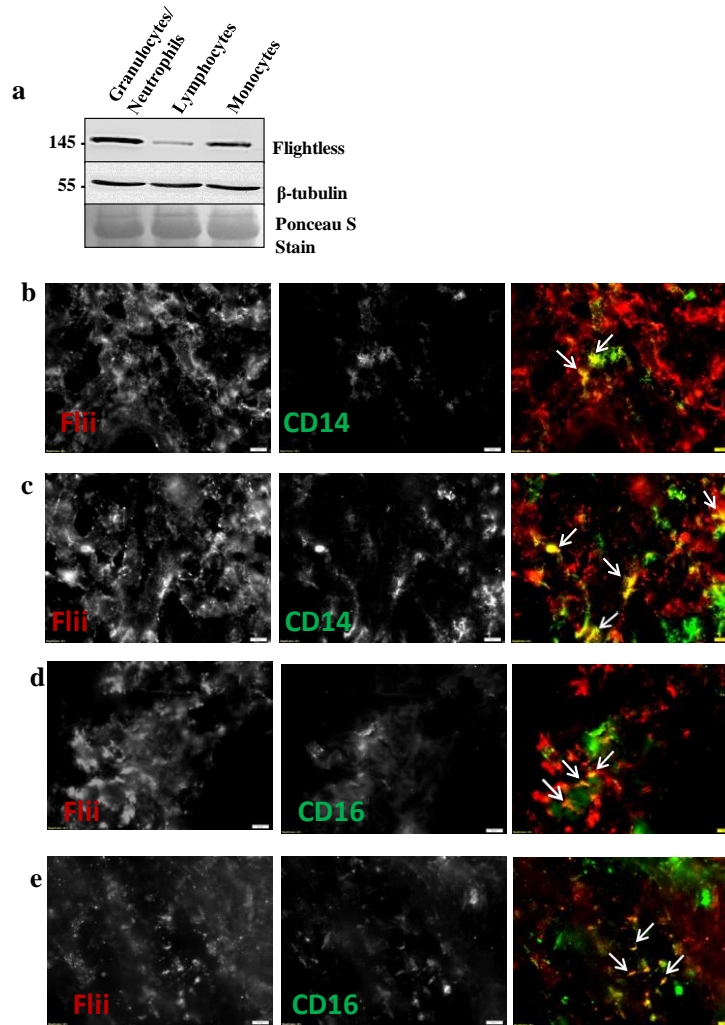


**Figure 4.3. Flii is present in wound fluid and human plasma.** Wound fluid was collected from patients with either acute or chronic wounds. Unwounded skin was collected from patients undergoing varicose vein ligation and stripping. (a) Protein was extracted and Western analysis performed showing the presence of Flii in acute and chronic wound fluid and in normal unwounded skin. (b) Graph showing quantification of Flii in wound fluid using integrated fluorescence intensity of band density. Peripheral blood was collected from patients undergoing abdominoplasty. (c) Western analysis showing the presence of Flii in plasma of human patients taken 0 (n=9), 1 (n=8) and 5 (n=5) day wounds post abdominoplasty. (d) Graph showing quantification of Flii in peripheral blood plasma using integrated fluorescence intensity of band density. Flii was normalized against albumin. Results represent mean  $\pm$  s.e.m. There were eight individuals in day 1 wound fluid and five in day 3 group. Six subjects were included in chronic wound fluid group.

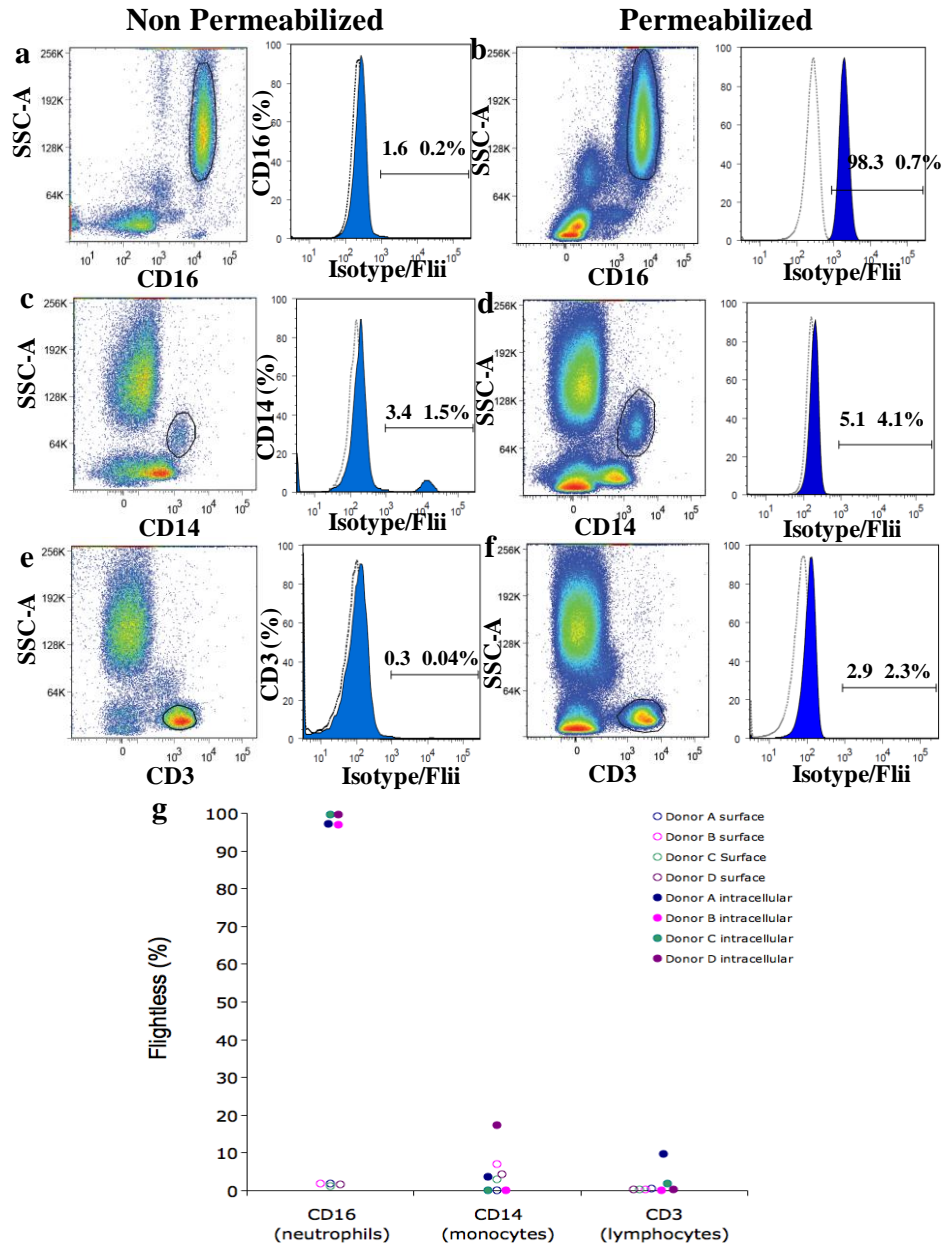
### **4.2.3 Flii is present in blood leukocytes and inflammatory cells**

To determine if Flii, which was detected in the plasma, was expressed by specific immune cells, sub-populations of leukocytes were isolated using FACS analysis. Granulocytes (neutrophils), monocytes and lymphocytes were sorted according to size and granularity based on their forward and side scatter. Cell fractions were analyzed for Flii expression using Western Blotting (Figure 4.4a). Flii was expressed in all three cell types, but was most abundant in granulocytes (neutrophils). Acute (Figure 4b) and chronic wounds (Figure 4.4c) were also co-stained with antibodies against Flii and CD14 (macrophage marker) to determine if Flii was present within tissue resident macrophages. Acute (Figure 4.4d) and chronic wounds (Figure 4.4e) were also co-stained with antibodies against Flii and CD16 (neutrophil marker) to determine if Flii was present within the neutrophils of acute and chronic wounds. These immunofluorescence studies showed that Flii was present in some but not all wound macrophages and neutrophils in chronic and acute wounds potentially reflecting the different activation states of these inflammatory cells (Figure 4.4b-e). We finally used four-coloured flow cytometry in conjunction with antibodies against CD16<sup>+</sup> (neutrophil marker) CD14 (monocyte marker) and CD3 (lymphocyte marker) to assess whether Flii was present within the cells (intracellular) or attached to the cells surface. High and bright signal was detected in permeabilised CD16<sup>+</sup> cells indicating high intracellular expression of Flii in these cells, which was not observed in other populations (Figure 4.5b and g). To determine if Flii protein was membrane bound we examined non-permeabilised CD3<sup>+</sup>, CD14<sup>+</sup> and CD16<sup>+</sup> cells and found that Flii was not expressed on the cell surface (Figure 4.5a, b, e and g).





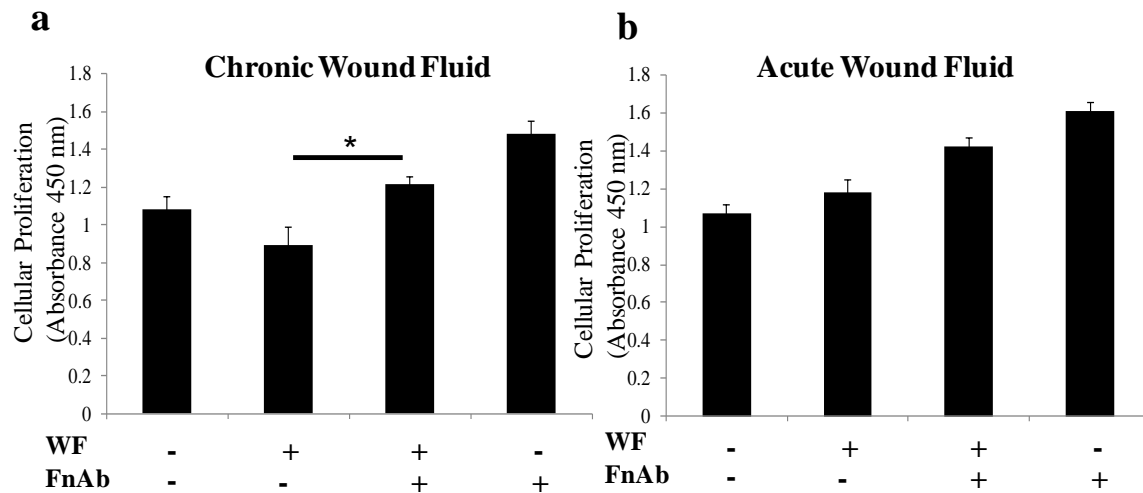
**Figure 4.4. Flii is present in blood leukocytes and wound neutrophils and macrophages.** Human granulocytes, monocytes and lymphocyte were isolated from peripheral blood leukocytes obtained from four healthy individuals and sorted by FACS to a purity of  $\geq 90\%$ . Cellular lysate samples (20  $\mu\text{g}$  of protein) were separated by 10% SDS-PAGE and presence of Flii determined by Western analysis (a). Equal loading of protein samples was confirmed using  $\beta$ -tubulin and Ponceau S staining. (b) Cryosections of biopsies taken from patients with acute wounds and (c) chronic venous leg ulcers were stained with Flii-Alexa 594 (red), CD14-Alexa-488 (green). This staining was performed for the detection of tissue resident macrophages. (d) Cryosections of biopsies taken from patients with acute wounds and (e) chronic venous leg ulcers were stained with Flii-Alexa 594 (red), CD16-Alexa-488 (green). This staining was performed for the detection of tissue resident neutrophils. Immunofluorescence analysis of CD14 positive macrophages and CD16 positive neutrophils co-expressing Flii was observed in the acute and chronic wounds. (n=6, scale bar = 10  $\mu\text{m}$ ; Magnification  $\times 60$ ).



**Figure 4.5. Flii is predominantly found in neutrophils within human blood.** Cell-surface expression of Flii protein was determined by staining whole blood with Flii, CD3, CD14 and CD16 antibodies and analyzed by flow cytometry (a, c, e). To investigate total, i.e. cell surface and intracellular expression of Flii protein, whole blood was permeabilised and stained with Flii, CD3, CD14 and CD16 antibodies. Significantly higher Flii expression was observed in permeabilised CD16-positive neutrophils (b) compared to notably lower Flii expression in CD14-positive monocytes (c, d) and CD3-positive T cells (e, f). Representative data from four independent experiments are shown (n=4). (g) Percentages of Flii-positive cells. Data are shown as means +/- s.e.m. of four independent experiments (n = 4).

#### **4.2.4 The inhibitory effect of chronic wound fluid on fibroblast proliferation is due in part to Flii**

Previous studies have identified Flii as a negative regulator of wound healing via mechanisms including reducing cellular proliferation, migration and adhesion (Cowin, AJ et al. 2007; Kopecki et al. 2009). Further studies have shown that wound fluid contains factors which are cytotoxic and can inhibit cellular proliferation (Cowin et al. 2006; Harding, Moore & Phillips 2005; Trengove et al. 1999). We therefore hypothesized that Flii present in chronic wound fluid may adversely affect the wound environment and inhibit cellular proliferation. Chronic and acute wound fluids were therefore pre-incubated with FnAbs for 30 minutes and then pre-treated wound fluid was assessed for its effect on fibroblast proliferation. When chronic wound fluid alone was added to the fibroblasts, a significant decrease in proliferation was observed (Figure 4.6a). When chronic wound fluid that had been neutralized for Flii activity was added to the fibroblasts the inhibitory effect of the wound fluid was ablated (Figure 4.6a). Interestingly, when acute wound fluid alone was added to fibroblast, no inhibition of proliferation was observed despite it containing Flii protein (Figure 4.6b).



**Figure 4.6. Neutralization of Flii using specific antibodies prevents the inhibitory effect of chronic wound fluid on fibroblast proliferation.** Chronic (a) and acute (b) wound fluid were preincubated with Flii neutralizing antibody (FnAb) (concentration = 20  $\mu\text{g/ml}$ ) for 30 minutes prior to applying to human foreskin fibroblasts. The effect on cell proliferation was determined using WST-1 proliferation assay at 24 hour. Results represent means  $\pm$  s.e.m. ( $n = 10$  for each group,  $*p < 0.05$ ).

#### 4.4 Discussion

Previous studies have shown that Flii is a negative regulator of wound healing and reducing its expression either at a genetic level or through using antibodies to neutralize protein activity improves wound outcomes (Cowin, AJ et al. 2007). Here we show that Flii is increased in both acute and chronic human wounds, that it is present in the wound fluid of these patients and also in their peripheral blood. Originally we speculated that Flii might be strongly upregulated in chronic non-healing wounds as this protein clearly has a negative influence on the healing process and may therefore be contributing to wound chronicity. However, our results showed that Flii was up-regulated in both acute (healing) and chronic (non-healing) wounds compared to unwounded skin and if anything was more highly expressed in acute wounds, although this was not statistically significant. Flii is a negative regulator of proliferation, migration and adhesion (Cowin, AJ et al. 2007) , it stimulates TGF- $\beta$ 1 expression (Adams et al. 2009) and increases collagen synthesis (Adams et al. 2009) all of which are important for wound healing. Our previous findings have shown that Flii is secreted by fibroblasts *in vitro* in response to scratch wounding (Cowin, AJ et al. 2007). Intracellular protein  $\beta$ -tubulin was not found in the media suggesting that this was not a consequence of damaged cells emptying their contents into the milieu, but the result of an active secretory process. Recent *in vitro* studies show that Flii is not secreted by the classical secretion pathway, but is instead released via a late endocytic/lysosomal pathway (see next chapter in this thesis) that is regulated by Rab7 and Stx11 (Lei et al. 2012).

Our current studies now show that Flii is present in both human acute and chronic wound fluid *in vivo*, which suggests that Flii is secreted *in vivo* and that it may not only affect cellular activities via its intracellular functions, but it may also have important extracellular activities. Gelsolin,

head of the family of actin remodelling proteins of which Flii is a member, is also released in response to injury (Lee, WM & Galbraith 1992). Tissue injury results in the release of the intracellular protein actin which is cleared from the circulation by the plasma proteins gelsolin and Gc-globulin, constituting the Extracellular Actin Scavenger System (EASS) (Dahl et al. 1999). The intravascular actin scavenger system depolymerizes and sequesters actin released after tissue injury. A study by Dahl and colleagues (Dahl et al. 1999) showed that plasma gelsolin levels were reduced in trauma patients compared with normal controls. Burn wound fluid also contains actin complexed with gelsolin and Gc protein, so it is likely that components of the actin scavenger system are functional in wound tissue (Grinnell et al. 1993). Flii has actin binding ability, (Lee, YH, Campbell & Stallcup 2004) therefore its presence in wound fluid and plasma may be similar to that observed for gelsolin, as a member of the extracellular actin scavenger system. The exact extracellular activities of Flii are yet to be determined, however application of neutralising antibodies to Flii significantly improve wound repair in both incisional and burn murine models suggesting that Flii may have effects beyond that of an actin scavenging protein.

Myeloid cells such as blood neutrophils and monocytes move from the local vascular network into wound sites, where they destroy pathogens as well as deliver molecules that help to repair the tissue loss, but may also cause tissue damage (Feng et al. 2010; McDonald et al. 2010). Phagocytic properties of neutrophils aid the clearance of non-viable and necrotic tissue, thus aiding healing (Kono & Rock 2008), however, exacerbated neutrophil recruitment can also damage and destruct viable tissue through its hydrolytic, oxidative and pore-forming molecules (Segal 2005). The high influx of neutrophils observed in chronic venous ulcers (Lundqvist,

Sorensen & Schmidtchen 2008) has been reported to contribute to the immunopathology and chronicity seen in these wounds (Peral et al. 2010). In peripheral blood, Flii was found to be present in granulocytes/neutrophils, lymphocytes and monocytes but was most abundant in granulocytes (neutrophils) suggesting that Flii may have an important function in inflammation. Our previous studies have shown that inflammation in wounded Flii overexpressing mice is prolonged (Adams et al. 2008) and Flii has also been shown to have an inhibitory effect on the TLR signaling pathway by competitively binding to MyD88 and switching off the NF- $\kappa$ B signalling pathway (Dai et al. 2009). Clearly much is still to be learnt regarding the role of Flii in the inflammatory response during wound healing. Most recently, Flii has been shown to be constitutively secreted from both macrophages and fibroblasts and this secreted Flii binds to LPS and dampens cytokine secretion (Lei et al. 2012). Acute inflammation is an important feature of normal wound healing, however excessive and chronic inflammation occurs in venous leg ulcers and diabetic wounds (Bermudez et al. 2011; Pukstad et al. 2010). Our current findings demonstrate that Flii is detected in both peripheral blood neutrophils and wound neutrophils. Acute and chronic wounds have a greater number of inflammatory cells compared to unwounded skin and we speculate that the elevated level of inflammatory cells in acute and chronic wounds contribute to the increased expression of Flii in these wounds which could possibly negatively impact on healing of these wounds. Interestingly, Flii was present in some, but not all wound macrophages and neutrophils in chronic and acute wounds potentially reflecting the different activation states of these inflammatory cells.

Given that previous studies have shown that chronic wound fluid contains cytotoxic cytokines and inhibits cellular proliferation (Harding, Moore & Phillips 2005) and Flii has been shown to

inhibit cellular proliferation we wanted to establish if the inhibitory effects of chronic wound fluid might be due in part to the presence of Flii. Pre-incubation of both chronic and acute wound fluid with neutralizing antibodies for Flii ablated the inhibitory effect of chronic wound fluid on fibroblast proliferation suggesting that Flii may have a negative effect on chronic wound healing *in vivo*. Acute wound fluid did not inhibit cell proliferation and when FnAb were added to acute wound fluid cell proliferation was further increased. Treatment of the acute wound fluid with FnAb lead to a significant increase in proliferation suggesting that acute wound may contain pro-proliferative factors as well as Flii, which are able to counteract the negative effects of Flii. While wound fluid is a complex mixture of serum, cytokines, growth factors and cell debris the fact that we could prevent chronic wound fluid from having its inhibitory effects on cell proliferation by applying FnAb suggests that application of Flii antibodies as a topical therapy to chronic wounds may promote improved wound outcomes. Flii is therefore a potential target for improving wound healing outcomes and future studies will investigate methods for reducing Flii directly in human wounds.



## *Chapter 5*

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# FLIGHTLESS I IS SECRETED VIA NON- CLASSICAL LATE ENDOSOME/LYSOSOME AND EXOSOME- ASSOCIATED PATHWAYS

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The results included in this chapter were published in the *Journal of Cell Science*. Nadira's contribution to this paper consisted of i) collection and analysis of wound fluid and plasma, ii) immunofluorescence staining and imaging of primary mouse fibroblasts with Cathepsin D, Flii, Vti1b and VAMP7, iii) immunofluorescence staining and imaging of HFFs with Cathepsin D, Flii, Vti1b and VAMP7.

**Title:** Flightless, secreted through a late endosome/lysosome pathway, binds LPS and dampens cytokine secretion

**Authors:** Nazi Lei, Linda Franken, Nadira Ruzehaji, Carolin Offenhäuser, Allison J Cowin, Rachael Z. Murray

Manuscript of the article is in Appendix 3

# FLIGHTLESS I IS SECRETED VIA NON-CLASSICAL LATE ENDOSOME/LYSOSOME AND EXOSOME-ASSOCIATED PATHWAYS

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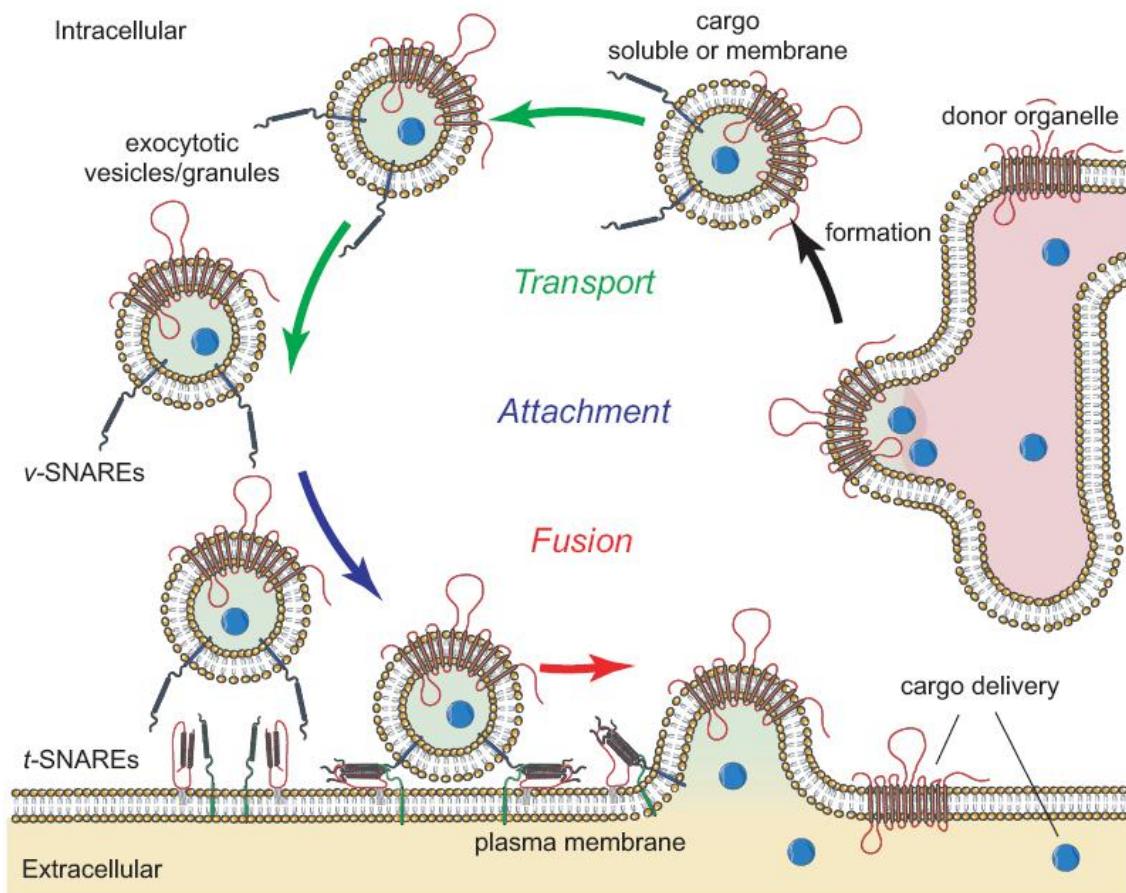
## 5.1 Introduction

Flii is a cytoskeletal protein with many intracellular functions (Kopecki & Cowin 2008). Flii binds and severs F-actin resulting in the remodelling of the actin cytoskeleton (Goshima et al. 1999). Apart from having a role within the nucleus (Lee, YH & Stallcup 2006), intracellular Flii is now emerging as a negative regulator of inflammation (Dai et al. 2009). In addition to its intracellular phenotype there is a cytoplasmic form of Flii. We previously showed that Flii is secreted *in vitro* and neutralization of extracellular Flii activity by FnAbs improves wound healing confirming that secreted Flii has important extracellular functions (Cowin, AJ et al. 2007). In the previous chapter we have shown that Flii is present in wound fluid and plasma. In this chapter we investigate the possible mechanisms involved in Flii trafficking and secretion.

The regulated trafficking or exocytosis of secretory vesicles into or across the plasma membrane is essential to all cells including skin fibroblasts (Burchfield et al. 2010). Exocytosis is a delivery mechanism of both lytic granule contents and secreted proteins such as cytokines (Griffiths, Tsun & Stinchcombe 2010). Proteins and lipids packaged into membrane-bound carrier vesicles bud off a donor compartment, and travel to, then dock and fuse with target membrane to deliver cargo to their final cellular or extracellular destination (Bonifacino & Glick 2004). Manufactured proteins including immune mediators are transported through the endoplasmic reticulum to the Golgi and packaged into exocytotic vesicles that undergo various steps of intracellular trafficking to the plasma membrane (Stow, Manderson & Murray 2006) (Figure 5.1). It has been convenient to describe this trafficking pathway as a three step process: transport, attachment and fusion (Burchfield et al. 2010). Exocytosis begins with the formation and transport of exocytotic vesicles to the destination site. Attachment is the next step and this is when membrane-bound

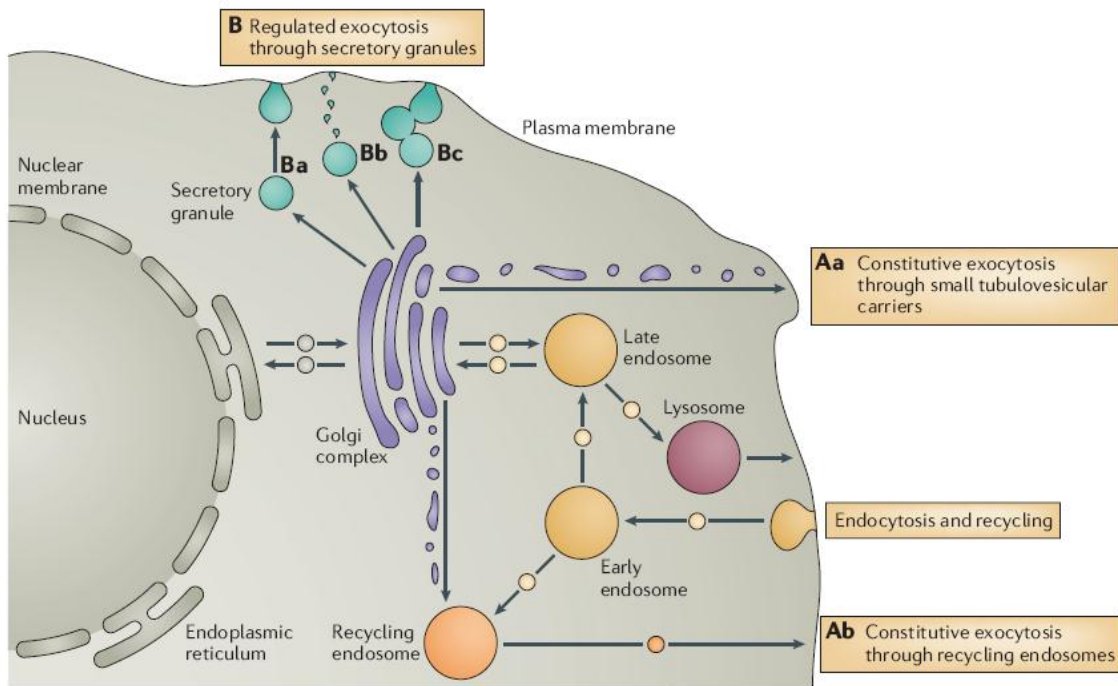
carrier vesicles stop moving and undergo attachment to the target membrane (Burchfield et al. 2010). Fusion is the final step of exocytosis and it involves the amalgamation of the two lipid membranes in preparation for the release of cargo.

Some molecules are released into the extracellular spaces via an alternative mechanism known as the exosome secretory pathway (Stoorvogel et al. 2002). Exosomes are small endocytic organelles, which once released induce the secretion of macromolecules.



**Figure 5.1 Model of exocytosis.** Membrane-bound vesicle originates from a donor organelle and is transported to a destination site where it undergoes attachment, followed by fusion and delivery of precious cargo to the plasma membrane or extra-cellular spaces. Adapted from (Burchfield et al. 2010).

Several other pathways for exocytosis exist, with constitutive exocytosis being the most common pathway of protein secretion (Figure 5.2). Constitutive exocytosis is a transport pathway which facilitates proteins transport either through small tubulovesicular carriers directly to the cell surface or through what is known as recycling endosome. (Stow, Manderson & Murray 2006). Early endosomes are the first an intracellular organelles in the endocytic pathway to receive proteins and lipids from the cell surface. Recycling endosomes are post-early-endosomes through which internalized molecules recycle to the cell surface (Bajno et al. 2000; Braun et al. 2004).



**Figure 5.2. Principle steps involved in intracellular transport of proteins.** Several secretory pathways are presently known, with each of them requiring fusion and docking of between cell organelles (carrier vesicles) and another membrane at their destination (Stow, Manderson & Murray 2006). Constitutive exocytosis occurs through small carrier vesicles going directly to the cell membrane (Aa) or through the recycling endosomes (Ab). The three variations of regulated secretion include direct fusion with the plasma membrane (Ba), delivery of cargo in small vesicles known as piecemeal exocytosis (Bb) and fusion of vesicles with each other, known as compound exocytosis (Bc).

The exact mechanisms involved in Flii secretion are poorly understood, however recent studies using specific agonist and antagonist-related pathways showed that Flii is trafficked via an endosomal/lysosomal pathway that is regulated by Rab7 and Stx11 (Lei et al. 2012). The aims of this study were (i) to characterize intracellular transport carriers associated with Flii, and (ii) delineate possible trafficking routes taken by Flii protein as it moves to the plasma membrane.

## **5.2 Results**

### **5.2.1 Flii is present in human plasma and wound fluid**

Human plasma samples collected from healthy volunteers were treated with TCA (trichloroacetic acid) to induce protein precipitation (Gruhler et al. 2005). To test whether Flii was present in these specimen unwounded skin and plasma samples were immunoblotted for Flii, the plasma marker albumin and the intracellular marker  $\beta$ -tubulin (Figure 5.3). A band corresponding to the 145kDa Flii protein was observed in both the skin and plasma samples suggesting Flii is present in the plasma and skin. Intracellular  $\beta$ -tubulin (negative control) was only detected in skin samples and not in the plasma (Figure 5.3). Flii was also detected by Western blotting in wound fluid samples collected from patients with acute and chronic wounds providing further evidence that Flii is present in human body fluids (Chapter 4, Figure 4.3a).

### **5.2.2 Endogenous Flii is expressed in both the nucleus and the cytoplasm**

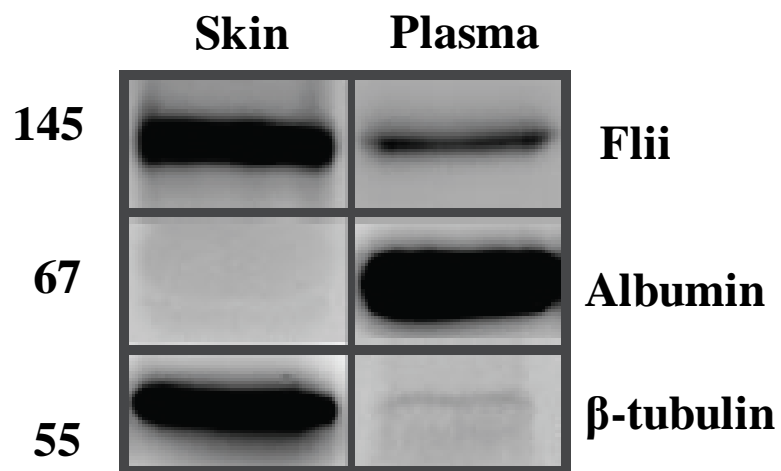
Having established the fact that Flii is present in culture media of scratched fibroblasts (Cowin, AJ et al. 2007) and in human plasma and wound fluid, we wanted to identify a pathway via which Flii may be secreted. The intracellular location of Flii protein provides an indication of how it might be secreted out of the cell. Fibroblasts are key cells typically found in wounds; hence a confluent layer of unwounded human skin fibroblasts was collected and fractionated into nuclear and cytosol compartments (Figure 5.4). Fractions were immunoblotted with antibodies specific for Flii, nucleoporin62 (nuclear marker) and  $\beta$ -tubulin (cytoplasmic marker). While Flii

was highly expressed in the cytoplasm, a similar proportion was also found in the nuclear fraction (Figure 5.4). Our results suggest that Flii is uniformly distributed within the cell and present in both nucleus and cytoplasm (Figure 5.4).

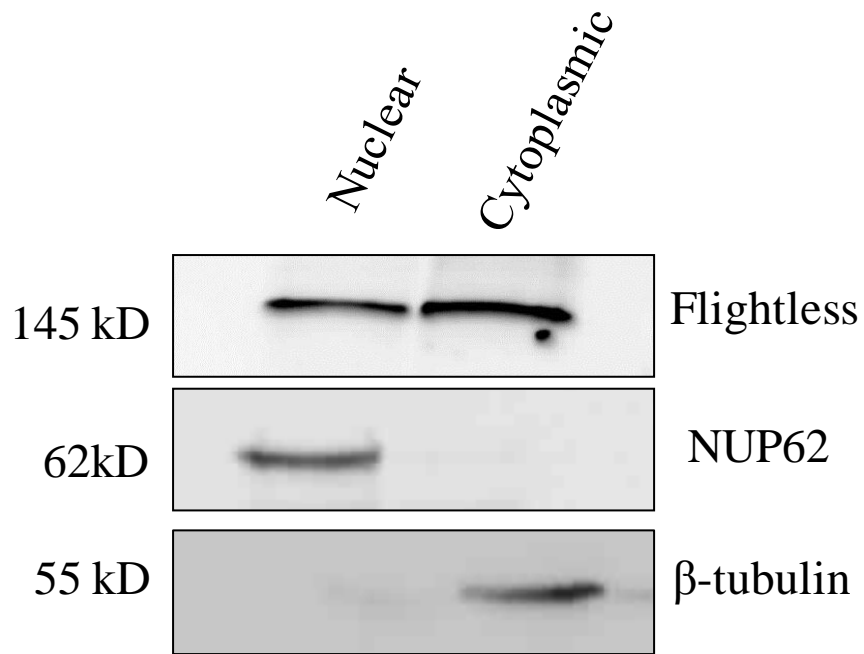
### **5.2.3 Flightless I is located on late endosomes/lysosomes and exported via a non-classical pathway of secretion**

Several pathways for secretion exist including a non-classical pathway where the delivery of proteins to the cell surface is trafficked via late endosomes/lysosomes (Nickel 2003). To identify possible binding partners and endocytic structures, immunostaining of Flii in combination with the late endosome/lysosome proteins was performed. Human skin fibroblasts (Figure 5.5) and primary mouse fibroblasts (Figure 5.6) were analysed under the confocal microscope to determine the potential Flii secretory pathways. Cathepsin D, VAMP7 and Vti1b are well known SNARE proteins (Stow, Manderson & Murray 2006) located on late endosomes/lysosomes (Offenhauser et al. 2011; Veale et al. 2011). Our studies showed that endogenous Flii associates with Cathepsin D, VAMP7 and Vti1b (late endosome/lysosome markers) pointing to an exocytotic route for Flii secretion (Figure 5.5). Dual immunofluorescence experiments revealed that Flii colocalised with the vesicular carriers whose morphology is consistent with that of the late endosome/lysosome (Lock & Stow 2005; Polishchuk et al. 2000). The association of Flii with the late endosomes could provide a working model explaining how Flii might be secreted.

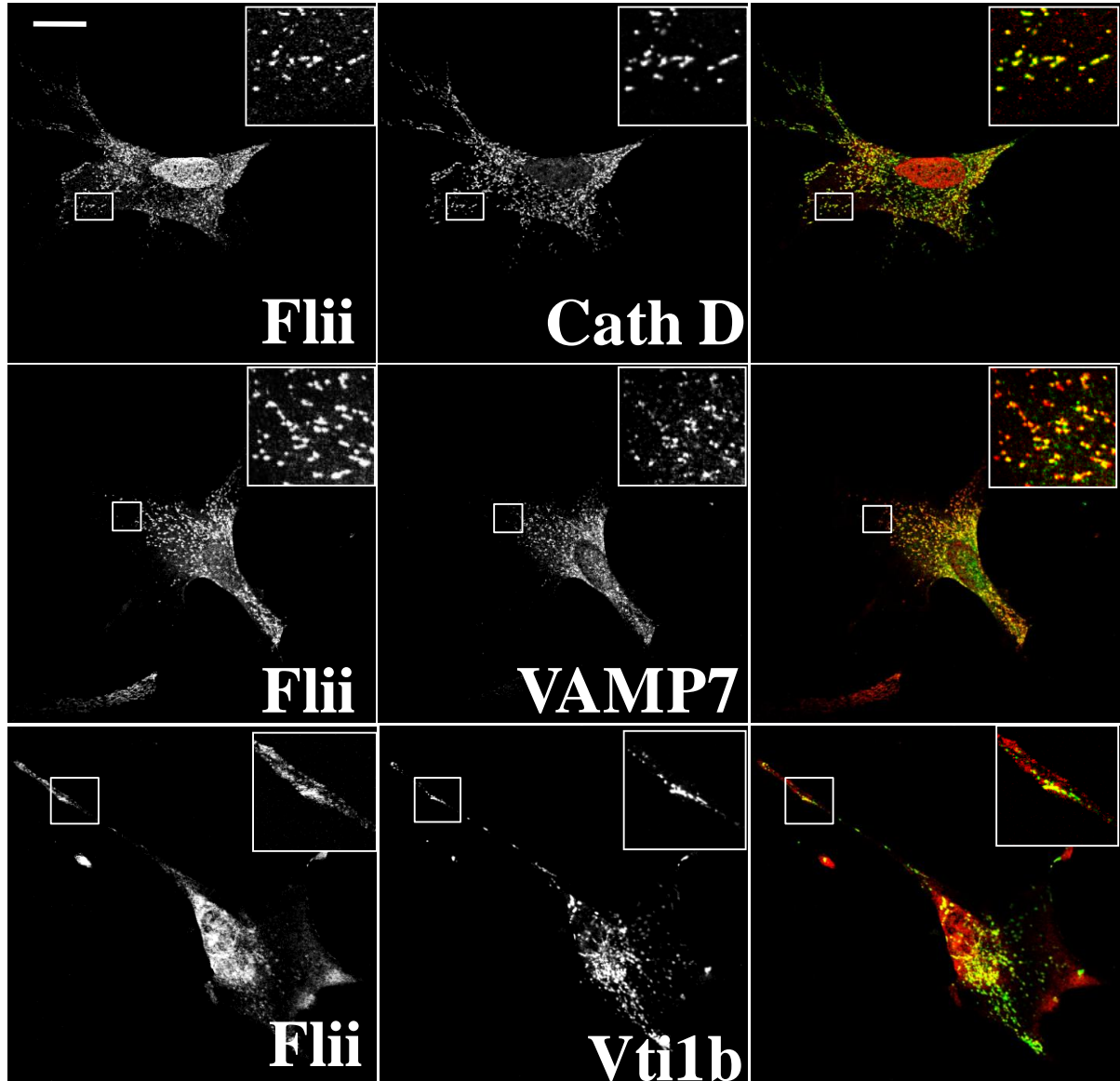




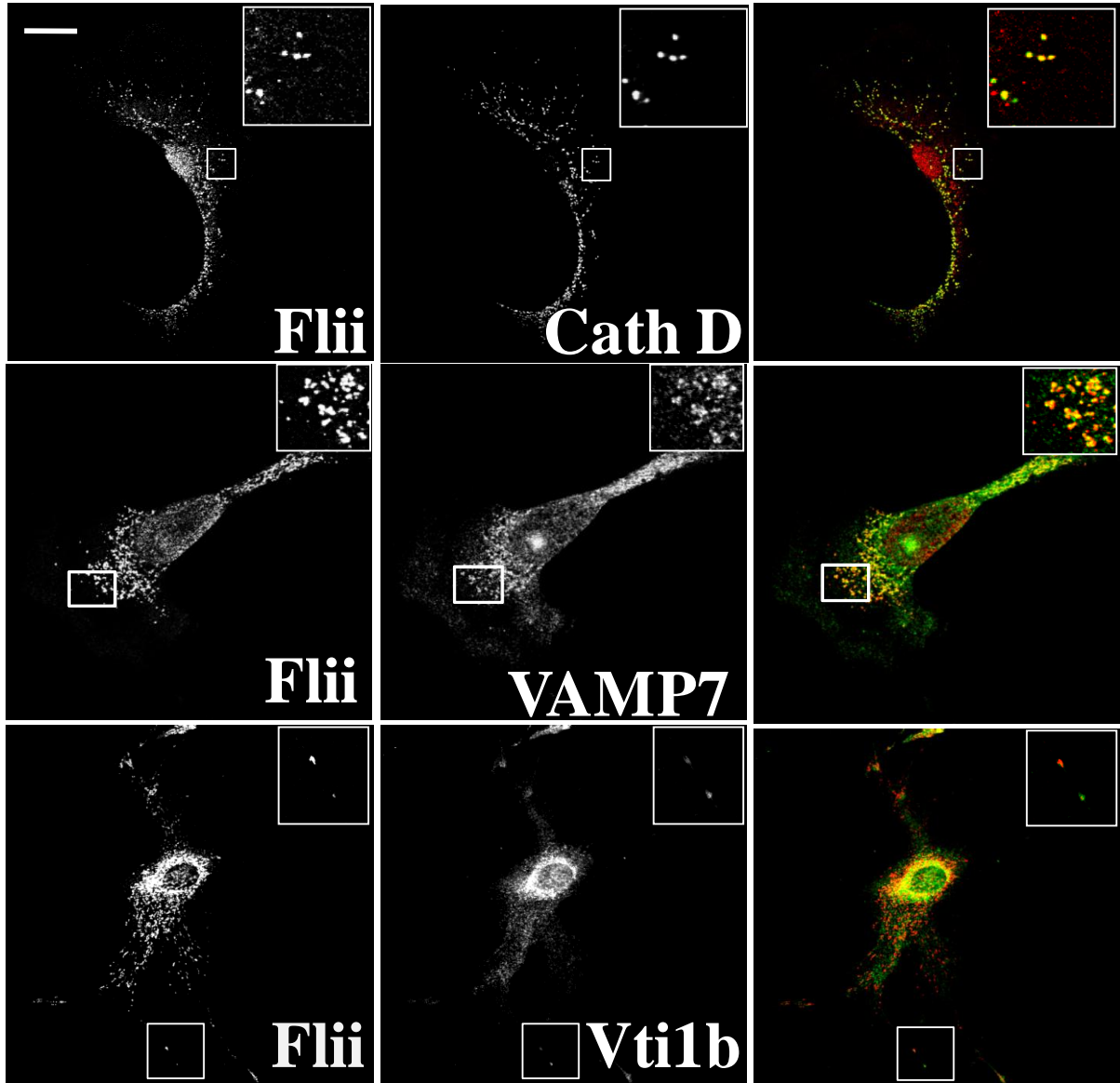
**Figure 5.3. Flii was detected in skin and plasma.** Protein was extracted from human skin and human plasma. The samples were immunoblotted for Flii,  $\beta$ -tubulin and albumin. Flii, but not  $\beta$ -tubulin was detected in plasma suggesting that Flii is present in plasma.



**Figure 5.4. Flii is present in both the nucleus and the cytoplasm.** Cytosol and nuclear fractions from human foreskin fibroblasts (HFF) were analysed by immunoblotting for the Flii protein as well as the cytoplasmic marker  $\beta$ -tubulin and the nuclear marker protein nucleoporin 62 (NUP62). The majority of Flii was present in both nucleus and cytoplasm.



**Figure 5.5. Flii is located on late endosomes/lysosomes in human foreskin fibroblasts.** HFFs were immunostained for endogenous Flii and incubated with three different antibodies each labelling a specific SNARE protein. Flii co-localizes with Cathepsin D (labels late endosomes and lysosomes), VAMP7 (labels late endosomes and lysosomes) and Vti1b (labels recycling endosomes and late endosomes/lysosomes) in HFFs. Scale bar is 5  $\mu$ m.



**Figure 5.6. Flii is associated with late endosomes/lysosomes in primary mouse fibroblasts.** Immunofluorescence staining for Cathepsin D, VAMP7, Vti1b and Flii was performed in primary mouse fibroblasts. Cathepsin D, VAMP7 and Vti1b belong to the SNARE family of proteins and label vesicles (late endosomes/lysosomes) known to be involved in the intracellular pathway and machinery responsible for protein secretion. When the pictures of Flii endogenous staining were merged with those of the markers of late endosomes/lysosomes, it was evident that Flii localizes with Cathepsin D, VAMP7, Vti1b suggesting that Flii is associated with the late endosomes/lysosomes in primary mouse fibroblasts. Scale bar is 5  $\mu\text{m}$ .

#### **5.2.4 Flii is not secreted via the classical secretory pathway**

Having established the fact that Flii has been shown to be a secreted protein and localises to proteins involved in the non-classical late endosome/lysosome mediated pathway we next looked to see if Flii might also be secreted via a well characterized classical pathway of secretion (Murray, Kay, et al. 2005; Murray, Wylie, et al. 2005). Flii was co-immunostained with two markers of intracellular organelles including GM130-positive Golgi complex and VAMP3-positive recycling endosome. Both GM130 and VAMP3 vesicles were located in the perinuclear region, but did not co-localise with Flii (Figure 5.7). Given that Flii did not co-localise with VAMP3 and GM130-labeled organelles our results suggest that Flii is unlikely to be transported via what is known as the classical pathway of secretion.

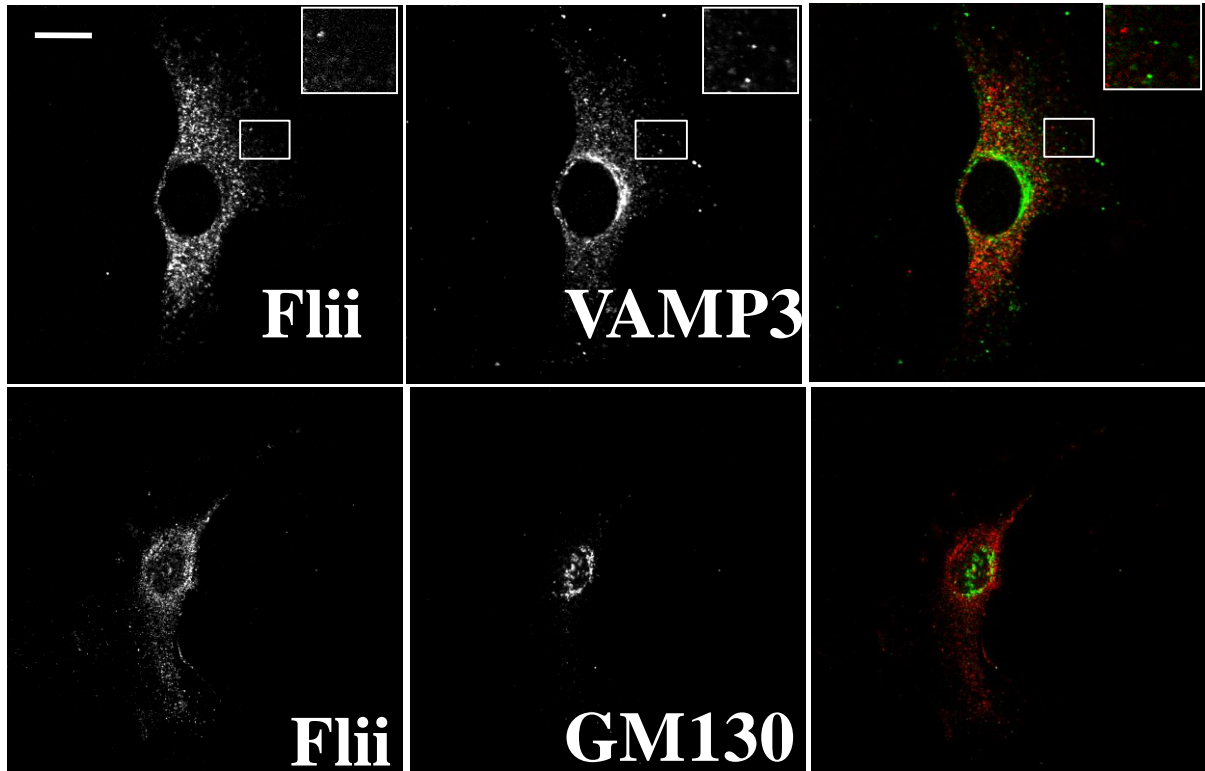
#### **5.2.5 Flii is transported via a novel exosome-associated secretory pathway**

Some cytosolic proteins are secreted via small vesicles known as exosomes, which once released into extracellular spaces function in signalling (Saelman et al. 1994), cell death (Peters et al. 1989) and antigen presentation (Zitvogel et al. 1998). To test whether Flii was present in exosomes, these small vesicles were isolated from the serum free media of a confluent layer of passage 1 primary mouse skin fibroblasts (Figure 5.8a). Exosomes were collected and protein samples immunoblotted for Flii, heat shock protein Hsc70 (exosome marker) and intracellular BiP/GRP78. A band corresponding to Flii was observed in the exosome fractions of all three samples (Figure 5.8a). BiP/GRP78 (negative control) was absent in exosomes showing that the extent was not contaminated by cell debris. As expected Flii, intracellular BiP/GRP78 and  $\beta$ -

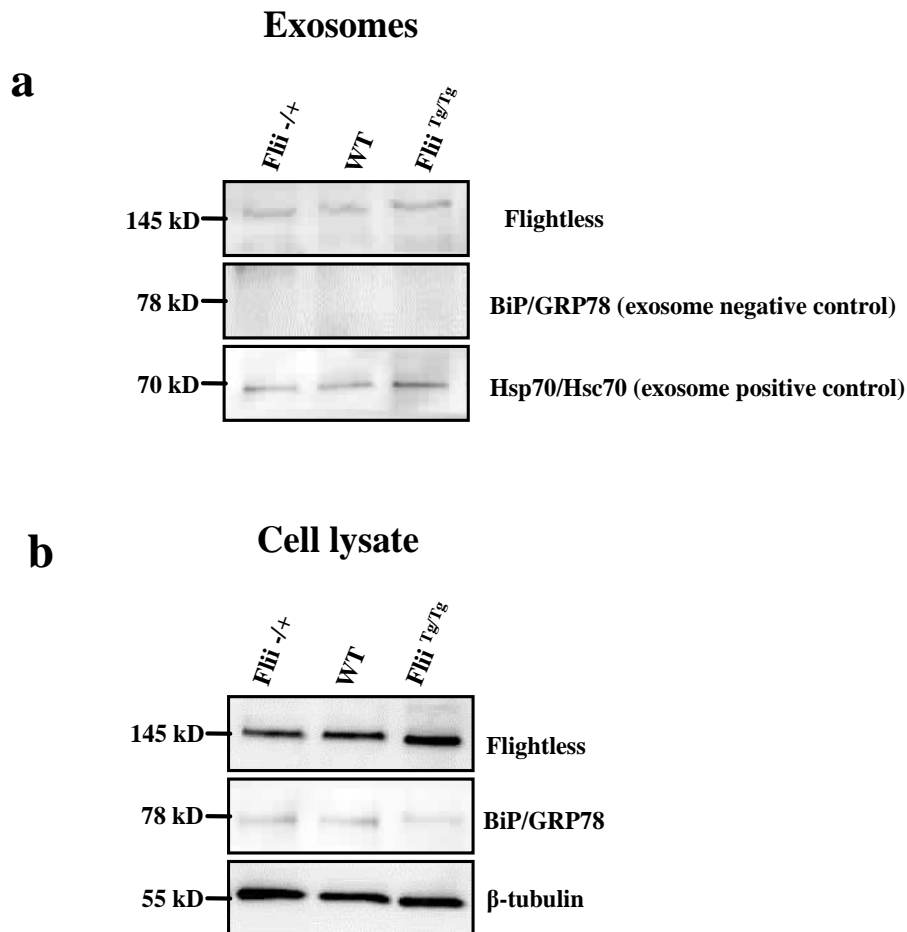
tubulin (loading control) were present in the whole of the cell lysates (Figure 5.8b). No difference was observed in total Flii levels seen in exosomes isolated from Flii<sup>+/-</sup>, WT and Flii<sup>Tg/Tg</sup> mouse skin fibroblasts. Cell extracts collected from Flii<sup>Tg/Tg</sup> skin fibroblasts were immunoblotted for Flii (Figure 5.8b). The levels of intracellular Flii were higher in Flii<sup>Tg/Tg</sup> compared to Flii<sup>+/-</sup> and WT genotype.

### **5.2.6 Flii secretory stimulus may depend on intact microtubules**

It is established that the cytoskeleton is critically involved in the trafficking of secretory vesicles from cells. Cells require intact microtubules to mediate transport of Golgi elements towards the plasma membrane (Schnekenburger et al. 2009). To investigate the role of microtubules in the secretion of Flii, human foreskin fibroblasts were incubated with 1  $\mu$ M colchicine – an inhibitor of microtubule formation (Abdel Bar et al. 2010; Joseph et al. 2012) for 7 hours and conditioned media was collected and concentrated for Western blotting analysis (Figure 5.9). Flii release from cells treated with colchicine was completely abolished while control cells cultured in the absence of colchicine were able to secrete Flii (Figure 5.9). These results support the hypothesis that microtubule-dependent mechanisms are important in Flii trafficking and exocytotic secretion.

