ROLES OF β-CATENIN IN DERMAL FIBROSIS

by

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LIST OF ABBREVIATIONS

ADAM12  Disintegrin and metalloproteinase domain-containing protein 12
APC    Adenomatous polyposis coli
CBP    CREB-binding protein
CK1-α  Casein kinase 1-α
CTGF   Connective tissue growth factor (CCN2)
Dkk    Dickkopf
Dlk1   Delta-like homologue 1
Dvl    Dishevelled
E      embryonic (day)
ECM    extracellular matrix
EMT    epithelial-to-mesenchymal transition
EndMT  endothelial-to-mesenchymal transition
FACS   fluorescence-activated cell sorting
FAK    Focal adhesion kinase
FGF    Fibroblast growth factor
Fz     Frizzled
GSK3-β Glycogen synthase kinase 3-β
GVHD   graft-versus-host disease
IGF    Insulin-like growth factor
IPF    idiopathic pulmonary fibrosis
Lef    Lymphoid enhancer factor
LOXL2  Lysyl oxidase-like 2
MEF    mouse embryonic fibroblast
Nov    Nephroblastoma over-expressed (CCN3)
NSF    nephrogenic system fibrosis
<table>
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<tr>
<td>P</td>
<td>postnatal (day)</td>
</tr>
<tr>
<td>PDGF(R)</td>
<td>Platelet derived growth factor (receptor)</td>
</tr>
<tr>
<td>rtTA</td>
<td>reverse tetracycline-regulated trans-activator</td>
</tr>
<tr>
<td>SFRP</td>
<td>Secreted frizzled-related protein</td>
</tr>
<tr>
<td>SSc</td>
<td>systemic sclerosis</td>
</tr>
<tr>
<td>α-SMA</td>
<td>α-smooth muscle actin</td>
</tr>
<tr>
<td>Tcf</td>
<td>T cell factor</td>
</tr>
<tr>
<td>TEM</td>
<td>transmission electron microscopy</td>
</tr>
<tr>
<td>TFBS</td>
<td>transcription factor binding site</td>
</tr>
<tr>
<td>TGF-β(R)</td>
<td>Transforming growth factor-β (receptor)</td>
</tr>
<tr>
<td>TKI</td>
<td>tyrosine kinase inhibitors</td>
</tr>
<tr>
<td>YFP</td>
<td>Yellow fluorescent protein</td>
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<td>WIF</td>
<td>Wnt inhibitory factor</td>
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Roles of β-Catenin in Dermal Fibrosis

ABSTRACT

by

EMILY J. HAMBURG-SHIELDS

Fibrosis is an end-stage response to tissue injury in which excess extracellular matrix production by fibroblasts results in loss of organ function. In skin, pathologic fibrosis is associated with keloid scar formation and scleroderma. Dermal fibroblasts in fibrotic skin exhibit increased activity of Wnt/β-catenin signaling; however, the precise role of dermal fibroblast β-catenin activity in fibrosis is not known. I have found that sustained expression of stabilized β-catenin in mouse dermal fibroblasts is sufficient for dermal fibrosis in vivo. The consequences of stabilized β-catenin expression in dermal fibroblasts include increased fibroblast proliferation, abundant collagen with abnormal fibril morphology, and elevated expression of matrix-encoding genes. Therefore, I have identified mediators of the pro-fibrotic effects of stabilized β-catenin and uncovered a matrix-regulatory role for fibroblast β-catenin activity. These findings support future investigation of β-catenin and its mediators as therapeutic targets for treatment of fibrotic skin disease.
1. INTRODUCTION*

1.1. Principles of fibrogenesis

When an organ is injured-- for example burning of the skin, ischemia of the heart, or poisoning of the liver or kidney-- a reactionary healing process aims to repair the wounded tissue. Unfortunately, the ability of adult human tissue to regenerate is limited, and the injured tissue is often replaced by scar tissue (Gurtner et al., 2008; Xin et al., 2013). Scar tissue, or fibrosis, maintains the structural integrity of the injured organ, but the function of the scarred organ is compromised (Wynn, 2008; 2007). Therapies to achieve reversal or resolution of scar tissue are extremely limited (Leask, 2012; Xin et al., 2013). Therefore, in the visceral organs, fibrosis is considered an end-stage pathological process: it is the final consequence of the healing process in these tissues, and is the harbinger of ultimately fatal loss of organ function.

Since fibrosis corresponds with loss of tissue function, it is highly desirable to attenuate or reverse fibrosis in order to preserve or restore organ function (Xin et al., 2013; Yates et al., 2013). Fibrosis can occur in virtually any visceral organ as well as in the skin (Friedman et al., 2013; Varga and Abraham, 2007). In addition, both benign and malignant tumor cells are associated with fibrous stroma (Kalluri

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* Portions of this chapter have been reproduced and slightly modified, with permission pending, from a chapter titled “Cutaneous fibrosis and normal wound healing” in Clinical and Basic Immunodermatology, 2nd ed. (Springer).
and Zeisberg, 2006). Therefore, the study of fibrosis has broad relevance for human disease. There are currently no therapies to reverse fibrosis (Leask, 2012). Strategies to slow the progression of fibrosis continue to be tested in clinical trials, particularly for treatment of idiopathic pulmonary fibrosis (Friedman et al., 2013). Better understanding of the common mechanisms of fibrosis are needed to continue developing therapies targeted against this pathologic process.

Fibroblasts are the key effector cell involved in fibrosis; therefore, it is essential to understand the behavior, physiology, and identity of fibroblasts in order to develop anti-fibrotic therapies. These cells are found throughout the body and their primary function is to produce extracellular matrix (ECM), which is the fabric of fibrous, woven proteins that gives strength and substance to tissues and organs (Watt and Fujiwara, 2011). Fibroblast proliferation and activation is one of the hallmark features of fibrosis (Phan, 2008). Another hallmark feature of fibrosis is the accumulation of abnormal ECM, which makes the whole tissue thickened and sclerotic (hardened) (Friedman et al., 2013; Watt and Fujiwara, 2011). Regardless of the particular tissue that has been injured and affected by fibrosis, these characteristic features are visible upon microscopic examination of the affected tissue, and are central to the definition of fibrosis.

Because of the central role of fibroblasts in fibrotic processes, regardless of the affected tissue, it follows that the major questions in fibrosis research are centered upon fibroblast behavior, physiology, and identity (including the heterogeneity of
fibroblasts of distinct embryonic origins and anatomic locations) (Friedman et al., 2013; Phan, 2008). My research has addressed three of these questions:

- *What are the cellular sources of fibroblasts that produce fibrosis?*
- *What are the molecular changes that occur in fibrotic tissues?*
- *Which signaling pathways drive fibrosis?*

### 1.2. Skin fibrosis

My research is focused on fibrosis of the skin (sections 1.2.1 and 1.2.2). Thickening and sclerosis of the skin is disfiguring and can lead to contracture of the skin over the joints, thus limiting mobility (Braverman and Cowper, 2010; Kamino et al., 2012; Shih and Bayat, 2010; Varga and Abraham, 2007). Like fibrosis of other organs, skin fibrosis is an end-stage pathology that occurs in the context of abnormal wound healing as well as systemic diseases (section 1.2.3). Skin fibrosis research has

![Figure 1. Architecture of the skin. Source: http://umm.edu/health/medical/ency/images/skin-layers](http://umm.edu/health/medical/ency/images/skin-layers)
benefited from the availability of multiple animal models and the accessibility of human tissue (section 1.2.4).

1.2.1. Anatomy of the skin

Human and mouse skin share similar architecture and include three layers: the epidermis, dermis, and hypodermis (Figure 1).

The epidermis is comprised of distinct layers of epidermal keratinocytes: the basal layer contains proliferating epidermal keratinocytes and is separated from the dermis by a basement membrane; the spinous and granular layers are made up of epidermal keratinocytes that differentiate and undergo apoptosis; and the outermost layer is the highly keratinized, acellular stratum corneum. The epidermis is also home to melanocytes (pigment cells) and Merkel cells (specialized mechanoreceptors), which reside in the basal layer, and Langerhans cells (antigen presenting dendritic cells) above the basal layer. In addition to these resident cells of the dermis, cells associated with the immune response and inflammation can migrate to the epidermis-- these include eosinophils, dermal macrophages, lymphocytes, and neutrophils (Norris, 2012).

The underlying dermis comprises the bulk of the skin: it is made up of resident dermal fibroblasts and an extensive extracellular matrix, which gives the skin its toughness and strength. The dermis is divided into upper and lower zones-- the papillary and reticular dermis, respectively-- which have contain distinct fibroblast
populations and characteristic extracellular matrix compositions (Driskell et al., 2013; Sorrell and Caplan, 2004). In the dermis reside the adnexal structures of the epidermis: hair follicles, sebaceous glands, and sweat glands. Blood vessels in the dermis supply the epidermal adnexa and the overlying epidermis, and small nerve fibers facilitate sensation and provide sympathetic nervous supply to blood vessels and the epidermal adnexal structures. Macrophages, T cells, dendritic cells, mast cells and plasma B cells also reside in the dermis (Norris, 2012). Beneath the dermis is the hypodermis, which is comprised mostly of adipocytes (white adipose tissue). The hypodermis is also vascularized and contains some fibroblasts. In mice, but not in humans, the hypodermis is associated with a thin layer of muscle called the panniculus carnosus, which separates the intradermal (hair follicle-associated) fat from true subcutaneous fat (Driskell et al., 2014).

1.2.2. How fibrosis affects the skin

As in other fibrotic organs, fibrotic skin experiences accumulation of extracellular matrix. Type I collagen, the predominant type of collagen in the skin, is substantially increased (Abergel et al., 1987; Rockwell et al., 1989). Fibrotic skin is thickened and stiff. There may be more fibroblasts present in the skin due to proliferation, or in the case of advanced fibrosis, the tissue may be hypocellular and mostly composed of matrix (Wynn, 2008). Fibrotic changes in the skin are not limited to the dermis. The epidermis can be affected in some fibrosing disorders, as can the vascular endothelium (section 1.3.3). In addition, fibrosis can extend into the hypodermis,
replacing the subcutaneous adipose tissue (section 1.3.2). Fibrotic skin lesions can be widespread over the body, limited, or focal, depending upon the causative disease process.

1.2.3. Fibrotic skin conditions and fibroproliferative tumors

The following diseases share the common feature of fibrosis, but their etiologies and clinical presentations vary widely. I have focused here on the fibrotic and fibroproliferative conditions that are most closely related to my research: scleroderma, nephrogenic systemic fibrosis, pathologic scar formation, and superficial and deep fibromatoses. Fibrotic skin disorders which are overtly inflammatory, such as eosinophilic fasciitis and the cutaneous manifestations of chronic graft-versus-host disease, are not discussed here.

Scleroderma

Scleroderma is the prototypical fibrotic skin disease. It is a collection of autoimmune disorders in which inflammation is accompanied by vasculopathy and progressive fibrosis of skin and often other organs (Varga and Abraham, 2007). The involvement of visceral organs and the extent of fibrosis occur in characteristic patterns lead to specific clinical diagnoses of:

- Localized scleroderma, which includes morphea (plaque-type lesions) and linear scleroderma (linear lesions); both only involve skin.
• Systemic sclerosis (SSc), in which cutaneous fibrosis can be limited (mostly involving the distal extremities and face) or diffuse (involving proximal extremities and trunk). SSc patients can also have fibrosis affecting the lungs, gastrointestinal tract, and kidneys.

• CREST syndrome, a subtype of limited SSc with characteristic features of calcinosis, Raynaud’s phenomenon, esophageal dysmotility, sclerodactyly, and telangiectasia.

Raynaud’s phenomenon, which is strongly associated with scleroderma (not only CREST syndrome), is caused by which occlusion of dermal microvessels. This results in extreme paleness of the fingertips in response to cold. Raynaud’s phenomenon is often observed before or soon after the diagnosis of scleroderma, and this association has led to the hypothesis that scleroderma is primarily a vascular disease (Fleming et al., 2009). However, how vascular abnormality might result in widespread dermal or systemic fibrosis is not understood. The pathogenic role of the various autoantibodies (including anti-nuclear antibodies and others) that are present in scleroderma patients is also not known (Sakkas et al., 2006).

Nephrogenic systemic fibrosis

Skin fibrosis is also associated with nephrogenic system fibrosis (NSF), which is caused by administration of gadolinium-containing contrast agent to patients with poor renal function. This is associated with gadolinium deposits in the skin and other affected organs (Braverman and Cowper, 2010; Cowper and Bucala, 2003), although exactly how this results in systemic fibrosis is not known. Identification of
the inciting agent for NSF has resulted in safety measures to prevent gadolinium exposure in susceptible patients, and so the incidence of NSF has dropped dramatically (Braverman and Cowper, 2010; Perez-Rodriguez et al., 2009).

*Wound healing and scar formation*

Fibrosis additionally occurs as a normal part of skin wound healing. Following the early inflammatory phase of wound healing, in the days and weeks following the initial injury, granulation tissue forms in the wound bed. Cells in the local inflammatory infiltrate produce growth factors that promote fibroproliferation and migration of fibroblasts, including myofibroblasts, from adjacent un-injured tissue into the wound (Eming, 2012; Gabbiani, 2003; Tomasek et al., 2002). Later, in the weeks to months following injury, the granulation tissue is replaced by mature scar tissue; this is considered a physiologic fibrotic process. Inflammation resolves and the myofibroblasts undergo apoptosis (Tomasek et al., 2002). The mature scar tissue is more collagenous than the preceding granulation tissue, better approximating the dermal extracellular matrix (Eming, 2012). However, mechanical and functional properties of the skin are not wholly restored to its original form (Corr and Hart, 2013).

Pathologic fibrosis during wound healing results from an exuberant response to the initial injury, and occurs during formation of keloid scars or hypertrophic scars. These are benign overgrown scars that are confined to the original wound borders (in the case of hypertrophic scars) or extend beyond the wound border (keloids).
Excessive scar formation is the most common fibrotic skin disorder. Keloid scars occur predominantly in darkly pigmented skin, and have an incidence of between 4.5% and 16% in black and Hispanic populations (Romanelli et al., 2012). A genetic predisposition to keloid formation is suspected, but the cause is not known.

**Superficial and deep fibromatoses**

Superficial and deep fibromatoses are fibroproliferative disorders often associated with distinct clinical syndromes. In superficial fibromatosis, benign fibroproliferation classically affects the palmar fascia of the hands (Dupuytren contracture), plantar fascia of the feet (Ledderhose disease), tunica albuginea of the penis (Peyronie disease), or the shoulder joint capsule (frozen shoulder syndrome; adhesive capsulitis) (Bowley et al., 2007). Superficial fibromatoses do not affect the skin, but rather the fascia lying beneath the hypodermis. Like other fibrotic conditions, they involve proliferation and activation of fibroblasts and excess deposition of collagen.

Deep fibromatoses, also called aggressive fibromatoses or desmoid tumors, are benign neoplastic tumors that are highly proliferative and invasive, although not metastatic (Bowley et al., 2007). They are comprised of monoclonal proliferations of fibroblastic cells (Alman et al., 1997b). Desmoid tumor formation in a mouse model correlates with mesenchymal stem cells (MSCs) as measured by the quantity of colony-forming units cultured from bone marrow extracts. In addition, human desmoid tumors exhibit characteristic cell surface markers and a gene expression
profile consistent with that of human MSCs (Wu et al., 2010). Together these data suggest that MSCs contribute to desmoid tumor formation, as a fibroblast progenitor and/or as a supportive cell during tumor formation. Definitive transplantation or lineage-tracing studies have not yet been performed and are required to demonstrate the cellular source of desmoid tumor fibroblasts in mice (Wu et al., 2010). Desmoid tumors do not characteristically involve the skin, but rather the muscular aponeurosis, and can occur anywhere in the body (Kamino et al., 2012). In a subset of patients, desmoid tumors are associated with familial adenomatous polyposis syndrome, in which an inactivating mutation in the APC gene causes early-onset colon cancer (Li et al., 2010). The important role of APC in the Wnt/β-catenin signaling pathway will be discussed in section 1.6.1, and the relationship of Wnt/β-catenin signaling to fibromatoses is detailed in section 1.6.4.

1.2.4. Experimental models of skin fibrosis

Fibrosis is studied in a variety of experimental systems which are based on tissues or cells from humans or mice. Each system has advantages and drawbacks which are summarized here. My research has relied on skin biopsies from patients with fibrotic diseases and a transgenic mouse model. Additionally, I will discuss others’ related findings from experiments using cultured fibroblasts from both humans and mice; the bleomycin-induced model of skin fibrosis; tight-skin mice; and multiple transgenic mice. Two additional animal models of skin fibrosis-- the cutaneous graft-versus-host disease (GVHD) murine model (Choi et al., 1987) and the avian
model of scleroderma (UCD200/206 chicken) (Gershwin et al., 1981) have yielded insight into the roles of inflammation and the vascular endothelium in fibrosis, but are not commonly used in contemporary experiments and so are not discussed in detail here.

Patient skin biopsies

Skin biopsies from patients with fibrotic diseases, as well as excised keloid scars, are used for microarray profiling as previously described, as well as immunohistochemical and histological studies. The advantage of skin biopsies is that they enable direct observation of cellular involvement, gene and protein expression, and possibly signaling pathway activity at a particular point in time. However, these tissues poses many challenges. The anatomic location of the biopsy is rarely constant across patients, and this variation may affect gene expression (Chang et al., 2002). There is also variation in the duration of disease and in previous and current treatments, which may affect the observations being made. And although these tissues are relatively easy to obtain in comparison to samples from visceral fibrotic organs, they are not in unlimited supply, especially for rare diseases like scleroderma and NSF. They are useful for direct observation of events or activities that can then be experimentally tested in another model.

Cultured dermal fibroblasts

Live dermal fibroblasts can be extracted from skin biopsies of patients and healthy humans, then maintained in tissue culture for multiple passages and/or established
as cell lines. Alternatively and more readily available, human foreskin fibroblasts can also be used. Mouse dermal fibroblasts are not as widely used in tissue culture-based experiments; mouse embryonic fibroblasts (MEFs) are more common. Regardless of the source, cultured dermal fibroblasts are amenable to manipulation of gene expression, signaling pathway activity, and extracellular environment. It is also relatively easy to generate many replicates, unlike experiments using animal models. However, culture conditions (including environmental conditions and mechanical tension of the substrate) can substantially affect fibroblast behavior and gene expression compared to their in vivo counterparts, and so it is important to confirm cell culture-based findings in animal models (Gardner et al., 2006; Olsen et al., 2011). Researchers are beginning to adopts methods for fibroblast culture that better mimic the in vivo environment (Booth et al., 2012; Hubmacher and Apte, 2013; Trappmann et al., 2012). Another issue with in vitro experiments is that they use heterogeneous populations of fibroblasts that are not well-characterized or defined. Fibroblast extraction protocols are typically based on enzymatic and mechanical digestion of tissue, followed by adhesion of cells to a substrate. This process selects for fibroblasts but does not ensure inclusion of all fibroblasts, and

![Figure 2](image-url)

**Figure 2.** Bleomycin-induced skin fibrosis. H&E stains skin injected with (A) PBS for four weeks; (B) 1mg/mL doxycycline for 2 weeks; (C) 1 mg/mL doxycycline for 4 weeks. Adapted by permission from Macmillan Publishers Ltd: *Journal of Investigative Dermatology* (Yamamoto et al., 1999), copyright 1999.
may include non-fibroblastic cells. Specific culture conditions can be applied to enrich the cultured cells for fibroblasts. Cell morphology is used to confirm that the cultured cells are indeed fibroblasts. However, these systems ignore the heterogeneity of fibroblasts that exists in vivo, for example the differences between papillary and reticular fibroblasts (Sorrell and Caplan, 2004).

**Bleomycin-induced murine skin fibrosis**

Repeated subcutaneous injection of bleomycin over several weeks causes skin fibrosis with inflammation, skin thickening, increased collagen deposition, and vasculopathy (Smith and Chan, 2010; Yamamoto et al., 1999) (Figure 2). The contributing fibroblasts likely arise predominantly from resident fibroblasts or fibroblast progenitors, with a numerically small contribution from bone marrow-derived collagen-expressing cells (Higashiyama et al., 2011), although definitive lineage tracing for all potential contributors has not been performed in this model. This is a common model of skin fibrosis since it shares many features of scleroderma. It is particularly useful because it can be used in transgenic mice to test whether genetic insufficiency of a profibrotic factor can protect against bleomycin-induced fibrosis. In addition, small molecule inhibitors can be

**Figure 3.** Tight-skin mouse skin. H&E demonstrating hypodermal thickening (*) in the dorsal skin of Tsk1/+ mouse, below, compared to wild-type, above. Reproduced from (Matsushita et al., 2007). Copyright 2007, the American Association of Immunologists, Inc.
tested in this system either by co-administration with the bleomycin (to test for a protective effect) or administration after the bleomycin-induced fibrosis has developed (to test for a therapeutic effect). The major limitation of this model, however, is that it requires repeated injections of bleomycin, and the fibrosis may regress several weeks after cessation of bleomycin. Therefore, bleomycin-induced fibrosis may be most similar to earlier stages of the fibrotic response to injury, limiting its applicability of these experiments to late-stage pathology (Yamamoto et al., 1999).

Tight-skin mice

Tight-skin mice (Tsk-1/+) have fibrotic skin resulting from an in-frame duplication in the Fbn1 gene, which encodes the microfibrillar protein fibrillin-1 (Green et al., 1976; Siracusa et al., 1996). A similar mutation in humans causes a congenital form of scleroderma called stiff-skin syndrome (Loeys et al., 2010). This is distinct from the point mutations in Fbn1 that cause Marfan syndrome. Tsk-1/+ mice have thickened skin with fibrotic expansion of the hypodermis (Matsushita et al., 2007) (Figure 3). Homozygosity for the mutation is embryonically lethal by embryonic day E7 or E8 (Green et al., 1976). Tsk-1/+ mice are used for testing anti-fibrotic therapies, since the fibrosis is progressive and does not resolve on its own. In addition, since skin fibrosis in these mice results from a change in a single protein (fibrillin-1), studies of Tsk-1/+ mice have yielded important insights into the biology of the fibrotic extracellular matrix. Tsk-2/+ mice are distinct from Tsk-1/+ mice; in Tsk-2/+ mice, skin fibrosis results from a mutation in Col3a1, the gene that encodes
the α-1 chain of type III collagen. Tsk-2/+ skin fibrosis predominantly affects the dermis, not the hypodermis (Long et al., 2014a).

Transgenic mice

Transgenic mice enable powerful and precise manipulation of gene expression in vivo. In particular, mice can be engineered such that expression or deletion of a transgene depends upon excision of loxP DNA sequences by bacterial Cre recombinase. Expression of the Cre recombinase is driven by a transgenic promoter. (Alternatively, Cre expression can be introduced by adenoviral gene transfer.) Use of a tissue-restricted promoter enables tissue-restricted Cre expression and excision activity. In addition to this spatial control of gene expression, this system can also be designed to allow temporal control. Temporal control is accomplished by use of an inducible Cre such as the Cre-estrogen receptor(ER) fusion protein. Cre-ER requires tamoxifen in order to translocate to the nucleus and excise loxP sequences. Therefore, tamoxifen can be administered at any point in time (as long as the promoter-Cre-ER transgene is being expressed) to induce permanent and heritable expression or deletion of the target transgene.

Temporal and spatial restriction of the genetic manipulation are significant benefits of using transgenic mice. In addition, the power to precisely manipulate expression of a single gene cannot be understated, since this allows the researcher to observe the consequences of the manipulation over time in vivo. With respect to skin fibrosis, the major limitation of this technology is the lack of well-characterized
tissue-restricted promoters that are expressed in cell types of interest (Derrett-Smith et al., 2009). A fibroblast specific enhancer of Col1a2 has been used to drive Cre and CreER expression in mice (Col1a2-Cre and Col1a2-CreERT constructs) (Bou-Gharios et al., 1996; Zheng et al., 2002). The Col1a2-Cre construct is active in embryonic day E15.5 tissues including dermis and skull bones (Bou-Gharios et al., 1996). The Col1a2-CreERT construct with postnatal tamoxifen induction drives transgene expression in fibroblasts and “fibroblastic” cells in the dermis, intestinal wall, lung, and blood vessel walls, and also in membranously ossified bone in the skull (Zheng et al., 2002). The Col1a2-CreERT construct has been used extensively for transgenic studies in mouse dermis, although its contribution to distinct fibroblast populations in the dermis and hypodermis have not been assessed (Derrett-Smith et al., 2009) (section 1.3.2). Other potentially interesting fibrogenic cell types in the skin, pericytes and adipocytes, have also proven difficult to target, although new reagents are becoming available for use in adipocytes (Armulik et al., 2011; Jeffery et al., 2014). For my research, I have used transgenic mice in which CreER expression is driven by the HoxB6 promoter, which with late fetal tamoxifen induction is restricted to the ventral dermis and limb mesenchyme (Nguyen et al., 2009).

1.3. Extracellular and cellular changes in skin fibrosis

Fibrotic tissue has substantial changes in the extracellular environment and in the behavior of several involved cell types. When evaluating a fibrotic skin phenotype
in a new mouse model, it is useful to determine which of these changes have occurred and to what extent. Fibrotic skin has excess accumulation of extracellular matrix proteins compared to healthy skin, including collagens, microfibrillar proteins, and proteoglycans (section 1.3.1). The primary cellular contributors to cutaneous fibrosis include fibroblasts and fibroblast progenitors (section 1.3.2). In addition, fibrosis can be associated with vascular abnormalities and changes in endothelial cell biology (section 1.3.3). Inflammation, which has important roles during normal wound healing, is also present in fibrotic tissues (section 1.3.4).

1.3.1. Extracellular matrix, adhesion, and mechanical stresses

Dermal extracellular matrix composition and function

The ECM of the adult dermis is predominated by type I and type III collagens, and
includes other proteins with diverse extracellular functions: elastin, fibrillins, laminins, fibulins, latent TGFβ binding proteins (LTBPs), proteoglycans, glycoproteins, and integrins (Bruckner-Tuderman, 2012). Non-structural/adhesion proteins such as regulatory/modifying enzymes such as matrix metalloproteinases (MMPs) and adamalysin family proteinases (ADAMs and ADAMTSs), ADAMTSls, secreted cytokines, and growth factors also exist in the matrix (Bruckner-Tuderman, 2012; Fowlkes and Winkler, 2002). These proteins collectively maintain a complex extracellular environment that gives the dermis its mechanical properties and facilitates intercellular signaling and regulation of cell behaviors (Bruckner-Tuderman, 2012; Lu et al., 2011) (Figure 4).

