Published in final edited form as:

Am J Surg. 2008 February ; 195(2): 144–148. doi:10.1016/j.amjsurg.2007.10.003.

Use of Organotypic Co-culture to Study Keloid Biology

Paris D. Butler, MD^{1,2}, Daphne P. Ly, MD¹, Michael T. Longaker, MD, MBA, FACS¹, and George P. Yang, MD, PhD, FACS^{1,3,4}

¹Departments of Surgery at Stanford University School of Medicine, Stanford, CA

²University of Virginia School of Medicine, Charlottesville, VA

³Palo Alto VA Health Care System

Abstract

Background—Keloids are pathologic scars afflicting a large segment of our population for which there is no definitive therapy. The lack of an animal model for keloid formation has hampered study. We developed an in vitro organotypic skin model to simulate normal keloid biology that may allow us to study keloid formation without an animal model.

Methods—Normal (NF) and keloid (KF) human fibroblasts were cultured in a collagen matrix to create a three-dimensional dermal structure. Normal human keratinocytes (NK) were cultured as a second layer on top and exposed to an air-fluid interface to allow differentiation into a mature keratinocyte layer. The organotypic skin was maintained for 28 days in DMEM with 10% FBS. Samples were collected, processed, sectioned, stained with hematoxylin and eosin, and then measured for qualitative analysis. α -smooth muscle actin was also evaluated by immunoblotting.

Results—KF/NK organotypic skin showed increased collagen deposition based upon significantly denser collagen staining, with increased dermal thickness when compared to NF/NK organotypic skin. We saw increased contracture in the KF/NK construct, and this was correlated with increased organization of α -smooth muscle actin fibers in the dermal layer of the KF/NK organotypic skin, when compared to NF/NK.

Conclusions—We have shown that co-culture of KFs with KFs leads to an increased collagen production and dermal contracture when compared to NFs and NKs, consistent with known keloid behavior. Given the lack of an animal model, we believe organotypic skin culture can serve as a surrogate to study keloid formation.

Summary/Table of Contents—Keloids are pathologic scars afflicting a large segment of our population for which there is no definitive therapy. The lack of an animal model for keloid formation has hampered study. We developed an in vitro organotypic skin model to simulate normal keloid biology that may allow us to study keloid formation without an animal model.

Keywords

Keloid; Organotypic co-culture; Fibroblast; Keratinocyte; Scar; Wound Healing

⁴To whom correspondence is to be addressed: George P. Yang, MD, PhD, 257 Campus Dr., M/C 5148, Stanford, CA 94305-5148, Phone: (650) 725-5644, Fax: (650) 736-1705, Email: gpyang@stanford.edu

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Keloids represent a form of pathologic wound healing which affects a significant segment of the US population. Among African-, Asian-, and Latin-American ethnicities with darker skin, it is estimated that up to 15% of the population are at risk to form a keloid, although lighter skinned individuals may develop them as well.¹⁻³ They are fibrous overgrowths occurring at sites of cutaneous injury that do not regress and grow continuously beyond the original margins of the scar.² The majority of keloids leads to considerable cosmetic defects but can grow large enough to become symptomatic by causing deformity or limiting joint mobility.

By definition, keloids are scars that continue to grow and extend beyond the confines of the original wound.⁴ In contrast to hypertrophic scars, which stay within the boundaries of the original wound and increase in size by pushing out the edge of the scar, keloids invade the skin beyond the perimeter of the original wound with a leading edge that is often erythematous and pruritic.^{2, 4} The growth can persist for years beyond the time of the initial injury, and has led to the characterization of keloids as a form of a benign tumor.¹

Current mainstays of therapy have not changed much over the decades and include steroid injections^{5, 6}, single fraction radiation^{7, 8}, pressure dressings⁹, and surgical excision.⁹⁻¹² Unfortunately, in all instances the rate of recurrence is high and due to this lack of an effective clinical treatment regiment, keloids represent a major burden to the health care system. The high recurrence rate and failure of these treatments clearly suggests that the underlying problems creating the keloid have not been addressed and that there has yet to be a clear molecular mechanism defined for their development.

