Effect of Collagen Nanotopography on Keloid Fibroblast Proliferation and Matrix Synthesis: Implications for Dermal Wound Healing

Lalitha Muthusubramaniam, PhD,^{1,2} Tatiana Zaitseva, PhD,³ Michael Paukshto, PhD,³ George Martin, PhD,⁴ and Tejal Desai, PhD^{1,2}

Keloids are locally exuberant dermal scars characterized by excessive fibroblast proliferation and matrix accumulation. Although treatment strategies include surgical removal and intralesional steroid injections, an effective regimen is yet to be established due to a high rate of recurrence. The regressing center and growing margin of the keloid have different collagen architecture and also differ in the rate of proliferation. To investigate whether proliferation is responsive to collagen topography, keloid, scar, and dermal fibroblasts were cultured on nanopatterned scaffolds varying in collagen fibril diameter and alignment—small and large diameter, aligned and random fibrils, and compared to cells grown on flat collagen-coated substrates, respectively. Cell morphology, proliferation, and expression of six genes related to proliferation (cyclin D1), phenotype (α -smooth muscle actin), and matrix synthesis (collagens I and III, and matrix metalloproteinase-1 and -2) were measured to evaluate cell response. Fibril alignment was shown to reduce proliferation and matrix synthesis in all three types of fibroblasts. Further, keloid cells were found to be most responsive to nanotopography.

Introduction

KELOIDS are raised dermal scars that extend beyond the boundaries of the initial injury. They are unique to humans, with no good animal models available.¹ They are more common in darker skinned individuals and affect about 4–16% of the darker skinned population.² They are often incapacitating with intense pain and cause both functional and aesthetic problems in affected individuals. Keloid fibroblasts (KF) have a higher rate of proliferation, more excessive deposition of extracellular matrix (ECM) proteins such as collagens I and III, and increased expression of the myofibroblast marker, α -smooth muscle actin (SMA), compared with normal dermal fibroblasts.³ The major drawback of current treatments such as surgical excision and intralesional steroid injections is the high rate of recurrence.⁴

The aggressive nature of a keloid is demonstrated by the fact that the center of the keloid is in clinical regression, and it has a reduced rate of cell proliferation, while fibroblasts in the growing margin are highly proliferative and often invade the surrounding healthy tissue.^{5,6} Collagen arrangement in keloid scar tissue differs from those in normal skin and in both normal and hypertrophic scar tissues.⁷ Healthy skin has

a three-dimensional basketweave-like network of distinct collagen bundles, each formed by a parallel array of closely packed collagen fibrils. In contrast, keloid scars show thicker collagen bundles⁸ arranged more loosely^{7,9} and mostly parallel to the epithelial surface^{7,10} and the skin tension line.¹⁰ Earlier descriptions of the pattern of collagen organization in keloids fluctuated, ranging from haphazard or random^{10,11} to parallel.^{7,8} A key to solving this discrepancy could be found in a study by Lee *et al.*¹⁰ The authors observed that organization of the fibrous tissue was rather variable from lesion to lesion, and suggested that the variability could be due to the way the specimen was sectioned. When a keloid specimen was cut in three different orientations with reference to the skin surface and the tension line of the scar, they found that the orientation of the fibrous tissue was mostly parallel to the skin surface and the skin tension line. The orientation of cutting the specimens with respect to epithelial surface was inconsistent between the studies, that is, perpendicular,⁷ parallel and perpendicular,^{9,10} or oblique,¹¹ or the details of routine procedures were not clarified,^{8,10} which may interfere with interpretation of the data. The fact that the keloid scar has a regressing center and growing margins,^{5,6} each with a possibly different collagen architecture, could further complicate the comparison.

¹Department of Bioengineering and Therapeutic Sciences, University of California, San Francisco, San Francisco, California.

²Joint Graduate Group in Bioengineering, University of California, San Francisco—University of California, Berkeley, California.

³Fibralign Corporation, Sunnyvale, California.

⁴National Institutes of Health, Bethesda, Maryland.

Furthermore, a detailed and extensive study by Verhaegen *et al.*,⁷ showed that in keloids, collagen bundles are mostly parallel in the superficial dermis, but more random and loosely arranged in the deeper dermal layers. In addition, Chung *et al.*¹² demonstrated the formation of keloid-like constructs in nude mice transplanted with human keloid-derived fibroblasts. These organotypic constructs were composed of collagen fibrils deposited in an aligned manner. Thus, gaining a better understanding of the effect of collagen architecture on the behavior of keloid-derived fibroblasts could enhance our basic understanding of these cells and serve to inform future therapeutic interventions.