Homeostasis of the ECM relies on continuous production and degradation of matrix proteins (Lu et al., 2011). Turnover of collagen is particularly relevant to the study of normal wound healing/scar formation and pathologic fibrosis. In the dermis, type I and III collagens are produced primarily by dermal fibroblasts (Bruckner-Tuderman, 2012) and are extruded extracellularly as pro-collagen. Formation into mature collagen fibrils requires extracellular processing by ADAMTS2 (type I collagen) and BMP1 (type I and III), and cross-linking by lysyl oxidase and tissue transglutaminase (Bruckner-Tuderman, 2012; Lu et al., 2011). There is also evidence that collagen fibrillogenesis and cross-linking is regulated by selected small leucine-rich proteoglycans (SLRPs) such as decorin and biglycan (Kalamajski and Oldberg, 2010). Collagen catabolism is carried about by specific members of the matrix metalloproteinase family, a large group of proteins (there are 23 MMPs
encoded by 24 genes in humans) that serve several roles based on their proteinase function (Pardo, 2006).

Multiple ECM proteins play active roles in the regulation of intercellular signaling. Specifically, ECM proteins can bind to extracellular signaling molecules to regulate their ability to bind to cognate receptors in cell membranes (Chaudhry et al., 2007; Chen and Birk, 2013; Fowlkes and Winkler, 2002; Schaefer and Iozzo, 2008; Wells and Discher, 2008). For example, members of the TGFβ ligand family physically interact with LTBPs, integrins, fibrillin, and proteoglycans (Bruckner-Tuderman, 2012; Chaudhry et al., 2007; Hildebrand et al., 1994; Schaefer and Iozzo, 2008). TGFβ ligand bound to LTBP is sequestered in a large latent complex (LLC) that limits the bioavailability of the ligand to interact with cell surface receptors. Release of TGFβ (and thus, its bioavailability) can potentially be regulated by interactions with fibrillin (Chaudhry et al., 2007) and SLRPs including decorin, biglycan, asporin, and fibromodulin (Hildebrand et al., 1994; Schaefer and Iozzo, 2008). In addition, MMPs may participate in releasing TGFβ from some of these interactions (Fowlkes and Winkler, 2002). The release of TGFβ from LTBP may also be sensitive to the stiffness of the ECM (Wells and Discher, 2008). Therefore, the signaling activity of TGFβ may be influenced by direct physical interactions in the ECM as well as by proteinase activity and matrix stiffness— and so altered ECM in fibrosis may result in an abnormal signaling environment via multiple mechanisms.
Abnormal matrix composition in fibrosis

Abnormalities in amounts and proportions of extracellular matrix proteins are established characteristics of fibrosis (Abergel et al., 1987; Rockwell et al., 1989; Uitto and Kouba, 2000; Uitto et al., 1989). In particular, increased collagen is the hallmark, and also most thoroughly characterized, feature of fibrosis in any tissue. Scleroderma skin has elevated type III collagen early during the disease, and elevated type I collagen at later stages (Carwile Leroy, 1981), and cultured dermal fibroblasts from scleroderma patients have increased collagen synthesis in vitro (Leroy, 1974). Reticular and papillary fibroblasts from the dermis of scleroderma patients both display increased collagen synthesis compared to normal dermal fibroblasts, but this elevated synthesis is more pronounced in the reticular dermal fibroblasts (Carwile Leroy, 1981). In a separate experiment that did not distinguish between reticular and papillary dermal fibroblasts, scleroderma and normal fibroblasts did not demonstrate any difference in metalloproteinase activity; so it is possible that altered collagen catabolism in the fibrotic dermis is not mediated by dermal fibroblasts but instead by another cell type (Uitto et al., 1979). Similarly to scleroderma fibroblasts, cultured keloid fibroblasts have elevated expression of type I collagen mRNA which is associated with increased collagen production in vitro (Abergel et al., 1985; Ala-Kokko et al., 1987).

It is generally thought that the excess collagen in fibrotic tissue is a consequence of increased synthesis coupled with decreased degradation to result in greater accumulation of mature collagen fibrils (McKleroy et al., 2013). Elevated expression
of collagen genes and increased activity of prolyl hydroxylase, lysyl hydroxylase, and cross-linking enzymes could contribute to the increased synthesis, and reduced activity of MMPs (possibly by increased activity of their inhibitors, TIMPs) could be responsible for decreased collagen catabolism (Frantz et al., 2010; Minor, 1980). Although not all collagen-regulating enzymes have been examined in in the fibrotic dermis, there is support for a role of elevated prolyl hydroxylase activity in fibrosis that may correlate with collagen synthesis. During normal wound healing, elevated activity of prolyl hydroxylase (an enzyme that converts proline to hydroxyproline resides as part of the normal post-translational modification of pro-collagen) is increased in the wound granulation tissue of both humans and rats (Stein and Keiser, 1971) compared to non-wounded dermis. The dermis of scleroderma patients has significantly elevated prolyl hydroxylase activity compared to healthy dermis, indicating increased collagen synthesis in the disease state (Stein and Keiser, 1971). With respect to collagen catabolism, there are reports of elevated MMP expression in fibrotic skin diseases, which appears to be inconsistent with a model relying on decreased collagen degradation (Denys et al., 2004). Perhaps increased collagen synthesis is the predominant force in favor of fibrosis, or the elevated MMPs are responsible for alternate, pro-fibrotic effects rather than collagen catabolism (Parks, 1999).

Abnormal ECM content in dermal fibrosis is certainly not limited to collagen and its modifying enzymes. For example, increased fibrillin-1 microfibril assembly is correlated with fibrosis in scleroderma and the tight-skin mouse model (Lemaire et
Furthermore, abnormal expression of just a single gene encoding a component or regulator of extracellular matrix can result in altered fibroblast biology and/or a fibrotic phenotype in mice (Bader et al., 2010; Hattori et al., 2011; Long et al., 2014b; Yang et al., 2012; Yu et al., 2012). However, lack of appropriate classification/categorization of all ECM-encoding genes has, until recently, hampered the ability of fibrosis researchers to evaluate global changes in ECM gene expression (Hynes and Naba, 2012; Naba et al., 2012a; 2012b). Therefore, thorough examination of matrix gene expression is fundamental for understanding the mechanisms of fibrosis and the consequences of ECM alterations in vivo.

**Functional consequences of altered ECM in fibrosis**

Fibrotic ECM has substantial effects on cell behaviors and intercellular signaling. It has been proposed that fibrotic ECM promotes fibroblast contractility and continued matrix synthesis as part of a feed-forward loop linking matrix composition with a continued pro-fibrotic response (Blaauboer et al., 2014; Shinde et al., 2014). These pro-fibrotic effects of the matrix may be exerted by controlling bioavailability of pro-fibrotic signaling molecules such as TGFβ; via biomechanical effects on fibroblasts; and direct interactions with membrane-bound receptors. For example, decreased decorin in keloid scars may lead to reduced sequestration and increased bioavailability of TGFβ (Carrino et al., 2012). In addition, in vitro studies of fibroblasts cultured on surfaces of varying stiffness have demonstrated that a stiffer matrix promotes greater bioavailability of TGFβ (Wells and Discher, 2008). These
effects may contribute to elevated TGFβ signaling that is observed in fibrosis (see also section 1.5.1 and section 4.4.3).

The altered mechanical properties of the fibrotic extracellular matrix compared to normal tissue results in altered forces on fibroblasts and other cells (Olsen et al., 1996). Elevated matrix stiffness in vitro results in increased conversion of dermal fibroblasts to a myofibroblast phenotype, and elevated expression of fibrosis-related genes such as COL1A1 and CTGF (Achterberg et al., 2014). The mechanoresponsiveness of fibroblasts may be via signaling pathways that have already been established to be pro-fibrotic, such as TGFβ/Smad2/3 and Wnt/β-catenin pathways (Huang and Ogawa, 2012). The effects of environmental stiffness are on cell behavior and gene expression in vivo remain to be fully elucidated, especially in the context of fibrosis and wound healing (Tschumperlin et al., 2013).

Individual proteins that mediate cell-cell and cell-matrix interactions are of great importance in fibrotic tissues. Adhesion molecules, namely integrins, act as the mediators between the extracellular environment and cells, including fibroblasts. Focal adhesion kinase (FAK), the downstream signaling transducer of integrins, is required in dermal fibroblasts for mechanosensation during scar formation, and mice lacking FAK in dermal fibroblasts have reduced fibrosis in a hypertrophic scar model (Wong et al., 2011). In addition, FAK is required for dermal fibroblast expression of fibrosis-related genes including ACTA2, CTGF, and COL1A1 (Kennedy et al., 2008). Therefore, integrin-mediated signaling is of potential therapeutic
interest in skin fibrosis, especially since integrin blockade therapies have had recent therapeutic success in mouse models of pulmonary and skin fibrosis (Gerber et al., 2014; Henderson et al., 2013).

1.3.2. Fibroblasts and other fibrogenic cells

Fibroblasts are a heterogeneous group of cells that are responsible for the production of the extracellular matrix (ECM). The dermis contains resident fibroblasts which, are defined by their ability to produce type I collagen (the predominant type of collagen in skin) and other matrix proteins including type III collagen, fibronectin, fibrillin, and elastin (Uitto et al., 1989). Dermal fibroblasts have important roles in embryonic development of the skin and during adult wound healing and pathologic fibrosis. Many different stimuli, both signaling factors and mechanical forces, can regulate the activities and behaviors of fibroblasts.

Dermal fibroblast development

During embryonic development, dermal fibroblasts in different parts of the body are derived from a variety of embryonic tissues, including lateral plate mesoderm, the dermomyotome, paraxial mesoderm, and neural crest (Yoshida et al., 2008) (Atit et al., 2006; Driskell and Watt, 2014; Ohtola et al., 2008). In addition to forming the nascent dermis, dermal fibroblasts are important for positional skin patterning and hair follicle initiation (Chang et al., 2002; Dhouailly, 1978; Eames, 2005). During embryonic development of the dorsal skin, dermal fibroblast progenitors can be
identified beneath the ectoderm at E12.5. These are common fibroblast progenitors that express characteristic markers PDGFR-α, Dlk1, and Lrig1 (Driskell et al., 2013). By E16.5, this population gives rise to two distinct fibroblast lineages— one which contributes to the papillary dermis, and the other than contributes to the reticular dermis and hypodermis (Driskell et al., 2013). The papillary lineage additionally is the source of the dermal papilla and arrector pili muscle; the reticular lineage additionally is the source of pre-adipocytes and adipocytes which reside in the hypodermis (Driskell et al., 2013). Other mesenchymal populations in the skin may include pericytes and bone marrow-derived mesenchymal stem cells, although the extent of their contribution is not yet clear (Driskell and Watt, 2014). The mechanism of lineage selection has not been elucidated for these specific sub-populations of dermal progenitors. However, more generally, it has been shown that Wnt/β-catenin signaling is a key signal that regulates dermal progenitor cell fate in the dorsal, ventral, and craniofacial dermis (Atit et al., 2006; Goodnough et al., 2014; Ohtola et al., 2008; Tran et al., 2010).

Fibroblasts in tissue repair

Fibroblasts involved in adult tissue repair have been proposed to arise from pre-existing resident fibroblasts or from fibroblasts differentiated or trans-differentiated from a progenitor or precursor cell types. These potential sources of injury-responsive fibroblasts include pericytes, fibrocytes, endothelial cells, and epithelial cells. One of the key questions in fibrosis research is determining which of these cell types substantially contribute to the population of active, matrix-
producing fibroblasts during fibrogenesis. This knowledge is important for understanding the cellular mechanisms of fibrosis and is crucial for developing therapies that can be targeted to particular types of cells.

In human tissue with established fibrosis, it is difficult to assess the cellular source(s) of effector fibroblasts. Therefore, most of the knowledge about cellular contributors to fibrosis are from fate-mapping studies using transgenic mice. In these studies, a particular type of cell expresses a gene that encodes non-endogenous protein (for example, a fluorescent bacterial protein) that can be detected by tissue staining or immunohistochemistry. Labeled cells and all of their progeny will express this detectable protein throughout the animal’s life (Kretzschmar and Watt, 2012). Fibrosis is typically induced by some sort of injury (for example, bleomycin injection into the lungs to induce pulmonary fibrosis, or unilateral ureteral obstruction to induce renal fibrosis) and the contribution of the labeled cells to the fibrotic tissue is observed. These studies are challenging to perform well because it is difficult to restrict lineage labeling to the specific cell type of interest. In addition, to determine whether the labeled cells have contributed to the population of fibroblasts, the fibroblasts themselves must be accurately detected. Unfortunately, due to the heterogeneity of this population, the available molecular markers for fibroblasts are not highly sensitive or specific (Driskell and Watt, 2014; Phan, 2008).
In spite of these challenges, fate mapping of several different lineages and their contribution to fibroblasts in fibrosis has been performed in renal, cardiac, hepatic, and pulmonary fibrosis models (Humphreys et al., 2010; Lotersztajn et al., 2005; Rock et al., 2011; Zeisberg et al., 2008; 2007). In fibrotic human diseases as well as animal models, there is evidence for the involvement of local resident fibroblasts as well as fibroblasts differentiated or trans-differentiated from circulating fibrocytes and local pericytes, endothelial cells, epithelial cells, and adipocytes or adipocyte progenitors (Postlethwaite et al., 2004):

- Circulating fibrocytes are a circulating population of monocyte-lineage, bone marrow-derived mesenchymal progenitor cells (Bucala et al., 1994) that migrate in response to locally released factors to injured tissues and contribute to fibrosis in wounds, scars (including keloidal scars), and nephrogenic systemic fibrosis (Cowper and Bucala, 2003; Metz, 2003). They have also been demonstrated in a bleomycin-induced murine model of skin fibrosis (Higashiyama et al., 2011; Katebi et al., 2008).

- Pericytes are perivascular cells that, in injured tissue, can differentiate into fibroblasts and contribute to organ fibrosis in mice (Dulauroy et al., 2012; Greenhalgh et al., 2013; Humphreys et al., 2010) and in human scleroderma skin, where they express common markers with myofibroblasts (Rajkumar et al., 2005).

- Mesenchymal stem cells (MSCs) are bone marrow-derived stromal cells that migrate to the fibrotic skin in the bleomycin-induced mouse model
(Higashiyama et al., 2011) and which contribute to the formation of human and murine desmoid tumors (Wu et al., 2010) (section 1.2.3).

- Endothelial cells can trans-differentiate to fibroblasts via endothelial-to-mesenchymal transition (EndMT) (Zeisberg et al., 2007; 2008).

- Epithelial cells can trans-differentiate to fibroblasts via epithelial-to-mesenchymal transition (EMT) in developmental and cancer, but the most recent lineage-tracing studies have not supported a role for this phenomenon in the context of fibrosis (Hu and Phan, 2013).

- Subcutaneous adipocyte progenitors are possible contributors to skin fibrosis, particularly in SSC, in which adipose tissue is replaced by fibrotic tissue (Wei et al., 2011a; 2011b). Skin fibrosis in the bleomycin mouse model is associated with impaired adipogenesis (Ohgo et al., 2013). The loss of subcutaneous adipose tissue in scleroderma has been hypothesized to be associated with differentiation of a common fibro-adipoprogenitor cell to fibroblast fate as occurs in fibrosis of muscle (Uezumi et al., 2011), but this phenomenon requires further investigation (Driskell et al., 2014).

The extent of involvement of each of these fibrogenic sources varies between different diseases and organ systems. The contributions of resident fibroblasts in many of the visceral organs have not been examined due to a lack of appropriate fate-mapping reagents. Resident stromal cells, but not pericytes, contribute to injury-induced lung fibrosis (Rock et al., 2011). More recently, portal fibroblasts have been found to be involved in hepatic fibrosis, although this was assessed by gene expression profile rather than lineage tracing (Iwaisako et al., 2014). The role
of resident fibroblasts in fibrosis is of particular interest in the dermis of the skin, which is predominated by resident fibroblasts in the healthy state. Fate-mapping studies have measured a contribution of superficial and deep (papillary and reticular) resident fibroblasts to dermal wound healing (Driskell et al., 2013), but the contribution of these fibroblasts and other sources of fibroblasts to dermal fibrosis has not been assessed.

Some conditions have a characteristically elevated fibroblast proliferation rate. These include normal healing wounds, keloidal and hypertrophic scars, and superficial and deep fibromatoses (Eming, 2012; Li et al., 2010; Tuan and Nichter, 1998; Varallo et al., 2003). In other fibrotic conditions, increased fibroblast proliferation is not a typical feature at the time of diagnosis (i.e. SSc, morphea, and stiff skin syndrome).

*Myofibroblasts*

Myofibroblasts, a fibroblast subtype, are present in many fibrosing conditions and in normal dermal wound healing (Gabbiani, 2003; Tomasek et al., 2002). These cells are defined by the presence of stress fibers and prominent endoplasmic reticulum, features that are detected by electron microscopy. Expression of α-smooth muscle actin (α-SMA) is widely accepted as a marker for myofibroblasts, although it is not specific, since it is also expressed in endothelial cells, pericytes, and smooth muscle cells (Eyden, 2007). In addition, α-smooth muscle actin identifiable by confocal microscopy may not be readily seen in typical immunohistochemical preparations.
(Rajkumar et al., 2005). Myofibroblasts are typically present in healing or fibrosing conditions, although fibrosis can occur in their absence. For example, early stage SSc skin does not always have myofibroblasts (Kissin et al., 2006). Congenital disorders of glycosylation, which cause fibrosis in visceral organs, involves fibroblasts that lack α-SMA expression (Lecca et al., 2011).

1.3.3. Vascular changes

Fibrotic skin is often associated with vascular changes. Endothelial cells are crucial mediators of the wound healing process, and proper angiogenesis and vasculogenesis are critical for dermal wound healing (Eming, 2012). In scleroderma, endothelial cell or microvascular dysfunction may play an important role in early stages of injury response leading to fibrogenesis (Wynn, 2008), since scleroderma is accompanied by skin microvessel vasculopathy, and is often preceded by the appearance of Raynaud phenomenon (Manetti et al., 2010). Tight-skin mice also exhibit endothelial dysfunction (Richard et al., 2008). In addition to contributing to tissue hypoxia, endothelial cell abnormalities may also play a part in fibrosis via endothelial-to-mesenchymal transition (EndMT), a phenomenon that produces activated fibroblasts in injured tissue (Zeisberg et al., 2007; 2008). In contrast to SSc, keloidal and hypertrophic scars do not exhibit endothelial cell apoptosis (Sgonc et al., 1996). Dermal microvessels are more abundant in these scar tissues than in normal skin and tend to exhibit occlusions that may contribute to local tissue hypoxia (Amadeu et al., 2003; Kischer et al., 1982). How these
changes might contribute to scar formation in comparison to normal wound healing, however, is unclear. In summary, vascular changes are highly variable across different fibrotic conditions, and have different potential contributions to fibrotic processes.

1.3.4. Inflammation and autoimmunity in fibrosis

Inflammation occurs in response to injury and so is often present in fibrotic tissues. Depending on the nature of the injury that has precipitated the fibrotic response, there is variable involvement of inflammatory cells such as T lymphocytes and macrophages. Infiltration of these cells is generally observed in the fibrotic dermis and in granulation tissue of a healing wound (Eming, 2012; Gershwin et al., 1981; Wynn, 2007; Yamamoto et al., 1999). Generally, the role of inflammatory cells in the injury response is to stimulate fibrogenic cell types to proliferate and form scar tissue (Borthwick et al., 2013). Additionally, in autoimmune fibrovaliding disorders (systemic sclerosis and morphea), there is a pathogenic role for B lymphocytes.

T lymphocytes

T lymphocytes (T cells), which are bone marrow-derived cells that are recruited to tissues via the vasculature, are present in the inflammatory phase of normal wound healing as well as in keloid scars and lesions of systemic sclerosis and acute GVHD (Boyce et al., 2001; Brüggen et al., 2014; Eming, 2012; O'Reilly et al., 2012). T cell involvement in wound healing is predominated by Th1 and Th2 cells, which are
subsets of CD4+ T cells (Eming, 2012); there are also CD8+ T cells in normally healing wounds and in keloid scars (Boyce et al., 2001). There is histopathological evidence of an activated T cell infiltrate (CD3+ CD69+, without additional sub-typing into Th subsets) in scleroderma lesions (Kalogerou, 2005).

T cells have several proposed roles in fibrotic processes which are based on their observed in vitro behaviors. First, Th2-derived interleukin-4 (IL-4) stimulates fibroblasts to produce excess collagen (Ong et al., 1998). Second, Th2-derived interleukin-13 (IL-13) induces local macrophages to produce pro-fibrotic TGFβ1 (Wynn, 2008). In addition, CD4+ and CD8+ can promote vascular abnormality, for example in systemic sclerosis, by inducing apoptosis of vascular endothelial cells (Kahaleh, 2008).

Studies in animal models have not demonstrated a coherent role for T cells in skin fibrosis. Donor T cells appear to contribute to fibrosis in the murine model of sclerodermoid GVHD (Zhang et al., 2002). Conversely, SCID mice, which lack functional T cells and B cells, have a normal fibrotic response to subcutaneous injection of bleomycin (Yamamoto, 2009). Therefore, the role for T cells in fibrogenesis seems to vary between animal models and perhaps also human diseases. The balance between distinct subpopulations of T cells is likely of importance (Boyce et al., 2001; Murao et al., 2014).
Macrophages

Macrophages are involved in wound healing and in skin fibrosis conditions. Resident macrophages are present in the healthy dermis; their contribution to wound healing is in addition to that from recruited monocyte-derived macrophages, which are recruited to the wound via the vasculature in wound healing (Leibovich and Ross, 1975) and scleroderma lesions (Ho et al., 2014). Macrophages are present during the inflammatory phase of wound repair (Eming, 2012) and in skin lesions of scleroderma (Ho et al., 2014), acute graft-versus-host disease (Lampert et al., 1982), and keloid scars (Boyce et al., 2001).

Macrophages are activated by pro-fibrotic signaling factors including TGFβ, IL-13, and PDGFR. Functional studies in mouse models have suggested that macrophage activation is in response to factors secreted by Th2 cells (Wynn, 2008). In turn, macrophages contribute to fibrosis by secreting pro-fibrotic cytokines including angiotensin II, CCL3 (MCP1α), and TGFβ1 (Duffield, 2003; Smith et al., 1994; Wynn, 2008).

In addition to human fibrotic skin, macrophages are present in the dermis of the bleomycin and cutaneous GVHD mouse models of fibrosis (Yamamoto et al., 1999; Zhang et al., 2002). Recruited macrophages are required for bleomycin-induced skin fibrosis in mice (Ferreira et al., 2006) and perhaps also for normal wound healing (Leibovich and Ross, 1975). CCL3/MCP1α, which is produced by macrophages, is required for fibrosis in the bleomycin-induced pulmonary fibrosis
mouse model (Smith et al., 1994), so this cytokine may be particularly important for
the macrophage contribution to fibrogenesis.

**B Lymphocytes**

B lymphocyte (B cell) activation is of mechanistic and therapeutic interest
specifically in autoimmune fibrosing disorders SSc and morphea. B lymphocyte
infiltrates are present in the dermis of SSc skin lesions: they are visible upon
immunostaining for CD20 surface marker, and SSc skin has elevated expression of
signature genes that are consistent with the presence of B cells (Sakkas et al., 2006;
Whitfield et al., 2003).

The pro-fibrotic effects of B cell activation are likely via their production of
particular autoantibodies and cytokines. In SSc, B cells produce multiple
autoantibodies including anti-DNA topoisomerase I, anti-centromere, anti-
fibrillin-1, anti-PDGFR, anti-fibroblast, anti-endothelial cell, anti-nuclear antibodies,
and others (Sakkas et al., 2006). Some of these autoantibodies have pathogenic
roles (Mehra et al., 2013). In addition, B lymphocytes produce IL-6, a cytokine that
stimulates fibroblast growth and collagen production (François et al., 2013;
Hasegawa et al., 2005).

Determining the precise pathogenic roles of B cells in autoimmune fibrosis has been
facilitated by two mouse models of skin fibrosis which both possess B cell
abnormalities. Both the tight-skin mouse (Tsk-1/+), a genetic model of skin fibrosis,
and mice subcutaneously injected with bleomycin, an injury-induced model of skin fibrosis, have activated B cell phenotypes and autoantibody production (Saito et al., 2002; Yoshizaki et al., 2010). Depletion of CD19+ B cells in these mouse models, as well as inhibition of BAFF in tight-skin mice, abrogates the development of skin fibrosis (Hasegawa et al., 2005; Matsushita et al., 2007; Yoshizaki et al., 2010).

Ongoing studies in SSc patients are investigating the therapeutic efficacy of B cell depletion using rituximab, a chimeric anti-CD20 antibody. The most recent studies have shown a significant reduction in clinical measures of SSc severity with rituximab treatment (Jordan et al., 2014; Smith et al., 2013; Woodrick and Varga, 2010). In addition to being a potential therapeutic targets, B cells are of interest as a source for biomarkers of disease progression in SSc (Wutte et al., 2013).

1.4. Fibrosis-associated genetic changes

1.4.1. Altered gene expression in skin fibrosis

Fibrotic tissue and the involved cells experience substantial changes in gene expression compared to the healthy state. These changes can be in response to the pro-fibrotic environment and, in turn, can perpetrate fibrosis. For example, Col1a1 encodes the α1 chain of type I collagen; its expression is induced by pro-fibrotic signaling including the TGFβ pathway, and its increased expression can contribute to greater production of type I collagen protein. Commonly referenced pro-fibrotic
genes are typically those that encode matrix-related proteins, fibroblast markers, or components of pro-fibrotic signaling pathways (e.g. \textit{Col1a1}, \textit{Acta2}, and \textit{Tgfb1}, respectively). In addition to the up-regulation of these classic pro-fibrotic mediators, cells and tissues in fibrosing diseases and related animal models have characteristic gene signatures with many dys-regulated genes. These signatures have been defined by microarray-based studies of human fibrotic tissues and cells (Sargent \textit{et al.}, 2009; Smith \textit{et al.}, 2007) and animal models of fibrosis (Bayle \textit{et al.}, 2008) and yield a great deal of information about fibrotic processes.

Fibrotic gene signatures in scleroderma patients are stable over time (Pendergrass \textit{et al.}, 2012) and between lesional and non-lesional skin from the same patient (Whitfield \textit{et al.}, 2003). It is not known whether fibrotic gene signatures have similar consistencies in other fibrosing skin disorders, as serial studies have not been performed. Several factors could potentially contribute to the stability of fibrotic gene signatures in scleroderma and other diseases: (1) epigenetic regulation of gene expression, which is stable and heritable (Hinz \textit{et al.}, 2012; Mann and Mann, 2013; Zeisberg and Zeisberg, 2012); (2) feed-forward mechanisms by which particular pro-fibrotic signaling pathways positively regulate expression of genes that maintain activity of those pathways; and (3) influence of the extracellular environment (by ECM-receptor interactions or mechanical stresses transduced by cells) on signaling activity and gene expression. Additional studies are needed to determine the contributing factors to gene signature stability in scleroderma, and to identify whether other fibrosing skin disorders and animal models have similarly
stable changes in gene expression. Animal models will be particularly useful for determining the stability of gene expression in lesional tissue during early development of fibrosis (as opposed to the established fibrosis observed in diagnosed human disease).