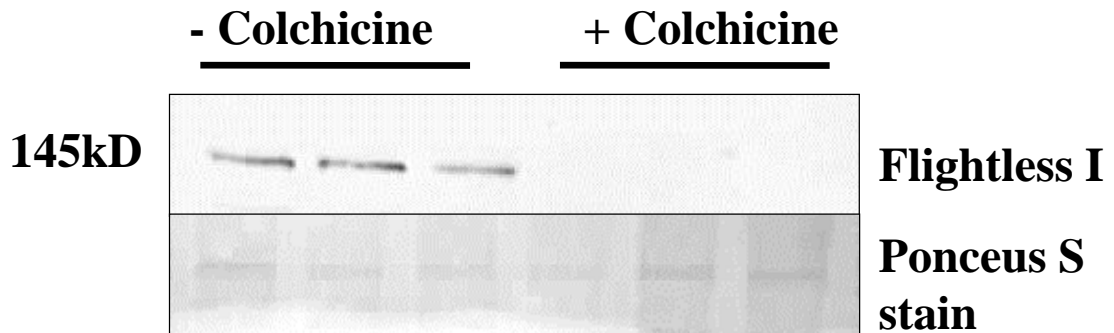


**Figure 5.7. Flii does not co-localise with either, GM130-labeled Golgi complex and VAMP3-labeled recycling endosome.** Fixed and permeabilised human fibroblasts were immunostained for endogenous Flii, VAMP3-positive recycling endosome and GM130-positive Golgi complex. VAMP3 (implicated in the classical secretory pathway) and GM130 (Golgi marker) did not accumulate in Flii-rich areas of cytoplasm. Flii did not co-localise with the GM130-positive Golgi complex or VAMP3-positive recycling endosome. Scale bar is 5  $\mu$ m.



**Figure 5.8. Flii is present in exosomes.** a) Primary fibroblasts isolated from the skin of *Flii*<sup>+/-</sup>, WT and *Flii*<sup>Tg/Tg</sup> mice were cultured in serum free media for 24 h. Secreted exosomes were collected from the media via a series of high speed centrifugation steps. Protein was extracted from the exosomal fractions of fibroblasts and probed for Flii, heat shock protein Hsc70 (exosome marker) and BiP/GRP78. Exosomes were positive for Flii and Hsp70 (positive control), but negative for BiP/GRP78 (negative control). (b) As expected Flii was expressed in the whole cell lysate isolated from a confluent layer of the same skin fibroblasts. Data are representative of at least three independent experiments.





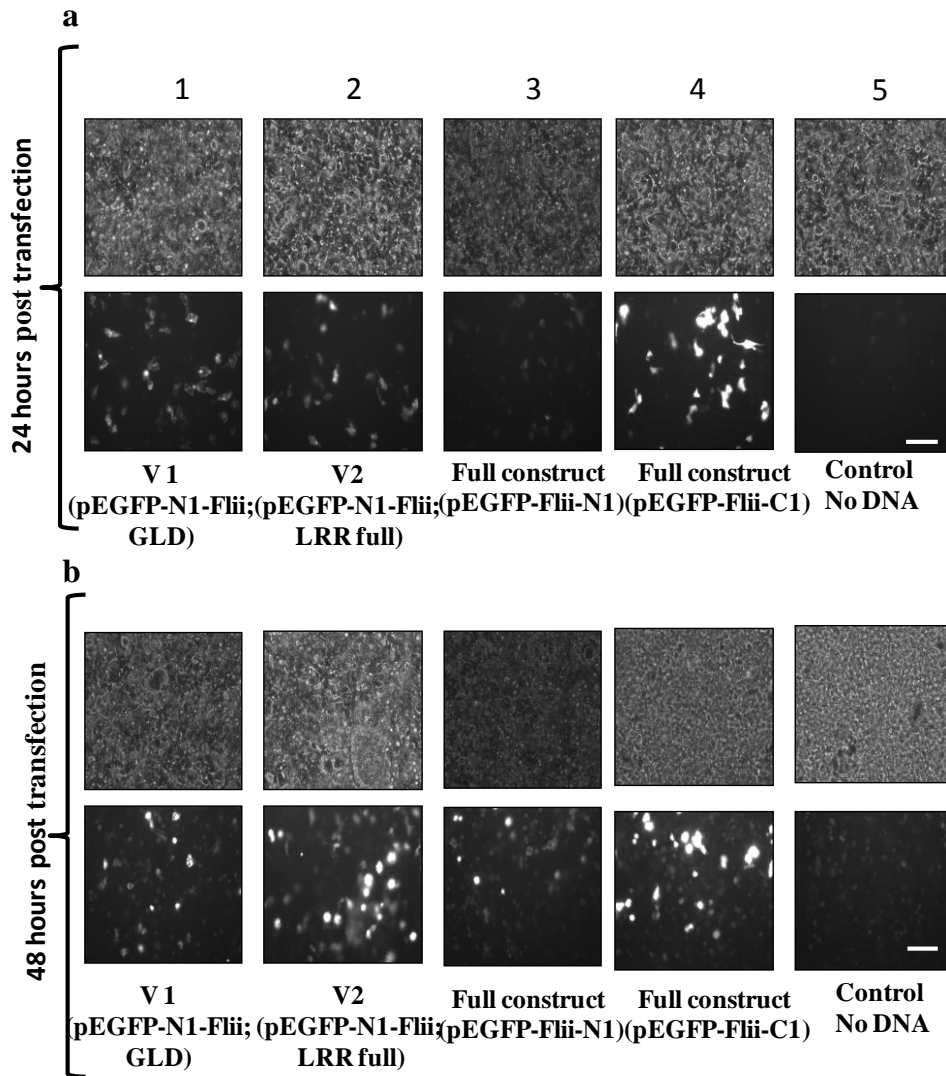
**Figure 5.9. Colchicine blocks the secretion of Flii from fibroblasts.** Human fibroblast cells were incubated with 1 $\mu$ M colchicine, which inhibits microtubule polymerization and disrupts exocytotic activity. Concentrated samples of conditioned medium were separated by SDS-PAGE, immunoblotted and probed with antibodies to Flii. Flii bands were detected in control (no colchicine) samples (n=3) and absent in colchicine-treated samples (n=3) suggesting that colchicine inhibited the release of Flii into the conditioned medium.

### **5.2.7 Overexpression of Flii protein in HEK293T using Flii-GFP constructs**

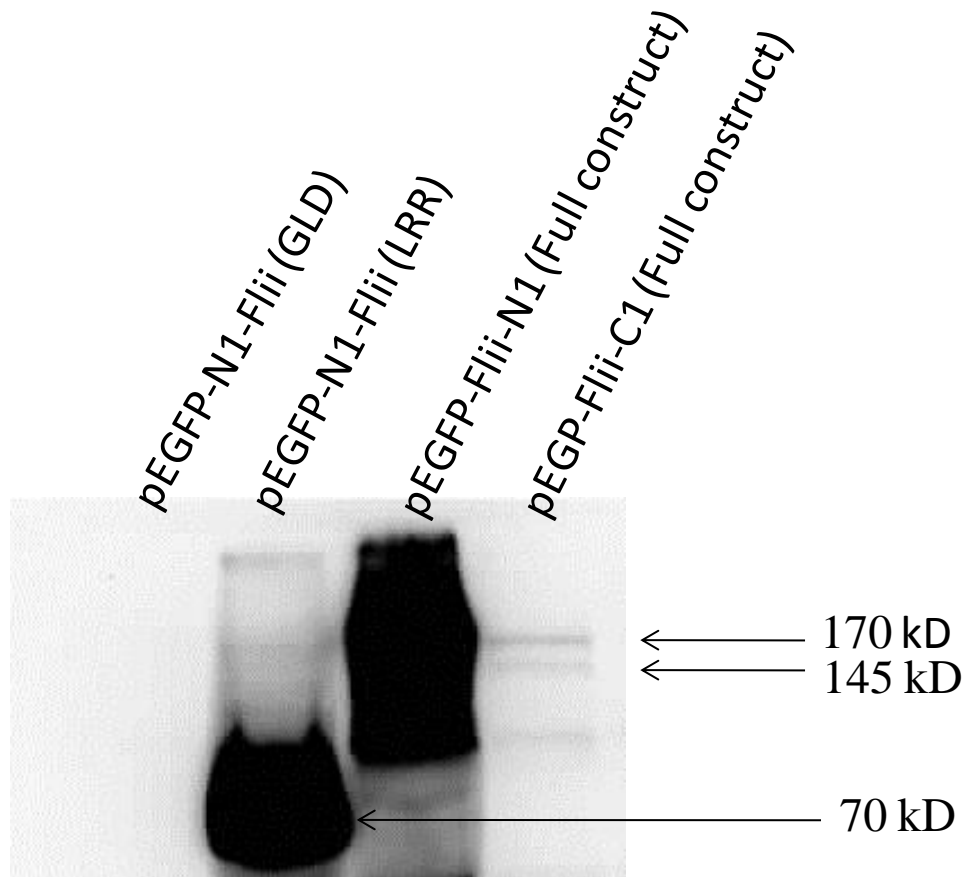
The efficiency of DNA vectors constructed and provided by our collaborator Dr Hugh Campbell (ANU) were tested in HEK293T cells. These vectors were designed to increase the expression of Flii protein. Four DNA vectors were used. For the construction of pEGFP-Flii-N1 vector, the human Flii coding region was fused with the N terminus of the coding region for the enhanced green fluorescent protein (EGFP). The human Flii coding region was fused in frame to the C terminus of the coding region for the enhanced green fluorescent protein EGFP in the making pEGFP-C1-Flii vector (Davy et al. 2001). In two remaining variants of Flii DNA vector only a portion of the human Flii coding region was fused. For example, pEGFP-N1-Flii (gelsolin domain) vector contained only the gelsolin domain of the human Flii coding region, whereas pEGFP-N1-Flii (LRR domain) vector contained only the LRR domain of the human Flii coding region. This separation of the functional regions of Flii allowed us to study the specific functions assigned to LRR and gelsolin domains of Flii protein. Lipofectamine<sup>TM</sup> 2000 assisted transient transfection of HEK293T cells was carried out in 6-well plates. Once transfected the cells were allowed to incubate overnight and the medium was then changed to serum free DMEM and left for 48 hours. EGFP-fluorescence was visualized with the FITC wavelength using fluorescent microscopy. Cells transiently expressing GFP were photographed at two time points (24 and 48 hours) and analysed for transfection efficiency (Figure 5.10). Our results showed that treatment with pEGFP-Flii-C1 resulted in approximately 20% transfection efficiency. Although, all four DNA vectors were able to be transfected, however pEGFP-Flii-C1 vector yielded the best results and produced the most number of GFP positive cells. Incubating the cells for 48 hours post transfection produced greater numbers of GFP-positive cells than leaving them for 24 hours post transfection (Figure 5.10).

### **5.2.8 LRR, but not gelsolin domain is important in Flii secretion**

In addition to its actin-binding, gelsolin-like domain, Flii has a 380 amino acid leucine-rich repeat region (LRR) (Kobe & Deisenhofer 1995), which characterizes the N-terminal domain of the Flii protein. LRR domains mediate protein-protein interactions (Kobe & Kajava 2001). To examine the role of LRR and gelsolin-like domains of Flii protein in transport and secretion we transfected HEK293T cells with vectors pEGFP-N1-Flii (gelsolin domain) or pEGFP-N1-Flii (LRR domain) (Figure 5.11). Extracellular pEGFP-N1-Flii (LRR domain) protein, but not pEGFP-N1-Flii (gelsolin domain) was detected in the media (Figure 5.11). This suggests that LRR domain, but not gelsolin domain is important for the secretion of Flii. The expressed product of two variants with the full-length Flii/GFP was also detected by immunoblotting. HEK293T cells were transfected with pEGFP-Flii-N1 (full-length construct) and pEGFP-Flii-C1 (full-length construct) as described previously (Davy et al. 2001). The product from full-length Flii/GFP plasmid was detected in the media, meaning that these proteins are secreted (Figure 5.11).



**Figure 5.10. Transfection of different Flii vectors.** HEK293T cells were treated with no DNA control and 4 different Flii vectors: pEGFP-N1-Flii (gelsolin domain), pEGFP-N1-Flii (LRR domain), pEGFP-Flii-N1 (full construct), pEGFP-Flii-C1 (full construct). Cells were incubated for 24 hour (a) and 48 hours (b) post-transfection and analysed for transfection efficiency by fluorescence microscope. The ratio of the percentage GFP-positive cells to the starting percentage was determined. HEK293T cells transfected with pEGFP-Flii-C1 (full construct) and incubated for 48 hours post-transfection gave the best transfection efficiency. Scale bar in (a) and (b) is 100  $\mu$ m.



**Figure 5.11. Exogenous pEGFP-N1-Flii (LRR domain) protein, but not pEGFP-N1-Flii (gelsolin domain) was secreted into media.** HEK293T cells were treated with no DNA control and transfected with 4 different Flii vectors: pEGFP-N1-Flii (gelsolin domain), pEGFP-N1-Flii (LRR domain), pEGFP-Flii-N1 (full construct) and pEGFP-Flii-C1 (full construct). The transfected cells were incubated in conditioned media for 48 hours. Media was collected and immunoblotted for Flii. A corresponding band was detected in media taken from pEGFP-N1-Flii (LRR), but not in pEGFP-N1-Flii (gelsolin) suggesting LRR, but not gelsolin domain is important in Flii secretion. Immunoprecipitation of the other two samples resulted in detection of a corresponding band indicating that transfection was efficient and both of the expressed EGFP-Flii fusion proteins are secreted into media.

### 5.3 Discussion

In this chapter we provide evidence for likely pathways through which Flii may be secreted in both live and fixed cells. VAMP-3-positive vesicles and Golgi-related GM130 organelles are implicated in classical secretory pathway (Murray, Kay, et al. 2005). Our intracellular localization studies revealed that Flii does not associate with VAMP3-positive vesicles and Golgi-related GM130 organelles. These observations suggest that Flii does not travel directly to the plasma membrane from the trans-Golgi network. Instead, Flii potentially transits via a late endocytic/lysosomal compartment with Cathepsin D, VAMP7 and Vti1b regulating this trafficking pathway in both primary mouse fibroblasts and human skin fibroblasts.

Cathepsin D protein is secreted via a late endosome/lysosome pathway (Gardella et al. 2001). Early endosomes are intracellular organelles involved in the endocytotic uptake of protein (Mellman 1996) where they are recycled back to the plasma membrane or proceed to late endosomes and eventually to lysosomes (Gu & Gruenberg 1999). Lysosomes are membrane-bound organelles, which have been implicated in secretory pathways whereby the contents of these secretory organelles are released into the extracellular environment (Holt, Gallo & Griffiths 2006). Using primary mouse and human fibroblasts, we showed that Flii co-localises with lysosomal protease Cathepsin D. It has been established that Cathepsin D protein itself is also secreted via a late endosome/lysosome pathway (Gardella, Andrei et al. 2001), which suggests that Flii, which localises to the same compartments as Cathepsin D-positive lysosomes could be secreted via a late endosome/lysosome pathway. Flii also co-localises with SNARE proteins (VAMP7 and Vti1b) which could potentially facilitate secretion through the

endosome/lysosome pathways. It is possible to speculate that trafficking of Flii in skin fibroblast may occur via a non-classical pathway, where secretory organelles such as lysosomes fuse with the plasma membrane and release Flii into the extracellular space.

Unlike conventional secretory cells such as mast cells, which are endowed with specific structures for storage and release, skin fibroblasts use secretory late endocytic/lysosomal compartments to secrete proteins (Stow, Manderson & Murray 2006). Several studies have implicated the importance of SNARE (soluble-N-ethylmaleimide-sensitive-factor accessory-protein (SNAP) receptor) proteins in membrane fusion and protein secretion (Das, Nal et al. 2004; Menager, Menasche et al. 2007; Marcet-Palacios, Odemuyiwa et al. 2008; Veale, Offenhauser et al. 2011). With 38 known members of the mammalian SNARE family (Stow, Manderson et al. 2006), these proteins are present in both the donor and target membranes and have been described as the engines for membrane fusion (Jahn and Scheller 2006). In immune cells, the SNARE proteins are implicated in a number of different trafficking pathways demonstrating that SNARE proteins are important for cytokine secretion (Murray, Kay et al. 2005; Murray, Wylie et al. 2005; Dressel, Elsner et al. 2010). Consistent with SNARE proteins being important for transport of proteins to the plasma membrane and regulation of exocytosis, we explored the possibility of SNARE proteins being involved in the secretory pathway for the trafficking of Flii.

Protein secretion can also be achieved via an unconventional pathway of transport involving microvesicles termed exosomes. These small (50-200 nm) organelles are functionally and phenotypically different to other intracellular vesicles (Murk et al. 2002) and are derived from a specialised compartment of the endosomal-lysosomal pathway (Soo et al. 2012). Exosomes

released into extracellular spaces appear to function in a range of biological processes including the transport and delivery of proteins to recipient cells. For example, the exosomes released into circulation from maturing red cells are the principal source of the soluble, circulating transferrin receptor (TFR) (Johnstone 1996). To explore the possibility that Flii might also be secreted via an exosome-associated pathway, we isolated these microvesicles through multiple cycles of ultracentrifugation (Savina et al. 2003). These studies showed that Flii was present in exosomes suggesting that in addition to the late endosome/lysosome mediated pathway (Lei et al. 2012) Flii may also be secreted via exosomes.

To determine if microtubule formation was important for Flii secretion colchicine was used as an antagonist of microtubule assembly (Abdel Bar et al. 2010). Microtubules form an essential network of filaments in all cells, enabling organelle and protein movement throughout the cell. They are known to function in a variety of cellular processes including exocytosis (Abdel Bar et al. 2010). Given that microtubules are involved in exocytosis and colchicine is a potent antimicrotubular agent (Schnekenburger et al. 2009), we treated skin fibroblasts with colchicine and collected the supernatant. Flii was undetected in conditioned media collected from colchicine-treated cells, suggesting that colchicine inhibited Flii exocytosis. It still remains to be established to what extent microtubules participate in Flii secretion. However, it is tempting to speculate that the inhibition of Flii secretory process occurred via a disruption of microtubular network.

In addition to its actin-binding gelsolin-like domain, Flii has a leucine-rich repeat (LRR) domain, which is thought to facilitate protein-protein interactions (Davy, Ball et al. 2000). Our results suggest that the LRR domain is important for the secretion of Flii as cells transfected with GFP-



tagged Flii-LRR vector, which express Flii-LRR protein, but not Flii-gelsolin protein were secreted into the conditioned media. The N-terminal sequence of Flii is structurally similar to the extracellular leucine-rich repeat (LRR) domain of TLR4 (Wang et al., 2006). The LRR regions of TLRs are important in innate immunity as they detect pathogen molecules such as the lipopolysaccharide (LPS) derived from Gram-negative bacteria (Kumar, Kawai & Akira 2011). Secreted Flii, with its TLR4 like N-terminus binds LPS and negatively regulates cytokine production suggesting that extracellular Flii also has the ability to alter inflammation. (Lei et al. 2012). Other functions of extracellular Flii are still to be described. Gelsolin, when secreted, reduces the immune response by scavenging bacterial wall components (Bucki et al. 2008; Bucki et al. 2005). It remains to be seen if secreted Flii can also act as a scavenger protein that reacts with components of a range of pathogens, which in turn can influence the inflammatory response. In conclusion, the identification of the mechanism via which Flii is secreted from cell will help to reveal the true *in vivo* nature of Flii and assist in understanding its function in wound repair and regeneration.

## *Chapter 6*

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# FLIGHTLESS I PROTEIN DEFICIENCY IMPROVES DIABETIC WOUND REPAIR THROUGH INCREASED ANGIOGENESIS

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Results in this chapter will be included in the manuscript currently in preparation for submission to *Diabetes*

**Title:** Attenuation of Flightless I protein improves healing in a murine model of type 1 diabetes through increased angiogenesis in the wounds

**Authors:** Ruzehaji N, Kopecki Z, Appleby SL, Bonder CS, Fitridge R, Cowin AJ

# FLIGHTLESS I PROTEIN DEFICIENCY IMPROVES DIABETIC WOUND REPAIR THROUGH INCREASED ANGIOGENESIS

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## 6.1 Introduction

Chronic hyperglycaemia in diabetes poses a significant risk for the development of micro and macrovascular complications (Tapp et al. 2003). Because of this, and the association with other known pathological changes including Metabolic syndrome, diabetes driven vascular changes cause reduced nerve perfusion and endoneurial hypoxia leading to neuropathy (Cameron, NE et al. 2001). The prevalence of peripheral neuropathy and peripheral vascular disease (PVD) in the diabetic population interferes with the successful wound closure resulting in skin ulceration (Brem et al. 2007). Up to 15% of all diabetic individuals are predicted to develop foot ulcers (Eldor et al. 2004) and estimated 35% of diabetic ulceration cases are caused by peripheral vascular disease (Boulton 2004).

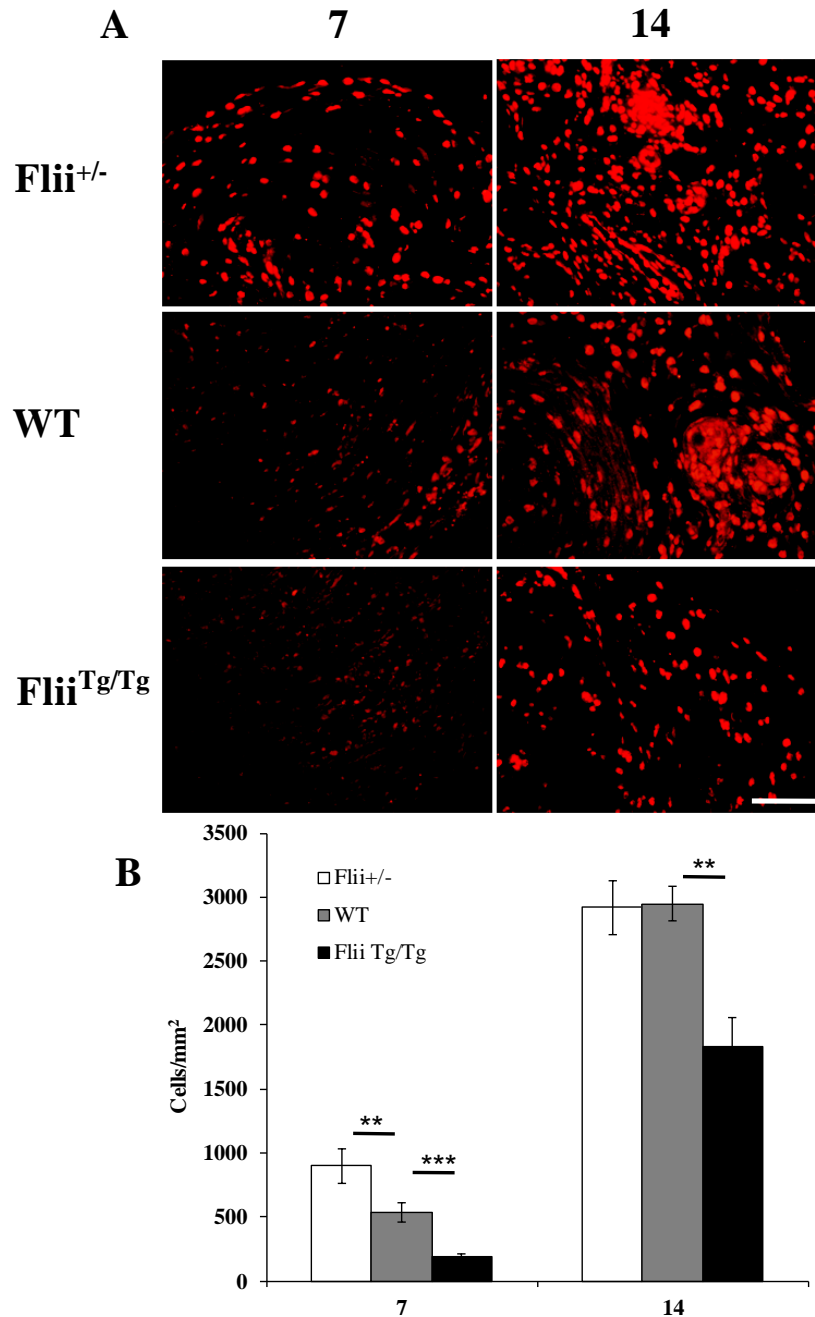
Diabetes impairs angiogenesis, the formation of new blood vessels from pre-existing vessels, which may explain in part the delayed wound healing seen in diabetic individuals and in experimental animal models of diabetes (Carmeliet & Jain 2011; Xue et al. 2010). Angiogenesis plays a key role in wound repair by nourishing local skin tissue with oxygen and nutrients and disposing of metabolic waste. Given that deviations from normal vessel growth contribute to skin ulceration and pathological changes in angiogenesis are involved in the causal pathway to diabetic foot ulceration, in this chapter we investigated the role of Flii in angiogenesis. To determine whether Flii influences angiogenesis and alters healing outcomes we modulated Flii expression by two means: i) genetic manipulation of Flii gene using knockout and transgenic mouse models; ii) Flii-specific neutralising antibodies (FnAb), which bind to extracellular Flii and reduce its local expression. In this chapter, we aimed to establish whether attenuation of Flii within the wound site modulated the angiogenic response. Using *in vitro* and *in vivo*

angiogenesis models we investigated whether FnAbs displayed angiogenic characteristics and whether exogenous application of FnAbs promoted angiogenesis within the diabetic wound.

## 6.2 Results

### 6.2.1 Reducing Flii increases the number of endothelial cells in wounds

To determine the effect of Flii on endothelial cell recruitment, healing wounds from Flii<sup>+/-</sup>, WT and Flii<sup>Tg/Tg</sup> were stained for von Willebrand factor. Von Willebrand factor is a marker of endothelial cells and blood vessels (Sardella et al. 2012). Day 7 wounds in Flii-deficient mice (Flii<sup>+/-</sup>) showed a 1.7-fold increase in the expression of von Willebrand factor compared to WT as determined by immunohistochemistry (Figure 6.1A) suggesting that reducing Flii may promote the formation of new blood vessels. Day 7 wounds from Flii-overexpressing (Flii<sup>Tg/Tg</sup>) mice showed a significantly decreased overall number of von Willebrand factor-positive cells compared with WT and Flii<sup>+/-</sup> wounds (2.8 and 4.7-fold decrease respectively) (Figure 6.1B). Day 14 wounds from Flii<sup>+/-</sup> mice showed significantly increased new blood vessel formation as evidenced by increased von Willebrand factor-positive endothelial cell recruitment compared to Flii<sup>Tg/Tg</sup> wounds.

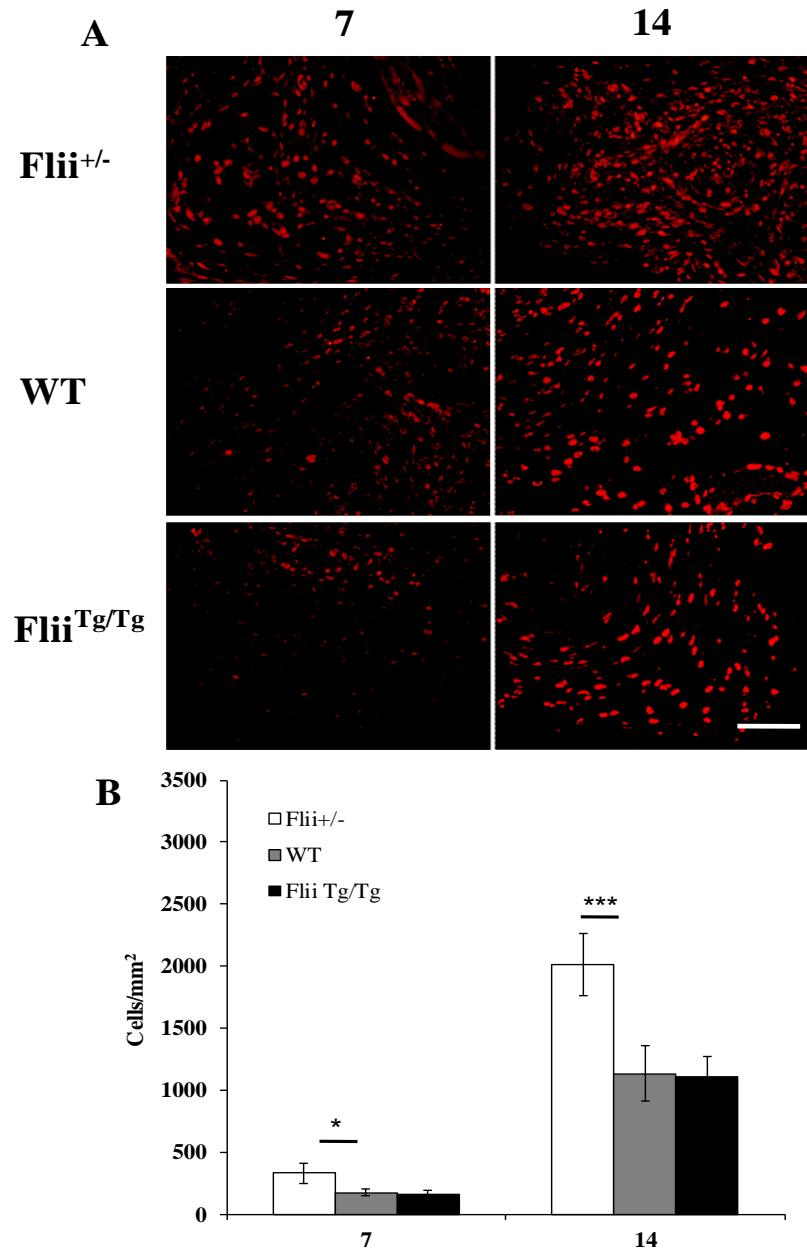


**Figure 6.1. Effects of Flii gene expression on von Willebrand factor expression in excisional wounds.** (A) Representative 7 and 14 days images are shown. Flii<sup>+/-</sup>, WT, Flii<sup>Tg/Tg</sup> wounds were stained with anti-von Willebrand factor antibody. Scale bar 100  $\mu$ m (40 $\times$  magnification). (B) At day 7 Von Willebrand factor staining is highest in the Flii<sup>+/-</sup> wounds. The number of von Willebrand factor-positive cells is significantly reduced in Flii<sup>Tg/Tg</sup> wounds compared to WT and Flii<sup>+/-</sup> (2.8-fold and 4.8-fold respectively). \*\* $p \leq 0.01$  in WT vs. Flii<sup>+/-</sup> wounds; \*\*\* $p \leq 0.001$  in WT vs. Flii<sup>Tg/Tg</sup> wounds; n=6. At day 14 Flii<sup>Tg/Tg</sup> wounds showed significantly decreased levels of von Willebrand factor compared to WT and Flii<sup>+/-</sup> wounds (\*\* $p \leq 0.01$ ; WT vs. Flii<sup>Tg/Tg</sup>; n=6).



### 6.2.2 Increased levels of Flii attenuate the number of endothelial cells

Von Willebrand factor, a glycoprotein produced uniquely by endothelial cells, is used routinely to identify vessels in tissue sections (Zanetta et al. 2000). We examined the effect of attenuating Flii gene expression on endothelial cell numbers by staining Flii<sup>+/-</sup>, WT and Flii<sup>Tg/Tg</sup> wounds for von Willebrand factor. Under normal conditions, Flii overexpression lead to significantly reduced new blood vessel formation as evidenced by reduced von Willebrand factor protein expression (Figure 6.1B). Diabetes reduced new blood vessel formation causing a 2.7 fold decrease in von Willebrand expression in day 7 diabetic wounds with reduced Flii (Flii<sup>+/-</sup>) compared to day 7 non-diabetic Flii<sup>+/-</sup> wounds (Figure 6.1B and 6.2 B). Defective recruitment of von Willebrand factor-positive endothelial cells in diabetic mouse wounds has previously been described (Albiero et al. 2011). Low Flii lead to markedly increased new blood vessel formation as evidenced by increased endothelial cell numbers in diabetic wounds compared to diabetic WT controls suggesting that Flii may play a role in endothelial cell recruitment and angiogenesis (Figure 6.2B).

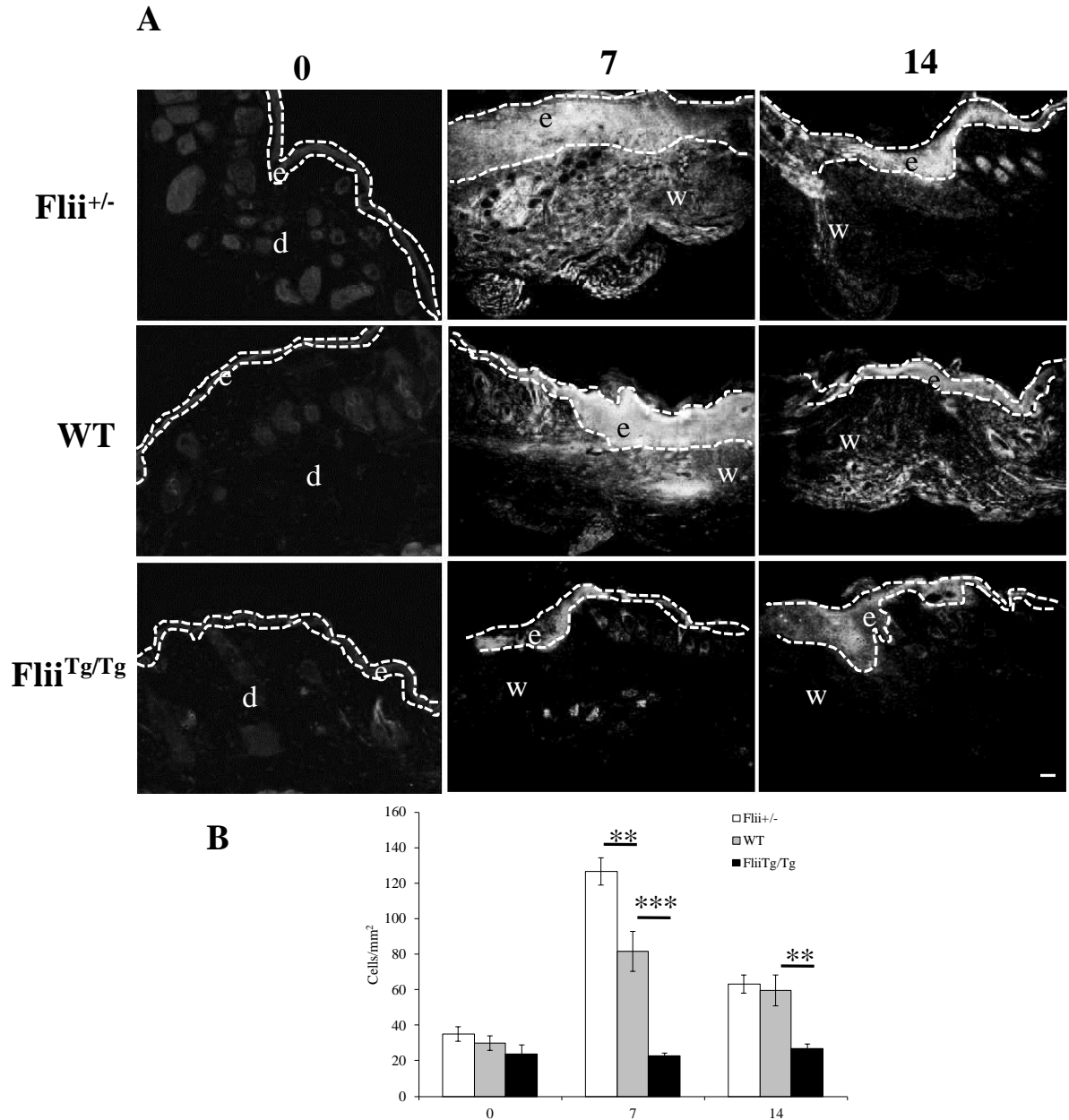


**Figure 6.2. The effects of Flii and diabetes on von Willebrand factor expression.** (A) Representative sections of diabetic wounds harvested 7 and 14 days post-wounding. Flii<sup>+/-</sup>, WT, Flii<sup>Tg/Tg</sup> wounds were stained with anti-von Willebrand factor antibody. Scale bar 100  $\mu$ m (40 $\times$  magnification). (B) Immunohistochemical analysis of von Willebrand factor-positive vasculature in diabetic wounds. Diabetes changed the expression of von Willebrand factor in Flii<sup>+/-</sup>, WT, Flii<sup>Tg/Tg</sup> wounds. The difference was associated with a 2.7 decrease in endothelial cell recruitment in diabetic wounds compared to non-diabetic wound. Flii<sup>+/-</sup> diabetic wounds showed the highest Von Willebrand factor staining at day 7 and day 14. (\*  $p \leq 0.05$ , day 7 diabetic Flii<sup>+/-</sup> vs. day 7 diabetic WT; \*\*\*  $p \leq 0.001$ , day 14 diabetic Flii<sup>+/-</sup> vs. day 14 diabetic WT; n=6).

### 6.2.3 Low levels of Flii enhance VEGF expression during wound healing

Angiogenesis is an essential component of wound healing resulting in the formation of blood vessels which supply oxygen and other nutrients to the disturbed tissue. VEGF regulates angiogenesis, stimulates vascular development and promotes vessel branching (Carmeliet & Jain 2011). Because VEGF is a predominant stimulator of angiogenesis, day 0, 7 and 14 wounds  $Flii^{+/-}$ , WT,  $Flii^{Tg/Tg}$  were stained to identify VEGF expression (Figure 6.3A). Although insignificant, Flii deficiency lead to a small increase in VEGF expression in day 0 WT non-diabetic wounds compared to day 0  $Flii^{+/-}$  non-diabetic wounds (Figure 6.3B and Table 6.1).

At 7 days post-wounding VEGF was observed throughout the granulation tissue within the wounds, with the distribution being highest in the wound centre (Figure 6.3A). Flii overexpression caused a significant reduction of VEGF expression at both day 7 and day 14 post-wounding with VEGF expression in  $Flii^{Tg/Tg}$  wounds being 5-fold higher compared to WT at 7 days post-wounds (Figure 6.3B). The overall expression of VEGF was lower at day 14 post-wounding compared to day 7 post-wounding. Flii overexpression caused a 2.4-fold decrease in VEGF expression at 14 days post-wounding suggesting that Flii may play a role in angiogenesis (Figure 6.3B).

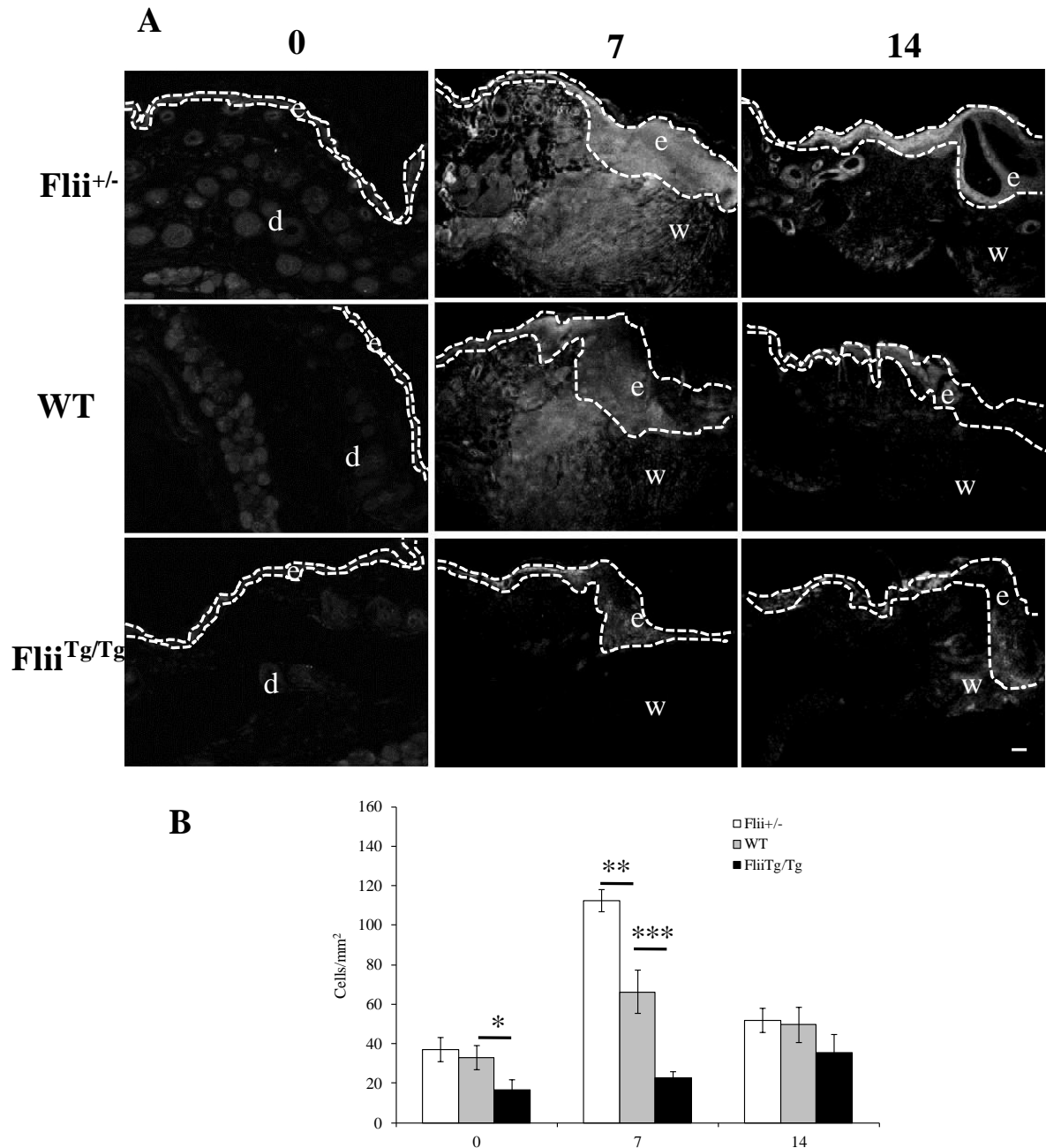


**Figure 6.3. Effects of Flii on VEGF expression in acute wounds.** (A) Representative images of wounds stained with anti-VEGF antibody when viewed under the fluorescent microscope. Displayed images were photographed at lower magnification ( $\times 10$ ) and the boxed regions (smallest box inside the image) are displayed at higher magnification ( $\times 40$ ) in the lower left corner. Scale bar =  $100 \mu\text{m}$  ( $\times 10$  magnification); scale bar =  $50 \mu\text{m}$  ( $\times 40$  magnification). (B) Sections of day 0, 7 and day 14 wounds were stained with anti-VEGF antibody and images taken with  $\times 10$  objective lens were used for immunofluorescence analysis. The images were evaluated and the number of VEGF-positive cells (expressed as cells per  $\text{mm}^2$ ) was identified. Flii overexpression was associated with significant downregulation of VEGF ( $*** p \leq 0.001$  at day 7 for Flii<sup>Tg/Tg</sup> vs. WT;  $** p \leq 0.01$  at day 14 for Flii<sup>Tg/Tg</sup> vs. WT;  $n=6$ ).

#### **6.2.4 Flii deficiency up-regulates pro-angiogenic VEGF expression in diabetics**

Diabetes causes pathological angiogenesis and restricted activity of VEGF (Carmeliet and Jain 2011). To determine the effect of Flii on VEGF production in diabetic mouse wounds we investigated the expression of this pro-angiogenic growth factor using immunohistochemistry. (Figure 6.4A and Table 6.1).

Flii deficiency was associated with a significant increase in VEGF expression in day 0 WT diabetic wounds compared to day 0 Flii<sup>Tg/Tg</sup> diabetic wounds (Figure 6.4B). Although insignificant, diabetes lead to a small decrease in VEGF expression in day 7 WT diabetic wounds compared to day 7 WT non-diabetic wounds (Figure 6.3B and 6.4B). However, we observed a marked increase of VEGF expression in Flii<sup>+/-</sup> wounds compared to WT at day 7 post-wounding. Flii deficiency was associated with a 1.7-fold increase compared to WT. By contrast, Flii overexpression resulted in a 3-fold decrease in VEGF expression compared to WT at day 7 post-wounding suggesting that reducing Flii, at least partially, may enhance diabetes-impaired VEGF expression in wounds. Day 14 Flii<sup>Tg/Tg</sup> diabetic wounds showed lower levels of VEGF expression compared to WT, however did not reach significance.



**Figure 6.4. VEGF expression in diabetic wounds of Flii<sup>+/-</sup>, WT, Flii<sup>Tg/Tg</sup> mice.** (A) Full-thickness excisional wounds were created on the backs of diabetic Flii<sup>+/-</sup>, WT, Flii<sup>Tg/Tg</sup> mice. Wound harvested at 7 and 14 days post-wounding were stained with anti-VEGF antibody. Representative images of VEGF at 0, 7 and 14 days post-wounding. Displayed micrographs were taken with  $\times 10$  objective and the boxed regions represent higher magnification ( $\times 40$ ) images. Scale bar = 100  $\mu\text{m}$  ( $\times 10$  magnification); scale bar = 50  $\mu\text{m}$  ( $\times 40$  magnification). (B) Graph showing the number of VEGF-positive cells per  $\text{mm}^2$  in diabetic wounds. Results represent means  $\pm$  SEM,  $n = 6$  wounds per mice group per time-point. \*\*  $p \leq 0.05$  Flii<sup>Tg/Tg</sup> vs. WT (day 0); \*\*  $p \leq 0.01$  Flii<sup>+/-</sup> vs. WT (day 7); \*\*\*  $p \leq 0.001$  Flii<sup>Tg/Tg</sup> vs. WT (day 7).

	<b>Controls</b>		
<b>Day</b>	<b>0</b>	<b>7</b>	<b>14</b>
Flii <sup>+/-</sup>	35±4.1	126.5±7.6	63.2±5
WT	30±4.2	81.4±11.2	59.5±8.6
Flii <sup>Tg/Tg</sup>	24±5.1	22.5±1.6‡	26.6±2.9§

	<b>Diabetic</b>		
<b>Day</b>	<b>0</b>	<b>7</b>	<b>14</b>
Flii <sup>+/-</sup>	37±6.1	112.5±5.6‡	51.6±6.2
WT	33±6.2	66±10.9	49.5±8.8
Flii <sup>Tg/Tg</sup>	16.5±5.2*	22.5±3.3‡	35.5±9

Values are mean ±S.E.M.

\* $p \leq 0.05$  different to WT in the same group (T-test)

§  $p \leq 0.01$  different to WT in the same group (T-test)

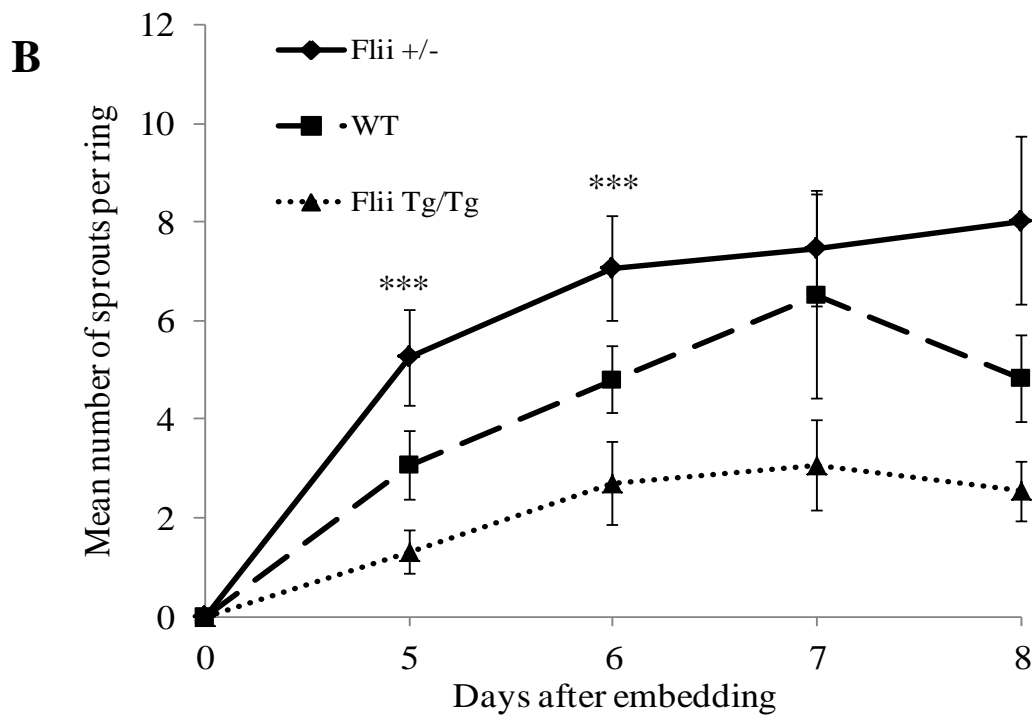
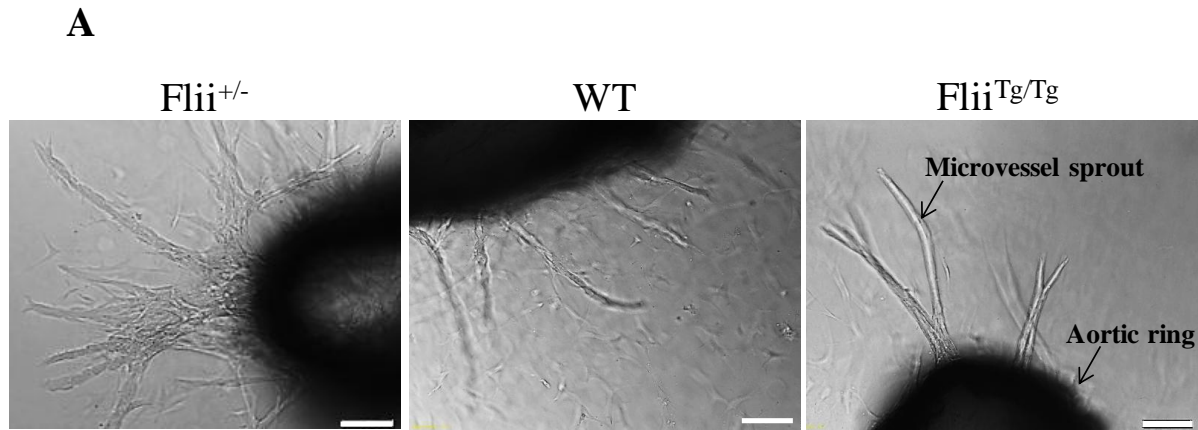
‡  $p \leq 0.001$  different to WT in the same group (T-test)

**Table 6.1. Comparison of VEGF protein expression in mouse non-diabetic control and diabetic wounds at 0, 7 and 14 days post-wounding.**

### 6.2.5 Pro-angiogenic effects of reduced Flii expression in *ex vivo* aortic ring assay

To investigate the relative role of Flii in angiogenesis we examined the effect of altering Flii gene expression using Flii<sup>+/-</sup>, WT, Flii<sup>Tg/Tg</sup> mice in a quantitative three-dimensional *ex vivo* mouse aortic ring angiogenesis assay. The aortic ring assay enables the assessment of various steps of angiogenesis including cellular proliferation, migration, quantification of developing microvessels and vessel branching (Baker et al. 2012). Aortae collected from 4 week old Flii<sup>+/-</sup>, WT and Flii<sup>Tg/Tg</sup> mice (n = 6 in each group) were cut into rings (15 rings per mouse aorta) and embedded in type 1 collagen (Figure 6.5A). Endothelial microvessel sprouts grown from the rings were counted at days 0, 5, 6, 7 and 8 post-embedding. Significant increases in microvessel sprouting were observed in Flii<sup>+/-</sup> rings compared with WT controls at day 5 and day 6 post-embedding (\*\*\*  $p \leq 0.001$ ; day 5 and day 6 for Flii<sup>+/-</sup> vs. WT). Significantly reduced sprouting was observed in Flii<sup>Tg/Tg</sup> rings isolated from aortae of Flii<sup>+/-</sup> and WT mice (\*\*\*  $p \leq 0.001$ ; days 5 and 6 for Flii<sup>Tg/Tg</sup> vs. WT). The results of this physiologically relevant assay, which mimics the stages of angiogenesis in a timescale similar to that observed *in vivo*, showed that Flii inhibits angiogenesis and that reducing Flii leads to a pro-angiogenic response (Figure 6.5B).

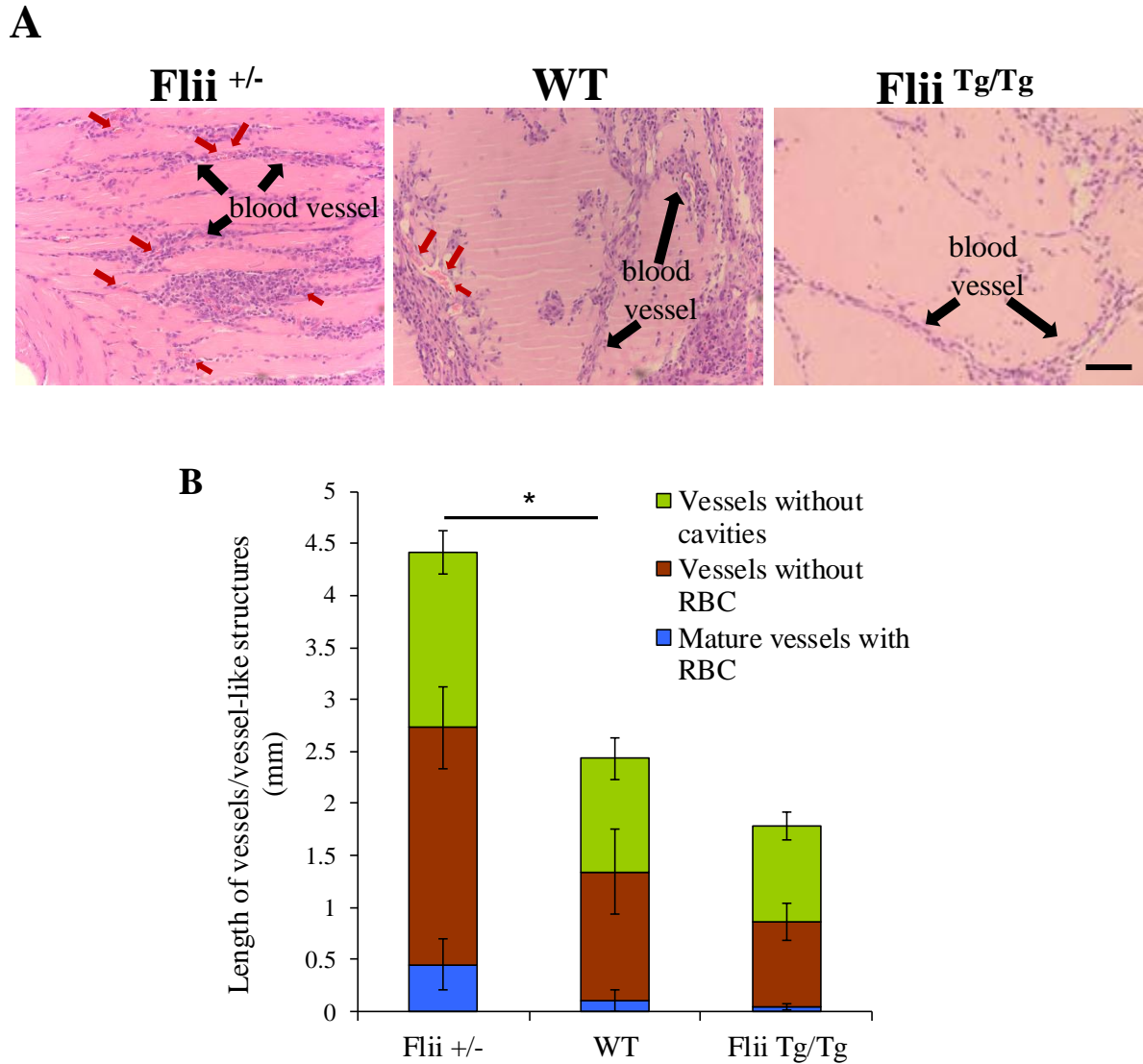




**Figure 6.5. Flii inhibits endothelial microvessel sprouting.** (A) Phase-contrast images of aortic rings embedded in type I collagen showing microvessel outgrowth at day 6 after embedding. Scale bar 100  $\mu$ m. original magnification 10  $\times$ . (B) Time course of microvessel sprouting from aortic rings collected from aortae of Flii<sup>+/-</sup>, WT, Flii<sup>Tg/Tg</sup> mice aged 4 weeks. Microvessel sprouts were counted from days 5 to 8 after embedding in type 1 collagen. Bars represent the mean number of microvessel sprouts  $\pm$  s.e.m. Assay quantification (n = 6; 15 rings per mouse aorta). \*\*\*  $p \leq 0.001$ ; Flii<sup>+/-</sup> vs. WT on days 5 and 6.

