KERATINOCYTE- RELEASABLE FACTORS MODULATE EXTRACELLULAR MATRIX COMPONENTS IN DERMAL FIBROBLASTS

by

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Abstract

A fine balance between the synthesis and degradation of extracellular matrix (ECM) is required in maintaining the structural integrity of healing tissue. An imbalance in ECM expression leads to fibrotic conditions such as hypertrophic scars (HS). It has been demonstrated that keratinocyte-releasable factors can function as ECM modulating factors (MMP-1 and type I collagen) in fibroblasts. We have shown that Stratifin (SFN) is an MMP-1 stimulatory factor in fibroblasts that failed to suppress the expression of type I collagen in fibroblasts. SFN is an intracellular protein that lacks a signal peptide. As such, it is critical to explore its mechanism of release and identify the keratinocyte-derived collagen-inhibiting factor(s) for dermal fibroblasts. In this doctoral research project I hypothesize that keratinocyte-releasable factor(s) function as a stop signal(s) for wound healing by modulating the expression of key ECM components such as MMP-1 and type I collagen in fibroblasts.

Two specific objectives were accomplished to address these issues. Under objective 1, the mechanism by which SFN is released has been explored. The findings demonstrate that SFN is released via exosomes in a Ca\textsuperscript{2+} dependant fashion. Moreover, only differentiated keratinocytes release SFN. Exosome-associated SFN exhibits a potent MMP-1 stimulatory effect. Under objective 2, using a series of systematic protein purification methods followed by mass spectroscopy, two proteins (SPARC and SFN) that inhibited collagen production by dermal fibroblasts were identified in keratinocyte-conditioned media. Using co-immunoprecipitation and 3D modeling, we determined that SFN
and SPARC form a complex thereby regulating type I collagen expression in fibroblasts. The levels of these proteins in fibrotic tissues (animal and human) were also evaluated and a differential expression of these proteins between normal and fibrotic tissue confirmed their potential role in development of fibrotic condition.

In conclusion, the identification of the mechanism of release of SFN and the identification of SPARC/SFN complex contribute to the understanding of how these factors are involved in the wound healing process. Also, SPARC/SFN complex provides us with another anti-fibrogenic factor that may be used to generate an effective therapeutic agent to treat HS frequently developed following burn injury, deep trauma and/or surgical incisions.
Preface

The work presented in this thesis has already been published or submitted for publication. This is to confirm that Claudia I. Chavez-Muñoz is the first author in all publications included in this thesis as shown below:


Dr. Aziz Ghahary was the principal investigator of the research project and the original identification of stratfin 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. Claudia I. Chavez-Munoz 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: Claudia Chavez-Munoz and Jennifer Morse contributed equally to the data presented in this manuscript. Jennifer Morse established the exosome purification method.

Chapter 4: Hans Adomat performed the mass-spectroscopy.

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 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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<tbody>
<tr>
<td>1MT</td>
<td>1-Methyl-Tryptophan</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>Calcium</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebro-Spinal Fluid</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle's Medium</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic Acid</td>
</tr>
<tr>
<td>EGF</td>
<td>Epithelial Growth Factor</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethylene Glycol Tetraacetic Acid</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic Reticulum</td>
</tr>
<tr>
<td>ESS</td>
<td>Engineered Skin Substitute</td>
</tr>
<tr>
<td>ESSIDO</td>
<td>Engineered Skin Substitute-Indoleamine 2,3 Dioxygenase</td>
</tr>
<tr>
<td>FBPA</td>
<td>Fructose-Biphosphate Aldolase</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast Growth Factor</td>
</tr>
<tr>
<td>FGF-BP</td>
<td>Fibroblasts Growth Factor-Binding Protein</td>
</tr>
<tr>
<td>FPLC</td>
<td>Fast Protein Liquid Chromatography</td>
</tr>
<tr>
<td>GAGs</td>
<td>Glycosaminoglycans</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3 Phosphate Deshydrogenase</td>
</tr>
<tr>
<td>HS</td>
<td>Hyperthrophic Scar</td>
</tr>
<tr>
<td>Hsp</td>
<td>Heat Shock Protein</td>
</tr>
<tr>
<td>hSPARC</td>
<td>Human Secreted Protein Acidic and Rich in Cystein</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukins</td>
</tr>
<tr>
<td>IONO</td>
<td>Ionomycin</td>
</tr>
<tr>
<td>KCM</td>
<td>Keratinocyte Conditioned Medium</td>
</tr>
<tr>
<td>KD-CIF</td>
<td>Keratinocyte-Derived Collagen Inhibiting Factor</td>
</tr>
<tr>
<td>KSFM</td>
<td>Keratinocyte Serum Free Medium</td>
</tr>
<tr>
<td>LAMP</td>
<td>Lysosomol-Associated Membrane Protein 2</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate Dehydrogenase</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-Activated Protein Kinase</td>
</tr>
<tr>
<td>MFG</td>
<td>Milk-fat Globule</td>
</tr>
<tr>
<td>MMPs</td>
<td>Matrix Metallo-Proteinases</td>
</tr>
<tr>
<td>MON</td>
<td>Monencin</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>MTT</td>
<td>3-4,5-Dimethylthiazol-2-yl</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular Weight</td>
</tr>
<tr>
<td>PAI-1</td>
<td>Plasminogen Activator Inhibitor Type 1</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet-Derived Growth Factor</td>
</tr>
<tr>
<td>q-PCR</td>
<td>Quantitative-Polymerase Chain Reaction</td>
</tr>
<tr>
<td>rhSFN</td>
<td>Recombinant Human Stratifin</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse Transcriptase-Polymerase Chain Reaction</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>SCC</td>
<td>Squamous Cell Carcinoma</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>SFN</td>
<td>Stratifin</td>
</tr>
<tr>
<td>SPARC</td>
<td>Secreted Protein Acidic and Rich in Cystein</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission Electromicroscopy</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming Growth Factor-Beta</td>
</tr>
<tr>
<td>TIMP</td>
<td>Tissue Inhibitor of Metallo-Proteases</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor Necrosis Factor-Alpha</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Cell Growth Factor</td>
</tr>
</tbody>
</table>
Acknowledgements

I want to start this section by stating that there are not enough words to express how grateful I am and I will always be to my supervisor Dr. Aziz Ghahary, who has not only provided me with invaluable knowledge but also with exceptional support throughout my degree. He has not only been a mentor, but also a father figure and an inspiration for life.

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Finally, but most importantly none of these accomplishments would have been possible without the unconditional support, friendship and love from my
beloved husband Dr. Omar Herrera. His strength and energy is my daily inspiration. The most heartfelt thanks to my parents Dr. Rene E. Chavez and MSc. Guadalupe Munoz and siblings Aynara C. Wulsin and Carlos Chavez-Munoz that without their continuous guidance support and love I would not have become the person that I am now.
Dedication

To my beloved husband, parents, siblings and Medicine
Chapter 1. Introduction

Background

Wound Healing

Wound healing in the skin is a dynamic process involving tissue response to different types of insults. This process involves a continuous sequence of signals and responses in which platelets, fibroblasts, epithelial, endothelial and immune cells come together outside their usual domains to orchestrate a complex event that results in tissue repair. These signals, which are mainly growth factors and cytokines, orchestrate the initiation, continuation and termination of wound healing.

When the epidermis is wounded, a set of events takes place in a predictable fashion to repair the damage. These events overlap in time (1, 2) and can be arbitrarily categorized into separate steps: the inflammatory, proliferative and remodeling phases. Some authors consider healing to take place in four stages, by splitting different parts of inflammation or proliferation into separate steps (Figure 1-1) (2).

The normal healing response begins the moment the tissue is injured. As the blood components arrive to the site of injury, the platelets come into contact with exposed collagen and other elements of the extracellular matrix. This contact triggers the platelets to release clotting factors as well as essential growth factors and cytokines such as platelet-derived growth factor (PDGF) and transforming growth factor beta (TGF-β) within 48-96 hours after injury (3-5).
Following hemostasis, the neutrophils enter the wound site and begin the critical task of removal of foreign materials, bacteria and damaged tissue. As part of this inflammatory phase, the macrophages appear and continue the process of phagocytosis as well as releasing more PDGF and TGF-β. Once the wound site is cleaned, fibroblasts migrate into the wound site and begin the proliferative phase and deposit new extracellular matrix.

TGF-β is considered to be a master control signal that regulates a host of fibroblasts functions, particularly extracellular matrix deposition (6). First, it increases transcription of the genes for collagen, proteoglycans and fibronectin thus increasing the overall production of matrix proteins. At the same time TGF-β decreases the secretion of proteases responsible for the breakdown of the matrix and it also stimulates the protease inhibitor, tissue inhibitor of metallo-proteases (TIMP) (7). Other cytokines, which are also considered to be important, are interleukins (IL), fibroblast growth factors (FGF) and tumor necrosis factor-alpha (TNF-α). As healing progresses several other important biological responses are activated.

The process of epithelization is stimulated by the presence of epithelial growth factor (EGF) and transforming growth factor-alpha (TGF-α) that are produced by activated wound macrophages, platelets and keratinocytes (8-10). Once the epithelial bridge is complete, enzymes are released to dissolve the attachment at the base of the scab resulting in removal.

Due to the high metabolic activity of the wound site, there is increased demand for oxygen and nutrients. Local factors in the wound microenvironment
such as low pH, reduced oxygen tension and increased lactate actually initiate
the release of factors needed to bring in a new blood supply (11, 12). This
process, angiogenesis, is stimulated by vascular endothelial cell growth factor
(VEGF), basic FGF and TGF-β. As the proliferative phase progresses the
predominant cells in the wound site are the fibroblasts. This mesenchymal-origin
cell is responsible for producing the new matrix needed to restore the structure
and function to the injured tissue. Fibroblasts attach to the provisional fibrin
matrix and begin to produce collagen (13). The new collagen matrix then
becomes cross-linked and organized during the final remodeling phase. Collagen
fibers in scar tissue are initially much smaller and possess a less organized
structure; scar tissue is always weaker and will break apart before the
surrounding normal tissue. However, it does increase in strength during
remodeling. Nevertheless, the regained tensile strength in a wound will never
approach normal. In fact, the maximum tensile strength that a wound can ever
achieve is approximately 80% of normal skin.

Finally, in the phase of collagen remodeling, collagen degradation is an
essential component of the remodeling phase of wound healing (14, 15). Specific
collagen enzymes in fibroblasts, neutrophils and macrophages clip the molecule
at a specific site, and break it down to a characteristic three-quarter and one-
quarter pieces. These collagen fragments undergo further denaturation and
digestion by other proteases. Despite this orderly sequence of events
responsible for normal wound healing, pathologic responses can lead to fibrosis
(hypertrophic scars (HS) and keloids) or chronic ulcers if any part of the healing sequence is altered.

**Figure 1-1 Phases of normal wound healing**

**A** Representation of the timeline for normal wound healing. Authors divided the process into four stages: hemostasis, inflammation, proliferation and remodeling phases.

**B**

1. **Inflammatory phase**
2. **Proliferative phase**
3. **Remodeling phase**
Diagram representing the three arbitrary phases of normal wound healing. (1) Inflammation, (2) Proliferative Phase and (3) Remodeling phases.

**Extracellular Matrix**

The extracellular matrix (ECM) in the skin, serves as support and anchorage for cells, segregating tissues from one another and regulating intracellular communication. It is composed of an interlocking mesh of fibrous proteins and glycosaminoglycans (GAGs) that regulate the dynamic behavior of the cells. ECM sequesters a wide range of cellular growth factors and acts as a local storage for them. The components of the ECM are produced intracellularly by resident cells and secreted into the ECM via exocytosis. Once secreted they then aggregate with the existing matrix.

GAGs are polymers that attach to the ECM and form proteoglycans, which have the capacity to attract water molecules through osmosis, keeping the ECM and resident cells hydrated. They may also help to trap and store growth factors. Hyaluronic acid (a polysaccharide) is another component of the ECM. It provides tissues the ability to resist compression by providing a counteracting turgor force by absorbing significant amounts of water.

Type 1 collagen is the most abundant protein in the extracellular matrix and it is the most fibrous form of all collagens produced by fibroblasts. At least 23 individual types of collagen have been identified to date. Collagen is responsible for providing the skin with tensile strength. Dermal collagen on a per weight basis approaches the tensile strength of steel. It is a strong and highly organized molecule. Type 1 collagen is composed of two polypeptide chains, proα1 (I) and
proα2 (I) in the ratio of 2:1 respectively. The synthesis of these two polypeptide chains is under the control of two separate genes, COL1A1 and COL1A2. Collagen is released in a precursor form (procollagen), which is then cleaved by procollagen proteases to allow extracellular assembly. The other common forms of collagen in normal skin are type III, V and VI. 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. Fibroblasts degrade collagen through either extracellular or intracellular pathways (16, 17). Collagen fibers are extremely resistant to proteolytic breakdown and their degradation is mediated by matrix metalloproteinases (MMPs), primarily by collagenases. MMPs represent a group of diverse proteolytic enzymes involved in ECM turnover and connective tissue remodeling during physiological conditions such as embryonic growth and development, uterine involution, bone growth and resorption, and wound healing (18).

The MMP family consist of 25 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 (19). Collagenases, which include MMP-1, MMP-8 and MMP-13 cleave the triple helix of fibrillar collagens of types I, II, III and V. They all cleave native type I collagen between Gly\textsuperscript{775}-Ile\textsuperscript{776} of α1 chain or Gly\textsuperscript{775}-Leu\textsuperscript{776} of α2 chain resulting in ¾ N-terminal and ¼ C-
terminal fragments, which then denature spontaneously in 37°C into gelatin and are further degraded by other MMPs such as gelatinases (MMP-2 and MMP-9) (18). In addition to extracellular degradation by MMPs, it has also been shown that in rapidly remodeling tissues, collagen fiber degradation can occur through an intracellular phagocytic pathway (17). The level of MMP expression in normal cells is low and this allows healthy connective tissue remodeling. However an imbalance in expression of MMPs has been implicated in a number of pathological conditions such as dermal fibrosis (20), rheumatoid arthritis, arteriosclerosis, pulmonary emphysema, and tumor invasion and metastasis (21).

**Cell-cell Communication in Wound Healing**

Epidermal/mesenchymal communication is critical in exchanging information between keratinocytes and fibroblasts in skin morphogenesis during development and probably also in the maintenance of the integumentary structure of skin in adults (22). It has been demonstrated that during the reepithelialization phase keratinocytes are dependent on signals coming from fibroblasts to reestablish a functional epidermis (23, 24). This communication between these two cells (keratinocytes/fibroblasts) is mostly regulated by secreted cytokines and other factors in a paracrine fashion (25). Disruption of this interaction can also affect the function of dermal fibroblasts.

Epidermal/mesenchymal interactions during wound healing process have a key function in controlling the expression of ECM components. It has been demonstrated that any disruption of these interactions can lead to an excessive
production of ECM, which can result in the development of fibrotic conditions such as, keloids and HS (20, 26, 27). Also, delays in epithelization during the process of wound healing increases the frequency of these fibrotic conditions (28). This suggests that in the absence of epithelization, ECM continues to accumulate until dermal fibroblasts receive signal(s) from epidermal cells to slow down the dynamic process of healing that leads to maturation and remodeling of the healing wound (29). Thus a fine balance between synthesis of ECM and degradation by MMPs are a key factor in maintaining the structural integrity of normal skin. Keratinocyte–releasable factors have been the subject of several studies that have provided evidence indicating that keratinocytes express many growth factors, cytokines and their receptors. One of the first growth factors, which has shown to be involved in mesenchymal/epithelial interactions, is KGF/FGF7 that is rapidly induced in fibroblasts after wounding and exerts its effects through binding to its receptor FGFR2IIIb on keratinocytes. Some growth factors are predominantly expressed in epidermal cells and exert their actions on mesenchymal cells, such as PDGF. For other growth factors, expression was detected in both keratinocytes and fibroblasts and the effects are observed in both cells in an autocrine and paracrine fashion. In fact, it has been demonstrated that when a cultured keratinocyte sheet is used as temporary wound coverage, it promotes wound healing and increases wound epithelialization (30).

Other studies have demonstrated that epidermal cell-derived factors regulate wound healing through stimulation of migration and proliferation of
keratinocytes from the sweat glands, hair follicles and wound edges. These factors also stimulate the contraction of collagen matrices by controlling numbers of fibroblasts or influencing their state (31, 32). Proliferation of fibroblasts and matrix modulation in response to keratinocyte-conditioned medium (KCM) has revealed that KCM significantly increases fibroblast replication and decreases collagen synthesis. This finding suggests that a paracrine control mechanism is involved in ECM modulation in these cell types during wound healing.

Previous studies have also suggested that the stimulation of wound healing as seen in wounds receiving cultured keratinocyte sheets was partially due to the expression of collagenase (33, 34). Many keratinocyte-derived factors have been identified showing an MMP stimulatory effect in fibroblasts. Therefore, understanding the role of factors involved in the cell-cell interactions will lead to the development of new and more promising therapies for pathological healing wounds.

**Stratifin as a Keratinocyte Releasing Factor**

Stratifin (SFN), also known as 14-3-3σ protein, belongs to the family of 14-3-3 proteins. Moore and Perez discovered these proteins in 1967 in bovine brain homogenate. Its name refers to the particular elution (Fraction 14) and migration pattern of these proteins on DEAE-cellulose chromatography and starch gel electrophoresis (position 3.3). These are a class of highly conserved molecular chaperones. They are a ubiquitous family of acidic eukaryotic proteins. The family consists of seven mammalian isoforms, β, ε, γ, η, σ, τ and ζ (35). 14-3-3 isoforms form homo- and heterodimers with highly rigid structures that can
induce conformational change in their binding partners as an adaptor or chaperone molecule (36). Each monomer consists of a bundle of 9 α-helices organized in anti-parallel fashion. The molecule has a cup-like shape. The structure has a highly conserved inner concave surface and a variable outer surface. In the concave surface it possesses an amphipathic groove, which is shown to mediate the binding of 14-3-3 to its client proteins. All 14-3-3 proteins bind to common phosphoserine/ phosphothreonine-containing peptide motifs corresponding to Mode-1 (R[SFYW]XpSXP) or Mode-2 (RX[SYFDWTQAD]p(S/T)X[PLM]) sequences, such as Raf-1, Cdc25, AKT1, IL-9R, Vimentin, Bcl-2, p53 and Bax (37). It has been reported that they also bind to cystein-rich proteins.

