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Evaluation of matricellular proteins as potential therapeutics for the treatment of human chronic skin wounds

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A thesis submitted in partial fulfillment of the requirements for the degree in Doctor of Philosophy

EVALUATION OF MATRICELLULAR PROTEINS AS POTENTIAL THERAPEUTICS FOR THE TREATMENT OF HUMAN CHRONIC SKIN WOUNDS

(Thesis format: Integrated Article)

by

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Abstract

There is currently an unmet need for treatments to enhance healing of human chronic skin wounds. Previously, therapy development has focused on growth factors and physical matrices, often resulting in disappointing clinical outcomes. In this thesis, we approached chronic skin wound treatment with a focus on fibrosis and matricellular proteins. Fibrosis is a pathological condition where tissue repair continues, unchecked, resulting in excess contraction, matrix accumulation and fibrogenic growth factor activity; features critically reduced in chronic skin wounds. Identifying factors that promote fibrosis may offer new therapeutic targets for use in chronic skin wounds. Two such factors are the matricellular proteins periostin and CCN2. As a group, matricellular proteins have established roles in acute wound healing; facilitating growth factor signaling, matrix production and contraction. However, as of yet, matricellular proteins represent an uninvestigated resource for modulating chronic skin wound healing. The objective of this thesis was to determine the potential of periostin and CCN2 as therapeutics for accelerating skin wound healing. Periostin is up-regulated during skin healing but its function was unknown. Using periostin knockout mice, we observed a delay in full-thickness excisional wound closure in the absence of periostin. This delay was attributed to a lack of myofibroblast differentiation, central to wound contraction, both *in vivo* and *in vitro*. Next we examined the expression patterns of periostin and CCN2 in tissue samples from human chronic skin wounds. Within these wounds CCN2 was not induced and periostin was decreased. These expression patterns were likely due to the environment of the wounds since fibroblasts cultured from wound tissue expressed periostin and CCN2, responded to TGFβ, proliferated and contracted collagen gels; consistent with a fibrotic phenotype. Using a mouse model of impaired diabetic skin healing, we found that delivery of recombinant periostin or CCN2 accelerated wound healing. The mechanisms through which periostin and CCN2 delivery influenced wound healing were distinct, and combination of the two treatments produced synergistic outcomes. These findings represent the first report of using matricellular proteins to enhance healing of diabetic skin wounds in an animal model, with an aim to improve healing of human chronic skin wounds.

Keywords

α-Smooth Muscle Actin, CCN2, Connective Tissue Growth Factor, Chronic Skin Wounds, Dermal Fibroblast, Fibrosis, Matricellular Protein, Myofibroblast, Periostin, Skin Healing, Transforming Growth Factor Beta, Wound Contraction, Wound Healing

Co-Authorship Statement

Chapter 1

Douglas W. Hamilton contributed to the design and concept of the review article. In addition, he provided critical feedback on the manuscript at all levels of completion.

Chapter 2

Jian Wang performed in situ hybridization for the detection of *Postn* mRNA in mouse wound tissue sections (Figure 2.2A). Xiaolei Guo produced the electrospun collagen, and periostin-containing collagen, scaffolds used in this study. Shi-wen Xu and Mark Eastwood performed and analyzed force generation experiments on $Postn^{+/+}$ and $Postn^{-/-}$ murine primary dermal fibroblasts (Figure 2.8A). Jianjun Guan supervised the production of, and provided expertise on, collagen scaffolds used in Figure 2.12. In addition, he provided critical feedback on the manuscript prior to its submission. Andrew Leask contributed to the design and concept of the study, as well as critical appraisal of the manuscript at all levels of completion. Simon J. Conway provided the $Postn^{-/-}$ mice used in this study. In addition, he provided critical feedback on the manuscript prior to its submission. Douglas W. Hamilton contributed to the design and concept of the study. He secured ethical approval for animal work. In addition, he provided critical feedback on the manuscript at all levels of completion.

Chapter 3

Xiaofei Li produced the electrospun collagen scaffolds used in this study. Jianjun Guan supervised the production of, and provided expertise on, collagen scaffolds used in animal work. In addition, he provided critical feedback on the manuscript prior to its submission. Joy Dunmore-Buyze provided technical expertise and carried out microCT scans of Microfil®-perfused animals (Figure 3.6). Maria Drangova contributed to the design of the microCT experiments (Figure 3.6). Thomas Forbes provided access to human tissues and obtained informed consent from participants. He contributed to study design and provided critical appraisal of the study, throughout. Andrew Leask

contributed to the design and concept of the study, as well as critical appraisal of the manuscript at all levels of completion. Douglas W. Hamilton contributed to the design and concept of the study. He secured ethical approval for both human and animal components of the work. In addition, he provided critical feedback on the manuscript at all levels of completion.

Chapter 4

Shawna S. Kim contributed to the design and concept of the commentary, which was incorporated into parts of the discussion. She contributed to the literature review and writing of the manuscript. Douglas W. Hamilton contributed to the design and concept of the commentary, which was incorporated into parts of the discussion. In addition, he provided critical feedback on the manuscript at all levels of completion.

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List of Abbreviations

ACTA2	alpha-smooth muscle actin (gene symbol)
ADSC	adipose derived stem cells
ANOVA	analysis of variance
α-SMA	alpha-smooth muscle actin
bTGFβ2	bovine transforming growth factor beta 2
CCN2	connective tissue growth factor
Col	collagen scaffold, no additives
DEJ	dermal epidermal junction
DMEM	Dulbecco's modified eagle medium
DMSO	dimethyl sulfoxide
ED-A FN	extra type-III domain-A fibronectin
ERK	extracellular signal-regulated kinases
FAK	focal adhesion kinase
FBS	fetal bovine serum
FDA	US Food and Drug Administration
FDR	false discovery rate
GO	gene ontology
HDFa	human dermal fibroblasts, adult
HIF-1α	hypoxia inducible factor-1, alpha subunit
IFNγ	interferon gamma
IHC	immunohistochemistry
IL-13	interleukin 13
JNK	c-Jun N terminal kinases
LAP	latency-associated proteins
LEA	lower extremity amputation
MEF	mouse embryonic fibroblast
MIP	maximum intensity projections
MMP	matrix metalloproteinase
PDGF	platelet-derived growth factor

PDL	periodontal ligament
PN	periostin
Postn	periostin (gene symbol)
PP2	4-Amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine
PRP	platelet rich plasma
PVD	peripheral vascular disease
rhCCN2	recombinant human CCN2
rhPDGF-BB	recombinant human platelet-derived growth factor B homodimer
rhPN	recombinant human periostin
RT-qPCR	reverse transcription quantitative polymerase chain reaction
SIS	small intestine submucosa
SSc	systemic sclerosis
TGFβ	transforming growth factor beta
T_h1	Type-1 helper T cells
T_h2	Type-2 helper T cells
TIMP	tissue inhibitors of matrix metalloproteinases
TNFα	tumor necrosis factor alpha
VEGF	vascular endothelial growth factor
Vegfa	vascular endothelial growth factor A (gene symbol)

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Chapter 1

1 General Introduction

1.1 Skin healing, chronic skin wounds and fibrosis

Development of non-healing skin lesions, which for simplicity will be referred to as chronic skin wounds, represent an increasing burden on today's health care systems. The burden of chronic skin wounds is further exacerbated by the increasing worldwide prevalence of diabetes. The need for novel and alternate therapeutics for the healing of chronic skin wounds is essential in order to reduce patient suffering and the significant costs associated with treatment. Chronic skin wounds result from a failure of the natural healing process to occur. In contrast, fibrosis results when the healing process continues, unchecked, to the point of scarring and impaired tissue function. As research in the fields of chronic skin wounds and fibrosis move forward, important parallels can be drawn between the cellular dysfunctions in fibrotic diseases and the apparent needs of chronic skin wounds, including increased proliferation, increased matrix synthesis and increased matrix contraction. Curiously, inflammation is a major contributing factor in both pathologies, but at this point they diverge. However, recent advances in our understanding of the processes underlying fibrosis may be applicable for the treatment of chronic skin wounds. Recently identified molecules in fibrosis, such as periostin or CCN2, could represent potential therapeutics for chronic skin wound healing. The theme of this introduction is to highlight the role of these molecules in the development of fibrosis and whether such cues could kick-start chronic skin wound healing. To identify the current limitations and potential for new therapeutics in the area of skin wound healing, it is first necessary to understand molecules that are essential for normal wound healing.

1.1.1 Acute wound healing

The process of cutaneous wound healing is very complex and dynamic, involving the co-ordination of multiple cell types and a plethora of growth factors, cytokines and their interactions. The aim of this introduction is not to review the intricate details of acute

wound healing. For such information, the reader is directed to comprehensive reviews on the subject (Clark, 1996; Singer and Clark, 1999; Diegelmann and Evans, 2004; Barrientos et al., 2008; Schultz and Wysocki, 2009).

Briefly, acute (normal) wound healing consists of three overlapping phases: inflammation, proliferation and tissue remodeling (Figure 1.1). Upon tissue injury, damage to blood vessels results in the aggregation of platelets and the formation of a fibrin clot. The clot is essential for restoring hemostasis but also acts as a provisional matrix for infiltrating cells (Clark, 1996). Platelets secrete several soluble factors including platelet-derived growth factor (PDGF), which initiates chemotaxis of neutrophils, macrophages and fibroblasts (Clark, 1996; Diegelmann and Evans, 2004). Neutrophils and macrophages debride the wound. Secretion of transforming growth factor (TGF) β by platelets and macrophages facilitates migration and activation of fibroblasts. Fibroblast infiltration of the granulation tissue is essential for transition from the inflammatory stage to the proliferation/tissue-building phase (Roberts and Sporn, 1993). Concurrently, keratinocytes proliferate and migrate from the wound edge, isolating the wound from the external environment (re-epithelialization).

The proliferation phase involves the formation of granulation tissue by simultaneous perfusion of the wound site with new vasculature and matrix turnover by fibroblasts (Singer and Clark, 1999). Fibroblast proliferation, migration and matrix synthesis is stimulated by PDGF and TGFβ (Clark, 1996). The fibrin clot is replaced by cellular fibronectin, collagen type-III and progressively more collagen type-1 (Midwood et al., 2004). Differentiation of fibroblasts into the α-smooth muscle actin (α-SMA)-positive contractile, myofibroblast phenotype allows the contraction and compaction of the granulation tissue into a matrix dense scar (Tomasek et al., 2002).

The transition from granulation tissue to scar formation marks the beginning of the remodeling phase. During this phase collagen is degraded, synthesized and rearranged at a slower rate. Increasingly, collagen is organized into large bundles and cross-linked. The resulting scar tissue is relatively acellular and achieves only about 80% of the breaking strength of normal skin (Levenson et al., 1965). Therefore, acute healing does not

Figure 1.1: Acute wound healing consists of three overlapping phases: inflammation, proliferation and remodeling. The inflammation phase is dominated by neutrophils and macrophages, which serve to remove foreign debris, bacteria and damaged tissue. The proliferative phase includes the formation of granulation tissue and reduced inflammatory signals. The dominant cell types are fibroblasts and myofibroblasts. Matrix turnover and contraction are key features of this phase. The remodeling phase serves to rearrange and strengthen the newly formed tissue, producing a matrix-dense, relatively acellular, scar. Development of chronic skin wounds and fibrotic lesions are both driven by increased inflammation. (A) However, in chronic skin wounds sustained inflammation and failed progression to proliferative and remodeling phases result. (B) Fibrosis results from failure of the remodeling process to terminate at an appropriate point. Instead, continued matrix secretion and contraction by myofibroblasts results in excessive scarring.

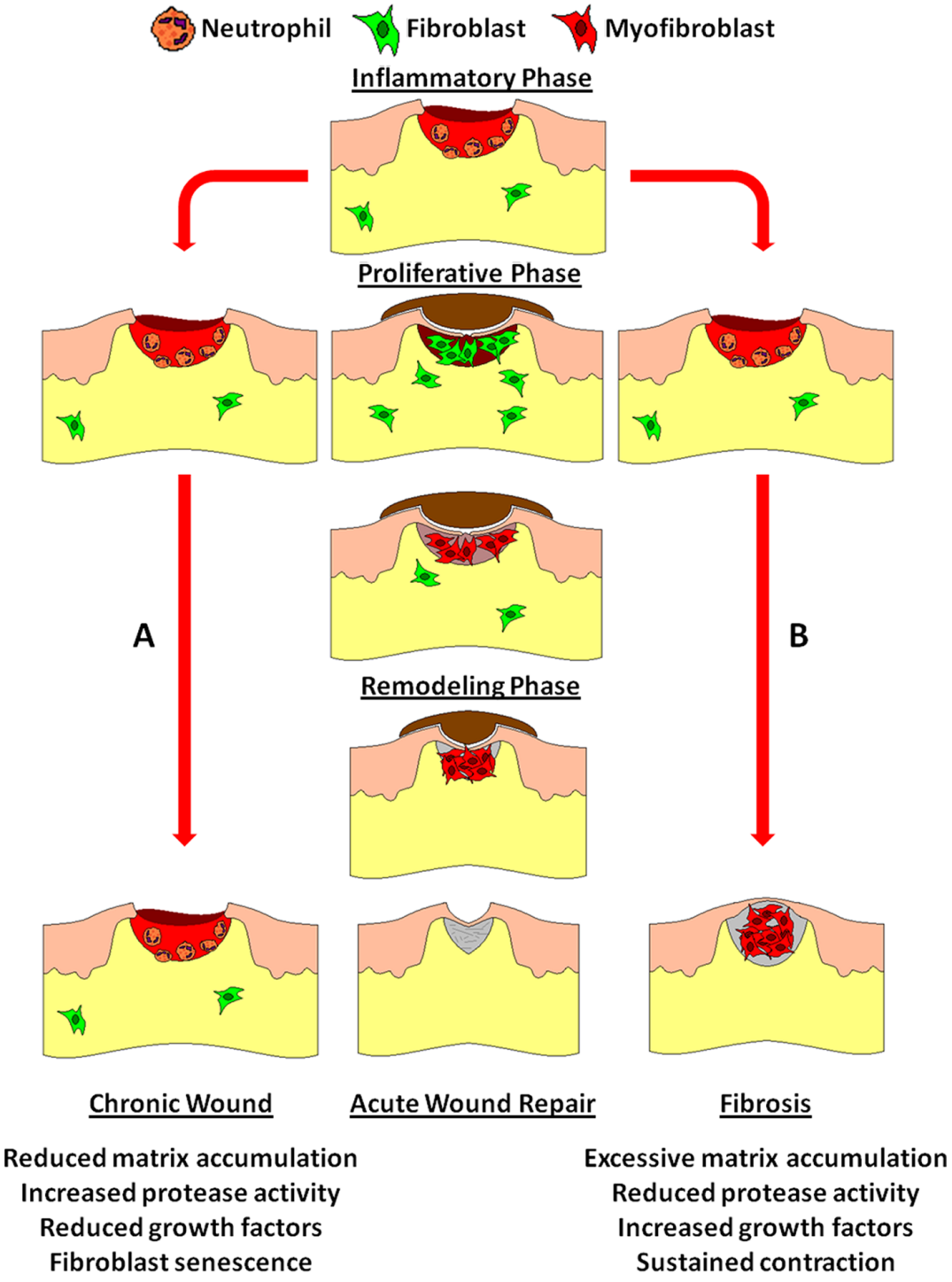

Figure 1.1: Acute wound healing consists of three overlapping phases: inflammation, proliferation and remodeling

perfectly regenerate the affected tissue, but instead strikes a favorable balance between a "good enough" repair and rapid wound closure. However, the inability of patients with existing medical conditions such as diabetes to heal skin wounds rapidly, or at all, is becoming more and more of a burden on healthcare systems.

1.1.2 The burden of chronic skin wounds

Chronic skin wounds are a diverse set of pathologies that are fundamentally defined by a severely compromised ability to heal. The majority of chronic skin wounds can be classified as either diabetic ulcers, venous ulcers or pressure sores (Mustoe et al., 2006), with arterial wounds being less prevalent. The underlying pathophysiologies of these wound types are quite distinct (extensively reviewed by Bryant and Nix, 2012). Yet they are all complicated by a combination of patient age, repeated ischemia-reperfusion injury, bacterial colonization and hypoxia (Mustoe, 2004; Schreml et al., 2010). Diabetic ulcers develop as a result of the neuropathy and compromised immune system present in people with diabetes. Neuropathy inhibits the perception of pain following minor injury or the discomfort following prolonged pressure. As a result there is repeated aggravation to the site, which, combined with reduced ability to fight infection, overwhelms the bodies ability to repair the tissue. Venous ulcers are the consequence of inadequate venous return due to faulty valves within the veins, leading to excess fluid build up and venous hypertension. In addition to impeding the flow of fresh blood, venous hypertension promotes leaking of blood proteins into the extravascular space. These proteins prevent oxygen and nutrients from reaching the tissue in addition to initiating a sustained immune response. Pressure sores, similarly to diabetic ulcers, result when tissue is starved of oxygen and nutrient rich blood as a result of prolonged pressure. Often referred to as bedsores, these wound are common in bed-ridded or paralyzed people. Arterial wounds are a result of impeded blood flow due to arterial dysfunctions. Blood vessel hardening and blockage leads to ischemia and tissue necrosis. The differences in the underlying causes for these wounds requires specialized and distinct corrective interventions (off-loading for diabetic and pressure, reperfusion for arterial, compression for venous ulcers) before wound healing can be expected to occur. Those wounds that persist even after

corrective interventions have been attempted become the focus of adjunct therapies, such as those described below.

In general, chronic skin wounds in healthy individuals are rare (Sen et al., 2009). However, in medically compromised patients, such as those suffering from diabetes, the risk of developing chronic skin wounds is greatly increased. The lifetime incidence of foot ulcers in diabetic patients has been estimated to be 15-25% (Reiber, 1996; Singh et al., 2005). The overall prevalence of pressure ulcers within Canadian healthcare institutions was estimated at 26% (Woodbury and Houghton, 2004). The estimated prevalence of chronic skin wounds encompassing all etiologies within the healthcare system is closer to 35.5% (Woodbury and Houghton, 2005). Unfortunately, failure of current treatment strategies to heal chronic skin wounds commonly leads to amputation of the effected limb (Cavanagh et al., 2005; Wu et al., 2007). In 1993, Siitonen and colleagues reported the occurrence of lower extremity amputation (LEA) to be 10 times higher in diabetic persons than in non-diabetics (Siitonen et al., 1993). Sixteen years later this trend had shown no signs of leveling off. In fact, LEA occurs 19 times as often in diabetic Canadians than in the general population (Canada Diabetes Report, 2009). Sixty percent of non-traumatic amputations in the US occurred in diabetic patients (CDC Diabetes Fact Sheet 2007), with 80% of amputations in diabetic patients being preceded by a chronic skin wound (Driver et al., 2010). The risks associated with LEA are of great clinical concern. Perioperative mortality rates following above knee LEA were reported to be as high as 18.6% in 2003 (Moxey et al., 2010) and 18% in 2005 (Ploeg et al., 2005). Recently, Aragón-Sánchez and colleagues reported 14.7% post-operative mortality following above knee amputations (Aragon-Sanchez et al., 2010). In England, a significant decrease occurred in mortality rates, from 18.6% in 2003 to 15.2% in 2007, supporting a promising trend (Moxey et al., 2010). Long term mortality following LEA, however, remains unacceptably high. Several recent reports place five-year survival rates following any LEA at approximately 50% (Hambleton et al., 2009; Papazafiropoulou et al., 2009). When major amputations (above knee) are considered alone, five-year survival rates are as low as 11% (Morbach et al., 2009).

An estimated 2.4 million Canadians were living with diabetes in 2009 (Public Health Agency of Canada, 2011). The prevalence of diabetes in Canada has increased steadily from 4.9 to 6.2% from 2002-03 to 2006-07, representing over 2 million Canadians (Canada Diabetes Report, 2009). Current estimates suggest that 347 million people worldwide have diabetes, surpassing previous projections of 336 million people living with diabetes by the year 2030 (Wild et al., 2004; Danaei et al., 2011). There is no indication that the burden of chronic skin wounds is shrinking. With the growing prevalence of diabetes, obesity and an aging population, the impact of chronic skin wounds is deserving of greater attention in healthcare research (Armstrong et al., 2007; Harding and Queen, 2010). Selection of target molecules for treatment of chronic skin wounds based on studies of acute wound healing can be difficult and time consuming due to the complexity of acute wound healing. To expedite the search for target molecules we can take advantage of the accumulating knowledge of fibrotic skin lesions, such as keloid scars, hypertrophic scars and scleroderma. Careful dissection of these conditions may offer hints towards treating chronic skin wounds.

1.1.3 Chronic skin wounds vs. fibrosis

Fibrosis is a general term describing pathological conditions in which the healing process has continued, unchecked, to the point where normal tissue is replaced by scar tissue, resulting in impaired or lost function. The central features of fibrotic diseases include increased growth factor activity, decreased protease activity and decreased fibroblast senescence (Figure 1.1) (Wynn, 2007). The culmination of these features is excess matrix deposition and scar formation. Although the etiology of chronic skin wounds is not fully understood, three major differences have been described between chronic and acute skin wounds; 1) reduced growth factor activity, 2) increased protease activity and 3) increased fibroblast senescence (Schultz and Mast, 1998; Harding et al., 2002). Interestingly, excessive inflammation is common to both chronic skin wounds (Cochrane, 1977; Trengove et al., 2000) and fibrosis (Abraham and Varga, 2005). The key difference is that chronic skin wounds become stalled in an inflammatory state, but fibrotic diseases progress beyond the initial inflammation and enter an aggressive fibrotic state (Abraham and Varga, 2005). In bleomycin-induced fibrosis, the initial stage of excessive

inflammation is characterized by increased numbers of macrophages (Kraling et al., 1995). Being a major source of the pro-fibrotic growth factors PDGF and TGFβ, increased macrophage numbers promote an elevated fibrotic response (Yamamoto and Nishioka, 2005). The elevated fibrotic response results in the increased and sustained activity of myofibroblasts, which are responsible for elevated collagen production, matrix contraction and continued secretion of TGFβ (Tomasek et al., 2002; Yamamoto and Nishioka, 2005). In contrast, chronic skin wounds suffer from reduced expression of key growth factors, such as PDGF and TGFβ, which impedes fibroblast activation and construction of the granulation tissue (Robson, 1997; Galkowska et al., 2006). Increased protease activity, originating from excessive neutrophil infiltration (Cochrane, 1977) and compounded by reduced protease inhibitor levels, serves to degrade important matrix components such as collagens (Wysocki et al., 1993; Vaalamo et al., 1999; Liu et al., 2009; Yang et al., 2009) and growth factors (Chen et al., 1997). These deficiencies are further exacerbated by the senescent state of fibroblasts within the wound, which impairs the response to the already scarce growth factors (Hasan et al., 1997; Stanley et al., 1997; Hehenberger et al., 1998; Mendez et al., 1998; Loot et al., 2002; Yang et al., 2009). A question of critical importance is why, or how, do fibrotic skin lesions progress beyond the initial excessive inflammation, whereas chronic skin wounds become stalled (Figure 1.1)?

1.2 Current targets for treatment of chronic skin wounds

1.2.1 Platelet-derived growth factor-BB

Does increased recruitment of macrophages in bleomycin-induced fibrosis provide the answer to progressing chronic skin wounds beyond inflammation? Fibrosis results from excessive extracellular matrix (ECM) production, primarily by a specialized type of activated fibroblasts, termed myofibroblasts, so-called as they highly express the protein α-SMA (Trojanowska, 2008). Macrophages release the pro-fibrotic growth factor PDGF (Yamamoto and Nishioka, 2005), which plays a key role in the expansion and persistence of myofibroblast populations (Bostrom et al., 1996; Heldin and Westermark, 1999; Trojanowska, 2008) by modulating fibroblast migration, proliferation and activation

(Barrientos et al., 2008). Elevated PDGF also contributes to TGFβ expression and thus represents a crucial initiator of granulation tissue formation (Heldin and Westermark, 1999).

PDGF includes a family of homo and heterodimeric proteins (PDGF-AA, PDGF-AB, PDGF-BB, PDGF-CC and PDGF-DD), which bind to transmembrane tyrosine kinase receptors (Bennett et al., 2003; Barrientos et al., 2008). In the fibrotic disorder systemic sclerosis (SSc), expression of PDGF-BB receptors is increased within and around dermal vasculature (Klareskog et al., 1990). Bronchoalveolar lavage fluid from SSc patients contains significantly elevated levels of PDGF-AA and -BB (Ludwicka et al., 1995). Fibroblasts isolated from SSc skin biopsies express elevated PDGF-B and PDGF receptor, compared to normal patients (Zheng et al., 1998). The c-Abl inhibitors imatinib (Distler et al., 2007), dasatinib and nilotinib (Akhmetshina et al., 2008), act downstream on non-receptor tyrosine kinases of both TGFβ and PDGF to block the production of ECM proteins (Daniels et al., 2004). Bleomycin-induced dermal thickening and myofibroblast accumulation is prevented by oral administration of these inhibitors in a mouse model (Distler et al., 2007; Akhmetshina et al., 2008), suggesting that PDGF may be an excellent target for the treatment of fibrosis (Beyer et al., 2010; Leask, 2010), but also highlighting PDGF as an obvious therapeutic agent for treating chronic skin wounds. As a chemoattractant for fibroblasts (Seppa et al., 1982), PDGF is important for the progression of wound healing beyond inflammation and into the proliferation phase (Gao et al., 2005). In animal models, delivery of PDGF to skin wounds increases fibroblast infiltration, TGFβ expression, collagen deposition and granulation tissue formation (Pierce et al., 1988; Pierce et al., 1989; Mustoe et al., 1990); resulting in accelerated wound healing. Moreover, these benefits are also achieved in models of chronic skin wounds (Mustoe et al., 1989; Mustoe et al., 1991). It has since been shown that PDGF-BB is absent in chronic skin wounds, but present in actively healing pressure ulcers (Pierce et al., 1995).

PDGF is currently the only growth factor approved for use by the FDA in the treatment of diabetic foot ulcers and is marketed under the name Regranex®. Application of rhPDGF-BB is preceded by debridement, or wound bed preparation, which is the attempt

to remove the infected and necrotic components of an ulcer and establish a pseudo-acute wound environment (Kirshen et al., 2006). Delivery of rhPDGF-BB to debrided wounds improved closure of non-diabetic chronic pressure ulcers (Robson et al., 1992) and deep pressure ulcers (Mustoe et al., 1994). In neurotrophic diabetic ulcers, treatment resulted in 48% of wounds closing compared to 25% of wounds closed in the placebo group (Steed, 1995) (Table 1.1). In a multi-center study, administration of 100 μg/g becaplermin (rhPDGF-BB) to diabetic ulcers resulted in closure of 50% vs. 35% (treatment vs. placebo) of wounds (Wieman et al., 1998). The smaller effect size reported by Wieman in comparison with Steed possibly reflects inherent variability in the larger, multi-center study, thus more closely representing a realistic effect size with wide spread use. The addition of rhPDGF-BB to diabetic wounds has therefore produced statistically significant, although modest, increases in wound healing over placebo. At best, 50% of wounds treated with rhPDGF-BB closed, the majority of which can be attributed to standard wound care (placebo) alone. Perhaps a limitation of PDGF based treatments is that the presence of PDGF results in recruitment of inflammatory cells such as macrophages and neutrophils (Deuel et al., 1982). Whereas macrophages are one of the major sources of TGFβ (Roberts and Sporn, 1993) and are required for wound healing (Leibovich and Ross, 1975), neutrophils are not required for wound healing in the absence of non-specific infection (Simpson and Ross, 1972). Neutrophils are a source of inflammatory cytokines (IL-1, -6 and TNFα) (Barrientos et al., 2008), reactive oxygen species and damaging proteolytic enzymes (Diegelmann and Evans, 2004). Without additional signals, which are absent in chronic skin wounds, it is possible that the pro-inflammatory role of PDGF may prevail. Thus complex interaction of the multitude of growth factors and matrix elements present in acute wounding may not be suitably recapitulated with rhPDGF-BB alone.

1.2.2 Platelet releasate

Platelet rich blood plasma contains multiple growth factors (Schultz and Grant, 1991) and the components of a fibrin matrix (Mosesson, 2005). Administration of autologous plasma to chronic skin wounds more closely mimics the complexity of the initial stages of natural wound healing, including structural matrix components. Platelet rich plasma

Table 1.1: Summary of reviewed therapeutic targets for chronic skin wounds

Factor	Role in acute skin wounds	Fibrosis	Chronic skin wounds	Treatment options and outcomes (treatment vs. control)*
PDGF-BB	Migration and activation of macrophages Migration, proliferation and activation of fibroblasts Granulation tissue formation	Increased	Decreased	Becaplermin: Steed *et al* 1995: 48% vs. 25% incidence of closure Wieman *et al* 1998: 50% vs. 35% incidence of closure
Platelet Releasate	Numerous due to inclusion of various growth factors and components of the fibrin matrix	N/A	N/A	Autologel™: Driver *et al* 2006: 81% vs. 42% incidence of closure (in a subset of patients) Margolis *et al* 2001: 50% vs. 41% incidence of closure
TGFβ	Decreases protease activity Migration, proliferation and activation of fibroblasts Granulation tissue formation	Increased	Decreased	Bovine TGFβ2: Robson *et al* 1995: open-label study increased closure rate, closed-label study no efficacy
CCN2	Not clear, may contribute to angiogenesis, cell recruitment and matrix accumulation	Increased	Unknown	No treatments currently target CCN2
Periostin	Unknown, but contributes to collagen fibrillogenesis, matrix accumulation, cell migration, proliferation	Increased	Unknown	No treatments currently target periostin

*Outcomes are listed as percent incidence of closure in the treatment group vs. percent incidence of closure in the control group.

(PRP) has been used for the treatment of chronic skin wounds for over 20 years. Autologel™, a PRP product, is prepared from a small sample of the patient's blood plasma mixed with a gel base, which is immediately applied to the wound. Despite its long history of use, very few randomized controlled clinic studies on the efficacy of PRP have been documented. Driver and colleagues carried out the first FDA approved prospective, randomized, blinded, placebo-controlled clinical trial of PRP (Autologel™) for diabetic foot ulcers (Driver et al., 2006). Although this study showed significance (81% vs. 42% of wounds closed, treatment vs. placebo) (Table 1.1), many of its participants were excluded from analysis for a variety of reasons. As the vast majority of persons with chronic skin wounds are likely to fall outside of the numerous exclusion criteria employed in this study, the applicability of this data would appear to be very limited. Margolis *et al*, in a retrospective, randomized, controlled study of nearly 27,000 patients (of which 21% were treated with platelet releasate), reported the overall proportion of chronic skin wounds healed at 50% vs. 41% (Margolis et al., 2001). This is likely a much more realistic outcome since it reflects the effectiveness of the treatment across a very large patient demographic, not the efficacy within a very idealized treatment group.

Use of platelet releasate for the treatment of chronic skin wounds offers benefits over placebo, however, similar to rhPDGF-BB, the increased incidence of wound healing is modest. The limited success of platelet releasate may be due to pro-inflammatory influences, which may undermine the pro-fibrotic effects of growth factors contained within. PDGF is a chemoattractant for fibroblasts (Seppa et al., 1982; Lin et al., 2006), however, it does not significantly increase expression of collagen type-1, a key component of granulation tissue and scars (Tan et al., 1995; Jinnin et al., 2005). Instead, PDGF increases expression of matrix metalloproteinase (MMP)-1, which contributes to collagen degradation (Tan et al., 1995). Interestingly, whereas the presence of PDGF-BB results in increased MMP-1 expression in human dermal fibroblasts, TGFβ1 in the matrix results in decreased MMP-1 expression and significantly increased collagen synthesis, contributing to matrix accumulation (Ignotz and Massague, 1986; Edwards et al., 1987;

Tan et al., 1995). Thus TGFβ is a logical alternative to PDGF as a pro-fibrotic therapeutic agent.

1.2.3 Transforming growth factor β

Transforming Growth Factor β expression during acute wound healing contributes to progression of granulation tissue formation and matrix deposition. Its presence in the matrix results in increased collagen production and elevated levels of TGFβ are associated with fibrosis (Barrientos et al., 2008). Platelets and macrophages are the major sources of TGFβ during the inflammatory phase (Clark, 1996). Fibroblasts are the key TGFβ target cells during the proliferation phase of wound healing. Cellular binding of PDGF and TGFβ promotes myofibroblast differentiation, however, PDGF binding does not stimulate α-SMA expression (Tomasek et al., 2002). Cellular binding to TGFβ significantly increases α-SMA expression and is required for differentiation of fibroblasts into myofibroblasts, the major cell type that produces collagen and remodels the matrix (Desmouliere et al., 1993; Tomasek et al., 2002; Desmouliere et al., 2005). Increased signaling in response to TGFβ results in decreased expression of proteases such as MMPs, and increased expression of tissue inhibitors of MMPs (TIMPs) in fibroblasts (Edwards et al., 1987), thereby further contributing to matrix accumulation. TGFβ signaling has been extensively reviewed elsewhere (Leask and Abraham, 2004; Varga and Abraham, 2007). Briefly, there are three isoforms of TGFβ: TGFβ1, TGFβ2 and TGFβ3. The actions of these isoforms are largely overlapping with the possible exception of TGFβ3, which has been shown to have anti-fibrotic influences (Shah et al., 1995). Activated TGFβ binds to heteromers of the TGFβ type-I and type-II receptors. Type-I receptors (ALK5) phosphorylate Smad2 and Smad3 which go on to bind Smad4 and translocate to the nucleus. Once in the nucleus, the Smad complex binds to Smad binding elements in target genes (Figure 1.2) (Massague and Wotton, 2000). TGFβ has also been known to signal through focal adhesion kinase (FAK), extracellular signal-regulated kinases (ERK), c-Jun N terminal kinases (JNK) and p38 (Leask, 2008). Target genes of TGFβ signaling include α-SMA, MMPs, TIMPs, periostin, connective tissue growth

Figure 1.2: Canonical pro-fibrotic TGFβ signaling is initiated by the binding of active TGFβ to TGFβ receptors. Type-I receptors phosphorylate receptor-Smads 2 and 3, which associate with Smad4 and translocate to the nucleus. In the nucleus the Smad complex interacts with Smad binding elements, promoting gene expression. Among the many genes influenced by TGFβ signaling are collagens I and III, α-SMA, fibronectin, TIMPs and periostin. Additionally, increased Smad7 expression creates a negative feedback loop, limiting pathway activation. The pro-fibrotic roles of periostin are summarized here. Via interactions with the ECM and BMP-1, periostin facilitates collagen crosslinking through activation of lysyl oxidase. Periostin's influences on cell behaviours include altered proliferation, migration and adhesion. Recent evidence suggests that periostin plays a role in liberation of TGFβ from the latency-associated proteins (LAP) in an MMP-2/9 dependent manner.

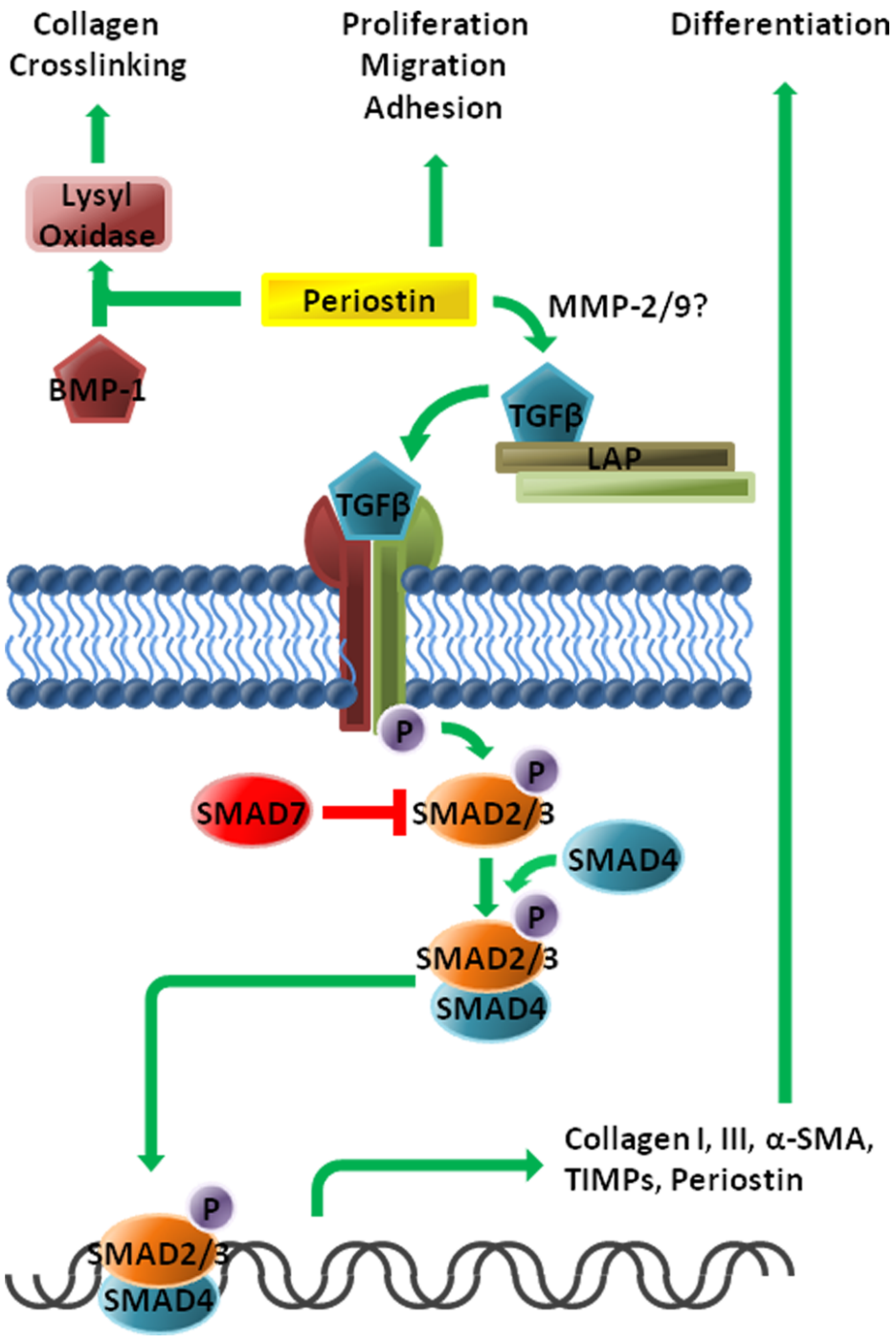

Figure 1.2: Canonical pro-fibrotic TGFβ signaling

factor (CCN2) and collagen type-I (Kocher and Madri, 1989; Igarashi et al., 1993; Takeshita et al., 1993; Holmes et al., 2001; Verrecchia et al., 2001).

TGFβ secretion and activation is central to development of fibrosis, where its cellular binding results in excessive collagen production and prolonged myofibroblast differentiation (Wei et al., 2010), leading to matrix accumulation and contraction. The involvement of TGFβ in fibrosis has received a lot of attention and is the topic of several reviews (Leask and Abraham, 2004; Pannu and Trojanowska, 2004; Varga and Pasche, 2009). Accumulating evidence implicates inappropriately elevated TGFβ signaling in the progression of SSc. Examples include: increased TGFβ receptor expression (Kawakami et al., 1998; Kubo et al., 2002; Pannu et al., 2004), increased Smad3 mRNA, protein levels and phosphorylation levels (Mori et al., 2003). Additionally, Smad3/4 nuclear localization is increased in SSc fibroblasts, both in the absence of TGFβ stimulation and in the presence of TGFβ blocking antibodies (Mori et al., 2003). The targeting of TGFβ directly in fibrosis has been successful in animal models. Injection of TGFβ neutralizing antibodies to the edges of dermal wounds in rats, results in reduced scar formation, where as addition of TGFβ increases scarring (Shah et al., 1992). Topical application of the P144 peptide, which interferes with TGFβ/receptor association, reduces bleomycin induced dermal thickening, Smad2/3 phosphorylation and α-SMA positive myofibroblast numbers (Santiago et al., 2005). Clinical strategies that target TGFβ in fibrosis are numerous (Varga and Pasche, 2009), but have so far had limited success (Denton et al., 2007).

TGFβ's pro-fibrotic role in acute skin wounds and in fibrosis makes it a realistic target for enhancing chronic skin wound healing. Indeed, TGFβ1 expression is reduced in diabetic and venous foot ulcers, compared to uninjured skin (Jude et al., 2002). In a rabbit ischemic ulcer model, topical application of rhTGFβ1 increased wound healing significantly (Beck et al., 1990; Zhao et al., 1994). These studies used young ischemic rabbits for their experiments. The combined effect of age and ischemia was addressed by

Wu *et al*. They found that 60-month-old ischemic rabbits did not response to application of rhTGFβ (Wu et al., 1999). From this observation they concluded that TGFβ signaling must be defective in the aged ischemic model and topical application of TGFβ may not benefit healing of human chronic skin wounds. However, due to the cartilage within the rabbit ear, the model chosen in this series of experiments does not allow for a significant contribution of contraction in healing (Mustoe et al., 1991). Perhaps this feature of the model masks the effects of TGFβ with respect to myofibroblast differentiation and activity. Nevertheless, the limited benefit of TGFβ treatments in animal models has been mirrored by the studies of Robson and colleagues, who investigated the application of TGFβ2 on human chronic venous ulcer healing. The study incorporated an open-label trial in which patients received either 0.5 μg/cm^2 bovine TGFβ2 (bTGFβ2) in a lyophilized collagen matrix or placebo (matrix only). Bovine TGFβ2 treatment resulted in a significant increase in wound healing after a 6-week regime (Robson et al., 1995). In the parallel closed-label trial, however, 2.5 μg/cm^2 bTGFβ2 treatment offered no benefit over the placebo (Table 1.1). The authors suggest that increased variation in the control groups undermined the effect of treatment in the closed-label study (Robson et al., 1995). With a small number of patients (n = 12/group) it is possible that sampling error played a significant role. We must be cautious, however, of how we interpret open-label trial data since the placebo effect has been shown in earlier examples to dominate the effectiveness of treatments. At a minimum, this study stresses the requirement for large-scale randomized, blinded, multi-center, placebo-controlled clinical trials.

Recent evidence suggests that the very limited efficacy of TGFβ in chronic skin wound treatment may be due to dysregulation of the TGFβ signaling cascades within the wounds and in wound fibroblasts (Hasan et al., 1997; Cowin et al., 2001; Jude et al., 2002; Pastar et al., 2010). Together these studies establish a trend towards reduced TGFβ receptor expression, although the data is somewhat conflicting when looking at the expression of specific receptors. Convincingly, TGFβ responsive genes are significantly reduced in venous ulcers (Pastar et al., 2010). A growing body of evidence now clearly identifies additional, or accessory, signaling pathways activated by TGFβ and necessary for appropriate context-specific TGFβ signaling. As will be discussed in the coming sections,

these non-canonical TGFβ signaling pathways are of particular importance to wound healing and fibrosis. Moreover, context-specific modulation of these pathways is increasingly becoming a role of matricellular proteins.