### 6.2.6 Attenuation of Flii gene expression improved new blood vessel formation *in vivo*

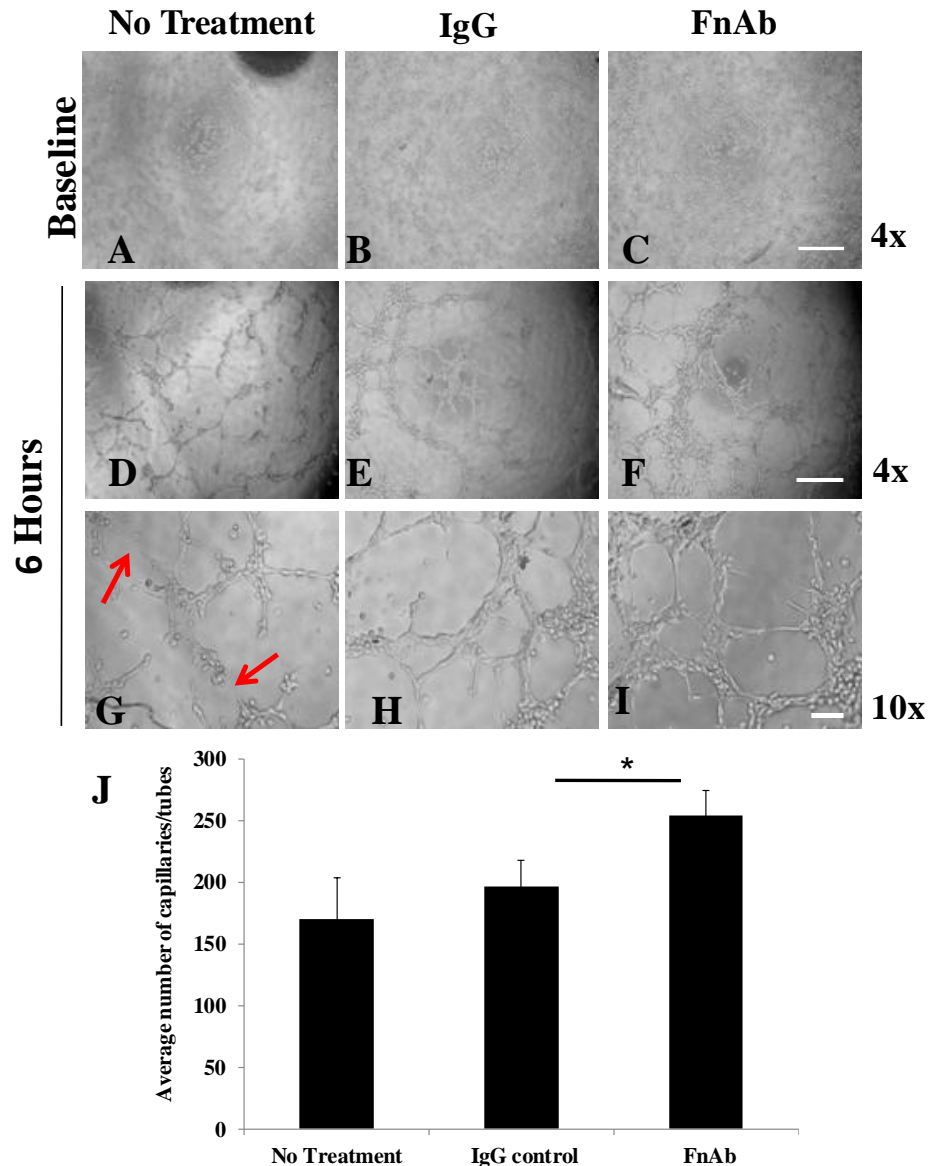
Given that Flii is involved in cellular migration we hypothesised that it could also be vital in the endothelial cell motility required during angiogenesis. To determine whether Flii affects angiogenesis *in vivo*, we inserted the Matrigel plugs under the skin of Flii<sup>+/-</sup>, WT and Flii<sup>Tg/Tg</sup> mice (Figure 6.6). In this *in vivo* angiogenesis model, the Matrigel plugs were examined for the ingrowth of new blood vessels. Newly formed vessels and vessel-like structures, which had grown into the Matrigel plugs were quantified by measuring the average length of new blood vessels (Figure 6.6A). The vessels were separated into 3 groups and classified as previously described (Kano, Morishita et al. 2005). The length of newly formed vessels was quantified and categorized into three groups: (1) vessels containing red blood cells (RBC), (2) vessels without RBCs, (3) vessel-like structure but without a cavity. In this *in vivo* model of angiogenesis, reduced expression of Flii (Flii<sup>+/-</sup>) resulted in 4-fold increase in the length of functional vessels containing RBCs compared to WT (Figure 6.6B). The length of vessels with cavities, but without the RBCs was also significantly higher in Flii<sup>+/-</sup> mice than their WT counterparts (\*  $p \leq 0.05$  in Flii<sup>+/-</sup> vs. WT; \*\*  $p \leq 0.01$  in Flii<sup>+/-</sup> vs. Flii<sup>Tg/Tg</sup>; n=6). Genetic overexpression of Flii substantially influenced the length of the newly formed vessels within the cavities suggesting that Flii may give a negative effect on angiogenesis and neovascular formation (\*  $p \leq 0.05$  in Flii<sup>+/-</sup> vs. WT; \*\*  $p \leq 0.01$  in Flii<sup>+/-</sup> vs. Flii<sup>Tg/Tg</sup>; n=6).



**Figure 6.6. Effect of altering Flii on angiogenesis using the Matrigel plug assay *in vivo*.** Matrigel plugs were subcutaneously injected into Flii<sup>+/-</sup>, WT and Flii<sup>Tg/Tg</sup> mice. (A) After 7 days Matrigel plugs were extracted, stained using H&E and examined under the light microscope. Representative H&E stained images of Matrigel plugs show newly formed vessels *in vivo*. Black arrow heads indicated the vessels containing the RBCs. Scale bar 100  $\mu$ m. (B) Quantification of vessel length (mm). Vessels and vessel-like structures were classified as follows: (1) mature blood vessels containing RBCs (blue), (2) vessels with cavities but without RBCs (red), (3) cells arraying in line but without cavities (green). \*  $p \leq 0.05$  for Flii<sup>+/-</sup> vs. WT (n=6) in 2 categories: (1) vessels with cavities but without RBCs and (2) vessels without cavities.

### **6.2.7 Pro-angiogenic effects of FnAb *in vitro***

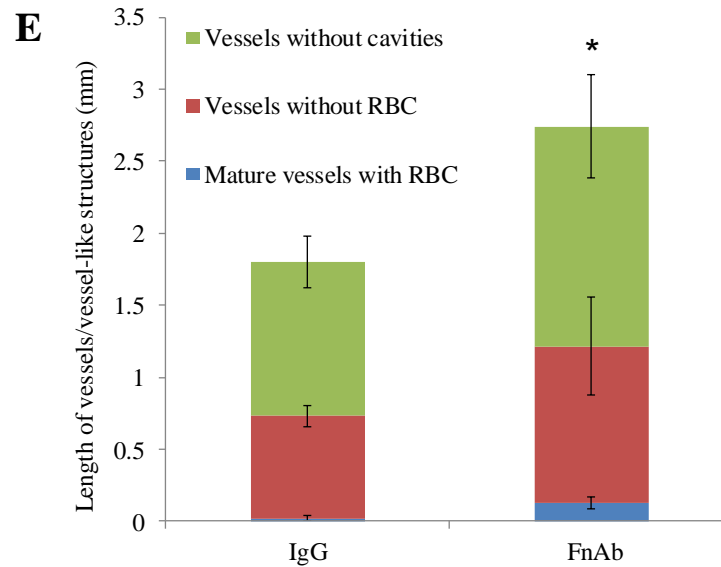
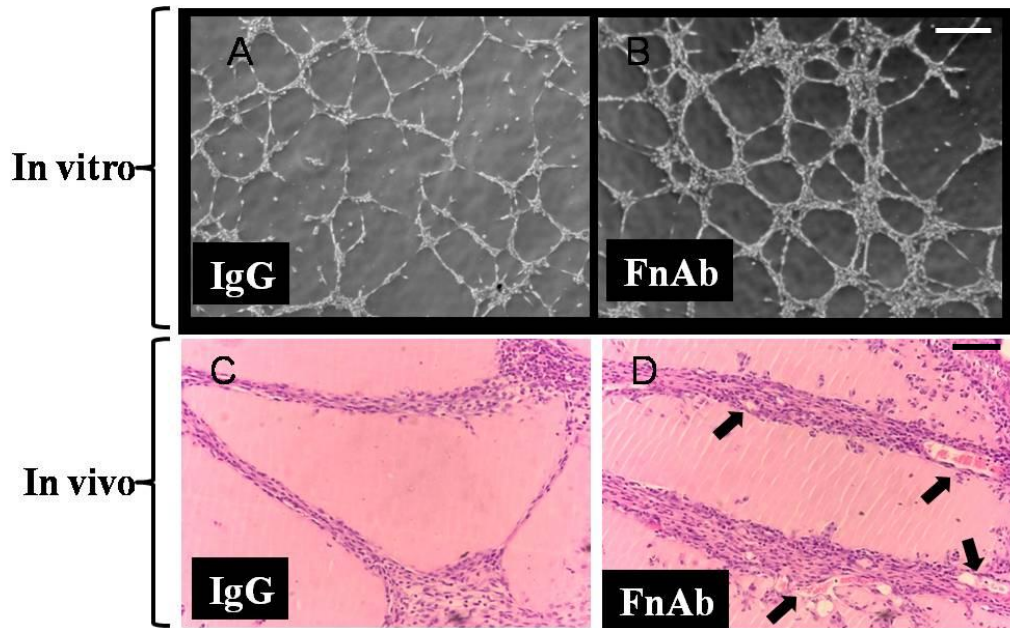
Having established the inhibitory effect of Flii on angiogenesis we speculated that administration of FnAb, which mops up Flii protein might promote vascularization in injured tissue. We first used an *in vitro* model of angiogenesis, in which the morphological events and the time course of changes have been characterized (West et al. 2010). To determine whether FnAb had the capacity to enhance capillary tube formation *in vitro*, we plated human vascular endothelial cells (HUVEC) on a 2D matrix of Matrigel in the presence of FnAb (50 µg/ml) or an isotype matched control (irrelevant IgG) at a final concentration of 50 µg/ml (Figure 6.7A-I). Assessment of capillary tube formation of the FnAb-treated HUVEC showed that these cells reorganized into large aggregates with subsequent capillary tubes (Figure 6.7I) suggesting an enhanced ability to reorganize the endothelial cell actin cytoskeleton. The FnAb-treated HUVEC formed significantly more capillary tubes compared to IgG-treated controls or no treatment (Figure 6.7A-I). The FnAb-treated cells showed a 1.3-fold increase (n=4) in the number of capillary tubes formed compared to IgG control (Figure 6.7J), thus confirming the importance of Flii in angiogenesis.



**Figure 6.7. Capillary tube formation is increased when Flii levels are reduced.** HUVECs were incubated with either FnAb (50  $\mu\text{g/ml}$ ) or dose-matched control antibody (IgG: 50  $\mu\text{g/ml}$ ) for 1 hour. The cells were harvested and plated onto Matrigel in the presence of either FnAb or IgG control. Capillary tube formation was monitored for 6 hours and images were taken to compare the number of capillary tubes formed for each treatment group. Representative phase-contrast microscopic images (A-C, 30 min after plating, 4 $\times$  magnification; D-F, 6 hours after plating, 4 $\times$  magnification; G-I, 6 hours after plating 10 $\times$  magnification) of capillary tubes formed *in vitro*. Scale bar A-f = 100  $\mu\text{m}$ ; scale bar in I refers to 200  $\mu\text{m}$ . (J) Quantification of number of capillary tubes based on 4 $\times$  images taken at 6 hours and grown on Matrigel in the absence of antibody (no treatment), in the presence of IgG control (50  $\mu\text{g/ml}$ ) or FnAb (50  $\mu\text{g/ml}$ ). \*  $p \leq 0.05$ ; IgG vs. FnAb; n=4.

### 6.2.8 Reducing Flii using FnAb enhances formation of mature blood vessels *in vivo*

To assess whether the pro-angiogenic properties of FnAb observed using the *in vitro* capillary tube formation assay (Figure 6.7) could be replicated *in vivo* we used an *in vivo* model of angiogenesis – Matrigel plug assay. Matrigel plugs were mixed with either IgG control (50 µg/ml) or FnAb (50 µg/ml) and injected under the skin of the abdominal area of WT mice. Each mouse received two Matrigel plugs – one plug contained IgG control (50 µg/ml) and the other contained FnAb (50 µg/ml). The Matrigel plugs were harvested 7 days post-injection. Formalin fixed and paraffin embedded sections of IgG control or FnAb-supplemented Matrigel plugs were stained using H&E and examined for the ingrowth of new blood vessels (Figure 6.8). New blood vessels and vessels and vessel-like structures in the Matrigel plugs were quantified by measuring their lengths. The vessels were categorized into three groups: (1) vessels containing red blood cells (RBC), (2) vessels without RBCs, (3) vessel-like structure but without a cavity. In an *in vivo* model of angiogenesis, FnAb resulted in a 4-fold increase of the length of functional vessels, which contained RBCs compared to IgG control (Figure 6.8C, D). Addition of FnAb at 50 µg/ml into Matrigel plugs in this *in vivo* assay enhanced the formation of new blood vessels (Figure 6.8E). The FnAb treatment was most successful in inducing functional blood vessels, which contained RBCs (Figure 8E). Significantly more RBC-containing vessels were formed with FnAb treatment than with stimulation with IgG control (Figure 6.8 E; \*  $p \leq 0.05$ ; n=6). Significantly longer vessels without cavities were formed in FnAb-treated Matrigel plugs compared with IgG-treated controls (Figure 6.8E; \*  $p \leq 0.05$ ; n=6).

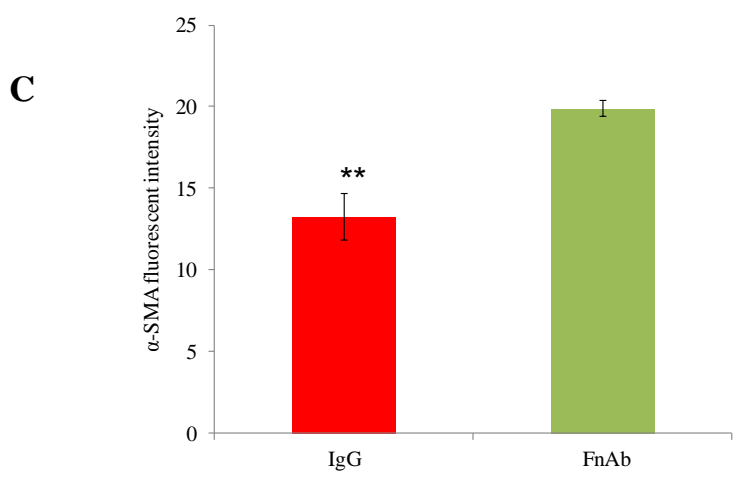
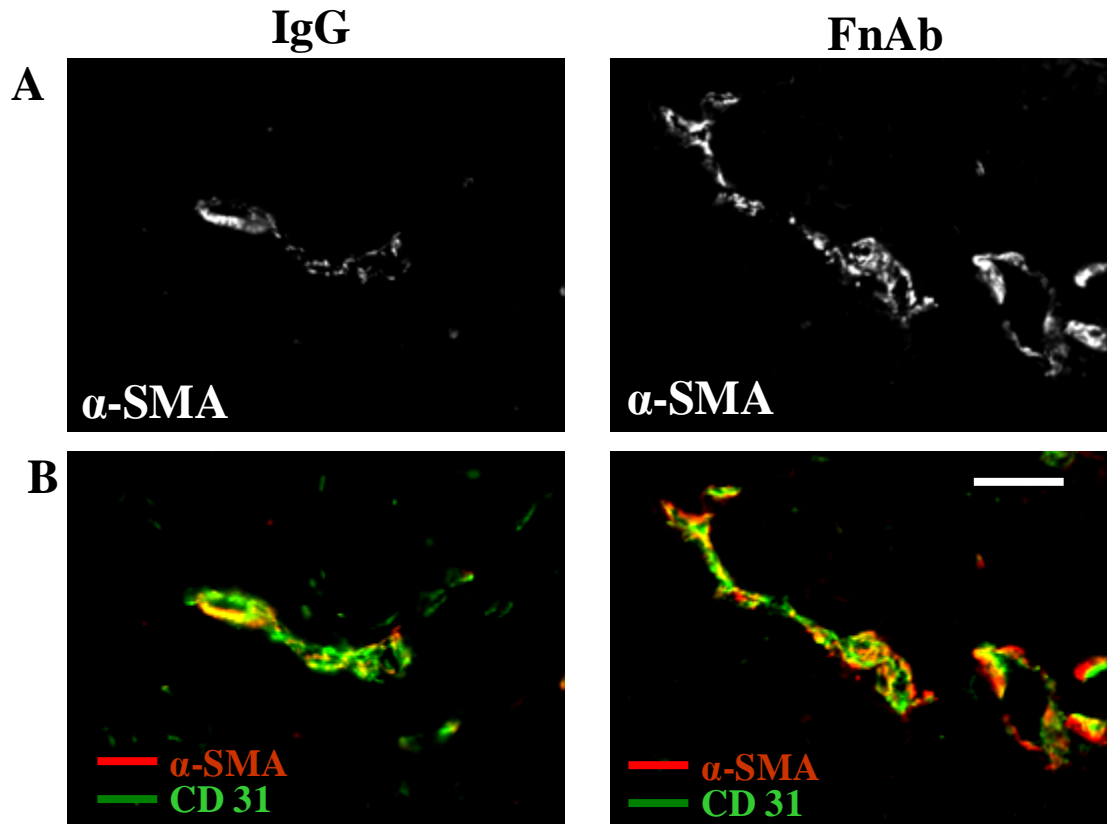


**Figure 6.8. Angiogenic efficacy of FnAb in mice using the *in vivo* Matrigel plug assay.** (A-B) Comparison of effects of IgG and FnAb stimulation *in vitro* (Matrigel tube formation assay) and (C-D) *in vivo* (Matrigel plug assay). Scale bar in A-B is 200  $\mu\text{m}$  ( $4 \times$  magnification); B-C is 100  $\mu\text{m}$  ( $10 \times$  magnification). (E) Quantification of the formation of new vessels in Matrigel plugs by their lengths. Stimulation with FnAb resulted in formation of significantly longer RBC-containing vessels and vessels without cavities compared with IgG control (\*  $p \leq 0.05$ ;  $n = 6$ )

### **6.2.9 FnAb contributes to wound vascularization and enhanced $\alpha$ -SMA<sup>+</sup>/CD31<sup>+</sup> cell recruitment *in vivo***

Based on the promising observation made *in vitro* and *in vivo* assays of angiogenesis, we proceeded to analyse the  $\alpha$ -SMA-positive cell expression in newly formed vessels growing in Matrigel plugs. Matrigel plugs supplemented with either IgG control (50  $\mu$ g/ml) or FnAb (50  $\mu$ g/ml) were injected under the skin of WT mice and harvested at day 7 post-injection. The Matrigel plugs were embedded in OCT medium, frozen and stored at  $-80^{\circ}\text{C}$ . Cryosections of Matrigel plugs were stained with anti-CD31/PECAM-1 (endothelial cell marker) and anti- $\alpha$ -SMA (pericyte marker) antibodies. Vessel stabilization is maintained by  $\alpha$ -SMA-positive pericytes (Carmeliet 2003). The measurement of pericyte markers such as  $\alpha$ -SMA (Baker et al. 2012) provides an indication of the level of vessel stability, because insufficient recruitment of these cells results in vessel fragility, leakiness and tendency to rupture and bleed (Carmeliet 2003). Matrigel plugs premixed with FnAb and IgG control antibodies (both at 50  $\mu$ g/ml) were stained with antibodies to establish the level of pericyte ( $\alpha$ -SMA) and endothelial cell (CD31/PECAM-1) recruitment (Figure 6.9A-B). The number of cells positive for  $\alpha$ -SMA were lower in IgG-treated Matrigel plugs compared to FnAb-treated Matrigel plug sections, suggesting that FnAb has the capacity to promote  $\alpha$ -SMA-positive pericyte recruitment and, hence enhance stabilization of nascent blood vessels *in vivo* (Figure 6.9B; \*\*  $p \leq 0.01$ ; n=6).

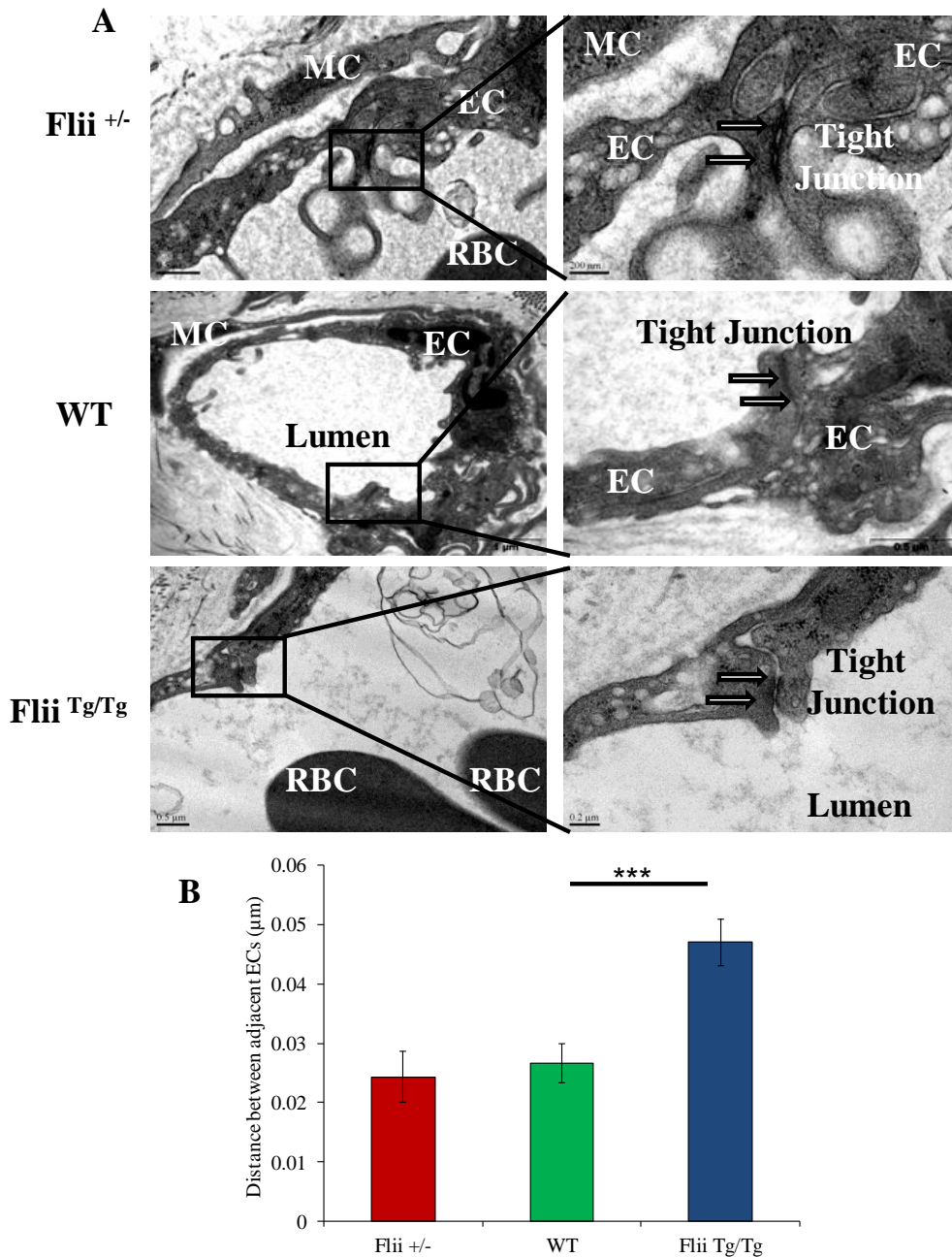




**Figure 6.9. Immunofluorescence staining of IgG and FnAb-treated Matrigel plugs.** (A) Two plugs (500  $\mu$ l each) of Matrigel pre-mixed with either IgG or FnAb (50  $\mu$ g/ml) were injected into the left and right side of the abdominal region of WT mice. Each plug was harvested on day 7 and snap frozen. (A-B) Formalin fixed cryosections of Matrigel plugs were stained with anti-murine PECAM1 (CD31) (green) and  $\alpha$ -SMA (red) antibodies. (C) Quantification of fluorescence intensity of  $\alpha$ -SMA expression in IgG and FnAb-treated Matrigel plugs. \*\*  $p \leq 0.01$ ; results represent mean  $\pm$  s.e.m. (n=6).

### 6.2.10 Increased Flii loosens endothelial cell interactions

Electron microscopy was employed to further examine the Matrigel plugs retrieved at day 7 post-subcutaneous injections from Flii<sup>+/-</sup>, WT and Flii<sup>Tg/Tg</sup> mice. The blood vessels in all Matrigel plugs were visualized at 3800× and 8800× magnification. Electron microscopic studies revealed that the endothelial cells in Flii<sup>Tg/Tg</sup> group failed to attach to each other and failed to form tight junctions (Figure 6.10A). The distance between the nearest edges of endothelial cells and their tight junctions were measured (Figure 6.10B). Partial knockout of Flii gene had a positive effect on tight junction formation and resulted in significantly smaller distances between adjacent endothelial cells compared to Flii<sup>Tg/Tg</sup> group (Figure 6.10B; \*\*\*  $p \leq 0.001$ ; Flii<sup>+/-</sup> vs. Flii<sup>Tg/Tg</sup>). Flii gene overexpression resulted in a 2-fold increase in distance between adjacent endothelial cells suggesting that Flii may play a role in loosening endothelial cell interaction and antagonizing vessel stabilization (Figure 6.10B).



**Figure 6.10. Electron microscopy and genetic manipulation of Flii *in vivo* Matrigel plug assay.** Matrigel plugs were retrieved from Flii<sup>+/-</sup>, WT and Flii<sup>Tg/Tg</sup> mice at day 7 post-injection and fixed in 2.5% glutaraldehyde. All sections were visualized under at 3800 × and 8800 × magnification. (A) Electron micrographs. Scales indicated in each photograph. MC, mural cells; EC, endothelial cells; RBC, red blood cells, black arrows, tight junctions formed between endothelial cells. (B) Quantification of the distance between adjacent endothelial cells. \*\*\*  $p \leq 0.001$ ;  $n = 6$  (Flii<sup>+/-</sup> vs. Flii<sup>Tg/Tg</sup> and Flii<sup>Tg/Tg</sup> vs. WT).

### **6.3 Discussion**

Diabetes mellitus is a risk factor for both microvascular and macrovascular changes in humans (Olieslagers et al. 2011). The risk of developing peripheral vascular disease is 5-fold higher in diabetic individuals and impaired wound healing which results in ulcers and subsequent amputations are severe complications of diabetes (Rivard et al. 1999). New vessel formation and vascular network expansion - collectively known as angiogenesis (Carmeliet & Jain 2011) promote tissue repair and accelerate wound closure (West et al. 2010). The underlying mechanisms of impaired angiogenesis in diabetic wounds remain largely unclear, hence identification of molecules involved in the pathogenesis of diabetic wound healing is an important step in the development of new therapies that will decrease the incidence of diabetes-related lower-limb amputation.

Actin cytoskeleton reorganization is vital in cellular migration and Flii-deficient skin cells showed improved migration (Cowin, AJ et al. 2007). Vascular cell migration is an important component of new vessel formation and vascular maturation, all of which are pivotal for wound healing (Francis-Goforth, Harken et al. 2010).

Our studies demonstrated that Flii gene overexpression hinders angiogenesis, whereas Flii gene knockdown increases vascular growth in mouse model of diabetes induced by STZ. Given that Flii-deficient mice showed improved angiogenesis, we proceeded to use Flightless neutralizing antibodies (FnAb) as a means of decreasing local expression of Flii in the wound environment. The most important finding of the current study was that FnAb administration was associated with improved diabetic wound healing through increased angiogenic responses and significantly improved vascular network formation.

This study has shown that low levels of Flii coincide with enhanced wound vascularization (as assessed by von Willebrand and VEGF staining), suggesting a role for Flii as an inhibitor of wound angiogenesis. In contrast to control wounds, wounds in diabetic mice showed decreased von Willebrand factor and VEGF levels confirming reduced angiogenesis was occurring in these diabetic wounds. In Flii-deficient diabetic wounds the levels of von Willebrand factor were elevated 2 fold on both day 7 and day 14 suggesting that reducing Flii in a diabetic wound could improve angiogenesis. Pro-angiogenic properties of VEGF are well established in genetic deletion and blockade of VEGF studies (Kano et al. 2005). In day 7 diabetic wounds low levels of Flii coincided with high levels of VEGF (VEGF increased fivefold in Flii<sup>+/-</sup> vs. Flii<sup>Tg/Tg</sup>). Whether changes in Flii directly affect VEGF levels are still to be determined. Addition of VEGF protein or gene therapy studies consistently demonstrate enhanced vessel growth in ischaemic tissues (Carmeliet & Jain 2011).

To distinguish the effect of Flii on microvessel formation an *ex vivo* aortic ring assay was used to study sprouting angiogenic responses. The aortic ring assay is a reliable and physiologically relevant model mimicking angiogenesis in a timescale similar to that observed *in vivo* (Baker et al. 2012). Aortic rings derived from Flii deficient (Flii<sup>+/-</sup>) mice induced robust sprouting of endothelial cells, whereas in contrast, aortic rings from Flii overexpressing mice (Flii<sup>Tg/Tg</sup>) showed minimal sprouting, suggesting that Flii inhibits microvessel sprouting.

A similar effect was observed using the *in vivo* Matrigel plug assay. Low levels of Flii significantly increased the length of functional blood vessels with lumen. The difference between the vasculature measured in Matrigel plugs retrieved from Flii<sup>+/-</sup> and Flii<sup>Tg/Tg</sup> mice was associated with a 2.7-fold increase in length of vessels with lumen. Lumen formation allows

perfusion of the neovessel (Carmeliet & Jain 2011). Moreover, in the Matrigel plug assay, vasculature in Flii<sup>+/-</sup> and WT mice was associated with tight cell to cell interactions producing physiologically normal tight junctions, whereas endothelial cells from Flii<sup>Tg/Tg</sup> mice failed to form these tight cell to cell interactions with significantly wider distances between adjacent endothelial cells being observed. These findings support previous observations of impaired hemidesmosome formation in Flii overexpressing mice (Kopecki et al. 2009) and our current electron microscopy studies suggest the importance of Flii in endothelial cell tight junction formation which is important for blood vessel stability and leakage prevention.

Flii has previous been shown to inhibit cellular migration and focal adhesion turnover in a Rac1 dependent manner (Kopecki, O'Neill, et al. 2011b). As enhanced cellular migration was observed in FnAb-treated fibroblasts (Adams et al. 2008; Cowin, AJ et al. 2007) we speculated that Ab-based neutralisation of Flii could also lead to improved motility of endothelial cell migration. Treatment with FnAb significantly increased the number of capillaries formed in Matrigel capillary tube formation assay.

To address the potential pro-angiogenic effect of FnAb *in vivo* we added FnAb into Matrigel compound and injected the plugs into WT mice. WT mice that received FnAb-treated Matrigel plugs showed longer and more mature blood vessel formation compared to IgG control. FnAb-treated Matrigel plugs showed longer vessels with RBC which indicated that these vessels were sufficiently mature to receive the blood flow with the flow being a critical determinant of vessel maintenance and durability (Carmeliet 2003).  $\alpha$ -SMA-positive pericytes ensheath endothelial cells and provide newly formed vessels with stability, prevent leakage and bleeding (Carmeliet & Jain 2011). In Matrigels treated with FnAb, the expression of SMA-positive cells was

significantly higher suggesting that FnAb may be able to promote pericyte migration towards endothelial cells, hence forming functionally mature blood vessels.

Having established that lowering the level of Flii is advantageous for wound angiogenesis we finally tested the pro-angiogenic effect of FnAb in WT diabetic wounds. Our findings showed that FnAb accelerated wound closure and improves diabetic wound re-epithelialisation via enhanced angiogenic responses. Our data demonstrate pro-angiogenic effects of FnAb, suggesting that it could be considered as a potential treatment strategy. Identification of novel angiogenic wound healing targets and knowledge of the molecular mechanisms involved in diabetic wound healing is the first step in the development of new therapies that will decrease the incidence of infection and lower-limb amputation, will improve the quality of life of patients with diabetes and reduce the costs involved in their treatments.

*Chapter 7*

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**DISCUSSION AND  
CONCLUSION**

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## DISCUSSION AND CONCLUSION

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## 7.1 Overview

The increasing prevalence of obesity due to high sugar, fat-rich diets and insufficient physical activity is leading to increased incidents of diabetes across the world. On average, nearly 8% of adults living in high-income countries have diabetes (Scully 2012). Both type 1 and type 2 diabetes are associated with an increased risk of foot ulceration. Elevated glucose levels provide an ideal environment for bacterial growth and coupled with profoundly retarded ability to heal wounds, the diabetic foot ulcer becomes a portal for infection often requiring a limb amputation. There is an urgent need for the development of new therapies and identification of molecules regulating wound repair is a first step to improving the healing of wounds in patients with diabetes and thereby preventing diabetes related lower limb amputation.

The cytoskeletal protein Flii is an important regulator of wound healing (Cowin, AJ et al. 2007). Flii was originally identified in *Drosophila melanogaster*, in which mutations in the gene caused defects in the flight muscles (Claudianos & Campbell 1995). Studies by Davy et al (Davy et al. 2001) demonstrated that Flii expression is mainly nuclear with the translocation to the cytoplasm occurring in the presence of the serum (Davy et al. 2001). Endogenous Flii acts as a nuclear receptor co-activator and binds to hormone-activated nuclear receptors and to transcriptional co-activators CARM1 and GRIP1 (Lee, YH, Campbell & Stallcup 2004). Flii also negatively modulates the TLR pathway with its endogenous reduction by small-interfering RNA enhancing the activation of NF- $\kappa$ B (Wang, XJ et al. 2006). Recent studies by Hayashi indicated that nucleoredoxin (NRX) subfamily of proteins forms a link between MyD88 and Flii to mediate negative regulation of the Toll-like receptor 4/MyD88 pathway (Hayashi et al.). Flii is involved in numerous cellular activities including regulating proliferation via interactions with

calmodulin-dependent protein kinase type II (Seward et al. 2008a), inflammation and cytokine production via caspase activation and IL-1 $\beta$  maturation (Li, JY, Yin, HL & Yuan, JY 2008).

Flii is a member of the gelsolin family of cytoskeletal proteins that regulate actin by severing pre-existing filaments and/or capping filament ends to enable filament reassembly into new cytoskeletal structures (Kopecki & Cowin 2008). Apart from being a fundamental component of the actin cytoskeleton network and an important regulator of actin filament severing, Flii is also involved in other cellular activities including cellular adhesion, spreading, migration and proliferation (Jackson et al. 2012; Mohammad et al. 2012). Reduction of Flii expression in primary murine fibroblasts and murine wounds *in vivo* (Adams et al. 2009; Jackson et al. 2012) significantly improves cellular functions and the rate of wound healing suggesting that decreasing Flii expression in wounds may be beneficial to improved diabetic wound healing. No studies to date have examined the effect of down-regulation and overexpression of Flii in diabetic wounds. Our current studies suggest that Flii is important in diabetic wound healing with its overexpression resulting in impaired wound healing. In both diabetic mouse and diabetic human wounds Flii expression increases compared to non-diabetic controls, hence reduction of Flii activity represents a possible therapeutic strategy for improved wound healing in humans.

## **7.2 Flii is elevated in diabetic wounds**

Human diabetic wounds were found to have significantly higher (2-fold) expression of Flii compared to non-diabetic acute wounds suggesting that Flii could be a contributing factor to the delayed healing associated with diabetic wounds. The pattern of Flii expression in human

diabetic wounds was distinctly different compared to non-diabetic chronic venous ulcers. Acute wounds, on the other hand, had a similar pattern of Flii expression to chronic venous ulcers. Several factors could have contributed to the disparate results between human diabetic and non-diabetic chronic wounds, including age, duration of disease, wound size and current medical history. Although, diabetic subjects were younger (mean age 64 y.o.) than non-diabetic study participants with a chronic venous wound (mean age 80 y.o.), diabetic subjects had other factors such as the presence of peripheral vascular disease and larger wound size – factors that further complicated wound healing and possibly contributed to the differences seen in the pattern of Flii expression in these wounds.

Using a murine model of streptozotocin-induced diabetes for assessing impaired wound healing we investigated the function of Flii protein in the diabetic wound repair. These animal studies showed that WT diabetic mouse wounds also have higher levels with a 7-fold increase observed after injury suggesting that high levels of Flii are present in these wounds. Studies have shown that Flii can inhibit the wound repair process (Cowin, AJ et al. 2007) so it is possible that high levels of Flii in diabetic wounds may contribute to their impaired healing.

Diabetic mice lacking one copy of the Flii gene (the double knockout being embryonically lethal (Campbell et al. 2002)) have lower levels of Flii and wounds created in these mice healed faster than Flii overexpressing wounds. Diabetic mice with two extra copies of Flii gene inserted into their genome had elevated levels of Flii and their wounds healed significantly slower than WT. Decreased Flii gene expression under diabetic conditions resulted in increased fibroblast growth and migration *in vitro*.

Similarly, *in vivo*, low levels of Flii resulted in improved wound outcomes with significantly higher rate of reepithelialisation. Conversely, mice with elevated Flii expression (i.e. Flii<sup>Tg/Tg</sup>) have impaired wound healing with larger wounds, reduced *in vitro* cellular proliferation and delayed reepithelialisation (Cowin, AJ et al. 2007). Studies within this thesis have identified Flii as being elevated in diabetic wounds indicating a potential negative effect on healing. Reducing Flii in diabetic wounds could be a promising approach to improving diabetic wound repair.

### **7.3 Antibodies raised against the cytoskeletal Flii (FnAbs) improve healing of diabetic wounds**

Flii is secreted by fibroblasts *in vitro* (Cowin, AJ et al. 2007) and in this current study we have shown that Flii is secreted *in vivo*. Human body fluids, including plasma and wound fluid contain secreted Flii. Immunostaining of Flii in both human and mouse fibroblasts revealed that Flii was located in the cytosol as well as in large endosomes located in the perinuclear region and throughout the cytoplasm. Late endosomes/lysosomes are organelles that have been found to perform a number of different functions, including secretion via fusion of these organelles with the cell surface to release its contents (Luzio, Pryor & Bright 2007). Dual immunostaining for Flii along with the late endosome/lysosome marker VAMP7 or cathepsin D, revealed that Flii localized to VAMP7 and cathepsin D-positive late endosomes/lysosomes in fibroblasts. The presence of Flii in late endosome/lysosomes suggests that Flii may be secreted via late endosomes/lysosomes - a known non-classical pathway of secretion (Lei et al. 2012). Flii was also detected in exosomes – small endocytic organelles involved in the exosome secretory

pathway, suggesting that Flii may also be secreted via exosome mediated mechanisms (Stoorvogel et al. 2002).

Given that Flii is secreted *in vivo* we proceeded to develop Flii monoclonal antibodies (FnAb) as a means of rendering extracellular Flii useless. We established that addition of FnAb to human fibroblasts in the presence of human chronic wound fluid increased proliferation *in vitro*. Previous studies have shown that chronic wound fluid contains cytotoxic cytokines and when added to cells in culture, chronic wound fluid can inhibit cellular proliferation (Harding, Moore & Phillips 2005). Flii was also inhibits cellular proliferation (Adams et al. 2008) so we wanted to establish if the inhibitory effects of chronic wound fluid could be due in part to the presence of Flii. Pre-treatment of the chronic wound fluid with FnAb resulted in a significant increase in fibroblast proliferation suggesting that secreted Flii present in the wound exudate inhibits proliferation and FnAbs were able to counteract the negative effects of Flii potentially by “mopping” up the Flii in the fluid. Previous work has shown that intradermal injection of FnAbs to murine burn wounds (Adams et al. 2009) and porcine excisional wounds (Jackson et al. 2012) significantly improve both the rate and quality of healing confirming that FnAbs can improve wound repair. To determine if neutralising Flii with FnAbs was an option for diabetic wounds we intradermally injected these antibodies to murine diabetic wounds and found that it lead to a significant improvement in the rate of healing confirming that neutralising Flii can improve healing of diabetic wounds.

#### 7.4 Flii inhibits angiogenesis in diabetic wounds

A healthy vasculature delivers oxygen and nutrients and removes carbon dioxide and metabolic waste products (Watt et al. 2010). New blood vessel formation is important for tissue repair and not surprisingly, vascular dysfunction including insufficient blood vessel formation is a contributing factor to delayed healing in diabetic wounds (Carmeliet & Jain 2011).

Flii-deficient mice have increased angiogenesis in their wounds on day 7 compared to WT as determined by the number of von Willebrand factor-positive cells and the expression of VEGF within diabetic wounds. VEGF is a key regulator of angiogenesis exerting multiple positive effects on endothelial cells (Estrach et al. 2011). VEGF protein or gene transfer stimulates vessel growth in ischaemic tissues (Carmeliet & Jain 2011). VEGF expression in Flii-deficient diabetic wounds was significantly higher compared to WT diabetic wounds at day 7 post-wounding. The physiological implications of these data are that elevated VEGF activities in Flii-deficient diabetic wounds may directly contribute to enhanced angiogenesis and acceleration of diabetic wound healing.

Neovascularization or new blood vessel formation is a key process for wound healing and impairments in neovascularization results in the development of chronic wounds (Bauer, Bauer & Velazquez 2005). The *in vivo* Matrigel plug assay showed that Flii-deficient mice had significantly higher numbers of functional blood vessels containing RBC compared to Flii overexpressing mice, suggesting that these would be enhanced oxygen delivery in Flii<sup>+/-</sup> wounds. Similarly, Flii-deficiency stimulated vessel growth resulting in the formation of longer vessels with or without cavities compared to WT, suggesting enhanced recruitment and differentiation of vascular progenitor cells to the area and stimulation of local endothelial cells to form neovessels,

all of which are important in diabetic wound repair. These results showed that increasing Flii in wounds resulting in reduced angiogenesis, whereas reducing the level of Flii lead to enhanced angiogenesis within wounds which is essential for efficient diabetic wound repair (Figure 7.1). It was therefore reasonable to hypothesize that excess Flii in the early stages of the diabetic repair process could impair healing by suppressing angiogenesis. We further showed that FnAbs could enhance angiogenesis using the *in vitro* capillary tube formation assay and the *in vivo* Matrigel plug assay. Not only did samples treated with FnAb form longer blood vessels *in vivo*, but they also appeared to be functional and mature, possibly, due to the presence of  $\alpha$ -SMA-positive cells. Establishment of this functional vascular network requires that immature vessels mature into durable vessels and pericyte or vascular smooth muscle cell (VSMC) recruitment is essential for the stabilization and maturation of blood microvessels. Enhanced  $\alpha$ -SMA –positive cell recruitment observed in FnAb treated samples of Matrigel plugs suggested that FnAbs may play a role in the formation of mature blood vessels (Jain 2003). Taken together, these results suggest that enhanced angiogenesis could be responsible for improved healing in FnAb-treated diabetic wounds compared to dose and isotype-matched controls.

### **7.5 Mechanisms of action of Flii within the wound site**

Flii is an important negative regulator of wound healing (Cowin, AJ et al. 2007) with multiple actions within the wound site (Figure 7.2). Wounding causes up-regulation of Flii in the skin (Cowin, AJ et al. 2007) and dermal fibroblasts are known to secrete Flii via non-classical pathway of secretion (Lei et al. 2012). Inflammatory cells including neutrophils and macrophages migrate to the wound from small blood vessels near the wound site, and actin-

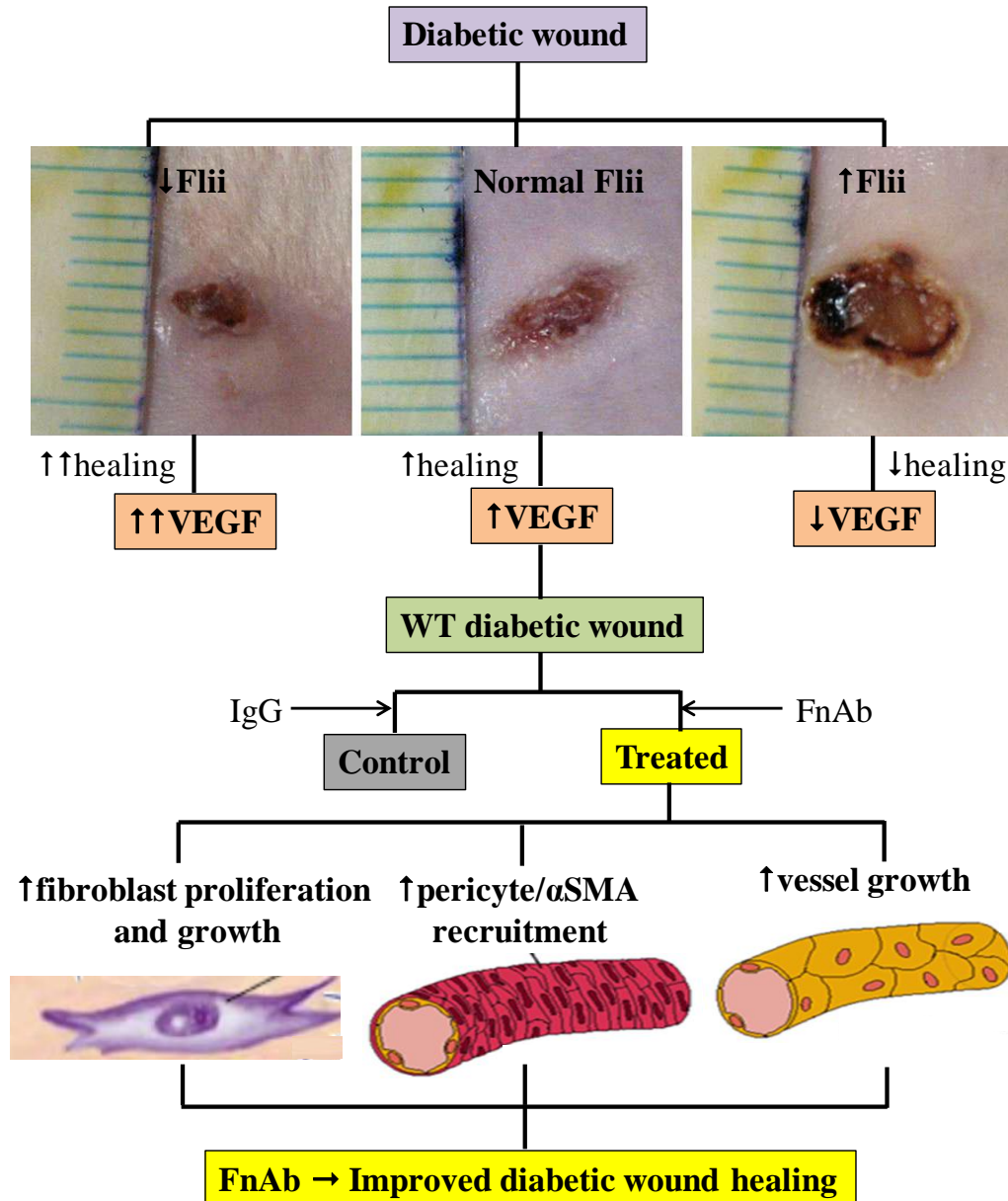


remodelling Flii is possibly involved in cytoskeletal reorganization (Campbell et al. 2002) of migrating leukocytes. Upon injury pathogens enter the wound and Flii binds to bacterial cell wall component lipopolysaccharide (LPS) (Lei et al. 2012). In macrophages, Flii inhibits the binding of MyD88 to TLR4 resulting in reduced activation of NF- $\kappa$ B leading to diminished cytokine secretion (Hayashi et al. 2010). Wounding causes the disruption of blood vessels and angiogenesis is stimulated by the release of growth factors such as vascular endothelial growth factor (VEGF). Flii impairs the angiogenic response by down-regulating pro-angiogenic VEGF expression. Flii is involved in keratinocyte migration (Mohammad et al. 2012) and cellular proliferation via interactions with calmodulin-dependent protein kinase type II (Seward, Easley et al. 2008). Flii alters the ability of fibroblasts to deposit (Cowin, AJ et al. 2007) and contract collagen I possibly via TGF- $\beta$ 1 (Kopecki, Arkell, et al. 2011). Endothelial cells are ensheathed by pericytes, which aid vessel maturation (Carmeliet & Jain 2011) and low Flii is associated with more pericytes suggesting that Flii is involved in VEGF-mediated new blood vessel formation and maturation.

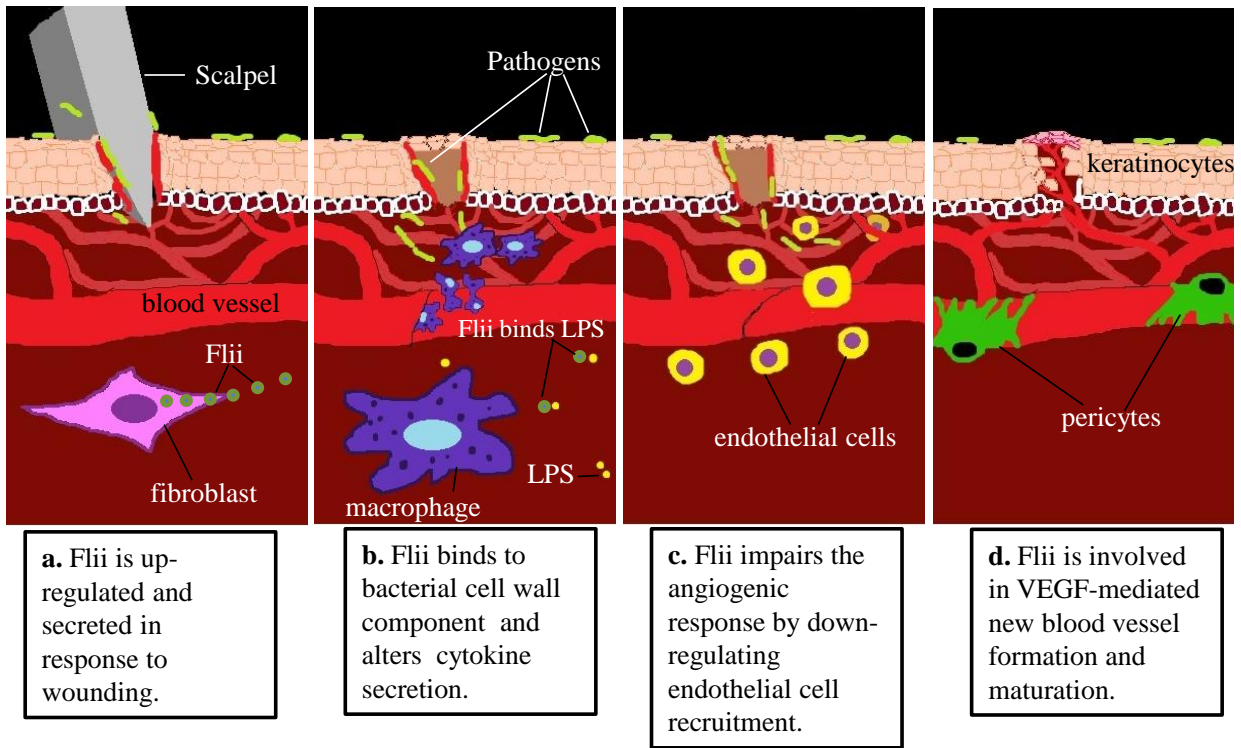
## **7.6 Conclusion**

Accumulative research to date portrays Flii as an important marker of impaired wound healing in different types of dermatological wounds. Our recent investigations suggest a role for Flii in diabetic wound repair and that application of Flii neutralising antibodies (FnAbs) *in vivo* significantly improved diabetic wound healing. While the exact mechanisms underpinning Flii effects in diabetic wound repair are still to be defined clear evidence now show that Flii significantly impairs the angiogenic response which is essential for a successful wound closure.

These observations may in part explain why reducing Flii results in the improvement of diabetic wound healing. Further studies using large animal models of diabetes are warranted. These studies could focus on investigating the effect of immobilising FnAbs onto dressings and into scaffolds aiming to increase cellular proliferation and angiogenesis within a porcine STZ-induced diabetic wound environment. In conclusion, we have identified Flii as a negative regulator of diabetic wound healing, demonstrated pro-angiogenic properties of FnAbs and confirmed their therapeutic usefulness for repairing diabetic wounds.



**Figure 7.1 Flii is a negative regulator of diabetic wound healing.** Experimental outline and schema of the proposed role of Flii in the regulation of diabetic wound healing emphasising the key findings of this thesis. Reduced Flii in diabetic wounds resulted in enhanced angiogenesis with the pro-angiogenic VEGF expression in Flii-deficient diabetic wounds being significantly higher compared to WT. Pro-angiogenic properties of Flii neutralising antibodies (FnAbs) may directly contribute to a significant improvement in the rate of healing of FnAb-treated diabetic wounds compared to IgG-treated controls.



