CROSSTALK BETWEEN KERATINOCYTES AND DERMAL FIBROBLASTS MODULATES THE RELEASE OF CRITICAL WOUND HEALING FACTORS

by

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Abstract

Epidermal-mesenchymal communication plays a critical role in cutaneous integrity during both normal and pathological wound healing processes. We have previously demonstrated that crosstalk between keratinocytes and dermal fibroblasts has the capacity to influence the expression of key extracellular matrix (ECM) components including members of the matrix metalloproteinase (MMP) and 14-3-3 protein families. In this Masters project, we hypothesized that bidirectional communication between these cell types plays a central role in regulating local expression of critical wound healing factors.

To address this question, two specific objectives were accomplished. Under Objective 1, we investigated whether fibroblasts are capable of modulating the expression of keratinocyte-releasable anti-fibrogenic 14-3-3 proteins by examining the levels of four 14-3-3 family members (β, η, γ, σ) by western blot and quantitative real-time polymerase chain-reaction (qPCR). Gene analysis revealed upregulation of all four 14-3-3 isoforms of interest in co-cultured compared to mono-cultured keratinocytes. Additionally, co-cultured keratinocytes expressed significantly higher levels of intracellular 14-3-3 γ and σ proteins.

Under Objective 2, we examined the impact of keratinocyte-releasable factors upon the fibroblast expression profile of key cytokines and growth factors using DNA microarray analysis. Fibroblasts co-cultured with keratinocytes demonstrated significantly upregulated colony-stimulating factor 3 (CSF3) expression, which was further verified by RT-PCR and western blot. Keratinocyte-conditioned medium (KCM) from undifferentiated keratinocytes was

observed to have higher CSF3-stimulatory potential than that of differentiated cells. Partial-characterization of the CSF3-stimulatory factor(s) was achieved by treating fibroblasts with KCM subjected to thermal stability and ammonium sulfate precipitation analyses. Additionally, to investigate the CSF3-stimulatory potential of interleukin-1 (IL-1) upon fibroblasts, we utilized an IL-1 receptor antagonist (IL-1ra) and revealed that keratinocyte-releasable IL-1 may be one of the factors responsible for stimulating CSF3 expression in fibroblasts.

Our studies concluded that dermal fibroblasts regulate the expression of several 14-3-3 isoforms (notably γ and σ) in keratinocytes, and conversely, that keratinocyte-releasable factors such as IL-1 modulate the expression of CSF3 in fibroblasts. These findings underline the critical nature of crosstalk between keratinocytes and fibroblasts, suggesting that bidirectional communication between these cells *in vivo* plays a prominent regulatory role in the cytokine production required for wound healing and maintaining a normal skin environment.

Preface

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

Chapter 2: Matthew Carr, Claudia Chavez-Muñoz, Amy Lai and Aziz Ghahary. Dermal Fibroblasts Influence the Expression Profile of 14-3-3 Proteins in Human Keratinocytes. Molecular and Cellular Biochemistry (2011) 353(1-2):205-14.

Chapter 3: Matthew Carr, Yunyuan Li, Claudia Chavez-Muñoz, Alireza-Moeen Rezakhanlou, Ryan Hartwell and Aziz Ghahary. Keratinocyte-releasable Factors Stimulate the Expression of Granulocyte Colony-stimulating Factor in Human Dermal Fibroblasts. (2012) (Submitted).

Dr. Aziz Ghahary was the principal investigator of the research projects and is credited with the original identification of stratifin as a keratinocyte-releasable factor. In addition, he supervised the experimental designs and analysis, and critically reviewed the manuscripts included in this thesis. Financial support for this thesis was provided by CIHR grants held by Dr. Ghahary. Matthew J. Carr was responsible for experimental designs, the research and data analysis, and wrote the manuscripts for both of the aforementioned projects. The co-authors assisted with approximately 15% and 10% of the experiments for Chapters 2 and 3 respectively.

The work described in this thesis was conducted with the approval of the University of British Columbia Biohazards Committee under the certificate number B09-0298.

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Abbreviations

Ca^{2+}	Calcium
CSF	Colony-stimulating Factor
DMEM	Dulbecco's Modified Eagle's Medium
ECM	Extracellular Matrix
EGF	Epithelial Growth Factor
FCM	Fibroblast-conditioned Medium
FBS	Fetal Bovine Serum
FGF	Fibroblast Growth Factor
G-CSF	Granulocyte Colony-stimulating Factor
GM-CSF	Granulocyte Macrophage Colony-stimulating Factor
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
HS	Hypertrophic Scar
IL	Interleukin
IL-1α	Interleukin-1 Alpha
IL-1β	Interleukin-1 Beta
IL-1ra	Interleukin-1 Receptor Antagonist
KCM	Keratinocyte-conditioned Medium
KDAF	Keratinocyte-derived Anti-fibrogenic Factor
KSFM	Keratinocyte Serum Free Medium
MAPK	Mitogen-activated Protein Kinase
MMPs	Matrix Metalloproteinases
mRNA	Messenger RNA
MW	Molecular Weight
PDGF	Platelet-derived Growth Factor
q-PCR	Quantitative-polymerase Chain Reaction
RT-PCR	Reverse Transcriptase-polymerase Chain Reaction
rhIL-1β	Recombinant Human Interleukin-1 Beta
SDS-PAGE	Sodium Dodecyl Sulfate-polyacrylamide Gel Electrophoresis
SFN	Stratifin
TGF-β	Transforming Growth Factor-beta
TNF-α	Tumor Necrosis Factor-alpha
VEGF	Vascular Endothelial Growth Factor

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Last, but certainly not least, heartfelt thanks are owed to my parents for their unquestioning support towards all of my endeavors.

This work is dedicated to my beloved parents, siblings & girlfriend.

Chapter 1. Introduction

Background

Wound Healing

The protective barrier provided by our skin is vital to our survival, as it acts as a first line of defense against pathogens and environmental factors such as ultraviolet light (1). Upon acute injury to the integument, the process of cutaneous wound healing begins with the initiation of hemostasis, followed by three overlapping stages – inflammation, proliferation and remodeling (Figure 1-1). These stages involve complex interactions between soluble mediators, extracellular matrix (ECM) components, and numerous important cell types including platelets, fibroblasts, epithelial, endothelial and immune cells (2-5). These cells collectively orchestrate the dynamic nature of the repair process by communicating with one another through the tightly mediated release of specific cytokines and growth factors.

The initial trauma to the skin is closely followed by the onset of hemostasis, which commences as platelets aggregate upon coming in contact with exposed extracellular matrix (ECM) components in order to form a hemostatic plug (3-7). Activated platelets and injured parenchymal cells also release clotting factors that further contribute to coagulation at the site of injury (5). In addition, platelets release critical growth factors and cytokines such as platelet-derived growth factor (PDGF) and transforming growth factor-beta (TGF-β) into the local wound microenvironment during the inflammatory phase of healing (Figure 1-2) (3-6, 8).

PDGF is known to be responsible for stimulating the chemotaxis of neutrophils, macrophages, smooth muscle cells, and fibroblasts; it also has the capacity to stimulate proliferation of these two latter cell types (4).

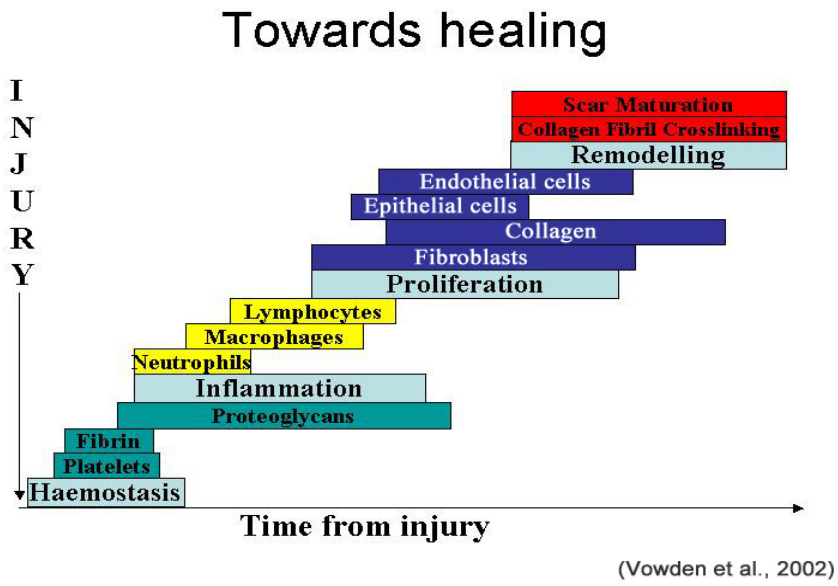

Figure 1-1. **The stages of normal acute wound healing (9).**

Timeline representation of acute cutaneous wound healing. Authors divided the process into four stages: *hemostasis*, *inflammation*, *proliferation* & *remodeling (Figure adapted from Vowden et al., Wound bed preparation, 2002) (9).*

Within hours of the initial trauma, polymorphonuclear neutrophils (PMNs) begin to infiltrate the wound site, phagocytizing bacteria and debris while simultaneously releasing proteases to degrade damaged tissue (3-5). As the number of neutrophils decreases, which typically occurs around 48 hours post-injury, macrophages derived from circulating monocytes begin to increase in number at the wound site until roughly 96 hours after the wound was incurred. Locally increased levels of TGF-β help to attract macrophages and also further

stimulate activated macrophages to continue the process of phagocytosis and to release more PDGF and TGF-β in addition to vascular endothelial growth factor (VEGF) (Figure 1-2) (3-5, 7). After sufficient debridement of the wound site has occurred, the proliferative phase commences as dermal fibroblasts, stimulated primarily by TGF-β, migrate into the wound and initiate matrix turnover through degradation, synthesis and deposition of ECM components (Figure 1-3) (3, 4).

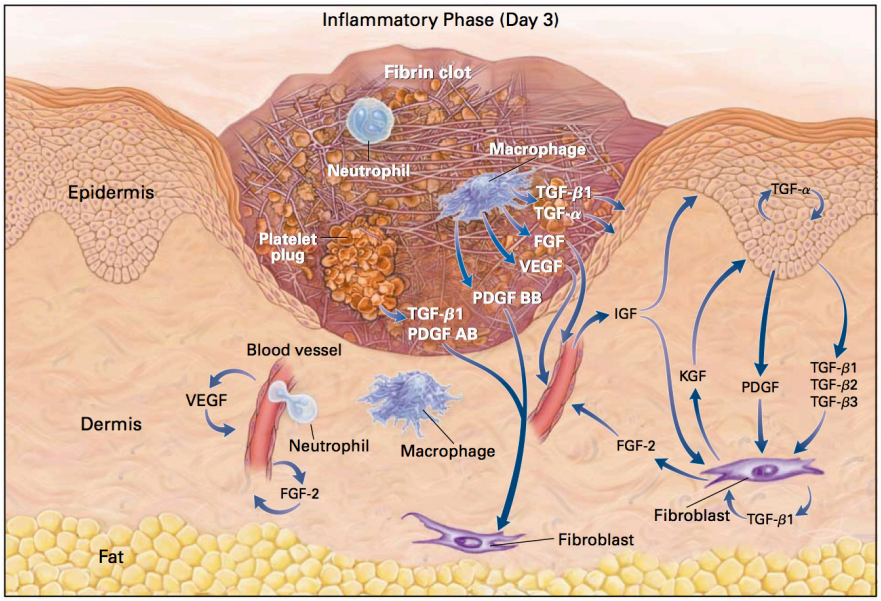

Figure 1-2. **A cutaneous wound three days after injury (3).**

Growth factors considered necessary for cell migration into the wound are shown *(Figure adapted from Singer et al., Cutaneous wound healing, 1999)* (3).

TGF-β plays a critical multifaceted role throughout the proliferative phase due to its role as a "master control signal" responsible for the regulation of numerous fibroblast functions (4, 10, 11). Firstly, it causes a net increase in the production of ECM components due to upregulation of fibroblast genes for

collagen, proteoglycans and fibronectin. Simultaneously, TGF-β limits the production of proteases and also stimulates tissue inhibitors of metalloproteinases (TIMPs) thus preventing further ECM degradation (12). Additionally, cytokines such as the interleukins (ILs), fibroblast growth factor (FGF) and tumor necrosis factor-alpha (TNF-α) are considered to be of importance (4, 13). Activated macrophages, platelets and keratinocytes release transforming growth factor-alpha (TGF-α) and epidermal growth factor (EGF) which in combination initiate the re-epithelialization process (14, 15). Once epithelial bridging is complete, specific enzymes are released locally in order to dissolve the basal connection of the scab and release it from the wound surface.

The newly formed fibrin provisional matrix acts as a conduit for cell migration, which facilitates the formation of granulation tissue (16). As healing continues, dermal fibroblasts become the predominant local cell type. These cells release numerous proteolytic enzymes including plasminogen activators (PA) and matrix metalloproteinases (MMPs) that help to cleave a path for cell migration into the wound site (Figure 1-3) (3). Fibroblasts bind to the provisional fibrin matrix with cell-surface integrin receptors and slowly replace it with collagenous matrix (17, 18). Under ideal circumstances, fibroblasts cease the production of collagen when ample matrix has been synthesized, however dysregulation of this process is highly implicated in the development of fibrotic disorders such as hypertrophic or keloid scarring (19, 20). Consequently, the heightened metabolic demands of the newly forming granulation tissue leads to a local reduction of oxygen levels and pH – these factors promote angiogenesis,

which is also stimulated in part by VEGF, basic FGF, and TGF-β (21, 22). The proteolytic breakdown of fibrin and ECM by fibroblasts allows endothelial cells stimulated by these factors to migrate through the fragmented basement membrane and commence neovascularization of the wound bed (Figure 1-3).

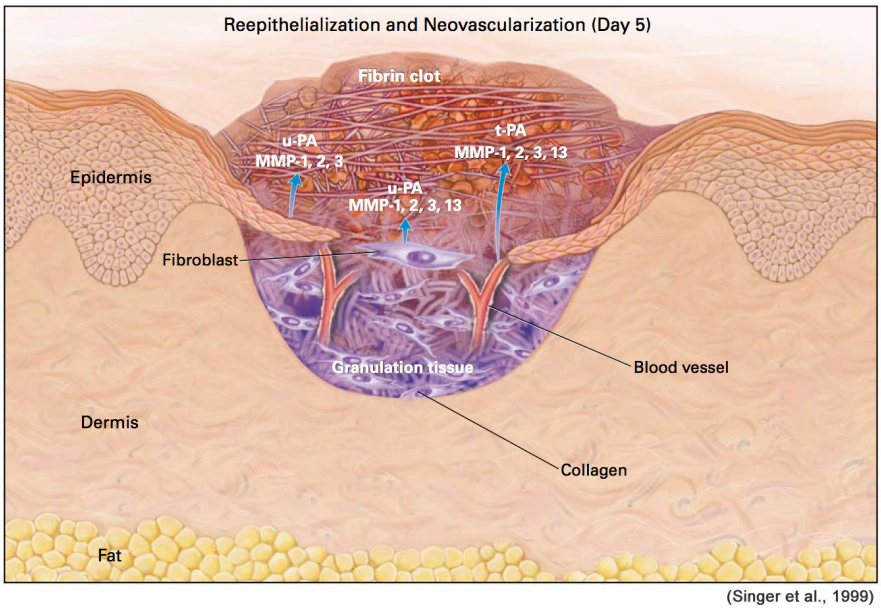

Figure 1-3. **A cutaneous wound five days after injury (3).**

Blood vessels are seen sprouting into the fibrin clot as epidermal cells resurface the wound. Proteases thought to be necessary for cell movement are shown *(Figure adapted from Singer et al., Cutaneous wound healing, 1999) (3)*.

In the final remodeling phase of wound healing, fibroblasts responsible for wound contraction acquire a contractile myofibroblast phenotype rich in actin filaments (23). The new collagen matrix gains in tensile strength as it is gradually cross-linked and organized – collagen degradation is an essential component of this process (24). This process is catalyzed by MMPs produced by epidermal and endothelial cells, macrophages and fibroblasts (3). Due to the inherent

complexity of the dynamic wound healing process, pathologic under or over-healing conditions may result when one or more of these processes go awry.

Extracellular Matrix

The components of the ECM are vital in maintaining skin integrity, as they provide essential support and anchorage for cells and also facilitate intercellular signaling in addition to numerous other functions. Furthermore, the capacity of the ECM to act as a “sink” for local storage of cytokines and growth factors plays an integral role in numerous physiological processes such as wound healing (25, 26). In the case of inadequate or defective ECM production, wounds exhibit an increased risk of extended inflammation and delayed epithelial closure (27).