Since the discovery of the first 14-3-3 protein in 1967 (38), the members of the 14-3-3 protein family have been re-discovered based on their new biological activities, primarily in signal transduction pathways. They have been identified as activators of tryptophan and tyrosine hydroxylase (39, 40) and PKC inhibitors (41). Subsequent studies identified 14-3-3 proteins as molecules that interact with PKCs, Raf family members, and now more than 100 other intracellular proteins with critical biological functions (42, 43), including cellular response to DNA damage and cell cycle regulation (44-46). SFN was reported to be vital in preventing mitotic catastrophe after DNA damage (45), also it has been reported to be a p53-regulator that inhibits G2/M progression (44). Nevertheless, all of these activities are regarded as being intracellular interactions and functions of 14-3-3 proteins.
A releasable form of 14-3-3 protein was shown to be present in cerebrospinal fluid (CSF) and reported to be associated with prion diseases such as Creutzfeldt-Jakob disease and other neurological disorders (47, 48). It has been also found in the conditioned media of various cells including human MDA-MB-231 breast carcinoma (49), mouse embryonic fibroblasts (50), human plasma (51), human uveal melanoma (52), activated human platelets (53), mouse astrocytes (54), dendritic cells (55), tumor-associated monocytes/macrophages (56), and adult human epidermis keratinocytes (57). SFN was also included in a catalogue of proteins found to be secreted by epidermal keratinocytes (57), but no physiological function was assigned to these proteins.

The first extracellular function for SFN was recently revealed, showing a potent MMP-1 stimulatory effect in fibroblasts (58). The role of extracellular SFN in signal transduction has never been addressed. Previously, our group explored the function of three MAPK pathways 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 (59). 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 might function as a ligand in a receptor-mediated transmembrane signaling event. Recently our
group has identified aminopeptidase N (APN) or CD13 as the cell surface receptor for extracellular SFN (60).

14-3-3 proteins are considered to be intracellular proteins with many critical biological activities. These proteins lack of known amino-terminal ER signal peptides and, as such, the mechanism by which these proteins become releasable remains unknown. It has been demonstrated that these proteins cannot pass through the classic endoplasmic reticulum secretory pathway in order to be released into the extracellular space (47, 61), and therefore, they must be released via a non-classical secretory pathway.

ER/Golgi-independent secretion has identified at least four different pathways. One of the most common mechanisms is exosome-dependent secretion (62). Exosomes are single membrane vesicles of endocytotic origin. These intralumenal vesicles are generated during endosome maturation by inward budding of the limiting membrane (63). Exosomes are nanovesicles (50-90nm), released from viable cells either constitutively or upon activation of cell secretion, but neither from lysed nor apoptotic cells. Initially exosomes were thought to be implicated in removing unneeded membrane proteins from cells that have poor capacities to degrade proteins with a lysosomal system, or cells which are located towards a drainage system such as tubule kidney or gut (64). Since then several cell types have been described to release exosomes in extracellular medium in vitro such as B and T cells, dendritic cells, mast cells, platelets, intestinal epithelial cells, Schwann cells, neuronal cells, adipocytes, tumor cells etc. Exosomes have been also found in
vivo in several biological fluids such as urine, plasma, epididymal fluid, amniotic liquid, malignant and pleural effusions of ascites, bronchoalveolar lavage fluid, synovial fluid and breast milk.

**SPARC Protein**

Secreted protein acidic and rich in cysteine (SPARC), also known as Osteonectin or BM-40 is a nonstructural glycoprotein secreted by platelets, leukocytes, endothelial cells, fibroblasts, and numerous other cell types into plasma and the extracellular matrix (ECM). It is expressed at high levels during processes such as development, angiogenesis, and wound healing that involve changes in cell shape, adhesion, and motility. SPARC protein has counter-adhesive properties, induces cell-rounding, inhibits cell spreading (65), mediates focal adhesions disassembly and the reorganization of actin stress-fibers (66), and delays cell cycle of many cell types in G1 phase (67). It was first isolated as the main non-collagenous component of the bone (68) where upon binding to collagen it induces calcium deposition. SPARC binds to collagens (I, III, IV and V) (30, 36, 50, 42, 51), acting as a chaperone affecting spontaneous fibril formation in vitro. It is also capable of binding to vitronectin, thrombospondin (54) and some fragments of fibrinogen (56).

Platelets, macrophages and fibroblasts start expressing SPARC in the inflammatory and proliferative phases of the wound healing process (69, 70). Its expression is often enhanced when attachment-dependent cells undergo a “culture shock” after tissue disruption (69, 71) and in cells subjected to various forms of stress (72). It is known to regulate the assembly, organization and
turnover of the ECM by binding and modulating the deposition of multiple structural components and attenuating the activity of extracellular proteases. SPARC can induce MMP-9 and MMP-1 in peripheral blood monocytes (48) and MMP-1, MMP-3 and MMP-9 in fibroblasts (49).

Previous studies have described a SPARC-null mice (73, 74). The data obtained from SPARC-null mice, indicates that the appropriate and regulated expression of SPARC is necessary for normal development. In the adult the expression of SPARC is limited to tissue undergoing repair or remodeling due to wound healing, disease or natural processes. Pathologies such as cancer metastasis, arthritis, diabetes and kidney disease are characterized by elevated expression of SPARC (75). However, the mechanism by which SPARC contributes to these pathologies has not been elucidated. Many studies have demonstrated that SPARC expression is associated with fibrosis (76, 77). Recently, it was reported that reduction of SPARC expression in scleroderma fibroblasts by RNA interference was sufficient to reduce collagen expression of SPARC and collagen I, in comparison to normal fibroblasts (78). SPARC therefore is an attractive target for the development of strategies to counteract fibrotic deposition of collagen.
Hypotheses and Objectives

Regardless of the site of injury a fine balance between the synthesis and degradation of ECM is required in maintaining the structural integrity of healing tissue. Driven by the deleterious consequences over-healing processes can have on the health and well being of patients, the goal of this research is to better understand the importance of mesenchymal-epithelial communication in the physiological and pathological process of dermal healing.

My working hypothesis has been that keratinocyte releasable factors might function as a stop signal(s) for wound healing by modulating the expression of key ECM components (such as collagenase (MMP-1) and collagen type I) in dermal fibroblasts (Figure 1-2). Recently we have identified keratinocyte-releasable stratifin as responsible for the MMP-1 stimulatory effect in dermal fibroblasts. However its mechanism of release is unknown. To further investigate its mechanism of release we tested the following hypothesis and specific objectives.

Hypothesis 1

Under this hypothesis, we propose that keratinocytes have the capacity to release stratifin through externalization of exosomes. To address this hypothesis, four specific objectives were pursued as described below.
Objective 1.1

Under this objective, in chapter 2, keratinocyte conditioned medium (KCM) was collected and exosomes were purified, confirming their presence by different techniques such as Transmission electron microscopy, western blotting and immunogold labeling. These findings revealed for the first time that human keratinocytes have the capacity to release exosomes and that stratifin is an exosomes-associated protein.

Objective 1.2

In order to evaluate whether exosome-associated stratifin had an MMP-1 stimulatory effect in dermal fibroblasts, these cells were treated with purified human keratinocyte exosomes. The result showed that keratinocyte exosomes were able to stimulate MMP-1 expression in fibroblasts.

Objective 1.3

Furthermore, we wanted to evaluate if keratinocyte differentiation played a role in the expression of stratifin. Our results shown in chapter 3, demonstrate that only differentiated keratinocytes release exosome-associated stratifin.

Objective 1.4

Finally, the mechanism by which differentiation of keratinocytes increases the levels of exosome-associated proteins was examined. To evaluate this, undifferentiated keratinocytes were treated with an ionophore (monensin) and exosomes were purified. The results showed a significant increase in the
exosome release when keratinocytes were treated with the ionophore. This finding suggests that keratinocyte exosome release pathway is Ca\(^{2+}\) dependent.

Previous studies have shown that expression of type I collagen in dermal fibroblasts is mediated by an unidentified paracrine factor secreted by keratinocytes. To further identify the keratinocyte-derived collagen inhibitory factor we tested hypothesis # 2 and specific objectives.

**Hypothesis 2**

Under this hypothesis, we suggest that keratinocyte-releasable factors modulate the expression of type I collagen in dermal fibroblasts. To address this hypothesis a series of experiments were conducted and the outline of these experiments are shown under objective 2.1.

**Objective 2.1**

To purify an unknown factor responsible for type I collagen suppression in fibroblasts was described in chapter 4, KCM was collected and subjected to protein purification assays. Finally mass spectroscopy peptide sequencing analysis identified SFN/SPARC as the complex responsible for the collagen inhibitory effect in fibroblasts. Co-IP, 3D modeling, and treatment of fibroblasts with the purified SFN/SPARC complex further confirmed our finding.
Figure 1-2 Cartoon representation of hypothesize keratinocyte-derived anti-fibrogenic factors and its mechanism of release.
Chapter 2. Primary Human Keratinocytes Externalize Stratifin Protein Via Exosomes

Introduction

Stratifin (SFN) is a member of the 14-3-3 family proteins which are conserved regulatory molecules expressed in all eukaryotic cells. They have been involved in a wide range of vital regulatory processes such as mitogenic signal transduction, apoptotic cell death, and cell cycle control (79). They are composed of seven isoforms in mammals (β, ε, γ, η, σ, τ, ζ). Recently, releasable form of 14-3-3σ, also known as stratifin, was found to have a potent matrix metalloproteinase 1 (MMP-1) stimulatory effect on dermal fibroblasts (80).

Although these proteins are primarily intracellular, there have been some reports indicating the presence of these proteins extracellularly such as those isoforms found in cerebrospinal fluid (CSF) of patients with certain neurological diseases (81, 82). Several reports have demonstrated that cell lysis is not the preliminary route of transfer this proteins to the extracellular space (80, 83, 84). The isoform compositions of degenerating neurons in Creutzfeldt-Jakob disease

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do not reflect those of the CSF suggesting that the increased presence of 14-3-3 proteins in CSF is not merely the result of neuronal cell lysis (82). Furthermore, lactate dehydrogenase (LDH), a strictly cytosolic protein, was not detected in keratinocyte-conditioned medium containing stratifin, confirming that the release of these proteins was not the result of keratinocyte lysis (80).

Secretion of these proteins is also unlikely as these proteins lack conventional signal peptides (85). Several other proteins such as, Hsp70 (86), IL-1β (87, 88), FGF-2 and galectins (85), are also released by non-classical (ER/Golgi independent) mechanisms.

ER/Golgi-independent secretion was discovered in the early 1990s and since then, at least four different pathways have been identified. One of the most common mechanisms is exosome-dependent secretion (85).

Exosomes are single membraened vesicles of endocytotic origin. These intralumenal vesicles are generated during endosome maturation by inward budding of the limiting membrane, a process during which a small portion of cytosol is trapped into the vesicle (89). Exosomes secretion has first been described for reticulocytes during their differentiation (90, 91). Later, other hematopoietic cells including B lymphocytes (92), dendritic cells (93), T lymphocytes (94, 95) and mast cells (96, 97) have been shown to release exosomes. Further, the results of several other studies have shown that non-hematopoietic cells such as intestinal epithelial cells (98), neuroglial cells(99), and tumor cells (100) also possess the ability to release exosomes. There are some specific characteristics related to exosomes that distinguish them from
other vesicles that originate from different cellular locations such as plasma membrane (101). Their size ranges from 50-90 nm in diameter and following a negative staining they display a unique “saucer-like” morphology (flattened sphere that is limited by a lipid bi-layer) by electron microscopy.

Biochemical and proteomic analysis of exosomes purified from the supernatants of several cells revealed the presence of common proteins (92, 93, 98, 102, 103). Depending on the cells examined, all or some of the following proteins such as cytosolic proteins such as hsp70, hsc73, hsp90, Tsg101, several annexins, Rab proteins, cytoskeletal proteins (actin, tubulin) and milk-fat globule (MFG)-E8 (or lactadherin) have been identified to be associated with exosomes, Membrane-bound proteins, such as, tetraspanins (CD9, CD63, CD81, CD82) and MHC class I and LAMP-2 molecules were also detected in exosomal proteins. Another specific characteristic feature of exosomes is that they float on sucrose gradient, and their density ranges from 1.13 g/ml to 1.22 g/ml. Contaminating material, such as protein aggregates or nucleosomal fragments are separated readily from exosomes by flotation on sucrose gradients (101, 102). Altogether, this specific composition distinguishes this population of vesicles from other vesicles or shed membranes. Exosomes are not the only type of secreted membrane. Larger membrane structures can be purified from supernatants of prostate cells (prostasome like granules), platelets, and activated neutrophils (called ectosomes). Such vesicles can be observed by con-focal microscopy and are greater than 100 nm in diameter. As such, they can not be considered as exosomes.
Although, we have previously demonstrated an easily detectable level of stratifin in keratinocyte conditioned medium, the mechanism by which this protein is externalized from keratinocytes is unknown. Here, we found that keratinocytes have the capacity to release some intracellular proteins such as stratifin via the externalization of exosomes into conditioned medium. In fact, a systematic proteomic analysis of exosomes obtained from dendritic cell conditioned medium revealed that three 14-3-3 isoforms (\(\eta\), \(\gamma\), and \(\gamma/\delta\)) are within a large group of proteins identified in exosomes (102). In order to explore this mechanism in keratinocytes, we conducted a series of experiments to test our working hypothesis that primary human keratinocytes release exosomes through which some associated cytosolic proteins, such as stratifin are released into conditioned medium. The findings of these series of experiments, demonstrate that: 1) keratinocytes have the capacity to produce and release exosomes and 2) exosomes contain stratifin with MMP-1 stimulatory activity in fibroblasts. This finding was then confirmed by depleting the exosomes from keratinocyte conditioned medium and showing a market reduction in expression of MMP-1 in exosome depleted KCM treated fibroblasts.
Materials and Methods

Antibodies

Rabbit anti-human stratifin antibodies were generously provided by Dr. Aitken (School of Biomedical and Clinical Sciences, University of Edinburgh, Scotland), and monoclonal anti-human stratifin was obtained from MEDICOR (Montreal, QB, Canada). Additionally, monoclonal anti-human LAMP-2 (CD170B) antibody was obtained from BD Pharmingen (San Diego, CA), mouse anti-human MMP-1 monoclonal antibody from R&D systems (Minneapolis, MN) and mouse anti-human β-actin monoclonal antibody from SIGMA (Saint Louis, MO). Horseradish peroxidase conjugated secondary antibodies against mouse and rabbit IgG were obtained from BioRad Lab. (Hercules, CA).

Cell Cultures

After informed consent, foreskin samples were obtained from patients undergoing elective circumcision. Samples were collected individually in Keratinocyte Serum Free Medium (KSFM) (GIBCO, Grand Island, NY), and washed several times in sterile PBS supplemented with antibiotic-antimycotic preparation (100 µg/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml amphotericin B) (GIBCO, Grand Island, NY). Skin was dissected free of fat and cut into small pieces 1cm in diameter and incubated in Dispase enzyme (25u/ml in KSFM) (GIBCO, Grand Island, NY) at 37 °C for 2 h in order to separate epidermis from dermis. Epidermis was incubated for 4 min in trypsin at 37 °C finger-vortexing every minute. Dulbecco’s Modified Eagle’s Medium (DMEM)
(GIBCO Grand Island, NY) with 10% Fetal Bovine Serum (FBS) was used to stop trypsin reaction. Centrifugation at 800 rpm at 4 °C for 8 min allowed us to obtain keratinocytes that were seeded into 25 cm² flasks and cultured with 5 ml of KSFM with growth supplement (GIBCO, Grand Island NY) and antibiotic-antimycotic preparation (GIBCO, Grand Island, NY). The dermal layer was minced into small pieces ~0.5 mm in diameter and distributed into 60x15 mm Petri dishes. Fibroblasts migrated from these minced pieces and were cultured with 10 ml of DMEM supplemented with 10% FBS and antibiotic-antimycotic preparation (GIBCO Grand Island, NY). Keratinocytes up to passage 4 and fibroblasts at passage 3-6 were used in all experiments conducted in this study.

**Exosome Purification**

Exosomes were purified from the supernatant of 2-day-old human keratinocyte cells (~5 x 10⁷ cells) cultured in KSFM with growth supplement (GIBCO, Grand Island, NY) at 37 °C. To purify exosomes, the mini-scale exosome purification protocol described by Lamparski (104) was used. Briefly, 100-150 ml of keratinocyte conditioned medium was cleared by centrifugation at 6,000 xg at 4 °C for 10 min in order to remove cell debris. The precleared medium was then concentrated to a volume of 1-2 ml using a 100 kDa MWCO Centricon Plus-20 filter capsule (Millipore Billerica, MA). The sample was then transferred to a 5ml ultracentrifuge tube and then underlayed with 300 µl of 30% sucrose-deuterium oxide (D₂O). The sample was ultracentrifuged at 100,000 xg for 1h at 4 °C. Three hundred and fifty µl of the cushion of sucrose from the bottom of the tubes was collected and diluted in 15 ml of PBS. The purified
exosomes were then washed and concentrated by centrifugation at 1000 xg for 10-25 min using 100 kDa Millipore Ultrafree-15 capsule filter (Millipore, Billerica, MA). Fresh exosomes were used in subsequent TEM imaging. The remaining fraction was stored at -20 °C until used for Western blot analysis.

**Transmission Electron Microscopy**

Exosomal preparation (5 µl) was fixed with 4% paraformaldehyde and deposited onto formvar-coated carbon EM grids (Canemco Inc., St. Laurent, Quebec) and incubated at room temperature for 10 min. Excess moisture was removed and the grids were negatively stained with 1% uranyl acetate for 15 sec. Excess stain was removed and the grids were air dried before viewing with a Hitachi H7600 Transmission electron microscope (TEM) with a side mount AMT Advantage (1 mega-pixel) CCD camera (Hamamatsu ORCA) (UBC Bioimaging Facility, Vancouver, BC, Canada).