1.3 Old players, but new rules in chronic skin wound healing

1.3.1 Non-canonical TGFβ signaling

In addition to Smad signaling, TGFβ is known to influence cellular functions through Smad independent, non-canonical pathways, which have been implicated in myofibroblast differentiation, matrix contraction and fibrosis. Serini and colleagues showed that adhesion to the ED-A splice variant of fibronectin (ED-A FN) is required for myofibroblast differentiation of human subcutaneous fibroblasts (Serini et al., 1998). Although binding to ED-A FN is not sufficient to cause differentiation, blocking specific ED-A FN binding disrupts TGFβ-induced α-SMA expression (Serini et al., 1998); demonstrating a role for adhesive signaling in the cell's response to TGFβ. Stimulation of adherent human lung fibroblasts with TGFβ results in increased FAK phosphorylation and α-SMA expression (Thannickal et al., 2003). TGFβ-induced α-SMA and collagen type-1 expression has been shown to require FAK and JNK activation in mouse embryonic fibroblasts (Liu et al., 2007b). When human fibroblasts are maintained in suspension or treated with the FAK/Src inhibitor, PP2, FAK phosphorylation is lost and TGFβ-induced α-SMA expression is abolished (Thannickal et al., 2003). Interestingly, phosphorylation of Smad2 following treatment with TGFβ is maintained, even when cells are maintained in suspension (Thannickal et al., 2003); suggesting non-canonical signaling critically regulates myofibroblast differentiation: one of the core features of fibrosis. Fibroblasts isolated from fibrotic lesions in patients with SSc show an increased ability to contract a collagen matrix. This increased contractility can be reduced by treatment with inhibitors of TGFβ type-1 receptor activation, ERK activation and proteoglycan synthesis (Chen et al., 2005). Furthermore, genetic deletion of the heparin sulphate proteoglycan, syndecan 4, results in loss of TGFβ-induced ERK

phosphorylation, α-SMA incorporation into stress fibers, and contractility (Chen et al., 2005).

Non-canonical TGFβ signaling has been suggested to modulate, or fine tune, the response to TGFβ in a gene and context specific manner (Leask and Abraham, 2004). As a result, these accessory pathways have gained the interest of fibrosis researchers since they offer potential targets for the treatment of fibrosis without disrupting the pleiotropic TGFβ/Smad pathway, which may be problematic for basal tissue functioning (Leask, 2008). Modulation of TGFβ's impact on healing via non-canonical signaling is a function that is becoming more and more prominent based on the identification of a group of proteins known as matricellular proteins. Moreover, the combination of TGFβ and matricellular proteins may represent a promising direction for development of new therapeutics for chronic skin wounds.

1.3.2 Matricellular proteins in fibrosis and acute wound healing

Matricellular proteins are non-structural ECM components which bind cell surface receptors to mediate interactions between the cell and the ECM, modulating essential events such as migration, proliferation and adhesion during wound healing (Bornstein, 1995; Midwood et al., 2004; Hamilton, 2008). Although genetic deletion of matricellular proteins typically results in very mild phenotypes (Hamilton, 2008), wound healing can be severely compromised (Midwood et al., 2004; Hamilton, 2008). Matricellular proteins have diverse roles that span all phases of wound healing. For example, efficient re-epithelialization is facilitated by tenascin-C, where keratinocyte migration is decreased in tenascin-C knockout mice (Matsuda et al., 1999). Thrombospondin-1 has been shown to activate latent TGFβ (Schultz-Cherry and Murphy-Ullrich, 1993). Macrophage infiltration is increased in the presence of the matricellular protein osteopontin (Denhardt et al., 2001).

The role of matricellular proteins in myofibroblast behaviour has received considerable attention. Osteopontin serves as a ligand for αvβ3 integrins where binding leads to activation of FAK and numerous downstream pathways (Sodek et al., 2000). Furthermore, osteopontin has been postulated to be required for TGFβ-induced

myofibroblast differentiation, where deletion of osteopontin attenuates TGFβ-dependent increases in α-SMA and ED-A FN expression (Lenga et al., 2008). CCN2 (further discussed below) promotes myofibroblast differentiation in the presence of TGFβ (Leask, 2008). Although CCN2 is considered to be a cofactor in fibrosis and not a fibrotic agent itself (Leask, 2010), it serves as a marker for severity of fibrosis in SSc (Takehara, 2003). CCN2 is required for maximal induction of α-SMA and collagen type-1 by TGFβ, where TGFβ-induced FAK and Akt activation is reduced in CCN2 null fibroblasts (Shi-wen et al., 2006). Furthermore, cellular binding to CCN2 results in activation of ERK through a syndecan-4-dependent mechanism (Kennedy et al., 2007). Syndecan-4, which was previously discussed as being required for many facets of TGFβ-induced myofibroblast behaviour, is considered by some to be a matricellular protein (Woods, 2001). In addition, human dermal fibroblasts are unable to contract a collagen matrix in the presence of neutralizing antibodies against the matricellular protein vitronectin (Sethi et al., 2002). Vitronectin incorporation into the ECM is inhibited by exogenous galectin-1, yet another matricellular protein (Moiseeva et al., 2003). Finally, CCN1 (CYR61) can bind integrins, leading to activation of ERK and p38 to cause myofibroblast senescence, thus serving as a natural brake on fibrotic tissue remodeling (Jun and Lau, 2010). Matricellular proteins can therefore both facilitate and inhibit critical events of acute and fibrotic healing. Despite mounting evidence for the importance of matricellular proteins in wound contraction and re-epithelialization, no clinical trials are currently employing these proteins for the treatment of chronic skin wounds. The various influences of matricellular proteins provide several potential targets for fibrosis and treatment of chronic skin wounds.

1.3.3 CCN2/Connective tissue growth factor

Connective tissue growth factor, otherwise known as CCN2, modulates TGFβ signaling, myofibroblast differentiation and collagen induction. Perhaps best known for its pervasive emergence in a multitude of fibrotic tissues and diseases, CCN2 represents a potential target for treatment of fibrosis (ClinicalTrial.gov #NCT01262001). Based on CCN2's role in fibrosis and evidence from several models of tissue repair, there is considerable support for CCN2 as a therapeutic for resolution of chronic skin wounds.

CCN2 was originally identified as a 38-kDa cysteine-rich mitogen and chemoattractant secreted from endothelial cells (Bradham et al., 1991). Structurally, CCN2 consists of a typical signal sequence and four conserved domains with sequence homology to 1) insulin-like growth factor-binding proteins, 2) the von Willebrand factor C domain, 3) thrombospondin type-1 repeat, and 4) a carboxy-terminal cysteine-knot (Figure 1.3) (Bork, 1993). The four domains each participate in complex interactions with other matrix elements and with cell surface molecules. These interactions, summarized in numerous review articles (Chen and Lau, 2009; Oliver et al., 2010; Arnott et al., 2011; Jun and Lau, 2011; Tran et al., 2013), are the basis of the influence CCN2 has on cellular functions such as adhesion, migration, proliferation, survival, apoptosis and angiogenesis. Of particular importance is CCN2's role in fibrosis.

CCN2 has been implicated in several fibrotic conditions affecting tissues including skin, lungs, kidneys, heart, liver and retina (Table 1.2). CCN2 has emerged as a reliable clinical marker for the degree of fibrosis (Dendooven et al., 2011). CCN2 is significantly induced by the presence of TGFβ and the presence of CCN2 seems to be required for the fibrotic effects of TGFβ. In rat kidney fibroblasts, CCN2 is required for TGFβ-induced collagen expression (Duncan et al., 1999). TGFβ-induced anchorage-independent growth is attenuated in the presence of an anti-CCN2 antibody or following expression of an antisense CCN2 gene, whereas CCN2 alone cannot induce anchorage-independent growth (Kothapalli et al., 1997). Similarly, CCN2 is required for TGFβ-induced myofibroblast differentiation and subsequent gel contraction (Garrett et al., 2004). Yet CCN2 alone is not sufficient to induce myofibroblast differentiation and gel contraction. CCN2 has thus been described as a cofactor in fibrosis, in that it is indispensible for fibrotic responses yet it alone cannot induce fibrosis. Additional support for the cofactor account of CCN2 comes from mouse models where injection of TGFβ into the subcutaneous tissue produced only transient skin fibrosis, whereas injections of TGFβ and CCN2 resulted in sustained skin fibrosis (Mori et al., 1999). Podocyte (Yokoi et al., 2008) and hepatocyte (Tong et al., 2009) specific over-expression of CCN2 in mice failed to induce spontaneous fibrosis and only after further insult do these mice exhibit enhanced fibrosis. There is, however, a growing body of evidence challenging the

Figure 1.3: Domain structures of human periostin and CCN2. (A) Human full length periostin (isoform 1) consists of a typical signal peptide (SP) sequence, an EMI domain responsible for binding to fibronectin, four tandem fasciclin-like domains that are responsible for integrin binding and a C-terminal region (CTR) where multiple splice variants originate. Tryptophan 65 within the EMI domain is required for fibronectin binding. The CTR has been shown to inhibit binding of periostin to several binding partners. **(B)** Human CCN2, shown on same scale as periostin first and then expanded for detail below, consists of a signal peptide (SP) sequence followed by four conserved domains with sequence homology to 1) insulin-like growth factor-binding proteins (IGFBP), 2) the von Willebrand factor C domain (VWC), 3) thrombospondin type-1 repeat (TSP_1) and 4) a carboxy-terminal cysteine-knot. Each domain comes with a compliment of binding sites for both cell surface receptors and matrix components. The diversity of these interactions may explain CCN2's diverse influences. The hinge region is vulnerable to proteolytic cleavage. Numbers represent amino acid residues flanking each domain.

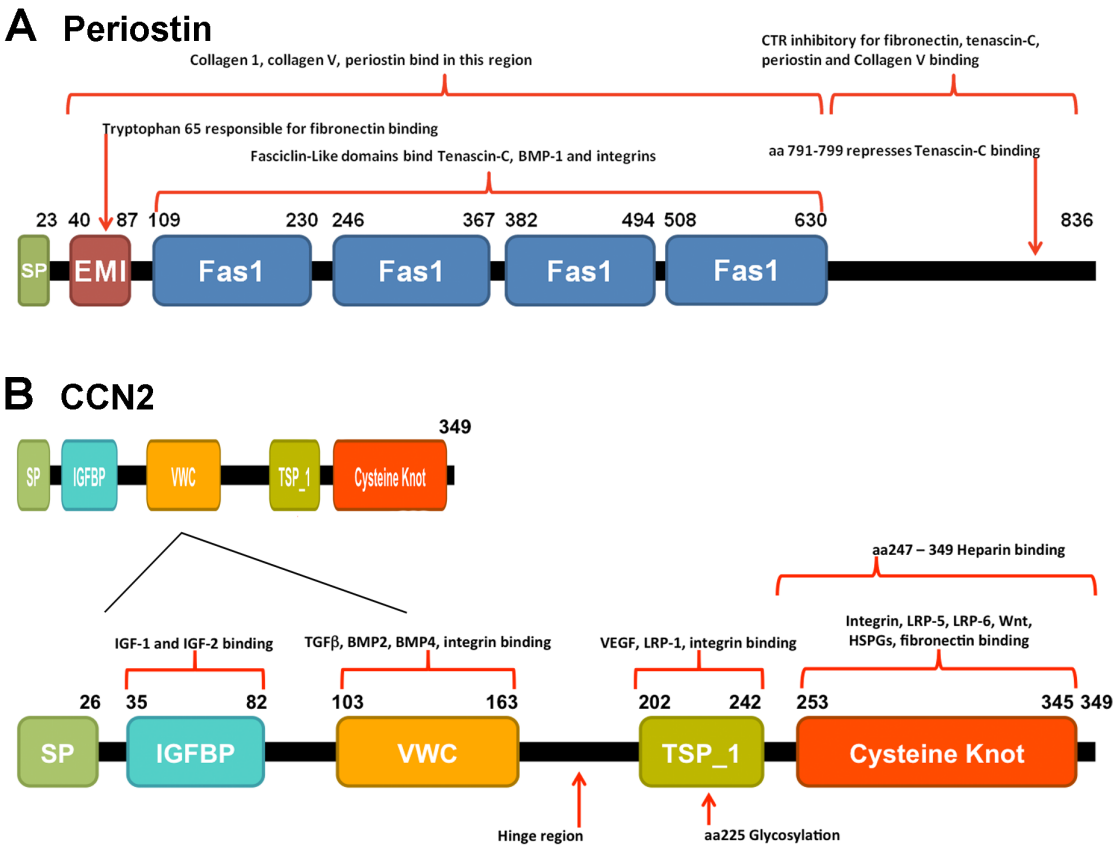

Figure 1.3: Domain structures of human periostin and CCN2

Table 1.2: Fibrotic conditions associated with periostin and CCN2

Periostin		CCN2, Connective Tissue Growth Factor	
Condition	**References**	**Condition**	**References**
Keloid scar	(Supp et al., 2012)	Keloid scar	(Smith et al., 2008)
	(Zhou et al., 2010)		(Khoo et al., 2006)
	(Wang et al., 2007)		(Igarashi et al., 1996)
	(Naitoh et al., 2005)	Hypertrophic scar	(Colwell et al., 2005)
Hypertrophic scar	(Zhou et al., 2010)	Systemic sclerosis	(Dziadzio et al., 2005)
	(Wang et al., 2007)		(Sato et al., 2000)
	(Naitoh et al., 2005)		(Shi-wen et al., 2000)
Systemic sclerosis	(Yamaguchi et al., 2013)		(Igarashi et al., 1996)
	(Yang et al., 2012)		(Igarashi et al., 1995)
Dupuytren's disease	(Shih et al., 2009)	Dupuytren's disease	(Igarashi et al., 1996)
	(Vi et al., 2009)	Pulmonary fibrosis	(Pan et al., 2001)
Pulmonary fibrosis	(Naik et al., 2012)		(Sato et al., 2000)
	(Uchida et al., 2012)	Kidney fibrosis	(Ito et al., 2010)
Sub-epithelial fibrosis	(Takayama et al., 2006)		(Tam et al., 2009)
Kidney fibrosis	(Guerrot et al., 2012)		(Cheng et al., 2006)
	(Sen et al., 2011)		(Nguyen et al., 2006)
Bone marrow fibrosis	(Oku et al., 2008)		(Andersen et al., 2005)
Fibrous dysplasia	(Kashima et al., 2009)		(Roestenberg et al., 2004)
Peritoneal fibrosis	(Braun et al., 2013)		(Gilbert et al., 2003)
Muscular dystrophy	(Lorts et al., 2012)	Cardiac fibrosis	(Koitabashi et al., 2008)
Cardiac fibrosis	(Stansfield et al., 2009)	Retinopathy	(Kuiper et al., 2008)
	(Litvin et al., 2006)		(Hinton et al., 2002)
Retinopathy	(Yoshida et al., 2011)	Liver fibrosis	(Morikawa et al., 2007)
	(Takada et al., 2010)		(Gressner et al., 2006)
			(Tamatani et al., 1998)
		Pancreatic fibrosis	(di Mola et al., 1999)

cofactor explanation of CCN2's influence. Fibroblast-specific over-expression of CCN2 leads to kidney fibrosis (basement membrane thickening and excess matrix accumulation around blood vessels), lung fibrosis and skin fibrosis, including an increased population of myofibroblasts in the dermis (Sonnylal et al., 2010). Fibroblast and smooth muscle-specific deletion of CCN2 protected mice from bleomycin induced skin fibrosis, including a reduction in the number of myofibroblasts present in the skin (Liu et al., 2011). However, fibroblasts isolated from these mice responded appropriately to TGFβ in terms of collagen and α-SMA expression. The conclusion was that CCN2 is required for bleomycin induced skin fibrosis but was not required for TGFβ response. Instead, the authors proposed that CCN2 was responsible for myofibroblast recruitment. Therefore, it is more accurate to say that CCN2 influences cellular activities in TGFβ-dependent and TGFβ-independent ways to promote fibrosis.

The role of CCN2 in acute wound healing has been difficult to assess since the *Ccn2*$^{-/-}$ genotype is lethal in mice. In 1993, Igarashi and colleagues demonstrated that CCN2 is up-regulated six and nine days following dermal wounding (Igarashi et al., 1993) and CCN2 up-regulation has since been confirmed in human skin wounds (Rittie et al., 2011). However, antisense inhibition of CCN2 mRNA in rabbit ear wounds had no detrimental effect on closure rate but did reduce hypertrophic scarring, suggesting CCN2 does not play a critical role in skin healing (Sisco et al., 2008). Despite this, there is mounting evidence that CCN2 is an important mediator of vascularization during tissue remodeling. Antibody blockade of CCN2 resulted in reduced granulation tissue formation and vascularity in mesenchymal stem cell-loaded sponges placed in excisional mouse wounds (Alfaro et al., 2013). Growth plates of *Ccn2*$^{-/-}$ mice exhibit defective vascularization, linked to reduced *Vegfa* expression in hypertrophic zones (Ivkovic et al., 2003). The presence of CCN2 has since been shown to up-regulate *Vegfa* at the transcriptional level by increasing HIF-1α activity in a chondrocytic cell line (Nishida et al., 2009). Hall-Glenn and colleagues have shown that CCN2 is required for stable association and retention of perivascular cells (pericytes) by endothelial cells (Hall-Glenn et al., 2012). By inducing PDGF-B expression in endothelial cells, CCN2 indirectly promotes pericyte recruitment and basement membrane formation during angiogenesis. Although the role of

CCN2 in skin healing is not clear, its influence on angiogenesis and matrix accumulation make it an interesting candidate for use in the treatment of chronic skin wounds.

To date, several groups have studied CCN2 as a therapeutic for aiding in tissue repair. Administration of rhCCN2 to a non-human primate burn wound model resulted in an increased wound closure rate, increased fibroblast and collagen content within the wound area and accelerated re-epithelialization (Liu et al., 2007a). CCN2 loaded fibrin glue has been shown to enhance meniscal repair by increasing *Col1a1*, *Col2a1* and *Vegfa* mRNA in the avascular zone of rabbit menisci following tearing defects (He et al., 2011). Furthermore, it was reported that 10 weeks after treatment, the density of capillaries was significantly higher in the defects of the CCN2 group. A patent has been filed on a polypeptide referred to as CTGF-2 (US Patent #20060052328 A1), which shares 49% identity and 67% similarity to mouse CCN2 (Pradhan et al., 2007), based on the observations of increased endothelial cell migration and pro-angiogenic outcomes in rabbit hind limb ischemia experiments. The authors of the patent suggest potential for CTGF-2 in enhancing wound healing. Therefore, there is clear evidence that CCN2 could be useful in the treatment of chronic skin wounds. In fact, recent evidence from a non-human primate model of diabetic wound healing showed that CCN2 is reduced in diabetic healing compared to that in control animals, concomitantly with delayed wound closure and reduced granulation tissue formation (Thomson et al., 2010). It is unknown, however, if the same is true for human chronic skin wounds. It is also unknown whether the reparative effects of recombinant CCN2 can be applied to human chronic skin wounds.

1.3.4 Periostin

The matricellular protein periostin is a molecule that has not yet been investigated for the treatment of fibrosis and chronic skin wounds. The presence of periostin can modulate many key aspects of acute healing and fibrosis. Collagen synthesis, fibril assembly and myofibroblast behaviour have all been increasingly linked to periostin. Furthermore, the expression pattern of periostin following tissue injury in various systems, including heart, bone and vasculature, suggests that it may play a common role in various models of acute wound healing (Hamilton, 2008).

Periostin is a secreted 90-kDa disulfide-linked, TGFβ-inducible protein, originally designated osteoblast specific factor 2 (Takeshita et al., 1993; Horiuchi et al., 1999). Structurally, periostin consists of a typical signal sequence, an EMI domain responsible for binding to fibronectin, four tandem fasciclin-like domains that are responsible for integrin binding (Kim et al., 2002) and a C-terminal region where multiple splice variants originate (Litvin et al., 2004) (Figure 1.3). Additionally, periostin has been shown to bind collagen, tenascin-C, BMP-1 and itself (Kii et al., 2006; Takayama et al., 2006; Norris et al., 2007; Kii et al., 2009; Maruhashi et al., 2010). The ability of periostin to interact with various integrin pairs (Gillan et al., 2002; Bao et al., 2004; Shao et al., 2004; Baril et al., 2007; Butcher et al., 2007) allows it to influence such biological effects as cell proliferation, cell migration, cell adhesion and epithelial to mesenchymal transformation (Horiuchi et al., 1999; Katsuragi et al., 2004; Lindner et al., 2005; Yan and Shao, 2006; Vi et al., 2009; Li et al., 2010).

Nakazawa and colleagues showed an increase in periostin mRNA following induced tibial fractures in mice. Periostin expression was significantly increased by day 3 and peaked at day 7 in preosteoblasts of the periosteum and undifferentiated mesenchymal cells within the soft callus (Nakazawa et al., 2004). Periostin expression was significantly increased following eight days of balloon injury to rat carotid arteries, eventually decreasing by 4 weeks post injury (Lindner et al., 2005). Li and colleagues confirmed that periostin peaks at 7 days post injury in this model (Li et al., 2006). To determine if periostin induction is a feature common in other connective tissue injuries, Lindner and colleagues created full-thickness incisional wounds in the skin of rats. Indeed, periostin expression was detected in the fibroblasts, but not keratinocytes, of the wound site (Lindner et al., 2005). The expression pattern of periostin during dermal wounding has since been further defined (Jackson-Boeters et al., 2009; Zhou et al., 2010). After incisional wounding, periostin is expressed in the dermis and basement membrane within the dermal-epidermal junction (DEJ) (Zhou et al., 2010). In full-thickness excisional wounds, in which the dermis and epidermis are completely removed, periostin expression is found throughout the newly formed granulation tissue (Jackson-Boeters et al., 2009). With expression beginning at day 3, peaking by day 7 and eventually returning to basal levels by 4 weeks (Jackson-Boeters et al., 2009), the temporal expression pattern of

periostin in skin mimics that seen in vascular balloon injury and bone fracture. These observations suggest that a common role for periostin may exist in all connective tissue repairs. Interestingly, periostin expression in skin healing coincides with the expression of α-SMA expression in the granulation tissue (Jackson-Boeters et al., 2009).

The functional role(s) of periostin are not fully understood. Several studies have highlighted a role in collagen deposition and myofibroblast differentiation. In murine models of myocardial infarction and ventricular hypertrophy (thickening of the myocardium), increased periostin expression results in increased collagen deposition (Katsuragi et al., 2004; Oka et al., 2007; Shimazaki et al., 2008; Stansfield et al., 2009). Genetic ablation of periostin results in increased incidence of ventricular rupture following myocardial infarction, which correlates with fewer α-SMA positive cells and decreased collagen fibril formation (Shimazaki et al., 2008). Addition of recombinant periostin to pancreatic stellate cells results in increased expression of key fibrotic proteins such as α-SMA, collagen type-1, fibronectin, TGFβ1 and periostin itself (Erkan et al., 2007). Collagen fibrils from the skin of periostin knockout mice are reduced in diameter and display decreased crosslinking (Norris et al., 2007). As a result, periostin knockout skin has a reduced tensile strength and an alteration of the visco-elastic properties (Norris et al., 2007). Recent insights into the functional role of periostin in collagen crosslinking implicate periostin as a scaffold protein, aiding in the incorporation of BMP-1 into the ECM where it can activate lysyl oxidase (an enzyme involved in collagen fibril crosslinking) (Maruhashi et al., 2010).

With respect to fibrosis, periostin has been strongly correlated with several conditions (Table 1.2). Periostin is the single most up-regulated gene in keloid scars, assessed using cDNA microarray analysis (Naitoh et al., 2005). Wang and colleagues reported increased periostin expression in keloid and hypertrophic scars, relative to normal human skin, where periostin expression was positively correlated with TGFβ1 expression (Wang et al., 2007). Periostin is highly expressed in tissues affected by Dupuytren's disease, a progressive disease that results in a scar-like, collagen-rich cord within the palmar fascia and permanent contracture of the hand (Vi et al., 2009). Fibroblasts isolated from diseased tissue have an increased ability to contract a collagen matrix, which is further

enhanced by addition of recombinant periostin (Vi et al., 2009). Periostin-induced contractility is accompanied by an increase in α-SMA protein. In addition to skin fibrosis, periostin has been associated with bone marrow fibrosis, where it correlates with the severity of fibrosis (Oku et al., 2008). Periostin has also been implicated with sub-epithelial fibrosis of bronchial asthma (Takayama et al., 2006), where recently it has been shown to facilitate TGFβ signaling (Sidhu et al., 2010). Over-expression of periostin in human bronchial epithelial cells results in increased periostin secretion, collagen synthesis and TGFβ expression/protein/activity (Sidhu et al., 2010). Addition of recombinant periostin to primary human bronchial epithelial cells increased collagen expression in a TGFβ-dependent manner. Finally, in several models of fibrosis, disease progression is attenuated in *Postn*$^{-/-}$ mice, compared to wild-type littermates (Naik et al., 2012; Uchida et al., 2012; Yang et al., 2012).

The role of periostin in acute wound healing and in fibrotic diseases has received a lot of attention in the past ten years (Table 1.2). However, research into its role in chronic skin wounds is lacking and very few studies have looked at periostin expression in this context. Of particular importance, expression of periostin following injury coincides with fibrotic, not inflammatory, stages of healing (Jackson-Boeters et al., 2009). In fact, chronically inflamed skin contains very little periostin, below that of normal skin (Zhou et al., 2010).

Future work must make use of the influences of matricellular proteins, like periostin and CCN2, on acute wound healing and apply them to the treatment of chronic skin wounds. Matricellular proteins are expressed during development, but are typically absent in the adult, except during tissue remodeling or repair (Bornstein, 1995). Their tight regulation during wound healing and absence in adult tissues makes matricellular proteins an ideal localized target for therapies (Midwood et al., 2004).

1.4 Future therapeutic design

Creating a favorable wound environment, via debridement, will certainly form the foundation of any future treatment strategies. To date, healing of chronic skin wounds via administration of growth factors has produced limited benefits. Future treatment

strategies must integrate the wealth of knowledge available from studies of acute and fibrotic healing, particularly the importance of cross talk between cells, structural matrix components, growth factors and matricellular proteins. The importance of the matrix on cell behaviour should not be underestimated in choosing alternative target molecules.

Historically, the introduction of physical matrices to chronic skin wounds has primarily focused on maintaining a moist environment to encourage healing (Queen et al., 2004). Examples of these modern dressings include hydrocolloid dressings, alginates and foam dressings (Qin and Gilding, 1996; Chaby et al., 2007). Despite weak evidence of clinical efficacy, these products have obtained widespread use (Chaby et al., 2007). One limitation of these dressings is their lack of biological activity, although hybrids containing biologically active components are now available (Donaghue et al., 1998; Murakami et al., 2010). Introduction of exogenous ECM components, such as collagens, fibronectin and fibrin, provide a scaffold on which cells can migrate into the wound area (Greiling and Clark, 1997), while also greatly influencing the behaviour of cells through binding of surface receptors and activation of signaling pathways (Schultz and Wysocki, 2009). One currently available ECM based product is de-cellularized porcine small intestine submucosa (SIS), which is marketed under the name OASIS® Wound Matrix (Mostow et al., 2005). In a randomized clinical trial, OASIS® Wound Matrix was shown to be at least as effective as Regranex® (rhPDGF-BB) in healing diabetic foot ulcers (Niezgoda et al., 2005). Interestingly, growth factors embedded within SIS can influence cell behaviours such as proliferation and cell morphology (Voytik-Harbin et al., 1997). It is proposed here that an ECM based bioactive scaffold can also provide a vehicle for delivery of matricellular proteins, where the choice of matricellular proteins can be tailored to the etiology of the target wound. Realization of such a treatment, however, depends on a greater understanding of the role of matricellular proteins in the pathogenesis of chronic skin wounds.

1.5 Rationale, Hypothesis and Objectives

Development of effective treatments to close chronic skin wounds has been hampered by a lack of thorough understanding of the dysfunctions present in these wounds. Matricellular proteins have received little attention despite their ability to modulate all aspects of wound healing. The overall hypothesis of this thesis is that the matricellular proteins periostin and CCN2, two matricellular proteins associated with fibrotic healing, can enhance the healing of chronic skin wounds.

The functional role of periostin is not fully understood, however, several lines of evidence have established a positive correlation between periostin expression and the extent of tissue repair. On the side of fibrosis, periostin has been implicated in bone marrow fibrosis (Oku et al., 2008), sub-epithelial fibrosis of bronchial asthma (Takayama et al., 2006), idiopathic pulmonary fibrosis (Naik et al., 2012; Uchida et al., 2012), systemic sclerosis (Yang et al., 2012; Yamaguchi et al., 2013), renal fibrosis (Sen et al., 2011) and fibrous dysplasia (Kashima et al., 2009). Moreover, *POSTN* is the single most up-regulated gene in keloid scars (Naitoh et al., 2005). In the context of acute healing, genetic ablation of periostin results in increased incidence of ventricular rupture following myocardial infarction due to reduced α-SMA positive cells and impaired collagen fibril formation (Shimazaki et al., 2008). Interestingly, periostin expression in dermal healing coincides with the expression of α-SMA expression within the granulation tissue (Jackson-Boeters et al., 2009). The causal relationship between periostin expression and a myofibroblast phenotype in skin healing remains to be established.

Therefore it is hypothesized that periostin is essential for normal dermal wound healing, contributing to myofibroblast differentiation, matrix production and matrix compaction. Furthermore, based on periostin's theorized role in wound healing, we hypothesize that delivery of rhPN to a model of impaired diabetic wound healing will enhance closure.

CCN2 represents an interesting adjunct or alternative to periostin in treating chronic skin wounds. CCN2 has been demonstrated to positively influence matrix production,

myofibroblast differentiation and angiogenesis, positioning it as an excellent candidate for enhancing wound healing. At least one model of diabetic wound healing has shown that CCN2 is inappropriately reduced in this pathological state, and previous attempts to deliver recombinant CCN2 to a variety of wound models have produced favorable results. It is therefore hypothesized that delivery of rhCCN2 to a model of impaired diabetic wound healing will enhance healing of said wounds.

The specific objectives of this project were:

1. To determine how genetic deletion of periostin alters dermal wound healing kinetics and the underlying changes in regulation of dermal and epithelial behaviours.

2. To determine mRNA and protein expression patterns of periostin and CCN2 in human chronic skin wound tissue.

3. To quantify the phenotypic response of human chronic skin wound fibroblasts by exogenous TGFβ and TNFα.

4. To assess the efficacy of local delivery of periostin and CCN2 containing electrospun scaffolds as a therapeutic for enhancing wound healing, using a diabetic murine model.

A version of this chapter has been published (Appendix A):

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Chapter 2

2 Periostin modulates myofibroblast differentiation during full-thickness cutaneous wound healing

Abstract

The matricellular protein periostin is expressed in the skin. Although periostin has been hypothesized to contribute to dermal homeostasis and healing, this hypothesis has not been directly tested. To assess the contribution of periostin to dermal healing, 6 mm full-thickness excisional wounds were created in the skin of periostin knockout and wild-type/sex-matched control mice. In wild-type mice, periostin was potently induced 5-7 days post-wounding. In the absence of periostin, day 7 wounds showed a significant reduction in myofibroblasts, as visualized by α-smooth muscle actin (α-SMA) expression within the granulation tissue. Delivery of recombinant human periostin via electrospun collagen scaffolds restored α-SMA expression. Isolated wild-type and knockout dermal fibroblasts did not differ in *in vitro* assays of adhesion or migration; however, in 3D culture, periostin knockout fibroblasts showed significantly reduced ability to contract a collagen matrix, and adopted a dendritic phenotype. Recombinant periostin, in a fashion which was sensitive to a neutralizing anti-β1-integrin and to the FAK/Src inhibitor PP2, restored the defects in cell morphology and matrix contraction displayed by periostin-deficient fibroblasts. We propose that periostin promotes wound contraction by facilitating myofibroblast differentiation and contraction.

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2.1 Introduction

Periostin is a secreted 90 kDa, disulfide-linked protein, which has structural similarity with the insect adhesion protein, fasciclin-1 (Takeshita et al., 1993). During development, periostin is expressed by cardiac fibroblasts in the embryonic heart (Kruzynska-Frejtag et al., 2001), where it facilitates organization of the extracellular matrix (ECM) (Snider et al., 2009) and differentiation of mesenchymal cushion progenitor cells into contractile myofibroblasts (Conway and Molkentin, 2008). Recent research has now associated increases in periostin expression with various disease states, including myocardial infarction (Iekushi et al., 2007; Oka et al., 2007; Shimazaki et al., 2008), and cancer (Gillan et al., 2002; Baril et al., 2007; Erkan et al., 2007). In particular, periostin is heavily implicated in fibrosis including in bone marrow (Oku et al., 2008), sub-epithelial fibrosis of bronchial asthma (Takayama et al., 2006), fibrous dysplasia (Kashima et al., 2009), and keloid and hypertrophic scarring of the skin (Naitoh et al., 2005; Wang et al., 2007a). Additionally, induction of periostin expression has been described following acute injury to numerous tissues (Goetsch et al., 2003; Nakazawa et al., 2004; Lindner et al., 2005; Li et al., 2006), including skin (Lindner 2005, Jackson-Boeters 2009, Zhou 2010), where expression peaks at 7-8 days and returns to basal levels by 4 weeks. Our previous reports on skin healing show that periostin expression is absent during inflammation, but instead corresponds with the proliferative and remodeling phases of healing (Jackson-Boeters et al., 2009; Zhou et al., 2010), suggesting a role for periostin during these later phases.

Fibroblasts are central to the proliferative and remodeling phases of skin (Ross, 1968). Dysregulation of fibroblast function in skin can result in inadequate healing (Shimazaki et al., 2008; Blumbach et al., 2010) or excessive matrix production (fibrosis) (Babu et al., 1992; Leask, 2010). Activated fibroblasts, or myofibroblasts, are fibroblasts which have differentiated into a contractile phenotype, characterized by the expression of α-SMA (Gabbiani et al., 1972). During wound healing they serve to expedite wound closure by drawing the edges of the wound together through generation of contractile forces (Gabbiani et al., 1971; Majno et al., 1971; Gabbiani et al., 1972), which are transmitted to the ECM through integrin-containing adhesion complexes known as focal adhesions

(Burridge and Chrzanowska-Wodnicka, 1996). Numerous studies have implicated periostin with α-SMA expression (Shimazaki et al., 2008; Jackson-Boeters et al., 2009; Vi et al., 2009), including in skin healing.

Induction of the myofibroblast phenotype depends on a combination of three major factors (Tomasek et al., 2002): engagement of integrin cell surface receptors with the ED-A splice variant of fibronectin (Serini et al., 1998), stimulation with transforming growth factor β (TGFβ) (Desmouliere et al., 1993) and mechanical tension (Hinz et al., 2001; Cevallos et al., 2006). However, recent work suggests that fine-tuning of myofibroblast differentiation involves a complex interaction of many other factors (Shi-wen et al., 2004; Blumbach et al., 2010; Liu et al., 2010), including matricellular proteins such as connective tissue growth factor (Liu et al., 2011), osteopontin (Lenga et al., 2008) and tenascin-C (Tamaoki et al., 2005). Matricellular proteins are a functionally related group of secreted proteins that have important roles in all phases of skin healing (Bornstein, 1995; Midwood et al., 2004). Often these proteins influence cell behaviour through adhesive signaling, typically through integrin binding and focal adhesion kinase (FAK) activation (Sodek et al., 2000; Shi-wen et al., 2006; Jun and Lau, 2010). Periostin is a matricellular protein which is known to modulate adhesive signaling through various integrins and FAK (Gillan et al., 2002; Bao et al., 2004; Shao et al., 2004; Baril et al., 2007; Butcher et al., 2007). Furthermore, periostin has been correlated with α-SMA expression in skin healing (Jackson-Boeters et al., 2009). However, the use of genetic deletion to determine the specific role of periostin in skin healing has not previously been attempted.

We therefore hypothesized that periostin facilitates myofibroblast differentiation during dermal wound healing and that the loss of periostin wound impede wound resolution. By use of a periostin knockout (*Postn*$^{-/-}$) mouse (Rios et al., 2005), we show that the loss of periostin results in altered wound closure kinetics, corresponding to the time of peak periostin expression. Histological analysis of wound tissue reveals that the granulation tissue of *Postn*$^{-/-}$ wounds is deficient in α-SMA. Furthermore, dermal fibroblasts isolated from *Postn*$^{-/-}$ mice are unable to contract a collagen matrix. This deficiency is corrected

by addition of exogenous periostin, via a β1-integrin and Src/FAK dependent mechanism.

2.2 Results

2.2.1 Loss of periostin results in altered wound closure kinetics

Periostin is up-regulated following acute injury to skin in wild-type mice (Lindner et al., 2005; Jackson-Boeters et al., 2009; Zhou et al., 2010). To investigate the contribution of periostin to the dermal wound healing process, full-thickness excisional wounds were created in *Postn*$^{-/-}$ mice and their sex- and age-matched *Postn*$^{+/+}$ littermates. At 5 and 7 days post wounding, *Postn*$^{+/+}$ wound size had reduced to 30% and 17%, respectively, of their initial wound area (Figure 2.1). In *Postn*$^{-/-}$ mice, however, wounds were significantly larger than those in *Postn*$^{+/+}$ mice (64% at day 5 and 41% at day 7, $p < 0.001$) (Figure 2.1). Histological analysis of sections from the centre of day 7 wounds confirmed that *Postn*$^{-/-}$ wounds were significant larger than those of *Postn*$^{+/+}$ littermates ($p < 0.05$) (Table 2.1). Epithelial migration distance was not significantly different in day 7 wounds ($p = 0.25$) (Table 2.1). Wounds in both wild-type and knockout animals had closed by day 11.

We have previously reported that periostin protein is evident in granulation tissue of excisional wounds by day 3, peaking at day 7 (Jackson-Boeters et al., 2009), which we have confirmed in this study in *Postn*$^{+/+}$ mice (Figure 2.2A,B). Analysis of *in vivo Postn* gene expression by *in situ* hybridization and real-time quantitative polymerase chain reaction (RT-qPCR) confirmed that *Postn* mRNA is significantly and selectively increased during cutaneous wound healing at day 7 ($p < 0.01$) (Figure 2.2A,C). Inflammatory cell infiltration was similar between day 5 *Postn*$^{+/+}$ and *Postn*$^{-/-}$ wounds (Figure 2.3), suggesting that the influence of periostin is not here.

Figure 2.1: Loss of periostin results in altered wound closure kinetics. Full-thickness excisional punch wounds were created in the skin of *Postn*$^{+/+}$ and *Postn*$^{-/-}$ mice using a 6 mm biopsy tool. **(A)** Wounds were photographed at 0, 3, 5, 7 and 11 days post-wounding. Four pairs of mice (four wounds per mouse) were followed for the 11-day time course. **(B)** Quantification of wound area was from photographs. *Postn*$^{-/-}$ wounds are delayed in closure at days 5 and 7 ($p < 0.01$). Data is expressed as a fraction of the initial wound area; error bars represent s.d. (* = $p < 0.01$; two-way ANOVA).

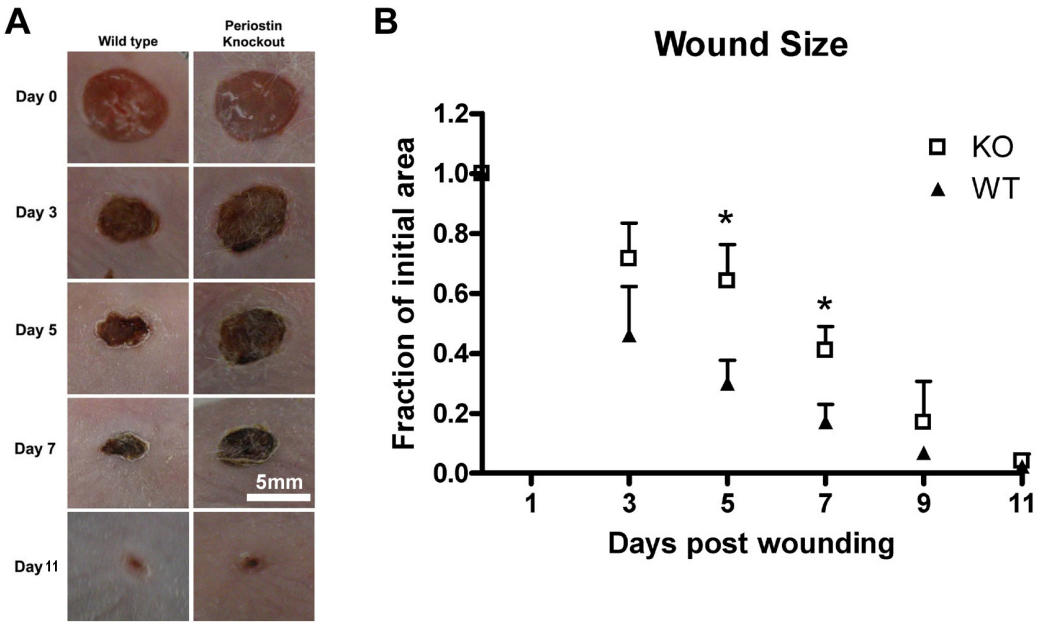

Figure 2.1: Loss of periostin results in altered wound closure kinetics

Table 2.1: Re-epithelialization of day 7 wounds in *Postn*$^{+/+}$ and *Postn*$^{-/-}$ mice

Measure	Genotype		p value
	Postn$^{+/+}$	*Postn*$^{-/-}$	
Wound size (mm)	2.036 ± 0.306	2.579 ± 0.319	0.041
Epithelial migration distance (mm)	0.723 ± 0.148	0.806 ± 0.134	0.254
Percent epithelialized (%)	82.2 ± 18.9	66.8 ± 12.6	0.358

Figure 2.2: α-SMA expression is reduced in the granulation tissue of *Postn*$^{-/-}$ mice. **(A)** *Postn* message detection in day 7 *Postn*$^{+/+}$ wounds via *in situ* hybridization showing periostin expression selectively in the wound. Arrowheads indicate the wound borders. **(B)** Histological analysis of day 7 wounds from *Postn*$^{+/+}$ and *Postn*$^{-/-}$ animals. Sections were incubated with antibodies for periostin or α-SMA. Detection was with peroxidase conjugated 2° antibodies and DAB. Day 7 *Postn*$^{-/-}$ wounds have reduced α-SMA staining (n = 5). **(C)** Healthy skin or wound tissue biopsies were analyzed for *Postn* and **(D)** *Acta2* mRNA via RT-qPCR. Target gene expression was normalized to *18S* using the ΔΔCt method. *Postn* expression is significantly increased at day 7 ($p < 0.001$, n = 5). *Postn* mRNA was not detected in *Postn*$^{-/-}$ specimens. Increased *Acta2* expression was observed at day 7 in *Postn*$^{+/+}$ wounds, but not in *Postn*$^{-/-}$ wounds ($p < 0.001$, n = 5). Data is expressed relative to day 0 *Postn*$^{+/+}$ expression; error bars represent s.e.m. (* = $p < 0.001$; two-way ANOVA).

