

Elucidating the Cellular Mechanism for E2-induced Dermal Fibrosis

DeAnna Baker Frost (✉ bakerde@musc.edu)

Medical University of South Carolina <https://orcid.org/0000-0002-9224-1391>

Alisa Savchenko

Medical University of South Carolina Department of Medicine

Adeyemi Ogunleye

University of North Carolina at Chapel Hill

Milton Armstrong

Medical University of South Carolina Department of Surgery

Carol Feghali-Bostwick

Medical University of South Carolina Department of Medicine, Division of Rheumatology

Research article

Keywords: Estrogen, Fibrosis, Dermal fibroblasts, Skin, TGF β

Posted Date: February 3rd, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-103581/v2>

License:  This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Version of Record: A version of this preprint was published on February 27th, 2021. See the published version at <https://doi.org/10.1186/s13075-021-02441-x>.

Abstract

Background: Both TGF β and estradiol (E2), a form of estrogen, are pro-fibrotic in the skin. In the connective tissue disease, systemic sclerosis (SSc), both TGF β and E2 are likely pathogenic. Yet, the regulation of TGF β 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 TGF β and investigated the extracellular matrix (ECM) components that are directly stimulated by E2-induced TGF β signaling.

Methods: We used primary human dermal fibroblasts *in vitro* and human skin tissue *ex vivo* stimulated with E2 or vehicle (ethanol) to measure *TGF β 1*, *TGF β 2* levels using quantitative PCR (qPCR). To identify the necessary cell signaling proteins in E2-induced *TGF β 1* and *TGF β 2* 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 TGF β receptor inhibitor, and ICI 182,780, an estrogen receptor α (ER α) inhibitor, to establish the effects of TGF β and ER α signaling on E2-induced collagen 22A1 (*Col22A1*) transcription.

Results: We found that expression of *TGF β 1*, *TGF β 2*, and *Col22A1*, a TGF β -responsive gene, are induced in response to E2 stimulation. Mechanistically, *Col22A1* induction was blocked by SB-431542 and ICI 182,780 despite E2 stimulation. Additionally, inhibiting E2-induced ERK/MAPK activation and early growth response 1 (*EGR1*) transcription prevents the E2-induced increase in *TGF β 1* and *TGF β 2* transcription and translation.

Conclusions: We conclude that E2-induced dermal fibrosis occurs in part through induction of *TGF β 1, 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 (TGF β) 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 TGF β as an inducer of fibrosis (1), but few studies explore the regulation of TGF β . Because TGF β is a major 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 TGF β isoforms to understand SSc disease pathogenesis and develop potential therapeutics.

Like TGF β , 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 TGF β , 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 TGF β 1 in a model of wound healing (22, 23), while the cell signaling molecules used by E2 to induce TGF β 1 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 demographic information of the donors is summarized in table 1. The *ex-vivo* human skin organ culture model was used as previously described (19, 24, 25). 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°C until further evaluation.

Cell Culture

Primary 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 mM of a mitogen-activated protein kinase/ extracellular signal-regulated kinase (MAPK/ERK) inhibitor, U0126 (26), estrogen receptor alpha (ER α) inhibitor fulvestrant (ICI 182,780) or TGF β 1 receptor inhibitor, SB-431542 (27), one hour before stimulation with E2 or ethanol (ETOH) as vehicle. U0126, SB-431542 and E2 were obtained from MilliporeSigma (St. Louis, MO), ICI 182,780 from Tocris (Minneapolis, MN), 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 *GAPDH*. 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 of four siRNAs) 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

Demographics

Table 1 includes the demographic information of healthy subjects who donated skin tissue used in the *ex vivo* human skin organ culture model experiments. The majority of the donors (63%) were women, with the donated tissue isolated from the abdomen (74%).

Table 1: Demographics of human skin donors

ID	Age	Sex	Anatomic Location
10	25	F	Breast
30	33	F	Abdomen
33	35	F	Abdomen
216	34	F	Abdomen
217	46	F	Arm
218	52	F	Abdomen
220	36	F	Buttocks
225	43	F	Abdomen
227	51	F	Abdomen
230	37	unknown	Abdomen
231	Unknown	unknown	Abdomen
237	38	unknown	Abdomen
268	36	M	Abdomen
275	35	M	Abdomen
277	66	M	Abdomen
278	36	F	Back
279	39	F	Abdomen
287	51	F	Abdomen
323	unknown	unknown	unknown

Table 1: Demographics of the human skin donors used in the *ex vivo* human skin organ culture model *ex vivo* experiments.

E2 increased transcription of pro-fibrotic mediators *in vitro*

Pro-fibrotic mediators and ECM components were measured in primary human dermal fibroblasts post-E2 stimulation. *TGFb1* and *TGFb2* 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).

TGFb1 and *TGFb2* transcript levels showed an increasing trend 48 hours post- E2 stimulation (supplementary figure 1, additional file 1). However, *TGFb3*, did not increase significantly at the same time point (additional file 2).

E2 activated ERK1/2 in primary human dermal fibroblasts

E2 induces MAPK cell signaling in uterine leiomyomas (28). 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 compared to total ERK1/2 ($p < 0.05$) (Fig 2a-b). We did not observe significant increases in phosphorylated ERK1/2 at earlier time points (additional file 3). Other MAPK family members, including p-38 and JNK, were examined and showed no evidence of activation (additional file 4).

MAPK/ERK inhibition reduced E2-induced *TGFb1* and *TGFb2* transcription and translation

We showed that E2 induced *TGFb1* and *TGFb2* transcription and ERK1/2 activation *in vitro*. Next, we tested the effects of blocking upstream, ERK activation on *TGFb1* and *TGFb2* transcription in primary human dermal fibroblasts, using the inhibitor U0126 (26). We first confirmed that U0126 inhibited E2-induced ERK activation (Fig 2c). Pre-treatment with the inhibitor one hour before E2 stimulation prevented induction of *TGFb1* and *TGFb2* transcription after 24 hours and *TGFb1* translation after 48 hours, compared to E2 and vehicle (Fig 2d-f).

