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Development of Biomimetic Tilapia Collagen Nanofibers for Skin Regeneration through Inducing Keratinocytes Differentiation and Collagen Synthesis of Dermal Fibroblasts

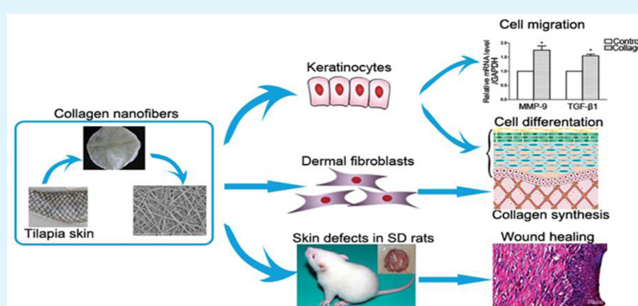
Tian Zhou,[†] Nanping Wang,[‡] Yang Xue,[†] Tingting Ding,[†] Xin Liu,[†] Xiumei Mo,[*,§] and Jiao Sun[*,†]

[†]Shanghai Biomaterials Research & Testing Center, Shanghai Key Laboratory of Stomatology, Ninth People's Hospital, Shanghai Jiaotong University School of Medicine, Shanghai 200023, China

[‡]Shanghai Fisheries Research Institute, Shanghai 200433, China

[§]College of Chemistry, Chemical Engineering and Biotechnology, Donghua University, Shanghai 201620, China

ABSTRACT: In this study, tilapia skin collagen sponge and electrospun nanofibers were developed for wound dressing. The collagen sponge was composed of at least two α-peptides, and its denaturation temperature was 44.99 °C. It did not change the number of spleen-derived lymphocytes in BALB/c mice, the ratio of $CD4^+/CD8^+$ lymphocytes, and the level of IgG or IgM in Sprague−Dawley rat. The contact angle, tensile strength, and weight loss temperature of collagen nanofibers were 21.2°, 6.72 ± 0.44 MPa, and 300 °C, respectively. The nanofibers could promote the viabilities of human keratinocytes (HaCaTs) and human dermal fibroblasts (HDFs), inducing epidermal differentiation through the gene expression of involucrin, filaggrin, and type I transglutaminase of HaCaTs, and they could also accelerate migration of HaCaTs with the expression of matrix metalloproteinase-9 and transforming growth factor-β1 (TGF-β1). Besides, the nanofibers could upregulate the protien level of Col-I in HDFs both via a direct effect and TGF-β1 secreted from HaCaTs, thus facilitating the formation of collagen fibers. Furthermore, the collagen nanofibers stimulated the skin regeneration rapidly and effectively in vivo. These biological effects could be explained as the contributions from the biomimic extracellular cell matrix structure, hydrophilicity, and the multiple amino acids of the collagen nanofibers.



KEYWORDS: *electrospinning tilapia collagen nanofibers, HaCaTs differentiation, TGF-β1, collagen synthesis, skin regeneration*

INTRODUCTION

It has been demonstrated in numerous studies that mammal collagen (especially porcine or bovine collagen) has excellent biocompatibility.[1] Therefore, this collagen has been extensively used for skin wound healing. However, mammal collagen still presents a risk of transmission of animal diseases, such as bovine spongiform encephalopathy and foot-and-mouth disease.[2,3] Additionally, the application of mammal collagen is restricted because of religious reason.[4] Recently, marine collagen has gradually attracted attention because of its abundance and low price. In 2008, Sankar et al.[5] first prepared collagen nanofibers from discarded fish scales (*Lates calcarifer*) that had enough tensile strength (2 MPa) for use as a wound-dressing material. After compounding with polymers or plant extracts, fish collagen can promote the proliferation of skin cells.[6−8] However, the immunogenicity of fish collagen and the pathway and mechanism underlying its biological functions for skin regeneration are still unknown. These issues are directly associated with future clinical applications of fish collagen as a wound-dressing material; therefore, they are worth exploring.

In recent years, studies focused on marine collagen have indicated that the denaturation temperature of tilapia fish scale collagen reached 48 °C, which was higher than body temperature and suggested good thermal stability.[9] In 2012, Terada et al. constructed a chitosan−collagen composite scaffold and found that this scaffold promoted the growth of oral mucosal keratinocytes in vitro.[10] Relative to fish scales, fish skin contains a higher level of protein (90.6%),[11] which is close to the structure of human skin collagen. Therefore, fish skin collagen has a higher application value. In 2014, preliminarily studies related to the biocompatibility of tilapia skin collagen were conducted by Yamamoto et al.[12] However, it is unclear whether tilapia skin collagen could be prepared as a wound dressing that produces excellent biological effects and effectively induces skin regeneration. It is a challenging subject with academic research value.

Human keratinocytes (HaCaTs) and human dermal fibroblasts (HDFs) are two types of key cells in skin wound healing. The viability, differentiation, and migration of keratinocytes play important roles in wound re-epithelialization. In addition, the adhesion, proliferation, and cytokine secretion of dermal fibroblasts can effectively promote dermal healing. It

Received: November 14, 2014
Accepted: January 19, 2015
Published: January 19, 2015

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had been reported by Fu et al. that mammal collagen could promote migration of keratinocytes though the expression of matrix metalloproteinase-9 (MMP-9).[13] Furthermore, a collagen bandage facilitated the proliferation of HDFs,[14] which was benifical for wound healing. Therefore, as a wound dressing, it is important to study its biological effects on these two types of skin cells and validate its healing effects in vivo, which are crucial to assess the potential of new materials for clinical application.

In the present study, high-purity fish collagen sponge was extracted from abandoned tilapia skin through a series of processing and purification technologies. Its amino acid composition and thermal denaturation temperature were analyzed, and the immunogenicity of collagen sponge was evaluated from the perspectives of humoral immunity and cellular immunity. In addition, collagen nanofibers with biomimic extracellular matrix (ECM) components and structures were further prepared by electrospinning, and their morphological structure, hydrophilicity, and mechanical properties were characterized. Furthermore, HaCaTs and HDFs were chosen to evaluate the effects of collagen nanofibers on cell adhesion and proliferation. The mechanisms that promote wound healing by collagen nanofibers at the gene and protein levels were discussed, focusing on the associations between HaCaTs differentiation and the expression of transforming growth factor-β1 (TGF-β1) and MMP-9 with epithelium regeneration. It was further investigated whether the expression of Col-I in HDFs, which was related to dermis regeneration, was affected by the collagen nanofibers. Finally, Sprague–Dawley (SD) rat models with full-thickness skin defects were used to confirm the ability of collagen nanofibers to accelerate skin regeneration. This research provides a scientific basis for the application of this novel electrospun tilapia collagen nanofibers as wound dressings.

