ROLE OF AMINOPEPTIDASE N IN WOUND HEALING

by

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Abstract

The dynamics and complexity of tissue repair are dominated by specific and intricately coordinated cellular events. Disruptions at the level of cellular communication are associated with imbalanced extracellular matrix (ECM) synthesis/degradation leading to fibrosis and chronic wounds. Our group has demonstrated that 14-3-3 sigma (also known as stratifin) functions as a stimulator of matrix metalloproteinase-1 (MMP-1) through interactions with aminopeptidase N (APN) on the surface of dermal fibroblasts. In this doctoral research project, it is hypothesized that APN functions as a receptor for keratinocyte-derived paracrine signals that control the expression of key ECM components in dermal fibroblasts.

Three specific objectives were accomplished in this project. Under Objective 1, the nature of APN expression in an environment of active epithelial-stromal communication was examined using an in vitro keratinocyte-fibroblast crosstalk model. The fibroblast expression of APN was significantly upregulated in the presence of keratinocyte-releasable soluble factors, of which stratifin was shown to be a potent stimulator. In light of the recent identification of APN as a receptor responsible for stratifin-mediated p38 MAPK activation leading to upregulation of MMP-1, the role of APN as a transmembrane mediator of signals that regulate ECM remodeling was investigated in Objective 2. Comparative analysis of the expression profiles of 118 ECM genes under conditions of keratinocyte stimulation and APN gene silencing revealed a group of key matrix proteases and adhesion molecules influenced by keratinocyte-derived signaling mediated
through APN. The aim of Objective 3 was to explore the therapeutic potential of targeting APN in cutaneous tissue repair. Topical application of an APN-neutralizing antibody on full-thickness skin wounds in a murine model had a positive outcome in healing. Acceleration of wound closure was accompanied by increased collagen deposition and fibroblast contractility.

Collectively, the findings presented herein confirmed our hypothesis that APN can be induced by keratinocytes and acts as a regulator of keratinocyte-derived stimuli in epidermal-dermal communication. Specifically, these findings support the receptor role of APN in mediating transmembrane signals derived from keratinocytes, and provide encouraging evidence for further investigations on the therapeutic use of APN agonist/antagonists in the field of tissue repair.
Preface

The work presented in this thesis has already been published or submitted for publication. This is to confirm that Amy M. Lai is the first author in all publications included in this thesis as shown below.


The principal investigator of the research critically reviewed all the published manuscripts included in this thesis. The financial support for this thesis was provided by CIHR grants held by Dr. Ghahary. Amy Lai is responsible for the experimental design, data analysis, and manuscript preparation for all the work described in this thesis with the exception of:

Chapter 2: Abdi Ghaffari performed the R18 pull-down assay. Yunyuan Li designed the construct for stratifin expression in mammalian cells.
Chapter 3: Abdi Ghaffari established the siRNA transfection protocol. Yunyuan Li provided helpful advice for the experimental design.

Chapter 4: Azadeh Hosseini-Tabatabaei and Ryan Hartwell assisted with animal handling and treatment. Elham Rahmani-Neishaboor prepared the gel for topical application. Ruhangiz Kilani tested the antibody used for immunoblot analysis.

The work described in this thesis has been conducted with the approval of the University of British Columbia Biohazards Committee under Certificate No. H05-0103. All animal studies have been conducted with close supervision of the University of British Columbia Animal Care Committee under Protocol No. A05-1211.
# Table of Contents

ABSTRACT ............................................................................................................................... II

PREFACE ............................................................................................................................... IV

TABLE OF CONTENTS ............................................................................................................. VI

LIST OF TABLES ........................................................................................................................ IX

LIST OF FIGURES ....................................................................................................................... X

LIST OF ABBREVIATIONS ........................................................................................................ XI

ACKNOWLEDGEMENTS ........................................................................................................... XIV

DEDICATION .............................................................................................................................. XV

CHAPTER 1. INTRODUCTION ............................................................................................. 1

Dermal Wound Healing ......................................................................................................... 1

The Dynamics of Extracellular Matrix during Dermal Repair ............................................. 4

Signal-mediated Intercellular Communication in Wound Healing ....................................... 6

Wound Contraction ................................................................................................................. 7

Matrix Metalloproteinases ...................................................................................................... 9

14-3-3 Proteins ....................................................................................................................... 15

Aminopeptidase N ................................................................................................................. 17

Hypotheses and Objectives ............................................................................................... 19

CHAPTER 2. PARACRINE REGULATION OF FIBROBLAST AMINOPEPTIDASE N/CD13 EXPRESSION BY KERATINOCYTE-RELEASABLE STRATIFIN ........................................ 24

Introduction .......................................................................................................................... 24

Materials and Methods .......................................................................................................... 27

Results ................................................................................................................................... 32

APN expression in dermal fibroblasts co-cultured with keratinocytes ................................... 32
Keratinocyte differentiation further enhances APN stimulation
14-3-3 proteins contribute to the paracrine regulation of APN
Releasable form of SFN increases APN at the gene and protein levels
siRNA knockdown of SFN compromises the ability of keratinocytes to stimulate APN
Bestatin treatment abolishes SFN-induced cell migration
Discussion

CHAPTER 3. MICROARRAY-BASED IDENTIFICATION OF AMINOPEPTIDASE N
TARGET GENES IN KERATINOCYTE CONDITIONED MEDIUM-STIMULATED DERMAL
FIBROBLASTS

Introduction
Materials and Methods
Results
ECM gene expression profiling following APN knockdown and KCM stimulation
APN modulates the keratinocyte-mediated regulation of matrix proteases
APN-mediated regulation of matrix proteases is independent of its enzymatic activity
APN-mediated downregulation of fibronectin production
APN modulates tenascin-C expression in an activity-dependent manner
Discussion

CHAPTER 4. TOPICAL APPLICATION OF AMINOPEPTIDASE N-NEUTRALIZING
ANTIBODY ACCELERATES WOUND CLOSURE

Introduction
Materials and Methods
Results
Topical application of APN mAb accelerated wound closure
Enhanced α-smooth muscle actin expression and contraction
Discussion
List of Tables

Table 1.1 Expression of MMPs and TIMPs during wound healing............................................... 11

Table 1.2 Substrate specificities of the members of the MMP family........................................... 14
List of Figures

Figure 1.1 Stages of wound healing................................................................. 3
Figure 1.2 Schematic summary of APN-mediated cellular activities in wound repair. ............... 23
Figure 2.1 Co-culturing human dermal fibroblasts with keratinocytes induces APN expression. 34
Figure 2.2 Keratinocyte differentiation has additional stimulatory effect on APN expression. ..... 36
Figure 2.3 14-3-3 depletion reduces the capacity of KCM to induce APN expression. ............... 39
Figure 2.4 SFN released by DsRed-SFN transfected 293T cells stimulates APN expression in fibroblasts................................................................. 41
Figure 2.5 siRNA knockdown of SFN compromises the ability of keratinocytes to stimulate APN expression in fibroblasts. ................................................................. 43
Figure 2.6 SFN-induced cell migration is dependent on the enzymatic activity of APN. ............... 45
Figure 3.1 Microarray-based ECM gene expression profiling of dermal fibroblasts following APN knockdown and KCM stimulation......................................................... 57
Figure 3.2 APN-mediated regulation of matrix proteases. ......................................................... 59
Figure 3.3 Effect of bestatin on the expression of MMPs in KCM-treated fibroblasts. ................. 60
Figure 3.4 APN-mediated downregulation of fibronectin production........................................ 62
Figure 3.5 APN-mediated regulation of tenascin-C expression. ............................................... 63
Figure 3.6 Bestatin-induced collagen gel contraction............................................................. 65
Figure 4.1 Healing of full-thickness skin wounds in mAb-treated mice. ..................................... 75
Figure 4.2 Analysis of α-SMA expression and contraction in skin wounds or dermal fibroblasts exposed to IgG, WM15, and bestatin. ................................................................. 76
# List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>APN</td>
<td>Aminopeptidase N</td>
</tr>
<tr>
<td>α-SMA</td>
<td>α-Smooth Muscle Actin</td>
</tr>
<tr>
<td>b-FGF</td>
<td>basic Fibroblast Growth Factor</td>
</tr>
<tr>
<td>CTNNA1</td>
<td>Catenin Alpha 1</td>
</tr>
<tr>
<td>DEAE</td>
<td>Diethylaminoethyl</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle's Medium</td>
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<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic Acid</td>
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<tr>
<td>EGF</td>
<td>Epidermal Growth Factor</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethylene Glycol Tetraacetic Acid</td>
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<tr>
<td>ER</td>
<td>Endoplasmic Reticulum</td>
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<td>EMPRIN</td>
<td>Extracellular MMP Inducer</td>
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<td>ETS</td>
<td>Erythroblastosis Twenty Six</td>
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<td>FAK</td>
<td>Focal Adhesion Kinase</td>
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<td>FBPA</td>
<td>Fructose-Biphosphate Aldolase</td>
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<td>FBS</td>
<td>Fetal Bovine Serum</td>
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<tr>
<td>FGF</td>
<td>Fibroblast Growth Factor</td>
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<td>FN</td>
<td>Fibronectin</td>
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<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3 Phosphate Dehydrogenase</td>
</tr>
<tr>
<td>Grb2</td>
<td>Growth factor receptor-bound protein 2</td>
</tr>
<tr>
<td>HGF</td>
<td>Hepatocyte Growth Factor</td>
</tr>
<tr>
<td>HUVEC</td>
<td>Human Umbilical Vein Endothelial Cell</td>
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<tr>
<td>IFN</td>
<td>Interferon</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<td>ITGA</td>
<td>Integrin Alpha</td>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>ITAM</td>
<td>Immunoreceptor Tyrosine-based Activation Motif</td>
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<td>KCM</td>
<td>Keratinocyte Conditioned Medium</td>
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<td>KGF</td>
<td>Keratinocyte Growth Factor</td>
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<tr>
<td>KSFM</td>
<td>Keratinocyte Serum Free Medium</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MAb</td>
<td>Monoclonal Antibody</td>
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<tr>
<td>MAPK</td>
<td>Mitogen-Activated Protein Kinase</td>
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<td>MMP</td>
<td>Matrix Metalloproteinase</td>
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<tr>
<td>mRNA</td>
<td>messenger RNA</td>
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<tr>
<td>PBS</td>
<td>Phosphate-Buffered Saline</td>
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<tr>
<td>PDGF</td>
<td>Platelet-Derived Growth Factor</td>
</tr>
<tr>
<td>PI3 Kinase</td>
<td>Phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>rSFN</td>
<td>Recombinant Stratifin</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse Transcriptase-Polymerase Chain Reaction</td>
</tr>
<tr>
<td>TN-C</td>
<td>Tenascin-C</td>
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<tr>
<td>SDS-PAGE</td>
<td>Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>siRNA</td>
<td>small-interfering RNA</td>
</tr>
<tr>
<td>SFN</td>
<td>Stratifin</td>
</tr>
<tr>
<td>SOS</td>
<td>Son of Sevenless</td>
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<tr>
<td>SPOCK</td>
<td>Sparc/osteonectin, cwcv and kazal-like domains proteoglycan</td>
</tr>
<tr>
<td>TCR</td>
<td>T Cell Receptor</td>
</tr>
<tr>
<td>TGEV</td>
<td>Transmissible Gastroenteritis Virus</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming Growth Factor-beta</td>
</tr>
<tr>
<td>TGFβRIII</td>
<td>Type III TGFβ Receptor</td>
</tr>
<tr>
<td>TIMP</td>
<td>Tissue Inhibitor of Metalloproteinase</td>
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<tr>
<td>TNF-α</td>
<td>Tumor Necrosis Factor-alpha</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>TACE</td>
<td>TNF-α Converting Enzyme</td>
</tr>
<tr>
<td>TSP-1</td>
<td>Thrombospondin-1</td>
</tr>
<tr>
<td>VEGFR</td>
<td>Vascular Endothelial Growth Factor Receptor</td>
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</table>
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Dedication

To my loving parents who have endured many hardships to provide me with the best education possible, and

my brothers George and Robert who have always stood by my side during all these years.
Chapter 1. Introduction

Dermal Wound Healing

Skin is the largest organ in the body and serves as the first line of defense against infection, dehydration, and physical distress. The ability to repair injured skin is essential for maintaining the integrity and functionality of the skin. Immediately following tissue injury and disruption of blood vessels, platelets extravasate from blood vessels and aggregate at the injured site to participate in the formation of a provisional matrix allowing cell attachment and migration [1]. This so called “hemostasis” is an initial event of the intricately orchestrated process of wound healing, followed by inflammation, proliferation and tissue remodeling. Interleukin-1 (IL-1), released from the reservoir in epidermis, is the first signal that alerts surrounding cells to the barrier disruption [2-4]. In response to platelet-released growth factors such as platelet-derived growth factor (PDGF) and transforming growth factor-β (TGF-β), neutrophils migrate to the wound bed, and become engaged in bacterial clearance and tissue debridement. Replacing the neutrophils, macrophages become the predominant cell type in the inflammatory phase, and secrete a cocktail of growth factors including epidermal growth factor (EGF), PDGF, and TGF-β, as well as cytokines such as IL-1 and IL-6 to recruit active players in the subsequent stage of wound healing [5]. Re-epithelialization is initiated shortly after injury, and keratinocyte proliferation and migration can be stimulated by the release of EGF [6], transforming growth factor-α (TGF-α) [7,8], and fibroblast growth factor (FGF) [9]. Once re-
epithelialization is complete, keratinocytes undergo differentiation and stratification to restore the skin barrier [10]. In the proliferative phase, granulation tissue is formed, and endothelial cells establish new blood vessels to provide oxygen and nutrients to cells at the wound site. PDGF induces fibroblast proliferation and its expression of proteoglycans which are required for fibroblast migration and attachment to the provisional matrix [11]. The binding of fibroblasts to fibronectin in the provisional matrix further stimulates their expression of proteoglycans as well as hyaluronic acid and collagen [12,13]. Wound maturation relies on fibroblasts to deposit type I collagen to replace type III collagen accumulated in the provisional matrix, and secrete proteases such as matrix metalloproteinases (MMPs) to continuously remodel the nascent tissue [14]. Fibroblasts can also differentiate into myofibroblasts which provide contractile force to mechanically reduce wound size [15]. TGF-β1 is an important control signal that impacts a range of cellular activities in the wound including fibroblast-mediated matrix deposition and contraction [16]. At the early stages of wound healing, it induces transcription of the genes for collagen, proteoglycans and fibronectin, increasing the overall production of matrix proteins for granulation tissue formation. At the same time, TGF-β1 suppresses protease-mediated matrix degradation by stimulating the expression of tissue inhibitors of metalloproteinase (TIMPs) [17]. Another feature that characterizes TGF-β1 as a pro-fibrotic factor is its ability to inhibit myofibroblast apoptosis [18] and induce myofibroblast differentiation, which is commonly accompanied by enhanced contraction as well as increased collagen deposition and α-smooth muscle actin
expression by fibroblasts [19,20]. Eventually the wound becomes less cellular and vascular, and scar tissue with reduced tensile strength is formed [21]. The various cell types and their main action in different phases of wound healing are summarized in Fig. 1.1.

