Decorin Isoform Expression in HTS Fibroblasts: the effects of TGF-β1 and IFN-α2b

By

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Abstract

Background: Hypertrophic scars (HTS) are a dermal fibroproliferative disorder that commonly occurs following burn injury. Due to the intricate nature of the wound healing process and an incomplete understanding of the molecular mechanisms involved in HTS formation, current treatments have significant limitations in their efficacy. HTS develop from the excessive synthesis and reduced degradation of structural proteins like collagen, and dysregulation of proteoglycans such as decorin in the extracellular matrix (ECM). Our group has demonstrated that decorin expression is significantly downregulated in HTS, deep dermal tissue, and thermally injured tissue, mitigating its ability to regulate pro-fibrotic transforming growth factor-beta 1 (TGF-β1) and normal fibrillogenesis. However, treatment of HTS fibroblasts with interferon-alpha 2b (IFN-α2b) has been shown to reduce excessive collagen synthesis and improve HTS by reducing serum TGF-β1 levels. The expression of decorin isoforms in HTS is currently unknown and the effects of TGF-β1 and IFN-α2b on decorin and decorin isoform expression are of great interest to our group. We hypothesize that there will be a differential expression of decorin and decorin isoforms in HTS fibroblasts relative to normal skin (NS) fibroblasts. By treating fibroblasts with TGF-β1 and/or IFN-α2b, TGF-β1 is expected to decrease the expression of decorin and decorin isoforms but increase type I collagen expression, whereas treatment with IFN-α2b will inhibit the pro-fibrotic effects of TGF-β1.

Methods: The gene expression of decorin and decorin isoforms were quantified in HTS fibroblasts and site-matched NS fibroblasts and in superficial dermal fibroblasts (SF) and deep dermal fibroblasts (DF) using a reverse transcription-quantitative polymerase chain reaction (RT-qPCR). To determine the effects of TGF-β1 and IFN-α2b, fibroblasts were treated with either 10 ng/mL of TGF-β1 only, 2000 U/mL of IFN-α2b only, both TGF-β1 and IFN-α2b, or PBS as a

control for 48 hours. The protein expression of decorin and type I collagen were quantified in fibroblasts with immunofluorescence (IF) staining. Decorin and procollagen type I protein secreted from the fibroblasts were measured in the cell culture media supernatant using an enzyme-linked immunosorbent assay (ELISA).

Results: The mRNA expression of decorin and each decorin isoform (A1, A2, B, C, D, and E) were significantly reduced in HTS fibroblasts relative to NS. TGF-β1 significantly decreased the mRNA expression of decorin and specific decorin isoforms, whereas IFN-α2b had the opposite effect, in all fibroblast populations. IFN-α2b did not inhibit the effect of TGF-β1 decreasing decorin or decorin isoform mRNA expression in any of the fibroblast populations. TGF-β1 significantly increased the mRNA expression of COL1A1 in all fibroblast populations, the protein synthesis of type I collagen in NS fibroblasts, and the amount of procollagen type 1 present in the cell culture media in both HTS and site-matched NS fibroblasts. Although not significant, IFN-α2b increased the protein synthesis of decorin in HTS and site-matched NS fibroblasts. There were no statistically significant differences in decorin protein secreted into the cell culture media between the different treatment groups in HTS or site-matched NS fibroblasts. IFN-α2b did not significantly reduce the mRNA expression of COL1A1 in any fibroblast population, but IFN-α2b did significantly inhibit the effect of TGF-β1 on the mRNA expression of COL1A1 in SF. The protein expression of type I collagen and the procollagen type 1 secreted into the cell culture media were not significantly reduced after treatment with IFN-α2b and the effects of TGF-β1 were not significantly inhibited by IFN-α2b in HTS or site-matched NS fibroblasts.

Conclusion: These data support that a further investigation into the structural and functional roles of decorin isoforms in HTS pathogenesis is warranted and that IFN-α2b is an important agent in reducing fibrotic outcomes.

Preface

This thesis is an original work by Elizabeth Eremenko. Portions from chapter 1 of this thesis have been published as Eremenko E, Ding J, Kwan P, Tredget EE. The Biology of Extracellular Matrix Proteins in Hypertrophic Scarring. Adv Wound Care (New Rochelle). 2022 May;11(5):234-254. doi: 10.1089/wound.2020.1257. Epub 2021 Jul 5. PMID: 33913776. I was responsible for the composition of the review article. Peter Kwan, Jie Ding, and Edward E. Tredget aided with review edits, concept formation, figures, and review modifications.

Chapter 2 is a manuscript-based portion. I, Elizabeth Eremenko was responsible for performing all experiments, data collection, analyses, and manuscript composition. Drs. Peter Kwan, Jie Ding, and Edward Tredget assisted with concept formation, data analyses, and thesis modifications/edits.

My committee members Drs. Adetoala Adesida and Gina Rayat assisted with concept formation and thesis edits. Dr. Sunita Ghosh assisted with statistical analysis of the experimental data.

Dedication

I would like to dedicate this thesis to my family Angela, Eric, Marc, and Emily Eremenko. Without their continuous love and support throughout the completion of this thesis, none of this would have been possible.

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List of Abbreviations

α-SMA	Alpha smooth muscle actin
ALK1	Activin receptor-like kinase 1
ALK5	Activin A receptor type II-like kinase
B2M	Beta-2-microglobulin
COL1A1	Collagen type I alpha 1
CS	Chondroitin sulfate
C_T	Comparative threshold cycle
DF	Deep dermal fibroblasts
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DS	Dermatan sulfate
ECM	Extracellular matrix
EDA	Extra domain A
EDB	Extra domain B
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
En-1	Engrailed-1
FAK	Focal adhesion kinase
FBS	Fetal bovine serum
GAG	Glycosaminoglycan
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HA	Hyaluronan

HS	Heparan sulfate
HTS	Hypertrophic scars
IF	Immunofluorescence
IFN-α2b	Interferon-alpha 2b
IL	Interleukin
KS	Keratan sulfate
LRRs	Leucine rich repeats
miRNA	Micro-ribonucleic acid
MMPs	Metalloproteinases
NS	Normal Skin
PDGF	Platelet derived growth factor
PDGFR	Platelet derived growth factor receptor
PBS	Phosphate buffered saline
SLRP	Small leucine rich proteoglycans
Smad	Small mothers against decapentaplegic
SF	Superficial dermal fibroblasts
TBSA	Total body surface area
TGF-β	Transforming growth factor-beta
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor-alpha
TS	Thrombospondin motif
RNA	Ribonucleic acid
RT-qPCR	Reverse transcription-quantitative polymerase chain reaction

RPL13A	Ribosomal protein L13a
VEGF	Vascular endothelial growth factor

Chapter 1. Introduction

1.1 Burn Injury and Hypertrophic Scars

Hypertrophic scars **(**HTS) are red, firm, raised lesions of the dermis, which are confined to the region of the original wound site (1). They are a major complication following injury to the deep dermis that cause aberrations in function, mobility, and appearance, often leading to psychosocial impairment, particularly following thermal injury (**Figure 1-1**) (2). The incidence of hypertrophic scarring after sustaining burn injuries is estimated to range from 32-72% of all burns (3). Skin grafting burn wounds is the primary method of treatment; however, suboptimal outcomes after skin grafts and donor site complications are significant limitations (4). Skin grafting does not reliably reduce scarring, as its main function is to close the wound. Current therapies for HTS include laser therapy, scar revision, cryotherapy, radiotherapy, compression therapy, topically applied silicone, and local corticosteroid injection; however, all current treatments have significant limitations and can result in undesirable side effects (5–7). With compression therapy, there are no comparative analyses to determine the most effective amount of pressure (6,8). Laser therapy can cause hyperpigmentation, hypopigmentation, and blister formation (6,8). Radiotherapy has an intrinsic risk of carcinogenesis, and local corticosteroid injection can cause skin atrophy, reduced wound strength, dehiscence, and granulomas (6,8).

To date, there are no drugs that have passed clinical trials and gained approval for the prevention or reduction of cutaneous scarring. There are several anti-scarring products that currently are, or have been, tested in clinical trials (8). A phase II clinical trial (clinicaltrials.gov: NCT00984646) was conducted with interleukin (IL)-10, which demonstrated its importance in reducing scar inflammation, accelerating wound healing, and reducing scarring in rats, mice, and humans (9); however, IL-10 showed no effect in reducing scar formation in humans of

continental African ancestral origin (10). Additional clinical trials aiming to mitigate scar formation include the drugs: DSC127, which is an analog of the naturally occurring peptide angiotensin 1-7 (11) (clinicaltrials.gov: NCT01830348), RXI-109 which is a modified small interfering ribonucleic acid (RNA) (clinicaltrials.gov: NCT02246465) and EXC 001 which are antisense oligonucleotides (clinicaltrials.gov: NCT01038297). Both RXI-109 and EXC 001 are designed to target and reduce connective tissue growth factor expression (12,13). RXI-109 has shown promising results for visibly reducing the appearance of HTS during phase II clinical trials, but work done with DSC127 and EXC 001 has ceased and their effects on scar reduction are inconclusive. Although RXI-109 shows efficacy in clinical trials, it requires multiple post-surgery injections, making it an expensive and a painful treatment for patients to endure (8). Patients with excessive hypertrophic scarring have increased levels of plasma N^T-methylhistamine and serum transforming growth factor beta (TGF-β) (14). A phase II clinical trial conducted by Tredget *et al*. tested the efficacy of interferon-alpha 2b (IFN-α2b) on the treatment of HTS (14). Treatment with subcutaneous recombinant IFN α-2b led to a reduction in plasma N^T-methylhistamine and serum TGF-β1 and an overall improvement of HTS (14). However, one patient in the trial failed to respond to IFN-α2b and showed minimal improvement in scar volume and scar assessments (14). This trial suggests the potential therapeutic benefit of IFN-α2b in the treatment of HTS, but it is not conclusive and requires further experimentation. As no phase III clinical trials have demonstrated efficacy with these drugs, there are currently no drugs that have been approved for treating or preventing cutaneous scarring.

1.2 Wound Healing and Hypertrophic Scarring

The extracellular matrix (ECM), as the largest component of skin, possesses a plethora of proteins that bind the epidermis to the dermis and regulate the signaling of growth factors and

cytokines. The macromolecular composition and specific structure of the ECM vary from tissue to tissue. The major components that constitute the ECM of skin are fibrous forming proteins such as: collagen, elastin, fibronectin, laminin, and proteoglycans/glycosaminoglycans (GAGs) (15). Specific proteoglycans that have pivotal roles in collagen fibrillogenesis, cellular signaling, and ECM assembly are small leucine-rich proteoglycans (SLRPs) such as: decorin, biglycan, fibromodulin, lumican, asporin, and keratocan, as well as hyalectan proteoglycans such as versican and aggrecan (**Figure 1-2**) (8,16,17). HTS develop from excessive synthesis and reduced degradation of structural proteins in the ECM, as well as dysregulated glycoproteins and proteoglycans that affect cellular signaling pathways (18). There are certain proteins and cytokines that are abnormally increased or decreased in wound healing, typically resulting in hypertrophic scarring (**Figure 1-3 A**) and histologically HTS are characterized by a thicker dermis and epidermis that loses its rete ridges, hypervascularity, hypercellularity, and a loss of normal collagen fibril structure/alignment (**Figure 1-3 B**) (19).

Following burn injury, wounds heal via four interrelated and overlapping steps: hemostasis, inflammation, proliferation, and remodelling (20). Ideally, regeneration and reformation of normal skin (NS) tissue is the optimal response following thermal burn injury. Hemostasis is initiated immediately following injury and is characterized by the formation of a clotting cascade and a provisional wound matrix (1,21). In the inflammatory phase, neutrophils and monocytes enter the wound to clear bacteria and cellular debris (22). The proliferation phase is characterized by the closure of the wound and consists of three major processes: granulation tissue formation, angiogenesis, and re-epithelialization (1,22). Lastly, the tissue remodelling phase includes additional wound contraction and re-epithelialization (22), which can last for several years as the ECM is remodelled.

The physiological process of normal wound healing will not result in hypertrophic scarring; however, abnormalities in this process disrupt the sensitive balance of ECM deposition and degradation, typically leading to HTS formation (1). Therapeutic approaches become very difficult to develop due to the complexity of wound healing, and the specificity that treatments would require for the many cellular processes involved.

Cutaneous wounds in mammalian fetuses heal with complete regeneration and reformation of normal dermal architecture and without scarring; whereas the inflammatory response is a crucial aspect of postnatal healing that influences the wound to heal with the development of fibrous scar (23). Embryonic wounds show a significantly reduced expression of inflammatory cells such as: neutrophils, macrophages, lymphocytes, and monocytes, and these cells are present for a much shorter duration within the wound, compared to adults (23). PU.1 is an erythroblast transformation specific-domain transcription factor that is coded for by the *SPI1* gene, and it has been demonstrated that PU.1 null mice are incapable of creating a standard inflammatory response due to an absence of macrophages and functioning neutrophils (24). PU.1 null mice display a mitigated inflammatory response that results in scar-free healing, similar to fetal wound healing (24).

Research has also demonstrated that TGF-β isoforms (TGF-β1, TGF-β2, and TGF-β3) play a crucial role in fetal wound healing. In adult wounds, TGF-β1 and TGF-β2 have an increased expression compared to TGF-β3, but in fetal wounds, the opposite is seen (25). Fetal wounds have an increased expression of TGF-β3 compared to TGF-β1 and TGF-β2 (25), and when exogenous TGF-β1 protein is added to human fetal skin wounds they form scar (26). The differential expression of these isoforms appears important in determining an outcome of either scar formation or scarless healing.

TGF-β isoforms also have a significant influence on the production of collagen in the ECM. Fibroblasts found in HTS secrete significantly more profibrotic cytokines, especially TGF-β1 (27). Overexpression of TGF-β1 and TGF-β2 effectively enhances the production of collagen by myofibroblasts, causing an excess of collagen accumulation in the ECM (28,29). In HTS and deep dermal fibroblasts (DF), TGF-β3, which antagonizes collagen production, is downregulated and unable to effectively counteract TGF-β1 stimulation (30). In a Phase II clinical trial (Clinicaltrials.gov: NCT00432211), intradermal avotermin (human recombinant TGF-β3) significantly improved the appearance of scars following scar revision surgery. The histological architecture of the ECM was more like that of NS and showed a normal basket-weave orientation of collagen fibers (31). However, even with a successful phase II clinical trial, avotermin failed to reach the required clinical endpoint in phase III trials (13). It is important to recognize that a complete deficiency of TGF-β1 in mouse tissue repair models result in a delayed and disrupted inflammatory response, and impairment of collagen deposition and angiogenesis (32). Thus, even if the effects of excessive TGF-β1 result in fibrotic scarring, its presence and activity are still essential for wound healing.

1.3 ECM Molecules Involved in Wound Healing and Hypertrophic Scar Formation

1.3.1 Collagen

Collagen accounts for ~75% of the dry weight of skin (33). 28 different types of collagens are divided into two categories, fibrillar and non-fibrillar (33), and most relevant to the ECM and wound healing are the fibrillar collagens. Collagen molecules are trimeric proteins that are formed via the interaction of three polypeptide chains, each encoded by a different gene (33,34). The main fibril-forming collagens are types I, II, and III, and the minor fibril-forming

collagens are types V and XI (34). Fibrillar collagens are differentially distributed across the variable types of connective tissue; however, they all contribute to structural stability and mechanical support.

During wound repair, collagen types I and III are the most prominent during the restoration of the damaged ECM and tissue. Type III collagen appears in the wound around days 2 to 3 and is followed by the more mature type I collagen emerging around days 6 to 7 (35). However, in abnormal wound healing, the balance between collagen types I and III is disrupted, as there is an excessive increase in the accumulation of type III collagen in the deep dermal layer (35). Fetal dermal fibroblasts synthesize more type III collagen that is rapidly deposited into a fine reticular network, leading to scarless wound healing that is indistinguishable from uninjured skin (36). Type I collagen is the most abundant collagen found in the ECM of both adults and fetuses, but fetal skin has a higher ratio of collagen type III to type I (34,37). Wang *et al.* showed that injecting exosomes derived from human adipose mesenchymal stem cells promotes healing of cutaneous wounds in mice and mitigates scar formation specifically by increasing the ratio of collagen type III to type I (38). Furthermore, treatment of cutaneous wounds in mice with human amniotic stem cells results in type I collagen being reduced and type III is increased (39). This leads to accelerated cutaneous wound healing and less fibrotic scarring, resembling fetal wound healing (39).

Fibroblasts produce collagen under the control of chemokines and growth factors, such as TGF-β, which dictates the rate of collagen synthesis and degradation. When fibroblasts become activated in the proliferation phase of wound healing they produce collagen, which transforms the provisional fibrin-fibronectin wound matrix into a more structurally stable ECM (40). As wound healing progresses, the provisional wound matrix becomes degraded and remodelled by

metalloproteases (MMPs) (20). MMPs mediate the degradation of type I and type III collagen (6). The activity of MMPs is regulated by tissue inhibitors of metalloproteinases (TIMPs); and increased levels of TIMPs (specifically TIMP-1 (41) and TIMP-2(6)) are involved in the formation of HTS. Fibroblasts in the granulation tissue respond to growth factors (e.g. TGF-β and platelet-derived growth factors (PDGF)) and fill the wound gap by initiating the formation of proteoglycans or glycosaminoglycans, fibronectin, and collagen (42).

HTS are composed of a higher concentration of collagen fibers and fiber bundles that are thinner, flatter, highly crosslinked, and arranged in a wavy pattern with whorls and nodules, as compared to the typical basket weave pattern of NS (43). These alterations in collagen morphology are related to changes in the amount and type of binding interfibrillar proteoglycans present in the ECM. Interfibrillar spaces become larger, irregular, and more prominent (44), which leads to an abnormal architecture of the ECM and fibrotic scarring.

1.3.2 Elastin

Elastin, like collagen, is a fibrillar protein that assists in maintaining the structural integrity of the ECM. Elastin is a polymeric protein that is assembled from monomeric tropoelastins to create a highly elastic tissue that can stretch and expand (45). The formation of covalent cross-links during elastin assembly aid in distributing strain and stress when deformation occurs (46). The physical properties of elastin make the dermis resilient and mechanically able to stretch. During the formation of granulation tissue in the proliferation phase, fibroblasts are responsible for producing fibrillar proteins that restore the structure and integrity of the ECM. However, in the context of HTS, fibroblasts have a diminished ability to produce elastin, reducing its levels in both the superficial and deep dermis (47,48). The elastin

content in the superficial dermis of HTS is reduced by 49% relative to NS (49). This reduction in elastin is in part what causes the tissue of HTS to be inflexible and restricted in its mobility (50).

Tropoelastin levels are controlled at the transcription level during mid to late embryonic stages and cease to be synthesized when tissue development is completed. Elastin production does not occur in maturity unless there is injury or disease present in the tissue (51). Tissue injury causes a surge of elastin deposition; however, it is aberrant and results in a disrupted elastic fiber network (51). In addition to the structural role of elastin in wound healing, it also functions in cell signaling. For example, tropoelastin supports the adhesion of a variety of cells such as, fibroblasts, chondrocytes, endothelial cells, and vascular smooth muscle cells (51). Elastin and tropoelastin peptides bind to elastin binding protein receptors and integrin receptors, activating intracellular signaling pathways such as focal adhesion kinases (FAK) and Ras/Raf/mitogen-activated protein kinases (52), leading to biological responses such as monocyte chemotaxis, fibroblast proliferation/migration, and protease production (51). Elastin also influences the biological activity of keratinocytes by promoting their migration. Even though elastin only constitutes 2-5% of the total protein content of the skin, it plays a crucial role in its overall structure and function (51). Collagen and elastin associate in the ECM, and this tight connection significantly limits elastin's ability to stretch. They are both pertinent in the ECM of skin, but collagen contributes to the tensile strength and elastin contributes to the extensibility and reversible recoil (53).

