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The Roles of Matrix-Associated Periostin in an In Vitro Model of Hypertrophic Scarring

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Graduate Program in Biochemistry

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

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THE ROLES OF MATRIX-ASSOCIATED PERIOSTIN IN AN *IN VITRO* MODEL OF HYPERTROPHIC SCARRING

(Thesis format: Monograph)

by

Justin David Crawford

Graduate Program in Biochemistry

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

The School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

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Abstract

Abnormal scarring is a type of benign fibrosis of the skin that can restrict mobility, dexterity and quality of life. There are few, if any, truly effective treatment options for these conditions. Hypertrophic scarring is a common form of abnormal scarring characterized by increased fibroblast proliferation and differentiation of apoptosis-resistant and hyper-contractile myofibroblasts that promote excessive deposition and contracture of the extracellular matrix (ECM). Periostin, a secreted ECM protein, is transiently expressed during normal cutaneous wound repair, but is abnormally abundant and persistent in abnormal scars and other benign fibroses that display enhanced fibroblast proliferation and myofibroblast differentiation. The objectives of this study were to elucidate the effects of periostin on fibroblast proliferation, myofibroblast differentiation and myofibroblast persistence in an in vitro human fibroblast model of hypertrophic scarring. Primary fibroblasts derived from patients with hypertrophic scars (HTS) or normal skin (NS) were cultured in two- and three-dimensional collagen cultures to more closely mimic their in vivo microenvironment. A series of in vitro techniques, including adenoviral transduction, WST-1 assays, stressed fibroblast populated collagen lattices (sFPCLs), western immunoblotting and immunofluorescence confocal microscopy were employed to assess periostin effects on fibroblast proliferation and myofibroblast differentiation under these culture conditions. Periostin treatment was shown to enhance HTS fibroblast proliferation in compliant 2D collagen cultures through Akt and Rho kinase dependent pathways. When subjected to isometric tension in sFPCLs, periostin enhanced myofibroblast differentiation, as evidenced by increases in collagen contraction, alpha smooth muscle actin (αSMA) and the formation of supermature focal adhesions. In contrast, no discernible effects of periostin treatment were evident in NS fibroblasts. Periostin signalling maintained αSMA levels in HTS fibroblasts in an environment of decreasing ECM tension, and this was correlated with changes in focal adhesion kinase, coflin and myosin light chain activities. These studies are the first to identify periostin as a mediator of excessive proliferation, myofibroblast differentiation and persistence in human fibroblasts derived from hypertrophic scars. The findings reported here suggest that novel therapeutic interventions designed to deplete periostin levels in the dermis of hypertrophic scars may have utility for attenuating fibroblast proliferation and depleting myofibroblast populations, and thereby enhance scar resolution.
Keywords

Hypertrophic scarring; periostin; fibroblasts; \textit{in vitro} human model; proliferation; myofibroblast differentiation and persistence; cell signalling; focal adhesions
Acknowledgments

“It’s good for the soul”

- Dr. David O’Gorman, 2009

First and foremost, I would like to thank my supervisor Dr. David O’Gorman. David it has been a great pleasure to work with you over the past six years. I am truly grateful for all your patience, support, and guidance and for giving me the opportunity to present my research on numerous occasions across Europe. Your advice and encouragement has been invaluable in my development as a scientist and I could not ask for a better mentor.

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<tbody>
<tr>
<td>2D</td>
<td>two-dimensional</td>
</tr>
<tr>
<td>3D</td>
<td>three-dimensional</td>
</tr>
<tr>
<td>αMEM</td>
<td>Alpha modified Eagle’s medium</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>αSMA</td>
<td>Alpha smooth muscle actin</td>
</tr>
<tr>
<td>Akti</td>
<td>Akt inhibitor VIII</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine-5’-triphosphate</td>
</tr>
<tr>
<td>AU</td>
<td>Arbitrary units</td>
</tr>
<tr>
<td>βig-h3</td>
<td>TGFβ-induced gene clone 3</td>
</tr>
<tr>
<td>bp</td>
<td>Base pairs</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>CM</td>
<td>Conditioned media</td>
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<tr>
<td>DI</td>
<td>Aspartic acid-Isoleucine</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EMI</td>
<td>Emilin-like</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial-mesenchymal transition</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>ERKi</td>
<td>Extracellular signal-regulated kinase inhibitor, PD98059</td>
</tr>
<tr>
<td>FA</td>
<td>Focal adhesion</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>FAK</td>
<td>Focal adhesion kinase</td>
</tr>
<tr>
<td>FAS1</td>
<td>Fasciclin 1</td>
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<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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<tr>
<td>GAP</td>
<td>GTPase activating protein</td>
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<td>GDP</td>
<td>Guanosine-5’-diphosphosphate</td>
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<td>GEF</td>
<td>Guanine nucleotide exchange factor</td>
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<tr>
<td>GSK-3β</td>
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<td>GTP</td>
<td>Guanosine-5’-triphosphate</td>
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<tr>
<td>hrPN</td>
<td>Human recombinant periostin</td>
</tr>
<tr>
<td>HTS</td>
<td>Hypertrophic scar</td>
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<tr>
<td>IP</td>
<td>Immunoprecipitation</td>
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<td>KS</td>
<td>Keloid scar</td>
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<tr>
<td>LAP</td>
<td>Latency-associated peptide</td>
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<tr>
<td>LC-MS/MS</td>
<td>Liquid chromatography tandem mass spectrometry</td>
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<tr>
<td>LTBP-1</td>
<td>Latent TGFβ-1 binding protein-1</td>
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<tr>
<td>MLC</td>
<td>Myosin light chain</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>MOI</td>
<td>Multiplicity of infection</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular weight</td>
</tr>
<tr>
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<td>Nickel</td>
</tr>
<tr>
<td>NLS</td>
<td>Nuclear localization signal</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
</tr>
<tr>
<td>---------</td>
<td>-----------</td>
</tr>
<tr>
<td>NS</td>
<td>Normal skin</td>
</tr>
<tr>
<td>Pa</td>
<td>Pascals</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<tr>
<td>pCofilin</td>
<td>Phosphorylated cofilin</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>pFAK</td>
<td>Phosphorylated focal adhesion kinase</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PI3Ki</td>
<td>Phosphatidylinositol 3-kinase inhibitor, LY294002</td>
</tr>
<tr>
<td>pMLC</td>
<td>Phosphorylated MLC</td>
</tr>
<tr>
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<td>Polymorphonuclear cells</td>
</tr>
<tr>
<td>PN</td>
<td>Periostin</td>
</tr>
<tr>
<td>RGD</td>
<td>Arginine-Glycine-Aspartic acid</td>
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<tr>
<td>rFPCL</td>
<td>Relaxed fibroblast populated collagen lattice</td>
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<tr>
<td>ROCK</td>
<td>Rho kinase</td>
</tr>
<tr>
<td>ROCKi</td>
<td>Rho kinase inhibitor, Y27632</td>
</tr>
<tr>
<td>RQ</td>
<td>Relative quantification</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate – polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>sFPCL</td>
<td>Stressed fibroblast populated collagen lattice</td>
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<tr>
<td>suFA</td>
<td>Supermature focal adhesion</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Transforming growth factor beta</td>
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<td>UV</td>
<td>Ultraviolet</td>
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xvii
<table>
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<tr>
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<td>Veh</td>
<td>Vehicle</td>
</tr>
<tr>
<td>WB</td>
<td>Western immunoblot</td>
</tr>
<tr>
<td>WST-1</td>
<td>Water soluble tetrazolium-1</td>
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Chapter 1

1  Introduction

1.1  The integumentary system

The integumentary system, which is comprised of the skin and its appendages, is the largest organ in the body and accounts for up to 15% of the total adult body weight (1, 2). The main function of the skin is to provide a physical, chemical, and adaptive barrier to protect the body from external damage (1, 3, 4). The skin also plays important roles in hydration, sensation, thermoregulation, vitamin D synthesis, immune function and ultraviolet radiation protection (2, 5). This unique tissue is composed of two morphologically and functionally distinct layers: the epidermis and the dermis (1, 6).

1.1.1  The epidermis

The superficial protective layer known as the epidermis is largely composed of stratified epithelium that continuously renews itself (1). The epidermis is mostly comprised of keratinocytes (90-95%) that originate from progenitor cells adjacent to the dermis. These cells undergo progressive differentiation resulting in flattened, anucleated, keratin-rich cells that form the physical barrier of the skin (1, 5). The epidermis also contains Langerhan’s cells, melanocytes, Merkel cells, and lymphocytes (1, 2). Immune function in the epidermis is maintained by lymphocytes and Langerhan’s cells, which are dendritic cells that process and present antigens to T-cells (1, 2). Melanocytes produce the protein melanin, which is responsible for skin colour and protection from ultraviolet radiation (1, 2). Lastly, Merkel cells are mechanoreceptors that make contact with sensory neurons and are involved with the sensation of touch (1, 2).

The epidermis is typically composed of four, and in some cases five, continuous layers known as stratum. From lower to upper epidermis, they are the stratum basale (the only layer capable of cell division), stratum spinosum, stratum granulosum, stratum luciderm (a layer only found in the palms and soles) and the stratum corneum (1, 2, 6). Since the epidermis is avascular, it relies on the vasculature in the adjacent dermis to provide nutrients. Therefore, as keratinocytes divide and move up through the epidermal layers
and away from the dermis, they receive fewer nutrients, undergo terminal differentiation and are ultimately shed by abrasion (1, 2). The epidermis is connected to the underlying dermis by the dermal-epidermal junction that facilitates the exchange of metabolic products between the stratum basale and the dermis (1, 2).

1.1.2 The dermis

The primary role of the dermis is to provide structure and tensile strength to the skin (1, 2, 6). The dermis is divided into 2 layers, the papillary and reticular dermis (1, 2, 6). The papillary region is adjacent to the epidermis and contains the vasculature that allows for the exchange of metabolic products between the epidermis and dermis (1, 2, 6). The papillary dermis is also rich in nerves and nerve endings, both of which are responsible for transmitting the sensation of heat and touch (1, 6). The deeper reticular dermis is composed of fibroblasts, blood vessels, lymphatic vessels and extracellular matrix (ECM) proteins including collagens and elastins (1, 6). Fibroblasts are the heterogeneous population of cells of mesenchymal origin that synthesize and secrete collagens, proteoglycans, fibronectin, matrix metalloproteinases (MMPs) and other ECM proteins that make up the papillary and reticular dermal layers (7). The organization of these ECM proteins provides tensile strength and structure to the skin (2). The reticular dermis also supports the skin’s appendages including hair follicles and various glands, including sweat glands (1, 2, 6).

Below the dermis is a layer known as the hypodermis that primarily consists of subcutaneous fat. Although not technically part of the skin, the hypodermis functions to anchor the skin to the underlying muscle and bone, and supplies the dermis with blood vessels and nerves (1, 6). Other than adipocytes, the hypodermis is also home to mast cells, lymphocytes, and fibroblasts, and contributes to thermoregulation, energy storage, immune response, and protection from mechanical injuries (1, 6).

Since the skin acts as a physical, chemical, and adaptive barrier to external insults, damage to the skin can leave an individual susceptible to a number of pathological conditions (8). Upon injury, the body needs to rapidly and effectively repair the wound to prevent infection and restore integrity to the skin (8).
1.2 Normal cutaneous wound repair

Integumental injuries are defined as any insult or disruption to the normal structure and function of the skin (9, 10). These injuries can range from a small break that disrupts epithelial integrity to injuries extending deep into the dermis and subcutaneous tissue (11, 12). The complex process required to repair injury, wound repair, is divided into 3 overlapping phases: the inflammatory, proliferation and remodelling phases (Figure 1.1, (10)). In each phase, several important molecular, humoral and cellular events occur in order to regenerate the protective barrier of the skin (10). The important features of each phase of normal cutaneous wound repair are described in the following sections.

1.2.1 Hemostasis and the inflammatory phase

Immediately after insult to the dermis, the inflammatory phase begins with a process known as hemostasis, characterized by the formation of a blood clot and cessation of bleeding (8). Fibrin, platelets and disrupted blood vessels play an important role in mediating this process. Minutes after injury, a clot is formed comprised mainly of fibrin and platelets (8). The formation of the clot not only prevents blood flow, it also acts as a provisional matrix for inflammatory cells and fibroblasts to infiltrate the wound (12). This provisional matrix is also a repository for growth factors and cytokines (12). During hemostasis, activated platelets in the provisional matrix excrete the growth factors and cytokines in their granules. One of these cytokines is transforming growth factor (TGF)β, a molecule that is utilized by numerous cell types in all phases of wound healing (8, 13, 14). In addition to the formation of a fibrin clot, vasoconstriction occurs at the site of injury, thereby preventing blood flow and contributing to hemostasis (8). Vasoconstriction lasts upwards of 15 minutes and is mediated by the release of vasoactive amines from injured cells (15, 16). Local vasoconstriction, in concert with activated platelets, allows for the accumulation of growth factors and cytokines in the provisional matrix and promotes the recruitment of inflammatory cells and fibroblasts to the wound (10).

Once hemostasis is achieved, vascular changes occur that result in vasodilation and increased capillary permeability (8). The increased blood flow into the wound facilitates
Normal cutaneous wound repair progresses through three overlapping phases following insult to the dermis. (a) The inflammatory phase is characterized by clot formation, vascular changes and recruitment of inflammatory cells, including macrophages, to the wound. Inflammatory cells are a major source of growth factors and cytokines that promote the proliferation phase. (b) The proliferation phase is marked by reepithelialization, angiogenesis, fibroblast migration and proliferation, collagen deposition and myofibroblast-mediated wound closure. (c) During the remodelling phase, collagen synthesis, degradation and ECM remodelling occur in order to restore strength to the wound. As the wound heals and the mechanical tension in the ECM is reduced, myofibroblasts and inflammatory cells undergo apoptosis. (d) The net result is a mature scar with high tensile strength. (e) When wound healing signals persist, excessive fibroblast proliferation and myofibroblast differentiation promote “overhealing” characterized by excessive ECM deposition and remodelling leading to the formation of abnormal scars.
the migration of inflammatory cells (8), such as polymorphonuclear cells (PMNs) and macrophages, that are attracted to the wound by growth factors and cytokines such as TGFβ-1 (10, 12, 16, 17). PMNs and activated macrophages remove cellular debris, foreign particles and bacteria, and are a major source of the proinflammatory cytokines (13, 16, 18, 19) that regulate the inflammatory phase and initiate the proliferation phase (8, 13, 20, 21).

1.2.2 The proliferation phase
The proliferation phase is characterized by the formation of granulation tissue, dermal re-vascularization and the re-establishment of the protective barrier of the skin. Fibroblasts play a vital role in the proliferation phase as they produce the ECM proteins, including collagens, that make up the granulation tissue (8). The provisional matrix is initially devoid of fibroblasts, therefore fibroblast migration, proliferation, and ECM deposition are vital for the regeneration of a functional dermis (8). Plasma-derived fibronectin (22) and TGFβ-1 are components of the provisional matrix that stimulate fibroblast migration to the wound (13, 23). Once within the wound, fibroblasts secrete components of the ECM, including collagens and fibronectin, and growth factors that regulate the function of other cells in the matrix (8). The ECM provides a scaffold for cell adhesion and regulates the growth, migration, and differentiation of the cells within it (24, 25).

Collagen production begins approximately 3-5 days after injury and is stimulated by a number of cytokines including TGFβ-1 (13, 16). In normal skin and mature scars, dermal collagen is composed of 80-90% type I collagen and 10-20% type III collagen. In the early stages of wound healing, the proportion of type III collagen increases to approximately 30% (8). Collagen deposition not only provides strength to the wound but also provides structural support for capillary formation and re-epithelialization (8).

The revascularization of the wound is a complex, but important, component of the proliferation phase. Cytokines, including TGFβ-1 (26), activate endothelial cells at the wound site, resulting in the release of proteolytic enzymes, such as MMPs, that dissolve the basal lamina (10). This allows the activated endothelial cells to proliferate and migrate within the wound. These newly built vessels interconnect with each other
forming a vessel loop (10). These new vessels differentiate into arteries and venules and restore blood flow to the newly formed granulation tissue (10).

Re-epithelialization is another important process in the proliferation phase and is required to restore the protective barrier of the skin. Keratinocyte migration across the provisional matrix and newly formed granulation tissue is stimulated by a lack of contact inhibition and growth factor stimulation (8, 10). As migration proceeds, keratinocyte proliferation occurs at the wound edge to replace the cells and provide more cells for the migrating sheet (8, 10). Migration ceases when cells from opposing sides of the wound come in contact (8, 10). Once contact inhibition is established, the keratinocytes proliferate and differentiate to re-establish the stratified epithelium, thus restoring the skin’s protective barrier (8, 10).

1.2.3 Myofibroblast differentiation
A major event in the proliferation phase is the differentiation of fibroblasts into contractile myofibroblasts. Myofibroblasts are the primary ECM producing cells in normal cutaneous wound healing and are responsible for the contracture of the granulation tissue during repair (27, 28). The myofibroblast was first identified in granulation tissue over forty years ago as a fibroblastic cell with a prominent endoplasmic reticulum and contractile microfilament bundles (29). The primary distinguishing feature of myofibroblasts is the neo-expression of alpha smooth muscle actin (αSMA) in stress fibres, allowing these cells to exhibit a hyper-contractile phenotype (30). Myofibroblasts are generally negative for smooth muscle myosin heavy chain, desmin, h-caldesom, and smoothelin, thereby distinguishing myofibroblasts from smooth muscle cells (31). In addition to fibroblasts, pericytes, vascular smooth muscle cells, cells undergoing epithelial-mesenchymal transition (EMT) and fibrocytes have all been implicated as precursors for myofibroblasts (32-37). Myofibroblasts combine the contractile features of smooth muscle cells with the extensive ECM production of fibroblasts (29, 31) thus allowing for deposition, contraction, and remodelling of the granulation tissue.

Fibroblasts in unwounded skin are stress shielded by the highly organized and cross-linked ECM that surrounds them. Following insult to the dermis, the initial contraction of
the wound by the tractional forces imposed by migrating fibroblasts, increases the mechanical tension in the granulation tissue (29, 38). This increase in ECM tension promotes the differentiation of fibroblasts into “proto”-myofibroblasts, characterized by the formation of stress fibres containing β and γ actin, mature focal adhesion formation, and de novo expression of the fibronectin splice variant, ED-A fibronectin (38, 39). Stress fibres connect to the ECM through integrin-associated focal adhesion complexes, while cell-cell interactions occur through de novo N-cadherin expression. The net result is an environment under high mechanical tension that allows the fibroblasts to stabilize their proto-myofibroblast phenotype (37).

Three events are required for the differentiation of proto-myofibroblasts into differentiated myofibroblasts characterized by the de novo expression of αSMA in their stress fibres (Figure 1.2, (30)). The first is the accumulation of cytokines such as active TGFβ-1, which induces αSMA expression (40, 41). Platelet granules, immune cells, and fibroblasts secrete TGFβ-1 in a latent form that is activated by increased mechanical tension within the ECM and by ECM-associated proteases (13, 42). Second, an accumulation of ED-A fibronectin in the ECM is required for myofibroblast differentiation (39). Lastly, the cells need to be stimulated by the increase in ECM tension generated by ECM remodelling (30). Specialized cell-ECM junctions termed fibronexus (in vivo) or “supermature” focal adhesions (in vitro) mediate this mechanoperception (43). The rigidity of the ECM determines the size of these cellular attachments to the ECM (43). αSMA can only become incorporated into pre-existing actin stress fibres once the ECM is stiff enough to allow the formation of supermature focal adhesions (suFAs, (43)). The formation of suFAs in combination with αSMA induction allows myofibroblasts to exert a 2-4 fold higher contractile stress on the ECM (43). A feedback loop between enhanced suFA formation and an ECM tension that is above threshold levels can maintain myofibroblasts in a differentiated state (43, 44).

In addition to secreting ECM proteins into the granulation tissue, myofibroblasts utilize their contractile machinery to achieve wound closure. This contractile force is generated by contractile stress fibres composed of actin bundles, non-muscle myosin, and actin-binding proteins, and is regulated by myosin light chain (MLC) phosphorylation
Figure 1.2 Schematic representation of the events required for myofibroblast differentiation. Fibroblasts in unwounded tissue are void of stress fibers and focal adhesions. Upon wounding, initial closure of the wound by migratory fibroblasts increases ECM tension leading to the formation of proto-myofibroblasts. Proto-myofibroblasts are characterized by the formation of stress fibers, mature focal adhesions and \textit{de novo} expression of the fibronectin splice variant, ED-A fibronectin. Contractile forces generated by proto-myofibroblasts increase the mechanical tension in the ECM resulting in the formation of supermature focal adhesions and increased levels of active TGFβ-1 and ED-A fibronectin. The presence of mechanical tension, TGFβ-1 and ED-A fibronectin leads to myofibroblast differentiation characterized by \textit{de novo} expression and incorporation of alpha smooth muscle actin into their stress fibers, giving these cells a hypercontractile phenotype. Figure adapted with permission from the authors from Tomasek et al (30).
Myofibroblasts utilize two signalling pathways to regulate MLC phosphorylation, the calcium dependent MLC kinase pathway and the Rho kinase (ROCK) pathway. An increase in intracellular concentrations of calcium activates MLC kinase, which promotes myofibroblast contraction through direct phosphorylation of MLC. Since increased calcium levels are often transient, this contraction is rapid and short-lived and is actively inhibited by the effects of MLC phosphatase on MLC. In the ROCK pathway, guanosine-5'-triphosphate (GTP) bound RhoA activates ROCK, which leads to increased MLC phosphorylation by two mechanisms. The first mechanism is by direct phosphorylation of MLC. The second mechanism is through MLC phosphatase inhibition. Since MLC phosphatase activity is required for terminating myofibroblast contraction, its inhibition results in sustained contraction. Myofibroblast contractility in combination with ECM synthesis and degradation leads to connective tissue remodelling that includes the irreversible and long-term contracture events that result in wound closure and increased wound strength.

1.2.4 Remodelling phase
The remodelling phase represents a balance between collagen synthesis, collagen degradation, and collagen remodelling with the net result of increasing the strength of the wound. Net collagen production by fibroblasts peaks around three weeks after wounding, after which collagen production decreases. As collagen production decreases, collagen degradation increases and several remodelling processes are initiated to enhance the strength of the wounded skin. Type III collagen and proteoglycan levels decrease while fibroblasts secrete increasing amounts of type I collagen, which imposes greater mechanical strength. In the early stages of wound repair, the collagen is arranged haphazardly, resulting in the visual appearance of a “scar”. As the scar matures, wound strength increases as the collagen fibers become shorter, thicker, more organized and cross-linked due to contraction by myofibroblasts. A mature scar has approximately 80% of the strength of unwounded skin, indicating that wound repair falls short of true skin “regeneration”. As the wound heals and mechanical stress of the ECM is relieved, the number of myofibroblasts and macrophages is reduced via apoptosis. Capillary growth ceases and blood flow to the
wound site decreases, resulting in reduced metabolic activity within the wound site (12). The net result is a relatively acellular and avascular mature scar with high tensile strength that approaches, but does not achieve, that of unwounded skin.

1.3 Abnormal cutaneous wound healing

Potentially, non-healing chronic wounds or excessive cutaneous scarring can arise from any aberration of processes that contribute to normal cutaneous wound healing. Abnormalities in cell migration, proliferation, inflammation, ECM secretion, cytokine synthesis, and apoptosis can prevent normal wound repair (54) or conversely, contribute to excessive scar formation (55). Factors that promote excessive fibroblast proliferation and myofibroblast persistence leads to an “overhealing” phenomenon characterized by excessive collagen deposition at the site of injury, termed fibrosis (10), and contracture of the ECM (56, 57) that result in raised, collagenous scars. The associated pathological contracture of the skin can cause major clinical problems, and there are few treatment options. Abnormal cutaneous scarring can be divided into two broad categories, hypertrophic scarring and keloid scarring. The following sections will discuss the clinical, histological, and epidemiological characteristics of both hypertrophic and keloid scars and their treatment options.

1.3.1 Clinical features of abnormal cutaneous wound healing

Hypertrophic scars are clinically defined as raised collagenous scars that are confined to the borders of the initial wound (58). These scars occur 4-8 weeks following insult to the dermis, have a rapid growth phase of up to six months, then gradually regress over a period of years, eventually leading to a flat scar (59). In contrast, keloid scars extend beyond the borders of the initial wound, may develop several years after injury, or spontaneously, and do not regress over time (Figure 1.3, (58, 59)). Hypertrophic scars tend to occur at areas of high tension such as the shoulders, neck, pre-sternum, knees and ankles (59). In comparison, keloid scars tend to form on the shoulders, anterior chest, ear lobes, upper arms and the cheeks (59). Recurrence of hypertrophic scars following surgical incision is rare, however recurrence rates are high with keloid scars (59). Other than imposing a financial burden, these conditions impose morbidity that is difficult to
Figure 1.3 Clinical presentation of hypertrophic and keloid scars. (a) Clinical presentation of hypertrophic scarring following lymph node resection in the inguinal region. Hypertrophic scars present as raised collagenous scars that are confined to the borders of the initial wound. (b) Clinical presentation of keloid scarring on the anterior chest and the neck. In contrast to hypertrophic scars, keloid scars are able to extend beyond the borders of the initial wound. Photographs c/o Roth|McFarlane Hand and Upper Limb Centre.
quantify, including pain and loss of mobility, especially if the scar is associated with a joint (60). Furthermore, these scars are disfiguring and can adversely affect a patient’s quality of life both physically and psychologically (60, 61).

1.3.2 Histological features of abnormal cutaneous wound healing

Histologically, both hypertrophic and keloid scars contain excessive amounts of collagen (59). Hypertrophic scars contain primarily type III collagen fibres arranged in a wavy pattern parallel to the epidermal surface (58). These scars also contain abundant nodules of myofibroblasts, small blood vessels, and large extracellular collagen filaments (58, 59). In contrast, keloid scars are composed of disorganized type I and III collagen fibres randomly oriented to the epidermal surface (59). These scars have poor vasculature, contain hypocellular collagen bundles and lack the excessive numbers of myofibroblasts evident in hypertrophic scars (59). Proliferating cell nuclear antigen, p53 and adenosine-5'-triphosphate (ATP) levels are elevated in keloid scars, suggesting that they are primarily the result of excessive fibro-proliferation and potentially explaining why they grow beyond the site of the original injury (62, 63). Both hypertrophic and keloid scars display increased immune cell infiltration, increased levels of TGFβ-1 and myofibroblast persistence, however these features decline over time in hypertrophic scars and may explain why hypertrophic scars regress (58, 59, 62).

1.3.3 Epidemiological features of abnormal cutaneous wound healing

Hypertrophic and keloid scars have equal sex distribution and have the highest incidence rates in the second and third decade of life (59). Hormones may affect hypertrophic and keloid scar formation, as these scars tend to occur during puberty and pregnancy and the scars can decrease in size following menopause (59, 64, 65). The incidence of hypertrophic scars is probably higher than keloid scars, however there is currently insufficient data available to make robust comparisons (62). Incidence rates for hypertrophic scars vary from 40-70% following surgical incision and up to 91% following burns (66, 67). With the exception of albino individuals, keloid scars can develop in people of any skin color, however, dark skinned individuals are more susceptible with incidence rates as high as 16% in African populations (59). The
formation of hypertrophic scars does not appear to have any association with skin pigmentation (68). The notion of genetic predisposition has been suggested, as patients with keloid scars report a positive family history that is strongly associated with keloid formation at multiple anatomical sites (59, 69). In contrast, patients with hypertrophic scars typically report no family history of scar development (59).

1.3.4 Treatments

Increased tension at wound margins has been implicated in the development of hypertrophic scars, and several prophylactic approaches have been implemented on this basis to reduce hypertrophic scar development (59). Pressure therapy and silicone gel sheeting have been used to apply continuous pressure to the scar during wound repair. These approaches have shown little success and cause discomfort leading to reduced patient compliance (70). TGFβ-3, which has been shown to reduce connective tissue deposition and is induced during the late stages of wound healing (71-73), has been used as a prophylactic approach to prevent scar formation. While initial studies showed evidence for the benefits of recombinant TGFβ-3, further independent studies revealed that this treatment was unsuccessful in improving scar resolution (74). Current treatment strategies include corticosteroid injections, cryotherapy, surgical resection, radiotherapy, and laser therapy. These treatment strategies have varied success rates in the resolution of hypertrophic and keloid scars and can be associated with painful and disfiguring side effects, risk of malignancy (in the case of radiotherapy), and high recurrence rates (surgical and laser therapy) (59, 70). Emerging treatment strategies include interferon injections, which reduces collagen synthesis, and 5’-fluorouracil treatment to increase fibroblast apoptosis. These treatments have also demonstrated varied success rates and can present painful and harmful side effects (59, 70).

Although some treatments have been shown to be effective in reducing hypertrophic and keloid scar formation in individuals, few of these studies have been supported by well-designed studies with adequate control groups (59). Despite numerous studies, both in vitro and in vivo, there is limited information available regarding the cellular mechanisms that induce hypertrophic and keloid scar formation (59). One reason for our lack of knowledge of the cellular and molecular mechanisms involved in the development of
abnormal scars is a lack of representative and physiologically relevant animal models of hypertrophic scar formation (59). Therefore, gaining a better understanding of the biochemical processes involved in abnormal wound healing is crucial if we hope to achieve normal skin repair or, optimally, true skin regeneration in patients prone to abnormal scarring.

1.4 TGFβ-1 is a difficult therapeutic target

As highlighted in the previous sections, TGFβ-1 plays essential roles in normal cutaneous wound healing. In addition to these roles, excessive signalling by this cytokine has been implicated in hypertrophic and keloid scar formation (58, 59, 62). TGFβ-1 has proven to be a difficult therapeutic target in many disease processes including abnormal scar formation, as it can exert pleiotropic effects on different cell types that can be diverse and, in some cases, seemingly paradoxical (75, 76). While TGFβ-1 is one of several cytokines that attract PMNs and macrophages to the wound (10, 12, 16, 17), depletion of TGFβ-1 in Tgfb null mouse models results in a lethal and widespread inflammatory response, revealing an essential anti-inflammatory role for this cytokine (77, 78).

Knockdown of TGFβ-1, its receptor, or signalling components in mouse models inhibits normal repair in the dermis (79-82), whereas TGFβ-1 promotes matrix formation for keratinocyte migration while inhibiting keratinocyte proliferation (83, 84). In vitro studies demonstrate that TGFβ-1 can promote fibroblast proliferation without affecting myofibroblast differentiation or myofibroblast differentiation without affecting proliferation, in the same cell line (75). These apparently paradoxical outcomes are likely to be the result of concurrent activation of other signalling pathways that modify and supplement the downstream effects of TGFβ-1 signalling intermediates. For this reason, the identification of TGFβ-1-inducible proteins that supplement, enhance or modify TGFβ-1 signalling to promote pathological, rather than normal, fibroblast proliferation and/or myofibroblast differentiation may have greater utility as therapeutic targets than TGFβ-1 itself.
1.5 Identification of TGFβ-1-inducible proteins in abnormal wound healing

During cutaneous wound healing, TGFβ-1 is released into the ECM by platelet granules, inflammatory cells and fibroblasts (13, 83) in an inactive form in association with latency-associated peptide (LAP) and latent TGFβ-1 binding protein-1 (LTBP-1) (31). Extracellular matrix proteins associate with LTBP-1 creating a reservoir of latent TGFβ-1 (85-87). Latent TGFβ-1 bound in the ECM can interact with cells through integrin binding sites located in the LAP (85, 88). Stress applied to these integrins, either by stretching the ECM or by inducing cellular contraction, results in a conformational change in the large latent complex allowing the release of TGFβ-1 in an active form (42). Mechanical activation of TGFβ-1 enhances tissue remodelling by translating the level of ECM stiffness into pro-fibrotic signals (31). Therefore, the ECM is an important regulator of TGFβ-1 activation in both normal and abnormal wound healing.