At the center of the scar, cells reside on a matrix of aligned collagen fibrils⁷ and show decreased proliferation;^{5,6} whereas at the active margin, cells encounter a random network of collagen fibrils as seen in normal skin⁷ and exhibit higher rates of proliferation.^{5,6} Not only is the rate of proliferation very different in the two regions of the keloid, but also there is a difference in collagen fibril organization, suggesting a correlation between collagen architecture and keloid growth. Al-Attar *et al.* suggested that mechanical tension during wound healing results in the misalignment of collagen fibrils and causes the formation of aberrant scars such as keloids.¹³ In addition, it has been shown that cryotherapy, which induces a greater parallelization of collagen fibers, is accompanied by a decrease in keloid scar volume.¹⁴ Therefore, we were interested in exploring the relationship between nanoscale collagen fibril organization and KF behavior.

There is abundant evidence in the literature indicating that topography affects cell proliferation and matrix synthesis. A previous study using aluminum oxide membranes showed that nanopore size influences keratinocyte proliferation and migration, both of which affect wound repair and reconstruction.¹⁵ Another study using fibroblasts cultured on polymer microrods showed that microtopography affected cell proliferation and downregulated synthesis of matrix proteins involved in scar formation.¹⁶ Additionally, previous work in our group using corneal fibroblasts cultured on collagen nanofibrillar substrates has shown that both fibril diameter and alignment promote the quiescent keratocyte phenotype and downregulate matrix synthesis.¹⁷

The objective of this study was to evaluate the effect of collagen fibril topography on KF proliferation, morphology, and matrix synthesis, all of which are indicators of wound healing in the skin. To this end, we cultured human fibroblasts from the following types of tissue: normal skin (human dermal fibroblasts [HDF]), normal scar (scar fibroblasts [SF]), and keloid scar (KF) on four collagen nanofibrillar substrates similar to those used in our previous study.¹⁷ The four substrates selected for this study were glass slides coated with nanopatterned bovine collagen type I (Advanced BioMatrix). The collagen coatings have the following average fibril diameter and orientation: small diameter aligned (SA; 55 ± 14 nm), small diameter random (SR; 74 ± 12 nm), large diameter aligned (LA; 165 ± 31 nm), and large diameter random (LR; 192 ± 23 nm) fibrils, respectively. Human dermis is made up of randomly arranged collagen fibers 50–60 nm in diameter.¹⁸ The SA and SR fibrils, therefore, represent the collagen fiber arrangement seen in scar (both normal and keloid scar) and skin tissue, respectively. Further, it has been reported that collagen fiber diameter changes during the

course of wound healing in the skin.¹⁸ The LA and LR substrates were therefore selected to investigate the effect of change in fibril diameter on dermal wound healing. Collagen-coated glass substrates were used as flat controls (FC) with no predetermined topography.

Fibroblast morphology was visualized using confocal microscopy and cell proliferation was measured using the CyQuant assay. Quantitative polymerase chain reaction (qPCR) was performed on six targets related to proliferation (cyclin D1), phenotype (SMA), and matrix synthesis (collagens I and III, and matrix metalloproteinase MMP-1 and MMP-2) to examine the effect of nanotopography at the level of gene expression. This work may lead to a better understanding of how the cellular microenvironment, specifically topographical cues at the nanoscale, can influence both normal and keloid-derived fibroblasts.

Materials and Methods

Cell culture

KF and SF were a gift from Dr. Shirley Russell at Vanderbilt University. For both KF and SF, fibroblasts isolated from three different donors were used in the study. The fibroblast isolation and propagation procedures have been described in earlier publications.^{19,20} HDF were obtained from Invitrogen. All cells were cultured in complete medium consisting of Dulbecco's modified Eagle's medium with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Gibco) in a humidity controlled incubator maintained at 37°C and 5% CO₂. Collagen nanofibrillar scaffolds (SA, SR, LA, and LR) and FC controls were seeded with KF, SF, and HDF at a density of 10,000 cells/cm² in complete medium. The complete medium was changed to serum-free medium 24 h after seeding, as FBS has been shown to act as an external wounding agent.^{21,22} At the end of 2 days, cells were fixed or lysed for further studies. The cells used in these experiments were passaged less than five times.

Scaffold characterization

The collagen nanofibrillar scaffolds were fabricated using bovine collagen type I and purchased under the trademark, AlignCol® Matrix (Catalog Nos. 5054, 5053, 5137, and 5136; Advanced BioMatrix). Scaffolds were air-dried and imaged using atomic force microscopy (AFM) to measure fiber diameter and orientation. All experiments were performed using the AFM Ntegra Prima and Solver Next (NT-MDT). AFM topographs were recorded using silicon tips NSG01 with typical radius <10 nm and spring constant 5.1 N/m (K-Tek Nanotechnology) in the semi-contact mode. The average fibril diameter was evaluated using Image Analysis software (NT-MDT, 2007) and was based on AFM measurements. Diffraction studies were also performed to evaluate fibril alignment on substrates. The diffraction patterns were produced using a red (630 nm) laser beam focused to a diameter of about 0.3 mm on the collagen layers deposited on glass substrates.