**Figure 7.2. Schematic illustration of possible mechanisms of action of Flii within the wound site.** (a) Fibroblasts secrete Flii via non-classical pathway of secretion (Lei et al. 2012). (b) Pathogens (light green) enter the wound and Flii (dark green) binds to lipopolysaccharide (LPS) (yellow) (Lei et al. 2012). In macrophages, Flii inhibits the binding of MyD88 to TLR4 resulting in reduced activation of NF- $\kappa$ B leading to diminished cytokine secretion (Hayashi et al. 2010). (c) Flii impairs the angiogenic response by down-regulating pro-angiogenic VEGF expression. (d) Endothelial cells are ensheathed by pericytes (green) which aid vessel maturation (Carmeliet & Jain 2011) and low Flii is associated with more pericytes suggesting that Flii is involved in VEGF-mediated new blood vessel formation and maturation.

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**Appendix 1. Primary antibodies used in this thesis.**

The concentration of the primary antibodies and incubation times are shown below.

<b>Antibody</b>	<b>Dilution</b>	<b>Incubation Time</b>	<b>Manufacturer</b>	<b>Host species</b>
Flightless I	1:200	overnight	Santa Cruz 21716	mouse
Flightless I	1:200	overnight	Santa Cruz 30046	rabbit
CD16	1:100	1 hour	BD Biosciences 550383	mouse
CD3 APC-Cy7	1:100	1 hour	BD Biosciences 557832	mouse
CD45 FITC	1:100	1 hour	BD Biosciences 555482	mouse
CD14 APC	1:100	1 hour	BD Biosciences 555399	mouse
CD16 PE	1:100	1 hour	BD Biosciences 555407	mouse
B-tubulin	1:10,000	overnight	Sigma-Aldrich T 7816	mouse
Albumin	1:20,000	overnight	Santa Cruz 51515	mouse

Nucleoporin P62 (NUP62)	1:200	Overnight	BD Biosciences 610497	mouse
Cathepsin D	1:100	1 hour	Millipore IM03-100UG	mouse
VAMP7	1:100	1 hour	Synaptic Systems 6186	rabbit
Vti1b	1:100	1 hour	Synaptic Systems 164002	rabbit
VAMP3	1:100	1 hour	Synaptic Systems 104103	rabbit
GM130	1:100	1 hour	BD Transduction 610822	mouse
Hsp70	1:100	overnight	Pharmlngen 551188	mouse
BiP/GRP78	1:200	overnight	BD Transduction 610978	mouse
F4/80	1:200	overnight	Santa Cruz 25830	rabbit
NIMP-R14	1:200	overnight	Santa Cruz 59338	rat
VEGF	1:100	overnight	Abcam 46154	rabbit
Von Willebrand Factor	1:200	overnight	Abcam 6994	rabbit

**Appendix 2. Secondary antibodies used in this thesis.** The concentration of the antibodies and the secondary antibody incubation time are shown below.

**Secondary antibodies**

<b>Antibody</b>	<b>Dilution</b>	<b>Incubation Time</b>	<b>Manufacturer</b>	<b>Host species</b>
anti-rabbit CY3	1:200	1 hour	Invitrogen 10520	goat
Anti-mouse CY3	1:200	1 hour	Invitrogen A10521	goat
Anti-mouse Alexa Fluor 488	1:200	1 hour	Invitrogen A-11001	goat
Anti-rabbit Alexa Fluor 488	1:200	1 hour	Invitrogen A-11008	goat
Anti-mouse Alexa Fluor 488	1:200	1 hour	Invitrogen A-11054	rabbit
Biotinylated anti-mouse IgG	1:200	1 hour	Vector BA-2000	horse
Biotinylated anti-rabbit IgG	1:200	1 hour	Vector BA- 1000	goat
IgG2a PE	1:200	1 hour	BD Biosciences 555574	mouse
IgG2a	1:200	1 hour	Dako X0943	mouse
Anti-mouse HRP	1:1000	1 hour	DakoCytomation P0447	goat

**Attenuation of Flightless I improves healing in a murine model of type 1 diabetes through increased angiogenesis in the wounds.**

Nadira Ruze Haji <sup>1,2</sup>, Zlatko Kopecki <sup>1</sup>, Elizabeth Melville <sup>1</sup>, Sarah L. Appleby<sup>3</sup>, Claudine S. Bonder <sup>3</sup>, Ruth M. Arkell <sup>4</sup>, Robert Fitridge <sup>5</sup>, Allison J. Cowin <sup>1,2,6</sup>

<sup>1</sup> Women's and Children's Health Research Institute, South Australia, Australia

<sup>2</sup> The University of Adelaide, Department of Paediatrics, South Australia, Australia

<sup>3</sup> The University of Adelaide, School of Medicine and the Centre for Stem Cell Research, South Australia, Australia

<sup>4</sup> Research School of Biology, Australian National University, Australian Capital Territory, Australia

<sup>5</sup> The University of Adelaide, Department of Surgery, South Australia, Australia

<sup>6</sup> Centre for Regenerative Medicine, Mawson Institute, University of South Australia

Corresponding author: Professor Allison J. Cowin, Centre for Regenerative Medicine, Mawson Institute, University of South Australia, 5095 Australia; telephone: +61 83025018; fax: +61 882390267; Email: allison.cowin@unisa.edu.au

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**Abstract** (191 words)

Skin lesions and ulceration are severe complications of diabetes often resulting in leg amputation. Overexpression of Flightless I (Flii) inhibits wound healing and modulation of this protein using specific neutralizing monoclonal antibodies (FnAb) enhances cellular proliferation and migration. Using a streptozotocin induced model of diabetes we investigated the effect of altered Flii expression through either Flii genetic knockdown or overexpression and treatment with FnAb. Full-thickness dorsal wounds were created on the back of diabetic mice and it was found that reduced Flii expression improved healing and promoted a robust pro-angiogenic response with significantly elevated von Willebrand Factor (vWF) and Vascular Endothelial Factor (VEGF)-positive endothelial cell infiltration. Diabetic mouse wounds treated intradermally with FnAb also showed improved healing and significantly increased rate of re-epithelialisation. FnAb improved the angiogenic response through enhanced formation of capillary tubes *in vitro* and functional neovasculature *in vivo*. Flii deficiency was associated with increased numbers of mature blood vessels as determined by an *in vivo* Matrigel plug assay with increased recruitment of  $\alpha$ -SMA-positive cells and improved tight junction formation. Manipulating Flii may therefore be a potential approach for therapeutic intervention in the treatment of the diabetic foot ulcers.



## Introduction

Diabetic foot ulceration is a leading cause of lower limb amputation (1). Up to 15% of people with diabetes can expect to develop a non-healing ulcer (2) and 3% will have a lower-limb amputation (3). Despite numerous approaches the therapeutic results are still very limited. Actin-remodelling proteins of the gelsolin family (4) including Flii (5; 6) regulate the dynamic remodelling of the actin cytoskeleton (7) by severing, capping, nucleating or bundling actin filaments (8). The defining feature of Flii is its homology with two families of proteins, the gelsolin protein family and leucine-rich repeat (LRR) protein family (9). The bipartite domain structure of Flii provides the capacity to transduce cell signalling events into a remodelling of the actin cytoskeleton and is proposed to be involved in a variety of other signalling pathways (6; 7; 10; 11). Flii downregulates IL-1/TLR4 signalling of Toll-like receptor (TLR) pathway suggesting that Flii is involved in the regulation of innate immune response (12). Flii interacts directly with MyD88, an intracellular adaptor protein immediately downstream of most Toll-like receptors, and modulates the activity of NF- $\kappa$ B (12-14), pro-inflammatory caspases (15) and TNF- $\alpha$  (9). Flii has also been identified as an important regulator of wound repair, affecting cell proliferation, motility and matrix deposition (16). Acute wounds in mice heterozygous for Flii gene knockout showed significantly improved healing, whereas Flii overexpressing mice had significantly impaired healing with reduced cell proliferation and delayed re-epithelialization (16). Neutralizing antibodies to Flii (FnAbs) have the capacity to increase cellular proliferation and migration *in vitro* (16; 17) and promote the rate of healing in cutaneous wounds *in vivo* (18). To date, FnAbs have been tested in murine (16) and porcine (19) models.

Chronic hyperglycaemia in diabetes poses a significant risk for the development of micro and macrovascular complications (20). Diabetes impairs angiogenesis, which may explain in part the delayed wound healing seen in patients with diabetes and in experimental animal models of diabetes (21; 22). Given that deviations from normal vessel growth contribute to skin ulceration and pathological changes in angiogenesis are involved in the causal pathway to diabetic foot ulceration, we investigated the role of Flii in angiogenesis. To determine whether Flii influences angiogenesis and alters healing outcomes we modulated Flii expression by two means: i) genetic manipulation of Flii gene using knockout and transgenic mouse models; ii) Flii-specific neutralizing antibodies (FnAb), which bind to extracellular Flii and reduce its local expression. In this study, we aimed to establish whether attenuation of Flii within the wound site modulated the angiogenic response. These studies highlight Flii as a novel marker of impaired wound healing and suggest an innovative Flii neutralizing antibody therapy approaches, which are currently in preclinical development.

## **Research Design and Methods**

### **Human tissue collection**

The collection of human skin tissue used in this study was given ethical clearance from the relevant Human Ethics Committee and consent was obtained from all subjects in accordance with the 'Declaration of Helsinki'. Patients attending the Queen Elizabeth Hospital (Adelaide, South Australia) were screened for exclusion and inclusion criteria. Exclusion criteria included an active infection, drugs that impair wound healing such as steroids and immunosuppressive drugs, ulcer of malignant nature and renal failure/dialysis with Glomerular Filtration Rate (GFR) less than 30 ml/min. Inclusion criteria included the existence of a diabetic wound ( $\geq 6$  weeks), presence of pulses and/or Ankle Brachial Index (ABI) more than 0.7 and toe pressure (TP) more than 40mmHg. Non-diabetic acute wounds were obtained from patients with trauma wounds ( $\leq 6$  weeks). Non-diabetic unwounded skin (control) was obtained from the leg of healthy individuals.

### **Reagents and antibodies**

Mouse monoclonal anti-Flii antibody was obtained from Santa Cruz, CA, USA. Rat anti-mouse CD31 antibody (BD Pharmingen™, North Ryde, Australia), anti- $\alpha$ -SMA, rabbit polyclonal anti-VEGF and rabbit polyclonal anti-von Willebrand factor antibodies were obtained from Abcam (Cambridge, UK). Alexa-488 and Alexa-594-tagged secondary antibodies were obtained from Invitrogen, Mulgrave, Australia) and mouse monoclonal anti-Flii neutralizing antibodies (FnAb) (19) were obtained from MAbSA (Adelaide, Australia).

### **Murine model of type 1 diabetes**

Flii heterozygous null (*Flii*<sup>Tm1Hdc</sup>; *Flii*<sup>+/-</sup>) (8) and transgenic over-expressing (*Tg(FLII)2Hdc*; *FLII*<sup>Tg/Tg</sup>) (16; 23) mice on a BALB/c background were generated as described previously (23). Wild type animals (*Flii*<sup>+/+</sup>) were obtained from the BALB/c colony used for maintenance of the null strain. All animal experiments were approved by the relevant Animal Ethics Committees. Female *Flii*<sup>+/-</sup>, WT and *FLII*<sup>Tg/Tg</sup> mice of 12-16 weeks of age, weighing 20-35g were given one intraperitoneal (IP) injection of streptozotocin (STZ) (Sigma-Aldrich, St. Louis, MO, USA) at 50mg/kg for 5 consecutive days as described previously (24). Age matched non-diabetic control animals were treated with an equivalent dose of vehicle (Online Supplemental Data; Figure 1C).

### **Murine model of diabetic wound healing**

Two 6 mm wounds, one on each side of the midline were created on the shaved dorsum of mice. Digital photographs were taken of the wounds at 0, 7, 14 and 21 days post-wounding. Wounds were harvested at 0, 7, 14 and 21 days and fixed in 10% buffered formalin. In a separate cohort, wounded diabetic WT mice were (n=10; 20 wounds in total) were injected intradermally around the wound margins with 200 µl of 50 µg/ml of FnAb at day 1, 2 and 3. Control diabetic mice were treated with an equivalent dosage of IgG antibodies (n=10, 20 wounds in total).

### **Skin explant outgrowth assay**

2 mm punch biopsies were taken from the excised skin of diabetic and non-diabetic mice. Biopsies were collected in ice-cold Nutrient Mixture F12 (Sigma-Aldrich, St. Louis, MO, USA)

and 1% Penicillin-Streptomycin Solution Stabilized (Sigma-Aldrich, St. Louis, MO, USA) and cultured as described previously (25) in either normal (5.5 mM) or high (25 mM) glucose media.

### **Immunohistochemistry**

Sections were deparaffinised in 2 changes of xylene and re-hydrated in 2 changes of absolute alcohol, 1 minute each, after which they were transferred into 70% and 30% alcohol for 1 minute each. Sections were brought to water, rinsed in 1 x PBS and placed into 250 ml TRS as described previously (18). Primary antibodies (1:100) were applied and incubated overnight at 4°C. Detection was by species-specific secondary antibodies (1:200). AnalySIS software package (GmbH, Munster, Germany) was used to measure fluorescence intensity per unit area as described previously (18). Control immunostaining was carried out by omitting primary or secondary antibodies for verification of staining and non-specific binding.

### **Aortic ring assay**

Dissection of the aortae, serum starvation, embedding and feeding of the aortic rings, imaging and quantifying microvessel sprouts was as described previously (26). A total of 15 rings per mouse were obtained. The microvessel growth from the aortae rings was quantified on days 5, 6, 7 and 8-post embedding by live phase-contrast microscopy. Emerging microvessels were counted and the data was plotted as mean microvessel numbers per ring as described previously (26).

### **Isolation and culture of human umbilical-vein endothelial cells (HUVEC)**

The collection of primary HUVEC for use in this study was given ethical clearance from the Royal Adelaide Hospital (RAH), Adelaide, South Australia and consent was obtained from all subjects in accordance with the 'Declaration of Helsinki'. HUVEC were isolated from umbilical veins by collagenase digestion as previously described (27). HUVEC were cultured on gelatin-coated flasks in HUVE media (Medium 199: Sigma-Aldrich, Sydney, Australia) supplemented with 20% fetal bovine serum, endothelial cell growth supplement (BD Biosciences, North Ryde, Australia). HUVEC were used at passage two for experiments.

### **Matrigel tube formation assay**

HUVEC were isolated from the umbilical vein of neonates born at the Adelaide's Women's and Children's Hospital as described previously (27). Passage 1-2 HUVEC were cultured on gelatine plated T25 flasks and maintained in culture media containing 10% FCS in EGM®-2 BulletKit (Lonza, Basel, Switzerland). HUVEC were seeded on angiogenesis plate (1  $\mu$ -Slide Angiogenesis ibiTreat, Ibidi, Munich, Germany) coated with 12  $\mu$ l of Matrigel (354234, BD Biosciences, Bedford, U.S.A.). The number of tubes was counted as previously described (28; 29) and data was derived from average of 4 donors.

### **Matrigel plug assay**

Two plugs containing different ligands (FnAb or IgG antibodies were mixed by pipetting; 500  $\mu$ l at 50  $\mu$ g/ml) were injected into each mouse to avoid differences between the individual mice

(30). The plug on the left side of the abdomen contained Matrigel compound (354248, BD Biosciences, Bedford, U.S.A.) mixed with 50 µg/ml of FnAb and the plug on the right (control) contained an equivalent dose of rabbit IgG. The Matrigel plugs were removed after 7 days with the half of the plug snap frozen in liquid nitrogen and used for blood vessel visualisation by immunofluorescence and the other half fixed in 10% formalin and processed for histology. Quantification in Matrigel plug assay was as described previously (30) using Image ProPlus 5.1 (Media Cybernetics, Rockville, MD, USA). Matrigel plugs retrieved from *Flii*<sup>+/-</sup>, WT and *FLII*<sup>Tg/Tg</sup> mice were fixed in 4% paraformaldehyde/1.25% glutaraldehyde and processed for electron microscopy.

### **Electron Microscopy**

After washing, specimen were dehydrated in a graded series of ethanol (70–100%) followed by further dehydration in propylene oxide for 30 minutes and treated further as previously described (25). Electron micrographs of six samples of Matrigel plugs harvested from *Flii*<sup>+/-</sup>, WT and *FLII*<sup>Tg/Tg</sup> mice were captured at two different magnifications (× 3,800 and × 88,000). Blood capillaries were examined focusing on the tight junction region, which seals the intercellular space between adjacent endothelial cells. The overall width of the outer leaflets of the cell membrane of adjacent endothelial cells forming tight junctions was measured using Analysis5 iTEM software (SIS, Japan).

## **Western Blotting**

Protein was extracted from day 7 WT diabetic mouse wounds treated with either IgG control antibodies or FnAb using standard protein extraction protocols (31). 50  $\mu$ g of protein was subjected to SDS-PAGE separation and immunoblotting. Densitometry was performed on bands within the linear range and fold changes in levels calculated from this data as previously described (9).

## **Statistical analysis**

Statistical significance was calculated using a paired student t-test. A *p* value of 0.05 or less was considered significant.



## Results

### Flii deficiency improves diabetic wound healing

The influence of Flii gene expression on diabetic vs. non-diabetic wound healing was determined by creating two 6 mm full-thickness excisional wounds on the dorsum of mice with low (*Flii*<sup>+/-</sup>), normal (*Flii*<sup>+/+</sup>), and high (*FLII*<sup>Tg/Tg</sup>) level of Flii expression. Representative digital images of 0, 7, 14 and 21 day non-diabetic and diabetic wounds are shown in Figure 1A, and 1C respectively. Decreased Flii gene expression resulted in significant reduction in wound size as determined by the planimetric analysis of surface wound area (Figure 1A, B, D and F). Histological analysis of diabetic wounds revealed that the distance between the dermal wound margins was longer in wounds with high levels of Flii at day 7 compared to WT and Flii-deficient wounds (Figure 1H). The percentage of wound re-epithelialization was evaluated by measuring the length of the neoepidermis at day 7 post-wounding (Figure 1G).

### Inhibitory effect of Flii on cellular growth and migration

Skin explants (2 mm) were collected from diabetic and non-diabetic mice and incubated in normal (5.5 mM D-glucose) or high (25 mM D-glucose) glucose supplemented media for 7 days (Figure 2A). On day 2 post-culture a halo appeared around the explants. The cellular growth and migration potential was determined by measuring the distance between the explant and the end of the growing halo. Diabetic explants displayed a significant reduction in their capacity to grow and migrate compared to non-diabetic skin tissue (Figure 2A and B).

## **Diabetes increased Flii expression in response to wounding**

Given that Flii levels are elevated in human diabetic wounds compared to control non-diabetic wounds (Online Supplemental Data; Figure 1A, B), the influence of diabetes and effect of wounding on Flii protein expression were determined in non-diabetic and diabetic *Flii*<sup>+/-</sup>, WT and *FLII*<sup>Tg/Tg</sup> mice, at 0, 7 and 14 days post-wounding (Figure 3A, B). In unwounded skin, diabetes did not change Flii expression when compared to non-diabetic conditions. In diabetic mice, wounding up-regulated Flii protein expression by 83% in *Flii*<sup>+/-</sup> mice, by 86% in WT and by 89% in *FLII*<sup>Tg/Tg</sup> mice (Figure 3D). Wounding increased Flii expression in all three mice lines peaking at day 7 post-wounding and with the expression reducing by 37.5% in diabetic *FLII*<sup>Tg/Tg</sup> wounds on day 14 post-wounding. Significantly less Flii staining was observed in diabetic *Flii*<sup>+/-</sup> wounds at days 7 and 14 post-wounding compared with diabetic *FLII*<sup>Tg/Tg</sup> wounds (Figure 3D).

## **Flii deficiency up-regulates pro-angiogenic VEGF expression in diabetic wounds**

Day 0, 7 and 14 wounds from non-diabetic (Figure 4A) and diabetic (Figure 4B) *Flii*<sup>+/-</sup>, WT, *FLII*<sup>Tg/Tg</sup> mice were stained for VEGF expression. Flii overexpression caused a significant reduction in VEGF expression at both day 7 and day 14 post-wounding with VEGF expression in *FLII*<sup>Tg/Tg</sup> non-diabetic wounds being 5-fold lower compared to non-diabetic WT at 7 days post-wounds (Figure 4C). Flii deficiency was associated with a significant increase in VEGF expression in day 0 WT diabetic wounds compared to day 0 *FLII*<sup>Tg/Tg</sup> diabetic wounds (Figure 4B, D). A significant increase of VEGF expression in diabetic *Flii*<sup>+/-</sup> wounds compared to diabetic WT at day 7 post-wounding was observed (Figure 4D).

### **Increased levels of Flii leads to increased numbers of endothelial cells in diabetic wounds**

To determine the effect of Flii on endothelial cell recruitment, healing wounds from non-diabetic and diabetic *Flii*<sup>+/-</sup>, WT and *FLII*<sup>Tg/Tg</sup> mouse wounds were stained for vWF. Day 7 non-diabetic mice *Flii*<sup>+/-</sup> wounds showed a 1.7-fold increase in the expression of vWF compared to WT as determined by immunohistochemistry (Figure 4F). Low Flii lead to markedly increased new blood vessel formation as evidenced by increased endothelial cell numbers in diabetic wounds compared to diabetic WT controls (Figure 4E-G).

### **Attenuation of *Flii* gene expression improves new blood vessel formation *in vivo***

Matrigel plugs were injected under the skin of *Flii*<sup>+/-</sup>, WT and *FLII*<sup>Tg/Tg</sup> mice to determine the effect of Flii on angiogenesis (Figure 5A-C). Average length of newly formed vessels and vessel-like structures, which had grown into the Matrigel plugs were quantified (Figure 5B). The length of newly formed vessels was quantified and categorized into three groups as previously described (30): (1) vessels containing red blood cells (RBC), (2) vessels without RBCs, (3) vessel-like structure but without a cavity. In this *in vivo* model of angiogenesis, reduced expression of Flii resulted in 4-fold increase in the length of functional vessels containing RBCs compared to WT (Figure 5B). The length of vessels with cavities, but without the RBCs was significantly higher in *Flii*<sup>+/-</sup> mice than their WT counterparts (Figure 5B). Electron microscopy revealed that the endothelial cells in *FLII*<sup>Tg/Tg</sup> group showed disrupted tight junction formation (Figure 5A). The distance between the nearest edges of endothelial cells and their tight junctions were measured (Figure 5C). Partial knockout of Flii gene had a positive effect on tight junction formation and resulted in significantly smaller distances between adjacent endothelial cells

compared to  $Flii^{Tg/Tg}$  group (Figure 5C). Finally, aortae collected from 4 week old  $Flii^{+/-}$ , WT and  $FLII^{Tg/Tg}$  mice were cut into rings and embedded in type 1 collagen (Figure 5D). Endothelial microvessel sprouts grown from the rings were counted at days 0, 5, 6, 7 and 8 post-embedding. Significant increases in microvessel sprouting were observed in  $Flii^{+/-}$  rings compared with WT controls at day 5 and day 6 post-embedding (Figure 5E). Significantly reduced sprouting was observed in  $FLII^{Tg/Tg}$  rings isolated from aortae of  $Flii^{+/-}$  and WT mice (Figure 5E).

### **Pro-angiogenic effects of Flii neutralising antibody (FnAb) *in vitro***

To determine whether FnAb had the capacity to enhance capillary tube formation *in vitro*, we plated HUVEC on a 2D matrix of Matrigel in the presence of FnAb (50  $\mu$ g/ml) or an isotype matched control (irrelevant IgG) at a final concentration of 50  $\mu$ g/ml (Figure 6A-B). Assessment of capillary tube formation of the FnAb-treated HUVEC showed that these cells reorganized into large aggregates with subsequent capillary tubes (Figure 6A-B). The FnAb-treated HUVEC formed significantly more capillary tubes compared to IgG-treated controls or no treatment (Figure 6E).

### **Reducing Flii using FnAb enhanced formation of mature blood vessels *in vivo***

Matrigel plugs were mixed with either IgG control (50  $\mu$ g/ml) or FnAb (50  $\mu$ g/ml) and injected under the skin of the abdominal area of WT mice (Figure 6C, D and F). The vessels were categorized into three groups: (1) vessels containing red blood cells (RBC), (2) vessels without RBCs, (3) vessel-like structure but without a cavity. FnAb resulted in a 4-fold increase of the

length of functional vessels, which contained RBCs compared to IgG control (Figure 6F). Addition of FnAb (50 µg/ml) into Matrigel plugs enhanced the formation of new blood vessels (Figure 6F).

### **FnAb contributes to wound vascularization and enhanced $\alpha$ -SMA<sup>+</sup>/CD31<sup>+</sup> cell recruitment *in vivo***

Matrigel plugs supplemented with either IgG control (50 µg/ml) or FnAb (50 µg/ml) were injected under the skin of WT mice and harvested at day 7 post-injection and subsequently stained with antibodies to establish the level of pericyte ( $\alpha$ -SMA) and endothelial cell (CD31/PECAM-1) recruitment (Figure 6G). The number of cells positive for  $\alpha$ -SMA were lower in IgG-treated Matrigel plugs compared to FnAb-treated Matrigel plug sections (Figure 6H).

### **Flii neutralising antibodies increases VEGF expression in diabetic wounds**

The effect of Flii deficiency on VEGF expression in diabetic wounds was assessed by immunoblotting and immunostaining of day 7 WT diabetic wounds treated with either IgG control antibodies or FnAb with anti-VEGF antibody (Figure 6I). FnAb-induced Flii deficiency resulted in significant up-regulation of VEGF expression (Figure 6J and K).

### **Accelerated diabetic wound closure is induced by FnAb *in vivo***

FnAbs were injected intradermally into WT mice and marked differences were recorded in the macroscopic appearance of healing wounds at 0, 1, 2, 3 and 7 days post-wounding (Figure 7A-E). By the end of the first week the control wounds were still covered with a non-viable necrotic tissue which included the clot (Figure 7A). In contrast, the appearance of wounds treated with FnAb was markedly improved compared to control wounds, i.e. faster clearance of non-viable tissue and rapid appearance of granulation tissue, which was promptly replaced by shiny epidermal layer (Figure 7A). Intradermal administration of FnAb (200  $\mu$ l of 50 $\mu$ g/ml injected at day 1, 2 and 3 post-wounding), but not control antibodies (irrelevant affinity purified IgG; 200  $\mu$ l of 50 $\mu$ g/ml injected at day 1, 2 and 3 post-wounding), resulted in a 1.9-fold decrease in wound healing (Figure 7C). Microscopic analysis of wound biopsies showed that intradermal injection of FnAb accelerated wound closure compared to their IgG-treated counterparts. FnAb-treated wounds had significantly smaller wound length and gape than IgG-treated control wounds on day 7 after wounding (Figure 7D-E).

## Discussion

A growing body of evidence suggests that Flightless I (Flii) protein plays an important role in cutaneous wound healing process and decreasing its activity in incisional and burn wounds improves healing, whereas increasing Flii impairs wound repair (18). No studies to date have examined the effect of differential levels of Flii in diabetes. Given the role of Flii as a negative regulator of wound healing, we embarked in our studies seeking to establish whether augmented Flii gene expression has a similar effect on diabetic wound healing as it does on incisional and partial-thickness burn injury repair. Diabetes was induced by STZ, confirmed by measuring blood glucose levels, and subsequently, excisional wounds were created on mice with low *Flii*<sup>+/-</sup>, normal (WT) and high (*FLII*<sup>Tg/Tg</sup>) expression of *Flii* gene. Our results indicate that a subset of diabetic mice left for 6 weeks have successfully developed a delayed wound healing phenotype. Reduced Flii significantly improved wound healing in both non-diabetic and diabetic wounds. Genetic overexpression of Flii reduced the rate of re-epithelialisation under both diabetic and non-diabetic conditions. Healing of *FLII*<sup>Tg/Tg</sup> diabetic wounds was significantly impaired with wounds gaping wider, healing slower and having significantly delayed rate of re-epithelialisation suggesting that increased expression of Flii in diabetic wounds may be partly responsible for delayed healing in these wounds. Given that rapid epithelialisation and closure of disrupted dermal matrix are key determinants of successful wound healing our results suggest that Flii-deficiency in wounds may exert a positive effect on diabetic wound closure.

Angiogenesis promotes tissue repair (22) and accelerates wound closure (32). Actin cytoskeleton reorganisation is vital in cellular migration and Flii-deficient skin cells showed improved migration (16). Vascular cell migration is an important component of new vessel formation and vascular maturation (33). Our studies demonstrated that *Flii* gene overexpression hinders

angiogenesis, whereas partial knockdown of *Flii* gene increases vascular growth in murine model of type 1 diabetes. Low levels of *Flii* coincide with enhanced wound vascularization suggesting a role for *Flii* as an inhibitor of wound angiogenesis. In *Flii*-deficient diabetic wounds the levels of vWF were elevated 2 fold on both day 7 and day 14 suggesting that reducing *Flii* in diabetic wounds could improve angiogenesis. In day 7 diabetic wounds low levels of *Flii* coincided with high levels of pro-angiogenic VEGF. Whether changes in *Flii* directly affect VEGF levels are still to be determined. *Flii* deficiency was associated with enhanced cell to cell interactions producing physiologically normal tight junctions, whereas *Flii* overexpression coincided with disrupted cell to cell interaction with significantly wider distances between adjacent endothelial cells. These findings support previous observations of impaired hemidesmosome formation in *Flii* overexpressing mice (25) and our current electron microscopy studies suggest the importance of *Flii* in endothelial cell tight junction formation which is important for blood vessel stability and leakage prevention. To distinguish the effect of *Flii* on microvessel formation an *ex vivo* aortic ring assay was used to study sprouting angiogenic responses. Aortic rings derived from *Flii*<sup>+/-</sup> mice induced robust sprouting of endothelial cells, whereas aortic rings from *FLII*<sup>Tg/Tg</sup> mice showed minimal sprouting suggesting that *Flii* inhibits microvessel sprouting.

Given that *Flii*-deficient mice showed improved angiogenesis, we proceeded to use Flightless neutralising antibodies (FnAb) as a means of decreasing local expression of *Flii* within the wound environment. As enhanced cellular migration was observed in FnAb-treated fibroblasts (16) we speculated that antibody-based neutralisation of *Flii* could also lead to improved motility of endothelial cell migration. Treatment with FnAb significantly increased the number of capillaries formed in Matrigel tube formation assay. To address the potential pro-angiogenic



effect of FnAb *in vivo* we added FnAb into Matrigel compound and injected the plugs into WT mice. WT mice that received FnAb-treated Matrigel plugs showed longer and more mature blood vessel formation compared to IgG controls. FnAb-treated Matrigel plugs showed longer vessels containing RBC which indicated that these vessels were sufficiently mature to receive the blood flow with flow being a critical determinant of vessel maintenance and durability (34). In Matrigels treated with FnAb, the expression of  $\alpha$ -SMA-positive cells was significantly higher suggesting that FnAb may be able to promote pericyte migration towards endothelial cells, hence forming functionally mature blood vessels.

Given that low level of *Flii* is advantageous for wound angiogenesis we proceeded to test the pro-angiogenic effect of FnAb in WT diabetic wounds. Our findings showed that FnAb accelerated wound closure and improved diabetic wound re-epithelialisation via enhanced angiogenic responses. Our data demonstrate pro-angiogenic effects of FnAb, suggesting that it could be considered as a potential candidate in the treatment of diabetic wounds. Having established that (i) reduced *Flii* gene expression enhances diabetic wound closure, (ii) *Flii* is up-regulated in response to wounding and (iii) *Flii* is secreted, we applied neutralising antibodies to show that diabetic wounds positively respond to treatment with *Flii* specific monoclonal antibodies (FnAb). Diabetic wounds treated with FnAb show accelerated wound closure, which may be promoted by the positive effect of FnAb on cellular proliferation. Dose dependent effects of neutralising antibodies to *Flii* (FnAb) were demonstrated previously (16) where addition of FnAb *in vitro* reduced the inhibitory effect of chronic wound fluid on proliferation (17). We next examined the extent to which FnAbs stimulate wound healing in diabetic wounds. It was revealed that FnAb-treated diabetic wounds developed an overtly superior wound healing phenotype compared to IgG-treated controls. The difference was associated with a 1.9-fold

decrease in average wound size in day 3 and day 7 WT FnAb-treated diabetic wounds. Similar effects were reported in previous studies where FnAbs increased the rate of incisional (16), excisional (19) and burn wound repair (18).

In summary, the current study provided evidence that reduced activity of the actin-remodelling protein Flii is beneficial in diabetic wound healing. Our findings showed that FnAb accelerated wound closure and improves diabetic wound re-epithelialisation via enhanced angiogenic responses. Our data demonstrate pro-angiogenic effects of FnAb, suggesting that it could be considered as a potential treatment strategy. Identification of novel angiogenic wound healing targets and knowledge of the molecular mechanisms involved in diabetic wound healing is the first step in the development of new therapies that will decrease the incidence of lower-limb amputation, improve the quality of life of patients with diabetes and reduce the costs of diabetic wound management.

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NR designed experiments, researched data and wrote the manuscript. ZK researched data and reviewed the manuscript. EM and SLA researched data. CSB assisted with the design of *in vivo* assay of angiogenesis and reviewed the manuscript. RMA generated and provided the mice. RF reviewed the manuscript. AJC designed the study, contributed to discussion and reviewed/edited manuscript. AJC is supported by the NHMRC Senior Research Fellowship (#1002009). ZK is supported by the NHMRC Early Career Fellowship (#1036509). CSB is supported by a Heart Foundation Fellowship. No potential conflicts of interest relevant to this article were reported. Prof. Allison Cowin is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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## Figure legends

**Figure 1. Flii overexpression impairs healing in mouse diabetic wounds.** Two full-thickness excisions were made on the dorsal skin of (A) non-diabetic control and (C) diabetic mice expressing low (*Flii*<sup>+/-</sup>), normal (*Flii*<sup>+/+</sup>), and high (*FLII*<sup>Tg/Tg</sup>) levels of Flii. Representative images of (A) non-diabetic and (C) diabetic wounds taken on days 0, 7, 14 and 21 post-surgery. Scale bar = 1mm. Representative images of H&E stained sections of (B) non-diabetic and (D) diabetic wounds at 7 and 21 days post-wounding. Arrows indicate wound margins. Scale bar = 100  $\mu$ m. Surface wound area at 0, 7, 14 and 21 days post-wounding in *Flii*<sup>+/-</sup>, WT and *FLII*<sup>Tg/Tg</sup> in (E) non-diabetic and (F) diabetic mice. Results represent mean  $\pm$  S.E.M. (n=6 mice, 12 wounds each group/time-point). (E) \*\* $p \leq 0.01$  non-diabetic WT vs. non-diabetic *FLII*<sup>Tg/Tg</sup>; \*\* $p \leq 0.01$  non-diabetic *Flii*<sup>+/-</sup> vs. non-diabetic *FLII*<sup>Tg/Tg</sup>. (F) Diabetic WT wounds were significantly smaller than diabetic *FLII*<sup>Tg/Tg</sup> wounds (\* $p \leq 0.05$  in diabetic WT vs. diabetic *FLII*<sup>Tg/Tg</sup>; \* $p \leq 0.05$  in diabetic *Flii*<sup>+/-</sup> vs. diabetic *FLII*<sup>Tg/Tg</sup>). (G) Wound re-epithelialisation was determined by measuring the percentage of the wound that had epidermal covering at day 7 (\* $p \leq 0.05$  non-diabetic *Flii*<sup>+/-</sup> vs. non-diabetic *FLII*<sup>Tg/Tg</sup>; \*\* $p \leq 0.01$  diabetic *Flii*<sup>+/-</sup> vs. diabetic *FLII*<sup>Tg/Tg</sup>). (H) Histological dermal wound gape of day 7 wounds from *Flii*<sup>+/-</sup>, *Flii*<sup>+/+</sup> and *FLII*<sup>Tg/Tg</sup> diabetic mice was determined by measuring the distance between the dermal wound margins. Results represent mean  $\pm$  S.E.M. (n=6 mice, 12 wounds each group/time-point); \* $p \leq 0.05$  diabetic WT vs. diabetic *FLII*<sup>Tg/Tg</sup>; \*\* $p \leq 0.01$  diabetic *Flii*<sup>+/-</sup> vs. diabetic *FLII*<sup>Tg/Tg</sup>.

**Figure 2. Flii deficiency downregulates cellular migration.** (A) Representative day 7 micrographs showing cellular migration upon *in vitro* incubation of skin explants from diabetic (n=4) and non-diabetic mice (n=4). Cellular outgrowth was higher under (B) non-diabetic conditions than (C) diabetic (\* $p \leq 0.02$ ; day 6; WT diabetic vs. WT non-diabetic). Cellular outgrowth was significantly slower in (B) non-diabetic *FLII*<sup>Tg/Tg</sup> cells compared to non-diabetic *Flii*<sup>+/-</sup> and WT fibroblasts on days 5, 6, 7 (\*\* $p \leq 0.01$ ; non-diabetic *FLII*<sup>Tg/Tg</sup> vs. non-diabetic *Flii*<sup>+/-</sup>). The worst phenotype was recorded in (C) diabetic *FLII*<sup>Tg/Tg</sup> cells, with the cellular outgrowth being the least efficient. (C) *FLII*<sup>Tg/Tg</sup> diabetic cells migrated significantly slower than their *Flii*<sup>+/-</sup> counterparts on days 5, 6 and 7 post-culture, suggesting that Flii gene has a negative effect on cellular growth and migration (\*\* $p \leq 0.01$ ; diabetic *FLII*<sup>Tg/Tg</sup> vs. diabetic *Flii*<sup>+/-</sup>). Scale bar = 100  $\mu$ m. All values represent means and S.E.M.

**Figure 3. Flii is up-regulated in response to wounding.** (A) Immunofluorescence analysis of Flii protein was performed on excisional non-diabetic wounds in *Flii*<sup>+/-</sup>, WT and *FLII*<sup>Tg/Tg</sup> mice. (A) Representative images are shown for 0, 7, and 14 day non-diabetic wounds. (B) Immunohistochemistry was performed on *Flii*<sup>+/-</sup>, WT and *FLII*<sup>Tg/Tg</sup> diabetic mouse wounds. Representative images are shown for 0, 7 and 14 day wounds. In all images, e denotes epidermis and w indicates position of wound. Scale bar in (A) and (B) refers to all images and = 50  $\mu$ m. (C) Flii fluorescence intensity was quantified in epidermis and dermis of day 0, 7 and 14 non-diabetic wounds (\*\* $p \leq 0.01$ ; \* $p \leq 0.05$ ). (D) Fluorescence intensity quantification in *Flii*<sup>+/-</sup>, WT and *FLII*<sup>Tg/Tg</sup> diabetic mouse excisional wounds (\* $p \leq 0.05$ ). Results represent mean  $\pm$  S.E.M. (n=6 mice, 12 wounds each group/time-point).

**Figure 4. Effects of Flii on VEGF expression in non-diabetic and diabetic wounds.** (A) Representative images of (A) non-diabetic and (B) diabetic wounds stained with anti-VEGF antibody. Scale bar = 100  $\mu\text{m}$ . VEGF-stained sections of (C) non-diabetic and (D) diabetic wounds were evaluated and the number of VEGF-positive cells (expressed as cells per  $\text{mm}^2$ ) was recorded. Flii deficiency was associated with significant up-regulation of VEGF. In (C)  $***p \leq 0.001$  at day 7 for  $FLII^{Tg/Tg}$  vs. WT;  $**p \leq 0.01$  at day 14 for  $FLII^{Tg/Tg}$  vs. WT;  $n=6$ . In (D)  $**p \leq 0.01$   $FLII^{Tg/Tg}$  vs. WT (day 0);  $**p \leq 0.01$   $Flii^{+/-}$  vs. WT (day 7);  $***p \leq 0.001$   $FLII^{Tg/Tg}$  vs. WT (day 7). (E) Representative images of diabetic  $Flii^{+/-}$ , WT,  $FLII^{Tg/Tg}$  wounds harvested 7 and 14 days post-wounding and stained with anti-von Willebrand factor (vWF) antibody. (F) Graphical representation of the number of vWF-positive cells in (F) non-diabetic and (G) wounds. In (F)  $**p \leq 0.01$  in non-diabetic WT vs. non-diabetic  $Flii^{+/-}$  in day 7 wounds;  $***p \leq 0.001$  in WT vs.  $FLII^{Tg/Tg}$  in day 7 wounds;  $n=6$ . Diabetes reduced the expression of vWF in diabetic vs. non-diabetic wounds. The difference was associated with a 2.7 decrease in endothelial cell recruitment in diabetic wounds compared to non-diabetic wounds ( $*p \leq 0.05$ , day 7 diabetic  $Flii^{+/-}$  vs. day 7 diabetic WT;  $***p \leq 0.001$ , day 14 diabetic  $Flii^{+/-}$  vs. day 14 diabetic WT;  $n=6$ ).

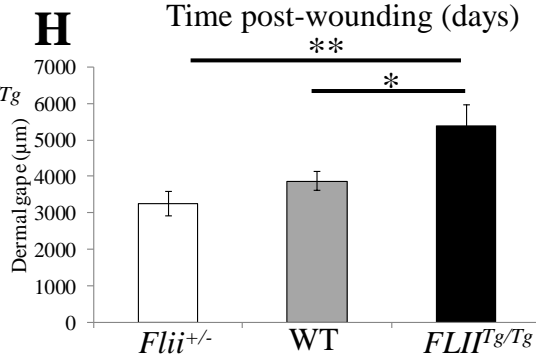
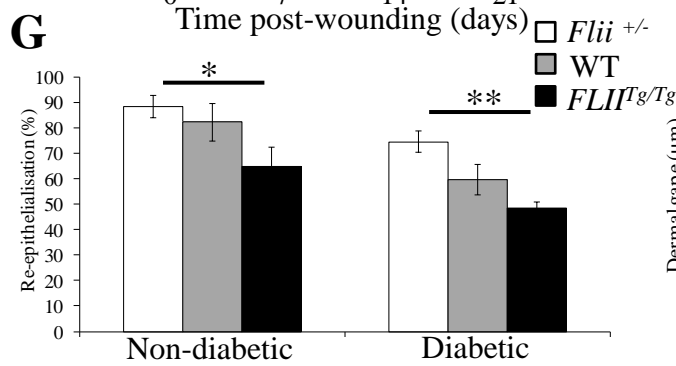
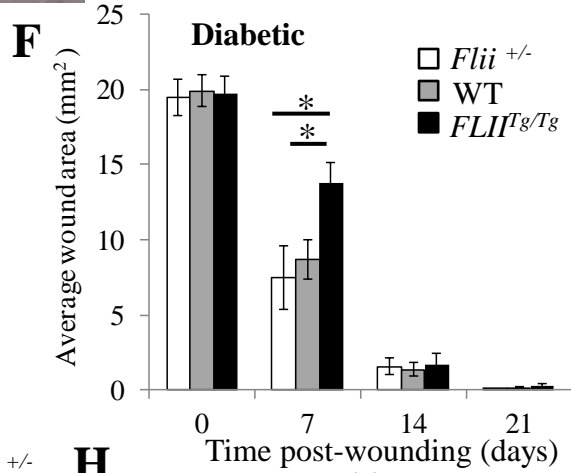
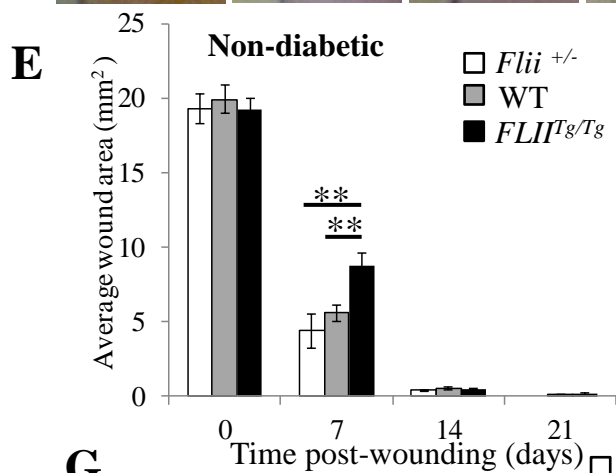
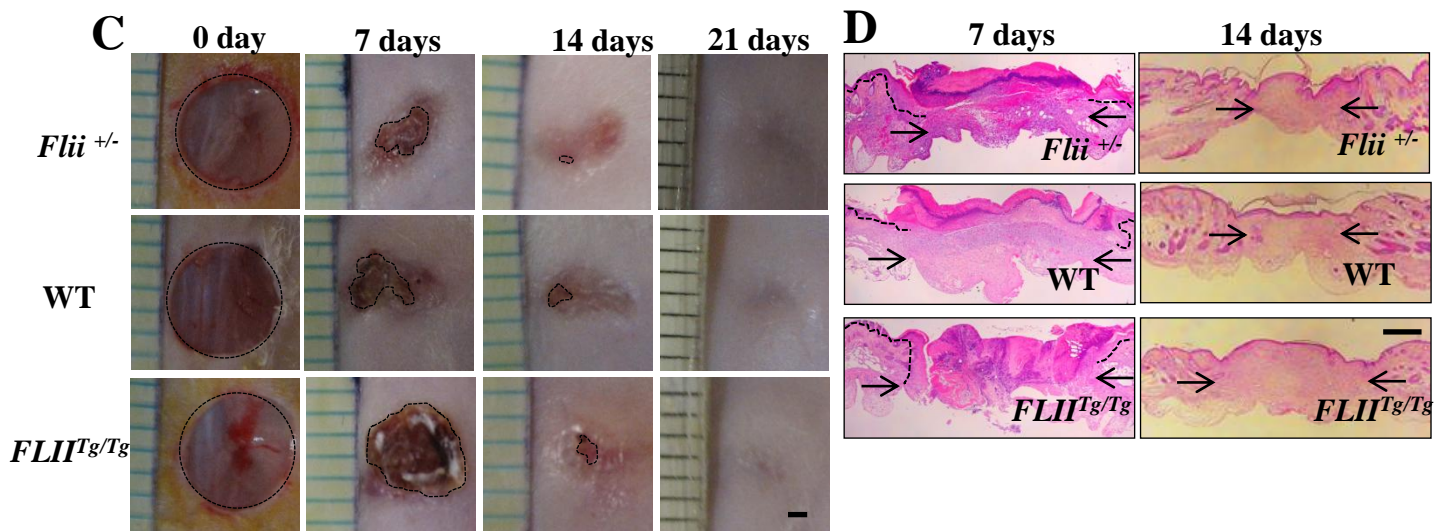
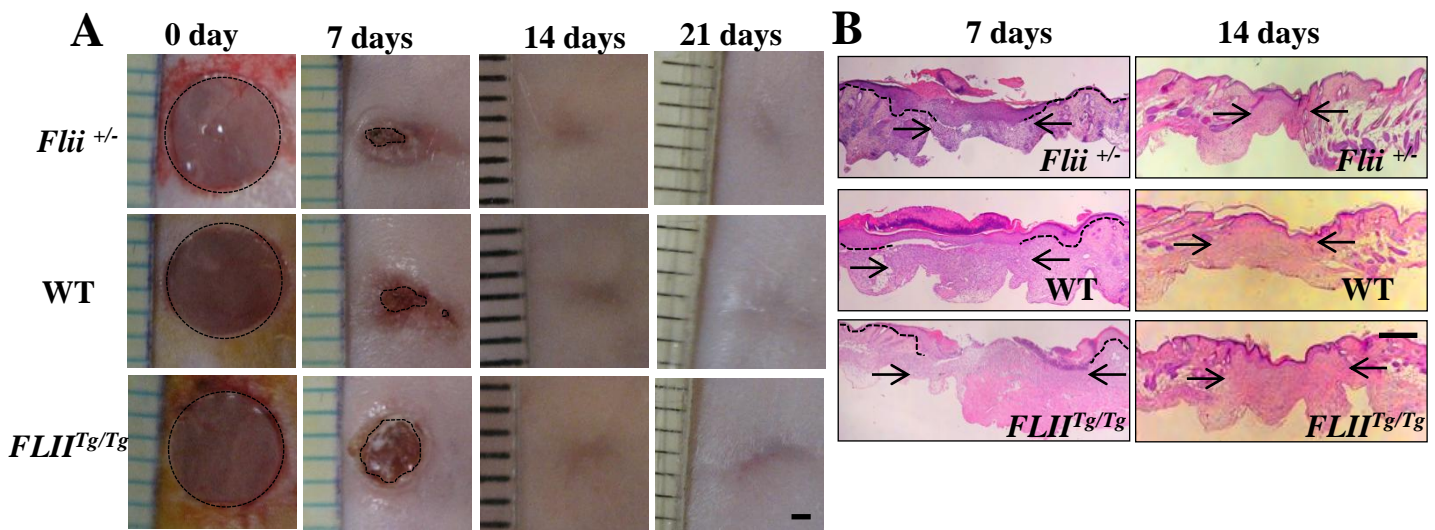
**Figure 5. The effect of Flii gene manipulation on angiogenesis.** Matrigel plugs were injected subcutaneously into  $Flii^{+/-}$ , WT and  $FLII^{Tg/Tg}$  mice. After 7 days Matrigel plugs were extracted, stained using H&E and examined under the light microscope. (A) Representative H&E stained images of Matrigel plugs show newly formed vessels *in vivo*. Black arrow heads indicate functional vessels containing RBCs. Scale bar 100  $\mu\text{m}$ . (A) Electron microscopy images of Matrigel plugs retrieved from  $Flii^{+/-}$ , WT and  $FLII^{Tg/Tg}$  mice at day 7 post-injection. All sections were visualized under at 3800 $\times$  and 8800 $\times$  magnification. Scales are indicated in each photograph. MC = mural cells; EC = endothelial cells; RBC = red blood cells, black arrows indicate tight junctions formed between endothelial cells. (B) Quantification of vessel length (mm). Vessels and vessel-like structures were classified as follows: (i) mature blood vessels containing RBCs (blue), (ii) vessels with cavities but without RBCs (red), (iii) cells arraying in line but without cavities (green).  $*p \leq 0.05$  for  $Flii^{+/-}$  vs. WT ( $n=6$ ) in 2 categories: (1) vessels with cavities but without RBCs and (2) vessels without cavities. (C) Quantification of the distance between adjacent endothelial cells.  $***p \leq 0.001$ ;  $n = 6$  ( $Flii^{+/-}$  vs.  $FLII^{Tg/Tg}$  and  $FLII^{Tg/Tg}$  vs. WT). (D) Phase-contrast images of aortic rings embedded in type I collagen showing microvessel outgrowth at day 6 after embedding. Scale bar 100  $\mu\text{m}$ . Original magnification 10  $\times$ . (E) Time course of microvessel sprouting from aortic rings collected from aortae of  $Flii^{+/-}$ , WT,  $FLII^{Tg/Tg}$  mice aged 4 weeks. Microvessel sprouts were counted from days 5 to 8 after embedding in type 1 collagen. Bars represent the mean number of microvessel sprouts  $\pm$  S.E.M. Assay quantification ( $n = 6$ ; 15 rings per mouse aorta).  $***p \leq 0.001$ ;  $Flii^{+/-}$  vs. WT on days 5 and 6.