The ECM is comprised of both structural and non-structural proteins (89, 90). Structural proteins, such as collagens and fibronectin, provide strength to the dermis and act as scaffolds for migrating cells and as reservoirs for growth factors (8). Non-structural proteins within the ECM include cytokines like TGFβ-1 and the family of “matricellular” proteins that modify cell signalling in response to extracellular stimuli, such as changes in mechanical tension of the ECM (40, 42, 90). Given the well-established roles of the ECM in scar formation, studies were focused on the roles of non-structural proteins in the ECM that might be activated by and/or act in combination with TGFβ-1 to promote excessive scarring.

Initial studies in our laboratory showed increased expression of genes encoding ECM proteins in Dupuytren’s Disease, a fibro-contractile disease of the palmar fascia that has many similarities to abnormal scarring. Microarray analyses of tissues derived from patients with or without Dupuytren’s disease identified POSTN, encoding the matricellular protein periostin, as a very highly upregulated transcript (91). Subsequent in vitro experiments demonstrated that periostin induced fibroblast survival and myofibroblast differentiation in primary fibroblasts derived from Dupuytren’s disease.
tissue and induced proliferation of fibroblasts derived from “pre-diseased” palmar fascia (91). As excessive fibroblast proliferation and increased myofibroblast differentiation and persistence are also characteristics of hypertrophic scarring (56, 57, 92-94), further studies were performed to investigate whether periostin expression was abundant in hypertrophic scar tissue. Western immunoblotting, immunohistochemistry and in-situ hybridization data performed by our laboratory (95, 96) and reports by other groups (97-99) demonstrated that POSTN expression was upregulated in the basal stratum of hypertrophic and keloid scar tissue relative to mature normal scar tissue. Levels of the translated product of POSTN mRNA, periostin, were also upregulated in the dermis (but not the basal stratum) of hypertrophic and keloid scar tissue relative to normal mature scars. Based on these studies, it was proposed that periostin might contribute to excessive fibroblast proliferation and myofibroblast differentiation in hypertrophic scarring.

1.6 Periostin

1.6.1 Gene and protein structure
Periostin was originally identified as osteoblast-specific factor 2, a novel cell-adhesion molecule, in a mouse osteoblastic cell line (100, 101). Using murine cDNAs as probes, human homologs encoding a protein with a molecular weight of up to 93 kDa were subsequently identified in placental and osteosarcoma cDNA libraries (100, 102). Osteoblast-specific factor 2 was later renamed periostin due to its preferential expression in cells of mesenchymal origin located in the collagen rich ECM surrounding the periosteum and periodontal ligament (101). The murine periostin gene, Postn, located on chromosome three and the human gene, POSTN, located on chromosome 13, are highly homologous. Amino acid identity between the two species is 89.2% for the entire protein and 90.1% for the mature processed forms (100). POSTN encodes 23 protein-coding exons over a genetic footprint of 36 kilobases (102). POSTN expression is upregulated by a number of factors involved in wound healing including TGFβ-1, interleukins, platelet-derived growth factor and fibroblast growth factor (101, 103, 104).
The N-terminal signal sequence of periostin consists of 23 amino acids and lacks a transmembrane domain, suggesting that periostin is a secreted protein (100). This is consistent with pronounced immunoreactivity to periostin in the ECM surrounding the periosteum and periodontal ligaments (101). Following the signal sequence is a cysteine-rich Emilin-like (EMI) domain encoded by exons two and three (100, 102). EMI domains are typically involved in protein-protein interactions (105) and protein multimerization, and periostin dimers have been reported in some studies (106). Adjacent to the EMI domain are four repeated regions of 150 amino acids encoded by exons 3-14 (100, 102). These repeats share homology with the insect axon guidance protein fasciclin 1 (FAS1), an adhesion module (100, 107). Since periostin contains FAS1 domains, it has been assigned to the fasciclin family of proteins that includes stabilin 1 and 2, and TGFβ-induced gene clone 3 (βig-h3) (108). Each FAS1 domain contains two conserved regions of 13-14 amino acids that have high sequence similarity. However, the conserved repeats between the FAS1 domains have relatively low sequence homology (100). Each FAS1 domain contains glutamate residues and contains N-terminal recognition sites for γ-glutamylcarboxylase, an enzyme responsible for posttranslational modification of glutamate residues (109). The FAS1 domains are believed to be involved in cell adhesion and protein-protein interactions, as bone morphogenic protein-1, tenascin C, and integrins have been suggested to bind this region of periostin (110-112). Interestingly, periostin does not contain any canonical Arg-Gly-Asp (RGD) integrin binding motifs, suggesting that integrin binding by periostin is independent of these motifs (102). βig-h3, which has a domain structure similar to periostin (102), has been demonstrated to bind α3β1 integrins at sites that are independent of the RGD motif (112). This interaction occurs by two pentapeptides containing a central Asp-Ile (DI) dimer located in the second and fourth FAS1 domain (112). The DI dimer has a similar conformation to the RGD binding motif (113) and suggests a mechanism for periostin-integrin interactions (102).

Lastly, exons 16-23, accounting for 182 amino acids, make up the hydrophilic or C-terminal domain (102). This region is devoid of known domains, contains few known sequence motifs and contains a C-terminal nuclear localization signal at exon 22-23 (102), suggesting that periostin may also have intracellular functions (114). Exons 17-21
**Figure 1.4 Schematic representation of the human POSTN gene and domain structures.** The human *POSTN* gene contains 23 protein-coding exons. Exon 1 encodes a signal sequence (SS), characteristic of secreted proteins. Exons 2-3 encode for the Emilin-like (EMI) domain involved in protein-protein interactions and protein multimerization. Exons 3-14 encode four repeated fasciclin 1 (FAS1) domains involved in cellular adhesion and protein-protein interactions. Purple arrows represent recognition sites for γ-glutamylcarboxylase and vertical black bars represent proposed integrin binding sites in FAS1 repeats 2 and 4. Exons 17-21 present themselves as exon cassettes that can be present or deleted in mature mRNA through alternative splicing events. Exons 22-23 encode a C-terminal nuclear localization signal (NLS). Red oval represents heparin binding site in exon 23.
can be considered as “exon cassettes” that can be included or deleted in mature mRNA in various combinations through alternative splicing events (100-102). This results in several periostin isoforms ranging in size from 83-93 kDa (115). The number of nucleotide residues in each exon cassette is a multiple of three, and therefore deletion or inclusion of any of these cassettes in the mature mRNA does not result in frameshift mutations (100). Initial studies identified four periostin isoforms in mouse and five isoforms in human tissues (100, 101), with additional studies expanding the latter to eight isoforms (116, 117). These isoforms appear to be differentially expressed during development and injury and appear to be tissue, developmental stage or disease specific (114, 118-121). However, the function(s) of each individual isoform remain unclear. Secondary structure predictions demonstrate consecutive beta strands as the predominant structure in the C-terminal domain (102). βig-h3, which lacks this domain, has not been reported to interact with collagen or fibronectin. Therefore it has been proposed that the C-terminal domain’s beta strand structure may facilitate periostin binding to collagen and fibronectin (102). Periostin contains similar repeats to those found in various bacteria that are able to bind fibronectin through an extended beta zipper (102, 122). Therefore, the strength of periostin’s interaction with the ECM would be influenced by the number of beta strands present in the mature protein determined by alternative splicing (102). Lastly, the C-terminal domain contains a heparin binding site in exon 23, creating a potential binding site for glycoproteins and proteoglycans (Figure 1.4, (101)).

1.6.2 Classification as a matricellular protein
Periostin has been classified as a matricellular protein (123), i.e. one of a family of structurally unrelated ECM proteins that do not serve a structural role in the ECM but instead serve as a link between the ECM and cells and modify cell behavior in response to external stimuli (90, 124). Originally, this family of proteins consisted of thrombospondin, tenascin-C and SPARC but recent studies have classified osteopontin, periostin and members of the CCN family, including CCN2, as matricellular proteins (125). Several characteristics have been identified for the classification of matricellular proteins. 1) Matricellular proteins interact with structural components of the ECM,
receptors, growth factors and proteases thereby allowing these proteins to regulate cellular signalling cascades (125). 2) Matricellular proteins promote an intermediate cellular adhesive state (126) that activates survival signals and increases expression of genes associated with adaptation and repair (125). 3) In normal adult tissue, expression of matricellular proteins is low, but is upregulated in response to injury or during development (125). 4) Mice with targeted disruption in matricellular genes have subtle abnormalities in the absence of injury. However, loss of these proteins results in a wide range of alterations in injured and remodelled tissue (125).

The first characteristic of matricellular proteins is their ability to bind ECM proteins, proteases and growth factors and to transmit external signals to the cell to modulate cellular phenotypes through receptor-mediated activation. Periostin has been demonstrated to bind several structural ECM proteins including type I and V collagen and fibronectin as well as other matricellular proteins including tenascin-C (104, 106). Bone morphogenic protein-1, a metalloproteinase, interacts with periostin inducing the cleavage and activation of lysyl oxidase required for collagen cross linking (127). Periostin has been shown to bind integrins αvβ3, αvβ5, α5β1, β1 and α6β4 in various systems (127-135), presumably through its FAS1 domains. Integrins mediate cell-ECM interactions and transmit signals from the ECM to modulate cell behavior (136, 137). Periostin signalling through αvβ3 and α6β4 has been shown to promote tumour cell survival through PI3K/Akt in human colon cancer cells and pancreatic ductal adenocarcinoma cells cultured on tissue culture plastic (129, 130). Periostin signalling through αvβ3 and αvβ5 can promote the migration of human ovarian epithelial cells, pancreatic ductal adenocarcinoma cells and mouse smooth muscle cells through Akt and FAK dependent pathways (128, 130, 134). Additionally, periostin has been shown to signal through αvβ3, αvβ5 and β1 integrins to induce the proliferation of rat cardiomyocytes (132). It should be noted, however, that very few of these signalling intermediates and pathways were assessed on physiologically relevant culture substrates or within an ECM, where periostin could act as a matricellular molecule.
The second characteristic of matricellular proteins is their ability to promote cellular de-adhesion that activates survival signals and induces expression of genes associated with repair. Periostin has been implicated in promoting a “de-adhesive” state in the stroma of cancers and its presence is correlated with malignant tumor invasion and metastasis (128, 129, 138-141). In the presence of periostin, tumour cells enhance their invasive activity by forming fewer stress fibres and increasing motility (128). Periostin transfected cancer cells demonstrate morphological changes and increased mesenchymal cell markers suggesting that periostin may induce cancer cell invasion through EMT (142). Periostin has also been shown to promote survival through ECM-receptor activation in cancers, bone and periodontal ligaments. Enhanced survival induced by periostin in cancers is mediated through PI3K and Akt activation (129, 130). In the periosteum and periodontal ligament, periostin-null mice have reduced expression of Notch 1 and its downstream effector Bcl-xL (143, 144). Notch proteins are transmembrane receptors that play an important role in cell survival under mechanical stress (145). Periostin binding to notch under stressed conditions increases expression of Bcl-xL leading to inhibition of apoptosis (121). Periostin expression has also been correlated with proteins associated with repair. Studies utilizing periostin-null mice have demonstrated reduced collagen fibrillogenesis and aSMA levels in cardiac repair and normal cutaneous wound healing (106, 120, 133). Collagen remodelling and myofibroblast differentiation are important processes in normal wound repair.

The expression of matricellular proteins is usually low in most normal adult tissues but is upregulated during embryonic development and in response to injury. Periostin is highly expressed by fibroblasts in the embryonic myocardium and developing neonatal heart. In adult hearts, periostin expression is decreased and is primarily localized to the supporting structures of the heart valves that are subjected to mechanical tension. (123, 146, 147). In mouse skin, periostin is highly expressed during embryonic development in keratinocytes, at the basement membrane and in the dermis, however expression significantly decreases in adult mice (97). Unlike most matricellular proteins that are expressed at low levels in adult tissue, periostin expression in adult tissue is high in collagen-rich tissues under mechanical stress such as the periosteum, periodontal
ligaments, tendons, cardiac valves, and the skin \((98, 101, 106, 123)\). A marked increase in periostin is seen in patients with myocardium infarction, advanced heart failure, and atherosclerotic and rheumatic value disease \((120, 148, 149)\). Up-regulation of periostin in myocardium infarction plays an important role in early scar formation by promoting migration, myofibroblast differentiation and collagen deposition thereby preventing cardiac rupture \((120, 150)\). Periostin expression is also upregulated during cutaneous wound healing \((98, 151, 152)\) and its roles in this process will be discussed in more detail in the following section.

The final unifying characteristic of matricellular proteins is that their deletion in mouse models generally results in subtle abnormalities in the absence of injury, but much more obvious abnormalities following injury. Despite high levels of periostin during embryonic development in the periosteum, periodontal ligament and heart valves, periostin-null mice are not embryonic-lethal and appear grossly normal at birth \((150, 153)\). Three to four weeks after birth, periostin-null mice display growth retardation and dental defects \((153)\). Approximately 15\% of periostin-null mice die in the first three weeks due to cardiac defects \((153, 154)\). Alterations in repair are evident following injury in periostin-null mice. Following myocardium infarction, periostin-null mice have increased rates of cardiac rupture resulting from impaired collagen fibrillogenesis and myofibroblast differentiation \((120, 150)\). Defects in collagen fibrillogenesis and osteoclast inactivation are evident in the periosteum resulting in decreased bone formation and mass \((121, 155)\). In normal cutaneous wound healing, periostin-null mice displayed delayed wound healing caused by defective re-epithelialization and decreased myofibroblast populations \((133, 151, 152)\). These studies suggest that periostin fulfills the requirements of a matricellular protein and has roles in tissue repair.

### 1.7 Periostin in normal cutaneous wound healing

Several groups have assessed the expression of \textit{POSTN} and periostin during normal cutaneous wound repair using murine excisional wound healing models. \textit{POSTN} expression is detectible in the wound at Day 1 after injury, with peak expression at Day 7
with an ongoing decrease thereafter approaching baseline levels by Day 18 (152). Periostin expression is evident in the granulation tissue and in migrating keratinocytes as early as three days post wounding (98, 151). As increased periostin expression correlates with increased αSMA levels in the granulation tissue at Day 7 post wounding, some authors have suggested that periostin may contribute to normal myofibroblast differentiation (98). By Day 21, periostin expression is absent in keratinocytes and is only evident in the remodeled ECM (98, 152) and levels return to baseline by Day 28 (98). The expression pattern of POSTN and periostin over time are currently unclear in human cutaneous wound healing, due to obvious ethical restrictions in using humans as in vivo wound healing models.

Only three groups have utilized periostin-null mice in excisional wounding studies to investigate the roles of periostin during cutaneous wound repair. Periostin-null mice display delayed wound closure (133, 151, 152) suggesting that periostin may have a role in re-epithelialization or granulation tissue formation. As periostin-null mice displayed decreased re-epithelialization despite evidence that periostin does not affect keratinocyte proliferation or migration (151), it is likely that loss of periostin impairs re-epithelialization through an indirect mechanism.

Of the three groups to report excisional wound healing studies in periostin-null mice, only one has investigated the effects of periostin in the dermis (133). This group demonstrated that the granulation tissue in periostin-null mice displayed decreased expression of αSMA and its gene, Acta2, at Day 7 post wounding relative to wild type controls (133). Similar results were identified in vitro, with decreased αSMA levels observed in periostin-null fibroblasts cultured in anchored collagen lattices, which resembles the matrix stiffness of granulation tissue (133, 156). The decrease in myofibroblast populations in the granulation tissue in these in vivo studies was not due to impaired fibroblast migration or proliferation (133). However, in vitro studies using embryonic mouse fibroblasts provided conflicting data, as periostin-null fibroblast exhibited decreased proliferation, while over expression of periostin induced their proliferation (152). It is difficult to draw conclusions from these studies as they lacked
parallel in vivo experiments and utilized embryonic fibroblasts that may have characteristics that are distinct from adult murine fibroblasts (157). In vitro and in vivo experiments demonstrated that periostin-null dermal fibroblasts were significantly less contractile than wild type fibroblasts and that the addition of recombinant periostin was able to rescue this effect (133). This effect was blocked in the presence of inhibitors against integrin β1 and FAK and addition of periostin induced FAK phosphorylation (133, 152). These studies suggest that periostin contributes to normal murine cutaneous wound healing by facilitating myofibroblast differentiation and contraction through an integrin-focal adhesion dependent pathway (133).

While these studies suggest that periostin can facilitate myofibroblast differentiation in murine wound healing models, the substantial differences between mouse and human skin make it unclear if these findings can be translated to normal human cutaneous wound healing. Mouse skin is covered in dense hair, lacks rete ridges and adipose sweat glands, has a thinner epidermis and dermis, has a faster keratinocyte turnover rate and is significantly more compliant than human skin (158, 159). In contrast, human skin has a thicker epidermis and dermis, less hair follicles, is relatively stiffer and is adherent to the underlying subcutaneous tissue (158-161). Mice and humans also heal cutaneous wounds by different mechanisms. Mice have a muscle layer below the hypodermis in the flanks, known as the panniculus carnosus, that is absent in most areas of human skin (158). This muscle layer allows mice to rapidly contract wounds following injury and enhances skin regeneration with minimal scarring. In contrast, human cutaneous wounds lacking a similar muscle layer heal through the formation of granulation tissue and re-epithelialization, typically leading to the formation of a scar (158, 160, 162). These profound differences in wound healing mechanisms between mice and humans make it unclear if the roles of periostin in murine excisional wound healing can be translated to normal, or particularly abnormal, cutaneous repair in humans.
1.8 Periostin in abnormal wound healing

Currently, there are no studies in the literature investigating the roles of periostin in the development of hypertrophic scars in humans. The molecular mechanisms implicated in the development of excessive scarring remain poorly understood. One reason for this is a lack of truly representative and physiological relevant animal models of human hypertrophic scar formation (59). Hypertrophic scarring does not normally occur in animals (163), and our current understanding of the mechanisms that promote excessive scarring are largely based on studies of chemically and/or genetically manipulated mice (164-166). These models can be very useful for investigating the cell types and molecules involved in scar formation at specific time points. More recently, animals with skin that more closely resembles human skin, such as the red Duroc pig (167), have been used as models with and without chemical or other treatments to induce scarring. While more physiologically relevant than mice, they cannot capture the multifactorial influences, such as age, sex, wound site and genetic predisposition that promote abnormal scarring in human patients. Since using humans as wound healing models is not ethically feasible, the next best option is analyze human scar tissue from patients undergoing surgical resections. By utilizing primary samples derived from human normal and abnormal scar tissue, we are able to perform a “top down” approach to identify factors that are elevated during abnormal scar formation relative to normal skin. Upon identification of such factors, in vitro studies can be performed on primary human fibroblasts that exhibit many clinically relevant features of both normal and abnormal scarring. As an in vitro model, these cells have the disadvantage of being obtained after scar formation has occurred, and are therefore unlikely to be informative of the initial stages of scar development, such as those induced by inflammation (59). However, primary fibroblasts derived from surgically resected hypertrophic tissue can provide clinically relevant models of the cellular processes that cause the abnormal persistence of scars for months or years, compared to normal skin wounds that typically heal in weeks.

In situ hybridization studies performed by our lab demonstrated that POSTN expression was upregulated and persistent in the basal epithelium of human hypertrophic scar (Figure 1.5a) and keloid scar tissue relative to normal mature scars (96), consistent with
previous reports (99). Immunohistochemistry studies performed by our laboratory (95, 96) and other groups (97, 98) have shown that periostin levels are abnormally abundant and localized to dermis in hypertrophic scars (Figure 1.5b and c) and keloid scars. Periostin is absent in the epidermis, despite clear evidence of POSTN expression, suggesting that the epidermis may be an additional source of dermal periostin in abnormal scarring conditions. Based on these findings, we hypothesize that the increased and persistent levels of periostin in the dermis of hypertrophic and keloid scars may contribute to excessive fibroblast proliferation and myofibroblast differentiation and persistence in these fibrotic conditions.

1.9 Assessing the function of periostin in hypertrophic scar formation in an in vitro model system

In order to assess the role(s) of a matricellular molecule like periostin in abnormal scar formation in vitro, studies must be performed in a model system that closely resembles the granulation tissue during wound healing in vivo. Excisional wound healing models and in vitro studies have been utilized to measure the stiffness of the ECM during wound healing. Matrix stiffness can be represented by Young’s modulus, which is the ratio of stress to strain of an elastic substrate. The Young’s modulus of the provisional matrix is 10-1000 Pascals (Pa), indicating that it is very compliant (31). The proto-myofibroblast phenotype is only evident when matrix stiffness reaches 3000 Pa (168). The stiffness of granulation tissue in seven day old rat granulation tissue is approximately 18 000 Pa, consistent with αSMA being incorporated into stress fibers at a Young’s modulus of 16 000 - 20 000 Pa (31). Fibrotic tissue and contracting granulation tissues can exhibit a matrix stiffness of 25 000 – 50 000 Pa (169). Tissue-culture plates provide an extremely stiff substrate (~3 gPa, or 3 000 000 000 Pa (170)). A culture substrate of this stiffness inevitably induces robust and non-physiological influences on myofibroblast differentiation (31) that has the potential to overshadow any other effects of potential inducers of myofibroblast formation (133). Despite its routine use for culturing fibroblasts, tissue culture plastic is a very non-physiological model system in which to assess cellular processes that are sensitive to matrix tension. To address this issue, our
**Figure 1.5** POSTN expression and periostin levels are increased in hypertrophic scars. (a) *In situ* hybridization studies were performed using $S^{35}$ labeled sense and antisense transcripts derived from a 391-bp fragment from the 5’ end of a *POSTN* cDNA. Paraffin-embedded mixed scar derived from a patient with a phenotypically normal scar at one edge transitioning to a hypertrophic scar at the opposite edge was exposed to antisense *POSTN* mRNA probe to compare *POSTN* expression across the normal-abnormal scarring transition zone (indicated with an arrow). Silver grains deposited due to exposure to $S^{35}$ labeled transcripts are evident as white specks under dark field microscopy. As shown, *POSTN* expression is much more abundant in the basal epithelium along the hypertrophic scar section of the mixed scar relative to the normal scar area. Immunohistochemistry of paraffin-embedded (b) hypertrophic scar and (c) normal scar tissue with a periostin polyclonal antibody. Periostin immunoreactivity is evident as brown staining from di-amino precipitation. Periostin is more abundant in hypertrophic scars relative to normal scars and expression extends deep into the dermis. Images c/o Cell and Molecular Biology Laboratory, Roth|McFarlane Hand and Upper Limb Centre.
laboratory takes the approach of routinely culturing primary fibroblasts on type I collagen substrates. Type I collagen is the most abundant collagen in the skin and mature scars (8) and our laboratory has demonstrated that the change in substrate stiffness induced by culture on collagen substrates can modify gene expression and protein levels in primary fibroblasts relative to the same cells cultured on stiff tissue culture plastic (91, 171). Based on these observations, the effects of periostin on fibroblast proliferation and myofibroblast differentiation were assessed in two- (2D) and three-dimensional (3D) collagen-based cultures in this thesis to more closely mimic the ECM and substrate stiffness experienced by these fibroblasts in vivo.

Currently, three dimensional collagen lattices are utilized for examining in vitro the mechanical interactions that occur between cells and the ECM and how these interactions induce myofibroblast differentiation (47, 156). When cultured within a collagen lattice, fibroblasts experience a more complex environment and geometry compared to cells cultured on plastic (172). Fibroblast attachment, spreading, and migration through the collagen lattice generate mechanical signals that can modulate cellular phenotype. If the collagen lattice is mechanically restrained, such as in stressed Fibroblast Populated Collagen Lattices (sFPCLs) (47), tension develops as tractional forces compact the collagen fibers (30, 172). As the mechanical tension progressively increases in the collagen lattice, the fibroblasts will form stress fibers and focal adhesions associated with proto-myofibroblast and myofibroblast differentiation (156) similar to ECM remodelling in vivo (30). This tension is sustained in the lattice until it is released from its points of attachment (30). Newly polymerized 2D and 3D collagen gels have a matrix stiffness of 300-400 Pa (173), which is similar to that seen in the provisional fibrin matrix (31). The Young’s modulus has not been reported for sFPCLs; however, it is believed to closely mimic the mechanical environment of the granulation tissue in Day 7 rat wounds, i.e. approximately 18 000 Pa (31, 44, 156). Using collagen substrates, periostin can be incorporated into collagenous ECM matrices to mimic its localization in vivo and provide a reproducible culture system to investigate periostin effect(s) on fibroblast proliferation and myofibroblast differentiation and persistence under physiological relevant conditions.
1.10 Hypothesis and objectives
When wound healing signals become abnormal, excessive fibroblast proliferation and myofibroblast persistence can be induced, resulting in excessive ECM deposition and remodelling and hypertrophic scarring (56, 57, 92-94). TGFβ-1 is a potent inducer of myofibroblast differentiation in this context, however it is also implicated in regulating many of the processes that are essential for normal wound healing (13, 41, 174-177), making this cytokine a difficult therapeutic target. Therefore, TGFβ-1-inducible proteins that act in combination with and/or sequentially to TGFβ-1 signalling to promote pathological, rather than normal, fibroblast proliferation and/or myofibroblast differentiation may have greater utility as therapeutic targets. Periostin is a novel matricellular protein that is upregulated in the ECM of hypertrophic scars and may have potential as such a target. Although several studies have demonstrated that periostin regulates fibroblast proliferation and myofibroblast differentiation in other systems (91, 132, 133, 152, 178-180), the roles of periostin in hypertrophic scar formation in human fibroblasts has not been assessed. Therefore, this thesis focuses on identifying the role(s) of periostin on hypertrophic scar fibroblast proliferation and myofibroblast differentiation and persistence in 2D and 3D collagen cultures.

The central hypothesis of this thesis is that ECM-associated periostin contributes to hypertrophic scar formation by promoting excessive fibroblast proliferation and myofibroblast differentiation and by maintaining myofibroblasts in a differentiated state.

