Nanofiber scaffolds attenuate collagen synthesis of human dermal fibroblasts through TGF-β1/TSG-6 pathway

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Abstract

Nanofiber scaffolds are promising patches for skin tissue regeneration as they provide favorable environment for the adhesion, infiltration and proliferation of skin dermal fibroblasts. However, the effects of nanofiber scaffolds on scar formation remain to be elucidated. The aim of this study was to find out the relationship between nanofiber scaffolds and scar formation, along with the underlying mechanism. We found that polycaprolactone (PCL)/gelatin nanofiber scaffolds attenuated the mRNA expression of fibrosis-associated genes in fibroblasts, including collagen I (collagen type I alpha 1), collagen III (collagen type III alpha 1) and fibronectin. Specifically thicker scaffolds displayed stronger fibrosis inhibitory effect than thin scaffolds. The mechanism relied on TGF-β1/TSG-6 pathway, and overexpression of TSG-6 impaired the anti-fibrosis effect of nanofiber scaffolds, which decreased TGF-β1 expression with thickness-dependency. Moreover, in vivo study demonstrated that nanofiber scaffolds remarkably accelerated the wound healing process by reducing the ratios of collagen I/collagen III and TGF-β1, eventually decreased the deposition of collagens. Taken together, our results suggested that the attenuation of fibrosis by PCL/gelatin nanofiber scaffolds was TGF-β1-dependent and through TGF-β1/TSG-6 pathway. Nanofiber scaffold of appropriate thickness would accelerate skin wound healing, stimulate re-epithelialization and form cutaneous skin appendages in skin trauma. Thus, PCL/gelatin nanofiber scaffolds could be adopted for scar-free skin wound healing and skin cosmetics applications.

1. Introduction

Wound healing is a complex and dynamic process that involves three overlapping phases: an inflammation phase, a proliferation phase, and a remodeling phase. The three phases contain distinctive cellular and molecular cascades [1–3]. In the process of scar formation, skin fibroblast plays important roles, including migrating to the damaged areas and producing matrix to restore the structure of skin. However, excessive wound repair would result in hypertrophic scar or keloid scar [4, 5]. The abnormal architecture of collagen that results following the remodeling phase is the main cause of scar formation [6, 7].

Fibroblast is the most abundant cell type in normal connective tissue, which plays an important role in the synthesis, degradation and remodeling of extracellular matrix (ECM) both in physiological and pathological tissue. The most extensively characterized factor influencing scar formation is transforming growth factor-beta (TGF-β) [8]. Specifically, TGF-β1 and TGF-β2 induce cutaneous scarring, while TGF-β3 is found to inhibit this process [9]. Previous studies suggest that blocking TGF-β1/Smad signaling pathway may inhibit the proliferation and ECM expression of dermal fibroblasts, thus preventing the development of skin fibrosis

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Additionally, activation and transformation of fibroblasts to myofibroblasts is essential for the synthesis of a collagen-rich scar. During this process, accumulation of a pericellular matrix of hyaluronan and interactions with hyaladherins, such as Inter-alpha-Inhibitor and Tumor necrosis factor-stimulated gene-6 (TSG-6) are essential for differentiation. Thus, TSG-6 was essential for fibroblasts differentiation and inhibition of TSG-6 strongly abrogates the phenotypic change of fibroblasts to myofibroblasts.