Keloid formation is believed to occur due to an abnormal inflammatory component of the wound healing process, involving both the secretory and responsive properties of keloid-derived fibroblasts.¹³ When compared to normal fibroblasts, numerous studies have revealed that keloid fibroblasts have increased receptor expression of many proinflammatory cytokines including transforming growth factor beta (TGF- β)^{14,15}, platelet derived growth factor (PDGF)¹⁶, and connective tissue growth factor (CTGF).¹⁷

One of the major difficulties in keloid research has been the lack of an animal model. Mice and other small traditional lab animals do not grow keloids, thus, investigators have been attempting to develop alternative in vitro models that would mimic in vivo biology. This led to our surgical research group to devise an organotypic skin model for keloids. Essentially, it consists of growing a keratinocyte cell layer upon a fibroblast cell layer to mimic an epidermal-dermal interface. Although not meant to fully replicate skin, it does allow for the typical epithelial-mesenchymal interactions that characterize skin. Similar systems have been used successfully to model skin developmental biology, regulation of growth factor expression, and transcriptional pathways in epithelial-mesenchymal interactions including wound healing.¹⁸⁻²¹ There is a clear role for these same interactions in keloid biology and we hypothesized that we could develop an organotypic cell culture system as an in vitro means to allow us to further study keloid physiology. This skin organotypic cell culture system will then be applied to examine the findings that have previously been elucidated between normal and keloid fibroblasts in culture.

METHODS

Cell culture

Keloid fibroblasts (KFs) were obtained from human earlobes keloid samples that had never been treated prior to excision. Normal fibroblasts (NFs) are from normal specimens discarded after repair of torn earlobes and represent site-matched controls. All specimens were obtained following informed consent and with Human Subjects IRB approval from Stanford University in accord with the ethical standards of the Helsinki Declaration of 1975. NFs and KFs were

maintained in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal calf serum (FCS), and passages 5 thru 8 were used for this study. Normal keratinocytes (NKs) were also primarily cultured from human earlobes, maintained in Keratinocyte Growth Medium (KGM), and passages 2 thru 4 were used.

Preparation of organotypic skin

The method is adapted from the published protocol by Maas-Szabowski, et al.²² NFs and KFs were grown to near confluence, trypsinized, and counted. The fibroblasts were resuspended in FCS at a concentration of 4×10^7 cells/ml. A master mixture of 8ml of collagen solution (5.9 mg/ml in 0.1% acetic acid, Nutragen collagen, Inamed Biomaterials, Fremont, CA) combined with 1ml of 10x Hanks buffer and 1ml of fibroblast suspension was made, with the pH adjusted to 7.4 by adding 2 N NaOH in a dropwise fashion. 2.5 ml of the master mixture containing 1×10^6 NFs or KFs, were dispensed into Falcon filter inserts (3.0 μ m pores, BD Biosciences, Bedford, MA) placed in BioCoat six-well plates (BD Biosciences, Bedford, MA) and allowed to gel for 1 hour at 37°C. Dermal equivalents were fed by diffusion of DMEM with 10% FCS placed in the base of the six-well plates and in contact with the lower portion of the insert. Glass rings with same diameter as the internal diameter of the inserts were placed over the dermal equivalent and 1×10^6 normal keratinocytes in 1ml of KGM were placed on top of each of the fibroblast containing collagen gels. After allowing the keratinocytes to attach for 48 hours, the medium and glass ring were removed. DMEM with the addition of 10% FCS and ascorbic acid were then added to the six-well plates to promote proliferation and differentiation of the co-cultured cells. The culture medium was changed every other day with the liquid level just high enough to contact the collagen gel, but leaving the keratinocyte layer exposed to air (Figure 1).

Analysis of the organotypic cultures

Organotypic skin cultures were maintained for 28 days after the keratinocyte layer had been added. We chose this time point empirically by looking for gross and microscopic differences between the two constructs. Our goal was to identify specific differences in the constructs that could be quantitated for future manipulations and studies. Upon reaching 28 days of grown the skin constructs were harvested. Specimens were fixed in formaldehyde, embedded in paraffin blocks, and sectioned. The sections were then stained with hematoxylin and eosin for histologic evaluation. To determine the thickness of the dermal and epidermal equivalents, three separate measurements were made using the internal calipers of the AxioVision software on a Zeiss Axioplan microscope. The values for each organotypic culture were averaged to give an average thickness for each specimen. Organotypic cultures were repeated in triplicate.