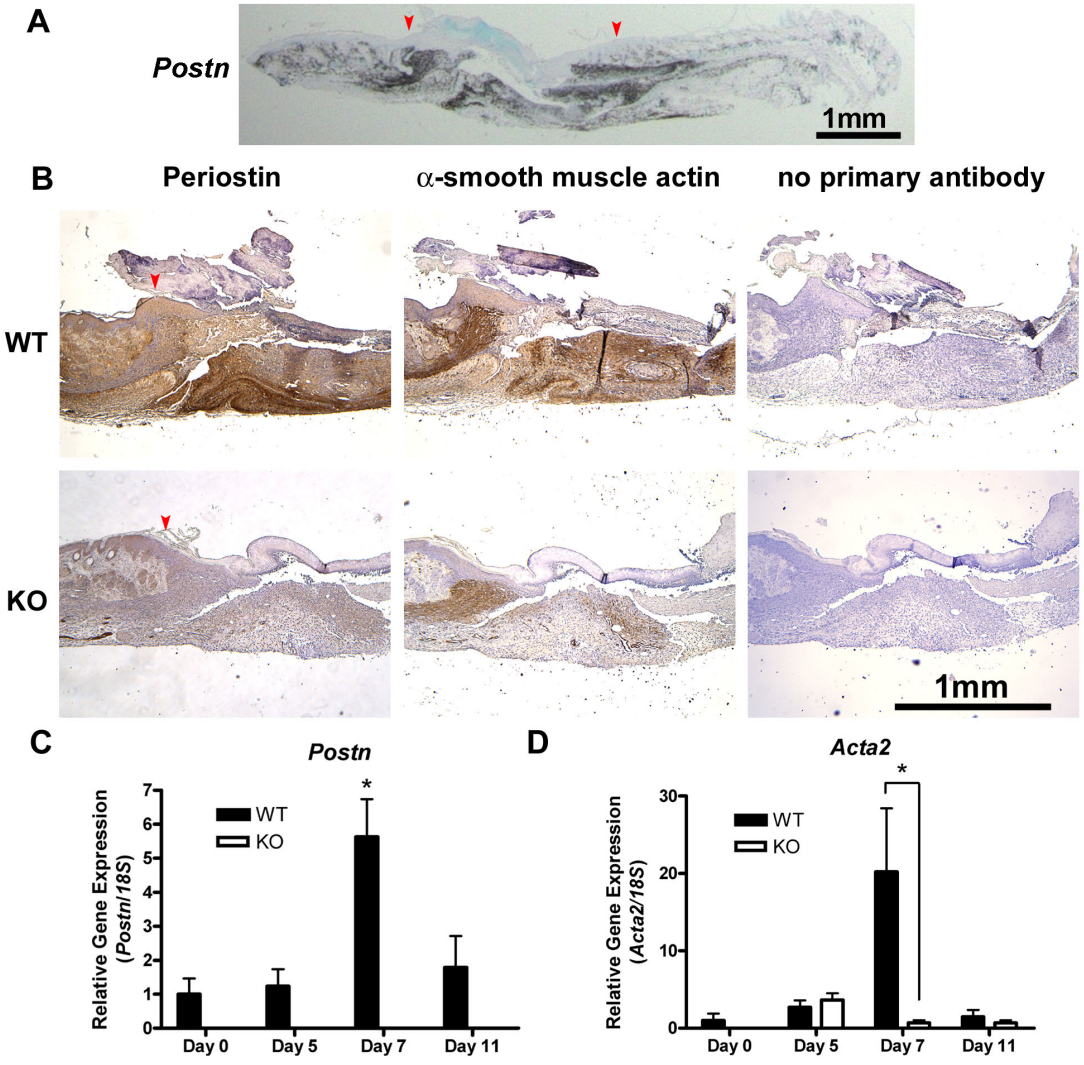

Figure 2.2: α-SMA expression is reduced in the granulation tissue of *Postn*$^{-/-}$ mice

Figure 2.3: Inflammatory cell infiltration is not altered in *Postn*$^{-/-}$ wounds. Histological analysis of day 5 wounds from *Postn*$^{+/+}$ and *Postn*$^{-/-}$ animals. Sections were incubated with antibodies for CD68 (macrophage marker) or neutrophil elastase (neutrophil marker). Detection was with peroxidase conjugated 2° antibodies and DAB. Day 5 *Postn*$^{+/+}$ and *Postn*$^{-/-}$ wounds do not differ with respect to inflammatory cell infiltration (n = 5).

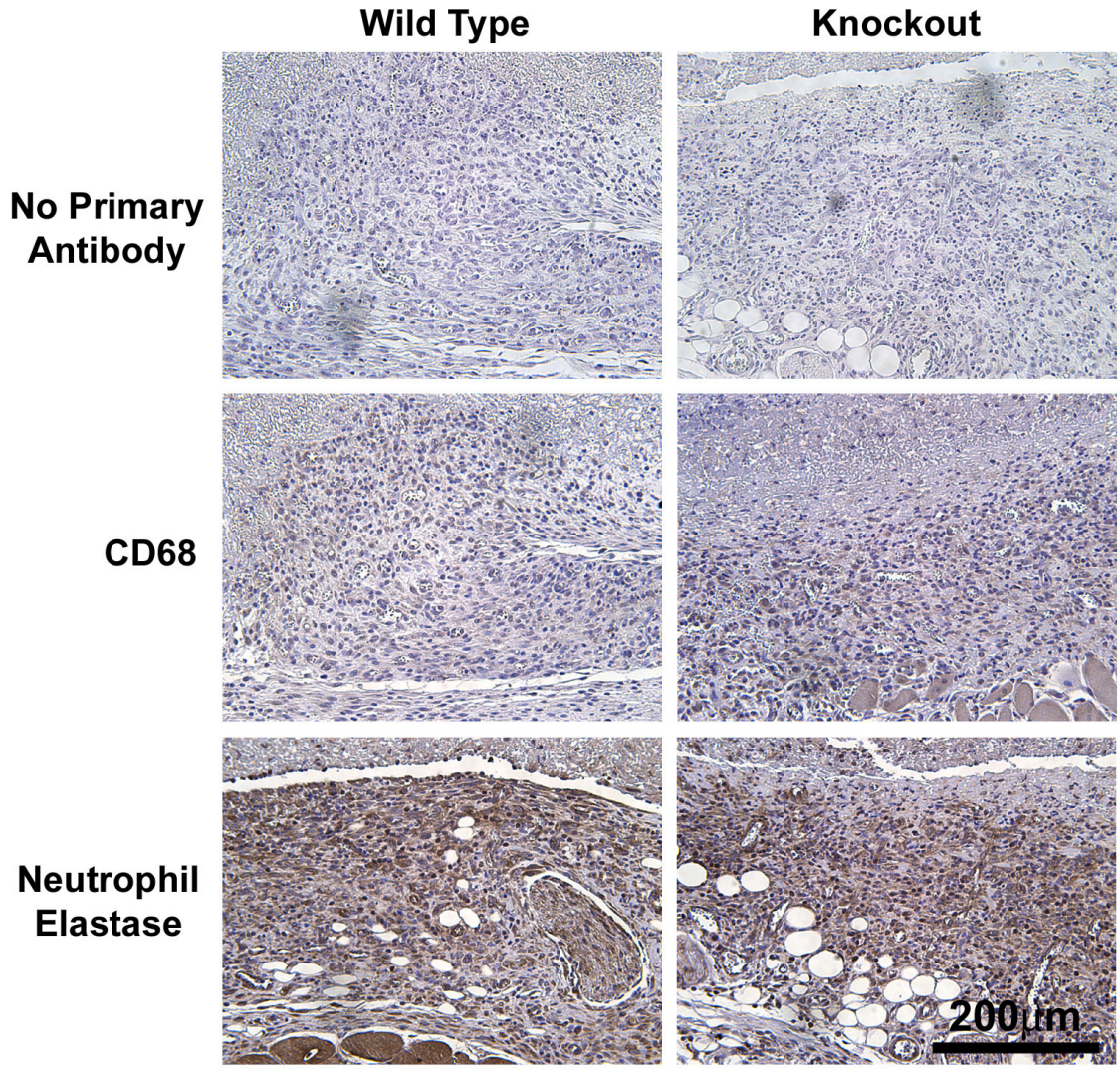

Figure 2.3: Inflammatory cell infiltration is not altered in *Postn*$^{-/-}$ wounds

2.2.2 α-Smooth muscle actin expression is reduced in the granulation tissue of *Postn*$^{-/-}$ mice

Our previous work has shown that increased levels of periostin protein in the granulation tissue of excisional punch wounds is paralleled by an increase in α-SMA protein (Jackson-Boeters et al., 2009). It is not clear, however, whether periostin is required for α-SMA expression. Therefore, we assessed the level of α-SMA in *Postn*$^{+/+}$ and *Postn*$^{-/-}$ wounds via immunohistochemistry (IHC). Immunoreactivity for α-SMA was evident at the wound edge and within the granulation tissue of *Postn*$^{+/+}$ wounds at day 5 (Figure 2.4). At day 7, increased levels of α-SMA were detected at the wound border, throughout the granulation tissue and in blood vessel walls (Figure 2.2B). In day 7 *Postn*$^{-/-}$ wounds, α-SMA immunoreactivity was significantly reduced when compared to sex-matched littermate controls (Figure 2.2B). Reduced α-SMA (*Acta2*) expression in *Postn*$^{-/-}$ wounds was confirmed by RT-qPCR, where excised day 7 *Postn*$^{-/-}$ wound tissue contained significantly less *Acta2* mRNA than day 7 *Postn*$^{+/+}$ wounds (Figure 2.2D) ($p < 0.01$).

This deficit in α-SMA immunoreactivity was specific to the granulation tissue of *Postn*$^{-/-}$ wounds (Figure 2.5A), with wound borders and vasculature positive for α-SMA in both *Postn*$^{+/+}$ and *Postn*$^{-/-}$ wounds. To determine if the reduction in α-SMA immunoreactivity was due to impaired fibroblast recruitment into the granulation tissue, sections were labeled for fibroblast-specific protein-1 (Figure 2.5B). Indeed, fibroblasts dominated the granulation tissue of both *Postn*$^{+/+}$ and *Postn*$^{-/-}$ day 7 wounds (Figure 2.5B). Additionally, tissue sections were stained for nuclei with 4',6-diamidino-2-phenylindole (DAPI) (Figure 2.5C). No significant differences in cell number were found between *Postn*$^{+/+}$ and *Postn*$^{-/-}$ wounds at the wound borders or within the granulation tissue (Figure 2.5C) ($p = 0.28$). To further rule out a defect in fibroblast recruitment we assessed fibroblast migration *in vitro* using a scratch wound assay. Scratch wounds in monolayers of *Postn*$^{+/+}$ and *Postn*$^{-/-}$ were resolved by 20 hours. No difference in migratory ability was observed between cell types (Figure 2.6) ($p = 0.34$).

Figure 2.4: *Postn*$^{-/-}$ wounds contain reduced α-SMA protein. Histological analysis of wounds from *Postn*$^{+/+}$ and *Postn*$^{-/-}$ animals. Sections were incubated with a primary antibody α-SMA. Detection was with peroxidase conjugated 2° antibodies and DAB. *Postn*$^{-/-}$ wounds display reduced α-SMA immunoreactivity at all time-points, relative to *Postn*$^{+/+}$ littermates (n = 4).

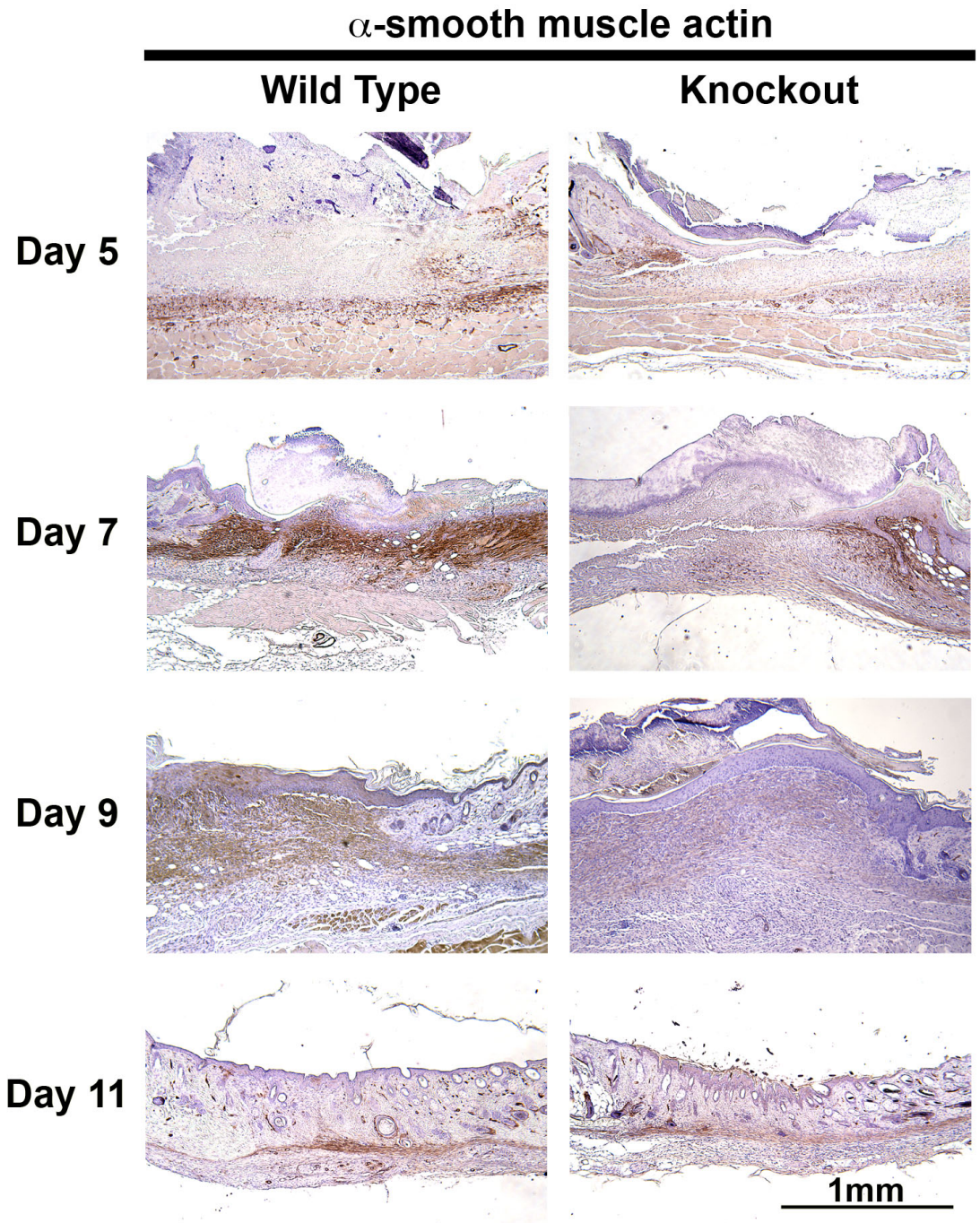

Figure 2.4: *Postn*$^{-/-}$ wounds contain reduced α-SMA protein

Figure 2.5: Decreased α-SMA expression is restricted to the granulation tissue of *Postn*$^{-/-}$ mice. (A) Histological analysis of day 7 wounds from *Postn*$^{+/+}$ and *Postn*$^{-/-}$ animals. Sections were incubated with an antibody for α-SMA **(B)** or fibroblast-specific protein-1. Detection was with peroxidase conjugated 2° antibodies and DAB. α-SMA is absent in the granulation tissue of *Postn*$^{-/-}$ wounds, but is present at the wound border (n = 4). Granulation tissue is dominated by fibroblasts in both *Postn*$^{+/+}$ and *Postn*$^{-/-}$ day 7 wounds. **(C)** Quantification of cell number at the wound border and within the granulation tissue from high power fields of view. Cell number was not different between *Postn*$^{+/+}$ and *Postn*$^{-/-}$ wounds at either location ($p = 0.28$). Data is expressed as mean number of cells per field of view; error bars represent s.d. (two-way ANOVA).

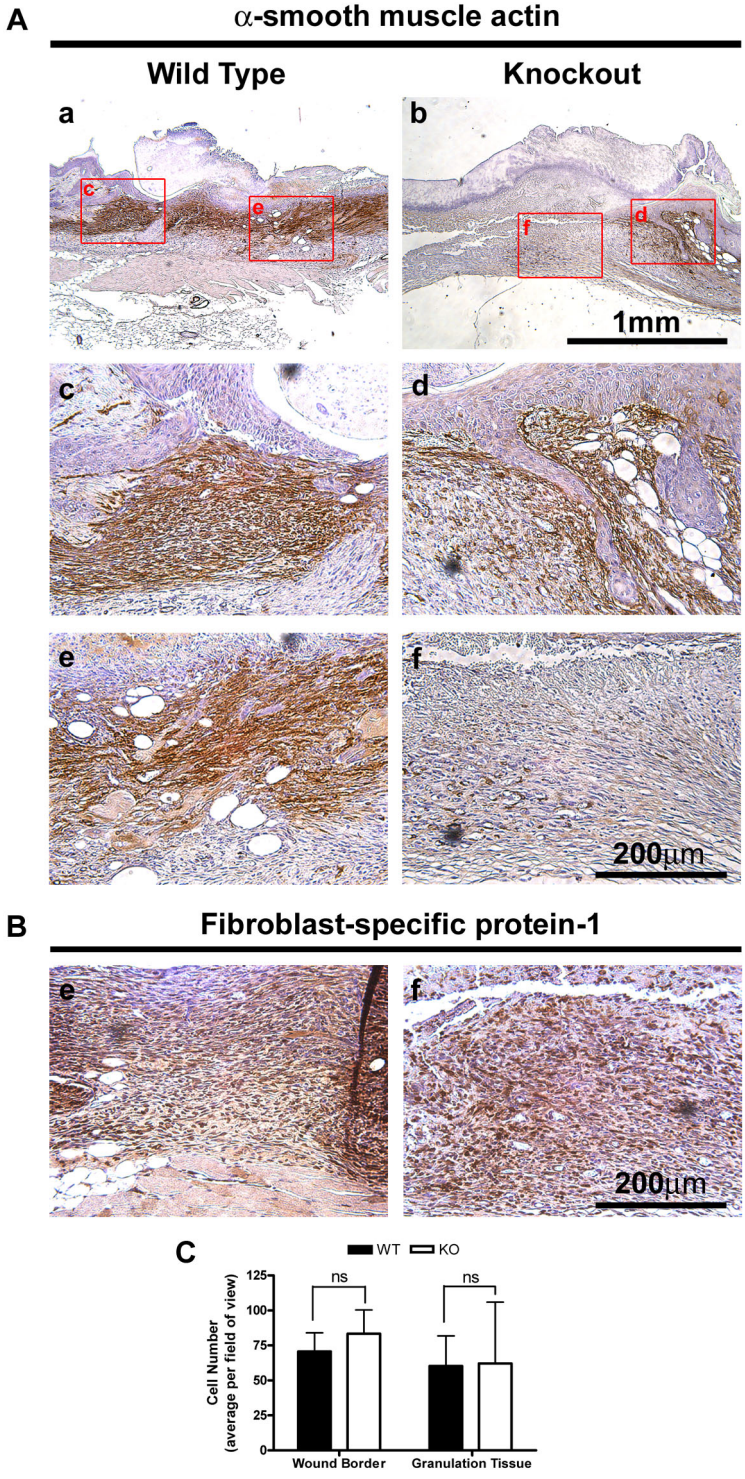

Figure 2.5: Decreased α-SMA expression is restricted to the granulation tissue of *Postn*$^{-/-}$ mice

Figure 2.6: Migration is not altered in *Postn*$^{-/-}$ fibroblasts. Dermal fibroblasts were isolated from *Postn*$^{+/+}$ and *Postn*$^{-/-}$ healthy skin biopsies. **(A)** Migration was assessed using the scratch wound method (n = 4). Scratch wounds were made in a confluent monolayer of *Postn*$^{+/+}$ or *Postn*$^{-/-}$ fibroblasts on glass bottom culture dishes. Migration was documented via time-lapse video microscopy. Images were generated from frames at 0, 8 and 16 hours. Yellow lines indicate the cell front. **(B)** Quantification of scratch area measured from images at four-hour intervals. No difference in fibroblast migration was detected ($p = 0.34$; two-way ANOVA). Data is expressed as a fraction of the initial scratch area; error bars represent s.d.

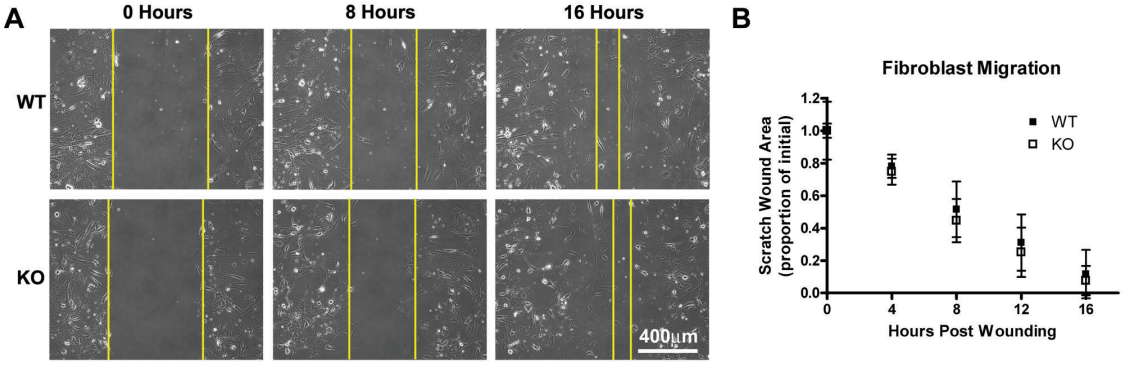

Figure 2.6: Migration is not altered in *Postn*$^{-/-}$ fibroblasts

2.2.3 Canonical TGFβ signaling is not altered in *Postn*$^{-/-}$ fibroblasts

TGFβ is known to cause an increase in α-SMA expression, via Smad3 phosphorylation (Gu et al., 2007), and plays a major role in myofibroblast differentiation (Desmouliere et al., 1993). Therefore, to determine if the reduction in α-SMA expression and immunoreactivity in *Postn*$^{-/-}$ granulation tissue was due to defective TGFβ/Smad3 signaling, we assessed the number of nuclei positive for phosphorylated Smad 2/3 (p-Smad2/3) within the granulation tissue. The number of p-Smad2/3 positive nuclei was similar in both *Postn*$^{+/+}$ and *Postn*$^{-/-}$ wounds (Figure 2.7A,B) ($p = 0.25$), suggesting canonical TGFβ signaling is active in *Postn*$^{-/-}$ wounds. This assay was limited in that it could not discriminate between subtle quantitative differences in p-Smad2/3 levels. However, collagen production in the granulation tissue of both *Postn*$^{+/+}$ and *Postn*$^{-/-}$ wounds, as evidenced by hydroxyproline content, was not significantly different (Figure 2.7C,D) ($p = 0.69$), a process for which TGFβ signaling is of great importance (Ignotz and Massague, 1986; Roberts et al., 1986; Wang et al., 2007b). These observations, combined with fibroblast-specific protein-1 staining and in vitro migration data, strongly refute a migratory explanation for the absence of α-SMA staining in day 7 *Postn*$^{-/-}$ granulation tissue.

2.2.4 Exogenous periostin is sufficient to induce a contractile phenotype

The reduced α-SMA expression observed *in vivo* indicated that a defect might exist in differentiation of fibroblasts to myofibroblasts and induction of contraction in *Postn*$^{-/-}$ wounds. *Postn*$^{+/+}$ and *Postn*$^{-/-}$ primary dermal fibroblasts were isolated and assayed for their ability to contract a floating collagen gel, as well as exert contractile forces across a collagen gel matrix (Figure 2.8A). *Postn*$^{+/+}$ cells were able to generate contractile forces across the collagen gel lattice, but *Postn*$^{-/-}$ fibroblasts showed a significant reduction in ability to contract the lattice. The anchored matrix gel contraction assay was then used, as it more closely resembles the mechanical environment of granulation tissue (Grinnell, 1994). Quantification of contraction through measurement of gel weight confirmed that *Postn*$^{+/+}$ fibroblasts were able to significantly contract the collagen matrix (Figure 2.8B,

Figure 2.7: Canonical TGFβ signaling is not altered in *Postn*$^{-/-}$ fibroblasts. (A) Histological analysis of day 7 wounds from *Postn*$^{+/+}$ and *Postn*$^{-/-}$ animals. Sections were incubated with an antibody for p-Smad2/3. Detection was with peroxidase conjugated 2° antibodies and DAB (n = 3) **(B)** Number of positively stained nuclei per high power field of view were not significantly different between *Postn*$^{+/+}$ and *Postn*$^{-/-}$ wounds (p = 0.37; Student's t-test). **(C)** Masson's trichrome staining of day 7 wound sections. **(D)** Hydroxyproline content (g/100 g dry tissue) of excised day 7 wounds was not different between *Postn*$^{+/+}$ and *Postn*$^{-/-}$ animals (p = 0.60; Student's t-test). Data is expressed as means; error bars represent s.d.

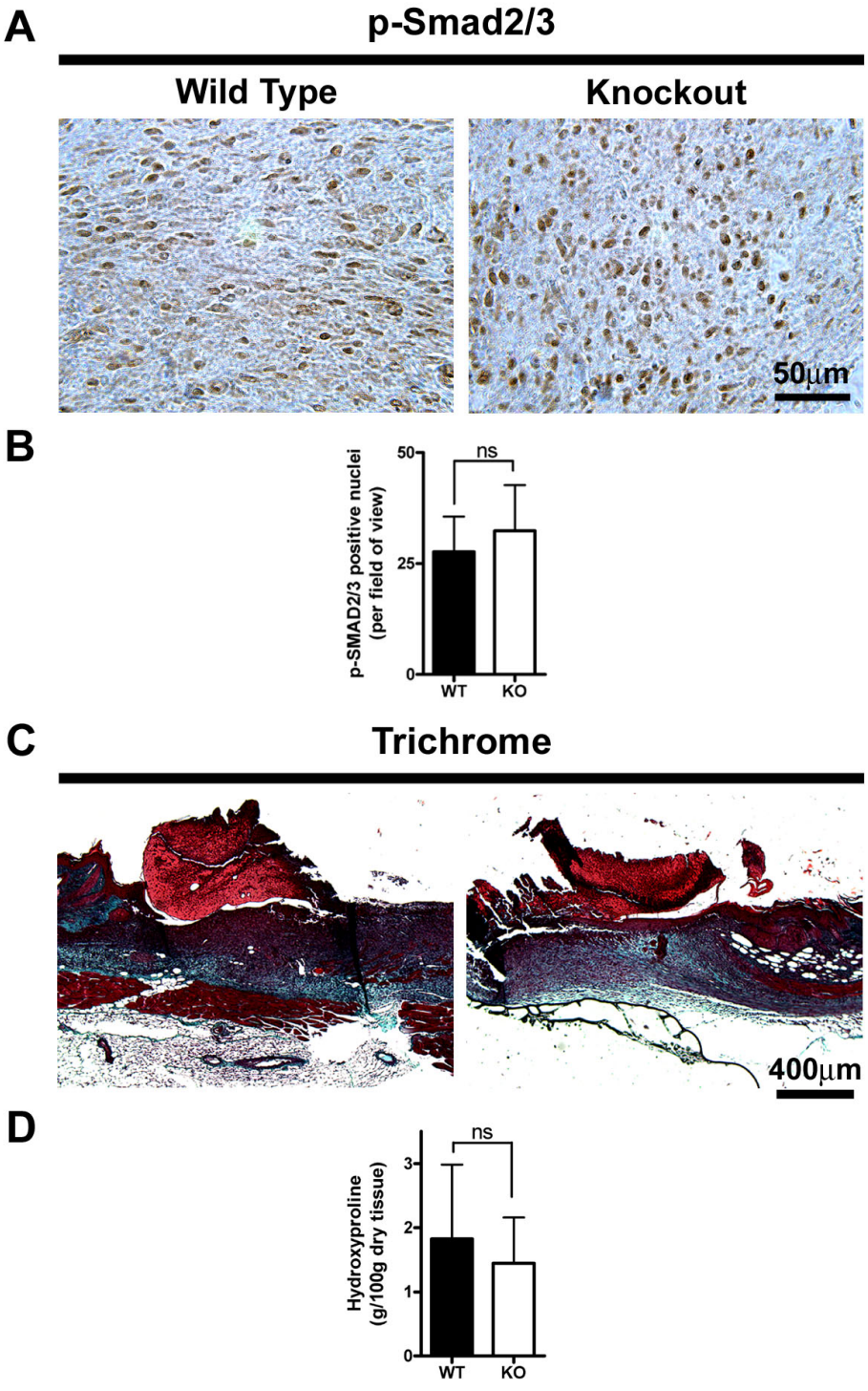

Figure 2.7: Canonical TGFβ signaling is not altered in *Postn*$^{-/-}$ fibroblasts

Figure 2.8: Exogenous periostin is sufficient to induce a contractile phenotype. (A) The effect of periostin deletion on the ability of dermal fibroblasts to exert contractile force in a fixed, tethered floating collagen gel lattice was investigated using a Culture Force Monitor. Forces generated by fibroblasts were measured over 24 hours; a representative trace is shown (n = 3). Units are dynes (10^{-5} N) **(B)** Cells contracted collagen gels over an additional 24 hours at 37°C, 5% CO_2. **(C)** Gel contraction was quantified by loss of gel weight, compared to gels lacking cells. *Postn*$^{-/-}$ fibroblasts were unable to significantly contract collagen gels. Note that *Postn*$^{+/+}$ fibroblasts were able to contract collagen gels. Addition of 5 μg/mL rhPN to the collagen gels rescued the contractile ability of *Postn*$^{-/-}$ fibroblasts (n = 3). Data is expressed as a fraction of the initial gel weight; error bars represent s.d. (* = $p < 0.05$; two-way ANOVA). **(D)** Gels were treated with 10 μM PP2 (or DMSO vehicle) or 10 μg/mL β1-integrin blocking antibody (mouse IgG for controls). Data is expressed as a fraction of the initial gel weight; error bars represent s.d. (* = $p < 0.05$; one-way ANOVA, n = 3). A Dunnett's multiple comparison test was employed where KO + PN + DMSO was used as the reference group. Extracellular periostin influences contractility through a β1-integrin/FAK dependent mechanism *in vitro*. **(E)** Fluorescent labeling of fibroblast populated collagen gels for α-SMA (green) and nuclei (blue). α-SMA positive cells were counted from high power fields of view. Percentage of α-SMA positive cells was significantly reduced in *Postn*$^{-/-}$ fibroblast populated gels ($p < 0.01$, n = 3). Addition of 5 μg/mL rhPN to the collagen gels restored the percentage of α-SMA positive cells. Data is expressed as mean; error bars represent s.d. (# = $p < 0.01$; one-way ANOVA).

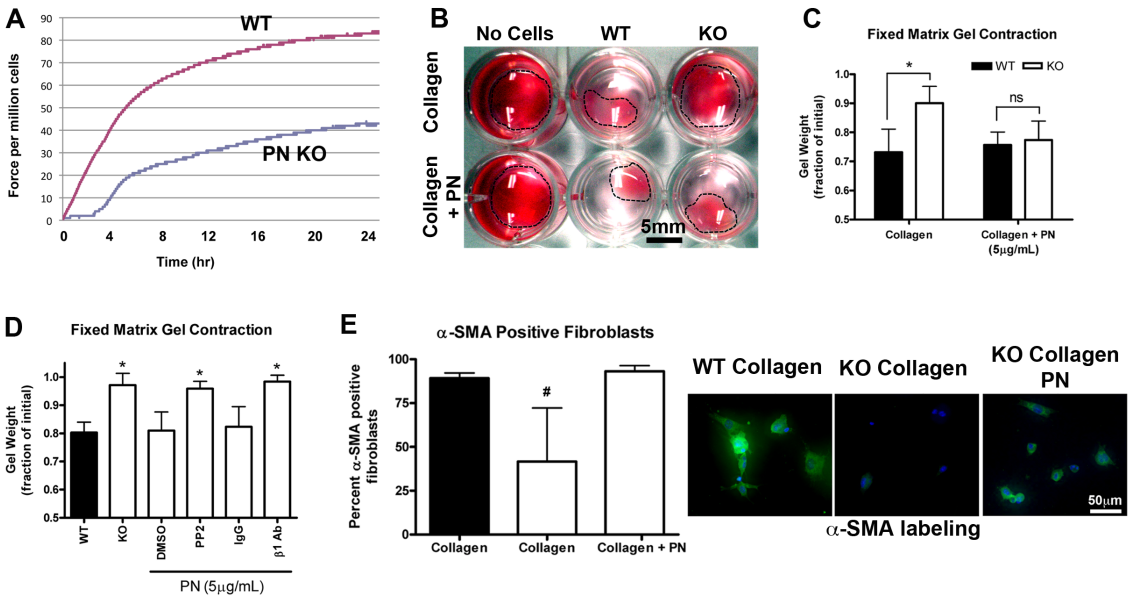

Figure 2.8: Exogenous periostin is sufficient to induce a contractile phenotype

C), in comparison with *Postn*$^{-/-}$ fibroblasts (Figure 2.8B), indicating that periostin is required for contraction of a collagen matrix by dermal fibroblasts ($p < 0.01$). To further investigate this finding, recombinant human periostin (rhPN) was added to the collagen matrix and was sufficient to induce contraction of the gels by *Postn*$^{-/-}$ dermal fibroblasts. Addition of 5 μg/mL rhPN fully recovered the contractile ability of *Postn*$^{-/-}$ cells (Figure 2.8C), supporting the notion that periostin facilitates wound healing by promoting wound contraction.

In order to understand the mechanism by which periostin induces contraction we attempted to reverse the effect of rhPN on *Postn*$^{-/-}$ fibroblast contraction with various inhibitors of signal transduction. As in Figure 2.8C, *Postn*$^{-/-}$ fibroblasts were unable to contract the collagen matrix. With the addition of rhPN, however, these cells contracted gels to the same extent as *Postn*$^{+/+}$ fibroblasts (Figure 2.8D). Blockade of β1-integrin ligation by incorporation of a β1-integrin neutralizing antibody (10 μg/mL) completely reversed the rhPN-induced gel contraction (Figure 2.8D) ($p < 0.01$). As a negative control, non-specific mouse IgG was instead incorporated into gels at the same concentration. Gels containing IgG contracted to the same degree as *Postn*$^{-/-}$ fibroblast populated gels with rhPN. Additionally, inhibition of Src/FAK phosphorylation with PP2 completely reversed the influence of rhPN on contraction of *Postn*$^{-/-}$ fibroblast populated collagen gels ($p < 0.01$).

To assess whether the differences in collagen matrix contraction were the result of differences in α-SMA levels, fibroblast-populated collagen gels were immunolabeled for α-SMA (Figure 2.8E). *Postn*$^{+/+}$ fibroblasts were generally more spread and displayed strong α-SMA labeling. In comparison, the number of *Postn*$^{-/-}$ fibroblasts positive for α-SMA was significantly reduced (89% positive in *Postn*$^{+/+}$, 40% in *Postn*$^{-/-}$, $p < 0.01$) (Figure 2.8E). In line with gel contraction data, addition of 5 μg/mL rhPN to the collagen matrix resulted in an increase in the number of α-SMA positive *Postn*$^{-/-}$ cells (93%, $p < 0.01$) (Figure 2.8E).

Periostin is known to influence cell adhesion (Takeshita et al., 1993). Therefore, it was conceivable that the lack of matrix contraction by *Postn*$^{-/-}$ fibroblasts was simply due to a defect in adhesion. To determine the influence of periostin on dermal fibroblast adhesion, we conducted adhesion assays with fibronectin, collagen type-1 and periostin coated tissue culture plates. *Postn*$^{+/+}$ and *Postn*$^{-/-}$ cells adhered to all matrix molecules tested equally, following two hours of incubation (Figure 2.9). Adhesion was higher on fibronectin than on collagen type-1, as anticipated ($p < 0.01$). Adhesion on periostin was significantly lower than on either fibronectin or collagen type-1 ($p < 0.01$). Coating wells with a combination of rhPN and fibronectin had no effect on dermal fibroblast adhesion compared to fibronectin alone. Coating with rhPN/collagen type-1, however, greatly reduced adhesion of dermal fibroblasts (Figure 2.9) ($p < 0.001$). This effect was consistent between *Postn*$^{+/+}$ and *Postn*$^{-/-}$ cells. Therefore, it is unlikely that periostin's influence on contractility is simply due to cell adhesion.

2.2.5 Periostin influences fibroblast morphology in 3D, but not 2D, culture

The presence of α-SMA immunoreactivity in the borders of day 7 *Postn*$^{-/-}$ wounds (but not within the granulation tissue) implies that alternative signals for myofibroblast differentiation are at work in these regions. Differentiation of fibroblasts into myofibroblasts requires TGFβ dependent signaling but also depends heavily on matrix stiffness (Tomasek et al., 2002). To explore the contribution of matrix stiffness on myofibroblast differentiation, dermal fibroblasts were seeded onto either collagen type-1 coated tissue culture plates or anchored collagen gels. Fibroblasts seeded on collagen coated tissue culture plates adopted a planar, well-spread morphology typical of fibroblasts in culture (Figure 2.10A). These cells developed very distinct stress fibers, which often incorporated α-SMA, indicating myofibroblast differentiation. *Postn*$^{-/-}$ fibroblasts grown on collagen-coated tissue culture plates were indistinguishable from *Postn*$^{+/+}$ fibroblasts. Moreover, α-SMA expression, as assessed by western blot, was not significantly different between *Postn*$^{+/+}$ and *Postn*$^{-/-}$ cells. Addition of 5 ng/mL recombinant human TGFβ1 did not further increase α-SMA levels (Figure 2.10B), indicating that the cultures might have already become maximally differentiated.

Figure 2.9: Periostin is a modulator of dermal fibroblast attachment. Wells of a 96 well tissue culture plate were precoated with 10 μg/mL of fibronectin, collagen-1 or rhPN. Additionally, wells were coated with combination of fibronectin + rhPN or collagen-1 + rhPN (all at 10 μg/mL). Prior to seeding cells, wells were washed with PBS and blocked with BSA. Isolated dermal fibroblasts were seeded and incubated for two hours before non-adhering cells were washed away. Cell number was determined by methylene blue staining, followed by dye extraction and measurement of absorbance at 650 nm. Values were compared to a standard curve. Data is expressed relative to adhesion of *Postn*$^{+/+}$ fibroblasts to fibronectin, with BSA adhesion subtracted; error bars represent s.d. Letters indicate treatment that are statistically similar ($p < 0.01$; two-way ANOVA, $n = 3$).

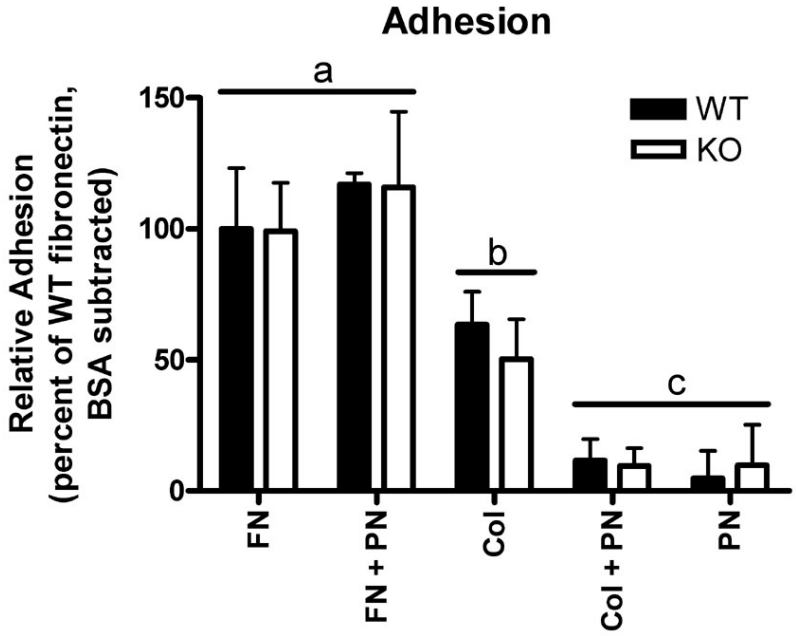

Figure 2.9: Periostin is a modulator of dermal fibroblast attachment

Figure 2.10: Periostin influences fibroblast morphology in 3D, but not 2D, culture. Isolated dermal fibroblasts were seeded on collagen coated tissue culture plates or onto precast collagen anchored gels. Cells were incubated for 48 hours before fixation or harvesting lysates. **(A)** Filamentous actin was visualized with rhodamine-conjugated phalloidin. Distinct stress fibers were observed in both *Postn*$^{+/+}$ and *Postn*$^{-/-}$ fibroblasts (n = 3). **(B)** Western blot analysis of lysates was carried out to quantify the level of α-SMA. Equal loading was confirmed by blotting for GAPDH. No difference was detected between *Postn*$^{+/+}$ and *Postn*$^{-/-}$ fibroblasts. **(C)** Fibroblasts seeded on 3D collagen gels were labeled with rhodamine-conjugated phalloidin and assessed for cell morphology (n = 3). *Postn*$^{-/-}$ fibroblasts were more likely to adopt a dendritic phenotype, characterized by a lack of stress fibers and extension of thin branching cytoplasmic extensions ($p < 0.01$). Addition of 5 μg/mL rhPN to the gels restored the percentage of dendritic fibroblasts to *Postn*$^{+/+}$ levels. Data is expressed as mean; error bars represent s.d. (* = $p < 0.01$; one-way ANOVA). **(D)** 3D collagen gels were homogenized and cells were lysed by sonication. α-SMA levels were assessed by western blot.

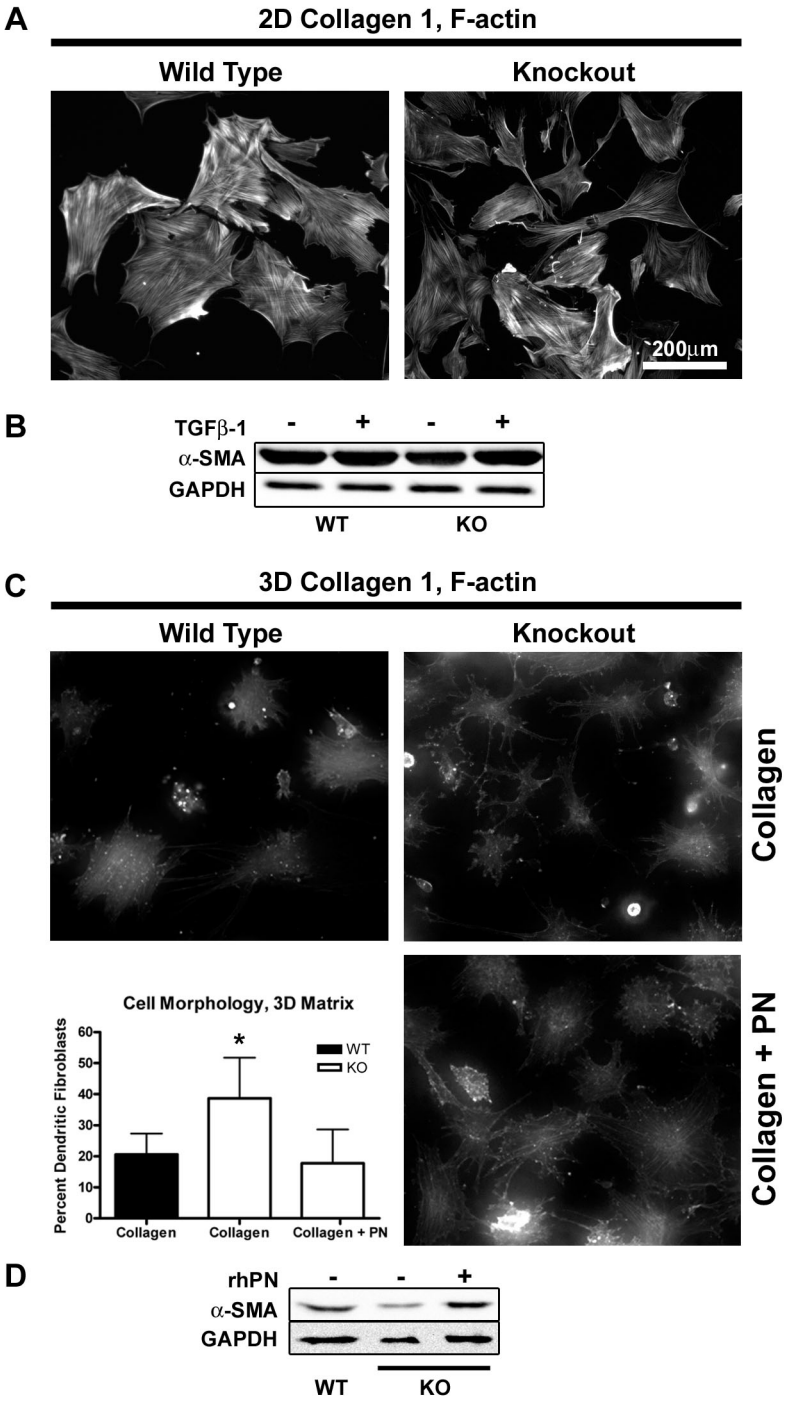

Figure 2.10: Periostin influences fibroblast morphology in 3D, but not 2D, culture

Tissue culture plastic provides an exceedingly stiff environment, whereas 3D collagen gels provide a much more compliant matrix, reminiscent of granulation tissue (Grinnell, 1994). The surface of the anchored collagen gels used in this study provides a mechanically intermediate environment (Arora et al., 1999), which produced mainly planar cells (Figure 2.10C). Twenty-one percent of *Postn*$^{+/+}$ cells, however, adopted a dendritic phenotype, characterized by poor spreading, lack of stress fibers and extension of numerous thin branching processes (Grinnell, 2003) (Figure 2.10C). Interestingly, the proportion of *Postn*$^{-/-}$ cells adopting the dendritic phenotype was significantly higher at 39% ($p < 0.01$). Furthermore, addition of 5 μg/mL rhPN to the collagen gels decreased the proportion of dendritic cells to the level of *Postn*$^{+/+}$ cultures. α-SMA protein was reduced in *Postn*$^{-/-}$ fibroblasts cultured on compliant collagen gels, but increased with the addition of rhPN to the gel (Figure 2.10D).

