PRS
GLOBAL OPEN

ORIGINAL ARTICLE

Research

Low Baseline Expression of Fibrotic Genes in an Ex Vivo Human Skin Model is a Potential Indicator of Excessive Skin Scarring

Joe E. Mouawad, BS*†
Jonathan Heywood, BS‡
Milton B. Armstrong, MD§
Adeyemi Ogunleye, MD¶
Carol Feghali-Bostwick, PhD†

Background: One of the challenges plastic surgeons face is the unpredictability of postoperative scarring. The variability of wound healing and subsequent scar formation across patients makes it virtually impossible to predict if a patient's surgery will result in excessive fibrosis and scarring, possibly amounting to keloids or hypertrophic scars. There is a need to find predictive molecular indicators of patients or skin location with high risk of excessive scarring. We hypothesized that baseline expression levels of fibrotic genes in the skin can serve as a potential indicator of excessive scarring.

Methods: An ex vivo model of skin fibrosis was used with abdominal and breast skin tissue from 45 patients undergoing breast reduction and/or abdominoplasty. Fibrosis was induced in skin explants in organ culture with transforming growth factor-β (TFGβ). Fibrotic gene response was assessed via quantitative real-time polymerase chain reaction and correlated with skin location, age, and baseline levels of fibrotic genes.

Results: The increase in TFGβ-induced fibronectin1 *(FN1)* gene expression in skin explants was significantly higher than for Collagen 1A1, alpha smooth muscle actin, and connective tissue growth factor. Also, *FN1* expression positively correlated with donor age. Moreover, lower expression of the fibrotic genes *FN1*, Collagen 1A1, and alpha smooth muscle actin correlated with a more pronounced fibrotic response, represented by higher induction levels of these genes.

Conclusions: Skin sites exhibit different baseline levels of profibrotic genes. Further, low baseline expression levels of fibrotic genes FN1, Collagen 1A1, and alpha smooth muscle actin, in donor skin may indicate a potential for excessive scarring of the skin. *(Plast Reconstr Surg Glob Open 2022;10:e4626; doi: 10.1097/GOX.0000000000004626; Published online 15 November 2022.)*

INTRODUCTION

When skin is injured, it restores itself through the process of wound healing, reestablishing the integrity of the different layers.[1] Yet, the outcome is not identical to uninjured skin. Cutaneous wound healing is a complex phenomenon that follows a coordinated sequence of processes, involving multiple cell types and signaling pathways.[2] It starts with an inflammatory phase marked by vasodilation, elimination of foreign bodies, and cell migration. The following proliferative phase consists of deposition of extracellular matrix (ECM) by resident dermal fibroblasts. Finally, in the maturation phase, the ECM is organized into a scar, the normal end-result of wound healing, restoring most of the skin's tensile strength.[3–5] Minor and superficial wounds tend to heal with little to no visible trace. However, scarring, or fibrosis of the skin, often results from the excessive deposition of ECM following tissue injury or in pathologic conditions such as

From the *Medical Scientist Training Program, Medical University of South Carolina, Charleston, S.C.; †Department of Medicine, Division of Rheumatology, Medical University of South Carolina; Charleston, S.C.; ‡College of Engineering, Computing and Applied Sciences, Clemson University; Clemson, S.C.; §Department of Surgery, Division of Plastic and Reconstructive Surgery, Medical University of South Carolina; Charleston, S.C.; and ¶Department of Surgery, Division of Plastic and Reconstructive Surgery, University of North Carolina; Chapel Hill, N.C.

Disclosure: *The authors have no financial interest to declare in relation to the content of this article.*

Related Digital Media are available in the full-text version of the article on www.PRSGlobalOpen.com.

keloids and hypertrophic scars.[6,7] Scarring carries a lot of stigma in our society as it is seen to fall short of beauty standards portrayed by the media.[8] In addition, patients are reminded of their trauma through their visible scars, causing them to avoid social interactions and intimacy, which increases their propensity to develop clinical depression.[8,9]

The extent of scarring is variable across population race and age, but also across different skin locations in each patient.[10,11] This unpredictability poses a challenge for plastic surgeons as it often compromises the postoperative management of surgical scars, especially in cases of keloids and hypertrophic scars.[12] Even in fibrotic conditions like systemic sclerosis (SSc), the extent of skin fibrosis differs across races and skin location.[13,14] It is not well understood why the variability in scarring occurs, and, as a result, predicting the extent of a patient's scarring after a surgical incision is virtually impossible. There is a need to understand the cause behind the variability in scarring and find predictive factors or potential biomarkers to identify patients or even skin locations with high risk for excessive scarring. The ability to better predict the risk of excessive scarring will allow surgeons to tailor preoperative patient selection, technique selection, and patient counseling to reduce such risk. The discovery of important biomarkers or mediators will also be expected to guide development of drug and non-drug therapies to reduce such risk.

The common denominator in scar formation, whether from surgical excision or a disorder, is fibrosis. Fibrosis results from the excessive secretion and accumulation of connective tissue components forming the ECM.[15] To close a wound, fibroblasts in the dermis are activated into myofibroblasts and secrete ECM proteins to help fill the wound gap.[16] These ECM proteins, like collagens and fibronectin (FN), make up much of a scar's content, and their excessive deposition and non-reticular alignment accentuate a scar's visibility on the skin surface.[17,18] In fact, while studies have indicated that many genes are differentially expressed in keloids and hypertrophic scars, FN and type I collagen (Collagen 1α1) have consistently been shown to be overproduced in these scar tissues.[19,20] Moreover, activated myofibroblasts express α-smooth muscle actin (*ACTA2*) that aids wound closure via its contractile property, but in scar tissue, *ACTA2* is overexpressed in myofibroblasts resulting in over-deposition of scar-forming ECM.[21,22] The secretome of activated myofibroblasts also plays a role in scar formation, especially connective tissue growth factor (*CTGF*), which has been shown to be intrinsically upregulated in hypertrophic scar fibroblasts. *CTGF* promotes fibrosis directly[23] and via TGFβ-induced transcription.[24] Thus, we focused on FN, collagen 1α1, *ACTA2*, and *CTGF* in our study as we hypothesized that their expression pattern could serve as an indicator for excessive scarring potential.