■ EXPERIMENTAL SECTION

Preparation of Tilapia Collagen Sponge. Tilapia skin (provided by Shanghai Fisheries Research Institute) was washed, chopped, and stirred in 0.1 M NaOH solution for 1–2 days. The samples were then soaked in 0.5–1 M acetic acid for 4–8 h with continuous stirring. The supernatant was collected by centrifugation at 10 000*g* followed by the addition of 0.1–0.5% pepsin with stirring for 24–48 h. Next, 0.4 M ammonium sulfate was added and the precipitate was collected by centrifugation at 10 000*g*. The precipitate was then dissolved in 0.5–1 M acetic acid, dialyzed, and lyophilized (Labconco Freeze-Dryer FreeZone 6 L) to obtain collagen sponges for subsequent use.

Characterization of Tilapia Collagen Sponge. The purity and molecular weight of the tilapia collagen sponge were determined using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (FR-980; Furi Science and Technology Co., Ltd., Shanghai, China). The amino acid content of the collagen sponge was determined using high-performance liquid chromatography (HPLC) (Agilent 1100). The denaturation temperature of the collagen sponge was measured with differential scanning calorimetry (DSC) (TAS-100, Rigaku Co., Tokyo, Japan).

Lymphocyte Proliferation Assay. The experimental protocol was approved by the Animal Care and Experiment Committee of the Ninth People's Hospital affiliated to the School of Medicine, Shanghai Jiao Tong University. BALB/c mice were anesthetized using sodium pentobarbital, and the spleens were collected under sterile conditions. The lymphocyte suspension was collected by adding red blood cell lysis buffer and diluting to 1×10^6 cells/mL. Lymphocytes were seeded on tilapia collagen sponges in 24-well plates. They were cultured in RPMI 1640 medium supplemented with 10% FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin. In addition, lymphocytes seeded on cover slips with or without the addition of concanavalin A (ConA) were used as negative and positive controls, respectively. After culture for 7 d, Cell Counting Kit-8 (CCK-8) solution was added, and the lymphocytes were incubated for 3 h. The optical density (OD) values at 570 nm were measured using a spectrophotometer.

Subcutaneous Implantation Assay in SD Rats. A total of eight healthy male 6–8-week-old SD rats (200–250 g) were used in this study. The experimental protocol was approved by the Animal Care and Experiment Committee of the Ninth People's Hospital affiliated to the School of Medicine, Shanghai Jiao Tong University. Eight rats were randomly divided into two groups. After anesthetization by sodium pentobarbital, the dorsal skin was longitudinally transected under sterile conditions. The subcutaneous tissues were separated, a tilapia collagen sponge with an area of approximately 1×1 cm^2 was implanted, and the skin was then sutured. The control group received a sham operation without the implantation. Twenty-eight days after the operation, the SD rats were anesthetized, and blood samples were collected by cardiac puncture. The concentrations of IgG and IgM in the serum were detected using enzyme-linked immunosorbent assay (ELISA) reagent kits (R&D), and CD4$^+$ and CD8$^+$ T lymphocytes (BioLegend) were counted by flow cytometry (FCM).

Fabrication of Electrospinning Tilapia Collagen Nanofibers. The tilapia collagen sponge was dissolved in a hexafluoroisopropanol (HFP) solution (Fluorochem Ltd.). The polymer was then placed in a plastic syringe and inserted in a syringe pump (789100C, Cole-Parmer). The electrospun tilapia collagen nanofibers formed membranes under high voltage. The collagen nanofibers were cross-linked using glutaraldehyde vapor and stored in a vacuum-drying oven.

Characterization of Tilapia Collagen Nanofibers. The chemical structure and phase composition of tilapia collagen nanofibers were determined by Fourier transform infrared spectroscopy (FTIR) (Avatar 380) and X-ray diffraction (XRD) (D8 ADVANCE, Bruker). The morphology of the collagen nanofibers was observed using scanning electron microscopy (SEM) (JEOL JSM-5600). The mean fiber diameters were determined with image analysis software (Image-J, National Institutes of Health) and calculated by selecting 100 fibers randomly. The pore size of the collagen nanofibers was measured using a specific surface and pore size analyzer (JW-BK122T-B). The contact angle was measured using a contact angle-measuring instrument (OCA40, Dataphysics). The mechanical strength was analyzed using a universal materials testing machine (H5K-S, Hounsfield). The weight loss temperature was determined with a thermogravimetric analyzer (Pyris 1, PerkinElmer).

Cell Viability Assay. HaCaTs (purchased from Kunming Animal Institute) were seeded on tilapia collagen nanofibers in 24-well plates, at a density of 2.5×10^4 cells/well in 500 μL of DMEM high-glucose medium supplemented with 10% FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin. After culture for 24 h, cell morphology was observed using SEM, fluorescence microscopy (Leica) and confocal laser-scanning microscopy (CLSM, Leica YCS SP2). HaCaTs were then seeded on the collagen nanofibers at 2.5×10^4 cells/well, and HaCaTs seeded on cover slips made of high borosilicate glass were used as a control. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed. After the HaCaTs were cultured for 1, 3, or 5 d, 5 mg/mL MTT (Sigma) was added, and the cells were incubated for 4 h. Dimethyl sulfoxide (DMSO) was then added to dissolve the crystals. The OD values at 570 and 630 nm were measured using a spectrophotometer (Labsystems Dragon Wellscan MK3). Cell viability was calculated according to the OD values. HDFs (purchased from Kunming Animal Institute) were also seeded on tilapia collagen nanofibers at a density of 0.5×10^4 cells/well in 500 μL of DMEM high-glucose medium supplemented with 10% FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin. Cell morphology was observed using SEM and fluorescence microscopy after culturing for 24 h. Cell viability was calculated with the MTT assay after 1, 3, and 5 d.

Real-Time Polymerase Chain Reaction (PCR). HaCaTs were seeded on tilapia collagen nanofibers at a density of 3×10^5 cells/well in 6-well plates. The cells seeded on cover slips were used as a control. After culturing for 24 h, total RNA was extracted using TRIZOL reagent (Invitrogen). RNA (1.0 μg) was reverse-transcribed into

DOI: 10.1021/am507990m
ACS Appl. Mater. Interfaces 2015, 7, 3253–3262

cDNA using a PrimeScript first strand cDNA synthesis kit (TaKaRa). The expression of differentiation-related genes, such as involucrin, filaggrin, and type I transglutaminase (TGase1), was detected using real-time PCR with SYBR Premix EX Taq (TaKaRa) in a MyiQ2 thermal cycler (Bio-Rad). In addition, HaCaTs and HDFs were seeded on collagen nanofibers for 3 d. The wound-healing-associated genes, such as TGF-β1 and MMP-9 in HaCaTs and Col-I in HDFs, were detected. Furthermore, the supernatant was collected from the HaCaTs cultured on collagen nanofibers for 24 h. The HDFs were then cultured with this supernatant for 24 h. The expression of Col-I in HDFs was detected using real-time PCR. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. The primers are listed in Table 1.

