# Fibroblast activation and pro-fibrotic phenotypes: modulation by FGF2 and MAPK signaling

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# Fibroblast activation and pro-fibrotic phenotypes: modulation by FGF2 and MAPK signaling


## Abstract

Fibrotic diseases are a leading cause of morbidity and mortality in the developed world. Despite this, the lack of therapies for fibrotic pathological disease states is severe. A large part of the reason for this lack of viable therapies is due to an incomplete understanding of the early processes driving tissue fibrosis, as well as the dismal results of pharmacologic monotherapies at the clinical trial stage in humans thus far. Therefore, better understanding of the upstream mechanisms driving tissue fibrosis is imperative. One of the common mechanisms underlying all fibroses is the presence and activity of the myofibroblast, a contractile mesenchymal cell that deposits high levels of extracellular matrix. Overpersistence of myofibroblasts in the wound site lead to deposition of an acellular, nonfunctional, mechanically aberrant scar that can result in loss of tissue function and, in severe cases, eventual organ failure. Here we investigate the mechanisms under which fibroblast growth factor 2 (FGF2), one member of the mammalian fibroblast growth factor family, antagonizes activation of fibroblasts to myofibroblasts. We identify a gene and protein expression signature induced by FGF2 that is antagonistic to activated myofibroblasts, and we demonstrate that induction of this antifibrotic gene expression paradigm is antagonized by inhibition of the mitogen-activated protein kinase pathways ERK and JNK, each of which lies canonically downstream of FGF2/FGFR signaling, suggesting that the antifibrotic effects of FGF2 as an antagonist to fibroblast activation are likely dependent at least in part upon activation of these cellular signaling pathways. We further demonstrated that, independent of exogenous FGF2 stimulation, inhibition of ERK or JNK signaling in proliferating human dermal fibroblasts was sufficient to induce fibroblast activation, accompanied by a pro-

fibrotic extracellular matrix gene expression paradigm. Inhibition of these pathways also resulted in distinct changes in transforming growth factor beta (TGF-β) gene expression paradigms, modulating the expression of both ligands and receptors involved in this pathway, and we verified that activation of fibroblasts via MAPK inhibition was dependent at least in part on activation of TGF-βR signaling. In contrast, inhibition of p38 MAPK was sufficient to antagonize fibroblast activation and subsequent fibrosis-associated extracellular matrix deposition, both in the presence and absence of exogenous TGF-β, via changes in gene expression antagonistic to pro-fibrotic TGF-β/TGF-βR signaling. Broadly, these data suggest that activation of ERK and JNK pathways broadly antagonize fibroblast activation and fibrosis, while activation of p38 drives fibroblast activation and pro-fibrotic fibroblast phenotypes. It is our hope that this information will lead to a better understanding of the way that cellular signaling pathways interact in order to drive fibroblast activation, and better inform the potential effects of kinase inhibitors or related therapeutics for use as anti-fibrotic therapies.

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**Abbreviations:**

**ADP**-Adenosine diphosphate

**PDGF**-Platelet-derived growth factor

**TGF**-Transforming growth factor

**VEGF**-Vascular endothelial growth factor

**MCP**-Macrophage chemotactic protein

**CCL**-Chemokine (C-C motif) ligand

**EGF**-Epidermal growth factor

**IGF**-Insulin-like growth factor

**ECM**-Extracellular matrix

**RHAMM**-Receptor for hyaluronic acid-mediated motility

**CD**-Cluster of differentiation

**FGF**-Fibroblast growth factor

**LTBP**-Latent TGF-β-binding protein

**TGF-βR**-TGF-β receptor

**α-SMA**-Alpha smooth muscle actin

**CTGF**-Connective tissue growth factor

**IL**-Interleukin

**LAP**-Latency-associated peptide

**MMP**-Matrix metalloproteinase

**IPF**-Idiopathic pulmonary fibrosis

**EMT**-Epithelial-to-mesenchymal transition

**bFGF**-Basic fibroblast growth factor

**FGFR**-Fibroblast growth factor receptor

**MAPK**-Mitogen-activated protein kinase

**mTOR**-Mechanistic target of rapamycin

**TUNEL**-Terminal deoxynucleotidyl transferase dUTP nick-end labeling

**ERK**-Extracellular signal-related kinase

**SMAD**-Homolog of mothers against decapentaplegic

**FRS-**Fibroblast growth factor receptor substrate

**EndMT**-Endothelial-to-mesenchymal transition

**SM22-**Smooth muscle protein, 22 alpha

**LOX**-Lysyl oxidase

**PI**-Propidium iodide

**JNK**-c-Jun N-terminal kinase

**MKK**-Mitogen-activated protein kinase kinase

**UTR**-Untranslated region

**miRNA**-Micro RNA

**SERPIN**-Serine protease

**RTK**-Receptor tyrosine kinase

**FITC**-Fluorescein isothiocyanate

**PVDF**-Polyvinylidene fluoride

**TBS-T**-Tris-buffered saline with tween

**HRP**-Horseradish peroxidase

**PBS**-Dulbecco's phosphate-buffered saline

**IgG**-Immunoglobulin G

**PBS-T**-Phosphate-buffered saline with tween

**BSA**-Bovine serum albumin

# Chapter 1-Introduction and review of literature

## 1.1 Fibrotic pathologies, facts and figures

Fibrosis, a pathological manifestation of the mammalian wound healing response, refers to the process by which tissue is damaged in such a manner that regeneration of native tissue architecture and function cannot be recapitulated from the healing process. Due to its visible nature and ubiquity, fibrosis has generally come to be associated with the skin. According to the United States Centers for Disease Control, 48.3 million surgical procedures were performed in the United States alone in the year 2010, most of which are expected to leave superficial scars (Hall, Schwartzman, Zhang, & Liu, 2017). However, nearly all organs are able to undergo fibrosis upon insult, and it is estimated that nearly half of all deaths in the developed world are due to diseases of fibroproliferation (Wynn, 2007). Thus, the need for anti-fibrotic drugs and therapies is self-evident. Broadly, Global Industry Analysts estimated the size of the annual wound care products market in the United States to be $16 billion by 2022 (Global Industry Analysts, 2016) and, more specifically, the life science-focused investment bank Nomura Code put forth a conservative estimate for the value of an anti-scarring drug at $12 billion solely for skin applications (Sen et al., 2009).

## 1.2 A dearth of effective anti-fibrotic therapies

The lack of effective anti-fibrotic drugs, particularly in the Western world, has not been a function of a lack of effort. Organismal processes and cellular pathways underlying fibrotic responses have been subject to a great deal of scrutiny over the past few decades, complete with optimization of fibrotic *in vitro* and animal models and elucidation of temporally-defined wound healing responses. Nevertheless, pharmacologic attempts to interfere with these pathways in

humans have been largely bereft of success. A case in point is the story of the Renovo Group plc, a UK-based pharmaceutical company with the goal of inventing the first scar prevention pharmaceutical drug in the United States and in Europe. Despite bringing four separate drugs to the stage of clinical trials, and one drug to pre-clinical development and evaluation, each one of them failed or was pulled due to lack of positive effect in the clinic. Perhaps the most widely-known of these failures was Juvista, a formulation of recombinant human TGF-β3. Despite promising data in pre-clinical studies of non-human animal fibrosis models and in Phase I and II clinical trials (Durani, Occleston, & O'Kane, 2008; Ferguson et al., 2009; Occleston et al., 2011), Juvista ultimately failed to reach its primary endpoints in a Phase III clinical trial in 2011, leading to a 75% decrease in market value of the Renovo Group (Lowe, 2011). One month after the failure of Juvista, Renovo announced a major restructuring plan, inspired in part by the decision of Shire LLC, the company's commercialization partner for Juvista, to terminate their licensing agreement. At this point, Renovo decided to halt any further development of the Juvista product, to downsize the advisory board substantially, and to halt further recruitment for an ongoing trial for another one of their drugs, Adaprev (Renovo, 2011). Despite the smaller than expected cohort, the Adaprev trial was allowed to continue. Thus, the failure of Juvista was allowed to be compounded by the failure of Adaprev, a solution of mannose-6-phosphate intended for reduction in scarring in patients having undergone tendon surgery (NIH, 2011). Renovo decided to halt further development of Adaprev not long after the decision to halt further development of Juvista, due to a failure of a phase II trial to meet clinical endpoints regarding range of motion in treated patients (Proactiveinvestors, 2011), despite continued demonstration of efficacy in mouse models years after its clinical failure (Wong et al., 2014).

Other failed anti-scarring drugs developed by Renovo included Prevascar, Juvidex, and RN1005. Prevascar was a formulation of recombinant human interleukin-10 that, despite initial promising data, demonstrated an absence of significant effects or even negative effects as per its clinical endpoints at the conclusion of its clinical trial. Renovo announced that it would halt all further development of the Prevascar product in April 2012 (Proactiveinvestors, 2012). Juvidex was another formulation of mannose-6-phosphate, similar to Adaprev, intended for topical application for split thickness skin grafts. In a phase II trial completed in 2008, Juvidex failed to meet its primary endpoint (NIH, 2008). RN1005 was a drug developed to target Wnt signaling to attenuate scar formation. It was awarded a Knowledge Transfer Partnership by the UK government, as Renovo announced in 2010 (Renovo, 2010), but despite this the drug never made it to clinical trials, due largely in part to the compounding failures of the company by this time. This myriad of disappointments in a short period of time eventually led to a near-complete layoff of the company's labor force. Renovo announced an agreement to sell Prevascar to ARMO Biosciences Inc. in December 2012, where it has since been repurposed as an anti-cancer immunotherapeutic (ARMO, 2013). In late 2013 Renovo acquired the financial solutions corporation known as Ultimate Finance Group, and on 6 August 2014 Renovo officially changed its name to Inspired Capital with, according to CEO Brian Cole, "a clear plan to become the UK's leading provider of financial solutions to SMEs [small and medium-sized enterprises] … whose small businesses serve as a cornerstone for British economic growth," (Renovo, 2014) clearly a far cry from its original goal of developing anti-fibrotic therapies, and the company was eventually taken private in August 2015 (Bloomberg, 2017). The Renovo story is just one of many examples of failed anti-fibrotic pharmaceutical ventures and highlights the disconnect between our understanding of the mechanisms underlying scarring using *in vitro* and pre-clinical

models, and the clinically relevant (and plausibly targetable) mechanisms occurring in humans *in vivo*. In order to develop genuinely effective anti-fibrotic therapeutics for the skin, to say nothing of other organs affected by pathophysiological fibroproliferation, a comprehensive understanding of the wound healing process is necessary, complete with pathway-specific, mechanistic, and compensatory information.

### 1.3 Stages of wound healing and fibrosis

The mammalian wound healing response proceeds via a highly regulated and carefully orchestrated set of mechanisms that can be broadly partitioned into four overlapping phases, the outcomes of which dictate the nature and effectiveness of the wound repair, including the deposition and degree of a fibrotic scar. The first phase of wound healing is hemostasis, which begins immediately after wounding occurs. Hemostasis can be delineated into the primary and secondary reactions. Primary hemostasis is initiated by leakage of adenosine diphosphate (ADP) from the wounded endothelium, attracting platelets that proceed to aggregate at the wound site in order to prevent further blood loss from the patient (Richard AF Clark, 1998). Secondary hemostasis is initiated by a cascade of tissue factors including factors 3 and 7, as well as the complex of factors 5 and 10 (Lansdown, 2002). During secondary hemostasis the blood plasma undergoes coagulation and the vasculature surrounding the wound site undergoes vasoconstriction in order to further limit blood loss at the wound site. The enzyme thrombin is released in the wound site, promoting degranulation of the platelets, resulting in the release of numerous proteins including fibrinogen. Thrombin catalytically promotes the cleavage of fibrinogen to mature fibrin, the primary material that is used to form a clot at the wound site. The fibrin clot serves to trap nearby blood cells including red blood cells, leukocytes, and additional platelets, leading to maturation of the clot via secretion of additional extracellular matrix proteins

that interface with the fibrin clot. These factors include fibronectin, vitronectin, and collagen types I, III, and IV (R. Clark, 2013; Lindblad, 2003). Not only does the clot prevent further blood loss at the wound site, it also serves as a chemical and structural support to which other cell types may migrate in order to further the wound healing response.

Towards the end of the hemostatic phase, the wound healing cascade proceeds via an inflammatory phase, the primary role of which is to kill any infectious agents that might have made their way into the wound site through the punctured skin barrier. The inflammatory phase is initiated upon degranulation of platelets from the hemostatic clot, resulting in release of platelet derived growth factor and transforming growth factor-beta 1 (PDGF and TGF-β1, respectively). These growth factors increase permeability of the capillaries, allowing for neutrophils, T-lymphocytes, and monocytes to leak into the clot in a process known as leukocyte extravasation, or diapedesis (Mamdouh, Chen, Pierini, Maxfield, & Muller, 2003; Riches, 1988; A. B. Roberts, Sporn, M.B., 1996). Fibronectin previously secreted into the clot in the hemostatic phase serves as a potent adhesive substrate for neutrophils and monocytes, the latter of which undergo differentiation at the wound site into macrophages and begin to secrete macrophage chemoattractant proteins MCP1, MCP2, and MCP3, macrophage inflammatory proteins CCL3 and CCL4, and additional PDGF and TGF-β1 (Haslett, 1996; Swift, Burns, Gray, & DiPietro, 2001). This secretion of a host of factors promoting inflammation and macrophage chemotaxis contributes to a positive feedback loop through which the inflammatory response is amplified via attraction of additional neutrophils and macrophages into the wound site. The neutrophils and monocytes that are chemoattracted to the wound site adhere to fibronectin secreted at the site of the clot, and neutrophils slow the re-epithelization process via promotion of keratinocyte differentiation at the wound edge, sustaining heightened levels of oxygen at the wound site. The

macrophages and neutrophils then utilize oxygen-dependent production of hydrogen peroxide and hypochlorous acid, as well as release of antimicrobial peptides, in order to kill invading pathogens (Richard AF Clark, 1998; Dovi, Szpaderska, & DiPietro, 2004). If sustained, these anti-microbial defenses can also cause tissue damage; prolonged inflammatory responses are associated with various tissue pathologies including, but not limited to, fibrosis. In order to prevent this, neutrophils undergo apoptotic cell death after which their remains are phagocytized by macrophages. The macrophages themselves undergo vascular endothelial growth factor (VEGF)-mediated apoptosis, from VEGF secreted by fibroblasts that begin to migrate to the wound site towards the end of the inflammatory phase, as the proliferation phase of wound healing is set to commence (Richard AF Clark, 1998; Dovi et al., 2004).

As the inflammatory phase tapers off, the proliferation and re-epithelialization phase of wound healing begins. A lack of cell-cell contact in the newly discontinuous, damaged tissue results in the stimulation of epidermal cells located near the wound edge to migrate towards the fibrin clot within the wound bed. This epidermal cell migration is also supported by macrophage-secreted cytokines including epidermal growth factor (EGF) and transforming growth factor alpha (TGF-α) (Barrientos, Stojadinovic, Golinko, Brem, & Tomic-Canic, 2008; Greenhalgh, 1996; Woodley, 1996). After migration, the epidermal cells are stimulated to proliferate by epiregulin, an EGF family member, in order to reform the damaged epidermal barrier (Draper, Komurasaki, Davidson, & Nanney, 2003). Concurrent with the inflammatory response, fibroblasts begin to migrate into the wound site from the surrounding dermis, where they attach to the clot fibronectin via integrin receptors (Grinnell, 1992). These fibroblasts that have attached to the clot begin to secrete specific extracellular matrix proteins including hyaluronic acid (W. J. Chen & Abatangelo, 1999), sulfated proteoglycans (Weitzhandler, 1992), and additional type III collagen

(Miller, 1992; Whitby & Ferguson, 1991) and fibronectin (McDonald, 1988). Clot fibroblasts are stimulated by chemical signals in the wound microenvironment including PDGF, TGF-β, EGF, and insulin-like growth factor-1 (IGF1). These chemical signals are initially secreted by macrophages that have migrated to the wound site during the inflammatory phase but, after the macrophages undergo apoptosis at the end of the inflammatory phase, these signals are maintained by autocrine and paracrine signaling from the clot-adherent fibroblasts themselves (Gottwald, Coerper, Schäffer, Köveker, & Stead, 1998; Grotendorst, 1992; Lawrence & Diegelmann, 1994; Pierce, 1991) and form new continuous tissue, termed "granulation tissue" by John Hunter in the 18th century due to its granular appearance under the microscope (Moore, 2009). In response to their new environment, these fibroblasts begin to synthesize and remodel the extracellular matrix (ECM) of the clot, depositing components such as hyaluronic acid, sulfated proteoglycans, collagens, and additional fibronectin (W. J. Chen & Abatangelo, 1999; McDonald, 1988; Miller, 1992; Weitzhandler, 1992; Whitby & Ferguson, 1991).

In early granulation tissue, hyaluronic acid and its receptors CD44 and receptor for HA-mediated motility (RHAMM) predominate in the extracellular matrix, and there is a large amount of type III collagen in comparison to type I collagen (R. A. Clark, 1996; McDonald, 1988). In late granulation tissue, however, sulfated proteoglycans begin to predominate; chondroitin sulfate and dertaman sulfate proteoglycans begin to replace hyaluronic acid, and type I collagen begins to predominate over type III collagen. This late-stage phenotypic switch is largely mediated by TGF-β1 (Pierce, 1991; A. B. Roberts, 1996), which stimulates the synthesis of sulfated proteoglycans and type I collagen, while inhibiting type I collagen degradation by downregulating transcription of collagenase genes and upregulating transcription of collagenase inhibitors (Jeffrey, 1992). As the granulation tissue is highly metabolically active, it needs to be

adequately provided with nutrients and oxygen, which diffuse poorly through tissue layers more than a few cells in thickness. The resultant hypoxia prompts fibroblasts to secrete pro-angiogenic factors including fibroblast growth factor 2 (FGF2) and VEGF in order to stimulate neighboring endothelial cells to regenerate capillaries, thereby supplying the wound bed with blood rich in oxygen and nutrients essential for cell survival and activity (Hunt, 1992).

Since granulation tissue is characterized by biomechanical and biochemical heterogeneity, net mechanical forces on fibroblasts due to mechanical strain from integrin-fibronectin and integrin-collagen interactions lead to the release of matrix-bound TGF-β1 from being immobilized by latent TGF-β binding proteins (LTBPs), resulting in subsequent participation of TGF-β in autocrine and paracrine responses via receptor-mediated TGF-β/TGF-βR signaling (Hinz, 2016) (**Fig 1.1**).

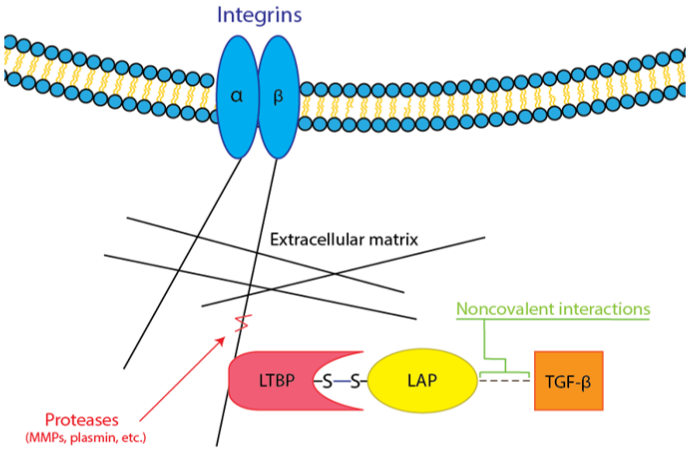


**Figure 1.1 Activation of latent TGF-ϐ**
TGF-ϐ is synthesized as a propeptide zymogen. Generally, the N-terminal latency-associated peptide (LAP) is cleaved off before cellular export, though TGF-ϐ remains associated with the LAP via noncovalent interactions. The LAP is connected via disulfide bonds to latent TGF-ϐ-binding proteins (LTBPs), which are embedded within ECM components. When the ECM is cleaved via protease activity, or strained upon mechanotransduction via integrin receptor signaling, TGF-ϐ can be liberated and be freed to interact with its cell surface receptor to initiate TGF-ϐ signaling.

In fibroblasts, stimulation by the release of matrix-bound TGF-β into the extracellular milieu results in fibroblast activation, a process by which fibroblasts differentiate into a contractile,

smooth muscle-like cell type referred to as the myofibroblast. Myofibroblasts differ from more plastic fibroblasts in their gene expression, morphology, and secretory profile. Myofibroblasts are characterized by their functional contractile ability, due to their expression of a set of contractile proteins generally restricted to smooth muscle myoblasts, including the functional contractile proteins smooth muscle alpha actin (α-SMA), calponin, and smooth muscle myosin heavy chain. Morphologically, myofibroblasts are characterized by a large spread area and prominent "supermature" focal adhesions (Goffin et al., 2006), as well as the presence of cytoplasmic stress fibers composed of filamentous actin transversing the cell body (Baum & Duffy, 2011) (**Fig 1.2**). The secretory profile of the myofibroblast consists of high levels of expression of extracellular matrix molecules including type I collagen, the profibrotic matricellular protein known as connective tissue growth factor (CTGF), and the extra domain A-containing splice variant of fibronectin (ED-A fibronectin) (Hinz et al., 2007). Particularly important to the myofibroblast phenotype is demonstrated contractile ability, which allows wound myofibroblasts to contract the wound site, closing the wound area and minimizing the amount of tissue needed to be deposited in order to recapitulate the native barrier function of the skin, or the physical boundary of the particular wounded organ in question. Ideally, after wound closure, myofibroblasts are cleared from the wound site via apoptosis mediated by interleukin 1 beta (IL-1β) (Baum & Duffy, 2011; Kaur, Chaurasia, Agrawal, Suto, & Wilson, 2009; Kissin & Korn, 2002; Zhang & Phan, 1999). However, myofibroblast persistence in the wound site and failure to undergo apoptosis is characteristic of the etiology of fibrotic pathologies (Sarrazy, Billet, Micallef, Coulomb, & Desmoulière, 2011; Van Amerongen et al., 2008).

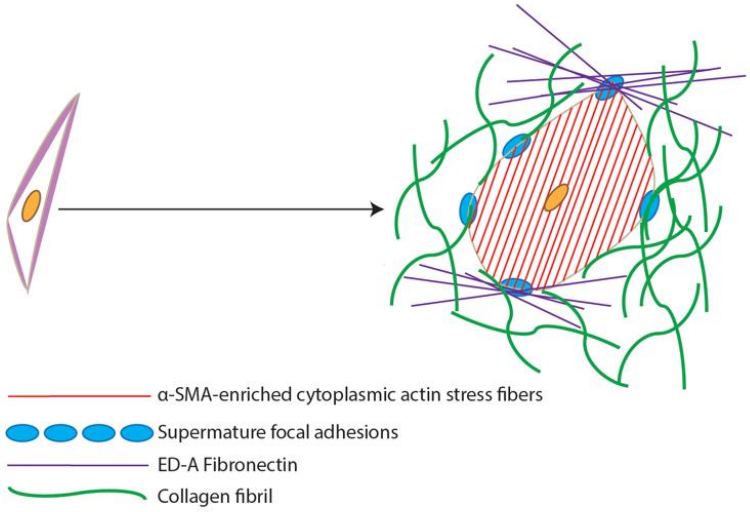


**Figure 1.2 Myofibroblast characteristics**
Fibroblast differentiation is characterized by acquisition of myofibroblast phenotypes. Myofibroblasts demonstrate a large spread area, with prominent cytoplasmic actin stress fibers enriched in alpha smooth muscle actin (α-SMA), supermature focal adhesions, and high levels of synthesis (and low levels of degradation) of ECM components including fibrilliar collagen, particularly type I collagen, as well as ED-A fibronectin.

Within the wound site, myofibroblasts serve several purposes based on their phenotypic characteristics and properties. Given that a wound results in separation between edges of intact, viable tissue, contraction by myofibroblasts in wound granulation tissue serves to bring these edges back together and minimize the need for synthesis of new tissue. In addition to their contractile properties, myofibroblasts also display a characteristic profile of synthesis and deposition of large amounts of extracellular matrix, particularly sulfated proteoglycans and type I collagen, a fibrillar structural protein that is the main constituent of scar tissue, as well as dysregulated expression of collagenases, collagenase inhibitors, and other ECM-remodeling enzymes (McAnulty, 2007; Phan, 2008).

The wound healing response culminates in the final phase, in which the granulation tissue is remodeled into a scar. The scar differs from the native dermis in several important ways. Unlike the native skin that the fibrotic tissue replaces, the scar lacks epidermal structures key to the

secondary, non-barrier functions of the skin such as pores and hair follicles (Aarabi, Longaker, & Gurtner, 2007; A. D. Metcalfe & M. W. J. Ferguson, 2007; Anthony D. Metcalfe & Mark W. J. Ferguson, 2007). Previously-deposited collagen fibers are broken down by matrix metalloproteinases (MMPs) and cross-linked into new fibers that run parallel to the wound surface, in opposition to the "basket weave" pattern found in uninjured, healthy dermis, while elastin fibers are also degraded in the scar tissue (Davidson, 1992; Linares, 1996; Miller, 1992). As a result, the abnormal composition of extracellular matrix leads to a decrease in the mechanical strength of scar tissue compared to normal skin and a greater propensity for subsequent breakage (Mast, 1992). The proteoglycan content of the scar differs from normal dermal tissue as well. Mature scar tissue displays lower levels of hyaluronic acid and decorin, as well as higher levels of chondroitin-4-sulfate proteoglycans, compared to healthy tissue. Over time, the scar continues to be remodeled as fibroblasts and capillary endothelial cells undergo apoptosis mediated by a lack of the survival cascade orchestrated by macrophage-secreted EGF, now largely absent from the wound site (Shao, Yi, & Wells, 2008). At some point, feedback mechanisms signal an end to the remodeling phase of wound healing, but this ending is nebulous, and the mechanisms that signal the end of the wound healing process are not well-understood (Stocum, 2012).

Generally, superficial skin wounds do not result in serious health problems; since the skin functions predominantly as a barrier organ, simple closure of an incision by deposition of scar tissue is sufficient to recapitulate the most important functions of the tissue, namely prevention of bleeding and maintenance of a protective boundary between internal physiological systems and extrinsic toxins and pathogens (Archer, 2010). However, in the event of damage that causes a fibrotic response in a large area of skin, such as that caused by burn wounds, the results of this

fibrosis can be far more serious. Additionally, aside from the skin, many other organs of the body are able to undergo pathophysiological fibroproliferative responses under particular conditions of stress, leading to tissue damage and often eventual organ failure.

