Deep and Superficial Keloid Fibroblasts Contribute Differentially to Tissue Phenotype in a Novel In Vivo Model of Keloid Scar

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Background: Keloids are thick fibrous scars that are refractory to treatment and unique to humans. The lack of keloid animal models has hampered development of effective therapies. The authors' goal was to develop an animal model of keloids using grafted engineered skin substitutes composed of keloid-derived cells. To demonstrate the model's utility, differences between deep and superficial keloid fibroblasts were investigated.

Methods: Engineered skin substitutes were prepared using six combinations of cells: 1, normal keratinocytes and normal fibroblasts; 2, normal keratinocytes and deep keloid fibroblasts; 3, normal keratinocytes and superficial keloid fibroblasts; 4, keloid keratinocytes and normal fibroblasts; 5, keloid keratinocytes and deep keloid fibroblasts; and 6, keloid keratinocytes and superficial keloid fibroblasts. Engineered skin substitutes stably grafted to athymic mice were evaluated for wound area, thickness, and gene expression.

Results: Deep keloid fibroblasts displayed elevated expression of type 1 collagen alpha 1 (*COL1A1*), transforming growth factor β-1, periostin, plasminogen activator inhibitor 2, and inhibin beta A compared with superficial keloid fibroblasts and normal fibroblasts. After grafting, engineered skin substitutes in group 5 were significantly thicker than controls and had increased *COL1A1* expression. Engineered skin substitutes in group 6 showed significantly increased area. Histologic analysis revealed abnormal collagen organization in engineered skin substitutes containing deep keloid fibroblasts or superficial keloid fibroblasts.

Conclusions: Aspects of the phenotypes of engineered skin substitutes prepared with keloid cells are analogous to thickening and spreading of human keloid scars. Therefore, use of keloid engineered skin substitutes is a valuable new tool for the study of keloid scarring. (*Plast. Reconstr. Surg.* 129: 1259, 2012.)

Keloids are raised dermal scars that result from an abnormal fibroproliferative response following skin injury. Keloid scars spread beyond the original wound boundary and tend to be refractory to treatment.[1] They are more common in darker pigmented populations, including African Americans, Asians, and Hispanics, and are believed to involve a genetic component.[1–5] Keloids share several features with hypertrophic scars, including excessive extracellular matrix deposition caused by unknown mechanisms[6]; significant morbidity caused by itching, pain, and decreased range of motion[7]; profoundly impacted psychosocial well-being; and impaired overall quality of life for affected patients.[7–10] Importantly, there are no real cures for either type

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of scar, despite the availability of multiple treatment options.[11–13] The fact that there are no universally accepted treatments for either keloids or hypertrophic scars underscores the heterogeneity of these debilitating lesions and indicates that the key mechanisms involved in abnormal scarring remain to be elucidated. Development of improved therapeutic options could be facilitated by both an increased understanding of the mechanisms of abnormal scar development and the use of appropriate in vivo models. However, the paucity of good animal models of abnormal scarring has significantly impeded research efforts.[14] Although there are a limited number of animal models that resemble some features of hypertrophic scars,[15–18] keloids are unique to humans[19] and there are currently no animal models for keloid scarring. Rodent models have been described that involve transplantation of human keloid tissue into athymic rats or, more commonly, athymic mice.[20,21] However, these models are limited because of the heterogeneous nature of the material transplanted, the limited time for which the transplants remain viable, and the inability to assess prophylactic therapies.[19]

In the absence of in vivo models, the role of growth factors in keloid scar formation has been studied in tissue culture models using cells derived from keloids. For example, keloid-derived fibroblasts secrete increased levels of collagen and enzymes involved in extracellular matrix remodeling, such as matrix metalloproteinases.[22] Because keratinocytes play an important role in paracrine regulation of fibroblast function,[23] their role in keloid formation has also been investigated. Several reports have described the influence of keloid-derived keratinocytes on normal or keloid-derived fibroblasts in culture.[24–29] Those studies indicate that keloid fibroblasts and keratinocytes secrete paracrine signals, distinct from signals in normal cells, to modulate gene expression and activity. Although these studies demonstrated the importance of keratinocytes in regulating fibroblast function, cells grown in monolayer cannot reproduce the cell-cell and cell-matrix interactions found in intact tissue. Recognizing the importance of these interactions, an organotypic method for studying keloid-derived fibroblasts was previously described[30] in which normal or keloid fibroblasts were embedded in bovine collagen gels overlaid with normal keratinocytes.[30] In that model, keloid fibroblasts caused increased gel contraction compared with normal fibroblasts. Although this model was more complex than previous two dimensional co-culture models, it was limited to in vitro analysis.

We hypothesized that engineered skin substitutes prepared using keloid-derived fibroblasts and keratinocytes grafted to mice could serve as an in vivo model of human keloid scarring. Engineered skin substitutes have been evaluated in clinical trials as an adjunctive treatment to achieve wound closure in burn patients with large wounds and limited donor sites for autografting.[31–34] For clinical application to burn patients, cultured autologous dermal fibroblasts and epidermal keratinocytes are combined with a collagen-based biopolymer matrix.[31,34–36] After 1 to 2 weeks of in vitro culture, engineered skin substitutes demonstrate skin-like tissue development, including differentiation of a stratified epidermal layer with a cornified surface, deposition of basement membrane, and remodeling of the dermal extracellular matrix by fibroblasts. Numerous preclinical studies have been performed for analysis of engineered skin substitutes using the athymic mouse model as a host,[37–42] permitting essentially permanent engraftment because of the absence of T cells in this immunodeficient mouse strain. The goal of the current study was to investigate the use of engineered skin substitutes as an in vivo model for the study of keloid-derived cells. Other investigators have identified differences in gene expression when comparing deep and superficial fibroblasts cultured from normal skin[43] or fibroblasts isolated from different regions of keloid scars.[44] We investigated transplantation of engineered skin substitutes containing normal and/or keloid cells for analysis of deep (reticular) and superficial (papillary) fibroblasts, to assess the validity of engineered skin substitutes as an organotypic in vivo model of keloid scarring. When combined in engineered skin substitutes and transplanted to athymic mice, keloid keratinocytes and deep keloid fibroblasts resulted in grafts that were significantly thicker than controls. However, if superficial fibroblasts were used, the transplanted grafts significantly increased in area but not thickness. These results indicate differential contributions of deep and superficial fibroblasts to the phenotype of keloid scars, and suggest that keloid engineered skin substitutes represent a valuable in vivo model of keloid abnormality.