Gene expression changes in fibrotic tissues often correlate to particular features of fibrosis, suggesting that these features may be consequences of altered gene expression. Measuring gene expression has been used in many experimental systems to identify up- and down-regulated genes that encode novel mediators of fibrosis or that correspond with activity of pro-fibrotic signaling pathways. Gene expression studies can also yield insight into the pathogenesis of fibrotic disease, for example by the discovery of a B lymphocyte expression signature in SSc patient skin (Whitfield et al., 2003). In systemic sclerosis, gene signatures can be used to divide patients into subtypes that correlate with disease severity and clinical response to treatment (Hinchcliff et al., 2013; Milano et al., 2008; Pendergrass et al., 2012). Altered expression of single genes is used as a marker of fibrotic activity or to indicate the presence of particular cell types (for example, Acta2 for myofibroblasts). While informative, these studies bear several caveats. First, they are based on DNA microarrays of tissue biopsies and, in some cases, cultured fibroblasts. Therefore, they tell an incomplete story, since the genes assayed are only those detectable by probes which are determined by the microarray platform used. Only recently has next-generation sequencing technology, which is not limited by selection of specific gene probes, been applied to studying fibrotic human tissues.
(Arron et al., 2014). Second, in the case of biopsied tissue, high cellular heterogeneity may mask cell type-specific gene expression changes. And third, in the case of cultured fibroblasts, the measured gene expression changes may be influenced by environmental stresses and culture conditions, and so not truly reflective of the pathological state (Gardner et al., 2006).

The same caveats that exist for measurement of altered gene expression in human tissue apply to animal models of fibrosis. Even in studies of tissue-restricted and tissue-specific fibrosis models, gene expression changes have been only measured using heterogeneous tissues and cultured cells. Most published results have measured gene expression changes by microarray, although recently, next-generation sequencing been applied to an animal model of renal fibrosis (Liu et al., 2014). Gene expression profiling produced by RNA-sequencing has not been published in any other animal models of fibrosis.

1.4.2. Genetic variants associated with fibrotic skin disease

The tight skin-1 mouse and human stiff skin syndrome are single-gene disorders that result in skin fibrosis (section 1.2.4). In contrast, SSc, keloid scarring, and superficial fibromatoses are complex diseases with no single genetic cause. However, there are a handful of genetic variants, i.e. single nucleotide polymorphisms (SNPs), that are associated with these disease phenotypes and which will be discussed in this section. Genome-wide association studies (GWAS)
have been used to identify these genetic variations in intronic and intergenic regions of DNA. The significance of each of these variations is determined by its frequency in large populations in case versus control studies. Disease-associated loci can then be further investigated with respect to pathogenesis or disease and can be potentially used as biomarkers for disease risk or therapeutic response (Manolio, 2010; 2013). In contrast to gene expression studies (section 1.4), GWA studies tend to identify very few loci of interest, but the genetic variations at these loci are intrinsic to the individual’s or population’s DNA, and so are not subject to many of the caveats of gene expression-based studies.

In systemic sclerosis, GWA studies have consistently identified disease-associated loci in and near the HLA region and genes that encode proteins related to immune response and inflammation, consistent with the autoimmune etiology of SSc (Jin et al., 2014). The relative rarity of SSc, heterogeneity of phenotypes, and ethnic diversity of SSc patients have challenged efforts to identify significantly associated SNPs or loci (Zhou et al., 2009). Even before GWAS, this was the case; one of the earliest gene association efforts in SSc was limited to a Choctaw Indian population that has a high prevalence of SSc with a strong familial component (Zhou et al., 2003). Later GWAS studies continued the theme of analyzing well-defined, restricted patient populations in order to identify a small number of significantly associated genetic loci. Studies restricted to patients of particular ancestries (Korean, US Caucasian, and European ancestry) identified loci in the HLA region and genes related to immune response (Radstake et al., 2010; Zhou et al., 2009).
However, since the autoimmune contribution to SSc was already well-established, the impact of these findings with respect to mechanism of disease was limited. Division of the SSc patients into sub-types proved to be a higher-yield approach. In particular, Gorlova et al. re-examined the patients from Radstake et al. (Radstake et al., 2010) in groups depending on SSc phenotype (limited or diffuse cutaneous involvement) and autoantibody status. This study elucidated associations of loci in NOTCH4 and SOX5 with anti-centromere antibody-positive SSc, and of loci in IRF8 and GRB10 with the limited cutaneous subset of SSc (Gorlova et al., 2011). More recent GWAS of SSc have focused on elucidating autoimmunity-linked loci (Martin et al., 2013) and have not lent particular insight into the fibrotic or cell signaling components of this disease (Jin et al., 2014).

GWA studies in superficial fibromatoses such as Dupuytren disease and Peyronie disease are also limited by the rarity of these conditions. An early genome wide association scan identified three susceptibility regions in 40 Dupuytren disease patients compared to forty healthy controls; however, this study did not identify the precise loci (Ojwang et al., 2010). The first robust GWAS in Dupuytren disease found eleven associated SNPs in nine distinct loci (Dolmans et al., 2011). Six of the nine loci were associated with coding regions for Wnt pathway genes (WNT4, SFRP4, WNT2, RSPO2, SULF1, WNT7B). The SNP associated with WNT2 was later identified by the same group to be associated with Peyronie disease (Dolmans et al., 2012). Therefore, the overarching finding from GWAS in superficial fibromatoses is an association with SNPs in Wnt pathway genes.
Very few SNPs have been associated with keloid formation, although the ethnic predisposition to keloid scars is well-established. The first GWAS for keloids identified four SNPs at three loci in Japanese patients (Nakashima et al., 2010). Two of these loci were in regions that including protein-coding genes: FOXL2, a transcription factor, and NEDD4, an E3 ubiquitin ligase. An association with the SNP in NEDD4 was later confirmed in a Chinese population by SNP genotyping (Zhu et al., 2013), and in an African American population by admixture mapping (Velez Edwards et al., 2014). The admixture mapping study in African American patients also identified a susceptibility at MYO1E and MYO7A, both of which encode cytoskeletal proteins. A follow-up in vitro study has demonstrated a possible role for NEDD4 in influencing β-catenin signaling and expression of type I collagen and fibronectin genes in keloid fibroblasts (Chung et al., 2011).

In summary, GWAS of fibrotic skin diseases have not yet yielded breakthrough insights into the mechanism of fibrosis (either common or specific to a particular disease process). The mechanistic significance of individual susceptibility loci remain to be elucidated. It is likely that in the face of heterogeneous disease phenotypes such as SSc, further sub-classification of patients will facilitate identification of novel genetic variants that are significant in specific clinical populations.
1.5. Pro-fibrotic signaling pathways

Stimulation of fibrogenic cells by inflammatory cells and their local environment is via particular signaling pathways. Generally these signaling pathways involve secretion of an extracellular signaling protein by a recipient cell. This signal is then transduced by the recipient cell and results in a biological response (Borthwick et al., 2013; Sahin and Wasmuth, 2012). The extracellular signal in question is typically a growth factor or other cytokine, and its ability to be detected may be regulated by its binding to extracellular proteins (Schaefer and Iozzo, 2008; Schultz and Wysocki, 2009). The cellular response may involve altered transcription of specific genes (Grgic et al., 2014; Hannivoort et al., 2012). In this way, extracellular signals can control cell behaviors like proliferation and also production of important pro-fibrotic molecules like adhesion factors and extracellular matrix proteins (Abergel et al., 1987; Ghosh, 2002; Jinnin, 2010). There are several prototypic signaling pathways associated with fibrosis (Friedman et al., 2013). Increased activity of these pathways has been observed in fibrotic disease conditions, and they have been shown using animal models to be sufficient to cause fibrosis (Artlett, 2010; Derrett-Smith et al., 2009; Smith and Chan, 2010).

Because fibrosis in skin and other organs is very difficult to treat effectively, fibrosis research has focused on identifying pro-fibrotic factors and signaling pathways in hopes of finding novel therapeutic targets (Friedman et al., 2013; Wei et al., 2011a). Fibrosis involves altered signaling activity between all of the cell populations
described in section 1.3 (Denton et al., 2006; Hogaboam et al., 1998; White and Mantovani, 2012; Wynn, 2008; 2007). Inflammatory cells and fibroblasts, in particular, actively secrete signaling molecules that contribute to the development and maintenance of pathologic fibrosis (see section 1.3.4). It is important to determine how particular pro-fibrotic signaling pathways result in fibrosis since they provide opportune points for therapeutic intervention. Small molecule inhibitors and monoclonal antibodies directed against common signaling pathways are widely available (Beyer et al., 2012b; Friedman et al., 2013). Since effective therapies for fibrotic diseases are quite limited, identifying novel fibrotic signaling mediators and testing their importance in animal models is an active area of research (Wynn, 2007).

Determining which signaling pathways are important in human fibrotic disease is often based upon assays of human tissue, often by microarray profiling as described previously. Increased expression of members of a particular signaling pathway (or decreased expression of inhibitors of that pathway) in fibrotic tissue is evidence for a role of that pathway in fibrosis. Similarly, increased expression of known target genes for that pathway suggests that the pathway may have elevated activity. These correlative findings, however, cannot conclusively demonstrate that a signaling pathway has elevated activity in fibrotic tissue, nor can it elucidate the consequences of that activity. To determine whether the particular signaling pathway is actually causing fibrosis requires the use of mouse models in which the activity level of the signaling pathway is altered. Forced activation of the signaling
pathway is used to test whether its activity is sufficient to cause fibrosis.

Conversely, inhibition of the signaling pathway in an established model of fibrosis (for example, the bleomycin-injected pulmonary fibrosis model) is used to test whether the pathway is required for fibrosis. Such experiments have validated a pro-fibrotic role for several cytokines, including several interleukins and growth factors, in various tissues (Friedman et al., 2013). However, few of these fibrogenic signaling molecules have yielded therapeutic benefit when targeted by small molecule inhibitors or neutralizing antibodies. For example, neutralizing antibodies against transforming growth factor (TGF)-β have not been successful in treatment of systemic sclerosis (Varga and Pasche, 2009). A promising therapeutic strategy currently being investigated is tyrosine kinase inhibition, which inhibits activity of multiple pathways including platelet derived growth factor (PDGF) signaling. Due to the lack of established, effective anti-fibrotic therapies, there is great interest on the part of patients, physicians, and pharmaceutical companies in the continued investigation of novel pro-fibrotic signaling pathways as potential therapeutic targets.

1.5.1. Pro-fibrotic signaling molecules

Observations in fibrotic human tissues and functional studies in animal models of fibrosis have identified many pro-fibrotic signaling factors. These include interleukins and other inflammatory cytokines which have very important roles in fibrosis, but which are not closely related to my research and so are not discussed in
The pro-fibrotic roles of transforming growth factor (TGF)-β, platelet-derived growth factors (PDGFs), and connective tissue growth factor (CTGF/CCN2) are the most relevant to my research.

**Transforming growth factor (TGF)-β**

TGFβ is the prototypic pro-fibrotic signaling factor, and has been extensively studied in human disease conditions and animal models (see also section 1.3.1 and section 4.4.3). The TGFβ ligand family comprises three isoforms; TGFβ1 is the most abundant isoform in adult tissues. TGFβ3 is more abundant in embryonic tissues, and is associated with fetal scarless wound healing (Walraven *et al.*, 2014). The ligand is secreted in a complex with a latent binding protein, which must be proteolytically cleaved to yield active extracellular ligand (Ashcroft, 1999). The active ligand binds to cell surface receptors, which are heterotrimers of TGFβ receptor I and TGFβ receptor II (TGGBRI and TGFBRII), stimulating their serine-threonine kinase activity (Werner and Grose, 2003). Downstream intracellular transducers of TGFβ signaling activity include the canonical Smad2/Smad3 response as well as non-canonical pathways (Varga and Pasche, 2009).

The TGFβ ligand is expressed and secreted by platelets (Werner and Grose, 2003), activated T lymphocytes (Roberts *et al.*, 1986), monocytes and macrophages, fibroblasts, neutrophils, & epithelial cells (Ashcroft, 1999). It is detected by neutrophils, macrophages, and fibroblasts (Werner and Grose, 2003). The functions of TGFβ are pleiotropic and sometimes even contradictory; depending on
the cellular context, its effects may be both pro- and anti-fibrotic (Wynn, 2008) and pro- and anti-inflammatory (Ashcroft, 1999). In the settings of fibrosis and wound healing, the prominent functions of TGFβ include promoting monocyte activation, chemotaxis, and cytokine production (Ashcroft, 1999) and fibroblast proliferation, expression of ECM proteins and integrins, and myofibroblast conversion (Desmoulière et al., 1993; Werner and Grose, 2003).

Elevated expression of TGFβ ligand and/or receptors is broadly evident in fibrotic tissues including systemic sclerosis lesion skin, hypertrophic scars, and keloid scar fibroblasts (Gabrielli et al., 1993; Pakyari et al., 2013; Wang et al., 2000). In systemic sclerosis primary fibroblasts, a TGFβ-responsive gene signature is correlated with more severe disease, providing rationale for this signaling pathway as a therapeutic target (Sargent et al., 2009). In a transgenic mouse model, expression of constitutively active TGFβ type I receptor in fibroblasts is sufficient to cause dermal fibrosis (Sonnylal et al., 2006). Conversely, inhibition of TGFβ signaling results in reduced matrix deposition and scar formation in a rat model of wound healing (Shah et al., 1994), and prevents skin and lung fibrosis in the mouse model of sclerodermatous GVHD (McCormick et al., 1999). Deletion of TGFBR2 expression in fibroblasts of transgenic mice results in altered wound healing including reduced collagen deposition with increased macrophage infiltration and accelerated re-epithelialization and wound closure (Martinez-Ferrer et al., 2010). This complex phenotype highlights the importance of TGFβ signaling in regulating a variety of cellular behaviors in vivo.
Due to its many pro-fibrotic effects on both fibroblasts and macrophages, TGFβ signaling is of great therapeutic interest for treatment of fibrosis in skin and other organs. However, neutralizing antibodies against TGFβ have not been successful in clinical trials (Varga and Pasche, 2009). Alternative strategies for inhibiting the pro-fibrotic effects of TGFβ include precisely manipulating other mediators of this pathway in order to avoid off-target effects of ligand inhibition (Bhattacharyya et al., 2011) and combinatorial therapies involving inhibition of both TGFβ and other pro-fibrotic signaling pathways, including canonical Wnt signaling (Distler et al., 2014b).

**Platelet-derived growth factors (PDGFs)**

PDGFs are secreted growth factors that bind to cell surface receptors that are homo- or heterodimers of PDGFRα and PDGFRβ. The PDGFRs are receptor tyrosine kinases that, when activated, trigger downstream signaling cascades including the Ras/MAP kinase (Erk) pathway. PDGF ligands are produced by fibroblasts, endothelial cells, macrophages, and platelets (Sakkas et al., 2006). Fibroblasts express the PDGF receptor and respond to this signal with increased proliferation, migration, expression of extracellular matrix genes including collagen-encoding genes, and conversion to a myofibroblast phenotype (Oh et al., 1998). Murine dermal fibrosis is associated with activation of PDGF receptors, presumably due to increased production or availability of PDGF ligands (Kavian et al., 2012), and possibly downstream of TGFβ signaling (Distler et al., 2006). In systemic sclerosis, activation of PDGF receptors can occur in response to stimulatory autoantibodies.
against the receptor. These autoantibodies are able to stimulate tyrosine kinase activity, type I collagen gene expression, and myofibroblast conversion in human fibroblasts in vitro (Baroni et al., 2006) and therefore may be an important factor in the etiology of systemic sclerosis.

A study in transgenic mice found that expression of activated Pdgfra is sufficient to cause fibrosis in skin and other organs (Olson and Soriano, 2009). Many more studies have used readily available tyrosine kinase inhibitors (TKIs), which block PDGF signaling and other related pathways, to test for a possible therapeutic benefit of PDGF inhibition (Beyer et al., 2012b). Imatinib mesylate, a TKI, inhibits PDGF signaling and reduces fibroblast proliferation, migration, collagen gene expression, and myofibroblast conversion in mouse wound healing and fibrosis models (Rajkumar et al., 2006). However, some of the in vivo effects of imatinib and other TKIs may be due to inhibition of c-Abl or other targets (Akhmetshina et al., 2008; Daniels et al., 2004). The therapeutic effects of imatinib against pre-existing dermal fibrosis in mouse models (Akhmetshina et al., 2009) provided strong rationale for studies of its efficacy in scleroderma skin fibrosis. However, a phase II clinical trial did not demonstrate clinical efficacy over 6 months of treatment (Prey et al., 2012). Additional studies addressing the pro-fibrotic functions of specific tyrosine kinase receptors and the specificities of particular TKIs against these receptors will perhaps yield additional therapeutic options in the future.
Connective tissue growth factor (CTGF/CCN2)

CTGF is a matricellular protein that is typically secreted by mesenchymal cells, including fibroblasts and endothelial cells. Its expression is induced by TGFβ signaling as well as other pro-fibrotic signaling pathways (Leask et al., 2009). In the extracellular space, CTGF can bind to integrins as well as other secreted proteins, including TGFβ1 (Dziadzio et al., 2005). From these properties, it follows that CTGF can mediate fibroblast and endothelial cell adhesion and potentially TGFβ1 ligand bioavailability (Blom et al., 2002; Dziadzio et al., 2005). In human fibroblasts in vitro, CTGF is sufficient to cause increased collagen and fibronectin synthesis (Shi-Wen, 2000).

CTGF has elevated expression in lesional fibroblasts and plasma from systemic sclerosis patients, and its levels correlate with severity of fibrosis, suggesting possible utility as a biomarker of fibrotic disease (Dziadzio et al., 2005; Leask et al., 2009; Shi-Wen, 2000). The role of CTGF in fibrosis and wound healing has been tested using multiple animal models. In murine models of skin fibrosis, CTGF over-expression in fibroblasts is sufficient to cause dermal, renal, and pulmonary fibrosis; and deletion of CTGF in fibroblasts protects against bleomycin-induced dermal fibrosis (Liu et al., 2010; Sonnylal et al., 2013). CTGF has elevated expression in healing wounds in rats during granulation and vasculogenesis (Igarashi et al., 1993). Finally, inhibition of CTGF mRNA in a rabbit model of scar formation resulted in reduced hypertrophic scarring with lower numbers of myofibroblasts, but with
maintenance of normal wound healing with respect to inflammation and angiogenesis (Sisco et al., 2008).

1.6. Canonical Wnt signaling in skin fibrosis

Canonical Wnt signaling, like the pro-fibrotic pathways discussed above, is a pathway that links extracellular signals to context-specific changes in gene expression (section 1.6.1). Wnt signaling has a well-documented role in the embryonic development of dermal fibroblasts and in dermal wound healing, and its dys-regulation is associated with fibroproliferation in desmoid tumors (section 1.6.2). Involvement of the Wnt pathway in skin fibrosis was initially suggested by the altered expression of genes encoding Wnt ligands, pathway inhibitors, and other related proteins in scleroderma and keloid tissues (section 1.6.3). Its possible roles in the context of fibrosis include regulating fibroblast proliferation and pro-fibrotic gene expression, although until recently these roles had not been functionally tested (section 1.6.4).

1.6.1. Wnt/β-catenin signaling is a gene-regulatory pathway

My research focus is the pro-fibrotic role of β-catenin, which is the central transducer of the canonical Wnt signaling pathway. β-catenin is an intracellular protein with two primary functions: it participates in cell adhesion by direct interaction with membrane-associated cadherins, and acts as a signal transduction
molecule in gene-regulatory pathways (McEwen et al., 2012). β-catenin was originally discovered as a protein associated with E-cadherin in mouse, human, and fibroblast cell lines (Ozawa et al., 1989). *Xenopus* β-catenin was determined to be a homolog of the product of *Drosophila* gene *armadillo* (McCrea et al., 1991), which interacts with *wingless* in *Drosophila* development and pattern formation (Peifer et al., 1991). The gene *wingless* is the homolog of murine *int-1*, a proto-oncogene activated by an oncogenic retrovirus in a mouse breast cancer model (Nusse and Varmus, 1982; Rijsewijk et al., 1987). The mammalian gene *int-1* encodes a Wnt ligand and is now known as *Wnt1*; the expanding *int* gene family was renamed *Wnt* in 1991 (Nusse et al., 1991; Nusse and Varmus, 2012). The homology between *armadillo* gene product (Arm) and β-catenin provided early evidence that β-catenin might have a role in an intercellular signaling pathway. Epistasis experiments in *Drosophila* later showed that *armadillo* acts downstream of *wingless* and affects gene expression (Noordermeer et al., 1994; Siegfried et al., 1994). Further epistasis experiments and characterizations of protein-protein interactions during the 1990s have elucidated the Wnt/β-catenin signaling pathway as it is understood currently and described here (Nusse and Varmus, 2012).

Wnt/β-catenin signaling takes place as follows: In the cytoplasm, the pool of β-catenin available for Wnt signal transduction is regulated by a complex of proteins that includes Adenomatous polyposis coli (APC), Axin-2, Glycogen synthase kinase 3-β (GSK3-β), and Casein kinase 1 (CK1)-α (Archbold et al., 2011; Nelson and Nusse, 2004). In the absence of Wnt signaling activity, CK1-α and GSK3-β tag cytoplasmic
β-catenin for ubiquitylation-mediated degradation by phosphorylating key serine and threonine residues. Inhibition of β-catenin phosphorylation, therefore, is essential for maintaining its active presence in the cytoplasm.

β-catenin is “active” or stabilized in the presence of extracellular Wnt signals that result in inhibition of degradation of β-catenin in the cytoplasm (Figure 5). There are nineteen Wnt ligands in mice and humans (Miller, 2002; van Amerongen and Nusse, 2009). Specific Wnt ligands have previously been associated with

![Figure 5. Degradation (left) and stabilization (right) of β-catenin by canonical Wnt signaling. Reproduced from Subcellular Biochemistry 60 (McEwen et al., 2012) Figure 8.2, copyright 2012, with kind permission from Springer Science and Business Media.](image-url)
“canonical” or “non-canonical” signaling activity. For example, Wnt3a is commonly thought of as a canonical ligand, while Wnt5a is a prototypic non-canonical ligand. However, this view may be overly simplified, since individual Wnt ligands have diverse receptor interactions and intracellular consequences which do not always fall neatly into the “canonical” and “non-canonical” categories (Willert and Nusse, 1998). Thus, the “canonical” Wnt signaling cascade is not defined by the inciting Wnt ligands, but solely by its effect upon β-catenin (van Amerongen and Nusse, 2009).

The canonical (β-catenin-dependent) Wnt signaling cascade is initiated when extracellular Wnt ligands bind to Frizzled (Fz) family transmembrane receptors. These receptors interact with Lrp family co-receptors (Lrp-5 or -6) to transmit the Wnt signal to the intracellular compartment. Interactions between the intracellular domains of Fz and Lrp coreceptors and Fz binding partner Dishevelled (Dvl) result in inhibition of the APC/Axin-2/GSK3-β destruction complex and stabilization of cytoplasmic β-catenin (Fuerer et al., 2008; Nusse, 2008). GSK3-β can also be inhibited independently of Wnt signaling. Activation of receptor tyrosine kinases (RTKs) results in a signaling cascade (mediated by PI3 kinase, PDK1/2, and Akt) that results in an inactivating phosphorylation of GSK3-β by Akt. Therefore, in the presence of growth factors like PDGFs and insulin-like growth factors (IGFs) which signal via RTKs, β-catenin can be stabilized (Bowley et al., 2007) (Figure 5). Similarly, inhibition of GSK3-β can be mediated by activation of integrin-linked kinases, for example by binding of extracellular fibronectin to β1 integrins (Bielefeld
et al., 2011). Alternatively to GSK3-β inhibition, active β-catenin accumulates in the cytoplasm as a consequence of loss-of-function somatic mutations of APC (which causes familial adenomatous polyposis and is associated with formation of desmoid tumors) (Alman et al., 1997a; Li et al., 2010). Thus, β-catenin signal transduction activity can result from canonical Wnt signaling or from activation of other signaling pathways that inhibit members of the β-catenin degradation complex.

After β-catenin accumulates in the cytoplasm, it translocates to the nucleus and binds to T cell factor/lymphoid enhancer factor (Tcf/Lef) cofactors to regulate the transcription of target genes in a cell type-specific manner (Archbold et al., 2011). The cell type-specific response to Wnt/β-catenin activity is mediated at several levels within the signaling cascade, including by ligand and receptor specificity and binding of cofactors to the Wnt receptor complex (van Amerongen and Nusse, 2009). Wnt ligand secretion, which requires the transmembrane protein Wntless (Wls), and bioavailability for signaling, likely mediated by interaction with extracellular matrix proteins, provides additional opportunities for regulation of pathway activity (Willert and Nusse, 2012). Intracellularly, the dosage of β-catenin affects target gene expression (Rudloff and Kemler, 2012).

At the level of the nucleus, tissue-specific gene expression is mediated by β-catenin complex formation with specific Tcf/Lef transcription factors (Mao and Byers, 2011) and binding to CBP, p300, and other nuclear cofactors (MacDonald et al., 2009; Teo and Kahn, 2010). Wnt/β-catenin activity is also regulated by endogenous
inhibitors: Secreted frizzled-related proteins (SFRPs) and Wnt inhibitory factors (WIFs), which bind to and inhibit extracellular Wnt ligands, and Dickkopf (Dkk) proteins, which inhibit LRP coreceptor activity. Wnt/β-catenin activity can induce gene expression of its own ligands, receptors, and inhibitors, including members of the β-catenin destruction complex like Axin2, thereby regulating its own activity via context-specific positive or negative feedback (Clevers, 2006; Logan and Nusse, 2004; van Amerongen and Nusse, 2009). Wnt/β-catenin signaling can also be mediated by input from broader signaling networks; these networks include profibrotic signaling pathways such as canonical TGFβ signaling. Specifically, TGFβ signaling can induce formation of a nuclear complex of Smad3, β-catenin, and p300 in the context of epithelial-to-mesenchymal transition and idiopathic pulmonary fibrosis (Zhou et al., 2012). Cumulatively, these and other points of regulation converge on nuclear β-catenin activities to precisely regulate a cell type-specific transcriptional response.

1.6.2. Context-specific roles for β-catenin activity in dermal fibroblasts

β-catenin activity is present in fibroblasts in multiple physiological contexts, and has been shown to have a gene-regulatory role in some of these contexts. During embryonic development of the mouse dermis, β-catenin is required for dermal cell specification, survival, and proliferation, and also for Tcf/Lef reporter activity (Atit et al., 2006; Chen et al., 2012; Ohtola et al., 2008; Tran et al., 2010). This supports a gene-regulatory role for β-catenin in developing dermal fibroblasts. In adult mouse
dermis, Tcf reporter activity has been demonstrated during dermal wound healing (Cheon et al., 2004), and over-expression of β-catenin in skin is sufficient for increased fibroblast proliferation (Cheon et al., 2002). This supports a gene-regulatory role for β-catenin in the adult dermis in the context of wound healing. However, the wound-healing β-catenin over-expression study relied upon a globally expressed tetracycline-inducible β-catenin transgene, and so was not tissue-restricted to fibroblasts or any other particular cell type. In my research I have addressed several remaining questions regarding the role of β-catenin in adult dermal fibroblasts, including the activity level of Wnt/β-catenin signaling in healthy adult mouse dermis, the requirement for β-catenin in adult dermal fibroblasts, and consequences of elevated β-catenin activity specifically in dermal fibroblasts.