Table 1. Real-Time RT-PCR Primer Sets

gene/oligo name	oligo sequence
involucrin forward	TCAATACCCATCAGGAGCAAATG
involucrin reverse	GAGCTCGACAGGCACCTTCT
TGase1 forward	TCTTCAAGAACCCCCTTCCC
TGase1 reverse	TCTGTAACCCAGAGCCTTCGA
filaggrin forward	CCATCATGGATCTGCGTGG
filaggrin reverse	CACGAGAGGAAGTCTCTGCGT
TGF-β1 forward	ATTCCTGGCGATACCTCAG
TGF-β1 reverse	TAAGGCGAAAGCCCTCAAT
MMP-9 forward	GCCTGCAACGTGAACATCT
MMP-9 reverse	TCAAAGACCGAGTCCAGCTT
Col-I A1 forward	CCTGGAAAGAATGGAGATGA
Col-I A1 reverse	CCAAACCACTGAAACCTCTG
GAPDH forward	TGAACGGGAAGCTCACTGG
GAPDH reverse	TCCACCACCCTGTTGCTGTA

Enzyme-Linked Immunosorbent Assay (ELISA). The HaCaTs and HDFs were seeded on tilapia collagen nanofibers at a density of 3 $\times 10^5$ cells/well in 6-well plates. The cells seeded on cover slips were used as a control. After culturing for 24 h, the secretion of TGF-β1 from the HaCaTs and the secretion of Col-I from the HDFs into the supernatant were detected with ELISA reagent kits (Wuhan Huamei Biotech Co., Ltd.). Next, the supernatant was collected from the HaCaTs cultured on collagen nanofibers for 24 h. The HDFs were then cultured with this supernatant for 24 h, and the secretion of Col-I from HDFs was detected.

Skin-Wound-Healing Experiment in SD Rats. The experimental protocol was approved by the Animal Care and Experiment Committee of the Ninth People's Hospital affiliated to the School of Medicine, Shanghai Jiao Tong University. Twelve healthy 6−8-week-old male SD rats with a body weight of 200−250 g (each) were injected with sodium pentobarbital (Sigma-Aldrich, St. Louis, MO). Three full-thickness skin defects with a diameter of 1.8 cm were incised on the dorsal region of each rat. These wounds were covered with tilapia collagen nanofibers or Kaltostat (ConvaTec), which is commonly used clinically as a wound dressing. The control group was not covered with any material (n = 3). Adhesive Tegaderm (3M) polyurethane films were used to attach the dressings to the wounds. At 4, 7, 10, and 14 d after surgery, the morphology of the wounds was examined. Subsequently, the animals were sacrificed to collect skin tissues.

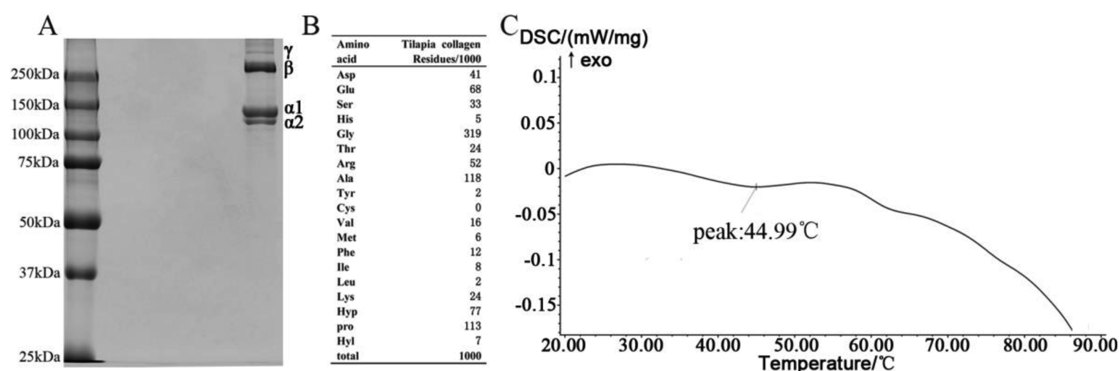
Histopathological Examination. The harvested samples collected on days 4, 7, 10, and 14 were fixed in 10% formaldehyde for 1 week, paraffin-embedded, and sectioned into 4-μm-thick sections using a microtome (Leica, Tokyo, Japan). The sections were stained with hematoxylin−eosin (H&E) and Masson's trichrome stain and then dehydrated, washed, mounted, and observed under a microscope.

Data Analysis. Data obtained from three separate experiments were expressed as the mean ± standard deviation. Statistically significant differences (p < 0.05) among the various groups were evaluated using one-way analysis of variance (ANOVA). All of the statistical analyses were performed using SPSS 11.0 software.

RESULTS AND DISCUSSION

Type I collagen accounts for approximately 70% of the total protein in fish skin.[15] In the present study, biomimetic electrospun fish collagen nanofibers were developed from tilapia skin through a series of extraction and purification techniques to investigate the potential for using fish collagen as a biomaterial. The immunogenicity of tilapia collagen sponge, the cellular effects of tilapia collagen nanofibers, and their effects on wound healing were evaluated. Additionally, we illuminated the pathway and mechanisms of skin regeneration induction by the collagen nanofibers and preliminarily assessed whether the collagen nanofibers could be used as wound dressings.

Characterization of Tilapia Collagen Sponge. Currently, the commonly used technologies to extract and purify collagen include acid dissolution and pepsin digestion. The former can maintain the triple helix structure of collagen to a maximal extent, and the latter can reduce the antigenicity of collagen by removing the N-terminal and C-terminal regions of collagen peptides. We combined these two methods and exploited their individual advantages to obtain a tilapia collagen sponge. The electrophoresis patterns (Figure 1A) showed that the extracted collagen sponge was composed of at least two α-peptides (α1 and α2) and contained high molecular weight β-chain and γ-chain bands that were cross-linked by α-chain molecules. The electrophoretic migration rates and composition of peptides suggested that the collagen sponge maintained its structural integrity, contained scarce amounts of impure



Amino acid	Tilapia collagen Residues/1000
Asp	41
Glu	68
Ser	33
His	5
Gly	319
Thr	24
Arg	52
Ala	118
Tyr	2
Cys	0
Val	16
Met	6
Phe	12
Ile	8
Leu	2
Lys	24
Hyp	77
pro	113
Hyl	7
total	1000

Figure 1. Characterization of tilapia collagen sponge. (A) SDS−PAGE pattern. (B) Amino acid composition. (C) Thermal denaturation curve.

