A comparative study of skin cell activities in collagen and fibrin constructs

Jia Xian Law[a], Faiza Musa[c], Ruszymah Bt Hj Idrus[a,b], Alicia J El Haj[c], Ying Yang[c,*]

[a]Tissue Engineering Centre, Universiti Kebangsaan Malaysia Medical Centre, Jalan Yaacob Latiff, 56000 Kuala Lumpur, Malaysia

[b]Department of Physiology, Faculty of Medicine, Universiti Kebangsaan Malaysia, Jalan Yaacob Latiff, 56000 Kuala Lumpur, Malaysia

[c]Institute for Science and Technology in Medicine, School of Medicine, Keele University, Stoke-on-Trent, ST4 7QB United Kingdom

Correspondence:

Dr. Ying Yang

Institute for Science & Technology in Medicine,

Keele University,

Stoke-on-Trent,

ST4 7QB United Kingdom

Phone: +44 (0)1782 674386

Email: y.yang@keele.ac.uk

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Abstract

Collagen and fibrin are widely used in tissue engineering due to their excellent biocompatibility and bioactivities that support in vivo tissue formation. These two hydrogels naturally present in different wound healing stages with different regulatory effects on cells, and both of them are mechanically weak in the reconstructed hydrogels. We conducted a comparative study by the growth of rat dermal fibroblasts or dermal fibroblasts and epidermal keratinocytes together in collagen and fibrin constructs respectively with and without the reinforcement of electrospun poly(lactic acid) nanofiber mesh. Cell proliferation, gel contraction and elastic modulus of the constructs were measured on the same gels at multiple time points during the 22 day culturing period using multiple non-destructive techniques. The results demonstrated considerably different cellular activities within the two types of constructs. Co-culturing keratinocytes with fibroblasts in the collagen constructs reduced the fibroblast proliferation, collagen contraction and mechanical strength at late culture point regardless of the presence of nanofibers. Co-culturing keratinocytes with fibroblasts in the fibrin constructs promoted fibroblast proliferation but exerted no influence on fibrin contraction and mechanical strength. The presence of nanofibers in the collagen and fibrin constructs played a favourable role on the fibroblast proliferation when keratinocytes were absent. Thus, this study exhibited new evidence of the strong cross-talk between keratinocytes and fibroblasts, which can be used to control fibroblast proliferation and construct contraction. This cross-talk activity is extracellular matrix-dependent in terms of the fibrous network morphology, density and strength.

Introduction

Tissue engineering is an emerging multidisciplinary field involving biology, medicine and engineering to restore or regenerate tissue or organ function (1). Tissue engineering consists of 3 core components: cell, scaffold and signaling molecule, that is generally referred to as the tissue engineering triad (2). As one of the main components, scaffold serves as a template for cell delivery and support for tissue remodeling, fills voids and controls the release of signaling molecules**.** A good scaffold for tissue engineering skin should be biocompatible, biodegradable, support cell growth and tissue regeneration, assists appropriate contraction, and possesses similar mechanical and physical properties as the original skin (3-5). In addition, it is also highly desirable that the scaffold is non-antigenic, non-toxic, readily available, has suitable microstructure, controllable degradation rate and can be stored for a long period of time.

The scaffold can be made of either natural or synthetic materials. The biggest advantage of natural materials is the excellent biocompatibility that supports cell bioactivities (e.g. attachment, migration, proliferation and differentiation), which in turn regulates and promotes tissue formation. Collagen and fibrin are two of the natural materials that have been widely used in tissue engineering for scaffold fabrication as they fulfill the majority of the desirable characteristics mentioned above. Collagen and fibrin can be easily tailored to form scaffolds that provide proper biological, chemical, structural and mechanical cues to the cells to guide tissue formation in vitro and in vivo (6,7).

Collagen is the major extracellular matrix protein of multiple tissues and organs. For example, approximately 70% of human skin extracellular matrices is collagen (8). Collagen mainly resides in the dermis, providing mechanical strength to skin (9). To date, more than 29 types of collagen consisted of no less than 46 distinct polypeptide chains have been identified (10,11). Due to its excellent flexibility, collagen has been made into various forms and shapes, including tubes, sponges, sheets, foams, fleeces, nanofibers, and injectable viscous solutions for tissue engineering applications (12).