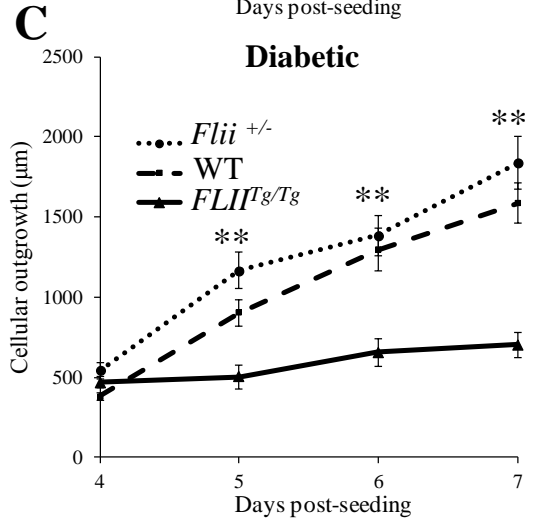
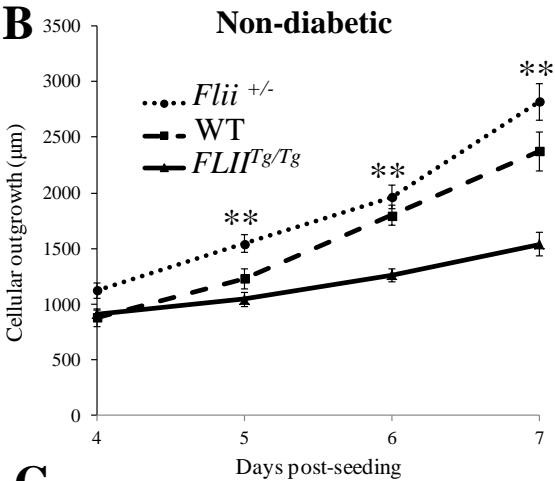
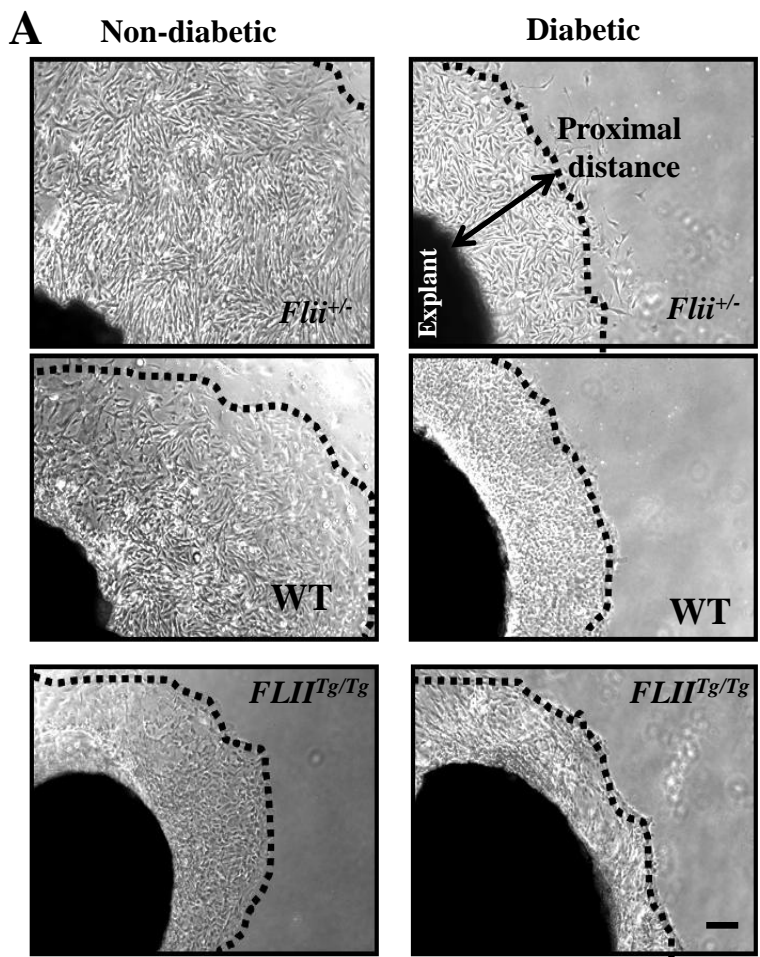
**Figure 6. New vessel formation is enhanced when Flii levels are reduced.** HUVEC were incubated with either FnAb or dose-matched control antibody IgG for 1 hour. The cells were harvested and plated onto Matrigel in the presence of either FnAb or IgG control. Capillary tube formation was monitored for 6 hours and images were taken to compare the number of capillary tubes formed for each treatment group. Representative phase-contrast microscopic images of (A) IgG-treated and (B) FnAb-treated HUVEC. Scale bar in (A) and (B) is 100  $\mu\text{m}$  (original magnification 10  $\times$ ). (C) H&E stained sections of Matrigel plugs stimulated with (C) IgG and

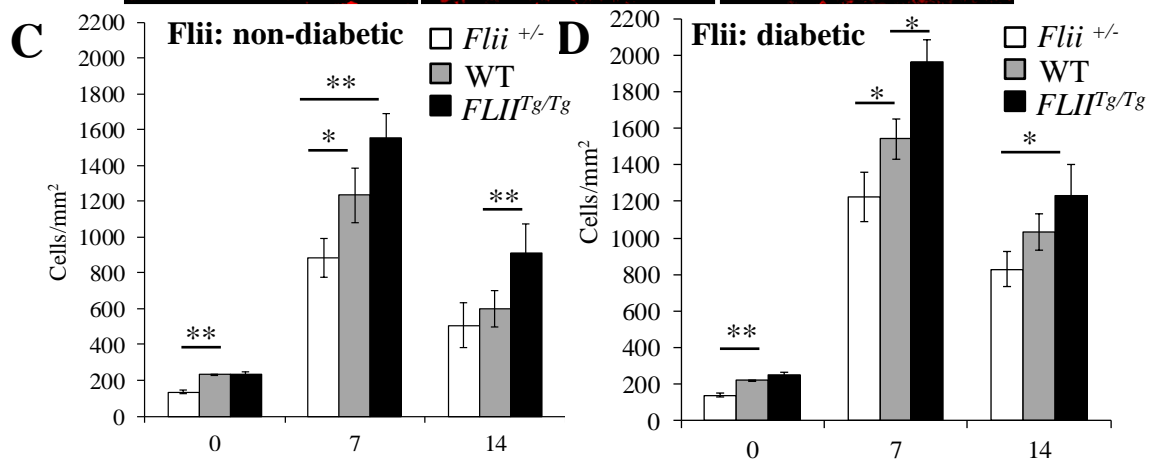
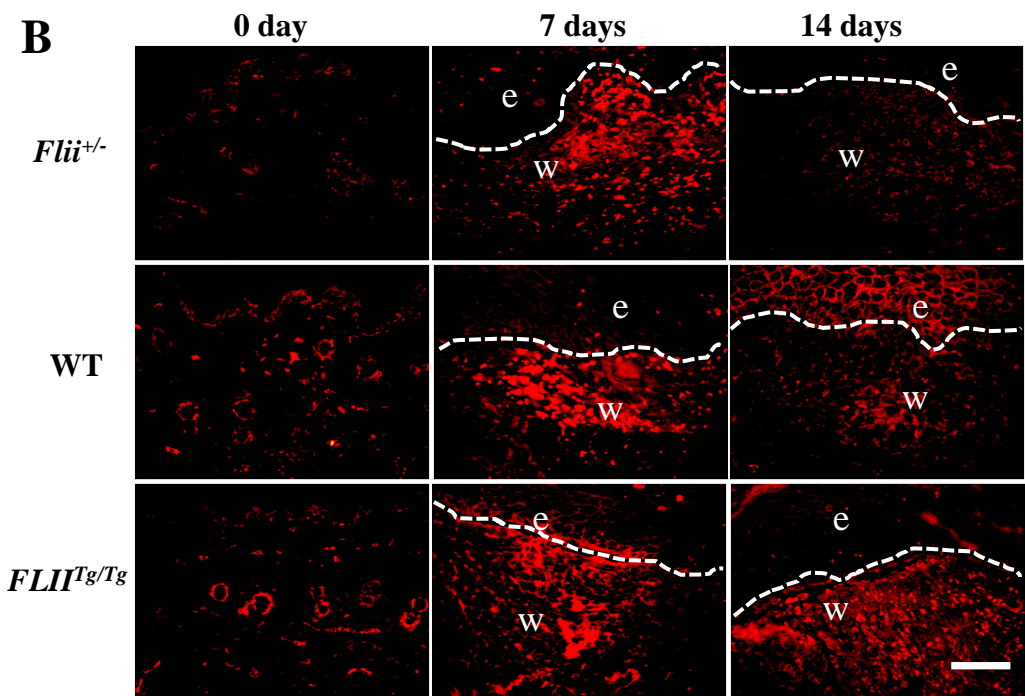
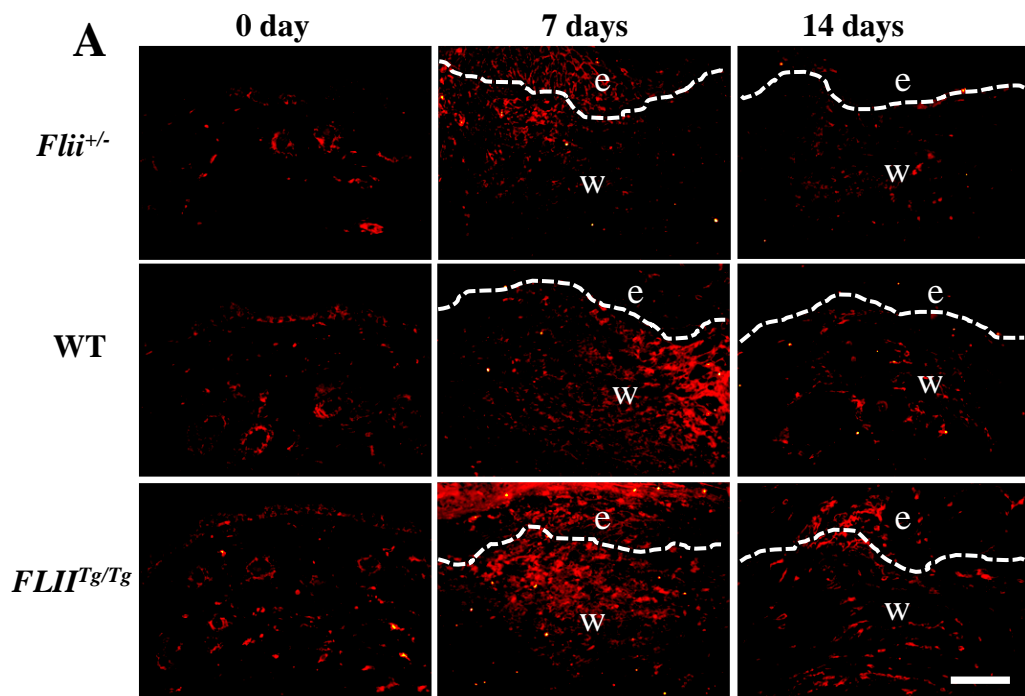


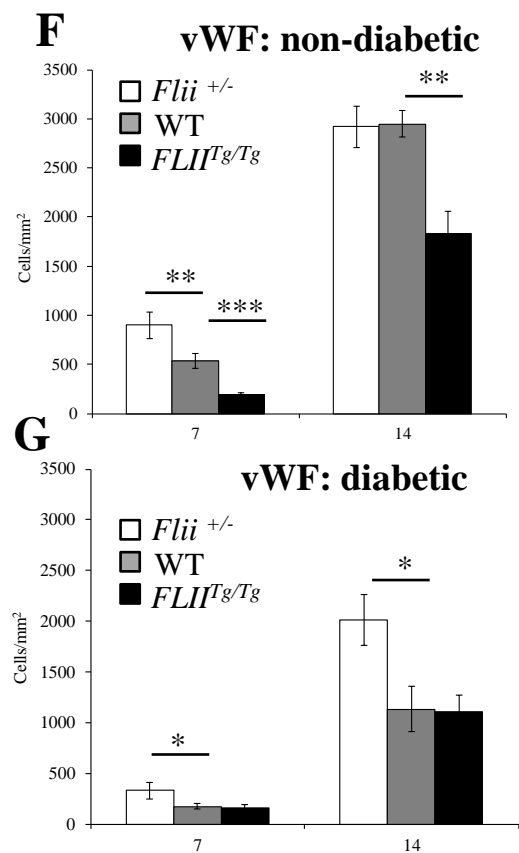
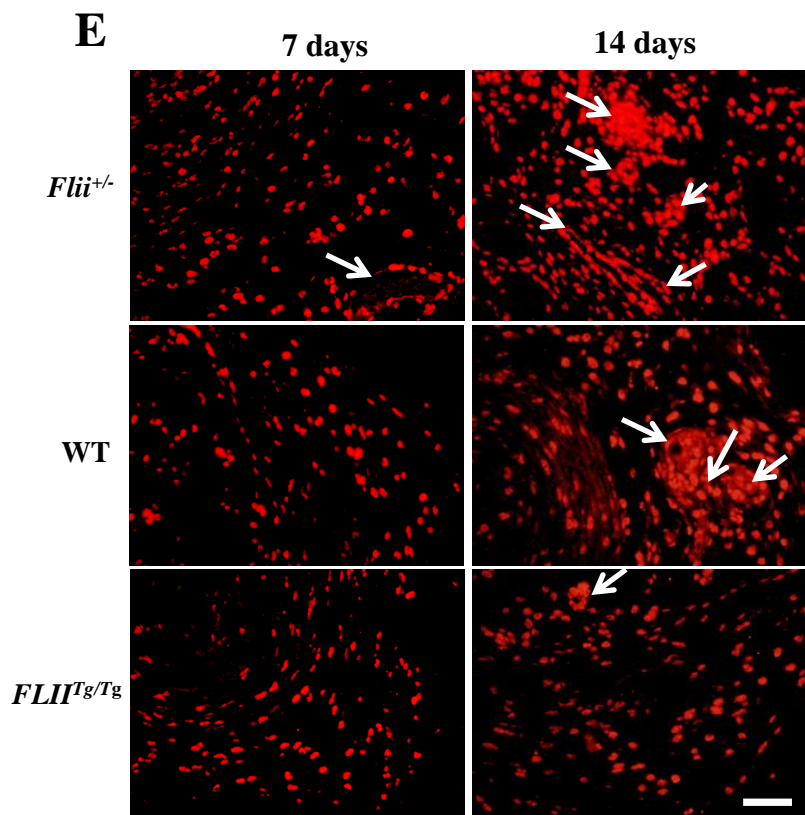
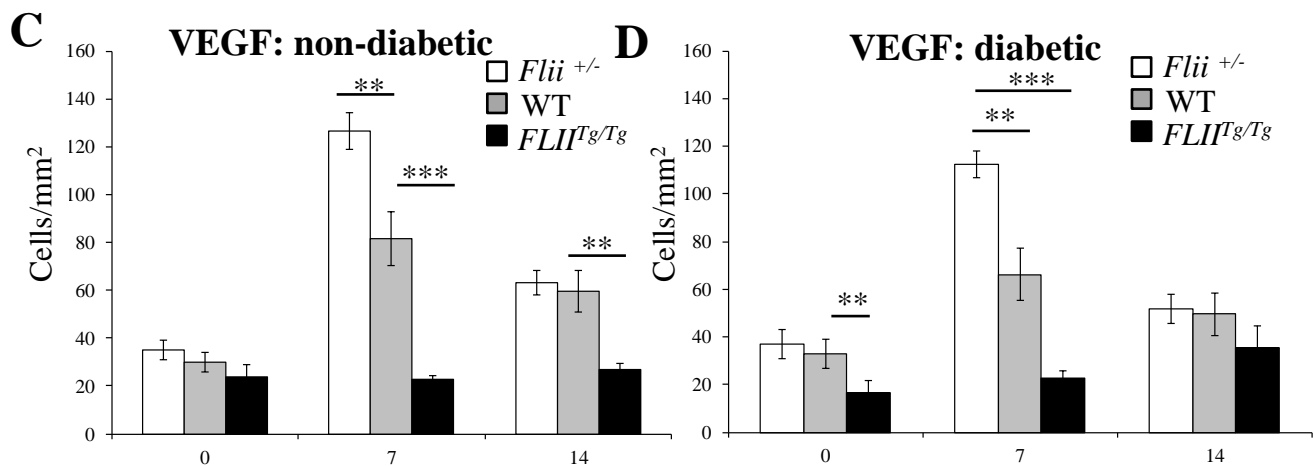
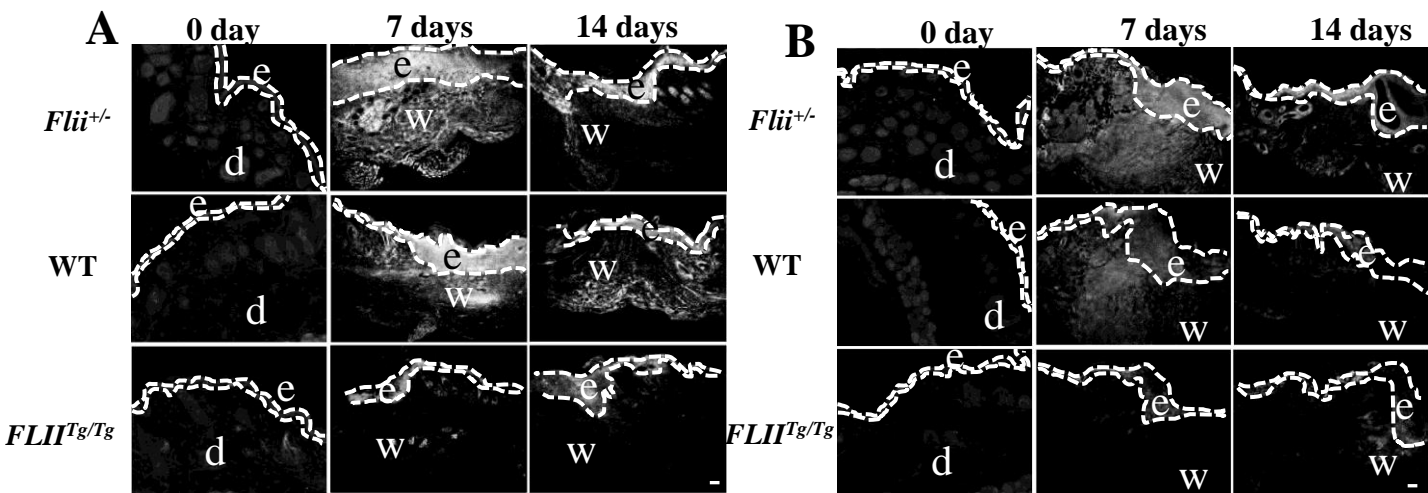
(D) FnAb. Scale bar in (C) and (D) is 100  $\mu\text{m}$  (original magnification 10 $\times$ ). (E) Quantification of number of capillary tubes based on 4 $\times$  images taken at 6 hours and grown on Matrigel substrate in the absence of antibody (no treatment), in the presence of IgG control (50  $\mu\text{g}/\text{ml}$ ) or FnAb (50  $\mu\text{g}/\text{ml}$ ).  $*p \leq 0.05$ ; IgG vs. FnAb; n=4. (F) Quantification of the formation of new vessels in Matrigel plugs by their lengths. Stimulation with FnAb resulted in formation of significantly longer RBC-containing vessels and vessels without cavities compared with IgG control ( $*p \leq 0.05$ ; n =6). (G) Two Matrigel plugs pre-mixed with either IgG or FnAb (50  $\mu\text{g}/\text{ml}$ ) were injected into WT mice. Each plug was harvested on day 7 and snap frozen. (G) Formalin fixed cryosections of Matrigel plugs were stained with anti-murine PECAM1 (CD31) (green) and  $\alpha$ -SMA (red) antibodies. (H) Quantification of fluorescence intensity of  $\alpha$ -SMA expression in IgG and FnAb-treated Matrigel plugs.  $**p \leq 0.01$ ; results represent mean  $\pm$  S.E.M. (n=6). (I) Representative immunofluorescence images of VEGF-stained sections of day 7 diabetic WT wounds treated with IgG and FnAb. (J) Graphical representation of VEGF staining of day 7 diabetic WT wounds treated with IgG and FnAb ( $*p \leq 0.05$ ; IgG vs. FnAb; n=6). (K) Western blotting analysis of VEGF expression of day 7 diabetic WT wounds treated with IgG and FnAb. (L) Bar graph with S.E.M. shows the fold change in the levels of VEGF in IgG and FnAb-treated day 7 diabetic WT wounds calculated from three replicates of immunoblot data ( $***p \leq 0.001$ ; n=4).

**Figure 7. Positive effect of FnAb treatment on diabetic wound healing.** (A) Images of day 0 and day 7 diabetic WT wounds treated IgG or FnAb (n=6; two wounds per mouse; 12 wounds). Dotted line delineates the wound edge. Scale bar = 1 mm. (B) Representative formalin fixed, paraffin embedded and H&E stained sections of day 7 diabetic WT wounds (n=6; 12 wounds per group). At least 2 histological sections from identical sites of day 7 wounds were studied to determine two important parameters and indicators of rated of wound repair, i.e. wound area, wound length and wound gape. (C) Digital images of IgG and FnAb treated diabetic WT wounds were taken at 0, 1, 2, 3, and 7 days post-wounding. (C) Quantification of wound surface area. Treatment with FnAb had a positive effect on diabetic wound closure *in vivo* by reducing the size of diabetic wounds treated with FnAb compared to IgG control on days 3 and 7 post-wounding. ( $*p \leq 0.05$ ; n=6; all values represent means and S.E.M.). (D) Microscopic wound length and (E) microscopic wound gape. Wound length was determined by manually drawing below the epidermis or clot between the wound margins. Dermal gape was recorded as the distance or a straight line between the dermal wound margins. Both parameters i.e. average wound length and gape were significantly smaller in FnAb-treated diabetic wounds than IgG-treated controls ( $*p \leq 0.05$ , IgG vs. FnAb; day 7 post-wounding).

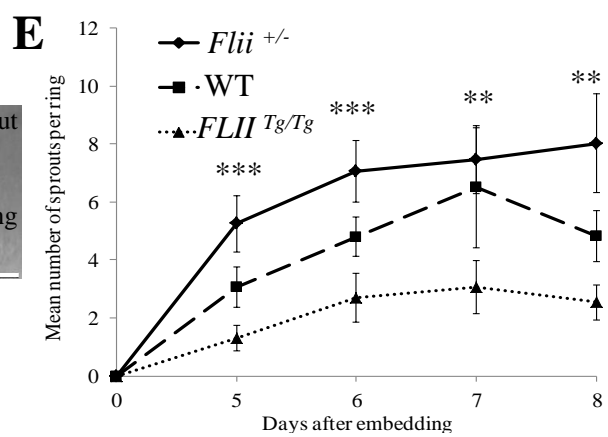
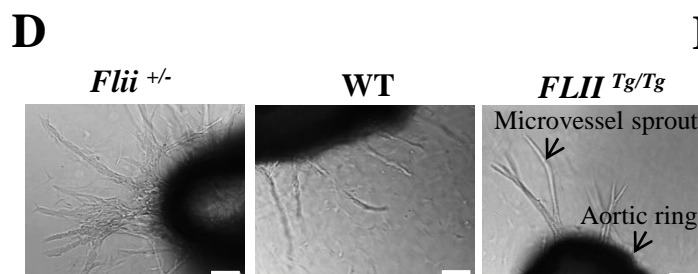
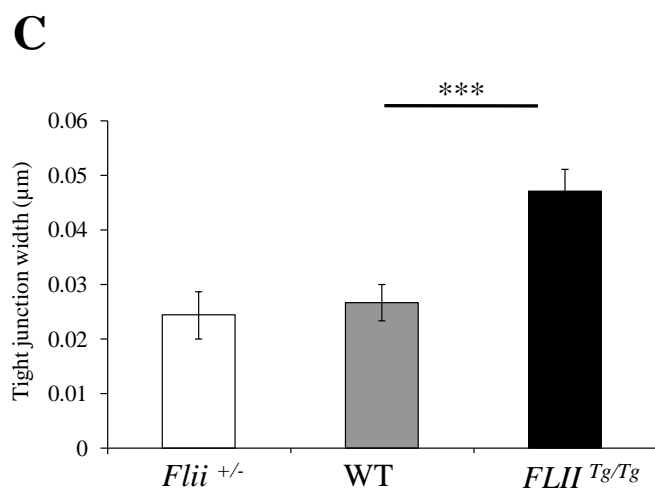
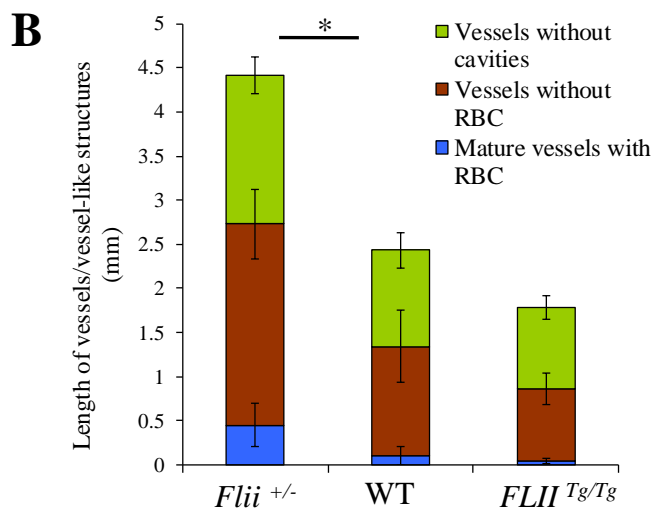
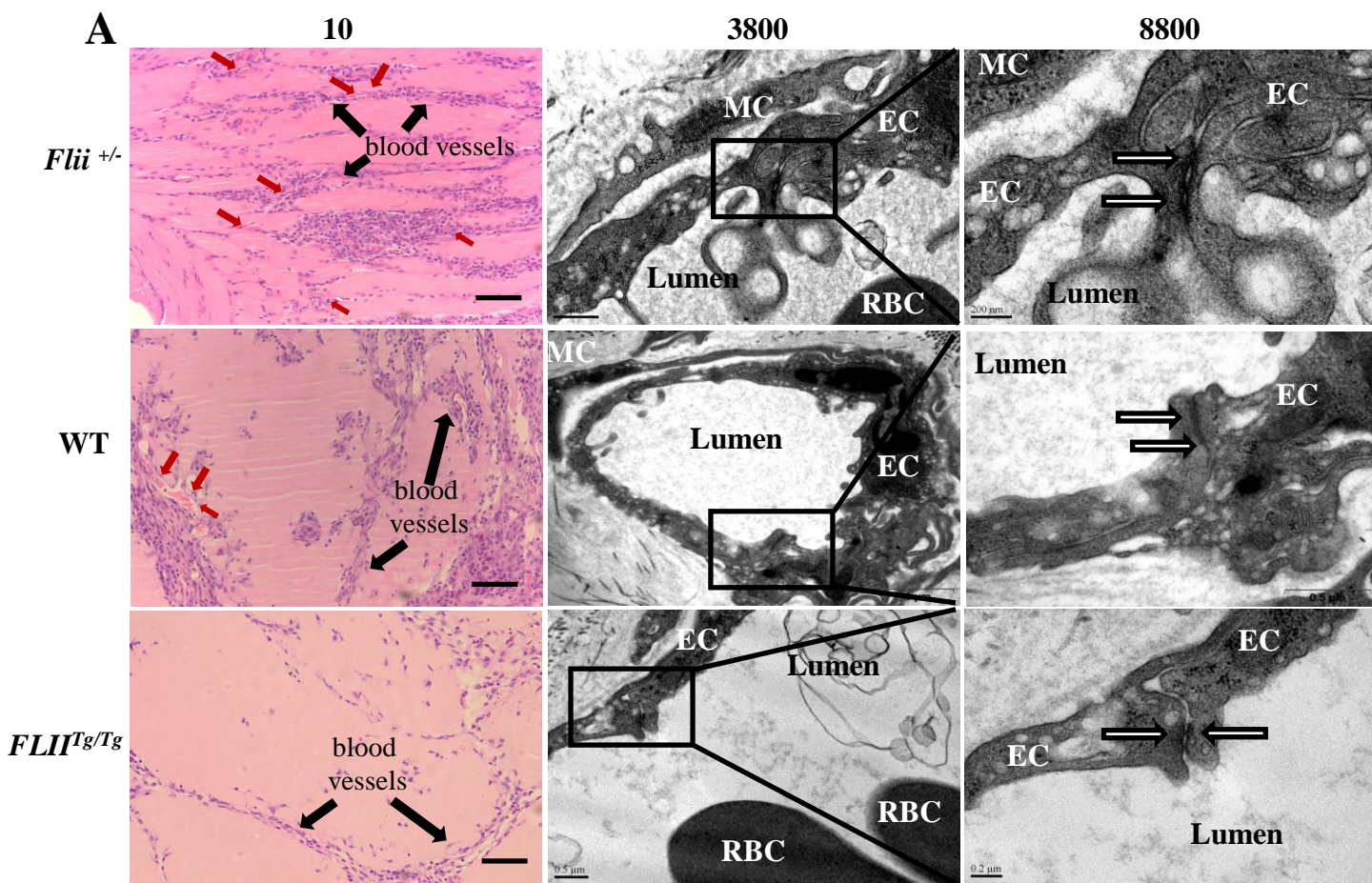


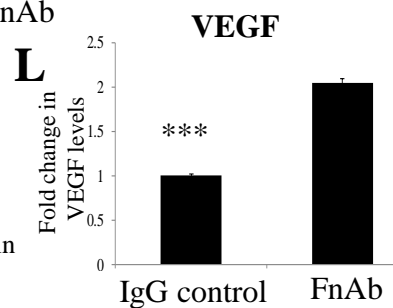
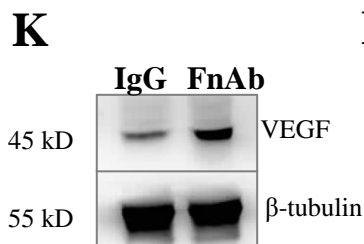
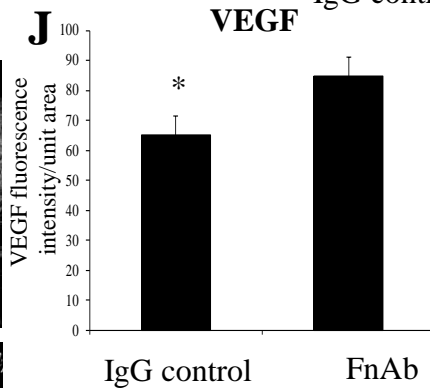
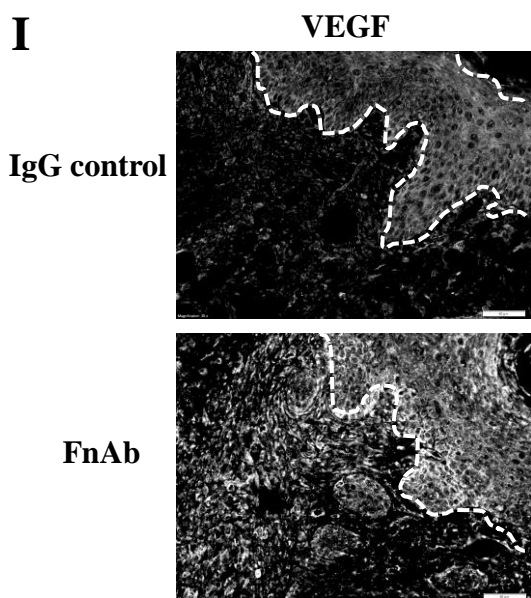
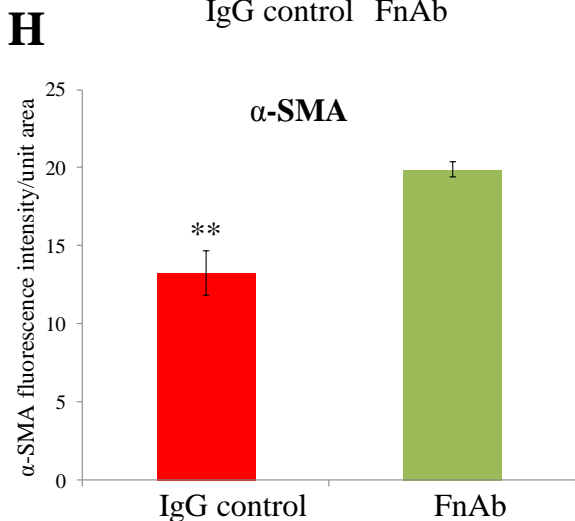
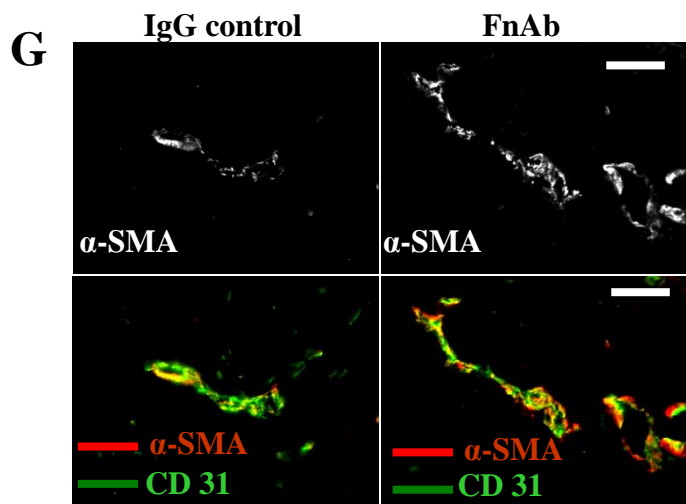
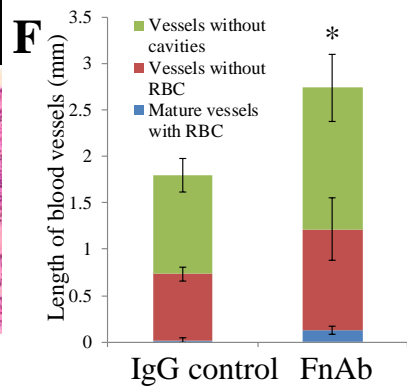
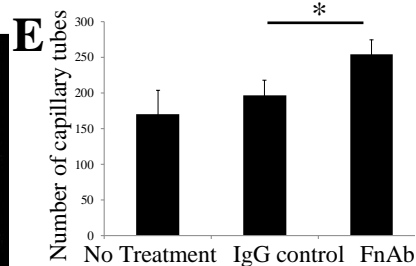
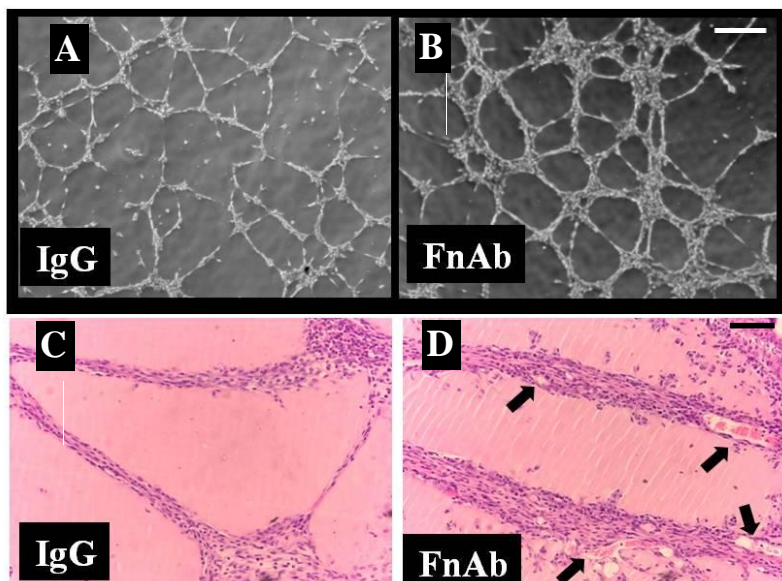


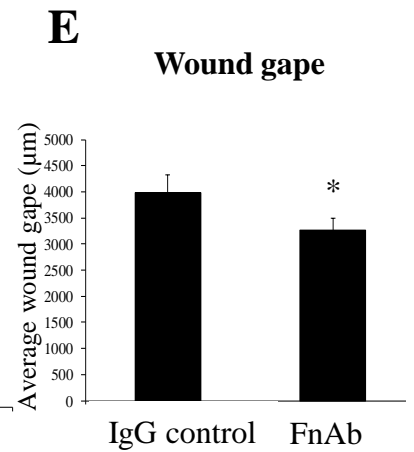
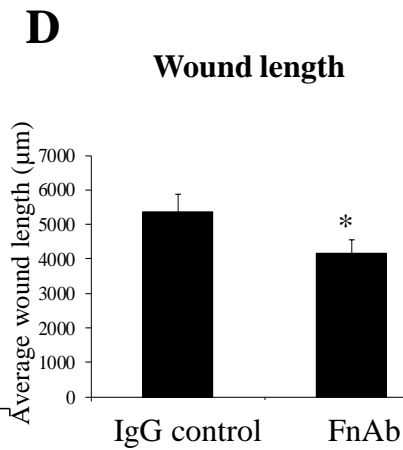
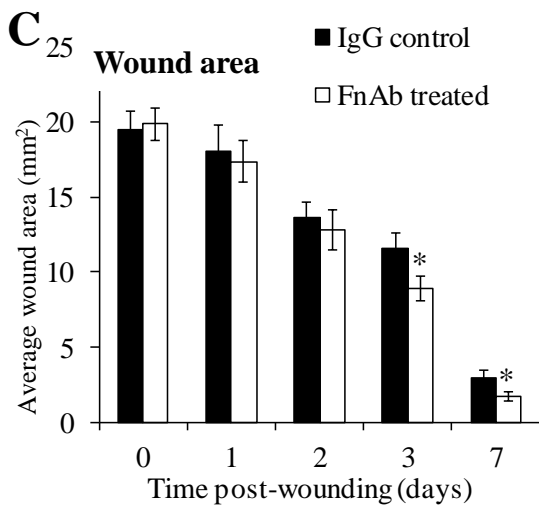
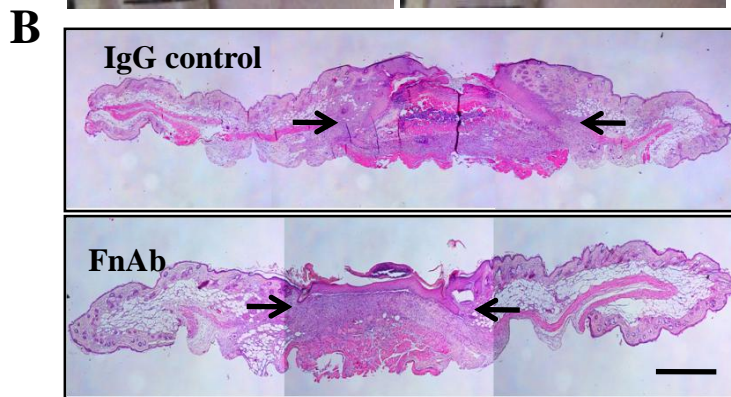
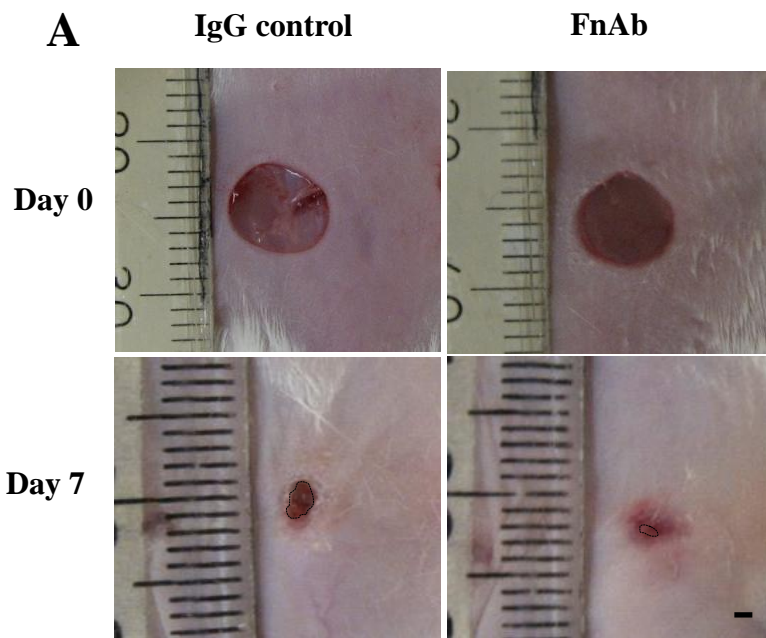




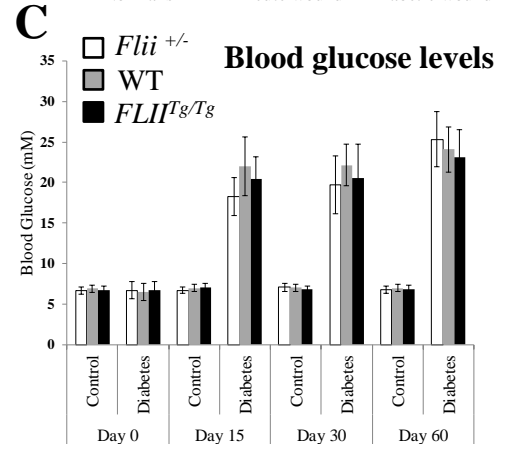
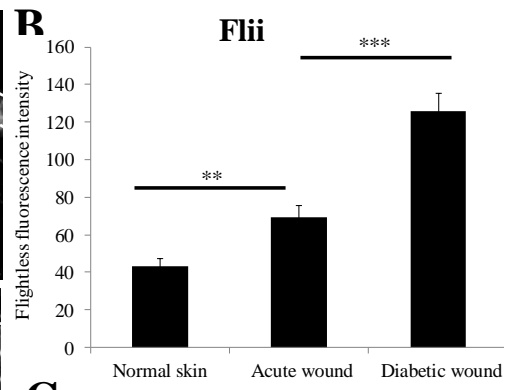
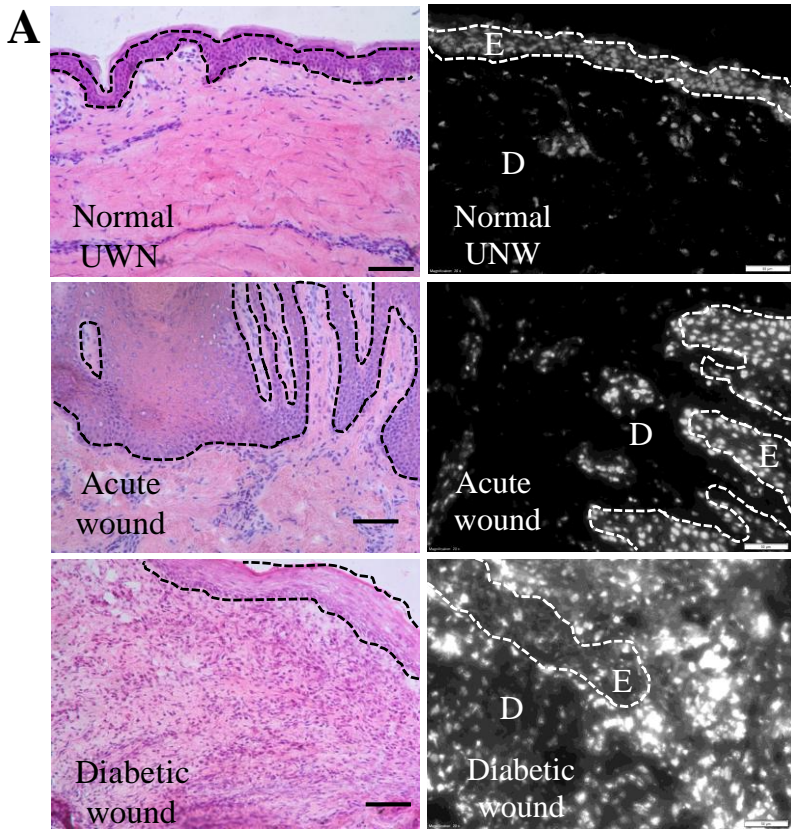












## **Flightless, secreted through a late endosome/lysosome pathway, binds LPS and dampens cytokine secretion**

Nazi Lei<sup>1,\*</sup>, Linda Franken<sup>1,\*</sup>, Nadira Ruzehaji<sup>2</sup>, Carolin Offenhäuser<sup>3</sup>, Allison J Cowin<sup>2,4</sup>, Rachael Z. Murray<sup>1,5‡</sup>

<sup>1</sup> CHBRI Wound Healing Laboratory, Kids Research Institute, Children's Hospital at Westmead, Sydney, NSW 2145, Australia.

<sup>2</sup> Women's and Children's Health Research Institute, North Adelaide, SA 5006, Australia.

<sup>3</sup> Institute for Molecular Bioscience, The University of Queensland, Brisbane, QLD 4072, Australia.

<sup>4</sup> Discipline of Paediatrics, The University of Adelaide, SA 5000, Australia

<sup>5</sup> Tissue Repair and Regeneration Program, Institute of Health and Biomedical Innovation, Queensland University of Technology, Brisbane, QLD 4095, Australia.

\*These authors contributed equally to this work.

‡Author for correspondence (rachael.murray@qut.edu.au)

**Short Title:** Secreted Flii dampens cytokine production

**Key words:** Flightless, late endosome, lysosome, secretion, TNF, cathepsin D, Rab7 and Stx11.

## Summary

Flightless (Flii) is upregulated in response to wounding and has been shown to function in wound closure and scarring. In macrophages intracellular Flii negatively modulates TLR signalling and dampens cytokine production. We now show that Flii is constitutively secreted from macrophages and fibroblasts and is present in human plasma. Secretion from fibroblasts is upregulated in response to scratch wounding and LPS-activated macrophages also temporally upregulate their secretion of Flii. Using siRNA, wild-type and mutant proteins we show that Flii is secreted via a late endosomal/lysosomal pathway that is regulated by Rab7 and Stx11. Flii contains 11 leucine rich repeat (LRR) domains in its N-terminus that have nearly 50% similarity to those in the extracellular pathogen binding portion of Toll-like receptor 4 (TLR4). We show secreted Flii can also bind LPS and has the ability to alter macrophage activation. LPS activation of macrophages in Flii depleted conditioned media leads to enhanced macrophage activation and increased TNF secretion compared to cells activated in the presence of Flii. These results show secreted Flii binds to LPS and in doing so alters macrophage activation and cytokine secretion, suggesting that like the intracellular pool of Flii, secreted Flii also has the ability to alter inflammation.

## Introduction

Flightless (Flii) is a member of the gelsolin superfamily of proteins and contains six gelsolin domains in its C-terminal end. Like other members of this family it binds actin and has F-actin severing activity via these domains (Goshima et al., 1999; Liu and Yin, 1998). As may be expected for an actin-remodelling protein, Flii regulates fibroblast and keratinocyte migration both in vitro and in vivo (Cowin et al., 2007). Many actin-binding proteins, including gelsolin family members, also have a role within the nucleus and Flii is no exception (Lee et al., 2004; Lee and Stallcup, 2006; Liu and Yin, 1998). Mice that lack other members of the gelsolin family are viable and fertile while the homozygous knockout of Flii in mice is embryonically lethal (Campbell et al., 2002). These results suggest other essential roles for Flii and its functions have now expanded well beyond the gelsolin-like actin remodelling and transcriptional regulator role found with other members of the family (Dai et al., 2009; Hayashi et al., 2010; Li et al., 2008; Wang et al., 2006).

Intracellular Flii is now emerging as an important negative regulator of inflammation (Dai et al., 2009; Li et al., 2008; Wang et al., 2006). Stimulation of Toll-Like Receptor (TLR) signalling pathways ultimately lead to transcription factor activation and the generation of cytokine and chemokine production (Kumar et al., 2011). This pathway is tightly regulated to prevent excessive and/or stimulation and increased signalling. Flii is a negative regulator of this pathway (Dai et al., 2009; Wang et al., 2006). Through its interaction with nucleoredoxin and MyD88 in macrophages Flii inhibits the binding of MyD88 to TLR4 resulting in reduced activation of NF- $\kappa$ B leading to diminished cytokine secretion (Dai et al., 2009; Wang et al., 2006). Flii has also been shown to inhibit cytokine secretion by other mechanisms (Li et al., 2008). Flii also binds to the proinflammatory caspases 1 and 11 and in doing so has been found to negatively regulate caspase-1 mediated interleukin-1 $\beta$  maturation and secretion (Li et al., 2008). Thus, Flii has the ability to dampen inflammation through a number of intracellular mechanisms.

The actions of Flii described to date involve intracellular Flii, however, results suggest that Flii may also be secreted (Cowin et al., 2007). We now show that Flii is secreted through a non-classical late endosome/lysosome-mediated pathway by at least two of the major cell types found in wounds: fibroblasts and macrophages. Secretion of Flii from fibroblasts is upregulated in response to wounding and a similar upregulation in secretion also occurs from LPS-activated macrophages. The N-terminal sequence of Flii shows nearly 50% similarity to the extracellular Leucine-Rich Repeat (LRR) domain of TLR4 (Wang et al., 2006). The LRR regions of TLRs play a key role in innate immunity by recognising structurally conserved molecules derived from microbes, with lipopolysaccharide (LPS) from Gram-negative bacteria being the archetypical TLR4 (Erridge, 2010; Kumar et al., 2011; Piccinini and Midwood, 2010). We show that secreted Flii, with its TLR4 like N-terminus can bind LPS and negatively regulate cytokine production from macrophages.

## **Results**

### **Flii secretion is both constitutive and regulated.**

To test whether Flii can be secreted from fibroblasts, extracts and media from a confluent layer of NIH3T3 fibroblasts (unwounded) were collected over an 8-hour time course. Samples were loaded equally on a SDS-PAGE gel (Fig 1A and

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supplementary material Fig. S1) and immunoblotted for Flii and actin (Fig. 1A). The anti-Flii antibody detected a band around 145 kDa both in the whole cell lysates and in media (Fig. 1). Little to no change in intracellular Flii levels were seen over the time course (Fig. 1A), however the level of Flii in the media increased with time suggesting it is constitutively secreted from fibroblasts (Fig. 1A,B). We have previously shown that Flii is upregulated in mouse skin wound tissue in response to injury (Adams et al., 2009; Cowin et al., 2007). Since Flii levels increase in the wounds of mice, scratch wounds were created in a confluent layer of NIH3T3 fibroblasts to determine whether scratch wounding these cells could alter Flii secretion. Cell extracts and media were also collected from these cells over the same 8-hour time course and immunoblotted for Flii and actin (Fig. 1A,B). The level of intracellular Flii was similar to that found in unscratched fibroblasts (Fig. 1A). However, the amount of secreted Flii was increased by 1.5 to 2.5 fold upon scratch wounding compared to media from unwounded cells incubated for the same time period suggesting its secretion is upregulated upon wounding (Fig. 1A,B). Immunoblotting of the same media samples for the cytosolic enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and for actin showed that the presence of Flii in the media is not due to general cytosol leakage or cell disruption (Fig. 1A). The activity of a known secreted lysosomal enzyme, beta-hexosaminidase, was also analysed in media from control and scratched fibroblasts (Fig. 1C). While the level of enzyme activity increased over the 8-hour time course no significant differences were seen between the amount of beta-hexosaminidase activity in the media from scratched and control fibroblasts (Fig. 1C). This data shows that beta-hexosaminidase is also secreted from these cells; however, unlike Flii its level of secretion is not significantly increase upon wounding. We next looked at the level of Flii secreted from cells over the 8-hour scratch wounded period (Fig. 1D). The levels of Flii secreted into the media 6 hours after scratching wound is equivalent to approximately 3% of the total intracellular pool of Flii (Fig. 1D). Thus, Flii is constitutively secreted by fibroblasts and its secretion can be upregulated in response to scratch wounding.

Immunoblotting of skin and plasma from healthy volunteers for Flii, the plasma marker albumin and the cellular marker tubulin showed that Flii is present in plasma as well as the skin (Fig. 1E). Flii has been found in macrophages where it can regulate TLR signalling and cytokine secretion (Dai et al., 2009; Hayashi et al., 2010;

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Wang et al., 2006). Monocyte/macrophages are present in high numbers both in wounds and plasma and so whether Flii could also be secreted from macrophages was next tested. Extracts from RAW264.7 macrophages were first immunoblotted alongside equal protein levels of NIH3T3 fibroblast extracts (Fig. 1F). Similar intracellular levels of Flii were seen in both cell types (Fig. 1F). To test whether Flii is also secreted by macrophages and whether this secretion is altered when macrophages are activated and secreting cytokines, RAW264.7 macrophages were stimulated with the bacterial cell wall component lipopolysaccharide (LPS) over a 6-hour time course. Cell extracts were immunoblotted for Flii, actin and the proinflammatory cytokine TNF (Fig. 1G,H). Thirty minutes post LPS stimulation we saw the characteristic upregulation of intracellular TNF protein levels, which peaked around 3 hours post stimulation, while the total levels of intracellular Flii appeared unaltered over the same time course (Fig. 1G) (Murray et al., 2005b). Immunoblotting of conditioned media collected from the same cells showed TNF secretion started 30 minutes post LPS stimulation and gradually increased (Fig. 1G,H). Moreover, Flii was also secreted by macrophages and in LPS-activated macrophages secretion peaked at around 30 minutes post-stimulation (Fig. 1G,H). These results together suggest Flii is constitutively secreted by both fibroblasts and macrophages and that Flii secretion can be upregulated from fibroblasts in response to scratch wounding and in macrophages in response to LPS-activation.

**Flightless is located in the nucleus, cytosol and in late endosomes/lysosomes.**

Since Flii has previously been found in both the nucleus and cytoplasm of Swiss 3T3 fibroblasts its location in unwounded NIH3T3 cells and scratch wounded fibroblasts was next analysed (Davy et al., 2001). Cell extracts from unwounded and scratch wounded confluent NIH3T3 fibroblasts were separated into nuclear and post-nuclear fractions (Fig. 2A). Equal protein was loaded and probed for Flii, the cytoplasmic marker protein tubulin and the nuclear marker protein HDAC1 (Fig 2A). Flii was predominantly located in the post-nuclear fraction in NIH3T3 fibroblasts and its location did not significantly alter upon wounding (Fig. 2A). RAW264.7 macrophages were incubated in the presence or absence of LPS for 3 hours and extracts were separated into nuclear and post-nuclear fractions to determine its location. Fractions were immunoblotted for Flii, the cytoplasmic marker protein

tubulin, the nuclear marker protein nucleoporin and the cytokine TNF (Fig. 2B). The majority of Flii was located in the post-nuclear fraction that contains membranes and cytosol in macrophages regardless of their activation state (Fig. 2B). Post-nuclear fractions from both NIH3T3 cells and RAW264.7 cells were next separated into membrane and cytosol using a high-speed centrifugation step. Membrane and cytosol fractions were probed for Flii, the trans-membrane protein LAMP1 and the cytoplasmic protein  $\beta$ -actin (Fig. 2C). In both fibroblasts and macrophages Flii is located both in the cytosol and the membrane fractions (Fig. 2C).

Immunostaining of Flii in both fibroblasts and macrophages revealed that Flii was located in the cytosol as well as in large endosomes located in the perinuclear region and throughout the cytoplasm (Fig. 3A,B). Some Flii could be detected in the nuclei of fibroblasts but its levels differed depending on the individual cell (Fig. 3A). Primary mouse fibroblasts were co-immunostained for Flii along with the late endosome/lysosome marker VAMP7 or cathepsin D, a late endosome/lysosomal protease that is also secreted by some cell types. Flii is located in the nucleus, cytosol and in late endosomes/lysosomes in fibroblasts (Fig. 4A). Immunostaining of RAW264.7 macrophages for Flii in combination with the late endosome/lysosome membrane proteins LAMP1 and VAMP7, as well as cathepsin D, showed these proteins colocalised in macrophages (Fig. 3B). Thus, Flii is located in late endosomes/lysosomes with cathepsin D in fibroblasts as well as macrophages and is potentially trafficked to the surface via late endosomes/lysosomes.

### **Flii is secreted via a late endosome/lysosomal pathway**

We next looked to see how Flii might be being secreted. Two major types of routes for secretion exist, classical and non-classical. In the classical pathway proteins are trafficked from the endoplasmic reticulum (ER) via the Golgi complex en route to the cell surface (Bonifacino and Glick, 2004). In this pathway secretory proteins in this pathway contain amino-terminal or internal signal peptides that direct their sorting to the endoplasmic reticulum (ER). Flii's amino acid sequence was analysed using SignalP 3.0 ([www.cbs.dtu.dk/services/SignalP](http://www.cbs.dtu.dk/services/SignalP)) and the results established that Flii lacks a secretory signal peptide (probability = 0.014). This suggested that Flii might not be secreted via the classical pathway. To confirm this, Flii was co-immunostained in RAW264.7 macrophages with the Golgi protein VAMP4, as well as with the



known classical secretory pathway trafficked cytokine TNF (Murray et al., 2005a; Murray et al., 2005b). Flii did not colocalize with the Golgi associated SNARE protein VAMP4 or the cytokine TNF (Fig. 2C). Brefeldin A (BFA) has been shown to cause disruption of the Golgi and prevent proteins trafficking from the ER to the Golgi through the classical pathway (Fujiwara et al., 1988). To confirm that Flii is not secreted via the classical pathway, RAW264.7 macrophages were incubated in the presence or absence of BFA (5ng/ml) for 60 min with LPS (100 ng/ml) for the last 30 min. Fixed cells, immunostained for the Golgi marker protein GM130, showed that in the presence of BFA the Golgi was disrupted as expected (Fig. 4B). Immunoblotting of extracts from these cells showed that the levels of Flii inside the cell and secreted in the media were not altered in the presence of BFA (Fig. 4C) confirming that Flii is not secreted via the classical pathway.