EGR1 regulated E2-induced *TGFb1* and *TGFb2* transcription

Several transcription factor binding sites are located in the promoter region of the *TGFb* isoforms (29, 30), including *EGR1* (31). *EGR1* is a known transcription factor that mediates fibrosis (32-34). Because E2 is known to activate transcription factors (35), we investigated whether E2 induces *EGR1* in primary human dermal fibroblasts. While *EGR1* transcript levels were not significantly increased 30 minutes post-E2 stimulation (supplementary figure 2, additional file 5), its transcription and translation were significantly increased 6 and 16 hours after E2 stimulation, respectively (Fig 3a-c). *EGR1* transcript levels remained elevated 8 hours post-E2 stimulation (supplementary figure 2, additional file 5). We then silenced *EGR1* to investigate whether *EGR1* regulates E2-induced *TGFb1* and *TGFb2* transcription in primary human dermal fibroblasts. Transfection of dermal fibroblasts with *EGR1*-targeted siRNA significantly diminished levels of *EGR1* and *TGFb2* in E2-stimulated cells, with a trend for *TGFb1* reduction (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 protein levels *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, *TGFb2* transcript levels increased in E2-stimulated vs. vehicle-treated skin (Fig 4a), and the effect subsided 48 hours post-E2 stimulation

(additional file 6). *Col22A1* is an early, TGF β -responsive gene that contributes to the ECM (36). E2 also induced *Col22A1* transcription at 48 hours post-stimulation (Fig 4b), not at 72 hours (additional file 6). Interestingly, similar increases were observed for steady-state mRNA levels of *TGF β 1* levels, albeit at 48 hours post-E2 stimulation (Fig 4c), while *Col IA2*, *Col IIIA1*, and *FN* transcription was significantly increased at 72 hours (Fig 4d-f) post-stimulation, but not at earlier timepoints of 24 or 48 hours (additional file 6). There was not a statistically significant induction in steady-state transcript levels of *Col IA1*, *CTGF* or *α -SMA* (additional file 7).

Inhibiting TGF β 1 signaling decreased E2-induced *Col22A1* transcription *ex vivo*

E2 induced *TGF β 1* and *TGF β 2* transcription earlier than other ECM components, suggesting that the ECM is induced through TGF β 1 and TGF β 2. To investigate whether TGF β signaling mediates the increase in *Col IA2*, *Col IIIA1*, *FN* and *Col22A1* post-E2 stimulation, we pre-treated human *ex vivo* skin samples with SB-431542, a type 1 TGF β receptor inhibitor (27), 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 suggesting that E2 induction of *Col22A1* is via TGF β .

Inhibiting E2 signaling through estrogen receptor alpha inhibited *Col22A1* transcription *ex vivo*

We demonstrated that E2-induced FN production and dermal thickness *ex vivo* are inhibited by fulvestrant (ICI 182,780), which inhibits estrogen signaling through one of its major receptors, estrogen receptor alpha (ER α) (19). We tested if E2 inhibition through ER α blockade would prevent the E2-induced increase in *Col22A1* transcription *ex vivo*. After 1 hour pre-treatment of human tissue with the ER α inhibitor, ICI 182,780, then subsequent ETOH and E2 treatment, E2-induced *Col22A1* transcription was significantly decreased (Fig 6).

Discussion

Both E2 and TGF β 1 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 TGF β . We are the first to report that, in the skin, E2 induces TGF β 1 and TGF β 2 through ER α signaling, MAPK activation and EGR1 inductions. TGF β 1 and TGF β 2, in turn, induce *Col22A1*. Based on these observations, we propose a cellular mechanism that mediates 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* (37). Additionally, whole exome sequencing of African American patients with SSc identified *Col22A1* as a rare variant that may increase African American susceptibility to SSc (38). 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 TGF β 1 stimulation. We also showed that *Col22A1* mediates the transition of fibroblasts to myofibroblasts (36). These data indicate

that Col22A1 is detected in patients with SSc and likely contributes to 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 TGFb, namely E2. Thus, E2 contributes to dermal fibrosis through inducing Col22A1.

We show that both *TGFb1* and *TGFb2* are induced by E2, as they are in a wound-healing model (22, 23), and both likely contribute to dermal fibrosis. Even though the *TGFb1* and *TGFb2* isoforms are encoded by different genes, both use the same signaling receptors and cascades (39-41) 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*, *TGFb1* and *TGFb2* 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 *ex vivo* skin tissue, such as keratinocytes, which are responsive to E2 and can contribute to ECM formation (42-44).

While primary human dermal fibroblasts produce less *Col IA1*, *Col IA2*, *FN* and *Col22A1* when pre-treated with the TGFb receptor inhibitor SB-431542 before stimulation with TGFb1 (36, 45), 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 (unpublished data), which can then increase collagen and FN levels through trans-signaling (46). IL-6 also activates STAT3, which promotes *Col IA2* synthesis post-transcriptionally (47). Thus, E2-induced fibrosis likely occurs through both the non-canonical TGFb-ERK and IL-6-STAT3 signaling pathways.

We investigated whether the MAPK pathway, which is central to E2-induced Col22A1 signaling, regulates TGFb1 and TGFb2. We report that E2-induced ERK1/2 phosphorylation in primary human dermal fibroblasts is vital to TGFb1 and TGFb2 expression, since blockade of the MAPK/ERK pathway was inhibitory. In apoptotic cells, TGFb transcriptional and translational regulation also occurs through the MAPK pathway, in addition to the RhoA and PI-3K/AKT pathways (48). Specifically, TGFb1 synthesis is induced by activation of CD36 on macrophages in response to apoptotic cells (49). Further studies are needed to elucidate the role of CD36 in E2-induced TGFb1 and TGFb2 transcription and translation in primary human dermal fibroblasts.

