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Influence of Periostin on Bone Marrow-Derived Cell Contribution to Dermal Wound Repair

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INFLUENCE OF PERIOSTIN ON BONE MARROW- DERIVED CELL CONTRIBUTION TO DERMAL WOUND REPAIR

A Dissertation
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy
Bioengineering

by
Suzanne Parks
May 2014

Accepted by:
Martine LaBerge, PhD, Committee Chair
Richard Visconti, PhD, Advisor
Delphine Dean, PhD
Hai Yao, PhD
ABSTRACT

Originally thought to function only as a scaffold, the extracellular matrix (ECM) is now understood to provide signals that regulate all aspects of cellular phenotype. Matricellular proteins are a subset of ECM-related molecules defined by their ability to modulate interactions between cells and the ECM. Periostin was recently classified as a matricellular protein based on its expression and function during development and wound repair. Periostin has been shown to influence cell behavior and collagen fibrillogenesis in several tissue types. In this study, we examined how periostin expression influences fibrocyte behavior after dermal injury using periostin null (Pstn −/−) and wild-type (WT) mice. Both periostin upregulation and fibrocyte infiltration have previously been shown to accelerate dermal closure after acute injury and to promote excessive scarring in fibrotic tissues. We found that periostin expression elevates fibrocyte levels in murine dermal wounds during wound contraction and at late stages of tissue remodeling. This suggests a novel mechanism by which periostin expression may accelerate the closure of dermal wounds as reported by other investigators and increase collagen accumulation in dermal scar tissue as demonstrated in this work. Periostin upregulation was also found to elevate circulating fibrocyte levels in the blood, suggesting that the peripheral circulation is a significant source of fibrocytes after dermal injury and that periostin expression influences fibrocyte behavior outside of the dermal wound bed. In vitro analysis of fibrocyte migration revealed that periostin expression also increases CXCL12-induced chemotaxis and integrin-mediated migration on periostin. CXCR4+/collagen type I+ (Col+) fibrocyte levels were also found to be elevated after dermal injury in WT mice.
compared to Pstn −/− mice, providing *in vivo* evidence indicating that periostin upregulation promotes fibrocyte participation in the CXCR4/CXCL12 signaling axis. Evaluation of our findings as well as data presented in previous publications suggests that periostin expression elevates fibrocyte levels in the dermal wound bed and peripheral blood by stimulating the fibroblastic differentiation of fibrocyte precursors and/or increasing the migration of fibrocytes and their precursors into these tissues. Collectively, the work presented in this dissertation demonstrates a novel role for periostin in promoting fibrocyte participation in wound repair, and also suggests that therapeutic strategies aimed at modulating periostin expression may be effective in regulating fibrocyte contribution to wound closure and tissue fibrosis.
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TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Component</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>TITLE PAGE</td>
<td>i</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>ii</td>
</tr>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>iv</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>ix</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>x</td>
</tr>
<tr>
<td>CHAPTER</td>
<td></td>
</tr>
<tr>
<td>I. INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>1.1 Clinical Significance</td>
<td>1</td>
</tr>
<tr>
<td>1.2 Project Objective and Specific Aims</td>
<td>4</td>
</tr>
<tr>
<td>1.3 References</td>
<td>6</td>
</tr>
<tr>
<td>II. LITERATURE REVIEW</td>
<td>9</td>
</tr>
<tr>
<td>2.1 Biology of Acute Dermal Wound Repair</td>
<td>9</td>
</tr>
<tr>
<td>2.1.1 Hemostasis and Inflammation</td>
<td>9</td>
</tr>
<tr>
<td>2.1.2 Proliferation</td>
<td>11</td>
</tr>
<tr>
<td>2.1.3 Remodeling</td>
<td>15</td>
</tr>
<tr>
<td>2.2 Introduction to Periostin</td>
<td>16</td>
</tr>
<tr>
<td>2.2.1 Protein Structure</td>
<td>17</td>
</tr>
<tr>
<td>2.2.2 Classification as a Matricellular Protein</td>
<td>18</td>
</tr>
<tr>
<td>2.2.3 Expression in Tissues and Pathologies</td>
<td>19</td>
</tr>
<tr>
<td>2.2.4 Phenotype of the Periostin Null Mouse</td>
<td>21</td>
</tr>
<tr>
<td>2.3 Functions of Periostin in Dermal Wound Repair</td>
<td>21</td>
</tr>
<tr>
<td>2.3.1 Expression in Healthy and Injured Skin</td>
<td>22</td>
</tr>
<tr>
<td>2.3.2 Proposed Mechanisms of Delayed Wound Closure in Periostin Null Mice</td>
<td>23</td>
</tr>
<tr>
<td>2.3.3 Role in Collagen Fibrillogenesis</td>
<td>31</td>
</tr>
<tr>
<td>2.4 Fibrocyte Participation in Wound Repair and Fibrosis</td>
<td>33</td>
</tr>
<tr>
<td>2.4.1 Differentiation and Trafficking</td>
<td>34</td>
</tr>
<tr>
<td>2.4.2 Contribution to Wound Repair</td>
<td>37</td>
</tr>
<tr>
<td>2.4.3 Contribution to Tissue Fibrosis</td>
<td>39</td>
</tr>
<tr>
<td>2.5 Opportunities for Research</td>
<td>45</td>
</tr>
</tbody>
</table>
Table of Contents (Continued)

2.6 References .................................................................................. 46

III. PERIOSTIN EXPRESSION PROMOTES COLLAGEN ACCUMULATION AND FIBROCYTE INFILTRATION IN EXCISIONAL DERMAL WOUNDS .............................................. 55

3.1 Introduction .................................................................................. 55
3.2 Materials and Methods ................................................................. 57
  3.2.1 Murine Model of Dermal Wound Repair .................................. 57
  3.2.2 Dermal Tissue Processing for Histological Examination ...... 58
  3.2.3 Modified Movat’s Pentachrome Staining ............................... 58
  3.2.4 Picro-sirius Red Staining for Collagen ............................... 59
  3.2.5 Immunofluorescence Labeling of Extracellular Matrix Proteins
  ........................................................................................................... 60
  3.2.6 Quantification of Fibrocytes ................................................. 61
3.3 Results ......................................................................................... 62
  3.3.1 Gross Tissue Morphology .................................................... 62
  3.3.2 Composition and Organization of the Reparative Extracellular
  Matrix .................................................................................................. 65
  3.3.3 Fibrocyte Infiltration into the Wound Bed .............................. 70
3.4 Discussion ..................................................................................... 72
3.5 Conclusions .................................................................................. 75
3.6 References ................................................................................... 76

IV. PERIOSTIN EXPRESSION ELEVATES FIBROCYTE LEVELS IN THE PERIPHERAL BLOOD AND SKIN AFTER DERMAL INJURY .......................................................... 80

4.1 Introduction .................................................................................. 80
4.2 Materials and Methods ................................................................. 81
  4.2.1 Murine Model of Dermal Repair ........................................... 81
  4.2.2 Isolation of Dermal Wound Bed Cells ................................. 82
  4.2.3 Isolation of Bone Marrow Cells .......................................... 83
  4.2.4 Isolation of Peripheral Blood Cells ................................. 83
  4.2.5 Flow Cytometry Analysis .................................................... 84
4.3 Results ......................................................................................... 85
  4.3.1 Fibrocyte Population in the Skin ......................................... 85
  4.3.2 Fibrocyte Population in the Bone Marrow .......................... 88
  4.3.3 Fibrocyte Population in the Blood ................................. 90
4.4 Discussion ..................................................................................... 93
4.5 Conclusions .................................................................................. 97
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table of Contents (Continued)</td>
<td></td>
</tr>
<tr>
<td>4.6 References ...........................................................................</td>
<td>97</td>
</tr>
<tr>
<td>V. PERIOSTIN EXPRESSION PROMOTES FIBROCYTE ADHESION AND MIGRATION ......</td>
<td></td>
</tr>
<tr>
<td>IN RESPONSE TO THE CXCR4/CXCL12 SIGNALING AXIS.</td>
<td>100</td>
</tr>
<tr>
<td>5.1 Introduction ..........................................................................</td>
<td>100</td>
</tr>
<tr>
<td>5.2 Materials and Methods ................................................................</td>
<td>101</td>
</tr>
<tr>
<td>5.2.1 Isolation and Culture of Bone Marrow Cells .......................</td>
<td>101</td>
</tr>
<tr>
<td>5.2.2 Immunolabeling of Cultured Bone Marrow Cells .....................</td>
<td>102</td>
</tr>
<tr>
<td>5.2.3 Chemotaxis of Bone Marrow Fibrocytes ................................</td>
<td>103</td>
</tr>
<tr>
<td>5.2.4 Bone Marrow Fibrocyte Adhesion .......................................</td>
<td>104</td>
</tr>
<tr>
<td>5.2.5 Integrin-mediated Migration of Bone Marrow Fibrocytes ..........</td>
<td>105</td>
</tr>
<tr>
<td>5.2.6 Flow Cytometry Analysis of circulating CXCR4+ fibrocytes ......</td>
<td>106</td>
</tr>
<tr>
<td>5.3 Results ..................................................................................</td>
<td>106</td>
</tr>
<tr>
<td>5.3.1 In Vitro Fibroblastic Differentiation of Bone Marrow-Derived Cells</td>
<td>106</td>
</tr>
<tr>
<td>5.3.2 Bone Marrow Fibrocyte Chemotaxis in Response to CXCL12 .......</td>
<td>108</td>
</tr>
<tr>
<td>5.3.3 Adhesion of Bone Marrow Fibrocytes ...................................</td>
<td>109</td>
</tr>
<tr>
<td>5.3.4 Integrin-mediated Migration of Bone Marrow Fibrocytes ..........</td>
<td>110</td>
</tr>
<tr>
<td>5.3.5 Quantification of CXCR4+ Fibrocytes in the Peripheral Blood</td>
<td>113</td>
</tr>
<tr>
<td>5.4 Discussion .............................................................................</td>
<td>115</td>
</tr>
<tr>
<td>5.5 Conclusions ............................................................................</td>
<td>121</td>
</tr>
<tr>
<td>5.6 References ............................................................................</td>
<td>122</td>
</tr>
<tr>
<td>VI. CONCLUSIONS ..........................................................................</td>
<td>125</td>
</tr>
<tr>
<td>6.1 Summary of Key Findings ................................................................</td>
<td>125</td>
</tr>
<tr>
<td>6.2 Suggested Mechanisms ..................................................................</td>
<td>130</td>
</tr>
<tr>
<td>6.3 Recommendations for Future Work ............................................</td>
<td>133</td>
</tr>
<tr>
<td>6.4 References ............................................................................</td>
<td>136</td>
</tr>
</tbody>
</table>
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Summary of three studies investigating the role of periostin in dermal wound repair</td>
</tr>
<tr>
<td>6.1</td>
<td>Generation of chimeric mouse models to study the influence of hematopoietic and structural sources</td>
</tr>
</tbody>
</table>
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Dermal wound repair is characterized by four overlapping phases .......... 9</td>
</tr>
<tr>
<td>2.2</td>
<td>Schematic showing key domains of periostin ................................ 18</td>
</tr>
<tr>
<td>2.3</td>
<td>Immunohistochemical staining of periostin in healthy skin and scar tissue .................................. 20</td>
</tr>
<tr>
<td>2.4</td>
<td>Immunohistochemical staining of periostin before and after dermal injury .................................. 23</td>
</tr>
<tr>
<td>2.5</td>
<td>Periostin gene deletion delays reepithelization and reduces keratinocyte proliferation .................................. 26</td>
</tr>
<tr>
<td>2.6</td>
<td>Periostin promotes α-SMA expression in the dermal wound bed .......... 28</td>
</tr>
<tr>
<td>2.7</td>
<td>Application of exogenous periostin stimulates dermal wound closure .................................. 29</td>
</tr>
<tr>
<td>2.8</td>
<td>TEM micrographs of dermal collagen fibrils and images of H&amp;E stained skin samples .................................. 32</td>
</tr>
<tr>
<td>2.9</td>
<td>Differentiation of CD14+ mononuclear cells into fibrocytes .......... 35</td>
</tr>
<tr>
<td>3.1</td>
<td>Murine model of dermal wound repair .................................. 57</td>
</tr>
<tr>
<td>3.2</td>
<td>Immunofluorescence images of Pstn−/− and WT skin sections labeled with anti-CTR periostin .................................. 63</td>
</tr>
<tr>
<td>3.3</td>
<td>Images of Pstn−/− and WT dermal wounds stained with modified Movat’s pentachrome (4x) .................................. 64</td>
</tr>
<tr>
<td>3.4</td>
<td>Measurements of epidermal and dermal thickness in Pstn−/− and WT wounds before and after dermal injury .................................. 65</td>
</tr>
<tr>
<td>3.5</td>
<td>Images of Pstn−/− and WT dermal wounds stained with modified Movat’s pentachrome (40x) .................................. 66</td>
</tr>
<tr>
<td>3.6</td>
<td>Analysis of dermal collagen content by picro-sirus red staining .......... 68</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
</tr>
<tr>
<td>--------</td>
<td>-------------</td>
</tr>
<tr>
<td>3.7</td>
<td>Immunofluorescence images of Pstn −/− and WT wounds labeled with anti-TNC</td>
</tr>
<tr>
<td>3.8</td>
<td>Immunofluorescence images of Pstn −/− and WT wounds labeled with anti-fibronectin</td>
</tr>
<tr>
<td>3.9</td>
<td>Quantification of fibrocytes in 21-day dermal wounds</td>
</tr>
<tr>
<td>4.1</td>
<td>Fibrocyte and fibroblast subsets in Pstn −/− and WT wounds</td>
</tr>
<tr>
<td>4.2</td>
<td>Quantification of fibrocyte and fibroblast levels in Pstn −/− and WT wounds</td>
</tr>
<tr>
<td>4.3</td>
<td>Quantification of bone marrow-derived cells in Pstn −/− and WT wounds</td>
</tr>
<tr>
<td>4.4</td>
<td>Fibrocyte subsets in bone marrow harvested from uninjured Pstn −/− and WT mice</td>
</tr>
<tr>
<td>4.5</td>
<td>Quantification of fibrocyte levels in the bone marrow of Pstn −/− and WT mice</td>
</tr>
<tr>
<td>4.6</td>
<td>Fibrocyte subsets in the peripheral blood of Pstn −/− and WT mice</td>
</tr>
<tr>
<td>4.7</td>
<td>Quantification of fibrocyte levels in the peripheral blood of Pstn −/− and WT mice</td>
</tr>
<tr>
<td>5.1</td>
<td>Use of the Neuro Probe ChemoTx system to study BM fibrocyte migration</td>
</tr>
<tr>
<td>5.2</td>
<td>Hematopoietic bone marrow-derived cells differentiate into fibrocytes in vitro</td>
</tr>
<tr>
<td>5.3</td>
<td>Chemotaxis of BM fibrocytes in response to CXCL12</td>
</tr>
<tr>
<td>5.4</td>
<td>BM fibrocyte adhesion to immobilized periostin and fibronectin</td>
</tr>
<tr>
<td>5.5</td>
<td>Chemotaxis of cultured BM fibrocytes on periostin and fibronectin-coated filters</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
</tr>
<tr>
<td>--------</td>
<td>-------------</td>
</tr>
<tr>
<td>5.6</td>
<td>Inhibition of BM fibrocyte migration on periostin-coated filters</td>
</tr>
<tr>
<td>5.7</td>
<td>Inhibition of BM fibrocyte migration on fibronectin-coated filters</td>
</tr>
<tr>
<td>5.8</td>
<td>CXCR4⁺/Col I⁺ fibrocyte subsets in the peripheral blood of Pstn −/− and WT mice</td>
</tr>
<tr>
<td>5.9</td>
<td>Quantification of CXCR4⁺/Col I⁺ cell levels in the peripheral blood of Pstn −/− and WT mice</td>
</tr>
<tr>
<td>6.1</td>
<td>Schematic outlining potential mechanisms by which periostin expression increases fibrocyte levels</td>
</tr>
</tbody>
</table>
CHAPTER ONE

INTRODUCTION

1.1 Clinical Significance

Dermal wound healing is a complex process characterized by coordinated cell activity in response to a milieu of molecular and environmental effectors. The wound repair process consists of four highly integrated and overlapping phases (hemostasis, inflammation, proliferation, and remodeling) that ultimately result in the formation of a fibrotic scar\(^1\). Alterations in the normal repair process can lead to significantly worse outcomes by preventing wound closure (chronic wounds) or by inducing excessive fibrosis (hypertrophic and keloid scars)\(^2\). These complications can ultimately result in disfigurement, disability due to loss of function, or even death\(^3\). The enormous demand for therapies that promote the closure of non-healing wounds and reduce the appearance of scars has led to the development of a global market for advanced wound care treatments exceeding $5 billion annually\(^4\). Despite recent innovations in therapeutic strategies, wound healing complications continue to represent a significant burden to the healthcare system due to the complexity of the repair process. The development of improved treatment technologies will require an increase in our understanding of the physiological processes that govern dermal wound repair.

Though much research has focused on the role of major structural proteins in wound repair (i.e. collagens, fibronectin, laminins, etc.), increasing attention is being given to a small group of glycoproteins termed matricellular proteins. Matricellular proteins do not contribute directly to the structural integrity of ECM elements (fibrils,
basement membrane\(^5\). Instead they bind to other matrix proteins, cell surface receptors, and molecules such as proteases and cytokines to modulate cell-matrix interactions and cell function\(^5\). Over the past two decades, the matricellular protein periostin has received much attention, primarily in the areas of cardiac development, cancer biology, and organ fibrosis\(^6\)\(^–\)\(^8\). Research in these fields has shown that periostin expression influences numerous biological processes, including cell differentiation, proliferation, and migration, as well as ECM maturation and remodeling.

Upregulation of periostin expression after acute dermal injury was first reported in 2007 by Jackson-Boeters and colleagues\(^9\). Three independent studies exploring the role of periostin expression in acute dermal repair have since demonstrated that periostin gene deletion leads to delayed wound closure\(^10\)\(^–\)\(^12\). By examining the behavior of resident cell populations, altered wound repair kinetics in \(\text{Pstn}^{-/-}\) mice were concluded to result from delayed reepithelialization, decreased wound contraction, and impaired fibroblast activity. Given the diverse effects of periostin expression on cell behavior in other systems, it is not surprising that periostin appears to influence multiple stages of dermal repair. Additionally, exogenous administration of periostin has been demonstrated to promote wound closure, suggesting that periostin may be a useful target for therapies aimed at accelerating dermal repair\(^12\). On the opposite end of the spectrum, periostin has also been implicated in dermal fibrosis, with scleroderma skin lesions and hypertrophic and keloid scars exhibiting increased levels of periostin expression\(^13\)\(^,\)\(^14\). Despite these observations, the role of periostin upregulation in ECM maturation and remodeling after acute dermal injury has not been analyzed.
Historically, resident fibroblasts have been portrayed as the key cellular effectors that direct wound contraction and ECM deposition after dermal injury. However, recent studies suggest that a population of circulating cells, termed fibrocytes, also play a critical role in these processes. Fibrocytes are a unique subset of collagen-producing hematopoietic-derived cells that home to sites of injury and participate in wound repair and fibrosis\textsuperscript{15}. Fibrocytes contribute to the myofibroblast population in injured skin and have been shown to participate in tissue remodeling through ECM protein production and matrix metalloproteinase secretion\textsuperscript{16,17}. As a significant source of cytokines, chemokines, and growth factors, fibrocytes also contribute to autocrine and paracrine signaling in injured tissues and have been suggested to regulate resident fibroblast activity by promoting proliferation, migration, myofibroblastic differentiation, and collagen production\textsuperscript{18}.

As the field of fibrocyte biology advances, a growing body of evidence suggests that fibrocytes play a critical role in wound repair and fibrotic disease pathogenesis. Therefore understanding the biological mechanisms that govern fibrocyte activity will be important for the development of treatment strategies that prevent fibrosis while also promoting wound repair. Interestingly, fibrocytes appear to promote tissue repair and fibrosis in many of the disease pathologies that also exhibit periostin upregulation, including dermal wound healing, keloid and hypertrophic scarring, pulmonary fibrosis, and interstitial myocardial fibrosis\textsuperscript{15,18-21}. Recently published reports have suggested a potential link between periostin expression and fibrocyte-facilitated tissue fibrosis in the lungs and heart\textsuperscript{22,23}. However, this relationship remains ill-defined and, to our knowledge,
has not been explored in the area of dermal wound healing.

1.2 Project Objective and Specific Aims

The goals of this research project are based on an extensive review of the literature as well as our lab’s interest in the bone marrow contribution of fibroblast-like cells to tissue repair. The matricellular functions of periostin in modulating cell-matrix interactions and cell behavior, as well as the overlapping influences of periostin expression and fibrocyte infiltration on wound repair and tissue fibrosis suggest periostin to be a potential regulator of fibrocyte activity during dermal repair. Thus, our overall objective is to explore how periostin expression influences fibrocyte behavior after dermal injury. Our over-arching hypothesis is that injury-induced periostin expression promotes wound closure and scar formation by stimulating the differentiation and/or engraftment of fibrocytes in dermal wounds. The aims used to complete this project are outlined below.

**Specific Aim I:** Identify the effects of periostin expression on the composition and structural organization of the reparative ECM after dermal injury.