Before use in cell culture, collagen scaffolds were rinsed in phosphate-buffered saline (PBS) and deionized water, and sterilized with 70% ethanol. To prepare the flat collagen-coated controls, glass slides were first washed in PBS and

deionized water and sterilized in 70% ethanol. The slides were then dipped in bovine collagen I solution (BD Biosciences; Catalog No. 354231, purity >95% per manufacturer's specifications) at a concentration of 50 $\mu\text{g/mL}$ in 0.01 M HCl at room temperature for 1 h according to the manufacturer's recommendations. Subsequently, slides were rinsed in PBS and used directly or stored in 70% ethanol at 2–8°C for up to 2 weeks before use.

Fluorescent microscopy

Substrates were fixed in 4% paraformaldehyde (Fisher Scientific) for 15 min, then permeated with 0.5% Triton X-100 for 5 min, and blocked with 1% BSA (Sigma) for 30 min. Finally, substrates were incubated with Alexa Fluor-568-conjugated phalloidin (Invitrogen) at 1:100 dilution for 60 min followed by Hoechst 33258 (Invitrogen) at 1:2000 dilution for 5 min. Samples were then visualized using confocal microscopy.

Proliferation assay

Two days after seeding, cell proliferation was measured using the CyQuant assay (Life Technologies). The CyQuant assay uses the CyQuant GR dye, which exhibits fluorescence upon binding to cellular nucleic acids. All the scaffolds were seeded at the same density (10,000 cells/ cm^2) and the initial adhesion of cells on all scaffolds was similar at day 0. Fluorescence was measured at 480 nm excitation and 520 nm emission, and compared to a standard curve to determine proliferation rates for the different cell types on

each scaffold. Three biological replicates were used for each cell type and the assay was performed in technical triplicates.

Quantitative polymerase chain reaction

The RNAqueous-4PCR kit (Applied Biosystems) was used for RNA extraction. The isolated RNA was reverse transcribed into complementary DNA (cDNA) using the Cells to C_T kit (Applied Biosystems). Primers were obtained from Integrated DNA Technologies. qPCR was performed on the StepOne Plus using Fast SYBR Green PCR Master Mix. The cDNA obtained from the target mRNA was normalized to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). GAPDH was used as a loading control to confirm equal loading of all samples. Three to five biological replicates were used per substrate. qPCR was performed in technical triplicates.

Statistical analysis

Analysis of variance (ANOVA) was used to compare group means both between the nanofibrillar scaffolds and between the three cell types. Differences were considered significant at the $p < 0.05$ level. If a significant difference was seen by ANOVA, then sequential Holm t -tests were conducted to detect specific differences between scaffolds or between cell types. In each case, the results were normalized to the flat control (FC) to exclude the effect of factors such as chemical composition and material stiffness and examine the effect of topography alone on cell response.

FIG. 1. Atomic force microscopy images of the nanopatterned collagen substrates and flat control. The five images represent (a) small diameter aligned fibrils (SA), (b) small diameter random fibrils (SR), (c) large diameter aligned fibrils (LA), (d) large diameter random fibrils (LR), and (e) flat collagen-coated control substrate (FC), respectively. Arrows represent fibril direction. The inset in (c) shows the LA substrate at a lower magnification to obtain an expanded view of the surface. Scale bars represent 1 μm . Color images available online at www.liebertpub.com/tea

FIG. 2. Diffraction patterns produced by red (630 nm) laser beam by the four collagen scaffolds: (a) SA, (b) SR, (c) LA, and (d) LR respectively. The two vertical petals in (a) indicate the uniform alignment of collagen fibrils in the SA matrix. The small circular spot with diffusive scattering in (b) corresponds to the SR matrix with small diameter, randomly arranged collagen fibrils. Highly aligned helical (crimped) fibrils of the LA matrix produce the cross-like pattern in (c). The large circular spot with diffusive scattering in (d) corresponds to the LR matrix with large diameter, randomly arranged collagen fibrils. Color images available online at www.liebertpub.com/tea

Results and Discussion

Characterization of nanofibrillar substrates

AFM analysis of the SA substrate revealed the presence of 55 nm aligned collagen fibrils representative of the collagen arrangement seen in both normal scar and keloid scar tissue (Fig. 1). AFM imaging of the SR substrate showed randomly organized fibrils, 75 nm in diameter, representing the collagen arrangement in healthy skin.¹⁸ AFM images of the LA and LR substrates showed thicker aligned and random fibrils, respectively. These were selected to investigate the effect of change in diameter on fibroblast proliferation and ECM production. The image of

the collagen-coated flat glass slide, FC, is also seen in Figure 1. The absence of fibrils on the surface makes this sample suitable for use as a flat “no topography” control in our study.