Transcription factors are crucial to comprehend E2-induced TGFb1 and TGFb2 transcription. EGR1 is a candidate transcription factor in this pathway because it has been reported downstream of TGFb1 signaling in human dermal fibroblasts (32), and the EGR1-TGFb1 relationship is important in a murine model of pulmonary fibrosis (50). We report that EGR1 is also upstream of E2-induced *TGFb1* transcription in primary human dermal fibroblasts, which is not surprising as EGR1 binds to and activates the TGFb1 promoter (31) and exogenous expression of *EGR1* in the cancer cell line HT1080 led

to increased secretion of TGFb1 (51). Additionally, EGR1 increased E2-induced *TGFb2* transcription in primary dermal fibroblasts (52, 53).

ERa is one of the classical estrogen receptors involved in E2 signaling (54). Fulvestrant is FDA-approved for use in breast cancer and has been shown effective at ameliorating bleomycin-induced lung fibrosis (55). We extend those findings in this report to suggest that ERa signaling blockade can also prevent E2-induced *Col22A1* transcription *ex vivo*. These results, in conjunction with our previous findings showing that circulating E2 levels are elevated in patients with SSc (20) suggest that fulvestrant is a promising therapeutic option in patients with SSc.

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 TGFb regulation. We found that E2-induced TGFb1 and TGFb2 directly increased *Col22A1* and contributed to ECM accumulation. Currently, several FDA-approved medications exist that inhibit 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), dimethyl sulfoxide (DMSO), systemic sclerosis (SSc), transforming growth factor b1 (TGFb1), transforming growth factor b2 (TGFb2), 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.

Funding

K24AR06029, UL1TR001450, KL2TR001452, T32AR050958, the Doris Duke Program to Retain Clinician Scientists, and the SmartState and Kitty Trask Holt Endowment. All mechanisms of funding enabled data collection, analysis, and interpretation.

Authors' contribution

DBF contributed to data collection, organization, analysis, and interpretation, figure preparation, and manuscript writing. AS assisted in data collection, organization, 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.

ACKNOWLEDGEMENTS

We would like to thank the donors who provided skin samples used in the experiments reported in this manuscript

References

1. Leask A, Abraham DJ. TGF-beta signaling and the fibrotic response. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*. 2004;18(7):816-27.
2. Lichtman MK, Otero-Vinas M, Falanga V. Transforming growth factor beta (TGF- β) isoforms in wound healing and fibrosis. *Wound repair and regeneration : official publication of the Wound Healing Society [and] the European Tissue Repair Society*. 2016;24(2):215-22.
3. Lafyatis R. Transforming growth factor beta—at the centre of systemic sclerosis. *Nature reviews Rheumatology*. 2014;10(12):706-19.
4. Gruschwitz M, Muller PU, Sepp N, Hofer E, Fontana A, Wick G. Transcription and expression of transforming growth factor type beta in the skin of progressive systemic sclerosis: a mediator of fibrosis? *The Journal of investigative dermatology*. 1990;94(2):197-203.
5. Falanga V, Gerhardt CO, Dasch JR, Takehara K, Ksander GA. Skin distribution and differential expression of transforming growth factor beta 1 and beta 2. *Journal of dermatological science*. 1992;3(3):131-6.
6. Gabrielli A, Di Loreto C, Taborro R, Candela M, Sambo P, Nitti C, et al. Immunohistochemical localization of intracellular and extracellular associated TGF beta in the skin of patients with systemic sclerosis (scleroderma) and primary Raynaud's phenomenon. *Clinical immunology and immunopathology*. 1993;68(3):340-9.

7. Denton CP, Merkel PA, Furst DE, Khanna D, Emery P, Hsu VM, et al. Recombinant human anti-transforming growth factor beta1 antibody therapy in systemic sclerosis: a multicenter, randomized, placebo-controlled phase I/II trial of CAT-192. *Arthritis and rheumatism*. 2007;56(1):323-33.
8. Peoples C, Medsger TA, Jr., Lucas M, Rosario BL, Feghali-Bostwick CA. Gender differences in systemic sclerosis: relationship to clinical features, serologic status and outcomes. *Journal of scleroderma and related disorders*. 2016;1(2):177-240.
9. Gharaee-Kermani M, Hatano K, Nozaki Y, Phan SH. Gender-based differences in bleomycin-induced pulmonary fibrosis. *The American journal of pathology*. 2005;166(6):1593-606.
10. Altinok AY, Yildirim S, Altug T, Sut N, Ober A, Ozsahin EM, et al. Aromatase inhibitors decrease radiation-induced lung fibrosis: Results of an experimental study. *Breast (Edinburgh, Scotland)*. 2016;28:174-7.
11. Chen X, Austin ED, Talati M, Fessel JP, Farber-Eger EH, Brittain EL, et al. Oestrogen inhibition reverses pulmonary arterial hypertension and associated metabolic defects. *The European respiratory journal*. 2017;50(2).
12. Rettberg JR, Yao J, Brinton RD. Estrogen: a master regulator of bioenergetic systems in the brain and body. *Front Neuroendocrinol*. 2014;35(1):8-30.
13. Meschia M. BF, Amicarelli F., Barbacini P., Monza G.C., Crosignani P.G. Transdermal hormone replacement therapy and skin in postmenopausal women, a placebo controlled study. *Menopause*. 1994;1:79-82.
14. Brincat M, Moniz CJ, Studd JW, Darby A, Magos A, Emburey G, et al. Long-term effects of the menopause and sex hormones on skin thickness. *British journal of obstetrics and gynaecology*. 1985;92(3):256-9.
15. Hall G, Phillips TJ. Estrogen and skin: the effects of estrogen, menopause, and hormone replacement therapy on the skin. *Journal of the American Academy of Dermatology*. 2005;53(4):555-68; quiz 69-72.
16. Kalogirou D, Aroni K, Kalogirou O, Antoniou G, Botsis D, Kontoravdis A. Histological changes induced by tibolone and estrogen/glucocorticoid on aging skin. *International journal of fertility and women's medicine*. 2000;45(4):273-8.
17. Castelo-Branco C, Duran M, Gonzalez-Merlo J. Skin collagen changes related to age and hormone replacement therapy. *Maturitas*. 1992;15(2):113-9.
18. Maheux R, Naud F, Rioux M, Grenier R, Lemay A, Guy J, et al. A randomized, double-blind, placebo-controlled study on the effect of conjugated estrogens on skin thickness. *American journal of obstetrics and gynecology*. 1994;170(2):642-9.
19. Aida-Yasuoka K, Peoples C, Yasuoka H, Hershberger P, Thiel K, Cauley JA, et al. Estradiol promotes the development of a fibrotic phenotype and is increased in the serum of patients with systemic sclerosis. *Arthritis research & therapy*. 2013;15(1):R10.
20. Baker Frost D, Wolf B, Peoples C, Fike J, Silver K, Laffoon M, et al. Estradiol levels are elevated in older men with diffuse cutaneous SSc and are associated with decreased survival. *Arthritis research*