1.6.3. Roles for elevated Wnt/β-catenin signaling in fibrotic tissues

Recently, dysregulation of canonical Wnt/β-catenin signaling has been observed in the skin of patients with fibrotic skin disorders as well as in animal models of skin fibrosis. The existing body of evidence is consistent with over-activation of Wnt signaling resulting in increased nuclear β-catenin localization. Canonical Wnt ligands are over-expressed in systemic sclerosis tissue (Beyer et al., 2012a; Wei et al., 2011b), and increased nuclear β-catenin by immunohistochemistry has also been shown in systemic sclerosis skin (Beyer et al., 2012a; Wei et al., 2012). In keloid scar tissue, increased nuclear and cytoplasmic β-catenin has been demonstrated by immunohistochemistry (Sato, 2006). Therefore, elevated β-
catenin nuclear localization could be a consequence of elevated Wnt ligand expression. Also consistent with over-activation of Wnt signaling, some negative regulators of Wnt/β-catenin signaling have decreased expression fibrotic skin disease. For example, microarray analysis of systemic sclerosis patient skin biopsies has shown a significant decrease in expression of Wnt inhibitory factor 1 (WIF1) (Gardner et al., 2006; Whitfield et al., 2003). There is also evidence of increased activation of Wnt/β-catenin signaling in animal models of fibrosis. Most recently, increased nuclear β-catenin has been described in dermal fibroblasts of the tight-skin (Tsk1/+) and bleomycin-induced models of skin fibrosis (Bergmann et al., 2011). In addition, the tight-skin mouse has increased expression of canonical ligands Wnt2a and -9 as well as endogenous pathway inhibitors SFRP2 and -4 (Bayle et al., 2008). Tight-skin mouse skin also demonstrates increased Wnt10b, another canonical ligand, by immunohistochemistry (Wei et al., 2011b).

Collectively, this dys-regulation of Wnt signaling components in fibrotic tissues provides strong rationale for a pro-fibrotic role of this pathway. I have contributed to the body of evidence for this rationale by further examining the extent and location of elevated β-catenin nuclear localization in multiple fibrotic skin diseases.

1.6.4. Consequences of elevated β-catenin signaling in skin fibrosis

My research has addressed the functional role(s) for the Wnt/β-catenin pathway in dermal fibroblasts and the sufficiency of stabilized β-catenin signaling in dermal fibroblasts to cause skin fibrosis. Previous experiments that tested of the
consequences of elevated β-catenin activity in cultured fibroblasts showed that β-catenin is sufficient for increased fibroblast proliferation, motility, and migration (Cheon et al., 2002). This is consistent with the fibroproliferative role for β-catenin in the embryonic dermis (Chen et al., 2012). Human desmoid tumors, which are markedly fibroproliferative, are associated with increased amounts of intracellular β-catenin and stabilizing somatic mutations in β-catenin (Alman et al., 1997a; Li et al., 2010; Tejpar et al., 1999). Therefore, it is possible that elevated β-catenin nuclear localization contributes to increased fibroblast proliferation in skin fibrosis.

The role of pro-fibrotic β-catenin might not be limited to solely regulating fibroblast proliferation, however. In cultured primary human dermal abdominal fibroblasts, increased canonical Wnt signaling (by addition of exogenous Wnt3a ligand) is sufficient for β-catenin translocation to the nucleus and significantly altered expression of 215 genes (Klapholz-Brown et al., 2007). The up- and down-regulated genes in this context encode secreted factors including fibroblast growth factors (FGFs), positional identity markers, and at least one marker of smooth muscle cell and myofibroblast differentiation (CALD1). Therefore, β-catenin may exert pro-fibrotic effects by increasing fibroblast proliferation and motility but also by regulating expression of other pro-fibrotic mediators.

1.7. Summary and hypothesis
Fibrosis is a pathologic response to injury characterized by altered fibroblast behavior and accumulation of abnormal extracellular matrix. In skin, fibrosis occurs as a result of several disease processes, including scleroderma and pathologic scarring. Tissues from these patients and experiments using murine models of skin fibrosis have demonstrated how complex abnormalities in the extracellular environment, fibroblast behavior, activity of pro-fibrotic signaling pathways, and gene expression contribute to fibrosis. Some of these characteristics of fibrosis may be regulated by the canonical Wnt/β-catenin signaling pathway, which has important roles in embryonic development of the dermis and altered expression levels in fibrotic skin.

I hypothesize that expression of stabilized β-catenin, the central mediator of canonical Wnt signaling, in dermal fibroblasts is sufficient to cause skin fibrosis. I have tested this hypothesis by inducing expression of stabilized β-catenin specifically in dermal fibroblasts of mice (Chapter 2). The resulting fibrotic phenotype suggests roles for β-catenin in regulating fibroblast proliferation (Chapter 2) and expression of extracellular matrix proteins (Chapter 3). In relation to the major questions outlined in section 1.1, my research has addressed the ability of a subset of resident dermal fibroblasts to contribute to experimental fibrosis; the dermal gene expression changes that occur as a result of β-catenin stabilization in dermal fibroblasts; and the interaction between β-catenin and classic pro-fibrotic signaling pathways.
2. SUSTAINED β-CATELIN ACTIVITY IN DERMAL FIBROBLASTS IS SUFFICIENT FOR SKIN FIBROSIS

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2.0.1. Contributions

This published manuscript was written in its entirety by EH, with substantial editorial assistance by RA. EH generated and assembled all figures. EH and RA conceived the experiments; EH was responsible for data acquisition with assistance from RA in obtaining signal counts in immunofluorescence/immunohistochemistry experiments. EH and RA analyzed and interpreted the data. All authors had final approval of the submitted version.

2.0.2. Manuscript review

Upon initial submission to the Journal of Investigative Dermatology, this manuscript received favorable reviews. The general suggestions from the three reviewers were to (1) improve the quality of the existing data and (2) add comments to specifically address the relevance to human disease, the role of TGFβ, and the role of the hypodermis in dermal fibrosis. The re-submitted and accepted version of the manuscript addressed these questions by the requested text discussion and updated figures with improved photographs and annotations for immunofluorescence and immunohistochemistry experiments.
2.1. Introduction

Fibrosis is a pathologic process in which deposition of abnormal extracellular matrix by fibroblasts leads to the loss of normal tissue structure and function. In skin, fibrosis is associated with several diseases, including systemic sclerosis, localized scleroderma, nephrogenic systemic fibrosis, and keloid scars. Currently, effective targeted therapies to reverse or prevent fibrosis are lacking.

Canonical Wnt/β-catenin signaling is essential for survival and specification of dermal fibroblasts during development (Ohtola et al., 2008) and has recently been implicated as a pro-fibrotic pathway in skin (Lam and Gottardi, 2011). Central to this pathway is the nuclear translocation of stabilized β-catenin for transcriptional regulation of cell type-specific target genes (van Amerongen and Nusse, 2009). Importantly, stabilization of β-catenin depends upon inhibition of GSK3-β via canonical Wnt signaling or indirect mechanisms (Bowley et al., 2007). Evidence for aberrantly increased Wnt/β-catenin signaling activity has been observed in systemic sclerosis skin, keloid scars, and desmoid tumors as well as in the tight-skin (tsk1/+) mouse models of skin fibrosis (Akhmetshina et al., 2012; Alman et al., 1997a; Beyer et al., 2012a; Bowley et al., 2007; Sato, 2006; Wei et al., 2012). Wnt signaling has also been implicated in thickening of the tsk1/+ mouse hypodermis region and in transdifferentiation of adipocytes to myofibroblasts leading to skin fibrosis (Akhmetshina et al., 2012; Wei et al., 2011b), but the role of hypodermal fibroblasts in skin fibrosis remains
unclear. Recent studies have uncovered a potential pro-fibrotic mechanism for Wnt signaling by which a complex interplay between β-catenin and TGFβ signaling regulates type I collagen α1 chain (Col1a1) mRNA expression in fibroblasts in vitro and collagen accumulation in the skin of experimental models of fibrosis (Akhmetshina et al., 2012; Wei et al., 2012). Collectively, these data demonstrate that the Wnt/β-catenin pathway can interact with TGFβ signaling to mediate fibrosis.

2.2. Results and discussion

Here, we show that skin affected by localized scleroderma (morphea) has an increased percentage of nuclear β-catenin-positive dermal fibroblasts compared to healthy control skin in papillary (n=4, p=0.04; Figure 6A and 6C) and reticular dermis (n=1; Figure 6B and 6C). This new finding is consistent with the recent observation of increased nuclear β-catenin in dermal fibroblasts of patients with systemic sclerosis (Beyer et al., 2012a; Wei et al., 2012). Thus, our data contribute to the growing body of evidence that supports a common role for β-catenin activity in dermal fibroblasts across multiple fibrotic skin diseases.

Based on this observation, we investigated the sufficiency of the Wnt signaling pathway for fibrogenesis. We used the tamoxifen-dependent HoxB6Cre (HoxB6Cre-ER7) line to conditionally activate the R26R-YFP lineage tracer and remove exon 3 of endogenous β-catenin (CatnbΔex3/+), resulting in the stabilization of β-catenin in
ventral dermal fibroblasts (Harada et al., 1999; Nguyen et al., 2009; Srinivas et al., 2001) (Figure 9A). Pregnant mice carrying control HoxB6CreER<sup>T</sup>/+; R26R-YFP/+ and HoxB6CreER<sup>T</sup>/+; R26R-YFP/+; Catnb<sup>Δex3</sup>/+ conditional mutant embryos were given one dose (3mg/40g body weight) of tamoxifen at E15.5, E16.5, or E17.5 to induce expression of stabilized β-catenin and YFP. YFP-positive recombined cells contributed to fibroblasts in the dermis and hypodermis and not to epidermis, skeletal muscle, adipose, or endothelial lineages in postnatal ventral skin (Figure 7A, Figure 10). HoxB6CreER<sup>T</sup>; R26R-YFP; Catnb<sup>Δex3</sup>/+ mutant cells showed a morphology consistent with fibroblasts in the dermis and the hypodermis until the latest time point analyzed (P50; Figure 8A).

HoxB6CreER<sup>T</sup>/+; R26-YFP/+; Catnb<sup>Δex3</sup>/+ mutant skin displayed a progressive increase in relative thickness of the mutant dermis and hypodermis compared to HoxB6CreER<sup>T</sup>/+; R26-YFP/+ control skin. While the thickened mutant dermis appeared histologically similar to age-matched controls, the mutant hypodermal tissue was characterized by invasion of adjacent subcutaneous muscle and adipose tissue by cords of spindle-shaped cells (Figure 7B and 8B). Measurements of mutant skin sections showed 1.4-fold thicker dermis and dramatic 3.8-fold expansion of hypodermal thickness compared to littermate controls (Figure 7C). There was a significantly greater percentage of nuclear β-catenin positive fibroblasts in mutant dermis and hypodermis compared to control dermis (Figure 7D and 8C). Fibroblast proliferation was significantly increased in mutant hypodermis compared to the control and mutant dermis (Figure 7E).
We next proceeded to characterize the thickened dermis and hypodermis in the stabilized β-catenin mutant skin. Quantification of collagen by hydroxyproline assay demonstrated 2.3- and 1.8-fold increases in collagen content of mutant skin at P50 and P100, respectively (Figure 7F). Accordingly, collagen accumulation was apparent in the mutant dermis and hypodermis by Masson’s trichrome stain (Figure 7G). CTGF/CCN2, a marker of fibrosis (Holmes, 2001), was expressed by dermal and hypodermal fibroblasts of P21 control and mutant skin, and was higher in the expanded mutant hypodermis (30.9 ±8.7% of fibroblasts) compared to the overlying dermis (17.8 ±5.1%; p=0.01) (Figure 7H). While there was no significant difference in the quantity or appearance of blood vessels between P50 control and mutant dermis (quantification not shown), there was a pronounced absence of blood vessels in the thickened mutant hypodermis by MECA-32 immunohistochemistry (Figure 7I).

In conclusion, taken together with previous observations in other fibrotic skin diseases, increased nuclear β-catenin immunoreactivity in skin affected by localized scleroderma strongly suggests a pro-fibrotic role for β-catenin in dermal fibroblasts. Our in vivo model of stabilized β-catenin in skin fibroblasts holds important implications for several human diseases that involve skin fibrosis, and our findings are consistent with recent studies (Akhmetshina et al., 2012; Beyer et al., 2012a; Wei et al., 2012). We have shown that restricted constitutive stabilization of β-catenin in a subset of mouse skin fibroblasts is sufficient for spontaneous,
progressive thickening of hypodermis and dermis accompanied by increased cell proliferation. In particular, the increased percentage of proliferating and CTGF-expressing fibroblasts in the hypodermal region compared to the overlying dermis of the stabilized β-catenin mutant skin demonstrates that expression of stabilized β-catenin in a distinct subpopulation of fibroblasts can substantially contribute to an overt skin fibrosis phenotype. This finding expands upon previous studies that have suggested a role for Wnt/β-catenin in the hypodermis of fibrotic skin (Akhmetshina et al., 2012; Bayle et al., 2008; Wei et al., 2011b). Additional studies are required to determine the genetic interactions between β-catenin and other pro-fibrotic signaling pathways during the development of β-catenin-dependent skin fibrosis. We will utilize the stabilized β-catenin model to define interactions between β-catenin and other important mediators of fibrosis like TGFβ/Smad2/3, CTGF, PTEN, and MAPK/ERK and to identify target genes that functionally mediate the pro-fibrotic effects of stabilized β-catenin. Ultimately, these studies will be critical for developing novel therapeutic approaches to prevent and treat fibrotic skin diseases.

2.3. Acknowledgements

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Award from the March of Dimes (R.A.), Scleroderma Research Foundation (R.A.), NIH T32 GM07250 (E.H.), and NIH TL1 RR02499 (E.H.). All animal work was done in compliance with IACUC regulations at Case Western Reserve University. Access to archived skin biopsies from localized scleroderma patients was obtained in compliance with Case Western Reserve University Institutional Review Board for Human Studies.
2.4. Figures

**Figure 6.** Nuclear β-catenin immunoreactivity in skin affected by localized scleroderma. Brightfield immunohistochemistry using anti-β-catenin antibody (BD Bioscience, 1:100) was performed on sections of affected skin from 4 localized scleroderma patients and normal skin from 4 healthy controls. (a) Representative images with insets showing detail from papillary dermis of healthy control and localized scleroderma affected skin. β-catenin-positive nuclei indicated by filled arrowheads. (b) Reticular dermis of control (representative image) and localized scleroderma skin that had fibroblasts in reticular dermis. (c) The percentage of nuclear β-catenin-positive fibroblasts in papillary and reticular dermis was calculated from 2-3 high-power fields for each sample. Scale bar length 50 μm.
Figure 7. Characterization of stabilized β-catenin phenotype. (a) Immunofluorescence using anti-GFP antibody against YFP, P50 (Aves Labs, 1:250). epi, epidermis; hf, hair follicle. (b) H&E-stained skin at P4, P22, and P50. hd, hypodermis; adip, adipose. (c) P50 dermal and hypodermal thickness quantified by Photoshop measurement tool; n = 15 controls, 15 mutants. (d) Percentage of nuclear β-catenin-positive fibroblasts in P50 skin; n = 7 controls, 7 mutants; p=0.002. HDF, hypodermal fibrosis. (e) P22 cell proliferation assessed by anti-Ki67 immunofluorescence (Abcam 15580, 1:500); n = 3 controls, 3 mutants. (f) Hydroxyproline content per 5-mm punch biopsy of P50 and P100 control and mutant ventral skin. (g) Masson’s trichrome stain of P50 skin. (h) Anti-CTGF (Abcam 6992, 1:400) brightfield immunohistochemistry performed on P21 skin (n=5 each controls and mutants). Arrowheads indicate CTGF-positive cells. (i) Brightfield immunohistochemistry of endothelial cells in P50 skin (anti-MECA-32 antibody, Developmental Studies Hybridoma Bank, 1:10). All corresponding images were photographed at same magnification. Quantification of marker expression based on 3 high-power fields/sample.
Figure 8. Recombination, histology, and β-catenin immunohistochemistry in stabilized β-catenin dermis and hypodermis. Indirect immunofluorescence using anti-GFP antibody against YFP showed genetic recombination of dermal fibroblasts (arrowheads) in P50 control dermis and mutant dermis and hypodermis. hf, hair follicle. (b) H&E staining of β-catenin-stabilized skin showing invasive fibrous tissue (arrowheads). (c) Nuclear β-catenin-immunoreactive fibroblast nuclei (brown stain) in P51 mutant dermis and hypodermis compared to age- and littermate-matched controls. Control and mutant skin sections were photographed at the same magnification for all corresponding images.
3. SUSTAINED β-CATENIN ACTIVITY IN DERMAL FIBROBLASTS PROMOTES FIBROSIS BY UP-REGULATING EXPRESSION OF EXTRACELLULAR MATRIX PROTEIN-CODING GENES

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3.0.1. Contributions

EH and RA conceived and carried out experiments and analyzed data. RL provided human tissue samples and data. GD performed genotyping and immunohistochemistry. NM collected and analyzed data. EH generated the figures. EH and RA interpreted the data. EH wrote the manuscript with substantial editorial contribution from RA. All authors had final approval of the submitted version.

3.0.2. Manuscript review

Upon initial submission to the Journal of Pathology, this manuscript received favorable reviews. The reviewers requested discussion of several points including the collagen fibril diameter results, presence of myofibroblasts, and clarification of the mechanism of fibrosis in this model; these points of discussion were added in the re-submitted manuscript. Per the reviewers’ suggestions, the re-submitted manuscript also included (1) examination of biglycan protein distribution in the mouse model (it has previously only been evaluated in human tissue); (2) additional negative controls for immunohistochemical staining for phosphorylated Smads, mouse biglycan, and human biglycan; and (3) validation of up-regulated genes by qPCR.
3.1. Abstract

Fibrosis is an end-stage response to tissue injury that is associated with loss of organ function as a result of excess extracellular matrix (ECM) production by fibroblasts. In skin, pathologic fibrosis is evident during keloid scar formation, systemic sclerosis (SSc), and morphea. Dermal fibroblasts in these fibrotic diseases exhibit increased activity of Wnt/β-catenin, a signaling pathway that is sufficient to cause fibrosis in mice. However, in the context of this complex pathology, the precise pro-fibrotic consequences of Wnt/β-catenin activity are not known. We found that sustained expression of stabilized β-catenin in mouse dermal fibroblasts resulted in spontaneous, progressive skin fibrosis with thickened collagen fibers and altered collagen fibril morphology. The fibrotic phenotype was predominated by resident dermal fibroblasts. Genome-wide profiling of the fibrotic mouse dermis revealed elevated expression of matrix-encoding genes, and the promoter regions of these genes were enriched for Tcf/Lef family transcription factor binding sites. Additionally, we identified 32 β-catenin-responsive genes in our mouse model which are also over-expressed in human fibrotic tissues and poised for regulation by Tcf/Lef family transcription factors. Therefore, we have uncovered a matrix-regulatory role for fibroblast β-catenin activity in vivo and have defined a set of β-catenin-responsive genes with relevance to fibrotic disease.
3.2. Introduction

Fibrosis is the formation of scar tissue in response to tissue injury by wounding or a disease process. It is characterized by excessive extracellular matrix (ECM) deposition by abnormal fibroblasts, which leads to tissue sclerosis and loss of organ function. This end-stage pathology is irreversible and is associated with substantial morbidity and mortality (Bitterman and Henke, 1991). Fibrosis is a consequence of diseases of visceral organs and connective tissues, including idiopathic pulmonary fibrosis, chronic kidney disease, chronic liver disease, and systemic sclerosis (SSc).

Fibroblasts are the key effector cell of fibrosis and can be derived from several potential sources: resident fibroblasts, pericytes, endothelial cells, epithelial cells, and circulating fibrocytes (Krenning et al., 2010; Postlethwaite et al., 2004). However, the relative contributions of these fibrogenic populations are not well understood and likely vary between diseases and affected tissues.

The skin is an advantageous organ in which to study fibrosis. The dermis of the skin is predominated by numerous resident fibroblasts, providing a population of healthy cells that can be directly compared to their abnormal counterparts in fibrotic skin. Resident dermal fibroblasts are a heterogeneous population of cells that are important for normal wound healing (a physiologic fibrotic process) (Driskell et al., 2013). Pathologic dermal fibrosis is evident in several diseases, including systemic sclerosis, nephrogenic systemic fibrosis, chronic graft versus host disease, and abnormal wound repair resulting in keloid or hypertrophic
scarring. For some of these fibrotic conditions, there are publicly available microarray datasets which allow comparative analysis of altered gene expression in patient skin biopsies and skin-derived fibroblasts. Such studies have used fibrotic gene expression signatures to subtype patients’ responses to therapy and to identify infiltrating cell populations and dysregulated signaling pathways (Hinchcliff et al., 2013; Milano et al., 2008; Pendergrass et al., 2012; Russell et al., 2010; Smith et al., 2007; Whitfield et al., 2003). However, little is known about the gene expression changes that result from manipulating specific cell types and signaling pathways in animal models of fibrosis.

Several pro-fibrotic signaling cascades are required and sufficient for fibrosis in animal models, most notably the canonical TGFβ pathway (Denton et al., 2003), but such findings have not yet successfully translated to clinical use. Consequently, there is ongoing interest in novel pro-fibrotic molecules as well as interactions between signaling pathways (Bhattacharyya et al., 2011; Distler et al., 2014b). We and others have shown that expression of a non-degradable form of β-catenin (stabilized β-catenin) in the mouse dermis is sufficient for spontaneous skin fibrosis with elevated fibroblast proliferation (Beyer et al., 2012a; Hamburg and Atit, 2012). In addition, inhibition of Wnt/β-catenin activity protects against fibrosis in kidney and skin (Beyer et al., 2013; Surendran, 2005). Specifically in fibroblasts, Wnt/β-catenin activity can drive proliferation and motility and induce broad changes in expression of genes that encode transcription factors and proteins involved in other signaling pathways (Cheon et al., 2002; 2004; Klapholz-Brown et al., 2007). Wnt/β-
catenin signaling is active in the presence of extracellular Wnt ligands, which induce a cascade of intracellular responses that result in stabilization of cytoplasmic β-catenin. Stabilized β-catenin, the central transducer of the pathway, then translocates to the nucleus and binds to Tcf/Lef family transcriptional cofactors to regulate target gene expression in a context-specific manner (Archbold et al., 2011). We are interested in the mechanism underlying the pro-fibrotic effects of canonical Wnt/β-catenin signaling in the context of dermal fibroblasts.

We hypothesized that expression of stabilized β-catenin in dermal fibroblasts is sufficient to cause fibrotic changes in the ECM via altered expression of ECM-encoding genes. Using our mouse model in which a restricted, traceable population of skin fibroblasts express a stabilized form of β-catenin resulting in spontaneous dermal fibrosis, we have examined matrix composition, cellular involvement, and signaling pathway activity for changes consistent with pathological fibrosis. With genomic analysis of β-catenin-induced fibrotic tissue, we have identified a set of ECM-encoding genes whose expression levels are responsive to expression of stabilized β-catenin. Finally, we related our findings to clinical fibrosis by determining which of the experimentally determined β-catenin-responsive genes are also over-expressed in human fibrotic tissues.

3.3. Materials and methods
Mice: *HoxB6CreER^T/+; R26-YFP/+; Catnb^Δex3/+* ("stabilized β-catenin") mice were generated and genotyped as previously described (Hamburg and Atit, 2012). Induced triple-transgenic stabilized β-catenin pups and double-transgenic *HoxB6CreER^T/+; R26-YFP/+* littermate controls were administered tamoxifen (Sigma-Aldrich, St. Louis, MO, USA; T5648) dissolved in corn oil via oral gavage to the pregnant dam at embryonic day E16.5 (3 mg tamoxifen/40 g maternal weight), born and aged to 3 weeks except where otherwise noted. All analyses were performed on four or more samples except where otherwise noted.

**Ethics:** All animal experiments were approved by Case Western Reserve University Institutional Animal Care and Use Committee and all procedures were in accordance with AVMA guidelines (Protocol # 2013-0156 approved 11/21/2014, Animal Welfare Assurance number A3145-01).

**Statistical analysis:** Unless noted otherwise, all statistical comparisons used two-tailed Student’s t-test and were carried out using GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA). Graphs show mean values with standard deviations.

**Histology and immunohistochemistry:** Tissue was fixed in 4% paraformaldehyde then dehydrated for cryoembedding and sectioning or fixed in 10% formalin with 1:1000 phosphatase inhibitor (Sigma-Aldrich, St. Louis, MO, USA; P5726) for paraffin embedding and sectioning. Immunohistochemical stains on paraffin sections were performed following heat-mediated citrate buffer antigen retrieval.
Paraffin sections were used for picrosirius red and Masson’s trichrome staining, performed by CWRU Cancer Center Tissue Resources Core; for immunohistochemistry using antibodies against: α-smooth muscle actin (Dako, Carpinteria, CA, USA; 1A4, 1:100), phosphoSmad2 (Cell Signaling, Beverly, MA, USA; 3101, 1:600), and phosphoSmad3 (Cell Signaling; 9520, 1:200); and for indirect immunofluorescence using anti-fibrillin-1 (kindly provided by Dr. Dieter Reinhardt, 1:300) (Tiedemann et al., 2001). Cryosections were used for indirect immunofluorescence using antibodies against: PDGFRα (R&D Systems, Minneapolis, MN, USA; AF1062, 1:150) (Driskell et al., 2013), PECAM/CD31 (BD Biosciences, San Jose, CA, USA; 550274, 1:10), perilipin (Abcam, Cambridge, MA, USA; ab3526, 1:800) and GFP for detection of YFP (Abcam; ab13970, 1:250). Appropriate species-specific secondary antibodies were used. Immunohistochemical staining was detected by ABC kit (Vector Labs, Burlingame, CA, USA) and DAB (Amresco, Solon, OH, USA). Nuclei were detected by DAPI staining for immunofluorescence (Sigma-Aldrich; Fluoroshield F6057) and hematoxylin counterstain for immunohistochemistry. Brightfield sections were viewed and photographed using a Leica DM2000 microscope and DFC490 camera (Leica, Wetzlar, DE). Fluorescent sections were viewed and photographed using an Olympus BX60 microscope and UTVIX2 camera (Olympus, Tokyo, JP). Confocal images were obtained with a Leica spinning disk confocal microscope.

