The Role of Decorin and Factors Influencing its Expression in Wound Healing and Hypertrophic Scarring

by

Peter Oliver Kwan

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Abstract

Hypertrophic scarring following deep burn injury is a significant problem for many burn patients, and can cause lifelong morbidity including impaired physical function, and reduced psychosocial function from poor cosmesis. While a significant body of research exists, there is still no gold standard effective clinical treatment—a reflection of the complexity of both wound healing and hypertrophic scar formation.

Decorin, a small leucine-rich proteoglycan, has been demonstrated in various models of fibrosis to counteract the effects of transforming growth factor-β, the prototypic profibrotic cytokine. In addition, it also has numerous effects on various other cell surface receptors, and collagen type I (a main constituent of extracellular matrix). Given its multiple important roles in wound healing, the use and role of decorin in modulating wound healing serves as a springboard from which to explore hypertrophic scar formation in burn patients.

The first paper presented in this thesis is a general review of wound healing, and explores the roles of various known factors from cells to cytokines. It serves as the background to the experimental work conducted in this thesis, and lays the foundation for appreciating the complexity of wound healing as a whole, and hypertrophic scar formation in particular.

The second paper presented explores the role of decorin and other cytokines in predicting the formation of hypertrophic scar, and explores their role in creating both local and systemic profibrotic environments. Efforts to predict those patients at risk of hypertrophic scar formation have been previously limited to the use of clinically available

factors such as age, sex, and burn size. However, given the previously established importance of decorin, interleukin-1β, and transforming growth factor-β1 in wound healing and hypertrophic scar, it was hypothesized their serum levels could aid in predicting the risk of hypertrophic scar formation. It was found that early serum levels of decorin and interleukin-1β, and late levels of transforming growth factor-β1 were predictive of hypertrophic scar formation. Furthermore, these temporal combinations were found to create both local and systemic profibrotic environments, and prime the homing of fibrocytes to burn wounds.

The third paper presented explores the downregulation of decorin by transforming growth factor-β1, and methods of reversing this downregulation to reduce the profibrotic effects of transforming growth factor-β1. It was hypothesized that there could be a microRNA upregulated by transforming growth factor-β1, which in turn downregulated decorin. A number of potential miRNA were screened, and it was found that miR-181b was upregulated by transforming growth factor-β1, and that miR-181b downregulated decorin expression through three binding sites. This effect on decorin could be reversed using an antagomiR to miR-181b, and this both increased decorin expression and reduced myofibroblast conversion in the face of transforming growth factor-β1 stimulation *in vitro*. Thus suggesting that antagomiR-181b could be a potential therapy for hypertrophic scar.

The fourth paper presented explores the upregulation of decorin using an adenoviral gene therapy vector to treat deep dermal fibroblasts used to populate collagen scaffolds. These collagen scaffolds serve as the base platform for the creation of cultured skin substitutes, which are a promising therapy for burn patients with large wounds and

limited donor sites. It was hypothesized that the collagen scaffold remodeling behavior of deep dermal fibroblasts, which are profibrotic, could be altered to more closely match that of superficial dermal fibroblasts, which are regenerative. As there are significantly more deep dermal fibroblasts present in dermal biopsies used to grow cultured skin substitutes, this has the potential to significantly increase the number of regenerative fibroblasts available for tissue engineering. It was found that an adenoviral decorin vector significantly upregulated decorin production by deep dermal fibroblasts, and this altered their collagen scaffold remodeling to match that of superficial dermal fibroblasts, as measured by collagen fibril thickness and collagen orientation index.

By using decorin as a basis for investigating hypertrophic scar, this thesis has explored opportunities for improving the prediction of hypertrophic scar formation, treating hypertrophic scar using microRNA modulation, and preventing hypertrophic scar formation by improving cultured skin substitutes using an adenoviral gene vector. It is hoped that these promising avenues of future research will ultimately improve the clinical outcomes of burn patients, and perhaps others suffering from fibrotic diseases as well.

Preface

(Mandatory due to collaborative work and research ethics approval)

All of the research design and experimentation conducted for this thesis was performed directly by myself, except for the measurement of hydroxyproline using mass spectrometry which was performed by Takashi Iwashina, a laboratory technician in the Wound Healing Research Group, Department of Surgery, University of Alberta. Dr. Keijiro Hori, a fellow graduate student, wrote part of the section on hypertrophic scar therapy in my review paper on wound healing, which forms the basis of chapter 2. I was otherwise responsible for performing all experiments, data collection, analysis, and manuscript composition. Dr. Edward Tredget and Dr. Jie Ding proofread my manuscripts, and made suggestions for modifications.

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The relevant research projects in the Wound Healing Research Laboratory, under which portions of this thesis were conducted, received research ethics approval from the University of Alberta Research Ethics Board under several related projects. Namely, "A double-blind, placebo-controlled trial in the treatment of partial-thickness donor site wounds with Interferon α2b", "Peripheral blood fibrocyte levels in burn patients", and "Investigation into Wound Healing, Fibrosis, Tissue Engineering and Regeneration".

Dedication

I would like to dedicate this thesis to my family. Without their support, its undertaking and completion would have been impossible. And with their support, even this has been possible. To my parents, Frederick and Linda, who instilled in me a sense of continual curiosity, the belief that any problem could be solved, and who gave me the skills and support to achieve my goals—in many ways this doctorate is as much for you as for me. To my brother, David, who is a co-adventurer in life, and supportive collaborator in many endeavors—your quiet insight and inventiveness have always inspired me. To my wife and partner, Ashley-Mae, who has put up with many long days and untold other tribulations while I spent long hours in the laboratory and hospital—you make every day worthwhile, and I look forward to a further lifetime of adventures with you. And finally, to my children Nathaniel and Elizabeth, your inquisitive natures, happy smiles, and wonderfully unique personalities add a whole new dimension of meaning to my life.

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First, I would like to thank Dr. Robert Cartotto and Dr. Joel Fish for their mentorship and inspiring me to pursue a career in plastic surgery, caring for burn patients, and seeking to research and better understand the problems burn patients face in pursuit of improving their outcomes. To have met and been mentored by these physicians early in my career was a privilege and did much to set me on the path I continue to pursue today.

Second, I would like to thank Dr. Edward Tredget, my supervisor, for mentoring me and providing the opportunity and resources to pursue basic science research at the University of Alberta. The opportunity to work on the basic biology of wound healing and hypertrophic scarring, learn a whole range of research techniques, and participate in the ongoing projects in his laboratory has been a privilege.

Third, I would like to thank my committee, Dr. Jie Ding, Dr. John Elliott, and Dr. Hasan Uludag, for many fruitful committee meetings and much patience as I have completed my degree. I would like to thank Dr. Adetola Adesida for serving on my candidacy and defense committees, and his helpful suggestions and comments. And I would also like to thank Dr. Thomas Churchill for his support and advice, and Dr. Gina Rayat for her open office and friendly ear. The guidance, questions, and insights provided by these individuals have been invaluable.

Fourth, I would like to thank the other individuals in my laboratory and the core facilities, who were a ready source of feedback and assistance, and taught me much of what I needed to accomplish my goals: Dr. Adil Ladak, Heather Shankowsky, Dr. JianFei Wang, Dr. Kunimasa Suzuki, Takashi Iwashina, and Yvonne Marcoux.

Lastly, I would like to thank the Division of Plastic Surgery and the Department of Surgery for allowing me the time off from my residency to pursue research, and the Edmonton Firefighters Burn Trust for supporting the research of the laboratory, including my projects. And I would like to thank the many anonymous patients and research participants who generously aided in making these studies possible.

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List of Abbreviations

α-SMA	α-smooth muscle actin
antagomiR	Antagonist to micro ribonucleic acid
APC	Antigen presenting cells
BGN	Biglycan
BM-MSC	Bone marrow-derived mesenchymal stem cells
CCN	Connective tissue growth factor, cysteine rich protein, nephroblastoma overexpressed gene
cDNA	Complementary deoxyribonucleic acid
COI	Collagen orientation index
CSS	Cultured skin substitute
CTGF	Connective tissue growth factor
CXADR	Coxsackie virus and adenovirus receptor
CXC	Chemokine C-X-C motif
CXCL	Chemokine C-X-C motif ligand
CXCR	Chemokine C-X-C motif receptor
DAMP	Damage associated molecular patterns
DAPI	4',6-diamidino-2-phenylindole
DCN	Decorin
DF	Deep dermal fibroblast
DMEM	Dulbecco's modified Eagle medium
ECM	Extracellular matrix
EGFR	Epithelial growth factor receptor

ELISA	Enzyme linked immunoassay
Er:YAG	Erbium:yttrium aluminum garnet
FBS	Fetal bovine serum
FFT	Fast Fourier transformation
FPCL	Fibroblast-populated collagen lattice
GAG	Glycosaminoglycan
H&E	Hematoxylin and eosin
HGFR	Hepatocyte growth factor receptor
HPRT1	Hypoxanthine phosphoribosyltransferase 1
HSc	Hypertrophic scar
HUVEC	Human umbilical vascular endothelial cells
IFN	Interferon
IGF	Insulin-like growth factor
IGF1R	Insulin-like growth factor 1 receptor
IHC	Immunohistochemistry
IL-1β	Interleukin 1β
ITGAV	Integrin alpha V
ITGB3	Integrin beta 3
ITGB5	Integrin beta 5
KDAF	Keratinocyte-derived anti-fibrogenic factor
LTBP	Latent TGF-β binding protein
microRNA	Micro ribonucleic acid
miR	Micro ribonucleic acid
miRNA	Micro ribonucleic acid

MMP	Matrix metalloproteinase
MOI	Multiplicity of infection
mRNA	Messenger ribonucleic acid
NCBI	National Center for Biotechnology Information
Nd:YAG	Neodymium doped:yttrium aluminum garnet
NS	Normal skin
P	P-value
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
SAP	Serum amyloid P
SDF-1	Stromal cell-derived factor 1
SEE	Standard error of the estimate
SEM	Standard error of the mean
SF	Superficial dermal fibroblast
siRNA	Silencing RNA / Small Inhibitory RNA
SLRP	Small leucine-rich proteoglycan
SMAD	Sma and Mad related protein family
TBSA	Total body surface area
TGF-β	Transforming growth factor-beta

Th	T helper
TIMP	Tissue inhibitor of matrix metalloproteinase

Chapter 1

Introduction

1.0 Introduction

Hypertrophic scarring (HSc) following deep burn injury is a significant problem for many burn patients, and can cause lifelong morbidity including impaired physical function, and reduced psychosocial function from poor cosmesis [1]. While a significant body of research exists [2-4], the pathophysiology of HSc is still not well understood and there is currently no gold standard treatment [2, 5]. What is known is that decorin (DCN), a small, leucine-rich proteoglycan has significant interactions with a number of profibrotic factors including transforming growth factor-β (TGF-β) [6], and connective tissue growth factor (CTGF) [7], and that it can prevent or reduce fibrosis in a wide variety of environments [8-10]. Therefore, DCN was chosen, for the purposes of this thesis, as the signature factor around which to base investigations into HSc formation following deep burn injuries.

This thesis begins with an overview of wound healing with a specific focus on the problem of HSc development in burn patients. The importance of superficial and deep dermal fibroblast subpopulations is reviewed, as well as the underlying actions of various pro- and anti-fibrotic cytokines, the roles that peripheral blood mononuclear cells (fibrocytes) and cells of the immune system play, and interactions between fibroblasts and keratinocytes in the formation of HSc. Current and potential therapies ranging from non-surgical to surgical options are also reviewed.

Although the clinical entity of HSc is well described, and a number of clinical factors predictive of its development have been studied, existing predictive models are still quite limited [11]. Therefore, it was hypothesized that measurable serum factors such as DCN (with a focus on biomarkers of inflammation, fibrosis, and extracellular matrix) could improve existing prediction models. It was also hypothesized that the temporal relationship of these factors, based on multiple linear regression analysis, would provide insight into the creation of local and systemic profibrotic environments, which are conducive to the formation of HSc. The exploration of these hypotheses is covered in the third chapter.

Recent research into the activity of microRNA in regulating gene expression suggests important roles in gene networks [12], and skin wound healing and fibrosis [13-15]. Therefore, it was hypothesized that DCN could also be regulated by profibrotic factors such as TGF-β or CTGF through a microRNA pathway. Furthermore, it was hypothesized that blocking specific microRNA using antagomiRs could serve as a potential treatment for HSc. The screening of candidate microRNA, confirmation of activity, and evaluation of antagomiR therapeutic potential is covered in the fourth chapter.

Finally, several studies show that cultured skin substitutes (CSS) hold great promise in reducing morbidity and mortality in patients with large burns [16]. To date, the best-studied CSS are based on collagen scaffolds, which are seeded with dermal fibroblasts derived from the burn patients on whom they will be engrafted [16]. Given previous research on the unique differences between superficial (regenerative) and deep (fibrotic) dermal fibroblasts [17, 18], and research demonstrating differential remodeling of collagen scaffolds by these subpopulations [19, 20], it was hypothesized that increasing DCN expression by deep dermal fibroblasts using an adenoviral gene vector could alter collagen scaffold remodeling to mimic that of superficial dermal fibroblasts. This work is covered in the fifth chapter.

Taken together, these hypothesis provide new insights into HSc formation following burn patients, and serve to provide a springboard for future work in this field.

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Chapter 2

Wound Healing, Scarring, Fibrosis, and Contractures Following Burn Injury

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2.0 Abstract

Dysregulated wound healing and pathologic fibrosis cause abnormal scarring leading to poor functional and aesthetic results in hand burns. Understanding the underlying biological mechanisms involved allows the hand surgeon to better address these issues, and suggests new avenues of research to improve patient outcomes. In this chapter, we review the biology of scar and contracture by focusing on potential causes of abnormal wound healing, including: depth of injury, cytokines, cells, the immune system, and extracellular matrix, and explore therapeutic measures designed to target the various biological causes of poor scar.

2.1 Introduction

As the primary interface between humans and their environment, the hands are constantly exposed to danger. As a result, burn injuries of the hand are common. In toddlers these are often scald burns that occur during exploration of the environment [1], whereas in adults these are often flame or flash burns resulting from occupational or recreational injuries [2, 3]. In both these groups the potential for lifelong morbidity resulting from loss of function is enormous. According to the Centers for Disease Control over 400,000 nonfatal burn injuries occurred in the United States of America in 2007 [4]. Of these, 45% involve the arm and hand [5]. The resulting potential for significant functional impairment and hypertrophic scarring (HSc) is high [6], making a hand burn one of the American Burn Association criteria for mandatory referral to a burn center [7].

While many of these hand burns are superficial and often heal without sequelae, deeper burns are prone to increased scarring and contracture [8-10], as shown in Figure 2.1. Those hand injuries that lead to poor scar are also prone to scar contracture. It is often this secondary contracture that leads to the greatest functional impairment, as seen in Figure 2.2. As the primary means of interaction with the physical environment, function and appearance of the hand are crucial [11, 12]. Scarring and contracture both lead to impaired hand function [13]. Understanding the underlying biological mechanisms involved allows the hand surgeon to better

address these issues, and suggests new avenues of research to improve patient outcomes [14].

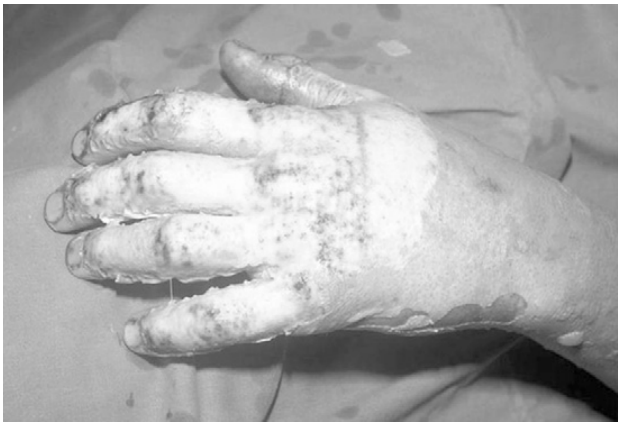

Figure 2.1 Superficial and deep hand burns.

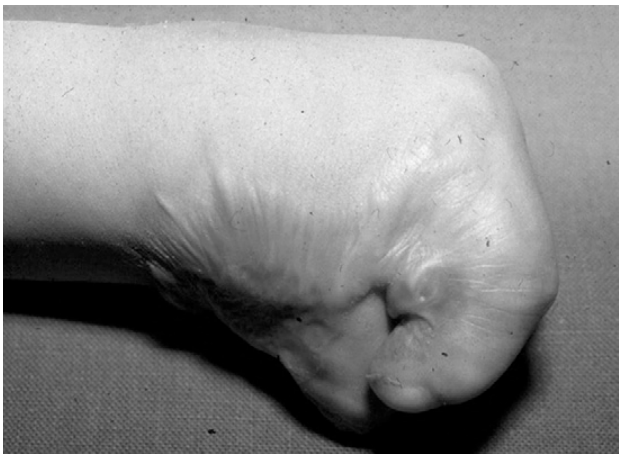

Figure 2.2 Scar contractures in a hand burn.

The stages of normal wound healing have been well described by several authors [15-19]. In this chapter, we review the underlying biology of scar and contracture by focusing on potential causes of abnormal wound healing and explore therapeutic measures designed to target the various biological causes of poor scar.

2.2 Pathophysiology of Scar

2.2.1 Injury Beyond a Critical Depth Leads to Scar Formation Rather than Regeneration

While determination of the depth of injury is beyond the scope of this article, it is well known that the difference between superficial and deep burns is of great clinical importance and largely determines how these injuries heal and the degree of scarring to be expected [9, 10]. Traditionally, superficial wounds are those expected to heal within two weeks without surgical intervention [10]. Deep wounds are prone to HSc and contracture [20] and surgical intervention, including the application of split thickness skin grafts, is typically used in an attempt to avoid these sequelae [21]. On the other hand, superficial wounds usually heal with a minimum of scarring [20] and are generally managed with dressings to facilitate the body's natural reparative mechanisms [22]. These clinical observations correlate with the wound healing seen in an experimental dermal scratch model developed by Dunkin, *et al*. [23]. In this model, a jig was used to create a linear skin wound that increased in depth along its length from no injury to full thickness injury [23]. The authors observed that superficial injury less than 0.56 mm in depth (or 33.1 % of normal hip skin thickness) resulted in regeneration rather than scar whereas deeper injury resulted in increasing scar formation [23]. This suggests that injury beyond a critical depth leads to scar formation rather than regeneration, Figure 2.3. The reasons for this are currently unclear, however two major hypothesis have been proposed: 1) selective proliferation of fibroblast subpopulations resulting from fibrogenic cytokines, and 2) thermal injury destroying a subpopulation of fibroblasts thus leaving a distinct phenotype of deeper fibroblasts to repopulate the wound [24]. Indeed, several studies have shown that superficial and deep dermal fibroblasts respond differently to injury [25-33]. This difference, to be discussed further, may be one of the keys to understanding HSc formation following burn injury [24]. Thus, not only does depth of injury play a crucial role in dictating clinical management, it also suggests new aspects of the pathophysiology of wound healing to be explored.

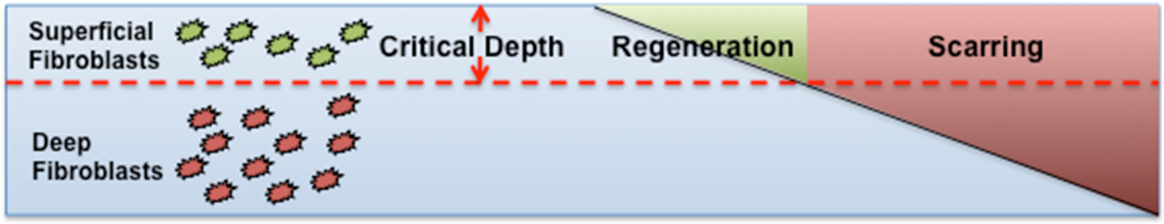

Figure 2.3 Regeneration occurs in superficial wounds while scarring occurs in deeper wounds.

2.2.2 Fibrogenic and Antifibrogenic Cytokines Modulate Fibroblasts

The local cellular environment exerts great control over the healing process. Local cytokines serve as the signaling molecules that modulate the activity of fibroblasts and keratinocytes, causing them to close and heal wounds or become overactive leading to HSc [34]. The balance between profibrotic and antifibrotic cytokines, as in Figure 2.4, has a great impact on the eventual wound healing outcome.

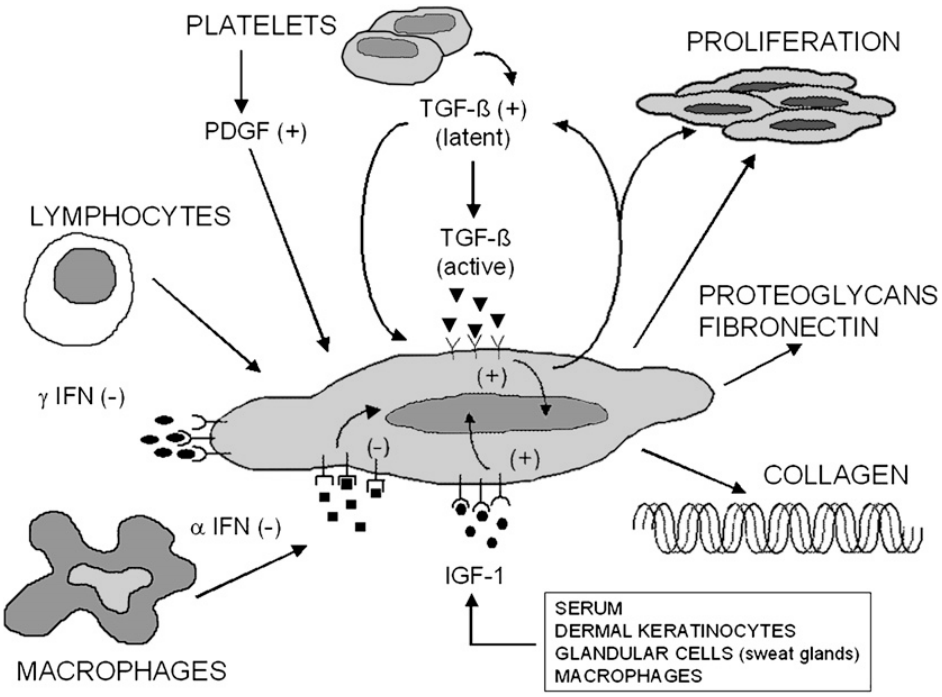

Figure 2.4 Balance of profibrotic and antifibrotic factors in wound healing [24].

Platelet-derived growth factor (PDGF) is produced in wound healing by platelets from the injured capillaries. Four subtypes have been described which form dimers to activate two structurally related tyrosine kinase receptors [35]. Activation causes cellular proliferation and actin reorganization making PDGF a potent mitogen on mesenchymal cells including fibroblasts and induces their transformation into myofibroblasts [36]. PDGF increases extracellular matrix (ECM) production and inhibits myofibroblast apoptosis [37]. It has been implicated in scleroderma, pulmonary fibrosis, hepatic fibrosis, and various renal diseases [38]. Dermal fibroblasts not only respond to PDGF but produce it as well, resulting in an autocrine loop, and PDGF also increases transforming growth factor-β (TGF-β) receptors in scleroderma fibroblasts [39]. The effect of PDGF is increased in HSc and keloid fibroblasts [40], all suggesting that PDGF may play a role in fibrosis and abnormal scarring of skin. This suggests that blocking PDGF via tyrosine kinase inhibitors could reduce fibrosis and improve clinical outcomes, which it does in a several murine models of radiation induced pulmonary fibrosis [41] and scleroderma [42].

TGF-β is the prototypic profibrotic cytokine and belongs to a large superfamily of cytokines sharing a conserved cysteine knot structure [43, 44]. When initially produced it is usually bound to an associated latent TGF-β binding protein (LTBP) in an inactive form. This bond is cleaved, to activate TGF-β, by a number of enzymes present in blood or released during cell injury, including matrix metalloproteinase-2 (MMP-2), MMP-9, and plasmin [45]. Three known isoforms exist in mammals: β1, β2, and β3 [44] and are produced by a multitude of sources including: degranulating platelets, macrophages, T-lymphocytes, endothelial cells, keratinocytes, and fibroblasts [46]. TGF-β1/2 acts via the Smad pathway to regulate several cellular processes related to fibrosis [47]. TGF-β acts as a chemoattractant for monocytes [48] and fibroblasts [49], stimulates fibroblasts to produce ECM [50], and modulates production of several proteinases and their inhibitors [51]. TGF-β is upregulated locally in tissue and systemically in serum in burn patients with HSc [52], and fibroblasts from HSc synthesize greater amounts of TGF-β than normal dermal fibroblasts [53]. Fetal wounds, which normally regenerate rather than form scar, can be induced to form scar tissue by exposure to TGF-β1 [54]. Taken together these results suggest TGF-β appears to be a key initiator of fibrosis and HSc. Interestingly, while most isoforms of TGF-β are profibrotic, several studies have shown

improved wound healing when exposed to TGF-β3 [55, 56]. TGF-β3 is strongly induced in the later stages of wound healing and reduces ECM deposition [57] which may be one factor in its mechanism of action.

Connective tissue growth factor (CTGF) is one of the original members of the CCN family [58]. It is a downstream regulator of fibrosis that is induced by TGF-β [59, 60] and upregulates ECM production by fibroblasts [61]. Interestingly, it appears that CTGF and TGF-β independently induce only transient fibrosis; whereas, when combined, they lead to prolonged fibrosis [62]. CTGF is upregulated in scleroderma, HSc [63], and many other fibrotic conditions [64]. In these chronic conditions fibrosis continues due to CTGF, even though TGF-β becomes downregulated [60]. This suggests that although TGF-β is important in initiating pathologic scarring, it is CTGF that sustains the fibrotic process. CTGF appears to be induced by TGF-β via the Ras/MEK/ERK pathway and blocking this pathway using iloprost reduces fibrosis [65] as does anti-CTGF antibody or CTGF siRNA [66].

Insulin-like growth factor-1 (IGF-1) modulates growth hormone effects on various tissues including dermal fibroblasts [67]. IGF-1 is a fibroblast [68] and endothelial cell [69] mitogen and induces collagen production in osteoblasts [70], human pulmonary fibroblasts [71], and human dermal fibroblasts [72]. It is expressed locally in injured tissue and this parallels granulation tissue formation up to 5 weeks post-injury [73]. Not only does IGF-1 stimulate glycosaminoglycan and collagen production, but it also reduces collagenase mRNA levels and activity by dermal fibroblasts [74]. This adversely impacts the critical balance of collagen production and degradation that is crucial to ECM remodeling. Both TGF-β and IGF-1 are increased in post-burn HSc compared to matched normal dermis from the same patients [75], in a similar fashion to other fibrotic conditions including: scleroderma, pulmonary fibrosis, and hepatic fibrosis [24]. Interestingly, it has been shown that in skin IGF-1 is restricted to epidermal sweat and sebaceous glands where dermal fibroblasts are not exposed to it [24, 75]. In wounds, such as burns, where these structures are damaged dermal fibroblasts would be exposed to IGF-1 and this could contribute to HSc formation. Once the epidermal wound has healed this exposure would cease and could account for the observation that wounds reepithelializing within 2 weeks are less prone to HSc than those taking longer to heal and expose fibroblasts to IGF-1 for extended periods of time [24]. In addition, IGF-1 has been shown to act as a TGF-β

stimulating factor [72]. Although IGF-1 may not be the only cause of fibrotic growth factor in HSc, these findings suggest it has an important role in concert with TGF-β in the pathogenesis of abnormal scarring.