**Rationale:** The final architecture of the reparative dermal matrix is determined by interactions between cells, soluble mediators, and ECM proteins. As a matricellular protein, periostin uniquely functions as a bridge between cells and the surrounding ECM. However, the functional role of periostin expression in scar formation following acute dermal injury has not been described. Therefore, histological staining and
immunofluorescence labeling were used to visualize gross tissue morphology and the deposition of important ECM proteins after dermal injury in Pstn −/− and WT mice. Periostin expression was observed to most significantly promote the accumulation of thick collagen fibers in dermal wounds, a process characteristic of dermal scar formation.

**Specific Aim II:** Determine the effects of periostin expression on fibrocyte levels following dermal injury.

**Rationale:** Fibrocytes contribute substantially to wound repair and fibrosis in a variety of organs. This knowledge has motivated research studying factors that modulate fibrocyte behavior. Based on the intersecting roles of injury-induced periostin expression and fibrocyte infiltration, as well as previous publications investigating heart and lung fibrosis, we hypothesized that periostin expression stimulates fibrocyte contribution to dermal repair. Immunofluorescence microscopy and flow cytometry were used to analyze the influence of periostin on fibrocyte activity in response to dermal injury. Periostin gene deletion was shown to significantly reduce the number of CD45+/Col I+ fibrocytes in the peripheral blood and in dermal wounds. This suggests a potential mechanism by which loss of periostin delays wound closure and reduces collagen accumulation in the reparative matrix (Aim I).

**Specific Aim III:** Establish the effects of periostin expression on fibrocyte trafficking.

**Rationale:** Chemokine-chemokine receptor interactions stimulate the mobilization, homing, and engraftment of fibrocytes and their precursors after tissue injury. Based on
our previous results (Aim II), we hypothesized that in addition to promoting fibroblastic differentiation, periostin expression also promotes injury-induced fibrocyte migration. *In vitro* migration and adhesion assays were performed to determine the effects of periostin expression on CXCR4/CXCL12-induced fibrocyte chemotaxis and integrin-mediated migration. Flow cytometry was also used to quantify *in vivo* differences due to loss of periostin in the number of fibrocytes expressing the chemokine receptor CXCR4. Periostin gene deletion was observed to significantly reduce fibrocyte adhesion and migration *in vitro* and to decrease the number of circulating CXCR4⁺/Col I⁺ fibrocytes *in vivo*. These findings suggest a novel role for periostin in promoting fibrocyte trafficking in dermal wounds, specifically with regards to the CXCR4/CXCL12 signaling axis.

1.3 References


CHAPTER TWO
LITERATURE REVIEW

2.1 Biology of Acute Dermal Wound Repair

Dermal wound repair is characterized by a complex series of overlapping phases, including hemostasis and inflammation, proliferation (reepithelialization, angiogenesis, fibroplasia, and wound contraction), and tissue remodeling (Figure 2.1). The key molecular and cellular effectors that regulate each of these phases will be reviewed in the following sections. The purpose of this review is to provide context for later sections, which analyze studies exploring periostin upregulation and fibrocyte infiltration in dermal wound repair.

Figure 2.1 Dermal wound repair is characterized by four overlapping phases: hemostasis, inflammation, proliferation, and remodeling. A complex profile of cellular and molecular effectors coordinate each phase (from Greaves et al., 2013).²

2.1.1 Hemostasis and Inflammation

Tissue injury is characterized by the mechanical disruption of blood vessels and the immediate extravasation of blood into the wound. To achieve rapid homeostasis,
vasoconstriction is induced by the release of prostaglandins and thromboxanes from injured cells and platelets. Platelet activation is mediated by contact with subendothelial collagen and von Willebrand factor. Upon activation, platelets release proinflammatory mediators and adhesive proteins, which recruit and activate additional platelets and induce platelet aggregation, leading to the formation of a platelet plug. Through the intrinsic and extrinsic coagulation pathways, the platelet plug is strengthened by the formation of a fibrin clot, composed of largely of cross-linked fibrin, fibronectin, vitronectin, and thrombospondin. The fibrin clot acts as a scaffold for the attachment and migration of leukocytes, keratinocytes, fibroblasts, and endothelial cells during subsequent stages of wound repair. It also serves as a reservoir of platelet-secreted growth factors that promote tissue repair. In addition to its deposition, timely degradation of the fibrin clot by fibrinolytic enzymes is essential for wound repair. Inadequate removal of the provisional fibrin matrix impedes normal healing and can cause excessive fibrosis in dermal wounds.

After hemostasis is achieved, an autonomic nervous response and the secretion of vasoactive cytokines from mast cells induces vasodilation and increases capillary permeability. This allows polymorphonuclear cells (PMNs) and monocytes to migrate into the wound bed. PMNs clean the wound bed through phagocytosis of bacteria and foreign particles and also secrete numerous proinflammatory cytokines. Circulating monocytes infiltrate the wound bed shortly after PMNs in response to platelet-secreted growth factors and extracellular degradation products such as collagen and fibronectin fragments and thrombin. Monocytes are transformed into macrophages in the fibrin-
based ECM of the wound bed. There they clear the wound bed of remaining foreign particles, tissue debris, bacteria, and apoptotic PMNs. Macrophages maintain the inflammatory process by secreting additional proinflammatory cytokines and also produce numerous growth factors that stimulate reepithelialization, angiogenesis, fibroblast migration and collagen production, and myofibroblast differentiation\textsuperscript{5,10}. Large burns and infected wounds often exhibit a prolonged inflammatory response. This results in an increased concentration of profibrotic cytokines such as transforming growth factor-β (TGF-β) and platelet derived growth factor (PDGF), which can induce excess fibrosis as seen in hypertrophic and keloid scars\textsuperscript{11}.

\textbf{2.1.2 Proliferation}

The proliferative phase begins around day 3 and lasts 2-4 weeks after injury\textsuperscript{2}. Through the migration and subsequent proliferation of keratinocytes, endothelial cells, and fibroblasts, the provisional ECM produced during hemostasis and inflammation is reepithelialized and replaced by granulation tissue. The major events of the proliferative phase are reepithelialization, granulation tissue formation (fibroplasia and angiogenesis), and wound contraction.

\textit{Reepithelialization}

Within 24 hours, keratinocytes from the free edges of the wound bed and remaining hair follicles begin regenerating the epidermis by migrating across the provisional matrix formed during hemostasis\textsuperscript{1,12}. As reepithelialization progresses, a wedge-shaped mass of keratinocytes is formed, with migrating keratinocytes at the
leading edge, followed by a stratified layer of proliferating cells. Integrins, growth factors, and matrix metalloproteinases (MMPs) are the main molecular effectors that regulate the migration and proliferation of keratinocytes during wound repair. TGF-β1 and macrophage stimulating protein facilitate keratinocyte migration by activating specific integrin receptors that allow keratinocytes to interact with ECM proteins in the wound bed. MMPs promote the degradation of the provisional fibrin matrix, which also enables keratinocyte migration and the reconstruction of the basement membrane. Epidermal thickness is restored through rapid keratinocyte proliferation, which is stimulated primarily by growth factors TGF-α, epidermal growth factor (EGF), and keratinocyte growth factor. Integrin binding as well as the processing of mitogens and cleavage of ECM proteins by MMPs also promotes keratinocyte proliferation by increasing the activity of growth factor receptors and their downstream mediators.

**Angiogenesis**

Angiogenesis refers to the process of new vessel growth through the sprouting of intact capillaries located adjacent to the wound bed. Newly formed blood vessels provide nutrients and oxygen and facilitate cell migration into the granulation tissue. Angiogenesis consists of the following steps: proteolytic degradation of the basement membrane by MMPs, endothelial cell migration towards angiogenic stimuli to form capillary sprouts, proliferation of endothelial cells behind leading migrating cells, reconstruction of the basement membrane, and organization of endothelial cells into stable capillary tubes. Angiogenesis is regulated by signals from the serum and surrounding ECM. Growth factors and cytokines, such as TGF-β, angiopoietin, vascular
endothelial growth factor (VEGF), and fibroblast growth factors 1 and 2 (FGF1 and 2), regulate angiogenesis by modulating endothelial cell proliferation, migration, integrin receptor expression, and tubule formation\textsuperscript{14}. Various ECM components, including collagen, fibronectin, vitronectin, and laminin, provide structural support for invading capillaries and migrating endothelial cells\textsuperscript{15}.

\textit{Fibroplasia}

In response to injury, fibroblasts transform from quiescent cells to migrating proliferative biosynthetically active cells. Fibroplasia describes the migration of fibroblasts into the wound bed and their subsequent proliferation and production of a new collagenous ECM\textsuperscript{7}. Using integrin receptors, fibroblasts migrate through the fibrin matrix by binding and releasing provisional matrix components such as fibrin, fibronectin, vitronectin, and hyaluronic acid. They also secrete MMPs to degrade obstacles that impede their migration. Fibroblast migration and integrin receptor expression are stimulated by macrophage-secreted growth factors PDGF and TGF-\textbeta\textsuperscript{5}. Fibronectin has also been shown to promote fibroblast migration into the wound bed\textsuperscript{16}. Once in the wound bed, fibroblasts gradually adopt a proliferative profibrotic phenotype and begin producing a more permanent ECM composed largely of collagens, proteoglycans, and elastin. Fibroblast proliferation and collagen production are also stimulated by macrophage-secreted growth factors PDGF and TGF-\textbeta, as well as EGF and FGF-2\textsuperscript{5}. Dysregulation of fibroblast function can significantly alter wound repair leading to delayed closure or excessive fibrosis. In chronic wounds of diabetic patients, dermal fibroblasts exhibit significantly reduced levels of proliferation\textsuperscript{17,18}. In contrast, dermal
fibroblasts isolated from keloid lesions overproduce collagen and fibronectin, leading to excessive matrix accumulation\textsuperscript{11,19}.

\textit{Wound Contraction}

Wound contraction is a cell directed process that begins approximately 4 to 5 days after injury and corresponds to the differentiation of fibroblasts into myofibroblasts\textsuperscript{15}. Myofibroblast differentiation is stimulated by the combined action of mechanical tension, TGF-\(\beta\) expression, and EDA-containing fibronectin\textsuperscript{20}. Myofibroblasts contain \(\alpha\)-smooth muscle actin (\(\alpha\)-SMA) filaments that form an intracellular contractile apparatus. These filaments terminate at the cell membrane to form an adhesion complex called the fibronexus, which links intracellular actin to extracellular fibronectin\textsuperscript{21}. The contraction of \(\alpha\)-SMA filaments generates tension across the ECM, which subsequently reduces the size of the wound bed. Contractile forces are stabilized by myofibroblast deposition of ECM collagen. Myofibroblasts normally undergo apoptosis after wound closure. However, in some situations they persist and are considered to contribute to pathological scar formation. Decreased apoptosis of myofibroblasts is thought to lead to the excessive ECM deposition and contractures associated with hypertrophic scar formation\textsuperscript{22}. Dysregulation of myofibroblast function has also been implicated in other fibrotic disorders, such as pulmonary fibrosis, renal fibrosis, and systemic scleroderma\textsuperscript{23,24}.

Historically, myofibroblasts have been considered to originate from resident fibroblast populations that are stimulated to develop a contractile phenotype by injury-induced growth factors and environmental changes\textsuperscript{25}. However, fibrocytes and hair follicle progenitor cells have been proposed to represent alternative sources of
myofibroblasts in dermal wounds\textsuperscript{26-28}. Fibrocytes are circulating hematopoietic stem cell (HSC)-derived progenitor cells that extravasate into the wound bed following injury\textsuperscript{26,29}. They have been found to contribute to wound repair by differentiating into myofibroblast-like cells and secreting collagen type I and inflammatory cytokines\textsuperscript{30,31}. Fibrocytes have also been found to be present in high numbers in post-burn hypertrophic scars\textsuperscript{32}. The role of fibrocytes in dermal wound repair and fibrosis will be discussed in greater detail later in this chapter.

2.1.3 Remodeling

Remodeling is characterized by a gradual change in the composition of the ECM through balanced degradation and synthesis of ECM components over time. Specifically, granulation tissue, which is highly vascular and rich in collagen type III, is replaced by an ECM composed largely of collagen type I with reduced vascular density\textsuperscript{1}. Fibroblasts, neutrophils, and macrophages produce various MMPs that degrade structural ECM proteins, specifically collagen type III\textsuperscript{15}. Collagen type III degradation is accompanied by an increase in fibroblast synthesis of collagen type I. As remodeling progresses, type I collagen fibrils increase in diameter, undergo increased interfibril binding, and are organized into parallel fiber bundles\textsuperscript{15,33}. Cellular and vascular density is also reduced\textsuperscript{15}. In combination, these changes result in the formation of a relatively avascular and acellular scar composed largely of parallel collagen fiber bundles.

Proteoglycans such as decorin, fibromodulin, and lumican are known to regulate collagen fibril diameter by binding to collagen fibrils in the ECM to either inhibit or
promote their aggregation\textsuperscript{34}. There is also a growing body of evidence suggesting that matricellular proteins play an important role in collagen fibril formation and maturation. Rentz et al. postulated that through the surface modification of procollagen fibrils, osteonectin mediates the association of procollagen fibrils with cells and promotes their aggregation and integration into the ECM\textsuperscript{35}. Based on \textit{in vivo} evidence, Egging et al. suggested tenascin-X (TNX) to be an important regulator of matrix maturation and stabilization during wound healing\textsuperscript{36}. Most recently, Maruhashi et al. proposed periostin to promote collagen crosslinking by serving as a scaffold for BMP-1 mediated proteolytic activation of lysyl oxidase (LOX)\textsuperscript{37}. An increased understanding of how these matricellular proteins modulate the deposition and maturation of ECM components during wound repair may assist in the development of therapeutics that promote normal wound repair in diseased tissues. The specific functions of periostin in modulating collagen fibrillogenesis will be discussed later in this review.

\subsection*{2.2 Introduction to Periostin}

The ECM, which continually changes in composition during wound repair, is composed largely of collagen and elastic fibers, glycosaminoglycans, proteoglycans, and glycoproteins. Collectively, these ECM components modulate cell migration, adhesion, differentiation, proliferation, and apoptosis by serving as a scaffold for cells and as a reservoir for a number of growth factors and cytokines. Historically, research efforts have focused primarily on the role of major structural proteins in wound repair, such as fibronectin and various types of collagen. However in recent years, this focus has
expanded to include a small group of glycoproteins termed matricellular proteins. A growing body of evidence suggests that periostin, the most recently classified matricellular protein, contributes significantly to dermal wound repair. The following sections broadly review research publications examining the expression and function of periostin in a variety of tissue types and pathologies. The more specific functions of periostin in dermal wound repair will be discussed after these sections.

2.2.1 Protein Structure

Periostin, originally termed osteoblast specific factor-2, was first identified as an 811-amino acid protein secreted by osteoblasts. The protein was later renamed periostin because of its expression in the periodontal ligament and the periosteum. Periostin is a 90kDa glycoprotein composed of an amino-terminal EMI domain, four fasciclin-1 (Fas1) domains with structural homology to the Drosophila Fas1 gene, and a carboxyl-terminal domain (CTR) (Figure 2.2). The cysteine-rich EMI domain acts as a site for protein-protein interactions, enabling periostin to interact directly with ECM proteins including collagen type I and fibronectin. The four Fas1 domains have been shown to bind proteins such as tenascin-C (TNC) and bone morphogenic protein-1 (BMP-1). In addition to binding ECM proteins, these domains have also been shown to promote integrin-dependent cell adhesion and motility by serving as ligands for αvβ3, αvβ5, and β1 integrins. The CTR domain of periostin contains a heparin binding site and proteolytic cleavage sites that can give rise to different splice variants of periostin.

17
Through these binding domains, periostin is uniquely able to interact with both cells and the ECM environment during dermal wound repair.

![Diagram showing key domains of periostin](https://example.com/diagram.png)

**Figure 2.2** Schematic showing key domains of periostin: amino-terminal EMI domain binds collagen type I and fibronectin, Fas1 domains bind TNC and BMP-1, as well as cell surface integrins, and CTR domain binds heparin and contains proteolytic cleavage sites (from Kudo, 2011)\(^{48}\).

### 2.2.2 Classification as a Matricellular Protein

In 1995 Bornstein introduced the term ‘matricellular’ to denote a subset of secreted proteins whose complex functions are derived from their ability to interact with structural ECM proteins as well as cell surface receptors and other molecules, such as proteases and cytokines\(^{49}\). Matricellular proteins do not contribute directly to the structural integrity of ECM elements, but instead act as modulators of cell-matrix interactions and cell function\(^{49}\). Matricellular proteins are highly expressed during development, growth, and after injury\(^{38}\). However, despite high levels of expression during development, the disruption of most matricellular proteins produces viable mice with only subtle differences in phenotype. These phenotypic differences are exacerbated
upon injury, with the wound healing responses of knockout mice typically differing from their WT counterparts\textsuperscript{50}. Examples of previously classified matricellular proteins include thrombospondins 1 and 2, TNC, TNX, osteonectin, and osteopontin.

In 2008 Norris and colleagues proposed the classification of periostin as a matricellular protein based on its functions in the developing and diseased murine heart\textsuperscript{51}. Periostin is highly expressed in the heart during embryonic development, where it promotes the differentiation of mesenchymal progenitor cells into cardiac fibroblasts. After cardiac injury, periostin promotes tissue remodeling by stimulating the integrin-mediated migration of cardiac fibroblasts toward the infarct region\textsuperscript{52,53}. Additional research has further demonstrated periostin’s function as a bridge between cells and the ECM. For example, periostin expression stimulates vascular smooth muscle cell migration \textit{in vitro}, as well as the integrin-mediated migration of epithelial ovarian cancer cells\textsuperscript{46,54}. Periostin also facilitates the maturation and assembly of collagen type I fibers in the skin and promotes the incorporation of TNC into the ECM by calvarial osteoblasts\textsuperscript{44,45}. The expression patterns of periostin during development and after injury, as well as the phenotype of the Pstn \texttext{-/-} mouse, also correspond to the characteristics of matricellular proteins described by Bornstein. These will be discussed in the following sections.

2.2.3 Expression in Tissues and Pathologies

The expression of periostin has been confirmed in numerous tissue types and pathologies. Periostin plays a pivotal role in the development of the heart, where it is
expressed by cardiac fibroblasts and aids in the organization of the ECM and in the differentiation of the cushion mesenchyme into myofibroblastic valve tissue\textsuperscript{55-57}. It has also been found in healthy adult connective tissues, specifically in the periosteum of bone, the periodontal ligament, and in the skin, where it is expressed by keratinocytes and dermal fibroblasts (Figure 2.3)\textsuperscript{43,58}. Periostin is prominently upregulated during ECM remodeling after dermal injury, myocardial infarction, bone fracture, muscle injury, and vascular injury\textsuperscript{58-62}. Recent studies have also shown that periostin is highly expressed in various human cancers, including colorectal cancer, epithelial ovarian cancer, and breast cancer\textsuperscript{46,63,64}. These studies also suggest that periostin may promote tumor growth and metastasis. Furthermore, periostin expression has been heavily implicated in fibrotic conditions including keloid and hypertrophic scarring, scleroderma, idiopathic pulmonary fibrosis, subepithelial fibrosis of bronchial asthma, bone marrow fibrosis, and fibrous dysplasia (Figure 2.3)\textsuperscript{65-70}.

\textbf{Figure 2.3} Immunohistochemical staining of periostin in healthy skin and scar tissue. Periostin is expressed in the basal lamina and hair follicles by keratinocytes and dermal fibroblasts in healthy skin (black arrows). Periostin expression is associated with thick collagen bundles in hypertrophic scar tissue (black arrows) and thinner collagen fibers in keloid scar tissue (from Zhou et al., 2011)\textsuperscript{70}.
2.2.4 Phenotype of the Periostin Null Mouse

The development of three periostin knockout mouse models has led to numerous discoveries regarding the role of periostin in development, disease, and wound repair\textsuperscript{71-73}. Periostin gene deletion is not lethal and Pstn −/− pups exhibit a grossly normal phenotype. However approximately 14\% of Pstn −/− mice die within 2-3 weeks of birth\textsuperscript{71}. Rios et al. hypothesized that this was due to valvular insufficiency resulting from large acellular deposits of ECM and ectopic clusters of smooth muscle cells in the valve leaflets\textsuperscript{71}. Norris et al. observed a reduction in the diameter and crosslinking of type I collagen fibers in the skin of adult Pstn −/− mice as well as a corresponding decrease in skin stiffness and tensile strength\textsuperscript{44}. Adult Pstn −/− mice were also found to exhibit significant growth retardation, early onset periodontal disease, incisor enamel defects, and incisor eruption disturbance\textsuperscript{71,72}. Collectively, these phenotypic differences suggest roles for periostin in fibroblastic differentiation and ECM maturation and organization. Placing Pstn −/− mice on a soft diet to alleviate mechanical strain on the periodontal ligament partially rescued their growth retardation, periodontal disease, and incisor enamel defects\textsuperscript{71}. This indicates that mechanical forces play a role in the function of periostin.

2.3 Functions of Periostin in Dermal Wound Repair

Interest in the role of periostin during dermal wound repair has increased considerably over the last few years. Initial publications reported changes in the spatiotemporal expression of periostin after excisional dermal injury\textsuperscript{58,70}. These findings,
as well as the development of the Pstn −/− mouse, motivated and enabled additional research exploring the mechanisms by which periostin facilitates dermal wound healing. The findings of these studies will be discussed in the following sections.