**Treatment of Fibroblast with Purified Keratinocyte Exosomes and Conditioned Medium**

Dermal fibroblasts were seeded in 6-well plates (2 x 10^5 cells per well) and treated in duplicate with either KCM, exosome depleted KCM or with purified keratinocyte exosomes. After 24 h of treatment, cells were lysed and the protein concentration was determined by Bradford assay. Western blot was used to assess MMP-1 levels using β-actin as control for equal protein loading.
**Western Blotting**

Human keratinocytes and dermal fibroblasts were lysed in lysis buffer (50 mM Tris-HCL, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.025% NaN3, 1% Triton-X100, 0.5% IGEPAL CA-630 and 1:100 protease inhibitor) for 1 h at 4 °C, then nuclei and cell debris were removed by centrifugation. Total proteins of the cell lysate were quantified by Bradford assay and subjected to SDS-PAGE analysis on either a 12% or 10% of acrylamide gel to visualize either stratifin or MMP-1 proteins, respectively. The gels were electrotransferred onto polyvinylidene difluoride membranes (Millipore Billerica, MA). Non-specific proteins on membranes were blocked with 5% skim milk powder in TBS containing 0.05% Tween 20 for 90 min at room temperature. Immunoblotting was performed using rabbit anti-stratifin antibody (1:1000 dilution) from Dr. Atiken’s and commercially available respectively, anti-human β-actin (1:1000), LAMP-2 (1:1000 dilution), and MMP-1 monoclonal antibodies (1:250 dilution). The membranes were then incubated with the appropriate horseradish peroxidase-conjugated secondary antibody (1:2500 dilution). Immunoreactive proteins were then visualized using Western blotting luminol reagent (Santa Cruz Biotechnology, Inc. Santa Cruz, CA). To show the specificity of the polyclonal anti-human stratifin used, the experiments related to those blots shown in Figure 2-2, Figure 2-3 and Figure 2-4 were repeated by using a monoclonal anti-human stratifin antibody purchased from MEDICOR, (Montreal, QB, Canada) (1:1000 dilution).
Quantitative Analysis of the Exosome Associated Stratifin

The quantity of the exosome associated stratifin was evaluated by purifying keratinocyte exosomes and comparing the level of stratifin in 10 µg of total exosomal proteins with that of various concentrations (0, 0.06, 0.12, 0.25, 0.5 and 1.0 µg/lane) of recombinant stratifin loaded in a 12% acrylamide gel using western blot analysis (n=3). The densities of the signals were then compared by densitometry and their values were calculated and quantified using a linear regression model.
Results

Human Keratinocytes Release Exosomes into Conditioned Medium

To examine whether keratinocytes release exosomes, 100-150 ml of 48 h conditioned medium was collected, concentrated and the presence of exosomes was examined using the exosome purification method developed by Lamparski (104) (described in Materials and Methods). To examine the presence and visualize the ultrastructure of the purified keratinocyte exosomes, fresh samples were obtained and subjected to analysis by a transmission electron microscopy (TEM). TEM images shown in Figure 2-1 identified many clusters of small vesicles under 100nm in diameter with the unique “saucer-like” shape that are salient features of exosomes. The morphology of these exosomes were very similar to those first demonstrated by Pan (91) in sheep reticulocytes, and then by Raposo (92) in human B cells.
Figure 2-1 TEM observation of membrane particles (exosomes) purified from keratinocyte conditioned medium

Purified exosomes were incubated briefly on formvar-coated carbon grids and excess moisture removed. The exosomes were negatively stained using 1% uranyl acetate to visualize ultrastructure immediately before examination by TEM. Cup-shaped structures 50-100 nm in diameter were identified as being exosomes.

**Purified Exosomes from Keratinocyte Conditioned Medium Contain Stratifin**

The level of exosome associated stratifin was evaluated by western blot analysis (Figure 2-2). The intensity of stratifin band (lane E) in keratinocyte exosomes was then compared with that present in either human keratinocyte lysate (lane K), concentrated keratinocyte conditioned medium (lane CM) or
concentrated keratinocyte un-conditioned medium (lane UM). The blot related to the same samples was then evaluated for exosomal markers such as lysosomal-associated membrane protein 2 (LAMP-2) and heat shock cognate 70 (hsc70) previously used by other investigators (102). The results showed the presence of stratifin in keratinocyte exosomes (lane E), concentrated KCM (lane CM), but not in concentrated unconditioned culture medium (lane UM). Further evaluation of the same samples revealed that a cytosolic protein, β-actin, was only detectable in keratinocyte cell lysate (lane K). This finding indicates that the presence of exosome associated stratifin is unlikely to be due to cellular disruption.

Figure 2-2 Western blot analysis of stratifin in exosomes

To purify exosomes, keratinocyte conditioned medium was collected, concentrated and exosomes were separated based on their unique size and density by ultracentrifugation using a sucrose-D$_2$O cushion. The exosome fraction was collected, rinsed once with PBS and concentrated. Fifteen µg of the total proteins associated with isolated
exosomes (lane E) were analyzed by western blot analysis using rabbit anti-human stratifin, mouse anti-human LAMP-2, and monoclonal anti-human hsc70. Ten µg of total proteins from either keratinocyte cell lysate (lane K), keratinocyte conditioned medium (lane CM) were used as positive controls; while, the same amount of total protein of non-conditioned medium (lane UM) served as negative control. The same blots were also probed with β-actin antibody to show that detection of stratifin in KCM is not due to cell lysis.

**Exosome-Associated Stratifin Stimulates the Expression of MMP-1 in Fibroblasts**

To examine whether the presence of exosome associated stratifin in keratinocyte conditioned medium is responsible for its MMP-1 stimulatory effect in fibroblasts, the effect of the exosome depleted and un-depleted KCM on expression of MMP-1 in fibroblasts was evaluated (Figure 2-3) Prior to achieving this, the level of stratifin in these conditioned medias were evaluated to be sure that stratifin level is reduced or depleted from the KCM. As shown in Figure 2-3A, the finding of this experiment revealed the presence of stratifin in KCM containing exosomes (lane KCM+) in a greater amount compared to KCM depleted of exosomes (lane KCM -). The levels of stratifin in keratinocyte and fibroblast lysate were also evaluated and used as positive (lane K) and negative controls (lane F), respectively. Confirming that stratifin can be reduced from KCM by exosomal depletion, the MMP-1 stimulatory effect of the same samples in 24 h treated and untreated fibroblasts were then evaluated using western blot analysis (Figure 2-3B). The results showed that KCM stimulates the expression of MMP-1
protein in treated fibroblasts (KCM+) compared to, either untreated fibroblasts (lane F) or fibroblasts treated with exosomes depleted KCM (KCM-). In parallel, squamous cell carcinoma lysate (7 µg/lane) was run and evaluated as a positive control for MMP-1 expression (lane SCC). The finding of this experiment suggested that exosome associated stratifin, at least in part, is responsible for MMP-1 stimulatory effect of KCM in dermal fibroblasts.

Figure 2-3 **Exosome depleted keratinocyte-conditioned medium lacks SFN protein and MMP-1 activity for fibroblasts**

(A) Keratinocyte-conditioned medium was collected and a 10 mL aliquot was set aside and concentrated. The rest was used for exosome preparation. After exosome purification and filtration, the retentate and filtrate were separately collected and used as exosome enriched (KCM+) and depleted (KCM-) conditioned media, respectively.
Western blot analysis was then used to evaluate the level of stratifin in 10 µg of total proteins from exosome enriched KCM (KCM +) and exosome depleted KCM (lane KCM -), keratinocyte lysate (K, positive) and fibroblasts (F, negative) were used as controls. The procedure of western blot analysis was the same as that described in the Materials and Methods. (B) To evaluate the stimulatory effect of exosome associated stratifin in fibroblasts, western blot analysis was used to detect the level of MMP-1 in fibroblasts treated with either nothing (lane F), exosome enriched (KCM+) or depleted (KCM-) conditioned medium using 7 µg of total squamous cell carcinoma lysate (lane SCC) as positive control for detection of MMP-1.

For further confirmation and statistical evaluation, dermal fibroblasts were treated with keratinocyte purified exosomes (15 µg of total proteins) prepared from three different strains of human keratinocytes (Figure 2-4A). The levels of MMP-1 in fibroblasts treated with recombinant stratifin (5 µg/mL) and untreated fibroblasts were also evaluated in parallel and used as positive (C+) and negative (C-) controls, respectively. The results of western blot analysis clearly showed that fibroblasts treated with purified keratinocyte exosomes (Lanes 1-3) expressed 7.4 times more MMP-1 protein compared to that of untreated fibroblasts (0.66 ± 0.15 vs 0.09 ± 0.09, n=3, p<0.05 ) (Figure 2-4B). This finding further confirmed that exosomal stratifin released from keratinocytes is biologically active and can significantly stimulate the production of MMP-1 protein in dermal fibroblasts.
Figure 2-4 Quantitative analysis of the MMP-1 stimulatory effect of exosome associated stratifin in fibroblasts

(A) Fibroblasts were either treated with nothing (C-), 5 µg of recombinant stratifin (C+) or 15 µg of total exosomal proteins prepared from each of the three different strains of keratinocytes (exosome, lanes 1, 2 and 3). Fibroblasts were then harvested, lysed and 20 µg of total proteins extracted from treated and untreated fibroblasts was used to evaluate the level of MMP-1 protein by western blot analysis using a 10% acrylamide gel. The level of β-actin as a loading control was also evaluated. (B) For quantitative
analysis, the signals for MMP-1 and β-actin were quantified by densitometry and the mean ± SD of MMP-1/β-actin ratio was calculated for three independent experiment and depicted in panel B. * indicates the significant difference (p<0.05) in the level of MMP-1/β-actin ratio between exosome treated and untreated fibroblasts.

To estimate the amount of stratifin in total exosomal proteins in an obtained keratinocyte exosome preparation, we conducted western blot analysis (n = 3) in which we loaded and compared the stratifin signal of 10 mg of total protein of keratinocyte exosomes with those of a serial dilution of the recombinant stratifin ranging from 0-1 mg/sample (0, 0.06, 0.12, 0.25, 0.5 and 1 mg/sample). When the intensities of corresponding bands were quantified by densitometry we created a standard curve generating a linear regression model (y = 10634 x + 301.42 with r² = 0.9954) in order to quantify the amount of stratifin protein in our exosome sample. The results showed that approximately 1% of the total exosomal proteins is related to stratifin (0.1 μg of stratifin in 10 μg of total exosomal proteins loaded) (Figure 2-5).
Figure 2-5 Estimation of the percentage of stratifin in total keratinocyte exosomal proteins

Ten µg of total proteins from keratinocyte exosomes (Lane E) was run along with different concentrations of recombinant stratifin (from 0, 0.06, 0.12, 0.25, 0.5 to 1µg/lane) (n=3). The intensity of corresponding signals were then quantified by densitometry and compared. A linear regression model was generated (y = 10634 x + 301.42 with r² = 0.9954) in order to quantify the amount of stratifin protein in keratinocyte exosomes sample. The results show that approximately 1% of all exosomal proteins correspond to stratifin (0.1 µg of stratifin in 10 µg of total exosomal proteins loaded).
Discussion

Stratifin was previously found to be secreted by epidermal keratinocytes (105), but no physiological function was assigned. We previously identified stratifin, also named as keratinocyte-derived anti-fibrogenic factor (KDAF), in keratinocyte-conditioned medium (80). The mechanism by which this protein is released into the extracellular environment is not known. In this study, we used the exosome purification method developed by Lamparski (104) and demonstrated that the vesicles purified from primary human keratinocyte conditioned medium were in fact exosomes. There are several reasons for this claim. Firstly, consistent with previous TEM exosome images (90-92, 106), the ultrastructure examination of the exosomal preparation by TEM showed small vesicles less than 100 nm in diameter with the unique “saucer-like” shape, which are characteristic features of exosomes. Secondly, the result of the western blot analysis of the exosomal fraction not only identified stratifin, it contained LAMP-2 and hsc70 which are two well established exosomal markers. Thirdly, preliminary results of a proteomic analysis of the purified keratinocytes exosomes revealed the presence of stratifin and other exosomal related common proteins such as cytoskeletal, cytosolic, annexins, Rab, tetraspanins and MHC class I proteins (data not shown). Further, the method established by Lamparski (104) used to purify exosomes from KCM consists of several steps that exclude other non-exosomal structures and cell debris. The first centrifugation procedure excludes cell debris and subsequent use of filters with large pore size (100 kDa) during concentration stages of exosome preparation allow free stratifin to pass into the
elute, while exosomes associated stratifin retains in the filtrate. Finally, the ultracentrifugation with a sucrose gradient allows only vesicles with exosome characteristics (density and size) to be purified and that excludes other types of vesicles such as apoptotic blebs (102).

Upon characterization of the purified keratinocyte exosomes, we then evaluated the level of exosome associated stratifin along with exosomal markers in the same exosomal preparation using specific antibody raised against human stratifin. Although, there is ~ 60% of homology amongst all seven 14-3-3 isoforms, stratifin (14-3-3 α) is the only isoform that shows less than 43% homology with other isoforms. Further, according to the previous report by Aitken’s group (107), this antibody was raised against acetylated N-terminal peptide of the non-homologous region of the stratifin. Using this antibody, we were able to easily detect the level of stratifin in KCM isolated exosomes using keratinocyte lysate as a positive control.

Although, the level of keratinocyte releasable exosome associated stratifin was easily detectable by western blot analysis, its value was less than that found in the keratinocyte conditioned medium. For this reason and for a better comparison, we had to load 1.5 times more of total exosomal proteins than that of concentrated KCM (15 vs 10 µg / lane of total concentrated protein in exosomes and KCM, respectively). In theory, if exosome externalization is the only mechanism by which stratifin is released, one expects to see a higher stratifin/total protein ratio in purified exosomes than that of KCM derived from the same numbers of cells or protein content. However, this was not the case in this
study. This is because, as stated in the introduction, in addition to exosome externalization, there are other pathways of non-classical externalization of proteins through which stratifin can also be released into conditioned medium. In fact, the result of the experiment on exosomal depletion shown in Figure 2-3A confirms this theory. Having said that, our finding revealed that at least 90% of the stratifin present in KCM is released by the mechanism of exosomal externalization. It remains to be seen what other mechanism(s) is involved in stratifin release other than exosomal externalization. Further more, we wanted to know what percentage of stratifin was associated with exosomal proteins, and we found that approximately 1% of the total exosomal proteins correspond to stratifin. Considering this, the increase on the expression of MMP-1 protein in dermal fibroblasts might be due to other proteins associated with exosomes including other 14-3-3 isoforms. In fact, our group recently reported data showing that not only stratifin increases MMP-1 protein expression in dermal fibroblasts, but also recombinant 14-3-3 η (108) and 14-3-3 β isoforms (Data not shown).

According to the previous published study (101), 14-3-3 and its isoforms were identified to be inside exosomes externalized from dendritic cells. Although, we speculate that this might also be the case for the keratinocyte exosome associated stratifin, a systematic study is needed to confirm this point. Considering this, one may ask how intra-exosomal stratifin can stimulate the expression of MMP-1 in fibroblast. There are two possibilities to explain this issue. The first possibility is that stratifin is released from exosomes sometime after their externalization and interacts directly with fibroblasts and stimulates
MMP-1 expression. The second possibility is that stratifin containing exosomes are taken up by the fibroblasts and by an unknown mechanism this would result in an increase in MMP-1 expression in fibroblasts. The results of the previous reports are in favour of the first theory. This is because our previous published paper (109) showed that recombinant stratifin directly stimulates MMP-1 mRNA expression in fibroblasts through p38 mitogen-activated protein kinase (MAPK). This finding was supported by showing that SB203580 (specific inhibitor of p38MAPK activity) inhibits the MMP-1 stimulatory effect of stratifin in fibroblasts. We further showed that treatment of dermal fibroblast with SFN resulted in a rapid and transient up-regulation of c-jun and c-fos mRNA levels. This finding reveals that stratifin may interact with fibroblasts and stimulate MMP-1 expression possibly by its release due to disruption of exosomes sometimes after their externalization from keratinocytes. In fact, according to our experience, the overnight keratinocyte conditioned medium used to evaluate the exosomal shape (Figure 2-1) by TEM did not show any intact exosomal structures until we have used fresh KCM instead. This finding indicates that exosomes are fragile and have tendency to rupture upon their release. However, a systematic study is needed to confirm this finding.

In conclusion, by purification and characterization of the exosomes from keratinocyte conditioned medium, we provided evidence that keratinocytes have the capacity to externalize exosomes in which stratifin is associated with. Further, the finding shows that exosome associated stratifin has a potent MMP-1 stimulatory effect for fibroblasts and this effect can be abrogated by depletion of
exosomes from KCM. Thus, the findings of this study open a new insight into the biological role of exosome associated proteins such as stratifin in keratinocytes / fibroblast interaction.
Chapter 3. Profile of Exosomes Related Proteins Released by Differentiated and Undifferentiated Human Keratinocytes

Introduction

The epidermis undergoes continuous self-renewal through proliferation of stem cells in the basal layer. Periodically, the basal layer cells leave the stratum basale, stop proliferating, and express a new set of structural proteins and enzymes. These cells move upwards as they undergo a process of terminal differentiation that finishes with the anucleated cells of the stratum corneum. The whole renewal process takes approximately one month (110). A number of autocrine and paracrine factors drive keratinocyte differentiation. Ca$^{2+}$ and 1,25-dihydroxyvitamin D3, an active metabolite of vitamin D, play important roles in this process (111). Cytosolic Ca$^{2+}$ signalling is well known to control a wide range of cell functions, from short term responses such as contraction and secretion to longer term regulation of cell growth and proliferation (112). The epidermis has a Ca$^{2+}$ gradient, with the lowest concentration in the stratum basale and the

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highest in the stratum granulosum, where critical proteins for barrier functions are produced (113, 114). In vitro, human keratinocytes respond to extracellular Ca\textsuperscript{2+} inducing differentiation (115).

Exosomes are single membrane vesicles of endocytotic origin. These intralumenal vesicles are generated by inward budding of the limiting membrane (89). They have been isolated from different cell types such as, reticulocytes (106, 116), platelets (117), B and T lymphocytes (92, 95), intestinal epithelial cells (98), mast cells (97, 118, 119) and tumor cells (100) and from clinical human samples: broncho-alveolar lavage fluid (120), breast milk (121), plasma (89), urine (122) and human mesothelioma cells (118). They have specific features that distinguish them from other plasma membrane-derived vesicles. Their size ranges from 50-90 nm in diameter and following a negative staining they display a unique “saucer-like” morphology by electron microscopy (101). Biochemical and proteomic analysis of exosomes purified from the conditioned media of several cultured cells revealed the presence of common proteins (92, 93, 98, 102, 123). Depending on the cells examined, all or some of the following cytosolic proteins such as, hsp70, hsc73, hsp90, Tsg101, several annexins, Rab proteins, cytoskeletal proteins (actin, tubulin) and milk-fat globule (MFG)-E8 (or lactadherin) have been identified to be associated with exosomes. Membrane-bound proteins such as, tetraspanin (CD9, CD63, CD81, CD82), LAMP-2 and MHC class I or class II molecules were also detected. A density ranging from 1.13 to 1.22 g/ml is another specific characteristic that allow exosomes to float on sucrose gradient. Thus, contaminating material such as protein aggregates or
nucleosomal fragments are easily separated from exosomes by using sucrose gradients (101, 102).

