IDENTIFICATION AND MECHANISM OF ACTION OF KERATINOCYTE-RELEASABLE FACTORS IN REGULATION OF EXTRACELLULAR MATRIX EXPRESSION

by

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Abstract

The complexity of multi-cellular organisms demands a dynamic reciprocity between neighboring cells in any given tissue. The disruption in keratinocytes and fibroblasts cross-talk in skin has been linked to an imbalance in extracellular matrix (ECM) expression leading to the onset of fibrosis such as hypertrophic scarring. Our group has recently identified 14-3-3σ or stratifin (SFN) as a potent MMP-1 stimulatory factor in fibroblasts. In this doctoral research project, we hypothesized that SFN can modulate other ECM components and execute its transmembrane signaling through interaction with a receptor on the surface of fibroblasts.

Three specific objectives were accomplished in this project. Under objective 1, ECM gene expression profile of fibroblasts treated with SFN or co-cultured with keratinocytes was characterized by an ECM-pathway specific gene array and revealed that SFN upregulates a wider range of MMPs such as MMP-3, 8, 10, and 24 other than MMP-1. As SFN was not responsible for the keratinocyte-mediated decrease in collagen expression, under objective 2 attempts were made to characterize the nature of a collagen inhibitory factor in keratinocyte-conditioned medium (KCM). Analysis of keratinocyte/fibroblast co-culture and KCM revealed a 30-50 kDa keratinocyte-derived collagen inhibitory factor with stable activity at high temperature (56 ºC) and acidic environment (pH=2, 30 min). Under objective 3, SFN's transmembrane signaling mechanism was investigated by utilizing a combination of receptor ectodomain biotin labeling, serial affinity purification, and MS/MS to identify aminopeptidase N or CD13 (APN) as a potential SFN receptor in fibroblasts. APN/SFN binding was further confirmed by immunoprecipitation, cross-linking, and co-distribution studies. Expression of APN and SFN increased after wound closure in a rabbit ear fibrotic

model as well as a longitudinal study in rats. The transient knockdown of APN blocked SFN-mediated p38 MAPK activation and MMP-1 expression.

Collectively, the findings presented in this thesis provide further support for the importance of keratinocyte-releasable factors in the regulation of ECM and MMP expression in fibroblasts. We also identify APN as a novel cell surface receptor for SFN. Therefore, our findings may provide additional therapeutic tools for the regulation of MMP expression in dermal fibrosis and chronic wound healing disorders.

Preface

The work presented in this thesis has already been published or accepted for publication as co-authored works. This is to confirm that Abdi Ghaffari is the first author in all of the publications included in this thesis as shown below:

Chapter 2: Abdi Ghaffari, Yunyaun Li, Ali Karami, Mazyar Ghaffari, Edward E. Tredget, and Aziz Ghahary. Fibroblast Extracellular Matrix Gene Expression in Response to Keratinocyte- Releasable Stratifin. Journal of Cellular Biochemistry (2006) 98:383–393.

Chapter 3: Abdi Ghaffari, Ruhangiz T. Kilani, and Aziz Ghahary. Keratinocyte-Conditioned Media Regulate Collagen Expression in Dermal Fibroblasts. Journal of Investigative Dermatology (2009) 129, 340–347.

Chapter 4: Ghaffari, Abdi; Li, Yunyaun; Kilani, Ruhangiz T.; Ghahary, Aziz. 14-3-3σ associates with cell surface aminopeptidase N in the regulation of matrix metalloproteinase-1. Journal of Cell Science (2010) 123, 2996-3005.

Dr. Aziz Ghahary was the principal investigator of the research project and the original identification of stratifin as a keratinocyte-releasable factor is credited to him. In addition, he supervised all experimental designs and analysis and 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. Abdi Ghaffari has been responsible for the identification and design, performing the research, data analysis, and manuscript preparation for all the work described in this thesis with the exception of:

Chapter 2: Dr. Tredget and Dr. Li advised on the approach and design of the study. Mr. Karami assisted with expression and purification of recombinant stratifin. Mr. Mazyar Ghaffari assisted with MMP-3 analysis in keratinocyte/fibroblast co-culture studies.

Chapter 3: Dr. Kilani and Abdi Ghaffari equally contributed to the data presented in this manuscript. Dr. Kilani performed all the northern blot experiments shown in this manuscript and the repeat of some of the western blot experiments.

Chapter 4: Dr. Li expressed the stratifin deletion fragments in bacteria previously and prepared the clones, carried out the western blot analysis on phosphorylated amino acid residue in APN (Figure 3.6, panel C), and consulted on approach for purification of APN. Dr. Kilani performed the receptor binding assay with $^{[125]}$I-labeled stratifin (Figure 3.1, panel B).

The work described in this thesis has been conducted with the approval of the University of British Columbia Biohazards Committee under the certificate number H05-0103. All the animal studies have been conducted with the close supervision of the University of British Columbia Animal Care Committee and under the protocol number A05-1211.

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Abbreviations

AP	Alkaline Phosphatase
AP-1	Activator Protein-1
APN	Aminopeptidase N
ATP	Adenosine Triphosphate
BSA	Bovine Serum Albumin
Col	Collagen
CPM	Count Per Minute
CTGF	Connective Tissue Growth Factor
Cyt	Cytosol
DMEM	Dulbecco's Modified Eagle's Medium
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
EMMPRIN	Extracellular MMP Inducer
ET-1	Endothelin-1
ETS	Erythroblastosis Twenty Six
FAK	Focal Adhesion Kinase
FGF	Fibroblast Growth Factor
FPCG	Fibroblast Populated Collagen Gel
GAPDH	Glyceraldehydes 3 Phosphate Dehydrogenase
GST	Glutathione S Transferase
HTS	Hypertrophic Scar
IFN	Interferon
IL-1	Interleukin-1
IL-1R	Interleukin-1 Receptor
IP	Immunoprecipitation
KCM	Keratinocyte Conditioned Medium
KD-CIF	Keratinocyte-Derived Collagen Inhibitory Factor
KDAF	Keratinocyte-Derived Antifibrogenic Factor
KGF	Keratinocyte Growth Factor
KSFM	Keratinocyte Serum Free Medium
LDH	Lactate Dehydrogenase
mAb	Monoclonal Antibody
MAPK	Mitogen Activated Protein Kinase
MMP	Matrix Metalloproteinase
MS	Mass Spectroscopy
MTT	Methyl Thiazolyl Tetrazolium
NCM	Non-Conditioned Medium
PBS	Phosphate-Buffered Saline
PDGF	Platelet-Derived Growth Factor
PFA	Paraformaldehyde
PM	Plasma Membrane
RT-PCR	Reversed Transcriptase-Polymerase Chain Reaction
SCC	Squamous Cell Carcinoma
SFN	Stratifin
siRNA	Small Interfering RNA

SMA	Smooth Muscle Actin
TGF	Transforming Growth Factor
TIMP	Tissue Inhibitors of MMP
TNF	Tumor Necrosis Factor
VEGF	Vascular Endothelial Growth Factor

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I am eternally grateful to Dr. Aziz Ghahary not only for being an exceptional supervisor, but also for being a friend, mentor, and father figure in my life. I am forever indebted to him for his continuous support, guidance, patience, and inspiration. It is with a bittersweet feeling that I complete my degree and part from his group. However, his positive influence will be forever a guide wherever my career takes me.

I am greatly thankful to the members of my advisory committee, Dr. Vince Duronio, Dr. Michael Cox, and Dr. Gang Li for their valuable guidance and insight. Isaac Newton is quoted for saying “if I have seen further it is only by standing on the shoulders of giants.” I am by no means comparing myself to Newton, however the small progress that I made in my field would not have been possible without the support of my committee members and supervisor.

I have been fortunate to work with an amazing group of people in Dr. Ghahary’s lab over these years whose friendship, support, and wisdom has improved the quality of my work and life. I am forever grateful to Dr. Ruhangiz Kilani, Dr. Yunyuan Li, Dr. Reza Jalili, Dr. Abeladro Medina, Dr. Claudia Chavez, Dr. Elham Rahmani, Ali Karami, Amy Lai, Darya Habibi, Dr. Alireza Moeen, Azadeh Tabatabai, and Ryan Hartwell.

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Finally and most importantly, none of my accomplishments would have been possible without the love and support of my family. The most heartfelt thanks to my parents for their continuous love, support, and strength; to my brother and best friend Mazyar for his endless support and love; to my kids, Sina and Soha who bring joy, hope, and daily dose of energy into my life; and my deepest love and thanks to my

wife, Susan, for her unconditional love, friendship, strength, and inspiration during all these years.

Dedication

This work is dedicated to:

My parents who endured many hardships so that their children can have access to the best education possible.

To science and scientists everywhere who illuminate the path of our journey with knowledge and truth.

Chapter 1. Introduction

Dermal Wound Healing

Skin is the largest organ in the human body and functions as our first line of defense. Skin also plays a critical role in immune surveillance, sensory detection, and emotional expression and interaction. Therefore, the ability to repair injured skin is essential to the continuity and quality of human life. Following an injury, skin can repair itself by re-epithelialization, scar formation, and contraction. When injury is only isolated to the epidermis, skin can heal by re-epithelialization where epithelial cells from the wound edge or skin adnexa migrate on the surface of the wound to cover the damaged area (Fuchs, 2007). When trauma extends beyond the epidermal layer into the skin dermis, all mammals repair the wound by formation of a scar tissue.

The wound healing process is divided into four major overlapping phases: hemostasis, inflammatory, proliferation, and tissue remodeling. Immediately following tissue injury and disruption of blood vessels, the blood clot reestablishes hemostasis and provides a temporary matrix scaffold as well as several mediators such as PDGF, EGF, and TGFβ1 to facilitate the activation and migration of immune cells, keratinocytes, fibroblasts, and endothelial cells in the injured site. During this inflammatory phase, Neutrophils and monocytes are then recruited from the circulating blood and initiate bacterial clearance, phagocytosis, and tissue debridement. Activated macrophages also release a complex cocktail of growth factors and cytokines essential for the activation and further recruitment of keratinocytes, fibroblasts, endothelial cells, as well as other inflammatory cells. In proliferation phase, epithelial cells, fibroblasts, and endothelial cells continue their migration into the wound. During this phase, collagen and other extracellular matrix (ECM) proteins released mainly from fibroblasts

start to accumulate and form the newly deposited granulation tissue. During the prolonged maturation phase, fibroblasts continue to remodel the newly accumulated collagen and ECM by an increase in the expression of proteases such as matrix metalloproteinases (MMPs) in order to increase the tensile strength of the injured tissue and recover the skin function. Eventually the wound becomes less cellular and vascular and a scar tissue is formed (Rhett et al., 2008). The injured skin can also heal by contraction, which is defined by mechanical reduction in size of the wound as the result of contracting α-smooth muscle actin-expressing fibroblasts called myofibroblasts (Hinz, 2007).

Hypertrophic Scarring

Development of scar tissue is an expected end result of wound healing in almost all mammalian species. In humans, some skin injuries, in particular burn patients, may develop into an elevated, red, pruritic and rigid mass of tissue called hypertrophic scar (HTS). Common risk factors for HTS formation in a skin wound are delayed closure, large area, depth, and tension in the injured site. Unlike keloids, which also elevate above skin level, HTS do not grow beyond the confines of the original lesion (Atiyeh et al., 2005). The physical properties of HTS are due to the presence of large amount of ECM which is of altered composition and organization in comparison to normal skin or mature scar. Although the etiology of HTS is not completely understood, its adverse properties have been attributed to prolongation of the inflammatory phase, delayed reepithelialization, excess production of ECM, hyperactive dermal cells, augmented neovascularization, atypical extracellular matrix remodeling, and reduced apoptosis. In a majority of cases, the HTS enlarges for a period of months and eventually regresses

spontaneously over a few years (Scott et al., 2000). If left untreated however, the scar tissue may undergo a reorganization leading to the development of contractures which further contribute to the functional impairment, discomfort, and cosmetic problems suffered by the patient (Ladak and Tredget, 2009).

Composition and Organization of the Extracellular Matrix

Collagen is the most abundant ECM protein in normal skin, mature, and hypertrophic scar. It is largely responsible for providing tensile strength in skin to allow it to serve as a protective barrier against external trauma. The major genetic form of collagen in skin is type I, which is a product of two genes (Col1A1 and Col1A2) and composed of two α1(I) and one α2(I) polypeptide chains assembling into thick fibrils, fibers, and fiber-bundles. The other common forms of collagen in normal skin are type III, V, and VI. Fibrils formed by types III and V collagen are significantly reduced in diameter when compared with type I collagen fibrils. In fact, heterotypic interactions between type I, III, and V collagens plays a regulatory role in the control of fibril diameter in tissue (Birk et al., 1990). Histological analysis of HTS revealed the presence of rounded whorls of immature collagen that consist of thin fibrils and rich in type III collagen. These nodules are clearly in contrast with the surrounding scar tissue, which usually is composed of mature thick collagen fibers oriented parallel to the skin surface (Scott et al., 2000; Verhaegen et al., 2009).

In physiological wound healing, the formation and degradation of collagen is primarily initiated by fibroblasts. This degradation and removal is necessary for the reorganization and alignment of collagen fibers with the tensional forces in skin to regain tissue strength and integrity. Fibroblasts degrade collagen through either

extracellular or intracellular pathways (Page-McCaw et al., 2007; Evert et al., 1996). Collagen fibers are extremely resisteant to proteolytic breakdown and their degredation is mediated by matrix metalloproteinases (MMPs) that include MMP-1, MMP-13, and MMP-14. These enzymes cleave native type I collagen at Gly-Ile and Gly-Leu within the helical region and generate ¾- and ¼-fragments, which unfold at physiological temperatures and further digested by gelatinase such as MMP-2 and MMP-9 (Nagase and Woessner, 1999). In addition to extracellular degradation by MMPs, it has also been shown that in rapidly remodelling tissues, collagen fiber degradation can occur through an intracellular phagocytic pathway (Evert et al., 1996). Using electron microscopy analysis, the collagen fragments can be localized within membrane-bound phagocytic vesicles which later fuse with lysosomes and degraded under acidic conditions by cathepsin enzymes such as cathepsin K (Li et al., 2004; Segal et al., 2001). Collagen phagocytosis appears to be receptor-mediated and recent studies have identified a critical role for MT1-MMP or MMP-14 in preparation of collagen fibrils for phagocytic degredation (Lee et al., 2006).

The interfibrillar space in dermal connective tissue is primarily filled by glycoproteins and proteoglycans. Glycoproteins such as fibronectin, vitronectin, and tenascin play a role in cell-matrix adhesion and providing a provisional matrix during wound healing. It has been reported that the level of glycoproteins including fibronectin is elevated in HTS, which might play a role in cell attachments and survival (Ghahary et al., 1993; Shetlar et al., 1971). Proteoglycans such as decorin, glycosaminoglycans, versican, and biglycan affect physical properties of the tissue such as elasticity, turgor, and resistance to compression by playing an important role in the orientation, organization, and size of the collagen fibrils produced in the tissue. Anionic polysaccharide glycosaminoglycans are mainly responsible for water-holding ability of

the tissue. It is not surprising that the increased turgor, rigidity, and thickness of HTS correlates with an elevated content of glycosaminoglycans and large proteoglycans such as versican (Scott et al., 1996).

Matrix Metalloproteinase

The precise breakdown and reorganization of ECM proteins such as collagen, proteoglycan, glycoprotein, and elastin is essential to normal development as well as tissue repair. Most ECM proteins exhibit resistance to general proteolysis and require specific enzymes for their degradation. Nearly 50 years ago, Gross and Lapiere observed that “collagen, representing a major fraction of animal protein, is removed rapidly during remodeling processes; yet a true animal collagenase has not been found.” They went on to characterize, for the first time, collagenase activity in animal tissue during metamorphosis in tadpoles (Gross and Lapiere, 1962). This report opened a new field of research that led to the discovery of the 23 members of matrix metalloproteinase (MMP) family humans. MMPs represent a group of diverse zinc-dependent proteolytic enzymes with the traditional role of ECM catabolism during embryonic growth and development, bone growth and resorption, tissue homeostasis, tumor metastasis, and wound healing (Nagase and Woessner, 1999). As new substrates continued to be identified, several groups have broadened the functional role of MMPs by including regulation of signaling networks through cleavage of ECM and bioactive molecules (Morrison et al., 2009; Page-McCaw et al., 2007).

MMPs are members of the metzincin group of proteases and can be categorized into at least five different subfamilies according to their substrate specificity, primary structure, and cellular localization. These are collagenases, gelatinases, stromelysins,

matrilysins, and membrane-type MMP. Mammalian MMPs possess two key conserved domains that consist of an autoinhibitory pro-domain and a catalytic domain (Nagase et al., 2006). All MMPs are expressed as inactive pro-proteins that require cleavage of the pro-domain in order to expose the catalytic site to the substrates. A majority of MMPs also possess a C-terminal hemopexin domain responsible for protease localization, substrate recognition, and degradation (Overall, 2002). In addition to classical regulatory processes such as RNA transcription, protein translation and secretion, MMPs can also be regulated at levels of intracellular or extracellular localization, pro-enzyme activation, protein inhibitors such as tissue inhibitors of MMPs (TIMPs), ECM binding, and protease degradation (Brinckerhoff and Matrisian, 2002; Page-McCaw et al., 2007).

The expression of MMP genes is primarily regulated by promoters containing the activator protein-1 (AP-1) enhancer element and/or erythroblastosis twenty six (ETS) site (Chakraborti et al., 2003). The constitutive expression of MMPs in intact tissue is very low. Their expression and function is upregulated in various physiologic and pathologic conditions such as reproductive cycle, embryonic development, wound healing, tumor metastasis and arthritis (Ravanti and Kahari, 2000). In wound healing, the expression of MMPs is mainly regulated by activation of mitogen-activated protein kinase (MAPK), Smad, or NF-κB pathways by growth factors and cytokines abundant in wounds such as transforming growth factor β (TGF-β), platelet-derived growth factor (PDGF), tumor necrosis factor α (TNF-α), interleukin-1β (IL-1β), basic fibroblast growth factor (b-FGF), epidermal growth factor (EGF), and keratinocyte growth factor (KGF). In addition, activation of β-catenin by cadherins and focal adhesion kinase (FAK) by integrin can also regulate MMP expression (Yan and Boyd, 2007). The expression and

cellular source of MMPs and TIMPs in acute dermal wound healing is summarized in Table 1.

Proliferating Keratinocyte	Migrating Keratinocyte	Fibroblast	Neutrophil	Macrophage	Endothelial Cell
MMP-3	MMP-1	MMP-1	MMP-8	MMP-12	MMP-9
MMP-19	MMP-10	MMP-2	MMP-9	MMP-19	MMP-2
MMP-28	MMP-9	MMP-3			MMP-19
TIMP-1	MMP-26	MMP-19			MT1-MMP
TIMP-2	TIMP-1	MT1-MMP			
TIMP-3		TIMP-1			
		TIMP-2			
		TIMP-3			

Table 1.1. Expression of MMPs and TIMPs in wound healing (Toriseva & Kahari, 2009).

Collagen type I and III, synthesized and deposited by fibroblasts, are the predominate ECM proteins in both normal skin and hypertrophic scar. Therefore their timely and precise breakdown by collagenases is an essential step of the remodeling phase in the wound healing process. In human skin, MMP-1 is the key collagenase responsible for breakdown of collagen in ECM and is almost entirely produced and secreted by dermal fibroblast during the remodeling phase (Bauer et al., 1970; Stricklin et al., 1993). Fibroblasts cultured from HTS tissue show reduced levels of MMP-1 mRNA (Ghahary et al., 1996) and transient over-expression of human MMP-1 in the liver attenuated experimental hepatic fibrosis (Iimuro et al., 2003). Therefore, evidence indicates that MMP-1 plays an important role in the progress of HTS although the exact nature of this involvement is not clearly understood.

Cell-Cell Communication in Wound Healing

The wound healing process involves a continuous sequence of cellular interactions in which platelets, fibroblasts, epithelial, endothelial, and immune cells

come together outside of their usual domains in order to orchestrate a very complex event to repair the injured tissue. These signals, which are mainly growth factors and cytokines, coordinate the initiation, continuation and termination of wound healing.

Platelets are the first cells recruited to the injured site, not only to block the loss of blood from damaged vessels, but also to activate local skin cells by the release of growth factors such as TGFβ, PDGF, EGF, and vascular endothelial growth factor (VEGF) (Barrientos et al., 2008). A massive influx of inflammatory phagocytes into the site of inflammation occurs as the local concentration of chemotactic cytokines increases. Neutrophils arrive at the site of injury very early as part of the innate immunity in order to kill invading microorganisms. As long as the conditions are kept sterile, neutrophils do not appear to play an important role in the repair process and their absence does not affect the function of other cells involved in wound healing (Simpson and Ross, 1972). The presence of macrophages is crucial in directing the behaviour of local cells involved in the progress of wound healing. The depletion of macrophages with antisera results in failure in debridement, fibroplasia, and significant impairment of the healing process (Leibovich and Ross, 1975). Macrophages produce numerous signalling molecules that are involved in the regulation of fibroblast proliferation, epithelial migration, and angiogenesis by endothelial cells (Eming et al., 2009; Martin and Leibovich, 2005). Some of the key macrophage-derived signals involved in cell-cell interaction during wound healing include EGF, TGFβ, VEGF, IL-1, IL-6, and tumor necrosis factor-α (TNF-α) (Barrientos et al., 2008).

In the mid phase of wound healing, cellular interactions become primarily dominated by the cross-talk between skin epithelial cells or keratinocytes and fibroblasts in the dermis, which gradually shifts the wound environment away from inflammatory phase into the proliferative- and ECM synthesis phase. Fibroblasts are

the primary stromal cells in the skin dermis responsible for ECM production, growth factors and cytokine signalling, and tissue remodeling. Keratinocytes are the major constituent of the skin epidermis and represent a constantly renewing cellular compartment, which is responsible for wound re-epithelialization following an injury. Epithelial and stromal interactions are crucial during skin morphogenesis in development as well as for maintaining the skin integrity in adult tissue (Martin and Parkhurst, 2004; Tickle, 2006).

The importance of epithelial-stromal interaction was clearly demonstrated by the feeder cell-culture system, where only the fibroblast feeder cells were able to provide an environment that supports proliferation and stem cell phenotype of keratinocytes (Rheinwald and Green, 1975). During the re-epithelialization phase of wound healing, keratinocytes are dependent on signals received from fibroblasts in order to restore a functional epidermis. Migrating keratinocytes induce fibroblasts to release KGF and HGF which in turn stimulate keratinocytes proliferation and migration (El et al., 2002; el-Ghalbzouri et al., 2002; Szabowski et al., 2000; Werner et al., 2007). In turn, fibroblast activation during wound healing is regulated by keratinocytes via the release of cytokines and growth factors. Keratinocytes stimulate the differentiation of fibroblasts into myofibroblasts by inducing α-smooth muscle actin (α-SMA) and collagen expression. Co-culture studies have revealed that keratinocyte-releasable TGF-β and IL-1 as well as keratinocyte-mediated release of endothelin-1 (ET-1) by fibroblasts play a role in regulation of myofibroblasts differentiation (Shephard et al., 2004a; Shephard et al., 2004b). Keratinocyte-derived factors also exhibit MMP stimulatory effects in dermal fibroblasts. Several groups have reported induction of fibroblast MMP-1 production stimulated by keratinocytes (Johnson-Wint, 1980; Moon et al., 2001). The candidate keratinocyte-derived factors responsible for the induction of MMP-1 in

fibroblasts are IL-1 (Johnson-Wint and Bauer, 1985; Mackay et al., 1992), extracellular MMP inducer (EMMPRIN) (DeCastro et al., 1996), and 14-3-3σ (SFN) (Ghahary et al., 2004). Co-culture with keratinocytes also appears to reduce collagen synthesis in fibroblasts, whereas fibroblast conditioned medium has no effect. It has also been shown that keratinocyte-conditioned medium alone can mimic the co-culture results by decreasing collagen production in fibroblasts (Garner, 1998; Harrison et al., 2006). However, the exact nature of collagen mediatory factors released by keratinocyte is not yet fully understood.

The disruption in epithelial-stromal communication has been shown to affect the function of dermal fibroblasts in wound healing. Increasing evidence suggests that delay in epithelialization increases the chance of developing HTS, where fibroblast continue to deposit new granulation tissue and delay remodeling of ECM due to low expression and release of MMPs (Deitch et al., 1983; Ghahary et al., 1996; Machesney et al., 1998; Niessen et al., 2001). The role of keratinocytes in HTS can be observed in wounds that receive meshed epithelial grafts, where raised scars often develop within the existing gaps (Hunt et al., 2000). Overall, the application of cultured epithelial autografts as well as split-thickness skin grafts have been very promising in significantly decreasing the HTS formation (Rudolph and Klein, 1973; Wood and Stoner, 1996) and inducing myofibroblasts apoptosis (Garbin et al., 1996). By delaying the reepithelialization of the wound in dominant-negative KGF receptor transgenic mice, Werner and colleagues were able to observe dermal hyperthickening and fibrosis (Werner et al., 1994) following injury to the skin. It is clear that epithelial-stromal communication plays a key role in maintaining and rebuilding the tissue integrity in healthy as well as injured skin. Therefore understanding the exact nature of this interaction becomes critical in improving clinical outcomes in skin disorders.

14-3-3 Proteins

14-3-3σ is a member of a highly conserved and abundant family of 28-33 kDa acidic polypeptides called 14-3-3 proteins (β, γ, ε, ζ, η, σ, τ isoforms). It was first described by Moore and Perez in their search of acidic proteins of the nervous system by 2D chromatography (Moore et al., 1967). The atypical name of 14-3-3 originates from their systemic classification of protein's migration pattern. The human 14-3-3 protein was first purified from brain tissue by Boston and colleagues. They later acknowledged that "The functional significance of this neuronal protein remains obscure" (Boston et al., 1982). Almost two decades after original discovery, cellular functions for 14-3-3s started to emerge (Ichimura et al., 1988; Toker et al., 1990). Aitken's group later identified 14-3-3s as novel chaperone proteins that interacts with components of signal-transduction pathways (Aitken, 1996). In mid 1990s, a surge in research activity revealed that 14-3-3 family members are expressed in all eukaryotes, with over 100 binding partners in all cellular compartments, and great functional diversity which includes signal transduction, cell-cycle control, apoptosis, cellular trafficking, cell proliferation and differentiation, and protein folding and processing (Aitken et al., 1995; Dougherty and Morrison, 2004).