Tissue engineering is a method of regenerating damaged tissues by presenting cell scaffolds and generating an environment in hope of facilitating cell adhesion and proliferation and mediating the biological functions to the cells. Nano-scaled morphology has been considered as one of the main structural characteristics of native ECM. To develop ECM-like fibrous structures, electrospinning technology has been widely utilized because of specific advantages, such as large-scale processing and easy control of fiber diameter and orientation. So far a large number of synthetic biocompatible polymers have been applied in tissue engineering, such as polycaprolactone (PCL), poly (lactic acid), poly (glycolic acid), poly ( acrylic acid), and poly (vinyl alcohol). As a synthetic polymer, PCL lacks bioactive sites for cellular interactions. Gelatin is derived from native collagen, and has been widely used to develop wound dressings owing to its biocompatibility and biological activities. Therefore, a combination of gelatin and PCL is feasible to obtain a biocompatible composite scaffolds, which have been proved effectively in tissues reconstruction, such as skin, bone, nerves and blood vessel. For skin regeneration, nanofiber system could accelerate the wound healing process by promoting angiogenesis, increasing re-epithelialization and controlling granulation tissue formation. However, the particular effects of nanofiber scaffolds on collagen synthesis and scar formation remain to be elucidated. In addition, some studies also emphasized the importance of thickness of nanofiber scaffolds in application. Wang et al demonstrated that thick-fiber electrospun PCL vascular grafts could enhance the vascular regeneration and remodeling process by mediating macrophage polarization into M2 phenotype, which facilitated the immunomodulatory and tissue remodeling. Moreover, the appropriate thickness of mesh influences the drug release rate, with thinner fiber meshes exhibited faster drug release. Here, we fabricated PCL/gelatin nanofiber scaffolds with varied thickness and investigated the effects of scaffold thickness on the behaviors of skin dermal fibroblasts as well as the scar formation in a rat full-thickness wound model. Throughout the process of wound healing and scar formation, we evaluated the behavioral, histological/physiological and molecular changes in the cells. This work demonstrates that nanofiber scaffolds reduced the progression of fibrosis through inhibiting TGF-β1/TSG-6 pathway. These findings shed light on previously unrecognized roles of nanofiber scaffolds in inhibiting scar formation and the optimization of nanofiber scaffolds thickness for tissue engineering applications.

2. Materials and methods

2.1. Fabrication and characterization of PCL/gelatin scaffolds

PCL/gelatin nanofibrous scaffolds of different thicknesses were fabricated by electrospinning technique according to the procedure we described previously. Briefly, 1 g of PCL (MW = 120 000, Sigma Aldrich, USA) and 1 g of gelatin (porcine skin origin, Sigma Aldrich, USA) were separately dissolved in 10 ml of 2,2,2-Trifluoroethanol (purity 99.0%, Fluka, Switzerland) to generate 10% w/v PCL and gelatin solution, respectively. After shaking overnight, 400 μl of acetic acid was added into PCL/gelatin mixture solution to form PCL/gelatin solution. The mixed polymer solution was loaded into a 5 ml syringe attached with a 26 G needle, and dispensed using a syringe pump. A voltage of about 12 kV and a flow rate of 2 ml h⁻¹ were applied to the system and the fibers were collected on a 22.5 mm diameter glass coverslip placed on a static flat collector. The samples were ventilated in a biosafety cabinet for at least two days before further experiments. Scanning electron microscope (SEM) observation was conducted to check the diameters of fibers and the thickness of scaffolds. Fiber diameters and scaffold thickness were determined by using Image-J software.

2.2. Cell culture on PCL/gelatin scaffolds

Human dermal fibroblasts were provided by Xiangya Hospital of Central South University. The cells were subsequently cultured in Dulbecco’s modified Eagle’s medium (DMEM) (HyClone, USA) supplemented with 10% fetal bovine serum (Gibco, USA) and 1% antibiotic/antimycotic (Invitrogen, USA) in a humidified incubator at 37 °C with 5% CO₂. Fibroblasts (passages 3–8) were seeded on non-adherent cell culture 12-well plates (10⁵ cells per well) pre-loaded with nanofiber scaffolds of different thicknesses, with regular tissue culture 12-well plates as substrate control. Prior to seeding, the ventilated PCL/gelatin nanofiber scaffolds were transferred to 12-well plates pre-loaded with 75% ethyl alcohol and incubated for 30 min, followed by UV sterilization (356 nm). After 24 h of incubation, fibroblasts were further treated with or without 10 ng ml⁻¹ of TGF-β1 (PeproTech, USA) for another 48 h.
For cell proliferation testing, 50 μl of MTT solution was introduced to each well at a final concentration of 0.5 mg ml⁻¹ at specific time intervals. After 4 h of incubation, 500 μl of DMSO was introduced into each well to dissolve the resultant crystals and then the absorbance of samples was measured at 490 nm using an EnSpire multimode plate reader (PerkinElmer EnSpire 2300, USA). Cell growth relative to control (%) was calculated and compared between different groups.