In the previously discussed research we demonstrated that human keratinocytes have the capacity to release exosomes through which 14-3-3σ protein was released into keratinocyte conditioned medium (124). We also illustrated that exosomal 14-3-3σ functions as an MMP-1 stimulating factor for dermal fibroblasts. We quantified the amount of 14-3-3σ protein associated to human keratinocyte exosomes and found that 1% of the total exosomal protein correspond to 14-3-3σ. This finding suggests that keratinocytes may have the capacity to release other proteins by exosomal externalization, which would not be possible through conventional secretory pathways. Therefore, the main questions asked in this study are whether: 1) Keratinocytes release other proteins through the same mechanism of exosome externalization and if so what are these proteins? 2) In addition to 14-3-3σ what other 14-3-3 isoforms are among these exosome-related releasable proteins? And, is there any difference in the profiles of these proteins between differentiated and undifferentiated keratinocytes? Finally, 3) Are there other 14-3-3 protein isoforms with MMP-1 stimulatory effect in dermal fibroblasts?
**Materials and Methods**

**Cell Cultures**

After informed consent, foreskin samples were obtained from patients undergoing elective circumcision. Samples were collected individually in Keratinocyte Serum Free Medium (KSFM) (GIBCO, Grand Island, NY), and washed several times in sterile PBS supplemented with antibiotic-antimycotic preparation (100 µg/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml amphotericin B) (GIBCO, Grand Island, NY). Skin was dissected free of fat and cut into small pieces (1 cm in diameter) and incubated in Dispase enzyme (25 U/ml in KSFM) (GIBCO, Grand Island, NY) at 37 °C for 2 h in order to separate epidermis from dermis. Epidermis was incubated for 4 min in trypsin at 37 °C finger-vortexing every minute. Dulbecco’s Modified Eagle’s Medium (DMEM) (GIBCO Grand Island, NY) with 10% Fetal Bovine Serum (FBS) was used to stop trypsin reaction. Centrifugation at 800 rpm at 4 °C for 8 min allowed us to obtain keratinocytes that were seeded into 25 cm² flasks and cultured with 5 ml of KSFM with growth supplement (GIBCO, Grand Island NY) and antibiotic-antimycotic preparation (GIBCO, Grand Island, NY). The dermal layer was minced into small pieces ~2 mm in diameter and distributed into 60x15 mm Petri dishes cultured with FBS for 24 h and DMEM 10% FBS after. Fibroblasts migrated from these minced pieces were cultured with 10 ml of DMEM supplemented with 10% FBS and antibiotic-antimycotic preparation (GIBCO Grand Island, NY). Keratinocytes up to passage 4 and fibroblasts at passage 3-6
were used in all experiments conducted in this study. Human keratinocytes were seeded in 75 cm² flask using KSFM with 0.07 mM calcium and considered undifferentiated up to 60% of cells confluency. After this stage, keratinocyte conditioned medium was changed to KSFM/DMEM medium (50:50) (GIBCO, Grand Island NY) with 1.8 mM calcium. In this study, differentiated keratinocytes were used after day 12 of being exposed to 50:50 medium. From either group, undifferentiated and differentiated keratinocyte conditioned media were collected for exosome purification and the cells were lysed for western blotting analysis.

**Antibodies**

Antibody raised in rabbit against different human 14-3-3 isoforms (α, β, γ, ε, ζ, τ, η) were generously provided by Dr. Aitken (School of Biomedical and Clinical Sciences, University of Edinburgh, Scotland). Additionally, anti-involucrin and anti-human β-actin monoclonal antibodies were purchased from SIGMA (Saint Louis, MO); anti-heat shock cognate 70 (Hsc70) polyclonal antibody from STRESSGEN Bioreagents Corp. (Victoria, BC Canada); anti-human MMP-1 monoclonal antibody from R&D systems (Minneapolis, MN) and IRDye secondary antibodies, goat anti-rabbit IgG and anti-mouse IgG were obtained from LI-COR Biosciences (Lincoln, NE). Gold-conjugated goat anti-rabbit IgG secondary antibody (5 nm gold size) obtained from Ted Pella, Inc. (Redding, CA).
Western Blotting

Human keratinocytes and dermal fibroblasts were lysed with lysis buffer (50 mM Tris-HCL, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.025% NaN3, 1% Triton-X100, 0.5% IGEPAL CA-630 and 1:100 protease inhibitor), frozen at -80 °C, thawed at 37 °C, rotated for 20 min at 4 °C and sonicated for 4 sec, then nuclei and cell debris were removed by centrifugation at 10,000 rpm for 10 min at 4 °C. Total proteins of the cell lysate were quantified by Bradford assay and subjected to SDS-PAGE analysis on a 12% of acrylamide gel (unless stated). The gels were electrotransferred onto polyvinylidene difloride membranes (Millipore Billerica, MA). Non-specific proteins on membranes were blocked with TBS containing 5% skim milk and 0.05% Tween 20 for 60 min at room temperature. Immunoblotting was performed using anti-14-3-3σ (1:1000 dilution), anti-human β-actin (1:25,000), anti- Hsc70 (1:1000 dilution), anti-MMP-1 (1:250 dilution) and anti-Involucrin (1:1000 dilution) antibodies. The membranes were then washed and incubated with the appropriate IRDye secondary antibody (1:10,000 dilution). Immunoreactive proteins were then visualized using Odyssey Infrared Imaging System (Li-Cor Biosciences Lincoln, NE) for either 700 or 800 nm wavelengths, depending on the type of antibodies used.

Exosome Purification

Exosomes were purified from the conditioned media of human keratinocyte cells (~5 x 10⁷ cells) cultured in either KSFM (GIBCO, Grand Island, NY) for undifferentiated keratinocytes or in KSFM/DMEM (50:50) for differentiated keratinocytes. To purify exosomes, the mini-scale exosome
puriﬁcation protocol described by Lamparski (104) was used. Briefly, 100-150 ml of keratinocyte conditioned medium was cleared by centrifugation at 6,000 xg at 4 °C for 10 min in order to remove cell debris. The precleared medium was then concentrated to a volume of 1-2 ml using a 100 kDa MWCO Centricon Plus-20 filter capsule (Millipore Billerica, MA). The sample was then transferred to a 5 ml ultracentrifuge tube and then underlayered with 300 µl of 30% sucrose-deuterium oxide (D₂O). The sample was ultracentrifuged at 100,000 xg for 1 h at 4 °C. Three hundred and ﬁfty µl of the cushion of sucrose from the bottom of the tubes was collected and diluted in 15 ml of PBS. The puriﬁed exosomes were then washed and concentrated by centrifugation at 1000 xg for 10-25 min using 100 kDa Millipore Ultrafree-15 capsule ﬁlter (Millipore, Billerica, MA). Fresh exosomes were used in subsequent immunogold labelling assay. The remaining fraction was stored at -20 °C until used for Western blot analysis.

Immunogold Labelling

After exosomes puriﬁcation, fresh exosomal preparation (10 µl) was ﬁxed with 4% paraformaldehyde and gently washed with 0.05% Tween 20 in PBS for 10 min. To immunogold label the exosomes for 14-3-3σ protein, we used a protocol described by Weaver L. (116). Briefly, 10 µl of samples were deposited onto formvar-coated carbon EM grids (Canemco Inc., St. Laurent, Quebec). Grids were washed twice with PBS 1 min each, and blocked for 10 min in 2% BSA in PBS solution. Excess moisture was removed. Grids were incubated with 14-3-3σ primary antibody (1:1000 dilution) for 1 h and washed 3 times for 5 min each with 0.2% BSA in PBS. Afterwards, grids were incubated with 5 nm gold-
conjugated goat anti-rabbit secondary antibody (Ted Pella, Inc.) (1:100 dilution) for 1 h and washed 3 times 5 min each with 0.2% BSA in PBS. Excess moisture was removed and samples were fixed with 1% glutaraldehyde in PBS for 5 min. Then, grids were washed with distilled water, and negatively stained with 1% uranyl acetate for 15 sec. Excess stain was removed and the grids were air dried before viewing with a Hitachi H7600 Transmission electron microscope (TEM) with a side mount AMT Advantage (1 mega-pixel) CCD camera (Hamamatsu ORCA) (UBC Bioimaging Facility, Vancouver, BC, Canada).

**Proteomics (HPLC/MS/MS)**

Fifteen µg of total exosomal proteins from undifferentiated keratinocytes and differentiated keratinocytes were loaded in a 12% acrylamide gel and run along with a molecular weight marker (Fermentas Life Science Burlington, ON). The gel was stained with Coomassie Blue (Fermentas Life Science Burlington, ON), and bands from 26 to 43 kDa from both samples were cut and sent to UBC MSL/LMB Proteomics Core Laboratory for proteomic analysis along with purified exosomes from undifferentiated keratinocytes and differentiated keratinocytes in solution (Fig 3A and Table 1). Samples were trypsin digested. The protein signals were recorded using AB MDS-SCIEX API QSTAR Pulsar i mass spectrometer (Sciex, Thornhill, ON). Peptides were separated by reverse phase C18 column on an Ultimate HPLC system (LC Packings, Amsterdam, Netherlands) interfaced with the mass spectrometer.

For LC/MS experiments, proteolytic digests of proteins were loaded onto a C18 column (LC Packings, 75 µm i.d. X 150 mm PepMap) and eluted with a
gradient of 2 to 40% solvent B over the course of 60 min at a flow rate of 0.2 µl/min (Solvent A, 0.1% formic acid and 2% acetonitrile in water; solvent B, 0.1% formic acid and 85% acetonitrile in water). The time of flight (TOF) mass analyzer was scanned over a mass-to-charge ratio range of 360-2000 amu, with a step size of 0.1 amu and the scan time of 1 s. The ion source potential was set at 2.2 KV; the orifice energy was 50 V. To determine the amino acid sequence, the mass spectrometer operated in a IDA (Information Determined Acquisition) MS/MS mode. A survey scan for one second was first done from 360 to 1500 m/z. The three most intense doubly and triply charged ions were selected to undergo MS/MS fragmentation in 3 second scans from 70 to 2000 m/z. The collision energies were determined automatically by the instrument based on the m/z values and charged states of the selected peptides. Carbamido methyl C and a variable N-terminal acetyl, Moxidation, N deamidation and C propionamide were selected as permitted. The search was carried out using Mascot database, human species.

**Probability Based Mowse Score**

Ions score is -10* Log(P), where P is the probability that the observed match is a random event. Individual ion scores > 40 indicate identity or extensive homology (p < 0.05). Protein scores are derived from ion scores as a non-probabilistic basis for ranking protein hits.
**Treatment of Fibroblast with Purified Keratinocyte Exosomes and Conditioned Medium**

Dermal fibroblasts were seeded in 6-well plates (2 x 10^5 cells per well) with DMEM 2% FBS and treated in duplicate with either 1 ml of keratinocyte conditioned medium (KCM) or with 15 µg of total keratinocyte exosome proteins from undifferentiated keratinocyte and differentiated keratinocyte cells. After 24 h of treatment, dermal fibroblasts were harvested, lysed and the protein concentration was determined by Bradford assay. Western blot was used to assess MMP-1 levels using β-actin as loading control.

**MMP-1 Expression in Fibroblasts Treated with Different 14-3-3 Isoforms**

To evaluate whether other 14-3-3 isoforms also stimulate MMP-1 expression in fibroblasts, we used two different approaches. First, we have used northern blot to examine the level of MMP-1 mRNA in response to 14-3-3 β isoform (another abundant 14-3-3 isoform present in keratinocyte exosomes). To achieve that, dermal fibroblasts were seeded in 6-well plates (2 x 10^5 cells per well) with DMEM 2% FBS and treated in duplicate with different concentrations of recombinant 14-3-3β protein. The concentrations used were 0.25, 0.5, 0.75, 1, 2 and 4 µg/ml. After 24 h of treatment, dermal fibroblast conditioned medium was removed, fibroblasts were washed with PBS and harvested with 400 µl of 4 M guanidium isothiocyanate (GITC) solution and total RNA was isolated by the acid-guanidium-phenol-chloroform method (125). Total RNA from each individual fibroblast culture was then separated by electrophoresis (10 µg per lane) on a 1% agarose gel containing 2.2 M formaldehyde and was transferred onto a
nitrocellulose membrane (Bio-Rad Laboratories, Hercules, California). The blots were baked for 2 h at 80 °C under vacuum and prehybridized for 4 h at 45 °C in a prehybridization solution. Hybridization was performed at 45 °C in the same solution, using collagenase (MMP-1), or 18S ribosomal RNA cDNA probes. The cDNA probes were labeled with α-32p-dCTP by nick translation. The membranes were then washed initially at room temperature with 2 X sodium citrate/sodium chloride buffers and 0.1% sodium dodecylsulfate (SDS) for 1 h and finally washed for 20 min at 65 °C in 0.1 X sodium citrate/sodium chloride buffer and 0.1% SDS. Autoradiography was performed by exposing Kodak X-Omat film to nitrocellulose filters at –80 °C in the presence of an intensifying screen. The cDNA probe for MMP-1 was obtained from American Type Culture Collection (Rockville, MD). Quantitative analysis of autoradiograms was accomplished by densitometry.

Second, to confirm this finding, we then used western blot analysis to evaluate the levels of MMP-1 protein not only in response to 14-3-3 β but also in response to other two isoforms, 14-3-3 η and σ. Dermal fibroblasts were treated in duplicate with 2 µg/mL of recombiant 14-3-3 (η, σ and β) isoforms. After 24 h fibroblasts were washed with PBS, harvested, lysed and the protein concentration was determined by Bradford assay. Detection of β-actin was used as loading control. Quantitative analysis of autographs was measured by densitometry.
Treatment of Human Keratinocytes with Ionophores, Monensin (MON) and Ionomycin (IONO), and Functional Assay

Knowing that Ca\(^{2+}\) play a role in the differentiation process (111, 115), we asked whether a Na\(^+\)/Ca\(^{2+}\) exchanger stimulates the release of keratinocyte exosomes. In order to achieve this, undifferentiated keratinocyte cells (~3 x 10\(^6\) cells), were incubated with either 7 µM Monensin (MON) purchased from eBioscience (San Diego, CA) or 1 µM Ionomycin (IONO) purchased from SIGMA-ALDRICH (St. Louis, MO) for 6 h at 37 °C. Keratinocyte cells without any treatment were used as a control. KCM was collected and exosomes were isolated and quantified by determining the levels of hsc70 protein by Western Blot analysis. Forty µl of keratinocyte exosomes from the 3 groups (either treated with MON, IONO or without treatment) were loaded in a 10% polyacrylamide gel, and western blot procedure was performed as previously described. The intensity of detected bands was quantified by densitometry. To demonstrate that exosome associated proteins are functional; fibroblasts were treated with 150 µl of purified keratinocyte exosomes from either keratinocytes treated with MON, or from control. After 24 h of treatment, dermal fibroblasts were harvested, lysed and the protein concentration was determined by Bradford assay. Western blot was used to asses the MMP-1 levels using β-actin as loading control.

Statistical Analysis

Results are presented as mean ± SD. The t-test was used to compare the mean values between different groups. P-values less than 0.05 were considered statistically significant.
Results

Differences in 14-3-3σ Protein Expression in Undifferentiated and Differentiated Keratinocytes

Human keratinocytes kept in keratinocyte serum free medium (KSFM) containing 0.07 mM Ca\(^{2+}\) and up to 60% of cell confluency were considered undifferentiated keratinocytes (Figure 3-1A), whereas those kept in KSFM/DMEM (50:50) containing 1.8 mM Ca\(^{2+}\) and up to day 12 were considered differentiated keratinocytes (Figure 3-1B). To confirm that culturing keratinocytes in high Ca\(^{2+}\) medium resulted in keratinocyte differentiation, the level of involucrin protein, which served as a differentiation-specific keratinocyte marker (Figure 3-1C middle panel) was evaluated by western blot analysis. Undifferentiated and differentiated keratinocyte lysates were loaded; 14-3-3σ recombinant protein and dermal fibroblast lysate were used as positive and negative control respectively. The result showed the expression of involucrin only in differentiated keratinocytes but not in undifferentiated keratinocytes or in fibroblast (control) lysate.

Confirming the differentiation status of these cells, the level of 14-3-3σ protein in undifferentiated and differentiated keratinocytes was also evaluated. The expression of this protein by differentiated keratinocytes was 2.2 times more compared to that of undifferentiated ones (Figure 3-1C top panel lanes KU and KD). β-actin protein was used as loading control (Figure 3-1C bottom panel).
Figure 3-1 Microscopic images and western blot analysis of differentiated and undifferentiated keratinocytes

(A). Human keratinocytes, which were cultured in KSFM with supplement up to 60% confluence, were considered undifferentiated keratinocytes. (B). After this stage, keratinocyte conditioned medium was changed to KSFM/DMEM medium (50:50), which contains 1.8 mM Ca²⁺ and kept until become differentiated, day 12. (C). For western blot analysis, 10 µg of total proteins of undifferentiated and differentiated keratinocytes was loaded in a 12% SDS-PAGE along with 5µg of recombinant 14-3-3σ and 10 µg of dermal fibroblast lysate, as positive and negative control, respectively. Samples were analysed
by western blot using rabbit anti-human 14-3-3σ and anti-involucrin and β-actin monoclonal antibodies.

