Apoptosis in Wound Repair

BY

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THESIS

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Apaf1	Apoptotic peptidase activating factor 1
Bax	BCL2-associated X protein
Bcl2	B-cell CLL/lymphoma 2
bFGF	Basic fibroblast growth factor
Casp	Caspase
CM	Conditioned media
COL1	Collage type I
COL3	Collagen type III
CPT	Camptothecin
CTGF	Connective tissue growth factor
Cycs	Cytochrome C
CYR61	Cysteine rich inducer 61
DR	Death receptors
ECM	Extracellular matrix
ECs	Endothelial cells
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
Fadd	Fas associated death domain
FasR	TNF receptor superfamily 6
FB	Fibroblasts
HMEC-1	Human microvascular endothelial cells
HSP47	Serpin H1,
IL-1beta	Interleukin 1beta
IP-10	Interferon inducible protein 10
MMP	Matrix metalloproteinase
MMP	Matrix metalloproteinase
NHDF	Normal human dermal fibroblasts
NS	Not significant
NT	No treatment (supplemented media)
PCR	Polymerase chain reaction
ROS	Reactive oxygen species
RT-PCR	Reverse transcriptase Polymerase chain reaction
SDF-1	Stromal derived growth factor
SEM	Standard error of the mean
SS	Serum starved
STMN1	Stathmin 1
TGFbeta1	Transforming growth factor beta 1
TGFbeta3	Transforming growth factor beta 3
TIMP	Tissue inhibitor metalloproteinase
TNF-alpha	Tumor necrosis factor alpha
Tnfrsf1b	Tumor necrosis factor receptor gene super family 1b
Tradd	TNF receptor associated death domain
Trp53	Transformation related protein 53
UV	Ultra violet
VEGF	Vascular endothelial growth factor

Summary

Tissue fibrosis can be caused by radiation, trauma, medications, or toxins, and fibrosis can occur anywhere in the body including the oral mucosa, lung, kidney, liver, and skin. Fibrosis in the skin can result from normal or pathologic wound healing. One characteristic of fibrosis is an increase in the synthesis of collagen type I (COL1) and decreases in both synthesis of COL3 and degradation of COL1. The presence of contractile fibroblasts (FB), termed myofibroblasts (myoFB), is considered a hallmark of the fibrotic response. Another hallmark of fibrosis is increased cell death of hepatocytes, alveolar epithelial cells, and endothelial cells (ECs). The level of apoptosis and degree of fibrosis have been related in hepatic, pulmonary, and renal fibrosis, suggesting that apoptotic cells directly influence the development of fibrosis. The mechanisms that dictate the interaction of apoptotic cells and fibrosis are poorly understood.

Wound healing is made up of 4 distinct but overlapping phases: hemostasis, inflammation, proliferation, and remodeling. During wound healing efficient elimination of cells is required to progress through the phases. Many different cell types undergo apoptosis over the course of wound healing. A large percent of apoptotic cells are ECs, particularly during vessel regression in the remodeling phase of wound healing. Dermal and mucosal healing are mechanistically similar. However, scarring and closure rates are dramatically improved in mucosal healing, possibly due to differences in apoptosis. EC apoptosis is a prominent feature in the latter stages of oral and dermal wound healing, as apoptosis is requisite to the pruning of the robust vascular bed that is produced during wound healing. The presence of high levels of apoptotic ECs in the

resolving wound suggests that endothelial-fibroblast interactions may play a role in scarring and fibrotic outcomes.

In renal, hepatic, and bleomycin-induced fibrosis, potent paracrine signaling factors secreted by apoptotic ECs have been identified. These factors can act directly on FBs to induce proliferation, myoFB differentiation, and collagen synthesis. The long term goal of the proposed research is to uncover the mechanisms that apoptotic cells use to influence scar formation and fibrosis in skin. This project has the potential to have a significant impact on the understating of fibrosis in scar formation as well as other related conditions, such as renal, hepatic, pulmonary, and cardiac fibrosis. Elucidation of these mechanisms will identify opportunities for therapeutic intervention in fibrosis.

CHAPTER 1

INTRODUCTION: Linking Angiogenesis and Apoptosis with Fibrosis

1.1 Overview

Fibrosis can occur in many different types of tissue such as lung, liver, heart, kidney, and skin. Several types of fibrosis are ranked in the top 20 Global Burdens of Disease (GBD) in the World Health Organization's (WHO) most recent GBD publication; burns, which often result in severe fibrotic scarring, ranked 15th and liver fibrosis/cirrhosis from hepatitis viral infections ranked 18th in the GBD [1]. Despite an international and cross-organ system prevalence, a functional anti-fibrotic treatment has not been developed [2]. This chapter focuses on fibrosis in other organ systems, the current understanding of the involvement of apoptosis and angiogenesis in these systems, and draws correlations between other models of fibrosis and scarring which results from wound healing.

1.2 Fibrosis

The hallmarks of fibrosis: increased presence, differentiation, and persistence of myofibroblasts (myoFBs), increased collagen deposition, and decreased collagen degradation, are conserved in most models of organ fibrosis. The normal progression of scar formation in a healing wound is similarly characterized by an increase in collagen deposition and decrease in collagen degradation, and the presence and persistence of myoFBs resuuting in a scar. Four types of fibrosis (liver, lung, transplant vasculopathy, and wound healing/skin) illustrate commonalities in fibrosis and the mechanisms that drive this process (Table I).

1.2.1 Liver Fibrosis

Liver fibrosis has been studied extensively because the liver is the only known organ to regenerate after a pro-fibrotic insult, and because of the clinical importance of fibrosis in this organ. As of 2004, liver fibrosis/cirrhosis from hepatitis viral infections ranked 18th in the GBD [1]. Liver fibrosis is characterized by chronic activation of the wound healing response and an increase in the differentiation of hepatic stellate cells (HSCs) to myoFB-like cells. In addition to HSCs other cell types contribute to liver fibrosis, specifically, resident portal FBs, bone marrow-derived, or circulating fibrocytes, and epithelial cells that have under gone epithelial-to-mesenchymal-transition (EMT). Perturbations in the expression of collagen, matrix metalloproteinases (MMPs), and tissue inhibitor metalloproteinases (TIMPs) have also been described in the fibrotic response of the liver. Changes in the vasculature are also a hallmark of liver fibrosis, as the microvascular fenestrations that are critical to healthy liver function are occluded by deposition of ECM.

Liver fibrosis is unique in the ability to repair fibrosis and regenerate normal liver tissue. Regeneration and reversal of fibrosis generally occurs when the pro-fibrotic stimuli are removed. A well characterized model of liver fibrosis in mice is carbon tetrachloride (CCl_4) administration. Cessation of the CCl_4 administration is accompanied by spontaneous resolution over longer periods of time. Most researchers agree that the spontaneous resolution of liver fibrosis is related to apoptosis of hepatic myoFBs. Another hypothesis relates to the increased transition of activated HSCs to quiescent HSCs [3]. Altogether liver fibrosis displays many of the common threads of fibrosis in

other organs, but is unique in the ability to regenerate the liver parenchyma after removal of the injurious stimuli.

1.2.2 Lung Fibrosis

Fibrosis in the lung can be derived from several different interstitial lung diseases. All of the interstitial lung diseases have similar pathologies, and varying etiologies. Idiopathic pulmonary fibrosis (IPF) is a type of interstitial lung disease and is diagnosed when the cause of the lung fibrosis is unknown. There are approximately 5 million cases of IPF globally [4] with more than 48,000 more being diagnosed annually.

Lung fibrosis is characterized by heavy collagen deposition. The collagen deposition is centered around fibrotic foci which contain ECs. Idiopathic pulmonary fibrosis (IPF) is initiated by an injury to the alveolar epithelium which is followed by a mild to moderate inflammatory response. This damage can cause a wound clot to form. After the formation of a wound clot, neighboring alveolar epithelial cells migrate to the “wound” site and are activated. FBs are recruited from the surrounding tissue, resident FBs, or fibrocytes from the bone marrow. Another mechanism for the increased presence of FBs and myoFBs is EMT. EMT in this case occurs as the protein microenvironment changes from being pro-healing to pro-fibrotic. These cellular and biochemical changes signal the initiation of normal tissue repair, similar to the normal wound healing response, however, in IPF the wound healing process never ceases. Chronic activation of the healing response results in fibrosis, similar to the chronic activation of the wound healing response in the liver.

1.2.3 Transplant vasculopathy

Another serious fibrotic condition presents in chronic transplant vasculopathy (CTV). The two most common types of CTV occur in cardiac transplants, termed cardiac allograft vasculopathy (CAV), and renal transplants, termed renal allograft vasculopathy (RAV). In CAV fibrosis of the neo-intima results in narrowing of the vessels. Fibrosis in CAV is diagnostic of a failing heart transplant and decreasing transplant recipient survival, and accounts for 30% of deaths 5 years after transplant. CAV is seen in 43% of cardiac allograft recipients after 8 years [5]. Fibrosis in RAV occurs in the arteries, glomerular tufts, and peritubular capillaries [6]. Accumulation of myoFB and fibrillar collagens will ultimately lead to renal allograft failure; similar to CAV.

Chronic transplant vasculopathy (CTV) is allograft rejection due to fibrosis of the transplanted organ. CTV in cardiac as well as renal allografts leads to failure of the transplanted organ and may lead to death. Cardiac allograft vasculopathy (CAV) leads to cardiac fibrosis and ultimately sudden death, silent myocardial infarction, or heart failure. Unlike the prior mentioned models of fibrosis, the immune response plays a crucial role in CAV. One of the characteristics of CAV is mononuclear cell infiltration and accumulation of vascular smooth muscle cells (VSMCs) in the neo-intima of the mid and dorsal coronary vessels. In addition there is accumulation of fibrillar collagens, collagen type I, III, and IV in the neo-intima. These are all diagnostic of intimal thickening. As rejection progresses so does cardiac stiffness. This is thought to be related to cellular infiltration and differentiation of FB to myoFB [7]. This response in allograft rejection is called intimal hyperplasia.

1.2.4 Wound Healing

Normal wound healing progresses through the four phases, hemostasis, inflammation, proliferation, and remodeling without pause, but still results in scar formation. When wound healing is aberrant ulcers, chronic wounds, keloids, and hypertrophic scars can develop. Keloids and hypertrophic scars are dermal proliferative disorders which occur following trauma, inflammation, surgery, and burns. The over exuberant scarring that occurs in keloid and hypertrophic scars results typically in disfigurement, contractures, and physical and psychological pain. Hypertrophic scarring is characterized as raised, red, fibrous lesions, which typically remain within the original wounded area, and usually undergo at least partial spontaneous resolution over long periods of time [8]. Hypertrophic scars unlike keloids can also be excised and treated with vascular-specific laser irradiation which can improve the persistent redness present in most hypertrophic scars [8]. Keloids, however, can enlarge and extend beyond the margins of the original wound, rarely regress, and have a significant increase in the FB population when compared to normal skin [9]. Researchers have identified a genetic predisposition but, the specific gene has not been determined [9]. Keloids can occur following minimal skin trauma and result in physical pain due to contractures and psychological stress [9]. Contractures generally limit the functional mobility of the patient not to mention the psychological implications of severe contractures [10]. Lastly, and arguably most important clinically, scarring can also result in wound dehiscence. The basic science behind wound dehiscence is not completely understood. Some researchers and physicians relate it to the reduced tensile strength of scar tissue due to distinct differences in collagen composition and cross-linking [10]. Excessive production

and deposition of extracellular matrix (ECM), in particular, type I and III collagen, FB hyperproliferation and apoptosis resistance, and differentiation into myoFB, and a decrease in collagen degradation have all been identified in hypertrophic and keloid scars and more importantly in a normal healing wound. Another hallmark of fibrosis is apoptosis or cell death. In particular, we consider the possible role of apoptotic cells as drivers of the fibrotic process.

1.3 Apoptosis

Apoptosis is nature's pre-programmed form of cell death. Apoptosis occurs throughout development, when damaged tissues are repaired, and as an ongoing event when tissues turn over in the human body. The classical understanding of apoptosis suggests that it provides a benign means for the necessary clearance of cells that are no longer needed or no longer functional. The attendant model holds that apoptotic cells do not elicit an immune response nor have any effect on surrounding cells. As described later, recent findings now challenge this paradigm of apoptosis as a process without consequence to neighboring tissue.

1.3.1 Pathways in apoptosis

There are 2 main pathways that initiate apoptosis, termed intrinsic and extrinsic. Extrinsic signaling pathways involve transmembrane death receptors of the tumor necrosis factor (TNF) receptor gene superfamily, FasR, TNFR1, and death receptor (DR) 3, 4, and 5 all contain a death domain that transmits the death signal from the cell surface to intracellular signaling pathways. In the mitochondrial or intrinsic pathway, the balance of pro- and anti-apoptotic Bcl-2 (Bcl-2 inhibits apoptosis and Bax promotes it) family proteins as well as caspase-2 determines cytochrome c release from

mitochondria. There are numerous intracellular mediators for both pathways that are necessary for formation of death receptor complexes and caspase cleavage and activation. Once inside the cell the caspases involved in apoptosis can be broken down into 3 broad categories: initiators (caspase-2,-8,-9,-10), executioners (caspase-3,-6,-7) and inflammatory caspases (caspase-1,-4,-5) [11, 12]. Caspase 3 is the classical caspase for all apoptosis pathways. Specifically, the extrinsic pathway recruits TNFR associated death domain (TRADD), fas associated death domain (FADD), and caspase 8; signaling is propagated by the cleavage and activation of caspase 8 which cleaves and activates caspase 3. While activation of the intrinsic pathway by mitochondrial membrane disruption releases cytochrome c. Cytochrome C binds to the cytosolic protein, apoptosis protease-activating factor-1 (APAF-1), which recruits caspase-9 for cleavage and activation. Caspase-9 in turn cleaves and activates caspase 3 similar to the extrinsic pathway. (Figure 1)

1.3.2 Mechanism of caspase activation

All caspases are originally produced in cells as catalytically inactive zymogens. Effectors caspases (Caspases 3, 6 and 7) are activated by proteolytic cleavage by initiator caspases (Caspases 2, 8, 9, and 10) at internal X-GLu-X-Asp residues that separate the effector caspases into a large and a small subunit [13]. Recent studies have identified that cleavage of the initiator procaspases is neither necessary nor sufficient to induce downstream caspase activity and cleavage of the executioner caspases [14]. Initiators are autoactivated via dimerization of zymogen monomers. Caspase 1 for instance is activated when a homodimer of two large and two small subunits is formed. Other initiator caspases are autoactivated by the protein-protein

interaction motifs on the death effector domains, or the caspase activator recruitment domains. Mature caspase 9 is as active as pro-caspase9 because of proximity induction to the apoptosome [13]. Recent studies have identified that the apoptosome and DISC merely stabilize the initiator homodimers for downstream cleavage and activation of the executioner caspases. Executioner caspases exist as inactive dimers in the cytosol and are only activated after cleavage from the initiator caspases.

1.4 Potential mechanisms for apoptotic cell involvement in fibrosis

For decades apoptotic cells were thought to be inconsequential to the final outcome of processes such as wound healing, and viewed simply as a necessary occurrence in the function of the human body. This model has recently been challenged, as apoptosis has been identified as a key player in the initiation, propagation, and resolution of organ fibrosis. Nearly all types of fibrosis exhibit high levels of apoptosis, and multiple possibilities by which these cells might dictate fibrotic outcomes have been suggested. The three most notable pathways that dictate the severity of the fibrotic outcomes include apoptosis resistance of FB and myoFBs, increased myoFB differentiation, and increased FB and myoFB proliferation. These effects can be conferred from apoptotic cells via secreted factors resulting from macrophages, neutrophils, and other leukocytes engulfment of apoptotic cells, and direct paracrine signaling from early and late apoptotic cells themselves (Figure 2).

1.4.1 Immune modulation – Indirect signaling

The modulation of the immune response by apoptotic cells is a concept that has gained support somewhat recently. Investigators have identified that phagocytosis of apoptotic cells by macrophages, a process termed efferocytosis, can define the

inflammatory state of macrophages and the resolution of inflammation in general. Most of the research on this topic has been to identify the initiating event in the macrophage phenotype switch from pro-inflammatory to pro-healing. During this switch macrophages begin secreting different cytokines and growth factors. However, yet another possibility is a pro-fibrotic phenotype where the secreted cytokines and growth factors contribute to the severity of the fibrotic outcome via FB and myoFB proliferation, myoFB differentiation, and FB and myoFB apoptosis resistance. Macrophages with the pro-healing phenotype are known to secrete lower levels of TNF alpha, IL-6 and increased levels of IL-10, TGF-beta1, and insulin-like growth factor 1 [15-17]. Importantly, IL-10, TGF-beta1, and insulin-like growth factor 1 have also been show to promote cell survival in the surrounding cells. In doing so, these signals may also be initiating a pro-fibrotic response by conferring apoptosis resistance and promoting proliferation in FB and myoFB and encouraging differentiation of FB and myoFB.

1.4.2 Secreted factors – Paracrine/Direct signaling

In addition to the modulation of macrophage function, recent studies suggest that apoptotic cells may act directly upon FBs and other cell types, enhancing cellular proliferation and pro-fibrotic phenotypes. The connection between fibrosis and EC apoptosis has been shown in cardiac and renal allograft rejection, pulmonary fibrosis and now in the skin [18-26].Recent studies suggest that apoptotic ECs can increase focal adhesion formation and gene expression of alpha-smooth muscle actin (alpha-SMA), a myoFB marker, in *in vitro* FB culture. The fibrotic effect of this CM was demonstrated both *in vivo* and *in vitro*, and was shown to derive from the presence of connective tissue growth factor (CTGF), a protein that has been previously implicated in

fibrosis [23]. These studies suggest that apoptotic EC (and perhaps CTGF) might mediate critical paracrine signals that influence fibrosis. However, the study was limited by the mode of apoptosis induction, serum starvation, and the use of human umbilical vein ECs (HUVECs) as opposed to a more globally relevant cell type. Nonetheless, these studies propose paracrine signaling mechanisms from apoptotic ECs and even a possible factor, CTGF, for the initiation of the fibrotic response *in vivo* and *in vitro*.

1.4.2.1 CTGF

CTGF is made up of 4 highly conserved structural modules; insulin-like growth factor binding protein (IGFBP) domain, von Willebrand type C repeats (vWC) domain, the thrombospondin type 1 repeat (TSR) domain, and a C-terminal domain (CT) with a cysteine knot motif. CTGF is capable of interaction with VEGF through TSP1 and cysteine knot modules, the IGFBP module interacts with IGFs; and the vWC module interacts with members of TGF-beta family and BMP to regulate cell proliferation and differentiation [27]. Given this wide array of interactions, it comes as no surprise that CTGF is capable of facilitating activation of many different signaling pathways [27]. Elevated levels of CTGF have been identified in human sclerotic, keloid and other fibrotic lesions [28, 29], liver fibrogenesis [30], and in bleomycin-induced and idiopathic pulmonary fibrosis [31]. Aberrant levels of CTGF were observed in liver fibrosis associated with hepatitis C infection [32]. CTGF has also been identified as critical to the development and maintenance of keloids, kidney fibrosis, and atherosclerosis [28, 33, 34].

1.4.2.2 CYR61

Also known as CCN1, CYR61 is a member of the CCN family of ECM associated signaling proteins [35, 36]. Like CTGF, CYR61 is known to associate with integrin receptors on ECs, macrophages, monocytes and platelets, and heparin sulfate proteoglycans on FBs and smooth muscle cells, and is important during development and embryogenesis and in inflammation and tissue repair [37-42]. With the varied binding partners, the downstream effects are cell type specific, but CYR61 promotes adhesion in all of them. In ECs CYR61 is known to be pro-angiogenic, while in FBs it increases cellular senescence and increases the capacity for TNF-alpha induced apoptosis [43-45]. In wound healing CYR61 is highly expressed by myoFBs and when high enough levels are accumulated in the developing ECM the secreted CYR61 has been shown to induce myoFB senescence and apoptosis [46, 47]. CYR61 is also elevated infarct zones following myocardial infarction [48]. In IPF, CYR61 was found to begin upregulation after lung injury and return to normal levels, however the combination of CYR61 and TGF-beta treatment increased alpha-SMA expression in pulmonary FBs in vitro [49]. Interestingly in renal pathologies CYR61 is decreased compared to normal, healthy tissue levels [50]. In vascular systems similar to chronic transplant vasculopathy, CYR61 is overexpressed in neointimal fibrosis [48, 51-54]. Extrinsic apoptosis pathway activators, TNF-alpha, FasL, and TRAIL activity is increased in the presence of CYR61 [43, 55, 56]. The M1 (pro-inflammatory) phenotype of macrophages is also perpetuated when CYR61 is overexpressed [37].

1.4.2.3 HSP47

Collagen synthesis is upregulated in all models of fibrosis. In order for the procollagen protein that is synthesized to be effectively utilized by the cell it undergoes a series of posttranslational modifications. HSP47 acts a molecular chaperone that facilitates interactions between procollagen and its posttranslational modifiers, aiding in folding, assembly, and transport from the endoplasmic reticulum, and potentially in the ultimate secretion of mature collagen [57, 58]. HSP47, a 47kDa serpin, or serine protease inhibitor [59], is known to interact with procollagen at the Pro-Pro-Gly peptide sequence [60]. HSP47 preferentially binds triple helical collagen over monomers [60-62]. In CCl4 induced liver fibrosis, bleomycin-induced pulmonary fibrosis, and age-associated renal fibrosis, HSP47 is concomitantly upregulated with collagen [63-66]. In a study using antisense for HSP47, bleomycin-induced pulmonary fibrosis measured by collagen accumulation was attenuated [64]. In keloids, HSP47 was upregulated in addition to collagen I and collagen III upregulation, suggesting a role in dermal fibrosis as well [67].

1.4.2.4 STMN1 and other factors

Stathmin I was identified as a secreted protein in the conditioned media from apoptotic ECs that was not secreted by non-apoptotic cells (Chapter 4). Stathmin 1 is a known microtubule destabilizing protein. In its active form, stathmin binds two molecules of free tubulin and prevents these molecules from participating in microtubule formation; it also promotes microtubule destabilization at the ends of each microtubule [68-70]. Stathmin has also been identified in cancer. In a mutated form it promotes cellular proliferation because it is unable to bind to free tubulin or regulate microtubule

dynamics, therefore the cell progresses through the cell cycle unregulated, much like cancer cells [68]. Very little is known about stathmin's contribution to the pro-fibrotic outcomes seen in its presence. However, unregulated cellular proliferation much like cancer cells could lead to increased fibrosis through steady-state collagen synthesis by an increased number of cells[71, 72].

Other factors associated with fibrosis include the TGF-beta family of secreted proteins which range from bone morphogeneic proteins, activins, and the transforming growth factor beta's themselves. The Th-2-type cytokines (IL-4, 5, 13, and 21), chemokines (MCP-1), pro-angiogenic factors (VEGF), growth factors (PDGF, TNF-alpha), peroxisome proliferator activated receptors (PPAR-gamma), caspases, and angiotensins (ANG2) [73]are also known promoters of fibrosis [73, 74]. All of these factors have also been examined in the aforementioned models of fibrosis and all are found to play an active role in the progression of fibrosis.

1.5 Sources of apoptotic cells in fibrotic responses

As previously mentioned, apoptotic cells may promote fibrosis through direct (paracrine - from apoptotic cells) or indirect (immune modulation - from macrophages) signaling. The sources of apoptotic cells depend largely on the tissue type where fibrosis is occurring. This section identifies several cell types that are known to contribute to the apoptotic cell load and the roles these cells play in the development of the fibrotic response.

1.5.1 Inflammatory cells

The presence and persistence of apoptotic cells may contribute to the fibrotic outcome via immune modulation and paracrine signaling, but identification of the source

of the apoptotic cells is also important. Several studies have identified apoptotic inflammatory cells contributing to fibrotic lesions [75, 76]. In a mouse model of lung fibrosis introduction of apoptotic macrophages via intratracheal administration resulted in increased macrophage recruitment and increased collagen deposition and contributed to the overall induction and propagation of pulmonary fibrosis [75, 76]. In early wound healing, the hemostasis and inflammatory phases, the highest level of apoptotic cells is neutrophils [12]. These cells undergo apoptosis and recruit more inflammatory cells, specifically macrophages, to the wound bed ensuring the movement into the inflammatory stage. Throughout the processes of wound healing, macrophages are recruited to the site of injury and undergo apoptosis as well. The precise mechanism of apoptosis in wound healing has not been determined, but the downstream effects are thought to promote exit from the inflammatory stages into the proliferative phases [12, 77]. Immune cells are involved in the clearance of apoptotic cells. The inflammatory state of macrophages (M1- pro-inflammatory, M2- pro-healing) and the resolution of inflammation can be determined by the amount and type of apoptotic cells macrophages are engulfing. [78]. Another potential state of macrophages is pro-fibrotic. In this state, macrophages potentially contribute to the severity of the fibrotic outcome by modulating the amount and type of collagen synthesized, and the degradative potential of the surrounding FBs.

1.5.2 Endothelial cells

As the wound progresses out of the proliferative phase FBs and ECs from abundant proliferation and vascularity also undergo apoptosis for efficient elimination from the wound bed [12, 79]. A vigorous angiogenic response is frequently associated

with fibrotic outcomes, and anti-angiogenic therapies have been proposed to modulate fibrosis. The role of dynamic angiogenesis in fibrosis and scar formation has been generally considered to involve the need for oxygen and nutrient delivery to support fibrotic processes. Recent studies in wound healing, however, suggest that the angiogenic response may influence fibrosis via the generation of apoptotic EC [80-83]. As the angiogenic process resolves, the vascular bed is pruned back via apoptosis of ECs. The resolution of robust angiogenesis thus creates a significant apoptotic burden. The increased levels of apoptotic cells may contribute via immune modulation and paracrine signaling to the resulting fibrotic outcomes.

1.5.3 Apoptotic cellular involvement in initiation, propagation, and resolution of fibrosis

Apoptosis in the initiation of liver fibrosis is characterized by high levels of apoptotic hepatocytes. Interestingly, the hepatic stellate cells (HSC) are induced to develop into myoFB like cells in proximity to the apoptotic hepatocytes. Fas and CD40, extrinsic apoptosis signaling receptors, are upregulated in the development and persistence of liver fibrosis [84]. Importantly, activated HSCs are resistant to apoptosis especially after sustained activation [85]. Apoptosis resistance aids in maintaining the pro-fibrotic environment and sustains the presence of activated HSCs. Inducing apoptosis of HSCs has been a major therapeutic target in liver fibrosis, and has been described as the major impediment to resolution of liver fibrosis [3, 86]. Interestingly in non-alcoholic fatty liver disease (NAFLD) and nonalcoholic steatohepatitis (NASH), hepatocyte apoptosis is increased and the response is inflammatory. After the initiation of inflammation and activation of HSCs cirrhosis and fibrosis develop [87-89]. In this

situation there is no toxin to remove to reverse the fibrotic response. Therefore, in some cases of liver fibrosis, specifically NAFLD and NASH, the initiation of fibrosis may be related to the apoptotic load of hepatocytes in the liver which are above and beyond the phagocytic clearance levels and therefore can directly contribute to fibrosis, via paracrine signaling to the surrounding HSCs.