DOI: 10.1021/am507990m
ACS Appl. Mater. Interfaces 2015, 7, 3253−3262

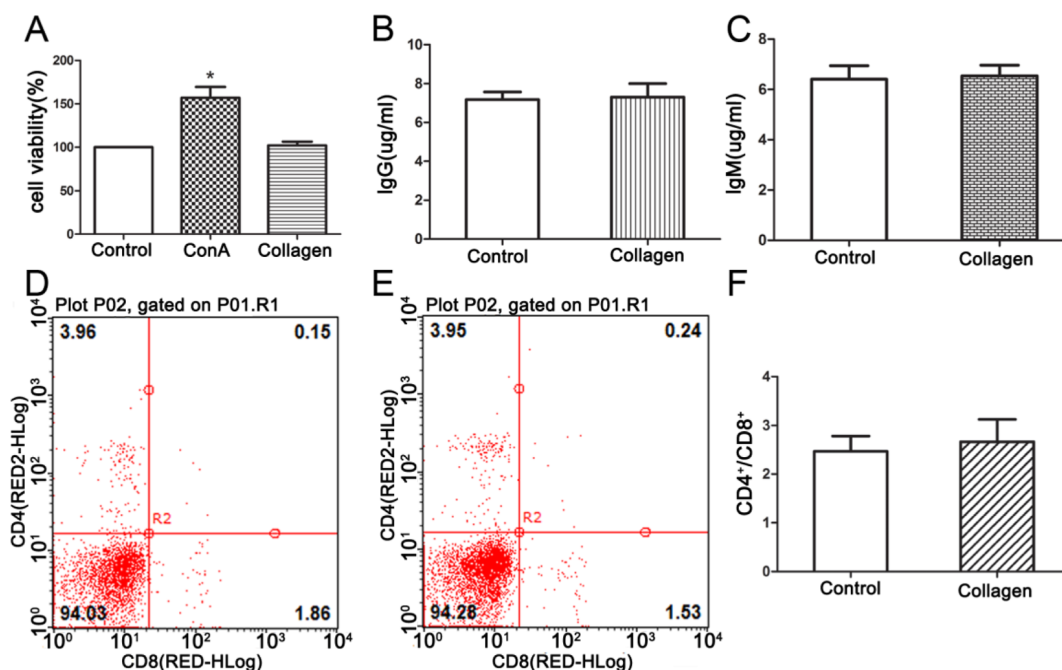


Figure 2. Immunogenicity analysis of tilapia collagen sponge and control groups. (A) Proliferation of lymphocytes cultured on the collagen sponge for 7 days. (B) The level of IgG in the serum of rat over 28 d. (C) The level of IgM. (D) Flow cytometry analysis of the control group showing the levels of $CD4^+$ (upper left quadrant) and $CD8^+$ lymphocytes (lower right quadrant) in the blood of rat over 28 days. (E) Flow cytometry analysis of the collagen sponge group. (F) The ratio of $CD4^+/CD8^+$ lymphocytes.

proteins of low molecular weight, and had the typical type I collagen structure.

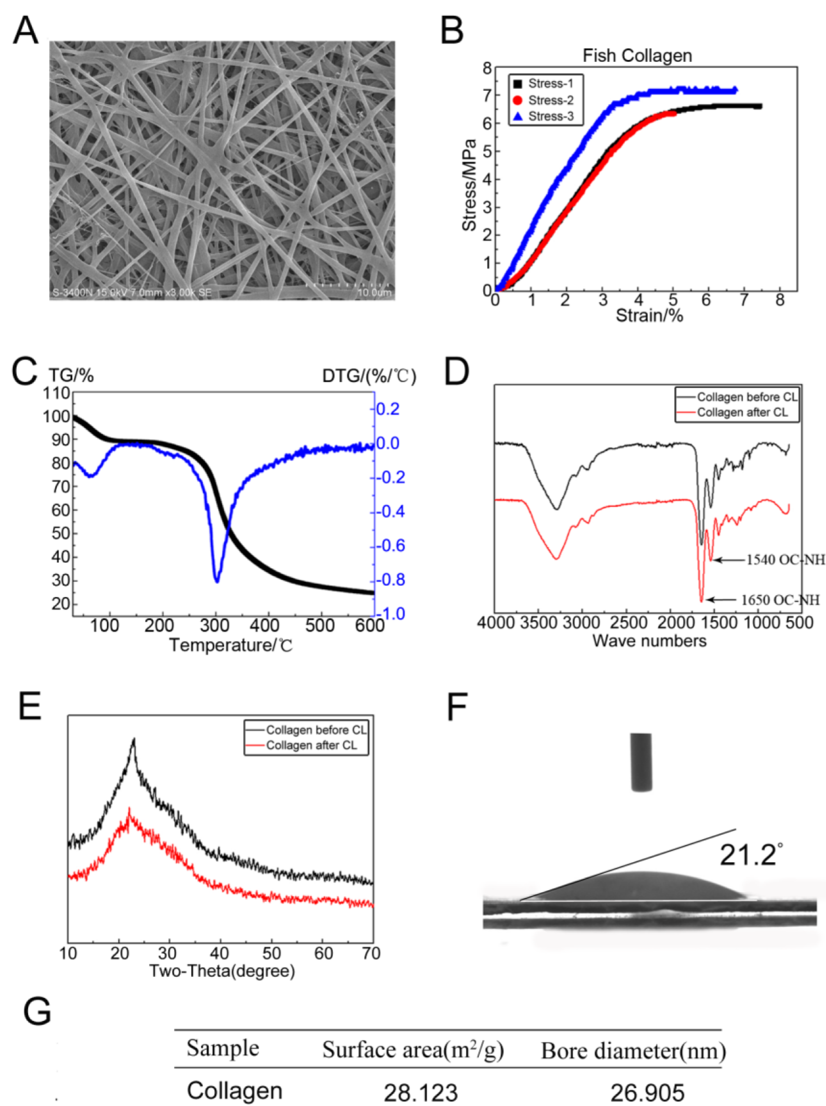
To further analyze the purity of tilapia collagen, the amino acid composition of the collagen sponge was measured. The results showed that the collagen sponge contained 19 different amino acids (Figure 1B); glycine (Gly) was the most abundant (31.9%), and hydroxyproline (Hyp) and proline (Pro) accounted for 7.7% and 11.3%, respectively. Thus, the tilapia collagen sponge was consistent with type I collagen and was extracted with high purity. Because the hydrogen bonds formed by Hyp and Pro play a key role in maintaining the triple-helix structure of collagen, the total amounts of these two amino acids determine the denaturation temperature of collagen, which is important for the clinical application of collagen. So far, the application of fish collagen has been limited in humans because of its low denaturation temperature. Although the denaturation temperatures of fresh water fish collagen are generally higher than those of deep-sea fish collagen (10 °C-20 °C), they are still lower than that of mammals due to the low living temperature and the low Hyp content.[16] However, it was shown by DSC that the denaturation temperature of tilapia collagen sponge was 44.99 °C (Figure 1C), which was similar to that reported by Chen et al. (higher than 40 °C) and was also close to that of mammal collagen (41 °C).[17,18] These features indicate that tilapia collagen sponge has the potential for medical applications.