Figure 1.1 Stages of wound healing.

Courtesy of Pilonidal Support Alliance (http://www.pilonidal.org).
The Dynamics of Extracellular Matrix during Dermal Repair

The major components of dermal extracellular matrix (ECM) are fibrous structural proteins, consisting of 80-85% type I collagen, 8-11% type III collagen, and smaller amounts of elastin [22]. While collagens provide tensile strength to the skin, elastin confers resiliency allowing the skin to stretch and return to its original shape. Type 1 collagen is composed of two \( \alpha_1 \) (COL1A1) and one \( \alpha_2 \) (COL1A2) polypeptide chains. Collagen is synthesized and secreted in a precursor form (procollagen), which is then cleaved by procollagen proteases to allow extracellular assembly [23].

Apart from the high content of structural proteins, the ECM contains a complex mixture of matricellular proteins and non-fibrous proteoglycans, as well as adhesive and adaptor proteins such as fibronectin which serves as a substrate for migratory cells in wound healing [24]. In addition to major protein fibrils, glycosaminoglycans (GAG) and proteoglycans fill void spaces, enhance mechanical strength, and ensure cell adhesion. GAG’s such as dermatan sulfate, heparin sulfate, chondroitin sulfate and the non-sulfated hyaluronic acid are long chain polysaccharides containing either N-acetylglucosamine or N-acetylgalactosamine [24]. Similarly, proteoglycans are glycosaminoglycans.

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covalently bound to core proteins and are responsible for mediating cell adhesion and signaling.

Matricellular proteins including the galectins, osteopontin, SPARC, tenascins, thrombospondins, and vitronectin associate with the ECM but do not perform a structural role within tissues [25]. During dermal repair, these proteins are expressed temporarily and spatially to provide signals that coordinate cellular activities within the wound. For instance, thrombospondin-1 (TSP-1) associates with the provisional matrix via binding to fibrinogen and fibronectin, and functions through activation of TGF-β1 [26]. Through interactions with α3β1 and α5β3 integrin receptors [27], TSP-1 activates intracellular signals relevant to cell adhesion, smooth muscle cell migration, and fibroblast migration through phosphorylation of proteins such as phosphatidylinositol 3-kinase (PI3 kinase) and focal adhesion kinase (FAK) [28]. Tenascin-C (TN-C) is another large extracellular glycoprotein, comprising four distinct domains: an assembly domain, epidermal growth factor-like repeats, fibronectin type III-like repeats, and a C-terminal fibrinogen-like globe [29]. At sites of injury, TN-C is specifically expressed at the wound edge and promotes fibroblast migration [30,31]. Deletion studies showed that the fibronectin type III-like repeats domain is as effective as full-length TN-C in promoting fibroblast migration [29]. TN-C also modulates cell adhesion by inhibiting the binding of syndecan-4 to fibronectin [32]. In addition to these activities, TN-C inhibits contraction of a fibrin-fibrinogen matrix by downregulating FAK phosphorylation, and this is thought to prevent premature
contraction of the matrix before collagen can be properly deposited for granulation tissue formation [33].

Signal-mediated Intercellular Communication in Wound Healing

Healing of wounds is one of the most complex biological events after birth, and involves a carefully orchestrated network of cellular interactions mediated by soluble mediators. The first instance is the chemokine-dependent recruitment of infiltrating cells to the wound site. The infiltrating cells, namely neutrophils, macrophages, mast cells, and lymphocytes, not only serve as immunological effector cells but also provide inflammatory cytokines and mitogenic growth factors [34]. The action of chemokines also influences re-epithelialization, matrix remodeling, and angiogenesis as indicated by the presence of chemokine receptors on keratinocytes [35], fibroblasts [36], and endothelial cells [37].

As the inflammatory response subsides, cellular interactions become dominated by crosstalk between skin epithelial cells (or keratinocytes) and fibroblasts in the dermis. Keratinocytes are the primary cell type in the epidermis, and contribute to the barrier function of the skin. During wound healing, keratinocytes perform a critical task in restoring a functional epidermis in a timely manner [38]. This helps to maintain a moist environment for cells to regenerate new tissue while preventing potential pathogenic infection. The importance of epithelial-dermal interaction was clearly demonstrated by the fact that epithelial keratinization requires soluble factors released from fibroblasts [39,40]. Delay in
re-epithelialization is often associated with higher frequency of hypertrophic scar development [41]. The underlying fibroblasts contribute to the process of re-epithelialization by releasing keratinocyte growth factor (KGF) and hepatocyte growth factor (HGF) which promote keratinocyte proliferation and migration [39,40,42]. In return, keratinocytes have been reported to induce MMP-1 production in fibroblasts [43,44] through soluble mediators such as interleukin-1 [45], extracellular MMP inducer (EMPRIN) [46], and 14-3-3 σ (SFN) [47]. Epidermal influence is also evident in dermal collagen production. For instance, normal fibroblasts co-cultured with keloid-derived keratinocytes secrete collagen in a keloid-like manner [48].

**Wound Contraction**

The two fundamental processes that critically affect the closure of skin wounds are re-epithelialization which involves replication and movement of keratinocytes across the open wound bed, and contraction of granulation tissue which is primarily mediated by myofibroblasts. Morphologically, myofibroblasts exhibit features of smooth muscle (SM) cells such as actin microfilaments [49], and are connected via gap junctions [50]. In addition to expressing β- and γ-cytoplasmic actins found in fibroblasts, myofibroblasts express α-smooth muscle actin (α-SMA) which remains as the most reliable marker of myofibroblasts [51]. It is generally accepted that the appearance of proto-myofibroblasts which only express β- and γ-cytoplasmic actins marks the start of myofibroblastic modulation
of fibroblasts populating the granulation tissue of the wound [52,53]. Wound myofibroblasts are assumed to originate from local fibroblasts and circulating bone marrow-derived progenitor cells (or fibrocytes) [54,55]. Induction of the myofibroblastic phenotype involves growth factors such as TGF-β1 [56,57], and specialized ECM molecules such as the cellular fibronectin splice variant ED-A [58] in a mechanically stressed environment [59].

In wound healing, myofibroblasts generate contractile force which pulls on the surrounding matrix to facilitate contraction of the granulation tissue. The force is generated by contractile stress fibers composed of actin microfilaments associated with myosin and actin-binding proteins [60,61]. Differently from the regulation of SM cell contraction which depends on the calcium-dependent myosin-light-chain kinase [62,63], the tension produced by myofibroblasts is regulated by a Rho/Rho kinase-mediated inhibition of myosin phosphatase [64,65].

Of the many factors that influence development of hypertrophic scar, persistence of myofibroblasts after completion of epithelialization is associated with contracture and overproduction of extracellular matrix [66-68]. Generally viewed as a subpopulation of differentiated fibroblasts, the myofibroblast is shown to be the predominant source of type I collagen and fibrogenic cytokines in fibrotic lesions [69,70]. Despite advances in understanding the myofibroblast biology, it remains challenging to modulate myofibroblast activities that regulate the evolution of fibrotic disease. Potential treatment avenues may include manipulations to intervene with myofibroblast apoptosis, collagen production, or
proteolytic enzyme production. For instance, inhibition of α-SMA incorporation into stress fibers by administering the N-terminal sequence AcEEED has been shown to reduce tension and collagen synthesis by cultured myofibroblasts [71].

Matrix Metalloproteinases

The precise breakdown and reorganization of ECM proteins such as collagen, proteoglycan, glycoprotein, and elastin is essential to tissue repair and requires specific enzymes. The matrix metalloproteinase family represents a group of zinc-dependent proteolytic enzymes with a traditional role in ECM catabolism during embryonic growth and development, bone growth and resorption, tissue homeostasis, tumor metastasis, and wound healing [72]. The growing list of substrates identified has broadened the known functionality of MMPs to include regulation of signaling networks through cleavage of ECM and bioactive molecules [73,74]. The 28 members of the MMP family are categorized into five subfamilies according to their substrate specificity, primary structure, and cellular localization, namely, collagenases, gelatinases, stromelysins, matrilysins, and membrane-type MMPs.

All MMPs are expressed as inactive proteases that require cleavage of the autoinhibitory pro-domain to expose their catalytic site to substrates [72]. The majority of MMPs also possess a C-terminal hemopexin domain responsible for protease localization, substrate recognition, and degradation [75]. The functional
diversity and impact of MMPs demands tight regulation at the levels of RNA transcription, protein translation and secretion.

The availability of active MMPs also depends on protein localization, proenzyme activation, protease degradation, and ECM binding, as well as the presence of tissue inhibitors of metalloproteinases (TIMPs) [74,76]. The gene expression of MMPs is primarily regulated by promoters containing the activator protein-1 (AP-1) enhancer element and/or erythroblastosis twenty six (ETS) site [77]. The constitutive expression of MMPs in intact tissue is generally low. Their expression and activity is upregulated in restricted physiological processes such as the reproductive cycle and embryonic development, as well as pathologic conditions such as wound healing, tumor metastasis and arthritis [77,78]. In wound healing, the expression of MMPs is mainly regulated via activation of mitogen-activated protein kinase (MAPK), Smad, or NF-κB pathways by growth factors and cytokines abundant in wounds such as TGF-β, PDGF, tumor necrosis factor α (TNF-α), interleukin-1β (IL-1β), basic fibroblast growth factor (b-FGF), epidermal growth factor (EGF), and KGF. The expression and cellular source of MMPs and TIMPs in acute wounds is summarized in Table 1 [79].
Table 1.1 Expression of MMPs and TIMPs during wound healing.

<table>
<thead>
<tr>
<th>Proliferating Keratinocyte</th>
<th>Migrating Keratinocyte</th>
<th>Fibroblast</th>
<th>Neutrophil</th>
<th>Macrophage</th>
<th>Endothelial Cell</th>
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As demonstrated in Table 1.1, the implications of MMPs and TIMPs in wound healing are extensive. An important aspect of the inflammatory response is the influx and activation of leukocytes which is largely mediated by chemokines. Many MMPs have been shown to regulate chemokine activity, either by direct proteolysis or by establishing chemokine gradient. For instance, upon processing by MMP-1, CCL2, 7, 8, and 13 which primarily regulate monocyte chemotaxis form receptor antagonists that inhibit downstream signaling [80]. Mice lacking both \textit{Mmp-2} and \textit{Mmp-9} have fewer neutrophils and eosinophils present in bronchoalveolar lavage, and this is shown to be due to an altered CCL7, CCL11, and CCL17 gradient [81]. In the re-epithelialization phase, cells at the wound edge loosen their cell-cell and cell-ECM contacts, and begin to migrate across the wound [34]. As keratinocytes assume the migratory phenotype and encounter a type I collagen-rich dermal matrix, MMP-1 facilitates keratinocyte migration by weakening the contact of collagen with the $\alpha 2\beta 1$ integrin [82]. Other matrix proteases that have been shown to influence cell migration are MMP-9 which is indispensable for EGF- and HGF-stimulated keratinocyte migration [83], and MMP-10 which co-localizes with MMP-1 [84]. Transgenic mice expressing a constitutive mutant of Mmp-10 had disorganized wound epithelium as a result of aberrant keratinocyte migration and altered laminin-5 expression [85]. Meanwhile, \textit{Mmp-3} knockout mice showed severely impaired wound contraction which ultimately led to delayed healing [86]. While the primary role of most MMPs is considered to be processing bioactive molecules such as cytokines and growth factors, they were originally discovered
for their functions in ECM degradation. Table 1.2 shows the members of the MMP family and their representative substrates [87].