We optimized a model of skin in organ culture to assess the efficacy and relevance of profibrotic and antifibrotic factors in a human tissue.[25–30] Our findings demonstrate that human tissue responds to these factors while in culture and is thus a viable model of direct relevance to human disease. We used this model to investigate the extent of *FN1*, *COL1A1*, *ACTA2*, and *CTGF* gene response

Takeaways

Question: Are there molecular markers that can potentially indicate propensity for excessive skin scarring?

Findings: Baseline expressions of fibrotic genes in skin tissue, such as Collagen 1A1, Fibronectin1, and alpha smooth muscle actin, are inversely correlated to their induced expression levels in response to a fibrotic stimulus.

Meaning: Low baseline expression of fibrotic genes may indicate a potential risk for excessive scarring of the skin, allowing surgeons to tailor preoperative patient selection, technique choice, and patient counseling to minimize such risk.

in skin obtained from two different locations (abdomen and breast) from patients undergoing skin resection surgeries. To this purpose, we used an ex vivo model of fibrosis to assess different patterns and correlations in fibrotic gene response in relation to skin location, age, as well as baseline gene expression of these fibrotic proteins. Because increased expression of these genes is a proxy for excessive scarring, a significant correlation between the continuous variables would suggest a causal relationship. Such correlations could guide surgeons in preoperative patient selection and counseling as well as technique choice to minimize risk of excessive scarring. We thus hypothesized that baseline gene expression levels may correlate with the response to TGFβ, and this may occur in an age-dependent manner.

METHODS

Ex Vivo Human Skin Organ Culture and Stimulation

Human skin was obtained from residual breast or abdominal tissue, due to availability of tissue during plastic surgery, from 45 donors who underwent breast reduction and/or abdominoplasty (Table 1). All tissues were obtained without identifiers according to the Medical University of South Carolina institutional review board guidelines under protocol #00063011 and following informed consent. Consecutive patients meeting inclusion and exclusion criteria were recruited over the time frame of the study (single institution study). Inclusion criteria included age above 18 years, undergoing skin resection procedures, and ability to give informed consent. Exclusion criteria included no history of fibrosing condition such as systemic sclerosis, or chronic steroid

Table 1. Demographic Characteristics of Donors

	Donors (n = 45)
Age (y)	39.71 ± 12.59
Range	17–64
Sex	
Masculine	1
Feminine	44
Surgery*	
Bilateral breast reduction	29
Abdominoplasty	29

Age expressed as mean ± SD.
*Thirteen patients had both surgeries.

or immunosuppressive medication, and inability to give informed consent. Laboratory analysis was performed with blinding until study completion. Skin was processed and maintained in organ culture as we previously described.[25,26,31,32] Briefly, immediately following procurement, subcutaneous fat tissue was removed. The skin was then cut into equal-sized explants with a 3-mm disposable biopsy punch. Skin areas with evidence of stretch marks were avoided. The explants were cultured in an air–liquid interface in Dulbecco's Modified Eagle's Medium (DMEM) (Mediatech, Herndon, Va.) supplemented with penicillin, streptomycin, and antimycotic agent (Invitrogen, Carlsbad, Calif.). Skin explants in organ culture were treated with TGFβ (10 ng/ml) (R&D Systems, Minneapolis, Minn.) or a vehicle control (0.1% BSA, 4 mM HCL) for 48 hours (four replicates per treatment group), after which explants were snap frozen. Skin explants harvested at 120 hours were snap frozen or fixed in 10% formalin and then embedded in paraffin for sectioning and staining. Frozen sections were stored at –80°C.

Measurement of Skin Dermal Density

Five-micrometer sections of paraffin-embedded skin tissues were stained through AML Labs (St. Augustine, Fla.) with Hematoxylin and eosin (H&E) (StatLab, McKinney, Tex.) following manufacturer's protocol. Images were taken on an Axio Observer Microscope (Carl Zeiss Microscopy GmbH, Oberkochen, Germany) using 10× magnification. Identical camera settings were used for all images. For each section, the density of the ECM bundles in the dermal layer was measured in a blinded manner by assessing the collective area of the ECM bundles via the thresholding method with the ImageJ software.[33] Briefly, pictures were converted to binary black and white, where H&E-stained ECM bundles in the layer beneath the epidermis were white against a black background. Then, the white area was measured as an indication of ECM bundle density as previously described.[34] Measurements from six random fields per section were averaged for each donor.

Quantitative Real-Time Polymerase Chain Reaction

Total RNA was extracted from the human skin samples using the TRIZOL Lysis Reagent (Life Technologies, Carlsbad, Calif.) and RNeasy kit (Qiagen Inc., Valencia, Calif.). Superscript IV (Invitrogen) was used for reverse transcription. Gene messenger RNA (mRNA) expression levels were then assessed using quantitative real-time polymerase chain reaction (RT-qPCR) on a TaqMan Gene Expression Assays Step One Plus (Life Technologies), following the manufacturer's protocol. TaqMan probes and premixed PCR primers for the human genes Collagen 1A1 (*COL1A1:* Hs00164004_m1), Fibronectin1 (*FN1:* Hs00365052_m1), actin alpha 2, smooth muscle (*ACTA2:* Hs00426835_g1), connective tissue growth factor *(CTGF: Hs01026927_g1)*, and Glyceraldehyde 3-phosphate dehydrogenase *(GAPDH:* Hs02758991_g1) were obtained from Life Technologies. Gene expression levels were normalized to GAPDH and compared using the $2^{-\Delta Ct}$ method. Fold change (FC) in ΔΔCt in response to TGFβ was used to assess magnitude of the fibrotic response.

Statistical Analysis

All continuous variables were expressed as the mean ± standard error of the mean (SEM). Recorded variables were skin location, donor age, baseline gene expression, and TGFβ-induced response. Statistical comparisons between two groups were performed using Student *t* test, and between three or more groups using one-way ANOVA. The Tukey correction was used to correct for multiple comparisons. Correlation studies were evaluated using Pearson's correlation coefficient. Outlier data points were identified using the ROUT method (Q = 1%) and were excluded from the analysis. All statistical analyses were done using GraphPad Prism 9. A *P* value of less than 0.05 was considered statistically significant.

