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ORIGINAL ARTICLE BASIC SCIENCE/EXPERIMENTAL

Effect of Keratinocytes on Myofibroblasts in Hypertrophic Scars

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Abstract Myofibroblasts play a central role in matrix formation and wound contraction during wound healing and undergo apoptosis at the end of the healing. Hypertrophic scarring is a pathologic condition in which myofibroblasts persist in the tissue. It has been hypothesized that abnormalities in epidermal–dermal crosstalk underlie this pathology. Therefore, in this study, we investigated whether myofibroblasts are affected by keratinocytes. Transforming growth factor beta-induced myofibroblasts (Imyo) and myofibroblasts from hypertrophic scar tissue (Hmyo) were characterized using microarrays. Keratinocytes were co-cultured with myofibroblasts, and quantitative PCR analysis was performed. We found that numerous extracellular matrix- and smooth muscle cell-associated genes were upregulated in Imyo and Hmyo respectively, and these findings suggest that Hmyo are fully differentiated myofibroblasts and that Imyo are less differentiated than Hmyo. Decreased collagen type 1 gene expression was found in keratinocytes co-cultured with Imyo and Hmyo; further, α-smooth muscle actin expression in Imyo increased in the presence of keratinocytes. These observations indicate that keratinocytes play a role in the development of pathological fibrosis in hypertrophic scar tissue by regulating the behavior of dermal fibroblasts and myofibroblasts. We believe that this study provides the basis for understanding the pathophysiology of hypertrophic scarring and identifying new therapeutic approaches for this dysfunction.
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Keywords Myofibroblast · Fibroblast · Keratinocyte · Hypertrophic scar

Introduction

Scarring results in cosmetic problems and, importantly, leads to various complications such as pain, itchiness, and motor impairment, caused by mismatch at the interface of the scar and normal tissues, and recurrence of the wound [1] Abnormal scarring, such as that in fibrosis, and keloid and hypertrophic scars, is a pathological process, distinctive from the normal physiological process of wound healing (Fig. 1). The general scope of research on scars is broad, encompassing the pathological states of scarring, the functional impairments resulting from scarring in internal organs, and the complications associated with this process, some of which can be lethal. Additionally, ongoing research efforts focus on the prevention of scarring by identifying and neutralizing the causes of this pathological process. During the wound-healing process, myofibroblasts play a critical role in extracellular matrix (ECM) synthesis and wound contraction. Normally, myofibroblasts undergo apoptosis once healing is completed. However, if they remain active at the

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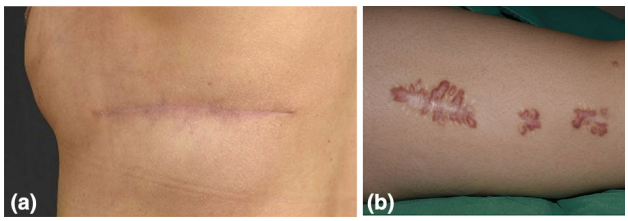

Fig. 1 Normal and abnormal (hypertrophic or keloid) scars. **a** Normal scar at the donor site for breast reconstruction with extended latissimus dorsi flap. **b** Hypertrophic or keloid scar on lower leg

wound site, a hypertrophic scar may develop. Multiple hypotheses have been proposed to explain this pathological state, that involves a balance between ECM synthesis and degradation, as well as to describe the involvement of cells and cytokines in this process. For example, it is considered that the elevated activation of platelets, macrophages, myofibroblasts, or fibronectin increases the migration of fibroblasts [2]; elevated histamine secretion by mast cells increases the synthesis of fibroblast collagen [3, 4], or the elevated expression of transforming growth factor beta (TGF-β) or platelet-derived growth factor (PDGF) from platelets and giant cells increases the proliferation and differentiation of fibroblasts. These processes result in increased ECM deposition and wound contraction [5–7]. Moreover, the increased migration of fibrocytes is known to elevate myofibroblast density [8, 9]. Further, the elevated expression of interleukin (IL)-4 and IL-13 by T lymphocytes increases ECM deposition [10, 11], and decreased fibroblast apoptosis increases ECM deposition and wound contraction [12, 13].

We hypothesized that scarring is induced by the interaction between the epidermis and dermis. Here we investigated the effect of co-culturing myofibroblasts from the dermis and keratinocytes from the epidermis on hypertrophic scarring, with the aim of identifying a potential therapeutic strategy for minimizing scars. Accordingly, we compared and analyzed the biological properties of TGF-β1-induced myofibroblasts (Imyo), taking into account that TGF-β1 plays an important role in myofibroblast formation [14] and hypertrophic scar myofibroblasts (Hmyo). The two types of myofibroblasts were then co-cultured with keratinocytes to identify their role in epidermal cell stimulation.

Materials and Methods

The present study was a prospective single-center study. The study protocol was approved by the institutional review board (IRB) on June 1, 2017 (Approval No.: 2017-04-029-001), at Kyungpook National University Hospital (www.e-irb.com).