HSc is not simply due to the presence of fibrogenic cytokines alone as many are present in both normal scar and HSc, albeit in differing quantities. Likely just as important is the relative decrease in a number of anti-fibrotic cytokines and the delicate balance between the two. Two anti-fibrotic cytokines of great interest are interferon-α2b (IFN-α2b) and IFN-γ. IFN-α is produced by leukocytes and fibroblasts, while IFN-γ is produced by Th1 T helper cells [76] all of which are known to play a role in wound healing. IFNs decrease ECM production by dermal fibroblasts from fibroproliferative lesions to normal levels [24]. IFN-α2b also increases dermal fibroblast collagenase expression and decreases tissue inhibitor of matrix metalloproteinase (TIMP-1) [77]. A prospective clinical trial by our research group in post burn patients showed reductions in HSc volume, normalized TGF-β levels, and reduced scar angiogenesis after treatment with subcutaneous IFN-α2b [52]. This suggests that, in the treatment of abnormal scar and contracture, increasing antifibrotic cytokines is just as important as reducing profibrotic ones.

2.2.3 Dermal Fibroblast Subpopulations Including Myofibroblasts Behave Differently in Wound Healing

Fibroblasts are one of the key players in wound healing and serve as the primary mesenchymal cell of scar formation and contraction. They participate in the physical aspects of wound closure and also produce and remodel ECM [15]. What has become increasingly clear is that fibroblasts from different tissues: lung, heart, kidney, and even different parts of the same tissue, including skin, behave quite differently [32, 78]. Recent studies have demonstrated that superficial and deep dermal fibroblasts, derived from the papillary and reticular layers behave quite differently. Compared to superficial fibroblasts, deeper fibroblasts produce more collagen [25], proliferate more slowly [26, 27], produce greater contraction of collagen gels [29], produce less decorin (DCN) [30], induce more irregular keratinocyte proliferation [31], and are not as supportive of the formation of capillaries by vascular endothelial cells [33]. This heterogeneity of fibroblasts may account for the different patterns of healing seen with varying depths of

injury.

It has been proposed that when superficial fibroblasts are destroyed by deep thermal injuries the deeper fibroblasts remain to repopulate and heal the wound, contributing to HSc formation [24]. In a mouse model where human skin was grafted onto animals and subsequently injured, deep dermal fibroblasts were found to initially close the experimental wounds which were then remodeled by superficial fibroblasts [79]. It is possible that insufficient numbers of superficial fibroblasts to remodel the ECM contributes to HSc formation. Certainly, HSc fibroblasts appear similar in behavior to deep dermal fibroblasts, as compared to superficial fibroblasts, when their production and response to cytokines, as well as production of ECM is examined [80]. Studies in our laboratory show that TGF-β and CTGF, two key profibrotic cytokines, are produced in greater quantities by deeper fibroblasts [80] which mirrors their increased production demonstrated in HSc fibroblasts [53, 63]. This suggests the biology of HSc fibroblasts is directly related to that of deep dermal fibroblasts, and that models and therapeutic measures targeted at deep dermal fibroblasts, which are simpler to obtain and more easily studied, will provide insight into HSc.

Myofibroblasts are a particular phenotype of fibroblasts, initially described by Majno *et al*. [81], and widely associated with contraction [82]. Myofibroblasts differ from fibroblasts by their expression of α-smooth muscle actin (α-SMA) [83] and several other aspects of their behavior. Myofibroblasts produce more collagen and less collagenase than fibroblasts [84] and are more numerous in HSc than normal scar [85, 86]. HSc myofibroblasts are less sensitive to apoptotic signals and this, coupled with their increased production of ECM, may be a direct factor in HSc formation [87].

2.2.4 Fibrocytes are Both a Systemic Source of Fibroblasts and Myofibroblasts and also Regulators of Preexisting Wound Healing Cells

Fibrocytes are a leukocyte subpopulation similar to monocytes but expressing collagen and participating in the regulation of fibroblasts and wound healing [88]. Fibrocytes were first described by Bucala *et al*. in 1994 who observed a blood-borne cell that behaved like a fibroblast in wound chambers implanted on the backs of mice [88]. Fibrocytes are uniquely identified by double staining for procollagen I, and CD34 [88] or leukocyte specific protein-1 [89]. Since

their initial description, fibrocytes have been found in both normal healing [90], and a number of fibroproliferative diseases including: pulmonary fibrosis [91, 92], nephrogenic fibrosis [93], atherosclerotic lesions [94], chronic pancreatitis [95], and chronic cystitis [96], as well as hypertrophic burn scars [97-99]. Abe *et al*. showed that secondary lymphoid chemokine (SLC), a C-C chemokine ligand of CCR7, promotes fibrocyte migration to wounds and is produced by the vascular endothelium in wounds, suggesting SLC plays a role in fibrocyte trafficking to wounds [90]. It has been shown that fibrocytes are upregulated in burn patients [97] and they are hypothesized to contribute to abnormal scarring through several different mechanisms [100]. When exposed to profibrotic cytokines, fibrocytes produce large amounts of ECM and differentiate into myofibroblasts via activation of the Smad2/3 and SAPK/JNK MAPK pathways [101]. Pilling *et al*. identified serum amyloid P (SAP), a constitutive plasma protein related to C-reactive protein (CRP), as an inhibitor of fibrocyte differentiation from CD14+ peripheral blood monocytes [102]. They show that sera from patients with scleroderma, low in SAP, does not inhibit fibrocyte differentiation [102] and in a murine bleomycin model of pulmonary fibrosis used SAP injections to inhibit fibrocyte differentiation and reduce collagen production, fibrocytes, and leukocytes in the lung [103].

Fibrocytes may be more than simply another source of fibroblasts and myofibroblasts in healing wounds. Fibrocytes may be a crucial link between the immune system and healing wounds and serve as regulators of preexisting wound healing fibroblasts and other cells. Fibrocytes, true to their leukocyte lineage, are capable of acting as antigen presenting cells (APC) and can prime naïve T cells [104]. They also express toll-like receptors (TLR) on their cell surfaces, allowing them to respond as part of the innate immune system to a large variety of invading pathogens [105]. Furthermore, a study by Wang *et al*. in burn patients suggests that fibrocytes regulate the activity of preexisting fibroblasts by producing TGF-β and CTGF, Figure 2.5 [89]. Fibrocytes may also play an important role in revascularization of healing wounds by secreting MMP-9 which degrades matrix and promotes endothelial cell invasion and also producing several proangiogenic factors including vascular endothelial growth factor (VEGF) [106].

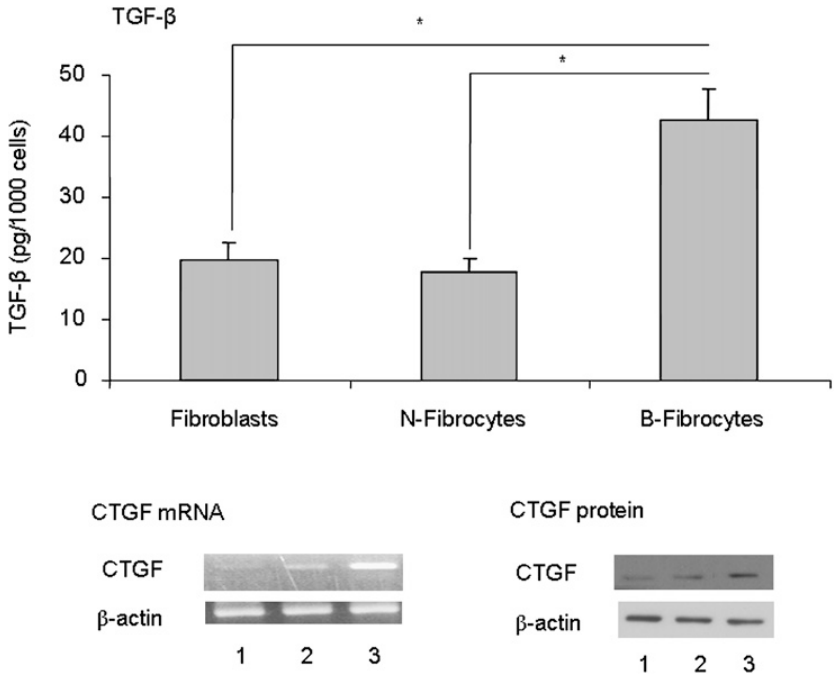

Figure 2.5 TGF-β and CTGF production by fibrocytes in normal and burn patients [89].

2.2.5 Keratinocytes Interact with Fibroblasts to Control Wound Healing

Keratinocytes play a crucial role in wound healing, with reepithelialization serving as a key endpoint in scar formation. Wounds taking longer than two weeks to reepithelialize are at increased risk of HSc [107]. This suggests that keratinocytes interact with fibroblasts to control scarring [108, 109]. Keratinocytes do regulate the action of fibroblasts and vice versa [110, 111]. Keratinocytes suppress TGF-β and CTGF production by fibroblasts [112]. Keratinocyte co-culture and keratinocyte conditioned media modulate fibroblasts by increasing proliferation but simultaneously decrease ECM synthesis, in part, via keratinocyte-derived anti-fibrogenic factor (KDAF), subsequently identified as stratifin (14-3-3 Sigma) [113]. This may occur via increased MMP-1 production from KDAF stimulated fibroblasts [113]. Conversely, several authors have demonstrated that keratinocytes from HSc induce cocultured fibroblasts to produce increased ECM as compared to keratinocytes from normal skin [114]. This suggests that abnormal regulation of keratinocyte-fibroblast crosstalk may be an important component of HSc formation.

2.2.6 Immune System and T Lymphocytes Regulate Wound Healing

Clinical observation suggests that injuries leading to a prolonged immune response appear to increase the risk of fibroproliferative scar. However, recent research suggests that the type of immune response rather than degree of inflammation is the predisposing factor [115]. Mast cells, neutrophils, and macrophages all play a role in the initial inflammatory state of wound healing [116]. Macrophages, in particular, are involved in the transition from inflammation to proliferation. Macrophages produce proinflammatory cytokines including interleukin-1 (IL-1), IL-6, and tumor necrosis factor-α (TNF-α) which control inflammatory cell adhesion and migration, and also stimulate keratinocytes and fibroblasts to proliferate [36]. Macrophages also produce known profibrogenic factors including PDGF, TGF-β, and IGF-1 [117]. In a CXCR3 knockout mouse model, it was shown that CXCR3 is a key receptor used by macrophages to infiltrate healing wounds, and that its inactivation leads to reduced wound healing [118]. This suggests that macrophages are a key component of the transition from inflammation to proliferation, and could also play a role in the initial stages of HSc formation.

T helper cells (CD4+) appear to act as immunoregulators that produce various cytokines to control the wound healing process (Figure 2.6) [100]. In burn injury, once activated by macrophages [119], dendritic cells [120] or APC, naïve T helper cells become polarized toward a Th1 or Th2 type [121] and produce specific cytokine profiles [122]. Samples of HSc dermis demonstrated increased CD4+ T lymphocyte infiltration compared to normal skin in the same patients [123]. Although, Th1 cells are classically considered the primary actors in cell-mediated immunity, they also produce mainly antifibrotic cytokines (IL-2, IFN-γ, and IL-12), induce fibroblast pro-apoptotic genes, and activate nitric oxide synthase (NOS) expression, which promotes collagenase activity [124]. In contrast, while Th2 cells are classically associated with antibody-mediated immunity, they also produce primarily profibrotic cytokines including IL-4, which has twice the profibrotic potency of TGF-β in fibroblasts; IL-5; and IL-13. Th2 linked genes upregulate ECM production and include pro-collagens I, III, and V, arginase-1, MMP-2, MMP-9, and tissue inhibitor of TIMP-1 [125]. In burn patients, serum samples show elevated Th2 cytokine levels of IL-4 and IL-10, and reduced Th1 cytokine levels of IFN-γ and IL-12 for over one year post-injury [126, 127]. Cultured fibroblasts treated with serum from burn patients resulted in Th2 polarized responses including: increased cellular proliferation, TGF-β

upregulation, and expression of α-SMA suggesting a transformation to the myofibroblast phenotype [123].

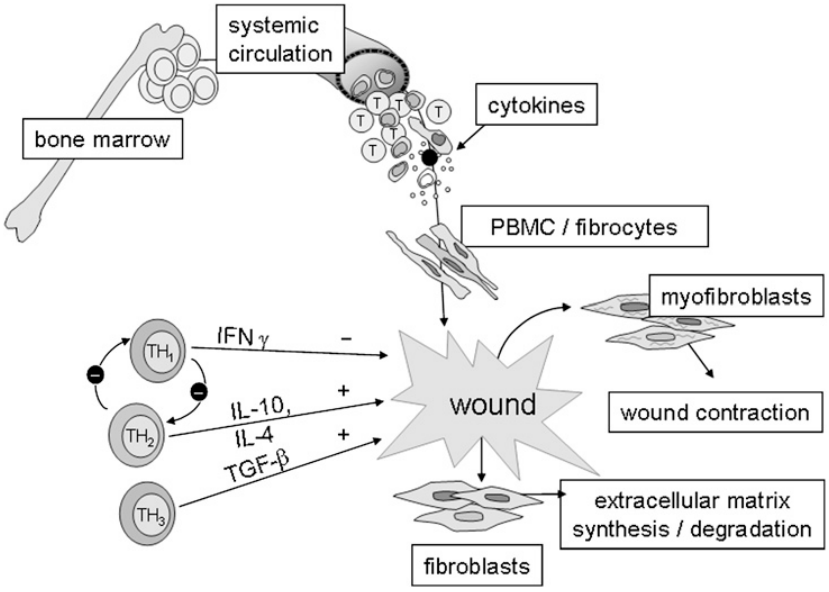

Figure 2.6 Hypothetical diagram of the role of Th1/Th2/Th3 cells in stimulating bone marrow stem cells to healing wounds [115].

2.2.7 Extracellular Matrix Influences Cellular Behavior

Ultimately, the ECM formed during initial wound closure is remodeled as wound healing and scar formation occurs. A complex interplay exists between fibroblasts and the ECM environment with which they interact [82, 128]. Conceptually, ECM has two major components: collagen fibrils that are responsible for tensile strength, and glycosaminoglycans that contribute to tissue osmotic pressure and provide resistance to compression [24]. Clinically HSc is raised, erythematous, and firm to the touch [129]. Structurally, HSc ECM contains dense nodules of poorly organized, thin collagen fibrils in whorl-like patterns, which appear encapsulated in more normal appearing collagen. In contrast, normal dermis contains thick parallel collagen bundles and fibers [130, 131]. ECM formed in HSc is thicker, is hyper-hydrated, and has overlying epidermis that is also often thicker [24]. This disorganization may be due in part to alterations in the collagen fibers and proteoglycans present in HSc as compared to normal tissue.

Although the dry-weight of HSc collagen is reduced in comparison to normal dermis or mature scar, because of its increased thickness HSc does have more collagen per unit surface area [24]. Abnormal collagen fibrils may be due in part to alterations in the relative proportions of the various collagen types. While normal dermis and scar are predominantly 80% type I, 10-15% type III, and minimal type V, the ratios in HSc are very different with ~33% type III and up to 10% type V [132-134]. Both type III and type V collagen have been shown to alter the fibril diameter of type I collagen bundles and the different ratios in HSc may account for some morphologic changes in ECM structure [135-137].

The relative content of several glycosaminoglycans is also altered in HSc [138]. Overall, there is an over two-fold increase in glycosaminoglycans, leading to greater hydration and likely causing the increased clinical firmness of HSc [24]. Proteoglycans also influence collagen fibril morphology, cell-matrix interactions, and cellular behavior. The most plentiful proteoglycan in normal dermis is DCN, which is reduced by 75% in HSc [138]. This is significant in that DCN has been shown to have a multitude of roles including modulation of collagen fibrils [139, 140], regulation of TGF-β [141, 142], and reduction of fibrosis [143, 144] and contraction [145, 146]. Two other proteoglycans, versican (VCAN) and BGN, are upregulated in HSc to compensate for the lack of DCN. In particular, VCAN is increased six-fold above normal and from its position

between collagen fibrils may contribute to increased tissue turgor and an expanded collagen network of ECM leading to the increased scar volume seen in HSc [24].

2.3 Pathophysiology of Contracture

2.3.1 Contracture Affects both Healing Wounds and Scars

When discussing contracture a key distinction must be drawn between the wound contracture that occurs as a part of the initial closure and healing process, and the scar contracture that occurs as the scar matures. Wound contraction is the biological means whereby the edges of an open wound are pulled together by forces resulting from the wound healing process. In contrast, scar contraction is the shrinkage that occurs in an already healed scar [84, 147].

Although most contraction research continues to occur in animal models, wound contraction plays a greater role in wound healing in animals as compared to humans [148, 149]. Scar contractures result from HSc over joints and mobile surfaces that contract secondarily, but HSc only occur in humans [84], making discovery of a good *in vivo* experimental animal model crucial to further research [150]. Wound contraction is usually assessed by photographic analysis of standardized wounds created on the backs of animals. Alternatively, *in vitro* contraction models consist of fibroblast-populated collagen lattices (FPCL); where collagen is solubilized, seeded with fibroblasts, and polymerized at 37 °C before measuring the change in surface area or diameter of the lattice over time [151].

It is generally accepted that the fibroblast and myofibroblast are involved in wound and scar contraction, although the relative roles of each vary depending on the theory. Two theories on the mechanism of contraction have been proposed: 1) myofibroblasts, and 2) fibroblast locomotion and shape changes [84]. The first theory involves myofibroblasts, a specialized type of fibroblast, that produce α-SMA and possess thick cytoplasmic stress fibers [82]. It has been hypothesized that wound contraction involves cell shortening via α-SMA that then rearranges the surrounding connective tissue due to cell-to-cell contact [152]. As previously discussed, it appears that myofibroblasts contribute to the excessive ECM present in HSc. This excess ECM

probably contributes to the rigidity of HSc and reduces movement leading to scar contracture [84, 153]. The second theory suggests that fibroblasts cause contraction by exerting a traction force as they propel themselves through connective tissue with filipodia [154, 155]. This theory is supported by the delayed predominance of myofibroblasts in healing wounds until a week after the majority of wound contraction has already occurred [84]. It has been suggested that myofibroblasts function not to cause contraction but rather to maintain a static equilibrium that already exists within tissue [156]. This is consistent with experiments demonstrating that tension applied to skin [157] and scar [86] caused the appearance of myofibroblasts. This suggests myofibroblasts may not play a large role in wound contracture, but may instead be primarily responsible for scar contracture.

2.3.2 Wound Contraction is Primarily Caused by Fibroblasts

Wound contraction is affected by the same variety of factors as scar formation. In FPCL the rate of contraction is accelerated by increased fibroblast density, or decreased collagen concentration [158]. The cytoskeleton is another key factor in contraction of FPCL, and presumably healing wounds. While fibroblasts in FPCL are initially spherical they subsequently form a bipolar configuration [159, 160], following rearrangement of their microfilaments, considered necessary for contraction [161, 162]. When FPCL are examined shortly after contraction begins there appear to be two cell subpopulations present. Fibroblasts with numerous cytoplasmic microfilaments, characteristic of myofibroblasts, are localized to the edges while bipolar fibroblasts are predominant at the center [84]. When the relative contractile abilities of these subpopulations are compared the bipolar fibroblasts are far more contractile, again suggesting that fibroblasts instead of myofibroblasts are the major cell responsible for wound contraction [156, 163]. The environmental cytokines also exert great influence on the contractile process. TGF-β increases the rate and degree of contraction without upregulating proliferation [164, 165], and it has been postulated this may be through the induction of PDGF [166]. Conversely, IFN-α2b reduced contraction of FPCL by fibroblasts possibly by downregulating cytoplasmic actin filaments [167] or increasing apoptosis [168]. In the FPCL model the type of collagen also has a great influence on contraction rates. Lattices with increased type III collagen [169], similar to HSc, display increased contraction rates, as did lattices of collagen taken from HSc [156]. Increased DCN expression by transfection inhibited

FPCL contraction [145]. Normally, DCN modulates collagen fibril formation [139, 140] and neutralizes TGF-β [141, 142]. Thus, in HSc, where DCN is significantly reduced, these factors may also add to wound contraction. The influence of ECM on wound contraction is further reinforced by the observation that contraction does not occur in frostbite injuries where cells are necrotic but ECM remains intact [170].

2.3.3 Scar Contraction is Primarily Caused by Myofibroblasts

Scar contraction is still a poorly understood process. Well known clinical risk factors include HSc [76]. The most predominant theories involve myofibroblasts [84]. It is well known that primary split thickness skin grafting is more effective in inhibiting wound contraction than delayed grafting [171], and more rapid scar maturation in animal models is associated with a more rapid reduction in myofibroblast population [172] secondary to the induction of apoptosis [173, 174]. Using fibroblasts and myofibroblasts isolated from HSc it was found that myofibroblasts played a greater role in scar contracture [175]. This suggests that myofibroblasts are the primary cell involved in scar contracture while fibroblasts are the primary cell involved in wound contracture. TGF-β is upregulated in HSc and increases the contractile forces of HSc fibroblasts leading to scar contracture [176]. Clinically, scar contractures appear most frequently over joints and mobile surfaces [84]. In burn wounds, HSc tensile forces induce transdifferentiation of fibroblasts into myofibroblasts [86]. Mechanical stress has also been shown to downregulate proapoptotic genes in fibroblasts [177]. This helps explain the predominance of myofibroblasts in scar contractures, why they do not undergo apoptosis as in regular scar, and suggests that modulation of myofibroblast behavior is likely key to reducing scar contracture.

2.4 Pathophysiology of Treatments

2.4.1 Non-Surgical Treatment

2.4.1.1 Pressure Garments

Pressure garments have been the major treatment modality for HSc since the early 1970s

[178]. The garments must be worn continuously for at least 23 hours a day and must be applied until the scar is mature, which can take 2-3 years [179]. The exact pressure required for effective treatment has never been scientifically established but most authors believe that pressures should exceed capillary pressure and recommend that pressure be maintained between 24-30 mmHg [180]. It is thought that, pressure may accelerate scar maturation and reduce the incidence of contractures. As well, pressure garments may help to alleviate the itchiness and pain associated with active HSc. A systematic meta-analysis of the evidence for use of pressure garment therapy revealed that there was a small, but statistically significant, improvement in scar height. However there was no significant difference for the outcomes of scar pigmentation, vascularity, pliability and color [181]. In terms of pathophysiology, pressure garments controls collagen synthesis, reduce collagen production and encourage realignment of collagen bundles already present [179]. While the exact mechanism of pressure garment therapy for the treatment of HSc is not fully understood, some of the possible mechanisms are increased myofibroblast apoptosis, a decrease in collagen synthesis, ischemic cell damage, and an increase in MMP-9 activity [115].

2.4.1.2 Silicone Gel Sheets

Silicone gel sheeting has been used for treatment of immature burn scars since being introduced by Perkins *et al*. in 1982 [182]. Silicone gel, which is cross-linked polymer of dimethylsiloxane, needs to be in place for at least 12 hours a day for 3-6 months [183]. Silicone gel sheets may accelerate scar maturation and improve pigmentation, vascularity, pliability and itchiness associated with HSc [184]. While the mechanism of silicone-based products in the treatment of HSc management has not been completely determined, some of the mechanisms of action suggested include an increase of skin temperature, development of a static electrical field, increased stratum corneum hydration, decreased TGF-β2 levels, increased fibroblast apoptosis, decreased mast cell numbers, and a decrease in fibroblast-mediated contraction [115].

2.4.1.3 Splinting

Patients with severe hand burns are subject to joint contractures which can lead to claw hand deformities (Figure 2.2). This intrinsic-minus position of the hand is due to increased fluid accumulation in the joints capsule, swelling of the collateral ligaments by fluid imbibition and subsequent ligament contraction [185]. In order to avoid the contracture, optimal positioning of

the hand is important [186]. Splints should be applied in intrinsic-plus position in which the wrist is slightly extended to 20-30°, the metacarpophalangeal joint should be flexed with approximately 80°, interphalangeal joints are completely extended and the thumb is placed in maximum abduction. Splinting is sufficient at night and active and passive exercises with the hand should be carried out twice a day. Range of motion should be avoided in patients with deep dermal or full thickness burns, where there is suspicion of an imminent injury to the extensor tendon apparatus to prevent rupture of the tendons [185].

2.4.2 Immunologic and Biomolecular Therapies

2.4.2.1 Corticosteroids

Intralesional corticosteroid injections have been used for the treatment of pathological scars since the mid-1960s [187] and remain the first line treatment. Triamcinolone acetonide is the most commonly used to treated concentration with injection of 10-40 mg/mL at 2-6 week intervals [188]. Injection should be confined to papillary dermis to avoid subcutaneous atrophy [189]. The mechanisms involved are complex and remain unclear. However, it is understood that corticosteroids inhibit the proliferation and contraction of fibroblasts, suppress inflammation by inhibition of leukocyte and monocyte migration and phagocytosis, increase hypoxia by vasoconstriction, increase collagenase production by inhibition of α2-macroglobulin, and inhibit growth factors such as TGF-β and IGF-1 [115, 189, 190].

2.4.2.2 5-Fluorouracil

5-fluorouracil (5-FU) is a pyramidine analog with antimetabolite activity, which is one of the antimitotic agents and is used as a chemotherapy drug. It has also been used as an antifibrotic adjunct to glaucoma surgery [191], in the treatment of basal cell carcinoma and keratoacanthoma [192], and for treatment of keloids and HSc [193, 194]. Intralesional injection of 5-FU (50 mg/mL) alone or in combination with corticosteroids and pulsed dye laser decrease the size of, soften, and flatten the abnormal scar [192-195]. 5-FU targets rapidly proliferating fibroblasts in dermal wounds which leads to inhibition of fibroblast proliferation and decreases fibroblast collagen production [196].

2.4.2.3 Interferon

The IFN family is an antifibrotic cytokine which consists of type 1 IFN (including IFN-α and IFN-β) and type 2 IFN (IFN-γ). They are capable of decreasing the excessive production of collagen and glycosaminoglycans by scar-forming fibroblasts and normalizing the subnormal level of collagenase activity [197]. IFN-α and IFN-γ have been antagonized TGF-b protein production [198]. IFN-α decreases cell proliferation, collagen and fibronectin synthesis, fibroblast-mediated wound contracture [76, 167], which relates to a decrease of TGF-β, and mast cell histamine production. IFN-α also reduces the collagenase inhibitor TIMP1 (tissue inhibitor of metalloproteinases) [77]. IFN-γ increases myofibroblast apoptosis [199] and inhibits collagen synthesis but decreases collagenase activity [77, 200]. Subcutaneous IFN-α2b injection significantly improved scar quality and volume and sustained reduced serum TGF-β levels even after treatment [52]. However, intralesional interferon injections have not been found to be effective in scar modulation [201].