For immunoblotting, sections were incubated with normal horse serum to block non-specific binding, and then incubated overnight at 4°C with monoclonal anti- α -smooth muscle actin (α -SMA) antibody (clone 1A4) (1:800 dilution, Sigma, St. Louis, MO). Thereafter, the sections were incubated with anti-mouse Ig biotinylated antibody (Vector Laboratories, Burlingame, CA) and detection was performed using streptavidin-biotin-peroxidase complex (Vector Laboratories, Burlingame, CA) followed by the addition of 3,3'-diaminobenzidine tetrahydrochloride (5mg in 10ml PBS with 5 μ l hydrogen peroxide) as substrate. Sections were counterstained with hematoxylin. Negative controls were performed by omitting the primary antibody.

Statistical analysis

Student's t-test analysis was performed to evaluate for the significance of difference between the thickness dermal and epidermal layers in organotypic cultures made with KFs compared to NFs. Differences were considered significant at $p < 0.05$.

RESULTS

To establish our model, we prepared organotypic skin cultures with either NFs or KFs with an epidermal layer of NKs. We reasoned that if organotypic culture would be helpful in studying keloids, we should detect distinct differences between the two that would reflect keloid biology. After 28 days of culture, we noted that there was greater contracture of the epidermal layer of the organotypic cultures prepared with KFs compared to those with NFs although the overall size of the construct was similar (Figure 2). We harvested the specimens and examined them with standard histological stains. The thickness of the dermal and epidermal layers was measured at three random points for quantitative comparison (Figure 3).

The experiment was performed in triplicate and the average fibroblast layer width of the KF/NK constructs was significantly thicker than the NF/NK constructs ($260.5 \mu\text{m} \pm 29.9 \mu\text{m}$ compared to $203.71 \mu\text{m} \pm 2.68 \mu\text{m}$, $p < 0.05$) (Figure 4). Interestingly, we also found that the average width of the epidermal layer of the KF/NF constructs was significantly thicker than the NF/NK constructs ($285.3 \mu\text{m} \pm 35.5 \mu\text{m}$ compared to $122.8 \mu\text{m} \pm 4.5 \mu\text{m}$, $p < 0.05$) (Figure 4).

It is well documented that within scars, myofibroblasts are responsible for the majority of wound contraction.²³⁻²⁷ Within scars, increased expression of α -SMA can be detected in fibroblasts that is contributing to the process of contraction.²⁸ Previous studies from the dermatological literature have described that KFs have elevated levels of α -SMA expression when compared to NFs.²⁹ We used immunohistochemistry for α -SMA to see if we could detect differences in the KF/NK constructs compared to the NF/NK constructs. We recognize that immunohistochemistry is not a quantitative assay and further experiments would need to be performed to demonstrate that there is indeed more α -SMA. However upon evaluation, the appearance of greater organization of α -SMA fibers in the KF/NK constructs compared to the NF/NK constructs was markedly striking (Figure 5). The staining for α -SMA appears to be more linear in the plane of the dermal layer and in a more organized arrangement. Once again, no quantitative information can be deduced from this; however, the results are intriguing and may help to reveal more about keloid biology with further experimentation.

DISCUSSION

The lack of an animal model continues to be one of the main reasons that further advancements in the understanding of keloid pathophysiology have stalled. The establishment of an alternative skin equivalent provides a platform for evaluating the effects of molecular changes on phenotype in a model system that can mimic normal skin architecture. We have determined that fibroblasts and keratinocytes can be successfully grown in co-culture as an in vitro model to grossly and microscopically assess keloid tissue biology. Using our co-culture system as an instrument to evaluate the differences between NFs and KFs, our initial experiments have revealed that KFs co-cultured with NKs for 28 days yield thicker fibroblast and keratinocyte layers than the NFs co-cultured with NKs. This increased thickness of the fibroblast layer of the keloid containing constructs is consistent with what we know in the literature. Evaluation of keloid scars when compared to normal skin and normal scars reveals a much thicker dermal layer which is thought to be due to increased proliferation of these keloid fibroblasts as well as the additional ECM that they produce.^{30, 31} In our current experiments, we do not demonstrate whether it is due to increased proliferation, ECM production or both. We suspect that there are multiple factors contributing to it and we are currently performing experiments to address the underlying mechanisms.