Fibril alignment was also evaluated by diffraction studies using a 630 nm (red) laser beam. The two vertical petals seen in SA (Fig. 2a) and the cross-like pattern seen in LA (Fig. 2c), both indicate fibril alignment on these surfaces. The small circular spot with diffusive scattering on the SR (Fig. 2b) represents small diameter, random collagen fibrils, while the corresponding large circular spot with diffusive scattering on LR (Fig. 2d) is indicative of large diameter, random collagen fibrils.

FIG. 3. Confocal microscopy images of fibroblasts on collagen nanofiber substrates and flat controls (FC). F-actin is stained using phalloidin and the nucleus is stained using Hoechst. Scale bars represent 100 μ m. Color images available online at www.liebertpub.com/tea

Fibroblast morphology on collagen nanofibrillar substrates

Previously, we have shown that fibril arrangement affects corneal fibroblast morphology and adhesion to nanopatterned collagen scaffolds.¹⁷ To determine whether this effect is also seen in fibroblasts related to dermal wound healing, KF, SF, and HDF were grown on the nanostructured scaffolds and controls and stained to image f-actin (phalloidin) and the nucleus (Hoechst). As seen in Figure 3, fibroblasts on the SA substrate exhibited a more dendritic and elongated morphology compared with cells on the other nanofibrillar substrates and FC control, which were larger, flatter, and more spread out with the appearance of numerous stress fibers (Supplementary Fig. S1 shows image at higher magnification; Supplementary Data are available online at www.liebertpub.com/tea). Cell spreading has been shown to be important for viability²³ and proliferation²⁴ of anchorage-dependant cells such as fibroblasts. A previous study using keratinocytes grown on aluminum oxide nanoporous membranes also showed that greater cell spreading correlated with higher levels of proliferation.¹⁵ Therefore, we hypothesized that decreased cell spreading will lead to lower proliferation on the SA samples compared with the other substrates in the study.

Collagen nanotopography reduces fibroblast proliferation

Excessive proliferation is a hallmark of keloid scars.^{25,26} To determine the effect of collagen nanotopography on proliferation, fibroblast proliferation was measured using the CyQuant assay. As seen in Figure 4, fibril alignment reduced KF proliferation (0.51 ± 0.35 -fold on the SA substrate and 0.65 ± 0.19 on the LA substrate, relative to FC, $p < 0.05$ in each case). Fibril alignment (SA) also downregulated SF proliferation (0.49 ± 0.15 on SA compared to FC, $p < 0.05$). Fibril alignment (SA) reduced HDF proliferation as well (0.62 ± 0.31 on SA compared to FC), although not to statistically significant levels. Therefore, fibril alignment (SA)

showed a tendency to reduce proliferation of all three types of fibroblasts. These results are in accordance with our fluorescent microscopy data, which showed that the SA substrate had reduced cell spreading that is linked to decreased proliferation.^{23,24} Furthermore, in the case of KF, the LA also showed reduced proliferation, while this effect was not seen with the corresponding random fibrils (LR). This suggests that the presence of aligned fibrils decreased KF proliferation regardless of fibril diameter.

Additionally, fibroblasts from scar tissue (both keloid and normal scar) appear to be more responsive to collagen nanotopography compared with fibroblasts from normal skin. Previous studies have reported that KF are more responsive to serum stimulation³ and show increased DNA and collagen production in response to transforming growth factor-beta (TGF- β).²⁷ It has been speculated that increased expression and phosphorylation of signal transducer and activator of transcription 3 (STAT-3), due to induction by cytokines and growth factors in KF, could possibly explain their enhanced response to chemical cues such as serum and TGF- β .³ Here, we have shown that KF also show increased sensitivity to physical cues such as matrix topography. To understand the mechanism underlying this response, we performed qPCR on six genes involved in fibroblast proliferation, phenotype expression, and ECM synthesis, all of which are markers of keloid wound healing.

Collagen nanotopography reduces cyclin D1 expression

It has been suggested that the increase in KF proliferation is due to their lower barrier for entry into S phase.²⁶ Cyclin D1 is a protein that forms complexes with cyclin-dependent kinase and acts as a switch regulating entry of cells into S phase.²⁸ Hence, expression of cyclin D1 mRNA was measured to investigate its role in fibroblast response to topography. As seen in Figure 5, cyclin D1 expression in KF was significantly reduced on three of the four nanostructured scaffolds as compared with FC (0.65 ± 0.29 on SA, 0.41 ± 0.20 on SR, and 0.36 ± 0.21 on LA, compared to FC respectively, $p < 0.05$ in each case). Cyclin D1 levels were also reduced on LR, although not to statistical significance. This indicates that the presence of collagen fibrils, regardless of their arrangement, affects cyclin D1 gene expression in KF.