RESULTS

TGFβ Successfully Established A Fibrotic Phenotype in Ex Vivo Skin Organ Culture

We treated skin explants with TGFβ or vehicle control in organ culture, as we previously described, to induce fibrosis[25] (Fig. 1). At 48 hours, TGFβ-treated explants showed significant induction of the major ECM genes associated with scarring, *COL1A1* (1.83±0.11-fold; *P* < 0.0001) (Fig. 2A) and *FN1* (8.8±1.20-fold; *P* < 0.0001) (Fig. 2B), as well as the profibrotic response gene, *CTGF* (1.36±0.09-fold; *P* < 0.001) (Fig. 2C), while the myofibroblast marker, *ACTA2*, which encodes alpha smooth muscle actin (αSMA), showed an increasing trend that did not achieve statistical significance (1.24±0.13-fold; *P* = 0.1263) (Fig. 2D). To further assess the skin explant response to TGFβ, we analyzed histology sections of explants harvested at 120 hours (Fig. 2E and 2F). Measurement of ECM bundles density using the threshold method[34] demonstrated a significant increase in ECM bundle density of the dermal layer in the TGFβ-treated explants as compared to the vehicle-treated explants (1.22±0.03-fold; *P* < 0.01) (Fig. 2G).

FN1 Shows Differences in Baseline Expression Based on Skin Location

We next compared vehicle control-treated skin tissues between the two different skin locations (the breast and abdomen) to measure baseline gene expression. Baseline expression levels of *FN1* were significantly higher in breast skin compared with abdominal skin (0.001809±0.0005815 dCt versus 0.0004604±0.0001657 dCt; *P* < 0.05) (Fig. 3A). In contrast, *COL1A1* baseline expression levels showed the opposite trend with higher expression levels, albeit not statistically significant, in abdominal skin compared with breast skin (0.008155±0.001274 dCt versus 0.0056±0.0007238 dCt; *P* = 0.0953) (Fig. 3B). *CTGF* and *ACTA2* had comparable baseline expression levels irrespective of skin site (data not shown).

FN1, *COL1A1*, and *ACTA2* Show Differences in Fibrotic Responses Based on Skin Location

To investigate the magnitude of fibrotic response in skin tissue and whether it differs based on skin location, we compared the FC in expression of *FN1*, *COL1A1*, *CTGF*, and *ACTA2* in TGFβ versus vehicle-treated tissues.
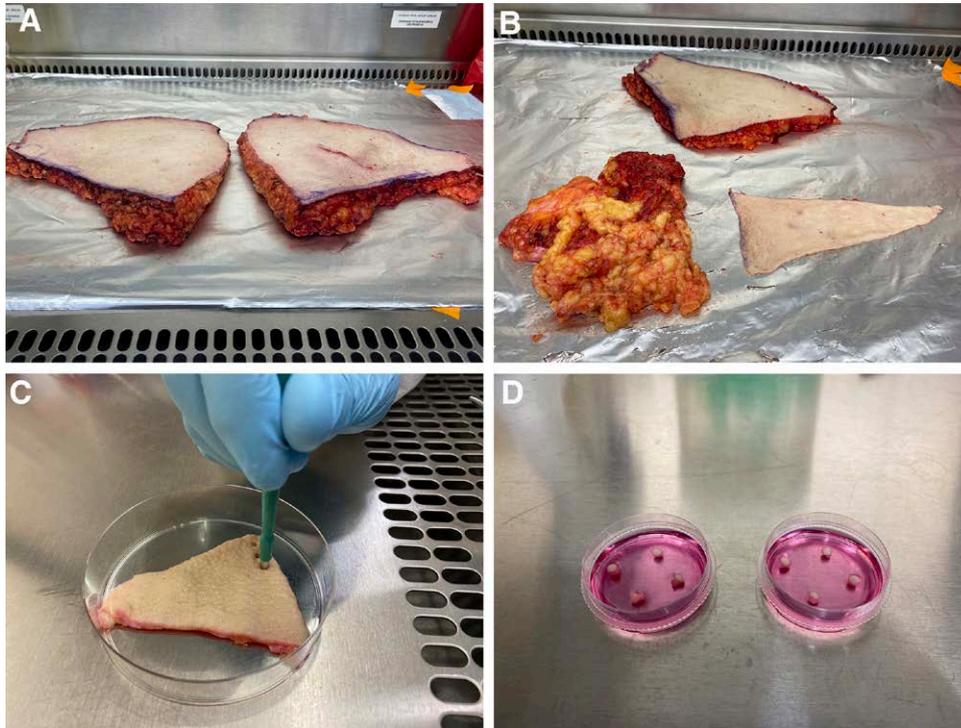
Fig. 1. Skin processing steps followed to set up organ culture. A, Skin was obtained from skin resection (abdominoplasty in image). B, Adipose tissue was removed to isolate the skin layer containing the epidermal and dermal layers. C, Equal-sized punches were taken from the skin layer using a 3-mm biopsy punch. D, Skin explants were placed in a 35-mm culture dish with cell culture media, forming an air–liquid interface, in which treatments with TGFβ or vehicle control were added.

In contrast to baseline expression data, the FC in *FN1* expression was significantly higher in abdominal skin compared with breast skin (13.66±3.098-fold versus 5.097±1.153-fold; *P* < 0.05) (Fig. 4A). Also, *COL1A1* showed a modest but significantly higher FC in expression in abdominal skin versus breast skin (1.988±0.2273-fold and 1.399±0.1553-fold; *P*< 0.05) (Fig. 4B). *ACTA2* showed an opposite trend with a lower, but not statistically significant, FC in expression in abdominal skin compared with breast skin (1.157±0.1830-fold versus 2.189±0.4952-fold, *p* = 0.066, data not shown). *CTGF* showed no difference in expression FC between skin from breast and abdominal areas (data not shown).

Magnitude of Fibrotic *FN1* Induction Is Directly Correlated with Age

We investigated a potential correlation between age (range: 17–64 years) and baseline or FC gene expression of *FN1, COL1A1, CTGF,* and *ACTA2* expression. We found no significant correlation between age and baseline expression of *FN1, COL1A1, CTGF,* or *ACTA2* (data not shown). However, when comparing TGFβ-induced FC with age, *FN1* expression showed a significant positive correlation with donor age (*r* = 0.4592, *P* < 0.01), which was not observed for *COL1A1, CTGF,* or *ACTA2* (**See figure 1, Supplemental Digital Content 1**, which shows correlation analysis between age and fibrotic response (FC in expression) of *FN1* (Above, left), *COL1A1* (Above, right), *ACTA2* (Below, left), and *CTGF* (Below, right), all normalized to

housekeeping gene *GAPDH*. Red font denotes significance (*P* < 0.05). **http://links.lww.com/PRSGO/C229**.)