As mentioned earlier, relative to NFs, KFs have increased expression of many potent inflammatory cytokines such as transforming growth factor beta (TGF- β)^{14, 15} and connective tissue growth factor (CTGF).¹⁷ In addition to increased cytokine production, KFs also increase

transcription of many of the receptors for these factors to a significant degree over NFs. Theoretically, this leads to aberrant healing as the response to these factors enhances cellular recruitment, causing excess synthesis of collagen, proteoglycans, and other ECM components, and thus, yielding a thicker dermal layer.

Interestingly, the finding of the increased thickness of the keratinocyte layer is not consistent with what is described in the literature about keloid scars. We suspect it is due to the increased contracture of the epidermal layer in the KF/NK constructs leading to the same cell mass distributed over a smaller area. Additionally, we believe that this increase in the epidermal layer is a byproduct of the increased contracture of the layer that we see in our organotypic cultures and is consistent with the qualitative changes we see on immunohistochemistry for α -SMA. The presence of increased α -SMA in keloids has been described, but our particular finding of qualitative differences in the arrangement of cells expression α -SMA has not been. It is not clear that if this finding is unique to this model or may reflect actual keloid biology, and we are planning experiments to address that. We do find the majority of these findings to be consistent with what has been previously cited in the literature^{28, 30, 31}, and we believe organotypic culture is a promising means of examining keloid biology.

Using isolated cells in culture, we have defined a number of specific differences in how keloid derived cells respond to the cellular stimuli present in wounds. In response to either mechanical strain or serum stimulation, they respond with a more pronounced transcriptional up-regulation of genes involved in fibrosis. However, it has been difficult to test whether manipulation of the signaling pathways involved can cause changes in the eventual growth of the keloid. In our previous studies with co-culture, fibroblasts and keratinocytes were co-cultured but there was no direct contact between the cell lines and their only connection was the medium in which they were suspended.^{30, 31} However, the organotypic skin model used in this study allows the cells to be grown in a three dimensional construct allowing for direct cell-cell contact, which is more similar to *in vivo* biology. This model is not necessarily better than the previous co-culture model, but does provide different information and adds factors, such as direct cell-cell contact, that were not present in the other model. We now have the ability to test whether changes in the appropriate signaling pathways can cause organotypic cultures made with KFs to decrease in thickness, or whether NFs can be induced to produce thicker dermis as the KFs. In addition to allowing us to determine potential therapeutic agents, it can also provide clues as to how mice can be engineered to develop keloids and create a true animal model.

Our future plans are to evaluate all combinations of NFs and KFs with NKs and KKs. It has been previously demonstrated that the combination of KFs with KKs leads to the most pronounced differences in terms of cell proliferation and collagen production. Future experiments are directed at identifying other features of the organotypic constructs that mirror known keloid biology including increased collagen and ECM production. Our goal will be to see how well the organotypic skin is able to mimic actual keloid biology. Ultimately, if these constructs prove faithful enough to known keloid biology, we can attempt to use them as a method for testing therapeutics. Keloid scarring remains a significant problem for the surgical community and having a more adequate instrument to decipher their pathobiology could pave the way for establishing more effective means of prevention and/or treatment.

Acknowledgements

This work was supported by grants from the NIH (R01 GM65213 to MTL, K08 GM069977 to GPY) and the Oak Foundation (to MTL and GPY).

Abbreviations/Acronyms

KF, Keloid Fibroblast; NF, Normal Fibroblast; NK, Normal Keratinocyte; DMEM, Dulbecco's Modified Eagle Medium; KGM, Keratinocyte Growth Medium.