2.4.2.4 Transforming Growth Factor-β

Transforming growth factor (TGF)-β has been implicated in wound healing and HSc [180]. There are 3 mammalian isoforms of TGF-β (TGF-β1, TGF-β2, and TGF-β3). TGF-β1 and TGF-β2 have been identified as promoters of collagen synthesis and scarring, whereas TGF-β3 has been linked with scar prevention [189, 190]. A number of studies have targeted TGF-β effects by reducing Smad-3 and increasing Smad-7 with neutralizing antibodies. Natural inhibitors of TGF-β, which do not block wound healing and the immune system, such as LTBP-1 latency-associated protein, DCN, and biglycan (BGN) have been also studied for inhibiting TGF-β mediated biological effects [115, 189]. Mannose 6-phosphate has been injected into wounds to inhibit proteolytic activation of TGF-β from its latent form [202, 203]. In a rat cutaneous wound model, Shah *et al*. demonstrated significant reduction of scarring with exogenous addition of neutralizing antibody of TGF-β1 and TGF-β2 or exogenous addition of recombinant TGF-β3 [204]. Further investigation of TGF-β3 injection to the wound demonstrated the improvement of extracellular matrix deposition [55]. Recently recombinant human TGF-β3 intradermal injection (50-500 ng/100μL/cm) in the wound margin around the time of surgery, showed significant improvements in scar appearance [205, 206].

2.4.2.5 Decorin

As previously discussed, DCN has multiple antifibrotic effects through its interactions with growth factors TGF-β [142] and CTGF [207], and multiple cell surface receptors including: epidermal growth factor receptor [208], insulin like growth factor 1 receptor [209], and hepatocyte growth factor receptor [210]. It is also a key component of ECM [138], and modulates collagen fibril thickness [211]. In numerous animal models, DCN has been shown to reduce cancer metastasis [212], reduce renal [213], pulmonary [144], and cardiac [214] fibrosis, and promote spinal cord regeneration [215]. Several of these studies have utilized adenoviral DCN gene vectors to increase DCN production [144, 214], suggesting that DCN gene therapy may one day have a clinical role in HSc treatment or prevention.

2.4.3 Surgical Treatment

2.4.3.1 Scar Revision Surgery

HSc resulting from excessive tension or delay of wound closure can be treated effectively with surgery. There are many surgical options that include intramarginal excision, skin grafts, local flaps and free flaps although the surgical method used will depend on the degree, the part of scar and contracture as well as the size of the tissue defect after the abnormal scar has been excised. Generally, these techniques are however not appropriate for immature HSc [180].

2.4.3.2 Laser Therapies

Laser therapies were introduced for HSc by Apfelberg *et al*. and Castro *et al*. in the mid-1980s. There are two major kinds of lasers: ablative nonselective lasers such as the carbon dioxide (CO_2) and erbium:yttrium aluminum garnet (Er:YAG) lasers, and non-ablative selective lasers such as the pulsed-dye and neodymium doped:YAG (Nd:YAG) lasers. CO_2 and Er:YAG lasers have a high affinity for water. These lasers cause thermal necrosis which promotes wound contraction and collagen remodeling [216]. Pulsed-dye lasers are effective in the improvement of scar texture, redness, size and pliability [217, 218]. The mechanism of this laser therapy is based on selective photothermolysis, in which the light energy emitted from a vascular laser is absorbed by oxyhemoglobin, generating heat and leading to coagulation necrosis [219]. Kuo *et al*. found suppression of fibroblast proliferation and collagen type III deposition and down-

regulation of TGF-β1 expression correlated with up-regulation of MMP-13 (collagenase-3) activity [219].

2.4.3.3 Cryosurgery

Shepherd and Dawber were the first to apply cryosurgery as a monotherapy regimen for treating HSc and keloids in 1982. Cryosurgery required up to 20 treatment sessions with 2-3 spray or contact freeze-thaw cycles of 15-30 seconds each [189]. Cryosurgery reduces the volume and helps to soften the lesions but the side effect of permanent hypopigmentation is a major handicap [190]. However, recently an intralesional needle cryoprobe method has been developed to improve the efficacy and avoid the side effects [220]. The mechanism of cryosurgery is based on the low temperatures, which cause blood stasis, cell anoxia, and necrosis, leading to an increase of fibroblast apoptosis and decrease of vascularity [115, 189].

2.4.3.4 Stem Cells and Tissue Engineering

Early excision of burn tissue and early wound closure improve HSc and joint contracture [221]. Because of the limitations inherent in donor sites, dermal substitutes have been developed. Autologous and allogeneic skin substitutes that are composed of keratinocytes or fibroblasts, in part combined with allogenic (fibrin) or xenogeneic (collagen, hyaluronan) matrix substances [222]. Cultured epithelial autografts has been used since 1981 for the treatment of full thickness burn [223] but they are fragile and the graft take rate can be relatively low without a dermal component. The non-cellular components of the dermis, which primarily consist of ECM proteins and collagen [224], have been developed and attempts have been made to improve the rapid vascularization of dermal substitutes where growth factors such as VEGF and fibroblast growth factor have been used to accelerate vascularization but with modest benefit [225]. In a mouse model, Kataoka *et al*. demonstrated that potential of bone marrow-derived cells to be differentiated into cells composing the skin such as epidermal keratinocytes, sebaceous gland cells, follicular epithelial cells, dendritic cells and endothelial cells [226]. In another study, bone marrow-derived mesenchymal stem cells (BM-MSC) accelerated cutaneous wound healing which is thought to occur by the transdifferentiation of human BM-MSC into epithelial cells [227].

2.5 Conclusion

Fibroproliferative disorders underlie a wide variety of human diseases spanning most major organ systems. In hand burns, HSc continues to be a major source of morbidity. Increased understanding of the basic biology and pathophysiology of abnormal scarring, as reviewed here, is providing new and exciting avenues of research and potential clinical therapeutics to a difficult problem. Hopefully advances in fibroproliferative research will ultimately provide better outcomes for those with hand burns, and a multitude of other diseases.

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Chapter 3

Serum Decorin (DCN), Interleukin 1 beta (IL-1β), and Transforming Growth Factor beta (TGF-β) Post-Burn Predict Hypertrophic Scar Formation and can Exert both Local and Systemic Effects that Promote Fibrosis: A Pilot Study

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3.0 Abstract

Hypertrophic scar following burn injury is a significant problem. Previous studies have examined roles for decorin, interleukin-1 beta, and transforming growth factor-beta 1 in hypertrophic scar formation locally, but few have considered their systemic influence. We conducted a pilot study to examine whether serum levels of these molecules could predict hypertrophic scar formation. Serum levels were measured using enzyme linked immunoassay and hypertrophic scar formation determined from chart reviews. Peripheral blood mononuclear cells and fibroblasts were stimulated with decorin, interleukin-1 beta, and transforming growth factor-beta 1, and expression of profibrotic molecules examined using flow cytometry, immunofluorescence microscopy, quantitative polymerase chain reaction, and mass spectrometry. Multiple linear regression analysis suggested early serum levels of decorin and interleukin-1 beta, and late serum levels of transforming growth factor-beta 1 were predictive of hypertrophic scar formation. Decorin upregulated toll like receptor 4 and C-X-C receptor 4 expression in peripheral blood mononuclear cells, and interleukin-1 beta upregulated fibroblast production of C-X-C ligand 12. Transforming growth factor-beta 1 upregulated, and interleukin-1 beta downregulated, production of profibrotic cytokines, collagen, and myofibroblast differentiation. Thus, our pilot model predicting hypertrophic scar formation is supported by clinical results and limited in vitro experiments.

3.1 Introduction

Hypertrophic scar (HSc) is a fibroproliferative disorder occurring after deep burns and other deep dermal injuries [1]. HSc causes significant morbidity in patient function and cosmesis, and has few treatments with limited effectiveness [1]. As one of many fibroproliferative disorders, HSc shares underlying pathophysiologic features with pulmonary fibrosis, renal fibrosis, and scleroderma [2].

Therapies for preventing and treating HSc are areas of active investigation, since current modalities are complex, expensive, or can have significant side-effects [1].

Therefore, predicting HSc development is crucial in selecting patients in whom to study HSc, and those most likely to benefit from therapy.

Investigations into HSc following burns demonstrate that clinical factors such as burn size, depth, location, and time-to-heal all influence its formation [3]. Additional basic science work in our laboratory, and others, demonstrates that deep dermal fibroblasts give rise to HSc [4], and that small leucine rich proteoglycans (SLRP) such as decorin (DCN), and cytokines such as transforming growth factor-beta 1 (TGF-β1) are significant regulators of HSc development [1]. Other cytokines, such as interleukin-1 beta (IL-1β), have also been shown to be affected by total body surface area (TBSA) burn size and are predictors of overall outcome in burn patients [5].

The potential exists for significant interactions between DCN, IL-1β, and TGF-β in HSc development. First, studies have shown DCN upregulation by IL-1β stimulation in some cellular populations [6]. Second, IL-1β and TGF-β can play both opposing [7] and supporting [8] roles depending on their relative concentrations and time course of exposure [9]. Third, DCN and TGF-β interact due to DCN's ability to bind and neutralize TGF-β, and TGF-β's ability to downregulate DCN [1]. Finally, recent studies suggest that DCN may also have a profibrotic role in unique contexts, potentially related to interactions with toll-like receptor 4 (TLR4) [10].

Previous studies examining the cytokine response to burn injury have noted a two phase response: early elevation of serum IL-1β [11], and later elevation of serum TGF-β [12, 13]. While this late phase increase in TGF-β has been tied to the development of HSc [14], the potential role of serum DCN and IL-1β in the development of HSc is unclear. This systemic cytokine response may have an effect on circulating peripheral blood mononuclear cells (PBMC) and fibrocytes which are known to have an important role in the development of HSc [15, 16]. Given that recent literature in other disease states, such as systemic lupus erythematous, demonstrates changes in PBMC toll-like receptor levels in response to stimulation with known ligands [17], it is possible a similar systemic response occurs in burn HSc formation.

Given the significance of DCN, IL-1β, and TGF-β1 in burn patients and HSc formation, and the potential complexity of their interactions we conducted a pilot study to explore their potential relationships. In this study we were interested in determining whether systemic circulating DCN, IL-1β, and TGF-β1 correlated with the degree of injury in burn patients and resulting HSc formation, and whether any temporal relationships between them might be significant in promoting fibrosis. Furthermore, we wanted to explore relationships between these factors in terms of their potential systemic and local effects on wound healing pathways. We also wanted to develop a model allowing early prediction of the degree of HSc formation in patients, which is likely to be of utility in selecting patients for prophylactic treatment to prevent HSc formation. We hope these experiments provide some direction for future research for ourselves and others to investigate the causes of HSc formation in burn patients, and highlight the importance of the systemic response to burn injury which may influence HSc formation.

3.2 Materials and Methods

3.2.1 Human Specimens and Ethics Approval

Serum samples from eight post-burn patients and three controls (Table 3.1), human dermal fibroblasts from abdominoplasty skin specimens, skin biopsies from four burn patients and three controls, and PBMC from healthy human whole blood phlebotomy specimens were collected with informed patient consent under protocols approved by the Health Research Ethics Board, University of Alberta Hospital, Edmonton, Alberta, Canada. Scars were clinically diagnosed as normotrophic or hypertrophic by an experienced burn surgeon, and all clinically relevant details were abstracted from standard burn patient medical records.

Table 3.1 Patient information (P# = Patient, C# = Control, M = Male, F = Female).

Patient	**Age** (years)	**Gender**	**Burn** (%TBSA)	**Hypertrophic Scar** (%TBSA)
P1	25	M	53	18
P2	60	F	15	5
P3	21	M	54	10
P4	26	M	42	1
P5	29	F	20	0
P6	25	M	18	4
P7	48	F	40	0
P8	62	M	25	4
C1	53	F	0	0
C2	32	M	0	0
C3	45	F	0	0

3.2.2 Cell Isolation

Human dermal fibroblasts were isolated from abdominoplasty specimens using a dermatome to separate dermis into superficial and deep layers for enzymatic extraction of fibroblasts [18]. Fibroblasts were propagated in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen) and antibiotic-antimycotic (Invitrogen) in a cell culture incubator at 37 °C at 1 atmosphere of air with 5% CO_2. Fibroblasts from passages 3-5 were used for all experiments.

Human PBMC were isolated from normal individual's peripheral whole blood specimens by layering on Lymphocyte Separation Medium (Mediatech, Manassas, VA) in 50 mL conical tubes and centrifugation according to the manufacturer's protocol. PBMC were then frozen in DMEM with 20% FBS and stored at -80 °C until used for further experimentation.

3.2.3 Enzyme-Linked Immunosorbent Assay for IL-1β, DCN, and TGF-β1 in Serum and Cell Culture Supernatant

Serum samples were isolated from burn patient's and normal individual's peripheral blood, centrifuged, divided into aliquots, and frozen at -80 °C in the usual fashion until analysis. Human IL-1β, DCN, and TGF-β1 ELISA kits (R&D Systems,

Minneapolis, MN) were used to analyze serum samples according to the manufacturer's instructions.

Human dermal fibroblasts, human umbilical vascular endothelial cells (HUVEC) (Invitrogen), and human PBMC were cultured in DMEM with 2% FBS, Medium 200 (Invitrogen) with Low Serum Growth Supplement (Invitrogen), and Roswell Park Memorial Institute (RPMI) 1640 (Invitrogen) with 10% FBS respectively. Cells were stimulated with varying concentrations (0, 50, 100, 200, and 500 pg/mL) of recombinant human IL-1β (R&D Systems) for 48 hours and DCN was measured in cell culture supernatant using ELISA as previously described.

3.2.4 Immunofluorescence for DCN Expression in Burned Human Skin

Matched normal and burned skin from four burn patients was fixed in Z-Fix (Anatech Limited, Battle Creek, MI) for 24 hours then processed into paraffin blocks, cut into 5 μm sections, and mounted on glass slides by the Alberta Diabetes Institute Histology Core Lab (University of Alberta, Edmonton, Canada). Sections were deparaffinized using sequential xylene and ethanol baths, then blocked with Image-iT FX (Invitrogen), and 10% bovine serum albumin (Sigma-Aldrich Corporation, St. Louis, MO). Sections were stained using goat anti-human DCN polyclonal antibodies (R&D Systems) diluted in 1% bovine serum albumin or diluent with primary antibody omitted for controls overnight at 4 °C and Alexa Fluor 488 chicken anti-goat secondary antibody (Invitrogen) diluted 1:200 at room temperature in the dark for 1 hour, then mounted in ProLong Gold with DAPI (Invitrogen) under glass cover slips. As a control, matched skin samples from three abdominoplasty specimens were dipped into 20°C (unburned) or 60°C (burned) water for 60 seconds [19] and processed and stained as described. Images were taken using a Zeiss Colibri microscope (Carl Zeiss MicroImaging, Thornwood, NY) and immunofluorescence was quantified using ImageJ (National Institutes of Health, Bethesda, MD).

3.2.5 Flow Cytometry Analysis for TLR4 and CXCR4 in PBMC, and α-Smooth Muscle Actin in Dermal Fibroblasts

Human PBMC were cultured in RPMI 1640 with 10% FBS and stimulated with recombinant human DCN (5 μg/mL) (R&D Systems) for 48 hours.

PBMC were stained using an allophycocyanin conjugated mouse monoclonal anti-human TLR4 antibody (eBioscience, San Diego, CA) and a phycoerythrin-cyanine 7™ mouse monoclonal anti-human C-X-C chemokine receptor type 4 (CXCR4) antibody (BD Pharmingen, San Diego, CA), fixed using formaldehyde, then immediately quantified using a FACSCanto II (BD Biosciences, San Jose, CA) flow cytometer (counting 10^4 events, dead cells gated out), and data analyzed with FACSDiva software (BD Biosciences).

Fibroblasts were cultured in DMEM with 2% FBS and antibiotics and stimulated for 48 hours with various combinations of IL-1β (200 pg/mL) and TGF-β1 (10 ng/mL) (Figure 3.1).

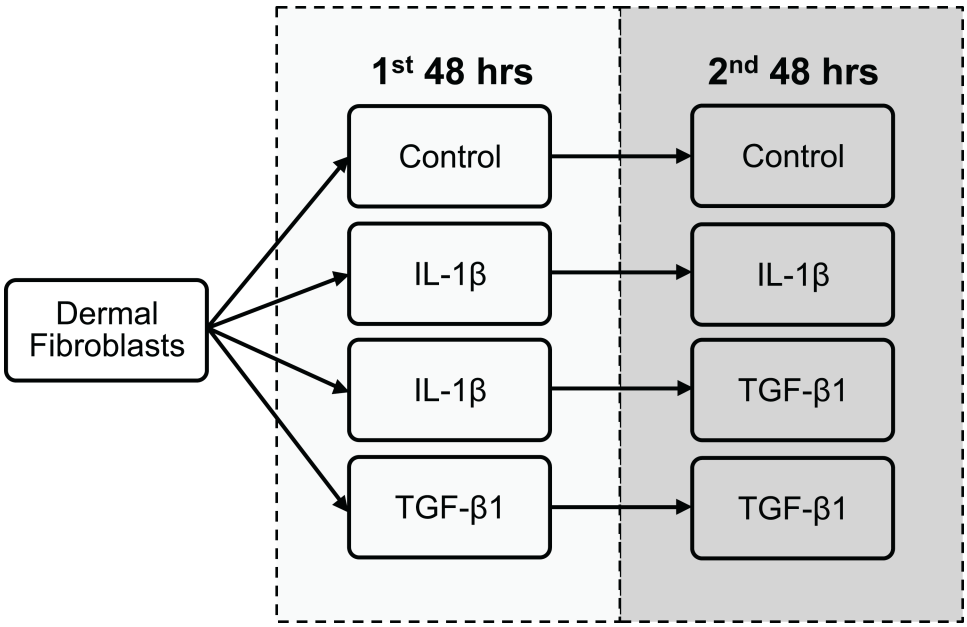


Figure 3.1 Experimental combinations of IL-1β and TGF-β1 used to stimulate dermal fibroblasts.

Fibroblasts were harvested using trypsin (Sigma-Aldrich), permeabilized using saponin (Sigma-Aldrich), then stained using a phycoerythrin conjugated mouse monoclonal anti-human α-smooth muscle actin antibody (R&D Systems) and fixed in formaldehyde. Myofibroblasts were quantified using a FACSCanto II flow cytometer (counting 10^4 events, dead cells gated out) and data analyzed with FACSDiva software

3.2.6 Quantitative Polymerase Chain Reaction for TLR4, IL6, IL8, IL-1β, and CXCR4

Human PBMC were cultured in RPMI 1640 with 10% FBS and stimulated with recombinant human DCN (5 μg/mL) (R&D Systems) for 48 hours.

Fibroblasts were cultured in DMEM with 2% FBS and antibiotics and stimulated for 48 hours with various combinations of IL-1β (200 pg/mL) and TGF-β1 (10 ng/mL) (Figure 3.1).

Cells were harvested for reverse transcription quantitative polymerase chain reaction (RT-qPCR) by centrifugation followed by total RNA isolation using Trizol (Invitrogen) according to the manufacturer's protocol. Reverse transcription using High Capacity cDNA Reverse Transcription Kit (Invitrogen) according to the manufacturer's protocol. Quantitative PCR (RT-qPCR) using RT^2 SYBR Green / ROX qPCR Master Mix (QIAGEN) with primers (Table 3.2) was conducted and results expressed as fold changes to reference gene hypoxanthine phosphoribosyltransferase 1 (HPRT1).

Table 3.2 PCR primers (F = Forward, R = Reverse).

Gene	**Direction**	**Sequence** (5' to 3')
TLR4	F	TGAGACCAGAAAGCTGGGAG
	R	CAGGTCCAGGTTCTTGGTTG
IL6	F	AGTGAGGAACAAGCCAGAGC
	R	CATTTGTGGTTGGGTCAGG
IL8	F	CGGAAGGAACCATCTCACTG
	R	AGCACTCCTTGGCAAAACTG
CXCR4	F	CGTGGAACGTTTTTCCTGTT
	R	AGGTGCTGAAATCAACCCAC
CXCL12	F	GTGGTCGTGCTGGTCCTC
	R	TTTGAGATGCTTGACGTTGG
IL1B	F	GAAGCTGATGGCCCTAAACA
	R	AAGCCCTTGCTGTAGTGGTG
TGFB1	F	CCCTGGACACCAACTATTGC
	R	CTTCCAGCCGAGGTCCTT
CTGF	F	TGGAGATTTTGGGAGTACGG
	R	TACCAATGACAACGCCTCCT
HPRT1	F	CTCCGTTATGGCGACCC
	R	CACCCTTTCCAAATCCTCAG

3.2.7 Liquid Chromatography and Mass Spectrometric Analysis of 4-Hydroxyproline

Fibroblasts were cultured in DMEM with 2% FBS and antibiotics and stimulated for 48 hours with various combinations of IL-1β (200 pg/mL) and TGF-β1 (10 ng/mL) (Figure 3.1).

Culture media was centrifuged and supernatant frozen and stored at -80 °C until analysis. Collagen production by fibroblasts was determined by quantifying 4-hydroxyproline levels in cell supernatant by liquid chromatography/mass spectrometry as previously described [20].

3.2.8 Statistical Analysis

All statistical analysis performed using STATA 10 (StataCorp, College Station, TX). Groups were compared with Students T-Test and ANOVA as appropriate. Statistical significance defined as P-value < 0.05, and Bonferroni correction used as appropriate.

For simple linear regression analysis DCN, IL-1β, TGF-β1, and %TBSA burned were analyzed as continuous variables and %TBSA HSc considered the dependent variable.

Multiple linear regression analysis was used to determine the most effective models predicting dependent variable %TBSA HSc based on measured serum values of DCN, IL-1β, and TGF-β1 taken at two different time points defined as early (first two weeks post injury), and late (two to four weeks post injury), as well as clinical variables such as age, gender, and burn size.

3.3 Results

3.3.1 Serum Levels of DCN, and IL-1β Correlate with Burn Size Whereas TGF-β1 Does Not

Serum from eight burn patients and three controls (Table 3.1) was collected within the first two weeks (early) and the following two weeks (late) post-burn and analyzed by ELISA for DCN, IL-1β, and TGF-β1. Both DCN and IL-1β are elevated following burn injury and this increase correlates significantly with the percent total body surface area (TBSA) burned, whereas TGF-β1 does not (Figure 3.2). This is confirmed by Pearson correlation coefficients showing a high degree of correlation between serum DCN and TBSA burned ($r = 0.81$, $P = 0.0026$), and serum IL-1β and TBSA burned ($r = 0.75$, $P = 0.0079$), but not between serum TGF-β1 and TBSA burned ($r = 0.35$, $P = 0.29$).

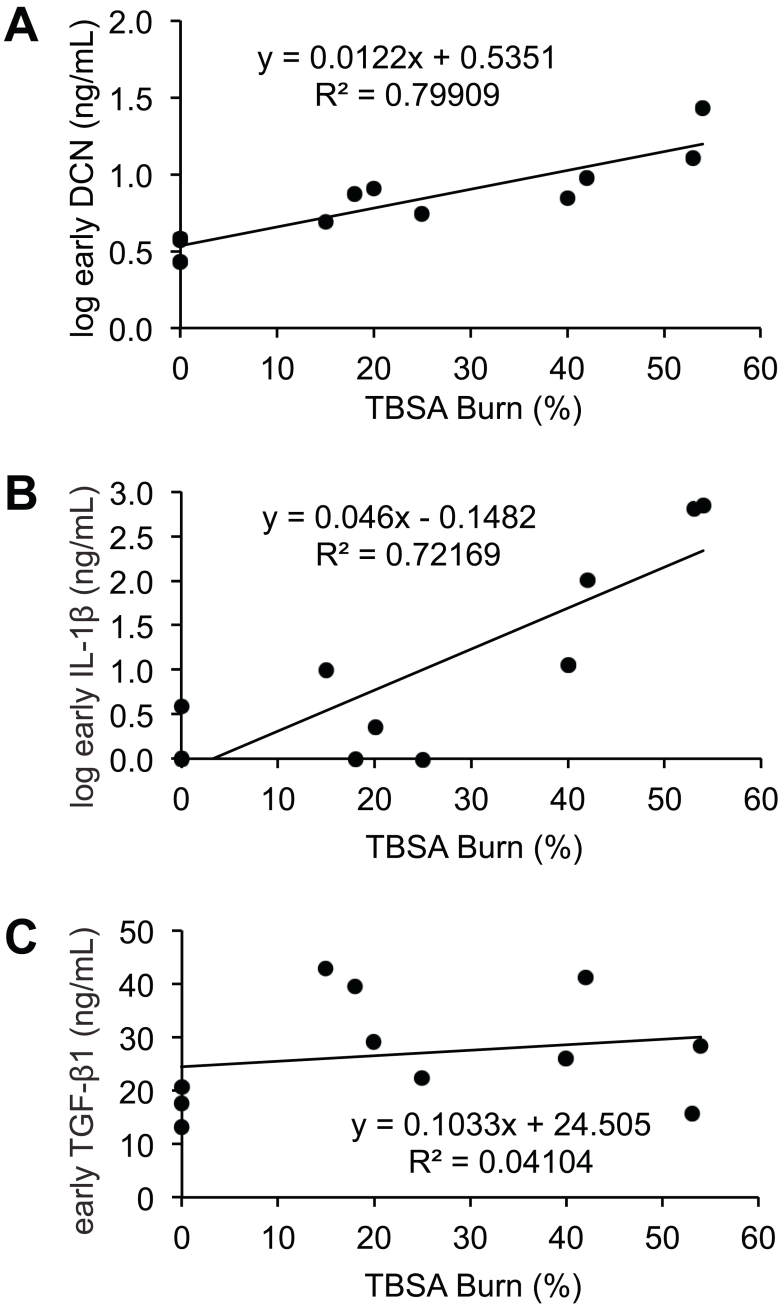


Figure 3.2 Linear regression of serum factors versus TBSA burned showing significant relationships between (A) log transformed early DCN and TBSA, and (B) log transformed early IL-1β and TBSA, but not between (C) early TGF-β1 and TBSA. (Data from 8 patients, 3 controls.)

3.3.2 Simple and Multiple Linear Regression Modeling of HSc Development Suggests Specific Temporal Combinations of DCN, IL-1β, and TGF-β1 Predict HSc Formation

To determine the role of serum DCN, IL-1β, and TGF-β1 in predicting HSc following burns, we performed simple and multiple linear regression using early and late serum values in addition to clinical variables including: gender, age, and burn size. In these models early DCN, early IL-1β, and late TGF-β1 emerged as significant predictors ($P = 0.0002$) of HSc formation while gender ($P > 0.38$), age ($P > 0.64$), and burn size ($P > 0.16$) did not. This predicted model of early IL-1β, and late TGF-β1 matches the observed pattern of an early serum IL-1β peak [11] and late serum TGF-β1 peak [12, 13] seen in the burn literature.