14-3-3 subunits form homo- and heterodimers with highly rigid structures that can induce conformational change in their binding partners as an adaptor or chaperone molecule (Morrison, 2009). The overwhelming number of binding partners is partly due to 14-3-3's specific phospho-serine/phospho-threonine binding activity. Phosphorylation plays a significant role in cellular biology and two high affinity binding motifs in target proteins are recognized by all 14-3-3 isotypes: RSXpSXP (mode 1) and RXXXpSXP

(mode 2), where pS represents phospho-serine (Rittinger et al., 1999; Yaffe et al., 1997). It is important to note that other phosphorylation-dependent recognition motifs exist with significantly different consensus sequences (Aitken, 2002) in addition to binding partners that do not depend on phosphorylation (Henriksson et al., 2002).

The sigma isoform of 14-3-3 is also known as stratifin (SFN), due to its high expression in stratifying keratinocytes. Leffers et al. first identified SFN as an 'epithelial marker' in Golgi apparatus, cytoplasm, and conditioned medium of cultured human keratinocytes. It's presence in these compartments suggested possible secretion of SFN by these cells (Leffers et al., 1993). Although no function was assigned to extracellular SFN, it has been unexpectedly but consistently found in the 'secretome' of various cells including human MDA-MB-231 breast carcinoma (Butler et al., 2008), mouse embryonic fibroblasts (Dean and Overall, 2007), human plasma (Anderson et al., 2004), human uveal melanoma cells (Pardo et al., 2006), activated human platelets (Coppinger et al., 2004), mouse astrocytes (Lafon-Cazal et al., 2003), dendritic cell exosomes (Thery et al., 2001), tumor-associated monocytes/macrophages (Kobayashi et al., 2009), and adult human epidermis keratinocytes (Katz and Taichman, 1999). Secretion of 14-3-3 proteins through the classical ER/Golgi-dependent pathway was unlikely due to the fact that these proteins lack the signaling peptide needed to localize to ER during synthesis. Interestingly, several other well-known extracellular proteins such as IL-1 and FGF-2 also lack such signaling peptide and non-classical mechanisms such as exosomes, ectosomes, transmembrane flipping activity, and ATP-binding cassette (ABC) transporters have been suggested for their release from cytoplasm (Nickel, 2009; Butler and Overall, 2009). Findings from our group have revealed that keratinocytes have the capacity to produce and release exosomes containing SFN as well as other six isoforms of 14-3-3. In fact, depleting exosomes

from KCM lead to a significant reduction in KCM-mediated MMP-1 expression (Chavez-Muñoz et al., 2008, 2009).

In the search for keratinocyte-derived anti-fibrogenic factors (KDAF) with collagen and MMP-1 regulatory activity in dermal fibroblasts, our group has consistently found SFN in the secretome of keratinocyte and in the affinity chromatography purified fraction that displayed MMP-1 stimulatory activity. It was later shown that purified recombinant SFN was also capable of inducing MMP-1 in fibroblasts (Ghahary et al., 2004). In a longitudinal study, where keratinocyte differentiation was induced by high calcium content, marked increase in SFN mRNA and protein levels were observed in the conditioned medium of differentiated keratinocytes in comparison to that of proliferating cells. In contrast to keratinocytes, fibroblasts that are highly responsive to SFN were unable to express this 14-3-3 isoform. Interestingly, SFN mRNA expression was significantly higher in keratinocyte co-cultured with fibroblasts relative to keratinocytes cultured alone (Ghahary et al., 2005). Further studies revealed the presence of all seven isoforms of 14-3-3 in cultured keratinocyte, conditioned medium, and epidermal layer of normal skin. The expression of SFN was absent in the dermal layer of skin and only low levels of ε, γ, and η were detectable in fibroblast conditioned medium (Kilani et al., 2008).

Signalling proteins in MAPK pathways (p38, JNK, ERK) are frequently activated during the induction of MMPs gene expression with the ability to turn on transcription factors such as AP-1, ETS, and C/EBP commonly found in MMPs promoter region (Vincenti and Brinckerhoff, 2007). As the role of extracellular SFN in signal transduction has never been addressed previously, our group explored the function of three MAPK pathways by utilizing specific inhibitor for each in the presence or absence of SFN stimulation. 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 (Lam et al., 2005). Further investigation revealed that SFN markedly increases the expression of Elk4/Sap1 and c-fos/c-jun transcription factors which are components of AP-1 transcription complex. The rapid and transient expression of these signaling molecules following SFN stimulation implied that extracellular SFN may function as a ligand in a receptor-mediated transmembrane signaling event. While many isoforms of 14-3-3 have been reported to associate with the cytoplasmic domains of plasma membrane receptors such as IGF-1 receptor (Craparo et al., 1997), BP180 or Collagen XVII (Li et al., 2007), TGFβ receptors (McGonigle et al., 2001) and EGF receptors (Oksvold et al., 2004), characterization of a functional cell surface receptor had not yet been reported for extracellular SFN prior to identification of aminopeptidase N (APN) or CD13 described in chapter 4 of this thesis.

Aminopeptidese N

Aminopeptidase N (APN) or CD13 is a 150 kDa type II membrane protein and an ecto-metalloproteinase belonging to the M1 family. In humans, its gene (ANPEP) is located on chromosome 15q25-q26 and it is widely expressed in a variety of cells such as myeloids, fibroblasts, renal and intestinal epithelial, and endothelial cells (Favaloro, 1991; Nomura et al., 2004; Riemann et al., 1999). Structurally, APN consists of a seven-domain organization with a short cytoplasmic domain I (8 amino acid residues) and an active site between domain V and VI (Sjöström et al., 2000).

APN has been referred to as a ‘moonlighting’ protein due to its wide range of functions such as the enzymatic regulation of peptides, viral receptor, tumor-homing

peptide receptor, tumor cell invasion, proliferation and apoptosis, motility, antigen presentation, cholesterol uptake, and signal transduction (Mina-Osorio, 2008). It is important to note that some of these functions are independent of APN's enzymatic activity. Activation of signal transduction pathways has been proposed as a potential mechanism of action for APN's role in homotypic aggregation of monocytes (Mina-Osorio et al., 2006). It was found that ligation of APN with monoclonal antibodies induces a rapid and transient calcium influx as well as phosphorylation of ERK, JNK, and p38 MAPK in monocytes (Santos et al., 2000). In addition, antibodies with no effect on enzymatic activity of APN exhibit a high capacity to induce cell adhesion in monocytes and this effect can be inhibited by SB203580, a p38 MAPK inhibitor. In the same study, Mina-Osorio and colleagues demonstrated the co-immunoprecipitation of Grb2 and Sos with APN from lysate of monocytic cells, suggesting a possible link between APN, Ras, and the ERK1/2 MAPK signaling pathway (Mina-Osorio et al., 2006).

In skin, APN or CD13 in combination with CD34 and collagen have been used as a cell surface marker for fibroblast-like peripheral blood-borne cells called fibrocytes commonly found at the site of dermal injury (Abe et al., 2001; Bucala et al., 1994). However, Sorrell and colleagues have demonstrated that the frequency and distribution of these cells do not account for the APN pattern in adult skin, underlining fibroblasts as another potential source of APN (Sorrell et al., 2003). The direct function of APN in wound healing has not previously been investigated. However, Mori et al. demonstrated an increase in the APN levels in wounded versus uninjured skin of balb/c mice (Mori et al., 2005). The findings presented here in chapter 4, identified a novel function for APN by suggesting it acts as a cell surface receptor in SFN-mediated activation of p38 MAPK and MMP-1 expression in dermal fibroblasts.

Hypotheses and Specific Objectives

Interactions between epithelial and stromal cells in skin plays an important role in regulation of ECM production and remodeling during development and wound repair in adult tissue. We had previously shown that keratinocyte-releasable stratifin acts as a potent MMP-1 stimulatory factor in dermal fibroblasts. Evidence also suggested that collagen type I expression in dermal fibroblasts is mediated by unidentified paracrine factors secreted from keratinocytes. To further investigate the role of stratifin in regulation of ECM components and to characterize potential keratinocyte-derived collagen inhibitory factors, we tested the hypothesis #1 and specific objectives described below.

Previous studies suggested a link between stratifin and p38 MAPK, Elk4/Sap1, and c-jun/c-fos transcription factors in induction of MMP-1 in dermal fibroblasts. This pointed toward a potential transmembrane signaling mechanism for stratifin-mediated MMP-1 expression. Preliminary results from cell- and receptor-binding assays also demonstrated a specific interaction between stratifin and fibroblasts cell surface. To investigate the nature of a potential stratifin cell surface receptor involved in p38 MAPK activation and MMP-1 induction, we tested the hypothesis #2 and specific objectives stated below.

Hypothesis 1

We first hypothesized that keratinocyte-releasable factors modulate the expression of MMPs as well as type I collagen in dermal fibroblasts. To address this hypothesis, two specific objectives were pursued as described below.

Objective 1.1

To address our hypothesis, in chapter 2, we pursued to outline the expression profile of ECM components in dermal fibroblasts treated with stratifin or co-cultured with human keratinocytes using an ECM pathway-specific gene array technology. Our findings revealed that stratifin is capable of inducing a wider range of MMPs such as MMP-3, 8, 10, and 24 in addition to MMP-1.

Objective 1.2

In order to examine the nature of keratinocyte-derived paracrine factors with collagen inhibitory activity, we treated dermal fibroblasts with keratinocyte-conditioned medium under various conditions. Our result, described in chapter 3, further confirmed the presence of a keratinocyte-derived collagen inhibitory factor(s) with a molecular weight of 30 – 50 kDa as well as stable activity at high temperature (56^{o}C) and acidic pH (pH=2, 30 min). The collagen inhibitory activity was present in conditioned medium collected from both basal and differentiated keratinocytes.

Hypothesis 2

Secondly, we hypothesized the presence of a stratifin cell surface receptor on dermal fibroblasts with links to signal transduction pathways leading to p38 MAPK activation and MMP-1 expression. The following objective describes our approach in addressing this hypothesis.

Objective 2.1

To purify an unknown cell surface binding protein for recombinant stratifin, in chapter 4 we describe an integrative approach which included biotin-labeling of cell surface protein ectodomains, serial affinity column purification with stratifin-Sepharose and Avidin-Agarose beads, and mass spectroscopy peptide sequencing analysis. This strategy led us to the identification of aminopeptidase N (APN) or CD13 as a specific and high-affinity binding integral protein for stratifin ligand. Our finding was further confirmed by co-IP, cross-linking analysis, and co-distribution by immunofluorescence. The in vivo expression and co-distribution of APN and stratifin was also confirmed in a rabbit ear fibrotic model as well as a longitudinal wound healing model in rats. In order to examine the link between APN and signal transduction pathway activated by stratifin, an RNA interference approach was used. The transient knockdown of APN blocked stratifin-mediated p38 MAPK phosphorylation and MMP-1 induction. The binding affinity between APN and various stratifin deletion fragments was examined to narrow down the APN binding site on stratifin ligand. The role of phosphorylation in APN/stratifin binding was also investigated.

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Chapter 2. Fibroblast Extracellular Matrix Gene Expression in Response to Keratinocyte-Releasable Stratifin[1]

Introduction

The ability to generate or repair injured tissue is vital to the continuity of human life. A fine balance between synthesis and degradation of extracellular matrix (ECM) is a key factor in repair of injured skin as well as maintenance of its structural integrity. Epidermal-mesenchymal communication is critical in exchanging the information between keratinocytes and fibroblasts in order to maintain the balance between ECM synthesis and breakdown in integument (Johnson-Wint, 1980). It is well established that any delay in epithelialization by keratinocytes increases the frequency of developing fibrotic conditions, as seen in hypertrophic scarring, characterized by excessive ECM deposition and altered remodeling (Ghahary et al, 2005).

MMPs represent a group of diverse proteolytic enzymes involved in ECM turnover and connective tissue remodeling during embryonic growth and development, bone growth and resorption, tumor metastasis, and wound healing (Nagase and Woessner, Jr., 1999). The MMP family consists of 23 zinc- and calcium-dependent proteinases in the mammalian system. According to their substrate specificity, primary structure, and cellular localization, these enzymes can be divided into at least five different subfamilies of closely related members known as collagenases, gelatinases, stromelysins, matrilysins, and membrane-type MMPs (Murphy et al 2002). Collectively, MMPs are capable of degrading essentially all components of ECM (Kahari and Saarialho-Kere, 1997), including type I, III, IV collagen, fibronectin, elastin, and

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proteoglycans which are commonly over-expressed in fibrotic tissues (Scott et al, 2000). It has been shown that ECM not only acts as a support for resident cells, it also provides a reservoir for embedded growth factors and cytokines. Cell surface ECM receptors allow cells to sense their microenvironment and react to stimuli (Schenk and Quaranta, 2003). Therefore, MMPs not only promote tissue remodeling by precise cleavage of ECM components but also indirectly influence cellular behavior by liberating bioactive fragments and changing ECM architecture (Mott and Werb, 2004). An imbalance in expression of MMPs has been implicated in a number of pathological conditions such as dermal fibrosis (Ghahary et al, 1996), rheumatoid arthritis, atherosclerosis, pulmonary emphysema, and tumor invasion and metastasis (Birkedal-Hansen et al, 1993; Nagase and Woessner, Jr., 1999).

Epidermal-mesenchymal interactions play a critical role in controlling the expression of MMPs during development and healing of skin. An increase in MMPs expression has been implicated with tissue degradation and remodeling during tumor invasion and wound healing. In both conditions, cell interactions between either fibroblasts and tumor cells or fibroblasts and keratinocytes results in an increase in MMP production (Ghahary et al, 2004; Folgueras et al, 2004). Several studies on the effect of keratinocyte-conditioned medium (KCM) on dermal fibroblasts and keratinocyte-fibroblast co-culture systems revealed epidermal growth factor (EGF), interleukin-1alpha (IL-1α) and interleukin-1beta (IL-1β) as potential mediators of fibroblast MMP-1 expression (Mackay et al, 1992;Moon et al, 2001). Further studies showed that MMP-1 production in dermal fibroblasts was only partially inhibited by IL-1 receptor antagonists (Moon et al, 2001) and EGF plays a very small role in upregulation of this protease (Moon et al, 2002; Stoll et al, 1997). Therefore, the

precise nature of epidermal-mesenchymal interactions and keratinocyte-derived factors that influence fibroblast MMP production remains to be elucidated.

Stratifin is a member of a large family of highly conserved dimeric 14-3-3 proteins expressed ubiquitously in all eukaryotic cells. Although more than 100 proteins have been reported to interact with intracellular members of 14-3-3 family (Pozuelo et al, 2004), functional activities of the releasable forms of these proteins in general and 14-3-3 σ, in particular, are completely unknown. In fact, the presence of 14-3-3 proteins in cerebral spinal fluid (Satoh et al, 1999) as well as keratinocyte-conditioned medium (Katz and Taichman, 1999) has been previously reported, without designating any function. Our group has recently identified and characterized a keratinocyte-releasable anti-fibrogenic factor (KDAF) for dermal fibroblasts, which was discovered to be a releasable form of stratifin. Both KDAF and recombinant stratifin demonstrated potent MMP-1 stimulatory activity in dermal fibroblasts (Ghahary et al, 2004; Ghahary et al, 2005). In this study, we further investigated the regulatory role of keratinocyte-releasable stratifin on fibroblast ECM expression using a microarray approach. Our data revealed that, in addition to upregulation of MMP-1, stratifin significantly increased the expression of other fibroblast MMPs, including stromelysin-1 (MMP-3), which is essential in ECM and scar remodeling.

Materials and Methods

Clinical Specimens and Cell Culture

Following informed consent, skin punch biopsies were obtained from patients undergoing elective reconstructive surgery, under local anesthesia, according to a protocol approved by the University of Alberta Hospitals Human Ethics Committee. Biopsies were collected individually and washed three times in sterile Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, New York) supplemented with antibiotic–antimycotic preparation (100 mg/ml penicillin, 100 mg/ml streptomycin, 0.25 mg/ml amphotericin B) (Gibco). Specimens were dissected free of fat and minced into small pieces less than 0.5 mm in diameter, washed six times with DMEM, and distributed into 60x15 mm Petri dishes. Cultures of fibroblasts were established as previously described (Karimi-Busheri et al, 2002). Upon reaching confluence, the cells were released by trypsinization, split for subculture at a ratio of 1:6, and reseeded into 75 cm^2 flasks. Fibroblasts at passages 3–7 were used in all experiments conducted in this study.

To establish cultured keratinocytes, the procedure of Rheinwald and Green (1975) was used for cultivation of human foreskin keratinocytes using serum-free keratinocyte medium (Gibco) supplemented with bovine pituitary extract (50 mg/ml) and EGF (5 ng/ml) (Rheinwald and Green, 1975). These additives were used only to establish keratinocytes in cultures. Thus, to eliminate any effects of EGF and/or pituitary extract on our findings, obtained from co-culturing keratinocytes with fibroblasts, keratinocyte serum-free medium (KSFM) supplemented with EGF and pituitary extract was exchanged with our test medium consisting of 49% DMEM, 49% KSFM, and 2% FBS with no additives, a medium in which keratinocytes undergo

differentiation with time. In this system, both keratinocytes and fibroblasts remain viable and any factor released from keratinocytes can diffuse through the 0.4 mm porous membrane separating the two chambers. Primary cultured keratinocytes at passages 3–5 were used.

Keratinocyte / Fibroblast Co-culture System

In order to study the effect of 14-3-3 proteins on fibroblasts during keratinocyte-fibroblast cross-talk, a previously established (Karimi-Busheri et al, 2002; Ghahary et al, 2004) keratinocyte/fibroblast co-culture system in which keratinocytes and fibroblasts were grown in the upper and lower chambers of the system, respectively was used. For the co-culture experiments, 30 mm Millicell-CM (Millipore) culture plate inserts with 0.4 mm pore size were coated with fetal bovine skin collagen (3 mg/ml). Subsequently, 0.5 x 10^6 keratinocytes were seeded on the collagen-coated inserts with KSFM supplemented with BPE (50 mg/ml) and EGF (5 mg/ml). In a separate experiment, 0.5 x 10^6 fibroblasts were seeded in each well of a six-well culture plate containing DMEM with 10% FBS. The cells were incubated in a cell culture incubator for 24 h and the conditioned medium was collected and cells were washed with phosphate-buffered saline (PBS). The co-culture system was then assembled, with the upper chamber being the collagen-coated insert with keratinocytes and the bottom chamber being the fibroblasts grown on a plastic six-well plate. The controls were inserts either alone, with keratinocytes, or with fibroblasts. Each chamber received 2.5 ml of our test medium consisting of 49% KSFM without additive and 49% DMEM plus 2% FBS. As only a permeable membrane separates these cells, fibroblasts in the lower chamber can be exposed to any soluble factor that may be released from keratinocytes. As an index for the anti-fibrogenic effects of keratinocyte-derived factor(s) on dermal fibroblasts, total RNA was extracted from fibroblasts grown in the

lower chamber and the expression of collagenase mRNA was evaluated by Northern analysis.

Preparation of Human Recombinant Stratifin (14-3-3 σ)

Procedures for human recombinant 14-3-3σ protein preparation were established as described previously (Ghahary et al, 2004). Briefly, the cDNA of 14-3-3σ from human keratinocytes was cloned into pGEX-6P-1 expression vector (Amersham / Pharmacia Biotech, Piscataway, New Jersey) and transformed into protein expressing bacteria, BL-21(DE3) (Novagene, Madison, Wisconsin). To purify the protein, bacteria were centrifuged and lysed with 50 mM Tris-HCl (pH 7.4) containing 10 mM EDTA, 5 mM EGTA, protease inhibitor cocktail (Sigma), 1% Triton X-100, and 0.5% IGEPAL CA630. GST-fused 14-3-3σ was purified by using a glutathione sepharose-4B affinity column and subsequently digested using PreScission protease according to the manufacturer's procedure (Amersham/Pharmacia Biotech). To evaluate the efficacy of our recombinant protein, confluent culture of fibroblasts were treated with various concentrations (0, 0.5, 1.0, 2.0, 4.0 ug/ml) of GST-fused 14-3-3 σ, GST-free 14-3-3σ, and GST alone. Cells were then harvested at 24 h and the expression of MMP-1 mRNA was evaluated by Northern blot analysis according to procedure described here. Furthermore, colorimetric methyl thiazolyl tetrazolium (MTT) assay was used to evaluate the effect of recombinant 14-3-3σ on fibroblasts' proliferation. Experiments were performed at least three times with similar results.

RNA Isolation and Northern Blot Analysis

Fibroblasts were harvested with 400 ml of 4 M guanidium isothiocyanate (GITC) solution, and total RNA from each group was isolated by the acid–guanidium–phenol–chloroform method (Ghahary et al, 1998). Total RNA from each individual fibroblast culture was then separated by electrophoresis (10 μg/lane) and was blotted onto a

nitrocellulose membrane (Bio-Rad Laboratories, Hercules, California). Following 2 h incubation in prehybridization solutions, blots were hybridized using MMP-1, stromelysin-1 (MMP-3), or 18S ribosomal RNA radioisotope labeled cDNA probes. Autoradiography was performed by exposing a Kodak X-Omat film to nitrocellulose filters at -20^{o}C in the presence of an intensifying screen. Each experiment was performed at least twice to ensure reproducibility of the results. The cDNA probes for MMP-1 and 18S ribosomal RNA were obtained from the American Type Culture Collection (Rockville, Maryland). The cDNA probe for stromelysin-1 was obtained by extracting fibroblast total RNA and was amplified by RT-PCR. The PCR product was then purified and ligated into a pGEX-6P-1 vector (Amersham/Pharmacia Biotech). The ligated products were then transformed to competent DH5α cells with the regular heat-shock transformation method. Positive clones were identified by the size of restriction enzyme-digested products. DNA sequence was confirmed by fluorescence dNTP sequence analysis.

Detection of Extracellular 14-3-3 σ and MMP Proteins by Western Blotting

To determine whether the release of stratifin (14-3-3σ) is unique to keratinocytes, fibroblasts, or both, conditioned media from fibroblast/fibroblast (F/F), keratinocyte/fibroblast (K/F), and keratinocyte alone (K) co-culture systems were collected for 48 h. It should be noted that the original KSFM media supplemented with BPE and EGF was replaced with test medium without FBS. FBS was removed as serum proteins interfere with electrophoresis of proteins present in conditioned medium.

To evaluate MMP-1 and MMP-3 production in dermal fibroblast, original conditioned media was replaced with FBS-free DMEM following overnight incubation and cells were treated with various concentrations of recombinant stratifin for 24 h and

conditioned medium collected for analysis. All samples of conditioned medium were centrifuged to pellet the debris and passed through a Centricon filter device (Millipore) with molecular weight cut of 5 kDa. Protein content of concentrated conditioned medium was determined and an equal amount (50 μg/sample) of total protein was subjected to SDS-PAGE analysis with 12% (wt/vol) acrylamide gel, and electro-transferred onto polyvinylidine difloride membranes (Millipore). Non-specific proteins on membranes were blocked in 5% skim milk powder in PBS–0.1% Tween-20 overnight. Immunoblotting was performed using polyclonal goat anti-human 14-3-3σ (Santa Cruz Biotechnology, Santa Cruz, USA), monoclonal mouse anti-human MMP-1 (R&D Systems), and monoclonal mouse anti-human MMP-3 (R&D Systems, Minneapolis, USA) antibodies. The membranes were then incubated with the appropriate secondary horseradish peroxidase-conjugated anti-goat IgG (Sigma, St Louis, USA) or anti-mouse IgG (Bio-Rad Laboratories, Hercules, USA) antibodies (1:2500 dilution). Immunoreactive proteins were then visualized using the ECL-plus western blotting detection system (Amersham Biosciences, Buckinghamshire, England).

Gene Expression Analysis by ECM-specific Microarray

To examine whether keratinocytes-derived or recombinant stratifin induced other ECM genes in dermal fibroblasts, GEArray pathway-specific gene expression array systems were purchased from SuperArray Bioscience Corporation (Bethesda, Maryland). Each GEArray Q Series Human ECM and Adhesion Molecules gene array consists of 96 genes known to be involved in cell adhesion, ECM deposition and degradation, as well as control sequences (PUC18 plasmid DNA as negative control; β-actin, PPIA, and glyceraldehydes 3 phosphate dehydrogenase (GAPDH) for loading). Using different arrays, we compared the gene expressions of keratinocyte-co-cultured, stratifin-treated, and untreated dermal fibroblasts. For these experiments, total cellular

RNA was isolated by a modification of the GITC technique. The integrity of RNA was assessed by visualization of ethidium bromide-stained gels and it was ensured that 260/280 nm absorbance ratio does not exceed 1.8. The microarrays were used according to the manufacturer's instructions. In brief, using the reagents provided, cDNA was prepared from total RNA by reverse transcription with MMLV reverse transcriptase, biotinylated with Biotin-16-dUTP (Roche, Indianapolis, Indiana), and then hybridized under precisely specified conditions to a positively charged nylon membrane containing the arrayed DNA. The arrays were visualized using the chemiluminescent detection system purchased from GEArray (Bethesda, Maryland). Loading was adjusted based on the intensity of hybridization signals to the housekeeping gene, PPIA, and then gene expression was quantified by scanning densitometry.

Reversed Transcriptase - Polymerase Chain Reaction (RT-PCR)

To validate the variations observed in MMP gene expression in the mircoarray experiment, a multigene RT-PCR profiling system was utilized. Total RNA was isolated from either monolayer of untreated fibroblasts (control), cells treated with 2.0 μg/ml stratifin (14-3-3σ), or co-cultured with keratinocytes (K/F). A260/A280 nm did not exceed 1.8 and integrity of RNA sample was checked by visualization of ethidium-bromide 28S and 18S bands. A total of 5 μg from each sample was reverse transcribed using the first-strand cDNA synthesis kit from SuperArray (Frederick, MD, USA). RNA samples from three separate experiments were pooled for use in each cDNA synthesis reaction. Samples were then processed for PCR using the MultiGene-12 reverse transcriptase PCR profiling kit for 11 human MMP genes plus a human GAPD housekeeping gene (SuperArray, Frekerick, MD) according to the manufacturer's instructions. Following completion of PCR, 10 μl of each sample was separated by agarose gel electrophoresis and stained and scanned as a digital image.