REFERENCES

1. Russell SB, Trupin KM, Rodriguez-Eaton S, Russell JD, Trupin JS. Reduced growth-factor requirement of keloid-derived fibroblasts may account for tumor growth. *Proc Natl Acad Sci U S A* 1988;85(2):587–591. [PubMed: 3422443]
2. Atiyeh BS, Costagliola M, Hayek SN. Keloid or hypertrophic scar: the controversy: review of the literature. *Ann Plast Surg* 2005;54(6):676–680. [PubMed: 15900161]
3. Nirodi CS, Devalaraja R, Nanney LB, Arrindell S, Russell S, Trupin J, Richmond A. Chemokine and chemokine receptor expression in keloid and normal fibroblasts. *Wound Repair Regen* 2000;8(5):371–382. [PubMed: 11115149]
4. De Felice B, Wilson RR, Nacca M, Ciarmiello LF, Pinelli C. Molecular characterization and expression of p63 isoforms in human keloids. *Mol Genet Genomics* 2004;272(1):28–34. [PubMed: 15248062]
5. Kiil J. Keloids treated with topical injections of triamcinolone acetonide (kenalog). Immediate and long-term results. *Scand J Plast Reconstr Surg* 1977;11(2):169–172. [PubMed: 345427]
6. Davison SP, Mess S, Kauffman LC, Al-Attar A. Ineffective treatment of keloids with interferon alpha-2b. *Plast Reconstr Surg* 2006;117(1):247–252. [PubMed: 16404275]
7. Ragoowansi R, Cornes PG, Moss AL, Glee JP. Treatment of keloids by surgical excision and immediate postoperative single-fraction radiotherapy. *Plast Reconstr Surg* 2003;111(6):1853–1859. [PubMed: 12711944]
8. Meythiaz A, de Mey A, Lejour M. Treatment of keloids by excision and postoperative radiotherapy. *European Journal of Plastic Surgery* 1992;15:13–16.
9. Mustoe TA, Cooter RD, Gold MH, Hobbs FD, Ramelet AA, Shakespeare PG, Stella M, Teot L, Wood FM, Ziegler UE. International clinical recommendations on scar management. *Plast Reconstr Surg* 2002;110(2):560–571. [PubMed: 12142678]
10. Niessen FB, Spauwen PH, Schalkwijk J, Kon M. On the nature of hypertrophic scars and keloids: a review. *Plast Reconstr Surg* 1999;104(5):1435–1458. [PubMed: 10513931]
11. Slemp AE, Kirschner RE. Keloids and scars: a review of keloids and scars, their pathogenesis, risk factors, and management. *Curr Opin Pediatr* 2006;18(4):396–402. [PubMed: 16914994]
12. Berman B, Bielek HC. Adjunct therapies to surgical management of keloids. *Dermatol Surg* 1996;22(2):126–130. [PubMed: 8608373]
13. Sandulache VC, Parekh A, Li-Korotky H, Dohar JE, Hebda PA. Prostaglandin E2 inhibition of keloid fibroblast migration, contraction, and transforming growth factor (TGF)-beta1-induced collagen synthesis. *Wound Repair Regen* 2007;15(1):122–133. [PubMed: 17244328]
14. Xia W, Longaker MT, Yang GP. P38 MAP kinase mediates transforming growth factor-beta2 transcription in human keloid fibroblasts. *Am J Physiol Regul Integr Comp Physiol* 2006;290(3):R501–508. [PubMed: 16467496]
15. Chin GS, Liu W, Peled Z, Lee TY, Steinbrech DS, Hsu M, Longaker MT. Differential expression of transforming growth factor-beta receptors I and II and activation of Smad 3 in keloid fibroblasts. *Plast Reconstr Surg* 2001;108(2):423–429. [PubMed: 11496185]
16. Haisa M, Okochi H, Grotendorst GR. Elevated levels of PDGF alpha receptors in keloid fibroblasts contribute to an enhanced response to PDGF. *J Invest Dermatol* 1994;103(4):560–563. [PubMed: 7930682]
17. Xia XKW, Phan TT, Lim IJ, Longaker MT, Yang GP. Increased CCN2 transcription in keloid fibroblasts requires cooperativity between AP-1 and Smad binding sites. *Annals of Surgery*. Accepted for publication 2007
18. Ikuta S, Sekino N, Hara T, Saito Y, Chida K. Mouse epidermal keratinocytes in three-dimensional organotypic coculture with dermal fibroblasts form a stratified sheet resembling skin. *Biosci Biotechnol Biochem* 2006;70(11):2669–2675. [PubMed: 17090939]