2.2.6 Periostin facilitates α-SMA expression on compliant substrates, but is not required with increasing substrate stiffness

Since *Postn*$^{-/-}$ fibroblasts showed altered spreading and a deficit in α-SMA protein when cultured on compliant collagen gels but not on collagen coated plastic, we sought to further assess periostin-induced myofibroblast differentiation in the context of matrix stiffness. *Postn*$^{+/+}$ and *Postn*$^{-/-}$ fibroblasts were seeded on collagen-coated flexible polyacrylamide gels of varying stiffness. On soft substrates (Young's modulus of 4800 Pa), 64% of *Postn*$^{+/+}$ fibroblasts were positive for α-SMA (Figure 2.11B). The proportion of α-SMA positive *Postn*$^{+/+}$ fibroblasts peaked on 19,200 Pa substrates at 90%, with no additional increase on the stiff 50,000 Pa substrates. The percentage of α-SMA positive *Postn*$^{-/-}$ fibroblasts increased with an increase in substrate stiffness ($p < 0.01$). Compared with *Postn*$^{+/+}$ fibroblasts, however, the proportion of α-SMA positive *Postn*$^{-/-}$ fibroblasts was significantly lower on 4,800 Pa and 19,200 Pa substrates, 29% and 57% respectively ($p < 0.05$) (Figure 2.11B). On 50,000 Pa substrates, the proportion of α-SMA positive *Postn*$^{-/-}$ fibroblasts was equivalent to that of *Postn*$^{+/+}$ fibroblasts (*Postn*$^{+/+}$ 87%, *Postn*$^{-/-}$ 84%). To further investigate the mechanism by which periostin facilitates α-SMA expression, we focused on the soft, 4,800 Pa substrates. *Postn*$^{-/-}$ fibroblasts showed an

Figure 2.11: Periostin facilitates α-SMA expression on a soft substrate, but is compensated for by increased substrate stiffness. Isolated dermal fibroblasts were seeded on collagen-coated flexible polyacrylamide substrates with Young's moduli of 4,800, 19,200 or 50,000 Pa. Cells were incubated for 48 hours before fixation. **(A)** Fibroblasts were fluorescently labeled for α-SMA (green), filamentous actin (red) and nuclei (blue). α-SMA positive cells were counted from high power fields of view. **(B)** Percentage of α-SMA positive cells was significantly reduced in *Postn*$^{-/-}$ fibroblasts grown on the soft 4,800 and 19,200 Pa substrates ($p < 0.05$, $n = 3$). On the stiff 50,000 Pa substrates, however, the proportion of α-SMA positive *Postn*$^{-/-}$ fibroblasts was equivalent to that of *Postn*$^{+/+}$ fibroblasts. Data is expressed as mean; error bars represent s.d. (* = $p < 0.05$; two-way ANOVA). **(C)** Western blot analysis of lysates from *Postn*$^{-/-}$ fibroblasts grown on 4,800 Pa substrates was carried out to quantify the level of pFAK^{Y397} and α-SMA. Loading was corrected by blotting for total FAK and GAPDH. pFAK^{Y397}/FAK and α-SMA/GAPDH indicate results of densitometry, relative to control. Incorporation of rhPN on 4,800 Pa substrates resulted in an increase in pFAK^{Y397} and α-SMA protein. Increased α-SMA was attenuated by 10 μM PP2.

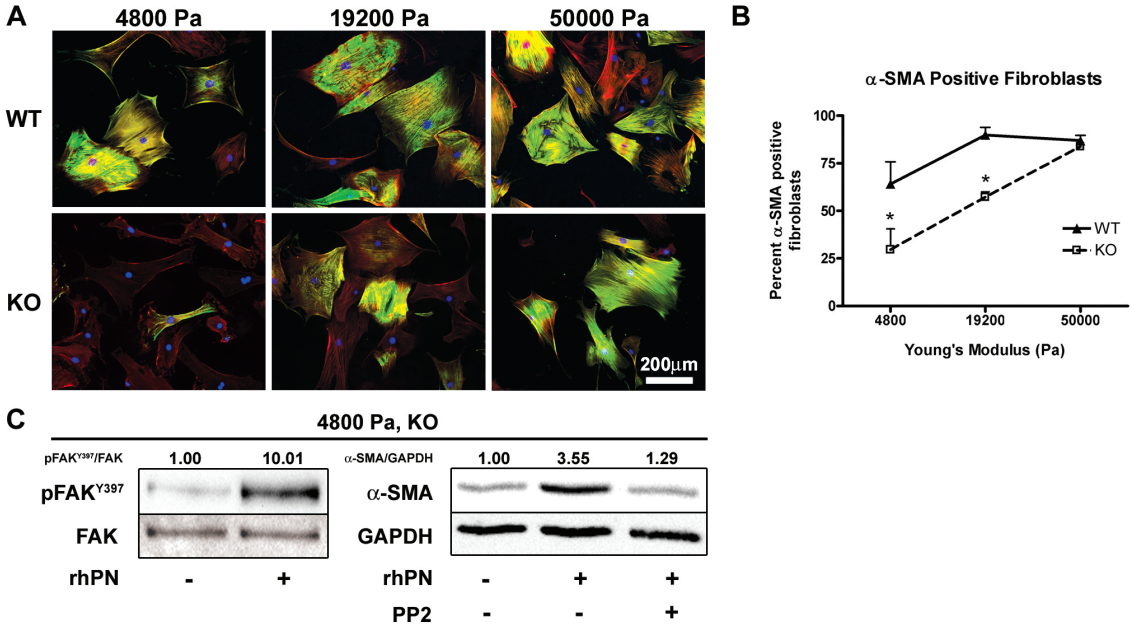

Figure 2.11: Periostin facilitates α-SMA expression on a soft substrate, but is compensated for by increased substrate stiffness

increase in α-SMA protein and phosphorylated FAK^{Y397} when polyacrylamide gels were coated with 100 μg/mL collagen + 5 μg/mL rhPN, compared to gels coated with 100 μg/mL collagen alone (Figure 2.11C). This increase was attenuated by 10μM PP2.

2.2.7 Delivery of recombinant periostin via electrospun collagen scaffolds stimulates α-SMA expression

Since rhPN was sufficient to restore α-SMA expression and contractility in *Postn*$^{-/-}$ fibroblasts *in vitro*, we attempted to reintroduce periostin into *Postn*$^{-/-}$ wounds *in vivo*. We incorporated rhPN into electrospun collagen scaffolds (Figure 2.12A,B) and these scaffolds (or control scaffolds lacking periostin) were laid into *Postn*$^{-/-}$ wounds immediately following wounding. Immunohistochemical analysis of day 7 wounds revealed a marked increase in α-SMA immunoreactivity within the wounds that received collagen/periostin scaffolds, compared to collagen-only controls (Figure 2.12C). Moreover, α-SMA immunoreactivity was detected throughout the granulation tissue of collagen/periostin treated wounds, mimicking the wild-type expression pattern.

2.3 Discussion

In this report we show that the loss of periostin, by use of the periostin knockout mouse (Rios et al., 2005), results in altered dermal wound closure kinetics, specifically during the pro-fibrotic phase of wound healing. The alteration in wound closure corresponds with the onset and peak of periostin expression in *Postn*$^{+/+}$ animals. Immunohistochemistry and RT-qPCR reveal that α-SMA is strikingly reduced in the granulation tissue of day 7 *Postn*$^{-/-}$ wounds. Altered wound closure kinetics in *Postn*$^{-/-}$ mice may therefore be due to a reduction in α-SMA positive myofibroblasts within the granulation tissue, and thus a reduction in wound contraction. Other phases of wounds healing appeared to be unaffected by the loss of periostin. Infiltration of inflammatory cells (macrophages and neutrophils) was similar between *Postn*$^{+/+}$ and *Postn*$^{-/-}$ wounds. The eventual closure of both *Postn*$^{+/+}$ and *Postn*$^{-/-}$ wounds by day 11 indicates that *Postn*$^{-/-}$ animals are able to close dermal wounds by a method other than contraction, possibly re-epithelialization. Recently, Nishiyama and colleagues reported reduced re-

Figure 2.12: Delivery of recombinant periostin via electrospun collagen scaffolds recovers α-SMA expression in PN KO mice. (A) Collagen type-1 electrospun scaffolds, with and without rhPN, were cut into 6 mm disks to match the size of punch wounds. **(B)** Periostin (green) protein was detected throughout the collagen + periostin scaffolds, but not the control collagen scaffolds. **(C)** Punch wounds were created in *Postn*$^{-/-}$ animals and scaffolds were immediately laid into the wounds. Half of the wounds received two control (collagen only) scaffolds and the other half of the wounds received two collagen + periostin scaffolds. Wounds were harvested for IHC at day 7. Addition of periostin to *Postn*$^{-/-}$ wounds via electrospun collagen scaffolds resulted in a marked increase in α-SMA immunoreactivity throughout the granulation tissue, when compared to wounds receiving control collagen scaffolds (n = 3).

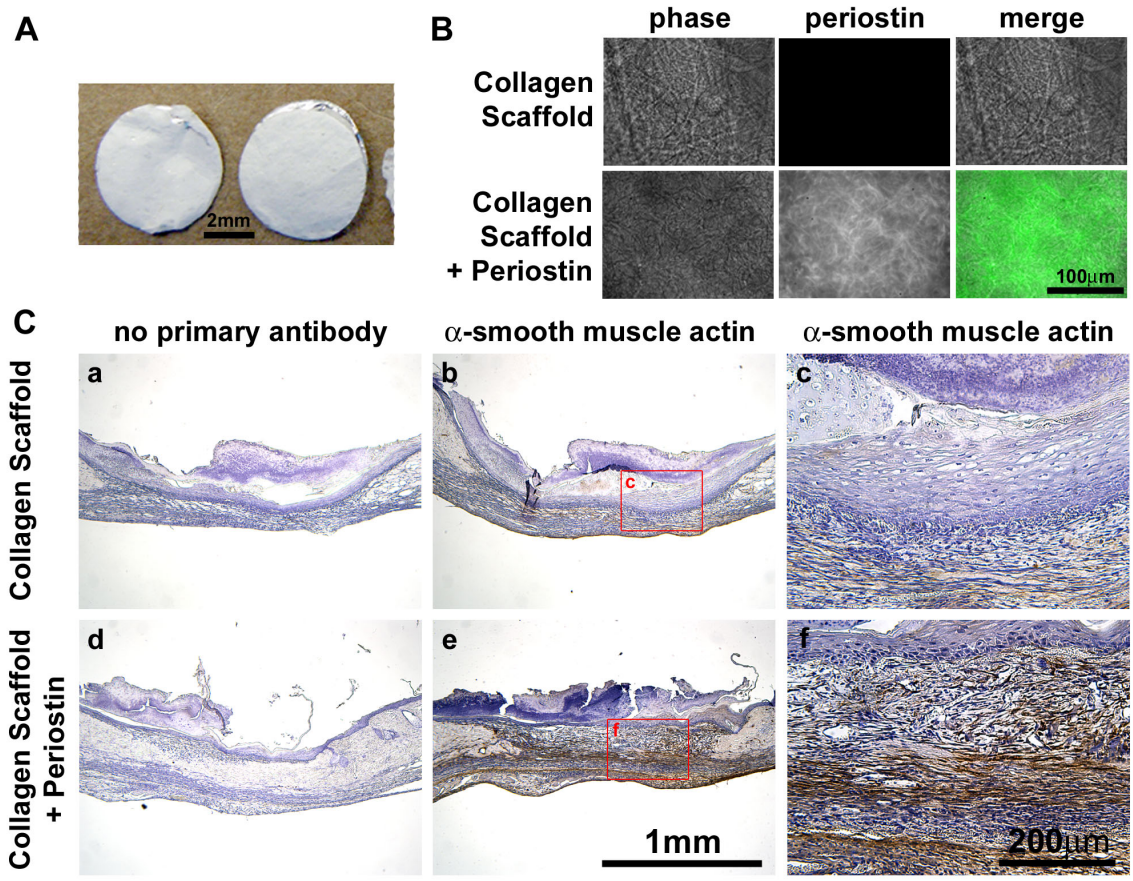

Figure 2.12: Delivery of recombinant periostin via electrospun collagen scaffolds recovers α-SMA expression in PN KO mice

epithelialization following dermal wounding in an independently derived *Postn*$^{-/-}$ mouse (Nishiyama et al., 2011). We observed no significant difference in re-epithelialization of *Postn*$^{-/-}$ wounds, when compared to *Postn*$^{+/+}$ controls. As would be expected, wound size (measured from histological sections) was greater in *Postn*$^{-/-}$ animals. Due to this difference in wound size, the calculated percentage of epithelialization appeared to be lower in *Postn*$^{-/-}$ wounds (not significant), yet the epithelial migration distance was actually higher. Therefore we feel that percent epithelialization is an inappropriate measurement for wounds of different sizes (Gorin et al., 1996) and epithelial migration distance is the more reliable method. Using this more reliable method we did not detect a difference in re-epithelialization.

Reduced α-SMA specifically within the granulation tissue of *Postn*$^{-/-}$ wounds, with wound borders and vasculature positive for α-SMA, raised the question of whether fibroblast recruitment was deficient in *Postn*$^{-/-}$ wounds. In fact, fibroblasts were observed, via immunohistochemical detection of fibroblast-specific protein-1, to dominate the newly formed granulation tissue of both *Postn*$^{+/+}$ and *Postn*$^{-/-}$ wounds. Cell number was also similar for *Postn*$^{+/+}$ and *Postn*$^{-/-}$ wounds in the granulation tissue and at wound borders. Moreover, fibroblast migration, as assessed by scratch wound assays *in vitro*, revealed no difference in migration between *Postn*$^{+/+}$ and *Postn*$^{-/-}$ fibroblasts. Together these results suggest fibroblast recruitment is not the underlying cause for the pattern of α-SMA expression in *Postn*$^{-/-}$ wounds. Collagen content from day 7 wounds, as assessed by hydroxyproline content, was not significantly different between *Postn*$^{+/+}$ and *Postn*$^{-/-}$ wounds. Therefore, we suggest that the granulation tissue of *Postn*$^{-/-}$ wounds harbors a synthetic fibroblast population, which is deficient, however, in contractile machinery.

As fibroblast recruitment did not appear to be altered in *Postn*$^{-/-}$ wounds, we sought to determine if reduced α-SMA within *Postn*$^{-/-}$ granulation tissue was the result of a defect in differentiation of fibroblasts into myofibroblasts. Supporting this hypothesis, isolated dermal fibroblasts from *Postn*$^{-/-}$ animals were unable to significantly contract anchored collagen gels, which assess contractility rather than tractional forces of migration (Grinnell, 1994). However, addition of exogenous rhPN fully rescued the phenotype of

the *Postn*$^{-/-}$ fibroblasts. Immunocytochemistry revealed that the degree of gel contraction corresponded to the number of α-SMA positive cells within the gel. Therefore, we conclude that periostin is required for gel contraction and its presence in the extracellular matrix is sufficient to induce a contractile myofibroblast phenotype. Previous reports support a role for periostin in gel contraction. Incorporation of purified periostin increased the ability of atrioventricular cushion mesenchymal cells to contract a collagen gel (Butcher et al., 2007). Similarly, addition of rhPN to collagen gels increased the contractility of fibroblasts isolated from patients suffering from Dupuytren's disease, a disease of connective tissue contracture (Vi et al., 2009). In both of these studies, increased contraction was associated with increased α-SMA protein.

Culturing isolated dermal fibroblasts on collagen type-1 coated tissue culture plates resulted in abundant α-SMA production, irrespective of periostin expression, showing that *Postn*$^{-/-}$ fibroblasts are capable of α-SMA expression. The pattern of α-SMA expression within day 7 *Postn*$^{-/-}$ wounds must, therefore, be due to the environment provided by the granulation tissue itself. Mechanical tension (Hinz et al., 2001; Cevallos et al., 2006) and TGFβ (Desmouliere et al., 1993) induce α-SMA expression and promote myofibroblasts differentiation (Tomasek et al., 2002). Induction of α-SMA by TGFβ is primarily through activation of canonical TGFβ signaling, specifically Smad3 (Gu et al., 2007). Interestingly, within the granulation tissue of *Postn*$^{+/+}$ and *Postn*$^{-/-}$ wounds, nuclear p-Smad2/3 was detected at equal levels. Although we cannot rule out Smad-independent TGFβ pathways (Derynck and Zhang, 2003), nor can we rule out subtle quantitative differences in p-Smad2/3 levels between *Postn*$^{+/+}$ and *Postn*$^{-/-}$ wounds, TGFβ signaling appears to be active within the granulation tissue of *Postn*$^{-/-}$ wounds. Therefore, we suspected that the mechanical environment might be the determining factor for periostin-induced myofibroblast differentiation.

Mathematical models of fibroblast-driven dermal wound healing predict that the wound border is a region of peak matrix stiffness (Murray, 2003). Of particular importance, the tangential stiffness modulus of this region is greatly elevated over that of the central granulation tissue (Murray, 2003). The potential correlation between predicted tissue stiffness and α-SMA immunoreactivity in *Postn*$^{-/-}$ wounds prompted us to test if periostin

influences fibroblast behaviour in a mechanically dependent manner. On collagen coated tissue culture plates, no difference in cell morphology or α-SMA expression was apparent. In contrast to 2D culture, when isolated dermal fibroblasts were cultured on the more compliant collagen gels, a greater percentage of *Postn*$^{-/-}$ cells adopted a dendritic phenotype and α-SMA protein was reduced. Incorporating rhPN into the collagen gels reversed both of these effects. Tissue culture plates provide an enormously stiff environment for cell growth, with a reported elastic modulus of 2.78 GPa (Callister and Rethwisch, 2000), whereas 2 mg/mL collagen gels have an elastic modulus of approximately 300-400 Pa (Paszek et al., 2005; Marenzana et al., 2006). The elastic modulus of anchored collagen gels has not been reported. However, they are believed to more closely represent the mechanical environment of the granulation tissue (Grinnell, 1994), which has been reported as having an elastic modulus of 18.5 kPa in day 7 rat wounds (Goffin et al., 2006).

We propose that periostin facilitates myofibroblast differentiation and matrix contraction in a compliant 3D environment, such as in the granulation tissue. However, in a rigid environment such as in 2D culture or at the wound border, the effects of periostin are overshadowed by the influence of mechanical tension. Multiple studies looking at the effect of matrix stiffness on fibroblasts morphology agree that above 3-6 kPa fibroblasts assemble stress fibers and that above 15 kPa α-SMA incorporation occurs (Wells, 2005; Yeung et al., 2005; Solon et al., 2007). To refine our assessment of myofibroblast differentiation in the context of matrix stiffness, we adopted the widely used matrix-coated flexible polyacrylamide substrate (Aplin and Hughes, 1981; Pelham and Wang, 1997). While the stiffness of collagen gels can be modulated by increasing or decreasing the collagen concentration, this method introduces ligand density as a confounding variable (Paszek et al., 2005). In this study, all substrates were coated with the same concentration of collagen, thereby ensuring that differences in cell differentiation were due to substrate stiffness and not ligand density. Using collagen-coated polyacrylamide gels of varying stiffness, we observed a significant reduction in the proportion of *Postn*$^{-/-}$ fibroblasts positive for α-SMA compared to wild-type controls. This reduction was present on the more compliant 4,800 Pa and 19,200 Pa substrates but not on the stiff

50,000 Pa substrates, thus clearly showing that the lack of periostin can be compensated for by matrix stiffness. Furthermore, this supports the hypothesis that increased matrix stiffness is responsible for α-SMA expression at the borders of day 7 *Postn*$^{-/-}$ wounds.

Interestingly, Sidhu and colleagues recently reported that incorporation of periostin, in the absence of any cell type, was sufficient to increase the elastic modulus of collagen type-1 gels (Sidhu et al., 2010). Based on this evidence it is possible that periostin itself may contribute to the stiffness of the granulation tissue via collagen cross-linking, thereby indirectly promoting myofibroblast differentiation. Such an effect would surely be a confounding variable in the collagen gel contraction assays employed here, although the concentrations of periostin used by Sidhu and colleagues were substantially higher (20 and 200 μg/mL) than the concentration used in our assays (5 μg/mL) (Sidhu et al., 2010). If the mechanism by which periostin facilitates myofibroblast differentiation is simply by increasing matrix stiffness, we would expect that *Postn*$^{+/+}$ and *Postn*$^{-/-}$ fibroblasts would behave similarly on 2D matrix-coated polyacrylamide substrates, where stiffness is controlled independent of collagen cross-linking. Our results however do not support such a mechanism, as *Postn*$^{-/-}$ fibroblast differentiation was significantly lower on soft substrates. Our results instead favour a mechanism where periostin facilitates myofibroblast differentiation in compliant substrates and its influence is overshadowed by increased matrix stiffness.

Although we had established a "niche" in which periostin promotes myofibroblast differentiation in compliant matrices, the mechanism for periostin's influence remained unclear. In gel contraction assays, we found that inhibition of β1-integrin ligation and inhibition of Src/FAK signaling reversed the periostin-induced increase in gel contraction. Moreover, coating polyacrylamide substrates with a combination of collagen and rhPN increased the level of α-SMA protein and phosphorylation of FAK^{Y397}. The rhPN-induced increase in α-SMA protein was attenuated by PP2. In addition to providing attachment to the ECM, integrin ligation is known to activate numerous signaling pathways (Giancotti and Ruoslahti, 1999). We cannot rule out the possibility that β1-integrin functioning is required for gel contraction and myofibroblast differentiation through a mechanism parallel to, but independent of, periostin. However, periostin has

been shown to bind β1-integrins, an event that is required for cushion mesenchyme cell invasion into collagen type-1 gels (Butcher et al., 2007).

Periostin was initially classified as an adhesion molecule (Takeshita et al., 1993; Horiuchi et al., 1999). However, more recent work (Katsuragi et al., 2004), as well as adhesion data presented in this report do not support the notion that periostin is an adhesion molecule per se. We suggest instead that the binding of periostin to surface receptors *in vivo* serves to modulate intracellular signaling, and that periostin's role is not strictly to increase attachment. Ligation of most integrin pairs results in activation of FAK (Giancotti and Ruoslahti, 1999). Indeed, periostin has been shown to influence intracellular FAK activation in an integrin dependant manner (Shimazaki et al., 2008; Li et al., 2010). Our data favours a mechanism by which periostin influences intracellular signaling of myofibroblast differentiation in a β1-intergrin/FAK dependent manner.

Myofibroblasts are at the core of many fibrotic diseases including systemic sclerosis (Leask, 2010b), hypertrophic scars (Baur et al., 1975) and sub-epithelial fibrosis in bronchial asthma (Brewster et al., 1990). To date, periostin has been implicated in sub-epithelial fibrosis (Takayama et al., 2006) and hypertrophic scars (Wang et al., 2007a; Zhou et al., 2010). The role of periostin in these diseases is not fully understood, however, our data supports the hypothesis that periostin facilitates myofibroblast differentiation, thereby contributing to disease progression. The ability of periostin-containing electrospun collagen scaffolds to increase α-SMA immunoreactivity in *Postn*$^{-/-}$ wounds may have important implications for treatment of non-healing skin lesions, or chronic skin wounds. By definition, chronic skin wounds are unable to close and, therefore, represent a massive burden on today's healthcare systems (Elliott and Hamilton, 2011). There is therefore an immediate need for therapeutics capable of expediting wound closure. Using recombinant material and collagen scaffolds, we have demonstrated the feasibility of using periostin to influence wound healing. Future work will focus on the employment of periostin-containing electrospun collagen scaffolds to determine if the increased α-SMA immunoreactivity translates into increases in wound closure rates.

2.4 Materials and Methods

2.4.1 Animals

All animal procedures were in accordance with protocols approved by the University Council on Animal Care at The University of Western Ontario. Periostin knockout mice (*Postn*$^{-/-}$) were generated and described previously (Rios et al., 2005). Heterozygous mice were crossed with C57BL/6J (JAX® Mice and Services, Bar Harbor, Maine) for a minimum of six generations to ensure an incipient congenic strain. Backcrossed heterozygous mice were used for breeding and offspring were genotyped as described previously (Rios et al., 2005). *Postn*$^{-/-}$ mice and sex-matched littermate *Postn*$^{+/+}$ control mice were weaned at 3 weeks and provided with powdered food to reduce the effects of tooth defects on growth rate (Rios et al., 2005). All animals were subjected to 12 h light/dark cycle and temperature in accordance with the guidelines of the Canadian Council on Animal Care.

2.4.2 Punch Wounds

For experiments, *Postn*$^{-/-}$ and sex-matched littermate *Postn*$^{+/+}$ mice (12 weeks of age weighing approximately 25 g) were anesthetized with an intraperitoneal injection of buprenorphine (50 μg/kg), followed by an injection of ketamine (100 mg/kg) and xylazine (5 mg/kg). Backs were shaved, depilated and sterilized with iodine. Two full-thickness excisional wounds were made on each side of the dorsal midline with a 6 mm punch biopsy. Removed tissue was considered day 0 and was retained for further analysis. Wounds were photographed immediately after wounding and again at 3, 5, 7, 9 and 11 days post wounding. Wound area was assessed from photographs using Northern Eclipse v7.0 software (Empix Imaging Inc., Mississauga, Ontario) and expressed as a fraction of initial area. Mice were caged individually following wounding and were sacrificed at day 11 for histology and gene expression assays.

2.4.3 Tissue Preparation and Immunohistochemistry

Additional animals were wounded as described above and sacrificed at days 5 and 7. At various time-points wounds were excised and either snap frozen in liquid nitrogen, or

fixed in 10% neutral buffered formalin (Sigma Aldrich, St. Louis, Missouri). Tissues were stained as previously described (Jackson-Boeters et al., 2009). Sections were blocked with 10% horse serum and incubated with goat anti-periostin (sc49480, Santa Cruz Biotechnology, Santa Cruz, California) primary antibody overnight at 4°C. For negative controls, periostin primary antibody was pre-absorbed for 1 hour with the immunizing peptide. Detection was by ImmPRESS Ig peroxidase kits (Vector Laboratories, Burlingame, California) and visualized with 3,3-diaminobenzidine (Vector Laboratories). Sections were counterstained with haematoxylin. Staining of α-SMA was with the rabbit anti-α-SMA primary antibody (ab5694, Abcam, Cambridge, United Kingdom), fibroblast-specific protein-1 was with anti-FSP1/S100A4 (Millipore, Billerica, Massachusetts), CD68 was with MCA1957 (AbD Serotec, Oxford, United Kingdom), neutrophil elastase was with ab68672 (Abcam). Negative controls excluded the primary antibody. For immunofluorescence, nuclei were labeled with DAPI (Vector Laboratories). Detection of p-Smad2/3 (sc11769-R, Santa Cruz Biotechnology) positive nuclei on paraffin sections was carried out as above excluding haematoxylin counterstaining. Trichrome staining was carried out as previously described (Liu et al., 2008). For assessment of re-epithelialization, sections from the centre of the wounds were stained with haematoxylin and photographed. Wound size and epithelial migration distance were measured using Northern Eclipse v7.0 software. Epithelial migration distance was defined as the unilateral distance between the wound border and the migrating front of keratinocytes. Percent epithelialization was determined from bilateral epithelial migration distance, normalized to wound size.

2.4.4 *In Situ* Hybridization

In situ hybridization for periostin message was performed on 10 μm paraffin serial skin sections using [^{35}S]-labeled riboprobe transcribed from a 574 bp fragment of mouse periostin cDNA (Kruzynska-Frejtag et al., 2001). Hybridization with sense probe was performed in parallel as negative control.

2.4.5 RT-qPCR

Snap frozen tissue samples were homogenized in 1 mL of TRIzol® reagent (Invitrogen, Carlsbad, California). Total RNA was extracted as per the manufacturer's recommendations. Real-time quantitative PCR was carried out on 50 ng of total RNA using TaqMan® One-Step RT-PCR Master Mix and gene-specific TaqMan® probes (Applied Biosystems, Carlsbad, California). *Postn* and *Acta2* gene expression was normalized to the endogenous control gene *18S*. PCR efficiency was verified to fall between 90 and 110%, via dilution series, and relative expression was calculated using the ΔΔCT method (Livak and Schmittgen, 2001).

2.4.6 Hydroxyproline

Hydroxyproline content of excised dermal wounds was determined as an indicator of collagen content essentially as previously described (Samuel, 2008). Hydroxyproline content was determined using a standard curve and normalized to tissue dry weight. Values were expressed as grams of hydroxyproline per 100 g of tissue.

2.4.7 Isolation of Primary Dermal Fibroblasts

Excised tissue from punch wounds were immediately transferred to Dulbecco's Modified Eagle Medium (High Glucose) supplemented with 10% fetal bovine serum and 2% AA (200 units penicillin/200 μg streptomycin/0.5 μg/mL amphotericin B) (Gibco, Carlsbad, California). Skin was washed with five changes of media then incubated at 37°C, 5% CO_2 to allow fibroblasts to migrate onto the culture surface. For use, skin was removed and cells were cultured for two to three passages.

2.4.8 Gel Contraction

Gel contraction assays were conducted essentially as previously described (Shi-wen et al., 2004). Collagen was prepared as follows: 10% 0.2 M HEPES (pH 8), 40% bovine collagen type-1 (Advanced BioMatrix Inc., San Diego, California) and 50% 2X Dulbecco's Modified Eagle Medium (High Glucose). Dermal fibroblasts were suspended in 0.5% FBS DMEM and mixed 1:1 with the collagen preparation to a final density of 100,000 cells/mL. Either 5 μg/mL rhPN (R&D Systems Inc., Minneapolis, Minnesota) or

an equivalent volume of PBS was incorporated into the collagen/cell mix. 24 well tissue culture plates were pre-coated with BSA overnight then washed with PBS. Collagen/cell mix (0.5 mL) was added to each well and allowed to set at 37°C. Following polymerization, wells were flooded with 1 mL 0.5% FBS DMEM. After 24 hours, gels were separated from the surface of the plate and incubated for an additional 24 hours. To ensure that contraction of gels horizontally and vertically was accounted for, quantification of gel contraction was assessed by loss of gel weight, whereby contraction of the collagen matrix excluded growth media, thus reducing the weight of the gel (Tingstrom et al., 1992).

The Src/FAK inhibitor, PP2 (Calbiochem, Darmstadt, Germany), was added at 10 μM to the collagen/cell mix during preparation and to the media following polymerization. An equal volume of dimethyl sulfoxide (DMSO) (Sigma Aldrich) was added to untreated control gels. Additionally, integrin β1 signaling was inhibited by addition of 10 μg/mL blocking antibody (MA2253, Millipore) or mouse IgG (PP100, Millipore) to the collagen/cell mix..

2.4.9 Fibroblast Populated Collagen Lattice (FPCL) Contraction Assay

Experiments were performed essentially as previously described (Shi-wen et al., 2004). Briefly, cells (1×10^6 cells/ml) were seeded into a collagen gel (First Link Ltd., Birmingham, United Kingdom) floated in DMEM, 0.5% FBS. Gels were anchored at one end and attached to a force transducer at the other end. Forces generated across the collagen lattice were measured over a 24 hour period. Graphical readings are produced every 15 seconds providing continuous measurements of generated forces (Dynes: 1×10^{-5} N) which are logged into a personal computer.

2.4.10 Adhesion

Tissue culture treated 96 well plates were coated overnight at 4°C with 10 μg/mL human fibronectin (Sigma Aldrich), 10 μg/mL bovine collagen type-1 (Advanced BioMatrix), 10 μg/mL rhPN (R&D Systems) or a combination of periostin and collagen or fibronectin. Plates were subsequently blocked with 3% BSA at 37°C for 2 hours. Dermal fibroblasts

were suspended in serum free media, seeded and allowed to attach for 2 hours. Adherent cells were fixed with 10% neutral buffered formalin (Sigma Aldrich) and stained with methylene blue. Adhesion was quantified by dye extraction and measurement of absorbance at 650 nm (Oliver et al., 1989). Cell number was determined from a standard curve.

2.4.11 Migration

Migration was assessed by scratch wound assays essentially as previously described (Liang et al., 2007). Dermal fibroblasts were seeded on glass bottom culture dishes and allowed to reach confluence. Scratches were created with a P200 tip and disrupted cells were washed away with PBS. Growth media was replaced with 0.5% FBS DMEM to reduce proliferation. Closure of scratches was documented by time-lapse video microscopy using a Zeiss Axio Observer Z.1 inverted microscope equipped with a temperature and CO_2 controlled incubation chamber (Carl Zeiss, Oberkochen, Germany).

2.4.12 Immunocytochemistry

Dermal fibroblasts were suspended in 0.5% FBS DMEM and seeded at 30,000 cells/well on pre-coated collagen type-1 6-well plates (BD Biosciences, Franklin Lakes, New Jersey). Cells were left to attach overnight before treatment with 5 ng/mL recombinant human TGFβ1 (R&D Systems) for 24 hours. Alternatively, cells were seeded on top of polymerized collagen gels (prepared as described above) in 24-well plates and incubated for 48 hours. Cells were fixed with 4% paraformaldehyde, permeablized with 0.1% Triton X-100 and blocked with 3% BSA. Filamentous actin was visualized using rhodamine-conjugated phalloidin (Molecular Probes, Carlsbad, California). α-SMA was labeled with a mouse monoclonal primary antibody (A5228, Sigma Aldrich) and detected with a goat anti-mouse IgG conjugated to Alexa Fluor 488 secondary antibody (Molecular Probes). Fibroblast morphology was assessed as dendritic or planar by a blinded observer from random fields of view. Fibroblasts were considered as displaying the dendritic morphology based on the lack of stress fibers and extension of thin branching cytoplasmic extensions. Cells displaying planar morphology were well-spread and showed prominent stress fibers.

2.4.13 Western Blotting

Cell lysates were harvested at 48 hours with RIPA buffer (Sigma Aldrich) containing protease and phosphatase inhibitor cocktails (Sigma Aldrich). Protein concentration was determined by BCA assay (Pierce, Waltham, Massachusetts). For 3D collagen gels, gels were homogenized and cells were lysed by sonication. Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. Membranes were washed with tris-buffered saline containing 0.05% tween-20 (TBST). Membranes were blocked with 5% milk TBST. Primary antibodies were anti-α-SMA (ab5694, Abcam), anti-FAK^{Y397} (BD611722, BD Biosciences), anti-FAK (sc-558, Santa Cruz Biotechnology) and anti-GAPDH (MAB374, Millipore). Detection was with appropriate peroxidase-conjugated secondary antibodies (Jackson ImmunoReaserch, West Grove, Philadelphia) and enhanced chemiluminescence (Pierce). Bands were quantified using Image J software, using GAPDH to correct for loading.

2.4.14 Polyacrylamide Substrates

Matrix-coated flexible polyacrylamide substrates were created on glass cover slips using methods described previously (Pelham and Wang, 1997; Bhana et al., 2010; Tilghman et al., 2010). Polyacrylamide gels of varying stiffness were prepared with 7% (4,800 and 19,200 Pa) or 15% (50,000 Pa) acrylamide (Sigma Aldrich) and 0.05% (4,800 Pa), 0.24% (19,200 Pa) or 0.3% (50,000 Pa) N,N'-methylenebis(acrylamide) (Sigma Aldrich) (Bhana et al., 2010; Tilghman et al., 2010). Gels were coated with either 100 μg/mL collagen type-1 (Advanced BioMatrix Inc) or 100 μg/mL collagen type-1 and 5 μg/mL rhPN (R&D Systems) using the heterobifunctional crosslinker Sulfo-SANPAH (Pierce). Gels were washed with PBS and equilibrated with growth media before seeding cells.

2.4.15 Scaffolds

Collagen type-1 (Sigma-Aldrich) was dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol to make a 15% (w/v) solution. Periostin (R&D Systems) was dissolved in PBS to make a 1 mg/ml solution. 20 μl of the periostin (concentration of 100 μg/ml) was mixed with 3 ml of collagen solution, and the mixture injected at a speed of 1 ml/h by a syringe pump into

a capillary charged with a voltage of +15 kV. The generated nanofibers were collected on a negatively charged (-10 kV) rotation mandrel. Control scaffolds contained 20 μl of PBS. To crosslink the scaffolds, they were immersed in 5% glutaraldehyde/ethanol solution for 30 min. Scaffolds were spun onto aluminum foil, and a 6 mm biopsy punch is used to cut the scaffolds to ensure they were the same size as the wound. Each scaffold was sterilized in 100% ethanol and rinsed 3 times with PBS. Two 6 mm diameter scaffolds were inserted into each wound immediately following wounding.

2.4.16 Statistical Methods

Statistical analysis was by one-way or two-way ANOVA, as appropriate, followed by a Bonferroni correction, using Graphpad Software v4 (Graphpad Software, La Jolla, California) ($p \leq 0.05$ was considered significant). Student's t-tests were used for p-Smad2/3 and hydroxyproline data. A Dunnett's multiple comparison test was used for gel contraction assays including inhibitors to compare all treatments to the reference group, KO + PN + DMSO. In total, 16 *Postn*$^{-/-}$ and 16 *Postn*$^{+/+}$ wounds were tracked for wound area over the 11-day time course. Data is expressed as a fraction of the original wound area (mean ± s.d.). *In vivo* gene expression data represents the mean ± s.e.m. of at least 5 *Postn*$^{-/-}$ and 5 *Postn*$^{+/+}$ wounds for each time point. *In vitro* data is expressed as the mean ± s.d. of three individual experiments with three independent sex-matched littermate primary cultures. Individual experiments included at least three replicates.

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Chapter 3

3 Evaluation of periostin and CCN2 as potential therapeutics for the treatment of human chronic skin wounds

Abstract

Development of effective treatments to close non-healing skin lesions, or chronic skin wounds, has been hampered by a lack of thorough understanding of the dysfunctions present in these wounds. Matricellular proteins have received little attention despite their known role in modulating many important aspects of normal wound healing. The matricellular proteins periostin and CCN2 promote fibrotic aspects of wound healing but their state in chronic skin wounds is unknown. The objective of this study was to determine the expression patterns of periostin and CCN2 in human chronic skin wounds. Periostin and CCN2 were reduced within the wound bed of human chronic skin wounds. This might be due to the inflammatory microenvironment since wound edge fibroblasts showed no deficits in inducible fibrotic phenotype *in vitro*. Administration of rhPN or rhCCN2, electrospun with collagen, accelerated wound closure in a model of impaired diabetic healing. We propose that matricellular proteins, particularly periostin and CCN2, are promising therapeutic options for the treatment of human chronic skin wounds.

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3.1 Introduction

The skin's ability to heal is fundamentally important to maintaining proper barrier function and ultimately our survival. However, certain human populations including the elderly and those with systemic conditions such as diabetes often demonstrate an impaired ability to heal skin wounds. Failed wound healing results in what is broadly termed a chronic skin wound. As of 2009/10, 2.4 million Canadians (Public Health Agency of Canada, 2011) and 25.8 million Americans were living with diabetes (Centers for Disease Control and Prevention, 2011). It is estimated that 25% of people with diabetes will develop a chronic skin wound in their lifetime (Singh et al., 2005). The consequences of developing a chronic skin wound are severe, with complications including increased risk of infection, sepsis, osteomyelitis, amputation, and even death (Sen et al., 2009). The financial costs of chronic skin wounds are estimated to be as high as $25 billion annually in the United States alone (Brem et al., 2007). With the incidence of diabetes and its associated clinical complications projected to rise significantly, it is imperative that new treatment modalities are developed. However, developing effective treatments has been hindered by our limited knowledge of the dysfunctions present in chronic skin wounds.

Chronic skin wounds, regardless of etiology, are classified based on an absence of healing after 4 weeks (Sheehan et al., 2003). They are complicated by a combination of patient age, repeated ischemia-reperfusion injury, bacterial colonization and hypoxia (Mustoe, 2004; Schreml et al., 2010). Common features of chronic skin wounds include reduced growth factor activity, decreased matrix accumulation, increased fibroblast senescence and a general failure to progress beyond the inflammatory phase of healing (Elliott and Hamilton, 2011). Many different treatment strategies have been investigated for the treatment of chronic skin wounds; including protease inhibitors, numerous growth factors, decellularized matrices, skin grafts, negative pressure, hyperbaric-oxygen, stem cell delivery and even ultrasound (Game et al., 2012). Of these, very few are supported by strong evidence of efficacy (Hinchliffe et al., 2008; Game et al., 2012). One of the most promising treatments evaluated in clinical trials, rhPDGF-BB (marketed under the name Regranex®) has received criticism due to the limited applicability and moderate

effectiveness compared to placebo (Hinchliffe et al., 2008; Elliott and Hamilton, 2011). Trials of the profibrotic growth factor TGFβ showed no efficacy in closed label trials (Robson et al., 1995), despite promising results in animals (Beck et al., 1990; Zhao et al., 1994). Perhaps the somewhat disappointing results from growth factor based therapies may be explained, in part, by another group of proteins; the matricellular proteins.

Matricellular proteins are secreted non-structural matrix components which have been shown to influence all aspects of wound healing (Midwood et al., 2004; Hamilton, 2008; Elliott and Hamilton, 2011). They modulate the interactions between the cell and its extracellular environment, further regulating cellular behaviour including growth factor response. The matricellular proteins periostin and CCN2 have been heavily implicated in the fibrotic and proliferative stages of tissue repair; including collagen production, matrix contraction and fibroblast differentiation (Elliott and Hamilton, 2011). Moreover, it has been shown that extracellular periostin (Sidhu et al., 2010; Lorts et al., 2012) and CCN2 (Kothapalli et al., 1997; Duncan et al., 1999; Shi-wen et al., 2006) are required for proper TGFβ signaling in a context-dependant manner. We have shown that a loss of periostin results in a delay in wound closure attributed to attenuation of wound contraction (Elliott et al., 2012). Additionally, periostin may have a positive influence on re-epithelialization and fibroblast proliferation (Nishiyama et al., 2011; Ontsuka et al., 2012). CCN2 is required for appropriate myofibroblast recruitment in skin (Sonnylal et al., 2010; Liu et al., 2011) and positively influences angiogenesis in a variety of tissues (Hall-Glenn et al., 2012; Alfaro et al., 2013).