MATERIALS AND METHODS

Cell Culture and Engineered Skin Substitute Preparation

Discarded human scar and skin samples were obtained with University of Cincinnati Institutional Review Board approval and in accordance with the 1975 Declaration of Helsinki, as revised in

1983. Primary cultures of fibroblasts and keratinocytes were isolated from a thick keloid scar excised from the face of a 10-year-old male African American burn survivor. The dermal component of the keloid was dissected into "superficial" dermis, which was approximately 0.5 cm thick and adjacent to the epidermis, and "deep" dermis, also approximately 0.5 cm thick, before primary culture. Normal fibroblasts and keratinocytes were isolated from full-thickness normal breast skin of a 17-year-old African American female patient undergoing elective breast reduction surgery; the entire dermal layer was used for fibroblast culture without subdivision. Fibroblasts and keratinocytes were isolated and cultured separately in specific growth medium as described in detail elsewhere.[45] Briefly, tissue samples were cleansed using 5% Dettol and then rinsed several times in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid–buffered saline. Tissue was cut into 2- to 3-mm-wide strips and incubated overnight at 4°C in 30 ml of Dispase II [2.4 units/ml in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid–buffered saline; Roche Applied Science, Indianapolis, Ind.], followed by manual separation of epidermis from dermis. Deep dermis tissue was processed immediately, without Dispase II incubation, for fibroblast isolation. Dermal strips were minced finely and incubated at 37°C for 1 hour in 30 ml of collagenase (625 units/ml; Worthington Biochemical Corp., Lakewood, N.J.) with occasional mixing. Fibroblasts were rinsed with culture medium[46] and cells and tissue pieces were pelleted and inoculated into flasks. To isolate keratinocytes, epidermal strips were incubated for 5 minutes at 37°C in 0.025% trypsin (Invitrogen, Carlsbad, Calif.) plus 0.01% ethylenediaminetetraacetic acid (Invitrogen); the mixture was neutralized with 10% fetal bovine serum (Invitrogen) and filtered through a BD-Falcon 70-μm cell strainer (BD Biosciences, Bedford, Mass.). Keratinocytes were pelleted by centrifugation and inoculated into flasks. Culture media were refreshed every 48 hours, and cells were passaged before reaching confluence. All cells were harvested for preparation of engineered skin substitutes at passage 2.

For preparation of engineered skin substitutes, fibroblasts were grown to near-confluence, harvested, and inoculated onto rehydrated bovine collagen-glycosaminoglycan polymer substrates (approximately 40 cm^2 starting area) at a density of $5 \times 10^5/cm^2$.[45,47] Two days later, keratinocytes were harvested at subconfluent densities and inoculated onto the dermal substrates at a density of $1 \times 10^6/cm^2$. Engineered skin substitutes were incubated at the air-liquid interface for 14 days with daily medium changes.[42] Six groups of engineered skin substitutes were prepared ($n = 3$ per group): group 1, normal keratinocytes and normal fibroblasts; group 2, normal keratinocytes and deep keloid fibroblasts; group 3, normal keratinocytes and superficial keloid fibroblasts; group 4, keloid keratinocytes and normal fibroblasts; group 5, keloid keratinocytes and deep keloid fibroblasts; and group 6, keloid keratinocytes and superficial keloid fibroblasts.

Grafting to Mice

All animal studies were performed with University of Cincinnati Institutional Animal Care and Use Committee approval and following U.S. National Institutes of Health guidelines. Homozygous nude athymic female mice, aged 6 to 8 weeks, were obtained from Harlan Laboratories (Indianapolis, Ind.). Engineered skin substitutes were cut to 2 × 2-cm squares and transplanted to full-thickness excisional wounds cut on the right flank of each mouse, to the depth of the panniculus carnosus, as described in detail elsewhere ($n = 8$ per group).[45,48] Grafts were sutured to the wounds at the corners and sides and covered with multiple layers of gauze coated with antimicrobial ointment, and opposing sutures were tied over the gauze to stent the wound and limit contraction for 2 weeks after surgery.[45] The grafted areas were covered with OpSite occlusive dressing (Smith & Nephew, London, United Kingdom), and the mice were wrapped using Coban bandages (3M Health Care, St. Paul, Minn.) until the dressing materials were removed at 2 weeks.

Mice were photographed every 2 weeks, and beginning at week 4, grafted areas were traced onto sterile frosted Mylar sheets for calculation of wound areas using planimetry (ImageJ software; National Institutes of Health, Bethesda, Md.; http://rsbweb.nih.gov/i/). Mice were killed at 12 weeks after surgery and biopsy specimens of engineered skin substitutes were collected for histologic analyses, immunohistochemistry, and RNA isolation.