There is also a tremendous amount of evidence that demonstrates importance of the inhibitors of metalloproteinases in wound repair. TNF-α is one of the most prominent cytokines involved in acute inflammation stimulates MMP-9 expression through activation of NFκB and p38 MAPK pathways [88]. Activation of TNF-α requires cleavage by TNF-α converting enzyme (TACE) which is primarily inhibited by TIMP-3 [89,90]. In the absence of TIMP-3, the level of TNF-α rises which causes an increase of inflammatory cell infiltration into the liver [91]. Timp-3 knockout mice develop enlarged airspaces, and this is accompanied by increased MMP activity in the lungs, as well as altered collagen remodeling and reduced fibronectin [92,93]. In addition, TIMP-3 has been shown to directly bind to vascular endothelial growth factor receptor (VEGFR)-2, thereby inhibiting its interaction with VEGF and the subsequent downstream signaling required for endothelial cell proliferation and angiogenesis [94].
<table>
<thead>
<tr>
<th>Common Name</th>
<th>MMP Number</th>
<th>Substrates</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Collagenases</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Interstitial</td>
<td>MMP-1</td>
<td>Col I(^2), II, III, VII, X, entactin, TN, aggrecan, proGel A, proGel B</td>
</tr>
<tr>
<td>Neutrophil</td>
<td>MMP-8</td>
<td>Col I, II, II(^2), III, aggrecan</td>
</tr>
<tr>
<td>Collagenase 3</td>
<td>MMP-13</td>
<td>Col I, II, III(^2), IV, IX, X, XIV, TN, FB(^18), FN(^15), gelatin(^3), proGel B(^2)</td>
</tr>
<tr>
<td>Collagenase 4(^8,22)</td>
<td>MMP-18(^8)–(^9,22)</td>
<td>TN, aggrecan, (gelatin)(^22)</td>
</tr>
<tr>
<td><strong>Gelatinases</strong></td>
<td></td>
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</tr>
<tr>
<td>Gelatinase A</td>
<td>MMP-2</td>
<td>Col I, IV, V, VII, X, gelatins, FB(^18), FN, LM, aggrecan, elastin, proGel B, proColl(^3), LM-5(^26)</td>
</tr>
<tr>
<td>Gelatinase B</td>
<td>MMP-9</td>
<td>Col I(^9), II(^9), IV, V, gelatins, elastin, FB(^18), entactin, aggrecan</td>
</tr>
<tr>
<td><strong>Stromelysins, matrilysin, metalloelastase</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stromelysin 1</td>
<td>MMP-3</td>
<td>Col II, IV, IX, X, XI, gelatin, LM, FB(^18), FN, elastin, TN, aggrecan, proCol, proGel B, neutrophil proCol</td>
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<tr>
<td>Stromelysin 2</td>
<td>MMP-10</td>
<td>Col IV, LM, FN, elastin, aggrecan</td>
</tr>
<tr>
<td>Stromelysin 3</td>
<td>MMP-11</td>
<td>Col IV, FN, LM, aggrecan</td>
</tr>
<tr>
<td>Matrilysin</td>
<td>MMP-7</td>
<td>Col IV, gelatin, LM, FN, elastin, elastin, aggrecan, TN, proGel A, proGel B, proCol</td>
</tr>
<tr>
<td>Matrilysin 2(^31)</td>
<td>MMP-26</td>
<td>Gelatins, casein(^31), FN, Flb, VN(^32), (\alpha)-proteinase inhibitor(^33)</td>
</tr>
<tr>
<td>Macrophage metalloelastase</td>
<td>MMP-12</td>
<td>Elastin, FB(^18), Col IV, gelatin, FN, LM, VN, PG(^28)</td>
</tr>
<tr>
<td><strong>Membrane-type metalloproteinases</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MT1-MMP</td>
<td>MMP-14</td>
<td>Progel A; gelatin, casein, elastin, FN, LM, VN, DSPG(^4); Col I, II, III(^1), TN, nidogen, aggrecan, perlecan, proTNF(\alpha)(^19), proColl(^-3)(^23)</td>
</tr>
<tr>
<td>MT2-MMP(^5)</td>
<td>MMP-15</td>
<td>ProGel A(^16); FN, TN, LM, aggrecan, perlecan, proTNF(\alpha)(^19)</td>
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<tr>
<td>MT3-MMP(^5)</td>
<td>MMP-16</td>
<td>ProGel A</td>
</tr>
<tr>
<td>MT4-MMP(^5)</td>
<td>MMP-17</td>
<td>Gelatin, proGel A(^29)</td>
</tr>
<tr>
<td>MT5-MMP(^25)</td>
<td>MMP-24</td>
<td>ProGel A</td>
</tr>
<tr>
<td>MT6-MMP(^26)</td>
<td>MMP-25</td>
<td>Gelatin</td>
</tr>
<tr>
<td><strong>Others</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Envelysin</td>
<td>MMP-20</td>
<td>Gelatin; amelogenin(^23)</td>
</tr>
<tr>
<td>Enamelysin(^10,11)</td>
<td>MMP-21/22(^20)</td>
<td></td>
</tr>
<tr>
<td>MMP-23(^24)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Epilysin(^34)</td>
<td>MMP-28</td>
<td></td>
</tr>
<tr>
<td><strong>Activities now identified as MMPs</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Telopeptidase(^12)</td>
<td>MMP-4 (likely MMP13)</td>
<td>Col I, FN</td>
</tr>
<tr>
<td>3/4-collagen endopeptidase(^13)</td>
<td>MMP-5 (rat MMP-2)</td>
<td>Col I, gelatin; 3/4-fragments col I, II, III</td>
</tr>
<tr>
<td>Acid MMP(^14)</td>
<td>MMP-6 (MMP-3)</td>
<td>Cartilage proteoglycan, insulin B chain</td>
</tr>
</tbody>
</table>
14-3-3 Proteins

14-3-3 is a highly conserved, ubiquitously expressed protein family, and consists of seven mammalian isoforms (β, γ, ε, σ, τ, η, ζ). The atypical name of 14-3-3 originates from the particular elution and migration patterns of these proteins on DEAE-cellulose chromatography and starch-gel electrophoresis. Since their initial discovery in 1967 [95], the 14-3-3 proteins remain an area of interest due to their role as molecular chaperones and adaptor molecules that interact with components of signal-transduction pathways [96-98]. Through association with over 100 binding proteins, the functionality of 14-3-3 proteins has expanded to include signal transduction, cell-cycle control, apoptosis, cellular trafficking, cell proliferation and differentiation, and protein folding and processing [99,100]. Most of these functions have been described for 14-3-3 proteins intracellularly; however, these proteins lack the signaling peptide needed for secretion through the classical ER/Golgi-dependent pathway. Recently, it has been demonstrated by our group that 14-3-3 proteins can be released via exosomes by keratinocytes [101]. Exosomes are a rather homogenous population with a size ranging from 30 to 120 nm [102]. They are stored as vesicles within the late endosome and are released when the multivesicular bodies of the late endosome fuse with the cell membrane. There are different ways that exosomes can mediate cell-to-cell communication, for example, by acting as signaling complex through surface-expressed ligands, transferring receptors between cells, delivering functional proteins to target cells, or transferring genetic information [103].
The molecular mechanism of 14-3-3 action involves formation of homo- and heterodimers with sufficient structural rigidity to induce conformational change in their binding partners [104]. The diverse and overwhelming binding capacity of 14-3-3’s can be attributed to their ability to recognize and bind two phospho-serine and phospho-threonine motifs RSXpSXP and RXXXpSXP in target proteins [105,106]. Not only these two recognition motifs can be found in different consensus sequences [107], 14-3-3 can also bind in a phosphorylation-independent manner [108].

Emerging evidence has shown that 14-3-3 sigma (also called stratifin) is unique among the isoforms due to its preference for homodimerization and specific selectivity for target binding proteins. In search for keratinocyte-derived factors with collagen or MMP-1 regulatory activity in dermal fibroblasts, our group has isolated stratifin (SFN) from the affinity purified fraction that displayed MMP-1 stimulatory activity. It was later confirmed that purified recombinant SFN was also capable of inducing MMP-1 in fibroblasts [47]. Differentiated keratinocytes are known to have different gene expression profiles from proliferating keratinocytes [109-111]. In fact, the amount of SFN secretion is significantly higher in differentiated keratinocytes than in proliferating keratinocytes. This has been demonstrated by the comparative analysis of protein profiles in exosomes released from differentiated and undifferentiated keratinocytes [112]. The keratinocyte production of SFN appears to receive paracrine input from fibroblasts, based on the observation that keratinocytes co-cultured with fibroblasts have higher SFN mRNA expression relative to keratinocytes cultured
alone [113]. Further studies revealed that rSFN also induces the expression of MMP-1, -3, -8, and -24, and downregulates fibronectin expression [114]. The induction of MMP expression is mediated via MAPK pathways (p38, JNK, ERK) which ultimately result in the activation of transcription factors such as AP-1, ETS, and C/EBP which are commonly found in the MMP promoter region [77]. Inhibitor assay in the presence of SFN stimulation revealed that a specific p38 MAPK inhibitor (SB203580) was able to significantly block the SFN-mediated induction of fibroblast MMP-1 mRNA, suggesting that extracellular SFN may function through the p38 signaling pathway [115]. Further investigation revealed that SFN markedly increases the expression of Elk4/Sap1 and cfos/c-jun transcription factors which are components of the AP-1 transcription complex. The gene modulatory effect of extracellular SFN requires a functional receptor on the cell surface to transmit the signal across the membrane, and this integral protein was later on identified to be an ectoenzyme called aminopeptidase N [116].

**Aminopeptidase N**

Aminopeptidase N (APN) or CD13 is a 150 kDa type II membrane glycoprotein with a zinc-dependent catalytic activity that preferentially cleaves neutral amino acids from the N-terminal end of oligopeptides. Structurally, APN consists of a large extracellular domain containing the active site, a transmembrane domain, and a short cytoplasmic tail (8-10 amino acid residues) with no known signaling motif [117]. The human APN gene (ANPEP) is widely
expressed in a variety of cells such as monocytes/macrophages, fibroblasts, epithelial cells, and endothelial cells [118-120]. APN has been referred to as a ‘moonlighting’ protein because of its wide range of functions including enzymatic regulation of peptides, viral receptor, tumor-homing peptide receptor, tumor cell invasion, proliferation and apoptosis, motility, antigen presentation, and signal transduction [121,122]. However, many of these functions are independent of its enzymatic activity. Ligation of APN molecules with monoclonal antibodies induces a rapid and transient calcium influx in monocytes via phosphorylation of ERK1/2 and p38 MAPK [123]. Activation of signal transduction has also been proposed as a mechanism of action for the role of APN as a mediator of homotypic aggregation in monocytes [124]. Specifically, Grb2 and Sos were found to co-immunoprecipitate with APN in monocytic cells, which linked APN to the Ras/ERK1/2 MAPK pathway.

Recent identification of APN as a functional cell surface receptor for extracellular SFN extends its already diverse functionality to wound healing. The expression of APN is minimal under normal physiological conditions, and appears to be induced upon tissue injury. Analysis of day 16 rabbit ear skin wound tissues revealed a significant increase of APN expression in the dermis, and this change coincides with the increase of SFN expression in the epidermis [116]. The APN-expressing cell population is also positive for vimentin, indicating that fibroblasts are likely to be responsible for the elevated APN expression in the dermis. In a rat model of skin wound healing, similar expression patterns of APN and SFN were observed, and their protein levels were highest at days 11 and 30.
Co-distribution of APN and SFN was noted in migrating fibroblasts, although less obvious in stationary cells. These studies provide strong evidence of a tight connection between SFN and its candidate receptor. Further characterization of the SFN/APN-mediated signaling will enhance our understanding of epithelial-dermal communication, and provide valuable information needed for deciphering the intricacy of tissue regeneration and wound repair.

**Hypotheses and Objectives**

Tissue repair in the skin demands active communication between keratinocytes and fibroblasts, which heavily relies on the release of soluble mediators. Apart from the well-known cytokines and growth factors, SFN has been recently isolated as a keratinocyte-releasable factor that stimulates MMP-1 expression in dermal fibroblasts via cell surface receptor APN [115,116]. *In vivo*, topical application of recombinant SFN ameliorates fibrosis in the rabbit ear hypertrophic scar model [125]. A recent study also showed that APN expression is upregulated at specific stages of dermal wound healing [116]. In this study, we will examine and test the general hypothesis that APN plays an active role in transmitting paracrine signals delivered to dermal fibroblasts and impacts the cell behavior during wound healing.

Specifically, we proposed that in an environment of active epidermal-mesenchymal communication, the fibroblast expression of APN is modified in
response to epidermal stimulation (hypothesis #1); and that similar to its ligand SFN, the cell surface receptor modulates ECM production (hypothesis #2). In light of the promising results from in vivo administration of SFN in a fibrosis model, we hypothesized that APN may have application as a therapeutic target in wound healing (hypothesis #3). Our experimental findings confirmed these hypotheses. Specifically, we provide evidence to demonstrate that APN can be induced by epidermal keratinocytes, and acts a regulator of keratinocyte-derived signals that modulate the production of key matrix molecules.

**Hypothesis 1**

To test the hypothesis that APN expression in dermal fibroblasts is responsive to keratinocyte modulation the following two objectives were pursued.

**Objective 1.1**

Under this objective, we investigated the mode of APN regulation by proliferating keratinocytes and keratinocyte-releasable soluble factors. We then examined whether differentiated keratinocytes regulate APN differently. Our findings demonstrated that keratinocytes modulate APN expression via release of soluble factors, and the state of differentiation enhances keratinocyte-mediated paracrine stimulation.

**Objective 1.2**

The aim of the second objective was to characterize the effect of SFN-binding on APN expression. Dermal fibroblasts were treated with recombinant
SFN, or keratinocyte-conditioned medium depleted of SFN by protein pull-down or gene knockdown. Our results demonstrated that SFN functions as a potent stimulator of APN expression.

**Hypothesis 2**

The second hypothesis of this study was that aminopeptidase N acts as a receptor to paracrine signals originating from keratinocytes, and that its presence is necessary for the transduction of these signals in dermal fibroblasts. The following objective describes our approach in addressing this hypothesis.

**Objective 2.1**

In this objective, we investigated whether APN serves as a receptor for signals other than those that upregulate MMP-1 expression. Specifically, the expression of APN in dermal fibroblasts was suppressed by siRNA-mediated gene silencing, and these cells were then stimulated with keratinocyte-conditioned medium. The combined approach of knocking down APN expression and providing epidermal stimuli to fibroblasts allowed us to identify downstream targets of signals mediated by APN. Microarray analysis of the ECM expression profile in these cells revealed that APN is indispensable for the transmission of signals into fibroblasts that influence a wide range of extracellular matrix components such as MMP-3, MMP-12, fibronectin, and tenascin-C in addition to MMP-1.
Hypothesis 3

In light of the newly identified function of APN in mediating regulatory signals for key ECM components, we hypothesized that it might be valuable as a therapeutic target in wound repair. The following objectives describe our effort in addressing this hypothesis.

Objective 3.1

In this objective, we investigated whether antagonizing the enzymatic activity of APN has any effect on wound healing. A monoclonal antibody (WM15) that blocks the APN enzymatic activity was topically applied to full-thickness skin wounds created on the back of BALB/c mice. Our results revealed that in comparison to control and IgG isotype antibody, the neutralizing antibody WM15 accelerated wound closure.

Objective 3.2

In this objective, we examined the underlying causes accountable for the favorable outcome in WM15-treated wounds. Immunohistochemical analysis showed that increases in the collagen deposition and fibroblast contractility led to accelerated wound closure.
Figure 1.2 Schematic summary of APN-mediated cellular activities in wound repair.