High levels of endothelial and alveolar epithelial apoptosis in lung fibrosis occur before, during, and after chronic activation of the wound healing response. Most researchers agree that the presence of apoptotic cells initiates the wound healing response and ultimately the fibrotic response. However, there are several other hypotheses that relate apoptosis to the progression of lung fibrosis: 1) apoptosis resistant FBs and myoFB perpetuate the fibrotic response or, 2) impaired apoptotic cell clearance by macrophages results in the release of the potentially toxic or pro-fibrotic cellular contents of apoptotic cells and pro-fibrotic activation of FBs [90, 91]. In experimental models of lung fibrosis, such as bleomycin induced fibrosis, the fibrotic response can be bolstered by concurrent administration of apoptotic cells and fibrosis can be induced by exposure to apoptotic cells alone [75]. Another study used an antibody that constitutively activated the Fas receptor ligand binding pathway, activating the extrinsic apoptosis pathway, which also resulted in a fibrotic response. Interestingly, in bleomycin induced lung fibrosis there is increased expression of Fas on alveolar epithelial cells [92, 93] suggesting that the extrinsic pathway is activated in chemically induced lung fibrosis. This research supports the hypothesis that the presence of apoptotic cells in the injured lung is sufficient to induce a fibrotic response. Interestingly, the other side of this hypothesis, is apoptosis necessary for induction of fibrosis, has not

been examined as extensively. Two studies have examined the necessity of apoptosis in the induction of lung fibrosis. The same study that examined administration of apoptotic cells and concurrent administration of apoptotic cells with bleomycin also examined the fibrotic response after bleomycin induced lung fibrosis and blocking the extrinsic apoptosis pathway with ANG-converting enzyme inhibitor or the pan-caspase inhibitor Z VAD-fmk [75]. The results were consistent with those from Kuwano et al., who used aerosol administration of Z VAD-fmk in bleomycin induced lung fibrosis [94]. Both studies showed significant reduction in the fibrotic response with concurrent administration of Z VAD-fmk, and subsequent downstream inhibition of apoptosis.

Histologically, apoptotic alveolar epithelial cells have been found in close proximity to the heaviest myoFB activity and collagen accumulation in both clinical cases of IPF and in experimental models of bleomycin induced pulmonary fibrosis. There is no doubt that apoptosis resistant FBs and myoFBs contribute to the pathogenesis and progression of lung fibrosis. This observation supports the hypothesis that cell type specific apoptosis contributes to the fibrotic response differently. In a study that utilized dexamethasone to induce apoptosis of macrophages, neutrophils, and other leukocytes in the bleomycin model of lung fibrosis. The authors found that the fibrotic response was significantly reduced [95], while other researchers argue that apoptosis of FB and myoFB may be necessary for resolution of fibrosis. Lastly, the effect of alveolar epithelial cell apoptosis has been studied to determine the role it plays in lung fibrosis. Researchers have identified several signaling pathways that control alveolar epithelial cell apoptosis: Ang-converting enzyme inhibitor which blocks Fas signaling, TNF-alpha, or antisense oligonucleotides for ANGEN mRNA [76, 96].Taken

together these observations and results suggest that apoptosis is the initiating event in lung fibrosis, and apoptosis may occur via extrinsic signaling pathways, specifically, TNF-alpha or Fas receptor signaling.

Apoptosis has been correlated with the initiation and propagation of the fibrogenic reaction in CTV. In both CAV and renal allograft vasculopathy (RAV) EC apoptosis is the initiator of hyperplasia of the neo-intima and the downstream fibrotic response seen in CTV. In any transplant the ECs lining the blood vessels of the donor organ are the first cells that directly contact the host's immune system [97]. Fas signaling has been implicated in graft-versus-host immune mediated vascular injury. The circulating immune cells of the host present the ECs lining the vasculature in the donor organ with Fas ligand. Recent studies have shown that blocking Fas signaling prevents vascular fibrogenesis [98]. Also, when apoptosis of ECs is blocked the capacity for immune cell infiltration is maintained, but there is significantly less fibrogenesis [99]. Another group has looked at sustained levels of apoptotic EC, they found that maintaining a sustained level of apoptotic ECs correlated with the development of CAV in heart transplant in pigs [6]. Alpha-SMA positive cells accumulate in close proximity to apoptotic ECs in the neo-intima and acquire apoptotic resistance, very similar to what has been observed in other models of fibrosis. In CTV, EC apoptosis plays a central role in the progression of vasculopathy and fibrogenesis by inducing a hyperadhesive and thrombogenic state in the interior of the vessels of the donor organ. In addition, many of the apoptotic ECs are phagocytized by macrophages; macrophages increase the production of TGF-beta in close proximity to the vessel wall and likely aids in initiation of the neo-intima formation. Lastly EC apoptosis can trigger

proteolysis and production of fibrogenic mediators in the surrounding tunica intima and tunica media which sends out recruitment signals for FBs and encourages FB to myoFB differentiation.

Some of the most suggestive evidence for a role of apoptotic cells in fibrosis comes from studies of healing wounds. During wound healing apoptosis maintains the homeostasis of the wound environment by balancing cell elimination with cell proliferation. Early apoptosis in wound healing involves primarily neutrophils and macrophages, cells that are eliminated as inflammation resolves. In the later phases of repair, both FB and ECs undergo apoptosis during the remodeling phase. However, recent studies suggest apoptotic ECs are the predominant apoptotic cell type in the resolving wound.

1.6 Angiogenesis

Very broadly, angiogenesis is the formation of new blood vessels via proliferation of resident ECs [100, 101]. Vasculogenesis is characterized by the growth of new vessels via incorporation of circulating bone marrow-derived endothelial progenitor cells (EPCs) at the site of new growth [100, 101]. For the purposes of this dissertation the term angiogenesis includes the classical definition of angiogenesis and vasculogenesis. Angiogenesis is a complex process that involves pro-angiogenic factor binding on the endothelial cell surface followed by degradation of the basement membrane, proliferation and “sprouting” of new vessels from the original larger vessel. These sprouts are generally transient and will collapse after the original pro-angiogenic stimuli are removed or when the pro-angiogenic stimuli are outweighed by anti-angiogenic stimuli.

1.6.1 Factors involved in regulating angiogenesis

Normal vasculature is maintained by balancing pro- and anti-angiogenic factors. When an event like the creation of a wound occurs, the balance is immediately shifted toward formation of new vessels through an increase in the pro-angiogenic stimuli. These stimuli include VEGF, bFGF, FGF-2, PDGF, CCN1, CCN2, TGF-beta and Angiopoietins [102-104]. When these factors are produced in quantities that outweigh the activity of anti-angiogenic mediators the result is proliferative, new blood vessel formation. As the wound resolves and the pro-angiogenic stimuli are no longer being produced at high levels, upregulation of anti-angiogenic stimuli will result in vessel collapse and regression. The known anti-angiogenic factors are TSP1 and 2, PEDF, endostatin, vasostatin, and IP10 and the upregulation of these factors are associated with increase vessel collapse, vessel regression, and endothelial cell apoptosis [102, 103].

1.6.2 Angiogenesis in Fibrosis

Many of the signaling pathways in angiogenesis overlap with those in fibrosis [105]. Recent literature identifies a linkage in many models of fibrosis between pathologic angiogenesis and the initiation and propagation of fibrosis. These common pathways play critical and differential roles in angiogenic proliferation and stability and the development of fibrosis in the liver, lung, heart, kidney and skin.

Recent studies, literature, and clinical observations have identified angiogenesis in the liver as a favorable condition for fibrogenesis. This has been identified as an aberrant angiogenic response similar to that seen in the wound healing response in the skin. Upregulation of angiogenesis is concurrent with increased VEGF expression and

hypoxia-signaling from hepatocytes being starved for nutrients by the activation of HSCs and the transition of sinusoidal to contiguous vessels, termed capillarization. During the resolution of fibrosis in the liver not much is known about vessel regression. Unfortunately angiogenesis and vessel regression in liver fibrosis are very complicated because the normal vasculature in a healthy liver is sinusoidal. The chronic wound healing response is activated and capillarization of sinusoidal vessels occurs simultaneously with angiogenesis and fibrogenesis, and as fibrosis is resolved there is a decrease in capillarization, angiogenesis, and fibrosis. Therefore, the general hypothesis is that the development liver fibrosis is related to angiogenesis during fibrogenesis and that the ingrowth of vessels contributes to the fibrotic response. This hypothesis is supported by the increased VEGF production in the activated HSC and myoFB like hepatocytes which would be chemotactic for new vessel growth [106]. The conflicting hypothesis that as fibrosis progresses angiogenesis is responding to the cellular phenotype changes is not as extensively supported. However, it is worth examining

Angiogenesis in experimental lung fibrosis is characterized by an increase in blood vessel density. Clinical observation has shown increased vascular remodeling in the normal parenchyma while the fibrotic regions have fewer vessels, similar to that seen in the fibrotic outcomes of the wound healing response, liver fibrosis, and tumorigenesis. The fibrotic regions contain both apoptotic as well as proliferating ECs and result in aberrant vascular architecture and anastomoses [107]. The changes in vascular architecture can be attributed to an imbalance of pro-angiogenic and anti-angiogenic mediators [108], [109], [110]. Expression of some of the mediators that

related to the control of angiogenesis in lung fibrosis have been examined; results have shown increased angiotensin 2, angiostatin, PEDF, TGF-beta, endostatin, and decreased CXCL8, VEGF, FGF-2, FGF-2, and angiotensin 1. Therefore, the increase of vascular remodeling can be attributed to dynamic pro-anti- angiogenic mediators. Additionally, the reduction in the ability of fibrotic lung tissue to induce an angiogenic response in vitro is likely due to the decrease in VEGF expression. Several studies have suggested that following epithelial cell damage or injury the mediators that are released such as TGF-beta, PEDF, and decreased VEGF have a pro-fibrotic effect via increases in ECM deposition and myoFB differentiation, while at the same time these factors may lead to EC injury and apoptosis which results in vessel loss and/or vasoconstriction. The other outcome of the EC injury could be EC proliferation from the aberrant growth factor regulation which results in vascular remodeling such as intima proliferation, plexiform lesions, media hypertrophy, and adventitia fibrosis all resulting in pulmonary hypertension and exacerbating the pulmonary fibrotic response [110].

Chronic transplant vasculopathy leads to transplant failure and caused by an increase in fibrosis and is initiated by an increase in the level of apoptotic ECs. Given the two similarities to the wound healing response the question becomes what is the angiogenic response in CTV? CTV has been characterized by an increase in the expression of adhesion molecules, chemokines, and growth factors, specifically VEGF [111-116]. In both CAV and RAV neovascularization and angiogenesis take place as the vasculopathy progresses to fibrosis and organ failure [114]. The angiogenesis that occurs in CTV is not related to hypoxia, but rather related to leukocyte-associated angiogenesis [113]. One study found increased capillary density was associated with

immune cell infiltration into the parenchyma of cardiac transplants [117]. Another study examined the role angiogenesis plays in the progression and ultimate outcome of CTV. This study found that angiogenesis was not associated with the initiation of CAV, but is associated with the progression. This group inhibited EC proliferation concurrent with the original transplant or later as CAV was developing. They found that concurrent inhibition had no response and that if EC proliferation was inhibited later CAV progression was halted [111]. Another group used endostatin, a physiologic angiogenic regulator, to determine its effect on the outcome of CAV and found that administration of endostatin inhibited angiogenesis and halted the progression of CAV in mice [113]. Similarly in renal allograft vasculopathy and rejection there is an increase in the vascular density and VEGF expression. Several studies suggest that the control of vascular density may also be related to increased expression of other angiogenic mediators or decreased expression of anti-angiogenic mediators [111, 112, 114].

The modulation of vessel density is an important component of wound repair; angiogenesis peaks during the proliferative phase and decreases gradually into the remodeling phase, termed vessel regression. As the wound progresses through the remodeling phase, there is a shift from a predominance of pro-angiogenic mediators to anti-angiogenic mediators [81, 83]. When the anti-angiogenic mediators outweigh the pro-angiogenic mediators the result is increased levels of apoptotic ECs and vascular regression [80, 82]. The balance between pro and anti-angiogenic mediators determines the apoptotic level of ECs in wound healing [80, 82]. Since persistent and high apoptotic loads are related to the etiology of several other types of fibrosis, it follows that the increase in the apoptotic load during vessel regression in normal wound

healing may influence FB function, effectively preventing perfect regeneration. The end result would then be a fibrotic scar.

1.6.3 Decreased angiogenesis in wound healing

Recent studies by us and others now suggest that a reduction in angiogenesis may actually be beneficial to healing outcomes. While this vigorous response was once thought to be essential for tissue repair, we and others have shown that a partial blunting of the angiogenic response does not impair wound closure. For example, neutralization of vascular endothelial growth factor (VEGF) via antibody treatment has been used to reduce the angiogenic response in adult skin wounds by about 50%.(unpublished data Guo, 2010) These treated wounds close normally. More importantly, this artificial reduction in wound angiogenesis yields a reduction in scar formation, and an outcome more closely resembling regeneration. The results from this study support the hypothesis that reducing angiogenesis also reduces the apoptotic load that occurs during vascular regression and the reduction in the apoptotic load may lead to reduced fibrosis.

1.6.4 Increased angiogenesis in wound healing

Additional evidence for a role for angiogenesis in wound scar formation comes from studies of a very specific and unfavorable outcome, that of hypertrophic scars. Hypertrophic scarring is characterized as raised, red, fibrous lesions, which typically remain within the original wounded area, and usually undergo at least partial spontaneous resolution over long periods of time. Hypertrophic scars can occur following minimal skin trauma, and result in physical pain and limited mobility due to contractures in addition to psychological stress due to the physical appearance of the

scar. Because the collagen architecture of hyptertrophic scars is abnormal, the scar tissue has less strength than normal skin, which contributes to high levels of wound dehiscence. Due to limited therapeutic treatments, hypertrophic scars continue to be a significant clinical problem. A link of robust angiogenesis with hypertrophic scars has now been made. Hypertrophic scars were recently demonstrated to be associated with higher levels of angiogenesis, suggesting scar formation will be reduced by anti-angiogenic therapy [88] [91, 118].

1.7 Linking Angiogenesis, Apoptosis, and Fibrosis

Modulation of vessel density is an important component of wound repair; angiogenesis peaks during the proliferative phase and decreases gradually into the remodeling phase, termed vessel regression. With this in mind it is important to note that the role of vascular regression in liver fibrosis, the only organ known to regenerate, has not been examined thoroughly. We believe that the vascular regression due to the chronic activation of the wound healing response is a very slow, controlled process and that in order to achieve regeneration in other models of fibrosis a slow, controlled apoptosis induction will be required, in addition to the induction of apoptosis in myoFB like HSCs. One mechanism of increased apoptosis in a healing wound is vascular regression. Our lab and others have identified that the typical angiogenic response in a normal healing wound is over-exuberant and unnecessary, and we have determined that blunting the angiogenic response results in a reduction in scar formation, and an outcome more closely resembling regeneration. There are several hypotheses regarding the reduction in scar formation resulting from the reduction in VEGF. The two most predominant relate either to the permeation of new vessels or the level of

apoptosis required during vessel regression. The first hypothesis, relates the improved scarring outcome to the reduction in the infiltration of vessels into the wound bed. The second hypothesis relates the reduction in blood vessel density to the improved scarring outcome via the ultimate reduction in the apoptotic load since the amount of vessel regression needed to return to normal vascular density is also reduced. Aberrant angiogenesis also occurs in hypertrophic and keloid scars characterized by a peak in angiogenesis much later in hypertrophic scars compared to normotrophic scars and an overall increase in blood vessel density over the time course of healing. Changes in vascularity or angiogenesis have also been identified in the previously discussed models of fibrosis.

Recent studies, literature, and clinical observations have identified angiogenesis in the liver as favorable for fibrogenesis. Researchers identified aberrant angiogenesis similar to that seen in the dermal wound healing response during the initiation and propagation of liver fibrosis. Upregulation of angiogenesis is concurrent with increased VEGF expression and hypoxia-signaling from hepatocytes. Hypoxic hepatocytes are oxygen and nutrient starved by the activation of HSCs and the transition of sinusoidal to contiguous vessels, termed capillarization. During liver fibrogenesis, the chronic wound healing response is activated and capillarization of sinusoidal vessels occurs simultaneously with angiogenesis and fibrogenesis. The role of vascular regression in the resolution of liver fibrosis has not been examined thoroughly. Unfortunately understanding angiogenesis and vessel regression in liver fibrosis is further complicated by the structure of the vessels in a healthy liver. Unlike normal vasculature in skin, the normal vasculature in a healthy liver is sinusoidal. Chronic activation of the wound

healing response in the liver results in contiguous vessels as opposed to the normal, leaky vasculature present in healthy liver. However, as fibrosis is resolved there is a concurrent decrease in capillarization, angiogenesis, and fibrosis.

Most researchers agree that the development of liver fibrosis is related to angiogenesis during fibrogenesis and that the ingrowth of vessels contributes to the fibrotic response. This hypothesis is supported by the increased VEGF production from activated HSC and myoFB-like hepatocytes which is chemotactic for new vessel growth [106]. However, to date no one has examined the influence of vessel regression in the resolution of liver fibrosis. An alternative hypothesis is that during the initiation and progression of liver fibrosis there is an increase in vessel density, but during the resolution of fibrosis the vessels slowly, and in a controlled manner undergo apoptosis. The low apoptotic load during the resolution of liver fibrosis contributes to the regenerative phenotype by maintaining a level of apoptosis that can be efficiently cleared by macrophages, promoting a pro-healing macrophage phenotype and preventing pro-fibrotic paracrine signaling from apoptotic cells.

Experimental lung fibrosis is also characterized by an increase in blood vessel density, in addition to heavy collagen deposition. Clinical observation has shown increased vascular remodeling in the normal parenchyma while the fibrotic regions have fewer vessels, similar to that seen in the fibrotic outcomes of the wound healing response, liver fibrosis, and tumorigenesis. The fibrotic regions contain both apoptotic as well as proliferating ECs and result in aberrant vascular architecture and anastomoses [107]. The changes in vascular architecture can be attributed to an imbalance of pro-angiogenic and anti-angiogenic mediators [108, 109, 119]. Expression

of some of the mediators that related to the control of angiogenesis in lung fibrosis have been examined; results have shown increased angiotensin 2, angiostatin, PEDF, TGF-beta, endostatin, and decreased CXCL8, VEGF, FGF-2, FGF-2, and angiotensin 1.

The increase of vascular remodeling can be attributed to dynamic pro- and anti-angiogenic mediators. Additionally, the reduction in the ability of fibrotic lung tissue to induce an angiogenic response in vitro is likely due to the decrease in VEGF expression. Several studies have suggested that following epithelial cell damage or injury, TGF-beta, PEDF, anti-angiogenic mediators, are released, and VEGF expression and production is downregulated. The regulation of the balance of pro and anti-angiogenic mediators may have a pro-fibrotic effect via increases in ECM deposition and myoFB differentiation. At the same time these factors may lead to EC injury and apoptosis which results in vessel loss and/or vasoconstriction. The other outcome of the vascular injury could be EC proliferation from the aberrant growth factor regulation which results in vascular remodeling such as intimal proliferation, plexiform lesions, media hypertrophy, and adventitia fibrosis all resulting in pulmonary hypertension and exacerbating the pulmonary fibrotic response [119].

1.8 Conclusion

In summary, apoptosis has been identified in several different models of fibrosis during initiation, progression, and/or resolution. Apoptosis that results from vascular regression could play an important role in the fibrosis as a result of activation of the wound healing response. The chronic activation of the wound healing response has been identified in liver, lung, CTV, and skin fibrosis. In liver fibrosis, apoptosis plays a critical role in the initiation and propagation of liver injury to liver fibrosis. In lung fibrosis,

apoptosis has been identified as the initiator of the fibrotic response and is both necessary and sufficient to induce a fibrotic response. In CTV, EC apoptosis as a result of graft versus host interactions is the initiator of the fibrotic response that will ultimately lead to transplant rejection. In wound healing, the role of apoptosis in the development of fibrosis is not well understood, however, fibrosis as a result of dermal wound healing can and should be compared to other models of fibrosis. Skin fibrosis, such as hypertrophic scars, is similarly characterized by excessive production and deposition of extracellular matrix (ECM), in particular, type I and III collagen, FB hyper-proliferation and apoptosis resistance, and differentiation into myoFB, and a decrease in collagen degradation.

One mode of modulating the apoptotic load in wound healing is regulation of vessel regression. Aberrant vessel regression and angiogenesis has already been identified in exaggerated fibrotic outcomes of wound healing such as hypertrophic or keloid scarring. The balance of pro and anti-angiogenic mediators controls angiogenesis and vessel regression and has been identified as pathogenic in liver fibrosis, lung fibrosis, and CTV. The chronic activation of the wound healing response and the imbalance of pro and anti-angiogenic mediators together may contribute to the increased apoptotic load and ultimately to fibrosis. The increased apoptotic load seen in vascular regression during normal wound healing may be necessary and sufficient to induce and promote the normotrophic scarring response, and aberrant angiogenic control may promote the formation of hypertrophic scars. In the future, by controlling vessel regression and the apoptotic load, researchers may be able to halt the chronic

activation of the wound healing response and promote tissue regeneration instead of tissue repair.

We hypothesize that in order to achieve regeneration, like the liver, other models of fibrosis such as a healing wound must also have a slow, controlled induction of vascular regression that results in a low apoptotic load. By controlling the induction of vascular regression we may be able to control the apoptotic EC load present during resolution, and thus the effect of the apoptotic load on the surrounding tissue.

1.9 Purpose of the Present Study

The purpose of the current study is to examine the role apoptotic cells play in the regulation of fibrosis in wound healing. **The hypothesis of this study is that apoptotic ECs contribute to fibrosis and scar formation by secreting factors that increase dermal myoFB differentiation, collagen synthesis, and FB proliferation, while decreasing collagen degradation in FBs.** The current goal of this research is to identify the contribution of apoptotic cells and specifically apoptotic endothelial cells arising from vessel regression and elucidate the mechanisms by which apoptotic ECs contribute to the final outcome of fibrosis. Our long-term goal is to identify secreted factors and signaling pathways involved in the initiation, propagation, and resolution of fibrosis and suggest therapeutic directions for the reduction of fibrotic scarring outcomes in wound healing and potential in other fibrotic tissues.

I. Establish a role for apoptotic EC as mediators of fibrosis *in vivo*.

A. *In vitro* and *in vivo systems* examine the relationship between EC apoptosis and FB function in fibrosis

B. Identify differential apoptosis in models of improved healing (mucosal) and normal healing (dermal).

II. Determine the mechanism of interaction between apoptotic ECs and FB function.

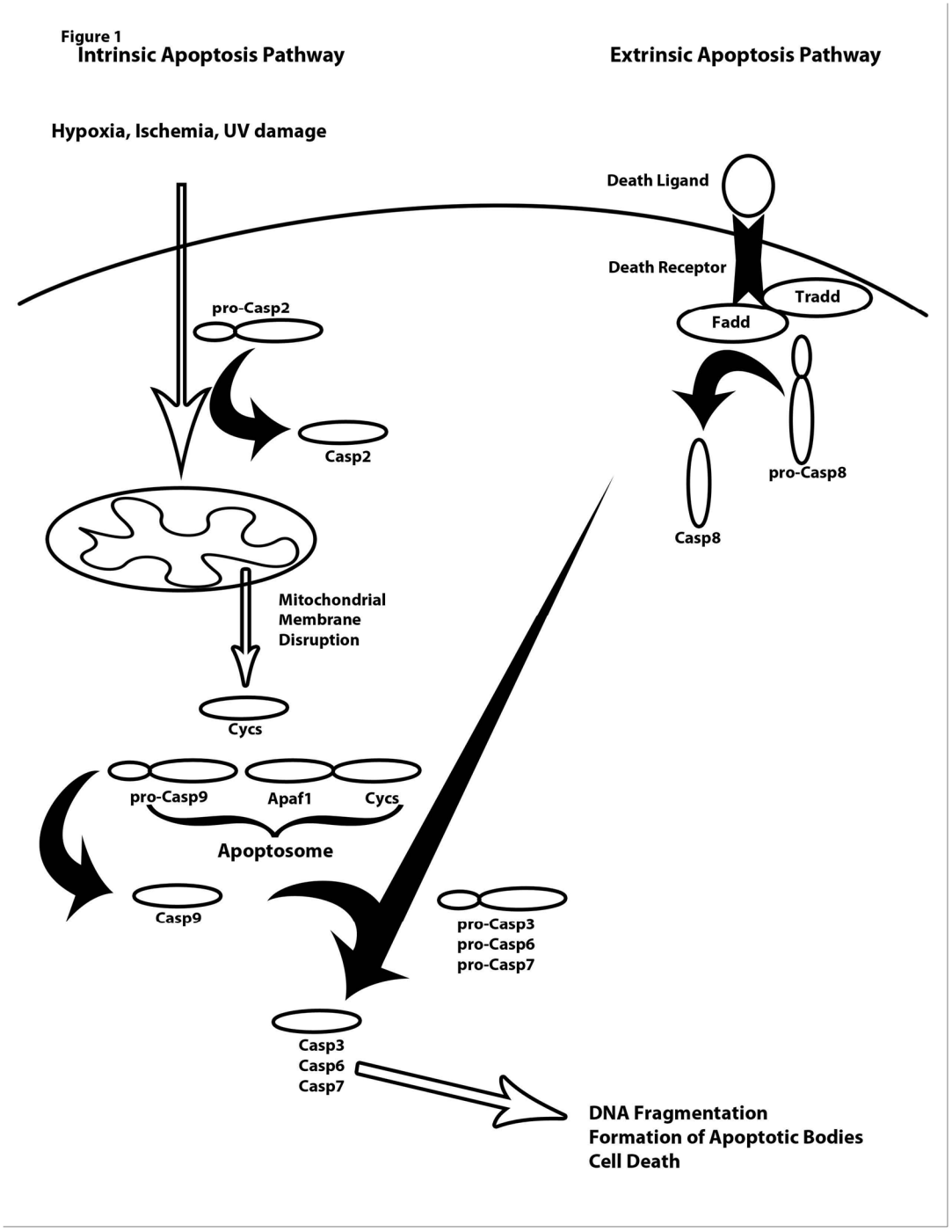
A. Examine the interaction between apoptotic ECs, secreted factors from apoptotic ECs, and FBs *in vitro* using a conditioned media treatment model.

B. Identify secreted factors in the conditioned media from apoptotic ECs and the effect of siRNA knockdown in pre-apoptotic ECs on collagen synthesis and degradation *in vitro*.