Local delivery of both of these proteins as therapeutics for the treatment of chronic skin wounds shows potential. Delivery of periostin into full-thickness excisional wounds of periostin knockout mice increases both closure rate (Ontsuka et al., 2012) and myofibroblast differentiation (Elliott et al., 2012). Administration of recombinant CCN2 in a burn model of wound healing results in increased closure rate and fibroblast recruitment (Liu et al., 2007). However, the relative expression levels of periostin and CCN2 in human chronic skin wound tissue have never been investigated. We hypothesize that these profibrotic matricellular proteins are significantly reduced in human chronic skin wounds. In the current study we show that in human chronic skin wound tissue

periostin expression is significantly reduced and CCN2 expression is not induced. Additionally, we demonstrate that fibroblasts from the edge of chronic skin wounds can be induced to express the profibrotic phenotype required for wound healing *in vitro*. Finally, in a model of impaired diabetic healing, we show that recombinant human periostin (rhPN) and CCN2 (rhCCN2) electrospun with collagen can rescue wound closure rates in diabetic db/db mice.

3.2 Results

3.2.1 Periostin and CCN2 are not induced in human chronic skin wounds

Immunohistochemical detection of periostin in human tissue samples (Table 3.1) showed strong reactivity within the dermis of the skin proximal to the wound edge (within 2 cm), as well as skin from a non-involved region of the limb (Figure 3.1a). At the wound edge, a sharp drop in periostin immunoreactivity occurred, which was also evident in the wound bed. CCN2 immunoreactivity was noted in the vasculature and epidermis of non-involved and proximal skin. CCN2 was present in the dermis/granulation tissue at the wound edge but was diminished within the wound bed. α-SMA immunoreactivity was restricted to blood vessels and exocrine glands in the non-involved and proximal tissue. At the wound edge, immunoreactivity was present in the blood vessels and dermis. Within the wound bed, both vasculature and stromal immunoreactivity of α-SMA was absent, indicating a lack of myofibroblast populations that would be expected within a remodeling wound. Furthermore, the density of blood vessels (visualized by α-SMA) was increased in proximal and wound edge tissue compared to non-involved skin. Masson's trichrome staining demonstrated a lack of collagen accumulation within the wound. A semi-quantitative comparison of periostin and CCN2 staining on the proximal and distal sides of the leading epithelial front (Figure 3.1b) confirmed that CCN2 was not increased beyond the epithelial front ($n = 14$, $p = 0.50$, two-tailed paired t-test). The percentage of area positively stained for periostin was significantly decreased distal to the leading epithelial front, compared to the intact skin on the proximal side of the epithelial front ($n = 15$, $p = 0.0004$, two-tailed paired t-test).

Table 3.1: Patient samples and demographics

	Sex	Age	Diabetic	Diagnosis	Location (amputation, wound location, cells obtained)
01	Male	74	No	PVD	Above knee, heel, wound cells
02	Female	79	No	PVD	Above knee, site of previous amp, wound cells
03	Male	34	Type One	Infection	Below knee, back of heel
04	Female	46	Type Two	PVD	Below knee, side of foot
05	Male	55	Type Two	PVD	Above knee, site of previous amp, wound cells
06	Male	70	Type Two	PVD	Below knee, side of foot, wound edge cells
07	Female	83	Type Two	PVD	Below knee, side of foot, wound edge cell
08	Male	65	Type Two	PVD	Above knee, side of calf
09	Male	78	Type Two	PVD	Foot amputated, sole of foot
10	Male	66	Type Two	PVD	Below knee, anterior ankle/foot
11	Male	56	Type Two	PVD	Below knee, previous toe amp
12	Male	86	Type Two	PVD	Below knee, base of big toe
13	Male	58	Type Two	PVD	Below knee, side of heel
14	Male	47	Type Two	PVD	Below knee, site of previous amp mid foot
15	Male	73	Type Two	PVD	Below knee, side of foot
16	Male	79	Type Two	PVD	Below knee, side of foot
17	Male	88	Type Two	PVD	Above knee, heel of foot, wound edge cells
18	Female	87	Type Two	PVD	Below knee, between toes, wound edge cells

PVD: Peripheral vascular disease

Figure 3.1: Periostin and CCN2 are not induced in human chronic skin wounds. Human chronic skin wound tissues were examined with **(a)** Masson's trichrome for collagen, IHC for periostin, α-SMA and CCN2 on sections from regions varying in distance from the wound edge (top). The Proximal region is approximately 2 cm from the wound edge. Scale bar = 200 μm. **(b)** To quantify periostin and CCN2 staining, images were masked for background or empty space (yellow) and positive DAB staining (red). Periodontal ligament (top), stained for periostin, is shown for illustration of the masking technique. The area of each mask was recorded separately for the wound bed and the intact tissue, demarcated by a vertical line at the leading edge of the epidermis (red arrowheads). Percent tissue area positively stained was calculated as per Methods ($n = 15$, * = $p < 0.001$, red arrows indicate the median, paired t-test). **(c)** RNA was extracted from human tissues and analysis of gene expression via RT-qPCR was carried out with probes specific for *POSTN, COL1A2, ACTA2* and *CCN2*, normalized to the endogenous control gene, *18S*. *POSTN* expression was significantly lower in wound edge tissue compared to non-involved tissue ($n = 10$, $p = 0.046$, Friedman test). *COL1A2, ACTA2* and *CCN2* expression were not increased at the wound edge.

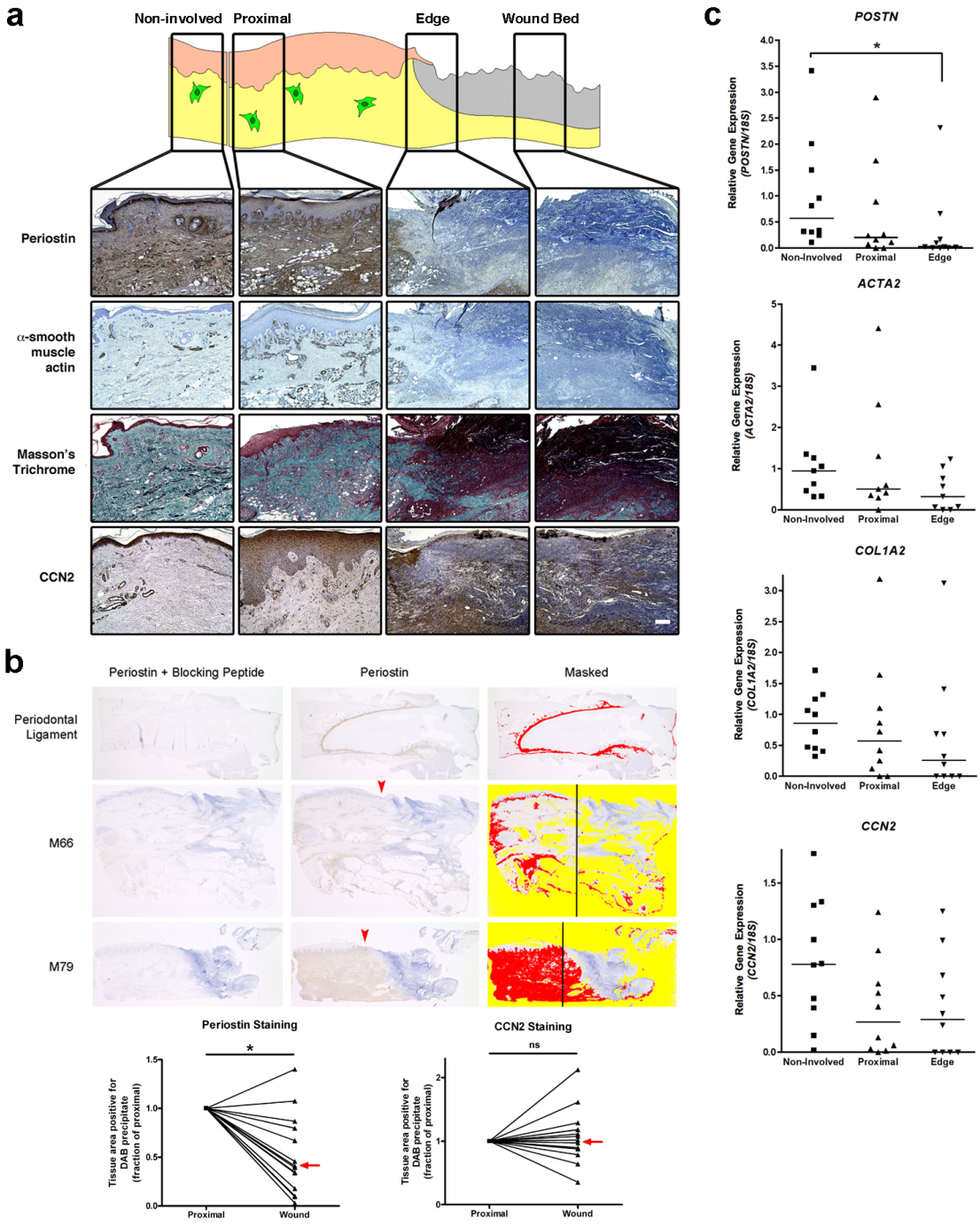


Figure 3.1: Periostin and CCN2 are not induced in human chronic skin wounds

To confirm our histological findings, we performed RT-qPCR on frozen human tissue samples (Figure 3.1c). *ACTA2* (n = 9, p = 0.28, Freidman test), *COL1A2* (n = 10, p = 0.37, Freidman test) and *CCN2* (n = 10, p = 0.32, Freidman test) transcripts showed a downward trend with proximity to the wound, although not significantly decreased. *POSTN* was significantly decreased at the wound edge, compared to non-involved tissue (n = 10, p = 0.046, Friedman test).

3.2.2 Human Chronic skin wounds are stalled in an inflammatory stage of wound healing

Previous studies with mouse models have shown that both periostin and CCN2 should be significantly induced during skin healing (Igarashi et al., 1993; Elliott et al., 2012), however, this was not evident in the human chronic skin wound tissue used in this study (Figure 3.1). To understand why, we next investigated cellular components and inflammatory mediators in the tissue samples. Immunoreactivity for TNFα, CD68 (macrophages) and neutrophil elastase all increased progressively with proximity to the wound bed (Figure 3.2). The profibrotic cytokine, TGFβ (using a pan-specific antibody), was detected at increasingly higher levels towards the wound bed. In non-involved skin immunoreactivity for TGFβ, neutrophil elastase and CD68 was largely absent. In addition, TNFα immunoreactivity remained present, albeit at much lower levels than in the wound bed tissue. Vimentin, a marker for mesenchymal cells such as fibroblasts and smooth muscle, was sparsely distributed throughout the dermis of non-involved and proximal regions, with increased density near the dermal-epidermal junction and the wound edge. Beyond the leading epithelial front there was a sharp decline in vimentin-positive cells, indicating a deficit in fibroblast infiltration into the wound bed. Periostin was strongly associated with mesenchymal cells at the dermal-epidermal junction up until the wound edge where periostin immunoreactivity abruptly declined (Figure 3.2). Small populations of mesenchymal cells were found beyond the wound edge, however they were not associated with periostin immunoreactivity.

Figure 3.2: Human chronic skin wounds are stalled in an inflammatory stage of wound healing. Human chronic skin wound tissues were examined with IHC for TNFα, CD68 (macrophage marker, ED-1), neutrophils elastase, TGFβ1 and vimentin on sections from regions varying in distance from the wound edge (top). The chronically inflamed granulation tissue showed markers for macrophages and neutrophils. TGFβ1 was present within the wound yet various fibrotic TGFβ1 target genes were not induced. Scale bar = 200 μm. Immunofluorescence for periostin and vimentin (bottom) was used to determine the extent of mesenchymal cell infiltration beyond the wound edge. A population of mesenchymal cells persisted beyond the wound edge but did not associate with periostin. Relative spatial relationship of these images is indicated diagrammatically (top).

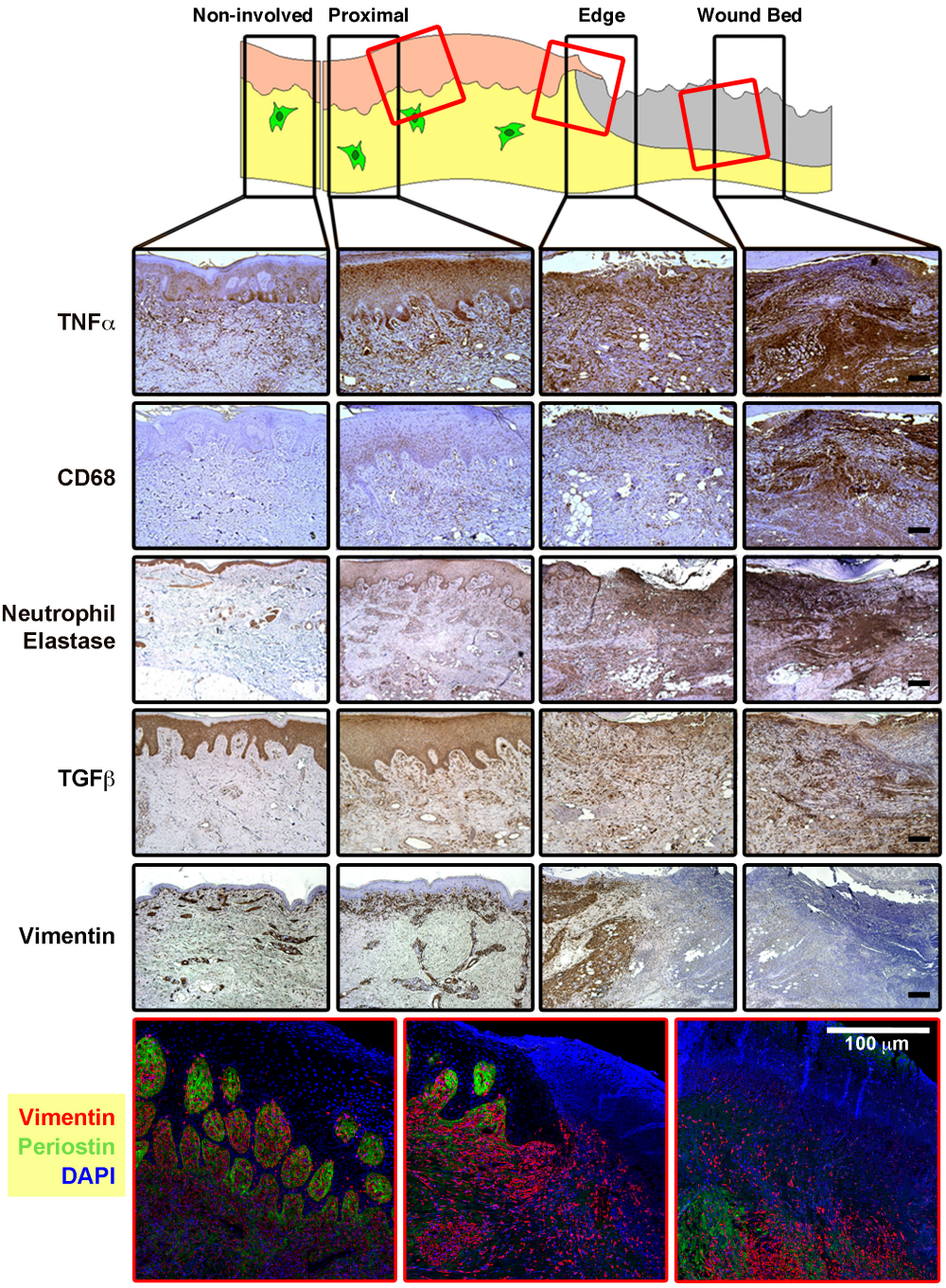


Figure 3.2: Human chronic skin wounds are stalled in an inflammatory stage of wound healing

3.2.3 Fibroblasts isolated from human chronic skin wound tissue can be induced to adopt a pro-fibrogenic phenotype

To determine if fibroblasts from human chronic skin wound tissue were inherently dysfunctional we cultured fibroblasts from non-involved and wound tissue explants. Initially, cells were grown from the wound tissue samples that spanned the proximal region (Figure 3.3a). These cells responded to stimulation with TGFβ1 similarly to non-involved fibroblasts (Figure 3.3a). Based on histological observations indicating that the proximal tissue was very similar to non-involved skin (Figure 3.1a,b and 3.2) we chose to focus our attention on fibroblasts cultured exclusively from the wound edge (Figure 3.3a).

Wound edge fibroblasts were more responsive to TGFβ1 with respect to *POSTN* ($n = 4$, cell source $p = 0.023$, treatment $p = 0.012$, interaction $p = 0.071$, two-way ANOVA) and *ACTA2* ($n = 4$, cell source $p = 0.047$, treatment $p = 0.0033$, interaction $p = 0.049$, two-way ANOVA) induction, compared to non-involved fibroblasts (Figure 3.3b). TNFα treatment depressed *POSTN* and *ACTA2* expression in both non-involved and wound edge fibroblasts. *COL1A2* ($n = 4$, treatment $p = 0.011$, interaction $p = 0.4734$, two-way ANOVA) and *CCN2* ($n = 4$, cell source $p = 0.71$, treatment $p = 0.0004$, interaction $p = 0.73$, two-way ANOVA) gene expression was similarly affected by TGFβ1 and TNFα stimulation between non-involved and wound edge fibroblasts. However, *COL1A2* expression was reduced in wound edge, compared to non-involved fibroblasts ($n = 4$, cell source $p = 0.0001$, two-way ANOVA). TNFα treatment attenuated the response to TGFβ1.

We next compared wound edge fibroblasts to healthy adult human fibroblasts (HDFa) (Figure 3.3c). *POSTN*, *COL1A2* and *CCN2* expression patterns in HDFa cells treated with TGFβ1, TNFα, or both were similar to those of the wound edge cells. TGFβ1 increased expression of these genes and TNFα attenuated its effects ($n = 3$, $p < 0.01$, one-way ANOVA). *ACTA2* expression was not increased in HDFa cells following TGFβ1 treatment, but instead was decreased ($n = 3$, $p = 0.0001$, one-way ANOVA). To confirm that this decrease was not simply due to initial high basal expression levels, we

performed the same experiments and measured α-SMA protein levels (Figure 3.3d). Western blots confirmed the lack of α-SMA induction in HDFa cells and clearly demonstrated that this was not a consequence of high basal expression (n = 4, cell source p = 0.0004, two-way ANOVA).

Next, we conducted proliferation assays (Fig. 6c) comparing isolated fibroblasts to HDFa. After 10 days in culture, HDFa cultures underwent 3.55 population doublings (n = 3). Non-involved and wound fibroblasts (n = 4) underwent 4.09 and 4.11 population doublings, respectively (cell source p = 0.0017, time p = 0.0001, interaction p = 0.91, two-way ANOVA).

Finally, we sought to determine if isolated wound fibroblasts were capable of contracting a collagen matrix. Non-involved and wound fibroblasts were similarly capable of contracting a fixed matrix collagen gel. Treatment with TGFβ1 provided no additional increase in gel contraction but treatment with TNFα was found to inhibit gel contraction (Figure 3.3f). A combination of TGFβ1 and TNFα resulted in an intermediate level of contraction (n = 4, cell source p = 0.76, treatment p = 0.0001, interaction p = 0.83, two-way repeated measure ANOVA). To determine if the degree of contraction was comparable to healthy adult fibroblasts, we conducted similar assays with HDFa cells (Figure 3.3g). HDFa (n = 3) cells contracted collagen gels to 65% of their original mass (measured from gels lacking cells), whereas wound cells (n = 5) contracted gels to 39% of their original mass. This difference was found to be significant (p = 0.034, two-tailed t-test). From this series of experiments we concluded that wound fibroblasts were not inherently dysfunctional.

3.2.4 Excisional wound healing in the genetically diabetic db/db mouse closely mimics human chronic skin wounds

Based on defined role of periostin and CCN2 in the proliferative phase of wound healing and their inappropriate expression patterns in human chronic skin wound tissue (Figure 3.1), we hypothesized that addition of rhPN and rhCCN2 could be used to push chronic skin wounds into the proliferative phase (Figure 3.4a). To test the effectiveness of adding rhPN and rhCCN2 to chronic skin wounds, we adopted the widely documented db/db

Figure 3.3: Fibroblasts isolated from human chronic skin wound tissue can be induced to adopt a pro-fibrogenic phenotype. (a) Human dermal fibroblasts were cultured *in vitro* from skin samples near the site of amputation (non-involved) and an area extending 2 cm from the wound boarder (wound). Alternatively, the source of cells was restricted to the tissue directly at the wound edge ($n = 4$, * = $p < 0.05$ compared to non-involved, two-way ANOVA). Cells wound tissue responded similarly to cells from non-involved tissue, prompting a focus on wound edge cells. **(b)** Cells (P_1) were treated with 5 ng/mL TGFβ1, 1 ng/mL TNFα or both for 24 hours. Quantitative RT-qPCR was carried out with probes specific for *POSTN, COL1A2, ACTA2* and *CCN2*. Data was normalized to the endogenous control gene, *18S*. *COL1A2* expression at baseline was reduced in cells cultured from the wound edge compared to cells cultured from non-involved skin (# = $p < 0.05$, two-way ANOVA). For *POSTN* and *ACTA2*, cells harvested from the wound edge were found to be more robust in response to TGFβ1 (* = $p < 0.05$). Treatment with 1 ng/mL TNFα decreased *POSTN* expression and attenuated the response to TGFβ1 for *POSTN, COL1A2, ACTA2* and *CCN2*. **(c)** Adult healthy human dermal fibroblasts were treated and assessed as above. Letters indicate statistically similar treatments ($n = 3$, $p < 0.05$, one-way ANOVA). **(d)** Response of α-SMA to TGFβ1 was confirmed at the protein level by western blot ($n = 4$). **(e)** Wound, non-involved and healthy human dermal fibroblasts were assessed for proliferation. Wound edge and non-involved fibroblasts were significantly more proliferative than healthy adult human dermal fibroblasts ($n = 4$, $p = 0.0017$ for cell source, two-way ANOVA). **(f)** Cells (P_1) were assessed for their ability to contract a fixed matrix collagen gel. **(g)** Wound cells contracted collagen gels to a further extent than healthy human dermal fibroblasts ($n = 4$, $p < 0.05$, t-test).

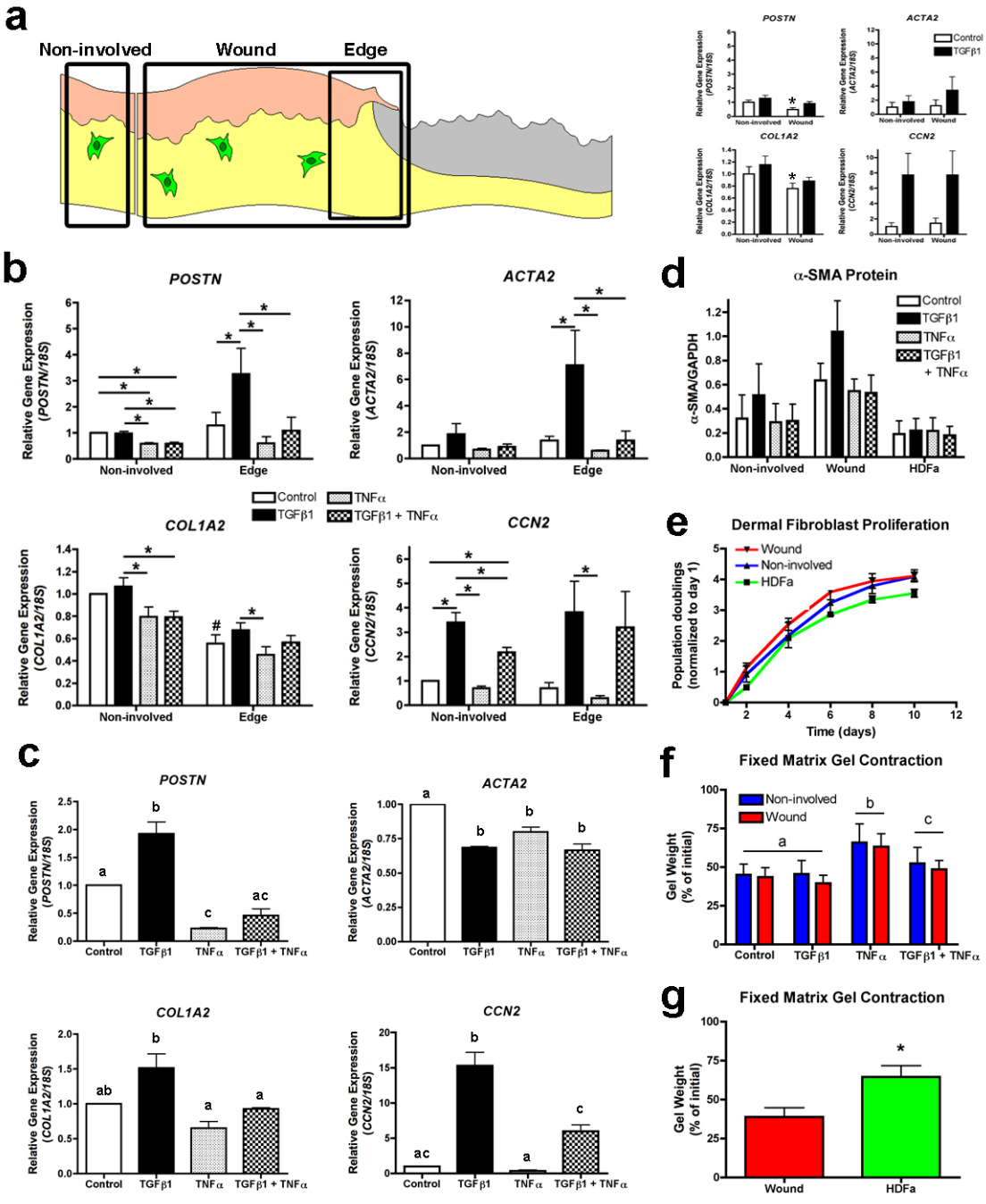


Figure 3.3: Fibroblasts isolated from human chronic skin wound tissue can be induced to adopt a pro-fibrogenic phenotype

Figure 3.4: Excisional wound healing in the genetically diabetic db/db mouse closely mimics human chronic skin wounds. (a) Proposed treatment approach for human chronic skin wounds using **(b)** electrospun collagen scaffolds containing rhPN or rhCCN2. Scaffolds were cut to size and inserted into excisional wounds on the backs of db/db mice and visualized via sulpho-rhodamine conjugation at day 1 post wounding. Scale bar = 200 μm. **(c)** Mice homozygous for the *Lepr*^{db} allele (db/db), on a C57BL/6 background were obese and hyperglycemic at 12 weeks of age (n = 10, * = $p < 0.0001$, t-test). **(d)** Adult wild-type (C57BL/6J) and db/db (B6.BKS(D)*Lepr*^{db}/J) mice were subjected to excisional cutaneous wounding and compared at day 7 to human chronic skin wound samples. The expression pattern of periostin, α-SMA, collagen and TNFα in db/db wounds closely mimicked that of human chronic skin wounds. Scale bar = 200 μm. **(e)** Total RNA was extracted from wild-type and db/db full-thickness excisional punch wounds at 7 and 11 days post wounding. Excised tissue was used for day 0 samples. Quantitative RT-qPCR was carried out with probes specific for *Postn, Col1a2, Acta2*, *Ccn2* and *Fn1*. Data was normalized to the endogenous control gene, *18s*. Compared to wild-type mice, db/db mice show a failure to appropriately induce fibrotic genes during wound healing (n = 5, # = $p < 0.05$ compared to day 0, * = $p < 0.05$ compared to wild-type, two-way ANOVA).

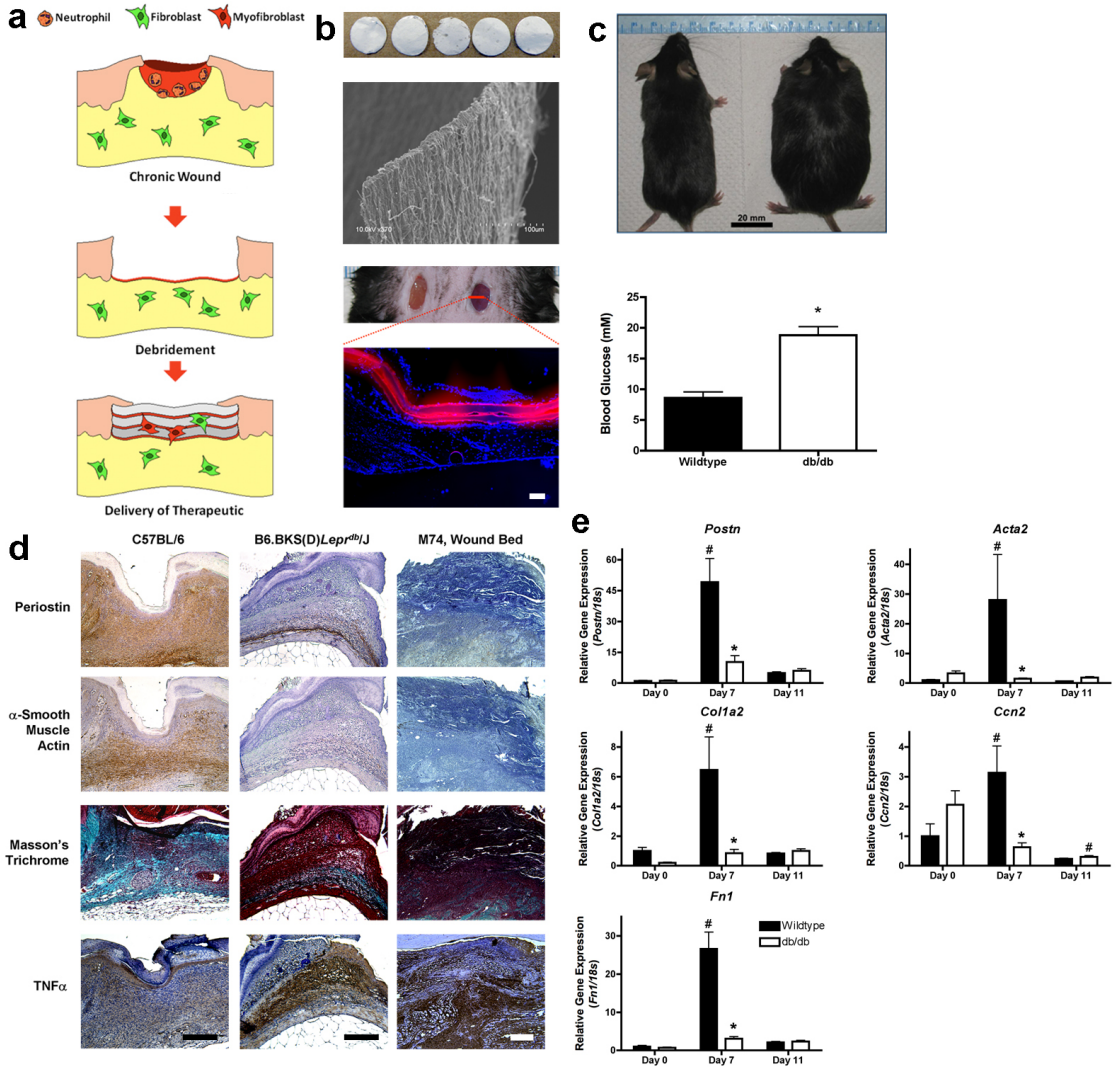


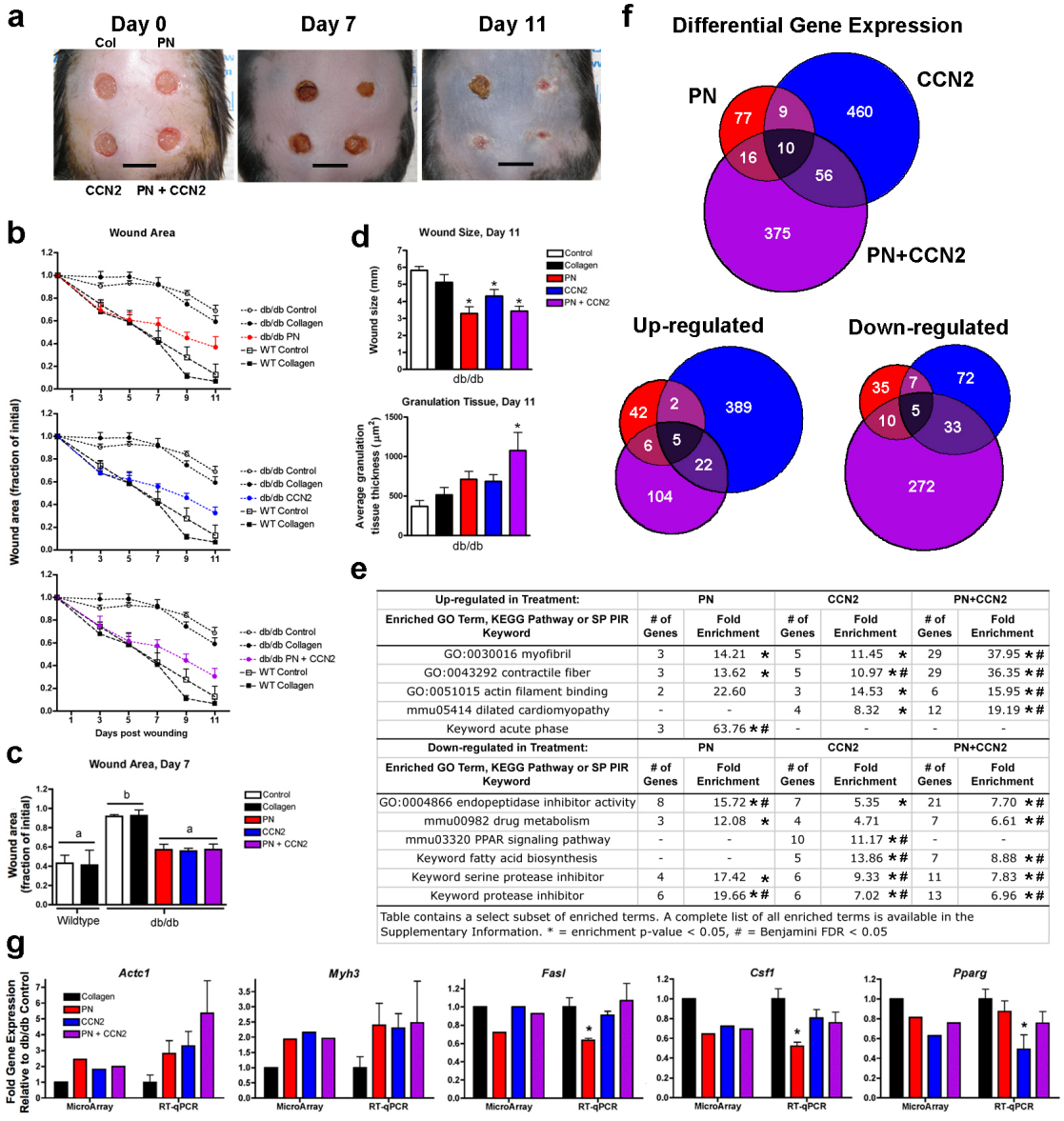
Figure 3.4: Excisional wound healing in the genetically diabetic db/db mouse closely mimics human chronic skin wounds

mouse model of type-two diabetes and employed our previously described electrospun collagen scaffold delivery mechanism (Figure 3.4b) (Elliott et al., 2012). Compared to C57BL/6J control mice (wild-type), db/db mice quickly became obese (wild-type 25.0 g, db/db 46.1 g at 12 weeks, n = 10, p = 0.0001, two-tailed t-test) and displayed an elevated fasting blood glucose level (wild-type 8.59 mM, db/db 18.78 mM, n = 10, p = 0.0001, two-tailed t-test) (Figure 3.4c). Day 7 wounds in db/db mice displayed lower periostin, α-SMA and collagen, but increased TNFα, compared to wild-type wounds. Day 7 db/db wounds closely resembled the histology of human chronic skin wounds with respect to these proteins (Figure 3.4d). RT-qPCR analysis of day 7 db/db wounds revealed a failed progression of the profibrotic phase of healing (Figure 3.4e).

3.2.5 Addition of recombinant human periostin or CCN2 increases the closure rate of excisional skin wounds in db/db mice

Healing of full-thickness excisional punch wounds (Figure 3.5a) was substantially delayed in db/db mice, compared to wild-type mice (Figure 3.5b). Addition of collagen scaffolds to either wild-type or db/db wounds did not significantly change the closure kinetics compared to untreated control wounds. Addition of scaffolds containing rhPN or rhCCN2 resulted in a significant reduction in wound area size by day 5 post wounding, and through to day 11 (time p = 0.0001, treatment p = 0.0001, interaction p = 0.0001, two-way ANOVA) (Figure 3.5b). An analysis of day 7 wound area measurements shows that collagen scaffolds alone did not affect closure rates in wild-type (n = 5) or in db/db mice (n = 11) (Figure 3.5c). Treatment with PN-containing or CCN2-containing scaffolds resulted in increased closure, similar to that in wild-type animals (n = 8, p = 0.0001, one-way ANOVA). Addition of one PN-containing scaffold and one CCN2-containing scaffold to the same wound (PN+CCN2) resulted in increased closure over controls, but not over PN or CCN2 alone (n = 6) (Figure 3.5c). At day 11, wound size was found to be significantly smaller in PN- ($p < 0.01$), CCN2- ($p < 0.05$) and PN+CCN2-treated wounds ($p < 0.01$) compared to control db/db wounds, as measured on tissue sections from the centers of the wounds (n = 5, one-way ANOVA) (Figure 3.5d). Granulation tissue

Figure 3.5: Addition of recombinant human periostin or CCN2 increases the closure rate of excisional skin wounds in db/db mice. Fully thickness excisional punch wounds were created in the skin of wild-type and db/db mice using a 6 mm biopsy tool. Wounds received either no treatment (control), electrospun collagen scaffolds (Collagen, Col), scaffolds containing rhPN (PN), scaffolds containing rhCCN2 (CCN2) or one scaffold of each (PN+CCN2). A total of two scaffolds were added per wound. **(a)** Wound area was assessed from photographs and expressed as a fraction of initial area. Scale bar = 10 mm. **(b)** Quantification of wound area from photographs showed a recovery of wound closure rates in the db/db wounds treated with PN, CCN2 or PN+CCN2. **(c)** At day 7, collagen alone is not sufficient to increase closure rate. PN, CCN2 and PN + CCN2 treated wounds exhibited accelerated healing. **(d)** At day 11, wound size measured from sections of the centre of the wound was significantly reduced in PN, CCN2 and PN+CCN2 treated wounds compared to db/db control wounds. Granulation tissue thickness was significantly increased in day 11 PN+CCN2 treated wounds compared to db/db control wounds. **(e)** Total RNA from the Collagen, PN, CCN2 and PN+CCN2 treated wounds of 3 db/db mice were used to generate cDNA, which was analyzed with Mouse Gene 2.0 ST arrays. Gene Ontology (GO), KEGG Pathway and SwissProt and Protein Information Resource (SP PIR) keyword enrichments were generated using DAVID Bioinformatics Resources 6.7, NIAID/NIH. Enrichments were filtered by a p-value and the Benjamini-Hochberg method was used to control for false discovery rate (FDR). **(f)** Differentially expressed genes were filtered based on an ANOVA p-value of less than 0.05 and 1.5 fold increase or decrease from db/db Collagen samples. **(g)** Genes that were differentially expressed and occurred in highly enriched terms were used to verify gene array data via RT-qPCR (n = 5).



e

Up-regulated in Treatment:	PN		CCN2		PN+CCN2	
Enriched GO Term, KEGG Pathway or SP PIR Keyword	# of Genes	Fold Enrichment	# of Genes	Fold Enrichment	# of Genes	Fold Enrichment
GO:0030016 myofibril	3	14.21 *	5	11.45 *	29	37.95 *#
GO:0043292 contractile fiber	3	13.62 *	5	10.97 *#	29	36.35 *#
GO:0051015 actin filament binding	2	22.60	3	14.53 *	6	15.95 *#
mmu05414 dilated cardiomyopathy	-	-	4	8.32 *	12	19.19 *#
Keyword acute phase	3	63.76 *#	-	-	-	-
Down-regulated in Treatment:	**PN**		**CCN2**		**PN+CCN2**	
Enriched GO Term, KEGG Pathway or SP PIR Keyword	**# of Genes**	**Fold Enrichment**	**# of Genes**	**Fold Enrichment**	**# of Genes**	**Fold Enrichment**
GO:0004866 endopeptidase inhibitor activity	8	15.72 *#	7	5.35 *	21	7.70 *#
mmu00982 drug metabolism	3	12.08 *	4	4.71	7	6.61 *#
mmu03320 PPAR signaling pathway	-	-	10	11.17 *#	-	-
Keyword fatty acid biosynthesis	-	-	5	13.86 *#	7	8.88 *#
Keyword serine protease inhibitor	4	17.42 *	6	9.33 *#	11	7.83 *#
Keyword protease inhibitor	6	19.66 *#	6	7.02 *#	13	6.96 *#

Table contains a select subset of enriched terms. A complete list of all enriched terms is available in the Supplementary Information. * = enrichment p-value < 0.05, # = Benjamini FDR < 0.05

Figure 3.5: Addition of recombinant human periostin or CCN2 increases the closure rate of excisional skin wounds in db/db mice

thickness was significantly increased in PN+CCN2-treated wounds compared to db/db control wounds, but not in either PN or CCN2 treatments ($n = 5$, $p = 0.0087$, one-way ANOVA) (Figure 3.5d).

To investigate the mechanisms through which these scaffolds were influencing wound healing, we isolated total RNA from wounds treated with collagen-only scaffolds, as well as PN-treated, CCN2-treated and PN+CCN2-treated wounds. Total RNA from day 7 wounds was subjected to whole genome transcriptional analysis. Genes for which expression levels changed by 1.5 fold (up or down, with a p-value ≤ 0.05) relative to db/db collagen wounds were filtered and analyzed for enriched Gene Ontology, KEGG Pathway and SwissProt and Protein Information Resource keywords (Figure 3.5e, Appendices C and D). The number of differentially expressed genes in the PN-treated wounds was 112 compared to the CCN2-treated wounds, where 535 genes changed (Figure 3.5f). In the PN+CCN2-treated wounds, a large number of differentially regulated genes were identified that were not identified in either PN- or CCN2-treated wounds alone (375) (Figure 3.5f), suggesting some interaction or synergistic effect of the combined treatment. Gene annotation analysis confirmed an enrichment of up-regulated genes associated with contractile machinery in all treatments (Figure 3.5e), with further enrichment still in PN+CCN2-treated wounds. Down-regulated gene lists were enriched for protease inhibitor terms, with higher enrichments for PN-treated wounds (Figure 3.5e). A large number of genes that were differentially regulated in the PN or CCN2 treatments were lost from the PN+CCN2 list (Figure 3.5f), indicating a loss of some of the effects of the singular treatments. Two such enrichments are the SP PIR Keyword acute phase, up-regulated in the PN treatment, and down-regulation of the PPARγ KEGG pathway with CCN2 treatment (Figure 3.5e). Genes that were differentially expressed and occurred in highly enriched terms were selected to verify microarray data via RT-qPCR (Figure. 3.5g). *Actc1*, *Myh3*, *Fasl*, *Csf1* and *Pparg* were chosen based on their relevance to tissue repair processes and were present in the most highly enriched terms from various treatments.