- & therapy. 2019;21(1):85.
21. Harlow CR, Bradshaw AC, Rae MT, Shearer KD, Hillier SG. Oestrogen formation and connective tissue growth factor expression in rat granulosa cells. *The Journal of endocrinology*. 2007;192(1):41-52.
 22. Stevenson S, Nelson LD, Sharpe DT, Thornton MJ. 17beta-estradiol regulates the secretion of TGF-beta by cultured human dermal fibroblasts. *Journal of biomaterials science Polymer edition*. 2008;19(8):1097-109.
 23. Ashcroft GS, Dodsworth J, van Boxtel E, Tarnuzzer RW, Horan MA, Schultz GS, et al. Estrogen accelerates cutaneous wound healing associated with an increase in TGF-beta1 levels. *Nat Med*. 1997;3(11):1209-15.
 24. Yamaguchi Y, Takihara T, Chambers RA, Veraldi KL, Larregina AT, Feghali-Bostwick CA. A peptide derived from endostatin ameliorates organ fibrosis. *Science translational medicine*. 2012;4(136):136ra71.
 25. Yasuoka H, Larregina AT, Yamaguchi Y, Feghali-Bostwick CA. Human skin culture as an ex vivo model for assessing the fibrotic effects of insulin-like growth factor binding proteins. *The open rheumatology journal*. 2008;2:17-22.
 26. Favata MF, Horiuchi KY, Manos EJ, Daulerio AJ, Stradley DA, Feeser WS, et al. Identification of a novel inhibitor of mitogen-activated protein kinase kinase. *The Journal of biological chemistry*. 1998;273(29):18623-32.
 27. Inman GJ, Nicolas FJ, Callahan JF, Harling JD, Gaster LM, Reith AD, et al. SB-431542 is a potent and specific inhibitor of transforming growth factor-beta superfamily type I activin receptor-like kinase (ALK) receptors ALK4, ALK5, and ALK7. *Molecular pharmacology*. 2002;62(1):65-74.
 28. Yu L, Moore AB, Castro L, Gao X, Huynh HL, Klippel M, et al. Estrogen Regulates MAPK-Related Genes through Genomic and Nongenomic Interactions between IGF-I Receptor Tyrosine Kinase and Estrogen Receptor-Alpha Signaling Pathways in Human Uterine Leiomyoma Cells. *J Signal Transduct*. 2012;2012:204236.
 29. Birchenall-Roberts MC, Ruscetti FW, Kasper J, Lee HD, Friedman R, Geiser A, et al. Transcriptional regulation of the transforming growth factor beta 1 promoter by v-src gene products is mediated through the AP-1 complex. *Molecular and cellular biology*. 1990;10(9):4978-83.
 30. Geiser AG, Busam KJ, Kim SJ, Lafyatis R, O'Reilly MA, Webbink R, et al. Regulation of the transforming growth factor-beta 1 and -beta 3 promoters by transcription factor Sp1. *Gene*. 1993;129(2):223-8.
 31. Liu C, Calogero A, Ragona G, Adamson E, Mercola D. EGR-1, the reluctant suppression factor: EGR-1 is known to function in the regulation of growth, differentiation, and also has significant tumor suppressor activity and a mechanism involving the induction of TGF-beta1 is postulated to account for this suppressor activity. *Critical reviews in oncogenesis*. 1996;7(1-2):101-25.
 32. Bhattacharyya S, Chen SJ, Wu M, Warner-Blankenship M, Ning H, Lakos G, et al. Smad-independent transforming growth factor-beta regulation of early growth response-1 and sustained expression in fibrosis: implications for scleroderma. *The American journal of pathology*. 2008;173(4):1085-99.