An intertwined network of long polysaccharides called glycosaminoglycans and fibrous proteins forms the primary structure of the ECM. These components are synthesized by local cells and bind to existing matrix components once exocytosed into the extracellular microenvironment. Accounting for more than 30% of the total, collagen is the most abundant protein in the human body (28, 29). Formed by a triple-helix of polypeptides arranged in a 2:1 ratio, Type I collagen is the major component of the dermal matrix (30), and imparts our skin with the majority of its tensile strength, which on a per weight basis is comparable to that of steel.

Due to the inherent ability of collagen to resist proteolytic degradation, enzymes known as MMPs are highly involved in catalyzing its breakdown. In addition to wound healing, this diverse family of enzymes is highly implicated

throughout an array of other physiological processes including embryonic development, uterine involution and bone growth/resorption (31). Within the MMP family, several specific subfamilies have been identified based on structure, cellular localization and substrate specificity (32). Among these classifications, the collagenase subset includes MMP-1, MMP-8 and MMP-13. These specifically cleave native type I collagen into smaller fragments that are accessible for further breakdown by other MMPs (31). Under normal circumstances, MMP expression is minimal in order to promote a healthy degree of ECM turnover; however, significant dysregulation of MMP expression has been observed under pathological conditions such as dermal fibrosis or chronic ulcers (19, 33). It is well understood that adherence to a delicate balance between ECM synthesis and deposition versus degradation by MMPs is essential for the maintenance of normal cutaneous integrity.

Keratinocyte-fibroblast Communication in Wound Healing

Keratinocyte-fibroblast communication is critical for exchanging information between keratinocytes and fibroblasts during skin morphogenesis, and is also likely to be important in maintaining the integumentary structure of adult skin (34). These epidermal-mesenchymal interactions have been examined under a wide range of circumstances. Notably, one of the first of such studies demonstrated that upon cutaneous injury, induction of keratinocyte growth factor (KGF) expression in fibroblasts occurs, resulting in increased epithelial mitogenic activity (35). Some growth factors exhibit predominantly single-paracrine effects, such as PDGF, which is largely expressed by keratinocytes but exerts its actions

upon fibroblasts. Conversely, other cytokines such as activins are expressed by both cell types, and are capable of exerting their effects in both paracrine and/or autocrine fashions (36, 37). In a related context, it has also been demonstrated that *in vitro*, fibroblast expression of numerous growth factors, cytokines/receptors, adhesion receptors, cell cycle regulators, ECM components and MMPs is mediated by keratinocyte-releasable factors (38) – this suggests that *in vivo*, similar paracrine mechanisms may be the driving force responsible for stimulating tissue repair.

At numerous stages throughout the intricate process of wound healing, particularly with respect to ECM deposition and degradation, epidermal-mesenchymal communication between keratinocytes and dermal fibroblasts serves a critical role. In the case of injury to the integument, disruption of the double-paracrine crosstalk between these cells has been shown to increase the risk of developing fibrotic conditions at the wound site such as hypertrophic or keloid scars (19, 20, 33). It has also been demonstrated that delays in epithelial closure can increase the occurrence of fibrotic over-healing disorders (39). The theory behind this clinical phenomenon is that in the absence of adequate wound coverage, extracellular matrix continues to accumulate until dermal fibroblasts receive signal(s) from epidermal keratinocytes to slow down the dynamic healing process, leading to the eventual maturation and remodeling of the closed wound (40).

Keratinocyte-derived Anti-fibrogenic Factor

Stratifin, also known as 14-3-3 σ, is a member of a highly conserved family of acidic dimeric 14-3-3 proteins comprised of seven mammalian isoforms (β, γ, ε, η, σ, τ, and ζ). First discovered in 1967 by Moore and Perez, this family of proteins derives its name from their original elution (fraction 14) and migration patterns (positions 3.3) on DEAE-cellulose and starch-gel electrophoresis. These predominantly cytoplasmic proteins are able to form homodimers or heterodimers and these frequently serve as chaperone molecules for a variety of binding partners. They have been recognized as important regulators of signal transduction pathways, apoptosis (*BAD/Bax* pro-apoptotic proteins), and cellular adhesion, proliferation, differentiation & survival (41-43). The affinity of 14-3-3 proteins to such a diverse array of binding partners is largely due to their specific phospho-serine/phospho-threonine binding activity, associating with two known high-affinity binding motifs on target proteins (42). Under occasional circumstances they are also known to interact in a phosphorylation-independent manner (*Bax*) (41). Phosphorylation-dependent binding to 14-3-3 proteins has been demonstrated in more than 100 small molecules – such interactions are highly involved in their role as protein kinase C (PKC) inhibitors (44) in addition to tryptophan & tyrosine hydroxylase activators (45, 46). They have also been recognized as key components of cellular response to damage – specifically, 14-3-3 σ is highly involved in preventing mitotic catastrophe that may be a consequence of DNA damage (47). It has been shown that in response to DNA damage 14-3-3 σ is subject to regulation by the p53 tumor suppressor protein,

ultimately causing a downstream inhibitory effect upon G2/M cell cycle progression (48).

Most isoforms within this family of acidic eukaryotic proteins are ubiquitously expressed in different cell types (49, 50). However, our group previously demonstrated that the expression of the 14-3-3 σ isoform appears to be restricted to epithelial cells. Specifically, it was revealed that all seven 14-3-3 isoforms are expressed in both cultured keratinocytes and intact epidermal layers of skin – in contrast, expression of the 14-3-3 σ isoform is absent in fibroblasts and within the dermis (51).

Despite the range of signal transduction pathways that involve 14-3-3 proteins, they have also been identified to play an important role in an extracellular environment. Our group originally demonstrated that keratinocyte-releasable stratifin/14-3-3 σ functions as a potent MMP-1, 3, 8, 10 and 24 stimulating factor for dermal fibroblasts (52, 53). Due to its inherent ability to rapidly induce collagenase (MMP-1) expression in dermal fibroblasts, 14-3-3 σ was eventually coined “keratinocyte-derived anti-fibrogenic factor” (KDAF). Exosome-dependent secretion was revealed to be the mechanism underlying externalization of 14-3-3 σ by keratinocytes (54, 55). Further investigations showed that a specific p38 MAPK inhibitor (SB203580) was capable of significantly blocking 14-3-3 σ induced MMP-1 expression in fibroblasts, alluding to the potential importance of the p38 signaling pathway throughout the extracellular mechanism of 14-3-3 σ (56). Furthermore, our research group

recently demonstrated that 14-3-3 σ binds to the fibroblast cell-surface receptor aminopeptidase N, also known as CD13 (57).

Granulocyte Colony-stimulating Factor

Granulocyte colony-stimulating factor (G-CSF), also known as colony-stimulating factor 3 (CSF3), was originally identified in the early 1980's. This glycoprotein, growth factor and cytokine is produced by numerous cell types such as epithelial, endothelial, and immune cells in addition to macrophages in order to stimulate bone marrow to produce granulocytes and stem cells. It is also known to stimulate the survival, proliferation, differentiation and function of both precursor and mature neutrophils (58, 59).

The early phases of wound healing involve coordinated cell-cell interactions that require the sequential release of growth factors – these consequentially lead to the recruitment of inflammatory cells that are necessary for tissue repair. In this context, G-CSF has been shown to dramatically increase the recruitment, migration, and function of bone marrow derived cells and other immune cells, leading to improved granulation tissue formation and ulcer healing in several pathological conditions such as microvascular inflammatory states (60). It has further been shown that G-CSF is capable of driving the proliferation, survival, and differentiation of bone marrow derived progenitor cells by activating receptors within the hematopoietin receptor superfamily that subsequently trigger downstream intracellular signaling mechanisms (61, 62).

Of the numerous cell types known to express G-CSF, monocytes and endothelial cells are among the most predominant producers. In addition to increasing granulopoiesis in the bone marrow (62, 63), in the case of trauma, G-CSF has the potential to induce bone marrow derived cells to migrate into the peripheral bloodstream where they can be mobilized to the site of injury (60, 63-65). Furthermore, the recombinant protein has been shown to significantly increase the proliferation of keratinocytes (66, 67); this characteristic may contribute significantly towards the possible therapeutic wound healing potential of G-CSF. Granulocyte-macrophage colony-stimulating factor (GM-CSF), also known as CSF2, has previously been demonstrated to possess therapeutic wound healing effects (68-74). Although less study has been done into the similar potential application of G-CSF, it has been shown that the application of recombinant G-CSF may yield similar beneficial effects to the wound healing process compared to that of GM-CSF (75-79).

Hypotheses and Objectives

In order to further our understanding of epidermal-mesenchymal crosstalk during the wound healing process, the goal of this research project has been to examine paracrine communication between keratinocytes and dermal fibroblasts. To evaluate the capacity of these cells to bidirectionally regulate wound healing factor expression, the following hypotheses and specific objectives were addressed.

Hypothesis 1

Our research group has previously shown that keratinocyte-derived factors, 14-3-3 sigma (σ) in particular, could modulate ECM protein expression in dermal fibroblasts. Given the unexplored capacity of fibroblasts to influence, in a reciprocal manner, keratinocyte-derived 14-3-3 proteins, we hypothesized that in a double-paracrine fashion fibroblast-releasable factors may possess the capacity to influence the 14-3-3 expression profile in keratinocytes.

Objective 1.1

In order to evaluate the ability of fibroblasts to influence the expression profiles of four 14-3-3 isoforms (β, η, γ, and σ) in keratinocytes, fibroblasts and keratinocytes were co-cultured and samples were analyzed by western blot and qPCR. The results shown in Chapter 2 revealed a significant increase in the expression of extracellular 14-3-3 σ and intracellular 14-3-3 γ and σ proteins by keratinocytes when cultured with fibroblasts, in addition to a significant upregulation of all four 14-3-3 isoforms (β, η, γ, σ) at the gene level.

Objective 1.2

In order to elucidate the mechanism responsible for 14-3-3 σ induction in keratinocytes stimulated with fibroblast-conditioned medium, cells were cultured in the presence of several MAPK inhibitors. The results suggest that various MAPK pathways play a potentially significant role in the regulation of 14-3-3 σ induction and expression in keratinocytes.

Hypothesis 2

Continuing our assessment of crosstalk between these two cell types, we examined whether keratinocytes possess the capacity to modulate the expression of key cytokines and growth factors in fibroblasts. We hypothesized that keratinocyte-releasable factor(s) have a significant impact upon the expression of such factors in dermal fibroblasts.

Objective 2.1

In order to assess the ability of keratinocytes to regulate the expression of critical cytokines and growth factors in fibroblasts, the two cell types were co-cultured and samples were analyzed by DNA microarray, RT-PCR, and western blot. The results presented in Chapter 3 demonstrate that fibroblasts cultured in the presence of keratinocytes express significantly higher levels of CSF3/G-CSF compared to fibroblasts cultured alone.

Objective 2.2

To evaluate the impact of keratinocyte differentiation-state upon their ability to stimulate CSF3 expression in fibroblasts, keratinocyte-conditioned

medium (KCM) was collected at several time points after inducing keratinocytes to begin differentiating. These findings show that KCM from undifferentiated keratinocytes is the most potent CSF3-stimulator for dermal fibroblasts.

Objective 2.3

To partially-characterize the keratinocyte-releasable factor(s) responsible for stimulating CSF3 expression in fibroblasts, KCM samples were subjected to thermal stability testing and ammonium sulfate precipitation techniques prior to being used for treating fibroblasts.

Objective 2.4

To investigate the role of keratinocyte-releasable IL-1 as a potential candidate for the CSF3-stimulating factor for dermal fibroblasts, we utilized an IL-1 receptor antagonist (IL-1ra) combined with KCM or IL-1 beta protein (rhIL-1β) to treat fibroblasts. The results demonstrate that keratinocyte-releasable IL-1 plays an important role in stimulating the upregulation of CSF3 in dermal fibroblasts.

Chapter 2. Dermal Fibroblasts Influence the Expression Profile of 14-3-3 Proteins in Human Keratinocytes[1]

Introduction

Epidermal-mesenchymal communication plays an integral role in the process of epithelialization and wound healing. In the case of burns, wounds or other damage to the integument, it is predominantly disruption in keratinocyte-fibroblast communication that leads to an increased frequency of developing fibrotic conditions at the wound site. This is mainly because this interaction is critical for exchanging information between keratinocytes and fibroblasts during skin morphogenesis, and is also likely to be important in maintaining the integumentary structure of adult skin (34). It has been well established that any delays in the epithelialization process lead to an increased frequency of developing fibrotic conditions. When keratinocytes form an epithelial layer on the wound site within 2-3 weeks, only one-third of the anatomically site matched wounds become fibrotic; whereas this disorder increases to 78% when it takes an excess of 21 days (39). This signifies that in the absence of adequate wound coverage, extracellular matrix continues to accumulate until dermal fibroblasts

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[1] A version of this chapter has been published. Matthew Carr, Claudia Chavez-Muñoz, Amy Lai and Aziz Ghahary. Dermal fibroblasts influence the expression profile of 14-3-3 proteins in human keratinocytes. Mol Cell Biochem 353(1-2):205-14 (2011).

receive signal(s) from epidermal keratinocytes to slow down the dynamic healing process that leads to maturation and remodeling of the healing wound (40).

In normal healthy skin, structural integrity is maintained by adhering to a fine balance between the synthesis and degradation of the ECM by a large family of enzymes known as the matrix metalloproteinases (MMPs). Our previous work has shown that keratinocyte releasable 14-3-3 σ functions as a potent MMP-1, 3, 8, 10 and 24 stimulating factor for dermal fibroblasts (52, 53). 14-3-3 σ is a member of a highly conserved family of acidic dimeric 14-3-3 proteins consisting of seven various isoforms (β, γ, ε, η, σ, τ, and ζ). While most members of this acidic eukaryotic family of proteins appear to be ubiquitously expressed in different cell types (49, 50), 14-3-3 σ expression seems to be specific to stratified epithelial cells (51). Later studies revealed the presence of other 14-3-3 isoforms in the dermal and epidermal layers, demonstrating that all seven isoforms are expressed in both cultured keratinocytes and intact epidermal, but not dermal layers of skin (51).

Although previous publications demonstrated that keratinocyte releasable 14-3-3 proteins modulate the expression of MMPs in fibroblasts (52, 53, 80), little work has been done with respect to the potential ability of fibroblasts to influence the expression of 14-3-3 proteins in keratinocytes. Here, we hypothesize that in a double-paracrine fashion, fibroblast-derived factors might also possess the capacity to influence the expression of 14-3-3 proteins in keratinocytes. In order to address this hypothesis, a keratinocyte/fibroblast co-culture system was

established, in which keratinocytes were seeded on 6-well plates, co-cultured with dermal fibroblasts seeded on plate inserts. Findings showed that when keratinocytes were co-cultured with fibroblasts they express significantly higher levels of intracellular 14-3-3 γ and σ proteins in addition to showing up-regulation of all four 14-3-3 genes of interest (β, η, γ, σ). Furthermore, the level of 14-3-3 σ in keratinocyte-conditioned medium was significantly increased in the presence of fibroblasts. Under similar experimental settings, the addition of several MAPK inhibitors was shown to reduce the expression of 14-3-3 σ in keratinocytes, alluding to the potential importance of these pathways for regulation of 14-3-3 expression. The findings of this study suggest that fibroblasts are able to influence 14-3-3 expression in keratinocytes, supporting the hypothesized double-paracrine relationship between these two cell types.

Materials and Methods

Clinical specimens and cell cultures

Following informed consent, human skin samples were obtained from adult patients undergoing elective reconstructive surgery. This study was carried out in accordance with the principles of the Declaration of Helsinki (World Medical Association of Helsinki, Somerset West, 1996). Samples were collected individually in Keratinocyte Serum Free Medium (KSFM) (GIBCO, Grand Island, NY), and then separately washed 3 times in sterile PBS supplemented with 1% antibiotic-antimycotic preparation (100 μg/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B) (GIBCO). Specimens were dissected free of fat and cut into small pieces (~1 cm in diameter) and incubated in Dispase enzyme (25 μg/ml in KSFM) (GIBCO) at 37°C for 2 h in order to separate the dermal and epidermal layers. Epidermis was incubated for 4 minutes in trypsin at 37°C, interspersed with finger-vortexing every minute. Dulbecco's Modified Eagle's Medium (DMEM) (GIBCO) with 10% Fetal Bovine Serum (FBS) (GIBCO) was used to deactivate the trypsin reaction. Centrifugation at 800 rpm at 4°C for 8 minutes allowed us to obtain keratinocytes that were seeded into 25 cm^2 flasks (BD Biosciences, Mississauga, ON) and cultured with 5 ml of KSFM with supplements (BPE, 50 μg/ml and EGF, 5 μg/ml) (GIBCO) and 1% antibiotic-antimycotic preparation. Once keratinocytes achieved approximately 80-90% confluence, they were released via trypsinization as described above, and re-seeded at a ratio of 1:5 into 75 cm^2 BD Falcon tissue-culture treated flasks (BD Biosciences). The additives were used only to establish keratinocytes in cultures.