Results

Efficacy of Stratifin on MMP-1 mRNA Expression and Fibroblast Proliferation

It was previously shown that the recombinant stratifin protein produced in bacteria is more than 95% pure and has strong MMP-1 stimulatory effect in fibroblasts (Ghahary et al, 2004). To ensure that recombinant Glutathione-S-transferase (GST) or any residual bacterial product is not contributing to MMP-1 expression, confluent cultures of fibroblasts were treated with various concentrations of GST, GST-stratifin fusion protein, or stratifin (0, 0.5, 0.1, 2.0, 4.0 μg/ml) for 24 h (Fig 2.1A). The results showed a marked increase in the expression of MMP-1 mRNA in treated fibroblasts for both GST-stratifin fusion protein and purified stratifin. This increase reached significance at around 1.0 μg/ml of stratifin used. No significant changes in MMP-1 mRNA expression was observed when cells were treated with GST alone, confirming that the MMP-1 stimulation is caused by stratifin alone. In addition, proliferation of dermal fibroblasts was monitored by MTT incorporation assays. Various concentrations of exogenous recombinant stratifin did not affect the proliferation rate of fibroblasts in culture, indicating the MMP expression in cells is not stress induced (Fig 2.1B).

Detection of Stratifin in Keratinocyte / Fibroblast Co-culture System

To determine if keratinocyte-releasable stratifin in the upper chamber diffused through collagen coated membrane, conditioned medium from the lower chamber was collected at 48 h and analyzed by western blot. Pooled conditioned media were concentrated about 30 times through a Centricon filter (5 kDa cut-off) and 10 μl of each sample was loaded on the gel. Various amounts of recombinant stratifin (0, 0.2, 0.5, 1.0 μg) were run on the SDS gel as standard. As shown in Figure 2.2A, only keratinocytes co-cultured with fibroblasts (K/F) released significant amount of stratifin. Since the 0.4

μm membrane pores coated, our results indicate that stratifin released from keratinocytes can diffuse through epidermis-dermis barrier to reach its target fibroblasts in the dermis. Based on the density of standard, loaded volume of protein, and concentration factor, the physiological level of stratifin was estimated to be 1.6 μg/ml of conditioned media in our co-culture system. No stratifin was detected in fibroblast/fibroblast co-culture condition medium. To measure the activity of stratifin on dermal fibroblasts, total RNA was extracted from fibroblasts co-cultured with keratinocytes (K/F) or treated with 2.0 μg/ml of the recombinant protein (σ). The expression of MMP-1 mRNA was evaluated by Northern blot (Fig 2.2B). MMP-1 mRNA expression was significantly increased in both groups, whereas little or no detectable level of MMP-1 mRNA was found in fibroblast/fibroblast co-culture (F/F) or monolayer fibroblasts (C). To further confirm the efficacy of our recombinant protein, the levels of extracellular MMP-1 protein in fibroblasts stimulated with different doses of stratifin (0, 0.5, 1.0, 2.0, 4.0 μg/ml) was observed by western blot. Figure 2.2C shows significant increase in MMP-1 protein levels in the conditioned medium at 1.0-2.0 μg/ml after 24 h of treatment.

Microarray Analysis of Fibroblast ECM Gene Expression

Results shown in Figure 2.3 display cDNA array results of human ECM and adhesion molecules gene expression in K/F co-culture system (panel A) and stratifin-treated fibroblasts (panel B). In both sets, total RNA was pooled from 3 independent experiments for cDNA preparation by RT-PCR and microarray hybridization. Final expression was normalized for cyclophilin A (PPIA). Genes with more than 2-fold increase or decrease in their expression were considered significant in selection

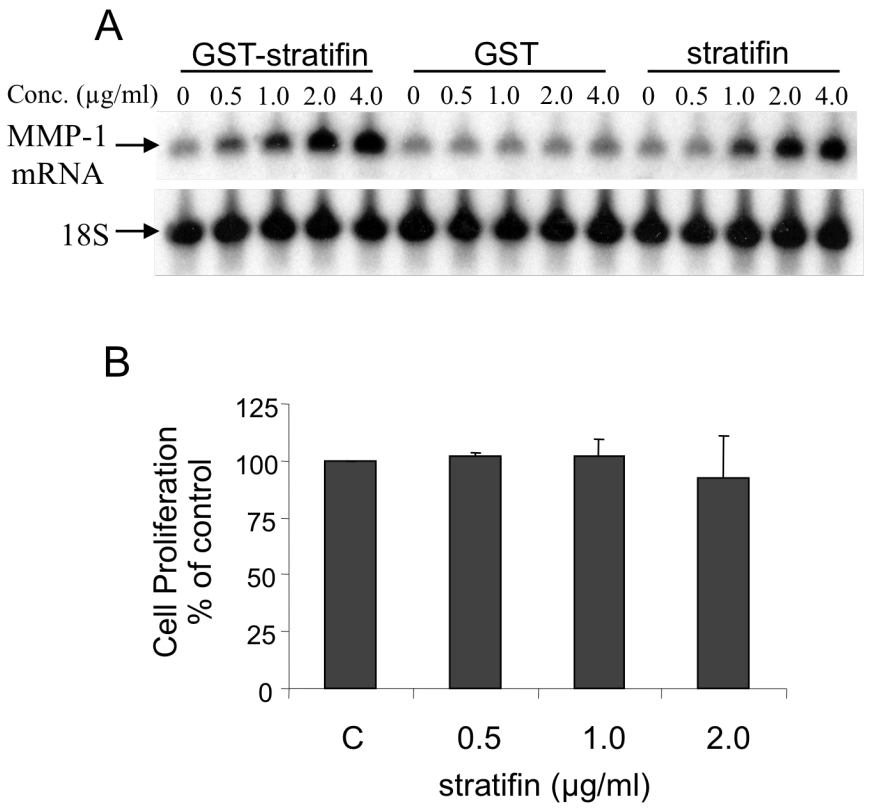


Figure 2.1. Recombinant stratifin protein increases the expression of MMP-1 mRNA in dermal fibroblasts.

Cells were treated with various concentrations (0, 0.5, 1.0, 2.0, 4.0 µg/ml) of GST-stratifin, GST, or purified stratifin for 24 h. Expression of MMP-1 and 18S ribosomal RNA (loading control) was assessed by Northern Analysis (panel A). To evaluate any potential cytotoxicity from the recombinant stratifin, confluent cultures of dermal fibroblasts were treated with either nothing or various concentration of purified protein for 24 h. The results of an MTT proliferation assay are presented in panel B as a relative percentage of the control group (untreated cells). Data represent means ± standard deviations for three separate experiments. Statistical significance ($\rho < 0.05$) was tested with Student unpaired two-tailed t test.

criteria. Microarray experiments were repeated twice with the representative images shown in figure 2.3. Comparison of 96 ECM genes in K/F showed increased expression of 12 genes compared with F/F, while the expression of 4 genes decreased (table in Fig 2.3, panel A). Majority of upregulated ECM genes in co-cultured fibroblasts belong to the MMP family.

There were 10 genes specific to key ECM components in dermal fibroblast that showed an increase by stratifin (2.0 μg/ml 14-3-3σ) treatment (Fig 2.3, panel B), with 2 genes demonstrating a significant decrease. Interestingly, as observed with fibroblasts co-cultured with keratinocytes, majority of ECM genes upregulated in stratifin-treated fibroblasts also belonged to the MMP family. Significant increase in expression of MMP-1, stromelysin-1 (MMP-3), stromelysin-2 (MMP-10), neutrophil collagenase (MMP-8), and membrane type MMP-24 (MT5-MMP) were observed in both K/F and stratifin-treated dermal fibroblasts.

In order to confirm the cDNA array findings, the level of expression of 12 selected MMP genes was evaluated by RT-PCR. As shown in figure 2.4, PCR analysis demonstrated significant increase in expression of MMP-1, -3, -8, and 24 in both K/F and stratifin-treated fibroblasts. MMP gene expression in untreated fibroblasts was used as experimental control and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the housekeeping gene for normalizing expression.

Effect of Stratifin on Expression and Secretion of MMP-3 by Fibroblasts

To further confirm some of the findings by cDNA array and RT-PCR, we examined the effect of stratifin on stromelysin-1 (MMP-3) expression and production in

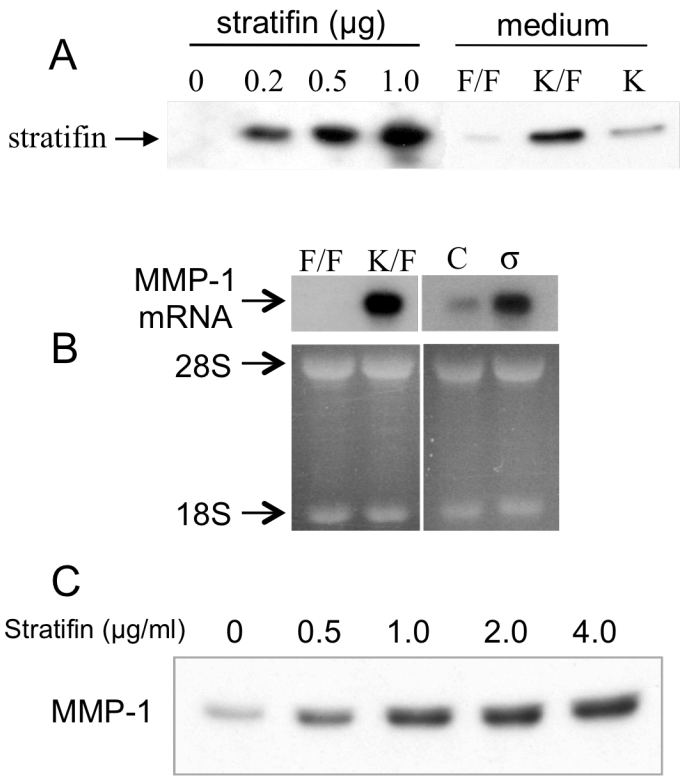
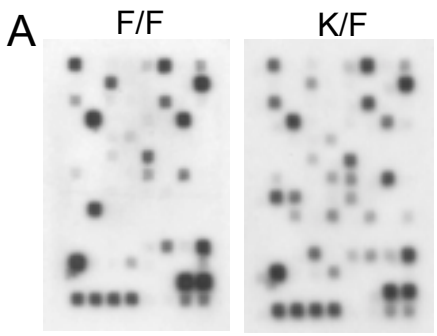


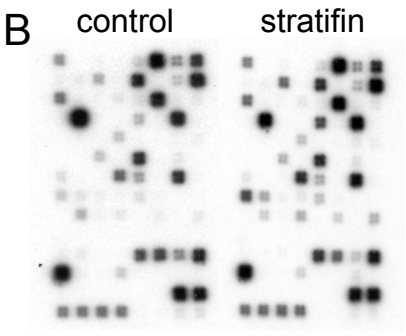
Figure 2.2. Physiological level of stratifin released by keratinocytes in a co-culture system.

Conditioned medium from fibroblast (F/F), fibroblast co-cultured with keratinocyte (K/F), or keratinocyte alone (K) was collected from the lower chamber following 48 h of incubation. Purified stratifin (0, 0.2, 0.5, 1.0 μg) was used as a standard in western blot analysis of concentrated conditioned medium (panel A). To validate the activity of releasable stratifin, expression of MMP-1 mRNA was analyzed in the co-culture system as well as fibroblasts treated with recombinant protein. As shown in panel B, fibroblasts co-cultured with keratinocytes in the upper chamber (K/F) and cells treated with stratifin (2.0 μg/ml) showed significant increase in MMP-1 expression compared with their corresponding controls (F/F and C, respectively). Pattern of 28S and 18S ethidium bromide-stained ribosomal RNA were used as a loading control. Panel C shows western blot analysis of MMP-1 secretion in fibroblast treated with various concentrations of stratifin. Fibroblast conditioned medium was concentrated by 5 kDa Centricon filter and 50 μg of total protein was loaded for each sample.

fibroblasts at the protein level. Confluent cultures of fibroblasts were either treated with nothing or various concentrations of stratifin (0.5, 1.0, 2.0, 4.0 μg/ml) for 24 h. Conditioned media from untreated or treated fibroblasts were collected, concentrated, and subjected to western blot analysis. The result of the dose-response experiment showed that stratifin stimulates secretion of MMP-3 protein in fibroblasts in a concentration-dependent fashion (Fig 2.5A). Significant increase in extracellular MMP-3 protein was detected in fibroblasts conditioned media starting at 0.5 μg/ml of stratifin and reached a peak at 2.0 μg/ml. Fibroblasts were also treated with 2.0 μg/ml of stratifin for different periods of time, and the release of MMP-3 into conditioned medium was determined by western blot analysis. The results showed a marked increase in MMP-3 protein level as early as 24 h post treatment and it reached its maximum following 48 h incubation with stratifin (Fig 2.5B). In another experiment, the lasting effect of stratifin in MMP-3 expression and production in dermal fibroblasts was evaluated. Following the initial treatment with 2.0 μg/ml stratifin for 24 h, original conditioned media was collected and replaced with fresh culture media. The results showed a marked increase in expression of MMP-3 in fibroblasts at 24 h after treatment. Following removal of stratifin, the expression remained high for another 24 h and then gradually reduced to normal levels within 96 h (Fig 2.6A). Level of MMP-3 protein released into fibroblast condition medium collected at different time points after removal of stratifin demonstrated a similar pattern to its gene expression (Fig 2.6B). The effect of stratifin on MMP-3 production in fibroblasts was also studied using various strains of primary fibroblasts harvested from different patients. All 4 different strains of fibroblasts demonstrated higher levels of MMP-3 in their conditioned medium following treatment with 2.0 μg/ml of stratifin for 24 hr (data not shown).



Pos	Gene Name	Fold Increase
22	Cathepsin L	2.5
45	Integrin β1	1.6
55	Hyaluronidases	3.0
57	Collageanse-1	12.0
58	Stromelysin-2	8.0
61	Collagenase-3	4.0
68	MMP-24	5.0
70	Stromelysin-1	6.0
72	Neutrophil Collagenase	3.5
83	PAI-2	12.0
87	Osteopontin	3.0
92	TIMP-2	2.0
11	Collagen type I, α1	0.4
26	Fibronectin-1	0.7
33	Integrin α11	0.5
86	SPARC	0.6



Pos	Gene Name	Fold Increase
29	Integrin α1	2.5
52	Integrin β8	2.0
55	Hyaluronidases	2.0
57	Collageanse-1	4.0
58	Stromelysin-2	2.5
68	MMP-24	3.0
70	Stromelysin-1	2.0
72	Neutrophil Collagenase	4.0
85	PAI-1	2.5
88	TSP-1	2.0
26	Fibronectin-1	0.5
86	SPARC	0.7

Figure 2.3. Microarray analysis of mRNA extracted from keratinocyte-co-cultured or stratifin-treated fibroblasts.

Fibroblasts were either co-cultured with keratinocytes (K/F) or treated with 2.0 μg/ml of stratifin (14-3-3 σ) for 24 h in separate experiments. Fibroblast / fibroblast co-culture (F/F) or monolayer culture of fibroblast (control) were used as control for each experiment. Total RNA was extracted, reverse-transcribed, and the corresponding cDNA biotin-labeled according to the manufacturer's directions. Images in panel A (co-culture) and panel B (stratifin-treated) demonstrated the expression profile of human ECM and adhesion molecules in dermal fibroblasts. The tables below each image indicate the fold increase of ECM gene expression relative to the control in each group. Cyclophilin A (PPIA) expression was used as negative control.

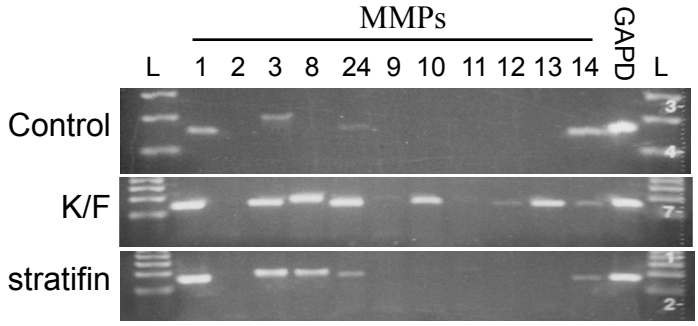


Figure 2.4. Stimulation of MMP gene expression in dermal fibroblast determined by RT-PCR.
Fibroblast were treated either with nothing (control), co-cultured with keratinocyte (K/F), or stratifin (14-3-3 σ) and were then harvested for total RNA. Three microgram of RNA was characterized using a customized human MMP gene family RT-PCR profiling kit (SuperArray). The resulting PCR products were characterized by agarose gel electrophoresis. From left to right, L: ladder; 1: collagenase; 2: gelatinase A; 3: stromelysin-1; 8: neutrophil collagenase; 24: MT5-MMP; 9: gelatinase B; 10: stromelysin-2; 11: stromelysin-3; 12: macrophage elastase; 13: collagenase-3; 14: MT1-MMP; GAPD: Glyceraldehyde-3-phosphate dehydrogenase (housekeeping gene); L: ladder.

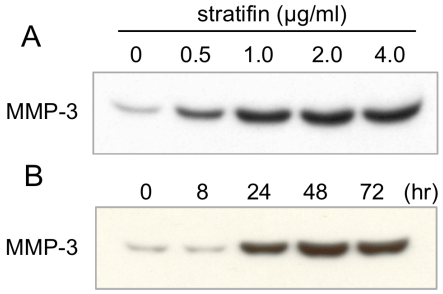


Figure 2.5. Confirmation of array results by Northern analysis and Western blot of MMP-3.
Dermal fibroblasts were treated with various concentrations of stratifin for 24 h. In panel A, fibroblast conditioned medium was collected post treatment and concentrated with a 5 kDa cut-off Centricon filter. Total protein concentration was determined using Bradford protein assay and 50 μg of each sample was subjected to SDS-PAGE analysis. In a similar experiment, fibroblasts were treated with 2.0 μg/ml of stratifin for different periods of time as indicated (panel B). The extracellular MMP-3 protein levels were determined by western blot analysis using specific antibodies.

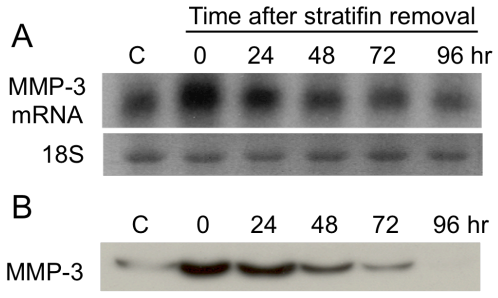


Figure 2.6. Recombinant stratifin protein increases the expression of stromelysin-1 (MMP-3) in dermal fibroblasts.
To determine the lasting effect of stratifin on MMP-3 expression, fibroblasts were treated with either nothing (c) or 2.0 μg / ml of stratifin for 24 h. the medium was replaced with fresh medium without stratifin and cells were then harvested at 0, 24, 48, 72, and 96 h post stratifin removal. Fibroblast conditioned medium was collected at each time point, concentrated using 5 kDa Centricon filter, and subjected to SDS-PAGE. The expression of MMP-3 gene and extracellular MMP-3 protein in dermal fibroblasts were then evaluated by Northern blot (panel A) and Western blot (panel B) analysis, respectively.

Discussion

The purpose of this study was to explore the regulatory effect of keratinocyte-releasable stratifin on ECM production in dermal fibroblasts. The expression of a wide range of MMPs was inducible in fibroblasts treated with the recombinant stratifin or co-cultured with primary keratinocytes. Furthermore, stromelysin-1 (MMP-3) gene expression and protein secretion was characterized in fibroblasts treated with stratifin protein. Our study showed that stratifin induced production of MMP-3 by fibroblasts in a dose- and time-dependent fashion.

Following full- or partial-thickness burns, wound healing may lead to formation of hypertrophic scars and keloids in predisposed patients. These scars are generally characterized by an overabundance of dermal collagens, proteoglycans, fibronectin, and other connective tissues, which are of altered composition and organization compared to normal skin or mature scar (Scott et al, 2000; Swann et al, 1985; Niessen et al, 1999). As fibroblasts are the main source of the excess scar tissue, most attempts have been focused on the dermal component of the scar. However, clinical evidence points toward irregularity in mesenchymal-epithelial signaling, as observed in delayed reepithelialization, as a possible mechanism for development of hypertrophic scarring (Niessen et al, 2001; Deitch et al, 1983; Ghahary et al, 2004). Wound repair involves cell proliferation, migration, and tissue remodeling. These highly orchestrated processes are facilitated by matrix-degrading proteases or MMPs. In search of keratinocyte-releasable anti-fibrogenic factors that mediate MMPs production in dermal fibroblasts, we have recently demonstrated that extracellular form of stratifin secreted by differentiated keratinocytes acts as a strong stimulator of MMP-1 in dermal fibroblasts (Ghahary et al, 2004; Ghahary et al, 2005). MMP-1, as all other

collagenases, cleaves fibrillar collagens into ¾ and ¼ fragments, which in turn denature into gelatin at body temperature (Kahari and Saarialho-Kere, 1997). However, MMP-1 degrades collagen type III more specifically, whereas MMP-8 and MMP-13, other members of the collagenase family, express more specificity toward type I and type II collagen, respectively (Knauper et al, 1996; Mitchell et al, 1996). Given that hypertrophic scars demonstrate excessive production of a wide range of fibrous proteins such as type I and III collagen, proteoglycans, fibronectin, elastin, and tenascin (Scott et al, 2000), it is unlikely that augmented expression of MMP-1 alone, independent of other proteases, can lead to optimum remodeling of the scar tissue. Our findings here suggest that extracellular stratifin released from keratinocytes possesses a potent MMP stimulatory activity for dermal fibroblasts. An increase in the expression of collagenase-1, stromelysin-1, stromelysin-2, neutrophil collagenase, and MT5-MMP, following stimulation by stratifin, can synergistically degrade type I, II, III, IV collagen, fibronectin, and proteoglycans, with high specificity, in scar tissue and promote tissue remodeling (Kahari and Saarialho-Kere, 1997;Wang et al, 1999;Dong et al, 2001;Abe et al, 2001).

Fibroblast stromelysin-1 (MMP-3) is a protease known to degrade mainly the noncollagenous portion of the ECM such as fibronectin, proteoglycans, and laminin (Kahari and Saarialho-Kere, 1997). Increase in MMP-3 expression and release by fibroblasts in response to extracellular stratifin, together with MMP-1 upregulation shown previously, can initiate degradation of almost all major components of the ECM. In fact, the results from a pilot rabbit ear hypertrophic scar study show significant ECM remodeling and reduction in size of scars treated with stratifin (data not shown). Interestingly, MMP-3 and MMP-1 expression appear to be coordinately modulated in synovial fibroblast cultures (Saus et al, 1988). Our results also support this finding, with

fibroblast MMP-1 and MMP-3 expression, at gene and protein level, showing dose- and time-dependent response to stratifin treatment. It is possible that promoter regions of these genes respond to stratifin stimuli through a common signaling pathway. Lam et al. has previously characterized that stratifin modulates fibroblast MMP-1 levels through the p38 mitogen-activated protein kinase (MAPK) signaling pathway, c-Fos gene transcription and formation of AP-1 transcription factor complex (Lam et al, 2005). The majority of inducible MMPs, including MMP-3, are regulated through AP-1 or PEA3 cis-elements in their promoter region (Gaire et al, 1994; Pendas et al, 1997). It remains to be seen if MMP-3 or other MMPs stimulated here by stratifin are also activated through the same signaling transduction pathway as MMP-1.

Our data shows a higher expression of MMP-1 mRNA in fibroblasts co-cultured with keratinocytes compared to cells treated with recombinant stratifin. This is due to the fact that stratifin is probably not the only signaling factor released by keratinocyte with collagenase-inducing activity. It has been shown that interleukin-1α (IL-1α) also stimulates matrix degradation and is mainly derived from keratinocytes during the later stages of wound healing (Moon et al, 2001;Maas-Szabowski et al, 2000). In fact, much lower epidermal IL-1α expression has been reported in hypertrophic compared with normal scars (Niessen et al, 2001). Therefore, it is not unreasonable to postulate that reduced expression of keratinocyte-derived factors such as stratifin and IL-1α, and subsequent reduction in MMP levels, may be involved in the development of dermal fibrotic conditions. Our results also reveal that fibroblasts co-cultured with keratinocytes show a significant reduction in mRNA expression of type I collagen and fibronectin, found in excess in fibrous connective tissue of hypertrophic scars or keloids (Scott et al, 2000). Whereas, cells treated with stratifin revealed a reduction in fibronectin gene expression only. It will be important to determine whether 14-3-3σ is the primary factor

released by keratinocytes that reduces fibronectin expression in fibroblasts and if the measured gene expression correlates with changes at the protein level.

Although presence of 14-3-3 proteins has been reported outside of the cell, they are generally considered to be intracellular proteins without the amino-terminal ER signal peptides. Therefore, the mechanism by which stratifin is secreted by keratinocytes has yet to be explored. We have previously assayed for the presence of lactate dehydrogenase (LDH) in the medium, as a marker for cell lysis, and established that the release of stratifin was not the result of cell lysis (Ghahary et al, 2004; Karimi-Busheri et al, 2002). Evidence indicates the cells can also release membrane vesicles, called exosomes, in the extracellular environment and a proteomic analysis revealed presence of various isoforms of 14-3-3 within these exosomes in dendritic cells (Thery et al, 2001). Studies are underway to investigate the mechanism of stratifin secretion from keratinocytes.

In conclusion, the findings of this study represent an important advance in understanding the role of keratinocyte-releasable stratifin as a potent promoter for ECM degradation and scar tissue remodeling, through stimulation of MMPs in dermal fibroblasts. Characterization of stratifin levels in normal and hypertrophic scar tissue might shed further light on the role of this factor in the development of dermal fibrotic conditions.

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Chapter 3. Keratinocyte Conditioned Media Regulates Collagen Expression in Dermal Fibroblasts[2]

Introduction

The production of extracellular matrix (ECM) in response to injury and its timely cessation are an essential part of normal healing process. Excessive ECM production often leads to deleterious fibrotic conditions such as hypertrophic scarring (HTS) and keloids in the skin (Scott et al., 2000). Type I collagen is the predominant form of collagen in human skin and is mainly produced by dermal fibroblasts (Cutroneo, 2003). Transcription of the genes encoding both collagen α1(I) and α2(I) chains have shown significant increases in severe fibrotic conditions (Uitto et al., 1979; Ghahary et al., 1996). An increasing body of evidence shows that epidermal-mesenchymal interactions during wound healing play a major role in controlling the expression of ECM components including type I collagen by dermal cells. It is well established that any delay in re-epithelialization by keratinocytes increases the frequency of developing fibrotic conditions in the skin (Deitch et al., 1983). Werner et al. reported that inhibition of keratinocyte growth factor (KGF) receptor signalling reduced the proliferation rate of epidermal keratinocytes at the wound edge, leading to dermal hyper-thickening and fibrosis in skin of transgenic mice post injury (Werner et al., 1994). In clinical setting, application of cultured epithelial auto grafts (CEAs) as well as split-thickness skin grafts have resulted in significant decreases in hypertrophic scar (HTS) formation (Wood and Stoner, 1996; Rudolph and Klein, 1973).