Fibrotic Response Is Inversely Correlated to Baseline ECM Expression

The divergent data between *FN1* baseline and FC expression in each of breast and abdominal tissue prompted us to investigate a potential inverse correlation between baseline expression of ECM genes and the magnitude of their TGFβ-induced fibrotic response, in breast and abdominal skin. We analyzed collective baseline expression levels of *COL1A1, FN1, ACTA2,* and *CTGF* (*COL1A1*: 0.006279±0.0005495 dCt; *FN1*: 0.001153±0.000254 dCt; *ACTA2*: 0.009406±0.0008673 dCt; *CTGF*: 0.0005266±0.0000544 dCt) (Fig. 5A) and their TGFβ-induced FC in all the skin samples (*COL1A1*: 1.91±0.1419-fold; *FN1*: 9.155±1.317-fold; *ACTA2*: 1.280±0.1657-fold; *CTGF*: 1.373±0.08738-fold) (Fig. 5B). We found that while *FN1* expression at baseline was significantly lower than *COL1A1* (*P* < 0.0001) and *ACTA2* (*P* < 0.0001), the FC in *FN1* expression following TGFβ1 stimulation was significantly higher than that of *COL1A1* (*P* < 0.0001) or *ACTA2* (*P* < 0.0001), which supports the possibility of an inverse relationship. In contrast, *CTGF* expression levels were relatively low both at baseline and upon TGFβ1 stimulation. To this end, we performed correlation analysis and expectedly found a significant inverse correlation between baseline expression and TGFβ-induced fibrotic response of *FN1* (Fig. 6A),
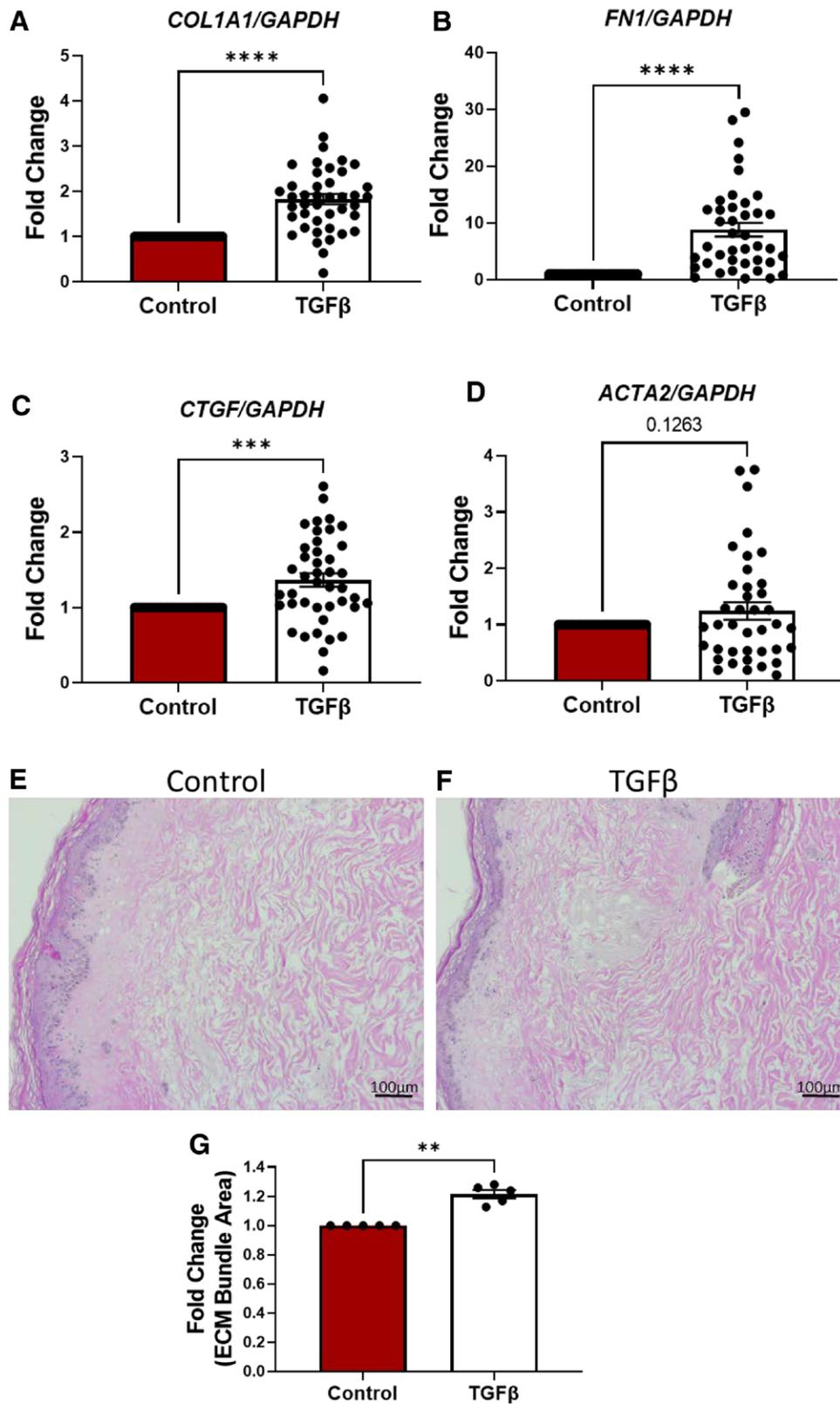
Fig. 2. TGFβ successfully established a fibrotic phenotype in ex vivo human skin in organ culture. RT-qPCR was used to measure TGFβ-induced FC in gene expression of *COL1A1* (A), *FN1* (B), *CTGF* (C), and *ACTA2* (D) at 48 h, compared with vehicle control (normalized to 1), all normalized to the housekeeping gene *GAPDH*. Microscopic images of H&E-stained sections of skin explants treated with vehicle control (E) or TGFβ (F) for 120 h, with graphical presentation of their ECM bundle density (G). Data are shown as individual values along with the mean ± SEM. Scale bar = 100 μm. **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.
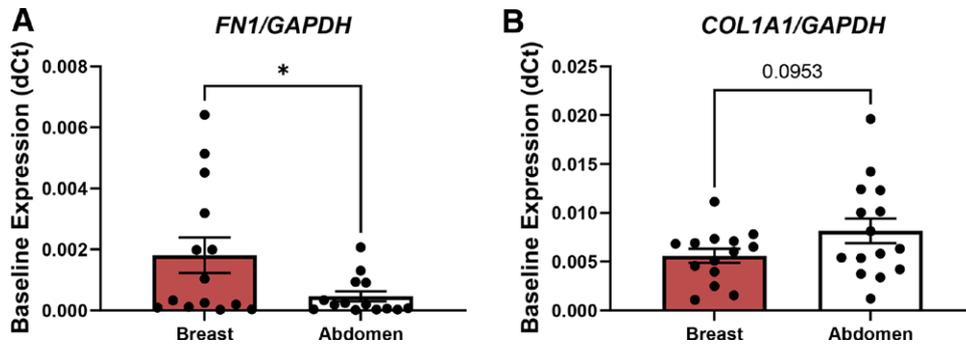
Fig. 3. FN1 levels show a difference in baseline gene expression based on skin location. RT-qPCR was used to measure baseline gene expression of *FN1* (A) and *COL1A1* (B), both normalized to the housekeeping gene *GAPDH*, in breast skin vs abdominal skin. Data are shown as individual values along with the mean ± SEM. *$P < 0.05$.
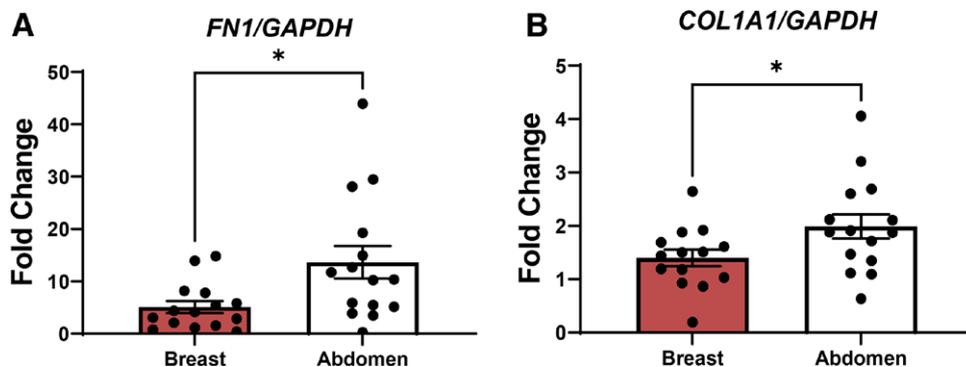