2.3. Cell proliferation assay

2.4. Construction of TSG-6 overexpression vector

The TSG-6 overexpression plasmid was generated in p3XFLAG-CMV-13 (Sigma Aldrich, USA). The total RNA of human dermal fibroblasts stimulated with TGF-β1 was purified using Trizol (Invitrogen, USA) as per manufacturer’s instructions, and the corresponding cDNA was obtained by using a reverse transcription kit (Takara, Japan). The TSG-6 gene was amplified from cDNA and quantified by PCR assay. The TSG-6 primers 5'-GGGGTACC (Kpn I) TTACTTCACTAACAATGAGAT-3' (sense) and 5'-GCTCTAGA (Xba I) TAAAGTCGTAATCTCCTAGCT-3' (anti-sense) yielded an 853-nucleotide product specific to the TSG-6 coding gene (GenBank: BC030205.1). The PCR products were ligated into the p3XFLAG-CMV-13 vector after digested with Kpn I and Xba I by using T4 DNA ligase (Beiyotime, China) and the recombinant plasmid was transformed into Escherichia coli DH5α strain. The expected clone was confirmed by DNA sequencing.

2.5. Gene transfection assay

Human dermal fibroblasts were transfected with plasmid DNA at the aid of Lipofectamine™ 2000 (Invitrogen, USA) according to the manufacturer’s protocol. Briefly, 2.5 μg of as-prepared plasmid DNA and 6 μl of Lipofectamine™ 2000 were separately diluted in 100 μl of DMEM culture medium. After 5 min, these two solutions were mixed in a 1.5 ml Eppendorf tube and rested for another 15 min. For transfection, the final mixture was added into 6-well plates with 2 ml of fresh culture medium. After incubation for 48 h, the cells were harvested for RT-PCR and western blotting analysis.

2.6. Real-time PCR analysis

2.7. Western blotting analysis

After the indicated treatments, cells were lysed by RIPA lysis buffer (Beiyotime, China) containing 1 mM of phenylmethanesulfonyl fluoride (Beiyotime, China). The concentration of total protein was measured with BCA protein assay kit (Beiyotime, China). Equivalent amounts of protein samples separated by SDS-PAGE were then transferred onto PVDF membranes pre-activated in methanol. The membranes were blocked in blocking buffer for western blotting (Beiyotime, China), and then incubated for 1 h with specific primary antibodies of interest for protein detection at room temperature. Horseradish peroxidase-conjugated secondary antibodies were then added to each membrane and incubated for 1 h at room temperature. The target proteins were visualized by ECL.
solution and detected by FluorChem E System. Each band on western blotting was quantified with β-actin as internal reference.

2.8. In vivo wound healing studies
8 week-old Sprague-Dawley rats were employed for full-thickness skin wound healing studies. All animal experiments were performed with approval from the Hunan University Animal Ethics Committee (No. 00083913, No. 00080815, No. 00080816 and No. 00080817). Animals were anesthetized with chloral hydrate (40 mg kg$^{-1}$), and the back was shaved. A 5 mm diameter biopsy puncher was used to create wounds along the dorsal side of the skin. Four wounds were created in each rat, and subsequently nanofiber scaffolds of different thicknesses were placed on the wound sites randomly, with open wound as blank control. Changes of wound areas were measured using a caliper daily after surgery until day 14. After 7 and 14 d, animals were euthanized by exposure to ether, and then the wound area and surrounding skin plus muscle were removed and fixed in 4% paraformaldehyde. Tissue samples were embedded in paraffin and sectioned in a microtome. Hematoxylin-eosin (H and E) staining, Masson’s trichrome staining, and Picro-sirius red staining were performed to evaluate the skin tissue sections. For each group, at least eight wounds were assigned at each time point.

2.9. Statistical analysis
All the experimental results were presented as mean ± standard deviation. Three independent experiments were carried out, and at least three samples per each test were taken for statistical analysis. Data were analyzed using SPSS Statistics 17.0 for one-way analysis of variance, followed by Tukey’s HSD post hoc test. Differences were considered statistically significant when $p < 0.05$.

3. Results and discussion
3.1. Characterization of PCL/gelatin fibers
Electrospinning method was employed to prepare nanofiber scaffolds of different thicknesses. Generally, a longer electrospinning time would result in a thicker fibrous scaffold when other parameters were kept constant. In the current study, electrospinning proceeded for 5 and 60 min were chosen to obtain the thin and thick fibrous scaffolds, then the morphology and structures of nanofibrous scaffolds were observed by SEM. We found that both the thin and thick scaffolds were characteristic of uniform fiber threads with smooth surfaces and random orientations. The diameter of the thin and thick fibrous scaffold were comparable with an average diameters of 432.65 ± 86.48 nm (figures 1(A)), and 434.41 ± 85.63 nm (figure 1(C)), corresponding to an average thickness of 42.78 ± 0.86 μm (figures 1(B)) and 441.72 ± 9.95 μm (figure 1(D)), respectively. These indicated that deposition time had almost no impact on the fiber fineness but strongly increase the thickness of PCL/gelatin nanofiber scaffolds (see table S1 of the supplementary data, available online at stacks.iop.org/JPMATER/2/044001/mmedia). Thus, the thickness of PCL/gelatin nanofiber scaffolds can be controlled with ease by regulating the deposition time when other parameters are kept constant.
3.2. PCL/gelatin fibers promoting cell proliferation and decreasing expression of ECM related genes in vitro