Fibrin is the matrix protein accumulated at wounds after injury to initiate hemostasis and healing (13). Fibrin is formed via the polymerization of fibrinogen monomers in the presence of thrombin. The presence of fibrin as a transitional wound healing matrix during the healing process is crucial, as it has been found to promote haemostasis, angiogenesis, fibroblast proliferation and re-epithelialization, with a potential role in reducing wound contraction and risk of infection (13-16). In addition, fibrin degradation products also have been found to play a profound role in wound healing by inducing fibroblast proliferation, extracellular matrix deposition and angiogenesis (17-19).

Collagen and fibrin have been widely used in skin tissue engineering to fabricate tissue-engineered skin substitutes. However, the two hydrogels have different gelation mechanisms. Fibrin network is initiated by thrombin-catalyzed cleavage of fibrinopeptides from fibrinogen to form fibrils. Collagen fibrils formation is through fibrillogenesis by self-assembly of triple-helical protocollagen molecules. Thus, the collagen fibers exhibit characteristic long bundling with twisted networks, whilst the

fibers in the fibrin appear straighter and more individual (20). Furthermore, collagen and fibrin gels have low mechanical properties initially, and collagen tends to contract, resulting in slower tissue regeneration and less favorable scar quality upon healing.

In contrast to natural materials, mechanical properties, microstructure and degradation time of synthetic polymers can be easily tailored and controlled to meet the requirement (21). However, synthetic polymers lack cell-recognition signals. This undesirable characteristic can be altered via the addition of chemical functional groups on the polymer surface (22). Another easier and probably more common alternative is the mixing of synthetic and natural materials. The combination of the advantages of both materials renders it more suitable for tissue engineering applications.

In this study, we intent to compare the regulatory effect of two hydrogels, collagen and fibrin, on skin regeneration and also the regulatory effect of keratinocytes on fibroblasts when grown in a different matrix environment. The poly(lactic acid) (PLA) nanofibers were incorporated into the collagen and fibrin constructs to improve their mechanical properties. We hypothesize that such a comparison study of the comprehensive matrix combination will provide a valuable communication for better selection of scaffolds in skin generation.

Materials and methods

Isolation and culture of murine epidermal keratinocytes and dermal fibroblasts

Murine dermal fibroblasts and epidermal keratinocytes were isolated using a method described previously (23). In brief, the skin from 4-6 month-old Sprague-Dawley rats was cleaned from fats and hairs before cutting into 1-2 mm^2 pieces. The rats were killed by approved Schedule 1 methods, following guidelines from the UK Animals, Scientific procedures Act, 1986 and authorization from Keele Universities's local ethics committee. Then, the sample was digested with 0.6% (v/w) collagenase type I (Sigma, USA) at 37°C for 2-3 hours under constant agitation, followed by 0.05% (w/v) trypsin-EDTA (TE; Lonza, Belgium) for 10 minutes to dislodge the cells. Isolated cells were cultured in Epilife medium (Gibco, UK) and F12:DMEM medium (Gibco) supplemented with 15% fetal bovine serum (FBS; Lonza) at equal volume. The cells were cultured at 37°C and 5% CO_2. The medium was changed three times per week. Upon 80% confluence, fibroblasts were separated by exposing the culture to TE for 4 minutes. Separated fibroblasts were cultured with F12:DMEM medium supplemented with 15% FBS, whereas remaining keratinocytes were cultured with Epilife medium.

Electrospinning of PLA nanofibers

A 2% PLA solution was prepared by dissolving PLA (Sigma) in chloroform (Sigma) and dimethylformamide (Sigma) in ratio 7:3. The process of electrospinning follows the established protocol (24). In detail, the PLA solution was placed in a 10 ml glass syringe fitted with 18G blunt end stainless steel needle. Random nanofibers were collected using round stainless steel wire ring of diameter 9 cm. Electrospinning was performed using the following processing parameters: ±6kV, 18 cm air gap, 0.025 ml/min flow rate and 0.200

ml volume. Collected nanofibers were air dried overnight and sterilized by UV radiation for 3 times, 90 seconds each, before use.