Fig. 4. *FN1* and *COL1A1* show differences in fibrotic responses based on skin location. RT-qPCR was used to measure TGFβ-induced FC in gene expression of *FN1* (A) and *COL1A1* (B), both normalized to the housekeeping gene *GAPDH*, in breast skin vs abdominal skin. Data are shown as individual values along with the mean ± SEM. *$P < 0.05$.

COL1A1 (Fig. 6B), and *ACTA2* (Fig. 6C), but not *CTGF* (*FN1*: $r = –0.4143$, $P < 0.05$; *COL1A1*: $r = –0.3465$, $P < 0.05$; *ACTA2*: $r = –0.3874$, $P < 0.05$, *CTGF*: data not shown). We further investigated the correlation based on skin location and found a similar trend of an inverse correlation in all the genes except *CTGF* in breast tissue, with only *ACTA2* levels achieving significance in abdominal skin ($r = –0.5898$, $P < 0.05$) (**See figure 2, Supplemental Digital Content 2,** which displays graphs showing correlation analysis between baseline expression and fibrotic response (FC in expression) of *FN1* (Above, left), *COL1A1* (Above, right), *ACTA2* (Below, left), and *CTGF* (Below, right), all normalized to housekeeping gene *GAPDH*, in breast skin tissue. http://links.lww.com/PRSGO/C230.)

(**See figure 3, Supplemental Digital Content 3,** which displays graphs showing correlation analysis between baseline expression and fibrotic response (FC in expression) of *FN1* (Above, left), *COL1A1* (Above, right), *ACTA2* (Below, left), and *CTGF* (Below, right), all normalized to housekeeping gene *GAPDH*, in abdominal skin tissue. Red font denotes significance ($P < 0.05$). http://links.lww.com/PRSGO/C231.)

DISCUSSION

The unpredictability of scarring in response to surgery is a challenge to plastic surgeons and a point of concern for the psychological well-being of patients.[35] Predicting the risk of patients for excessive scarring allows surgeons to tailor patient selection preoperatively, improve patient counseling, and guide technique selection to reduce such risk. The visibility and degree of scarring after completion of wound healing is directly related to the extent of fibrosis in the scar.[36] In this study, we used an ex vivo model of human dermal fibrosis to assess potential molecular markers indicative of patients with high risk for excessive scarring. We searched for patterns in baseline expression, age, and skin location, and how they correlate to the magnitude of the fibrotic response.

TGFβ has been extensively studied as the master regulator and driver of fibrosis in multiple organs and tissues.[37,38] TGFβ is the prototypical experiment fibrotic factor whose signaling plays an important role in pathologic scar formation.[39] In fact, compared with normal skin tissue, hypertrophic scar tissues express higher levels of TGFβ, which drive the excessive scar formation.[40] Therefore, we used TGFβ to successfully induce a fibrotic phenotype in our ex vivo explants that is biologically representative of pathological scarring in human skin.