Immunogenicity of Tilapia Collagen Sponge. Although tilapia collagen has an appropriate denaturation temperature for application in human preparations, its immunogenicity should also be evaluated, because it is a heterogenic protein. Previous reports have found that bovine collagen applied to human skin could cause hypersensitivity and increase the immunoglobulin level in 3−5% of patients,[19] and that porcine collagen could increase immunoglobulin IgG.[20] Whether tilapia collagen has immunogenicity is still unclear and has never been confirmed. The spleen is the largest immune organ in the body and contains a large number of immunocompetent B and T lymphocytes, which have the ability to recognize antigens and mediate humoral and cellular immunity, thus playing a primary role in immune response. Therefore, mixed lymphocytes were extracted from mouse spleen to evaluate the immunogenicity of tilapia collagen sponge in vitro. The results showed that the collagen sponge did not induce the proliferation of lymphocytes (Figure 2A).

Additionally, tilapia collagen sponge was implanted into the subcutaneous tissue of rats to investigate the immune response caused by the collagen sponge in the original state and small molecules produced during the process of its degradation. The two major antibodies, IgG and IgM, which play important roles in humoral immunity, were detected after 28 days. It was found that the collagen sponge did not induce significant changes in the level of IgG or IgM (Figure 2B,C). The ratio of $CD4^+$ and $CD8^+$ lymphocytes was further evaluated. $CD4^+$ lymphocytes participate in antigen recognition and signal transduction, whereas $CD8^+$ lymphocytes kill infected cells. A relatively stable ratio between $CD4^+$ and $CD8^+$ lymphocytes is important for coordinating cellular immunity. The results showed that the ratio of $CD4^+/CD8^+$ lymphocytes in the collagen sponge group was similar to that of the control group (Figure 2D−F). This finding indicated that the collagen sponge did not induce humoral or cellular immunity, which may be because the collagen sponge contains primarily nonaromatic amino acids. The immunogenicity of protein is largely caused by aromatic amino acids,[21] and the proportion of aromatic amino acids in tilapia collagen is significantly lower than that of mammal collagen.[22]

Characterization of Electrospun Tilapia Collagen Nanofibers. On the basis of the results that the tilapia collagen sponge did not generate immune responses, biomimetic wound dressings, which could be applied to the

DOI: 10.1021/am507990m
ACS Appl. Mater. Interfaces 2015, 7, 3253−3262



Sample	Surface area(m^2/g)	Bore diameter(nm)
Collagen	28.123	26.905

Figure 3. Characterization of tilapia collagen nanofibers. (A) SEM images. (B) Stress/strain curves. (C) TG spectra. (D) FTIR spectra before and after cross-linking. (E) XRD diagram before and after cross-linking. (F) Contact angle. (G) Physical characterization.

human body, were chosen to be developed in this study. ECM is mainly composed of collagen and has a reticular structure. In order to biomimic ECM, tilapia collagen nanofibers were fabricated as membranes by electrospinning and were cross-linked using glutaraldehyde vapor. SEM showed that the collagen nanofibers were smooth with a diameter of 310 ± 117 nm (Figure 3A), which was similar to the diameter of collagen fibers in human ECM.[23] Because the mechanical strength and thermal stability of wound dressings can be effectively enhanced by cross-linking,[24] the tensile strength of the collagen nanofibers was analyzed using a universal material testing machine. The results showed that the tensile strength of the collagen nanofibers was 6.72 ± 0.44 MPa (Figure 3B), which met the requirements for human skin (2.5−16 MPa).[25] Furthermore, the primary dehydration temperature of the collagen nanofibers and main weight-loss temperature of protein were 70 and 300 °C, respectively (Figure 3C), indicating that its thermal stability was suitable for human applications. These parameters may have been influenced via the increased interaction between collagen molecules induced by cross-linking to stabilize the triple-helix structure of collagen, which enhanced the mechanical strength and thermal stability of the collagen nanofibers. To further determine whether the chemical structure of the collagen nanofibers was altered after cross-linking, FTIR and XRD were performed to analyze changes in the characteristic absorption peaks of the amide groups and the diffraction peaks after cross-linking. It was found that the collagen nanofibers maintained α-helical structures after cross-linking. (Figure 3D,E).

Viabilities of HaCaTs and HDFs on Tilapia Collagen Nanofibers. It was further investigated whether the essential cells in wound healing, HaCaTs and HDFs, would be affected by tilapia collagen nanofibers with biomimetic ECM structure. First, the effects of the collagen nanofibers on the adhesion and proliferation of these two types of cells were studied in vitro. The results illustrated that after HaCaT cells and HDFs were seeded on the collagen nanofibers for 24 h, the cells were firmly attached and equally distributed. They were also cross-linked with excellent morphology (Figure 4). After being cultured on the collagen nanofibers for 5 d, the proliferation rates of the HaCaTs and HDFs were 114% (Figure 4) and 132% (Figure 5), respectively, indicating that the collagen nanofibers promoted cell adhesion and growth. These results may originate from the nanostructure (Figure 3A) and high specific