Histologic Analysis and Immunohistochemistry

Biopsy specimens of engineered skin substitutes for histology were processed and sectioned by the Shriners Hospitals for Children–Cincinnati Histology Core Facility. Samples for histologic analysis were fixed in 10% buffered neutral formalin, embedded in paraffin, sectioned, and stained using Gomori's One Step Trichrome Method for Connective Tissue (Light Green), according to the manufacturer's instructions (Poly

Scientific R&D Corp., Bay Shore, N.Y.). Engraftment of human cells was confirmed by immunohistochemical localization of human leukocyte antigen-ABC in frozen nonfixed engineered skin substitute sections using a fluorescein-labeled mouse monoclonal anti–human leukocyte antigen class I antibody[49] (Accurate Chemical & Scientific Corp., Westbury, N.Y.). Only animals with grafts that displayed positive human leukocyte antigen-ABC immunostaining, indicating engraftment of human cells in engineered skin substitutes, were included for further analyses.

Image Analysis

Histologic sections were examined and photographed using a Nikon Eclipse 90i microscope (Nikon Instruments, Inc., Melville, N.Y.). Thickness of dermal and epidermal regions was quantified using image analysis (NIS-Elements AR3.1; Nikon). To avoid biasing the thickness measurements because of nonlinear dermal-epidermal junctions, quantification was performed by separately calculating the areas of the epidermis (beneath the stratum corneum to the dermal-epidermal junction) and dermis (dermal-epidermal junction to the panniculus carnosus) in microscopic fields photographed at 4× magnification. Two to three low-power fields per section were measured.

Expression Analyses

Fibroblasts were harvested and pelleted, and cells were disrupted using Qiashredders (Qiagen, Inc., Valencia, Calif.); engineered skin substitute tissue biopsy specimens from mice were homogenized in lysis buffer using a rotor-stator homogenizer. RNA was isolated using RNeasy Mini Kits (Qiagen). Quantitative real-time polymerase chain reaction analysis was used for analyses of gene expression levels. cDNA was prepared using the SuperScript VILO cDNA synthesis kit (Invitrogen), and amplification was performed using gene-specific primers (RT^2 qPCR Primer Assays; Qiagen), and the iCycler iQ system (BioRad Inc., Hercules, Calif.). The comparative $\Delta\Delta C_t$ method was used to calculate the fold differences between the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) and the genes of interest.[50] The following genes were selected for analysis because they were previously implicated in keloid scarring: alpha 1 chain of type I collagen (*COL1A1*), transforming growth factor beta-1 (*TGF-β1*), periostin (*POSTN*), plasminogen activator inhibitor 2 (*PAI2*), inhibin beta A (*INHBA*), follistatin (*FST*), and secreted frizzled-related protein 2 (*SFRP2*).[44,46,51] All samples were analyzed in triplicate and the mean expression levels, normalized to expression in normal fibroblasts or engineered skin substitutes containing normal fibroblasts and keratinocytes, are presented.

Statistical Analysis

Statistical analyses were performed to identify any significant differences in *COL1A1* expression, grafted area, dermal thickness, or epidermal thickness among groups. Gene expression data obtained from normal, deep dermal, and superficial dermal fibroblasts were not analyzed statistically because technical triplicates, not biological replicates, were examined. Statistical analyses were performed using SigmaStat software version 3.1 (Systat Software Corp., Chicago, Ill.). For analysis of *COL1A1* expression in vitro and in vivo, and thickness at week 12 after grafting, comparisons between groups were performed by one-way analysis of variance, and subsequent pairwise comparisons were performed using the Student-Newman-Keuls method. For analysis of graft areas at sequential times after grafting, two-way repeated measures analysis of variance was used; variable factors were time (weeks) and group. Subsequent pairwise comparisons were performed using the *t* test. Differences were considered statistically significant at values of $p < 0.05$.

RESULTS

Engineered Skin Substitutes Containing Normal and Keloid-Derived Cells

To confirm regional differences between deep keloid fibroblasts and superficial keloid fibroblasts, expression levels of multiple genes previously implicated in keloid scarring[44,46,51] were analyzed (Fig. 1). These included *COL1A1*, *TGF-β1*, *POSTN*, *PAI2*, *INHBA*, *FST*, and *SFRP2*. As expected, all were differentially expressed between normal and keloid-derived fibroblasts. In addition, *COL1A1*, *TGFB1*, *POSTN*, *PAI2*, and *INHBA* were expressed at higher levels in deep keloid fibroblasts compared with superficial keloid fibroblasts. *FST* and *SFRP2* were expressed at slightly higher levels in superficial keloid fibroblasts compared with deep keloid fibroblasts. The differential expression of these genes suggested that there may be functionally relevant differences that could be evaluated further in engineered skin substitutes grafted to mice. Therefore, preparation of engineered skin substitutes containing keloid-derived keratinocytes and either deep or superficial keloid fibro-

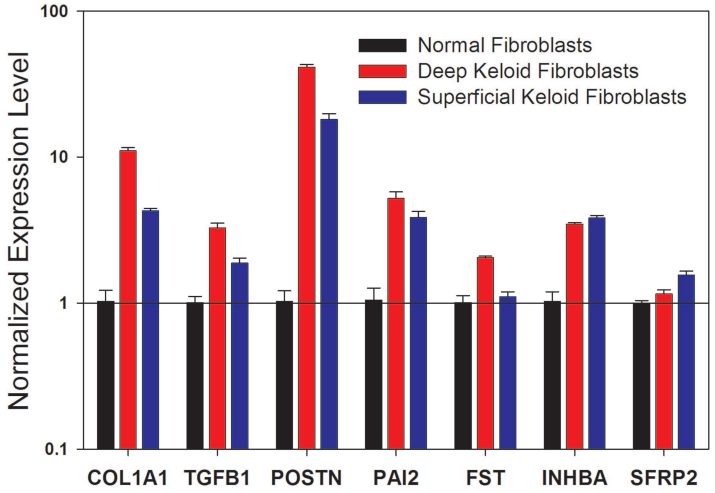

Fig. 1. Expression of genes implicated in keloid scarring in fibroblasts from normal skin, deep keloid dermis, and superficial keloid dermis. Multiple genes previously shown to be differentially expressed between normal and keloid fibroblasts were examined by quantitative real-time polymerase chain reaction in normal fibroblasts (*black bars*), deep keloid fibroblasts (*red bars*), and superficial keloid fibroblasts (*blue bars*). For most of these genes, differences in relative expression were observed, not only between normal and keloid cells, but between deep keloid fibroblasts and superficial keloid fibroblasts. Plotted are the means of technical triplicates ± SEM, normalized to the mean level for each gene in normal fibroblasts.

blasts was performed to test the validity of using the engineered skin model for investigation of keloid-derived cells.