### 2.3.1 Expression in Healthy and Injured Skin

The composition of the dermal ECM changes significantly between the processes of development, tissue homeostasis, and wound healing. Understanding the specific changes that occur provides insight regarding the biological effectors that guide each of these processes. Periostin is highly expressed in the dermis, basement membrane, and hair follicles during embryonic development, which suggests that it may facilitate the initial formation of these structures. In healthy adult skin, periostin is localized to the basement membrane and the hair follicles. It is also expressed by keratinocytes and fibroblasts, but is not present in the ECM (Figure 2.4, Day 0). This indicates that periostin expression in the ECM is not required for the maintenance of tissue homeostasis.

Several studies have shown significant upregulation of periostin after dermal injury. Periostin can be detected in the hair follicles and in the basement membrane 1 to 3 days after injury (Figure 2.4, Day 0). Localization of periostin then shifts from the cells to the granulation tissue beginning approximately 3 days after injury and peaking at 7 days (Figure 2.4, Day 7). High levels of periostin expression have also been identified in the ECM of fibrous dermal pathologies, including keloid and hypertrophic scars as well as scleroderma lesions (Figure 2.3). The change in
periostin localization from cells in healthy skin to the ECM during dermal remodeling and in fibrous dermal pathologies suggests that periostin plays a significant role in the skin’s remodeling response to injury.

![Figure 2.4 Immunohistochemical staining of periostin before and after dermal injury. White arrows indicate nuclear localization of periostin before and 3 days after injury. Periostin expression shifts from the cells to the ECM 3 days after injury and remains present through 21 days, peaking at 7 days (from Jackson-Boeters et al., 2009)\(^58\).](image)

### 2.3.2 Proposed Mechanisms of Delayed Wound Closure in Periostin Null Mice

To date, three papers that explore the role of periostin in dermal wound repair have been published\(^{39-41}\). Despite using different derivations of the Pstn \(−/−\) mouse, each describes delayed wound closure in mice lacking periostin. In addition, one of these papers reports accelerated closure of wounds treated with recombinant periostin\(^{41}\). These
findings suggest that periostin plays an important role in wound closure and may have therapeutic applications for the treatment of non-healing wounds. Because wound repair is a complex process characterized by coordinated cellular activity in response to a milieu of molecular and environmental effectors and periostin is known to modulate various cell behaviors as well as matrix deposition in numerous tissue types, it is not surprising that each paper investigates a different mechanism for the function of periostin in dermal wound healing. These mechanisms include: keratinocyte proliferation, myofibroblast differentiation, and fibroblast proliferation and migration. Table 1.1 outlines the experimental setup and proposed mechanism of delayed closure in Pstn −/− mice for each publication.

<table>
<thead>
<tr>
<th>Pstn −/− mouse model</th>
<th>Nishiyama et al., 2011⁴⁰</th>
<th>Elliot et al., 2012⁴¹</th>
<th>Onsuka et al., 2012⁴²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wound dimensions</td>
<td>3mm biopsy punch</td>
<td>6mm biopsy punch</td>
<td>8 or 10 mm biopsy punch</td>
</tr>
<tr>
<td>Time points of delayed closure in Pstn −/− mice</td>
<td>Days 3, 5, and 8</td>
<td>Days 5 and 7</td>
<td>Days 3, 5, 7, and 11</td>
</tr>
<tr>
<td>Proposed cause of delayed closure in Pstn −/− mice</td>
<td>Delayed reepithelialization due to reduced keratinocyte proliferation</td>
<td>Reduced wound contraction due to decreased myofibroblast differentiation and contraction</td>
<td>Impaired proliferation and migration of dermal fibroblasts</td>
</tr>
</tbody>
</table>

Table 1.1 Summary of three studies investigating the role of periostin in dermal wound repair.

*Keratinocyte Proliferation*

Nishiyama and colleagues were the first to report delayed dermal wound closure in Pstn −/− mice⁴⁰. They attributed this to a defect in reepithelialization caused by reduced keratinocyte proliferation. Hematoxylin and eosin stained sections of Pstn −/− wounds were found to exhibit a significantly lower percent reepithelialization than WT
wounds at 3 and 5 days after injury (Figure 2.5, A). In a later publication however, Elliot et al. reported no significant differences in reepithelialization at day 7\textsuperscript{40}. This discrepancy may be due to the analysis of wounds at different time points (3 and 5 days versus 7 days) or to different methods of measurement. Nishiyama et al. reported their results as percent reepithelialization, which is influenced by the size of the wound, whereas Elliot et al. measured epithelial migration distance, which is not dependent on wound size. Elliot et al. reported Pstn\textsuperscript{−/−} wounds to be significantly larger than WT wounds at 5 and 7 days after injury\textsuperscript{40}. Therefore, the lower percent reepithelialization reported by Nishiyama et al. may not reflect an actual delay in wound reepithelialization, but rather the increased area of Pstn\textsuperscript{−/−} wounds.

Nishiyama and colleagues also measured the proliferation of keratinocytes in the wound beds of Pstn\textsuperscript{−/−} and WT mice 3 days after injury using an antibody against the proliferation marker Ki67\textsuperscript{39}. In Pstn\textsuperscript{−/−} and WT mice, a similar number of Ki67-positive cells was found around the hair follicles of uninjured skin and in the granulation tissue and area of migrating keratinocytes of wounded skin. However, the number of Ki67-positive cells surrounding the hair follicles of injured skin was approximately twice as high in WT mice compared to Pstn\textsuperscript{−/−} mice (Figure 2.5, B). This result suggests that early periostin expression around the hair follicles and in the basement membrane may induce keratinocyte proliferation, leading to faster reepithelialization. The authors also studied keratinocyte proliferation \textit{in vitro} using cells from a human keratinocyte cell line (HaCaT cells). Because they do not express significant levels of periostin, HaCaT cells were transfected with a mouse periostin-HA construct. After one week of culture at
confluence, overexpressing HaCaT cultures showed increased bromodeoxyuridine uptake and higher numbers of phospho-NF-κB positive cells, suggesting that overexpression of periostin increases human keratinocyte proliferation.

Figure 2.5 Periostin gene deletion delays reepithelialization and reduces keratinocyte proliferation. A) H&E stained sections show delayed reepithelialization of Pstn −/− wounds. Arrowheads and arrows represent the original wound border and the leading edge of the reepithelialized area, respectively. The dotted line marks the area of the newly formed epidermis. Scale bar: 50µm. B) The percentage of proliferating keratinocytes in the bulge region of hair follicles is reduced in Pstn −/− mice after dermal injury (from Nishiyama et al., 2011)\textsuperscript{39}.

Nishiyama et al. proposed the following mechanism for periostin-induced keratinocyte proliferation: Soon after dermal injury, periostin is expressed in the hair follicles and basement membrane of the skin. There it acts as a scaffold protein by binding to fibronectin, laminin 5γ2, and BMP-1\textsuperscript{39}. Periostin, which has been previously shown to enhance the proteolytic activity of BMP-1, then facilitates the cleavage of laminin 5γ2 by BMP-1\textsuperscript{37}. This is followed by the induction of keratinocyte proliferation through NF-κB phosphorylation. Previous reports, which demonstrate that keratinocyte
adhesion to laminin 5 plays a significant role in NF-κB-mediated keratinocyte proliferation, support this proposal.

Myofibroblast Differentiation

Elliot et al. also reported delayed dermal wound closure in Pstn −/− mice 5-7 days after injury. This delay corresponded with peak periostin expression in the granulation tissue of WT mice. At day 7, Pstn −/− wounds were found to contain significantly fewer myofibroblasts as evidenced by reduced α-SMA expression (Figure 2.6, A and B). Fibroblast recruitment and collagen expression appeared to be unaltered. In vivo application of recombinant human periostin on electrospun collagen scaffolds increased the expression of α-SMA in the wound beds of Pstn −/− mice (Figure 2.6, C and D). Based on these results, Elliot and colleagues proposed that the delay in wound closure was due to decreased wound contraction caused by reduced myofibroblast differentiation. Additional support for this hypothesis was obtained through in vitro experiments. Pstn −/− fibroblasts showed a deficit in their ability to contract a collagen matrix and reduced α-SMA actin expression in three-dimensional and compliant two-dimensional (2D) culture systems. Delivery of recombinant human periostin restored the ability of Pstn −/− fibroblasts to contract collagen gels and increased their expression of α-SMA. Additionally, the inhibition of β1 integrin binding as well as Src and focal adhesion kinase (FAK) signaling reversed periostin-induced gel contraction by Pstn −/− fibroblasts. From this data, the authors proposed that by binding cell surface receptors
periostin is able to modulate intracellular signaling to facilitate myofibroblast differentiation.

Figure 2.6 Periostin promotes α-SMA expression in the dermal wound bed. A and B) Immunohistochemical staining reveals that Pstn −/− wounds exhibit reduced α-SMA expression 7 days after injury relative to WT controls. C and D) Treatment of Pstn −/− wounds with electrospun collagen scaffolds containing recombinant periostin increases α-SMA expression in the wound bed (from Elliot et al., 2012).

Fibroblast Proliferation and Migration

Ontsuka and colleagues also examined the role of periostin in dermal wound repair using Pstn −/− mice. Similar to previous publications, they observed robust periostin expression in the granulation tissue of WT mice 6-9 days after injury and delayed wound closure in Pstn −/− mice. Ontsuka also reported that intradermal injection of recombinant periostin restored normal wound closure kinetics in Pstn −/− mice and accelerated the closure of WT wounds (Figure 2.7). Based on the results of several in vitro experiments, Ontsuka and colleagues attributed the delayed closure of Pstn −/−
wounds to impaired dermal fibroblast proliferation and migration. Using fibroblast cultures established from the skin of newborn mice, they demonstrated that Pstn −/− fibroblasts exhibit a decreased rate of proliferation. Treatment with recombinant periostin slightly enhanced the proliferation of fibroblasts from Pstn −/− and WT mice, as well as normal human dermal fibroblasts. Ontsuka et al. also performed a scratch wound assay with mouse embryonic fibroblasts (MEFs) and showed that MEFs derived from Pstn −/− mice had a slightly lower migratory ability compared to MEFs from WT mice.

![Figure 2.7 Application of exogenous periostin stimulates dermal wound closure. Periostin knockout mice (KO) exhibit delayed dermal wound closure relative to WT controls (A). Intradermal injection of recombinant human periostin (PN) every 2 days increases the rate of wound closure in KO (B) and WT (C) mice. *p<0.05, **p<0.01, ***p<0.001, n=10 for each group (from Ontsuka et al., 2013)41.](image-url)
Unfortunately the authors did not show corresponding \textit{in vivo} evidence for reduced fibroblast migration and proliferation, making it difficult to determine whether delayed wound closure in Pstn $-/-$ mice is caused by these deficits. In addition, contradictory results published by Nishiyama et al. and Elliot et al. raise questions as to the validity of their conclusions regarding \textit{in vivo} wound repair\textsuperscript{39,40}. In tissue sections labeled with anti-Ki67 antibody, Nishiyama observed no difference in the number of proliferating cells in the granulation tissue of adult Pstn $-/-$ and WT wounds\textsuperscript{39}. Elliot and colleagues analyzed the proliferation of adult murine fibroblasts \textit{in vitro} and similarly found no difference between Pstn $-/-$ and WT cells\textsuperscript{40}. Elliot also performed a scratch wound assay using adult dermal fibroblasts and, unlike Ontsuka, observed no difference in the migratory ability between Pstn $-/-$ and WT fibroblasts\textsuperscript{40}. One reason for these discrepancies may be the use of different cell sources. Ontsuka used newborn dermal fibroblasts and MEFs while Elliot used adult dermal fibroblasts. The pattern of periostin expression is different in developing and mature skin. Similarly, newborn and embryonic dermal fibroblasts may respond differently than adult dermal fibroblasts to periostin. In conclusion, while the \textit{in vitro} evidence presented by Ontsuka et al. demonstrates reduced proliferation and migration of Pstn $-/-$ fibroblasts, the cell sources used for the \textit{in vitro} assays must be further considered and additional \textit{in vivo} evidence must be obtained before it can be concluded that periostin accelerates wound repair through fibroblast activation.
2.3.3 **Role in Collagen Fibrillogenesis**

As discussed previously, periostin is expressed in a number of collagen-rich connective tissues during development, growth, and wound repair. Because of this, Norris and colleagues investigated the role of periostin in collagen fibrillogenesis and its effect on the biomechanical properties of connective tissues\textsuperscript{44}. Using co-immunoprecipitation assays and immunogold transmission electron microscopy (TEM), Norris et al. demonstrated that periostin interacts directly with collagen type I. The functional importance of this interaction was further established using Pstn\textsuperscript{−/−} mice. Skin samples from Pstn\textsuperscript{−/−} mice showed reduced dermal thickness and decreased collagen fibril diameter (Figure 2.8). Thermal denaturation temperature, which reflects the level of collagen crosslinking in tissues, was measured using differential scanning calorimetry. Tendons from Pstn\textsuperscript{−/−} mice exhibited lower denaturation temperatures, indicating that loss of periostin expression reduces collagen crosslinking. Furthermore, biomechanical testing revealed that periostin gene deletion also decreases the modulus of elasticity and ultimate tensile strength of murine skin. Collectively, this data indicates that periostin is a regulator of collagen fibrillogenesis and an effector of the biomechanical properties of dermal tissue.
Norris et al. suggested that periostin may facilitate collagen type I fibril maturation and assembly by stabilizing adjacent collagen fibrils during crosslinking or by acting as a substrate for enzymes involved in crosslink formation. In 2010, Maruhashi and colleagues proposed a more specific mechanism for periostin action in collagen crosslink formation. Periostin was shown to promote the proteolytic activation of LOX by BMP-1. LOX is an enzyme that is activated by BMP-1 and plays a critical role in ECM maturation by initiating the crosslinking of collagens and elastin. Maruhashi et al. demonstrated that levels of active LOX protein are decreased in Pstn−/− calvarial osteoblasts and increased in periostin-overexpressing mouse embryonic C3H10T1/2 cells. Additionally, co-immunoprecipitation assays revealed that periostin interacts directly with BMP-1, and immunofluorescence microscopy and western blot analyses showed that periostin promotes the deposition of BMP-1 onto fibronectin. Collectively, these results suggest that periostin facilitates collagen crosslinking by enhancing BMP-1 deposition onto fibronectin in the ECM, which in turn promotes the proteolytic activation of LOX.
2.4 Fibrocyte Participation in Wound Repair and Fibrosis

It appears that periostin contributes significantly to the dermal repair process through its influence on cell behavior and collagen fibrillogenesis. In vivo and in vitro experiments exploring the functions of periostin have focused on resident keratinocytes and dermal fibroblasts, two cell types traditionally understood to direct dermal wound repair\textsuperscript{39-41}. However, recent studies indicate that cells from the bone marrow not only contribute to the inflammatory phase, but also give rise to a population of fibroblast-like cells, often referred to as fibrocytes, that participate in dermal wound repair and remodeling\textsuperscript{26,29}. The effect of periostin expression on fibrocyte behavior in the skin has not been studied. However researchers have proposed a link between periostin expression and fibrocyte contribution to fibrotic lung and heart pathologies\textsuperscript{53,73,77}.

Fibrocytes were first described in 1994 by Bucala and colleagues as a distinct population of blood-borne fibroblast-like cells that migrate into regions of tissue injury\textsuperscript{29}. They were detected in the inflammatory exudate extracted from subcutaneously implanted wound chambers. Fibrocytes uniquely co-express mesenchymal (collagen types I and III, vimentin, and fibronectin) and hematopoietic (CD34, CD45) cell markers, and display an adherent, spindle-shaped morphology when cultured in vitro\textsuperscript{29}. In healthy hosts, fibrocytes are estimated to comprise 0.1% to 0.5% of the nucleated cells in the peripheral blood\textsuperscript{26}. By using chimeric mice transplanted with donor bone marrow, a number of independent studies have established that fibrocytes and their precursors are of bone marrow origin\textsuperscript{30,78-81}. These studies also demonstrate injury-induced recruitment of fibrocytes from the bone marrow into various organs, including the skin, heart, liver, and
lungs. Fibrocytes have also been implicated in numerous fibrotic disorders such as idiopathic pulmonary fibrosis, post-infarct myocardial fibrosis, and hypertrophic and keloid scarring. The following sections will review our current understanding of fibrocyte biology, with a focus on fibrocyte contribution to dermal wound repair and fibrotic disorders.

2.4.1 Differentiation and Trafficking

The differentiation of fibrocytes from their hematopoietic bone marrow-derived precursors appears to be regulated by a complex milieu of chemokines, cytokines, and plasma proteins. Pilling and Gomer proposed four stages during which fibrocyte differentiation was most likely to be regulated by such factors: 1) during the production of fibrocyte precursors in the bone marrow, 2) in the peripheral circulation, 3) as cells traverse the endothelium, and 4) in tissues, where the final maturation and activation of fibrocytes occurs. They also categorized fibrocytes based on their level of differentiation. Early fibrocytes are lineage committed fibrocyte precursors that have not yet elongated. Mature fibrocytes are elongated fibroblast-like cells that have migrated into injured tissues, where they express collagen types I and III. Myofibrocytes are derived from mature fibrocytes. They express α-SMA and appear to function similarly to myofibroblasts in wound contraction and ECM protein production.

Fibrocytes appear to mature in culture from a subpopulation of CD14 mononuclear cells that can be found in the bone marrow, the peripheral blood, and at sites of tissue injury. Several monocyte activators and profibrotic cytokines have been
identified to influence the differentiation of CD14⁺ mononuclear cells into mature fibrocytes and myofibrocytes (Figure 2.9). The profibrotic cytokines interleukin (IL)-4 and IL-13 promote the differentiation of CD14⁺ peripheral blood monocytes into fibrocytes. In contrast, the proinflammatory cytokines interferon gamma (IFN-γ) and IL-12, as well as serum amyloid P (SAP) inhibit fibrocyte differentiation. Administration of SAP after dermal injury has been shown to reduce the number of myofibroblasts in the wound bed and delay wound closure. Naik-Mathuria et al. hypothesized that these changes were due to the inhibition of fibrocyte and myofibrocyte differentiation by SAP. After fibrocytes differentiate from their mononuclear precursors, further differentiation into myofibrocytes can be stimulated by TGF-β and endothelin-1 (ET-1). Myofibrocytes produce higher levels of collagen and fibronectin, express the myofibroblast marker α-SMA, and gradually lose their expression of CD34 and CD45.

Figure 2.9 Differentiation of CD14⁺ mononuclear cells into fibrocytes is regulated by a milieu of injury-induced factors. As mononuclear precursors differentiate they lose CD45 and CD34 expression and produce higher levels of collagen types I and III, fibronectin, and α-SMA.
In vivo cell-tracking experiments in murine models of dermal injury and chronic asthma have demonstrated that intravenously injected fibrocytes migrate rapidly to sites of tissue injury\textsuperscript{26,89}. Circulating cells such as monocytes, neutrophils, and T lymphocytes migrate into injured tissues in response to chemokine signaling. This observation led investigators to study the chemokine receptor expression profile of fibrocytes\textsuperscript{26}. It was determined that cultured human fibrocytes express CCR3, CCR5, CCR7, and CXCR4, and that cultured murine fibrocytes express CCR7, CXCR4, and CCR2\textsuperscript{26,81}. Through in vivo and in vitro studies, Abe and colleagues demonstrated that fibrocytes migrate in response to secondary lymphoid chemokine (SLC)\textsuperscript{26}. SLC is a ligand for CCR7 and has been shown to be expressed at sites of chronic inflammation\textsuperscript{26,90}. Additionally, Phillips et al. demonstrated that circulating CXCR4\textsuperscript{+} fibrocytes migrate toward CXCL12 in vitro and traffic to murine lungs after bleomycin-induced fibrosis\textsuperscript{88}. CXCL12 is a ligand for CXCR4 and has been shown to be upregulated in fibrotic lung tissue and dermal blister burns\textsuperscript{88,91}.

Though our understanding of the mechanisms that govern fibrocyte differentiation and migration are not complete, current data suggests that both processes are regulated by a complex profile of injury-induced factors. Increasing our understanding of the molecular effectors that govern fibrocyte behavior may enable the development of novel therapeutics that accelerate wound closure and reduce tissue fibrosis. Specifically studying matricellular proteins like periostin, which appear to influence myofibroblast differentiation and collagen fibrillogenesis, may further our understanding of how fibrocytes differentiate and migrate into injured tissues.
2.4.2 Contribution to Wound Repair

Bucala et al. first identified fibrocytes while using implanted wound chambers to study the acute cellular response to injury. Fibrocytes, which appeared concurrently with circulating inflammatory cells, accounted for approximately 10% of the cells in wound chambers and were hypothesized to contribute to scar formation based on the increased presence of CD34+ cells in murine and human scar tissue. An abundance of information regarding the contribution of fibrocytes to wound repair has since been obtained from murine and human clinical investigations. Evidence suggests that fibrocytes contribute to wound repair through multiple mechanisms, including by serving as antigen presenting cells (APCs), promoting angiogenesis, and facilitating wound contraction and remodeling.

Human fibrocytes express surface proteins required for antigen presentation, including class II major histocompatibility molecules, the co-stimulatory molecules CD80 and CD86, and the adhesion molecules CD11a, CD54, and CD58. Human fibrocytes stimulate APC-dependent T cell proliferation in vitro and antigen-pulsed murine fibrocytes migrate to proximal lymph nodes to prime naïve T cells in vivo. Additionally, fibrocytes secrete a number of inflammatory cytokines and are an abundant source of potent T cell chemoattractants, including macrophage-inflammatory protein (MIP)-1α, MIP-1β, and monocyte chemoattractant protein-1 (MCP-1). Collectively this information suggests that fibrocytes promote the activation and recruitment of T cells in inflamed tissues, thus playing an important role in initiating the immune response during wound repair and in fibrotic disorders associated with inflammation.
Fibrocytes also secrete several proangiogenic factors, including VEGF, b-FGF, IL-8, and PDGF. Hartlapp et al. demonstrated that endothelial cells adopt an angiogenic phenotype characterized by increased cell migration, proliferation, and tube formation when cultured in fibrocyte-conditioned medium containing these factors. Additionally, the inclusion of fibrocytes or fibrocyte-conditioned medium in matrigel plugs was shown to increase in vivo blood vessel formation relative to fibroblast controls. Fibrocytes were also found to secrete high levels of MMP-9, a proteinase found in wound exudate that mediates endothelial cell invasion during angiogenesis.