Keratinocytes modulate the aminopeptidase N (APN) expression in dermal fibroblasts via release of soluble factors such as stratifin. APN functions as a cell-surface receptor for keratinocyte-derived regulatory signals that modulate the expression of key matrix proteins involved in extracellular matrix remodeling and contraction. (Inset) Topical application of an APN-neutralizing antibody accelerated wound closure as a result of increased collagen deposition and fibroblast contractility. The dotted lines indicate edges of the wound.
Chapter 2. Paracrine regulation of fibroblast aminopeptidase N/CD13 expression by keratinocyte-releasable stratifin²

Introduction

Cutaneous tissue repair is a dynamic and well orchestrated process that involves various cell types, growth factors and matrix molecules at the injured site. The repair process is generally divided into four overlapping phases: homeostasis, inflammation, proliferation (granulation tissue formation and re-epithelialization), and remodeling. As the inflammatory phase ends, epidermal keratinocytes and dermal fibroblasts emerge as dominant cell types that coordinate granulation tissue formation, re-epithelialization, and tissue remodeling, highlighting keratinocyte-fibroblast crosstalk as an important aspect of wound healing. Given the semi-permeable nature of the basement membrane which separates the epidermis from the dermis, the mode of communication between keratinocytes and fibroblasts is likely to engage diffusible, soluble factors secreted by these cells. Apart from the well-established signaling molecules in the skin such as TGF-β, KGF/fibroblast growth factor-7 (FGF7),

² A version of this chapter has been published. Lai A., Ghaffari A., Li Y., Ghahary A. Paracrine regulation of fibroblast aminopeptidase N/CD13 expression by keratinocyte-releasable stratifin. J Cell Physiol. 2011 Feb 1.
FGF2, and EGF [126-129], our group has successfully isolated an MMP-1-stimulating factor, stratifin (SFN) which is preferentially secreted by differentiated keratinocytes and acts as a signal mediator of keratinocyte-fibroblast crosstalk [130,131]. Microarray analysis of SFN-treated fibroblasts revealed that this protein functions as a modulator of important ECM molecules such as MMPs, integrins and fibronectin [132]. Recent evidence suggests that the SFN-mediated ECM changes may involve a ligand-receptor interaction. Specifically, APN has been proposed to act as a cell surface receptor for SFN, with its transmembrane signal transduction leading to the expression of MMP-1 in fibroblasts via activation of the p38 MAPK pathway [133].

Studies that employ different cell and tissue systems have shown that APN is involved in cell adhesion, migration, differentiation, proliferation, and angiogenesis (reviewed in [122]). Many of these functions may impact the wound healing process when applied in the context of the skin. The diverse physiological actions of APN signify the importance of controlled APN gene expression. Indeed, pathologically dysregulated expression of APN is associated with tumors including melanoma [134], squamous cell carcinoma [135], breast [136], colon [137], prostate [138] and thyroid [139] cancers, as well as inflammatory diseases such as rheumatoid arthritis [140], multiple sclerosis [141], and CNS inflammation [142]. However, in light of its first discovery as a myeloid marker, most of the studies on APN regulation have focused on the molecules of the immune system, in particular, TGF-β, lipopolysaccharide (LPS), and cytokines such as IL-1β, IL-4, IL-6, IL-10, and IFN-γ [143-148]. In addition to
regulation by growth factors and cytokines, APN expression can also be influenced by neighboring cells. For instance, Riemann et al. [149] demonstrated that APN expression in T and B lymphocytes is induced upon adhesion to fibroblast-like synoviocytes, which explains why synovial fluid T cells from arthritis patients are APN positive even though lymphocytes of peripheral blood do not express APN. Also, Saho et al. [150] suggested that gingival fibroblasts might be responsible for the presence of APN-positive T lymphocytes in inflamed gingival tissues. Both of these studies have shown that APN in immune cells can be induced by incubation with cells of different origin, suggesting that APN may be directly involved in cell-cell communications and facilitating immune response at the sites of lesion.

The recent identification of APN as a receptor for SFN implicates its importance in wound repair, and more specifically its potential function in epidermal-mesenchymal communication and ECM modulation. The first step in assessing the role of APN in wound repair is to understand how APN expression is regulated in the cutaneous environment, specifically, whether the fibroblast production of APN is affected by neighboring cells such as keratinocytes. To address this issue, we cultured dermal fibroblasts with keratinocytes or in the presence of keratinocyte-conditioned medium (KCM), and detected an upregulation of APN expression as a result of keratinocyte stimulation. Treatment of fibroblasts with 14-3-3-depleted KCM led to reduced APN induction, suggesting that the 14-3-3 proteins might be responsible for the KCM-induced APN expression. This was confirmed by testing the ability of SFN, a proposed
ligand for APN and one of the most abundant 14-3-3 isoforms released by keratinocytes, to induce APN expression. By incubating fibroblasts with recombinant SFN (rSFN) or conditioned medium of SFN-overexpressing mammalian cells, we showed that SFN up-regulates APN expression in a dose-dependent manner and promotes cell migration. The stimulatory effect of SFN on fibroblast motility can also be successfully abolished by addition of bestatin (a potent inhibitor of APN enzymatic activity).

Materials and Methods

Cell culture

Skin punch biopsies were obtained with informed consent from patients undergoing elective circumcision. The study was approved by the University of British Columbia Hospital Human Ethics Committee and conducted according to the Declaration of Helsinki Principles. The detailed protocol of harvesting fibroblasts and keratinocytes from fetal foreskin is described previously [114]. Fibroblasts were grown in DMEM with 10% FBS and keratinocytes in KSFM (Invitrogen Life Technologies, Carlsbad, CA, USA) supplemented with bovine pituitary extract (50 μg/ml) and EGF (0.2 μg/ml). Fibroblasts of passages 3-6 and keratinocytes of passages 3-5 were used in this study.
Keratinocyte-conditioned media and induction of keratinocyte differentiation

Primary human keratinocytes were cultured in KSFM without growth supplements, and their conditioned media were collected and centrifuged prior to addition to fibroblasts. To induce differentiation in vitro, primary human keratinocytes were incubated in a medium mixture of 50% KSFM and 50% DMEM in the absence of growth supplements or FBS [131]. The level of differentiation was confirmed by involucrin expression using immunoblot analysis. Conditioned media were collected after varying periods of incubation in the 50:50 medium, and used to treat primary human fibroblasts.

14-3-3 protein pull-down assay

A biotin-conjugated R18 (Enzo Life Sciences, Plymouth Meeting, PA, USA) was used to deplete all seven isoforms of the 14-3-3 family (namely β, γ, ε, σ, τ, η, and ζ) from KCM. Concentrated KCM was incubated for 1 hr with biotin-R18, followed by 1 hr incubation with Avidin agarose beads (Sigma-Aldrich, Oakville, ON, Canada) under constant rotation at 4°C. The Avidin-biotin-R18-14-3-3 complex was then pulled down by centrifugation at 550 x g and the supernatant (14-3-3-depleted KCM) was added to fibroblasts. Following 24 hr incubation, cell lysates were collected from the control and treated fibroblasts, and immunoblotted for APN expression by immunoblot analysis.
Immunoblot analysis

Immunoblotting was performed with a mouse monoclonal anti-APN/APN (1:1000 dilution; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), mouse monoclonal anti-involucrin (1:2,000 dilution; Sigma-Aldrich, Oakville, ON, Canada), or mouse monoclonal anti-human β-actin antibody (1:20,000 dilution; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), followed by a horseradish peroxidase-conjugated IgG antibody (1:3,000 dilution; Bio-Rad, Mississauga, ON, Canada).

Generation of recombinant SFN protein, construction of mammalian expression vector, and transient transfection

Recombinant SFN was prepared according to the previously described method [130]. The mammalian expression vector encoding full-length human SFN was fused N-terminally with DsRed using an existing SFN plasmid [130] or the pLP-Adeno-X-XMV-E3-DsRed-rexpress plasmid (Clontech Laboratories, Palo Alto, CA, USA) as template by 30 cycles of PCR conditions: 95°C 1 min, 55°C 1 min, and 72°C 1 min. The PCR products were purified by the QIAEX II Gel Extraction Kit (QIAGEN, Valencia, CA, USA) and digested with either HindIII/BamH1 (for SFN) or BamH1/EcoR1 (for DsRed). The digested PCR products of SFN and DsRed were then cloned into the pDNR-CMV expression vector (Clontech Laboratories, Palo Alto, CA, USA). Positive clones containing the DsRed-SFN gene were identified by restriction enzyme digestion and further confirmed by DNA sequencing. The positive plasmid was transformed into DH5α.
competent cells and purified using the PureLink™ HiPure Plasmid Maxiprep Kit (Invitrogen Life Technologies, Carlsbad, CA). The respective PCR primers used in the cloning are 5’ DS EcoR1: gcgaattcatggcctcctcgagaacgtcat; 3’ DS BamH1: ggttcaggaacaggtggtggcggccct; 5’ SFN BamH1: ggttcaggaacaggtggtggcggccct; and 3’SFN HindIII: caagctttggcgggcaacactcagctc.

Transfection of the pDNR-CMV DsRed-SFN plasmid into 293T cells was performed using FuGene HD Transfection Reagent (Roche Diagnostics Canada, Laval, QC, Canada). Following transfection, 293T cells were incubated in DMEM supplemented with 10% FBS for 72 hours, and conditioned media were collected and concentrated using Amicon Ultra centrifugal filters (Millipore, Billerica, MA, USA). The SFN expression in transfected 293T cells was confirmed by immunoblot analysis and fluorescence microscopy using the Motic MHG 100B microscope and associated imaging software.

RNA extraction and PCR

Total RNA was isolated from treated cells and their controls using the RNeasy Mini Kit (QIAGEN, Valencia, CA, USA), and reverse transcribed into cDNA using the SuperScript cDNA Synthesis Kit (Invitrogen Life Technologies, Carlsbad, CA, USA). A 324 bp fragment of human APN was amplified using the primers described in [151] by standard PCR analysis.

siRNA knockdown of SFN

SFN knockdown was tested using four different siRNA oligonucleotide sequences: Hs_SFN_1 FlexiTube siRNA, SI00084854; Hs_SFN_4 FlexiTube
siRNA, SI00084875; Hs_SFN_5 FlexiTube siRNA, SI02653637; Hs_SFN_6 FlexiTube siRNA, SI02653679, purchased from QIAGEN (Valencia, CA, USA). A non-silencing siRNA was used as the negative control. Primary human keratinocytes were transfected with 25 nM siRNA oligonucleotide using HiPerfect Transfection Reagent according to the manufacturer's recommendations (QIAGEN, Valencia, CA, USA). Keratinocytes were harvested 96 hours post-transfection and analyzed for intracellular SFN expression. Prior to cell harvest, KCM was collected 48-96 hours post-transfection and used to treat different strains of primary human fibroblasts.

**In vitro scratch assay**

An in vitro scratch assay was performed as described in [152]. Briefly, primary human dermal fibroblasts of passages 3-5 were seeded on 12-well plates in 10% FBS/DMEM. At 95% confluency, the cells were washed twice with PBS and a scratch wound was made across each well with a 200-μl pipette tip. The cells were treated with rSFN protein (4 μg/ml) or 100 μM bestatin hydrochloride (Sigma-Aldrich, Oakville, ON, Canada) in serum-free DMEM and incubated at 37 °C. Photographs were taken immediately and 24 hours after treatment using a Zeiss HB-100 fluorescence microscope, and analyzed using the Northern Eclipse image analysis software (Carl Zeiss Canada, Toronto, ON, Canada).
Statistical analysis

Data were expressed as mean ± SD, and the mean values of replicated wells between different treatments in three independently conducted experiments were compared using ANOVA. *P* values of < 0.05 were considered statistically significant in this study.

Results

APN expression in dermal fibroblasts co-cultured with keratinocytes

To test whether keratinocytes regulate fibroblast APN expression, we evaluated the level of APN in fibroblasts co-cultured with keratinocytes using an *in vitro* two-chamber system. In this system, keratinocytes and fibroblasts were separated by a semi-permeable membrane that allowed transfer of soluble macromolecules between the upper and lower chambers without physical contact of the cells. Cells cultured in the lower chamber were harvested and analyzed for their protein expression. As shown in Figure 2.1A, when fibroblasts were seeded in the upper chamber and keratinocytes in the lower chamber (lane F/K), the protein expression of APN was significantly elevated in fibroblasts as a result of keratinocyte co-culturing in comparison with fibroblast/fibroblast co-culture (lane F/F). Similarly, a slight increase of APN expression was observed in keratinocytes co-cultured with fibroblasts in the upper chamber (lane K/F) as compared to keratinocyte/keratinocyte co-culture (lane K/K). The APN
expression pattern correlated with that of MMP1 which was shown in our previous studies to increase upon co-culturing with keratinocytes. The keratinocyte-induced APN production was further confirmed by a dose-dependent study, in which fibroblasts were co-cultured with an increasing number of keratinocytes. The results showed that the level of APN increased in proportion to the number of keratinocytes in the co-culture (Figure 2.1B), confirming a direct regulation of APN expression by keratinocytes. The question was then to determine whether the induction of APN expression requires a continuous interaction with keratinocytes or if it can be sustained by merely the presence of keratinocyte-derived soluble factors. To address this issue, we treated fibroblasts with various volumes of keratinocyte-conditioned medium and evaluated the protein expression of APN in fibroblasts. As shown in Figure 2.1C, the level of APN expression was increased in parallel to the relative concentration of KCM, suggesting that keratinocyte-released soluble factors alone are sufficient to promote APN expression.
Figure 2.1 Co-culturing human dermal fibroblasts with keratinocytes induces APN expression.

Panel A: Primary human dermal fibroblasts were co-cultured with an equal number of keratinocytes (F/K) or fibroblasts of strain no. F41 (F/F), and vice versa (K/K and K/F).

Panel B: 1.0 x 10^6 fibroblasts were co-cultured with 1.0 x 10^6 fibroblasts of strain no. F3, or varying numbers of keratinocytes (0.1, 0.5 or 1.0 x 10^6 per well). Panel C: Primary human fibroblasts were incubated with different volumes of KCM. The percentage indicates the relative concentration of KCM in the total volume of medium used for the treatment. The protein expressions of APN and MMP-1 in fibroblasts were examined by immunoblot analysis, and β-actin was used as a loading control.
Keratinocyte differentiation further enhances APN stimulation

In view of the generally acknowledged impact of keratinocyte differentiation on fibroblast phenotype and gene expression profile, we investigated whether APN expression is regulated by differentiated keratinocytes. Human epidermal keratinocytes were cultured in a test medium containing 50% KSFM and 50% DMEM which reportedly induces keratinocyte differentiation \textit{in vitro} as a result of increasing calcium concentration in the medium [153]. A marked increase in the expression of involucrin, a differentiation marker, was observed in keratinocytes harvested after 2, 4, 6 and 8 days of incubation in the test medium relative to that of control (Figure 2.2A). The ability of differentiated keratinocytes to stimulate APN was then determined by treating dermal fibroblasts with conditioned medium collected at these time points and evaluating APN expression by immunoblot analysis. As shown in Figure 2.2B, the KCM-induced APN expression in fibroblasts increased as the cells progressed to a more differentiated state (as demonstrated by the gradual increase of involucrin expression). This indicates a direct impact of keratinocyte differentiation on the paracrine activation of fibroblast APN expression. The upward trend of APN expression as keratinocyte differentiation progresses is consistent with the MMP-1 expression pattern observed in an earlier report [131]. Signals from the APN and \(\beta\)-actin immunoblots were quantified by densitometry, and the ratios are presented in Figure 2.2C.
Figure 2.2 Keratinocyte differentiation has additional stimulatory effect on APN expression.