33. Bhattacharyya S, Wu M, Fang F, Tourtellotte W, Feghali-Bostwick C, Varga J. Early growth response transcription factors: key mediators of fibrosis and novel targets for anti-fibrotic therapy. *Matrix Biol.* 2011;30(4):235-42.
34. Yasuoka H, Hsu E, Ruiz XD, Steinman RA, Choi AM, Feghali-Bostwick CA. The fibrotic phenotype induced by IGFBP-5 is regulated by MAPK activation and egr-1-dependent and -independent mechanisms. *The American journal of pathology.* 2009;175(2):605-15.
35. Marino M, Galluzzo P, Ascenzi P. Estrogen signaling multiple pathways to impact gene transcription. *Current genomics.* 2006;7(8):497-508.
36. Watanabe T, Baker Frost DA, Mlakar L, Heywood J, da Silveira WA, Hardiman G, et al. A Human Skin Model Recapitulates Systemic Sclerosis Dermal Fibrosis and Identifies COL22A1 as a TGFbeta Early Response Gene that Mediates Fibroblast to Myofibroblast Transition. *Genes.* 2019;10(2).
37. Mak AC, Tang PL, Cleveland C, Smith MH, Kari Connolly M, Katsumoto TR, et al. Brief Report: Whole-Exome Sequencing for Identification of Potential Causal Variants for Diffuse Cutaneous Systemic Sclerosis. *Arthritis & rheumatology (Hoboken, NJ).* 2016;68(9):2257-62.
38. Gourh P, Remmers EF, Boyden SE, Alexander T, Morgan ND, Shah AA, et al. Brief Report: Whole-Exome Sequencing to Identify Rare Variants and Gene Networks That Increase Susceptibility to Scleroderma in African Americans. *Arthritis & rheumatology (Hoboken, NJ).* 2018;70(10):1654-60.
39. Chang H, Brown CW, Matzuk MM. Genetic analysis of the mammalian transforming growth factor-beta superfamily. *Endocrine reviews.* 2002;23(6):787-823.
40. Attisano L, Carcamo J, Ventura F, Weis FM, Massague J, Wrana JL. Identification of human activin and TGF beta type I receptors that form heteromeric kinase complexes with type II receptors. *Cell.* 1993;75(4):671-80.
41. ten Dijke P, Yamashita H, Ichijo H, Franzen P, Laiho M, Miyazono K, et al. Characterization of type I receptors for transforming growth factor-beta and activin. *Science (New York, NY).* 1994;264(5155):101-4.
42. Nikitorowicz-Buniak J, Shiwen X, Denton CP, Abraham D, Stratton R. Abnormally differentiating keratinocytes in the epidermis of systemic sclerosis patients show enhanced secretion of CCN2 and S100A9. *The Journal of investigative dermatology.* 2014;134(11):2693-702.
43. Perzelova V, Sabol F, Vasilenko T, Novotny M, Kovac I, Slezak M, et al. Pharmacological activation of estrogen receptors-alpha and -beta differentially modulates keratinocyte differentiation with functional impact on wound healing. *International journal of molecular medicine.* 2016;37(1):21-8.
44. McCoy SS, Reed TJ, Berthier CC, Tsou PS, Liu J, Gudjonsson JE, et al. Scleroderma keratinocytes promote fibroblast activation independent of transforming growth factor beta. *Rheumatology (Oxford, England).* 2017;56(11):1970-81.
45. Mori Y, Chen SJ, Varga J. Expression and regulation of intracellular SMAD signaling in scleroderma skin fibroblasts. *Arthritis and rheumatism.* 2003;48(7):1964-78.
46. Le TT, Karmouty-Quintana H, Melicoff E, Le TT, Weng T, Chen NY, et al. Blockade of IL-6 Trans signaling attenuates pulmonary fibrosis. *Journal of immunology (Baltimore, Md : 1950).*

- 2014;193(7):3755-68.
47. Papaioannou I, Xu S, Denton CP, Abraham DJ, Ponticos M. STAT3 controls COL1A2 enhancer activation cooperatively with JunB, regulates type I collagen synthesis posttranscriptionally, and is essential for lung myofibroblast differentiation. *Molecular biology of the cell*. 2018;29(2):84-95.
 48. Xiao YQ, Freire-de-Lima CG, Schiemann WP, Bratton DL, Vandivier RW, Henson PM. Transcriptional and translational regulation of TGF-beta production in response to apoptotic cells. *Journal of immunology (Baltimore, Md : 1950)*. 2008;181(5):3575-85.
 49. Xiong W, Frasch SC, Thomas SM, Bratton DL, Henson PM. Induction of TGF-beta1 synthesis by macrophages in response to apoptotic cells requires activation of the scavenger receptor CD36. *PLoS one*. 2013;8(8):e72772.
 50. Chen CC, Lee WR, Safe S. Egr-1 is activated by 17beta-estradiol in MCF-7 cells by mitogen-activated protein kinase-dependent phosphorylation of ELK-1. *Journal of cellular biochemistry*. 2004;93(5):1063-74.
 51. Liu C, Adamson E, Mercola D. Transcription factor EGR-1 suppresses the growth and transformation of human HT-1080 fibrosarcoma cells by induction of transforming growth factor beta 1. *Proceedings of the National Academy of Sciences of the United States of America*. 1996;93(21):11831-6.
 52. Gaut L, Robert N, Delalande A, Bonnin MA, Pichon C, Duprez D. EGR1 Regulates Transcription Downstream of Mechanical Signals during Tendon Formation and Healing. *PLoS one*. 2016;11(11):e0166237.
 53. Guerquin MJ, Charvet B, Nourissat G, Havis E, Ronsin O, Bonnin MA, et al. Transcription factor EGR1 directs tendon differentiation and promotes tendon repair. *The Journal of clinical investigation*. 2013;123(8):3564-76.
 54. Yasar P, Ayaz G, User SD, Gupur G, Muyan M. Molecular mechanism of estrogen-estrogen receptor signaling. *Reproductive medicine and biology*. 2017;16(1):4-20.
 55. Elliot S, Periera-Simon S, Xia X, Catanuto P, Rubio G, Shahzeidi S, et al. MicroRNA let-7 Downregulates Ligand-Independent Estrogen Receptor-mediated Male-Predominant Pulmonary Fibrosis. *American journal of respiratory and critical care medicine*. 2019;200(10):1246-57.