Together, these findings demonstrate the ability of fibrocytes to promote each stage of angiogenesis, from the dissolution of the basement membrane to the formation of capillary tubes, through the production of proangiogenic factors.

Synthetically active fibroblasts and myofibroblasts play a critical role in wound closure and ECM remodeling. These cells have traditionally been understood to arise from resident dermal fibroblasts. However, a growing body of evidence suggests that fibrocytes contribute to a unique population of bone marrow-derived fibroblast- and myofibroblast-like cells that infiltrate dermal wounds. Abe at al. observed that fibrocytes exhibit a TGF-β-dependent fibrotic response in vitro. Treatment of fibrocyte cultures with TGF-β enhanced fibrocyte proliferation, myofibroblastic differentiation, and collagen production. Fibrocytes treated with TGF-β also exhibit increased contractile activity in collagen gels. Mori and colleagues confirmed that fibrocytes contribute to the myofibroblast population in vivo by showing that between 4 and 7 days after dermal injury over 60% of fibrocytes in the wound bed also express α-SMA. In addition to
contributing to wound contraction and collagen production, fibrocytes also secrete growth factors, such as connective tissue growth factor (CTGF) and TGF-β, which promote the differentiation of resident dermal fibroblasts into myofibroblasts\textsuperscript{94}.

A recently published study by Kao and colleagues demonstrates the ability of cultured fibrocytes to enhance dermal wound healing\textsuperscript{89}. After making full-thickness excisional wounds, cultured fibrocytes were injected into the tail veins of diabetic mice. Wound healing kinetics and the expression of soluble factors and ECM proteins were measured. Treatment with fibrocyte injection was found to improve the rate of wound closure through increased reepithelialization and wound contraction. Wounds of fibrocyte-treated mice also exhibited higher blood vessel density and increased basal keratinocyte proliferation. Real time PCR quantification of RNA expression showed that treatment with fibrocyte injection upregulated the expression of growth factors (TGF-β, PDGF-A, FGF-7, VEGF, and b-FGF), chemokines (MCP-1 and MIP-1α), collagen type I, and α-SMA in dermal wounds. Collectively, these findings suggest that fibrocytes accelerate impaired dermal wound healing by promoting reepithelialization, wound contraction, and angiogenesis. Of note, periostin expression has also been suggested to stimulate reepithelialization and wound contraction after dermal injury, and to promote tumor angiogenesis\textsuperscript{39,40,95}. However, the relationship between periostin expression and fibrocyte contribution to dermal wound repair has not been examined.

\textbf{2.4.3 Contribution to Tissue Fibrosis}

By producing numerous injury-induced cytokines and ECM proteins, fibrocytes
play an important role in the balance between normal wound repair and pathological fibrosis. Despite their therapeutic usefulness in cases of impaired dermal healing, fibrocytes, like periostin, have been implicated in numerous fibrotic conditions. The contribution of fibrocytes to fibrotic disorders of the lungs, heart, and skin will be discussed. Additionally, our current understanding of how periostin expression affects the function of fibrocytes in each of these conditions will be reviewed.

Lungs

Pulmonary fibrosis, which is characterized by the accumulation of ECM proteins within the interstitial space of the lungs, leads to the progressive destruction of lung tissue architecture and impaired gas exchange. Historically, activated resident fibroblasts have been considered to be the main source of ECM proteins in pulmonary fibrosis. However, recent evidence from several independent studies suggests that fibrocytes also contribute to the pathogenesis of this disease. Using a mouse model of bleomycin-induced pulmonary fibrosis, Phillips et al. demonstrated that intravenously injected human CD45^+Col I^+CXCR4^+ fibrocytes and endogenous murine CD45^+Col I^+CXCR4^+ fibrocytes preferentially migrate to the lungs of bleomycin-challenged mice. The bone marrow and peripheral blood of bleomycin-challenged mice were also found to contain a higher number of fibrocytes relative to saline-treated controls. Collagen deposition and CXCL12 expression were significantly higher in murine lungs after bleomycin exposure. Treatment with a neutralizing antibody against CXCL12 inhibited fibrocyte infiltration and decreased collagen deposition in the lungs, significantly attenuating bleomycin-induced fibrosis. Additionally, data from human studies has
shown that patients with fibrotic interstitial lung disease have a significantly higher number of circulating CD45^+Col I^+CXCR4^+ fibrocytes. Fibrocytes were also found in the lung tissue of 8 out of 9 patients with idiopathic pulmonary fibrosis (IPF), while none were identified in healthy human lungs. CXCL12 was also increased in the plasma of human patients with IPF. Collectively, these results suggest that circulating fibrocytes contribute significantly to pulmonary fibrosis by infiltrating the lungs in response to a CXCL12 gradient and participating in ECM deposition.

Naik et al. recently reported that periostin expression is also increased in the lungs of IPF patients. Lung fibroblasts from IPF patients were shown to produce approximately 3.5 times more periostin than lung fibroblasts from healthy controls. Interestingly, increased periostin levels were also detected in the plasma of IPF patients, suggesting an additional source of periostin, likely from peripheral blood cells. Naik et al. determined that a higher percentage of circulating monocytes in IPF patients secrete periostin and that fibrocytes cultured from the peripheral blood of IPF patients express periostin mRNA, while fibrocytes from healthy individuals do not. Additionally, Pstn^−/− mice were protected from bleomycin-induced pulmonary fibrosis, exhibiting decreased collagen deposition and improved lung architecture. To determine whether structural cell- or hematopoietic cell-derived periostin contributes to pulmonary fibrosis, Naik et al. generated chimeric mice through the transplantation of WT bone marrow into WT recipients (WT → WT) or Pstn^−/− recipients (WT → Pstn^−/−). Complementary chimeras were also created [(Pstn^−/− → Pstn^−/−) and (Pstn^−/− → WT)]. Bleomycin-exposure revealed that both structural and hematopoietic sources of periostin promote
pulmonary fibrosis, as evidenced by changes in collagen deposition. Though the relationship between periostin expression and fibrocyte behavior is not completely understood, the data discussed above suggests a potential link between periostin expression and fibrocyte contribution to pulmonary fibrosis.

Heart

Although connective tissue deposition and remodeling are critical to cardiac repair, the accumulation of collagen after injury results in the formation of a non-functioning scar, which can lead to cardiac dysfunction\textsuperscript{73}. A number of studies have demonstrated that bone marrow-derived fibroblast-like cells, or fibrocytes, contribute to this process of fibrotic remodeling. In a closed chest murine model of ischemic/reperfusion cardiomyopathy (I/RC), daily 15-minute vessel occlusion episodes were found to result in cardiac fibrosis and global left ventricular dysfunction\textsuperscript{99}. Using this model, researchers also observed a prolonged elevation of the mononuclear chemokine MCP-1, and the infiltration of small spindle-shaped cells into the myocardium\textsuperscript{80,99}. These cells expressed markers consistent with fibrocytes, including CD34, CD45, collagen type I, and α-SMA. The administration of SAP, which is known to inhibit fibrocyte differentiation, largely eliminated the presence of fibrocytes in the myocardium and also prevented I/RC induced fibrosis and ventricular dysfunction\textsuperscript{80}. Similar results were later reported in a model of angiotensin-II (Ang-II) infusion, which induced interstitial cardiac fibrosis, hypertension, and cardiac hypertrophy in mice\textsuperscript{100}. Ang-II infusion also promoted the infiltration of spindle-shaped cells that expressed CD34, CD45, collagen type I, and the cardiac fibroblast marker DDR2. Genetic deletion
of MCP-1 prevented Ang-II-induced interstitial fibrosis and eliminated fibrocyte infiltration. Together, these results suggest that interstitial cardiac fibrosis is promoted by the induction of MCP-1 and infiltration of fibrocytes into the myocardium.

After myocardial infarction, periostin expression is highly upregulated and the number of fibroblasts in the infarcted tissue increases substantially\(^{60,73}\). Oka et al. demonstrated that global abrogation of periostin expression reduces the size of the infarct scar\(^{73}\). Though periostin gene deletion increased ventricular rupture, surviving animals were found to exhibit improved long term cardiac function. Interestingly, significantly fewer fibroblasts were found in the infarcted myocardium of Pstn \(-/-\) mice, suggesting that periostin modulates fibroblast behavior during cardiac remodeling. In this study, fibroblasts were identified by immunolabeling with vimentin, a mesenchymal marker that is also expressed by fibrocytes. Visconti et al. subjected clonal EGFP\(^+\) HSC-engrafted mice to myocardial infarction by permanent ligation of the left anterior descending coronary artery\(^{53}\). A dense population of elongated bone marrow-derived cells surrounded by a periostin-rich ECM was observed in the infarcted tissue. These cells exhibited a fibroblast-like morphology and, through RNA analysis, were shown to express collagen type I, leading Visconti et al. to propose that bone marrow-derived progenitors contribute to the formation of the post-infarct scar. Periostin was suggested to be a potential regulator of bone marrow-derived cell fibroblastic differentiation. However, the question of whether periostin specifically alters the contribution of circulating fibrocytes or resident fibroblasts to myocardial scar formation remains to be fully answered.
Skin

Hypertrophic scar tissue has been shown to contain a greater number of fibrocytes than mature scar tissue\(^{32}\). Additionally, mononuclear cells isolated from patients with keloid scars exhibit a higher propensity to differentiate into fibrocytes in vitro and are more resistant to the differentiation-limiting effects of SAP\(^{101}\). Hypertrophic and keloid scars often form after severe thermal injury. In response to cutaneous injury, resident fibroblasts migrate from the adjacent uninjured tissue to participate in wound repair and remodeling. However when large areas of skin are burned long migration distances can prevent fibroblasts from migrating to the center of the wound bed. Based on these observations, Yang et al. hypothesized that circulating fibrocytes represent an additional source of cells that supplement the fibroblast population and contribute to the severe scarring often associated with thermal injury\(^{102}\). Yang et al. isolated mononuclear cells from the blood of burn patients and normal individuals. Adherent cells cultured from burn patients were observed to differentiate into fibrocytes more efficiently and to produce higher levels of TGF-β than fibrocytes derived from healthy individuals\(^{102}\). Additional studies conducted by Wang et al. suggest that fibrocytes also regulate the activity of resident fibroblasts during cutaneous repair\(^{94}\). When treated with medium conditioned by burn patient fibrocytes, dermal fibroblasts exhibited increased cell proliferation, motility, and α-SMA expression. Wang et al. attributed these changes in fibroblast behavior to the higher levels of profibrotic factors TGF-β1 and CTGF produced in vitro by fibrocytes from burn patients.
As previously discussed, periostin expression is also significantly upregulated after dermal injury and remains elevated in hypertrophic and keloid scar tissue. However, despite previous research in fibrotic lung and heart disorders, the relationship between periostin expression and fibrocyte contribution to dermal repair and scar formation has not been investigated.

2.5 Opportunities for Research

The upregulation of periostin expression and the infiltration of fibrocytes are two distinct biological processes that have been shown to play an important role in the repair and fibrosis of wounded and diseases tissues. As a matricellular protein, periostin functions uniquely as a bridge between cells and the surrounding ECM during tissue repair and remodeling. Previous research has demonstrated that periostin facilitates (myo)fibroblastic differentiation and modulates ECM maturation and organization. Periostin has also been shown to promote the proliferation and migration of several cell types. Fibrocytes, derived from hematopoietic progenitor cells, differentiate and migrate from the peripheral circulation into tissues in response to injury-induced factors. They contribute positively to wound repair through multiple mechanisms, including by facilitating wound contraction and remodeling, serving as APCs, and promoting angiogenesis. Fibrocytes have also been linked to severe scar formation in organs such as the lungs, heart, and skin. Despite their overlapping roles in tissue repair and fibrosis, the relationship between injury-induced periostin expression and fibrocyte contribution to wound healing remains poorly understood. This gap in knowledge represents an
opportunity for significant advancement in our understanding of how injury-induced matricellular proteins influence cell behavior during wound healing. Studying the specific relationship between periostin expression and fibrocyte behavior may enable the development of therapeutics aimed at treating non-healing wounds and preventing severe tissue fibrosis.

2.6 References


CHAPTER THREE
PERIOSTIN EXPRESSION PROMOTES COLLAGEN ACCUMULATION AND FIBROCYTE INFILTRATION IN EXCISIONAL DERMAL WOUNDS

3.1 Introduction

Periostin is highly upregulated after injury and in fibrotic pathologies\textsuperscript{1-4}. It has the unique ability to interact with several ECM proteins, including collagen type I, fibronectin, and TNC, as well as with multiple cell surface receptors, such as $\alpha_v\beta_3$, $\alpha_v\beta_5$, and $\beta_1$\textsuperscript{5-8}. Like other matricellular proteins, the functional consequences of abrogation of periostin expression are not fully appreciated during normal tissue homeostasis but become apparent in injured or diseased tissues. Through the use of knockout mice, periostin has been shown to modulate collagen deposition in fibrotic heart and lung pathologies, and to be a regulator of collagen fibrillogenesis in the skin\textsuperscript{3,5,9}. Despite its important role in scar formation, we have a limited understanding of how periostin expression influences the reparative ECM after dermal injury. To date, three independent studies have demonstrated that the global abrogation of periostin expression delays dermal wound closure\textsuperscript{10-12}. These studies examined the effects of periostin expression on the behavior of local keratinocyte and fibroblast populations. Based on their observations, altered wound repair kinetics were concluded to result from delayed reepithelialization, decreased wound contraction, and impaired fibroblast activity.

While periostin expression has been shown to influence resident cell behavior, its effects on circulating cells, specifically fibrocytes, have not been studied. Fibrocytes are a distinct population of blood-borne fibroblast-like cells that migrate into regions of tissue
injury. They contribute to wound repair and fibrosis in several tissue types, many of which also express periostin under pathological conditions. Previously discussed examples of pathological processes that are influenced by both periostin upregulation and fibrocyte infiltration include: dermal wound healing, keloid and hypertrophic scarring, pulmonary fibrosis, and interstitial myocardial fibrosis. Data published by Phillips et al., Naik et al., and Visconti et al. suggest a possible link between periostin upregulation and fibrocyte-facilitated tissue fibrosis in the lungs and heart. However, the relationship between periostin upregulation and fibrocyte activity remains incompletely understood, particularly in the area of dermal wound repair.

In this study, we used Pstn−/− and WT mice to examine how periostin expression after dermal injury influences the composition and architecture of the reparative ECM. Histological staining and immunolabeling techniques were used to visualize gross tissue morphology and the deposition of important ECM proteins after dermal injury. Because we observed reduced collagen deposition in Pstn−/− wounds and previous publications have suggested a link between periostin upregulation and fibrocyte-facilitated tissue fibrosis, we also quantified the number of infiltrating fibrocytes in Pstn−/− and WT wounds to determine whether differences in the reparative ECM may result from changes in fibrocyte contribution to dermal wound healing. Pstn−/− wounds were found to contain significantly fewer fibrocytes at late stages of wound healing, suggesting a novel role for periostin in promoting fibrocyte contribution to dermal wound repair.
3.2 Materials and Methods

3.2.1 Murine Model of Dermal Wound Repair

All animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) at the Medical University of South Carolina (MUSC). Pstn−/− mice were generated using a murine model of global periostin abrogation developed by Oka et al. A targeting vector was used to replace exons 4 through 10 in the periostin gene of C57BL/6 mice (Figure 3.1, A). Age-matched WT mice were used as controls for all experiments. Mice were classified as Pstn−/− or WT through PCR genotyping.

Figure 3.1 Murine model of dermal wound repair. A) A targeting vector designed to replace exons 4-10 of the periostin gene with neomycin was electroporated into mouse embryonic stem cells, which were subsequently injected into C57/BL6 blastocysts to generate chimeric mice. Homozygous Pstn−/− mice were bred from these chimeric mice. B) Full-thickness 4 mm excisional wounds were created on the dorsa of Pstn−/− and WT mice for dermal injury studies.

Full-thickness excisional wounds were created on the dorsa of 2-4 month Pstn−/− and WT mice. Mice were anesthetized by 2% isofluorane nose-cone inhalation and administered analgesia (buprenorphine, 0.1mg/kg) by intraperitoneal injection. The dorsum of each mouse was depilated using a commercial hair removal cream (Nair,
Church & Dwight Company Inc., Ewing, NJ) and the skin was sterilized with 10% povidone-iodine solution and 70% ethanol. Two full-thickness excisional wounds were made on each side of the dorsal midline at the level of the scapulae using a 4 mm biopsy punch (Figure 3.1, B). Prior to wounding, the skin was confirmed to be in the telogen phase of the hair cycle as evidenced by pink colored skin\textsuperscript{22}. Wounds were allowed to heal uncovered.

3.2.2 Dermal Tissue Processing for Histological Examination

Mice were euthanized by isofluorane inhalation followed by cervical dislocation at 7, 11, and 21 days after excisional dermal injury. Tissue samples were fixed in aqueous zinc formalin (Anatech LTD, Battle Creek, MI) overnight at 4°C and embedded in paraffin using an automatic tissue processor (Tissue-Tek\textsuperscript{®} VIP, Miles Scientific). Serial sections (5 μm) were cut using a rotary microtome and placed on positively charged glass slides for staining and immunofluorescence labeling.

3.2.3 Modified Movat’s Pentachrome Staining

A modified Movat’s pentachrome stain was used to study general tissue morphology at different stages of dermal repair. This method stains nuclei and elastic fibers black, collagen and reticular fibers yellow, ground substance and mucin blue, and muscle and fibrin red. Tissue sections from the center of Pstn\textsuperscript{−/−} and WT wounds at 7, 11, and 21 days after injury were deparaffinized in Histo-Clear II (National Diagnostics, Atlanta, GA), rehydrated in a graded ethanol series, and mordanted in Bouin’s solution
for one hour. After washing thoroughly, sections were stained in 1% Alcian blue for 20 minutes, placed in alkaline alcohol for 1 hour, and stained in Verhoeff’s hematoxylin for 15 minutes. Sections were then differentiated in 2% ferric chloride, stained in Woodstain Scarlet-Acid Fuchsin for 90 seconds, rinsed in 0.5% acetic acid water, and differentiated in 5% phosphotungstic acid for 10 minutes. Finally, sections were rinsed in 0.5% acetic acid followed by 100% ethanol and stained in alcoholic saffron solution for 20 minutes before being mounted in a xylene-based mounting medium. All staining solutions were purchased from Electron Microscopy Sciences in Hatfield, PA. Movat’s stained sections were imaged at 4x and 40x using an Olympus BX20 microscope (Olympus America, Center Valley, PA). At least four wounds per genotype and time point were analyzed. The dermal and epidermal thicknesses of skin sections from Pstn −/− and WT mice before and 7, 11, and 21 days after injury were measured using ImageJ software (National Institutes of Health, Bethesda, MD). Measurements, which were taken at the center of each wound bed, were analyzed using a Student’s t-test for independent samples (IBM SPSS Statistics, IBM Corporation, Armonk, New York), where p-values <0.05 were considered significant.

### 3.2.4 Picro-sirius Red Staining for Collagen

Picro-sirius red staining enhances the intrinsic birefringence of collagen fibers and can be used to analyze collagen content and fiber morphology in connective tissues. Tissue sections from the center of Pstn −/− and WT wounds at 7, 11, and 21 days after injury were deparaffinized in xylene and rehydrated in a graded ethanol series. Nuclei
were stained in iron hematoxylin for 10 minutes and collagen fibers were stained in picro-sirius red (Sigma Aldrich, St. Louis, MO) for one hour at room temperature. Sections were rinsed in 1% glacial acetic acid, dehydrated in ethanol, and cleared in xylene before mounting with coverslips. An Olympus BX63 microscope (Olympus America) was used to image sections from each wound at 40x magnification under polarized light. At least four wounds per genotype and time point were imaged. The percent areas of thick and thin collagen fibers in each image were measured using ImageJ software. Mean percent areas were calculated for each time point and analyzed using a Student’s t-test for independent samples, where p-values <0.05 were considered significant.

3.2.5 Immunofluorescence Labeling of Extracellular Matrix Proteins

Immunofluorescence microscopy was used to observe the deposition of specific extracellular matrix proteins over the course of wound repair. Tissue sections from the center of four Pstn −/− and four WT wounds at 7, 11, and 21 days after injury were deparaffinized in Histo-clear II and rehydrated in a graded ethanol series. Antigen retrieval was accomplished by placing slides in a pressure cooker containing a citric acid based antigen-unmasking solution (Vector Laboratories, Burlington, CA) for 5 minutes. After permeabilization in 0.1% Triton X-100 and blocking with Background Buster (Innovex Biosciences, Richmond, CA), tissues were incubated overnight at 4°C with primary antibodies [rabbit anti-mouse CTR-periostin and rabbit anti-mouse TNC (provided by Dr. Stanley Hoffman at MUSC) and rabbit anti-mouse fibronectin
(Cedarlane Laboratories, Burlington, NC). Primary antibodies were then localized by incubation with Cy5-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) for one hour at room temperature. Nuclear counterstaining was achieved with Yo-Pro-1 (Molecular Probes/Life Technologies, Eugene, OR) and sections were mounted in Fluoro-Gel (Electron Microscopy Sciences, Hatfield, PA). A Leica SP5 laser scanning confocal microscope (Leica Microsystems Inc., Exton, PA) was used to image sections at 10x and 63x magnification. Images were processed for qualitative analysis using ImageJ software and Adobe Photoshop CS5 (Adobe Systems Inc., San Jose, CA).