Cell Cultures

Isolation of Fibroblasts from Normal Skin and Myofibroblast from Hypertrophic Scar Tissue

Normal skin and hypertrophic scar tissue from a patient was washed several times with phosphate-buffered saline (PBS, pH 7.4) and incubated with 2.4 units of Dispase® (Sigma, St. Louis, MO, USA) for 30 min to 1 h and 12 h, respectively. After washing with PBS, the dermis and epidermis were separated using forceps. The separated dermal tissue was cut into small pieces, and the cells were precipitated using centrifugation at 1000 rpm for 5 min. After several washes, the cell suspension was filtered through a cell strainer (pore size 75 μm, Nunc, Roskilde, Denmark). The filtered cells were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco/BRL, Gaithersburg, MD, USA) containing 100 units/mL penicillin G, 100 μg/mL streptomycin sulfate (Sigma), and 10% fetal bovine serum (Gibco/BRL) in 100-mm culture dishes in an incubator at 37 °C and 5% CO_2. Once the fibroblasts and myofibroblasts had reached 70–80% confluence, they were treated with EDTA at a concentration of 2×10^5/mL for separation.

Induction of Fibroblasts from Normal Skin to Imyo

Induction of fibroblasts from normal skin was performed by treating with 10 ng/mL of TGF-β1 (Invitrogen, Carlsbad, CA, USA) for 48 h in a six-well plate.

Comparison of the Biological Properties of Imyo and Hmyo

Microarray Analysis of cDNA

A microarray experiment was performed for the genetic analysis of Imyo and Hmyo. cDNA was retro-transcribed from the extracted mRNA using a Dynabead mRNA purification kit (Dynal AS, Norway). cDNA from Imyo was labeled with Cy5-dUTP (for red fluorescence) and that from Hmyo with Cy3-dUTP (green fluorescence, both from Amersham, Little Chalfont, UK). Printed DNA microarray images were scanned and analyzed using the GenePix software (version 3.0, Axon Instruments, Inc., Union City, CA, USA). Analysis of the microarray data using the fold change criteria ≥ 2 revealed that, among the 5,761 genes analyzed, more than 600 genes were upregulated in Imyo and Hmyo relative to the control group; among these, 83 genes that showed significant increase in expression were selected for further analysis. The biological meaning and correlation of each gene were investigated using Cytoscape, David, NCBI and UCSC Genome Browser.

Clusters and Pathway Analysis of Upregulated Genes in Imyo and Hmyo

Hierarchical clustering of the 83 upregulated genes was performed using the MeV software tool. The logarithm intensities of genes were mapped on a color scale with two dendrograms. Each colored element in the heatmap corresponds to one gene, and the dendrograms provide a qualitative means of assessing the similarity between genes and between patient samples. The interactions between gene products identified in this study were evaluated with the Search Tool for the Retrieval of Interacting Genes/Proteins database, STRING, version 10.5. All prioritized candidate susceptibility genes are selected from Table 1.

Co-culture with Keratinocytes

Isolation and Culture of Keratinocytes

Keratinocytes were isolated from neonatal foreskin and grown in a monolayer using the Rheinward and Green method [15]. The keratinocytes were subcultured up to seven times using 0.25% trypsin (Gibco/BRL) and 0.02% EDTA (Sigma), ensuring that cellular senescence was not induced.

Co-culture of Myofibroblasts and Cultured Keratinocytes

The co-culture experiment was performed with normal fibroblasts, Imyo, and Hmyo. Keratinocyte growth medium (Keratinocyte-SFM, Invitrogen) and DMEM were added in a 1:1 ratio, and cells were incubated together for 48 h before analysis.

Real-Time Quantitative PCR (qPCR)

qPCR was performed using the SYBR Green PCR master mix (Applied Biosystems, Foster City, CA, USA) and cDNA obtained from cultured cells as described above. The genes investigated by qPCR were collagen type 1 (*COL1A1*) and alpha smooth muscle actin (*αSMA*) gene. Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was used as a house-keeping gene. The PCR primers were as follows: *GAPDH* forward primer 5′-AGGGGTCTACATG GCAACTG-3′ and reverse primer 5′-GGCCTCCAAGGA GTAAGACC-3′; *COL1A1* forward primer 5′-CTCCG GCTCCTGCTCCTCTTA-3′ and reverse primer 5′-GCAC AGCACTCGCCCTCCC-3′; *αSMA* forward primer 5′-TT CAATGTCCCAGCCATGTA-3′ and reverse primer 5′-GA AGGAATAGCCACGCTCAG-3′. qPCR was performed in a total volume of 20 μL containing 3 μL of cDNA diluted to 1/20, 10 μL of 2 × SYBR green master mix, and 2 pmol/μL (2 μL) of each primer, in distilled water. For all genes, the melting temperature was 60 °C, and 40–50 cycles of amplification were performed. After extension, the fluorescence values were recorded, and the results were analyzed with ABI software (Applied Biosystems, Foster City, CA, USA). Relative quantity (RQ) values were obtained, and statistical significance was verified using the Kruskal–Wallis test. Significance was set at $P < 0.05$.