### 1.4 Burn wound-induced fibrosis

According to the World Health Organization, burn wounds account for approximately 180,000 deaths each year, mostly occurring in low- and middle-income countries, predominantly in Africa and Southeast Asia. In addition to fatal burns, non-fatal burns are considered a leading cause of morbidity, due to the fact that they result in substantial terms of hospitalization, occurrences of disfigurement and social rejection, as well as the fact that non-fatal burns result in one of the highest sum totals of disability-adjusted life years of all conditions. Far from being an issue only in the developing world, approximately 11 million people worldwide annually are burned severely enough to require medical attention (WHO, 2017). In the United States, more than 500,000 people per year seek medical treatment for burns resulting in about 40,000 hospitalizations, 4,000 deaths, and one billion dollars in direct treatment costs, without taking into account the costs of rehabilitation and/or disability (Colohan, 2010). The pathophysiology of burn wounds can manifest itself in several manners. Severe thermal injury over a large area of the body can lead to acute systemic responses known as burn shock, which is characterized by hypovolemia and decreased cardiac output and can be fatal if not addressed by resuscitation via fluid infusion. Even when the patient is successfully treated with infusion, the follow-up period of large burns is characterized by chronic inflammation, hypermetabolism, wasting of lean muscle, and hypoproteinemia in neighboring tissue due to protein flux into the wound site (Pham, Cancio, & Gibran, 2008; Porter, Hurren, Herndon, & Børsheim, 2013). Even in cases of survival of serious burns and subsequent discharge from the hospital, patients often suffer

lifelong physical and social issues including but not limited to hypertrophic scarring, contracture of burn scar (the pressure of which may later have to be relieved by surgical intervention), chronic pain, depression, and extreme social anxiety due to disfigurement. Additionally, due to the inability of the fibrotic response to recapitulate native tissue architecture and secondary structure, burnt tissue is unable to re-form hair follicles, resulting in a lack of hair growth at the burn site, as well as a lack of pores, inhibiting the ability of the sweating response to regulate internal body temperature as intended (Wiechman, 2012). Thus, even when burn wounds do not prove acutely fatal, fibrosis resulting from the subsequent wound healing response can result in severe morbidity and loss of quality of life, particularly in patients with burn wounds covering large areas of their body.

### 1.5 Hepatic fibrosis

Despite its renowned endogenous regenerative potential, the liver is also able to undergo fibroproliferation, resulting in fibrotic disease. Thus, hepatic fibrosis refers to a class of fibroproliferative diseases that occur due to acute or chronic liver damage, leading to a perturbation in liver homeostasis and a subsequent fibrotic response. The diseases that can lead to hepatic fibrosis and end-stage liver failure are numerous; wildly varying agents can serve as initiators or drivers of these pathologies. In pediatric patients, onset of hepatic fibrosis can be induced by a number of genetic defects, including Alagille syndrome, progressive familial choleostasis, and bile acid synthetic disorders, among others. In adults, common factors underlying hepatic fibrosis include alcohol consumption, autoimmune disease, infection with hepatitis B or, more commonly, hepatitis C, Wilson’s disease, and hemochromatosis, among others (Wells, 2017). Cirrhosis, a form of late-stage liver dysfunction, was reported based on data from 2001 to be the sixth leading cause of death in developed countries, and the ninth

leading cause of death in developing countries, and the estimated prevalence of cirrhosis worldwide ranges from 4.5-9.5 percent, representing a significant health burden (Y.-S. Lim & Kim, 2008).

### 1.6 Kidney fibrosis

Virtually every type of chronic kidney disease results eventually in kidney fibrosis, the replacement of functional kidney tissue with pathophysiological non-functional scar tissue, thus leading to functional decline and organ failure, generally referred to as end-stage renal disease. Chronic kidney disease has an estimated worldwide prevalence of 8-16%, and in the United States alone, treatment of chronic kidney disease burdens the healthcare system at an annual cost of approximately $48 billion (Jha et al., 2013). Kidney disease, and subsequently kidney fibrosis, most commonly emerges as a complication of diabetes mellitus, or of hypertension, the incidence of each of which has been markedly on the rise in developed countries (Caramori, 2017). Other causes of kidney disease include genetic conditions such as polycystic kidney disease or Alport syndrome, infections, or drug-induced nephrotoxicity, among others (NIH, 2017). Matrix-producing, myofibroblast-like cells can be derived from activation of renal fibroblasts, as well as from mesangial cells or from endothelial-to-mesenchymal transition of the renal tubules of the nephron, leading to similar fibroproliferative pathophysiological responses (Liu, 2006). End-stage renal disease can only be treated by dialysis, which must be undertaken frequently and at high cost and burden, or by kidney transplant, for which there exist severe donor limitations (Lovisa, Zeisberg, & Kalluri, 2016).

### 1.7 Cardiac fibrosis

Heart disease is the predominant cause of mortality in the United States, accounting for around 800,000 deaths per year, with estimated associated medical costs expected to reach over $900 billion per year by the year 2030. (MEMBERS et al., 2014). Practically all heart disease etiologies result in pathophysiological myocardial remodeling by cardiac fibroblasts, including dysregulation of the balance between matrix synthesis and degradation, leading to progressive deposition of fibrotic extracellular matrix. This in turn leads to development of non-native, pathophysiological stiffness and other aberrations of mechanical properties of the myocardium, a reduction in tissue compliance, and acceleration of progression to heart failure. Thus, cardiac fibrosis represents an important therapeutic challenge with great potential payoff in terms of lives and money saved. The tendency of the heart to undergo fibrosis in response to insult is due in part to the ready presence and essential structural roles of activation-prone cardiac fibroblasts in the cardiac interstitium, as well as the epicardial and perivascular regions, as cardiac fibroblasts make up a large portion of the adult heart, but also due to the notoriously poor endogenous regenerative capacity of the adult myocardium, at least under pathological conditions (Travers, Kamal, Robbins, Yutzey, & Blaxall, 2016). Thus, accumulation of pathophysiological cardiac fibroblast activation, followed by an increase in cardiac stiffness and an inhibition of communication among healthy cardiac myocytes, leads naturally to a decline in cardiac function, a decline in cardiac output, and, eventually, congestive heart failure, frequently leading to patient mortality.

### 1.8 Pulmonary fibrosis

Pulmonary fibroses refer to a collection of fibroproliferative diseases of the lungs that can be caused by various insults, but all of which result in progressive stiffening of the lung and eventual loss of organ function. Causal factors for pulmonary fibroses are numerous and include

inflammatory responses induced in response to cystic fibrosis-mediated infection of the lung, repeated inhalation of pulmonary toxins including silica particles or asbestos, and treatment with chemotherapeutics, among others factors. In the event that the cause of a particular case of fibrosis cannot be determined definitively, the diagnosis falls under the umbrella category of idiopathic pulmonary fibrosis, commonly referred to as IPF (King, Pardo, & Selman, 2011). Since treatments for patients with lung fibroses have generally been limited to the paradigm of treating causal factors, for example antibiotic courses to treat pulmonary infection in patients with cystic fibrosis, a diagnosis of IPF generally carries minimal treatment options and an exceptionally poor prognosis, with a median life expectancy of 2.5-3.5 years from the point of diagnosis (Ley, Collard, & King Jr, 2011). A historical paradigm of treatment with corticosteroids, azathioprine, and N-acetylcysteine was revealed to not only lack benefit but also to be potentially harmful with the publication of the results of the PANTHER trial (Network, 2012), and thus the most recent guidelines for treatment of IPF published jointly by the American Thoracic Society, the European Respiratory Society, the Japanese Respiratory Society, and the Latin American Thoracic Association contained no recommendations for pharmaceutical therapies for patients with IPF (Raghu et al., 2011).

Only very recently has progress been made in the anti-fibrotic drug development space for pulmonary fibrosis, with the FDA approval of two drugs, pirfenidone and nintendanib, for patients with IPF. Early pulmonary protective evidence in animal models in the 1990s of pirfenidone led to broad clinical exploration of this drug for treatment of IPF, particularly at the hands of the Japanese medical community. Subsequently a large, multi-site phase III trial known as the ASCEND trial demonstrated that oral pirfenidone was effective in reducing disease progression and mortality, while increasing progression-free survival, in patients diagnosed with

IPF (King Jr et al., 2014). Around the same time, two replicate phase III trials known as the INPULSIS trials demonstrated that twice daily oral intake of nintendanib, a broad spectrum tyrosine kinase inhibitor, resulted in a reduction of disease progression in patients diagnosed with IPF (Richeldi et al., 2014). Despite the relatively small magnitudes of the effects of these drugs, and the fact that most patients that undergo treatment will still succumb to the disease, the development and clinical successes of these pharmaceutical therapies provide some hope for continued antifibrotic drug development for other organs and other fibroproliferative pathologies.

### 1.9 The ubiquity of the myofibroblast

Fibroproliferative diseases have been described in organs as varied as the lungs, kidney, liver, eye, heart, and skin, among others. Though each fibrotic pathology has causal factors and pathological manifestations specific to that organ and that condition, it appears that there are common mechanisms underlying many fibrotic pathologies, lending potential translational value to research focused on any specific fibrosis. Due to the myriad organs that can respond to insult with a fibrotic response, and due to the prevalence of risk factors including heart disease and high blood pressure in the developed world contributing to many of these pathologies, it is reasonable to estimate that around half of all deaths worldwide come as a result of fibrotic disease states (Wynn, 2007).

One common mechanism underlying fibrotic pathology is contraction of the wound area and subsequent deposition of large quantities of collagen by a cell type known as the myofibroblast (Baum & Duffy, 2011). Myofibroblasts are characterized by their morphology, functional properties, and gene expression. Compared to non-activated fibroblasts, myofibroblasts demonstrate formation of supermature focal adhesions, which are larger in area and thus are able

to exert more stress, contributing to the maintenance of myofibroblastic phenotypes (Goffin et al., 2006). Myofibroblasts contain contractile bundles, known as stress fibers, rich in smooth muscle α-actin (α-SMA), which augments their ability to generate contractile force in the wound site. Myofibroblasts are also characterized as producing high quantities of extracellular matrix, particularly type I collagen and the fibronectin isoform known as extra domain A (ED-A) fibronectin (Hinz, 2016).

Fibroblast activation to myofibroblasts is a normal part of wound healing. During the proliferative phase of wound healing, myofibroblasts are derived from activation of local connective tissue fibroblasts, and they can also arise from other sources including mesenchymal progenitor cells from the injured tissue or bone marrow, circulating fibrocytes, or even from neighboring epithelial cells via the epithelial-to-mesenchymal transition (EMT), among other sources depending on the tissue in question, including hepatic stellate cells in the liver or mesangial cells in the kidney (Micallef et al., 2012) (**Figure 1.3**). Understanding the different progenitor cells that differentiate into myofibroblasts under various pathophysiological fibroses, in both a tissue-specific and etiology-specific manner, is key to understanding not only the cellular determinants of different pathological manifestations of various fibrotic pathologies, but also for understanding and formulating new anti-fibrotic therapies. Ideally, at the resolution of the wound healing process, myofibroblasts undergo apoptosis to clear the wound site. However, under pathological conditions, myofibroblasts are retained at the site of the wound, where they partake in overproduction of collagen and deposition of a fibrotic scar (Baum & Duffy, 2011; Sarrazy et al., 2011). Many cytokines and growth factors have been interrogated for their roles in activation and resolution of fibrosis at the wound site. One of the most well-studied and promising of these is the cytokine known as fibroblast growth factor 2 (FGF2).

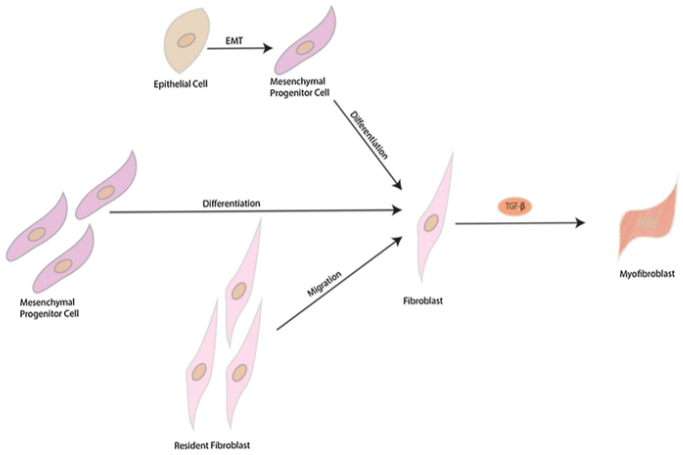


**Figure 1.3 Varied sources of wound myofibroblasts**
Depending on the tissue, wound myofibroblasts can be derived from various sources.
Fibroblasts in tissues nearby the wound may undergo chemotaxis to reach the wound site. Alternatively, mesenchymal progenitor cells may migrate to the wound site and differentiate into myofibroblasts to contribute to wound contraction and collagen deposition as well. Transdifferentiation can also contribute to the pool of myofibroblasts, as neighboring cells may undergo the epithelial-to-mesenchymal (EMT) or endothelial-to-mesenchymal transition (EndMT) in order to become myofibroblast precursors and differentiate into functional myofibroblasts.

## 1.10 FGF2 and FGF2 signaling

FGF2, also known as basic fibroblast growth factor (bFGF), is one of the family of mammalian fibroblast growth factors consisting of at least 23 members (22 identified in humans to date). Fibroblast growth factors have been implicated in a wide array of cellular and organismal processes including embryonic development, pathogenesis, oncogenesis, and tissue repair, among many others. At the cellular level, the roles of fibroblast growth factors prove every bit as myriad, where they contribute to processes as diverse as differentiation, metabolism, self-renewal, and proliferation, among others (D. M. Ornitz & Itoh, 2015).

FGF2 is encoded by the *FGF2* gene, which is located on chromosome 4 in humans (D. M. Ornitz & Itoh, 2001). *FGF2* codes for a single transcript, which is translated into 5 different protein isoforms by the use of alternative, in-frame start codons. The shortest isoform, translation of which is initiated by a canonical 5'-AUG-3' start codon, is known as the low molecular weight or 18kDa FGF2 isoform. Each of the other isoforms (22kDa, 22.5kDa, 24kDa, and 34kDa, known as the high molecular weight isoforms) is a co-linear, N-terminal extension of the low molecular weight 18kDa FGF2 isoform, with translation initiated at one of four non-canonical 5'-CUG-3' start codons (Delrieu, 2000) (**Fig 1.4**). While the high molecular weight FGF2 isoforms have attracted significant attention from researchers in recent years, the overwhelming majority of the data, both clinical and mechanistic, regarding the antifibrotic effects of FGF2 in particular have been garnered from experimentation with low molecular weight FGF2. Thus from here onward, "FGF2" will refer to the low molecular weight 18kDa isoform of FGF2 unless explicitly stated otherwise.

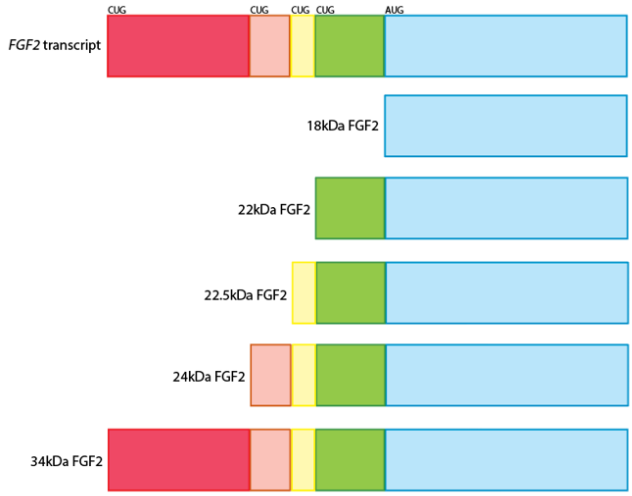


**Figure 1.4 *FGF2* structure**
The *FGF2* gene encodes one transcript that can be translated into five different protein isoforms, either from the canonical 5'-AUG-3' start codon (18kDa isoform, referred to as the low molecular weight isoform), or from one of four noncanonical 5'-CUG-3' start codons (22, 22.5, 24, and 34kDa isoforms, referred to as the high molecular weight isoforms). The high molecular weight isoforms are all collinear, N-terminal extensions of the 18kDa isoform.

From cells that produce it, FGF2 is secreted via an unconventional mechanism that is not entirely understood, but that appears to act via direct translocation across the plasma membrane, rather than via vesicle-mediated exocytosis as is typical with most other secreted proteins (Steringer, Müller, & Nickel, 2015). As FGF2 is generally secreted, it thus acts predominantly in an autocrine or paracrine manner via its cell surface receptors, the fibroblast growth factor receptors (FGFRs). There are four genes encoding FGFRs, *FGFR1, FGFR2, FGFR3,* and *FGFR4*. Each of these genes encodes for an FGF-specific receptor tyrosine kinase. With the exception of *FGFR4*, each of the *FGFR* genes contains two receptor isoforms, denoted *FGFRx* IIIb or IIIc, where "x" refers to the gene from which the transcript is transcribed, and "IIIb" or "IIIc" refer to inclusion of mutually exclusive exons (exon IIIb or exon IIIc) in the particular transcript variant of the receptor (Turner & Grose, 2010). Alongside the diversity present in the family of FGF ligands, this variable inclusion of alternate exons in FGFRs imparts an additional degree of receptor-ligand specificity to FGF signaling. While FGF1, the prototypic member of the FGF family, is able to bind and activate all of the FGF receptor variants, it is the only family member to exhibit this degree of promiscuity; FGF2 is unable to activate FGFR2 IIIb and FGFR3 IIIb, accounting at least in part for the differences in action of FGF2 and FGF1 under certain contexts (D. M. Ornitz et al., 1996). When FGF2 comes into contact with its receptor, an interaction mediated by heparin sulfate proteoglycans on the cell surface (D. Ornitz & Leder, 1992; D. M. Ornitz et al., 1992; Schultz et al., 2017), it induces structural changes in FGFR dimers leading to increased intracellular receptor tyrosine kinase activity (Sarabipour & Hristova, 2016). Intracellular adaptor and effector proteins are recruited to transduce the growth factor signal, leading to stimulation of myriad pathways, most notably the mitogen-activated protein kinases (MAPKs) (Ahn, Lee, Kwack, & Kwon, 2009; Heffron & Mandell, 2005; Liao et al., 2007; Maher, 1999),

but also other pathways such as Akt/mTOR (Eiselleova et al., 2009; Lau, So, & Leung, 2013; Lin et al., 2011), STAT (Dudka, Sweet, & Heath, 2010; Hart et al., 2000; Su, Kitagawa, Xue, & Xie, 1997), and PLCγ (Y. Chen, Li, Berezin, & Bock, 2010; Fearon & Grose, 2014; Ma, Ponnusamy, Song, Ming, & Song, 2009; Maffucci et al., 2009) (**Fig 1.5**). The myriad combinations of variant-specific and isoform-specific FGF/FGFR interactions, coupled with a variety of downstream signal transduction pathways, is responsible for the pleiotropy of FGF2 signaling specifically, and of fibroblast growth factor signaling more generally.

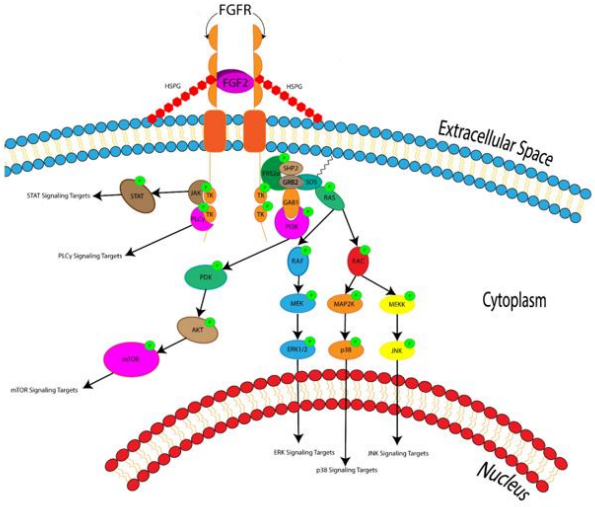


**Figure 1.5 Schematic of canonical FGF2/FGFR signaling**
Schematic overview of FGF2/FGFR signaling. The extracellular FGF2 ligand binds its receptor, FGFR, in conjunction with cell surface heparin sulfate proteoglycans (HSPGs). This causes receptor dimerization and activation of intracellular tyrosine kinase (TK) domains. These tyrosine kinase domains can directly phosphorylate target signaling proteins such as JAK, leading to activation of JAK/STAT signaling, or PLCγ. Alternatively, the tyrosine kinase domains can phosphorylate adaptor proteins like FRS2α, leading to activation of myriad downstream pathways. Adaptor protein-mediated signal transduction through PI3K leads to activation of AKT and, subsequently, mTOR. In parallel, activation of RAS transduces a signal that activates mitogen-activated protein kinases (MAPKs). RAS can activate RAF, which leads to phosphorylation and activation of ERK and subsequent ERK signaling targets, or RAC, which leads to activation of the MAPKs p38 and JNK. The diversity of FGF2/FGFR signal effectors accounts for much of the complexity and the pleiotropy of FGF2 signaling.

Of all of the fibroblast growth factors, FGF2 is one of the most well-studied members, particularly for its roles in osteogenesis (Byun et al., 2014; Lei et al., 2015; S. Lim et al., 2016; D. M. Ornitz & Marie, 2015), stem cell self-renewal (Eiselleova et al., 2009; Ishii, Kanatsu-Shinohara, Toyokuni, & Shinohara, 2012; Kole et al., 2017; Levenstein et al., 2006; Zaragosi, Ailhaud, & Dani, 2006), and tissue repair. Specifically, FGF2 has been recognized as a potent

antifibrotic agent when applied to wounds of both non-human animals (Akasaka, Ono, Yamashita, Jimbow, & Ishii, 2004; McGee et al., 1988; Shi et al., 2013; Spyrou & Naylor, 2002) and humans (Akita, Akino, Imaizumi, & Hirano, 2008; Akita et al., 2006; Nissen, Polverini, Gamelli, & DiPietro, 1996; Nunes, Li, Sun, Kinnunen, & Fernig, 2016; Ono et al., 2007). Though the role of FGF2 as an efficacious antifibrotic is clear, the mechanisms underlying its therapeutic effects are not entirely understood. In order to better ask unanswered questions regarding the mechanisms by which pharmacologic FGF2 acts as an antifibrotic agent, it is helpful to first understand what is known about these mechanisms, particularly through its actions on myofibroblasts and on cells that have the potential to become them, as myofibroblast activity is an integral paradigm underlying fibrotic pathology, and a paradigm in which FGF2 has been directly implicated.

### 1.11 FGF2 in clinical treatment of wounds

In addition to decades of extensive literature in pre-clinical, non-human animal models, FGF2 has shown a substantial amount of efficacy in improvement of the healing process and wound outcome in human patients for treatment of various types of wounds, including but not limited to suture wounds, split-thickness skin grafts, vocal cord injury, chronic wounds including pressure ulcers and diabetic foot ulcers, burn wounds, corneal abrasions, avulsions, periodontitis, oral mucositis, tympanic membrane perforation, and cardiovascular disease, reviewed extensively in (Nunes et al., 2016) and (Spaccapelo, 2016). In addition to promotion and acceleration of wound closure in several pathophysiological tissue scenarios, FGF2 has demonstrated potent antifibrotic potential in burn wounds (Akita et al., 2008), surgical incisions (Lu, Jin, & Pang, 2006; Ono et al., 2007), vocal cord injury (Kanazawa et al., 2015) and skin graft wounds (Matsumine, 2015; Xu, Li, & Fan, 2000), among others. Clinically, Kaken Pharmaceutical Company obtained

exclusive licensing rights for recombinant human FGF2 in 1988. Following more than a decade of continued research and development work, culminating in human clinical trials, a solution of recombinant human FGF2 (under the product name Fiblast) was approved in 2001 for treatment of ulcers. In 2005, Kaken acquired the rights for worldwide development of manufacturing and marketing the drug for all other therapeutic purposes, due to its recognized potential for other regenerative medicine applications (Kaken). In China, recombinant bovine FGF2 has been formulated into several products for corneal and topical cutaneous wound healing, as well as other indications such as bone fractures and haemorrhagic stroke (Spaccapelo, 2016). At the pharmacologic doses utilized in clinical care, no serious adverse effects have been reported from the use of FGF2. However, it is worth mentioning that the use of growth factor therapies entails malignancy risks. Several pre-clinical animal studies demonstrate promotion of neoplastic, neovascular, and metastasic potential for FGF2 in cancer models (Fabris et al., 2010; Giulianelli et al., 2008; Tsunoda, Nakamura, Sakurai, & Saiki, 2007), suggesting possible wisdom in contraindication of the use of FGF2 for patients who previously had cancer, currently have cancer, or are genetically or environmentally predisposed to cancer. Concerns about malignant side-effects of growth factor therapies are not limited to the use of FGF2. In a post-market follow-up study the FDA describes a randomized, controlled trial in which clinical use of Regranex ® (generic name becaplermin), a gel formulation of recombinant human PDGF-BB indicated for treatment of diabetic neuropathic ulcers, resulted in an increased rate of remote malignancy development and an increased rate of death from systemic malignancies. Thus, the FDA cautions the healthcare provider to carefully weigh the risks and benefits of becaplermin treatment prior to prescription (FDA, 2008). The European Medicines Agency describes a review conducted by the Agency's Committee for Medicinal Products for Human Use. A small

observational study of Regranex users and a control group of patients did not find a significant difference in the overall risk of developing cancer between these groups, but it did describe that patients who used three or more tubes of becaplermin and did develop cancer were more likely to die from it, possibly due to the well-established role of PDGF as an angiogenic and mitogenic agent. The committee noted that, despite the lack of firm evidence linking Regranex and cancer, nor was there sufficient evidence to rule out a link (EMA, 2010).

Given aforementioned concerns about growth factor therapies and malignancies, as well as general difficulties associated with using growth factors as therapeutic agents including short half-life and restricted absorption (Park, Hwang, & Yoon, 2017), a more comprehensive understanding of the downstream effectors and molecular mechanisms by which FGF2 acts as an antifibrotic may yield novel pharmaceutical targets or treatment paradigms to inhibit or reverse fibroproliferative disease phenotypes while minimizing off-target effects including those related to malignant transformation or promotion of metastasis.

### 1.12 Mechanistic evidence for anti-fibrotic effects of FGF2

Though the anti-fibrotic mechanisms of FGF2 are not entirely understood, there is a substantial body of literature investigating pharmacologic application of FGF2 *in vitro* and *in vivo*.

In a rat skin wound model, FGF2 promoted wound healing and acceleration of wound closure concurrent with an alleviation in scar formation. FGF2-treated animals demonstrated reduced accumulation of types I and III collagen in the wound relative to control animals, and wounded rat skin stained positive by the apoptotic TUNEL assay in $\alpha$-SMA$^{+}$ cells (a common marker indicative of myofibroblasts) in FGF2-treated but not untreated rats, likely indicating the

existence of FGF2-mediated apoptosis in wound myofibroblasts *in vivo* (Shi et al., 2013). Concordantly, other reports detailed that administration of FGF2 to rat incisional wounds resulted in an increase in apoptosis of granulation tissue fibroblasts, and a subsequent decrease in tissue fibrosis (Akasaka et al., 2004; Ishiguro et al., 2009). Interestingly, since most of the reports of FGF2-induced promotion of myofibroblast apoptosis have been performed *in vivo*, and based on reports in which FGF2 fails to induce myofibroblast apoptosis *in vitro* (Svystonyuk et al., 2015), it is likely that FGF2-mediated induction of apoptosis in myofibroblasts occurs at least in part cell-nonautonomously, via an indirect pathway involving other cell types. Taken together, evidence from these reports suggests that FGF2 has the ability to reduce the presence of aberrant myofibroblasts in the wound site through promotion of myofibroblast apoptosis.