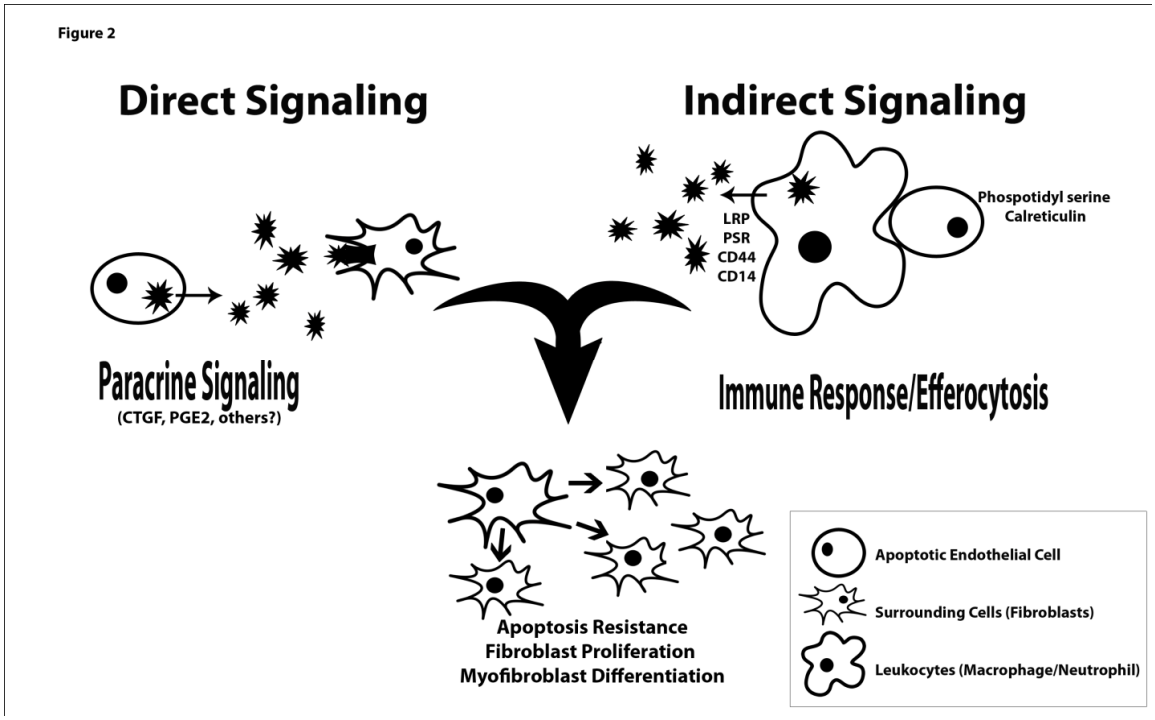
1.10 Figures, Tables, Figure Legends

Table I
Common attributes of fibrosis in different models of fibrosis

	Wound Healing	Liver Fibrosis	Lung Fibrosis	CTV -CAV -RAV
Fibrosis				
○ **Collagen synthesis**	Increase	Increase	Increase	Increase
○ **Presence and persistence of fibrotic cells**	Increase	Increase	Increase	Increase
○ **Fibrotic cell type**	Myofibroblasts	Hepatic stellate cells	Pneumocytes	Myofibroblasts
○ **Fibrotic outcome**	Scarring	Cirrhosis	Idiopathic pulmonary fibrosis	Intimal hyperplasia
○ **Collagen Degradation**	Decrease	Decrease	Decrease	Increase
Sources of apoptotic cells	○ Neutrophils/ Macrophages (early) ○ Endothelial cells (late)	○ Hepatocytes	○ Endothelial cells ○ Alveolar epithelial cells	○ Cardiomyocytes ○ Donor organ endothelial cells
Angiogenesis	Higher than necessary	Increase	Increase	Increase



1.10.2 Figure 1 - Diagram of the intrinsic and extrinsic apoptosis signaling pathways (adapted from Johnson et al., 2013).



1.10.3 Figure 2 - Roles of apoptosis in fibrosis: Immune response modulation and paracrine signaling

Figure Legends

Table I – Common attributes of fibrosis in different models of fibrosis. This table depicts common attributes in fibrosis: collagen synthesis, fibrotic cells, fibrotic outcomes, and collagen degradation. In addition sources of apoptotic cells are outlined as well as the levels of angiogenesis, in each of the 4 models of fibrosis discussed extensively in this dissertation.

Figure 1.11.2 – Diagram of the intrinsic and extrinsic apoptosis signaling pathways (adapted from Johnson et al., 2013). Intrinsic apoptosis (left side) is usually the result of hypoxia, ischemia, or UV damage, but may also be induced via stress from low levels of growth factors. These damage signals are propagated through Casp2- and Trp53-mediated disruption of the mitochondrial membrane. Cytochrome C (Cycs) is released following mitochondrial membrane disruption and recruited to form the apoptosome with Apaf1 and pro-Casp9. Pro-Casp9 is cleaved and activates the caspase cascade resulting in cleavage and activation of Casp3, Casp6, and Casp7. The downstream effect of activation of the caspase cascade is DNA fragmentation, formation of apoptotic bodies, and cell death. The extrinsic apoptosis signaling pathway (right side) requires binding of the death ligand (FasL or TNF-α) to its respective receptor (FasR or Tnfrsf1b). Binding of the death ligand to the death signals the recruitment of Tradd, Fadd, and pro-Casp8. Pro-Casp8 is cleaved by the complex and begins the caspase cleavage cascade resulting in the cleavage and activation of Casp3, Casp6, and Casp7. Similar to the intrinsic pathway,

the result of the caspase cleavage cascade is DNA fragmentation, formation of apoptotic bodies, and cell death.

Figure 1.11.3 –Roles of apoptosis in fibrosis: Immune response modulation and paracrine signaling. Apoptotic cells, such as, apoptotic ECs can effect surrounding cells through paracrine signaling directly from the apoptotic ECs or through immune modulation of leukocytes that efferocytose apoptotic ECs. The downstream effects on the surrounding cells include apoptosis resistance, increased proliferation, and differentiation.

CHAPTER 2

Differential Apoptosis in Mucosal and Dermal Wound Healing

2.1 Introduction

Wound healing is a complex process that requires succinct yet overlapping phases of hemostasis, inflammation, proliferation, and remodeling. Our lab and others have extensively examined the differences between mucosal and dermal healing. The differences range from macroscopic differences in wound closure rates and scarring outcomes to the microscopic differences in inflammatory cell infiltrates and rates of re-epithelialization, and differential pro-healing and pro-angiogenesis protein production [120-123]. Mucosal healing has several key features that mimic regeneration. Mucosal wounds are faster to re-epithelialize, have a decreased inflammatory response, and have a blunted angiogenic response with concomitant reduction in vascular endothelial growth factor (VEGF) gene and protein expression [123]. Altogether, the phases of wound healing following mucosal injury are shortened in duration and generally have reduced gene and protein expression changes in comparison to skin wound healing [124].

Apoptosis is an important mechanism for cellular elimination during wound healing and maintains tissue homeostasis in normal, uninjured tissue. In a recent study, the overall gene expression of mucosal and skin wounds was compared via microarray analysis [124]. Among the multiple differences that were noted, the data suggested that wound healing in these two tissues might exhibit differential signatures of apoptosis related genes, such as tumor necrosis factor-hat wound healing in these two tissues might exhibit[124].

Apoptosis can be induced via two main pathways, termed intrinsic and extrinsic. The intrinsic pathway is related to DNA damage from UV light, chemotherapy, ischemia, and oxidative stress. The extrinsic pathway requires extracellular input, specifically, activation of the intracellular portion of the death receptor by binding of a death ligand. Apoptosis in general is associated with an intracellular caspase cleavage cascade. Caspases involved in apoptosis can be broken down into 3 broad categories: the initiators of apoptosis (caspase-2, -8, and -9), executioners of apoptosis (caspase-3 and -7), and inflammation-related (caspases-1, -4, -5, and -12) [12, 125, 126].

In the intrinsic pathway caspase-2 (Casp2), the balance of Bcl-2 and Bax, and the levels of p53 (Trp53) determine cytochrome-c (Cycs) release from mitochondria following mitochondrial membrane disruption. Cycs then forms the apoptosome with Apaf1, which cleaves and activates Casp9. The resulting caspase cleavage cascade ends with Casp3 cleavage and activation. Casp3 cleavage and activation represents the point of convergence for the intrinsic and extrinsic pathways and is the final step in initiation of cell death through further DNA fragmentation, and cleavage of cytoskeletal proteins (Figure 1).

The extrinsic apoptotic signaling pathway involves transmembrane death receptors of the tumor necrosis factor (TNF) receptor gene superfamily, FasR, TnfR1, and death receptors (DR) 3, 4, and 5. Upon binding of the death receptor ligand, Fas ligand (FasL) or TNF-α, the TnfR-associated death domain (Tradd) is activated and recruits Fas-associated death domain (Fadd) to the intracellular portion of the death receptor. This begins the intracellular signaling cascade of recruitment and cleavage of pro-caspase 8 (Casp8) and ultimately cleavage and activation of Casp3 (Figure 1).

The purpose of this study was to determine if differential apoptotic responses occur in oral and skin wound healing. Equally sized wounds from the oral mucosa and the dorsal skin were compared at 5 different time points (6h, 24h, D3, D5, and D7) over the course of wound healing for changes in gene expression of key factors in the apoptosis pathways. We hypothesized that apoptosis would be initiated through different pathways in the oral wounds compared to skin.

2.2 Clinical problem addressed

In the skin, fibrosis or scarring can vary from normal to hypertrophic scars, keloids, or painful contractures. Effective anti-fibrotic or anti-scarring treatments are currently limited. Oral wound healing, like fetal wound healing, closely resembles optimal healing with very rare occurrences of keloids or hypertrophic scars. Further examination of the mechanisms involved in cellular clearance may direct the development of therapeutic tools to improve the healing process and in turn, patient scarring outcomes.

2.3 Materials and Methods

Animals and Wound Models

All animal procedures were approved by the University of Illinois at Chicago Institutional Animal Care and Use Committee. The standard skin and tongue wounding protocols were described previously [124]. Briefly, female six-week-old Balb/c mice (Harlan, Inc. Indianapolis, IN, USA) were anesthetized with intraperitoneal injection of 100mg/kg ketamine and 0.05mg/kg xylazine. For mice with dorsal wounds (n=3, per time point, 6 wounds per mouse) the dorsal skin was shaved and 6 excisional dermal wounds were placed using a 1mm punch biopsy (Acu-Punch, Acuderm Inc., Ft. Lauderdale, FL, USA) on opposing sides of the midline starting at the scapula level and continuing caudally. For mice with mucosal wounds (n=3, per time point, 1 wound per mouse) a 1mm biopsy punch (Acu-punch, Acuderm) was used to make wounds lateral to and equal distance from the midline of the tongue.

Tissue Harvesting and Fixation

All mice were euthanized via CO_2 inhalation combined with cervical dislocation. Dorsal skin wounds were excised by first cutting a 2cm x 2cm square encompassing all dorsal wounds (6 total) followed by 2mm biopsy punch (Acu-punch, Acuderm) of the original wound sites. Oral wound tissue was harvested by excision of the tongue as close to the base as possible. The tongue was then bisected laterally, followed by 2mm biopsy punch at the site of the original wound on each half of the bisected tongue. Uninjured tissue was harvested in a similar manner from 2mm biopsy punches taken at the beginning of the experiment in euthanized mice. The wounds and surrounding tissues were collected, and placed in 0.5mL RNAlater (Sigma, St. Louis, MO, USA) for

RNA isolation and stored at –20℃ prior to analysis, or snap frozen in OCT compound (Sakura Fine technical, Tokyo, Japan) for cryotome sectioning and immunofluorescence, and stored at -80℃ prior to analysis. For RNA analysis and isolation, wounds were harvested at 6h, 24h, D3, D5, and D7 (n=3 mice per time point, per tissue type) post-injury for RNA isolation. The small size of the mouse oral cavity and tongue only allow for 1mm wounds, making it the standard protocol for mucosal injury in mice. Although these small wounds heal quickly, significant site-specific patterns of healing have been identified in this model [120, 123, 124, 127, 128].

Real time RT-PCR

Total RNA was isolated from 3 wounds per time point per group using TriZol (Invitrogen, Carlsbad, CA, USA) according to the instructions of the manufacturer. The concentration of RNA was determined with Nanodrop 1000 (Thermo Scientific Wilmington, DE, USA), and 1µg of RNA was used from each sample for the remainder of the RT-PCR protocol. RNA was treated with DNase I (Invitrogen), and reverse transcription performed with Retroscript kit (Ambion, Life Technologies, Grand Island, NY USA) according to the manufacturer's instructions. The cDNA was amplified on an ABI Step One Plus Real Time PCR System (Applied Biosystems, Life Technologies, Foster City, CA) in 96-well plate reactions with 3 reference gene wells and 3 target gene wells per sample. Primer sequences used for target genes analyzed are listed in Table II. To quantify relative differences in mRNA expression, the comparative C_T method ($\Delta\Delta C_T$) was used to determine relative quantity (RQ) [129]. All target genes were normalized to Gapdh expression in uninjured tongue tissue. Gene expression patterns of 18S RNA, β-actin, and ribosomal protein large, p0 (Rplp0) were also examined and

Gapdh was determined to be the most stable reference gene. In order to compare expression over the time, and to assess differences between the uninjured tongue and skin, gene expression was normalized to the reference uninjured tongue as a single baseline. Results were analyzed with two-way ANOVA to analyze the time and tissue effects comparing skin and tongue at each time point to each other and to uninjured tissue followed by a Bonferroni's post-test with $\alpha=0.05$.

Immunofluorescence

For immunofluorescence, tissue (n=2 per group) was sectioned with a cryotome (Leica 3050CS, Buffalo Grove, IL, USA) at a 8 µm thickness and placed on UltraStick glass slides (Gold Seal, Portsmouth, NH, USA).Tissue sections were fixed with ice cold acetone for 5min and then washed 2 x 5min with Tris-Buffered Saline (TBS) 0.025% Triton X-100, followed by 1X TBS wash 3 x 5min. The slides were then blocked with normal goat serum (10% Normal Goat Serum in 0.1% BSA 1X PBS) for 2h. The primary antibody, rabbit anti-active Casp3 (1:100, Abcam, Cambridge, MA, USA) diluted with 1% BSA in PBS was applied in a humidified chamber overnight at 4℃. Slides were rinsed 3 x 5min with TBS. Secondary antibody, Alexafluor 488 conjugated goat anti rabbit (1:1000, Invitrogen Molecular Probes, Grand Island, NY, USA) with 0.1µg/mL Hoescht nuclear stain (Immunochemistry, Bloomington, MN USA) diluted with 1% BSA in PBS was applied in a humidified chamber at room temperature for 2h in the dark. Slides were rinsed 3 x 5min with TBST (1X TBS and 0.5% Tween 20). Slides were then mounted in aqueous mounting media (VectaMount AQ, Vector Laboratories, Burlingame, CA, USA) followed by a coverslip and sealed with nail polish. All slides

were visualized on a Carl Zeiss fluorescence microscope using AxioVision LE (Thornwood, NY, USA).

2.4 Results

Apoptosis in skin and tongue wounds

To identify the relative amounts of apoptosis occurring over the course of wound healing, the levels of gene expression of Casp3 and Casp7, the executioner caspases involved in the final steps of both intrinsic and extrinsic apoptosis pathways, were examined. Casp3 expression was higher in uninjured skin and over the time course of wound healing in skin, significantly at D5 (Figure 2 a) as compared to tongue wound healing. Casp3 expression in skin wounds showed a significant increase compared to uninjured skin at D3 and D5 (Figure 2 a). When compared to tongue, Casp7 expression was significantly higher in uninjured skin (Figure 2 b). Over the time course of wound healing, skin wounds exhibited significantly increased levels of Casp7 at D5 (Figure 2 b), when compared to tongue wounds.

Casp3 protein expression was also qualitatively examined by immunofluorescence staining of active (cleaved) Casp3 in uninjured, D3, and D5 skin and tongue tissue (Figure 3). These time points were identified as having significant differences in Casp3 gene expression. Qualitatively, active Casp3 protein was seen in both uninjured skin and skin wounds; minimal expression was seen in tongue. These results support the concept that, as compared to skin, both normal tongue tissue and tongue wounds exhibit significantly less active, cleaved Casp3.

Intrinsic apoptosis pathway

To determine if differential involvement of the intrinsic apoptosis pathway occurs in wound healing of the oral mucosa and the dermis, gene expression of Trp53, Casp2, Casp9, Cycs, and Apaf1 was examined in skin and oral wounds. Significantly lower

gene expression of Trp53 was seen in oral wounds at 24h and D5 (Figure 4 a). Compared to uninjured skin, levels of Trp53 showed a significant increase in skin wounds through D7 (Figure 4 a). Casp2 expression was initially higher in uninjured tongue compared to skin, followed by a peak at D3 in oral wound healing. In contrast, skin wounds demonstrated little change in Casp2 expression (Figure 4 b). Casp9 expression was significantly higher in uninjured skin compared to tongue (Figure 4 c). In tongue wounds, the expression of Casp9 significantly increased at 24h (Figure 4 c) followed by a decrease to baseline levels by D7. Skin wounds, however, exhibited a significant decrease in Casp9 expression at 6h post-wounding (Figure 4 c) and then increased back to baseline levels by D7. Cycs expression was not significantly different for uninjured tissues. In skin wounds Cycs levels increased at 6h after injury (Figure 4 d), while a significant increase in tongue wound healing did not occur until 24h (Figure 4 d) compared to uninjured tongue tissue. Apaf1 expression in uninjured tissues was not significantly different. Both skin and tongue wounds showed significantly increased Apaf1at 24h after injury (Figure 4 e); Apaf1 levels remained elevated through D7. Overall, the pattern of expression of the signaling factors of the apoptosis pathway suggested that intrinsic apoptosis may play a more significant role in oral wound healing compared to skin wound healing.

Extrinsic apoptosis pathway

To assess differences in the contribution of the extrinsic pathway to apoptosis in skin and oral wound healing, we examined the relative gene expression of Tnfrsf1b, FasR, Casp8, Tradd, and Fadd. Tnfrsf1b expression was similar in uninjured skin and tongue, however, a significant increase in expression was observed in skin wounds at

24h (Figure 5 a) followed by a return to baseline levels. FasR expression was higher in uninjured skin, and significantly increased by 24h post-injury in skin wounds (Figure 5 b). No corresponding increase in FasR was seen in oral wounds, and in fact FasR expression was significantly lower in oral wounds at 24h, D3, and D5 (Figure 5 b) compared to skin wounds. Casp8 expression was higher in uninjured skin, and significantly increased by 24h post-injury in both skin and tongue wound healing compared to uninjured tissues (Figure 5 c),with skin showing greater levels than tongue at 24h (Figure 5 c). Casp8 expression also decreased significantly by D7 in skin wound healing compared to uninjured skin (Figure 5 c). Tradd expression was not significantly different in uninjured tissues, however, by 24h a significant increase in expression was seen in oral but not skin wounds (Figure 5 d) compared to uninjured tongue and 24h skin wounds. No corresponding peaks in skin wound healing were observed over the course of wound healing. Fadd expression was higher in uninjured skin (Figure 5 e), and increased over the time course of the experiment, trending to higher than baseline levels (Figure 5 e). When Fadd expression in healing wounds was examined, no significant differences were seen (Figure 5 e). Overall, the expression of genes involved in the extrinsic pathway tended to be significantly increased in skin wound healing as compared to oral wound healing, suggesting more involvement of the extrinsic apoptosis pathway in skin wound healing.

2.5 Discussion

Oral mucosal wound healing has previously been shown to exhibit reduced scar formation, a faster rate of re-epithelialization, lower levels of inflammation, and lower levels of angiogenesis compared to wound repair in the skin [120, 123]. However, very little attention has been given to the mechanisms that regulate cell death in wounds of these two tissues. Cell death and the mechanism of cell elimination may play an important role in the scarring outcome via paracrine signaling or immune modulation. Recent studies have suggested that apoptotic cells secrete factors that can modulate immune cell phenotypes to affect myoFB differentiation, FB and myoFB proliferation, and apoptosis resistance [130]. Since increased levels of myoFBs, increased FB and myoFB proliferation, and increased apoptosis resistance are known to influence scarring and fibrosis, apoptotic cells may play an important role in determining the final result of wound healing. The current study demonstrates that the dominant mechanisms of apoptosis differ for wounds of the oral mucosa and skin. Given the differential scar formation in these two anatomic sites, these results suggest possible connections for apoptotic mechanisms and scarring outcomes.

The mechanism of apoptosis is known to derive from the local environment of pre-apoptotic cells. The intrinsic apoptotic pathway is generally initiated by ischemia, DNA damage, and a reduction in the levels of growth factors, cytokines, or hormones. In oral wound healing the predominance of the intrinsic apoptosis pathway is early, generally peaking at 24h. This early peak may have to do with lower levels of pro-survival growth factors in oral wounds such as VEGF, EGF, and TGF- β1. Previous studies have shown lower levels of these key anti-apoptotic growth factors in oral

mucosal wounds [120, 128]. The lower levels of important anti-apoptotic growth factors in oral wound healing may be responsible for triggering the intrinsic apoptosis pathway by reducing pro-survival signaling. In contrast, the extrinsic apoptosis pathway requires extracellular input to initiate cell death. Skin wound healing is characterized by robust growth factor production, and hyperproliferation, effectively preventing the initiation of the intrinsic apoptosis pathway. In this situation, activation of the extrinsic apoptosis signaling pathway may be required to induce cell death.

Several other characteristics of oral wound healing have been suggested to play a role in the increased healing rate: faster re-epithelialization, increase proliferation of oral keratinocytes, decreased immune response, increased oxygen availability in the oral cavity, the moist wound environment, temperature, saliva flow, and local microflora. Previous studies have determined that the saliva-based, moist-wound environment plays a role in oral healing [131, 132]; however, the presence of saliva seems to be more important in larger wounds. Smaller mucosal wounds heal at similar rates independent of salivary influence [133]. Saliva contains growth factors, including EGF and VEGF, both of which have been suggested to important to oral wound healing [134, 135]. The tissue levels of these growth factors, however, is low when compared to skin wound healing. Correspondingly, skin that is transplanted into the oral cavity and shows a healing response that more closely resembled that of skin rather than that of oral mucosa [136, 137]. Together these studies suggest that environmental factors have a somewhat limited role in healing of oral mucosa. Intrinsic differences between oral mucosa and skin tissue seem likely to play an important part in defining the improved healing of oral wounds.

Our observations indicate that there are distinct and often significant differences in the gene expression of key mediators of both the intrinsic and extrinsic apoptosis pathways in oral wound healing compared to skin wound healing. Overall, our results show that the gene expression of the mediators of both intrinsic and extrinsic apoptosis pathways generally maintain low levels over the course of oral wound healing and return to baseline levels faster (Figures 2-5) than skin wounds. This observation leads to the conclusion that apoptosis in oral wounds occurs via rapid and concise mechanisms. Although changes in gene expression levels do not necessarily translate to protein expression or function, our findings show that cleaved Casp3 protein levels follow similar trends to that of gene expression (Figure 3). Our work does not address the protein levels and activation status of the remaining elements of the apoptotic cascade. Further studies will be necessary to quantitatively determine how translational regulation, post-translational modification, and release of intracellular stores influence the many other elements of the apoptotic pathways.

The data here suggest that overall, expression of genes related to the intrinsic pathway are generally higher in oral wound healing compared to skin wound healing (Figure 1). The timing of the peak of the gene expression related to intrinsic apoptosis in oral wound healing was most commonly seen at 24h (Figure 4 b, c, d, and e). This peak may correspond with the particular events occurring at that time. Specifically, inflammatory cells in an oral wound peak at around 24h and the peak in intrinsic apoptosis may be related to the resolution of inflammation and the elimination of inflammatory cells present in the wound bed.

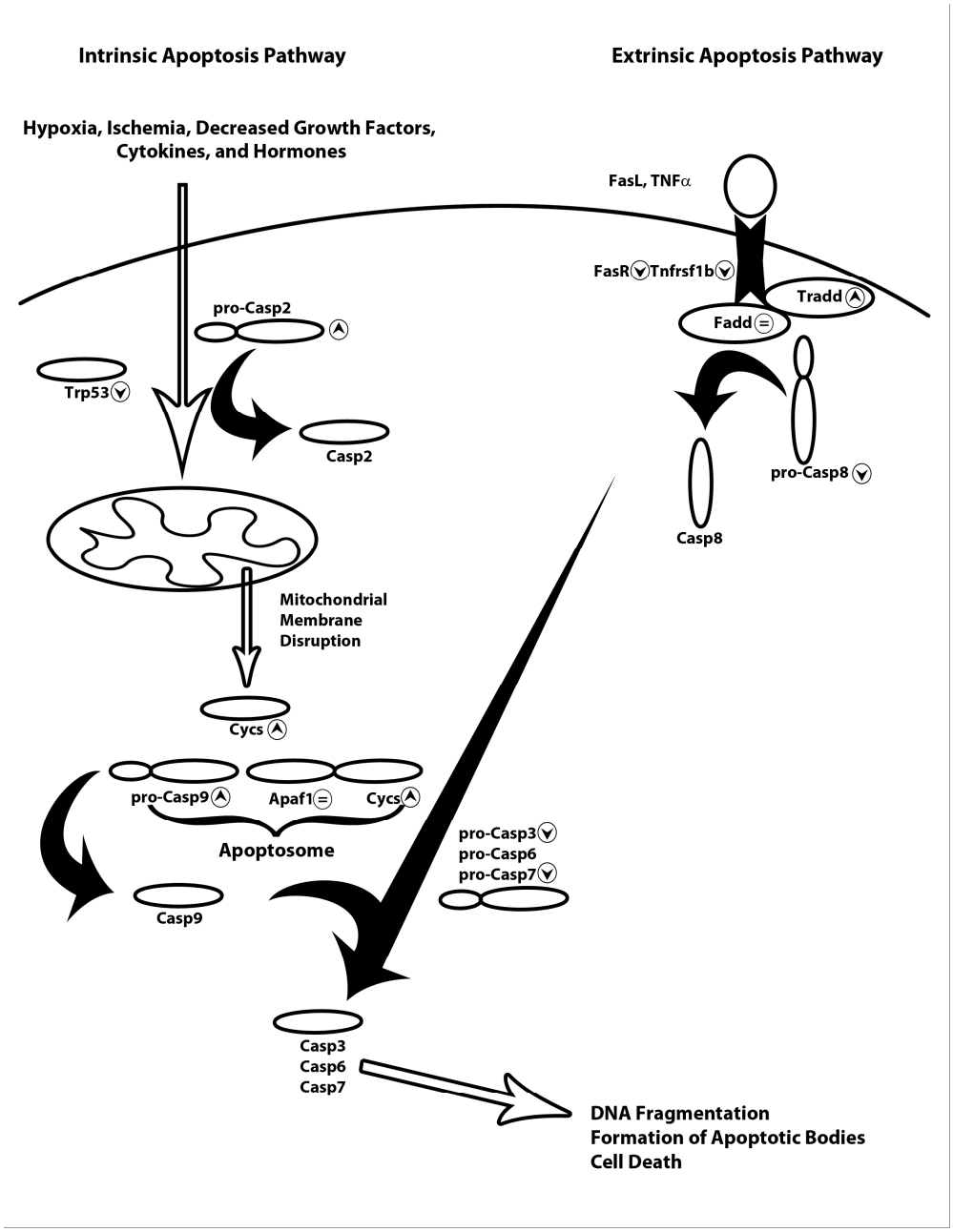
In contrast to the intrinsic pathway, our studies suggest that mediators of the extrinsic pathway are significantly increased in skin versus oral wound healing. Here again the timing of the peak may be related to the other events occurring in the wound. For example, the peak gene expression of Tnfrsf1b and Casp 8 occurs at 24h for both oral and skin wound healing (Figure 5 a and 5 c, respectively), although the levels are significantly higher in skin wounds. Apoptosis occurring at 24h may again be related to the elimination of inflammatory cells and the resolution of inflammation. Interestingly, FasR gene expression also peaks at 24h in skin wound healing, but there is no corresponding peak in oral wound healing (Figure 5 b). This phenomenon may be due to a more significant role of Fas-mediated apoptosis in skin wound healing, both in general and at 24h. Also of interest, Tradd gene expression peaks in oral wound healing at 24h, but there is no corresponding peak in skin wound healing (Figure 5 d). Tradd may be the rate limiting mediator in extrinsic apoptosis in skin wound healing, while it may be in excess in oral wound healing. The significant differences later on in skin wound healing (FasR at D5 and D7, Figure 5 b) could correspond to apoptosis occurring during vessel regression. As opposed to oral mucosa, the robust angiogenesis seen in skin wounds requires a pruning of the overabundant new vessels. As normal skin wound healing progresses into the remodeling phase, large numbers of ECs undergo apoptosis as unnecessary vessels regress.