In SF, cyclin D1 expression was significantly reduced on SA alone (0.58 ± 0.21 compared to FC, $p < 0.05$). This corresponds very well with the results of the CyQuant assay where SF proliferation is downregulated only on SA (Fig. 4). The trend in cyclin D1 expression in SF on the other scaffolds also correlates to their proliferation profiles as seen with the CyQuant assay. In the case of HDF, similar to KF, cyclin D1 levels were reduced on all of the nanopatterned substrates compared with FC. This difference reaches statistical significance for SR and LA (0.47 ± 0.31 for SR and 0.40 ± 0.18 for LA, compared to FC, $p < 0.05$) (Fig. 5). Also, cyclin D1 levels were significantly downregulated in KF compared with SF for the SR and LA samples, respectively ($p < 0.05$) (Fig. 5). In summary, SA is most effective in reducing cyclin D1 levels across all three cell types, and KF appear to be more sensitive to the effect of collagen nanotopography compared to SF, with respect to cyclin D1 expression.

FIG. 4. Fibril alignment (SA) reduced cell proliferation for keloid fibroblasts (KF), scar fibroblasts (SF), and human dermal fibroblasts (HDF) as shown by CyQuant assay. Data are presented as mean $\pm$ SD, using three replicates per substrate. * $p < 0.05$ versus corresponding values in the FC control of each cell type. The dashed reference line indicates the FC control value (control value equals one for experimental data normalization).

FIG. 5. Fibril alignment (SA) reduced gene expression of cyclin D1 and alpha-smooth muscle actin (SMA) most effectively in KF. Data are presented as mean $\pm$ SD, using three to five replicates per substrate. * $p < 0.05$ versus corresponding values in the FC control of each cell type. # $p < 0.05$ versus corresponding values in KF. The dashed reference line indicates the FC control value (control value equals one for experimental data normalization). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the loading control.

Collagen nanotopography attenuates the fibrotic myofibroblast phenotype

SMA is an established marker of the myofibroblast phenotype.²⁹ Immunohistochemical studies of SMA expression in biopsy samples of keloid tissue are not consistent, with some studies showing that SMA is absent in keloids,^{8,30} and other works demonstrating the presence of SMA in keloids.^{10,31,32} This discrepancy may be due to variable sample sizes available for analysis and/or due to the analysis of different lesional sites within the keloid. It has been shown that the active margin of keloids have a different gene expression pattern compared with the regressing center of the keloid.^{5,19} However, there is agreement that KF isolated

from fresh keloid samples express SMA when grown in culture,^{8,29} and further, the expression of SMA is increased in KF.²⁹ These data support the fact that KF present the myofibroblast phenotype, especially under *in vitro* conditions. As shown in Figure 3, fibroblasts on SA substrates display an elongated morphology, while fibroblasts on other substrates were spread out with more prominent appearance of stress fibers, indicative of the myofibroblast phenotype. Hence, SMA gene expression was measured to determine the effect of nanotopography on phenotype. SMA expression in KF was decreased on SA, LA, and LR compared to FC (Fig. 5). The decrease was statistically significant for LA and LR (0.47 ± 0.15 and 0.40 ± 0.23 compared to FC respectively, $p < 0.05$). Therefore, SMA expression in KF is

FIG. 6. Fibril alignment (SA) reduced matrix synthesis most effectively in KF as seen by gene expression of collagen I, collagen III, and matrix metalloproteinase-2 (MMP-2), respectively. Data are presented as mean $\pm$ SD, using three to five replicates per substrate. * $p < 0.05$ versus corresponding values in the FC control of each cell type. # $p < 0.05$ versus corresponding values in KF. The dashed reference line indicates the FC control value (control value equals one for experimental data normalization). GAPDH was used as the loading control.

TABLE 1. FIBRIL ALIGNMENT REDUCED PROLIFERATION AND TARGET GENE EXPRESSION MOST EFFECTIVELY IN KF

	Aligned fibrils			Random fibrils		
	KF	SF	HDF	KF	SF	HDF
Small fibrils						
SA						
Proliferation	↓	↓				
Cyclin D1	↓	↓		↓		↓
SMA						
Collagen I	↓	↓		↓		
Collagen III	↓					
MMP-2	↓					
Large fibrils						
LA						
Proliferation	↓					
Cyclin D1	↓		↓			
SMA	↓					
Collagen I	↓			↓		
Collagen III				↓		
MMP-2	↓			↓		

Downward arrows (↓) indicate statistical significance compared to corresponding FC values ($p < 0.05$).