Aside from resident fibroblasts, several other cell types in the body can differentiate to form myofibroblasts at the wound site and thus may contribute to the fibroproliferative response *in vivo.* Recent evidence has demonstrated the ability of FGF2 to antagonize myofibroblast differentiation from some of these progenitor populations as well, while inhibiting pro-fibrotic TGF-β signaling (**Fig 1.6**).

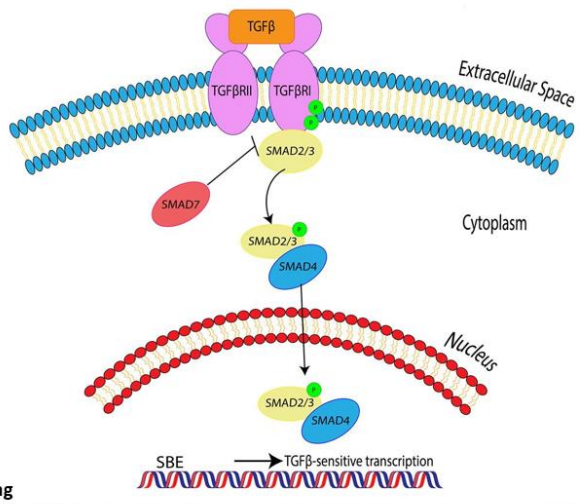


**Figure 1.6 Canonical TGF-ß signaling**
Schematic overview of canonical TGF-β/SMAD signaling. The extracellular TGF-β ligand binds to the TGF-βRII receptor, which subsequently phosphorylates TGF-βRI and propagates the growth factor signal. In the canonical signaling pathway, TGF-βRI phosphorylates serine residues in the C-terminal, MH2 region of SMAD2 and SMAD3. When phosphorylated, these residues are able to associate with SMAD4 and translocate to the nucleus, where the SMAD complex associates with other protein co-factors (not shown) at SMAD binding elements (SBEs) located in the promoters of particular genes, driving expression of these genes. The inhibitory protein SMAD7 inhibits the interaction between TGF-βRI and SMAD2, thus preventing SMAD2 phosphorylation and subsequent association with SMAD4, which is necessary for nuclear translocation and promotion of further TGF-β-mediated signal transduction

Culture of retinal pericytes in the presence of exogenous FGF2 maintained a highly proliferative, non-contractile phenotype devoid of α-SMA expression, whereas removal of FGF2 or culture in the presence of exogenous TGF-β1 induced withdrawal from the cell cycle and promoted a contractile phenotype (Papetti, Shujath, Riley, & Herman, 2003). Li et al. demonstrated that seeding of FGF2-producing, bone marrow-derived mesenchymal stem cells into a graphene scaffold and implantation into a dorsal excision wound model resulted in faster healing and a decrease in expression of fibrosis-associated genes encoding α-SMA and TGF-β1, and an increase in expression of the gene encoding the transforming growth factor family member TGF-β3 (Li, Wang, Yang, Sun, & Huo, 2015). TGF-β3 has demonstrated antifibrotic activity *in vivo* (Chang, Kishimoto, Hasan, & Welham, 2014), and elevated TGF-β3/TGF-β1 ratios have been associated with scarless healing phenotypes including those of oral mucosa (Schrementi, Ferreira, Zender, & DiPietro, 2008) and those induced by tattooing (Ferguson & O'Kane, 2004). In human valvular interstitial cells, which are of mesenchymal origin and are largely

myofibroblastic in phenotype, addition of exogenous FGF2 reduced cell area, contractile activity, and a host of myofibroblast markers and deposition of ECM proteins (Latif et al., 2015). Valvular interstitial cells demonstrate a classical activation response to TGF-β, and it has been reported that FGF2 has the ability to impede this TGF-β response in an ERK-dependent manner, likely via a decrease in SMAD-driven promoter activity through inhibition of SMAD3 nuclear accumulation (Cushing, Mariner, Liao, Sims, & Anseth, 2008). Though the mechanisms underlying the inhibition of nuclear translocation of SMAD3 by FGF2 are not entirely clear, it may the case that MAPK signaling induced by FGF2 regulates the phosphorylation status of the linker region of SMAD signal transducers, the effects of which are context-dependent and not entirely understood, but are known to affect SMAD localization and, intriguingly, extracellular matrix synthesis in various contexts (Burch, Zheng, & Little, 2011; Derynck & Zhang, 2003; G. Wang, Matsuura, He, & Liu, 2009). In vascular smooth muscle cells, FGF2 promotes proliferation, which is associated with a synthetic, non-contractile phenotype compared to a contractile phenotype, analogous to the phenotypic switch between more highly proliferative, less contractile fibroblasts and less proliferative, more contractile and matrix-synthetic myofibroblasts (Chua, Rahimi, Forsten-Williams, & Nugent, 2004). In primary human aortic smooth muscle cells, knockdown of FGFR1 or of its adaptor protein FRS2α potentiated TGF-β signaling and induced a contractile phenotype. In contrast, addition of exogenous FGF2 or overexpression of constitutively active FGFR1 each maintained smooth muscle cells in a proliferative, noncontractile state, suggesting that signaling downstream of FGFR1 is responsible, at least in part, for the antagonism of contractile phenotype in these cells demonstrated upon addition of exogenous FGF2, and may provide hints to the identity of the pathways underlying the anti-contractile properties witnessed in other cell types such as valvular

interstitial cells or fibroblasts (P.-Y. Chen, Qin, Li, Tellides, & Simons, 2016). Similarly, in human airway smooth muscle cells, exogenous FGF2 demonstrated ERK-dependent attenuation of TGF-β-induced contractile activity and expression of smooth muscle/myofibroblast-associated contractile proteins α-SMA, calponin and SM22α. This antagonism of contractile activity may be independent of direct effects on canonical TGF-β signaling, or at least may be downstream of canonical SMAD phosphorylation, as exogenous FGF2 did not affect the levels of p-SMAD2/3 induced by TGF-β. FGF2 was able to both inhibit *de novo* TGF-β-mediated contractile protein expression and to reverse existing contractile protein expression, when added concurrently with, or 24 hours after, TGF-β respectively. This effect was demonstrated even in the presence of TGF-βRI inhibitor, suggesting that the anti-contractile effect of FGF2 is unlikely to be time sensitive, at least in this context, and is independent of TGF-βRI activity (Schuliga et al., 2013). Taken together these data suggest that FGF2 can inhibit contractile activity and myofibroblast phenotypes in cell types other than fibroblasts, likely at least in part by interference with signal transduction via TGF-β and SMADs.

Evidence also exists that FGF2 has the ability to suppress acquisition of mesenchymal markers and myofibroblastic phenotypes in other cell types. In primary mouse lens epithelial cells, FGF2 antagonized TGF-β2-mediated epithelial-to-mesenchymal transition (EMT), a form of transdifferentiation involved in the establishment of a fibrotic eye disease known as posterior capsule opacification. FGF2-treated lens epithelial cells demonstrated diminished presence of stress fibers and α-SMA expression, a process reversed by ERK inhibition (Kubo et al., 2017). In lung epithelial cells, FGF1 (the closest family member to FGF2) contributed to the maintenance of epithelial morphology and phenotypes in contrast to TGF-β1 treatment, as assessed by an increase in expression of E-Cadherin and decrease in expression of α-SMA, in a manner also

dependent on ERK (Ramos et al., 2010). Recently, Wang et al. reported that FGF2 attenuated the differentiation of rat epidermal stem cells into myofibroblasts, as assessed by downregulation of Collagen I, Collagen III, and α-SMA, in a manner dependent on both FGFR activation and Notch/Jagged signaling. *In vivo*, pharmacologic application of FGF2 to a rabbit ear wound model resulted in accelerated wound closure and better scar quality as determined by histological analysis. FGF2 treatment resulted in a greater proportion of proliferative epidermal cells and a lesser proportion of proliferative mesenchymal (α-$SMA^{+}$) cells, suggesting that FGF2 may promote retention of epidermal stem cells in their progenitor state *in vitro* by antagonizing differentiation to myofibroblasts or myofibroblast-like cells (P. Wang et al., 2017). Similarly, in mouse lymphatic endothelial cells, FGF2 suppressed mesenchymal phenotypes and preserved endothelial phenotypes, while depletion of FGF2 or addition of TGF-β resulted in promotion of the endothelial-to-mesenchymal transition (EndMT). Overexpression of H-Ras rescued the EndMT-suppressing effects of FGF2 depletion via inhibition of TGFβ-mediated SMAD2 activation, implicating FGF2/FGFR/Ras in TGFβ signaling and maintenance of endothelial cellular identity. Knockdown of endogenous Ras reduced endothelial markers, suggesting that baseline Ras activity maintains endothelial and suppresses mesenchymal phenotypes in endothelial cells (Ichise, Yoshida, & Ichise, 2014). Taken together, these data suggest that FGF2 is able to prevent or reverse transdifferentiation to myofibroblasts from precursors such as endothelial and epithelial cells.

### 1.13 Conclusions

There is ample evidence that pharmacologic FGF2 has the potential to attenuate fibrotic phenotypes and drive more desirable, regenerative resolution of wound healing in damaged tissue, characterized by lesser severity of scar deposition, though the mechanisms through which

this therapeutic activity arises, particularly as they relate to antagonism of fibroblast activation, are not entirely understood. With a better understanding of the mechanisms by which FGF2 antagonizes fibrotic processes in vivo, and a more intimate understanding of the molecular pathways involved in FGF2-mediated and MAPK-mediated antagonism of these processes, we hope to gain insight into the basic biology of the wound resolution process, as well as into possible ventures that may yield much-needed molecular antifibrotic therapies.

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# Chapter 2-FGF2 antagonizes fibroblast activation and myofibroblast phenotypes

## 2.1 Abstract

Previous human and non-human animal studies have demonstrated the ability of exogenously administered basic fibroblast growth factor (FGF2) to act as an antifibrotic agent, particularly in the skin. Though the activity of FGF2 as an anti-fibrotic agent is well-established, in particular for fibroproliferative skin wound responses, the mechanisms by which FGF2 exerts these actions are not entirely understood. Here we describe a general effect of FGF2 signaling in human dermal fibroblasts that maintains proliferative, rather than activated, fibroblast phenotypes *in vitro*. FGF2 dampens basal expression of myofibroblast markers *in vitro*, as well as attenuates TGF-β-mediated activation of these myofibroblast markers at the level of both the transcript and the protein. FGF2-mediated antagonism of myofibroblast marker expression is also accompanied by a concomitant decrease in myofibroblast-associated extracellular matrix production, including a decrease in the expression of type I collagen and ED-A fibronectin, and a decrease in functional contractile activity. Taken together, these data suggest that the mechanisms of FGF2-mediated inhibition of fibroblast activity *in vitro*, and likely *in vivo*, occur at least in part via FGF2-mediated antagonism of fibroblast activation into myofibroblasts, resulting in lesser pathological wound contracture and a reduction in deposition of nonfunctional, fibrotic extracellular matrix.

## 2.2 Introduction

Fibrosis is an undesirable result of wound healing and subsequent tissue repair that affects myriad tissues in the body. Rather than replacing damaged tissue with functional replacement, the fibrotic response results in deposition of a collagenous scar that preserves barrier function but fails to recapitulate the native function and mechanical properties of the tissue. As various tissue-specific diseases result in fibrotic outcomes, this suggests that common cellular and molecular mechanisms, at least in part, underlie tissue fibrosis across various organs (Rockey, Bell, & Hill, 2015). One of the most important of these mechanisms is the presence and activity of the myofibroblast, an activated fibroblast denoted by the presence of actin stress fibers, contractile capability, and high levels of collagen production and deposition (Hinz et al., 2012). It has recently been demonstrated that myofibroblasts arise from multiple different cell types *in vivo*, including fibrocytes, mesenchymal stem cells, and smooth muscle cells (Hinz et al., 2007). However, one of the most important paradigms in skin fibrosis, in particular, is the activation of resident granulation tissue fibroblasts by transforming growth factor beta (TGF-β) signaling. Mechanical heterogeneity of the granulation tissue leads to the release of TGF-β1 from the provisional extracellular matrix, leading to the differentiation of resident wound fibroblasts into myofibroblasts (termed “fibroblast activation”) (Hinz, 2016). These myofibroblasts, with their contractile potential and high levels of collagen deposition, are important to wound closure, but if they persist aberrantly in the wound past the phase of wound resolution they contribute to formation of a nonfunctional hypertrophic scar (Sarrazy, Billet, Micallef, Coulomb, & Desmoulière, 2011).

Fibroblast growth factor 2 (FGF2) is one of the most well-studied members of the fibroblast growth factor superfamily, and it has been implicated in cellular processes and paradigms as

diverse as mitogenesis, differentiation, proliferative lifespan, survival, oncogenesis, and stem cell self-renewal, among others (Bailly, Soulet, Leroy, & Amalric, 2000; Dolivo, Hernandez, & Dominko, 2016; Eiselleova et al., 2009; Fang et al., 2003). FGF2 activates its target receptor tyrosine kinases, the FGFRs, on the cell surface in order to activate numerous downstream pathways, including several mitogen activated protein kinase (MAPK) pathways (Ornitz & Itoh, 2015). Importantly, it has been determined that application of exogenous FGF2 has both accelerative and anti-fibrotic effects in various types of skin wounds, reviewed extensively in (Nunes, Li, Sun, Kinnunen, & Fernig, 2016). This has been demonstrated in humans for acute incisional wounds, avulsions, and burn wounds (Akita, Akino, Imaizumi, & Hirano, 2008; Matsumine, 2015; Ono et al., 2007), as well as in several animal models (Eto et al., 2012; Shi et al., 2013). The mechanisms and signaling pathways by which FGF2 inhibits the fibrotic response have been investigated previously but remain incompletely understood. Previously, our lab and others have described anti-fibrotic gene expression paradigms in fibroblasts in response to FGF2 treatment, including downregulation of inflammatory cytokines, upregulation of interstitial collagenases, and inhibition of pro-fibrotic integrin signaling (Delrieu, Jean-Charles, Bayard, & Maret, 1999; Grella, Kole, Holmes, & Dominko, 2016; Kashpur, LaPointe, Ambady, Ryder, & Dominko, 2013; Newberry, Willis, Latifi, Boudreaux, & Towler, 1997; Shi et al., 2013; Yasui et al., 2004). Additionally, FGF2 has been demonstrated under certain circumstances to antagonize TGF-β signaling, including reports in which FGF2 has demonstrated the potential to antagonize TGF-β-mediated myofibroblast phenotypes (Chen, Qin, Li, Tellides, & Simons, 2016; Correia, Moonen, Brinker, & Krenning, 2016; Eto et al., 2012; Grella et al., 2016; Ichise, Yoshida, & Ichise, 2014; Ito, Sawada, Fujiwara, & Seyama, 2007; Shi et al., 2013). Thus, since FGF2 is known to act directly on fibroblasts to produce anti-fibrotic and anti-myofibroblastic effects, and

since myofibroblasts are one of the most important effectors of the fibrotic response in the skin, we set out to better understand the effects of FGF2 as an antagonist to TGF-β in human dermal fibroblasts.

## 2.3 Results

We first hypothesized that FGF2 attenuates fibroblast activation and maintains fibroblasts in an undifferentiated state. We thus set out to determine whether the phenotypic effects of FGF2 on fibroblasts *in vitro* were congruent with what would be expected from inhibition or reversal of fibroblast activity. **Table 2.1** lists some well-defined phenotypic characteristics frequently used to distinguish fibroblast activation.

| Undifferentiated fibroblast | Activated myofibroblast |
|---|---|
| Narrow, spindle-shaped cells | Wide cells with large spread area |
| Highly proliferative | Less proliferative |
| Lack of cytoplasmic stress fibers | Stress fibers enriched with α-SMA |
| Standard focal adhesions | Large, "supermature" focal adhesions |
| Normal produciton of collagen and ED-A Fn | Enhanced production of collagen and ED-A Fn |
| High metalloproteinase activity | Low metalloproteinase activity |
| Minimal contractile activity | Potent contractile activity |

**Table 2.1 Defining phenotypic characteristics of undifferentiated fibroblasts versus activated myofibroblasts**

### 2.3.1 FGF2 enhances fibroblast proliferation

As proliferation is generally associated with maintenance of cellular plasticity, and due to the well-established inverse relationship between cellular proliferation and differentiation (Ruijtenberg & van den Heuvel, 2016), we expected that a factor that antagonizes fibroblast activation, and thus FGF2, would be likely to induce cellular proliferation. Indeed, stimulation of CRL-2097 human neonatal foreskin fibroblasts and CRL-2352 human adult dermal fibroblasts resulted in a substantial increase in proliferation as assessed by direct cell count at each passage (**Figure 2.1**). These data fit with the wealth of existing literature that FGF2 induces proliferation

in fibroblasts (among other cell types), and further suggest that FGF2 maintains fibroblasts in an inactivated state.

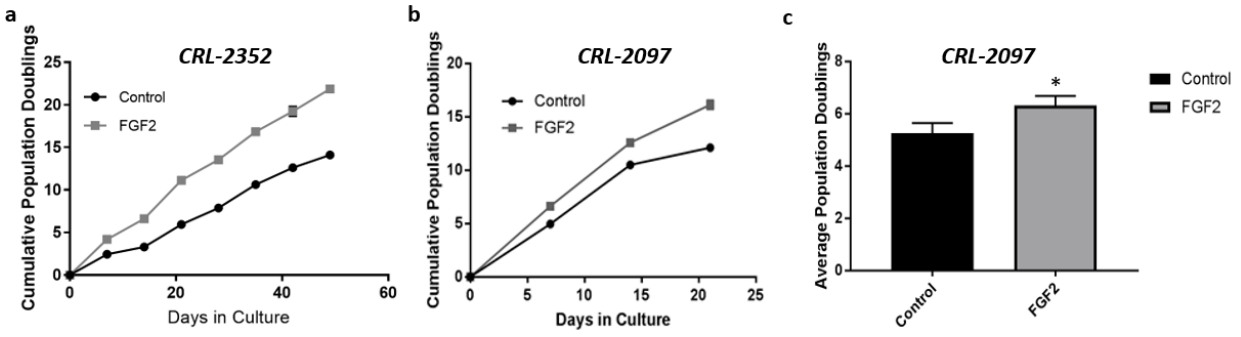


**Figure 2.1 Effects of FGF2 on fibroblast growth**

**(a)** CRL-2352 and **(b)** CRL-2097 human dermal fibroblasts were grown in the presence or absence of 4ng/mL FGF2 for 49 and 21 days in culture, respectively. Population doublings were calculated at each passage and used to construct growth curves reflected by cumulative population doublings as a function of time in culture. n=2 biological replicates per time point per condition. Error bars=standard deviation. **(c)** CRL-2097 fibroblasts were cultured for four days in the presence or absence of 4ng/mL FGF2 and the number of population doublings was calculated for each treatment condition. n=4 biological replicates/condition. Error bars=standard deviation. Statistical significance was determined using Student's t-test. *=p<.05

### 2.3.2 FGF2 treatment maintains undifferentiated fibroblastic phenotype

Since myofibroblasts are characterized by large spread area and prominent F-actin stress fibers, we decided to visually inspect fibroblasts cultured in the presence or absence of exogenous FGF2 in order to determine the effects of FGF2 on superficial fibroblast phenotype. Treatment of fibroblasts with exogenous FGF2 maintained fibroblasts in a narrow, spindle-like shape and suppressed the formation of F-actin stressed fibers as assessed qualitatively by fluorescent microscopy (**Figure 2.2**). These data further suggest that FGF2 maintains fibroblasts in an unactivated state, as characterized by the maintenance of fibroblastic phenotypes in culture of fibroblasts cultured in the presence of exogenous FGF2.

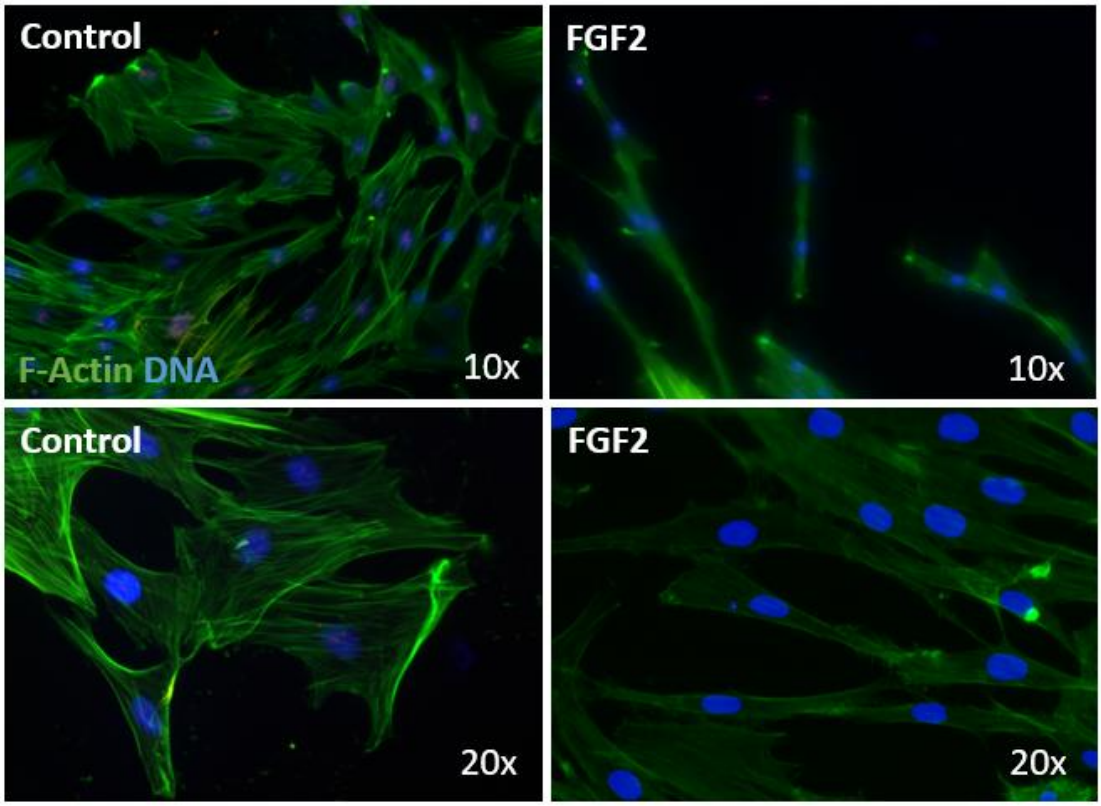


**Figure 2.2 Effects of FGF2 on fibroblast morphology**
CRL-2352 adult human dermal fibroblasts were grown in the presence or absence of 4ng/mL FGF2 until day 4 in culture, fixed and stained with fluorescent phalloidin, and imaged at 10x and 20x in order to visualize F-actin and cell shape. Images between treatment conditions were taken with identical magnifications and exposure times.

### 2.3.3 FGF2 inhibits collagen gel contraction by fibroblasts

Since contractile activity is a hallmark of myofibroblast phenotype, and since it is critically important to myofibroblast phenotype and fibroproliferative etiology *in vivo*, we wished to determine the effects of FGF2 on fibroblast contractile potential. CRL-2097 fibroblasts treated with exogenous FGF2 demonstrated reduced contraction of a collagen lattice after 24 hours in culture (**Figure 2.3a**), and this reduction in contraction corresponded with a decrease in protein expression of the canonical myofibroblast maker α-SMA, as determined by Western blot (**Figure 2.3b**). These data suggest that FGF2 inhibits fibroblast contractile activity, possibly by downregulation of functional contractile proteins including α-SMA.

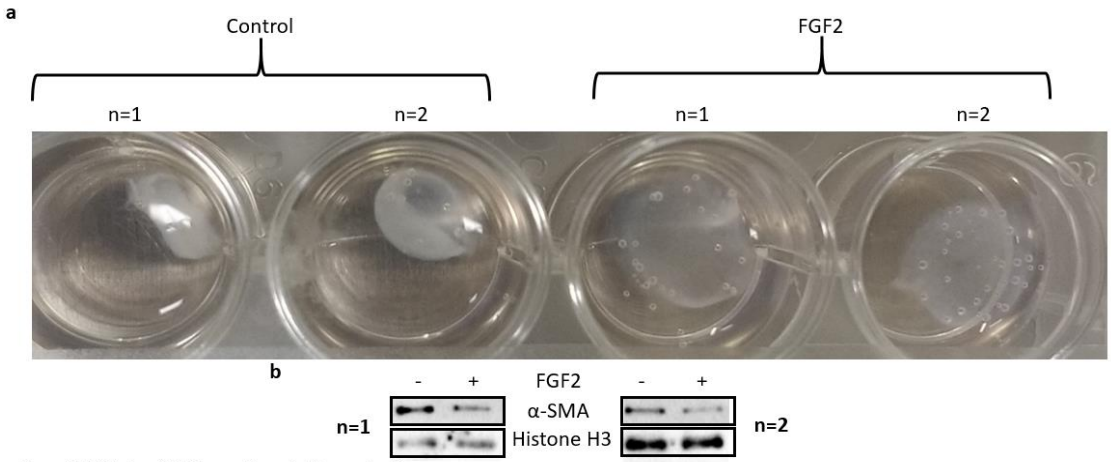


**Figure 2.3 Effects of FGF2 on collagen lattice contraction**
**(a)** CRL-2097 fibroblasts were seeded into a collagen I lattice and cultured for 24 hours in the presence or absence of 4ng/mL exogenous FGF2. Lattices were photographed at t=24 hours post-treatment. 2 replicates are shown in the figure. **(b)** Cell-seeded collagen lattices were digested and subject to Western blot analysis for myofibroblast marker smooth muscle alpha α- actin. Histone H3 was used as a loading control.

### 2.3.4 FGF2 robustly downregulates basal and TGF-β1-induced expression of α-SMA

Since treatment with FGF2 antagonized contractile function of fibroblasts, and since this antagonism of contractile function was associated with decreased α-SMA protein in fibroblasts, we wished to determine whether FGF2 was able to decrease expression of α-SMA, particularly α-SMA induced by TGF-β. CRL-2097s were cultured until day 4 in the presence or absence of exogenous FGF2 and TGF-β1, and expression of *ACTA2* transcript and α-SMA protein were determined by several assays. Analysis by qRT-PCR determined that TGF-β1 induced expression of *ACTA2* transcript, and that treatment with co-treatment with exogenous FGF2 antagonized this induction substantially. Fibroblasts treated with FGF2 also demonstrated a decrease in basal expression of *ACTA2* (**Figure 2.4a**). Analysis by Western blot determined that FGF2 also downregulated substantially both basal and TGF-β-induced expression of α-SMA protein, in agreement with the Western blot data from **Figure 2.3**. Analysis of α-SMA expression using a monoclonal antibody and indirect flow cytometry determined that FGF2 substantially decreased both the percentage of cells expressing α-SMA (**Figure 2.4c**) and the average α-SMA expression per cell (**Figure 2.4d**), and that FGF2 also antagonized TGF-β1-mediated induction of α-SMA expression at both the population and individual cell levels by these same metrics.