Recent studies have suggested differences in the mechanism of apoptosis in fetal wound healing compared to adult wound healing. Cleavage of Casp7 and PARP were significantly increased in scarless fetal wound healing (embryonic day 15) compared to fetal wound that resulted in a scar (embryonic day 18) [138] Similar to oral

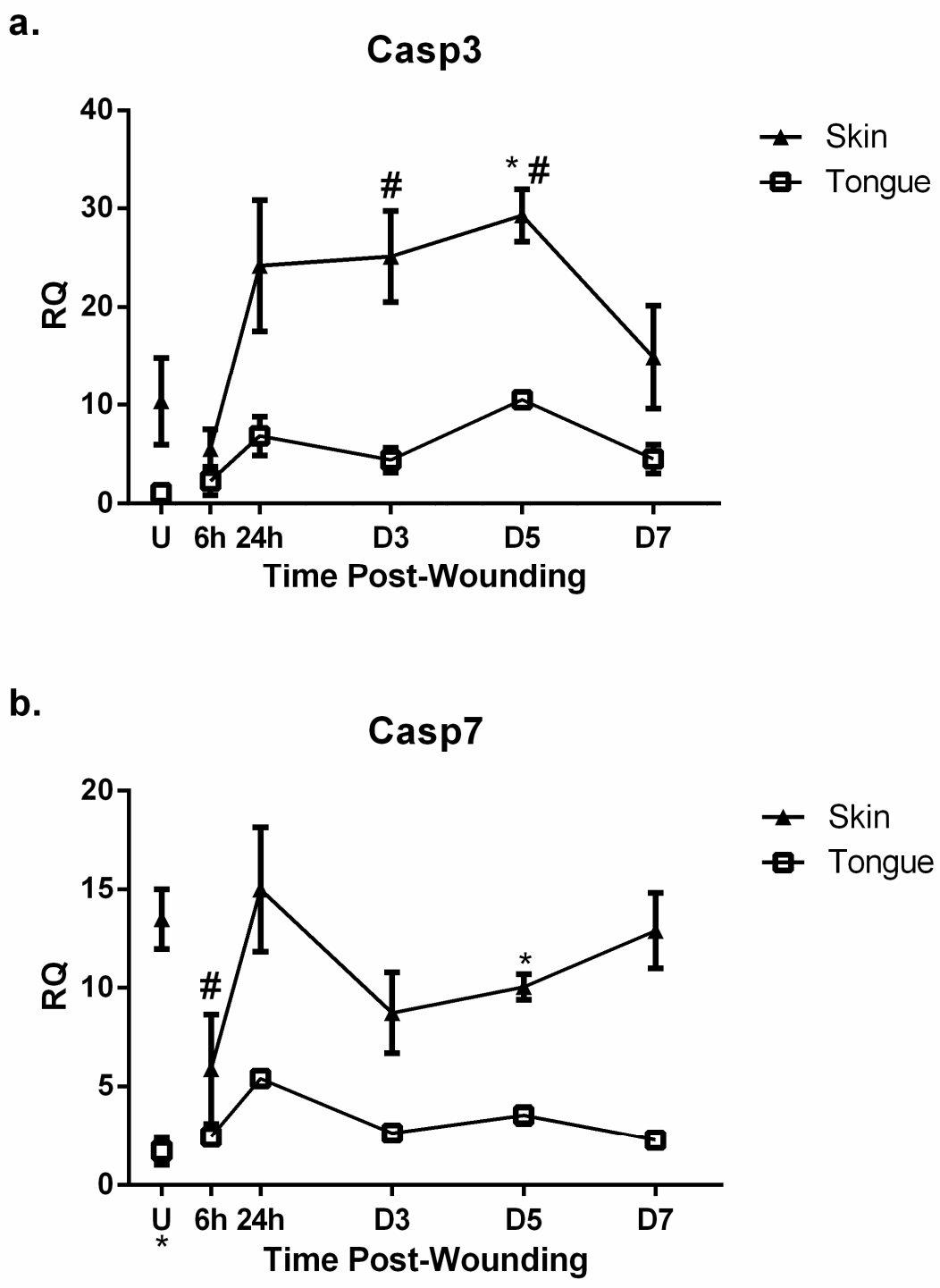
mucosal wound healing, regeneration or improved scarring outcomes have been identified in fetal wound healing; several observations suggest potential reasons for the similarities. First, similar to oral wound healing, the immune response in fetal wound healing is significantly lower than adult wound healing [139]. Second, the extracellular matrix in fetal and oral wounds have lower collagen I to collagen III ratios compared to normal adult skin wound healing [140-148]. Third, the presence and persistence of myoFBs during fetal and oral healing are lower compared to adult skin wound healing [149, 150]. On that same thread, the growth factors that stimulate myoFB differentiation have been identified as differentially regulated, with predominance of transforming growth factor (TGF)-β3 in fetal wound healing and TGF-β1 in adult wound healing [151]. TGF- β1 protein levels are significantly lower in oral wound healing compared to tongue [128], in vitro, oral FBs exhibit a decreased fibrotic response to the same levels of TGF-beta [152]. Lastly, similar to oral wound healing, the angiogenic response in fetal wound healing is significantly lower than adult wound healing [120]. Given the numerous similarities between fetal and oral wound healing, the finding of differences in the levels and mechanisms of apoptosis in both oral and fetal wounds [138] suggests that apoptosis may play a significant role in the determination of scarring outcomes.

In summary, our results indicate that intrinsic apoptosis may be the predominant mechanism of induction of apoptosis in oral wound healing, while extrinsic apoptosis may play a more significant role in skin wound healing. The differences in the pathways for apoptosis induction may provide potential targets for modifying skin wound healing outcomes to resemble the regeneration seen in oral and fetal wound healing.

2.6 Figures, Tables, and Figure Legends

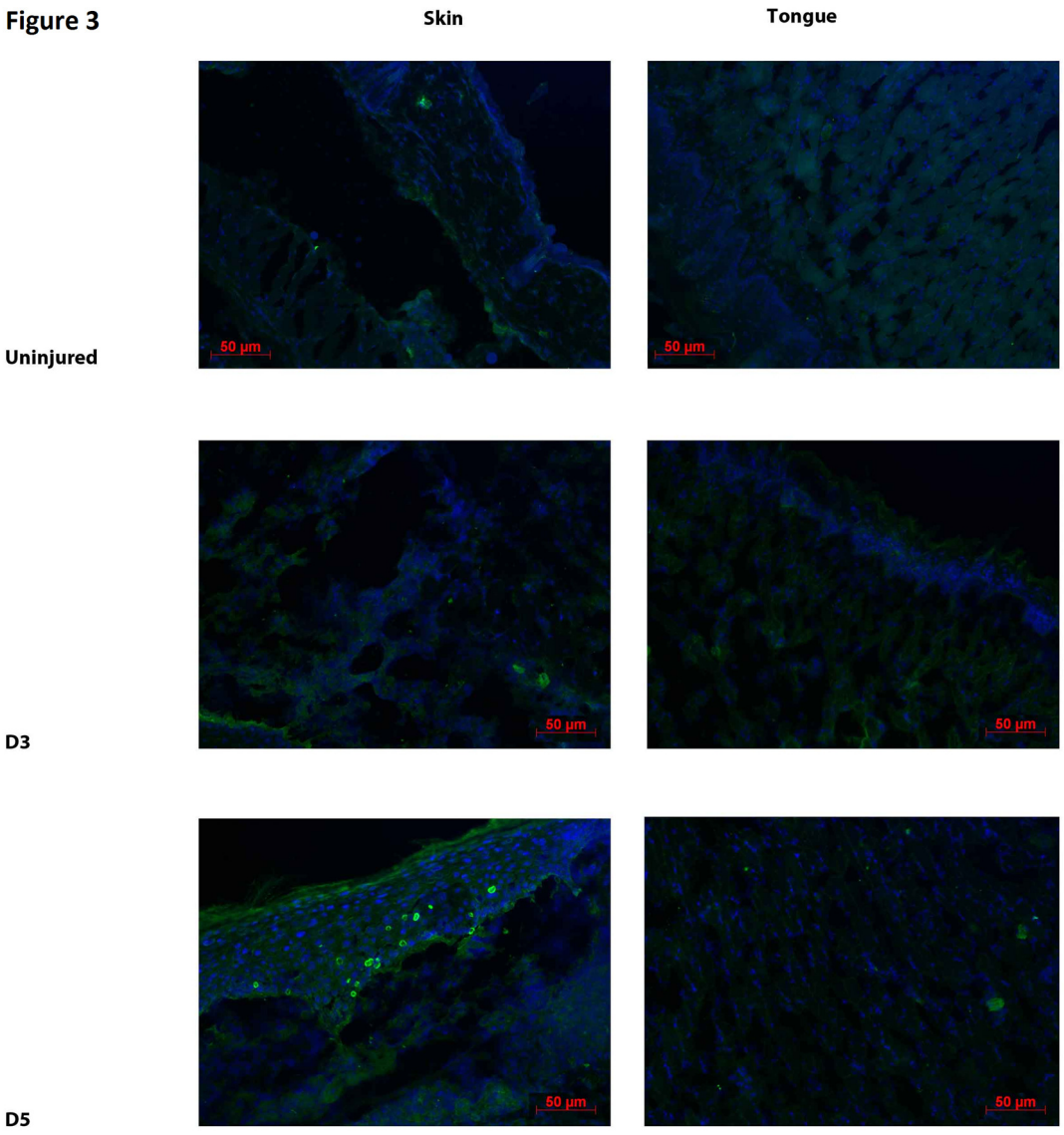


2.6.1 Figure 1 - Diagram of the intrinsic and extrinsic apoptosis signaling pathways

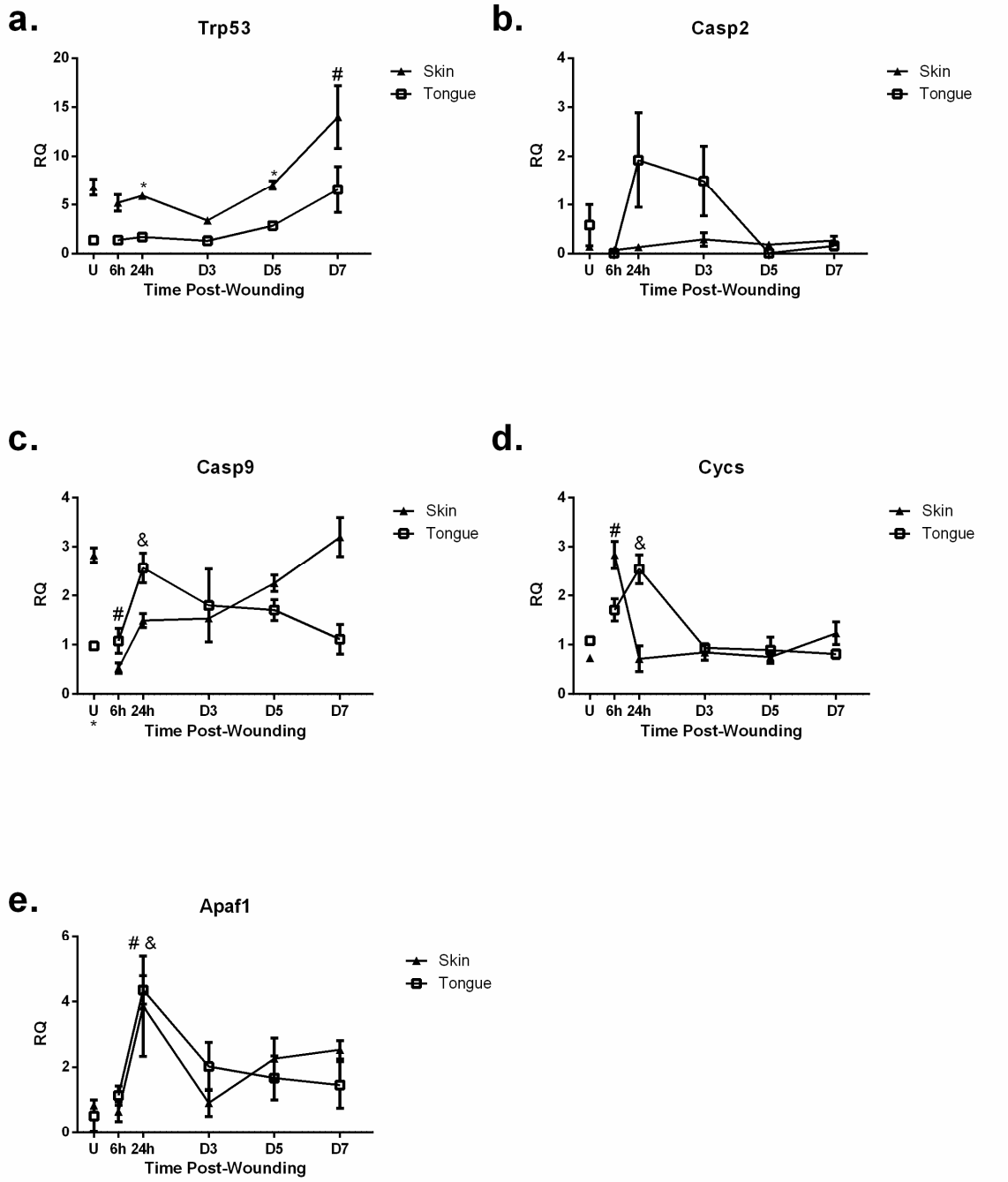


2.6.2 Figure 2 - Apoptosis Markers in Skin and Tongue

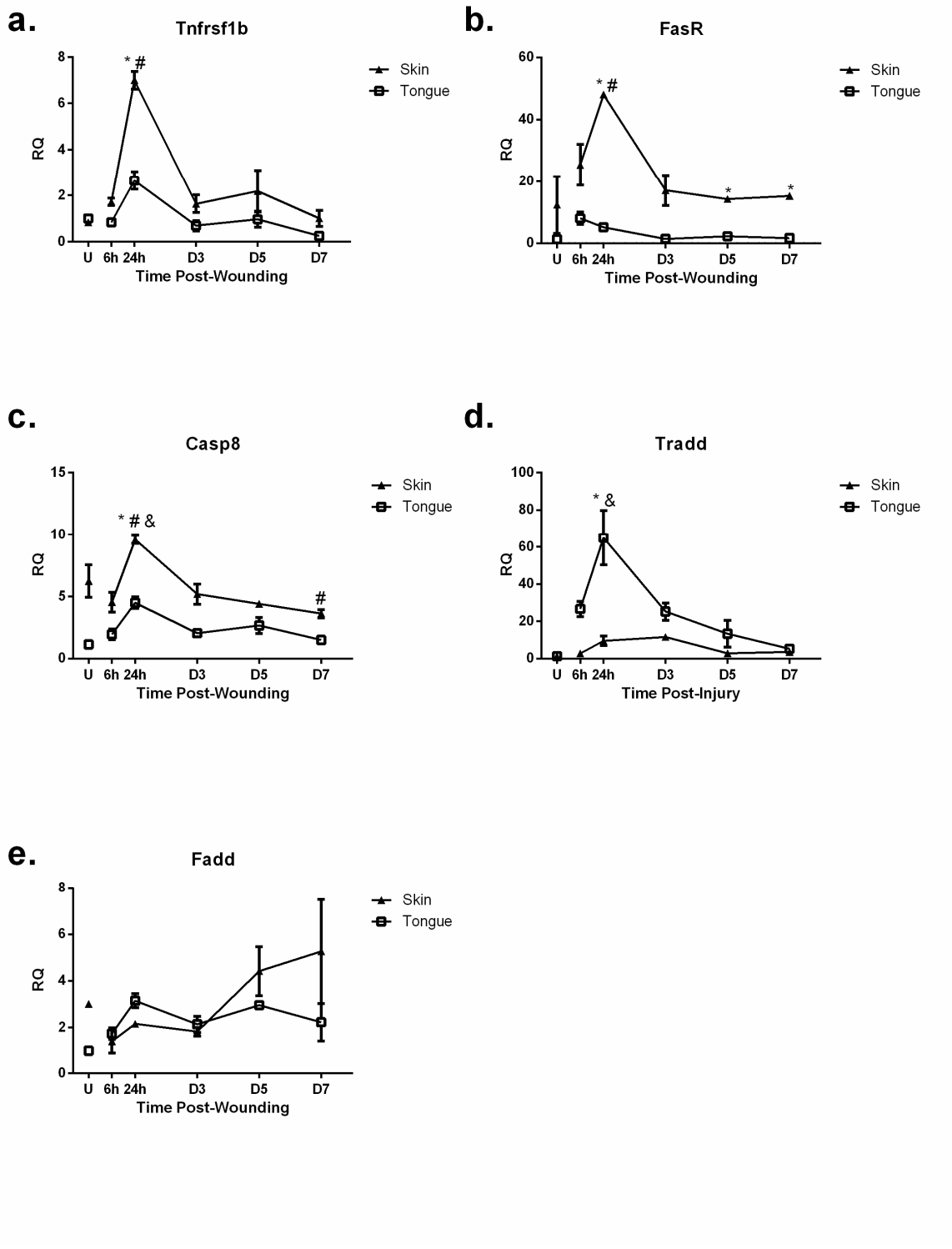
Figure 3



2.6.3 Figure 3 - Active Casp3 Protein Expression



2.6.4 Figure 4 - Intrinsic Pathway Markers



2.6.5 Figure 5 - Extrinsic Pathway Markers

Table II
Mouse RT-PCR Primer sequences

Gene	Forward Primer Sequence	Reverse Primer Sequence
Gapdh	TCACCACCATGGAGAAGGC	GCTAAGCAGTTGGTGGTGCA
Tnfrsf1b	ACTCCAAGCATCCTTACATCG	TTCACCAGTCCTAACATCAGC
FasR	AAGTCCCAGAAATCGCCTATG	GGTATGGTTTCACGACTGGAG
Tradd	ACGAACTCACTAGTCTAGCAGAG	AATACCCCAACAGCCACC
Fadd	GCAAGAGTGAGAATATGTCCCC	TCATGGTGTGATCAAGTCCAC
Casp8	AACTTCCTAGACTGCAACCG	TCTCAATTCCAACTCGCTCAC
Casp3	GACTGATGAGGAGATGGCTTG	TGCAAAGGGACTGGATGAAC
Casp7	CCCACTTATCTGTACCGCATG	GGTTTTGGAAGCACTTGAAGA G
Trp53	ATGTTCCGGGAGCTGAATG	CCCCACTTTCTTGACCATTG
Apaf1	GATGTGGAGGTGATCGTGAAG	TACTGGATGGTGCTGTGATG
Cycs	AAGGGAGGCAAGCATAAGAC	ATTCTCCAAATACTCCATCAGGG
Casp9	TGTGTCAAGTTTGCCTACCC	CCACTTTTCTTGTCCCTCCAG
Casp2	CAAGTCTCCCTTTCTCGGTG	AGTGTGCCTGGTAAAACTCAG

Figure legends

Figure 2.1: Diagram of the intrinsic and extrinsic apoptosis signaling pathways. Intrinsic apoptosis (the left side of the figure) is usually the result of hypoxia, ischemia, or UV damage. These induce cell stress which is propagated through Casp2 and Trp53 disruption of the balance of the mitochondrial membrane. Cytochrome C (Cycs) is released following mitochondrial membrane disruption and recruited to form the apoptosome with Apaf1 and pro-Casp9. Pro-Casp9 is cleaved and activates the caspase cascade resulting in cleavage and activation of Casp3, Casp6, and Casp7. The downstream effect of activation of the caspase cascade is DNA fragmentation, formation of apoptotic bodies, and cell death. The extrinsic apoptosis signaling pathway (the right side of the figure) requires binding of the death ligand (FasL or TNF-α) to its respective receptor (FasR or Tnfrsf1b). Binding of the death ligand to the death signals the recruitment of Tradd, Fadd, and pro-Casp8. Pro-Casp8 is cleaved by the complex and begins the caspase cleavage cascade resulting in the cleavage and activation of Casp3, Casp6, and Casp7. Similar to the intrinsic pathway, the result of the caspase cleavage cascade is DNA fragmentation, formation of apoptotic bodies, and cell death. (▲, significantly increased in oral mucosa versus skin; ▼significantly decreased in oral mucosa versus skin; =, no significant difference in oral mucosa versus skin gene expression).

Figure 2.2: Apoptosis Markers in Skin and Tongue

Real time RT-PCR of Casp3 and Casp7 were performed on RNA isolated from uninjured tissue (U) and wound samples at 6h, 24h, D3, D5, and D7 post-injury. To

determine relative quantity (RQ) of mRNA levels during wound healing, all samples were normalized to Gapdh expression in uninjured tongue tissue. The results are shown as the mean ± SEM; n=3. Data were analyzed by two-way ANOVA and Bonferroni's posttest (* $p<0.05$ for skin versus tongue wounds, # $p<0.05$ for skin versus uninjured skin, & $p<0.05$ for tongue versus uninjured tongue).

Figure 2.3: Active Casp3 Protein Expression

Immunofluorescence for cleaved (active) Casp3 was performed on uninjured, D3, and D5 post-injury tissues (n=2). The images were not quantified, merely observed to detect the presence of active Casp3 protein.

Figure 2.4: Intrinsic Pathway Markers

Real time RT-PCR of Trp53, Casp2, Casp9, Cycs, and Apaf1 were performed on RNA isolated from uninjured tissue (U) and wound samples at 6h, 24h, D3, D5, and D7 post-injury. To determine relative changes in mRNA levels during wound healing, all samples were normalized to Gapdh expression in uninjured tongue tissue. The results are shown as the mean ± SEM; n=3. Data were analyzed by two-way ANOVA and Bonferroni's posttest (* $p<0.05$ for skin versus tongue wounds, # $p<0.05$ for skin versus uninjured skin, & $p<0.05$ for tongue versus uninjured tongue).

Figure 2.5: Extrinsic Pathway Markers

Real time RT-PCR of Tnfrsf1b, FasR, Casp8, Tradd, and Fadd were performed on RNA isolated from uninjured tissue (U) and wound samples at 6h, 24h, D3, D5, and D7 post-

injury. To determine relative changes in mRNA levels during wound healing, all samples were normalized to Gapdh expression in uninjured tongue tissue. The results are shown as the mean ± SEM; n=3. Data were analyzed by two-way ANOVA and Bonferroni's posttest (* $p<0.05$ for skin versus tongue wounds, # $p<0.05$ for skin versus uninjured skin, & $p<0.05$ for tongue versus uninjured tongue).

CHAPTER 3

Inhibition of apoptosis during the remodeling phase of wound healing results in increased vessel density.

3. 1 Introduction

Wound healing is a complex process that can be broken down into four distinct phases of hemostasis, inflammation, proliferation, and remodeling. During the proliferative phase there is an over exuberant angiogenic response that creates a vascular bed that is several fold more dense than uninjured tissue. These excess vessels must then be removed during the remodeling phase. One widely accepted view is that optimal wound healing requires a robust and vigorous angiogenic response during the proliferative phase [153, 154]. There is emerging evidence challenging the idea that a robust angiogenic response is required for optimal healing outcomes. At least five separate studies, including several from our lab, show that full thickness wounds heal well even when the angiogenesis is reduced [155-159]. For example, oral mucosal wounds, which are known to heal very rapidly and with minimal scar formation, exhibit a less robust angiogenic response in comparison to skin wounds [160]. In more recent studies, we used neutralization of vascular endothelial growth factor (VEGF) via antibody treatment which reduces the peak of wound vascularity by approximately 50% in adult skin wounds. Wound scar width was significantly reduced and even wound closure was normal [159]. Recent studies of human scars further support the concept that hypertrophic scar formation may be linked to robust angiogenesis [161]. The use of anti-angiogenic therapy to reduce scar formation has been suggested by us [159] and others [162]. Inhibition of angiogenesis through anti-angiogenic therapy also results in

decreased apoptotic ECs during vessel regression in the remodeling phase. It is unclear whether the reduction of scarring due to anti-angiogenic therapy is mediated through decreased vessel density or decreased levels of apoptosis in vessel regression.

During vascular regression, unnecessary blood vessels are removed via caspase 3 (CASP3)-dependent apoptosis of ECs [12, 61, 63, 163-169]. Until recently, it was assumed that apoptotic ECs had no effect on the surrounding tissue. However, current studies demonstrate that apoptotic ECs have a lasting effect on myoFB (myoFB) differentiation and generation of fibrosis. In particular, a connection between fibrosis and EC apoptosis has been shown in cardiac and renal allograft rejection, pulmonary and liver fibrosis and now in the skin.

The specific contributions of angiogenesis and vascular regression to scarring in normal wound healing have not been determined. In order to examine the role apoptosis plays in wound healing we inhibited apoptosis during the critical remodeling stage of wound healing. In the present study we examined the effect of benzyloxycarbonyl-Val-Ala-Asp (OMe) fluoromethylketone (Z VAD-fmk), a pan caspase inhibitor, on the outcome of wound healing. During the remodeling phase of wound healing cell elimination is necessary to produce tissue homeostasis. The predominant mechanism of cellular elimination at this stage is apoptosis [12, 61, 63, 163-169]. We hypothesized that prevention of apoptosis could have lasting effects on wound healing outcomes similar to the effects seen in other tissue types such as pulmonary, cardiac, and renal tissues.

3.2 Materials and Methods

Animals and wounding

All animal procedures were approved by the University of Illinois at Chicago Institutional Animal Care and Use Committee. Standard skin wounds were prepared as described previously [64].[64]. Briefly, female, six-week-old, Balb/c mice (Harlan, Inc. Indianapolis, IN, USA) were anesthetized via intraperitoneal injection of 0.1cc ketamine/xylazine. Dorsal wounds (n=3 mice per group, per time point) were created immediately after shaving the dorsum of the mice. Two 2cm long skin incisions were made through the dermis and panniculus carnosus in contralateral, paraspinal locations and closed with surgical clips (MikRon 9mm AutoClip, BD Biosciences, Franklin Lakes, New Jersey, USA). Surgical clips were removed on postoperative day 5. Wounds were harvested at 7, 10, and 14 days post injury, the animals were sacrificed and the dorsal skin harvested for analysis.

Intradermal injections of Z VAD-fmk

Each 2 cm incisional wound (2 per mouse) was injected daily in 3 locations: caudally, cephalically, and medially. Mice were separated into 2 groups: saline or Z VAD-fmk (ProMega, Madison, WI, USA) injected. Each injection (both control and treatment) was approximately 25uL. The concentration of Z VAD-fmk was 0.2 µg/µL. Briefly, mice were anesthetized with isoflurane (Isothesia, Butler Schein, Dublin, OH, USA) until eye touch and toe pinch negative, at which point intradermal injections were made with a hypodermic needle (1cc Insulin Syringe, BD, USA) lateral to the wounds, directed centrally, ensuring that the treatment was applied to the dermis of the wound

bed. Injections began on day 5, when the surgi-clips were removed, and continued every day until tissue was harvested at days 7, 10, and 14.