The delivery of cargo to the cell surface via late endosomes/lysosomes is a known non-classical pathway of secretion and the presence of Flii in late endosome/lysosomes suggest that Flii may be secreted via this same pathway (Nickel, 2003). The small G-protein Rab7 regulates traffic through the late endosome (Bucci et al., 2000; Vanlandingham and Ceresa, 2009). To determine whether Flii is indeed trafficked through the late endosome on route to the cell surface RAW264.7 macrophages were transiently transfected with GFP, GFP-Rab7 or a GFP-tagged dominant negative form of Rab7 (GFP-Rab7T22N) known to inhibit traffic through this organelle (Bucci et al., 2000). Extracts from these macrophages were immunoblotted for Flii, cathepsin D, GFP and actin (Fig. 5A). The intracellular levels of both Flii and the mature form of cathepsin D were decreased by 25% and 21% respectively in cells transiently overexpressing GFP-tagged wild-type Rab7 as compared to cells expressing GFP alone (Fig 5A,B). This decrease in intracellular Flii in cells coincided with a 1.7-fold increase in Flii secreted into the media (Fig. 5A,B). Detection of cathepsin D in media by immunoblotting was obscured due to the large albumin band in the media so the same conditioned media was also analysed for the secreted lysosomal enzyme, beta-hexosaminidase (Fig. 5C). Similar to Flii, the level of secreted beta-hexosaminidase is increased (approximately 1.2 fold) in the conditioned media of cells transiently overexpressing GFP-Rab7 (Fig. 5C). Macrophages transiently transfected with the dominant negative form of Rab7 (GFP-Rab7T22N) showed an increase (approximately 1.2 fold) in intracellular Flii and



mature cathepsin D as compared to cells transfected with GFP alone (Fig. 5A,B). The increased intracellular Flii levels corresponded with a reduction in secreted Flii in the media by almost half compared with control GFP expressing cells suggesting secretion was blocked by expression of the dominant negative form of Rab7 (Fig 5A,B). Similarly a reduction in beta-hexosaminidase secreted into the media was seen in conditioned media from cells expressing the dominant negative form of Rab7 (Fig. 5C). These results together suggest that Flii is trafficked through a late endocytic/lysosomal compartment on route to the cell surface for secretion.

**Increasing fusion of a late endosome/lysosome compartment with the cell surface results in increased Flii secretion.**

We have recently shown that the loss of the SNARE protein Stx11 in macrophages leads to an increased fusion of a LAMP1-positive late endosomal/lysosomal compartment with the cell surface (Fig. 5D) (Offenhäuser et al., 2011). If Flii were trafficked via this same Stx11-regulated pathway then it would be expected that a reduction in Stx11 levels would deplete intracellular Flii pools while increasing the level of Flii secreted into the media. To test this, macrophages were treated with small interfering RNAs (siRNAs) to reduced the level of Stx11 (Fig. 6A,B). Cells treated with control or Stx11 siRNA were fixed, permeabilised and immunostained live for surface LAMP1 (Fig. 5D). As previously shown, surface levels of LAMP1 are greatly increased on macrophages treated with Stx11 siRNA as compared to control treated cells, indicative of an increase in the fusion of LAMP1-positive organelles with the cell surface (Fig. 5D). Cell extracts from these macrophages were immunoblotted for Stx11, Flii, cathepsin D and actin and media from the same cells was immunoblotted for Flii (Fig. 5E). Compared to cells treated with control siRNA cells treated with siRNA to Stx11 showed a reduction in intracellular Flii and cathepsin D (approximately 50% and 20% respectively) and a concomitant increase (1.5 fold) in secreted Flii levels (Fig. 5E,F). An increase in the lysosomal enzyme beta-hexosaminidase secreted into the media was also seen when cells were treated with Stx11 siRNA compared to control treated cells (Fig. 5G). These results together suggest that the pool of Flii located in a LAMP1-positive late endosome/lysosome compartment is trafficked to the cell surface and released into the extracellular milieu when this LAMP1-positive late endosome/lysosome-related compartment fuses with

the cell surface.

### **Secreted Flii binds to LPS and alters macrophage activation**

Eleven leucine-rich repeat (LRR) domains, located at the N-terminus between amino acids 57 to 362 in Flii, were identified using ScanProsite and InterPro Scan domain mapping tools (<http://expasy.org/tools/>) (Fig 6A). This LRR region shares 29% sequence identity and 42% similarity to the LRR domains found in the extracellular portion of TLR4 (Wang et al., 2006). The binding of the bacterial cell wall component LPS to this same domain in TLR4 leads to the signal transduction events that ultimately result in cytokine secretion (Bell et al., 2003). Thus, whether Flii, like TLR4, could also bind LPS was next tested. Conditioned media containing high levels of secreted Flii from scratch wounded NIH3T3 cells was incubated with LPS and antibodies to GFP, LPS or Flii bound to protein A-agarose. Co-immunoprecipitated proteins were immunoblotted for Flii and results confirmed the anti-Flii antibody was able to immunoprecipitate Flii as expected (Fig. 6B). Moreover, Flii from the conditioned media was immunoprecipitated with LPS (Fig. 6B). Addition of an antibody specific for the first LRR domain of Flii (Cowin et al., 2007), added to conditioned medium for 1 hour prior to the above immunoprecipitation experiment, inhibited the binding of Flii to LPS (Fig. 6C). These results suggest Flii might bind LPS via its LRR domain.

We next tested whether the binding of Flii to LPS could influence LPS-activation of macrophages, looking at the effect of altering Flii levels on the downstream TNF production and secretion. Conditioned media, containing high levels of secreted Flii from scratch wounded NIH3T3 cells, was depleted of Flii using anti-Flii antibodies bound to protein A-agarose. Immunoblotting showed Flii levels in the medium were greatly depleted (Fig. 6D). Macrophages grown on coverslips were incubated in conditioned media containing high levels of Flii or in the Flii depleted media and stimulated with LPS for 3 hours then fixed, permeabilised and stained for TNF and F-actin. Both the level and the number of cells expressing TNF were greatly reduced in the presence of high levels of Flii, suggesting Flii may inhibit LPS activation of macrophages (Fig. 6E). To confirm this, extracts from LPS stimulated macrophages incubated in conditioned media containing Flii or in Flii depleted media were immunoblotted for TNF and actin. In the presence of high levels of Flii the total

TNF levels were reduced by approximately 30% compared to cells stimulated in Flii depleted media (Fig. 6F,H). A 40% reduction in secreted TNF was seen when cells were stimulated with LPS in the presence of high levels of Flii (Fig. 6G,H). These results suggest that secreted Flii may sequester LPS, preventing it from activating macrophages.

## Discussion

Intracellular Flii has been found to be an important negative regulator of inflammation (Dai et al., 2009; Li et al., 2008; Wang et al., 2006). We now show that Flii is also secreted and the secreted form can also regulate inflammation. Flii is constitutively secreted by both fibroblasts and macrophages and can be found in plasma samples from healthy volunteers. Secretion of Flii can be upregulated by either scratch wounding fibroblasts or LPS stimulating macrophages. Flii is secreted via a late endocytic/lysosomal compartment and both Rab7 and the SNARE Stx11 regulate this trafficking pathway in macrophages. Flii also localises to the same compartment in fibroblasts, suggesting Flii is secreted via a similar pathway in both cell types. Inhibition of this trafficking pathway results in increased intracellular Flii and a reduction in secretion, while increasing fusion of late endosomes/lysosomes increases Flii secretion. Once secreted Flii can bind to LPS and this binding can be inhibited with an antibody specific for the LRR domains at the N-terminus of Flii. Macrophages stimulated with LPS in the presence of media depleted of Flii synthesise and secrete more TNF, suggesting secreted Flii may act to sequester LPS and alter cytokine secretion during an inflammatory response.

Biochemical and localisation studies in NIH3T3 fibroblasts revealed that Flii is located in the nucleus, cytosol and in late endosomes/lysosomes. In RAW264.7 macrophages the majority of Flii is located in the cytosol and in late endosomes/lysosomes. Flii has previously been found in the nucleus, cytosol and to be associated with the cytoskeleton in Swiss 3T3 cells (Davy et al., 2001). The slight differences in localisation may reflect the different cell types and perhaps the functions of Flii in these cells. Flii has been found to play a number of different roles in cells (Cowin et al., 2007; Davy et al., 2001; Kopecki and Cowin, 2008; Wang et al., 2006). In macrophages the cytosolic pool of Flii has been found to bind to MyD88 and prevent its binding the cytoplasmic tail of TLR4 (Wang et al., 2006). Flii also

binds to caspase-1 in macrophages where it can inhibit the maturation of interleukin-1 $\beta$  maturation and leading to a reduction in its secretion (Li et al., 2008). Interestingly pro-interleukin-1 $\beta$  and caspase-1 have both been shown to be found in late endosomes with Cathepsin D and to be secreted from this compartment (Andrei et al., 1999; Eder, 2009).

Lysosomes or lysosome-related organelles have been found to perform a range of functions, some common to all cell types, such as degradation, whilst others are more specialised and tend to involve fusion of this organelle with the cell surface to release its contents (Holt et al., 2006; Luzio et al., 2007). We have used Rab and SNARE proteins, located on specific membranes that regulate transport at distinct sites in the late endosome/lysosome pathway, to show Flii is secreted from this compartment (Stow et al., 2006; Zerial and McBride, 2001). Rab7 is located on late endosomes, where it regulates traffic to lysosomes (Bucci et al., 2000; Vanlandingham and Ceresa, 2009). Similarly the SNARE protein Stx11 is located on late endosomes and lysosomes and regulates traffic between these organelles and the cell surface (Offenhäuser et al., 2011). Loss of either Rab7 or Stx11 results in enlarged endosomes and loss of Stx11 leads to increased fusion of this late endocytic/lysosomal compartment with the cell surface (Offenhäuser et al., 2011; Vanlandingham and Ceresa, 2009). We now show that Rab7 and Stx11 regulate Flii secretion from a late endocytic/lysosomal compartment in macrophages. Similar results were seen for cathepsin D, which is known to be secreted via a late endocytic/lysosomal compartment (Gardella et al., 2001).

Lysosomal secretion can be either from distinct subsets of lysosome related organelles, for example melanosomes, or from organelles that are indistinguishable from lysosomes themselves (Holt et al., 2006; Luzio et al., 2007). Secretion from these organelles is often switched on by external stimuli, such as the recognition of a foreign antigen (Holt et al., 2006; Luzio et al., 2007). Our results suggest that in both fibroblasts and macrophages secretion of Flii from a late endosome/lysosome compartment is constitutive and can be upregulated in response to specific external stimuli. In response to scratch wounding fibroblasts secrete up to 2.5 times more Flii than unwounded cells, whilst in macrophages LPS activation leads to a temporal increase in secreted Flii 30 minutes after activation. No increase in intracellular Flii was seen after scratch wounding to match the increase in secretion, however the level

of Flii secreted is very low (3% over 6 hours) in comparison to the total cellular levels. These results suggest that secretion from this compartment may be continual but can be amplified in response to external stimuli. It is currently unclear how Flii enters the late endosomal/lysosomal compartment but future studies will address this question.

Mice with increased levels of Flii have impaired wound healing compared to control mice, while mice with low levels of Flii have significantly improved wound healing responses (Cowin et al., 2007). However, Flii is upregulated in tissue in response to injury in mice and we now show fibroblasts increase secretion of Flii in response to wounding (Adams et al., 2009; Cowin et al., 2007). The upregulation of Flii in response to injury seems counterproductive since increasing the levels of Flii leads to reduced cell migration, impaired healing and increased scar formation. The results shown here suggest the answer may lie in part in Flii's ability to also alter the inflammatory response. Whilst mounting a successful immune response is important to clear an infection, a hyper-activated immune response leads to overproduction of tissue damaging cytokines, which could potentially be detrimental to the wound, as well as systemically where it can lead to sepsis (Murphy et al., 2004). Intracellular Flii has previously been shown to negatively regulate cytokine secretion by inhibiting MyD88 binding to TLR4 and effectively acts to reduce signalling for cytokine production and dampen the inflammatory response (Dai et al., 2009; Wang et al., 2006). Our results now suggest that Flii is also secreted and that extracellular Flii can also alter cytokine secretion by binding to LPS. The binding of Flii to LPS and the timing of Flii secretion suggest that Flii may act as a scavenger to help mop up excess LPS and prevent hyper-activation of the immune system. These results suggest that the upregulation of Flii in wounds might serve to alter inflammation in the wound and prevent excess stimulation of cytokine production.

The related protein gelsolin is also secreted and the extracellular form has been shown to dampen the immune response by binding to LPS and lipoteichoic acid (LTA), released from the outer walls of Gram-negative and Gram-positive bacteria respectively (Bucki et al., 2008; Bucki et al., 2005). Thus, it has been proposed that extracellular gelsolin may act as an extracellular scavenger for these bacterial cell wall components (Bucki et al., 2008). The region in gelsolin that binds to LPS is not present in Flii (data not shown); however, Flii does have an LRR domain at its N-

terminus, not present in other gelsolin family members (Kopecki and Cowin, 2008; Mintzer et al., 2006). The LRR domain in Flii shares 29% sequence identity and 42% similarity to the LRR domain in TLR4 (Wang et al., 2006). The LRR domain of TLRs is located on the extracellular side of the plasma membrane and has been shown to be involved in the binding of pathogen-associated molecular patterns (PAMPs) from a range of pathogens, including LPS, as well as damage-associated molecular patterns (DAMPs) found in some host molecules released upon injury (Bell et al., 2003; Erridge, 2010; Kumar et al., 2011). The binding of Flii to LPS and the timing of Flii secretion suggest that Flii may also act as a scavenger to help mop up excess LPS and prevent hyper-activation of the immune system. Since many proteins are released upon injury, a number of which also can activate TLR signalling, it remains to be seen whether secreted Flii can also act as a general scavenger protein that binds to the DAMPs in host intracellular proteins released upon injury (Erridge, 2010). In summary, Flii may be acting both in side and outside of the cell to dampen inflammation by negatively regulating intracellular TLR signalling as shown previously or as we now show by binding LPS and preventing activation of TLR signalling.

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### **Materials and Methods**

#### **Antibodies and reagents**

Antibodies specific for Vti1b, VAMP3, VAMP4 and VAMP7 were purchased from Synaptic Systems (Goettingen, Germany) and antibodies specific for cathepsin D and actin were purchased from Millipore (Kilsyth, Vic, Australia). Anti-mouse Flii (IgG Sc-21716), anti-rabbit Flii (IgG Sc-30046), anti-HDAC1 and anti-albumin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-GAPDH antibodies were purchased from Ambion (Austin, Texas, USA). Antibodies specific for LAMP1 were purchased from Southern Biotech (InVitro, Noble Park, Vic, Australia), while antibodies specific for nucleoporin and GM130 were purchased

from BD Transduction Laboratories (North Ryde, NSW, Australia). Antibodies specific for TNF were purchased from Calbiochem (Merck, Kilsyth, Vic, Australia). Anti-LPS (*E. coli*) antibodies were purchased from Abcam (Sapphire Biosciences, Waterloo, NSW, Australia). Rabbit anti-GFP antibodies were purchased from Invitrogen (Mulgrave, Vic, Australia) and mouse anti-GFP antibodies were purchased from Roche (Castle Hill, NSW, Australia). HRP and CY3-tagged secondary antibodies were purchased from Jackson Laboratories (West Grove, PA, USA), while Alexa-488 and Alexa-647-tagged secondary antibodies and Alexa-488 phalloidin were purchased from Molecular Probes (Invitrogen, Mulgrave, Vic, Australia). Anti-Flii LRR (IgM) antibodies made to amino acids 56-69 located in the first LRR domain of Flii as described previously (Adams et al., 2009). Anti- $\beta$ -tubulin antibodies, LPS from *Salmonella minnesota* Re595 and LPS from *Escherichia coli* J5 (Rc mutant) (used in the immunoprecipitation experiments) and 4-nitrophenyl N-acetyl- $\beta$ -D-glucosaminide (20  $\mu$ l) were purchased from Sigma (Sydney, NSW, Australia). Rabbit polyclonal anti-Stx11 antibodies were a kind gift from Prof Jenny Stow (University of Queensland, Australia) and have been described elsewhere (Offenhäuser et al., 2011). GFP-Rab7 and GFP-Rab7T22N were a kind gift from Prof Zerial (Max Planck Institute of Molecular Cell Biology and Genetics, Dresden).

### **Preparation of human skin samples and plasma**

The collection of human skin and peripheral blood samples from patients undergoing abdominoplasty was approved by the Central Northern Adelaide Health Service Ethics of Human Research Committee. Peripheral blood collected in EDTA treated blood collection tubes was centrifuged at 300 x *g* for 10 min at 4°C and the plasma transferred to a new tube. Samples were mixed with trichloroacetic acid at 1:10 sample volume and centrifuged for 1.5 h at 6500 x *g*. Samples were resuspended in 800  $\mu$ l of -20°C acetone, incubated overnight at -20°C and centrifuged at 6500 x *g* at 4°C for 10 minutes. The pellet was then resuspended in -20°C acetone, incubated at -20°C for 30 minutes, recentrifuged, the acetone discarded and the pellet dried. The pellet was then resuspended in 1% SDS prior to separation on an SDS-PAGE gel. Human skin samples dissected into 0.5 mm x 0.5 mm pieces were lysed in RIPA buffer containing protease inhibitors (50 mM Tris, pH 7.2, containing 150 mM NaCl, 2 mM EDTA, 1% TritonX-100, 1% nadeoxycholate, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM NaF, 2



mM Perfabloc and complete protease inhibitors (Roche, Castle Hill, NSW, Australia)). Samples were homogenised on ice using a Heidolph Diax 600 homogenizer (Sigma, Sydney, NSW, Australia) at 22000 rpm for 1 min. Homogenised samples were incubated for 30 minutes at 4°C, then centrifuged at 12000 rpm for 15 min and the supernatant collected for further analysis.

### **Cell culture and transfection**

RAW264.7 murine macrophages were cultured and in some experiments the cells were activated for the times indicated with LPS (100 ng/ml) as described previously (Murray et al., 2005a). Primary fibroblasts from BALB/c mice were cultured as previously described (Kopecki et al., 2009). NIH3T3 fibroblasts were cultured according to the ATCC instructions in DMEM supplemented with 4 mM L-glutamine, 1.5 g/L sodium bicarbonate 4.5 g/L glucose and 10 % FCS. Macrophages were transfected with cDNAs using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen, Mulgrave, Vic, Australia). In some experiments RAW264.7 cells were incubated with 5 µg/ml Brefeldin A (BFA) for 30 minutes and then LPS (100 ng/m) was added for a further 30 minutes. For siRNA knockdown macrophages were transfected using Lipofectamine 2000 (Invitrogen, (Mulgrave, Vic, Australia), cultured for 24 hours, retransfected under the same conditions and then cultured for a further 24 hours prior to use as previously described (Veale et al., 2010).

### **Cell fractionation**

To separate nuclear and post nuclear fractions cells were detached from plates using 0.25 % Trypsin/EDTA (Invitrogen, Mulgrave, Vic, Australia) for NIH3T3 cells or 0.5 mM EDTA in PBS for RAW264.7 cells. The cells were washed once in ice cold PBS, resuspended in buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.15% NP-40 and Complete protease inhibitors (Roche, Castle Hill, NSW, Australia) and incubated on ice for 15 minutes with occasional mixing. The lysed cells were centrifuged for 30 sec at 14,000 x g and the supernatant was the post nuclear fraction. The pellet containing intact nuclei was washed three times in Buffer A, resuspend in Buffer B (20 mM HEPES, pH 7.9, 0.4 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.5% NP-40 and Complete protease inhibitors (Roche, Castle Hill, NSW,



Australia)), sonicated and centrifuged for 30 min at 14,000 x g and the supernatant is the nuclear fraction. Samples (100  $\mu$ g) were separated by SDS-PAGE. Membranes and cytosol were prepared as previously described (Offenhäuser et al., 2011). The membrane volume was adjusted to the same as the cytosol and equal volumes loaded on SDS-PAGE gel.

### **SDS-PAGE, immunoprecipitation, immunoblotting and immunofluorescence**

Cell extracts were prepared as previously described (Veale et al., 2010). Briefly, cells were washed three times with ice-cold PBS and lysed in Buffer C (10 mM Tris, pH 7.4, containing 1 mM EDTA, 150 mM NaCl, 1% Triton X-100, and Complete protease inhibitors (Roche, Castle Hill, NSW, Australia)) by passage through a series of successively smaller needles. The supernatant was then collected after centrifugation for 30 min at 14,000 x g. Protein content in the supernatant was assayed using the BioRad protein assay kit according and 50  $\mu$ g of protein subject to SDS-PAGE separation and immunoblotting. Densitometry was performed on bands within the linear range and fold changes in levels calculated from this data. For immunoprecipitation, the media was incubated with 5  $\mu$ g of antibody bound to protein A-agarose (Sigma, Sydney, NSW, Australia) for 2 h at 4°C with constant mixing. The protein A-agarose was washed five times in excess Buffer C and the bound proteins were solubilised in SDS-PAGE sample buffer. Immunofluorescence staining was performed as described previously (Murray et al., 2005b). Briefly, cells were fixed in either ice-cold methanol (for subsequent immunostaining with the mouse anti-Flii antibody) for 5 minutes at -20°C or 4% paraformaldehyde in PBS (for subsequent immunostaining with the rabbit anti-Flii antibody) for 60 minutes and washed in phosphate buffered saline (PBS). Paraformaldehyde fixed cells were permeabilised with 0.1% Triton X-100 in PBS for 4 minutes. Cells were washed and blocked with 0.5% bovine serum albumin (BSA) in PBS for 10 minutes, incubated with anti-Flii antibody (1:1000 in 0.5 % BSA in PBS) for 60 minutes and then washed with 0.5 % BSA in PBS. Cells were incubated with the appropriate fluorophore-tagged secondary for 60 minutes, washed with 0.5 % BSA in PBS and the coverslips mounted in DABCO. Surface staining of LAMP1 was performed on live cells on ice, cells were then fixed with 4% paraformaldehyde at room temperature as previously described (Offenhäuser et al., 2011). Confocal microscopy was performed using a

Leica (North Ryde, NSW, Australia) TCS SP2 Confocal Microscope equipped with a 100X oil objective. Confocal images were pseudocolored where appropriate and overlaid for publication using Adobe Photoshop CS3.

#### **Measurement of beta-hexosaminidase activity**

Conditioned and unconditioned medium (20  $\mu$ l) was incubated with 4.8 mM 4-nitrophenyl N-acetyl- $\beta$ -D-glucosaminide (20  $\mu$ l) (Sigma-Aldrich) in citrate buffer (0.1 M, pH 4.5) at 37°C for 90 min. The reaction was halted by adding 150  $\mu$ l carbonate buffer (0.1M, pH 9.0) and the absorption read at A415 (nm) and the activity in unconditioned media subtracted.

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## Figure legends

### **Figure 1. Flii is expressed in human skin as well as being secreted into plasma.**

A) Scratch wounded and unwounded confluent monolayers of NIH3T3 fibroblasts were incubated over an 8-hour time course. Extracts and media from these cells were immunoblotted for Flii and the cytoplasmic proteins actin and GAPDH. No change in total intracellular Flii was seen, however, the level of Flii increased with time suggesting Flii is constitutively secreted and this secretion can be upregulated upon scratch wounding. WCL, whole cell lysate. B) Bar graph with SEM shows the increase in Flii in the media, calculated from three replicates of immunoblot data, over the time course (\*\*,  $P < 0.01$ ; \*,  $P < 0.05$ ). C) Media from scratch wounded and unwounded confluent monolayers of NIH3T3 fibroblasts incubated over an 8-hour time course was analysed for beta-hexaminidase activity. Bar graph with SEM (n=3) shows the activity of beta hexaminidase in the media increases over the time course but does not further increase upon scratch wounding. D) Scratch wounded confluent monolayers of NIH3T3 fibroblasts were incubated over an 6-hour time course. Media (1/40) and cell extracts (1/1300) from these cells were immunoblotted for Flii. E) Extracts from human skin and plasma were immunoblotted for Flii,  $\beta$ -tubulin and albumin. Flii is expressed in both skin and plasma. F) Extracts from NIH3T3 fibroblasts and RAW264.7 macrophages were immunoblotted for Flii and Actin. Flii was present at similar levels in macrophages and fibroblasts. G) RAW264.7 macrophages were stimulated with 100 ng/ml LPS over a 6 hour time course. Extracts and media were probed for Flii, TNF and actin.

### **Figure 2. Flii is located in the nucleus and cytosol and is associated with a membrane fraction.**

A) Confluent NIH3T3 fibroblasts were scratch wounded and incubated for 0, 3 or 6 hours. Nuclear and cytoplasmic cell extracts prepared from these cells were immunoblotted for Flii, HDAC1 (nuclear marker) and tubulin (cytoplasmic marker). The majority of Flii was located in the cytoplasm and its translocation into the nucleus was not altered upon wounding. B) Nuclear and cytoplasmic cell extracts were prepared from RAW264.7 macrophages treated in the presence or absence of 100 ng/ml LPS for 3 hours and immunoblotted for Flii, the cytokine TNF, nucleoporin (nuclear marker) and  $\beta$ -tubulin (cytoplasmic marker). Flii

was predominantly found in the cytoplasm of macrophages. C) Membrane (M) and cytosol (C) prepared from RAW264.7 macrophages and NIH3T3 fibroblasts were immunoblotted for Flii, LAMP1 and actin. Flii is located both in the cytosol and in membrane fractions.

**Figure 3. Flii is located in the nucleus, cytoplasm and in late endosomes/lysosomes.** A) Primary fibroblasts were fixed with methanol, immunostained for Flii (mouse anti-Flii antibody) and the late endosomal/lysosomal proteins cathepsin D or VAMP7. Flii localises to the nucleus, cytosol and late endosomes/lysosomes in fibroblasts. B) RAW264.7 macrophages fixed with 4% paraformaldehyde (upper panels) or methanol (middle and lower panels) were immunostained for Flii with the mouse (middle and lower panels) or rabbit (upper panel) anti-Flii antibody, the late endosome/lysosome marker proteins LAMP1, VAMP7 and Cathepsin D (Cath D). Flii localises to the cytosol and late endosomes/lysosomes in macrophages. Scale bar is 5  $\mu$ m.

**Figure 4. Flii is not secreted via the classical secretory pathway.** A) RAW264.7 macrophages fixed with 4% paraformaldehyde were immunostained for Flii (mouse anti-Flii antibody) along with VAMP4 (Golgi complex) or TNF, a cytokine that traffics through the classical secretory pathway via the Golgi complex. Flii did not localise to the Golgi complex or with TNF in the classical secretory pathway. Scale bar is 5  $\mu$ m. B) RAW264.7 macrophages incubated in the presence or absence of brefeldin A (BFA) for 60 minutes in the presence of LPS for the final 30 minutes were fixed in 4% paraformaldehyde and stained for F-actin and the Golgi marker GM130. The Golgi complex is disrupted in the presence of BFA. C) Extracts and media from the same cells were immunoblotted for Flii. There is no change in the level of Flii secreted in the presence of Flii suggesting Flii is not secreted via the classical pathway.

**Figure 5. Rab7 and Stx11 regulate the trafficking and secretion of Flii from LAMP1 positive late endosomes/lysosomes.** A) Extracts and media from macrophages transiently transfected with GFP, GFP-Rab7 or GFP-Rab7T22N were immunoblotted for Flii, cathepsin D, GFP and actin. Overexpression of wild-type



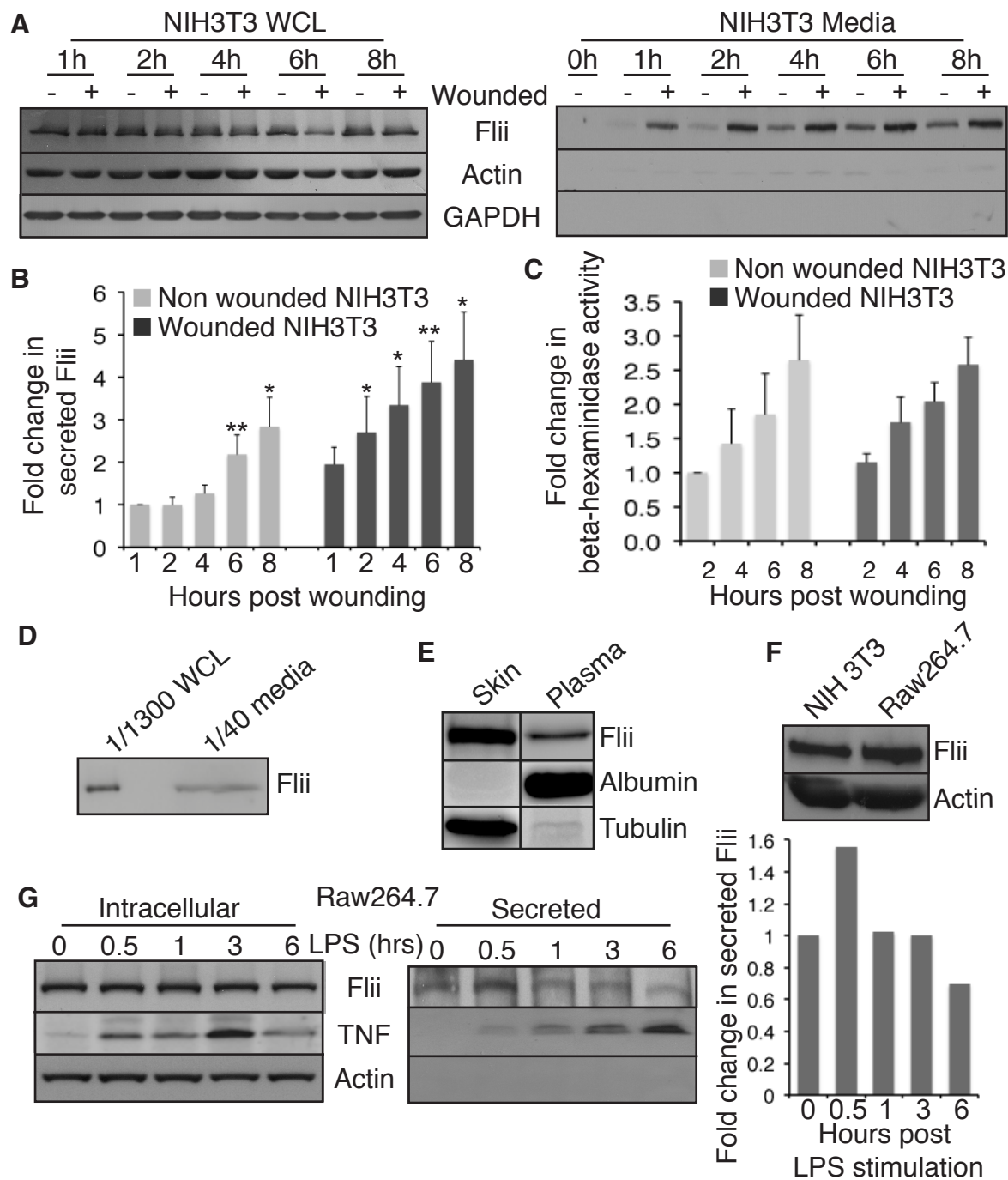
Rab7 results in a reduction in intracellular Flii and cathepsin D and a concomitant increase in secreted Flii. Conversely, expression of dominant negative form of Rab7 (Rab7T22N) results in an increase in intracellular Flii and cathepsin D with a reduction in secreted Flii. B) Bar graph with SEM shows the fold change in the levels of intracellular Flii and cathepsin D and secreted Flii in cells transiently expressing GFP, GFP-Rab7 and GFP-Rab7T22N, calculated from three replicates of immunoblot data (\*\*,  $P < 0.01$ ; \*,  $P < 0.05$ ). C) Media from RAW264.7 macrophages transiently transfected with GFP, GFP-Rab7 or GFP-Rab7T22N was analysed for beta-hexaminidase activity. Bar graph with SEM shows the activity of beta hexaminidase in the media increases when Rab7 is overexpressed and decreases when the dominant negative form is expressed (n=3). D) RAW264.7 macrophages were transfected with control or Stx11 siRNA. Macrophages were immunostained live on unpermeabilised cells for surface LAMP1 then fixed at room temperature. The knockdown of Stx11 levels results in increased delivery of LAMP1 to the cell surface as previously seen. Scale bar is 10  $\mu\text{m}$ . E) Cell extracts and media from macrophages transfected with control or Stx11 siRNA were immunoblotted for Flii, Stx11 and cathepsin D, along with actin as a loading control. Cells depleted of Stx11 have reduced levels of intracellular Flii and cathepsin and a concomitant increase in secreted Flii. F) Bar chart with SEM shows the reduction in intracellular Flii and cathepsin D in cells treated with Stx11 siRNA and the increase in secreted Flii, calculated from three replicates of immunoblot data (\*\*,  $P < 0.01$ ; \*,  $P < 0.05$ ). G) Media from RAW264.7 macrophages treated with control or Stx11 siRNA was analysed for beta-hexaminidase activity. Bar graph with SEM shows the activity of beta hexaminidase in the media increases in cells treated with Stx11 siRNA compared to control cells (n=3).

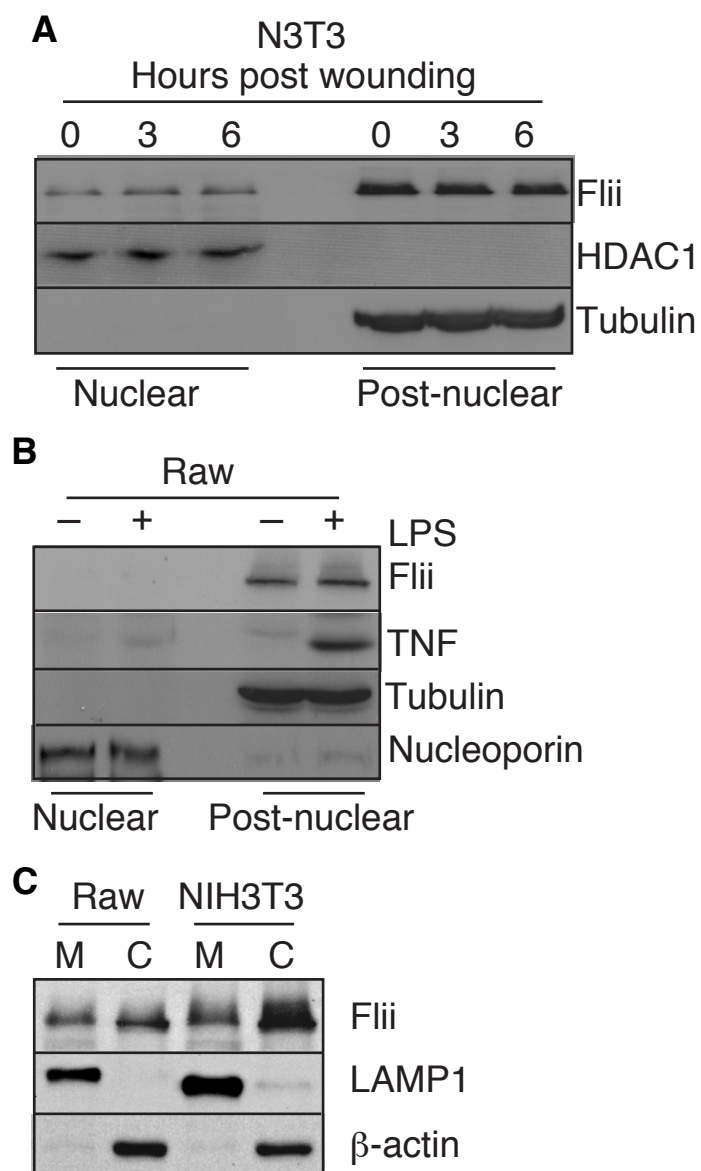
**Figure 6. Flii forms a complex with LPS and regulates TNF secretion**

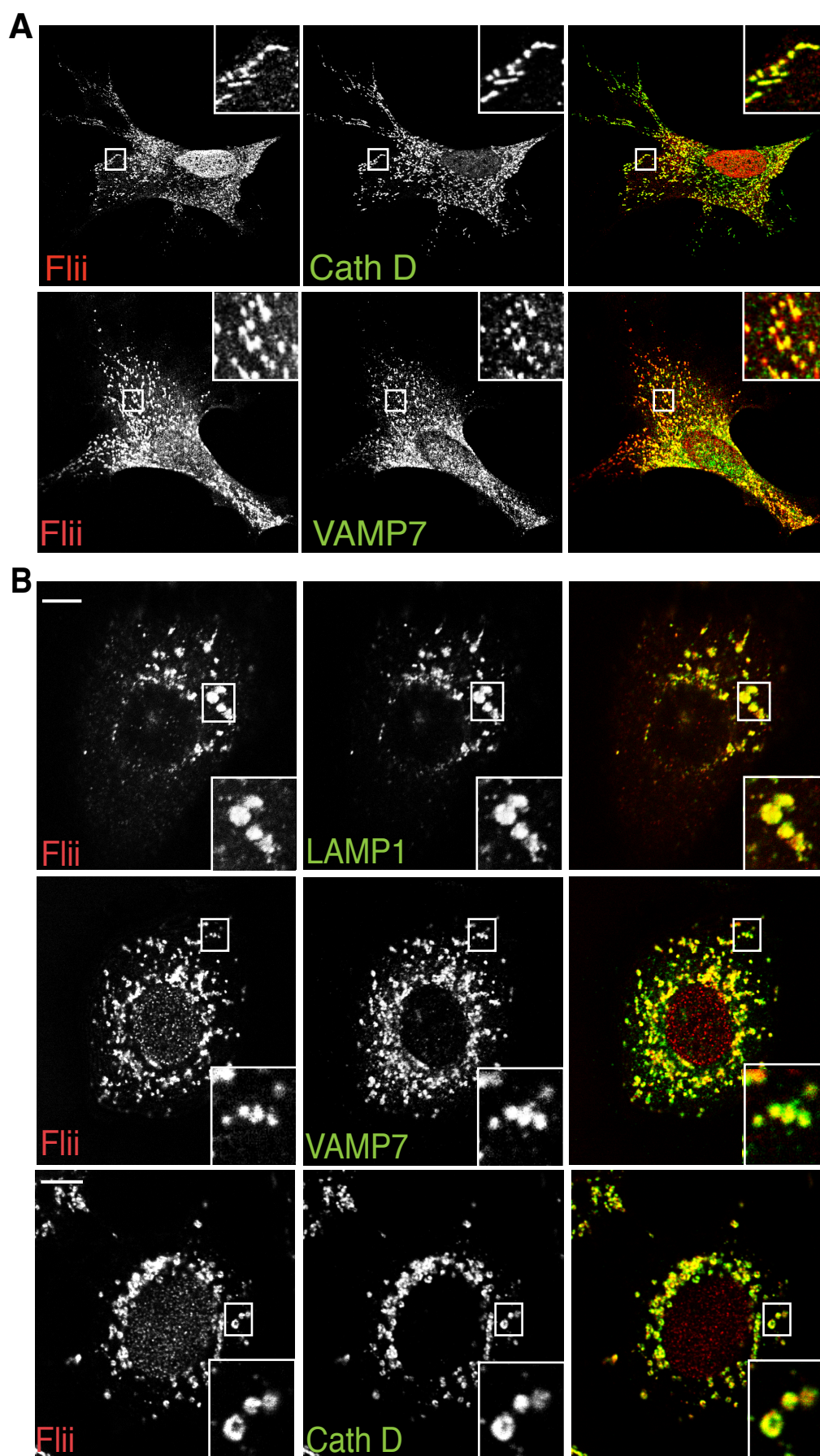
A) Schematic representation of the domain structures of Flii showing the two major domains: gelsolin and leucine-rich repeat domains. B) NIH3T3 fibroblast conditioned media containing LPS (1  $\mu\text{g/ml}$ ) was incubated with antibodies (IgGs) to GFP, LPS and Flii bound to protein A-agarose. Precipitates were analysed by immunoblotting. IP, immunoprecipitated; WCL, whole cell lysate. C) NIH3T3 fibroblast conditioned media was pre-incubated in the presence or absence of antibodies specific to the LRR

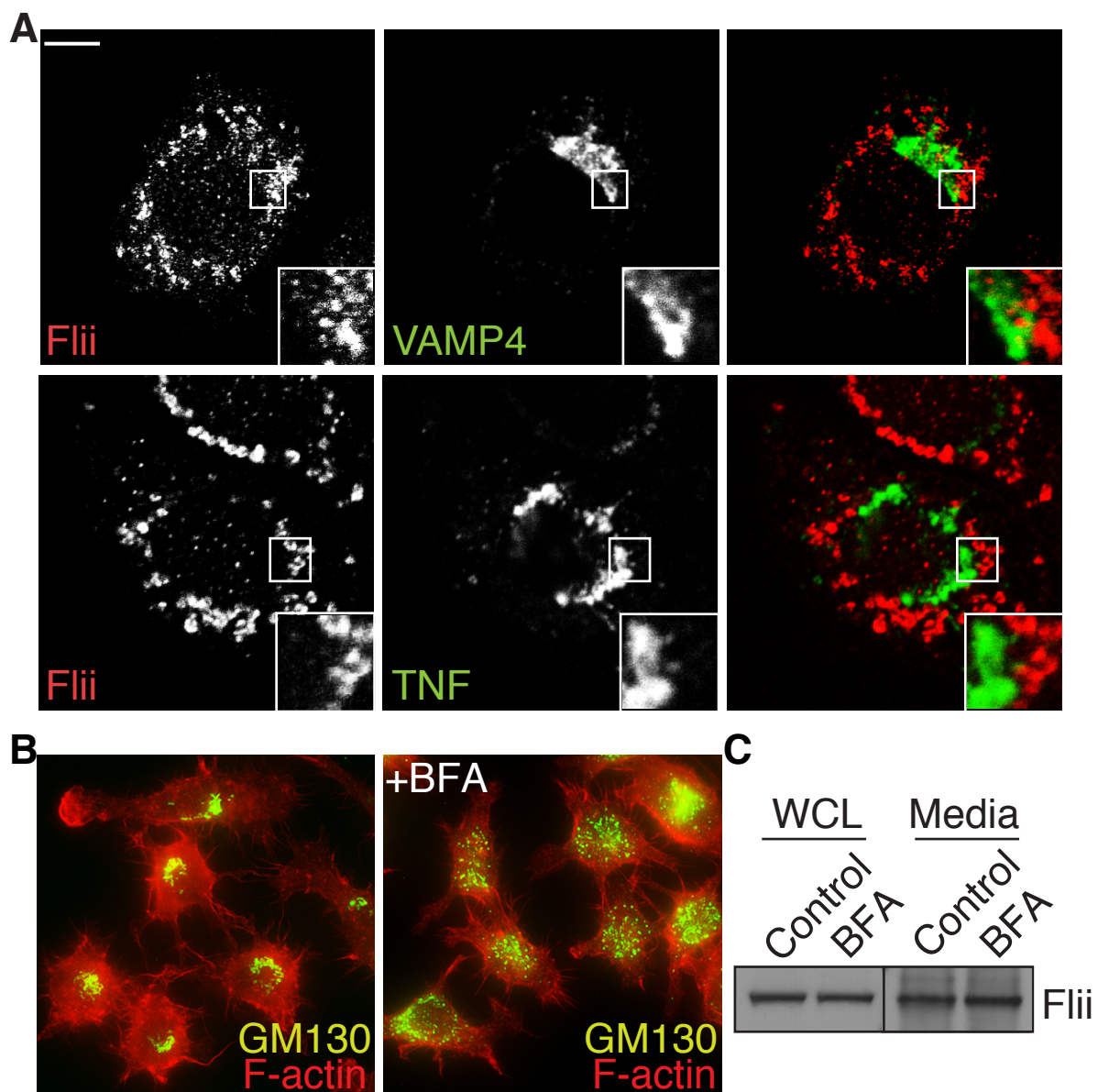


domain of Flii (IgM antibodies, which do not bind protein A-agarose). The media was then incubated with LPS and antibodies specific for LPS bound to protein A-agarose and precipitates were immunoblotted for Flii. Flii is no longer precipitated with LPS in the presence of anti-LRR Flii antibodies. IP, immunoprecipitated. D) NIH3T3 fibroblast conditioned media was pre-incubated in the presence or absence of Flii antibody bound to protein A-agarose and the precipitated proteins discarded. The remaining media was immunoblotted for Flii. Flii is depleted from conditioned media by antibodies specific for Flii. E) RAW264.7 macrophages grown on coverslips were incubated with NIH3T3 fibroblast conditioned media or Flii depleted NIH3T3 fibroblast conditioned media in the presence of LPS (100 ng/ml) for 3 hours. Cells were fixed, permeabilised and stained for TNF and F-actin. Both the number and level of cells containing TNF was increased when Flii is depleted from media. F) Extracts from macrophages incubated with NIH3T3 fibroblast conditioned media or Flii depleted NIH3T3 fibroblast conditioned media in the presence of LPS (100 ng/ml) for 3 hours were immunoblotted for TNF and actin. Levels of intracellular TNF are increased when Flii is depleted from the media of LPS-stimulated macrophages. WCL, whole cell lysate. G) Media from the same cells was also immunoblotted for Flii. Coomassie blue staining of the membrane shows equal loading of proteins. TNF secretion is increased from macrophages activated with LPS in the presence of Flii depleted media. H) Bar graph with SEM shows the fold change in both intracellular and secreted Flii, calculated from three replicates of immunoblot data (\*\*,  $P < 0.01$ ; \*,  $P < 0.05$ ).

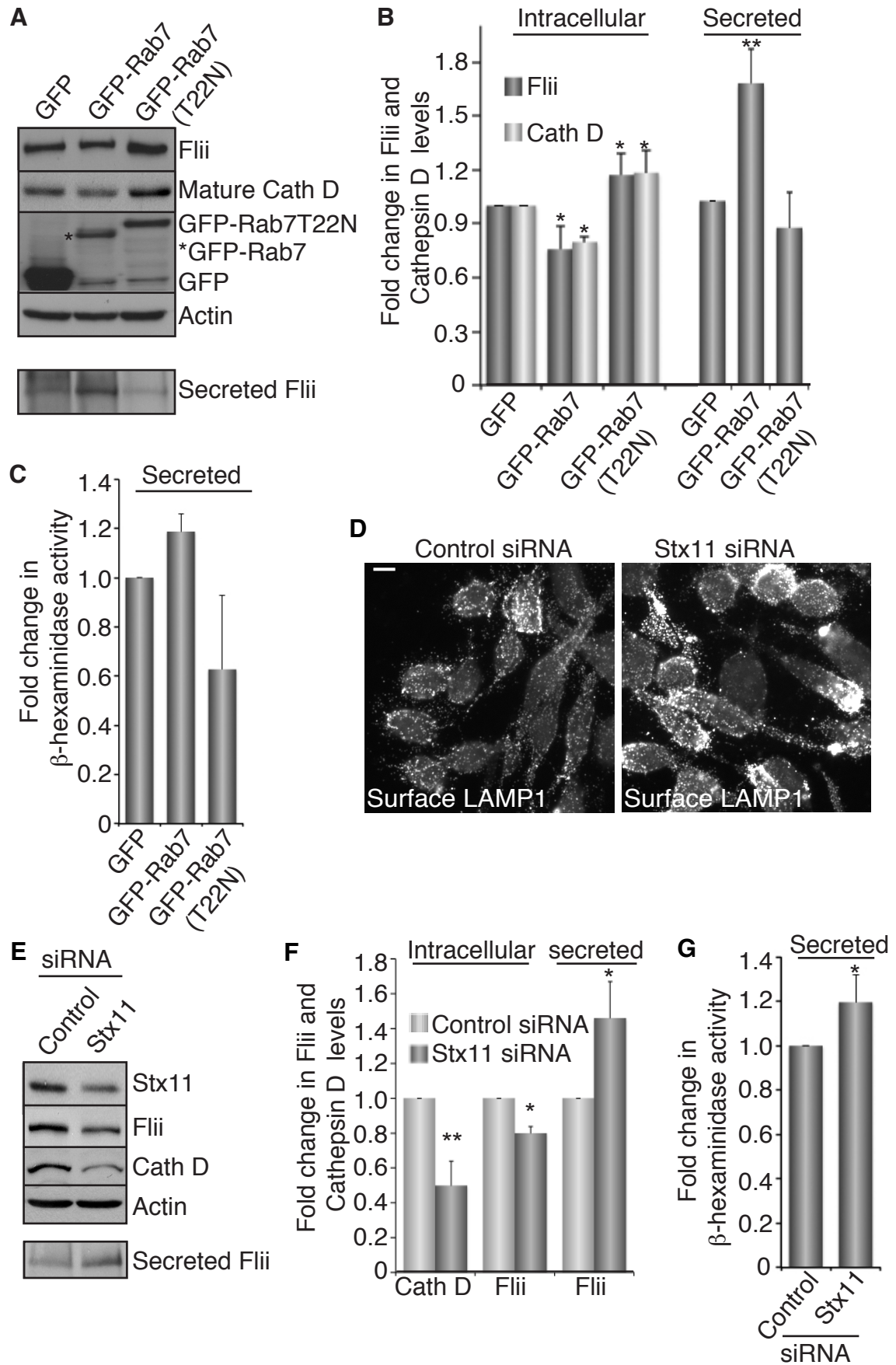


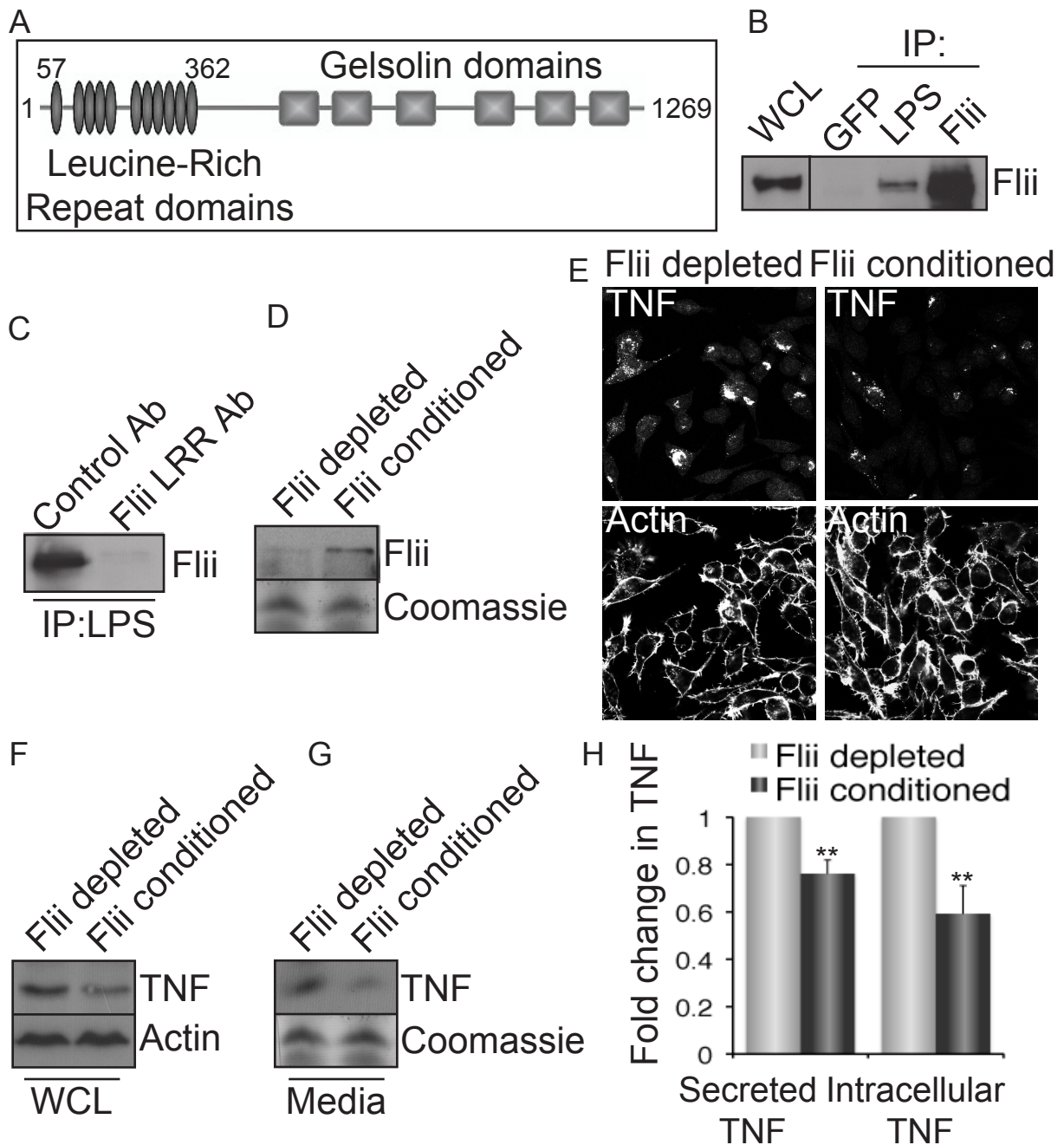












**Influence of Flightless I on Toll-Like Receptor-Mediated Inflammation in a Murine  
Model of Diabetic Wound Healing**

**Nadira Ruzehaji<sup>1,2</sup>, Stuart J. Mills<sup>1</sup>, Elizabeth Melville<sup>1</sup>, Ruth Arkell<sup>3</sup>, Robert Fitridge<sup>2</sup>,  
Allison J Cowin<sup>1,2</sup>**

<sup>1</sup> Women's and Children's Health Research Institute, Wound Healing Laboratory, 72 King William Road, North Adelaide, South Australia, Australia 5006; Email: stuart.mills@adelaide.edu.au; nadira.ruzehaji@adelaide.edu.au; elizabeth.melville@adelaide.edu.au; allison.cowin@adelaide.edu.au

<sup>2</sup> Faculty of Health Sciences, University of Adelaide, SA, Australia, 5005; Email: allison.cowin@adelaide.edu.au; robert.fitridge@adelaide.edu.au

<sup>3</sup>Early Mammalian Development Laboratory, Research School of Biological Sciences, Australian National University, ACT, 2601, Australia; Email: ruth.arkell@anu.edu.au

Correspondence should be addressed to Allison Cowin; allison.cowin@adelaide.edu.au

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**Abstract (202 words)**

Impaired wound healing and ulceration represent a serious complication of both type 1 and type 2 diabetes. Cytoskeletal protein Flightless I (Flii) is an important inhibitor of wound repair and reduced Flii gene expression in fibroblasts increased migration, proliferation and adhesion. As such it has the ability to influence all phases of wound healing including inflammation, remodelling and angiogenesis. Flii has the potential to modulate inflammation through its interaction with MyD88 which is an adaptor protein for TLR4. To assess the effect of Flii on the inflammatory response of diabetic wounds we used a murine model of streptozocin-induced diabetes and Flii genetic mice. Increased levels of Flii were detected in Flii transgenic murine wounds resulting in impaired healing which was exacerbated when diabetes was induced. When Flii levels were reduced in diabetic wounds of Flii deficient mice, healing was improved and decreased levels of TLR4 were observed. In contrast, increasing the level of Flii in diabetic mouse wounds led to increased TLR4 and NF $\kappa$ B production. Treatment of murine diabetic wounds with neutralising antibodies to Flii led to an improvement in healing with decreased expression of TLR4. Decreasing the level of Flii in diabetic wounds may therefore reduce the inflammatory response and improve healing.

## Introduction

Up to 25% of people with diabetes can expect to develop a foot ulcer at some point in their lives [1]. Due to poor outcomes of existing therapies, lower extremity amputation is a common complication, affecting 15% of diabetics with foot ulcers, with one major amputation occurring every 30 seconds worldwide and over 2500 limbs lost a day [2]. The effectiveness of current treatments for diabetic foot ulcers is limited, and many patients with chronic, unhealed wounds need continual care. Understanding the processes involved in impaired wound healing will help to develop new therapeutic targets and tools for improving wound repair.