While the large family of different collagen types obtain their functional and architectural diversity by being produced from over 40 collagen genes, there is only a single gene for tropoelastin and its isoforms (54). There are at least 13 tropoelastin isoforms that have been identified as a result of alternative splicing of various domains (55). The various isoforms of

tropoelastin differ in structure, potentially leading to alterations and variability in the biological functions of elastin. The amino acid sequence of tropoelastin is divided into alternating hydrophobic and hydrophilic domains (56). The hydrophobic domains are responsible for phase separation, self-assembly, and elastomeric properties, whereas the hydrophilic domains are utilized for covalent cross-linking (55). The structure of tropoelastin gives it a predictable nature that allows insight into which isoforms could potentially affect the architecture and functional properties of a polymeric elastin matrix. For example, Reichheld *et al*. proposes in a review that excising hydrophilic (cross-linking) domains could alter the density of cross-linking, possibly affecting the elastic modulus (a measure of the stiffness), and elastomeric properties of the polymer (54). Miao *et al*. demonstrated that elastic modulus is significantly decreased in tropoelastin when exons 23 and 32 are removed from the protein (55). Furthermore, specific domain variants within tropoelastin give rise to significant changes in the hydrodynamic radius (the shape or compactness of the monomer), resilience (efficiency), and stress relaxation (the ability of the material to undergo molecular rearrangements to relieve stress) of *in vitro* generated polymeric elastin (55). Information regarding the differential effects of isoforms is crucial for understanding how aberrant variations could contribute to a reduction in the structural integrity of the elastic matrix and differences in the overall architecture of the ECM.

1.3.3 Laminin

Laminins comprise a family of high molecular weight glycoproteins that function structurally and affect cellular behaviours in the ECM. Laminin is necessary for the migration and proliferation of epidermal cells that provide immunoregulatory functions when tissues are damaged (57). In the skin, laminin interacts with the proteoglycans syndecan and perlecan, which facilitates keratinocyte migration and cell attachment/recruitment during tissue

development/remodelling (17). In wound healing, it is essential that the provisional ECM is remodelled, which includes the hydrolysis of collagen, elastin, fibronectin, and laminin via MMPs (43). Once laminin is cleaved by MMPs it exposes a cryptic site that binds to the epidermal growth factor (EGF) receptor and enhances the migration and proliferation of keratinocytes and fibroblasts by creating a promigratory track (47).

Mammalian laminins are heterotrimeric proteins that are composed of five α subunits, four β chains, and three γ chains that differentially interact to create 16 unique isoforms that differ structurally and functionally (58,59). Individuals with mutations in the genes coding for the laminin-332 isoform develop junctional epidermolysis bullosa, which is phenotypically characterized as generalized blistering of the skin starting at birth, due to an interruption of the dermal-epidermal junction (58). Furthermore, individuals with junctional epidermolysis bullosa have keratinocytes that display altered migration patterns and an aberrant morphology (58). Injury of the epidermis leads to the transcription and deposition of laminin-332 into the basement membrane of the provisional matrix by keratinocytes that migrate into the wound bed just hours after injury (60). The deposition of laminin-332 by keratinocytes is thought to be essential in regulating cell spreading and signaling that is unique from that of collagen and fibronectin (60). Again, there is a significant relationship between the form and biological function of laminin and its isoforms that lead to a variety of different phenotypes being expressed.

1.3.4 Fibronectin

Fibronectin is a structural and cell regulating protein that consists of two monomers linked together by a pair of disulfide bonds and specific functional domains. When fibroblasts enter the wound and release fibronectin to form granulation tissue, its expression is regulated predominantly by TGF-β (40). Wound healing consists of both plasma and tissue fibronectin.

Plasma fibronectin is formed by hepatocytes and endothelial cells and tissue fibronectin is formed by fibroblasts and keratinocytes (61). In the early phases of wound healing, plasma fibronectin is more prominent as it binds to fibrin and platelets to strengthen the fibrin clot; however, in the later proliferation and remodelling phases, tissue fibronectin becomes more pronounced (61). Fibronectin is essential in wound healing as it is a key constituent of the ECM that binds to integrins, collagen, and fibrin, and acts as a reservoir for growth factors to form growth factor gradients (48,62). Compared to normal dermis and mature scars, HTS have an increased expression of fibronectin (48). Furthermore, fibronectin is believed to play a crucial role in directing scarless fetal wound healing. In an *in vivo* full-thickness excision wound healing study, fibronectin dressings effectively enhanced wound healing and mimicked the microenvironment conditions of regenerative fetal wound healing, ultimately improving the experimental outcome of tissue repair in the model (63).

Fibronectin is composed of types I, II, and III repeating units, and in humans the different transcript variants of fibronectin can encode 20 isoforms that are either plasma or tissue derived (64). Protein diversity is attributed to alternative splicing of two type III exons named extra domain A and B (EDA and EDB) (65). Plasma fibronectin lacks the alternatively spliced EDA and EDB sequences and tissue fibronectin has variable portions of these sequences (65). EDA fibronectin enhances the differentiation of lipocytes into myofibroblasts, and in combination with TGF-β1, EDA fibronectin also induces the differentiation of fibroblasts into myofibroblasts (65). Experimentally, EDA deficient mice are unable to generate smooth muscle actin (α-SMA) expressing myofibroblasts, hindering the formation of tissue fibrosis (65). This relationship highlights the importance that EDA fibronectin has in tissue fibrosis, as it is required for the fibroblast to myofibroblast transition. The EDA domains are upregulated specifically in tissue

repair, tissue fibrosis, angiogenesis, and cell migration (65). The EDB domain does not stimulate the conversion of lipocytes into myofibroblasts, rather it aids in regulating fibronectin matrix assembly and fibronectin matrix-dependent cell growth and is more readily incorporated into the ECM (66). Deleting both the EDA and EDB exons from the fibronectin gene leads to embryonic lethality, cardiovascular defects, and a reduction in the amount of α-SMA expression (67), again highlighting the varying physical and pathological roles of fibronectin isoforms.

1.4 Proteoglycans

There are 6 different proteoglycans found within the ECM of skin that are predominant in the research of HTS and wound healing. The first being decorin, which has the highest expression in skin, followed by biglycan, fibromodulin, lumican, versican, and aggrecan (68). Decorin and fibromodulin expression are significantly downregulated in HTS tissue compared to NS tissue, biglycan expression is significantly upregulated, and lumican expression is not altered (**Figure 1-4**) (69). Many of the proteoglycans found in the ECM of skin have various alternative isoforms (**Figure 1-5**) (70), but currently, information and research on the biological functions of these isoforms is minimal. There are studies that show the functional diversity and influence that versican isoforms have in wound healing, indicating that research into the functional diversity of other proteoglycan isoforms may provide a more robust and specific understanding of wound healing and HTS development.

Proteoglycans consist of a core protein with one or more covalently attached GAG (**Figure 1-6 A and B**). The attached GAGs are long, linear, negatively charged, and are typically sulfated (e.g., chondroitin (CS), dermatan (DS), keratan (KS), or heparan sulfate (HS)) making them extremely polar and able to attract water. There are five classes of small leucine-rich proteoglycans (SLRP) based on homologies from both the gene and protein levels (71). SLRPs

are classified based on the number of tandem leucine rich repeats they have, the unique function of the modified N-terminus, and the organization of the C-terminus (71). In skin, proteoglycans serve varying biological functions, such as, influencing its physiological structure, hydrating the ECM and its cells by attracting water, and controlling the bioavailability of cytokines and growth factors within the ECM (17,43). Many proteoglycans bind to collagen fibrils and aid in forming normal collagen matrix alignment, indicating their critical role in fibrillogenesis and matrix assembly in the skin (71). Collagen molecules aggregate in parallel to form an elongated cable-like fibril structure with a cross striated, D-periodic banding pattern (D=67 nm) (33,34,72). The mature collagen molecules are oriented into characteristic D-staggered interactions to form fibrils by interacting with integrins, fibronectins, and SLRPs (34,71). Decorin specifically has a high affinity for collagen, which depends primarily on decorin's core structure. Scott *et al*. determined that the crystal structure of decorin forms a dimer (**Figure 1-7**) (73). Each monomer has a right-handed "banana" shaped curve that is composed of 12 leucine rich repeats (LRRs), and the two monomers interact via their concave faces (73). The amino acid sequences located between LRRs III and IV bind to type I collagen, and the GAG chain of decorin extends laterally from adjacent collagen fibrils, maintaining an appropriate interfibrillar distance in the ECM of the dermis (74). A single collagen triple helix (1.5 nm) can fit inside the arc shape of decorin (2.5 nm) (75). Decorin encloses one triple helix of collagen in the gap between molecules at the D band, with a secondary binding site located near the amino terminus of an adjacent collagen molecule (75). The presence of decorin at these specific binding sites aids in the correct positioning of collagen molecules within the staggered orientation of the fibril (75). Appropriate expression of decorin, and its coordinated interaction with collagen may regulate matrix assembly. Biglycan, a structurally similar SLRP to decorin, poses controversy regarding its

interaction with collagen because of its differential behaviours in various experimental conditions (76). Schönherr *et al*. has shown that biglycan competes with decorin to bind to type I collagen at the gap zone of the fibrils (76,77). However, research conducted by Svenson *et al.* has shown that biglycan does not bind to type I collagen in a collagen gel assay (76,78). These contradictory findings demonstrate the lack of understanding regarding the mechanism of biglycan binding to collagen (76). Fibromodulin can bind type I collagen at LRR 11 and LRR 7, whereas LRR 11 has a higher affinity (79). Lumican, which is structurally similar to fibromodulin, can also bind collagen at its LRR 7 (80). Therefore, because both class II SLRPs bind type I collagen at LRR 7, they compete with one another. However, fibromodulin has a higher affinity for collagen due to its additional collagen binding site at LRR 11 and its ability to sterically hinder lumican binding (80). Thus, fibromodulin can bind two collagen monomers and align two triple helices for an ordered fibril assembly, whereas lumican can only bind one monomer (76). Alterations in expression, localization, and type of proteoglycans present in the skin and wound site are crucial to the morphology and integrity of the collagen fibrils in the ECM and HTS (30).

1.4.1 The Functional Role of Decorin in the ECM

Decorin is a class I SLRP that plays a role in both regulating collagen fibril structure (**Figure 1-8**) (81) and cellular proliferation/activity in the skin. Decorin can bind and regulate the bioavailability of TGF-β1, which stimulates dermal fibroblasts to produce collagen and enhance wound contraction, by influencing its expression *in vivo* and *in vitro* (30,82). The specific binding site for TGF-β to decorin is found within the leucine-155 valine-260 sequence in the core of decorin (83). The interaction of decorin with TGF-β1 is crucial for ECM assembly. *In vitro,* by using a fibroblast-populated collagen lattice assay, basal contraction of the collagen gel

is increased by HTS fibroblasts compared to normal fibroblasts and is even further enhanced with the addition of TGF-β1 (84). Experimentally, decorin inhibits the basal and TGF-β1 mediated contraction of the collagen gel by HTS fibroblasts (84). Decorin reduces contraction by inhibiting both the basal and enhanced levels of F-actin, which generates forces in wound contraction, after stimulation with TGF-β1 (84). Furthermore, decorin reduces α-SMA and plasminogen activator inhibitor-1 expression, which are both significantly upregulated in HTS fibroblasts stimulated by TGF-β1 (84). Contraction of the wound decreases the wound area; however, excessive contraction leads to abnormal scarring. Therefore, decorin shows potential for preventing and treating excessive skin contraction, because of its ability to antagonize TGF-β1 activation and subsequent profibrotic proteins induced by TGF-β1. Compared to NS fibroblasts, HTS fibroblasts express a higher concentration of TGF-β1 per cell (85). Zhang *et al*. have shown that when HTS fibroblasts are treated with decorin (10, 50, 100, 200 nM) the concentration of TGF-β1 is significantly reduced and decorin inhibits the secretion of type I collagen from HTS fibroblasts (85). The presence of decorin in the wound environment is essential for regulating the profibrotic effects of TGF-β1.

Decorin expression is significantly higher in the superficial dermis of NS tissue compared to the deep dermis and both the superficial and deep dermis of HTS tissue (**Figure 1-9 A and B**) (86). By comparing burned and unburned tissue, immunofluorescence staining shows that decorin intensity is significantly decreased in thermally injured tissue (**Figure 1-10 A and B**) (87). Therefore, the decreased production of decorin by DF suggests that fibroblasts in the deep dermal layer are more similar to HTS fibroblasts than are superficial dermal fibroblasts (SF) (82). Research conducted in our laboratory determined *in vitro* that miRNA-181b is a potential down regulator of decorin (86). A comparison of site matched HTS and NS biopsies and

matched deep and superficial dermis showed that miRNA-181b expression is significantly increased in fibroblasts from the deep dermis and HTS fibroblasts as compared to the superficial dermis and NS fibroblasts, respectively (86). Additionally, experiments done by Driskell *et al.* show that DF mediate the initial point of dermal repair and more SF are only recruited during re-epithelialization in full thickness wounds (88). These experiments provide evidence that DF have a significant influence on the formation of HTS.

It has become increasingly recognized that fibroblasts are a heterogeneous cell population, composed of numerous subtypes that vary in their morphology and biological function. The behaviour of these heterogeneous fibroblast populations, with different biophysical and biochemical characteristics, are crucial for determining the outcome of wound healing and the extent of fibrotic scarring (89,90). Guerrero-Juarez *et al.* studied fibroblast heterogeneity in a mouse model for wound-induced regeneration and found that 12-day post-wounding fibroblasts can be classified into two different categories based on their transcription factor signatures and platelet derived growth factor receptor (PDGFR) expression patterns (91). PDGFRA signaling is a known contributor to fibrosis, as cells that have a high expression for PDGFRA also display increased activation of the TGF-β pathway (91). One population represents ~24% of wound fibroblasts and consists of three subclusters that express low levels of TGF-β2, TGF-β3, and PDGFA receptors, but high levels of PDGFB receptors (91). This population localizes throughout the wound. The other population of wound fibroblasts represents the remaining ~76% and consists of nine subclusters that range in their expression of TGF-β2 and TGF-β3 from intermediate to high levels, and highly express PDGFA, but not PDGFB (91). In addition, the fibroblast lineage that is defined by the embryonic expression of engrailed-1 (En-1) is the primary contributor to connective tissue secretion and organization during cutaneous wound

healing (92). However, En-1 positive fibroblasts produce scar tissue and are a primary secretor of collagen in the healing wound (92). Specific ablation of the En-1 positive fibroblast lineage reduced cutaneous scarring during wound healing and upheld the structural integrity of the healed skin (92). Current evidence then warrants further investigation into the expression of proteoglycans and ECM proteins in various fibroblast populations to better define these molecules and gain a more rigorous understanding of their influence on wound healing and HTS.

The role of decorin in regulating inflammation is very sophisticated. Decorin plays a role in the innate immune response as a ligand of toll-like receptors (TLR)-2 and -4, and stimulates the production of pro-inflammatory molecules, such as programmed cell death 4, tumor necrosis factor-alpha (TNF-α), and IL-12 in macrophages, which enhance the pro-inflammatory effects of lipopolysaccharides (93). Contrary to this, decorin has also been shown to have anti-inflammatory effects in inflammatory diseases associated with fibrosis (94). Research conducted on the genetic knockout of decorin and studies utilizing decorin gene therapy both consistently demonstrate the anti-inflammatory effects of the proteoglycan (94). Research done in a rat model of liver fibrosis showed that when decorin was coupled to a lecithin-based nanoliposome and administered through bile ducts, the expression of pro-inflammatory cytokines IL-6, TNF-α, and IL-1β were significantly downregulated, ultimately reducing liver inflammation and fibrosis (95). Moreover, decorin has been shown to act as both an inhibitor and promoter of angiogenesis, depending on the physiological and pathological conditions it is present in (96). In cutaneous wound healing models, a lack of decorin increases fibrovascular inclusion and enhances the formation of blood vessels (96), which may contribute to the hypervascularity seen in fibroproliferative disorders.

A significant decrease in the concentration of decorin results in collagen fibrils being irregular in diameter, structure, and tensile strength, causing increased interfibrillar spaces, a decreased average diameter, and an increased size range of fibrils (97,98). In brief, decorin has been studied for its therapeutic potential in wound healing and scarring due to its ability to delay collagen fibrillogenesis and sequester the activity of TGF-β by directly binding to it. Wang *et al.* tested the *in vivo* effect of decorin in an osteomyelitis rat model and found that rats treated with decorin had a gradual reduction of blood vessels, fibroblasts, collagen fibers, and ordered arrangement of collagen fibers, due to decorin's ability to inhibit TGF-β1 (99). Human recombinant decorin is currently available and manufactured in CGMP form, making it accessible for research working towards clinical implementation (100).

Bacterial keratitis is an infection of the cornea that can cause injury and ultimately lead to a corneal wound healing response. Similar to the dermis, this process can lead to scarring as a result of dysregulated tissue remodelling and aberrant collagen fibril orientation (100). Hill *et al.* evaluated the anti-scarring capability of human recombinant decorin in a gellan-based fluid gel using mice that were treated with *Pseudomonas keratitis* to induce corneal infection and injury (100). The research group demonstrated that treatment with human recombinant decorin improved the restoration of normal eye anatomy and reduced corneal opacity. Treatment also reduced stromal edema, thickness, ECM deposition, and enhanced the rate of re-epithelialization. However, the effect of decorin on inhibiting inflammation and angiogenesis, regulating autophagy, and sequestering TGF-β in this model needs to be investigated further as effects on these biological mechanisms were inconclusive.

Even with positive results of decorin reducing scarring and improving wound healing in animal models, it has yet to reach the clinic (101). Järvinen and Ruoslahti hypothesized that

enhancing the activity of decorin, to achieve desirable results at a lower dosage, could be possible by adding a wound-targeting function to decorin (101). The wound-homing peptide used was CARSKNKDC (CAR). The CAR-decorin fusion protein was utilized for the targeted delivery of decorin into wounded tissues and it was shown that a wound targeted, systemic treatment with decorin significantly reduced scar formation in wounded dermal tissue of mice by neutralizing TGF-β1 and TGF-β2 and having no effect on TGF-β3 (101). Decorin also accumulated in the deep layers of the wounded dermis where profibrotic, HTS-inducing fibroblasts are more prominent (101). Decorin is an extensively studied proteoglycan, but translational research on decorin in scar formation and its full therapeutic potential, as well as the potential of its isoforms, currently remains untapped.

1.4.2 Biglycan

Biglycan is a class I SLRP that plays a role as a pro-inflammatory signaling molecule. During tissue injury, biglycan is released from the ECM and turns into a danger-associated molecular pattern that is recognized by the innate immune system (102). Although experiments have not been conducted specifically in skin wound healing models, biglycan has been shown to be a pro-inflammatory factor that acts in macrophages as an endogenous ligand of TLR-2 and TLR-4 both *in vivo* in mice and *in vitro* in mouse peritoneal macrophages (103). Biglycan activates the TLR signaling cascades, ultimately inducing a pro-inflammatory response by enhancing the signaling of TNF-α and macrophage inflammatory protein-2 (103). Induction and amplification of the inflammatory response is a key step in skin wound healing as the phagocytosis that occurs is necessary to establish conditions for the reparative synthesis of the ECM.