The following objectives were designed to test this hypothesis:

Objective 1: To assess the effects of periostin on the proliferation of normal skin and hypertrophic scar derived fibroblasts

Objective 2: To assess the effects of periostin on the differentiation of fibroblasts derived from normal skin and hypertrophic scar tissues

Objective 3: To identify the mechanism(s) utilized by periostin to induce and maintain hypertrophic scar myofibroblasts in a differentiated state
Chapter 2

2 Materials and Methods

2.1 Materials

2.1.1 Biochemicals

<table>
<thead>
<tr>
<th>Material</th>
<th>Supplier</th>
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</thead>
<tbody>
<tr>
<td>Bovine serum albumin (BSA)</td>
<td>Sigma-Aldrich, St. Louis, MO</td>
</tr>
<tr>
<td>Glycine</td>
<td>Fisher Scientific, Hampton, NH</td>
</tr>
<tr>
<td>Sodium hydroxide</td>
<td>Sigma-Aldrich, St. Louis, MO</td>
</tr>
<tr>
<td>Hydrochloric acid</td>
<td>Merck KGaA, Darmstadt, GER</td>
</tr>
<tr>
<td>Sodium dodecyl sulfate (SDS)</td>
<td>Sigma-Aldrich, St. Louis, MO</td>
</tr>
<tr>
<td>Tris Base</td>
<td>Fisher Scientific, Hampton, NH</td>
</tr>
<tr>
<td>Sodium chloride (NaCl)</td>
<td>Fisher Scientific, Hampton, NH</td>
</tr>
<tr>
<td>Glacial acetic Acid</td>
<td>Fisher Scientific, Hampton, NH</td>
</tr>
<tr>
<td>Potassium chloride (KCl)</td>
<td>Fisher Scientific, Hampton, NH</td>
</tr>
<tr>
<td>Potassium phosphate (KH₂PO₄)</td>
<td>Fisher Scientific, Hampton, NH</td>
</tr>
<tr>
<td>Sodium phosphate, monobasic (NaH₂PO₄)</td>
<td>Fisher Scientific, Hampton, NH</td>
</tr>
<tr>
<td>Sodium phosphate, dibasic (Na₂HPO₄)</td>
<td>Fisher Scientific, Hampton, NH</td>
</tr>
<tr>
<td>Imidazole</td>
<td>Sigma-Aldrich, St. Louis, MO</td>
</tr>
<tr>
<td>Glycerol</td>
<td>Fisher Scientific, Hampton, NH</td>
</tr>
<tr>
<td>Bromophenol blue</td>
<td>Fisher Scientific, Hampton, NH</td>
</tr>
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</table>

2.1.2 Cell culture

<table>
<thead>
<tr>
<th>Material</th>
<th>Supplier</th>
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</thead>
<tbody>
<tr>
<td>100 mm and 24-well tissue culture plates</td>
<td>Becton Dickinson, Franklin Lakes, NJ</td>
</tr>
<tr>
<td>Alpha Modified Eagle’s medium (αMEM)</td>
<td>Life Technologies, Carlsbad, CA</td>
</tr>
</tbody>
</table>
Dulbecco’s modified Eagle’s medium (DMEM)

Life Technologies, Carlsbad, CA

100x L-glutamine

Life Technologies, Carlsbad, CA

100x antibiotic-antimycotic

Life Technologies, Carlsbad, CA

Fetal bovine serum (FBS)

Life Technologies, Carlsbad, CA

Tryspin-EDTA

Life Technologies, Carlsbad, CA

Type I rat tail collagen

Made in our laboratory

Waymouth medium

Sigma-Aldrich, St. Louis, MO

Dimethyl sulfoxide (DMSO)

Sigma-Aldrich, St. Louis, MO

Recombinant human periostin

R&D systems, Minneapolis, MN

Recombinant human TGFβ-1

R&D systems, Minneapolis, MN

Hank’s balanced salt solution

Sigma-Aldrich, St. Louis, MO

Collagenase

Sigma-Aldrich, St. Louis, MO

Nalgene® cryogenic vials

Thermo Scientific, Waltham, MA

Sircol collagen quantification assay

Biocolor Ltd., Carrickfergus, UK

<table>
<thead>
<tr>
<th>Buffer/medium</th>
<th>Application</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphate buffered saline (PBS)</td>
<td>Wash buffer</td>
<td>8 g NaCl, 0.2 g KCl, 1.44 g Na₂HPO₄, 0.24 g KH₂PO₄, reverse osmosis (R.O) H₂O up to 1 L, pH 7.4</td>
</tr>
<tr>
<td>Freezing Medium</td>
<td>Primary cell storage</td>
<td>50% FBS, 35% αMEM, 15% DMSO</td>
</tr>
</tbody>
</table>
2.1.3 Cell proliferation assay

Cell Proliferation Reagent WST-1  
Roche Diagnostics, Mannheim, GER

96-well tissue culture plates  
Becton Dickinson, Franklin Lakes, NJ

Table 2.2 Inhibitors for cell proliferation assays

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Mode of Action</th>
<th>Concentration</th>
<th>Vehicle</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>PI3K inhibitor, LY294002</td>
<td>Inhibition is competitive with respect to ATP binding site</td>
<td>10 μM</td>
<td>DMSO</td>
<td>Cell Signaling Technologies, Danvers, MA</td>
</tr>
<tr>
<td>AKT inhibitor VIII</td>
<td>Binds pleckstrin homology domain and prevents activation by PI3K</td>
<td>0.5 μM</td>
<td>DMSO</td>
<td>Merck KGaA, Darmstadt, GER</td>
</tr>
<tr>
<td>GSK3β inhibitor VIII</td>
<td>Inhibition is competitive with respect to ATP binding site</td>
<td>1 μM</td>
<td>DMSO</td>
<td>Merck KGaA, Darmstadt, GER</td>
</tr>
<tr>
<td>Rho Kinase inhibitor, Y27632</td>
<td>Inhibition is competitive with respect to ATP binding site</td>
<td>10 μM</td>
<td>H2O</td>
<td>Sigma Aldrich, St. Louis, MO</td>
</tr>
<tr>
<td>ERK 1/2 inhibitor, PD98059</td>
<td>Binds to ERK specific MAP kinase, MEK, preventing activation of ERK 1/2 by MEK</td>
<td>20 μM</td>
<td>DMSO</td>
<td>Sigma Aldrich, St. Louis, MO</td>
</tr>
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## 2.1.4 Protein chemistry

<table>
<thead>
<tr>
<th>Item</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>RIP A lysis buffer</td>
<td>Teknova Inc., Hollister, CA</td>
</tr>
<tr>
<td>Proteinase inhibitor cocktail</td>
<td>Sigma-Aldrich, St. Louis, MO</td>
</tr>
<tr>
<td>Phenylmethanesulfonylfluoride (PMSF)</td>
<td>Sigma-Aldrich, St. Louis, MO</td>
</tr>
<tr>
<td>Sodium fluoride (NaF)</td>
<td>Sigma-Aldrich, St. Louis, MO</td>
</tr>
<tr>
<td>Sodium orthovanadate (Na(_3)VO(_4))</td>
<td>Sigma-Aldrich, St. Louis, MO</td>
</tr>
<tr>
<td>Pierce® BCA protein assay kit</td>
<td>Thermo Scientific, Waltham, MA</td>
</tr>
<tr>
<td>30% Acrylamide/Bis solution (29:1)</td>
<td>BioRad, Hercules, CA</td>
</tr>
<tr>
<td>Ammonium persulphate</td>
<td>BioRad, Hercules, CA</td>
</tr>
<tr>
<td>N,N,N',N'-tetramethylethylenediamine (TEMED)</td>
<td>BioRad, Hercules, CA</td>
</tr>
<tr>
<td>Dithiothreitol (DTT)</td>
<td>Sigma-Aldrich, St. Louis, MO</td>
</tr>
<tr>
<td>polyvinylidene difluoride (PVDF) – plus, 0.45 micron transfer membranes</td>
<td>Santa Cruz Biotechnology, Santa Cruz, CA</td>
</tr>
<tr>
<td>Tween®20</td>
<td>Merck KGaA, Darmstadt, GER</td>
</tr>
<tr>
<td>Novex sharp molecular weight markers</td>
<td>Life Technologies, Carlsbad, CA</td>
</tr>
<tr>
<td>Immobilon™ western chemiluminescent HRP substrate</td>
<td>Millipore Corporation, Billerica, MA</td>
</tr>
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</table>
Table 2.3 Buffers for protein analysis

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Application</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>RIPA lysis buffer</td>
<td>Cell lysis</td>
<td>150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl (pH 7.5), 2 mM EDTA (pH 8.0)</td>
</tr>
<tr>
<td>SDS gel-loading buffer</td>
<td>SDS-PAGE</td>
<td>R.O. H₂O with 50 mM Tris-HCl (pH 6.8), 100 mM DTT, 2% SDS, 0.1% bromophenol blue, 10% glycerol</td>
</tr>
<tr>
<td>1.0 M Tris</td>
<td>SDS-PAGE stacking gel</td>
<td>121.0 g Tris base dissolved in R.O. H₂O to 1 L, pH 6.8</td>
</tr>
<tr>
<td>1.5 M Tris</td>
<td>SDS-PAGE resolving gel</td>
<td>181.65 g Tris base dissolved in R.O. H₂O to 1 L, pH 8.8</td>
</tr>
<tr>
<td>5x electrophoresis running buffer</td>
<td>SDS-PAGE</td>
<td>15.1 g Tris Base, 94.0 g glycine, 25 ml 20% (w/v) SDS, R.O. H₂O up to 1 L</td>
</tr>
<tr>
<td>10x electrophoresis transfer buffer</td>
<td>SDS-PAGE</td>
<td>30.3 g Tris base, 144.4 g glycine, R.O. H₂O up to 1 L</td>
</tr>
<tr>
<td>1x electrophoresis transfer buffer</td>
<td>SDS-PAGE</td>
<td>1 part 10x electrophoresis transfer buffer, 2 parts methanol, 7 parts R.O. H₂O</td>
</tr>
<tr>
<td>10x Tris buffered saline (TBS)</td>
<td>Western immunoblotting</td>
<td>80.8g NaCl, 60.6 g Tris Base, R.O. H₂O up to 1 L, pH 7.4</td>
</tr>
<tr>
<td>TBS/tween20</td>
<td>Western immunoblotting</td>
<td>1x TBS with 0.1% Tween®20</td>
</tr>
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</table>
### Table 2.4 Primary antisera for western immunoblotting

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Description</th>
<th>Antibody dilution (western blotting)</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phospho-Akt</td>
<td>Rabbit anti-phospho-Akt (ser 473), polyclonal</td>
<td>1:1000 in 5% BSA, TBS/tween20</td>
<td>Cell Signaling Technology, Danvers, MA</td>
</tr>
<tr>
<td>Total Akt</td>
<td>Rabbit anti-Akt, polyclonal</td>
<td>1:1000 in 5% BSA, TBS/tween20</td>
<td>Cell Signaling Technology, Danvers, MA</td>
</tr>
<tr>
<td>Phospho-GSK-3β</td>
<td>Rabbit anti-phospho-GSK-3β (ser 9), polyclonal</td>
<td>1:1000 in 5% BSA, TBS/tween20</td>
<td>Cell Signaling Technology, Danvers, MA</td>
</tr>
<tr>
<td>Total GSK-3β</td>
<td>Mouse anti-GSK-3β, clone 7/GSK-3β, monoclonal</td>
<td>1:1000 in 5% non-fat milk, TBS/tween20</td>
<td>BD Biosciences Canada, Mississauga, ON</td>
</tr>
<tr>
<td>β-catenin</td>
<td>Rabbit anti-β-catenin, polyclonal</td>
<td>1:1000 in 5% non-fat milk, TBS/tween20</td>
<td>Millipore Corporation, Billerica, MA</td>
</tr>
<tr>
<td>Phospho-ERK 1/2</td>
<td>Rabbit anti-phospho-p44/42 MAPK (Thr 202, Tyr 204)</td>
<td>1:1000 in 5% BSA, TBS/tween20</td>
<td>Cell Signaling Technology, Danvers, MA</td>
</tr>
<tr>
<td>Total ERK 1/2</td>
<td>mouse anti-p44/42 MAPK (ERK1/2), clone L34F12, monoclonal</td>
<td>1:1000 in 5% non-fat milk, TBS/tween20</td>
<td>Cell Signaling Technology, Danvers, MA</td>
</tr>
<tr>
<td>Alpha smooth muscle actin (αSMA)</td>
<td>Mouse anti-αSMA, monoclonal</td>
<td>1:1000 in 5% non-fat milk, TBS/tween20</td>
<td>Sigma-Aldrich, St. Louis, MO</td>
</tr>
<tr>
<td>OB cadherin</td>
<td>Mouse anti-Cadherin 11, clone 5B2H5, monoclonal</td>
<td>1:1000 in 5% non-fat milk, TBS/tween20</td>
<td>Life Technologies, Carlsbad, CA</td>
</tr>
<tr>
<td>Periostin</td>
<td>Rabbit anti-periostin, clone H-300, polyclonal</td>
<td>1:1000 in 5% non-fat milk, TBS/tween20</td>
<td>Santa Cruz Biotechnology, Santa Cruz, CA</td>
</tr>
<tr>
<td></td>
<td>Antibody Description</td>
<td>Dilution</td>
<td>Supplier</td>
</tr>
<tr>
<td>------------------------</td>
<td>-------------------------------------------------------</td>
<td>------------------</td>
<td>----------------------------------------</td>
</tr>
<tr>
<td>Phospho-Focal adhesion Kinase (FAK)</td>
<td>Rabbit anti-phospho-FAK (Tyr 397), polyclonal</td>
<td>1:500 in 5% BSA, TBS/tween20</td>
<td>Cell Signaling Technology, Danvers, MA</td>
</tr>
<tr>
<td>Total FAK</td>
<td>Rabbit anti-FAK, polyclonal</td>
<td>1:500 in 5% BSA, TBS/tween20</td>
<td>Cell Signaling Technology, Danvers, MA</td>
</tr>
<tr>
<td>Phospho-myosin light chain (MLC)</td>
<td>Rabbit anti-phospho-MLC 2 (ser 19), polyclonal</td>
<td>1:500 in 5% BSA, TBS/tween20</td>
<td>Thermo Scientific, Rockford, IL</td>
</tr>
<tr>
<td>Total MLC</td>
<td>Rabbit anti-MLC 2, polyclonal</td>
<td>1:500 in 5% BSA, TBS/tween20</td>
<td>Thermo Scientific, Rockford, IL</td>
</tr>
<tr>
<td>Phospho-cofilin</td>
<td>Rabbit anti-phospho-cofilin (Ser 3), clone 77G2, monoclonal</td>
<td>1:500 in 5% BSA, TBS/tween20</td>
<td>Cell Signaling Technology, Danvers, MA</td>
</tr>
<tr>
<td>Total cofillin</td>
<td>Rabbit anti-cofilin, clone D3F9, monoclonal</td>
<td>1:500 in 5% non-fat milk, TBS/tween20</td>
<td>Cell Signaling Technology, Danvers, MA</td>
</tr>
<tr>
<td>Alpha V integrin (αv)</td>
<td>Rabbit anti-αv, clone Q-20, polyclonal</td>
<td>1:1000 in 5% non-fat milk, TBS/tween20</td>
<td>Santa Cruz Biotechnology, Santa Cruz, CA</td>
</tr>
<tr>
<td>Alpha 5 integrin (α5)</td>
<td>Rabbit anti-α5, polyclonal</td>
<td>1:1000 in 5% non-fat milk, TBS/tween20</td>
<td>Cell Signaling Technology, Danvers, MA</td>
</tr>
<tr>
<td>β-actin</td>
<td>Mouse anti-β-actin, HRP-linked, clone C4, monoclonal</td>
<td>1:2500 in 5% non-fat milk, TBS/tween20</td>
<td>Santa Cruz Biotechnology, Santa Cruz, CA</td>
</tr>
</tbody>
</table>
Table 2.5 Secondary antisera for western immunoblotting

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Description</th>
<th>Antibody dilution (western blotting)</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse secondary</td>
<td>Horse anti-mouse IgG, HRP-linked</td>
<td>1:1000 in 5% non-fat milk, TBS/tween20</td>
<td>Cell Signaling Technology, Danvers, MA</td>
</tr>
<tr>
<td>Rabbit Secondary</td>
<td>Goat anti-rabbit IgG, HRP-linked</td>
<td>1:1000 in 5% non-fat milk, TBS/tween20</td>
<td>Cell Signaling Technology, Danvers, MA</td>
</tr>
</tbody>
</table>

2.1.5 Polymerase Chain Reaction (PCR)

- RNeasy mini kit: Qiagen, Venlo, Netherlands
- High-capacity cDNA archive kit: Life Technologies, Carlsbad, CA
- 10X PCR buffer, minus Mg: Life Technologies, Carlsbad, CA
- 50 mM Magnesium chloride: Life Technologies, Carlsbad, CA
- Custom primers: Sigma Aldrich, St. Louis, MO
- Platinum® Taq DNA polymerase: Life Technologies, Carlsbad, CA
- 100 mM dNTP set, PCR grade: Life Technologies, Carlsbad, CA
- UltraPure™ Agarose: Life Technologies, Carlsbad, CA
- QIAEX® II Gel extraction kit: Qiagen, Venlo, Netherlands
- MicroAmp Optical 384-well reaction plate: Life Technologies, Carlsbad, CA
- TaqMan universal PCR master mix: Life Technologies, Carlsbad, CA
- TaqMan gene expression assay primers: Life Technologies, Carlsbad, CA
## Table 2.6 TaqMan gene expression assay primers

<table>
<thead>
<tr>
<th>Primer</th>
<th>Protein Encoded</th>
<th>Assay ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>POSTN</td>
<td>Periostin</td>
<td>Hs00170815_m1</td>
</tr>
<tr>
<td>CCND1</td>
<td>Cyclin D1</td>
<td>Hs00765553_m1</td>
</tr>
<tr>
<td>BCL2</td>
<td>Bcl2</td>
<td>Hs00608023_m1</td>
</tr>
<tr>
<td>BAX</td>
<td>Bax</td>
<td>Hs00180269_m1</td>
</tr>
<tr>
<td>GAPDH</td>
<td>GAPDH</td>
<td>Hs99999905_m1</td>
</tr>
<tr>
<td>ACTB</td>
<td>β-actin</td>
<td>Hs99999903_m1</td>
</tr>
</tbody>
</table>

### 2.1.6 Flow cytometry

- **Guava ViaCount® reagent**
  - Millipore, Billerica, MA
- **MCDB 105 medium**
  - Sigma-Aldrich, St. Louis, MO

### 2.1.7 Immunoprecipitation

- **Protein A/G magnetic beads**
  - Thermo Scientific, Waltham, MA
- **Coomassie brilliant blue R-250**
  - Fisher Scientific, Hampton, NH
- **Amicon® Ultra centrifugal 10 kDa filters**
  - Millipore Corporation, Billerica, MA

## Table 2.7 Antisera for immunoprecipitation

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Description</th>
<th>Antibody Concentration</th>
<th>Suppliers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Periostin</td>
<td>Rabbit anti-periostin, clone H-300, polyclonal</td>
<td>2 μg / 500 μg total cell lysate</td>
<td>Santa Cruz Biotechnology, Santa Cruz, CA</td>
</tr>
<tr>
<td>IgG</td>
<td>Rabbit anti-human IgG3, clone H-270, polyclonal</td>
<td>2 μg / 500 μg total cell lysate</td>
<td>Santa Cruz Biotechnology, Santa Cruz, CA</td>
</tr>
<tr>
<td>IgG</td>
<td>Anti-human IgG from rabbit serum</td>
<td>2 μg / 500 μg total cell lysate</td>
<td>Sigma-Aldrich, St. Louis, MO</td>
</tr>
</tbody>
</table>
**Table 2.8 Buffers and solutions for immunoprecipitation**

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Application</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysis buffer</td>
<td>Cell lysis</td>
<td>R.O. H₂O with 20 mM Tris-HCl (pH 8.0), 137 mM NaCl, 1% Triton® X-100, 2 mM EDTA</td>
</tr>
<tr>
<td>Wash buffer</td>
<td>Wash buffer</td>
<td>TBS with 0.05% Tween®20 and 0.5 M NaCl</td>
</tr>
<tr>
<td>Elution buffer</td>
<td>Protein elution</td>
<td>4 parts lysis buffer, 1 part SDS gel-loading buffer</td>
</tr>
<tr>
<td>Gel fixation solution</td>
<td>Fixing polyacrylamide gels</td>
<td>50% methanol, 10% acetic acid, 40% R.O. H₂O</td>
</tr>
<tr>
<td>Coomassie brilliant blue concentrated stain solution</td>
<td>Protein staining</td>
<td>12.0 g coomassie brilliant blue, 300 ml methanol, 60 ml acetic acid</td>
</tr>
<tr>
<td>Coomassie brilliant blue staining solution</td>
<td>Protein staining</td>
<td>500 ml methanol, 30 ml coomassie brilliant blue concentrated stain solution, 400 ml R.O H₂O, 100 ml acetic acid, filtered using 0.22 μm filter</td>
</tr>
<tr>
<td>Destaining solution</td>
<td>Wash solution</td>
<td>45% methanol, 10% acetic acid, 45% R.O. H₂O</td>
</tr>
<tr>
<td>Gel storage solution</td>
<td>Polyacrylamide gel storage</td>
<td>R.O. H₂O with 5% acetic acid</td>
</tr>
</tbody>
</table>

**2.1.8 Nickel magnetic bead precipitation**

- **37% formaldehyde**  
  Merck KGaA, Darmstadt, GER

- **10x glycine solution**  
  Cell Signaling Technology, Danvers, MA

- **Nonidet P40 (NP-40)**  
  Merck KGaA, Darmstadt, GER

- **PureProteome™ nickel magnetic beads**  
  Millipore, Billerica, MA
### Table 2.9 Buffers for Nickel Magnetic Bead precipitation

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Application</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysis buffer</td>
<td>Cell lysis</td>
<td>R.O. H₂O with 50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole (pH 8), 0.1% NP-40</td>
</tr>
<tr>
<td>Wash buffer</td>
<td>Wash buffer</td>
<td>R.O. H₂O with 50 mM NaH₂PO₄, 300 mM NaCl, 20 mM imidazole (pH 8), 0.1% NP-40</td>
</tr>
<tr>
<td>Elution Buffer</td>
<td>Protein elution</td>
<td>R.O. H₂O with 50 mM NaH₂PO₄, 300 mM NaCl, 300 mM imidazole (pH 8)</td>
</tr>
</tbody>
</table>

### 2.1.9 Loss-of-function studies

### Table 2.10 Adenoviral shRNA constructs

<table>
<thead>
<tr>
<th>Construct</th>
<th>Promoter</th>
<th>Targeting sequence</th>
<th>Multiplicity of infection (MOI)</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ad-GFP-U6-h-POSTN-shRNA</td>
<td>U6</td>
<td>CGGTGACAGTATA ACAGTAA</td>
<td>25</td>
<td>Vector Biolabs, Philadelphia, PA</td>
</tr>
<tr>
<td>Ad-U6-RNAi-GFP</td>
<td>U6</td>
<td>GACACGCGACTTG TACCACT</td>
<td>25</td>
<td>Vector Biolabs, Philadelphia, PA</td>
</tr>
</tbody>
</table>

### 2.1.10 Immunofluorescence confocal microscopy

- 35 mm, poly-D-lysine coated glass bottom MatTek Corporation, Ashland, MA
- tissue culture plates (No. 1.5 coverslip, 10 mm glass diameter)
- Paraformaldehyde 16% solution Electron Microscopy Sciences, Hatfield, PA
Background Sniper  Biocare Medical, Concord, Ca
Dako antibody diluent  Dako Canada Inc., Burlington, ON
Negative control mouse IgG1  Dako Canada Inc., Burlington, ON
Hoeschst 33342  Life Technologies, Carlsbad, CA
ProLong® Gold antifade reagent  Life Technologies, Carlsbad, CA
18 x 18 mm, No.1 coverslips  Fisher Scientific, Hampton, NH

Table 2.11 Buffers for immunofluorescence confocal microscopy

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Application</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immunofluorescence phosphate buffered solution (IF-PBS)</td>
<td>Wash buffer</td>
<td>0.2 g NaH₂PO₄, 1.375 g Na₂HPO₄, 8.8 g NaCl, R.O. H₂O up to 1 L</td>
</tr>
<tr>
<td>Cell permeabilization solution</td>
<td>Cell permeabilization</td>
<td>PBS with 0.1% Triton® X-100</td>
</tr>
</tbody>
</table>

Table 2.12 Antisera for immunofluorescence confocal microscopy

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Description</th>
<th>Dilution</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vinculin</td>
<td>Mouse anti-vinculin, clone VIN-11-5, monoclonal</td>
<td>1:200 in Dako antibody diluent</td>
<td>Sigma-Aldrich, St. Louis, MO</td>
</tr>
<tr>
<td>Negative control mouse IgG1</td>
<td>Mouse anti-Aspergillus niger glucose oxidase, clone DAK-GO1, monoclonal</td>
<td>4 μg/ml in Dako antibody diluent</td>
<td>Dako Canada Inc., Burlington, ON</td>
</tr>
</tbody>
</table>
Table 2.13 Spectral properties of molecular probes and LSM 510 Duo Vario confocal microscope

<table>
<thead>
<tr>
<th>Molecular Probe</th>
<th>Dilution</th>
<th>LSM Laser Lines for Excitation</th>
<th>LSM Emission Filters</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>αSMA-cy3</td>
<td>1:100 in Dako antibody diluent</td>
<td>30 mW 514 nm Argon/2 Ion laser</td>
<td>BP 560-615 IR</td>
<td>Sigma-Aldrich, St. Louis, MO</td>
</tr>
<tr>
<td>Alexa Fluor 647</td>
<td>1:100 in Dako antibody diluent</td>
<td>5 mW 633 nm HeNe</td>
<td>LP 650</td>
<td>Life Technologies, Carlsbad, CA</td>
</tr>
<tr>
<td>Alexa Fluor 594</td>
<td>1:100 in Dako antibody diluent</td>
<td>30 mW 514 nm Argon/2 Ion laser</td>
<td>BP 560-615 IR</td>
<td>Life Technologies, Carlsbad, CA</td>
</tr>
<tr>
<td>Hoechst</td>
<td>1:10 000 in PBS</td>
<td>50 mW 405-50 nm Diode</td>
<td>BP 420-480</td>
<td>Life Technologies, Carlsbad, CA</td>
</tr>
</tbody>
</table>

2.2 Methods

2.2.1 Clinical specimen collection
Surgically resected normal skin, hypertrophic scar and keloid scar tissue samples were collected from patients in the clinics of the Roth|McFarlane Hand and Upper Limb Centre or in the operating rooms of St. Joseph’s Hospital, London, Ontario. All subjects provided written informed consent and specimens were collected with the approval of Western University Research Ethics Board for Health Sciences Research involving Human Subjects (HSREB protocol # 08222E).

2.2.2 Primary cell culture
Primary cells were harvested from surgically resected hypertrophic scar, keloid scar and normal skin tissue as previously described (181). A total of eight patient-derived hypertrophic scar samples, three keloid scar samples and six normal skin samples were
used to derive primary fibroblasts for analyses in this thesis. In brief, tissue samples were finely minced, adhered to the bottom of 100 mm dishes and cultured in αMEM supplemented with 10% fetal bovine serum (FBS), 1x L-glutamine and 1x antibiotic-antimycotic solution at 37°C in 5% CO₂. Explant cultures (passage 0) were incubated for 2-4 weeks allowing for the migration, and subsequent proliferation, of fibroblasts from the tissue to the 100 mm dish. Upon reaching 50% confluency, primary cultures were sub-cultured using 0.05% Trypsin-EDTA. Primary fibroblast cultures at passage 1 were frozen down in freezing medium (Table 2.1) in Nalgene® cryogenic vials at -80°C overnight then stored in liquid nitrogen. All primary cell lines were used up and until a maximum of four passages, during which no changes in cell behavior or morphology attributable to serial passage were evident.

2.2.3 Preparation of rat tail type I collagen
Frozen rat tails were obtained from the laboratories of Drs. Ruud Veldhuizen and Ting-Yim Lee (Western University, London, ON). Frozen rat tails were thawed in 70% ethanol for one hour. Rat tails were skinned and collagen fibers were mechanical extracted from rat tail tendons. Collagen fibers were soaked in 70% ethanol for 30 minutes then placed under ultraviolet light overnight in a tissue culture cabinet. Under sterile conditions, collagen fibers were incubated in sterile 0.017M acetic acid with mechanical stirring for seven days at 4°C. Undissolved collagen fibers were removed from the solution by centrifugation at 18,000 x g at 4°C overnight. The supernatant was collected into sterile bottles and stored at 4°C. Collagen concentration was determined using the Sircol collagen quantification assay (Biocolor Ltd., Carrickfergus, UK) in accordance with the manufacturer’s instructions.

2.2.4 WST-1 cell proliferation assay
The WST-1 proliferation assay was utilized to measure the proliferation of hypertrophic scar and normal skin fibroblasts grown in collagen cultures. Collagen lattices were cast in 96-well tissue culture trays. For each well, 48 μl of type I rat tail collagen (final concentration of 1.8 mg/ml) was quickly mixed with 12 μl of neutralization solution (3 parts Waymouth media: 2 parts 0.34 M NaOH), and 2 μg/ml human recombinant periostin or vehicle (0.1% BSA-PBS). Collagen lattices were incubated at 37°C in 5%
CO₂ for 20 minutes to allow for collagen polymerization. 1x10⁵ cells in 100 μl of 2% FBS-αMEM ± inhibitors (Table 2.2) were seeded onto the collagen lattices and incubated at 37°C in 5% CO₂. 10 μl of WST-1 reagent was added at days 1, 3, 5 and 7 post seeding and incubated for 2 hours at 37°C to allow for the cleavage of tetrazolium salt to formazan by the succinate-tetrazolium reductase system. Equal volumes of media (90 μl) were transferred to an additional 96-well tissue culture tray and absorbencies were measured at 450 and 650 nm (reference wavelength) using an iMARK Microplate Reader (Bio-Rad, Hercules, CA). Difference scores were calculated relative to day 1 absorbance readings.

### 2.2.5 Fibroblast Populated Collagen Lattices (FPCLs)

Collagen contraction assays were carried out using primary normal skin, hypertrophic scar and keloid scar fibroblasts based on Tomasek and Rayan (182). Collagen lattices were cast in 24-well tissue culture trays. For each well, 1x10⁵ cells were quickly mixed with 400 μl of type I rat tail collagen (final concentration of 1.8 mg/ml), 100 μl of neutralization solution and 2 μg/ml human recombinant periostin or vehicle. In a subset of experiments, normal skin fibroblasts were primed with 5 ng/ml recombinant TGFβ-1 or vehicle (PBS with 1% BSA and 4 mM HCl) in 2% FBS-αMEM for 72 hours prior to sFPCL setup. Collagen lattices were incubated at 37°C in 5% CO₂ for 45 minutes to allow for collagen polymerization. Wells were then flooded with 1 ml of 2% FBS-αMEM. In a subset of experiments, wells were flooded with fibroblast-conditioned media (section 2.2.9.2). For stressed FPCLs (sFPCLs), collagen cultures were maintained for 72 hours. During this time, the primary fibroblasts in three-dimensional cultures respond to stress within the tethered, polymerized collagen lattice and differentiate into contractile myofibroblasts (183). After three days in culture, collagen lattices were simultaneously released from the wells using a metal spatula, thereby allowing the differentiated myofibroblasts to contract the untethered lattice. Floating lattices were digitally scanned within two minutes (designated time 0), 0.5, 2, 6, and 24 hours after release and the area of each lattice was determined using the freehand tool in ImageJ software (National Institutes of Health, Bethesda, MD). Sequential area calculations were normalized to the area of the well prior to release.
For relaxed FPCLs (rFPCLs), lattices were cultured for one hour prior to being mechanical released from the wells. Fibroblasts cultured in rFPCLs are not subjected to mechanical tension and do not differentiate into myofibroblasts. Therefore, rFPCLs undergo gradual lattice contraction over a 24 hour period due to fibroblast attachment, spreading and migration (183). Floating lattices for rFPCLs were digitally scanned at 24 hours after release.

2.2.6 Western immunoblotting

2.2.6.1 Protein extraction

For total protein extraction from fibroblasts cultured on collagen substrates, cultures were washed in PBS and incubated with 1 ml collagenase solution (final concentration of 0.25 mg/ml) for 30 minutes with gentle rocking at 37˚C using a thermal rocker, in order to detach the fibroblasts from the collagen substrates. The collagenase solution was collected from each well and centrifuged at 600 x g for 4 minutes to pellet the cells. Cell pellets were washed with PBS and centrifuged at 16 000 x g for 1 minute. Cell pellets were resuspended in 100 μl of ice cold RIPA lysis buffer supplemented with proteinase inhibitor cocktail, 0.1M sodium fluoride (NaF), 10 mM phenylmethanesulfonyfluoride (PMSF) and 10 mM sodium orthovanadate (Na₃VO₄). Cell pellets were incubated on ice for 30 minutes then subjected to needle aspiration. Cell lysates were centrifuged at 16 000 x g for 10 minutes and the supernatant was stored at -80°C.

For total protein extraction from sFPCLs, lattices were collected immediately prior to collagen lattice release (after 72 hours in culture), 24 and 48 hours after collagen lattice release and transferred to a 2 ml microcentrifuge tube containing 100 – 400 μl of ice cold RIPA lysis buffer supplemented with proteinase inhibitor, NaF, PMSF and Na₃VO₄. Collagen lattices were homogenized using an Ultra-Turrax T25 tissue homogenizer (IKA® Works Inc., Wilmington, NC) at 16 000 x g for 30 seconds. Cell extracts were incubated on ice for 30 minutes then subjected to needle aspiration. Cell extracts were centrifuged at 16 000 x g for 10 minutes to remove insoluble material and the supernatant was stored at -80°C.
2.2.6.2 Bicinchoninic acid (BCA) total protein assay

Bovine serum albumin (BSA) standards were prepared by serial dilution with RIPA lysis buffer to concentrations of 2000, 1000, 500, 250, 125, 62.5, 31.25 and 0 μg/ml. Total cell lysates were prepared in a 1/5 and 1/10 dilution in RIPA lysis buffer and 10 μl of each dilution and standard was added in triplicate to 96-well tissue culture trays. 200 μl of Pierce® BCA protein assay reagent A:B (49:1) was added to each well and samples were incubated for 30 minutes at 37°C in 5% CO₂. The colourimetric reaction was analyzed at 595 nm using an iMARK Microplate Reader. The data was used to construct a standard curve and protein concentrations were calculated from the obtained absorbance values to ensure equal loading into polyacrylamide gels. We were unable to perform BCA analyses on sFPCL lysates due to the high collagen concentration in the lysates and phenol red in the media. Therefore, 40 μl of total cell lysate was used for western immunoblotting and densitometry was performed to correct for unequal loading.