**Transmission electron microscopy and collagen fibril measurement:** Tissue was fixed in 4% paraformaldehyde and 2.5% glutaraldehyde in 0.1M cacodylate
buffer, embedded, and prepared for transmission electron microscopic (TEM) imaging at the Cleveland Clinic Foundation Lerner Research Institute imaging core facility. Sections of 85nm thickness were stained with uranyl acetate, then observed with a Tecnai G2 SpiritBT electron microscope operated at 60kV (FEI, Hillsboro, OR, USA). TEM images were acquired at 54,000X total magnification. Collagen fibril diameters were measured from between 5 and 13 images per sample (depending on how many fields within the section contained cross-sectional fibrils) using MetaMorph 7.5.5 image analysis software (MDS Analytical Technologies, Sunnyvale, CA, USA).

**Dermal dissociation, fluorescence-activated cell sorting, and flow cytometry:**

Hair was clipped and ventral skin removed and held in cold calcium- and magnesium-free HBSS (Life Technologies; 14175095). The skin was minced into 1-mm² fragments using spring scissors. These fragments were digested using Liberase TL Research Grade collagenase (Roche, Basel, CH; 250 mU/mL) and hyaluronidase (Sigma-Aldrich; H3884, 0.1 mg/mL) in DMEM (Life Technologies; 11995065) for 45 minutes to 1 hour at 37°C with occasional trituration by pipetting. After digestion, the collagenase was inhibited using EDTA, and the remaining fragments of skin were removed by passing the solution through a 40-μm nylon mesh cell strainer.

For fluorescence-activated cell sorting (FACS), the extracted cells were pelleted, washed, and resuspended in 3% BSA in calcium- and magnesium-free HBSS.
containing 7-aminoactinomycin (Life Technologies, Carlsbad, CA, USA; A1310, 1:1000) to assess cell viability. Fluorescence-activated cell sorting (FACS) was carried out using iCyt Reflection (Sony Biotechnology, Champaign, IL, USA) and BD FACSARia (BD Biosciences) cell sorters. For flow cytometry, the extracted cells were pelleted, washed, and resuspended in 3% BSA/HBSS containing rat anti-PDGFRβ (Abcam; ab91066, 1 μg/100 μL) or rat anti-CD45 (Abcam; ab23910, 0.025 μg/100 μL) and incubated for 30 minutes at 4°C. The cells were pelleted, washed, and resuspended in 3% BSA/HBSS containing donkey anti-rat conjugated with Alexa Fluor 594 (Molecular Probes; A21209, 1:500) and incubated for 30 minutes at 4°C. After pelleting and washing, the cells were resuspended in 3% BSA/HBSS containing 1:1000 7-aminoactinomycin. Flow cytometry was carried out using a BD LSR II flow cytometer (BD Biosciences) with post-analysis using WinList 7 (Verity Software House, Topsham, ME, USA). Equipment, software, and technical assistance were provided by CWRU Cancer Center Cytometry Core.

Quantitative PCR: Total RNA was extracted from FACS-purified cells or whole dermis using standard Trizol/isopropanol protocol followed by RNeasy MinElute cleanup kit with DNase treatment (Qiagen, Venlo, NL; 74204, 79254). RNA was converted to cDNA using the High Capacity RNA-to-cDNA Reverse Transcription Kit (Life Technologies; 4387406) according to manufacturer's instructions. Relative mRNA quantities were determined using a StepOnePlus Real-Time PCR System (Life Technologies) and calculated using the ΔΔCt method. Taqman assays were used for detection of Axin2 (Mm00443610_m1), Col1a1 (Mm00801666_g1), Serpine1
(Mm00435860_m1), *Thbs4* (Mm03003598_s1), *Cc3/Nov* (Mm00456855_m1), and *Fbln1* (Mm00515700_m1) mRNA, measured relative to *Actb* (Mm00607939_s1) (Life Technologies). *Fbn1* mRNA quantity was measured relative to *Gadph* using SYBR Green reagents (Life Technologies). Primer sequences for detection of *Fbn1* mRNA (Bayle *et al.*, 2008): (F) CCAGACTACATGCAAGTGAACGG and (R) CCTTTCCCTGGTAACATAGGAAAGC; for *Gapdh* mRNA: (F) GCACAGTCAAGGCGAGAAT and (R) GCCTTCTCCATGGTGTTGAA.

**Dermal RNA sequencing and differential expression analysis:** Hair was clipped and ventral skin removed and held in cold PBS until all tissue was collected. The skin samples were incubated with 0.25% trypsin-EDTA (Life Technologies; 25200056) in 1.5-mL microcentrifuge tubes at 4°C overnight (14-18 hours) and then at 37°C for 45 minutes to 1 hour. The skin was rinsed in cold PBS and the epidermis removed by scraping. The intact dermis was flash-frozen in dry ice, mechanically homogenized, and lysed in Trizol (Life Technologies; 15596026) for RNA extraction according to manufacturer’s instructions.

Libraries were prepared in the CWRU Genomics Sequencing Core using TruSeq Stranded Total RNA kit (Illumina, San Diego, CA, USA; RS-122-2301). Paired-end sequencing was carried out on the Illumina HiSeq 2500 platform. The 101-base paired-end reads were mapped to mouse genome release mm10 that was downloaded from the UCSC genome database using the GSNAP program (Wu and Nacu, 2010) with 2 mismatches allowed for the full length of the reads. Only reads
that were mapped to a unique location in the mm10 genome were retained for further analysis. The numbers of raw reads that were mapped to mouse genes annotated in the RefSeq database were counted using HTSeq program (Anders et al., 2014) and subsequently used for differential gene expression analysis after normalizing the values to the total of mapped reads in each condition. Genes were considered statistically significant if they had p-values less than 0.05 after adjusting for false discovery rate with the Benjamini and Hochberg method (Benjamini and Hochberg, 1995) in a negative binomial test and showed 1.5 fold-changes between compared conditions. Normalized mapped reads are publicly accessible via Gene Expression Omnibus using accession GSE60569 (Barrett et al., 2013; Edgar et al., 2002).

**Bioinformatic analysis of differentially expressed genes:** The list of 176 differentially expressed genes was analyzed using DAVID and oPOSSUM (Huang et al., 2009a; 2009b; Kwon et al., 2012). DAVID Functional Annotation Clustering was used for gene ontology analysis of the 172 of 176 differentially expressed genes that were annotated in the DAVID database at the time of inquiry (Huang et al., 2009a; 2009b). This analysis was carried out using default settings. Transcription factor binding site (TFBS) over-representation analysis was completed using oPOSSUM mouse single-site analysis (Kwon et al., 2012). All genes in the oPOSSUM database were used as background. Using oPOSSUM default settings, we calculated the over-representation Z-scores and Fisher scores for all transcription factors with JASPAR PBM and CORE profiles (Mathelier et al., 2014) within 5000 base pairs of
transcription start sites for 173 of the 176 differentially expressed genes. GraphPad Prism 6 was used to plot corresponding Fisher and Z-scores for each TFBS profile to visualize which TFBS had the highest statistical scores, corresponding to relative enrichment of that TFBS within our set of differentially expressed genes. The PANTHER classification system (Mi et al., 2013a; 2013b) and the proteomically-defined matrisome (Naba et al., 2012a) were used to manually assign 175 of the 176 genes to descriptive categories.

**Human fibrotic tissue microarray analysis:** Microarrays publicly available via Gene Expression Omnibus (GEO) were analyzed using the built-in GEO2R tool using sample groups indicated in Table 3 (Barrett et al., 2013; Edgar et al., 2002). Over-expressed genes (fold change>1.5, p<0.05 with Benjamini and Hochberg adjustment for false discovery rate) (Benjamini and Hochberg, 1995) were compared with a list of human genes homologous to the mouse genes of interest (Huang et al., 2009b; 2008; Sayers et al., 2012). The oPOSSUM human single-site analysis tool was used to determine the number of JASPAR PBM and CORE Tcf/Lef transcription factor binding sites (TFBS) for the human gene set (Kwon et al., 2012; Mathelier et al., 2014).

3.4. **Results**

3.4.1. **Expression of stabilized β-catenin in skin fibroblasts results in fibrosis**
To test the consequences of increased canonical Wnt signaling in fibroblasts \textit{in vivo}, we induced expression of a non-degradable form of \( \beta \)-catenin (“stabilized \( \beta \)-catenin”) in a restricted, traceable population of mouse ventral skin fibroblasts (Figure 9A). We measured a >5-fold increase in relative expression of \( Axin2 \), a \( \beta \)-catenin-responsive gene, in mutant YFP-labeled fibroblasts purified by FACS (Figure 9B), consistent with increased \( \beta \)-catenin nuclear localization (Jho \textit{et al.}, 2002).

There was no difference in the relative number of YFP-labeled cells between control and mutant skin (Table 1). In control and mutant mice, the YFP-labeled cells were restricted to the ventral skin and contributed to the dermis and hypodermis. All of the YFP+ cells expressed PDGFR\( \alpha \), a pan-fibroblast marker in mouse skin (Figure 9C, Figure 10) (Driskell \textit{et al.}, 2013). We did not detect any YFP-labeled adipocytes, endothelial cells, or pericytes (Figures 10 and 11). Therefore, we define the YFP-labeled population of cells as fibroblasts that reside in the dermis and hypodermis within the skin.

In this transgenic mouse model, expression of stabilized \( \beta \)-catenin in ventral skin fibroblasts resulted in spontaneous, progressive dermal fibrosis (Hamburg and Atit, 2012). Stabilized \( \beta \)-catenin mice at 3 weeks of age had a thickened hypodermis which stained blue for collagen with Masson’s trichrome stain (Figure 9D). The hypodermis and dermis of stabilized \( \beta \)-catenin mutant animals was hypercellular compared to control dermis, consistent with our previous observation of elevated fibroproliferation in these tissues (Figure 7, Figure 9D) (Hamburg and Atit, 2012).
Therefore, expression of stabilized β-catenin in ventral dermal fibroblasts causes a skin fibrosis phenotype in the absence of any injury or other stimulus.

3.4.2. Non-dermal fibroblast cell types do not have measurable involvement in stabilized β-catenin-induced skin fibrosis

Next, to determine whether the stabilized β-catenin-induced skin fibrosis phenotype involved other cell types, we examined for the presence of myofibroblasts, which are often observed in fibrotic tissues (Hinz et al., 2010); pericytes, which have been hypothesized to contribute to fibrosis of skin and other organs (Postlethwaite et al., 2004); and monocytes, which include fibrocytes, a potential fibrogenic cell population, as well as macrophages and other hematopoietic cells (Postlethwaite et al., 2004). First, we performed immunohistochemical staining for α-smooth muscle actin (α-SMA), a marker for myofibroblasts. We found comparable expression of α-SMA in control and stabilized β-catenin mutant blood vessels, arrector pili, and hair follicles, and did not detect α-SMA+ fibroblasts in skin at age 3 weeks or 4 months (Figure 11A, Figure 12). Next, we performed flow cytometry on suspensions of single cells extracted from whole skin for PDGFRβ, a marker for pericytes, and found no statistically significant difference in the relative number of PDGFRβ+ cells between control and mutant skin at one week of age (before the appearance of the fibrotic phenotype) or 3 weeks of age (after fibrosis had developed) (Figure 11B). Similarly, we tested for infiltration of bone marrow-derived monocyte-lineage cells in the fibrotic tissue. Flow cytometry for CD45, a surface marker for monocytes,
showed no statistically significant difference in the relative number of marker-positive cells between stabilized β-catenin mutant skin and control skin at one week or 3 weeks of age (Figure 11C). These data suggest that the stabilized β-catenin-induced fibrosis phenotype is predominated by resident dermal fibroblasts, without infiltration or increased numbers of other classically pro-fibrotic cell types.

3.4.3. Stabilized β-catenin-induced skin fibrosis occurs without altered canonical TGFβ signaling

We next investigated potential crosstalk between stabilized β-catenin and canonical TGFβ signaling, which is the prototypic pro-fibrotic signaling pathway (Leask and Abraham, 2004). We found ~1.5-fold increased expression of PAI-1 mRNA, a gene responsive to TGFβ and Wnt/β-catenin signaling (He et al., 2010; Verrecchia et al., 2006), in stabilized β-catenin dermis compared to control dermis by qPCR (Figure 13A). To directly test for activation of TGFβ signaling, we performed immunohistochemical staining for phosphorylated (active) Smad2 (pSmad2) and Smad3 (pSmad3), the intracellular mediators of canonical TGFβ signaling. Comparable percentages of dermal and hypodermal cells expressed nuclear pSmad2 and pSmad3 in both control and stabilized β-catenin mutant skin at 1 week and 3 weeks of age (Figure 13B-C and Figure 14). These data suggest that β-catenin-induced fibrosis occurs independently of altered canonical TGFβ signaling.
3.4.4. Sustained expression of stabilized β-catenin in dermal fibroblasts is sufficient for abnormal ECM in vivo

The key pathological feature of fibrosis in any organ, including skin, is the accumulation of excess ECM with aberrant morphology (Hayes and Rodnan, 1971; Rockwell et al., 1989). We investigated for altered ECM morphology in the stabilized β-catenin skin at 3 weeks of age, when excess collagen is first evident in the mutant hypodermis. We found that thickened collagen fibers, visualized by picrosirius red stain viewed under crossed polarized filters, predominated in the mutant skin, with very few visible thin green birefringent fibers (Figure 15A). We measured cross-sectional fibril thickness in the dermis and hypodermis in images obtained by TEM (Figure 15B, representative images from n = 2 control and mutant animals). In the dermis, average collagen fibril diameter was reduced by 16% in the stabilized β-catenin mutant compared to control tissue (mutant diameter 86.91 +/- 0.49 nm from n = 1896 fibrils versus control diameter 103.6 +/- 0.48 nm from n = 2128 fibrils, p < 0.0001). In contrast, in the stabilized β-catenin mutant hypodermis, average collagen fibril diameter was increased by 12% compared to control tissue (mutant diameter 78.38 +/- 0.33 nm from n = 3735 fibrils versus control diameter 70.10 +/- 0.33 nm from n = 1374 fibrils, p < 0.0001). In FACS-purified stabilized β-catenin mutant cells, we measured a 1.8-fold increase in relative expression of Col1a1 mRNA, which encodes the α1 chain of type I collagen, the predominant type of collagen in skin (Figure 15C, n = 4 control and mutant animals). In addition, we found altered distribution of fibrillin-1 protein by immunofluorescent staining;
rather than being limited to the lower border of hypodermal connective tissue as in the control, fibrillin-1 immunoreactivity was observed throughout the thickened mutant hypodermis (Figure 15D). However, relative levels of Fbn1 mRNA were not significantly different between control and mutant cells (Figure 15E) (Bayle et al., 2008). Collectively, these data suggest that the stabilized β-catenin-induced fibrotic response is not only via altered expression of genes that encode ECM proteins such as Col1a1, but also by altered activity of regulators of matrix protein post-translational assembly or turnover.

3.4.5. Fibrosis resulting from expression of stabilized β-catenin is associated with increased expression of matrisome-encoding genes

To determine which matrisome-encoding genes are responsive to dermal fibroblast β-catenin levels in vivo, we performed whole transcriptome sequencing of RNA extracted from the dermis of control and stabilized β-catenin mice at 3 weeks of age (n = 3). There were 176 annotated genes that showed significantly different expression in the stabilized β-catenin dermis versus control. We confirmed increased expression of 3 of these genes, Thbs4, Ccn3/Nov, and Fbln1, in 2 additional biological replicates by qPCR (Figure 16A), and corresponding increased protein expression of CCN3/NOV by immunohistochemistry (Figure 16B). All differentially expressed genes had increased expression in the mutant dermis (fold change > 1.5, adjusted p-value < 0.05). This set of β-catenin-responsive genes was strongly enriched for gene ontology terms related to ECM and cell adhesion (Table 2). We
categorized 175 of these genes and found that 36 (20.6%) encode matrisome proteins as defined by Naba et al. (Naba et al., 2012a) (Figure 17A-B) (Table 5 in Appendix). These matrisome-encoding genes include the three whose increase expression we verified by qPCR: Thbs4, Ccn3/Nov, and Fbln1, which encode glycoproteins thrombospondin-4, CCN3/NOV, and fibulin-1. We performed over-representation analysis of the promoter regions of the 36 matrisome-encoding genes and found relative enrichment of predicted binding sites for Tcf7l2, Tcf1 (HNF1-α), Sox8, Junm2, and Irf4 transcription factors. Two predicted Smad3 binding sites were also queried in the over-representation analysis and were depleted in our matrisome-encoding gene set relative to binding sites for other transcription factors (Figure 17C). Of the 36 β-catenin-responsive matrisome-encoding genes, 16 have predicted Tcf/Lef family transcription factor binding sites (TFBSs) in their promoter regions (Figure 17D). These data show that expression of stabilized β-catenin in skin fibroblasts is sufficient for elevated dermal expression of several genes related to the ECM, and a subset of these β-catenin-responsive genes is poised for regulation via Tcf/Lef family transcription factors.

3.4.6. β-catenin-responsive genes in mouse dermis have elevated expression in human fibrotic tissues

Next, we used publicly available microarray datasets comparing fibrotic and healthy human tissues to determine which β-catenin-responsive genes were over-expressed in human disease. We generated lists of differentially expressed genes (fold change
> 1.5, adjusted p-value < 0.05) in breast and prostate cancer stroma, systemic sclerosis (SSc) skin biopsies, idiopathic pulmonary fibrosis (IPF) lung biopsies, and cultured fibroblasts from SSc skin and lung, morphea, keloid scar, IPF lung, and Dupuytren contracture (superficial fibromatosis) (Table 3). Separately, we converted our list of 176 β-catenin-responsive mouse genes to their human homologs; 5 of the mouse genes did not have corresponding human homologous gene in the two databases that we queried and were dropped from further analysis (Table 6 in Appendix). Comparison of the list of 171 remaining genes to the list of differentially expressed genes from each microarray experiment yielded an overlap of 41 genes (Table 7 in Appendix). All the overlapping genes were over-expressed in microarrays from biopsied tissue, not from cultured cells. Therefore, we have identified a list of genes whose expression responds to expression of stabilized β-catenin in the dermis and which are over-expressed in at least one type of human fibrotic tissue. Of these 41 genes, 32 have predicted Tcf/Lef family TFBSs in their promoter regions (Table 4). These disease-associated genes are both β-catenin-responsive in vivo and poised for regulation via Tcf/Lef family transcription factors. These findings suggest that increased Wnt/β-catenin signaling in fibrotic conditions such as IPF, diffuse SSc, and tumor stroma may indirectly influence broader gene expression changes by regulating expression of transcription factors Sp5 and Osr2. In addition, stabilized β-catenin may directly regulate expression of matrisome genes that encode proteins with diverse roles in the extracellular matrix, including glycoproteins such as fibulin-1, thrombospondin-4, and CCN3/NOV; proteoglycans such as biglycan; and regulatory enzymes such as matrix metalloproteinase-16.
Of the disease-associated, β-catenin-responsive, matrisome-encoding genes that we identified, we next focused on Bgn, which encodes biglycan, a small leucine-rich proteoglycan. *Bgn* is over-expressed in tumor stroma (Table 4; Table 7 in Appendix) and systemic sclerosis skin (Gardner et al., 2006). Biglycan binds to type I collagen and is associated with thick collagen fibrils (San Martin and Zorn, 2003; Schönherr et al., 1995). We found altered distribution of biglycan protein expression in the reticular dermis of diffuse SSc and in the nodules of keloid scars (Figure 18) by immunohistochemistry using antiserum that detects biglycan and its precursor, pro-biglycan. Biglycan protein expression is variably increased in the hypodermis of stabilized β-catenin mice (Figure 19). Since both SSc dermis and keloid scar tissue have elevated fibroblast nuclear β-catenin (Figure 20) (Sato, 2006; Wei et al., 2012), this finding supports a model in which nuclear β-catenin in fibroblasts up-regulates the expression of matrix-encoding genes such as *Bgn* to contribute to aberrant, fibrotic ECM.

### 3.5. Discussion

Numerous studies in human tissues and animal models have yielded many insights into the pathobiology of fibrosis. These investigations have revealed both common and distinct features of fibrosis between various tissues and diseases. By developing a tractable and cell-type restricted model to study the mechanism underlying the pro-fibrotic effects of β-catenin, we have uncovered a specific role for
β-catenin signaling in the regulation of ECM-encoding gene expression. We found that expression of stabilized β-catenin in dermal fibroblasts are sufficient for abnormal ECM morphology in the skin with concomitant increase in the expression levels of matrisome-encoding genes. This defines a novel pro-fibrotic role for elevated dermal fibroblast nuclear β-catenin. Future studies are required to confirm the pro-fibrotic role of stabilized β-catenin in mature dermal fibroblasts, since our manipulation of fetal dermal fibroblasts may have altered their development.

Studies using genetically modified mice and cell lineage tracing are able to delineate the roles of specific cell types and the relationships between these cell types in fibrotic tissue. By manipulating the stability of a single signal transduction molecule specifically in dermal fibroblasts, we have dissected out a specific role for β-catenin: to promote the expression of matrix-encoding genes that contribute to the fibrotic ECM. This broad regulatory role for β-catenin activity in fibrosis is consistent with the finding that inhibition of β-catenin is sufficient for reversal of bleomycin-induced skin fibrosis (Beyer et al., 2013). We have demonstrated that stabilized β-catenin-induced dermal fibrosis does not involve expansion of pericyte or monocyte populations, or the presence of detectable α-SMA-expressing myofibroblasts. Our result contrasts with the increase of α-SMA+ cells reported in the skin of mice with post-natal induction of stabilized β-catenin expression in fibroblasts, suggesting that the age or identity of resident dermal fibroblasts may affect the appearance of α-SMA+ cells in skin fibrosis (Beyer et al., 2012a). Myofibroblasts are well-documented in fibrosis but not universally present during pathologic fibrosis, and
may correlate with earlier disease (Hinz *et al.*, 2012; Kissin *et al.*, 2006; Lecca *et al.*, 2011). Our findings show that, in clinical fibrosis, β-catenin signaling activity in dermal fibroblasts is likely a downstream event from the involvement of these other cell types.

Several studies have addressed the question of interaction between Wnt/β-catenin signaling and canonical TGFβ signaling, showing that each pathway mediates the other pathway’s induction of collagen gene expression in whole skin (Beyer *et al.*, 2012a; Wei *et al.*, 2012). By examining expression of phosphorylated Smad2 and Smad3 proteins, we were unable to identify any changes in canonical TGFβ signaling activity in stabilized β-catenin-induced fibrosis. Moreover, the matrisome-encoding gene set that we identified in our model is also depleted for predicted Smad3 binding sites. Additional studies are required to rigorously distinguish whether β-catenin nuclear localization specifically in fibroblasts is a downstream or parallel event to canonical TGFβ signaling in fibrotic disease.

The predominant effect of expression of stabilized β-catenin in dermal fibroblasts in our system is altered ECM morphology and gene expression. This is in contrast to other mouse models of fibrosis which demonstrate cellular infiltrates, myofibroblasts, and TGFβ signaling activity in addition to increased type I collagen (Beyer *et al.*, 2012a; Derrett-Smith *et al.*, 2009; Smith and Chan, 2010). The discrepant changes in collagen fibril thickness between dermis and hypodermis suggest that β-catenin may have distinct, specific regulatory consequences between
dermal and hypodermal fibroblasts. Our results support a model of fibrosis in which the primary role for nuclear β-catenin, in addition to its known role in dermal fibroblast proliferation (Cheon et al., 2002; 2004), is to regulate expression of specific matrix-encoding genes, resulting in altered morphology, distribution, and amounts of ECM proteins. Several of the β-catenin-responsive matrisome genes that we identified in this model encode proteoglycans, glycoproteins, and matrix metalloproteinases, each of which may have distinct contributions to the fibrotic phenotype. These contributions likely include structural contribution to the bulk extracellular matrix, regulation of matrix protein turnover, regulation of collagen fibrillogenesis, sequestration or potentiation of secreted signaling factors, and altered crosslinking of extracellular proteins, which collectively result in a progressive fibrotic phenotype. Some of the genes we identified, such as those encoding matrix metalloproteinases that enzymatically degrade matrix proteins, and CCN3/NOV, which is anti-fibrotic when over-expressed in mouse embryonic fibroblasts (Lemaire et al., 2010), may comprise a negative feedback loop in response to pro-fibrotic β-catenin.

We found that the promoter regions of the β-catenin-responsive matrix-encoding genes are enriched for predicted Tcf/Lef binding sites. Chromatin-immunoprecipitation studies and functional analysis of candidate targets are required to demonstrate which of these genes are directly regulated by β-catenin, and how their gene products contribute to fibrosis. Due to technical limitations, we measured expression levels of genes in the whole dermis, not just in fibroblasts;
therefore, we only were able to detect the most robustly over-expressed transcripts. Future efforts to define gene expression changes in stabilized β-catenin mutant dermal fibroblasts will likely reveal novel mediators of fibrosis whose expression levels are diluted out when evaluated in whole tissue samples (Liu et al., 2014).

Increased Wnt/β-catenin signaling has been implicated in various fibrosing tissues including fibrous tumors (Cheon et al., 2002; Chilosi et al., 2003; Kalluri and Zeisberg, 2006; Ng et al., 2005; Smith et al., 2007; Wei et al., 2012). In an attempt to understand if the β-catenin-responsive genes that we identified in our mouse model might be relevant to fibrotic disease, we compared the mouse dermal over-expressed genes to the genes that are over-expressed in fibrotic human tissues and cells and tumor stroma. We found 32 β-catenin-responsive genes that are over-expressed in fibrotic human tissues, including genes that encode matrisome proteins and transcription factors. As a proof-of-principle, we verified that biglycan, a proteoglycan encoded by one of the these 32 genes, has altered distribution of protein expression in the reticular dermis of SSc skin and in keloid nodules (Hesselstrand et al., 2002; Hunzelmann et al., 1996). Expression of biglycan protein positively correlates with skin thickness in cultured dSSc fibroblasts (Hesselstrand et al., 2002), and biglycan protein and mRNA levels are elevated in keloids (Hunzelmann et al., 1996). Since biglycan binds to type I collagen and is associated with thicker collagen fibrils (San Martin and Zorn, 2003; Schönherr et al., 1995), the over-expression of Bgn in stabilized β-catenin dermis and in SSc may be linked to the altered fibril morphology in these tissues (Gardner et al., 2006; Hayes and
Rodnan, 1971; Rockwell et al., 1989). Future studies to test whether β-catenin activity is required for cell-autonomous expression of Bgn and other ECM protein-coding genes will help establish a role for β-catenin control of the aberrant matrix in fibrotic disease.

Together, our findings suggest that the increased nuclear β-catenin that has been observed in fibrotic human tissue may both directly regulate the production of fibrotic ECM, and also exert broader effects via up-regulation of specific transcription factors. Since ECM accumulation is the feature of fibrosis that is explicitly involved in loss of organ function, our findings strengthen the rationale for testing inhibitors of Wnt/β-catenin signaling in amelioration of fibrosis (Dees and Distler, 2013; Lafyatis, 2012). In addition, the β-catenin-responsive, disease-associated genes that we identified encode proteins that may have utility as new targets for therapeutic intervention and as biomarkers for tracking the response to Wnt/β-catenin inhibition in vivo.