One area of research which has been shown to be integral to the wound repair process is that of the actin cytoskeleton which is a filamentous network found in all cells and facilitates processes such as cellular adhesion, migration and contraction [7-8]. One member of the actin cytoskeleton is Flightless I (Flii) which is a member of the gelsolin family of actin remodelling proteins [3]. Flii colocalizes with actin and microtubule based structures, is required for normal actin distribution and possesses  $\text{Ca}^{2+}$  independent G-actin binding activity as well as F-actin binding and severing activities [4-7]. In addition to its role as a regulator of the cytoskeleton, the LRR domain allows Flii to bind a number of other proteins unrelated to actin including LRR Flii interacting protein 1 and 2 (LLRFIP1 and LLRFIP2) and the double stranded RNA binding protein TRIP [8, 9]. Flii is involved in numerous cellular activities including regulating transcription via co-activation of nuclear hormone receptors [10, 11] and regulation of beta-catenin dependent transcription [12], important signalling pathways including the TLR pathway [13, 14], cellular polarity, asymmetric cell division [15], proliferation via interactions with calmodulin-dependent protein kinase type II [16], inflammation and cytokine production via caspase activation and IL-1 $\beta$  maturation [17]. Flii-deficient mice have improved reepithelialisation post wounding while Flii

overexpressing mice have impaired healing with larger wounds with reduced contraction, cellular proliferation and delayed reepithelialisation [18].

Increased inflammation is an important contributing factor in the failure to heal of diabetic foot ulcers [19]. Inflammation is an integral part of the wound healing process and is regulated by toll-like receptors (TLRs). TLRs are key innate immune receptors that alert the immune system to tissue damage and mediate the inflammatory response. The human TLR family consists of 10 members structurally characterised by the presence of a leucine-rich repeat (LRR) domain in their extracellular domain and Toll/interleukin (IL)-1 receptor (TIR) domain in their intracellular domain. Through their intracellular TIR domain TLRs activate or deactivate signalling pathways that generate cytokine and chemokine production and thereby regulate inflammatory responses. TLR signalling is tightly regulated to control the intensity and duration of inflammation [12]. TLRs are expressed on a wide variety of cells including macrophages and neutrophils and they respond to an array of viral, bacterial and fungal ligands as well as cellular debris [13]. The receptors convey their specificity through the utilization of different adaptor proteins such as myeloid differentiation factor 88 (MyD88), TIR-associated protein (TIRAP), TIR-domain containing adaptor protein-inducing IFN- $\beta$  (TRIF) and TRIF-related adaptor molecule (TRAM) [13-15]. Upon activation, the adaptor proteins promote signalling to result in the expression of pro-inflammatory cytokines, growth factors and interferons. MyD88 has been shown to play an important role during wound healing as MyD88 knockout mice have impaired wound healing [16] with wounds showing reduced contraction, decreased and delayed granulation tissue formation and reduced blood vessel density [16].

The LRR region of Flii shares 29% sequence identity and 42% similarity to TLR4 [20]. Through its interaction with MyD88, it has been suggested that Flii can modulate inflammation by suppressing TLR4-MyD88-mediated activation of NF- $\kappa$ B [21]. Conversely,

a reduction in Flii level may enhance activation of NFkB and increase cytokine secretion[20]. Several studies investigating the effect of Flii on TLR signalling in murine macrophages suggest that Flii can sequester activator proteins such as LPS and adaptor proteins such as MyD88 resulting in reduced cytokine expression [10-11, 16-17]. In this study we used mice with low (Flii<sup>+/-</sup>), normal (WT) and high (Flii<sup>Tg/Tg</sup>) Flii gene expression to investigate the function of Flii in a murine wound healing model of streptozocin-induced type 1 diabetes. We also investigated whether modulation of Flii by exogenous application of Flii neutralising antibodies improved diabetic wound healing via effects on TLR-mediated inflammation.

## **Materials and Methods**

### **Antibodies**

Mouse monoclonal anti-Flii antibodies raised to the LRR domain of the human Flii protein and the rabbit anti-human MyD88 antibody were obtained from Santa Cruz Biotechnology (VIC, Australia). Rabbit anti-human TLR4 and TLR9 antibodies were obtained from Imgenex (SA, Australia), mouse anti-human CD14 antibody and mouse anti-human CD16 antibody from BD Biosciences (NSW, Australia) and rabbit anti-human NF- $\kappa$ B antibody from Abcam (NSW, Australia). All antibodies were used at a 1:100 dilution. The appropriate secondary antibodies were used depending on the fluorescence required – goat anti-mouse Alexa Fluor 488 and goat anti-mouse Alexa Fluor 594 were obtained from Life Technologies (VIC, Australia) and also used at a 1:100 dilution. Flii is a highly conserved protein with 95% homology between mice and humans [19]. The Flii neutralising antibodies (FnAb) used *in vivo* diabetic mouse trials were mouse monoclonal anti-Flii antibodies raised against the N-terminus of the LRR domain of the human Flii protein [22] and obtained from Monoclonal Antibody SA Technologies (SA, Australia). Mouse IgG antibody used as a control *in vivo* diabetic mouse was obtained from Sigma (MO, U.S.A.)

### **Animal studies**

All experiments were approved by the Women's and Children's Health Network Animal Ethics Committee following the Australian Code of Practice for the Care and the Use of Animals for Scientific Purposes. Studies were performed using mice with a BALB/c background. Three strains of mice were used in this study with low (Flii<sup>+/-</sup>), normal (WT) and high (Flii<sup>Tg/Tg</sup>) Flii gene expression. Mice lacking one copy of the Flii gene, the double knockout being embryonically lethal [23], were made as described previously [23] and will

be written as  $Flii^{+/-}$ . Transgenic mice carrying two extra copies of human *Flii* gene incorporated into mouse genome will be written as  $Flii^{Tg/Tg}$ .

### **Murine model of diabetic wound healing**

Six female  $Flii^{+/-}$ , WT and  $Flii^{Tg/Tg}$  mice of 12-16 weeks of age and weighing 20-35g, were used for the induction of diabetes. Streptozocin (STZ) was used to induce type 1 diabetes (Sigma-Aldrich, MO, USA). STZ is toxic to the pancreatic beta-islet cells, rendering the mouse unable to produce adequate amount of insulin. Mice were given one intraperitoneal (IP) injection of STZ for 5 consecutive days (STZ:50 mg/kg) in citrate buffer pH 6.5. This dose was chosen based on previously reported studies [24]. Age matched non-diabetic control animals were treated with an equivalent dose of vehicle (citrate buffer alone). Diabetic symptoms were observed closely and non-fasting blood glucose levels (BGL) were tested weekly by tail vein sampling. To maintain body weight and prevent ketoacidosis, animals with confirmed diabetes were maintained with subcutaneous injection of insulin (1 IU, Mixtard 30/70, Novo-Nordisk, NSW, Australia). Mice were tested for sufficient levels of hyperglycaemia at 6 weeks after the last STZ injection and only those with blood glucose levels greater than 15 mmol/L were wounded. Diabetic animals were wounded using a 6 mm biopsy punch (Stiefel Laboratories, NSW, Australia). Anaesthesia was induced by inhalation of isoflurane (5% induction at 2 L/min and 2% maintenance at 500 ml/min). To expose the skin, hair was removed by shaving then application of hair removal cream (Veet®, Reckitt Benckiser, NSW, Australia). Two 6 mm full thickness wounds, one on each side of the midline were created on the dorsum of the mouse. Temgesic (buprenorphine 0.05 mg/kg) was administered post-operatively to provide analgesia for up to 8 hours. The mice were euthanized at 7 days post-wounding. Digital photographs were taken of the wounds at 0 and 7

days post-wounding. A ruler was aligned next to the wound to allow direct wound area measurements to be made. Wounds were fixed in formalin and processed for histology and immunohistochemistry.

An additional cohort of female WT diabetic mice were injected intradermally around the wound margins with 200  $\mu$ l of FnAb (50  $\mu$ g/ml; n=10) or mouse IgG control (50  $\mu$ g/ml; n=10) immediately after surgery and at 1 and 2 days post-wounding. Digital photographs of wounds were taken at day 0 and 7 post-wounding. All animals were euthanized at day 7 post-wounding with the wounds harvested, fixed in formalin and processed for histological analysis.

### **Histology, immunohistochemistry and image analysis**

Histological sections (4  $\mu$ m) of mouse wounds were cut and stained with haematoxylin and eosin or subjected to immunohistochemistry following antigen retrieval as described previously [25]. Briefly, following antigen retrieval, sections were blocked in 3% normal goat serum, primary antibodies against Flii (1:100), TLR9 (1:100), TLR4 (1:100), MyD88 (1:100) and NF- $\kappa$ B (1:100) were applied and incubated overnight at 4°C. Appropriate Alexa Fluor 488-conjugated secondary antibodies (1:100) were used and incubated for 1 hour. Fluorescence intensity was determined using AnalySIS software package (Soft Imaging System GmbH, Munster, Germany) and optical fluorescence in the epidermis and dermis of the wounds was analysed as previously described [25]. Negative controls included replacing primary antibodies with normal mouse or normal rabbit IgG. Primary or secondary antibodies were omitted to verify the staining and detect nonspecific binding. All control sections had negligible immunofluorescence.

### **Histological image analysis**

Histological image analysis was performed using the Digital Microscope Camera ProgRes® C5 (JENOPTIK Laser, Jena, Germany). Wound size was determined by manually drawing below the clot or the portions of the wound that were not covered by epidermis. Dermal wound gape was determined by measuring between the dermal wound margins. Fluorescent images were taken using an Olympus IX81 (Olympus Australia, VIC, Melbourne, Australia) at a magnification of  $\times 20$ . The fluorescent intensity of the staining was calculated using Image Pro-Plus software (Media Cybernetics, MD, USA).

### **Statistical Analysis**

Statistical significance was calculated using a paired student t-test or analysis of variance. A *p* value of 0.05 or less was considered significant.



## Results

### Diabetic wounds heal faster in mice with low levels of Flii gene expression

To assess the biological function of Flii and determine the effect of Flii gene modulation on diabetic wound healing, three lines of mice were used expressing low (Flii<sup>+/-</sup>), normal (Flii<sup>+/+</sup>), and high (Flii<sup>Tg/Tg</sup>) levels of Flii. Representative digital images of wounds at 7 days post-wounding are shown in Figure 1A. Overexpression of Flii in diabetic and non-diabetic wounds resulted in delayed wound closure at day 7 post-wounding (Figure 1A). In contrast, wound area was decreased significantly when Flii levels were reduced (Flii<sup>+/-</sup>;  $p=0.01$ ) compared to Flii<sup>Tg/Tg</sup> mice at day 7 post-wounding (Figure 1B (i)). Representative microscopic images of day 7 wounds are shown in Figure 1A. Histological assessment of these diabetic mouse wounds showed that at day 7 the dermal wound gape was significantly smaller in Flii deficient mice (Flii<sup>+/-</sup>) compared with Flii overexpressing (Flii<sup>Tg/Tg</sup>) mice (Figures 1B (ii);  $p=0.05$ ).

### Treatment of diabetic wounds with Flii neutralising antibodies (FnAb) improves wound healing

Previous studies have shown that intradermal injection of Flii neutralising antibodies reduce the level of Flii in wounds and improve healing [26]. FnAbs were injected intradermally at day 0, 1 and 2 and representative images of macroscopic (at days 0 and 7) and microscopic (day 7) appearances of diabetic wounds are shown in Figure 2A. Intradermal administration of FnAb to diabetic wounds resulted in a 1.9-fold decrease in average wound area (Figure 2B(i)) and histological wound gape (Figure 2B(ii)) compared to IgG-treated WT diabetic controls (Figure 2B (i and ii);  $p \leq 0.05$ ; IgG vs. FnAb).

### **Elevating Flii gene expression increases TLR4 in diabetic mouse wounds**

Immunofluorescence staining of day 7 diabetic wounds in Flii<sup>+/-</sup>, WT and Flii<sup>Tg/Tg</sup> mice shows increased Flii in Flii<sup>Tg/Tg</sup> diabetic wounds > WT > Flii<sup>+/-</sup> wounds (Figure 3A) with significantly less Flii staining being observed in day 7 diabetic Flii<sup>+/-</sup> and WT wounds (p=0.04; Flii<sup>+/-</sup> vs. Flii<sup>Tg/Tg</sup>; p=0.05; WT vs. Flii<sup>Tg/Tg</sup>; Figure 3B(i)). The presence of foreign molecules and pathogens was detected by a family of receptors known as Toll-Like Receptors (TLRs), which contribute to prolonged inflammation [27]. To assess if altered levels of Flii affected TLR4 expression, diabetic wounds were stained for TLR4. Increasing Flii lead to a concomitant increase in TLR4 expression (Figure 3A(d-f)). Flii deficiency caused a significant reduction in TLR4 expression (Figure 3B(ii)) at day 7 post-wounding which was significantly lower than WT and Flii<sup>Tg/Tg</sup> wounds (p ≤ 0.01 WT vs. Flii<sup>+/-</sup>; p ≤ 0.001 WT vs. Flii<sup>Tg/Tg</sup>). Given that the inflammatory response in diabetic wounds was associated with increased TLR4 expression we next proceeded to test if the downstream molecule NF-κB production was also effected. Figure 3A shows that NF-κB expression was also increased in Flii overexpressing wounds (5-fold higher than WT diabetic wounds) (Figure 3B(iii); p ≤ 0.001 Flii<sup>Tg/Tg</sup> vs. WT). A reduction in NF-κB was observed between Flii<sup>+/-</sup> wounds and WT but this was not statistically significant (Figure 3B(iii)).

### **Flii neutralising antibodies reduce TLR4 expression in diabetic wounds**

Given that intradermal application of FnAb resulted in improved healing of diabetic wounds (Figure 2A) we proceeded to test whether this was in part due to modulated levels of TLR4-mediated inflammation. TLR4 and NF-κB expression were quantified and representative images are shown in Figure 4A. Treatment with FnAbs resulted in a significant decrease in

TLR4 expression (Figure 4B;  $p \leq 0.05$  IgG vs. FnAb) whereas NF- $\kappa$ B expression in day 7 FnAb-treated wounds remained unchanged compared to IgG controls (Figure 4B (ii)).

## Discussion

Flii has been identified as a protein that can inhibit the rate of healing by reducing the migration of keratinocytes and fibroblasts and limiting the degree of wound contraction [18, 22, 28]. Flii deficiency is associated with improved reepithelialisation in acute wounds [18] while Flii overexpressing mice have impaired wound healing with delayed re-epithelialisation. Here we have investigated if Flii is involved in the impaired healing associated with diabetic wounds. Wound healing was impaired as Flii levels increased and this impairment was exacerbated when diabetes was induced. Flii is upregulated during the wound repair process [18, 25] and is constitutively secreted by two of the major cell types found in wounds: fibroblasts and macrophages in response to wounding both *in vitro* [29] and *in vivo* [22, 30]. Recent studies show that Flii is also secreted through a non-classical late endocytic/lysosomal pathway of secretion by fibroblasts and macrophages [29]. Addition of Flii monoclonal neutralising antibodies as a means of reducing the Flii protein in the wound environment were able to counteract the negative effect of Flii on wound healing [18] and treatment of murine diabetic wounds with neutralising antibodies to Flii lead to an improvement in healing suggesting that high levels of Flii in diabetic wounds contributes to wound chronicity.

Inflammation is an essential component of the normal wound healing process, however excessive inflammation is detrimental to this process [31]. Disproportionate inflammation is one of the major contributing factor to the formation of diabetic ulcers as these chronic wounds often have an unregulated and excessive inflammatory reaction [31, 32]. The innate immune system detects foreign particles such as bacteria, fungi and viruses via Pathogen-Associated Molecular Pattern (PAMPs) molecules leading to the activation of the inflammatory response via Toll-like receptors (TLRs) which recognise PAMPs [33, 34]. TLRs have also been linked to diabetes with studies showing TLR-immune activation can

result in activation of pro-inflammatory pathways leading to autoimmunity which may cause the onset of diabetes [27]. Previous reports suggested that stimulation of TLR4 leads to activation of the downstream transcriptional regulator NF- $\kappa$ B resulting in cytokine secretion [21] and both TLR4 expression and pro-inflammatory cytokine NF $\kappa$ B were elevated in the murine diabetic wounds which would further contribute to inflammation and chronicity. In Flii deficient diabetic mouse wounds, decreased TLR4 and NF $\kappa$ B were observed and when Flii neutralising antibodies were administered to diabetic wounds, a decrease in TLR4 expression was seen suggesting a dampening of the inflammatory response when Flii levels were reduced. Interestingly, the effect of FnAb was specific to TLR4 and did not appear to affect the production of the pro-inflammatory cytokine NF- $\kappa$ B suggesting that alternative pathways may still be active in these wounds.

*In vitro* studies have previously shown that the LRR region of Flii shares 29% sequence identity and 42% similarity to TLR4 [20] suggesting that Flii may influence TLR signalling. However, while *in vitro* studies showed that through its interaction with MyD88, Flii was able to negatively regulate the TLR4-MyD88-mediated activation of NF- $\kappa$ B and the subsequent cytokine secretion in macrophages [20, 21], our studies show that *in vivo*, in mouse diabetic wounds increased levels of Flii appear to correlate with an increase in the expression of TLR4 and its signalling protein NF- $\kappa$ B. This is clearly in opposition to the findings in these cell based studies but may be accounted for by the differing *in vivo* and *in vitro* environments. The time points investigated also differ with the *in vitro* studies looking at responses from 3-6 hours whereas this wounding study investigated time point which were measured in days rather than hours. It is, however, not inconceivable that Flii may have a dual role in wounding and in the inflammatory response which depends on the time point investigated.

In conclusion, inflammation is an integral component of the normal wound healing process and occurs even in the absence of infection, however excessive and prolonged inflammation impairs healing. Flii is a multifunctional protein and is currently emerging as a regulator of inflammation, however, whether it is pro-or anti-inflammatory is still to be determined. Our *in vivo* studies show that reducing the expression of Flii in diabetic murine wounds improves healing and reduces the pro-inflammatory response. Being able to manipulate the level of inflammation in a wound would greatly improve the wound healing outcomes of patients with diabetes and it remains to be elucidated whether neutralisation of Flii in human diabetic wounds could help improve healing of these chronic non-healing ulcers.

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**Disclosure Policy**

The authors know of no potential conflicts of interest.

## Figure Legends

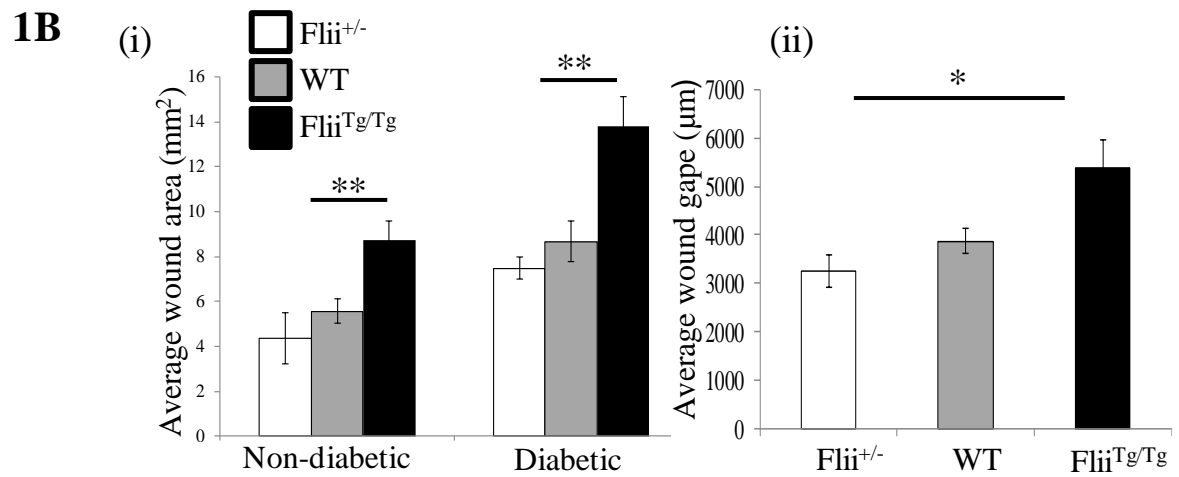
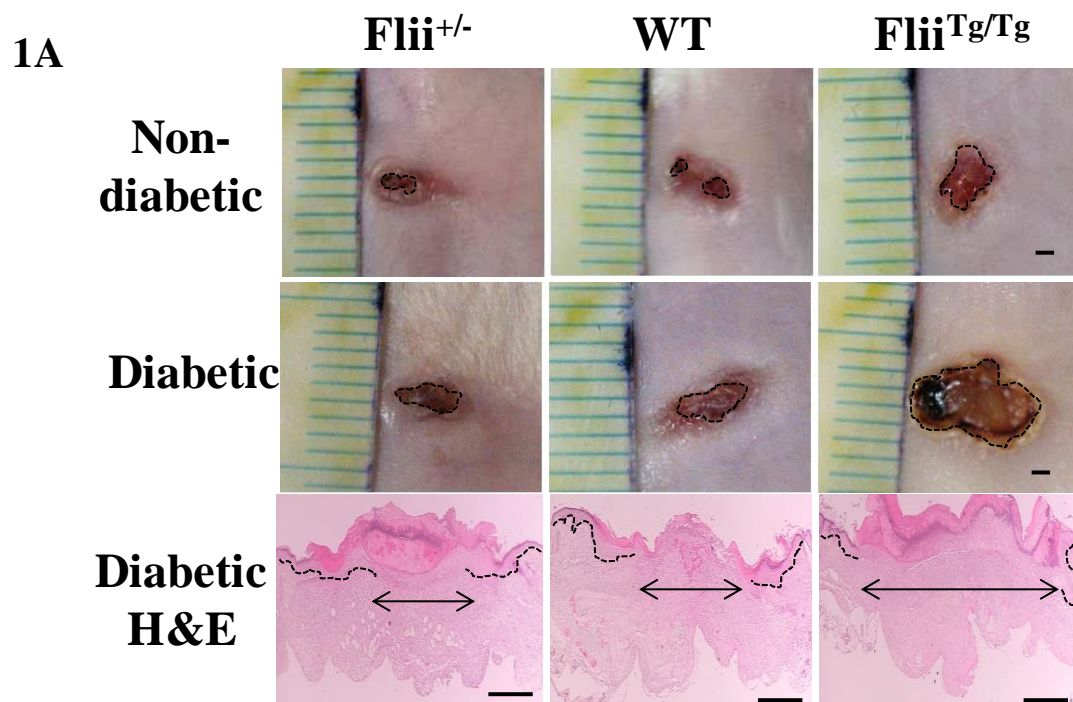
**Figure 1. Flii delays wound healing in diabetic murine wounds after 7 days.** (A) Macroscopic images of day 7 non-diabetic and diabetic mouse wounds in the Flii<sup>+/-</sup>, WT and Flii<sup>Tg/Tg</sup> mice and representative histological H&E staining of day 7 diabetic wounds. Scale bars = 1 mm for macroscopic pictures and for the histology magnification was x 4 and scale bars = 100  $\mu$ m. (B) Shows graphical representations of the wound areas of the day 7 (a) wound areas of the three Flii genotype diabetic wounds (n=6) and b) the microscopic dermal wound gape of the day 7 diabetic wounds where \*\*  $p \leq 0.01$  and \*  $p \leq 0.05$  (n=6).

**Figure 2. Healing can be improved by the application of Flii neutralizing antibodies.** (A) Shows macroscopic images of day 0 and day 7 excisional WT diabetic wounds treated with IgG isotype control (n=10) and Flii neutralizing antibody (FnAb) (n=10). Representative pictures of H&E staining of day 7 diabetic wounds treated with IgG and FnAb. (B) Graphical representation of the (i) wound areas and (ii) histological wound gapes of the day 0 and 7 IgG control-treated and FnAb-treated WT diabetic wounds where \*  $p \leq 0.05$  (n=10).

**Figure 3. Concomitant increase in TLR4 and NF- $\kappa$ B staining occurs with increasing levels of Flii.** (A) Representative images for Flii (a-c), TLR4 (d-f) and NF- $\kappa$ B (g-i) immunostaining of the three Flii genotypes (Flii<sup>+/-</sup>, WT and Flii<sup>Tg/Tg</sup>). (B) Graphical representation of i) Flii, ii) TLR4 and iii) NF- $\kappa$ B in Flii<sup>+/-</sup>, WT and Flii<sup>Tg/Tg</sup> day 7 diabetic wounds. \*  $p \leq 0.05$ ; \*\*  $p \leq 0.01$ ; \*\*\*  $p \leq 0.001$  (n=6) and scale bar = 100 $\mu$ m.

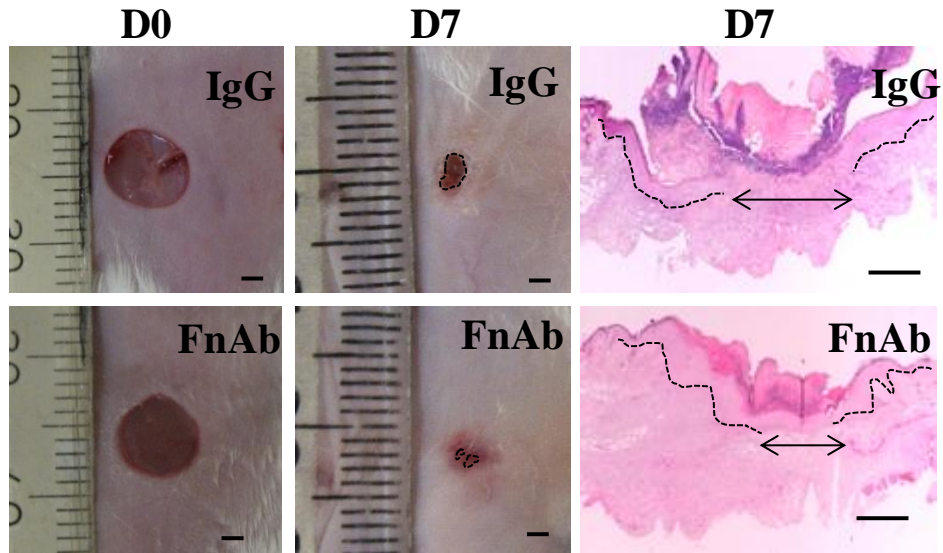
**Figure 4. Modulation of Flii by exogenous application of Flii neutralising antibodies (FnAb) reduces TLR4 expressing in diabetic wounds.** (A) Representative images of TLR4 and NF- $\kappa$ B staining of day 7 diabetic wounds treated with IgG and FnAb. (B) Graphical representation of (i) TLR4 and (ii) NF- $\kappa$ B staining of day 7 diabetic wounds treated with IgG and FnAb. \*  $p \leq 0.05$

**Figure 1**

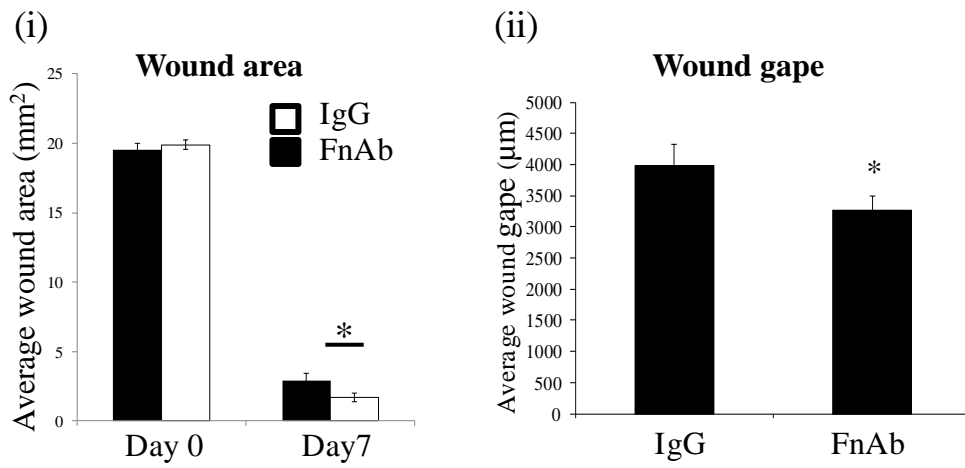


**Figure 2**

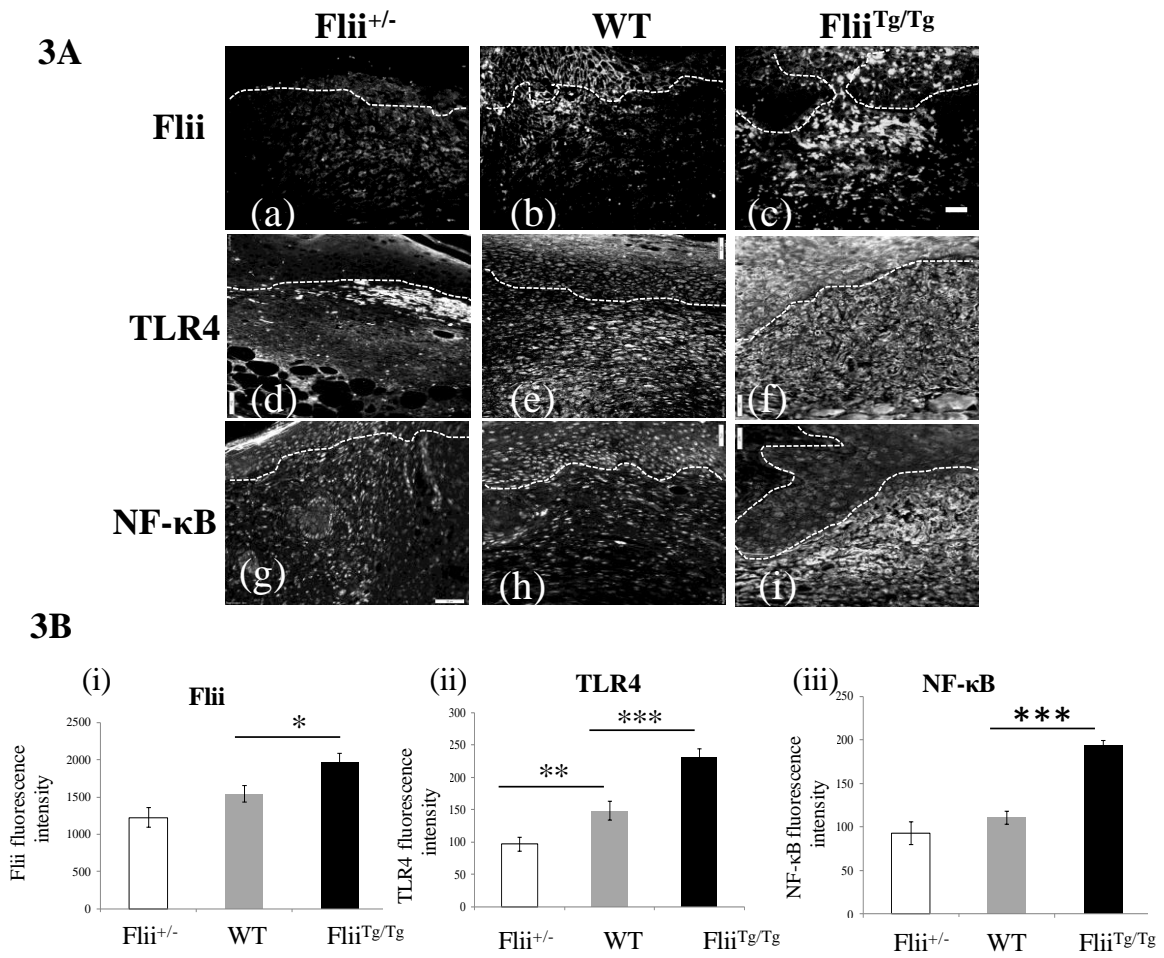
**2A**



**2B**

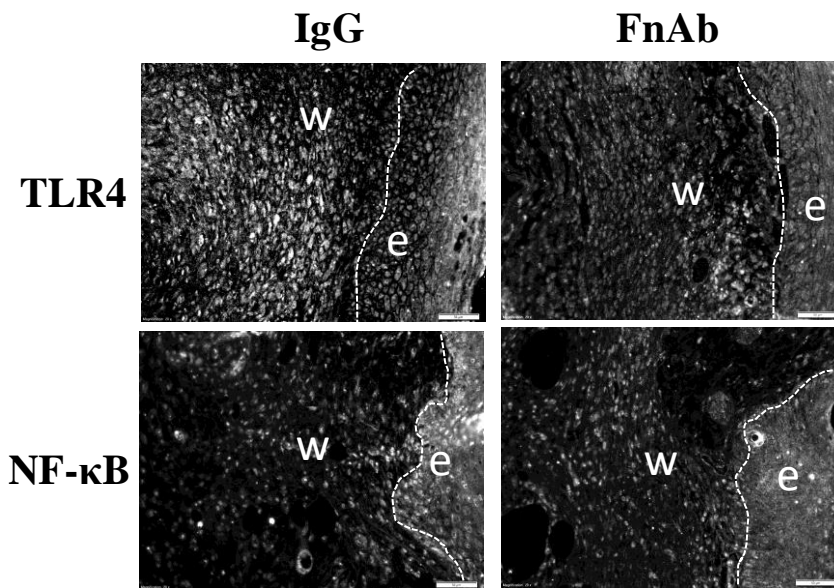


**Figure 3**

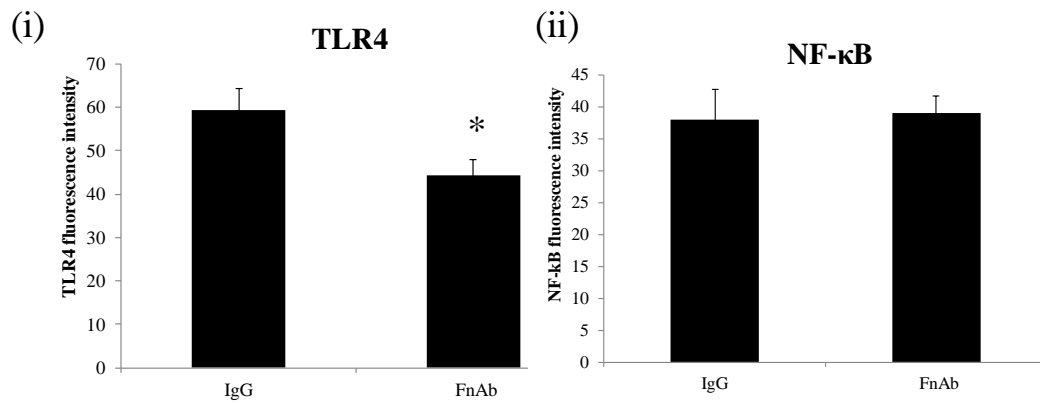


**Figure 4**

**4A**



**4B**



## **The Expression of Flightless I and Toll-Like Receptors in Human Diabetic Wounds**

Nadira Ruzehaji<sup>1,2</sup>, Stuart J. Mills<sup>1</sup>, Elizabeth Melville<sup>1</sup>, Robert Fitridge<sup>2</sup>, Allison J Cowin<sup>1,2</sup>

<sup>1</sup> Women's and Children's Health Research Institute, Wound Healing Laboratory, 72 King William Road, North Adelaide, South Australia, Australia 5006; Email:

stuart.mills@adelaide.edu.au; nadira.ruzehaji@adelaide.edu.au;

elizabeth.melville@adelaide.edu.au; allison.cowin@adelaide.edu.au

<sup>2</sup> Faculty of Health Sciences, University of Adelaide, SA, Australia, 5005; Email:

allison.cowin@adelaide.edu.au; robert.fitridge@adelaide.edu.au

Correspondence should be addressed to Allison J. Cowin; allison.cowin@adelaide.edu.au

Key words: Flightless I, inflammation, diabetes, Toll-like receptors

## **Abstract**

Cytoskeletal protein Flightless I (Flii) is a negative regulator of cellular migration, proliferation and adhesion. As such it has the ability to influence all phases of wound healing including inflammation, matrix deposition, remodeling and angiogenesis. Flii has the potential to modulate inflammation through its interaction with myeloid differentiation factor 88 (MyD88), which is an adaptor protein for Toll-like receptor 4 (TLR4). In order to investigate the role of Flii during the inflammatory response of diabetic wounds we used human diabetic wound samples. In human diabetic wounds we observed increased levels of Flii, which were associated with increased levels of CD14<sup>+</sup> and CD16<sup>+</sup> inflammatory cell infiltrate. Flii co-localization with MyD88 was reduced in diabetic wounds compared with non-diabetic wounds, which may have contributed to the pro-inflammatory profile in diabetic wounds. Given that up-regulation of Flii in diabetic human wounds coincides with weaker interaction between Flii and MyD88 together with increased levels of pro-inflammatory TLR4 and NF- $\kappa$ B production, it is reasonable to hypothesize that decreasing the level of Flii in diabetic wounds may reduce the inflammatory response and improve diabetic wound outcomes.



## **Introduction**

Diabetes is the fastest growing chronic disease worldwide (1). The global estimates for the years 2010 and 2030, using data from 216 countries of the United Nations, indicate that the world prevalence of diabetes among adults was 6.4% in 2010, and will increase to 7.7% by 2030 (2). The increased risk for foot ulcers and amputation amongst individuals with diabetes is well established (3). Diabetic foot ulcers are likely to occur in up to 25% of people with diabetes mellitus at some time in their life (4). With an impaired ability to fight wound infections, individuals with diabetes become largely unable to elicit an adequate inflammatory response and the diabetic foot ulcer becomes a portal for infection that can lead to a life-threatening sepsis and amputation (3). With one Australian losing a lower limb every 3 hours due to diabetes-related foot disease (5), the financial burden of treating diabetic foot ulcers is high for both the individual and society.

The process of wound repair is a normal reaction to injury and actin cytoskeleton reorganisation plays a vital role in cellular migration – a key component of reepithelialisation, which is pivotal for successful wound closure (6). Actin-remodelling proteins of the gelsolin family (7), which includes Flightless I (Flii) (8), have been identified as regulators of the dynamic remodelling of the actin cytoskeleton (9). Flii has been identified as a negative regulator of wound healing affecting both cellular migration and proliferation (6). Mice with reduced expression of Flii have improved reepithelialisation while Flii-overexpressing mice have impaired healing with larger wounds and reduced fibroblast proliferation (10). Flii is a protein containing Leucine Rich Repeat (LLR) sequence, which was recently detected in wound fluid and plasma (11). Secreted Flii binds to lipopolysaccharide (LPS) through its LRR region in activated macrophages (12). Flii is a multifunctional protein and in addition to its actin remodelling role Flii is involved in

transcription via co-activation of nuclear hormone receptors (13), regulation of beta-catenin dependent transcription (14), important signalling pathways including the TLR pathway (15), cellular polarity, asymmetric cell division (16), cellular migration (17) and proliferation via interactions with calmodulin-dependent protein kinase type II (18), inflammation and cytokine production via caspase activation and IL-1 $\beta$  maturation (19).

Resolution of inflammation is necessary during acute stages of wound repair (20), however prolonged and excessive inflammation is detrimental to the healing process preventing the closure of diabetic foot ulcers (21). An intact immune system and an appropriate inflammatory response during wound healing is important for generation of soluble mediators that modulate and activate proliferation and extracellular matrix production of skin fibroblasts (20). The presence of infection within the human body is recognised via Pathogen-Associated Molecular Pattern (PAMPs) and Damage-Associated Molecular Pattern (DAMPs) molecules (22). LPS – a well known PAMP is present in wounds and can be recognised by Toll-like receptors (TLRs) found on the surface of immune cells (22). TLRs expressed by macrophages and neutrophils respond to an array of pathogens and mediate the inflammatory response (23). Binding of PAMPs and DAMPs to TLR receptors activates TLR signaling pathways via interaction between different adaptor proteins such as myeloid differentiation factor 88 (MyD88), TIR-associated protein (TIRAP), TIR-domain containing adaptor protein-inducing IFN- $\beta$  (TRIF) and TRIF-related adaptor molecule (TRAM) (15, 16, 24). Activation of TLR signaling pathways triggers a cascade of event leading to the secretion of cytokines and initiating a pro-inflammatory response (25).

Cytoskeletal protein Flii contains 11 LRR domains in its N-terminus that share 42% similarity to the LRR domain in TLR4 (12, 15). Flii has a number of intracellular binding partners including a

common adaptor protein MyD88 (15, 26). The interaction of Flii with MyD88 leads to modulation of inflammation by suppressing TLR4-MyD88-mediated activation of NF- $\kappa$ B (24). Down-regulation of Flii levels may enhance activation of NF $\kappa$ B and promote cytokine secretion (15). In contrast, numerous studies investigating the effect of Flii on TLR signalling suggested that Flii can suppress the activity of molecules such as LPS and common adaptor proteins such as MyD88 resulting in down-regulation of pro-inflammatory cytokines (13, 14, 18, 19). In this study using human samples of diabetic wounds we have investigated the effect of diabetes on inflammation, TLR expression and Flii activity.

## **Materials and Methods**

### **Antibodies**

Mouse monoclonal anti-Flii antibodies raised to the LRR domain of the human Flii protein (sc-21716) and the rabbit anti-human MyD88 antibody (sc-11356) were obtained from Santa Cruz Biotechnology (VIC, Australia). Rabbit anti-human TLR4 (IMG-579A) and TLR9 (IMG-431) antibodies were obtained from Imgenex (SA, Australia), mouse anti-human CD14 antibody (347490) and mouse anti-human CD16 antibody (Clone 3G8) from BD Biosciences (NSW, Australia) and rabbit anti-human NF- $\kappa$ B antibody from Abcam (NSW, Australia). All antibodies were used at a 1:100 dilution. The appropriate secondary antibodies were used depending on the fluorescence required – goat anti-mouse Alexa Fluor 488 (A11001) and goat anti-mouse Alexa Fluor 594 (A11012) were obtained from Life Technologies (VIC, Australia) and also used at a 1:100 dilution.

### **Human wound and skin collection**

Informed consent was obtained from each patient and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in approval by the Health Service Human Research Ethics Committee and Central Northern Adelaide Health Service Ethics of Human Research Committee. Patients attending the outpatient clinic, multidisciplinary foot clinic, leg ulcer clinic and those admitted to the Queen Elizabeth Hospital (South Australia, Australia) were screened for suitability to participate in this study. All potential subjects were screened for inclusion and exclusion criteria. During the screening period all relevant results from investigations such as the blood tests, quantitative microbiology, duplex and advanced

wound diagnostics in the form of toe pressure, ankle brachial index and transcutaneous oxygen pressure were checked to ensure that no exclusion criteria were met. Once assigned a randomised code, the patients were entered into one of the three study groups: diabetic ulcer, acute wound and normal skin (Table 1.1). As part of the inclusion criteria all members of groups 1 displayed presence of pulses and/or Ankle Brachial Index (ABI) more than 0.7, had a diabetic ulcer and toe pressure (TP) more than 40mmHg. The diabetic ulcer had to be present for at least 6 weeks. All members of group 2 (acute wound group) displayed the presence of pulses and Ankle Brachial Index (ABI) more than 0.9 and had a wound for the duration of less than 6 weeks. All members of group 3 (normal skin group) displayed presence of pulses and Ankle Brachial Index (ABI) more than 0.9 and did not have signs of venous insufficiency or diabetes. Exclusion criteria included an active infection or cellulitis in the area biopsied, drugs that impair wound healing such as steroids and immunosuppressive drugs, ulcer of malignant nature and renal failure/dialysis with Glomerular Filtration Rate (GFR) less than 30 ml/min. Wound and skin biopsies were collected following the guidelines of ulcer biopsy technique mandated to exclude malignancy of some chronic ulcers and routinely practiced by the medical staff. The patients were made comfortable on an examining couch and bupivacaine 0.5% with adrenaline 1:200,000 was used for local anaesthesia. This was injected subcutaneously into the edge of the ulcer and advanced to include the ulcer base. Wound biopsies were collected using a 6 mm biopsy punch (Stiefel Laboratories, NSW, Australia). The skin and wound biopsies were bisected longitudinally. Half the biopsy was snap frozen and used for biochemical/molecular analysis. The other half was further divided into two sections with one being fixed in 10% formalin for paraffin embedding and the other was embedded in Optimal Cutting Temperature (OCT)

compound (Sakura Finetek, CA, U.S.A) and stored at -80°C until further use in histological analysis.

<b>Group</b>	<b>Specimen collected</b>	<b>Inclusion criteria</b>
<b>1 (diabetic ulcer; n=6)</b>	Diabetic ulcer (n=6)	Type 1 or Type 2 diabetes, diabetic ulcer ( $\geq 6$ weeks old)
<b>2 (acute wound; n=6)</b>	Acute wound biopsy (n=6)	Acute trauma wounds such as those seen in the emergency department ( $\leq 6$ weeks old)
<b>3 (normal skin; n=6)</b>	Normal skin (n=6)	Unwounded skin from healthy volunteers

**Table 1.1. Overview of the inclusion criteria and recruitment parameters for the human biopsy collection.**

### **Histology, immunohistochemistry and image analysis**

Human skin and wound samples embedded in OCT were cut at 7  $\mu\text{m}$  and mounted onto slides. Sections were stained with haematoxylin and eosin or subjected to standard immunohistochemical techniques used previously (11). Briefly, sections were fixed in methanol for 5 minutes at -20°C and washed with PBS before staining. 3% normal goat serum was applied to prevent non-specific staining before the application of the primary antibody and incubated for 30 min. The sections were washed in PBS and then incubated with the appropriated primary (1 hour) and secondary antibodies (1 hour). Sections were then mounted using Fluorescent Mounting Media (Dako, VIC, Australia). Histological image analysis was performed using the Digital Microscope Camera ProgRes® C5 (JENOPTIK Laser, Jena, Germany). Fluorescent

images were taken using an Olympus IX81 (Olympus Australia, VIC, Australia). The fluorescent intensity of the staining was calculated using Image Pro-Plus software (Media Cybernetics, MD, USA) as described previously (11).

### **Statistical Analysis**

Statistical significance was calculated using a paired student t-test or analysis of variance. A *p* value of 0.05 or less was considered significant.

## Results

### **Inflammatory infiltrate in diabetic wounds is increased in comparison to acute wounds.**

Wound biopsies were collected from patients diagnosed with diabetic foot ulcers with duration  $\geq$  6 weeks. All wounds studied showed features characteristic of diabetic ulcers with loss of epithelium that extended into the dermis and deeper layers of skin. The epithelial margin was characterized by marked hyperkeratosis. Acute wounds ( $\leq$  6 weeks old) were collected from 6 patients with acute trauma wounds such as those seen in the emergency room. Unwounded skin specimens were collected from 6 healthy donors and used as controls. Representative H&E stained views of a diabetic ulcer, acute wound and normal skin are shown in Figure 1A.

Immunohistochemical analysis of macrophage and neutrophil infiltrate was assessed by staining the wound sections with anti-CD14 (macrophage) and anti-CD16 (neutrophil) antibodies. Figure 1A shows that in the uninjured skin there was little or no presence of macrophages or neutrophils (b-c) whereas in the acute wounds there was a significant increase in both the number of macrophages ( $p=0.026$ ) and neutrophils ( $p=0.05$ ) (Figure 1B). An increase in the numbers of inflammatory cells within the diabetic wounds was also observed with highly significant increases in the numbers of macrophages when compared to the acute wounds ( $p=0.0009$ ). A significant increase in neutrophil numbers in the diabetic wounds was also observed when compared to the acute wounds ( $p=0.038$ ).



**The increase in inflammatory cell numbers seen in the diabetic wounds is concurrent with an increase in TLR4 expression and signalling.**

The presence of foreign molecules and pathogens is detected by a family of receptors known as Toll-Like Receptors (TLRs), which contribute to prolonged inflammation. To assess the effect of diabetes on the level of TLR4 expression, human diabetic wounds were analysed using immunohistochemical staining of TLR4 (Figure 2A). A significant increase ( $p=0.009$ ) in expression of TLR4 was observed in the acute wounds (b) when compared to the normal unwounded skin (a). There was also a large and significant up-regulation of TLR4 in the diabetic wounds (Figure 2B) when compared to the acute wounds ( $p=0.04$ ). No significant difference was seen in the expression of TLR9 between the acute and diabetic wounds suggesting that the increase in inflammatory response in the diabetic wounds may be driven more through TLR4 signalling rather than TLR9 (Figure 2B).

**Increased TLR4 expression in diabetic wounds correlates with an increase in NF- $\kappa$ B release**

Stimulation of TLR4 leads to activation of the downstream transcriptional regulator NF- $\kappa$ B resulting in cytokine secretion (23). Given that the inflammatory response in diabetic wounds was associated with increased TLR4 expression we next proceeded to test if downstream molecule NF- $\kappa$ B production was also elevated. Figure 3A shows that diabetic wounds also show an increase in the expression of NF- $\kappa$ B in the epidermis and dermis when compared to the acute wounds where the staining was less intense and contained within the dermis. This increase in fluorescent intensity was highly significant ( $p=0.0003$ ; Figure 3B) suggesting that along with

increased expression of TLR4 there was also an increase in TLR4 signalling within the diabetic wounds (27).

### **Cytoskeletal protein Flightless I (Flii) is increased in diabetic wounds.**

Cytoskeletal protein Flii binds to intracellular MyD88/TRIF signalling complexes that mediate TLR signalling (15, 26). Furthermore Flii has also been shown to be increased in wounds and to adversely affect wound healing. To assess the role of Flii in diabetic wounds we first stained human acute, diabetic and normal skin for Flii protein. Flii was observed to be present in the epidermis and dermis of unwounded skin (Figure 4A). An increase in Flii expression was seen in acute wounds when compared to unwounded skin (Figure 4B;  $p=0.005$ ) however, a much greater and more statistically significant increase in Flii was observed in diabetic wounds when compared to acute non-diabetic wounds ( $p=0.0008$ ; Figure 4B).

### **Up-regulation of Flii coincided with a parallel increase in MyD88 expression in wounds**

Complex formation between Flii and MyD88 is important in regulation of TLR4-MyD88 pathway (26), which can be suppressed by Flii (15). To determine if Flii localization in the wound was associated with that of MyD88, wounds were dual stained using anti-Flii and anti-MyD88 antibodies. Flii and MyD88 were up-regulated upon wounding (Figure 5) in both acute and diabetic wounds. Unlike unwounded skin, where the expression of both of these proteins appeared distinct and separate (Flii: green, MyD88: red), after wounding strong co-expression of Flii and MyD88 was observed (yellow) in non-diabetic acute wounds, but not in diabetic chronic

wounds where co-expression was much weaker (Figure 5). This was evidenced by the presence of green rather than intense yellow color in merged images (Figure 5). Our results indicate that weak co-expression of Flii and MyD88 in diabetic wounds could be, in part, responsible for driving the pro-inflammatory profile in these wounds.

## **Discussion**

The cytoskeletal protein Flii is involved in cellular processes including migration, proliferation and contraction (6, 17). As such it has the potential to influence all phases of wound healing including inflammation, matrix deposition, remodelling and angiogenesis. Flii has been identified as a protein that can inhibit the rate of healing by reducing the migration of keratinocytes and fibroblasts and limiting the degree of wound contraction (6, 28, 29). Here we have investigated if Flii is involved in the impaired healing associated with diabetic wounds and observed that human diabetic wounds are associated with increased levels of Flii. The majority of actions of Flii described to date involve intracellular Flii, however, recent studies show that Flii is constitutively released by fibroblasts and macrophages via a non-classical late endosome/lysosome pathway of secretion (12).

The regulation and resolution of the inflammatory phase is a critical component of the wound healing response (30). Inflammation plays a major role in the formation of diabetic ulcers as these chronic wounds often have an unregulated and excessive inflammatory reaction (30, 31). The innate immune system activates the inflammatory response via TLRs which recognise and bind Pathogen-Associated Molecular Pattern (PAMPs) molecules including bacteria, fungi and viruses as well as cellular debris which occurs in response to wounding (23, 32). TLRs have also been linked in diabetes with studies showing TLR-associated immune stimulation can result in activation of pro-inflammatory pathways leading to autoimmunity which may cause the onset of diabetes (27). As expected there was an increase in the inflammatory response of diabetic human wounds with greater numbers of neutrophils and macrophages in the chronic vs. acute wounds. Additionally, expression of TLR and pro-inflammatory cytokine NF- $\kappa$ B were also elevated in diabetic wounds, which could explain the observed increase in inflammation and chronicity in

these wounds. Alongside the increased inflammatory response we also saw a concomitant increase in the expression of Flii in human diabetic wounds suggesting a negative role for Flii in these chronic non-healing wounds.

Dual staining of human non-diabetic acute wounds for Flii and MyD88 showed that Flii appeared to be associated with this adaptor protein. Diabetic wounds, on the other hand, failed to demonstrate a strong association between Flii and MyD88, which is necessary for previously reported down-regulation of inflammatory pathway and cytokine production (15). It is therefore reasonable to speculate that increased inflammatory profile in diabetic wounds could be, in part, due to weak interaction between Flii and MyD88. *In vitro* studies have previously shown that Flii can interact with MyD88 and the LRR region of Flii shares 29% sequence identity and 42% similarity to TLR4 (15) suggesting that Flii may influence TLR signalling. Through its interaction with MyD88, Flii was able to negatively regulate the TLR4-MyD88-mediated activation of NF- $\kappa$ B and the subsequent cytokine secretion in macrophages (15, 24). Our studies show that *in vivo*, in human diabetic wounds increased levels of Flii correlated with an increase in the expression of TLR4 and its signalling protein NF- $\kappa$ B. Taken together our results suggest that increased TLR4 expression and weak interaction between Flii and MyD88 within the diabetic wound could be suppressing previously reported (15) potential for Flii to reduce inflammation. We don't know whether Flii is a positive or negative regulator of inflammation, it is, however, not inconceivable that Flii may have a dual role in wounding and in the inflammatory response which depends on the time point and wound type investigated.

Interestingly, our studies showed no difference in the expression of TLR9 in acute and chronic diabetic human wounds. TLR9 is expressed by numerous cells of the immune system such as dendritic cells, B lymphocytes, monocytes and natural killer (NK) cells. TLR9 is expressed

intracellularly, within the endosomal compartments and functions to alert the immune system of viral and bacterial infections by binding to DNA rich in CpG motifs. It has been suggested that TLR9 may aid the healing process by promoting a resolution of the inflammatory response (33-35). An increase in the expression of TLR9 within diabetic wounds would, therefore, be desirable to facilitate the resolution of the inflammatory phase of wound healing.

In summary, inflammation is an integral part of the normal wound healing process (36), however a prolonged inflammatory response is a significant contributing factor to impaired wound healing (20, 37). Flii is emerging as a regulator of inflammation, however whether it is pro-or anti-inflammatory is still to be determined. Our *in vivo* studies show that Flii is up-regulated in chronic diabetic wounds along with TLR4 and NF- $\kappa$ B. We don't know whether modulation of the levels of extracellular Flii would have the capacity to alter inflammation, however, mechanisms that prevent excessive stimulation of TLRs would greatly improve the wound healing outcomes of patients with diabetes.