Experimentally, biglycan levels are increased in HTS fibroblasts compared to fibroblasts cultured from NS, which is the opposite pattern to that seen with decorin and fibromodulin (69). This relationship of the class I SLRPs suggests that when one is downregulated the other is upregulated to compensate (71). Thus, although biglycan is increased post-injury in the dermis, it does not completely functionally compensate for the loss of decorin. Moreover, fibroblasts in the deep dermis internalize more biglycan than fibroblasts in the superficial dermis of normal human skin (104). It is unclear whether higher internalization of biglycan by DF is related to the profibrotic or inflammatory response observed in deep dermal injury (103,104). Further experimentation is required to elucidate the relationship between the high levels of biglycan present in DF and wound healing.

Studies pertaining to skeletal muscle differentiation have shown that like decorin, biglycan is also a regulator of the bioavailability of TGF-β1 (105). During skeletal muscle regeneration, which occurs as a damage-induced regenerative response, mature myofibers are produced from the influence of growth factors and cytokines. Similar to wound healing, the ECM plays a pertinent role in this process and disorganization of the ECM affects the proper progression of myoblast formation. TGF-β1 shows a strong inhibitory effect on myogenesis; however, exogenous biglycan shows an attenuating effect on TGF-β1 by its ability to sequester and limit its binding to transducing receptors (105). The ability of biglycan to modulate TGF-β1 indicates that it may be pertinent in determining the overall effect that fibrotic TGF-β has in skin wound healing.

Biglycan contains codon phases that are identical to decorin, and the genomic structure of these two SLRPs is highly conserved, each containing 8 exons (106). Tufvesson *et al*. demonstrated that a different isoform of biglycan is secreted from human lung fibroblasts (107).

The predominant form of biglycan has a 40-kDa core protein with two attached CS/DS GAGs. In this study, the isoform had a smaller core protein, causing a reduction in molecular weight, and a mixture of GAG chains that differed in length. The amount of iduronic acid in the predominant form of biglycan DS chains is ~71%, but experimentally this decreased to ~61% in the isoform (107). It is speculated that decorin and biglycan GAG chains that have differing proportions of CS and DS could potentially have altered affinities for collagen types I, II, and III (72). Higher molecular weight CS and DS GAGs bind to collagen more; therefore, the higher molecular weight chains of biglycan may potentially enhance its binding ability compared to decorin (72). Experimentally, the molecular weight of the GAG chains in the biglycan isoform are lower than the predominant form (107). This change in structure may alter biglycan's functional properties and its ability to bind to certain ECM molecules, changing the overall effect that biglycan has in the ECM.

1.4.3 Fibromodulin

Fibromodulin is a class II SLRP that is secreted primarily by dermal fibroblasts and is the most abundant proteoglycan in the ECM. Delayed wound closure, resulting from deficient dermal cell migration and granulation tissue formation, occurs in fibromodulin null mice. Interestingly, these changes are associated with excessive TGF-β3 levels (108). Typically, fibromodulin attenuates the antimotility effects of TGF-β3 on fibroblasts (108). However, in fibromodulin null mice, dermal cell migration by fibroblasts is significantly reduced, because of an increased susceptibility to TGF-β3's antimotility effects (108). In this experiment, TGF-β3's effect on mitigating dermal cell migration outweighs its previously observed positive implications of reducing dermal scarring. In a subsequent study, Zheng *et al.* show that fibromodulin modulates the expression of TGF-β isoforms in a temporospatial manner during

wound healing (109). Epidermal levels of TGF-β1 are significantly increased in fibromodulin null mice 1-day post dermal injury and in the ECM of granulation tissue 7-days post injury, compared to wild type controls (109). Total dermal TGF-β2 is significantly increased in fibromodulin null mice half a day post wounding but is significantly decreased 7- and 14-days post injury, relative to wild type controls (109). Lastly, TGF-β3 increases 1-and 2-days post injury in fibromodulin null mice but is negligible 14-days post injury (109). Wild type mice express TGF-β3 14-days post injury (109). Additionally, in preclinical animal models, exogenous fibromodulin significantly promotes wound healing by selectively augmenting early pro-migratory and pro-contractile canonical TGF-β1 signaling, and reduces scar size, increases tensile strength, and improves dermal collagen organization by reducing TGF-β1 stimulated fibrotic gene expression (110).

Zheng *et al.* also demonstrated that fibromodulin elicits fetal-like cellular and molecular phenotypes in adult dermal fibroblasts *in vitro* and in adult cutaneous wounds *in vivo* (111). Fibromodulin treatment of adult wounds induces the expression of fibrosis-associated genes, increases myofibroblast differentiation and contraction, and leads to rapid clearing of myofibroblasts from the wound, which are features that resemble fetal scarless repair (110). Fibroblasts from superficial wounds that heal with little to no scarring in adults show a higher level of fibromodulin production compared to fibroblasts from deep wounds; therefore, reduced fibromodulin is believed to contribute to the formation of HTS (30). Further supporting this notion, expression of fibromodulin is significantly reduced in HTS tissue, compared to NS (69), and fibromodulin deficient mice exhibit reduced angiogenesis, extended inflammation, and elevated epithelial migration (112).

Fibromodulin null mice have collagen fibrils that are irregular in shape and size leading to less orderly packing, a disorganized ECM, increased interfibrillar space, a loss of structural complexity, and fusion of larger abnormal fibrils with smaller ones (113). These alterations observed in the fibril structure of fibromodulin null mice result in a higher collagen density, smaller collagen bundle sizes, and a larger area of fibrotic scar (113). This provides significant evidence that fibromodulin is essential for regular fibril organization and restoration of the ECM.

1.4.4 Versican

Versican is an extremely hydrophilic hyalectan proteoglycan that contains up to 30 CS chains. Versican occupies the interfibrillar space of the ECM, influencing collagen composition, growth factor activity, cellular behaviour, and tissue turgor (114,115). DF produce significantly more versican than SF (82), and it is significantly upregulated in HTS (114). Versican's upregulation in HTS causes an expansion of the ECM and an increased volume of scar, due to its hydrophilic nature (114). Data suggest that versican has a profound effect on the formation of HTS and warrants further investigation.

Versican and hyaluronan (HA) are found at high concentrations in the provisional pericellular matrices, which allows cells to change shape and guides their proliferation and migration (116). When the composition of the pericellular matrix is altered by the proteoglycan content, it can result in altered cell phenotypes (116). For example, dermal fibroblasts that lack a versican degrading protease have thick pericellular coats and display a myofibroblast phenotype that is indicated by increased α-SMA expression, enhanced contractility, and increased phosphorylation of small mothers against decapentaplegic (Smad) 2 (116,117). Dermal fibroblasts in mice that experimentally express partial versican isoforms have a significantly

reduced ability to contract collagen gel, although they have normal pericellular coats and a normal phenotype (116,117).

Versican influences immune cell phenotypes through its ability to bind to HA. Incubating versican with HA inhibits its ability to bind to T-lymphocytes, which ultimately blocks their migratory capability during matrix formation (118). Furthermore, versican and versican fragments can bind to TLR-2 and TLR-4 receptors on macrophages and induce the signal transduction pathways that release inflammatory cytokines (116).

Modifications in versican's structure influences the mechanical properties of the provisional matrix. The presence of different versican isoforms can lead to either a pro-inflammatory or anti-inflammatory ECM. For example, overexpression of V3 leads to a reduced expression of HA, V1, and V0, which causes an increase in elastic fiber assembly and a decreased capacity for the remodelled ECM to bind and activate macrophages (116).

Carthy *et al*. determined *in vitro* the effect of V1 on mouse fibroblasts (119). After being transfected with the gene for the human V1 isoform, the mouse fibroblasts proliferated faster, demonstrated increased cell adhesion, and migrated slower than control cells, due to an increase in N-cadherin and integrin beta-1 expression and FAK phosphorylation (119). Western blot and immunofluorescence experiments showed that V1 transfected fibroblasts significantly increased the expression of α-SMA, suggesting an increase in myofibroblast differentiation. Dysregulated and excessive myofibroblast activity causes tissue deformation and a loss of function by excessive contracture (120). V1 transfected fibroblasts have an increased capacity to contract collagen lattices and increase collagen III mRNA expression (119). V1 does this by increasing TGF-β signaling through Smad 2, as V1 increases the accumulation of Smad 2 in fibroblasts

(119). These results indicate that the V1 isoform of versican has an influence on the fate of wound healing and scar contracture.

1.4.5 Lumican

Lumican is another class II SLRP found in the ECM of skin. Lumican is similar in structure and function to fibromodulin; however, it acts independently in the process of wound healing. Lumican null mice have significant alterations in their collagen fibril diameters and interfibrillar spacing, which result in skin fragility and laxity, similar to Ehlers-Danlos syndrome phenotypes (121). Fibril diameters are thicker, lateral fibril fusion is enhanced, and non-uniform fibril spacing becomes more frequent, leading to expansions of interfibrillar spacing and altered biomechanics of the ECM (122). The altered biomechanics of the collagen fibrils leads to a reduction in tensile strength of the skin as there is a disruption in load transmission from the matrix to the collagen fibrils (122). Lumican-deficient mice, given a circular full-thickness wound on the dorsal skin, show a delay in wound healing and gene expression of TGF-β1, type I collagen, and fibronectin (121). Furthermore, lumican null mice have decreased skin thickness, particularly in the dermal layer, from a decrease in collagen fiber abundance, and an increased number of inflammatory macrophages present in dermal wounds (3 and 12 days after injury) (121).

Experimentally, topical application of lumican significantly improves the healing of full-thickness skin excision wounds in mice (123). Moreover, fibroblasts isolated from HTS show a markedly reduced production of lumican compared to normal fibroblasts. The experimental upregulation of lumican in HTS mitigates the excessive production and degradation of the ECM and it impedes cell cycle progression of HTS fibroblasts, interrupting their excessive proliferation (124). Lumican shows its effect in fibrosis through its interaction with the collagen-

integrin FAK signaling pathway. Upregulation of lumican leads to enhanced binding of lumican to $\alpha_2\beta_1$ integrin, causing a reduction in its expression, as well as reduced phosphorylation of FAK. Ultimately, inhibition of $\alpha_2\beta_1$ integrin and FAK shows significant efficacy in reducing HTS fibroblast proliferation (124). Saika *et al.* conducted *in vitro* and *in vivo* wound healing studies with the corneal epithelia of mice. The group found that the addition of anti-lumican antibodies *in vitro* mitigates closure of the epithelial defect in healing wounds and lumican null mice display a delay in re-epithelialization of corneal epithelium defects *in vivo* (125). 4-month-old lumican null mice displayed abnormally thick collagen fibers in the posterior corneal stromata; however, these thicker fibers were not observed in younger lumican null mice (125). The group proposes that lumican may modulate epithelial cell migration during corneal wound healing via direct interactions with these cells.

In skin wounds, contraction is primarily facilitated by fibroblasts and re-epithelialization is primarily facilitated by keratinocytes. Both *in vivo* and *in vitro*, lumican has no effect on the activation of keratinocytes, but does promote fibroblast activation in the early phases of wound healing (123). Lumican does not affect the proliferation of fibroblasts but does enhance their contractility via its interaction with integrin α_2 (123). Therefore, lumican facilitates wound healing by enhancing contractility of fibroblasts, not by influencing the progression of re-epithelialization. The interaction of lumican with collagen influences ECM rigidity and its ability to modulate the mechanical contractile activity of fibroblasts demonstrates its role in restoration of the ECM.

Like the inverse relationship in the concentration of class I SLRPs in HTS, class II SLRPs also show this pattern. Thus, as fibromodulin mRNA expression is decreased in HTS, lumican's expression is increased, possibly as a compensatory effect (69). Like the other SLRPs,

the role of lumican in the reconstruction of the dermis and wound healing has not been fully elucidated. Interestingly, skin aging is characterized by its functional impairment of fibroblasts, and concentrations of specific SLRPs that change over one's lifespan are thought to influence this. A study conducted by Vuillermoz *et al*. demonstrated a steady decline in lumican transcriptional levels in fibroblasts from human skin as age increases (126). This inverse correlation is the opposite of what is seen with decorin which becomes slightly increased with aging. Biglycan levels remain constant and do not significantly change with aging (126). Taken together, the increase in decorin expression and the decrease in lumican expression could potentially be involved in the functional alterations that occur in aging skin such as aberrant collagen homeostasis, skin thinning, and a diminished ability to heal wounds (127).

1.4.6 Aggrecan

Aggrecan is a high molecular weight hyalectan proteoglycan with roughly 100 CS chains. The CS chains create an extremely high fixed charge density that produces an osmotic environment crucial for retaining water within tissue (128,129). Of the 6 proteoglycans mentioned, aggrecan is found at the lowest concentration in the skin; however, it accumulates in scar tissue (17,68). Aggrecan proteins and transcripts increase in mice during dermal regeneration, following excisional dermal wounding, but aggrecan that accumulates in the granulation tissue is removed 8 days after injury (130). Aggrecanases are proteolytic enzymes that cleave the substrate aggrecan. The removal of aggrecan by aggrecanases must occur for there to be wound contraction and collagen deposition (130). Aggrecan deposition is found in the dermal wounds of aggrecanase knockout mice, resulting in impaired healing (130).

The most common GAG found in the ECM of mammals is HA, which transfers signals from outside the cell to inside by binding to its main receptor CD44 (131). Interestingly, CD44

exists as several isoforms, giving it the ability to interact with a plethora of macromolecules such as collagen, fibronectin, growth factors, cytokines, and MMPs (131). The interaction of HA with CD44 prevents PDGF-BB-induced migration of human dermal fibroblasts (131). Aggrecan forms extremely large aggregates with HA via its G1 interglobular domain and binds other ECM molecules via its G3 domain (i.e., tenascin-R, fibulin-1/fibulin-2, and fibrillin-1), which may be important for reparative synthesis (17,129).

Thrombospondin motif (TS)-5 is required for the removal of aggrecan. $TS5^{-/-}$ mice cannot heal dermal wounds, whereas $TS4^{-/-}$ mice show essentially normal wound contracture and fibrillogenesis (130). In $TS5^{-/-}$ mice, aggrecan does not accumulate in the granulation phase, but does so when contraction and dermal regeneration are occurring, forming cellular aggregates. The formation of these cellular aggregates is dependent on the presence of CD44 (130). It is proposed that fibroblast progenitor cells are stimulated by growth factors in the granulation tissue to produce aggrecan and HA, and aggrecan is eventually degraded by TS5 (130). The removal of aggrecan alters the environment of the ECM because the high negative charge diminishes. The alteration of charge in the ECM is believed to promote binding of the TGF-β1/TGFβRII complex to the activin A receptor type II-like kinase (ALK5) which in turn promotes phosphorylation of the Smad 2/3 signaling pathway (130), characteristic of fibrotic phenotypes. Alternatively, when TS5 is absent, aggrecan stays bound to CD44 and is not degraded (130). Aggrecan continues to accumulate, resulting in a highly negative charged matrix that hinders the binding of the TGF-β1/TGFβRII complex to ALK5 and promotes its binding to ALK1 (130). This instead leads to Smad 1/5/8 phosphorylation and a reduction of profibrotic signaling (130). The specific role that aggrecan and its isoforms play in wound healing and HTS formation is currently not fully understood. The affinity of different aggrecan isoforms binding

to ECM ligands has been researched (129); however, their relevance regarding hypertrophic scarring and wound healing has not been studied.

1.5 IFN-α2b and TGF-β1 in Hypertrophic Scarring

TGF-β1 is one of the most important profibrotic cytokines in wound healing and as mentioned previously, HTS fibroblasts express significantly more TGF-β1 (85). TGF-β1 is upregulated locally in HTS tissue and systemically in serum from burn patients presenting with HTS (132). DF, which has similar phenotypes to HTS fibroblasts, also overexpress TGF-β1, providing evidence that DF may be critical in the formation of HTS (82). It is well accepted that TGF-β/Smad signaling is linked to many different types of fibrosis such as renal, hepatic, and pulmonary (133). TGF-β1 exerts its effects by activating Smad 2 and Smad 3 through its type I and II serine/threonine kinase receptors, but it is negatively regulated by Smad 7 (133). The pathway of TGF-β is inhibited by Smad 7 binding to TGF-β receptor 1 and blocking the recruitment and phosphorylation of Smad 2 and Smad 3. Smad 7 also recruits numerous proteins such as E3 ubiquitin ligase Smad ubiquitination regulatory factor and homologous to the E6-associated protein carboxyl terminus that bind to the N-terminus region of Smad 7 and causes degradation of TGF-β receptor complex 1 (133). By acting through the Smad 2/3 signaling pathway, TGF-β1 drives the expression of key profibrotic ECM genes like collagen and fibronectin (134). Therefore, Smad 7 is pertinent for reducing fibrosis that is dependent on the TGF-β/Smad signaling pathway (135).

The family of IFNs are cytokines that have antiproliferative activities. IFN-γ is secreted by activated T lymphocytes whereas leukocytes produce IFN-α and fibroblasts produce IFN-β.

Normal and HTS fibroblasts treated with IFN-α2b both show a reduced expression of type I procollagen, but the effects are less prominent in HTS fibroblasts (136). The effects of IFN-α2b on HTS and NS fibroblasts appear after 48 hours, which is different from the reduced 12-hour onset of effects that occur after IFN-γ treatment (136). This indicates that there are likely different mechanisms of signal transduction for the two different IFNs. Furthermore, NS fibroblasts that were treated with IFN-α2b and then allowed to recover without the presence of IFN-α2b, maintained a 60% reduced expression of type I collagen compared to control cells, whereas HTS fibroblasts recovered mRNA expression of type I collagen more quickly and completely following cessation of IFN-α2b exposure (136). IFN-α2b increases collagenase mRNA and leads to increased collagenase activity, whereas IFN-γ does not have this effect (14). Both IFN-α2b and IFN-γ antagonize the effects of TGF-β on fibroblast proliferation and TGF-β stimulated collagen production, and after 96 hours of treatment, both IFNs significantly inhibit the amount of TGF-β1 secreted by NS and HTS fibroblasts (14). Additionally, IFN-α2b decreases fibroblast mediated wound contracture and the collagenase inhibitor TIMP1 (114,137).

As mentioned previously, our IFN-α2b clinical trial in burn patients with HTS resulted in significant improvements in scar assessments in 7 out of 9 patients and systemic administration of IFN-α2b improves HTS through the induction of scar remodelling and maturation (14,138). Our group has also demonstrated that patients who presented clinically with HTS and received IFN-α2b treatment for 24 weeks, showed a reduction in fibroblasts cell density, and a statistically significant reduction in myofibroblast cells density in 4 out of 9 patients, possibly due to an increase in apoptosis of these cells (139). In more recent studies, our group has reported that IFN-α2b treatment may improve scarring in post burn patients by reducing fibrocytes, decreasing angiogenesis in HTS tissue *in vivo*, and by inhibiting endothelial cell

proliferation, vascular endothelial growth factor (VEGF) mediated cellular proliferation, and endothelial cell tube formation (which represents the angiogenic process *in vivo*) *in vitro.* (140,141). Based on these findings from our lab, IFN-α2b shows promising results as a therapeutic agent in the treatment and reduction of HTS in burn patients.