Figures

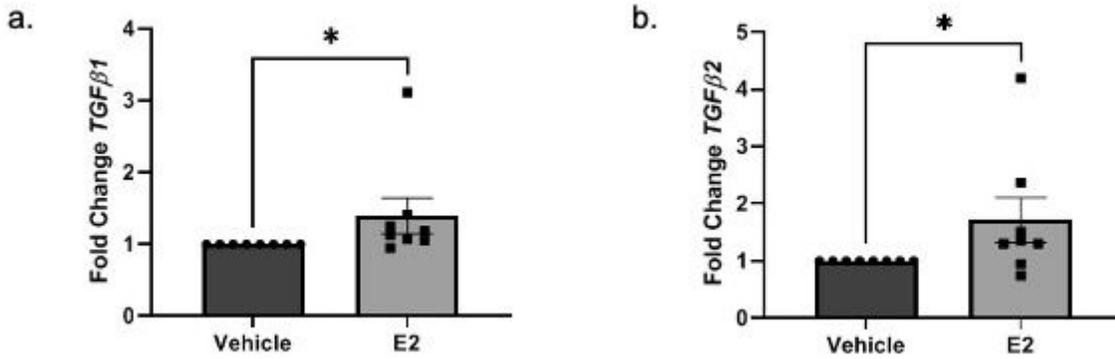


Figure 1

E2-induced TGFβ1 and TGFβ2 transcription in vitro. Steady-state mRNA levels 24 hours post-vehicle vs. E2-stimulation in vitro. Steady state mRNA levels of TGFβ1 (a), TGFβ2 (b). Normalized to B2M. Data shown is from 8 independent experiments using dermal fibroblasts from 8 different donors. Bars = mean +/- SEM. Statistical test: Two-tailed, Wilcoxon matched-pairs signed rank test. * $p \leq 0.05$.

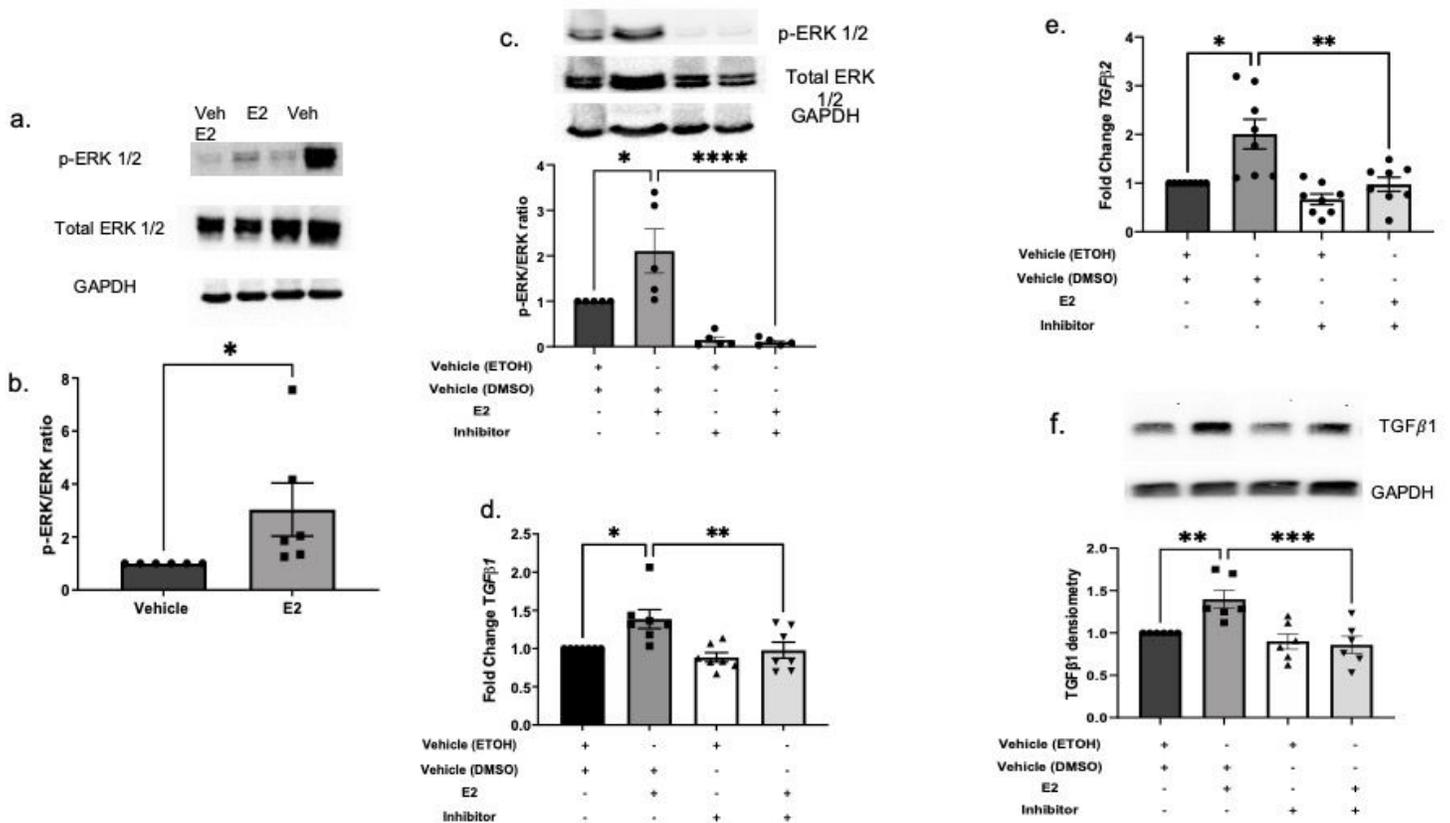


Figure 2

E2-induced activation of pERK and its effects on TGFβ1 and TGFβ2 transcription and translation in vitro. a. Representative 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 ERK1/2 ratio. c. Representative immunoblot and densitometry of p-ERK1/2:total ERK ratio after 1 hour pre-treatment with the mitogen-activated protein kinase (MAPK)/ERK inhibitor, U0126, and subsequent 1 hour of E2-stimulation. d and e:

Steady-state transcript levels of TGFβ1 (d) and TGFβ2 (e) in primary human dermal fibroblasts following a 1-hour pre-treatment with U0126 and subsequent 24 hours post E2-stimulation. Representative immunoblot of TGFβ1 (f) in primary human dermal fibroblast lysates during a 1-hour pre-treatment with U0126 and subsequent 48 hours post E2 stimulation. ethanol = ETOH, estradiol = E2, Glyceraldehyde 3-phosphate dehydrogenase = GAPDH, Dimethyl Sulfoxide = DMSO. Normalized to GAPDH (b, c and f) or B2M (d and e). 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, **** p ≤0.0001.

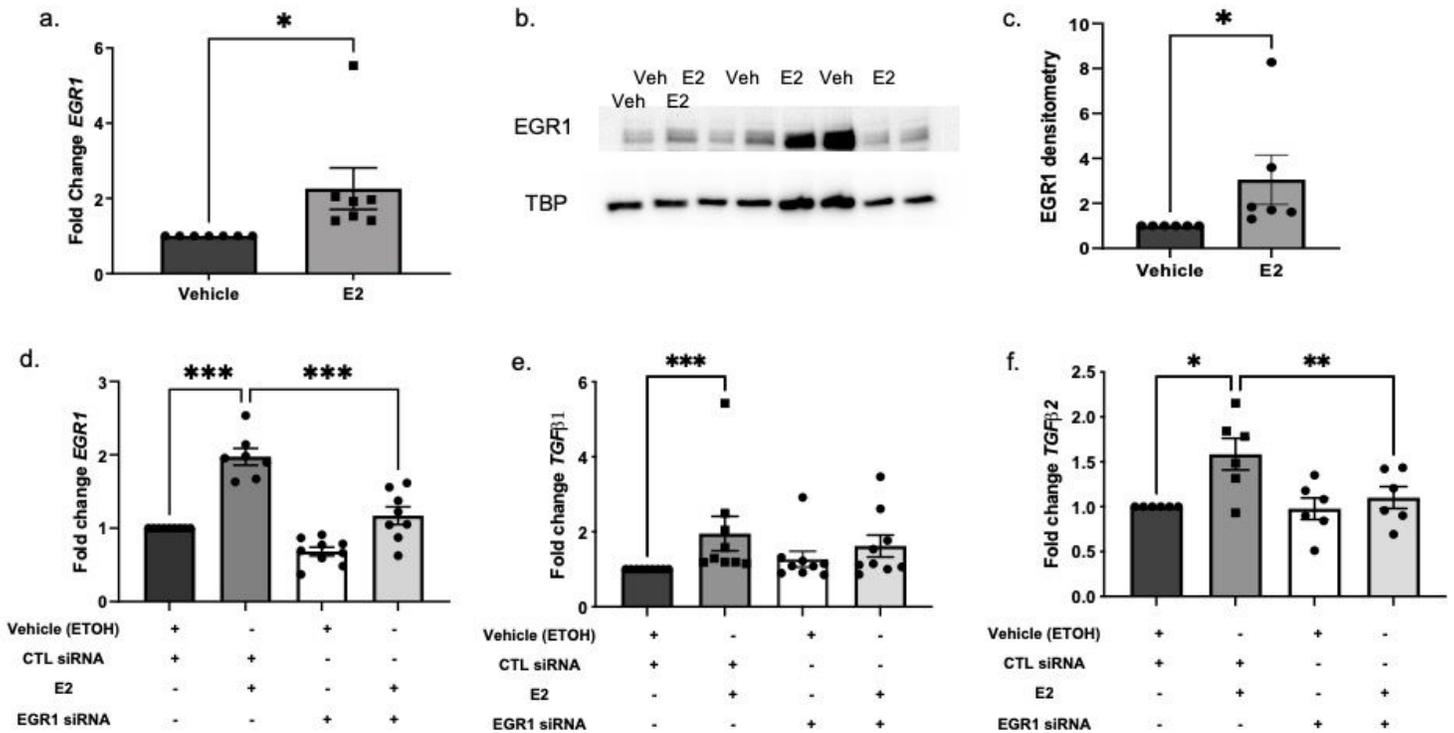


Figure 3

E2-induced EGR1 transcription and translation and its effects on TGFβ1 and TGFβ2 transcription in vitro. a. Steady-state transcript levels of EGR1 6 hours post vehicle vs. E2-stimulation in vitro. b. Representative immunoblot of EGR1 protein 16 hours post-E2 stimulation. c. Densitometry of EGR1 immunoblot. d-f. Steady-state transcript levels of EGR1 (d) TGFβ1 (e) and TGFβ2 (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 from a-f includes ≥ 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.