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With minimal direct cell-to-cell contact, keratinocyte-fibroblast communication is mainly regulated by releasable factors acting in an autocrine/paracrine loop. In fact, several studies have demonstrated the regulatory role of releasable factors within keratinocyte conditioned medium (KCM) in expression of ECM genes in dermal fibroblasts (Ghaffari et al., 2006; Harrison et al., 2005; Nowinski et al., 2004; Ralston et al., 1997). These studies suggest that following epithelialization, the presence of keratinocytes directs fibroblasts to a phenotype more suited for ECM remodeling rather than synthesis (Ghahary et al., 1995; Harrison et al., 2006). In search for keratinocyte-releasable antifibrogenic factors, our group has recently identified stratifin as a potent mediator of matrix metalloproteinases (MMPs) expression in dermal fibroblasts (Ghahary et al., 2004; Ghaffari et al., 2006). Other studies on keratinocyte-fibroblast co-culture system or KCM have also identified tumour necrosis factor-alpha (TNF-α), interleukin-1α (IL-1α), and IL-1β as mediators of MMPs in fibroblasts, confirming a potential role for keratinocytes in regulation of matrix degradation and remodeling (Mackay et al., 1992; Moon et al., 2002).

Fibroblasts harvested from keloid or HTS demonstrate higher expression of collagen in culture (Phan et al., 2002; Ghahary et al., 1993). Interestingly, normal fibroblasts also show significant increase in collagen production in the presence of keloid- or HTS-derived keratinocytes (Bellemare et al., 2005; Lim et al., 2002). However, co-culture studies on the role of normal keratinocytes in fibroblast collagen expression are controversial, demonstrating either inhibition (Harrison et al., 2006; Garner, 1998) or up-regulation (Bellemare et al., 2005; Shephard et al., 2004; Phan et al., 2002) of collagen synthesis. Although the nature of collagen mediatory factors secreted by keratinocytes is not well understood, it is clear that cellular communication at epidermal-dermal interface is important in physiological and pathological processes

of dermal healing. To explore this further, we have investigated the characteristics of potential keratinocyte-derived collagen inhibitory factors (KD-CIFs) in a keratinocyte-fibroblast co-culture as well as KCM by monitoring type I collagen expression at both mRNA and protein levels.

Results

Keratinocyte-releasable Factor(s) Inhibit Collagen Expression

We evaluated the steady state level of type I collagen in dermal fibroblasts in the presence or absence of keratinocytes in a co-culture system. Keratinocyte (upper chamber) and fibroblasts (lower chamber) were separated by a semi-permeable membrane allowing diffusion of macromolecules while preventing cell migration. As shown in Figure 3.1A, a marked reduction in the steady state level of type I collagen mRNA (COL1A1) was observed in fibroblasts co-cultured with keratinocytes (lane K/F) compared to the fibroblast-fibroblast co-culture (lane F/F). As expected, high steady state level of COL1A1 was observed in a fibroblast monolayer cell culture (F). For control of total RNA loading and quantitative analysis, all corresponding blots were re-hybridized with a cDNA specific for 18S ribosomal RNA. The signals were then quantified by densitometry and the ratio of COL1A1 mRNA/18S RNA was determined as shown in Figure 3.1B. The finding clearly indicates a marked reduction in the ratio of the steady state level of type I collagen mRNA/18S for fibroblast co-cultured with keratinocytes relative to fibroblast-fibroblast co-cultured (F/F). A similar collagen inhibitory effect was also observed in a three-dimensional skin substitute model, where fibroblasts populated in a bovine collagen lattice expressed lower levels of collagen when keratinocytes were layered on top of the lattice (Figure 3.1, panel C). To take into account the effect of keratinocyte on fibroblast's cell proliferation, we have normalized the level of type I procollagen measured in both conditioned media and cell lysate

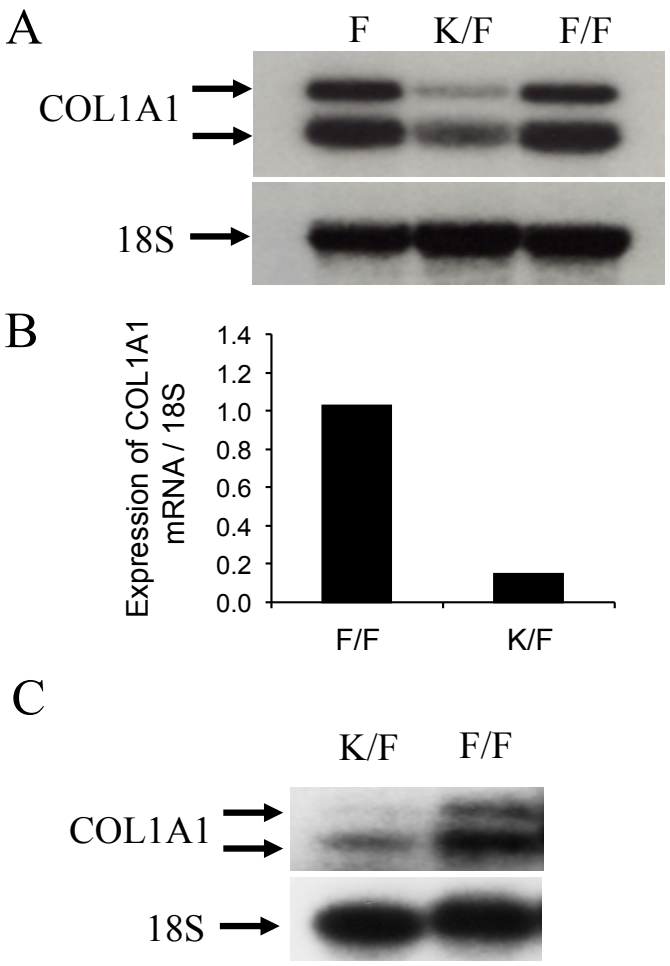

Figure 3.1. Keratinocyte-releasable factor(s) reduces the expression of type I collagen in dermal fibroblasts.

In panel A, the level of 4.8 and 5.8 kb transcripts of pro α1(I) collagen mRNA (COL1A1) in fibroblast alone (F), fibroblasts co-cultured with keratinocytes (K/F) and fibroblasts co-cultured with fibroblasts (F/F) were evaluated by northern analysis. The two COL1A1 bands represent the two different polyadenylyation sites on human collagen gene. The same blot was re-hybridized for 18S ribosomal RNA and used as a control for RNA loading. The signals were then quantified by densitometry and the ratio of COL1A1/18S was determined. The means of COL1A1/18S in K/F and F/F groups from two separate experiments were then calculated and depicted as a histogram as shown in panel B. COL1A1 mRNA expression in fibroblast populated collagen gel was evaluated in presence of a keratinocyte (K/F) or a fibroblast (F/F) top layer (panel C).

against the cell number and observed similar reductions in level of collagen per number of cell in each case (data not shown).

Keratinocyte-conditioned Medium Reduce Collagen Expression in a Time Dependent Fashion

The results of a time-response experiment revealed that KCM, similar to keratinocyte co-culture, also reduced the steady state level of collagen mRNA in dermal fibroblasts after 24 hrs of treatment (Figure 3.2A). The steady state levels of 18S ribosomal RNA in the same samples was also evaluated and used as a loading control. To determine whether changes in mRNA are reflected at the level of protein translation, pro α1 (I) collagen protein level was evaluated by western blot using a specific antibody. Considering the lag time between mRNA transcription and protein translation, the reduction in the steady state level of pro α1(I) collagen in 36 hrs following KCM treatment was consistent with the disappearance of the COL1A1 mRNA signal at 24 hrs. For quantitative analysis, the mean ratio ± SD of type I collagen protein/β-actin (percent of control) was determined (Figure 3.2B bottom panel). The results shown in this panel revealed a significant reduction in the level of collagen protein after 36 hrs in KCM treated cells (49.2±9.28, n=4, $p < 0.01$) relative to the controls (C-24 and C-48). These findings indicate that the expression and release of KD-CIFs by keratinocytes is independent of a co-culture system, as KCM from monolayer keratinocytes was also capable of reducing the steady state level of collagen mRNA and protein in fibroblasts.

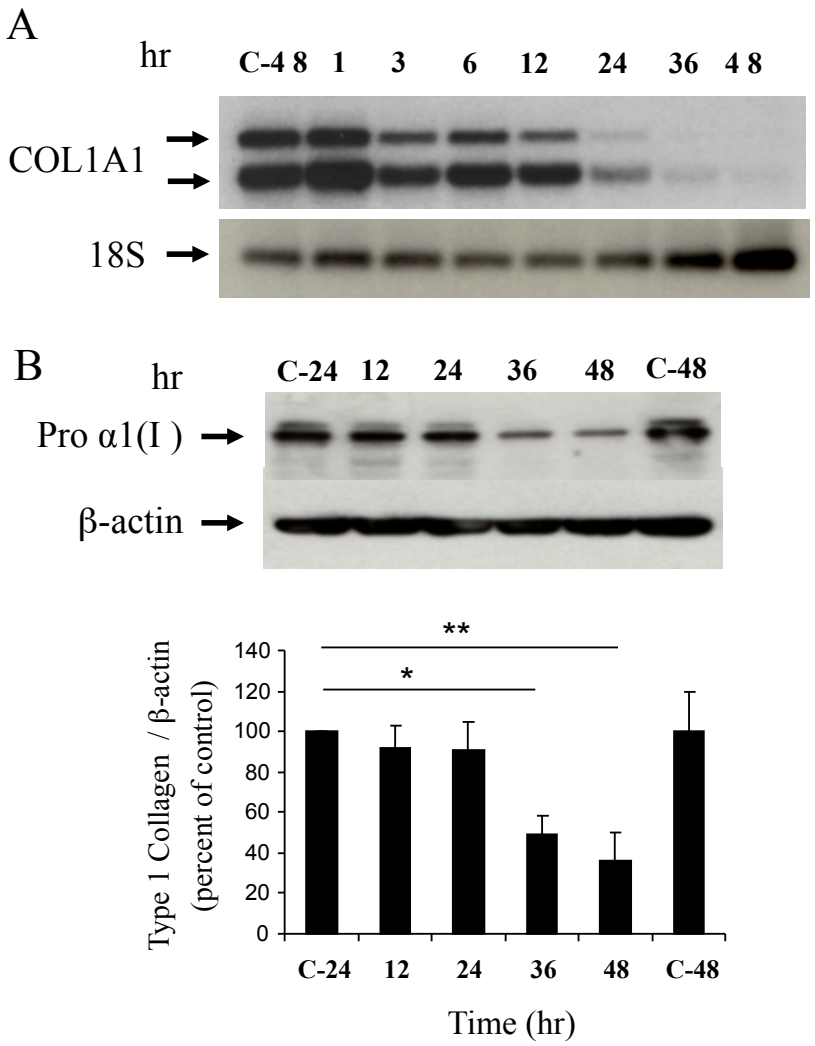

Figure 3.2. Collagen inhibitory effect of KCM in dermal fibroblasts is time dependent.

In Panel A, fibroblasts were treated with KCM for either of 1, 3, 6, 12, 24, 36 and 48 hrs. Total RNA was then extracted and subjected to northern analysis (panel A). The blots were initially hybridized with cDNA specific for COL1A1 and subsequently with cDNA for 18S ribosomal RNA used as a loading control. Panel B shows similar time-dependent experiments where fibroblasts were treated with KCM for 12, 24, 36, and 48 hrs, with control for 24 (C-24) and 48 (C-48) hrs. The level of intracellular type I procollagen protein was evaluated by western blot using β-actin expression as a loading control. For quantitative analysis, this experiment was repeated four times and the ratio of type I collagen/ β-actin (percent of control, C-24) was calculated and the mean±SD was depicted and shown in lower panel. (*) and (**) represent a $p < 0.01$ and $p < 0.001$ between the control (C-24) and cells treated with KCM for 36 and 48 hrs, respectively.

Molecular Characteristic of KD-CIF

In order to determine the approximate molecular weight of KD-CIFs, we fractionated the total KCM collected every 48 hrs up to day 10 post differentiation. Upon fractionation of the KCM by 50 and 30 kDa centrifugal filters, the corresponding filtrate and retentate were collected and analyzed for their collagen inhibitory activity in dermal fibroblasts with total KCM as the positive control. As shown in Figure 3.3A, the steady state levels of type I collagen mRNA was markedly reduced in fibroblasts treated with either total KCM (Lane 1) or the filtrate of the 50 kDa cut-off fraction (Lane 3, <50 kDa) relative to the retentate fraction (Lane 2, >50 kDa). As a control, non-conditioned medium (NCM) was also processed as described above and the results showed no alteration in the steady state level of collagen mRNA in cells treated with either of these fractions. To further narrow down the size of the KD-CIFs in KCM, a 30 kDa cut-off filter was used in a second set of experiments. The results shown in Figure 3.3B, indicate that the 30 kDa cut-off filtrate (Lane 3, <30 kDa) did not reduce the steady state level of collagen mRNA in fibroblasts. In contrast, the level of collagen mRNA was significantly ($p< 0.01$) reduced in both the total KCM (Lane 1) and the 30 kDa cut-off retentate (Lane 2, >30 kDa) compared to that of fractionated NCM treated cells used as negative controls. These findings placed the apparent molecular weight of the KD-CIF between 30 and 50 kDa.

For quantitative analysis, the intensity of signals were evaluated by densitometry and the mean±SD of the steady state level of pro α1(I) collagen mRNA/18S was plotted for fibroblasts treated with either nothing (C, control), total KCM (KCM), 50 KDa retentate (>50 kDa), 30-50 kDa fraction (30-50) or 30 kDa filtrate (<30 kDa), as shown in Figure 3.3C. A significant reduction in the steady state level of collagen mRNA was

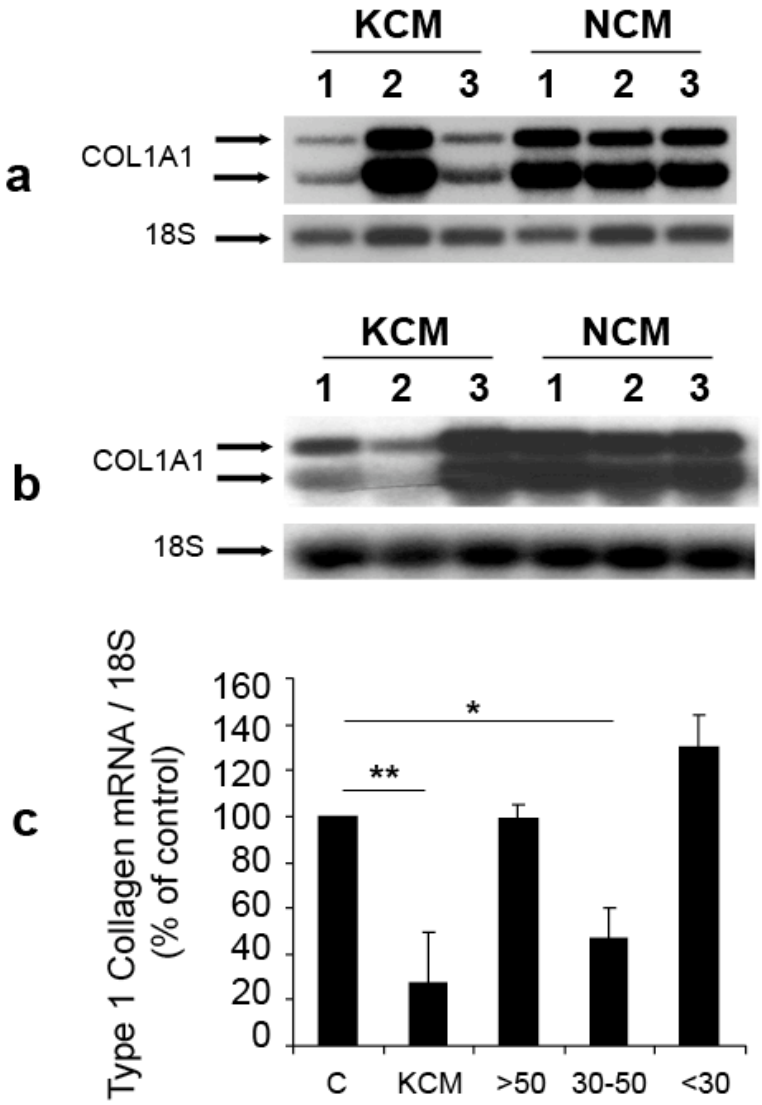

Figure 3.3. Crude determination of KD-CIFs molecular weight.

Panel A: KCM was passed through a 50 kDa cut-off centrifugal filter and corresponding filtrate and retentate were used to treat dermal fibroblasts. Total KCM and non-conditioned medium (NCM) were also used as positive and negative controls, respectively. COL1A1 expression level was determined by northern blot analysis. Total KCM (lane 1), retentate (Lane 2, >50 kDa) and filtrate (lane 3, <50 kDa) of 50 kDa cut-off filter as well as the same fractions for NCM are shown here. **Panel B** shows the same experimental protocol as above only with a 30 kDa cut-off filter. Total KCM (Lane 1), retentate (Lane 2, >30 kDa) and filtrate (Lane 3, <30 kDa) of 30 kDa cut-off filter and corresponding fractions of NCM are presented. Expression of 18S ribosomal RNA was used as a control for loading. Graph in **panel C** represents the mean ratio of COL1A1/18S RNA (percent of control, which is NCM treated cells) ± SD of four separate experiments. (*) and (**) represent a $p < 0.01$ and $p < 0.001$ between control and KCM or KCM fraction of 30–50 kDa treated cells, respectively.

seen in cells treated with either total KCM (27.0 ± 22.06, n=4, p <0.001) or the fraction containing proteins with apparent MW of 30-50 kDa. (47.0 ± 12.7, n=4, p <0.01) relative to that of NCM cells. The results also revealed no significant difference in the steady state level of collagen mRNA between control and that of cells treated with either >50 KDa or <30 KDa fractions of KCM. To ensure that the reduction in the level of collagen is not caused by the suspension of total protein synthesis, we also evaluated and showed a marked increase in the steady state level of MMP-1 mRNA and protein in cells treated with KCM (data not shown). The differential effect of KCM on collagen and MMP-1 expressed by fibroblasts indicated the specificity of matrix modulating proteins present in KCM.

Stability of Keratinocyte-derived Collagen Inhibitory Factor(s)

To characterize the stability of factors responsible for collagen inhibition, KCM was subjected to acidification (pH 2) for 30 minutes followed by neutralization (pH 7.4) with a second sample heated at 56ºC for 30 minutes. Dermal fibroblasts were then incubated with the above samples for 24 (mRNA) and 48 hrs (protein) and the steady state levels of collagen mRNA and protein expression were evaluated by northern and western blot analysis, respectively. The results shown in Figure 3.4A revealed a marked reduction in the steady state level of collagen mRNA in KCM treated cells (Lane 1) and this activity was not effected by either acidification/neutralization (lane 2) or heating of KCM at 56^{o}C (lane 3). Consistent with that of COL1A1 mRNA, Figure 3.4B revealed that pro α1(I) collagen was also markedly reduced at the protein level in fibroblasts treated with KCM (Lane 1) relative to that of the control (C). This reduction was more pronounced in cells treated with either acidified/neutralized (Lane 2) or pre-heated (Lane 3) KCM. For quantitative analysis, the mean±SD of pro α1(I) collagen/β-

actin obtained from three separate experiments was graphed (Figure 3.4B, lower panel). Interestingly, the level of collagen protein was lower in acidified/neutralized (Lane 2) or pre-heated (Lane 3) KCM treated cells relative to that of cells treated with untreated KCM. This might be due to the fact that some collagen stimulating factors such as TGF-β1 in KCM, sensitive to both acidification and heat, are deactivated at the same time.

To further characterize the KD-CIFs, the total KCM was saturated with 80% ammonium sulfate in order to precipitate total proteins in the conditioned media. Precipitated proteins were then used to treat fibroblasts. As seen in Figure 3.4C, ammonium sulfate precipitation completely removed the collagen inhibitory activity of KCM in dermal fibroblasts (lane: PPT). The pattern of β-actin expression in the same samples revealed that this blockage is neither due to protein loading nor cytotoxicity of this treatment. In fact, western blotting with anti-MMP-1 antibody revealed that the MMP-1 stimulatory effect of KCM was not suppressed as a result of ammonium sulfate precipitation (not shown). This finding confirmed that the loss of KD-CIFs activity was not due to total breakdown or deactivation of all protein functional groups during ammonium sulfate precipitation. Dialyzed supernatant from the same precipitation experiment also failed to suppress collagen production in treated fibroblasts, indicating that the collagen inhibitory activity was not retained in the supernatant fraction of KCM (not shown). For quantitative analysis, the mean±SD of pro α1(I) collagen protein/β-actin (percent of control) from three separate experiments was then calculated and graphed as shown in Figure 3.4C, bottom panel.

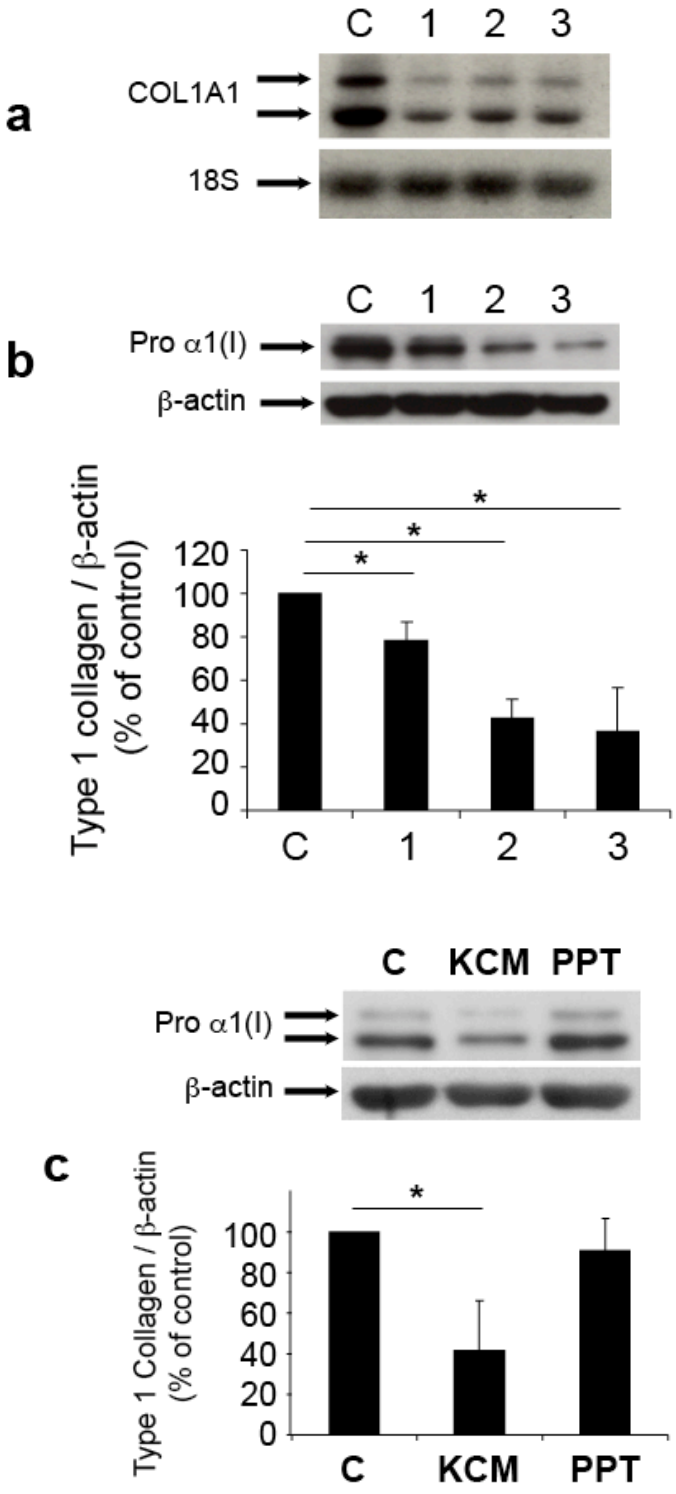

Figure 3.4. Stability of the keratinocyte derived collagen inhibitory factor(s).

Panel A, dermal fibroblasts were treated with medium alone (Lane C) or KCM which has been collected and subjected to either nothing (lane 1), acidification/neutralization (lane 2) or heat at 56^{o}C for 30 min (lane 3). Total RNA was extracted after 24 hrs and subjected to northern blot analysis for COL1A1 mRNA. The corresponding blots were subsequently re-hybridized with 18S ribosomal RNA and used as a loading control. **Panel B**, in a similar set of experiments, the levels of pro a1(I) collagen protein and b-actin were evaluated after 48 hrs by western blot analysis. For quantitative analysis, the mean±SD of pro a1(I) collagen/b-actin obtained from three separate experiments was then determined and the corresponding histogram depicted in lower panel. (*) indicates the significant difference between test and control groups ($p<0.05$). **Panel C**, cells were treated with either nothing (C), KCM, or ammonium sulfate precipitated proteins from KCM (PTT). Total proteins were extracted and evaluated for pro a1(I) collagen protein and b-actin by western blot analysis. The ratio of mean±SD of pro a1(I) collagen/b-actin was then determined from three different experiments and corresponding data were depicted in the lower panel. (*) indicates the significant difference between test (KCM) and control (C) ($p<0.05$). No significant difference was observed between control and PPT ($P>0.05$).