Engineered skin substitutes were prepared using different combinations of normal or keloid keratinocytes and fibroblasts: group 1, normal keratinocytes and normal fibroblasts; group 2, normal keratinocytes and deep keloid fibroblasts; group 3, normal keratinocytes and superficial keloid fibroblasts; group 4, keloid keratinocytes and normal fibroblasts; group 5, keloid keratinocytes and deep keloid fibroblasts; and group 6, keloid keratinocytes and superficial keloid fibroblasts. Contraction of the collagen-based dermal substrate was observed during in vitro culture, but there were no differences in contraction among groups (data not shown). Collagen expression was analyzed in engineered skin substitutes at the end of the 2-week in vitro incubation. *COL1A1* expression was elevated in engineered skin substitutes prepared with deep keloid fibroblasts and either normal keratinocytes or keloid keratinocytes (Fig. 2); however, the differences were not statistically significant, probably because of a relatively high degree of variability in each group.

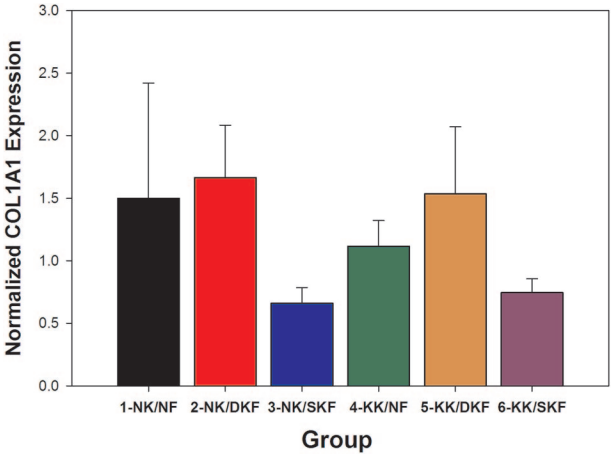

Fig. 2. Expression of the gene encoding alpha chain of type 1 collagen (*COL1A1*) in engineered skin substitutes prepared with normal and/or keloid derived cells. Relative expression levels were determined using quantitative real-time polymerase chain reaction and were normalized to the mean level for *COL1A1* in engineered skin substitutes prepared with all normal cells (group 1). Technical triplicates were performed for each skin substitute sample, and the means for engineered skin substitutes in each group ($n = 3$ per group) are plotted ± SEM. Differences between groups were not statistically significant. *NK*, normal keratinocytes; *NF*, normal fibroblasts; *DKF*, deep keloid fibroblasts; *SKF*, superficial keloid fibroblasts; *KK*, keloid keratinocytes.

Phenotypic Differences in Engineered Skin Substitutes after Transplantation to Mice

Engineered skin substitutes were transplanted to full-thickness excisional wounds in athymic mice and evaluated up to 12 weeks after grafting (Fig. 3). Differences in pigmentation were observed between groups prepared with normal keratinocytes or keloid keratinocytes, but these were attributable to different amounts of melanocytes contaminating the keratinocyte cultures used for preparation of engineered skin substitutes. Melanocytes have been observed to persist at variable but low levels in the epidermal keratinocyte cultures and remain as nonspecific "passengers" in engineered skin substitutes.[52] On healing, passenger melanocytes can result in pigmented foci that reach a finite size and persist long term, a phenomenon observed in engineered skin substitutes in both clinical and preclinical studies.[48,52]

Beginning at week 4, differences in wound area were noted between groups, and wound area measurements were therefore collected. During the first 2 weeks after surgery, the wounds were stented by dressing materials and thus differences in wound contraction were not expected at early time points. Historically, normal skin-derived engineered skin substitutes grafted to mice contract after dressing removal, stabilizing at 30 to 40 percent of the original wound area by 6 to 8 weeks after grafting.[42,53] In contrast, engineered skin substitutes prepared with keloid keratinocytes and superficial keloid fibroblasts (group 6) increased in area, and by week 6 after grafting were significantly larger than engineered skin substitutes in the other groups (Fig. 4).

At 12 weeks after surgery, mice were killed, and immunohistochemistry with anti–human leukocyte antigen-ABC antibody was used to quantify engraftment (data not shown). Although the number of engineered skin substitutes staining positive for human leukocyte antigen-ABC varied among groups (Table 1), the differences were not statistically significant. Grafts that were negative for human leukocyte antigen-ABC antigen staining were not included in the histologic, quantitative, or gene expression analyses.

Histologic sections of engineered skin substitutes from 12 weeks after transplantation showed differences in collagen organization between engineered skin substitutes prepared with normal keratinocytes and normal fibroblasts compared with engineered skin substitutes prepared using keloid cells (Fig. 5). At this time point after grafting, the bovine collagen from the biopolymer substrate used for in vitro incubation has been replaced by newly synthesized human collagen.[54] In normal engineered skin substitutes, collagen fibers were well organized and were roughly parallel to the epidermis. In contrast, thick, disorganized collagen bundles were observed in engineered skin substitutes prepared with either deep keloid fibroblasts or superficial keloid fibroblasts (Fig. 5). In addition, the dermal compartments of engineered skin substitutes prepared with keloid cells appeared thicker than normal engineered skin substitutes.