Preparation of collagen and fibrin constructs

Collagen construct**s** were prepared using 3.6 mg/ml rat tail collagen type I solution (BD Bioscience, USA). A total of 0.5×10^6 fibroblasts (F, labeled with PKH 2, green fluorescence (Sigma)) were seeded per construct. The components of the final collagen constructs were 83.33% collagen type I solution, 10% 10× DMEM, 1.92% 1 N NaOH and 4.75% dH_2O. The final collagen concentration was 3 mg/ml. Collagen construct**s** were formed by placing 0.5 ml collagen mixture solution on top of a hollow filter paper ring of diameter 25 mm to prevent lateral contraction. To prepare collagen construct**s** with random nanofibers (NF), 0.1 ml of collagen gel mixture was used to form the base before random PLA nanofibers were placed on top of it and sealed with 0.4 ml collagen gel mixture (S1). The collagen mixture construct was incubated at 37°C for 45 minutes for complete gelation before F12:DMEM medium supplemented with 15% FBS, 1% antibiotic-antimycotic (AA; Gibco) and 50 µg/ml ascorbic acid was added.

Fibrin construct**s** were prepared using human plasma fibrinogen (Calbiochem, USA). The final fibrin construct**s** contained 5 mg/ml fibrinogen, 1 U/ml thrombin (Calbiochem) and 2 mg/ml aminocaproic acid (ACA, Sigma). Each fibrin construct consisted of 0.5 ml fibrin solution with 0.5×10^6 fibroblasts (labeled with PKH 2). Fibrin construct**s** were formed by placing 0.5 ml fibrin gel solution on top of a hollow filter paper ring. To prepare fibrin construct with NF, NF were placed on top of filter paper ring, followed by

0.5 ml fibrin solution. Fabricated construct**s** were incubated at 37°C for 1 hour before F12:DMEM medium supplemented with 15% FBS, 1% AA, 50 µg/ml ascorbic acid and 2 mg/ml ACA was added. ACA is a lysine analog that promotes rapid dissociation of plasmin and is thus an inhibitor of fibrinolysis. 1×10^5 keratinocytes (K; labeled with PKH 26, red fluorescence (Sigma)) were seeded on top of the collagen and fibrin construct**s** on day 2. Fabricated construct**s** were cultured at 37°C and 5% CO_2 with medium changed every 3 days.

For both hydrogels, four groups of samples have been constructed respectively as indicated in follows: NF^-K^-: constructs with fibroblasts but without nanofibers and keratinocytes; NF^-K^+: constructs with fibroblasts and keratinocytes but without nanofibers; NF^+K^-: constructs with fibroblasts and nanofibers but without keratinocytes and NF^+K^+: constructs with fibroblasts, keratinocytes and nanofibers

Labeling of fibroblasts with PKH 2 and keratinocyte with PKH 26 were performed according to manufacturer's recommendation. In brief, trypsinized cells were washed in serum-free medium before suspended in 300 µl of Diluent C and 300 µl of 4 µM PKH dye (for the staining of 6×10^6 cells). The cells were incubated in dark for 10 minutes and washed 3 times before mixing with the gels for construct fabrication.

Confocal microscopy

Three-dimensional fluorescence imaging of the collagen and fibrin constructs was performed to observe the cell distribution after the samples were fixed with

paraformaldehyde (Sigma) overnight. Images were captured at XYZ-axis (20 μm step size in Z-axis) using a 10x dry objective lens. The 3-D image was built by stacking of the captured 2-D images.

Cell proliferation

The number of cells within the cultured constructs at selected days was determined using alamarBlue® cell viability reagent. In brief, spent culture medium was replaced with fresh culture medium with 10% alamarBlue® reagent. The cultures were incubated at 37°C for 3 hours before fluorescence measurement was performed using a microplate reader with fluorescence excitation wavelength of 530 nm and the fluorescence emission was read at wavelength 590 nm.