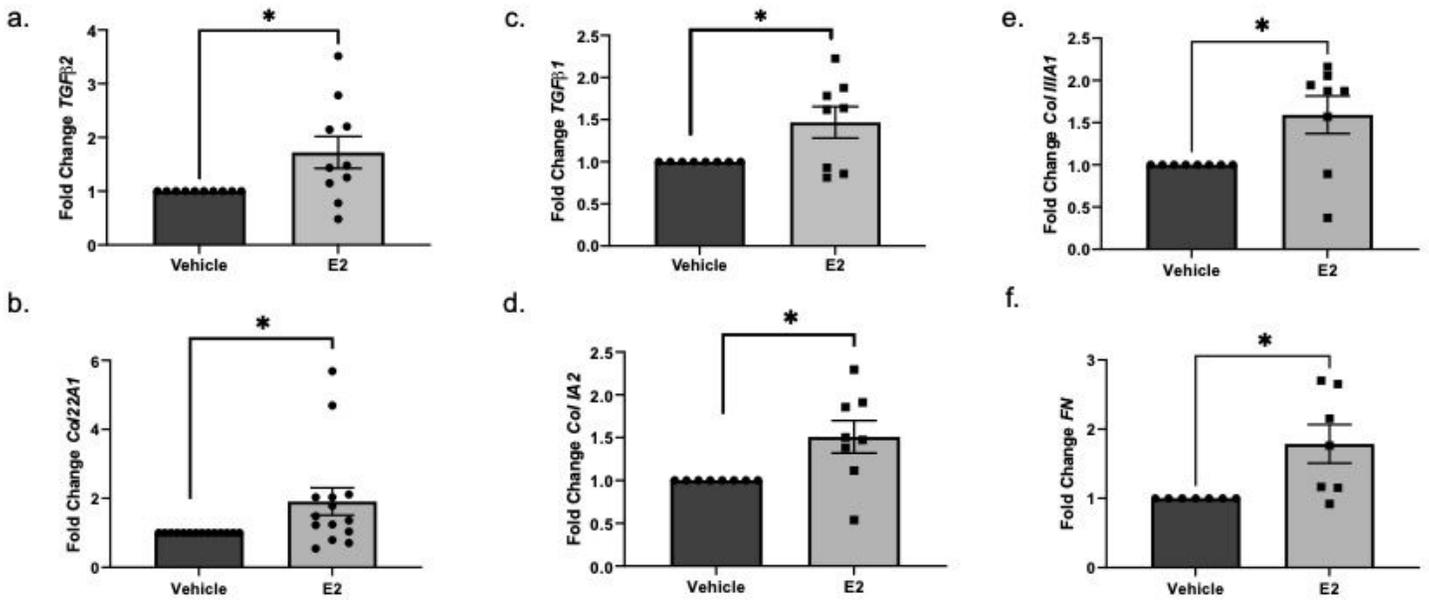


Figure 4

E2-induced transcription of ECM components and pro-fibrotic mediators ex vivo. Steady-state mRNA levels of TGFβ2 (a), Col22A1 (b), TGFβ1 (c), Col I A2 (d), Col IIIA1 (e), and FN (f) at 24 (a) 48 (b-c) or 72 (d-f) hours post-vehicle vs. E2-stimulation of human skin ex vivo. Bars = mean +/- SEM. Normalized to B2M (a-e) and GAPDH (f). Data shown is from ≥ 8 independent experiments using human skin tissue from ≥ 8 different donors. Statistical test: Two-tailed, parametric, paired t-Test (a-d, f), Wilcoxon matched-pairs signed rank test (e). *p ≤ 0.05.

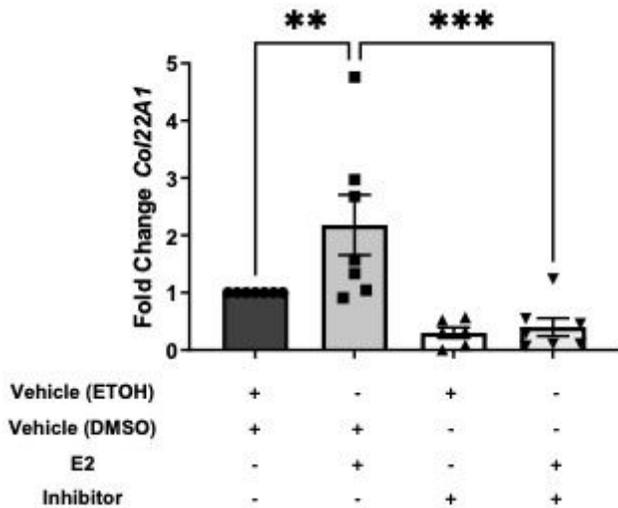


Figure 5

Col22A1 transcription post TGFβ1 signaling blockade ex vivo. Steady-state transcript levels of Col22A1 during a 1-hour pre-treatment with the TGFβ 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 human skin from ≥ 6 different donors.

Statistical test: parametric one-way ANOVA with Sidak's multiple comparisons post-hoc test. ** $p \leq 0.01$, *** $p \leq 0.005$.

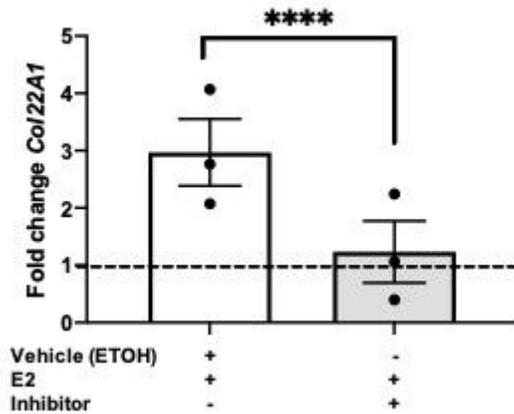


Figure 6

Col22A1 transcription post E2 signaling blockade ex vivo. Steady-state transcript levels of Col22A1 following a 1-hour pre-treatment with the ER α inhibitor, fulvestrant, 24 hours post-E2 stimulation of human skin ex vivo. Dotted line indicates normalization to baseline treatment with ETOH alone. ethanol = ETOH, estradiol = E2. Normalized to B2M, Bars = mean \pm SEM. Data shown is from human skin tissue of one donor performed in triplicate independent skin punches. Statistical test: Two-tailed, parametric, paired t-Test. **** $p \leq 0.001$.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [additonalfig1.jpg](#)
- [additionalfig2.jpg](#)
- [additionalfig3.jpg](#)
- [additionalfile4.jpg](#)
- [additionalfigure5.jpg](#)
- [additionalfig6.jpg](#)
- [additionalfig7.jpg](#)