Tissue Harvesting and Fixation

All mice were euthanized via CO2 inhalation followed by cervical dislocation. Dorsal skin wounds were excised by first cutting a 2cm x 2cm square encompassing both dorsal wounds followed by three 5mm diameter biopsies (Acu-punch, Acuderm, Ft. Lauderdale, FL, USA) centered down the length of the original incisional wound sites. For wound tensiometry a specialized excisional template was utilized (described in "Wound Tensiometry" section). The wounds and surrounding tissues were collected, and either immediately tested for wound breaking strength, placed into 0.5mL RNAlater (Sigma, St. Louis, MO, USA) and stored at –20°C for RNA analysis, embedded in cryostat freeze media (Histoprep, Fischer Scientific, Fairlawn, NJ, USA) and stored at -80°C for immunofluorescence, snap frozen and stored at -80°C for protein expression and hydroxyproline content analyses, or fixed in formalin and then embedded in paraffin for histological analysis.

Assessment of Wound Vascularity via Immunofluorescence

Tissue (n=3 per group per time point) was sectioned with a cryotome (Leica 3050CS, Buffalo Grove, IL, USA) at a 8 µm thickness and placed on UltraStick glass slides (Gold Seal, Portsmouth, NH, USA).Tissue sections were fixed with ice cold acetone for 5min and then washed 2 x 5min with Tris-Buffered Saline (TBS) 0.025% Triton X-100, followed by 1X TBS wash 3 x 5min. The slides were then blocked with normal goat serum (10% Normal Goat Serum in 0.1% BSA 1X PBS) for 2h. The primary antibody, rabbit anti-Cd31 (1:100, BD Biosciences, San Jose, California, USA) diluted

with 1% BSA in PBS was applied in a humidified chamber overnight at 4℃. Slides were rinsed 3 x 5min with TBS. Secondary antibody, Alexafluor 488 conjugated goat anti rabbit (1:1000, Invitrogen Molecular Probes, Grand Island, NY, USA) with 0.1µg/mL Hoescht nuclear stain (Immunochemistry, Bloomington, MN USA) diluted with 1% BSA in PBS was applied in a humidified chamber at room temperature for 2h in the dark. Slides were rinsed 3 x 5min with TBST (1X TBS and 0.5% Tween 20). Slides were then mounted in aqueous mounting media (VectaMount AQ, Vector Laboratories, Burlingame, CA, USA) followed by a coverslip and sealed with nail polish. All slides were visualized on a Carl Zeiss fluorescence microscope using AxioVision LE (Thornwood, NY, USA) and analyzed using ImageJ software to determine vessel density.

Picrosirius Red Staining

Picrosirius red staining was used to identify mature and immature collagen. Manufacturer's instructions were followed for the picrosirius red kit (Electron Microscopy Sciences, Hatfield PA, USA). Briefly, paraffin embedded tissue (n=3 per group, per time point) was sectioned with a microtome (Jung Histocut 820, Leica) to 5um thickness and placed on glass slides (Fischer Scientific, Pittsburgh, PA, USA). Tissue sections were rehydrated step-wise through 100-70% EtOH and ddH20 for 5-10 min each step. The sections were then treated with Phosphomolybidic Acid (0.2% aq, Electron Microscopy Sciences) for 3 min, rinsed in ddH20, stained with Sirius Red Acid (0.1% Electron Microscopy Sciences) for 90 min, and washed with 0.01 N HCl (Electron Microscopy Sciences) two times for 1 min. The sections were then dehydrated and mounted with permount (Histo mounting media, Fischer) and a coverslip. Wounds were first located

under white light and then viewed for imaging under polarized light with a gold filter using a Carl Zeiss microscope with AxioVision LE. Collagen maturity (red = mature collagen, green/yellow = immature collagen) of each wound was determined using ImageJ. Images were analyzed to determine the pixel-level for each color threshold, and expressed as % mature or immature collagen/pixel^2.

Active and Pro Caspase 3

Active and pro Caspase3 protein levels were determined using manufacturers' instructions for a DUPLEX ELISA kit (Abcam, Cambridge, MA, USA). Briefly, standards were prepared for both active Casp3 and Pro Casp3. HeLa-Vehicle treated standards, used to interpolate pro-Casp3 protein expression, were prepared in a 12-800 µg/mL working range. HeLa-staurosporine treated standards, used to interpolate active-Casp3, were prepared in a 12-800 µg/mL working range. Samples were prepared by tissue homogenization of a 5mm diameter biopsy punch centered over the incisional wound, including surrounding tissue (Power Gen 125, Fischer). The homogenate is suspended in PBS at 10mg/mL and equal volume of 2X extraction buffer. After 20 min incubation on ice the samples were centrifuged at 18,000 xg for 20 min at 4 ℃. The supernatant was analyzed in a pro-caspase 3 and active caspase 3 pre-coated, sandwich ELISA well strip (Abcam). 50 µL of each sample or standard was added per well and the plate was incubated for 2 h at room temperature. Each well was washed twice with 1X Wash Buffer (Abcam). A 1X Caspase 3 detector antibodies mixture was added to each well and the plate incubated for 1h at room temp. The plate was washed 3 times with 1X Wash Buffer (Abcam) and Development Solution (Abcam) was added to each well. Immediately following this addition the plate was read in a fluorescence plate reader

(Victor X5, Perkin Elmer, Downer's Grove, IL, USA). The plate was read in kinetic mode for AP detection at 360nm, HRP detection at 540nm, for 15 min, every 60 sec, with shaking between readings. All samples and standards were tested in duplicate Data were analyzed using raw relative fluorescence units (RFU) at each reading time point. Standard curves for pro- and active- Casp3 were generated using four parameter algorithm from the corresponding HeLa standards; HeLa-vehicle and HeLa-staurosporine, respectively. Relative protein concentrations were interpolated from the standard curves using GraphPad Prism (version 6.01, GraphPad Software, San Diego, CA).

Hydroxyproline Analysis

The hydroxyproline content of wound biopsies (n = 3 mice per group, per time point, one biopsy per mouse) was determined according to a standard protocol [38]. Each wound was harvested with a 3-mm biopsy punch. Uninjured skin was harvested at the time of wound harvesting to use as a control. Tissue was processed as previously described [38].

Gene Expression Analysis

Total RNA was isolated from wounds stored in RNAlater (Sigma) by means of TriZol (Invitrogen, Carlsbad, CA, USA) according to the instructions of the manufacturer. The concentration of RNA was determined with Nanodrop 2000 (Thermo Scientific Wilmington, DE, USA), and 1µg of RNA was used from each sample for the RT-PCR protocol. RNA was treated with DNase I (Invitrogen), and reverse-transcribed with Retroscript kit (Ambion, Life Technologies, Grand Island, NY USA) according to the manufacturer's instructions. The cDNA was amplified on the ABI Step One Plus Real

Time PCR System (Applied Biosystems, Life Technologies, Foster City, CA) in 96-well plate reactions with 3 control gene wells and 3 target gene wells per sample. *Col1a2* and *Col3a1* were both analyzed for gene expression. Primer sequences used for target genes analyzed are listed in Table III. To quantify relative differences in mRNA expression, the comparative C_T ($\Delta\Delta C_T$) method was used to obtain relative quantities. All target genes were normalized to *GapDH*, the endogenous control, in uninjured skin. Relative levels were calculated by determining the ratio of Col:Col3 gene expression.

Wound Tensiometry

Wound breaking strength was measured as previously described [39]. Skin strips for testing were cut at right angles to the incisional wound with specially designed biopsy punches. The biopsy punches are shaped like a dog bone with a narrow portion encompassing the wound site approximately 4mm across, between to larger portions on each end. The larger end portions of the skin biopsy were pulled apart by a motorized tensiometer (Mark-10, Copiague, NY). The tensiometer separates the larger portions at a constant rate at a constant rate (3 cm/min) until the wound site is broken. A digital output of the wound breaking strength was recorded for each strip per wound. One skin strip per wound was subjected to analysis and the wound breaking strength was averaged for all 3 mice per group per time point.

Statistical Analyses

Data were analyzed using GraphPad Prism (version 6.01, GraphPad Software). The data were calculated as means ± SEM for each treatment group per time point. Results were analyzed with two-way ANOVA and Multiple t-tests using Bonferroni's Post-test with $\alpha=0.05$.

3.3 Results

Inhibition of apoptosis significantly increased vessel density but did not significantly decrease cleavage of Caspase 3.

To determine if apoptosis would affect vessel density, we inhibited apoptosis in wounds using intradermal administration of 0.1 µg/µL of Z VAD-fmk. Z VAD-fmk inhibits the cleavage of caspases thus preventing the propagation of apoptosis through the intrinsic and extrinsic apoptosis pathways. A large portion of apoptotic cells during wound remodeling derive from regressing vessels. Daily injections of 120ul total in 3 location surrounding the wound (caudal, medial, and cephalic) of Z VAD-fmk prevented vessel regression, significantly increasing vessel density at D7 (Figure 1a, * $p<0.05$). The vessel density continued to decline with daily injections of Z VAD-fmk, reaching levels similar to control by D14. In addition to significant increase in vessel density, histologically the vessels appeared larger and more convoluted in D7 wounds treated with Z VAD-fmk compared to those treated with saline (data not shown). Despite the observed effect on capillaries, intradermal administration of 120uL of 0.1 µg/µL Z VAD-fmk per wound was not sufficient to significantly alter the ratio of active to pro Casp3 in incisional wound healing (Figure 1b, $p>0.05$). The trend in active to pro Casp3 expression was approaching uninjured skin on D14 (Figure 1b) in the control group. The Z VAD-fmk treatment started at levels similar to uninjured tissue at D7, peaked at D10 (failed to reach significance), and then began decreasing again by D14 (Figure 1b). These results suggest that D7 post-wounding inhibition of apoptosis modulates vessel density; this may be due to changes in the active to pro Casp3 levels.

Formation of fibrous scar tissue is not significantly altered when apoptosis is inhibited

Previous studies in other organ systems have shown that decreasing the vessel density resulted in decreased fibrosis. In order to determine if ECM production in wounds was altered by treatment with Z VAD-fmk, we measured hydroxyproline (total collagen) of wound tissue. Biopsies of the wounds were obtained from different areas (caudal, cephalic, or medial) to control for any site-specific differences. Results were calculated as µmol hydroxyproline/mg tissue wet weight. Daily Z VAD-fmk injections did not significantly alter the hydroxyproline content (Figure 2a). In fact, the pattern of collagen content was nearly identical for control and treated wounds. To determine the maturity of collagen present in healing wounds which had been subjected to inhibition of apoptosis, picrosirius red staining was used. The data showed a significant decrease in the % immature collagen between D7 and D14 (Figure 2b, *$p<0.05$) in wounds treated with Z VAD-fmk. This change over time was not observed in the control wounds. The % immature collagen was significantly greater than the % mature collagen at D7 in both control and treated wounds (Figure2b, *$p<0.05$). This is representative of patterns observed in normal healing. There was also a significant decrease in % immature collagen from D10 to D14 in control treated wounds (Figure 2b, *$p<0.05$). Control and treated wounds follow similar trends in decreasing % immature collagen and increasing % mature collagen over the course of wound healing and daily injections, however significant differences over time and between treatment groups was not observed. Taken together, this data suggests that inhibition of apoptosis did not affect total collagen levels and did not influence collagen maturity.

Gene expression of Col1 (mature) and Col3 (immature) was not significantly modified with anti-apoptosis treatment

In order to determine if treatment with Z VAD-fmk affects gene expression of collagen in wounds, we performed RT-PCR for collagen type 1a2 (Col1) and collagen type 3a1 (Col3) using RNA isolated from control and treated wound tissue. Our data shows a decrease in the percent of Col1 and an increase in percent of Col3 gene expression at D10 in treated compared to control wounds (Figure 3a), however this difference is not statistically significant. This data along with the percent mature and immature collagen suggests that inhibition of apoptosis does not have a significant effect on collagen organization and gene expression.

Wound breaking strength increases more quickly with anti-apoptosis treatment

Improvements in wound breaking strength are generally correlated to improved wound healing and decreased scar tissue. To assess how treatment with Z VAD-fmk would influence wound breaking strength, we examined the wound breaking strength using a wound tensiometer. Wound breaking strength significantly improved from D7 to D14 (Figure 3b, *$p<0.05$) in the treatment group, with treated wounds gaining strength more quickly than control. . Z VAD-fmk treated wounds reached a high level of wound breaking strength by D14; in contrast, control treated wounds did not reach this higher level until D21. These results suggest that treatment with apoptosis inhibitors improves wound breaking strength more quickly than untreated wounds.

3.4 Discussion

Clinical trials are underway for the use of caspase cleavage inhibition for fibrosis in several different organs [170-182]. In idiopathic pulmonary fibrosis, caspase cleavage inhibition has been used to decrease the extent of fibrosis and improve lung function [174, 179, 181] [46-48, 51]. Recently clinical research has examined inhibition of apoptosis in cardiac fibrosis, allograft vasculopathy, and myocardial infarcts. Treatment with apoptosis inhibitors following a myocardial infarct decreases the size of the infarct and the corresponding cardiac remodeling, in addition to decreasing cardiac allograft vasculopathy in cardiac transplants [37, 40, 42-45, 170, 177]. Hepatic fibrosis is characterized by increased collagen deposition, hepatocyte apoptosis, and activation of collagen-producing hepatic stellate cells. Inhibition of apoptosis in models of hepatic injury and fibrosis resulted in attenuated liver fibrosis, improved liver function tests, decreased hepatic stellate cells, decreased collagen deposition [173, 176, 178, 182] and improved liver regeneration [50, 52-56]. In renal ischemia reperfusion injury, injection of apoptosis inhibitors resulted in improved organ function, decreased inflammation, and prevention of the spread of ischemia-reperfusion injury [35, 36, 41, 49, 183, 184]. In renal fibrosis, which occurs as a result of renal allograft rejection, Z VAD-fmk has been used to increase renal function and prolong graft acceptance [171, 172, 175]. Fibrosis and scarring of the skin as a result of wound healing has many parallel facets to fibrosis in liver, lung, kidney and cardiac tissues. Given the similarities relating to fibrosis in other tissue types, we examined the effect of caspase cleavage inhibition on wound healing and scarring in the remodeling phase.

Caspase cleavage inhibition via Z VAD-fmk is commonly used to prevent apoptosis. Z VAD-fmk is a fluoromethyl ketone that inhibits cysteine protease activity which is the predominant mechanism of caspase activation [185]. Caspase inhibitors are composed of 3 major subunits: an active site recognition element, an aspartic acid to confer caspase specificity, and an electrophilic thiol reactive site [174, 186]. Z VAD-fmk contains a valine-alanine- aspartate active site recognition element, with the aspartate conferring caspase specificity, and a fluoromethyl ketone which acts as the electrophilic thiol reactive site [174, 186]. In Figure 1b, the level of apoptosis (active:pro Casp3 protein expression) was increased in the treatment group at D10, however prior to that point the treatment appeared to be preventing the cleavage of Casp3 (a low active:pro Casp3 ratio). The largest caveat to our study is that the injected volume and concentration of Z VAD-fmk may not have been sufficient to maintain a low level of apoptosis. However, given that Z VAD-fmk treatment led to significant changes in vessel density, it seems likely that an anti-apoptotic effect was achieved by D7 time point. One unexpected result was that the vessel density in wounds of the control group showed increasing levels over time (Figure1a). This is atypical, as peak of vessel density in 2cm incisional wounds has been shown to occur at 5 days post-wounding [187, 188]. One possible explanation for this result is the increased hydrostatic pressure that occurs with intradermal injections. Previous studies have shown that increased hydrostatic pressure can significantly alter the dermal architecture mediated by connective tissue FB regulation of tissue homeostasis [189, 190]. To reduce any effect of increased hydrostatic pressure, we performed injections every other day. Under these conditions, the vessel density in the control group followed the expected

decreasing pattern over time, while the vessel density in the treatment group was significantly higher at D14 (data not shown).

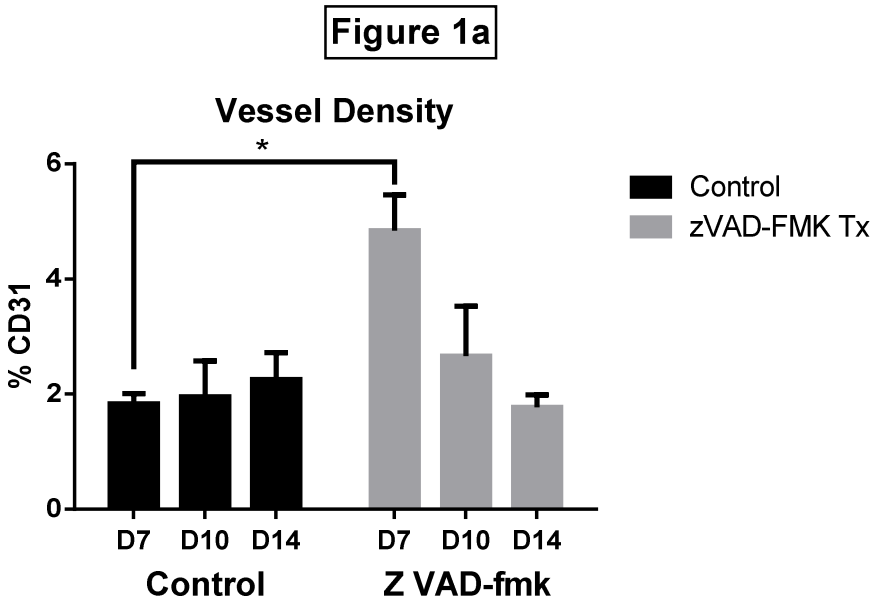
In addition to changes in total vascularity, treatment with anti-apoptotic agents also led to an observed change in vessel architecture, as the vessels appeared larger and more convoluted (Figure 4). Studies have identified structural breakdown of the vessels as a critical primary step to vascular regression and apoptosis of ECs [191]. By inhibiting apoptosis, vessel regression may have been prevented and the levels of pro-angiogenic cytokines and chemokines may have been maintained, encouraging EC proliferation, inducing increased size and density of vessel in the wound bed, and decreasing cellular turnover normally seen in vessel regression.

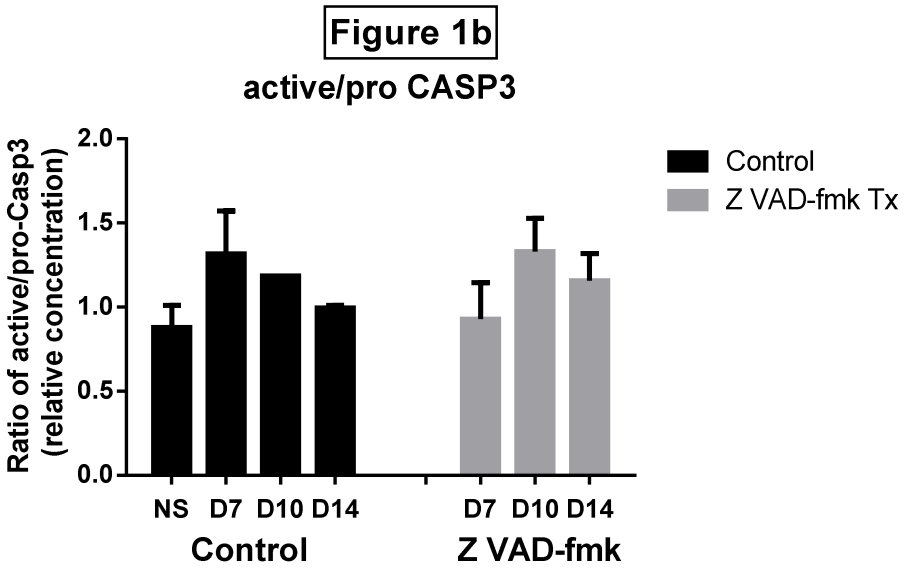
Our studies also examined how changes in apoptosis might influence scar formation using collagen synthesis and structure as a measure of scarring and fibrosis. In this study the changes to the collagen architecture were determined via changes in total collagen content (Figure 2a), collagen maturity (Figure 2b), Col1 and Col3 gene expression (Figure 3a), and wound breaking strength (Figure 3b). In both control and treatment groups the total levels of collagen were not altered, and showed a trend similar to previously described values for incisional wound healing. In other models of fibrosis such as pulmonary, cardiac, hepatic, and renal, inhibition of apoptosis decreased the total collagen present, the collagen type, and maturity [170-182]. In fetal wound healing, a model of regeneration when wounds are made in the first trimester, collagen type 3 and immature collagen are prevalent in the healing wound and the ultimate scarlessly healed wound [192]. Taken together, apoptosis inhibition in wound healing should resemble regeneration seen in fetal wound healing. In our model of

incisional wound healing the dosages applied to the wound bed may have been too low to produce significant responses. Additionally, the inclusion of the surrounding uninjured tissue may have obscured any differences occurring within the very narrow, healing incisional wounds. Wound breaking strength is a macroscale measurement of collagen content, type, and remodeling (i.e. crosslinking). Another measurement that may have yielded more substantive results regarding the improvement in scarring is trans-epidermal water loss or dermal capacitance. These measurements identify the return of the barrier properties of the epidermal layer and electrical capacitance and deep tissue damage [193, 194], respectively. They may have been added as clinically-relevant measures of the regenerative/anti-fibrotic effects of treatment with the caspase cleavage inhibitor.

We inhibited apoptosis in 2 cm incisional wound healing in the remodeling phase, in an attempt to prevent vascular regression, and execution of apoptosis through caspase cleavage. Inhibition of apoptosis with Z VAD-fmk significantly increased vascular density 7 day post wounding, but did not significantly inhibit caspase activation or alter the total collagen present in healing wounds. A slight but non-significant advance in wound breaking strength was seen in Z VAD-fmk treated wounds, as the treatment group showed an earlier gain of wound breaking strength. In addition, collagen maturity and type of collagen being synthesized appeared to show some changes under apoptosis inhibition, but the differences were not significant. Additional studies are needed to determine if other caspase inhibitors, specifically those being used in clinical trials to prevent fibrosis may yield improved outcomes in wound healing.

3.5 Figures and Figure Legends





3.5.1 Figure 1 Effect of inhibition of apoptosis on vessel density and caspase 3 activation in wounds

Figure 2a

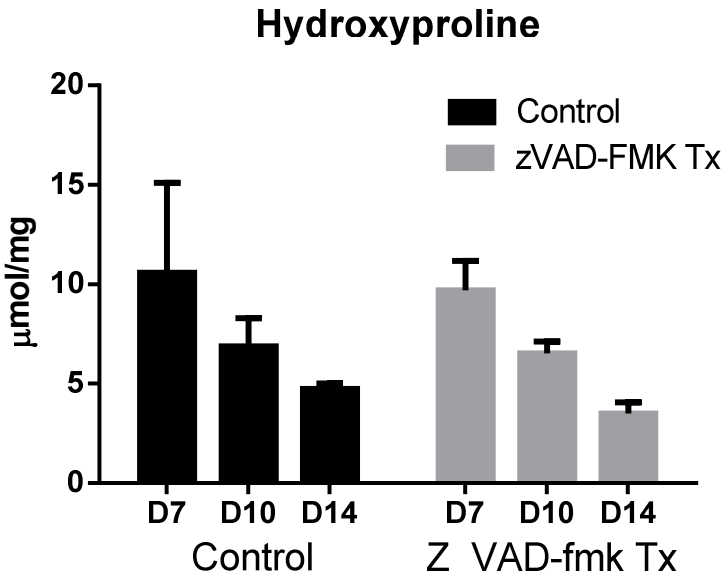
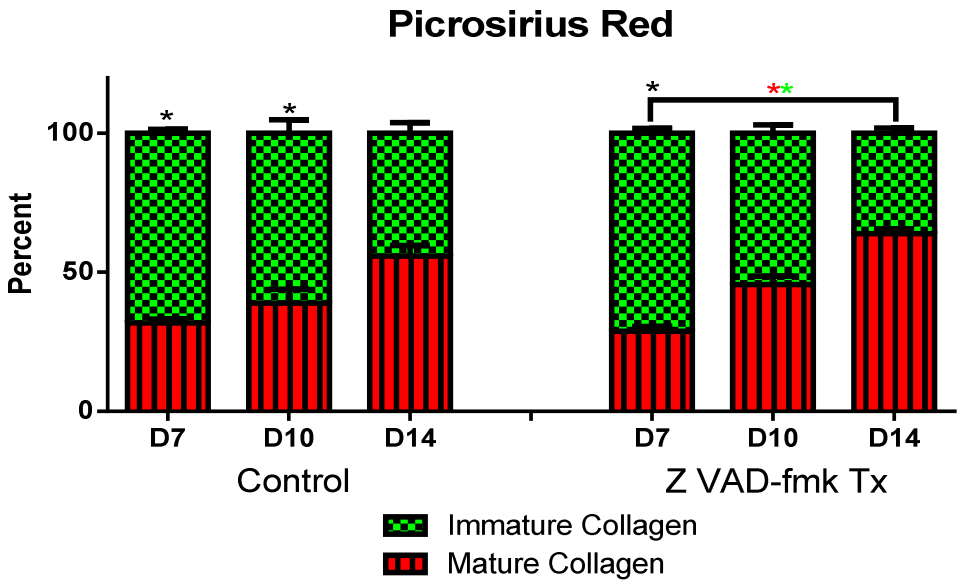


Figure 2b



3.5.2 Figure 2 Formation of fibrous scar tissue is not significantly altered when apoptosis is inhibited

Figure 3a

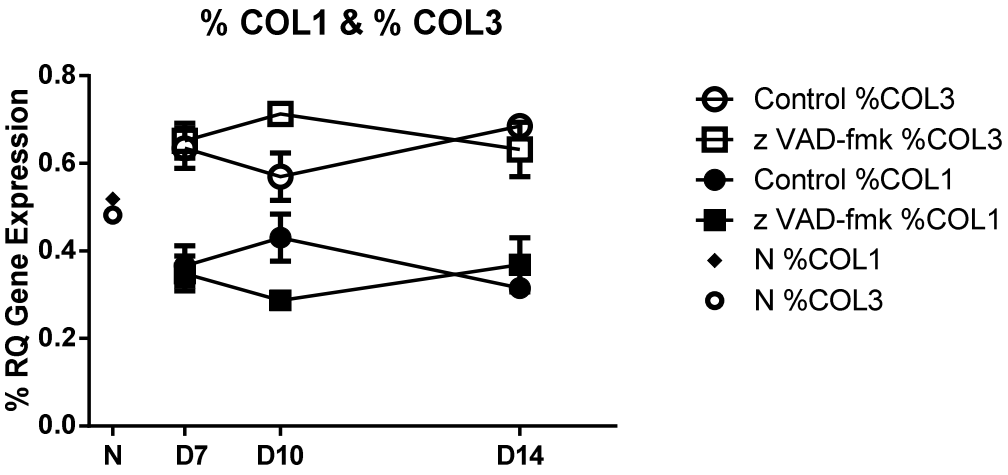
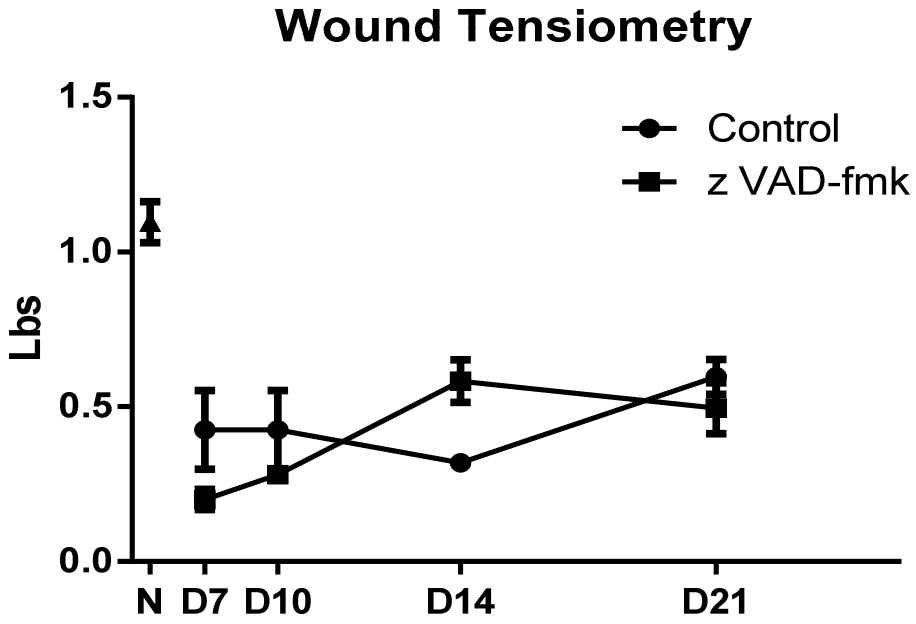
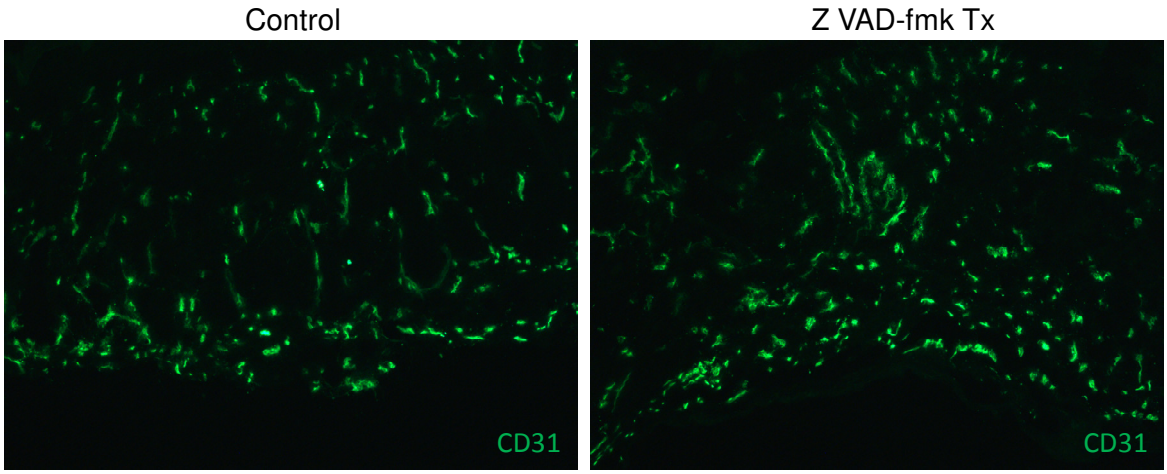


Figure 3b



3.5.3 Figure 3 Inhibition of caspase cleavage did not significantly alter collagen gene expression and wound breaking strength

Figure 4



3.5.4 Figure 4 Caspase cleavage inhibition confers a mircovessel architectural change

Figure Legends

Figure 3.5.1 – Effect of inhibition of apoptosis on vessel density and caspase 3 activation in wounds. Inhibition of apoptosis significantly increased vessel density but did not significantly decrease cleavage of Caspase 3. (a)Vessel density was determined by quantification of immunofluorescent images. Wound tissue was cryosectioned and the sections immunofluorescently labeled for CD31. Immunofluorescent images were obtained and quantified with ImageJ software for %CD31/wound area at D7, D10, and D14, in both control and z VAD-fmk treated wounds. The results are shown as the mean ± SEM; n=3. Data were analyzed by two-way ANOVA and Bonferroni's posttest (* $p<0.05$). (b)Cleavage of caspase 3 was determined by analysis with a DUPLEX ELISA kit from Abcam for active and pro-caspase 3. Wounds were harvested, and tissue processed according to the manufacturer's instructions. The harvested wounds were from D7, D10, and D14 in both control and z VAD-fmk treated groups. The results are shown as the mean ± SEM; n=3. Data were analyzed by two-way ANOVA and Bonferroni's posttest.