2.2.6.3 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

8-10% polyacrylamide resolving gels were made with a 5% stacking gel and set up in the Bio-Rad Mini Protean III apparatus (Biorad, Hercules, CA). 8% polyacrylamide resolving gels were used for the analysis of αSMA, OB cadherin, periostin, integrins, β-catenin, FAK, Akt, ERK1/2 and GSK-3β. 10% polyacrylamide resolving gels were used for the analysis of myosin light chain and cofilin levels. Samples were diluted to load either 25 μg of total protein or 40 μl of sFPCL lysate per well and SDS gel-loading buffer (Table 2.3) was added to each sample at a 1:4 (v/v) ratio. Samples were boiled at 95°C for 5-10 minutes, placed on ice for 2 minutes and centrifuged at 16 000 x g for 1 minute. Samples were loaded into 1.5 mm wells and subjected to SDS-PAGE in 1x electrophoresis running buffer (Table 2.3) using a PowerPac 3000 (Bio-Rad, Hercules, CA) set to 80 V though the stacking gel and 120 V through the resolving gel.

2.2.6.4 Transfer, blocking, incubation, detection and densitometry

Proteins were transferred to polyvinylidene difluoride (PVDF) – plus, 0.45 micron transfer membranes in ice cold 1x electrophoresis transfer buffer (Table 2.3) on ice at 225 mA for two hours. The PVDF membranes were blocked for one hour in 5% non-fat milk (w/v) or 5% BSA (w/v) in TBS-0.1% Tween®20 (TBS/tween, Table 2.3) depending on
the primary antibody (Table 2.4). Membranes were washed with TBS/tween (3 x 5 minutes) and incubated with primary antibody overnight at 4°C with gentle rotation. Membranes were washed with TBS/tween (3 x 5 minutes) and probed with the corresponding horseradish peroxidase (HRP)–conjugated secondary antibody (Table 2.5) for 90 minutes at room temperature with gentle rotation. Membranes were washed with TBS/tween (3 x 5 minutes) and incubated with 1 ml Immobilon™ western chemiluminescent HRP substrate (1:1 ratio of luminal to HRP solution) and imaged using the ChemiGenius² Bio Imaging System (Syngene, Frederick, MD). Images were captured using Gene Snap (Syngene, Frederick, MD) and densitometry analysis was performed using the manual band quantification analysis function in Gene Tools (Syngene, Frederick, MD).

2.2.7 PCR

2.2.7.1 RNA extraction and cDNA synthesis

Cell pellets were collected from sFPCLs or two-dimensional collagen substrates using collagenase treatment (2.2.6.1). Cell pellets were lysed and total RNA was extracted using the RNeasy mini kit in accordance with the manufacturer’s instructions. Total RNA was eluted in 50 μl of RNase-free water. Total RNA concentration and quality was assessed using the NanoDrop-1000 spectrophotometer (Thermo Scientific, Waltham, MA). 2 μg of high quality total RNA was reversed transcribed into cDNA first strand using the High-Capacity cDNA archive kit in accordance with the manufacturer’s instructions.

2.2.7.2 Real time PCR

cDNA samples were diluted to a final concentration of 10 ng/μl in RNase/DNase free water and 20 ng of cDNA was loaded in triplicate into MicroAmp Optical 384-well reaction plates. TaqMan gene expression assays were used to measure POSTN, CCND1, BAX and BCL2 expression in accordance with the manufacturer’s instructions (Table 2.6 for primers). POSTN and BAX expression was measured relative to GAPDH endogenous control and CCND1 and BCL2 expression was measured relative to ACTB using the ΔΔCt method (relative quantification, RQ) after confirmation of parallel PCR
amplification efficiencies using the 7900HT Sequence Detection System (Life Technologies, Carlsbad, CA). PCR reactions were carried out under the following conditions: Initial denaturation at 95°C for 5 minutes followed by cycles of denaturation (95°C for 15 seconds), primer annealing (60°C for 1 minute) and transcript extension (50°C for 2 minutes) for 40 cycles. The relative quantification for each gene normalized to vehicle controls was analyzed using RQ Manager 1.2.1 software (Life Technologies, Carlsbad, CA).

2.2.7.3 Reverse-transcription PCR

20 ng of cDNA was subjected to PCR analysis with Platinum®Taq DNA polymerase in accordance with the manufacturer’s instructions. PCR reactions were carried out under the following conditions: initial denaturation at 95°C for 3 minutes, followed by cycles of denaturation (95°C for 30 seconds), primer annealing (58°C for 30 seconds) and transcript extension (72°C for 2 minutes) for 40 cycles, with a final extension at 72°C for 7 minutes. The primers (Forward 5’- ACAACGGGCAATACTGGAA-3’, Reverse 5’- AACTTCCTCACGGGTGTGTCA-3’) were designed to have 100% sequence identity with four POSTN mRNA isoforms originally reported ([101], NCBI Reference Sequences: NM_006475.2, NM_001135934.1, NM_001135935.1 and NM_001135936.1). PCR products were visualized by agarose gel electrophoresis, amplicons were isolated using the QIAEX® II Gel extraction kit and sequenced at the Robarts Research Institute DNA sequencing facility (Western University, London, ON).

2.2.8 Flow Cytometry

Cell viability was assessed using the Guava ViaCount® assay that distinguishes between viable and non-viable cells based on the differential permeability of DNA-binding dyes in the ViaCount® reagent. Primary fibroblasts were cultured in sFPCLs treated with 2 μg/ml human recombinant periostin or vehicle for 72 hours. Cells were extracted from sFPCLs using collagenase (2.2.6.1). Cell pellets were resuspended in 1 ml of MCDB medium and cell number was assessed using a hemocytometer. Accurate cell counting on the Guava system occurs at a cell concentration of 1x10^4 - 5x10^5 cells/ml. Cell suspensions of appropriate concentrations were diluted to 300 μl in MCDB media and incubated with 100 μl of Guava ViaCount reagent for 5 minutes. Flow cytometry analysis
was performed using the Guava EasyCyte Mini (Millipore, Billerica, MA). Using a
stained cell sample, threshold levels were set to separate nucleated cells from debris and
viable cells from non-viable cells using CytoSoft 4.2.1 software (Millipore, Billerica,
MA). Once threshold levels were set, samples were analyzed in triplicate for 1000
cellular events. Viability data was exported to FlowJo 7.6.5 (Tree Star Inc, Ashland, OR)
and plotted with cell viability on the y-axis and forward scatter on the x-axis to separate
collagen debris from the viable and non-viable cell populations. Manual gates were set
and applied to all the samples within the experiment. Viability was assessed by
calculating the percentage of viable versus non-viable cells from the total cell population.

2.2.9 Immunoprecipitation (IP)

2.2.9.1 Fixation
Primary hypertrophic scar fibroblasts were cultured on 100 mm tissue culture plates in
10% FBS-αMEM. When 85% confluency was achieved, fibroblasts were fixed with
formaldehyde (final concentration of 1%) for 10 minutes at room temperature. 25 mM
glycine solution (final concentration of 2.5 mM) was added to each plate and incubated
for an additional five minutes at room temperature to quench the formaldehyde. Plates
were washed with ice-cold PBS (5 x 1 minute), incubated with 100 µl non-denaturing
lysis buffer (Table 2.8) and cells were lysed using a cell scraper. Total protein extracts
were collected in a 1.5 ml microcentrifuge tube, incubated on ice for 30 minutes and
subjected to needle aspiration. Cell extracts were centrifuged at 16 000 x g for 10 minutes
and the supernatant was stored at -80°C.

2.2.9.2 Generation of conditioned media
Primary hypertrophic scar or normal skin fibroblasts were cultured on 100 mm tissue
culture plates in 10% FBS-αMEM until the cells achieved 80% confluence. Cells were
washed overnight in serum free αMEM then cultured in 5 ml fresh serum-free αMEM for
96 hours. Conditioned media was collected, supplemented with proteinase inhibitor
cocktail and stored at -80°C. For immunoprecipitation studies, fresh conditioned media
was concentrated 50x using 10 kDa Amicon® Ultra centrifugal filters and the
concentration was assessed by BCA assay (2.2.6.2).
2.2.9.3  Immunoprecipitation

1 mg of hypertrophic scar-conditioned media or total cell lysate (2.2.9.1), in a final volume of 1 ml, was incubated with 4 µg of periostin-specific antibody or IgG control (Table 2.7) overnight at 4°C with gentle rotation. Protein A/G magnetic beads were washed twice with wash buffer (175 µl and 1 ml for 1 minute each, Table 2.8), and incubated with the antigen/antibody mixture for 1 hour at room temperature with gentle rotation. Magnetic beads were washed with 500 µl wash buffer (3 x 5 minutes) and 500 µl purified water (1 x 2 minutes). Magnetic beads were resuspended in 80 µl lysis buffer (Table 2.8) and 20 µl SDS gel-loading buffer (Table 2.3) and boiled at 95°C for 5 minutes (conditioned media samples) or 70°C for 45 minutes to reverse formaldehyde crosslinks (total cell lysate samples). The supernatant was collected and 50 µl was loaded into 1.5 mm wells and subjected to SDS-PAGE. PVDF membranes were probed with antibodies against periostin and integrins αV and α5 (Table 2.4). 50 µg of total cell lysate or conditioned media (input samples) was analyzed to confirm the presence of periostin and integrins αV and α5 in the pre-immunoprecipitated sample.

2.2.9.4  Gel fixation and coomassie brilliant blue staining

Immunoprecipitated samples were subjected to SDS-PAGE and stained with coomassie brilliant blue for liquid chromatography tandem mass spectrometry analysis. Polyacrylamide gels were fixed for 30 minutes at room temperature with gentle rocking in gel fixation solution (Table 2.8). Gels were stained with coomassie brilliant blue staining solution (Table 2.8) for 30 minutes at room temperature with gentle rocking. Gels were then destained in destaining solution (Table 2.8) for 2-3 hours at room temperature with gentle rocking. Destaining solution was replaced every 45 minutes. Gels were imaged using the ChemiGenius Bio Imaging System and images were captured using Gene Snap. Gels were stored at 4°C in 5% acetic acid.

2.2.9.5  Spot picking, in-gel digestion and mass spectrometry

Differential protein bands in periostin immunoprecipitated samples relative to IgG controls were isolated using the Ettan spot picker (GE Healthcare, Little Chalfont, UK) at the Functional Proteonomic Facility (FPF) at Western University (London, ON). Isolated samples were stored in R.O H2O with 45% methanol and 5% acetic acid and submitted to
the FPF for in-gel digestion. In-gel digestion was performed using a MassPREP automated digester station (PerkinElmer, Waltham, MA). In brief, gel pieces were coomassie brilliant blue destained using 50 mM ammonium bicarbonate and 50% acetonitrile. Proteins were reduced using 10 mM DTT, followed by alkylation using 55 mM iodoacetamide and tryptic digestion. Peptides were extracted using a solution of 1% formic acid and 2% acetonitrile and lyophilized.

Lyophilized samples were submitted to the Biological Mass Spectrometry Laboratory (Western University, London, ON) for quadrupole time-of-flight (Q-TOF) liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis. In brief, lyophilized fractions were reconstituted in 0.1% formic acid prior to injection. For gel-enhanced analysis, excised band samples were identified as having low, medium, or high complexity based on clear, light, or dark coomassie staining, respectively. Depending upon the anticipated complexity of the sample, ~1/2, 1/5, or 1/8 of each fraction was analyzed using a 60-, 90-, or 150-min LC method, respectively. Separation using LC (5–40% acetonitrile, 0.1% formic acid gradient) was performed on a NanoAcquity Ultra Performance Liquid Chromatography (UPLC, Waters Corporation, Milford, MA) with a 25 cm x 75 μm C18 reverse phase column. Peptide ions were detected in data-dependent acquisition mode by tandem MS using a Q-ToF Ultima-Global mass spectrometer (Waters Corporation, Milford, MA). Raw data was refined, deNovo sequenced and searched using Peaks 6.0 (Bioinformatics Solutions Inc., Waterloo, ON) against NCBInr – human database. Results were filtered using a false discovery rate of 1% and a minimum of two unique peptides were required for positive protein identification.

### 2.2.10 Nickel magnetic bead precipitation

Primary hypertrophic scar fibroblasts were cultured on 100 mm tissue culture plates in 10% FBS-αMEM until 80% confluency was achieved. Cultures were then treated with 2 μg/ml human recombinant periostin or vehicle in 2%FBS-αMEM overnight. Samples were fixed and lysed as described 2.2.9.1. PureProteome™ Nickel Magnetic Beads (Millipore, Billerica, MA) were used to precipitate the histidine-tagged human recombinant periostin from total cell lysates. Nickel magnetic beads were placed in a magnetic stand to remove the storage buffer and washed for 1 minute with gentle rotation
with 500 µl lysis buffer (Table 2.9). 1 mg of total cell lysate, in a final volume of 1 ml, was added to the magnetic beads and incubated at room temperature with gentle rotation for 2 hours. Magnetic beads were washed for 10 minutes with sterile water, 2.5 hours with wash buffer (Table 2.9), twice for 10 minutes with wash buffer then quickly in sterile water. Magnetic beads were incubated with 100 µl elution buffer (Table 2.9) for 5 minutes at room temperature with gentle rotation. 25 µl of SDS gel-loading buffer (Table 2.3) and 1 µl proteinase inhibitor cocktail was added to the supernatant and samples were incubated at 70°C for 45 minutes to reverse the formaldehyde crosslinks. 50 µl of the supernatant was loaded into 1.5 mm wells and subjected to SDS-PAGE. PVDF membranes were probed with antibodies against periostin and integrins αV and α5 (Table 2.4). 50 μg of total cell lysate (input sample) was analyzed to confirm the presence of periostin and integrins αV and α5 in the pre-precipitated samples.

2.2.11 Loss-of-function studies
Adenoviral shRNA constructs were purchased from Vector Biolabs (Philadelphia, PA). Both POSTN shRNA and scrambled control vectors have an adenoviral-type 5 (dE1/E3) backbone, are regulated by the U6 promoter and co-express green fluorescent protein (GFP), allowing for visualization of transduction efficiency by epifluorescent microscopy. POSTN and scrambled shRNA sequences (Table 2.10) were analyzed by BLAST to confirm that the POSTN shRNA sequence was present in all POSTN variants and that the scrambled shRNA sequence was not directed against any known human target.

2.2.11.1 Amplification of adenoviral shRNA constructs
Adenoviral POSTN and scrambled shRNA vectors were amplified using HEK 293 cells (Gift from Dr. Lina Dagnino, Western University, London, ON). HEK 293 cells were cultured in 100 mm tissue culture plates to confluency in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 4% FBS. Adenovirus, at a multiplicity of infection (MOI) of 2, was added to fresh 4% FBS-DMEM and HEK 293 cells were incubated in this inoculum for 4-5 days. When 70% of the cells floated off the plate, cells were harvested by rinsing the plate with media. Cells were pelleted by centrifugation at 1300 x g for 5 minutes. The media was aspirated and cell pellets were resuspended in 1 ml sterile
10% glycerol-PBS. Cell pellets were subjected to four rounds of freeze/thaw cycles using liquid nitrogen and a warm water bath. Cell pellets were vortexed gently after each thaw. Cellular debris was removed from the samples by centrifugation at 16 000 x g for 10 minutes and the supernatant containing the virus was stored at -80°C.

2.2.11.2 GFP adenovirus titering
Primary hypertrophic scar fibroblasts were seeded in 10% FBS-αMEM in 24-well trays at a cell concentration of 100 000 cells/well and cultured overnight at 37°C in 5% CO₂. Viral dilutions of the amplified stocks were prepared by serially diluting virus in αMEM supplemented with 2% FBS. Viral dilutions were added to each well at 300 μl/well in triplicate and incubated for 48 hours. After 48 hours, non-transduced cells were collected following trypsin digestion and counted using a hemocytometer to obtain a total cell count per well. The cells transduced with virus were fixed in 4% paraformaldehyde for 10 minutes at room temperature. Wells were washed with PBS (3 x 5 minutes) and stained with Hoescht for 5 minutes. Wells were then washed in PBS (2 x 5 minutes) and imaged using the Olympus IX81 deconvolution microscope (Olympus Corporation, Tokyo, Japan). Cell numbers per field were assessed by counting the number of Hoescht positive cells. The number of GFP positive-viral transduced cells were counted in the same field. The percentage of virus transduced cells was calculated in the lowest viral dilution where at least 70% of the cells were transduced. The percentage of viral transduced cells per field was multiplied by the total number of cells present in the well to determine the total number of cells transduced per well. Using the dilution factor and the number of transduced cells, the concentration of the virus was determined.

2.2.11.3 Multiplicity of infection (MOI) optimization studies
Primary hypertrophic scar fibroblasts were cultured on 100 mm tissue culture plates in 10% FBS-αMEM until the cells reached 60% confluence. The media was replaced with 2% FBS –αMEM containing adenoviral POSTN or scrambled shRNA constructs at an MOI of 0, 10, 25, 50 and 100. Cells were cultured for 72 hours at 37°C in 5% CO₂. After 72 hours, cell pellets were collected following digestion with trypsin (5 minutes at 37°C). Total protein and RNA was extracted from cells using methods described in 2.2.6 and 2.2.7. POSTN expression and periostin levels were assessed in POSTN shRNA treated
fibroblasts relative to scrambled shRNA controls using real time PCR and western immunoblotting, respectively.

2.2.11.4 Viral transduction
Primary fibroblasts were cultured in 10% FBS-αMEM on 100 mm tissue culture plates. When 60% confluency was achieved, fibroblasts were transduced with adenovirus constructs expressing shRNAs against POSTN or scrambled shRNA controls at an MOI of 25 in 2% FBS-αMEM. After 72 hours in culture, fibroblasts were assessed for GFP expression using an Olympus IX81 deconvolution microscope to determine transduction efficiency. If transduction efficiency was greater than 90%, the cells were collected by trypsin digestion and seeded into sFPCLs.

2.2.12 Immunofluorescence (IF) confocal microscopy
2.2.12.1 sFPCL setup for immunofluorescence confocal microscopy
Miniature collagen lattices were cast in 35 mm, poly-D-lysine coated glass bottom tissue culture plates with a No.1.5 cover slip with a glass diameter of 10 mm. For each glass bottom plate, 1 x10^4 cells were quickly mixed with 40 μl of type I rat tail collagen (final collagen concentration of 1.8 mg/ml), 10 μl of neutralization solution and 2 μg/ml human recombinant periostin or vehicle. Collagen lattices were incubated at 37°C in 5% CO₂ for 45 minutes to allow for collagen polymerization. Wells were then flooded with 1 ml 2% FBS-αMEM and incubated for 72 hours to achieve a stressed collagen matrix. After 72 hours, the media was aspirated and lattices were fixed with 4% paraformaldehyde for 10 minutes at room temperature. Plates were washed with PBS (3 x 5 minutes) and stored in 70% ethanol at 4°C.

2.2.12.2 Immunofluorescence staining with two primary antibodies from the same host species
Glass bottom plates were washed with IF-PBS (Table 2.11, 5 x 5 minutes) to remove the ethanol. Cell membranes were permeabilized (5 minutes, Table 2.11), washed with IF-PBS (3 x 5 minutes) and blocked with Background Sniper for 5-10 minutes. Incubation with Background Sniper for longer than 10 minutes resulted in loss of signal. Samples were washed briefly with IF-PBS and incubated with a primary vinculin antibody (Table
2.12) for 1 hour at room temperature in a covered humidity chamber. Samples were washed with IF-PBS (3 x 5 minutes) then incubated with an Alexa Fluor 647 conjugated secondary antibody (Table 2.13) for 1 hour at room temperature in a covered humidity chamber in the dark. Samples were washed with IF-PBS (3 x 5 minutes) then blocked with negative control mouse IgG1 (Table 2.12) for 45 minutes at room temperature in a covered humidity chamber in the dark. Samples were washed with IF-PBS (3 x 5 minutes) and incubated with a primary αSMA antibody conjugated to cy3 (Table 2.13) for 1 hour at room temperature in a covered humidity chamber in the dark. Samples were washed with IF-PBS (3 x 5 minutes), incubated with Hoechst (Table 2.13) for 5 minutes in the dark then washed with IF-PBS(2 x 5 minutes). Prolong Gold anti-fade reagent was added to the samples and a cover slip was applied over top. Samples were incubated overnight at 4°C then coverslips were sealed with nail polish. Samples were stored at 4°C in the dark.

2.2.12.3 Imaging and data analysis
Triple immunofluorescence-labelled cells were observed with a Zeiss LSM 510 Duo Vario confocal microscope (Zeiss Canada Inc., Toronto, ON) equipped with three lasers used simultaneously; an Argon/2 ion laser, a HeNe laser and a Diode laser (Table 2.13 for LSM laser lines for excitation and emission filters). All images were captured using a 40x/1.3 Oil Plan Apochromat objective lens. The pinhole was set to match 1 AU (airy unit) to ensure the best signal to noise ratio. Each channel was adjusted for gain and digital offset to achieve optimal fluorescent signal using Zen 2009 Software (Zeiss Canada Inc., Toronto, ON). Serial optical sections of individual cells were taken in a total depth of 3-6 μm with an optimal z-step of 0.38 μm determined by Zen 2009 Software. A minimum of 30 images from three different experiments (10 images per experiment) were captured per treatment. Images were exported as TIFFs using Zen Software and extended depth of field composite images of maximum intensity were generated for vinculin stained images using Image-Pro Plus 7.0 (Media Cybernetics, Inc., Rockville, MD). Length of focal adhesions (FA), determined by vinculin staining, was manually measured using the straight line tool in Image J. The length of vinculin-stained FAs were defined by their longest axis regardless of orientation. Total FA numbers, average FA
length and the classification of FAs were calculated using Excel. Focal adhesions were arbitrarily classified into three categories: immature FAs (≤ 2 μm), mature FAs (2-6 μm) and supermature FAs (> 6 μm, (184)).

2.2.13 Statistical analyses
Statistics were calculated using SPSS Statistics 17.0 software (IBM Corporation, Armonk, NY) and Microsoft Excel 2007 (Microsoft, Redmond, WA). All data sets consist of data obtained from a minimum of three patients from three independent experiments assessed in triplicate unless otherwise stated. ANOVA of repeated measures were used to assess significant treatment effects and significant treatment/time interactions to distinguish between overall treatment-induced effects from treatment-induced effects that become significant over time. When a significant treatment/time interaction was observed, simple main effects analyses were further performed to determine at which time point treatment had a significant effect. Significant treatment effects at individual time points were assessed by paired t-tests. Results were deemed significant when p<0.05.
Chapter 3

3 In vitro analysis of the effects of periostin on fibroblast proliferation in hypertrophic scarring

3.1 Rationale

Excessive fibroblast proliferation is one of the hallmarks of “overhealing” conditions like hypertrophic scarring (55, 185, 186). Periostin has been demonstrated to induce proliferation in several cell types including human dermal fibroblasts (152), mouse embryonic fibroblasts (152), phenotypically normal palmar fascia fibroblasts from patients with Dupuytren’s disease (91), human periodontal ligament fibroblasts (178), mouse mesenchymal cells (179), colorectal cancer cells (180), human retinal pigment epithelial cells (187) and rat cardiomyocytes (132). Of these previous in vitro studies, only two groups (132, 187) have investigated the signalling pathways utilized by periostin to induce cellular proliferation. These studies have demonstrated that periostin-induced human retinal pigment epithelial cell and rat cardiomyocyte proliferation was dependent on phosphatidylinositide 3-kinases (PI3K)/Akt activation. However, there have been no studies in the literature investigating the effects of periostin on hypertrophic scar-derived fibroblast proliferation or the signalling intermediates activated by it, nor have these pathways been assessed in fibroblasts grown on physiologically relevant culture substrates. Since periostin has been demonstrated to induce proliferation in other cell types, it was hypothesized that periostin contributes to the excessive fibroblast proliferation characteristically observed in hypertrophic scarring.

The aim of this chapter is to describe the effects of periostin on hypertrophic scar (HTS)- and normal skin (NS)-derived fibroblast proliferation and to identify potential signalling pathways utilized by periostin to elicit these effects.

3.2 Assessment of POSTN expression and periostin levels in fibroblasts derived from hypertrophic scar tissue

Previous in situ hybridization studies performed in our laboratory demonstrated that POSTN expression was upregulated and persistent in keratinocytes in the basal stratum
and in dermal fibroblasts in hypertrophic scar tissue relative to normal scar tissue (96), consistent with a previous report (99). To confirm the increased expression of POSTN in primary HTS-derived fibroblasts relative to fibroblasts derived from NS, real time PCR for POSTN expression was performed as described in 2.2.7.2. POSTN expression was increased approximately six fold in primary fibroblasts derived from HTS tissue relative to NS tissue (Figure 3.1a, N=1 patient, n=1). These findings were consistent with our previous in vivo studies (96) and previously published data (99).

In parallel, western immunoblotting studies were performed to assess the levels of periostin in primary fibroblasts derived from HTS and NS tissue. Alternative splicing in the C-terminal region of POSTN results in periostin isoforms that range in size from 83-93 kDa (115). Western immunoblotting of total cell lysates with a periostin-specific antibody revealed two bands approximately 85 and 90 kDa in size. Densitometry analysis of both bands revealed that periostin levels were increased approximately five fold in primary fibroblasts derived from HTS tissue relative to NS tissue (Figure 3.1b, N=2 patient, n=1). These findings are consistent with previous immunohistochemistry studies performed by our group (95, 96) and others (97) that demonstrated that periostin was abnormally abundant in the dermis of hypertrophic and keloid scar tissue.

3.3 Identification of POSTN variants in fibroblasts derived from hypertrophic scar tissue

Up to eight human POSTN variants have been reported in various systems to date (101, 102, 116, 117, 188-190). To determine which POSTN variants were present in abnormal scar tissue, total RNA was extracted from HTS fibroblasts and amplified by reverse-transcription PCR. Forward and reverse primers designed to anneal to regions outside of the variable region in the C-terminal domain, as described in 2.2.7.3 (Figure 3.2a), revealed four amplicons (variants 1-4) representing four human POSTN variants originally reported by Horiuchi et al., (101) in human placental and osteosarcoma cDNA libraries (Figure 3.2b, N=2 patients, n=1). The predicted amplicon sizes were 1120 base pairs (bp) for variant 1, 946 bp for variant 2, 955 bp for variant 3 and 865 bp for variant 4. The identity of each POSTN variant was confirmed by DNA sequencing. Although not
Figure 3.1 POSTN expression and periostin levels are abundant in primary fibroblasts derived from hypertrophic scar tissue. Primary hypertrophic scar (HTS)- and normal skin (NS)-derived fibroblasts were cultured on two-dimensional collagen substrates for 72 hours in 10% FBS-αMEM. (a) POSTN expression was assessed in total RNA by real time PCR as described in 2.2.7.2. As shown, POSTN expression was increased approximately five to six fold in HTS fibroblasts relative to NS fibroblasts. Expression data are normalized to NS (N=1 patient, n=1). (b) Periostin levels were assessed in total cell lysates by western immunoblotting and densitometry as described in 2.2.6. Periostin levels were increased five to six fold in HTS fibroblasts relative to NS fibroblasts. Values were normalized to NS patient 11 (N=2 patients, n=1). β-actin immunoreactivity was assessed to ensure equal protein loading. Human recombinant periostin (1 µg/ml, hrPN) was used as a positive control. Data are represented by mean values and SEM. RQ= relative quantification, AU= arbitrary units.
Figure 3.2 *POSTN* variant 1 is the most abundant transcript in hypertrophic scar fibroblasts. (a) Schematic representation of *POSTN* variants 1–4 and location of PCR primers within the cDNA (red arrows). Breaks in black bars represent deleted exon cassettes through alternative splicing. (b) Primary hypertrophic scar (HTS)-derived fibroblasts were cultured on two-dimensional collagen substrates for 72 hours in 10% FBS-αMEM. *POSTN* variants were assessed by reverse-transcription PCR of total RNA using primers designed to amplify the alternatively spliced C-terminal region. The identity of the largest MW amplicon was confirmed as isoform 1 by DNA sequencing (N=2 patients, n=1). MW= molecular weight ladder, bp= base pairs.
quantitative, these PCR analyses were performed using a single primer set and the relative intensity of each amplicon was predicted to approximate the amount of each variant in the sample. Based on this assumption, POSTN variant 1 was identified as the most abundant POSTN variant in hypertrophic scars. Western immunoblotting studies confirmed the most abundant periostin isoform in HTS cells was the same molecular weight as human recombinant periostin variant 1 (Figure 3.1b, N=2 patients, n=1).

3.4 Assessment of the basal proliferation rates of hypertrophic scar- and normal skin-derived fibroblasts in 2D collagen cultures

The “basal” (unstimulated) proliferation rates of primary fibroblasts derived from HTS and NS tissue were assessed on type I collagen substrates using the water soluble tetrazolium-1 (WST-1) proliferation assay as described in 2.2.4. Primary fibroblasts were seeded onto a thin two-dimensional (2D) type I collagen substrate in 2% FBS-αMEM and proliferation was assessed over a seven day period. As shown in Figure 3.3a, no significant differences between the basal proliferation rates of HTS and NS fibroblasts were observed over the course of the assay (N=3 patients, n=3).

3.5 Assessment of the effects of periostin on hypertrophic scar and normal skin fibroblast proliferation in 2D collagen cultures

To determine the effects of periostin on fibroblast proliferation, primary HTS and NS fibroblasts were seeded onto a type I collagen substrate containing 2 μg/ml human recombinant periostin or vehicle (PBS with 0.1% BSA) and proliferation was assessed over a seven day period using the WST-1 proliferation assay (2.2.4). A significant interaction between periostin treatment and time was observed (p<0.05) and the presence of periostin in the culture substrate enhanced HTS fibroblast proliferation over a seven day period relative to vehicle controls with significant differences evident at day five and seven (Figure 3.3b, p<0.05, N=3 patients, n=3). In contrast, exogenous periostin treatment had no discernible effect on the proliferation of normal skin fibroblasts under identical culture conditions (Figure 3.3c, N=3 patients, n=3).
Figure 3.3 Periostin enhances the proliferation of hypertrophic scar-derived fibroblasts. Primary hypertrophic scar (HTS) and normal skin (NS)-derived fibroblast proliferation was assessed using the WST-1 proliferation assay described in 2.2.4. Primary fibroblasts were seeded onto a two-dimensional type 1 collagen substrate containing periostin (PN, 2µg/ml) or vehicle (veh, 0.1% BSA in PBS) and proliferation was assessed over seven days. (a) No significant differences between the basal proliferation rates of HTS and NS fibroblasts were observed (N=3 patients each, n=3). (b) Incorporation of exogenous periostin into two-dimensional collagen lattices significantly enhanced HTS fibroblast proliferation relative to vehicle controls over a seven day period (N=3 patients, n=3). (c) Exogenous periostin treatment had no discernible effect on NS fibroblast proliferation under identical culture conditions (N=3 patients, n=3). Significant treatment/time interactions are denoted by #p<0.05 (ANOVA of repeated measures). Significant treatment effects at individual time points are denoted as *p<0.05 (simple main effects analyses). Data are presented by mean difference scores relative to day one absorbance readings and SEM.
3.6 Identification of the signalling pathway(s) utilized by periostin to induce hypertrophic scar fibroblast proliferation in 2D collagen cultures

A central aim of this chapter was to identify the signalling intermediates utilized by periostin to induce the proliferation of HTS fibroblasts. To achieve this, a series of pharmacological inhibitors were assessed. These inhibitors were targeted against signalling intermediates previously shown to be activated by periostin or other mitogens.