Visualization of α-SMA protein was performed using immunofluorescence, and these data were consistent with the flow cytometry data; FGF2 decreased both basal and TGF-β1-induced expression of α-SMA at the single cell and the population levels, and FGF2 antagonized the formation of α-SMA-containing filamentous actin stress fibers, similar to that which was observed in **Figure 2.2**. Taken together, these data demonstrate that FGF2 decreases basal and TGF-β1-induced α-SMA expression and antagonizes formation of α-SMA-containing F-actin stress fibers in fibroblasts, in a manner that is at least in part a result of downregulation of the transcript for *ACTA2*, the gene encoding the α-SMA protein.

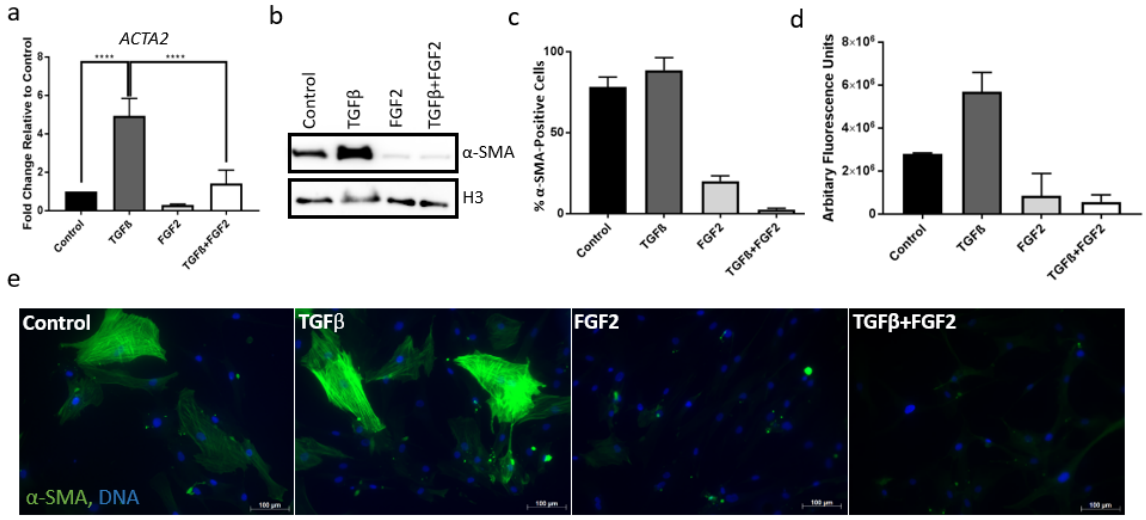


**Figure 2.4 Effects of FGF2 on expression of α-SMA**
CRL-2097 fibroblasts were cultured in the presence or absence of 4ng/mL exogenous FGF2 and/or 10ng/mL exogenous TGF-β1. Analysis on day 4 was perfomed for all assays. **(a)** qRT-PCR was used to analyze transcript level of *ACTA2*, the gene encoding α-SMA. n=4 biological replicates per experimental condition. Error bars=standard deviation. ****=p<.0001. One-way ANOVA was performed with Tukey's multiple comparison post-hoc test in order to determine significance. Data are normalized to *GAPDH* and fold change calculated using the $\Delta\Delta C_t$ method and expressed as fold change relative to control fibroblasts cultured in the absence of exogenous FGF2 and TGF-β1. **(b)** Western blot was used to measure total α-SMA protein. Histone H3 was used as a loading control. **(c,d)** Indirect flow cytometry was performed to detect expression of α-SMA at the level of **(c)** the population and **(d)** the individual cell. Data are expressed as percentage of α-SMA positive cells and fluorescence per cell, respectively. n=2 biological replicates per condition. Error bars=standard deviation. **(e)** Immunofluorescence was performed in order to visualize the expression and localization of α-SMA. DNA was counterstained with Hoechst. Error bars=100μm.

### 2.3.5 FGF2 antagonizes basal and TGF-β1-induced expression of myofibroblast markers

Since FGF2 decreased contractile activity and robustly downregulated α-SMA expression in human dermal fibroblasts, we next wished to determine whether FGF2 also inhibited the effects of other myofibroblast marker proteins involved with the contractile apparatus, or whether this

inhibitory effect was specific to α-SMA. Analysis by qRT-PCR demonstrated that FGF2 downregulated both basal and TGF-β1-induced expression of *CNN1* (**Figure 2.5a**) and *TAGLN* (**Figure 2.5b**), the genes encoding calponin and SM22α, respectively, in CRL-2097 fibroblasts. Concordantly, analysis by Western blot determined that FGF2 downregulated both basal and TGF-β1-induced expression of calponin and SM22α in addition to α-SMA, as was demonstrated previously **(Figure 2.5c**). We recognized that there was a possibility that the ability of FGF2 to antagonize myofibroblast protein expression may be idiosyncratic. Thus, we performed Western blot analysis for the same myofibroblast markers on two other lines of human dermal fibroblasts, CRL-2352s and CT-1005s, and witnessed a qualitatively similar pattern of FGF2-mediated downregulation of basal and of TGF-β1-induced expression of α-SMA, calponin, and SM22α protein. Taken together, these data suggest that FGF2 robustly antagonizes fibroblast activation, as demonstrated by a reduction in canonical contractile myofibroblast markers, and that this antagonism is due at least in part to a decrease in expression of the transcripts encoding these proteins.

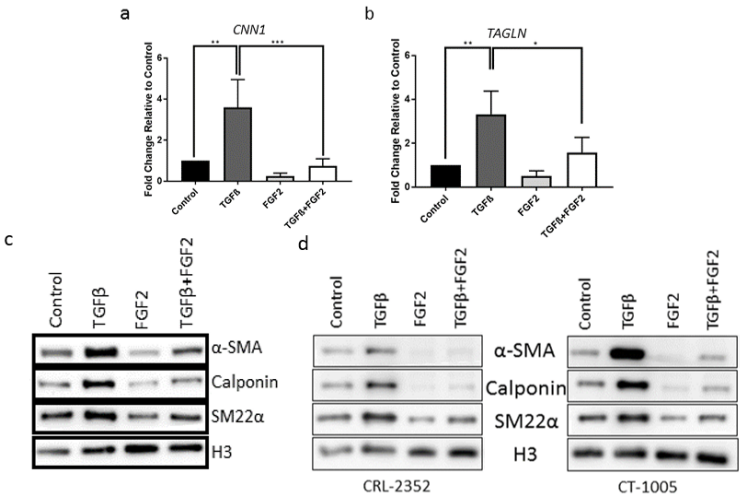


**Figure 2.5 Effects of FGF2 on myofibroblast markers**

CRL-2097 fibroblasts were cultured in the presence or absence of 4ng/mL exogenous FGF2 and/or 10ng/mL exogenous TGF-β1. Analysis on day 4 was perfomed for all assays. (**a**,**b**) qRT-PCR was used to analyze transcript level of **(a)** *CNN1*, the gene encoding Calponin and **(b)** *TAGLN*, the gene encoding SM22α. n=4 biological replicates per experimental condition. Error bars=standard deviation. *=p<.05, **=p<.01, ***=p<001, ****=p<.0001. One-way ANOVA was performed with Tukey's multiple comparison post-hoc test in order to determine significance. Data are normalized to *GAPDH* and fold change calculated using the $\Delta\Delta C_t$ method and expressed as fold change relative to control fibroblasts cultured in the absence of exogenous FGF2 and TGF-β1. (**c**,**d**) Western blot analysis was performed to analyze protein expression of myofibroblast markers α-SMA, Calponin, and SM22α in (**c**) CRL-2097 and (**d**) CRL-2352 and CT-1005 human dermal fibroblasts. Histone H3 was used as a loading control.

### 2.3.6 FGF2 antagonizes pro-fibrotic ECM and ECM-related protein production

Since FGF2 inhibited fibroblast activation into myofibroblasts as assessed by common myofibroblast markers, we next wished to assess whether FGF2 also was able to attenuate expression of fibrotic effectors expressed and secreted by myofibroblasts, including myofibroblast-associated ECM gene expression. Analysis of transcript expression by qRT-PCR demonstrated that FGF2 antagonized both basal and TGF-β1-induced transcript expression of *CCN2* (**Figure 2.6a**) and of the ED-A splice variant of *Fn* (**Figure 2.6b**), the transcripts encoding for CTGF and ED-A fibronectin, respectively. In addition, FGF2 downregulated basal and TGF-β1-mediated expression of *MYOCD*, the gene encoding myocardin (**Figure 2.6c**), a transcription factor causally implicated in the expression of myofibroblast marker genes, particularly in smooth muscle cells (Wang, Wang, Pipes, & Olson, 2003). FGF2 also downregulated basal expression of *IL6* (**Figure 2.6d**), the gene encoding interleukin 6, a cytokine that has been causally implicated in fibrotic pathophysiology and the fibrotic response *in vivo* (Fielding et al., 2014; Liechty, Adzick, & Crombleholme, 2000). Immunofluorescent

analysis (**Figure 2.6e**) also demonstrated that treatment of FGF2 attenuated expression of both collagen I (green) and ED-A fibronectin (magenta), and attenuated TGF-β1-mediated expression of these ECM proteins, as would be expected from fibroblasts prompted to maintain or revert to an undifferentiated state. Western blot analysis was concordant with the immunofluorescent data and transcript expression data, demonstrating that treatment with FGF2 resulted in a downregulation of collagen I, ED-A fibronectin, and CTGF protein expression in both CRL-2097 and CRL-2352 human dermal fibroblasts (**Figure 2.6f**). The exception to this is that CTGF expression was unable to be detected at the protein level in CRL-2352 fibroblasts, so the effects of FGF2 on expression of this protein could not be determined in this cell line. The presence of two immunoreactive CTGF bands in the CRL-2097 Western blot is likely indicative of the presence of both glycosylated and non-glycosylated forms of the protein, as has been reported previously (Yang, Kim, Wilson, Rosenfeld, & Oh, 1998). Taken together, these data indicate that FGF2 is able to inhibit both basal and TGF-β1-induced expression of ECM and ECM-associated matricellular proteins in fibroblasts, and that this inhibition is due at least in part to downregulation of ECM protein transcripts. Additionally, FGF2 has the ability to downregulate other pro-fibrotic genes in fibroblasts as well.

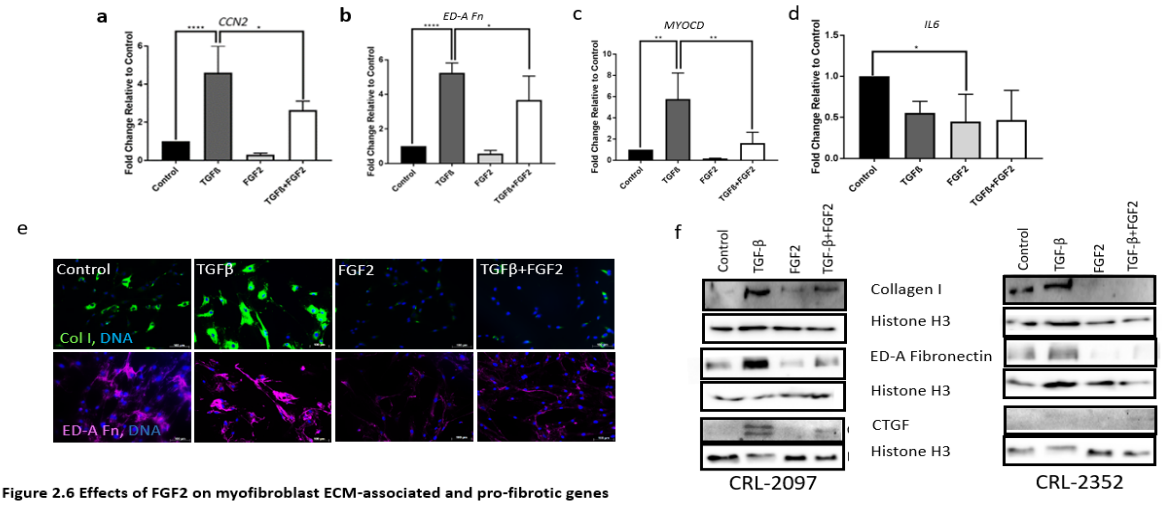


**Figure 2.6 Effects of FGF2 on myofibroblast ECM-associated and pro-fibrotic genes**
CRL-2097 fibroblasts were cultured in the presence or absence of 4ng/mL exogenous FGF2 and/or 10ng/mL exogenous TGF-β1. Analysis on day 4 was perfomed for all assays. (**a-d**) qRT-PCR was used to analyze transcript level of **(a)** *CCN2*, the gene encoding connective tissue growth factor (CTGF) and **(b)** *ED-A Fn*, the gene encoding for the ED-A splice variant of fibronectin, **(c)** *MYOCD*, the gene encoding myocardin, and **(d)** *IL6*, the gene encoding interleukin 6. n=4 biological replicates per experimental condition. Error bars=standard deviation. *=p<.05, ****=p<.0001. One-way ANOVA was performed with Tukey's multiple comparison post-hoc test in order to determine significance. Data are normalized to *GAPDH* and fold change calculated using the $\Delta\Delta C_t$ method and expressed as fold change relative to control fibroblasts cultured in the absence of exogenous FGF2 and TGF-β1. (**e**) Immunofluorescent analysis was performed to analyze protein expression of myofibroblast ECM proteins Collagen I and ED-A fibronectin. DNA was counterstained with Hoechst. Scale bar=100μM. (**f**) Western blot analysis was performed in order to determine expression of myofibroblast ECM proteins Collagen I and ED-A fibronectin, and matricellular pro-fibrotic protein CTGF in CRL-2097 and CRL-2352 human dermal fibroblasts. Histone H3 was used as a loading control.

In addition to ECM transcript and protein expression, we also wished to investigate the expression of genes involved in maturation and degradation of ECM proteins. Treatment of CRL-2097 fibroblasts with FGF2 attenuated (p=.07) TGF-β1-mediated upregulation of the *LOX* transcript (**Figure 2.7a**), the gene encoding lysyl oxidase, a protein responsible for cross-linking immature collagen proteins into mature collagen fibrils. In comparison, FGF2 failed to significantly downregulate *LOXL2*, a functional homologue of *LOX* (**Figure 2.7b**). FGF2 also both upregulated basal transcript expression and rescued TGF-β1-mediated decrease in transcript expression of *MMP1* (**Figure 2.7c**), the gene encoding matrix metalloproteinase 1 (MMP1), a collagenase responsible for degradation of fibrillar collagen. Since FGF2 upregulated *MMP1* expression, and since previous data from our lab demonstrates other dysregulation in expression of proteases and their inhibitors (Kashpur et al., 2013), we wished to determine whether FGF2-mediated decrease in type I collagen, a major constituent of scar tissue and the dominant collagen in the fibrotic pathophysiological response, was dependent in part on metalloproteinase

activity. We utilized a broad spectrum MMP inhibitor, ilomastat (also known as GM6001) in order to inhibit metalloproteinase activity. The structure and pharmacodynamic values of this compound are included in **Figure 2.7d**. Co-treatment of CRL-2097 fibroblasts with ilomastat inhibited the collagen I depletion effects witnessed upon culture in the presence of exogenous FGF2 **(Figure 2.7e**). In comparison, FGF2 failed to decrease the protein level of type IV collagen in cultured fibroblasts, congruent with data we had published previously (Grella et al., 2016), suggesting that the MMP-mediated decrease in collagen in fibroblasts is not general to all collagens but rather is likely specific to particular collagens, including type I collagen.

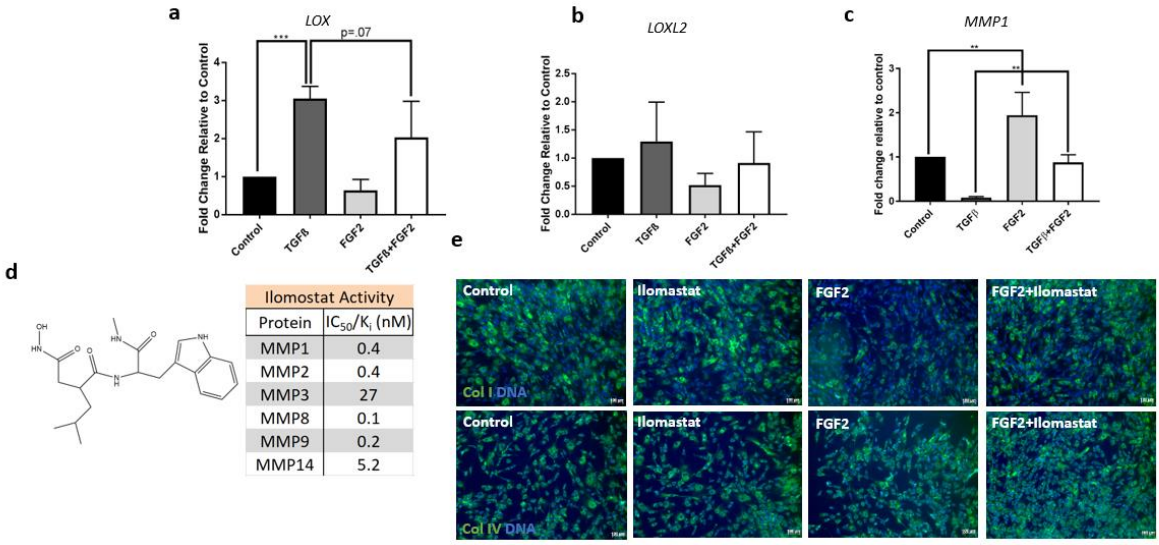


**Figure 2.7 Effects of FGF2 on matrix maturity and degradation**
CRL-2097 fibroblasts were cultured in the presence or absence of 4ng/mL exogenous FGF2 and/or 10ng/mL exogenous TGF-β1 and/or 10μM ilomastat. Analysis on day 4 was perfomed for all assays. (**a-c**) qRT-PCR was used to analyze transcript level of **(a)** *MMP1*, the gene encoding matrix metalloproteinase 1, **(a)** *LOX*, the gene encoding lysyl oxidase **(b)**, *LOXL2*, the gene encoding lysyl oxidase-like 2, **(c)**. n=4 biological replicates per experimental condition. Error bars=standard deviation. **=p<.01, ***=p<.001. One-way ANOVA was performed with Tukey's multiple comparison post-hoc test in order to determine significance. Data are normalized to *GAPDH* and fold change calculated using the $\Delta\Delta C_t$ method and expressed as fold change relative to control fibroblasts cultured in the absence of exogenous FGF2 and TGF-β1. (**d**) Chemical structure of ilomastat, also known as GM6001, as well as its *in vitro* pharmacodynamic characterization. **(e)** Immunofluorescent analysis was performed to analyze expression of Collagen I and Collagen IV. DNA was counterstained with Hoechst. Scale bar=100μM.

### 2.3.7 FGF2 fails to induce fibroblast apoptosis *in vitro* and to reverse fibroblast activation at short time points

One possible mechanism by which FGF2 might reduce fibroblast activation is by specific apoptotic induction in activated fibroblasts, a mechanism not unlike that which happens in healthy wound resolution (Darby, Laverdet, Bonté, & Desmoulière, 2014). In order to test this

hypothesis, we decided to culture cells in the presence or absence of exogenous FGF2 and TGF-β1, and determine the level of apoptotic cells by Annexin V/Propidium iodide co-stained flow cytometry on day 4 in culture (**Figure 2.8a**). Culture of CRL-2097 fibroblasts in the presence of FGF2 and/or TGF-β1 failed to induce any substantial amount of cell death **(Figure 2.8b**) or, more specifically, apoptosis (**Figure 2.8c**) in cultured fibroblasts. These data suggest that the mechanism of inhibition of fibroblast activation by FGF2 is not due to stimulation of fibroblast apoptosis *in vitro* and, if the mechanism of action *in vivo* is in part due to myofibroblast apoptosis, then the effect is likely not a cell-autonomous response of the myofibroblast.

As there was no evident apoptotic response from human fibroblasts cultured in the presence of exogenous FGF2, and since most of our assays performed up until this point had investigated the effects of fibroblasts treated until day 4, we next wanted to determine whether FGF2 had the ability to reverse fibroblast activation at comparably short time points. Culture of human fibroblasts in the presence of exogenous FGF2 for time points of up to 6 hours failed to reduce α-SMA protein levels to any noticeable degree (**Figure 2.8d**). Thus, it appears that the inhibitory effects of FGF2 on fibroblast activation likely either occur over a longer time frame, or are more potent at preventing activation of fibroblasts in the first place, rather than reversing activation.

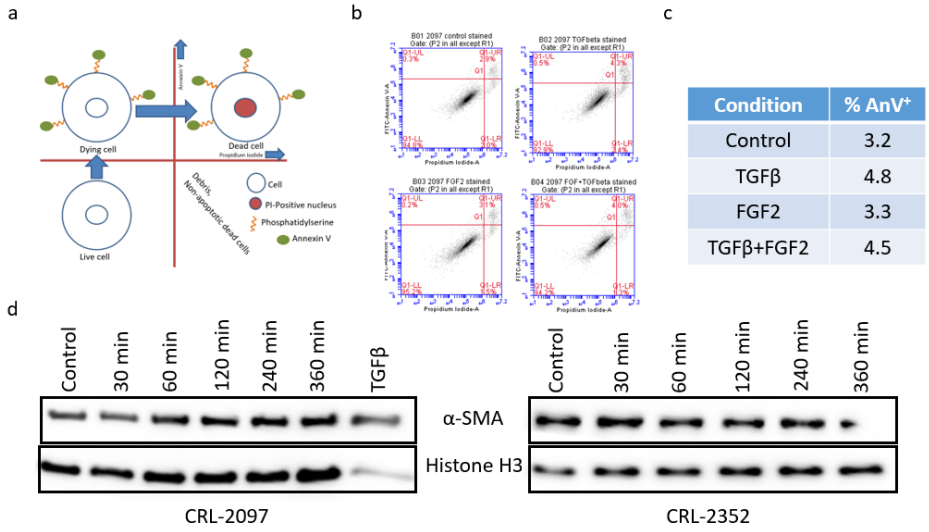


**Figure 2.8 Effects of FGF2 on apoptosis and reversal of differentiation**
**(a)** Schematic representing the interpretation of different populations of cells in Annexin V/Propidium Iodide staining by flow cytometry. **(b)** CRL-2097 fibroblasts were cultured in the presence or absence of 4ng/mL exogenous FGF2 and/or 10ng/mL exogenous TGF-β1, and cells were fixed, stained, and analyzed by flow cytometry for Annexin V and Propidium iodide on day 4. **(c)** Data from **(b)** were quantified and represented as the % Annexin V$^+$ cells in the population. **(d)** Human fibroblasts were cultured in the presence of 4ng/mL exogenous FGF2, 10ng/mL of exogenous TGF-β1, or neither for the indicated periods of time, and Western blot analysis was performed for α-SMA. Histone H3 was used as a loading control.

### 2.3.8 FGF2 fails to attenuate TGF-β1-mediated induction of pro-fibrotic integrin β1

Since fibroblast activation is heavily tied to the cellular mechanical microenvironment, we decided to investigate whether FGF2 had notable effects on pro-fibrotic integrin expression. Our lab has previously demonstrated that FGF2 substantially attenuates expression of integrin α11 (Grella et al., 2016), a relatively poorly studied integrin but with potent effects and causal associations regarding fibroblast activation (Navab et al., 2016; Rodriguez et al., 2009; Talior-Volodarsky, Connelly, Arora, Gullberg, & McCulloch, 2012). Since integrin α11 signals in conjunction with its heterodimeric binding partner, integrin β1, and since abrogation of integrin β1 signaling (which is known to be upregulated under multiple fibrotic conditions) has recently been demonstrated to block or reverse fibrotic phenotypes and gene expression paradigms in myofibroblastic activated hepatic stellate cells *in vitro*, we wished to determine whether FGF2 could mediate expression of integrin β1 in addition to that of integrin α11. We first utilized flow cytometry to investigate whether there was a correlation between expression of integrin β1, a known pro-fibrotic integrin, with α-SMA, a canonical myofibroblast marker. Flow cytometry

analysis demonstrated that there was a strong positive correlation between expression of α-SMA and expression of Integrin β1 on the per-cell level (**Figure 2.9a**), suggesting likely involvement of integrin β1 in establishment of α-SMA positive myofibroblasts. We then wished to investigate expression of integrin β1 as a function of treatment with exogenous growth factors. In fibroblasts cultured in the presence or absence of 4ng/mL FGF2 and/or 10ng/mL TGF-β1, greater than 96 percent of all cells analyzed stained positive for integrin β1 for each treatment condition (**Figure 2.9b**). We then analyzed integrin β1 expression per cell. TGF-β1 modestly increased the expression of integrin β1, and FGF2 modestly decreased the expression of integrin β1, per positive cell (~33.8% increase and ~ 33.0% decrease in expression, respectively), but treatment of fibroblasts with exogenous FGF2 did not appear to attenuate the increase in integrin β1 expression induced by treatment with exogenous TGF-β1 (**Figure 2.9c**). Taken together, these data suggest that, though FGF2 may decrease basal integrin β1 expression, it appears unable to rescue the heightened expression of integrin β1 induced by treatment with TGF-β1.

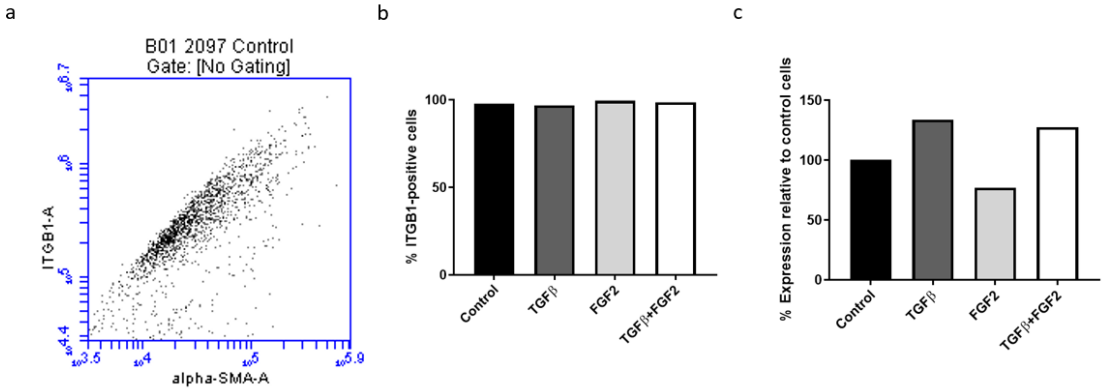


**Figure 2.9 Effects of FGF2 and TGF-β1 on Integrin β1 expression**
**(a)** Two-color flow cytometry analysis demonstrating a strong positive correlation between expression of α-SMA and expression of integrin β1 in CRL-2097 fibroblasts grown under control conditions in the absence of exogenous growth factor supplementation. **(b-c)** CRL-2097 fibroblasts were cultured in the presence or absence of 4ng/mL exogenous FGF2 and/or 10ng/mL exogenous TGF-β1, and cells were fixed, stained, and analyzed by flow cytometry for Integrin β1 on day 4. **(b)** Percentage of cells displaying a positive signal for expression of Integrin β1 as determined by flow cytometry. **(c)** Percent expression of Integrin β1 per positively-stained cell relative to fibroblasts cultured under control conditions.