Thus, in order to eliminate any effects of FBS, EGF and/or pituitary extract on our findings, when assembling the co-culture systems, the KSFM with EGF and pituitary extract was exchanged with the test medium consisting of 49.5% DMEM+ 49.5% KSFM + 1% Antibiotic with no supplements. Only primary cultured keratinocytes at passages 2-4 were used in the experiments.

In order to isolate fibroblasts, following removal of the epidermal layer, all remaining pieces of dermal tissue were minced into small pieces (~1-2 mm in diameter), washed six times with sterile PBS, and distributed into 60x15 mm Petri dishes. Cultures of dermal fibroblasts were established as previously described (81). Upon achieving confluence, the fibroblasts were released by trypsinization and split for subculture at a ratio of 1:5 and re-seeded into tissue-culture treated 75 cm^2 flasks. Only fibroblasts from passages 3-6 were used in the experiments.

Fibroblast/keratinocyte co-culture system

Co-culture systems were conducted as previously described by our group (52, 81). Briefly, fibroblasts or keratinocytes were either seeded on tissue-culture treated 30 mm 6-well tissue-culture plate inserts with 0.4 μm pore size (BD Biosciences) as the “top” chamber or on the tissue-culture treated 6-well plate (BD Biosciences) as the “bottom” chamber. Cells were grown in either DMEM with 10% FBS (for fibroblasts) or KSFM with supplements (for keratinocytes). Both cell types were incubated independently for 24-48 h at 37ºC with 5% CO_2 until approximately 80-90% of confluence was reached.

Prior to assembling the co-culture systems, cells were counted using a hemocytometer in order to verify that approximately $1x10^6$ of each cell type would be present in the top and bottom chambers of each system. Cells were also washed 3 times with PBS to remove remaining traces of supplements (ie. FBS, BPE, and EGF). The treatment groups consisted of fibroblasts on the insert co-cultured with keratinocytes growing on the bottom of the 6-well plate (*lane F/K*). The two co-culture control groups consisted of keratinocytes (*lane K/K*) and fibroblasts (*lane F/F*). Each combined well and insert in the 6-well culture plate received 2.0 ml of the test medium mentioned above.

Antibodies

Rabbit anti-human 14-3-3 γ antibody was generously provided by Dr. Aitken (School of Biomedical and Clinical Sciences, University of Edinburgh, Scotland). Rabbit anti-human 14-3-3 β and 14-3-3 η polyclonal antibodies were purchased from BioMol International, LP (Plymouth Meeting, PA), mouse anti-human 14-3-3 σ monoclonal antibody was obtained from NeoMarkers (Fremont, CA), and mouse anti-human β-actin monoclonal antibody was purchased from SIGMA (Saint Louis, MO). Goat anti-rabbit IgG and anti-mouse IgG IRDye secondary antibodies were obtained from LI-COR Biosciences (Lincoln, NE).

Western blotting

In order to evaluate both the intra- and extracellular expression of some of the 14-3-3 protein isoforms, cell lysate and conditioned medium (CM) samples were collected from cells in both mono- and co-culture systems. In both the

mono- and co-culture systems, the CM for western blot analysis was collected after 48 h of incubation time along with the corresponding cell lysates from the bystander cells. 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). Following freezing & thawing, the samples were sonicated and the nuclei and cell debris were removed by centrifugation at 10,000 rpm for 10 minutes at 4°C. The protein concentration of the lysates was determined using a Bradford protein assay and 10 µg of total protein was loaded on 12% (wt/vol) acrylamide gel and subjected to electrophoresis (SDS-PAGE). However, the samples of CM were loaded by volume (20 µl each), representative of an equivalent number of cells. The proteins were then electrotransferred from the acrylamide gel onto nitrocellulose membranes using the iBlot electrotransfer system (Invitrogen, Burlington, ON). Non-specific proteins on the membranes were blocked in 5% skim milk powder in TBS – 0.5% Tween-20 overnight at 4°C. Immunoblotting was performed using either anti-14-3-3 σ monoclonal antibody (1:1000 dilution), anti-14-3-3 β, anti-14-3-3 η, anti-14-3-3 γ polyclonal antibodies (1:1000 dilution), as well as anti-β-actin (1:25,000 dilution). Following 4 washes with TBS – 0.1% Tween-20 for 5 minutes each, the membranes were then incubated accordingly with the appropriate IRDye secondary antibody (1:10,000 dilution). Immunoreactive proteins were then visualized using the Odyssey Infrared Imaging System (LI-COR Biosciences) for either 680 nm (anti-rabbit) or 800 nm (anti-mouse) wavelengths. The digital images were further subjected to

quantification by means of densitometry using ImageJ64 software (Research Service Branch, National Institutes of Health).

Quantitative analysis of intracellular levels of 14-3-3 σ protein in keratinocytes

The quantity of intracellular 14-3-3 σ in co-cultured keratinocytes was evaluated by comparing 20 µg of total protein from the cell lysate of the treated cells (*lane F/K*) against various concentrations (0, 0.06, 0.12, 0.25, 0.5, and 1.0 µg/lane) of recombinant 14-3-3 σ loaded in a 12% (wt/vol) acrylamide gel using western blot analysis. The densities of the signals were then measured by densitometry and compared.

Quantitative real-time polymerase chain reaction (qPCR)

For qPCR analysis, following 24 h of incubation time for the combined top and bottom chambers, inserts were discarded and the bystander cells from controls *K/K* and *F/F* and treatment *F/K* were released via trypsinization. Following centrifugation, cell pellets were washed with PBS and total RNA was extracted using RNeasy mini kit (Qiagen, Crawley, UK). Only RNA with an A_{260}/A_{280} ratio between 1.9-2.2 was used in the experiments. A total of 1 µg of total RNA from each sample was reverse transcribed using a Superscript II First Strand cDNA Synthesis kit (Invitrogen, Burlington, ON), and quantitative real-time PCR analysis was carried out on the Applied Biosystems 7900HT Fast Real-Time PCR System (Applied Biosystems, Warrington, UK) using the SYBR Green PCR Master-Mix kit (Applied Biosystems). Each sample was tested in triplicate and cDNA was amplified using real-time qPCR that measured the relative levels

of 14-3-3 gene expression (β, η, γ, σ) using the comparative cycle threshold (Ct) method. The values for β-actin were used as a housekeeping gene to normalize the gene expression data for each sample. The relative value of target gene expression was calculated from the standardized target gene and β-actin amplified curves. The cycling conditions were 95°C/15 min with 40 cycles of 95°C/1 min, 55°C/30 s and 72°C/30 s. The following sense and anti-sense primers were used: h14-3-3 β (sense: ACCCAATTCGTCTTGGTCTG; anti-sense: TCCGATGTCCACAGAGTGAG); h14-3-3 η (sense: CCATAGCTGAGCTGGACACA; anti-sense: ATTGTGGCAAGGAAGAATCG); h14-3-3 γ (sense: TTCATGCAAGTGGAGCTGTC; anti-sense: ACCCTGGAAAACATGCAGAG); h14-3-3 σ (sense: GGATCCCACTCTTCTTGCAG; anti-sense: CTGCCACTGTCCAGTTCTCA); and hβ-actin (sense: GCTCTTTTCCAGCCTTCCTT; anti-sense: CGGATGTCAACTTCACACTT) (Invitrogen, Burlington, ON).

Statistical evaluation of extracellular 14-3-3 protein expression in keratinocyte/fibroblast co-cultures

After 48 h of co-culturing *K/K*, *F/F*, and *F/K*, the conditioned media (CM) was collected and centrifuged at 10,000 rpm at 4°C for 10 min to remove any cellular debris. Samples were evaluated using western blot analysis as mentioned previously. All blots were tested for the four 14-3-3 isoforms (β, η, γ, σ) and also blotted for β-actin. The absence of β-actin indicates that the presence of these 14-3-3 proteins is unlikely to be due to cellular disruption. In this co-culture

system, any releasable factors were able to freely exchange across the membrane that separates the upper and lower chambers.

In order to compare the extracellular protein expression of the co-culture control groups *K/K* and *F/F* (*Control*) versus that of the co-cultured treatment group *F/K* (*Treated*) in an equitable manner, results from *K/K* and *F/F* co-culture experiments are presented in Table 2-1 as 14-3-3 expression (densitometry units) per $1x10^6$ cells (Table 2-1, *KCM* (keratinocyte-conditioned medium) and *FCM* (fibroblast-conditioned medium) columns) or $2x10^6$ cells (Table 2-1, *Control* and *Treated* columns). These same data are visually represented in Fig.2-5, with FCM and KCM shown combined, corresponding to the extracellular protein expression of the control co-culture group (black/white *"Control"* columns in Fig.2-5) compared to that of our treated group *F/K ("Treated"* columns).

Treatment of keratinocytes

In order to investigate the mechanism of 14-3-3 σ induction in keratinocytes, protocols were utilized as previously established by our group (56). Briefly, keratinocytes were seeded on 25 cm^2 tissue-culture flasks (Corning) and grown in KSFM with supplements. Cells were incubated for 24-48 h at 37ºC with 5% CO_2 until approximately 80-90% of confluence was reached. For the inhibition of ERK1/2, p38, and JNK MAPK, 10 μM of specific inhibitors for each kinase (PD98059, SB203580 and SP600125 respectively) were added 1 h prior to stimulation. MEK1/2 inhibitor PD, p38 inhibitor SB, and JNK inhibitor SP were purchased from Calbiochem (San Diego, California). After 1 h of incubation with the respective inhibitors, keratinocytes were stimulated with 30 μl of FCM in order

to induce 14-3-3 σ expression (the volume of FCM applied was collected from an equivalent number of fibroblasts to the number of keratinocytes treated). Cells were harvested 24 h after stimulation and cell lysates were analyzed by western blot for 14-3-3 σ expression.

Statistical analysis

Results are presented as the mean ± the standard deviation from a set of three to five experiments. T-tests were used in order to compare the average values between two populations of data. P-values less than 0.05 were considered statistically significant in this study.

Results

Intra- and extracellular expression of four 14-3-3 isoforms (β, γ, η, σ) in mono-cultured keratinocytes and dermal fibroblasts

In addition to our primary protein of interest, 14-3-3 σ, three other 14-3-3 isoforms (β, γ, and η) were selected for further investigation. This selection was based on our previous studies which demonstrated that these three isoforms are most highly expressed in both cell types (51). In order to evaluate the constitutive expression of these proteins regardless of any treatment, keratinocytes and dermal fibroblasts grown in mono-cultures, in addition to their respective conditioned media, were collected and processed for further analysis as described in the Materials & Methods. As seen in Fig.2-1, western blotting reveals that all four 14-3-3 isoforms of interest are constitutively expressed by both cell types, with the exception of the σ isoform. As we have previously demonstrated (51), dermal fibroblasts do not express any detectable levels of 14-3-3 σ intra- or extracellularly; however, keratinocytes express it strongly intracellularly, and release it in even higher levels. β-actin was used as a loading control for the cell-lysates. The absence of this protein extracellularly indicates that the presence of 14-3-3 proteins is unlikely to be due to cellular disruption.

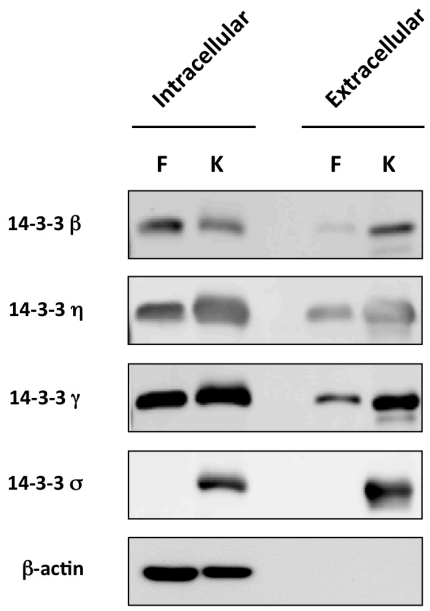


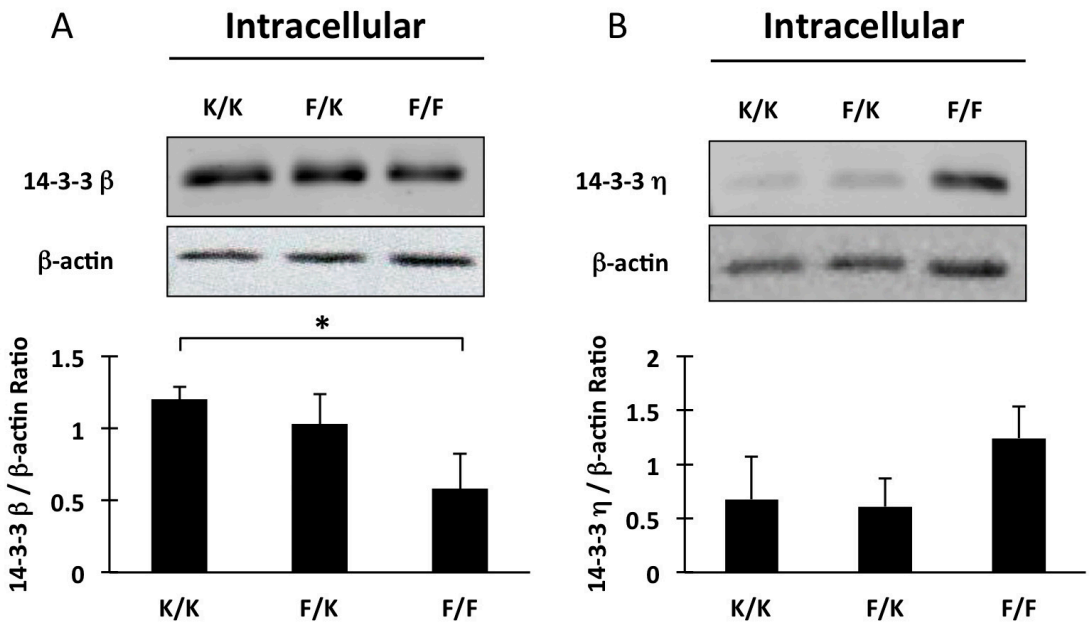
Figure 2-1. **Western blot analysis of four 14-3-3 isoforms (β, γ, η, σ) in lysates and conditioned media of keratinocytes and dermal fibroblasts.**

Independently mono-cultured keratinocytes and dermal fibroblasts were allowed to proliferate until an approximately 90% confluent monolayer was observed. In order to analyze extracellular levels of the 14-3-3 isoforms, conditioned medium was collected from both cell types and concentrated using acetone precipitation (as described in Materials & Methods). Likewise, for detection of intracellular protein levels, the corresponding cells were lysed and centrifuged to remove dead cells & debris. Total protein concentrations were determined via Bradford assay, and 10 µg of each sample was subjected to SDS-PAGE analysis. Blots were exposed to the housekeeping protein β-actin to serve as a loading control.