3.6.1 Assessment of PI3K inhibition effects on periostin-induced HTS fibroblast proliferation.

LY294002 is a small molecule inhibitor that competes with the ATP binding site in the catalytic domain of PI3K. To determine if periostin signalling required PI3K activity to enhance proliferation, LY294002 and periostin (2 µg/ml) were included in a subset of cultures of HTS fibroblasts and proliferation was assessed using WST-1 assays. 10 µM LY294002 was used in these experiments as this concentration has been demonstrated to inhibit human fibroblast proliferation in other systems (191-193). LY294002 has modest off-target effects at this concentration (194). As shown in Figure 3.4a, a significant interaction between LY294002 treatment and time was observed (p<0.05) and PI3K inhibition attenuated periostin-induced HTS fibroblast proliferation over seven days relative to vehicle controls with significant differences evident at day three and seven (p<0.01, N=3 patients, n=3). Similarly, a significant LY294002 treatment and time interaction was observed (p<0.05) and LY294002 treatment was found to attenuate basal HTS fibroblast proliferation over the course of the assay relative to vehicle controls with significant effects observed at day five and seven (Figure 3.4b, p<0.05, N=3 patients, n=3).

3.6.2 Assessment of Akt inhibition effects on periostin-induced HTS fibroblast proliferation

Akt inhibitor VIII is a small molecule inhibitor that binds to the pleckstrin homology domain of Akt and prevents activation by PI3K. To determine if periostin signalling required Akt activity to enhance HTS fibroblast proliferation, Akt inhibitor VIII and
Figure 3.4 Inhibition of phosphatidylinositol 3-kinases attenuates periostin-induced and basal hypertrophic scar fibroblast proliferation. Primary hypertrophic scar (HTS)-derived fibroblasts were cultured onto two-dimensional collagen substrates containing periostin (PN, 2 µg/ml) or vehicle (veh). Culture medium was supplemented with 10 µM LY294002 (PI3Ki) or DMSO control and proliferation was assessed in WST-1 assays. As shown in (a), PI3K inhibition significantly attenuated PN-induced HTS fibroblast proliferation relative to vehicle controls (N=3 patients, n=3). (b) PI3K inhibition significantly attenuated basal proliferation over a seven day period relative to vehicle controls (N=3 patients, n=3). Significant treatment/time interactions are denoted by #p<0.05 (ANOVA of repeated measures). Significant treatment effects at individual time points are denoted by *p<0.05, **p<0.01 and ***p<0.001 (simple main effects analyses). Data are presented by mean difference scores relative to day one absorbance readings and SEM.
Figure 3.5 Inhibition of Akt attenuates periostin-induced hypertrophic scar fibroblast proliferation. Primary hypertrophic scar (HTS)-derived fibroblasts were cultured on two-dimensional collagen substrates containing periostin (PN, 2 µg/ml) or vehicle (veh). Culture medium was supplemented with 0.5 µM Akt inhibitor VIII (Akti) or DMSO and proliferation was assessed in WST-1 assays. (a) Akt inhibition significantly attenuated PN-induced HTS fibroblast proliferation over seven days relative to vehicle controls (N=3 patients, n=3). (b) Akt inhibition had no discernible effects on basal HTS fibroblast proliferation over seven days relative to vehicle controls (N=3 patients, n=3). Significant treatment/time interactions are denoted by #p<0.05 (ANOVA of repeated measures). Significant treatment effects at individual time points are denoted by *p<0.05 (simple main effects analyses). Data are presented by mean difference scores relative to day one absorbance readings and SEM. (c) Primary HTS fibroblasts were cultured on two-dimensional collagen cultures for 72 hours, washed overnight in serum-free medium and treated with 2 µg/ml PN or vehicle for 60 minutes in serum-free medium. Total cell lysates were assessed for phosphorylated (ser 473) and total Akt immunoreactivity by western immunoblotting as described in 2.2.6. Exogenous PN treatment had no effect on Akt activation (N=1 patient, n=3). β-actin immunoreactivity was assessed to ensure equal protein loading. Representative immunoblots are shown.
periostin (2 µg/ml) were included in a subset of cultures of HTS fibroblasts and proliferation was assessed using WST-1 assays. 0.5 µM Akt inhibitor VIII was used in these experiments based on previous studies investigating the effect of Akt inhibition on B-cell proliferation (195). At 1 µM, Akt inhibitor VIII does not significantly inhibit other kinases (194). A significant interaction between Akt inhibitor VIII treatment and time was observed (p<0.05) and Akt inhibition attenuated periostin-induced HTS fibroblast proliferation over the course of the assay relative to vehicle controls with significant differences evident at day seven (Figure 3.5a, p<0.05, N=3 patients, n=3). In contrast, Akt inhibition had no discernible effects on the proliferation of untreated HTS fibroblasts (Figure 3.5b, N=3 patients, n=3). In addition to WST-1 proliferation assays, western immunoblotting was performed to determine if exogenous periostin treatment induced the phosphorylation of Akt at serine 473. As shown in Figure 3.5c, addition of periostin had no discernible effects on the phosphorylation of serine 473 in HTS fibroblasts after treatment with periostin in media for 60 minutes relative to vehicle controls (N=1 patient, n=3).

3.6.3 Assessment of Glycogen synthase kinase - 3 beta (GSK-3β) inhibition effects on periostin-induced HTS fibroblast proliferation

GSK-3β inhibitor VIII is a small molecule inhibitor that competes with the ATP binding site in the catalytic domain of GSK-3β. To determine if periostin signalling required GSK-3β activity to promote HTS fibroblast proliferation, GSK-3β inhibitor VIII and periostin (2 µg/ml) were included in a subset of cultures of HTS fibroblasts and proliferation was assessed using WST-1 assays. 1 µM GSK-3β inhibitor VIII was used in these experiments as this concentration has been demonstrated to enhance prostate cancer cell proliferation (196). GSK-3β inhibitor VIII has no discernible off-target effects at concentrations up to 10 µM (194). GSK-3β inhibition had no discernible effects on periostin-induced or basal HTS fibroblasts proliferation relative to vehicle controls (Figure 3.6a and b, N=3 patients, n=3). In addition to WST-1 proliferation assays, western immunoblotting studies were performed to determine if exogenous periostin treatment increased β-catenin levels or the phosphorylation of GSK-3β at serine 9. As shown in Figure 3.6c, addition of periostin did not enhance β-catenin levels or induce
Primary hypertrophic scar (HTS)-derived fibroblasts were cultured onto two-dimensional collagen substrates containing periostin (PN, 2 µg/ml) or vehicle (veh). Culture medium was supplemented with 1 µM GSK-3β inhibitor VIII (GSK-3βi) or DMSO and proliferation was assessed in WST-1 assays. GSK-3β inhibition had no discernible effect on (a) PN-induced or (b) basal HTS fibroblast proliferation relative to vehicle controls (N=3 patients, n=3). Data are presented by mean difference scores relative to day one absorbance readings and SEM. (c) Primary HTS fibroblasts were cultured on two-dimensional collagen cultures for 72 hours, washed overnight in serum-free medium and treated with 2 µg/ml PN or vehicle for 60 minutes in serum-free medium. Total cell lysates were extracted and assessed for β-catenin and total and phosphorylated (ser 9) GSK-3β immunoreactivity by western immunoblotting as described in 2.2.6. Exogenous PN treatment did not affect β-catenin levels or induce GSK-3β phosphorylation relative to vehicle controls (N=1 patient, n=3). (d) Positive control demonstrating that 1 µM GSK-3β inhibitor VIII was effective in inhibiting GSK-3β activity by increasing β-catenin levels. β-actin immunoreactivity was assessed to ensure equal protein loading. Representation immunoblots are shown.
GSK-3β phosphorylation at serine 9 in HTS fibroblasts after exposure to periostin in media for 60 minutes relative to vehicle controls (N=1 patient, n=3).

3.6.4 Assessment of Rho kinase (ROCK) inhibition effects on periostin-induced HTS fibroblast proliferation.

Y27632 is a small molecule inhibitor that competes with the ATP binding site in the catalytic domain of ROCK. To determine if periostin signalling required ROCK activity to enhance HTS fibroblast proliferation, Y27632 and periostin (2 µg/ml) were included in a subset of cultures of HTS fibroblasts and proliferation was assessed using WST-1 assays. 10 µM Y27632 was used in these experiments which has been demonstrated to inhibit NIH 3T3 and human gingival fibroblast proliferation (197, 198). Y27632 has modest off-target effects at this concentration (194). A significant interaction between Y27632 treatment and time was observed (p<0.05) and ROCK inhibition significantly attenuated periostin-induced HTS fibroblast proliferation over seven days relative to vehicle controls with a strong trend (p=0.051) towards decreased proliferation evident at day seven (Figure 3.7a, N=3 patients, n=3). In contrast, ROCK inhibition had no discernible effects on the basal proliferation of HTS fibroblasts cultured under identical conditions (Figure 3.7b, N=3 patients, n=3). In parallel, western immunoblotting was performed to determine if exogenous periostin treatment induced the phosphorylation of myosin light chain (MLC), an established downstream target of ROCK. MLC phosphorylation at serine 19 was not detected under these culture conditions (data not shown).

3.6.5 Extracellular signal-regulated kinase (ERK) inhibition effects on periostin-induced HTS fibroblast proliferation

ROCK has previously been shown to enhance proliferation through ERK (197-201). Therefore, the effects of ERK inhibition were assessed on periostin-induced HTS fibroblast proliferation. PD98059 is a small molecule inhibitor that prevents binding and activation of ERK1/2 by the ERK specific mitogen-activated protein kinase, MEK. To determine if periostin signalling required ERK1/2 activity to promote HTS fibroblast proliferation, PD98059 and periostin (2 µg/ml) were included in a subset of cultures of
Figure 3.7 Rho kinase inhibition attenuates periostin-induced hypertrophic scar fibroblast proliferation. Primary hypertrophic scar (HTS)-derived fibroblasts were cultured onto two-dimensional collagen substrates containing periostin (PN, 2 µg/ml) or vehicle (veh). Culture medium was supplemented with 10 µM Y27632 (ROCKi) or vehicle and proliferation was assessed in WST-1 assays. (a) ROCK inhibition significantly attenuated PN-induced HTS fibroblast proliferation relative to vehicle controls over the course of the assay (N=3 patients, n=3). (b) In contrast, ROCK inhibition had no discernible effect on basal HTS fibroblast proliferation (N=3 patients, n=3). Significant treatment/time interactions are denoted by #p<0.05 (ANOVA of repeated measures). Data are presented by mean difference scores relative to day one absorbance readings and SEM.
Figure 3.8 Extracellular signal-regulated kinase inhibition had no effect on basal or periostin-induced hypertrophic scar fibroblast proliferation. Primary hypertrophic scar (HTS)-derived fibroblasts were cultured on two-dimensional collagen substrates containing periostin (PN, 2 µg/ml) or vehicle (veh). Culture medium was supplemented with 20 µM PD98059 (ERKi) or DMSO and proliferation was assessed in WST-1 assays. ERK inhibition had no discernible effect on (a) PN-induced or (b) basal HTS fibroblast proliferation over the course of the assay relative to vehicle controls (N=3 patients, n=3). Data are presented by mean difference scores relative to day one absorbance readings and SEM. (c) Primary HTS fibroblasts were cultured on two-dimensional collagen cultures for 72 hours, washed overnight in serum-free medium and treated with 2 µg/ml PN or vehicle for 60 minutes in serum-free medium. Total cell lysates were assessed for total and phosphorylated (thr 202/tyr 204, thr185/tyr187) ERK immunoreactivity by western immunoblotting as described in 2.2.6. Exogenous PN treatment did not enhance ERK phosphorylation relative to vehicle controls (N=1 patient, n=3). (d) Positive control demonstrating that 20 µM ERK inhibitor, PD98059, was effective at inhibiting TGFβ-1-induced ERK1/2 phosphorylation. β-actin immunoreactivity was assessed to ensure equal protein loading. Representative immunoblots are shown.
HTS fibroblasts and proliferation was assessed using WST-1 assays. 20 µM PD98059 was used in these experiments based on previous studies investigating the effects of ERK inhibition on human cardiac and dermal fibroblast proliferation (195). At 50 µM, PD98059 has no discernible off-target effects (202). Inhibition of ERK activity had no discernible effects on periostin-induced or basal HTS fibroblast proliferation relative to vehicle controls (Figure 3.8a and b, N=3 patients, n=3). In parallel, western immunoblotting studies were performed to determine if exogenous periostin treatment induced the phosphorylation of ERK1/2. As shown in Figure 3.8c, periostin treatment did not increase ERK1/2 phosphorylation relative to vehicle controls after exposure to periostin in media for 60 minutes (N=1 patient, n=3).

3.7 Discussion

A central hypothesis of this thesis was that periostin contributes to excessive fibroblast proliferation in hypertrophic scarring. The in vitro studies presented in this chapter demonstrate that exogenous periostin treatment was able to enhance the proliferation of fibroblasts derived from abnormal scar tissue, supporting the central hypothesis. Periostin appears to elicit this effect in HTS fibroblasts through Akt and ROCK dependent pathways. In contrast, exogenous periostin had no discernible effects on the proliferation of NS fibroblasts cultured under identical conditions on type I collagen substrates.

One of the molecular features of hypertrophic scarring is the increased expression of POSTN and the abnormal abundance and persistence of its protein product, periostin. Real time PCR and western immunoblotting studies indicated that POSTN expression and periostin levels were increased in primary fibroblasts derived from HTS tissue relative to NS tissue in in vitro cultures. These data are consistent with previous in vivo studies performed by our laboratory (95, 96) and by other groups (97, 99) which demonstrated that POSTN expression is markedly increased in the basal epithelium and in dermal fibroblasts, and that periostin is abnormally abundant in the dermis of hypertrophic scar tissue relative to normal scar tissue. These findings indicate that POSTN expression and periostin levels in primary fibroblasts derived from NS and HTS tissues reflect the tissues from whence they were derived and these cells are therefore likely to be representative in vitro models of scarring.
As discussed in section 1.6.1, *POSTN* is alternatively spliced and up to eight human *POSTN* variants have been reported (101, 102, 116, 117, 188-190). The expression and function of these variants is currently unclear and the identity of specific *POSTN* variants expressed in hypertrophic scarring has not been previously reported. With reverse-transcription PCR, four amplicons were identified in total RNA extracted from HTS fibroblasts, and these amplicons corresponded to four of the *POSTN* variants previously identified (101). The largest and most abundant of these amplicons was identified as *POSTN* variant 1 by DNA sequencing. In addition, three additional splice variants (2, 3 and 4) were detected in HTS fibroblasts and sequenced. To our knowledge, this is the first study to identify different *POSTN* variants in hypertrophic scarring. Comparisons between the immunoreactivity of recombinant periostin and the endogenously produced periostin in human hypertrophic scar tissue lysates revealed that 2 µg/ml of recombinant periostin approximated the levels of periostin in hypertrophic scars *in vivo* (91, 96). Based on these findings, the effects of treating HTS and NS fibroblasts with recombinant periostin isoform 1 at 2 µg/ml were investigated in the remainder of the thesis.

A small number of *in vitro* studies have assessed the effects of periostin on proliferation in cells cultured on substrates that mimic the microenvironment (91, 203). However, the majority of *in vitro* studies (132, 152, 178-180, 187) have assessed periostin effects on proliferation in cells cultured on rigid tissue culture plastic. Tissue culture plastic provides an extremely stiff substrate that greatly exceeds the stiffness of the ECM of contracting or fibrotic tissues *in vivo* (169, 170) and invariably leads to robust myofibroblast differentiation in fibroblast cultures (31). Therefore, culturing primary fibroblasts on plastic substrates may provide misleading information derived from the differences in fibroblast and myofibroblast sensitivities to treatments. For this reason, the effects of periostin on HTS fibroblast proliferation was assessed on type I collagen substrates, the most abundant collagen in normal skin and mature scar tissue (8). As a matricellular protein, periostin serves as a link between cells and their collagenous ECM, and it can modify cellular behavior in response to external stimuli, including changes in ECM stiffness (90). Using collagen substrates, periostin can be incorporated into a collagenous ECM matrix to mimic its localization *in vivo* and provide a model system to assess the effects of ECM-associated periostin on HTS fibroblast proliferation under
physiological relevant conditions. Compliant 2D collagen gels have a Young’s modulus of 300-400 Pa, which is equivalent to the ECM stiffness found in the provisional matrix in vivo (31, 173). Since fibroblasts are recruited to a similarly compliant provisional matrix during cutaneous wound healing (8), 2D collagen substrates provide a more physiologically representative in vitro model.

Fibroblast proliferation was assessed using the WST-1 proliferation assay, which measures cellular mitochondrial activity as an indicator of net cellular proliferation (the combined effects of growth and apoptosis). While this approach provides an indirect measure of proliferation, it is an optimal approach in this context because it avoids the need to disrupt cellular interactions with periostin embedded into the collagen substrate. Optimization studies in our laboratory have demonstrated a linear correlation between manual cell counts and WST-1 absorbance over a range of 0.03 to 0.4 absorbance units at 450 nm with every 0.038 nm correlating to approximately 1000 cells (data not shown). In addition, WST-1 absorbance values plateau in HTS fibroblasts cultured in the presence of mitomycin C, an established inhibitor of cellular proliferation (204), on 2D collagen substrates (data not shown). These findings indicate that this assay is a representative measure of cellular proliferation under these culture conditions.

Periostin has been demonstrated to induce the proliferation of various cell types in other culture systems (91, 132, 152, 178-180, 187). The data presented in this chapter indicates that exogenous ECM-associated periostin significantly enhances HTS fibroblast proliferation on 2D compliant collagen cultures. Based on these findings and the accumulation of periostin in the dermis of excisional mouse wounds as early as three days post wounding (98, 151), periostin may act as a growth factor during the initial stages of wound healing to increase fibroblast numbers at the wound site. Fibroblast proliferation in the provisional matrix and newly formed granulation tissue is vital for the regeneration of a functional dermis (8). Therefore, the persistent and abundant levels of periostin observed in the dermis of hypertrophic scars may continue to promote excessive fibroblast proliferation after wound closure. Excessive fibroblast proliferation at the wound site would be predicted to enhance the dermal density of the wound to abnormal
levels and promote excessive myofibroblast differentiation, thereby enhancing hypertrophic scar formation.

In contrast to HTS fibroblasts, exogenous periostin treatment had no effect on the proliferation of NS fibroblasts. The mechanisms that explain these differences in sensitivity to periostin between HTS and NS fibroblasts have yet to be elucidated. One possibility is that a combination of fibrosis-associated growth factors is required to “activate” NS fibroblasts to induce periostin sensitivity in these cells. This activation may include the expression of a receptor(s) or other interacting molecules not normally present in unwounded NS fibroblasts, or activation of a sequential or parallel pathway(s) required for periostin signalling. These possibilities are further addressed in Chapter 4.6. As NS fibroblasts appear relatively insensitive to ECM-associated periostin under these culture conditions, that may suggest that periostin promotes pathological, rather than normal, fibroblast proliferation. It remains unclear if periostin-induced fibroblast proliferation is a normal or abnormal component of cutaneous wound healing in humans, as normal scar tissue samples could not be obtained for primary cell derivation due to ethical constraints. Collectively, these studies indicate that periostin specifically enhances the growth of abnormal scar-derived fibroblasts cultured on 2D collagen substrates. Therefore, periostin may be an attractive therapeutic target for therapies designed to inhibit excessive fibroblast proliferation characteristically observed in hypertrophic scarring.

The second component of this chapter was to identify the signalling intermediates utilized by periostin to promote HTS fibroblast proliferation on 2D collagen cultures. As periostin has been demonstrated to signal through the PI3K/Akt pathway to elicit tumour growth (129-131) and proliferation of several cell types in vitro (132, 187), initial studies focused on these intermediates. PI3K inhibition was shown to significantly attenuate both basal and periostin-induced HTS fibroblast proliferation. These data were interpreted to indicate that PI3K inhibition attenuates multiple pathways involved in cellular proliferation and is therefore unlikely to be helpful for distinguishing the specific effects of periostin from other signalling pathways that also induce proliferation in a PI3K
dependent manner. Therefore, the effects of Akt inhibition, an established downstream target of PI3K (205), were assessed on periostin-induced HTS fibroblast proliferation.

Akt inhibition significantly attenuated periostin-induced HTS fibroblast proliferation while having no discernible effects on basal proliferation. These data are consistent with previous reports demonstrating that periostin promoted the proliferation of rat cardiomyocytes and retinal pigment epithelial cells through a PI3K/Akt dependent pathway (132, 187). Although significant, the effects of Akt inhibition on periostin-induced HTS fibroblast proliferation were quite modest and proliferation was attenuated by approximately 19% over seven days relative to vehicle controls. The modest cumulative reduction in proliferation across seven days led to the conclusion that Akt was not the major signalling pathway intermediate utilized for periostin-induced HTS fibroblast proliferation under these culture conditions.

While Akt inhibition significantly attenuated periostin-induced HTS fibroblast proliferation using WST-1 assays, no evidence of periostin-induced Akt activation was observed by western immunoblotting. These findings are inconsistent with previous reports demonstrating that periostin enhanced Akt activation in other systems (130, 152, 187). The contrasting findings between the WST-1 and western immunoblotting studies may be explained by the technical limitations of assessing matricellular interactions by western immunoblotting. Unlike the WST-1 assays, where periostin was incorporated into a collagen-rich matrix where it was constantly available to stimulate proliferation, it was necessary to add periostin directly to the culture medium to assay for rapid changes in Akt phosphorylation by western immunoblotting. Fibroblast attachment and spreading onto collagen substrates after seeding takes several hours. If periostin was incorporated into the collagen substrates prior to seeding, HTS fibroblasts would be exposed to periostin throughout the attachment and spreading process. Once these fibroblasts had achieved normal morphology, any effects of periostin on Akt activation may no longer be detectable, as Akt phosphorylation is typically rapid and transient. Therefore, periostin was added to the culture medium after the fibroblasts achieved normal morphology (130, 152, 187) in order to assess its effects on Akt activation. As it is a matricellular protein, periostin interacts with cells while attached to the surrounding ECM and modifies cellular
signalling and phenotype in response to changes in the microenvironment (90). It is plausible that soluble periostin (added to the culture medium) is functionally distinct from ECM-associated periostin and this may explain why no changes in kinase phosphorylation were detected.

To identify additional signalling pathway(s) utilized by periostin to induce HTS fibroblast proliferation, a series of pharmacological inhibitors targeted against GSK-3β, ROCK and ERK1/2, each of which are established signalling intermediates in cellular proliferation (201, 206, 207), were assessed. These studies demonstrated that inhibition of GSK-3β and ERK had no discernible effects on basal or periostin-induced HTS fibroblast proliferation in 2D collagen cultures, whereas ROCK inhibition significantly attenuated periostin-induced HTS fibroblast proliferation.

In addition to its well established roles in mediating stress fiber formation, focal adhesion formation and contraction of myofibroblasts (49-53, 208, 209), ROCK has also been shown to mediate proliferation in corneal epithelial cells (200, 210), neuroblastoma cells (211), hepatic stellate cells (201) and NIH 3T3 mouse fibroblasts (197). The ROCK specific inhibitor, Y27632, used for the studies in Chapter 3 had no effect on basal levels of proliferation relative to vehicle treated HTS fibroblasts, consistent with previous reports in corneal epithelial cells and neuroblastoma cells (210, 211). These data demonstrate that ROCK signalling is not essential for basal HTS fibroblast proliferation. However, ROCK inhibition significantly attenuated periostin-induced HTS fibroblast proliferation and this effect was more pronounced than that observed with Akt inhibition (37% decrease vs 19% decrease in proliferation over seven days). These data indicate that periostin also promotes HTS fibroblast proliferation in 2D collagen cultures through a ROCK dependent pathway. To our knowledge, this is the first study to identify ROCK as a signalling intermediate in periostin-induced proliferation in any model system.

Previous studies have demonstrated that ROCK induces the expression of cell cycle progression proteins, including cyclin D1, through a Ras/ERK mediated pathway (197-201). However, ERK inhibition had no discernible effect on periostin-induced HTS fibroblast proliferation. Parallel studies of CCND1 expression, encoding cyclin D1, were
performed by culturing HTS fibroblasts in serum free media for 72 hours in order to
synchronize the fibroblasts in G₀ prior to stimulation with serum and soluble periostin.
No effects on CCND1 expression were evident in periostin treated samples relative to
serum controls after 3 hours (data not shown). As described for the western
immunoblotting assays for Akt activity, periostin was not incorporated in the collagen
substrates in these experiments, as it was necessary to synchronize the cells in G₀ prior to
periostin stimulation. HTS fibroblasts secrete high levels of endogenous periostin, so it is
very likely that these cells were exposed to periostin during the synchronization process
prior to treatment, making these experiments difficult to interpret. It is currently unclear
if soluble and ECM-associated periostin have differential effects on the behavior of HTS
fibroblasts. Exposure to periostin during the synchronization process and treatment with
soluble periostin may explain why CCND1 expression was not altered in these
experiments. The downstream mechanisms involved in ROCK-dependent periostin-
induced HTS fibroblast proliferation will be a focus of future studies.

Previous studies have reported conflicting data in regards to the regulation of ROCK
activity by PI3K activation (130, 212, 213), and this interaction may be dependent on
particular cell type and/or specific treatment conditions (214). As PI3K inhibition
significantly attenuated HTS fibroblast proliferation, it is conceivable that PI3K
activation may be involved in periostin-induced ROCK activation in 2D collagen
cultures. Future studies, outside the scope of this thesis, will investigate whether PI3K or
a parallel pathway activates ROCK to promote periostin-induced HTS fibroblast
proliferation in 2D collagen cultures.

In summary, periostin enhances HTS fibroblast proliferation in 2D collagen cultures that
mimic the mechanical tension of the provisional matrix in the early stages of cutaneous
wound healing in vivo (31, 173). As a hallmark of abnormal scarring, excessive fibroblast
proliferation increases cell density and ECM deposition in the wound thereby increasing
the number of cells available for myofibroblast differentiation and promoting abnormal
dermal density. These studies demonstrate that periostin promotes HTS fibroblast
proliferation through an Akt dependent pathway, consistent with previous reports (132,
187). Furthermore, periostin was shown for the first time to enhance HTS fibroblast
proliferation through a ROCK dependent pathway. As ROCK is better known as an essential component of myofibroblast differentiation and contraction (30), and since myofibroblasts are the cellular cause of excessive contraction and remodelling of the dermis during scar formation, the remainder of this thesis focuses on the roles of periostin on myofibroblast differentiation and persistence.
Chapter 4

4  *In vitro* analysis of the effects of periostin on myofibroblast differentiation and persistence in hypertrophic scarring

4.1 Rationale

The myofibroblast is a major cellular contributor in both normal cutaneous wound healing and abnormal scar formation (27, 28, 56-58). Myofibroblasts combine the contractile features of smooth muscle cells with the extensive ECM production of fibroblasts, thereby allowing them to deposit, contract and remodel granulation tissue (29, 31). In normal cutaneous wound healing, myofibroblast numbers increase during the proliferation phase and gradually undergo apoptosis in the remodelling phase. However, in abnormal scarring conditions, myofibroblast populations are maintained at high levels (56, 57, 92-94) due, at least in part, to a prolonged proliferation phase (59). An abnormal excess of myofibroblasts results in an “overhealing” phenomenon characterized by excessive collagen production and contracture of the ECM (56, 57, 92-94). The molecules that promote excessive myofibroblast differentiation and their persistence during hypertrophic scar formation are unclear.

Previous studies have demonstrated that periostin induces myofibroblast differentiation in animal models of normal cutaneous wound repair (133) and in primary human palmar fascia fibroblasts (91). In addition, changes in periostin levels over time have been demonstrated to parallel αSMA levels in murine models of cutaneous wound healing (98) suggesting a role for periostin in myofibroblast differentiation. These studies led to the hypothesis that periostin promotes excessive myofibroblast differentiation and persistence in hypertrophic scars.
4.2 Assessment of the basal contractility of hypertrophic scar- and normal skin-derived fibroblasts in stressed fibroblast populated collagen lattices (sFPCL)

The basal contractility of primary fibroblasts derived from HTS and NS tissue was assessed and compared using sFPCL contraction assays as described in 2.2.5. In brief, primary fibroblasts were seeded into 3D collagen lattices and maintained in culture for 72 hours in 2% FBS-αMEM to promote their tension-mediated differentiation into myofibroblasts, modelling their differentiation during wound closure in vivo \( (30, 183) \). After 72 hours, the collagen lattices were released from the surrounding well and lattice contraction was measured over a 24 hour period. As shown in Figure 4.1a, HTS fibroblasts were significantly more contractile than NS fibroblasts \( (p<0.01) \) with significant increases in contraction observed immediately after release (designated as 0 hr), 6 hours and 24 hours after release \( (p<0.05, \text{N=6 patients, n=3}) \). Similarly, fibroblasts derived from keloid scar (KS) tissue, an abnormal scarring condition with similarities to hypertrophic scarring including increased periostin levels \( (58, 59, 62, 97, 215) \), displayed a non-significant trend \( (p=0.118) \) towards increased contractility relative to NS fibroblasts (Figure 4.1b, N=3 patients, n=3).

4.3 Assessment of the effects of periostin on the contractility of HTS and NS fibroblasts in sFPCLs

To assess the effects of increased periostin levels in the dermis of hypertrophic scar tissue on collagen lattice contraction, primary fibroblasts were seeded in sFPCLs with 2 μg/ml human recombinant periostin or vehicle incorporated into the collagen lattice. A significant interaction between periostin treatment and time was observed in HTS fibroblasts \( (p<0.01) \) and periostin enhanced HTS fibroblast contractility over 24 hours relative to vehicle controls with significant differences evident at all time points assessed (Figure 4.2a, \( p<0.05, \text{N=8 patients, n=3} \)). Periostin also significantly increased the contractility of KS fibroblasts above basal levels \( (p<0.05) \) with significant differences evident at lattice release (Figure 4.2b, \( p<0.05, \text{N=3 patients, n=3} \)). In contrast, a periostin-enriched matrix had no discernible effects on the contractility of NS fibroblasts relative to basal levels (Figure 4.2c, N=6 patients, n=3).
Figure 4.1 Hypertrophic scar-derived fibroblasts have enhanced contractility relative to normal skin-derived fibroblasts. To assess the basal contractility of primary fibroblasts derived from hypertrophic scar (HTS), keloid scar (KS) and normal skin (NS) tissue, stressed fibroblast populated collagen lattices were used as described in 2.2.5. Primary fibroblasts were cultured within three-dimensional, tethered collagen lattices for 72 hours to induce myofibroblast differentiation. After 72 hours, collagen lattices were mechanically released from the surrounding wells allowing for myofibroblast-mediated contraction of the free floating collagen lattices. Area measurements were taken over a 24 hour period following release. (a) HTS fibroblasts significantly contracted collagen lattices to a greater extent than NS fibroblasts (N=6 patients, n=3). (b) A non-significant trend (p=0.118) towards enhanced collagen lattice contraction was observed in KS-derived fibroblasts relative to NS fibroblasts (N=3 patients, n=3). Significant overall treatment effects are denoted by ^^p<0.01 (ANOVA of repeated measures). Significant treatment effects at individual time points are denoted by *p<0.05 and **p<0.01 (t-tests). Data are presented by mean values and SEM.
**Figure 4.2 Periostin enhances collagen lattice contraction in abnormal scar-derived fibroblasts.** To assess the effects of periostin (PN) on collagen lattice contraction, primary hypertrophic scar (HTS)-, keloid scar (KS)- and normal skin (NS)-derived fibroblasts were cultured in stressed fibroblast populated collagen lattices containing 2 µg/ml PN or vehicle (veh, 0.1% BSA in PBS). (a) Exogenous PN treatment significantly enhanced HTS fibroblast collagen lattice contraction over 24 hours relative to vehicle controls (N=8 patients, n=3). (b) Exogenous PN treatment also significantly enhanced KS fibroblast collagen lattice contraction relative to vehicle controls (N=3 patients, n=3). (c) Exogenous PN treatment had no discernible effect on NS fibroblast collagen lattice contraction (N=6 patients, n=3). Significant overall treatment effects are denoted by ^p<0.05 and significant treatment/time interactions are denoted by ##p<0.01 (ANOVA of repeated measures). Significant treatment effects at individual time points are denoted by *p<0.05 and **p<0.01 (t-tests and simple main effects analyses). Data are presented by mean values and SEM.
4.4 Identification of the mechanisms utilized by periostin to enhance HTS fibroblast contractility

These findings suggested that, unlike NS fibroblasts, HTS fibroblasts were sensitive to periostin in their ECM and responded to its presence by increasing collagen lattice contraction. Periostin could potentially induce collagen lattice contraction through several mechanisms. These include increasing fibroblast proliferation and/or viability in the collagen lattice, increasing fibroblast migration and adhesion within the lattice, or by enhancing myofibroblast differentiation. Each of these mechanisms was assessed in turn to identify the molecular mechanism(s) utilized by periostin to induce collagen lattice contraction.

