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Knockdown of electron transfer flavoprotein β subunit reduced TGF-β-induced α-SMA mRNA expression but not COL1A1 in fibroblast-populated three-dimensional collagen gel cultures

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ABSTRACT

Background: The inhibition of transforming growth factor β (TGF-β)-induced myofibroblast differentiation is a key objective for the treatment of hypertrophic scarring. We previously reported that knockdown of the electron transfer flavoprotein β subunit (ETFB) reduced mechanoregulated cell number in fibroblast-populated collagen gel cultures [1].
Objective: To characterize the effects of ETFB knockdown, we investigated gel contraction, TGF-β-induced collagen, α-SMA mRNA expression and stress fiber formation.
Methods: Fibroblasts were transfected with negative control or ETFB-specific siRNAs and embedded in collagen gels in an attached or detached condition. The gel contraction assay was performed in three different concentrations of collagen (0.5, 1.0 or 1.5 mg/mL) and was analyzed by measuring the changes in the gel area throughout the culture period. The attached collagen gel culture was performed in the presence of rTGF-β and the mRNA levels of α-SMA and COL1A1 were measured by qRT-PCR. The effect of ETFB knockdown on proliferation and stress fiber organization in monolayer cultures was investigated by conducting AlamarBlue assays and phalloidin staining.
Results: The transfection of ETFB siRNA did not alter gel contraction compared to the negative control in all collagen concentrations. When the cells were treated with TGF-β under mechanical stress conditions, ETFB knockdown attenuated α-SMA mRNA expression to a level comparable to that observed in the absence of TGF-β. However, no inhibitory effect on COL1A1 mRNA levels was observed. The AlamarBlue assay indicated that the knockdown had no effect on the proliferation of cells cultured on plastic. Phalloidin staining of a monolayer culture showed that ETFB knockdown weakened the stress fiber organization induced by rTGF-β.
Conclusion: ETFB knockdown can affect TGF-β-induced tissue remodeling and/or fibrotic processes *in vitro*.

1. Introduction

Granulation tissue is formed as part of the initial response to wounding during the healing and repair process in the dermis and is composed of small vessels, abundant fibroblasts and variable numbers of inflammatory cells [2]. When the dermis is injured, the fibroblasts acquire a migratory phenotype and generate comparably small traction forces to promote wound closure in response to changes in the composition, organization and mechanical properties of the ECM. The fibroblasts also respond to cytokines released locally by inflammatory and other resident cells by proliferating and producing ECM components such as fibronectin and collagen [3]. In response to the increasing ECM stress resulting from these

Abbreviations: α-SMA, alpha smooth muscle actin; bFGF, basic fibroblast growth factor; COL1A1, collagen type I α I; CTGF, connective tissue growth factor; ETFB, electron transfer flavoprotein β subunit; ECM, extracellular matrix; IFN-γ, interferon gamma; PDGF, platelet-derived growth factor; PPAR, peroxisome proliferator-activated receptor; rTGF-β, recombinant transforming growth factor β; 2DE, two-dimensional gel electrophoresis; 2D, two-dimensional; 3D, three-dimensional.

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remodeling activities, some fibroblasts differentiate into myofibroblasts and upregulate α-SMA, the most widely used myofibroblast marker [3]. As myofibroblasts exhibit higher contractile forces and ECM production, it is generally accepted that the fibroblast-to-myofibroblast differentiation represents a key event during wound healing and tissue repair. The high contractile force generated by myofibroblasts is beneficial for physiological tissue remodeling but detrimental for tissue function when it becomes excessive, as in hypertrophic scars, which are histologically characterized by an overabundance of dermal collagen and myofibroblasts [4].

Many studies have evaluated candidate anti-fibrosis factors or reagents by assessing their impact on TGF-β-induced collagen and α-SMA expression in fibroblasts. For example, a Rac1 inhibitor [5], IFN-γ [6], a p38 inhibitor [7], Vitamin D [8], SB431542 (TGF-β1 inhibitor) [9], a PPAR-γ agonist [10] and CTGF antisense oligonucleotides [11] can inhibit the increase in collagen and α-SMA expression induced by TGF-β. PPAR-γ activation and CTGF inhibition were also effective in *in vivo* models of fibrosis [12,13]. Recently, bFGF was shown to reduce excessive fibrosis by inhibiting the differentiation of fibroblasts into myofibroblasts [14] and may serve as a therapeutic tool for hypertrophic scarring [15].

Fibroblast-populated three-dimensional (3D) collagen gel culture has been widely accepted as an *in vitro* model of wound closure or fibrosis [16–19]. In the detached-free-floating culture condition, tractional forces generated by fibroblasts cause the collagen matrix to contract, making this culture method a useful *in vitro* wound closure model [16,18,20]. Taken together, in the attached-tethering culture condition, the cells are proliferative, morphologically elongated and bipolar, and some populations differentiate into α-SMA-expressing myofibroblasts. This condition has therefore been recognized to mimic fibrosis [17,19].

To find new pharmaceutical targets for the treatment of hypertrophic scars, we performed a 2DE differential display comparison of 3D collagen gel cultures in the attached and detached conditions to identify factors that reduce mechanoregulated fibroblast cell proliferation. The electron transfer flavoprotein β subunit (ETFB) was one of the candidates identified [1]. In the present report, we verified the inhibitory effect of ETFB on fibroblast cell number in our 3D collagen culture system and determined the ETFB mRNA expression pattern. To further validate ETFB as an anti-fibrosis factor, we examined the effects of ETFB knockdown on the induction of collagen and α-SMA mRNA expression by rTGF-β in attached collagen gel cultures.