Intracellular expression of 14-3-3 β, η, γ, and σ proteins in co-cultured keratinocytes/fibroblasts

In order to evaluate the ability of fibroblasts to influence the intracellular expression profile of four 14-3-3 isoforms (β, η, γ, and σ) in keratinocytes, co-culture systems were assembled as described in the Materials & Methods. Following 48 h of co-culture, bystander cells were lysed and samples were subjected to western blot analysis. As illustrated in Fig.2-2 panel A, intracellular expression of 14-3-3 β in keratinocytes was not significantly altered by the presence of dermal fibroblasts in co-culture (*lane F/K*) relative to that of the keratinocyte control (*lane K/K*) (1.03 ± 0.21 vs. 1.20 ± 0.09; n=3; $p>0.05$). However, the level of this protein was significantly lower in the fibroblast control (*lane F/F*) compared to that of the keratinocyte control (*lane K/K*) (0.58 ± 0.24 vs. 1.20 ± 0.09; n=3; $p<0.05$). When the levels of intracellular 14-3-3 η were evaluated, similarly the result shown in Fig.2-2 panel B reveals that fibroblasts do not appear to significantly influence the intracellular expression of 14-3-3 η in co-cultured keratinocytes (*lane F/K*) compared to that of the keratinocyte control (*lane K/K*) (0.61 ± 0.26 vs. 0.68 ± 0.40; n=4; $p>0.05$). In the case of 14-3-3 η, the data shown in Fig.2-2 panel B also demonstrate that the fibroblast control (*lane F/F*) appears to express markedly higher levels of the protein compared to the keratinocyte control (*lane K/K*) (1.24 ± 0.29 vs. 0.68 ± 0.40; n=4; $p>0.05$). The findings in Fig.2-2 panel C reveal that the expression of intracellular 14-3-3 γ in co-cultured keratinocytes (*lane F/K*) is significantly increased, by 54.3% compared to that of the keratinocyte control (*lane K/K*) (0.84 ± 0.04 vs. 0.55 ±

0.14; n=3; p<0.05). In addition, the fibroblast control (*lane F/F*) expressed significantly higher levels of 14-3-3 γ in comparison to the keratinocyte control (*lane K/K*) (1.01 ± 0.17 vs. 0.55 ± 0.14; n=4; p<0.05). Our findings also demonstrated a significant increase in the level of intracellular 14-3-3 σ in keratinocytes when co-cultured with fibroblasts (*lane F/K*) relative to that of the keratinocyte control (*lane K/K*). The result shown in Fig.2-2 panel D reveals a 21.6% increase of intracellular 14-3-3 σ expression in co-cultured keratinocytes (*lane F/K*) compared to that of keratinocytes control (*lane K/K*) (2.58 ± 0.18 vs. 2.13 ± 0.13; n=3; p<0.05). As expected based on our previous findings, it was confirmed that fibroblasts (*lane F/F*) do not express any detectable levels of intracellular 14-3-3 σ.



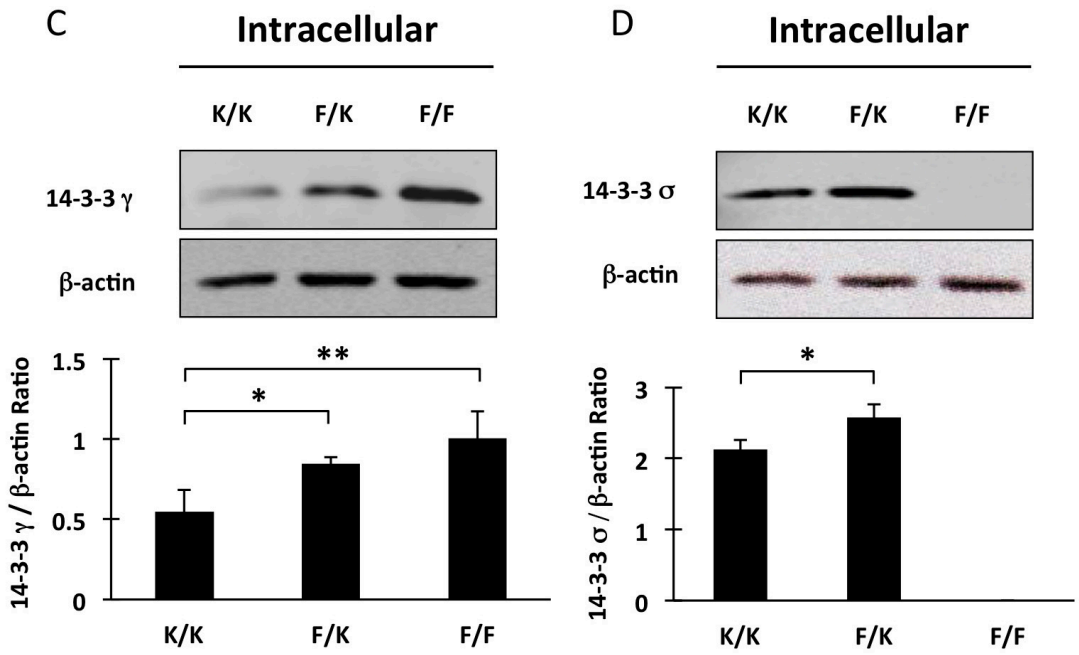


Figure 2-2. **Western blot analysis of intracellular levels of 14-3-3 β, η, γ, and σ in co-cultured keratinocytes and dermal fibroblasts.**

Following 48 h of co-culture time, cells in the bottom-chambers were lysed and 10 µg of total protein from each sample was subjected to SDS-PAGE analysis. The blots were exposed to either anti-human 14-3-3 β (panel **A**), 14-3-3 η (panel **B**), 14-3-3 γ (panel **C**), or 14-3-3 σ (panel **D**) antibodies. The intensities of the bands for each set of experiments shown in panels A-D were individually determined and are presented as graphs representing the 14-3-3 / β-actin ratio. Comparing co-cultured keratinocytes to the keratinocyte control (*lane F/K vs. K/K respectively*) revealed: panel **A** (n=3, p>0.05), panel **B** (n=4, p>0.05), panel **C** (n=3, * p<0.05), and panel **D** (n=3, * p<0.05). Marked differences were also observed when comparing the expression of the keratinocyte and fibroblast controls (*lane K/K vs F/F respectively*): panel **A** (n=3, * p<0.05), panel **B** (n=4, p>0.05), and panel **C** (n=4, ** p<0.05). Panel **D** also confirms that fibroblasts do not

express any detectable level of 14-3-3 σ. β-actin antibody was used as a loading control across all replicates.

Quantification of 14-3-3 σ protein in co-cultured keratinocytes

Quantitative analysis of 14-3-3 σ expressed intracellularly in keratinocytes was achieved by performing serial dilutions. A known total protein concentration (20 µg) of the treated keratinocyte lysate (*lane F/K*) was loaded into a 12% (wt/vol) acrylamide gel along with a series of diluted recombinant 14-3-3 σ (as described in Materials & Methods). Blots were then exposed to anti-human 14-3-3 σ antibody and the intensities of the bands were quantified by densitometry. We created a standard curve generating a linear regression model (y=14123x + 765.7; r^2=0.97806) in order to quantify the amount of 14-3-3 σ in our treated sample (*F/K*). The results presented in Fig.2-3 reveal that 20 µg of the treated keratinocyte lysate contains approximately 0.8 µg of 14-3-3 σ. This implies that approximately 4.0% of the total proteins present in the co-cultured treated keratinocytes (*lane F/K*) are 14-3-3 σ isoform. Therefore, based on the observed 21.6% increase of intracellular 14-3-3 σ in the co-cultured keratinocytes compared to that of the keratinocyte control (*lane K/K*), it was determined that approximately 3.3% of the total proteins present in the keratinocyte control (*lane K/K*) correspond to 14-3-3 σ.

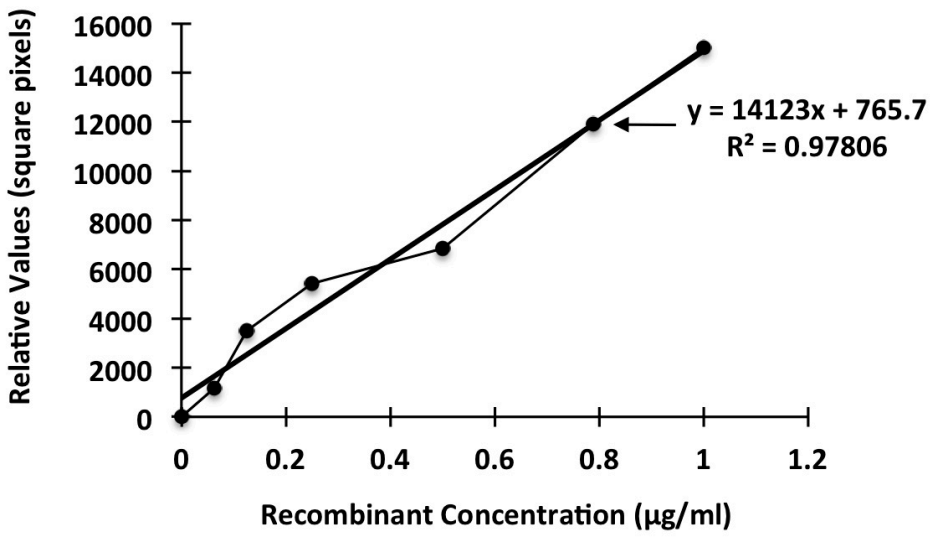


Figure 2-3. **Quantification of 14-3-3 σ in co-cultured keratinocyte lysates.**

In order to approximate the amount of 14-3-3 σ present, a serial dilution of recombinant 14-3-3 σ protein was run in an SDS-PAGE gel against 20 µg of total protein from the treated cell-lysates (*lane F/K*). The blot was exposed to anti-human 14-3-3 σ antibody and the intensity of the bands was quantified by densitometry, using a serial-dilution of recombinant 14-3-3 σ as a reference standard.

Gene expression of 14-3-3 β, η, γ, and σ in co-cultured keratinocytes

To investigate the effects of fibroblasts upon the gene expression profile of the four 14-3-3 isoforms (β, η, γ, σ) in keratinocytes, total RNA was harvested from cells following 24 h of co-culture time. Samples were reverse transcribed to cDNA as described in the Materials and Methods, and gene expression was assessed by qPCR analysis. Genes of interest were amplified along with β-actin in order to serve as an internal control. Results presented in Fig.2-4 reveal that, upon co-culturing with fibroblasts, the expression of all four genes of interest is

upregulated in treated keratinocytes relative to that of keratinocytes cultured alone. The respective increases in 14-3-3 gene expression in treated keratinocytes are as follows: 14-3-3 β gene expression increased by 1.6-fold versus that of the control (1.64 ± 0.28; n=3; p>0.05); 14-3-3 η expression increased by approximately 1.1-fold relative to keratinocytes alone (1.09 ± 0.20; n=3; p>0.05); 14-3-3 γ expression increased by 1.5-fold (1.50 ± 0.03; n=3; p<0.001), and 14-3-3 σ gene expression increased by 2.0-fold (2.00 ± 0.27; n=3; p<0.05).

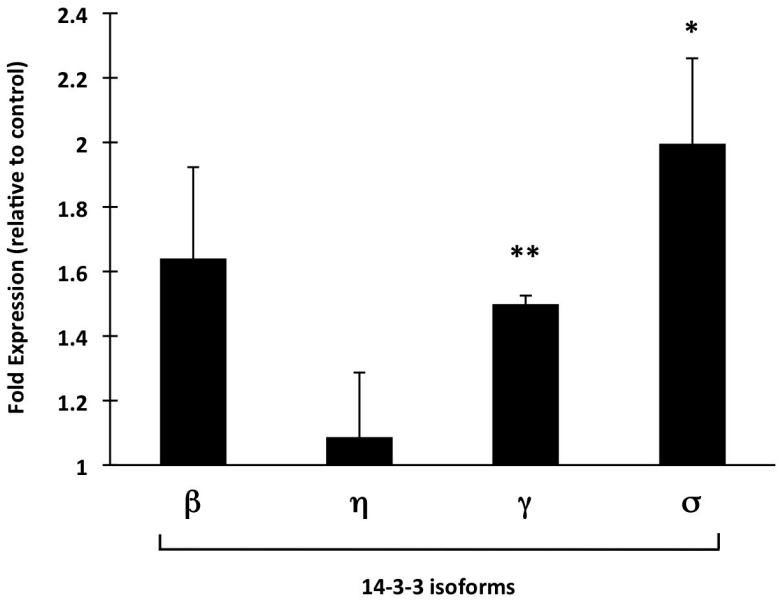


Figure 2-4. **qPCR analysis of 14-3-3 gene expression (β, η, γ, σ) in keratinocytes co-cultured with dermal fibroblasts.**

Total RNA was extracted from bystander cells after 24 h across all treatment/control groups and qPCR was performed according to manufacturers directions. Using primers for our 14-3-3 genes of interest (β, η, γ, σ), samples were analyzed in triplicates in order

to quantify the expression of these genes in our treatment and control groups. β-actin was used as an internal reference. The graph shown represents the fold increase of 14-3-3 gene expression in keratinocytes when co-cultured with fibroblasts relative to that of control keratinocytes cultured alone (n=3, ** $p<0.001$, * $p<0.05$).

Extracellular expression of 14-3-3 β, η, γ, and σ proteins in co-cultured keratinocytes/fibroblasts

Following assessment of the effects of fibroblasts upon 14-3-3 expression (β, γ, η, σ) in keratinocytes at gene and intracellular protein levels, conditioned media was analyzed for levels of the same proteins. The objective was to assess any changes in the release of 14-3-3 β, γ, η, and σ after the two cell types were co-cultured. Fibroblasts co-cultured with fibroblasts and keratinocytes co-cultured with keratinocytes were utilized as controls. The absence of β-actin was verified in all blots in order to exclude the possibility of cellular disruption. The values presented in Table 2-1 represent the densitometry values acquired from our western blot analyses, displayed in terms of expression per $1x10^6$ cells (Table 2-1, *KCM* and *FCM* columns) or $2x10^6$ cells (Table 2-1, *Control* and *Treated* columns). These data, visually represented in Fig.2-5, reveal that the co-cultured treatment group (*Treated*) releases approximately 12% higher levels of extracellular 14-3-3 β than the control (*Control*) (7.33 ± 0.89 vs. 6.55 ± 0.92; n=3; $p>0.05$). The results shown in Fig.2-5 also reveal a 36.7% decreased level of extracellular 14-3-3 η in the co-culture treated setting compared to that of the control (2.33 ± 0.73 vs. 3.68 ± 1.58; n=3; $p>0.05$). The findings presented in Fig.2-5 additionally show that the treatment group undergoes a 12.7% decrease

in the expression of extracellular 14-3-3 γ compared to that of the control (6.36 ± 0.29 vs. 7.28 ± 1.33; n=5; p>0.05). The data shown in Fig.2-5 further reveal an observed 63.2% increase in the extracellular release of 14-3-3 σ in our co-cultured treatment group compared to that of the control (5.49 ± 0.99 vs. 3.36 ± 0.44; n=3, p<0.05). Again in support of our previous findings, it was established that control fibroblasts do not release any 14-3-3 σ.

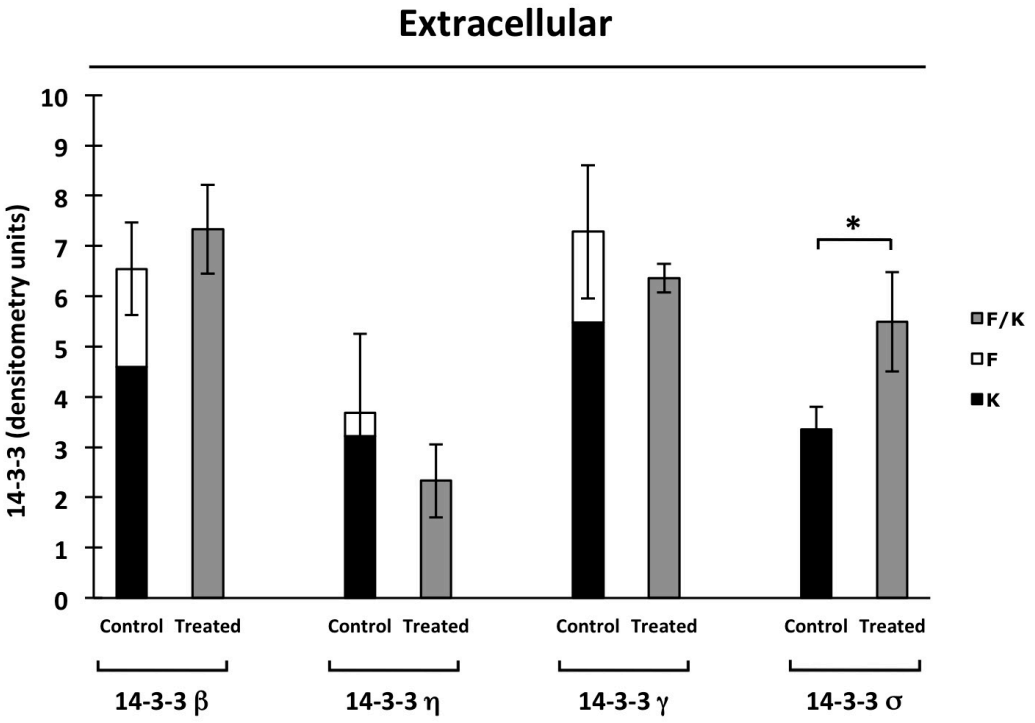


Figure 2-5. **Western blot analysis of extracellular levels of 14-3-3 β, η, γ, and σ in co-cultured keratinocytes and dermal fibroblasts.**

Following 48 h of co-culture time, conditioned medium was collected from both the bottom and top chambers of the co-culture system and their combined proteins were concentrated using acetone precipitation (as described in Materials & Methods). An equal volume (20 µL) of each sample was loaded. Blots were exposed to either anti-

human 14-3-3 β, η, γ, or σ antibodies. Based on the data presented in Table 2-1, the graph shown represents 14-3-3 densitometry units, with the two control groups combined (*F* and *K* respectively) such that comparisons could be made based on equal numbers of each cell type: for 14-3-3 β (n=3, p>0.05); for 14-3-3 η (n=3, p>0.05); for 14-3-3 γ (n=5, p>0.05); and for 14-3-3 σ (n=3, * p<0.05). These blots were also exposed to β-actin in order to exclude the possibility of cell-lysate contamination within the conditioned media.