## 2.4 Discussion

Though FGF2 is well-established as an anti-fibrotic compound *in vivo* and in the clinic, as has recently been reviewed thoroughly by (Nunes et al., 2016), the mechanisms by which FGF2 acts therapeutically to inhibit the fibrotic response *in vivo* are not entirely clear. We recently reviewed the available literature in order to surmise at least some of the likely mechanisms by which FGF2 modulates the wound repair process in order to drive regenerative wound repair in lieu of the fibroproliferative deposition of scar tissue (D. Dolivo, S. Larson, & T. Dominko, 2017). Namely, we explain that FGF2 serves to not only antagonize pro-fibrotic gene expression paradigms and to antagonize myofibroblast differentiation via epithelial-to-mesenchymal and endothelial-to-mesenchymal transition (EMT and EndMT, respectively) in a tissue-specific manner, but we also proposed that FGF2 inhibits or reverses differentiation of fibroblasts and myofibroblasts in the wound site, based on much of the data presented above, as well as that contributed by other labs (D. M. Dolivo, S. A. Larson, & T. Dominko, 2017). In this chapter we have demonstrated that FGF2 has the ability to shift the gene expression paradigm of human dermal fibroblasts to a less fibrotic phenotype, concurrent with antagonism of activation of fibroblasts to myofibroblasts, as assessed by common markers of myofibroblast phenotype including α-SMA, calponin, and SM22α, as well as of the transcripts that encode them. The phenotypes of FGF2-treated fibroblasts are more reminiscent of undifferentiated mesenchymal cells as well; FGF2-treated fibroblasts proliferate more quickly and are thinner and more spindle-shaped, compared to more slowly proliferating myofibroblasts that are wider and have larger spread area. Demonstration of this inhibition of fibroblast activation in several healthy primary fibroblast cell lines confirms that this effect is not limited to one cell line and is rather a generalized response of dermal fibroblasts, and likely of fibroblasts more generally, to exogenous FGF2 treatment and FGF signaling.

In addition to antagonizing both basal and TGF-β1-induced expression of myofibroblast markers, FGF2 also downregulates expression of myofibroblast-associated extracellular matrix and extracellular matrix-associated genes, resulting in deposition of a less dense, less mature extracellular matrix, characterized by lower levels of collagen I, ED-A fibronectin, and lysyl oxidase, and higher levels of MMP1. This gene expression and ECM profile is akin to what would be expected to be induced by an antifibrotic compound, as deposition of large amounts of type I collagen and ED-A fibronectin, and high expression of cross-linking enzymes including lysyl oxidase by wound myofibroblasts, is well-established as a conserved pathophysiological mechanism in various fibrotic pathologies across various tissues and organs *in vivo*.

We examined whether FGF2-mediated antagonism of fibroblast activation was due in part to induction of myofibroblast apoptosis. The motivation for this examination was due to the natural course of myofibroblast differentiation and apoptosis in the proliferation and resolution phases of wound healing (Darby et al., 2014), but also due to the evidence that, *in vivo*, FGF2 induces apoptosis in granulation tissue myofibroblasts in animal models of wound healing (Akasaka, Ono, Yamashita, Jimbow, & Ishii, 2004; Ishiguro et al., 2009; Shi et al., 2013). However, due to the complex nature of the biomechanical and biochemical environment of the myofibroblast (Hinz, 2016), the generally accepted paradigm that myofibroblast apoptosis is generally promoted by interleukin-1β at the wound site (Kissin & Korn, 2002; Zhang & Phan, 1999), a parallel report describing a lack of myofibroblast apoptosis induced by FGF2 *in vitro* (Svystonyuk et al., 2015), and the flow cytometry data we have presented in this chapter (**Figure 2.8b-c**), we feel fairly confident that the promotion of apoptosis in granulation tissue myofibroblasts is due to mechanisms that are not fibroblast-autonomous, and likely include cytokines and effector molecules from other cell types in the complex wound environment.

This chapter has revealed a host of fibroblast-mediated, cell-autonomous effects induced by FGF2 signaling that promote regenerative healing and oppose fibrotic wound resolution, shedding light on some of the mechanisms underlying the already-established anti-fibrotic effects of pharmacologic FGF2 in the process of wound healing.

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# Chapter 3-FGF2-mediated antagonism of fibroblast activation is reversed or attenuated by MAPK inhibition

## 3.1 Abstract

FGF2 has demonstrated antifibrotic potential *in vitro* and *in vivo*, but the pathways by which FGF2 transduces a signal that leads to induction of an antifibrotic gene expression paradigm are not understood. Given the pleiotropic nature of FGF2, and the well-characterized severity of off-target effects associated with therapeutic intervention at the level of the growth factor or the tyrosine kinase, it would be beneficial to better understand specific mechanisms and pathways through which FGF2 exerts its antifibrotic effects. Since we had previously demonstrated that FGF2 antagonizes activation of human dermal fibroblasts, we set out to use small molecule-mediated inhibition to determine which mitogen activated protein kinase (MAPK) pathways were responsible for these effects. Here we demonstrate that inhibition of either ERK or JNK is sufficient to reverse FGF2-mediated inhibition of fibroblast activation, as indicated by proliferative capacity, cellular morphology, expression of key markers of the myofibroblast phenotype, and deposition of extracellular matrix. This suggests that FGF2-mediated activation of ERK and JNK may negatively regulate fibroblast activation We also demonstrated that small molecule-mediated inhibition of p38 potentiated FGF2-mediated inhibition of fibroblast activation, and that inhibition of p38 was also sufficient to inhibit TGF-β-mediated fibroblast activation, suggesting that activation of p38 by FGF2 or even by other cytokines may promote fibroblast activation.

### 3.2 Introduction

The knowledge of the effects of FGF2 as a potent antifibrotic in multiple organs has led to the intriguing possibility of using FGF2, or using knowledge garnered from understanding its molecular mechanisms, for development of antifibrotic therapies (D. M. Dolivo, S. A. Larson, & T. Dominko, 2017). FGF2 is a highly pleiotropic growth factor that acts canonically via signaling through its transmembrane receptors, the fibroblast growth factor receptors (FGFRs), or non-canonically through other mediators such as syndecans and integrins in processes that are not nearly as well-understood (Murakami, Elfenbein, & Simons, 2007). Unlike FGF1, which is able to activate all known FGFR variants, FGF2 only has the ability to activate FGFR1IIIb, FGFR1IIIc, FGFR2IIIc, FGFR3IIIc, and FGFR4 (D. M. Ornitz et al., 1996). Downstream of FGFR signaling, FGF2 activates a myriad of cellular pathways including AKT/mTOR, JAK/STAT, and the MAPKs ERK, JNK, and p38.

Most of the antifibrotic effects of FGF2 have not been demonstrated to depend on particular molecular pathways, limiting the understanding of the intricacies of the effects of pharmacologic FGF2 on fibrosis. Given the highly pleiotropic effects of FGF signaling across multiple tissue types and physiological processes (David M Ornitz & Itoh, 2015), and keeping in mind the numerous known off-target effects and toxicities associated with modulating tyrosine kinase pathways (Hartmann, Haap, Kopp, & Lipp, 2009), we set out to better understand the pathways through which FGF2 exerts its antifibrotic effects, with a focus on MAPK signaling associated with canonical FGF/FGFR signal transduction.

## 3.3 Results

### 3.3.1 FGF2-mediated proliferation of human fibroblasts is attenuated by inhibition of ERK/JNK but enhanced by inhibition of p38

Since FGF2 is a well-characterized mitogen, and since proliferation of cells tends to correlate with plasticity rather than differentiated phenotypes (Ruijtenberg & van den Heuvel, 2016), we decided to use proliferative ability as a proxy to investigate fibroblast activation generally. Stimulation of CRL-2097 fibroblasts with exogenous FGF2 resulted in an increase in proliferation as evidenced by both increased reduction of resazurin (**Figure 3.1a**), and by direct cell count (**Figure 3.1b**). Co-treatment of fibroblasts with FGF2 and an inhibitor towards either ERK (10μM U0126) or JNK (10μM SP600125) resulted in attenuation of this proliferative response, suggesting that activation of these pathways may be required for FGF2-mediated proliferation in fibroblasts, and thus may also potentially be responsible for FGF2-mediated inhibition of fibroblast activation. Additionally, small molecule-mediated inhibition of p38 (10μM SB202190) promoted fibroblast proliferation cooperatively alongside co-treatment with exogenous FGF2, suggesting that inhibition of p38 may cooperatively enhance attenuation of fibroblast activation, and possibly even inhibit fibroblast activation on its own.

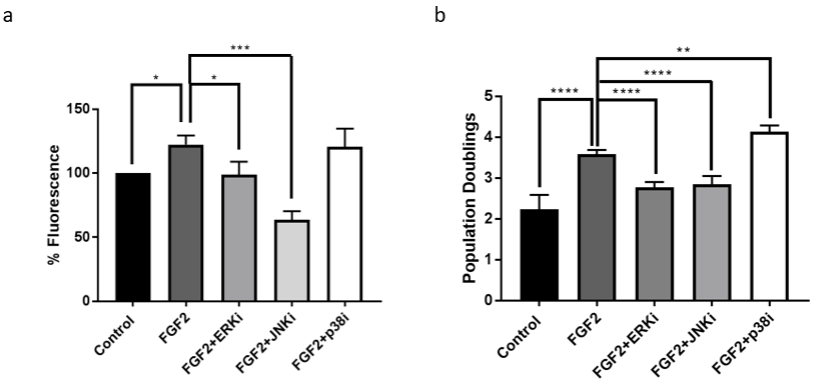



**Figure 3.1 Effects of MAPK inhibitors on FGF2-induced fibroblast proliferation**
CRL-2097 human dermal fibroblasts were grown in the presence or absence of 4ng/mL FGF2 and/or 10μM U0126 (ERKi), 10μM SP600125 (JNKi) or 10μM SB202190 (p38i) until day 4 in culture. **(a)** Resazurin was used in order to determine cell count relative to control cells. Proliferative index was expressed as percentage relative to control cells grown in the absence of exogenous FGF2 or MAPK inhibitor. n=4 biological replicates per condition. **(b)** Raw cell counts were used to determine number of population doublings in culture per condition. Error bars=standard deviation. Statistical significance was determined using a One-Way ANOVA with Tukey's post-hoc analysis. *p<.05, **p<.01, ***p<.001, ****p<.0001.

### 3.3.2 FGF2-mediated change in cell phenotype is attenuated by inhibition of ERK/JNK but not by inhibition of p38

Since we had previously determined that FGF2 decreased spread area and formation of F-actin stress fibers in fibroblasts, we wished to determine which MAPK pathways were responsible for this phenotypic switch, as a proxy to determine the ability to attenuate fibroblast activation. As demonstrated previously, treatment of CRL-2097 fibroblasts with exogenous FGF2 resulted in smaller, more elongated cells with reduced spread area and reduced F-actin stress fibers. However, when fibroblasts were co-treated with FGF2 and an inhibitor of ERK or JNK, the change to a more elongated, spindle shape was attenuated, and the resulting cells resembled the control fibroblasts more closely. In contrast, fibroblasts cultured in the presence of both FGF2 and p38 inhibitor maintained an elongated, spindle shape and lacked prominent F-actin stress fibers, thus more closely resembling the FGF2-treated cells compared to the control cells or TGF-β-treated cells (**Figure 3.2**). Taken together, these data suggest that the FGF2-mediated effects on cell morphology can be ablated by inhibition of ERK or JNK, but not by inhibition of p38, suggesting that activation of ERK and JNK pathways may be important for FGF2-mediated attenuation of fibroblast activation.

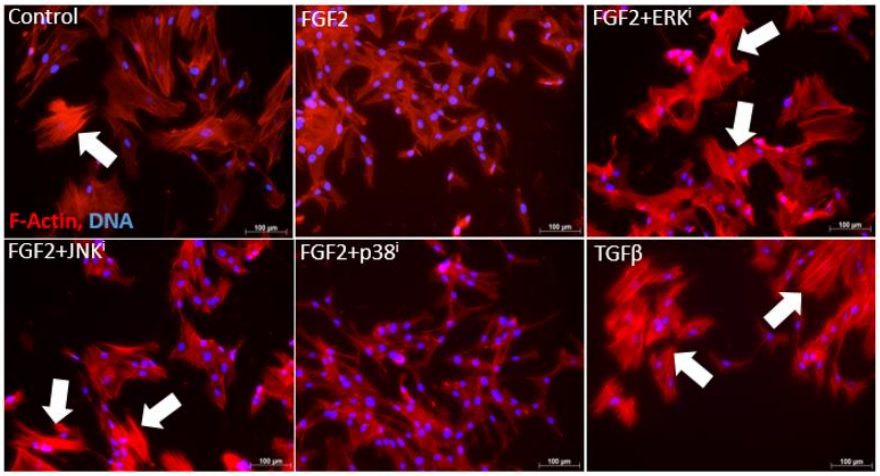



**Figure 3.2 Effects of MAPK inhibition on FGF2-mediated changes in fibroblast morphology**
CRL-2097 human dermal fibroblasts were cultured in the presence or absence of 4ng/mL exogenous recombinant FGF2, 10ng/mL exogenous recombinant TGF-ß1 and/or 10μM U0126 (ERKi), 10μM SP600125 (JNKi) or 10μM SB202190 (p38i) until day 4 in culture. Cells were then fixed and stained with fluorescent phalloidin and Hoechst, and then imaged in order to visualize F-actin, stress fiber formation, and cell shape. White arrows designate fibroblasts with densely-packed F-actin stress fibers. Scale bar=100 μm.

### 3.3.3 FGF2-mediated inhibition of myofibroblast marker and ECM protein expression is antagonized by inhibition of ERK/JNK, but not by inhibition of p38

Since we had previously demonstrated that FGF2-mediated antagonism of fibroblast activation was demonstrated by reduction in expression of myofibroblast markers, and since we were beginning to implicate specific FGF2-associated MAPK pathways in other myofibroblast phenotypes, we set out to determine which of these MAPK pathways were involved in these FGF2 effects as well. As demonstrated previously, treatment of CRL-2097 human dermal fibroblasts with exogenous recombinant FGF2 resulted in downregulation of myofibroblast-associated contractile proteins α-SMA, calponin, and SM22α. This downregulation was antagonized by co-treatment with ERK inhibitor or JNK inhibitor, but not by co-treatment with p38 inhibitor. These findings were also recapitulated in CRL-2352 human dermal fibroblasts, in which qualitatively similar effects of FGF2 and of MAPK inhibitors were observed, although with more difficulty due to the low basal expression of myofibroblast markers in these fibroblasts, as observed previously (**Figure 3.3**).

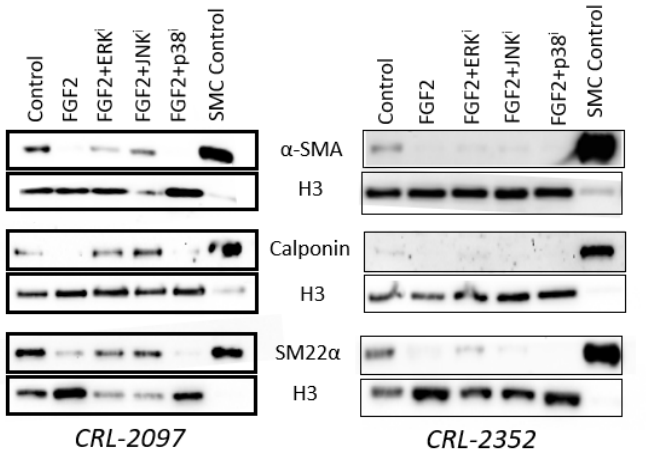



**Figure 3.3 Effects of MAPK inhibitors on FGF2-mediated antagonism of myofibroblast marker expression**
CRL-2097 (left) and CRL-2352 (right) human dermal fibroblasts were cultured in the presence or absence of 4ng/mL exogenous recombinant FGF2 and/or 10μM U0126 (ERKi), 10μM SP600125 (JNKi) or 10μM SB202190 (p38i) until day 4. Western blot analysis was performed to analyze protein expression of myofibroblast markers α-SMA, Calponin, and SM22α. Histone H3 was used as a loading control. Protein lysate from contractile human vascular smooth muscle cells (SMC control) was used as a positive control.

Since we determined that inhibition of ERK or JNK was sufficient to antagonize FGF2-mediated attenuation of myofibroblast marker expression, we then wished to determine whether or not these effects were consistent with the effects of MAPK inhibition on FGF2-mediated effects on expression of myofibroblast-associated ECM proteins. As expected, immunofluorescent analysis demonstrated that treatment with ERK inhibitor or JNK inhibitor but not p38 inhibitor antagonized FGF2-mediated attenuation of α-SMA expression and stress fiber formation, qualitatively validating the Western blot data observed in **Figure 3.3**. Immunofluorescent analysis also demonstrated that treatment with ERK inhibitor or JNK inhibitor but not p38 inhibitor antagonized FGF2-mediated attenuation of ED-A fibronectin, and of type I collagen, two extracellular matrix proteins secreted at high levels by myofibroblasts and causally associated in fibroproliferative pathologies *in vivo* (**Figure 3.4**). Taken together, these data demonstrate that the antifibrotic effects of FGF2 as an antagonist of fibroblast activation and of fibrosis-associated ECM protein expression is antagonized substantially by inhibition of ERK or JNK, but not by inhibition of p38.

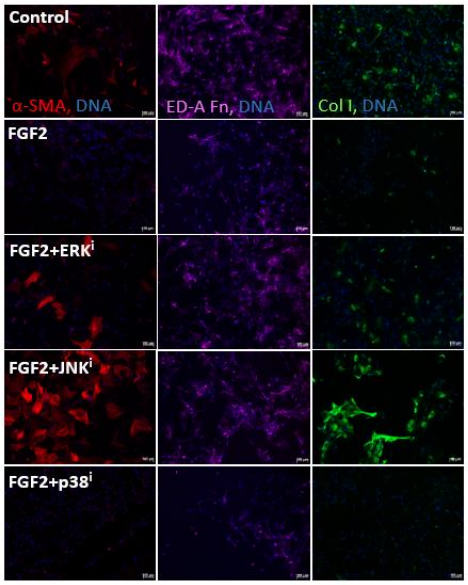



**Figure 3.4 Effects of MAPK inhibitors on FGF2-mediated antagonism of myofibroblast ECM protein expression**
CRL-2097 human dermal fibroblasts were cultured in the presence or absence of 4ng/mL exogenous recombinant FGF2 and/or 10μM U0126 (ERK$^{i}$), 10μM SP600125 (JNK$^{i}$) or 10μM SB202190 (p38$^{i}$) until day 4 in culture. Immunofluorescent analysis was performed to analyze expression of myofibroblast ECM proteins Collagen I and ED-A fibronectin. DNA was counterstained with Hoechst. Scale bar=100μM.

### 3.3.4 Inhibition of p38 antagonizes fibroblast activation

Since inhibition of p38 appeared to cooperate with FGF2 as indicated by both fibroblast proliferation (**Fig 3.1b**) and expression of myofibroblast marker proteins (**Fig. 3.3-3.4**) we wondered whether inhibition of p38 was sufficient to antagonize fibroblast activation even in the absence of exogenous FGF2. Western blot analysis **(Fig. 3.5a**) demonstrated that culture in the presence of p38 inhibitor was sufficient to not only reduce baseline levels of expression of myofibroblast markers α-SMA, calponin, and SM22α, but also to antagonize increases in expression of these protein markers by exogenous recombinant TGF-β1. Immunofluorescent analysis also demonstrated p38 inhibition-mediated antagonism of basal and of TGF-β1-induced expression of myofibroblast markers α-SMA and calponin, as well as of expression of myofibroblast-associated ECM proteins collagen I and ED-A fibronectin **(Fig 3.5b**). Inhibition of p38 also inhibited TGF-β1-mediated increase in total F-actin and of change in cell

morphology, as determined by phalloidin staining and fluorescent imaging. Taken together, these data demonstrate that p38 inhibition is sufficient to attenuate both basal and TGF-β1-mediated induction of fibroblast differentiation in human dermal fibroblasts.

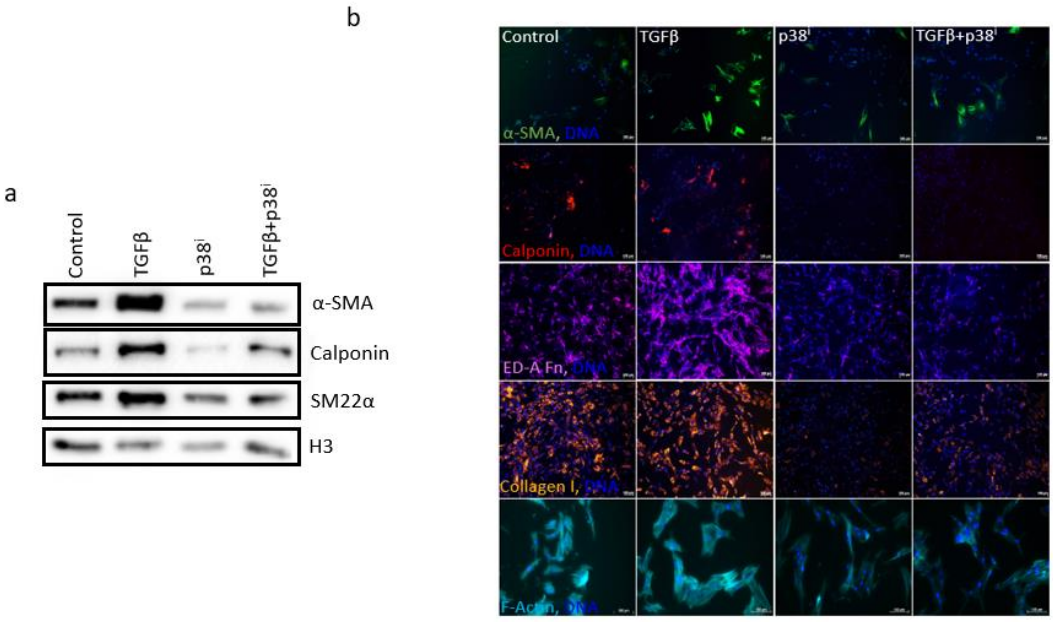
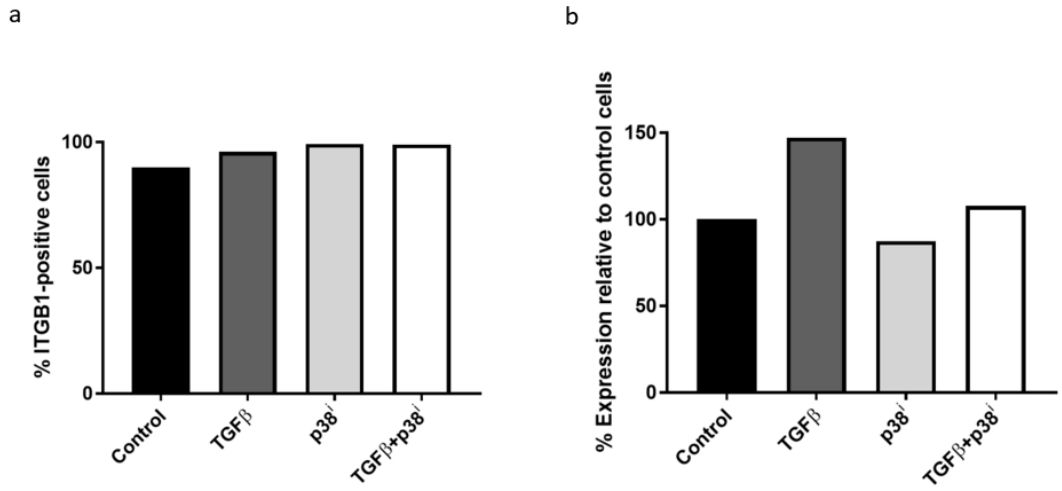



**Figure 3.5 Effects of p38 inhibition on TGFß-mediated expression and deposition of myofibroblast-associated proteins**
CRL-2097 fibroblasts were cultured in the presence or absence of TGF-ß1 and/or 10 µM SB202190 (p38ⁱ) until day 4. **(a)** Cells were harvested and protein lysates were used for Western blot analysis for expression of α-SMA, calponin, and SM22α. Histone H3 was used as a loading control. **(b)** Cells were fixed and stained for immunofluorescence for α-SMA, calponin, collagen I, ED-A fibronectin, and fluorophore-conjugated phalloidin for visualization. DNA was counterstained with Hoechst. Scale bar = 100µm.

### 3.3.5 Inhibition of p38 may attenuate TGF-β1-mediated induction of pro-fibrotic integrin β1

As explained previously (see **Section 2.2.8**), fibroblast activation is associated with expression of pro-fibrotic integrin heterodimers, including α11β1, which was recently reported to be necessary for myofibroblast formation and activity (Martin et al., 2016). Thus, we decided to investigate whether p38 inhibition affected expression of integrin β1 in human dermal fibroblasts. Fixation and immunostaining followed by flow cytometry demonstrated that, regardless of treatment condition, a vast majority (≥90%) of fibroblasts stained positive for integrin β1 (**Figure 3.6a**).

However, analysis on a per cell basis demonstrated that treatment with p38 inhibitor modestly decreased the expression of integrin β1 on a per cell basis, as well as partially antagonized TGF-β1-mediated induction of integrin β1 on a per cell basis **(Figure 3.6b**). Thus, these data suggest that p38 inhibitor-mediated antagonism of fibroblast activation may be due at least in part to inhibition of pro-fibrotic integrin β1 expression.




**3.4**

**Figure 3.6 Effects of p38 inhibition on basal and TGF-ß1-induced Integrin β1 expression**
CRL-2097 fibroblasts were cultured in the presence or absence of TGF-ß1 and/or 10 μM SB202190 (p38$^{i}$), and cells were fixed, stained, and analyzed by flow cytometry for Integrin β1 on day 4. **(a)** Percentage of cells displaying a positive signal for expression of Integrin β1 as determined by flow cytometry. **(b)** Percent expression of Integrin β1 per positively-stained cell relative to fibroblasts cultured under control conditions.

## Discussion

Given the potent nature of FGF2 as a compound with pro-regenerative capacity in numerous different kinds of wounds and in different organs (D. M. Dolivo et al., 2017; Nunes, Li, Sun, Kinnunen, & Fernig, 2016; Spaccapelo, 2016), and given the potential and demonstrated deleterious effects associated with drugging highly pleiotropic signaling pathways too far upstream, particularly those involving tyrosine kinases (Caldemeyer, Dugan, Edwards, & Akard, 2016; Hartmann et al., 2009; Joensuu, Trent, & Reichardt, 2011; Richeldi et al., 2014; Sodergren et al., 2014), there is potentially great value in understanding which FGF2-associated signaling pathways are involved in the antifibrotic and pro-regenerative effects of this growth factor on cell types relevant to wound healing pathologies. Thus, given the importance of fibroblast

phenotypes including proliferation and activation in wound healing and fibrosis in particular (Hinz, 2016), we set out to determine which pathways were involved in, or at least necessary for, the effects of FGF2 as an antagonist of fibroblast differentiation (D. Dolivo, S. Larson, & T. Dominko, 2017).