1.6 Research Summary and Formulation of Thesis

The development of HTS following a cutaneous thermal injury is heavily dependent on remodelling the ECM through its synthesis and degradation during wound healing. This process is very sensitive to variations in the ECM molecules involved, as discussed, but requires further investigation to identify key proteins and their isoforms that may direct the normal wound healing process towards fibrogenesis and HTS formation seen clinically. This suggests that researching isoforms of various proteoglycans, such as decorin, may increase our understanding of dermal fibrosis and HTS formation.

The outcome of fibrotic wound healing following burn injury is the formation of HTS, which are characterized by an accumulation of structural proteins like collagen, and the dysregulation of proteoglycans such as decorin. TGF-β1 is a pro-fibrotic cytokine that stimulates the myofibroblast phenotype responsible for generating excessive collagen deposition (28,29), but decorin directly binds to TGF-β1 and regulates its profibrotic effects (83–85). However, previous research conducted in our lab has shown that decorin expression is significantly downregulated in HTS, deep dermal tissue, and thermally injured tissue, mitigating its ability to regulate TGF-β1 (69,86,87). The cytokine IFN-α2b has been shown to reduce angiogenesis, TGF-β1 expression, type I collagen, wound contracture, and fibrocytes (14,132,136–141) whereas clinically it has been shown to downregulate serum TGF-β1 and N^T-methylhistamine,

improving HTS in patients (14). Therefore, IFN-α2b has potential therapeutic benefits that need to be further researched and understood.

The profile for the gene expression of decorin isoforms in HTS and DF has not been identified, so determining this will enhance our understanding of the similarities that these fibroblast populations potentially share. Furthermore, the effects of TGF-β1 and IFN-α2b on decorin isoform expression has not yet been researched. Analysis of the effects of TGF-β1 and IFN-α2b on decorin isoforms in HTS fibroblasts and DF will contribute to understanding their potential role in dermal fibrosis. Specifically, determining the effect that IFN-α2b has on the expression of decorin and decorin isoforms may further elucidate its therapeutic potential.

1.6.1 Hypothesis and Objectives

In this thesis, we hypothesize that there will be a differential expression of decorin and decorin isoforms in HTS fibroblasts relative to NS fibroblasts. By treating fibroblasts with TGF-β1 and/or IFN-α2b, we hypothesize that TGF-β1 will decrease the expression of decorin and decorin isoforms but increase type I collagen expression, whereas treatment with IFN-α2b will counteract the effects of TGF-β1.

Our experimental objectives are:

1. Quantify and compare the gene expression of decorin isoforms in fibroblasts from NS and HTS tissue, and fibroblasts from superficial and deep dermal tissue by reverse-transcription quantitative polymerase chain reaction (RT-qPCR).
2. Determine the effects of TGF-β1 and IFN-α2b on the gene expression of decorin isoforms and type I collagen in NS and HTS fibroblasts, and in SF and DF via RT-qPCR.

3. Quantify the protein expression of decorin and type 1 collagen from NS and HTS fibroblasts by immunofluorescence (IF) staining and enzyme-linked immunosorbent assays (ELISA).

1.7 Figures

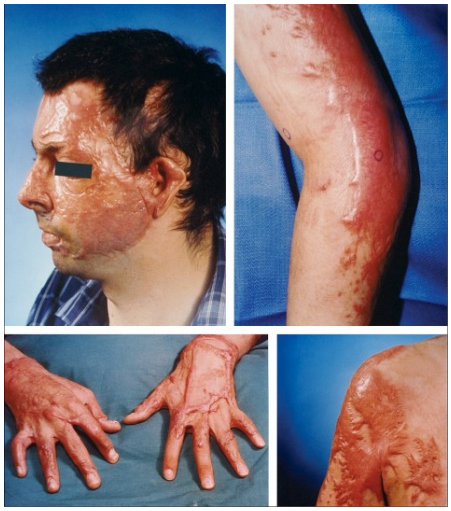

Figure 1-1 34-year-old male with HTS development 8 months after a burn injury to 60% of the total body surface area (2).

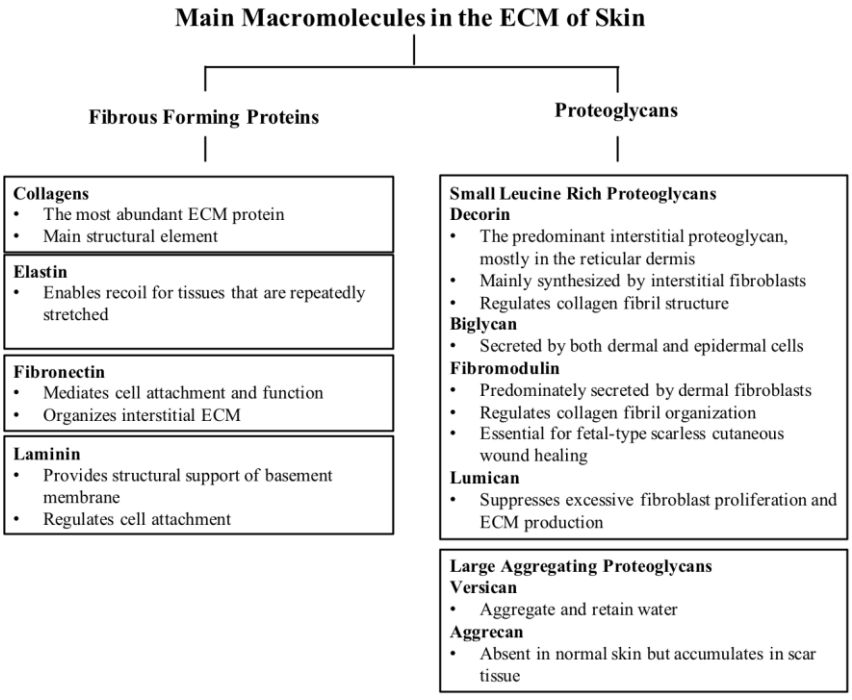

Figure 1-2 Fibrous forming proteins and proteoglycans in the ECM that are related to wound healing.

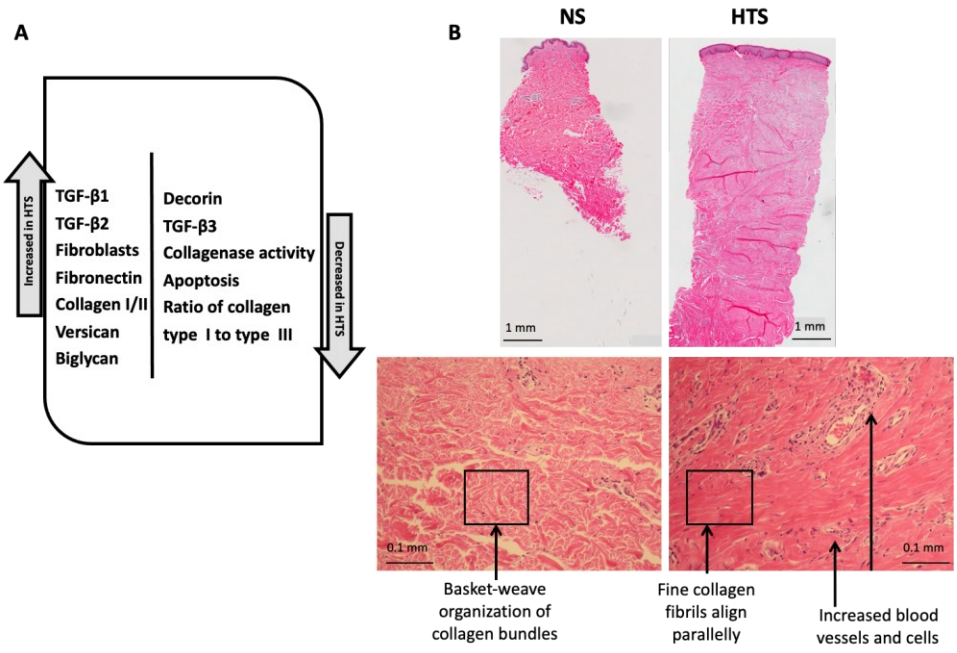

Figure 1-3 A) Alterations in the expression of various proteins and cytokines as features of HTS from dysregulated wound healing. **B)** Hematoxylin and eosin stains for NS and HTS (19).

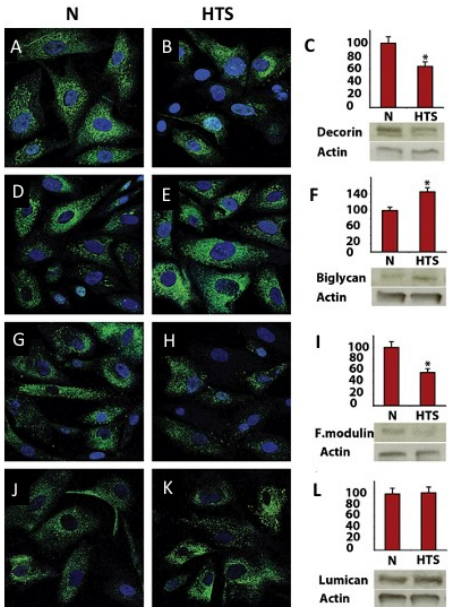

Figure 1-4 A-B) Immunofluorescence staining for decorin, **D-E)** biglycan, **G-H)** fibromodulin, **J-K)** and lumican in NS **(A, D, G, J)** and HTS fibroblasts **(B, E, H, K).** Western blot analysis of **C)** decorin, **F)** biglycan, **I)** fibromodulin, and **L)** lumican in NS and HTS fibroblasts (69).

Proteoglycans	Number of Transcript Variants	Isoform Variants
Decorin	6	A1, A2, B, C, D, and E
Biglycan	2	BGN and X1 (predicted)
Fibromodulin	2	1 and 2 (non-coding)
Versican	4	V0, V1, V2, V3
Lumican	1	LUM
Aggrecan	5	1, 2, 3, X2 and X3 (predicted)

Figure 1-5 Transcripts and isoforms for six proteoglycans in the ECM of skin (70).

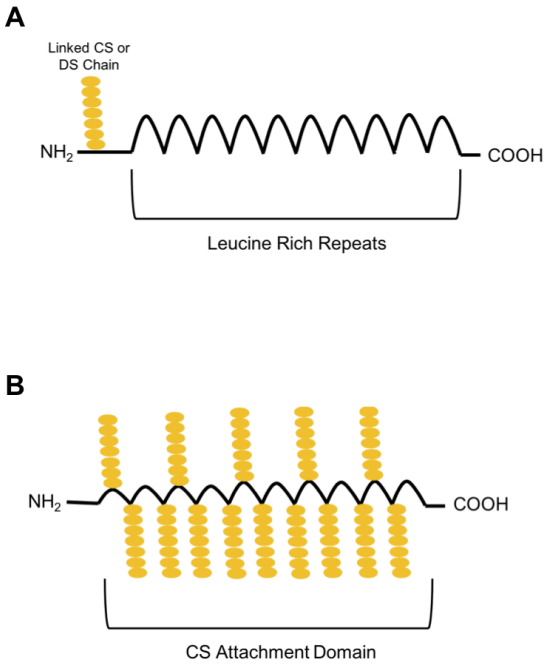

Figure 1-6 A) Basic structure of decorin. Decorin has one attached CS, or dermatan sulfate (DS) glycosaminoglycan chain and its core is composed of 12 leucine rich repeats. **B)** Basic structure

of the large aggregating hyalectan proteoglycan versican. Versican has up to 30 attached CS glycosaminoglycan chains.

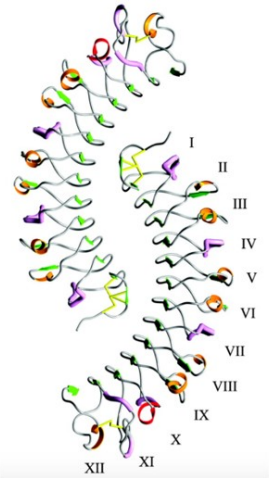

Figure 1-7 Ribbon structure of the dimeric leucine rich repeat protein core of bovine decorin (73).

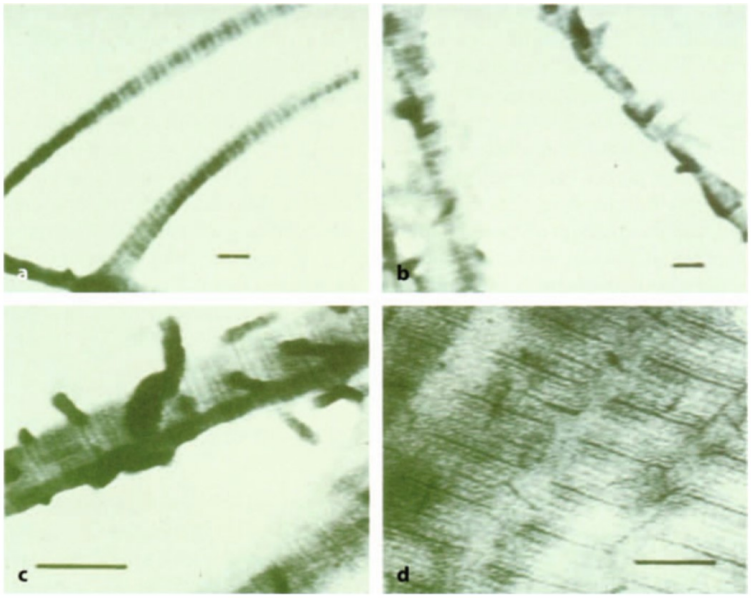

Figure 1-8 Scanning electron microscope images of decorin binding to type I collagen and resulting in tightly packed fiber bundles (81).

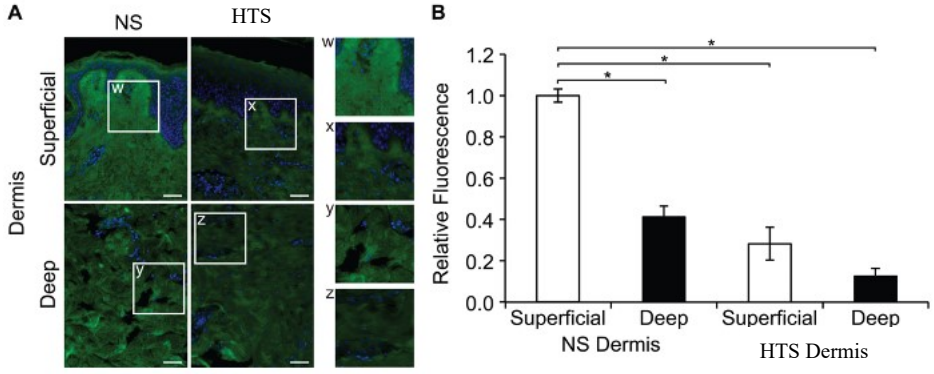

Figure 1-9 A) Immunofluorescence staining for decorin in HTS and site matched NS. **B)** Summary of the relative fluorescence of decorin in superficial and deep dermal tissue of NS and HTS (86).

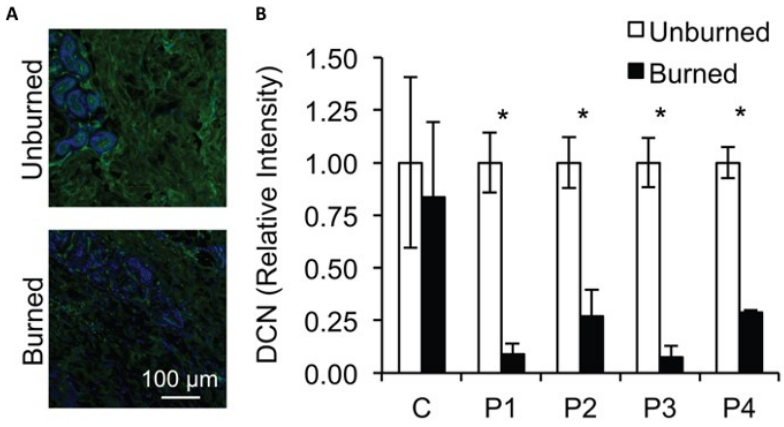

Figure 1-10 A) Immunofluorescence staining for decorin in unburned and burned skin**. B)** Relative fluorescence intensity from unburned and burned skin of burn patients and normal controls (87).

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Chapter 2: The Effects of TGF-β1 and IFN-α2b on Decorin Isoforms and Type I Collagen in Hypertrophic Scar Dermal Fibroblasts

2.1 Introduction

Hypertrophic scars (HTS) are a fibroproliferative skin disease that often occurs following deep dermal injury to the tissue from trauma or burn injury (1). HTS result in psychosocial, aesthetic, and physiological abnormalities that cause limitations in joint mobility and function (1,2). The development of HTS occurs from an excessive deposition and reduced degradation of structural extracellular matrix (ECM) proteins like type I and III collagen, excessive wound contraction, and aberrant biological activity of multiple proteins, cytokines, and growth factors (3–6).

Dermal fibroblasts are crucial cells involved in HTS formation. Fibroblasts from the papillary (superficial) dermis and the reticular (deep) dermis are heterogeneous and have different lipid compositional states (7). This lipid heterogeneity results in diverse responses to extracellular stimuli and creates dynamic cell identities that can possess unique functions in wound healing and dermal fibrosis (7). Furthermore, fibroblasts from the deep aspect of the dermis phenotypically and genotypically resemble HTS fibroblasts and are critical in the formation of HTS (8,9). Their resemblance suggests that HTS fibroblasts arise from deep dermal fibroblasts (DF).

Decorin is a small leucine rich proteoglycan that binds collagen with high affinity and aids in the correct positioning of collagen molecules within the staggered orientation of the fibril (10–12). The prototypic fibrotic cytokine transforming growth factor-beta 1 (TGF-β1) stimulates dermal fibroblasts to produce collagen and enhance wound contraction, where decorin binds and regulates TGF-β1 by inhibiting its interaction with its receptor complexes (13–15). Research has shown that decorin expression is significantly downregulated in HTS tissue and fibroblasts,

thermally injured tissue, and in the deep dermis, whereas TGF-β1 is increased in HTS and DF fibroblasts, and in the serum of patients presenting with HTS (8,9,13,16,17). Therefore, the expression of decorin and TGF-β1 and their complex regulatory relationship appears to be very important in the pathogenesis of HTS.

Previous research conducted in our laboratory has shown that interferon-alpha 2b (IFN-α2b) significantly inhibits the amount of TGF-β1 secreted by normal skin (NS) and HTS fibroblasts and antagonizes type I collagen production (17,18). In clinical trials, our group has also shown that IFN-α2b treatment in burn patients with HTS leads to significant improvements in scar assessments and is a promising therapeutic agent in the prevention and remodelling of HTS (17,19). However, the effect of IFN-α2b on decorin expression in HTS fibroblasts or DF has not been determined.

Most genes can generate multiple mRNA isoforms, many of which can have diverse functions (20). Intron retention, alternative splicing, and alternative transcription start and stop sites generate mRNA isoforms that differ in their protein products (20). Ultimately, these differences in protein structure can lead to major differences in their biological functions and roles. For example, similar isoforms of the proteoglycan versican can possess completely opposite biological functions, as seen with isoforms V1 and V2 (21). However, the structural and functional differences of proteoglycan isoforms in wound healing and fibrosis currently remain underexplored.