19. Maas-Szabowski N, Stark HJ, Fusenig NE. Keratinocyte growth regulation in defined organotypic cultures through IL-1-induced keratinocyte growth factor expression in resting fibroblasts. *J Invest Dermatol* 2000;114(6):1075–1084. [PubMed: 10844548]
20. Maas-Szabowski N, Szabowski A, Stark HJ, Andrecht S, Kolbus A, Schorpp-Kistner M, Angel P, Fusenig NE. Organotypic cocultures with genetically modified mouse fibroblasts as a tool to dissect molecular mechanisms regulating keratinocyte growth and differentiation. *J Invest Dermatol* 2001;116(5):816–820. [PubMed: 11348477]
21. Stark HJ, Szabowski A, Fusenig NE, Maas-Szabowski N. Organotypic cocultures as skin equivalents: A complex and sophisticated in vitro system. *Biol Proced Online* 2004;6:55–60. [PubMed: 15103399]
22. Maas-Szabowski N, Fusenig NE, Stark HJ. Experimental models to analyze differentiation functions of cultured keratinocytes in vitro and in vivo. *Methods Mol Biol* 2005;289:47–60. [PubMed: 15502169]
23. Paul Ehrlich H, Sun B, Kainth KS, Kromah F. Elucidating the mechanism of wound contraction: rapid versus sustained myosin ATPase activity in attached-delayed-released compared with free-floating fibroblast-populated collagen lattices. *Wound Repair Regen* 2006;14(5):625–632. [PubMed: 17014676]
24. Grinnell F. Fibroblasts, myofibroblasts, and wound contraction. *J Cell Biol* 1994;124(4):401–404. [PubMed: 8106541]
25. Berry DP, Harding KG, Stanton MR, Jasani B, Ehrlich HP. Human wound contraction: collagen organization, fibroblasts, and myofibroblasts. *Plast Reconstr Surg* 1998;102(1):124–131. [PubMed: 9655417]discussion 132-124
26. Sewall GK, Robertson KM, Connor NP, Heisey DM, Hartig GK. Effect of topical mitomycin on skin wound contraction. *Arch Facial Plast Surg* 2003;5(1):59–62. [PubMed: 12533141]
27. Moulin V, Castilloux G, Jean A, Garrel DR, Auger FA, Germain L. In vitro models to study wound healing fibroblasts. *Burns* 1996;22(5):359–362. [PubMed: 8840034]
28. Campaner AB, Ferreira LM, Gragnani A, Bruder JM, Cusick JL, Morgan JR. Upregulation of TGF-beta1 expression may be necessary but is not sufficient for excessive scarring. *J Invest Dermatol* 2006;126(5):1168–1176. [PubMed: 16498396]
29. Chipev CC, Simon M. Phenotypic differences between dermal fibroblasts from different body sites determine their responses to tension and TGFbeta1. *BMC Dermatol* 2002;2:13. [PubMed: 12445328]
30. Lim IJ, Phan TT, Song C, Tan WT, Longaker MT. Investigation of the influence of keloid-derived keratinocytes on fibroblast growth and proliferation in vitro. *Plast Reconstr Surg* 2001;107(3):797–808. [PubMed: 11304607]
31. Xia W, Phan TT, Lim IJ, Longaker MT, Yang GP. Complex epithelial-mesenchymal interactions modulate transforming growth factor-beta expression in keloid-derived cells. *Wound Repair Regen* 2004;12(5):546–556. [PubMed: 15453837]

Figure 1. Schematic of the organotypic co-culture construct. (A) Fibroblasts are suspended in a collagen matrix and placed into a Falcon well insert. (B) Keratinocytes are then placed on top of this fibroblast containing collagen matrix to mimic an epidermal-mesenchymal interface. (C) DMEM containing 10% FBS and ascorbic acid are then placed in the bottom of the well to provide nourishment to the cells via simple diffusion through the microporous bottom of the Falcon well insert.

Figure 2. Gross photographs of organotypic cultures. Images were taken at Day 0 (A, C) and at Day 28 (B, D) of NF/NK constructs (A, B) and KF/NK constructs (C, D). All images are at 7.5x magnification with the exception of D at 15x magnification to better demonstrate the contracted epidermal layer.

Figure 3.

Basic histology of organotypic cultures. NF/NK (A) and KF/NK constructs (B) were harvested at Day 28, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Magnification is 100x.

Construct	Fibroblast Layer Width (μm)	Keratinocyte Layer Width (μm)
NF/NK	A 203.71	C 122.83
KF/NK	B *260.5	D *285.31

Figure 4.

The thickness of the dermal and epidermal layers was measured at three random points in each construct using internal calipers in the AxioVision software and averaged for qualitative analysis. Values represent the average of 3 separate constructs. * $p < 0.05$.

Figure 5.

Immunohistochemistry with antibody to α -SMA. NF/NK (A) and KF/NK (B) constructs were harvested at Day 28, embedded in paraffin, sectioned and assayed by immunohistochemistry for α -SMA. Slides were counterstained with hematoxylin to demonstrate construct architecture. Magnification is 200x.