Results

Characterization of TGF-β1-Induced Myofibroblasts (Imyo)

TGF-β1 is known to induce the differentiation of normal fibroblasts into myofibroblasts [16, 17]. In this study, TGF-β1 treatment (10 ng/ml for 48 h) was used to establish the Imyo from the patient-derived dermal fibroblasts. To verify the cellular state of the Imyo, the gene expressions of the representative myofibroblast markers, COL1A1, and αSMA, were quantified using qPCR analysis. As shown in Fig. 2, the Imyo exhibited significantly elevated expressions of both COL1A1 and αSMA compared with the non-treated normal fibroblasts (Fig. 2).

Comparison of the Biological Properties of Imyo and Hmyo

The Hmyo cells are myofibroblasts isolated from hypertrophic scars of patients. Although both Imyo and Hmyo represent characteristics of myofibroblasts, their physiological state differs. Imyo may represent early stage myofibroblasts, whereas the Hmyo should represent the matured myofibroblast from the existing hypertrophic scar. Based on the microarray data, among the selected pool of 83 upregulated genes, 21 genes (including GNAS, ATCG1, SRI, and others) showed similar expression levels, suggesting that these genes are linked with genes of the stage-independent processes in myofibroblasts. In contrast, the 62 remaining genes, whose expression showed a greater than twofold difference between the Imyo and Hmyo, may reflect stage-specific differences in myofibroblasts.

Many of the genes with distinctively elevated expression in Imyo were found to be those whose products play a role in extracellular matrices such as fibulin 1 (*FBLN1*) transcript variant D, elastin (*ELN*), biglycan (*BGN*), and collagen type I alpha 2 (*COL1A2*). In contrast, many of the genes with elevated expression in the Hmyo were those whose products are related to muscle components, such as actin alpha 2 smooth muscle aorta (*ACTA2*), tubulin alpha ubiquitous (*K-ALPHA-1*), myosin light polypeptide 6 alkali smooth muscle and non-muscle, tubulin alpha 3 (*TUBA3*), transcript variant 2, and tropomyosin 1 alpha (*TPM1*) (Fig. 3, Table 1). Gene

Table 1 Genes whose expression was significantly upregulated in TGF β1-induced myofibroblasts (Imyo) and hypertrophic scar myofibroblasts (Hmyo)

Imyo-upregulated genes	Hmyo-upregulated genes
Fibulin 1 (FBLN1), transcript variant D	Karyopherin alpha 2 (RAG cohort 1, importin alpha 1) (KPNA2), mRNA
Platelet-derived growth factor receptor, alpha polypeptide (PDGFRA),	Small nuclear ribonucleoprotein polypeptide G (SNRPG), mRNA
Osteoblast-specific factor 2 (fasciclin I-like) (OSF-2), mRNA	Pyruvate dehydrogenase (lipoamide) beta (PDHB), mRNA
Enah/Vasp-like (EVL), mRNA	*Actin, alpha 2, smooth muscle, aorta (ACTA2), mRNA*
cDNA FLJ42181 fis, clone THYMU2031368	*Tubulin, alpha, ubiquitous (K-ALPHA-1), mRNA*
WD repeat and FYVE domain containing 1 (WDFY1), transcript variant 1, mRNA	H2A histone family, member Z (H2AFZ), mRNA
Glycoprotein (transmembrane) nmb (GPNMB)	*Myosin, light polypeptide 6, alkali, smooth muscle and non-muscle (MYL6)*
Intraflagellar transport protein IFT20 (LOC90410), mRNA	Interleukin 27 (IL27),
Insulin-like growth factor binding protein 4 (IGFBP4), mRNA	Crystallin, alpha B (CRYAB), mRNA
Elastin (supravalvular aortic stenosis, Williams-Beuren syndrome) (ELN), mRNA	*Tubulin, alpha 3 (TUBA3), mRNA*
Biglycan (BGN), mRNA	*Myosin, light polypeptide kinase (MYLK), transcript variant 8, mRNA*
Decorin (DCN), transcript variant A1, mRNA	Voltage-dependent anion channel 3 (VDAC3), mRNA
Serine (or cysteine) proteinase inhibitor, clade E (SERPINE1)	UPF3 regulator of nonsense transcripts homolog A (yeast) (UPF3A), mRNA
Trinucleotide repeat containing 3 (TNRC3)	Integral membrane protein 1 (ITM1), mRNA
Low-density lipoprotein-related protein 1 (LRP1), mRNA	Tumor rejection antigen (gp96) 1 (TRA1), mRNA
Collagen, type VI, alpha 1 (COL6A1), mRNA	Chloride intracellular channel 1 (CLIC1), mRNA
EGF-containing fibulin-like extracellular matrix protein 1 (EFEMP1),	*Tropomyosin 3 (TPM3), mRNA*
Collagen, type VI, alpha 3 (COL6A3), transcript variant 1, mRNA	AHA1, activator of heat shock 90 kDa protein ATPase homolog 1 (yeast), mRNA
Ferritin, heavy polypeptide 1 (FTH1), mRNA	Neural precursor cell expressed, developmentally down-regulated 8 (NEDD8), mRNA
Fibronectin 1 (FN1), transcript variant 1, mRNA	*ARP3 actin-related protein 3 homolog (yeast) (ACTR3), mRNA*
BCL2/adenovirus E1B 19 kDa interacting protein 3 (BNIP3), nuclear gene ……	Sorcin (SRI), transcript variant 1, mRNA
Collagen, type V, alpha 1 (COL5A1), mRNA	v-fos FBJ murine osteosarcoma viral oncogene homolog (FOS), mRNA
Proteinnexin1	*Actin, gamma 1 (ACTG1), mRNA*
Integral membrane protein 2B (ITM2B), mRNA	*Myosin IC (MYO1C), mRNA*
Transportin 2 (importin 3, karyopherin beta 2b) (TNPO2), mRNA	Annexin A2 (ANXA2), mRNA
Carbonyl reductase 3 (CBR3), mRNA	LIM and SH3 protein 1 (LASP1), mRNA
Glucose phosphate isomerase (GPI), mRNA	PDZ and LIM domain 1 (elfin) (PDLIM1), mRNA
Phosphogluconate dehydrogenase (PGD), mRNA	Peptidylprolyl isomerase A (cyclophilin A) (PPIA), mRNA
Tissue inhibitor of metalloproteinase 1 (TIMP1)	18S ribosomal RNA pseudogene (LOC359724) on chromosome Y
Matrix metalloproteinase 2 (gelatinase A) (MMP2), mRNA	Cysteine-rich, angiogenic inducer, 61 (CYR61), mRNA
DnaJ (Hsp40) homolog, subfamily B, member 6 (DNAJB6), mRNA	Glyceraldehyde-3-phosphate dehydrogenase (GAPD), mRNA
Glypican 3 (GPC3)	Cysteine-rich, angiogenic inducer, 61 (CYR61), mRNA
Fibronectin 1 (FN1), transcript variant 2, mRNA	Glyceraldehyde-3-phosphate dehydrogenase (GAPD), mRNA
Actin, beta (ACTB), mRNA	*Myosin, light polypeptide 9, regulatory (MYL9), transcript variant 2, mRNA*
cDNA FLJ36544 fis, clone TRACH2006378	GNAS complex locus (GNAS), transcript variant 2, mRNA
Fibronectin 1 (FN1), transcript variant 1, mRNA	*Tropomyosin 1 (alpha) (TPM1), mRNA*
Collagen, type I, alpha 2 (COL1A2), mRNA	