As a mimic to natural ECM, nanofiber scaffolds have been found to provide a favorable matrix for cell proliferation and formation of ECM products [16, 32]. Cell proliferation assay suggested that the nanofiber scaffolds were cytocompatible, with significantly higher cell growth rates on nanofiber scaffolds than on TCP control (figure 2(A)). As observed, cells were unable to attach well on blank non-adherent coverslips (data not shown). Therefore, the role of non-adherent coverslips beneath thick or thin nanofiber scaffolds in inducing cell proliferation was ignorable. Furthermore, human dermal fibroblasts grew faster on thick scaffolds than on thin scaffolds (figure 2(A)). Pathological scars, including hypertrophic scars and keloids, are significant skin fibrosis problems resulting from an abnormal tissue repair process after skin trauma [33]. The major characteristic of hypertrophic scars and keloids is a metabolism disorder of collagen-based ECM proteins [4, 34]. The effect of nanofibers on fibrosis-associated genes [35], such as collagen I, collagen III and fibronectin, are important for understanding the cellular responses to scaffolds and particularly the effects on scar formation [8]. The expression of collagen I, collagen III and fibronectin in the cells cultured on nanofiber scaffolds were down-regulated compared to TCP control (figure 2(B)). More importantly, a stronger suppression effect of these genes was observed when the thickness of scaffold was increased. The aforementioned findings indicated that nanofiber scaffolds promoted the proliferation of human dermal fibroblasts but decreased the gene expression of collagen I, collagen III and fibronectin. Moreover, the attenuation of the fibrosis-associated genes was positively correlated to the thickness of the scaffolds.

3.3. TGF-β1-dependent attenuation of fibrosis-related genes expression induced by nanofiber scaffolds

A number of key pro-fibrotic cytokines are responsible for driving the process of fibrogenesis. Among these pro-fibrotic cytokines, TGF-β1 is considered to be the main pro-fibrogenic mediator and recognized as the major inducer of dermal fibroblast proliferation [36, 37]. We also found that TGF-β1 receptor inhibitor A83-01 decreased collagen I, collagen III and fibronectin expression in human dermal fibroblasts in a dose-dependent manner (figure S1). To confirm whether TGF-β1 involved in the inhibition of fibrosis-related genes induced by PCL/gelatin nanofiber scaffolds, we compared the TGF-β1 expression level in the cells cultured on indicated substrates after culturing for 2 d. Real-time PCR showed that PCL/gelatin nanofiber scaffolds down-regulated the expression of TGF-β1 compared with control (figure 3(A)), and exhibited a negative correlation between TGF-β1 expression and the thickness of nanofiber scaffolds, which was similar to the attenuation patterns of fibrosis related genes. Thus, we proceeded to investigate whether PCL/gelatin nanofiber scaffolds-induced attenuation of fibrosis-related genes expression is TGF-β1-dependent. TGF-β1 treatment led to a significant expression of collagen I, collagen III and fibronectin in cells compared with untreated group (figures 3(B)–(D)). While, the enhanced effect on collagen I, collagen III and fibronectin expression was attenuated by nanofiber scaffolds, and the inhibitory effect also was thickness dependent, with thicker scaffolds leading to stronger attenuation. Interestingly, the depressed effect on collagen I, collagen III and fibronectin induced by nanofiber scaffolds was offset by TGF-β1 treatment. These data suggest that nanofiber scaffolds inhibits fibrogenesis in TGF-β1-stimulated human dermal fibroblasts through suppression of phosphorylation of Smad3. Overall, these results proved that nanofiber scaffolds attenuated of fibrosis-related genes expression through inhibiting TGF-β1.
3.4. TSG-6-dependent attenuation of fibrosis-related genes expression induced by nanofiber scaffolds

Fibroblasts are involved in wound healing and fibrosis through TGF-β1-triggered differentiation into contractile and α-SMA-positive myofibroblasts. TSG-6 had been reported to facilitate the myofibroblastic differentiation of fibroblasts, which was TGF-β1-dependent [15]. Our results showed that TGF-β1 treatment remarkably enhanced the expression of α-SMA and TSG-6, which were two markers for myofibroblastic differentiation in human dermal fibroblasts. However, nanofiber scaffolds obviously abrogated the expression of α-SMA and TSG-6 with or without the stimulation of TGF-β1 (figures 3(E) and (F)). These findings indicated that TSG-6 might play an important role in inhibiting the conversion of fibroblasts to myoblasts by nanofiber scaffolds.