Image analysis of histologic sections of engineered skin substitutes was performed to quantify epidermal and dermal thickness. No differences in epidermal thickness were observed among groups (Fig. 6). However, engineered skin substitutes prepared with keloid keratinocytes and deep keloid fibroblasts (group 5) had significantly thicker dermal compartments compared with engineered skin substitutes prepared with all normal cells (group 1). Group 2 engineered skin substitutes, prepared with normal keratinocytes and deep keloid fibroblasts, also appeared thicker, but the difference was not statistically significant. Differences in *COL1A1* expression in vivo were observed among groups, and the trends were similar to the differences in dermal thickness observed in vivo (Fig. 7). *COL1A1* expression was significantly higher in groups 2 and 5, prepared using deep keloid fibroblasts, compared with controls, but was not increased in grafts prepared using superficial keloid fibroblasts.

DISCUSSION

The results presented here suggest that engineered skin substitutes can serve as a model for investigation of keloid scarring in vivo. After transplantation to athymic mice, phenotypic differences were observed between engineered skin substitutes prepared using normal and keloid cells and, in particular, between engineered skin substitutes prepared with deep or superficial keloid fibroblasts. Engineered skin substitutes prepared with keloid keratinocytes and deep keloid fibroblasts formed a thicker dermal layer in vivo compared with controls, whereas engineered skin substitutes prepared with keloid keratinocytes and superficial keloid fibroblasts increased in area over time after grafting. These grafts initially contracted after transplantation but by 4 weeks began to increase in area and were significantly different from normal engineered skin substitutes. The fact

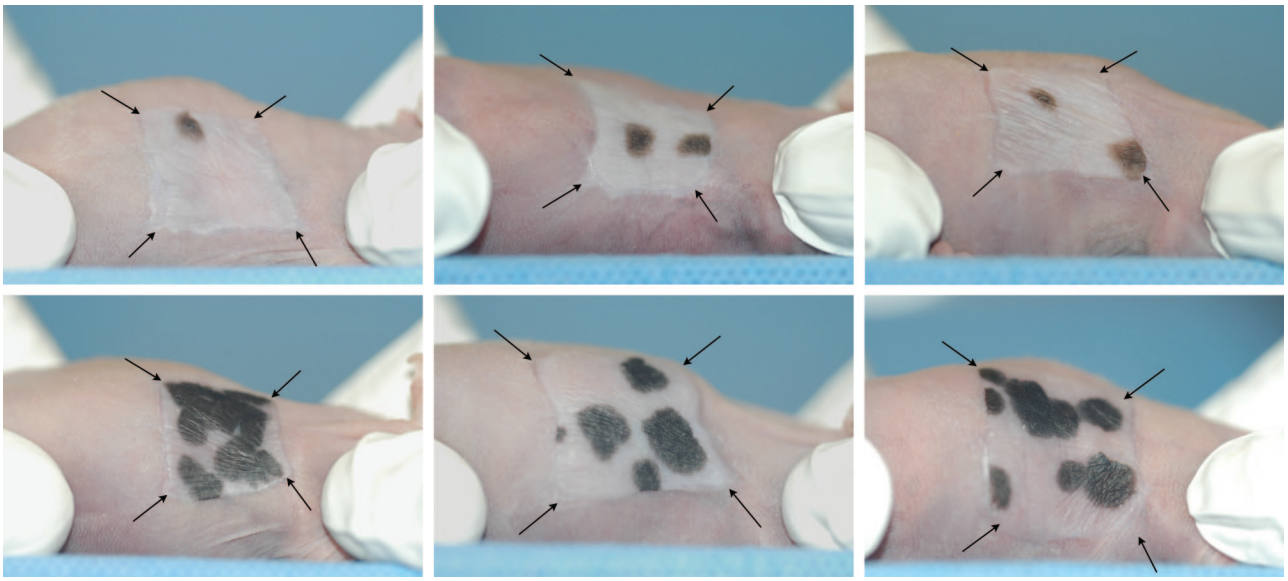

Fig. 3. Appearance of engineered skin substitutes containing normal and/or keloid-derived cells grafted to athymic mice. Shown are representative mice photographed at 12 weeks after surgery. (*Above*, *left*) Group 1, engineered skin substitutes prepared with normal keratinocytes and normal fibroblasts. (*Above*, *center*) Group 2, engineered skin substitutes prepared with normal keratinocytes and deep keloid fibroblasts. (*Above*, *right*) Group 3, engineered skin substitutes prepared with normal keratinocytes and superficial keloid fibroblasts. (*Below*, *left*) Group 4, engineered skin substitutes prepared with keloid keratinocytes and normal fibroblasts. (*Below*, *center*) Group 5, engineered skin substitutes prepared with keloid keratinocytes and deep keloid fibroblasts. (*Below*, *right*) Group 6, engineered skin substitutes prepared with keloid keratinocytes and superficial keloid fibroblasts. The corners of the grafted areas are indicated by *arrows*.