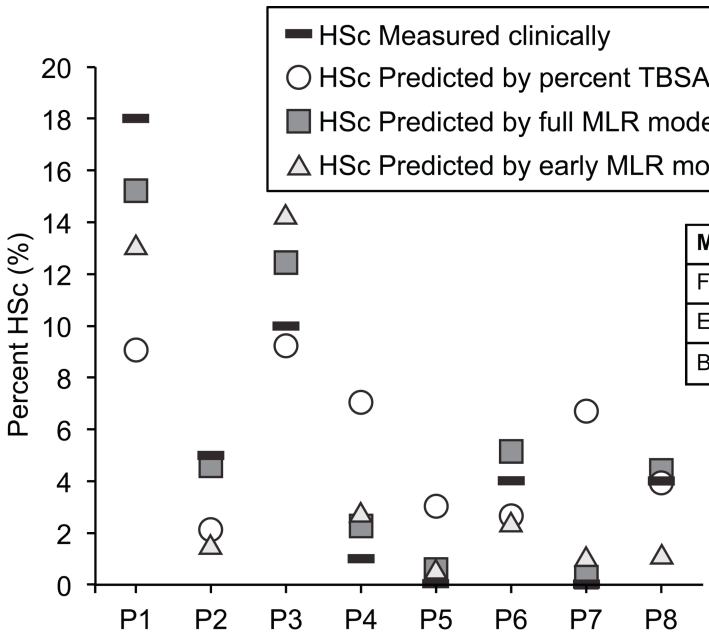
Interestingly, while a simple linear regression model of DCN alone in HSc formation suggests that DCN is profibrotic, the multiple linear regression model of DCN, IL-1β, and TGF-β1 suggests that DCN is antifibrotic. This suggests that DCN may play both pro- or anti-fibrotic roles depending on the context in which it acts—an idea investigated further in this study.

3.3.3 Multiple Linear Regression Modeling of HSc is More Accurate than Models Based on TBSA Alone, and Can Predict HSc Risk Early

Since percent TBSA is readily measured clinically and frequently cited as a predictor of HSc formation [21] we developed a simple linear regression model predicting HSc formation based on percent TBSA burn alone and compared this to our multiple linear regression model (Figure 3.3). In this model early DCN, early IL-1β, and late TGF-β1 predicted HSc formation more accurately than percent TBSA burn (adjusted $R^2 = 0.91$ versus adjusted $R^2 = 0.43$, all model parameters $P < 0.05$, and standard error of the estimate = 1.0 versus 5.0) (Figure 3.3).

In clinical practice a model based only on early serum values would be useful in guiding surgical management or prophylactic treatment. Therefore a multiple linear regression model based only on early serum DCN and IL-1β was also created, and it

compared favorably with the TBSA alone model (adjusted $R^2 = 0.70$ versus adjusted $R^2 = 0.43$, model P = 0.0035, and standard error of the estimate = 3.0 versus 5.0) (Figure 3.3).



Model	Adjusted R^2	P-Value	SEE
Full	0.908	0.0002	1.0
Early	0.697	0.0035	3.0
Burn Size	0.371	0.0276	5.0

Figure 3.3 Comparison of regression models in predicting HSc formation (patients P1-P8, details in Table 3.1). A full model of early DCN, early IL-1β, and late TGF-β1 (SEE 1.0) performs better than an early model of early DCN, and early IL-1β only (SEE 3.0), and both perform better than a model using %TBSA burn only (SEE 5.0).

3.3.4 Serum DCN is Produced by Dermal Fibroblasts in Response to IL-1β and Detectable in Burn Wound ECM

Serum IL-1β and DCN are highly correlated (Figure 3.4A) ($r = 0.97$, $P < 0.0001$). Two possible causes include IL-1β acting on the DCN gene promoter to drive its expression [6], and release of DCN from damaged tissue into the circulation, similar to the release of IL-1β following burn injury [5]. This correlation appears related to the degree of tissue injury as both are also correlated with percent TBSA burned, as discussed previously. Since it is unclear where serum DCN originates we considered two

possible sources: cells capable of producing DCN and releasing it into serum, and the ECM of burn wounds.

Since conflicting reports in the literature suggest not all cells produce DCN in response to IL-1β stimulation [22], we investigated specific cell populations involved in the systemic circulation: human dermal fibroblasts, human umbilical vascular endothelial cells (HUVEC), and PBMC, to determine if they produced DCN in response to IL-1β stimulation. Only DCN production by fibroblasts increased significantly in response to IL-1β ($P < 0.05$), while no increase was seen in either HUVEC ($P > 0.17$) or PBMC ($P > 0.16$), which both also had low basal levels of DCN expression (Figure 3.4B).

To examine the potential release of DCN from burned skin ECM, matched biopsies were taken from normal and burned skin showing a significant decrease in DCN in burned versus unburned skin (approximately 5 fold decrease, $P < 0.01$), whereas control skin burned *ex vivo* did not show this decrease (Figures 3.4C and 3.4D). This suggests serum DCN may be derived from the burn wound through a combination of production by surviving dermal fibroblasts stimulated by serum IL-1β, and release from proteolysis of burned ECM [23]. It is also possible that decorin in ECM is degraded via inflammatory proteolysis [24, 25], and that serum decorin is derived from some other, unknown, source.

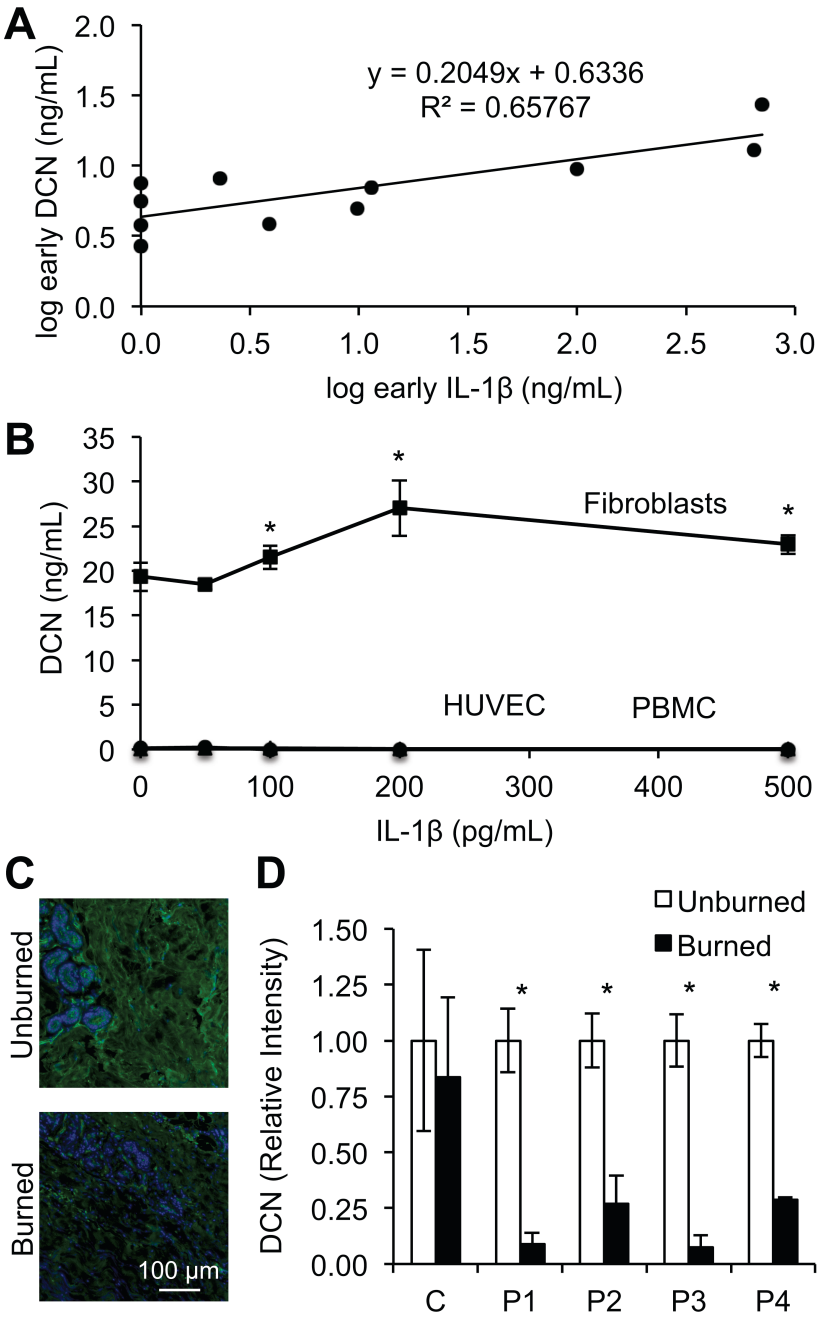


Figure 3.4 Potential sources of DCN in serum. (A) Log transformed DCN versus log transformed IL-1β levels, (B) DCN production with varying concentrations of IL-1β stimulation in HUVEC, PBMC, and dermal fibroblasts (mean ± SEM, n = 3 for each cell type at each time point, * $P < 0.05$). (C) Representative IHC of matched unburned and burned skin biopsies stained for DCN. (D) Quantitation of DCN present in matched unburned and burned skin biopsies from three controls and four patients (mean ± SEM, n = 3 sections per biopsy, * $P < 0.01$).

3.3.5 DCN Upregulates TLR4 and CXCR4 Expression by PBMC Contributing to a Systemic Profibrotic Response

While numerous publications have investigated the local effects of DCN in wound healing [1], few have considered its potential systemic effects. Multiple authors have investigated the profibrotic role of PBMC and their derivative fibrocytes in wound healing [26] and HSc development [16]. While the sequential stimulation of PBMC by IL-1β and then TGF-β has been shown to increase fibrocyte numbers [26], the potential role of serum DCN in PBMC regulation is unclear.

To investigate DCN's systemic effects we stimulated PBMC with DCN and measured changes in the expression of TLR4 and CXCR4 by RT-qPCR, and flow cytometry. We found DCN had two major profibrotic effects on PBMC. First, DCN significantly upregulated TLR4 protein expression by 41.4 ± 5.5 % ($P = 0.005$) as measured by mean fluorescence intensity (Figure 3.5A) and significantly increased the number of cells expressing TLR4 from 5.6 ± 0.6 to 8.3 ± 0.7 % ($P = 0.04$) (Figure 3.5B). Second, DCN significantly upregulated CXCR4 gene expression ($P < 0.03$) (Figure 3.5C) and protein expression by 12.0 ± 3.2 % ($P = 0.03$) as measured by mean fluorescence intensity. This increases PBMC sensitivity to profibrotic TLR4 ligands, and upregulates the CXCR4 receptor for CXCL12 increasing their ability to home to sites of injury.

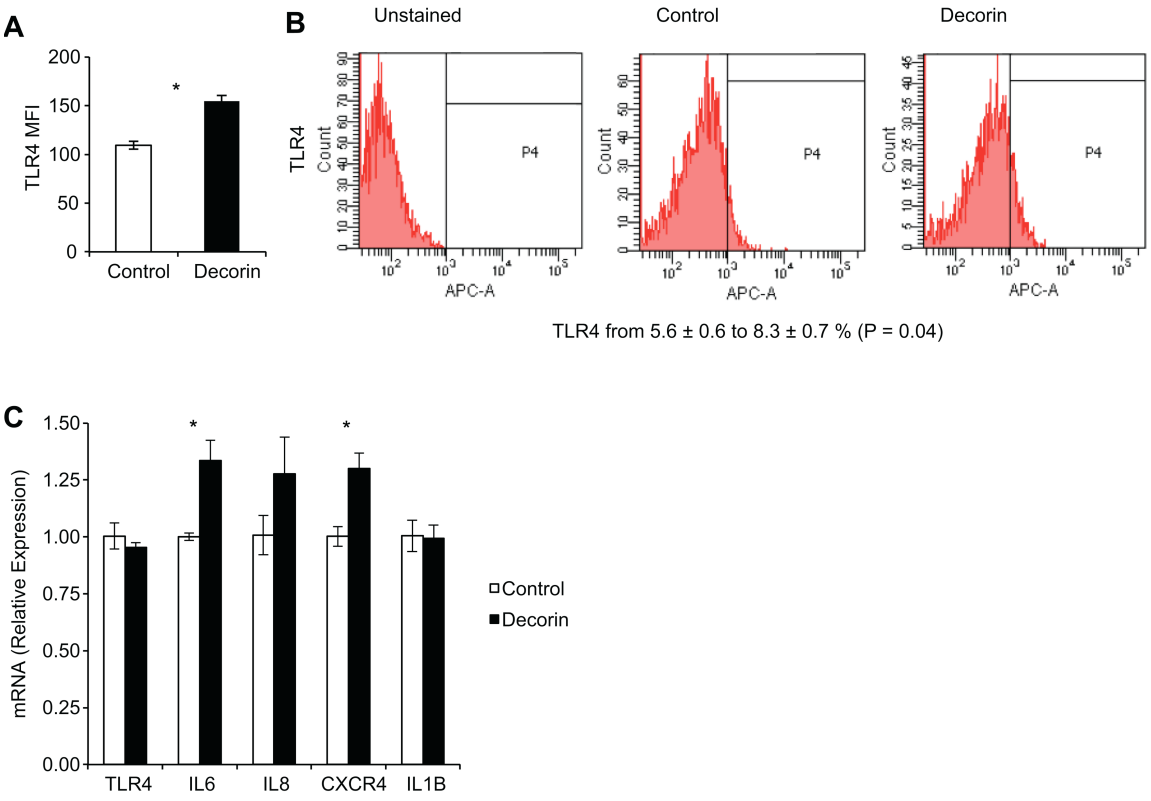


Figure 3.5 Effects of DCN on PBMC. (A) TLR4 expression with DCN stimulation of PBMC as measured by mean fluorescence intensity (mean ± SEM, n = 3, * P = 0.005). (B) Percentage of PBMC expressing TLR4 following DCN stimulation. (C) Relative expression of mRNA in PBMC for various cytokines following DCN stimulation, with significant upregulation of IL-6 and CXCR4 (mean ± SEM, n = 3, * P < 0.03).

3.3.6 IL-1β Upregulates CXCL12 Expression by Dermal Fibroblasts Contributing to a Systemic Profibrotic Response

Having established upregulation of the CXCR4 receptor on PBMC in response to DCN, we investigated production of its ligand, CXCL12, by dermal fibroblasts in response to IL-1β and TGF-β1 stimulation. CXCL12 is significantly upregulated by IL-1β ($P < 0.0025$), and this response is abolished by TGF-β1 (Figure 3.6).

This is consistent with the finding that early serum IL-1β is profibrotic and suggests that in the early phase of wound healing both serum IL-1β and serum DCN act in concert to activate PBMC and increase their homing to sites of injury via the CXCR4-

CXCL12 axis. As time progresses those cells local to the wound, such as dermal fibroblasts and previously recruited PBMC, increase their TGF-β1 production [27] with subsequent downregulation of CXCL12 and a damping of the CXCR4-CXCL12 axis, which may serve as a negative feedback mechanism. Those PBMC recruited to burn wounds also undergo a transition from PBMC to fibrocytes under TGF-β1 stimulation [26]. This temporal change could help explain the ordered progression seen in wound healing from inflammation to proliferation [27], and then development of HSc.

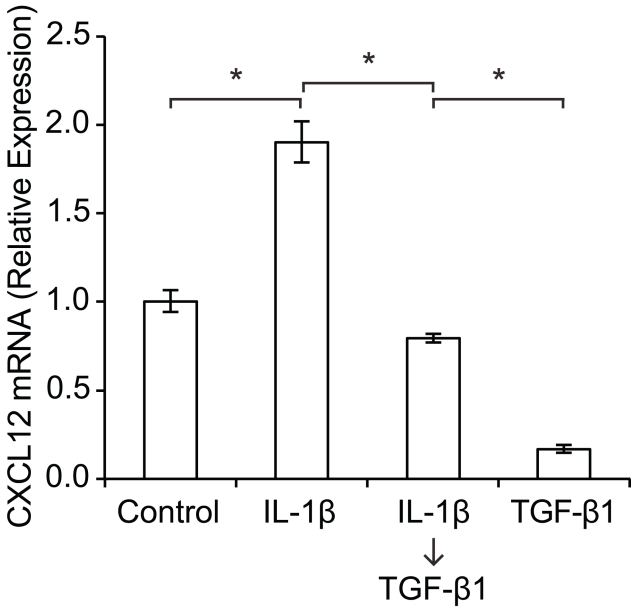


Figure 3.6 Effects of IL-1β and TGF-β1 on dermal fibroblast expression of CXCL12. IL-1β significantly upregulates CXCL12 whereas TGF-β1 significantly downregulates CXCL12 (mean ± SEM, n = 3, * $P < 0.001$).

3.3.7 TGF-β1 Stimulation of Dermal Fibroblasts Increase Production of Profibrotic Cytokines TGF-β1 and CTGF

Interactions of IL-1β and TGF-β1 on cellular behavior are varied based on cell type and duration of stimulation [7-9], with reports that acute IL-1β stimulation

downregulates TGF-β1 induced profibrotic behavior [7], but that chronic IL-1β stimulation upregulates TGF-β1 induced fibrosis [9].

As previously discussed, studies of post-burn serum IL-1β and TGF-β1 demonstrate an early IL-1β peak [11] and late TGF-β1 peak [12, 13]. Therefore effects of IL-1β and TGF-β1 stimulation on local wound fibroblasts were modeled by stimulating dermal fibroblasts following the temporal combination of IL-1β then TGF-β1 observed in the literature, and measuring production of key profibrotic cytokines TGF-β1 and CTGF using RT-qPCR.

IL-1β had minimal effects on TGF-β1 production and significantly decreased CTGF production ($P < 0.01$) (Figure 3.7). These effects were overcome by treatment with TGF-β1, which led to significant upregulation of these profibrotic cytokines ($P < 0.002$) (Figure 3.7). This demonstrates that IL-1β does not reduce the ability of TGF-β1 to induce profibrotic cytokines in dermal fibroblasts. It is also possible that other factors present *in vivo* but not in our *in vitro* model are required to maximize IL-1β's profibrotic effects [28]. This behavior supports our proposed model of HSc prediction.

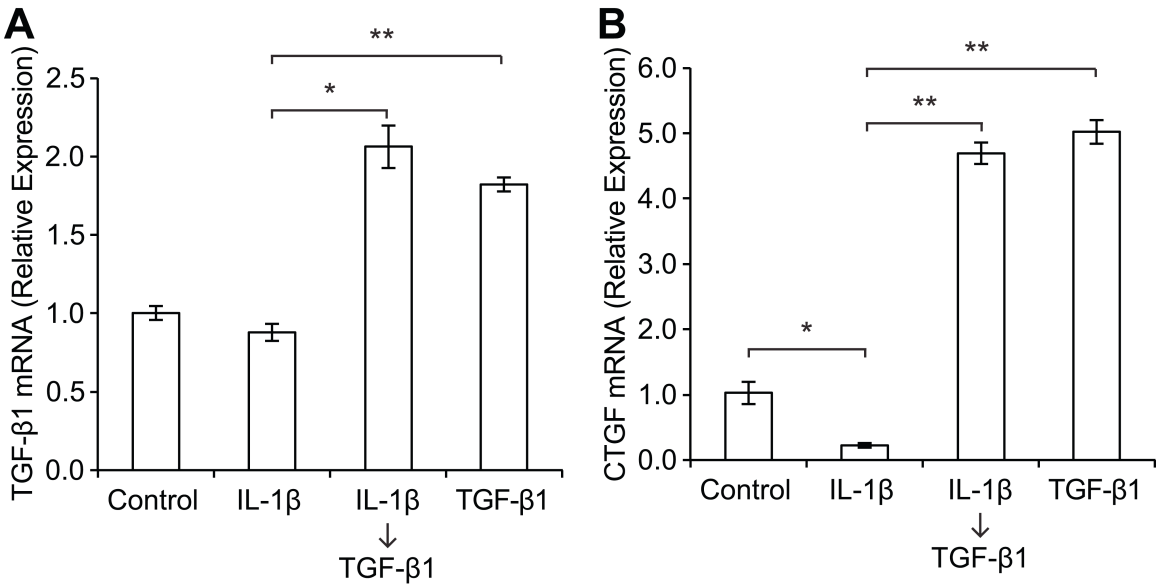


Figure 3.7 Effects of IL-1β and TGF-β1 on dermal fibroblast expression of TGF-β1 and CTGF. TGF-β1 significantly upregulates (A) TGF-β1 (mean ± SEM, n = 3, * P = 0.001, ** P = 0.0002), and (B) CTGF (mean ± SEM, n = 3, * P < 0.01, ** P < 0.0001).

3.3.8 TGF-β1 Stimulation of Dermal Fibroblasts Induces Collagen Production and Myofibroblast Differentiation

In the literature IL-1β and TGF-β1 appear to have opposing roles with respect to collagen production and myofibroblast differentiation [1, 29, 30]. While IL-1β decreases collagen production and myofibroblast differentiation, and TGF-β1 increases both, the effect of combining both cytokines in this regard is relatively unexplored.

To examine changes in profibrotic phenotype induced in dermal fibroblasts by IL-1β and TGF-β1 we measured collagen type I production using mass spectrometry of hydroxyproline, and myofibroblasts using flow cytometry. We found that IL-1β downregulates collagen, a finding supported by others [29], and that TGF-β1 significantly upregulates collagen production ($P < 0.04$), overcoming the effects of IL-1β (Figure 3.8A).

Since myofibroblasts are a histologic hallmark of HSc formation we measured the change in phenotype from fibroblast to myofibroblast following stimulation. As expected

[31], treatment with TGF-β1 significantly increased myofibroblast transformation ($P < 0.01$) (Figure 3.8B), whereas treatment with IL-1β had no effect (Figure 3.8B). This finding supports our model of late serum TGF-β stimulation promoting HSc development, and is in agreement with previous literature demonstrating the role of serum TGF-β in HSc formation [14].

Interestingly, although the percentage of myofibroblasts seen with stimulation of dermal fibroblasts by IL-1β followed by TGF-β1 was lower than for TGF-β1 treatment alone, the quantity of Type I collagen produced was similar. This suggests the collagen produced is not necessarily directly proportional to the number of myofibroblasts, but that a minimum number of myofibroblasts may be necessary for HSc formation.

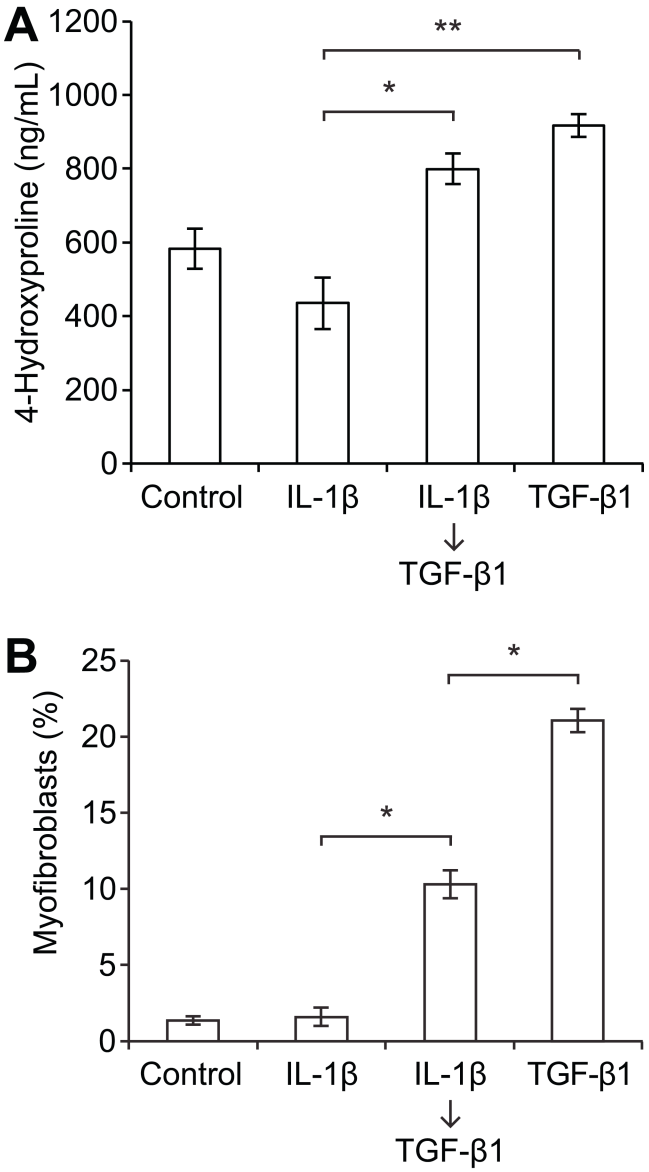


Figure 3.8 Effects of IL-1β and TGF-β1 on dermal fibroblast production of collagen type I and myofibroblast transformation. TGF-β1 significantly upregulates (A) 4-hydroxyproline as measured by liquid chromatography mass spectrometry (mean ± SEM, n = 3, * P = 0.01, ** P = 0.003), and (B) myofibroblasts as measured by flow cytometry (mean ± SEM, n = 3, * P < 0.002).

3.4 Discussion

Burn injury has long been observed clinically to have both local and systemic effects [32]. After survival, one of the main functional goals of burn care is prevention and treatment of HSc [1] which accounts for much of the morbidity suffered by burn survivors, and predicting its occurrence is of great importance. Although many authors have proposed models of HSc prediction [21], in general these use only clinical variables such as age, gender, percent TBSA, and anatomic location, and have limited accuracy.

HSc is a local wound healing response that occurs as a result of both local and systemic profibrotic effects [1]. By investigating circulating factors previously implicated in the response to burn injury (DCN, IL-1β, and TGF-β1) and their effects on both systemic PBMC and local dermal fibroblasts, we have developed a model predicting HSc formation that is supported both by *in vivo* clinical data and limited *in vitro* cellular experiments. This leads us to speculate that HSc may result, in part, from the interaction of local and systemic effects of DCN, IL-1β, and TGF-β1 on cells known to play key roles in HSc formation, namely fibroblasts and PBMC/fibrocytes. We have outlined a potential model of these interactions in Figure 9. As a pilot study our investigation has a limited sample size, however we believe the unique *in vivo* and *in vitro* combination used in developing our model warrants further investigation. Since HSc results from both local and systemic effects, efforts to identify burn wounds likely to develop HSc should not be based purely on depth of injury, but could be enhanced by measuring circulating factors as well. This may lead to development of a more comprehensive and clinically applicable model of HSc formation allowing burn surgeons to select those patients most at risk of developing HSc and treating them more aggressively.

The temporal significance noted in our multiple linear regression model is not unprecedented. Wound healing generally follows an orderly progression from an inflammatory phase to a proliferative phase and then a remodeling phase, with subsequent changes in both the types of cells involved and the cytokines and ECM they produce. Although our burn patient sample size is small, we believe this pilot project does suggest an important role for the systemic response to burns in contributing to HSc formation.