3.2.6 Quantification of Fibrocytes

Fibrocytes can be identified by the co-expression of a hematopoietic marker (CD45, CD34) and a mesenchymal marker (collagen type I, vimentin)\textsuperscript{24-26}. To quantify the percentage of fibrocytes in 21-day Pstn \textsuperscript{−/−} and WT wounds, skin sections were double labeled with goat anti-mouse CD45 (R&D Systems Inc., Minneapolis, MN) and rabbit anti-mouse collagen type I (Cedarlane Laboratories) using the methods described above. Primary antibodies were localized by incubation with Cy3-conjugated donkey anti-goat IgG (Jackson ImmunoResearch Laboratories) and Cy5-conjugated donkey anti-rabbit IgG. High-powered images from at least three sections per wound and four wounds per genotype were taken using a Leica SP5 laser scanning confocal microscope. Total cells, CD45\textsuperscript{+} cells, and CD45\textsuperscript{+}/Col I\textsuperscript{+} cells were counted using Adobe Photoshop CS5 software. Cell counts from each of the three sections per wound were summed and used
to calculate the average percentages of CD45$^+$ cells and CD45$^+$/Col I$^+$ cells in Pstn $^{−/−}$ and WT wounds. Results were analyzed using a Student’s t-test for independent variables, where p-values <0.05 were considered significant.

3.3 Results

3.3.1 Gross Tissue Morphology

Previous publications have demonstrated that periostin expression is induced after excisional dermal wounding and is maximally upregulated 7 days after injury$^{1,10-12}$. We confirmed these results in our murine model of dermal repair through immunofluorescence labeling of WT wounds with an antibody against the CTR domain of periostin. Images taken at 10x magnification show that periostin is expressed in the granulation tissue of WT wounds at 4, 7, and 11 days after injury, with peak expression occurring at 7 days (Figure 3.2, A, B, and C). Additionally, images taken at 63x magnification confirm that periostin is not expressed after dermal injury in Pstn $^{−/−}$ mice (Figure 3.2, D and E).
Figure 3.2 Immunofluorescence images of Pstn −/− and WT skin sections labeled with anti-CTR periostin (green) and counterstained with Yo-Pro-1 nuclear stain (red). A, B, and C) Periostin is expressed in the granulation tissue after injury, with maximal expression occurring at 7 days. Scale bar: 500 µm. D and E) High-powered images confirm global abrogation of periostin expression in Pstn −/− mice. Scale bar: 50 µm.

Though periostin is prominently upregulated during ECM remodeling in a variety of tissues and has been implicated in numerous fibrotic pathologies, its influence on dermal scarring has not been studied. To compare the effects of periostin expression on the composition and architecture of the ECM at different stages of wound repair, Pstn −/− and WT wounds were stained with a modified Movat’s pentachrome stain. At 4x magnification, only slight differences in gross tissue morphology were observed between Pstn −/− and WT mice at 7, 11, and 21 days (Figure 3.3). Most notably, Pstn −/− wounds appeared to exhibit reduced epidermal thickness, particularly at early stages of repair (Figure 3.3, 7 days).
Figure 3.3 Images of Pstn −/− and WT dermal wounds stained with modified Movat’s pentachrome. Scale bar: 0.5 mm.

To quantify this difference and to determine whether a loss of periostin also decreases dermal thickness, we measured the epidermal and dermal thicknesses of Pstn −/− and WT wounds using Movat’s pentachrome stained sections (Figure 3.4, A and B). Dermal thickness measurements were not statistically different between Pstn −/− and WT mice before or at any time point after skin injury (Figure 3.4, B). However at 7 and 21 days, epidermal thickness values were significantly lower in Pstn −/− mice relative to WT mice (Figure 3.4, A). Average epidermal thickness values were approximately 110 µm and 23 µm in Pstn −/− mice and 189 µm and 49 µm in WT mice at 7 and 21 days, respectively (p= 0.022 and p= 0.021). These results substantiate previous findings by Nishiyama et al., which demonstrate that Pstn −/− mice exhibit delayed wound
reepithelialization and suggest reduced keratinocyte proliferation in the absence of periostin to be the cause\textsuperscript{10}.

![Graph A](image1)

![Graph B](image2)

Figure 3.4 Measurements of epidermal and dermal thickness in Pstn $-/-$ and WT wounds before and after dermal injury. A) Epidermal thickness is reduced in Pstn $-/-$ mice at 7 and 21 days. B) Dermal thickness is not altered by periostin gene deletion during cutaneous wound repair. *p<0.05

3.3.2 Composition and Organization of the Reparative Extracellular Matrix

To further study whether periostin expression influences the composition and architecture of the reparative ECM, Movat’s stained sections were also imaged at 40x magnification. No consistent differences were observed in the reparative ECM of Pstn $-/-$ and WT wounds at 7 or 11 days after injury (Figure 3.5, 7 and 11 days). However at 21 days, collagen content and fiber thickness appeared to be reduced in Pstn $-/-$ wounds compared to WT wounds (Figure 3.5, 21 days).
Figure 3.5 Images of Pstn −/− and WT dermal wounds stained with modified Movat’s pentachrome. At 40x magnification, 21-day WT wounds appear to contain higher collagen content and thicker collagen fibers than 21-day Pstn −/− wounds. Scale bar: 100 µm.

Periostin gene deletion has been shown to reduce collagen fibril diameter and decrease collagen crosslinking in healthy connective tissues\textsuperscript{5}. To quantitatively assess whether periostin expression influences collagen content and fiber morphology after dermal injury, skin sections from Pstn −/− and WT wounds were stained with picro-sirius red and imaged under polarized light. Under polarized light, thicker collagen fibers appear red/orange and thinner collagen fibers appear green/yellow. Some researchers have suggested that fiber color can be used to determine collagen type, with red/orange fibers representing collagen type I and green/yellow fibers representing collagen type III\textsuperscript{27}. However, though collagen type I fibers are generally thicker than type III fibers, immature collagen type I fibers may also appear green/yellow, especially during early wound remodeling\textsuperscript{28}. Thus when analyzing picro-sirius red stained sections, we will
describe collagen fibers as thick or thin, with the understanding that collagen type I fibers are typically, but not always represented by thick or red/orange fibers.

Similar to Movat’s stained sections, collagen content and fiber diameter appeared to be reduced in 21-day Pstn −/− wounds stained with picro-sirius red. Representative images of 21-day picro-sirius red stained sections are shown in Figure 3.6, A and B. Collagen content was quantified using ImageJ software to measure the percent area of each fiber type in images taken at 40x magnification. In the absence of periostin, thick collagen fiber content was significantly reduced at 21 days after injury, with the average percent area of thick collagen fibers in Pstn −/− wounds equaling 9.4% versus 15.8% in WT wounds (p=0.045, Figure 3.6, C). Differences between Pstn −/− and WT wounds in thick fiber content at earlier time points and in thin fiber content throughout dermal repair were not significant (Figure 3.6, D). Collectively, these results suggest that periostin expression increases the accumulation of thick fibers, which likely represent collagen type I fibers, in later stages of dermal repair.
In addition to interacting directly with collagen type I, periostin also binds to TNC and fibronectin. In calvarial osteoblast cultures, periostin has been shown to play a critical role in incorporating these proteins into the architecture of the ECM. To our knowledge however, the influence of periostin expression on TNC and fibronectin deposition in dermal wounds has not been investigated. To study this, tissue sections from Pstn −/− and WT mice at 7, 11, and 21 days after injury were labeled with...
antibodies against TNC and fibronectin. In general, Pstn −/− wounds were observed to express slightly lower levels of TNC than WT wounds at 7 and 11 days after injury, as indicated by reduced fluorescent signals (Figure 3.7). TNC deposition also appeared to be more granular and less associated with ECM fibers in a majority of Pstn −/− wounds at these time points. At 7 days, fibronectin expression was observed to be higher in 3 out of 5 WT wounds relative to Pstn −/− wounds. No differences were observed in the morphology of fibronectin fibrils between Pstn −/− and WT mice. Representative images of 7-day Pstn −/− and WT wounds labeled with fibronectin are shown in Figure 3.8.

![Immunofluorescence images of Pstn −/− and WT wounds](image)

Figure 3.7 Immunofluorescence images of Pstn −/− and WT wounds labeled with anti-TNC (green) and counterstained with Yo-Pro-1 nuclear stain (red). Minimal TNC expression was observed at 21 days (data not shown). TNC expression appeared to be slightly reduced and more granular at 7 and 11 days after injury in Pstn −/− wounds compared to WT wounds. Scale bar: 50 µm.
Figure 3.8 Immunofluorescence images of Pstn $-/-$ and WT wounds labeled with anti-fibronectin (green) and counterstained with Yo-Pro-1 nuclear stain (red). Minimal fibronectin expression was observed at 11 and 21 days (data not shown). Higher levels of fibronectin expression were observed in a majority of WT wounds compared to Pstn $-/-$ wounds. Scale bar at 10x: 500 µm, at 63x: 50 µm.

3.3.3 Fibrocyte Infiltration into the Wound Bed

Periostin has been described as a profibrogenic protein that mediates both ECM protein synthesis and fibroblast differentiation$^{1,29}$. Our results suggest that periostin expression increases collagen content in dermal scar tissue. The purpose of this experiment was to determine whether bone marrow-derived cells represent a source of this increase. Fibrocytes are commonly identified by the co-expression of CD45 and collagen$^{26}$. To establish the effects of periostin expression on fibrocyte infiltration into the wound bed, tissue sections from the center of 21-day Pstn $-/-$ and WT wounds were
labeled with antibodies against CD45 and collagen type I. Sections were imaged at 63x magnification and the number of total cells, CD45+ cells, and CD45+/Col I+ cells were counted for each wound. Representative images of labeled tissue sections are shown in Figure 3.9, A. The average percentage of CD45+/Col I+ cells was calculated to be over four times higher in WT wounds than in Pstn −/− wounds (2.52% in Pstn −/− and 10.16% in WT, p= 0.00013, Figure 3.9, B). This indicates that periostin expression increases the number of fibrocytes present in dermal wounds. The average percentage of CD45+ cells in WT wounds was slightly lower than in Pstn −/− wounds. However, this difference was not statistically significant (17.93% in Pstn −/− and 23.80% in WT, p= 0.566, Figure 3.9, B).

Figure 3.9 Quantification of fibrocytes in 21-day dermal wounds. A) Pstn −/− and WT wounds were immunolabeled with anti-CD45 (red), anti-collagen type I (green), and Yo-Pro-1 nuclear stain (blue). White arrows indicate CD45+/Col I+ cells. Scale bar: 50 µm. B) The percentages of CD45+ cells and CD45+/Col I+ cells were calculated. At 21 days, WT wounds were found to have a significantly higher number of CD45+/Col I+ cells relative to Pstn −/− wounds. ***p<0.001
3.4 Discussion

Impaired dermal wound healing can manifest either as delayed wound closure (non-healing chronic wounds) or as excessive wound fibrosis (keloid or hypertrophic scars). Previous studies have focused on the function of periostin in dermal wound closure, demonstrating its potential applications for the treatment of non-healing dermal lesions. However, periostin expression has also been implicated in dermal fibroproliferative disorders, which due to an overabundant deposition of collagen fibers result in excessive scarring. In this study, we showed that periostin gene deletion alters scar tissue composition by reducing collagen content during later phases of wound remodeling. We also investigated bone marrow-derived cells as potential contributors to this change and demonstrated that the global abrogation of periostin reduces fibrocyte contribution to dermal wound healing.

Periostin can interact directly with several ECM proteins that are secreted during dermal wound repair, including collagen type I, TNC, and fibronectin. We studied the expression of these proteins in Pstn−/− and WT mice after excisional dermal wounding to determine whether periostin influences their expression in the reparative matrix. Analysis of Movat’s pentachrome and picro-sirius red stained sections revealed that thick collagen fiber content is significantly reduced in the scar tissue of 21-day Pstn−/− wounds. Recent studies have shown that loss of periostin expression reduces collagen accumulation and improves tissue architecture in murine hearts after myocardial infarction and in murine lungs after bleomycin-induced pulmonary fibrosis. Periostin gene deletion has also been observed to reduce collagen fibril diameter and crosslinking
in murine skin and tendons, suggesting that periostin plays an important role in collagen fibrillogenesis. Collectively, these observations correspond with our results, which demonstrate that loss of periostin expression reduces the accumulation of thick collagen fibers in dermal scar tissue.

Periostin gene deletion has also been shown to alter the meshwork architecture of the ECM in the periosteum and in calvarial osteoblast cultures, specifically with respect to TNC and fibronectin deposition. To determine whether similar changes occur in the reparative dermal ECM, we used immunofluorescence microscopy to localize TNC and fibronectin expression at different stages of dermal repair. Previously, Kii et al. reported that loss of periostin expression significantly reduces TNC deposition in the periosteum. In this study, Pstn−/− mice were also observed to express lower levels of TNC than WT mice after dermal injury. However, the reduction in fluorescent signal was not as significant as previously reported in the periosteum. TNC deposition also appeared to be more granular and less associated with the dermal ECM in Pstn−/− mice, a difference also detected in Pstn−/− calvarial osteoblast cultures. Periostin has been proposed to function as a bridge between TNC and the ECM through its EMI and fasciclin-1 domains, which interact with collagen type I and fibronectin, respectively. Reduced deposition of TNC on the reparative dermal ECM in Pstn−/− mice is likely a result of this function of periostin. Interestingly, periostin is expressed under homeostatic conditions in the periosteum, but not in the dermis. Homeostatic expression of periostin may cause periostin gene deletion to affect the ECM architecture of the periosteum to a greater degree than that of injured skin.
Kii et al. also observed the fibronectin network produced by Pstn $\sim\sim-$ calvarial osteoblasts to contain thicker fibers with fewer branched connections relative to the fine fibronectin meshwork produced by WT cells. In contrast, we did not observe differences in fibronectin fibril morphology after dermal injury. As with TNC, fibronectin expression did however appear to be slightly reduced in Pstn $\sim\sim-$ wounds. Decreased fibronectin expression after dermal injury has previously been observed in TNC $\sim:\sim$ mice. This suggests that reduced TNC expression due to periostin gene deletion may also decrease fibronectin expression in dermal wounds. Collectively, our results indicate that a loss of periostin slightly alters the deposition of both TNC and fibronectin in the reparative ECM. However, these changes are less dramatic than those observed in the periosteum and in osteoblast cell cultures. Overall, global abrogation of periostin appears to most significantly alter collagen accumulation in the reparative dermal ECM.

Research investigating the role of periostin in dermal wound healing has focused largely on the activity of local keratinocyte and fibroblast populations. However, an increasing body of evidence suggests that bone marrow-derived fibrocytes play an important role in dermal wound repair and fibrosis. To determine whether reduced collagen accumulation at 21 days may result from changes in fibrocyte contribution to dermal wound repair, 21-day Pstn $\sim\sim$ and WT wounds were labeled with antibodies against CD45 and collagen type I. The total percentage of bone marrow-derived cells murine wounds was not significantly altered by periostin gene deletion. This indicates that periostin expression does not substantially influence the inflammatory response at 21 days after dermal injury. Interestingly however, periostin gene deletion
was found to significantly reduce the number of fibrocytes in 21-day dermal wounds. Fibrocytes have been shown to participate in tissue remodeling through ECM protein production and to promote fibroblast proliferation, migration, and collagen production through the secretion of several soluble factors. Therefore, the elevated number of fibrocytes observed in 21-day WT wounds may contribute to the increase in collagen accumulation either directly through the production of ECM components or indirectly by influencing fibroblast behavior through the production of profibrotic factors.

Periostin has previously been shown to promote the fibroblastic differentiation of mesenchymal progenitor cells in the heart, as well as the myofibroblastic differentiation of dermal fibroblasts after injury\textsuperscript{11,29}. Several research groups have also demonstrated that periostin enhances the motility of different cell types including murine embryonic fibroblasts, ovarian epithelial cells, cardiac fibroblasts, and vascular smooth muscle cells\textsuperscript{7,8,12,37}. In light of these reports, our results suggest that periostin expression after dermal injury may influence fibrocyte behavior by promoting the fibroblastic differentiation of fibrocytes from their bone marrow-derived precursors and/or by stimulating the migration of fibrocytes and their precursors into dermal wounds. Both mechanisms would result in the increased fibrocytes levels that we observed in WT mice 21 days after dermal injury.

3.5 Conclusions

In this study, we demonstrate that periostin is a profibrogenic matricellular protein that enhances collagen accumulation in the reparative ECM after dermal injury. We also
establish that periostin expression increases the contribution of fibrocytes to dermal wound repair. Fibrocytes represent an additional source of collagen and profibrotic factors in the reparative ECM and, in response to periostin expression, appear to contribute significantly to dermal remodeling. Although the involvement of periostin in tissue fibrosis has been well demonstrated in various diseases, the physiological functions of periostin in dermal scar formation are just beginning to be explored. The results presented in this chapter demonstrate a novel role for periostin in promoting fibrocyte contribution to late phases of dermal wound repair.

3.6 References


37. Li, G. et al. Periostin mediates vascular smooth muscle cell migration through the integrins αvβ3 and αvβ5and focal adhesion kinase (FAK) pathway. Atherosclerosis 208, 8–8 (2010).
CHAPTER FOUR

PERIOSTIN EXPRESSION ELEVATES FIBROCYTE LEVELS IN THE
PERIPHERAL BLOOD AND SKIN AFTER DERMAL INJURY

4.1. Introduction

Fibrocytes were first described in 1994 by Bucala and colleagues when they identified a unique population of blood-borne fibroblast-like cells in subcutaneously implanted wound chambers\(^1\). Through the use of chimeric mouse models, several independent studies have since demonstrated that fibrocytes originate from the bone marrow and migrate into various tissues in response to injury\(^2\)\(^-\)\(^6\). Though our understanding of the mechanisms that govern fibrocyte differentiation and trafficking are not complete, current data suggests that these processes are regulated by a complex profile of chemokines, cytokines, and plasma proteins produced in response to injury\(^7\)\(^-\)\(^10\). Fibrocyte infiltration into tissues affected by acute injury or fibrosis has been well established in numerous organs\(^1\),\(^5\),\(^10\)\(^-\)\(^13\). Moreover, increased fibrocyte levels have also been observed in the bone marrow of mice with bleomycin-induced pulmonary fibrosis and in the peripheral blood of patients with fibrotic conditions such as hypertensive heart disease, pulmonary fibrosis, and systemic sclerosis\(^10\),\(^14\)\(^-\)\(^17\). Collectively, these reports suggest that regulation of fibrocyte behavior by injury-induced factors is likely to occur at multiple stages, affecting fibrocytes and their precursors in the bone marrow, in the peripheral circulation, and at sites of tissue injury.

As previously described in Chapter 3, we observed fewer fibrocytes in the wound beds of Pstn\(^{-/-}\) mice and a corresponding decrease in thick collagen fiber content at late
phases of wound repair. Although these findings suggest a novel role for periostin in promoting fibrocyte contribution to dermal remodeling, the impact of periostin expression on fibrocyte levels during early wound healing and outside of the wound bed has not previously been studied. In this chapter, we measured the number of CD45+/Col I+ fibrocytes present at early phases of repair in the skin, bone marrow, and peripheral blood of WT mice. Dermal injury was shown to increase fibrocyte levels in murine wound beds and in the peripheral blood. Pstn −/− mice were then used to determine whether periostin expression contributes to this increase. Periostin gene deletion was observed to significantly reduce fibrocyte levels present in the skin and peripheral blood after dermal injury. Similar to our previous findings, the work presented in this chapter demonstrates that periostin expression stimulates fibrocyte contribution to early phases of dermal repair. Our results also suggest that periostin expression influences bone marrow-derived cell behavior outside of the dermal wound bed.

4.2. Materials and Methods

4.2.1. Murine Model of Dermal Wound Repair

All animal studies were approved by IACUC at MUSC. As discussed previously, Pstn −/− mice were generated using a murine model of global periostin abrogation developed by Oka et al. Age-matched WT C57BL/6 mice were used as controls for all experiments. Mice were classified as Pstn −/− or WT through PCR genotyping. Full-thickness excisional wounds were created on the dorsa of 2-4 month Pstn −/− and WT mice using methods described previously in Section 3.2.1. Briefly, two 4 mm excisional
dermal wounds were made on either side of the dorsal midline following anesthetization and administration of appropriate analgesia. A depilatory cream was applied prior to wounding and the skin was confirmed to be in the telogen phase of the hair cycle as evidenced by pink colored skin. Wounds were allowed to heal uncovered.