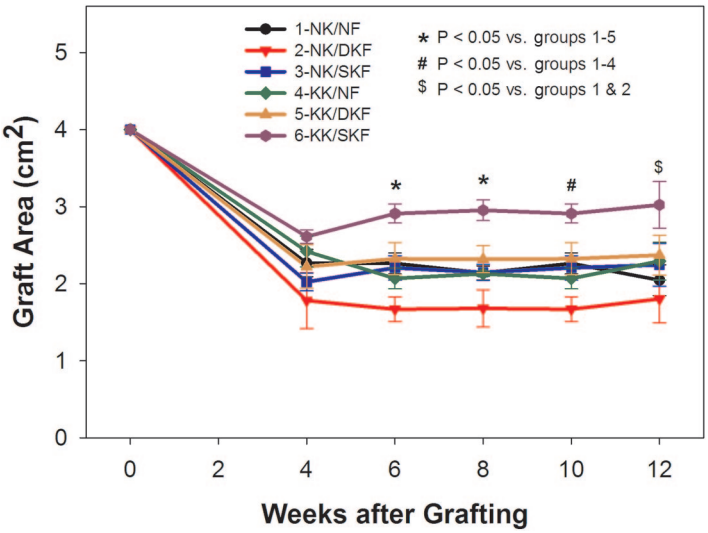

Fig. 4. Area of engineered skin substitutes following transplantation to athymic mice. Plotted are mean areas ± SEM. All grafts were 4 cm^2 at the time of transplantation (week 0). Significant differences between group 6 and other groups are indicated. *NK*, normal keratinocytes; *NF*, normal fibroblasts; *DKF*, deep keloid fibroblasts; *SKF*, superficial keloid fibroblasts; *KK*, keloid keratinocytes.

Table 1. Engraftment of Engineered Skin Substitutes Determined by HLA-ABC Immunohistochemistry

Group	Cells	HLA-Positive ESS
1	NK/NF	6/8
2	NK/DKF	5/8
3	NK/SKF	6/8
4	KK/NF	5/8
5	KK/DKF	6/8
6	KK/SKF	4/8

HLA, human leukocyte antigen; ESS, engineered skin substitutes; NK, normal keratinocytes; NF, normal fibroblasts; DKF, deep keloid fibroblasts; SKF, superficial keloid fibroblasts; KK, keloid keratinocytes.

that different phenotypes were observed when different keloid fibroblast populations were used for preparation of engineered skin substitutes, and that these phenotypes were significantly different from controls, indicates that keloid engineered skin substitutes grafted to athymic mice can serve as a valuable in vivo model of keloid scarring. In humans, keloid scars spread beyond the original wound margin and often bulge out over adjacent normal tissue. This bulging was not observed in keloid engineered skin substitutes grafted to mice. This may be attributable, in part, to the fact that mouse skin is thinner and looser than human skin. Rather than bulging over the top of adjacent mouse skin, the grafted engineered skin substitutes may displace the mouse skin as it increases in area. It is possible that insufficient time was allowed in this study for bulging scars to develop, because keloids in humans can appear several months after injury. In addition, wounding of the healed engineered skin might be required to trigger a wound healing response after establishment of the keloid engineered tissue. Therefore, future studies should include much later time points, investigation of wounding in vivo, and analysis of additional donor-derived cell strains, to fully evaluate these possibilities.

Alternatively, the different results observed in engineered skin substitutes prepared with the two distinct populations of fibroblasts may reflect different phenotypes of keloid scars that have been described in human populations.[5] In familial keloids described in African tribes, two different inherited phenotypes of keloids have been ob-