TSG-6 has been proved to be essential in the TGF-β1-dependent phenotypic activation of fibroblasts [38, 39], as well as in the myofibroblastic differentiation. To further confirm whether nanofiber scaffolds down-regulated collagen I and collagen III expression through TSG-6, we constructed p3XFLAG-cmv-TSG-6 plasmid (pTSG-6), and an optimal DNA/Lipo2000 ratio of 1.0/2.4 (specifically 2.5 μg p3XFLAG-cmv-13 (pVector) or pTSG-6 and 6 μl Lipo2000) was chosen in following experiments, and RT-PCR results showed that the expression of collagen I and collagen III were increased in pTSG-6 transfected cells, with mock transfected cells as control (figure S2). When pTSG-6 transfected cell were cultured on fibers, the cells showed significantly higher expression of TSG-6 (figures 4(A) and (D)) as well as collagen I (figure 4(B)) and collagen III (figure 4(C)) when compared with the mock transfected cells, in which the expression of abovementioned genes were strongly inhibited by fiber compared to blank control. Under TGF-β1 stimulation, similar trends were maintained, with partial inhibition of the genes in cases of fiber treatment but the expression of these genes was improved when TSG-6 was over-expressed (figures 4(E) and (F)).

Figure 4(D) confirmed that the mRNA expression level of TSG-6 was enhanced following TGF-β1 stimulation, which was similar to the result reported by John [15]. Furthermore, the overexpression of TSG-6 partially impaired the inhibitory effect on collagen I and collagen III expression induced by nanofiber scaffolds treatment (figures 4(E) and (F)). However, overexpression of TSG-6 did not affect the mRNA expression level of TGF-β1 in cells neither with (figure 4(H)) nor without (figure 4(G)) nanofiber scaffolds treatment. Additionally, with different concentrations of A83-01 treatment suppressed TSG-6 mRNA expression in a dose-dependent manner (figure 4(I)). Collectively, these results suggested that TSG-6 should be the downstream of TGF-β1 in enhancing the expression of fibrosis-related genes, and nanofiber scaffolds abrogated the mRNA expression levels of the genes through down-regulation of TGF-β1 and TSG-6.

3.5. Nanofiber scaffolds accelerating wound healing in a full-thickness skin defect model

We prepared nanofiber scaffolds with different thicknesses to find out the scar formation effects of nanofiber scaffolds on cutaneous wounds involve injury to both epidermis and the underlying dermis of collagen-rich
connective tissue. Three thicknesses of nanofiber scaffold (400, 800 and 1200 μm) with the same diameter as wounds were selected based on the thickness of rat’s full skin, which is up to 1200 μm. In the current study, the gross morphology of the wounds was monitored through the healing process (figure 5(A)), and it was found that the wounds were repaired with time in all groups, 14 d after surgery, all wounds were healed. However, the wound areas were significantly reduced in 400 and 800 μm groups compared to open control and 1200 μm group (figure 5(B)). Furthermore, during the healing process scab has fallen off happened twice. The first round was at day 4 in 400 and 800 μm groups but at nearly day 7 in open control and 1200 μm group. The second round was observed at about day 10 in 400 and 800 μm groups, while it was at about day 12 in open control and 1200 μm group. In conclusion, 400 and 800 μm groups enabled an accelerated wound healing process and earlier removal of scar debris from wound sites when compared with control and 1200 μm group.

