Elucidating the Cellular Mechanism for E2-induced Dermal Fibrosis

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Abstract

Background: Both TGFb and estradiol (E2), a form of estrogen, are pro-fibrotic in the skin. In the connective tissue disease, systemic sclerosis (SSc), both TGFb and E2 are likely pathogenic. Yet, the regulation of TGFb in E2-induced dermal fibrosis remains ill-defined. Elucidating those regulatory mechanisms will improve the understanding of fibrotic disease pathogenesis and set the stage for developing potential therapeutics. Using E2-stimulated primary human dermal fibroblasts *in vitro* and human skin tissue *ex vivo*, we identified the important regulatory proteins for TGFb and investigated the extracellular matrix (ECM) components that are directly stimulated by E2-induced TGFb signaling.

Methods: We used primary human dermal fibroblasts *in vitro* and human skin tissue *ex vivo* stimulated with E2 or vehicle (ethanol) to measure *TGFb1, TGFb2* levels using quantitative PCR (qPCR). To identify the necessary cell signaling proteins in E2-induced *TGFb1* and *TGFb2* transcription, human dermal fibroblasts were pre-treated with an inhibitor of the extracellular signal-regulated kinase/ mitogen-activated protein kinase (ERK/MAPK) pathway, U0126. Finally, human skin tissue *ex vivo* was pre-treated with SB-431542, a TGFb receptor inhibitor, to establish the effects of TGFb signaling on E2-induced collagen 22A1 (*Col22A1*) transcription. Statistical tests used included parametric and non-parametric ANOVA, student's T-test and Wilcoxon matched-pairs signed rank test. Significance was defined as p-value ≤ 0.05.

Results: We found that TGFb1, TGFb2, and collagen 22A1 (Col22A1), a TGFb-responsive gene, are induced in response to E2 stimulation. Mechanistically, Col22A1 induction is blocked by the TGFb receptor inhibitor SB-431542, despite E2 stimulation. Additionally, inhibiting E2-induced ERK/MAPK activation and early growth response 1 (*EGR1*) transcription prevents E2-induced increase in TGFb1 and TGFb2 transcription and translation.

Conclusions: We conclude that E2-induced dermal fibrosis occurs in part through induction of TGFb1, 2 and Col22A1, which is regulated through EGR1 and the MAPK pathway. Thus, blocking estrogen signaling and/or production may be a novel therapeutic option in pro-fibrotic diseases.

Background

Transforming growth factor beta (TGFβ) is central to the production of extracellular matrix (ECM) (1), which promotes wound healing (2) but can also lead to organ fibrosis, especially in the connective tissue disease, systemic sclerosis (SSc) (3–6). Several studies focus on TGFβ as an inducer of fibrosis (1), but few studies explore the regulation of TGFβ. Because TGFβ is a mjor inducer of fibrosis, its inhibition was the emphasis for a potential pharmacologic intervention in SSc, without success (7). Since there are limited treatment options for SSc, it is important to understand the regulation of TGFβ isoforms to understand SSc disease pathogenesis and develop potential therapeutics.

Like TGFβ, the steroid hormone estrogen augments fibrosis. Because SSc has a female predominance (8), estrogen is hypothesized to be pathogenic in fibrosis and to contribute to sex differences noted in

animal models of fibrosis. Female rats treated with bleomycin had more severe fibrosis and higher mortality than male rats (9). In radiation-induced lung fibrosis, rats had less lung fibrosis if treated with an aromatase inhibitor that blocks estrogen production (10). Likewise, mice with a heritable form of pulmonary hypertension that were treated with either an estrogen receptor inhibitor or an aromatase inhibitor showed significant disease improvement (11). These results imply estrogen is influential in lung fibrosis and its blockade is a conceivable therapeutic target.

Estradiol (E2) is the most bioactive and abundant form of estrogen in non-pregnant women (12). In the skin, E2 promotes ECM production. Women on hormone replacement therapy (HRT) have increased dermal thickness and collagen content than women not taking HRT (13–18). Our group has shown that E2 levels are increased in post-menopausal women with SSc, and E2 induced dermal thickening and increased fibronectin (FN) in an *ex vivo* human skin organ culture model and in primary human dermal fibroblasts (19). Elevated E2 levels in patients with SSc are associated with reduced survival (20), suggesting a role for E2 in SSc-fibrosis.

As with TGFβ, most studies regarding E2-induced fibrosis have centered on ECM production. However, few experiments have focused on the production of E2-induced pro-fibrotic mediators or the transcriptional regulation of these proteins. E2 induced the pro-fibrotic mediators connective tissue growth factor (CTGF) in rats (21) and TGFβ1 in a model of wound healing (22, 23), while the cell signaling molecules used by E2 to induce TGFβ1 remain undefined. In this study, we seek to characterize E2-induced dermal fibrosis by describing the underlying cellular mechanisms that promote fibrosis.