Using this model, we first compared baseline and TGFβ-induced gene expression of *COL1A1, FN1, CTGF* and *ACTA2*, all of which are profibrotic components involved in scarring.[24,41,42] We found that baseline expression of
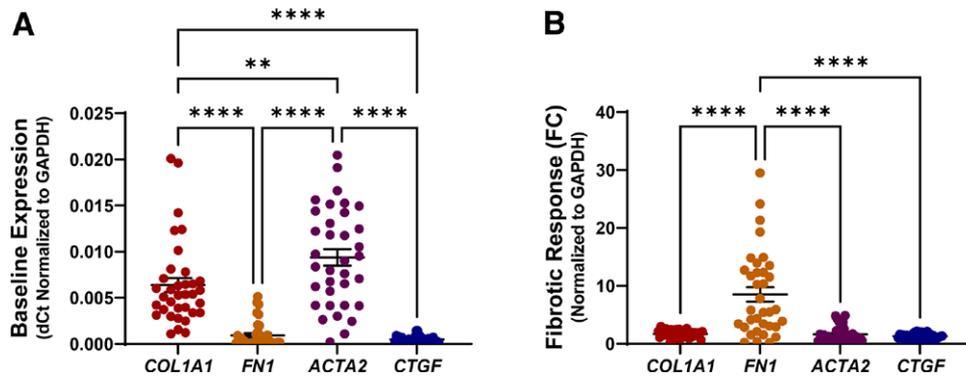
Fig. 5. Collective data from breast and skin tissue show opposing trends in baseline expression versus fibrotic response of *COL1A1*, *FN1*, and *ACTA2*. Baseline expression (A) and fibrotic response (FC in expression) data (B) for *COL1A1*, *FN1*, *ACTA2,* and *CTGF* from both breast and abdominal tissue were pooled and compared. Data are shown as individual values along with the mean ± SEM. **$P < 0.05$, ****$P < 0.0001$.
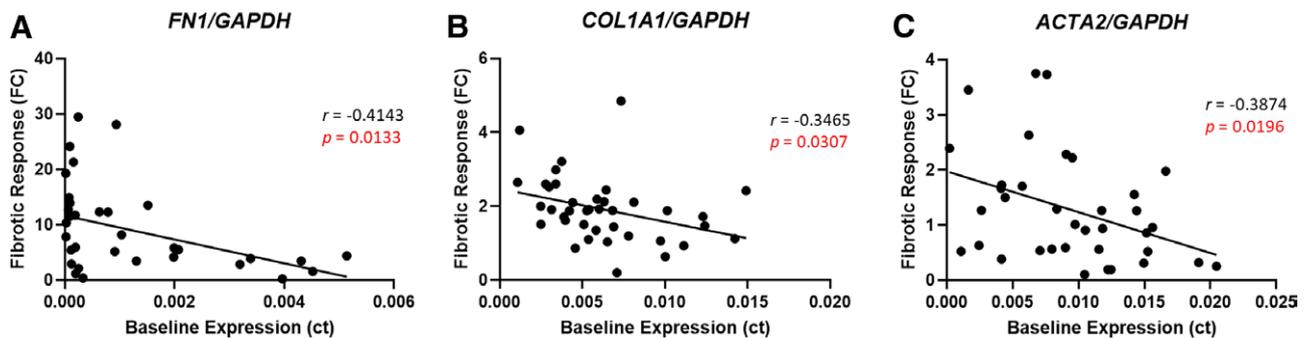

Fig. 6. Fibrotic responses of *FN1*, *COL1A1*, and *ACTA2* are inversely correlated to their baseline expression. Correlation analysis was performed using Pearson correlation coefficient to investigate the relationship between baseline expression and fibrotic response (FC in expression) of *FN1* (A), *COL1A1* (B), and *ACTA2* (C). *P* values in red show significance ($P < 0.05$).

FN1 is significantly higher in breast skin compared with abdominal skin. Yet, the magnitude of the fibrotic induction of *FN1* shows the opposite, being significantly blunted in breast skin compared with abdominal skin. This indicates that while quiescent expression levels of *FN1* show location-based variation, the fibrotic response of the *FN1* gene favors skin location with a lower expression at baseline. In fact, the severity and recurrence of pathologic scarring are very much related to anatomical location.[43] Hypertrophic scars and keloids are prone to develop in certain anatomic locations such as the chest and abdomen.[44,45] At the molecular level, fibronectin was shown to be constantly overproduced in both hypertrophic scars and keloid human skin samples, suggesting a failure to proceed with the healing stages or "maturation arrest," where wound healing signals persist or their downregulation fails.[44,46] While studies show that macroscopically both chest and abdominal areas are susceptible to pathological scarring, we suggest there could be a difference at the molecular level, where FN production plays a more direct role in scarring in the abdominal region when compared with that in the chest.

Moreover, we found that the magnitude of fibrotic induction of only *FN1* is positively correlated with age. Studies have shown the importance of FN in the deposition and stability of ECM.[47] During the proliferative phase

of wound healing, FN molecules create fibrils, which serve as a mesh for collagen deposition and consequently wound contracture.[48] However, with age, the ECM deposition and hence wound closure are delayed.[11] As such, our data suggest that older individuals likely increasingly rely on FN expression to effectively stabilize the slower deposition of ECM and successfully heal a wound.

Interestingly, when we looked at collective data from both skin locations together, we found that *COL1A1*, *ACTA2*, and *CTGF* had comparable fibrotic induction levels across donors, yet *FN1* expression showed significantly higher and more variable induction levels following TGFβ treatment. This further supports the notion that induction levels of FN during wound healing might be a decisive factor in the severity of scar formation. In fact, FN deposition is induced in response to other known fibrotic stimuli as well, such as insulin-like growth factor binding protein 5, insulin-like growth factor binding protein 3, and insulin-like growth factor II, all of which are implicated in aberrant wound healing and pathologic scarring.[49–53]

Intriguingly, baseline expression of *ACTA2*, *COL1A1*, and *FN1* showed a clear opposite pattern compared with the TGFβ-induced expression levels, similar to what we observed with *FN1* expression when comparing breast with abdominal skin. Therefore, we hypothesized that an inverse correlation might exist between baseline

expression levels of these genes and their fibrosis-induced levels. As expected, we showed that this inverse correlation is significant in *ACTA2*, *COL1A1*, and *FN1*, but not in *CTGF*. *ACTA2* is a marker of myofibroblast activation, which explains the inverse relationship being mirrored in *COL1A1* and *FN1* because the expression and deposition of these ECM proteins are dependent on myofibroblast activation.[54] Fibroblasts play a fundamental role in the proliferative phase of wound healing by depositing ECM for wound closure.[4,55] In that sense, the inverse relationship implies that fibroblasts respond more potently to a fibrotic inducer in areas of low ECM expression, making that area more susceptible to excessive scarring. To this end, low baseline expression of *ACTA2*, or ECM genes such as *FN1* or *COL1A1*, in a certain skin location could potentially be an indicator of high scarring propensity in that specific location, making it susceptible to more visible and potentially pathologic scarring, like keloids or hypertrophic scars. Alternatively, resident fibroblasts in areas of low ECM abundance could express dysregulated wound healing. In fact, Eto et al showed that fibroblasts isolated from hypertrophic scars no longer showed increased production of ECM components, namely Collagen type 1 and 3, in culture.[56] This supports our findings, suggesting that gene expression in skin fibroblasts is directly affected by their microenvironment, where a low abundance of ECM could sensitize resident fibroblasts into overproducing ECM proteins when activated. We believe this may also explain the etiology behind the fibrotic variation between diffuse and limited cutaneous SSc, suggesting that patients diagnosed with SSc may develop more extensive cutaneous fibrosis if their skin's baseline expression levels of these genes are generally lower.[13,57]