4.2.2. Isolation of Dermal Wound Bed Cells

Uniformly sized skin samples from 4 and 7-day Pstn −/− and WT wound beds were obtained using a 4 mm biopsy punch. Three mice per time point and genotype were used for this experiment and mice were euthanized by isofluorane inhalation followed by cervical dislocation prior harvesting skin samples. As described by Wilson et al., the skin samples were cut into 1 mm² pieces and incubated overnight at 4°C in Solution A [1 ml HBSS containing 1 mg/ml Dispase I (Sigma Aldrich), 3% FBS, and 1x antibiotic-antimycotic solution]. The remaining tissue was then removed from Solution A, further minced, and incubated while shaking at 37°C in Solution B [4 ml of high glucose DMEM containing 1 mg/ml hyaluronidase I (Sigma Aldrich), 1 mg/ml collagenase D (Roche Diagnostics, Mannheim, Germany) and 150 U/ml DNAse]. After approximately 1 hour, the cell suspension was agitated with a 1 ml pipette and combined with Solution A, passed through a 70 µm cell strainer, and washed twice in complete media [high glucose DMEM (Gibco/Life Technologies, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS, Atlanta Biologicals, Atlanta, GA), 10% mouse serum (Atlanta Biologicals), 1x GlutaMAX (Gibco/Life Technologies), 1% penicillin streptomycin (Gibco/Life Technologies), and 1x antibiotic-antimycotic solution (Sigma Aldrich)].
Cells were allowed to recover in complete media at 37°C for 45 minutes. Cells were then centrifuged at 1500 rpm for 5 minutes and resuspended in ice-cold fluorescence activated cell sorting (FACS) buffer [1% BSA (Sigma Aldrich) in PBS with DNase (Thermo Fisher Scientific Inc., Waltham, MA)] for antibody labeling.

4.2.3. Isolation of Bone Marrow Cells

Bone marrow cells were isolated from a minimum of three Pstn−/− and three WT mice before and 4, 7, and 11 days after dermal injury. Mice were euthanized by isofluorane inhalation followed by cervical dislocation. Using a 1 cc insulin syringe, bone marrow cells were flushed from the femurs and tibiae of mice with complete media. The cells in complete media were aspirated through a 22-gauge needle and triturated several times to generate a single cell suspension. The homogenized cell suspension was then filtered through a 70 µm cell strainer, centrifuged at 1500 rpm for 5 minutes, and resuspended in ice-cold FACS buffer for antibody labeling.

4.2.4. Isolation of Peripheral Blood Cells

Blood samples (~50 µl) were obtained by retro-orbital bleeding of Pstn−/− and WT mice before and 4, 7, and 11 days after dermal wounding. Red blood cells were lysed with lysis buffer (BD Pharm Lyse, BD Biosciences, San Jose, CA) and the remaining peripheral blood cell suspension was centrifuged at 1500 rpm for 5 minutes. After dumping the supernatant, cells were resuspended in ice-cold FACS buffer for antibody labeling. Peripheral blood was obtained from a minimum of four mice per genotype at
each time point. Mice were maintained under isofluorane-induced anesthesia throughout the procedure.

4.2.5. Flow Cytometry Analysis

Cells isolated from the skin, bone marrow, and peripheral blood of Pstn −/− and WT mice were analyzed by flow cytometry using previously published methods with modifications\textsuperscript{10,12}. Cells were incubated in allophycocyanin-Cy7-conjugated (APC-Cy7) CD45 (BD Biosciences) in FACS buffer at 4°C for 40 minutes. Subsequently, cells were fixed and permeabilized using a cytofix/cytoperm kit (BD Biosciences) to enable intracellular staining of collagen type I. Cells were then labeled for 30 minutes at 4°C with rabbit anti-collagen type I (Rockland Immunocbes Inc., Gilbertsville, PA) followed by fluorescein isothiocyanate-conjugated (FITC) donkey anti-rabbit IgG (Jackson ImmunoResearch). Cells were washed twice in FACS buffer and analyzed on a MoFlo Atrios cell sorter using Summit v6.1 software (Beckman Coulter, Indianapolis, IN). A minimum of 10,000 cells was analyzed per condition. The average percentage of CD45\textsuperscript{+}/Col I\textsuperscript{+} fibrocytes was calculated for each time point and genotype. Data was analyzed using a Student’s t-test for independent samples, where p-values <0.05 were considered significant.
4.3. Results

4.3.1. Fibrocyte Population in the Skin

Our previous results show that at late stages of dermal repair, WT wounds contain a significantly higher number of fibrocytes compared to Pstn −/− wounds. To determine whether periostin expression also increases the number of fibrocytes in the wound bed at early stages of dermal repair, we used flow cytometry to measure the percentage of fibrocytes in Pstn −/− and WT wounds at 4 and 7 days after injury. At these time points, we observed distinct CD45+/Col I+ and CD45−/Col I+ cell populations in the wound beds of both Pstn −/− and WT mice. These populations, which denote fibrocytes and resident fibroblasts, respectively, can be clearly seen in the representative 2D plots shown in Figure 4.1.

We used these plots to calculate the average percentage of fibrocytes and resident fibroblasts in Pstn −/− and WT wounds at each time point. The percentage of fibrocytes in both Pstn −/− and WT wounds was found to increase significantly between days 4 and 7, from 4.22% to 14.69% in Pstn −/− wounds (p=0.044) and 4.28% to 24.38% in WT wounds (p=0.00016, Figure 4.2, A). These results support previously published data, which shows the percentage of fibrocytes in human dermal wounds to increase substantially between 4 and 14 days after injury\textsuperscript{21}. The percentage of resident fibroblasts also increased significantly between 4 and 7 days in both groups, from 4.83% to 22.85% in Pstn −/− wounds (p=0.004) and 4.10% to 20.90% in WT wounds (p=0.003, Figure 4.2, B).
Figure 4.1 Fibrocyte and fibroblast subsets in Pstn −/− and WT wounds. Primary data demonstrates that fluorescence levels in unstained controls (top) are readily distinguished from fluorescence levels provided by cells labeled with anti-CD45 and anti-collagen I antibodies (bottom). Distinct populations of CD45+/Col I+ and CD45−/Col I+ cells, which represent fibrocytes and fibroblasts, can also be observed in 7-day wounds.

Interestingly, while no differences in fibrocyte number were observed between Pstn −/− and WT mice at 4 days after injury, loss of periostin expression was found to significantly reduce the percentage of fibrocytes present in 7-day dermal wounds. At 7 days, fibrocytes comprised 24.38% of cells in WT wounds and only 14.69% of cells in Pstn −/− wounds (p=0.025, Figure 4.2, A). Periostin gene deletion did not alter the percentage of resident fibroblasts at either time point (Figure 4.2, B). To determine whether a loss of periostin reduces total bone marrow-derived cell infiltration into the
wound bed, we also calculated the average percentage of CD45$^{+}$ cells in Pstn$^{-/-}$ and WT wounds. The average percentage of CD45$^{+}$ cells at 7 days was lower in Pstn$^{-/-}$ mice than in WT mice (34.81% versus 48.90%, p=0.127). However, this difference was not statistically significant (Figure 4.3).

Figure 4.2 Quantification of fibrocyte and fibroblast levels in Pstn$^{-/-}$ and WT wounds. A) The average percentage of CD45$^{+}$/Col I$^{+}$ cells was significantly higher in WT wounds than in Pstn$^{-/-}$ wounds at 7 days after dermal injury. B) Loss of periostin did not alter the average percentage of CD45$^{-}$/Col I$^{+}$ cells at 4 or 7-day wounds. *p<0.05

Figure 4.3 Quantification of bone marrow-derived cells in Pstn$^{-/-}$ and WT wounds. A and B) Gates were set based on fluorescence levels in unlabeled control samples. Representative histograms of unlabeled cells (A) and cells labeled with an antibody against CD45 (B) are shown. C) Loss of periostin did not significantly reduce the number of CD45$^{+}$ cells in Pstn$^{-/-}$ wounds at 4 or 7 days.
Collectively, these results demonstrate that a loss of periostin reduces the number of fibrocytes in injured dermal tissue, but does not alter the number of resident fibroblasts in the wound bed at early stages of repair. While differences in the percentage of CD45$^+$ cells were not significant, reduced migration of bone marrow-derived cells into Pstn $^{-/-}$ wounds may contribute to the observable decrease in fibrocyte number. In the next experimental sections we examined the effects of periostin gene deletion on fibrocyte levels in the bone marrow and peripheral blood to determine whether periostin expression influences fibrocyte behavior outside of the injured tissue.

4.3.2. Fibrocyte Population in the Bone Marrow

Bleomycin-induced pulmonary fibrosis has been shown to dramatically increase the number of fibrocytes in murine bone marrow$^{10}$. Based on this finding, investigators have proposed the bone marrow to be a significant source of circulating fibrocytes that migrate into injured lung tissue$^{10}$. To our knowledge, the effects of dermal injury, as well as periostin expression, on fibrocyte levels in the bone marrow have not been studied. To examine these effects, bone marrow was recovered from Pstn $^{-/-}$ and WT mice before and 4, 7, and 11 days after dermal injury, labeled with antibodies against CD45 and collagen type I, and analyzed by flow cytometry. We identified a small population of CD45$^+/\text{Col I}^+$ cells in the bone marrow of Pstn $^{-/-}$ and WT mice (Figure 4.4). The average percentage of this population did not change significantly before or at any time point after dermal injury (Figure 4.5). This indicates that our model of excisional dermal wounding does not stimulate the same profibrotic response in the bone marrow as
bleomycin-induced pulmonary fibrosis. Additionally, no differences in the number of fibrocytes present in the bone marrow were observed between Pstn −/− and WT mice (Figure 4.5). These results suggest that periostin expression does not influence fibrocyte differentiation in the bone marrow after dermal injury. However because our injury model did not stimulate an increase in bone marrow fibrocyte number, using an injury model that does may provide a better perspective on how periostin expression influences fibrocyte formation in the bone marrow.

![Figure 4.4 Fibrocyte subsets in bone marrow harvested from uninjured Pstn −/− and WT mice (R16). Primary data demonstrates that fluorescence levels in unstained controls (top) are readily distinguished from fluorescence levels provided by cells labeled with anti-CD45 and anti-collagen I antibodies (bottom).](image-url)
Figure 4.5 Quantification of fibrocyte levels in the bone marrow of Pstn −/− and WT mice before and after dermal injury. Dermal injury did not induce a significant increase in the number of CD45+/Col I+ cells. No differences in fibrocyte levels were observed between Pstn −/− and WT mice.

4.3.3. Fibrocyte Population in the Blood

In healthy hosts, fibrocytes are estimated to comprise 0.1% to 0.5% of the nucleated cells in the peripheral blood⁴. However, this percentage has been shown to be elevated in patients with several fibrotic disorders, suggesting that injury-induced factors also affect fibrocytes and their precursors in the peripheral circulation. To determine whether dermal injury also causes the number of circulating fibrocytes to increase, peripheral blood was sampled from WT mice before and after dermal injury and labeled with antibodies against CD45 and collagen type I for flow cytometry analysis. Representative 2D plots of labeled peripheral blood cells before and 4 days after injury are shown in Figure 4.6. CD45+/Col I+ cells are located in region R20.
Figure 4.6 Fibrocyte subsets in the peripheral blood of Pstn−/− and WT mice before and 4 days after injury (R20). Representative histograms show a visible increase in the percentage of CD45+/Col I+ fibrocytes after dermal wounding in Pstn−/− and WT mice.

These 2D plots were used to calculate the average percentage of CD45+/Col I+ cells in the peripheral blood at each time point. Dermal injury was found to induce a significant increase in the number of circulating fibrocytes in WT mice (Figure 4.7). The percentage of fibrocytes in the peripheral blood was highest at 4 days, increasing from 0.62% before injury to 4.2% 4 days after injury (p=2.62x10⁻⁵). After 4 days, the number of circulating fibrocytes gradually decreased before returning to uninjured levels at 11 days. As with previous studies, we also used Pstn−/− mice to determine if these changes were related to injury-induced periostin expression. The average percentage of circulating fibrocytes in Pstn−/− mice at 4 days after injury was also significantly higher than in
uninjured controls (0.42% to 2.07%, p=0.013). Prior to dermal injury, Pstn −/− and WT mice were found to contain a similar percentage of peripheral blood fibrocytes. In WT mice however, dermal injury induced a significantly higher increase in the number of circulating fibrocytes. At 4 days, fibrocytes comprised 4.20% of cells in WT blood and only 2.07% of cells in Pstn −/− blood (p=0.008). Additionally, though the overall percentage of fibrocytes was decreased in both genotypes at 7 days, the percentage of circulating fibrocytes in WT mice remained statistically higher, comprising 1.47% of cells in WT blood and only 0.34% of cells in Pstn −/− blood (p=0.0003). These results indicate that periostin expression induced by dermal injury significantly enhances fibrocytes levels in the peripheral blood at early stages of wound repair.

Figure 4.7 Quantification of fibrocyte levels in the peripheral blood of Pstn −/− and WT mice before and after dermal injury. Dermal injury induced a significant increase the percentage of circulating fibrocytes in both Pstn −/− and WT mice, with fibrocyte levels peaking at 4 days after injury. WT mice also exhibited a significantly higher percentage of circulating fibrocytes compared to Pstn −/− mice at 4, 7, and 11 days after dermal injury. *p<0.05, **p<0.01
4.4. Discussion

In human dermal wounds fibrocytes appear as early as 4 days after injury, and their number has been shown to increase substantially with wound age, peaking at approximately 12-14 days\textsuperscript{21}. As remodeling occurs, the number of fibrocytes in the wound bed decreases\textsuperscript{21}. However in hypertrophic scars, fibrocytes persist in high numbers and are thought to promote overabundant collagen fiber deposition\textsuperscript{11}. Interestingly, levels of periostin expression also remain elevated in hypertrophic and keloid scar tissue\textsuperscript{22}. We previously demonstrated that loss of periostin expression reduces fibrocyte infiltration into dermal scars, making periostin a potential therapeutic target for the treatment of fibrotic pathologies associated with elevated fibrocyte levels.

On the opposite end of the spectrum, periostin expression and fibrocyte infiltration have also been shown to independently promote wound closure. Periostin upregulation enhances wound contraction by increasing $\alpha$-SMA expression in the wound bed. Infiltrating fibrocytes also have the ability to promote wound contraction by differentiating into myofibroblast-like cells and by secreting molecular effectors that stimulate the myofibroblastic differentiation of resident fibroblasts. To establish whether periostin expression enhances fibrocyte participation in early stages of wound repair, we measured fibrocyte levels in Pstn \textsuperscript{−/−} and WT wounds at 4 and 7 days after dermal injury. Periostin gene deletion did not alter the number of resident fibroblasts present in dermal wounds at either time point. However at 7 days, WT wounds were found to contain a significantly higher percentage of fibrocytes than Pstn \textsuperscript{−/−} wounds. Interestingly, the expression of periostin and $\alpha$-SMA in the reparative ECM also peaks at
this time point. These results correspond with our previous findings in 21-day dermal wounds and also indicate that periostin expression may promote wound closure by increasing the number of fibrocytes that participate in early dermal repair. Though a specific mechanism for this change has not been identified, our results suggest that periostin expression may either promote fibrocyte differentiation within the wound bed or increase the number of fibrocytes that migrate into injured dermal tissue. Other investigators have previously suggested that periostin functions as a profibrogenic mediator, promoting the differentiation of mesenchymal progenitor cells into cardiac fibroblasts and the maturation of dermal fibroblasts into myofibroblasts. Through integrin-mediated cell adhesion, periostin has also been shown to enhance the migratory ability of several cell types, including ovarian epithelial cells, cushion mesenchymal cells, and vascular smooth muscle cells.

We measured the number of fibrocytes present in the bone marrow and peripheral blood of Pstn−/− and WT mice before and after dermal injury to determine whether periostin expression also influences fibrocyte behavior outside of the dermal wound bed. In contrast to our observations in skin biopsies, excisional dermal wounding did not elicit an increase in the number of fibrocytes in the bone marrow of either Pstn−/− or WT mice. Similarly, the percentage of fibrocytes was also unaffected by periostin gene deletion. Because dermal injury did not induce any changes in fibrocyte number, it is difficult to determine whether or not injury-induced periostin expression affects the formation of fibrocytes in the bone marrow. For future studies, we recommend combining the use of Pstn−/− mice with the model of bleomycin-induced pulmonary
fibrosis described by Phillips et al., in which bleomycin treatment was observed to significantly increase the number of bone marrow fibrocytes\textsuperscript{10}. Because periostin is rapidly upregulated in bleomycin-treated mice, comparing the number of fibrocytes in the bone marrow of Pstn $-/-$ and WT mice after bleomycin-treatment would provide valuable information regarding how periostin expression influences fibrocyte formation in the bone marrow\textsuperscript{28}.

In the peripheral blood, we observed a significant increase in the number of fibrocytes present after dermal injury. The percentage of circulating fibrocytes in both Pstn $-/-$ and WT mice peaked at 4 days after injury and decreased gradually before returning to basal levels at 11 days. Elevated numbers of circulating fibrocytes have been found in patients with various fibrotic disorders\textsuperscript{10,12,14-17}. To our knowledge however, this is the first study to demonstrate that dermal injury significantly increases fibrocytes levels in the peripheral blood. This suggests that injury-induced factors like periostin influence fibrocyte behavior in the circulation, rather than only at sites of dermal injury, and that the peripheral circulation may be a significant source of fibrocytes that invade injured dermal tissue.

We also observed the percentage of fibrocytes in the peripheral blood to be significantly lower in Pstn $-/-$ mice compared to WT mice after dermal injury. At 4 and 7 days after injury, periostin gene deletion reduced the number of fibrocytes in the peripheral blood by over fifty and seventy-five percent, respectively. These results indicate that periostin expression promotes the injury-induced increase in circulating fibrocytes, likely by one or more of the following mechanisms: increasing the formation
of fibrocyte precursors in the bone marrow, promoting the mobilization of fibrocytes and their precursors from the bone marrow into the peripheral circulation, and/or stimulating the differentiation of fibrocytes in the peripheral blood. Increased numbers of fibrocytes in the peripheral blood may contribute to the higher numbers of fibrocytes we observed in the wound beds of WT mice compared to Pstn −/− mice.

Interestingly, Naik et al. recently demonstrated that fibrocytes and monocytes of patients with pulmonary fibrosis produce higher levels of periostin in the peripheral blood relative to healthy controls. They also showed that, in addition to structural sources of periostin, hematopoietic cell-derived periostin promotes injury-induced fibrosis in the lungs. Further studies examining whether periostin is expressed by circulating cells after dermal injury and, if so, how this source of periostin affects fibrocyte numbers in the peripheral blood and skin would provide valuable information regarding the mechanisms that govern fibrocyte differentiation and migration after dermal injury.

Collectively, our results indicate that regulation of fibrocyte behavior by injury-induced periostin expression is likely to occur at multiple stages, involving fibrocytes and their precursors in the bone marrow, peripheral circulation, and dermal wound bed. While our findings do not demonstrate modulation of fibrocyte levels to be the sole mechanism by which periostin gene deletion delays wound closure and reduces collagen content in the dermal wound bed, they do clearly indicate that abrogation of periostin expression can be used to reduce fibrocyte contribution to both early and late phases of dermal wound repair.
4.5. Conclusions

Investigators have previously established that fibrocyte infiltration can contribute to both timely dermal wound repair as well as excessive scar formation. These observations have motivated additional research exploring factors that modulate fibrocyte behavior for the development of therapeutics aimed at either stimulating wound closure or reducing tissue fibrosis. In this chapter we demonstrate, for the first time, that periostin expression significantly enhances fibrocyte levels in both the skin and peripheral blood at early stages of wound repair. In combination with the results described in Chapter 3, these findings contribute significantly to our understanding of fibrocyte biology by establishing periostin to be an effector of fibrocyte levels at both early and late stages of dermal repair. Our results also demonstrate that periostin expression influences fibrocyte levels outside of the dermal wound bed, which raises questions regarding the effects of hematopoietic and structural sources of periostin on fibrocyte behavior.

4.6. References


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CHAPTER FIVE
PERIOSTIN EXPRESSION PROMOTES FIBROCYTE ADHESION AND MIGRATION IN RESPONSE TO THE CXCL14/CXCR4 SIGNALLING AXIS

5.1 Introduction

The mobilization, homing, and engraftment of HSCs and their leukocyte subpopulations involve a complex profile of adhesion molecules, chemoattractant cytokines, and chemokine receptors. Recent work in the area of chemokine-chemokine receptor interactions suggests that there is substantial overlap between the mechanisms that govern these processes and fibrocyte migration\textsuperscript{1}. Human fibrocytes express several chemokine receptors, including CCR3, CCR5, CCR7, and CXCR4, and murine fibrocytes express CCR2, CCR7, and CXCR4\textsuperscript{2-4}. The chemokine receptor CXCR4 and its conjugate ligand stromal-derived factor 1 (CXCL12) play a critical role in HSC retention in the bone marrow and also in HSC homing to sites of tissue injury\textsuperscript{5}. Consistent with their bone marrow origin, fibrocytes expressing CXCR4 have been shown to migrate in response to a CXCL12 gradient\textsuperscript{4}. CXCL12 secretion is associated with tissue damage caused by events such as excisional and thermal burn injuries to the skin, pulmonary fibrosis, and myocardial infarction\textsuperscript{4,6-10}. The CXCR4/CXCL12 signaling axis has been shown to promote pulmonary fibrosis by increasing fibrocyte infiltration into the lungs and to enhance wound healing through the recruitment of bone marrow-derived mesenchymal stem cells into dermal excisional and burn wounds\textsuperscript{4,6,7}.