4.4.1 Assessment of the effects of periostin on cell viability in sFPCLs

To determine the effects of periostin on HTS fibroblast viability in sFPCLs, cell membrane permeability and $BCL2/BAX$ mRNA ratio, independent markers of cell viability, were independently assessed using flow cytometry (2.2.8) and real time PCR (2.2.7), respectively. Exogenous periostin treatment had no discernible effect on HTS fibroblast membrane permeability in sFPCLs after 72 hours in culture (Figure 4.3a, N=4 patients, n=3) or $BCL2/BAX$ ratio after 48 hours in sFPCL culture relative to vehicle controls (Figure 4.3b, N=3 patients, n=3). Similarly, exogenous periostin treatment had no discernible effect on the $BCL2/BAX$ ratio in KS fibroblasts after 48 hours in sFPCL culture relative to vehicle controls (Figure 4.3c, N=3 patients, n=3).

4.4.2 Assessment of the effects of periostin on proliferation in sFPCLs

To determine if periostin induced HTS fibroblast proliferation in 3D cultures, $CCND1$ expression was measured by real time PCR 48 hours after culture in a sFPCL, with or without periostin treatment. In contrast to periostin-induced HTS fibroblast proliferation in compliant 2D cultures over a seven day period, periostin had no effect on $CCND1$ expression in HTS fibroblasts cultured under mechanical tension (Figure 4.4a, N=3 patients, n=3). Exogenous periostin treatment also had no discernible effect on $CCND1$
Figure 4.3 Periostin had no effect on hypertrophic scar fibroblast viability in stressed fibroblast populated collagen lattices. Primary hypertrophic scar (HTS) and keloid scar (KS) fibroblasts were cultured in stressed fibroblast populated collagen lattices (sFPCLs) containing periostin (PN, 2 µg/ml) or vehicle (veh). (a) The effects of PN on HTS fibroblast membrane permeability, an indicator of cellular viability, was assessed after 72 hours in sFPCL culture using flow cytometry as described in 2.2.8. PN had no discernible effect on HTS fibroblast viability relative to vehicle controls (N=4 patients, n=3). (b and c) The effects of PN on BCL2/BAX expression, a marker of apoptosis, were assessed in HTS and KS fibroblasts cultured in sFPCLs for 48 hours by real time PCR (2.2.7). PN had no effect on the BCL2/BAX ratio in (b) HTS or (c) KS fibroblasts relative to vehicle controls when cultured under mechanical tension. Values were normalized to vehicle controls (N=3 patients, n=3). Ultraviolet (UV) irradiation of HTS cells for 60 minutes was utilized as a positive control to induce cell death. Data are presented by mean values and SEM. RQ=relative quantification.
Figure 4.4 Periostin had no effect on hypertrophic scar fibroblast proliferation in stressed fibroblast populated collagen lattices. Primary hypertrophic scar (HTS) and keloid scar (KS) fibroblasts were cultured in stressed fibroblast populated collagen lattices (sFPCLs) containing periostin (PN, 2 µg/ml) or vehicle (veh) for 48 hours. *CCND1* expression, a marker of proliferation, was assessed in total RNA by real time PCR as described in 2.2.7. Exogenous periostin treatment did not affect *CCND1* expression in (a) HTS or (b) KS fibroblasts cultured under mechanical tension relative to vehicle controls. Values were normalized to vehicle controls (N=3 patients, n=3). Transforming growth factor (TGF)β-1 (5 ng/ml) was utilized as a control to inhibit *CCND1* expression. Data are presented by mean values and SEM. RQ=relative quantification.
expression in KS fibroblasts cultured under identical conditions (Figure 4.4b, N=3 patients, n=3).

4.4.3 Assessment of the effects of periostin on adhesion and 3D migration in rFPCLs

Relaxed FPCLs (rFPCLs), described in 2.2.5, were utilized to assess the effects of periostin on HTS fibroblast adhesion and migration in 3D collagen cultures. Fibroblasts cultured in rFPCLs are not subjected to mechanical tension and do not undergo culture-induced myofibroblast differentiation (183). Instead, lattice contraction is induced by tractional forces generated by cellular adhesion to the collagen and migration within the lattice (183, 216, 217). Periostin treatment had no discernible effect on HTS or KS fibroblast contractility in rFPCLs (Figure 4.5a and b, N=3 patients, n=3).

4.4.4 Assessment of the effects of periostin on myofibroblast differentiation in sFPCLs

To determine if periostin-induced HTS fibroblast contraction of sFPCLs was the result of enhanced myofibroblast differentiation, cell lysates were derived from sFPCLs immediately prior to collagen lattice release (after 72 hours of culture in the restrained lattice). αSMA and OB cadherin levels, established markers of myofibroblast differentiation (30, 218), were assessed by western immunoblotting and densitometry. Exogenous periostin treatment significantly increased αSMA levels in HTS fibroblasts relative to vehicle controls under identical culture conditions (Figure 4.6a, p<0.05, N=5 patients, n=3). A non-significant trend (p=0.066) towards increased OB cadherin levels was observed in periostin treated HTS fibroblasts relative to vehicle controls (Figure 4.6b, N=5 patients, n=3). Periostin had no discernible effects on αSMA or OB cadherin levels in KS fibroblasts relative to vehicle controls (Figure 4.6c and d, N=3 patients, n=3).
Figure 4.5 Periostin had no effect on hypertrophic scar fibroblast adhesion and migration in three dimensional collagen cultures. To assess the effects of exogenous periostin (PN) treatment on fibroblast adhesion and migration in three-dimensional (3D) collagen cultures, primary hypertrophic scar (HTS) and keloid scar (KS) fibroblasts were cultured in relaxed fibroblast populated collagen lattices containing PN (2 µg/ml) or vehicle (veh) as described in 2.2.5. Area measurements of the contracted collagen lattices were performed 24 hours after collagen lattice release. PN had no discernible effect on (a) HTS or (b) KS fibroblast migration in 3D collagen cultures (N=3 patients, n=3). Platelet-derived growth factor (PDGF) treatment (100 ng/ml) was utilized as a positive control to induce fibroblast migration based on previous studies (219). Data are presented by mean values and SEM.
Figure 4.6 Periostin enhances myofibroblast differentiation in hypertrophic scar-derived fibroblasts. Primary hypertrophic scar (HTS) and keloid scar (KS) fibroblasts were cultured in stressed fibroblast populated collagen lattices containing periostin (PN, 2µg/ml) or vehicle (veh) for 72 hours as described in 2.2.5. Total cell lysates were assessed for alpha smooth muscle actin (αSMA) and OB cadherin immunoreactivity, markers of myofibroblast differentiation, by western immunoblotting and densitometry, as described in 2.2.6. (a) Exogenous PN treatment significantly increased αSMA levels in HTS fibroblasts cultured in sFPCLs relative to vehicle controls (N=5 patients, n=3). (b) A non-significant trend (p=0.066) towards increased OB cadherin levels were observed in HTS fibroblast cultured in sFPCLs embedded with PN relative to vehicle controls (N=5 patients, n=3 patients). Exogenous PN treatment had no discernible effects on (c) αSMA and (d) OB cadherin levels in KS fibroblasts relative to vehicle controls. Values were normalized to vehicle controls. β-actin immunoreactivity was assessed to ensure equal protein loading. Significant treatment effects are denoted by *p<0.05 (t-tests). Data are presented by mean values and SEM. Representative immunoblots of the mean densitometry data are shown. AU=arbitrary units.
4.5 Assessment of the effects of periostin-depletion of HTS myofibroblast differentiation in sFPCLs

As shown in Figure 3.1b, HTS fibroblasts exhibit increased endogenous periostin levels relative to NS cells. The effects of exogenous periostin treatment on HTS fibroblasts described in the previous sections were therefore over and above the effects of endogenous periostin signalling. To obtain further support for the central hypothesis of this section, loss of function studies were performed in HTS fibroblasts by transducing them with an adenoviral vector containing a shRNA targeted against POSTN.

4.5.1 Assessment of POSTN expression and periostin levels in HTS fibroblasts following adenoviral transduction with shRNAs against POSTN

HTS fibroblasts were transduced with adenoviral vectors expressing either shRNAs against POSTN or scrambled control shRNAs. Transduction was at a multiplicity of infection (MOI) of 25 and the transduced fibroblasts were cultured on plastic tissue culture dishes for 72 hours in 2% FBS-αMEM. Transduction efficiency was then determined by assessing the percentage of cells expressing green fluorescent protein (GFP) by epifluorescence microscopy. As shown in Figure 4.7a and b, adenovirus transduction efficiency approximated 100% for all experiments assessed. Transduced HTS fibroblasts were then seeded into sFPCLs and POSTN and periostin levels were assessed after 72 hours in culture under 3D tension by real time PCR and western immunoblotting, respectively. As shown in Figure 4.7c and d, adenoviral transduction with shRNAs against POSTN significantly reduced POSTN expression by 92% and periostin levels by 60% relative to scrambled shRNA controls (p<0.001, N=4 patients, n=3).

4.5.2 Assessment of the effects of periostin depletion on HTS myofibroblast differentiation in sFPCLs

To assess the effects of periostin depletion on HTS myofibroblast differentiation in sFPCLs, HTS fibroblasts were transduced with adenovirus expressing shRNAs against POSTN or scrambled control shRNAs for 72 hours in 2% FBS-αMEM prior to seeding in
Figure 4.7 POSTN expression and periostin levels are significantly depleted in hypertrophic scar fibroblasts transduced with adenovirus expressing shRNAs against POSTN. Primary hypertrophic scar (HTS)-derived fibroblast were transduced with adenoviral vectors encoding shRNAs against POSTN or scrambled control shRNAs at a multiplicity of infection of 25. The adenoviral vector co-expresses green fluorescent protein, allowing transduction efficiency to be assessed by epifluorescence microscopy. (a) Comparisons of epifluorescence and (b) differential interference contrast microscopy indicate that transduction efficiency approximated 100% in all experiments. (c and d) Following adenoviral transduction, HTS fibroblasts were cultured in sFPCLs for 72 hours. (c) POSTN expression was assessed in total RNA by real time PCR as described in 2.2.7. Periostin depletion by shRNA treatment significantly decreased POSTN expression in HTS fibroblasts by 92% relative to scrambled controls (N=3 patients, n=3). (d) Total cell lysates were assessed for periostin immunoreactivity by western immunoblotting and densitometry as described in 2.2.6. Periostin depletion by shRNA treatment significantly decreased periostin levels by 60% relative to scrambled controls (N=4 patients, n=3). Values were normalized to scrambled shRNA controls. β-actin immunoreactivity was assessed to ensure equal protein loading. Significant treatment effects are denoted by ***p<0.001 (t-tests). Data are presented by mean values and SEM. Representative immunoblots of the mean densitometry data are shown. RQ=relative quantification, AU=arbitrary units.
sFPCLs. No significant effects on the ability of HTS fibroblasts to contract sFPCLs were observed in periostin-depleted HTS or KS fibroblasts relative to scrambled controls (Figure 4.8a and b, N=3 patients, n=3 for each). Periostin depletion by shRNA treatment had no discernible effect on αSMA levels in HTS fibroblasts relative to scrambled shRNA controls (Figure 4.9a, N=4 patients, n=3). However, a significant decrease in αSMA levels was observed in periostin-depleted fibroblasts relative to scrambled shRNA controls in two of the four HTS patient fibroblast lines assessed (p<0.05, Figure 4.9b). Periostin depletion by shRNA treatment had no effect on OB cadherin levels in HTS fibroblasts relative to scrambled shRNA controls (Figure 4.9c, N=4 patients, n=3). Additionally, periostin depletion by shRNA treatment had no significant effect on αSMA or OB cadherin levels in KS fibroblasts relative to scrambled shRNA controls (Figure 4.10a and b, N=3 patients, n=3).

4.6 Assessment of NS fibroblast responses to periostin

The contraction data presented in section 4.3 demonstrates that periostin induces collagen lattice contraction in HTS fibroblasts but not in NS fibroblasts. As NS fibroblasts are unlikely to be exposed to the growth factors and cytokines that promote abnormal wound healing, it was proposed that NS fibroblasts might need to be “activated” by fibrosis-associated factors before they can respond to periostin treatment. While not a major focus of this thesis, the following section describes pilot studies to identify some of the factors required for NS fibroblast “activation” in this model system.

4.6.1 Assessment of the effects of TGFβ-1 priming on NS fibroblast contractility

During cutaneous wound healing, TGFβ-1 is released following injury to the dermis and plays a critical role in many processes essential to normal wound repair (8, 13, 41, 174-177, 220). With relevance to the roles of periostin described in the previous sections, TGFβ-1 signalling is required for myofibroblast differentiation (40, 41). Therefore, it was proposed that priming with TGFβ-1 might be required to sensitize NS fibroblasts to the presence of periostin. To assess this, cultures were pretreated with 5 ng/ml human
Figure 4.8 Periostin depletion had no effect on collagen lattice contraction in hypertrophic scar-derived fibroblasts. Primary hypertrophic scar (HTS) and keloid scar (KS)-derived fibroblasts were transduced with adenoviral vectors expressing shRNAs targeted against POSTN or scrambled control shRNAs for 72 hours on tissue culture plastic. To assess the effects of periostin depletion on collagen lattice contraction, shRNA treated HTS and KS fibroblasts were cultured in stressed fibroblast populated collagen lattices described in 2.2.5. Collagen lattice contraction was assessed over a 24 hour period. Periostin depletion by shRNA treatment had no effect on (a) HTS or (b) KS fibroblast collagen lattice contraction relative to scrambled shRNA controls (N=3 patients, n=3). Data are presented by mean values and SEM.
**Figure 4.9 Periostin depletion significantly decreases αSMA levels in fibroblasts derived from a subset of HTS patients.** Primary hypertrophic scar (HTS)-derived fibroblasts were transduced with adenoviral vectors expressing shRNAs targeted against *POSTN* or scrambled control shRNAs for 72 hours and then seeded into stressed fibroblast populated collagen lattices. Total cell lysates were assessed for alpha smooth muscle actin (αSMA) and OB cadherin immunoreactivity, by western immunoblotting and densitometry, immediately prior to collagen lattice release. (a) Periostin-depletion had no significant effects on αSMA levels in HTS fibroblasts assessed as a group (N=4 patients, n=3), (b) however, significant decreases in αSMA levels were observed following periostin depletion in a subset of individual patient-derived fibroblast cultures. Shown is a representative immunoblot and densitometry data for HTS patient #5. (c) No discernible effects on OB cadherin levels were observed in periostin-depleted HTS fibroblasts relative to scrambled shRNA controls (N=4 patients, n=3). Values were normalized to scrambled shRNA controls. β-actin immunoreactivity was assessed to ensure equal protein loading. Significant treatment effects are denoted by **p<0.01 (t-tests). Data are presented by mean values and SEM. Representative immunoblots of the mean densitometry data are shown. AU=arbitrary units.
Figure 4.10 Periostin depletion had no significant effects on myofibroblast markers in keloid scar fibroblasts. Primary keloid scar (KS)-derived fibroblasts were transduced with adenoviral vectors expressing shRNAs targeted against POSTN or scrambled control shRNAs for 72 hours on tissue culture plastic then seeded into stressed fibroblast populated collagen lattices. Total cell lysates were assessed for alpha smooth muscle actin (αSMA) and OB cadherin immunoreactivity, by western immunoblotting and densitometry, immediately prior to collagen lattice release. Periostin-depletion had no significant effects on (a) αSMA or (b) OB cadherin levels in HTS fibroblasts relative to scrambled shRNA controls (N=3 patients, n=3). Values were normalized to scrambled shRNA controls. β-actin immunoreactivity was assessed to ensure equal protein loading. Data are presented by mean values and SEM. Representative immunoblots of the mean densitometry data are shown. AU=arbitrary units.
recombinant TGFβ-1 for 72 hours prior to being seeded into sFPCLs. As shown in Figure 4.11a, a significant increase in sFPCL contraction was evident in TGFβ-1 primed NS fibroblasts in collagen lattices containing periostin (p<0.05) relative to TGFβ-1 primed NS fibroblasts in the absence of periostin. This effect was only evident at release and no significant effects were observed at any time point assessed after release (N=3 patients, n=3).

4.6.2 Assessment of the effects of HTS-conditioned media on NS fibroblast contractility in sFPCLs

TGFβ-1 is likely to be only one of many growth factors and cytokines secreted by HTS fibroblasts. To determine if additional fibrosis-associated signalling molecules were required to induce periostin sensitivity in NS fibroblasts, the effects of exposing NS fibroblasts to HTS fibroblast-conditioned culture media were assessed. HTS fibroblasts were grown to 80% confluency in serum-containing media, washed and cultured for 96 hours in serum free media, after which the conditioned media was collected. NS fibroblasts were then cultured in sFPCLs in the presence of HTS-conditioned media for 72 hours, with or without periostin addition to the collagen matrix. The addition of HTS-conditioned media had no effects on NS fibroblast contraction relative to fibroblasts cultured in the presence of NS-conditioned media (Figure 4.11b, N=1 patient, n=5). However, a significant periostin treatment and time interaction (p<0.01) was observed and the presence of exogenous periostin significantly induced the contractility of NS fibroblasts cultured in HTS-conditioned media over 24 hours relative to vehicle controls. Significant increases in periostin-induced NS fibroblast contraction were observed at collagen lattice release, 0.5 and 2 hours after release (Figure 4.11c, p<0.05, N=1 patient, n=5).

4.6.3 Identification of potential periostin interactors in HTS-conditioned media

A combination of periostin and HTS-conditioned media was found to significantly induce NS fibroblast contractility, whereas neither of these treatments alone had discernible effects on the ability of NS fibroblasts to contract collagen lattices. These data were interpreted to suggest that periostin needed to interact with one or more molecules in
Figure 4.11 Fibrosis-associated growth factors are required to induce periostin sensitivity in normal skin fibroblasts. (a) Primary normal skin (NS) fibroblasts were cultured on tissue culture plastic for 72 hours in 10% FBS-αMEM supplemented with 5 ng/ml transforming growth factor (TGF)β-1. TGFβ-1 primed NS fibroblasts were then seeded into stressed fibroblast populated collagen lattices (sFPCLs) containing 2 µg/ml periostin (PN) or vehicle (veh) and cultured for 72 hours. Collagen lattice contraction was assessed over 24 hours. PN significantly enhanced TGFβ-1-primed NS fibroblast contraction at collagen lattice release (N=3 patients, n=3). (b,c) Primary NS fibroblasts were seeded into sFPCLs embedded with PN or vehicle and cultured for 72 hours in hypertrophic scar (HTS)- or NS fibroblast-conditioned media (CM). (b) Culture in HTS CM had no effect on NS fibroblast collagen lattice contraction relative to NS fibroblasts cultured in NS CM (N=1 patient, n=3). (c) Exogenous periostin treatment significantly enhanced NS fibroblast contractility when cultured in HTS CM over 24 hours relative to vehicle controls (N=1 patient, n=5). Significant treatment/time interactions are denoted by ##p<0.01 (ANOVA of repeated measures). Significant treatment effects at individual time points are denoted by *p<0.05 and **p<0.01 (t-tests and simple main effects analyses). Data are presented by mean values and SEM.
HTS fibroblast-conditioned media to enhance the contraction of NS fibroblasts. In order to identify potential periostin interactors, immunoprecipitation experiments were performed on HTS-conditioned media. In brief, HTS-conditioned media, in the absence of exogenous periostin, was concentrated 50 times and 1 mg of total protein was incubated with an antibody specific to periostin and precipitated with protein A/G magnetic beads. Eluted samples were separated using SDS-PAGE and analyzed by coomassie staining and western immunoblotting. Coomassie staining identified bands at approximately 85 and 90 kDa in periostin immunoprecipitated samples (Figure 4.12a) and western immunoblotting confirmed the identity of these bands as endogenous periostin (Figure 4.12b). Two additional bands were evident in the periostin immunoprecipitated samples that were absent or extremely faint in the IgG control lane (Figure 4.12a). These bands were excised and analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS). They were identified as keratin-14 (user contamination) and fragments of IgG and periostin. Optimization experiments utilizing a variety of protocol modifications failed to identify potential periostin interactors in HTS-conditioned media.

4.7 Assessment of the effects of periostin on HTS myofibroblast persistence in sFPCLs

Normally, as cutaneous wounds heal and ECM stress is relieved, the myofibroblasts that promote wound closure undergo apoptosis (8, 12). However, in abnormal scarring, myofibroblasts fail to undergo apoptosis and persist, resulting in excessive collagen production and contracture of the ECM (56, 57, 92-94) that promotes hypertrophic scar formation. When sFPCLs are released, a rapid contraction of the collagen lattices decreases the tension in the ECM. This release in ECM tension correlates with disassembly of stress fibers in myofibroblasts (47). To determine if periostin promotes myofibroblast persistence in HTS fibroblasts, αSMA levels were assessed in sFPCL cultures immediately prior to release (under mechanical tension for 72 hours) and up to 48 hours after collagen lattice release (in the presence of decreased ECM tension) by western immunoblotting and densitometry. HTS fibroblasts with high levels of
Figure 4.12 Identification of potential periostin interactors in hypertrophic scar-conditioned media. Hypertrophic scar (HTS) conditioned media was generated (2.2.9.2) and concentrated 50 times. 1 mg of total protein was incubated with a periostin (PN)-specific antibody and immunoprecipitated (IP) with protein A/G magnetic beads as described in 2.2.9.3. IP samples were separated using SDS-PAGE and samples were analyzed by (a) coomassie staining (2.2.9.4) and (b) western immunoblotting (2.2.6). (a) Coomassie staining identified 4 bands at molecular weights of 90 kDa, 85 kDa, 65 kDa and 60 kDa that were absent or extremely faint in IgG control samples. The 65 and 60 kDa bands were identified as keratin-14 and fragments of IgG and PN by liquid chromatography tandem mass spectrometry (not shown). (b) The 90 and 85 kDa bands were identified as endogenous PN by western immunoblotting with a PN-specific antibody. Protein A/G magnetic beads (beads) and PN-specific antibody (ab) were used as controls. Bands at 55 kDa in both images correlate to the size of IgG heavy chain. Representative images are shown. MW= molecular weight ladder, WB= western immunoblot.
Figure 4.13 Periostin maintains myofibroblasts in a differentiated state under conditions of decreasing ECM tension. Primary hypertrophic scar (HTS) fibroblasts were cultured in stressed fibroblast populated collagen lattices (sFPCLs) and total cell lysates were derived immediately prior to collagen lattice release (after 72 hours of culture), 24 and 48 hours after collagen lattice release. The effects of periostin (PN) addition or depletion on alpha smooth muscle actin (αSMA) levels were assessed by western immunoblotting and densitometry. (a) HTS fibroblasts maintained αSMA levels following collagen lattice release in the presence of endogenous PN. αSMA levels were significantly increased in PN treated HTS fibroblasts relative to vehicle controls and αSMA levels were maintained for at least 48 hours after lattice release. Values were normalized to vehicle controls immediately prior to lattice release (N=5 patients, n=3). (b) Periostin depletion by shRNA treatment resulted in a significant and ongoing decrease in αSMA levels in HTS fibroblasts following collagen lattice release relative to scrambled shRNA controls. Values were normalized to scrambled controls immediately prior to lattice release (N=4 patients, n=3). β-actin immunoreactivity was assessed to ensure equal protein loading. Significant overall treatment effects are denoted by \(^{p<0.05}\) (ANOVA of repeated measures). Significant treatment effects at individual time points are denoted by *\(p<0.05\) and **\(p<0.01\) (t-tests). Data are presented as mean values and SEM. Representative immunoblots of the mean densitometry data are shown. AU=arbitrary units.
endogenous periostin expression (vehicle treated fibroblasts) maintained αSMA levels following collagen lattice release. HTS fibroblasts treated with exogenous periostin had significantly higher αSMA levels (p<0.05) relative to vehicle treated fibroblasts cultured under mechanical tension and were able to maintain these high levels of αSMA following the release of ECM tension (Figure 4.13a, N=5 patients, n=3). In contrast, periostin-depleted HTS fibroblasts had significantly decreased αSMA levels (p<0.05) relative to scrambled shRNA controls with significant decreases observed at 24 and 48 hours after collagen lattice release (Figure 4.13b, p<0.05, N=4 patients, n=3).

4.8 Discussion

In order to assess periostin effects on myofibroblast differentiation and persistence, it was necessary to perform studies in a model system that mimicked in vivo conditions of 3D stress in a collagen-enriched environment. The stressed fibroblast populated collagen lattice (sFPCL) assay was chosen on this basis. When initially seeded into sFPCL, the fibroblasts experience an ECM environment similar to that of the provisional matrix in vivo (31, 173). During ECM remodelling, tractional forces generated by migrating fibroblasts increase the mechanical tension of the ECM thereby inducing proto-myofibroblast and myofibroblast differentiation (30, 156). This mechanical tension is sustained until the collagen lattice is released from its points of attachment (30). Upon release, myofibroblast-mediated contraction rapidly contracts the collagen lattice.

To investigate periostin effects on myofibroblast differentiation in HTS fibroblasts, three markers of myofibroblast differentiation were assessed: contractility, αSMA and OB cadherin levels. The neo-expression of αSMA in their stress fibres is a distinguishing feature of myofibroblasts and facilitates their hyper-contractile phenotype (30).

Incorporation of αSMA into stress fibers is specific to differentiated myofibroblasts, as the stress fibers in proto-myofibroblast are composed solely of β- and γ-actins (38, 39). OB cadherin, a membrane protein involved in adherins junctions (221), also enhances collagen lattice contraction by myofibroblasts (218) and its expression is reported to be upregulated in differentiated myofibroblasts relative to proto-myofibroblasts (218).
The findings reported in this chapter indicate that periostin enhances myofibroblast differentiation in human dermal fibroblasts derived from hypertrophic scar tissue. The evidence supporting this conclusion includes significant increases in HTS fibroblast contractility and αSMA levels in the presence of exogenous periostin. In addition, a non-significant trend towards increased OB cadherin levels in periostin treated HTS fibroblasts was noted. The absence of any discernible effects of periostin treatment on fibroblast proliferation, viability, adhesion or migration in 3D cultures suggests that they are unlikely to explain periostin-induced HTS collagen lattice contraction. Furthermore, periostin significantly enhanced KS fibroblast contractility above highly contractile basal levels. These data suggest that periostin effects on myofibroblast differentiation may not be specific to HTS fibroblasts but may be a common feature of fibrotic conditions of the skin. Periostin also induces myofibroblast differentiation of fibroblasts derived from Dupuytren’s disease, a fibrosis of the palmar fascia with similarities to scarring (91), suggesting that these findings may be extendible to other tissues.

Since HTS fibroblasts express increased endogenous levels of periostin relative to NS cells and as exogenous periostin addition promoted increased myofibroblast differentiation in HTS-derived fibroblast, loss of function studies were performed to confirm these findings. Adenoviral transduction was chosen as an optimal method for shRNA delivery for several reasons. Transient transfection efficiency is typically low in primary fibroblasts and selection for stable transfection is impractical in primary fibroblasts that lose their in vivo characteristics after a limited number of passages. Previous studies in our laboratory have demonstrated that, unlike transient transfection, viral transduction efficiency approximates 100% in primary fibroblasts under optimal conditions. An adenoviral transduction system was used in preference to a lentiviral system because adenovirus remains episomal and does not integrate into the host genome, thereby limiting the potential for non-specific disruption of gene expression caused by viral DNA integration into the genome.

In contrast to the gain-of-function studies where exogenous periostin significantly enhanced HTS myofibroblast differentiation, no significant effects of periostin depletion on myofibroblast differentiation were evident in the loss-of-function studies. As markers
of myofibroblast differentiation, a significant decrease in collagen lattice contraction, αSMA or OB cadherin levels would have provided independent support for the gain-of-function studies. The lack of significant findings in these loss-of-function studies may be explained in part by inter-patient variability. Factors including age, gender and race may affect the sensitivity of periostin depletion between individual patients and make it difficult to identify consistent effects. Future analyses of additional patients may be required to overcome inter-patient variability and allow us to identify consistent effects in all, or even a majority of, patients assessed. It is also a possibility that a 60% reduction in endogenous periostin levels in HTS fibroblasts following POSTN shRNA treatment was insufficient to induce detectible effects on contraction and myofibroblast differentiation. Periostin is proposed to be a very stable protein with a long half-life that is difficult to deplete under conditions of high ECM tension (222). Therefore, depletion of intracellular periostin in HTS fibroblasts may not correlate with depletion of secreted and potentially very stable ECM-associated periostin. Another possibility is that, while periostin can modestly enhance myofibroblast differentiation above basal levels, its primary role is to maintain, rather than induce, myofibroblast differentiation. Therefore, increased matrix tension, such as that experienced by fibroblasts cultured in sFPCLs, may be sufficient to induce HTS myofibroblast differentiation and periostin may play a modestly stimulatory, but dispensable, role in this process.