Decorin transcripts can undergo alternative splicing that yields various isoforms. Isoform A contains the complete amino acid coding sequence and results from transcript variant A1 from exon 1a in the 5' untranslated region and from transcript variant A2 from exon 1b in the 5' untranslated region (22,23). Isoform B lacks exons 3-4, C lacks 3-5, D lacks 4-7, and E lacks 3-7

(22). The loss of amino acid sequences alters the structure of the protein and can lead to functional variations. The roles of decorin isoforms have not been investigated in relation to dermal fibrosis. Therefore, the mRNA expression of different decorin isoforms was investigated as an initial step in understanding their potential variable functions in HTS development. It is our objective to determine the mRNA expression of decorin isoforms in NS and HTS fibroblasts and to determine the effects of TGF-β1 and IFN-α2b on decorin isoforms and type I collagen expression.

It is our hypothesis, that there will be a differential expression of decorin isoforms in HTS fibroblasts relative to NS fibroblasts. By treating fibroblasts with TGF-β1, the expression of decorin isoforms are expected to decrease while type I collagen expression will increase, whereas treatment with IFN-α2b will counteract the pro-fibrotic effects of TGF-β1.

2.2 Materials and Methods

2.2.1 Fibroblast Isolation, Propagation, and Storage

The protocols for obtaining human tissue samples for fibroblast isolation were previously approved by the University of Alberta Health Research Ethics Board. Following informed consent, tissue specimens were obtained from patients at the University of Alberta Firefighters' Burn Treatment Unit who clinically presented with HTS. Site-matched NS tissue samples were also obtained from the patients as controls. Demographic information of the patients that the HTS and site-matched NS samples were obtained from are presented in Table 1. Briefly, two pairs of HTS and site-matched NS samples used in this study were obtained from the same patient, so they have identical demographic information (**Table 1**). These samples, however, were collected at different time points following burn injury, so they are considered as separate and independent from each other.

Human dermal fibroblasts were also previously cultured from discarded abdominoplasty skin specimens obtained from patients undergoing elective surgery, following informed consent. Superficial dermal fibroblasts (SF) and DF were obtained from the appropriate regions of the skin as previously described (8). Following the removal of the epidermis, SF are isolated from the most superficial aspect of the papillary dermis, and DF are isolated from the deepest portion of the reticular dermis. Fibroblast isolation and culture from all tissue samples were performed previously via established protocols in our laboratory (18). Briefly, fibroblasts were cryopreserved by suspending the cells in Dulbecco's Modified Eagle Medium (DMEM) containing 20% fetal bovine serum (FBS) and 10% dimethyl sulfoxide (DMSO) (Sigma Aldrich, St. Louis, MO). Cells were frozen in cryovials to -80°C then transferred to liquid nitrogen tanks for prolonged storage after 24 hours.

For the following experiments, fibroblasts were thawed on ice and cell viability was determined using an automated cell counter (Bio-Rad Laboratories, Hercules, CA). Fibroblast samples that indicated a live cell count of approximately 70% or more were propagated in DMEM (Invitrogen, Waltham, MA) supplemented with 10% FBS (Invitrogen, Waltham, MA) and 1% Antibiotic-Antimycotic-100x (antibiotics) containing streptomycin sulfate and penicillin G sodium (Invitrogen, Waltham, MA) in a cell culture incubator at 37°C with 5% CO_2. The growth medium was changed every 2 days, and sub-culturing was conducted at 90% confluence until fibroblasts were propagated to passages 4-7. Fibroblasts were lysed with 1 mL of TRIzol reagent (Invitrogen, Waltham, MA) and frozen at -20°C.

2.2.2 IFN-α2b and TGF-β1 Treatment of Dermal Fibroblasts

Previous experiments have shown that treatment with 2000 U/mL of IFN-α2b significantly reduces the expression of type I collagen mRNA and increases collagenase and

tissue inhibitor of metalloproteinase 1 mRNA in HTS fibroblasts (18,24). Furthermore, dose-dependency treatments with recombinant human TGF-β1 (R&D Systems, Minneapolis, MN) showed that 10 ng/mL was a sufficient dose for reducing decorin isoform expression (**Appendix Figure A-1**). HTS and site-matched NS fibroblasts and matched SF and DF were grown and seeded into 6-well plates. Once attachment and confluency were reached, fibroblasts were treated with either 10 ng/mL of TGF-β1 (R&D Systems, Minneapolis, MN) or 2000 U/mL of IFN-α2b (Merck & Co., Kenilworth, NJ) only, or both TGF-β1 and IFN-α2b, where phosphate buffer saline (PBS) served as a control. All treatments were done in DMEM with 2% FBS and 1% antibiotics for 48 hours.

2.2.3 Reverse Transcription-Quantitative Polymerase Chain Reaction (RT-qPCR) for Decorin, Decorin Isoforms, and Type I Collagen Alpha I (COL1A1)

Five different pairs of HTS and site-matched NS fibroblasts from separate primary patient cell lines were used to measure the mRNA expression of decorin and decorin isoforms with no treatments, via RT-qPCR. For the mRNA expression of decorin, decorin isoforms, and COL1A1 to be quantified following TGF-β1 and/or IFN-α2b treatments, previously characterized primary cell lines consisting of site matched pairs of HTS and NS fibroblasts, and site matched pairs of SF and DF were cultured and treated, with three separate independent samples of each cell line used. Fibroblasts were lysed with TRIzol (Invitrogen, Waltham, MA), and total RNA was isolated and purified using a PureLink^{TM} RNA Mini Kit (Invitrogen, Waltham, MA), according to the manufacturer's protocol. The total RNA concentration and quality (260 nm/280 nm ratio) of each sample was quantified using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). RNA samples that displayed a 260 nm/280 nm ratio value between 1.7 and 2.5 were considered high quality and were used for

further experimentation. Reverse transcription of 2 μg of total RNA extract was done using a High-Capacity cDNA Reverse Transcription Kit (Applied biosystems, Waltham, MA), according to the manufacturer's protocol. cDNA was diluted 1:10 in RNase/DNase-free H_2O and stored at 4°C.

RT-qPCR was performed using PowerUp™ SYBR™ Green Master Mix (Applied Biosystems, Waltham, MA) and gene-specific forward and reverse primers for each isoform (Eurofins Genomics LLC, Louisville, KY). The forward and reverse primers for each isoform were designed using NCBI's primer designing tool to ensure product amplification was highly specific (**Table 2**) (25). Additional primers used are also in Table 2. The RT-qPCR reaction plate was prepared to a volume of 20 μL/well, consisting of 10 μL of PowerUp™ SYBR™ Green Master Mix (Applied Biosystems, Waltham, MA), 1 μL of both the forward and reverse primers (20 μM), 1 μL of cDNA, and 7 μL of RNase/DNase-free H_2O. Analysis and amplification were conducted using the StepOnePlus RT-PCR system (Applied Biosystems, Waltham, MA) and conditions used were: UDG activation at 50°C for 2 minutes, Dual-Lock™ DNA polymerase at 95°C for 2 minutes, followed by 40 cycles each consisting of 95°C for 15 seconds (denaturation) and 60°C for 1 minute (annealing). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), beta-2-microglobulin (B2M) and ribosomal protein L13a (RPL13A) were all tested and compared for their use as a potential reference gene. Ultimately, GAPDH served as the normalization standard (**Appendix Figure A-2**). The comparative threshold cycle (C_t) method was conducted using the formula $2^{-\Delta\Delta Ct}$. Values were reported as the mean fold change ± SE.

2.2.4 Immunofluorescence (IF) Staining and Relative Quantification of Decorin and Type 1 Collagen

Three different pairs of HTS and site-matched NS fibroblasts from separate primary patient cell lines were used. NS and HTS fibroblasts were grown and treated with TGF-β1 and/or IFN-α2b in 12-well plates with coverslips. Coverslips containing the attached fibroblasts were fixed in 100% methanol (chilled at -20°C) for 5 minutes. All steps were completed at ambient temperature. To permeabilize the cells, the coverslips were incubated in PBS containing 0.2% Triton X-100 (Thermo Fisher Scientific, Waltham, MA) for 10 minutes. A blocking solution composed of 1% bovine serum albumin (Sigma-Aldrich, St. Louis, MO), glycine (Sigma-Aldrich, St. Louis, MO), PBS, and 0.1% Tween-20 (Thermo Fisher Scientific, Waltham, MA) was added to the cells for 30 minutes to block non-specific binding of the antibodies. Following this, cells were sequentially stained for decorin and type I collagen. The coverslips were incubated with a 1:400 dilution of anti-human collagen I polyclonal primary rabbit antibody (PA1-27396, Thermo Fisher Scientific, Waltham, MA) for 1 hour in a humidified chamber, followed by incubation with a 1:1000 dilution of Alexa Flour 594® goat anti-rabbit secondary antibody (A-11012, Thermo Fisher Scientific, Waltham, MA) for 1 hour in the dark. The cells were then incubated with a 1:200 dilution of anti-human decorin monoclonal primary mouse antibody (sc-73896, Santa Cruz Biotechnology, Dallas, TX) followed by incubation with a 1:1000 dilution of Alexa Flour 488® goat anti-mouse secondary antibody (A-11017, Thermo Fisher Scientific, Waltham, MA) under the same conditions as described above. Mounting of the slides was done with ProLong® Gold Antifade with DAPI to stain the nuclei (Thermo Fisher Scientific, Waltham, MA), prior to image analysis. Fibroblasts were imaged using NIS Elements Imaging Software on a Nikon Eclipse T*i*-E inverted microscope. Briefly, ImageJ (National Institutes of Health, Bethesda, MD) was used to quantify the fluorescent density per cell by measuring the signal intensity in a high-powered field and dividing this signal by the number of

cells present in that field. This was replicated in four separate high-powered fields and the values were averaged to obtain the fluorescent density/cell for decorin and type I collagen.

2.2.5 Enzyme Linked Immunosorbent Assay (ELISA) for Decorin and Procollagen Type I

Three different pairs of HTS and site-matched NS fibroblasts from separate primary patient cell lines were used. NS and HTS fibroblasts were grown and treated with TGF-β1 and/or IFN-α2b, as described previously, and both decorin and procollagen type 1 were measured in the cell culture media using an ELISA. The proteins secreted by fibroblasts were measured using a sandwich ELISA specific for human decorin (EHDCN, Invitrogen, Waltham, MA) or human procollagen type 1 (ab210966, Abcam, Cambridge, UK), according to the manufacturer's protocol. The samples were diluted 200-fold for measuring decorin and 1000-fold for procollagen type 1, and all subsequent steps were conducted at ambient temperature.

For decorin measurement, 100 µL of each sample were added to a 96-well plate precoated with decorin capture antibody for 2.5 hours. This was followed by a 1-hour incubation with 100 µL of human decorin biotin conjugated antibody and then a 45-minute incubation with 100 µL of streptavidin-horseradish peroxidase. For procollagen type 1 protein measurement, 50 µL of each sample were added to a 96-well precoated plate with 50 µL of a prepared antibody cocktail consisting of human procollagen type 1 capture antibody, human procollagen type 1 detector antibody with horseradish peroxidase, and antibody diluent for 1 hour. 100 µL of TMB substrate was added to all samples in the dark (30 minutes for decorin and 10 minutes for procollagen type 1), before stop solution, composed of phosphoric acid, was added to each sample and the optical density was recorded at 450 nm using a Varioskan Lux plate reader (Thermo Fisher Scientific, Waltham, MA). Decorin and procollagen type 1 concentrations were

calculated based on a standard curve from a serial dilution of recombinant human decorin protein and procollagen type 1 protein, respectively.

2.2.6 Statistical Analysis

All statistical analysis were preformed using STATA for Macintosh version 28.0 (StataCorp, College Station, TX). A Shapiro-Wilk normality test was performed for each dataset, and the appropriate parametric or non-parametric tests were used according to the normality test outcome. Two-tailed tests were used in all instances. Comparisons between matched samples (HTS/NS fibroblasts and SF/DF) for normally distributed datasets were done with an independent samples T-Test. Multiple group comparisons that were normally distributed were evaluated using one-way ANOVA followed by Tukey's post-hoc test and datasets that were not normally distributed were evaluated using a Kruskal-Wallis H Test with pairwise comparisons. A p-value of ≤ 0.05 was considered statistically significant.

2.3 Results

2.3.1 mRNA Expression of Decorin and Decorin Isoforms in HTS and Site-Matched NS Fibroblasts

We observed that the mRNA expression of decorin was significantly decreased in HTS fibroblasts relative to NS fibroblasts (**Figure 2-1 A**). The mRNA expression of decorin A1, A2, B, C, D, and E were variable in NS and HTS fibroblasts and the expression of each isoform was significantly decreased in HTS fibroblasts relative to NS fibroblasts (**Figure 2-1 B**).

2.3.2 mRNA Expression of Decorin Isoforms After Treatment with TGF-β1 and/or IFN-α2b

TGF-β1 significantly decreased the expression of decorin in NS and HTS fibroblasts and in SF and DF, relative to the PBS control (**Figure 2-2 A-B**). The expression of decorin was

significantly less in NS than HTS fibroblasts treated with TGF-β1. IFN-α2b significantly increased the expression of decorin in NS fibroblasts only, but there was a relative increase in SF and DF. Decorin expression was significantly greater in NS fibroblasts than in HTS fibroblasts and in SF than DF treated with IFN-α2b. All fibroblast populations treated with both TGF-β1 and IFN-α2b showed a significant reduction in their expression of decorin and did not differ from the TGF-β1 treatment only group. Therefore, IFN-α2b did not significantly inhibit the effects of TGF-β1 in any instance.

In NS and HTS fibroblasts, TGF-β1 significantly decreased the expression of decorin A1, A2, B, and E (**Figure 2-2 C**) and showed a trend of decreasing the expression of D. Isoform C expression significantly decreased in HTS fibroblasts and relatively decreased in NS fibroblasts. The expression of decorin A1 and B were significantly decreased, and E was relatively decreased in NS fibroblasts treated with TGF-β1, when compared to HTS fibroblasts. IFN-α2b significantly increased the expression of decorin A1, A2, and E in NS fibroblasts and B in HTS fibroblasts. There was also a noticeable relative increase of B and C in NS fibroblasts and A2, C, and E in HTS fibroblasts treated with IFN-α2b. The expression of decorin A1, A2, D, and E were significantly greater in NS fibroblasts treated with IFN-α2b than in HTS fibroblasts and relatively greater in B and C. Fibroblasts treated with both TGF-β1 and IFN-α2b showed a significant decrease in the expression of decorin A1, A2, B, and E in NS and HTS fibroblasts. Isoform C was significantly decreased in HTS fibroblasts and relatively decreased in NS fibroblasts treated with both TGF-β1 and IFN-α2b. Since there were no differences relative to the TGF-β1 only treatment group, IFN-α2b did not significantly inhibit the effects of TGF-β1 on decorin isoform expression.

TGF-β1 significantly decreased the expression of decorin A1, A2, B, C, and E in SF and relatively decreased their expression in DF, apart from A1 which was significantly decreased in DF (**Figure 2-2 D**). TGF-β1 relatively decreased D in both SF and DF. Expression of A2 was significantly less in TGF-β1 treated DF than SF. IFN-α2b significantly increased the expression of isoforms A2 and E in SF fibroblasts but had no significant effect on any isoforms in DF. There was a noticeable relative increase in the expression of all other isoforms in SF treated with IFN-α2b and decorin A1, A2, B, D, and E in DF. The expression of A2 and C in SF treated with IFN-α2b were significantly greater than in DF. Apart from isoform D, the expression of all other isoforms treated with IFN-α2b were significantly greater in SF than DF. When treatments of TGF-β1 and IFN-α2b were combined, there were no statistically significant differences in the expression of any isoform in SF or DF relative to the TGF-β1 only treatment group. Therefore, IFN-α2b was not able to inhibit the effects of TGF-β1 decreasing isoform expression in SF or DF under the conditions used.

2.3.3 mRNA Expression of COL1A1 After Treatment with TGF-β1 and/or IFN-α2b

TGF-β1 increased the expression of COL1A1 in NS and HTS fibroblasts, whereas the effect was significantly greater in NS fibroblasts (**Figure 2-3 A**). IFN-α2b had no significant effect, relative to the PBS controls in either NS or HTS fibroblasts. When fibroblasts were treated with both TGF-β1 and IFN-α2b, there was a reduction in the expression of COL1A1 relative to the TGF-β1 treatment group alone. Therefore, IFN-α2b showed a trend of inhibiting the effects of TGF-β1 in NS and HTS fibroblasts, but this effect was not statistically significant.

In both SF and DF, TGF-β1 significantly increased the expression of COL1A1 relative to the PBS controls, whereas IFN-α2b had no significant effect on its expression (**Figure 2-3 B**). In

SF, IFN-α2b significantly inhibited the effect of TGF-β1, as the expression of COL1A1 was significantly less than that of the TGF-β1 treatment group alone. This effect was not seen in DF and the combined treatments of TGF-β1and IFN-α2b showed no relative differences when compared to the TGF-β1 only treatment group.

2.3.4 Decorin and Type 1 Collagen Protein Expression in Fibroblasts After Treatment with TGF-β1 and/or IFN-α2b

Decorin and type 1 collagen in NS and HTS fibroblasts were stained using IF, and representative images from one patient were included for each treatment group (**Figure 2-4 A)**. There were no statistically significant differences in the fluorescent density/cell of decorin between the different treatment groups in NS or HTS fibroblasts (**Figure 2-4 B-C)**. There were however pronounced relative differences after IFN-α2b treatment. In NS and HTS fibroblasts, IFN-α2b increased the fluorescent density/cell of decorin relative to the PBS control group. In NS fibroblasts, IFN-α2b showed a trend of inhibiting the effect of TGF-β1 as there was an increase in the fluorescent density/cell of decorin when the treatments were combined, relative to the TGF-β1 treatment group alone. In the combined treatment group in HTS fibroblasts, decorin fluorescent density/cell was relatively lower than that of the TGF-β1 treatment group alone.

In NS fibroblasts, TGF-β1 and the combined treatment group both significantly increased the fluorescent density/cell of type I collagen relative to the PBS control group (**Figure 2-4 D)**. Therefore, IFN-α2b did not significantly inhibit TGF-β1. In HTS fibroblasts there were no notable differences between the different treatment groups (**Figure 2-4 E**).

2.3.5 Decorin and Procollagen Type I Secreted from Fibroblasts After Treatment with TGF-β1 and/or IFN-α2b

There were no statistically significant differences in decorin secreted between the different treatment groups in NS or HTS samples measured by ELISA (**Figure 2-5 A-B**). TGF-β1 and the combined treatment group both relatively decreased the secretion of decorin in NS and HTS samples, showing therefore, that IFN-α2b did not inhibit TGF-β1's downregulation of decorin.

In NS and HTS fibroblasts, TGF-β1 significantly increased the amount of procollagen type 1 present in the cell culture media (**Figure 2-5 C-D**). IFN-α2b had no significant effect relative to the PBS control, and it did not significantly inhibit the effect of TGF-β1 on procollagen type 1. However, when combined with TGF-β1, IFN-α2b did show a trend of inhibiting its effect by decreasing the amount of procollagen type 1 present when compared to the TGF-β1 treatment group alone in NS and HTS fibroblasts.

2.4 Discussion and Conclusion

The intricate and complex nature of the wound healing processes, coupled with the plethora of molecules that are involved, makes it difficult to understand the pathogenesis of HTS formation completely. However, researchers continue investigating a broad spectrum of cells and molecules that may be key to the prevention and treatment of fibrotic scarring. Specifically, our group has great interest in the influence of decorin, collagen, and cytokines such as TGF-β1 and IFN-α2b on the development and treatment of HTS (8,9,18,19,24,26–29). Broadening our understanding of the roles and interactions between these molecules gives insight into their potential as therapeutic agents and understanding the effects of specific isoforms in relation to dermal fibrosis pathogenesis is a promising new avenue of research.