**Panel A:** Keratinocyte differentiation was induced by culturing keratinocytes for 0, 2, 4, 6 and 8 days in a medium mixture of 50% KSFM and 50% DMEM. The involucrin expression in keratinocytes harvested at these time points was analyzed. β-actin was used as a loading control.

**Panel B:** Human dermal fibroblasts were incubated with conditioned medium from keratinocytes induced to differentiation for various periods of time. The levels of APN and β-actin were evaluated by immunoblot analysis after 24-hr incubation and determined by densitometric quantification. **Panel C:** The relative fold changes of APN expression normalized by the β-actin loading control are shown. Data represent means ± standard deviations for three independently conducted experiments. *P* < 0.05, significant difference between day 0 sample and day 4, 6, and 8 samples.
14-3-3 proteins contribute to the paracrine regulation of APN

A number of growth factors and cytokines have been shown to stimulate APN production. In this study, we specifically examined the influence of 14-3-3 proteins on APN based on our previous findings that the 14-3-3 proteins are produced and released by keratinocytes [154] and that APN acts as a cell surface receptor for 14-3-3. First, KCM was depleted of 14-3-3 by pull-down with biotinylated R18 and avidin agarose. R18 is a short peptide with high binding affinity to all 14-3-3 isoforms [155]. To test the success of the 14-3-3 pull-down assay, SFN levels were analyzed by immunoblotting. Figure 2.3A shows that approximately 60% of the SFN released by keratinocytes was removed by R18 pull-down assay. As shown in Figure 2.3B, compared with the stimulation of fibroblast APN expression by full KCM, the APN-stimulating effect of 14-3-3-depleted KCM was markedly reduced to approximately 50%. To exclude any undesirable effect that may result from the non-specific binding of avidin-agarose in the 14-3-3 pull-down assay, fibroblasts were treated with KCM pre-incubated with avidin-agarose alone. Despite the slight increase of APN expression in the avidin-treated sample, the R18/avidin-treated sample showed reduced APN stimulation and this was solely due to removal of the 14-3-3 proteins. Further, in view of our earlier observation on the parallel expression of APN and MMP-1 in keratinocyte-fibroblast co-culture, MMP-1 expression in the treated cells was also examined. The result showed that 14-3-3 depletion reduced the KCM-induced MMP-1 expression by 25%, confirming the correlation between levels of APN and MMP-1.
It is well established that SFN, which is an epithelial marker, induces MMP-1 expression in dermal fibroblasts and is found in great abundance in the conditioned medium of keratinocytes, in particular, those that have differentiated. Depletion of the 14-3-3 proteins has markedly compromised the capacity of KCM to induce APN expression; however, it remains to be confirmed whether SFN per se can induce APN expression. In that regard, we cloned and produced rSFN as previously described [47], and incubated dermal fibroblasts with various doses of rSFN protein. As shown in Figure 2.3C, the ability of rSFN to stimulate APN expression was prominently observed at 2 and 4 μg/ml.
Figure 2.3 14-3-3 depletion reduces the capacity of KCM to induce APN expression.

Panel A: KCM was depleted of 14-3-3 proteins in a pull-down assay using biotinylated R18 peptide and avidin-agarose beads. The content of SFN in full KCM and the 14-3-3 depleted KCM was evaluated by immunoblot analysis. Panel B: Primary human dermal fibroblasts were treated with KCM, avidin agarose-cleared KCM, or 14-3-3-depleted KCM. Intracellular expressions of APN and MMP-1 in the fibroblasts were evaluated after 24-hr incubation by immunoblot analysis. Panel C: Dermal fibroblasts were treated with various doses of rSFN (1, 2 and 4 μg/ml) for 24 hours, and the expression level of APN was examined by immunoblot analysis. β-actin was used as a loading control. Data are representative of three independently conducted experiments.
**Releasable form of SFN increases APN at the gene and protein levels**

To exclude the possibility that the observed APN stimulation might be due to LPS contamination or bacterial by-product in the production of rSFN protein, we constructed a mammalian expression plasmid carrying a fusion gene of full-length SFN and the DsRed fluorescent protein (Figure 2.4A). The plasmid was transfected into HEK293T cells, and SFN expression was confirmed by fluorescence microscopy (Figure 2.4B) and immunoblot analysis (Figure 2.4C). Extracellular release of soluble SFN was confirmed by subjecting conditioned medium of SFN-overexpressing cells to immunoblot analysis (Figure 2.4C). For positive control of immunoblotting, cell lysate and conditioned medium from differentiated keratinocytes were used (lanes DK). After it was confirmed that the cell lysate and conditioned medium collected from DsRed-SFN transfected cells (lanes SFN) contained SFN protein, the conditioned medium was used to treat human dermal fibroblasts. Cell lysates of fibroblasts incubated with conditioned medium from non-transfected (lanes NT) or DsRed-SFN transfected HEK293T cells were collected after 24 hours, and APN expression was examined by both quantitative PCR and immunoblot analyses. As shown in Figures 2.4D and 2.4E, APN expression was enhanced both at the gene and protein levels.
Figure 2.4 SFN released by DsRed-SFN transfected 293T cells stimulates APN expression in fibroblasts.

Panel A: Vector construction scheme. Panel B: The upper panel shows fluorescent images of non-transfected (NT) and transfected (SFN) 293T cells, and the lower panel presents bright field images of the same fields. Panel C: The production and release of DsRed-SFN was confirmed by analyzing the presence of DsRed-SFN (≈ 60 kDa) in the cell lysate and conditioned medium (CM) of transfected 293T cells. Differentiated keratinocytes (DK) were used as positive control (endogenous SFN ≈ 26 kDa). Panels D-E: The gene and protein expressions of APN in fibroblasts incubated with conditioned medium of non-transfected or transfected 293T cells were analyzed and shown in panels D and E, respectively. Data are representative of three independent experiments.
siRNA knockdown of SFN compromises the ability of keratinocytes to stimulate APN

To confirm that keratinocyte-released SFN protein directly contributes to the induction of APN production in fibroblasts, SFN expression was suppressed by transfection of primary human keratinocytes with SFN-specific siRNA oligonucleotides. As shown in Figure 2.5A, the level of intracellular SFN expression normalized to β-actin was reduced to 53%, 69%, 60%, and 64% of the control due to transfection with SFN siRNA #1, 4, 5, or 6, respectively. Analysis of conditioned medium collected from control and transfected keratinocytes also showed reduced release of SFN protein as a result of the siRNA transfection. Consistent with Figure 2.3, fibroblasts treated with KCM containing a reduced amount of SFN showed decreased APN induction (Figure 2.5B).
Figure 2.5 siRNA knockdown of SFN compromises the ability of keratinocytes to stimulate APN expression in fibroblasts.

**Panel A:** Levels of both the intracellular and releasable forms of SFN following siRNA knockdown were quantified by immunoblot analysis. Cell lysates and conditioned medium of untreated keratinocytes (C) and keratinocytes transfected with 25 nM of non-silencing siRNA (siC) or different SFN siRNA oligonucleotides (#1, 4, 5 and 6) were collected and analyzed for SFN protein. **Panel B:** APN expression in fibroblasts treated with conditioned medium collected from untreated keratinocytes (C), or keratinocytes transfected with the non-silencing siRNA (siC) or SFN siRNA #1 (siSFN) was analyzed by immunoblot analysis.
Bestatin treatment abolishes SFN-induced cell migration

Accumulating evidence have shown that APN is involved in tumor cell invasion [156,157]. While SFN is known to promote MMP1 expression and accelerate ECM remodeling, the effect of SFN on cell migration is yet to be investigated. Based on the evidence that SFN stimulates APN production, we hypothesized that SFN may also play a role in cell migration. Treatment of primary human fibroblasts with rSFN protein (4 μg/ml) showed an approximately 40% increase in cell migration in a 24-hour scratch assay (Figure 2.6A). To examine whether this stimulatory effect is mediated by APN, fibroblasts were treated with bestatin (a chemical inhibitor of APN enzymatic activity) for 30 minutes prior to addition of the rSFN protein. The result showed that bestatin successfully offset the stimulatory effect of SFN on cell migration, reducing it to a level not significantly different from that of untreated cells (Figure 2.6B).
Figure 2.6 SFN-induced cell migration is dependent on the enzymatic activity of APN.

Panel A: Images of human dermal fibroblasts taken immediately and 24 hours after addition of recombinant SFN or bestatin in an *in vitro* wound scratch assay. Scale bar: 10 μm. Panel B: Abolition of SFN-induced migration by bestatin treatment. Fibroblast migration was evaluated by counting the number of cells migrated from the edges of the wound into the denuded area after the 24-hour treatment, and expressed as the percentage of cells present in the open wound area relative to that of the control well at the end of the 24-hour incubation (n = 5, \( p < 0.01 \)). Data represent means ± standard deviations for three independently conducted experiments.
Discussion

Epidermal-mesenchymal interactions have been proven to affect the outcome of wound healing. Disruption of this crosstalk leads to skin disorders such as psoriasis, hypertrophic scarring, keloid and chronic wounds [158-161]. Several groups have investigated the gene expression profiles of fibroblasts co-cultured with keratinocytes, providing compelling evidence that the epidermal cells control ECM composition, tissue remodeling and cell adhesion through modulation of the fibroblast phenotype and gene expression [162-164]. The current study reveals that the fibroblast expression of APN, which is a membrane-bound aminopeptidase and a candidate receptor for keratinocyte-releasable SFN, can be induced in keratinocyte-fibroblast co-culture or by KCM treatment. This suggests involvement of APN in keratinocyte-fibroblast crosstalk and its potential role in wound repair. Indeed, some of the known secretory molecules implicated in wound healing, particularly those released by keratinocytes including TGF-β, IL-1β, IL-4, IL-6, IL-10, IFN-γ have been shown to influence APN production [146,165-167]. Through modulating the expression of APN which has a wide range of functions, these signaling molecules may elicit additional phenotypic or gene expression changes apart from the downstream signaling initiated by binding to their intrinsic receptors. Thus, the current finding may also help to rationalize some of the fibroblast phenotypic changes observed in keratinocyte-fibroblast co-culture such as enhanced cell migration. In support of the recently proposed role of APN in signal transduction, the consistent
correlation between levels of APN and MMP-1 observed in the present study implicates a role of this membrane-bound enzyme in keratinocyte-mediated paracrine regulation of ECM molecules. In fact, our preliminary analysis showed that APN gene knockdown in dermal fibroblasts abolished many of KCM-induced gene expression changes in these cells. At the same time, the reciprocal induction of keratinocyte APN expression by fibroblasts illustrates the importance of epidermal-mesenchymal interaction in skin diseases hallmarked by abnormal APN expression in keratinocytes [168], and provides supporting evidence for the proposed role of APN in nkeratinocyte growth and differentiation which are profoundly influenced by the presence of fibroblasts [169,170].

Under normal physiological conditions, basal keratinocytes undergo a dynamic and well regulated program of differentiation accompanied by numerous biochemical and morphological changes. According to the degree of differentiation, keratinocytes have different gene expression and secretome profiles [101,112,131,171-174], producing differential effects on fibroblast gene expression [131,171,172]. As wound repair enters the remodeling phase, the ratio of differentiated to undifferentiated keratinocytes increases, indicating enhanced input and participation of differentiated keratinocytes in modulating matrix deposition and tissue remodeling. The amplified stimulation of APN expression as a result of keratinocyte differentiation implies that APN may serve as a coordinator of signaling events specifically promoted by differentiated keratinocytes, and facilitate the transition from granulation tissue formation to extracellular matrix remodeling. This hypothesis can be further supported by the
observed upregulation of APN by SFN, which is preferentially expressed and released by differentiated keratinocytes [109,113], and induces changes in the gene expression of ECM proteins and proteases synthesized by fibroblasts [114]. In particular, SFN is a potent stimulator of MMP-1 which initiates degradation of accumulated collagen [47], and has recently been proven to be therapeutically effective for ameliorating scarring in a rabbit ear fibrotic model [175].

In addition to the cell signaling mediated by direct binding of SFN (ligand) to APN (receptor), an alternate mechanism of SFN-mediated ECM modulation may involve direct regulation of APN expression in fibroblasts. The stimulatory effect of SFN on APN expression was observed both at the gene and protein levels. The SFN-induced APN expression appears to be mediated through a different signal transduction pathway from the p38 MAPK pathway which was shown to be responsible for the SFN-induced MMP-1 expression [115], since treatment of dermal fibroblasts with MAPK inhibitors did not block the SFN induction of APN (unpublished data). Further, while preincubation of dermal fibroblasts with bestatin abolished SFN-induced cell migration, SFN-mediated MMP-1 production requires the presence of APN but does not depend on its enzymatic activity [176]. The increase of APN expression and therefore its enzymatic activity induced by SFN might have contributed to the enhanced migratory activity of SFN-treated cells. The exact mechanism of how APN regulates cell migration is still unclear; however, its enzymatic activity is important for the chemotactic migration of leukocytes [177], and invasive potential of lung cancer cells and osteocarcinoma cells [166,178]. Meanwhile, emerging evidence have demonstrated that not all
APN-associated functions require its enzymatic activity, and might be mediated via signal transduction [123,124,179,180]. Our group has recently shown that APN mediates the SFN-induced MMP-1 expression possibly through the p38 MAPK pathway, as transient knockdown of APN expression blocked both the SFN-mediated p38 activation and MMP-1 induction [116]. The implication of signaling was reinforced by the observation that inhibition of the APN enzymatic activity by bestatin did not affect the stimulatory effect of SFN on MMP-1.