Keratinocyte Differentiation Has no Effect on KD-CIF Activity

To investigate the effect of keratinocyte differentiation on the release and activity of KD-CIFs, we collected KCM from keratinocyte culture for up to 12 days. We made use of a high calcium test medium (49% KSFM + 49% DMEM + 2% FBS) in order to induce differentiation of cultured keratinocytes. As keratinocytes differentiate, cells start to migrate on top of each other forming a multilayer cell culture in vitro (Figure 3.5, panel A). We further confirmed the differentiation by evaluating the intracellular level of involucrin protein (Figure 3.5, panel B), a marker of keratinocyte differentiation. As seen in this panel, the expression of involucrin was easily detectable as early as two days after switching to a high calcium medium and reached its peak on day 10. To evaluate the collagen suppressive effect of conditioned media collected from un-differentiated and differentiated cells, dermal fibroblasts were then treated with total KCM collected from day 0, 4, 8, 10 and 12 for 48 hrs. Western blot analysis revealed that all KCM collections markedly reduced the expression levels of pro α1(I) collagen, but not β-actin in treated fibroblasts. Therefore, keratinocyte differentiation did not alter synthesis, release, or activity of KD-CIFs (Figure 3.5, panel C). To ensure that the reduction was not due to suppression of total protein synthesis in KCM-treated cells, the same samples were evaluated for expression of MMP-1. As shown in Figure 3.5C, in contrast to the collagen inhibitory factors present in KCM collected from differentiated cells, the stimulatory effect of KCM on MMP-1 steadily increased as keratinocytes differentiated.

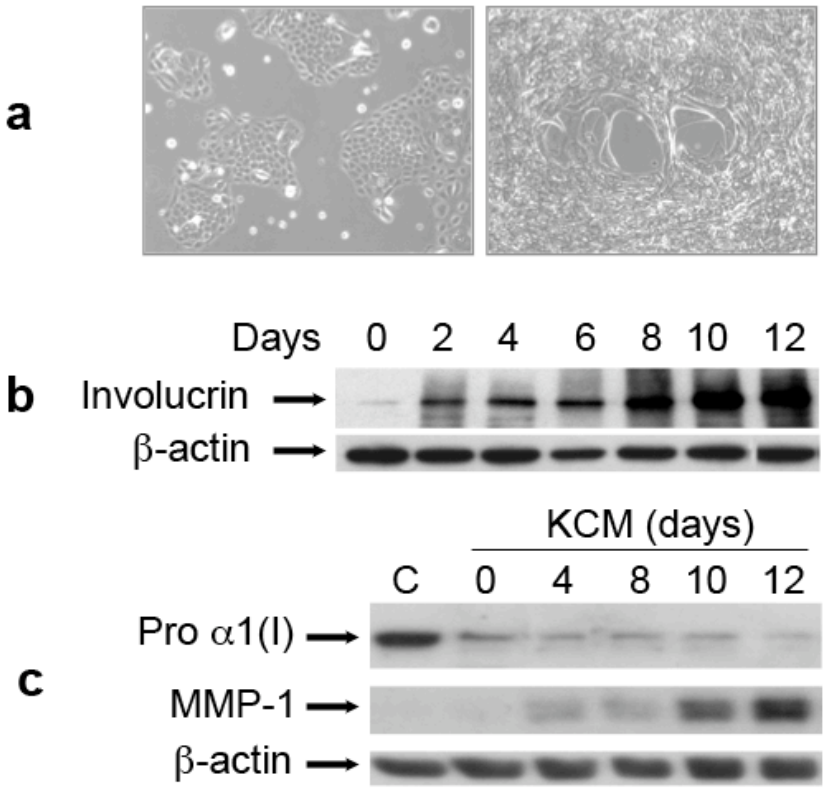

Figure 3.5. Keratinocyte differentiation has no effect on KD-CIFs activity.

Panel A shows the multilayer formation by differentiated keratinocyte on day 14 (right panel) compared with monolayer of cells on day 0 (left panel). **Panel B**: Total proteins from keratinocyte lysate after 0, 2, 4, 6, 8, 10, and 12 days of culture were extracted and subjected to western blot analysis for involucrin protein used as a marker of keratinocyte differentiation. The level of b-actin was tested as a loading control. **Panel C**: Dermal fibroblasts were treated with KCM collected on day 0, 4, 8, 10, and 12 of keratinocyte culture. Non-conditioned medium was used as control (lane C). Total cell extracts were prepared and intracellular type I procollagen and MMP-1 protein levels were determined by western blot analysis. β-actin is shown as loading control.

Discussion

Dermal fibrotic conditions, such as keloids and HTS, result in bulky, itchy, and inelastic scars that pose serious health and functional problems for recovering patients. The characteristic excess ECM, which is of altered composition and organization, is most likely the end product of series of events involving several cell types and cytokines released during the process of healing (Scott et al., 2004). Following an injury, keratinocytes migrate and proliferate over the injured site and demonstrate paracrine effects on the underlying fibroblasts (Werner et al., 2007).

The current study presents evidence for production of soluble KD-CIFs, independent of a paracrine loop mechanism between keratinocyte and fibroblasts, with stable activity at high temperature and low pH, and an estimated molecular size of 30-50 kDa. To characterize such KD-CIFs for dermal fibroblasts, a keratinocyte/fibroblast co-culture system was established to resemble the human skin, where these cells are separated by a porous basal membrane. In a series of experiments, it was shown that the steady state level of pro α1(I) collagen was significantly suppressed in fibroblasts co-cultured with keratinocytes compared to fibroblasts co-cultured with fibroblasts. This suppression was found at both the level of mRNA and protein. This concurs with data from Harrison et al. (Harrison et al., 2006) and Garner (Garner, 1998), in which amino terminal propeptide of type I collagen in conditioned medium or incorporation of [3H]-proline were used as markers for collagen production. To ensure that the pro α1(I) collagen inhibitory effect of KCM in fibroblasts was not caused by suppression of protein synthesis in these cell, the same samples collected from treated fibroblasts were evaluated for MMP-1 expression. In contrast to the inhibitory effect of KCM for pro α1(I) collagen, an increase in the level of MMP-1 mRNA and protein was observed

in the same fibroblasts. These findings are in agreement with the clinical observations where timely epithelialization or application of cultured epithelial autografts significantly reduces the development of fibrosis in injured skin tissue (Deitch et al., 1983; Wood and Stoner, 1996). These results further support the role of keratinocytes in regulation of fibroblasts into a phenotype more suitable for ECM remodeling as opposed to ECM deposition.

The range of growth factors or cytokines reported to be expressed by keratinocytes, either constitutively or upon stimulation, includes (IL)-1, -6, -7, -8, -10, -12, -15, -18, and -20, tumour necrosis factor (TNF)-α, interferons (IFN) α, β, and γ (Grone, 2002), transforming growth factor-beta1 (TGF-β1) (Ghahary et al., 2001), and platelet-derived growth factor (PDGF) (Ansel et al., 1993). Among these, IL-1α and β, TNF-α (Mauviel et al., 1991; Diaz et al., 1993), and IFN-α, β and γ (Granstein et al., 1990; Ghahary et al., 2000b; Ghosh et al., 2006) have been reported to directly or indirectly inhibit expression of type I collagen in fibroblasts. It is important to note that among these factors, only IL-1α was found in measurable levels in KCM from primary keratinocytes (Nowinski et al., 2002). In the present study, we demonstrated the removal of KD-CIFs activity in KCM upon exclusion of factors between 30 and 50 kDa. Based on our findings, keratinocyte-derived IL-1-α and -β (~ 18kDa), soluble form of TNF-α (~ 25 kDa), and IFN-α and -β (~ 20kDa) are unlikely to be the KD-CIFs characterized in this study. Soluble TNF-α forms a homotrimer structure of about 75 kDa in size and would be excluded in the fractionation process by 50 kDa filter (Jones et al., 1989). In contrast to the monomeric Type I IFNs (IFN-α and –β), IFN-γ forms a homodimer with molecular weight of about 32 kDa (Pestka et al., 2004). IFN-γ has been shown to suppress the collagen synthesis by human dermal fibroblasts, synovial fibroblast-like cells, chondrocytes, and rat myofibroblasts (Granstein et al., 1990).

However, it is also unlikely that KD-CIFs' effect is due to the dimeric form of IFN-γ because cytokines such as IFN-γ show sensitivity to heat and pH treatment (Sareneva et al., 1995). Collagen inhibitory activity in KCM was not only blocked, but also increased when KCM was treated with either 56ºC heat or acidic pH. The mechanism by which heat or acidity increase the KCM collagen suppressive effect in fibroblasts is not clear at this time. We speculate that the effects of fibrogenic factors produced by keratinocytes may have been reduced due to heat or acid treatment of KCM. As a result, the efficacy of KD-CIFs which are stable under extreme conditions became more pronounced in our experiment.

It is plausible that keratinocytes act indirectly by inducing expression and release of an autocrine anti-fibrogenic factor from fibroblasts, which in turn will suppress collagen production in the same cells. In fact, evidence indicates that keratinocyte-releasable factors can inhibit expression of fibrogenic factors produced by fibroblasts and released into the culture media (Kilani et al., 2007). TGF-β1 and connective tissue growth factor (CTGF) are fibrogenic growth factors produced by various cells including fibroblasts in the skin and play a significant role in induction of ECM deposition and scar formation during wound healing (Martin, 1997; Leask et al., 2002). It has been previously demonstrated that expression of TGF-β1 (Le Poole and Boyce, 1999; Amjad et al., 2007) and CTGF (Nowinski et al., 2002; Khoo et al., 2006) by fibroblasts are downregulated via paracrine action of keratinocytes in a skin substitute or a co-culture model. Interestingly, higher expression of secreted CTGF was observed in normal and keloid fibroblasts co-cultured with keloid keratinocytes as compared with normal keratinocyte (Khoo et al., 2006). Although these studies did not directly investigate collagen expression, it is plausible that KD-CIFs characterized in the current study suppresses the production of TGF-β1 or CTGF by fibroblasts and therefore attenuates

collagen induction by the same cells. Interestingly, Nowinski et al. reported IL-1α as the major isoform of IL-1 detectable in KCM and responsible for inhibiting the expression of CTGF in fibroblasts (Nowinski et al., 2002). Others have reported that IL-1β stimulates the type I collagen mRNA synthesis and inhibits type I collagen protein synthesis in dermal fibroblasts, suggesting regulation at the posttranscriptional or translational level (Mauviel et al., 1991). As discussed earlier, the collagen inhibitory factor(s) released by keratinocyte in our study was shown to be larger than 30 kDa, excluding both isoforms of IL-1 from KD-CIFs described here. It should be noted that unprocessed precursor for human IL-1 is about 31 kDa, but its role in regulation of collagen in dermal fibroblasts is not known at this time. Further systematic studies are needed to address the possible role IL-1 and its precursor play in regulation of collagen expression in fibroblasts.

Differential expression of fibrogenic factors between differentiated and un-differentiated keratinocytes have been reported previously. It appears that the level of TGF-β1 is gradually reduced as keratinocytes become differentiated (Ghahary et al., 2001). On the other hand, the level of stratifin, recently identified as an MMP-1 stimulating factor, markedly increases during the course of keratinocyte differentiation (Ghahary et al., 2005). For this reason, we also evaluated the activity of KD-CIFs in KCM obtained from differentiated keratinocytes on pro α1 (I) collagen synthesis. In contrast to TGF-β1 and stratifin, the level of KD-CIFs in KCM was not affected by keratinocyte differentiation. To further confirm this finding, we also evaluated the level of involucrin, a keratinocyte differentiation marker, in the same samples. The result showed an increase in the level of this factor as early as two days post induction of differentiation. Notably, the KD-CIFs activity of KCM was removed following protein precipitation with 80% ammonium sulfate. It appears that KD-CIF(s) tertiary structure and activity was irreversibly altered as a result of interaction with charged salt ions and

did not recover after PBS dialysis. Interestingly, the MMP-1-stimulatory effect of KCM was not affected by precipitation at the same salt saturation level (not shown). Testing the supernatant of ammonium sulfate precipitation did not reveal any collagen inhibitory activity, therefore excluding the possibility of a non-protein macromolecule (e.g. lipid) remaining in the supernatant fraction during the salting-out phase.

The present study therefore advances our understanding of keratinocyte-fibroblast interaction and confirms the presence of keratinocyte-derived factors involved in regulation of ECM factors such as type I collagen. Identification of anti-fibrogenic keratinocyte-derived factors with the ability to suppress scar formation could prove valuable in development of novel therapeutics for fibrotic disorders. Further studies are required to identify the exact nature of the KD-CIFs and its potential role in wound healing and fibrotic conditions.

Materials and Methods

Cell Culture

Following informed consent, skin punch biopsies were obtained from patients undergoing elective surgery under the local anesthesia. Written informed consent was obtained from each participant and the study was approved by the University of British Columbia Hospital Human Ethics Committee and conducted according to Declaration of Helsinki Principles. Biopsies were then collected individually and washed five times in sterile Dulbecco's modified Eagle's medium (DMEM) supplemented with antibiotic-antimycotic preparation (100 U/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml amphotericin B). Epidermal and dermal layers were then separated by treating samples with dispase (Roche Applied Science, Laval., QC, Canada). Cultures of fibroblasts and keratinocytes were established as described previously (Ghahary et al., 2000a) (Ghahary et al., 2004). Fibroblasts were grown in DMEM with 10% fetal bovine serum and keratinocytes in serum-free keratinocyte medium (KSFM) (Invitrogen Life Technologies, Carlsbad, CA) supplemented with bovine pituitary extract (50 µg/ml) and EGF (0.2 µg/ml).

Keratinocyte / Fibroblast Co-culture System

The co-culture model is described in Ghaffari et al. (2006). In brief, 30-mm Millicell-CM (Millipore) culture plate inserts with 0.4-mm pore size were coated with fetal bovine skin collagen (3 mg/ml) overnight. Subsequently, either 0.25×10^{6} keratinocytes or fibroblasts were seeded on the collagen-coated inserts with either KSFM supplemented with BPE and EGF or DMEM plus 10% FBS, respectively.

Fibroblasts were seeded (0.5×10^6) in the bottom chamber of each well of a six-well culture plate containing DMEM with 10% FBS. Cells were incubated for 24 hrs and the conditioned medium was collected and cells were washed with phosphate-buffered saline (PBS). To assemble the co-culture system, collagen-coated inserts with either keratinocytes (K/F) or fibroblasts (F/F) (upper chamber) were placed on top of the fibroblasts (bottom chamber) in six-well plates. The test medium used in co-culture consisted of 49% KSFM without supplements and 49% DMEM plus 2% FBS for K/F and F/F. The co-culture study was also performed on a 3D human skin equivalent. Fibroblasts populated in collagen gel (FPCG) were prepared by casting 2×10^6 cells in 1.0 ml of bovine type I procollagen as described previously (Sarkhosh et al., 2003). Following overnight incubation, 5×10^5 keratinocytes (K/F) or fibroblasts (F/F) were seeded on the surface of FPCG and grown in 49% KSFM without supplements and 49% DMEM plus 2% FBS. Fibroblasts were harvested from the FPCG by digesting the collagen lattice with bacterial collagenase (Sigma) as described by Sarkhosh et al. (Sarkhosh et al., 2003).

Treatment of Fibroblasts with Keratinocytes Conditioned Medium

To collect KCM from keratinocyte culture alone, our test medium consisted of 50% DMEM plus 50% KSFM in the absence of FBS or growth supplements. To evaluate the effect of KCM on fibroblasts collagen type I expression the following experiments were performed: 1) Fibroblasts were treated with KCM or fresh media for up to 48 hrs, 2) To examine the stability of keratinocyte derived collagen inhibitory factors (KD-CIFs): (2A) KCM was subjected to acidic condition (pH 2.0) for 30 min by adding hydrochloric acid and then neutralized back to pH 7.4 with addition of sodium hydroxide solution. (2B) In order to observe the effect of heat on activity of KD-CIFs,

samples of KCM were subjected to 56ºC for 30 minutes prior to treating fibroblasts. In another set of experiments the secreted proteins in KCM were concentrated using ammonium sulfate precipitation. Following collection of KCM from the cells, proteins were precipitated by addition of ammonium sulfate at 80% saturation for 30 min at 4°C under slow stirring. Precipitated proteins were pelleted by centrifugation (30 min, 3000 × g at 4^{o}C) and resuspended in phosphate buffer (20 mM, pH 7.2). Ammonium sulfate was removed by dialysis against same phosphate buffer containing 1 mM DTT, overnight at 4°C. Fibroblasts were treated with the above conditioned media for 24 or 48 hrs in triplicate experiments. 3) To determine the effect of Keratinocyte differentiation, KCM collected from keratinocyte at different time points (0, 4, 8, 10, 14 days) were examined for collagen inhibitory effect on fibroblasts as well as MMP-1 expression after 48 hrs of treatment. Expression of differentiation marker (involucrin) was evaluated at the same time points in keratinocyte lysate (by western analysis). Results of triplicate experiments are presented.

Molecular Weight Estimation of KD-CIF

To estimate the MW of KD-CIFs, KCM was passed through 50 and 30 kDa cut-off Centricon filters (Amicon) and portions of total KCM, filtrate, and retentate were individually analyzed. Each fraction was used to treat fibroblasts and the level of pro α1 (I) collagen was determined by northern analysis after 24 hrs in duplicate experiments for each of 50 and 30 kDa cut off filter products. Total KCM and non-conditioned medium (NCM) (medium alone) and its fractions were also used as positive and negative controls, respectively.

RNA Isolation and Northern Blot Analysis

Total RNA was isolated from treated cells and their controls as previously described (Ghahary et al., 1998). Total RNA was then separated by electrophoresis (10 µg per lane) and blotted onto a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA). Following 2 hrs incubation in prehybridization solutions, blots were hybridized using type I collagen, collagenase-1 (MMP-1), or 18S ribosomal RNA (loading control) radioisotope-labeled cDNA probes. Autoradiography was performed by exposing a Kodak X-Omat film to nitrocellulose filters at -20°C in the presence of an intensifying screen. The cDNA probes for α-1 chain of type I procollagen [Pro α1(I)], MMP-1, and 18S ribosomal RNA were obtained from the American Type Culture Collection (Rockville, MD).

Western Blot Analysis

Western blot technique was carried out as previously described (Ghaffari et al., 2006). Immunoblotting was performed with 1:5000 dilution of rabbit anti-human procollagen type I (Rockland, Gilbertsville, PA, USA), 1:2000 dilution of mouse monoclonal anti-involucrin (Sigma), 1:500 dilution of mouse monoclonal anti-human MMP-1 (R&D Systems, Minneapolis), or 1:20000 dilution of mouse anti-human β-actin mAbs (Santa Cruz Biotechnology Inc.). The appropriate secondary antibody (horseradish peroxidase-conjugated IgG) was used at a 1:3000 dilution (Bio-Rad, Mississauga, ON, Canada).

Statistical Analysis

Data were expressed as mean±SD and analyzed with one-way ANOVA with Tukey-Kramer Multiple Comparison Test among different groups of each cell type where indicated. P-values less than 0.05 are considered statistically significant in this study.

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Chapter 4. 14-3-3σ Associates with Cell Surface Aminopeptidase N in the Regulation of Matrix Metalloproteinase-1[3]

Introduction

The complexity of multicellular organisms demands extensive communication between cells of different germ layers in order to direct tissue development and repair. Increasing evidence suggests that bidirectional signaling between stromal and epithelial cells plays a critical role in maintaining the extracellular matrix (ECM) integrity and that a disruption in this communication can lead to fibrogenesis (Deitch et al., 1983; Machesney et al., 1998; Niessen et al., 2001) or predispose cells to malignancy (Bissell and Radisky, 2001; Radisky and Bissell, 2004). Matrix metalloproteinases (MMPs) represent a group of diverse proteolytic enzymes which are responsible for degradation of ECM and are involved in variety of biological processes such as tissue repair and remodeling, embryonic development, bone growth/resorption, and tumor metastasis (Butler and Overall, 2009b; Nagase et al., 2006; Rodriguez et al., 2009). The MMPs synthesis by stromal fibroblasts is partially regulated by neighboring epithelial cells primarily through diffusible cytokines and growth factors (Gabison et al., 2005; Garner, 1998; Harrison et al., 2006).

Stratifin (SFN) or 14-3-3σ is a member of a large family of highly conserved 14-3-3 proteins which are known to function as intracellular chaperons in signal transduction, apoptosis and cell cycle regulation (Hermeking, 2003; Pozuelo et al.,

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2004). Several groups have reported on the presence of 14-3-3 proteins in the extracellular space (Butler and Overall, 2009a;Katz and Taichman, 1999; Kobayashi et al., 2009; Leffers et al., 1993; Satoh et al., 1999). Our group has recently identified a novel function for the keratinocyte-releasable form of SFN as a potent MMP-1 stimulatory factor in dermal fibroblasts (Ghahary et al., 2004). It was found that SFN released from keratinocytes induces MMP1, 3, 8, and 24 expressions in fibroblasts through the p38 MAPK signaling pathway (Ghaffari et al., 2006a; Ghahary et al., 2004; Lam et al., 2005). Furthermore, high levels of 14-3-3 eta (η) and gamma (γ) isoforms were detected in the synovial fluid of patients suffering from rheumatoid arthritis. The levels of 14-3-3η strongly correlated with the serum levels of MMP-1 and MMP-3 and the purified protein possessed MMP-1 stimulatory activity (Kilani et al., 2007).

Data presented indicate that releasable 14-3-3 may function as a ligand in a receptor-mediated transmembrane signaling pathway, which leads to the induction of MMP-1 expression. Isoforms of 14-3-3 proteins have been reported to interact with the cytoplasmic domain of various plasma membrane proteins such as bullous pemphigoid antigen-2 or BP180 (Li et al., 2007); insulin-like growth factor I receptor (Craparo et al., 1997); muscle specific tyrosine kinase receptor (Strochlic et al., 2004); epidermal growth factor receptor (Oksvold et al., 2004); and type I transforming growth factor β receptor (McGonigle et al., 2001). However, no interaction between 14-3-3 proteins and the ectodomain of a cell surface receptor has been identified to date. In the current study, we report on the association between SFN and aminopeptidase N (APN) or CD13, a type II integral membrane protein, and highlight a transmembrane signaling mechanism for SFN-mediated MMP-1 expression in fibroblasts.

Results

Recombinant SFN Exhibits Cell Surface Binding Activity

Human primary dermal fibroblasts - which are highly responsive to SFN activity (Ghahary et al., 2004) - were used in a cell-binding assay to test the potential receptor binding activity of rSFN. To avoid nonspecific detection of intracellular 14-3-3 isoforms, a biotin-labeled rSFN (biotin-SFN) was prepared. Cells were incubated with biotin-SFN at 4ºC for 15 minutes and analyzed with fluorescent-conjugated streptavidin. The results revealed that biotin-SFN efficiently binds to the fibroblasts' cell surface (Fig. 4.1A, top panels). To ensure specific binding, the assay was repeated by competing biotin-SFN with excessive amount of unlabeled rSFN. This nearly eliminated the binding activity of biotin-SFN (Fig. 4.1A, middle panels). The assay was also repeated with a biotin-labeled GST protein (biotin-GST) expressed by the same plasmid as rSFN (Ghahary et al., 2004). No cell binding activity was detected in the presence of biotin-GST (Fig. 4.1A, bottom panels). SFN binding to the cell surface was further evaluated by using a ^{125}I-labelled SFN (^{125}I-SFN) in a ligand/receptor binding assay. Cells treated with ^{125}I-SFN were incubated in the presence of an increasing concentration of unlabelled SFN for 2 hours and then harvested by lysis buffer to measure the total binding in a gamma counter. As shown in figure 4.1B, more than 90% of the ^{125}I-SFN binding was replaced with competing and unlabelled SFN, demonstrating a specific SFN binding to the cell surface. A plasma membrane (PM) isolation technique was utilized to investigate the localization of exogenous rSFN which had been added to cells. Following a short incubation with biotin-SFN at 4ºC, the PM and cytoplasmic fractions were separated on SDS-PAGE and analyzed by immunoblotting to identify

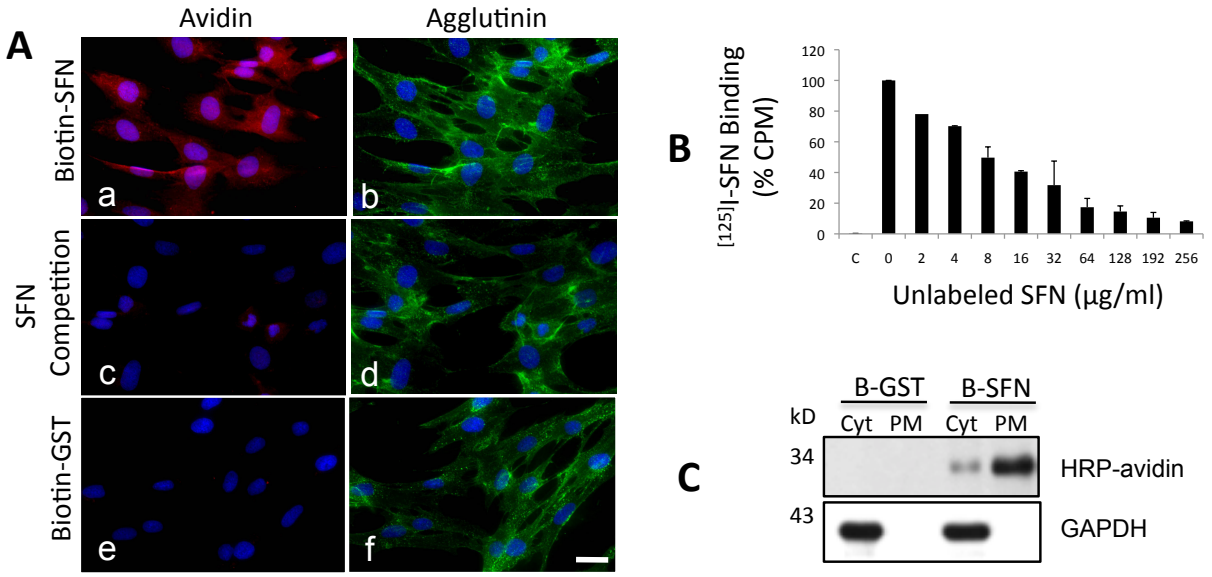

Figure 4.1. rSFN cell surface binding activity.