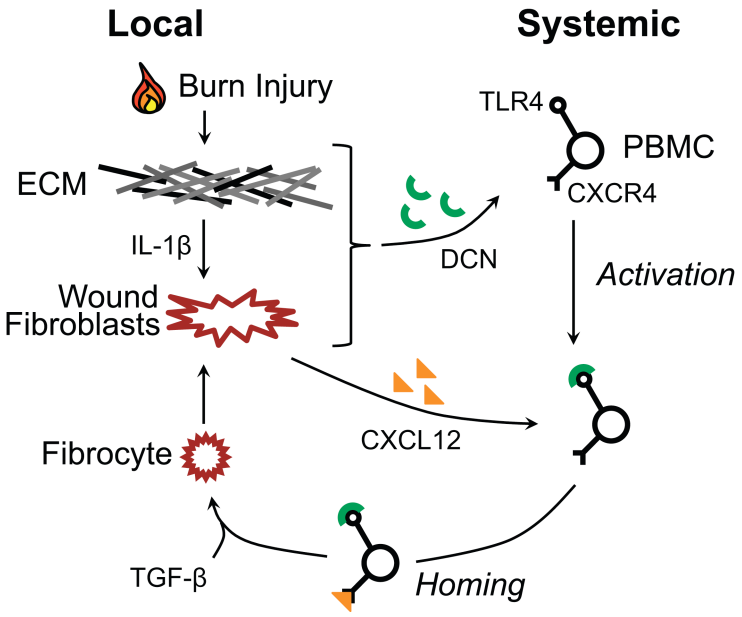


Figure 3.9 Proposed local and systemic roles of DCN, IL-1β, and TGF-β in contributing to the development of HSc.

Several research groups have demonstrated that IL-1β increases proliferation of fibrocytes and TGF-β1 increases fibrocyte differentiation into myofibroblasts [26]. Thus a temporal sequence of IL-1β followed by TGF-β after thermal injury, as predicted by our model, would lead to the increased numbers of profibrotic fibrocytes seen in post-burn HSc [16]. Similarly, late, long term TGF-β stimulation locally in wounds contributes to HSc formation [33]. Based on these results we investigated the potential local and systemic effects of these factors in creating a profibrotic environment that would promote HSc formation.

Although many studies suggest DCN is antifibrotic [1], this study suggests DCN may be profibrotic in certain unique contexts. This may be due to DCN activation of TLR4 in a similar manner to its closest related SLRP biglycan [10]. This would be consistent with previous reports that TLR activation of dermal fibroblasts is also profibrotic [34]. Although the pro- and anti-fibrotic properties of DCN appear at odds with each other, it is possible that ECM DCN from burn wounds has been fundamentally

altered or denatured and while retaining the ability to stimulate TLR4 systemically is no longer able to provide antifibrotic functions such as inactivating TGF-β locally. While the source of serum DCN in burn patients is currently unknown, it is potentially derived from a combination of burn wound fibroblasts and proteolytic degradataion of burn wound ECM [23, 25]. Ongoing research into the importance of damage associated molecular patterns (DAMP) in innate immune system activation and HSc formation [34], and the potential role of burn wounds in as a source of DAMPs may help in understanding the significant immunomodulatory effect of burn wounds [35]. It would appear that stimulation of PBMC by DCN does upregulate the CXCL12-CXCR4 axis, which has previously been shown to play a role in HSc formation in burn patients [15]. We demonstrated that the CXCL12-CXCR4 axis is also upregulated by IL-1β stimulation of dermal fibroblasts and subsequently downregulated by TGF-β1 stimulation of dermal fibroblasts, corresponding with the importance of early IL-1β and late TGF-β1 levels in our model predicting HSc.

While our clinical prediction model for HSc formation is based on human *in vivo* data, our subsequent experimental approach exploring the role of DCN, IL-1β, and TGF-β1 in the potential systemic and local response to burn injury is based on *in vitro* experiments using primary human cells and thus has inherent limitations. Despite these limitations, we believe our experiments suggest a potential role for DAMPs in the CXCL12-CXCR4 trafficking of PBMC to burn wounds. In the future, we plan to continue to exploring potential effects of this pathway on trafficking of PBMC and fibrocytes to burn wounds, and the role this plays in HSc development [15, 16].

As HSc develops more frequently in those wounds taking longer than two weeks to heal [36], the ability to predict those patients at increased risk of developing HSc during the initial two weeks following burn injury could be crucial in targeting patients most likely to benefit from prophylactic therapy or surgery. Future research in this area will likely benefit from combining both local burn wound evaluation by experienced burn surgeons or laser Doppler imaging [37], and systemic serum analysis for factors such as DCN, IL-1β, and TGF-β1.

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Chapter 4

MicroRNA 181b Regulates Decorin Production by Dermal Fibroblasts and may be a Potential Therapy for Hypertrophic Scar

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4.0 Abstract

Hypertrophic scarring is a frequent fibroproliferative complication following deep dermal burns leading to impaired function and lifelong disfigurement. Decorin reduces fibrosis and induces regeneration in many tissues, and is significantly downregulated in hypertrophic scar and normal deep dermal fibroblasts. It was hypothesized that microRNAs in these fibroblasts downregulate decorin and blocking them would increase decorin and may prevent hypertrophic scarring. Lower decorin levels were found in hypertrophic scar as compared to normal skin, and in deep as compared to superficial dermis. A decorin 3' un-translated region reporter assay demonstrated microRNA decreased decorin in deep dermal fibroblasts, and microRNA screening predicted miR-24, 181b, 421, 526b, or 543 as candidates. After finding increased levels of mir-181b in deep dermal fibroblasts, it was demonstrated that TGF-β_1 stimulation decreased miR-24 but increased miR-181b and that hypertrophic scar and deep dermis contained increased levels of miR-181b. By blocking miR-181b with an antagomiR, it was possible to increase decorin protein expression in dermal fibroblasts. This suggests miR-181b is involved in the differential expression of decorin in skin and wound healing. Furthermore, blocking miR-181b reversed TGF-β_1 induced decorin downregulation and myofibroblast differentiation in hypertrophic scar fibroblasts, suggesting a potential therapy for hypertrophic scar.

4.1 Introduction

The genetic regulation underlying wound healing and its dysregulation in hypertrophic scar (HSc) is complex and incompletely understood [1, 2]. HSc following burns share many features with fibroproliferative disorders like pulmonary fibrosis, renal fibrosis, and scleroderma [3]. Unfortunately current therapies for HSc are of limited efficacy [4]. Clinically HSc is red, raised, pruritic, and inelastic scar in the original zone of injury [5]. It impairs function [6], and its disfiguring effects can cause lifelong psychosocial morbidity [7]. Histologically, HSc is characterized by increased

myofibroblasts and mast cells, hypervascularity, excessive extracellular matrix (ECM) [8], whorls or nodules [9], and significantly decreased decorin (DCN) [10].

DCN is a small, leucine-rich proteoglycan [11] that plays key roles in ECM where it inactivates profibrotic transforming growth factor beta (TGF-β) [12] and connective tissue growth factor (CTGF/CCN2) [13], and antagonizes multiple cell surface receptors, including epidermal growth factor receptor [14], insulin like growth factor 1 receptor [15], and hepatocyte growth factor receptor [16]. In animal models DCN reduces cancer metastases [17], decreases renal [18] and pulmonary [19] fibrosis, improves post-infarction myocardial remodeling [20], and induces spinal cord regeneration [21]. DCN has been proposed as a treatment for HSc based on its *in vitro* ability to reduce collagen gel contraction by HSc fibroblasts [22], decrease cellular proliferation, reduce TGF-β_1 production, and decrease collagen synthesis [23]. Previous work demonstrates that DCN is significantly downregulated in HSc versus normal skin (NS) fibroblasts [24], and in deep dermal fibroblasts (DF) versus superficial dermal fibroblasts (SF) [25]. In a linear scratch model of increasing dermal depth Dunkin *et al*. found superficial injury regenerated and deeper injury scarred [26]. These observations suggest DCN production by SF is important for dermal regeneration and decreased production by DF contributes to scarring. Furthermore, it has been proposed that HSc arises from DF [25, 27].

MicroRNA (miRNA) are short, endogenous RNA, predicted to post-transcriptionally regulate approximately two thirds of human protein encoding genes [28]. They bind to the 3'UTR (un-translated region) of mRNA through seed region base pairing and decrease protein expression via effects on mRNA stability or translation [29]. The importance of miRNAs in skin development, homeostasis, and disease has been recently highlighted [30, 31], as has their role in fibrosis [32], and regulation of the proteoglycan versican [33].

Our hypothesis is that since miRNA often regulate related cell signaling networks [34], determining ones regulating DCN could indicate miRNA with roles in other fibrotic pathways and provide therapeutic targets with diverse effects. Based on differences between HSc and NS, and DF and SF, it is possible that increased expression of miRNA

targeting DCN in HSc and DF might help explain their reduced DCN expression and provide insight into HSc pathophysiology.

4.2 Materials and Methods

4.2.1 Primary Human Cells and Tissue Specimens

HSc and site-matched NS biopsies from burn patients, and matched SF and DF from human abdominoplasty specimens were obtained with written informed consent under protocols approved by the University of Alberta Hospital Health Research Ethics Board and conducted according to the Declaration of Helsinki Principles. Patient information is given in Table 4.1. Dermal fibroblasts were cultured from NS and HSc using explantation [35], or from abdominoplasty specimens using a dermatome to separate dermis into superficial and deep layers for enzymatic extraction of fibroblasts [25, 35]. Fibroblasts were propagated in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen) and antibiotic-antimycotic (Invitrogen) in an incubator at 37 °C in atmospheric air with 5% CO_2. Fibroblasts at passages 3-5 were used.

Table 4.1 Patient information.

Patient	**Sex**	**Age**	**Total Body Surface Area Burn** (%)	**Injury**
P1	Male	23	0	None
P2	Male	23	0	None
P3	Female	37	0	None
P4	Male	23	18	Burn
P5	Male	37	35	Burn
P6	Male	46	20	Burn
P7	Male	42	50	Burn
P8	Male	27	40	Burn

4.2.2 DCN Immunohistochemistry

Biopsies of site-matched HSc and NS were fixed in Z-Fix (Anatech Limited, Battle Creek, MI) for 24 hours then processed into paraffin blocks, cut into 5 μm sections, and mounted on glass slides by the Alberta Diabetes Institute Histology Core Laboratory (University of Alberta, Edmonton, Canada). Sections were deparaffinized using sequential xylene and ethanol baths, then blocked with Image-iT FX (Invitrogen), and then 10% goat serum (Jackson ImmunoResearch Laboratories, West Grove, PA) and 1% bovine serum albumin (Sigma-Aldrich Corporation, St. Louis, MO). Sections were incubated at 4 °C overnight with primary polyclonal goat anti-human DCN antibody (R&D Systems, Minneapolis, MN) diluted in 1% bovine serum albumin or diluent with antibody omitted as a negative control. Sections were then incubated with a secondary Alexa Fluor 488 chicken anti-goat antibody (Invitrogen) diluted 1:200 at room temperature in the dark for 1 hour. Specimens were mounted in ProLong Gold with DAPI (Invitrogen) under glass cover slips, imaged using a Zeiss Colibri microscope (Carl Zeiss MicroImaging, Thornwood, NY), and fluorescence measured using ImageJ (National Institutes of Health, Bethesda, MD).

4.2.3 DCN 3' UTR Reporter Assay

The DCN 3'UTR was cloned from a DCN cDNA (accession # BC005322) plasmid pDNR-LIB-DCN (Open Biosystems Products, Huntsville, AL) and inserted into pCAG-DsRed2 [36] from Addgene plasmid 15777 (Addgene, Cambridge, MA) between the stop codon and poly(A) sequence using Sticky-End PCR [37] to form pCAG-DsRed2-D3U (all primer sequences in Table 4.2A). pCAG-EmGFP was generated by replacing DsRed2 in pCAG-DsRed2 with EmGFP from pRSET-EmGFP (Invitrogen) using Sticky-End PCR. Plasmids were verified by sequencing at The Applied Genomics Centre (University of Alberta). SF or DF were grown on glass cover slips in DMEM + 10% FBS. Equimolar amounts of pCAG-DsRed2 and pCAG-EmGFP, or pCAG-DsRed2-D3U and pCAG-EmGFP were transfected using Lipofectamine LTX (Invitrogen), according to the manufacturer's instructions, then cultured for a further 48 hours in DMEM + 2% FBS. Cells were fixed in fresh 2% formaldehyde for 5 minutes,

mounted on glass slides in ProLong Gold with DAPI, imaged using a Zeiss Colibri microscope, and relative intensities calculated using ImageJ.

Table 4.2 Primer sequences used for (A) Sticky-end PCR, (B) miRNA qPCR screening, and (C) RT-qPCR of mRNA (F = forward, R = reverse).

Primer	**Sequence (5'-3')**
A	
DCN 3'UTR A	GGCCGCTTCTCAAGAAAGCCCTCATT
DCN 3'UTR B	CGTTCTCAAGAAAGCCCTCATT
DCN 3'UTR C	GGCCGCAGCTTTACTAAATATTGACATATATATTTACT
DCN 3'UTR D	CGAGCTTTACTAAATATTGACATATATATTTACT
EmGFP A	AATTCGCCACCATGGTGAGCAAG
EmGFP B	CGCCACCATGGTGAGCAAG
EmGFP C	GGCCGCATCAAGCTTCTCGAG
EmGFP D	GCATCAAGCTTCTCGAGTTACTTGTACAG
B	
anchor	ATGTGTCTACGTGCGCTCTG
+ve Control	CCATCTGGATTTGTTCAGAACGCTCGGTTGCC
-ve Control	TAGCACCATTTGAAATCAGTGTT
miR-24	TGGCTCAGTTCAGCAGGAACAG
miR-181b	AACATTCATTGCTGTCGGTGGGT
miR-191	CAACGGAATCCCAAAAGCAGCTG
miR-218	TTGTGCTTGATCTAACCATGT
miR-299-3p	TATGTGGGATGGTAAACCGCTT
miR-421	ATCAACAGACATTAATTGGGCGC
miR-491-3p	CTTATGCAAGATTCCCTTCTAC
miR-526b	CTCTTGAGGGAAGCACTTTCTGT
miR-543	AAACATTCGCGGTGCACTTCTT
miR-590-3p	TAATTTTATGTATAAGCTAGT
miR-875-3p	CCTGGAAACACTGAGGTTGTG
C	
DCN F	GGCTTCTTATTCGGGTGTGA
DCN R	CAGAGCGCACGTAGACACAT
HPRT1 F	CTCCGTTATGGCGACCC
HPRT1 R	CACCCTTTCCAAATCCTCAG

4.2.4 miRNA Screening

To determine potential miRNA regulating DCN we used prediction algorithms TargetScan [38], and miRanda [39]. Results were manually curated to select miRNA predicted to interact with other wound healing and fibrosis genes.

To further screen miRNA interactions a DCN 3'UTR qPCR screening protocol was developed. Briefly, qPCR primers (Table 4.2B) were designed as follows and ordered from Eurofins MWG Operon (Huntsville, AL). A forward primer was designed with perfect homology to a sequence of the plasmid pDNR-LIB-DCN upstream of the DCN 3'UTR. A positive control reverse primer with perfect homology to a sequence downstream of the DCN 3'UTR and a negative control reverse primer with a scrambled sequence were design. miRNA primers were designed using mature miRNA sequences from miRBase [40]. qPCR was performed with 5 ng of pDNR-LIB-DCN as template, appropriate primers, RT^2 SYBR Green / ROX qPCR Master Mix (SABiosciences, Frederick, MD), and the PCR cycles in Figure 4.1A. To determine the relative efficiency (E) of the miRNA as a reverse primer the equation in Figure 4.1B was used. The amplification efficiency (E) of the positive control primers was set to 2.0, the same quantity (Q) of pDNR-LIB-DCN was used as a template for all reactions, and calculations of amplification efficiency for remaining primer combinations were performed as outlined in Figure 4.1B, thus allowing the relative efficiencies of the miRNA primer to be calculated. The efficiency of the negative control primer was verified to be less than 1.35 as expected. Primer combinations with efficiencies ≥ 1.35 were selected as potential interactions based on acceptable efficiencies [41], and the remainder considered non-interactions.

4.2.5 Reverse Transcription Quantitative Polymerase Chain Reaction

Total RNA was isolated from cell culture using TRIzol (Invitrogen) according to manufacturer protocols with addition of GlycoBlue (Invitrogen) during isopropanol precipitation. Tissue for RNA extraction was flash frozen in liquid nitrogen, stored at -80 °C until it was ground to a fine powder in a chilled pestle and mortar, then dissolved in TRIzol and total RNA was isolated. Total RNA was reverse transcribed to cDNA using

miScript (QIAGEN, Valencia, CA). RT-qPCR was performed using RT^2 SYBR Green / ROX qPCR Master Mix (QIAGEN). RT-qPCR of miRNA was performed using miRNA specific primers (QIAGEN) according to manufacturer protocols and relative expression calculated using the comparative C_T method [42] with reference gene RNU6B. RT-qPCR of mRNA was performed using primers listed in Table 4.2C with reference gene HPRT1.

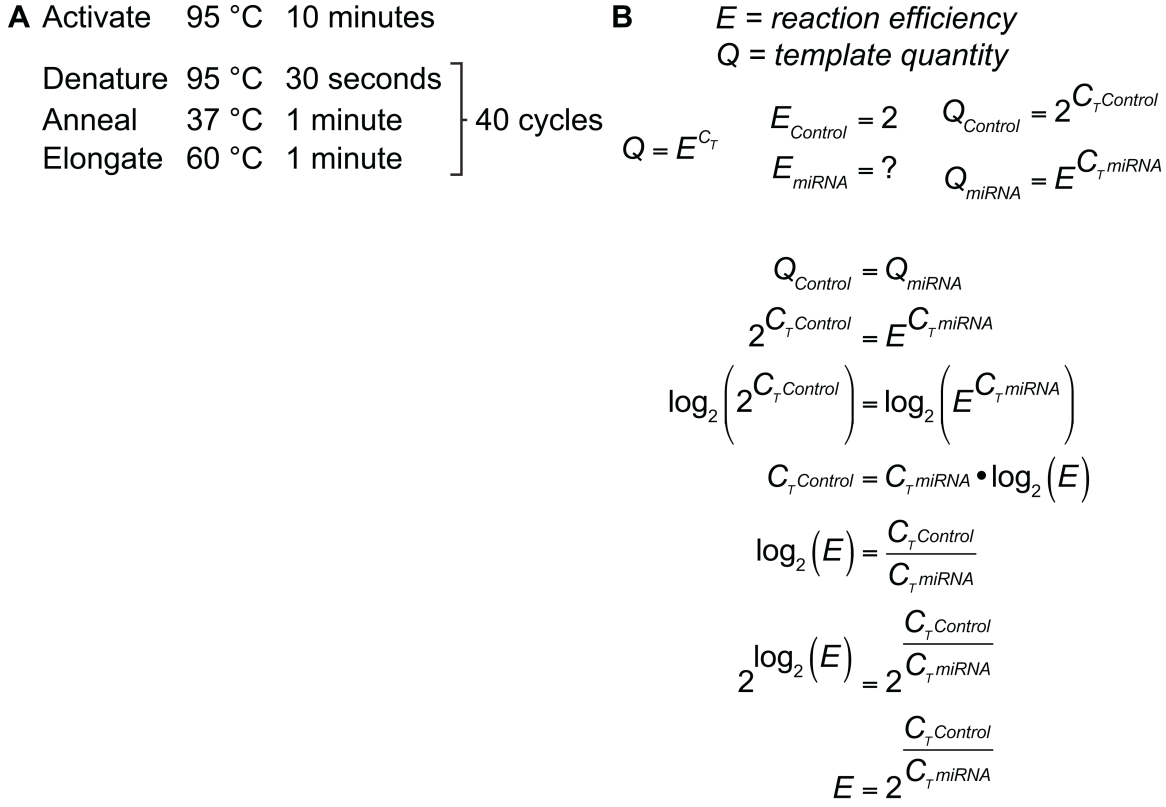

Figure 4.1 (A) PCR cycle parameters allowing nonspecific binding of mature miRNA at physiologic temperatures. (B) Derivation of the efficiency equation to determine interacting and non-interacting miRNA.

4.2.6 TGF-β_1 and CTGF Stimulation of Dermal Fibroblasts and Measurement of miRNA by RT-qPCR and DCN Protein by Enzyme-Linked Immunosorbent Assay

Matched SF and DF in DMEM + 2% FBS were stimulated with recombinant human TGF-β_1 (10 and 20 ng/mL) or CTGF (5 and 10 ng/mL) (R&D Systems). Total RNA was harvested and RT-qPCR of miR-24 and miR-181b was performed.

Recombinant human TGF-β_1 was used to stimulate site-matched NS and HSc fibroblasts in DMEM + 2% FBS at various concentrations for 48 hours. AntagomiR-181b (QIAGEN) was transfected into HSc fibroblasts using HiPerFect (QIAGEN). Cell culture supernatant was collected and DCN was measured using a human DCN ELISA kit (R&D Systems) according to manufacturer protocols.

4.2.7 Dual Luciferase Reporter Assay to Measure miR-181b Interactions with Potential Binding Sites from DCN 3'UTR

A dual luciferase reporter assay, pmirGLO (Promega, Madison, WI), had potential miRNA binding sites (Table 4.3) inserted using the manufacturer's protocol, to create reporters: pmirGLO-miR181b, pmirGLO-scramble, pmirGLO-DCN1, pmirGLO-DCN2, and pmirGLO-DCN3. Plasmids were verified by sequencing. HEK293A cells (American Type Culture Collection, Manassas, VA) were cultured in a 96 well plate using DMEM + 2% FBS and transfected with pmirGLO, pmirGLO-181b, pmirGLO-scramble, pmirGLO-DCN1, pmirGLO-DCN2, or pmirGLO-DCN3, and synthetic miR-181b (QIAGEN) using HiPerFect. After 48 hours relative luminescence was measured using the Dual-Luciferase Reporter Assay System (Promega) and an EnVision 2104 Multilabel Reader (PerkinElmer, Waltham, MA).

Table 4.3 Sequences used for miRNA binding sites in pmirGLO dual luciferase reporter plasmid.

Plasmid	**Sequence (**5'-3')
pmirGLO	No insert
pmirGLO-181b	ACCCACCGACAGCAATGAATGTT
pmirGLO-scramble	TGGGCGTATAGACGTGTTACAC
pmirGLO-DCN1	AACCTAACTGCAATGTGGATGTT
pmirGLO-DCN2	CATTACTGGTAAAGCCTCATTTGAATGTG
pmirGLO-DCN3	TTATGTCATCTATGTTGAATGTA

4.2.8 Synthetic miR-181b, DCN siRNA, and antagomiR-181b Treatment of Dermal Fibroblasts and Measurement of DCN mRNA by Reverse Transcription Quantitative Polymerase Chain Reaction and DCN Protein by Enzyme-Linked Immunosorbent Assay

Untreated, or control miRNA (QIAGEN), synthetic miR-181b, or DCN siRNA, was transfected into SF using HiPerFect according to manufacturer protocols. After 48 hours cell culture supernatant was collected and DCN measured using ELISA. Total RNA was harvested and RT-qPCR for DCN mRNA performed. Untreated, or control miRNA, or antagomiR-181b was transfected into DF using HiPerFect. After 48 hours supernatant was collected and DCN measured using ELISA.

4.2.9 TGF-β_1 Stimulation of Dermal Fibroblasts and Measurement of Myofibroblast Differentiation by Flow Cytometry

Recombinant human TGF-β_1 was used to stimulate site-matched NS and HSc fibroblasts in DMEM + 2% FBS at various concentrations for 48 hours. Control antagomiR or antagomiR-181b was transfected into HSc fibroblasts using HiPerFect. Cells were harvested using trypsin (Sigma-Aldrich) then permeabilized with saponin (Sigma-Aldrich) and stained using a phycoerythrin conjugated mouse monoclonal anti-human α-smooth muscle actin antibody (R&D Systems). Myofibroblasts were quantified using a FACSCanto II (BD Biosciences, San Jose, CA) flow cytometer and data analyzed using FACSDiva software (BD Biosciences).

4.2.10 Statistical Analysis

All statistical analysis performed with Stata 10 (Stata Corportation, College Station, TX). Student's t-test with Bonferroni correction, Wilcoxon signed-rank test, and Kruskal-Wallis rank test were used. $P < 0.05$ was considered significant.

4.3 Results

4.3.1 DCN Expression is Lower in HSc as Compared to Site-matched NS, and Deep as Compared to Superficial Dermis

To determine *in vivo* tissue DCN expression, immunohistochemistry was used to compare DCN in site-matched HSc and NS biopsies from burn patients, as shown in Figure 4.2A. DCN was significantly lower in HSc versus NS ($P < 0.001$), and deep versus superficial dermis in NS ($P < 0.001$), but not HSc ($P = 0.055$) (Figure 4.2B).

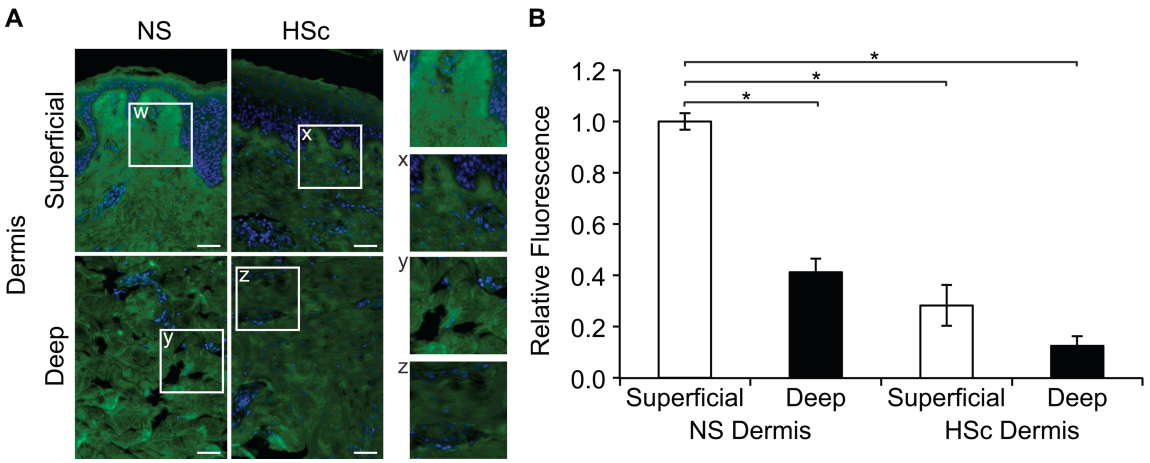

Figure 4.2 Immunohistochemical DCN expression in HSc and site-matched NS from burn patients. (A) Immunohistochemistry using a polyclonal goat anti-human DCN antibody and Alexa Fluor 488 secondary antibody (green fluorescence), and counterstained with DAPI (blue fluorescence) in representative site-matched sections of NS and HSc (scale bar = 50 μm). (B) Relative expression of DCN in matched superficial and deep NS and HSc sections was calculated from fluorescence using ImageJ (mean ± SEM, n = 4 patients, * $P < 0.001$).