In Chapter 4, we described our findings that periostin expression contributes to the elevated number of fibrocytes present in the peripheral blood and skin after dermal
injury. These results suggest that periostin expression affects fibrocyte behavior at multiple stages, potentially influencing fibrocytes and their bone marrow-derived precursors in the bone marrow, peripheral circulation, and dermal wound bed. Other investigators have previously demonstrated that periostin expression enhances the migratory ability of several cell types. In this study, we examined how periostin expression specifically affects fibrocyte migration in response to the CXCR4/CXCL12 signaling axis. Using in vitro methods, we demonstrated that periostin gene deletion significantly reduces bone marrow fibrocyte (BM fibrocyte) chemotaxis and integrin-mediated migration. Subsequent in vivo assays revealed that loss of periostin also reduces the number of circulating CXCR4⁺/Col I⁺ fibrocytes in mice after dermal injury. Collectively, our findings suggest that periostin expression plays an important role in fibrocyte trafficking in response to the CXCR4/CXCL12 signaling axis. Periostin gene deletion may reduce the ability of fibrocytes to migrate from the bone marrow into the peripheral circulation and then into skin after dermal injury, leading to the decrease in fibrocyte numbers previously observed in the blood and skin of Pstn −/− mice.

5.2 Materials and Methods

5.2.1 Isolation and Culture of Bone Marrow Cells

Cells were harvested from the bone marrow of adult Pstn −/− and WT mice. Pstn −/− mice were generated as described previously in Section 3.2.1. Mice were euthanized by isofluorane inhalation followed by cervical dislocation. Using a 1 cc insulin syringe, bone marrow cells were flushed from the femurs and tibiae with complete medium. Bone
marrow cells in medium were aspirated through a 22-gauge needle and triturated several times to generate a single cell suspension. The homogenized cell suspension was then filtered through a 70 µm cell strainer, centrifuged at 1500 rpm for 5 minutes, and resuspended in ice-cold FACS buffer. To separate hematopoietic cells from non-hematopoietic cells, bone marrow cells were labeled with PE-conjugated mouse-anti-mouse CD45 (BD Pharmingen) for 30 minutes at 4°C and sorted on a MoFlo Atrios cell sorter using Summit v6.1 software. After sorting, CD45+ cells were washed and plated on fibronectin-coated 24-well plates at 2.5x10^6 cells per well in complete medium. As previously described, cells were cultured at 37°C, 5% CO_2 for 12 days with regular medium changes.

5.2.2 Immunolabeling of Cultured Bone Marrow Cells

Cultured bone marrow cells were detached using TrypLE (Life Technologies) and transferred to an 8-well chamber slide at a seeding density of 1.5x10^4 cells per well. After 24 hours of culture, cells were fixed in zinc formalin, permeabilized with Triton X-100, and blocked with Background Buster. To determine whether they co-expressed hematopoietic and mesenchymal markers, cultured bone marrow cells were incubated at 4°C overnight in diluted primary antibodies [goat anti-mouse CD45 and rabbit anti-collagen type I (Rockland Immunochemicals Inc.)]. Primary antibodies were then localized by incubation with coordinate secondary antibodies (Cy3-conjugated donkey anti-goat IgG and Cy5-conjugated donkey anti-rabbit IgG) for one hour at room temperature. Nuclear counterstaining was achieved with DAPI and slides were mounted.
in Fluoro-Gel. Cells were imaged at 20x magnification on a Leica DMI 4000B inverted microscope (Leica Microsystems Inc.). Images were processed for qualitative analysis using Adobe Photoshop CS5 software.

5.2.3 Chemotaxis of Bone Marrow Fibrocytes

The migration of BM fibrocytes was assessed *in vitro* using the Neuro Probe ChemoTx System (Neuro Probe, Gaithersburg, MD) with a 5 µm pore-size polycarbonate filter. After 12 days of culture, Pstn −/− and WT BM fibrocytes were detached using TrypLE and allowed to recover for 45 minutes in complete medium at 37°C. Cells were then suspended in migration medium [serum-free DMEM with 2% bovine serum albumin (BSA)] at 6x10⁵ cells/ml. A quantity of 50 µl of cell suspension was pipetted onto the filter site above each well (Figure 5.1). Cells were allowed to migrate to lower wells containing migration medium in the presence or absence of the chemoattractant CXCL12 (100 ng/ml, R&D Systems) for three hours at 37°C. Filter sites were fixed in methanol, stained with DAPI, and mounted on glass slides in Fluoro-gel. The cells that migrated to the lower side of the filter were imaged in 10 fields per filter site at 20x magnification using a Leica DMI 4000B inverted microscope and counted using ImageJ software. A minimum of four filter sites per condition was evaluated.
Figure 5.1 Use of the Neuro Probe ChemoTx system to study BM fibrocyte migration. BM fibrocytes in suspension were pipetted onto each filter site and allowed to migrate toward the lower chambers, which contained migration medium in the presence or absence of the chemoattractant CXCL12.

5.2.4 Bone Marrow Fibrocyte Adhesion

The adhesion of BM fibrocytes to immobilized periostin and fibronectin was quantified using previously published methods with modifications\textsuperscript{12}. Wells of a round-bottom 96-well plate were coated with recombinant murine-derived periostin (20 µg/ml, R&D Systems) or bovine-derived fibronectin (3 µg/ml, Sigma Aldrich) for one hour at room temperature and then blocked with 1% BSA for one hour. After 12 days of culture as described in Section 5.2.1, Pstn −/− and WT BM fibrocytes were detached using TrypLE and allowed to recover for 30 minutes in complete medium at 37°C. Cells were centrifuged and resuspended in serum-free DMEM, added to coated wells (30,000 cells in 100 µl per well), and allowed to adhere for one hour at 37°C. Round-bottom 96-well plates were washed in warm PBS and the remaining adherent cells were fixed and stained in 10% ethanol/2% crystal violet for 5 minutes. After washing out unbound dye, bound crystal violet was solubilized by adding 0.1M sodium acetate in 50% ethanol. A
spectrophotometer was used to record the absorbance at 560 nm of 9 wells per condition.

5.2.5 Integrin-mediated Migration of Bone Marrow Fibrocytes

The migration of BM fibrocytes on periostin and fibronectin-coated filters was evaluated using the methods described in Section 5.2.3. Polycarbonate filters were coated for one hour per side at 37°C with recombinant murine-derived periostin (10 µg/ml) or bovine-derived fibronectin (3 µg/ml). Coated filters were allowed to dry at 4°C overnight prior to use. A minimum of four filter sites for each condition was evaluated.

Additionally, function blocking antibodies against αv, β1, and β3 integrins (Biolegend, San Diego, CA) were used to target integrin receptors involved in BM fibrocyte migration on periostin and fibronectin-coated filters. After detachment with TrypLE and 45 minutes of recovery in complete medium, Pstn −/− and WT BM fibrocytes were incubated for 30 minutes at 4°C in migration medium containing function blocking antibodies at 25µg/ml. Control cells were incubated without the addition of function blocking antibodies. Migration assays were then conducted as previously described using periostin and fibronectin-coated filters. Three filters sites per condition were evaluated. The fold change was calculated for each condition (no blocking antibody, anti-αv antibody, anti-β1 antibody, and anti-β3 antibody), where:

\[
\text{Fold change} = \frac{\# \text{ cells that migrate in response to CXCL12 for each condition}}{\# \text{ cells that randomly migrate (no CXCL12 and no blocking antibodies)}}
\]

A fold change of one indicates that the anti-integrin antibody used completely blocks CXCL12-induced migration so that only random migration occurs.
5.2.6 Flow cytometry analysis of circulating CXCR4\(^+\) fibrocytes

To detect circulating CXCR4\(^+\) fibrocytes \textit{in vivo}, peripheral blood cells were analyzed for co-expression of CXCR4 and collagen type I by flow cytometry using the methods described in Sections 4.2.4 and 4.2.5. Nucleated cells were isolated from the peripheral blood of Pstn \(^{-/-}\) and WT mice before and 4 and 7 days after dermal injury. Briefly, cells were labeled with phycoerythrin-conjugated (PE) CXCR4 (BD Biosciences) at 4\(^\circ\)C for 40 minutes. After fixation and permeabilization, cells were incubated with rabbit anti-collagen type I (Rockland Immunochemicals Inc.) for 30 minutes at 4\(^\circ\)C and labeled with FITC-conjugated donkey anti-rabbit IgG. A minimum of 10,000 cells was analyzed for each condition on a MoFlo Atrios cell sorter using Summit v6.1 software. The average percentage of CXCR4\(^+\)/Col I\(^+\) cells was calculated for each time point and genotype. Data was analyzed using a Student’s t-test for independent samples, where p-values <0.05 were considered significant.

5.3 Results

5.3.1 \textit{In vitro} fibroblastic differentiation of bone marrow-derived cells

Fibrocytes can be identified by the co-expression of a hematopoietic marker (CD45, CD34) and a mesenchymal marker (collagen type I, vimentin)\(^{14-16}\). Methods employed for the isolation, growth, and characterization of fibrocytes commonly rely on the derivation of fibrocytes from mononuclear cells in the peripheral blood\(^{2,17}\). Generally, Ficoll-Paque density-gradient centrifugation is conducted to remove red blood cells from the leukocyte-richuffy coat fraction. Cells in the buffy coat fraction are then plated and
cultured in DMEM supplemented with serum for 10-14 days. Over time non-adherent cells are washed off through medium changes and fibrocytes appear as clusters of spindle-shaped cells.

Given our lab’s focus on the bone marrow contribution of fibroblast-like cells to tissue repair, we chose to isolate and culture hematopoietic cells from the bone marrow rather than the peripheral blood\textsuperscript{18}. Bone marrow cells were aspirated from the femurs and tibiae of Pstn \textsuperscript{−/−} and WT mice. To remove non-hematopoietic cells, bone marrow cells were labeled and sorted for CD45 expression using flow cytometry. CD45\textsuperscript{+} cells were then cultured for 12 days in complete medium. As with cultured peripheral blood fibrocytes, adherent bone marrow cells adopted a fibroblast-like spindle-shaped morphology (Figure 5.2, A and B). Cultured bone marrow cells were also confirmed to co-express CD45 and collagen type I, as indicated by fluorescence microscopy (Figure 5.2, C, D, and E). Collectively, these observations demonstrate the ability of hematopoietic bone marrow precursors to differentiate into fibrocytes \textit{in vitro}. 
Figure 5.2 Hematopoietic bone marrow-derived cells differentiate into fibrocytes *in vitro*. A and B) Phase contrast images show that after 12 days of culture Pstn−/− and WT bone marrow cells exhibit a fibroblast-like spindle-shaped morphology. C and D) Cultured bone marrow cells co-express CD45 (red) and collagen type I (green). DAPI stained nuclei are blue. E) Negative control samples incubated with only secondary antibodies confirm specific labeling by primary antibodies against CD45 and collagen type I.

5.3.2 Bone marrow fibrocyte chemotaxis in response to CXCL12

Fibrocytes derived from peripheral blood mononuclear cells have previously been shown to migrate in response to the CXCR4/CXCL12 signaling axis\(^4\). We performed chemotaxis assays on polycarbonate filters to confirm that BM fibrocytes also migrate in the presence of a CXCL12 gradient and to determine whether periostin gene deletion affects their migration. We observed significant chemotaxis of Pstn−/− and WT BM fibrocytes toward wells containing CXCL12 compared to controls with no added chemoattractant (p=0.007 and p=0.010, respectively, Figure 5.3). Interestingly, periostin gene deletion also was found to significantly reduce BM fibrocyte migration, with an
average of 1337 WT cells counted per filter versus only 742 Pstn −/− cells counted per filter (p=0.004, Figure 5.3).

![Chemotaxis of BM fibrocytes on polycarbonate filters](image)

Figure 5.3 Chemotaxis of BM fibrocytes in response to CXCL12 (100 ng/ml). The presence of a CXCL12 gradient stimulated Pstn −/− and WT BM fibrocyte migration across polycarbonate filters. Periostin gene deletion significantly reduced CXCL12-induced BM fibrocyte migration. **p<0.01, n=8.

### 5.3.3 Adhesion of bone marrow fibrocytes

Cell migration relies not only on the expression of paracrine chemokines such as CXCL12, but also on interactions between cell integrin receptors and various components of the surrounding ECM. To determine whether peristin gene deletion alters fibrocyte interactions with the ECM, we quantified differences in BM fibrocyte adhesion to immobilized peristin and fibronectin, two adhesion proteins that are abundantly expressed in the ECM during dermal repair. WT BM fibrocytes exhibited a significantly greater ability to adhere to both peristin and fibronectin-coated wells compared to Pstn −/− BM fibrocytes (p=0.044 and p=0.001, respectively, Figure 5.4).
Figure 5.4 BM fibrocyte adhesion to immobilized periostin and fibronectin. Pstn −/− cells exhibit a reduced ability to adhere to periostin and fibronectin relative to WT cells. *p<0.05, **p<0.01, n=9.

5.3.4 Integrin-mediated migration of bone marrow fibrocytes

We also examined whether periostin gene deletion affects integrin-mediated migration of BM fibrocytes. Chemotaxis assays were performed using polycarbonate filters coated with either periostin or fibronectin. No significant differences in migratory behavior were observed between Pstn −/− and WT BM fibrocytes on fibronectin-coated filters (Figure 5.5, A). In contrast, on periostin-coated filters significantly fewer Pstn −/− BM fibrocytes migrated randomly and in response to the CXCL12 gradient (p=0.017 without chemoattractant and p=0.005 with added CXCL12, Figure 5.5, B). Of note, random migration of WT cells was also significantly higher on periostin-coated filters than on uncoated polycarbonate filters, with an average of 551 WT cells counted on periostin-coated filters and only 140 WT cells counted on uncoated filters (p=0.009,
Figures 5.3 and 5.5, B). Coating filters with periostin did not increase the random migration of Pstn −/− BM fibrocytes (p=0.485, Figures 5.3 and 5.5, A).

Figure 5.5 Chemotaxis of cultured BM fibrocytes on periostin and fibronectin-coated filters. A) Pstn −/− cells exhibit reduced migration on periostin-coated filters with and without the presence of a CXCL12 gradient. *p<0.05, **p<0.01, n=5. B) On fibronectin-coated filters, no significant differences are observed between Pstn −/− and WT cells. n=4.

Periostin contains four Fas-1 domains that, in addition to binding proteins such as TNC and BMP-1, promote integrin-dependent cell adhesion and motility by serving as ligands for various integrin receptors. To identify integrin receptors on BM fibrocytes that interact with periostin during chemotaxis, cells were incubated with function blocking antibodies against αv, β1, and β3 integrin subunits. Periostin has been previously shown to interact directly with integrins containing these subunits11,12. Blocking antibodies against αv and β1 subunits were found to significantly inhibit the chemotactic migration of BM fibrocytes on periostin, reducing the fold change from 2.60 to 1.32 and
1.26 for Pstn −/− cells and from 2.32 to 1.11 and 1.10 for WT cells, respectively (p<0.05 for all conditions, Figure 5.6).

![Inhibition of chemotaxis by anti-integrin antibodies on periostin-coated filters](chart)

**Figure 5.6** Inhibition of BM fibrocyte migration on periostin-coated filters with integrin blocking antibodies. Antibodies against αv and β1 integrin receptors significantly reduced the chemotactic migration of BM fibrocytes on periostin. *p<0.05, n=3.

Fibronectin is an adhesive protein that is also highly expressed during early stages of wound repair. BM fibrocytes were incubated with function blocking antibodies against αv, β1, and β3 integrin subunits to determine whether they also play a critical role in CXCL12-induced fibrocyte migration on fibronectin. Unique from periostin, antibodies against β1 and β3 integrin subunits were found to significantly inhibit the chemotactic migration of BM fibrocytes on fibronectin, reducing the fold change from 1.82 to 0.94 and 1.34 for Pstn −/− cells and from 2.14 to 1.24 and 1.55 for WT cells, respectively (Figure 5.7, p<0.05 for all conditions).
5.3.5 Quantification of CXCR4⁺ fibrocytes in the peripheral blood

To determine whether periostin expression influences fibrocyte participation in the CXCR4/CXCL12 signaling axis *in vivo*, we used flow cytometry to measure the number of circulating CXCR4⁺/Col I⁺ fibrocytes in Pstn −/− and WT mice before and 4 and 7 days after dermal injury. Though not traditionally used as a hematopoietic marker for fibrocytes, investigators agree that CXCR4 is a hematopoietic cell marker and have previously used dual expression of CXCR4 and collagen type I to identify fibrocytes in the peripheral blood\textsuperscript{15}. Representative 2D plots of labeled peripheral blood cells are shown in Figure 5.8, with CXCR4⁺/Col I⁺ cells located in region R11.
Figure 5.8 CXCR4⁺/Col I⁺ fibrocyte subsets in the peripheral blood of Pstn⁻/⁻ and WT mice before and 4 days after injury (R11). Representative histograms show a visible increase in the percentage of CXCR4⁺/Col I⁺ cells after dermal wounding in Pstn⁻/⁻ and WT mice.

These 2D plots were used to calculate the average percentage of CXCR4⁺/Col I⁺ fibrocytes in the peripheral blood of Pstn⁻/⁻ and WT mice at each time point. Dermal injury was observed to induce a significant increase in the number of circulating CXCR4⁺/Col I⁺ cells in both Pstn⁻/⁻ and WT mice (Figure 5.9). The percentage of circulating CXCR4⁺/Col I⁺ cells was highest at 4 days in both genotypes, increasing from 0.19% to 0.67% in Pstn⁻/⁻ mice (p=0.187) and from 0.16% to 1.54% in WT mice (p=0.007). Prior to dermal wounding, Pstn⁻/⁻ and WT mice were observed to contain a similar percentage of circulating CXCR4⁺/Col I⁺ cells. However, dermal injury induced a
significantly higher increase in the number of circulating CXCR4⁺/Col I⁺ cells in WT mice compared to Pstn −/− mice. At 4 days, CXCR4⁺/Col I⁺ cells comprised 1.54% of cells in WT blood and only 0.67% of cells in Pstn −/− blood (p=0.026). Similarly, at 7 days CXCR4⁺/Col I⁺ cells comprised 0.55% of cells in WT blood and only 0.16% of cells in Pstn −/− blood (p=0.003). These differences correspond with the results described in Chapter 4, which show that CD45⁺/Col I⁺ fibrocyte levels also peak at 4 days after injury and are significantly higher in the presence of periostin expression.

![Figure 5.9 Quantification of CXCR4⁺/Col I⁺ cell levels in the peripheral blood of Pstn −/− and WT mice before and after dermal injury. Periostin gene deletion significantly reduced the percentage of circulating CXCR4⁺/Col I⁺ cells at 4 and 7 days after dermal injury. *p<0.05, **p<0.01](image)

5.4 Discussion

A growing body of research focused on the contribution of periostin to numerous biological processes, such as wound healing, tissue fibrosis, and tumor metastasis, has
shown that periostin expression influences many aspects of cell behavior, including cell
differentiation, proliferation, adhesion, and migration\textsuperscript{19-23}. Our results described in
Chapters 3 and 4 demonstrate that periostin expression increases the number of
CD45\textsuperscript{+}/Col I\textsuperscript{+} fibrocytes that contribute to dermal repair. Based on the diverse effects of
periostin on cell behavior, these results suggest that periostin expression promotes the
fibroblastic differentiation of bone marrow progenitors and/or increases the trafficking of
fibrocytes and their precursors into dermal wounds. To improve our understanding of
how periostin expression influences fibrocyte mobilization, homing, and engraftment to
sites of tissue injury, we studied the effects of periostin gene deletion on fibrocyte
adhesion and migration \textit{in vitro}. Our findings demonstrate that periostin gene deletion
significantly reduces BM fibrocyte adhesion to periostin and fibronectin-coated surfaces.
Correspondingly, loss of periostin also reduces fibrocyte chemotaxis and integrin-
mediated migration in response to the CXCR4/CXCL12 signaling axis. These results
suggest that increased fibrocyte trafficking may be one mechanism by which periostin
expression promotes fibrocyte contribution to dermal repair.

Methods used to generate fibrocytes \textit{in vitro} typically rely on the derivation of
fibrocytes from mononuclear cells in the peripheral blood\textsuperscript{2,17}. However, recent
publications have identified fibrocytes in the bone marrow and have reported the
successful \textit{in vitro} derivation of fibrocytes from bone marrow cells\textsuperscript{4,18,24,25}. Scholten et al.
isolated CD45\textsuperscript{+}collagen\textsuperscript{+} cells from the bone marrow using flow cytometry and, after 7
days of culture, observed these cells to give rise to classic collagen-expressing spindle-
shaped fibroblast-like cells\textsuperscript{25}. Using a murine model of clonal engraftment of cells
derived from a single EGFP⁺ HSC, Ebihara et al. also demonstrated that EGFP⁺ bone marrow cells adopt a fibroblast-like phenotype and express mesenchymal markers in culture. Based on these publications, as well as our lab’s interest in the bone marrow contribution of fibroblast-like cells to tissue repair, we chose to isolate and culture murine fibrocytes from CD45⁺ bone marrow cells rather than nucleated peripheral blood cells. As with peripheral blood fibrocyte cultures, within 12 days adherent bone marrow cells adopted a fibroblast-like spindle-shaped morphology and were confirmed to co-express CD45 and collagen type I through immunofluorescence microscopy. According to the minimum criteria used to define fibrocytes of co-expression of hematopoietic and mesenchymal markers, our observations demonstrate that hematopoietic bone marrow cells have the ability to differentiate into fibrocytes in vitro.