3.3 Discussion

The burden of chronic skin wounds is a large and growing concern for healthcare systems around the world. However, development and implementation of new and effective treatment options has not kept up. Most growth factor treatments have had limited efficacy when tested in clinical trials and those that do have effects provide only a moderate benefit (Hinchliffe et al., 2008; Game et al., 2012). Physical matrices, skin substitutes and cell-based treatments (including stem cell therapies) can be extremely costly but have thus far shown no competitive advantage for treatment of these skin wounds. It is becoming widely recognized that combination approaches will become the successes of the future and that single factor approaches simply do not address the complexities of these chronic skin wounds. However, in designing these treatments, matricellular proteins have not yet been included in the discussion. Matricellular proteins are critically important in facilitating the profibrotic behaviours of cells and their response to growth factors (Brekken and Sage, 2000; Garrett et al., 2004; Shi-wen et al., 2006; Lorts et al., 2012). Inclusion of matricellular proteins in therapeutic design may provide the missing links to facilitate growth factor signaling, cell differentiation and other important steps in wound healing. Unfortunately, very little is known about the expression and function of matricellular proteins in chronic skin wounds.

We demonstrate here that the matricellular proteins periostin and CCN2 are inappropriately expressed in human chronic skin wounds. Studies in mice indicate that both of these proteins should be induced by day 3 following wounding (Igarashi et al., 1993; Elliott et al., 2012). Although CCN2 was not reduced at the wound edge compared to non-involved tissue, it did not significantly increase either, as would be expected. Interestingly, previous research has shown that CCN2 is reduced in venous ulcers of greater than 6 month duration, compared to acute wounds of less than 6 weeks duration in otherwise healthy adults (Minhas et al., 2011). Therefore, depending on the etiology of the wounds, CCN2 may show different expression patterns but overall it is not up-regulated as in acute mouse wounds. We observed an abrupt decrease in periostin immunoreactivity at the wound edge, confirmed by RT-qPCR. Interestingly, periostin transcript has been shown to be dramatically up-regulated in laser-captured blood vessels

from the edge of chronic skin wounds, compared to blood vessels from intact human skin (Roy et al., 2007). However, it is unclear from that study from exactly where, with respect to the wound edge, the samples were obtained and how that compares to the regions we describe surrounding the wound edge.

The underlying cause(s) for reduced expression levels of periostin and CCN2 in chronic skin wounds are not currently known. We hypothesize that their expression pattern is a consequence of the inflammatory state of these wounds. We detected increased markers of inflammation beyond the wound edge, including neutrophil elastase, CD68 and TNFα. This is in agreement with previous reports showing increased neutrophil elastase in chronic compared with acute wound fluid (Trengove et al., 1999). Several groups have documented elevated TNFα levels in chronic skin wound fluid (Wallace and Stacey, 1998; Trengove et al., 2000; Cowin et al., 2006). Furthermore, systemic anti-TNFα or anti-macrophage treatments have been shown to rescue delayed wound closure in the leptin deficient ob/ob mouse model of type-2 diabetes (Goren et al., 2007). The anti-fibrotic influence of TNFα is well documented, including a recent study demonstrating its ability to inhibit periostin expression both in periodontal ligament and gingival fibroblasts (Arancibia et al., 2013; Padial-Molina et al., 2013). We show that TNFα suppresses periostin gene expression and attenuates TGFβ1 induction of periostin and CCN2 in dermal fibroblasts, *in vitro*. These results may explain the pattern of periostin and CCN2 expression found in human chronic skin wound edge and wound bed tissue. However, based on its role in the normal immune response, inhibition of TNFα for the treatment of chronic skin wounds is not an option (Wolbing et al., 2009).

An alternative explanation for the expression pattern of periostin and CCN2 (as well as other fibrotic genes) is that the fibroblasts surrounding the chronic skin wound are senescent and therefore inherently dysfunctional. There is considerable conflicting evidence in the literature concerning the physiology of these wound edge fibroblasts. Cultured chronic skin wound fibroblasts have been shown to produce less collagen, have lower growth rates, reduced responses to PDGF-BB and TGFβ, reduced ability to contract collagen gels and exhibit higher levels of the senescence marker SA-beta-Gal (Telgenhoff and Shroot, 2005). Yet these indicators of senescence are highly dependent

on where the fibroblasts were harvested from around the wound. Brem and colleagues cultured fibroblasts from the edge of venous ulcers and compared those to fibroblasts from adjacent skin (closer to the wound edge than our proximal region). They found that wound edge cells migrated significantly slower and assumed a flattened polygonal morphology typically associated with senescence (Brem et al., 2007). In a subsequent study, they subdivided the wound edge into tissue removed during debridement and tissue that remained after debridement. Fibroblasts were isolated from these tissues and again assessed for morphology and migration (Brem et al., 2008). They found that wound edge fibroblasts from the tissue remaining after debridement migrated faster in response to GM-CSF and adopted a spindle shape. We have found that wound edge fibroblasts are responsive to TGFβ1 and TNFα (significantly increased compared to non-involved fibroblasts with respect to *POSTN* and *ACTA2*), and that they proliferate and contract collagen gels significantly more than HDFa. From our data, and the work of others, we hypothesize that fibroblasts from the open wound likely are few and exhibit senescent qualities. The wound edge however seems to contain a population of fibroblasts that are highly capable of up-regulating profibrotic genes and assuming a myofibroblastic phenotype. The samples we collected likely spanned both wound edge regions described by Brem and colleagues, and it is possible that that our populations represent an enrichment of those fibroblasts that were able to migrate and proliferate quickly. Nevertheless, those fibroblasts are a resource that should be harnessed in future treatment designs.

Previous reports have documented dysregulation of other matricellular proteins in chronic skin wounds, including thrombospondin 1, tenascin-X (Shih et al., 2012) and galectin-3 (Pepe et al., in press). However, to our knowledge the use of matricellular proteins as a therapeutic for chronic skin wounds has never been previously investigated. We have shown here that local delivery of rhPN and rhCCN2 electrospun with collagen are potential therapeutics for the treatment of chronic skin wounds. Both periostin and CCN2 significantly increased would closure rates, an effect that could not be attributed to the electrospun collagen scaffold vehicle alone. Although we only employed one dose of PN and CCN2, these doses were carefully chosen to represent physiologically relevant concentrations. Our scaffolds, cut to 8 mm disks, contained approximately 50 ng rhPN or

25 ng rhCCN2 per disk. With two scaffolds per wound, treatments consisted of 100 ng rhPN or 50 ng rhCCN2. Serum concentrations of periostin in patients with skin sclerosis ranged from 95.6 to 146.8 ng/mL depending on the severity of the disease (Yamaguchi et al., 2013). Using the same PN scaffolds, we have previously demonstrated a rescue of α-SMA immunoreactivity in *Postn*$^{-/-}$ mouse wounds (Elliott et al., 2012). Others have used a higher concentration of periostin (2 μg) to demonstrate therapeutic effects in *Postn*$^{-/-}$ mice (Ontsuka et al., 2012), however such a high dose is not only expensive, it is on par with that found in fibrotic pathologies such as Dupuytren's contracture (Vi et al., 2009), raising questions of safety. Serum concentrations of CCN2 in systemic sclerosis vary considerably but their central tendency ranges from 42 to 83 ng/mL depending on severity (Dziadzio et al., 2005). At 100 ng/cm^2 CCN2 produced favourable outcomes in a model of burn wound healing (Liu et al., 2007). Our 8 mm disks have a surface area of 0.503 cm^2, representing a 100 ng/cm^2 dose with 2 disks. For the combination treatment, one scaffold of each was added to the wounds, thereby halving the dose of both PN and CCN2.

At the doses of PN and CCN2 used in this study, we observed significant changes in gene expression patterns, including robust enrichments of contractile gene sets. CCN2 produced a larger and very distinct list of differentially regulated genes compared to PN, indicating that they function through different mechanisms despite their similar effects on wound closure rate. PN had a strong influence on genes associated with early inflammation, the defense response, and surprisingly, down-regulation of several genes associated with protease inhibitors. It is not completely unexpected that periostin can influence inflammatory processes. In models of allergic airway inflammation periostin is up-regulated by IL-13 and periostin-deficiency leads to reduced eosinophil infiltration and a bias towards type-1 helper T cell (T_h1) cytokine profiles, including stimulation of IFNγ (Blanchard et al., 2008; Sehra et al., 2011). T_h1-type inflammation is associated with cell death and tissue damage, whereas T_h2-type inflammation is associated with wound healing and fibrosis (Wynn, 2004). The balance between these profiles may be of critical importance in understanding how increased inflammation can lead to the divergent pathologies of both chronic skin wounds and fibrosis. In a model of allergy-

induced atopic dermatitis fibroblast-derived periostin is not just induced by IL-13, a key T_h2 cytokine, it goes on to shift the cytokine profile towards a T_h2 balance. Periostin does this by stimulating keratinocytes to produce various T_h2 cytokines but not the potent T_h1 cytokine, IFNγ (Masuoka et al., 2012). In our microarray data we saw a modest but statistically significant increase in IL-13 (1.2 fold) in all treatments. In the PN treatment we saw reduced expression of the key inflammatory mediators FasL and Csf1. FasL is a well-known inducer of apoptosis and is elevated in T_h1 cells (Suda et al., 1995). Csf1 (M-CSF) is an important modulator and chemoattractant for monocytes and macrophages. It is curious that PN treatment did not induce α-SMA expression, as was the case in *Postn*$^{-/-}$ mice (Elliott et al., 2012). It is possible that this is a consequence of the complex inflammatory state of db/db wounds compared to *Postn*$^{-/-}$ wounds. Certainly elevated TNFα could be the involved in this observation (Goldberg et al., 2007). Further research is needed to sort through the specific interactions of periostin and the inflammatory cells, but there is considerable evidence to suggest that periostin can be utilized as a modifier of early inflammation in skin healing.

Addition of CCN2-containing scaffolds caused a down-regulation of a number of genes associated with fatty acid synthesis, obesity and PPARγ signaling. PPARγ expression is dramatically reduced in systemic sclerosis and its signaling directly apposes the profibrotic actions of TGFβ. In dermal fibroblasts, TGFβ has been shown to reduce PPARγ expression and block the PPARγ-dependent adipogenic differentiation of mesenchymal cells (Wei et al., 2010). We did not see an up-regulation of TGFβ in our microarray data despite the fact that CCN2 is an important downstream mediator of TGFβ. It is possible that exogenously added CCN2 might act as a surrogate for TGFβ with respect to PPARγ regulation. When CCN2- and PN-containing scaffolds were combined in the same wound, a pattern of differential gene expression was observed which included a large number of genes that were not changed in either of the individual treatments, indicating a synergistic effect of PN and CCN2 delivery. Granulation tissue thickness also increased significantly more with the combined treatment than with either individual treatment. Conversely, a large number of genes that were differentially expressed in the individual treatments were lost in the presence of the combination

scaffold. The likely explanation for this is that halving the dose of both PN and CCN2 meant their individual effects were diminished. Future work should include higher doses of PN and CCN2 in the combined treatments to determine if all of these effects can be preserved. Still, many of the gene clusters enriched in the individual treatments were further enriched in the combination treatment, including those associated with contractile machinery.

With the diversity of roles matricellular proteins play in a multitude of tissues, developing them into effective treatment options for chronic skin wounds will require additional research. Unraveling the context-dependent nature of their functions is a challenge in itself. In this report we have shown that periostin and CCN2, two matricellular proteins intimately tied to fibrosis, are reduced in human chronic skin wounds that are stalled in an inflammatory state. We attribute this to the environment of the chronic skin wound, as fibroblasts removed from that environment express both periostin and CCN2, and exhibit a myofibroblast phenotype in response to TGFβ1. Addition of periostin or CCN2 to a model of impaired diabetic healing via a biologically compatible collagen scaffold resulted in recovery of wound closure. The mechanisms employed by PN and CCN2 were largely distinct but by combining both treatments in the same wound we saw a synergistic effect. We believe this to be the first account of matricellular proteins being used to aid in the closure of chronic skin wounds. Based on their apparent biological functions, it will be of great interest to further explore combination treatments of various matricellular proteins with other established therapeutics, such as growth factors. The outcome of TGFβ signaling has been shown to depend on both periostin and CCN2, where deletion of these proteins attenuates or redirects TGFβ-induced cell behaviours (Garrett et al., 2004; Shi-wen et al., 2006; Lorts et al., 2012). Reduced matricellular protein expression might be why clinical trials of TGFβ treatment did not work. More work is needed to confirm this.

3.4 Materials and Methods

3.4.1 Human Skin Sample Collection

Procedures involving human tissue were approved by the University of Western Ontario Review Board for Health Sciences Research Involving Human Subjects and are in accordance with the 1964 Declaration of Helsinki. Skin samples were obtained with informed consent from patients exhibiting non-healing skin wounds and undergoing elective lower extremity amputation for the affected limb (Table 3.1). Eighteen patients were enrolled with a median age of 71.5 years (ranging from 34 to 88). Of these, four were female. The majority of patients were type-two diabetic (n = 15) and one was type-one diabetic. Diagnosis of the patients' condition was almost exclusively peripheral vascular disease, making it likely that the samples collected were representative of arterial wounds. Sets of skin samples were collected from the wound site as well as from a non-involved region of the limb (Figure 3.1). At each site, samples were collected for histology, RNA isolation and cell culture, which were immersed in 10% neutral buffered formalin (Sigma Aldrich, St. Louis, Missouri), RNAlater® (Ambion, Carlsbad, California) or growth media, respectively, until they could be further processed. For RNA, tissues were snap frozen in liquid nitrogen and stored at -86°C. To culture human dermal fibroblasts, tissue samples were washed extensively in Dulbecco's Modified Eagle Medium (High Glucose) supplemented with 10% fetal bovine serum and 2% AA (200 units penicillin/200 μg streptomycin/0.5 μg/mL amphotericin B) (Gibco, Carlsbad, California). Skin was then incubated at 37°C, 5% CO_2 to allow fibroblasts to migrate onto the culture surface. For use, skin was removed and cells were cultured for two to three passages. Healthy adult human fibroblasts (HDFa) were from a commercial source (C-013-5C, Invitrogen, Carlsbad, California).

3.4.2 Tissue Preparation and Immunohistochemistry

Tissues were processed to paraffin and sectioned at 5 μm. Tissue sections were stained as previously described (Jackson-Boeters et al., 2009). Sections were blocked with 10% horse serum followed by primary antibody overnight at 4°C (Table 3.2). Detection was by ImmPRESS Ig peroxidase kits (Vector Laboratories, Burlingame, California) and

visualized with 3,3-diaminobenzidine (Vector Laboratories). Sections were counterstained with haematoxylin. Trichrome staining was carried out as previously described (Liu et al., 2008). To quantify periostin and CCN2 staining, slides were stained in one batch and photographed at low magnification with identical exposure settings. Images were masked for background/empty space (yellow) and positive DAB staining (red) in Image Pro Plus v7.0 (Media Cybernetics Inc., Rockville, Maryland). The same masking parameters were then applied to all images. The area occupied by these masks

Table 3.2: Antibodies and dilutions used in this study

Target	Product #	Dilution	Antigen Retrieval	Supplier
Periostin	sc49480	1/200	No	Santa Cruz Biotechnology, Santa Cruz, CA
α-Smooth Muscle Actin (IHC-P)	ab5694	1/100	No	Abcam plc, Cambridge, UK
α-Smooth Muscle Actin (WB, IF)	A5228	1/500	No	Sigma Aldrich, St. Louis, MO
CCN2	ab6992	1/100	No	Abcam plc, Cambridge, UK
TNF-α	ab6671	1/200	Heat Mediated	Abcam plc, Cambridge, UK
CD68	MCA1957	1/200	Heat Mediated	AbD Serotec, Oxford, UK
Neutrophil Elastase	ab68672	1/2000	Heat Mediated	Abcam plc, Cambridge, UK
TGF-β	ab66043	1/100	Heat Mediated	Abcam plc, Cambridge, UK
Vimentin	ab92547	1/400	No	Abcam plc, Cambridge, UK
GAPDH	MAB374	1/1000	No	Millipore, Billerica, MA

was recorded separately for the wound bed and the intact tissue, demarcated by a verticalline at the leading edge of the epidermis. Percent positively stained tissue area was calculated as:

$$\% = [\text{Area of red mask}/(\text{area selected} - \text{area of yellow mask})] \times 100$$

Immunofluorescent labeling of human tissue was carried out as above except detection was with appropriate fluorescently labeled secondary antibodies (Jackson ImmunoReaserch, West Grove, Philadelphia). Granulation tissue thickness and wound size were measured from images of sections from the centre of the wounds using Image Pro Plus v7.0.

3.4.3 RT-qPCR

Pieces of snap frozen tissue samples were homogenized in 1 mL of TRIzol® reagent (Invitrogen). Total RNA was extracted as per the manufacture's recommendations. Real-time quantitative PCR was carried out on 50 ng of total RNA using TaqMan® One-Step RT-PCR Master Mix and gene-specific TaqMan® probes (Applied Biosystems, Carlsbad, California). Gene expression was normalized to the endogenous control gene, *18S*. PCR efficiency was verified to fall between 90 and 110%, via dilution series, and relative expression was calculated using the ΔΔCT method (Livak and Schmittgen, 2001).

3.4.4 *In Vitro* Gene Expression and Western Blotting

Primary human dermal fibroblasts ($P_{2\text{-}3}$) were seeded at 30,000 cells/well in 6 well plates. 24 hours prior to treatment, cells were starved in serum free media. Treatment was with 5 ng/mL TGFβ1 (R&D Systems Inc., Minneapolis, Minnesota), 1 ng/mL TNFα (R&D Systems) or both for 24 hours in serum free media. RNA was harvested with 1 mL of TRIzol® reagent (Invitrogen). Protein was with RIPA buffer containing protease and phosphatase inhibitor cocktails (Sigma Aldrich), and concentration was determined by BCA assay (Pierce, Waltham, Massachusetts). Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. Membranes were washed with tris-buffered saline containing

0.05% tween-20 (TBST). Membranes were blocked with 5% milk TBST. Unequal loading was controlled for with GAPDH (Millipore, Billerica, Massachusetts). Detection was with appropriate peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch) and enhanced chemiluminescence (Pierce). Bands were quantified using Image J software.

3.4.5 Proliferation

Primary human dermal fibroblasts were seeded at 2,000 cells/well in 24 well plates in 10% FBS supplemented media. Media was changed every 48 hours throughout the course of the experiments. At the desired time-points, media was completely aspirated and the plate was frozen at -86°C. Once all time-points were captured, the CyQUANT cell proliferation assay kit (Invitrogen) was used to determine cell number as per the manufacturer's protocol. A standard curve was used to obtain cell number. Cell number was transformed to a Log_2 scale to calculate population doublings.

3.4.6 Gel Contraction

Gel contraction assays were conducted essentially as previously described (Shi-wen et al., 2004; Elliott et al., 2012). Collagen was prepared as follows: 10% 0.2 M HEPES (pH 8), 40% bovine collagen type-1 (Advanced BioMatrix Inc., San Diego, California) and 50% 2X Dulbecco's Modified Eagle Medium (High Glucose). Dermal fibroblasts were suspended in 0.5% FBS DMEM and mixed 1:1 with the collagen preparation to a final density of 100,000 cells/mL. 24 well tissue culture plates were pre-coated with BSA overnight then washed with PBS. Collagen/cell mix (0.5 mL) was added to each well and allowed to set at 37°C. Following polymerization, wells were flooded with 1 mL 0.5% FBS DMEM. After 24 hours, gels were separated from the surface of the plate and incubated for an additional 24 hours. To ensure that contraction of gels horizontally and vertically was accounted for, quantification of gel contraction was assessed by loss of gel weight, whereby contraction of the collagen matrix excluded growth media, thus reducing the weight of the gel (Tingstrom et al., 1992).

3.4.7 Scaffolds

Collagen type-1 (Sigma-Aldrich) was dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol to make a 15% (w/v) solution. Recombinant hPN (R&D Systems) was dissolved in PBS to make a 1 mg/mL solution. Twenty μL of the periostin solution was mixed with 20 μL BSA solution (100 mg/mL in PBS) and 3 mL of collagen solution. The mixture was injected at a speed of 1 mL/h by a syringe pump into a capillary charged with a voltage of +15 kV. The generated nanofibers were collected on a negatively charged (-10 kV) rotation mandrel. Control scaffolds contained 20 μL of PBS. To crosslink the scaffolds, they were immersed in 5% glutaraldehyde/ethanol solution for 30 min. Scaffolds were spun onto aluminum foil, and an 8 mm biopsy punch was used to cut the scaffolds. Each scaffold was sterilized in 100% ethanol and rinsed 3 times with PBS. The fabrication conditions for CCN2 scaffolds were the same except that the rhCCN2 (R&D Systems) solution was 0.5 mg/mL.

3.4.8 Electron Microscopy

Collagen scaffolds were mounted on 15 mm SEM stubs with adhesive disks and were then sputter coated with palladium-gold for 5 minutes at 8 mV using a Hummer VI sputter coater (Anantech Ltd., Battle Creek, Michigan). Images were obtained with a 3400-N Variable Pressure Scanning Electron Microscope (Hitachi Ltd., Tokyo, Japan) using an accelerating voltage of 10 kV.

3.4.9 Animals

All animal procedures were in accordance with protocols approved by the University Council on Animal Care at The University of Western Ontario. Wild-type C57BL/6J (000664, JAX® Mice and Services, Bar Harbor, Maine) and genetically diabetic db/db (B6.BKS(D)*Lepr*^{db}/J, 000697, JAX®) mice were housed in conventional caging and provided water and food ad libitum. All animals were subjected to 12 h light/dark cycle and temperature in accordance with the guidelines of the Canadian Council on Animal Care. For experiments, wild-type and db/db sex-matched adult mice (12 weeks of age) were anesthetized with an intraperitoneal injection of buprenorphine (50 μg/kg), followed by an injection of ketamine (100 mg/kg) and xylazine (5 mg/kg). Backs were shaved,

depilated and sterilized with iodine. Full-thickness excisional wounds were made on each side of the dorsal midline with a 6 mm punch biopsy. Removed tissue was considered day 0 and was retained for RNA analysis. Some wounds were left untreated as controls while others received: 2 x collagen, 2 x rhPN-containing (PN), 2 x rhCCN2-containing (CCN2) or 1 x rhPN- and 1 x rhCCN2-containing (PN+CCN2) scaffolds. Eight mm diameter scaffolds were inserted into each wound immediately following wounding. The larger 8 mm scaffolds aided in retention of the scaffolds within the wound by partially slipping under the surrounding skin. Wounds were photographed immediately after wounding and again at 3, 5, 7, 9 and 11 days post wounding. Wound area was assessed from photographs using Northern Eclipse v7.0 software (Empix Imaging Inc., Mississauga, Ontario) and expressed as a fraction of initial area. Mice were caged individually following wounding.

3.4.10 Blood Glucose

Animals were euthanized following 4 hours of fasting. Blood was collected via cardiac puncture and glucose was measured using a OneTouch® Ultra®2 blood glucose monitoring system (LifeScan, Inc., Milpitas, California).

3.4.11 Microarray

Total RNA from the Collagen-, PN-, CCN2- and PN+CCN2-treated wounds of 3 db/db mice and Collagen-treated wounds were used to generate cDNA. All sample labeling and GeneChip processing was performed at the London Regional Genomics Centre (Robarts Research Institute, London, Ontario, Canada; http://www.lrgc.ca). RNA quality was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Palo Alto, California) and the RNA 6000 Nano kit (Caliper Life Sciences, Mountain View, California). Single stranded complimentary DNA (sscDNA) was prepared from 200 ng of total RNA as per the Ambion WT Expression Kit for Affymetrix GeneChip Whole Transcript WT Expression Arrays (Applied Biosystems) and the Affymetrix GeneChip WT Terminal Labeling kit and Hybridization (Affymetrix, Santa Clara, California). Total RNA was first converted to cDNA, followed by *in vitro* transcription to make cRNA. Single stranded cDNA (5.5 μg) was synthesized, end labeled and hybridized for

16 hours at 45°C to Mouse Gene 2.0 ST arrays. A GeneChip Fluidics Station 450 performed all liquid handling steps and GeneChips were scanned with the GeneChip Scanner 3000 7G (Affymetrix) using Command Console v3.2.4. Probe level (.CEL file). Data were summarized to gene level data in Partek Genomics Suite v6.6 (Partek, St. Louis, MO) using the RMA algorithm (Irizarry et al., 2003). Partek was used to determine gene level ANOVA p-values and fold changes. Gene Ontology (GO), KEGG Pathway and SwissProt and Protein Information Resource (SP PIR) keyword enrichments were generated using DAVID Bioinformatics Resources 6.7, NIAID/NIH (Huang da et al., 2009b; Huang da et al., 2009a). Enrichments were filtered by p-value and the Benjamini-Hochberg method was used to control for false discovery rate (FDR). Differentially expressed genes were selected based on an ANOVA p-value of less than 0.05 and 1.5 fold increase or decrease from db/db Collagen samples.

3.4.12 Statistical Methods

Statistical analysis was by Graphpad Software v4 (Graphpad Software, La Jolla, California) ($p \leq 0.05$ was considered significant). Normality was assessed with the Kolmogorov–Smirnov test where sufficient replicates allowed. Unless stated otherwise data was found to be normally distributed. Two-tailed t-tests were used for periostin and CCN2 tissue staining quantification (paired) and for blood glucose measurements (unpaired). *In vivo* human tissue gene expression data was not normally distributed so the non-parametric paired Friedman test with a Dunn's multiple comparisons test was applied. A one-way ANOVA followed by a Bonferroni correction was used to analyze HDFa *in vitro* gene expression data. Comparative gene expression, protein expression and proliferation analysis of isolated non-involved and wound fibroblasts (and HDFa), as well as *in vitro* mouse wound gene expression were assessed with a two-way ANOVA followed by a Bonferroni correction. Gel contraction data was analyzed with a repeated measure two-way ANOVA for comparison of non-involved vs. wound cells (followed by Bonferroni correction), a two-tailed t-test was used to compare wound cells to HDFa. Wound closure kinetics in mouse punch wounds were analyzed with a two-way ANOVA (with Bonferroni correction). Wound size and granulation tissue thickness were analyzed with a one-way ANOVA (with Bonferroni correction).

3.5 References

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Chapter 4

4 Discussion

The overall research focus of this thesis was to investigate the specific roles of two matricellular proteins in skin pathology and the wound healing process, and to determine if local delivery of these proteins could be used as novel therapeutics for enhancing wound healing. Specifically, the focus was on periostin and CCN2, matricellular proteins that are associated with many types of tissue fibrosis, including hypertrophic and keloid scarring of the skin. In fibrotic conditions, periostin and CCN2 have been shown to regulate cellular processes associated with matrix accumulation, increased myofibroblastic phenotype and increased profibrotic growth factor signaling; all of which are reduced in chronic skin wounds. The role of CCN2 in skin healing had been previously documented, but it was necessary to identify the specific molecular roles of periostin in skin healing.

The specific objectives of this project were:

1. To determine how genetic deletion of periostin alters dermal wound healing kinetics and the underlying changes in regulation of dermal and epithelial behaviours.

2. To determine mRNA and protein expression patterns of periostin and CCN2 in human chronic skin wound tissue.

3. To quantify the phenotypic response of human chronic skin wound fibroblasts by exogenous TGFβ and TNFα.

4. To assess the efficacy of local delivery of periostin and CCN2 containing electrospun scaffolds as a therapeutic for enhancing wound healing using a diabetic murine model.

The results from objective one are documented in Chapter 2. In summary, deletion of periostin resulted in a delay in wound closure rate, which coincided with peak periostin

expression in wild-type animals. Delayed closure was attributed to a defect in myofibroblast differentiation based on failed α-SMA induction *in vivo* and a compromised myofibroblast phenotype *in vitro*. Addition of rhPN to *Postn*$^{-/-}$ wounds recovered α-SMA immunoreactivity, demonstrating potential for the delivery of extracellular periostin to dermal wounds to modulate fibroblast function.

Objectives two, three and four are presented in Chapter 3. In human chronic skin wound tissue, CCN2 was not increased compared to non-involved skin and periostin was significantly reduced within the wound. Inappropriate expression of these matricellular proteins may be a result of the inflammatory environment of the wounds, since fibroblasts isolated from wound tissue, and therefore removed from that environment, were able to respond to TGFβ, proliferate and contract collagen gels. Furthermore, the proinflammatory cytokine TNFα, which was abundant in the wound tissue, suppressed induction of periostin and CCN2 in human dermal chronic skin wound fibroblasts, supporting the contention that the wound environment was responsible for reduced periostin and CCN2.

The expression patterns of periostin and CCN2 in human chronic skin wounds, combined with the fibrotic potential of wound edge fibroblasts, indicated that delivering these proteins as therapeutics designed to recruit fibroblasts and induce their differentiation might be beneficial for would healing. Delivery of rhPN and rhCCN2 electrospun with collagen to dermal wounds in a mouse model of impaired diabetic skin healing resulted in rescue of wound closure to rates similar to wild-type mice. Whole genome transcriptional analysis indicated that the two proteins functioned through distinct mechanisms. A combination of the rhPN- and rhCCN2-containing scaffolds added to a single wound showed synergistic effects in gene expression changes, and also showed up-regulation of additional genes neither protein did on their own. This work represents the first matricellular protein-based biomaterial therapeutic designed with an aim to enhance chronic skin wound healing.

4.1 Periostin and wound healing in skin: a multi-faceted player?

This section contains excerpts modified from our published work (Appendix E):

Elliott. C.G., Kim, S.S., and Hamilton, D.W. (2012) Functional Significance of Periostin in Excisional Wound Healing: Is the Devil in the Detail? Cell Adh Migr. 6(4): 319-26

Although originally implicated in skin healing in 2005 (Lindner et al., 2005), the first report describing the impact of periostin deletion on healing was not described until 2011. Within one year, three independent papers were published describing potential roles for periostin in dermal wound healing from experiments performed using different derivations of periostin knockout mice (*Postn*$^{-/-}$). Beginning with Nishiyama and colleagues (Nishiyama et al., 2011), followed by our report (Elliott et al., 2012b) and finally Ontsuka et al (Ontsuka et al., 2012), all three independent studies confirm that periostin is significantly up-regulated following dermal wounding in mice, peaking at day 7. In normal skin periostin is localized at the dermal-epidermal junction (DEJ) and in hair follicles. Following excisional wounding in *Postn*$^{+/+}$ mice, periostin is predominantly expressed in the granulation tissue and in neighboring hair follicles. The absence of periostin results in delayed wound healing, which is most pronounced between days 3 and 7. The similarities in the studies begin to diverge, however, when we begin to look at the underlying mechanisms proposed by each of these reports (Table 4.1). The three studies proposed three very different mechanisms to explain the delay in *Postn*$^{-/-}$ wound healing, each supported by *in vitro* evidence.

It is surprising that three independent groups could come up with three divergent accounts of how the deletion of periostin resulted in delayed wound healing. The relevance of each proposed mechanism (contraction, keratinocyte proliferation and fibroblast migration/proliferation) to the *in vivo* wound healing phenotype is debatable. But the *in vitro* results are not and there is certainly support in the literature for each proposed cellular mechanism individually (Table 4.2). The differences were likely a consequence of the experimental variables between each study (discussed below). The diversity of cellular behaviours affected by the presence of periostin can serve as a lesson for the importance of the context dependent nature of matricellular protein influence in

Table 4.1: Major findings from three reports on skin healing in *Postn*$^{-/-}$ mice

	Elliott et al 2012	Nishiyama et al 2011	Ontsuka et al 2012
Wound Model	6 mm punch biopsy	3 mm punch biopsy	8 mm or 10 mm punch biopsy
Main Effect	KO delay at D5 and D7	KO delay at D3, D5 and D7	KO delay at D3, D5, D7 and D11
Proposed *In vivo* Cause	Myofibroblast differentiation	Re-epithelialization via keratinocyte proliferation	Fibroblast proliferation and migration
In vivo Evidence	Reduced α-smooth muscle actin gene expression and immunoreactivity in KO	Measurements from H&E stained sections Reduced Ki-67 immunoreactivity around KO hair follicles	No data
In vitro Support	Adult KO fibroblasts showed reduced: Force generation, Collagen gel contraction, α-smooth muscle actin immunofluorescence, α-smooth muscle actin protein	No data	Newborn KO fibroblasts show reduced proliferation KO MEFs show reduced migration
Rescue Tool(s) (*in vitro*)	Recombinant full-length human periostin (R&D systems) produced in a mouse myeloma cell line (NS0)	Expression vector for mouse periostin	Recombinant full-length mouse periostin (R&D systems) produced in an insect ovarian cell line (Sf21) Expression vector for full-length mouse periostin
Rescue? (*in vitro*)	Adult KO fibroblasts showed restored: Collagen gel contraction, α-smooth muscle actin staining, α-smooth muscle actin protein. (Force generation not tested for rescue)	Conflicting results: Over-expression of Ms *Postn* in human keratinocyte cell line (HaCaT) resulted in no difference in cell number when cultured for 96 hours. However, the same cells cultured for one week beyond confluence showed an increase in BrdU labelling.	Proliferation of newborn mouse fibroblasts (vector and recombinant) Proliferation of normal human dermal fibroblasts (recombinant)
Rescue Tool (*in vivo*)	Recombinant full-length human periostin (R&D systems) incorporated into an electrospun collagen scaffold	No *in vivo* rescue	Recombinant full-length mouse periostin (R&D systems) added directly onto wounds
Rescue? (*in vivo*)	Increased α-smooth muscle actin immunoreactivity at D7 No wound closure kinetics	No *in vivo* rescue	Restored wound closure kinetics No evidence for mechanism
Additional Findings	No difference in fibroblast migration No difference in re-epithelialization	Similar Ki-67 numbers in granulation tissue and migrating keratinocytes	

Table 4.2: Evidence from the literature supports all three mechanisms

Myofibroblast	Proliferation	Migration
Shimazaki 2008: *Postn*$^{-/-}$ mice had an increased incidence of ventricular rupture following myocardial infarction due to reduced α-SMA positive cells and impaired collagen formation (Shimazaki et al., 2008).	Ben 2011: Transfection of pancreatic cancer cell lines (BxPC-3 and Panc-1) with a periostin expression vector (Ad5-PN) promoted anchorage-independent growth (Ben et al., 2011).	Shimazaki 2008: Recombinant periostin (ΔbΔe splice variant) enhanced chemotaxis of cardiac fibroblasts from *Postn*$^{-/-}$ mice. This increase was attenuated by an anti-periostin antibody (Shimazaki et al., 2008).
Erkan 2007: Addition of rhPN to pancreatic stellate cells resulted in increased expression of α-SMA, collagen 1, fibronectin, TGFβ1 and periostin. Silencing periostin decreased α-SMA expression (Erkan et al., 2007).	Erkan 2007: Under serum deprivation, rhPN stimulated growth of Panc1, SU86.86, and T3M3 (pancreatic cancer cell lines) (Erkan et al., 2007).	Ben 2011: Cells (BxPC-3 or Panc-1) infected with Ad5-PN migrated and invaded faster than controls in transwell assays (Ben et al., 2011).
Vi 2009: Fibroblasts isolated from Dupuytren's disease (DD) over-expressed periostin and had an increased ability to contract a collagen matrix, which was further enhanced by addition of rhPN. DD cells in 3D culture induced α-SMA in response to rhPN (Vi et al., 2009).	Vi 2009: Growth on rhPN coated plates resulted in an increase in proliferation of palmar fascia fibroblasts (Vi et al., 2009).	
Sidhu 2010: Transfection of bronchial epithelial cells (BEAS2B) with a rhPN expression vector resulted in increased α-SMA protein and mRNA (Sidhu et al., 2010).	Liu 2011: Periostin-silenced gastric cancer cells exhibited reduced cell proliferation (Liu and Liu, 2011).	Liu 2011: Periostin-silenced gastric cancer cells exhibited reduced invasion using a Boyden chamber invasion assay (Liu and Liu, 2011).
Bozyk 2012: Hyperoxia exposure increased α-SMA positive myofibroblasts in the lungs of *Postn*$^{+/+}$, but not *Postn*$^{-/-}$, mice (Bozyk et al., 2012). Bozyk 2012: Periostin treatment increased α-SMA expression in neonatal lung mesenchymal stromal cells (Bozyk et al., 2012).	Bozyk 2012: Periostin induced human mesenchymal stromal cell DNA synthesis in the presence of TGFβ1 (Bozyk et al., 2012).	
Hakuno 2010: High fat diet-induced α-SMA in cardiac valve complexes is attenuated in *Postn*$^{-/-}$ mice (Hakuno et al., 2010).	Kuhn 2007: rhPN induced proliferation of neonatal cardiomyocytes in a PI3K/Akt dependent manner. Injecting rhPN into the myocardium induced DNA synthesis and division of nearby differentiated cardiomyocytes (Kuhn et al., 2007).	Hakuno 2010: Conditioned media from periostin transfected cells increased migration of human coronary artery endothelial cells (Hakuno et al., 2010).
Yoshida 2011: Silencing of periostin splice variant III attenuated TGFβ2 induced α-SMA production in primary human retinal pigment epithelial cells (Yoshida et al., 2011).	Zhu 2011: Neutralizing monoclonal antibody to periostin inhibited anchorage-independent growth of the periostin-expressing ovarian cancer cell line A2780 (Zhu et al., 2011).	Zhu 2011: Neutralizing monoclonal antibody to periostin inhibited periostin-induced cancer cell migration and invasion (Zhu et al., 2011).

Table 4.2: Evidence from the literature supports all three mechanisms, continued

Myofibroblast	Proliferation	Migration
Jackson-Boeters 2009: Periostin expression coincides with α-SMA expression within the granulation tissue of excisional wounds of mice (Jackson-Boeters et al., 2009).	Liu 2010: Inhibition of periostin expression via RNA interference suppressed proliferation of a human osteosarcoma cell line (U2OS) (Liu et al., 2010).	Liu 2010: Inhibition of periostin gene expression via RNA interference suppressed migration and invasion of U2OS cells in transwell assays (Liu et al., 2010).
Lindner 2005: Acquisition of a smooth muscle cell phenotype (α-SMA expression) correlated with acquisition of periostin expression both *in vitro* and *in vivo* (Lindner et al., 2005).	Yan 2006: Mice that received periostin-producing 293T cells at the mammalian fat pad tissue had significantly larger local tumours than did mice receiving control cells (Yan and Shao, 2006).	Lindner 2005: Periostin over-expressing C3H10T1/2 cells had greater migratory response to serum, which was attenuated by a periostin-blocking antibody (Lindner et al., 2005).
Hong 2010: Periostin over-expressing A549 cells expressed higher levels of vimentin mRNA (Hong et al., 2010).	Hong 2010: Periostin over-expressing A549 cells displayed increased proliferation (Hong et al., 2010).	Hong 2010: Periostin over-expressing A549 cells migrated and closed scratch wounds at an increased rate (Hong et al., 2010).
Kikuchi 2008: Close approximation of periostin immunoreactivity to α-SMA positive cells (periocryptal fibroblast) in normal colonic mucosa. Decreased periostin immunoreactivity preceded a decrease of α-SMA positive cells (Kikuchi et al., 2008).	Kikuchi 2008: Ki-67–positive epithelial cells were significantly decreased in the colonic crypts of *Postn*$^{-/-}$ mice (Kikuchi et al., 2008).	Yan 2006: Using transwell assays, significantly more periostin-expressing 293T cells migrated into the membrane relative to control cells (Yan and Shao, 2006).

general. For example, we did not detect a difference in migration (Figure 2.6) or proliferation (Elliott et al., 2012a) between *Postn*$^{+/+}$ and *Postn*$^{-/-}$ adult dermal fibroblasts. Ontsuka reported significantly reduced proliferation in newborn *Postn*$^{-/-}$ fibroblasts and significantly reduced migration in *Postn*$^{-/-}$ mouse embryonic fibroblasts (MEF), compared to their respective *Postn*$^{+/+}$ controls. We attributed these discrepancies to the use of adult vs. embryonic or newborn cells (Table 4.1).

We have previously reported that periostin expression/localization is very different in developing and newborn mouse skin when compared to that of skin in the adult animal (Zhou et al., 2010). From embryonic day 13.5 forward, periostin is expressed in the developing skin. At 2 and 9 days old, periostin is heavily expressed at the dermal epidermal junction (DEJ) and in the dermis of the newborn skin. This pattern changes dramatically by day 19 where periostin expression in the dermis is largely absent. By day 60, periostin is restricted to hair follicles and the DEJ, although at a reduced level. Perhaps the role of periostin in development, and thus newborn fibroblasts or MEFs, is vastly different from its role in adult skin and the residing dermal fibroblast. If this is true then we must be careful when choosing our *in vitro* tools to confirm *in vivo* findings. To emphasize this, we have shown that in 2D culture *Postn*$^{-/-}$ fibroblasts do differentiate into myofibroblasts and it is only when the substrate rigidity is reduced (Figure 2.11) or 3D culture employed (Figure 2.8 and 2.10), that the periostin deficiency and loss of the myofibroblast phenotype manifests in knockout cells.

Deletion of periostin consistently resulted in delayed wound healing across the three studies. But *in vitro* explanations for this are difficult to interpret due to the use of different tools and differing experimental designs. Understanding how the presence of periostin effects wound healing may be facilitated through *in vivo* rescue experiments. We have shown that the addition of rhPN to *Postn*$^{-/-}$ wounds results in increased α-SMA protein at day 7, demonstrating that rhPN can modulate fibroblast to myofibroblast transition during wound healing. Whether this increase in α-SMA translates into accelerated wound closure kinetics was reserved for future publication, but is now available (Figure 3.5) and will be commented on below. Ontsuka also recognized the

importance of *in vivo* rescue experiments using recombinant periostin. In their experiments, they documented the closure kinetics of *Postn*$^{+/+}$ and *Postn*$^{-/-}$ wounds, with and without addition of recombinant periostin. Their findings were very encouraging; showing a complete restoration of normal closure kinetics in *Postn*$^{-/-}$ wounds and accelerated closure in *Postn*$^{+/+}$ wounds. Together these two outcomes paint a very promising picture for periostin as a therapeutic. Interestingly, in both rescue attempts periostin was added to the wounds earlier than the endogenous peak (day 0 in our report, day 1 and every second day to day 9 for Ontsuka). The wound closure kinetics provided by Ontsuka show an immediate response to exogenous periostin, statistically significant at day 3. We now know that addition of rhPN to the wounds of db/db mice results in a similar increase in wound closure rate (Figure 3.5), including an early departure from the control kinetics. We observed an increase in closure rate as early as day 3, although not statistically significant until day 5.

We show that in *Postn*$^{-/-}$ wounds exogenous periostin increases α-SMA by day 7 (Figure 2.12), but can myofibroblast differentiation be a major contributor to wound closure at day 3? At such an early time point fibroblast migration into the wound is a key event. There is support for the hypothesis that tractional forces of fibroblast migration alone, as opposed to myofibroblast based contraction, can generate sufficient force to initiate wound contraction (Ehrlich et al., 1999; Au and Ehrlich, 2010). As resistance in the newly formed matrix increases, fibroblasts differentiate into myofibroblasts to complete closure. It is possible that the presence of exogenous periostin influences fibroblast migration at earlier time points. However, there is no *in vivo* evidence to support this and our data argues against a migratory role for periostin *in vivo*.