Our data demonstrated that the potent mitogenic effects of FGF2 in human dermal fibroblasts, as well as the effects of FGF2 on cell morphology, could be reversed by co-treatment with MAPK inhibitors of ERK and JNK. This suggested that fibroblast activation may be antagonized at least in part by FGF2-mediated activation of ERK and JNK. We also demonstrated that these phenotypes induced by FGF2 could be potentiated by co-treatment with p38 inhibitor, suggesting that FGF2-mediated activation of p38 may actually promote fibroblast activation. As these data suggested dependencies of FGF2-mediated proliferation and effects on cell morphology on particular MAPK pathways, we thus decided to determine whether these same pathways were responsible for FGF2-mediated antagonism of myofibroblast differentiation. As expected, small molecule-mediated inhibition of ERK or JNK was sufficient to antagonize FGF2-mediated attenuation of myofibroblast phenotypes, as assessed by traditional myofibroblast markers and myofibroblast-associated extracellular matrix protein expression, including the fibrotic type I collagen. It is important to note, however, that the data in this chapter do not definitively prove that the antifibrotic effects of FGF2 proceed directly via ERK and/or JNK pathways, as these pathways have numerous activators within a cellular context and exist in a complicated cellular signaling environment. That being said, our data strongly suggest that the antifibrotic effects of FGF2 at least involve, if not require, activation of these pathways (**Figure 3.7**).

In addition to the possibility that MAPK inhibition affects FGF2/FGFR signaling directly in order to antagonize (or potentiate, in the case of p38 inhibition) the effects of FGF2 signaling, it is also possible that the MAPK inhibitors have other effects that exhibit cross-talk with TGF-β signaling. It has previously been reported that ERK activation can antagonize phosphorylation and activation of the TGF-β effector protein SMAD2, as well as active nuclear accumulation of SMAD2/3, suggesting a potential role for ERK activity in modulating TGF-β signaling (Zhang, Min, Liggett, & Baek, 2013). Additionally, ERK has been demonstrated to be able to phosphorylate serines and threonines in the linker region of SMAD2 and SMAD3, and mutation of these ERK-targeted residues in SMAD3 has been demonstrated to potentiate SMAD3 signaling, suggesting a possible mechanism by which ERK activation, and growth factors that signal through ERK, may antagonize pro-fibrotic TGF-β signaling (Kretzschmar, Doody, Timokhina, & Massagué, 1999). Previous reports also demonstrate the ability of JNK activation to antagonize TGF-β/SMAD signaling as well (Lin et al., 2013;

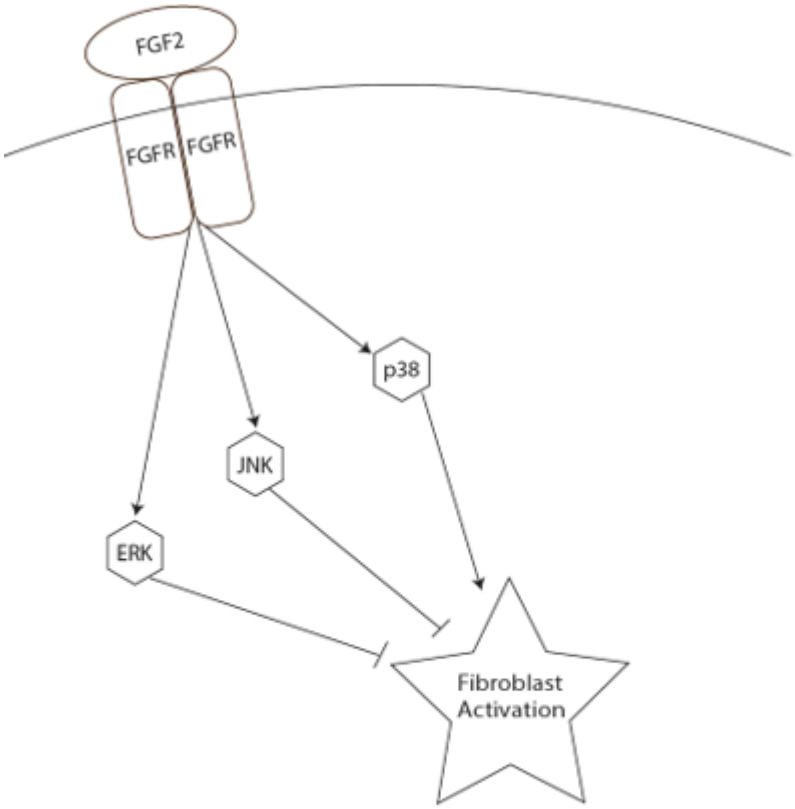



**Figure 3.7 Model of MAPK dependence on FGF2-mediated antagonism of fibroblast activation**
Based on the data from chapter 3, FGF2 is proposed to antagonize fibroblast activation via activation of ERK and JNK but likely not via activation of p38.

Verrecchia, Tacheau, Wagner, & Mauviel, 2003; Wu, Kasisomayajula, Peng, & Bancalari, 2009).

In addition to our data regarding ERK and JNK pathways, we also demonstrated that small molecule-mediated inhibition of p38 was sufficient to antagonize *in vitro* basal and TGF-β-mediated fibroblast activation and subsequent expression of myofibroblast-associated extracellular matrix proteins. Though our data do not allow us to conclude a specific mechanism by which p38 positively regulates fibroblast activation and pro-fibrotic fibroblast phenotypes, we propose several possibilities based on the literature. Previously, it has been demonstrated that TGF-β signaling can lead directly to phosphorylation of p38 in a manner distinct and separable from its ability to activate canonical SMAD signaling (Galliher & Schiemann, 2007), and that TGF-β-mediated phosphorylation of p38 mediates certain TGF-β-governed phenotypes including apoptosis and the epithelial-to-mesenchymal transition in a SMAD-independent manner (Yu, Hébert, & Zhang, 2002). Additionally, p38 has been demonstrated to modulate the transcriptional activity of TGF-β signaling (Hanafusa et al., 1999; Kimoto, Nakatsuka, Matsuo, & Yoshioka, 2004; Yu et al., 2002), and p38 has also been implicated in the regulation of serum response factor, an important transcription factor driving attainment of the myofibroblastic gene expression program (Liang, Ahlqvist, Pannem, Posern, & Massoumi, 2011; Thuerauf et al., 1998). Our findings find favor among recent reports demonstrating therapeutic potential of p38 inhibition in prevention or treatment of fibroses of the kidney (Choi et al., 2016; Nishida, Okumura, Sato, & Hamaoka, 2008; Stambe et al., 2004; Sugiyama, Kohno, & Yokoyama, 2011) and the heart (Molkentin et al., 2017; See et al., 2004).

This chapter has revealed potential roles and mechanistic information for specific MAPK pathways in the antagonistic effects of FGF2 on fibroblast activation and subsequent expression

of fibrotic ECM proteins. It also has suggested a role for p38 signaling in fibroblast activation, and demonstrated that small molecule-mediated inhibition of p38 was sufficient to antagonize the potent differentiation and pro-fibrotic effects of TGF-β on human fibroblasts.

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# Chapter 4-Cross-talk between MAPK inhibitors and TGF-β signaling results in variable activation of human dermal fibroblasts

## 4.1 Abstract

Fibroblast activation is a key step in the establishment of skin fibrosis induced by acute injury, and it is characterized by the differentiation of plastic resident tissue fibroblasts into contractile, ECM-secreting myofibroblasts. As fibroblast activation must be carefully regulated *in vivo*, fibroblasts receive signals from the surrounding environment that initiate their fibrotic program. Thus, we set out to explore the effects of MAPK signaling pathways on fibroblast activation. We demonstrated in primary human dermal fibroblasts that small molecule-mediated inhibition of ERK and JNK potentiated fibroblast activation, and that small molecule-mediated inhibition of p38 antagonized fibroblast activation suggesting that, in proliferating fibroblasts, ERK and JNK activity antagonize fibroblast activation and p38 activity promotes fibroblast activation. ERK and JNK inhibition cooperatively enhanced fibroblast activation mediated by treatment with exogenous TGF-β1, and p38 inhibition antagonized ERK inhibitor-mediated or JNK-inhibitor mediated fibroblast activation. Transcript analysis demonstrated that ERK and JNK inhibitor-mediated fibroblast activation was accompanied by distinct changes in expression of TGF-β-associated ligands and receptors, and that p38 inhibitor-mediated antagonism of fibroblast activation was accompanied by a distinct expression paradigm of TGF-β-associated genes, notably including upregulation of betaglycan. Both ERK inhibitor-mediated and JNK inhibitor-mediated fibroblast activation was partially antagonized by small molecule-mediated inhibition of TGF-βRI, suggesting that ERK/JNK activity-mediated antagonism of fibroblast activation is dependent at least in part on TGF-β/TGF-βR signaling. These data collectively demonstrate and provide partial explanations of the varied effects and pathway dependencies of MAPK-mediated effects on fibroblast activation.

## 4.2 Introduction

The wound environment is defined by a complex, non-homeostatic summation of cellular agents, biochemical effectors, extracellular signaling mediators, perceived and real mechanical forces in the wound microenvironment, and countless other stimuli and variables. Additionally, each phase in the wound healing response requires multiple types of effector cells integrating various environmental cues, displaying various patterns of gene expression, and fulfilling various roles in the wound healing cascade in concert. Thus, an understanding of the interplay of various stimulating factors in the wound microenvironment is paramount to the understanding of healthy and pathological wound healing. Despite this, the manner in which complex environmental signals are integrated into a coherent wound healing response, characterized by defined actions of particular effectors cells (including myofibroblasts), is not well-understood.

As myofibroblast contractile activity and overdeposition of fibrotic extracellular matrix can frequently be detrimental to the health and function of an organ, activation of fibroblasts must be carefully regulated by the cellular environment, such that fibroblasts only become activated during scenarios in which tissue repair is necessary. One class of pathways responsible for transducing cellular signals from the extracellular environment is known as the mitogen activated protein kinases (MAPKs) (Cargnello & Roux, 2011). MAPK pathways have been implicated in the development or prevention of fibrotic phenotypes at the cellular level and in various tissues, in part through their ability to propagate signaling initiated by growth factors in the extracellular milieu, including the ubiquitous pro-fibrotic transforming growth factor beta (TGF-β) pathway (Dolivo, Larson, & Dominko, 2017; Furukawa et al., 2003; Grella, Kole, Holmes, & Dominko, 2016; Li et al., 2006; Meyer-ter-Vehn et al., 2006; Molkentin et al., 2017; Mulsow, Watson, Fitzpatrick, & O'connell, 2005; See et al., 2004; Sousa et al., 2007; Stambe et al., 2004; Wu,

Muchir, Shan, Bonne, & Worman, 2011). Thus, an understanding of the interplay of various stimulating factors in the wound microenvironment is paramount to the understanding of healthy and pathological wound healing. Despite this, the manner in which complex environmental signals are integrated into a coherent wound healing response, characterized by defined actions of particular effectors cells, is not well-understood. Thus, we set out to better understand the effects of MAPK pathways through the use of chemical inhibitors, in conjunction with each other and with TGF-β signaling mediators, in order to better understand some of the signaling processes leading to fibroblast activation.

## 4.3 Results

### 4.3.1 Fibroblast activation is promoted by inhibition of ERK or JNK and antagonized by inhibition of p38

We first wished to investigate the effects of culture in the presence of MAPK inhibitors on activation of human fibroblasts. Treatment of CRL-2097 human neonatal foreskin fibroblasts with 10μM U0126 (ERK inhibitor) or 10μM SP600125 (JNK inhibitor) was sufficient to induce upregulation of three myofibroblast-associated transcripts, *ACTA2*, *CNN1*, and *TAGLN*, the genes that encode α-smooth muscle actin (α-SMA), calponin, and transgelin (SM22α), respectively. In contrast, treatment with 10μM SB202190 (p38 inhibitor) was sufficient to downregulate expression of these transcripts below basal levels (**Figure 4.1a**). Analysis by Western blot coincided with the qRT-PCR data and demonstrated that treatment with these same inhibitors upregulated or downregulated expression of the myofibroblast markers encoded by these genes as well in a qualitatively similar manner (**Figure 4.1b**). In order to better understand the effects of MAPK inhibition on expression of α-SMA, the major myofibroblast marker, at the per cell level, indirect flow cytometry was performed. Analysis of the data revealed that the

average level of α-SMA protein expression per cell was increased in cells cultured in the presence of ERK or JNK inhibitor, or in the presence of exogenous TGF-β1, and decreased in the presence of p38 inhibitor, compared to fibroblasts cultured under control conditions (**Figure 4.1c**). Immunofluorescent imaging revealed that fibroblasts treated with ERK or JNK inhibitor expressed higher levels of filamentous actin, arranged into stress fibers, and that more of these cells stained positive for α-SMA compared to control fibroblasts cultured in the absence of MAPK inhibitors. In contrast, fibroblasts cultured in the presence of p38 inhibitor demonstrated a notable lack of filamentous actin stress fiber formation, as well as a lack of expression of α-SMA protein (**Figure 4.1d**). We next wished to ensure that the effects of MAPK inhibition on fibroblast activation were generalizable across multiple lines of human dermal fibroblasts, rather than specific to one cell line. We demonstrated by Western blot that two primary adult human dermal fibroblast lines, CRL-2352 and CT-1005, each demonstrated a qualitatively similar increase in α-SMA expression when treated with ERK or JNK inhibitor, and a qualitatively similar decrease in α-SMA expression when treated with p38 inhibitor, as was demonstrated in CRL-2097 fibroblasts (**Figure 4.1e)**. Taken together, these data demonstrate that culture of fibroblasts in the presence of inhibitors for ERK or JNK is sufficient to induce fibroblast activation, and that culture of fibroblasts in the presence of an inhibitor of p38 is sufficient to

antagonize fibroblast activation, and that these effects are generalizable across multiple lines of primary human dermal fibroblasts.

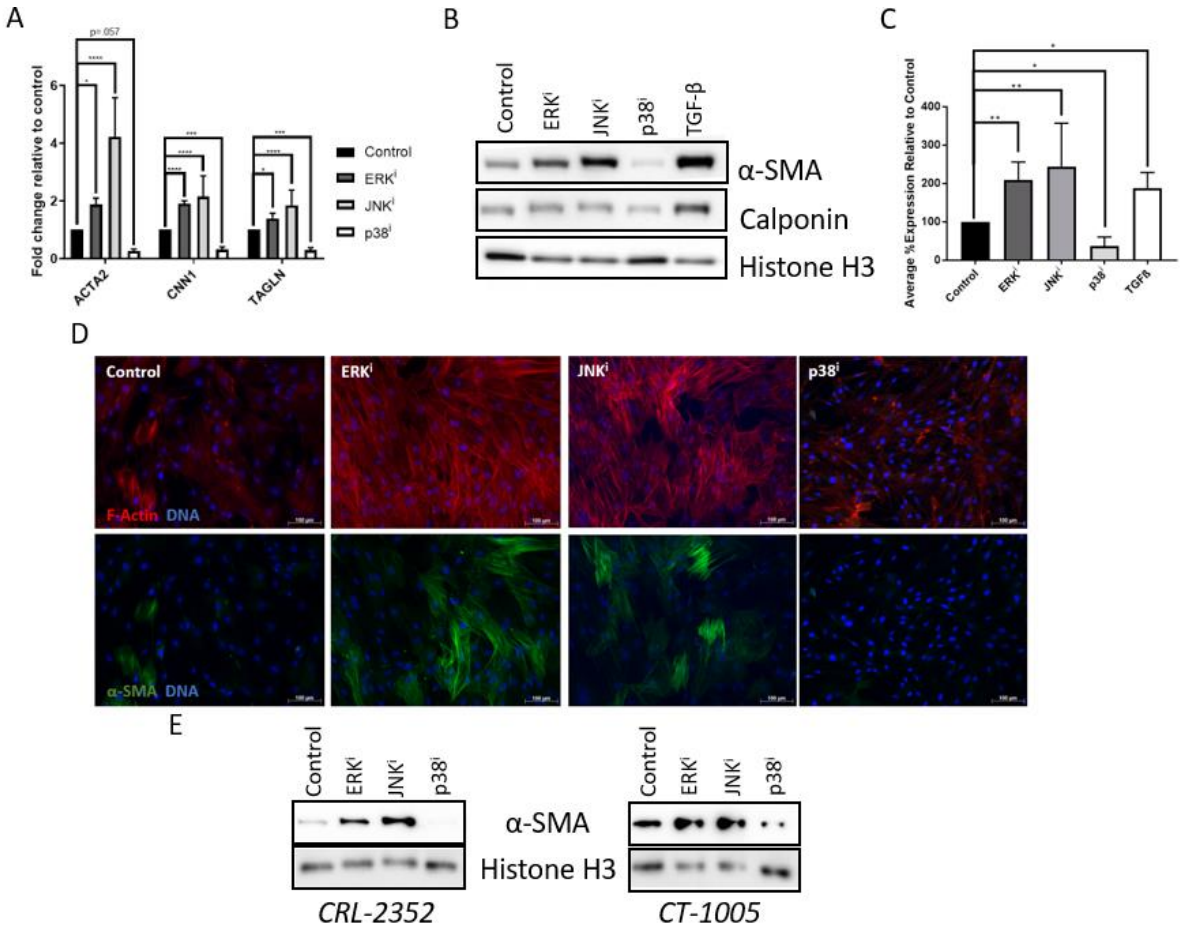



**Figure 4.1: Effects of MAPK inhibitors on fibroblast activation**
(**a-d**) CRL-2097 human dermal fibroblasts were cultured under control conditions or in the presence of 10μM U0126 (ERK$^i$), 10μM SP600125 (JNK$^i$), or 10 μM SB202190 (p38$^i$), or 10ng/mL exogenous recombinant human TGF-β1 and harvested at day 4 in culture. **(a)** Expression of myofibroblast marker transcripts *ACTA2*, *CNN1*, and *TAGLN* were determined by qRT-PCR, normalized to expression of *GAPDH*, and expressed as fold change relative to expression of control samples. n=4 biological replicates/condition. Error bars=standard deviation. Statistics were performed by one-way ANOVA with multiple comparisons using the Holm-Sidak post-hoc test. *p<.05, ***p<.001, ****p<.0001. **(b)** Western blot analysis was performed on myofibroblast markers α-SMA and calponin on whole cell lysates. Histone H3 was used as a loading control. **(c)** Fibroblasts were fixed and subject to indirect flow cytometry, and per cell expression of α-SMA was compared to expression in control fibroblasts. n=4 biological replicates per condition. **(d)** Fibroblasts were fixed and stained by immunofluorescence for α-SMA, and F-actin was visualized using fluorescently-labeled phalloidin. Nuclei were counterstained with Hoechst. Scale bars=100μm. **(e)** CRL-2352 and CT-1005 fibroblasts were cultured under control conditions or in the presence of MAPK inhibitor and harvested at day 4 in culture. Western blot analysis was performed for detection of α-SMA. Histone H3 was used as a loading control.

### 4.3.2 A pro-fibrotic gene expression paradigm is induced by inhibition of ERK or JNK and antagonized by inhibition of p38

Since we demonstrated that MAPK inhibition variably affected fibroblast activation as determined by expression of canonical myofibroblast markers, we next wished to determine whether this inhibition also affected expression of other genes associated with extracellular

matrix deposition and fibroblast activation. Analysis by qRT-PCR demonstrated that inhibition of ERK or JNK resulted in upregulation of *Col1A1* and *Col1A2*, the genes encoding both chains of type I collagen, the major extracellular matrix component of fibrotic scar tissue, as well as *Col3A1*, the gene encoding type III collagen. Concordantly, inhibition of ERK or JNK also resulted in downregulation of transcription of the interstitial collagenase *MMP1*, further suggesting an activated fibroblast phenotype associated with overdeposition of pathologic extracellular matrix (**Figure 4.2a**). Inhibition of ERK or JNK also resulted in increased expression of the gene encoding the myofibroblast-specific, extra domain A variant of fibronectin (*ED-A Fn*) as well as *MYOCD* and *CCN2*, the genes encoding the myogenic transcription factor myocardin and the pro-fibrotic matricellular protein connective tissue growth factor, respectively (**Figure 4.2b**). In contrast, inhibition of p38 resulted in a decrease in expression of each of these myofibroblast-associated genes, consistent with its apparent ability to antagonize fibroblast activation. Inhibition of p38 also resulted in downregulation of *LOX*, the transcript encoding lysyl oxidase, a copper-dependent protein that is responsible for the crosslinking of immature collagen precursors into mature collagen fibrils (Kagan & Li, 2003) (**Figure 4.2a**). Additionally, immunofluorescent analysis demonstrated that inhibition of ERK or JNK resulted in higher levels of type I collagen and ED-A fibronectin deposition, and that inhibition of p38 resulted in diminished levels of type I collagen and ED-A fibronectin deposition (**Figure 2c**). Taken together, these data suggest that inhibition of ERK or JNK leads to increased deposition of fibrotic ECM, through both upregulation of transcription of fibrosis-associated and collagen-encoding genes, as well as through downregulation of expression of the metalloproteinase-encoding gene *MMP1*. Additionally, these data suggest that inhibition of p38 leads to decreased deposition of fibrotic ECM, through both downregulation of fibrosis-

associated and collagen-encoding genes, as well as through downregulation of gene encoding the collagen maturation enzyme LOX.

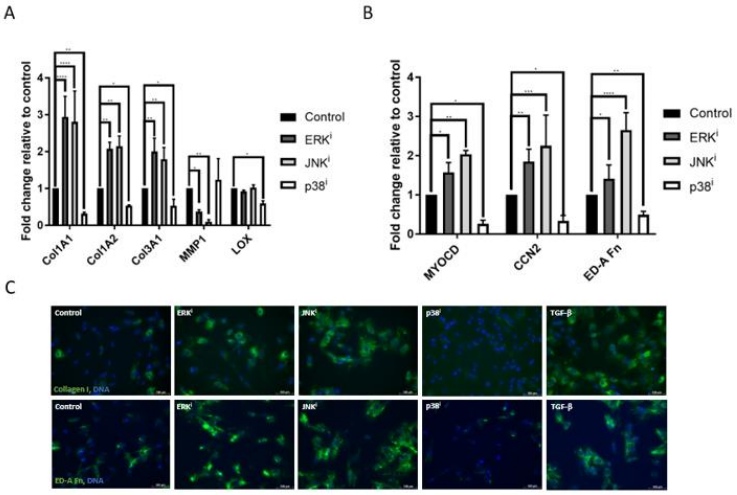



**Figure 4.2 Effects of MAPK inhibitors on extracellular matrix**
CRL-2097 human dermal fibroblasts were cultured in the presence or absence of 10μM U0126 (ERK$^i$), 10μM SP600125 (JNK$^i$), or 10 μM SB202190 (p38$^i$) and harvested at day 4. Expression levels of extracellular matrix-associated transcripts **(a)** *Col1A1*, *Col1A2*, *Col3A1*, *MMP1*, and *LOX* and **(b)** *MYOCD*, *CCN2*, and *ED-A Fn* were determined relative to fibroblasts cultured under control conditions by qRT-PCR. *GAPDH* expression was used as a loading control. Expression levels per transcript were compared using a one-way ANOVA and post-hoc Holm-Sidak analysis. *p<.05, **p<.01, ***p<.001, ****p<.0001. 2≤n≤6 biological replicates per condition. **(c)** Fibroblasts were fixed and stained by immunofluorescence for Collagen I or ED-A Fibronectin. Nuclei were counterstained with Hoechst. Scale bars=100μm.

### 4.3.3 ERK or JNK inhibition cooperatively enhances TGF-β-mediated fibroblast activation

As TGF-β1 is a fibrogenic cytokine and canonical myofibroblast activator, and specific paradigms of noncanonical TGF-β signaling have been demonstrated to proceed via activation of MAPKs including ERK and JNK, we wished to determine whether ERK and JNK inhibition would antagonize or enhance TGF-β1-mediated fibroblast activation. Western blot analysis demonstrated that treatment with exogenous TGF-β1, as well as treatment with just ERK inhibitor or JNK inhibitor, was sufficient to induce expression of myofibroblast maker proteins α-SMA, calponin, and SM22α above the baseline expression levels of fibroblasts. Additionally,

treatment of fibroblasts with TGF-β1 and one of the demonstrably fibrogenic MAPK inhibitors (either ERK inhibitor U0126 or JNK inhibitor SP600125) enhanced myofibroblast activation as assessed by a substantial increase in the expression of the same myofibroblast marker proteins over the levels of expression observed in cells treated with either exogenous TGF-β1 or pro-fibrotic MAPK inhibitor alone (**Figure 4.3a**). These findings were confirmed by immunofluorescence, in which co-treatment with ERK inhibitor of JNK inhibitor increased the number and intensity of α-SMA$^{+}$ cells compared to treatment with TGF-β1 alone (**Figure 4.3b**). Taken together, these data suggest that inhibition of ERK or JNK can cooperatively enhance the effects of exogenous TGF-β1 on fibroblast activation.

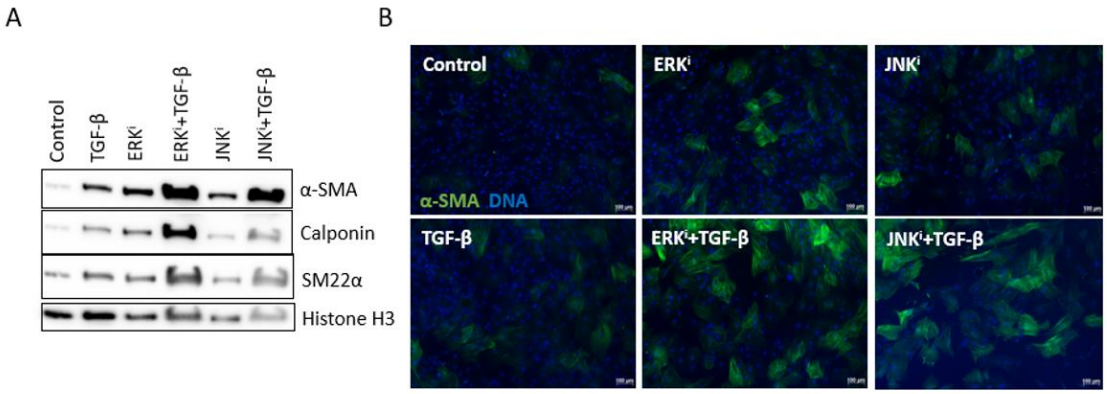



**Figure 4.3: Cooperative effects of MAPK inhibition and exogenous TGF-β on fibroblast activation**
CRL-2097 human dermal fibroblasts were cultured under control conditions or in the presence of 10μM U0126 (ERK$^{i}$), 10μM SP600125 (JNK$^{i}$), and/or 10ng/mL exogenous recombinant human TGF-β1 and analyzed at day 4 in culture. **(a)** Protein lysates were examined for expression of myofibroblast associated protein markers α-SMA, calponin, and SM22α by Western blot. Histone H3 was used as a loading control. **(b)** Fibroblasts were fixed and stained for the myofibroblast marker protein α-SMA and DNA was counterstained with Hoechst. Scale bar=100μm.