## **Acknowledgement**

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## **Disclosure Policy**

The authors know of no potential conflicts of interest.

## Figure Legends

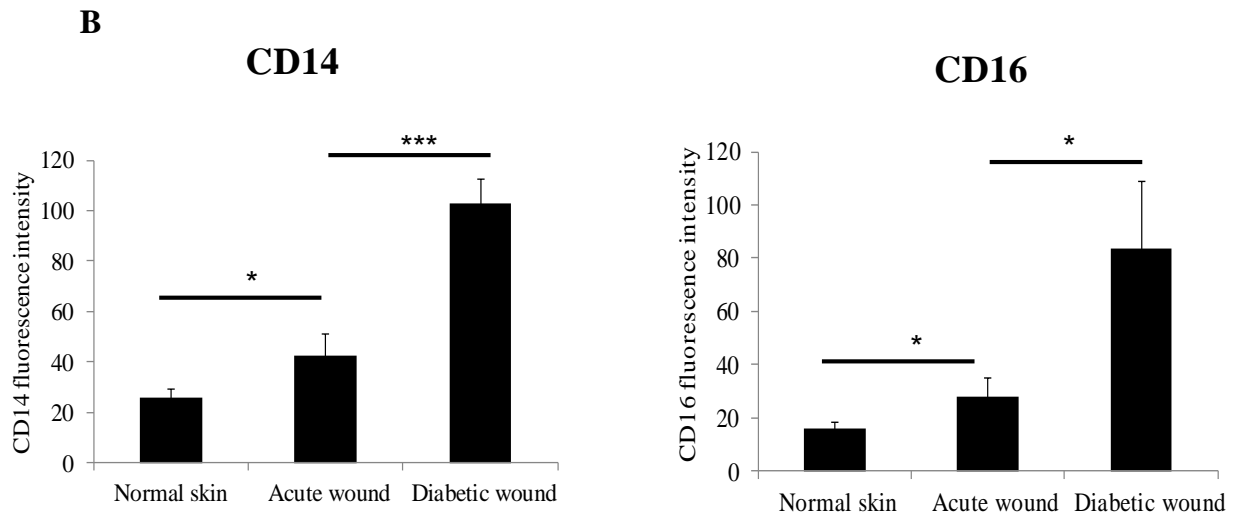
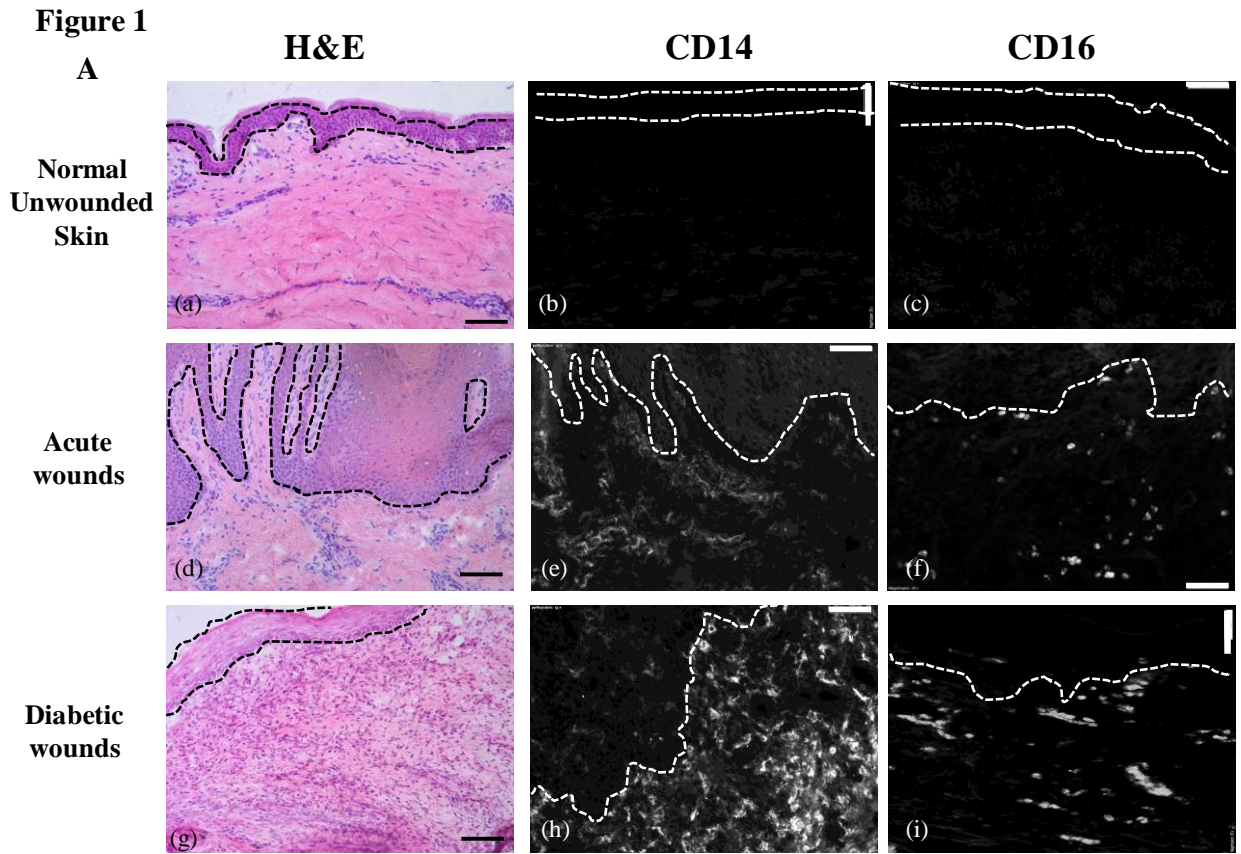
**Figure 1. Inflammatory cell infiltrate is increased in human diabetic wounds.** Figure 1A shows representative staining of H&E, CD14 and CD16 of normal unwounded skin (a-c), acute wounds (d-f) and diabetic wounds (g-i). E denotes epidermis and D denotes dermis in these figures. Pictures were taken at x 20 magnification and scale bars = 50  $\mu$ m. Figure 1B is a graphical representation of the fluorescent intensity of CD14 and CD16 in the different groups. \* $p \leq 0.05$  and \*\*\* $p \leq 0.001$ ; n=6

**Figure 2. TLR4 but not TLR9 is increased in human diabetic wounds.** Figure 2A shows representative staining of TLR4 and TLR9 in unwounded normal skin (a and d), acute wounds (b and e) and diabetic wounds (c and f). E denotes the epidermis and D the dermis. Scale bars = 50 $\mu$ m. Figure 2B show graphical data of the fluorescent intensity of the TLR4 and TLR9 staining in different wound types. \* $p \leq 0.05$  and \*\*  $p \leq 0.01$ ; n=6.

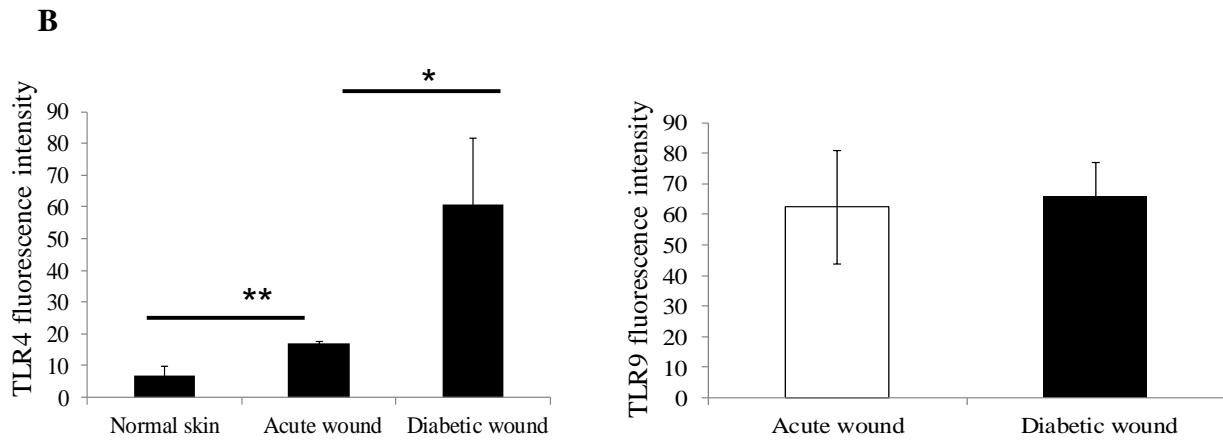
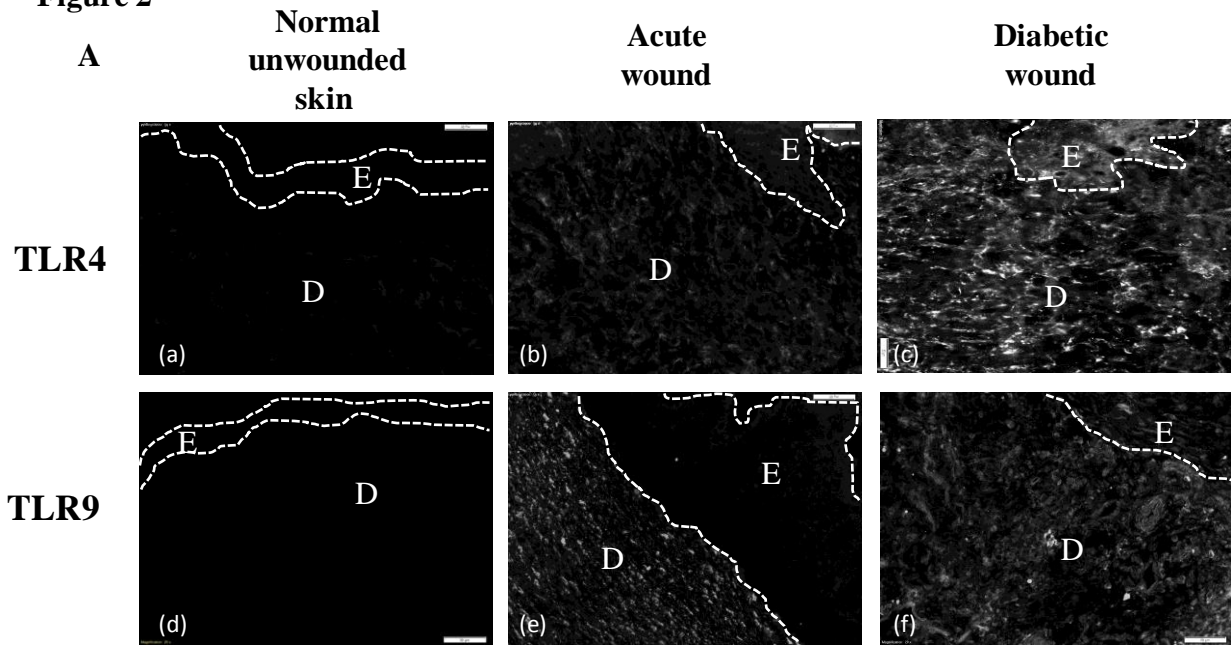
**Figure 3. NF- $\kappa$ B is increased in human diabetic wounds when compared to human acute wounds.** Figure 3A shows representative staining of NF- $\kappa$ B in both acute (a) and diabetic (b) wounds. E denotes the epidermis and D the dermis. Scale bars = 50 $\mu$ m. Figure 3B is a graphical representation of the fluorescent intensity of the NF- $\kappa$ B staining within the acute and diabetic wounds. \*\*\*  $p \leq 0.001$ ; n=6.

**Figure 4. Flii is increased in human diabetic wounds.** Figure 4A shows representative staining of Flii in normal unwounded skin (a), acute wounds (b) and diabetic wounds (c). E denotes the epidermis and D the dermis. Scale bars = 50 $\mu$ m. Figure 4B is a graphical representation of the intensity of Flii staining in the three groups. \*\*  $p \leq 0.01$  and \*\*\* $p \leq 0.001$ ; n=6.

**Figure 5. Co-staining of Flii and MyD88 in normal skin, acute wounds and diabetic wounds.** Representative staining of Flii (green) and MyD88 (red) in the three types of human wounds; chronic diabetic (n=6), acute non-diabetic (n=6) and normal unwounded skin (n=6). Staining of wounds showed that there was an up-regulation of both Flii and MyD88 in acute and diabetic wounds and that there was strong co-expression (yellow) in the acute, but weak co-expression in diabetic wounds. E denotes epidermis, D denotes dermis and W denotes wound in these figures. Pictures were taken at x 20 magnification and scale bars = 50  $\mu$ m.

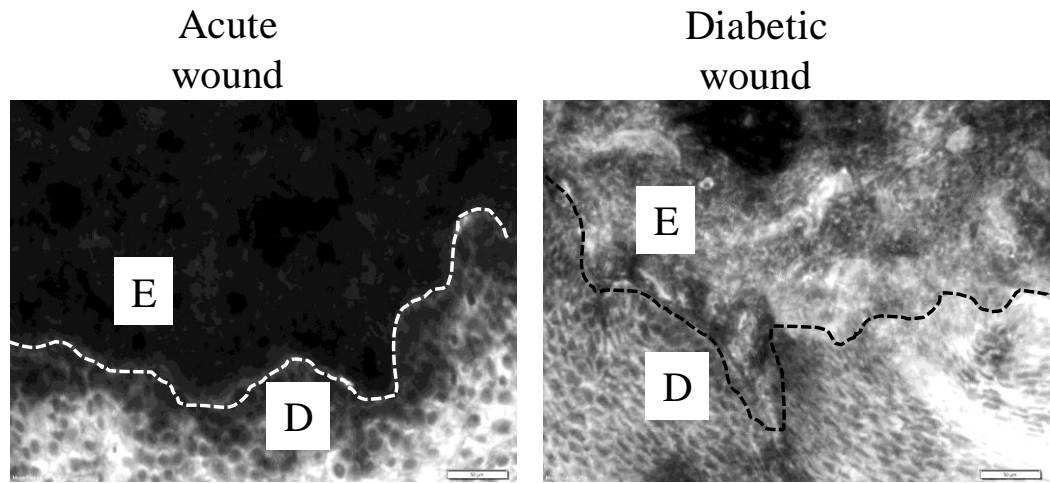


**Figure 2**

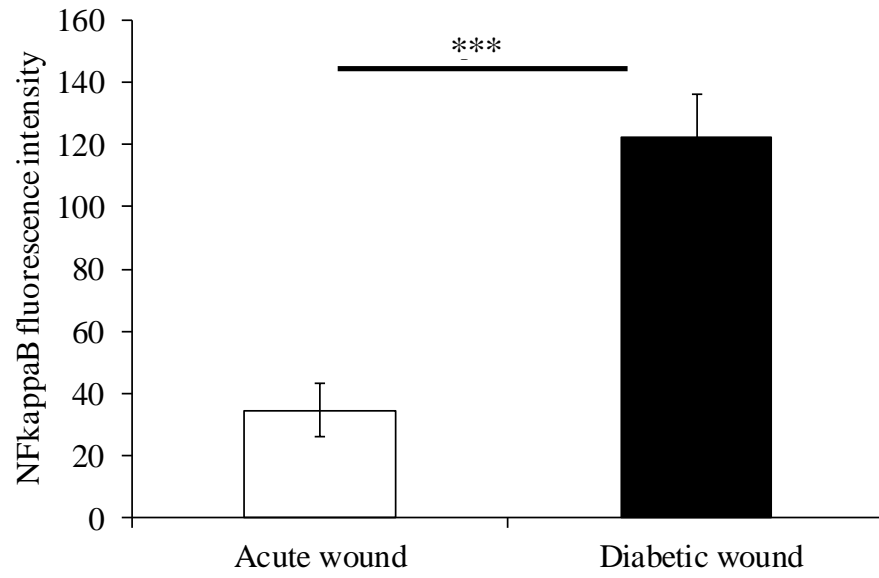


**Figure 3**

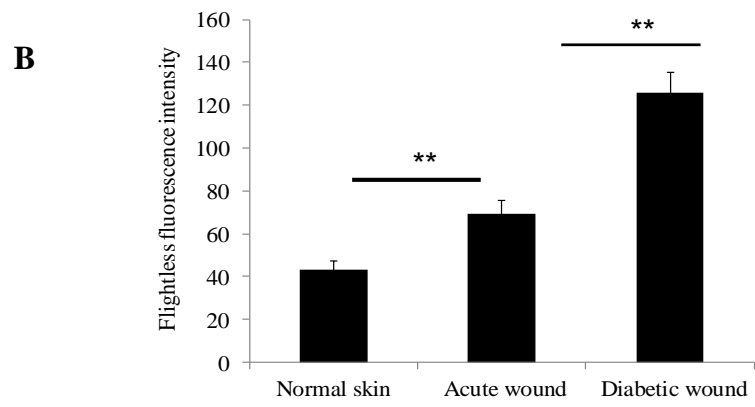
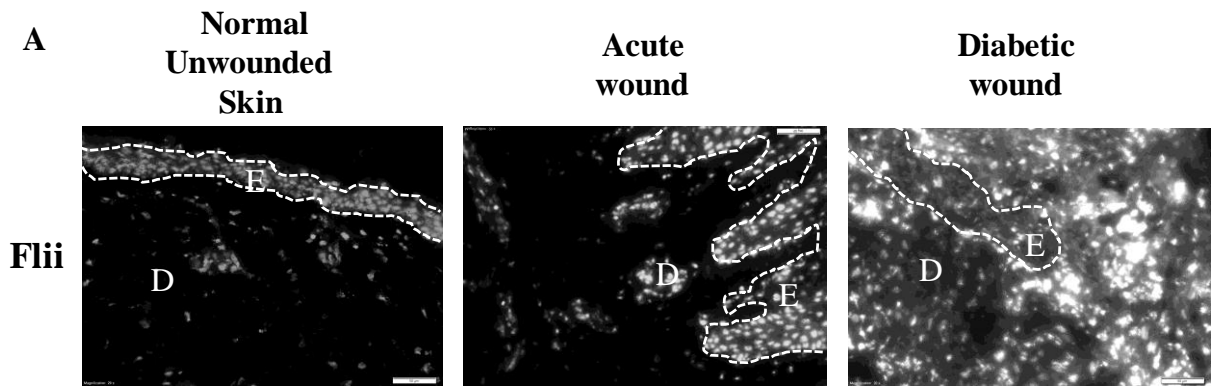
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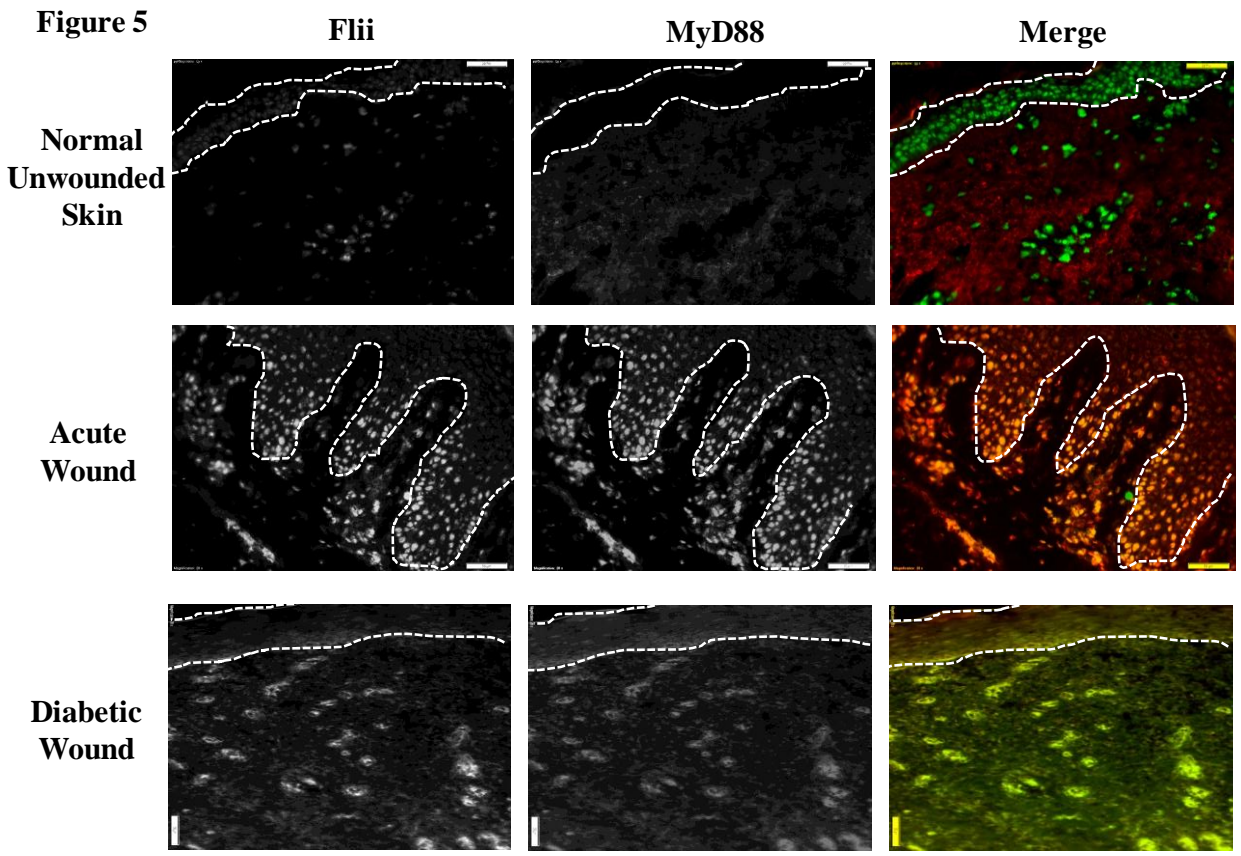


**B**



**Figure 4**







**Cytoskeletal protein Flightless (Flii) is elevated in chronic and acute human wounds and wound fluid: neutralizing its activity in chronic but not acute wound fluid improves cellular proliferation**

Nadira Ruzehaji, BSc<sup>1,2</sup>; Randall Grose, PhD<sup>1</sup>; Doreen Krumbiegel, PhD<sup>1</sup>; Heddy Zola, PhD<sup>1</sup>; Pallave Dasari, PhD<sup>1</sup>; Hilary Wallace, PhD<sup>2</sup>; Michael Stacey, DS, FRACS<sup>3</sup>; Robert Fitridge MSc, MBBS, FRACS<sup>2</sup>; Allison J. Cowin, PhD<sup>1,2</sup>

1. Women's and Children's Health Research Institute, 72 King William Road, North Adelaide, SA, Australia
2. Faculty of Health Sciences, University of Adelaide, SA, Australia
3. School of Surgery, University of Western Australia, Perth, Australia.

**\*Correspondence:** A.J. Cowin, Women's and Children's Health Research Institute, 72 King William Road, North Adelaide. Tel +61 881617077, Fax +61 82390267. Email:

[allison.cowin@adelaide.edu.au](mailto:allison.cowin@adelaide.edu.au)

**Short Running Title:** Presence of Flii in chronic and acute wounds

## **ABSTRACT**

Chronic non healing wounds form a medical need which will expand as the population ages and the obesity epidemic grows. Whilst the complex mechanisms underlying wound repair are not fully understood, remodelling of the actin cytoskeleton plays a critical role. Elevated expression of the actin cytoskeletal protein Flightless I (Flii) is known to impair wound outcomes. To determine if Flii is involved in the impaired healing observed in chronic wounds its expression in non-healing human wounds from patients with venous leg ulcers was determined and compared to its expression in acute wounds and unwounded skin. Increased expression of Flii was observed in both chronic and acute wounds with wound fluid and plasma also containing secreted Flii protein. Inflammation is a key aspect of wound repair and fluorescence-activated cell sorting (FACS) analysis revealed Flii was located in neutrophils within the blood and that it colocalised with CD16+ neutrophils in chronic wounds. The function of secreted Flii was investigated as both chronic wound fluid and Flii have previously been shown to inhibit fibroblast proliferation. To determine if the inhibitory effect of wound fluid was due in part to the presence of Flii, wound fluids were depleted of Flii using Flii-specific neutralizing antibodies (FnAb). Flii depleted chronic wound fluid no longer inhibited fibroblast proliferation suggesting that Flii may contribute to the inhibitory effect of chronic wound fluid on fibroblast function. Application of FnAbs to chronic wounds may therefore be a novel approach used to improve the local environment of non-healing wounds and potentially improve healing outcomes.

**Key Words:** Flightless, cytoskeleton, chronic wound, wound fluid, antibodies

## INTRODUCTION

Chronic wounds are an area of growing concern within the health care community.<sup>1</sup> The sharp rise of the aged population and the prevalence of diabetes and obesity within the community is leading to an increase in the numbers of chronic, non-healing wounds.<sup>2</sup> Understanding the processes involved in impaired healing will help the development of new approaches to address these hard to heal wounds. Acute wounds heal in an efficient manner involving dynamic interaction between various cells such as fibroblasts, keratinocytes and immune cells. Apart from numerous cellular interactions, acute wound healing is characterized by dynamic reciprocity of extracellular matrix (ECM), cytokines, chemokines, growth factors, inhibitors and their receptors.<sup>3,4</sup> Chronic wounds, on the other hand, fail to proceed through the normal stages of healing and become arrested in a pathological inflammatory state subsequently resulting in delayed healing with poor anatomical and functional outcomes.<sup>5</sup>

The actin cytoskeleton is an essential network of filaments found in all cells.<sup>6,7</sup> Reorganisation and remodeling of the actin cytoskeleton is fundamental to all processes of wound repair including cellular adhesion<sup>8</sup>, migration, contraction and motility.<sup>9</sup> Actin-remodeling proteins of the gelsolin family<sup>10</sup> are instrumental in reorganizing the cytoskeleton by severing, capping, nucleating or bundling actin filaments<sup>11</sup> and our previous studies have revealed their importance in murine incisional wounds<sup>12</sup>, age-related impaired wound healing<sup>9</sup> and partial-thickness burn wounds.<sup>13</sup> One highly conserved member of the gelsolin family is Flightless I (Flii).<sup>14</sup> Flii was originally identified in *Drosophila melanogaster*, in which mutations in the gene caused defects in the flight muscles.<sup>15</sup> Studies by Davy et al<sup>16</sup> demonstrated that Flii expression is mainly nuclear with the translocation to the cytoplasm occurring in the presence of the serum.<sup>16</sup>

Endogenous Flii acts as a nuclear receptor co-activator and binds to hormone-activated nuclear receptors and to transcriptional co-activators CARM1 and GRIP1.<sup>17</sup> Flii also negatively modulates the TLR pathway with its endogenous reduction by small-interfering RNA enhancing the activation of NF- $\kappa$ B.<sup>18</sup> Recent studies by Hayashi indicated that nucleoredoxin (NRX) subfamily of proteins forms a link between MyD88 and Flii to mediate negative regulation of the Toll-like receptor 4/MyD88 pathway.<sup>19</sup> We have previously shown that Flii is an important negative regulator of wound healing. In mice with reduced expression of the *FLII* gene, healing is enhanced<sup>12</sup>, whereas Flii overexpressing mice have significantly impaired healing with deficient keratinocyte and fibroblast migration, adhesion, and spreading.<sup>8</sup> Our studies also revealed that Flii is secreted by fibroblasts in response to wounding *in vitro* and that application of Flii neutralizing antibodies to incisional murine wounds improves their healing.<sup>12</sup> Here we aim to investigate the expression of Flii – a known inhibitor of wound healing, in chronic venous ulcers and wound fluid. Identifying inhibitory factors in chronic wounds could lead to the development of new therapies aimed at improving chronic wound repair.

## **MATERIALS AND METHODS**

### **Reagents and antibodies**

Mouse monoclonal anti-Flii (sc-21716) and mouse monoclonal anti-albumin (sc-51515) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Mouse monoclonal anti-human CD16 (550383), mouse monoclonal anti-human CD14 (347490), mouse monoclonal anti-human CD3 APC-Cy7 (557832), mouse monoclonal anti-human CD14 APC (555399) and mouse monoclonal anti-human CD16 PE (555407) were obtained from BD Biosciences (North Ryde, NSW, Australia). Cy3-streptavidin conjugate and mouse monoclonal anti- $\beta$ -tubulin (T 7816) were purchased from Sigma-Aldrich (Saint Louis, Missouri, U.S.A.). Alexa-488 and Alexa-594-tagged secondary antibodies were purchased from Molecular Probes (Invitrogen, Mulgrave, Victoria, Australia). Biotinylated anti-mouse antibody was obtained from Vector (Burlingame, CA, U.S.A.). Anti-Flii neutralizing antibodies (FnAb) were made to the LRR domain of Flii as described previously.<sup>13,16</sup> A New Zealand White rabbit was injected subcutaneously and serum collected following clot retraction and stored at -70°C. The FnAb were affinity-purified using 1 mL, NHS-activated, HiTrap affinity columns (Pharmacia, Uppsala, Sweden) coupled with the peptide described above, according to the manufacturer's instructions.

### **Skin biopsy, wound fluid and plasma collection**

Human tissue collection was approved by the Fremantle Hospital and Health Service Human Research Ethics Committee and Central Northern Adelaide Health Service Ethics of Human Research Committee. 12 patients (8 women, 4 men, mean age of 80 years) with chronic venous leg ulcers were included in this study. 6 patients with chronic venous ulcers were used for the

compilation of chronic wound fluid and another six for the collection of wound biopsies. The venous ulcers were covered with polyurethane film (Op Site, Smith and Nephew, Mile End, SA, Australia) for 3 hours and wound fluid was collected by aspiration using a 1 ml syringe. Day 0 plasma was collected prior to surgery from patients undergoing abdominoplasty (6 women, mean age 49 years). Acute wound (AW) fluid was obtained from surgical drainages (twenty two patients, mean age 52 years, undergoing abdominoplasty and breast reduction as part of the weight loss surgery). In six patients with chronic venous ulcers, 6 mm biopsies were taken from the wound edge. Acute wound biopsies were collected from six patients with acute trauma wounds such as those seen in the emergency room (4 women, 2 men, mean age of 40 years). Control biopsies were taken from healthy skin of the leg of patients undergoing varicose vein ligation and stripping (4 women, 2 men, mean age 42 years). Human white blood cells (WBC) were isolated from EDTA treated peripheral blood of health volunteers generously provided by the Australian Red Cross Blood Service, Adelaide, Australia (1 woman, 3 men, mean age of 33 years).

### **Histology, immunohistochemistry and image analysis**

Cryosections (5  $\mu\text{m}$ ) were cut from Optimal Cutting Temperature (OCT) compound embedded frozen tissue samples. These sections were stained with haematoxylin and eosin and subjected to fluorescence immunohistochemistry. Acetone fixed cryosections of human skin biopsies were blocked in 1% BSA for 1 hour at room temperature. Primary antibodies against Flii (1: 200) (Santa Cruz, CA, U.S.A.) were applied and incubated overnight at 4°C. Anti-mouse biotinylated antibodies (1:200) were used and detected with Cy3-conjugated streptavidin (1:200) (Sigma-Aldrich, NSW, Australia). AnalySIS software package (Soft Imaging System GmbH, Munster,

Germany) was used to measure fluorescence intensity per unit area. Control immunostaining was carried out using the secondary antibody against immunoglobulins of the respective species only for verification of staining and non-specific binding. Negligible immunofluorescence was detected in all negative controls.

### **Immunofluorescence staining of Flii, CD14 and CD16**

5 µm methanol fixed cryosections of chronic venous ulcers were co-stained with Flii (Santa Cruz, CA, U.S.A.), CD14 (347490 BD Biosciences, CA, U.S.A.) and CD16 (550383 BD Biosciences, CA, U.S.A.). Immunofluorescence staining was performed by blocking the sections with 1% BSA and incubating with primary antibodies against Flii (1:100), CD14 (1:100) and CD16 (1:100) at 4°C overnight. The secondary antibodies were applied for 1 hour with Alexa Fluor 488 (1:100) goat anti-mouse (Invitrogen) being used to detect CD14 and CD16. Alexa Fluor 594 (1:100) goat anti-rabbit (Invitrogen) antibodies were used to detect Flii. Coverslips were mounted onto slides using Dako mounting medium. Immunofluorescent microscopy was performed using Olympus IX81 microscope. Immunofluorescent microscope equipped with a 60× oil objective was used to capture immunofluorescent images using CellSens Dimension Imaging Software.

### **Western Blotting**

Protein was extracted from human chronic and acute wounds and normal skin biopsies using standard protein extraction protocols.<sup>8</sup> Briefly, biopsies were micro dissected and placed in lysis buffer (50mM Tris pH 7.5, 1mM EDTA, 50mM NaCl, 0.5% Triton X-100) containing protease inhibitor cocktail tablet (1 per 10ml; Complete, Mini (Roche Products Pty Ltd, Dee

Why, NSW, Australia)) and samples were homogenized briefly. Samples were centrifuged (16,000 x g, 10min, at 4°C), with supernatants being transferred to fresh tubes and centrifuged for a further 10 minutes and the supernatants stored at -20°C. Sample protein (10µg) was run on a 10% SDS-PAGE gels at 100V for 1 hour and then transferred to nitrocellulose by wet transfer (Bio-Rad Laboratories, Regents Park, NSW, Australia) using standard Towbins Buffer with 20% Methanol at 100V for 1 hour. Membranes were blocked in 5% milk blocking buffer for 1 hour and primary antibodies added in PBS containing 5% skimmed milk powder, 0.3% Tween. Appropriate species-specific secondary horse radish peroxidase-conjugated antibodies were added for a further 1 hour at room temperature. Stringent washes for 1 hour were then performed before detection of horse radish peroxidase by Super Signal West Femto Maximum Sensitivity Substrate (Pierce Biotechnology, Rockford, USA) and capture using GeneSnap analysis program (SynGene, Maryland, USA). Membranes were stripped and re-probed for albumin (Sigma-Aldrich, Sydney, Australia) for normalization of protein levels.

Samples of plasma, acute and chronic wound fluid were also collected and centrifuged to precipitate cellular debris. The supernatant was used to isolate proteins by precipitation with trichloroacetic acid (TCA).<sup>20</sup> Flii protein levels were quantified by Western Blotting as previously described.<sup>21</sup> Western blots were probed with Flii (1:200), albumin (1:20,000) and  $\beta$ -tubulin (1:10,000) antibodies. Protein bands were visualized and captured using Syngene G:BOX Chemi HR16 and GeneSnap image capture software (Syngene, Cambridge, UK). Quantification of protein bands was carried out using the Image Quant program by Molecular Dynamics; Amersham Pharmacia Biotech. (GE Healthcare, NSW, Australia).



## **Isolation of granulocytes, monocytes and lymphocytes from peripheral blood**

Human peripheral blood was collected from the brachial artery of healthy volunteers (n=4). Granulocytes, monocytes and lymphocytes were sorted by fluorescence-activated cell sorting (FACS) to a purity of  $\geq 90\%$ . Sorted cells were placed in lysis buffer (50mM Tris pH 7.5, 1mM EDTA, 50mM NaCl, 0.5% Triton X-100) containing protease inhibitor cocktail tablet (1 per 10ml; Complete, Mini (Roche Products Pty Ltd, Dee Why, NSW, Australia)). The samples were homogenized briefly and incubated for 30 min at 4°C. Following cell lysis, the samples were centrifuged (16,000 x g, 10min, at 4°C), with supernatants being transferred to fresh tubes and centrifuged for a further 10 minutes and the supernatants stored at -20°C. The whole cell lysates were used to quantify total protein using Pierce BCA protein assay (Quantum Scientific, Paddington, Australia). Flii (1:200) and  $\beta$ -tubulin (1:10,000) antibodies were used to probe the Western Blot. Western blotting analysis was carried out in the same manner as described above.

## **Fluorescence-activated cell sorting (FACS)**

Peripheral blood was collected from healthy adult individuals by venipuncture in EDTA-treated collection tubes and analyzed by flow cytometry using BD FACSAria™ II (BD Biosciences, San Jose, CA, U.S.A). For intracellular phenotyping assay, 50  $\mu$ l of whole blood was blocked with 10  $\mu$ l of human serum for 30 minutes. Primary antibody (mouse monoclonal anti-Flii FITC, IgG2a, sc-21716) applied for 1 hour followed by washes with 0.1% w/v PBS/Azide. Red cell lysis was performed using FACS lysing solution (BD Biosciences, San Jose, CA, U.S.A., 349202). Control immunostaining was carried out using FITC mouse IgG2a isotype control antibody (BD Biosciences, 553456) and used as a negative isotype control. FITC conjugated

anti-cytochrome C (11660182, eBioscience) antibody was used as a positive intracellular control. FITC mouse anti-human CD 45 (BD Biosciences, 555482) antibody was used as a positive cell surface control. The percentage of Flii positive cells within the CD4, CD14 and CD16 leukocyte population was determined by implementing a four coloured flow cytometry assay: Flii FITC (sc-21716), CD3 APCCy7 (BD Biosciences, 557832) , CD14 APC (BD Biosciences, 555399), CD16 PE (BD Biosciences).

### **Cell proliferation**

Human foreskin fibroblasts (HFF) were used in the *in vitro* assays. 96-wells plate (n=6 for each group) was used to seed HFF at  $2 \times 10^3$  cells/well. On day 1, the cells were seeded and incubated overnight in 10% FCS/DMEM, 2% Penicillin Streptomycin, 2% Fungizone (Sigma-Aldrich) at 37°C, 5 % CO<sub>2</sub>. On day 2, serum free DMEM, 2% Penicillin Streptomycin, 2% Fungizone (Sigma-Aldrich) were mixed with acute and chronic wound fluid at a ratio of 1:1. Flightless neutralizing antibodies (FnAb) were added to wound fluid with the final antibody concentration of 20 µg/ml. Wound fluid was incubated with FnAb at 37°C, 5 % CO<sub>2</sub> for 30 min to counteract the negative effects of Flii. 100 µl of the mixture containing wound fluid, DMEM and FnAb were added to each well with HFF and incubated for 24 hours at 37°C, 5 % CO<sub>2</sub>. On day 3, the cell proliferation reagent WST-1 was added to determine spectrophotometric value of cell growth and viability at 24 hours post-addition of FnAb. The metabolic substrate WST-1 was used in accordance with the manufacturer's protocols (Roche Applied Science, Munich, Germany).

### **Statistics**

Statistical differences were determined using the 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

### **Flii expression is up-regulated in chronic and acute human wounds**

Chronic wound biopsies were collected from 6 patients diagnosed with venous insufficiency and with venous ulcers  $\geq 6$  weeks old (4 women, 2 men, mean age of 80 years). All wounds studied showed features characteristic of chronic venous ulcers. The ulcer wound base was characterized by areas of vascular granulation tissue overlying disorganized collagen bundles. A prominent inflammatory infiltrate was present in most wounds, often extending into the surrounding dermis. Characteristic hemosiderin deposits and capillary cuffing were noted. The epithelial margin was characterized by a marked hyperplastic acanthosis. Acute wounds ( $\leq 6$  weeks old) were collected from 6 patients with acute trauma wounds such as those seen in the emergency room (4 women, 2 men, mean age of 40 years). Unwounded skin specimens were collected from 6 healthy donors (4 women, 2 men, mean age 42 years) and used as controls. Representative H&E stained views of a chronic ulcer, acute wound and normal skin are shown in Fig 1a, c, e, respectively and represent the views shown in the subsequent immunofluorescent studies (Fig 1b, d, f).

Immunofluorescence staining revealed that Flii was increased in response to wounding with both acute and chronic wounds (Fig 1b, d) having higher Flii expression than unwounded skin (Fig 1 f). Flii expression was particularly strong at the leading edge of acute wounds (Fig 1b). Although statistically insignificant, chronic wounds showed reduced Flii staining compared to acute wounds, particularly in the dermis (Fig 1b, d). Quantitation of Flii immunofluorescence showed significantly elevated expression in both wound types compared to control unwounded skin (Fig

1g). Western analysis showed increased levels of Flii in both chronic and acute wounds compared to unwounded skin with acute wounds having higher Flii levels than chronic wounds (Fig 1h). To determine which specific cell types Flii was expressed in, dual immunofluorescence was performed using Keratin 14 as a marker of keratinocytes and Von Willibrand factor as a marker of endothelial cells. Flii was observed colocalised with Keratin 14 positive cells in both acute (Fig 2a) and chronic (Fig 2b) wounds particularly in the cytoplasm of these keratinocytes. However, Flii was not observed to colocalise with endothelial cells associated with blood vessels in these types of wounds. Instead, Flii positive cells (red) could clearly be seen distinct from the green endothelial cells around blood vessels in both acute (Fig 2c) and chronic (Fig 2d) wounds. These Flii positive cells could be pericytes, which are cells that surround endothelial cells and provide contractile properties to vessels.

### **Flii is present in chronic, acute wound fluid and human plasma**

Our previous studies have identified that Flii is secreted by fibroblasts in response to wounding in vitro.<sup>12</sup> To determine if Flii was present in the wound fluid, we collected wound exudate from patients undergoing abdominoplasty (collected on day 1 and day 3 post-surgery) and those with venous ulcers. These samples were analysed using Western blotting and representative blots are shown in Fig 3. Flii was present in both acute and chronic wound fluids (Fig 3a), but no statistical difference was observed between the levels of Flii in these different wound fluids (Fig 3b). To determine if Flii was also present in peripheral blood, plasma samples were collected from patients undergoing abdominoplasty at day 0 (prior to surgery), 1 and 5, and assessed using Western Blotting. Flii was present in all samples of plasma (Fig 3c). Flii levels were decreased in plasma at day 1 and 3 post surgery compared to those collected prior to surgery (day 0), but

again this was not statistically significant. All blots were stripped and re-probed for albumin (loading control) and  $\beta$ -tubulin (negative control), which is an intracellular protein and therefore a marker of cell lysis. The absence of  $\beta$ -tubulin indicates that the presence of Flii in the wound fluid and plasma is through an active secretory process and not just through cellular debris.

### **Flii is present in blood leukocytes and inflammatory wound cells**

To determine if Flii, which was detected in the plasma, was expressed by specific immune cells, sub-populations of leukocytes were isolated using FACS analysis. Granulocytes (neutrophils), monocytes and lymphocytes were sorted according to size and granularity based on their forward and side scatter. Cell fractions were analyzed for Flii expression using Western Blotting (Fig 4a). Flii was expressed in all three cell types, but was most abundant in granulocytes (neutrophils). Acute (Fig 4 b) and chronic wounds (Fig 4c) were also co-stained with antibodies against Flii and CD14 (macrophage marker) to determine if Flii was present within tissue resident macrophages. Acute (Fig 4d) and chronic wounds (Fig 4e) were also co-stained with antibodies against Flii and CD16 (neutrophil marker) to determine if Flii was present within the neutrophils of acute and chronic wounds. These immunofluorescence studies showed that Flii was present in some but not all wound macrophages and neutrophils in chronic and acute wounds potentially reflecting the different activation states of these inflammatory cells (Fig 4b-e). We finally used four-coloured flow cytometry in conjunction with antibodies against CD16<sup>+</sup> (neutrophil marker) CD14 (monocyte marker) and CD3 (lymphocyte marker) to assess whether Flii was present within the cells (intracellular) or attached to the cells surface. High and bright signal was detected in permeabilised CD16<sup>+</sup> cells indicating high intracellular expression of Flii in these

cells, which was not observed in other populations (Fig 5b and g). To determine if Flii protein was membrane bound we examined non-permeabilised CD3+, CD14+ and CD16+ cells and found that Flii was not expressed on the cell surface (Figure 5a, b, e and g).

### **The inhibitory effect of chronic wound fluid on fibroblast proliferation is due in part to Flii**

Previous studies have identified Flii as a negative regulator of wound healing via mechanisms including reducing cellular proliferation, migration and adhesion.<sup>8,12</sup> Further studies have shown that wound fluid contains factors which are cytotoxic and can inhibit cellular proliferation.<sup>22-24</sup> We therefore hypothesized that Flii present in chronic wound fluid may adversely affect the wound environment and inhibit cellular proliferation. Chronic and acute wound fluids were therefore pre-incubated with FnAbs for 30 minutes and then pre-treated wound fluid was assessed for its effect on fibroblast proliferation. When chronic wound fluid alone was added to the fibroblasts, a significant decrease in proliferation was observed (Fig 6a). When chronic wound fluid that had been neutralized for Flii activity was added to the fibroblasts the inhibitory effect of the wound fluid was ablated (Fig 6a). Interestingly, when acute wound fluid alone was added to fibroblast, no inhibition of proliferation was observed despite it containing Flii protein (Figure 6b).

## DISCUSSION

Previous studies have shown that Flii is a negative regulator of wound healing and reducing its expression either at a genetic level or through using antibodies to neutralize protein activity improves wound outcomes.<sup>12</sup> Here we show that Flii is increased in both acute and chronic human wounds, that it is present in the wound fluid of these patients and also in their peripheral blood. Originally we speculated that Flii might be strongly upregulated in chronic non-healing wounds as this protein clearly has a negative influence on the healing process and may therefore be contributing to wound chronicity. However, our results showed that Flii was up-regulated in both acute (healing) and chronic (non-healing) wounds compared to unwounded skin and if anything was more highly expressed in acute wounds, although this was not statistically significant. Flii is a negative regulator of proliferation, migration and adhesion<sup>12</sup>, it stimulates TGF- $\beta$ 1 expression<sup>13</sup> and increases collagen synthesis<sup>13</sup> all of which are important for wound healing. Our previous findings have shown that Flii is secreted by fibroblasts *in vitro* in response to scratch wounding.<sup>12</sup> Intracellular protein  $\beta$ -tubulin was not found in the media suggesting that this was not a consequence of damaged cells emptying their contents into the milieu, but the result of an active secretory process. Recent *in vitro* studies show that Flii is not secreted by the classical secretion pathway, but is instead released via a late endocytic/lysosomal pathway that is regulated by Rab7 and Stx11.<sup>25</sup>

Our current studies now show that Flii is present in both human acute and chronic wound fluid *in vivo*, which suggests that Flii is secreted *in vivo* and that it may not only affect cellular activities via its intracellular functions, but it may also have important extracellular activities. Gelsolin, head of the family of actin remodeling proteins of which Flii is a member, is also released in



response to injury.<sup>26</sup> Tissue injury results in the release of the intracellular protein actin which is cleared from the circulation by the plasma proteins gelsolin and Gc-globulin, constituting the Extracellular Actin Scavenger System (EASS).<sup>27</sup> The intravascular actin scavenger system depolymerizes and sequesters actin released after tissue injury. A study by Dahl and colleagues<sup>27</sup> showed that plasma gelsolin levels were reduced in trauma patients compared with normal controls. Burn wound fluid also contains actin complexed with gelsolin and Gc protein, so it is likely that components of the actin scavenger system are functional in wound tissue.<sup>28</sup> Flii has actin binding ability,<sup>17</sup> therefore its presence in wound fluid and plasma may be similar to that observed for gelsolin, as a member of the extracellular actin scavenger system. The exact extracellular activities of Flii are yet to be determined, however application of neutralising antibodies to Flii significantly improve wound repair in both incisional and burn murine models suggesting that Flii may have effects beyond that of an actin scavenging protein.

Myeloid cells such as blood neutrophils and monocytes move from the local vascular network into wound sites, where they destroy pathogens as well as deliver molecules that help to repair the tissue loss, but may also cause tissue damage.<sup>29,30</sup> Phagocytic properties of neutrophils aid the clearance of non-viable and necrotic tissue, thus aiding healing<sup>31</sup>, however, exacerbated neutrophil recruitment can also damage and destruct viable tissue through its hydrolytic, oxidative and pore-forming molecules.<sup>32</sup> The high influx of neutrophils observed in chronic venous ulcers<sup>33</sup> has been reported to contribute to the immunopathology and chronicity seen in these wounds<sup>34</sup>. In peripheral blood, Flii was found to be present in granulocytes/neutrophils, lymphocytes and monocytes but was most abundant in granulocytes (neutrophils) suggesting that Flii may have an important function in inflammation. Our previous studies have shown that

inflammation in wounded Flii overexpressing mice is prolonged<sup>9</sup> and Flii has also been shown to have an inhibitory effect on the TLR signaling pathway by competitively binding to MyD88 and switching off the NF- $\kappa$ B signaling pathway<sup>35</sup>. Clearly much is still to be learnt regarding the role of Flii in the inflammatory response during wound healing. Most recently, Flii has been shown to be constitutively secreted from both macrophages and fibroblasts and this secreted Flii binds to LPS and dampens cytokine secretion<sup>25</sup>. Acute inflammation is an important feature of normal wound healing, however excessive and chronic inflammation occurs in venous leg ulcers and diabetic wounds.<sup>36,37</sup> Our current findings demonstrate that Flii is detected in both peripheral blood neutrophils and wound neutrophils. Acute and chronic wounds have a greater number of inflammatory cells compared to unwounded skin and we speculate that the elevated level of inflammatory cells in acute and chronic wounds contribute to the increased expression of Flii in these wounds which could possibly negatively impact on healing of these wounds. Interestingly, Flii was present in some, but not all wound macrophages and neutrophils in chronic and acute wounds potentially reflecting the different activation states of these inflammatory cells.

Given that previous studies have shown that chronic wound fluid contains cytotoxic cytokines and inhibits cellular proliferation<sup>22</sup> and Flii has been shown to inhibit cellular proliferation we wanted to establish if the inhibitory effects of chronic wound fluid might be due in part to the presence of Flii. Pre-incubation of both chronic and acute wound fluid with neutralizing antibodies for Flii ablated the inhibitory effect of chronic wound fluid on fibroblast proliferation suggesting that Flii may have a negative effect on chronic wound healing *in vivo*. Acute wound fluid did not inhibit cell proliferation and when FnAb were added to acute wound fluid cell

proliferation was further increased. Treatment of the acute wound fluid with FnAb lead to a significant increase in proliferation suggesting that acute wound may contain pro-proliferative factors as well as Flii, which are able to counteract the negative effects of Flii. While wound fluid is a complex mixture of serum, cytokines, growth factors and cell debris the fact that we could prevent chronic wound fluid from having its inhibitory effects on cell proliferation by applying FnAb suggests that application of Flii antibodies as a topical therapy to chronic wounds may promote improved wound outcomes. Flii is therefore a potential target for improving wound healing outcomes and future studies will investigate methods for reducing Flii directly in human wounds.

#### **CONFLICT OF INTEREST**

The authors state no conflict of interest.

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## FIGURE LEGENDS

**Figure 1. Flii expression is up-regulated in the dermis of acute wounds.** Cryosections of skin biopsies (5µm thick) were used for histology and to perform immunohistochemistry for Flii protein on human acute wounds ( $\leq 6$  weeks old) (b), human chronic wounds ( $\geq 6$  weeks old; skin ulcers secondary to venous stasis) (d) and human unwounded skin (f). In all images, E denotes the position of the epidermis, D denotes the position of the dermis. Representative H&E images (40×) (a, c, e). Graph showing fluorescence optical density of Flii immunofluorescence is shown in (g). Western blotting analysis of skin samples from acute, chronic and normal unwounded skin is shown in (h) (n = 6 for each group). \*Denotes significance in (g)  $p = 0.02$  acute wounds vs. unwounded skin;  $p = 0.04$  chronic wounds vs. unwounded skin. Data are expressed as mean  $\pm$  s.e.m. (n=6). Scale bar is 50 µm.

**Figure 2. Flii is expressed by wound keratinocytes.** (a) Acute and (b) chronic wounds were dual immunostained for Flii and K14 (keratinocyte marker). Flii localizes mainly to the cytosol in keratinocytes in both wound types. (c) Acute and (d) chronic wounds were dual immunostained for Flii with the endothelial cell marker von Willebrand factor. Flii localizes to the nucleus and cytosol of some, but not all endothelial cells and possibly pericytes. Scale bar is 50 µm.

**Figure 3. Flii is present in wound fluid and human plasma.** Wound fluid was collected from patients with either acute or chronic wounds. Unwounded skin was collected from patients undergoing varicose vein ligation and stripping. (a) Protein was extracted and Western analysis performed showing the presence of Flii in acute and chronic wound fluid and in normal unwounded skin. (b) Graph showing quantification of Flii in wound fluid using integrated

fluorescence intensity of band density. Peripheral blood was collected from patients undergoing abdominoplasty. (c) Western analysis showing the presence of Flii in plasma of human patients taken 0 (n=9), 1 (n=8) and 5 (n=5) day wounds post abdominoplasty. (d) Graph showing quantification of Flii in peripheral blood plasma using integrated fluorescence intensity of band density. Flii was normalized against albumin. Results represent mean  $\pm$  s.e.m. There were eight individuals in day 1 wound fluid and five in day 3 group. Six subjects were included in chronic wound fluid group.

**Figure 4. Flii is present in blood leukocytes and wound neutrophils and macrophages.**

Human granulocytes, monocytes and lymphocyte were isolated from peripheral blood leukocytes obtained from four healthy individuals and sorted by FACS to a purity of  $\geq 90\%$ . Cellular lysate samples (20  $\mu$ g of protein) were separated by 10% SDS-PAGE and presence of Flii determined by Western analysis (a). Equal loading of protein samples was confirmed using  $\beta$ -tubulin and Ponceau S staining. (b) Cryosections of biopsies taken from patients with acute wounds and (c) chronic venous leg ulcers were stained with Flii-Alexa 594 (red), CD14-Alexa-488 (green). This staining was performed for the detection of tissue resident macrophages. (d) Cryosections of biopsies taken from patients with acute wounds and (e) chronic venous leg ulcers were stained with Flii-Alexa 594 (red), CD16-Alexa-488 (green). This staining was performed for the detection of tissue resident neutrophils. Immunofluorescence analysis of CD14 positive macrophages and CD16 positive neutrophils co-expressing Flii was observed in the acute and chronic wounds. (n=6, scale bar = 10  $\mu$ m; Magnification  $\times 60$ ).

**Figure 5. Flii is predominantly found in neutrophils within human blood.** Cell-surface expression of Flii protein was determined by staining whole blood with Flii, CD3, CD14 and



CD16 antibodies and analyzed by flow cytometry (a, c, e). To investigate total, i.e. cell surface and intracellular expression of Flii protein, whole blood was permeabilized and stained with Flii, CD3, CD14 and CD16 antibodies. Significantly higher Flii expression was observed in permeabilized CD16-positive neutrophils (b) compared to notably lower Flii expression in CD14-positive monocytes (c, d) and CD3-positive T cells (e, f). Representative data from four independent experiments are shown (n=4). (g) Percentages of Flii-positive cells. Data are shown as means +/- s.e.m. of four independent experiments (n = 4).

**Figure 6. Neutralization of Flii using specific antibodies prevents the inhibitory effect of chronic wound fluid on fibroblast proliferation.** Chronic (a) and acute (b) wound fluid were preincubated with Flii neutralizing antibody (FnAb) (concentration = 20 µg/ml) for 30 minutes prior to applying to human foreskin fibroblasts. The effect on cell proliferation was determined using WST-1 proliferation assay at 24 hour. Results represent means +/- s.e.m. (n = 10 for each group, \**p* < 0.05).