**Presence of 14-3-3σ in Exosomes from Differentiated Keratinocytes**

To demonstrate whether the level of releasable 14-3-3σ in exosomes from differentiated and undifferentiated keratinocytes reflects intracellular levels of this protein, conditioned media were collected and exosomes were purified using the Lamparski mini-scale purification method (104). The level of 14-3-3σ protein in exosomes from differentiated and undifferentiated keratinocytes was evaluated by western blot analysis (Figure 3-2A). The results showed a significant increase in the level of 14-3-3σ in both KCM (Figure 3-2A lane KCM-D) and exosomes released from differentiated keratinocytes (Figure 3-2A lane EXO-D) compared to those of KCM (Figure 3-2A lane KCM-U) and exosomes (Figure 3-2A lane EXO-U) released from undifferentiated keratinocytes. A quantitative analysis of these data showed a 2.6 time increase in the level of 14-3-3σ in exosomes from differentiated keratinocytes to that of undifferentiated keratinocytes (4.4 ± 1.16 vs. 1.7 ± 0.5; n=4; p= 0.007). Furthermore, we performed immunogold labelling for 14-3-3σ associated with keratinocyte exosomes from differentiated and undifferentiated keratinocytes. The results showed many clusters of small vesicles under 100 nm in diameter with the unique “saucer-like” shape, characteristic of exosomes visualized under TEM. Interestingly, as shown by TEM image, 14-3-3σ immunogold-particles were only detected in exosomes released from differentiated keratinocytes (Figure 3-2B image B and C) relative to those of undifferentiated keratinocytes (Figure 3-2B image A). This finding is
consistent with that of western blot analysis showing a markedly higher level of 14-3-3σ in both differentiated keratinocyte lysate and exosomes released from these cells. The lack of gold particles in the background demonstrates the highly specific association of these particles to the exosomes. Quantitative analysis showed that ~60% of the exosomes had gold particles associated with them (500 exosomes were scored).

Figure 3-2 *Presence of 14-3-3σ in keratinocyte exosomes*
Ten µg of total proteins of KCM and purified human keratinocyte exosomes from undifferentiated keratinocytes (Lane U) and differentiated keratinocytes (Lane D) was analysed by western blot using rabbit anti-human 14-3-3σ antibody. Bands were quantified using densitometry and the mean ± SD was calculated for 4 independent experiments. * and ** represents p value < 0.05 and 0.001, respectively and were considered to be statistically significant. (B) For electron microscopic evaluation of 14-3-3σ, 10 µl of fresh purified exosomes from undifferentiated keratinocytes and differentiated keratinocytes were fixed and deposited onto formvar-coated carbon EM grids. They were first incubated with rabbit anti-human 14-3-3σ primary antibody and later with 5 nm gold-conjugated goat anti-rabbit secondary antibody. Grids were negatively stained and viewed using transmission electron microscope. (Left image) Electron microscopic evaluation of 14-3-3σ in exosomes from undifferentiated keratinocytes. The absence of gold-beads confirms the lack of 14-3-3σ in exosomes from undifferentiated keratinocytes (Bar = 100 nm); (Centre image) Electron microscopic evaluation of 14-3-3 in exosomes from differentiated keratinocytes. The arrow shows the gold-beads associated to exosomes, confirming the presence of 14-3-3σ. (Bar = 100 nm) (Right image) High magnification showing the gold-beads associated with exosomes from differentiated keratinocytes. (Bar = 10 nm)

Profile of Undifferentiated and Differentiated Keratinocyte Exosome-Associated Proteins

To further evaluate whether other proteins particularly the rest of the 14-3-3 isoforms were also associated with exosomes, exosomes from differentiated and undifferentiated keratinocytes in solution were sent for proteomic analysis. The profile of exosomal proteins was compared between undifferentiated and
differentiated keratinocytes. Proteins with > 40 MOWSE score were considered statistically significant (p < 0.05) and listed in Table 3-1. The profile of detected proteins was categorized based on protein function and compared to those previously reported from other cell types. The proteomics analysis showed that keratinocyte exosomes from undifferentiated and differentiated keratinocytes contain a wide variety of proteins (more than 100 proteins each), such as metabolic enzymes like: glyceraldehyde 3 phosphate deshydrogenase (GAPDH), fructose-biphosphate aldolase (FBPA), α-enolase, etc.; cytoskeletal proteins: actin, moesin, coflin, etc.; chaperones: hsc70, hsp90, cyclophilin, etc.; signalling proteins: 14-3-3 proteins, Ras, etc.; trafficking and adhesion proteins: cadherin, annexin 2, Rab proteins, etc., showing consistency with exosomes from other cell types previously published (89, 101, 121) (Table 3-1). Interestingly, the content of exosomes from differentiated and undifferentiated keratinocytes was different. They showed similarities regarding the proteins mentioned above, however proteins related to keratinocyte differentiation were mainly found in exosomes isolated from differentiated keratinocyte conditioned medium. More than 10 proteins shown with an asterisk in Table 3-1 are specific or involved in cell differentiation particularly in keratinocytes.

Furthermore, some proteins such as fibroblasts growth factor binding protein (FGF-BP), MMP-1 proprotein, MMP-3/TIMP-1 complex, MMP-8 and MMP-9 were found in exosomes from either undifferentiated or differentiated keratinocytes. These findings suggest that keratinocyte exosomes might function as communicating vesicles between keratinocytes and fibroblasts. The results
also revealed that not all isoforms of 14-3-3 proteins are present in exosomes from undifferentiated keratinocytes. As shown in Figure 3-3A, exosomes from undifferentiated keratinocytes do not contain 14-3-3α, ε, and τ isoforms, whereas exosomes from differentiated keratinocytes contain all 7 isoforms (σ, β, η, ε, ζ, γ and τ).

To further confirm this finding, we performed western blot analysis of differentiated and undifferentiated keratinocyte lysate as well as the corresponding releasable exosomes (Figure 3-3B). The results showed that all seven isoforms are detectable in differentiated and undifferentiated keratinocyte lysate (Figure 3-3B lane K-U and K-D) but with different intensities. The results of the 14-3-3 protein profile between differentiated and undifferentiated keratinocytes evaluated by western blot analysis (Figure 3-3B) were consistent with those obtained from proteomic analysis. It is worth mentioning that the level of 14-3-3 η isoform was higher in exosomes from undifferentiated keratinocytes compared to that of differentiated cells (Figure 3-3B lanes EXO U and EXO D 3rd row). On the other hand, under the same experimental condition, the level of releasable 14-3-3 β was higher in keratinocyte exosomes (Figure 3-3B, 2nd row) than that from the cell lysate. This was particularly pronounced in exosomes from differentiated keratinocytes than that of undifferentiated keratinocytes.
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Table 3-1 Identification of exosome-associated proteins from undifferentiated and differentiated keratinocytes using proteomic analysis

Proteins with > 40 MOWSE score were considered statistically significant (p < 0.05) and listed in this table.\textsuperscript{1,2}Mast, \textit{Mast cell} (4); B, \textit{B cell} (33,35); MC, \textit{Mesothelioma cell} (19); IEC, \textit{Intestinal Epithelial cell} (41); DC, \textit{Dendritic cell} (38, 39); HM, \textit{Human Milk} (2); R, \textit{Reticulocytes} (22); TC, \textit{Tumor cells} (42). * Proteins involved in cell differentiation.
Figure 3-3 Presence of other 14-3-3 isoforms in exosomes from undifferentiated and differentiated keratinocytes

For proteomic analysis, 15 µg of total exosomal proteins was loaded in a 12% SDS-PAGE from undifferentiated and differentiated keratinocytes. Gels were stained with Coomassie Blue and bands from 26 to 43kDa were excised and sent for proteomic analysis. (A) The table shows the presence or absence of different 14-3-3 isoforms in exosomes from undifferentiated keratinocytes (Lane EXO U) and differentiated keratinocytes (Lane EXO D) using proteomic analysis. (B) Ten µg of total keratinocyte
lysate was loaded along with 15 µg of total exosomal proteins from undifferentiated keratinocytes (Lane U) and differentiated keratinocytes (Lane D) in a 12% SDS-PAGE. Western blot analysis was used to confirm the presence of 14-3-3 isoforms in both samples. β-actin was used as a loading control for the lysates.

**Expression of MMP-1 in Fibroblasts after Treatment with Keratinocyte Exosomes, KCM and 14-3-3η, σ and β Recombinant Protein**

To evaluate the MMP-1 stimulatory effect of exosomes-associated 14-3-3 on dermal fibroblast, cells were treated with 15 µg of total protein extracted from exosomes released from either undifferentiated or differentiated keratinocytes. Fibroblasts without treatment and treated with 5 µg of recombinant 14-3-3σ were used as negative and positive controls, respectively. The results of western blot analysis clearly showed that dermal fibroblasts treated with exosomes from differentiated keratinocytes expressed 2 times more MMP-1 protein compared to that of those treated with exosomes from undifferentiated keratinocytes (0.9 ± 0.08 vs 0.453 ± 0.213, n = 3, p= 0.027) (Figure 3-4A and B). These differences were not due to variation in protein loading as the level of β-actin, used as a loading control, was used to determine the ratio of MMP-1/β-actin protein, which is depicted in Figure 3-4A. We also found that in spite of the lack of 14-3-3σ in exosomes from undifferentiated keratinocytes, the expression of MMP-1 in dermal fibroblast was increased upon treatment with these exosomes (Figure 3-4A EXO U). As we reported previously, the other 14-3-3 isoform (η) which is present in exosomes also increases the level of MMP-1 in dermal fibroblasts.
(126). As the level of 14-3-3 β isoform was highly expressed in exosomes evaluated by western blot analysis, here, we tested the MMP-1 stimulatory effect of this isoform in dermal fibroblasts. To achieve this, fibroblasts were treated with different concentrations of recombinant 14-3-3 β isoform and the expression of MMP-1 mRNA was examined. The Northern blot analysis showed a marked dose dependent increase in the level of MMP-1 mRNA in fibroblasts treated with 14-3-3 β isoform relative to that of untreated control cells (Figure 3-4C). Detection of 18S ribosomal mRNA was used as a loading control (0.3 ± 0.16; 0.43 ± 0.28; 0.64 ± 0.23; 1.005 ± 0.57; 1.04 ± 0.03; 1.20 ± 0.20 and 1.19 ± 0.23; n = 2). At the protein level, MMP-1 expression in dermal fibroblasts was evaluated by treating fibroblasts with three different isoforms of 14-3-3 recombinant proteins (η, σ and β) in two independent experiments. β-actin was used as a loading control (MMP-1/β-actin ratio shown in Figure 3-4D). The western blot analysis showed the increased expression of MMP-1 in dermal fibroblasts after treatment with all three isoforms tested compared to that of control (0.925 ± 0.015 vs. 0.439 ± 0.2, n = 2; 0.8 ± 0.05 vs. 0.439 ± 0.2, n = 4, p = 0.04; n = 2).
Figure 3-4 Expression of MMP-1 in fibroblasts treated with keratinocyte exosomes and keratinocyte conditioned medium

(A) Fibroblasts (2 x 10^5 cells per well) in 1 ml of DMEM were treated with either 1 ml of KCM or with 15 µg of total exosome proteins from undifferentiated keratinocytes (Lane U) and differentiated keratinocytes (Lane D) 2% of FBS was added to all groups. Cells were harvested after 24 h, lysed and total proteins were extracted and quantified. Western blot was used to assess the MMP-1 levels using β-actin as a loading control. (B)
For quantitative analysis, the signals for MMP-1 and β-actin were quantified by densitometry and the mean ± SD of MMP-1/β-actin ratio was calculated for three independent experiments. * represents p value < 0.05 considered to be statistically significant. Expression of MMP-1 in dermal fibroblasts treated with 14-3-3 isoforms using both northern and western blot (C) Dermal fibroblasts were treated with different concentrations (0.25, 0.5, 0.75, 1, 2 and 4 µg/ml) of 14-3-3 β protein. Cells were harvested, RNA was isolated and separated by electrophoresis on a 1% agarose gel. Hybridization was performed using MMP-1 and 18S ribosomal RNA cDNA probes using procedure described under the Materials and Methods. Quantitative analysis of autoradiograms was accomplished by densitometry. MMP-1/18S ratio was calculated for two independent experiments. (D) To confirm the results of northern blot at the protein level, dermal fibroblasts were treated with 2 µg/ml of three different 14-3-3 isoforms (η, σ and β). Cells were harvested, lysed and total proteins were extracted and quantified. Western blot analysis was used to assess the MMP-1 levels using β-actin as a loading control. For quantitative analysis, the signals for MMP-1 and β-actin were quantified by densitometry and MMP-1/β-actin ratio was calculated and depicted in panel B (n=2 independent experiments).

Treatment of Undifferentiated Keratinocytes with Ionophores (MON and IONO) and Functional Assay

To explore the mechanism by which differentiation of keratinocytes increase the production of exosome-associated proteins, we evaluated the role of Ca²⁺ in the release of exosomes. It has been previously reported that exosome release from K562 cells (Human erythroleukemic cells) was increased when they
were treated with a ionophore like Monensin (MON), a Ca^{2+} entry inducer by reversed activity of the Na^{+}/Ca^{2+} exchanger (127-129). To explore the possibility of the same mechanism, we treated undifferentiated keratinocytes with MON at different concentrations ranging from 1-10 µM (data not shown) and found that the tolerable dose of MON for keratinocytes was 7 µM. Keratinocytes without treatment were used as control. Hsc70 was used as an exosomal marker. After 6 h of treatment, conditioned media from both groups were collected and exosomes were purified. The results showed 4.2 times increase in the level of Hsc70 exosome-associated protein release in keratinocytes treated with 7 µM MON compared to that of untreated controls (6.4 ± 1.8 vs 1.5 ± 0.521, n= 7, p= 0.0002) (Figure 3-5A).

To examine whether an increase in exosome release produced an MMP-1 stimulatory effect, dermal fibroblasts were treated with the same volume of exosomes released from MON treated and untreated keratinocytes. The results of 4 independent experiments revealed that keratinocytes treated with MON not only released more exosomes, but that these exosomes were also functional demonstrated by an increase in the expression of MMP-1 in dermal fibroblasts (Figure 3-5B lanes EXO MON and EXO C).

To confirm the effects of MON, we also treated undifferentiated keratinocytes with 1 nM of Ionomycin (IONO), a ionophore that is widely used for increasing the intracellular levels of calcium. After 6hr treatment conditioned media were collected from untreated (control) and treated cells and exosomes purification was performed. The results of five independent experiments showed
that IONO increased the release of Hsc70 exosome-associated protein 2.25-fold compared to that of control (29.68 ± 2.16 vs. 13.19 ± 1.85, n= 5, p= 0.0004). This finding confirms the important role that Ca$^{2+}$ plays in the mechanism of exosome release.

Figure 3-5 Treatment of undifferentiated keratinocytes with ionophores (MON and IONO) and functional assay
(A and B) Human keratinocytes were incubated with either 7 μM MON or 1 μM IONO for 6 h at 37 °C. Keratinocytes without any treatment were used as control. KCM was collected and exosomes were isolated. Forty μl of purified exosomes from treated and untreated keratinocytes were loaded in a 10% SDS-PAGE. Exosomes were quantified by determining the levels of Hsc70 proteins by Western blot analysis. The intensity of detected bands was quantified by densitometry. The mean ± SD of Hsc70 was calculated for more than 5 independent experiments. * represents the p value ≤ 0.0002 considered to be statistically very significant. (C) Dermal fibroblasts were treated with 150 μl of purified exosomes from MON treated and untreated keratinocytes. Cells were harvested after 24 h, lysed and total protein were extracted and quantified. Twenty five μg of total proteins from each sample was loaded in a 10% SDS-PAGE. Western blot was used to asses the MMP-1 levels using β-actin as a loading control. For quantitative analysis, the signals for MMP-1 and β-actin were quantified by densitometry and the mean ± SD of MMP-1/β-actin ratio was calculated for 4 independent experiments. * and ** represents p value < 0.001 and < 0.005 respectively, which considered to be statistically significant.


**Discussion**

In our previous report, we demonstrated that human keratinocytes have the capacity to release exosomes containing 14-3-3σ protein (124). This finding set the stage for the current study to ask the question of whether keratinocytes release other proteins through the same mechanism and if so, what are these proteins? We further asked whether other 14-3-3 isoforms are amongst these exosome-related releasable proteins and if so, is there any difference in the profiles of these proteins between differentiated and undifferentiated keratinocytes?

It is well established that different intracellular 14-3-3 isoforms possess multifunctional roles in signal transduction pathways controlling cell proliferation and differentiation (130, 131). For instance, 14-3-3σ is an intracellular protein, which is involved in a wide range of vital regulatory processes. When 14-3-3σ binds to KRT17 it regulates protein synthesis and epithelial cell growth.

Keratinocytes from the basal layer (undifferentiated keratinocytes) possess different functions and protein profile compared to those of differentiated epidermal cells (132). In this study, keratinocyte differentiation was induced by using an increased (still physiological levels) Ca²⁺ concentration in the medium (1.8 mM). As it is well known, Ca²⁺ is one of the essential factors inducing this process due to the natural Ca²⁺ gradient in the epidermis (111). Using this system, we found that differentiated keratinocytes expresses an easy detectable level of 14-3-3σ; however the expression of this protein was barely detectable in undifferentiated ones, as well as in releasable exosomes from these cells.
To address the question of whether other cellular proteins are also released by exosomes externalization, proteomic analysis was carried out and the results showed that keratinocyte exosomes contain a variety of proteins such as metabolic enzymes, cytoskeletal, cytosolic, chaperones, signalling, trafficking and adhesion proteins, consistent with exosome proteins previously described from other cell types (89, 101, 121). For instance, proteins like Hsp70 family and annexin 2 are most likely involved in exosome function and or biogenesis, whereas proteins like Mac-2BP are potentially involved in their association to target cells (Amigorena S. et al, 1999).

Surprisingly, we found that keratinocytes also release plasminogen activator inhibitor type 1 (PAI-1) through exosomes. PAI-1 has been suggested to play a role in the formation of a provisional matrix in wound healing (133) and also stimulates keratinocytes adhesion and migration for tissue repair (134). L-lactate dehydrogenase (LDH) was also found in keratinocyte exosomes. This molecule produces L-lactate (lactic acid) which can stimulate endothelial cells growth and proliferation in angiogenesis (135). Transforming growth factor-β induced (TGF-β induced) was also found to be secreted through keratinocyte exosomes. This growth factor is a chemoattractant for fibroblasts (136), stimulating them to express collagen and other ECM proteins (137). Matrix metalloproteinase-9 (MMP-9) was also found to be released by keratinocytes via exosomes. This MMP might be involved in keratinocyte migration, knowing that they can only migrate over living tissue (138, 139). Proteins such as, epithelial cell growth factor, involucrin, kallikrein-7, keratinocyte differentiation-associated
protein, MAPK3 protein are involved in keratinocyte differentiation. Interestingly, there were proteins that suggest communication with fibroblasts such as, transmembrane protein jagged-1 which is suggested to stimulate fibroblast growth factor in vitro \(140\).