Fibroblasts, keratinocytes, T and B cells, and mast cells express transmembrane Toll-like receptors (TLR), which are innate immune receptors that recognize pathogen-associated

molecular patterns and damage-associated molecular patterns (30). TLRs function as activators of the innate immune system but have been shown to play a role in the transition from normal wound healing to fibrosis (31). TLR activation leads to NF-kB and mitogen-activated protein kinase activation, cytokine gene transcription, and co-stimulatory molecule expression such as CD40 (32). Specifically, TLR4 dependent fibrosis in the liver is stimulated by lipopolysaccharides that cause chemokines to be released and macrophage-like Kupffer cells to be activated (31). This pathway results in aberrant TGF-β-mediated activation of hepatic stellate cells, liver fibrosis, and an excessive deposition of extracellular matrix. Our lab has demonstrated that the expression of TLR4 is significantly increased in HTS fibroblasts, implicating its role in the development of this fibrotic disorder (32). Decorin exhibits a dose dependent effect on the stimulation of TLR4, so this finding becomes contradictory to the anti-fibrotic properties that decorin has typically been show to display (30). It is possible, however, that decorin from the extracellular matrix of thermally injured tissue has been denatured, causing its anti-fibrotic function of inactivating TGF-β1 to be abated while it still retains the ability to stimulate TLR4 (16). Therefore, the structure of decorin is crucial in determining its resulting biological functions.

Previously, it has been determined that the expression of decorin is significantly decreased in HTS fibroblasts relative to NS fibroblasts (9,33,34) and our RT-qPCR experiments measuring the mRNA expression of decorin corroborates these findings. In this study, we report for the first time that there is differential expression of each decorin isoform in NS and HTS fibroblasts, and that the expression of each isoform is significantly decreased in HTS fibroblasts relative to NS fibroblasts. This provides preliminary insight into how the differential expression of decorin isoforms may be a potential indicator of dermal fibrosis.

TGF-β1 has been shown to downregulate the expression of decorin via a complex relationship (9). Our findings show that TGF-β1 significantly decreases the expression of decorin mRNA in all fibroblast populations analyzed. Previous research has shown TGF-β1's role in negatively regulating decorin in dermal fibroblasts via its promoter sequence, and our observations that TGF-β1 has a more pronounced effect on decorin expression at the mRNA level than the protein level adds additional support for this complex relationship (9,35,36).

The effect of IFN-α2b on decorin expression appears to be more pronounced at the protein level within the NS and HTS fibroblasts than it does on decorin protein being secreted from the fibroblasts. Although not significant, the fluorescent density/cell of decorin shows a 3-fold increase in NS fibroblasts and a 2-fold increase in HTS fibroblasts, following IFN-α2b treatment. In our RT-qPCR experiments we show that IFN-α2b only significantly increases the expression of decorin mRNA in NS fibroblasts, but there is a trend of IFN-α2b relatively increasing its expression in SF and DF. Notably, in our experiments there was high biological variability present between the samples from different patients. Previous research done in a nude mouse model with HTS implanted into the subcutaneous space has shown that an intralesional injection of IFN-α2b increases the mRNA expression of decorin (37). This effect, however, was measured following 4 weeks of treatment. Thus, the longer treatment duration could be why that study showed a significant increase in decorin mRNA expression in their HTS model and our experiment did not.

The effect of TGF-β1 significantly decreasing decorin isoform mRNA expression was variable in all fibroblast populations examined. In both NS and SF, the mRNA expression of A1, A2, B, and E were all significantly decreased. There was, however, a trend of TGF-β1 decreasing the mRNA expression of each isoform in all fibroblast populations. Similar results were

observed with IFN-α2b which significantly increased the expression of certain isoforms in the different fibroblast populations. Again, there was a relative trend of IFN-α2b increasing the mRNA expression of each isoform in all fibroblast populations. Interestingly, IFN-α2b significantly increased the mRNA expression of A2 which is the longest transcript variant (alongside A1), and E which is the shortest transcript variant in both NS and SF. This preliminary evidence may indicate that these isoforms harbor unique structural and functional differences that cause their variable responses to the effects of TGF-β1 and IFN-α2b and warrants further investigation.

Based on these experiments, the decrease in mRNA expression of decorin and decorin isoforms after treatment with 10 ng/mL of TGF-β1 could not be restored with 2000 U/mL of IFN-α2b. A dosage of 2000 U/mL of IFN-α2b for 48 hours has been effective in reducing type I collagen in previous studies (18), however, a higher dosage for a longer duration may be required to counteract TGF-β1's effect on decorin and decorin isoform expression. Furthermore, 400 U/mL of IFN-α2b has been shown to reduce the mRNA expression of α-SMA in TGF-β1 stimulated fibrocytes (29), but this was after 7 days of treatment, so again a longer duration of treatment may be required to see more significant effects.

There is currently limited research on the specific interaction between IFN-α2b and TGF-β1 in fibrotic environments, but it has recently been shown that IFN-α2b has an inhibitory effect on the TGF-β1/Smad signaling pathway (38). Researching if IFN-α2b is influencing TGF-β1 via inhibition of the TGF-β1/Smad signaling pathway in dermal fibrosis would be an important future avenue to explore why our study did not show IFN-α2b significantly inhibiting the fibrotic effects of TGF-β1. Briefly, this could be done by measuring the mRNA and protein expression

of Smad 2 and 3, phosphorylated Smad 2 and 3, and TGF-β1 receptor types I and II in HTS and site-matched NS fibroblasts, following treatment with IFN-α2b.

Due to the phenotypic similarities between HTS fibroblasts and DF observed in previous research, it could be expected that they would be influenced similarly following TGF-β1 and IFN-α2b treatments (8). The effect of IFN-α2b increasing the gene expression of decorin was significantly less in both HTS and DF, relative to NS and SF, respectively. Furthermore, the effect of TGF-β1 increasing the mRNA expression of COL1A1 was significantly less pronounced in HTS and DF, relative to NS and SF, which supports previous findings (18). Regarding isoform expression, IFN-α2b had less of an effect on increasing specific isoform expression in HTS and DF, when compared to NS and SF, respectively. Further evidence of the similarity between HTS fibroblasts and DF is shown by the reduced effect that TGF-β1 and IFN-α2b have on altering their gene expression patterns.

TGF-β1 induces type I collagen synthesis in a Smad 2/3-dependent fashion (39). Specifically, TGF-β1 has been shown to have a stimulatory effect on type I collagen alpha 2 transcription via the interaction of transcription factor Sp1 and Smad 3, which bind to an upstream sequence designated the TGF-β1 responsive element (40). Whereas IFN-α suppresses the activation of the type I collagen alpha 2 promoter (40). COL1A1 mRNA expression increased significantly with TGF-β1 treatment in all fibroblast populations, but IFN-α2b only significantly inhibited this effect in SF. In NS and HTS fibroblasts, a relative trend of IFN-α2b inhibiting TGF-β1 can also be seen. However, to acquire further evidence of IFN-α2b inhibiting the effect of TGF-β1 increasing COL1A1 mRNA expression, more samples would need to be analyzed. In this study, the mRNA expression of COL1A1 was significantly increased by TGF-β1, which supports previous research that TGF-β1 is having its effect at the promoter.

Relative protein quantification from NS fibroblasts showed similar results to the mRNA experiments. Procollagen type 1 and type I collagen measured by ELISA and IF, respectively, increased significantly with TGF-β1 treatment. IFN-α2b did not significantly counteract TGF-β1, but it did reduce the procollagen type 1 secreted by NS and HTS fibroblasts. Interestingly, TGF-β1 and IFN-α2b treatments did not show any significant effects on the type I collagen measured by IF in HTS fibroblasts, but procollagen type 1 secreted from HTS fibroblasts showed similar results to the mRNA experiments. The expression inside the HTS fibroblasts, as measured by IF, following treatment appears to be different than what is measured in the cell culture media using an ELISA.

Procollagen type 1 is flanked by N- and C- terminal propeptides, making it soluble in solution. Once the N- and C- terminal propeptides are cleaved by procollagen N- and C-proteinase, mature triple helices can self-assemble and crosslink in the extracellular matrix. The ELISA used in this study measured soluble type I procollagen that was secreted into the cell culture media via an antibody for the procollagen I N-terminal propeptide. Although this ELISA does not specifically measure intracellular or matrix bound collagen, we accept that this measure of soluble procollagen type 1 is an accurate reflection of recently synthesized type I collagen protein.

The pro-fibrotic nature of TGF-β1 is shown again through this series of experiments, and the importance of the dosage and duration of IFN-α2b treatment is made apparent from the minimal impact that it had on significantly inhibiting the effects of TGF-β1 in this work. While IFN-α2b showed a relative trend of mitigating TGF-β1's fibrotic effects, it only significantly counteracted it increasing the mRNA expression of COL1A1 in SF. Pro-fibrotic TGF-β1 had more profound effects than the anti-fibrotic cytokine IFN-α2b, which could possibly be due to

the basal levels of TGF-β1 being increased in DF, NS, and HTS fibroblasts. Although NS fibroblasts were used as a control and we expected to see similar results to what was observed in SF, they were obtained from patients who presented with HTS. Due to the burn injury and hypertrophic scarring that these patients experienced, it is possible that the basal levels of TGF-β1 were systemically elevated throughout the patient's body, even in the NS regions. Notably, a clinical trial by Tredget et al., demonstrated that TGF-β1 concentrations in the serum of burn patients with HTS are increased significantly above normal levels (17). The SF, however, were obtained from healthy individuals, so it would be expected that the basal levels of TGF-β1 would not be elevated. This could be why we observed IFN-α2b significantly inhibiting TGF-β1 in SF but not in NS fibroblasts. Therefore, it is possible that the current dosage of IFN-α2b used was ineffective against the combination of the increased basal level and added dose of TGF-β1. This speculation would need to be confirmed by measuring the mRNA and protein expression of TGF-β1 both before and after treatment.

Regulating the expression of different cytokines has been shown to depend on the rates of transcription, splicing, message turnover, translation, protein processing, export, and protein degradation, where each step is influenced by different stimuli or inhibitors (41). A similar phenomenon could be occurring in the NS and HTS fibroblasts where any one of these processes could be differentially affected, causing the observed differences in decorin and type I collagen expression intracellularly, in the cell culture media, and at the mRNA level. Furthermore, broad biological variability between samples and the use of either one or three primary cell lines could account for the differences in significance observed between the different experiments (i.e., RT-qPCR, IF, and ELISA). Further experiments with more samples will need to be conducted to make any significant observations.

We recognize that the mRNA expression of molecules does not necessarily equate to their protein expression following translation. The effects of TGF-β1 and IFN-α2b on the expression of decorin isoforms were measured only with RT-qPCR, which is a significant limitation. There are currently no commercially available antibodies that are specific to each isoform, making analysis at this point difficult. A future direction would be the creation of specific antibodies for protein analysis as well as synthesizing the protein for each isoform and observing their biological functions in the ECM environment. For example, quantifying the affinity each isoform has for type I and III collagen fibrils could elucidate if they have differential roles in matrix alignment and fibrillogenesis. This structure and function relationship would add insight into the biological roles of decorin isoforms and their specific influence on the pathogenesis of HTS.

Briefly, the importance of the structure function relationship has been shown with isoforms of the proteoglycan versican (21). Versican has a chondroitin sulfate (CS) attachment sequence that is alternatively spliced into two domains, termed CSα and CSβ, ultimately creating four isoforms (21). Isoform V1 only possess the CSα domain and isoform V2 only possess the CSβ domain, which results in the isoforms having vastly different biological functions. Isoform V1 increases cellular proliferation, upregulates epidermal growth factor receptor expression, and reduces apoptosis, whereas isoform V2 displays the opposite effects (21). Furthermore, an increased expression of V2 after a central nervous system injury inhibits axon regeneration and expression of isoforms V0 and V1 increases in tumors (42). Since it has been demonstrated that an abnormal expression of versican isoforms is associated with various pathological conditions, it is possible that this could be seen with the isoforms of other ECM proteoglycans, such as decorin.

In conclusion, there was variable expression of each decorin isoform in NS and HTS fibroblasts and the reduced expression for each in HTS fibroblasts was shown for the first time. Pro-fibrotic TGF-β1 and anti-fibrotic IFN-α2b showed variable influence on decorin isoforms in all fibroblast populations, but overall trends showed that TGF-β1 decreased their expression and IFN-α2b increased their expression. With each isoform being differentially affected by these cytokines, it warrants further investigation into their structural and functional roles in HTS pathogenesis. We believe that a higher dose of IFN-α2b for a longer duration would have a more significant effect on inhibiting TGF-β1.

In congruence with previous research, TGF-β1 treatment leads to profibrotic genotypes and phenotypes whereas IFN-α2b treatment leads to anti-fibrotic genotypes and phenotypes. Treatment with 2000 U/mL of IFN-α2b for 48 hours is not sufficient in significantly inhibiting the effects of 10 ng/mL of TGF-β1, but it does show a relative trend of mitigating its effect on decorin, decorin isoforms, and type I collagen expression. IFN-α2b may be a useful form of treatment for HTS, but the dosage and treatment duration are important factors to consider.

2.5 Figures and Tables

Table 1. Patient demographic information for HTS and site-matched NS samples.

Patient	Age (years)	Sex	% Total Body Surface Area of Burn
Patient 1	37	M	75%
Patient 2	46	M	3%
Patient 3*	37	M	4%
Patient 4	24	M	25%

* Two pairs of HTS and site-matched NS samples used in this study came from the same patient (patient 3). These samples have identical demographic information associated with them, but the samples were collected at different time points following burn injury. Although the two samples came from the same patient, they are considered as separate and independent because they were obtained at different times.

Table 2. Sequences for forward and reverse primers used for RT-qPCR.

Primers for Decorin Isoforms	Forward Primer Sequence (5'-3')	Reverse Primer Sequence (5'-3')
A1	GGGTGCTTCACATAAAAGGTTGGA	GGCTGTACTGAAGACAGAAAGTGG
A2	AGGGACAGACCAAGCACGCAA	GTGGGCTTCTCTTCTGTGTCCCC
B	GTTTCCTGGGCTGGACCGTT	TGGTGCCCAGTTCCAAATCAG
C	GTGTTCTGATTTGGGTCTTCCTCC	CGTGTTGGCCAGAGAGCCAT
D	TCCGCTGTCAATGCCATCTT	ATGAAGGTAGACAACGTGAAGG
E	GTGTTCTGATTTGGGTTGTCTACCT	CGAGTTGAATGGCAGAGCGCA
Universal Decorin	GGCTTCTTATTCGGGTGTGA	TCCGAGTTGAATGGCAGAGC
COL1A1	CGAAGACATCCCACCAATCAC	TCATCGCACAACACCTTGC
GAPDH	TCCTGTTCGACAGTCAGCCGC	CCAGGCGCCCAATACGACCA

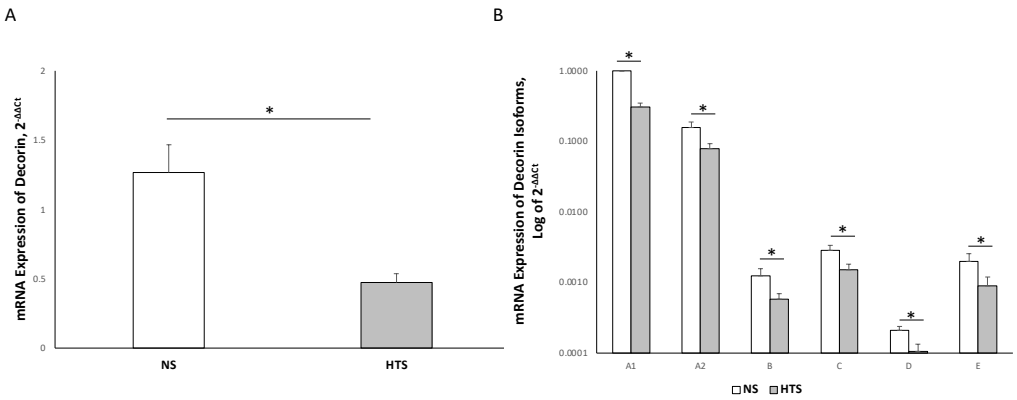


Figure 2-1 Expression of decorin and decorin isoforms in HTS and site-matched NS fibroblasts.

Total RNA was extracted from HTS and site-matched NS fibroblasts. cDNA was synthesized from 2 μg of total RNA, and the mRNA expression of targets were measured using RT-qPCR. Five different pairs of HTS and site-matched NS fibroblasts from separate primary patient cell lines were used. **A)** Decorin mRNA expression is displayed as the mean fold value ($2^{-\Delta\Delta Ct}$) ± SE. **B)** mRNA expression of isoforms A1, A2, B, C, D, and E are displayed as a mean fold value relative to isoform A1 (*$p \leq 0.05$, n=5).

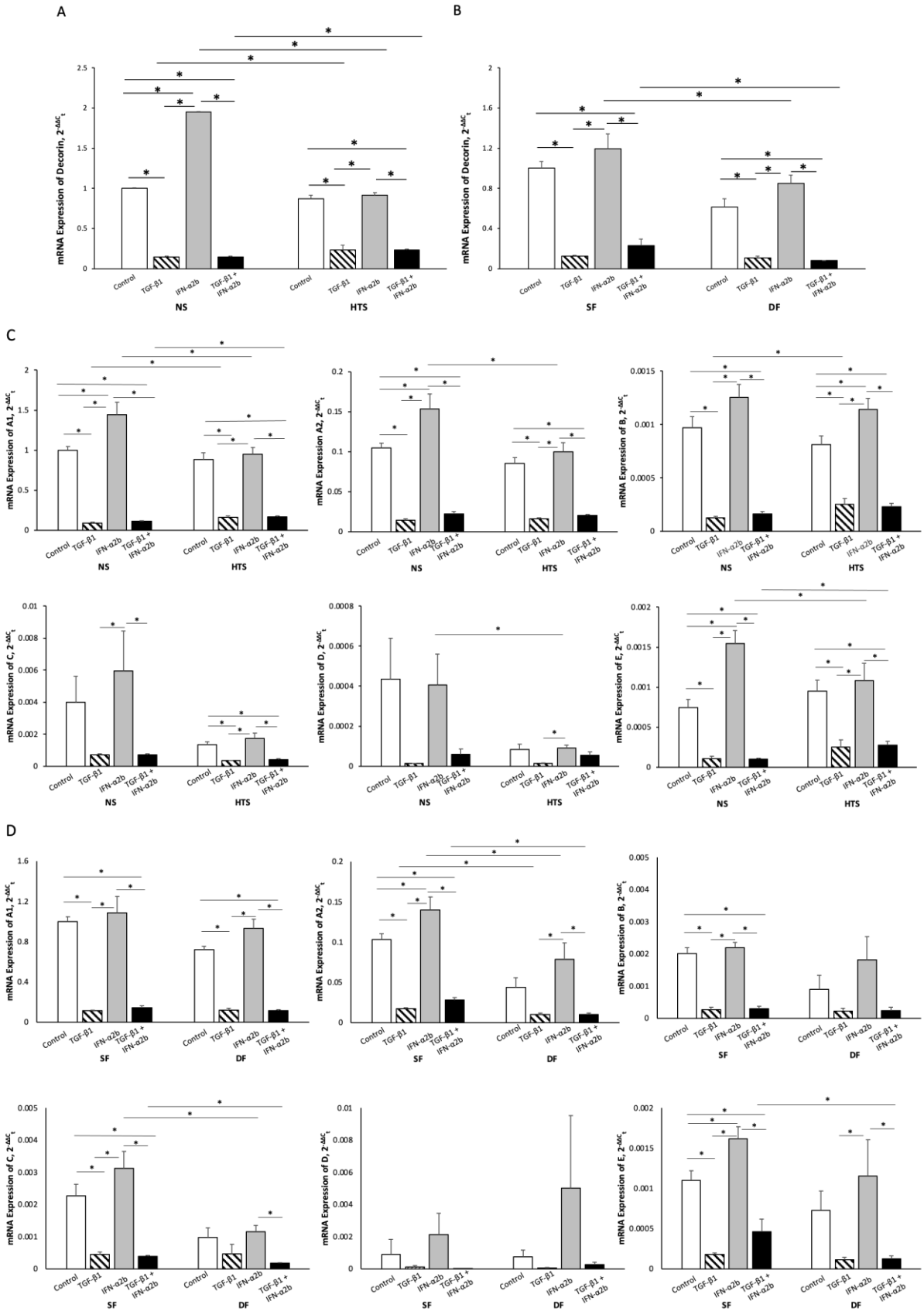

A
B
C
D
mRNA Expression of Decorin, 2-ΔΔCt
mRNA Expression of A1, 2-ΔΔCt
mRNA Expression of A2, 2-ΔΔCt
mRNA Expression of B, 2-ΔΔCt
mRNA Expression of C, 2-ΔΔCt
mRNA Expression of D, 2-ΔΔCt
mRNA Expression of E, 2-ΔΔCt
Control
TGF-β1
IFN-α2b
TGF-β1 + IFN-α2b
NS
HTS
SF
DF

Figure 2-2 Effects of TGF-β1 and/or IFN-α2b on the mRNA expression of decorin and decorin isoforms in HTS and site-matched NS fibroblasts and in matched SF and DF.