Methods

Ex vivo human skin organ culture

We received skin samples from healthy donors of various ages who underwent skin-resection procedures in the Division of Plastic Surgery at the Medical University of South Carolina under an approved IRB protocol. The *ex-vivo* human skin model organ culture was used as previously described (19). For experimentation, we used a six-well tissue culture dish (Costar, Corning, NY) with six 3-mm punches/wells placed dermal side down in serum-free, phenol-red-free DMEM (Corning, Corning, NY). In all experiments, skin punches and supernatants were harvested at the specified times and stored at -80^{0}C until further evaluation.

Cell Culture

Human primary dermal fibroblasts were isolated from human dermal tissue using the previously described outgrowth method (19). Primary dermal fibroblasts (passages 3–8) were plated in six-well culture dishes (Costar, Corning, NY) at a concentration of 2.0×10^5 cells/mL. Prior to stimulation, the cells were serum-starved in serum-free, phenol-red-free DMEM (Hyclone, South Logan, UT). Cells were then treated with 10 μM of a mitogen-activated protein kinase/ extracellular signal-regulated kinase

(MAPK/ERK) inhibitor, U0126 (24), or TGFβ1 receptor inhibitor, SB-431542 (25), one hour before stimulation with E2 or ethanol (ETOH). U0126, SB-431542 and E2 were obtained from MilliporeSigma (St. Louis, MO), and the ETOH from Hyclone (South Logan ,UT). For subcellular fractions, cells were grown in 10-cm culture dishes and maintained in complete media (DMEM supplemented with 1x antibiotic and antimycotic) from Hyclone. Once 90–95% confluent, the cells were serum-starved for at least eight hours in phenol red-free media then stimulated with ETOH or E2 for the indicated timepoints. The subcellular fractions were isolated using a subcellular protein fractionation kit for cultured cells (Thermofisher Scientific, Rockford, IL).

Measurement of steady-state mRNA levels

Total RNA was isolated from human skin punches through homogenization using TRIzol (Invitrogen, Carlsbad, CA), with further purification using a RNeasy isolation kit (Qiagen, Hilden, Germany). RNA from primary dermal fibroblasts was isolated with a RNeasy isolation kit. Steady-state mRNA levels were measured using quantitative PCR (qPCR) and levels are shown as fold change over vehicle following normalization of signal to *B2M* and G*APDH*. Primers specific for *FN*, *Col22A1*, *TGFβ1, TGFβ2, TGFβ3, collagen IA2 (Col IA2), collagen IIIA1 (Col IIIA1)* and *early growth response 1 (EGR1)* were all purchased from Thermofisher Scientific (Rockford, IL).

siRNA Transfection

Primary dermal fibroblasts were plated at 1.5×10^5 cells/mL in six-well culture dishes. After reaching 70% confluency, the cells were transfected with siRNA targeted to *EGR1* or a negative control pool (composed four siRNA) of non-targeting siRNA (CTL) (Horizon Discovery, Cambridge, UK) at 100 nM for 24 hours and then serum-starved in phenol red-free media for eight hours before treatment with ETOH or E2 for 24 hours.

Immunoblot Analysis

Whole-cell protein lysates or cellular fractions from dermal fibroblasts were subjected to immunoblot analysis. Equal volumes (20 µL/lane) were resolved on 10% SDS PAGE gel and transferred to a nitrocellulose membrane (GE Healthcare Life Sciences, Pittsburgh, PA). Following blocking with 5% non-fat dry milk, membranes were incubated with antibodies against phosphorylated ERK (Rabbit polyclonal, catalog # 9101, 1:1000 dilution, Cell Signaling Technology, Danvers, MA), total ERK (Rabbit monoclonal, catalog # 4695 S, 1:1000 dilution, Cell Signaling Technology, Danvers, MA), TGFβ1, (Rabbit monoclonal, clone EPR18163, 1:1000 dilution, Abcam, Cambridge, UK), EGR1 (Rabbit monoclonal, clone 15F7, 1:1000 dilution, Cell Signaling Technology, Danvers, MA), or GAPDH (Mouse monoclonal, catalog # sc-47724, dilution 1:5000, Santa Cruz, Santa Cruz, CA), followed by horseradish peroxidase–conjugated secondary antibodies. After being washed, immunoblots were developed with chemiluminescence reagents according to the manufacturer's protocol (Pierce, Rockford, IL). Total MAP kinases were examined in the same immunoblots as their phosphorylated forms. Signal intensities of the phosphorylated MAP kinase bands were quantitated by densitometry, and the results, which were normalized against the intensities of

the corresponding total MAP kinase bands in each sample, were expressed as the magnitude of increase compared with controls. Densitometry was calculated using Image J.