This study also revealed that the isoform profile of 14-3-3 proteins in exosomes from differentiated keratinocytes were different from those of undifferentiated ones. Exosomes from undifferentiated keratinocytes only showed the presence of 4 (\(\beta, \eta, \zeta, \gamma\)) of the 7 different 14-3-3 isoforms. On the other hand, exosomes released from differentiated keratinocytes contained all seven isoforms (\(\alpha, \beta, \eta, \varepsilon, \zeta, \gamma\) and \(\tau\)). These findings were further confirmed by western blot analysis. 14-3-3 \(\eta\) isoform was detected at a higher density in exosomes from undifferentiated keratinocytes relative to those of differentiated keratinocytes; whereas \(\gamma, \alpha, \varepsilon\) and \(\zeta\) isoforms were easily detectable in exosomes from differentiated keratinocytes compared to those of undifferentiated ones.

Each of the 14-3-3 protein isoforms are involved in different functions. For example: 14-3-3 \(\sigma\) isoform is involved in the regulation of protein synthesis and epithelial cell growth; it is also known as epithelial cell marker \(141\) and our studies have shown to be a collagenase stimulating factor for dermal fibroblasts \(80\). 14-3-3 \(\gamma\) and \(\beta\) isoforms are known as protein kinase C inhibitor proteins; 14-3-3 \(\varepsilon\) has activity related to signal transduction and cell division and along with 14-3-3 \(\zeta\) both are involved in the regulation of insulin sensitivity. 14-3-3 \(\tau\) is also known as 14-3-3 protein T-cell as well as a protein kinase regulator \(130\).
In order to reveal the mechanism by which differentiated keratinocytes increase the expression of exosome-associated proteins, we used two ionophores (MON and IONO) to study the effect of Ca\(^{2+}\) in exosomes. Both are known to increase the level of intracellular calcium in cells. It is also known that treatment with MON leads to the generation of multivesicular bodies. Our results demonstrated that the release of exosomes from undifferentiated keratinocytes was increased by treatment with MON (4.2 fold) and IONO (2.25 fold). This finding might explain the physiological Ca\(^{2+}\) gradient found in the epidermis and its relation with the increase in the expression of exosome-associated proteins.

The results of the present study demonstrate an in depth profile of keratinocyte exosomes with specific characterization of the 14-3-3 protein profile differences between exosomes coming from differentiated and undifferentiated keratinocytes. We further demonstrated that not only 14-3-3\(\sigma\) increases the levels of MMP-1, 14-3-3 \(\beta\) and 14-3-3\(\eta\) isoforms (126) also stimulated the expression of MMP-1 in fibroblasts. This finding suggests a potential role for these proteins released from keratinocytes upon epithelialization. The MMP-1 stimulating effect of 14-3-3\(\sigma\) free exosomes from undifferentiated keratinocytes may be explained by the presence of 14-3-3\(\eta\) and \(\beta\). This finding may explain why delay in wound closure and lack of keratinocyte-fibroblasts interaction would result in accumulation of key ECM components such as collagen at the wound site during the late stage of the healing process. As such, one may propose to therapeutically use these factors to prevent and/or improve fibroproliferative
conditions such as keloid and hypertrophic scarring which frequently develop upon thermal injury, severe trauma and incisional surgery.
Chapter 4. SPARC/SFN, a Novel Complex that Suppresses Type I Collagen Expression in Dermal Fibroblasts

Introduction

Dermal fibrotic conditions, such as, hypertrophic scars (HS), are characterized by large amounts of extracellular matrix (ECM) deposition that is of altered composition and organization compared with normal dermis or mature scar (142). However the molecular changes taking place in HS are not well understood. Delayed epithelialization during the process of wound healing also increases the frequency of development of these fibrotic conditions (28). These observations suggest that signals derived from epithelial cells slow down the mid-stage process of healing, leading to maturation and remodeling of the healing wound (29).

Type I collagen is the most abundant and fibrous form of all collagens produced by fibroblasts (143). Excessive deposition of collagen results in

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excessive scarring and dermal fibrosis. An increasing body of evidence suggests that keratinocytes are able to regulate the expression of type I collagen in dermal fibroblasts (58, 144, 145). This regulation is primarily mediated by releasable factors acting in an autocrine/paracrine loop and to a lesser extent direct cell to cell contact. In fact, several studies have shown the regulatory role of releasable factors in keratinocyte-conditioned media (KCM) in the expression of ECM genes by dermal fibroblasts (25, 146-148).

In the search for keratinocyte-derived anti-fibrogenic factors, our group has previously identified stratifin (SFN) as a potent stimulator of matrix metalloproteinase 1 (MMP-1) expression in dermal fibroblasts (58). We have also reported a significant decrease in the expression of type I collagen in human fibroblasts that have been either co-cultured with keratinocytes or treated with KCM (at the mRNA and protein level) (58, 149). Further characterization of this keratinocyte-derived collagen-inhibitory factor (KD-CIF) revealed a >30kDa molecular weight protein, which has shown stability up to 56°C and in an acidic environment of pH 2.0 (149). In this study, we identified two proteins that are released by keratinocytes, which form a novel complex with a significant collagen-inhibiting effect on dermal fibroblasts. In this study we also evaluated the presence of these two proteins in a fibrotic animal model and human HS tissue. In conclusion, these findings provide evidence that type I collagen expression in fibroblasts is modulated by a novel keratinocyte-derived SPARC/SFN complex.
**Materials and Methods**

**Cell Culture**

Following informed consent, foreskins were obtained from neonates undergoing circumcision. The study was approved by the University of British Columbia Hospital Human Ethics Committee and conducted according to the Declaration of Helsinki Principals. Written informed consent was obtained from the parents of each participant. Foreskins were washed with PBS 1% antibiotic-antimycotic preparation (100 U/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B) (GIBCO, Carlsbad, CA). Epidermal and dermal layers were then separated by treating samples with dispase (Roche Applied Sciences, Laval, QC, Canada). Keratinocytes and fibroblasts were cultured as previously described (150). Fibroblasts were grown in Dulbecco's Modified Eagle Medium (DMEM) with 10% Fetal Bovine Serum (FBS) (GIBCO, Carlsbad, CA) and keratinocytes in Keratinocyte Serum-Free Medium (KSFM) supplemented with bovine pituitary extract (50 µg/ml) and epidermal growth factor (0.2 µg/ml) (GIBCO, Carlsbad, CA).

**Keratinocyte Fibroblast Co-Culture System**

The co-culture model was previously described in Ghaffari et al. (2006). Briefly, either keratinocytes or fibroblasts (0.25 x 10^6) were seeded on a 30 mm Millicell-CM culture plate inserts (Millipore, Billerica, MA) with 0.4 mm pore size with either KSFM with supplement or DMEM 10% FBS, respectively. Fibroblasts (0.5 x 10^6) were seeded on the bottom chamber of a 6-well culture plate.
containing DMEM 10% FBS. Cells were incubated separately for 24 h, after which the conditioned media were collected and cells were washed with PBS. To assemble the co-culture system, inserts containing either keratinocytes or fibroblasts (upper chamber) were placed on top of the fibroblasts (K/F or F/F respectively) (bottom chamber) seeded in 6-well plates. The test medium used in co-culture consisted of 49% KSFM without supplements, 49% DMEM and 2% FBS for all samples. Fibroblasts were harvested at either 24 h for RNA extraction or 48 h for protein extraction, and analyzed using q-PCR or western blot analysis.

**Purification of Keratinocyte-Derived Collagen Inhibiting Factor**

Keratinocytes were grown as described above, and when they reached 90-95% confluency, medium was changed to 49.5% KSFM no supplements, plus 49.5% DMEM, plus 1% antibiotic-antimycotic preparation. The conditioned media collected from keratinocytes every 24h for a period of 10 days, were centrifuged to remove any cell debris and concentrated. Five hundred mg of total protein was subjected to fast protein liquid chromatography system (FPLC) ÄKTAFPLC™ (General Electric, Piscataway, NJ). First an ion-exchange column (anion) Mono Q™ (General Electric, Piscataway, NJ) was used and then a size-exclusion column Superdex™ 75 HR gel filtration column (General Electric, Piscataway, NJ). The fractions from the ion-exchange column were desalted using Zeba desalting spin columns (Pierce, Rockford, IL). In order to evaluate the collagen-inhibiting effects, fibroblasts were treated with desalted fractions complemented with test medium for 24 h if analyzed using q-PCR or 48 h if analyzed by western blot. Only active fractions were subjected to the size exclusion column. The
active fractions were subsequently examined by electrophoresis on a gradient 4-20% Mini-PROTEAN® TGX™ Precast Gel (BioRad, Mississauga, ON) and stained with SYPRO Ruby (Invitrogen, Carlsbad, CA). Bands were cut and analyzed by proteomics.

**Proteomics**

Active fraction (in solution) and their bands were analyzed using mass spectrometry. All samples were trypsin digested. The resulting peptides were separated using a 75 µm x 100 mm 1.7 µm BEH130 C18 column using a 3-40% linear acetonitrile gradient, with 0.1% FA present, at 0.3 µl/min over 40 min using a NanoAcquity™ LC (Waters, Milford, MA). The column was re-equilibrated for 20 min between runs. Column eluate was directed into a Synapt™ mass spectrometer through a 20 µm capillary held at 3.2 kV. Instrument calibration was carried out using glu-fibrinogen fragments and Glu-fib was also used as a lock mass to compensate for any calibration drift. The instrument was run in V-mode with a mass resolution of approximately 10000. A data dependent method was used with a 1 s scan followed by up to 3 fragment scans, using ion intensity and charge state as the main selection criteria. The accumulated data was analysed using ProteinLynx Global Server software (PLGS) using peptide and fragment mass accuracies of 25 ppm and 0.1 Da respectively. Uniform carbamido methyl C and variable N-terminal acetyl, M oxidation, N deamidation and C propionamide were selected as permitted modifications with a maximum protein MW of 250 K. This search engine was applied to the full Uniprot
database, human species. A search with similar parameters was also carried out using Mascot using the .pkI peak list files generated in PLGS.

**Probability Based Mowse Score**

Ions score is -10*Log(P), where P is the probability that the observed match is a random event. Individual ions scores > 40 indicate identity or extensive homology (p<0.05). Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.

**Western Blot Analysis**

After 48 h, cell samples were lysed using lysis buffer (50 mM Tris-HCL, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.025% NaN3, 1% Triton-X100, 0.5% IGEPAL CA-630 and 1% protease inhibitor), frozen at -80 °C, thawed at 37 °C, rotated for 20 min at 4 °C and finally centrifuged at 10,000 rpm for 10 min at 4 °C to remove cell debris. Total proteins were quantified using Bradford assay (BioRad, Mississauga, ON). Ten to twenty µg were loaded into polyacrylamide gels. KCM samples were centrifuged at 10,000 rpm for 10 min at 4 °C to remove cell debris and 30 µl was loaded into polyacrylamide gels. Gels were run using Tris/Glycin/SDS Running Buffer at 115 V for 1 h. For proteins >72 kDa a 7% SDS-PAGE was used and transferred using Tris/Glycin/0.375% SDS transfer buffer at 80 V for 1 h. For proteins <72 kDa a 12% SDS-PAGE was used and transferred using a Tris/Glycine transfer buffer at 75 V for 90 min. Proteins were transferred to a PVDF membrane (Millipore, Billerica, MA). Non-specific binding sites were blocked using TBS-t 5% skim milk over night and incubated with either
anti-collagen type I mAb (1:100) SP1.D8 (Developmental Studies Hybridoma Bank, U Iowa, Iowa city, IA), or anti-SPARC mAb (1:10,000) (Haematologic Technologies Inc., Essex, VT), or anti-Stratifin mAb (1:1000) (NeoMarkers, Fremont, CA), or anti-Stratifin polyclonal Ab (1:1000 dilution) generously provided by Dr. Aitken (School of Biomedical and Clinical Sciences, University of Edinburgh, Scotland) or anti-β actin mAb (1:30,000) (SIGMA, Saint Louis, MO) for 90 min at 37°C. Then membranes were incubated with goat anti-mouse or goat anti-rabbit IgG IRDye secondary antibody from LI-COR Biosciences (Lincoln, NE) and analyzed using an Odyssey system (LI-COR Biosciences, Lincoln, NE) with two different wavelengths (800 and 680nm, respectively).

**Modeling Methods**

The ClusPro (151-154) web-based server was used in order to obtain the protein-protein docking model, where the PDB X-ray structures of 1SRA (155) (SPARC protein) and 1YZ5 (156) (SFN protein) where used as ligand-receptor, respectively. The most populated and balanced structure was chosen as the best protein-protein interaction model (Fig. 6A). Then, by means of the Molecular Operating Environment (MOE) (157), the residues of the ligand (receptor) closer than 2.5, 3.5 or 5.0 Å of the receptor (ligand) where computed.

**Co-Immunoprecipitation (Co-IP)**

Co-IP was performed as previously described (158). Briefly, 500 µg of total proteins from KCM was incubated with 1 µg of mouse anti-SFN (NeoMarkers, Fremont, CA) at 4°C overnight. Immunoprecipitates (IP) were
washed three times with PBST, resuspended in 40 µl of sample buffer and subjected to western blot analysis. Membranes were probed for anti-SPARC and anti-SFN. The remaining supernatants from IP were kept frozen for further functional assay.

For the functional assay, fibroblasts were treated with 200µl of the remaining IgG and IP supernatants. After 48h, fibroblasts were harvested, lysed and analyzed using western blot.

**Validation of Type I Collagen-Inhibiting Effect of hSPARC/rhSFN Complex**

Fibroblasts cultured in DMEM 2% FBS were treated with either various concentrations of hSPARC (Haematologic Technologies Inc., Essex, VT) (0.5, 1, 1.5, 2, 5, 10 µg/ml) or with various concentrations of rhSFN (58) (0.5, 1, 1.5, 2, 5, 10 µg/ml) separately, and analyzed for type I collagen expression at mRNA and protein levels. Untreated fibroblasts (C), or treated with 49% KCM (KCM) were used as negative and positive control, respectively.

To demonstrate whether hSPARC/rhSFN complex functions as a collagen-inhibitory factor in fibroblasts, fibroblasts cultured in DMEM 2% FBS were treated with a constant amount of hSPARC (1.5 µg) plus various concentrations of rhSFN (0.5, 1, 1.5, 2, 5, 10 µg/ml). After either 24 h or 48 h fibroblasts were harvested for either mRNA or protein. Untreated fibroblasts (C) were used as negative control. Fibroblasts treated with 49% of KCM (KCM) or 1.5µg/ml of hSPARC (SPARC) or 5µg of rhSFN (SFN) served as positive controls.
Quantitative-PCR (q-PCR)

Following 24 h of treatment, cell samples were pelleted and washed with PBS and total RNA was extracted. A total of 1 µg of RNA from each sample was reverse transcribed using the Superscript® first-strand synthesis system for RT-PCR (Invitrogen, Carlsbad, CA). For amplification process, the following sense and anti-sense primers shown in Table 4-1 were used on an Applied Biosystems 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA) mixed with SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA). Target gene expression was normalized to β-actin levels, and the comparative cycle threshold (C_t) method was used to calculate relative quantification of target mRNAs. Each assay was performed in triplicate with an n of 3-5 independent experiments.

<table>
<thead>
<tr>
<th>Species</th>
<th>Primer</th>
<th>Sense</th>
<th>Anti-sense</th>
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</thead>
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<tr>
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<td>SFN</td>
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<td>CTGCCACTGTCCAGTTCTCA</td>
</tr>
<tr>
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<td>ATGGCCTCTCGGAGCTGTT</td>
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<tr>
<td>Human</td>
<td>SPARC</td>
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<td>B-actin</td>
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<td>CTTCTGCAATGCAGTCGAGCGA</td>
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</table>

Table 4-1 Primers used for q-PCR analysis
Tissue Samples

To evaluate fibrotic scar formation in vivo, we utilized a well-established rabbit ear model (159). Four female adult New Zealand white rabbits (2.5 kg to 3 kg) were used in this study. They were housed and treated under a protocol approved by the Animal Ethical Committee from The University of British Columbia (A-051211). Animals were anesthetized with intramuscular injection of ketamine (22.5 mg/kg) and xylazine (2.5 mg/kg) followed by isoflurane gas through orotracheal intubation. One 8mm in diameter excisional wounds were prepared on the ventral surface of each ear using a punch biopsy device. The punch involved the removal of a circular, full-thickness tissue sample down to but not including the cartilage. Two wounds per animal were left open for 28 days. After 28 days wounds were totally epithelialized and scars were elevated. Animals were sacrificed and wounds were harvested (HS). Normal skin from each ear was used as controls (C). Thirty µg of tissue was used for mRNA extraction using Mini RNeasy Fibrous Tissue Kit (Qiagen, Mississauga, ON).

For human samples, punch biopsies were collected following informed consent from patients undergoing hypertrophic scar excision. Tissues were fixed in 10% formalin and sent for immunohistochemistry.

Immunohistochemistry

Human tissue samples were fixed with 10% formalin prior to paraffin embedding. Sections (4 µm) were then prepared and mounted on slides for staining. Antigen retrieval was performed using Citrate Buffer (pH 6.0).

Immunohistochemical staining of SPARC and SFN was achieved using a
mAb anti-SPARC (Haematologic Technologies Inc., Essex, VT) and mAb anti-SFN (NeoMarkers, Fremont, CA) at 1:100 dilution for 16 h at 4 °C. Biotinylated goat anti-mouse secondary (Vector Laboratories, Inc., Burlingame, CA) at 1:500 dilution was used followed by signal detection using Vector VIP substrate kit (Vector Laboratories, Inc., Burlingame, CA) according to the manufacturers instructions. Counterstaining of nucleus was achieved using methylene green (Vector Laboratories, Inc., Burlingame, CA). Images were captured using a Nikon DSRi1 camera on a Nikon Eclipse 80i upright bright field microscope and processed using Nikon NIS Elements software.