HDF, human dermal fibroblasts; KF, keloid fibroblasts; LA, large diameter aligned; LR, large diameter random; MMP, matrix metalloproteinase; SA, small diameter aligned; SF, scar fibroblasts; SR, small diameter random; SMA, alpha smooth muscle actin.

decreased in the presence of collagen fibrils, similar to the expression of cyclin D1. This decrease was, however, not seen in the case of SF or HDF, thus suggesting that KF are more responsive to nanotopography with respect to SMA gene expression. Furthermore, SMA levels on the LR substrate in KF were more significantly reduced compared with corresponding values in HDF ($p < 0.05$).

Collagen nanotopography reduces matrix synthesis

Excessive collagen accumulation is a histological characteristic of keloid scars. This is due to both excessive collagen synthesis^{33,34} and abnormal collagen turnover by matrix degrading enzymes such as MMP-1 and MMP-2.²² Cells from the active margin of keloid scars have been shown to have increased expression of collagen I and III mRNA.³³ Additionally, the levels of MMP-1, an interstitial collagenase, and MMP-2, which breaks down denatured collagens, are elevated in KF compared with HDF.²² It has been suggested that MMP-1 and MMP-2 may be involved in KF migration, and therefore, their increased production contributes to the invasive growth of keloid scar into the surrounding healthy skin. Hence, we measured the gene expression of collagens I and III, and MMP-1 and MMP-2, to determine the effect of nanotopography on ECM accumulation.

As seen in Figure 6, collagen I gene expression in KF was significantly downregulated on all four nanofibrillar scaffolds compared to FC (0.46 ± 0.23 on SA, 0.22 ± 0.21 on SR, 0.28 ± 0.04 on LA, and 0.31 ± 0.20 on LR, as compared to FC, respectively, $p < 0.05$ in each case). Thus, the presence of collagen fibrils, regardless of their diameter or orientation, decreases collagen I expression in KF, following the trend seen with cyclin D1. In the case of both SF and HDF, collagen I gene expression was reduced only on the SA substrate, with SF showing statistically significant reduction (0.48 ± 0.14 on SA compared to FC, $p < 0.05$). Also, the decrease in collagen I expression in KF is greater compared

to corresponding values in HDF for both SR and LR, respectively ($p < 0.05$). A similar trend was observed with collagen III wherein SA is the only scaffold to show reduced collagen III expression across all the three cell types compared to FC (Fig. 6). Furthermore, this reduction is statistically significant only in KF (0.63 ± 0.09 on SA compared to FC, $p < 0.05$). In summary, SA scaffold is most effective in reducing expression of both collagens I and III across all three cell types. Also, KF are more responsive to nanotopography compared to SF and HDF.

Expression of MMP-1 in KF was slightly decreased on the nanofibrillar scaffolds compared to FC, although not to statistically significant levels (Supplementary Fig. S2). However, the same trend is seen as with most of the other markers, wherein KF is more responsive to nanotopography as there was no decrease in MMP-1 expression with both SF and HDF, respectively. On the other hand, MMP-2 levels in KF were significantly reduced on three of the four nanofibrillar scaffolds— 0.70 ± 0.11 on SA, 0.20 ± 0.03 on LA, and 0.42 ± 0.28 on LR—compared to FC, respectively ($p < 0.05$ in each case) (Fig. 6). The difference in KF response between MMP-1 and MMP-2 suggests that nanotopography differently acts on the different members of the MMP family. Interestingly, immunohistochemistry studies on keloid tissue showed higher expression of MMP-2 and not that of MMP-1, when compared with healthy skin controls,³⁵ indicating that MMP expression in keloids is also altered *in vivo*. Additionally, there was no change in MMP-2 expression on the nanopatterned substrates for both SF and HDF, showing that nanotopography modulates MMP-2 expression only in the case of KF, similar to the trend seen with some of the other proteins (SMA, collagen III, and MMP-1).

Conclusions

Table 1 summarizes the effect of collagen fibril diameter and alignment on the three cell types used in the study.

Overall, we observed that fibril alignment (SA and LA) reduced cell proliferation and expression of genes related to proliferation (cyclin D1), fibrotic phenotype (SMA), and ECM synthesis (collagen I and III and MMP-2), respectively. Further, KF showed enhanced responsiveness to nanotopography. This may be desirable from a therapeutic standpoint to control keloid proliferation and inhibit scar growth, while still allowing dermal fibroblasts to repopulate the region and complete wound healing. Further studies exploring the molecular mechanisms involved in cell response, and alternative nanotopographies, may also aid in identifying an effective approach for keloid management.

Acknowledgments

We would like to thank Dr. Russell for the kind gift of KF and SF used in the study. We also thank Dr. Yuri Bobrov for AFM measurements, Dr. Vuk Uskokovic for help with scanning electron microscopy measurements, Dr. Kurt Thorn at the UCSF Nikon Imaging Center for assistance with confocal microscopy, and Laura Walsh and Kristina Krebs for help with qPCR. Funding for this work was provided by the NIH Training Grant 5T32GM008155-26.