3.5.1. Nanofiber scaffolds stimulating re-epithelialization and formatting skin appendages
Effective recovery of skin wounds depends not only on the closure of the wound itself, but also on the replacement of damaged ECM with freshly deposited collagen and the formation of new skin appendages. Structure of the epidermis and dermis had been damaged in the full-thickness wound model and obvious hyperplasia was noticed in all the samples at week 1 (figures 6(A)–(D)). In open control group, obvious clot tissue was observed, which might be caused by inflammation response after surgery. On contrast, no obvious clot tissue was observed in experimental groups at week 1 post surgery (figures 6(A’–(D’)), which indicated an anti-inflammation effect of nanofiber scaffolds [40]. Eschar was a collection of dead tissue that was tightly connected with the damaged tissue and falls faster in the 800 μm group. In parallel, hyperproliferative epithelial tissue formed in all groups, however, longer epithelial tongues were observed in 400 and 800 μm groups compared
with control and 1200 μm groups. In addition, full coverage of new epithelium was formed after 1 week of treatment in 400 and 800 μm groups, suggesting epithelial regeneration rates was faster in both the 400 and 800 components. It was reported that the formation of new skin appendages is an important hallmark of decreased scarring [8, 38]. After 2 weeks of treatment, repaired tissue in 400 and 800 μm groups generated more skin appendages compared to the open control, while in 1200 μm group nearly no skin appendages was found around the wound sites (figures 6(E′)–(H′)). Thus, the better healing and skin appendages formation in 400 μm and 800 μm groups suggested that the thickness of nano fiber scaffolds played an important role in regulating the formation of scar in vivo.

3.5.2. Nano fiber scaffolds attenuating the collagen deposition and TGF-β1 expression in vivo

Hypertrophic scarring is mainly caused by excessive collagen production by fibroblasts [4, 5]. Masson’s trichrome staining was performed to assess the collagen deposition (stained in blue color) in wound sites at days 7 and 14. 800 μm group displayed remarkably less amount of collagen deposition than other groups, while no obvious morphology difference was observed among the rest three groups (figures 7(A) and (B)). Compared to the open control group, 400 and 800 μm groups showed lower amounts of collagen I (stained in red or bright yellow) and collagen III (stained in green) by Picro-sirius red staining. Furthermore, more mature collagen fibers were observed in 800 μm group with the morphology of the newly formed collagen I, while the shape of the

Figure 5. Nanofiber scaffolds treatment affected wound healing and removal of scabs. (A) Representative gross morphology of wounds in different groups at specific time intervals. Scale bar: 3 mm. (B) Quantification of wound area in different groups at specific time intervals. Data are presented as mean ± SD of four samples and compared with Tukey’s HSD post hoc test, \( p^* < 0.05 \).

Figure 6. Nanofiber scaffolds treatment modulated re-epithelialization and formation of skin appendages. H and E staining of cutaneous wound sites treated with nanofiber scaffolds of different thicknesses at week 1 (A)–(D) and week 2 (E)–(H). A′–H′, the higher-power images of the areas of skin wounds in A–H, respectively. C, Clot; ES, eschar; G, granulation tissue; HE, hyperproliferative epithelium (A′)–(D′). Black arrowhead indicate hair follicle and yellow arrowhead indicate sweat glands (E′), (F′), and (G). Scale bar: 500 μm.
newly formed collagen I seemed slenderer in other three groups at day 7 (figure 7(C)). However, there was no obvious difference between each group at day 14 (figure 7(D)). Furthermore, RT-PCR results showed the lowest expression levels of collagen I and collagen III in 800 μm group at days 7 and 14 (figures 7(E) and (F)). Moreover, the ratio of collagen I to collagen III in 800 μm samples was significantly decreased compared with control at day 7 (figure 7(G)). These results demonstrated that 400 and 800 μm nanofiber scaffolds could attenuated collagen deposition and decreased the expression ratio of collagen I/III, which contributed to prevent scarring [41]. As we had proved that TGF-β1 led to the expression of fibrosis-related genes, to further confirm whether TGF-β1 plays an important role in the process of wound healing and scar formation in vivo, the mRNA expression level of TGF-β1 in cutaneous wounding sites was analyzed at days 7 and 14. The expression of TGF-β1 was increased
in 400 μm group and 1200 μm group compared to open control group, while the mRNA expression of TGF-β1 was decreased in 800 μm group. While, the TGF-β1 expression levels in the three scaffolds were all down-regulation at day 14 compared with control (figure 7(I)), which was consistent with our in vitro observations. Taken together, nanofiber scaffolds could decrease the scar formation through inhibiting TGF-β1 and attenuating collagen deposition, and the thickness of PCL/gelatin nanofiber scaffolds should be appropriately optimized for application.