### 4.3.4 Inhibition of p38 attenuates fibroblast activation induced by ERK or JNK inhibition

Previous work has implicated MAPK pathways in fibroblast activation, and has demonstrated the ability of MAPKs to act as signal transduction mediators of growth factors demonstrating both fibrotic and antifibrotic activities. Since we had established at this point that different MAPK inhibitors have varied effects on fibroblast activation, and since we had previously demonstrated

that inhibition of p38 was sufficient to attenuate fibroblast activation induced by exogenous TGF-β1 (Dolivo et al., 2017), we set out to determine whether these MAPK inhibitors had the ability to antagonize the activity of each other. Measurement of myofibroblast-associated transcripts *ACTA2*, *CNN1*, and *TAGLN* demonstrated that treatment with p38 inhibitor antagonized fibroblast activation induced by treatment with ERK or JNK inhibitor (**Figure 4.4a**). Similarly, Western blot analysis demonstrated that treatment with p38 inhibitor antagonized expression of α-SMA, calponin, and SM22α protein induced by treatment with ERK or JNK inhibitor (**Figure 4.4b**). Immunofluorescent analysis corroborated the Western blot data, demonstrating that treatment with p38 inhibitor decreased the number and intensity of α-SMA$^+$ cells (**Figure 4.4c**). Taken together, these data suggest that inhibition of p38 is sufficient to antagonize fibroblast activation induced by inhibition of ERK or JNK at the level as assessed by both transcript and protein levels of canonical myofibroblast markers.

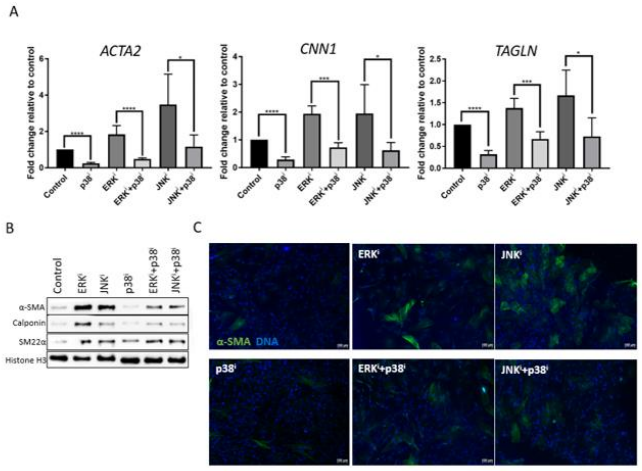



**Figure 4.4: Antagonism of p38 inhibitor towards ERK and JNK inhibitor-mediated fibroblast activation**
CRL-2097 human dermal fibroblasts were cultured under control conditions or in the presence of 10μM U0126 (ERK$^i$), 10μM SP600125 (JNK$^i$), and/or 10μM SB202190 (p38$^i$) and analyzed at day 4 in culture. **(a)** Expression levels of myofibroblast-associated transcripts *ACTA2, CNN1,* and *TAGLN* were determined relative to fibroblasts cultured under control conditions by qRT-PCR. *GAPDH* expression was used as a loading control. T-tests were performed in order to compare expression between each culture condition ± its p38$^i$-treated counterpart. *p<.05, ***p<.001, ****p<.0001. n≥4 biological replicates per condition. **(b)** Protein lysates were examined for expression of myofibroblast-associated marker proteins α-SMA, calponin, and SM22α by Western blot. Histone H3 was used as a loading control. **(c)** Fibroblasts were fixed and stained for the myofibroblast marker protein α-SMA and DNA was counterstained with Hoechst. Scale bar=100μm.

### 4.3.5 Similar effects of ERK inhibitor and JNK inhibitor on fibroblast activation are not a function of non-specific inhibition of phospho-ERK by JNK inhibitor

Due to our findings that ERK inhibitor-mediated and JNK inhibitor-mediated fibroblast activation were associated with qualitatively similar expression changes for every gene we had analyzed, and in consideration of previously reported activity of SP600125 as a modest inhibitor of MKK1 (Bain et al., 2007), we wanted to ensure that the reason for these similarities was not due to nonspecific inhibitory activity of ERK signaling by the JNK inhibitor. Western blot analysis determined that, while ERK inhibitor treatment decreased ERK phosphorylation at 15-60 minutes post-treatment as expected, treatment with JNK inhibitor failed to inhibit ERK phosphorylation at these same time points post-treatment (**Figure 4.5a**).

### 4.3.6 MAPK inhibitors variably affect transcription of genes encoding TGF-β ligands and receptors

Given the importance of TGF-β signaling as the ubiquitous central modulator of myofibroblast activation, we wished to determine whether the observed effects of MAPK inhibitors on fibroblast activation were accompanied by changes in expression of TGF-β-associated genes. Analysis of transcript expression (**Fig 5b**) demonstrated that treatment with ERK inhibitor resulted in upregulation of *TGFB2* and *TGFBR1*, the genes encoding TGF-β2 and TGF-βRI respectively, while treatment with JNK inhibitor led to upregulation of *TGFB1* and downregulation of *TGFBR3*, the genes encoding TGF-β1 and betaglycan respectively, as well as an unexpected downregulation of *TGFBR2*, the gene encoding TGF-βR2. These data also demonstrated that treatment with p38 inhibitor decreased expression of *TGFB2* and induced

expression of *TGFBR3*, in accordance with our expectation that p38 antagonize expression of fibrotic TGF-β signaling mediators as suggested by our previous results. The TGF-β/TGF-βR pathway transcript expression data are summarized schematically in **Figure 4.5c**. Taken together, these data suggest that the effects of MAPK inhibitor treatments on fibroblast activation are likely due at least in part to their effects on expression of TGF-β signaling-associated genes.

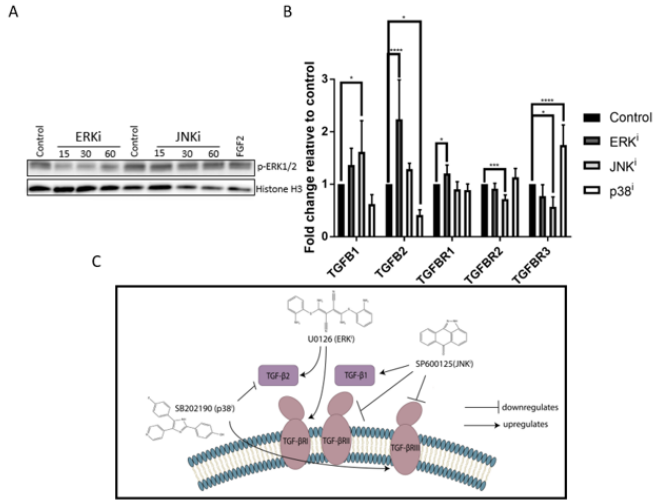


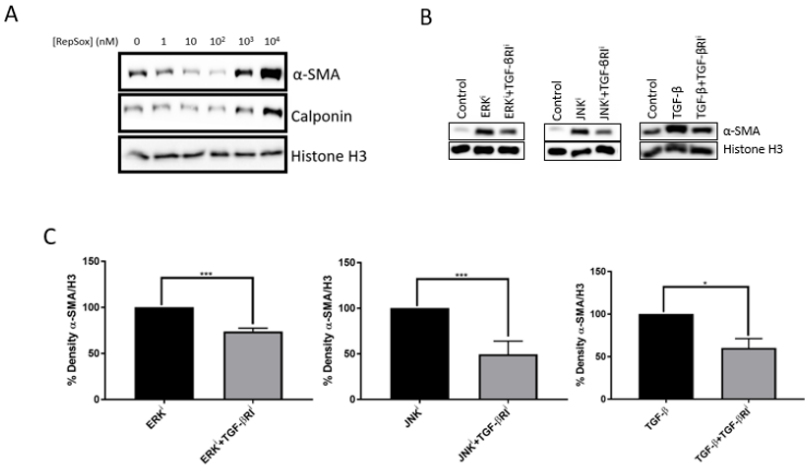

***Figure 4.5: TGF-β-related gene expression profiles induced by treatment with MAPK inhibitors***
**(a)** CRL-2097 human dermal fibroblasts were cultured for 15-60 minutes in the presence or absence of 10μM U0126 (ERKi) and 10μM SP600125 (JNKi) and subjected to analysis by Western blot for p-ERK1/2. Histone H3 was used as a loading control. Fibroblasts incubated with 50ng/mL recombinant human FGF2 for 30 minutes were used as a positive control for p-ERK1/2 expression. **(b)** CRL-2097 human dermal fibroblasts were cultured in the presence or absence of 10μM U0126 (ERKi), 10μM SP600125 (JNKi), or 10μM SB202190 (p38i) until day 4. Expression levels of TGF-β-associated transcripts *TGFB1*, *TGFB2*, *TGFBR1*, *TGFBR2*, and *TGFBR3* were determined relative to fibroblasts cultured under control conditions by qRT-PCR. *GAPDH* expression was used as an internal control. Expression levels per transcript were compared using a one-way ANOVA and post-hoc Holm-Sidak analysis. *p<.05, ***p<.001, ****p<.0001. n=6 biological replicates/condition. Error bars=standard deviation.
**(c)** Schematic of the TGF-β/TGF-βR interaction depicting the changes in gene expression induced by various MAPK inhibitors.

### 4.3.7 Fibroblast activation induced by ERK inhibition or JNK inhibition is antagonized by co-inhibition of TGF-βRI

Since ERK and JNK inhibition resulted in fibroblast activation and were associated with modulation of expression of genes encoding TGF-β ligands and receptors, we wished to determine whether ERK inhibitor-mediated or JNK inhibitor-mediated fibroblast activation could be antagonized by inhibition of TGF-β signaling. A dose response curve demonstrated that treatment of fibroblasts with 100nM Repsox, a TGF-βRI inhibitor, was sufficient to decrease

basal expression of myofibroblast markers α-SMA and calponin (**Figure 4.6a**). Thus, 100nM Repsox was chosen for use in subsequent experiments. Interestingly, increasing concentrations of Repsox up to 10μM were determined to substantially induce fibroblast activation, as demonstrated by notable upregulation of myofibroblast-associated proteins α-SMA and calponin, in contrast to intended effects as an antagonist of TGF-β signaling.

Analysis by Western blot demonstrated that treatment with 100nM TGF-βRI inhibitor partially antagonized ERK inhibitor-mediated and JNK inhibitor-mediated fibroblast activation as indicated by expression levels of α-SMA protein (**Figure 4.6b**). Densitometry and subsequent statistical analysis confirmed that the antagonistic effects of TGF-βRI inhibitor on ERK inhibitor-mediated fibroblast activation, JNK inhibitor-mediated fibroblast activation, and exogenous TGF-β1-mediated fibroblast activation were statistically significant (**Figure 4.6c**). Taken together, these data demonstrate that fibroblast activation induced by small molecule-mediated inhibition of ERK or JNK is dependent at least in part on signaling via TGF-βRI.




**4.4**

**Figure 4.6 MAPK inhibitor-mediated fibroblast activation is antagonized by inhibition of TGF-βRI**
**(a)** CRL-2097 human dermal fibroblasts were cultured in the presence or absence of an indicated concentration of the TGF-βRI inhibitor RepSox. Expression of myofibroblast-associated proteins α-SMA and calponin were determined by Western blot. Histone H3 was used as a loading control. **(b)** CRL-2097 human dermal fibroblasts were cultured in the presence or absence of 10μM U0126 ($ERK^i$), 10μM SP600125 ($JNK^i$), and/or 100nM RepSox, and expression of α-SMA was determined by Western blot. Histone H3 was used as a loading control. **(c)** Western blots were analyzed by densitometry for α-SMA relative to histone loading control for each treatment condition, and percent inhibition was calculated and analyzed for statistical significance using a two-tailed t-test between each activated sample ($ERK^i$, $JNK^i$, or TGF-β) and its repsox-treated partner. 2≤n≤4 percondition. *p<.05, ***p<.001

**Discussion**

One of the dominant mechanisms by which a cell receives signals from its extracellular environment and converts these signals to actionable responses is through signaling via growth factors (Barrientos, Stojadinovic, Golinko, Brem, & Tomic-Canic, 2008; Johnson & Wilgus, 2014; Maddaluno, Urwyler, & Werner, 2017; Werner & Grose, 2003). Growth factors are one vehicle used to relay signals containing information about the extracellular environment in order to induce a functional response in other cells. Growth factor signaling pathways are defined by the ligands that initiate the cellular signal, the growth factor receptors that transduce this signal from the extracellular environment across the plasma membrane, and the intracellular effector proteins that work to initiate a functional response in the target cell. The mechanisms by which growth factor/growth factor receptor complexes transduce a signal and lead to cellular action vary among different pathways, but one of the most common paradigms is transduction via mitogen-activated protein kinase (MAPK) signaling (Krishna & Narang, 2008; Plotnikov, Zehorai, Procaccia, & Seger, 2011). As myofibroblasts are one of most important effector cells in the mammalian wound healing response, and as fibroblasts are activated to myofibroblasts by TGF-β signaling, we decided to investigate the effects of MAPK signaling on TGF-β signaling and on fibroblast activation more generally.

Our data demonstrate that treatment of fibroblasts with ERK inhibitor or JNK inhibitor is sufficient to induce fibroblast activation, and to upregulate expression of myofibroblast marker proteins, and that treatment with p38 inhibitor is sufficient to antagonize fibroblast activation (**Figure 4.1**), in accordance with data we have published previously (Dolivo et al., 2017). These fibroblast activation dynamics also largely predicted the changes we witnessed of extracellular matrix-associated gene and protein expression (**Figure 4.2**), where we determined that treatment

of fibroblasts with ERK or JNK inhibitor induced a relative gene expression profile associated with fibroblast activation and conducive to fibrotic ECM deposition, and that treatment of fibroblasts with p38 inhibitor induced a relative gene expression profile contrary to fibroblast activation and averse to fibrotic ECM deposition. This suggests that ERK and JNK activity serve to antagonize fibroblast activation and pro-fibrotic fibroblast phenotypes, and that p38 activity promotes fibroblast activation (**Figure 4.7**).

There is precedent for the role of p38 in fibroproliferative diseases. Constitutive activation of p38 was demonstrated in fibroblasts derived from scleroderma patients, and inhibition of p38 activation via either small molecule inhibition or transient transfection with a p38 dominant negative mutant was sufficient to reduce the high levels of type I collagen and fibronectin associated with scleroderma fibroblasts (Ihn, Yamane, & Tamaki, 2005). Cardiac fibroblast-specific deletion of *Mapk14*, the gene encoding p38α resulted in a reduction of fibrotic damage induced at a later time in a murine ischemic model and, additionally, deletion of *Mapk14* in existing myofibroblasts resulted in increased tolerance to myocardial infarct injury. In concordance with these findings, cardiac fibroblast-specific overexpression of MKK6, which is responsible for activation of p38 signaling, led to enhanced fibrotic response (Molkentin et al., 2017). In a mouse model of nephropathic fibrosis, small molecule-mediated inhibition of p38 reduced intestinal fibrosis, both alone and cooperatively alongside administration of a TGF-βRI inhibitor (Li et al., 2006). This suggests that the pro-fibrotic effects of p38 signaling are, at least in part, distinct from canonical TGF-β/TGF-βR/SMAD signaling. This seems plausible, given our understanding of the ability of TGF-βRI and TGF-βRII to directly contribute to MAPK phosphorylation, (Galliher & Schiemann, 2007; Lee et al., 2007), as well as in light of the numerous examples of cross-talk between TGF-β and MAPK signaling (Chapnick, Warner,

Bernet, Rao, & Liu, 2011; Gui, Sun, Shimokado, & Muragaki, 2012; Kolosova, Nethery, & Kern, 2011; Selvamurugan, Kwok, Alliston, Reiss, & Partridge, 2004; Terai et al., 2011; Xiao et al., 2002; Xie et al., 2004). Taken together with other reports demonstrating the antifibrotic effects of p38 inhibition (Gao et al., 2016; Nishida, Okumura, Sato, & Hamaoka, 2008; Raia et al., 2005; Sugiyama, Kohno, & Yokoyama, 2011; Wilde et al., 2016), these data suggest that p38 activation is necessary for the differentiation into and the maintenance of myofibroblasts, such that inhibition of p38 may be a viable therapeutic option for certain fibroproliferative pathological states.

We also demonstrated that treatment with ERK or JNK inhibitor cooperatively enhanced fibroblast activation induced by treatment with exogenous TGF-β1 (**Figure 4.3**), and that treatment with p38 inhibitor partially inhibited fibroblast activation induced by treatment with either ERK inhibitor or JNK inhibitor (**Figure 4.4**), suggesting cross-talk between MAPK and TGF-β pathways, as well as between individual MAPK pathways. We determined that fibroblasts treated with MAPK inhibitors demonstrated distinct and sometimes conflicting gene expression profiles of TGF-β-associated genes relative to control fibroblasts **(Figure 4.5**). Interestingly, these changes in gene expression spanned both TGF-β ligands and receptors, including the non-signal-transducing receptor TGF-βRIII, also known as betaglycan, which has the ability to sequester pro-fibrotic TGF-β ligands (Eickelberg, Centrella, Reiss, Kashgarian, & Wells, 2002; López-Casillas, Payne, Andres, & Massagué, 1994). Treatment with JNK inhibitor downregulated betaglycan transcript expression, and treatment with p38 inhibitor upregulated betaglycan transcript expression, both of which seem intuitive given our findings that JNK inhibition induces and p38 inhibition antagonizes fibroblast activation. Accordingly, a synthetic peptide derived from the ligand-binding domain of betaglycan has been demonstrated to

attenuate fibrosis in *in vivo* models of myocardial (Hermida et al., 2008), hepatic (Ezquerro et al., 2003), and dermal fibrosis (Santiago et al., 2005), and stable overexpression of betaglycan attenuates TGF-β signaling (both SMAD-dependent and SMAD-independent) in lung fibroblasts (Ahn, Park, Yun, & Song, 2010). We also found that the activation of fibroblasts mediated by treatment with ERK or JNK inhibitor was partially antagonized by co-treatment with a small molecule inhibitor of TGF-βRI, suggesting that fibroblast activation induced by these inhibitors is due at least in part to their effects on TGF-β/TGF-βR expression, as demonstrated in **Figure 4.6** and **Figure 4.7,** suggesting cross-talk between MAPK and TGF-β signaling in fibroblasts.

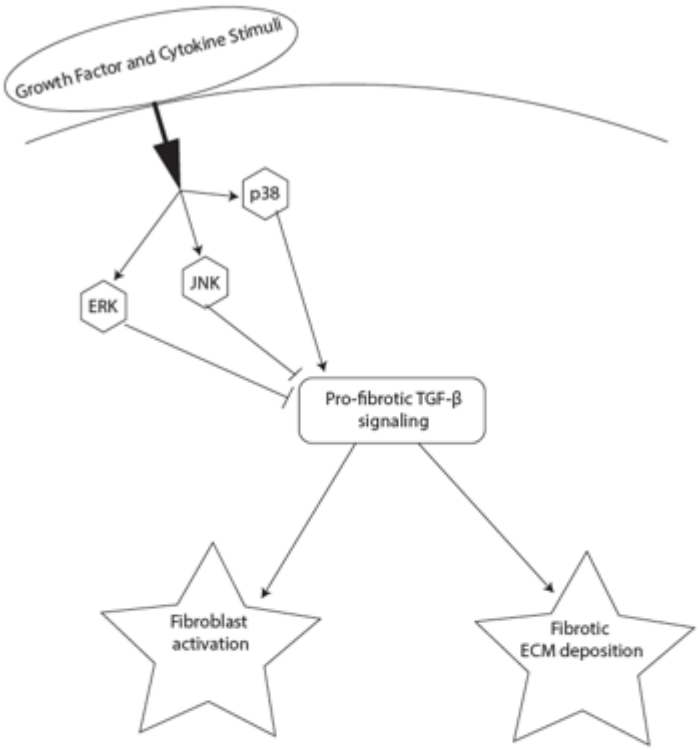



**Figure 4.7 Model of MAPK modulation of fibroblast activation and pro-fibrotic ECM deposition**
Based on the data from chapter 4, MAPK activation is proposed to mediate fibroblast activation via modulation of TGF-β signaling, resulting in changes in fibroblast phenotype and ECM deposition profile

The data presented here shed some light on the effects of MAPK inhibition on human fibroblasts, in the context of fibroblast activation and subsequent profibrotic, myofibroblast-associated gene expression. Developing an understanding of MAPK effects on fibroblast phenotype is important, particularly given interest in tyrosine kinase inhibitors as therapeutics for various diseases, including fibrotic diseases (Hartmann, Haap,

Kopp, & Lipp, 2009; Kompa, 2016; Qu et al., 2016; Raghu & Selman, 2015). As further research yields a more complete understanding of the effects of MAPK signaling and MAPK inhibitors on fibroblasts, and the mechanisms through which these activities are induced, we can better assess the potential benefits and pitfalls of drugging these highly pleiotropic pathways for treatment or prevention of fibroproliferative diseases.

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# Chapter 5: Summary and future directions

## 5.1 Summary

The work presented in this thesis has described in some detail mechanisms and shifts in gene expression profiles underlying the antifibrotic effects of the cytokine FGF2, through its activity antagonistic to fibroblast activation and pro-fibrotic matrix deposition (D. Dolivo, S. Larson, & T. Dominko, 2017). This work has also been considered in the greater understanding of the field of the antifibrotic effects of FGF2/FGFR signaling, and a general picture has emerged of FGF2 signaling being critical for antagonism of pro-fibrotic phenotypes across multiple organs and tissue types and in a variety of myofibroblast progenitors, including not only fibroblasts but also epithelial cells and mesenchymal myofibroblast progenitors (D. M. Dolivo, S. A. Larson, & T. Dominko, 2017). To complement our understanding of the role of canonical FGF/FGFR signaling in the process of fibroblast activation and profibrotic phenotypes and gene expression profiles, we also set out to determine the effects of inhibition of MAPK signaling on fibroblast activation. Through this work we discovered that small molecule-mediated inhibition of specific MAPKs could either promote or antagonize fibroblast activation, and that the effects of MAPK signaling fibroblast activation were at least in part due to cross-talk with, and modulation of, TGF-β/TGF-βR signaling. It is our hope that this progress in understanding another facet of the roles of FGF/FGFR and MAPK signaling to pathophysiological fibroblast activation will lead to more well-informed design of antifibrotic therapeutics and a better understanding of the inherent risks of modulating RTK and/or MAPK signaling in order to achieve therapeutic effects in treatment of fibrotic disease.

## 5.2 Future Directions

At this point, the mechanisms underlying the anti-fibrotic effects of FGF2 through the lens of inhibition of myofibroblast progenitor differentiation, reversion of myofibroblast phenotypes, or induction of myofibroblast apoptosis are fairly well-understood (D. M. Dolivo et al., 2017), and some of the molecular mechanisms of FGF2-mediated inhibition of fibroblast phenotypes have also been elucidated, by our lab and by others (D. Dolivo et al., 2017; Kashpur, LaPointe, Ambady, Ryder, & Dominko, 2013; Shi et al., 2013; Suga et al., 2009).

A minor scientific point, though one worth mentioning, is the incomplete understanding of the full mechanism by which FGF2/FGFR signaling antagonizes TGF-β. The fact that FGF2 *does* antagonize TGF-β signaling is undeniable, a case we have recently put forth in (D. M. Dolivo et al., 2017) but the level(s) at which this antagonism occurs is open to debate. In various contexts and experimental models, FGF2 has been proposed and demonstrated to inhibit TGF-β ligand transcription and translation, inhibit receptor transcription and translation, downregulate the TGF-β signaling mediator CTGF, upregulate miRNA-20a (which contains consensus sequences in the 3'UTRs of several TGF-β-related genes including the genes encoding TGF-βRI/II and SMAD2-4), mediate mechanical tension by modulating integrin expression profiles, upregulate anti-fibrotic TGF-β3, and upregulate the TGF-β inhibitor SMAD7, among demonstrations of other effects. One intriguing paradigm that we have suggested but, to the best of our knowledge, has never been investigated explicitly is the possibility of FGF2/FGFR signaling directly phosphorylating the linker regions of SMAD proteins, thereby inhibiting their ability to act as transcription factors by process of nuclear exclusion (**Figure 5.1**).

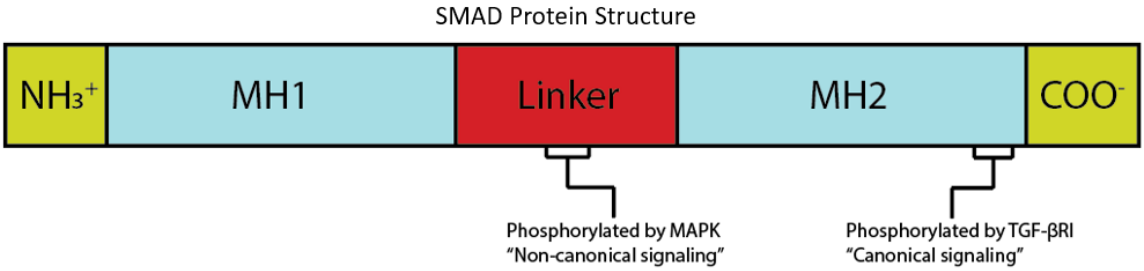


**Figure 5.1 Structure of SMAD proteins**
SMAD proteins contain 3 domains, an amino-terminal MH1 domain, a carboxy-terminal MH2 domain, and a linker region connecting the two. Canonical SMAD phosphorylation, such as that induced by TGF-βRI or BMP-RI, generally occurs via phosphorylation of serines and threonines at the extreme C-terminus of the MH2 domain. However, recent evidence suggests that phosphorylation of serine or threonine residues in the linker domain by other kinases including MAPKs and CDKs can further regulate SMAD localization and, subsequently, function.

Generally, the literature on SMAD linker phosphorylation as it relates to fibroblast activation and organ fibroses is relatively sparse; what we do know about SMAD linker phosphorylation from a biochemical perspective comes predominantly from the field of cancer biology. That being said, there is a growing body of very recent literature demonstrating the effects of SMAD linker phosphorylation on regulation of extracellular matrix synthesis, deposition, and degradation, reviewed expertly in (Burch, Zheng, & Little, 2011). Crucially, though, the adoption of SMAD linker phosphorylation biology to the field of fibropathology should be met with a degree of caution. While the rule of thumb in cancer biology has been that phosphorylation of the linker region of SMAD proteins results in nuclear exclusion and subsequent negative regulation of their activity (Derynck & Zhang, 2003), more complexity has emerged in examining these paradigms in other contexts. A recent report detailed ERK-mediated phosphorylation of the linker region of SMADs located in the nucleus, potentiating SMAD signaling and target gene expression (Hough, Radu, & Doré, 2012). Also in contrast to the notion that SMAD linker phosphorylation leads to strict antagonism of TGF-β signaling it has been reported that, in rat hepatic stellate cell-derived

myofibroblasts, noncanonical TGF-β-mediated activation of p38 led to the phosphorylation of the linker region of SMAD3, leading to heterodimerization of SMAD3 and SMAD4 and subsequent transcriptional activation of the TGF-β target gene *SERPINE1* (Furukawa et al., 2003). The demonstrated ability of not only FGF/FGFR signaling but also (noncanonical) TGF-β/TGF-βR signaling to directly phosphorylate ERK, JNK, and p38 MAPKs, among other protein kinases (Burch et al., 2011), leads to another complex level of regulatory potential to aid in explanation, at least in part, of the myriad data suggesting crosstalk between FGF and TGF-β signaling. Taken together, these reports suggest that phosphorylation of the linker region of SMAD proteins is likely involved in the regulation of TGF-β by FGF signaling, but further elucidation of the specifics of this cross-talk between these pathways would allow for better therapeutic usage of this understanding and a better understanding of how other growth factors in the wound environment might further complicate the effects of FGF2/TGF-β on fibroblast activation.