A) Fibroblasts were treated with biotin-SFN in absence (a, b) or presence of 200 times excess unlabeled rSFN (c, d), or biotin-GST as negative control (e, f). Cells were then analyzed by immunofluorescent microscopy by anti-biotin stretavidin-alexa 568 or agglutinin-alexa 488 as a plasma membrane marker. **B**) Receptor-ligand binding assay was performed by incubating cells with ^{125}I-labeled SFN in competition with increasing concentration of unlabeled SFN. Radioactive count per minute (CPM) was displayed as a percentage of ^{125}I-SFN binding in the absence of unlabeled SFN. **C**) plasma membrane (PM) and cytosolic (Cyt) fractions were isolated following incubation of cells with biotin-SFN (B-SFN) and biotin-GST (B-GST) and analyzed for the presence of biotin. GAPDH was used as a cytosolic marker to test the purity of PM fraction. (Scale bars, 20 µm).

biotin-SFN. As expected, Biotin-SFN was detected predominantly in the membrane fraction (Fig. 4.1C). The GAPDH was not observed in the PM fraction, which indicated that the membrane preparation was devoid of cytosolic protein contamination. Exogenous biotin-GST was used in a separate group to control for the potential nonspecific binding of a biotin-labeled protein.

APN: A Cell Surface SFN Receptor

To identify a candidate receptor for SFN, we employed a cell surface biotin labeling technique followed by serial SFN-Sepharose and Avidin-Agarose affinity purification. The ectodomains of PM proteins were labeled with a membrane impermeable and non-cleavable biotin (sulfo-NHS-biotin), which binds covalently with the exposed primary amine groups. The cell membrane was disrupted by a mild lysis buffer and passed through an SFN- or blank-Sepharose (control) batch column to isolate any potential SFN binding partners. To further purify potential candidates, the proteins retained by SFN-Sepharose were eluted at acidic pH (Glycine pH=2), neutralized, and then passed through an immobilized avidin affinity column. Final purified proteins were eluted by the SDS sample buffer, separated on SDS-PAGE, and visualized by silver staining. As shown in fig. 4.2A, silver staining of affinity-purified complex revealed several potential SFN binding proteins. Streptavidin immunoblotting - of the same sample - identified two main biotinylated proteins at 170 and 150 kDa. As the 170 kDa band was also seen in the control group (blank Sepharose), the 150 kDa protein was the only biotinylated membrane protein with binding affinity to SFN. To identify this protein, the SDS-PAGE band was subjected to trypsin digestion and liquid chromatography-mass spectrometry (LC/MS/MS) protein identification. The peptide

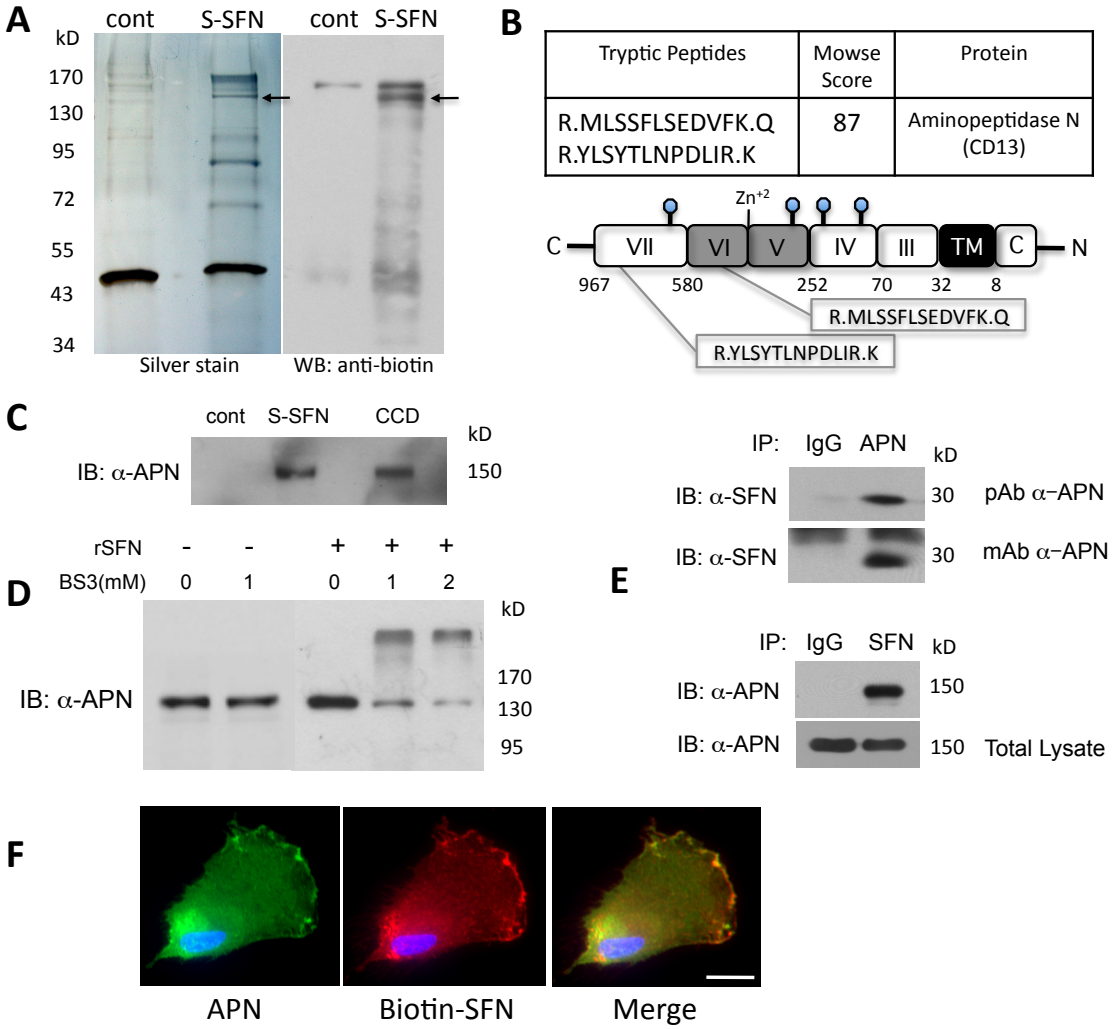

Figure 4.2. Identification of the APN/SFN complex.

A) Silver stain and western blot of cell surface SFN-binding proteins isolated by serial affinity purification. Total cell lysate was initially passed through Sepharose alone (Cont) or SFN-Sepharose (S-SFN) before further purification by an avidin-agarose column. A 150 kDa biotin-labeled protein (arrow) was identified by western blot and the equivalent band from the silver stain was subjected to trypsin digestion and MS analysis. **B**) The alignment of the tryptic peptides, identified by MS sequencing, within the full APN sequence is shown. A Mouse score of 87 indicated a significant alignment by MASCOT search engine. **C**) Cell lysate was passed through SFN-Sepharose column and purified protein mixture immunoblotted with anti-APN antibody. APN-expressing CCD-1064 cell lysate (CCD) was used as positive control. **D**) Cells were incubated with rSFN prior to treatment with 0, 1.0, 2.0 mM BS^3 cross-linker. After separation of total lysate on SDS-PAGE, the shift in APN migration was revealed by immunoblotting. **E**) Total cell lysate from rSFN-treated fibroblasts were immunoprecipitated (IP) with IgG or anti-APN polyclonal (pAb) and monoclonal (mAb) antibodies and immunoblotted with the anti-SFN antibody (top panels). The reverse IP by anti-SFN antibody pulled down APN from the total cell lysate (bottom panel). Level of APN in total cell lysate of both IgG and SFN IP groups is shown as loading control. **F**) Immunofluorescent microscopy displaying the co-distribution of biotin-SFN and APN in fixed cells. (Scale bar, 20 μm).

sequences were analyzed with the MASCOT search engine. The peptides were aligned with the amino acids 485 to 498 and 843 to 855 of the human aminopeptidase N (APN) or CD13 (Fig. 4.2B). The probability-based Mowse score of 87 indicated a statistically significant alignment.

To confirm the results from the MS protein identification, we repeated the SFN-Sepharose affinity binding using fibroblast cell lysate and separated the eluted complex by SDS-PAGE and subjected it to immunoblotting using an anti-APN antibody. Consistent with our original finding, the APN protein demonstrated a high binding affinity to immobilized SFN, as shown in fig. 4.2C. To further demonstrate that rSFN is a specific binding partner for APN, we used membrane-impermeable bis-sulfosuccinimidyl suberate (BS^3) to crosslink cell surface binding partners. Cells were incubated with rSFN prior to lysis and then cross-linked with BS^3. Immunoblotting by the anti-APN antibody revealed a clear shift in the APN position only in the presence of rSFN and the BS^3 cross-linker (Fig. 4.2D). We also performed immunoprecipitation experiments with antibodies against each protein. Fibroblasts were incubated with rSFN, washed extensively, and lysed prior to performing the immunoprecipitation. Two different anti-APN antibodies co-immunoprecipitated SFN as detected by western blot analysis using an antibody against SFN (Fig. 4.2E, top panels). A reverse immunoprecipitation experiment further revealed that the anti-SFN antibody was able to co-precipitate APN protein, as shown in the lower panel of fig. 4.2E. To study the co-distribution of APN and SFN, fixed cell were incubated with biotin-SFN at 4ºC for 30 minutes prior to immunofluorescence analysis with anti-APN monoclonal antibody and fluorescent-conjugated streptavidin. As shown in fig. 4.2, panel F, it appears that biotin-SFN co-distributed with APN in the cultured fibroblasts. Interestingly, this co-distribution was more pronounced at the leading edge of the cell.

APN Expression In Vivo

To test the presence of candidate SFN cell surface receptor in vivo, we examined the APN and SFN expression in the normal and wounded rabbit ear by immunofluorescence. Dermal wound samples were collected on 16 days after the injury with the non-injured area in the back of the same ear used as control. As expected, minimal expression of APN and SFN were observed in the uninjured skin (Fig. 4.3, panel a and c). However, examination of the wound revealed a large number of APN-positive cells throughout the dermis on day 16 post injury (Fig. 4.3, panel b), with the majority of the positive cells exhibiting a spindle-shape fibroblast-like morphology. This coincided with an increase in the expression of SFN in the epidermis (Fig. 4.3, panel d) confirming the co-expression of the ligand in the wound. Immunofluorescence analysis of vimentin, a mesenchymal marker, was used to reveal that a majority of the cells in the dermis of the wounded skin exhibit fibroblast phenotype (Fig. 4.3, insert in panel b). To study the co-distribution of SFN and its receptor in the skin at different stages of tissue repair, a longitudinal rat wound healing model was utilized where tissue extract from days 8, 11, 14, 17, and 30 post wounding were analyzed by western blot. Although both SFN and APN were detected in the wound following re-epithelialization (day 11), there was a strong overlapping peak on day 30 post-wounding (Fig. 4.3, panel b). This provided evidence for the co-expression of this ligand and receptor during the remodeling phase of wound healing.

APN Knockdown Blocks SFN-mediated MMP-1 Expression

Small interfering RNAs (siRNAs) were employed to knockdown APN expression in fibroblasts in order to examine the role of APN/SFN interaction in SFN-mediated

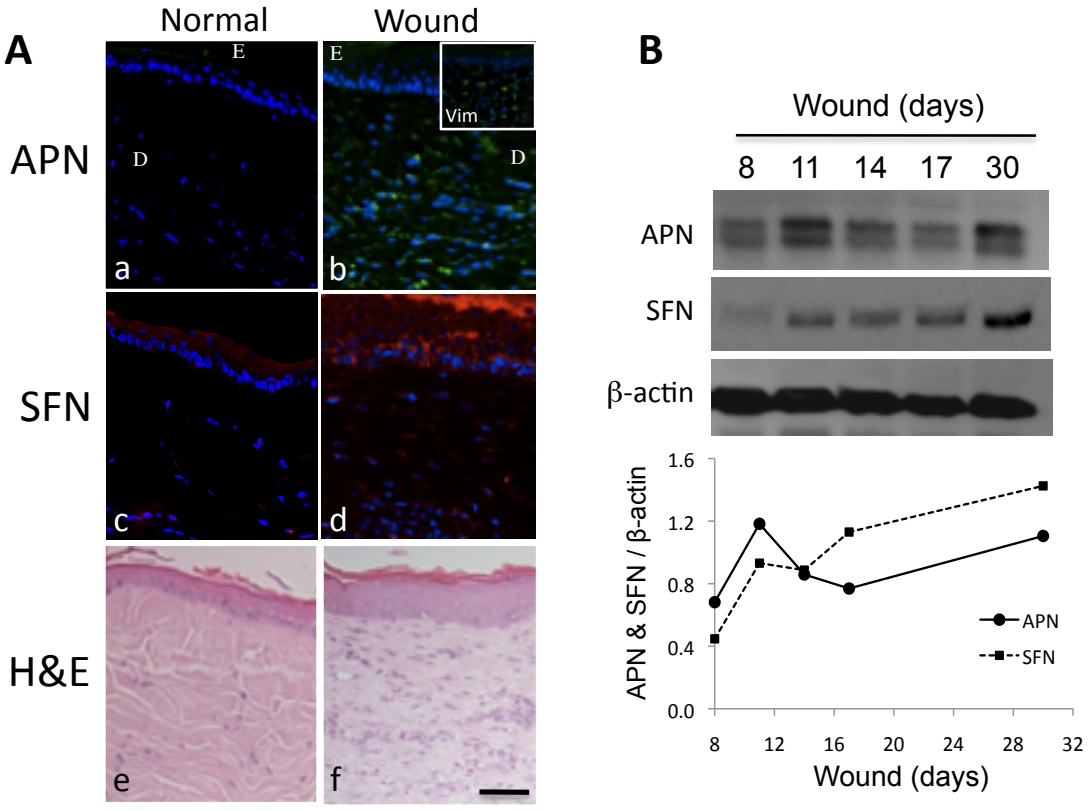

Figure 4.3. APN expression in vivo.

A) Skin tissue samples were collected from the rabbit ear 16 days post injury and analyzed for APN (panel b) and SFN (panel d) expression by immunofluorescent microscopy. Uninjured tissue on the back of the same ear was used as control (panel a and c). In the injured tissue (wound), APN was widely expressed in fibroblast-like cells in the dermis confirmed by the expression of vimentin as shown by the insert in panel b. Panels e and f represent the H&E stain of the sections shown in panel a and b, respectively. DAPI stain was used to mark the nucleus in panels a-d. E: epidermis; D: Dermis; **B**) skin tissue biopsies from a rat wound healing model were taken on days 8, 11, 14, 17, and 30 post wounding. The protein levels of APN and SFN were determined in the total tissue lysate by western blotting. β-actin level was used as a loading control. To evaluate their co-distribution in tissue, the quantified expression of SFN and APN normalized against β-actin (by densitometry) is shown by the line graph in the lower panel. (Scale bar, 50 μm).

MMP-1 expression. Two different siRNA sequences were used against the APN mRNA (Gene bank accession number: NM_001150). Fig. 4.4A shows the inhibitory effect of siRNA on APN expression as detected by immunofluorescence analysis. The quantification of the APN fluorescence intensity - which was normalized against the number of nuclei in each field (DAPI stain) - revealed 50% and 80% inhibition at 10 and 50 nM siRNA concentrations, respectively (Fig. 4.4A, right panel). The cells were then transfected with either non-silencing or APN siRNAs for 72 hours and then treated with rSFN to examine the MMP-1 expression. The siRNA knockdown of APN sufficiently blocked the SFN-mediated MMP-1 expression in contrast to the controls (Fig. 4.4B). The non-silencing siRNA concentration was matched with the highest dose of APN siRNA at 50 nM. To exclude the possibility that APN siRNA would directly suppress MMP-1 expression, fibroblasts were treated with recombinant human interleukin-1 (rIL-1), a potent MMP-1 stimulatory cytokine (Mackay et al., 1992). As expected, the knockdown of APN by siRNA did not block the IL-1-mediated MMP-1 expression (Fig. 4.4C). In addition, we screened various strains of fibroblasts for APN expression. IMR-90, a human fetal lung fibroblasts, does not express APN protein at levels detectable by immunoblotting. Interestingly, IMR-90 was also the only tested strain that was not responsive to SFN stimulation (Fig. 4.4D), even though these cells have been shown to express MMP-1 when stimulated by different collagen substrates (Abraham et al., 2007).

APN in SFN-mediated P38 MAPK Activation

Previously, we have shown that stimulation with SFN leads to the transient activation of p38 MAPK (Lam et al., 2005). In the current study, we tested the intermediary role of APN in the activation of p38 by knocking down its expression prior

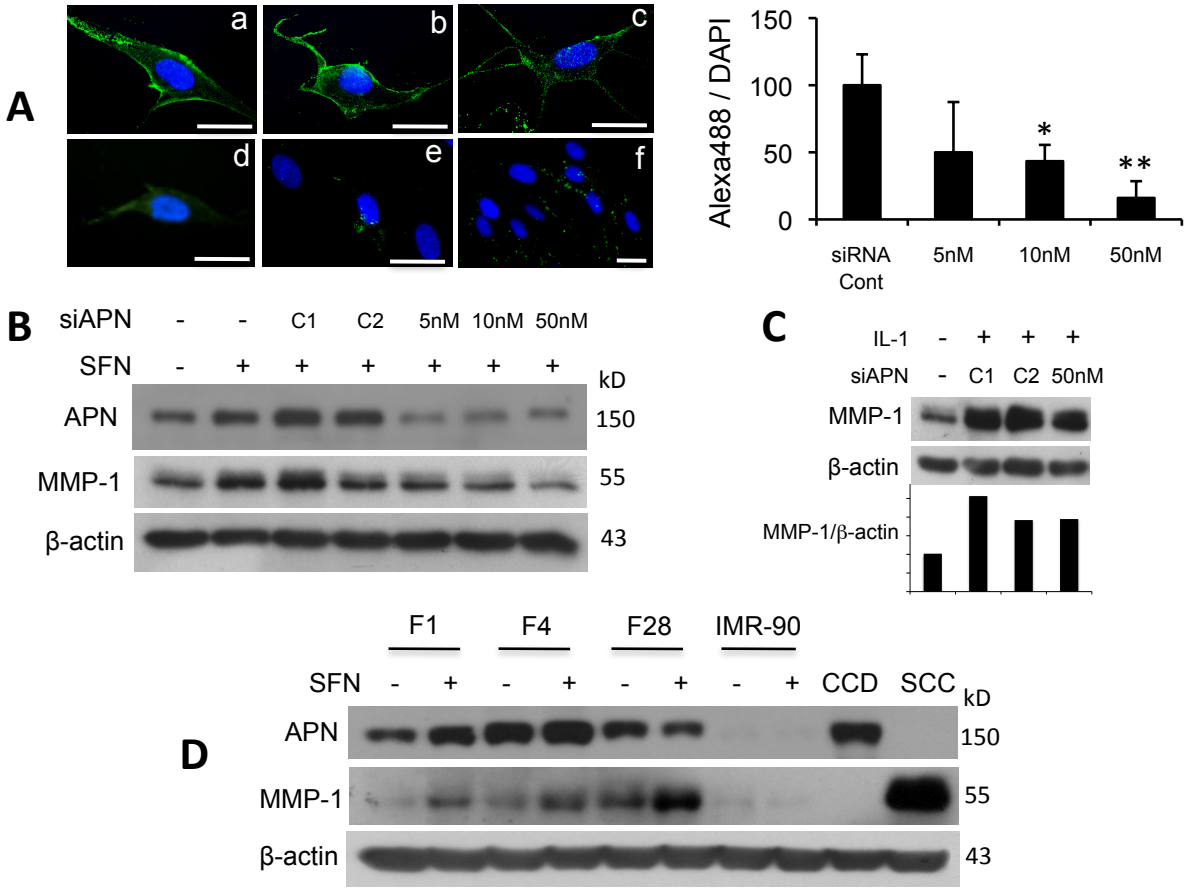

Figure 4.4. APN knockdown blocks rSFN-mediated MMP-1 expression.

A) APN knockdown following siRNA transfection was quantified by immunofluorescent microscopy. The intensity of APN immunofluorescence signal following transfection with (a) nothing, (b) nonsilencing siRNA, (c) 5 nM, (d) 10 nM, and (e) 50 nM of APN siRNA was quantified and normalized against the intensity of DAPI nucleus stain. At least 5 random fields were analyzed in each group. Transfection with 10 and 50 nM of APN siRNA lead to 57% ($p<0.05$) and 84% ($p<0.001$) reduction of APN protein expression, respectively (graph). **B**) Fibroblasts were transfected with increasing dose of APN siRNA prior to treatment with rSFN and then analyzed for MMP-1 expression. For control, cells were transfected with nothing (-), transfection buffer alone (C1), or nonsilencing siRNA (C2). **C**) Fibroblasts were incubated with transfection buffer alone (C1), nonsilencing siRNA (C2), and 50nM of APN siRNA (siAPN) in the presence of rIL-1 and examines for the expression of MMP-1 (left panel). The densitometry analysis of the same blots displays the ratio of MMP-1 to β-actin intensity. **D**) APN and SFN-mediated MMP-1 expressions were analyzed in the primary human skin fibroblasts (F1, F4, and F28) as well as a human fetal lung fibroblast (IMR-90). IMR-90 was the only strain that did not expression APN and was also non-responsive to SFN treatment. CCD-1064 cell (CCD) and squamous cell carcinoma (SCC) lysates were used as APN and MMP-1 positive control, respectively. β-actin level was used as a loading control. (Scale bars, 20 μm).

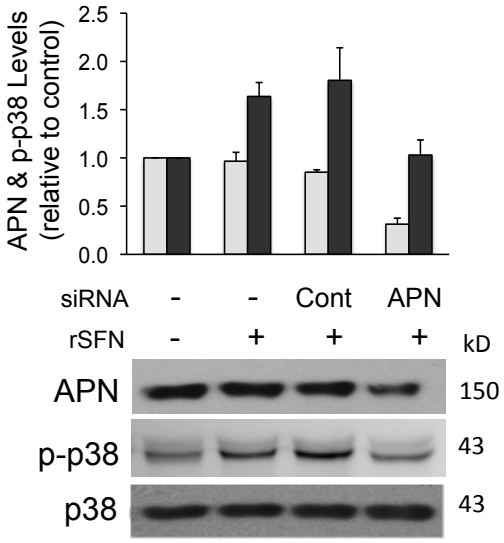

Figure 4.5. APN knockdown blocks SFN-mediated p38 activation.

Fibroblasts were transfected with non-silencing (cont) and APN siRNA (APN) prior to stimulation with rSFN and analyzed by western blot against total p38 MAPK (p38) and phosphorylated form of p38 (p-p38). The relative intensity of APN (open bar) and p-p38 (solid bar) proteins were quantified by densitometry (top panel). The expression level of both proteins was normalized against the control (lane 1) in the absence of siRNA and rSFN.

to rSFN stimulation. The phosphorylation of p38 was analyzed by immunoblotting using a phospho-p38 specific antibody. Both non-transfected and control siRNA-transfected cells demonstrated phosphorylation of p38 following 90 minutes of stimulation by rSFN, whereas the APN knockdown significantly blocked the p38 activation by rSFN (Fig. 4.5). The relative APN and p-p38 levels from two separate experiments were quantified by densitometry analysis (Fig. 4.5, upper panel). This finding suggests that SFN interaction with APN is required to activate the p38 MAPK pathway and to induce MMP-1 expression in the dermal fibroblasts.

SFN / APN Interaction

All 14-3-3 proteins, including SFN, are composed of a dimerization region at the amino-terminus as well as a target-binding region primarily concentrated at the carboxyl-terminal (Tzivion et al., 2001). To examine whether a specific region within SFN is responsible for its association with APN, a number of GST-fusion SFN fragments with deletions in the amino- or carboxyl-terminal were expressed in bacteria and purified with Glutathione-Sepharose (Fig. 4.6A, top panel). The binding capacity of

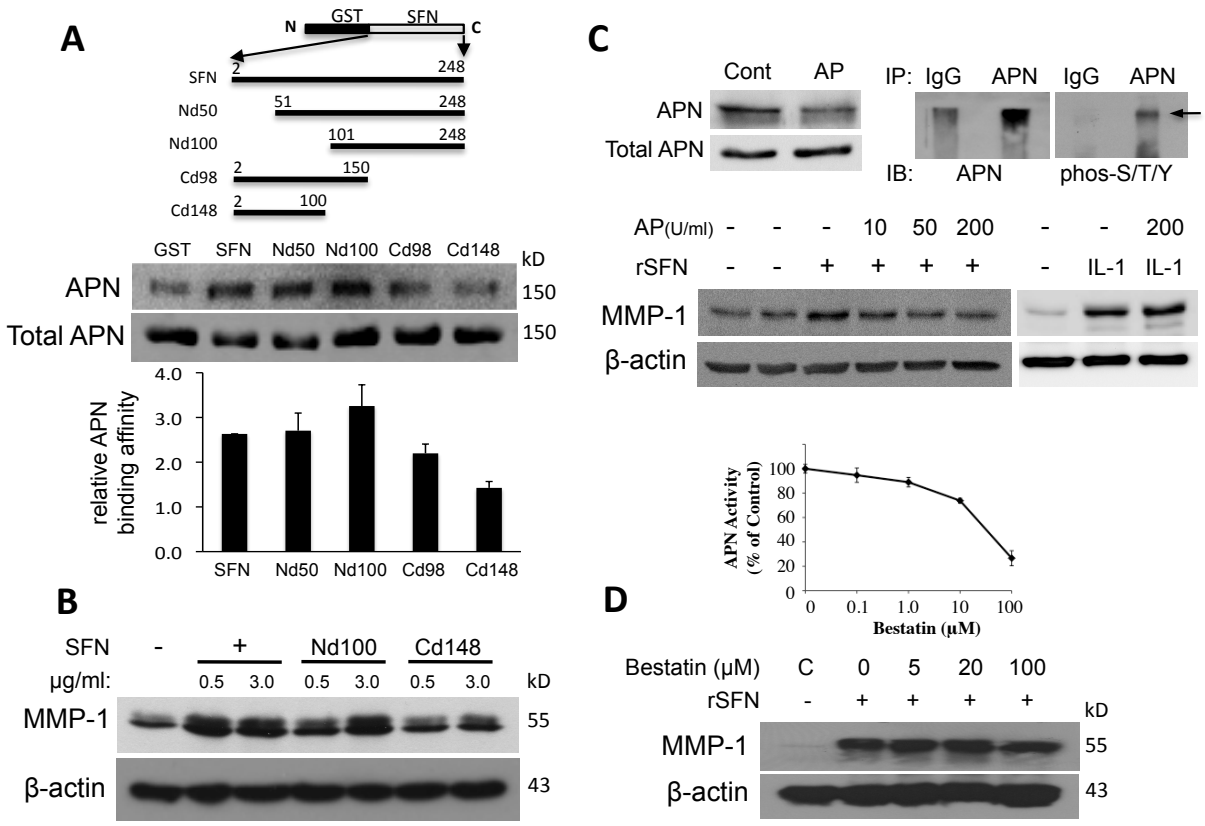

Figure 4.6. The APN/SFN binding characteristics.