Our study has some limitations that can be addressed in future studies. We used skin mainly from the breast and abdomen of female donors; therefore, further studies are needed to confirm our results in skin from additional skin sites, male donors, and the response to skin explants to different profibrotic and inflammatory factors documented in scarring. Further, our study is based on skin derived from different individual donors. Therefore, future approaches should analyze potential confounding variables inherent to human studies. Finally, our future directions will also focus on studying gene expression in scars of participants who require secondary surgery for scar revision of hypertrophic scars or keloids.

CONCLUSIONS

In conclusion, baseline expression of the myofibroblast marker, *ACTA2*, and the ECM genes, *COL1A1* and *FN1*, in the skin are inversely correlated to their TGFβ-induced levels. Thus, skin areas with low expression of these genes could potentially be prone for excessive or pathologic scarring.

Carol Feghali-Bostwick, PhD
96 Jonathan Lucas St., MSC 637
Charleston, SC 29425
E-mail: feghalib@musc.edu

REFERENCES

1. Gonzalez AC, Costa TF, Andrade ZA, et al. Wound healing – a literature review. *An Bras Dermatol.* 2016;91:614–620.
2. Rodrigues M, Kosaric N, Bonham CA, et al. Wound healing: a cellular perspective. *Physiol Rev.* 2019;99:665–706.
3. Sorg H, Tilkorn DJ, Hager S, et al. Skin wound healing: an update on the current knowledge and concepts. *Eur Surg Res.* 2017;58:81–94.
4. Rippa AL, Kalabusheva EP, Vorotelyak EA. Regeneration of dermis: scarring and cells involved. *Cells.* 2019;8:E607.
5. Grabowski G, Pacana MJ, Chen E. Keloid and hypertrophic scar formation, prevention, and management: standard review of abnormal scarring in orthopaedic surgery. *J Am Acad Orthop Surg.* 2020;28:e408–e414.
6. El Ayadi A, Jay JW, Prasai A. Current approaches targeting the wound healing phases to attenuate fibrosis and scarring. *Int J Mol Sci.* 2020;21:E1105.
7. Berman B, Maderal A, Raphael B. Keloids and hypertrophic scars: pathophysiology, classification, and treatment. *Dermatol Surg.* 2017;43 (Suppl 1):S3–S18.
8. Ngaage M, Agius M. The psychology of scars: a mini-review. *Psychiatr Danub.* 2018;30(Suppl 7):633–638.
9. Ziolkowski N, Kitto SC, Jeong D, et al. Psychosocial and quality of life impact of scars in the surgical, traumatic and burn populations: a scoping review protocol. *BMJ Open.* 2019;9:e021289.
10. Visscher MO, Bailey JK, Hom DB. Scar treatment variations by skin type. *Facial Plast Surg Clin North Am.* 2014;22:453–462.
11. Bonifant H, Holloway S. A review of the effects of ageing on skin integrity and wound healing. *Br J Community Nurs.* 2019;24 (Suppl 3):S28–S33.
12. Lee Peng G, Kerolus JL. Management of surgical scars. *Facial Plast Surg Clin North Am.* 2019;27:513–517.
13. Denton CP, Khanna D. Systemic sclerosis. *Lancet.* 2017;390:1685–1699.
14. Ferreli C, Gasparini G, Parodi A, et al. cutaneous manifestations of scleroderma and scleroderma-like disorders: a comprehensive review. *Clin Rev Allergy Immunol.* 2017;53:306–336.
15. Wynn TA, Ramalingam TR. Mechanisms of fibrosis: therapeutic translation for fibrotic disease. *Nat Med.* 2012;18:1028–1040.
16. Darby IA, Hewitson TD. Fibroblast differentiation in wound healing and fibrosis. *Int Rev Cytol.* 2007;257:143–179.
17. Dallon J, Sherratt J, Maini P, et al. Biological implications of a discrete mathematical model for collagen deposition and alignment in dermal wound repair. *IMA J Math Appl Med Biol.* 2000;17:379–393.
18. Dallon JC, Sherratt JA, Maini PK. Modeling the effects of transforming growth factor-beta on extracellular matrix alignment in dermal wound repair. *Wound Repair Regen.* 2001;9:278–286.
19. Zhang L, Qin H, Wu Z, et al. Identification of the potential targets for keloid and hypertrophic scar prevention. *J Dermatolog Treat.* 2018;29:600–605.
20. Sible JC, Eriksson E, Smith SP, et al. Fibronectin gene expression differs in normal and abnormal human wound healing. *Wound Repair Regen.* 1994;2:3–19.
21. Chen Y, Shi-Wen X, van Beek J, et al. Matrix contraction by dermal fibroblasts requires transforming growth factor-beta/activin-linked kinase 5, heparan sulfate-containing proteoglycans, and MEK/ERK: insights into pathological scarring in chronic fibrotic disease. *Am J Pathol.* 2005;167:1699–1711.

22. Tai Y, Woods EL, Dally J, et al. Myofibroblasts: function, formation, and scope of molecular therapies for skin fibrosis. *Biomolecules.* 2021;11:1095.

23. Lian N, Li T. Growth factor pathways in hypertrophic scars: molecular pathogenesis and therapeutic implications. *Biomed Pharmacother.* 2016;84:42–50.

24. Colwell AS, Phan TT, Kong W, et al. Hypertrophic scar fibroblasts have increased connective tissue growth factor expression after transforming growth factor-beta stimulation. *Plast Reconstr Surg.* 2005;116:1387–1390; discussion 1391.

25. Yamaguchi Y, Takihara T, Chambers RA, et al. A peptide derived from endostatin ameliorates organ fibrosis. *Sci Transl Med.* 2012;4:136ra71.

26. Yasuoka H, Larregina AT, Yamaguchi Y, et al. Human skin culture as an ex vivo model for assessing the fibrotic effects of insulin-like growth factor binding proteins. *Open Rheumatol J.* 2008;2:17–22.