Bold text indicates genes whose products play a role in the cytoskeleton in the Imyo group, and italic text indicates genes whose products are muscle components in the Hmyo group

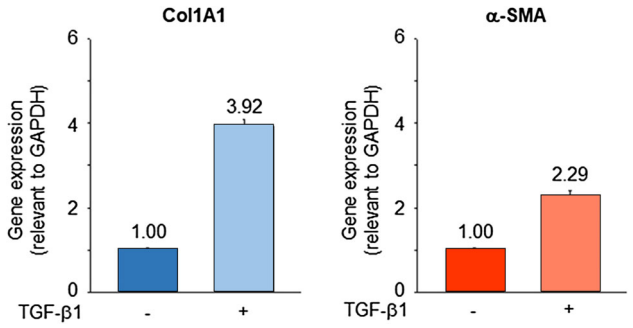

Fig. 2 qPCR results of dermal fibroblasts w/wo TGF-β1 treatment. TGF-β1-induced fibroblasts are defined as Imyo

network analysis was performed to confirm the association between upregulated genes (Fig. 4). In the Imyo group, among the 34 upregulated genes, 20 genes (58.8%) were interconnected to form a protein–protein interaction network based on the prediction results obtained using the STRING database. Moreover, several core genes in the network (FBLN1, MMP2, COL6A1, COL6A3, COL1A2, COL5A1, TIMP1, FN1, SERPINE, LRP1, GPC3, DCN, and BGN) were highly associated with extracellular matrix organization and blood vessel development categories. In the Hmyo group, among the 34 upregulated genes, 24 genes (70.6%) were highly interconnected. Moreover, interestingly, the hubs in this network were related to muscle structure development and muscle contraction (ACTG1, MYL9, TPM1, MYL6, TPM3, and MYLK). Consistent with the microarray data, PCR results confirmed the elevated expressions of ECM-related COL1A1 in the Imyo and of muscle-related αSMA in the Hmyo (Fig. 5).

Co-culture with Keratinocytes

The changes in the genes expressed in normal fibroblast, Imyo, and Hmyo upon co-culture with keratinocytes were quantitatively analyzed by qPCR. Specifically, we selected *COL1A1* and *αSMA* as a representative ECM-related gene marker and muscle-related gene marker, respectively (Fig. 6). When normal fibroblast cells were co-cultured with keratinocytes, the *COL1A1* gene expression was suppressed significantly to 0.41 ± 0.27 compared with that of the normal fibroblast-alone sample. However, αSMA gene expression was enhanced by the presence of keratinocyte in the culture (Fig. 6a). When TGF-β1-treated fibroblasts (Imyo), which are considered early myofibroblasts, were co-cultured with keratinocytes, the expression levels of both *COL1A1* and αSMA were greatly reduced (Fig. 6b). In the case of Hmyo, which are myofibroblasts from hypertrophic scars, the presence of keratinocytes had a smaller effects and elicited a slight increase and decrease in *COL1A1* and αSMA, respectively (Fig. 6c).