Although SFN appears to regulate the expressions of MMP-1 and APN via separate mechanisms, it is clear from the current finding that APN participates in epidermal-mesenchymal communication and may potentially serve as a mediator of the signal transduction initiated by keratinocyte-releasable SFN. In combination with our previous findings, the current study provides evidence that SFN modulates wound repair through a ligand-receptor interaction with APN as well as by regulating the level of APN production in fibroblasts. As a future perspective, modulating APN expression may provide a therapeutic tool for treatment of ECM-related skin diseases.
Chapter 3. Microarray-based identification of aminopeptidase N target genes in keratinocyte conditioned medium-stimulated dermal fibroblasts

Introduction

The extracellular matrix serves as a scaffold for tissue regeneration and repair by providing structural support to cells, regulating intercellular communication, and maintaining a constant flux of growth factors. In the skin, the composition of the matrix is dynamically modulated by dermal fibroblasts, which continuously synthesize and degrade extracellular molecules and their receptors in response to signaling molecules released by keratinocytes and neighboring cells. Apart from the well-characterized signaling molecules at the epidermal-dermal junction, our group has successfully isolated stratifin (also known as 14-3-3 sigma) which functions as a keratinocyte-releasable stimulating factor of matrix metalloproteinases [130,130,132]. Stratifin belongs to the 14-3-3 family of phospho-serine/threonine-binding proteins which normally function as intracellular chaperones in signal transduction, cell cycle regulation, molecular

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transport, and apoptosis [181-183]. Upon release by keratinocytes, stratifin (SFN) stimulates the expression of MMP-1, -3, -8, and -24 in fibroblasts via the p38 MAPK signaling pathway [115,130,132]. In vivo SFN treatment in a fibrotic rabbit ear model showed improvement of hypertrophic scar in reducing scar thickness and cellularity [184]. Further studies indicated that the SFN-mediated MMP-1 modulation depends on the presence of aminopeptidase N/CD13 in fibroblasts [116].

Aminopeptidase N (APN) is a type II transmembrane metalloprotease implicated in a myriad of biological processes including cell adhesion, motility, differentiation, proliferation, chemokine processing, tumor invasion, and angiogenesis (reviewed in [122]). Most of these functions depend on the activity of this membrane-bound enzyme to cleave neutral amino acids from the N terminus of peptides [185]. Recent studies have shown that APN modulates certain cellular processes independently of its catalytic activity and may play a role in signal transduction as a co-regulator of signaling pathways [116,123,124]. Thus, while some APN-associated functions can be controlled by modulating its enzymatic activity, others require manipulation at the level of gene expression. An example of such is the paracrine regulation of the cell-surface APN expression on fibroblasts by keratinocyte-derived signals such as SFN [186]. In light of the recent identification of APN as a candidate receptor responsible for SFN-mediated MMP-1 upregulation [116], its induction by SFN strongly suggests a regulatory role of APN in keratinocyte/fibroblast-mediated ECM remodeling. It was therefore of interest to examine whether APN has any influence on the
keratinocyte-mediated regulation of ECM production in fibroblasts. To address this question, we knocked down APN expression by siRNA-mediated gene silencing, and then evaluated by DNA microarray analysis the expression of key ECM components in APN-knocked down fibroblasts upon keratinocyte stimulation. Specifically, this study aimed to identify targets of APN-mediated signal transduction in epidermal-dermal interactions.

Materials and Methods

Cell culture and reagents

Skin punch biopsies were obtained with informed consents from patients undergoing elective circumcision. The study was approved by the University of British Columbia Hospital Human Ethics Committee and conducted according to the Declaration of Helsinki Principles. The detailed protocol of harvesting fibroblasts and keratinocytes from fetal foreskin has been described previously [131]. Three strains of cells harvested from skins of different patients were used in this study. Fibroblasts were grown in DMEM with 10% FBS and keratinocytes in KSFM (Invitrogen Life Technologies, Carlsbad, CA, USA) supplemented with bovine pituitary extract (50 μg/ml) and EGF (0.2 μg/ml). Fibroblasts of passages 3-6 and keratinocytes of passages 3-5 were used in this study. KCM was collected from keratinocytes cultured in KSFM without supplements. Bestatin hydrochloride (Sigma Chemicals, Oakville, ON, Canada) was used as a competitive inhibitor of APN enzymatic activity.
**siRNA knockdown of aminopeptidase N**

For APN knockdown, the siRNA oligonucleotide sequence, Hs_ANPEP_5 FlexiTube siRNA (SI02780211), purchased from QIAGEN (Valencia, CA, USA) was used. A non-silencing siRNA with the same GC content as the APN siRNA was used as a negative control. HiPerfect transfection reagent was used according to the manufacturer’s recommendations (QIAGEN, Valencia, CA, USA). Fibroblasts were seeded at 1x10⁵ cells/well and transfected with 25 nM of the siRNA oligonucleotide. The medium was replaced 24 hours later and the cells were treated with KCM at 72 hours post-transfection.

**Gene expression analysis by ECM-specific microarray**

To examine the impact of APN modulation on keratinocyte-regulated ECM gene expression in dermal fibroblasts, Oligo GEArray pathway-specific gene expression arrays were purchased from SuperArray Bioscience Corporation (Fredrick, MD, USA). Each gene array consists of 113 genes of human ECM and adhesion molecules involved in cell adhesion, ECM deposition, and degradation, as well as sequences for loading control such as β-actin and glyceraldehyde 3 phosphate dehydrogenase (GAPDH). The arrays were used according to the manufacturer’s instructions. In brief, total RNA was isolated from cells using the RNeasy Mini Kit (QIAGEN, Valencia, CA, USA). cDNA was then prepared from the total RNA using MMLV reverse transcriptase, biotinylated with Biotin-16-dUTP (Roche, Indianapolis, IN, USA), and hybridized to a positively charged nylon membrane containing the arrayed DNA. The arrays were visualized using
the chemiluminescent detection system provided (SuperArray Bioscience Corporation, USA). Loading was adjusted based on the intensity of hybridization signals to the housekeeping gene, GAPDH, and then gene expression was quantified by densitometric analysis using the ImageJ software available from NIH.

Collagen gel contraction assay

Analysis of fibroblast-populated collagen gel contraction was performed as described [187] in collagen gels containing 200,000 cells per gel treated with bestatin. Changes in surface area were measured every 24 hours for three days. Ultrapure bovine collagen solution (3 mg/ml) was used (Sigma Chemicals, Oakville, ON, Canada).

Immunoblot analysis

Immunoblotting technique was carried out as previously described [188]. The following antibodies were used: mouse monoclonal anti-APN/clone 3D8 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), rabbit polyclonal anti-FN/clone H300 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), mouse monoclonal anti-procollagen type I/clone SP1.D8 (Developmental Studies Hybridoma Bank, maintained by the University of Iowa, Department of Biological Science, Iowa City, IA, USA), rabbit monoclonal anti-MMP-1 (Epitomics, Burlingame, CA, USA), rabbit monoclonal anti-MMP-3 (Epitomics, Burlingame, CA, USA), rabbit polyclonal anti-MMP-12 (Millipore, Billerica, MA, USA), rabbit polyclonal anti-TN-C (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA),
rabbit monoclonal anti-α-SMA (Epitomics, Burlingame, CA, USA), or mouse monoclonal anti-β-actin antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA).

**Statistical analysis**

Data were expressed as mean ± SD, and the mean values of replicated wells between different treatments in three independently conducted experiments were compared using ANOVA. *P* values of < 0.05 were considered statistically significant in this study.

**Results**

**ECM gene expression profiling following APN knockdown and KCM stimulation**

Epidermal keratinocytes release a myriad of signaling molecules that act as stimuli of cell growth, migration, adhesion, and ECM production for the underlying fibroblasts. To investigate the keratinocyte-mediated changes of ECM gene expression that occur downstream of APN receptor signaling and identify its potential targets in dermal fibroblasts, fibroblasts were transiently transfected with an APN-specific siRNA and exposed to KCM 72 hours post-transfection. As shown in Figure 3.1A, immunoblot analysis of cell lysates collected 24 hours after KCM addition confirmed that KCM potently stimulated APN expression, and the KCM-induced APN expression was not affected by the introduction of a scramble
siRNA (siC), but it was effectively suppressed by an APN-specific siRNA (siAPN) that had been previously shown to reduce its basal expression [116].

After confirmation of the siRNA efficiency, a pathway-focused oligonucleotide array was used to monitor the changes of ECM gene expression in fibroblasts. As shown in Figure 3.1B, arrays were individually incubated with the cDNA of fibroblasts that were untreated, KCM-treated, scramble siRNA-transfected and KCM-treated (siC/KCM) or APN-specific siRNA-transfected and KCM-treated (siAPN/KCM). Of the genes that responded to KCM treatment, those affected by APN knockdown were selected for further analysis and categorized in Figure 3.1C according to their main functionality in the skin. These genes were considered targets of APN-mediated signaling in keratinocyte-stimulated fibroblasts because their KCM-induced expression changes were partially or completely offset by APN knockdown.

Of the genes upregulated in KCM-treated fibroblasts, the induction of MMP-1, MMP-3, MMP-12, versican, tenascin-C (TN-C), integrin alpha 1 (ITGA1) and catenin alpha 1 (CTNNA1) was abrogated by APN knockdown. Of particular interest is the 7.2-fold increase of MMP-12 expression by KCM treatment and the significant reduction that resulted from suppressing the APN expression. Meanwhile, the KCM-mediated inhibition of fibronectin (FN), Sparc/osteonectin, cwcv and kazal-like domains proteoglycan (SPOCK) and integrin alpha 3 (ITGA3) was successfully rescued by APN knockdown.
Figure 3.1 Microarray-based ECM gene expression profiling of dermal fibroblasts following APN knockdown and KCM stimulation.

Fibroblasts were transfected with a non-silencing siRNA (siC/KCM) or an APN-specific siRNA (siAPN/KCM), and exposed to KCM. Untreated fibroblasts (Un) and KCM-treated fibroblasts (KCM) were used as comparisons. **Panel A**: APN protein expression. **Panel B**: Microarray images showing the expression profile of human ECM and adhesion molecules in fibroblasts. Data are representative of two separate experiments. **Panel C**: List of ECM genes whose expression is modulated by both KCM treatment and APN knockdown, and their gene array positions. The KCM column shows differences in gene expression between untreated cells and KCM-treated cells; and the APN knockdown column shows differences in gene expression between siC/KCM-treated cells and siAPN/KCM-treated cells. Genes selected for further analysis are boxed in panel B and shown in bold in panel C.
APN modulates the keratinocyte-mediated regulation of matrix proteases

The array results on the matrix proteases were validated by immunoblot analysis of cell lysates obtained above using an antibody specific to each of the MMP proteins (Figure 3.2). In correlation with the array data and our previous finding, the level of MMP-1 protein (panel A) was stimulated by KCM treatment. However, the increase of MMP-1 expression was significantly diminished by APN knockdown, leading to an 80% blockage of the keratinocyte-mediated MMP-1 induction. Similarly, it was confirmed that APN suppression abolished the keratinocyte stimulation of MMP-3 at the protein level (panel B).

The expression of MMP-12 was stimulated by KCM treatment, although slightly higher in the siC/KCM sample (Figure 3.2C). Despite the slight increase that occurred as a result of the siRNA transfection, suppression of the APN expression significantly blocked the keratinocyte-mediated stimulation of MMP-12. To the authors’ knowledge, the expression of MMP-12 (commonly known as macrophage elastase) or its regulation was never studied in detail in dermal fibroblasts. To further confirm this observation, MMP-12 expression was examined in dermal fibroblasts co-cultured with different strains of primary human keratinocytes. As shown in Figure 3.2D, the presence of keratinocytes significantly increased MMP-12 expression (F/K), validating the influence of epidermal regulation on the protein.
Figure 3.2 APN-mediated regulation of matrix proteases.

Cell lysates of untreated, KCM-, siC/KCM-, siAPN/KCM-treated fibroblasts were collected, and examined by immunoblot analysis. **Panel A:** MMP-1 protein expression. **Panel B:** MMP-3 protein expression. **Panel C:** MMP-12 protein expression. **Panel D:** MMP-12 protein expression in fibroblasts co-cultured with fibroblasts (F/F) or keratinocytes (F/K). Signals from the MMP-1, MMP-3, MMP-12, and β-actin immunoblots were quantified by densitometry, and the ratios were calculated respectively (n = 3, p < 0.02). Data represent means ± standard deviations for three independently conducted experiments.
APN-mediated regulation of matrix proteases is independent of its enzymatic activity

While APN has the structural features of an integral membrane protein, most of the biological functions discovered so far are associated with the catalytic activity of its extracellular domain rather than with its receptor-mediated intracellular signaling. Thus, it is important to determine whether the APN-mediated regulation of MMPs requires its enzymatic activity. Bestatin is a potent inhibitor of aminopeptidase activity and was used to abolish the catalytic activity of APN. A previous activity inhibition assay of APN in dermal fibroblasts showed that 50 μM concentration of bestatin was effective to block more than 50 % of APN activity without cytotoxicity. As shown in Figure 3.3, bestatin treatment (50 μM) alone did not affect the expression of any of MMP-1, -3, and -12, and nor was it able to block the KCM-mediated induction of the MMPs.

![Figure 3.3](image)

**Figure 3.3 Effect of bestatin on the expression of MMPs in KCM-treated fibroblasts.**

The cells were treated with 50 μM bestatin for one hour prior to KCM addition. Immunoblot analysis of cell lysates collected after 24 hours of KCM incubation showed the expression of MMP-1, MMP-3, and MMP-12 in untreated, bestatin-, KCM-, bestatin/KCM-treated fibroblasts. Data are representative of three separate experiments.
**APN-mediated downregulation of fibronectin production**

Of the adhesion-associated ECM genes affected by APN modulation, FN plays a crucial role in wound healing, and altered FN production has been associated with fibrosis [189-191]. To validate the microarray data, cells were treated as described above, and their lysates were collected and immunoblotted with an anti-FN antibody (Figure 3.3A). Densitometric analysis of the signals showed that the protein expression of FN was reduced by 27% following KCM treatment, and APN knockdown completely reversed the inhibition of KCM on FN. In fact, it increased the level of FN expression by 40% when compared to the untreated sample. To examine whether the APN-mediated suppression of FN depends on its enzymatic activity, fibroblasts were treated with KCM in the presence of bestatin. As shown in Figure 3.3B, inhibition of APN activity did not interfere with the epidermal regulation of FN expression.
Figure 3.4 APN-mediated downregulation of fibronectin production.

