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# Regulation of migratory activity of human keratinocytes by topography of multiscale collagen-containing nanofibrous matrices

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ABSTRACT

Nanofibrous matrices hold great promise in skin wound repair partially due to their capability of recapturing the essential attributes of native extracellular matrix (ECM). With regard to limited studies on the effect of nanofibrous matrices on keratinocytes, the present study was aimed to understand how the topographical feature of nanofibrous matrices regulates keratinocyte motility by culturing keratinocytes on polycaprolactone (PCL)/collagen nanofibrous matrices (rough surface with fiber diameters of 331 ± 112 nm) or the matrices coated with a thin layer of collagen gel to form a secondary ultrafine fibrous network (smooth surface with ultrafine fiber diameters of 55 ± 26 nm). It was found that the PCL/collagen nanofibrous matrices alone did not stimulate cell migration, while collagen gel coating could significantly increase cell motility. Further studies demonstrated that the ultrafine fibrous network of collagen gel coating significantly activated integrin β1, Rac1 and Cdc42, facilitated the deposition of laminin-332 (formerly called laminin-5), and promoted the expression of active matrix metalloproteinases (MMPs) (*i.e.*, MMP-2 and 9). Neutralization of integrin β1 activity abrogated the gel coating-induced keratinocyte migration. These findings provide important evidence on the role of topographical features of nanofibrous matrices in regulating the phenotypic alteration of keratinocytes and suggest the possible utility of collagen-containing nanofibrous matrices for skin regeneration especially in re-epithelialization.

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## 1. Introduction

In skin wound repair, rapid restoration of the epithelial barrier function is crucial to minimize potential threats from the environment such as bacterial invasion and dehydration [1]. Extensive efforts have been made to regenerate epidermis by transplanting cultured epithelial cell sheets [2–5], grafting meshed split skin [6,7], using tissue-engineered skin grafts with an epidermal layer [8–10], or stimulating the re-epithelialization from wound edge or intact hair follicles [1,11]. In either approach, complete re-epithelialization cannot be achieved until further migration of keratinocytes from the graft and wound edge to bridge the open wound surface.

In recognition of the importance of keratinocyte migration in wound re-epithelialization, it is highly desirable to create a microenvironment with the ability to promptly recruit autologous keratinocytes from the intact wound edge and promote their migration for faster re-epithelialization. Electrospun nanofibrous matrices, featuring high surface-to-volume ratio, high porosity and good interconnectivity, have great potential to mimic the skin ECM in both morphology and composition [12,13]. It has been reported that nanofibrous matrices support the adhesion and spreading of normal human keratinocytes [14]. However, the most critical behavior of keratinocyte in wound healing, *i.e.*, migration on electrospun nanofibrous matrices has never been studied.

The activation of keratinocyte migration can initiate soon after injury, while its migration rate significantly relies on the wound bed and underlying ECM [15–17] and involves the synergistic coordination among several cellular events including cell attachment/detachment, cytoskeletal reorganization, ECM degradation and redistribution of membrane integrins [16,18,19]. It has been found that ECM molecules like collagen type I and type IV, laminin, fibronectin and vitronectin can regulate keratinocyte migration by

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interacting with various integrins on the cell surface [16,17,20–22]. In this regard, a plausible strategy for creating skin grafts is to incorporate relevant ECM molecules in the scaffolds, for example, either directly incorporated into electrospun nanofibers or coated onto the fiber surface post-electrospinning [23], to promote keratinocyte proliferation and migration. Furthermore, transplantation of such grafts to the wound bed can also formulate a stimulatory microenvironment to recruit autologous keratinocytes for an accelerated re-epithelialization. Upon injury, keratinocytes of the epidermis are activated and migrate onto dermis, which is rich with type I collagen fibrils [16,17,24], suggesting the stimulatory effect of type I collagen on keratinocyte migration. Previous results showed that type I collagen-containing electrospun nanofibers promote the adhesion and spreading of human epidermal keratinocytes and support the formation of epidermal layers [25]. However, electrospun collagen fibers normally have diameters ranging from 100 to 1200 nm [25,26], much larger than collagen fibrils in dermis (~50 nm in diameter), which would result in different surface roughness and subsequently induce differential keratinocyte migratory response. In this regard, it becomes necessary to determine whether electrospun nanofibers are optimal for wound repair, especially for rapid re-epithelialization, and if not, what kind of modification should be made to further improve them.

