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FGF2-mediated attenuation of myofibroblast activation is modulated by distinct MAPK signaling pathways in human dermal fibroblasts

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ABSTRACT

Background: Previous human and animal studies have demonstrated the ability of exogenously administered basic fibroblast growth factor (FGF2) to act as an antifibrotic agent in the skin. Though the activity of FGF2 as an anti-scarring agent is well-established for fibrotic skin wounds, the mechanisms by which FGF2 exerts these actions are not entirely understood. Canonical FGF2 signaling proceeds in part via FGFR/MAPK pathways in human dermal fibroblasts, and FGF2 has been described to prevent or reverse the fibroblast-to-myofibroblast transition, which is driven by TGFβ signaling and understood to be an important step in the formation of a fibrotic scar *in vivo*. Thus, we set out to investigate the antagonistic effects of FGF2 on TGFβ signaling as well as the broader effects of MAPK inhibition on the TGFβ-mediated induction of myofibroblast gene expression.

Objective: To better understand the effects of FGF2 signaling pathways on myofibroblastic gene expression and cell phenotypes.

Methods: Human dermal fibroblasts were cultured *in vitro* in the presence of FGF2, TGFβ, and/or MAPK inhibitors, and the effects of these agents were investigated by molecular biology techniques including qRT-PCR, immunofluorescence, Western blot, and flow cytometry.

Results: FGF2 inhibited TGFβ-mediated fibroblast activation, resulting in more rapidly proliferating, spindle-shaped cells, compared to the more slowly proliferating, flatter TGFβ-treated cells. Treatment with FGF2 also attenuated TGFβ-mediated increase in expression of myofibroblast markers smooth muscle α-actin, calponin, transgelin, connective tissue growth factor, ED-A fibronectin, and collagen I. FGF2-mediated antagonism of the TGFβ-mediated fibroblast-to-myofibroblast transition was reversed by small molecule inhibition of ERK or JNK, and it was potentiated by inhibition of p38. MAPK inhibition was demonstrated to have qualitatively similar effects even in the absence of exogenous FGF2, and small molecule inhibition of p38 MAPK was sufficient to attenuate TGFβ-mediated fibroblast activation.

Conclusions: Inhibition of select MAPK signaling pathways can reverse or potentiate anti-fibrotic FGF2 effects on human dermal fibroblasts, as well as exert their effects independently of exogenous FGF2 supplementation.

1. 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 [1]. 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 [2]. It has recently been demonstrated that myofibroblasts arise from multiple different cell types *in vivo*, including fibrocytes, mesenchymal stem cells, and smooth muscle cells [3]. 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 (TGFβ) signaling. Mechanical heterogeneity of the granulation tissue leads to the release of TGF-β1 from the provisional extracellular matrix,

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leading to the differentiation of resident wound fibroblasts into myofibroblasts (termed "fibroblast activation") [4]. 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 [5].

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 [6–9]. 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 [10]. 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 [11]. This has been demonstrated in humans for acute incisional wounds, avulsions, and burn wounds [12–14], as well as in several animal models [15,16]. 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 [16–21]. Additionally, FGF2 has been demonstrated under certain circumstances to antagonize TGFβ signaling, including reports in which FGF2 has demonstrated the potential to antagonize TGFβ-mediated myofibroblast phenotypes [15,16,18,22–25]. 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 TGFβ in human dermal fibroblasts, as well as to understand the effects on these same phenotypes of inhibition of specific MAPK pathways known to be activated by FGF2/FGFR signaling.

2. Materials and methods

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-365970 CTGF, sc-136987 Calponin (all from Santa Cruz Biotechnology), and VPA00048KT SM22α (from Bio-Rad). The secondary antibodies used were Alexafluor488-conjugated or Alexafluor568-conjugated (Invitrogen) for immunofluorescence and HRP-conjugated for Western blotting (Bio-Rad).

2.2. 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 4 mM 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 4 ng/mL FGF2 (Cell Signaling Technology), 10 ng/mL TGF-β1 (Peprotech), 10 μM U0126 (MEK1/2 inhibitor; Cell Signaling Technology), 10 μM SP600125 (JNK inhibitor; Santa Cruz Biotechnology), and/or 10 μM SB202190 (p38 MAPK inhibitor; Santa Cruz Biotechnology). Cells were processed for analysis on day 4.

2.3. Proliferation studies

CRL-2097 human dermal fibroblasts were plated in 60 mm tissue culture plastic dishes at 15,000 cells/dish and cultured in the presence or absence of 4 ng/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.

2.4. 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), 5 ng cDNA per reaction, and 500 nM concentration per primer. Primer sequences are listed in Table 1. Fold change was calculated using the $\Delta\Delta C_t$ method [26].

2.5. 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+0.1% Tween-20, pH = 8.0) and incubated overnight at 4 °C in the primary antibody solution at a pre-determined, antibody-specific

Table 1
Primer sequences used for qRT-PCR.

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
GAPDH	GAPDH	GAGTCCACTGGCGTCTTCAC	TTCACACCCATGACGAACAT	119

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