Statistical Analysis

All data were tested for normal distribution using the Shapiro-Wilk normality test. If the data were normally distributed, a one-way ANOVA with Sidak's multiple comparisons post-hoc test or paired, two-sample T-test were used to determine statistical significance. For non-normally distributed data, non-parametric one-way ANOVA using Dunn's multiple comparisons post-hoc test or Wilcoxon matched-pairs signed rank tests were used to determine statistical significance, which was defined as a p-value ≤ 0.05 using GraphPad.

Results

E2 increased transcription of ECM components and pro-fibrotic mediators in vitro

Pro-fibrotic mediators and ECM components were measured in primary human dermal fibroblasts post-E2 stimulation. *TGFβ1* and *TGFβ2* transcript levels were 1.4- and 1.7-fold higher in E2-stimulated cells, respectively, than vehicle-treated cells at 24 hours, which reached statistical significance (Fig. 1a-b). However, other pro-fibrotic components, including *Col 1A2*, *Col IIIA1*, and *TGFβ3*, did not increase significantly (data not shown).

E2 activated ERK1/2 after E2 stimulation in primary human dermal fibroblasts

E2 induces MAPK cell signaling proteins in organ-specific fibrosis, namely uterine leiomyomas (26). Therefore, we investigated if E2-treated primary human dermal fibroblasts induce ERK1/2 phosphorylation, signifying MAPK activation. After one hour, E2 stimulation led to a 3.7-fold greater induction in phosphorylated/activated ERK1/2 than total ERK1/2 ($p < 0.05$) (Fig. 2a-b). We did not observe significant increases in phosphorylated ERK1/2 at earlier time points (data not shown). Other MAPK family members, including p-38 and JNK, were examined and were not phosphorylated above vehicle-treated cells (data not shown).

MAPK/ERK inhibition reduced E2-induced TGFβ1 and TGFβ2 transcription and translation

We showed that E2 induced *TGFβ1* and *TGFβ2* transcription and ERK1/2 activation *in vitro*. Next, we tested the effects of blocking upstream, MAPK/ERK activation on *TGFβ1* and *TGFβ2* transcription in primary human dermal fibroblasts, using the inhibitor U0126 (24). Pre-treatment with the inhibitor one hour before E2 stimulation prevented induction of *TGFβ1* and *TGFβ2* transcription after 24 hours and TGFβ1 translation after 48 hours, compared to vehicle (Fig. 2c-e).

EGR1 regulated E2-induced TGFβ1 and TGFβ2 transcription

Several transcription factor binding sites are located in the promoter region of the TGFβ isoforms (27, 28), including EGR1 (29). EGR1 is a known transcription factor that mediates fibrosis (30–32). Because E2 is known to activate transcription factors (33), we investigated whether E2 induces EGR1 in primary human dermal fibroblasts. EGR1 transcription and translation doubled 6 and 16 hours after E2 stimulation, respectively (Fig. 3a-c). We then silenced *EGR1* to investigate whether EGR1 regulates E2-induced *TGFβ1* and *TGFβ2* transcription in primary human dermal fibroblasts. Transfection of dermal fibroblasts with *EGR1*-targeted siRNA significantly diminished differences between *EGR1* and *TGFβ2* transcription levels in E2-stimulated cells at 24 hours, with a trend for *TGFβ1* transcription (Fig. 3d-f) .

E2 increased transcription of ECM components and pro-fibrotic mediators ex vivo

Even though both *in vitro* and *in vivo* evidence suggests that E2 induces *FN ex vivo* (19), its effects on the steady-state mRNA levels of other ECM products are unknown. We measured steady-state mRNA levels of known genes implicated in fibrosis in an *ex vivo* human skin organ culture model 24, 48 and 72 hours after E2 stimulation. At 24 hours post E2-stimulation, we found *TGFβ2* transcript increased in E2-stimulated vs. vehicle-treated cells (Fig. 4a). Col22A1 is an early, TGFβ-responsive gene that contributes to the ECM (34). We discovered E2-induced *Col22A1* transcription 24 hours post-stimulation (Fig. 4b). Interestingly, similar increases were also observed for steady-state transcript mRNA levels of *TGFβ1* levels 48 hours post-E2 stimulation (Fig. 4c), while *Col IA2, Col IIIA1*, and *FN* transcription occurred at 72 hours (Fig. 4d-f) post-stimulation. There was not a statistically significant induction in steady-state transcript levels of *Col IA1, CTGF* or *α-SMA* (data not shown).

Inhibiting TGFβ1 signaling decreased E2-induced Col22A1 transcription ex vivo

E2 induced *TGFβ1* and *TGFβ2* transcription earlier than other ECM components, suggesting that the ECM is induced through TGFβ1 and TGFβ2. To investigate whether TGFβ signaling mediates the increase in *Col IA2, Col IIIA1, FN* and *Col22A1* transcripts post-E2 stimulation, we pre-treated human *ex vivo* skin samples with SB-431542, a type 1 TGFβ receptor inhibitor (25), before stimulating with E2 for an additional 24, 48 or 72 hours. SB-431542 reduced steady-state mRNA transcript levels of *Col22A1* despite E2 treatment (Fig. 5), but not *Col IA2, Col IIIA1*, and *FN* transcription to below those of the E2-treated skin samples (data not shown), suggesting that E2 induction of *Col22A1* is via TGFβ.