Figure 3.5.2 - Formation of fibrous scar tissue is not significantly altered when apoptosis is inhibited. (a) Hydroxyproline measures total collagen. The levels of hydroxyproline were not significantly different between the control and z VAD-fmk treatment groups at D7, D10 and D14. The results are shown as the mean ± SEM; n=3. Data were analyzed by two-way ANOVA and Bonferroni's posttest, however no significance was reached. (b) Picrosirius red staining was used to measure the changes in collagen maturity at D7, D10 and D14 in control and z VAD-fmk treated wounds.

Images were analyzed for %red and %green/yellow, for mature and immature collagen, respectively. The results are shown as the mean± SEM; n=3. Data were analyzed by two-way ANOVA and Bonferroni's posttest (*p<0.05).

Figure 3.5.3 – Inhibition of caspase cleavage did not significantly alter collagen gene expression and wound breaking strength. Gene expression of Col1 (mature) and Col3 (immature) and wound breaking strength were not significantly modified with anti-apoptosis treatment (a) Real time RT-PCR of Col1 and Col3 were performed on RNA isolated from uninjured tissue (UI) and wound samples at D7, D10, and D14 post-injury. To determine relative changes in mRNA levels during wound healing, all samples were normalized to Gapdh expression in uninjured tissue. The results are shown as the mean ± SEM; n=3. Data were analyzed by two-way ANOVA and Bonferroni's posttest (*p<0.05). (b) Wound tensiometry was performed on tissue at the time of euthanasia by obtaining a biopsy in a dog bone shape and stretching it on a wound tensiometer to the point of breaking for D7, D10, and D14 post injury in the control and z VAD-fmk treatment groups. All samples were normalized to uninjured tissue and expressed as a % of wound breaking strength. The results are shown as the mean ± SEM; n=3. Data were analyzed by two-way ANOVA and Bonferroni's posttest, however no significance was reached.

Representative photos of CD31 immunofluorescence in D10 wounds

Figure 3.5.6: Caspase cleavage inhibition confers a mircovessel architectural change

Immunofluorescence for CD31 (Pecam) was performed on D7, D10, and D14 control and Z VAD-fmk treated wounds. Images were taken of immunofluorescent staining of the wound bed. The images were quantified (data quantified in Figure 1a). These are representative images of showcasing the larger, more convoluted vessel lumens present in the Z VAD-fmk treated wound.

CHAPTER 4
Apoptotic endothelial cells: factors that influence the fibrotic response

4.1 Introduction

Fibrosis may occur in nearly any organ system, with fibrosis in the lung, liver, kidney, and heart receiving significant experimental attention. In the skin, fibrosis or scarring can vary from normal scarring to hypertrophic scars, keloids, and painful contractures. In particular, scarring can create serious functional problems including limited mobility, restricted skeletal growth, and weakened tissue resulting in wound dehiscence.

Fibrosis in any organ is characterized by increased presence, differentiation and persistence of myoFBs. Generally, myoFB presence and persistence is associated with increased collagen deposition, increased ratios of collagen type 1 to collagen type 3, (termed fibrotic collagen profile) and decreased collagen degradative capacity, a modified balance of matrix metalloproteinases (MMP) with their inhibitors, tissue inhibitor metalloproteinases (TIMP) [195]. The normal progression of a healing wound is similarly characterized by an increase in collagen deposition and decrease in collagen degradation. In wounds, the presence and persistence of myoFBs is associated with scar formation. There are several striking commonalities among fibrosis in the liver, lung, kidney, heart, and skin, including the altered collagen profile, collagen synthesis, and degradation. Additional commonalities among fibrotic conditions include increased apoptotic cells and increased vascular density. The type of apoptotic cell varies, but high levels of apoptosis are consistent across many experimental and clinical models of fibrosis.

Up until recently, apoptotic cells were considered inconsequential to the final outcome of fibrosis, and apoptosis was viewed predominantly as a necessary function of development and the standard mechanism of cell death. Recently, however, apoptosis has been identified as a key player in the initiation, propagation, and resolution of organ fibrosis [196]. Two major mechanisms for apoptotic cell signaling have been identified: indirect and direct. Indirect signaling is a consequence of macrophage, neutrophil or other leukocyte stimulation after phagocytosis, or efferocytosis of apoptotic cells. Direct signaling occurs after cell death is initiated and the apoptotic cells begin secreting factors that act in a paracrine fashion on surrounding cells [197].

Apoptosis is a naturally occurring process, in which cells undergo efficient elimination during development, when damaged tissue is repaired, and as normal tissue maintains a consistent turnover rate in the human body. Apoptosis occurs through two major pathways in the human body; intrinsic and extrinsic. In intrinsic apoptosis, UV damage, radiation, ischemia, or other types of damage to the cell result in caspase 2 autoactivation. Caspase 2 regulates cytochrome c release from the mitochondria. After cytochrome c is released from the mitochondria, it is recruited along with caspase 9 and apoptosis protease activating factor-1 (Apaf-1) to form the apoptosome. The formation of the apoptosome results in cleavage of caspase 9 which in turns cleaves and activates the executioner caspases, 3, 6, and 7, and results in DNA fragmentation and cell death. In the extrinsic pathway a death ligand binds to a death receptor. Common death ligands and receptors include the TNF-alpha receptor, the TNF receptor gene super family, Fas ligand and receptor, and death receptors 3, 4, and 5. After ligand-

receptor binding, the intracellular portion of the death receptor recruits the TNF receptor associated death domain (Tradd), Fas associated death domain, and caspase 8. These 3 components make up the death inducing signaling complex (DISC). Formation of the DISC results in cleavage and activation of caspase 8. Cleaved caspase 8 starts the caspase cleavage cascade which ultimately results in cleavage of the executioner caspases, and cell death [11, 12].

The purpose of this study was to determine whether direct paracrine signaling occurs from apoptotic ECs, and to identify paracrine factors that might be produced. Candidate factors secreted by apoptotic ECs were examined for their ability to promote fibrotic collagen synthesis (COL1+COL3), increase the fibrotic collagen profile (COL1:COL3), and decrease the collagen degradative capacity (MMP1:TIMP1) of FBs, *in vitro*. We hypothesize that apoptotic ECs contribute to fibrosis and scar formation by secreting factors that increase collagen synthesis, increase the fibrotic collagen profile, and decrease collagen degradation in FBs *in vitro*, and that blocking synthesis and secretion of these factors will prevent ameliorate the fibrotic effect of apoptotic ECs.

4.2 Materials and Methods

4.2.1 Cell culture

Human microvascular ECs (HMECs-1), obtained from CDC (Atlanta, GA, USA), were grown in EBM-2 (endothelial basal media-2, Lonza, Walkersville, MD, USA) supplemented with 10% fetal calf serum, 10ng/mL Epidermal Growth Factor (BD, Bedford, MA, USA), 100 U/ml penicillin and 100 µg/ml streptomycin. The THP-1 monocyte/macrophage cell line (ATCC, Manassas, VA, USA), was grown in RPMI-1640 (Gibco, Grand Island, NY, USA) culture medium supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 µg/ml streptomycin. THP-1 monocytes were differentiated or "activated" to become macrophages at a seeding density of 5×10^5 cells per ml in serum-free RPMI-1640 (Gibco) with 200 nM PMA (Sigma, St. Louis, MO, USA) for 24 h [198]. After incubation, non-adherent cells were removed by aspiration, and the adherent cells were washed with supplemented RPMI three times. Normal human dermal fibroblasts (NHDF) were obtained from ATCC, and grown in DMEM culture medium supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 µg/ml streptomycin. Apoptosis was induced in both HMEC-1 and activated THP-1 macrophages using serum starvation (basal media, EBM-2 and RPMI, respectively) and 5 µM camptothecin (Sigma) after 24h of culture under normal (supplemented media) conditions.

4.2.2 siRNA knockdown and generation of and treatment with conditioned media

siRNAs (negative control, positive control (GAPDH with a FITC tag), CTGF, CYR61, HSP47, and STMN1) were purchased from Ambion (Grand Island, NY, USA). siRNAs were transfected using the Lipofectamine RNAiMAX (Ambion) at 4µL of 20 nM siRNA per 400µL OPTI-mem (Gibco) and 5µL Lipofectamine RNAiMAX. The siRNA

lipofectamine solution was allowed to set for 15 minutes at room temperature prior to removing the normal media and washing the cells. The siRNA complex was incubated with the cells for 6 hours. At this point the transfection stability was checked with immunofluorescence and determined to be 95% efficient. The transfection media was replaced with supplemented endothelial media for 18 hours. The cells were washed and the treatment media (no treatment – supplemented media or serum starved – basal media) was added to each well for 24 hours to generate apoptotic and non-apoptotic conditions. After several washes with PBS, the treatment media was replaced with basal media for 24 hours to generate conditioned media. At the time the conditioned media was harvested, RNA was isolated from HMEC-1 for gene expression analysis. Conditioned media was mixed in a 1:1 ratio with DMEM and incubated with NHDF for 24 hours. After 24 hours the RNA was isolated from the NHDF and analyzed for gene expression analysis.

4.2.3 Real time RT-PCR

Total RNA was isolated from 6 wells per treatment group point per siRNA knockdown target using TriZol (Invitrogen, Carlsbad, CA, USA) according to manufacturer instructions. The concentration of RNA was determined with Nanodrop 2000 (Thermo Scientific Wilmington, DE, USA), and 1µg of RNA was used from each sample for the remainder of the RT-PCR protocol. RNA was treated with DNase I (Invitrogen), and reverse transcription performed with Retroscript kit (Ambion, Life Technologies, Grand Island, NY USA) according to the manufacturer's instructions. The cDNA was amplified on an ABI Step One Plus Real Time PCR System (Applied Biosystems, Life Technologies, Foster City, CA) in 96-well plate reactions with 3

reference gene wells and 3 target gene wells per sample. Primer sequences used for target genes analyzed are listed in Table III. To quantify relative differences in mRNA expression, the comparative C_T method ($\Delta\Delta C_T$) was used to determine relative quantity (RQ) [129]. All target genes examined in HMEC-1 were normalized to ribosomal protein large, p0 (*RPLP0*) expression in negative control. In NHDF all target genes were normalized to *GAPDH*. The ratio of relative quantities of gene expression of COL1 and COL3 is defined as the fibrotic collagen profile. Total collagen expression is defined as the sum of the relative quantities of COL1 and COL3. Collagen degradation capacity is defined as the ratio of relative quantities of MMP1 to its inhibitor TIMP1. Results were analyzed with two-way ANOVA to analyze the induction of apoptosis (No treatment and serum starved) and knockdown (+/- siRNA transfection) effects comparing no treatment and serum starved conditions with and without siRNA transfection followed by a Bonferroni's post-test with $\alpha=0.05$.

4.2.4 Immunofluorescence

For immunofluorescence, HMEC-1 and activated THP-1 were plated on 4 chamber slides (Lab-Tek, Nunc, Roskilde, Denmark). The cells were treated according to section 4.2.2. After treatment a caspase 3 detection kit (ImmunoChemistry technologies, Bloomington, MN, USA) was used to immunofluorescently label the active caspase 3, following manufacturer's instructions. Briefly, adherent cells cultured to 50-60% confluence at which time apoptosis was induced with serum starvation and 5μM camptothecin; supplemented media was used as a negative control with non-apoptotic cells. The cells were washed several times with 1X PBS followed by incubation in 1X FAM-FLICA (Caspase 3 assay kit, ImmunoChemistry technologies) in supplemented

media for 1 hour. Cells were washed several times with 1X Apoptosis Wash Buffer (Caspase 3 assay kit, ImmunoChemistry technologies). Cell nuclei were labeled with Hoescht 33342 (Caspase 3 assay kit, Immunochemistry technologies). At this point the chambers were removed and the slides were then mounted in aqueous mounting media (VectaMount AQ, Vector Laboratories, Burlingame, CA, USA) followed by a coverslip and sealed with nail polish. All slides were visualized immediately on a Carl Zeiss fluorescence microscope using AxioVision LE (Thornwood, NY, USA) and analyzed with ImageJ (NIH, Bethesda, MD, USA).

4.2.5 Proteomic analysis

Proteins present in conditioned media from apoptotic and non-apoptotic HMEC-1 were determined with proteomic analysis by liquid chromatography and tandem mass spectroscopy (LC-MS/MS). 10 mL of each treatment condition (non-apoptotic and apoptotic ECs) was concentrated on a reverse phase liquid chromatography column to remove salts and buffers. The proteins were eluted with 500 µL of acetonitrile. The acetonitrile was removed via speedvac centrifugation for 30 minutes. At which point the proteins digested with trypsin. The samples were brought to the proteomics core at the Research Resource Center for reverse phase column (75 150 mm Zorbax SB300 C-18, Agilent Technologies) chromatogrphay (Ultimate 3000 HPLC, Dionex, CA, USA), in tandem with a mass spectrometer through a nanospray interface (Finnigan LTQ-FT, Thermo Fisher, CA, USA). 2 MS/MS spectra for each detected ion were recorded for quantitative analysis. Data files were created and processed to produce an intermediate file containing the peaks detected and fragmented; these intermediate files are transferred to a server where sequence database searching and de-novo sequencing

may be performed. The data was imported into and analyzed with Scaffold 3.0 viewer (Proteome Software, Inc., Portland, OR, USA) for differential proteins and relative quantities.

4.3 Results

4.3.1 Serum starvation and camptothecin is sufficient to induce apoptosis in HMEC-1 and activated THP-1 macrophages

To determine an efficient, reproducible method for induction of apoptosis in HMEC-1 and THP-1 activated macrophages, cells were exposed to serum starvation and 5μM camptothecin conditions and the amount of caspase 3 protein expression was measured with immunofluorescence. After exposure to serum starvation and camptothecin conditions, active/cleaved caspase 3 protein levels were examined using fluorescently labeled inhibitors of caspases 3 which covalently bind to the active protein. The immunofluorescent cells were quantified for %Casp3 positive staining and for number of cells/field. (Figure 1 a, b, c, d, n=4 wells, *$p<0.05$). Serum starvation induced an increase in %Casp3 positive staining (Figure 1a, n=4 wells), but also induced a significant decrease in the number of cells per field (Figure 1c, n=4 wells, *$p<0.05$) in HMEC-1. While camptothecin treatment induced a decrease in the %Casp3 positive staining (Figure 1a,, n=4 wells), but also induced a significant decrease in the number of cells per field (Figure 1c, n=4wells, *$p<0.05$). The values of %Casp3 and number of cells were used to calculate the %Casp3+ cells per field, which is increased in both serum starved and camptothecin treated HMEC-1 (Figure 1e, n=4 wells). This suggests that camptothecin induces high levels of apoptosis, but that cells may become non-adherent after 24h (since the total number of cells was low), while serum starvation also induces high levels of apoptosis, and maintains a significantly higher number of cells (Figure 1c, *$p<0.05$). Casp3 positive staining in activated THP-1 macrophages showed similar results with an increase in %Casp3 (Figure 1b, n=4 wells, NS) but maintains the

cells per field (Figure 1d, n=4wells, NS) with serum starvation and camptothecin treatment. The values of %Casp3 and number of cells were used to calculate the %Casp3+ cells per field, which is increased in both serum starved and camptothecin treated, activated THP-1 macrophages (Figure 1f, n=4 wells). This data suggests that serum starvation and 5μM camptothecin are sufficient to induce Casp3 mediated apoptosis in HMEC-1 and activated THP-1 macrophages.

4.3.2 Conditioned media from apoptotic and non-apoptotic HMEC-1 and apoptotic and non-apoptotic activated THP-1 macrophages induces a significant fibrotic response

To determine the effect of paracrine factors secreted by apoptotic cells on NHDF we combined conditioned media from apoptotic and non-apoptotic HMEC-1 or apoptotic and non-apoptotic, activated THP-1 macrophages with basal media (DMEM) in a 1:1 ratio, and incubated it with NHDF for 24h. Conditioned media from serum starved HMEC-1 induced a slight increase in the fibrotic collagen profile while conditioned media from camptothecin treated HMEC-1 induced a significant increase in the fibrotic collagen profile (Figure 2a, n=4 wells, *$p<0.05$). Conditioned media from serum starvation and camptothecin treated HMEC-1 induced a slight decrease in total collagen (Figure 2b, n= 4 wells, NS). Interestingly, conditioned media from serum starved HMEC-1 induced a decrease in the collagen degradation capacity, while CM from camptothecin treated HMEC-1 had no effect on the collagen degradation capacity (Figure 2c, n= 4 wells, NS). This data suggests that conditioned media from serum starved, apoptotic HMEC-1 does not induce a significant fibrotic effect, however the fibrotic collagen profile is increased, and the total collagen expression is slightly decreased, meaning that the

majority of the lower level of collagen being synthesized is type 1 collagen. Conditioned media from camptothecin treated, apoptotic HMEC-1 significantly increases the fibrotic collagen profile, a slight insignificant decrease in total collagen, and no change in collagen degradative capacity. Taken together this data suggests that conditioned media from apoptotic HMEC-1 (serum starved and camptothecin treated) induces variable fibrotic effects, some of which are significant. Conditioned media from serum starved, and camptothecin treated, activated THP-1 macrophages induced no change in the fibrotic collagen profile (Figure 2d, n=4 wells, NS). Total collagen expression was not changed with conditioned media from serum starved, activated THP-1 macrophages, but conditioned media from camptothecin treated, activated THP-1 macrophages resulted in significantly decreased levels of total collagen expression (Figure 2e, n=4 wells, *$p<0.05$). Collagen degradative capacity was slightly, but not significantly decreased with conditioned media from serum starved, activated THP-1 macrophages, but was significantly decreased with conditioned media from camptothecin treated, activated THP-1 macrophages (Figure 2f, n=4 wells, *$p<0.05$). This data suggests that conditioned media from camptothecin treated, apoptotic, activated THP-1 macrophages induces a significant fibrotic effect by significantly decreasing total collagen synthesized but increasing the fibrotic collagen profile and significantly decreasing the collagen degradative capacity. These effects were not seen in serum starved, apoptotic, activated THP-1 macrophages. Taken together this suggests that serum starvation of THP-1 cells does not induce significant amounts of apoptosis (Figure 1f) and therefore does not elicit the fibrotic response in NHDF, while high levels of apoptosis with camptothecin treatment in activated THP-1 macrophages

results in significant fibrotic effects. The modulation in conditioned media from apoptotic HMEC-1 and apoptotic, activated THP-1 macrophages in the fibrotic response in NHDF is likely due to secreted paracrine factors.

4.3.3 Proteomic analysis of conditioned media from apoptotic and non-apoptotic HMEC-1 revealed several secreted paracrine factors

To identify the secreted paracrine factors present in the conditioned media from apoptotic HMEC-1 that may be eliciting the fibrotic effect, CM from non-apoptotic, serum starved, and camptothecin-treated HMEC-1 was subjected to LC-MS/MS analysis. The analysis showed several different proteins present in both apoptotic and non-apoptotic conditioned media; however several proteins were identified as only present in apoptotic conditioned media (Table IV). CTGF, CYR61, and STMN1 were all observed in conditioned media from serum starved, apoptotic HMEC-1. While HSP47 was present only in conditioned media from camptothecin treated, apoptotic HMEC-1. CTGF, CYR61, and HSP47 were also present in non-apoptotic CM, however for each of these, a higher quantity of peptide fragments were observed in apoptotic CM. This data suggests that CTGF, CYR61, HSP47, and STMN1 may contribute to the fibrotic effect of apoptotic CM.

4.3.4 siRNA is capable of efficient knockdown of target gene expression in HMEC-1

In view of the proteins identified in apoptotic and non-apoptotic conditioned media, we identified 4 candidate factors for further study: CTGF, CYR61, HSP47, and STMN1. These factors were selected primarily due to prior observations that described a role for these proteins in fibrosis *in vivo* [29-32, 34, 36, 38, 39, 42, 44, 45, 49-51, 53,

54, 63-70, 163-165, 169, 184, 191, 199-206]. To determine the contribution of each factor to the paracrine fibrotic effect of the CM, siRNA knock down of each was performed prior to induction of apoptosis. Given that proteomics identified 3 of the 4 factors of interest in conditioned media from serum starved, apoptotic HMEC-1, and that serum starvation is a more physiologically relevant approach to inducing apoptosis; we used serum starved conditions instead of camptothecin to induce apoptosis and examine the importance of each candidate factor. Under conditions of serum starvation induced apoptosis, we used siRNA knockdown to independently reduce the level of each of the 4 candidate factors. GAPDH siRNA treatment was used as a positive control. siRNA knockdown resulted in significantly decreased gene expression of three of the proteins (CTGF, CYR61, HSP47) as well as the control GAPDH in both apoptotic and non-apoptotic treatment conditions (Figure 3a, b, c, and d, n=6-wells, *$p<0.05$). While STMN1 gene expression was significantly decreased with siRNA treatment under apoptotic conditions, STMN1 expression was significantly increased with siRNA treatment under normal conditions (Figure 3e, n=6 wells, *$p<0.05$). The increased STMN1 expression in non-apoptotic culture conditions could be a result of efficient knockdown early, followed by rapid hyperproliferation and loss of the siRNA effect after numerous cell divisions. The loss of the siRNA effect early after siRNA transfection would result in an increased number of cells expressing normal STMN1. This data suggests that siRNA knockdown significantly and effectively reduced gene expression of GAPDH, CTGF, CYR61, HSP47, and STMN1 under apoptotic conditions.

4.3.5 Targeted gene knockdown alters the effects of conditioned media from apoptotic cells on NHDF

After confirmation of efficient target gene knockdown with siRNA, the conditioned media from apoptotic and non-apoptotic HMEC-1 with siRNA knockdown was placed on 60-70% confluent NHDF culture (1:1 ratio with basal DMEM) for 24 hours. When the fibrotic collagen profile was examined, knockdown of CYR61 and STMN 1 each led to a significant change in response to the CM from apoptotic cells, with little change seen in the non-apoptotic conditions (Figure 4b, 4d, *$p<0.05$). In contrast, knockdown of CTGF and HSP47 each had no effect on the fibrotic profile following exposure to CM (Figure 4a, 4c, 4d, NS). Total collagen expression was also examined; significant decreases in the total collagen expression were observed following siRNA knockdown of CTGF, CYR61, and STMN1 under apoptotic conditions (Figure 5a, 5b, 5d, n=4 wells, *$p<0.05$); no such effect was observed in CM derived from non-apoptotic conditions. Knock-down of HSP47 had no significant effect the ability of CM to modify total collagen production under any condition (Figure 5c, n=4 wells, NS). Collagen degradation profiles were also modified in CM derived from siRNA knockdown conditions. Increases in the collagen degradative capacity were observed under siRNA knockdown of all four candidate factors in CM from apoptotic HMEC-1 (Figure 6a, 6b, 6c, 6d, n=4 wells, NS). This data suggests that siRNA knockdown of the candidate secreted proteins results in significant effects on the fibrotic response in NHDF *in vitro*.