Interestingly, no evidence of periostin-induced myofibroblast contraction was observed in NS fibroblast cultured in sFPCLs. These findings suggest that periostin induces pathological, rather than normal, collagen lattice contraction, as the effects were specific to fibroblasts derived from abnormal scar tissue. It remains unclear if periostin-induced contraction is a component of normal cutaneous wound healing, as, due to ethical constraints, we were unable to obtain samples of normal human scar tissue from which to derive primary fibroblasts. The mechanisms that explain the differences in sensitivity to periostin between HTS and NS fibroblasts are currently unclear. It is feasible that NS fibroblasts may need to be “activated” in order to respond to periostin. This activation could include induction or activation of a receptor(s) not normally present in unwounded NS fibroblasts or exposure to fibrosis-associated growth factors and cytokines required for periostin signalling.
Preliminary western immunoblotting studies demonstrated that both HTS and NS fibroblasts express integrins αν, β3 and β5 subunits (data not shown) through which periostin has been shown to signal in other systems (127-129, 131-134). Although HTS and NS fibroblasts had similar expression profiles of individual integrin subunits, it is unclear if HTS and NS fibroblasts express different integrin heterodimers and the activation state of these dimers.

The hypothesis that exposure to fibrosis-associated growth factors and cytokines are required to activate and induce periostin sensitivity in NS fibroblasts was also tested. During cutaneous wound healing, TGFβ-1 is released following injury to the dermis and plays a critical role in many processes essential to normal wound repair, including myofibroblast differentiation (8, 13, 40, 41, 174-177, 220). Therefore, TGFβ-1 signalling may be required to activate NS fibroblasts and induce periostin sensitivity in these cells. These studies indicated that priming NS fibroblasts with recombinant TGFβ-1 was only partially effective in inducing NS fibroblast sensitivity to periostin. It is likely that a combination of fibrosis-associated growth factors that are expressed at high levels by HTS fibroblasts (for example, CCN2 (223)), might be required to induce periostin sensitivity in NS fibroblasts.

Since a large number of growth factors are secreted by fibroblasts during cutaneous wound healing (8), it is feasible that HTS fibroblasts might secrete factors that can activate NS fibroblasts. The data presented in this chapter supports this notion as periostin significantly enhanced NS fibroblast contractility when cultured in HTS-conditioned media. As HTS-conditioned media had no discernible effect on NS fibroblast contractility in the absence of exogenous periostin, these data imply that periostin is interacting with a component in the conditioned media to enhance contraction of NS fibroblasts. Despite multiple optimization experiments, no periostin interacting molecules that were specific to HTS-conditioned media were identified by immunoprecipitation analyses. Future studies, beyond the scope of this thesis, will be required to identify the elusive molecular interactions that induce periostin sensitivity in NS fibroblasts.
The second component of this aim was to assess periostin effects on myofibroblast persistence. Normally, as cutaneous wounds heal and ECM stress is relieved, the myofibroblasts that promote wound closure undergo apoptosis (8, 12). However, in abnormal scarring conditions, myofibroblasts fail to undergo apoptosis and persist, and continue to remodel the ECM and increase dermal density to abnormal levels. Many factors, most notably TGFβ-1 (41), have been demonstrated to induce myofibroblast differentiation in combination with increased ECM tension (30, 38, 42). However, much less is known about the factors that maintain myofibroblasts in a differentiated state in hypertrophic scars that persist for month or years. Since periostin promotes myofibroblast differentiation in HTS fibroblasts and is abundant and persistent in the dermis of hypertrophic scars, it was hypothesized that periostin promotes myofibroblast persistence in hypertrophic scarring.

To test this hypothesis, αSMA levels in HTS fibroblasts in sFPCLs cultured under mechanical tension (pre-release) and up to 48 hours after collagen lattice release were assessed. This model was designed to mimic the loss of ECM stress that induces apoptosis in normal scar myofibroblasts (8, 12). While the rapid loss of ECM tension induced by collagen lattice release in sFPCLs is much more abrupt than the physiological process it is designed to mimic (183), it does provide a reproducible assay system for myofibroblast persistence under rapidly diminishing ECM tension. These studies demonstrate that HTS fibroblasts are able to maintain αSMA levels at constant levels under conditions of decreasing ECM tension in the presence of endogenous periostin and that these levels are significantly increased and maintained by the addition of exogenous periostin. In contrast, αSMA levels were significantly decreased in periostin-depleted HTS fibroblasts under conditions of decreasing ECM tension relative to scrambled controls. It is unclear if this loss in αSMA levels following collagen lattice release was due to the onset of myofibroblast apoptosis, myofibroblasts dedifferentiation or a combination of both. These findings support the hypothesis that periostin maintains myofibroblast differentiation in HTS fibroblasts under conditions of decreasing ECM tension.
In summary, these studies demonstrated that periostin induces HTS fibroblast contraction and differentiation in 3D collagen lattices, a model that mimics the mechanical tension of granulation tissue during cutaneous wound healing. In addition, periostin maintained αSMA levels, a marker of myofibroblast differentiation, in sFPCL cultures exposed to reduced ECM tension, mimicking wound remodelling after closure. Together these data demonstrate novel roles for periostin in inducing and maintaining myofibroblast differentiation and persistence in HTS fibroblasts.
Chapter 5

In vitro analysis of the mechanisms utilized by periostin to induce myofibroblast differentiation and persistence in hypertrophic scarring

5.1 Rationale

Periostin was shown to induce dermal fibroblasts derived from hypertrophic scar tissue to express α smooth muscle actin (αSMA) and differentiate into myofibroblasts in Chapter 4. However, the signalling pathway(s) and mechanisms activated by periostin to induce and/or maintain myofibroblast differentiation had not been elucidated. Therefore, the focus of Chapter 5 is to identify these pathway(s). Periostin has been previously reported to signal through integrins in other cell types (127-134), and integrins have well-established roles in mediating signalling events that increase cellular attachment and contraction of the ECM (46). The integrin-associated complexes that form between cells and the matrix are known as focal adhesions (30, 46, 156). Previous studies have shown that αSMA is only incorporated into the stress fibers of these cells when the mechanical tension in the surrounding ECM is sufficient to induce the “maturation” of focal adhesions (FAs) into “supermature” focal adhesions (suFAs, (30, 43)). As periostin induces αSMA production and HTS myofibroblast contractility, the hypothesis for this chapter was that periostin signals through integrins in HTS fibroblasts, activates integrin-associated signalling pathways, enhances the formation of suFAs between HTS fibroblasts and the surrounding collagen matrix and promotes the incorporation of αSMA into stress fibres. This hypothesis is illustrated in Figure 5.1.

5.2 Immunoprecipitation analyses of receptor interactions with periostin in HTS fibroblasts.

To determine if periostin interacts with integrins in HTS fibroblasts, immunoprecipitation analyses with a periostin-specific antibody were performed on total cell lysates derived from HTS fibroblasts cultured on plastic as described in 2.2.9. Using this approach, periostin immunoreactivity was evident in samples immunoprecipitated with a periostin-
Figure 5.1 Schematic representation of the proposed signalling pathway utilized by periostin to induce myofibroblast differentiation and persistence. The central hypothesis of Chapter 5 is that ECM-associated periostin signals through integrins (Chapter 5.2) in HTS fibroblasts to activate focal adhesion kinase (FAK, Chapter 5.3.1), a tyrosine kinase that localizes to focal adhesion complexes. FAK activation promotes myofibroblast differentiation and focal adhesion formation through RhoA-GTP and ROCK (Chapter 5.3.2) and its downstream effectors cofolin (Chapter 5.3.3) and myosin light chain (MLC, Chapter 5.3.4). Cofilin inhibition and MLC-mediated stress fiber contraction promotes stress fiber maintenance, increased ECM tension and integrin clustering resulting in the formation of supermature focal adhesions (suFA, Chapter 5.4), a prerequisite for αSMA incorporation into stress fibers of differentiated myofibroblasts.
specific antibody and absent in samples immunoprecipitated with a non-specific IgG control, demonstrating the specificity of the antibodies for periostin. Western immunoblotting analyses revealed interactions between periostin and integrins αv and α5 however, these interactions were also consistently evident in samples immunoprecipitated with a non-specific IgG control. These data indicated that these interactions were not specific to periostin. Several modifications of standard immunoprecipitation protocols were trialed including the use of nickel (Ni) coated magnetic beads that bind specifically to histidine-tagged human recombinant periostin, shorter incubation times and more stringent washing conditions (described in detail in 2.2.10) to enhance the specificity of interactions between periostin and integrin subunits. The approach of using Ni coated magnetic bead to specifically isolate recombinant periostin from total cell lysates of HTS fibroblasts exposed to treatment was successful (Figure 5.2a). However, interactions between periostin and integrins αv and α5 were evident in HTS cell lysates in both the presence and absence of recombinant periostin treatment, indicating a high background level of non-specific interactions (Figures 5.2b and c). In addition to western immunoblotting analyses, immunoprecipitated samples were stained with coomassie to identify bands of unique molecular weight in comparisons between treated and untreated samples, potentially indicating novel periostin interactors. Despite substantial method optimization and multiple mass spectrometry analyses, no novel periostin interactors were identified using this approach (data not shown). Therefore, this research effort was refocused onto the identification of integrin-associated signalling intermediates that were modified by periostin during myofibroblast differentiation.

5.3 Assessment of the effects of periostin on integrin-associated signalling molecules in HTS-derived fibroblasts

The integrin-associated complexes that form between cells and the ECM are known as focal adhesions (30, 46, 156). Focal adhesion kinase (FAK) is a tyrosine phosphorylated protein that localizes to focal adhesions (224, 225) and its activation is required for adhesion-mediated myofibroblast differentiation (226) and suFA formation (43). FAK
Figure 5.2 Immunoprecipitation analyses of receptor interactions with periostin in HTS fibroblasts. Primary hypertrophic scar (HTS) fibroblasts were cultured to 85% confluency on tissue culture plastic trays and treated with 2 µg/ml periostin (PN) or vehicle (veh) in 2% FBS-αMEM overnight prior to formaldehyde fixation. 1 mg of total cell lysate was precipitated using nickel (Ni) coated magnetic beads to bind histidine-tagged human recombinant periostin as described in 2.2.10. Eluted precipitation complexes were subjected to SDS-PAGE and assessed for periostin, αv and α5 integrin immunoreactivity by western immunoblotting as described in 2.2.6. (a) Ni coated magnetic beads specifically precipitated recombinant periostin as demonstrated by the presence of a band at 90 kDa that is absent in the vehicle control lane. (b and c) Ni coated magnetic beads non-s specifically precipitated integrins αv and α5 as demonstrated by the presence of bands at (b) 140 kDa and (c) 150 kDa in both the periostin and vehicle treated samples. Representative immunoblots are shown. MW= molecular weight, WB=western immunoblot.
can promote myofibroblast differentiation and focal adhesion formation by modifying Rho Kinase (ROCK) activity and the activities of its downstream targets. These targets include coflinin and myosin light chain (MLC), which regulate stress fiber stabilization and stress fiber-mediated contraction, respectively. Therefore, it was hypothesized that periostin promotes myofibroblast differentiation and persistence through a FAK-ROCK dependent signalling pathway.

**5.3.1 Assessment of the effects of periostin on FAK activation in HTS fibroblasts cultured in sFPCLs**

To assess the contribution of FAK activation in periostin-induced HTS myofibroblast differentiation and persistence, phosphorylated FAK levels were assessed in sFPCL lysates immediately prior to release and up to 48 hours after release by western immunoblotting and densitometry. A significant interaction between periostin treatment and time was observed (p<0.01) and exogenous periostin treatment enhanced FAK phosphorylation following collagen lattice release relative to vehicle controls with a significant increase in FAK phosphorylation evident at 48 hours after lattice release (Figure 5.3a, p<0.05, N=3 patients, n=3). A significant overall treatment effect was also evident in periostin-depleted HTS fibroblasts (p<0.05) with periostin-depleted HTS fibroblasts displaying increased levels of phosphorylated FAK relative to scrambled shRNA controls. Significant increases in FAK phosphorylation were evident in periostin-depleted HTS fibroblasts immediately prior to collagen lattice release and at 48 hours after release (Figure 5.3b, p<0.05, N=3 patients, n=3). Periostin addition or depletion had no discernible effects on total FAK levels (data not shown, N=3 patients, n=3).

**5.3.2 Assessment of the effects of ROCK inhibition on periostin-induced HTS myofibroblast differentiation**

ROCK plays a central and essential role in activating and maintaining myofibroblast differentiation and contractility (30, 43, 51-53). To confirm that periostin signalling required ROCK activity to induce HTS myofibroblast-mediated collagen lattice contraction, a ROCK specific inhibitor, Y27632, was included in a subset of sFPCL cultures of HTS fibroblasts, with or without exogenous periostin treatment, and collagen
Figure 5.3 Periostin enhances focal adhesion kinase phosphorylation. Primary hypertrophic scar (HTS) fibroblasts were cultured in stressed fibroblast populated collagen lattices (sFPCLs) as described in 2.2.5 and total cell lysates were derived immediately prior to sFPCL release (72 hours after polymerization), 24 and 48 hours after release. Levels of phosphorylated focal adhesion kinase (pFAK, tyr397) were assessed in HTS lysates by western immunoblotting and densitometry as described in 2.2.6. (a) Periostin significantly enhanced FAK phosphorylation in HTS fibroblasts cultured in conditions of decreasing ECM tension relative to vehicle controls. Values were normalized to vehicle controls immediately prior to lattice release (N=3 patients, n=3). (b) Periostin depletion by shRNA treatment significantly increased FAK phosphorylation in HTS fibroblasts in a model of decreasing ECM tension relative to scrambled shRNA controls. Values were normalized to scrambled shRNA controls immediately prior to lattice release (N=3 patients, n=3). β-actin immunoreactivity was assessed to ensure equal protein loading. Significant overall treatment effects are denoted by ^p<0.05 and significant treatment/time interactions are denoted by ##p<0.01 (ANOVA of repeated measures). Significant treatment effects at individual time points are denoted as *p<0.05 (t-tests and simple main effect analyses). Data are presented by mean values and SEM. Representative immunoblots of the mean densitometry data are shown. AU=arbitrary units.
Figure 5.4 Rho kinase inhibition significantly attenuates hypertrophic scar fibroblast contraction of stressed fibroblast populated collagen lattices. To assess the effects of Rho kinase (ROCK) inhibition of hypertrophic scar (HTS) fibroblast contractility, primary HTS fibroblasts were seeded in stressed fibroblast populated collagen lattices containing periostin (PN) or vehicle (veh) as described in 2.2.5 and cultured in 2% FBS-αMEM supplemented with 10 µM ROCK inhibitor, Y27632 (ROCKi), or vehicle control (water) for 72 hours. Collagen lattice contraction was assessed over 24 hours. ROCK inhibition significantly attenuated basal HTS fibroblast collagen lattice contraction at all time points assessed. The addition of exogenous PN was unable to rescue HTS fibroblast contraction in the presence of Y27632 (N=1 patient, n=3). Significant treatment/time interactions are denoted by #p<0.05 (ANOVA of repeated measures). Significant treatment effects at individual time points are denoted by *p<0.05, **p<0.01, ***p<0.001 (simple main effect analyses). Data are presented by mean values and SEM.
lattice contraction was assessed over 24 hours. A significant interaction between Y27632 treatment and time was observed (p<0.01) and ROCK inhibition strongly attenuated HTS fibroblast contractility relative to vehicle controls with significant differences observed at all time points assessed (p<0.05). The addition of exogenous periostin did not rescue HTS fibroblast contraction in the presence of Y27632 (Figure 5.4, N=1 patient, n=3).

5.3.3 Assessment of the effects of periostin on cofilin activation in HTS fibroblasts cultured in sFPCLs

Cofilin is an actin-binding protein that is a downstream target of ROCK and promotes actin stress fiber depolymerization (227). ROCK activation leads to the phosphorylation and inhibition of cofilin activity, thereby preventing actin depolymerization (228). To assess the contribution of cofilin in periostin-induced HTS myofibroblast differentiation, phosphorylated cofilin levels were assessed in sFPCL lysates immediately prior to release and up to 48 hours after release by western immunoblotting and densitometry. Exogenous periostin treatment had no significant effect on cofilin phosphorylation relative to vehicle controls when HTS fibroblasts were assessed as a group (Figure 5.5a, N=3 patients, n=3). However, it was noted that periostin significantly increased cofilin phosphorylation at individual time points in two of the three individual patients assessed (Figure 5.5b, p<0.05).

Periostin depletion by shRNA treatment had no significant effects on cofilin phosphorylation in HTS fibroblasts when assessed as a group (Figure 5.5c, N=3 patients, n=3). However, a significant treatment effect was observed (p<0.05) in one of the three patient-derived fibroblast lines assessed and phosphorylated cofilin levels were found to be significantly decreased in periostin-depleted HTS myofibroblasts relative to scrambled shRNA controls. In addition, phosphorylated cofilin levels were found to be significantly decreased at individual time points in the other two patient-derived fibroblast lines assessed (Figure 5d, p<0.05). Periostin addition or depletion had no discernible effects on total cofilin levels (data not shown, N=3 patients, n=3).
**Figure 5.5 Periostin enhances cofilin phosphorylation in conditions of decreasing ECM tension in a subset of patients.** Primary hypertrophic scar (HTS) fibroblasts were cultured in stressed fibroblast populated collagen lattices (sFPCLs) and total cell lysates were derived immediately prior to sFPCL release (72 hours after polymerization), 24 and 48 hrs after release. Levels of phosphorylated cofilin (pCofilin, ser3) were assessed in HTS lysates by western immunoblotting and densitometry. (a) Periostin had no discernible effect on cofilin phosphorylation relative to vehicle controls when assessed as a group (N=3 patients, n=3 patient). (b) However, periostin significantly enhanced cofilin phosphorylation relative to vehicle controls in a subset of individual patients. Shown is a representative immunoblot and densitometry data for HTS patient #5. Values were normalized to vehicle controls immediately prior to lattice release. (c) Periostin depletion by shRNA treatment had no significant effects on cofilin phosphorylation relative to scrambled shRNA controls when assessed as a group (N=3 patients, n=3). (d) In contrast, periostin depletion by shRNA treatment significantly decreased cofilin phosphorylation relative to scrambled shRNA controls in a subset of individual patients. Shown is a representative immunoblot and densitometry data for HTS patient #5. Values were normalized to scrambled shRNA controls immediately prior to lattice release. β-actin immunoreactivity was assessed to ensure equal protein loading. Significant treatment effects at individual time points are denoted by *p<0.05 (t-tests). Data are presented by mean values and SEM. Representative immunoblots of the mean densitometry data are shown. AU=arbitrary units.
5.3.4 Assessment of the effects of periostin on MLC activation in HTS fibroblasts cultured in sFPCLs

The contractile forces induced by myofibroblasts on their ECM are generated by contractile stress fibres composed of actin bundles, non-muscle myosin, and actin-binding proteins and is regulated by the activity (phosphorylation) of MLC (30, 45-47).

To assess the contribution of MLC in periostin-induced HTS myofibroblast differentiation, phosphorylated MLC levels were assessed in sFPCL lysates immediately prior to release and up to 48 hours after release by western immunoblotting and densitometry. Exogenous periostin addition to sFPCL cultures had no significant effect on MLC phosphorylation in HTS fibroblasts cultured under mechanical tension or following the release of tension relative to vehicle controls (Figure 5.6a, N=3 patients, n=3). No significant changes in MLC phosphorylation were observed in periostin-depleted HTS fibroblasts relative to scrambled shRNA controls cultured under mechanical tension and following the release of tension (Figure 5.6b, N=3 patients, n=3). However, a significant treatment effect was observed (p<0.05) in one of three patient-derived fibroblast lines assessed with phosphorylated MLC levels being significantly lower in periostin-depleted HTS fibroblasts relative to vehicle controls (Figure 5.6c). Periostin addition or depletion had no discernible effects on total MLC levels (data not shown, N=3 patients, n=3).

As described earlier in this chapter, FAK activity enhances suFA formation and myofibroblast differentiation through the activation of ROCK and its downstream targets coflin and MLC. ROCK-mediated coflin inhibition prevents stress fiber depolymerization, whereas MLC activation leads to myofibroblast contraction that increases the mechanical tension in the ECM and induces integrin clustering resulting in the formation of larger focal adhesion complexes. Although no consistent effects of periostin on coflin inhibition and MLC activation could be discerned in HTS fibroblasts when assessed as a group, the significant effects noted in individual fibroblast lines were invariably consistent with periostin promoting suFA formation during myofibroblast differentiation. Therefore, the potential for periostin to enhance suFA formation was assessed in HTS fibroblasts as a group.
Figure 5.6 Periostin depletion reduces myosin light chain phosphorylation in HTS myofibroblasts cultured in sFPCLs in a subset of individuals. Primary hypertrophic scar (HTS) fibroblasts were cultured in stressed fibroblast populated collagen lattices (sFPCLs) and total cell lysates were derived immediately prior to sFPCL release (72 hours after polymerization), 24 and 48 hours after release. Levels of phosphorylated myosin light chain (pMLC, ser19) were assessed in HTS lysates by western immunoblotting and densitometry. (a) Periostin had no significant effect on MLC phosphorylation in HTS fibroblasts cultured in sFPCLs relative to vehicle controls. Values were normalized to vehicle controls immediately prior to collagen lattice release (N=3 patients, n=3). (b) Periostin depletion by shRNA treatment had no significant effects on MLC phosphorylation in HTS fibroblasts relative to scrambled shRNA controls when assessed as a group (N=3 patients, n=3). (c) While periostin treatment did not significantly affect MLC phosphorylation at any specific time point, an overall treatment effect indicating a significant decrease in MLC phosphorylation in periostin-depleted HTS fibroblasts relative to scrambled shRNA controls was detected in a subset of individual patients. Shown is a representative immunoblot and densitometry data for HTS patient #9. Values were normalized to scrambled shRNA controls immediately prior to collagen lattice release. β-actin immunoreactivity was assessed to ensure equal protein loading. Significant overall treatment effects are denoted by ^p<0.05 (ANOVA of repeated measures). Data are presented by mean values and SEM. Representative immunoblots of the mean densitometry data are shown. AU=arbitrary units.
5.4 Supermature focal adhesions

Focal adhesions are integrin-associated complexes that facilitate communication between cells and their surrounding ECM (30, 46, 156). Fibroblasts typically exhibit a mixture of immature and mature focal adhesions (FAs, (229)) and, when they differentiate into myofibroblasts, the formation of suFAs corresponds with the incorporation of αSMA into stress fibres (43, 184, 230). suFAs can be identified by the coexpression of αSMA with other proteins normally found in FAs including ED-A fibronectin, vinculin, paxillin and tensin (230). suFAs can also be distinguished by size, where suFAs are defined as FAs that are greater than 6 μm in length, mature focal adhesions are typically between 2 and 6 μm in length, and immature FAs are typically less than 2 μm in length (43, 184, 230, 231). The incorporation of αSMA in suFAs is essential to allow myofibroblasts to exert a greater (typically 2 to 4-fold) higher contractile force on the surrounding ECM thereby enhancing ECM tension and maintaining the myofibroblast phenotype (30, 43).

5.4.1 Assessment of the effects of periostin on the formation of supermature focal adhesions

To determine if periostin enhances suFA formation, a novel approach was developed to measure focal adhesion length in sFPCLs by immunofluorescence confocal microscopy based on modifications of protocols described in previous studies (184, 232). In brief, “mini” sFPCL cultures were designed to be thin enough to allow confocal microscopy of the cells within a three-dimensional matrix and while under mechanical tension. These cultures of HTS fibroblasts were prepared in the presence or absence of matrix-associated periostin and, after a 72 hour incubation to induce myofibroblast differentiation, the mini sFPCLs were co-stained with αSMA to confirm myofibroblast differentiation, and vinculin, a focal adhesion protein found in all three focal adhesion classes (Figure 5.7, (184)). Focal adhesion length was measured in serial optical sections of individual cells cultured under 3D matrix tension in collagen using Image-Pro Plus 7.0 and the straight-line tool in Image J as described in 2.2.12.3. The total number of focal adhesions, their average length and their classification as immature (≤ 2 μm), mature (2-6 μm) and supermature (≥ 6 μm) FAs were determined in 30 cells per treatment in HTS fibroblasts derived from three patients.
As shown in Figure 5.8a, periostin treatment had no significant effects on the total number of focal adhesions present in HTS fibroblasts cultured in sFPCLs relative to vehicle controls (53.5 FAs ± 10.8 vs 47 FAs ± 9.9, N=3 patients, n=30 cells per treatment). However, the average length of FAs were significantly increased in periostin treated HTS fibroblasts (4.55 μm ± 0.26 in length) compared to vehicle treated controls (4.02 μm ± 0.24 in length, Figure 5.8b, N=3 patients, n=30 cells per treatment). Vehicle treated HTS fibroblasts contained significantly more immature FAs (10.00 % ± 1.63 vs 7.04 % ± 1.61) and mature FAs (76.95 % ± 1.32 vs 72.91% ± 1.28) than periostin treated fibroblasts. Conversely, periostin treated HTS fibroblasts contained a significantly higher percentage of suFAs than vehicle treated cells (20.04 % ± 2.53 vs 13.12 % ± 2.58) cultured under identical conditions (Figure 5.8c, N=3 patients, n=30 cells per treatment).

5.5 Discussion

The objectives of this chapter were to identify the signalling pathways utilized by periostin to induce myofibroblast differentiation in hypertrophic scarring and to identify the structural consequences of activating this pathway. The in vitro studies presented here demonstrate that periostin promotes/maintains myofibroblast differentiation through the formation of supermature focal adhesions by activating FAK-ROCK mediated pathway(s).

The first objective of this chapter was to identify the receptor(s) utilized by periostin to induce myofibroblast differentiation. Changes in mechanical tension within the ECM are transmitted between the ECM and cells through integrin-adhesion complexes (46). Since periostin has been reported to signal through integrins in other systems (127-134), it was hypothesized that periostin promotes myofibroblast differentiation and persistence through an integrin-mediated pathway. Immunoprecipitation experiments were performed to address this question. When performing immunoprecipitation experiments for receptors, disruption of the cell membrane with harsh detergents, denaturing buffers or mechanical lysing can lead to receptor deactivation and loss of receptor-ligand interaction, especially if the interaction is weak. To account for this, immunoprecipitation experiments were performed in non-denaturing lysis buffers with gentle detergents and a
Figure 5.7 Periostin enhances supermature focal adhesion formation in hypertrophic scar-derived fibroblasts. (a) Primary hypertrophic scar (HTS) fibroblasts were cultured in “mini” stressed fibroblast populated collagen lattices (sFPCLs) for 72 hours in the presence or absence of periostin (PN, 2 µg/ml) as described in 2.2.12.1. Lattices were fixed and assessed for vinculin immunoreactivity (red), alpha smooth muscle actin (αSMA) immunoreactivity (green), co-stained with Hoescht (blue) to identify nuclei and imaged using immunofluorescence confocal microscopy as described in 2.2.12.2. Focal adhesion length was measured as described in 2.2.12.3 and supermature focal adhesions (<6 µm in length) are indicated by arrows. Representative photographs are shown. Scale bar = 20 µm.
Figure 5.8 Periostin enhances focal adhesion length and supermature focal adhesion formation in hypertrophic scar-derived fibroblasts. Primary hypertrophic scar (HTS)-derived fibroblasts were cultured in mini stressed fibroblast populated collagen lattices for 72 hours and focal adhesion numbers and length were assessed by immunofluorescence confocal microscopy as described in 2.2.12. (a) Exogenous PN treatment had no discernible effects on the total number of focal adhesions present in HTS fibroblasts cultured under mechanical tension (N=3 patients, n=30 cells per treatment). (b) The average focal adhesion length was significantly greater in PN treated HTS fibroblasts relative to vehicle (veh) controls (N=3 patients, n=30 cells per treatment). (c) Vehicle treated HTS fibroblasts had significantly more immature and mature focal adhesions relative to PN treated fibroblasts. However, PN treated HTS fibroblasts had significantly more supermature focal adhesions relative to vehicle treated cells under these culture conditions (N=3 patients, n=30 cells per treatment). Significant treatment effects are denoted by *p<0.05 (t-tests). Data are presented by mean values and SEM.
subset of samples were formaldehyde fixed prior to lysis to stabilize potential interactions (233, 234). Optimization studies demonstrated that formaldehyde fixation prior to cell lysis had no discernible negative effects on the ability of the periostin antibody or nickel coated magnetic beads to bind periostin in the samples (data not shown).

Multiple assay conditions were tested to optimize the detection of direct interactions between periostin and integrins. Initial immunoprecipitation studies were performed in sFPCLs to model 3D interactions in vivo, however neither endogenous nor recombinant periostin could be detected in the subsequent immunoprecipitation analyses. While the technical roadblocks that prevented periostin detection in these assays remains unclear, one possibility was that the abundance of collagen caused non-specific antibody binding and/or interfered with antibody-epitope binding. Therefore, despite being potentially less physiologically relevant, subsequent immunoprecipitation experiments were performed on HTS fibroblasts cultured on plastic dishes in the absence of a collagen substrate.

While periostin and integrin subunits were consistently co-immunoprecipitated in these analyses, apparently non-specific, yet consistent, integrin binding was also observed in samples immunoprecipitated with non-antigen specific IgG controls, making it impossible to demonstrate that these interactions were specific to periostin. Optimization studies were undertaken with the aim of reducing non-specific integrin binding in the IgG immunoprecipitated samples. These included 1) the use of different IgG controls from different suppliers, 2) the use of Ni coated magnetic beads to bind histadine tags at the amino terminus of the recombinant periostin, 3) various combinations of incubation times and 4) more stringent binding and washing conditions. None of these modifications proved to be effective for reducing non-specific binding between these integrin subunits and IgG controls.

To our knowledge, there are currently no examples of immunoprecipitation experiments demonstrating interactions between periostin and integrins in the literature. Previous studies investigating periostin interactions with integrins have utilized blocking antibodies that are targeted against specific integrins in functional assays (128, 131, 134). Experiments designed to assess these interactions with HTS fibroblasts in sFPCLs using blocking antibodies against integrin αvβ3 and β1 induced very modest decreases in lattice
contractility that were indistinguishable to the effects induced by non-specific IgG controls (data not shown). Co-localization experiments for periostin and integrins were also performed using immunofluorescence confocal microscopy. However, HTS fibroblasts that were fixed and probed with a fluorescently labeled periostin antibody exhibited abundant and saturated staining throughout the collagen matrix, making it impractical to use this approach to identify specific areas of co-localization with integrins in these cultures (data not shown).

In the event that periostin might interact with and signal through molecules other than integrins, multiple immunoprecipitation experiments were performed using a periostin-specific antibody and coomassie staining to detect differential banding patterns between periostin and IgG immunoprecipitated samples. No differential banding patterns were observed in these assays. Coomassie staining is insensitive relative to some other protein stains, such as silver nitrate, however these stains were incompatible with subsequent mass spectrometry analyses. In summary, no specific interactions between periostin and integrins or other molecules could be demonstrated. The development of substantially more sensitive and specific methodologies will be required for future studies assessing protein interactions with periostin in more physiologically relevant, 3D collagen matrices.