27. Aida-Yasuoka K, Peoples C, Yasuoka H, et al. Estradiol promotes the development of a fibrotic phenotype and is increased in the serum of patients with systemic sclerosis. *Arthritis Res Ther.* 2013;15:R10.

28. Yasuoka H, Yamaguchi Y, Feghali-Bostwick CA. The membrane-associated adaptor protein DOK5 is upregulated in systemic sclerosis and associated with IGFBP-5-induced fibrosis. *PLoS One.* 2014;9:e87754.

29. Su Y, Nishimoto T, Hoffman S, et al. Insulin-like growth factor binding protein-4 exerts antifibrotic activity by reducing levels of connective tissue growth factor and the C-X-C chemokine receptor 4. *FASEB Bioadv.* 2019;1:167–179.

30. Malaab M, Renaud L, Takamura N, et al. Antifibrotic factor KLF4 is repressed by the miR-10/TFAP2A/TBX5 axis in dermal fibroblasts: insights from twins discordant for systemic sclerosis. *Ann Rheum Dis.* 2022;81:268–277.

31. Watanabe T, Nishimoto T, Mlakar L, et al. Optimization of a murine and human tissue model to recapitulate dermal and pulmonary features of systemic sclerosis. *PLoS One.* 2017;12:e0179917.

32. Watanabe T, Baker Frost DA, Mlakar L, et al. A human skin model recapitulates systemic sclerosis dermal fibrosis and identifies COL22A1 as a TGFβ early response gene that mediates fibroblast to myofibroblast transition. *Genes (Basel).* 2019;10:E75.

33. Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. *Nat Methods.* 2012;9:671–675.

34. Hong JH, Kim DH, Rhyu IJ, et al. A simple morphometric analysis method for dermal microstructure using color thresholding and moments. *Skin Res Technol.* 2020;26:132–136.

35. Capon A, Iarmarcovai G, Gonnelli D, et al. Scar prevention using laser-assisted skin healing (LASH) in plastic surgery. *Aesthetic Plast Surg.* 2010;34:438–446.

36. Monavarian M, Kader S, Moeinzadeh S, et al. Regenerative scar-free skin wound healing. *Tissue Eng Part B Rev.* 2019;25:294–311.

37. Stewart AG, Thomas B, Koff J. TGF-β: Master regulator of inflammation and fibrosis. *Respirology.* 2018;23:1096–1097.

38. Meng XM, Nikolic-Paterson DJ, Lan HY. TGF-beta: the master regulator of fibrosis. *Nat Rev Nephrol.* 2016;12:325–338.

39. Penn JW, Grobbelaar AO, Rolfe KJ. The role of the TGF-β family in wound healing, burns and scarring: a review. *Int J Burns Trauma.* 2012;2:18–28.

40. Wang R, Ghahary A, Shen Q, et al. Hypertrophic scar tissues and fibroblasts produce more transforming growth factor-beta1 mRNA and protein than normal skin and cells. *Wound Repair Regen.* 2000;8:128–137.

41. Inui S, Shono F, Nakajima T, et al. Identification and characterization of cartilage oligomeric matrix protein as a novel pathogenic factor in keloids. *Am J Pathol.* 2011;179:1951–1960.

42. Rockey DC, Bell PD, Hill JA. Fibrosis — a common pathway to organ injury and failure. *N Engl J Med.* 2015;372:1138–1149.

43. Park TH, Chang CH. Location of keloids and its treatment modality may influence the keloid recurrence in children. *J Craniofac Surg.* 2015;26:1355–1357.

44. Gauglitz GG, Korting HC, Pavicic T, et al. Hypertrophic scarring and keloids: pathomechanisms and current and emerging treatment strategies. *Mol Med.* 2011;17:113–125.

45. Matsumura H, Engrav LH, Gibran NS, et al. Cones of skin occur where hypertrophic scar occurs. *Wound Repair Regen.* 2001;9:269–277.

46. Sephel GC, Woodward SC. *Repair, Regeneration, and Fibrosis.* Baltimore: Lippincott, Williams & Wilkins; 2001;84–117.

47. Ud-Din S, Foden P, Stocking K, et al. Objective assessment of dermal fibrosis in cutaneous scarring, using optical coherence tomography, high-frequency ultrasound and immunohistomorphometry of human skin. *Br J Dermatol.* 2019;181:722–732.

48. Lenselink EA. Role of fibronectin in normal wound healing. *Int Wound J.* 2015;12:313–316.

49. Garrett SM, Hsu E, Thomas JM, et al. Insulin-like growth factor (IGF)-II-mediated fibrosis in pathogenic lung conditions. *PLoS One.* 2019;14:e0225422.

50. Pilewski JM, Liu L, Henry AC, et al. Insulin-like growth factor binding proteins 3 and 5 are overexpressed in idiopathic pulmonary fibrosis and contribute to extracellular matrix deposition. *Am J Pathol.* 2005;166:399–407.

51. Russell SB, Russell JD, Trupin KM, et al. Epigenetically altered wound healing in keloid fibroblasts. *J Invest Dermatol.* 2010;130:2489–2496.

52. Smith JC, Boone BE, Opalenik SR, et al. Gene profiling of keloid fibroblasts shows altered expression in multiple fibrosis-associated pathways. *J Invest Dermatol.* 2008;128:1298–1310.

53. Xia W, Phan TT, Lim IJ, et al. Complex epithelial-mesenchymal interactions modulate transforming growth factor-beta expression in keloid-derived cells. *Wound Repair Regen.* 2004;12:546–556.

54. Garrett SM, Baker Frost D, Feghali-Bostwick C. The mighty fibroblast and its utility in scleroderma research. *J Scleroderma Relat Disord.* 2017;2:69–134.

55. Grabowski G, Pacana MJ, Chen E. Keloid and hypertrophic scar formation, prevention, and management: standard review of abnormal scarring in orthopaedic surgery. *J Am Acad Orthop Surg.* 2020;28:e408–e414.

56. Eto H, Suga H, Aoi N, et al. Therapeutic potential of fibroblast growth factor-2 for hypertrophic scars: upregulation of MMP-1 and HGF expression. *Lab Invest.* 2012;92:214–223.

57. Ostojić P, Damjanov N. Different clinical features in patients with limited and diffuse cutaneous systemic sclerosis. *Clin Rheumatol.* 2006;25:453–457.