4.3.2 DCN is Downregulated by miRNA in DF

HSc fibroblasts and DF produce less DCN than NS fibroblasts and SF *in vitro* [24, 25]. One possible explanation for decreased DCN production in deep dermal and HSc fibroblasts is increased levels of miRNA targeting DCN. To test this hypothesis a DCN 3'UTR fluorescent reporter assay was created. Production of fluorescent protein DsRed2 as normalized to fluorescent protein EmGFP was significantly downregulated to 0.52 ± 0.06 versus a baseline of 1.0 ± 0.06 ($P < 0.005$) in DF but not in SF ($P = 0.76$), suggesting DCN regulation by increased miRNA in DF targeting the DCN 3'UTR in the DsRed2 construct.

4.3.3 Several miRNA are Predicted to Regulate DCN in DF as Compared to SF

Potential miRNA regulating DCN were screened *in silico* and manually curated. Because many miRNA were predicted, a PCR protocol used to screen a cDNA library for

miRNA interactions [43] was modified for qPCR screening of mRNA 3'UTR-miRNA interactions. Testing of miRNA predicted to interact with the DCN 3'UTR fragment and several others not predicted to interact was performed (Figure 4.3). miR- 24, 181b, 421, 526b, and 543, had amplification efficiencies greater than 1.35 [41], as calculated using the formula in Figure 4.1B, and were further investigated.

miR- 24, 181b, 421, 526b, and 543 in matched SF and DF were measured using RT-qPCR (Figure 4.4). Significantly higher levels of miR-24 ($P < 0.05$) and miR-181b ($P < 0.05$) were found in DF versus SF suggesting one might be responsible for decreasing DCN. In contrast, although miR-421 was expressed at statistically higher levels ($P < 0.05$) in DF, its magnitude was low so it was not investigated further.

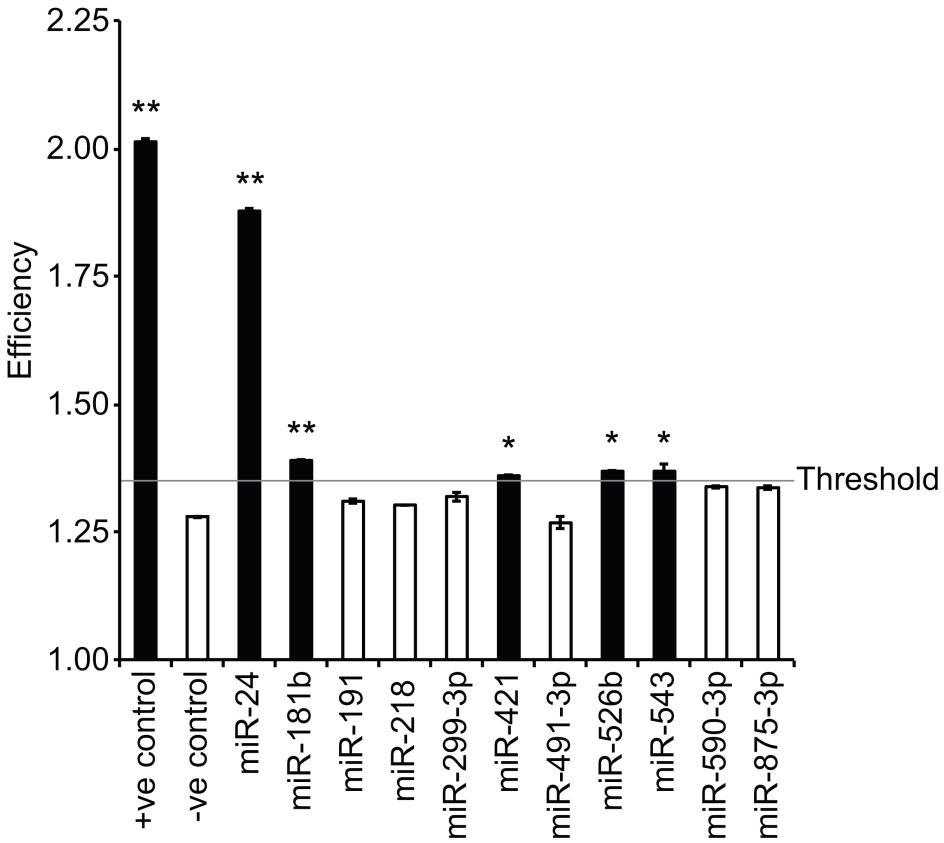

Figure 4.3 Results of miRNA qPCR screening suggest that miR- 24, 181b, 421, 526b, and 543 potentially target the DCN 3'UTR (mean ± SEM, n = 3, * $P < 0.005$, ** $P < 0.0005$).

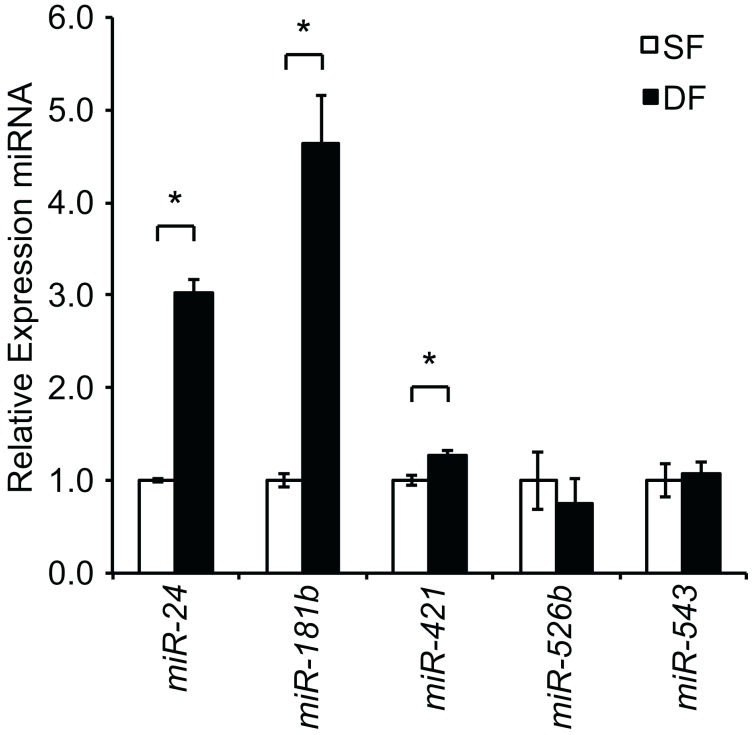

Figure 4.4 Evidence for the involvement of miRNA in DCN downregulation in DF. Total RNA was extracted from SF and DF cell culture after 48 hours and relative expression of selected miRNA quantitated using RT-qPCR (mean ± SEM, n = 3, * $P < 0.05$).

4.3.4 TGF-β_1 Upregulates miR-181b Expression in Dermal Fibroblasts but CTGF Does Not

Since TGF-β_1 is a key profibrotic cytokine in HSc development [44], its effects on miR-24 and miR-181b in SF and DF were examined using RT-qPCR. miR-24 was downregulated by TGF-β_1 in SF and DF in a dose-dependent manner (Figure 4.5A), in keeping with findings in myoblasts [45]. In contrast, miR-181b was significantly upregulated by physiologic levels of TGF-β_1 in SF and DF with a return to baseline at an extreme of 40 ng/mL (Figure 4.5B), and time-dependent manner (Figure 4.5C), similar to observations in hepatocytes [46]. A similar experiment using CTGF stimulation did not show changes in miR-181b expression. Based on these results miR-181b was selected for further investigation.

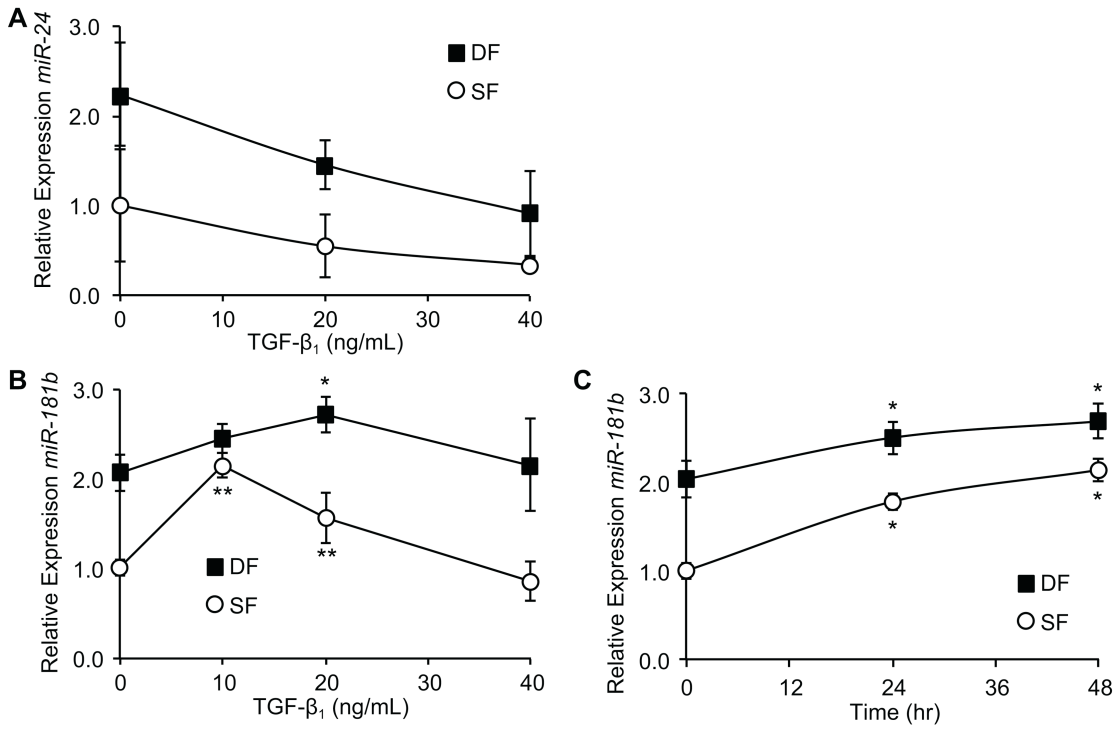

Figure 4.5 Regulation of miRNA expression by TGF-β_1 in SF and DF. Cells were cultured in DMEM + 2% FBS with the indicated treatment protocols and total RNA extracted for RT-qPCR. (A) Dose-response curve showing relative expression of miR-24 for SF and DF cultured in increasing concentrations of TGF-β_1 for 48 hours (mean ± SEM, n = 3). (B) Dose-response curve showing relative expression of miR-181b for SF and DF culutured in increasing concentrations of TGF-β_1 for 48 hours (mean ± SEM, n = 3, * $P < 0.05$, ** $P < 0.01$). (C) Time-response curve showing relative expression of miR-181b for SF and DF at fixed concentrations of TGF-β_1 (SF 10 ng/mL, DF 20 ng/mL) (mean ± SEM, n = 3, * $P < 0.03$).

4.3.5 miR-181b is Increased in HSc as Compared to Site-matched NS, and Deep as Compared to Matched Superficial Normal Dermis

After identifying miR-181b as a potential downregulator of DCN *in vitro*, its expression *in vivo* in tissues known to express less DCN was examined using RT-qPCR of miRNA isolated from site-matched HSc and NS biopsies, and matched deep and

superficial dermis. miR-181b was significantly increased in deep as compared to superficial dermis (Figure 4.6A), and HSc as compared to NS (Figure 4.6B).

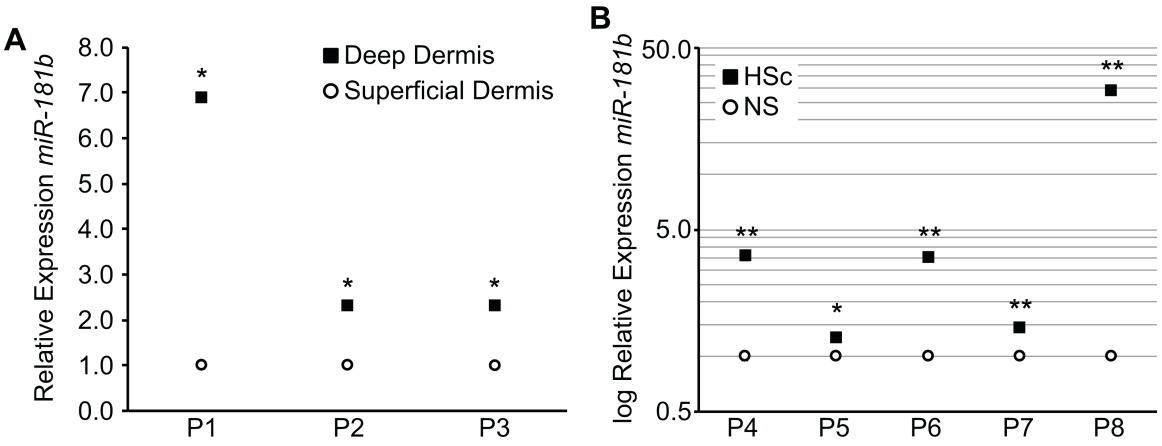

Figure 4.6 Relative expression of miR-181b in matched superficial and deep dermis and site-matched NS and HSc biopsies. Total RNA was extracted from tissue specimens using a chilled pestle and mortar and Trizol for relative quantitation using RT-qPCR. (A) Relative expression of miR-181b in matched superficial and deep dermis of NS (mean ± SEM, n = 3 samples per patient, * $P < 0.001$). (B) Relative expression of miR-181b in matched NS and HSc (mean ± SEM, n = 3 samples per patient, * $P < 0.05$, ** $P < 0.01$).

4.3.6 miR-181b Regulates DCN in Dermal Fibroblasts

To confirm predicted miR-181b binding sites from the DCN 3'UTR a series of dual luciferase reporter vectors based on pmirGLO were created and transfected into HEK293A cells (Figure 4.7A). There was no difference in regulation by miR-181b of reporters with no binding site or a scramble site ($P = 0.96$), however reporters with a perfect miR-181b site or one of three predicted miR-181b binding sites from the DCN 3'UTR (Figure 4.8) were all significantly downregulated by miR-181b ($P \leq 0.01$). One method to confirm miRNA regulation is to modulate miRNA levels and observe effects on its putative target [47]. Therefore, to confirm that miR-181b regulates DCN, synthetic miR-181b and antagomiR-181b were used to change miR-181b levels and changes in

DCN measured in dermal fibroblasts. SF were transfected with a synthetic miR-181b mimic which significantly reduced DCN protein by ELISA ($P < 0.03$) (Figure 4.7B), similar to DCN siRNA ($P < 0.02$), but not DCN mRNA by RT-qPCR (Figure 4.7C). When DF were transfected with antagomiR-181b, DCN protein by ELISA was significantly increased ($P < 0.01$) (Figure 4.7D). DCN protein levels are expressed as fold changes to allow comparison despite variation between fibroblasts from different individuals.

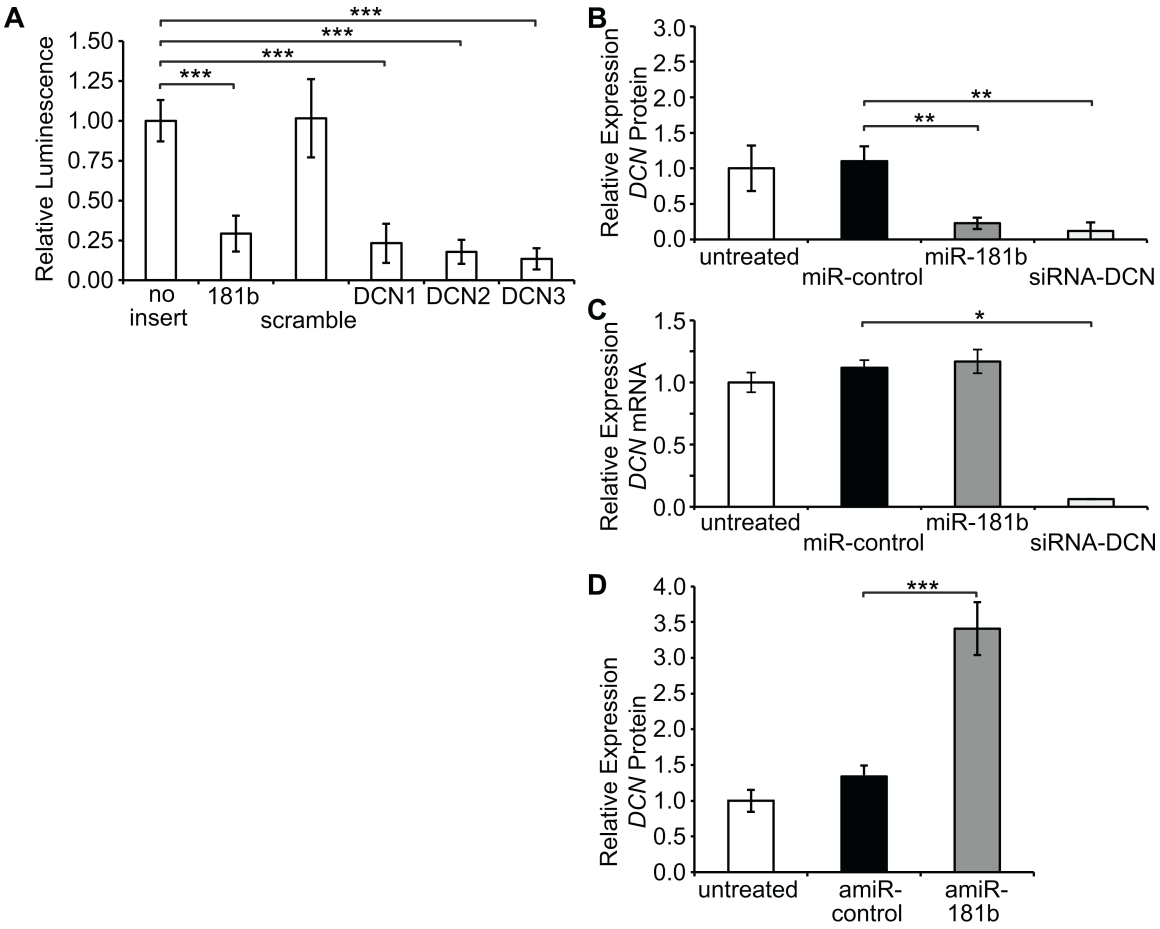

Figure 4.7 Regulation of DCN by miR-181b. HEK293A were cultured in DMEM + 2% FBS and transfected with pmirGLO constructs containing various miRNA binding sites and (A) relative fluorescence quantitated using a luminometer to determine relative knockdown by miR-181b (mean ± SEM, n = 4, *** $P \leq 0.01$). SF were cultured in DMEM + 2% FBS and transfected with miR-control, synthetic miR-181b or siRNA-DCN and (B) DCN protein in supernatant was measured by ELISA (mean ± SEM, n = 3, ** $P < 0.03$), and (C) DCN mRNA was measured using RT-qPCR on total RNA (mean ± SEM, n = 3, * $P < 0.05$). (D) DF were cultured in DMEM + 2% FBS and transfected with antagomiR-control (amiR-control) or antagomiR-181b (amiR-181b) and DCN protein in supernatant was measured by ELISA (mean ± SEM, n = 3, *** $P < 0.01$).

Figure 4.8 DCN 3'UTR (NCBI accession NM_001920.3) showing predicted miR-181b binding sites in bold with potential base pair alignment.

4.3.7 Blocking miR-181b Using antagomiR-181b Reverses TGF-β_1 Induced Downregulation of DCN and Upregulation of Myofibroblast Differentiation in HSc Fibroblasts

Based on prior results, blocking miR-181b might treat HSc, so this strategy was examined in matched NS and HSc fibroblasts treated with TGF-β_1. As shown in Figure 4.9A, TGF-β_1 stimulation significantly decreased DCN in both NS ($P < 0.02$) and HSc ($P < 0.02$) fibroblasts, and antagomiR-181b treatment reversed the decrease in DCN induced by TGF-β_1 in HSc fibroblasts, returning DCN to baseline ($P < 0.02$). Again, DCN protein levels are expressed as fold changes to allow comparison despite inter-individual

variation. As shown in Figure 4.9B, TGF-β_1 stimulation significantly increased myofibroblast differentiation (mean 7.99 fold increase compared to baseline, $P < 0.03$), and antagomiR-181b treatment reversed this effect, significantly decreasing the number of myofibroblasts (mean 3.01 fold increase compared to baseline, $P = 0.01$).

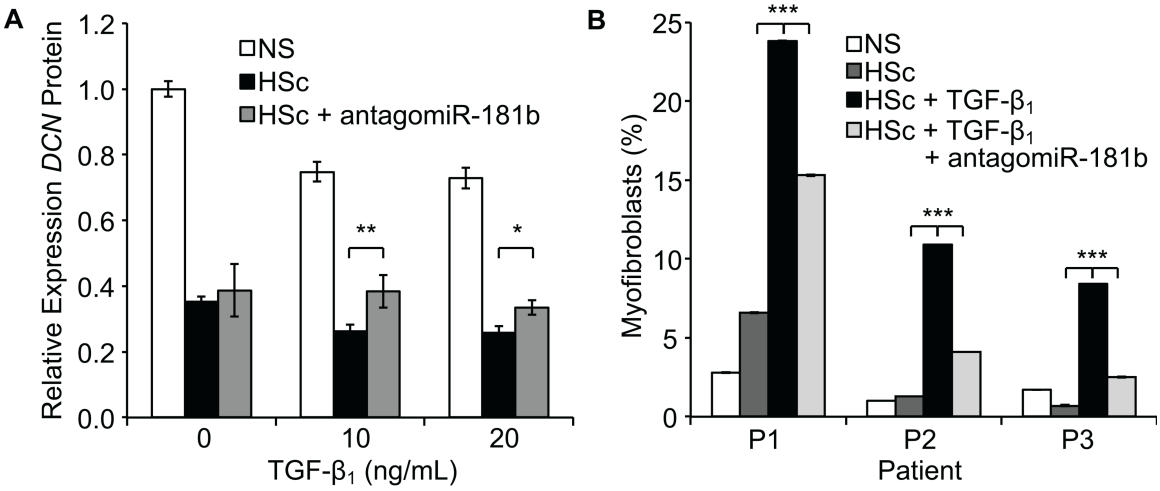

Figure 4.9 The effect of antagomiR-181b on TGF-β1 stimulated NS and HSc fibroblasts. (A) antagomiR-181b reversed DCN downregulation in HSc fibroblasts. Cells were stimulated by TGF-β1 at indicated concentrations and transfected with antagomiR-control or antagomiR-181b for 48 hours in DMEM + 2% FBS, and DCN protein was measured using ELISA on the supernatants (mean ± SEM, n = 3, * $P < 0.02$, ** $P < 0.006$). (B) antagomiR-181b reversed myofibroblast differentiation in HSc fibroblasts. Cells were stimulated by TGF-β1 10 ng/mL and transfected with antagomiR-control or antagomiR-181b for 48 hours in DMEM + 2% FBS then permeabilized and stained for α-smooth muscle actin and 10,000 cells per sample measured by flow cytometry (mean ± rSD, n = 3, *** $P < 0.03$).

4.4 Discussion

Finding DCN was significantly downregulated in HSc as compared to NS, and deep as compared to superficial dermis, adds to previous observations that HSc fibroblasts produce significantly less DCN than NS fibroblasts [24], and confirms *in vivo* tissue patterns which match *in vitro* observations of decreased DCN production by DF [25]. Similarities between superficial and deep HSc, suggests it arises from DF. Observations that miR-181b was significantly upregulated in DF *in vitro* and deep dermis and HSc *in vivo*, suggests comparing SF and DF *in vitro* mimics their *in vivo* behavior. Furthermore, similarities in miR-181b expression between HSc and DF add to publications supporting the hypothesis that HSc fibroblasts arise from DF [25]. DCN expression was altered by miR-181b modulation, thus demonstrating miR-181b regulates DCN. Previous work on DCN regulation explored TGF-β_1's role in negatively regulating DCN in dermal fibroblasts via its promoter sequence [48, 49], and our work demonstrating miR-181b, also induced by TGF-β_1, downregulates DCN adds further insight into this complex regulatory relationship.

Members of the miR-181 family, originally described as specific to hematopoetic tissues [50], are found in many tissues including muscle [51] and endothelial cells [52], as well as cancers including multiple myeloma [53] and hepatocellular carcinoma [54]. Depending on context miR-181b either inhibits or promotes differentiation of cells by regulating various transcription factors. Ji *et al*. found miR-181b was upregulated in hepatic stem cells, embryonic livers, and hepatocellular carcinoma where it inhibited differentiation by targeting NLK, GATA6, and CDX2 transcription factors [54]. In contrast, in myoblasts miR-181b promotes differentiation by targeting the transcription factor Hox-A11 [55]. Arnold *et al*. found increased miR-181b was characteristic of the transition from stem to proliferating non self-renewing cells [56], and Shi *et al*. found miR-181b was a tumor repressor in gliomas [57]. As the only adult organ known to undergo regeneration, the liver is unique as compared to other human organs [58], and this may explain the alternate function of miR-181b. In any case, miR-181b does regulate key transcription factors determining cellular differentiation and function, and may also do so in dermal wound healing. Furthermore, if miR-181b activation seen in

active muscle by Safdar *et al*. is due to repeated mechanical stress [51], one may speculate that this mechanism could be involved in the increased prevalence of HSc occurring in healing wounds over active joints [6]. Additionally, since miR-181b regulates a histone acetyl-transferase [53], it could also influence fibroblast epigenetics. It thus appears that many previously validated targets of miR-181b are transcription factors and signal transduction pathway components involved in regulating cellular behavior on a fundamental level, similar to the significant differences observed between NS and HSc fibroblasts, and suggesting that miR-181b regulation of DCN fits with the broad role DCN is already known to play in influencing cellular functions.

Knowing TGF-β levels are elevated in tissues following burn injury [8], suggests HSc fibroblasts exist in an environment of profibrotic TGF-β stimulation, and this promotes both DCN downregulation and myofibroblast differentiation. Based on these experiments it is possible to restore DCN production in TGF-β_1 stimulated HSc fibroblasts to their basal level by blocking miR-181b and significantly reverse the differentiation of HSc fibroblasts into myofibroblasts. Although this does not revert HSc fibroblast DCN production to that of NS fibroblasts, this likely a result of epigenetic changes occurring during HSc formation. As scar matures, DCN expression returns to NS levels, suggesting events delaying normalization contribute to the prolonged abnormalities seen in HSc. Blocking miR-181b removed this pathway of DCN downregulation and normalized DCN production. Perhaps this could restore balance between profibrotic (e.g. TGF-β_1) and antifibrotic (e.g. DCN) factors, thus encouraging scar maturation rather than fibroproliferation, and serving as a potential therapy for HSc.

The observations of increases in basal miR-181b expression in HSc and DF suggest miR-181b may serve an epigenetic role [59] in altered DCN expression levels in these cells and tissues. Given miR-181b's previously described roles in a wide variety of tissues and its regulation of several transcription factors and a histone acetyl-transferase, one may speculate that it could serve a broad regulatory role. Experiments to determine if miR-181b downregulates other antifibrotic signaling molecules or is upregulated by additional profibrotic cytokines would help further delineate its role in wound healing and fibrosis. And in fact, *in silico* prediction algorithms suggest that miR-181b targets

several other key factors involved in wound healing (Table 4.4). Given these findings, antagomiR-181b is a potential therapy for HSc and may either prevent its occurrence or accelerate its resolution through restoring targets of miR-181b, including DCN.