3.6. Acknowledgements

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GM07250 (EH), TL1 TR000441 (EH), NIA F30 AG045009 (EH); Scleroderma Research Foundation (RA), and Global Fibrosis Foundation (RA).
3.7. Tables

Table 1. Proportion of YFP-expressing cells detected during FACS of whole dermis of control and mutant mice at ages 1 week (n=6 controls and 6 mutants) and 3 weeks (n=10 controls and 8 mutants).

<table>
<thead>
<tr>
<th>Age (weeks)</th>
<th>YFP+ cells per 1000 detections (standard deviation)</th>
<th>p-value (Student’s t-test)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Stabilized β-catenin</td>
</tr>
<tr>
<td>1</td>
<td>5.51 (2.51)</td>
<td>3.90 (1.78)</td>
</tr>
<tr>
<td>3</td>
<td>1.67 (1.54)</td>
<td>0.73 (0.44)</td>
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Table 2. Enriched DAVID functional annotation clusters in stabilized β-catenin dermis.

<table>
<thead>
<tr>
<th>Annotation cluster</th>
<th>Representative annotation term(s)</th>
<th>Enrichment score</th>
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<tbody>
<tr>
<td>1</td>
<td>Glycoprotein/disulfide bond</td>
<td>18.04</td>
</tr>
<tr>
<td>2</td>
<td>Secreted/extracellular region</td>
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</tr>
<tr>
<td>3</td>
<td>Extracellular matrix</td>
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</tr>
<tr>
<td>4</td>
<td>Immunoglobulin</td>
<td>4.39</td>
</tr>
<tr>
<td>5</td>
<td>Cell adhesion</td>
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### Table 3. Publicly available microarray studies of fibrotic tissues and cells.

<table>
<thead>
<tr>
<th>GEO accession (publication)</th>
<th>Experimental setup</th>
<th>Tissues used</th>
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</thead>
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<tr>
<td>GSE26910 (Planche et al., 2011)</td>
<td>(1) Normal prostate v. prostate tumor</td>
<td>Stroma isolated from tissue sections by laser capture microdissection</td>
</tr>
<tr>
<td></td>
<td>(2) Normal breast v. breast tumor</td>
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</tr>
<tr>
<td>GSE45001 (Sulpice et al., 2013)</td>
<td>Tumor v. non-tumor stroma from intrahepatic cholangiocarcinoma patients</td>
<td>Stroma isolated from tissue sections by laser capture microdissection</td>
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<td>GSE9285 (Milano et al., 2008)</td>
<td>(1) Healthy control v. dSSc</td>
<td>Forearm skin biopsies</td>
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<td></td>
<td>(2) Healthy control v. lSSc</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(3) Healthy control v. morphea</td>
<td></td>
</tr>
<tr>
<td>GSE32413 (Pendergrass et al., 2012)</td>
<td>Healthy controls v. DSSc (untreated)</td>
<td>Skin biopsies</td>
</tr>
<tr>
<td>GSE2052 (Pardo, 2006; Wang et al., 2006)</td>
<td>Normal histology lung v. IPF</td>
<td>Lung biopsy or explant</td>
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<tr>
<td>GSE24206 (Meltzer et al., 2011)</td>
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<td>(2) Healthy donor v. advanced IPF</td>
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<td>(2) Healthy control v. morphea</td>
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<td>GSE1724 (Renzoni et al., 2004)</td>
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<td></td>
<td>(2) Control v. IPF (untreated)</td>
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<tr>
<td>GSE31356</td>
<td>Non-lesional v. lesional tissue from Dupuytren contracture patients</td>
<td>Cultured fibroblasts</td>
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</table>

IPF, idiopathic pulmonary fibrosis; SSc, systemic sclerosis (d, diffuse; l, limited)
Table 4. β-catenin-responsive genes over-expressed in human fibrotic tissue and containing Tcf/Lef predicted binding sites within 5kb of the transcription start site.

<table>
<thead>
<tr>
<th>Gene symbol</th>
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<th>IPF lung</th>
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Figure 9. Expression of stabilized β-catenin in mouse ventral dermal fibroblasts causes skin fibrosis. (A and top) Schematic of the experimental regimen showing tamoxifen administration at E16.5 to induce Cre-mediated expression of yellow fluorescent protein (YFP) and stabilized β-catenin in the ventral dermal fibroblasts of mutant animals. (B) Relative expression levels of Axin2 in FACS-purified YFP+ cells from stabilized β-catenin and control skin analyzed by real-time PCR (n=2). (C) Fluorescent immunohistochemistry showing co-expression of PDGFRα and YFP in control and stabilized β-catenin skin (view of Z-stack). (D) Transverse sections through ventral skin at 3 weeks of age stained with Masson's trichrome to show collagen in blue. Scale bar = 100 μm; arrows indicate expanded collagenous tissue in mutant skin compared to corresponding region in control skin. Rectangles indicate areas selected for higher-magnification images of dermis and hypodermis, below. Abbreviations: derm, dermis; hf, hair follicle; hd, hypodermis.
**Figure 10.** Manipulated cells express fibroblast markers. (A) Merged and single-channel images showing YFP+ PDGFRα+ cells. (B) Lack of double immunofluorescent staining for YFP and endothelial marker PECAM or adipose marker perilipin. (C) Flow cytometric scatter plot showing lack of YFP+ PDGFRβ+ cells; values indicate percent of detected cells. hf, hair follicle.
Figure 11. Stabilized β-catenin dermis and hypodermis do not have increased numbers of known pro-fibrotic cell populations. (A) Transverse sections through ventral skin at age 3 weeks with immunohistochemical staining showing comparable expression of α-smooth muscle actin (SMA) in control and stabilized β-catenin skin. Positive control expression in arrector pili muscle (open arrowhead, visible in cross-section in dermis), blood vessel walls (closed arrowhead, visible in subcutaneous fat region), and faintly in the dermal sheath of hair follicles (thin open arrowhead). Scale bar = 100 μm. (B, C) Comparable percentage of total cells express (B) PDGFRβ and (C) CD45 in control and stabilized β-catenin skin at both 1 week and 3 weeks of age (n≥6). Abbreviations: sg, sebaceous gland; hf, hair follicle.
**Figure 12.** α-SMA-expressing fibroblasts do not appear in advanced β-catenin-induced fibrosis. Immunohistochemical staining for α-SMA in 4-month-old control and stabilized β-catenin mice demonstrates α-SMA+ structures (labeled), but not α-SMA+ fibroblasts. hf, hair follicle; musc, muscle; adip, adipose tissue.
**Figure 13.** Canonical TGFβ signaling activity is not affected by expression of stabilized β-catenin in skin fibroblasts. Stabilized β-catenin mutant skin has (A) 1.5-fold increase in relative expression of *Serpine1 (PAI1)* but no change in percentage of fibroblasts that express nuclear (B) pSmad2 or (C) pSmad3 at 1 week or 3 weeks of age. Scale bar (in secondary-only control image) = 200 μm; all images photographed at same magnification.
Figure 14. Canonical TGFβ signaling activity is not affected by expression of stabilized β-catenin in skin fibroblasts. (A, B) Representative images of transverse sections through ventral skin of control and stabilized β-catenin mutants at age 3 weeks. Scale bars = 100 µm. Immunohistochemical staining for (A) phosphorylated Smad2 (pSmad2) or (B) phosphorylated Smad3 (pSmad3) followed by quantification of nuclear expression in fibroblast morphology cells shows comparable pSmad2 and pSmad3 activity in control and stabilized β-catenin skin at 1 week and 3 weeks of age.
Figure 15. Expression of stabilized β-catenin in skin fibroblasts is sufficient for abnormal extracellular matrix. (A) Transverse sections through ventral skin stained with picrosirus red stain and viewed under crossed polarized filters showing increased thick collagen fibres in stabilized β-catenin skin compared to control skin. (B) Electron microscopy of collagen fibrils and measurement of cross-sectional fibril diameter shows thicker hypodermal and thinner dermal fibrils in stabilized β-catenin skin compared to control skin (p<0.0001 for both comparisons). Scale bars = 1 μm. (C) Relative expression of Col1a1 mRNA is elevated in stabilized β-catenin mutant versus control FACS-purified YFP+ fibroblasts by real-time PCR (n=4). (D) Immunofluorescent staining for fibrillin-1 showing expanded expression in the thickened mutant hypodermis. Scale bars = 50 μm. (E) Relative expression of Fbn1 mRNA, measured by real-time PCR in whole skin, is not significantly different between control (n=2) and stabilized β-catenin mutant skin (n=4). epi, epidermis; hf, hair follicle; musc, muscle.
Figure 16. Validation of up-regulated genes in additional biological replicates. (A) Stabilized β-catenin mutant dermis has increased relative expression, measured by qPCR (n=2), of Thbs4, Ccn3, and Fbln1, genes which were also up-regulated upon differential expression analysis of whole transcriptome profiles. (B) Stabilized β-catenin mutant skin has increased CCN3 protein expression in dermis and hypodermis compared to control skin. Higher-magnification images correspond with dotted and solid outlined areas in corresponding lower-magnification image. Scale bars (in control images) = 200 μm; all images photographed at same magnification.
Figure 17. Stabilized β-catenin mouse dermis has elevated expression of matrix-encoding genes. (A) Over-expressed genes in stabilized β-catenin dermis compared to control, categorized using PANTHER categories and the proteomically defined matrisome. (B) The differentially expressed genes include 36 that encode matrisome proteins. (C) Transcription factor binding site over-representation analysis using oPOSSUM showing the promoter regions of the 36 over-expressed matrisome-encoding genes are enriched for predicted binding sites for transcription factors including Tcf7l2 (Tcf4). (D) Calculated log2(fold change) of the 16 β-catenin-responsive matrisome genes that have predicted Tcf/Lef transcription factor binding sites in their promoter regions.
Figure 18. Fibrotic human skin has altered distribution of biglycan. Immunohistochemical detection of biglycan showing elevated biglycan immunoreactivity in the keloid nodule compared to overlying papillary dermis and in the reticular dermis of early (< 2 years) and advanced (> 2 years) diffuse systemic sclerosis (dSSc). This is in contrast to homogenous staining throughout the papillary and reticular dermis in healthy control skin. Open arrowheads indicate elevated biglycan immunoreactivity just beneath the basal layer of the epidermis in advanced dSSc and keloid tissue. Representative images from n = 5 healthy controls, n = 6 each early dSSc, advanced SSC, and keloids.
Figure 19. Protein expression of biglycan in stabilized β-catenin mouse skin. Stabilized β-catenin mutant skin has variably increased biglycan protein expression in the hypodermis compared to control skin. Higher-magnification images correspond with dotted and solid outlined areas in corresponding lower-magnification image. Scale bars (in control images) = 200 μm; all images photographed at same magnification.
**Figure 20.** Keloid scar nodule has elevated cytoplasmic and nuclear β-catenin. Immunohistochemical detection of β-catenin in healthy control skin and three keloid scars demonstrating elevated cytoplasmic and nuclear β-catenin immunoreactivity in the keloid nodule.
4. DISCUSSION

I have shown that stabilized β-catenin in mouse dermal fibroblasts leads to elevated fibroblast proliferation, increased collagen deposition, and thickened skin. Dermal and hypodermal collagen fibril morphology are altered in the stabilized β-catenin mice, and genes encoding cell adhesion proteins and matrisome proteins have increased expression in the stabilized β-catenin dermis. These findings support my original hypothesis that expression of stabilized β-catenin in dermal fibroblasts is sufficient to cause skin fibrosis, and elucidate a specific role for β-catenin in production of fibrotic extracellular matrix. Here I will discuss the significance and limitations of these findings and their application to selected broader contexts.

4.1. Human fibrotic skin has elevated nuclear β-catenin in dermal fibroblasts

![Figure 21](image)

**Figure 21.** Immunohistochemical detection and quantification of percentage of fibroblasts expressing nuclear β-catenin in healthy control and systemic sclerosis (SSc) skin (Wei et al., 2012). Reproduced with permission by John Wiley and Sons; copyright 2012, American College of Rheumatology.
4.1.1. Significance

Keloid scars and affected skin from patients with SSc and morphea have an elevated percentage of dermal fibroblasts with nuclear β-catenin positivity by immunohistochemical staining (Figure 6, Figure 20, Figure 21) (Hamburg and Atit, 2012; Hamburg-Shields et al., 2014; Wei et al., 2012). Previously, microarray and single gene expression studies of whole tissue biopsies had shown altered expression of genes associated with the canonical Wnt signaling pathway, but whether or not this correlated with altered (increased or decreased) activity of Wnt signaling was not known (Bayle et al., 2008; Gardner et al., 2006; Russell et al., 2010; Smith et al., 2007; Whitfield et al., 2003). In addition, which cell types in the skin might be involved in transducing the hypothetically altered Wnt signals was not known. Our experiment queried for nuclear localization of β-catenin in tissue sections and provided two pieces of information: morphologic indication of which cell types in the skin exhibited a change, if any, in subcellular localization of β-catenin; and the ability to quantify the proportion of these cells with nuclear β-catenin versus without. This finding provides support for a pro-fibrotic role for β-catenin specifically in fibroblasts (as opposed to in vascular endothelium, epidermis, or other cell types in the skin). Similar results have been obtained for nuclear β-catenin immunohistochemical staining in keloid scars (Sato, 2006) and SSc (Beyer et al., 2012a).
4.1.2. Limitations and remaining questions

This finding is consistent with increased canonical Wnt signaling activity, although our experiment did not address the cause of the elevated nuclear β-catenin immunoreactivity in these human tissues. In human SSc and keloids and mouse models of skin fibrosis, expression levels of Wnt mRNAs are elevated, while expression levels of some endogenous Wnt inhibitors are decreased (Bayle et al., 2008; Gardner et al., 2006; Russell et al., 2010; Smith et al., 2007; Whitfield et al., 2003). Consistent with a role for increased Wnt ligands in dermal fibrosis, siRNA-mediated in vivo knockdown of Evi, which is required for Wnt ligand secretion, abrogates bleomycin-induced dermal fibrosis in mice (Distler et al., 2014a). However, the cellular source and upstream cause of increased Wnt ligand secretion in dermal fibrosis is not yet known. Similarly, the downstream consequences of nuclear β-catenin in human dermal fibroblasts in fibrotic disease have not been addressed by our examination of this tissue.

Another limitation of this experiment is the small sample size queried (as few as four patient tissue samples each for NSF and morphea) and the high variability in patient disease duration and treatment exposure. In addition, healthy controls were not age-, gender-, or site-matched. Perhaps not surprisingly, then, we observed substantial variability in the percentage of nuclear β-catenin-positive dermal fibroblasts in the patient tissue sections. This variation could possibly correlate with demographic variables, anatomic location of the biopsy (Chang et al., 2002), stage of
disease, or in the case of SSc, gene expression profile-based disease subset (Pendergrass *et al.*, 2012; Whitfield *et al.*, 2003), but we were not able to determine any correlation within our small sets of samples.

### 4.2. Labeling and manipulation of a subset of mouse dermal fibroblasts

#### 4.2.1. Comparison to other methods of manipulating dermal fibroblasts

We used the *HoxB6CreER* 

\(^T\)

driver to restrict the manipulation of β-catenin to dermal fibroblasts (Figure 9A). In addition to tissue restriction, this system provided the benefit of temporal induction by tamoxifen administration. In triple transgenic mutant animals, the deletion of the third exon of one allele of *Ctnnb* was a permanent and heritable alteration—therefore, even though *HoxB6CreER* 

\(^T\)

is not expressed in perinatal or adult tissues, we were able to generate perinatal and adult mice with stabilized β-catenin expression in dermal fibroblasts. Other reagents that enable manipulation of dermal fibroblasts lack tissue specificity. *Col1a2-CreER* 

\(^T\)

has been successfully used for manipulating adult dermal fibroblasts, although detailed lineage labeling studies have not been published to assess the anatomic location or distribution of these fibroblasts in the skin (Derrett-Smith *et al.*, 2009). This construct enables tamoxifen induction in perinatal and adult mice to drive transgene expression in dermal fibroblasts as well as fibroblasts/fibroblastic cells in the intestinal wall, lung, blood vessel walls, and sites of membranous ossification in the skull (Zheng *et al.*, 2002). Therefore, the results of experiments using *Col1a2*-
CreER[^T] could be confounded by effects on fibroblasts that reside in tissues other than skin.

Clonal and wound healing analyses of PDGFRACreER[^T] -expressing cells labeled in the embryonic dermis has shown contribution of these cells to papillary dermis, reticular dermis, and arrector pili muscle (Driskell et al., 2013). This driver is also expressed in adult oligodendrocytes (Rivers et al., 2008). PDGFRA continues to be expressed in adult fibroblasts (Figure 10), so PDGFRACreER[^T] could have potential use for manipulation of perinatal and adult dermal fibroblasts.

In comparison to other fibroblast-restricted reagents, then, HoxB6CreER[^T] has the benefit of being restricted to fibroblasts in the skin, and also has been adequately characterized in terms of labeling the manipulated fibroblasts and documenting their distribution within the skin.

4.2.2. Limitations and remaining questions

Limitations of the HoxB6CreER[^T] driver include the following: First, it limits the experiment to manipulating embryonic or fetal fibroblasts. By late embryonic/fetal stages (E15.5-18.5), it is restricted to fewer than half of the fibroblasts in the skin. Therefore it does not enable uniform targeting of all dermal fibroblasts, in contrast to PDGFRACreER[^T]. Additionally, the timing of induced transgene expression is likely to influence perinatal development of the manipulated dermal fibroblasts. In particular, the hypodermis continues to develop in perinates (Bayle et al., 2008) and
*HoxB6CreER\(^T\)* targets fibroblasts in the hypodermis. Therefore the phenotype may have a strong developmental component, in contrast to a phenotype resulting from purely postnatal manipulation of dermal fibroblasts.

Second, *HoxB6CreER\(^T\)* targets a heterogeneous population of fibroblasts within the skin. These fibroblasts reside in the upper and lower dermis (Figure 8) and occasionally contribute to the dermal sheath of hair follicles (unpublished observation). Because the stabilized \(\beta\)-catenin mutant mice exhibited hypodermal hypercellularity, it seems that the fibroblasts in the lower dermis were more sensitive (in terms of proliferation index) than upper dermal fibroblasts to expression of stabilized \(\beta\)-catenin. However, collagen accumulation and gross tissue thickening was apparent in both the upper and lower dermis (Figure 7, Figure 9). Therefore, the heterogeneous population of fibroblasts targeted by this driver seem to have distinct responses to expression of stabilized \(\beta\)-catenin. These distinct responses may be masking additional affects of stabilized \(\beta\)-catenin which may only be apparent in more restricted/homogenous populations of fibroblasts.

The final limitation of the *HoxB6CreER\(^T\)* driver is that its expression is anatomically restricted to dermis of the ventral body wall and limbs. This makes it challenging to compare to other mouse models of skin fibrosis, which typically rely on analysis of dorsal skin (*Akhmetshina et al.*, 2012 and others; *Gerber et al.*, 2014; *Yamamoto et al.*, 1999).
4.3. Cellular consequences of forced expression of stabilized β-catenin in mouse dermal fibroblasts

4.3.1. Fibroblast proliferation

The role of β-catenin in cellular proliferation also is well-documented in multiple tissues including tumors (MacDonald et al., 2009). The sufficiency of stabilized β-catenin to cause increased dermal fibroblast proliferation has been previously shown in vitro and in vivo (Cheon et al., 2002; 2004). However, that previous experiment was not tissue-specific, since it relied on reverse tetracycline-transactivated (rtTA)-controlled expression of stabilized β-catenin which was induced by subcutaneous doxycycline. Therefore all skin cell types, not just fibroblasts, were exposed to the experimental manipulation (Cheon et al., 2002). While our finding of increased fibroblast proliferation due to expression of stabilized β-catenin is not novel (Figure 7), it does demonstrate the sufficiency of stabilized β-catenin to drive fibroblast proliferation in vivo when expressed in a restricted population of fibroblasts.

4.3.2. Lack of α-SMA+ myofibroblasts

Demonstration of α-SMA+ myofibroblasts is a common readout for fibrosis in animal models. In spite of the fibrotic phenotype in stabilized β-catenin skin, however, α-SMA myofibroblasts were not apparent by immunohistochemistry at
any one of several time points (Figure 11, Figure 12). This is in contrast to the finding by Beyer et al (Beyer et al., 2012a) that induced stabilization of β-catenin in adult dermal fibroblasts results in increased number of α-SMA+ myofibroblasts in the skin. However, these published results are expressed as a fold-change value, without indication of the raw number of myofibroblasts observed, or any description of how these cells were distinguished from the α-SMA+ arrector pili muscle (Beyer et al., 2012a) (Figure 12). This discrepancy could be a consequence of the time point at which expression of stabilized β-catenin is induced. Perhaps the E16.5 HoxB6CreERT fibroblast population is precluded in some way from acquiring the myofibroblast phenotype. A common viewpoint is that myofibroblasts are a form of terminally differentiated fibroblast (Hinz et al., 2010) and so perhaps Wnt/β-catenin signaling, an important pathway in embryonic development of the dermis, maintains the dermal fibroblast in a less-differentiated state. The elevated expression of Twist2/Dermo1, which mediates Wnt/β-catenin signaling in E12.5 dermal precursors (Atit et al., 2006; Ohtola et al., 2008; Tran et al., 2010) may be an indicator that the stabilized β-catenin fibroblasts retain some “embryonic” features.

4.3.3. Lack of inflammatory infiltrate

Inflammation is associated with wound healing (Eming, 2012) and with fibrosis in SSc (section 1.3.4) and the bleomycin mouse model of skin fibrosis. However, no inflammatory infiltrate was detected in the stabilized β-catenin mouse skin. This was evaluated by anti-F4/80 immunofluorescence (unpublished result), anti-CD45
immunofluorescence and flow cytometry (Figure 11), and examination of H&E stained paraffin sections (unpublished observation). This finding supports a model for pro-fibrotic β-catenin that is dominated by its effect on fibroblast proliferation and extracellular matrix synthesis/accumulation. This is in contrast to the bleomycin mouse model, in which fibrosis occurs in response to an injurious event. This suggests that elevated β-catenin in dermal fibroblasts in fibrotic diseases occurs as a consequence of a perceived tissue injury.

4.3.4. Vascular endothelium

Vascular endothelial injury is associated scleroderma, and is thought to occur prior to fibrogenesis in this disease (Fleming et al., 2009; Ho et al., 2014). The lack of endothelial cell immunoreactivity in the hypodermal fibrosis of stabilized β-catenin mouse skin (Figure 7I) does not appear to recapitulate the vascular phenotype of scleroderma or any other fibrotic disease. It is not clear how the fibrotic tissue that lacked immunoreactive endothelial cells (Figure 7C) was adequately oxygenated. Further characterization of this aspect of the stabilized β-catenin skin phenotype could reveal the existence of an altered vascular endothelium that has severely abnormal surface marker expression, rendering it undetectable by our multiple methods. Although the altered vasculature in the fibrotic hypodermis does not mimic any particular human fibrotic disease, it does demonstrate that an isolated manipulation of local dermal fibroblasts resulting in skin fibrosis can result in vascular endothelial changes, perhaps mediated by changes in the ECM and/or
vascular basement membrane (Uitto et al., 1989). Etiologic models of scleroderma have positioned vascular endothelial injury as a purely causative event in this disease; however, the altered endothelial cell immunoreactivity in this model suggest that the fibrotic environment may have a role in maintaining or exacerbating the abnormal vasculature as fibrotic disease progresses.

4.3.5. Pericytes

Proliferation and differentiation of pericytes may contribute to pathologic fibroblasts in renal fibrosis (Dulauroy et al., 2012; Greenhalgh et al., 2013; Humphreys et al., 2010) (section 1.3.2). Pericytes are present in skin and may be involved in skin fibrosis in scleroderma (Rajkumar et al., 2005). However, the requirement of pericytes in dermal fibrosis has not been functionally tested in skin as it has in other organs. Our investigation did not reveal any expansion of the PDGFR-β+ pericyte population in stabilized β-catenin skin (Figure 11). However, especially given the vascular phenotype in the fibrotic hypodermis, it seems likely that perivascular cells such as pericytes could be affected somehow in this model. For example, their interaction with the vascular basement membrane could be affected by the generalized ECM alterations in stabilized β-catenin skin (Bergers and Song, 2005). A pericyte phenotype or response to dermal fibrosis in this model might be manifest as an altered secretory profile or differentiation of some pericytes without a change in the overall number/incidence in the fibrotic skin.
4.3.6. Wound healing

Over-expression of β-catenin in the skin is sufficient for increased fibroblast proliferation during wound healing (Cheon et al., 2002). After full-thickness wounding of mouse skin, Tcf reporter activity is evident in the healing dermis (Cheon et al., 2004). Wounding studies have not been performed in my stabilized β-catenin mouse model. This model is not ideal for full-thickness wounds since the expression of stabilized β-catenin is restricted to the ventral dermis, and so animal morbidity and contamination of the wound bed are of concern. Given the findings of Cheon et al., I expect that wounding of the stabilized β-catenin skin would result in a hyperproliferative response by dermal fibroblasts adjacent to the wound. In particular, the reticular and hypodermal fibroblasts which predominantly contribute to the healing dermis (Driskell et al., 2013) would be responsible for a hypertrophic/keloidal phenotype similar to that reported by Cheon et al. (Cheon et al., 2002). That being said, restricted expression of stabilized β-catenin in the mouse epidermis prior to wound healing results in expansion of papillary and reticular fibroblast populations (Driskell et al., 2013). Therefore, since the Cheon et al. experiment relied upon global over-expression of stabilized β-catenin, there could have been a more robust fibroproliferative response in this model due to the contribution of epidermal β-catenin during wound healing.
4.4. Molecular/signaling consequences of forced expression of stabilized β-catenin in mouse dermal fibroblasts

4.4.1. Changes in the extracellular matrix

Stabilized β-catenin mutant mice have thickened ventral skin with increased collagen and altered collagen fibril morphology (Figure 7, Figure 15A-B). In addition, the hypodermis contains an expanded domain of fibrillin-1 protein expression (Figure 15D). Increased hypodermal fibrillin-1 is a feature of the tight-skin mouse, and enhanced fibrillin-1 microfibril assembly occurs by fibroblasts in vitro in response to Wnt3a ligand (Bayle et al., 2008). The fibrillin-1 expanded domain observed in the stabilized β-catenin mutant skin provides further support for a role of Wnt/β-catenin in regulation microfibrillar assembly (Hamburg-Shields et al., 2014). Increased collagen (by hydroxyproline assay) has been shown in multiple other mouse models in response to increased Wnt signaling or stabilization of β-catenin, and is associated with elevated expression of Col1a1 and Col1a2 (Akhmetshina et al., 2012; Beyer et al., 2012a). Our additional finding that collagen fibril morphology is altered (increased average fibril cross-sectional diameter in the stabilized β-catenin hypodermis, and decreased fibril diameter in the dermis) suggests that stabilized β-catenin in dermal fibroblasts is not only sufficient for increased total amount of collagen, but also has some direct or indirect effect on post-translational modifications of collagen.
In addition to examining amount/distribution and mRNA expression for specific ECM components of interest, we found by whole transcriptome analysis that the bulk dermis of stabilized β-catenin mice has elevated expression of a variety of genes that encode components and regulators of the proteinaceous extracellular matrix. These findings support a model in which dermal fibroblast β-catenin is sufficient for synthesis of a fibrotic ECM by regulating expression of ECM genes (section 4.4.2). These genes encode proteins of all different ECM categories (Naba et al., 2012a) including proteoglycans, glycoproteins, and ECM regulators such as matrix metalloproteinases (Figure 17).