					*		
	1x10^6 cells				Control	Treated	
Protein:	**KCM:**	**FCM:**	**KCM + FCM:**	**SDev:**	**F/K:**	**SDev:**	**P-value:**
14-3-3 β	4.589	1.957	6.546	0.921	7.332	0.885	p>0.05
14-3-3 η	3.218	0.464	3.682	1.576	2.329	0.732	p>0.05
14-3-3 γ	5.48	1.801	7.281	1.329	6.359	0.289	p>0.05
14-3-3 σ	3.361	0.000	3.361	0.439	5.486	0.987	*** p<0.05**

Table 2-1. **Western blot analysis of extracellular levels of 14-3-3 β, η, γ, and σ in co-cultured keratinocytes and dermal fibroblasts.**

Following 48 h of co-culture time, conditioned media was collected from both the bottom and top chambers of the co-culture system and their combined proteins were concentrated using acetone precipitation (as described in Materials & Methods). An equal volume (20 µL) of each sample was loaded. Blots were exposed to either anti-human 14-3-3 β, η, γ, or σ antibodies. Values shown correspond to 14-3-3 expression (densitometry units) per either 1x10^6 cells (*KCM* and *FCM*) or 2x10^6 cells (*Control* and *Treated*).

Mechanism of 14-3-3 σ induction in keratinocytes stimulated with FCM

In order to investigate the possible signaling pathway by which fibroblasts increase the expression of 14-3-3 σ in keratinocytes, we explored three distinct MAPK pathways that could potentially be involved in the regulation of 14-3-3 expression: (a) ERK1/2 (Raf → ERK kinase (MEK)1/2 → ERK1/2); (b) JNK/SAPK (MEK kinase 1–3 → MEK kinase 4 and 7 → JNK/SAPK); and (c) p38 MAPK (MAPK kinase kinase → MAPK kinase 3 and 6 → p38). We explored the role of the specific MAPK pathways in the FCM-induced expression of 14-3-3 σ in keratinocytes by blocking MEK1/2 and p38 MAPK pathways with chemical inhibitors, including some previously shown to block 14-3-3 σ gene activation in other cell types (82). As seen in Fig.2-6, 14-3-3 σ expression and induction in keratinocytes was moderately inhibited in the presence of SB203580 (*SB*), a specific inhibitor of p38 activity, both with and without FCM stimulation, compared to that of the un-inhibited control samples (*Control* and *FCM*) (*without* FCM: 0.81 vs. 1.02; *with* FCM: 0.61 vs. 1.40; n=1). A similar degree of inhibition resulted following the addition of PD98059 (*PD*), a MEK1/2 specific inhibitor (*without* FCM: 0.67 vs. 1.02; *with* FCM: 0.51 vs. 1.40; n=1). Notably, the results shown in Fig.2-6 reveal a marked reduction in 14-3-3 σ expression in both FCM-stimulated and un-stimulated keratinocytes when cultured in the presence of SP600125 (*SP*), a specific inhibitor of JNK (*without* FCM: 0.30 vs. 1.02; *with* FCM: 0.23 vs. 1.40; n=1). These findings suggest that these MAPK pathways potentially have a significant involvement in the regulation of 14-3-3 σ induction and expression in keratinocytes.

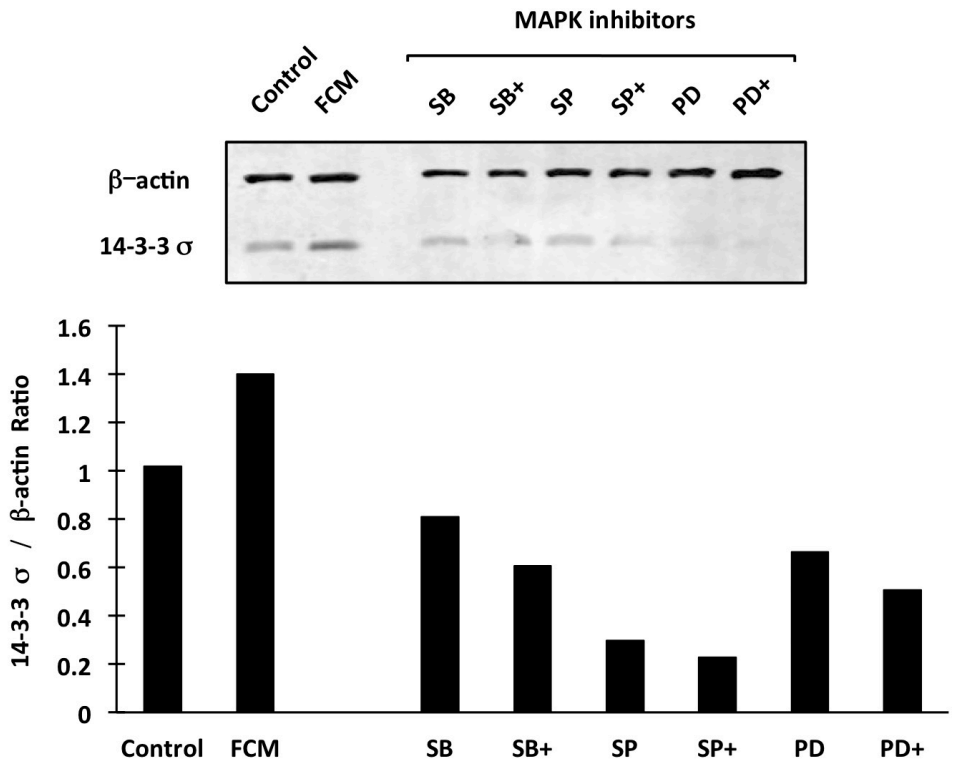


Figure 2-6. **Induction of 14-3-3 σ expression in keratinocytes stimulated with fibroblast-conditioned medium (FCM) is mediated by mitogen-activated protein kinase (MAPK) pathways.**

Keratinocytes cultured in 25 cm^2 flasks in KSFM with supplements (as described in Materials & Methods) were incubated in the presence of 10 μM of specific inhibitors for each MAPK kinase: SB203580 (*SB*), SP600125 (*SP*), and PD98059 (*PD*), for 1 h before stimulation with 30μl of FCM (for *SB*+, *SP*+, and *PD*+). Samples were harvested following 24 h and cell-lysates were analyzed by western blot for the presence of 14-3-3 σ.

Discussion

Since the original discovery of the first 14-3-3 protein, found in the brain with an unknown function, many members of the 14-3-3 protein family have been re-discovered based on new functions. The 14-3-3 proteins, primarily in the context of signal transduction pathways, have been identified as activators of tyrosine and tryptophan hydroxylase (46) and protein kinase C inhibitors (44). In this study we established a co-culture system in order to evaluate the potential ability of fibroblasts to influence the expression and release of some 14-3-3 isoforms in keratinocytes. It has already been shown by our group that human keratinocytes have the capacity to release exosomes (54) containing all seven isoforms of the 14-3-3 proteins (55). It has also been shown that many of these isoforms are intracellularly expressed in differential proportions (51). For example 14-3-3 σ, β, η, γ and ε are highly expressed in human keratinocytes (51) compared to that of ζ and τ isoforms. Based on previous results, we selected 14-3-3 σ, β, η and γ isoforms, and aimed to assess whether their levels of expression in keratinocytes are altered in response to fibroblast releasable factors.

The results suggest that intracellular levels of 14-3-3 β and η proteins in keratinocytes were not influenced by the presence of co-cultured dermal fibroblasts; however, intracellular levels of 14-3-3 γ in keratinocytes revealed an increase when co-cultured with fibroblasts. Furthermore, gene expression was quantified using qPCR, demonstrating a significant increase in the expression of 14-3-3 γ. These findings suggest that there might be a factor(s) that is released

by fibroblasts which stimulates 14-3-3 γ expression in human keratinocytes. Additional evaluation showed that exposure to a co-culture system did not significantly modify the release of 14-3-3 β, η and γ proteins by either cell type.

14-3-3 γ has been identified to be a protein kinase C inhibitor (44). Protein kinase C is involved in multiple functions such as: receptor desensitization, modulating membrane structure events, regulating transcription, in mediating immune responses, and in regulating cell growth. These functions are achieved by PKC mediated phosphorylation of other proteins. However, the substrate proteins present for phosphorylation vary since protein expression is different between different kinds of cells; thus, the effects of PKC are cell type specific. In tissue, PKC primarily has a contractile function. This observation suggests that 14-3-3 γ may function as an anti-contractile factor and thus as an anti-fibrogenic factor in wound healing.

Another novel finding of our current study included the observation that dermal fibroblasts were able to increase the 14-3-3 σ expression in keratinocytes. Previously it has been demonstrated that keratinocytes are able to stimulate MMP-1 expression in dermal fibroblasts by the release of 14-3-3 σ (52, 83); however, the possibility of dermal fibroblasts influencing the expression and release of 14-3-3 proteins in keratinocytes has never been explored. Our finding suggests that there might be a factor(s) that is released by fibroblasts which stimulates 14-3-3 σ expression in human keratinocytes.

In order to elucidate the mechanism(s) responsible for 14-3-3 σ induction in human keratinocytes when cultured in the presence of dermal fibroblasts, we conducted a number of experiments involving several MAPK-specific inhibitors. These inhibitors were selected based on previously established protocols utilized by our group (56), as well as work by other groups which support the role of MAPK in the regulation of 14-3-3 σ, specifically the ability of PD98059 to abolish 14-3-3 σ induction in PC12 cells stimulated with hydrogen peroxide (82). The findings of our study revealed that the three MAPK-specific inhibitors tested (SB, SP, and PD) all possess the capacity to moderately inhibit both the endogenous production of 14-3-3 σ by keratinocytes, as well as limit the induction of 14-3-3 σ when cultured in the presence of dermal fibroblasts. This suggests that the mitogen-activated protein kinases are involved in both the regulation of endogenous 14-3-3 σ production in keratinocytes as well the induction of 14-3-3 σ when co-cultured with fibroblasts.

One of our previous studies (83), briefly alluded to the possibility of up-regulated 14-3-3 σ gene expression in keratinocytes when co-cultured with dermal fibroblasts – an observation which had not been previously documented. In our present study, investigations at the gene level further supported this finding, revealing nearly a 2-fold increase in the gene expression of 14-3-3 σ in keratinocytes when co-cultured with fibroblasts compared to that of those in the keratinocyte control. These results lend support to the observed 21.6% increase in intracellular expression of this protein in co-cultured keratinocytes (*lane F/K*) relative to that of the keratinocyte control (*lane K/K*). Furthermore, this finding is

consistent with the observed 63.2% increase in the release of this protein in treated (*F/K*) versus control groups (*F+K*).

In conclusion, the findings of this study suggest that epidermal-mesenchymal communication, with specific respect to the 14-3-3 proteins, is in fact a double-paracrine scenario with crosstalk occurring between the two cell types. Our work revealed that fibroblasts do indeed possess the ability to influence the levels of protein and gene expression of at least some of the MMP-1 stimulating factors such as 14-3-3 σ in keratinocytes. These findings are likely to have strong implications both for the purpose of reducing fibrosis, and for simply gaining a more complete understanding of the complex tissue remodeling process.

Chapter 3. Keratinocyte-releasable Factors Stimulate the Expression of Granulocyte Colony-stimulating Factor in Human Dermal Fibroblasts[2]

Introduction

Double-paracrine communication between keratinocytes and dermal fibroblasts is essential for both wound healing and maintaining skin integrity (34). The critical nature of crosstalk between these two cell types has been firmly supported by clinical observations such as the established link between delays in re-epithelialization and increased development of fibrosis at wound sites (39, 40).

Under normal conditions, the integrity of skin is maintained through a strict balance between the synthesis and degradation of extracellular matrix components by regulating the expression of a large family of enzymes known as the matrix metalloproteinases. Our previous work has shown that epidermal-mesenchymal communication plays a central role in this process, revealing that keratinocyte-releasable 14-3-3 σ functions as a potent stimulator of several MMP isoforms in dermal fibroblasts (52, 53, 80). Similarly, we also demonstrated that in a converse fashion, fibroblasts also possess the capacity to modulate the expression of 14-3-3 proteins in keratinocytes (84).

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[2] A version of this chapter has been submitted for publication. Matthew Carr, Yunyuan Li, Claudia Chavez-Muñoz, Alireza-Moeen Rezakhanlou, Ryan Hartwell and Aziz Ghahary. Keratinocyte-releasable Factors Stimulate the Expression of Granulocyte Colony-stimulating Factor in Human Dermal Fibroblasts. (2012).

The process of cutaneous wound healing comprises three major stages – inflammation, proliferation and tissue remodeling/maturation (2). These overlapping phases involve a complex orchestration of soluble mediators, blood and parenchymal cells, and ECM. Regulation and coordination of the dynamic wound healing process occurs through the controlled release of numerous cytokines and growth factors by platelets and fibroblasts in addition to epithelial, endothelial and immune cells (3-5). Specifically, the early phases of wound healing involve an array of cytokines such as PDGF, TGF-β, FGF, VEGF, IGF, KGF, in addition to the interleukin (IL) and colony stimulating factor (CSF) families (7, 8, 13, 85, 86). Our recent studies on keratinocyte-fibroblast interactions revealed that keratinocytes are capable of potently stimulating the expression of several of these cytokines and growth factors in dermal fibroblasts – notably, the results showed a significant increase in colony-stimulating factor 3 (CSF3), also known as granulocyte-colony stimulating factor (G-CSF).

Numerous cell types such as monocytes and endothelial cells produce G-CSF in order to stimulate granulopoiesis in the bone marrow (63). In the case of trauma, G-CSF may also induce these bone marrow derived cells to migrate into the peripheral bloodstream where they can be mobilized to the site of injury (60, 63-65). Furthermore, G-CSF has been shown to dramatically promote the proliferation of keratinocytes (66, 67). Granulocyte-macrophage colony-stimulating factor (GM-CSF), also known as CSF2, has widely demonstrated therapeutic effects in the context of wound healing (68-74). Although the potential uses of G-CSF have been less studied, it has been shown that the clinical

application of recombinant G-CSF may yield similar beneficial effects to GM-CSF when utilized appropriately during the wound healing process (75-79).

As the potential therapeutic role of G-CSF in wound healing has been previously investigated (75-79), we aimed to further characterize the process by which CSF3 stimulation occurs in a co-culture environment, hypothesizing that keratinocyte-releasable factor(s) are potent stimulators of CSF3 expression in dermal fibroblasts. In order to test this hypothesis, we utilized keratinocyte/fibroblast co-culture systems in which fibroblasts were seeded on 6-well plates along with keratinocytes grown on plate inserts. The results demonstrated that keratinocytes stimulate fibroblasts to express significantly higher levels of CSF3 when the two cell types are co-cultured. The effects of keratinocyte differentiation-state were assessed by treating fibroblasts with keratinocyte-conditioned medium (KCM) collected at various stages of keratinocyte differentiation, revealing that undifferentiated keratinocytes are the most potent CSF3-stimulators for fibroblasts. By utilizing an interleukin-1 receptor antagonist (IL-1ra) we also identified keratinocyte-releasable IL-1 to be a major contributor to CSF3 stimulation in fibroblasts. The findings of this study support that keratinocyte-releasable factor(s), specifically IL-1, play a prominent role in regulating dermal fibroblast expression of CSF3.