The effects of periostin gene deletion on BM fibrocyte chemotaxis were studied using the CXCR4/CXCL12 signaling axis. CXCR4 is widely expressed on hematopoietic cells, including fibrocytes and their HSC and monocyte precursors. CXCL12, which binds the CXCR4 receptor and is an important chemoattractant for CXCR4⁺ cells, is significantly upregulated in the peripheral blood and blister fluid of patients with skin burns and at the margins of excisional dermal wounds. Upregulation of CXCL12 expression in the peripheral blood and tissues in response to disease or injury has been suggested to play a significant role in the mobilization of progenitor cells and fibrocytes from the bone marrow. In this study, a CXCL12 gradient was observed to induce Pstn −/− and WT BM fibrocyte chemotaxis across porous polycarbonate filters. Interestingly however, migration of WT cells was substantially greater than that of Pstn −/− cells. This
suggests that periostin expression may increase the sensitivity of BM fibrocytes to CXCL12, potentially by upregulating the expression of CXCR4 on BM fibrocytes in vitro. CXCR4 upregulation on fibrocytes has been shown to be induced by hypoxia and growth factors through the PI3-kinase/Akt/mTOR pathway\textsuperscript{26}. Of note, periostin expression has also been identified to activate the PI3-kinase/Akt pathway, inducing collagen type I expression and promoting the proliferation of murine dermal fibroblasts in vitro\textsuperscript{21,29}. To our knowledge however, the influence of periostin expression on CXCR4 receptor upregulation has not been studied. Future work investigating this, as well as the potential involvement of the PI3-kinase/Akt pathway in periostin-induced CXCR4 upregulation, would provide valuable information regarding the mechanisms that govern fibrocyte homing to sites of tissue injury.

Adhesion molecules, namely integrins, also play a central role in cell trafficking. Integrins are the major transmembrane receptors that mediate cellular interactions with the ECM during cell migration. By linking the ECM to actomyosin filaments, integrin-based adhesions generate traction for cell movement and also organize signaling networks that regulate cell migration, as well as other cellular processes including gene expression, differentiation, proliferation and apoptosis\textsuperscript{30}. Treatment of dermal fibroblasts with exogenous periostin has been shown to increase the phosphorylation of integrin-associated signaling molecules, such as FAK, STAT3, Akt, and p44/42MAPK, suggesting that interactions between periostin and cell-surface integrins activate key signaling pathways involved in wound repair\textsuperscript{21,29}.

In this study, we observed that periostin gene deletion significantly reduces BM
fibrocyte adhesion to periostin and fibronectin, two ECM proteins expressed after dermal injury that are also known to mediate cell migration. Based on these differences, we were interested to determine whether Pstn −/− BM fibrocytes also exhibit reduced integrin-mediated migration. We performed migration assays using periostin and fibronectin-coated polycarbonate filters. Migration on both surfaces was mediated by integrin-substrate interactions. Blocking antibodies against αv and β1 integrin subunits inhibited CXCL12-induced BM fibrocyte migration on periostin, while antibodies against β1 and β3 subunits inhibited migration on fibronectin. These findings highlight similarities between integrin function on fibrocytes and on endogenous fibroblasts. Additionally, on periostin-coated filters, periostin gene deletion was observed to significantly reduce both random and CXCL12-induced migration of BM fibrocytes. These differences were not observed on fibronectin-coated filters, suggesting changes in migratory behavior to be substrate specific.

The adhesive and migratory behavior of cells is heavily affected by cell type and integrin expression31. Our results suggest that periostin gene deletion alters the transcriptome of BM fibrocytes in culture, potentially affecting integrin expression and/or downstream signaling pathway activation. Further research investigating the influence of periostin on integrin expression and the activation of integrin-associated signaling molecules such as PI3-kinase will be important for understanding the mechanisms that govern fibrocyte adhesion and migration. Such knowledge will assist in the development of therapeutics aimed at modulating fibrocyte trafficking in vivo.

To supplement our in vitro findings with in vivo evidence of periostin’s influence
on fibrocyte participation in the CXCR4/CXCL12 signaling axis, we also studied how periostin gene deletion affects CXCR4⁺/Col ¹ fibrocyte levels in the blood. Tourkina et al. previously reported increased levels of both CD45⁺/Col ¹ and CXCR4⁺/Col ¹ fibrocytes in the peripheral blood of patients with systemic sclerosis. CD45⁺/Col ¹ cells were found to be more prevalent than CXCR4⁺/Col ¹ cells in this patient population. Our results reflect a similar trend. Dermal injury increased the number of circulating CXCR4⁺/Col ¹ cells in both Pstn −/− and WT mice. CXCR4⁺/Col ¹ fibrocytes levels were also slightly lower than the CD45⁺/Col ¹ fibrocyte levels reported in Chapter 4.

Most interestingly however, at 4 and 7 days after injury, a significantly greater number of CXCR4⁺/Col ¹ fibrocytes was observed in the peripheral blood of WT mice compared to Pstn −/− mice. This suggests that injury-induced periostin expression may increase circulating fibrocyte levels by stimulating the mobilization of CXCR4⁺ fibrocytes and their precursors from the bone marrow into the peripheral circulation or by promoting the fibroblastic differentiation of CXCR4⁺ precursors in the peripheral blood. Our in vitro data suggests that increased mobilization of fibrocytes and their precursors from the bone marrow into the peripheral circulation may occur through two mechanisms, including by enhanced sensitivity to injury-induced CXCL12 expression through CXCR4 upregulation or by increased integrin-mediated migration through altered expression of integrin receptors and/or activation of downstream signaling pathways. Additional in vitro and in vivo studies that quantify the effects of periostin on CXCR4 and integrin receptor expression as well as migration-related signaling pathway activation must be performed to determine whether these mechanisms specifically
contribute to the higher CXCR4⁺/Col I⁺ fibrocyte levels observed in injured WT mice relative to Pstn −/− mice.

5.5 Conclusions

The mechanisms that govern the mobilization, homing, and engraftment of fibrocytes in response to tissue injury remain to be fully understood. However, in this study we demonstrate a novel role for periostin in promoting fibrocyte adhesion and migration. Using in vitro assays, we show that periostin gene deletion reduces BM fibrocyte adhesion to both periostin and fibronectin and decreases integrin-mediated migration on periostin, as well as CXCL12-induced chemotaxis. These results suggest that periostin expression influences the transcriptome of fibrocytes in culture, potentially altering integrin and chemokine receptor expression and migration-related signaling pathway activation. Such changes could reduce the mobilization of fibrocytes and their precursors from the bone marrow into the peripheral circulation and decrease their infiltration into dermal wounds. We also present in vivo data that supports our in vitro findings and suggests that periostin expression positively influences fibrocyte participation the CXCL4/CXCL12 signaling axis. While periostin expression likely affects fibrocyte behavior through multiple mechanisms, our data provides original evidence suggesting that periostin plays a significant role in fibrocyte trafficking after dermal injury.
5.6 References


CHAPTER SIX

CONCLUSIONS

Dermal wound repair is a complex process guided by a dynamic interplay between various cell types and the surrounding extracellular environment. Understanding this interplay is important for designing effective therapies that promote the closure of non-healing wounds and prevent excessive scar formation. Periostin expression and bone marrow-derived fibrocyte infiltration have been shown by other investigators to independently contribute to dermal wound closure and tissue fibrosis. However, to our knowledge, the relationship between these components in the context of dermal repair has not been investigated. The present work demonstrates a novel role for periostin in promoting fibrocyte contribution to dermal wound healing and also suggests that therapeutic strategies aimed at modulating injury-induced periostin expression may be effective in controlling fibrocyte contribution to wound closure and tissue fibrosis.

6.1 Summary of Key Findings

The overall objective of this project was to identify how periostin expression influences fibrocyte behavior in response to dermal injury. Based on an extensive review of the literature, we hypothesized that injury-induced periostin expression enhances fibrocyte contribution to dermal wound closure and scar formation. The following three aims were used to study this hypothesis: (I) identify the effects of periostin expression on the composition and structural organization of the reparative ECM after dermal injury, (II) determine the effects of periostin expression on fibrocyte levels after dermal injury,
and (III) establish the effects of periostin expression on fibrocyte trafficking. In this section, we will summarize the conclusions we have drawn from this project as they apply to these aims and the overall project objective.

Several independent studies previously demonstrated that periostin gene deletion delays dermal wound closure, suggesting that exogenous application of periostin protein may be useful for the treatment of non-healing lesions\textsuperscript{1-3}. However, despite its elevated expression in dermal fibroproliferative disorders, the influence of periostin on the composition and architecture of the reparative ECM in dermal wounds has not been previously examined. In Chapter 3, we showed for the first time that loss of periostin most significantly alters scar tissue composition by reducing thick collagen fiber content at late stages of wound remodeling. Interestingly, periostin gene deletion has also been shown to reduce collagen accumulation in mice after myocardial infarction and bleomycin-induced pulmonary fibrosis, two fibrotic pathologies associated with significant levels of fibrocyte infiltration\textsuperscript{4,5}. Subsequent quantification of CD45\textsuperscript{+}/Col I\textsuperscript{+} fibrocytes through immunofluorescence labeling revealed that periostin gene deletion also reduces fibrocyte levels in dermal wounds at late stages of repair. Fibrocytes represent an additional source of collagen in dermal wounds. They also secrete numerous cytokines, chemokines, and growth factors that have been suggested to regulate resident fibroblast activity by promoting migration, proliferation, and collagen production\textsuperscript{6}. Together, these results suggest that injury-induced periostin expression may promote collagen accumulation in dermal scar tissue by increasing fibrocyte participation in late stages of dermal wound repair.
In addition to promoting tissue fibrosis, infiltrating fibrocytes have the ability to facilitate wound closure by differentiating into myofibroblast-like cells that participate directly in wound contraction and by secreting molecular effectors that promote the myofibroblastic differentiation of resident fibroblasts\textsuperscript{6,7}. In Chapter 4 we examined whether periostin expression also increases CD45\textsuperscript{+}/Col I\textsuperscript{+} fibrocyte levels in dermal wounds at early stages of repair, specifically during the wound contraction period. Using flow cytometry, we observed fibrocyte levels in dermal wounds to be significantly higher in WT mice than in Pstn\textsuperscript{−/−} mice at 7 days after injury. Interestingly, the expression of periostin and α-SMA also peaks at this time point. These results suggest that, in addition to promoting collagen accumulation in mature wounds, periostin expression may also stimulate wound closure by increasing the number of fibrocytes present in dermal wounds at early stages of repair.

To study fibrocyte behavior outside of the dermal wound bed, we also measured CD45\textsuperscript{+}/Col I\textsuperscript{+} fibrocyte levels in the bone marrow and peripheral blood before and after dermal injury. Dermal wounding did not increase bone marrow fibrocytes levels, indicating that factors produced in response to dermal injury do not significantly affect fibrocyte formation in the bone marrow. In the peripheral blood however, fibrocyte levels were significantly elevated, as has been previously observed in human patients with various fibrotic disorders. To our knowledge, this is the first time that excisional dermal wounding has been shown to stimulate an increase in circulating fibrocyte levels. In addition to demonstrating that dermal injury-induced factors influence fibrocyte behavior in the blood, these results also suggest that the circulation may be a significant source of
fibrocytes that infiltrate dermal wounds. In response to these observations, we studied how dermal injury-induced periostin expression specifically affects circulating fibrocyte levels. As observed previously in the dermal wound bed, we found that periostin expression also elevates fibrocyte levels in the peripheral blood. This demonstrates that periostin influences fibrocyte behavior beyond the site of tissue injury and raises important questions regarding the impact of structural and hematopoietic sources of periostin on fibrocyte behavior.

Collectively, the results described in Chapters 3 and 4 suggest a novel role for periostin in modulating fibrocyte behavior at both early and late stages of dermal repair and in multiple locations, including in the peripheral blood and dermal wound bed. Periostin expression may enhance the injury-induced increase in fibrocyte levels in the peripheral blood and dermal wound bed by promoting the fibroblastic differentiation of fibrocyte precursors and/or increasing the trafficking of fibrocytes and their precursors into these tissues. These possible mechanisms, which will be discussed in greater detail in Section 6.2, are supported by the findings of other investigators that show periostin expression to promote (myo)fibroblastic cell differentiation as well as cell adhesion and migration.

The mobilization, homing, and engraftment of fibrocytes after tissue injury is critical for their participation in wound repair and fibrosis. In Chapter 5, we compared the migratory ability of Pstn −/− and WT BM fibrocytes to determine whether fibrocyte trafficking is influenced by periostin expression. Using in vitro migration assays, we demonstrated that periostin gene deletion reduces CXCL12-induced chemotaxis and
integrin-mediated migration on periostin coated filters. These results suggest that periostin expression alters the transcriptome of BM fibrocytes *in vitro*, potentially reducing the expression of chemokine and integrin receptors, as well as the activation of migration-related signaling pathways. These changes could reduce the mobilization of fibrocytes from the bone marrow into the peripheral circulation and decrease their infiltration into dermal wounds, leading to the lower fibrocyte levels observed in Pstn −/− mice as reported in Chapters 3 and 4. We also presented data showing that periostin gene deletion reduces the number of CXCR4+/Col I+ fibrocytes in the peripheral blood. This supports our *in vitro* findings and indicates that periostin expression has a positive influence on fibrocyte participation in the CXCR4/CXCL12 signaling axis *in vivo*. Together, the results presented in Chapter 5 provide evidence that periostin plays an important role in fibrocyte trafficking and suggest that loss of periostin expression may reduce fibrocyte levels by decreasing their migratory ability.

Collectively, the clinical and scientific implications of the work presented in this dissertation are quite significant. For the first time, we demonstrate that injury-induced periostin expression positively influences fibrocyte behavior after dermal injury, as observed through increased fibrocyte levels in the peripheral blood and skin and increased fibrocyte migration *in vitro*. Other investigators have shown periostin expression and fibrocyte infiltration to individually promote wound closure and tissue fibrosis. In this work however, by relating periostin protein function to fibrocyte behavior, we provide original evidence suggesting that modulation of periostin expression can be used to control fibrocyte contribution to wound repair and fibrosis.
Because of the substantial burden that wound healing complications place on our healthcare system, this is a very exciting scientific discovery that represents a significant advancement in our understanding of how the extracellular environment influences cell function during tissue repair. This work also provides a foundation for future scientific research to explore the relationship between periostin expression and fibrocyte behavior in other organ systems, particularly those affected by severe tissue fibrosis.

6.2 Suggested Mechanisms

The present work raises important questions regarding the specific mechanisms by which periostin expression increases fibrocyte levels in the peripheral blood and skin after excisional dermal wounding. Based on a review of the current literature and the results presented in Chapters 3, 4, and 5, we propose the increase in fibrocyte levels to result from increased fibroblastic differentiation of fibrocyte precursors and/or increased trafficking of fibrocytes and their precursors. The schematic shown in Figure 6.1 outlines these mechanisms.

In 2006, Visconti et al. proposed the “periostin hypothesis,” which suggests that periostin promotes and maintains the fibroblastic differentiation of cells and/or inhibits their differentiation into other mesenchymal lineages. Research examining this function of periostin has focused primarily on cardiac development and repair, with periostin having been shown to direct the fibroblastic differentiation and collagen secretion of cardiac mesenchymal progenitor cells. More recently, periostin expression has been shown to promote myofibroblastic differentiation in dermal wounds, leading to enhanced
wound closure. The results presented in this dissertation suggest that periostin may also enhance the fibroblastic differentiation of bone marrow progenitor cells, as evidenced by increased fibrocyte levels in the peripheral blood and dermal wound bed of WT mice relative to Pstn −/− mice. To further test whether periostin is an active effector of fibroblastic differentiation in fibrocyte precursors, we recommended quantifying fibroblast-specific mRNA and protein expression in cultured BM fibrocytes and in fibrocytes and their CD14+ mononuclear precursors isolated from the peripheral blood and skin of WT and Pstn −/− mice after dermal injury.

The results described in Chapter 5 suggest that periostin expression also influences fibrocyte trafficking, specifically by increasing fibrocyte migration in response to the CXCR4/CXCL12 signaling axis. Such changes could increase the mobilization of fibrocytes and their precursors from the bone marrow into the peripheral blood and/or their infiltration into the wound bed after dermal injury. This would lead to the higher fibrocyte levels observed in the blood and skin of WT mice compared to Pstn −/− mice. Other studies have previously reported periostin expression to promote cell motility, namely through increased integrin-mediated activation of various signaling pathways. Additional insights into the possible role of periostin in promoting the trafficking of fibrocytes and their precursors could be provided by examining the phosphorylation of key signaling molecules such as FAK, Akt, and PI3-kinase in cultured BM fibrocytes and in fibrocytes and their CD14+ mononuclear precursors isolated from murine bone marrow, peripheral blood, and dermal wounds after injury. In addition to activating migration-related signaling pathways, our results suggest that periostin may
upregulate chemokine and integrin receptor expression on fibrocytes. Therefore, we also recommend quantifying the expression of the chemokine receptor CXCR4 and integrin receptor subunits $\alpha_v$, $\beta_1$, and $\beta_3$ on cultured BM fibrocytes as well as murine fibrocytes and their CD14$^+$ mononuclear precursors \textit{in vivo}. Such work would help to further elucidate the role that periostin plays in fibrocyte homing to dermal wounds.

![Figure 6.1 Schematic outlining potential mechanisms by which periostin expression increases fibrocyte levels in the peripheral blood and skin after dermal injury. Blue cells: fibrocyte precursors, Green cells: mature fibrocytes.](image)
6.3 Recommendations for Future Work

The work presented in this dissertation establishes a novel role for periostin in modulating fibrocyte behavior in response to dermal injury and provides a foundation for future studies aimed at controlling fibrocyte contribution to wound repair and tissue fibrosis in the skin and other organs. Having shown a positive relationship between periostin expression and fibrocyte levels, in addition to the work proposed in Section 6.2, we now recommend the following future studies to further elucidate the mechanisms that govern this relationship and to identify whether local modulation of periostin expression may be used to control fibrocyte activity for therapeutic applications.

1. Examine how hematopoietic and structural sources of periostin influence fibrocyte levels after dermal injury.

In a recent publication, Naik et al. showed that periostin levels are upregulated in both the lungs and peripheral blood of patients with IPF. Pulmonary fibroblasts were observed to be the primary source of periostin in the lungs, while monocytes and fibrocytes were found to produce periostin in the blood. Using chimeric mice, Naik et al. established that both sources contribute to lung fibrosis. However, the effect of each source on fibrocyte behavior was not specifically examined. We first suggest quantifying periostin levels in the peripheral blood of injured WT mice to determine whether excisional dermal injury also upregulates periostin expression in the circulation. Then, to establish whether hematopoietic and/or structural sources of periostin affect fibrocyte behavior, we recommend measuring fibrocyte levels in the
peripheral blood and dermal wound bed of chimeric mice generated by transplantation of WT or Pstn −/− bone marrow into WT or Pstn −/− recipients (Table 6.1)

<table>
<thead>
<tr>
<th>BM Donor</th>
<th>Recipient</th>
<th>WT</th>
<th>Pstn −/−</th>
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<tr>
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<td></td>
<td>WT x WT (control)</td>
<td>WT x Pstn −/− (hematopoietic sources of periostin)</td>
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<tr>
<td>WT</td>
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<tr>
<td>Pstn −/−</td>
<td>Pstn −/− x WT (structural sources of periostin)</td>
<td>Pstn −/− x Pstn −/− (control)</td>
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Table 6.1 Generation of chimeric mouse models to study the influence of hematopoietic and structural sources of periostin on fibrocyte behavior.

2. Determine whether modulation of periostin expression can be used to control fibrocyte contribution to wound closure and tissue fibrosis.

If structural sources of periostin (i.e. fibroblast-secreted periostin in the granulation tissue) are observed to enhance infiltrating fibrocyte levels and promote collagen accumulation in the dermal wound bed, we recommend conducting additional studies to determine whether local modulation of periostin expression can be used to control fibrocyte behavior for therapeutic applications. Recombinant periostin protein can be delivered to increase periostin levels for delayed wound repair applications and a neutralizing antibody against periostin or silencing RNAs (siRNAs) can be delivered to inhibit local expression of periostin for the reduction of dermal scarring.
To facilitate the clinical translation of this work, appropriate drug delivery systems should be tested to optimize effective modulation of periostin expression in dermal wounds. Delivery of periostin protein, neutralizing antibodies, and siRNAs can be achieved through multiple approaches, which broadly include topical application, intradermal injection, and incorporation into biodegradable scaffolds. The rate of wound closure, collagen content in the granulation tissue, and fibrocyte levels in the peripheral blood and skin should be measured to assess the efficacy of each treatment method.

3. Study how periostin expression influences fibrocyte participation in wound closure in hard-to-heal wounds and, at the opposite end of the spectrum, scarring in cases of excessive dermal fibrosis.

The model of excisional dermal wound repair used in this project followed a sequence of events associated with normal wound healing. However, dermal wounds requiring advanced therapeutic treatments typically exhibit impairments in the healing process that prevent timely wound closure and result in excessive tissue fibrosis. Therefore, we also recommend examining the effects of periostin expression on fibrocyte behavior in murine models characterized by delayed and fibrotic wound healing to determine whether the relationship established in this dissertation holds true in cases of impaired wound repair. Two specific murine models that we suggest for these studies include excisional dermal injury in diabetic (db/db) mice and bleomycin-induced skin fibrosis.
4. Explore the relationship between periostin expression and fibrocyte levels in other organ systems and pathologies

As discussed throughout this dissertation, periostin expression and fibrocyte infiltration have been shown to individually contribute to tissue fibrosis in several organs other than the skin. Given the devastating effects of excessive fibrosis in the heart and lungs, as well as the clear contribution of these elements to cardiac and pulmonary fibrogenesis, we recommend examining the relationship between periostin expression and fibrocyte infiltration in these organs as well.

6.4 References