4.4 Discussion

Wound healing is a complex process that involves 4 distinct yet overlapping phases: hemostasis, inflammation, proliferation, and remodeling. During these phases certain cell types become necessary and are recruited to the wound bed, while others become unnecessary or overly abundant at which point they are removed from the wound bed through migration or apoptosis. Previously, cells that undergo programmed cell death, were believed to not elicit an immune response and to have little effect on the surrounding cells. Recent studies, including this one, have shown that apoptotic cells can affect the surrounding environment and, in fact may have a significant effect on the outcomes of injury and fibrosis [87, 96, 97, 183, 191, 197, 207-211]. Research in fibrotic conditions such as liver, lung, kidney, cardiac, and skin fibrosis have identified high apoptotic levels in the initiation, propagation, and resolution of fibrosis. Apoptosis resistance of FBs and myoFBs, increased myoFB differentiation, and increased FB and myoFB differentiation develop after exposure to apoptotic cells. The secreted factors from apoptotic ECs may have a direct paracrine signaling effect, or may recruit macrophages, neutrophils, and other leukocytes which change the antigens they present or the factors they secrete following phagocytosis of apoptotic cells [197].

Several cell types have been identified as contributing to the apoptotic load in a healing wound depending on the phase. In early wound healing, the hemostasis and inflammatory phases, the highest level of apoptotic cells is neutrophils [12]. These cells undergo apoptosis and recruit more inflammatory cells, specifically macrophages, to the wound bed ensuring the movement into the inflammatory stage. Throughout the processes of wound healing, macrophages are recruited and undergo apoptosis as well. The precise mechanism of apoptosis in wound healing has not been determined, but

the downstream effects are thought to promote exit from the inflammatory stages into the proliferative phases [12, 77]. As the wound progresses out of the proliferative phase FBs and ECs from abundant proliferation and vascularity also undergo apoptosis for efficient elimination from the wound bed [12, 79].

The effects mediated by apoptotic cells are believed to be conferred through two predominant mechanisms: immune modulation (indirect) and paracrine signaling (direct). When immune cells engulf apoptotic cells, the inflammatory state of the macrophages and the resolution of inflammation can be altered. More specifically, phagocytosis of apoptotic cells has been shown to cause macrophages to switch from an M1 (pro-inflammatory) to an M2 (pro-healing) phenotype [78]. There is some disagreement regarding the precise changes in the macrophage phenotype; however, the majority of studies suggest that macrophages begin to express different cellular markers and secrete different cytokines and growth factors following the switch [78]. One potential phenotype change is from a pro-inflammatory or a pro-healing macrophage to a pro-fibrotic macrophage. In this scenario macrophages begin secreting cytokines and growth factors that contribute to the severity of the fibrotic outcome by increasing collagen synthesis, decreasing collagen degradation, and altering the collagen profile. Others have identified changes in collagen degradation as a downstream effect of increased apoptotic cells [212], but failed to determine if the effect on collagen degradation was mediated by macrophages that had ingested apoptotic cells, or by apoptotic macrophages themselves.

In addition to the effect mediated by apoptotic cells once they are engulfed by macrophages, another possible mechanism by which apoptotic cells can influence

tissue repair outcomes is direct paracrine signaling. Direct paracrine signaling may act by enhancing cellular proliferation and pro-fibrotic phenotypes. One cell that is likely to exert such paracrine effects in wounds is the endothelial cell.

Endothelial cell (EC) apoptosis is a prominent feature in the later stages of wound healing, suggesting that endothelial-FB interactions may play an important role in fibrotic wound healing outcomes. The connection between fibrosis and EC apoptosis has been shown in cardiac and renal allograft rejection, pulmonary fibrosis and now in the skin [18-26]. The current study demonstrates that conditioned media from apoptotic ECs (Figure 2a, b, and c, Table IV) contains secreted factors which influence the fibrosis *in vivo* and *in vitro.* One limitation of our design involves the use of unconcentrated conditioned media. Concentration of conditioned media from higher volumes may have elicited significant responses from NHDF where fibrotic responses derived from unconcentrated conditioned media elicited only minor responses. We identified several secreted factors present in conditioned media from apoptotic ECs in higher levels than conditioned media from non-apoptotic ECs (Table IV). Of these factors we chose to further examine the downstream effects of CTGF, CYR61, HSP47, and STMN1 in the conditioned media. 3 of the 4 factors were identified in conditioned media were derived from serum starved, apoptotic HMEC-1. Therefore we used serum starvation conditions to induce apoptosis in siRNA knockdown experiments.

CTGF, a member of the CCN family, has been identified in many different types of fibrosis [28, 33, 34]. Specifically, elevated levels of CTGF have been seen in human sclerotic, keloid and other fibrotic lesions [28, 29], liver fibrogenesis [30], and in bleomycin-induced and idiopathic pulmonary fibrosis [31]. The structure of CTGF allows

for interaction with several different families of growth factors [27]. CYR61, also a member of the CCN family, is known to associate with integrin receptors on ECs, macrophages, monocytes and platelets, and heparin sulfate proteoglycans on FBs and smooth muscle cells [184]. CYR61 and is important during development and embryogenesis and in inflammation and tissue repair [37-42]. CYR61 is also elevated in infarct zones following myocardial infarction [48], plays a significant role in the development of IPF after lung injury [49], and overexpressed in chronic transplant vasculopathy [48, 51-54], but is downregulated in renal pathologies [50]. HSP47 acts a molecular chaperone that facilitates interactions between procollagen and its posttranslational modifiers, aiding in folding, assembly, and transport from the endoplasmic reticulum, and potentially in the ultimate secretion of mature collagen [57, 58]. HSP47 is upregulated in liver fibrosis, bleomycin-induced pulmonary fibrosis, age-associated renal fibrosis, and in keloids [63-66], [67]. STMN1, unlike CTGF, CYR61, and HSP47 is known only for its regulation of microtubule dynamics, particularly in cell cycle control [68-70]. In a mutated form, STMN1 results in cellular hyperproliferation in the progression of cancer [68]. Translating cellular hyperproliferation to fibrosis we can see that STMN1 may also play a key role in the regulation of FB and myoFB hyperproliferation [71, 72].

In conclusion, we identified a variable pro-fibrotic response in normal human dermal FBs to conditioned media generated from apoptotic ECs and macrophages. Additionally we identified CTGF, CYR61, HSP47, and STMN1 proteins in conditioned media from apoptotic and non-apoptotic ECs at varying levels. When these factors are targeted for siRNA knockdown prior to induction of apoptosis we observed an

amelioration of at least one of the three aspects identified as pro-fibrotic; total collagen synthesis, fibrotic collagen profile, and collagen degradative capacity. This suggests that when the target genes are not expressed, the downstream pro-fibrotic effects of apoptotic ECs are diminished as well. This research confirms the involvement of CTGF, CYR61, HSP47, and STMN1 in the initiation and propagation of fibrosis *in vitro*, and further outlines their anti-fibrotic therapeutic potential.

4.5 Figures and Tables

Table III
Human RT-PCR primer sequences

Gene	Forward Primer Sequence	Reverse Primer Sequence
GAPDH	CAGGGCTGCTTTTAACTCTGG	TGGGTGGAATCATATTGGAACA
COL1A2	AGGACAAGAAACACGTCTGG	GGTGATGTTCTGAGAGGCATAG
COL3A1	AAGTCAAGGAGAAAGTGGTCG	CTCGTTCTCCATTCTTACCAGG
MMP1	GCACAAATCCCTTCTACCCG	TGAACAGCCCAGTACTTATTCC
TIMP1	TTCTGCAATTCCGACCTCG	TCATAACGCTGGTATAAGGTGG
CTGF	ACCTGTGCCTGCCATTAC	TCTCTCACTCTCTGGCTTCA
CYR61	GAAGCAGCGTTTCCCTTCTA	CTGACACTCTTCTCCCTTGTTT
HSP47	GTGAGACCAAATTGAGCTAGGG	TAGTTGGGAGAGGTTGGGATAG
STMN1	CCTGTCGCTTGTCTTCTATTCA	GGGCTGAGAATCAGCTCAAA
RPLP0	ATCAACGGGTACAAACGAGTC	CAGATGGATCAGCCAAGAAGG

Figure 1

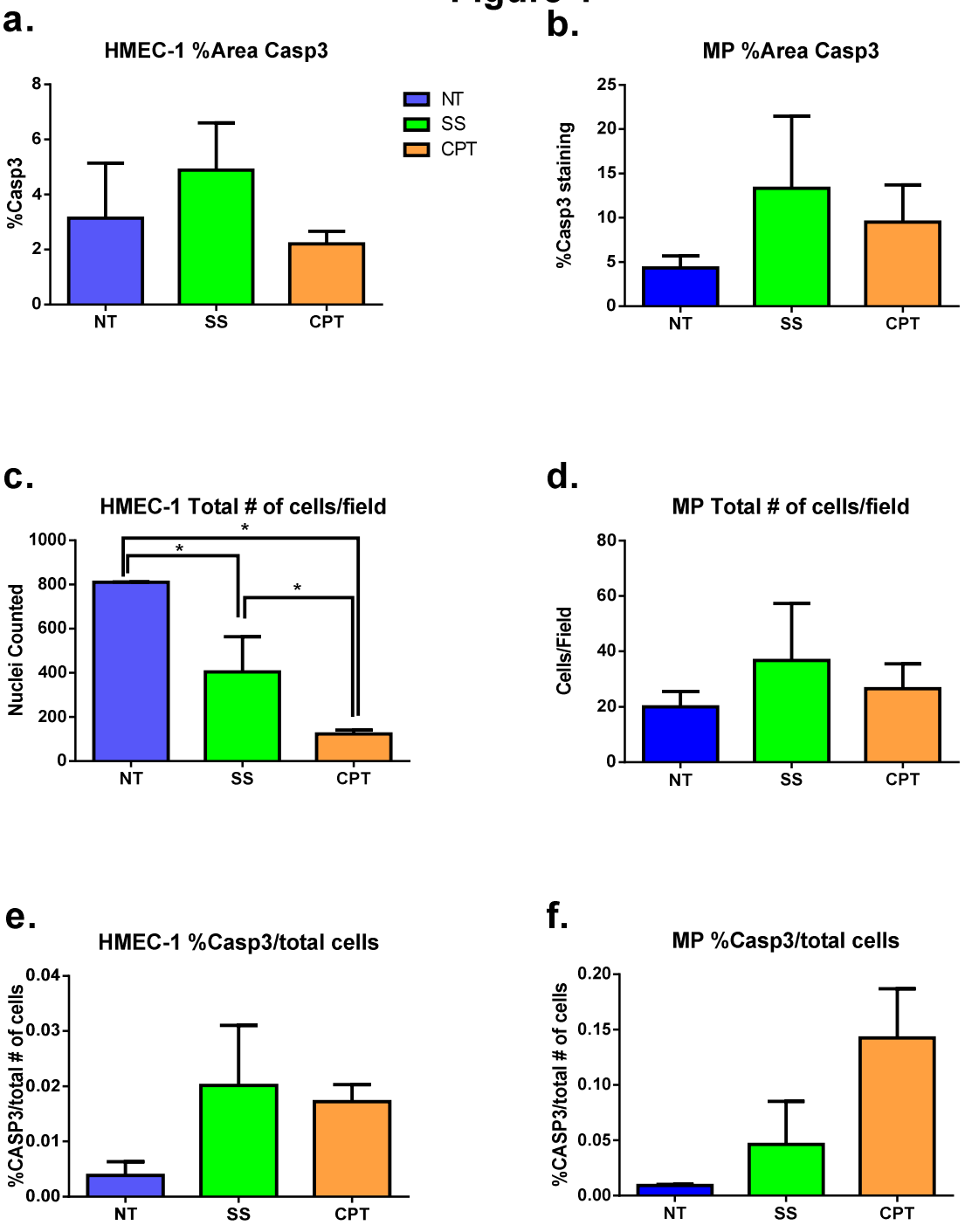


Figure 1 Serum starvation and camptothecin (CPT) treatment of HMEC-1 and activated THP-1 macrophages increased %Casp3 immunofluorescence, modulated total number of cells/field, and increased the number of Casp3+ cells/field

Figure 2

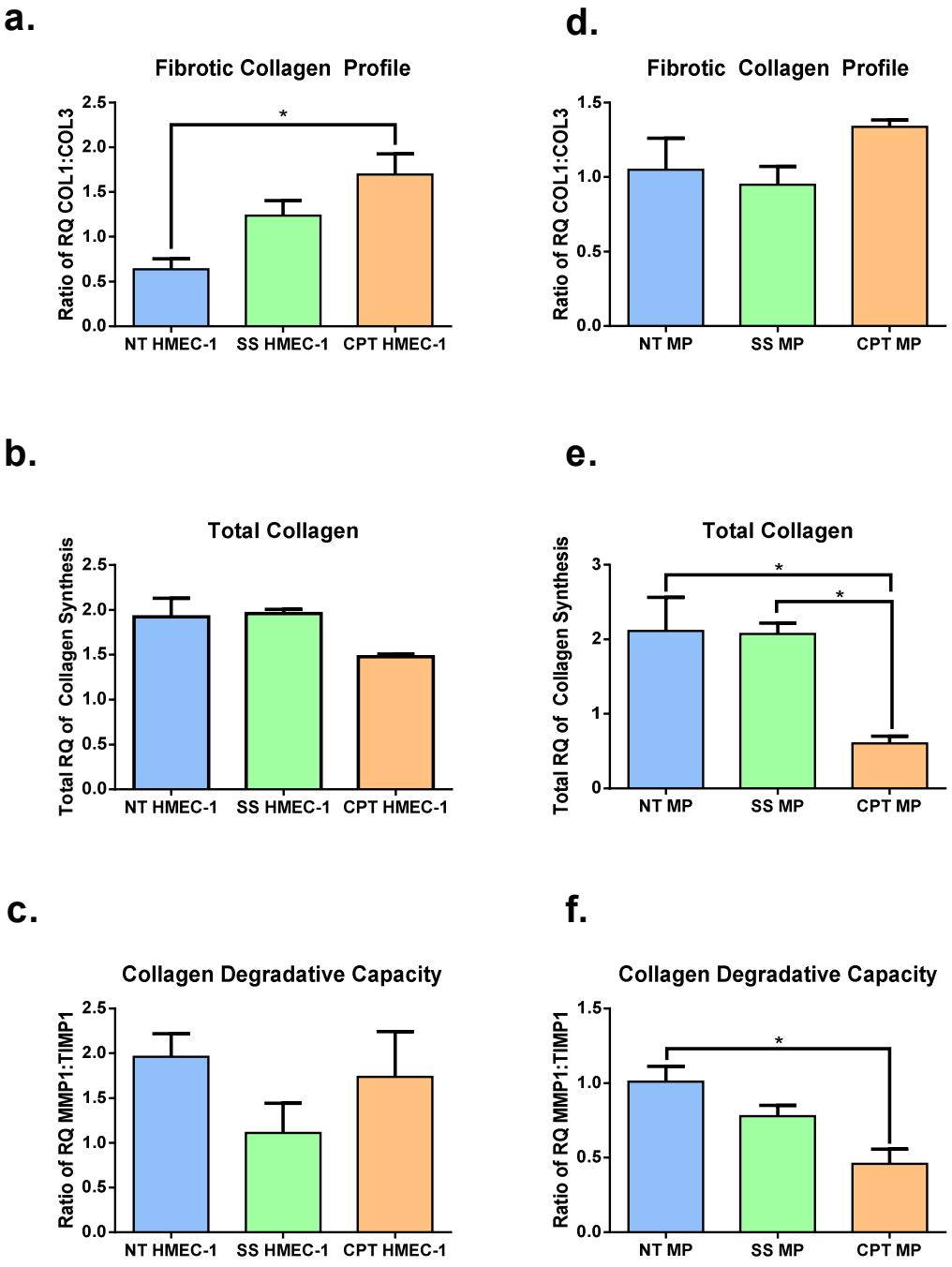


Figure 2 - Significant differences in fibrotic collagen profile, total collagen, and collagen degradative capacity was observed in NHDF treated with CM from apoptotic and non-apoptotic ECs

Table IV

Secreted factors present in conditioned media from apoptotic and non-apoptotic ECs

Factors in non-apoptotic conditioned media	Factors in both apoptotic and non-apoptotic conditioned media			Factors in apoptotic conditioned media
Voltage-dependent anion selective channel protein 1	Superoxide dismutase *	Filamin-A*	Protein CYR61*	Stathmin
ATP synthase subunit beta	Desmoplakin*	Calmodulin-like protein 5	Protein S100-A11	Neuroblast differentiation associated protein
Prolactin-inducible protein	Desmoglein-1	Cystatin-A	Fructose-bisphosphonate aldolase A *	
Integrin beta 1	Junction plakoglobin*	Connective tissue growth factor*	Serpin H1*	Clathrin heavy chain 1

Figure 3

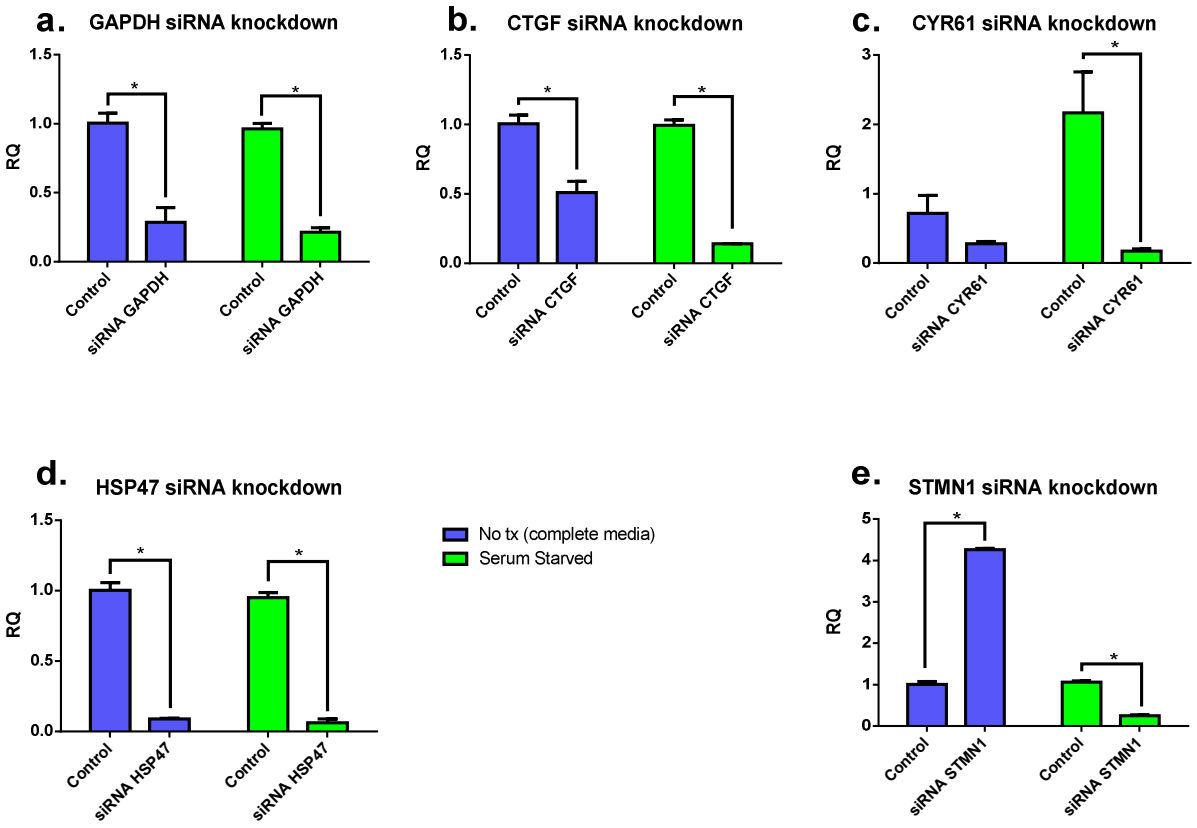


Figure 3 - siRNA transfection significantly altered gene expression of GAPDH, CTGF, CYR61, HSP47, and STMN1 in apoptotic and non-apoptotic ECs

Figure 4

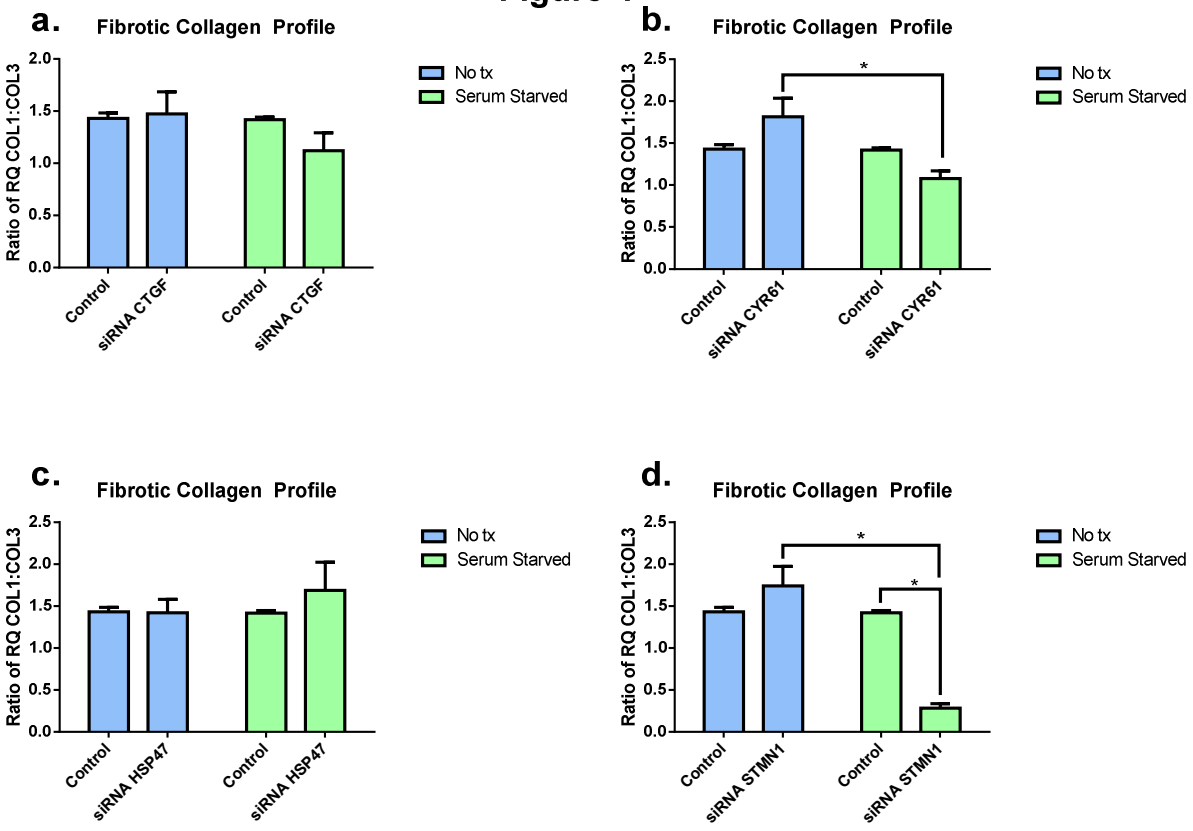


Figure 4 - The fibrotic collagen profile is significantly decreased in NHDF after exposure to CM from CYR61 and STMN1 siRNA treated apoptotic ECs

Figure 5

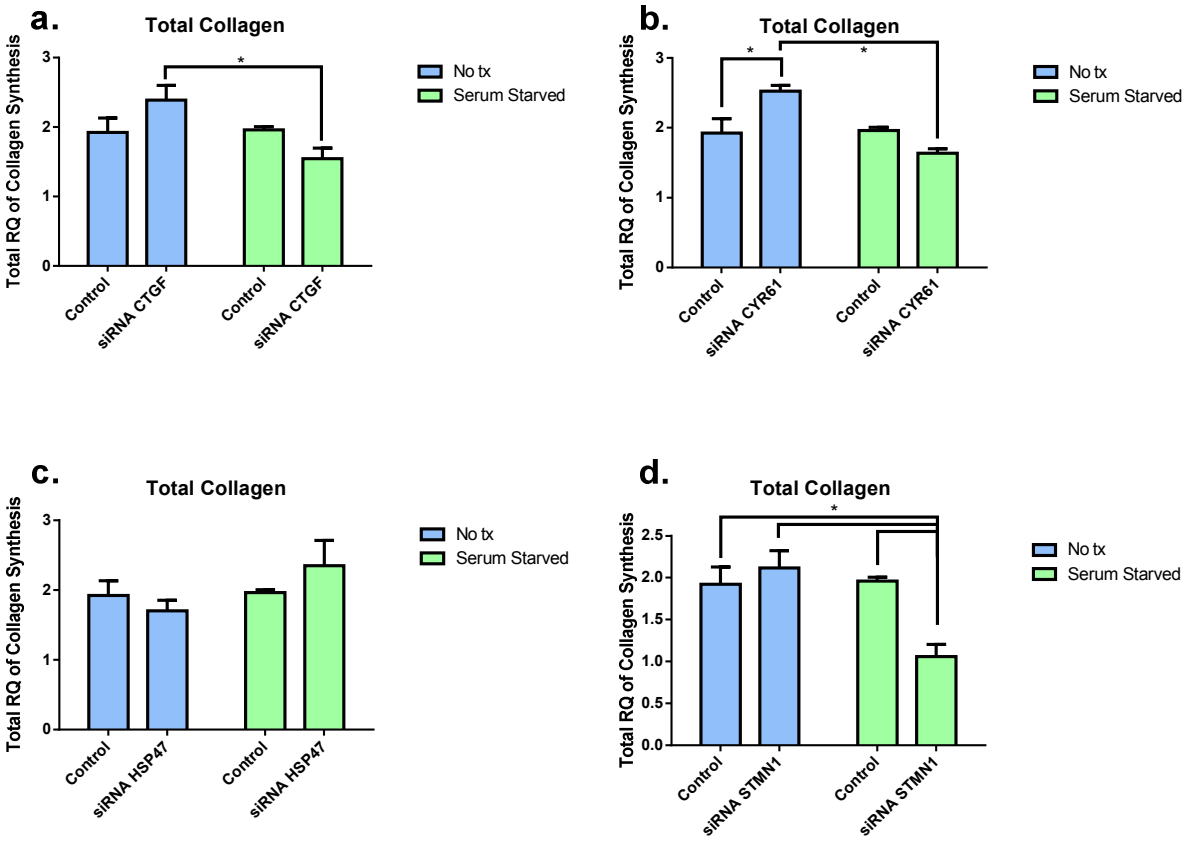


Figure 5 - Total collagen is significantly decreased in NHDF after exposure to CM from CTGF, CYR61, and STMN1 siRNA treated apoptotic ECs

Figure 6

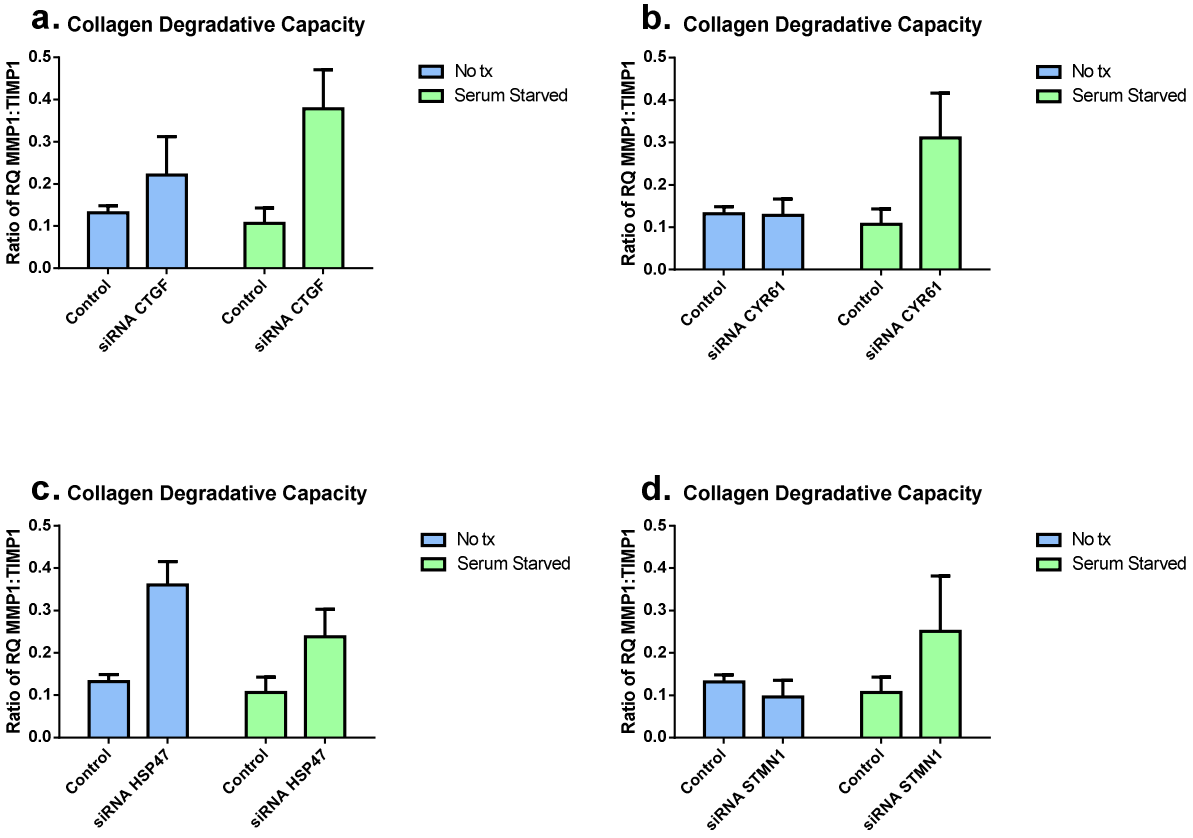


Figure 6 - Collagen degradative capacity in NHDF is increased after exposure to CM from siRNA treated apoptotic ECs

Figure and Table Legends

Table III – Human RT-PCR primer sequences. Real time PCR Primers for human genes analyzed.