Materials and Methods

Clinical specimens and cell cultures

Following informed consent, human skin samples were obtained from adult patients undergoing elective reconstructive surgery. This study was carried out in accordance with the principles of the Declaration of Helsinki (World Medical Association of Helsinki, Somerset West, 1996). Samples were collected individually in Keratinocyte Serum Free Medium (KSFM) (GIBCO, Grand Island, NY), and then separately washed 3 times in sterile PBS supplemented with 1% antibiotic-antimycotic preparation (100 μg/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B) (GIBCO). Specimens were dissected free of fat and cut into small pieces (~1 cm in diameter) and incubated in Dispase enzyme (25 μg/ml in KSFM) (GIBCO) at 37°C for 2 h in order to separate the dermal and epidermal layers. Epidermis was incubated for 4 minutes in trypsin at 37°C, interspersed with finger-vortexing every minute. Dulbecco's Modified Eagle's Medium (DMEM) (GIBCO) with 10% Fetal Bovine Serum (FBS) (GIBCO) was used to deactivate the trypsin reaction. Centrifugation at 800 rpm at 4°C for 8 minutes allowed us to obtain keratinocytes that were seeded into 25 cm^2 flasks (BD Biosciences, Mississauga, ON) and cultured with 5 ml of KSFM with supplements (BPE, 50 μg/ml and EGF, 5 μg/ml) (GIBCO) and 1% antibiotic-antimycotic preparation. Once keratinocytes achieved approximately 80-90% confluence, they were released via trypsinization as described above, and re-seeded at a ratio of 1:5 into 75 cm^2 BD Falcon tissue-culture treated flasks (BD Biosciences). The additives were used only to establish keratinocytes in cultures.

Thus, in order to eliminate any effects of FBS, EGF and/or pituitary extract on our findings, when assembling the co-culture systems, the KSFM with EGF and pituitary extract was exchanged with the test medium consisting of 49.5% DMEM+ 49.5% KSFM + 1% Antibiotic with no supplements. Only primary cultured keratinocytes at passages 2-4 were used in the experiments.

In order to isolate fibroblasts, following removal of the epidermal layer, all remaining pieces of dermal tissue were minced into small pieces (~1-2 mm in diameter), washed six times with sterile PBS, and distributed into 60x15 mm Petri dishes. Cultures of dermal fibroblasts were established as previously described (81). Upon achieving confluence, the fibroblasts were released by trypsinization and split for subculture at a ratio of 1:5 and re-seeded into tissue-culture treated 75 cm^2 flasks. Only fibroblasts from passages 3-6 were used in the experiments.

Spontaneously immortalized keratinocytes (HaCaT) were obtained from the American Type Cell Collection (Manassas, VA). The cells were cultured in KSFM with supplements (GIBCO) and 1% antibiotic-antimycotic preparation. Cells were passaged every 4 days at a ratio of 1:5.

Keratinocyte/fibroblast co-culture system

Co-culture systems were conducted as previously described by our group (52, 81). Briefly, fibroblasts or keratinocytes were either seeded on tissue-culture treated 30 mm 6-well tissue-culture plate inserts with 0.4 μm pore size (BD Biosciences) as the “top” chamber or on the tissue-culture treated 6-well plate (BD Biosciences) as the “bottom” chamber. Cells were grown in either DMEM

with 10% FBS (for fibroblasts) or KSFM with supplements (for keratinocytes). Both cell types were incubated independently for 24-48 h at 37ºC with 5% CO_2 until approximately 80-90% of confluence was reached.

Prior to assembling the co-culture systems, cells were counted using a hemocytometer in order to verify that approximately $1x10^6$ of each cell type would be present in the top and bottom chambers of each system. Cells were also washed 3 times with PBS to remove remaining traces of supplements (ie. FBS, BPE, and EGF). The treatment setting consisted of keratinocytes on the insert co-cultured with fibroblasts growing on the bottom of the 6-well plate (*lane K/F*). The control setting consisted of fibroblasts in both chambers (*lane F/F*). Each combined well and insert in the 6-well culture plate received 2.0 ml of the test medium mentioned above.

DNA microarray analysis of human cytokine expression

In order to examine the gene expression of human cytokines in co-cultured fibroblasts, we utilized the Oligo GEArray® DNA: Human Common Cytokines Microarray kit from SuperArray Bioscience Corporation (Fredrick, USA). Each DNA microarray profile consists of 114 genes including: interferons, interleukins, bone morphogenetic proteins, the TGF-beta family, the TNF superfamily, various growth factors, as well as control sequences such as loading controls β-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and negative blank controls. Gene expression was assessed in dermal fibroblasts cultured with keratinocytes (*lane K/F*) for 24 h under the same co-culture conditions as described above. In brief, total RNA was isolated by

using the RNeasy Mini Kit (Qiagen). The integrity of RNA was evaluated through direct visualization of ethidium bromide stained agarose gel. Only RNA with an A_{260}/A_{280} ratio between 2.0-2.2 was used in the microarray experiments. The procedure was conducted under the manufacturer's instructions. cRNA was generated from total RNA by using TrueLabeling-AMP 2.0 kit (SuperArray Bioscience Corporation, Fredrick, USA), biotinylated with Biotin-16-dUTP (Roche, Indianapolis, IN), followed by hybridization to a 2.2 x 3.8 cm nylon membrane which has the proprietary probes printed in rows and columns (8 x 12). Four micrograms of cRNA was used for each sample. The arrays were visualized using the Chemiluminescent Detection Kit (SuperArray Bioscience Corporation). Film was developed at different time points and scanned for further quantification analysis by using scanning densitometry (UN-SCAN-IT, Silk Scientific, Inc). The intensity of the housekeeping gene (GAPDH) was used as an internal control for normalization. Three separate experiments were repeated using three different fibroblast cell strains. Analysis of variance (ANOVA) testing was used to determine the statistical significance.

Real-time polymerase chain reaction (RT-PCR)

For RT-PCR analysis, following 24 h of co-culture incubation time, the upper inserts were discarded and the bystander cells from the control (*lane F/F*) and treatment (*lane K/F*) settings were released via trypsinization. Following centrifugation, cell pellets were washed with PBS and total RNA was extracted using RNeasy mini kit (Qiagen, Crawley, UK). Only RNA with an A_{260}/A_{280} ratio between 1.9-2.2 was used in the experiments. RNA integrity of each sample was

verified by direct visualization of SYBR® Safe DNA gel stained 28S and 18S bands. A total of 1 µg of total RNA from each sample was reverse transcribed using the Superscript II First Strand cDNA Synthesis kit (Invitrogen, Burlington, ON). RT-PCR was performed using the ThermoScript RT-PCR system (Invitrogen) according to the manufacturer's protocols, in combination with the following sense and anti-sense primers: hCSF-3 (sense: TATTTAAAGACAGGGAAGAGCAGAA; anti-sense: CATGTCCCAAAAGTCTTAAGAAGAA); and hβ-actin (sense: GCTCTTTTCCAGCCTTCCTT; anti-sense: CGGATGTCAACTTCACACTT) (Invitrogen). β-actin was utilized as a housekeeping gene for internal control purposes. A total of 10 µg of each sample was separated by agarose gel electrophoresis and their scanned digital images were quantitatively analyzed using ImageJ64 scanning densitometry software (Research Service Branch, National Institutes of Health).

Antibodies

Mouse anti-human granulocyte colony-stimulating factor (G-CSF) monoclonal antibody was purchased from Abcam (Cambridge, MA), and mouse anti-human β-actin monoclonal antibody was purchased from SIGMA (Saint Louis, MO). Goat anti-mouse IgG IRDye secondary antibody was obtained from LI-COR Biosciences (Lincoln, NE).

Western blotting

In order to evaluate the release of G-CSF by co-cultured fibroblasts, conditioned medium (CM) samples were collected from fibroblasts after 48 h of

co-culture incubation. Samples were then centrifuged at 10,000 rpm at 4°C for 10 min to remove any cellular debris. The samples of CM were loaded by volume (20 µl each), representative of an equivalent number of cells, on 12% (wt/vol) acrylamide gel and subjected to electrophoresis (SDS-PAGE). The proteins were then electrotransferred from the acrylamide gel onto nitrocellulose membranes using the iBlot electrotransfer system (Invitrogen, Burlington, ON). Non-specific proteins on the membranes were blocked in 5% skim milk powder in TBS – 0.5% Tween-20 overnight at 4°C. Immunoblotting was performed using anti-G-CSF monoclonal antibody (1:1000 dilution) as well as anti-β-actin (1:25,000 dilution). The absence of β-actin indicates that the presence of G-CSF is unlikely to be due to cellular disruption. Following 4 washes with TBS – 0.1% Tween-20 for 5 min each, the membranes were then incubated accordingly with the appropriate IRDye secondary antibody (1:10,000 dilution). Immunoreactive proteins were then visualized using the Odyssey Infrared Imaging System (LI-COR Biosciences) for either 680 nm (anti-rabbit) or 800 nm (anti-mouse) wavelengths. The digital images were further subjected to quantification by means of densitometry using ImageJ64 software (Research Service Branch, National Institutes of Health).

Differentiated keratinocyte-conditioned medium

As previously demonstrated by our group, high-calcium test medium can be utilized to induce differentiation in keratinocytes, with expression of involucrin protein by keratinocytes reaching its peak at 10 days post-induction (87, 88). Employing similar methodology for our experiments, keratinocyte-conditioned

medium (KCM) was collected from keratinocyte monocultures, using a test medium consisting of 50% DMEM plus 50% KSFM in the absence of FBS or growth supplements. Keratinocytes were seeded in 6-well plates using KSFM with 0.07 mM calcium and considered undifferentiated until cells reached 60% confluence. At this point, KCM was changed to KSFM/DMEM medium (50:50) (GIBCO) with 1.8 mM calcium. KCM was collected from the keratinocytes every 24 h in exchange for fresh KSFM/DMEM. Collection was continued until 10 days post-induction with high-calcium medium, at which point we considered the keratinocytes to be fully differentiated based on previous findings by our group (87, 88). Prior to treating dermal fibroblasts, KCM samples were centrifuged at 10,000 rpm at 4°C for 10 min to remove any cellular debris.

Treatment of fibroblasts with keratinocyte-conditioned medium

In order to evaluate the effects of KCM (differentiated and un-differentiated) on fibroblast expression of CSF3, fibroblasts were seeded in 6-well plates (2 x 10^5 cells per well) with DMEM 2% FBS and treated in duplicate with 1 ml of DMEM combined with 1 ml of KCM corresponding to either 1, 3, 5, 7 or 10 days post-induction with high-calcium medium. All treatment and control groups additionally received 1% antibiotic-antimycotic preparation (GIBCO) and 2% FBS. Experimental control groups consisted of fibroblasts treated with either 2 ml of DMEM + 2% FBS (*lane FC1*) or with 1 ml DMEM + 1 ml DMEM/KSFM (50:50, the KSFM used was “non-conditioned medium” (NCM)) + 2% FBS (*lane FC2*). Following 24 h of treatment, fibroblasts were harvested, RNA was extracted as

described above, and RT-PCR was utilized to analyze CSF3 expression. β-actin was used as a housekeeping gene for internal control purposes.

Ammonium sulfate precipitation of keratinocyte-releasable factor(s)

KCM was clarified by centrifugation at 3000 × g for 10 min and passing a 0.25 μm filter before precipitation by ammonium sulfate ($(NH_4)_2SO_4$). Solid powder of ammonium sulfate was then slowly added to KCM at a final concentration of 30%. The mixture was centrifuged at 3000 × g for 20 min after stirring at room temperature for 15 min. The supernatant was then transferred into a new 50 ml tube and the pellet was re-suspended by adding 4 ml of H_2O. Ammonium sulfate was added to the supernatant to make a final concentration of 50%. Mixture was stirred and centrifuged as described above. Supernatant was transferred into a new 50 ml tube and the pellet was re-suspended by adding 4 ml of H_2O. The supernatant was further used to precipitate proteins at final concentrations of 70% and >70% ammonium sulfate, respectively, by utilizing the same procedures described above. Re-suspended proteins were desalted by passing through a cut-off 10 kDa filter (Millipore); after washing 3 times with PBS, the retentate was collected and its concentration calculated based on the original volume prior to being used for dermal fibroblast treatments. Treatment of fibroblasts was conducted using the same protocols for KCM treatments described above.

Thermal stability of keratinocyte-releasable factor(s)

In our efforts to partially characterize the keratinocyte-releasable factor(s) responsible for CSF3 stimulation in fibroblasts, we examined the thermal stability

by exposing KCM to various thermal environments. KCM samples were collected as previously described, and were placed in an incubated water bath for either 30 min at 56°C or 5 min at 95°C. Heat treated samples were allowed to cool to 37°C prior to treating dermal fibroblasts. The preparation of samples for treatment of dermal fibroblasts employed the same aforementioned protocols; however, the control settings instead consisted of 1 ml DMEM + 1 ml DMEM/KSFM (50:50, DMEM:NCM) + 2% FBS (*negative control; lane NCM*) and 1 ml DMEM + 1 ml DMEM/KSFM (50:50, DMEM:KCM) + 2% FBS (*positive control; lane KCM*).

Recombinant human interleukin-1 and receptor antagonist

Recombinant human interleukin-1 beta (rhIL-1β) protein was purchased from SIGMA (Saint Louis, MO) and was applied to fibroblasts at a concentration of 50 pg/ml of DMEM:KSFM (50:50) test medium + 2% FBS. In order to neutralize the activity of both IL-1 alpha and beta proteins in the CM, we utilized an IL-1 receptor antagonist (IL-1ra) protein purchased from ProSpec (East Brunswick, NJ). In treatment conditions that involved the inclusion of IL-1ra, we applied the inhibitor at a concentration of 1 ng/ml of DMEM:KSFM (50:50) test medium + 2% FBS. Dose response experiments (not shown) revealed that this concentration provided ideal inhibition of endogenous IL-1 activity without compromising cell viability. Fibroblasts were treated in 6-well plates as described previously.

Statistical analysis

Results are presented as the mean ± the standard deviation from a set of three to five experiments. ANOVA testing was utilized for assessing the DNA

microarray results. For all other experiments t-tests were used in order to compare the average values between two populations of data. P-values less than 0.05 were considered statistically significant in this study.

Results

DNA microarray analysis of human cytokine expression in dermal fibroblasts co-cultured with keratinocytes

In order to evaluate changes in dermal fibroblast expression of key cytokines and growth factors when co-cultured with keratinocytes, a DNA microarray analysis of 114 genes was carried out. Dermal fibroblasts and keratinocytes were co-cultured according to previously established protocols (described in the Materials & Methods). Fig.3-1 panel A shows the results of the Oligo GEArray® DNA Human Common Cytokines Microarray kit. In addition, genes that demonstrated greater than a 2-fold increase were selected and presented in terms of fold-increase as a bar chart in Fig.3-1 panel B.

The results shown in Fig.3-1 panel B indicate that fibroblasts co-cultured with keratinocytes (*lane K/F*) expressed significantly higher levels of CSF3, VEGFB, ATP6AP1 and RPS27A. Most notably, the endogenous expression level of CSF3 in fibroblasts cultured alone (*lane F/F*) was nearly undetectable by microarray. In contrast, when fibroblasts were co-cultured with keratinocytes (*lane K/F*), fibroblast expression of CSF3 was significantly increased (45.00 ± 5.00 vs. 0.25 ± 0.05; $n=3$; $p<0.05$). Although modulated expression of other genes was also observed, in order to focus our investigations only CSF3 was further characterized in this chapter.