2. Materials and methods

2.1. Cell culture and reagents

CCD-1113sk cells (ATCC No. CRL2439), which are normal human skin fibroblasts isolated from a 46-year-old female, were purchased from the American Type Culture Collection at the fourth passage. The cells were cultured in DMEM (1000 mg/mL glucose, 0.584 g/L L-glutamine and 3.7 g/L $NaHCO_3$; D-6046, Sigma, MO) supplemented with 10% FBS and no antibiotics in a tissue culture flask (353024, BD Falcon, NJ). The experiments were performed before passage 13 because senescence was observed after 16 passages.

The collagen gel culture was performed as previously described [1]. Briefly, for one 24-well plate, a collagen matrix solution was prepared by mixing 7 mL of 5 mg/mL bovine dermis-derived, acid-solubilized type I collagen (Cellgen, Koken, Tokyo), 3.5 mL FBS, 7 mL of 5× DMEM (4500 mg/mL glucose, final concentration, HEPES(−), $NaHCO_3$(−); 12800-017, Invitrogen, CA), 3.5 mL of 200 mM HEPES, 3.5 mL of 2.2% $NaHCO_3$, 1.75 mL of 0.1 N NaOH and 5.25 mL of H_2O. The final concentrations of collagen and FBS were 1 mg/mL and 10% (v/v), respectively. For the gel contraction assay, the volume of collagen was adjusted with the volume of H_2O. The solution was stored at 12 °C before use. Before culturing, 250 μL of the solution was seeded into each well of a 24-well plate as a basal layer and incubated for 15 min. After mixing with 2.5 mL of suspended fibroblasts (1×10^6 cells/mL) and 22.5 mL of the collagen matrix solution, 1 mL of the mixture was seeded onto the basal layer. The final calculated cell number was 1×10^5 cells/well. For the groups cultured in the detached condition, the gel and basal layer were released from the well with a spatula after 2 h of incubation. For TGF-β treatments, 300 ng/mL rTGF-β (100-B-010, R&D Systems, MN) was mixed into the collagen solution.

To measure cell numbers, the matrices were incubated with 1000 U/mL collagenase (C-1889, Sigma, MO) in DMEM (2 mL collagenase solution/matrix) for 30 min with agitation at 37 °C. The cells were then harvested by centrifugation at 3000 rpm for 5 min, and the pellet was suspended in DMEM. The cell number was automatically counted by trypan blue staining using Vi-Cell XR (Beckman Coulter, CA). The statistical differences between the ETFB siRNA- and negative control siRNA-transfected cultures were evaluated by Student's *t*-test ($p < 0.05$) at each time point.

2.2. Transfection

The day before transfection, 7.25×10^5 cells were plated onto a 10-cm dish. Transfection was performed according to the manufacturer's instructions with Lipofectamine 2000 (Invitrogen, CA). Briefly, the siRNA and Opti-MEM (Invitrogen, Carlsbad, CA) were mixed to a final concentration of 33 nM siRNA. Lipofectamine 2000 and Opti-MEM were mixed to achieve a final concentration of 50 μL Lipofectamine 2000 per dish and then incubated for 5 min at room temperature. Equal volumes of the two mixtures were combined and incubated for 20 min at room temperature. During the incubation, the culture medium of the 10-cm dishes was replaced with 12.5 mL of fresh medium (DMEM supplemented with 10% (v/v) FBS). Next, 2.5 mL of the siRNA–Lipofectamine mixture was added to each dish and incubated for 24 h. At the end of the experiment, the transfected cells were trypsinized and subjected to collagen gel culture and monolayer culture.

The siRNA for ETFB was purchased from GE Healthcare (UK; D-010494-04). The AllStars Negative Control siRNA was used for negative controls (Qiagen, CA).

2.3. qRT-PCR

Total RNA was extracted from collagenase-dissolved cells (3 gels/extraction) using the RNeasy RNA extraction kit (Qiagen, CA) according to the manufacturer's instructions. The RNA concentration of each sample was measured with a NanoDrop 1000 (ThermoFisher Scientific, MA) and adjusted to 50 ng/μL with RNase-free H_2O. The concentration-adjusted RNA samples were visibly checked with agarose gel electrophoresis (E-gel System, Invitrogen, CA). The cDNA synthesis was performed with the QuantiTect cDNA synthesis kit (Qiagen, CA). The concentration of the RNA template was 250 ng per 20 μL reaction. Next, qRT-PCR was conducted using the SYBR green method (QuantiTect SYBR Green PCR Kit, Qiagen, CA) and an ABI7500 instrument (Applied Biosystems, CA). A total of 5 μL cDNA was used in a final volume of 25 μL. The PCR reaction consisted of 55 cycles of denaturation at 94 °C for 15 s, annealing at 60 °C for 30 s, and extension at 72 °C for 34 s.

The data were analyzed with 7500 SDS System Software (Applied Biosystems, CA). The relative mRNA expression of the samples compared to that of GAPDH was analyzed with the ddCt method. For statistical analysis, Student's *t*-test ($p < 0.05$) was performed using dCt values [21].