Total RNA was extracted from all fibroblast populations. cDNA was synthesized from 2 μg of total RNA, and the mRNA expression of targets were measured using RT-qPCR. Fibroblasts were grown from one pair of HTS and site-matched NS fibroblasts and one pair of matched SF and DF and cultured as three independent samples for experimentation. **A)** Decorin mRNA expression was measured in HTS and site-matched NS fibroblasts and **B)** in SF and DF following treatments. Results are displayed as the mean fold value ($2^{-\Delta\Delta Ct}$) ± SE relative to the respective NS or SF PBS control group. **C)** Decorin isoform mRNA expression (A1, A2, B, C, D, and E) in HTS and site-matched NS fibroblasts and **D)** in matched SF and DF following treatments. Results are displayed as the mean fold value ($2^{-\Delta\Delta Ct}$) ± SE relative to the respective NS or SF PBS control of isoform A1 (*$p \leq 0.05$, n=3).

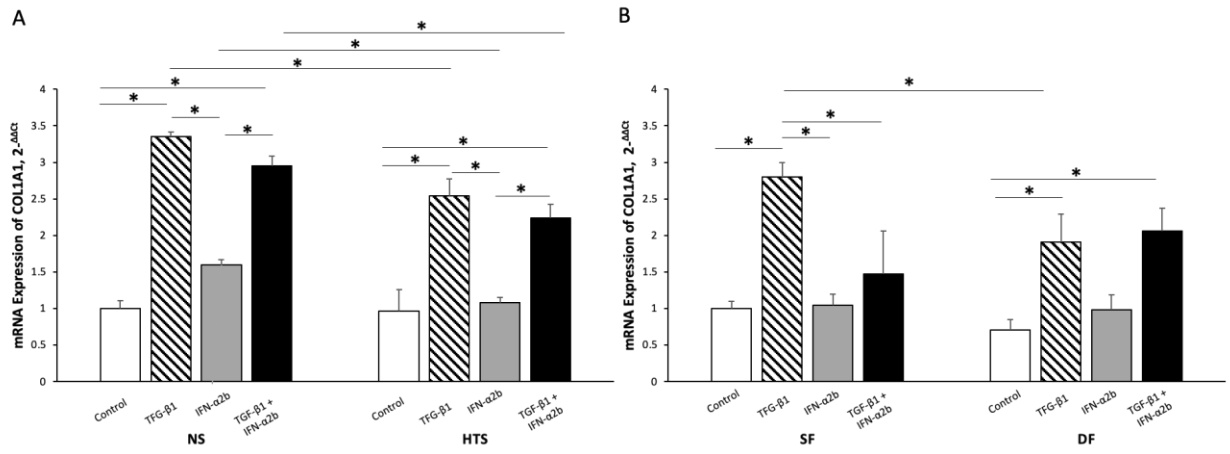
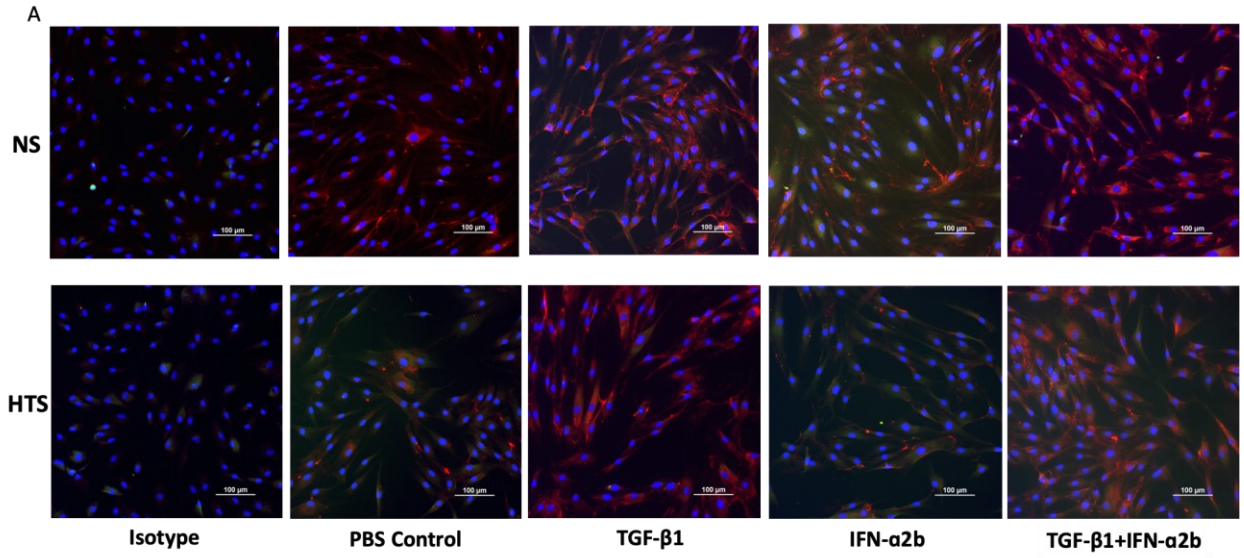
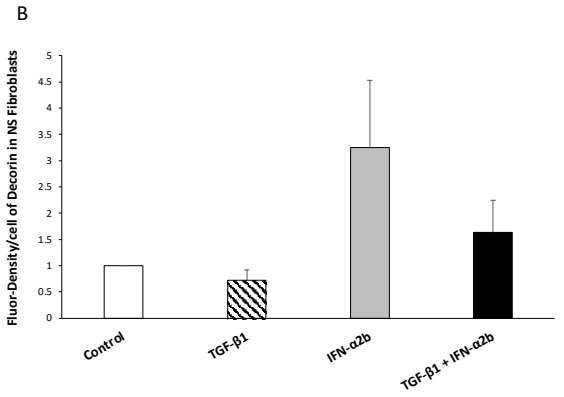


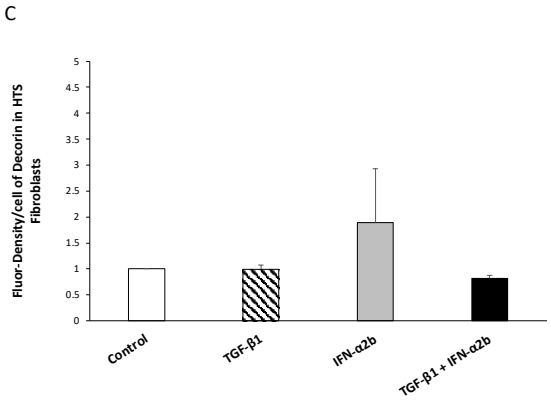
Figure 2-3 COL1A1 mRNA expression in HTS and site-matched NS fibroblasts and in matched SF and DF treated with TGF-β1 and/or IFN-α2b.

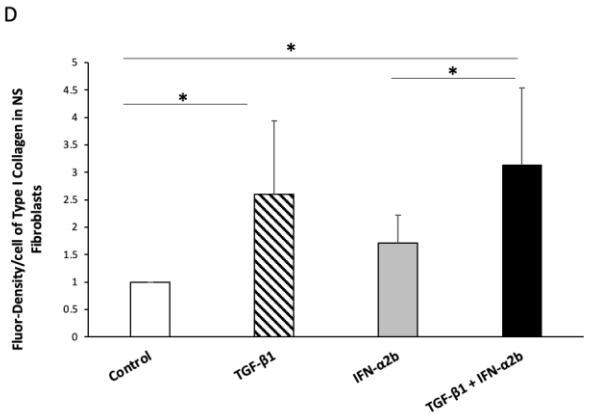
Total RNA was extracted from all fibroblast populations and cDNA was synthesized from 2 μg of total RNA. COL1A1 mRNA expression in targets was measured by RT-qPCR. Fibroblasts were grown from one pair of HTS and site-matched NS fibroblasts and one pair of matched SF

and DF and cultured as three independent samples for experimentation. Results are displayed as the mean fold value ($2^{-\Delta\Delta Ct}$) ± SE relative to the respective NS or SF PBS control group. **A)** COL1A1 mRNA expression in HTS and site-matched NS fibroblasts and **B)** COL1A1 mRNA expression in matched SF and DF following treatments (*$p \leq 0.05$, n=3).









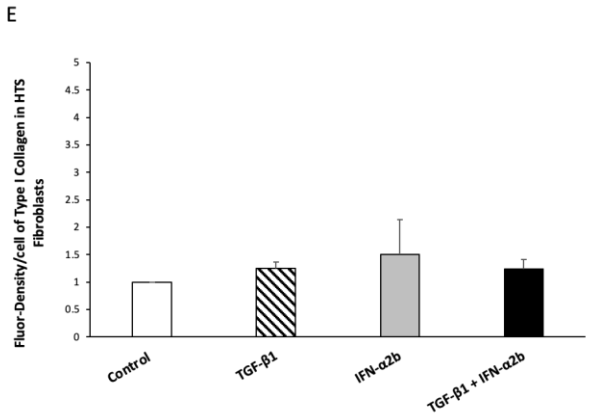


Figure 2-4 IF staining and relative fluorescent density/cell of decorin and type 1 collagen in HTS and site-matched NS fibroblasts treated with TGF-β1 and/or IFN-α2b.

HTS and site-matched NS fibroblasts were sequentially IF stained for decorin and type I collagen. Three different pairs of HTS and site-matched NS fibroblasts from separate primary patient cell lines were used. Results are displayed as the relative mean fluorescence/cell respective to the NS or HTS PBS control group ± SE **A)** Representative IF images of NS (top) and HTS (bottom) fibroblasts from one patient stained for type I collagen (red), decorin (green), and nuclei (blue) (Scale bar is 100 μm, 20X). **B)** Fluorescent density/cell of decorin in NS fibroblasts and **C)** HTS fibroblasts following treatments. **D)** Fluorescent density/cell of type I collagen in NS fibroblasts and **E)** HTS fibroblasts following treatments (*$p \leq 0.05$, n=3).

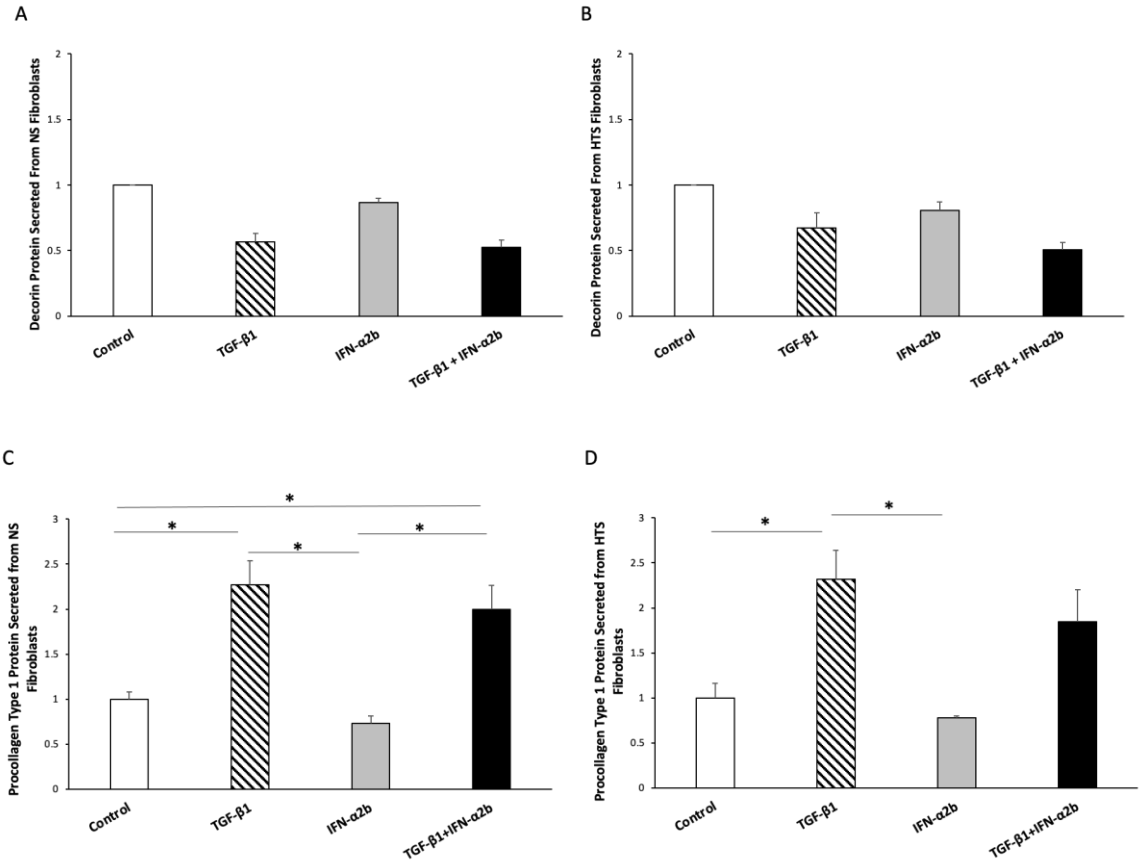


Figure 2-5 Decorin and procollagen type 1 secreted from HTS and site-matched NS fibroblasts treated with TGF-β1 and/or IFN-α2b.

Total cell culture media (1 mL) was collected and centrifuged. Decorin and procollagen type 1 protein secreted from HTS and site-matched NS fibroblasts after treatment with TGF-β1 and/or IFN-α2b were measured by ELISA. Three different pairs of HTS and site-matched NS fibroblasts from separate primary patient cell lines were used. Results are displayed as relative values to the respective NS or HTS PBS control group ± SE. **A)** Decorin secreted from NS fibroblasts. **B)** Decorin secreted from HTS fibroblasts. **C)** Procollagen type 1 secreted from NS fibroblasts. **D)** Procollagen type 1 secreted from HTS fibroblasts (*$p \leq 0.05$, n=3).

2.6 References

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Chapter 3 Conclusions and Future Directions

In this study, the mRNA expression of decorin and its isoforms were examined via RT-qPCR in site-matched NS and HTS fibroblasts. The effects of TGF-β1 and IFN-α2b on decorin, decorin isoforms, and type 1 collagen expression were also examined in site-matched NS and HTS fibroblasts and in matched SF and DF.

Based on our hypotheses, we anticipated that the mRNA expression of decorin and decorin isoforms would be decreased in HTS fibroblasts relative to NS fibroblasts. We also expected that treatment with TGF-β1 would decrease the expression of decorin and decorin isoforms but increase type I collagen expression. Whereas treatment with IFN-α2b would increase the expression of decorin and decorin isoforms but decrease type 1 collagen expression and inhibit the effects of TGF-β1.

For the first time, we show that the mRNA expression of each decorin isoform (A1, A2, B, C, D, and E) was different in NS and HTS fibroblasts, and that the expression of each was significantly decreased in HTS fibroblasts relative to NS fibroblasts. Interestingly, TGF-β1 and IFN-α2b displayed variable effectiveness on either significantly decreasing or increasing the mRNA expression of the isoforms, respectively. This preliminary evidence may indicate that these isoforms harbor unique structural and functional differences that cause their varying responses to the effects of TGF-β1 and IFN-α2b and warrant further investigation. It would also be of great interest and benefit for future studies to conduct single cell or long read RNA sequencing on the different fibroblast populations examined and compare this to the RT-qPCR data obtained for the isoforms.

We show that TGF-β1 is pro-fibrotic, corroborating existing literature, as it relatively decreased the expression of decorin and decorin isoforms and increased the expression and

secretion of procollagen type 1. The fact that TGF-β1 did not influence type I collagen expression quantified by IF staining in HTS fibroblasts is likely due to the large variability present between the small numbers of different samples. IFN-α2b showed relative anti-fibrotic effects by increasing the expression of decorin and decorin isoforms, but it had no effect on the secretion of decorin. This could be due in part to the biological variability of the different samples, or it could be due to IFN-α2b having its effect at the promotor of the transcript. The relationship between how IFN-α2b regulates decorin or decorin isoform expression is unknown, so researching this interaction would be crucial for understanding the mechanism by which it influences decorin expression and why there is minimal effect on decorin protein secreted.

In some instances, IFN-α2b did show a trend of mitigating TGF-β1's pro-fibrotic effects. Specifically, it was more effective at mitigating the increased expression of type 1 collagen than it was at restoring or increasing the reduced expression of decorin or decorin isoforms. In previous research, 2000 U/mL of IFN-α2b was shown to be effective at reducing type I collagen expression after 48 hours (1). However, a higher dosage for a longer duration may be required to significantly counteract the effects of exogenous TGF-β1 on type I collagen, decorin, and decorin isoform expression.

IFN-α2b significantly inhibited TGF-β1's effect of increasing the expression of type 1 collagen in SF only. It is possible that the basal levels of TGF-β1 were elevated in DF and in NS and HTS fibroblasts because of the fibrotic nature of the environments they were obtained from. NS fibroblasts are obtained from patients who sustained burn injury and presented with HTS, as they are a site-matched control, making it possible that the basal levels of TGF-β1 were elevated above those in SF. An elevated basal level of TGF-β1, coupled with the treatment dosage, could account for why we did not observe IFN-α2b significantly counteracting its effects. Measuring

the mRNA and protein expression or secretion of TGF-β1 both before and after treatment would determine if the basal levels of TGF-β1 are significant and need to be accounted for. These measurements could be done via RT-qPCR, a western blot, and an ELISA. Furthermore, IFN-α2b influences fibroblasts in a time and concentration dependent manner and shows variability in its effectiveness depending on which specific mRNA transcript or protein is being measured (2–6). Therefore, it would be beneficial to conduct a dose dependency experiment for variable durations to further determine IFN-α2b's effect on decorin mRNA expression.