Discussion

Both E2 and TGFβ1 are central to ECM production in the skin; however, little is known about the cellular mechanisms underlying E2-induced dermal fibrosis or the transcriptional and translational regulation of TGFβ. We are the first to report that, in the skin, E2 induces TGFβ1 and TGFβ2, and they are regulated through the MAPK pathway and EGR1. TGFβ1 and TGFβ2, in turn, induce Col22A1. Based on these observations, we propose a cellular mechanism to describe aspects of E2-induced dermal fibrosis.

Col22A1 has been identified in patients with SSc and is known to contribute to fibrosis. Whole exome sequencing of patients with diffuse cutaneous SSc showed an enrichment of genes in the ECM pathway, including Col22A1 (35). Additionally, whole exome sequencing of African American patients with SSc identified Col22A1 as a rare variant that may increase African American susceptibility to SSc (36). To characterize the regulation of Col22A1, our group reported that primary human dermal fibroblasts from patients with SSc and healthy controls release Col22A1 in direct response to TGFβ1 stimulation. We also showed that Col22A1 mediates the transition of fibroblasts to myofibroblasts (34). These data indicate that Col22A1 is detected in patients with SSc and likely promotes fibrosis. Our current study extends the understanding of Col22A1 induction by demonstrating that it can occur in direct response to a pro-fibrotic mediator that induces TGFβ, namely E2. Thus, E2 contributes to dermal fibrosis through inducing Col22A1.

We show that both *TGFβ1* and *TGFβ2* are induced by E2, as they are in a wound-healing model (22, 23), and both likely contribute to dermal fibrosis. Even though the *TGFβ1* and *TGFβ2* isoforms are encoded by different genes, both use the same signaling receptors and cascades (37–39) and likely contribute to E2-induced *Col22A1* transcription.

This report also extends the number of E2-induced ECM proteins and mediators known to impact dermal fibrosis to include *Col IA2*, *Col IIIA1*, *Col22A1*, *TGFβ1* and *TGFβ2* in our *ex vivo* human skin organ culture model. Yet, *Col IA2* and *Col IIIA1* steady-state transcript levels were not increased in primary dermal fibroblast single-cell culture *in vitro*. The discrepancy between *ex vivo* and *in vitro* results may be explained by the inclusion of other cell types in e*x vivo* skin tissue, such as keratinocytes, which are responsive to E2 and can contribute to ECM formation (40–42).

While primary human dermal fibroblasts produce less *Col IA1*, *Col IA2, FN and Col22A1* when pre-treated with the TGFβ receptor inhibitor SB-431542 before stimulation with TGFβ1 (34, 43), E2-induced *Col IA2, Col IIIA1* and *FN* transcription was not prevented by pre-treatment with the inhibitor. This is likely because E2 can induce other pro-fibrotic mediators to influence ECM production. Our unpublished findings suggest that E2 increases the transcript and protein levels of the pro-fibrotic cytokine IL-6 (unpubished data), which can then increase collagen and FN levels through trans-signaling (44). IL-6 also activates STAT3, which promotes *Col IA2* synthesis post-transcriptionally (45). Thus, E2-induced fibrosis likely occurs through both the non-canonical TGFβ-ERK and IL-6-STAT3 signaling pathways.

We investigated whether the MAPK pathway, which is central to E2-induced Col22A1 signaling, regulates TGFβ1 and TGFβ2. We report that E2-induced ERK1/2 phosphorylation in primary human dermal fibroblasts is vital to TGFβ1 and TGFβ2 expression, since blockade of the MAPK/ERK pathway was inhibitory. In apoptotic cells, TGFβ transcriptional and translational regulation also occurs through the MAPK pathway, in addition to the RhoA and PI-3K/AKT pathways (46). Specifically, TGFβ1 synthesis is induced by activation of CD36 on macrophages in response to apoptotic cells (47). Further studies are needed to elucidate the role of CD36 in E2-induced TGFβ1 and TGFβ2 transcription and translation in primary human dermal fibroblasts.

Transcription factors are crucial to comprehend E2-induced TGFβ1 and TGFβ2 transcription. EGR1 is a candidate transcription factor in this pathway because it has been reported downstream of TGFβ1 signaling in human dermal fibroblasts (30), and the EGR1-TGFβ1 relationship is important in a murine model of pulmonary fibrosis (48). We report that EGR1 is also upstream of E2-induced *TGFβ1* transcription in primary human dermal fibroblasts, because EGR1 binds to and activates the TGFβ1 promoter (29). As confirmation, exogenous expression of EGR1 in the cancer cell line HT1080 led to increased secretion of TGFβ1 (49). Additionally, EGR1 increased E2-induced *TGFβ2* transcription in primary dermal fibroblasts (50, 51).