Figure 1 – Serum starvation and camptothecin (CPT) treatment of HMEC-1 and activated THP-1 macrophages increased %Casp3 immunofluorescence, modulated total number of cells/field, and increased the number of Casp3+ cells/field. Immunofluorescence for cleaved (active) Casp3 was performed on non-apoptotic (supplemented media) and apoptotic (serum starved or CPT) HMEC-1 and activated THP-1 macrophages (n=4 chambers). The images were quantified using ImageJ to calculate %Casp3, total #cells/field, and %Casp3/total #cells/field. The results are shown as the mean ± SEM; n=4 chambers. Data were analyzed by two-way ANOVA and Bonferroni's post test (*$p<0.05$). (a and b) %Casp3 is increased in apoptotic HMEC-1 and THP-1 macrophages (NS). (c and d) A significant decrease in the number of cells/field was observed in HMEC-1 and no change was observed in THP-1 macrophages(NS). (e and f) Casp3/total cells is increased in apoptotic HMEC-1 and THP-1 macrophages (NS).

Figure 2 –Significant differences in fibrotic collagen profile, total collagen, and collagen degradative capacity was observed in NHDF treated with CM from apoptotic and non-apoptotic ECs. Real time RT-PCR of COL1, COL3, MMP1, and TIMP1 were performed on RNA isolated from NHDF treated with CM from apoptotic (SS or CPT) and non-apoptotic (NT) HMEC-1. To determine relative changes in mRNA levels, all samples were normalized to GAPDH expression in NHDF in basal media (DMEM) for 24h. The results are shown as the mean ± SEM; n=4 wells. Data were

analyzed by two-way ANOVA and Bonferroni's posttest (*p<0.05). (a, b, c, d, e, f) A significant increase in the fibrotic collagen profile was observed with CM from CPT treated apoptotic HMEC-1 (Figure a, n= 4 wells, *p<0.05). Total collagen was significantly increased in CM from CPT treated apoptotic, activated THP-1 macrophages (Figure 2e, n=4 wells, *p<0.05). A significant decrease in the collagen degradative capacity after incubation with CM from CPT treated apoptotic, activated THP-1 macrophages was observed (Figure 2f, n=4 wells, *p<0.05).

Table IV – Secreted factors present in conditioned media from apoptotic and non-apoptotic ECs. LC-MS/MS was used to identify proteins that were present in conditioned media from apoptotic and non-apoptotic ECs. Relative expression was determined based on number of unique peptides identified for each protein sequence. 3 proteins were identified only in apoptotic CM, 4 proteins were identified only in non-apoptotic CM, and 12 proteins were identified in both apoptotic an non-apoptotic CM.

Figure 3 siRNA transfection significantly altered gene expression of GAPDH, CTGF, CYR61, HSP47, and STMN1 in apoptotic and non-apoptotic ECs. Real time RT-PCR of GAPDH, CTGF, HSP47, and STMN1 were performed on RNA isolated from HMEC-1 treated with siRNA for each target gene. To determine relative changes in mRNA levels, all samples were normalized to RPLP0 expression in HMEC-1 treated with negative control (scramble) siRNA. The results are shown as the mean ± SEM; n=6 wells. Data were analyzed by two-way ANOVA and Bonferroni's posttest (*p<0.05).(a, b, d) Significant decreases occur in apoptotic and non-apoptotic ECs after siRNA treatment with GAPDH, CTGF, and HSP47. (c) CYR61 siRNA treatment resulted in a significant decrease in gene expression in apoptotic ECs only. (e) While STMN1 siRNA

treatment significantly decreased gene expression in apoptotic ECs, it significantly increased STMN1 expression in non-apoptotic ECs.

Figure 4 The fibrotic collagen profile is significantly decreased in NHDF after exposure to CM from CYR61 and STMN1 siRNA treated apoptotic ECs. Real time RT-PCR of COL1, COL3, MMP1, and TIMP1 were performed on RNA isolated from NHDF treated with CM from apoptotic (SS) and non-apoptotic (NT) HMEC-1. To determine relative changes in mRNA levels, all samples were normalized to GAPDH expression in NHDF in basal media (DMEM) for 24h. The results are shown as the mean ± SEM; n=4 wells. Data were analyzed by two-way ANOVA and Bonferroni's posttest (* $p<0.05$). (a and c) No significant differences were observed in the fibrotic collagen profile in NHDF after exposure to CM from CTGF and HSP47 siRNA treated ECs. (b) Significant decreases in fibrotic collagen profiles were identified in NHDF after incubation with CM from CYR61 siRNA treated ECs compared to non-apoptotic siRNA treated ECs. (d) CM from STMN1 siRNA treated ECs induced a significant decrease in the fibrotic collagen profile of NHDF compared to non-apoptotic siRNA treated ECs, and to apoptotic, negative control siRNA treated ECs.

Figure 5 Total collagen is significantly decreased in NHDF after exposure to CM from CTGF, CYR61, and STMN1 siRNA treated apoptotic ECs Real time RT-PCR of COL1, COL3, MMP1, and TIMP1 were performed on RNA isolated from NHDF treated with CM from apoptotic (SS) and non-apoptotic (NT) HMEC-1. To determine relative changes in mRNA levels, all samples were normalized to GAPDH expression in NHDF in basal media (DMEM) for 24h. The results are shown as the mean ± SEM; n=4 wells. Data were analyzed by two-way ANOVA and Bonferroni's posttest (* $p<0.05$). (c) No

significant differences were observed in the total collagen in NHDF after exposure to CM from HSP47 siRNA treated ECs. (a) Significant decreases in total collagen were identified in NHDF after incubation with CM from CTGF siRNA treated ECs compared to non-apoptotic siRNA treated ECs. (b) Significant decreases in total collagen were identified in NHDF after incubation with CM from CYR61 siRNA treated ECs compared to non-apoptotic siRNA treated ECs, while CM from siRNA treated, non-apoptotic ECs induced a significant increase in total collagen compared to non-apoptotic, negative control siRNA treated ECs. (d) CM from STMN1 siRNA treated ECs induced a significant decrease in the fibrotic collagen profile of NHDF compared to apoptotic, negative control siRNA treated ECs, non-apoptotic siRNA treated ECs, and to non-apoptotic, negative control siRNA treated ECs.

Figure 6 Collagen degradative capacity in NHDF is increased after exposure to CM from siRNA treated apoptotic ECs. Real time RT-PCR of COL1, COL3, MMP1, and TIMP1 were performed on RNA isolated from NHDF treated with CM from apoptotic (SS) and non-apoptotic (NT) HMEC-1. To determine relative changes in mRNA levels, all samples were normalized to GAPDH expression in NHDF in basal media (DMEM) for 24h. The results are shown as the mean ± SEM; n=4 wells. Data were analyzed by two-way ANOVA and Bonferroni's posttest (* $p<0.05$). (a, b, c, d) No significant differences were observed in the collagen degradative capacity in NHDF after incubation with CM from CTGF, CYR61, HSP47, and STMN1 siRNA treated ECs.

CHAPTER 5
DISCUSSION

Fibrosis may occur in nearly any organ system, with fibrosis in the lung, liver, kidney, and heart receiving the most clinical and experimental attention. In the skin, fibrosis or scarring can vary from normal scarring to hypertrophic scars or keloids. Several commonalities that are seen in nearly all types of fibrosis have been identified. These include 1) an increase in the fibrotic collagen profile identified as collagen I (Col1) to collagen III (Col3) ratio [213-215], 2) a decrease in the degradation of collagen commonly exhibited as low levels of Mmp1 and high levels of the inhibitor of Mmp1, tissue inhibitor metalloproteinase 1 (Timp1) [213-215], 3) high levels of angiogenesis, and 4) high levels of apoptosis. Despite this knowledge, effective anti-fibrotic treatments are currently limited. The magnitude of the clinical problem of scarring emphasizes the need for a further understanding of the biologic mechanisms that contribute to fibrotic outcomes in wounds, and similarly, for the exploration of factors that result in optimal healing. With this knowledge, therapeutic tools may be engineered to improve the healing process and in turn, improve patient outcomes. This dissertation research examined the relationship between apoptotic ECs and fibrosis, and suggested therapeutic directions for the reduction of fibrotic scarring outcomes in wound healing.

High levels of apoptosis are well-characterized in the development of fibrosis in the lung, liver, kidney, and heart. In scar formation from skin wounds, such an interaction has not been characterized. For decades apoptotic cells were thought to be inconsequential to the final outcome of processes such as wound healing, and viewed simply as a necessary occurrence in the function of the human body. This model has

recently been challenged, as apoptosis has been identified as a key player in the initiation, propagation, and resolution of organ fibrosis. Apoptotic cells can stimulate these fibrotic outcomes either indirectly or directly. Macrophages, neutrophils, and other leukocytes may be stimulated to secrete factors that mediate fibrotic effects following the engulfment of apoptotic cells. Direct paracrine signaling from early and late apoptotic cells themselves can also influence fibrotic outcomes (Figure 1.2). Apoptosis has also been examined in two models of wound healing that are considered regenerative or heal with little to no scarring: fetal wound healing and oral mucosal wound healing. One study has identified differential expression of cleaved caspase 7 and poly ADP-ribose polymerase (PARP) in regenerative, first trimester fetal wounds compared to scarring, late gestational wounds [216, 217], however, the cellular source of the differential apoptotic signals was not determined. Oral mucosal wound healing has previously been shown to exhibit reduced scar formation, a faster rate of re-epithelialization, lower levels of inflammation, and lower levels of angiogenesis compared to wound repair in the skin [120, 123]. However, very little attention has been given to the mechanisms that regulate cell death in skin and oral wounds. Cell death and the mechanism of cell elimination may play an important role in the scarring outcome.

Chapter 2, “Differential Apoptosis in Mucosal and Dermal Wound Healing,” demonstrated that the dominant mechanisms of apoptosis differ for wounds in the oral mucosa and skin. Given the differential scar formation in these two anatomic sites, these results suggest possible connections for apoptotic mechanisms and scarring outcomes. Our results indicated that the intrinsic apoptosis pathway may be the

predominant mechanism of induction of apoptosis in oral wound healing, while the extrinsic apoptosis pathway may play a more significant role in skin wound healing. The differences in the pathways for apoptosis induction suggest potential involvement of apoptosis regulation in scar formation and fibrotic outcomes.

The last two phases of wound healing are characterized by a significant increase in cell number (in the proliferative phase) and a significant decrease in cell number (during the remodeling phase. During the proliferative phase there is an over exuberant angiogenic response that creates a vascular bed that is several fold more dense than normal, and during the remodeling phase there are high levels of apoptotic cells, mostly due to vessel regression. One widely accepted view is that optimal wound healing requires a robust and vigorous angiogenic response [153, 154]. Yet there is emerging evidence challenging the idea that robust angiogenesis is required for optimal healing outcomes. At least five separate studies, including several from our lab, show that full thickness wounds heal well, even when angiogenesis is reduced [18, 218-221]. In recent studies, we used neutralization of vascular endothelial growth factor (VEGF) via antibody treatment to effect an approximately 50% reduction in peak wound vascularity in adult skin wounds. Surprisingly, the treated wounds closed normally, and also showed a significant reduction in scar width [218]. Beyond a reduction in capillaries, this treatment would be expected to reduce the apoptotic load in the wound.

Recent clinical studies in scarring further support the concept that hypertrophic scar formation is linked to robust angiogenesis [26], and the use of anti-angiogenic therapy to reduce scar formation has been suggested by us [218] and others [20]. The mechanism of this novel concept has yet to be determined, but several possible

mechanisms are the regulation of vascular invasion and regression. Aberrant vessel regression and angiogenesis has already been identified in exaggerated fibrotic outcomes of wound healing such as hypertrophic or keloid scarring. The balance of pro and anti-angiogenic mediators has been identified as pathogenic in liver fibrosis, lung fibrosis, and CTV. The chronic activation of the wound healing response and the imbalance of pro and anti-angiogenic mediators together may contribute to the increased apoptotic load and ultimately to fibrosis.

Clinical trials are underway for the use of caspase cleavage inhibition for treatment of fibrosis in several different organs [170-182]. In idiopathic pulmonary fibrosis, caspase cleavage inhibition has been used to decrease the extent of fibrosis and improve lung function [46-48, 51, 174, 179, 181]. Treatment with apoptosis inhibitors following a myocardial infarct decreases the size of the infarct and the corresponding cardiac remodeling, in addition to decreasing cardiac allograft vasculopathy in cardiac transplants [37, 40, 42-45, 170, 177]. Inhibition of apoptosis in models of hepatic injury and fibrosis resulted in attenuated liver fibrosis, improved liver function tests, decreased hepatic stellate cells, decreased collagen deposition [173, 176, 178, 182] and improved liver regeneration [50, 52-56]. In renal ischemia reperfusion injury, injection of apoptosis inhibitors resulted in improved organ function, decreased inflammation, and prevention of the spread of ischemia-reperfusion injury [35, 36, 41, 49, 183, 184]. The increased apoptotic load seen in vessel regression during normal wound healing may be necessary and sufficient to induce and promote the normotrophic scarring response, and aberrant angiogenic control may promote pathologic scarring.

Chapter 3, “Inhibition of apoptosis during the remodeling phase of wound healing results in increased vessel density,” examined the role apoptosis plays in scar formation during the remodeling phase of wound healing. Inhibition of apoptosis prevented vessel regression and appeared to promote the presence of larger, more convoluted vessels. However, only slight, non-significant differences were found in collagen content, architecture, or wound breaking strength. Therefore, the improved wound healing outcomes seen with inhibition of angiogenesis are likely not mediated directly by reducing the apoptotic load present in wound healing during the remodeling phase. However, as the remodeling phase progressed, vessel regression occurred regardless of inhibition of apoptosis. This suggests that vessel regression and cellular apoptosis may occur through another mechanism and that prevention of apoptosis through caspase cleavage, similar to differential apoptosis in oral and skin wound healing, may have a differential effect on the fibrotic outcomes. Additional studies are needed to determine if other caspase inhibitors, specifically those being used in clinical trials to prevent fibrosis in other tissue may yield improved outcomes in wound healing and to determine the precise mechanism of vessel regression when apoptosis is inhibited.

The interaction between apoptotic cells and the surrounding tissue is likely mediated by secreted factors. Recently, a recent study by Laplante et al. showed that conditioned media (CM) from CASP3- mediated, apoptotic ECs increased focal adhesions and gene expression of alpha-smooth muscle actin (alpha-SMA), a marker of myoFB, in FBs *in vitro*. The fibrotic effect of this CM was demonstrated both *in vivo* and *in vitro*, and was shown to derive from the presence of connective tissue growth factor (CTGF) in the CM, a protein that has been previously implicated in fibrosis [23]. While

these studies suggest that apoptotic EC (and perhaps CTGF) might send critical signals that influence fibrosis in healing wounds, this study was limited namely in its use of human umbilical vein ECs, a cell type that is not relevant to skin and wound repair. Nevertheless, these studies provide a basis for the elucidation of the connection between apoptotic ECs and fibrotic outcomes in wound healing.

In Chapter 4 “Apoptotic ECs: factors that influence the fibrotic response,” we determined that apoptotic cells secrete factors that directly affect the fibrotic response of FBs *in vitro*, some of the factors identified included several well-known fibrotic proteins, namely, connective tissue growth factor, cysteine-rich angiogenic inducer 61, serpin H1, and stathmin 1. The pro-fibrotic response identified in FBs treated with conditioned media from apoptotic ECs and macrophages was marginal. When these factors were targeted for siRNA knockdown we were able to impart a significant anti-fibrotic effect. These results suggest that the factors we identified are involved in the initiation and propagation of fibrosis *in vitro* and outlined potential anti-fibrotic treatment targets.

More research in the involvement of apoptosis in wound healing and scar formation is required. We have identified improved scarring outcomes with distinct differences in apoptosis pathways, however evidence to suggest differential apoptosis in pathologic scar formation has not been discovered. We have identified that prevention of vascular regression and apoptosis inhibition, at least short term, is not effective at reducing the scarring outcome *in vivo*. Research identifying the effect of vascular invasion on the surrounding tissue and the downstream fibrotic response will further elucidate the mechanism behind improved scarring outcomes seen with inhibition of angiogenesis. Lastly we have identified potential direct paracrine signaling

effects mediated by secreted factors from apoptotic cells on FBs. Research is underway to identify the role of indirect signaling via immune cell modulation following engulfment of apoptotic cells. The fibrotic effects mediated by apoptotic cells do not stop at the interaction with FBs; further research into the interactions of apoptotic cells with other cell types present in the wound such as, ECs, pericytes, and epithelial cells will paint a much clearer picture of the role of apoptotic cells in wound healing. Identification of overlapping secreted factors from all types of apoptotic cells will solidify anti-fibrotic therapeutic targets. In the future, by controlling vascular invasion and regression and the apoptotic load, researchers may be able to halt chronic activation of the wound healing response and promote tissue regeneration instead of tissue repair.

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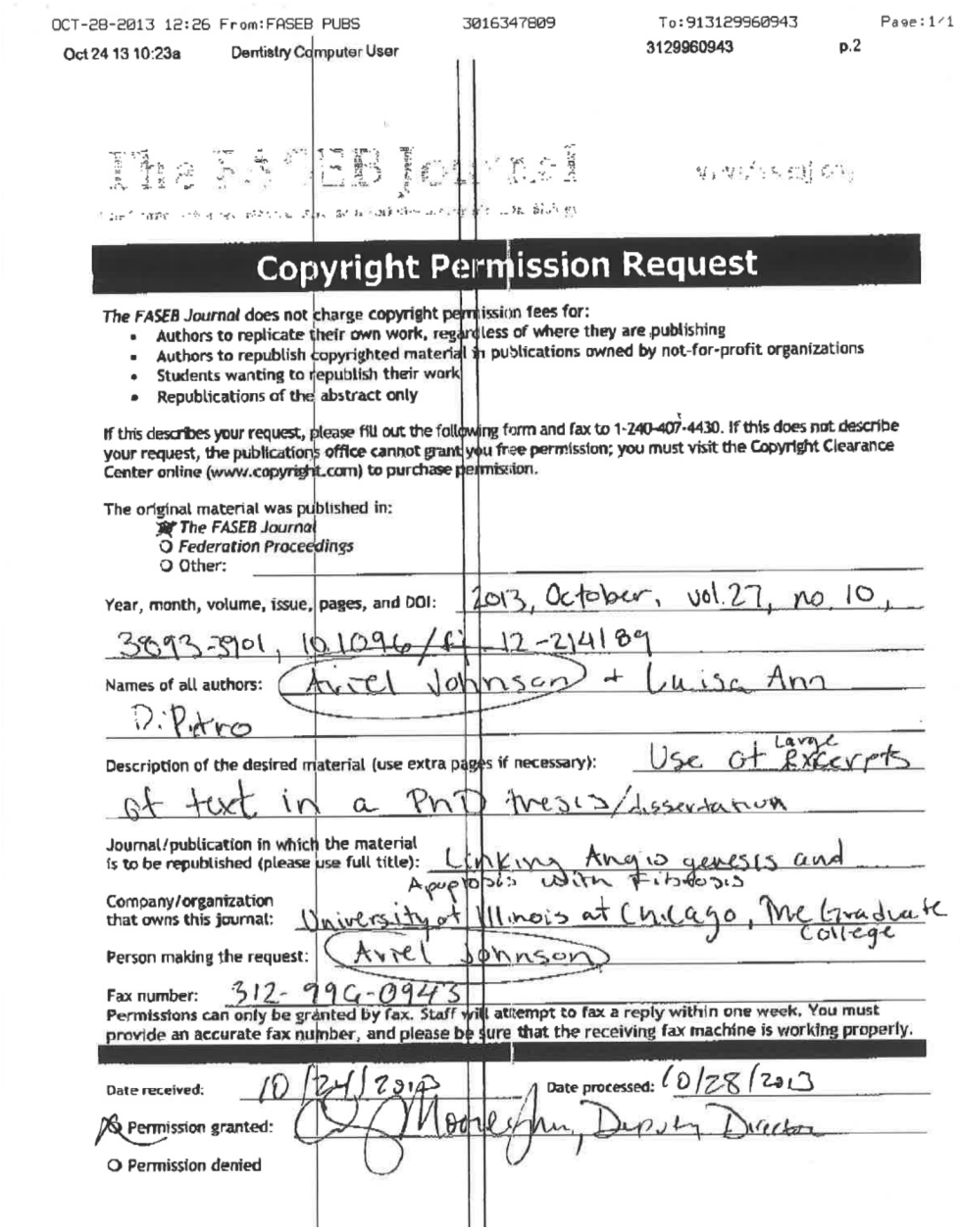

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CURRICULUM VITA

Relevant Employment

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Research

CENTER FOR WOUND HEALING AND TISSUE REGENERATION AUG 2009 –DEC2013
UNIVERSITY OF ILLINOIS AT CHICAGO, COLLEGE OF DENTISTRY
Vascular regression/endothelial cell apoptosis and scarring in wound healing (Linking angiogenesis and apoptosis with fibrosis)

- Basic Science Advisor – Dr. Luisa DiPietro DDS, PhD
- Clinical Advisor - Dr. William Ennis DO, MBA

- Vascular regression and endothelial cell apoptosis in scar formation – Determine the role of apoptotic endothelial cells present in the remodeling phase of wound healing related to scar formation and fibrosis.

- Awards and Honors
  - ThinkChicago Participant – Chicago Ideas Week program for elite students (10/2013)
  - NIH NRSA F31 Recipient – NIDCR F31DE023012-01A1 (05/2013-current)
  - Graduate Student Award – 3rd Place Basic Science Research (03/2013).
  - T32 Recipient – T32 DE018381 (08/2009-08/2011).
  - UIC College of Dentistry Student Research Group – President and AADR Representative (04/2011-06/2013)
  - American Associate for Dental Research, National Student Research Group – Special Assistant to the President (04/2010-04/2011)
  - Graduate Student Council Travel Award (03/2012 and 03/2013).
  - Chicago Chapter of AADR Travel Award (03/2012 and 03/2013).

- Publications
  - Apoptosis and angiogenesis: an emerging mechanism for fibrosis. FASEB Journal, 2013 Jun 19 (epub ahead of print)
  - Invited Discovery Express, Differential apoptosis in dermal versus oral mucosal wound healing. Advances in Wound Care, February 2013 (In Press).

- Seminars/Posters/Abstracts
  - **Johnson A.**, Chen L, DiPietro LA 2013 " Oral Abstract. Factors Secreted By Apoptotic Cells Involved in Fibrosis." ACWHTR December 2013. Chicago, IL.
  - Pre-Conference Seminar at ACWHTR 2012, Panelist: "Apoptosis and Wound Healing."
  - **Johnson A**, DiPietro LA. 2013. Effect of apoptotic human microvascular endothelial cells (HMEC-1) on collagen synthesis in vivo. Clinic and Research day 2013, University of Illinois at Chicago, College of Dentistry, Chicago IL.
  - **Johnson A,** Francis M, DiPietro, LA. 2013 Differential apoptosis in mucosal and dermal wound healing. Clinic and Research Day 2013, Midwestern Regional Dental Research Conference 2013.
  - **Johnson A**, DiPietro LA. 2012. Effect of apoptotic HMEC-1 on normal human dermal fibroblasts (NHDF) expression of collagen synthesis and degradation *in vitro*. Clinic and Research Day 2012, University of Illinois at Chicago, College of Dentistry, Chicago, IL.
  - Lagunas A, **Johnson A**, DiPietro LA. 2012. Gene expression differences in wound healing of tongue versus skin in mouse model. Clinic and Research Day 2012, University of Illinois at Chicago, College of Dentistry, Chicago, IL.

- Oral Abstract "Apoptosis, angiogenesis and healing: An evolving model." ACWHTR August 2012, Chicago, IL.

DEPARTMENT OF KINESIOLOGY **AUG 2009 – FEB 2011**
UNIVERSITY OF ILLINOIS AT CHICAGO, APPLIED HEALTH SCIENCES
Clinical diabetic wound healing research (Impaired Wound Healing and Inflammation)

- Advisor – Dr. Timothy J Koh PhD; Dr. William Ennis DO, MBA
  - Impaired Wound Healing and Inflammation – Clinical research examining inflammatory phenotypes of macrophages as it relates to delayed wound healing in diabetic patients.
- Posters/Abstracts
  - Mirza R, **Johnson A**, Koh TJ. 2011. Dysregulation of Monocyte/Macrophage phenotype in wound of Diabetic Mice. Gordon Research Conference Tissue Repair and Regeneration 2011, New London, NH.
  - Mirza R, **Johnson A**, Koh TJ. 2011. Dysregulation of Monocyte/Macrophage phenotype in wound of Diabetic Mice. American College of Wound Healing and Tissue Repair 1st Annual meeting 2011, Chicago, IL.

COLLEGE OF PHARMACY, SCHOOL OF MEDICINE **OCT 2007 – AUG 2009**
UNIVERSITY OF MINNESOTA, DULUTH, DEPARTMENT OF CHEMISTRY AND BIOCHEMISTRY
Blood brain barrier research (Engineering the cerebrovasculature)

- Advisor – Grant W. Anderson, PhD
  - Engineer the blood brain barrier via bone marrow transplant, and injection of endothelial cells and EPCs during or prior to exposure to hypoxic conditions.
- Awards
  - Graduate Research Assistantship (08/2007-08/2009)
  - Graduate Teach Assistantship (08/2008-08/2009)