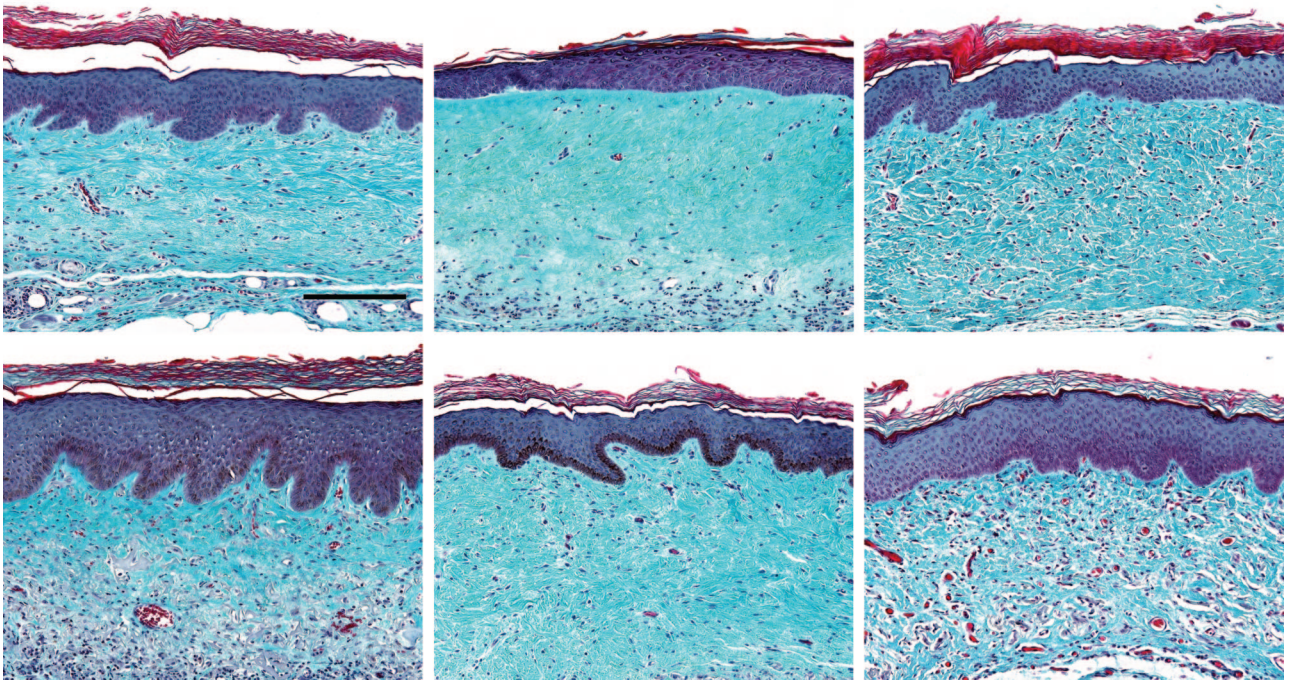

Fig. 5. Histologic sections of engineered skin substitutes in vivo. Shown are Masson trichrome–stained sections of engineered skin substitutes from 12 weeks after transplantation. (*Above*, *left*) Group 1, engineered skin substitutes prepared with normal keratinocytes and normal fibroblasts. (*Above*, *center*) Group 2, engineered skin substitutes prepared with normal keratinocytes and deep keloid fibroblasts. (*Above*, *right*) Group 3, engineered skin substitutes prepared with normal keratinocytes and superficial keloid fibroblasts. (*Below*, *left*) Group 4, engineered skin substitutes prepared with keloid keratinocytes and normal fibroblasts. (*Below*, *center*) Group 5, engineered skin substitutes prepared with keloid keratinocytes and deep keloid fibroblasts. (*Below*, *right*) Group 6, engineered skin substitutes prepared with keloid keratinocytes and superficial keloid fibroblasts. *Scale bar* (*above*, *left*) = 0.2 mm and is the same for all sections.

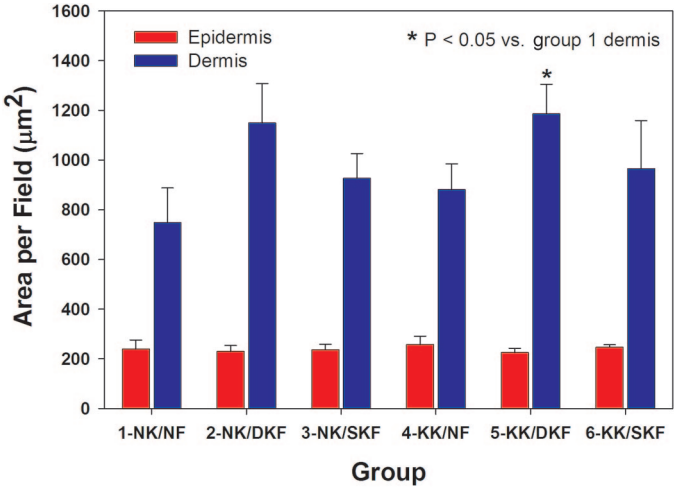

Fig. 6. Increased dermal thickness in engineered skin substitutes prepared with keloid keratinocytes and deep dermal keratinocytes. To calculate thickness while accounting for nonlinear dermal epidermal junctions, the areas of the epidermal (*red bars*) and dermal (*blue bars*) components of a fixed field length were calculated. Statistical analyses showed that dermal components of engineered skin substitutes in group 5 were significantly thicker than grafts in group 1 (*asterisk*). *NK*, normal keratinocytes; *NF*, normal fibroblasts; *DKF*, deep keloid fibroblasts; *SKF*, superficial keloid fibroblasts; *KK*, keloid keratinocytes.

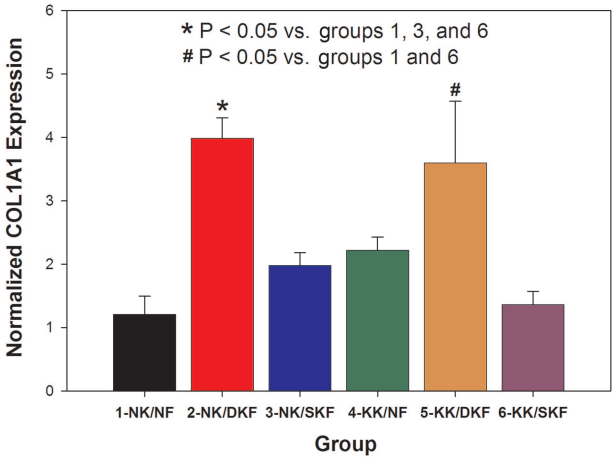

Fig. 7. Expression of *COL1A1* in engineered skin substitutes prepared with normal and/or keloid-derived cells at 12 weeks after transplantation to athymic mice. Shown are mean relative expression levels, normalized to the mean level for *COL1A1* in engineered skin substitutes prepared with all normal cells. Grafts that were negative for human leukocyte antigen-ABC immunostaining were not included in the analysis. *NK*, normal keratinocytes; *NF*, normal fibroblasts; *DKF*, deep keloid fibroblasts; *SKF*, superficial keloid fibroblasts; *KK*, keloid keratinocytes.

served: one involving superficial spreading, and one involving raised scars.[5] The authors of that study speculated that the heterogeneity observed in phenotypes of keloids may be attributable to different causative genetic lesions. Similarly, regional differences in gene expression may result in distinct phenotypes in engineered skin substitutes prepared with keloid cells, as described here.

Based on the phenotypes observed here, we propose the following model. After wounding, deep keloid fibroblasts cause thickening because of overproduction of extracellular matrix components or an imbalance between extracellular matrix production and degradation. Spreading, caused by superficial keloid fibroblasts, results in an increase in surface area of the upper dermis and overlying epidermis (Fig. 8). The result is a bulging phenotype, which can vary from person to person based on the relative contributions from deep keloid fibroblasts and superficial keloid fibroblasts in the dermis. Further in vivo analyses of keloid-derived cells from multiple different individuals in engineered skin substitutes, and combinations of deep and superficial fibroblasts, will be pursued in future studies to validate this model. Analysis of global gene expression patterns will be