The mRNA expression and the protein expression of macromolecules are not necessarily equivalent, so a significant limitation of this work is that the expression of decorin isoforms were only measured via RT-qPCR. There are currently no commercially available antibodies specific for each decorin isoform, making analysis at this point difficult. Therefore, creating specific antibodies for protein analysis and quantification would be a valuable future direction. Furthermore, the use of either one or three primary cell lines, coupled with the biological variability between different samples, could account for the inconsistencies in significance observed between the mRNA and protein expression/secretion data. Further experiments with more samples will need to be conducted to make any significant observations or correlations between the mRNA and protein data (**Appendix Figure A-3**).

The amino acid sequences located between leucine rich repeats III and IV of decorin bind to type I collagen (7,8) and the leucine-155 valine-260 sequence in the core of decorin is the specific binding site for TGF-β1 (9). When exons are spliced to create decorin isoforms, it could ultimately change the structure of the protein and the interactions that they have with type 1 collagen and TGF-β1. Therefore, determining the protein structure of each isoform would be valuable. Furthermore, quantifying the affinity each isoform has for type I and III collagen fibrils

and TGF-β1 could elucidate if they have differential roles in matrix alignment and fibrillogenesis, and ultimately show which isoforms are most effective in minimizing the formation of HTS.

There is limited research on the interaction between IFN-α2b and TGF-β1 in fibrotic environments. It was recently reported that treatment with IFN-α2b reduced fibroblast proliferation and epidural fibrosis in rats by inhibiting the TGF-β/Smad signaling pathway (6). Further understanding of whether IFN-α2b is influencing the Smad 2/3 signaling pathway or TGF-β1 at its promotor region would help explain why our experiments did not show IFN-α2b significantly counteracting TGF-β1 in most instances. It would also give greater insight into how IFN-α2b specifically acts as a therapeutic agent for fibrotic conditions.

This research demonstrates that decorin isoforms are differentially expressed in NS and HTS fibroblasts and that the expression of each isoform is reduced in HTS fibroblasts, warranting further investigation into their roles in hypertrophic scarring. Additionally, we corroborated previous research that TGF-β1 is pro-fibrotic and IFN-α2b is anti-fibrotic and have shown that the duration and concentration of IFN-α2b treatment is extremely important and basal levels of TGF-β1 may need to be accounted for.

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Appendices

Appendix 1. To test the dose of TGF-β1 used in subsequent experiments, HTS and site-matched NS fibroblasts and matched SF and DF were grown to 80% confluence in DMEM supplemented with 10% FBS and 1% antibiotics in a 75cm^2 flask. Fibroblasts were then equally distributed into a 6-well plate with 1 mL of DMEM with 10% FBS and 1% antibiotics. Following attachment and 80% confluency, DMEM with 10% FBS was changed for DMEM with 2% FBS and the fibroblasts were treated with either 10 ng/mL or 20 ng/mL of recombinant human TGF-β1 (R&D systems, Minneapolis, MN) for 24 hours. Fibroblasts were lysed and collected with 1 mL of TRIzol reagent (Invitrogen, Waltham, MA) and frozen at -20°C. When NS and HTS fibroblasts were treated with either 10 ng/mL or 20 ng/mL of TGF-β1, we observed that the expression of A1 decreased in NS and HTS fibroblasts with both treatment doses (**Figure A-1 A and B**). There was also a significant decrease in the expression of A2 in NS fibroblasts that were treated with 20 ng/mL of TGF-β1. TGF-β1 had a more profound effect in SF and DF. Treatment with either 10 ng/mL and/or 20 ng/mL of TGF-β1 significantly decreased the expression of all isoforms in both SF and DF, apart from isoform D in DF (**Figure A-1 C and D**). For all further experiments we used 10 ng/mL of TGF-β1.

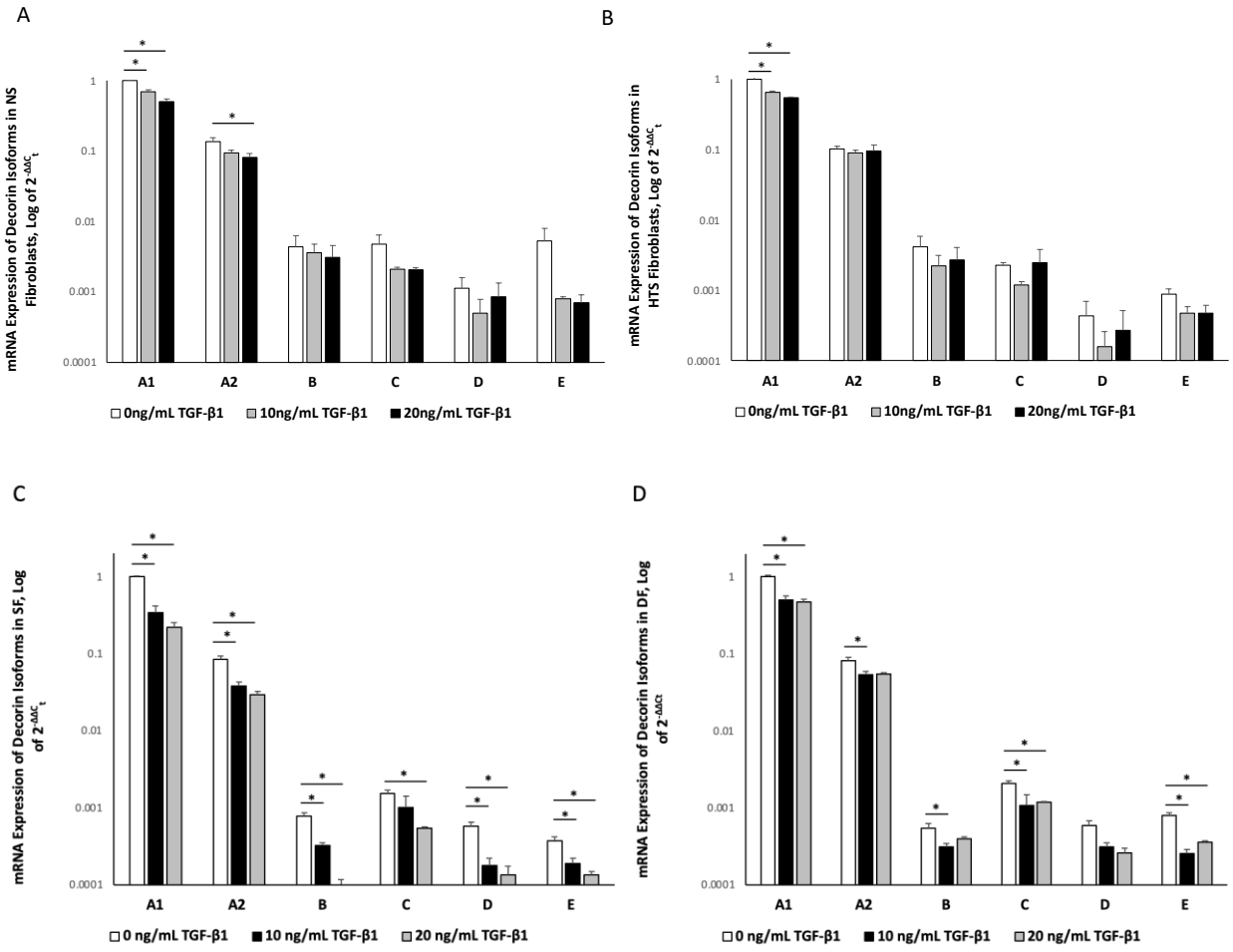


Figure A-1 Dose dependent effects of TGF-β1 on decorin isoform expression in HTS and site-matched NS fibroblasts and in matched SF and DF.

HTS and site-matched NS fibroblasts and matched SF and DF were treated with either 10 ng/mL or 20 ng/mL of TGF-β1 and total RNA was extracted. cDNA was synthesized from 2 µg of total RNA, and the mRNA expression of decorin isoforms were measured using RT-qPCR. Three pairs of HTS and site-matched NS fibroblasts from separate patients were used. mRNA expression is displayed as the mean fold value ($2^{-\Delta\Delta Ct}$) ± SE relative to isoform A1 in the respective 0 ng/mL TGF-β1 treatment group. **A)** TGF-β1 treatments in NS fibroblasts. **B)** TGF-β1 treatments in HTS fibroblasts. **C)** TGF-β1 treatments in SF. **D)** TGF-β1 treatments in DF (*$p \leq 0.05$, n=3).

Appendix 2. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), Beta-2-microglobulin (B2M) and ribosomal protein L13a (RPL13A) were all tested and compared for their use as a possible reference gene. The C_t method was conducted using the formula $2^{-\Delta\Delta Ct}$. Using RT-qPCR, we observed that there were no statistically significant differences in the expression of decorin when normalized to either GAPDH, B2M, or RPL13A in NS fibroblasts or in HTS fibroblasts (**Figure A-2**). GAPDH was used for all further experimentation.

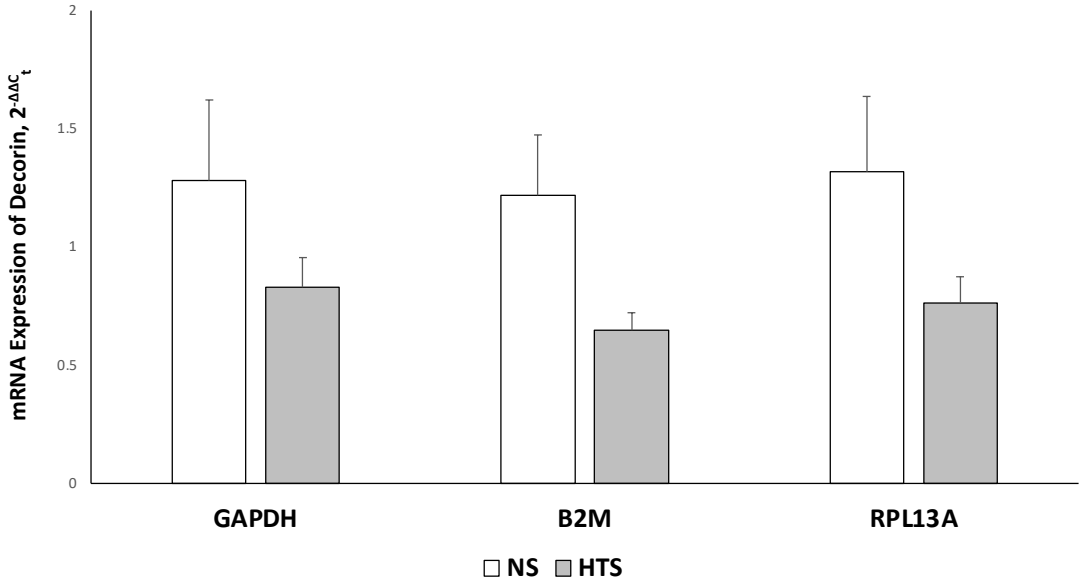
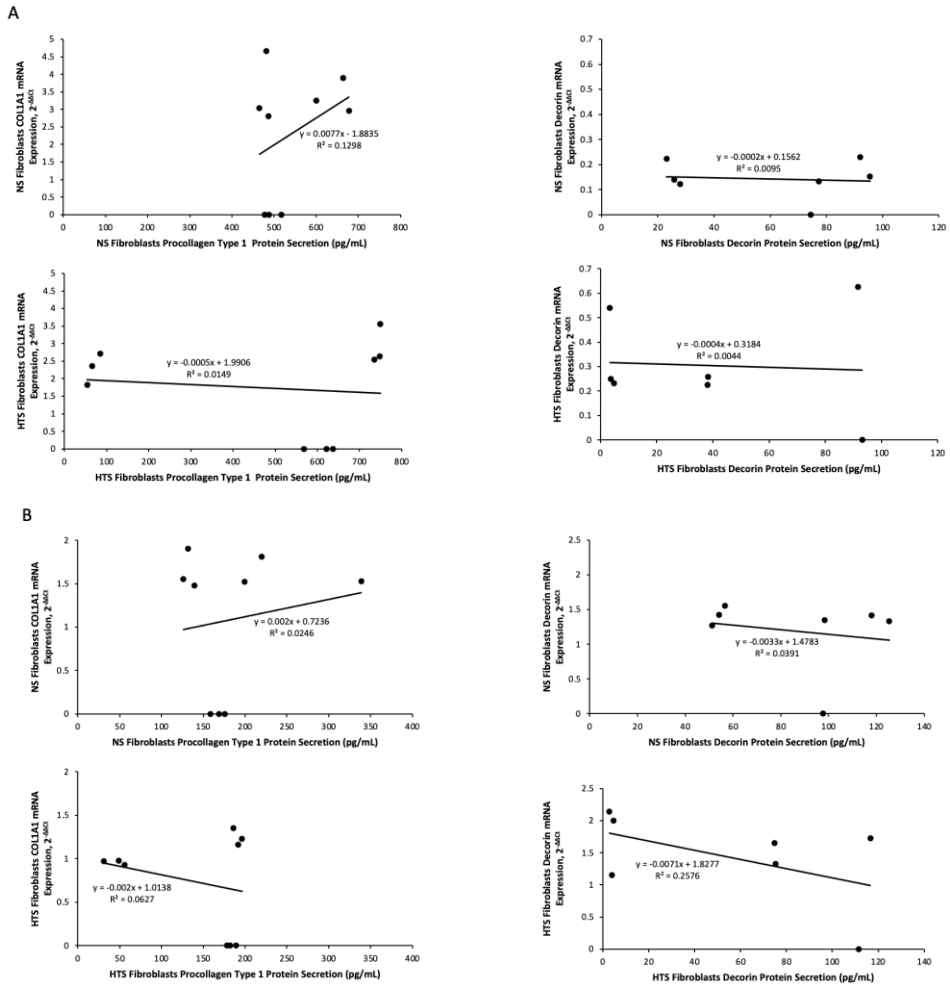


Figure A-2 mRNA expression of decorin in HTS and site-matched NS fibroblasts normalized to three housekeeping genes.

Total RNA was extracted from HTS and site-matched NS fibroblasts. cDNA was synthesized from 2 μg of total RNA, and the mRNA expression of decorin was measured by RT-qPCR. Five pairs of HTS and site-matched NS fibroblasts from separate patients were used. Comparison to the housekeeping genes GAPDH, B2M, or RPL13A were measured using the C_t method. Decorin mRNA expression is displayed as the mean fold value ($2^{-\Delta\Delta Ct}$) ± SE ($p \geq 0.05$, n=5).

Appendix 3. Correlations were analyzed between the mRNA expression and protein secretion of decorin and type 1 collagen in HTS and site-matched NS fibroblasts after TGF-β1 and/or IFN-α2b treatment. Decorin and COL1A1 mRNA expression were measured using RT-qPCR and the C_t method was conducted using the formula $2^{-\Delta\Delta Ct}$. Decorin and procollagen type 1 protein secreted from HTS and site-matched NS fibroblasts were measured with ELISA. There were no significant correlations between the mRNA expression and protein secretion of decorin in NS or HTS fibroblasts following treatment (**Figure A-3 A-D**). There were also no significant correlations between the mRNA expression of COL1A1 and protein secretion of procollagen type 1 in NS or HTS fibroblasts following treatment (**Figure A-3 A-D**).



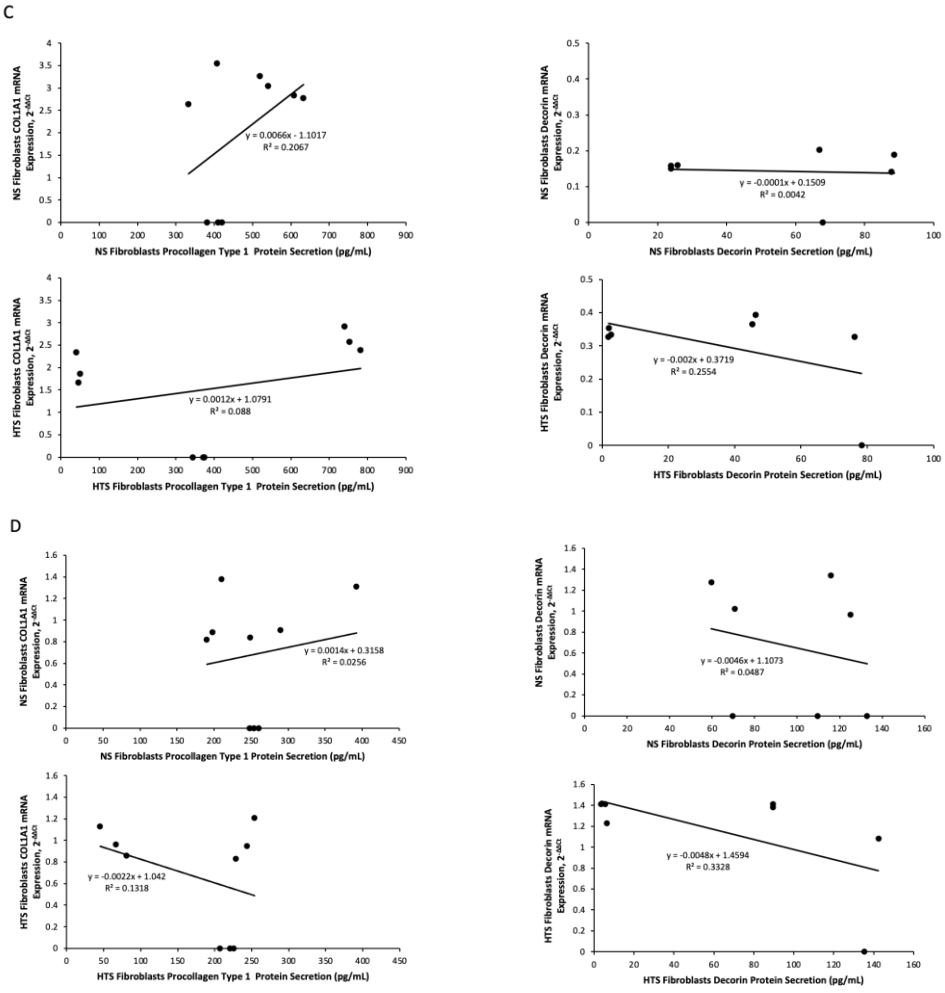


Figure A-3 Correlation between the mRNA expression and protein secretion of decorin or 1 collagen in HTS and site-matched NS fibroblasts treated with TGF-β1 and/or IFN-α2b.

Total RNA was extracted from HTS and site-matched NS fibroblasts. cDNA was synthesized from 2 μg of total RNA, and the mRNA expression of decorin and COL1A1 were measured by RT-qPCR. Decorin and COL1A1 mRNA expression are displayed as mean fold values ($2^{-\Delta\Delta Ct}$). Total cell culture media (1 mL) was collected and centrifuged. Decorin and procollagen type 1 protein secreted from HTS and site-matched NS fibroblasts were measured by ELISA. Decorin and procollagen type 1 protein secreted are displayed as the concentration present in the cell culture media (pg/mL). **A)** Correlations between the mRNA expression of decorin or COL1A1

and the protein secretion of decorin or procollagen type 1, respectively, in NS and HTS fibroblasts following TGF-β1 treatment **B)** IFN-α2b treatment **C)** TGF-β1 and IFN-α2b combined treatment and **D)** PBS control treatment.