Statistical Analysis

Data were expressed as mean ± SD and analyzed with one-way ANOVA using GraphPad Instat Software (GraphPad Software Inc., San Diego, CA). A P-value of less than 0.05 was considered statistically significant.
**Results**

**KCM Suppresses Type I Collagen Expression in Fibroblasts**

Collagen expression in dermal fibroblasts was determined following co-culture with keratinocytes in a Transwell system as previously described (58). To quantify the collagen expression in fibroblasts co-cultured with keratinocytes or treated with KCM, total RNA and protein was extracted from fibroblasts. Fibroblasts mono-cultures were used as control. As shown in Figure 4-1A, type I collagen protein expression is significantly decreased (78.1%) in fibroblasts co-cultured with keratinocytes (lane K/F*) and 87.1% reduction in fibroblasts when treated with KCM (lane KCM) when compared to that of control (lane F) (0.219 ± 0.04 vs 1; n= 4; p< 0.01 and 0.129 ± 0.012 vs 1; n= 4; p< 0.01, respectively). The mRNA expression is also significantly decreased by 43.5% in fibroblasts co-cultured with keratinocytes (lane K/F*) and 36.7% in fibroblasts treated with KCM (lane KCM) relative to that of fibroblasts grown alone (lane F) (0.565 ± 0.063 vs 1; n= 4; p< 0.05 and 0.633 ± 0.054 vs 1; n= 4; p< 0.05, respectively).

To evaluate if the state of keratinocyte differentiation influenced the collagen expression in fibroblasts, KCM was collected everyday from day 1 to day 6 and used individually to treat fibroblasts. Fibroblasts treated in triplicates with a pooled sample of KCM from days 1 to 6 and untreated fibroblasts were used as positive and negative controls, respectively. As shown in Figure 4-1B, type I collagen at the protein level is decreased in fibroblasts treated with KCM from day 1-6 (lanes 1-6 KCM days) as well as when treated with the pooled KCM
(lane C+) when compared to that of untreated fibroblasts (lane C-). We have previously demonstrated that a differentiation marker, involucrin, is expressed from day 4 of culturing keratinocytes in DMEM/KSF media (160).

To further confirm that keratinocytes were viable during the collection period (every 24h for 10 days) when cultured in KSFM/DMEM medium in the absence of supplements, MTT assay was performed to keratinocytes in culture for 10 days. MTT assay results did not demonstrate any notable change in viability relative to that keratinocytes cultured in KSFM with supplements (Figure 4-1C).
Figure 4-1 Keratinocytes regulate type I collagen in fibroblasts

(A) Protein and mRNA were extracted from fibroblasts co-cultured with keratinocytes (K/F*), and fibroblasts treated with KCM (KCM) as well as fibroblasts untreated (F) and subjected to western blot analysis and q-PCR. All samples were tested for type I collagen and β-actin as loading control. The results showed that type I collagen protein expression is significantly decreased (78.1%) in fibroblasts co-cultured with keratinocytes (0.219 ± 0.04 vs 1; n= 4; p< 0.01) (lane K/F*) and 87.1% reduction in
fibroblasts when treated with KCM (0.129 ± 0.012 vs 1; n= 4; p< 0.01) (lane KCM) when compared to that of control (lane F). The mRNA expression is also significantly decreased by 43.5% in fibroblasts co-cultured with keratinocytes (0.565 ± 0.063; n= 4; p< 0.05) (lane K/F*) and 36.7% in fibroblasts treated with KCM (0.633 ± 0.054; n= 4 p< 0.05) (lane KCM) relative to that of fibroblasts grown alone (lane F). (B) Fibroblasts were treated in triplicates with different KCM collection days (D1-D6). Fibroblasts treated with KCM D1-D6 pool (C+) and untreated (C-) were used as positive and negative control respectively. Samples were subjected to western blot analysis and proved for type I collagen. β-actin was used as a loading control. The results showed that type I collagen at the protein level is decreased in fibroblasts treated with KCM from day 1-6 (lanes 1-6 KCM days) as well as when treated with the pooled KCM (lane C+) when compared to that of untreated fibroblasts (lane C-). (Image representative of an n= 3). (C) Keratinocytes in culture with KSFM/DMEM media without supplements (K/D) as well as keratinocytes cultured in regular media (KSFM with supplements) (Control) throughout 10 days were subjected to MTT assay to evaluate viability of the cells.

Identification of KD-CIF

To identify keratinocyte-derived collagen-inhibiting factor(s), KCM was subjected to an ion-exchange column (anion) as described under Materials and Methods section. Twenty-one fractions were collected and fibroblasts were treated with pooled samples comprising 3 fractions (i.e. 1-3; 4-6; 7-9 etc.). The results showed that fibroblasts treated with fractions 13-15 exhibited a 33.2% decrease in the expression of type I collagen when compared to that of control (lane FC) (0.668 ± 0.046 vs 1 ± 0.016, respectively; n= 3; p< 0.05) (Figure 4-2A)
and these results were further confirmed at the protein level (Figure 4-2B). To evaluate which fractions contained KD-CIF, fibroblasts were treated with fractions 10-21 individually, and untreated fibroblasts were used as negative control (lane C). The q-PCR results showed that fractions 13 and 14 contained the collagen-inhibiting factor(s) as evident by 70% decrease of type I collagen when compared to that of control (Figure 4-2C). To further identify this factor(s), fractions 13 and 14 were passed through a size exclusion column using the same FPLC system. Forty-five fractions were collected and fibroblasts were treated individually with these fractions. The results of q-PCR showed that fraction 20 showed a significant collagen reduction (0.447 ± 0.103; n= 3, p< 0.01) when compared to that of the control (Figure 4-3A). These results were subsequently confirmed by western blotting (Figure 4-3B). The linear regression of a molecular weight standard curve showed that a LogMW of 4.7166 for fraction 20 represented approximately 52 kDa (Figure 4-3C).

In order to identify the keratinocyte-derived collagen-inhibiting factor(s), fractions 16-26 were run on a gradient gel, showing a strong band around the 60 kDa in fractions 19-21, and finally disappearing on fraction 26 (Figure 4-4A). Bands from 19 and 20 as well as the total fractions were sent for mass-spectrometry. The results revealed the presence of two known proteins in the bands as well as in the total fraction: secreted protein acidic cystein-rich (SPARC) also known as osteonectin (34 kDa), and 14-3-3σ also known as stratifin (SFN) (28 kDa). As shown in Figure 4-4B, mass-spectrometry revealed
only peptides that matched for SPARC and SFN proteins showing a high MOWSE score.

A

B

C
Figure 4-2  **Purification of KD-CIF using an ion exchanger column on a FPLC system**

KCM was passed through an ion exchanger column and 21 fractions were collected. (A, B) Fibroblasts were treated in triplicates with groups of these fractions and subjected to q-PCR and western blot analysis. (A) Fractions 13-15 presented a 40% decrease in the expression of type I collagen when compared to that of control (lane FC) (0.668 ± 0.046 vs 1 ± 0.016, respectively; n= 5; *p< 0.05). (B) Results were confirmed at the protein level. *(Image representative of an n= 3).* (C) Fibroblasts were then treated in triplicates with fractions 15-21 individually. The q-PCR results showed that fractions 13 and 14 contained the collagen-inhibiting factor(s) as evident by 70% decrease of type I collagen when compared to that of control.
Figure 4-3 Purification of KD-CIF using size exclusion column on a FPLC system

(A) From 45 fractions, only fractions 13 and 14 were passed through a size exclusion column. Fibroblasts were treated in triplicates with the fractions and subjected to q-PCR. The results showed that fraction 20 had the most significant collagen-inhibiting effect
(0.447 ± 0.103; n=3, **p<0.01) when compared to that of the control. (B) Samples were also analyzed using western blotting. (*Image representative of an n=3*). (C) A standard curve was created using a linear regression. A $\text{LogMW}$ of 4.7166 for fraction 20 represented approximately 52 kDa.

<table>
<thead>
<tr>
<th></th>
<th>Samples</th>
<th>MW (kDa)</th>
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<tr>
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<tr>
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<tr>
<td>Ribonuclease A</td>
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Table 4-2 *Standards used for size exclusion column*
Figure 4-4 **Mass-spectroscopy of active fractions reveals SFN and SPARC proteins** (A). Fractions 16 – 26 were run in a gradient gel and stained with SYPRO RUBY. (B) Bands from 19 and 20 as well as the total fractions were sent for mass-spectroscopy.
The results matched peptide specifically for SPARC and SFN with a high mouse score. [Scores > 40 indicate identity or extensive homology (p< 0.05)].

**Expression of SPARC/SFN in Keratinocytes and Fibroblasts**

Upon identification of SFN and SPARC proteins by mass-spectroscopy, we then evaluated the expression of these proteins in: keratinocytes (lane K), fibroblasts (lane F) and when co-cultured fibroblasts with either fibroblasts (lane F/F*) or keratinocytes (lane K/F*) at the mRNA and protein levels. The result of Figure 4-5A reveals the presence of SPARC at the mRNA and protein levels in K and F and when co-cultured together. Unlike SPARC, SFN is only present in keratinocytes but not in fibroblasts shown at the mRNA and protein level. β-actin was used as a loading control in both settings.
**Figure 4-5 Expression of SPARC and SFN in keratinocytes, fibroblasts and KCM**

(A) Keratinocytes (K) and fibroblasts (F) as well as fibroblasts co-cultured with keratinocytes (K/F*) and fibroblasts co-cultured with fibroblasts (F/F*) were evaluated for the presence of SPARC and SFN at the gene and at the protein level. β-actin was used as a loading control. *(Image representative of an n= 3).*

(B) To evaluate the possibility of these two protein forming a complex under natural conditions, KCM as well as fraction 20 were run on a native non-denaturing gel. The results show the co-migration of both proteins at a molecular weight between 52-72 kDa.
Evaluation of SPARC/SFN as a Complex in KCM

Knowing that both proteins were produced and released by keratinocytes, we then evaluated the possibility of the formation of a complex by these two proteins under native conditions in KCM. To achieve this, KCM as well as fraction 20 were run on a native non-denaturing gel. The result of Figure 4-5B reveals that both proteins co-migrated to the same molecular weight on the gel, as both were detected using mouse anti-SPARC and rabbit anti-SFN antibodies and visualizing them under two different wavelengths (SPARC 800 nm and SFN 680 nm). This result suggested that SFN and SPARC may form a complex in KCM.

To further validate our findings, a Co-immunoprecipitation (Co-IP) of KCM was performed. Using sepharose beads as described under Material and Methods section, SFN was precipitated using anti-SFN antibody, IgG and IP from input, IP and supernatant samples were run on a SDS-PAGE and blotted for anti-SPARC antibody. As shown in Figure 4-6A, the IP sample showed the presence of SPARC and SFN when immunoprecipitated for SFN. These results confirm the presence of both proteins as a complex in the KCM.

In order to model how these two proteins could indeed form naturally a complex, a 3-D modeling using X-ray structures was designed as described under Material and Methods section (Figure 4-6B). The predicted structure showed a populated and balanced structure with rms constraints of 9 Å suggesting high possibilities of the formation of this structure in physiological conditions. Furthermore, possible binding sites were predicted with rms of 2.5 Å according to the binding characteristics for SFN. SPARC, which is a cystein-rich
protein, possesses two exposed (surface) serines (S) and one threonine (T), which can easily be phosphorylated. These residues are found at a close proximity of 3.5 Å to the SFN binding site. In fact, it is well known that SFN binds to phosphorylated S/T on target proteins as well as cystein-rich proteins (Figure 4-6B and D). Furthermore it was found that the possible binding sites of SPARC (Figure 4-6C) binds to two of the well known residues that are part of the “binding groove” of SFN at 3.5 Å (161). Together these findings demonstrate the ability of SPARC and SFN to form a complex structure.
Figure 4-6 Evaluation of SPARC/SFN as a complex in KCM

(A) Co-immunoprecipitation (Co-IP) on KCM was performed. SFN was precipitated using mouse anti-SFN antibody, IgG and IP from input, IP and supernatant samples were run on a SDS-PAGE and blotted for anti-SPARC antibody. The results showed the presence of SPARC and SFN when immunoprecipitated for SFN. (Image representative of an n=
4). In order to predict if these two proteins could indeed form naturally a complex, a 3-D modeling using X-ray structures was designed. **(B) Protein-protein interaction model.** SFN protein is depicted in green and SPARC protein is depicted in blue. The Serine and Threonine residues are highlighted in red and the residues close than 3.5 Å to them are drawn in yellow (the central residues are draft as Van der Waals spheres). **(C) A closer look at these binding sites between SFN (depicted in green) and SPARC (depicted in blue).** Possible phosphorylation sites (S/T) depicted in yellow, and in purple the two well-known residues (K49 and R56), which are part of the SFN “binding groove” at < 3.5 Å. **(D) Residues of the ligand (receptor) closer than 2.5, 3.5 or 5.0 Å of the receptor (ligand).** The possible phosphorylated residues of the ligand (SPARC) are depicted in red, and their corresponding binding residues from SFN are depicted in yellow and purple matching panel B image.

**SPARC/SFN Complex Responsible for the Collagen-Inhibitory Effect in Fibroblasts**

To evaluate if the SPARC/SFN complex was in fact responsible for suppressing collagen expression, we treated fibroblasts with the supernatant of IgG and IP samples using untreated and KCM-treated fibroblasts as controls. As shown in Figure 4-7A, IgG supernatant revealed a 40% decrease in type I collagen expression (lane IgG) comparable to that seen when treated with KCM (40% decrease) (lane KCM). This suppression was restored when fibroblasts were treated with the IP supernatant (lane IP) when compared to that of IgG supernatant (lane IgG) (1.348 ± 0.255 vs. 0.807 ± 0.139; n= 4; p< 0.01). Notably collagen expression in fibroblasts treated with IP supernatant (lane IP) is
comparable to collagen expression in untreated fibroblasts (lane C). This finding suggests that when the SPARC/SFN complex is removed from the KCM, type I collagen levels return to baseline, loosing the collagen-inhibitory effect, however when fibroblasts are treated with the IgG supernatant containing SPARC/SFN complex we observed the collagen-inhibitory effect in fibroblasts. These results imply that SPARC/SFN complex may be responsible for collagen suppression in fibroblasts.

To further investigate the function of SPARC/SFN as a complex compared to their efficacy independent from one another, fibroblasts were treated with each protein separately. As mentioned under Materials and Methods section, fibroblasts were treated with different concentrations of rhSFN and hSPARC (0.5, 1, 1.5, 2, 5 and 10 µg/ml) and type I collagen at the mRNA and protein levels were evaluated. As observed in Figure 4-7B, when fibroblasts were treated with hrSFN at 0.5 µg/ml there was a significant reduction in collagen expression when compared to that of control (0.70 ± 0.05 vs 1 ± 0.03, respectively; n= 5; p< 0.05), which progressively returned to the baseline levels as the concentration of SFN is increased. A similar trend was observed when fibroblasts were treated with hSPARC, however at a 1.5 µg/ml concentration, type I collagen expression was stimulated and progressively increased from baseline in a dose dependent fashion. Similar trend was also found when evaluating collagen mRNA expression (1.48 ± 0.168 vs 1 ± 0.37, respectively; n= 5; p< 0.05) (Figure 4-7C).

Importantly, the significant collagen-inhibitory effect of KCM treatment was not observed at any concentration of these proteins when delivered
independently. Fibroblasts were then treated with SPARC and SFN in combination, to evaluate the potential collagen-inhibitory effect (Figure 4-7D). Fibroblasts were treated with a fixed dose of 1.5 µg/ml of hSPARC (according to above results), and with variable increasing doses of rhSFN (0.5, 1, 1.5, 2, 5, 10 µg/ml). Fibroblasts treated with 1.5 µg/ml of SPARC (lane 1.5 µg SPARC) and fibroblasts treated with 2 µg/ml of rhSFN (lane 2 µg SFN) as well as untreated fibroblasts (lane C) or treated with KCM (lane KCM) were used as controls. The results revealed a significant 70% decrease in type I collagen expression at both mRNA and protein levels when a combination of 1.5 µg/ml of SPARC plus 1.5 µg/ml SFN were added to fibroblasts (0.3636 ± 0.066; n= 3; p< 0.01) compared to that of untreated fibroblasts (lane C) or to fibroblasts treated with either 1.5 µg/ml SPARC or 2 µg/ml SFN (1.371 ± 0.043 and 1.052 ± 0.006; n= 3) (Figure 4-7D). This effect was maintained while SFN dosage was increased (2, 5 and 10 µg/ml) and SPARC dosage remained the same (1.5 µg/ml) (0.93 ± 0.01; 0.79 ± 0.02; 0.335 ± 0.057; n= 3; respectively). This finding also supports the earlier computerized docking data, whereby a specific ratio of SPARC to SFN suggests to be required to form the complex and influence collagen expression in dermal fibroblasts.
Figure 4-7 SPARC/SFN complex as the responsible factor for the collagen-inhibitory effect in fibroblasts

To evaluate if the SPARC/SFN complex was in fact responsible for suppressing collagen expression, (A) we treated fibroblasts with the supernatant of IgG and IP samples using untreated and KCM-treated fibroblasts as controls. The results showed that when fibroblasts were treated with IgG supernatant it significantly decreased type I collagen expression (40%) (lane IgG) comparable to that of when treated with KCM (40% decrease) (lane KCM). This suppression was reverted when fibroblasts were treated with the IP supernatant (lane IP) when compared to that of IgG supernatant (lane IgG) (1.348 ± 0.255 vs. 0.807 ± 0.139; n= 4; **p< 0.01). (B) Fibroblasts were treated in triplicate with different concentrations of rhSFN and hSPARC (0.5, 1, 1.5, 2, 5 and 10 µg/ml) and evaluated for type I collagen at the protein and (C) mRNA levels. Fibroblasts treated with 0.5 µg of rhSFN showed a significant reduction in collagen expression when compared to that of control (0.70 ± 0.05 vs 1 ± 0.03, respectively; n= 5; *p< 0.05), which progressively returned to the baseline levels as the concentration of SFN is increased. Similar trend was observed when fibroblasts were treated with hSPARC, however at a 1.5 µg/ml concentration, type I collagen expression was stimulated and progressively increased from baseline in a dose dependent fashion (1.48 ± 0.168 vs 1 ± 0.37, respectively; n= 5; *p< 0.05). (D) To evaluate the collagen-inhibitory effect, fibroblasts were treated with the combination of both proteins. Fibroblasts were treated with a fixed dose of 1.5 µg/ml of hSPARC, and with variable increasing doses of rhSFN (0.5, 1, 1.5, 2, 5, 10 µg/ml). Fibroblasts treated with 1.5 µg/ml of SPARC (lane 1.5 µg SPARC) and fibroblasts treated with 2 µg/ml of rhSFN (lane 2 µg SFN) as well as fibroblasts untreated (lane C) or treated with KCM (lane KCM) were used as controls. Samples were evaluated at the protein (top image) and mRNA levels (bottom Graph). The results revealed a significant 70% decrease in type I collagen expression at both mRNA and
protein levels when a combination of 1.5 µg/ml of SPARC plus 1.5 µg/ml SFN were added to fibroblasts (0.3636 ± 0.066; n= 3; **p< 0.01) compared to that of untreated fibroblasts (lane C) or to fibroblasts treated with either 1.5 µg/ml SPARC or 2 µg/ml SFN (1.371 ± 0.043 and 1.052 ± 0.006; n= 3; **p< 0.01). This effect was maintained while SFN dosage was increased.