In this study, collagen-containing nanofibrous matrices with two different topographical features, *i.e.*, electrospun polycaprolactone (PCL)/collagen nanofibrous matrices with an average fiber diameter of 331 ± 112 nm and electrospun PCL/collagen nanofibrous matrices coated with a thin layer of type I collagen gel to form a secondary ultrafine fibrous network (average diameter of 55 ± 26 nm) bridging the electrospun fibers, were fabricated. The proliferation, morphology and migration of human epidermal keratinocytes on both nanofibrous matrices were side-by-side studied.

## 2. Materials and methods

### 2.1. Materials

1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) was obtained from Oakwood products Inc (West Columbia, SC). Poly(epsilon-caprolactone) (PCL, Mw = 80,000) was purchased from Sigma–Aldrich (St. Louis, MO) and type I collagen (Coll) was obtained from Elastin Products Inc. (Owensville, MO). All other reagents and solutions were obtained from Invitrogen (Carlsbad, CA) except as indicated.

### 2.2. Nanofibrous matrix preparation, modification and characterization

Nanofibrous matrices of collagen or PCL/Coll were fabricated using the electrospinning technique following the same procedures as previously described [27]. Briefly, 8% (w/v) pure collagen or 3:1 PCL/Coll (w/w) blend solutions were prepared by dissolving PCL and collagen in HFIP. Then, the solution was loaded into a 5-mL syringe with a 20-gauge blunt-tip stainless steel needle and electrospun at 15 kV using a customized electrospinning apparatus. The polymer solution was dispensed using a syringe pump (Kdscientific, Holliston, MA) at 10 μL/min. Nanofibrous matrices collected onto round glass cover slips (Ø = 12 mm) under sterile conditions were used for the studies unless indicated. Then, PCL/Coll nanofibrous matrices were placed in 24-well culture plates.

To modify the electrospun nanofibrous matrices with collagen gel, fresh collagen solution was prepared by dissolving type I collagen in 0.01 N HCl and stored at 4 °C for further use. To modify the PCL/Coll nanofibrous matrices, prepared collagen solution was diluted in PBS at a ratio of 4:1 and then neutralized by adding 0.1 N NaOH on ice. The neutralized collagen solution with a final concentration of 3 mg/mL was added onto the PCL/Coll nanofibrous matrix surface and then immediately removed as much as possible. After incubation for 1 h at 37 °C for a complete gelation, the matrices were washed with PBS and then immediately used for cell culture or for characterization as described below. To better compare the effect of substrate surface on keratinocyte behavior, two additional groups were also included: collagen adsorbed PCL/Coll nanofibrous matrices and electrospun collagen nanofibrous matrices. For the former, a neutralized collagen solution of 50 μg/mL was added onto PCL/Coll nanofibrous matrix surface and incubated for 2 h at room temperature for adsorption. After adsorption, the meshes were washed with PBS and used for cell culture or further characterization.

Scanning electron microscopy (SEM) and atomic force microscopy (AFM) were used to characterize these nanofibrous matrices. For SEM examination, the dehydrated samples were sputter-coated with gold and then examined with a LEO 982 FEG SEM. To determine the diameter of nanofibers, images of five randomly selected areas were captured and analyzed by analysis software (NIS-elements BR 3.10, Nikon, Japan). The surface roughness of different nanofibrous matrices was determined by using Agilent NanoR AFM in a "dynamic force mode", that is, a "tapping mode." Three randomly selected areas of the surface with the size of 10 × 10 μm (*x*, *y* direction) were scanned, respectively. Close contact mounted cantilevers (Pacific Nanotechnology, Santa Clara, CA) were used. To describe the topography and roughness of the substrates, the roughness parameter for the surface, $R_{ms}$, the root-mean-square height of the surface, was calculated by NanoRule.