The up-regulation of proteoglycan genes *Aspn*, *Bgn*, *Lum*, and *Vcan* in the stabilized β-catenin dermis is intriguing (Table 5). These genes encode asporin, biglycan, lumican, and versican, respectively. Proteoglycans have a structural role in the extracellular matrix; in particular, versican is a large proteoglycan (265-370 kDa) with numerous glycosaminoglycan (GAG) side chains. Versican forms hydrophilic complexes with hyaluronic acid in order to bind water molecules and give skin its tautness (Bruckner-Tuderman, 2012). The proteoglycans, especially the small leucine-rich proteoglycans (SLRPs), also have roles in intercellular signaling. The SLRPs include biglycan, decorin, asporin, lumican, and fibromodulin (Schaefer and Iozzo, 2008). These proteins bind to a broad spectrum of other extracellular proteins, including growth factors, collagens, the extracellular portion of membrane-bound receptors, and even bacterial lipopolysaccharide (Chen and Birk, 2013; Iozzo and Schaefer, 2010; Kalamajski and Oldberg, 2010; Schaefer and Iozzo, 2008).
Generally, SLRPs can modulate the bioavailability of bound proteins by sequestering them in the extracellular matrix and, conversely, by facilitating receptor-ligand interactions (Iozzo and Schaefer, 2010). In addition, SLRPs are involved in collagen fibrillogenesis and cross-linking (Chen and Birk, 2013; Kalamajski and Oldberg, 2010). These roles and many of the precise protein interaction sites are ongoing areas of study. Knockout mice for distinct SLRP genes have weakened connective tissues, depending on the expression pattern of the specific protein (Kalamajski and Oldberg, 2010). Specific SLRPs are dys-regulated in diverse fibrotic conditions including tumor stroma, SSc, and IPF (Table 7); renal fibrosis (Stokes et al., 2001); and uterine fibroids and keloid scars (Carrino et al., 2012). The nature of the SLRP dys-regulation varies between different disease states. For example, uterine fibroids and keloid scars have decreased decorin and no apparent change in biglycan protein (Carrino et al., 2012); conversely, biglycan is up-regulated in tumor stroma and SSc (Table 7). To add to the complexity of SLRP interactions, they may share binding partners and have synergistic effects in some contexts (Corsi et al., 2002). Further studies are needed to determine the precise roles of specific SLRPs in skin fibrosis, which likely involve both regulation of collagen fibrillogenesis and as well as modulation of growth factor-mediated signaling.

The predominant limitation of the whole transcriptome (RNA-seq) analysis of stabilized β-catenin dermis is that this experiment measured gene expression in the bulk tissue, not just in the stabilized β-catenin fibroblasts. Therefore, it encompasses direct and indirect (or cell-autonomous and non-autonomous) effects
of β-catenin. In addition, any subtle gene expression changes specifically in the manipulated fibroblasts have likely been obscured by this analysis of the bulk tissue.

The comparison to microarray results from various fibrotic human diseases did not identify any particular gene that was up-regulated across the board for SSc, IPF, tumor stroma, and our mouse model (Table 7). Given the heterogeneity of the tissues being examined, this is not surprising, similar to our quantification of dermal fibroblast nuclear β-catenin in human skin biopsies. However, this comparison nevertheless has produced a short list of genes that have responded to stabilized β-catenin in vivo, which are putative direct targets of β-catenin via Tcf/Lef promoter region binding, and which are up-regulated in a human disease state.

4.4.2. Potential roles of specific β-catenin-responsive genes

We have identified ECM and other genes that are strong positive responders to stabilized β-catenin in our mouse model and poised for direct regulation via Tcf/Lef binding sites within their promoter regions (Figure 17). These genes include Bgn, Thbs4, and Twist1 (Table 4). Another gene, Nov, is up-regulated in stabilized β-catenin dermis and in the tight-skin mouse model (Bayle et al., 2008) and is also discussed below (Table 5). The variety of functions attributed to the β-catenin-responsive genes suggests a model in which stabilized β-catenin leads to fibrosis by initiating and maintaining expression of a pro-fibrotic “program”. The facets of this
program may be elucidated by further exploration of individual up-regulated genes, including the selected few that are discussed here.

\textit{Bgn}

Biglycan is a small leucine-rich proteoglycan (SLRP) encoded by \textit{Bgn} in mice. \textit{BGN} is over-expressed in human fibrotic tissues (Gardner \textit{et al.}, 2006; Honardoust \textit{et al.}, 2011) and the biglycan protein is abundant in keloid nodules (Figure 20) (section 5.3). The association between biglycan and thickened collagen fibrils in the uterine decidua (San Martin and Zorn, 2003) suggests that this gene product may contribute to the increased collagen fibril thickness in stabilized \(\beta\)-catenin hypodermis (Figure 15). \textit{Bgn}-deficient mice have thinner dermis containing collagen fibrils that have a broadened range of fibril diameters with irregular cross-sectional profiles (Corsi \textit{et al.}, 2002). In addition to its possible interaction with collagen, biglycan (like decorin, another SLRP) has been shown to bind to TGF\(\beta\) ligands \textit{in vitro} (Hildebrand \textit{et al.}, 1994). Such interactions may be influenced by the availability of SLRP binding sites due to varying degrees of glycosylation or size of glycosamingoglycan (GAG) side chains in different contexts (Carrino \textit{et al.}, 2012; Chen and Birk, 2013). The interaction between biglycan and TGF\(\beta\) has been proposed to functionally sequester TGF\(\beta\) ligand in the extracellular environment, preventing it from interacting with membrane-bound receptors (Hildebrand \textit{et al.}, 1994). However, while recombinant decorin can sequester and inhibit pro-fibrotic activity \textit{in vivo} in a lung fibrosis model, recombinant biglycan has no effect--therefore it seems that the TGF\(\beta\)-inhibitory functions of these two SLRPs are distinct and possibly context-dependent.
(Kolb et al., 2001). Indeed, biglycan-null bone marrow stromal cells appear to require biglycan expression in order to proliferate and synthesize collagen in response to TGFβ ligand in vitro, suggesting that TGFβ activity in this context requires or is potentiated by biglycan expression (Young et al., 2002). Because the stabilized β-catenin mouse model lacked a measurable response of TGFb/Smad2/3 signaling in the presence of elevated Bgn expression, it is not clear if interactions between biglycan and TGFβ ligand influenced the fibrotic phenotype.

**Thbs4**

*Thbs4* encodes thrombospondin-4, a glycoprotein. Although its human homolog *THBS4* is not up-regulated in any of the fibrotic conditions examined in our microarray comparison, it is up-regulated specifically in fibroblasts in murine renal fibrosis (Grgic et al., 2014) and murine hepatic fibrosis (Iwaisako et al., 2014). It is also over-expressed in the stromal cells and cancer-associated fibroblasts of invasive diffuse gastric adenocarcinomas (Förster et al., 2011) and invasive breast tumors (McCart Reed et al., 2013). Therefore, *THBS4* expression is associated with highly proliferative and invasive fibroblasts. In murine cardiac injury, thrombospondin-4 is expressed intracellularly and is required for the normal ER stress response to injury; transgenic over-expression of *Thbs4* is cardioprotective in this model (Lynch et al., 2012). Therefore, *Thbs4* may have both intracellular and extracellular roles in fibrotic tissue. Further studies are required to determine the function of *Thbs4* in dermal fibroblasts and skin fibrosis specifically, especially in benign fibroproliferative conditions such as keloid scars and superficial
fibromatoses. Its extracellular function in fibrotic tissue may be related to its direct binding of type I collagen (Narouz-Ott et al., 2000).

**Nov**

Nov (Nephroblastoma over-expressed)/CCN3 is a matricellular signaling protein that, although not detected by the microarrays we analyzed, has elevated expression in scleroderma skin and the tight-skin mouse (Bayle et al., 2008; Lemaire et al., 2010; Perbal, 2001). In the context of cultured mouse embryonic fibroblasts, it negatively regulates assembly of fibrillin fibrils and appears to antagonize the effects of exogenous Wnt3a (Lemaire et al., 2010). In cultured human dermal fibroblasts exposed to gadolinium-containing contrast agent (a model for NSF), exogenous CCN3 inhibits proliferation and blocks the fibroblast response to TGFβ (Riser et al., 2012). Therefore, in the stabilized β-catenin mouse, increased expression of Nov may be a negative feedback response to attenuate the effects of stabilized β-catenin. How Nov/CCN3 mediates this negative feedback loop remains to be elucidated (section 5.3).

**Twist1**

Twist1 is one of a few transcription factors that we found to be β-catenin-responsive in our mouse model and also up-regulated in human fibrotic disease. Specifically, it has increased expression in tumor stroma and IPF lung (Table 7). An independent microarray study identified Twist1 as the most highly up-regulated gene in IPF lung tissue (Bridges et al., 2010). Twist1 is expressed in response to viral-induced injury
of mouse lung epithelial cells in vitro and appears to be required for epithelial-to-mesenchymal transition in this model (Pozharskaya et al., 2009). Its over-expression in rat lung fibroblasts is protective against apoptosis (Bridges et al., 2010). While a role for epithelial-to-mesenchymal transition in skin fibrosis has not been ruled out, it seems more likely that the anti-apoptotic effect of Twist1 might predominate in the context of dermal fibroblasts. Another possible role for Twist1 in fibrosis is mediation of a downstream pro-fibrotic gene signature. In mouse embryonic cranial mesenchyme, β-catenin promotes cranial bone specification via Twist1 (Goodnough et al., 2012). Intriguingly, keloid scars exhibit strong up-regulation of osteoblastic markers (Naitoh et al., 2005). Further investigation of the “osteogenic” gene signature in keloid scars may uncover a role for Twist1 mediation of pro-fibrotic β-catenin in dermal fibroblasts.

4.4.3. Interactions between β-catenin and TGFβ signaling

TGFβ1 signaling via Smad2/3 has been implicated to have a fundamental role in skin fibrosis. TGFβ1 protein levels are increased in keloid scar fibroblasts (Fujiwara et al., 2005) and systemic sclerosis skin (Gabrielli et al., 1993), and expression of constitutively active TGFβ receptor 1 in fibroblasts is sufficient for skin fibrosis (Sonnylal et al., 2006) (section 1.5.1). Experiments in multiple models/systems have demonstrated interactions between Wnt/β-catenin and TGFβ/Smad signaling in the contexts of fibroblasts and dermal fibrosis.
Results from *in vitro* experiments demonstrate that both Wnt and TGFβ signaling are sufficient to up-regulate components of the other pathway: Wnt3a induces expression of TGFβ in cultured mouse and human fibroblasts (Carthy *et al.*, 2011; Wei *et al.*, 2012), while TGFβ treatment results in increased β-catenin protein in mouse fibroblasts (Cheon *et al.*, 2002), TOPFlash reporter activity in human dermal fibroblasts (Sato, 2006), and nuclear β-catenin in human dermal fibroblasts (Akhmetshina *et al.*, 2012). Epistasis experiments with respect to Wnt or TGFβ/Smad regulation of specific genes have yielded inconsistent results. Specifically, Wnt3a-induced expression of *COL1A1* depends upon Smad3 in human foreskin fibroblasts (Wei *et al.*, 2012); while, conversely, Wnt10b-induced expression of type I collagen appears to be independent of TGFβ/Smad2/3 signaling (human foreskin fibroblasts) (Wei *et al.*, 2011b) and Wnt3a-induced fibrillin matrix assembly is independent of Smad3 (MEFs) (Bayle *et al.*, 2008). Most recently, *in vivo* experiments have shown that treatment of bleomycin and tight-skin mouse models with an inhibitor of TGFβRI results in reduced nuclear β-catenin-expressing dermal fibroblasts (Akhmetshina *et al.*, 2012). Also, inhibition of Wnt/β-catenin signaling by multiple approaches is sufficient to ameliorate dermal fibrosis induced by expression of constitutively active TGFβRI (Akhmetshina *et al.*, 2012; Beyer *et al.*, 2013; Distler *et al.*, 2014a; 2014b).

In the stabilized β-catenin mouse model, I used phosphorylated Smad2 and phosphorylated Smad3 as readouts for canonical TGFβ signaling activity. I found very high baseline levels of nuclear pSmad2 and pSmad3 in dermal fibroblasts, with
no difference in the percent of dermal fibroblasts with nuclear pSmad2/3 between stabilized β-catenin and control skin (Figure 13, Figure 14). Therefore, if there is an interaction between β-catenin and TGFβ/Smad2/3 in this model, it does not appear to be mediated by nuclear localization of Smads. The effects of stabilized β-catenin in my model could be occurring independently of TGFβ/Smad2/3 signaling. This could be tested by treating stabilized β-catenin mice with neutralizing antibody directed against TGFβ1 (McCormick et al., 1999). Alternatively, interaction between β-catenin and TGFβ/Smad2/3 could be mediated by nuclear interactions between Smad2/3, β-catenin, and Tcf/Lef cofactors; a complex of these proteins has been demonstrated in Xenopus (Labbé et al., 2000). Such interactions could be detected by single gene ChIP (for genes that seem likely to be co-regulated, for example CCN3 and FBN1 (Lemaire et al., 2010) or comparison of ChIP-sequencing for these proteins in dermal fibroblasts.
5. FUTURE DIRECTIONS

My work has addressed three major questions in the field of fibrosis research, but further investigation into each of these areas is warranted:

- **What are the cellular sources of fibroblasts that produce skin fibrosis?** - By restricting the expression of stabilized β-catenin to dermal and hypodermal fibroblasts, I have shown that manipulating only a local fibroblast population is sufficient to result in skin fibrosis. In addition, this fibrotic process does not involve increased numbers of monocyte-lineage cells or pericytes, which have potential roles for fibrosis in other systems (section 1.3.2). In section 5.2, I propose robust lineage-tracing experiments that will accurately define the cellular contributions to skin fibrosis.

- **What are the molecular changes that occur in fibrotic tissues?** - I have measured increased expression of genes in stabilized β-catenin dermis, thus identifying that stabilized β-catenin in dermal fibroblasts regulates (directly or indirectly) expression of genes that encode matrix and cell adhesion proteins. I have also compared these molecular changes to those found by microarray analysis of fibrotic human tissues in order to identify a set of genes that responds to experimentally stabilized β-catenin and is associated with human fibrotic disease. In section 5.3, I propose functional testing of two of these genes and their protein products in the context of skin fibrosis.
• *Which signaling pathways drive fibrosis?* In addition to showing that stabilized 
β-catenin in dermal and hypodermal fibroblasts is sufficient to cause fibrosis, I 
have shown that the fibrotic phenotype develops without apparent 
involvement of canonical TGFβ signaling, which is a classic pro-fibrotic 
pathway. This finding supports a model of fibrotic disease in which pro-
fibrotic β-catenin activity is downstream or parallel to canonical TGFβ 
signaling. In section 5.1, I propose additional testing of the dermal fibroblast 
requirement for β-catenin in skin fibrosis.

In the following sections, I discuss my findings in the context of recently published 
research, identify unresolved questions, and propose specific experiments to 
address these questions. I have emphasized experimental approaches with cell-type 
specificity, since these studies are currently lacking in skin fibrosis research 
compared to other organs.

5.1. **Test the requirement of β-catenin for sustained fibrosis in adult dermal 
fibroblasts.**

5.1.1. **Rationale for a role for dermal fibroblast β-catenin activity in advanced 
fibrosis**

When developing anti-fibrotic therapies directed against particular pathways or 
signaling molecules, it is important to distinguish between factors that are
important for *initiation* versus *sustainment* of the fibrotic process. Most patients with fibrotic skin disease do not present in the clinic until they have established fibrosis; therefore, a pro-fibrotic factor that is required for established fibrosis is an advantageous therapeutic target. In contrast, a pro-fibrotic factor that is required only in the early stages of fibrosis may not be an effective target by the time of diagnosis. My research has shown that forced stabilization of β-catenin in fetal mouse dermal fibroblasts results in fibrosis that is evident by three weeks of age and maintained until at least 4 months of age (Figure 7, Figure 9). This demonstrates that elevated activity of β-catenin in dermal fibroblasts is sufficient to *initiate* fibrosis. However, it is not yet known whether β-catenin is required in dermal fibroblasts to *sustain* existing fibrosis.

There are multiple points of evidence that suggest that dermal fibroblast β-catenin has a role in sustained fibrosis. First, in human fibrotic diseases, an increased percentage of dermal fibroblasts have nuclear (active) β-catenin compared to healthy control skin (Hamburg and Atit, 2012; Sato, 2006; Wei *et al.*, 2012) (Figure 6, Figure 21). Therefore, β-catenin activity is elevated in clinically evident fibrosis, including in systemic sclerosis up to 4 years following diagnosis. Second, in mice, stabilized β-catenin-induced fibrosis has increasing skin thickening over time (Chapter 2). The progression of this phenotype supports a sustained pro-fibrotic role for β-catenin in existing experimental fibrosis. Third, established bleomycin-induced skin fibrosis in mice can be reversed by systemic treatment with small molecule inhibitors of nuclear β-catenin activity (Beyer *et al.*, 2013). This
demonstrates a functional requirement for nuclear β-catenin activity to maintain existing bleomycin-induced skin fibrosis. And last, genetic deletion of β-catenin by tamoxifen-induced Col1a2-CreER$^T$ expression prevents bleomycin-induced skin fibrosis (Beyer et al., 2012a). However, it is not known if these anti-fibrotic effect of β-catenin inhibition or deletion are specifically mediated by dermal fibroblasts. Since other cell types in the skin, for example hair-follicle-associated dermal papilla cells, also express nuclear β-catenin (Myung et al., 2013), it is valuable to investigate this question in a cell-type-specific manner. These three points of evidence support my hypothesis that elevated β-catenin activity is required specifically in dermal fibroblasts to sustain existing fibrosis.

![Timeline and tamoxifen/doxycycline induction scheme for inducible, reversible expression of stabilized β-catenin.](image)

**Figure 22.** Timeline and tamoxifen/doxycycline induction scheme for inducible, reversible expression of stabilized β-catenin.
5.1.2. Proposed experiments and expected results

To test this hypothesis requires an experimental system of established fibrosis in which β-catenin activity can be attenuated specifically in dermal fibroblasts. I propose a transgenic mouse model with reversible, inducible expression of stabilized β-catenin in dermal fibroblasts. This will allow induction of stabilized β-catenin expression in dermal fibroblasts, which I have shown is sufficient for skin fibrosis. Subsequently reversing the induction of stabilized β-catenin will directly test my hypothesis that elevated β-catenin activity is required in dermal fibroblasts to sustain fibrosis. One method of inducing and reversing transgene expression is by tetracycline-inducible transcriptional activation. This is a binary system in which expression of a transgene depends upon the absence or presence of tetracycline (“Tet-Off” or “Tet-On” systems, respectively). Specifically, I propose using the tamoxifen-responsive HoxB6CreER<sup>T</sup> driver (Hamburg and Atit, 2012; Nguyen et al., 2009) to restrict expression of a reverse tetracycline-regulated trans-activator (rtTA)-EGFP in the endogenous Gt(Rosa)26Sor locus (Belteki et al., 2005) to ventral dermal fibroblasts at E16.5. This is a “Tet-On” system in which the presence of doxycycline, a tetracycline analogue, will activate the expression of a tetracycline-responsive transgene, TETO-ΔN89β-catenin encoding stabilized β-catenin from a TetO-N89 β-catenin responder (Mukherjee et al., 2010) (Figure 22). The following experiments will compare triple-transgenic HoxB6CreER<sup>T</sup>/+; R26rtTA/++; TETO-ΔN89β-catenin/+ mutant mice and control HoxB6CreER<sup>T</sup>/+; R26rtTA/+ age- and gender-matched litter-mates. Following tamoxifen induction of rtTA expression in
dermal fibroblasts, doxycycline can be administered at any point (i.e. to fetal, perinatal, or adult mice) to induce expression of the tetracycline-responsive stabilized β-catenin.

Expression of stabilized β-catenin in a subset of dermal fibroblasts is sufficient to cause fibrosis in similar systems (Hamburg and Atit, 2012) (Chapter 2), including one that uses a fibroblast-restricted driver in adult mice (Beyer et al., 2012a; Zheng et al., 2002). Therefore, I expect that tamoxifen-induced, doxycycline-treated HoxB6CreER<sup>T</sup>/+; R26rtTA/+; TETO-ΔN89β-catenin/+ mice will develop skin fibrosis with thickened ventral skin, increased collagen content, and elevated fibroblast proliferation. It is also possible that this system could recapitulate the increased collagen fiber thickness and abnormal fibril morphology that I observed upon fetal dermal fibroblast induction of stabilized β-catenin (Figure 15). With this, I also expect to measure elevated dermal expression of β-catenin-responsive genes including Bgn and others similar to the gene signature of HoxB6CreER<sup>T</sup>/+; R26-YFP/+; Catnb<sup>dex3</sup>/+ fibrotic dermis. This phenotype could be evident by 3 weeks of doxycycline treatment, and will likely progress with continued exposure to doxycycline. Older mice could develop a more severe fibrotic phenotype than their younger counterparts, as there is evidence for increased fibrogenic potential in older animal tissue (Brack et al., 2007). This finding would validate my previous work in the new context of adult dermal fibroblasts.
After skin fibrosis is established in adult triple transgenic mice, cessation of doxycycline treatment will terminate the expression of stabilized β-catenin. In the days and weeks following doxycycline cessation, the sustainment of skin fibrosis can be analyzed by measuring skin thickness, collagen content, and fibroblast proliferation. This will test whether stabilized β-catenin in dermal fibroblasts is required to sustain established skin fibrosis. I anticipate that removal of stabilized β-catenin expression will normalize the elevated fibroblast proliferation and attenuate the excess collagen accumulation in triple transgenic mutant mice. Fibroblast proliferation is a known consequence of increased β-catenin activity, and I expect that the fibroblast proliferative index should be normalized within three days post-doxycycline (Cheon et al., 2002). Similarly, the elevated expression of β-catenin-responsive genes such as Bgn should normalize within days of doxycycline cessation. Genes whose expression responds early to the loss of stabilized β-catenin expression may be direct targets of β-catenin via Tcf/Lef transcription factor binding; however, elevated expression of some of the β-catenin-responsive genes could be maintained as part of a feed-forward loop maintained by the abnormal extracellular matrix (Bielefeld et al., 2011). Loss of excess collagen will require degradation of the existing fibrotic collagen, and will likely take weeks to achieve normalcy. I expect that if the excess collagen resolves, that the collagen fiber thickness and fibril morphology will also normalize over time. In addition, there may be loss of the excessive number of fibroblasts residing in the fibrotic tissue via apoptosis or transdifferentiation to adipocyte identity, a cell fate that is suppressed by Wnt/β-catenin signaling (Wei et al., 2011b).
5.1.3. Interpretation of results

Partial or complete attenuation of established fibrosis upon removal of stabilized β-catenin expression specifically in dermal fibroblasts will demonstrate a requirement for β-catenin in dermal fibroblasts to sustain fibrosis. It is possible that the requirement for β-catenin may only be observed for particular aspects of the fibrotic phenotype, for example expression of matrix-encoding proteins or increased fibroproliferation. Therefore, careful analysis of the stabilized β-catenin-induced and reversed phenotypes will be important for dissecting the context-specific role of β-catenin in adult dermal fibroblasts and established fibrosis.

If removal of stabilized β-catenin expression is successful in attenuating the fibrotic phenotype, then further analysis of tissue-wide gene expression changes will be helpful for identifying genes whose expression relies upon dermal fibroblast β-catenin activity. One benefit of the inducible-reversible system of β-catenin stabilization in dermal fibroblasts is that it is restricted to a population of dermal fibroblasts that have been shown to contribute to experimental fibrosis. Therefore, genes whose expression levels respond to induction and de-induction of stabilized β-catenin may turn out to be very closely tied to the mechanism of pro-fibrotic β-catenin activity in vivo. Such responders warrant further investigation: they may be direct targets of β-catenin via Tcf/Lef binding, which could be detected by chromatin immunoprecipitation-based experiments, and they may be potent pro-
fibrotic factors unto themselves, which could be tested using mouse models of skin fibrosis (as in section 5.2). Based on my gene expression profiling in fetal-induced stabilized β-catenin dermal fibroblasts, some of the responsive genes may encode integrin subunits and matrix cross-linking enzymes (Table 5). These are closely related to potential therapeutic targets that are undergoing investigation in clinical trials and animal models of fibrosis, including LOXL2 (Barry-Hamilton et al., 2010; Garber, 2013) and the αv integrin subunit (Henderson et al., 2013). These and other genes that are highly sensitive to expression of stabilized β-catenin may be fundamental mediators of pro-fibrotic β-catenin activity. Therefore, the results from this experiment could determine novel causative factors for fibrosis, thus elucidating detailed mechanisms for pro-fibrotic β-catenin activity in dermal fibroblasts, and identifying possible therapeutic targets for treatment of fibrotic disease.

5.2. **Determine whether stabilized β-catenin has a pro-fibrotic role in other fibrogenic cell types.**

5.2.1. **Rationale for a role for β-catenin in other fibrogenic cell types**

Contributions of different fibrogenic cell types has been shown to be important in pulmonary, renal, cardiac, and hepatic fibrosis models (Humphreys et al., 2010; Lotersztajn et al., 2005; Zeisberg et al., 2007; 2008). This knowledge has guided cell-type-specific investigations into mechanisms of fibrosis, including gene
expression profiling of five distinct cell types that contribute to injury-induced renal fibrosis (Grgic et al., 2014; Liu et al., 2014), and therapeutic targeting of integrins expressed by hepatic stellate cell-derived myofibroblasts in liver fibrosis (Henderson et al., 2013). In skin, the potential cellular sources of activated fibroblasts include resident dermal fibroblasts, pericytes, adipocytes or fibro-adipoprogenitor cells, and fibrocytes. However, the relative contributions of this different cell types to skin fibrosis is poorly understood. My work has shown that a single manipulation of a subset of resident dermal fibroblasts is sufficient to cause a fibrotic response. This fibrotic response is most robust in the hypodermis of the skin, although there are labeled manipulated fibroblasts in both the dermis and the hypodermis of stabilized β-catenin mutant and control mice (Figure 8). Similarly, fibrosis occurs robustly in the hypodermis of tight-skin mice (Lemaire et al., 2004a). Therefore, in addition to possible contributions from distinct cell lineages, fibrosis may also be differentially produced by distinct subsets of dermal fibroblasts. Specifically, there may be a greater contribution from fibroblasts in the deep dermis and hypodermis. The reticular dermis and hypodermis become fibrotic in advance of the papillary dermis in scleroderma (Wei et al., 2011a). During wound healing in mice, regeneration of the dermis and its extracellular matrix is dominated by fibroblasts originating from reticular and hypodermal fibroblasts adjacent to the wound bed (Driskell et al., 2013). Careful lineage-tracing studies are needed to track the contributions of resident fibroblasts and other fibrogenic cell types to fibrosis. In this section, I will propose specific lineage-tracing experiments of reticular dermal fibroblasts, adipocytes, and pericytes. Based on clinical features of
skin fibrosis that involve the hypodermis, subcutaneous fat, and vasculature, I believe that these two lineages are likely to have important roles in skin fibrosis.