4. Discussion

Scar formation is a pathogenesis of the dermis characteristic of the overcompensation of wound defect. In previous studies, PCL/gelatin nanofiber scaffolds distinctly induced the angiogenesis, collagen deposition and re-epithelialization in the wound site in diabetic mouse model, as well as inhibited the inflammation reaction [42]. Although PCL/gelatin nanofiber scaffolds provide great potential in the treatment of dermal wounds [24], the effects of fibrous scaffold on scar formation and the underlying mechanism that regulating fibrosis-associated ECM molecules remain elusive.

As the most extensively characterized factor influencing scar formation, TGF-β mediates the expression of collagen I and deposition of other fibrosis-associated ECM [8]. In the current study, it was found that nanofiber scaffolds attenuated the expression of fibrosis-relevant genes through suppressing TGF-β1 expression. Previous data proved that human amniotic epithelial cells attenuate TGF-β1-induced human dermal fibroblast transformation to myofibroblasts via TGF-β1/Smad pathway [43], and multiple studies documented that TGF-β1/Smad pathway had critical functions in promoting fibrosis and scar formation [44, 45].

In the investigation of myofibroblastic differentiation of fibroblasts, the expression of α-SMA in response to nanofiber scaffolds was similar to the expression pattern of fibrosis-related genes, suggesting that nanofiber scaffolds affected the transition of dermal fibroblasts to myofibroblasts. Our results demonstrated that TSG-6 was down-regulated by PCL/gelatin nanofiber scaffolds with or without treatment of TGF-β1, suggesting that TSG-6 was a key factor in the acquisition of myofibroblast phenotype. On the other hand, the overexpression of TSG-6 partially impairs the inhibitory effect on collagen I and collagen III expression induced by nanofiber scaffolds treatment. However, the mRNA expression level of TGF-β1 in cells cultured on TCP or nanofiber scaffolds was not affected by overexpression of TSG-6. Taken together, PCL/gelatin nanofiber scaffolds hindered conversion of fibroblasts to myofibroblasts by downregulating the expression levels of collagen I and collagen III through inhibiting TGF-β1/TSG-6 pathway.

In the full-thickness skin wound model, 400 and 800 μm nanofiber scaffolds-treated wounds repaired with newly formed skin appendages, quicker removal of cellular debris, higher re-epithelialization rates, and lower granulation tissue areas. Although faster wound healing was observed in all nanofiber-treated groups when compared with the control before day 10, the 400 and 800 μm nanofiber scaffolds exhibited enhanced fur growth and less scar tissue formation at day 14 post-surgery. H and E staining demonstrated that less clot tissue and faster re-epithelialization were found in 400 and 800 μm group at day 7 compared to open control group, suggesting that appropriate thickness of nanofibers possess a high surface-to-volume ratio and help to promote the adhesion and proliferation of epithelial cells [46]. Our Masson’s trichrome and Picro-sirius red staining results further confirmed that nanofiber scaffolds contributed to wound healing and skin appendages regeneration with 800 μm as the optimal thickness.

In summary, the present study demonstrated that PCL/gelatin nanofiber scaffolds helped to reduce scar formation by attenuating fibrosis-related genes expression through inhibiting TGF-β1/TSG-6, and the inhibitory effect also depended on the thickness of scaffold. Thus, proper thickness of PCL/gelatin nanofiber scaffolds may facilitate an optimal regeneration of full-thickness skin wound without scars formation. And our in vivo results proposed that nanofiber scaffolds modulated the expression of TGF-β1, and the decreased expression of TGF-β1 may be contributed to release from PCL/gelatin nanofiber scaffolds (data not shown) [30]. In the future, a more comprehensive investigation is warranted to validate this hypothesis and verify the linkage between release patterns and anti-scarring effects.

5. Conclusion

In this study, we obtained PCL/gelatin nanofiber scaffolds with different thicknesses, which inhibited the expression of fibrosis-related genes in a thickness-dependent manner in vitro. Moreover, the decreased expression of fibrosis-related genes through TGF-β1/TSG-6 pathway, which led to inhibited transition of fibroblasts to myofibroblasts in vitro and decreased deposition of collagen I and collagen III in a full-thickness skin wound model. These findings suggested that nanofiber scaffolds of appropriate thickness provided not only an ECM mimic but also attenuated the expression of TGF-β1, the latter of which may help prevent the
formation of scars. Thus, PCL/gelatin nanofiber scaffolds could be adopted for scar-free skin wound healing and skin cosmetics applications.

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