Panel A: Immunoblot analysis and desitometric quantification of FN protein expression in untreated, KCM-, siC/KCM-, siAPN/KCM-treated fibroblasts. The FN/β-actin ratios were calculated (n = 3, p < 0.03). Data represent means ± standard deviations for three independently conducted experiments. Panel B: Effect of bestatin (50 μM) on FN expression in KCM-treated fibroblasts.
APN modulates tenascin-C expression in an activity-dependent manner

Another important adhesion molecule influenced by keratinocyte stimulation is TN-C because of its regulatory role in cell migration and matrix contraction [33,192]. Microarray analysis showed KCM stimulation of TN-C expression and its reversal by APN knockdown. This was validated by immunoblot analysis using a TN-C-specific antibody (Figure 3.5). Consistent with the microarray data, KCM-treated fibroblasts showed an increase in TN-C expression when compared with untreated cells, and APN knockdown completely blocked the KCM stimulatory effect, reducing the protein expression of TN-C to the level of the untreated sample.

**Figure 3.5 APN-mediated regulation of tenascin-C expression.**

The levels of TN-C and β-actin in untreated, KCM-, siC/KCM-, and siAPN/KCM-treated fibroblasts were determined by densitometric quantification, and their ratios were calculated (n = 3, p < 0.01). Data represent means ± standard deviations for three independently conducted experiments.
Based on the role of TN-C in matrix contraction [33], and a strong body of evidence that shows direct correlation of alpha smooth muscle actin (α-SMA) expression with contraction [193,194], we evaluated α-SMA expression in relation to TN-C expression. KCM treatment in the presence of bestatin showed that bestatin suppressed the basal TN-C expression in fibroblasts but failed to completely abolish the KCM-induced expression of TN-C (Figure 3.6A, TN-C). That is, the TN-C expression can be suppressed by inhibiting the catalytic activity of APN; however, the inhibition was not sufficient to circumvent the effect of KCM stimulation. Inhibition of the APN activity also has a direct impact on the α-SMA expression which showed an inverse pattern to TN-C expression and was increased as a result of bestatin treatment (Figure 3.6A, α-SMA).

High level of α-SMA expression is generally associated with enhanced contractile activity [193,194]. Based on our earlier observation that bestatin induces α-SMA expression, we examined the effect of bestatin on the contractile activity of dermal fibroblasts embedded within collagen gels (Figure 3.6B). As shown in Figure 3.6C, the first day measurements demonstrated that the untreated gels (control) shrank 24% while the bestatin-treated gels shrank 45% from their original size. In other words, bestatin has reduced the surface area of fibroblast-populated collagen gel by 21% (relative to the control) during the first 24 hours of treatment, and the inhibitory effect gradually wore off as the incubation period extended to 48 and 72 hours.
Figure 3.6 Bestatin-induced collagen gel contraction.

Panel A: Immunoblot analysis of the protein expression of TN-C and α-SMA in dermal fibroblasts after bestatin (50 μM) treatment and 24-hr KCM incubation. Panels B-C: Evaluation of the effect of bestatin on fibroblast contractility by collagen gel contraction assay. The surface area of fibroblast-populated collagen gels was measured on days 0, 1, 2 and 3 after gel release. The relative surface area expressed as percentage of the original gel surface area was determined by calculating the surface area measured daily against the surface area of the original gel (*p < 0.03). Data represent means ± standard deviations of five gels in three independently conducted experiments.
Discussion

SFN is an ECM-modulating factor released by keratinocytes, and the recent identification of APN as a fibroblast surface receptor for SFN has raised attention to its potential role in regulation of cell behavior and matrix remodeling in the dermis. Although some physiological substrates are known, very few downstream targets have been identified for APN because of its short cytoplasmic domain and unknown binding partners. This is the first time that APN has been shown to directly regulate the expression of ECM proteins. Of the genes that responded to APN modulation in KCM-stimulated fibroblasts, the adhesion-associated gene (fibronectin), matrix-degrading proteases (MMPs), and contraction-associated gene (tenascin-C) are of special interest due to their implication in tissue remodeling during wound healing. More importantly, these candidate targets are involved in biological processes that are consistent with APN functions established from studies using cross-linking antibodies and activity inhibitors, such as tumor metastasis and extracellular matrix degradation [156,157,195].

The present study discovered that MMP-12 is present in dermal fibroblasts and its expression is under the influence of epidermal keratinocytes. Although commonly known as macrophage elastase, MMP-12 is also expressed in non-immune cells such as vascular fibroblasts, smooth muscle cells, and corneal fibroblasts [196,197]. MMP-12 is the most potent MMP against elastin [198], and can degrade many other ECM components because of its ability to initiate a
cascade of proteolytic events by activating pro-MMP-2 and pro-MMP-3 [199]. While the current study addressed APN-mediated regulation of MMP-12 only in dermal fibroblasts, it is likely to also occur in immune cells as suggested by the prominent role of APN in T cell response and cytokine production.

TN-C shows a temporo-spatial distribution in human adult skin and is specifically expressed near the wound edge within 24 hours of injury [200,201]. Given the dynamic and transient nature of TN-C expression, tremendous efforts have been made to study the mode of TN-C regulation, in particular, to identify molecules and regulatory pathways that govern the local expression of TN-C. Here we showed that TN-C expression is stimulated by KCM which contains a plethora of keratinocyte-released signaling molecules, and that the keratinocyte-mediated TN-C stimulation is abolished in fibroblasts with suppressed APN expression. The fact that the fibroblast expression of TN-C can be modulated by epidermal keratinocytes may explain the reported increase of TN-C expression in wound-edge fibroblasts [200,201]. In an open wound, as epithelial cells migrate towards the site of injury, fibroblasts at the wound edge become exposed to epithelial cells, and increase their TN-C expression in receipt of signals released by these cells.

In addition to its anti-adhesive and pro-migratory role, TN-C inhibits matrix contraction through downregulation of focal adhesion kinase (FAK) phosphorylation [33,192]. Induction of TN-C at the early stage of wound healing represses premature contraction, while persistent TN-C expression after granulation tissue formation in embryonic wound or oral mucosal wound is
thought to prevent excessive contraction and thus scar formation [200,202,203]. Inhibition of the APN enzymatic activity by bestatin was not sufficient to abrogate KCM stimulation on TN-C; however when used alone, bestatin clearly suppressed the basal expression of TN-C in fibroblasts, suggesting that APN may be involved in modulating fibroblast contractile activity. This was confirmed by the elevated α-SMA expression in bestatin-treated fibroblasts. As a transmembrane protease, APN is known to cleave various cytokines and growth factors, and in most situations the cleavage causes inactivation of bioactive molecules [204-208]. It is possible that bestatin suppresses the enzymatic activity of cell-surface APN to cleave and inactivate an unknown stimulating factor in the extracellular environment, thereby causing a reduction of TN-C production in fibroblasts. The importance of APN in regulation of fibroblast contractility was further evaluated using a fibroblast-populated collagen gel contraction assay which demonstrated that bestatin promotes contraction. The same phenomenon is observed in the gastric system, where aminopeptidase inhibitors have been proven to potentiate the enkephalin-stimulated contraction of gastric smooth muscle cells [209]. Under physiological conditions, APN degrades Leu- and Met-enkephalins, thereby limiting the number of available peptides to activate receptors [210-212]. Thus, inhibition of APN activity significantly increased the potency of Met-enkephalin to induce contraction [213].

In contrast to TN-C modulation, activity inhibition had no effect on the expression of FN or MMPs, suggesting that the APN-mediated regulation of these genes is likely to involve intracellular signaling events associated with its
receptor function. This hypothesis is further supported by our previous finding on SFN-mediated ECM modulation, which showed that SFN stimulates MMP-1 expression via the p38/MAPK pathway in an APN-dependent manner and that the stimulatory effect is unaffected by suppression of the APN catalytic activity [115,116].

In summary, the present study demonstrated that in the presence of a virtually complete repertoire of keratinocyte-derived growth factors and cytokines, the fibroblast production of certain ECM components relies on the availability of APN. The additional level of regulation conferred by the cell-surface APN receptor on fibroblasts is critical for the transmembrane signaling of certain keratinocyte-derived stimuli, including those that influence the expression of FN, MMPs, and TN-C. Accordingly, dysregulation of APN may result in a fibrotic phenotype due to over-accumulation of extracellular matrix and reduced matrix-degrading activity. In line with the proposed effect of its ligand SFN in ameliorating hypertrophic scarring [184], the direct impact of APN on ECM gene expression makes it an ideal therapeutic target in wound healing.
Chapter 4. **Topical application of aminopeptidase N-neutralizing antibody accelerates wound closure**

**Introduction**

Cutaneous tissue repair is a complex process that involves intricately coordinated cell-to-cell communications and controlled release of soluble mediators. Injury to the skin triggers activation of resident cells and influx of immune cells from the circulating blood. Among the resident cells within the wound microenvironment, dermal fibroblasts play an active role in tissue remodeling by synthesizing extracellular matrix proteins and matrix-degrading proteases [34]. The fibroblast-dominated matrix turnover is under the constant influence of surrounding cells, especially keratinocytes in the epidermis [47,214]. Apart from the well-characterized signaling molecules at the epidermal-dermal junction, our group has successfully isolated 14-3-3 sigma (also known as stratifin) which upon release from keratinocytes functions as a modulator of extracellular matrix production in the underlying fibroblasts [113]. Recently, it has been demonstrated that aminopeptidase N is responsible for the stratifin-
mediated p38 MAPK activation leading to upregulation of matrix metalloproteinase-1 [115,116]. Aminopeptidase N (APN) is a ubiquitously expressed membrane-bound zinc-dependent protease with a myriad of functions (reviewed in [122]), and has been recently implicated in epidermal-dermal communication and regulation of key matrix proteins [215]. In a rat skin wound model, APN expression was upregulated at the beginning and towards the end of healing [116]. In light of the growing importance of APN in modulation of the fibroblast phenotype, the present study evaluated the potential of targeting the ectoenzyme in cutaneous repair, and demonstrated that neutralization of APN led to acceleration of wound closure. This was attributed to at least in part the increase of collagen deposition and fibroblast contractility in the granulation tissue.

Materials and Methods

Wound creation and treatment scheme

All animal procedures have been conducted with the close supervision of the University of British Columbia Animal Care Committee under Protocol No. A05-1211. Full-thickness skin excision wounds (4 wounds per mouse) were created on the dorsal surface of Balb/c mice (8-12 wk old females; Jackson Laboratory, ME, USA) using a 4 mm punch biopsy tool (Dormer Laboratories, Mississauga, ON, Canada). One wound was left untreated as control, while the other wounds were treated with either WM15 or an IgG1 isotype antibody (5 μg/wound; Santa
Cruz Biotechnology, CA, USA). In each set of experiments, the order of treatment was changed to reduce the chance of bias in wound healing based on the position where the wounds were created on the animal. Treatment scheme began 24 hours post-wounding, and included alternate-day topical application of the antibodies prepared in pluronic colloidal gel (Poloxamer 407-polyoxyethylene/ polyoxypropylene block co-polymer) which was received as gift from BASF, USA. The pluronic polymer was dissolved in cold distilled water (20% w/w) and adjusted to pH 7.4. The antibodies were mixed with the gel under cold condition.

**Tissue preparation and immunohistochemistry**

Five mice were sacrificed on days 7 and 10 each, and skin tissues (6 mm) were excised, fixed in 10% neutral buffered formaldehyde, dehydrated, and embedded in paraffin. Five-micrometer-thick sections of the tissues (2 sections per sample) were prepared for immunohistochemical analysis. Collagen deposition in the tissue was visualized with Masson’s Trichrome (blue staining). Immunohistochemical staining of α-SMA was performed using a rabbit monoclonal antibody against human/mouse α-SMA (Epitomics, CA, USA).

**Immunoblot analysis of collagen and α-SMA**

The protein expression of collagen and α-SMA in dermal fibroblasts was detected using a specific antibody against human procollagen type Iα1 (mouse monoclonal; Developmental Studies Hybridoma Bank, IA, USA) or human/mouse
\( \alpha \)-SMA (rabbit monoclonal; Epitomics, CA, USA). GAPDH was used as a loading control.

**Collagen gel contraction assay**

Collagen gel contraction was performed as previously described using WM15 (2 \( \mu \)g/ml), IgG1 isotype antibody for negative control (2 \( \mu \)g/ml), and bestatin for positive control (50 \( \mu \)M; Sigma-Aldrich, ON, Canada).

**Wound closure analysis**

The wounds were traced on a transparent film during the 10-day treatment course, and the wound area was determined using the ImageJ software available from NIH.

**Statistical analysis**

Data were expressed as mean \( \pm \) SD. Statistical analysis was conducted using the ANOVA’s test, and \( p \) values of \(< 0.02 \) were considered significant.

**Results**

**Topical application of APN mAb accelerated wound closure**

In comparison to control and IgG1 isotype antibody, WM15 accelerated wound closure by approximately 24\( \pm \)2 \% (Figure 4.1 A, B) and increased
collagen deposition (Figure 4.1C). \textit{In vitro}, neutralization of APN by WM15 increased collagen production in dermal fibroblasts (Figure 4.1D).

**Enhanced \(\alpha\)-smooth muscle actin expression and contraction**

Analysis of day 7 tissue sections showed that the distribution and intensity of \(\alpha\)-SMA expression in the newly formed dermis was significantly increased in WM15-treated wounds relative to control and IgG1-treated wounds (Figure 4.2A). The relevance of APN with contractile activity was further evaluated by examining contraction of fibroblast-populated collagen gel and \(\alpha\)-SMA expression in mAb-treated dermal fibroblasts. In line with the in vivo observation, WM15 promoted fibroblast contractile activity (Figure 4.2B) and amplified \(\alpha\)-SMA expression in fibroblasts (Figure 4.2C).
Figure 4.1 Healing of full-thickness skin wounds in mAb-treated mice.

Panel A: Representative photographs of control, IgG, and WM15-treated wounds on days 0, 3, 5, 7, and 10 post-wounding. Panel B: Percent wound area relative to d0 wound area expressed as mean ± SD (n = 8, p < 0.02). Panel C: Masson’s trichrome staining of collagen in d10 skin wounds (2x and 10x magnifications; n = 4). Scale bars: 250 μm. Panel D: In vitro collagen expression in dermal fibroblasts exposed to IgG, WM15, and bestatin for 24h (n = 3, p < 0.01). Data represent means ± standard deviations for three independently conducted experiments.
Figure 4.2 Analysis of α-SMA expression and contraction in skin wounds or dermal fibroblasts exposed to IgG, WM15, and bestatin.