Conclusions

Dermal fibrosis is a key feature of some pro-fibrotic diseases, such as SSc, making it critical that we understand its underlying mechanism. Here, we suggest a cell signaling mechanism for E2-induced dermal fibrosis and TGFβ regulation. We found that E2-induced TGFβ1 and TGFβ2 directly increased Col22A1 and contributed to ECM accumulation. Currently, several FDA-approved medications exist that inhibt E2 signaling and production. However, these medications have been limited to use in hormonal cancers. Our data suggest that therapies that inhibit E2 signaling may reduce dermal fibrosis and present an interesting treatment alternative in pro-fibrotic diseases.

Abbreviations

estradiol (E2), ethanol (ETOH), systemic sclerosis (SSc), transforming growth factor β1 (TGFβ1), transforming growth factor β2 (TGFβ2), quantitative PCR (qPCR), collagen IA2 (Col IA2), collagen IIIA1 (Col IIIA1), collagen 22A1 (Col22A1), extracellular matrix (ECM), extracellular signal-regulated kinase (ERK), mitogen-activated protein kinase (MAPK), early growth response 1 (EGR1)

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

Not applicable

Competing interests

DBF received an honorarium from Atheneum Partners.

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Authors'contribution

DBF contributed to data collection, organization, analysis, and interpretation, figure preparation, and manuscript writing. AS assisted in data collection, bakerdeorganization, analysis, interpretation, figure preparation and manuscript preparation. AO contributed to study design and manuscript preparation. MA contributed to study design and manuscript preparation. CFB contributed to study design, data organization, analysis and interpretation and manuscript preparation. All authors read and approved the manuscript.

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Figures

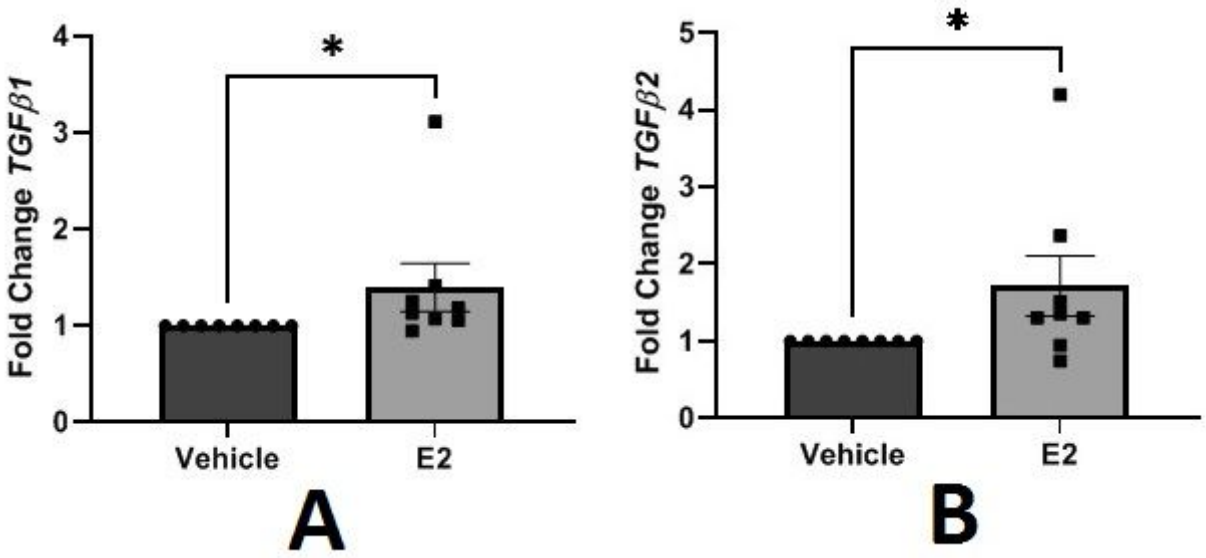

Figure 1

a-b: Steady-state mRNA levels 24 hours post-vehicle vs. E2-stimulation in vitro. Steady state mRNA levels of TGF 1 (a), TGF 2 (b). Normalized to B2M. Data shown is from 8 independent experiments using dermal fibroblasts from 8 different donors. Bars = mean +/- SEM, Stastical test: Two-tailed, Wilcoxon matched-pairs signed rank test.*p ≤ 0.05.