Despite impressive safety and efficacy of topical application of growth factors for wound healing (Nunes, Li, Sun, Kinnunen, & Fernig, 2016), there are inherent difficulties in utilizing growth factors for pharmacological utility internally. Like most growth factors, and indeed most proteins, FGF2 has a relatively short half-life *in vivo*, necessitating introduction of large quantities of growth factor in order to overcome this limitation, and leading to possible toxic effects due to high concentration of growth factor locally. Strategies to enhance protein stability and reduce the need for bolus administration of FGF2 include rational recombinant growth factor design to increase protein stability thermodynamically (Lehmann & Wyss, 2001), but such approaches are difficult, rely on challenging crystallographical determination of the folding patterns of the protein(s) in question. Even with good crystallographic data, however, these

methods are poorly deterministic, are computationally intensive, and are constrained by the biophysical parameters and the inherent nature of the dependence of protein activity on its tertiary and quaternary structures; sufficient modification to protein primary structure may adversely affect its specificity and its biochemistry, rendering it unable to perform its endogenous function. Thus, while there may be some value in protein engineering to enhance the stability and thus usefulness of FGF2 as an antifibrotic, the ratio of potential benefit to the effort likely necessary in order to realize said benefit is likely very low.

Controlled release of FGF2, and of other growth factors, has also been considered, often taking advantage of the native affinity of FGF2 for heparin, or its basic electrical charge at physiological pH, or simply by incorporation into biocompatible materials (Ding, Shendi, Rolle, & Peterson, 2017; Layman et al., 2007; Nakamura et al., 2008; Vashi et al., 2006; Zern, Chu, & Wang, 2010). The disadvantage of biomaterial-based methods of controlled release include the need for surgical insertion of the material of interest, as well as the subsequent complications associated with any association of a foreign body (cost of procedure, risk of infection, xenobiotic immune response, keeping the material fixed in place, biodegradability). Thus, surgical implantation is not ideal for treatment of conditions that would not otherwise require surgical intervention, though targeted drug delivery systems for specific organs may be an emerging option (Bartneck, Warzecha, & Tacke, 2014; Nastase, Zeng-Brouwers, Wygrecka, & Schaefer, 2017), though these methods are likely more suitable for delivery of small molecules than bioactive peptides.

Internal use of pharmacologic fibroblast growth factors are also problematic due to the ubiquitous nature of FGF/FGFR signaling across myriad cells, tissues, and organs, and in the context of various physiological and pathophysiological processes (Ornitz & Itoh, 2015).

Aberrant FGF and subsequent RTK signaling is associated with the development and progression of solid tumors and hematological malignancies (Akl et al., 2016), as well as metabolic and neurological diseases (Itoh & Ornitz, 2011), among others. Thus, usage of fibroblast growth factor-based therapeutics *in vivo*, even if released in a defined temporal manner, carries with it risk of off-target, pathophysiological consequences. Naturally, then, targeting of myofibroblast progenitors would be desirable, but this is complicated due to the numerous cell types that differentiate into myofibroblasts (Hinz et al., 2007), and the manner in which this varies from tissue to tissue and pathology to pathology. Reversal of fibrosis may be possible at least in theory for fibrotic organs, with demonstrated success in animal models of hepatic (Friedman, 2012; Kisseleva, Cong, & Paik, 2012) and pulmonary fibrosis (Zhou et al., 2013), for example, but the methods harnessed for fibrotic reversion have often been genetic in nature and thus impractical or ethically problematic to recapitulate in human experiments utilizing solely the paradigms of gene therapy available to us at the present time. Even with the benefit of genetic methods, however, specific targeting of myofibroblast progenitors is not considered feasible at the current time.

While targeting of myofibroblast *progenitors*, as mentioned above, is complicated by the diversity of cell types that are able to differentiate into mature, functional myofibroblasts, targeting of *mature* myofibroblasts is similarly rendered difficult to achieve due to the lack of specific myofibroblast markers, as well as the large overlap between the gene and surface protein expression profiles of myofibroblasts and other mesenchymal cells and of smooth muscle cells (Hinz, 2016), the proper functioning of which is critical to the integrity and physiology of many tissues. Thus, specific targeting of myofibroblasts for induction of apoptosis or reversion of pro-

fibrogenic phenotypes may not be a plausible strategy either, due to the likelihood of off-target tissue damage inherent to nonspecific targeting.

Even in the event that these hurdles could be overcome, recent data has emerged demonstrating that FGF/FGFR signaling, in addition to being protective, can also exacerbate fibrotic pathology in the lungs. In mice, bleomycin-induced pulmonary fibrosis was determined to depend on FGFR signaling in mesenchymal cell populations (Guzy et al., 2017). Further, though its mechanism of action is not entirely understood, the antifibrotic effects of pirfenidone, a drug recently approved for treatment of idiopathic pulmonary fibrosis, are attributed in part to antagonism of the increase in FGF2 expression associated with animal models of pulmonary fibrosis (Oku et al., 2008). Treatment with a mutant soluble ectodomain of FGFR2 IIIc, one of the receptor variants activated by FGF2, reduced lung fibrosis in a bleomycin model of murine pulmonary fibrosis, likely through desensitization of murine lung fibroblasts to the pro-fibrotic effects of TGF-β1 (Yu et al., 2012). FGF2 has also been demonstrated to cooperate with, rather than antagonize, TGF-β1, in rat lung fibroblasts (Tan, Tan, Wang, & Cheng, 2015), in contrast to a variety of literature concerning fibroblasts and fibroblast-like cells from various other organs (Cushing, Mariner, Liao, Sims, & Anseth, 2008; D. Dolivo et al., 2017; Grella, Kole, Holmes, & Dominko, 2016; Kashpur et al., 2013; Latif et al., 2015; Shi et al., 2013). However, there is also evidence that lack of expression of FGF2 antagonizes bleomycin-induced pulmonary fibrosis, and that overexpression of FGF2 can provide protection to pulmonary fibrosis as well. The key to understanding the diversity of effects of fibroblast growth factor effects in the lungs may come from understanding the critical role of TGF-β signaling in not only pulmonary fibroblasts (Hoyles et al., 2011) , but also in lung epithelial cells (Li et al., 2011), in the fibrotic response, and the different effects of FGF signaling (and FGF2 signaling more specifically) on TGF-β

signaling in these different cell types. Thus, due to an incomplete understanding of the pleiotropy of FGF2 signaling in the lungs, and the associated dangers suggested from *in vitro* and pre-clinical models concerning FGF signaling and pulmonary fibrosis, more research must be undertaken in order to understand whether FGF signaling can be drugged as a viable therapy for pulmonary fibroses.

Some of the issues associated with the use of FGF2 pharmacologically, such as stability, release kinetics, and pleiotropy may be circumvented by use of small molecule-based drugs instead. Indeed, our data have suggested that some of the antifibrotic effects of FGF2 may be recapitulated simply by agonism of ERK or JNK signaling (or antagonism of p38 signaling), processes that are less likely to cause damaging off-target effects due to being further downstream than FGF2/FGFR. However, the very real issue remains that these pathways are still ubiquitous at not only the cellular level, but the tissue and organ level as well, complicating any manipulation of these pathways internally. While genetic approaches harnessing these concepts, as mentioned earlier, have been largely successful in animal models, key advancements in the field of tissue-specific drug delivery must be made and validated in order to inspire confidence in the viability of such therapy.

With advances in drug delivery, we sincerely hope that pharmacologic therapies to reverse or prevent accumulation of fibrotic tissue lesions internally, through targeting the pathways that activate or sustain myofibroblast phenotypes, will be made viable, thus representing a monumental step forward in medicine and in the betterment of human health.

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# Chapter 6-Materials and methods

## 6.1 Chapter 2

### *6.1.1 Antibodies*

The primary antibodies used were the following: sc-32251 α-SMA, sc-8654-R Histone H3, sc-8783 Collagen I, sc-47778 β-Actin, sc-59826 ED-A Fn, sc-365970 CTGF, sc-136987 Calponin (all from Santa Cruz Biotechnology), VPA00048KT SM22α (from Bio-Rad), and 11311-50 FITC-CD29 (Bio-Gems). The secondary antibodies used were Alexafluor488-conjugated or Alexafluor568-conjugated (Invitrogen) for immunofluorescence and HRP-conjugated for Western blotting (Bio-Rad).

### *6.1.2 Cell culture*

CRL-2097 and CRL-2352 human dermal fibroblasts were obtained from ATCC, and CT-1005 human dermal fibroblasts were obtained from the University of Massachusetts Medical School tissue distribution program in Worcester, MA. All fibroblasts were cultured in 1:1 DMEM:Ham's F12 (Corning) supplemented with 4mM L-glutamine (Mediatech) and 10% Fetal Clone III (Hyclone) at 37°C, 5% $O_2$, 5% $CO_2$, and high humidity. When indicated, cells were treated with 4ng/mL FGF2 (Cell Signaling Technology), 10ng/mL TGF-β1 (Peprotech), or 10μM Ilomostat (Santa Cruz Biotechnology). Cells were processed for analysis on day 4 unless otherwise indicated.

### *6.1.3 Proliferation studies*

CRL-2097 human dermal fibroblasts were plated in 60mm tissue culture plastic dishes at 15,000 cells/dish and cultured in the presence or absence of 4ng/mL FGF2 (Cell Signaling Technology)

and MAPK inhibitors as listed in *2.2*. Media was changed every fourth day and cells were passaged on every seventh day.

*6.1.4 RNA isolation and qRT-PCR*

RNA was isolated from snap-frozen cell pellets with the E.Z.N.A. DNA/RNA Isolation Kit (Omega Bio-Tek) according to manufacturer's instructions. RNA concentration was analyzed using a Nanodrop 2000 spectrophotometer (Thermo Scientific). cDNA was synthesized using SuperScript VILO Master Mix (ThermoFisher) according to manufacturer's instructions and stored at -20°C. PCR reactions were carried out using established protocols using an AB 7500 (Applied Biosystems) with PowerUp SYBR Master Mix (ThermoFisher), 5ng cDNA per reaction, and 500nM concentration per primer. Primer sequences are listed in **Table 1**. Fold change was calculated using the $\Delta\Delta C_t$ method [26].

*6.1.5 SDS-PAGE and Western blotting*

Cells were lysed using Laemmli sample buffer and lysate proteins and separated by SDS-PAGE. Proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore) using a semi-dry transfer apparatus (GE Healthcare). The membrane was blocked with 5% fat-free dry milk in TBS-T buffer (1xTBS+.1%Tween-20, pH=8.0) and incubated overnight at 4°C in the primary antibody solution at a pre-determined, antibody-specific dilution into 1% fat-free dry milk in TBS-T. The membrane was washed and incubated in a species-specific HRP-conjugated secondary antibody diluted 1:5000 into 1% fat-free dry milk in TBS-T. Signal was visualized using a ChemiDoc XRS system (Bio-Rad) using SuperSignal West Dura Extended Duration Substrate (ThermoFisher).

*6.1.6 Immuno flow cytometry*

Cells were harvested with trypsin, washed twice in 1x Dulbecco's phosphate-buffered saline (DPBS), and fixed for 20 minutes in cold methanol. The cells were then washed in 1x DPBS, followed by a 30 minute incubation in a primary antibody solution (1:200 dilution of α-SMA antibody or 1:100 dilution of FITC-CD29 antibody) in PBS+.05% Tween-20 (PBS-T). Cells were then washed again and resuspended in a 1:500 dilution of secondary antibody solution (for indirect cytometry only) in PBS-T (Alexa Fluor 488-conjugated goat anti-mouse IgG, Invitrogen). Cell nuclei were counterstained with propidium iodide. Samples were analyzed on an Accuri C6 Flow Cytometer (BD Biosciences). Ten thousand events were collected per sample.

*6.1.7 Annexin V/propidium iodide flow cytometry*

Cells were plated under various treatment conditions and cultured until day 4 and harvested for analysis according to manufacturer's instructions. Briefly, cell samples were washed in PBS, centrifuged, and resuspended in assay buffer containing 500ng/mL FITC-Annexin V (Santa Cruz Biotechnology) and 10μg/mL propidium iodide (Invitrogen). Samples were vortexed briefly and incubated in the dark for 30 minutes. Samples were then analyzed by flow cytometry on an Accuri C6 Flow Cytometer (BD Biosciences). Ten thousand events were collected per sample.

*6.1.8 Immunofluorescence*

Cells were plated on glass coverslips for 4 days and cultured under various treatment conditions. At the time of analysis, cells were fixed in 4% paraformaldehyde at room temperature, and permeabilized with 0.1% Triton X-100 in 1xPBS, before blocking with 5% BSA in PBS-T. Cells were then incubated overnight at 4°C in primary antibody in PBS-T, washed and incubated in a 1:500 dilution of Alexa Fluor-conjugated secondary antibody or Alexa Fluor-conjugated

Phalloidin, (Invitrogen). Cell nuclei were counterstained with Hoechst 33342 and coverslips mounted onto glass slides using Prolong Gold (Life Technologies) and stored at 4°C until imaging. Images were collected using an Axiovert 200M (Zeiss) using identical exposure times and settings between treatments.

#### *6.1.9 Collagen Lattice Contraction*

Fifteen-thousand CRL-2097 fibroblasts per sample were resuspended in 500μL of a 1:1 solution of 3.1mg/mL bovine type I collagen (Advanced Biomatrix):growth media (DMEM/F12+10% FCIII) and added to a Nunclon Delta 24-well tissue culture plate (ThermoFisher). Collagen gels were allowed to polymerize at 37°C for one hour before mechanical detachment from the sides of the well and addition of 1mL growth medium with or without 4ng/mL exogenous FGF2 (Cell Signaling Technology). Tissue culture plate was imaged at 24 hours for visualization of gel contraction.

### ***6.2 Chapter 3***

#### *6.2.1 Antibodies*

The primary antibodies used were the following: sc-32251 α-SMA, sc-8654-R Histone H3, sc-8783 Collagen I, sc-47778 β-Actin, sc-59826 ED-A Fn, , sc-136987 Calponin (all from Santa Cruz Biotechnology), VPA00048KT SM22α (from Bio-Rad), and 11311-50 FITC-CD29 (Bio-Gems). The secondary antibodies used were Alexafluor488-conjugated or Alexafluor568-conjugated (Invitrogen) for immunofluorescence and HRP-conjugated for Western blotting (Bio-Rad).

#### *6.2.2 Cell culture*

CRL-2097 and CRL-2352 human dermal fibroblasts were obtained from ATCC. All fibroblasts were cultured in 1:1 DMEM:Ham's F12 (Corning) supplemented with 4mM L-glutamine (Mediatech) and 10% Fetal Clone III (Hyclone) at 37°C, 5% $O_2$, 5% $CO_2$, and high humidity. When indicated, cells were treated with 4ng/mL FGF2 (Cell Signaling Technology), 10ng/mL TGF-β1 (Peprotech), 10μM U0126 (MEK1/2 inhibitor; Cell Signaling Technology), 10μM SP600125 (JNK inhibitor; Santa Cruz Biotechnology), and/or 10μM SB202190 (p38 MAPK inhibitor; Santa Cruz Biotechnology). Cells were processed for analysis on day 4 unless otherwise indicated.

*6.2.3 Resazurin assay*

CRL-2097 fibroblasts were cultured in 96-well Nunclon Delta tissue culture plates (ThermoFisher) in growth media in the presence or absence of 4ng/mL FGF2 and/or 10μM specific MAPK inhibitor. On day 4, 100μL fresh quiescent media (1:1 DMEM/F12, .1% FCIII) was added to each well, and 20μL of .15mg/mL resazurin was added to each well. Plates were incubated at 37°C for 2 hours before fluorescent readings were made at Ex/Em=544nm/590nm.

*6.2.4 SDS-PAGE and Western blotting*

Cells were lysed using Laemmli sample buffer and lysate proteins and separated by SDS-PAGE. Proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore) using a semi-dry transfer apparatus (GE Healthcare). The membrane was blocked with 5% fat-free dry milk in TBS-T buffer (1xTBS+.1%Tween-20, pH=8.0) and incubated overnight at 4°C in the primary antibody solution at a pre-determined, antibody-specific dilution into 1% fat-free dry milk in TBS-T. The membrane was washed and incubated in a species-specific HRP-conjugated secondary antibody diluted 1:5000 into 1% fat-free dry milk in TBS-T. Signal was visualized

using a ChemiDoc XRS system (Bio-Rad) using SuperSignal West Dura Extended Duration Substrate (ThermoFisher).

*6.2.5 Immuno flow cytometry*

Cells were harvested with trypsin, washed twice in 1x Dulbecco's phosphate-buffered saline (DPBS), and fixed for 20 minutes in cold methanol. The cells were then washed in 1x DPBS, followed by a 30 minute incubation in a 1:100 solution of FITC-CD29 antibody) in PBS+.05% Tween-20 (PBS-T). Cells were then washed and cell nuclei were counterstained with propidium iodide. Samples were analyzed on an Accuri C6 Flow Cytometer (BD Biosciences). Ten thousand events were collected per sample.

*6.2.6 Immunofluorescence*

Cells were plated on glass coverslips for 4 days and cultured under various treatment conditions. At the time of analysis, cells were fixed in 4% paraformaldehyde at room temperature, and permeabilized with 0.1% Triton X-100 in 1xPBS, before blocking with 5% BSA in PBS-T. Cells were then incubated overnight at 4°C in primary antibody in PBS-T, washed and incubated in a 1:500 dilution of Alexa Fluor-conjugated secondary antibody or Alexa Fluor-conjugated Phalloidin, (Invitrogen). Cell nuclei were counterstained with Hoechst 33342 and coverslips mounted onto glass slides using Prolong Gold (Life Technologies) and stored at 4°C until imaging. Images were collected using an Axiovert 200M (Zeiss) using identical exposure times and settings between treatments.

***6.3 Chapter 4***

*6.3.1 Reagents*

The primary antibodies used were the following: sc-32251 α-SMA, sc-8783 Collagen I, sc-59826 ED-A Fn, sc-8654-R Histone H3 (all from Santa Cruz Biotechnology), clone: CALP Calponin (from Dako), VPA00048KT SM22α (from Bio-Rad), and #4370 p-ERK1/2 (Cell Signaling Technology). The secondary antibodies used were Alexafluor488-conjugated or Alexafluor568-conjugated (Invitrogen) for immunofluorescence and HRP-conjugated for Western blotting (Bio-Rad). MAPK inhibitors used were U0126, SP600125, and SB202190 (all from Santa Cruz Biotechnology). TGF-βRI inhibitor used was RepSox (Tocris). The recombinant human TGF-β1 used was purchased from Peprotech. The recombinant human FGF2 used was purchased from Cell Signaling Technology.

*6.3.2 Cell culture*

CRL-2097 and CRL-2352 human dermal fibroblasts were obtained from ATCC, and CT-1005 human dermal fibroblasts were obtained from the University of Massachusetts Medical School tissue distribution program in Worcester, MA. Fibroblasts were cultured in 1:1 DMEM:Ham's F12 (Corning) supplemented with 4mM L-glutamine (Mediatech) and 10% Fetal Clone III (Hyclone) on Nunclon Delta tissue culture plastic (ThermoFisher). Cultures were incubated at 37°C, 5% $O_2$, 5% $CO_2$, and high humidity, and cells were processed on day 4 in culture unless otherwise indicated for analysis.

*6.3.3 Immuno flow cytometry*

Cells were harvested with .05% trypsin and washed twice in DBPS. Cells were fixed for 20 minutes in cold methanol and washed in 1×DPBS, followed by a 30-minute incubation in a primary antibody solution (1:200 dilution of α-SMA antibody, sc-32251 from Santa Cruz Biotechnology) in PBS+0.05% Tween-20 (PBS-T). Cells were then washed again and

resuspended in a 1:500 dilution of secondary antibody solution in PBS-T (Alexa Fluor 488-Conjugated goat anti-mouse IgG, Invitrogen). Cells were counterstained with 500ng/mL propidium iodide for 15 minutes and analyzed on an Accuri C6 flow cytometer (BD Biosciences). Twenty thousand events were collected per sample.

*6.3.4 RNA isolation and qRT-PCR*

RNA was isolated from snap-frozen cell pellets with the E.Z.N.A. DNA/RNA Isolation Kit (Omega Bio-Tek) according to manufacturer's instructions. Concentration of RNA was determined using a Nanodrop 2000 spectrophotometer (Thermo Scientific). cDNA was synthesized using qScript cDNA Supermix (Quantabio) according to manufacturer's instructions and stored at -20°C. PCR reactions were carried out using manufacturer's protocols using an AB 7500 (Applied Biosystems) with PowerUp SYBR Master Mix (ThermoFisher), using 5ng cDNA per reaction and 500nM concentration per primer. Primer sequences are listed in **Table 1**. Relative expression fold changes were calculated using the $\Delta\Delta C_t$ method (36).

*6.3.5 SDS-PAGE and Western blotting*

Protein was isolated from cells using cold 2x Laemmli sample buffer supplemented with protease and phosphatase inhibitor cocktail (ThermoFisher). Cells were lysed on ice and DNA was sheared on ice using a Missonix CL-2000 ultrasonic cell disruptor. Samples were boiled for 10 minutes on a heat block and then separated on SDS-PAGE gels. For analysis by Western blot, protein from the gel was transferred to a PVDF membrane (Millipore) using a semi-dry transfer apparatus (GE Healthcare). The membrane was blocked in 5% fat-free dry milk in TBS-T buffer (1xTBS+.1%Tween-20, pH=8.0) for 1 hour at room temperature. The membrane was incubated and rotated overnight at 4°C in the primary antibody solution at a pre-determined, antibody-

specific dilution into 1% fat-free dry milk in TBS-T. The membrane was washed 4 x 10 minutes in TBS-T and incubated in a species-specific, HRP-conjugated secondary antibody diluted 1:5000 into 1% fat-free dry milk in TBS-T and rotated at room temperature for 2 hours. The membrane was washed 4 x 10 minutes in TBS-T, and signal was visualized using a ChemiDoc XRS system (Bio-Rad) using SuperSignal West Dura Extended Duration Substrate (ThermoFisher).

*6.3.6 Fluorescent microscopy*

Cells were plated on glass coverslips inside of 24 well tissue culture plates for 4 days in the appropriate treatment conditions. At the time of analysis, cells were fixed in 4% paraformaldehyde at room temperature for 20 minutes at 4°C, and then permeabilized with 0.1% Triton X-100 in 1xPBS. Blocking was performed in 5% BSA in PBS-T for 30 minutes at room temperature. Cells were incubated overnight at 4°C with agitation in 250µL/well of primary antibody in PBS-T using antibody-specific dilutions. Cells were then washed 3 x 5 minutes in PBS-T before addition of a 1:500 dilution of Alexa Fluor-conjugated secondary antibody (or Alexa Fluor-conjugated phalloidin, both purchased from Invitrogen), incubated at room temperature in the dark for 30 minutes, and protected from light thereafter. Cells were then washed 3 x 5 minutes in PBS-T before being incubated in 500ng/mL Hoechst 33342 in 1xPBS-T (ThermoFisher) for 15 minutes at room temperature to stain the nuclei. Cells were washed 2 x 5 minutes in PBS and coverslips were mounted onto glass slides using Prolong Gold (Life Technologies) and stored at 4°C until imaging. Images were collected using an Axiovert 200M (Zeiss) using identical exposure times and settings between treatments, to allow for direct qualitative comparison of protein expression between different treatments.

**Appendix**

*A.1 PCR primer sequences*

| Gene name | Protein encoded | Forward primer (5'-3') | Reverse primer (5'-3') | Amplicon size (bp) |
|---|---|---|---|---|
| *ACTA2* | α-SMA | ACTGCCTTGGTGTGTGACAA | CACCATCACCCCCTGATGTC | 120 |
| *CNN1* | Calponin | AGGTTAAGAACAAGCTGGCCC | GAGGCCGTCCATGAAGTTGT | 113 |
| *TAGLN* | SM22α | CACAAGGTGTGTGTAAGGGTG | GGCTCATGCCATAGGAAGGAC | 132 |
| *CCN2* | CTGF | GTGCCTGCCATTACAACTGTC | TCTCACTCTCTGGCTTCATGC | 98 |
| *ED-A Fn* | Fibronectin (ED-A) | CAGTGGAGTATGTGGTTAGTGTC | GTGACCTGAGTGAACTTCAGG | 119 |
| *Col1A1* | Collagen 1 (α1) | GTCAGGCTGGTGTGATGGG | GCCTTGTTCACCTCTCTCGC | 182 |
| *Col1A2* | Collagen 1 (α2) | CTGGAGAGGCTGGTACTGCT | AGCACCAAGAAGACCCTGAG | 62 |
| *Col3A1* | Collagen III (α1) | GGACACAGAGGCTTCGATGG | CTCGAGCACCGTCATTACCC | 190 |
| *MMP1* | Matrix metalloproteinase 1 | GCATATCGATGCTGCTCTTTC | GATAACCTGGATCCATAGATCGTT | 110 |
| *LOX* | Lysyl Oxidase | CTCTTGCTGTCCTCCGCTC | ATCTTGGTCGGCTGGGTAAG | 155 |
| *MYOCD* | Myocardin | AGAGGCCATAAAAGGTAACCAGG | GGGGGTCTTCACTTCGAGTC | 116 |
| *TGFB1* | TGF-β1 | CATTGGTGATGAAATCCTGGT | TGACACTCACCACATTGTTTTTC | 110 |
| *TGFB2* | TGF-β2 | GAGCGACGAAGAGTACTACG | TTGTAACAACTGGGCAGACA | 89 |
| *TGFBR1* | TGF-βR1 | GCAGACTTAGGACTGGCAGTAAG | AGAACTTCAGGGGCCATGT | 104 |
| *TGFBR2* | TGF-βR2 | TGGACCCTACTCTGTCTGTG | CTGGAGCCATGTATCTTGCAG | 72 |
| *TGFBR3* | Betaglycan | CTGGTGTGGCATCTGAAGAC | GGACCACAGAACCCTCAGAC | 79 |
| *GAPDH* | GAPDH | GAGTCCACTGGCGTCTTCAC | TTCACACCCATGACGAACAT | 119 |