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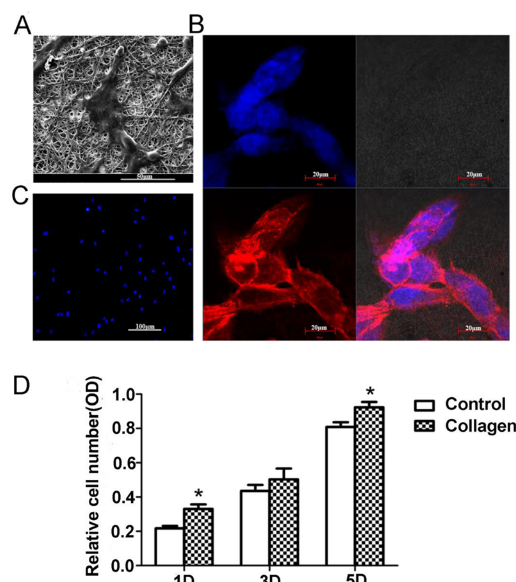

Figure 4. Adhesion and proliferation of HaCaTs cultured on tilapia collagen nanofibers. (A) SEM images of HaCaTs cultured for 1 day. (B) Fluorescence microscopy photographs of HaCaTs cultured for 1 day. The HaCaTs were stained with DAPI for nuclei (blue). (C) Confocal images of HaCaTs cultured for 1 day. The HaCaTs were double-stained with rhodamine-conjugated phalloidin for F-actin (red) and DAPI for nuclei (blue). (D) Cell viability of HaCaTs cultured for 1, 3, and 5 days. The control group was cultured on cover slips.

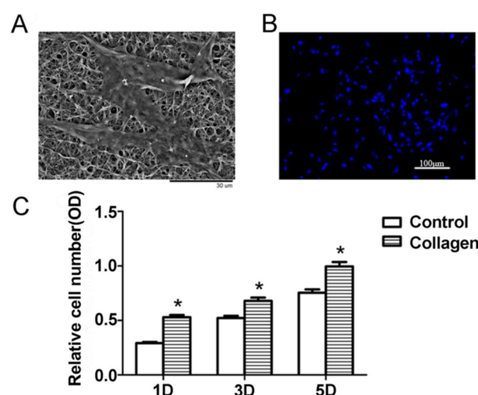

Figure 5. Adhesion and proliferation of HDFs cultured on tilapia collagen nanofibers. (A) SEM images of HDFs cultured for 1 day. (B) Fluorescence microscopy photographs of HDFs cultured for 1 day. The HDFs were stained with DAPI for nuclei (blue). (C) Cell viability of HDFs cultured for 1, 3, 5 days. The control group was cultured on cover slips.

surface area of collagen nanofibers (Figure 3G). It was reported that a greater number of cells adhered on the nanofibers than on the microfibers.[26] In addition, scaffolds with high specific surface area were more conducive to cell growth and the transportation of nutrients and metabolic products.[27] The viabilities of cells could also be related to the excellent hydrophilicity of the collagen nanofibers ($\theta = 21.2°$) (Figure 3F), because materials with good hydrophilicity have the ability to promote cell adhesion and proliferation.

Epidermal Differentiation Induced by Tilapia Collagen Nanofibers. In addition to confirming the cells-adhesion- and proliferation-inducing capacity of tilapia collagen nanofibers during wound healing, the differentiation of epidermal keratinocytes also reveals the speed and quality of re-epithelialization. Normal epidermis should include a basal layer, spinous layer, granular layer, and cornified layer. Whether collagen nanofibers can induce keratinocyte differentiation to each layer is a challenging research topic, and it has not been reported thus far. Therefore, keratinocyte (HaCaTs) differentiation influenced by collagen nanofibers was investigated without the addition of any growth factors. It was found that the collagen nanofibers significantly upregulated the expression of involucrin, filaggrin, and TGase1 genes (Figure 6A). Involucrin and filaggrin are independently expressed in spinous layer cells and granular layer cells, respectively. TGase1 is a key enzyme in the synthesis of cornified envelopes, which indicates the formation of cornified layer cells. The results demonstrated that the collagen nanofibers induced keratinocyte differentiation, which is required for the formation of integrated epidermis and might be closely associated with the amino acid composition of tilapia collagen. As shown in Figure 1B, collagen sponge contained significant amounts of Pro and limited amounts of tyrosine (Tyr) and methionine (Met). It has been reported that Pro and Tyr regulate the migration and differentiation of HaCaTs.[28,29] In addition, Met promotes the synthesis of proteins and nucleic acids as the most important methyl group donor, playing an important role in cell proliferation and differentiation. Our previous studies also found that the hydrolysis of tilapia collagen could induce cell differentiation because of its variety of amino acids.[30] These data further confirmed that the amino acids of tilapia collagen could effectively regulate HaCaTs differentiation.

Migration of HaCaTs Stimulated by Tilapia Collagen Nanofibers. Epidermal regeneration not only requires the differentiation of keratinocytes but also requires the migration of keratinocytes to the wound sites. Multiple genes, such as MMP-9 and TGF-β1, participate in wound healing. Therefore, the effect of tilapia collagen nanofibers on these two types of genes was considered. The results showed that the expression of MMP-9 and TGF-β1 was promoted by tilapia collagen nanofibers (Figure 6B). MMP-9 plays an important role in HaCaTs migration and re-epithelialization through the regulation of ECM.[29] Furthermore, TGF-β1 is an important regulatory factor that can directly regulate the deposition of ECM and stimulate the expression of effector molecules such as MMPs. These results suggested that the collagen nanofibers could accelerate re-epithelialization by promoting HaCaTs migration.

Col-I Protein Upregulated by Tilapia Collagen Nanofibers. The ability to form collagen fibers by dermal fibroblasts was an important parameter to measure for the evaluation of the effects of wound dressings on repairing full-thickness skin defects. To explore the dermis regeneration influenced by tilapia collagen nanofibers, we focused on dermal fibroblasts, which are essential cells of the dermis. Col-I plays a vital role in wound healing because it is involved in the synthesis of ECM during skin regeneration and can form abundant collagen fibers outside of cells. Therefore, the ability of HDFs to produce Col-I was examined. It was found that the collagen nanofibers could directly promote the Col-I gene expression and protein secretion from HDFs. The effects could also be achieved with the supernatant collected from the HaCaTs cultured on collagen nanofibers. Besides, the expression of TGF-β1 in HaCaTs was promoted by collagen nanofibers (Figure 7C). The dual effects demonstrated that the collagen nanofibers have the potential to induce skin regeneration. The paracrine