### 2.3. Cell and cell culture

Human keratinocytes immortalized by expressing the catalytic subunit of telomerase were a gift from Dr. James Rheinwald (NIH Harvard Skin Disease Research Center). Cells were plated in serum-free keratinocyte medium supplemented with recombinant EGF (2.5 μg/500 mL), bovine pituitary extract (25 mg/500 mL), 0.3 mM $CaCl_2$, 100 μg/mL streptomycin and 100 IU/mL of penicillin (Sigma, St Louis, MO) and cultured at 37 °C in a humidified 5% $CO_2$ atmosphere. Medium was replaced every 48 h until they reached 50–60% confluence.

Keratinocytes were seeded onto various nanofibrous matrices or glass cover slips at a density of $5 \times 10^3$ cells/cm$^2$ and allowed to attach for 60 min prior to the addition of fully supplemented medium. The cells were further cultured for up to 7 days. The effect of nanofibrous matrices on cell behaviors was determined throughout the 1-week culture period.

### 2.4. Cell migration assay

*In vitro* CytoSelect™ 24-Well Wound Healing Assay Kit purchased from Cell Biolabs Inc. (San Diego, CA) was used to evaluate the migratory capacity of keratinocytes. Briefly, $2 \times 10^5$ keratinocytes suspended in the culture media were seeded onto various matrix surfaces with inserts in place. After culturing for 12 h, the insert was removed to generate a consistent 0.9 mm wound gap among the cells. Cells were allowed to migrate into the wound gap for 24 and 48 h. After staining the cells with methylene blue, images of the wound gap were taken to analyze the migration distance of keratinocytes. 10 representative points along the "wound" of each sample were used to evaluate average migration ($n = 4$).

To determine the involvement of integrin β1 in regulating keratinocyte migration, anti-integrin β1 antibody (Millipore, Clone P4C10, 1:200) was added to the culture right after the removal of insert to neutralize the integrin β1 function.

### 2.5. Cell proliferation

Cell proliferation on various substrates was measured using CyQUANT® Cell Proliferation Assay Kit (Molecular Probes, Inc., Eugene, OR) following the protocol suggested by the manufacturer. Briefly, A standard curve over cell number was first established ($y = 0.007x - 2.977$, $R^2 = 0.995$). Samples were then harvested on day 1, 3 and 7 ($n = 4$ per group for each time point). After removal of medium and washing with PBS, the samples were frozen and stored at −80 °C. After collecting all the time-point samples, the samples were lysed in CyQUANT® cell-lysis buffer for 1 h at room temperature and then 200 μL of CyQUANT® GR dye/cell-lysis buffer was added to each sample and incubated for 2–5 min in the dark at room temperature. The fluorescence of cell lysates was measured using the multi-mode BioTek microplate reader at ~480 nm excitation and ~520 nm emission. Cell numbers were calculated based on the standard curve.

### 2.6. Immunofluorescent microscopy

Immunofluorescent staining of cells was performed following the procedures previously described [28]. Briefly, cultured cells were fixed in 4% paraformaldehyde for 10 min and then permeabilized with 0.2% Triton X-100 in PBS. Antibodies used in the study were as follows: phalloidin-TRITC (Sigma, 1:500), anti-vinculin-FITC (Sigma, 1:200), antibody against the active conformation of β1 integrin (Millipore, clone HUTS-4, 1:300) and anti-laminin-332 (also known as anti-laminin-5, Millipore, clone P3H9-2, 1:200). For those primary antibodies without fluorescent labels, the cells were further incubated with goat anti-mouse IgG-FITC conjugate secondary antibody (Caltag, 1:400). Cell nuclei were stained with DAPI (Sigma, 1:1000). The staining was examined under a Nikon Eclipse 80i epifluorescent microscope. The fluorescence intensity of the lysate of cells stained for active integrin β1 was also quantified by using a multi-mode microplate reader (Synergy™ HT, BioTek Instruments, Inc., Winooski, VT).

### 2.7. Determination of polarized keratinocytes

Keratinocytes were seeded on various matrices at a density of $2 \times 10^4$ cells/cm$^2$ and cultured for 24 h and then fixed with 4% formaldehyde. After staining the cells with TRITC-phalloidin and DAPI, images were taken from three randomly selected fields containing approximately 200 cells for each culture well. To score a cell as polarized, the cell needed to possess two defined morphological features, including (1) elongation and (2) the presence of long cytoplasmic extensions (lamellipodia and

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