The day 3 wound is largely a pool of inflammatory cells and relatively few fibroblasts. Periostin has been shown to regulate chemokine release in lung fibroblasts, thereby modulating neutrophil and macrophage recruitment (Uchida et al., 2012). Additionally, as discussed in Chapter 3, periostin has been linked to the balance of T-helper cell type cytokine profiles, where its presence shifts the cytokine profile towards a T_h2, profibrotic bias (Masuoka et al., 2012). In our microarray data we saw statistically significant reductions in the expression of the key inflammatory mediators FasL and Csf1 in the PN-

treated wounds (Figure 3.5). Therefore, several lines of evidence demonstrate that periostin can modulate the inflammatory response. The observation that PN-containing treatment did not induce α-SMA expression, as was the case in *Postn*$^{-/-}$ mice (Elliott et al., 2012b), may be a consequence of the complex inflammatory state of db/db wounds compared to *Postn*$^{-/-}$ wounds. One hypothesis is that TNFα inhibits myofibroblast differentiation in the db/db wounds (Figure 3.3) (Goldberg et al., 2007). More work is needed to sort through the specific interactions of periostin and the immune response. Whether these inflammatory effects or another discrete pathway are involved in periostin-dependant α-SMA expression has yet to be determined.

4.2 Are matricellular protein expression patterns and the models we used relevant to human healing?

Our understanding of human skin healing is largely based on what we have learned from mouse models of wound healing. However, there are known differences between murine skin and human skin that raise questions about the accuracy of mouse models. For example, murine skin is thin compared to human skin and contains an additional muscle layer known as the panniculosus carnosus (Wong et al., 2011). Human skin is relatively rigid, tight and is firmly anchored to the underlying tissue. Murine skin, on the other hand, is loose, compliant and freely moves independently of the underlying tissue (Wong et al., 2011). The consequences of these differences for wound healing is that murine wounds are free to close primarily by contraction, whereas human wound healing is largely accomplished through re-epithelialization and granulation tissue deposition (Wong et al., 2011). These differences contribute to difficulty in translating therapeutic developments validated in mice into effective treatments for use in the clinic. On a larger scale, these differences call into question how closely the expression pattern and function of factors such as matricellular proteins in mice mimic their homologues in humans. To attempt to address this, we can draw on examples from other tissues where matricellular proteins have been studied in both human pathology and the corresponding murine models.

Periostin is heavily expressed in the periodontal ligament (PDL) of mice (Horiuchi et al., 1999). In mice, periostin is essential for PDL integrity, where deletion of periostin results in degradation of the PDL (Hamilton, 2008; Rios et al., 2008). Removal of occlusal loading, however, is protective of the PDL in *Postn*$^{-/-}$ mice, suggesting periostin's role in PDL is associated with adapting to mechanical stress. Periostin is also heavily expressed in human PDL (Wen et al., 2010). Moreover, in human PDL from teeth where occlusal loading has been removed, periostin expression is reduced (Wen et al., 2010). Therefore, periostin expression in the PDL of both humans and mice seems to be mechanically regulated in a similar manner.

Five days after induced myocardial infarction, periostin expression is up-regulated in the murine ventricular wall (Shimazaki et al., 2008). Deletion of *Postn*$^{-/-}$ results in reduced fibrosis of the infarct area; characterized by reduced collagen fibril formation and myofibroblast differentiation. In infarct tissue from human hearts, periostin is increased compared with healthy tissue surrounding the infarct. Furthermore, elevated periostin expression in human infarct tissue is associated with fibrosis, suggesting, again that periostin expression is regulated similarly in mouse and human tissues (Shimazaki et al., 2008).

Confirming that periostin has a parallel role in murine and human skin healing would be difficult due to ethical concerns. However, periostin has been studied in human fibrotic skin conditions where its expression is congruent with murine models of skin fibrosis. In mice, periostin is significantly up-regulated in bleomycin-induced skin fibrosis, and deletion of periostin is protective against skin fibrosis (Yang et al., 2012). In human skin, elevated periostin expression is associated with keloid and hypertrophic scars (Naitoh et al., 2005; Zhou et al., 2010). In SSc, periostin expression correlates with the degree of skin fibrosis (Yamaguchi et al., 2013). The same is true for CCN2 in skin. CCN2 is required for bleomycin-induced skin fibrosis in mice (Liu et al., 2011), and the level of CCN2 correlates with the severity of SSc in human skin (Takehara, 2003). The roles of periostin and CCN2 in human skin healing are unknown, and ethical concerns prohibit determining their expression patterns following wounding. However, the similarities between their expression patterns in human pathologies and corresponding murine

models strongly suggests that the roles of periostin and CCN2 in murine models of skin healing are relevant to human healing.

Of particular importance for our study was selecting a model that was relevant to human chronic skin wounds. The majority of human samples obtained for this study were from patients with type-2 diabetes. A suitable model for studying our PN- and CCN2-containing treatments should therefore model the impaired skin healing associated with diabetes. For this, we chose the db/db model of type-2 diabetes. The db/db mouse was first described in 1966 (Hummel et al., 1966) and has since become the most widely studied mouse model of diabetes (Michaels et al., 2007). The db/db mouse carries a point mutation in the leptin receptor (*Lepr*) gene, termed db, which results in defective leptin signaling. Shortly after birth, homozygous $Lepr^{db}$ (db/db) mice become obese and exhibit hyperglycemia by four to eight weeks (Hummel et al., 1966). These mice are polyphagic, polydipsic, and polyuric. They are insulin resistant, hypertriglyceridemic, and have impaired glucose tolerance. Most importantly, they exhibit severely impaired skin wound healing. We have shown that the histology of day 7 skin wounds in db/db mice is similar with respect to periostin, α-SMA, collagen and TNFα (Figure 3.4d) to the human chronic skin wound tissues we examined (Figure 3.2). Additionally, *Ccn2* expression is not induced in day 7 db/db wounds (Figure 3.4e). Therefore, the db/db mouse represents an excellent model for the study of PN- and CCN2-containing scaffolds with an aim towards treating human chronic skin wounds.

There are, however, criticisms of the db/db mouse model. One such criticism is that the monogenetic etiology (defective leptin signaling) does not match the complexity of human diabetes (Fang et al., 2010). In humans the influence of leptin on obesity and metabolic disorders is far subtler than in mice. Therefore, the model is centered on the role of leptin more than on diabetes as a whole. Furthermore, leptin has been shown to influence skin healing independent of metabolic disorders. Topical application of leptin to wild-type and leptin-deficient ob/ob mice, but not db/db mice, accelerated wound healing (Frank et al., 2000; Ring et al., 2000; Stallmeyer et al., 2001). Leptin receptors have been detected on keratinocytes at the wound margins in mice and leptin can increase keratinocyte proliferation *in vitro*, far removed from the metabolic disorders present in

the animal (Frank et al., 2000). The direct effects of leptin on skin healing suggest that the impaired healing in db/db mice is independent of a diabetic state. However, topical administration of leptin to wounds in ob/ob mice did not completely correct impaired healing. Deficits in angiogenesis persist even though blood vessels in the granulation tissue of ob/ob mice present the leptin receptor (Ring et al., 2000; Stallmeyer et al., 2001), suggesting there is more to this model than leptin dependant effects.

The two most commonly used alternatives to the db/db model of diabetes are the streptozotocin-induced and Akita models (Michaels et al., 2007). These mice model type-1 diabetes and therefore were not an appropriate comparison for our human tissue samples. Moreover, the existence of wound healing impairments in these models is questionable and, when present, not as severe as in the db/db mice (Keswani et al., 2004; Michaels et al., 2007). For example, in a study comparing these models Akita mice did not show a closure defect following incisional wounding (Fang et al., 2010). Streptozocin mice showed an incisional defect (Fang et al., 2010) but in another study they did not show an excisional defect (Michaels et al., 2007). An alternative to murine models is the ischemic rabbit ear model of impaired healing (Sisco and Mustoe, 2003). However, this is not a diabetic model and the surgical procedures involved are complex and technically difficult. Additionally, rabbits are far more costly to maintain than rodents.

Alternatives to the standard excisional punch biopsy wound model used in this study are available. Examples include the ischemia/re-profusion injury model (Reid et al., 2004), which requires additional technical expertise and equipment, and the ischemic skin flap model (Wong et al., 2011), which produces difficult to control ischemic gradients. These more specialized models have the advantage of mimicking the ischemic environment that is important in human chronic skin wound pathogenesis. However, these wound models are technically demanding and do not lend well to comparison of multiple topically applied treatments. Furthermore, significantly increased tissue ischemia is one of the many defects that is well documented in db/db excisional wound healing (Ring et al., 2000; Stallmeyer et al., 2001; Botusan et al., 2008). Therefore, although there are criticisms of the db/db model of impaired diabetic healing, it remains an appropriate and cost effective choice. Our histological comparison of day 7 db/db wounds with human

chronic skin wounds, with respect to periostin and CCN2, underscore the relevance of the db/db model.

4.3 Matricellular proteins as therapeutics

The idea of targeting matricellular proteins in conditions of pathological remodeling was first suggested by Midwood and colleagues in 2004 following review of the many ways matricellular proteins influence wound healing (Midwood et al., 2004). Surprisingly, in the intervening years since that publication, no studies have actually investigated the efficacy of these proteins in skin healing.

Matricellular proteins are typically not expressed in the adult except during wound healing or tissue remodeling (Bornstein and Sage, 2002; Hamilton, 2008). Targeted deletion of matricellular proteins produces animals that are grossly normal or display a very subtle phenotype (Bornstein and Sage, 2002; Hamilton, 2008). As a result, matricellular proteins represent a highly localized, highly selective target for manipulating wound healing. Unlike the potentially life-threatening effects of systemic blockade of pleiotropic molecules like TNFα (Wolbing et al., 2009), systemic targeting of matricellular proteins produces few side effects. Intravenous delivery of an anti-CCN2 monoclonal antibody to patients with microalbuminuric diabetic kidney disease was well tolerated in Phase I clinical trials (Adler et al., 2010), as was subcutaneous administration of the anti-angiogenic thrombospondin-1 mimetic, ABT-510, to patients with advanced solid tumors (Gietema et al., 2006). Recently, thrombospondin-1 was identified as being consistently elevated in biopsies taken of venous ulcers, as well as biopsies of the same locations 7 and 14 days later. This was compared to acute wounds created in the same patient where the acute wounds were re-sampled at days 7 and 14 (Shih et al., 2012). Interruption of thrombospondin-1 signaling with an antibody against its receptor, CD47, was shown to be protective against ischemia-reperfusion injury in mice (Maxhimer et al., 2009). Perhaps it is time to test anti-thrombospondin-1 treatments on human chronic skin wounds.

Inhibiting or targeting the downstream effects of over-expressed matricellular proteins, which by their over-expression are imposing deleterious effects on wound healing (i.e.

thrombospondin-1), might enhance healing of chronic skin wounds. However, given that the skin is readily accessible, local delivery of matricellular proteins that promote wound healing can be easily achieved. This is the approach we have chosen to pursue. We selected periostin and CCN2 because they are functionally associated with the profibrotic aspects of wound healing, including matrix accumulation, myofibroblast differentiation and profibrotic growth factor signaling. There are, however, other potential choices that may be useful in targeting different aspects of wound healing. Deletion of angiopoietin-like 4 (ANGPTL4), a recent addition to the matricellular protein family, results in delayed re-epithelialization of wounds in mice (Goh et al., 2010b), suggesting ANGPTL4 has a role in positively effecting re-epithelialization. Furthermore, topical application of an anti-ANGPTL4 antibody significantly delayed re-epithelialization in wild-type mice, showing that its effects are initiated from outside the cell. Of particular interest, delivery of recombinant ANGPTL4 fully recovered delayed re-epithelialization in *Angptl4*$^{-/-}$ mice (Goh et al., 2010a). Future work should determine if addition of ANGPTL4 to human wounds could enhance re-epithelialization. The galectins are a large group of matricellular proteins with diverse roles, including modulating inflammation. Galectin-1 can induce T_h2 polarization by selectively killing T_h1 cells. Additionally, galectin-1 promotes IL-10 production and attenuates IFNγ (Liu et al., 2012), furthering a T_h2 bias. Galectin-3 also exerts its influence on the T_h1/T_h2 balance, favouring a T_h2 profile (Liu et al., 2012). The interplay of the galectins and inflammation is complex and their role(s) in chronic skin wounds is unclear. Recently, however, we have shown that galectin-3 is absent in the epidermis surrounding human chronic skin wounds but is abundant in the epidermis of non-involved skin (Pepe et al., in press). The implications of this are currently unknown.

The importance of modulating the inflammatory phase of wound healing was not a focus in our study. Clinically, debridement of a wound removes the bacterial load and necrotic tissue responsible for perpetuating the inflammatory response and aims to “reset” the chronic skin wound to an acute wound. We hypothesized that after debridement, addition of periostin and CCN2 to a chronic skin wound could immediately initiate fibrotic wound healing, by-passing the stalled inflammatory phase associated with chronic skin wounds. However, with systemic disorders like diabetes still present, it is possible that following

debridement the wound might revert to a state of excessive inflammation. It may therefore be necessary to include another matricellular protein (i.e. galectin-1 or -3) to modulate inflammation. Based on the matricellular proteins discussed here, a hypothetical therapeutic device may incorporate galectins to shift inflammation to a T_h2 profile, periostin and CCN2 to produce dermal fibrotic healing and ANGPTL4 to accelerate re-epithelialization.

4.4 Different types of chronic skin wounds

The majority of chronic skin wounds can be classified as either diabetic ulcers, venous ulcers or pressure sores (Mustoe et al., 2006), with arterial wounds being less prevalent. Understanding the differences between these wounds is essential in prevention and avoiding recurrence. Control of diabetic ulcers requires tight regulation of diet and blood glucose as well as disciplined use of offloading devices. Venous ulcer occurrence is best controlled with compression bandages. Pressure sores, like diabetic ulcers, require offloading but also close monitoring and frequent proactive repositioning. Surgical reperfusion and anti-platelet medications are the standard of care for arterial wound treatment, however compression is contraindicated. These treatments are very deliberate, specific and effective in controlling the different etiologies of chronic skin wounds.

There is evidence that once wounds have formed the different etiologies present differing molecular profiles. For example, myofibroblasts (α-SMA positive stromal cells) were detected at higher levels in venous ulcers greater than 3 months in duration compared to acute 7 day-old wounds (Trostrup et al., 2011). In the wounds we observed, however, α-SMA positive cells were restricted to the vasculature. The patients enrolled in our study were almost exclusively diagnosed with peripheral vascular disease (PVD) (Table 3.1), suggesting that the wounds we examined were primarily arterial in nature. The distinction between wound etiologies is not rigid, however, and there is considerable overlap between various chronic skin wounds (i.e. an arterial wound caused by large vessel atherosclerosis but complicated by diabetes and pressure). Moreover, the diagnosis of a patient's limb does not always match the complexity of the specific wound. For example, we have collected tissue from a wound on the heel of a diabetic patient's foot, for which

the diagnosis was PVD. Yet it is reasonable to expect that this wound may have been complicated by pressure and diabetic neuropathy. Thus, the importance of molecular differences between wound etiologies, once they have developed, may not be as important as the similarities.

Regardless of the etiology of the chronic skin wound, there are common overarching determinants of pathogenesis. They are: advanced patient age, repeated ischemia-reperfusion injury, bacterial colonization and hypoxia (Mustoe et al., 2006). The complex interaction of these factors results in an out-of-control, self-sustaining inflammatory response (Menke et al., 2007). We suggest that future treatments may not need to be tailored to the specific molecular profile of each chronic skin wound etiology. Successful widely applicable treatments may be those that address the common dysfunctions of all chronic skin wounds. With this in mind we did not employ exclusion criteria (i.e. etiology, smoking, medications, infection) in our study. We hypothesize that any factors consistently found to be inappropriately expressed in chronic skin wounds from a wide range of patients would offer greater therapeutic applicability then those restricted to a subset (i.e. venous ulcers in non-smoking patients). The wounds we examined were mostly from patients with type-2 diabetes and PVD. To determine if the expression patterns of periostin and CCN2 are consistent across a wider range of etiologies, we will need to examine chronic skin wounds specifically classified as venous ulcers, diabetic ulcers and pressure sores. It is possible that venous ulcers like those shown to contain high numbers of α-SMA positive myofibroblasts (Trostrup et al., 2011) may also contain high levels of periostin.

4.5 Future directions and implications

For the rhPN- and rhCCN2-containing scaffolds used in Chapter 3, the next step in developing these into a treatment for chronic skin wounds is to test their efficacy in larger animals, such as the pig. Mouse models of wound healing in skin are always difficult to translate to human use due to the loose skin of mice (Ansell et al., 2012). Pigs, like humans, have tight skin that heals primarily by re-epithelialization (Sullivan et al., 2001) rather than from contraction, as in mice (Galiano et al., 2004). Mechanical tension in

wound healing promotes scar formation, which creates cosmetic concerns but can also compromise skin function (Gurtner et al., 2011). It will be interesting to see if mechanical tension will work synergistically with rhPN or rhCCN2 to promote fibrosis. The presence of a scar may be an acceptable outcome for patients suffering from chronic skin wounds when the alternative is amputation. There are apparati (stents) available to mimic tight skin in mice, however, porcine skin is still the superior choice for its ability to accommodate wounds of a size comparable to human chronic skin wounds.

An important finding from our experiments with wound edge fibroblasts was that they did not display a senescent phenotype. There is debate in the literature about the state of chronic skin wound edge cells and their utility in wound healing. For years the evidence supported the theory that chronic skin wound fibroblasts are senescent and are not salvageable for wound healing. Brem and colleagues' attempt to map out differences in fibroblast senescence based on proximity to the wound bed challenged that theory (Brem et al., 2007; Brem et al., 2008). Their work defined two subpopulations of cells defined by the location they were harvested from. Those from the non-healing edge displayed migration deficits and a polygonal morphology consistent with senescent cells. Those from the healing edge (a few millimetres away from the wound bed) migrated faster and displayed a spindle shape. Our results confirm that wound edge cells, even if only a subpopulation, are capable of adopting an activated phenotype (Figure 3.3). The implication that Brem and colleagues realized was that debridement of the wound edge should be with care since there are valuable cell populations that could be lost. We agree, but more work is needed to make the non-healing wound edge identifiable and distinguishable from the healing wound edge in a surgical setting. Periostin may be a potential marker for the region containing usable fibroblasts but periostin is not readily detectable in a surgical setting. Our data shows that the localization of periostin coincides with an area of hyper-vascularization around the wound edge. Non-invasive imaging techniques, such as diffuse optical imaging, are available for detecting blood oxygen. Such modalities might be used to detect the hyper-vascularization as a surrogate for periostin, and therefore direction as to where debridement should occur.

In addition to guiding debridement, we propose that the wound edge is a valuable resource for use in cell-based therapies. Steinberg and colleagues recently compared the therapeutic efficacy of adipose derived stem cells (ADSC) and dermal fibroblasts in an ischemic rabbit model of wound healing (Steinberg et al., 2012). Both cell types significantly increased wound healing rates. Surprisingly, they found no statistical difference between the abilities of ADSC and fibroblasts to accelerate wound healing. In light of our results with wound edge fibroblasts, and knowing that repeated debridement is the standard of care for difficult-to-heal wounds, we propose that debrided wound edge skin is a minimally invasive source of dermal fibroblasts to be used in cell-based therapies.

The complexity of chronic skin wounds likely cannot be resolved by a single factor (i.e. PDGF-BB, periostin or CCN2). Previously, we discussed how various matricellular proteins could be combined in a treatment to influence many aspects of wound healing. Future work should go beyond this and combine matricellular proteins with other therapies like growth factor-based treatments. CCN2 is required for maximal induction of α-SMA and collagen type-1 by TGFβ (Shi-wen et al., 2006). Similarly, CCN2 is required for TGFβ induced myofibroblast differentiation and subsequent gel contraction (Garrett et al., 2004). Deletion of periostin redirects the influence of TGFβ from fibrosis to regeneration in a model of muscular dystrophy (Lorts et al., 2012). Perhaps the disappointing clinical outcomes of TGFβ application to venous ulcers (Robson et al., 1995) would have turned out differently if TGFβ was combined with rhCCN2 or rhPN. SPARC has been shown to bind to and inhibit the function of both PDGF and VEGF (Brekken and Sage, 2000). PDGF-BB combined with an anti-SPARC agent may increase efficacy over PDGF-BB alone. By optimizing currently available agent-based therapies through combinations with matricellular proteins, we may be able to produce a therapeutic that is effective and widely applicable. The advantages that an agent-based therapeutic has over negative pressure or cell-based therapies are a longer shelf life, ease of use, ease of storage and no requirement for additional equipment. With the high cost of chronic skin wounds around the world, these benefits should not be discounted.

4.6 Summary

The overall hypothesis of this thesis was that the matricellular proteins periostin and CCN2, two matricellular proteins associated with fibrotic healing, could enhance the healing of chronic skin wounds. We have demonstrated that in a murine model of impaired healing these proteins can enhance skin healing. Therefore, the potential exists for these proteins to be used as therapeutics for healing of human chronic skin wounds.

4.7 References

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Appendix C: Enrichment analysis of up-regulated genes from microarray

Term	-		PN			CCN2				PN + CCN2			
KEYWORDS	-	Count	P value	Fold Enrichment	Benjamini	Count	P value	Fold Enrichment	Benjamini	Count	P value	Fold Enrichment	Benjamini
heart	-					2	3.33E-02	58.35	3.24E-01	4	3.51E-05	57.92	1.02E-03
cardiac muscle	-					2	3.33E-02	58.35	3.24E-01	4	3.51E-05	57.92	1.02E-03
thick filament	-	2	2.45E-02	78.48	2.39E-01	2	4.77E-02	40.39	3.45E-01	5	2.18E-06	50.12	1.27E-04
skeletal muscle	-									5	2.18E-06	50.12	1.27E-04
muscle protein	-	3	4.07E-03	30.61	5.10E-02	7	3.10E-08	36.76	8.06E-07	17	2.02E-22	44.31	3.54E-20
sarcoplasmic reticulum	-									5	1.14E-05	34.30	5.00E-04
sodium/potassium transport	-									2	5.93E-02	32.58	4.31E-01
myogenesis	-									3	1.10E-02	18.62	1.29E-01
myosin	-	2	8.58E-02	21.71	4.99E-01	3	1.35E-02	16.76	2.09E-01	6	2.83E-05	16.64	9.89E-04
EF hand	-									3	1.91E-02	13.96	2.02E-01
LIM domain	-									6	2.36E-04	10.71	5.89E-03
actin-binding	-									17	1.74E-11	9.80	1.52E-09
kelch repeat	-									5	1.80E-03	9.58	2.82E-02
calcium binding	-									3	4.98E-02	8.32	3.92E-01
calmodulin-binding	-									6	1.15E-03	7.59	2.21E-02
motor protein	-									6	2.51E-03	6.36	3.33E-02
methylation	-	3	6.61E-02	6.92	4.43E-01					9	2.94E-04	5.31	6.42E-03
Immunoglobulin domain	-									11	2.11E-03	3.24	3.03E-02
cytoskeleton	-									13	1.68E-03	2.91	2.90E-02
ion transport	-									10	2.32E-02	2.40	2.27E-01
coiled coil	-									21	3.91E-02	1.58	3.37E-01
sensory transduction	-					4	4.39E-02	5.03	3.73E-01				
extracellular matrix	-					4	4.60E-02	4.93	3.59E-01				
leucine-rich repeat	-					5	1.98E-02	4.77	2.29E-01				
g-protein coupled receptor	-					25	1.02E-10	4.65	1.06E-08				
transducer	-					25	2.28E-10	4.47	1.19E-08				
receptor	-					30	3.68E-09	3.20	1.27E-07				
disulfide bond	-	13	1.36E-03	2.69	2.58E-02	17	1.85E-02	1.81	2.42E-01				
transmembrane	-					30	1.04E-02	1.50	1.95E-01				
acute phase	-	3	9.46E-04	63.76	2.40E-02								
Lectin	-	4	3.89E-03	12.22	5.82E-02								
Secreted	-	11	2.19E-04	3.95	8.38E-03								
signal	-	16	1.54E-04	2.75	1.18E-02								
glycoprotein	-	13	2.90E-02	1.84	2.46E-01								

Term			PN			CCN2				PN + CCN2			
KEGG PATHWAY		Count	P value	Fold Enrichment	Benjamini	Count	P value	Fold Enrichment	Benjamini	Count	P value	Fold Enrichment	Benjamini
mmu04260:Cardiac muscle contraction	-									12	1.27E-12	22.64	7.11E-11
mmu05410:Hypertrophic cardiomyopathy (HCM)	-									12	2.95E-12	21.02	8.27E-11
mmu05414:Dilated cardiomyopathy	-					4	1.08E-02	8.32	1.36E-01	12	8.25E-12	19.19	1.54E-10
mmu05416:Viral myocarditis	-									8	2.11E-06	12.52	2.95E-05
mmu05412:Arrhythmogenic right ventricular cardio	-									6	1.20E-04	11.77	1.34E-03
mmu04960:Aldosterone-regulated sodium reabsorp	-									3	3.11E-02	10.51	1.99E-01
mmu04530:Tight junction	-									5	1.16E-02	5.45	8.94E-02
mmu04020:Calcium signaling pathway	-									6	7.97E-03	4.62	7.20E-02
mmu04740:Olfactory transduction	-					19	4.96E-08	3.79	1.34E-06				

Term		PN				CCN2				PN + CCN2			
GO TERM CELLULAR COMPARTMENT		Count	P value	Fold Enrichment	Benjamini	Count	P value	Fold Enrichment	Benjamini	Count	P value	Fold Enrichment	Benjamini
GO:0005859~muscle myosin complex	-									5	9.13E-07	59.54	9.52E-06
GO:0005863~striated muscle thick filament	-									4	3.04E-05	59.54	2.61E-04
GO:0033018~sarcoplasmic reticulum lumen	-									2	3.29E-02	59.54	1.77E-01
GO:0031430~M band	-									3	1.40E-03	51.04	1.07E-02
GO:0016460~myosin II complex	-									5	2.12E-06	49.62	2.07E-05
GO:0032982~myosin filament	-	2	3.31E-02	57.49	3.09E-01					6	1.02E-07	47.63	1.24E-06
GO:0031674~I band	-					3	2.59E-02	11.80	2.01E-01	21	5.85E-29	47.18	1.71E-27
GO:0030018~Z disc	-									17	1.26E-22	44.01	3.06E-21
GO:0030017~sarcomere	-					4	6.34E-03	10.42	1.19E-01	27	1.53E-35	40.19	7.46E-34
GO:0031672~A band	-									4	1.16E-04	39.70	9.44E-04
GO:0016012~sarcoglycan complex	-									2	4.89E-02	39.70	2.45E-01
GO:0030016~myofibril	-	3	1.75E-02	14.21	3.85E-01	5	8.80E-04	11.45	5.15E-02	29	1.64E-37	37.95	2.40E-35
GO:0044449~contractile fiber part	-					4	7.74E-03	9.69	1.10E-01	27	1.39E-34	37.39	5.06E-33
GO:0043292~contractile fiber	-	3	1.90E-02	13.62	2.96E-01	5	1.03E-03	10.97	3.06E-02	29	6.65E-37	36.35	4.86E-35
GO:0016529~sarcoplasmic reticulum	-									10	5.91E-12	35.03	1.23E-10
GO:0005916~fascia adherens	-									2	5.68E-02	34.02	2.71E-01
GO:0032155~cell division site part	-									2	5.68E-02	34.02	2.71E-01
GO:0032153~cell division site	-									2	5.68E-02	34.02	2.71E-01
GO:0016011~dystroglycan complex	-									2	5.68E-02	34.02	2.71E-01
GO:0033017~sarcoplasmic reticulum membrane	-									2	5.68E-02	34.02	2.71E-01
GO:0016528~sarcoplasm	-									10	1.05E-11	33.08	1.91E-10
GO:0016010~dystrophin-associated glycoprotein cc	-									3	4.28E-03	29.77	3.08E-02
GO:0014704~intercalated disc	-									2	8.78E-02	21.65	3.81E-01
GO:0030315~T-tubule	-									3	8.59E-03	21.02	5.56E-02
GO:0042383~sarcolemma	-									8	1.07E-07	20.27	1.21E-06
GO:0016459~myosin complex	-					3	3.35E-02	10.25	2.03E-01	10	1.62E-09	19.52	2.16E-08
GO:0015629~actin cytoskeleton	-									16	1.32E-10	9.29	2.14E-09
GO:0016323~basolateral plasma membrane	-									5	3.00E-02	4.22	1.69E-01
GO:0005856~cytoskeleton	-									33	2.27E-10	3.50	3.32E-09
GO:0044430~cytoskeletal part	-									15	4.76E-03	2.31	3.27E-02
GO:0043228~non-membrane-bounded organelle	-									35	7.43E-06	2.17	6.78E-05
GO:0043232~intracellular non-membrane-bounded	-									35	7.43E-06	2.17	6.78E-05
GO:0005783~endoplasmic reticulum	-									14	2.14E-02	1.99	1.28E-01
GO:0044421~extracellular region part	-	6	2.69E-02	3.34	3.12E-01	9	2.81E-02	2.42	1.92E-01				
GO:0016021~integral to membrane	-					36	2.39E-02	1.31	2.15E-01				
GO:0031224~intrinsic to membrane	-					37	2.35E-02	1.30	2.48E-01				
GO:0005576~extracellular region	-	12	5.95E-04	3.08	3.22E-02								

Term		PN				CCN2				PN + CCN2			
GO TERM MOLECULAR FUNCTION		**Count**	**P value**	**Fold Enrichment**	**Benjamini**	**Count**	**P value**	**Fold Enrichment**	**Benjamini**	**Count**	**P value**	**Fold Enrichment**	**Benjamini**
GO:0008307~structural constituent of muscle	-									4	2.32E-05	65.14	1.70E-03
GO:0005391~sodium:potassium-exchanging ATPas	-									3	2.47E-03	39.08	5.88E-02
GO:0005523~tropomyosin binding	-									2	5.20E-02	37.22	6.24E-01
GO:0005200~structural constituent of cytoskeleton	-									4	1.35E-03	17.97	4.83E-02
GO:0051015~actin filament binding	-	2	8.15E-02	22.60	8.55E-01	3	1.75E-02	14.53	3.37E-01	6	3.36E-05	15.95	1.85E-03
GO:0003779~actin binding	-									19	4.85E-12	8.59	5.34E-10
GO:0008092~cytoskeletal protein binding	-									22	3.64E-12	6.92	8.02E-10
GO:0005516~calmodulin binding	-									6	1.75E-03	6.86	5.36E-02
GO:0015662~ATPase activity, coupled to transmem	-									3	6.97E-02	6.86	6.54E-01
GO:0003774~motor activity	-									7	6.36E-04	6.61	2.76E-02
GO:0005262~calcium channel activity	-									3	9.44E-02	5.75	7.02E-01
GO:0042625~ATPase activity, coupled to transmem	-									3	9.68E-02	5.66	6.92E-01
GO:0003712~transcription cofactor activity	-									6	1.20E-02	4.34	2.15E-01
GO:0005198~structural molecule activity	-									11	2.20E-03	3.18	5.88E-02
GO:0008134~transcription factor binding	-									6	6.84E-02	2.71	6.72E-01
GO:0005509~calcium ion binding	-									15	4.40E-03	2.33	9.25E-02
GO:0043169~cation binding	-									38	6.51E-02	1.27	6.80E-01
GO:0043167~ion binding	-									38	7.63E-02	1.26	6.64E-01
GO:0046872~metal ion binding	-									37	8.66E-02	1.25	6.90E-01
GO:0016503~pheromone receptor activity	-					5	2.33E-03	8.79	7.83E-02				
GO:0004984~olfactory receptor activity	-					24	2.79E-11	5.02	1.95E-09				
GO:0005529~sugar binding	-	4	3.60E-03	12.24	2.18E-01								
GO:0030246~carbohydrate binding	-	4	1.67E-02	6.99	4.36E-01								

Term		PN				CCN2				PN + CCN2			
GO TERM BIOLOGICAL PROCESS		**Count**	**P value**	**Fold Enrichment**	**Benjamini**	**Count**	**P value**	**Fold Enrichment**	**Benjamini**	**Count**	**P value**	**Fold Enrichment**	**Benjamini**
GO:0055003~cardiac myofibril assembly	-									3	6.98E-04	71.52	1.59E-02
GO:0014823~response to activity	-									2	4.08E-02	47.68	3.68E-01
GO:0055013~cardiac muscle cell development	-									4	1.10E-04	40.87	3.49E-03
GO:0055006~cardiac cell development	-									4	1.10E-04	40.87	3.49E-03
GO:0045214~sarcomere organization	-									3	2.50E-03	39.01	4.94E-02
GO:0030239~myofibril assembly	-									5	7.69E-06	37.64	3.24E-04
GO:0031032~actomyosin structure organization	-									5	2.43E-05	28.61	8.78E-04
GO:0006936~muscle contraction	-									11	8.72E-12	26.22	2.21E-09
GO:0006941~striated muscle contraction	-									4	5.84E-04	23.84	1.40E-02
GO:0003012~muscle system process	-									11	2.76E-11	23.48	4.65E-09
GO:0002026~regulation of the force of heart contra	-									2	8.63E-02	22.00	5.51E-01
GO:0007512~adult heart development	-									2	8.63E-02	22.00	5.51E-01
GO:0055002~striated muscle cell development	-									8	8.22E-08	21.19	6.93E-06
GO:0055007~cardiac muscle cell differentiation	-									4	9.27E-04	20.43	2.02E-02
GO:0010927~cellular component assembly involvec	-									5	1.07E-04	19.87	3.60E-03
GO:0019229~regulation of vasoconstriction	-									2	9.89E-02	19.07	5.85E-01
GO:0055001~muscle cell development	-									8	1.95E-07	18.76	1.41E-05
GO:0048738~cardiac muscle tissue development	-									7	2.55E-06	17.57	1.43E-04
GO:0035051~cardiac cell differentiation	-									4	1.51E-03	17.34	3.13E-02
GO:0014706~striated muscle tissue development	-									13	6.55E-11	14.64	8.29E-09
GO:0006937~regulation of muscle contraction	-									4	2.83E-03	13.95	5.16E-02
GO:0060537~muscle tissue development	-									13	1.47E-10	13.67	1.49E-08
GO:0009408~response to heat	-									3	2.05E-02	13.41	2.72E-01
GO:0007517~muscle organ development	-									16	1.01E-12	13.00	5.14E-10
GO:0051146~striated muscle cell differentiation	-									8	2.63E-06	12.86	1.33E-04
GO:0007519~skeletal muscle tissue development	-									6	1.38E-04	11.92	4.10E-03
GO:0060538~skeletal muscle organ development	-									6	1.57E-04	11.60	4.18E-03
GO:0048747~muscle fiber development	-									3	2.83E-02	11.29	3.18E-01
GO:0001756~somitogenesis	-									3	3.26E-02	10.47	3.43E-01
GO:0042692~muscle cell differentiation	-									8	1.62E-05	9.78	6.29E-04

Term		PN				CCN2				PN + CCN2			
GO TERM BIOLOGICAL PROCESS, Continued		Count	P value	Fold Enrichment	Benjamini	Count	P value	Fold Enrichment	Benjamini	Count	P value	Fold Enrichment	Benjamini
GO:0008016~regulation of heart contraction	-									3	4.68E-02	8.58	3.97E-01
GO:0009266~response to temperature stimulus	-									3	4.85E-02	8.41	4.02E-01
GO:0030029~actin filament-based process	-									10	3.46E-06	8.13	1.59E-04
GO:0035282~segmentation	-									3	5.37E-02	7.95	4.04E-01
GO:0003007~heart morphogenesis	-									4	1.46E-02	7.73	2.13E-01
GO:0007507~heart development	-									12	3.80E-07	7.70	2.40E-05
GO:0055074~calcium ion homeostasis	-									5	4.23E-03	7.53	7.38E-02
GO:0030036~actin cytoskeleton organization	-									8	1.45E-04	6.93	4.06E-03
GO:0055065~metal ion homeostasis	-									5	6.23E-03	6.75	1.03E-01
GO:0046034~ATP metabolic process	-									4	2.44E-02	6.36	3.08E-01
GO:0006874~cellular calcium ion homeostasis	-									4	2.51E-02	6.29	3.01E-01
GO:0006875~cellular metal ion homeostasis	-									4	3.20E-02	5.72	3.45E-01
GO:0009205~purine ribonucleoside triphosphate m	-									4	3.28E-02	5.66	3.38E-01
GO:0009199~ribonucleoside triphosphate metaboli	-									4	3.37E-02	5.61	3.38E-01
GO:0009144~purine nucleoside triphosphate metat	-									4	3.71E-02	5.40	3.53E-01
GO:0044057~regulation of system process	-									7	2.69E-03	4.98	5.11E-02
GO:0009141~nucleoside triphosphate metabolic pr	-									4	4.55E-02	4.98	3.94E-01
GO:0055066~di-, tri-valent inorganic cation homeo	-									5	1.85E-02	4.90	2.56E-01
GO:0009150~purine ribonucleotide metabolic proce	-									4	4.94E-02	4.81	3.95E-01
GO:0006816~calcium ion transport	-									4	5.15E-02	4.73	4.02E-01
GO:0009259~ribonucleotide metabolic process	-									4	5.57E-02	4.58	4.10E-01
GO:0006814~sodium ion transport	-									4	5.57E-02	4.58	4.10E-01
GO:0007010~cytoskeleton organization	-									10	4.19E-04	4.39	1.05E-02
GO:0030005~cellular di-, tri-valent inorganic cation	-									4	6.58E-02	4.27	4.60E-01
GO:0055080~cation homeostasis	-									5	3.87E-02	3.89	3.59E-01
GO:0030003~cellular cation homeostasis	-									4	8.83E-02	3.76	5.54E-01
GO:0006813~potassium ion transport	-									4	9.93E-02	3.58	5.80E-01
GO:0006163~purine nucleotide metabolic process	-									4	9.93E-02	3.58	5.80E-01
GO:0050801~ion homeostasis	-									6	5.25E-02	2.93	4.02E-01
GO:0030001~metal ion transport	-									9	1.14E-02	2.91	1.76E-01
GO:0032989~cellular component morphogenesis	-									7	3.48E-02	2.85	3.41E-01
GO:0019725~cellular homeostasis	-									6	8.92E-02	2.50	5.51E-01
GO:0006812~cation transport	-									9	2.61E-02	2.50	3.03E-01
GO:0006811~ion transport	-									11	2.51E-02	2.21	3.08E-01
GO:0042592~homeostatic process	-									9	4.90E-02	2.20	3.98E-01
GO:0007608~sensory perception of smell	-					24	3.10E-11	5.03	3.19E-09				
GO:0007606~sensory perception of chemical stimu	-					25	1.53E-11	4.91	2.36E-09				
GO:0007600~sensory perception	-					25	4.55E-10	4.18	3.51E-08				
GO:0050890~cognition	-					25	1.38E-09	3.96	7.09E-08				
GO:0007186~G-protein coupled receptor protein si	-					31	4.11E-12	3.87	1.27E-09				
GO:0050877~neurological system process	-					26	3.22E-09	3.62	1.42E-07				
GO:0007166~cell surface receptor linked signal tra	-					32	1.05E-09	3.00	6.47E-08				
GO:0006953~acute-phase response	-	3	1.06E-03	59.08	1.72E-01								
GO:0002526~acute inflammatory response	-	3	7.50E-03	21.88	3.60E-01								
GO:0006954~inflammatory response	-	3	5.07E-02	7.88	9.01E-01								
GO:0006952~defense response	-	5	5.32E-03	6.59	3.78E-01								

Appendix D: Enrichment analysis of down-regulated genes from microarray

Term	PN				CCN2				PN + CCN2			
KEYWORDS	Count	P value	Fold Enrichment	Benjamini	Count	P value	Fold Enrichment	Benjamini	Count	P value	Fold Enrichment	Benjamini
keratinization	-								6	2.92E-05	15.91	5.93E-04
gap junction	-								4	6.79E-03	10.15	6.68E-02
tandem repeat	-								3	4.11E-02	9.21	2.65E-01
Fatty acid biosynthesis	-	4	1.48E-03	17.42	5	4.45E-04	13.86	1.35E-02	7	1.25E-04	8.88	2.06E-03
Serine protease inhibitor	-			8.62E-02	6	4.49E-04	9.33	1.19E-02	11	1.32E-06	7.83	6.96E-05
lipid synthesis	-				10	7.74E-08	12.88	8.25E-06	13	1.22E-07	7.66	1.08E-05
Intermediate filament	-								9	3.75E-05	7.10	7.07E-04
protease inhibitor	-	6	1.19E-05	19.66	1.45E-03	6	1.63E-03	7.02	2.45E-02	13	3.56E-07	6.96	2.35E-05
electron transfer	-								3	7.72E-02	6.48	4.12E-01
keratin	-					5	1.04E-02	5.85	9.25E-02	10	1.05E-04	5.35	1.85E-03
lipid degradation	-					7	3.48E-05	11.30	1.85E-03	7	2.31E-03	5.17	2.74E-02
lipid metabolism	-					12	9.08E-09	11.25	1.93E-06	12	2.21E-05	5.15	5.31E-04
antibiotic	-								5	1.62E-02	5.12	1.26E-01
microsome	-	3	7.45E-02	6.53	5.76E-01	4	4.32E-02	5.10	2.69E-01	8	1.64E-03	4.67	2.04E-02
Serine protease	-								13	2.58E-05	4.63	5.67E-04
Antimicrobial	-								5	2.90E-02	4.29	2.04E-01
multifunctional enzyme	-	3	5.79E-02	7.54	5.98E-01	4	1.04E-02	8.80	8.88E-02	4	7.67E-02	4.02	4.17E-01
nadp	-					7	8.56E-04	6.29	1.81E-02	9	3.08E-03	3.70	3.34E-02
Acyltransferase	-					5	3.30E-02	4.11	2.40E-01	9	5.23E-03	3.39	5.39E-02
heme	-	3	5.79E-02	7.54	5.98E-01	7	8.56E-04	6.29	1.81E-02	8	1.11E-02	3.29	9.38E-02
cell junction	-								22	4.20E-06	3.27	1.38E-04
Monooxygenase	-								6	3.78E-02	3.24	2.52E-01
nad	-					7	2.10E-03	5.28	2.94E-02	9	8.62E-03	3.11	8.12E-02
lyase	-					4	7.00E-02	4.18	3.49E-01	6	5.83E-02	2.87	3.41E-01
oxidoreductase	-	6	1.98E-02	3.75	4.57E-01	20	9.95E-08	4.46	7.06E-06	28	1.99E-06	2.86	8.75E-05
cell adhesion	-								16	2.37E-03	2.46	2.68E-02
iron	-	4	5.79E-02	4.45	6.46E-01	10	9.32E-04	3.97	1.64E-02	13	9.45E-03	2.36	8.28E-02
cleavage on pair of basic residues	-								9	5.20E-02	2.21	3.17E-01
calcium	-								27	3.58E-04	2.16	5.54E-03
endoplasmic reticulum	-					16	2.63E-04	3.01	9.29E-03	25	6.35E-04	2.15	8.79E-03
Signal-anchor	-								14	2.25E-02	2.02	1.67E-01
Secreted	-	11	4.59E-03	2.77	1.71E-01	24	6.02E-04	2.16	1.42E-02	49	3.94E-06	2.01	1.48E-04
Protease	-								17	1.45E-02	1.95	1.17E-01
signal	-	14	5.34E-02	1.68	6.72E-01	36	6.48E-03	1.55	6.69E-02	88	1.17E-07	1.73	1.55E-05
disulfide bond	-	12	6.78E-02	1.74	5.76E-01	31	8.01E-03	1.60	7.84E-02	72	5.05E-06	1.70	1.48E-04
cell membrane	-					20	7.28E-02	1.49	3.53E-01	47	1.35E-03	1.60	1.77E-02
hydrolase	-					19	4.32E-02	1.62	2.77E-01	39	9.23E-03	1.52	8.37E-02
membrane	-					64	2.22E-04	1.48	9.41E-03	143	4.83E-09	1.52	1.28E-06
glycoprotein	-					40	1.56E-02	1.42	1.25E-01	93	1.88E-05	1.51	4.97E-04
transmembrane	-					57	3.61E-03	1.39	4.42E-02	117	6.14E-04	1.30	8.97E-03
obesity	-					3	8.84E-04	63.76	1.70E-02				
diabetes mellitus	-					2	3.83E-02	51.01	2.65E-01				
oxygen carrier	-					2	3.83E-02	51.01	2.65E-01				
blood	-					2	4.58E-02	42.51	2.75E-01				
lipid droplet	-	2	2.71E-02	71.42	4.89E-01	3	2.60E-03	38.26	3.41E-02				
erythrocyte	-					2	6.06E-02	31.88	3.32E-01				
oxygen transport	-					2	8.24E-02	23.19	3.75E-01				
vldl	-					2	8.24E-02	23.19	3.75E-01				
chromoprotein	-					4	3.70E-03	12.75	4.29E-02				
metalloprotein	-					5	9.44E-04	11.39	1.54E-02				
pyridoxal phosphate	-					3	7.95E-02	6.38	3.72E-01				
chloride	-					3	9.84E-02	5.63	4.16E-01				
peroxisome	-					4	3.91E-02	5.31	2.62E-01				
manganese	-					6	5.53E-03	5.28	6.03E-02				
Symport	-					4	5.34E-02	4.68	3.06E-01				
polymorphism	-					5	6.71E-02	3.25	3.45E-01				
transmembrane protein	-					9	8.48E-03	3.10	7.91E-02				
mitochondrion	-					11	8.91E-02	1.78	3.91E-01				
transport	-					19	6.24E-02	1.54	3.32E-01				
thiol protease inhibitor	-	2	5.87E-02	32.46	5.60E-01								