Mechanical testing

Mechanical testing was performed using non-destructive ball indentation technique that allows time series analysis as described previously (25). In brief, constructs were circumferentially clamped in between 2 transparent plastic circular rings that were held in place by tightly screwed thin stainless steel plates (Figure 1A). The constructs were deformed by placing a PTFE sphere of weight 0.072 g and diameter 4 mm in the centre. Images of deformed constructs were acquired 5 minutes after placing the sphere at day 1, 4, 10 and 22 using a long working distance microscope system. The extent of the deformation (δ) was measured using Image J.

The cross-sectional thickness of each construct was measured using a home-built optical coherence tomography (OCT) (26). Construct contraction was measured as changes in thickness as the filter paper ring prevented contraction in all other directions. Thickness measurement was performed at day 1, 4, 10 and 22.

Illustration of hydrogel indentation by a sphere is shown in Figure 1B. Elastic modulus (E) was calculated using the following mathematical equation (27);

$$6wr = Eh\,(0.075\delta 2 + 0.78r\delta)$$

where h is the construct thickness, r is the radius of the sphere and w is the weight of the sphere. The measurement was ensured that the ratio of a/r was equal to 5 and δ/r below 1.7, where a was the radius of the clamped portion of the construct to meet the equation condition. This model also assumes that the ratio of thickness to the radius is low and the deformation is large, hence, stretching of the membrane dominates over bending.

Statistical analysis

The data are presented as mean±SEM (n=3) and analyzed using Statistical Package for Social Science (SPSS, version 20.0). Statistical analysis was performed using one-way analysis of variance (ANOVA). The differences were considered significant if $p<0.05$.

Results

Dermal fibroblast and epidermal keratinocyte isolation and culture

Murine dermal fibroblasts and epidermal keratinocytes were isolated via the sequential treatment with collagenase type I and trypsin-EDTA. Culture of the isolated cells showed

the presence of spindle-shaped fibroblasts and cobblestone-shaped keratinocytes (Figure 2A). Upon confluence (approximately 8-10 days), the keratinocytes formed colonies that were surrounded by fibroblasts (Figure 2B). The co-cultured cells were separated via differential trypsinization upon reaching 80% confluence to yield highly pure fibroblasts and keratinocytes (Figure 2C & D).

Fabrication of collagen and fibrin-based skin constructs

Collagen and fibrin constructs were fabricated with a round filter paper ring as support to prevent lateral contraction. This was important to maintain the shape and size for mechanical testing. Grossly, the collagen and fibrin constructs looked similar with smooth flat surface and translucent appearance at the early culture time point (Figure 3). Nanofibers within the collagen and fibrin constructs could not be seen due to the hydrogels' translucent characteristic and the low density of fine PLA nanofibers. The fiber diameter was measured ranging from 500-800nm and fiber line density of 182±8/mm (S2). To tract the cell migration within the constructs, fibroblasts and keratinocytes were labeled with PKH 2 (green) and PKH 26 (red), respectively. Keratinocytes did not migrate into the fibroblast layer as shown by the presence of a layer of red fluorescent keratinocytes on top of the green fluorescent fibroblasts after 22 days of culture (Figure 4).

Cell proliferation

Cell proliferation was examined via alamarBlue® assay. For the constructs without nanofibers, comparison between fibroblast only groups showed that the fibroblasts

number was significantly higher in the NF^-K^- collagen construct compared to the NF^-K^- fibrin construct at day 4 and 22. After 22 days in culture, the fibroblast number almost double in the NF^-K^- collagen construct, whereas the fibroblast number in the NF^-K^- fibrin construct maintained the same. For the constructs with keratinocytes, the NF^-K^+ collagen construct has significantly higher cell number (keratinocytes+fibroblasts) compared to the NF^-K^+ fibrin construct at day 4, but the opposite were detected at day 10 and 22. The cell number in the NF^-K^+ collagen construct gradually reduced with time, whereas the cell number in the NF^-K^+ fibrin construct increased dramatically (Figure 5A).

Analysis of the constructs with nanofibers showed that the NF^+K^- fibrin construct contained more fibroblasts compared to the NF^+K^- collagen construct at day 10, but the opposite was detected at day 22. The NF^+K^+ fibrin construct has significantly more cells compared to the NF^+K^+ collagen construct at day 10 and 22. The cell number in all the groups increased by 2-3 times after 22 days in culture, except the NF^+K^+ collagen construct (Figure 5B).