DOI: 10.1021/am507990m
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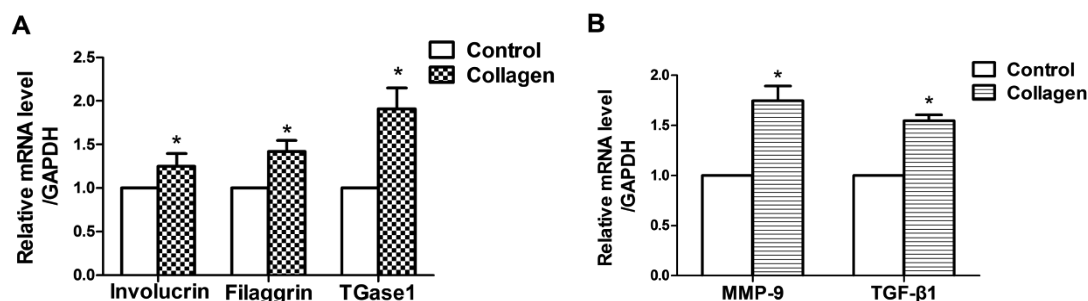

Figure 6. Gene expression profile of (A) differentiation-related genes involucrin, filaggrin, and TGse1 in HaCaTs cultured on tilapia collagen nanofibers for 1 day. The control group was cultured on cover slips. (B) wound-healing-related genes MMP-9 and TGF-β1 in HaCaTs cultured on the collagen nanofibers for 3 days. The control group was cultured on cover slips.

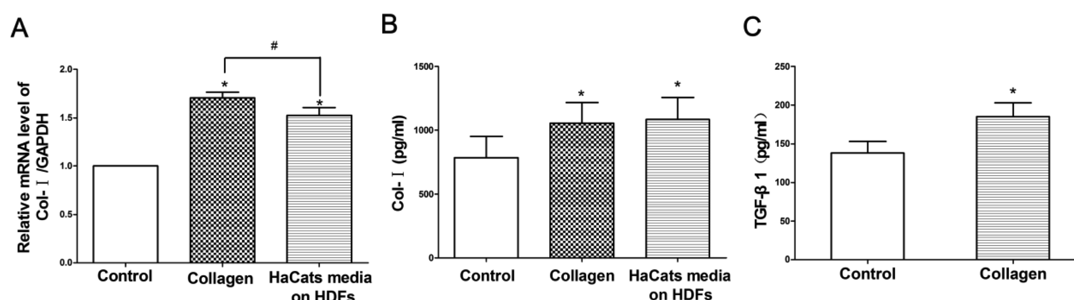

Figure 7. Gene and cytokines expression in HDFs and HaCaTs. (A) Col-I gene expression in HDFs cultured on tilapia collagen nanofibers and treated with the supernatants of HaCaTs cultured on collagen nanofibers for 3 days. (B) Col-I cytokine secretion from HDFs cultured on the collagen nanofibers and treated by the supernatants of HaCaTs cultured on the nanofibers for 1 day. (C) TGF-β1 cytokine secretion from HaCaTs cultured on the collagen nanofibers for 1 day. The control group was cultured on cover slips.

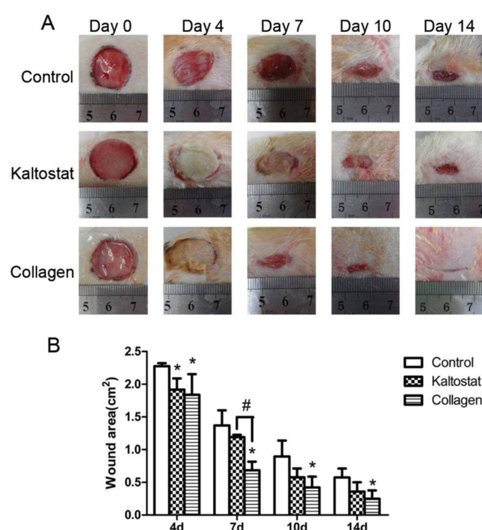

Figure 8. Wound healing in SD rats. (A) The representative images of skin wounds after treatment with tilapia collagen nanofibers or Kaltostat, with untreated wounds as control (n = 3). (B) Wound areas at different time points after treatment.

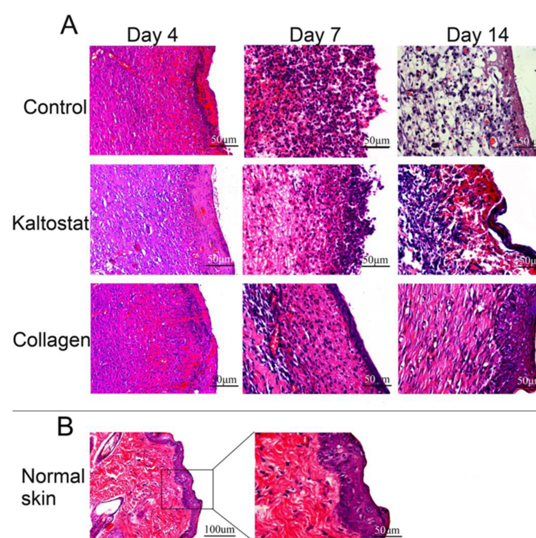

Figure 9. Representative images of H&E staining. (A) Wound sections treated with tilapia collagen nanofibers or Kaltostat, with untreated wounds as control group, at days 7 and 14. (B) Normal skin.

mechanism can be explained as Col-I transcription being activated through interactions with TGF-β1 receptors on the cell membranes of HDFs, which led to the promotion of Col-I secretion.[31] The above results suggested that the collagen nanofibers could accelerate wound healing in an autocrine or paracrine manner to stimulate HDFs to generate collagen fibers.

Skin Regeneration Induced by Tilapia Collagen Nanofibers. Ultimately, to validate the effects of tilapia collagen nanofibers on wound healing, dorsal full-thickness skin-defect models in SD rats were designed. Compared to the control groups, the wound-healing rate was significantly improved, crust started to disappear at day 7, and most of the wound area was covered with a continuous epidermis at day 14 in the collagen nanofibers group (Figure 8), while the skin wounds in the other two groups were not fully healed. The histopathological results confirmed that the collagen nanofibers caused the lowest degree of inflammatory response and induced the best growth status of new epidermis throughout the process of wound healing (Figure 9). The inflammatory response was