**Evaluation of Physiological Levels of SPARC and SFN in Fibrotic Tissues**

A fibrotic rabbit ear model was used to evaluate the physiological role of SPARC/SFN. Four, 8-mm diameter wounds were created on rabbits’ ears. On day 28 when hypertrophic scars (HS) were formed, tissues were harvested and evaluated at mRNA level. The tissue was analyzed using q-PCR for SPARC, SFN and type I collagen mRNAs. The results show (Figure 4-8A) that HS reveal a very significant amount of SPARC when compared to that of control (3.158 ± 0.707 vs. 1; n= 4, p< 0.01) and a very low level of SFN when compared to that of control (0.342 ± 0.0046 vs 1; n= 4, p< 0.01). On the other hand, type I collagen was very high (14.235 ± 0.535; n= 4, p< 0.01) when compared to that of control.

To further evaluate this pattern, human HS tissue samples and normal human tissue were also analyzed for SPARC and SFN using immunohistochemistry. The results shown in Figure 4-8B revealed an increased expression of SPARC and an almost absent expression of SFN in human HS when compared to that of control. These results may suggest that a balance is probably necessary between SPARC and SFN to maintain type I collagen normal levels.
Figure 4-8 Evaluation of physiological levels of SPARC/SFN in a fibrotic animal model

(A) Hypertrophic scar tissue was analyzed using q-PCR for SPARC, SFN and type I collagen mRNAs. The results show that HS present a very significant amount of SPARC when compared to that of control (3.158 ± 0.707 vs. 1; n= 4, **p< 0.01) and a very low level of SFN when compared to that of control (0.342 ± 0.0046 vs 1; n= 4, **p< 0.01).
Nonetheless, type I collagen is very high (14.235 ± 0.535; n= 4, **p< 0.01) when compared to that of control. (B) Human HS tissue samples and normal human tissue were analyzed for SPARC and SFN using immunohistochemistry. The results revealed an increased expression of SPARC and an almost absent expression of SFN in human HS when compared to that of control. Images were taken at low magnification of 10x (planfluor, 0.3 NA) with a scale bar of 100 μm and high magnification of 40x (planfluor, 0.75 NA) with a scale bar of 50 μm. All images were captured using a Nikon DSRI1 camera on a Nikon Eclipse 80i upright bright field microscope and processed using Nikon NIS Elements software.
Discussion

This study provides compelling evidence that keratinocyte-derived factors, such as SPARC/SFN, are modulators of the epithelial/mesenchymal interactions, crucial in the regulations of ECM components. Previously our group discovered a keratinocyte-releasable form of SFN that showed a significant MMP-1 stimulatory effect in dermal fibroblasts (58). Although it was also evident at that time that KCM could suppress the mRNA expression of type I collagen the reason of this effect remained unknown, as rhSFN alone did not reproduce this observation.

Here, we showed that when fibroblasts are co-cultured with keratinocytes or treated with KCM there was a significant reduction in type I collagen expression at the mRNA and protein levels (Figure 4-1A). This is consistent with previous data from Harrison and Gardner (33, 144), in which they used the N-terminal pro-peptide of type I collagen in conditioned media or the incorporation of (3H) proline as markers for collagen production, revealing also a role for keratinocyte-fibroblast cross talk in regulation of type I collagen.

To ensure that this collagen-inhibitory factor was not released as a consequence of keratinocyte death, an MTT assay was performed. This assay demonstrated that cells were as viable as compared with those from control.

Furthermore, we were able to identified by mass-spectroscopy that SFN and SPARC are the two proteins present in the active fraction.

SFN belongs to the family of 14-3-3 proteins, which are highly conserved molecular chaperones. It was originally thought to be an exclusive intracellular protein due to its lack of a signal peptide, but in 1982 Boston et al., (47) reported
the presence of this protein in cerebrospinal fluid in patients with neurological disorders. Later in 1999 Katz et al., (57) found that SFN was secreted into keratinocyte-conditioned media, however no physiological function was assigned to this protein until in 2004 when Ghahary et al., (58) demonstrated SFN to be a potent MMP-1 stimulatory protein in dermal fibroblasts.

SPARC also known as osteonectin, or BM-40, was first described by three different groups as a major constituent of bovine and human bone and as a protein secreted by proliferating cells in vitro (68, 162, 163). It was shown to be a Ca\(^{2+}\)- binding glycoprotein that functions as a counter adhesive protein, modulating cell shape, growth factor activity and as a cell-cell inhibitor. SPARC is expressed at significant levels in tissues undergoing repair or remodeling due to wound healing process, disease or physiological conditions. Fibroblasts and macrophages start expressing SPARC in healing wounds where it is also released by platelet degranulation (69). Pathological conditions such as cancer metastasis, arthritis, diabetes, kidney disease, and fibrotic conditions are characterized by elevated expression of SPARC (75).

We have previously showed that SFN does not inhibit collagen expression in dermal fibroblast (58). It has also been demonstrated that SPARC is able to stimulate the expression of type I collagen (76). This was further confirmed when, Francki et al., showed that SPARC-null cells exhibited significantly diminished expression of type I collagen and TGF-β1 in mouse mesangial cells (76). After treatment with rhSPARC, the levels of type I collagen and TGF-b1 were restored to 70% and 100%, respectively, compared to those produced by wild-type cells.
We further confirmed these results, by treating fibroblasts with either rhSFN or hSPARC alone, without observing any type I collagen-inhibitory effect (Figure 4-7B). In fact, fraction 20 containing SFN (a 28 kDa protein) and SPARC (a 34 kDa protein) corresponded to an approximate molecular weight of 52 kDa. This finding suggested that SFN and SPARC may form a complex in the conditioned media from keratinocytes, and possibly together inhibit type I collagen expression in fibroblasts. This was confirmed when a 3-D structure of the complex was created using known X-ray structures of both SFN and SPARC, revealing a balanced structure with a high probability of binding (rms 2.5 Å).

Moreover, three protein-protein binding sites were predicted suggesting to occur on phosphorylated Serine (S141, S231) and Threonine (T233) which coincide with the binding characteristics of SFN to other known proteins (79, 161). These data further underscore the probability of a naturally occurring SPARC/SFN complex *in vivo*. In addition, we demonstrated that when fibroblasts were treated with hSPARC/hrSFN complex the expression of type I collagen in fibroblasts was markedly decreased at the mRNA and protein levels. Likewise, when fibroblasts were treated with the supernatant from immunoprecipitation, the collagen-inhibitory effect was removed, suggesting that the complex is indeed responsible for this effect.

There are no reports in the literature suggesting that these two proteins interact, yet together they influence mRNA or protein expression. In fact, very few extracellular functional roles have been attributed to either protein.
It is well documented that 14-3-3 proteins, in general, are capable of binding to and sequestering or inhibiting other proteins (79). Although the mechanism for the suppression of collagen expression by the SPARC/SFN complex remains to be elucidated, it is reasonable to suggest that SFN might sequester SPARC in the extracellular environment and thus, interfere with ligand and receptor interaction. This interference may result from inhibiting the binding of SPARC to integrins or adaptor proteins and thereby suppressing collagen synthesis. Further studies are needed to clarify this mechanism. Although much progress has been made to understand the function of SPARC and SFN, many questions still remain unanswered.

Notably in the wound environment differentiated keratinocytes release SFN, whereas numerous skin cells and infiltrated immune cells can release SPARC and thus, our findings further support the observation that delay in wound epithelialization can result in fibrotic conditions such as HS (76, 164, 165). This was observed when we evaluated the physiological level of SFN, SPARC and type I collagen in a fibrotic animal model (Figure 4-8A). Similarly, sections from human HS showed a reduction in SFN and an increase in the amount of SPARC (Figure 4-8B). Based on our findings, a lack of SFN protein and an overexpression of SPARC cause a greater expression of type I collagen, which may in part, be the reason why these wounds develop HS.

In conclusion, our findings suggest that upon epithelialization, keratinocytes may in fact release SPARC and SFN and that may complex in the extracellular space modulating the expression of ECM components in fibroblasts
during the phase of tissue remodeling. The identification of this complex has further provided us with another potential therapeutic agent to treat HS, frequently developed following burn injury, deep trauma and some surgical incisions.
Chapter 5. Conclusion and Suggestions for Future Work

General Discussion

It is increasingly evident that cell-cell communication in the dermal healing process, for example the epithelial-mesenchymal cross talk by keratinocyte cells and fibroblasts, is central to any wound healing strategies including remodeling. Wound healing depends on a fine regulation of protein production by fibroblasts, as well as, matrix degradation, attributed essentially to proteolytic enzymes belonging to the matrix metalloproteinase (MMP) family (166).

It is been demonstrated that any delay in epithelialization during the process of wound healing, due to either infection or severity of injury, increases the frequency of developing fibrotic conditions (28). This suggests that in the absence of epidermis, the ECM continues to accumulate until fibroblasts receive signal(s) from the epidermis to slow down the dynamic process of healing that leads to maturation and remodeling of the healing wound (29). Furthermore, studies have shown that keratinocyte-derived factors are able to modulate a large number of genes in fibroblasts, coding for growth factors, cytokines and their receptors, ECM, adhesion receptors, and cell cycle regulators in vitro (25). These findings suggest that this kind of cell-cell communication also exist in vivo contributing to rebuilding of tissue integrity following injury.

Chronic wounds and dermal fibrosis are considered to be two extremes of wound healing. They represent serious pathologic conditions in which the
biomechanical properties of normal tissue are disrupted due to either differences or alterations in ECM composition and organization. Termination of wound healing, therefore, requires a fine balance between collagen deposition and its hydrolysis. This suggests that there must be a set of factors that gradually slow down and/or terminate the dynamic process at the late stage of wound healing. With the objective of identifying and characterizing such keratinocyte-derived ECM-modulating factors for dermal fibroblasts, our group demonstrated a significant increase in MMP-1 expression and decrease in type I collagen in dermal fibroblasts when treated with KCM or co-cultured with keratinocytes. Sequential experiments identified this keratinocyte-derived antifibrogenic factor as a releasable form of SFN also known as 14-3-3σ (58). However SFN, did not suppress the production of type I collagen in dermal fibroblasts, suggesting the role of another anti-fibrogenic factor in keratinocyte/fibroblast communication.

Stratifin (SFN) is a member of a large family of highly conserved, acidic dimeric 14-3-3 proteins. Leffers et al. (167), identified several members of this protein family in keratinocytes and KCM. However, secretion of this protein by conventional pathway is unlikely as these proteins lack signal peptides (62).

To address the question of how this cytosolic protein is released, as shown in chapter 2, a non-classical secretory pathway was studied. The results demonstrate that this protein is released through exosome-dependent secretion, one of the most common non-classical pathways. Several other studies have shown that other cells such as neuroglial cells (168), intestinal cells (169), dendritic cells (170) and tumor cells (171) have also the ability to release
exosomes. Following exosome purification protocols and imaging methods we were able to describe for the first time the capacity of keratinocytes to release exosomes (Figure 2-1). Moreover, we were also able to detect the presence of SFN in keratinocyte exosomes (Figure 2-2). In fact, other studies have demonstrated the presence of other members of the 14-3-3 family (η, γ and γ/δ) in exosomes from dendritic cells (55). Additionally, exosome-associated stratifin was able to stimulate MMP-1 expression in fibroblasts, as previously described by our group (58) (Figure 2-4).

As described in chapter 3, the second aim of this study was to identify differences in the release of exosome-associated stratifin from undifferentiated and differentiated keratinocytes. It is well known that keratinocytes from the basal layer (undifferentiated keratinocytes) possess different functions and protein profile from those of granuloseum layer (differentiated keratinocytes) (172).

Ca²⁺ is one of the essential factors that are able to induce the differentiation process. The epidermal layer possesses a natural Ca²⁺ gradient showing a higher level of Ca²⁺ in the upper layers and a lower level of Ca²⁺ in the basal layer (173). Being interested in identifying not only SFN but also other members of the 14-3-3 family as well as the full protein profile of exosomes from undifferentiated and differentiated keratinocytes, we performed proteomic analysis. The result revealed that keratinocyte exosomes contained a variety of proteins such as metabolic enzymes, cytoskeletal, cytosolic, chaperones, signaling, trafficking and adhesion proteins (Table 3-1). Furthermore, these results also showed differences in the isoform profile of 14-3-3 proteins in
exosomes from undifferentiated compared to those of differentiated keratinocytes (Figure 3-3). These findings were expected as it is well known that each of the 14-3-3 members are involved in different functions. SFN was only identified in exosomes from differentiated keratinocytes. Additionally we wanted to elucidate the mechanism by which differentiated keratinocytes increase the expression of exosome-associated proteins. We were able to demonstrate that induction of intracellular Ca\(^{2+}\) was responsible for increasing the release of exosomes (Figure 3-5). This finding may explain the physiological Ca\(^{2+}\) gradient found in the epidermis and its relation with the increase in the expression of exosome-associated proteins.

Previous observations from our group and from others have revealed a type I collagen-inhibiting effect on fibroblasts when co-cultured with keratinocytes or treated with KCM (33, 58, 144). Keratinocyte releasable SFN had no effect on type I collagen expression in fibroblasts.

In chapter 4, we were able to identify the factor responsible for the collagen inhibiting effect in fibroblasts. A combination of protein purification technique and proteomic analysis, identified two proteins (SPARC/SFN) that are released by keratinocytes, which form a novel complex showing a significant type I collagen-inhibiting effect in dermal fibroblasts.

SPARC, also known as osteonectin or BM-40 is a Ca\(^{2+}\) binding glycoprotein that functions as a counteradhesive protein, modulating cell shape, growth factor activity and as a cell-cell adhesion inhibitor. SPARC protein is known to be expressed at significant levels in tissue undergoing repair or
remodeling due to wound healing, disease or natural processes. It has been demonstrated that pathologies such as cancer metastasis, arthritis, diabetes, kidney disease and fibrotic conditions are characterized by elevated expression of SPARC (75).

Francki et al. (76), demonstrated that SPARC-null cells exhibited significantly diminished expression of type I collagen and TGF-β1 in mouse mesangial cells. After treatment with rhSPARC, the levels of type I collagen and TGF-β1 were restored. We further confirmed this result when we treated fibroblasts with hSPARC (Figure 4-7B and C). This finding suggested that SFN and SPARC were forming a complex in the conditioned medium and thereby, inhibiting collagen in fibroblasts. Moreover we were able to demonstrate that when fibroblasts were treated with SPARC/SFN complex type I collagen was suppressed (Figure 4-7D). Co-IP was also performed, revealing that when SFN was IP from the KCM, SPARC was also precipitated (Figure 4-6A). Further treatment with the supernatant after IP and 3-D modeling methods, confirmed our suggestions that these two proteins form a complex in the extracellular space and suppress type I collagen expression in dermal fibroblasts. There are no current reports in the literature suggesting that these two proteins interact. Moreover, this data indicates an unanticipated effect of the SPARC/SFN complex influencing collagen gene or protein expression. In fact, there are no reports showing the binding of SFN protein in the extracellular space.

In contrary, SPARC is an extracellular protein and is well known to bind to collagens, act as a chaperone and affect spontaneous fibril formation in vitro.
However, the mechanisms by which SPARC functions has not been identified. One may suggest the possibility of SFN sequestering SPARC in the extracellular environment and thus, interfere with downstream signaling. This interference may result from inhibiting the binding of SPARC to integrins or adaptor proteins and thereby suppressing collagen synthesis in fibroblasts. Although the exact nature of SFN and SPARC interaction in wound healing process remains to be investigated, an increased SPARC expression and a decreased SFN expression were observed in hypertrophic scar tissues (Figure 4-8). These findings suggest that a fine balance between these two proteins may orchestrate the process of normal wound healing. In addition, the identification of the SPARC/SFN complex may provide another avenue for therapeutic interventions to prevent the formation of hypertrophic scars, frequently observed in patients following burn injury, deep trauma and some surgical incisions.

In summary, in this thesis we were able to 1) describe the mechanism by which keratinocytes release SFN, 2) identify keratinocyte-derived SFN/SPARC complex that functions as a type I collagen inhibitory factor for fibroblasts.
Suggestions for Future Work

Although this body of work has contributed to a better understanding of the processes involved in wound healing, as well as, the mediating factors in epithelial/mesenchymal communication, it is apparent that this is only a starting point for additional research. Specifically, more investigation is required to understand how these proteins interact and potentially translate these findings into clinically applicable products. The following are some of the suggestions that may advance our current finding.

I. In chapter 2, we demonstrated that SFN is released from keratinocytes via exosomes, producing an MMP-1 stimulatory effect in dermal fibroblasts. The mechanism by which exosomes stimulate MMP-1 should be elucidated. A couple of possibilities were outlined under the discussion section of chapter 2.

II. In chapter 3, we revealed that keratinocyte exosome secretion occurs in a Ca\(^{2+}\) dependent-manner. The mechanism by which increased levels of intracellular calcium induce exosome release could be further elucidated. There are some evidence showing that Rab11 pathway regulates exosome release (175).

III. In chapter 4, we identified the keratinocyte-derived collagen inhibitor factor (SFN/SPARC complex). The mechanism by which SPARC/SFN inhibits
collagen production in fibroblasts is unknown. Suggestions for elucidating the mechanism of this effect are described in chapter 4.

IV. In chapter 4, we demonstrated physiological levels of SPARC/SFN in a fibrotic animal model. Hypertrophic scar tissue, exhibited an imbalance between these two proteins. The interactions of this complex in hypertrophic scarring/fibrosis could be further investigated in a fibrotic animal model. This may also present potential opportunities for clinical applications of this new understanding of the process of abnormal scar formation.

V. In chapter 4, we predicted potential binding sites for SFN and SPARC, which coincide with previous description of possible binding grooves. Mutating or deleting sections of SFN protein may assist in further characterization these binding sites.

VI. In chapter 4, we predicted the binding of SPARC/SFN complex. It would be useful to demonstrate the molecular ratio of this complex. This can be confirmed using quantitative mass spectroscopy. It is possible, as suggested above that since SFN is a chaperone protein, 4 or more monomers may bind to SPARC.
References


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