Term		PN				CCN2				PN + CCN2			
KEGG PATHWAY		Count	P value	Fold Enrichment	Benjamini	Count	P value	Fold Enrichment	Benjamini	Count	P value	Fold Enrichment	Benjamini
mmu00100:Steroid biosynthesis	-									4	1.54E-03	16.67	5.06E-02
mmu00910:Nitrogen metabolism	-									4	3.78E-03	12.32	7.37E-02
mmu00150:Androgen and estrogen metabolism	-									4	1.05E-02	8.59	1.63E-01
mmu00140:Steroid hormone biosynthesis	-					3	8.93E-02	5.89	4.72E-01	5	3.38E-03	7.87	8.20E-02
mmu00980:Metabolism of xenobiotics by cytochron	-					4	3.68E-02	5.35	4.01E-01	7	2.84E-04	7.51	2.83E-02
mmu00260:Glycine, serine and threonine metabolis	-									3	7.28E-02	6.64	5.00E-01
mmu00982:Drug metabolism	-	3	2.25E-02	12.08	5.70E-01	4	5.07E-02	4.71	3.77E-01	7	5.69E-04	6.61	2.83E-02
mmu00590:Arachidonic acid metabolism	-					4	6.48E-02	4.25	4.23E-01	5	2.81E-02	4.27	3.37E-01
mmu00830:Retinol metabolism	-					4	3.97E-02	5.19	3.40E-01	4	6.86E-02	4.17	5.12E-01
mmu05412:Arrhythmogenic right ventricular cardio	-									4	8.64E-02	3.78	5.32E-01
mmu04520:Adherens junction	-									4	8.91E-02	3.73	5.16E-01
mmu04360:Axon guidance	-									6	3.54E-02	3.24	3.65E-01
mmu04144:Endocytosis	-									7	6.17E-02	2.45	5.11E-01
mmu03320:PPAR signaling pathway	-					10	1.67E-07	11.17	1.37E-05				
mmu00565:Ether lipid metabolism	-					4	6.72E-03	10.09	1.29E-01				
mmu00561:Glycerolipid metabolism	-					5	1.75E-03	9.39	6.94E-02				
mmu00350:Tyrosine metabolism	-					3	6.97E-02	6.79	4.17E-01				
mmu00564:Glycerophospholipid metabolism	-					5	6.38E-03	6.59	1.60E-01				
mmu04920:Adipocytokine signaling pathway	-					4	3.82E-02	5.27	3.67E-01				
mmu04910:Insulin signaling pathway	-					6	1.83E-02	3.84	2.62E-01				

Term		PN				CCN2				PN + CCN2			
GO TERM CELLULAR COMPARTMENT		Count	P value	Fold Enrichment	Benjamini	Count	P value	Fold Enrichment	Benjamini	Count	P value	Fold Enrichment	Benjamini
GO:0005914~spot adherens junction	-									2	3.85E-02	51.25	2.82E-01
GO:0030057~desmosome	-									11	9.95E-13	28.19	1.76E-10
GO:0001533~cornified envelope	-									5	4.29E-04	13.49	9.44E-03
GO:0005913~cell-cell adherens junction	-									5	2.90E-03	8.27	3.88E-02
GO:0005922~connexon complex	-									3	5.18E-02	8.09	3.36E-01
GO:0005921~gap junction	-									4	1.50E-02	7.59	1.54E-01
GO:0016327~apicolateral plasma membrane	-									15	1.12E-08	7.46	6.59E-07
GO:0043296~apical junction complex	-									14	7.33E-08	7.10	2.60E-06
GO:0070161~anchoring junction	-									16	1.50E-08	6.67	6.64E-07
GO:0005911~cell-cell junction	-									20	1.03E-09	5.92	9.16E-08
GO:0045095~keratin filament	-					4	2.45E-02	6.35	5.17E-01	6	7.99E-03	4.80	9.03E-02
GO:0005912~adherens junction	-									8	4.58E-03	3.87	5.64E-02
GO:0005882~intermediate filament	-					5	4.50E-02	3.71	6.86E-01	10	1.41E-03	3.74	2.47E-02
GO:0045111~intermediate filament cytoskeleton	-					5	4.81E-02	3.63	6.62E-01	10	1.64E-03	3.66	2.61E-02
GO:0016324~apical plasma membrane	-									6	4.37E-02	3.11	3.02E-01
GO:0045177~apical part of cell	-									8	1.69E-02	3.01	1.63E-01
GO:0016323~basolateral plasma membrane	-									8	2.02E-02	2.91	1.82E-01
GO:0030054~cell junction	-									25	1.52E-05	2.73	3.83E-04
GO:0042598~vesicular fraction	-									9	2.58E-02	2.53	2.07E-01
GO:0005792~microsome	-									8	5.62E-02	2.33	3.47E-01
GO:0005576~extracellular region	-	13	1.84E-03	2.62	1.72E-01	26	1.93E-02	1.57	4.96E-01	61	1.37E-06	1.86	4.05E-05
GO:0019898~extrinsic to membrane	-									15	7.59E-02	1.63	4.28E-01
GO:0044459~plasma membrane part	-									51	6.38E-04	1.60	1.25E-02
GO:0005783~endoplasmic reticulum	-					21	1.66E-04	2.55	2.87E-02	26	2.14E-02	1.59	1.82E-01
GO:0005886~plasma membrane	-									77	2.30E-03	1.36	3.33E-02
GO:0012511~monolayer-surrounded lipid storage b	-	2	1.72E-02	112.65	5.86E-01	2	5.71E-02	33.89	6.84E-01				
GO:0005811~lipid particle	-	2	3.68E-02	51.99	7.21E-01	4	2.41E-04	31.28	2.10E-02				
GO:0005833~hemoglobin complex	-					2	7.55E-02	25.41	7.49E-01				
GO:0034385~triglyceride-rich lipoprotein particle	-					2	9.34E-02	20.33	7.35E-01				
GO:0034361~very-low-density lipoprotein particle	-					2	9.34E-02	20.33	7.35E-01				
GO:0042579~microbody	-					4	8.13E-02	3.91	7.43E-01				
GO:0005777~peroxisome	-					4	8.13E-02	3.91	7.43E-01				
GO:0005829~cytosol	-					12	1.83E-02	2.22	5.56E-01				
GO:0005740~mitochondrial envelope	-					8	8.80E-02	2.08	7.41E-01				
GO:0005739~mitochondrion	-					23	8.54E-03	1.77	3.95E-01				
GO:0031090~organelle membrane	-					13	9.61E-02	1.63	7.19E-01				

Term		PN				CCN2				PN + CCN2			
GO TERM MOLECULAR FUNCTION		Count	P value	Fold Enrichment	Benjamini	Count	P value	Fold Enrichment	Benjamini	Count	P value	Fold Enrichment	Benjamini
GO:0005007~fibroblast growth factor receptor activ	-									2	6.58E-02	29.53	5.78E-01
GO:0042153~RPTP-like protein binding	-									2	8.15E-02	23.62	6.25E-01
GO:0004030~aldehyde dehydrogenase [NAD(P)+]	-									2	8.15E-02	23.62	6.25E-01
GO:0017134~fibroblast growth factor binding	-									3	1.16E-02	17.72	2.91E-01
GO:0016628~oxidoreductase activity, acting on the	-					3	6.30E-03	24.61	1.63E-01	3	2.57E-02	11.81	3.77E-01
GO:0004089~carbonate dehydratase activity	-									3	3.63E-02	9.84	4.18E-01
GO:0004867~serine-type endopeptidase inhibitor a	-	6	2.60E-05	16.51	8.77E-04	6	2.35E-03	6.42	8.42E-02	16	8.52E-10	8.22	8.12E-08
GO:0016620~oxidoreductase activity, acting on the	-	2	8.58E-02	21.82	7.80E-01					4	1.25E-02	8.15	2.74E-01
GO:0016229~steroid dehydrogenase activity	-									4	1.25E-02	8.15	2.74E-01
GO:0004866~endopeptidase inhibitor activity	-	8	5.35E-07	15.72	7.22E-05	7	1.90E-03	5.35	7.71E-02	21	2.97E-12	7.70	5.66E-10
GO:0033764~steroid dehydrogenase activity, actin	-									3	5.68E-02	7.70	5.36E-01
GO:0004869~cysteine-type endopeptidase inhibitor	-									5	4.07E-03	7.57	1.32E-01
GO:0030414~peptidase inhibitor activity	-	8	9.76E-07	14.38	6.59E-05	7	2.97E-03	4.89	9.54E-02	22	1.84E-12	7.38	6.99E-10
GO:0004364~glutathione transferase activity	-									3	7.06E-02	6.81	5.93E-01
GO:0016836~hydro-lyase activity	-									4	3.35E-02	5.62	4.18E-01
GO:0016712~oxidoreductase activity, acting on pai	-									4	3.77E-02	5.37	4.19E-01
GO:0004857~enzyme inhibitor activity	-	8	8.30E-06	10.42	3.73E-04	7	1.37E-02	3.54	2.52E-01	22	8.45E-10	5.35	1.07E-07
GO:0004714~transmembrane receptor protein tyro	-									5	1.46E-02	5.27	2.67E-01
GO:0004252~serine-type endopeptidase activity	-									14	1.91E-05	4.37	1.04E-03
GO:0016298~lipase activity	-					7	5.78E-05	10.25	6.48E-03	6	1.37E-02	4.22	2.80E-01
GO:0008236~serine-type peptidase activity	-									15	1.44E-05	4.18	1.09E-03
GO:0050660~FAD binding	-									5	3.18E-02	4.16	4.14E-01
GO:0017171~serine hydrolase activity	-									15	1.51E-05	4.16	9.61E-04
GO:0015662~ATPase activity, coupled to transmem	-									4	7.13E-02	4.14	5.85E-01
GO:0020037~heme binding	-	3	7.27E-02	6.59	8.17E-01	7	1.07E-03	5.98	8.66E-02	9	3.05E-03	3.69	1.21E-01
GO:0046906~tetrapyrrole binding	-	3	7.89E-02	6.29	7.95E-01	7	1.37E-03	5.70	8.84E-02	9	4.07E-03	3.52	1.44E-01
GO:0019842~vitamin binding	-					5	1.65E-02	5.08	2.80E-01	7	1.66E-02	3.42	2.85E-01
GO:0004091~carboxylesterase activity	-					8	2.58E-05	9.11	4.33E-03	6	3.59E-02	3.28	4.27E-01
GO:0048037~cofactor binding	-					6	3.58E-02	3.27	4.59E-01	10	1.45E-02	2.61	2.79E-01
GO:0050662~coenzyme binding	-									7	5.38E-02	2.58	5.29E-01
GO:0005506~iron ion binding	-	4	8.86E-02	3.69	7.51E-01	10	1.78E-03	3.59	8.23E-02	14	5.49E-03	2.41	1.60E-01
GO:0004175~endopeptidase activity	-									15	1.22E-02	2.10	2.85E-01
GO:0005509~calcium ion binding	-									28	9.29E-04	1.97	4.33E-02
GO:0008233~peptidase activity	-									19	2.00E-02	1.78	3.19E-01
GO:0070011~peptidase activity, acting on L-amino	-									18	2.65E-02	1.76	3.72E-01
GO:0004977~melanocortin receptor activity	-					2	3.96E-02	49.21	4.77E-01				
GO:0004806~triacylglycerol lipase activity	-					5	3.74E-06	43.94	1.26E-03				
GO:0017040~ceramidase activity	-					2	5.50E-02	35.15	5.64E-01				
GO:0005416~cation:amino acid symporter activity	-					2	7.77E-02	24.61	6.36E-01				
GO:0015175~neutral amino acid transmembrane tr	-					3	7.16E-03	23.07	1.59E-01				
GO:0005344~oxygen transporter activity	-					2	8.51E-02	22.37	6.57E-01				
GO:0004467~long-chain-fatty-acid-CoA ligase activ	-					2	9.98E-02	18.93	7.05E-01				
GO:0015171~amino acid transmembrane transport	-					4	7.56E-03	9.84	1.57E-01				
GO:0005275~amine transmembrane transporter ac	-					5	1.64E-03	9.76	8.83E-02				
GO:0015370~solute:sodium symporter activity	-					3	5.29E-02	8.02	5.65E-01				
GO:0070279~vitamin B6 binding	-					3	7.02E-02	6.84	6.25E-01				
GO:0030170~pyridoxal phosphate binding	-					3	7.02E-02	6.84	6.25E-01				
GO:0015294~solute:cation symporter activity	-					4	2.10E-02	6.74	3.28E-01				
GO:0030145~manganese ion binding	-					6	6.86E-03	4.99	1.63E-01				
GO:0015293~symporter activity	-					5	2.19E-02	4.66	3.25E-01				
GO:0005179~hormone activity	-					4	6.30E-02	4.36	5.99E-01				
GO:0046983~protein dimerization activity	-					9	6.14E-03	3.26	1.72E-01				
GO:0042803~protein homodimerization activity	-					5	7.04E-02	3.19	6.12E-01				
GO:0004029~aldehyde dehydrogenase (NAD) activ	-	2	2.44E-02	79.10	4.87E-01								

Term	PN				CCN2				PN + CCN2			
GO TERM BIOLOGICAL PROCESS	Count	P value	Fold Enrichment	Benjamini	Count	P value	Fold Enrichment	Benjamini	Count	P value	Fold Enrichment	Benjamini
GO:0060529~squamous basal epithelial stem cell d	-								2	3.26E-02	60.66	6.70E-01
GO:0043616~keratinocyte proliferation	-								4	4.81E-04	24.26	3.38E-02
GO:0001736~establishment of planar polarity	-								2	7.94E-02	24.26	8.36E-01
GO:0060601~lateral sprouting from an epithelium	-								2	9.45E-02	20.22	8.46E-01
GO:0031424~keratinization	-								7	5.09E-06	15.17	6.06E-04
GO:0030216~keratinocyte differentiation	-								12	2.41E-10	15.17	8.61E-08
GO:0009913~epidermal cell differentiation	-								12	4.87E-10	14.27	1.04E-07
GO:0042573~retinoic acid metabolic process	-	2	4.29E-02	44.41	9.89E-01				3	3.10E-02	10.70	6.64E-01
GO:0043588~skin development	-								5	1.38E-03	10.11	8.36E-02
GO:0008544~epidermis development	-								20	1.65E-13	9.71	1.77E-10
GO:0007398~ectoderm development	-								20	5.24E-13	9.12	2.81E-10
GO:0016101~diterpenoid metabolic process	-	2	6.97E-02	26.96	9.84E-01	3	2.14E-02	13.12	3.65E-01	4	1.06E-02	8.67	3.78E-01
GO:0001523~retinoid metabolic process	-	2	6.97E-02	26.96	9.84E-01	3	2.14E-02	13.12	3.65E-01	4	1.06E-02	8.67	3.78E-01
GO:0006721~terpenoid metabolic process	-	2	7.21E-02	26.03	9.79E-01	3	2.29E-02	12.66	3.78E-01	4	1.16E-02	8.37	3.95E-01
GO:0045104~intermediate filament cytoskeleton or	-								3	4.99E-02	8.27	7.73E-01
GO:0048806~genitalia development	-								3	5.41E-02	7.91	7.92E-01
GO:0048730~epidermis morphogenesis	-								3	5.41E-02	7.91	7.92E-01
GO:0006633~fatty acid biosynthetic process	-				6	4.99E-04	9.07	3.29E-02	10	6.88E-06	7.49	7.37E-04
GO:0030855~epithelial cell differentiation	-								15	1.36E-08	7.40	2.43E-06
GO:0060688~regulation of morphogenesis of a bra	-								3	6.28E-02	7.28	8.17E-01
GO:0045103~intermediate filament-based process	-								3	6.28E-02	7.28	8.17E-01
GO:0006775~fat-soluble vitamin metabolic process	-	2	8.41E-02	22.20	9.77E-01	3	3.08E-02	10.80	4.42E-01	4	1.80E-02	7.14	5.26E-01
GO:0006776~vitamin A metabolic process	-	2	6.49E-02	29.03	9.86E-01	3	1.86E-02	14.12	3.62E-01	3	6.74E-02	7.00	8.24E-01
GO:0060512~prostate gland morphogenesis	-								3	7.20E-02	6.74	8.25E-01
GO:0007435~salivary gland morphogenesis	-								3	7.20E-02	6.74	8.25E-01
GO:0007431~salivary gland development	-								3	9.14E-02	5.87	8.46E-01
GO:0007156~homophilic cell adhesion	-								10	1.31E-04	5.18	1.16E-02
GO:0006694~steroid biosynthetic process	-				4	1.98E-02	6.90	3.55E-01	6	6.16E-03	5.13	2.60E-01
GO:0008610~lipid biosynthetic process	-				17	1.10E-09	7.30	1.10E-06	24	2.96E-10	5.11	7.93E-08
GO:0006720~isoprenoid metabolic process	-				3	5.97E-02	7.49	6.20E-01	4	4.63E-02	4.95	7.66E-01
GO:0046394~carboxylic acid biosynthetic process	-				7	9.93E-04	6.08	5.41E-02	11	1.10E-04	4.73	1.07E-02
GO:0016053~organic acid biosynthetic process	-				7	9.93E-04	6.08	5.41E-02	11	1.10E-04	4.73	1.07E-02
GO:0060429~epithelium development	-								21	2.08E-08	4.70	3.18E-06
GO:0016042~lipid catabolic process	-	3	4.65E-02	8.45	9.83E-01	11	1.26E-07	10.05	6.35E-05	10	3.63E-04	4.53	2.75E-02
GO:0009063~cellular amino acid catabolic process	-					3	7.08E-02	6.80	6.63E-01	4	5.87E-02	4.49	8.03E-01
GO:0045017~glycerolipid biosynthetic process	-					3	8.01E-02	6.33	6.89E-01	4	6.97E-02	4.18	8.28E-01
GO:0007498~mesoderm development	-									4	7.26E-02	4.11	8.21E-01
GO:0001892~embryonic placenta development	-									4	7.86E-02	3.98	8.39E-01
GO:0006665~sphingolipid metabolic process	-				5	1.58E-03	9.87	6.99E-02	4	8.16E-02	3.91	8.33E-01
GO:0050678~regulation of epithelial cell proliferati	-								4	8.47E-02	3.85	8.39E-01
GO:0006643~membrane lipid metabolic process	-				5	1.78E-03	9.56	7.50E-02	4	8.78E-02	3.79	8.44E-01
GO:0046395~carboxylic acid catabolic process	-				5	4.19E-03	7.56	1.40E-01	5	4.41E-02	3.74	7.59E-01
GO:0016054~organic acid catabolic process	-				5	4.19E-03	7.56	1.40E-01	5	4.41E-02	3.74	7.59E-01
GO:0009310~amine catabolic process	-				3	9.73E-02	5.65	7.29E-01	4	9.10E-02	3.73	8.50E-01
GO:0006631~fatty acid metabolic process	-				11	2.36E-06	7.32	5.94E-04	11	9.21E-04	3.63	5.99E-02
GO:0022612~gland morphogenesis	-								5	4.93E-02	3.61	7.78E-01
GO:0016337~cell-cell adhesion	-								14	1.43E-04	3.60	1.17E-02
GO:0048839~inner ear development	-								5	5.67E-02	3.45	7.99E-01
GO:0008202~steroid metabolic process	-				7	1.96E-03	5.32	7.90E-02	9	5.08E-03	3.39	2.29E-01
GO:0002009~morphogenesis of an epithelium	-								9	7.74E-03	3.16	3.04E-01
GO:0048729~tissue morphogenesis	-								12	1.93E-03	3.06	1.09E-01
GO:0043583~ear development	-								5	9.18E-02	2.92	8.42E-01
GO:0009967~positive regulation of signal transduct	-	3	7.22E-02	6.58	9.70E-01				8	2.34E-02	2.82	5.83E-01
GO:0055114~oxidation reduction	-	6	2.77E-02	3.37	9.92E-01	20	1.68E-06	3.64	5.64E-04	30	1.78E-06	2.71	2.39E-04
GO:0010647~positive regulation of cell communica	-	3	8.49E-02	5.99	9.70E-01					8	3.63E-02	2.57	6.99E-01
GO:0043065~positive regulation of apoptosis	-									10	2.14E-02	2.45	5.76E-01
GO:0043068~positive regulation of programmed ce	-									10	2.25E-02	2.43	5.81E-01
GO:0048812~neuron projection morphogenesis	-									7	7.01E-02	2.41	8.23E-01
GO:0010942~positive regulation of cell death	-									10	2.34E-02	2.41	5.71E-01
GO:0031175~neuron projection development	-									8	6.73E-02	2.23	8.31E-01
GO:0007155~cell adhesion	-									20	2.29E-03	2.16	1.22E-01
GO:0022610~biological adhesion	-									20	2.34E-03	2.16	1.18E-01
GO:0030030~cell projection organization	-									10	8.05E-02	1.90	8.34E-01
GO:0050995~negative regulation of lipid catabolic	-				2	2.41E-02	81.61	3.82E-01					
GO:0046503~glycerolipid catabolic process	-				4	1.01E-05	81.61	1.70E-03					
GO:0046464~acylglycerol catabolic process	-				4	1.01E-05	81.61	1.70E-03					

Term	PN				CCN2				PN + CCN2			
GO TERM BIOLOGICAL PROCESS, Continued	Count	P value	Fold Enrichment	Benjamini	Count	P value	Fold Enrichment	Benjamini	Count	P value	Fold Enrichment	Benjamini
GO:0044269~glycerol ether catabolic process					4	1.01E-05	81.61	1.70E-03				
GO:0032328~alanine transport					2	2.41E-02	81.61	3.82E-01				
GO:0019433~triglyceride catabolic process					4	1.01E-05	81.61	1.70E-03				
GO:0010889~regulation of sequestering of triglycer					2	2.41E-02	81.61	3.82E-01				
GO:0046461~neutral lipid catabolic process					4	1.76E-05	69.95	2.54E-03				
GO:0050994~regulation of lipid catabolic process					3	1.33E-03	52.46	6.17E-02				
GO:0006111~regulation of gluconeogenesis					2	4.76E-02	40.80	5.47E-01				
GO:0015909~long-chain fatty acid transport					2	5.53E-02	34.98	5.97E-01				
GO:0010883~regulation of lipid storage					2	5.53E-02	34.98	5.97E-01				
GO:0019432~triglyceride biosynthetic process					2	6.30E-02	30.60	6.29E-01				
GO:0020027~hemoglobin metabolic process	2	2.55E-02	75.49	9.99E-01	2	7.81E-02	24.48	6.90E-01				
GO:0050872~white fat cell differentiation					2	7.81E-02	24.48	6.90E-01				
GO:0045833~negative regulation of lipid metabolic					2	7.81E-02	24.48	6.90E-01				
GO:0043255~regulation of carbohydrate biosynthet					2	7.81E-02	24.48	6.90E-01				
GO:0042572~retinol metabolic process					2	7.81E-02	24.48	6.90E-01				
GO:0034284~response to monosaccharide stimulus					4	7.11E-04	22.26	4.12E-02				
GO:0015671~oxygen transport					2	8.56E-02	22.26	7.04E-01				
GO:0009749~response to glucose stimulus					4	7.11E-04	22.26	4.12E-02				
GO:0009746~response to hexose stimulus					4	7.11E-04	22.26	4.12E-02				
GO:0002793~positive regulation of peptide secretic					2	8.56E-02	22.26	7.04E-01				
GO:0050873~brown fat cell differentiation					5	8.26E-05	21.11	7.53E-03				
GO:0046463~acylglycerol biosynthetic process					2	9.30E-02	20.40	7.21E-01				
GO:0046460~neutral lipid biosynthetic process					2	9.30E-02	20.40	7.21E-01				
GO:0006641~triglyceride metabolic process					6	9.79E-06	20.40	1.97E-03				
GO:0002021~response to dietary excess					2	9.30E-02	20.40	7.21E-01				
GO:0009743~response to carbohydrate stimulus					4	1.17E-03	18.83	5.73E-02				
GO:0002791~regulation of peptide secretion					4	1.17E-03	18.83	5.73E-02				
GO:0006094~gluconeogenesis					3	1.12E-02	18.36	2.64E-01				
GO:0006639~acylglycerol metabolic process					6	2.39E-05	17.08	3.00E-03				
GO:0050796~regulation of insulin secretion					3	1.35E-02	16.69	2.90E-01				
GO:0006662~glycerol ether metabolic process					6	3.00E-05	16.32	3.35E-03				
GO:0006638~neutral lipid metabolic process					6	3.00E-05	16.32	3.35E-03				
GO:0018904~organic ether metabolic process					6	4.12E-05	15.30	4.14E-03				
GO:0019319~hexose biosynthetic process					3	1.73E-02	14.69	3.48E-01				
GO:0006090~pyruvate metabolic process					3	1.73E-02	14.69	3.48E-01				
GO:0046883~regulation of hormone secretion					4	3.03E-03	13.60	1.11E-01				
GO:0044242~cellular lipid catabolic process					6	8.73E-05	13.12	7.30E-03				
GO:0019216~regulation of lipid metabolic process					6	1.03E-04	12.66	7.97E-03				
GO:0045444~fat cell differentiation					6	1.32E-04	12.04	9.42E-03				
GO:0046364~monosaccharide biosynthetic process					3	2.59E-02	11.85	3.93E-01				
GO:0006672~ceramide metabolic process					3	3.42E-02	10.20	4.71E-01				
GO:0046165~alcohol biosynthetic process					3	3.60E-02	9.93	4.77E-01				
GO:0046519~sphingoid metabolic process					3	3.78E-02	9.66	4.88E-01				
GO:0034754~cellular hormone metabolic process					4	8.56E-03	9.42	2.25E-01				
GO:0034637~cellular carbohydrate biosynthetic pro					4	1.05E-02	8.74	2.55E-01				
GO:0032868~response to insulin stimulus					4	1.21E-02	8.30	2.69E-01				
GO:0043434~response to peptide hormone stimulu					6	1.03E-03	7.73	5.32E-02				
GO:0016051~carbohydrate biosynthetic process					5	4.57E-03	7.37	1.47E-01				
GO:0009894~regulation of catabolic process					3	6.19E-02	7.34	6.28E-01				
GO:0008203~cholesterol metabolic process					4	1.91E-02	7.00	3.57E-01				
GO:0006818~hydrogen transport					3	6.85E-02	6.93	6.56E-01				
GO:0042445~hormone metabolic process	3	2.24E-02	12.58	1.00E+00	5	6.09E-03	6.80	1.80E-01				
GO:0007188~G-protein signaling, coupled to cAMP					3	7.31E-02	6.68	6.70E-01				
GO:0046486~glycerolipid metabolic process					7	6.23E-04	6.64	3.85E-02				
GO:0040014~regulation of multicellular organism g					4	2.29E-02	6.53	3.73E-01				
GO:0016125~sterol metabolic process					4	2.45E-02	6.36	3.81E-01				
GO:0046942~carboxylic acid transport					5	8.49E-03	6.18	2.29E-01				
GO:0045761~regulation of adenylate cyclase activit					3	8.49E-02	6.12	7.06E-01				
GO:0015849~organic acid transport					5	8.79E-03	6.12	2.24E-01				
GO:0007187~G-protein signaling, coupled to cyclic					3	8.49E-02	6.12	7.06E-01				
GO:0019933~cAMP-mediated signaling					3	8.74E-02	6.02	7.07E-01				
GO:0051339~regulation of lyase activity					3	8.98E-02	5.92	7.13E-01				
GO:0031279~regulation of cyclase activity					3	8.98E-02	5.92	7.13E-01				
GO:0030817~regulation of cAMP biosynthetic proce					3	9.73E-02	5.65	7.29E-01				

Term	-	PN				CCN2				PN + CCN2			
GO TERM BIOLOGICAL PROCESS, Continued	-	Count	P value	Fold Enrichment	Benjamini	Count	P value	Fold Enrichment	Benjamini	Count	P value	Fold Enrichment	Benjamini
GO:0008217~regulation of blood pressure	-					3	9.73E-02	5.65	7.29E-01				
GO:0019935~cyclic-nucleotide-mediated signaling	-					3	9.98E-02	5.56	7.34E-01				
GO:0010740~positive regulation of protein kinase (	-					4	3.45E-02	5.56	4.68E-01				
GO:0015837~amine transport	-					4	3.86E-02	5.32	4.89E-01				
GO:0030534~adult behavior	-					4	3.96E-02	5.27	4.93E-01				
GO:0006006~glucose metabolic process	-					6	5.57E-03	5.25	1.71E-01				
GO:0009725~response to hormone stimulus	-					7	2.22E-03	5.19	8.55E-02				
GO:0051046~regulation of secretion	-					5	1.87E-02	4.90	3.57E-01				
GO:0010817~regulation of hormone levels	-	3	4.28E-02	8.85	9.96E-01	5	2.02E-02	4.78	3.54E-01				
GO:0010876~lipid localization	-					5	2.02E-02	4.78	3.54E-01				
GO:0009719~response to endogenous stimulus	-					7	3.80E-03	4.66	1.33E-01				
GO:0019318~hexose metabolic process	-					6	1.20E-02	4.35	2.75E-01				
GO:0005996~monosaccharide metabolic process	-					6	1.95E-02	3.85	3.56E-01				
GO:0060341~regulation of cellular localization	-					5	4.17E-02	3.80	5.05E-01				
GO:0051094~positive regulation of developmental	-					5	9.58E-02	2.86	7.27E-01				
GO:0010033~response to organic substance	-					11	7.79E-03	2.67	2.18E-01				
GO:0055085~transmembrane transport	-					8	7.96E-02	2.13	6.92E-01				
GO:0042168~heme metabolic process	-	2	4.79E-02	39.73	9.73E-01								
GO:0033013~tetrapyrrole metabolic process	-	2	6.49E-02	29.03	9.86E-01								
GO:0006778~porphyrin metabolic process	-	2	6.49E-02	29.03	9.86E-01								
GO:0002824~positive regulation of adaptive immur	-	2	8.41E-02	22.20	9.77E-01								
GO:0002821~positive regulation of adaptive immur	-	2	8.41E-02	22.20	9.77E-01								
GO:0042440~pigment metabolic process	-	2	9.11E-02	20.40	9.70E-01								
GO:0002708~positive regulation of lymphocyte me	-	2	9.82E-02	18.87	9.71E-01								
GO:0002705~positive regulation of leukocyte medi	-	2	9.82E-02	18.87	9.71E-01								

Appendix E: Copyright and use license from Cell Adhesion & Migration

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Signed for and on behalf of the Authors: _____ Date: _____

Please print name: _____

Appendix F: University Council on Animal Care ethics approval

Western

August 28, 2008

This is the Original Approval for this protocol
A Full Protocol submission will be required in 2012

Dear Dr. Hamilton:

Your Animal Use Protocol form entitled:
In Vitro and In Vivo Influence of Substratum Topography and Chemistry on Osteoblast, Osteoclast, and Fibroblast Physiology
Funding Agency CANADIAN INSTITUTES OF HEALTH RESEARCH - APPLIED; INTERNATIONAL TEAM FOR IMPLANTOLOGY Grant #538-2007; NATURAL SCIENCES & ENGINEERING RESEARCH COUNCIL - APPLIED ONTARIO CENTRE OF EXCELLENCE - Grant #R4091A02

has been approved by the University Council on Animal Care. This approval is valid from **August 28, 2008** to **August 31, 2009.** The protocol number for this project is **#2008-097**.

1. This number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this number.
3. If no number appears please contact this office when grant approval is received.
   If the application for funding is not successful and you wish to proceed with the project, request that an internal scientific peer review be performed by the Animal Use Subcommittee office.
4. Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.

ANIMALS APPROVED FOR 4 Years

Species	Strain	Other Detail	Pain Level	Animal # Total for 4 Years
Mouse	Periostin KO	addult M/F	D	2000
Rat	Sprague Dawley	200-600 gms M/F	D	50
Rabbit	NZW/SPF	2-4 kg M	D	27

REQUIREMENTS/COMMENTS
Please ensure that individual(s) performing procedures on live animals, as described in this protocol, are familiar with the contents of this document.

c.c. Approved Protocol - D. Hamilton, W. Lagerwerf
Approval Letter - W. Lagerwerf

Appendix G: Use of human subjects ethics approval

Office of Research Ethics
The University of Western Ontario
London, ON, Canada N6A 5C1
Telephone: Fax: Email:
Website: www.uwo.ca/research/ethics

Western

Use of Human Subjects - Ethics Approval Notice

Principal Investigator: Dr. D.W. Hamilton
Review Number: **16245E** **Review Level:** Expedited
Review Date: June 24, 2009
Protocol Title: Role of periostin in the repair of skin
Department and Institution: Anatomy, University of Western Ontario
Sponsor: CIHR-CANADIAN INSTITUTE OF HEALTH RESEARCH
Ethics Approval Date: July 10, 2009 **Expiry Date:** July 30, 2014
Documents Reviewed and Approved: UWO Protocol, Letter of Information and Consent.
Documents Received for Information:

This is to notify you that The University of Western Ontario Research Ethics Board for Health Sciences Research Involving Human Subjects (HSREB) which is organized and operates according to the Tri-Council Policy Statement: Ethical Conduct of Research Involving Humans and the Health Canada/ICH Good Clinical Practice Practices: Consolidated Guidelines; and the applicable laws and regulations of Ontario has reviewed and granted approval to the above referenced study on the approval date noted above. The membership of this REB also complies with the membership requirements for REB's as defined in Division 5 of the Food and Drug Regulations.

The ethics approval for this study shall remain valid until the expiry date noted above assuming timely and acceptable responses to the HSREB's periodic requests for surveillance and monitoring information. If you require an updated approval notice prior to that time you must request it using the UWO Updated Approval Request Form.

During the course of the research, no deviations from, or changes to, the protocol or consent form may be initiated without prior written approval from the HSREB except when necessary to eliminate immediate hazards to the subject or when the change(s) involve only logistical or administrative aspects of the study (e.g. change of monitor, telephone number). Expedited review of minor change(s) in ongoing studies will be considered. Subjects must receive a copy of the signed information/consent documentation.

Investigators must promptly also report to the HSREB:

a) changes increasing the risk to the participant(s) and/or affecting significantly the conduct of the study;
b) all adverse and unexpected experiences or events that are both serious and unexpected;
c) new information that may adversely affect the safety of the subjects or the conduct of the study.

If these changes/adverse events require a change to the information/consent documentation, and/or recruitment advertisement, the newly revised information/consent documentation, and/or advertisement, must be submitted to this office for approval.

Members of the HSREB who are named as investigators in research studies, or declare a conflict of interest, do not participate in discussion related to, nor vote on, such studies when they are presented to the HSREB.

Chair of HSREB: Dr. Joseph Gilbert

Ethics Officer to Contact for Further Information

☐ Janice Sutherland	☐ Elizabeth Wambolt	☑ Grace Kelly	☐ Denise Grafton

This is an official document. Please retain the original in your files.

cc: ORE File
LHRI

Curriculum Vitae

Christopher Gordon Elliott, CD

Education

1. Bachelor of Science (Honours) (2002-2007)
   Biomedical Biology Honours program
   Laurentian University, Sudbury, ON
2. PhD Candidate (2008-Present)
   Department of Anatomy and Cell Biology
   University of Western Ontario, London, ON

Scholarships and Awards

1. A.B. Lakshman Memorial Prize in Biology (2008)
2. Schulich Graduate Scholarship (2008-2013)
3. Western Graduate Research Scholarship (2008-2013)
4. Ontario Graduate Scholarship (2009-2010): Declined
5. National Science and Engineering Research Council of Canada,
   CGS-M (2009-2010)
6. Ontario Graduate Scholarship (2010-2011): Declined
7. National Science and Engineering Research Council of Canada,
   CGS-D3 (2010-2013)
8. Canadian Student Health Research Forum Silver Poster Award (2011)
9. ICS Travel Award, Institute of Musculoskeletal Health and Arthritis (2012)

Teaching Assistantships

1. Biology 281b - Genetics (2009)
2. Anatomy 3309 - Mammalian Histology (2010-2011)
3. Medical Sciences 4900F/G - Medical Sciences Laboratory (2011-2013 winter)

Publications

1. **Elliott, C.**, Ye, Z., Mojumdar, S., and Saleh, M. (2007) *A potential carrier for bioremediation, characterization of insoluble potato fiber.* Journal of Thermal Analysis and Calorimetry. 90: 707-11
2. Zhou, H., Wang, J., **Elliott, C.**, Wen, W., Hamilton, D. and Conway, S.J. (2010) *Spatiotemporal expression of periostin during skin development and incisional wound healing: lessons for human fibrotic scar formation.* J. Cell Commun. Signal. 4(2): 99-107
3. Wen, W., Chau, E., **Elliott, C.**, Daley, T. and Hamilton, D. (2010) *TGF-β1 and FAK regulate periostin expression in PDL fibroblasts.* J. Dent Res. 89(12): 1439-43
4. Parapuram, S., Shi-wen, X., **Elliott, C.,** Welch, I., Jones, H., Baron, M., Denton, C., Abraham, D., and Leask, A. (2011) *Loss of PTEN Expression by Dermal Fibroblasts Causes Skin Fibrosis.* J. Invest Dermatol. 131: 1996-2003
5. **Elliott, C.,** and Hamilton, D.W. (2011) *Deconstructing Fibrosis Research: Do Pro-fibrotic Signals Point the Way for Chronic Dermal Wound Regeneration?* J. Cell Commun Signal. 5(4): 301-15
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8. **Elliott, C.,** Kim, S.S., and Hamilton, D.W. (2012) *Functional Significance of Periostin in Excisional Wound Healing: Is the Devil in the Detail?* Cell Adh Migr. 6(4): 319-26
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10. Pepe, D., **Elliott, C.,** Forbes, T., Hamilton, D.W. (accepted) *Detection of Galectin-3 and Location of Advanced Glycation End Products (AGE) in Human Chronic Skin Wounds.* Histol Histopathol. B-4898

Other Contributions

1. Conference Presentation: *Characterization of Insoluble Potato Starch Fibers: A Potential Bacterial Carrier for Bioremediation*, Ontario Biology Day 2007, Hamilton, ON.
2. Poster Presentation: *Periostin knockout mice show a reduction in collagen production, wound contraction and TGFβ1 response in experimentally induced dermal wounds.* Murry Barr Research Day 2009, London, ON.
3. Poster Presentation: *Periostin knockout mice show a reduction in collagen production, wound contraction and TGF beta 1 response in experimentally induced dermal wounds.* Organization and Regulation of the Extracellular Matrix Poster Session, 49th ASCB Annual Meeting December 5th–9th, 2009, San Diego, CA.
4. Published Poster: *Periostin knockout mice show a reduction in collagen production, wound contraction and TGF beta 1 response in experimentally induced dermal wounds.* Faculty of 1000 Posters.
5. Poster Presentation: *Periostin knockout mice show a reduction in collagen production, wound contraction and TGF beta 1 response in experimentally induced dermal wounds.* Margret Moffat Research Day, 2010, London, ON.
6. Oral Presentation: *The role of periostin in cutaneous wound repair.* Murry Barr Research Day 2010, London, ON.
7. Poster Presentation: *Periostin modulates myofibroblast differentiation during full-thickness cutaneous wound repair.* Margret Moffat Research Day, 2011, London, ON.
8. Poster Presentation: *Periostin modulates myofibroblast differentiation during full-thickness cutaneous wound repair.* CIHR Canadian Student Health Research Forum, 2011, Winnipeg, MB

9. Oral Presentation: *Periostin modulates myofibroblast differentiation during full-thickness cutaneous wound repair.* International CCN Society Meeting, 2011, Vancouver, BC.
10. Poster Presentation: *Targeting matricellular proteins in human chronic skin wounds.* London Health Research Day, 2012, London, ON.
11. Oral Presentation: *Periostin in Skin Repair: Closing the Gap.* Department of Anatomy and Cell Biology Departmental Seminar, Western University of Canada, 2012, London, ON.
12. Oral Presentation: *Periostin in Skin Repair: Closing the Gap.* Demonstration for ACB5620/9620, Department of Anatomy and Cell Biology, Western University of Canada, 2012, London, ON.
13. Oral Presentation: *Targeting matricellular proteins in human chronic skin wounds.* European Tissue Repair Society Meeting, 2012, Athens, Greece
14. Oral Presentation: *We can rebuild him. We have the technology. But do we have to make him better than he was before?* Retiring with Strong Minds Seminar Series, 2013, London, ON
15. Oral Presentation: *Periostin in Skin Repair: Closing the Gap.* Guest Speaker for the Matrix Dynamics Group, Faculty of Dentistry, University of Toronto, 2013, Toronto, ON.

Leadership and Community Contributions

1. Let's Talk Science Volunteer (2010-Present)
2. Chair, Graduate Students' Council for the Department of Anatomy and Cell Biology, University of Western Ontario (2010-2012)
3. Volunteer Judge, Annual London District Science and Technology Fair (2011)
4. Event Organizer, Schulich Beach Volleyball Fundraiser (2011, 2012)
5. Event Organizer, Department of Anatomy and Cell Biology Welcome BBQ (2011, 2012)
6. Master of Ceremonies, Fundraiser Trivia Night (2011)
7. Chair, Graduate Students' Council for Schulich School of Medicine and Dentistry, University of Western Ontario (2011-2012)

8. Student Representative, Graduate Affairs Committee for Schulich School of Medicine and Dentistry, University of Western Ontario (2011-2012)
9. Master of Ceremonies, Speed Dating Fundraiser (2012)
10. Master of Ceremonies, Fundraiser Trivia Night (2012)