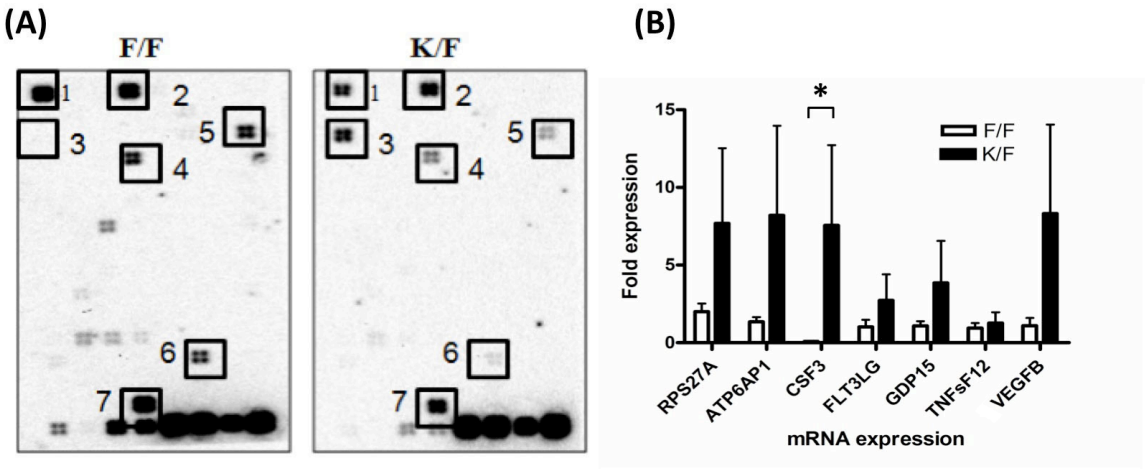

Figure 3-1. **DNA microarray analysis reveals upregulated cytokine expression in dermal fibroblasts co-cultured with keratinocytes.**

Following 24 h of co-culture time, RNA was extracted from fibroblasts in the bottom chambers. Co-culture settings (*lane K/F*) are comprised of keratinocytes grown on plate inserts with fibroblasts grown in the plate wells. Panel **A**: DNA microarray analysis was utilized to assess the expression of key human cytokines and growth factors. Panel **B**: Genes with more than a 2-fold increase are presented in a bar chart (n=3, * $p<0.05$). β-actin and GAPDH were utilized as internal controls across all replicates.

Analysis of CSF3/G-CSF expression in dermal fibroblasts co-cultured with keratinocytes

To further confirm the results of the DNA microarray, dermal fibroblasts and keratinocytes were seeded in co-culture systems as previously described. To analyze gene expression using RT-PCR, cells were harvested following 24 h of co-culture time, and total RNA was reverse transcribed to cDNA (as described in Materials & Methods). The CSF3 gene was amplified along with β-actin to serve as an internal control. The results presented in Fig.3-2 panel A reveal that, when

co-cultured with keratinocytes (*lane K/F*), fibroblasts express nearly 7-fold higher levels of the CSF3 gene compared to fibroblasts alone (*lane F/F*) (1.00 ± 0.07 vs. 0.15 ± 0.08; n=3; $p<0.01$).

In order to examine the level of fibroblast-releasable G-CSF protein, fibroblasts were co-cultured with keratinocytes for 48 h, at which point CM samples were collected and processed as described in the Materials & Methods. Samples were loaded by equal volume (10 µl) and subjected to western blot analysis. The results shown in Fig.3-2 panel B demonstrate that co-cultured fibroblasts (*lane K/F*) release markedly higher levels of G-CSF protein into their surrounding CM than fibroblasts in the control setting (*lane F/F*) (2.90 ± 1.25 vs. 0.58 ± 0.12; n=2). The absence of β-actin extracellularly indicates that the presence of G-CSF protein is unlikely to be due to cellular disruption.

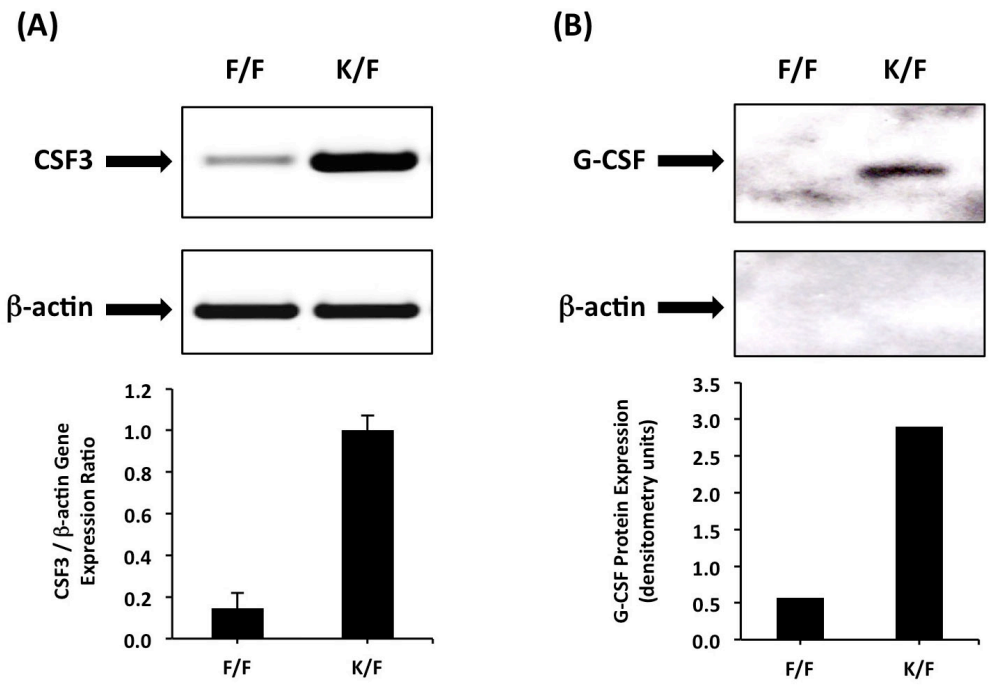

Figure 3-2. **Dermal fibroblasts express higher levels of CSF3/G-CSF when co-cultured with keratinocytes.**

Panel **A**: To analyze the gene expression level, keratinocytes and fibroblasts were co-cultured for 24 h and total RNA was extracted from fibroblasts. Co-culture settings (*lane K/F*) are comprised of keratinocytes grown on plate inserts with fibroblasts grown in the plate wells. Samples were processed and RT-PCR analysis was conducted using primers for CSF3 and β-actin as an internal reference (n=3, $p<0.01$). Panel **B**: For western blot analysis, CM was collected from co-culture systems following 48 h of co-culture time. A volume of 10 µl per sample was subjected to SDS-PAGE analysis (n=2). Blots were also exposed to β-actin in order to exclude the possibility of cellular disruption.

Differentiation-state of keratinocytes impacts their release of CSF3-stimulatory factor(s) for dermal fibroblasts

Firstly, in order to assess whether immortalized keratinocyte cell lines have the capacity to stimulate CSF3 expression in dermal fibroblasts, we treated dermal fibroblasts with CM collected from either immortalized HaCat keratinocytes or primary human keratinocytes. Treatment of fibroblasts and experimental control settings were conducted as described in the Materials & Methods. In brief, total RNA was harvested from fibroblasts following 24 h of treatment time, and samples were reverse transcribed to cDNA and subjected to RT-PCR analysis. The CSF3 gene was amplified in addition to β-actin to serve as an internal control. Fig.3-3 panel A reveals that CM collected from HaCat cells does not stimulate fibroblasts to express CSF3 ($n=3$; $p>0.05$). In contrast, KCM collected from primary human keratinocytes significantly increases the CSF3 expression in dermal fibroblasts compared to the control media (1.10 ± 0.13 vs. *undetectable*; $n=3$; $p<0.01$).

Next, to assess whether keratinocyte differentiation-state has an impact upon their capacity to release fibroblast CSF3-stimulating-factor(s), we cultured primary keratinocytes in high-calcium growth medium in order to stimulate the differentiation process (as described in Materials & Methods). As previously established by our group (87), keratinocytes achieve full differentiation at approximately 10 days post-induction. Accordingly, we chose to collect KCM every 24 h until day 10; the KCM samples from days 1, 3, 5, 7, and 10 were then used for treatment of dermal fibroblasts (as described in Materials & Methods).

Following 24 h of treatment time, total RNA was harvested from fibroblasts, and samples were reverse transcribed to cDNA then subjected to RT-PCR analysis using primers for CSF3 and β-actin as an internal control. Fig.3-3 panel B shows that KCM from day 1 (*lane D1*) appears to be the most potent stimulator of CSF3 expression in fibroblasts relative to the control (*lane FC2*) (1.11 ± 0.11 vs. *undetectable*; n=4; $p<0.01$). Interestingly, KCM collected from day 7 (*lane D7*) produced moderate stimulation of CSF3 activity in fibroblasts versus the control (*lane FC2*) (0.65 ± 0.30 vs. *undetectable*; n=4; $p<0.01$). KCM from day 10 (*lane D10*) was also observed to produce weak levels of CSF3 stimulation (0.26 ± 0.24 vs. *undetectable*; n=4; $p>0.05$).

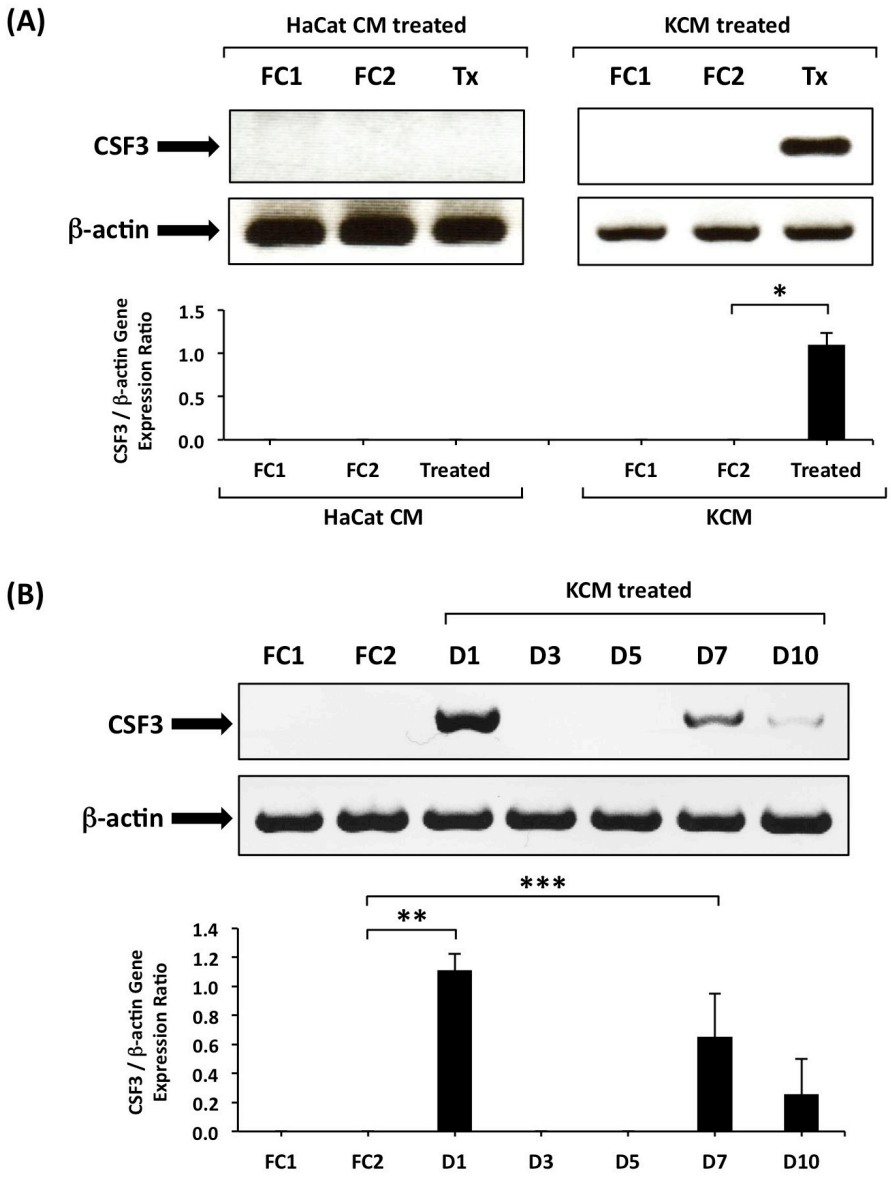

Figure 3-3. **Expression of keratinocyte-releasable CSF3-stimulatory factor(s) varies with keratinocyte differentiation-state.**

Following treatments with KCM for 24 h, total RNA was extracted from fibroblasts and RT-PCR was performed using primers for CSF3 and β-actin as an internal reference. Panel **A**: Fibroblasts were treated with conditioned medium collected from either immortalized HaCat keratinocytes (n=3, $p>0.05$) or primary human keratinocytes (n=3, * $p<0.01$). Panel **B**: Following inducing keratinocytes to differentiate using high-calcium

growth medium, KCM samples collected from days 1, 3, 5, 7, and 10 were used to treat dermal fibroblasts (n=4, ** $p<0.01$, *** $p<0.01$).

Partial characterization of keratinocyte-releasable CSF3-stimulatory factor(s) for dermal fibroblasts

In order to partially characterize the keratinocyte-releasable factor(s) responsible for stimulating CSF3 expression in dermal fibroblasts, we subjected KCM collected from primary keratinocytes to ammonium sulfate precipitation and heat treatment techniques prior to treating dermal fibroblasts (as described in Materials & Methods). Total RNA was harvested from fibroblasts following 24 h of treatment time, and samples were reverse transcribed to cDNA and subjected to RT-PCR analysis. The CSF3 gene was amplified in addition to β-actin to serve as an internal control.

Fig.3-4 panel A demonstrates that, when used to treat fibroblasts, a 50% concentration of ammonium sulfate (*lane 50*) provides the most effective precipitation of CSF3-stimulatory factor(s) in the CM compared to the positive control setting (*lane KCM*) (0.84 vs. 1.06; n=1).

Interestingly, Fig.3-4 panel B reveals that the keratinocyte-releasable factor(s) responsible for stimulating CSF3 expression in dermal fibroblasts significantly increased in potency when KCM was heat treated at 56°C for 30 min (*lane 56°C*) compared to the positive control (*lane KCM*) (0.99 ± 0.08 vs. 0.57 ± 0.09; n=5; $p<0.01$). However, as seen in Fig.3-4 panel C, the CSF3-stimulatory capacity of keratinocyte-releasable factors are abolished relative to the control

setting (*lane KCM*) (0.05 ± 0.02 vs. 1.02 ± 0.02; n=3; p<0.01) when KCM is treated for 5 min at 95°C (*lane 95°C*).

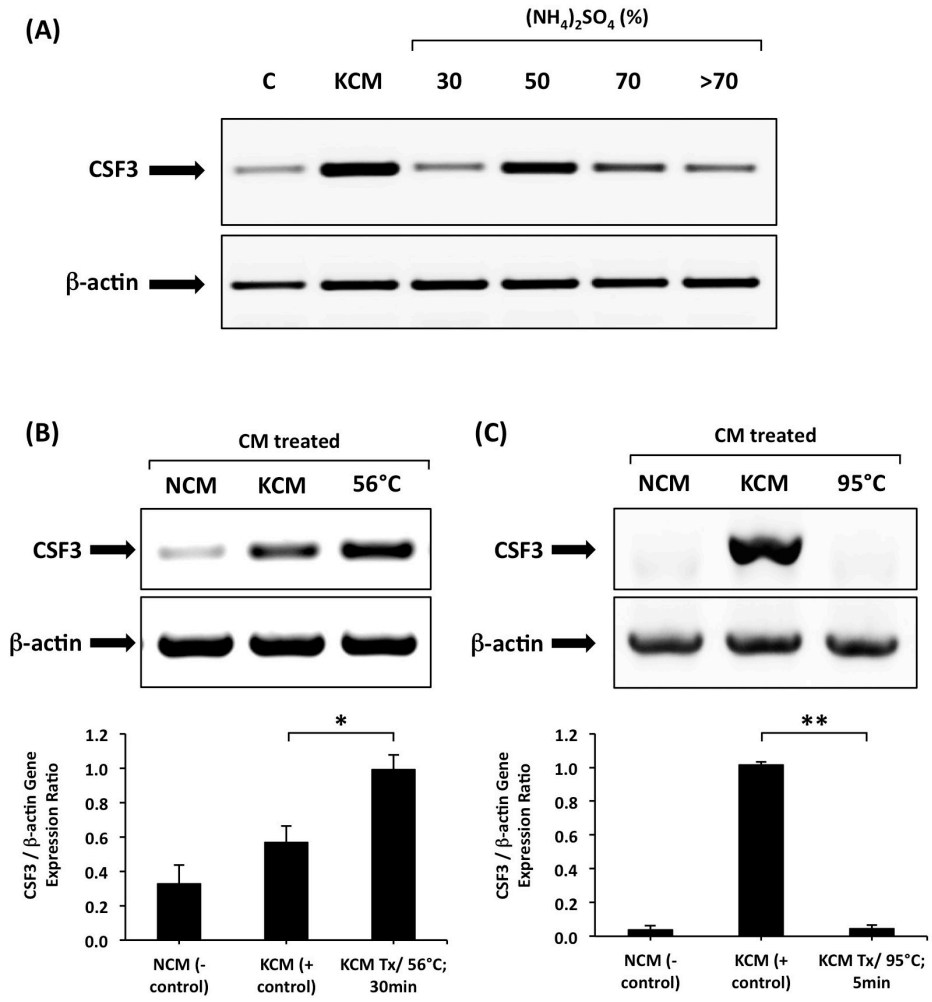

Figure 3-4. **Partial characterization of keratinocyte-releasable CSF3-stimulatory factor(s) for dermal fibroblasts.**

Dermal fibroblasts were treated with varying types of KCM. Total RNA was extracted from the cells after 24 h of treatment time and RT-PCR was performed using primers for CSF3 and β-actin as an internal reference. Panel **A**: Prior to treating fibroblasts with KCM, it was subjected to varying percentages of ammonium sulfate in order to evaluate the CSF3-stimulatory capacity of precipitated proteins (n=1). Panel **B**: KCM was heat

treated at 56°C for 30 min before treating fibroblasts (n=5, * p<0.01). Panel **C**: KCM used to treat fibroblasts was heat treated at 95°C for 5 min (n=3, ** p<0.01).