Cell proliferation pattern was different between the collagen and fibrin constructs. The NF^-K^- and NF^+K^- collagen constructs demonstrated slow cell proliferation from day 4 to day 10 and accelerated cell proliferation from day 10 to day 22. In contrast, cell proliferation of the NF^-K^+, NF^+K^- and NF^+K^+ fibrin constructs was fast from day 4 to day 10 but slowed down from day 10 to day 22.

Construct contraction

Construct contraction was measured in term of changes in thickness compared to day 1 using a home built OCT (S3 and S4). For the constructs without nanofibers, the collagen constructs showed a reduction in thickness, whilst fibrin constructs' thickness either no change or slightly increased. At day 4, significant different were detected between the $NF^{-}K^{-}$ collagen construct with the $NF^{-}K^{-}$ and $NF^{-}K^{+}$ fibrin constructs, and the $NF^{-}K^{+}$ collagen construct with the $NF^{-}K^{+}$ fibrin construct. At day 10 and 22, the thickness of the $NF^{-}K^{-}$ collagen construct reduced significantly more compared all the other constructs. In addition, the $NF^{-}K^{+}$ collagen construct also showed a significantly higher reduction in thickness compared to the fibrin constructs (Fig. 6A).

All the constructs with nanofibers demonstrated a slight reduction in thickness except for the $NF^{+}K^{-}$ collagen. Significant differences were detected between the $NF^{+}K^{-}$ collagen construct compared to all the other constructs at day 22 (Fig. 6B). Generally, the $NF^{-}K^{-}$ and $NF^{+}K^{-}$ collagen constructs' thickness reduced gradually with time, whereas all the others groups showed less change with time.

Mechanical property

Calculation of the elastic modulus showed that incorporation of nanofiber into the collagen and fibrin constructs insignificantly increased the mechanical strength. In all the experimental groups, only the $NF^{-}K^{-}$ and $NF^{+}K^{-}$ collagen constructs demonstrated significant increased in Young's modulus with time, whereby significant differences were detected between day 22 and all the earlier time points (Figure 7A & B). Comparison between the fibrin constructs showed that the presence of keratinocytes and nanofiber

297 mesh exerted no influence on the construct mechanical strength. For the collagen
298 constructs, it was found that constructs without keratinocytes were significantly stronger
299 compared to the constructs with keratinocytes regardless of the presence of nanofibers at
300 later culturing period.
301
302 **Discussion**
303 Both fibrin and collagen play an important role in wound healing. Fibrin is the
304 provisional matrix at the early phase of healing, replaced by collagen at the later phase for
305 scar tissue formation. Although both collagen and fibrin have been used as a scaffold for
306 skin tissue engineering, there was no systematical or comparison report of their different
307 regulatory effect on skin cells, also the regulatory effect on the cellular cross-talk when
308 keratinocytes and fibroblasts were co-cultured. The current comparative study has
309 generated interesting data by multiple non-destructive techniques and demonstrated that
310 hydrogel scaffolds can exert considerable influence on the skin cells and their cross-talk
311 activities.
312
313 The alamarBlue measurements indicated that collagen and fibrin have different influences
314 on cell proliferation. For the cell proliferation, comparisons were made between the
315 collagen and fibrin constructs that only contained fibroblasts and also between the
316 collagen and fibrin constructs that contained both fibroblasts and keratinocytes. It was
317 found that collagen and fibrin constructs supported and suppressed fibroblast proliferation,
318 respectively, in the absence of keratinocytes. However, keratinocyte and fibroblast co-
319 culture suppressed cell proliferation in collagen constructs but promoted the cell