Disclosure Statement

The authors state no conflict of interest.

References

- Shih, B., Garside, E., McGrouther, D.A., and Bayat, A. Molecular dissection of abnormal wound healing processes resulting in keloid disease. *Wound Repair Regen* **18**, 39, 2010.
- Atiyeh, B.S., Costagliola, M., and Hayek, S.N. Keloid or hypertrophic scar: the controversy: review of the literature. *Ann Plast Surg* **54**, 676, 2005.
- Ong, C.T., Khoo, Y.T., Mukhopadhyay, A., Do, D.V., Lim, I.J., Aalami, O., and Phan, T.T. mTOR as a potential therapeutic target for treatment of keloids and excessive scars. *Exp Dermatol* **16**, 394, 2007.
- Atiyeh, B. Nonsurgical management of hypertrophic scars: evidence-based therapies, standard practices, and emerging methods. *Aesthetic Plast Surg* **31**, 468, 2007.
- Seifert, O., Bayat, A., Geffers, R., Dienus, K., Buer, J., Löfgren, S., and Matussek, A. Identification of unique gene expression patterns within different lesional sites of keloids. *Wound Repair Regen* **16**, 254, 2008.
- Appleton, I., Brown, N.J., and Willoughby, D.A. Apoptosis, necrosis, and proliferation: possible implications in the etiology of keloids. *Am J Pathol* **149**, 1441, 1996.
- Verhaegen, P.D.H.M., Van Zuijlen, P.P.M., Pennings, N.M., Van Marle, J., Niessen, F.B., Van Der Horst, C.M.A.M., and Middelkoop, E. Differences in collagen architecture between keloid, hypertrophic scar, normotrophic scar, and normal skin: an objective histopathological analysis. *Wound Repair Regen* **17**, 649, 2009.
- Ehrlich, H.P., Desmouliere, A., Diegelmann, R.F., Cohen, I.K., Compton, C.C., Gamer, W.L., Kapanci, Y., and Gabbiani, G. Morphological and immunochemical differences between keloid and hypertrophic scar. *Am J Pathol* **145**, 105, 1994.
- Knapp, T.R., Daniels, J.R., and Kaplan, E.N. Pathologic scar formation: morphologic and biochemical correlates. *Am J Pathol* **86**, 47, 1977.
- Lee, J.Y., Yang, C.C., Chao, S.C., and Wong, T.W. Histopathological differential diagnosis of keloid and hypertrophic scar. *Am J Dermatopathol* **26**, 379, 2004.
- Da Costa, V., Wei, R., Lim, R., Sun, C.H., Brown, J.J., and Wong, B.J. Nondestructive imaging of live human keloid and facial tissue using multiphoton microscopy. *Arch Facial Plast Surg* **10**, 38, 2008.
- Chung, H.J., Steplewski, A., Chung, K.Y., Uitto, J., and Fertala, A. Collagen fibril formation: a new target to limit fibrosis. *J Biol Chem* **283**, 25879, 2008.
- Al-Attar, A., Mess, S., Thomassen, J.M., Kauffman, C.L., and Davison, S.P. Keloid pathogenesis and treatment. *Plast Reconstr Surg* **117**, 286, 2006.
- Har-Shai, Y., Amar, M., and Sabo, E. Intralesional cryotherapy for enhancing the involution of hypertrophic scars and keloids. *Plast Reconstr Surg* **111**, 1841, 2003.
- Parkinson, L.G., Rea, S.M., Stevenson, A.W., Wood, F.M., and Fear, M.W. The effect of nano-scale topography on keratinocyte phenotype and wound healing following burn injury. *Tissue Eng Part A* **18**, 703, 2012.
- Ayala, P., Lopez, J.L., and Desai, T. Microtopographical cues in 3D attenuate fibrotic phenotype and extracellular matrix deposition: implications for tissue regeneration. *Tissue Eng Part A* **16**, 2519, 2010.
- Muthusubramaniam, L., Peng, L., Zaitseva, T., Paukshto, M., Martin, G.R., and Desai, T.A. Collagen fibril diameter and alignment promote the quiescent keratocyte phenotype. *J Biomed Mater Res A* **100A**, 613, 2011.
- Schmults, C.D., Phelps, R., and Goldberg, D.J. Nonablative facial remodeling: erythema reduction and histologic evidence of new collagen formation using a 300-microsecond 1064-nm Nd:YAG laser. *Arch Dermatol* **140**, 1373, 2004.
- Smith, J.C., Boone, B.E., Opalenik, S.R., Williams, S.M., and Russell, S.B. Gene profiling of keloid fibroblasts shows altered expression in multiple fibrosis-associated pathways. *J Invest Dermatol* **128**, 1298, 2007.
- Russell, S.B., Russell, J.D., Trupin, K.M., Gayden, A.E., Opalenik, S.R., Nanne, L.B., Broquist, A.H., Raju, L., and Williams, S.M. Epigenetically altered wound healing in keloid fibroblasts. *J Invest Dermatol* **130**, 2489, 2010.
- Iyer, V.R., Eisen, M.B., Ross, D.T., Schuler, G., Moore, T., Lee, J.C.F., Trent, J.M., Staudt, L.M., Hudson, J., Boguski, M.S., Lashkari, D., Shalon, D., Botstein, D., and Brown, P.O. The transcriptional program in the response of human fibroblasts to serum. *Science* **283**, 83, 1999.
- Fujiwara, M., Muragaki, Y., and Ooshima, A. Keloid-derived fibroblasts show increased secretion of factors involved in collagen turnover and depend on matrix metalloproteinase for migration. *Br J Dermatol* **153**, 295, 2005.
- Chen, C.S., Mrksich, M., Huang, S., Whitesides, G.M., and Ingber, D.E. Micropatterned surfaces for control of cell shape, position, and function. *Biotechnol Prog* **14**, 356, 1998.
- Singhvi, R., Kumar, A., Lopez, G.P., Stephanopoulos, G.N., Wang, D.I., Whitesides, G.M., and Ingber, D.E. Engineering cell shape and function. *Science* **264**, 696, 1994.
- Toh, P.P., Li, J.J., Yip, G.W., Lo, S.L., Guo, C.H., Phan, T.T., and Bay, B.H. Modulation of metallothionein isoforms is associated with collagen deposition in proliferating keloid fibroblasts *in vitro*. *Exp Dermatol* **19**, 987, 2010.
- Calderon, M., Lawrence, W.T., and Banes, A.J. Increased proliferation in keloid fibroblasts wounded *in vitro*. *J Surg Res* **61**, 343, 1996.