Following this proper assessment of cellular contribution to skin fibrosis, I propose assessment of the pro-fibrotic role of β-catenin in particular fibrogenic cell types. The histology of the fibrotic phenotypes in tight-skin mice and in the HoxB6CreER\(^T\)/+; R26-YFP/+; Catnb\(^{Δe3}\)/+ mice suggest a robust contribution from hypodermal and reticular fibroblasts to skin fibrosis (Hamburg and Atit, 2012; Lemaire et al., 2004b). Beyer et al. have demonstrated that β-catenin expression in a broad population of fibroblasts is required for bleomycin-induced fibrosis (Beyer et al., 2012a), and it would be valuable to dissect out this requirement for β-catenin amongst the distinct subpopulations of fibroblasts in the dermis. In addition, β-catenin may also play a role in other fibrogenic cell types. For example, in pulmonary fibrosis, β-catenin may play a role in mediating EMT to produce myofibroblasts (Chilosi et al., 2003), and may have roles in pericyte migration (Ren et al., 2013) and myofibroblast activation (DiRocco et al., 2013) in renal fibrosis. Therefore, testing the requirement for β-catenin in other fibrogenic cell types may yield additional information about the pro-fibrotic role of canonical Wnt signaling in skin fibrosis.

5.2.2. Proposed experiments and expected results
I propose lineage tracing of different fibrogenic cell types to be performed in the bleomycin-injected model of skin fibrosis. This induction of skin fibrosis is easily applied to transgenic mice and is analogous to the injury-based models that have been used in lineage-tracing of fibrogenic cell types in renal, pulmonary, and hepatic fibrosis, allowing some comparison of results across organ systems.

**Lineage tracing of reticular dermal fibroblasts**

This experiment will test my hypothesis that reticular and hypodermal fibroblasts contribute substantially to skin fibrosis in this model. Dermal fibroblasts are a heterogenous population of cells whose behaviors and gene expression patterns vary with anatomic location (Chang et al., 2002) and position in the dermis (Janson et al., 2012). Until recently, there have not been robust methods of identifying particular subtypes of dermal fibroblasts, for example papillary versus reticular fibroblasts, other than by position in the upper or lower dermis, respectively. Driskell et al. assayed expression of several markers of dermal and hypodermal adipocyte subpopulations in embryonic and perinatal mice, then used classic lineage tracing methods to trace these subpopulations in the developing dermis and during dermal wound healing. Specifically, they found that dermal cells expressing Delta-like homologue 1 (Dlk1) at E16.5 contribute to reticular dermal and hypodermal fibroblasts and a small portion of mature adipocytes by postnatal day P21. The Dlk1+ cells are a subset of the PDGFR-α+ lineage which comprises the majority of the dermal mesenchymal compartment (Driskell et al., 2013). Unfortunately, since the E16.5 Dlk1+ lineage contributes to adipocytes as well as fibroblasts, it is not
ideal for lineage tracing the fibroblast-specific response to injury. However, it is possible that Dlk1 expression becomes more restricted after E16.5 (the latest point of lineage labeling induction by Driskell et al), especially after the perinatal development of the subcutaneous adipocyte layer, which lacks expression of Dlk1. Therefore, I propose use of postnatal Dlk1 lineage labeling to follow the response of reticular and hypodermal fibroblasts to bleomycin injury.

Injection of tamoxifen will result lineage labeling in Dlk1Cre-ER/+; R26-YFP/+ mice at any time point at which Dlk1 is expressed. This will induce YFP expression in all Dlk1-expressing cells and their progeny. Since the subcutaneous adipocyte layer is quite well established by postnatal day 4 (Figure 7B), I propose confirming that Dlk1 is expressed at P4 and restricted to reticular and hypodermal fibroblasts. Tamoxifen induction at P4 to Dlk1Cre-ER/+; R26-YFP/+ will label these fibroblast subpopulations. Then, after several weeks, the adult mice can be given subcutaneous bleomycin to induce skin fibrosis. I expect that the Dlk1+ lineage will be present in increased numbers in bleomycin-injected skin in comparison to vehicle-treated control skin due to elevated proliferation. In addition, I expect that these cells will have increased expression of fibrosis markers such as connective tissue growth factor (CTGF) and possibly α-SMA, and increased expression of matrix-encoding genes including Col1a1 and Fbn1. Relative expression of mRNA can be measured in YFP-expressing Dlk1+ cells in vehicle-treated and tamoxifen-induced animals after fluorescence activated cell sorting from the dermis, as shown in Figure 9B. In the case that Dlk1Cre-ER induction continues to label the adipocyte
lineage postnatally, thus confounding whether fibroblasts or adipocytes (or both) are contributing to the fibrotic response, I propose inducing lineage labeling with sub-maximal doses of tamoxifen such that clonal populations of cells can be followed after bleomycin injury (similarly to Driskell et al., who clonally labeled dermal fibroblasts during development). This approach would not conclusively exclude an adipocyte contribution to the fibrotic response, but may be informative if specific clonal expansions of Dlk1+ lineage cells are positioned some distance from the adipose tissue.

*Lineage tracing of adipocytes*

Bleomycin-induced skin fibrosis and other murine models of skin fibrosis experience atrophy of the subcutaneous adipose tissue (Ohgo et al., 2013; Smith and Chan, 2010; Wei et al., 2011b). To test my hypothesis that adipocytes contribute to the fibrogenic population of cells in bleomycin-induced skin fibrosis, I propose a similar approach as described above for reticular/hypodermal fibroblast lineage-tracing. Expression of the *Adipoq-CreER* driver is restricted to mature white adipose tissue, including subcutaneous adipocytes (Jeffery et al., 2014). Therefore, *Adipoq-CreER/*; *R26-YFP/+* mice will express YFP in all hypodermal adipocytes. Bleomycin-induced injury to the skin of mature mice will result in fibrosis, and the contribution of YFP-expressing, Adipoq+ lineage cells to the fibrotic skin will be measured. I anticipate that, concomitant with the loss of subcutaneous adipose tissue in bleomycin-induced fibrosis, a subset of Adipoq+ lineage cells will lose expression of adipocyte markers and acquire a fibroblast fate. Fibroblastic
characteristics of the Adipoq+ cells may include morphological appearance consistent with fibroblasts, dissociation from the layer of subcutaneous adipose tissue, and increased expression of matrix-encoding proteins, especially Cola1 and Col1a2, compared to Adipoq+ cells in vehicle-injected control mice. Complete transdifferentiation from adipocyte to fibroblast fate can be tested by measuring expression of adipocyte markers such as adiponectin and perilipin and fibroblast/myofibroblast markers such as desmin, vimentin, and α-SMA.

*Lineage tracing of pericytes*

PDGFR-β is a marker of pericytes, perivascular mesenchymal cells that contribute to fibrosis in multiple organ systems (Greenhalgh *et al.*, 2013). Lineage tracing of pericytes in fibrosis would be greatly assisted by development of an inducible *PDGFRBCre-ER* driver. PDGFRB-Cre targets myofibroblasts in other organs, and so bleomycin-induced skin fibrosis would result in lineage labeling of all de novo myofibroblasts, regardless of whether they developed from pericyte lineage cells (Henderson *et al.*, 2013). This issue could be avoided by inducing YFP expression in PDGFRB-CreER-expressing cells (i.e. pericytes) with a single tamoxifen injection prior to bleomycin injection. With this method, cells that acquire PDGFR-β expression only as a result of bleomycin injection will not be lineage-labeled (since they will not experience tamoxifen induction of the CreER); only progeny of the pre-existing PDGFRβ+ population will be labeled.
To test my hypothesis that pericytes contribute to the fibrogenic population of cells
in bleomycin-induced skin fibrosis, I propose generating PDGFRBCre-ER/+; R26-
YFP/+ mice and inducing skin fibrosis by subcutaneous bleomycin injury, similarly
to the two lineage-labeling experiments described above. Contribution to fibrosis
by PDGFRB+ lineage cells will be evident by dissociation from the dermal
vasculature and increased expression of fibrosis-associated genes like Col1a1,
Col1a2, and ACTA2 compared to PDGFRB+ cells in vehicle-injected control animals.
The PDGFRB-Cre driver has been used in other organ systems to track the pericyte
contribution to fibrosis (Foo et al., 2006; Henderson et al., 2013); however, it has
not been fully characterized in skin to accurately lineage-label pericytes. Therefore,
characterization of these cells in healthy control skin will be required as part of this
experiment. If the PDGFRB-CreER driver turns out to not be restricted to pericytes
in skin, then ADAM12+ cells may be labeled and followed as in Dulauroy et al.
ADAM12+ cells comprise a subset of pericytes that contribute to the acute injury
response in skin and muscle (Dulauroy et al., 2012).

Test the differential requirement for β-catenin expression in specific fibrogenic cell
populations

The consequences of β-catenin activity differ between cell types, and may even
differ between subtypes of cells. For example, papillary and reticular dermal
fibroblasts differ in their perinatal expression of canonical Wnt/β-catenin
transcriptional effectors Lef1, which have increased expression in the papillary
dermis, and Tcf3 and Tcf4, which have increased expression in the hypodermis
Differential expression of these transcriptional cofactors are just one possible mechanism by which β-catenin activity could lead to distinct cell-type specific consequences in gene expression (Rudloff and Kemler, 2012). I anticipate that β-catenin activity may have distinct roles in the different fibrogenic cell populations in skin. For example, I have shown that expression of stabilized β-catenin using the HoxB6CreER<sup>T</sup> driver in ventral dermal fibroblasts is sufficient for skin fibrosis that lacks the appearance of α-SMA+ myofibroblasts. In contrast, expression of stabilized β-catenin driven by Col1a2-CreER<sup>T</sup>, which targets mature fibroblasts in multiple organs, is sufficient for elevated α-SMA+ myofibroblasts in skin (Beyer et al., 2012a). One explanation for these contrasting results is that the manipulation of β-catenin expression has affected distinct subpopulations of dermal fibroblasts. Therefore, investigating the requirement for β-catenin in defined fibrogenic populations will yield precise insights into the pro-fibrotic role of β-catenin.

Based on my results showing that forced stabilization of β-catenin in dermal fibroblasts, including deep dermal and hypodermal fibroblasts, is sufficient for fibrosis, I anticipate that β-catenin expression will be required in this fibroblast subpopulation during fibrosis. To test this, I propose generating Dlk1Cre-ER/+; R26-YFP/+; Ctnnb1<sup>Δ/flox</sup> mice and inducing deletion of the floxed allele of Ctnnb1 at P7, similar to the lineage tracing experiment proposed above. This will result in β-catenin-null reticular and hypodermal fibroblasts. Then, subcutaneous injection of bleomycin will be used to induce skin fibrosis. Double-transgenic Dlk1Cre-ER/+;
R26-YFP/+ can receive the same tamoxifen and bleomycin treatment and serve as controls. I expect that the mice lacking β-catenin expression in reticular and hypodermal fibroblasts will have an impaired fibrotic response to bleomycin, with decreased dermal thickness, lower fibroblast proliferation, and reduced collagen accumulation compared to the bleomycin-injected double-transgenic control mice. I do not expect β-catenin deletion to cause any changes in fibroblast biology prior to bleomycin injection, since nuclear β-catenin is not expressed in this population normally (Figure 8C).

Similarly, I propose using the Adipoq-Cre driver to delete one allele of β-catenin in Adipoq-Cre/+; R26-YFP/+; Ctnnb1Δ/lox mice. This will result in β-catenin-null mature adipocytes. Canonical Wnt signaling is generally inhibitory of adipogenesis (Bennett et al., 2002; Wei et al., 2011b), and mouse subcutaneous adipocytes do not normally express nuclear β-catenin (Figure 8C). Therefore, I expect that deletion of β-catenin in adipocyte lineage cells will attenuate the fibrotic response to bleomycin injection with reduced skin thickness, reduced collagen accumulation, and maintenance of the subcutaneous adipose tissue, in comparison to bleomycin-injected control mice with intact expression of β-catenin. As with the targeted deletion of β-catenin in reticular and hypodermal fibroblasts, I do not expect β-catenin deletion to cause any changes in adipocyte biology prior to bleomycin injection, since nuclear β-catenin is not expressed in this population normally (Figure 8C).
Finally, I propose testing the consequences of deleting β-catenin expression specifically in pericyte lineage cells. Given the importance of β-catenin expression in the developing mesenchyme and dermis, deletion of β-catenin expression using PDGFRB-Cre could potentially affect pericyte identity or biology prior to induction of fibrosis. Although Wnt/β-catenin activity has not been assessed in dermal pericytes, Wnt reporter activity is present in a portion of healthy renal pericytes, suggesting that nuclear β-catenin may have a role in pericyte homeostasis (Ren et al., 2013). Whether this role for Wnt/β-catenin extends to dermal pericytes is not yet known. In addition, there is the potentially confounding expression of PDGFRβ in myofibroblasts following bleomycin injection. Because of these issues, it would be beneficial to use an inducible pericyte-restricted driver for pericytes in this experiment. Provided that an appropriate inducible pericyte-restricted driver such as PDGFRB-CreER is available, then, I propose deletion of β-catenin expression in this population, followed by bleomycin induction of skin fibrosis. I expect that pericytes may require β-catenin in the fibrotic response to bleomycin. Wnt/β-catenin signaling is present in pericytes in injury-induced renal fibrosis (Ren et al., 2013), although the requirement for β-catenin to respond to injury has not been tested in vivo. Pericyte targeted β-catenin deletion may result in an attenuated response to bleomycin-induced fibrosis, and could also help maintain small vessel patency compared to control mice with intact expression of β-catenin in pericytes.

5.2.3. Interpretation of results
The lineage-tracing experiments proposed in this section will demonstrate the response of potential fibrogenic cell populations to bleomycin-induced skin fibrosis. I have focused on analyzing the involvement of reticular and hypodermal fibroblasts, mature adipocytes, and pericytes in fibrotic skin. This will elucidate the distinct response of each of these three lineages to a fibrotic stimulus. If any single lineage makes a substantial contribution the fibrotic phenotype, this sets the stage for more precise, cell-type-specific investigation of pro-fibrotic signaling pathways and gene expression in that population of cells. For example, if reticular and hypodermal fibroblasts proliferate and comprise the majority of cells in the fibrotic lesion, then isolation of the mechanisms driving that response will be valuable in future experiments. Conversely, if this fibroblast population does not have a compelling role in bleomycin-induced fibrosis, then other fibrogenic cells types should be more closely scrutinized. Involvement of pericytes in fibrosis could be mediated by their interaction with the dermal vasculature, and so this finding would suggest investigation of the vascular reaction to bleomycin injury to determine which factors mediate the pericyte response. A contribution by adipocytes to the fibrotic response would at least partially explain the loss of these cells that occurs in skin fibrosis, and identify a novel source of fibroblasts associated with fibrosis. Cumulatively, if multiple lineages give rise to fibroblasts in bleomycin-induced fibrosis, this introduces the question of whether common or distinct mechanisms drive the contribution of each lineage. This could give rise to combinatorial therapies to modulate the individual cellular contributions, resulting in more effective inhibition of fibrosis.
Deletion of β-catenin in each of these lineages enables a precise assessment of the requirement for β-catenin in distinct fibrogenic populations of cells. It is likely that β-catenin is required for the fibrotic response by reticular and hypodermal fibroblasts. If β-catenin is also required by adipocytes and/or pericytes to produce fibrosis, then follow-up experiments should assess β-catenin-dependent gene expression changes in each cell type. This will begin to elucidate whether β-catenin has common or distinct pro-fibrotic roles in individual cell populations during the fibrotic response. For example, resident fibroblasts and pericyte-derived fibroblasts may require β-catenin for proliferation and matrix production, while adipocytes may require β-catenin in order to transdifferentiate to a fibroblast fate (Bennett et al., 2002; Wei et al., 2011b).

5.3. Test the roles of key mediators of pro-fibrotic Wnt/β-catenin activity

5.3.1. Rationale for investigating pro-fibrotic mediators of stabilized β-catenin

Canonical Wnt/β-catenin activity has important roles in normal physiology, for example dermal wound healing and maintenance of the intestinal epithelium (Cheon et al., 2004; Fevr et al., 2007). These roles suggest, in spite of its ability to drive key characteristics of fibrosis, β-catenin might not be a lucrative target for anti-fibrotic therapy, since systemic inhibition of β-catenin could have substantial side effects. Here, I propose experiments to identify and test effectors and
mediators of pro-fibrotic β-catenin signaling that may be more specific to the context of fibrosis, and could prove to be effective therapeutic targets. To determine the mechanism of pro-fibrotic β-catenin activity, it is worthwhile to investigate potential upstream effectors as well as downstream mediators of canonical Wnt/β-catenin signaling.

There are several potential causative factors that can result in increased Wnt/β-catenin signaling activity in human skin fibrosis conditions and animal models of skin fibrosis. These fibrotic states are associated with up-regulation of Wnt ligands and up/down-regulation of pathway inhibitors such as WIF, SFRP2, and DKK1 (Lemaire et al., 2010). However, of all the putative upstream effectors of increased Wnt/β-catenin activity, only Dkk1 has been directly shown to influence β-catenin activity in fibroblasts in fibrosis in vivo. This was demonstrated using Col1a1-Dkk1 transgenic mice, in which Dkk1 expression is presumably increased in dermal fibroblasts. Col1a1-Dkk1 mice were resistant to fibrosis in the bleomycin-induced and tight-skin models and had decreased dermal fibroblast expression of nuclear β-catenin (Akhmetshina et al., 2012). There is also functional support for a pro-fibrotic role for Wnt ligands: siRNA inhibition of Wntless (Wls) expression, which is required for secretion of both canonical and non-canonical Wnt ligands, is sufficient to abrogate experimental skin fibrosis (Distler et al., 2014b).

I have identified several potential downstream mediators of pro-fibrotic β-catenin activity in the dermis by transcriptome analysis (Chapter 3). Stabilized β-catenin
fibrotic dermis had increased expression of several genes that encode extracellular matrix proteins, adhesion proteins, and growth factors. Extracellular matrix and adhesion proteins are of great interest for developing anti-fibrotic therapies, since they may be more restricted to the abnormal/fibrotic tissue and may avoid the systemic side effects that can result from targeting signaling pathways or inflammatory mediators (Friedman et al., 2013; Gerber et al., 2014). In addition, normalization of the fibrotic extracellular matrix may help increase tissue bioavailability of cell-targeted therapies (Barry-Hamilton et al., 2010) and normalize myofibroblast activation, which is responsive to the fibrotic extracellular environment (Achterberg et al., 2014; Olsen et al., 2011).

5.3.2. Proposed experiments and expected results

*Test the requirement for biglycan, a putative mediator of β-catenin-induced skin fibrosis.*

I have measured increased expression of *Bgn*, which encodes the small leucine-rich protein biglycan, in fibrotic stabilized β-catenin mouse dermis (Chapter 3). Expression of *BGN* is elevated in scleroderma skin (Gardner et al., 2006) and hypertrophic scars (Honardoust et al., 2011), and biglycan protein is robustly expressed in the nodule of keloid scar tissue (Figure 18). Biglycan-deficient mice are viable but have defects in skeletal growth leading to generalized osteopenia (Corsi et al., 2002). Biglycan is associated with thickened collagen fibrils in uterine decidua (San Martin and Zorn, 2003) and could play a role in the altered collagen
fibril morphology that I observed in stabilized β-catenin dermis and hypodermis (Figure 15). To test my hypothesis that biglycan is required to mediate stabilized β-catenin skin fibrosis, I propose generating transgenic mice with inducible HoxB6CreER<sup>T</sup>/+; Catnb<sup>Δex3</sup>/+ in a Bgn-deficient background. Biglycan deficiency can be achieved by generating homozygous knockout females (Bgn<sup>-/-</sup>) or heterozygous knockout males (Bgn<sup>-/+</sup>), since the biglycan gene is located on the X chromosome (Young <i>et al.</i>, 2002). Comparison of the fibrotic phenotype between HoxB6CreER<sup>T</sup>/+; Catnb<sup>Δex3</sup>/+ mice with intact versus deficient Bgn expression will show whether Bgn is a required mediator of β-catenin-induced fibrosis. I anticipate that, while biglycan deficiency may not totally abrogate the β-catenin-induced fibrosis, mice lacking biglycan will have normalized collagen fibril diameter in the dermis and hypodermis.

In addition to its role in mediating collagen fibril thickness, biglycan may also enhance Wnt signaling activity by acting as an extracellular reservoir for Wnt ligands, thus participating in a feed-forward loop with stabilized β-catenin.

Overexpression of biglycan, Wnt3a, and Wnt co-receptor Lrp6 in HEK293T cells followed by immunoprecipitation and pull-down assays demonstrated the ability of these three proteins to bind to each other, suggesting formation of an extracellular complex that regulates availability of Wnt3a in the pericellular space. This interaction is required for expression of Runx2 in cultured mouse osteoprogenitor cells (Berendsen <i>et al.</i>, 2011). The existence of this feed-forward loop could be tested in the context of fibrosis by inducing skin fibrosis by bleomycin injection in
biglycan-deficient mice. Lack of biglycan in the dermis could impair the increased activity of dermal Wnt/β-catenin signaling that is normally observed during bleomycin-induced fibrosis. This would result in an attenuated fibrotic response compared to mice with intact biglycan expression, with reduced expression of nuclear β-catenin in dermal fibroblasts and a dampened effect on Wnt-responsive gene expression.

*Test the role of CCN3, a putative mediator of β-catenin-induced skin fibrosis.*

Another gene that is responsive to expression of stabilized β-catenin in the dermis is *Nov*, which encodes Nephroblastoma over-expressed (Nov)/CCN3. *Nov/CCN3* is a member of the Cyr-61/Ctgf/Nov family of matricellular signaling proteins (Perbal, 2001). It has elevated expression in scleroderma skin and tight-skin mouse skin, and negatively regulates assembly of fibrillin fibrils by cultured mouse embryonic fibroblasts (MEFs). Exogenous CCN3 blocks Wnt3a-induced gene expression changes in cultured MEFs (Lemaire *et al.*, 2010). I hypothesize that CCN3 mediates a negative-feedback response to increased β-catenin activity to act as an anti-fibrotic factor in skin fibrosis. To determine its role in β-catenin induced fibrosis, I propose generating transgenic mice with inducible *HoxB6CreER<sup>T</sup>/; Catnb<sub>dex3</sub>/* in a CCN3-deficient background, similar to the experiment proposed above for testing the requirement for *Bgn*. CCN3 deficiency can be achieved by generating CCN3-null mice, which are viable and develop cardiomyopathy and muscle atrophy by 5 months of age (Heath *et al.*, 2008). Comparison of the fibrotic phenotype between *HoxB6CreER<sup>T</sup>/; Catnb<sub>dex3</sub>/+ mice with intact versus deficient *CCN3* expression will
demonstrate the role for CCN3 downstream of β-catenin activity in dermal fibroblasts. I expect that, in the CCN3-null background, β-catenin-induced fibrosis will be exacerbated, with more robustly increased fibrillin deposition in the hypodermis compared to HoxB6CreER<sup>T</sup>/+; Catnb<sup>△ex3</sup>/+ control mice (Figure 15). The anti-fibrotic role of CCN3 could be further tested by administration of exogenous CCN3 by subcutaneous injection to mice with bleomycin-induced fibrosis and β-catenin induced fibrosis. Abrogation of the fibrotic phenotype in these mice would demonstrate an anti-fibrotic effect of CCN3 and validate its potential therapeutic benefit.

5.3.3. Interpretation of results

Recently, there has been increasing focus on developing anti-fibrotic therapies directed against extracellular molecules and interactions. For example, a monoclonal antibody against LOXL2, a collagen crosslinking enzyme, has entered clinical trials for treatment against idiopathic pulmonary fibrosis, and modulation of integrin subunits, which mediate cell-cell and cell-matrix interactions, have shown promise in pulmonary fibrosis and tight-skin mice (Barry-Hamilton <i>et al.</i>, 2010; Garber, 2013; Gerber <i>et al.</i>, 2014; Henderson <i>et al.</i>, 2013). Here, I have proposed investigating the fibrotic roles of two β-catenin-responsive genes, <i>BGN</i> and <i>CCN3</i>, and the proteins that they encode, biglycan and CCN3/NOV. If biglycan is required to mediate β-catenin-induced skin fibrosis, then its precise pro-fibrotic activity should be investigated further. Specifically, its potential roles in mediating collagen
fibril assembly and enhancing canonical Wnt signaling are of possible therapeutic interest. Disruption of these activities could have anti-fibrotic effects in vivo. Conversely, CCN3, a growth factor with increased expression in response to stabilized β-catenin, may have anti-fibrotic effects on fibrillin assembly and Wnt-responsive gene expression. Depending on the tissue specificity and expression levels of these two potential mediators of β-catenin-induced fibrosis, they may be potential therapeutic targets for treatment of skin fibrosis.

5.4. Conclusion

I have shown that forced stabilization of β-catenin in dermal and hypodermal fibroblasts is sufficient to cause spontaneous, progressive skin fibrosis. The pro-fibrotic effects of β-catenin in dermal fibroblasts include increased fibroblast proliferation, increased collagen deposition, and altered expression of genes that encode matrix proteins, growth factors, and adhesion proteins. Therefore, β-catenin is a potent pro-fibrotic molecule in a resident fibroblast population. The molecular response to stabilized β-catenin in the dermis comprises potential therapeutic targets and biomarkers of fibrotic disease. Future investigation into these β-catenin-responsive mediators of fibrosis may yield more effective anti-fibrotic therapies for fibrosis in skin and other organs.
Table 5. Categorization of 36 differentially-expressed matrisome-encoding genes.

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Table 6. List of homologous human genes corresponding to 171 of the differentially expressed genes from our mouse model.

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Table 7. List of the human homologous genes that are also over-expressed in fibrotic tissues, with corresponding log(fold change) and adjusted p-values from GEO2R analysis.

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