The second objective of this chapter was to identify the signalling pathway(s) utilized by periostin to induce myofibroblast differentiation and persistence. The ROCK signalling pathway was an obvious focus for these studies, as ROCK activation plays a central and essential role in activating and maintaining myofibroblast differentiation and contractility (30, 51-53). ROCK inhibition was shown to significantly attenuate basal HTS fibroblast contractility, and this effect could not be rescued by exogenous periostin treatment. Inhibiting such a central and essential regulator of myofibroblast differentiation and contraction is unlikely to be helpful for distinguishing the specific effects of periostin from multiple signalling pathways that also induce differentiation and contraction in a ROCK dependent manner. Therefore, gain- and loss-of-function studies were performed to assess the effects of periostin addition or depletion on upstream and downstream effectors of ROCK-mediated myofibroblast differentiation and contraction.
Integrin-associated complexes known as focal adhesions \((30, 46, 156)\) mediate communication between cells and the ECM. These complexes transmit external signals from the microenvironment to modulate cellular phenotypes \((235)\). Focal adhesion kinase (FAK) has been shown to localize to focal adhesions \((224, 225)\), regulate ROCK-dependent stress fiber formation and maintenance \((236)\), promote suFAs formation \((43)\) and to be involved in adhesion-mediated myofibroblast differentiation \((226)\). Since periostin has been shown to enhance FAK phosphorylation in other systems \((130, 133, 134, 187, 237, 238)\), it was hypothesized that periostin promotes HTS myofibroblast differentiation and maintenance through a FAK-ROCK dependent signalling pathway.

The findings presented in this chapter demonstrate that, when cultured under isometric tension, periostin had no significant additive effects on FAK phosphorylation in HTS fibroblasts. However, after the release of isometric tension, a significant increase in FAK phosphorylation was observed in periostin treated HTS fibroblasts for up to 48 hours relative to vehicle controls. While these results are not informative regarding FAK activation during myofibroblast differentiation, these data suggest that periostin enhances FAK activation in HTS fibroblasts under conditions of decreasing mechanical tension. Surprisingly, phosphorylated FAK levels were also significantly increased in periostin-depleted HTS fibroblasts 48 hours after collagen lattice release relative to vector transduced controls. These findings may be explained in the context of previous studies demonstrating that FAK signalling can both promote and inhibit ROCK activation and stress fiber maintenance \((236, 239-241)\).

FAK signalling can promote stress fiber formation and contraction through the phosphorylation and activation of p190 RhoGEF \((236, 241)\). p190 RhoGEF is a Rho specific guanine nucleotide exchange factor (GEF) that activates RhoA by promoting the dissociation of guanosine-5’-diphosphate (GDP) and the binding of GTP to RhoA \((242, 243)\). RhoA-GTP binding to ROCK induces a conformational change in ROCK that activates the protein \((244, 245)\). FAK signalling can also promote the phosphorylation and activation of p190 RhoGAP, a GTPase activating protein (GAP), which inhibits RhoA activity by promoting binding of GDP to RhoA \((236, 246)\). Inhibition of RhoA prevents downstream activation of signalling intermediates involved in stress fiber
maintenance and contraction. It is possible that, following the release of mechanical tension in sFPCLs, periostin signalling is able to maintain positive FAK-ROCK activation and thereby promote myofibroblast persistence.

This hypothesis could be tested by assessing the effects of periostin on the activity of RhoA and ROCK. The RhoA activity assays currently available utilize the Rho binding domain of the Rho effector protein, Rhotekin, to specifically bind RhoA-GTP. However, these analyses require the precipitation of Rhotekin-RhoA-GTP complexes using glutathione affinity beads. Unfortunately, the amount of total cell lysate required for this assay would be unattainable in practice for 3D collagen cultures. As ROCK is activated by a conformational change induced by RhoA-GTP binding, its activity cannot be assessed using western immunoblotting. Therefore, to gain additional insights into periostin effects on ROCK activation, the activation of downstream ROCK targets were assessed using gain- and loss-of-function studies.

ROCK activation can promote stress fiber maintenance, focal adhesion formation and myofibroblast differentiation through two divergent pathways. Cofilin, a downstream target of ROCK, is an actin-binding protein that promotes actin stress fiber depolymerization (227). ROCK-mediated cofilin phosphorylation inhibits cofilin activity and actin depolymerization, thereby promoting stress fiber maintenance (228). ROCK activation also induces myofibroblast-mediated contraction through phosphorylation and activation of the regulatory MLC (51-53). In combination, these signalling events promote the formation and maintenance of stress fibers and increase ECM tension through myofibroblast contraction, resulting in integrin clustering, the formation of suFA complexes and myofibroblast differentiation and/or persistence (247).

No significant changes in the phosphorylation states of cofilin or MLC were consistently detected in HTS myofibroblasts cultured under isometric tension or following the release of tension irrespective of treatment. It is likely that the lack of significant findings in these studies can be explained by inter-patient variability. Gender, age, scar location, scar maturity and additional co-morbidities may influence periostin sensitivity between individual patients and this variability can make it difficult to detect significant
differences when patients are assessed as a group. This was evident in several of these studies as statistically significant treatment effects were observed in a subset of individual patients but significance was lost when the patients were assessed as a group. Future analyses of much larger numbers of patient-derived fibroblast lines may be required to overcome this inter-patient variability, and sub-group analyses of those that yield significant results may shed new light on the source(s) of variability. These findings also illustrate the importance of assessing treatment effects in primary fibroblast lines derived from multiple patients, as such variability might not be observed in an immortalized cell line derived from a single patient.

Although inconclusive, these studies did not rule out the possibility that periostin signalled through these intermediates to enhance suFA formation, myofibroblast differentiation and/or persistence. Therefore, independent analyses of suFA formation were performed. As described previously in this chapter, αSMA is only incorporated into stress fibers of myofibroblasts when the mechanical tension in the ECM passes a critical threshold that allows for the formation of suFAs (30). The formation of suFAs and the de novo expression and incorporation of αSMA into stress fibers allows myofibroblasts to exert much greater contractile forces on the ECM (43), thereby contributing to the abnormal dermal density that characterizes scar formation. As periostin promotes myofibroblast differentiation and persistence in hypertrophic scarring (Chapter 4.4 and 4.7), potentially by activating focal adhesion signalling molecules (Chapter 5.3), it was hypothesized that exogenous periostin treatment would enhance the formation of suFAs.

To assess suFA formation, two markers of suFAs, αSMA and vinculin, were assessed by immunofluorescence confocal microscopy. The combination of αSMA and vinculin was optimal for confirming the differentiated state of the myofibroblasts while simultaneously visualizing all three FA classes (184). The length of each FA was assessed along its longest axis regardless of orientation as described in previous studies (184). Using this approach, periostin treated HTS fibroblasts cultured under mechanical tension were shown to exhibit significantly larger focal adhesions and a higher percentage of suFAs (> 6 μm in length) per cell relative to vehicle controls. A feedback mechanism between enhanced suFA formation and increased ECM tension above threshold levels promotes
myofibroblast differentiation and persistence (43, 44) leading to the excessive deposition and contracture of the ECM characteristically seen in hypertrophic scarring. These data suggest that periostin signalling may promote and maintain suFA formation and myofibroblast differentiation in patients prone to hypertrophic scarring.

In summary, periostin was shown to promote myofibroblast differentiation by enhancing suFA formation in HTS myofibroblasts. Although the receptor(s) utilized by periostin to induce suFA formation are yet to be identified, these findings are consistent with periostin enhancing myofibroblast differentiation and persistence in hypertrophic scarring through a FAK-ROCK dependent pathway. This is the first study to link periostin activation of these signalling pathways to suFA formation in hypertrophic scarring. As hypertrophic scarring and related benign fibrotic conditions are characterized by enhanced myofibroblast differentiation and the persistence of these highly contractile cells within the dermis, periostin may have potential as a therapeutic target for novel therapies designed to induce myofibroblast de-differentiation and/or apoptosis and, thereby, enhance hypertrophic scar resolution.
Chapter 6

6 Conclusion

Hundreds of millions of patients in developed countries develop scars each year following surgical procedures, with the incidence rate for hypertrophic scar formation varying between 40-70% in many centers (66, 67, 248). Hypertrophic scars and other abnormal scarring conditions are the result of aberrant wound healing after an insult to the deep dermis. Scarring imposes morbidity that is difficult to quantify, but includes pain and loss of mobility, especially if the scar is associated with a joint (60). As well as imposing a financial burden, scars are often disfiguring and can adversely affect a patient’s quality of life, both physically and psychologically (60, 61). Unfortunately, there are few, if any, truly effective treatment options for abnormal scarring conditions (58, 60). Prevention of hypertrophic scarring is also difficult, as abnormal skin remodelling may not become evident until 4-8 weeks after wound closure (59). Current treatment strategies have therefore focused on improving hypertrophic and keloid scar resolution, rather than on prevention.

The findings presented in this thesis suggest that increased and persistent levels of periostin in the dermis of human hypertrophic scar tissue contribute to excessive fibroblast proliferation and myofibroblast differentiation and persistence (Figure 6.1). Periostin depletion in the dermis of hypertrophic scars may therefore have utility as a therapeutic target for novel therapies designed to improve scar resolution by attenuating fibroblast proliferation and promoting myofibroblast apoptosis and/or dedifferentiation. In addition to hypertrophic scars, periostin is abnormally abundant in keloid scars and periostin may play a similar role in promoting and maintaining myofibroblast differentiation of keloid scar-derived fibroblasts. These data suggest that the effects of periostin described in this thesis may not be specific to HTS but may be a common feature of other benign fibroses. This concept is supported by findings that periostin promotes myofibroblast differentiation of primary fibroblasts derived from Dupuytren’s Disease, a benign fibrosis of the palmar fascia (91). Therefore, periostin may not only be
Figure 6.1 Schematic representation of the roles of periostin in hypertrophic scarring. Increased and persistent levels of ECM-associated periostin in the dermis of hypertrophic scar tissue contribute to hypertrophic scar formation by enhancing fibroblast proliferation and myofibroblast differentiation and persistence through a Rho kinase mediated pathway. Enhanced fibroblast proliferation and myofibroblast differentiation leads to excessive ECM deposition and remodelling leading to the formation of raised, dense collagenous scars.
an attractive therapeutic target to improve abnormal scar resolution but may have utility as a more universal target for treating a variety of benign fibrotic conditions.

Non-healing chronic wounds are a less obvious but potentially relevant application of these findings. Chronic non-healing wounds do not progress beyond the inflammatory phase of wound healing (54), display decreased growth factor activity and increased fibroblast senescence relative to normal healing wounds (249). The prevalence of non-healing wounds within the Canadian healthcare system is approximately 35% (250) and may result in amputation of the affected limb due to lack of effective treatment options (251). Since periostin expression is decreased in chronic non-healing wounds (97), exogenous periostin treatment may stimulate fibroblast proliferation and myofibroblast-mediated ECM deposition and remodelling resulting in wound closure.

In order to assess the role(s) of periostin in normal and abnormal cutaneous wound repair, an appropriate model system was required. Unfortunately, there is a lack of representative and physiological relevant *in vivo* animal models of hypertrophic scarring (59). Hypertrophic or any other forms of fibrotic dermal scarring do not normally occur in mice (163) as they can regenerate dermal wounds very efficiently (160). While several scarring-prone mouse models have been developed after genetic modifications or chemical treatments (165, 252), the physiological relevance of these models are unclear. There are significant anatomical differences between the inter-follicular area (161), epidermal thickness and turnover rate (159) and dermal thickness (160) of mouse and human skin, limiting our ability to translate findings between these species (253, 254). More recently, the red duroc pig has been utilized as a model for human cutaneous wound healing. Swine have a similar dermal and epidermal architecture (255-257) and heal wounds by re-epithelialization in a similar manner to humans (258). In contrast to mouse models, red duroc pigs readily form raised collagenous scars that are similar to hypertrophic scars following insult to the deep dermis (255, 259). Nonetheless, the clinical relevance of these extremely expensive models (257) remains unclear. Very little is known about the complex array of single nucleotide polymorphisms or other genetic or epigenetic changes that predispose some patients to scarring, and these details will need to be elucidated before any truly representative animal models can be developed. As it is
not ethically feasible to perform scarring experiments on humans, it can be argued that the best available models of hypertrophic scarring are the tissues or tissue-derived cells derived from surgical scar resection. In this thesis, primary human dermal fibroblasts derived from hypertrophic scar, keloid scar and normal skin from multiple patients were chosen to assess periostin effects in \textit{in vitro} models.

While the clinical relevance of analyzing primary dermal fibroblasts obtained from normal and abnormal human tissue is obvious, these cells have inherent characteristics that can make them challenging to interpret. Scars are generally resected months or years after their initial formation, making it difficult or impossible to identify the earliest signalling events that promote abnormal scar formation. The major challenge, however, is inter-patient variability. Differences in gender, race, age, scar location, and other co-morbidities may affect the sensitivity of individual patient-derived fibroblasts to treatment and can make it extremely difficult to identify consistent effects on fibroblast proliferation and/or myofibroblast differentiation in all, or even the majority, of patients. This was evident in several studies presented in this thesis, as significant treatment effects were evident in a subset of patients but absent in others. Additionally, variation can arise from selection between passages, as the fibroblasts derived from patient tissues are a heterogeneous population of cells. Therefore, there is the potential to select for different subsets of fibroblasts that have differing tolerances to \textit{in vitro} culture with each passage. With inter-patient and inter-passage variability, it can be difficult to identify statistically significant effects when assessing the mean data derived from a mixed population. Thus, several trends have been identified in this thesis that may or may not represent real effects in a subset of patient-derived fibroblasts. Assessment of very large numbers of patients may be required to identify the source of this inter-patient variability and yield significant findings within pre-identified sub groups of patients. However, such variability may also be impossible to avoid in practice due to the very large number of variables between individuals.

Another limitation of using primary cells is their limited replicative potential compared to immortalized cell lines. The pilot studies that led to this project in our laboratory demonstrated that HTS fibroblasts lose their initial characteristics after approximately
four passages. Due to their short “shelf life,” it is difficult to genetically modify primary fibroblasts by conventional transfection techniques and obtain meaningful results in four passages or less. It could be argued that an immortalized human HTS fibroblast cell line, which currently does not exist to our knowledge, would be beneficial in reducing experimental variability in these assays and in identifying and characterizing periostin-induced specific signalling pathways in more detail. However, the “trade off” with such a cell line is that it would be even more difficult to translate the findings in these immortalized cells to a clinical setting. A single cell line could only provide a detailed assessment of periostin effects in a single patient that may, or may not, be representative of the broad and variable population of individuals affected by abnormal scarring. Thus, despite their inherent limitations, this thesis focused on in vitro analyses of primary fibroblasts derived from surgically resected scar tissue of multiple patients, and on identifying and characterizing the signalling pathways that were activated by periostin in most or all of these patients. With further development, the approach of using primary cell “banks” derived directly from patients may have great potential for providing clinically relevant information about the cellular processes that cause the abnormal persistence of scars.

The molecular focus of this thesis was periostin, a TGFβ-1-inducible secreted ECM protein (101) that is transiently expressed during normal cutaneous wound repair (98, 151, 152), and is abnormally abundant and persistent in abnormal scars and other benign fibroses (91, 97-99, 148, 150, 179, 260-263). This protein is often described as a “matricellular” molecule, a term originally coined by Bornstein (90, 124) to describe extracellular proteins that do not contribute directly to the structure of the ECM but instead serve to modulate cell-matrix interactions and cell function. Periostin has been demonstrated to enhance fibroblast proliferation and myofibroblast differentiation in other models of variable physiological relevance to humans (91, 132, 133, 152, 178-180). To assess the functional consequences of enhancing or depleting any matricellular protein, it is essential that an appropriate 3D cell culture system be in place to assess signalling effects from the ECM that may or may not occur in the absence of a physiologically relevant ECM as a culture substrate. Previous studies have demonstrated that culturing fibroblasts on rigid tissue culture plastic dishes results in spontaneous
proto- and myofibroblast differentiation (31) and that substrate-induced effects can oversheat treatment effects on cellular phenotypes (133). Our laboratory is one of many that have shown that variations in substrate stiffness can modify gene expression and protein levels in primary human fibroblasts (171). Others have shown that, when cultured on tissue culture plastic, cells can adopt very different structures to in vivo and fail to respond to appropriate stimuli. However, if the same cells are cultured on substrates that mimic the in vivo microenvironment, they adopt in vivo-like structures and function (264-266). The effectiveness of therapeutic treatments has also been shown to differ between cells cultured on plastic and on in vivo-like matrices (267-269). Taken together, these studies highlight the importance of the microenvironment in regulating cellular phenotype in in vitro cultures, particularly in the context of matricellular signalling.

For these reasons, the primary human fibroblasts examined in this thesis were cultured on two- and three-dimensional type I collagen substrates, the most abundant collagen in the skin and mature scars (8), to more closely mimic the microenvironment and substrate stiffness experienced by these fibroblasts in vivo. Although the dermal ECM is much more complex than polymerized type I collagen, the use of collagen substrates allows for the incorporation of periostin within a collagenous ECM matrix, mimicking its localization in vivo, and provides a simple and reproducible physiological relevant in vitro culture system for assessing the effects of periostin on hypertrophic scar formation. To gain additional insight into the roles of periostin in promoting abnormal scar formation, future in vitro studies should focus on culturing fibroblasts directly on decellularized hypertrophic scar and normal skin tissue to achieve even better models of the microenvironments in normal and abnormal conditions. Alternatively, much more complex mixtures of purified collagens and other ECM molecules, or co-culture experiments with other cell types involved in cutaneous wound repair such as keratinocytes, may be used for in vitro analyses to enhance the physiological relevance of the cellular environment.

Despite the methodological challenges in achieving such complex culture systems, there is a clear and urgent need for better in vitro models that are physiologically relevant to
humans and representative of a genetically diverse population. Recent high profile reports (270) have described how the majority of peer reviewed medical research publications describing novel drug targets and similar findings cannot be independently reproduced. Amongst many other contributing factors, a lack of physiologically relevant and representative models is likely to have contributed significantly to this problem. We should not be surprised that novel drug targets identified in immortalized human cells derived from a single individual, and then “verified” in animal models with significant physiological differences to humans and with much less individual variability, fail to progress in clinical trials on human populations.

The identification of molecules that promote aberrant wound healing signals will improve our current understanding of the processes involved in abnormal scar formation. The findings presented in this thesis indicate that periostin enhances fibroblast proliferation in compliant collagen cultures, a model designed to mimic the low mechanical tension of the provisional matrix in the early stages of wound healing, through an Akt and ROCK dependent pathway. When subjected to isometric tension in sFPCLs, a model that mimics aspects of wound closure in vivo, periostin induces myofibroblast differentiation, as evidenced by an increase in αSMA levels, collagen lattice contraction and supermature focal adhesion formation. Additionally, periostin signalling maintained αSMA levels in myofibroblasts in an environment of decreasing ECM stress, indicating a role for periostin in myofibroblast persistence, potentially through a FAK-ROCK dependent pathway. Since periostin had no discernible effects on NS-derived fibroblast proliferation or differentiation, these data support the central hypothesis that periostin promotes pathological effects leading to abnormal scar formation.

The function of an organ, including the skin, is dependent on the numerous cell types that make up the organ and the organization of the surrounding extracellular matrix (235). Interactions between the cells and the surrounding ECM through integrins and cell-cell interactions through adherens junctions determine the gene expression and phenotype of the resident cells without altering the genotype (235, 271). Therefore, the microenvironment provides necessary signalling to maintain tissue homeostasis and suppress malignant or abnormal phenotypes. However, aberrant signalling from the
microenvironment can lead to loss of tissue homeostasis and promote abnormal or malignant behaviours in the resident cells (271).

Wounding events have been shown to be an effective stimulus for promoting malignancy (272). Stromal fibroblasts are responsible for the production, deposition and remodelling of the ECM (271) and increased stromal density (changing tissue microenvironment) has been correlated with an increase risk of cancer progression (273, 274). Increased myofibroblast differentiation within the stroma has been correlated with increased risk of cancer development and the induction of an inflammatory response by an established tumour can further promote a fibrotic stroma (271). Studies in mouse models of epithelial cancer progression have demonstrated that increased stromal density enhanced epithelial cell proliferation, tumourgenesis, tumour growth, migration and invasion (275-277), some of which may be induced by FAK and Rho signalling within epithelial cells (278-280).

These finding may be relevant to this thesis because, in addition to being an upregulated component of benign fibroses, periostin expression is also increased in malignant diseases. Periostin is upregulated in a variety of tumours and associated stroma and its increased expression is correlated with malignancy (108, 128, 129, 141, 180, 281). Periostin can interact with several integrins to induce tumour cell migration, growth, EMT and survival (128-131, 142). Over expression of periostin in colon cancer cells increased the size and number of metastatic colonies at secondary sites (129). A recent study by Malanchi et al., (281) demonstrated that metastatic colonies required periostin expression by stromal fibroblasts in order to successfully colonize at those locations. As periostin enhanced fibroblast proliferation and myofibroblast differentiation in fibroblasts derived from hypertrophic scar tissue, the findings in this thesis may enhance our understanding of periostin’s roles in tissue remodelling in metastatic conditions. Increased levels of periostin in the stroma of metastatic diseases may promote excessive fibroblast proliferation and myofibroblast differentiation, causing abnormal stromal density and enhanced tumour formation, growth and invasion. A normal microenvironment can induce some malignant cells to regain a normal phenotype in vitro (235, 282) stressing the importance of the microenvironment in tissue homeostasis and
disease progression. Therefore, periostin depletion in the stroma surrounding malignant cells may be an attractive therapeutic target for novel therapies designed to reduce stromal density.

It has become increasingly evident that achieving normal tissue homeostasis is the ultimate treatment outcome and that targeting the microenvironment may have profound clinical benefits (271). The findings presented in this thesis demonstrate that increased levels of periostin in the wound microenvironment of hypertrophic scar tissue results in excessive fibroblast proliferation and myofibroblast differentiation. Even in conditions of decreasing ECM tension, abundant and persistent levels of periostin in the microenvironment “shield” HTS myofibroblasts from the mechanotransduction signalling events associated with changes in ECM tension, thereby allowing these cells to maintain a differentiated state. Identification of the molecular components and mechanisms involved in periostin regulation and degradation may have profound clinical benefits for improving scar resolution in patients affected by abnormal scarring.

Periostin levels are increased and persistent in the ECM of benign fibrotic conditions such as hypertrophic scarring. It is plausible that the pathways that normally regulate periostin levels in the ECM are atypical in these conditions and that their identification may reveal attractive therapeutic agents for depleting periostin levels in the microenvironment of hypertrophic scar tissue. Periostin expression is reported to be regulated by a number of factors essential for normal cutaneous wound healing such as TGFβ-1, platelet derived growth factor, interleukins, fibroblast growth factor and mechanical tension (101, 103, 104, 283) and these would be difficult to target in a clinical setting. The factors that regulate periostin depletion in normal cutaneous wound healing are unclear. As periostin is proposed to be a very stable protein with a long half-life, and to be difficult to deplete under conditions of high ECM tension (222), active degradation of periostin in the dermis may be responsible for decreased periostin levels in the dermis during the remodelling phase of normal cutaneous wound healing.

Unfortunately, nothing is known about periostin degradation. Previous studies have shown that matricellular proteins thrombospondin-1, SPARC and CCN1 are targets of
several proteases including plasmin, MMP-1 and MMP-9 (284-286). As levels of plasmin, MMP-1 and MMP-9 are decreased in hypertrophic and keloid scarring (287-289), these or similar proteases may have roles in the degradation of the matricellular protein periostin in hypertrophic scarring. This hypothesis should be assessed initially in a cell free system to determine if individual proteases degrade periostin in solution, and then verified in 3D restrained collagen in vitro model systems like those described in this thesis. If periostin levels were significantly reduced following protease treatment relative to controls, addition of these proteases to sFPCL cultures should inhibit periostin-induced HTS myofibroblast differentiation and promote myofibroblast apoptosis and/or dedifferentiation in conditions of decreasing ECM tension.

Another intriguing possibility is that dermal periostin levels may be regulated by receptor-mediated endocytosis and lysosomal degradation. The matricellular protein SPARC has been shown to be regulated by interacting with stabilin-1 (290), a scavenger receptor involved in receptor-mediated endocytosis expressed in macrophages and endothelial cells (291). Periostin and stabilin-1 both belong to the fasciclin family of proteins characterized by the presence of FAS-1 domains involved in cell adhesion and protein-protein interactions (108). Therefore, it is possible that interactions between periostin and stabilin-1 may regulate periostin levels in normal and abnormal wound healing.

Identification of proteases and/or mechanisms involved in periostin degradation may have profound benefits as therapeutic treatments designed promote myofibroblast apoptosis and reduce dermal density in patients affected by hypertrophic scarring. Conversion of the disease microenvironment to a more homeostatic state should improve scar resolution and eliminate the need for costly surgical and therapeutic interventions. It is hoped that these studies will encourage research in the area of periostin regulation in excessive cutaneous wound repair with the goal of uncovering new therapeutic approaches for disease resolution.
References


Appendix

Appendix A - Ethics Approval Notice

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<thead>
<tr>
<th>Document Name</th>
<th>Comments</th>
<th>Version Date</th>
</tr>
</thead>
<tbody>
<tr>
<td>Addition of Co-investigator</td>
<td>A new co-investigator has been added - Dr. Roel Ophoff from UCLA.</td>
<td></td>
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<tr>
<td>Revised Study End Date</td>
<td>The researcher has requested a 5 year extension on this study - December 31, 2016.</td>
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<tr>
<td>Revised UWO Protocol</td>
<td>With this new co-investigator samples will now be sent off site. This has been revised in the letter of information.</td>
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<tr>
<td>Revised Letter of Information &amp; Consent</td>
<td>September 2011 - Controls</td>
<td></td>
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<tr>
<td>Revised Letter of Information &amp; Consent</td>
<td>September 2011 - Study Subjects</td>
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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 for 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 revision(s) or amendment(s) on the approval date noted above. The membership requirements for REBs 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.

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.

The Chair of the HSREB is Dr. Joseph Gilbert. The UWO HSREB is registered with the U.S. Department of Health & Human Services under the IRB registration number IRB 0000540.
Curriculum Vitae

Justin David Crawford

EDUCATION

September 2009 – August 2014

Doctor of Philosophy (PhD)
Department of Biochemistry
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September 2005 – April 2009

Bachelor of Medical Sciences (BMSc)
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EXPERIENCE

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Graduate Research Assistantship
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Supervisor: Dr. D.B. O’Gorman

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Graduate Teaching Assistant
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Biochemistry 3386b – Clinical Biochemistry
Course Coordinator: Dr. Norman Smith

September 2013 – December 2013

Graduate Teaching Assistant
University of Western Ontario, London, Ontario, Canada
Biochemistry 4450a – Molecular genetics of human cancer
Course Coordinator: Dr. David Rodenhiser

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CIHR Summer Student
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Undergraduate Research Student.
Cell and Molecular Biology Laboratory,
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St. Joseph’s Health Care, Lawson Health
Research Institute, London, Ontario, Canada
Supervisor: Dr. D.B. O’Gorman

DISTINCTIONS, HONORS, AWARDS RECEIVED

Institute Community Support Travel Award
Canadian Institutes of Health Research, August 2012
Perisotin: A potential mediator of myofibroblast contraction and survival in a 3-D model of hypertrophic scarring
Value: $1000

Young Investigators Award
European Tissue Repair Society. September 2010
Value: 500 €

Western Graduate Research Scholarship
University of Western Ontario, 2009 – 2012
Value: Covers tuition

Western Scholarship of Distinction
University of Western Ontario, 2005
Value: $1500

RESEARCH STUDENTSHIP AWARDS

September 2010 – August 2011

Frederick Banting and Charles Best Canada Graduate Scholarship – Master’s Award
Canadian Institutes of Health Research
The role(s) of peristin in abnormal wound healing
Supervisor: Dr. David O’Gorman
Value: $17,500

May 2009 – July 2009

IMHA Summer Studentship in Musculoskeletal Research
Canadian Institutes of Health Research
Generation of a lox-stop-lox POSTN cDNA transgene
Supervisors: Drs. D.B. O’Gorman and B.S. Gan
Value: $4,950

BOOK CHAPTERS

PUBLICATIONS


PUBLISHED ABSTRACTS

Crawford JD*, Gan BS and O’Gorman DB
Periostin: A mediator of myofibroblast contraction and survival in a 3-D model of hypertrophic scarring
22nd European Tissue Repair Society Congress, 5 October 2012, Royal Olympic Hotel, Athens, Greece

Crawford JD*, Gan BS and O’Gorman DB
Periostin induces the proliferation of primary hypertrophic scar fibroblasts and the GSK-3β/beta-catenin signaling pathway
21st European Tissue Repair Society Congress, 6 October 2011, Felix Meritis, Amsterdam, Netherlands

Raykha CN, Crawford JD, Gan BS and O’Gorman DB
Is Insulin-like Growth Factor Binding Protein-6 a ‘fibrosis suppressor’ in Dupuytren's Disease?
21st European Tissue Repair Society Congress, 6 October 2011, Felix Meritis, Amsterdam, Netherlands

Raykha CN, Crawford JD, Gan BS and O’Gorman DB
IGFBP-6 and IGF-II: novel mediators of myofibroblast differentiation in Dupuytren’s Disease?
Fifth International Congress of the GRS and IGF Society, 6 October 2010, Sheraton Hotel, New York, NY

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Periostin specifically induces fibroblast proliferation and myofibroblast differentiation in hypertrophic scarring
20th European Tissue Repair Society Congress, 17 September 2010, Het Pand, Gent, Belgium

Raykha CN, Crawford JD, Gan BS and O’Gorman DB
Insulin-like growth factor-II and insulin-like growth factor binding protein-6; previously unrecognized mediators of myofibroblast differentiation in Dupuytren’s disease
20th European Tissue Repair Society Congress, 17 September 2010, Het Pand, Gent, Belgium
Raykha CN, **Crawford JD**, Gan BS, O’Gorman DB
IGFBP-6: A novel mediator of myofibroblast differentiation in Dupuytren’s Disease?
International Dupuytren Symposium, InterContinental Hotel, 22 May 2010, Miami, FL

**ORAL PRESENTATIONS**

**Crawford JD**, Gan BS and O’Gorman DB
Periostin promotes and maintains myofibroblast differentiation in hypertrophic scarring

**Crawford JD**, Gan BS and O’Gorman DB
Periostin: A mediator of myofibroblast contraction and survival in a 3-D model of hypertrophic scarring
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O’Gorman DB, **Crawford JD**, Raykha CN, and Gan BS
Dupuytren's Disease: a model for dissecting the molecular components of fibrosis
Kolling Institute of Medical Research, 18th March 2009, Royal North Shore Hospital, St, Leonards, Sydney, Australia
POSTER PRESENTATIONS

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Periostin: a matricellular inhibitor of apoptosis in hypertrophic scars?

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Dr. Sandy Kirkley Musculoskeletal Research Day, Dr. Sandy Kirkley Centre for Musculoskeletal Research, 28 September 2011, London, ON, Canada

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Examining the Effects of Periostin on Hypertrophic Scar-Derived Fibroblasts
Margaret Moffat Research Day, University of Western Ontario, 30 March 2010, London, ON, Canada

Crawford JD*, Gan BS and O’Gorman DB
The Roles of Periostin in Keloid and Hypertrophic Scars
Western Research Forum, University of Western Ontario, 27 February 2010, London, ON, Canada

* Primary presenter