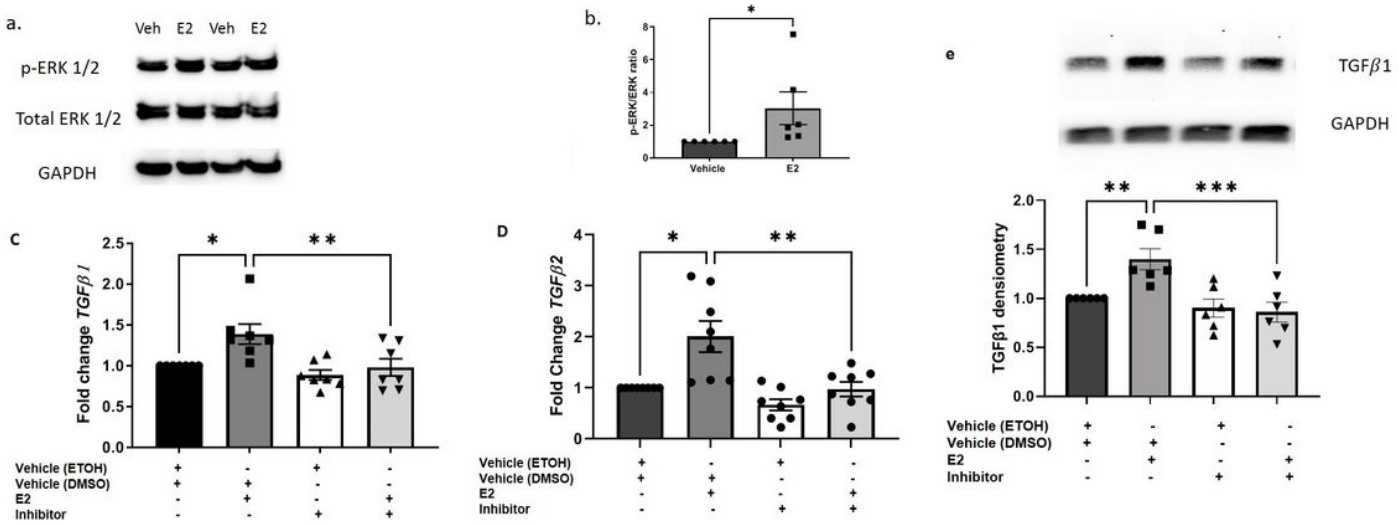

Figure 2

a-e: a. Immunoblot of extracellular signal-regulated kinase 1/2 (ERK1/2) activation 1-hour post E2 stimulation in vitro. b. Densitometry of p-ERK1/2:total ERK ratio. c and d: Steady-state transcript levels of TGF 1 (c) and TGF 2 (d) in primary human dermal fibroblasts during a 1-hour pretreatment with the mitogen-activated protein kinase (MAPK)/ERK inhibitor U0126 and 24 hours post E2-stimulation. Immunoblot of TGF 1 (e) in primary human dermal fibroblast lysates during a 1-hour pre-treatment with U0126 48 hours post E2 stimulation. ethanol = ETOH, estradiol = E2, Glyceraldehyde 3-phosphate dehydrogenase = GAPDH, Dimethyl Sulfoxide = DMSO. Normalized to GAPDH (b and e) or B2M (c-d). Bars = mean +/- SEM. Data shown is from 6 independent experiments using dermal fibroblasts from 6 different donors. Statistical analysis: Two-tailed, Wilcoxon matched-pairs signed rank test (b) and one-way ANOVA with Sidak's multiple comparisons post-hoc test (c-e). *p ≤0.05, ** p ≤0.01, ** p ≤0.005.