27. Bettinger, D.A., Yager, D.R., Diegelmann, R.F., and Cohen, I.K. The effect of TGF-beta on keloid fibroblast proliferation and collagen synthesis. *Plast Reconstr Surg* **98**, 827, 1996.
28. Stacey, D.W. Cyclin D1 serves as a cell cycle regulatory switch in actively proliferating cells. *Curr Opin Cell Biol* **15**, 158, 2003.
29. Chipev, C.C., Simman, R., Hatch, G., Katz, A.E., Siegel, D.M., and Simon, M. Myofibroblast phenotype and apoptosis in keloid and palmar fibroblasts *in vitro*. *Cell Death Differ* **7**, 166, 2000.
30. Theoret, C.L., Olutoye, O.O., Parnell, L.K.S., and Hicks, J. Equine exuberant granulation tissue and human keloids: a comparative histopathologic study. *Vet Surg* **42**, 783, 2013.
31. Santucci, M., Borgognoni, L., Reali, U.M., and Gabbiani, G. Keloids and hypertrophic scars of caucasians show distinctive morphologic and immunophenotypic profiles. *Virchows Arch* **438**, 457, 2001.
32. Lee, Y.S., and Vijayasingam, S. Mast cells and myofibroblasts in keloid: a light microscopic, immunohistochemical and ultrastructural study. *Ann Acad Med Singapore* **24**, 902, 1995.
33. Syed, F., Ahmadi, E., Iqbal, S.A., Singh, S., McGrouther, D.A., and Bayat, A. Fibroblasts from the growing margin of keloid scars produce higher levels of collagen I and III compared with intralesional and extralesional sites: clinical implications for lesional site-directed therapy. *Br J Dermatol* **64**, 83, 2011.
34. Friedman, D.W., Boyd, C.D., Mackenzie, J.W., Norton, P., Olson, R.M., Deak, S.B. Regulation of collagen gene expression in keloids and hypertrophic scars. *J Surg Res* **55**, 21, 1993.
35. Imaizumi, R., Akasaka, Y., Inomata, N., Okada, E., Ito, K., Ishikawa, Y., and Maruyama, Y. Promoted activation of matrix metalloproteinase (MMP)-2 in keloid fibroblasts and increased expression of MMP-2 in collagen bundle regions: implications for mechanisms of keloid progression. *Histopathology* **54**, 722, 2009.

Address correspondence to:

Tejal Desai, PhD

Department of Bioengineering and Therapeutic Sciences
University of California, San Francisco
Box 2520, Byers Hall Rm 203C
San Francisco, CA 94158-2330

E-mail: tejal.desai@ucsf.edu

Received: August 30, 2013

Accepted: April 2, 2014

Online Publication Date: May 19, 2014