Panel A: Immunohistochemical staining of α-SMA in d7 skin wounds (40x and 100x magnifications; n = 4). Scale bar: 100 μm. Panel B: Percent surface area of fibroblast-populated collagen gel relative to original gel surface area (n = 5, p < 0.01). Panel C: In vitro α-SMA expression in dermal fibroblasts exposed to IgG, WM15, and bestatin for 24h (n = 3, p < 0.01). Data represent means ± standard deviations for three independently conducted experiments.
Discussion

As the inflammatory response subsides, fibroblasts accumulate at the wound site and become the predominant cell type throughout the remainder of the wound healing process. In the proliferative phase, fibroblasts form a collagen-rich connective tissue matrix to replace the fibrin clot at the wound bed. The nascent collagen matrix serves as a scaffold for fibroblast migration and guides fibroblast-mediated matrix remodeling. In the present study, we showed that WM15 which is an APN-neutralizing antibody accelerated wound closure by increasing collagen production. WM15 has been shown to inhibit type IV collagen degradation in tumor cells [157]. The neutralizing antibody is likely to have blocked the binding of a potential ligand to the APN receptor and consequently the ligand-mediated signaling that normally leads to reduced collagen level. One such ligand might be stratifin. In one experiment where fibroblasts were treated with conditioned medium of keratinocytes with suppressed stratifin expression and release, the level of collagen increased significantly (unpublished data). Although the exact mechanism of APN in modulating collagen synthesis remains elusive, its enzymatic activity is unlikely to be involved. This is because bestatin (a potent chemical inhibitor of the APN enzymatic activity) showed no effect on the level of collagen production when added to fibroblasts (Figure 4.1D).

The increase in collagen deposition can also be attributed to an increase in the number of α-SMA-expressing fibroblasts in the dermis (Figure 4.2). The appearance of α-SMA represents a contractile myofibroblast phenotype, which is
responsible for the generation of mechanical forces that govern granulation tissue contraction [216]. The myofibroblast also contributes to tissue remodeling with its ability to produce extracellular matrix proteins and proteases [49]. In comparison to non-contractile fibroblasts, myofibroblasts secrete elevated levels of collagen [216,217]. The increased collagen production seems to be associated with acquisition of the myofibroblast phenotype and α-SMA expression [218], as suppression of α-SMA expression leads to reduced collagen level [219].

An increase in the α-SMA-positive cell population also means higher contractile activity which results in faster wound closure [15,193]. To date, there has been no evidence that directly links APN to the rho/rho kinase pathway involved in smooth muscle cell contraction. However, in a previous study, we found that APN enzymatically upregulates the expression of tenascin-C which has been shown to modulate matrix contraction via focal adhesion kinase- and rho-mediated signaling [33], and is upregulated at the early stage of adult wound healing to repress premature contraction [200]. By blocking APN enzymatic activity, WM15 was able to enhance α-SMA expression and fibroblast-mediated contraction.

While this is only a preliminary study that examined the potential of targeting APN in wound healing, these findings affirmed the important role of APN in wound contraction and post-injury matrix remodeling. A valuable insight gained from the present study is that not all APN-mediated biological events require catalytically active APN molecules. This feature can be advantageously applied to target diseases caused by impaired APN enzymatic activity without affecting
the activity-independent functions. At the same time, this raises concerns about use of catalytic antagonists of APN in treatment of pathological conditions where its enzymatic activity is not relevant.
Chapter 5. Conclusion and Suggestion for Future Work

General Discussion

Cell signaling during the wound healing process has been a topic of intensive research, and stromal-epithelial communication is of particular importance as it directly contributes to the pathogenesis of fibrotic diseases. With restricted access imposed by the basement membrane, the mode of communication between epidermis and dermis mainly involves soluble factors released by cells in these two tissue compartments.

In search for signaling molecules that contribute to the elevation of MMP-1 observed in dermal fibroblasts co-cultured with keratinocytes, SFN was identified as a keratinocyte-released factor that modulates the fibroblast production of key extracellular matrix proteins. Transmembrane signaling responsible for the SFN-mediated p38 MAPK activation and MMP-1 upregulation in dermal fibroblasts is mediated by cell-surface receptor APN [116]. The receptor is known for its zinc-dependent catalytic activity which preferentially cleaves neutral amino acids from the N-terminal end of oligopeptides. However, unlike other metalloproteinases such as MMPs which are released as proenzymes and need to be proteolytically activated, APN does not have a pro-form and is synthesized as an active enzyme. In Chapter 2, we demonstrated that SFN serves as a stimulus of APN expression. The constitutive level of this ectoenzyme in intact tissue is low, and its expression is upregulated in specific physiologic and pathologic situations.
In the context of epidermal-dermal communication, SFN functions as a paracrine mediator of extracellular matrix remodeling via APN. When the need for active communication arises, for instance, in skin injury, binding of SFN triggers transmembrane signaling to produce more matrix proteins. At the same time, a positive feedback cascade is initiated to increase the constitutively low cell-surface expression of the receptor and therefore its catalytic activity, which in turn amplifies the cellular responsiveness to extrinsic stimulation.

In intact skin, minimal cell communication is required to maintain tissue homeostasis. In response to physical distress, cells at the injured site undergo phenotypic changes and modify the expression of specific molecules such as receptors to enhance protective signaling [221-223]. The ability of keratinocytes to modulate the cell-surface expression of APN in the underlying dermal fibroblasts (Figure 2.1) signifies the importance of this receptor as part of the repair mechanism following skin injury. Under special circumstances when the communication between keratinocytes and fibroblasts is activated, APN is suggested to serve as a transmembrane mediator of keratinocyte-derived signals transmitted to the surface of fibroblasts. Further investigation of this APN-mediated paracrine signaling demonstrated that the epidermal modulation of several key matrix proteases and adhesion molecules depends on the availability of the receptor (Chapter 3). In fibroblasts with suppressed APN expression, signaling molecules released from keratinocytes failed to fulfill their physiological role of transmitting some of the signals destined to impact matrix production. This
ubiquitously expressed receptor possesses a myriad of functions ranging from antigen presentation to cell migration and adhesion [122]. The downstream targets of APN-mediated signaling identified in this study are functionally consistent with the ectoenzyme. For instance, upregulation of MMP-1 and MMP-3 may be partially responsible for the increased tumor invasion and metastasis associated with elevated APN expression [156,157,224,225]. Reinhold and colleagues showed that APN serves as a regulator of T cell immunity and inhibition of APN suppresses inflammatory immune response [226,227]. The immunotherapeutic potential of APN might be partly attributed to its regulatory effect on MMP-12 which has been proposed to function as a pro-inflammatory mediator [228]. We further demonstrated that enzymatic activity is not required for the APN-mediated regulation of these selective MMPs. In fact, many of the functions ascribed to the ectoenzyme including induction of calcium influx, proliferation of myeloid cells, adhesion of human umbilical vein endothelial cells (HUVECs), and viral infection are independent of its catalytic activity, and involve activation of signal transduction [121,179,229,230].

Despite ample evidence to the contrary, APN is structurally incompetent of transducing intracellular signaling events because of its extremely short cytoplasmic region [231]. In a study on homotypic aggregation of monocytic cells, Mina-Osorio and colleagues proposed that APN activates the Ras-MAPK pathway through interaction with adaptor molecules Grb2 and SOS [123,124]. Grb2 is an adaptor molecule that interacts with a receptor protein by binding to phosphorylated tyrosine sequences in its intracellular region [232]. However, in
the absence of a phosphorylation site or signaling motif, the cytoplasmic tail of APN is unlikely to be functional or able to engage in signal transduction, much less a phosphorylation-dependent collaboration. In fact, APN-mediated transmissible gastroenteritis virus (TGEV) infection still occurs when the enzyme is devoid of its cytoplasmic domain [233].

There are several signal transducing receptors that lack a functionally defined cytoplasmic region. One such example is T cell receptor (TCR), which induces activation of T cells in response to antigen presentation [234]. TCR itself has a very short cytoplasmic tail, and is incapable of transducing signals intracellularly. On the cell membrane, TCR is non-covalently linked to CD3 molecules which possess a characteristic sequence motif for tyrosine phosphorylation called ITAMs (Immunoreceptor Tyrosine-based Activation Motifs) [235]. The signals that lead to T cell activation are recognized by the antigen-binding site on TCR, and transduced through the CD3 molecules which become phosphorylated by the SRC family of kinases. An alternative mechanism of signal transduction is for APN to present its ligand to an auxiliary protein with the ability to activate signaling pathways intracellularly. For example, type III TGFβ receptor (TGFβRIII) sequesters TGF-β at the cell surface and presents the ligand to type II TGFβ receptor, leading to the activation of smad3 and smad4 transcription complex [236].

The use of APN as a therapeutic target has been under intense investigation. During 2006 - 2010, there are a total of 16 patent applications filed for inventions of APN inhibitors [237]. The majority of these inventions are directed to
applications in anti-tumor and anti-inflammatory therapies based on evidence of APN in tumor cell invasion and antigen presentation [156,220]. The novel role of APN as a signal receptor in stromal-epithelial communication established in our studies implies that the ectoenzyme may have some therapeutic potential in wound healing. In Chapter 4, the APN-neutralizing antibody WM15 displayed a beneficial effect on the healing of full-thickness skin wounds created on the back of BALB/c mice. Acceleration of wound closure was due to at least in part the increase of collagen deposition and fibroblast contractility. In a previous study, WM15 was shown to inhibit the degradation of type IV collagen in tumor cells [157]. While activity inhibition did not affect collagen production, the enzymatic activity of APN was indispensable for its regulation of α-SMA expression and fibroblast contractile activity. The importance of aminopeptidase activity in maintaining cell contractility has been previously demonstrated using gastric muscle cells [238]. Although the specific mechanisms of how APN modulates collagen content in the extracellular matrix or fibroblast-mediated contraction are still unknown, it can be concluded that APN engages both its peptidase activity and ligand-binding ability to modulate the phenotype of dermal fibroblasts and their surrounding matrix.

In the in vivo study, we specifically examined the regulatory role of APN with respect to matrix remodeling and fibroblast contractility; however, other previously identified APN functions such as inflammation and angiogenesis might have also influenced wound healing. For instance, elevated expression of the soluble or membrane-bound form of APN has been observed in
hyperinflammatory conditions such as rheumatoid arthritis [239], and vessels of inflammatory tissues [240]. Dual inhibition of dipeptidyl peptidase IV/CD26 and APN suppresses proliferation and effector functions of proinflammatory T cells [241], and DNA synthesis in mitogen-stimulated human T cells [227]. The implication of APN in inflammatory diseases is associated with its chemotactic activity for T lymphocytes [242], and its enzymatic activity of processing antigenic peptides on MHC class II molecules [243], as well as its proposed role as a regulator of T cell activation [226]. Chemical inhibitors of APN block angiogenesis at high dose in vitro [185], and in some types of cancer [244]. It pro-angiogenic property has been proven in APN-null mice which are characterized by impaired angiogenesis [245]. These mice have reduced retinal neovascularization under hypoxic conditions, and dampened angiogenic response to growth factors. Mechanisms of APN in promoting angiogenesis have been proposed to be independent of its enzymatic activity, and can be linked to its involvement in endothelial cell morphogeneis [246], association with the pro-angiogenic protein galectin-3 [247], or mediation of endothelial cell adhesion [244,248]. While the implications of APN in inflammation and angiogenesis can both affect healing, the outcome of targeting APN seems to depend on the method of interference, varying from gene manipulation, use of chemical inhibitors, and use of antibodies. For instance, migration and adhesion of human umbilical vein endothelial cells were significantly inhibited by APN siRNA, but not by the APN-specific antibody WM15 [121]. The multifunctional roles of APN have demonstrated it to be a powerful therapeutic target in cancer and inflammatory diseases; however, the
dependence (or independence) of its catalytic activity also requires special attention in choosing the tools of intervention in treatment.

In summary, in this thesis we were able to 1) confirm keratinocyte-mediated paracrine regulation of APN in dermal fibroblasts, 2) identify stratifin as a stimulator of APN expression, 3) characterize APN as a transmembrane receptor for signals that modulate the expression of key ECM proteins in fibroblasts, and 4) explore the therapeutic potential of targeting APN in wound healing.

**Suggestions for Future Work**

The ubiquitous distribution and extensive functionality of APN, as well as the unpredictability of activity dependence (or independence) associated with a particular function make it challenging to utilize APN as a therapeutic target. The current findings have provided encouraging evidence for future investigation on the therapeutic potential of APN agonists/antagonists for cutaneous wound repair, and can be improved upon with the work suggested below.

I. In Chapter 2, we demonstrated that keratinocytes release soluble factors that stimulate fibroblast APN expression. Pro-inflammatory cytokines have also been shown to induce APN expression. One of the most studied functions of APN is inflammation and regulation of T cell activation. In order to generate a more detailed overview of the physiological processes mediated by APN in wound healing, it would be
worthwhile to profile the cytokines and growth factors released by keratinocytes as well as other cell types present in the wound site including macrophages and endothelial cells according to their effects on APN expression.

II. In Chapter 3, we selectively studied genes that show changes in gene expression in the presence of keratinocyte stimulation and when fibroblast APN expression is suppressed. However, there were several genes including thrombospondin-1 that changed their expression in response to APN knockdown, but were unaffected by keratinocyte stimulation. Thrombospondin-1 is a matricellular protein that acts temporarily and spatially at sites of injury. Overexpression of thrombospondin-1 led to impaired healing due to a decrease in granulation tissue formation and angiogenesis [249]. It is also recognized for its role as a major activator of TGF-β1 [250], which is a pro-fibrotic factor implicated in all phases of wound healing. Further investigation of these genes is recommended.

III. In Chapter 3, we showed that APN regulates the expression of some matrix proteins independently of its enzymatic activity. In order to maximize the efficacy of targeting APN and minimize the associated downside risks, it is important to distinguish the functions that are dependent on the enzymatic activity of APN from those that do not. In addition to using a chemical inhibitor specific to APN, generating an enzymatically inactive mutant and comparing its activities with those of
wild-type APN can help determine whether a particular function is catalytically dependent.

IV. In Chapter 3, we proposed that some of the APN-associated functions are mediated via signal transduction. Identification of co-receptors or adaptor molecules associated with APN in those functions is highly recommended. This can be done by adding a crosslinker to cells to stabilize the interaction between APN and its potential binding partner, extracting intact cell membrane with mild detergents, and identifying the binding protein by mass spectrometry.

V. In Chapter 4, we assessed the therapeutic potential of an APN-neutralizing antibody in a murine model. To confirm the therapeutic efficacy for future clinical trials, repeat of the study using an animal model of non-healing wounds such as those used for venous ulcer and diabetes is recommended.
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