Table 4.4 Select *in silico* predicted miR-181b targets involved in fibrosis and wound healing using TargetScan [38].

Target Gene Symbol	Target Gene Name
IL2	Interleukin 2
TGFBRAP1	Transforming growth factor beta receptor associated protein 1
TIMP3	Tissue inhibitor of metalloproteinase 3
HSP90B1	Heat shock protein 90kDa beta 1
IL1A	Interleukin 1 alpha
BMP3	Bone morphogenetic protein 3
TGFBR1	Transforming growth factor beta receptor 1
SIRT1	Sirtuin 1
PLAU	Plasminogen activator urokinase
SMAD7	SMAD family member 7
TGFBR2	Transforming growth factor beta receptor 2
VCAN	Versican
SMAD2	SMAD family member 2
FGFR3	Fibroblast growth factor receptor 3

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Chapter 5

Decorin Gene Therapy Alters Deep Dermal Fibroblast Behavior to Mimic that of Superficial Dermal Fibroblasts in Remodeling Collagen Scaffold Used for Cultured Skin Substitutes

Submitted in part as:

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5.0 Abstract

The production and clinical use of cultured skin substitutes (CSS) can have a major impact on the survival and quality of life of major burn patients, and others. Unfortunately, skin biopsies used as a source of cells for CSS consist mainly of profibrotic deep dermal fibroblasts, rather than regenerative superficial dermal fibroblasts. We hypothesized that it would be possible to reduce fibrosis and improve remodeling of collagen scaffolds, which serve as the base for CSS production, by using an adenoviral gene vector for decorin (DCN), a small leucine-rich proteoglycan, to transduce deep dermal fibroblasts seeded onto the scaffolds. We compared the remodeling of these scaffolds with those seeded with superficial dermal fibroblasts, which regenerate rather than scar, deep dermal fibroblasts, which are profibrotic, and deep dermal fibroblasts treated with a control vector. We found the DCN vector had no adverse effects on dermal fibroblast proliferation or adhesion, and significantly reduced the production of profibrotic cytokines: transforming growth factor beta, and connective tissue growth factor in deep dermal fibroblasts to mimic levels seen in superficial dermal fibroblasts. We cultured the various fibroblasts in scaffolds for 14 days and then measured scaffold collagen fibril thickness, and analyzed scaffold morphology using a collagen orientation index. We found that scaffold remodeling by deep dermal fibroblasts was similar to that seen in hypertrophic scars, whereas scaffold remodeling by superficial dermal fibroblasts and deep dermal fibroblasts treated with the DCN vector were similar to normal skin. This suggests that DCN gene vector treatment of fibrotic deep dermal fibroblasts causes them to remodel collagen scaffolds similarly to regenerative superficial dermal fibroblasts, and may help in creating a CSS that promotes regeneration and inhibits fibrosis.

5.1 Introduction

Injury to skin as a result of trauma or surgery is a frequent occurrence. Of these, burn injuries are often the most severe in terms of both skin surface area affected, and resulting systemic effects, which can be life-threatening [1]. Recovery for patients with burns > 80% total body surface area is prolonged by the need for large quantities of autologous skin to close the

wounds and scarcity of donor sites, and complicated by the frequent development of hypertrophic scar (HSc) in deeper burns [2].

One approach to solving these problems has been the use of cultured skin substitutes (CSS)[3]. In this method a full thickness skin biopsy is taken from the patient's normal skin (NS) and separated into two main skin cell populations: keratinocytes and fibroblasts, which are then cultured and populated onto collagen based scaffolds where they form a limited autologous skin replacement [3]. As burn sizes increase the amount of available normal, unburned skin decreases. In some cases, there is so little unburned skin left, that partial thickness burns must be allowed to heal, a process often taking two to three weeks, so that the biopsy can come from these areas. Additionally, any full thickness skin biopsies are permanently unavailable as future donor sites for skin graft harvesting. Therefore, strategies to reduce the biopsy size needed, or the yield of useful cells from biopsies, may significantly improve patient outcomes. Furthermore, while promising, these CSS still form scar. We believe this may be due in part to the relative proportions of superficial and deep dermal fibroblast subpopulations used, although there has been little investigation of this issue.

Dermal fibroblasts can be subdivided into two distinct subpopulations: superficial and deep, with widely divergent responses to injury [4, 5]. In a dermal scratch model of increasing depth superficial injuries underwent regeneration whereas deep injuries resulted in scar [6]. There are several possible reasons superficial dermal fibroblasts are regenerative while deep dermal fibroblasts are fibrotic. Deep dermal fibroblasts produce more profibrotic cytokines such as transforming growth factor beta (TGF-β), and connective tissue growth factor (CTGF) [4]. They also produce more collagen, more tissue inhibitor of metalloproteinases (TIMP), and less matrix metalloproteinases (MMP) [4]. Another significant difference is that deep dermal fibroblasts produce much less decorin (DCN) than superficial dermal fibroblasts [4]. Previous research demonstrates that DCN, a small leucine-rich proteoglycan [7], binds and inactivates the profibrotic cytokines TGF-β [8] and CTGF [9], controls collagen morphology [10], and binds numerous profibrotic cell surface receptors including epithelial growth factor receptor (EGFR) [11], insuling-like growth factor 1 receptor (IGF1R) [12], and hepatocyte growth factor receptor (HGFR) [13]. Furthermore, DCN transduction with viral vectors shows promise in reducing fibrosis and inducing regeneration in several animal models of injury [14-16].

Since superficial dermal fibroblasts appear to cause skin regeneration and deep dermal fibroblasts appear to form scar, methods to alter deep dermal fibroblast behavior to be less fibrotic and more regenerative could provide greater numbers of desirable fibroblasts for CSS. Previous work in our laboratory suggests that CSS generated with superficial fibroblasts has desirable properties such as improved tensile strength [17], and improved basement membrane formation [18]. Therefore, we hypothesized that it would be possible to alter deep dermal fibroblast behavior to more closely mirror that of superficial dermal fibroblasts by use of a recombinant human DCN replication incompetent adenoviral vector, and that this would result in improved collagen scaffold remodeling.

5.2 Materials and Methods

5.2.1 Primary Human Cells and Tissue Specimens

Human dermal fibroblasts from abdominoplasty skin specimens, and site matched NS and HSc biopsies from burn patients were collected with written informed patient consent under protocols approved by the University of Alberta Hospital Health Research Ethics Board and conducted according to the Declaration of Helsinki Principles. Dermal fibroblasts were isolated from abdominoplasty specimens using a dermatome to separate dermis into superficial and deep layers for enzymatic extraction of fibroblasts [4, 19, 20]. Fibroblasts were propagated in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen) and antibiotic-antimycotic (Invitrogen) in a cell culture incubator at 37 °C at 1 atmosphere of air with 5% CO_2. Fibroblasts from passages 3-5 were used for all experiments.

5.2.2 Relative Distribution of Human Superficial and Deep Dermal Fibroblasts in Dermis

Surgical biopsies of NS were fixed in Z-fix (Anatech Limited, Battle Creek, MI) for 24 hours then processed into paraffin blocks, cut into 5 μm sections, and mounted on glass slides and stained with hematoxylin and eosin (H&E) by the Alberta Diabetes Institute Histology Core Laboratory (University of Alberta, Edmonton, Canada). The dermis was divided into superficial (< 0.56mm) and deep (> 0.56 mm) sections based on Dunkin *et al*. [6] and ImageJ (National

Institutes of Health, Bethesda, MD) used to aid in counting fibroblasts in the superficial and deep sections in three separate sections per patient to determine the number of superficial and deep dermal fibroblasts present.

5.2.3 Recombinant Decorin Adenoviral Vector Production

Using the AdMax™ Adenovirus Vector (Microbix Biosystems, Mississauga, Ontario, Canada), an E1/E3 deleted Cre-Lox replication incompetent recombinant adenovirus serotype 5 (rAd5) vector construction kit and standard molecular biology techniques, the coding domain sequence from a human DCN cDNA plasmid pDNR-LIB-DCN (accession # BC005322, Open Biosystems Products, Huntsville, AL) was cloned into the transfer plasmid pDC316 (Microbix Biosystems) to create pDC316-DCN and insertion confirmed by sequencing at The Applied Genomics Centre (University of Alberta, Edmonton, AB). The pDC316-DCN plasmid was co-transfected with plasmid pBHGloxΔ1,3Cre into HEK293A cells (Microbix Biosystems) using $CaCl_2$ precipitation according to the manufacturer's protocols. The resulting DCN adenoviral vector (rAd5-DCN) was titrated using multiplicity of infection (MOI) based on a plaque forming assay according to the manufacturer's protocols. A replication incompetent adenoviral vector (rAd5-DL70) (gift of Dr. Jack Gauldie, McMaster University, Hamilton, ON) was used as a control [21].

5.2.4 Collagen Scaffold Production

Scaffolds were prepared according to a standard protocol used in our laboratory where collagen-glycosaminoglycan matrices are prepared by freeze casting a co-precipitate of type I bovine collagen and chondroitin-6-sulfate, lyophilizing it, then using a vacuum oven for simultaneous dehydrothermal crosslinking and heat sterilization. Briefly, 0.5 wt% collagen powder (Devro Pty Limited, Bathurst, New South Wales, Australia) was solubilized in 0.5 M acetic acid (Sigma, St. Louis, MO) co-precipitated with 0.05 wt% chondroitin-6-sulfate (Sigma), and homogenized at 15000 rpm at 4 °C for 4 hours with an overhead blender (IKA, Wilmington, NC). The solution was degassed under vacuum for 2 hours at 20 °C, then cast between grade 316L stainless steel plates (Metal Supermarkets, Edmonton, Alberta, Canada) and frozen to -40 °C at a constant rate of -1 °C/minute in a refrigerated circulating ethanol bath (Haake Phoenix II, Thermo Scientific, Waltham, MA). The cast was then opened and the scaffold lyophilized

(FreeZone Plus 6, Labconco, Kansas City, MI) at -40 °C for 16 hours and then brought to 20 °C over 8 hours. Scaffolds were then cut into 20 mm discs and packaged in aluminum foil (Reynolds, Lake Forest, IL), and placed in a vacuum drying oven (APT.Line VD, BINDER GmbH, Tuttlingen, Germany) at 140 °C for 24 hours for dehydrothermal crosslinking and sterilization. Before use, scaffolds were rinsed twice in phosphate buffered saline for 15 minutes, then twice in cell culture medium for 15 minutes.

5.2.5 Adenoviral Vector Transduction and Effects on Dermal Fibroblast Decorin Production

Superficial and deep dermal fibroblasts were treated with rAd5-DCN in MOI ranging from 0 to 1000 for time points ranging from 0 to 24 hours. Decorin production was then measured in cell culture media after 48 hours using a DCN enzyme linked immunoassay (ELISA) as per the manufacturer's instructions (R&D Systems, Minneapolis, MN). For all adenoviral experiments, other than titration studies, fibroblasts were transduced for 24 hours at an MOI of 500.

5.2.6 Dermal Fibroblast Proliferation and Adhesion Following Adenoviral Vector Treatment

Mock transduced superficial and deep dermal fibroblasts, and rAd5-DL70 and rAd5-DCN treated deep dermal fibroblasts were plated at 5.0×10^3 cells per well in 96-well plates and cultured in DMEM with 10% FBS and antibiotics for varying times. The relative numbers of cells were determined using a previously published crystal violet assay [22]. Briefly, cells were fixed by adding 10 μL of 4% formaldehyde to the 100 μL of media in each well, followed by mixing with a rotary shaker at 500 cycles per minute for 15 minutes. Plates were then washed three times using deionized water and stained using 100 μL of 0.1% crystal violet dissolved in 200 mM boric acid for 20 minutes. Plates were again washed three times using deionized water, dried, and dye was then solubilized using 100 μL of 10% acetic acid. The optical density was measured at 590 nm using a microplate reader (THERMOmax, Molecular Devices, Sunnyvale, CA).

5.2.7 Reverse Transcription Quantitative Polymerase Chain Reaction

Superficial and deep dermal fibroblasts, and HEK293A cells (Microbix Biosystems) were cultured in DMEM with 10% FBS and antibiotics for 24 hours. Cells were harvested for reverse transcription quantitative polymerase chain reaction (RT-qPCR) by centrifugation followed by total RNA isolation using Trizol (Invitrogen) according to the manufacturer's protocol. Deep dermal fibroblasts were cultured in DMEM with 10% FBS and antibiotics for 0, 3, or 21 days after transduction with rAd5-DCN, then total RNA was extracted using Trizol (Invitrogen). And fibroblasts from the various treatment groups in collagen scaffold were harvested at the 14-day time point by extraction of total RNA using Trizol (Invitrogen).

Reverse transcription using the High Capacity cDNA Reverse Transcription Kit (Invitrogen) according to the manufacturer's protocol. Quantitative PCR using RT^2 SYBR Green / ROX qPCR Master Mix (QIAGEN) with primers for target genes (Table 5.1) was conducted and results expressed as fold changes to reference gene hypoxanthine phosphoribosyltransferase 1 (HPRT1).

Table 5.1 Polymerase chain reaction primers (F = forward, R = reverse).

Gene	**Direction**	**Sequence** (5' to 3')
Ad5-Fiber	F	GGAAATATCTGCACCCCTCA
	R	GCAGGGCTAGCTTTCCTTCT
DCN	F	GGCTTCTTATTCGGGTGTGA
	R	CAGAGCGCACGTAGACACAT
COL1A1	F	AAGAGGAAGGCCAAGTCGAG
	R	CACACGTCTCGGTCATGGTA
CTGF	F	TGGAGATTTTGGGAGTACGG
	R	TACCAATGACAACGCCTCCT
CXADR	F	GTGCTCCTGTGCGGAGTAGT
	R	GCATGGCAGATAGGCAGTTT
HPRT1	F	CTCCGTTATGGCGACCC
	R	CACCCTTTCCAAATCCTCAG
ITGAV	F	GTGACTGGTCTTCTACCCGC
	R	TCCAAACCACTGATGGGACT
ITGB3	F	TCTGGGCGACTGTGCTG
	R	TCATCAGAGCACCAGGCA
ITGB5	F	CCTTTCTGTGAGTGCGACAA
	R	TGTAACCTGCATGGCACTTG
MMP1	F	ACACATCTGACCTACAGGATTGA
	R	GTGTGACATTACTCCAGAGTTGG
TGFB1	F	CCCTGGACACCAACTATTGC
	R	CTTCCAGCCGAGGTCCTT

5.2.8 Collagen Scaffold Remodeling Measured by Collagen Fibril Thickness and Collagen Orientation Index

Collagen scaffolds, in triplicate, were seeded with 5.0×10^5 cells per cm^2 of superficial dermal fibroblasts, deep dermal fibroblasts, deep dermal fibroblasts transduced with rAd5-DL70, or deep dermal fibroblasts transduced with rAd5-DCN, and cultured for 14 days. Scaffolds were then harvested and divided into portions for microscopic examination and portions for RNA extraction.

Collagen scaffold sections, and matched NS and HSc sections were deparaffinized using sequential xylene and ethanol baths, then adjacent sections were stained using picrosirius red [23, 24] or hematoxylin and eosin (H&E). Picrosirius red stained sections were examined using a

circularly polarizing microscope (AxioImager.A1, Carl Zeiss MicroImaging Incorporated, Thornwood, NY), and photographed using a Canon PowerShot A640 (Canon Canada Incorporated, Mississauga, Ontario, Canada). ImageJ was used to measure collagen fibril thickness. Hematoxylin and eosin stained sections were examined and photographed using a Zeiss Colibri microscope (Carl Zeiss MicroImaging, Thornwood, NY). Sections were also imaged using a spinning disc confocal microscope to examine collagen fibrils (UltraVIEW VoX, PerkinElmer, Waltham, MA). Using ImageJ the collagen orientation index (COI) was calculated using the fast Fourier transformation (FFT) [25].

5.3 Results

5.3.1 The Majority of Dermal Fibroblasts in Normal Skin are Deep Dermal Fibroblasts

To determine the relative numbers of superficial and deep dermal fibroblasts present in skin we used the depth measured by Dunkin *et al*. in their linear scratch model (0.56 mm) as the dividing point between regeneration and scarring or superficial and deep dermal fibroblasts [6]. Comparing the number of superficial to deep dermal fibroblasts in H&E stained biopsies from three patients demonstrated that there are significantly more deep dermal fibroblasts than superficial fibroblasts ($77.6 \pm 2.0\%$ versus $22.4 \pm 2.0\%$, $P < 0.001$), as shown in Figure 5.1.

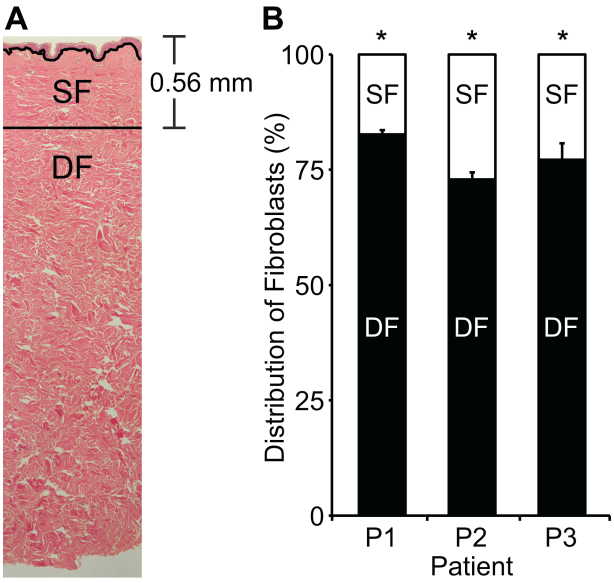

Figure 5.1 The majority of dermal fibroblasts in NS are deep dermal fibroblasts as seen on H&E section (77.6 ± 2.0% versus 22.4 ± 2.0%; mean ± SEM; n = 3 patients, 3 samples per patient; * P < 0.001).

5.3.2 Normal Skin has a Distinct Morphology and Thickness of Collagen Bundles as Compared to Hypertrophic Scar

To compare the morphology and thickness of collagen bundles in matched biopsies of NS and HSc we examined matched H&E and picrosirius red stained sections under the microscope, and noted several significant differences (Figure 5.2). Specifically on H&E sections one can see that there are more rete ridges, decreased cellularity, and a thinner epidermis in NS as compared to HSc. Furthermore, on picrosirius red sections one can see that collagen fibril morphology has a more linear and thinner appearance in HSc as compared to the "basket weave" seen in NS, and that in NS the deeper dermis has thicker collagen fibrils than in the superficial dermis. Finally, the COI of HSc was significantly higher than that of NS (0.34 ± 0.03 versus 0.07 ± 0.02, P <0.004). These findings are all consistent with those described in the literature [25-27].

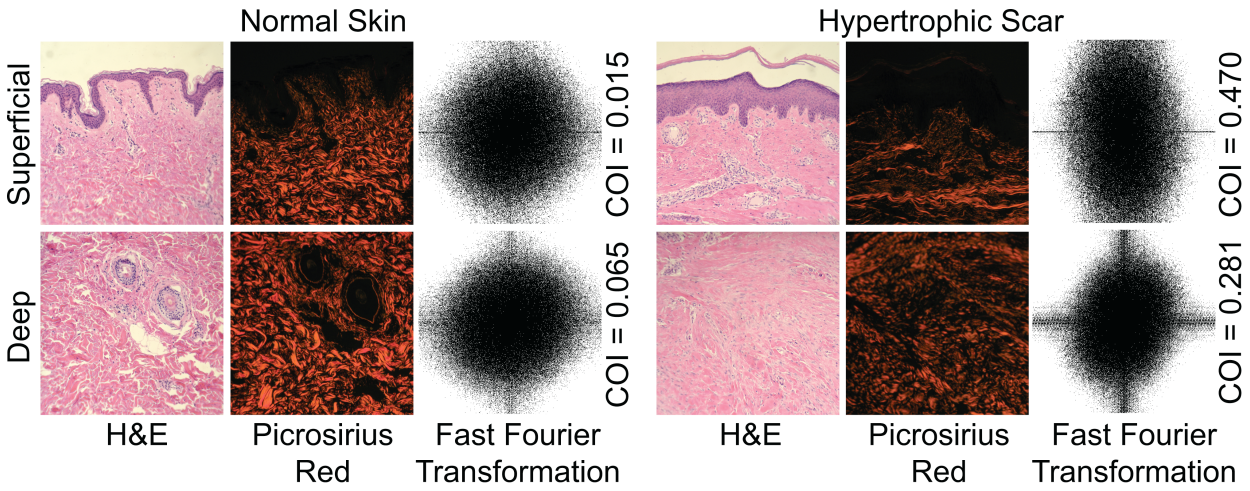

Figure 5.2 Histologic comparison of representative matched NS and HSc sections using H&E, and picrosirius red, with corresponding FFT and COI calculations.

5.3.3 Adenoviral Transduction Efficiency is Lower in Dermal Fibroblast Subpopulations than HEK293A cells, and this May be Due to Differential Expression of Adenoviral Cell Surface Receptors

Adenoviral transduction of HEK293A cells is virtually 100% for a MOI of 1-10 [28]. In contrast, superficial and deep dermal fibroblasts need a MOI of at least 500 to significantly increase DCN production (Figure 5.3A), suggesting they have lower transduction efficiency than HEK293A cells, a finding in keeping with the literature [29]. At an MOI of 500 deep dermal fibroblasts produce the same amount of DCN as untransduced superficial dermal fibroblasts, suggesting that this is the optimal MOI for deep dermal fibroblasts to mirror the DCN production of normal superficial dermal fibroblasts. With MOI of 1000 although there was an increase in DCN production, there was also greater variability between samples, which appeared to be due to increased cell death.

The serotype 5 adenoviral vectors with standard knobs require a number of cell surface moieties for efficient cell surface binding and transduction including Coxsackie virus and adenovirus receptor (CXADR), integrin alpha V (ITGAV), integrin beta 3 (ITGB3), and integrin beta 5 (ITGB5) [30]. It is possible that differences in expression of these adenoviral receptors between HEK293A and dermal fibroblasts could account for observed differences in

transduction efficiency [29]. Using RT-qPCR we compared these cell surface receptor levels between HEK293A cells, which are readily transfected with adenoviral vectors, and superficial and deep dermal fibroblasts. We found that while superficial and deep dermal fibroblasts express similar, and significantly higher levels of ITGAV, ITGB3, and ITGB5 than HEK293A cells (Figure 5.3B), they express significantly lower levels of CXADR than HEK293A cells (Figure 5.3C).

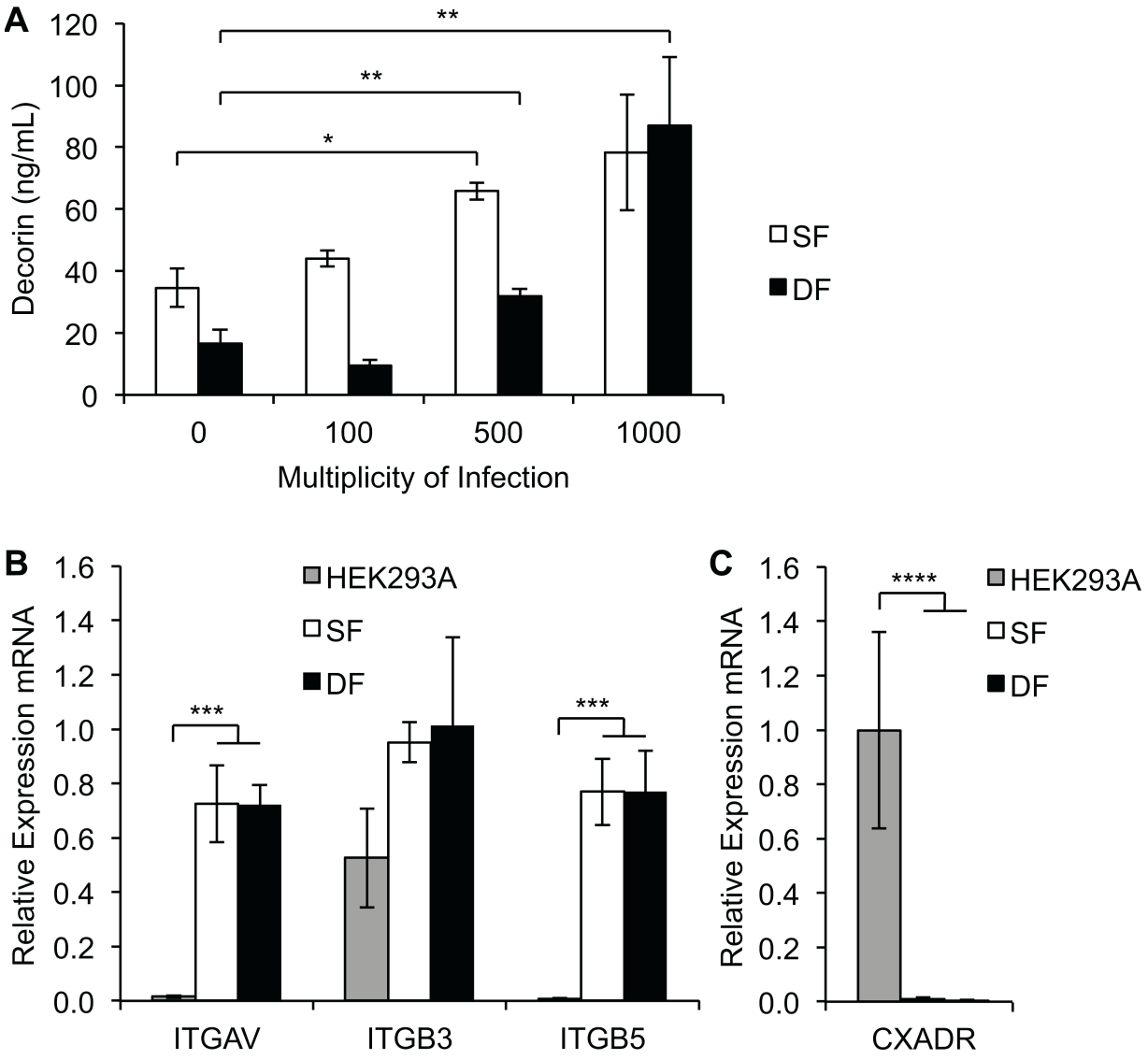

Figure 5.3 (A) Titration of rAd5-DCN vector transduction of deep dermal fibroblasts to determine optimal MOI for similar DCN production as superficial dermal fibroblasts (mean ± SEM; n = 3; * $P < 0.01$, ** $P < 0.05$). (B) Comparison of relative integrin ITGAV, ITGB3, and ITGB5 mRNA expression levels between readily transduced HEK293A cells, and superficial and deep dermal fibroblasts (mean ± SEM; n = 3; *** $P < 0.001$). (C) Comparison of relative CXADR mRNA expression levels between readily transduced HEK293A cells, and superficial and deep dermal fibroblasts (mean ± SEM; n = 3; **** $P < 0.005$).