DOI: 10.1021/am507990m
ACS Appl. Mater. Interfaces 2015, 7, 3253−3262

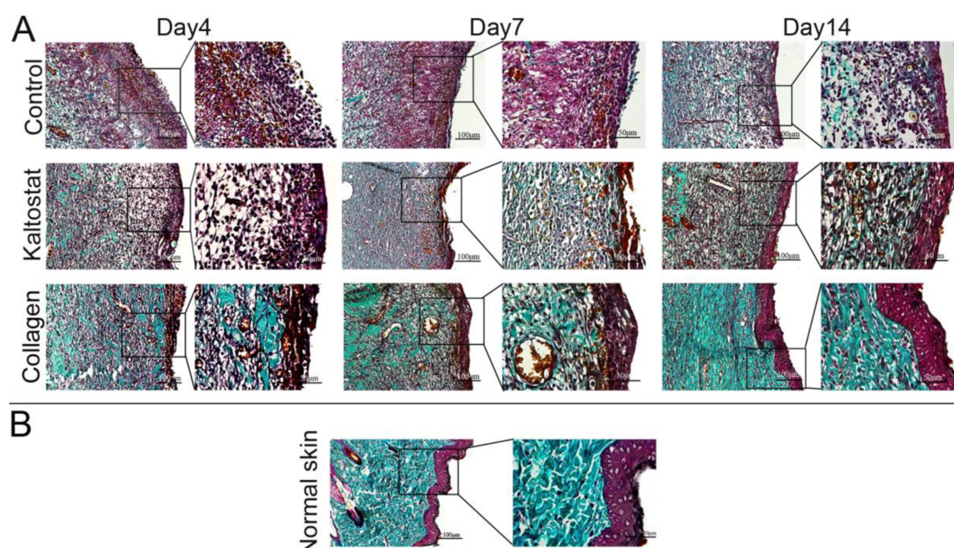

Figure 10. Microscope images of Masson staining. (A) Wound sections treated with tilapia collagen nanofibers or Kaltostat, with untreated wounds as control at days 7 and 14. (B) Normal skin.

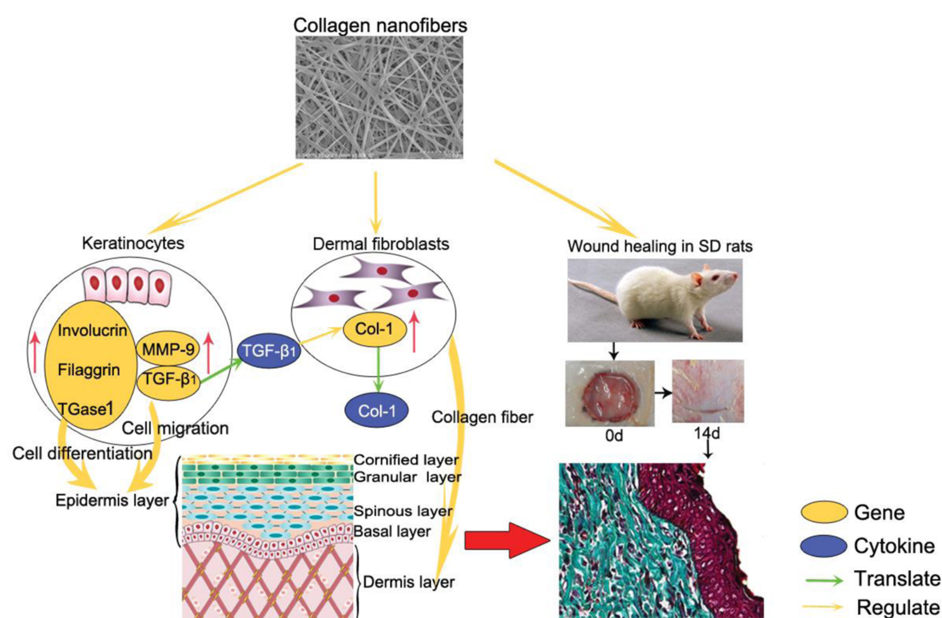

Figure 11. A schematic graph of the mechanisms involved in skin regeneration induced by tilapia collagen nanofibers.

significantly reduced at day 7, and new epidermis with intact structure and good continuity could also been found at day 14. The epidermal cells were fully differentiated, basal cells were closely arranged, the horny layer could be observed, and layers of keratinocytes were evident. Moreover, there was a large amount of fibroblasts present in the dermal layer. Figure 10 shows the formation of collagen fibers in each group at different time points. The regenerative collagen fibers in the collagen nanofibers group appeared at day 4, earlier than in the other two groups. The collagen fibers in this group were also arranged orderly at day 14 and their morphology was similar to that of normal skin (Figure 10). The above results indicated that collagen nanofibers could accelerate wound healing by promoting re-epithelialization and dermal reconstruction, which could be explained by the results of in vitro experiments (Figure 11). The collagen nanofibers developed in our study could effectively promote wound healing at a low price without generating immune responses. Therefore, they have great potential for clinical application.

■ CONCLUSION

In the present study, tilapia skin collagen sponge and the original electrospun collagen nanofibers were successfully fabricated. There nanofibers elicited no immune responses. The mechanical properties and thermal stability of the collagen nanofibers were suitable for application on human skin. It was demonstrated that the collagen nanofibers could promote the viability, migration, and differentiation of HaCaTs with increased expression of MMP-9, TGF-β1, involucrin, filaggrin, and TGase1. Additionally, collagen nanofibers upregulated the expression of Col-I in HDFs both via direct effect and the TGF-β1 secreted from HaCaTs. It was further confirmed in a rat model that the collagen nanofibers significantly stimulated re-epithelialization and dermal reconstruction in wound healing.

DOI: 10.1021/am507990m
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These effects were associated with the biomimetic structure, hydrophilicity, and multiple amino acids of the collagen nanofibers. This study indicated that the low-priced, novel biomimetic electrospun tilapia skin collagen nanofibers could induce skin regeneration rapidly and effectively without producing any immune responses, thus providing a scientific basis for the future application of tilapia collagen nanofibers in skin regeneration.

AUTHOR INFORMATION

Corresponding Authors

*X.M.: tel, +86 021 67792653; e-mail, xmm@dhu.edu.cn.

*J.S.: tel, +86 021 63034903; fax, +86 021 63011643; e-mail, jiaosun59@126.com.

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interests.

ACKNOWLEDGMENTS

This work was supported by grants from Natural Science Foundation of China (nos. 31470917, 31470941).

ABBREVIATIONS USED

HaCaTs = human keratinocytes
HDFs = human dermal fibroblasts
MMP-9 = matrix metalloproteinase-9
TGF-β1 = transforming growth factor-β1
SDS−PAGE = sodium dodecyl sulfate−polyacrylamide gel electrophoresis
HPLC = high-performance liquid chromatography
DSC = differential scanning calorimetry
FCM = flow cytometry
SEM = scanning electron microscope
FTIR = Fourier transform infrared spectroscopy
XRD = X-ray diffraction
ELISA = enzyme-linked immunosorbent assay
RT-PCR = real-time polymerase chain reaction

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DOI: 10.1021/am507990m
ACS Appl. Mater. Interfaces 2015, 7, 3253–3262