proliferation in fibrin constructs. The presence of nanofibers did not alter the cell proliferation activities as the same cell proliferation pattern was seen in the nanofiber incorporated constructs. These findings showed that the fibroblast proliferation in 3-D lattices and keratinocytes' regulatory effect on fibroblast proliferation was matrix dependent. Similarly, Eisinger et al. showed that epidermal cell-derived factors suppress fibroblast proliferation in collagen lattice and Sese et al. found that co-cultured keratinocytes and fibroblasts in fibrin construct proliferated faster than when they were incorporated alone (28,29). These observations reminiscent the natural wound healing process whereby keratinocytes secrete soluble factors that promote fibroblast proliferation during wound healing (wound bed rich in fibrin) and reduced the fibroblast number after healing (wound bed rich in collagen). We speculated that matrix proteins may influence the keratinocyte-mediated regulation of fibroblast proliferation. Furthermore, the alamarBlue data indicated that nanofibers increased the fibroblast proliferation in both collagen and fibrin constructs without keratinocytes. PLA nanofibers may provide mechanical or chemical cues to the fibroblasts to stimulate proliferation.

Thickness measurements showed that all the collagen constructs demonstrated reduced thickness, whereas the fibrin constructs without nanofibers increased in thickness and those with nanofibers decreased slightly in thickness. Keratinocyte-fibroblast co-culture reduced the collagen constructs' contraction. Previous studies by Chakrabarty et al. (30) and Isaac et al. (31) showed that keratinocyte-fibroblast co-culture increased collagen contraction. Furthermore, Souren et al. showed that co-existentance of keratinocytes and fibroblasts on top and in the collagen lattice, respectively, resulted in greater contraction

compared to the separate presence of both cell types (32). However, consistent with our results, Eisinger et al. found that epidermal cell-derived factor inhibits collagen contraction (28). The lower collagen contraction in our study was probably due to the suppression of fibroblast proliferation, migration and reduction of traction forces by the cytokines secreted by keratinocytes, supported by the findings of lower cell number in the co-cultured collagen constructs. Nien et al. found that fibrin appeared to inhibit contraction of matrix, which might explain our observation (33). Whether the fibrin could exert hypertrophic effects on the fibroblasts is not yet known. Interestingly, the presence of nanofibers in fibrin construct with or without keratinocytes triggered small amount contraction, implying that fibroblasts adhered to nanofiber rather than in fibrin could override the fibrin inhibition influence.

Mechanical testing showed that highly contracted constructs were mechanical stronger compared to the less contracted counterparts, as shown by the hike in Young's modulus of highly contracted $NF^{-}K^{-}$ and $NF^{+}K^{-}$ collagen constructs at day 22. Incorporation of nanofiber mesh insignificantly improved the Young's modulus of both collagen and fibrin constructs, probably due to the low density nature of the incorporated mesh. Several strategies can be used to improve the mechanical properties of the construct through PLA nanofiber mesh, including reinforcement with denser nanofiber mesh, increasing of the diameter of incorporated nanofibers, substituting random nanofibers with aligned nanofibers and crosslinking of nanofiber mesh (34-37). Nonetheless, each option has its own disadvantages, crosslinking and dense nanofiber mesh reduce the porosity, thus may hinder cell migration. Skin dermis consists of randomly oriented nanofibers (38).

Increasing the fiber diameter and nanofiber orientation may not provide adequate biochemical and biophysical cues needed by the cells to form tissue with proper architecture resembling the native skin.

Skin mainly consists of keratinocytes in the epidermis and fibroblasts in the dermis. The interaction between these cells is very important in the maintenance of tissue homeostasis and regeneration. Upon injury, cross-talk between keratinocytes and fibroblasts in a double paracrine manner has been found to regulate wound healing. For example, keratinocytes produce IL-1 which stimulates GM-CSF and KGF (that regulates the keratinocyte proliferation and differentiation) production by fibroblasts via the activation of IL-1R (39). In addition, keratinocyte-fibroblast interaction also influences the fibroblast phenotypical changes (fibroblasts to myofibroblasts) and extracellular matrix protein synthesis (40,41). The influences of keratinocytes on fibroblasts' activities in the fibrin and collagen constructs are clearly elucidated in this study. From the results, we found that keratinocyte-fibroblast interaction altered the cell proliferation, contraction and elastic modulus of collagen constructs. The collagen constructs with keratinocytes showed lower cell number, contraction and elastic modulus regardless of the presence of nanofibers. We speculated that decreased cell number created lower contraction force which reduced the contraction and mechanical strength of these constructs. For the fibrin constructs, keratinocyte-fibroblast co-culture increased the cell number. However, contrary to collagen constructs, higher cell number in fibrin constructs did not induced gel contraction and improved the mechanical property. These discrepancies revealed that influence of keratinocytes on fibroblasts cultured in 3-D lattice was greatly affected by