Discussion

Scars may be broadly classified as normal, hypertrophic, and keloid scars, and fibrosis [18] (Fig. 1). In the case of fibrosis, the cells do not regenerate the normal connective tissue. In internal organs, the deposition of functionless connective tissue may cause functional problems, and even death in severe cases. Fibrosis is a dysfunctional process that occurs in diseases such as systemic sclerosis [19, 20], Ehlers-Danlos syndrome [21], and diabetic disorders [22]. Abnormal scar formation (hypertrophic or keloid scars) occurs in the skin and causes a wide array of local symptoms, from motor limitation caused by scar contraction to dermatological problems such as pain and itching.

Among the multiple pathological mechanisms that have been implicated in abnormal scarring, the aberrant interaction between keratinocytes and myofibroblasts in the remodeling phase of wound healing is one of the most widely accepted [23, 24]. The pathogenesis of hypertrophic scar and keloids remains unknown; however, it is clear that the physiopathology is related to the regulation of inflammation that relies on various cell types. Among growth factors, TGF-β isoforms, which are upregulated in wound healing, play an important role in tissue regeneration, contributing to fibroblast versus myofibroblast differentiation and favoring the deposition of extracellular matrix. In addition, it is reported that degranulation triggered by an immunologic allergic reaction or hypersensitivity is elevated in hypertrophic scars [25]. Overall, numerous factors are considered to increase the inflammatory and proliferation phase during the course of wound healing, triggering hypertrophic scarring. Here, we aimed to investigate whether it is possible to inhibit abnormal scarring by targeting the interaction between keratinocytes and myofibroblasts.

Myofibroblasts appear in the final stage of wound healing—the remodeling stage—and differentiate from fibroblasts as a result of the interaction between the epidermis and the dermis. This interaction is evident during the embryonic development of the skin, and it is involved in the regulation of the skin's homeostasis during injuries or diseases [26]. In the interaction between the epidermis and the mesoderm that occurs in the early and middle phases of normal granulation tissue formation, keratinocytes play an important role in the differentiation of fibroblasts into myofibroblasts [27].

In the early phases of an injury, fibroblasts do not express αSMA; however, in the mid-phases, TGF-β1 and IL-1 secreted by keratinocytes decrease the inhibitory effect of keratinocytes on fibroblasts, while increasing the expression of genes related to TGF-β1. Consequently, the synthesis of constituents of the basal membrane by the