Assessing IL-1 as a potential keratinocyte-releasable CSF3-stimulatory factor for dermal fibroblasts

To investigate the role of IL-1 alpha or beta as potential candidates for the keratinocyte-releasable factor(s) responsible for stimulating CSF3 activity in dermal fibroblasts, we utilized an IL-1 receptor antagonist to block the function of IL-1 in the KCM samples used to treat fibroblasts. rhIL-1β was also used to treat fibroblasts both in the presence and exclusion of the IL-1ra. Treatment of fibroblasts and control settings were conducted as described in the Materials & Methods. Total RNA was harvested from fibroblasts following 24 h of treatment time, and samples were reverse transcribed to cDNA and subjected to RT-PCR analysis. The CSF3 gene was amplified in addition to β-actin to serve as an internal control. Fig.3-5 demonstrates that the addition of IL-1ra to KCM (*lane KCM + IL-1ra*) results in a dramatic reduction of CSF3 expression in dermal fibroblasts compared to unaltered KCM (*lane KCM*) (0.32 vs. 0.80; n=1). Likewise, when IL-1ra was added to rhIL-1β samples used to treat fibroblasts (*lane rhIL-1β + IL-1ra*), a similar reduction in CSF3-stimulatory capacity was observed versus rhIL-1β alone (*lane rhIL-1β*) (0.20 vs. 0.42; n=1). Interestingly, the results also revealed that the CSF3-stimulatory capacity for fibroblasts is markedly greater in KCM (*lane KCM*) compared to rhIL-1β applied at a dosage of 50 pg/ml (*lane rhIL-1β*) (0.80 vs. 0.42; n=1).

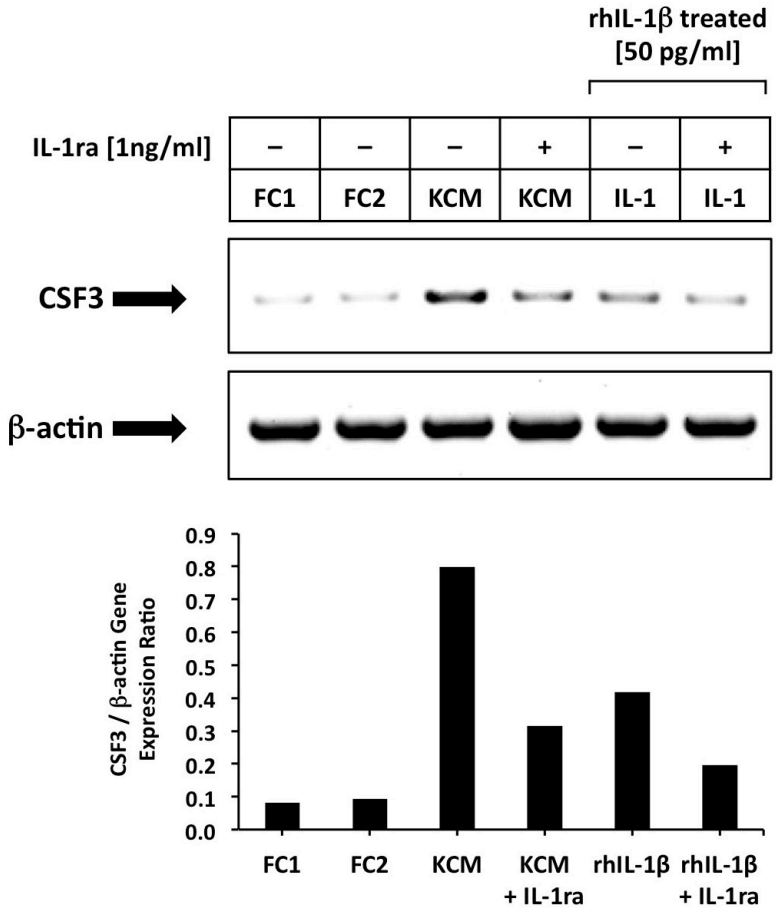

Figure 3-5. **CSF3 expression in dermal fibroblasts treated in the presence/absence of KCM, recombinant human IL-1β and IL-1 receptor antagonist.**

Dermal fibroblasts were treated with combinations of either KCM or rhIL-1β [50 pg/ml] in the presence/absence of IL-1ra [1 ng/ml]. Following 24 h of treatment time, total RNA was extracted from fibroblasts and RT-PCR was performed using primers for CSF3 and β-actin as an internal reference (n=1).

Discussion

CSF3, also known as granulocyte colony-stimulating factor (G-CSF), is a 19 KDa protein that can be produced by numerous cell types including monocytes (63, 89-91), lymphocytes (92), bone marrow stromal cells (BMSCs) (93), endothelial cells (94) and fibroblasts (63, 95, 96). While the main function of G-CSF is to induce the production of granulocytes in bone marrow (97), it is also known to stimulate mobilization of bone marrow derived cells into the peripheral bloodstream and recruit them to sites of traumatic injury (64). Secondary effects of G-CSF such as its ability to promote keratinocyte proliferation have also been recognized (66, 67). Although some cells such as peripheral blood monocytes and BMSCs constitutively express low levels of CSF3/G-CSF, endothelial cells and fibroblasts require stimuli such as lipopolysaccharide (LPS), TNF-α or IL-1 in order to produce detectable levels of the protein (95, 98-100). It has also been demonstrated that IL-1 is a more potent G-CSF-stimulatory factor for fibroblasts compared to LPS, while the converse is true for stimulating G-CSF expression in monocytes (100).

In this study we utilized a co-culture system in order to evaluate the capacity of keratinocytes to stimulate the expression of CSF3/G-CSF in dermal fibroblasts. Our results confirmed that fibroblasts are able to produce G-CSF under the appropriate stimuli (such as rhIL-1β), and further demonstrated in a novel fashion that fibroblasts co-cultured with keratinocytes express high levels of CSF3. To characterize the keratinocyte-releasable factor(s) responsible for stimulating CSF3 expression in fibroblasts, we selected IL-1 as a candidate due

to its ability to stimulate CSF3 expression in various cell types in addition to its important role in keratinocyte-fibroblast wound healing interactions. When the epidermal barrier is injured or disrupted, keratinocytes release pre-stored IL-1 into their surrounding environment which signals the damage to nearby cells – this stimulates host defense mechanisms in addition to promoting keratinocyte proliferation, fibrosis and scarring (69, 101, 102). In a related context, it has been reported that keratinocyte-derived IL-1 can induce fibroblasts to produce cytokines and growth factors such as CSF2/GM-CSF, which in turn modulates epithelial migration and proliferation (36, 60, 103). The findings of our study showed that IL-1 is predominantly responsible for the observed upregulation of CSF3 expression in fibroblasts co-cultured with keratinocytes. Furthermore, KCM continues to produce moderate induction of CSF3 expression in fibroblasts, even in the presence of an IL-1 receptor antagonist. This phenomenon can be attributed to the possible presence of other keratinocyte-releasable factors such as TNF-α that may partially contribute to the CSF3-stimulatory effect in dermal fibroblasts.

Our theory that keratinocyte-releasable IL-1 is the predominant CSF3-stimulating factor for fibroblasts was further supported by our investigations into keratinocyte differentiation-state. The findings of our study revealed that KCM collected from undifferentiated keratinocytes (*lane D1*, Fig.3-3 panel B) was the most potent stimulator of CSF3 expression in fibroblasts – this interestingly correlates with previous publications demonstrating that undifferentiated keratinocytes express significantly higher levels of IL-1α in comparison to

differentiated keratinocytes (101, 104, 105). This endogenous production of IL-1 is in contrast to other known CSF3-stimulatory factors that are producible by keratinocytes such as TNF-α, which is predominantly released in response to stimuli such as LPS or ultraviolet light (106).

Interestingly, the activity of KCM is stable at typical denaturing temperatures (56ºC), and remains stable through to 95ºC, at which point the activity of the CSF-stimulatory factors are lost. Heat treatment of KCM in fact augmented the efficacy of the treatment media to induce CSF3 in fibroblasts, which was contrary to what would be expected if IL-1α was the sole factor responsible. It is possible that IL-1α stabilizing co-factors are responsible for the increased heat-resistance – if released upon heating they could potentially render a more active form of interleukin-1 following heat treatment at 56ºC. This is similar to effects of heat shock proteins and HIF-1α stabilization intracellularly (107). In a similar fashion other cytokines have been known to rest in dormant states, extracellularly bound to matrix components and glycoproteins that are cleaved following trauma, and in this case heat treatment may denature any bound component while preserving active sites on IL-1 or other CSF3 stimulating factors and cytokines (108, 109).

We theorize that the versatile ability of G-CSF to induce: granulopoiesis, mobilization to the site of injury, and epithelial proliferation, are all potential mechanisms that may explain its beneficial effects when used therapeutically under clinical settings. In conclusion, our findings demonstrate that keratinocyte-

releasable IL-1 is a potent stimulator of CSF3/G-CSF expression in dermal fibroblasts. Considered in combination with the therapeutic potential of G-CSF, our results further underscore the importance of epidermal-mesenchymal communication throughout the stages of wound healing. The observation that keratinocyte-releasable IL-1 plays an important mechanistic role during CSF3 induction in co-cultured dermal fibroblasts has implications towards the development of novel wound healing strategies in addition to furthering our understanding of the dynamic wound healing process.

Chapter 4. Conclusion and Suggestions for Future Work

General Discussion

Communication between keratinocytes and fibroblasts plays an integral role in the process of epithelialization and wound healing. In the case of injury to the skin, it has been demonstrated that delays in the epithelialization process may lead to an increased risk of fibrosis (39). This signifies that without adequate wound coverage, extracellular matrix components continue to accumulate until dermal fibroblasts receive signal(s) from epidermal keratinocytes. These signals slow down the dynamic healing process and eventually lead to maturation and remodeling of the healing wound (40). Under normal conditions, structural integrity is maintained by adhering to a fine balance between the synthesis of ECM components and their respective degradation by MMPs.

Our group previously demonstrated that keratinocyte releasable 14-3-3 σ functions as a potent MMP-1, 3, 8, 10 and 24 stimulating factor for dermal fibroblasts (52, 53). However, the possibility of dermal fibroblasts influencing the expression and release of 14-3-3 proteins in keratinocytes had never been explored. Based on prior findings, we selected 14-3-3 σ, β, η and γ isoforms, and in a converse fashion, aimed to assess whether their levels of expression in keratinocytes are altered in response to fibroblast releasable factors.

The results shown in Chapter 2 suggest that intracellular levels of 14-3-3 β and η proteins in keratinocytes are not altered by the presence of co-cultured dermal fibroblasts. In contrast, intracellular levels of 14-3-3 γ were found to

increase in keratinocytes co-cultured with fibroblasts compared to that of mono-cultured keratinocytes. These findings were further confirmed by qPCR. Given the role of 14-3-3 γ as a PKC inhibitor in conjunction with the contractile effects of PKC in various tissue types, we theorize that keratinocyte-releasable 14-3-3 γ may possess important properties as an anti-contractile factor and thus as an anti-fibrogenic factor in wound healing.

It was also demonstrated that fibroblasts have the capacity to influence the expression of certain MMP-1 stimulating factors such as 14-3-3 σ in keratinocytes. Further investigations suggested that dermal fibroblasts regulate 14-3-3 σ expression in keratinocytes through several of the MAPK signaling pathways. Collectively, our findings suggest dermal fibroblasts may release factor(s) that cause upregulation of 14-3-3 γ and σ expression in human keratinocytes. The findings outlined in Chapter 2 suggest that epidermal-mesenchymal communication, with specific respect to the 14-3-3 proteins, is in fact a double-paracrine scenario with crosstalk occurring between the two cell types.

In Chapter 3 we evaluated the capacity of keratinocytes to stimulate the expression of CSF3/G-CSF in dermal fibroblasts. Our results demonstrated in a novel fashion that fibroblasts co-cultured with keratinocytes express high levels of CSF3. In order to characterize the keratinocyte-releasable factor(s) responsible for stimulating CSF3 expression in fibroblasts, we selected IL-1 as a candidate due to its CSF3-stimulatory capacity in various cell types. When the

epidermal barrier is injured or disrupted, keratinocytes release pre-stored IL-1 into their surrounding environment which signals the damage to nearby cells – this stimulates host defense mechanisms in addition to promoting keratinocyte proliferation, fibrosis and scarring (69, 101, 102). Additionally, it has been reported that keratinocyte-derived IL-1 can induce fibroblasts to produce cytokines and growth factors such as CSF2/GM-CSF, which in turn modulates epithelial migration and proliferation (36, 60, 103). We demonstrated that keratinocyte-releasable IL-1 is a potent stimulator of CSF3 expression in fibroblasts. Based on the observation that KCM still produces moderate induction of CSF3 expression in fibroblasts even in the presence of IL-1ra, we recognize that several other keratinocyte-releasable factors such as TNF-α may also contribute to CSF3 upregulation in dermal fibroblasts.

We theorize that the versatile ability of G-CSF to induce: granulopoiesis, mobilization to the site of injury, and epithelial proliferation, are all potential mechanisms that may explain its therapeutic effects on healing wounds. The results outlined in Chapter 3 demonstrate that keratinocyte-releasable IL-1 is a potent stimulator of CSF3/G-CSF expression in dermal fibroblasts. When considered along with the recognized therapeutic potential of G-CSF, this highlights the importance of epidermal-mesenchymal communication throughout the wound healing process.

The central objective to this research project has been to examine the intricacies of bidirectional communication between keratinocytes and dermal fibroblasts, specifically in regard to critical wound healing factors. Considered as

a whole, we believe that the results presented in this thesis have positive implications towards the development of novel wound healing strategies to reduce fibrosis, and also towards gaining a more complete understanding of the complex tissue remodelling process.

Suggestions for Future Work

Although this body of work has provided novel insight into several aspects of epidermal-mesenchymal crosstalk during wound healing, it is apparent that this may represent a mere starting point for future research. We acknowledge that further experimentation will be necessary in order to translate these findings towards potential wound healing therapies. The following are several suggestions that may advance our current conclusions:

I. In Chapter 2, we showed that keratinocytes co-cultured with dermal fibroblasts express significantly higher levels of 14-3-3 σ and γ compared to mono-cultured keratinocytes. Further mechanistic investigation (pathways other than MAPK) is warranted in order to better understand the cellular processes underlying the ability of fibroblasts to stimulate the expression of 14-3-3 σ and γ in keratinocytes. Partial-characterization of the fibroblast-releasable factor(s) responsible (i.e. size exclusion, pH & temperature stability analyses) could potentially lead to the eventual identification of the specific factor(s) that stimulate 14-3-3 production in keratinocytes.

II. In Chapter 3, we revealed that fibroblasts express CSF3/G-CSF when co-cultured with keratinocytes; specifically showing that keratinocyte-releasable IL-1 is an important part of this mechanism. In order to further characterize this paracrine process, a logical progression from our current findings would be to first evaluate the amount of IL-1 produced by keratinocytes, followed by dose-response experiments on fibroblasts

in order to determine the ideal concentration of IL-1 for stimulating G-CSF production in fibroblasts. Correlating the results of these follow-up studies would assist in determining the precise involvement of keratinocyte-releasable IL-1 throughout this process.

III. Additional mechanistic investigations using intracellular signaling inhibitors would improve our understanding in regards to the signal transduction events involved with stimulation of G-CSF production in dermal fibroblasts. Such information is likely to assist in characterization of the keratinocyte-releasable factors involved, and is also a necessary component of fully understanding the potential roles of G-CSF during wound healing and tissue remodeling.

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