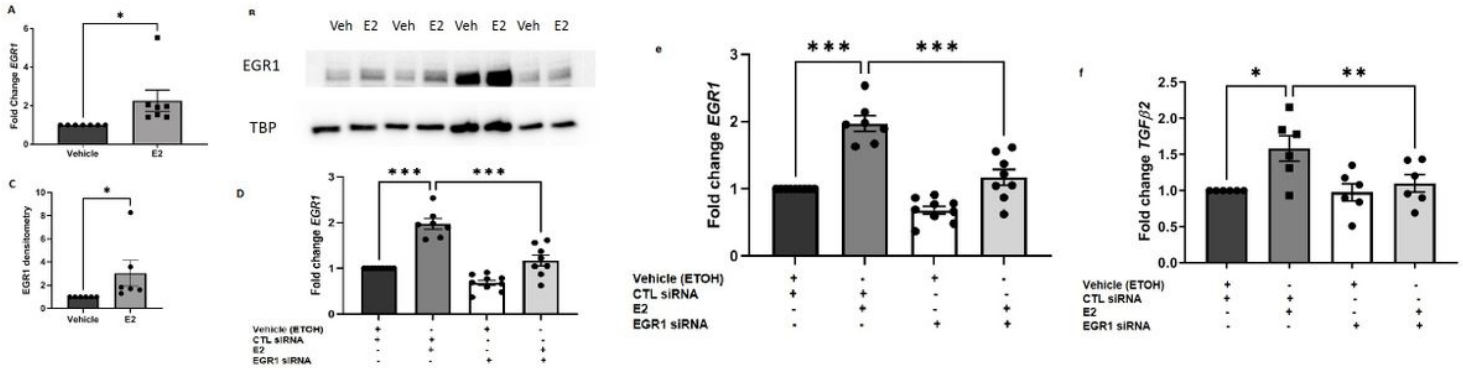

Figure 3

a. Steady-state transcript levels of Early Growth Response 1 (EGR1) 6 hours post vehicle vs. E2-stimulation in vitro. b. Immunoblot of EGR1 protein 16 hours post-E2 stimulation. c. Densitometry of EGR1 immunoblot. d-f. Steady-state transcript levels of EGR1 (d) TGF 1 (e) and TGF 2 (f) in primary human dermal fibroblasts transfected with siRNA against EGR1 24 hours post E2 stimulation. Control = CTL. Normalized to B2M (a, d-f) or Tata-binding protein (TBP) (c). Bars = mean +/- SEM. Data shown is

from ≥ 6 independent experiments using dermal fibroblasts from ≥ 6 different donors. Statistical tests: Wilcoxon matched-pairs signed rank test (a, c) and parametric, one-way ANOVA with Sidak's multiple comparisons post-hoc test (d, f), non-parametric one-way ANOVA using Dunn's multiple comparisons (e). *p ≤ 0.05, ** p ≤ 0.01, ** p ≤0.005.

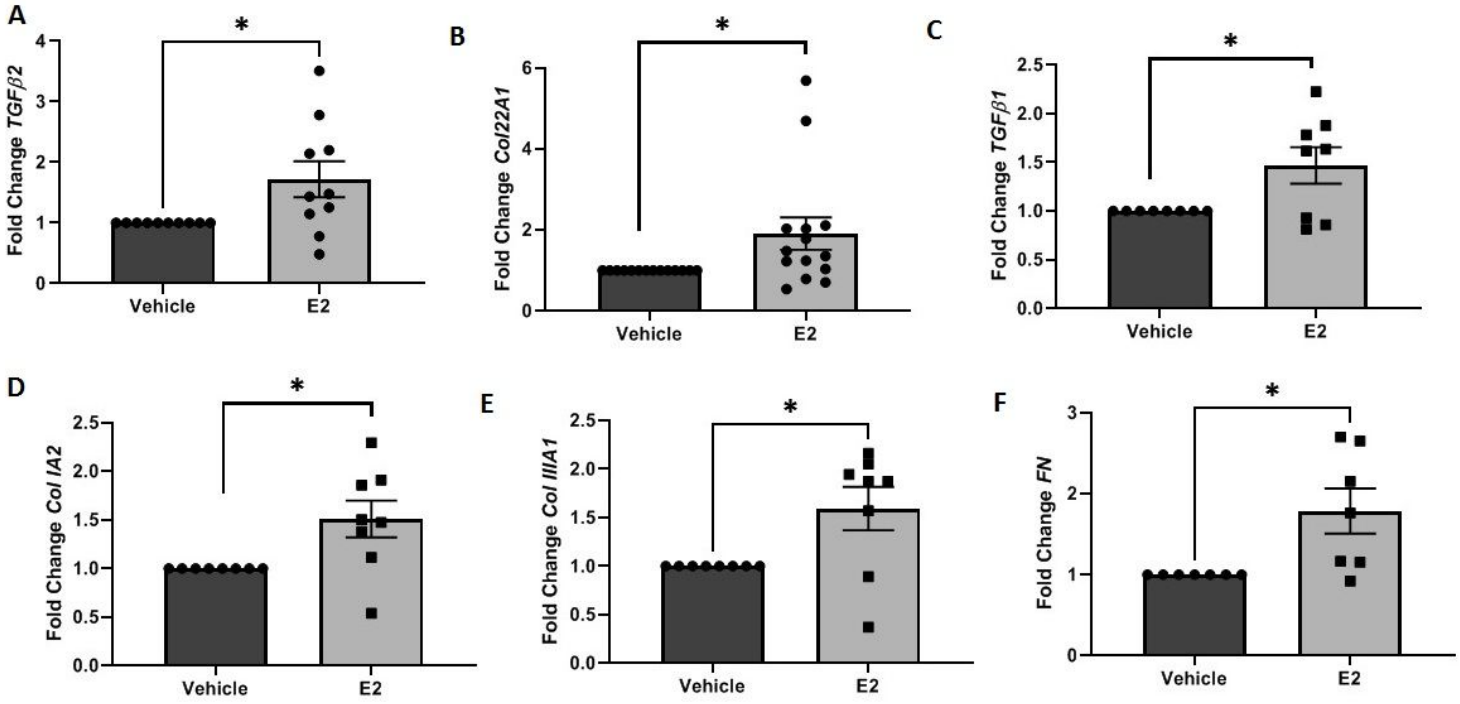

Figure 4

Steady-state mRNA levels of TGF□2 (a), Col22A1 (b), TGF□1 (c), Col I IA2 (d), Col IIIA1 (e), and FN (f) at 24 (a-b) 48 (c) or 72 (d-f) hours post-vehicle vs. E2-stimulation ex vivo. Bars = mean +/- SEM. Normalized to B2M (a-e) and GAPDH (f). data shown is from □ 8 independent experiments using dermal fibroblasts from □ 8 different donors. Stastical test: Two-tailed, parametric, paired t-Test (a-d, f), Wilcoxon matched-pairs signed rank test (e). *p ≤ 0.05.