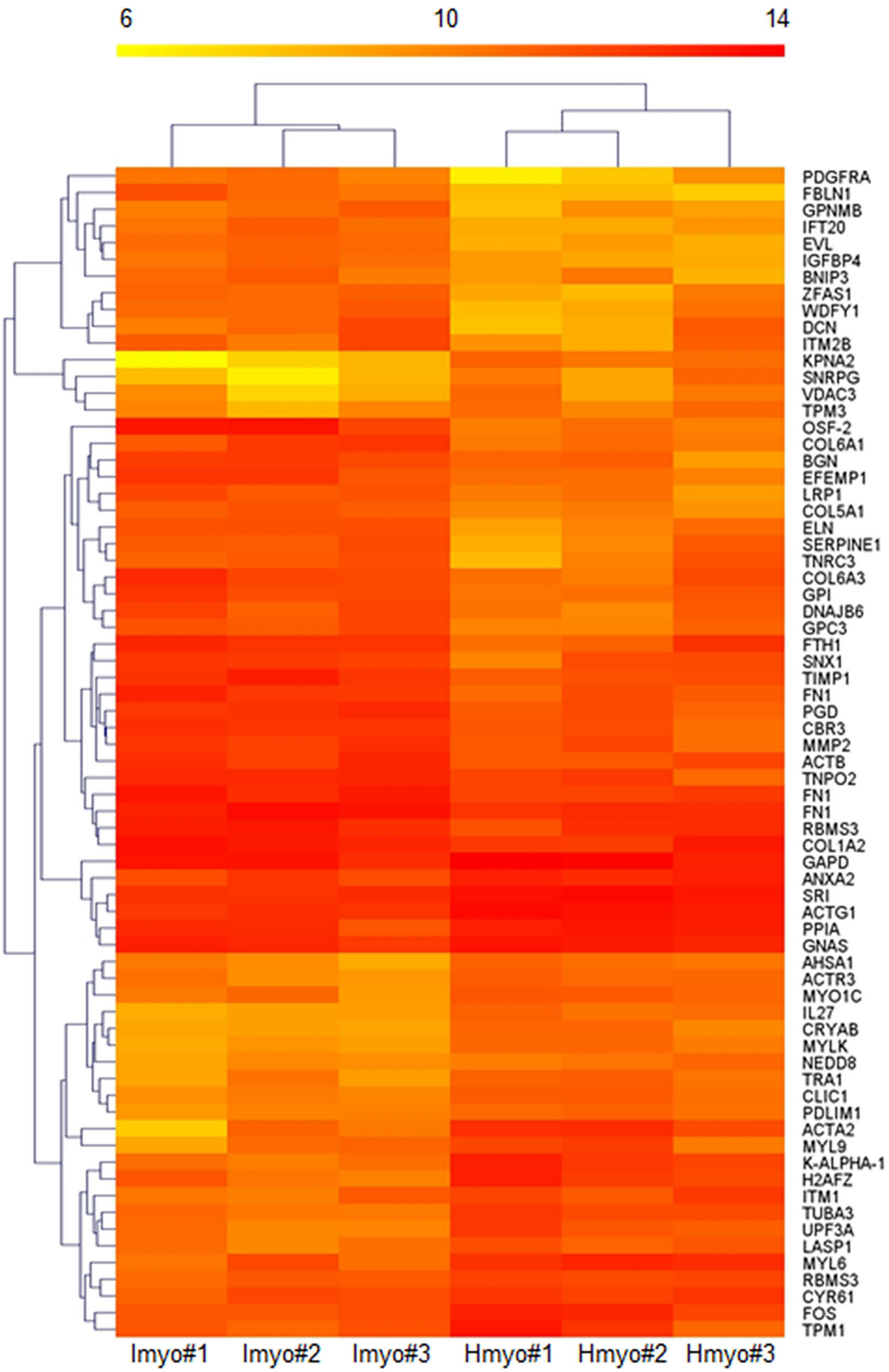

Fig. 3 Comparison between Imyo and Hmyo: Heatmap of logarithm intensity values built after data clustering. All data collected from the microarray platform test are subdivided by patients (*x* axis) and representative genes (*y* axis). The distances and relationships between clusters of patients and genes are expressed by dendrograms on their corresponding axis. The color code follows an non-dimensional scale. The positive log ratios are shown in red, whereas the negative log ratios are shown in yellow

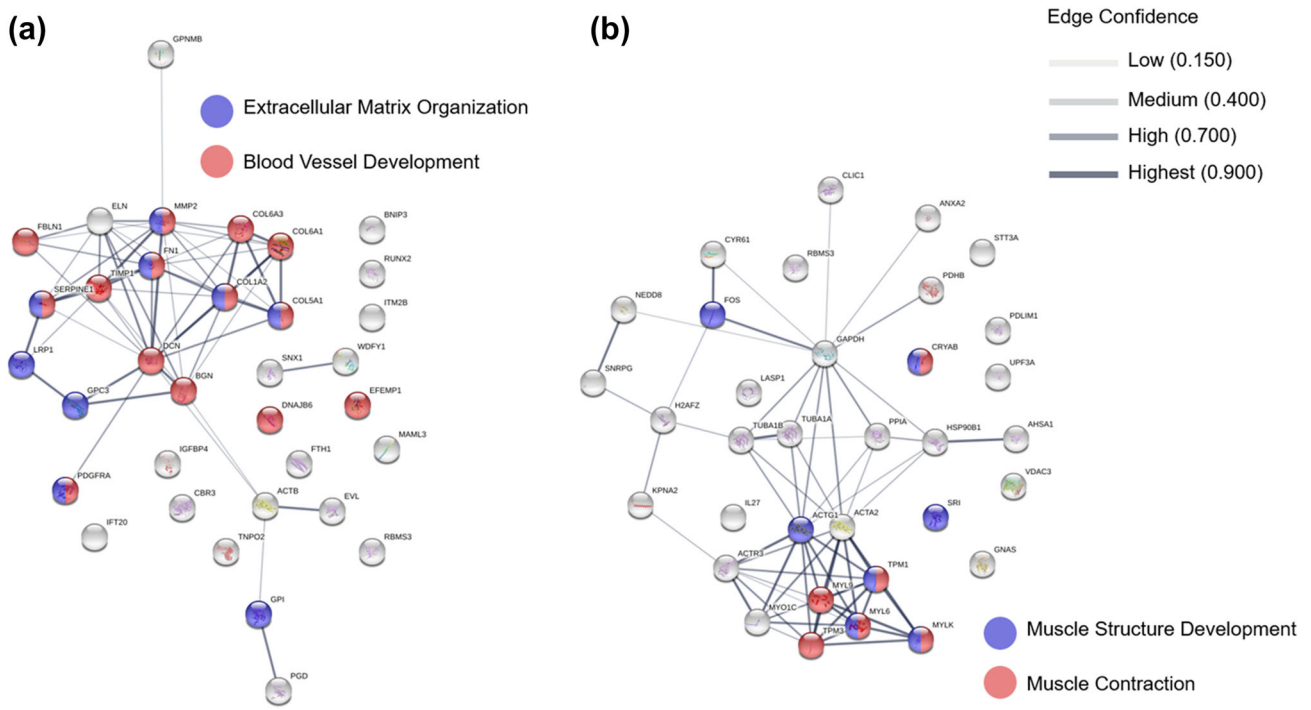

Fig. 4 Interactions between upregulated genes in the **a** Imyo and **b** Hmyo samples, respectively. The 34 total genes identified in the microarray screen were subjected to functional mapping for each group using the STRING database