the matrix proteins, as the initial modulus, fibrous network, fibril structure and bioactivities varies from one material to another. In this study, fibrin showed stiff fibril morphology and lower mechanical strength due to different gelation mechanism compared to collagen.

Uniquely, this study used three non-destructive monitoring techniques, ball indentation, OCT and cell proliferation, to continuously examine the same sample for prolonged culture period, which eliminated the sample preparation variation and enabled identification of the turning point in the cellular activities.

In summary, we showed that keratinocytes can regulate the fibroblasts' proliferation and fibroblast-mediated gel contraction in 3-D constructs. This regulation is greatly influenced by the matrix proteins, probably via the alternation of keratinocyte soluble factor secretory profile. Reinforcement with nanofibers in collagen and fibrin constructs slightly improved the mechanical property and fibroblast behavior in fibrin.

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Figure legends

Figure 1 Mechanical testing. (A) Schematic diagram of the ball indentation setup to measure mechanical property. (B) Illustration of construct indentation by a ball (Reproduced with permission from Royal Society Publishing).

Figure 2 Murine dermal fibroblast and epidermal keratinocyte cultures. (A) Co-culture at day 2. **(B)** Co-culture at day 8. **(C)** Passage 1 fibroblasts (Day 7). **(D)** Passage 1 keratinocytes (Day 8).

Figure 3 Appearance of collagen (A) and fibrin (B) constructs.

Figure 4 Confocal images showing cell distribution within the collagen and fibrin constructs at day 22. Fibroblasts and keratinocytes were labeled as green fluorescent and red fluorescent cells, respectively. Blue, green and red lines represent the x, y and z-axis, respectively. **(A)** $NF^{-}K^{+}$ collagen; **(B)** $NF^{-}K^{+}$ fibrin. (Scale in μm)

Figure 5 Changes in cell number with time. The graft shows the absorbance of alamarBlue® at wavelength 590nm. A higher absorbance corresponds to a higher metabolic activity, which is an indication of higher cell number. **(A)** Collagen and fibrin constructs without nanofibers. *, significant higher compared to the $NF^{-}K^{-}$ fibrin construct. $^{+}$, significant higher compared to the $NF^{-}K^{+}$ fibrin construct. $^{++}$, significant higher compared to the $NF^{-}K^{+}$ collagen construct. **(B)** Collagen and fibrin constructs with

549 nanofibers. $^{\Delta}$, significant higher compared to the $NF^{+}K^{-}$ collagen construct. $^{\#}$, significant
550 higher compared to the $NF^{+}K^{+}$ collagen construct. $^{\Delta\Delta}$, significant higher compared to the
551 $NF^{+}K^{-}$ fibrin construct.
552
553 **Figure 6 Changes in construct thickness along culture time. (A)** Collagen and fibrin
554 constructs without nanofibers. *, significant different compared to the $NF^{-}K^{-}$ and $NF^{-}K^{+}$
555 fibrin constructs. $^{+}$, significant different compared to the $NF^{-}K^{+}$ fibrin construct. **,
556 significant different compared to all the other constructs. $^{++}$, significant different
557 compared to the $NF^{-}K^{-}$ and $NF^{-}K^{+}$ fibrin constructs. **(B)** Collagen and fibrin constructs
558 with nanofibers. $^{\Delta}$, significant different compared to all the other constructs.
559
560 **Figure 7 Changes in gel elastic modulus along culture time. (A)** Collagen and fibrin
561 constructs without nanofibers. **(B)** Collagen and fibrin constructs with nanofibers. *,
562 $NF^{-}K^{-}$ and $NF^{+}K^{-}$ collagen constructs at day 22 demonstrated significantly higher
563 Young's modulus compared to all the earlier time points.