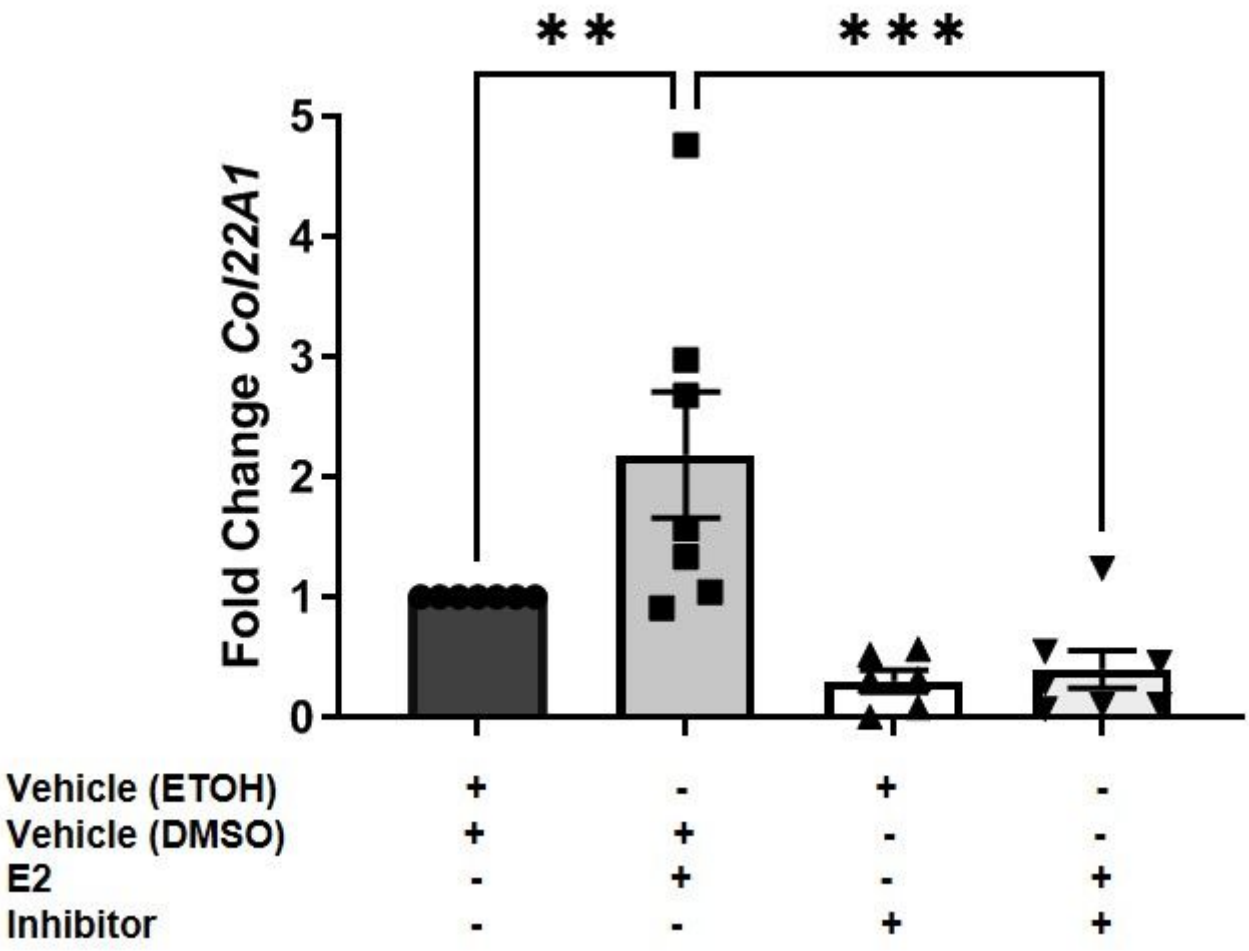

Figure 5

Steady-state transcript levels of Col22A1 during a 1-hour pre-treatment with the TGF type I inhibitor, SB-431542, 48 hours post-E2 stimulation, ex vivo. ethanol = ETOH, estradiol = E2, Dimethyl Sulfoxide = DMSO, Normalized to B2M, Bars = mean +/- SEM. Data shown is from 6 independent experiments using dermal fibroblasts from 6 different donors. Stastical test: parametric one-way ANOVA with Sidak's multiple comparisons post-hoc test. **p≤ 0.01, *** p≤ 0.005.