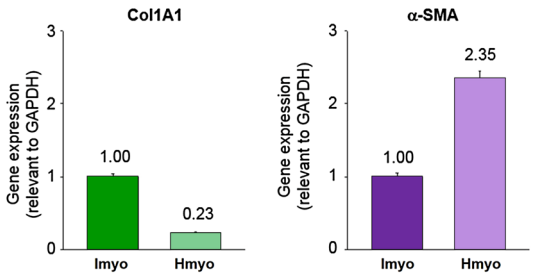

Fig. 5 The qPCR analysis comparison between Imyo and Hmyo for the representative ECM marker, Collagen 1A1 (*COL1A1*), and muscle marker, smooth muscle actin (*αSMA*)

fibroblasts increases. Although these fibroblasts are αSMA negative, they act as a physical barrier and are referred to as proto-myofibroblasts. When TGF-β1 action is prolonged, proto-myofibroblasts mature into myofibroblasts, which express αSMA, and the mechanical tension they offer is strengthened [28].

We performed microarray to compare the genes expressed in Imyo and Hmyo. Imyo are fibroblasts induced by treatment with TGF-β1 that, compared with fibroblasts isolated from normal dermis, show elevated expression of genes related to the extracellular matrices. In contrast, Hmyo, which are fibroblasts isolated from the dermis of hypertrophic scars, exhibit high levels of expression of genes related to muscle components (Figs. 3, 4). Results from gene expression mapping are represented using a heatmap, which makes it easier to evaluate changes in the expression of genes in Imyo and Hmyo—red represents genes expressed at high levels and yellow represents those expressed at low levels. Our results reveal that the ECM and blood vessel components were upregulated in Imyo, while the muscle-related genes were upregulated in Hmyo (Fig. 4). These findings suggest that the TGF-β1-treated fibroblasts represent myofibroblasts, which are less mature than Hmyo isolated from hypertrophic scars.

TGF-β is a growth factor with multiple functions in wound healing and is intimately associated with fibrotic scarring [29]. In particular, TGF-β1 has an important effect on myofibroblasts [30]. Notably, in uninjured skin, keratinocytes do not normally express TGF-β1; however keratinocytes that migrate to injured skin secrete this growth factor [31]. Interestingly, hypertrophic scarring has been reported to frequently occur when re-epithelialization is delayed [32]. Based on our findings, we believe that normal keratinocytes play an important role through the TGF-β1 they release. In an in vitro study comparing TGF-β1-treated fibroblasts and myofibroblasts in artificial skin models, these cell types were found to exhibit similar functions in terms of stimulating the differentiation of keratinocytes and increasing αSMA expression in dermal cells. Importantly, they do not induce re-epithelialization but induce the deposition of collagen type IV and laminin, thereby thickening the basal membrane; this effect is likely correlated with high elasticity and force resistance [33].