A) Deletion of SFN C-terminus abrogates its binding capacity to APN. Schematic representation of various rSFN deletions fused with GST protein is shown at the top. The binding affinity of APN to each GST-rSFN fragment was analyzed by Glutathione-Sepharose affinity column purification and immunoblotting (bottom panel). GST-Sepharose beads were used as the control (GST). The Mean relative APN binding affinity to each SFN fragment was quantified by densitometry (n=2), as displayed in the bar graph. Total APN in a small sample from each group is shown as the loading control. **B**) MMP-1 stimulatory activity of purified SFN, truncated Nd100, and Cd148 recombinant proteins was tested in fibroblasts **C**) Fibroblasts were incubated with alkaline phosphatase (AP) at 200 unit/ml for 30 minutes and the cell lysate was then subjected to SFN affinity column and western blot to analyze APN/SFN binding (top left panel). To test phoshphorylation of APN, IgG or APN immunoprecipitate from fibroblasts were separated on 7% SDS-PAGE. Immunoblotting with anti-phosphoSer/Thr/Tyr antibody detected a band corresponding to the size of APN (arrow). The same blot was used to immunoblot with anti-APN antibody (top right panel). In a separate experiment, cells were incubated with increasing concentration of the phosphatase (10, 50, 200 unit/ml) prior to analysis of SFN-mediated MMP-1 expression (bottom panel). IL-1-mediated MMP-1 expression was also observed in the presence of 200 unit/ml of phosphatase enzyme to evaluate the indirect effect of dephosphorylation on MMP-1 levels. **D**) Blocking APN enzyme active site did not inhibit the SFN-mediated MMP-1 expression. Bestatin inhibitory activity was first tested by an APN enzymatic assay (top panel). Cells were then incubated with various concentrations of bestatin (0, 5, 20, 100 μM) for 1 hour prior to treatment with rSFN and analyzed for MMP-1 expression (lower panel).

APN with each GST-SFN fragment was then examined by a GST pull down assay and analyzed by SDS-PAGE and western blot. The relative binding affinity of APN to each SFN fragment was quantified by densitometry analysis and normalized against the GST group (Fig. 4.6, panel A, bar graph). Deletions in the amino-terminal (Nd50 and Nd100) did not affect the APN binding in contrast to its binding with the full sequence SFN. However, SFN fragments with 98 (Cd98) and 148 (Cd148) amino acid deletions from the carboxyl-terminal displayed a reduced binding affinity to APN (Fig. 4.6A). Accordingly, the SFN MMP-1 stimulatory activity was only diminished in the Cd148 deletion in contrast to the full length or Nd100 deletion (Fig. 4.6B). These findings suggest that the SFN carboxyl terminal is the potential binding site for APN and the active site for MMP-1 stimulatory activity.

14-3-3 proteins regulate many cellular processes by binding to phosphorylated sites in target proteins (Pozuelo et al., 2004). We therefore tested the phosphorylation dependence of SFN binding to APN in the presence and absence of a phosphatase. Suspended intact cells were incubated with an alkaline phosphatase at 200 unit/ml for 30 minutes at 30^{o}C before lysis. Cell lysate of phosphatase-treated and control groups were then passed through a Sepharose-SFN affinity column and the eluate's APN level was analyzed by western blot. Surprisingly, the binding affinity of APN to SFN was reduced following the dephosphorylation of the cell surface proteins (Fig. 4.6C, top left panel). As phosphorylation of APN has not been reported previously, we immunoprecipitated APN protein from primary fibroblasts prior to its separation on SDS-PAGE and western blot analysis. As shown in figure 4.6C (top right panel), an anti-phoshphoSer/Thr/Tyr antibody clearly detected a band corresponding to the size of APN demonstrating the presence of a phosphorylated amino acid residue in this protein. Immunoblotting also detected APN in the same blot at a molecular weight that

matched the previously identified phosphorylated protein. In a separate experiment, live cells were incubated with phosphatase for 30 minutes at 37^{o}C and then washed extensively prior to being treated with rSFN. A western blot analysis of MMP-1 expression revealed that the SFN-mediated upregulation of MMP-1 was attenuated in a dose-dependent manner as a result of pre-incubation with the phosphatase enzyme (Fig. 4.6C, lower panel). To rule out the indirect effect of dephosphorylation, the IL-1-mediated MMP-1 expression was shown to be unchanged in the presence of the phosphatase enzyme.

APN is a zinc-dependent metalloproteinase and a type II integral membrane ectoenzyme (Riemann et al., 1999). Bestatin is a potent inhibitor of APN enzymatic activity that blocks the active site by directly binding with the zinc ion (Addlagatta et al., 2006). To test the role of APN enzymatic activity in SFN-mediated MMP-1 expression, fibroblasts were pre-incubated with various doses of bestatin prior to treatment with rSFN. APN enzymatic assay revealed a dose dependent inhibition by bestatin in fibroblasts (Fig. 4.6D, upper panel). However, the bestatin had no effect on the SFN-mediated MMP-1 expression (Fig. 4.6D, lower panel). This finding suggests that APN enzymatic activity does not play a role in regulation of MMP-1 expression and the enzyme active site is most likely not the binding site for SFN as the presence of bestatin did not block interaction with the ligand.

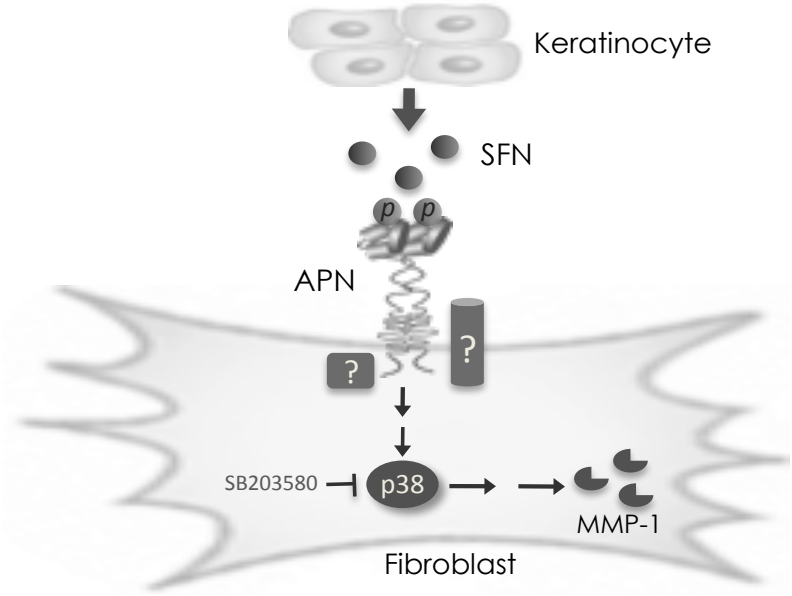

Figure 4.7. The proposed role of APN in the SFN-mediated MMP-1 signaling pathway.

Due to a short cytoplasmic domain, it is hypothesized that APN either acts as a co-receptor or binds with a peripheral plasma membrane signaling molecule prior to the activation of p38 MAPK pathway and MMP-1 expression.

Discussion

The 14-3-3 proteins interact with over 100 binding partners and therefore regulate numerous cellular processes, mainly by inducing conformational changes in the target protein, masking of specific binding or active sites, or the co-localization of two proteins (Yaffe, 2002). Although 14-3-3 proteins are mainly intracellular, their presence in the secretome of various cell lines (Butler and Overall, 2009a) as well as primary keratinocytes has been reported by others (Katz and Taichman, 1999; Leffers et al., 1993), followed by the unexpected discovery of its MMP-1 stimulatory activity by our group (Ghahary et al., 2004). It was recently suggested that due to a lack of conventional signaling peptide, keratinocyte-releasable SFN follows a non-classical secretory pathway via release of exosomes (Chavez-Munoz et al., 2008). In fact, several well-known extracellular proteins including IL-1β and FGF-2, are also released by non-classical (ER/Golgi independent) pathways (Keller et al., 2008; Nickel, 2007). Activation of p38 MAPK signal transduction pathway followed by a rapid increase in the expression of MMP-1 in response to SFN (Lam et al., 2005) as well as its association with fibroblasts cell surface, all suggest the existence of a receptor-mediated transmembrane signaling pathway. In this study, biotin labeling of extracellular domains, serial affinity purification, and LC/MS/MS protein sequencing have led us to identify APN or CD13 as a potential SFN cell surface receptor or co-receptor.

APN is an ectoenzyme metalloproteinase belonging to the M1 family and a type II integral membrane protein with a short N-terminal cytoplasmic domain. It is highly glycosylated, which contributes to at least 20% of its molecular weight. Further, it is expressed in a wide variety of cells, such as myeloid cells, fibroblasts, renal and intestinal epithelia, and endothelial cells (Favaloro, 1991; Nomura et al., 2004;

Riemann et al., 1999). APN has been referred to as a 'moonlighting' protein due to its wide range of functions such as the enzymatic regulation of peptides, viral receptor, tumor-homing peptide receptor, tumor cell invasion, proliferation and apoptosis, motility, antigen presentation, cholesterol uptake, and signal transduction (Mina-Osorio, 2008). Our findings introduce a novel function for APN as a cell surface receptor for the SFN ligand with a link to the signal transduction events leading to MMP-1 expression in fibroblasts. It will be interesting to see whether the interaction between APN and SFN also plays a role in APN's previously identified functions in fibroblasts and other human cells.

The transmission of extracellular signals to MMP-1 gene expression in various cell types is mainly mediated by the family of serine/threonine-specific mitogen-activated protein kinase (MAPK), including ERK1/2 and p38 MAPK subgroups (Nagase and Woessner, 1999). SFN stimulation of fibroblasts leads to a transient activation of p38 MAPK as well as an elevation in c-jun and c-fos - components of the heterodimeric AP-1 transcription factor – resulting in transcription of the MMP-1 gene (Lam et al., 2005). Our findings indicate that APN knockdown is sufficient to block the p38 activation in the presence of rSFN stimulus, suggesting a link between the APN and MAPK signaling cascade. In fact, signal transduction has been proposed as a mechanism of action for a number of APN functions which operate independently of its enzymatic activity (Mina-Osorio, 2008). Santos et al. found that the ligation of APN with monoclonal antibodies (mAb) increases the intracellular free calcium ions in monocytes and induces phosphorylation of ERK1/2, JNK, and p38 MAPK. The same study also demonstrates that inhibitors to tyrosine kinase and phosphatidylinositol 3-kinase block the increase in calcium influx as well as the phosphorylation of MAPK in the presence of the APN mAb (Santos et al., 2000). In addition, APN antibodies which have little

effect on enzymatic activity exhibit a high capacity to induce cell adhesion in monocytes; this effect can be inhibited by SB203580, a p38 MAPK inhibitor (Mina-Osorio et al., 2006). APN has a very short cytoplasmic domain without any known signaling motifs (Luan and Xu, 2007). Therefore, it is possible that the APN interacts with a yet unidentified integral or peripheral signaling molecule in the SFN-mediated activation of p38 MAPK. In fact, Mina-Osorio and her colleagues recently demonstrated the co-immunoprecipitation of Grb2 and Sos with APN from lysate of monocytic cells, suggesting a possible link between APN, Ras, and the ERK1/2 MAPK signaling pathway (Mina-Osorio et al., 2006).

Our finding regarding the phosphorylation-dependent nature of SFN and APN binding is unexpected, mainly because APN is a type II plasma membrane protein with no previously reported phosphorylation sites. Nevertheless, short incubation with a phosphatase is sufficient to block not only the binding between SFN and APN, but also the expression of SFN-mediated MMP-1. The effect also appears to be specific to SFN and APN interaction, as IL-1-mediated MMP-1 expression was not hindered by dephosphorylation of intact cells. Reports show that ECM proteins (osteopontin, sialoprotein, dentin matrix protein-1, phosphophoryn) as well as many secretory proteins (casein, enkephalin precursor, progastrin, chromogranin) become phosphorylated during their movement through the secretory pathway (Boskey et al., 2008; Turner et al., 1993). Therefore, it is possible for APN to become phosphorylated in the cytoplasm prior to its incorporation in the plasma membrane. The detection of a band following immunoprecipitation of APN by an anti-phosphosSer/Thr/Tyr antibody (Fig. 4.6C) provides evidence for a phosphorylated APN. In fact, the extracellular phosphorylation of integral protein's ectodomains is not unprecedented. It has been shown that CD36 ectodomain, a membrane receptor for thrombospondin and collagen,

is phosphorylated by PKC (Asch et al., 1993). Furthermore, Ehrlich and Redegeld have both argued for the presence of adenosine triphosphate (ATP) and ectoprotein kinases in the extracellular space (Ehrlich et al., 1990; Redegeld et al., 1997). Additional investigation is warranted to determine the exact site of phosphorylation on the ectodomain of APN.

Seven mammalian isoforms of 14-3-3 proteins have been described previously; all of which form dimers that bind to high affinity phosphorylation-dependent motifs R(S/X)X*p*SXP and RXXX*p*SXP (Yaffe et al., 1997) as well as atypical phosphorylated and unmodified proteins (Fuglsang et al., 1999; Furlanetto et al., 1997; Waterman et al., 1998). All 14-3-3 proteins are composed of a dimerization region located at the amino-terminus and a target-protein-binding region located primarily in the carboxyl terminus (Tzivion et al., 2001; Wang et al., 1999). The fragmentation study presented here reveals attenuation in the APN binding affinity following deletions in the SFN carboxyl terminus, which indicates a potential binding site for APN. The effect of 14-3-3 dimerization on binding to a target protein seems to be dependent on the strength of their binding affinity. It has been shown that partners with high-affinity binding sites are able to bind to 14-3-3 even in the absence of dimerization (Tzivion et al., 1998; Yaffe et al., 1997). Interestingly, the deletions in the SFN dimerization interface (amino terminus) did not appear to affect APN binding affinity in our study. This could indicate the presence of a high-affinity binding site within the extracellular domain of APN, allowing it to interact with the SFN monomer which lacks the amino terminal dimerization region.

The degradation of ECM by collagenases (e.g. MMP-1) is an essential component of pathophysiological events such as wound repair and tumor invasion. In wound repair, a significant portion of MMP production is carried out by stromal

fibroblasts, partly under the paracrine influence of local epithelial and immune cells (Toriseva and Kahari, 2009). Although the exact nature of APN and SFN interaction in wound healing remains unknown, we show an apparent co-distribution of SFN and APN in both the rabbit and rat tissue during the healing process. The rise in their expression appears to take place following re-epithelialization and during the early stages of the remodeling phase where MMPs play a significant role in reorganization of the newly deposited granulation tissue.

APN or CD13, in combination with CD34 and collagen, is used as a cell surface marker in the identification of fibrocytes. Fibrocytes are peripheral blood-borne fibroblast-like cells that rapidly enter the injured site and synthesizes ECM (Abe et al., 2001; Bucala et al., 1994). However, Sorrell and colleagues have demonstrated that the frequency and distribution of these cells do not account for the APN pattern in adult skin, underlining fibroblasts as another potential source of APN (Sorrell et al., 2003). In line with our in vivo findings, Mori et al. demonstrated an increase in the APN levels in wounded versus uninjured skin of balb/c mice (Mori et al., 2005). This effect might be attributed to the reported APN enzymatic activity in ECM degradation (Saiki et al., 1993) or APN'S role in cellular motility and migration (Chang et al., 2005; Fukasawa et al., 2006). Based on our findings, it is also reasonable to speculate that the increased expression of APN is a response which augments cellular sensitivity to MMP-stimulating factors such as keratinocyte-derived SFN.

In cancer, it is the neighboring stromal fibroblasts in addition to malignant epithelial cells that contribute to the MMPs repertoire responsible for the proteolytic breakdown of the peritumoral ECM (Egeblad and Werb, 2002; Overall and Lopez-Otin, 2002). Furthermore, stromal cells in tumors appear to be activated by paracrine factors released from malignant epithelial cells (Mueller and Fusenig, 2004). Cell surface APN

has been found to play a critical role in tumor cell metastasis and is over-expressed in wide range of solid tumors (Luan and Xu, 2007). Interestingly, an immunohistochemical examination of human lung carcinoma tissue revealed that the majority of APN-positive cells were in fact stromal fibroblasts (Ichimura et al., 2006). It is therefore tempting to speculate that epithelial-released factors such as SFN can act through the APN receptor to induce MMP production by neighboring stromal fibroblasts. If this hypothesis is correct, then the reported increase in both APN and MMPs expression in the tumor microenvironment are connected events and not a coincidence. In fact, our data shows a significant increase in fibroblasts' APN expression when co-cultured with keratinocytes or treated with keratinocyte-derived conditioned medium (unpublished).

In conclusion, the data presented in this paper indentifies APN as a novel cell surface receptor for SFN ligand and proposes a new function for this type II integral membrane protein in the SFN-mediated activation of p38 MAPK and MMP-1 expression in fibroblasts. As indicated in fig. 4.7, our findings also raise a few key questions about the exact mechanism of APN activation by SFN. Does APN act alone or as a co-receptor? Where is the SFN binding site on the APN ectodomain? Where is the exact phosphorylation sites on APN? Answers to these questions could pave the way for targeting APN in the treatment of disorders marked by an imbalance in MMP expression.

Materials and Methods

Cell Culture and Reagents

Following informed consent, foreskin samples were obtained from patients undergoing elective surgery under local anesthesia. Written informed consent was obtained from each participant, and the study was approved by the University of British Columbia Hospital Human Ethics Committee and conducted according to the Declaration of Helsinki Principles. Human primary dermal fibroblasts were harvested as previously described (Ghaffari et al., 2006b). Fibroblasts, balb/3T3, and 293T cells were grown in DMEM supplemented with 10% fetal bovine serum (FBS) and antibiotic-antimycotic preparation (100 U/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B). The human fetal lung fibroblast cell line IMR-90 (ATCC, Manassas, VA, USA) were cultured in DMEM plus 10% FBS. The CCD-1064Sk cell lysate (Santa Cruz Biotechnology, Santa Cruz, CA) was used as a positive control for APN protein expression. Calf intestinal alkaline phosphatase (AP) (New England Biolabs, Ipswich, MA) was used for protein dephosphorylation experiment according to manufacturer's instructions. Bestatin hydrochloride (Sigma Chemicals, Oakville, ON, Canada) was used as a competitive and specific inhibitor of APN enzymatic activity. Cell lines isolated from human squamous cell carcinoma (SCC) were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and used as positive control for MMP-1 expression.

Recombinant Protein Expression

Expression vectors for human stratifin have been described previously (Li et al., 2007). In brief, human full-length stratifin gene was subcloned into prokaryotic expression vectors pGEX-6P-1 (GE Healthcare Bio-Sciences, Quebec, Canada) and pET-28a(+) (Novagen, Madison, WI) for fusion protein tagged with glutathione S-transferase (GST) and histidine (his), respectively. The his-tagged SFN was purified using a Ni-Sepharose column according to manufacturer's instructions (GE Healthcare Bio-Sciences, Quebec, Canada). Different fragments of SFN fused to GST were also prepared as previously described (Li et al., 2007). Fusion proteins containing peptide fragments of SFN were referred as Nd50 (amino acid 51-246); Nd100 (amino acid 101-246); Cd98 (amino acid 1-150); and Cd148 (amino acid 1-100). GST-tagged SFN fragments bound to glutathione-Sepharose (GE Healthcare Bio-Sciences, Quebec, Canada) was then used in affinity binding experiments.

Cell and Ligand Binding Assays

To examine the cell binding activity of SFN protein, $5x10^4$ primary human fibroblasts were plated in a 12-well plate on a glass cover slip overnight. The cells were then serum starved for 3 hours prior to the addition of protein. Recombinant stratifin (rSFN) in PBS was labeled with Sulfo-NHS-LC-Biotin (Pierce, Rockford, IL) in a 3:1 ratio (Biotin:rSFN) as instructed by the manufacturer. Cells were blocked with 1.0% bovine serum albumin (BSA) in DMEM for 30 minutes prior to the addition of Biotin-SFN at 1.0 µg/ml for 15 minutes at 4^oC and then washed extensively with PBS. Biotin labeled glutathione S-transferase (biotin-GST) was used a negative control. Competition assay was performed by pre-incubating cells with 200 µg/ml of rSFN for 30

minutes at 4°C prior to adding biotin-SFN. Biotin was detected by immunofluorescence microscopy using streptavidin-alexa 568 (Invitrogen, Eugene, OR). To perform a ligand-receptor binding assay, rSFN was labeled with ^{125}I (New England Nuclear, Boston, MA) and Iodo-Gen® reagent (Pierce, Rockford, Illinois) according to the manufacturer's instructions and as previously described (Uludag et al., 1999). Following a wash with cold PBS, fibroblasts were incubated with ^{125}I-SFN ($5x10^5$ cpm) in the absence and presence of increasing concentration of unlabeled rSFN for 2 hours at 4°C. Free radioactive was then washed with cold PBS and cell were harvested by lysis buffer and total radioactive binding was measured in a liquid scintillation counter (Beckman coulter, Mississauga, ON, Canada).

Collection of Cell Lysate and Fractions

For total cell lysate, following an extensive wash with PBS, cells were disrupted in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1.0 mM EDTA, 1.0 mM EGTA, 0.025% NaN_3, 1% Triton X-100, 0.5% IGEPAL, protease inhibitor cocktail [Sigma Chemicals, Oakville, ON, Canada], at pH 7.5) and centrifuged at 13,000 rpm for 15 minutes. For preparation of the cell fractions, cells were released by EDTA and collected in tissue disruption buffer (150 mM NaCl, 20 mM Na_2HPO_4, 2 mM NaH_2PO_4, 20% glycerol, 2 mM sodium orthovanadate and protease inhibitor cocktail) and homogenized in a Dounce homogenizer glass (Fisher, Ottawa, ON, Canada). Homogenized preparation was further disrupted by sonication on ice and then centrifuged at 10,000 rpm to remove nuclei and cell debris. The supernatant was then centrifuges at 100,000 x g for 90 minutes at 4°C. The soluble cytoplasmic fraction (supernatant) was collected and the remaining pellet (total membrane fraction) was solubilized in lysis buffer.

Purification of Cell Surface Stratifin-Binding Proteins

Following release of fibroblasts with EDTA, cell surface proteins of intact cells were biotinylated using 2 mM water-soluble and membrane impermeable EZ-Link sulfo-NHS-LC-biotin reagent (Pierce, Rockford, IL) in PBS for 30 minutes at 4^{o}C. Free biotin was quenched and removed by three washes of cold PBS + 100 mM glycine. To obtain total cell lysate, cells were disrupted in lysis buffer (50mM Tris-HCl; 10mM EDTA; 5mM EGTA; 0.025% NaN_3; 1% Triton-X100; 0.5% IGEPAL CA-630; protease inhibitor) for 30 minutes by gentle rotation at 4^{o}C and then cleared by centrifugation at 18,000 x g for 10 min at 4^{o}C. Affinity purified his-SFN was dialyzed against PBS and then conjugated with CNBR-activated Sepharose 4B (GE Healthcare Bio-Sciences, Quebec, Canada) at a ratio of 10 μmole ligand to 1.0 ml of Sepharose, according to manufacturer's instructions. A deactivated and blank Sepharose-4B was used as control. Biotin-labeled cell lysate was then applied to 30 μl bed of Sepharose-SFN or Sepharose alone for 2 hours at 4^{o}C with gentle rotation. Beads were then washed 7 times with PBS + 1% Tween-20, eluted with 100 mM glycine at pH 2.0. The glycine sample was neutralized with 1 M Tris solution, applied to 30 μl of Avidin-Agarose (Sigma-Aldrich, Oakville, ON, Canada) for 1 hour at 4^{o}C, and washed 3 times with PBS + 1% Tween-20. Biotinylated proteins were then eluted by SDS loading buffer (0.35 M Tris-HCl, 10.28% (w/v) SDS, 36% (v/v) glycerol, 5% 2-mercaptoethanol, 0.012% (w/v) bromophenol blue) with 10 minutes incubation at room temperature followed by 5 minutes boil. Eluted proteins were resolved by 10% precast keratin-free SDS-PAGE (Bio-Rad, Hercules, CA) and either immunoblotted with streptavidin-HRP or stained with ProteoSilver silver (Sigma-Aldrich, Oakville, ON, Canada). For protein sequencing, purified biotinylated proteins excised from silver-stained gels were subjected to trypsin digestion and identified by

API Q STAR PULSARi Hybrid LC/MS/MS at University of British Columbia MSL/LMB Proteomics Core Facility. The peptide sequences were analyzed using Mascot search engine and Analyst software (Applied Biosystems, Foster City, CA, USA) against the non-redundant NCBI database.

Western Blot

Cell lysates, cell fractions, or purified proteins were transferred to PVDF membrane with Mini Trans-Blot Cell (Bio-Rad, Hercules, CA) after separation by SDS-PAGE. Immunoblotting was carried out with the following the antibodies: 1:5000 streptavidin-HRP (Pierce, Rockford, IL); 1:5000 anti-GAPDH (R&D Systems, Minneapolis, MN); 1:500 anti-APN [3D8] (Santa Cruz Biotechnology, Santa Cruz, CA); 1:1000 anti-stratifin (Medicorp, Montreal, QC, Canada); 1:500 anti-MMP-1 (R&D Systems, Minneapolis, MN); 1:1000 anti-phosphoserine/threonine/tyrosine (Abcam, Cambridge, MA); 1:20000 anti-β-actin (Sigma-Aldrich, Oakville, ON, Canada); 1:3000 of anti-mouse IgG-HRP and anti-rabbit IgG-HRP (Bio-Rad, Hercules, CA). Protein bands were visualized by an ECL detection system (Santa Cruz Biotechnology, Santa Cruz, CA).