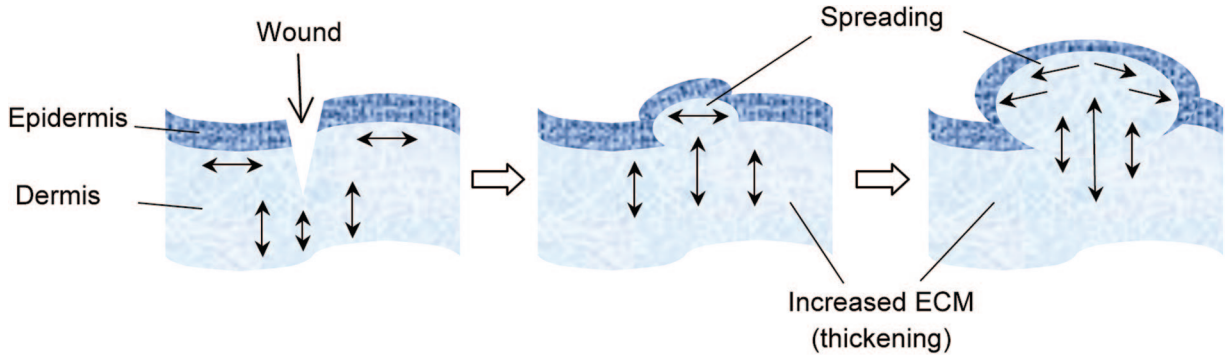

Fig. 8. Model for development of bulging keloid scars. (*Left*) Schematic diagram of cross-section of skin following a wound. During wound healing, fibroblasts proliferate, migrate, and deposit extracellular matrix. Based on the observations from the current study, fibroblasts in the deep dermis secrete extracellular matrix (*ECM*) and cause a thickening of the lower dermis, and fibroblasts in the upper dermis spread, causing an increase in area (*center*). With increasing time after injury, the combination of deep dermal thickening and superficial spreading results in a bulging phenotype (*right*).

required to begin to identify genetic pathways responsible for the observed phenotypes.

Fibroblast heterogeneity is well documented, and differences in gene expression in different regions of keloid scars have been described previously.[44] Similarly, differences in fibroblasts cultured from papillary and reticular dermis have been reported.[55,56] Deep dermal fibroblasts have been proposed to be critically important in formation of hypertrophic scars because they produce higher levels of collagen than superficial fibroblasts.[57] The mechanisms that govern fibroblast heterogeneity, and preserve it following dissection and in vitro culture, remain poorly understood. Inductive signals from epidermal keratinocytes are involved in regulation of fibroblast gene expression, and this may include assignment of positional identity to dermal fibroblasts that varies based on distance from the epidermis, resulting in stable differences in gene expression that are maintained following primary culture.

In contrast to some previously described reports of organotypic models containing deep or superficial fibroblasts, or keloid versus normal fibroblasts,[30,43] significant differences in morphology were not observed in engineered skin substitutes in vitro. This may result from differences in the methods used and timing of analysis. The model described by Butler et al. required 28 days of in vitro incubation for differences between groups to become apparent.[30] In our experiments, engineered skin substitutes were maintained in vitro for only 14 days, because this incubation has been shown to yield optimal tissue development following transplantation in vivo.[34] Previous studies demonstrated that engineered skin substitutes exhibit a hyperproliferative phenotype at day 14, with fibroblasts actively remodeling the dermal component.[47,48] Thus, extension of the in vitro incubation beyond 14 days may be required for significant differences in matrix deposition and contraction to be observed. Varkey et al. observed differences in contraction between collagen-glycosaminoglycan matrices inoculated with deep or superficial fibroblasts, but these constructs were smaller than those used in our study, did not contain an epidermal keratinocyte layer, and were not cultured in a lifted format.[43] These factors likely facilitated contraction of the matrix by fibroblasts. In contrast, the organotypic model described here consists of a relatively large substrate (approximately 40 cm^2 starting area), with a surface layer of keratinocytes; incubation at the air-liquid interface is achieved by culturing the engineered skin substitutes on a steel lifting platform covered with a cotton wick to permit nutrient transfer to cells within the engineered skin substitutes.[45] This format was developed in part to facilitate favorable epidermal differentiation and barrier formation for clinical application but may not be the best approach for revealing differences in contraction in vitro. However, this method can result in permanent engraftment following transplantation.

Differences in engraftment between groups were not statistically significant and therefore are not believed to result from the use of either deep or superficial fibroblasts. Although athymic mice are not expected to reject human cells, we have previously observed a low, variable rate of graft failure when engineered skin substitutes are analyzed several weeks to months after transplantation (data not shown). We attribute this to residual T-cell immunity in nude mice, which has been

documented by others and also found to increase as the mice age.[58–60] For future applications of this model, this minor limitation will necessitate the use of additional mice to overcome reduced numbers because of failure to engraft in a small percentage of mice.

SUMMARY

We describe an organotypic model for the study of keloid pathology in vivo. Although thick bulging scars were not observed at 12 weeks, there were significant differences between normal and keloid grafts, thereby permitting the use of this model for evaluation of therapeutic interventions for reduction of fibrosis and normalization of the keloid phenotype. This model contains both fibroblasts and keratinocytes in addition to biopolymers; thus, it can be used to study both cell-cell and cell-matrix interactions in a three dimensional skin-like tissue. Because engineered skin substitutes can be prepared using primary cells from any patient, it can be customized for investigation of patient-specific factors. It is easily scalable, so that large numbers of mice can be grafted, which represents a vast improvement over previous keloid rodent models that involved grafting of human keloid tissue.[21,61] Importantly, this model of keloid scar can be used for evaluation of therapeutic interventions, including novel treatments and preventative strategies, and is therefore considered a valuable in vivo model for the study of keloid scarring.

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As far as what is or is not ratable, the standard is to exclude basic science, bench work, animal, and cadaveric studies because the information gained from these studies is not something that can be applied directly to patient treatment decisions.

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