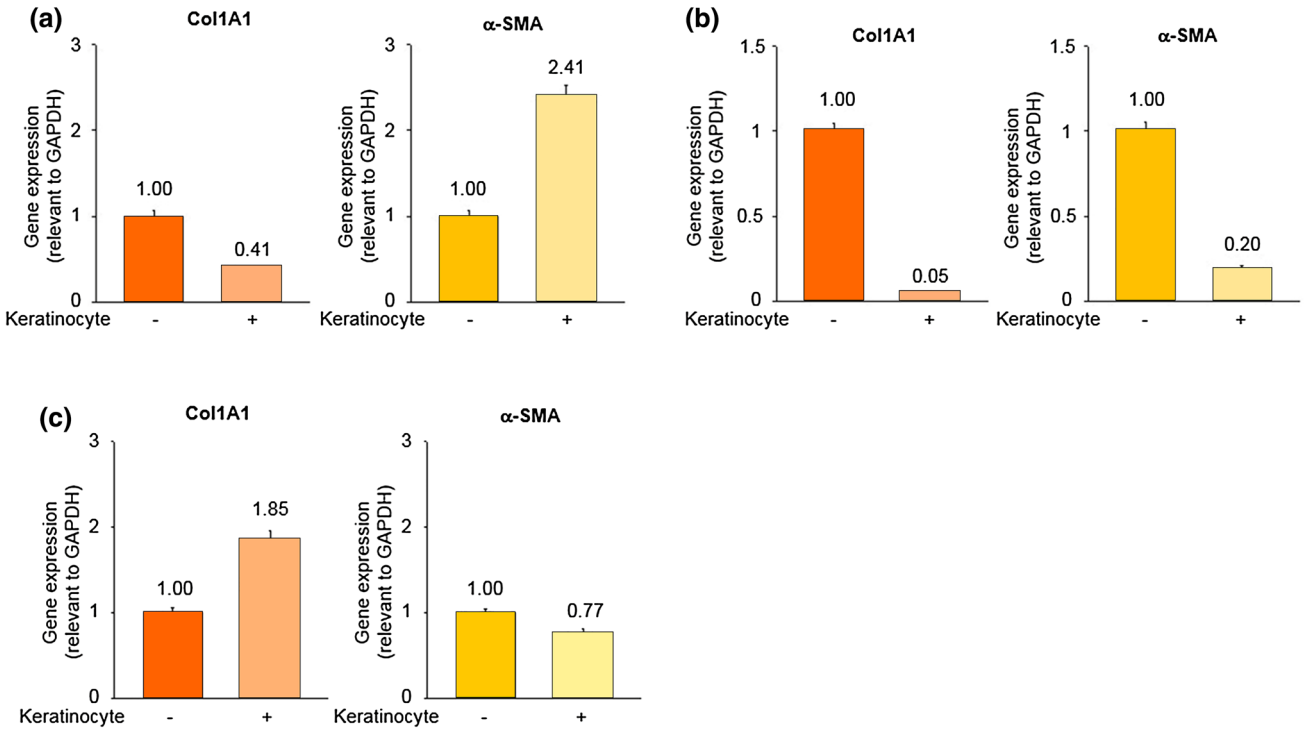

Fig. 6 qPCR analysis in keratinocyte co-culture experiments with **a** normal fibroblast **b** Imyo, and **c** Hmyo. The effects of keratinocytes on regulation of gene expression in fibroblasts, Imyo, and Hmyo differed strongly. All data were statistically significant

The exogenous application of applied mechanical force has been shown to accelerate scar formation; therefore, we speculate that the rapid re-epithelization of the wound facilitated ECM proteins synthesis by motile keratinocytes would inhibit the abnormal scarring. A study by Varkey et al. [34], demonstrating the reduction of fibrotic remodeling by keratinocytes supports this notion.

The present qPCR results showed that when keratinocytes were co-cultured with normal fibroblasts, *COL1A1* expression decreased and a SMA expression increased significantly compared with that in the fibroblast control (Fig. 6a). Based on these results, we found that co-culture of Imyo with keratinocytes led to markedly lower *COL1A1* expression and substantially lower *αSMA* expression (Fig. 6b). Similarly, Hmyo co-cultured with keratinocytes showed lower *αSMA* expression than the control Hmyo (Fig. 6c). SMA enhanced effects relative to collagen I, as shown in Fig. 6c, suggests that keratinocytes modulate mature hypertrophic scar formation. Yet collagen framework gets mature and difficult to give positive impacts. SMA, a muscle factor affecting contracture, actively promotes hypertrophic scar formation actively and is considered to reduce its effects.

The present results suggest that the presence of keratinocytes seems to inhibit the differentiation of Imyo into myofibroblasts and suppresses *COL1A1* expression, reducing collagen synthesis and α SMA levels. Furthermore, the addition of keratinocytes to more mature Hmyo significantly reduces the α SMA expressions of the muscle components in these cells (Fig. 6). Hmyo exhibits several properties of muscle, based on both PCR and αSMA analysis. The cellular states of the Imyo and Hmyo are very distinctive.

We speculate that keratinocytes inhibit abnormal scarring during the early stages of this process, when fibroblasts differentiate into proto-myofibroblasts, by reducing the expression of *COL1A1* and *αSMA*. Further, keratinocytes contribute to the amelioration of scar formations in the hypertrophic stage, when fibroblasts have already undergone differentiation, by reducing αSMA expression. Other signals are involved in the interactions of keratinocytes with fibroblasts or myofibroblasts. Therefore, we cannot generalize our findings to an in vivo scenario and additional studies are necessary to examine the complex mechanisms involved. Nevertheless, this study clearly suggests that the presence of healthy keratinocytes may potentially reduce abnormal scarring.

The interaction between the epidermis and dermis plays a significant role in abnormal scarring, and identification of the mechanisms involved in this process is important for the treatment of scars. In this study, we analyzed the genes expressed in Imyo and Hmyo relative to normal fibroblasts and found that Imyo can be considered proto-myofibroblasts, and Hmyo more

differentiated myofibroblasts based on the differences in their gene expression. When Imyo were co-cultured with keratinocytes, the expression of *COL1A1* and *αSMA* was markedly reduced. In addition, co-culture of Hmyo with keratinocytes also led to a decline in *αSMA* expression compared with that in Hmyo not co-cultured with keratinocytes. Based on these results, we believe that keratinocytes play a role in inhibiting abnormal scarring in the early stage of scar formation and may also contribute to improving already differentiated, mature hypertrophic scars. The present work provides novel insights into the mechanisms underlying abnormal scar formation during wound healing. The findings suggest that it is necessary to minimize infectious and allergic conditions early on during the treatment of traumatic or postoperative wounds in the plastic surgery, and also to use dressing material or collagen-based material made of homograft keratinocytes or cultured keratinocytes. Promoting wound healing by using biologic dressing material is considered a strategy to avoid the formation of an abnormal scar. And the suture of the epidermis to the wound edge is helpful in keratinocyte action and may reduce abnormal scar formation. Therefore, strategies that promote re-epithelization or the application of keratinocytes at cutaneous wound sites represent an alternative way to ameliorate scarring. Further research and clinical studies are necessary to identify additional factors that promote normal and rapid wound healing and minimize abnormal scar formation.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflicts of interest to disclose.

Statement of Animal Rights This article does not contain any studies with human participants or animals performed by any of the authors.

Informed Consent For this type of study, informed consent is not required.

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