Immunoprecipitation

Fibroblasts were released by EDTA and washed by PBS prior to incubating with rSFN for 30 minutes at 4^{o}C. Soluble cell lysate was collected in lysis buffer, cleared by centrifugation, and immunoprecipitated by 2 μg/ml monoclonal anti-APN [3D8], 2 μg/ml polyclonal anti-APN [H300] (Santa Cruz Biotechnology, Santa Cruz, CA), or anti-SFN antibody at 4 μg/ml for 1 hour at 4^{o}C with gentle rotation. Non-immunized rabbit and mouse IgG were used as controls. Immune complexes were collected on protein G-

Sepharose beads (50 µl/ml) (GE Healthcare Bio-Sciences, Quebec, Canada) for 1 hour at 4^{o}C, washed five times with PBS.T (0.1% Tween-20), eluted by boiling in sample buffer, and resolved on SDS-PAGE for western blot analysis.

siRNA Knockdown of Aminopeptidase N

APN protein knockdown was tested by two different siRNA oligonucleotide sequences; si_ANPEP_1, CCGAAATGCCACACTGGTCAA, and si-ANPEP_2, CCGGGTGAACTACGACGAAGA purchased from Qiagen (Valencia, CA). A non-silencing siRNA, with the same GC content as APN siRNA, conjugated with Alexa Fluor 488 was used as control and for monitoring transfection by fluorescent microscopy. HiPerfect transfection reagent was used according to the manufacturer's recommendations (Qiagen). Proliferating fibroblasts were harvested and 1x10^{5} cells plated in 6-well dishes containing 5, 10, or 50 nM of siRNA oligonucleotide. The medium was replaced 24 hours post-transfection and the cells were treated with rSFN (2.5 µg/ml) at 72 hours post-transfection.

In Vivo Wound Healing Models

All animals were cared for in accordance with the guidelines of the Canadian Council on Animal Care and with approval from the University of British Columbia Animal Welfare and Policy Committee. Female New Zealand white rabbits weighing approximately 4-5 Kg were used in the rabbit ear fibrotic wound model. After the rabbits were anaesthetized, 8-mm diameter full-thickness wounds down to the perichondrial membrane were created as previously described (Lee et al., 2005; Morris et al., 1997). On day 16 post wounding, when reepithelialization was complete, the rabbits were sacrificed and the wounds harvested and fixed in 2% PFA before

embedding in paraffin. Normal and non-injured skin on the back of the same ear was used as the control. Sprague-Dawley rats were used as described previously in a longitudinal wound healing study to monitor levels of APN and SFN at different stages of dermal repair (Li et al., 2006). In brief, six full-thickness excisional wounds (6.0 mm diameter) were made on the dorsal surface of rat skin and allowed to heal for up to 30 days. Animals were euthanized on post-wound day 8, 11, 14, 17, and 30 and a 4.0 mm punch biopsy was obtained from the injured area and frozen at -80^{o}C. Tissue samples were weighed out and homogenized in lysis buffer with a tissue homogenizer (Qiagen, Valencia, CA) and glass pestle followed by sonication. Bradford protein assay was performed and equal amount of tissue lysate (75 µg) was separated by SDS-PAGE as described above. Tissue lysates from three random wounds were pulled together for this experiment.

Immunofluorescence Microscopy

Cultured fibroblasts were grown on glass coverslips, fixed with 1% paraformaldehyde (PFA) for 15 minutes on ice, and blocked with 2% BSA in PBS. For staining the plasma membrane, wheat germ agglutinin-Alexa 488 (Invitrogen, Eugene, OR) was added (1:2000 dilution) prior to fixing the cells. APN was detected using mouse anti-APN 3D8 antibody (Santa Cruz) followed by Alexa 488-conjugated goat anti-mouse IgG (Invitrogen, Eugene, OR). Biotin-labeled SFN was detected with Alexa 568-conjugated streptavidin (Invitrogen, Eugene, OR). To observe the APN protein expression in the normal and injured rabbit skin tissue, paraffin-embedded sections fixed in 2% PFA were incubated with 1:50 dilution of 3D8 anti-APN antibody followed by goat anti-mouse Alexa 488 secondary (1:2500 dilution). Samples were mounted in ProLong Gold antiface reagent with DAPI (Invitrogen, Eugene, OR) and examined by a

Zeiss Axioplan-2 fluorescent microscope and Northern Eclipse image analysis software. A second tissue section was stained with haematoxylin and eosin (H&E) for histological analysis.

Enzymatic Assay

The quantitative assays for APN on cultured human dermal fibroblasts were performed according to previously published methods (Raynaud et al., 1992). In brief, all assays were performed in triplicate using Ala-4 nitroanilide (Ala-pNA, Sigma-Aldrich, Oakville, ON, Canada) in phosphate buffer pH 7.4 as chromogenic substrate. Ala-pNA (100 µl) was added in a concentration of 5mM to 10^5 cells. Reactions were stopped by adding 900 µl acetate buffer (1.0 M, pH 4.4) after 60 minutes of incubation at 37^{o}C. After centrifugation (2 minutes at 10,000xg), the absorbance of the supernatant was detected spectrophotometrically at 405 nm.

Statistical Analysis

Data were expressed as mean ± SD and analyzed with one-way analysis of variance with Tukey–Kramer multiple comparison test among different groups where indicated. P-values less than 0.05 were considered statistically significant in this study. All studies were repeated at least three times unless indicated otherwise.

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Chapter 5. Conclusion and Suggestions for Future Work

General Discussion

The complexity of multicellular organisms demands elaborate communication networks between cells of different compartments in order to direct tissue development and repair. Early tissue recombination studies elegantly demonstrated the presence of a 'dynamic reciprocity' between neighboring cells, where embryonic mesoderm or adult mesenchyme can influence the identity and function of embryonic ectoderm and adult epithelium, respectively (Nelson and Bissell, 2006; Parmar and Cunha, 2004). Equally in tissue repair, delay or disruption in communication between epithelial and stromal cells has been linked to the onset of fibrosis (Deitch et al., 1983; Machesney et al., 1998). In skin tissue, the interaction between skin epithelial and stromal cells is predominately governed by local paracrine soluble factors. Evidence suggests that epidermal-dermal interface has a significant impact in the regulation of collagen production and remodeling (Hartwell et al., 2009; Werner et al., 2007). In an attempt to identify keratinocyte-derived soluble factors involved in the regulation of key ECM protein in dermal fibroblasts, our group identified SFN as a potent MMP-1 stimulatory factor. Identification of keratinocyte-releasable SFN raised further questions on the effect of SFN in the regulation of other ECM proteins as well as the exact nature of its transmembrane signaling in fibroblasts.

To address the first question, as described in chapter 2, we utilized an ECM pathway-specific microarray to compare the gene expression profile in fibroblasts co-cultured with keratinocyte or stimulated with recombinant SFN. Majority of proteins upregulated by SFN belong to the MMP family. Analysis of several MMP gene promoters revealed the involvement of AP-1 binding site for transcriptional activation with jun and fos transcriptional factors being mainly responsible for interacting with the AP-1 binding site (Yan and Boyd, 2007). We have previously shown a transient upregulation of Elk4/Sap1 transcription factor leading to rapid expression of c-jun and c-fos in fibroblasts stimulated with SFN (Lam et al., 2005). Therefore, it is likely that upregulation of MMP-1, 3, and 10 by SFN are also mediated by c-jun/c-fos expression and AP-1 transcription factor complex.

Similarly, IL-1 released from keratinocytes has also been shown to induce the expression of MMP-1 and MMP-3 through activation of AP-1 transcription factor (Barchowsky et al., 2000; Hui et al., 1998; Maas-Szabowski et al., 2000). Our data indicates that fibroblasts co-cultured with keratinocytes or treated with KCM demonstrate a higher level of MMP-1 expression compared to cells treated with physiological levels of recombinant SFN. This could in part be explained by the presence of other MMP-1-inducing factors released by keratinocytes such as IL-1. To further clarify IL-1's contribution in KCM-mediated MMP-1 induction, we neutralized IL-1R with IL-1R antagonist (IL-1Ra) prior to stimulation with KCM and only managed to block about 50% of KCM-mediated MMP-1 expression in fibroblasts (Appendix, Figure A.I.1). Interestingly, it appears that the expression

of IL-1 (Maas-Szabowski et al., 2000) and SFN (Figure 3.3) significantly increases in the last stages of wound healing where dermal tissue is going through remodeling. In fact, lower epidermal IL-1 expression has been reported in HTS tissue compared with normal mature scar (Niessen et al., 2001). It remains to be determined if SFN also follows a similar pattern in HTS. Considering that HTS tissue is characterized by excessive deposition of collagen (mainly type I and III), fibronectin, and proteoglycans (Scott et al., 2000), it requires a wide range of MMPs to specifically target each of these ECM proteins in order to resolve the excessive scar tissue. It is therefore promising to observe SFN-mediated upregulation of MMP-1 and MMP-3 in fibroblasts, as they can collectively initiate the efficient breakdown of most ECM fibrous proteins found in HTS tissue. In fact, recent animal studies revealed the delivery of SFN can significantly reduce the scar elevation in a rabbit ear fibrosis model (Rahmani-Neishaboor et al., 2010).

Previous co-culture studies have indicated that presence of keratinocytes reduces the expression level of type I collagen in dermal fibroblasts (Garner, 1998; Harrison et al., 2006). Our findings also reveals a reduced expression of type I collagen in fibroblast treated with KCM (Figure 2.3). Keratinocyte-releasable SFN had no effect on the collagen expression by fibroblasts, although rSFN was able to reduce fibronectin expression in these cells. As the exact nature of collagen mediatory factors secreted by keratinocyte is not well understood at this time, in chapter 4 we made an attempt to characterize this factor in KCM. Our findings revealed a factor with molecular weight between 30

and 50 kDa responsible for down-regulation of collagen expression in fibroblast and provided further evidence for the role of keratinocytes-fibroblasts communication in control of ECM production in skin. Interestingly, a recent report conducted by Kim and colleagues revealed that interleukine-18 (IL-18) significantly inhibits collagen type I and III expression in dermal fibroblasts (Kim et al., 2010). As IL-18 is highly expressed in keratinocytes, it could potentially be the factor we previously termed keratinocyte-derived collagen inhibitory factor (KDCIF). As fibroblasts derived from HTS (Ghahary et al., 1993; Phan et al., 2002) as well as normal fibroblasts co-cultured with HTS-derived keratinocytes (Lim et al., 2002) display significant increase in collagen production, it is important to identify keratinocyte-derived factors involved in regulation of collagen production. A combination treatment with SFN and KDCIF can be more effective in reducing the excess collagen present in HTS tissue and induce the remodeling of existing granulation tissue.

To address the second question on the nature of transmembrane signaling by SFN, in chapter 3 we utilized biotin labeling of fibroblast cell surface protein ectodomains in combination with serial affinity purification in order to identify aminopeptidase N or CD13 (APN) as a cell surface receptor for SFN. APN has been shown to function as a viral receptor in infections by human coronavirus 229E (Kolb et al., 1998) and cytomegalovirus (Soderberg et al., 1993). Treatment of cells with an anti-APN antibody significantly reduced the uptake of virus in these studies. APN also acts as a receptor for tumor-homing peptide asparagine-glycine-arginine (NGR) in tumor blood vessels as well as

other vessels undergoing angiogenesis (Pasqualini et al., 2000). To our knowledge, the results presented in chapter 4 identified, for the first time, a novel function for APN as a receptor linking the signal from an extracellular ligand to p38 MAPK signaling pathway and MMP-1 expression in dermal fibroblasts.

A link between APN and signal transduction pathways has been proposed previously in homotypic aggregation of monocytic cells (Mina-Osorio et al., 2006; Santos et al., 2000). By using specific inhibitors, Mina-Osorio and colleagues have proposed a link between APN and Src kinase, PI-3K, MEK-1, and p38 MAPK in APN-mediated homotypic aggregation of monocytic cells induced by anti-APN monoclonal antibodies. Furthermore, the study reveals co-immunoprecipitaton of APN, Grb2, and Sos provinding a possible link between APN and MAPK through the activation of Ras (Mina-Osorio et al., 2006). Interestingly, a role for Ras has been previously suggested in the activation of p38 MAPK by IL-1 (Palsson et al., 2000). Our data show that the transient knockdown of APN by siRNA can block SFN-mediated activation of p38 MAPK. Therefore, a potential signaling path including APN, Grb2, Sos, Ras, and p38 MAPK can be implicated in SFN-mediate MMP-1 induction. Further investigation is warranted to test the role of Grb2, Sos, and Ras in the activation of p38 MAPK and upregulation of MMP-1 expression by SFN and APN.

Grb2 is an adapter protein that interacts with tyrosine phosphorylated motifs through its SH2 domain (Downward, 2001). In the absence of a phosphorylation site in APN short cytosolic domain, it is very likely that APN acts as an auxiliary or co-receptor in activation of Grb2 and Sos. In this thesis, we

proposed that APN acts as a potential receptor for extracellular SFN and the ligand-receptor complex take part in activation of p38 MAPK. However, the cytoplasmic domain of APN is comprised of only 8-10 amino acid residues with no known signaling motifs at this time (Luan and Xu, 2007). In the absence of a phosphorylation site in APN cytosolic tail, it is very likely that APN acts as a ligand-binding receptor within a larger receptor complex. There are several examples of receptors with short cytoplasmic tails that lack signaling domains but play a central role in the activation of signal transduction pathways. Probably the most recognized examples are the antigen receptors on B cells (BCR) and T cells (TCR), which are oligomeric complexes that can be separated into the ligand-binding and signal-transducing subunits (Weiss and Littman, 1994). In BCR complex, the antigen-binding receptor is a membrane-bound immunoglobulin (mIgM) with a very short cytoplasmic tail. In order to generate signal upon binding with its ligand (antigen), the heavy chain of mIgM has to interact with Igα and Igβ on the B-cell surface. The cytoplasmic domains of Igα and Igβ contain immunoreceptor tyrosine-based activation motifs (ITAMs) that become phosphorylated by Src-family tyrosine kinases in order to transmit the extracellular signal into the cell (Monroe, 2006). In T cells, α and β chains make up the antigen-binding subunits of the TCR complex and interact non-covalently with signal-transducing subunits CD3γ, δ, ε and TCRζ to form a functional receptor (Pitcher and van Oers, 2003).

An alternative mechanism is for APN to present its ligand to a functional cell surface receptor with the ability to activate signaling pathways in the

cytoplasm. For example, type III TGFβ receptor (TGFβRIII) or β-glycan is a transmembrane proteoglycan that binds and concentrates TGFβ at the cell surface and facilitates its binding to type II TGFβ receptor leading to the activation of smad3 and smad4 transcription complex (Gordon and Blobe, 2008). The TGFβRIII with its cytoplasmic domain deleted is still capable of binding to TGFβ and present to TGFβRII through interaction between their respective extracellular domains (Blobe et al., 2001).

The α subunit for IL-2 receptor (IL-2Rα) or CD25 is another example of a ligand-binding subunit of a larger complex responsible for binding to IL-2 cytokine. The cytoplasmic tail of IL-2Rα consists of 13 amino acids and is not capable of transducing a signal. IL-2R complex is comprised of three noncovalently linked chains which include IL-2Rα, IL-2Rβ or CD122, and IL-2Rγ or CD132 (Wang et al., 2005). Only IL-2Rβ and IL-2Rγ participate in signaling by interacting with enzymes such as Janus kinase 1 (JAK1) and JAK3 (Russell et al., 1994). IL-2Rα binds with IL-2 ligand with much higher affinity in comparison to the other two receptor subunits. However, the fact that IL-2Rα displays fairly weak interaction with IL-2Rβ and IL-2Rγ suggests that IL-2Rα serves as a ligand carrier. For efficient cell signaling, extracellular IL-2 must be concentrated at the cell surface to minimize its loss by diffusion. The high affinity of IL-2Rα for IL-2 as well as its large excess over IL-2Rβ and IL-2Rγ allows for IL-2 capture and presentation to IL-2Rβ/γ complex (Stauber et al., 2006). It has been suggested that a stepwise assembly might lead to a greater number of high-affinity signaling

complexes in comparison to a preformed and fully assembled IL-2R complex (Forsten and Lauffenburger, 1994).

The majority of studies in transcriptional upregulation of APN have been conducted in monocytes or endothelial cells. APN mRNA and protein expression has been shown to be upregulated in response to hypoxia, bFGF, VEGF, TGFβ, and down-regulated by IL-10 (Bhagwat et al., 2001; Kehlen et al., 2004). In fibroblasts, Sorrell and colleagues demonstrated increased APN enzymatic activity following stimulation with IL-1 and IFNγ, without confirming the transcriptional expression (Sorrell et al., 2003). Our results reveal that SFN not only binds with APN, but it also upregulates its expression at the protein level (Figure A.I.2, panel a). Fibroblasts co-cultured with keratinocytes or stimulated with KCM displayed the same increase in CD13 expression. Interestingly, SFN appears to be the primary keratinocyte-derived factor responsible for induction of APN expression in fibroblasts, as an SFN-depleted KCM did not increase the APN expression (Figure A.I.2, panel b and c). This finding is pointing to the presence of a potential positive feedback loop, where the presence of SFN (ligand) increases the number of receptors (APN) expressed at cell surface in order to maintain cellular sensitivity to the external stimuli leading to high levels of MMPs expressed by the target cell.

Although the exact nature of SFN and APN interaction in wound healing process remains to be investigated, an increase in protein expression as well as co-distribution of the receptor and ligand following wound epithelialization, represents a potential role in ECM remodeling. After wound closure, the task of

reorganizing the newly deposited granulation tissue falls mainly on dermal fibroblasts, as they are the primary source for synthesis of ECM as well as MMPs responsible for their degradation in the dermis. Therefore, it is likely that SFN and APN interaction in upregulation of MMPs plays an important role in remodeling phase of tissue repair. The same interaction may also exist between tumor epithelial cells and stromal fibroblasts not unlike what is observed in wound healing. In fact, in the past, tumors have been described as wounds that never heal (Dvorak, 1986). Considerable attention has been given to neighboring stromal fibroblasts in cancer for their role in production of MMPs and breakdown of peritumoral ECM (Overall and Lopez-Otin, 2002). Accordingly, most of the evidence for a link between APN and ECM degradation in tissue comes from cancer research. These include: 1) elevated levels of APN in highly invasive tumors (Luan and Xu, 2007), 2) expression of an enzymatically inactive APN mutant in lung cancer cells also enhanced cell migration and invasiveness (Chang et al., 2005), 3) APN transfection into human melanoma cells increased degradation of collagen and invasion in ECM (Fujii et al., 1995), and 4) APN-positive cells in lung carcinoma were mainly associated with the stroma (Ichimura et al., 2006). Therefore, identification of SFN as a ligand for APN may provide a link between the augmented APN levels in tumor cells and an increase in ECM degradation and invasiveness of the cancer.

In summary, in this thesis we were able to 1) confirm upregulation of MMPs with more specific focus on MMP-1 and MMP-3 in fibroblasts stimulated with SFN, 2) identify APN as a potential receptor for extracellular SFN and its

role in SFN-mediated p38 MAPK activation and MMP-1 upregulation, 3) further characterize the role of keratinocyte-releasable factors in the regulation of type I collagen production by dermal fibroblasts.

Suggestions for Future Work

Although, we believe that our work has shed some light into the nature of keratinocyte / fibroblast cross-talk and its role in skin homeostasis as well as wound healing, obviously considerable amount of work is needed to move this idea from bench to bedside. The following are some of the suggestions that may improve upon our current finding (in no particular order).

i. In chapter 2, we confirmed upregulation of MMPs following stimulation by SFN using microarray analysis. We further demonstrated a dose- and time-response in MMP-3 expression following induction by SFN. The SFN-mediated induction of other MMPs identified here (MMP8, 10, 24) should also be further characterized at transcriptional as well as functional level.

ii. In chapter 2, the gene expression profile of fibroblast induced with SFN revealed upregulation in integrins α1 and β8 along with down-regulation of fibronectin-1 genes. Both integrins and fibronectin play an important role in wound healing and the effect of SFN on their expression and function should be studied.

iii. In chapter 3, we characterized a keratinocyte-releasable collagen inhibitory factor with a molecular weight of 30-50 kDa. Further purification of the active fraction by ion-exchange and size-exclusion chromatography (FPLC) is recommended. A role for IL-18 in down-regulation of collagen

expression was suggested (Kim et al., 2010) and further examination on its activity in our system is also warranted.

iv. In chapter 4, we showed an increase in the expression as well as co-distribution of SFN and APN in rabbit ear and rat wound healing models. It would be interesting to investigate the expression of APN in human dermal fibrotic disorders such as HTS and keloids and compare with uninjured skin and mature scar tissue. Study the role of APN in wound healing and scarring by using an APN-null mice model, which recently was characterized by Dr. Pasqualini's group (Rangel et al., 2007).

v. In chapter 4, figure 4.4, we showed minimal expression of APN in lung IMR-90 cells. If the difficulty in transfecting these cells can be overcome, it would be an ideal model for the gain of functional study on the role of APN in SFN-mediated MMP-1 induction.

vi. In chapter 4, our data indicated that the c-terminal of SFN is important in binding with APN. In order to design small therapeutic peptides to induce MMP expression or block the receptor activity, the exact binding sites on SFN and APN must be characterized. More in depth deletion studies using different fragments for SFN and APN affinity binding are recommended. The structure of APN consists of 7 domains (Luan and Xu, 2007). Cloning and expression of each domain in a mammalian cell may be a good starting point for the SFN affinity studies.

vii. Phosphorylation dependence of APN in binding with SFN and detection of a phosphoSer/Thr/Tyr residue in the APN IP requires further attention. Phosphorylation of APN might also be confirmed by mass spectroscopy. In fact, a combination of site-directed mutagenesis, $^{(32)}$P labeling, and phosphorylation analysis by mass spectroscopy might be useful in identifying a phosphorylation site in APN. This may also help with previous recommendation, as phosphorylation site may be within the binding site of APN.

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Appendix I

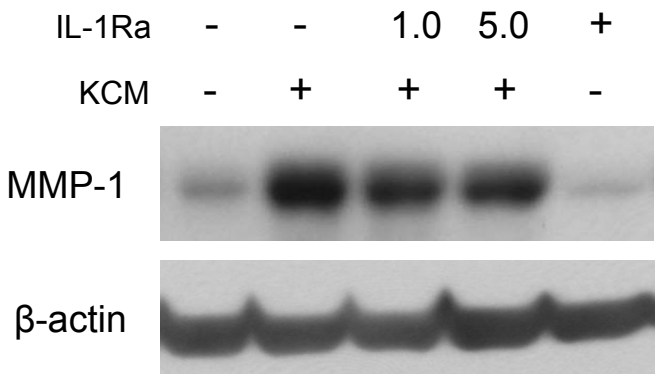

Figure A.I.1. IL-1R antagonist does not completely block the KCM-mediate MMP-1 expression in fibroblasts.

Cells were pre-incubated with IL-1Ra at 1.0 and 5.0 μg/ml, washed extensively with PBS buffer and then treated with KCM overnight. MMP-1 protein expression was analyzed by western blot and β-actin used as the loading control.

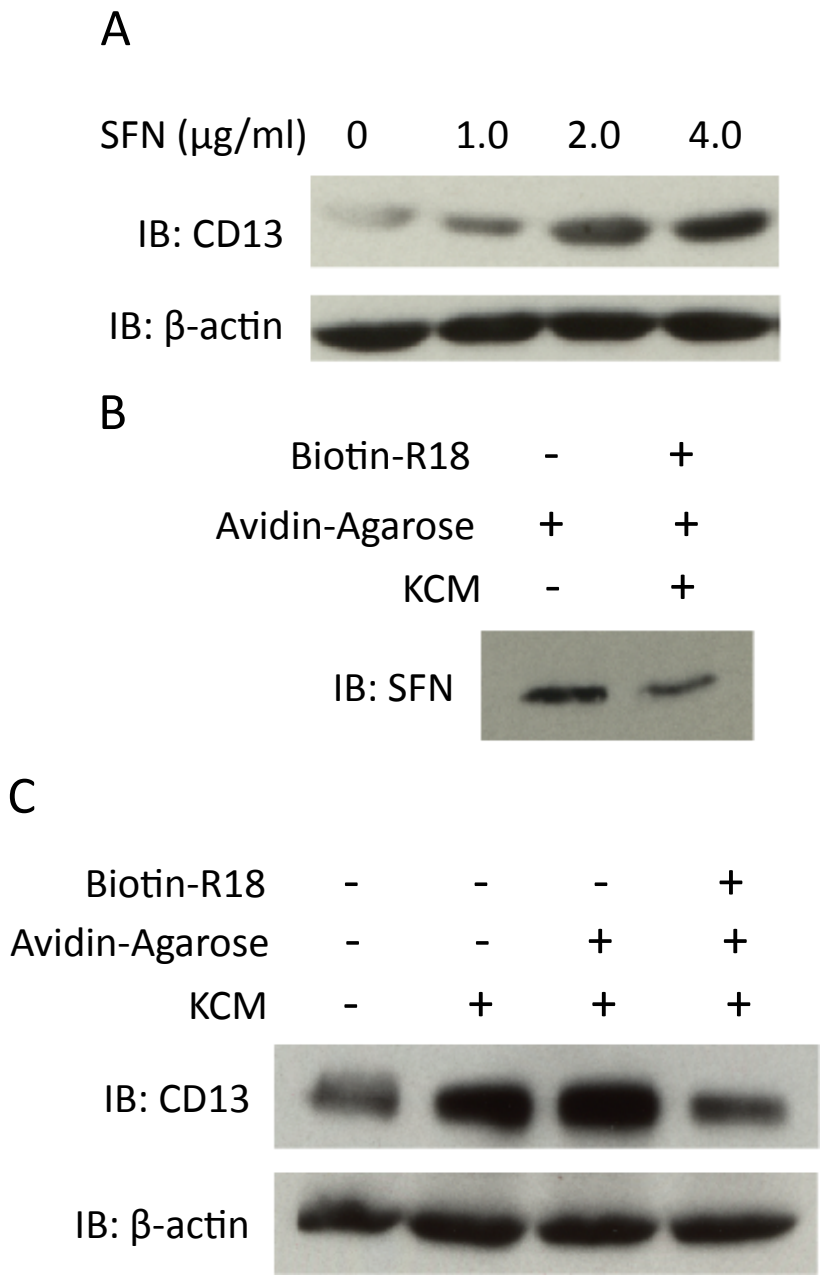

Figure A.I.2. SFN upregulates CD13 (APN) expression in fibroblasts.

(**A**) Fibroblasts were treated with various doses of SFN (1, 2 and 4 μg/ml) overnight and analyzed for the expression level of CD13 by Western blot analysis. (**B**) SFN was pulled down from total KCM using high affinity R18 binding protein. (**C**) The ability to induce CD13 expression in fibroblast was abrogated in SFN-depleted KCM. β-actin was used as a loading control.