5.3.4 rAd5-DCN Transfection Upregulates DCN Production, and Does Not Negatively Alter Dermal Fibroblast Proliferation or Adhesion

The rAd5-DCN and control rAd5-DL70 vectors were used to transduce deep dermal fibroblasts and the proliferation and adhesion was compared to mock transduced deep dermal fibroblast and superficial dermal fibroblast controls. There was no significant decrease in proliferation (Figure 5.4A) or adhesion (Figure 5.4B) of rAd5-DCN transduced deep dermal fibroblasts as compared to mock transduced superficial and deep dermal fibroblasts, suggesting that adenoviral vector transduction did not negatively alter fibroblast proliferation or adhesion.

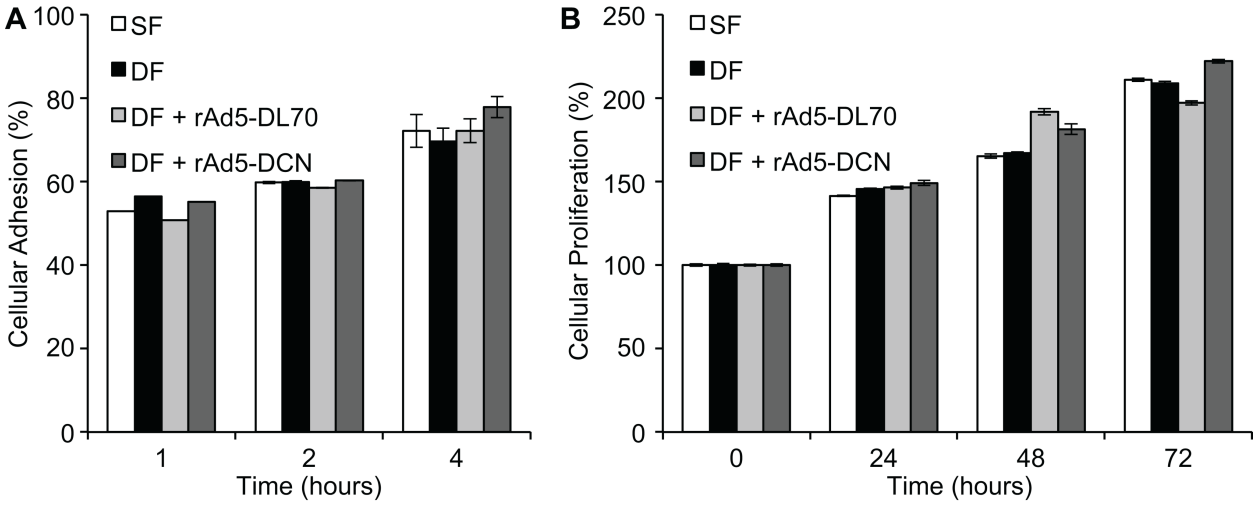

Figure 5.4 Effects of rAd5-DL70 and rAd5-DCN on (A) dermal fibroblast adhesion and (B) proliferation (mean ± SEM; n = 5 samples per group).

5.3.5 rAd5-DCN Transduction of Deep Dermal Fibroblasts Transiently Causes Transcription of Viral Genes but Causes a Sustained Increase in Decorin Production

One advantage of adenoviral gene vectors is non-integration into the target genome, although this leads to transient expression [31]. To follow viral genome presence in transduced cells, we performed PCR for the adenoviral fibre gene at 0, 3, and 21 days post-transfection (Figure 5.5A), demonstrating a significant reduction from day 3 to day 21 ($P < 0.035$). At corresponding time points, DCN relative expression significantly increased (Figure 5.5B) ($P < 0.02$), suggesting that although viral vector was decreasing, the increase in DCN expression was persistent.

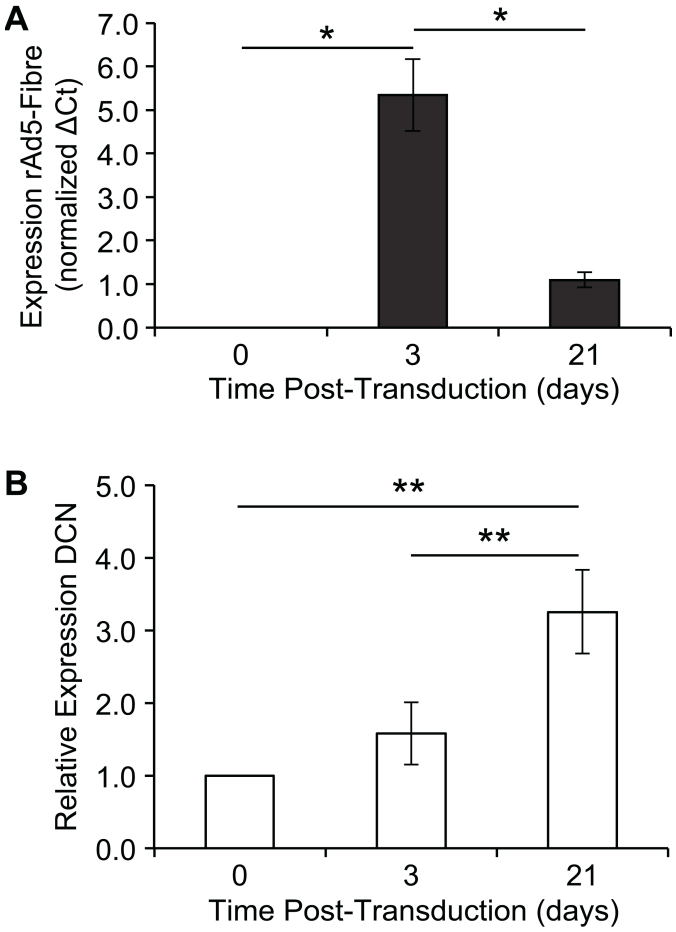

Figure 5.5 Persistence of (A) adenoviral vector fibre mRNA levels and (B) DCN relative mRNA levels at 0, 3, and 21 days post-transduction (mean ± SEM; n = 3; * $P < 0.04$, ** $P < 0.02$).

5.3.6 rAd5-DCN Transduction Alters Deep Dermal Fibroblast Behavior to Parallel Superficial Dermal Fibroblast Behavior as Measured by Alterations in Key Anti- and Pro-Fibrotic Factors

The expression of several genes relevant to the wound healing process: COL1A1, MMP1, TGFB1, and CTGF, were measured in superficial dermal, deep dermal, deep dermal treated with rAd5-DL70, and deep dermal treated with rAd5-DCN fibroblasts after culture in collagen scaffolds for 14 days (Figure 5.6). In comparison to superficial dermal fibroblasts, deep dermal fibroblasts had significantly higher levels of mRNA for COL1A1, MMP1, TGFB1, and CTGF. Deep dermal fibroblasts treated with rAd5-DL70 had significantly higher levels of COL1A1, TGFB1, and CTGF as compared with both superficial and deep dermal fibroblasts, potentially a reflection of fibroblast responses to control adenoviral vector transduction. In contrast, deep dermal fibroblasts treated with rAd5-DCN had a mixed picture. They had similar COL1A1 levels to deep fibroblasts treated with control rAd5-DL70, again likely a reflection of responses to adenoviral transduction, but significantly lower levels of TGFB1 and CTGF than both deep dermal fibroblasts and deep dermal fibroblasts treated with rAd5-DL70, similar to levels seen in superficial dermal fibroblasts, which is presumably a reflection of increased DCN activity.

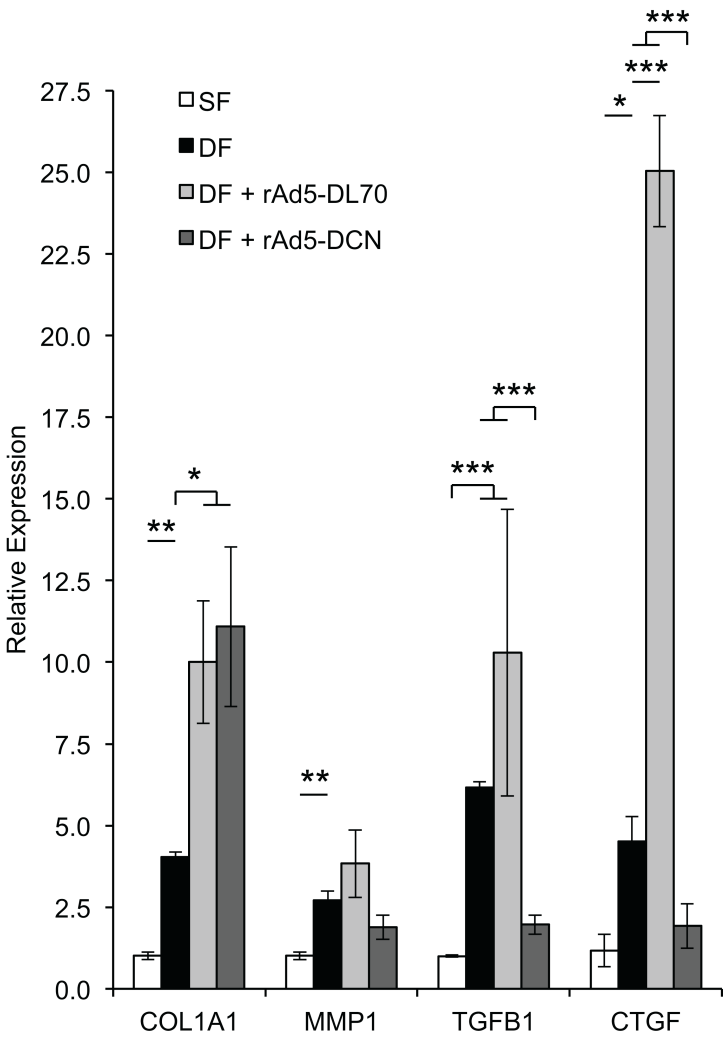

Figure 5.6 Effects of rAd5-DCN on dermal fibroblast COL1A1, MMP1, TGFB1, and CTGF mRNA relative expression (mean ± SEM; n = 3 per group; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

5.3.7 rAd5-DCN Reprograms Deep Dermal Fibroblast Remodeling to Parallel that of Superficial Dermal Fibroblasts

The rAd5-DCN and control rAd5-DL70 vectors were used to transduce deep dermal fibroblasts and these, or mock transduced deep dermal or superficial dermal fibroblasts, were seeded onto collagen scaffolds and cultured for 14 days. The effects of dermal fibroblast remodeling on the collagen scaffolds were investigated by examining the collagen fibril thickness (Figure 5.7A and 5.7B), and COI (Figure 5.7C and 5.7D) of the resulting scaffolds.

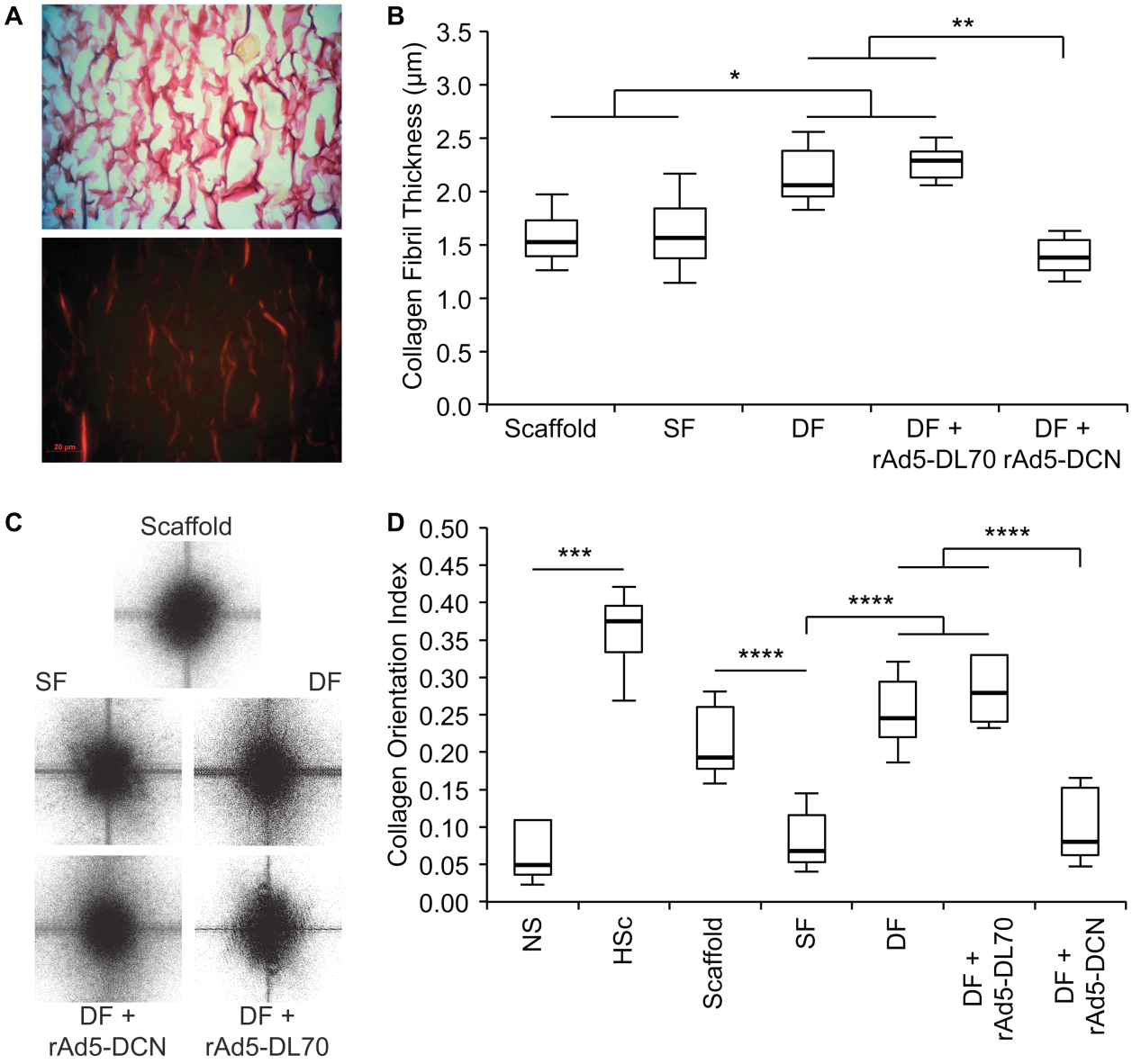

Figure 5.7 Effects of rAd5-DCN on collagen scaffold fibril thickness (A) as measured using picrosirius red staining, between (B) scaffolds remodeled by superficial dermal fibroblasts, deep dermal fibroblasts, and deep dermal fibroblasts treated with control vector rAd5-DL70 or rAd5-DCN (n = 3 collagen scaffolds per group, 6 samples per scaffold). Effects of rAd5-DCN treated deep dermal fibroblasts on collagen scaffold remodeling as measured by (C) COI, and (D) for the same treatment groups (for NS and HSc n = 5 patients; for scaffolds n = 3 scaffolds per group, 9 samples per scaffold; * $P = 0.001$, ** $P < 0.0001$, *** $P = 0.004$, **** $P < 0.0002$).

As can be seen in Figure 5.7B, mock and control rAd5-DL70 transduced deep dermal fibroblasts generated significantly thicker collagen fibrils in scaffold as compared to superficial dermal fibroblasts ($P = 0.001$). In contrast, rAd5-DCN transduced deep dermal fibroblasts generated significantly thinner collagen fibrils than deep dermal fibroblasts ($P < 0.0001$), which were instead similar in thickness to superficial dermal fibroblasts. This mirrors the *in vivo* situation where ECM associated with superficial dermal fibroblasts has significantly thinner collagen fibrils than ECM associated with deep dermal fibroblasts, as seen in Figure 2 and previous literature [26, 27].

As can be seen in Figure 5.7D, the COI of HSc is significantly higher than that of matched NS (0.050 versus 0.375, $P = 0.004$) which is similar to previous publications [25], whereas scaffold alone has an intermediate COI (0.193). Scaffold remodeled by mock transduced superficial dermal fibroblasts had a COI similar to that of NS, whereas scaffold remodeled by mock and control rAd5-DL70 transduced deep dermal fibroblasts had a significantly higher COI (0.068 versus 0.240 and 0.280, $P < 0.0002$), similar to that of HSc. In contrast, rAd5-DCN transduced deep dermal fibroblast remodeled scaffold had a significantly lower COI (0.080 versus 0.240 and 0.280, $P < 0.0002$), which was similar to the COI of superficial dermal fibroblast remodeled scaffold.

5.4 Discussion

Multiple studies have demonstrated the unique regenerative properties of superficial dermal fibroblasts as compared to the fibrotic properties of deep dermal fibroblasts [4-6]. Cultured skin substitutes show great promise in treating patients suffering from extensive burns, and have been shown to reduce donor sites and increase survival [3]. Unfortunately, as we have demonstrated, the vast majority of fibroblasts acquired from dermal biopsies are deep dermal and profibrotic [4].

Previous authors have used DCN adenoviral vectors, frequently *in vivo*, to upregulate DCN and reduce various aspects of fibrosis in animal models [15, 16]. Similar to other studies in dermal fibroblast populations [29], we found that superficial and deep dermal fibroblast subpopulations had reduced adenoviral vector uptake as compared to HEK293A cells. We

overcame this deficiency by increasing the MOI used for transduction, which is a widely used strategy in cells with low CXADR expression [32]. Other strategies include retargeting to alternate cell surface receptors such as integrins, which we have shown are abundantly expressed on superficial and deep dermal fibroblast subpopulations.

Use of high MOI for adenoviral vector transduction can result in cell toxicity, and reduced proliferation [33]. However, in our study the high MOI used for transduction had no adverse effects on fibroblast proliferation or adhesion. Messenger RNA levels for adenoviral genes were significantly reduced by 21 days post-transduction, suggesting this may be an optimal clinical time-point for treated CSS to be grafted onto patients with reduced stimulation of the immune system by adenoviral vector products—although this requires further testing in an animal model.

Gene expression levels of collagen (COL1A1) were significantly higher in deep dermal fibroblasts, as compared to superficial dermal fibroblasts, and even higher in adenoviral vector treated deep dermal fibroblasts. In contrast, while TGF-β_1 and CTGF mRNA levels were significantly higher in deep dermal and control vector rAd5-DL70 treated fibroblasts than superficial dermal fibroblasts, levels in rAd5-DCN treated deep dermal fibroblasts were significantly lower, and mirrored those of superficial dermal fibroblasts. This suggests that rAd5-DCN had antifibrotic effects on deep dermal fibroblast expression of these two key effectors of fibrosis, a finding which mirrors that seen by other researchers [34, 35]. Given the importance of TGF-β_1 and CTGF in fibrosis [36], it is possible the main effect of rAd5-DCN is through their blockade.

Treatment of deep dermal fibroblasts with our rAd5-DCN vector had significant effects on scaffold remodeling when compared to mock or control rAd5-DL70 treatment, and superficial dermal fibroblasts. Scaffold collagen fibril thickness was not significantly altered by superficial dermal fibroblasts. In contrast, scaffolds with deep dermal fibroblasts and those treated with control vector rAd5-DL70 had significantly thicker collagen fibrils, with this effect reversed in deep dermal fibroblasts treated with rAd5-DCN. A finding that mimics the morphologic characteristics seen in the matrix of matched superficial and deep dermal biopsies. These differences are also apparent in the COI of scaffold from these different groups. While the greatest difference in COI was between NS and HSc, similar to previous reports [25], there were

also significant differences between the other groups. Untreated scaffolds had intermediate COI, whereas superficial dermal fibroblast remodeled scaffolds had significantly lower COI, similar to NS. Scaffolds with deep dermal fibroblasts and those treated with control vector rAd5-DL70 had significantly higher COI, and those treated with rAd5-DCN had significantly lower COI, again similar to superficial dermal fibroblasts and NS. Taken together, this suggests that the scaffold remodeling behavior of deep dermal fibroblasts is significantly different from that of superficial dermal fibroblasts, a finding supported by previous research in our laboratory [17], and that treatment with rAd5-DCN alters the remodeling behavior of deep dermal fibroblasts to more closely mimic that of superficial dermal fibroblasts.

Clearly, an open question remains as to what degree of clinical improvement in scarring and regeneration will occur with CSS produced using mainly superficial dermal fibroblasts or rAd5-DCN transduced deep dermal fibroblasts. Even if there is less scarring and improved regeneration, CSS with such dermal fibroblasts may be less resilient or prone to greater degrees of damage from injury. Furthermore, it is possible that the optimal CSS construct consists of distinct layers of superficial and deep dermal fibroblasts, which would more closely mimic *in vivo* skin. One could argue that a view of superficial and deep dermal fibroblasts as regenerative and scarring may be overly simplistic, and that a distinct bilayer of superficial and deep dermal fibroblasts exists in nature so that superficial wounds, which have less threat to survival, can regenerate whereas deeper injuries benefit from the rapid formation of scar tissue. Certainly, clinical experience suggests that thicker split thickness skin grafts, which proportionally contain more deep dermal fibroblasts, tend to heal with superior results as compared to thinner split thickness skin grafts which contain mainly superficial dermal fibroblasts [37]. To what degree this is a factor of differences in the ECM physical structure, ECM contents, or the dermal fibroblast subpopulations is unknown. These are questions we look forward to exploring with further studies using our collagen scaffold and various dermal fibroblast subpopulations.

Future planned work includes exploration of alternate adenoviral vector targeting strategies, long-term culture of CSS incorporating both keratinocytes and rAd5-DCN treated fibroblasts, and animal experiments to explore effects on engraftment and wound healing.

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Chapter 6

Conclusion

6.0 Conclusion

This thesis has reviewed the factors involved in wound healing and hypertrophic scar (HSc) formation, and covered the exploration of three related aspects of HSc formation following deep burns: prediction of HSc formation using serum factors, treatment of HSc using an antagomiR, and prevention of HSc using an adenoviral decorin gene vector to influence collagen scaffold remodeling.

First, the goal of improving predictions of the risk of HSc formation following deep burn injuries was addressed. Investigations demonstrated that serum levels of decorin (DCN), interleukin-1β (IL-1β), and transforming growth factor-β1 (TGF-β1), can be used to improve existing methods for the prediction of HSc formation risk following deep burn injury, and the time course of these changes creates profibrotic local and systemic environments. This is important for three major reasons. First, predicting those patients at higher risk of HSc formation will aid in selecting these patients for longitudinal study starting early in their burn injury course, before the late manifestations of clinically evident HSc formation. Second, it helps select patients at high risk for HSc formation for earlier, potentially preventative, interventions and therapies to avoid HSc formation. This is important since most diseases can be more easily treated in their early stages, and potential treatments are likely to be complex, expensive, and have their own associated risks or side effects [1], making early patient selection crucial to appropriate treatment. Finally, this thesis introduces the idea that prediction of HSc formation can, and should, include measurable biochemical markers, not simply gross clinical variables such as age, sex, and burn size [2].

Second, the goal of expanding the understanding of how DCN is regulated by TGF-β1 and how this might present new therapeutic opportunities was addressed. Unfortunately, existing treatments for HSc have mixed and limited results, which is reflected in the wide variety of current therapeutic options [1]. Thus, new treatment avenues for HSc are needed which can target portions of the underlying pathophysiology. Investigations demonstrated that microRNA-181b is regulated by the profibrotic cytokine TGF-β1, and in turn regulates the antifibrotic proteoglycan DCN. Reversal using antagomiR-181b was able to block this interaction, and reduced TGF-β1 downregulation

of DCN, helping to restore DCN levels in HSc fibroblasts. This is significant since there is growing appreciation for the complexity of translational control and the importance of miRNA regulatory networks in skin wound healing [3-5], and this thesis expands the understanding of this promising area of research, and suggests a potential therapy for HSc.

Finally, the role of DCN gene therapy in prevention of HSc formation was studied. Patients with larger burns are more likely to develop HSc [2]. This may be due to the limited donor sites available, prolonged healing time and thus a prolonged inflammatory/profibrotic period, and necessity of multiple skin graft harvests from the same donor sites. Cultured skin substitutes (CSS) can significantly reduce the time to complete wound coverage, and reduce morbidity and mortality in the process [6]. However, current CSS use a mixed dermal fibroblast population derived from patient skin biopsies. Given the unique differences between superficial (regenerative) and deep (fibrotic) dermal fibroblasts, there is an opportunity to improve collagen scaffold remodeling in CSS by modulating the fibroblasts seeded onto the scaffold. Investigations demonstrated that an adenoviral DCN gene vector significantly upregulated DCN expression by deep dermal fibroblasts and this was accompanied by changes in remodeling behavior to mirror that of superficial dermal fibroblasts. This is significant since deep dermal fibroblasts are more abundant in skin, and give rise to HSc formation post-burn [7]. Thus, the ability to alter their behavior may help in preventing HSc formation and improve CSS for burn patients.

Although the field of wound healing research continues to expand, and understanding of the underlying pathophysiology of HSc formation is increasing, there is still no effective treatment available for patients. Each of these aspects of HSc formation are important both from their specific application to the significant problems faced by burn patients who develop HSc, and from their generalizability to the many related problems of fibrosis and scarring which underlie the pathophysiology of diseases as diverse as scleroderma [8], and pulmonary fibrosis [9]. As can be seen in this thesis, DCN plays a key role in the fibrotic pathway, and as reviewed earlier, has multiple independent interactions with a host of pro-fibrotic receptors and growth factors. All these diverse aspects of DCN highlight its unique role in wound healing, and present

opportunities to improve outcomes by modulating its expression, as was done in this thesis.

Based on the results of these studies, the opportunity for future work can be broadly divided into three main tracks. First, the systemic response to burn injury and the resulting circulating serum factors must be better defined both between patients, and during the longitudinal course of burn recovery. Larger cohorts of patients will allow validation and fine-tuning of HSc predictive algorithms, and may also inform other areas of interest in burn care such as improving resuscitation, predicting survival, and estimating duration of hospitalization. A large scale, multi-center study using biobanking to store patient serum samples from multiple time-points, and with post-burn follow-up for two years would aid in answering these important questions. Future work in this area should focus on building collaborations with other high-volume burn centres to facilitate such as study. Second, continued work on the underlying microRNA regulatory networks of wound healing and HSc formation, and epigenetic differences between superficial and deep dermal fibroblasts can provide insight into a wide variety of fibrotic diseases, and suggest potential therapies. Future experiments on the role of miRNA-181b, and other microRNA, in regulating other aspects of the transforming growth factor-β pathway will be important in exploring interconnections with other gene networks, and could suggest promising treatments. Future work here should take advantage of new advances in large scale screening of microRNA to determine appropriate targets for further study. Finally, although there is yet no clinically available culture skin substitute, this is still one of the most promising avenues of treatment for patients with large burns. Additionally, tissue engineered skin continues to serve as a proof-of-concept and highly useful test-bed for addressing the multiple problems that face tissue engineering in general. Therefore, future efforts that reduce scarring and fibrosis in tissue engineered skin, such as the use of decorin adenoviral vector gene therapy, will have benefits not only for the burn patients to which they are directly applicable, but may also help in efforts to tissue engineer other organs which are likely to face similar challenges. Future work should focus on adenoviral vector modifications to improve transduction efficiency, and limit toxicity, so that decorin adenoviral vector gene therapy can be tested in a

suitable animal model of HSc formation, and begin to approach the translational leap from bench to bedside, which is ultimately required for patient benefit.

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